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Francisco Massot

DOCTORAL DISSERTATION

Glyphosate Rhizoremediation Strategies for Soils Under Intensive Agricultural Use

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Abstract

The Argentinian Humid Pampa is the main agricultural region of the country and one of the most important land fields in South America. The agricultural production model that prevails entails, among other problems, an intensive use of inputs, mainly agrochemicals, meaning putting at risk both human and ecosystems health.

In the past years, the heavy dependence on glyphosate by the agricultural sector faced the growing concern in the environment, social and human health setting a growing dispute in the country.

Considering the wide extent of the territory, the need to preserve the soil texture and quality, while not interfering with the main economic activity, low-cost and on-site remediation techniques arises as the best option to remove glyphosate in soils and thus, avoid the contamination of water bodies. That is why rhizoremediation, which is the use of plants and root associated microorganisms to remove pollutants from soil, emerges as the most promising phytoremediation technology.

In this context, the main objective of this thesis work is to develop the knowledge to design a glyphosate rhizoremediation system to reduce its associated impact in the Argentinean Humid Pampa soils under intensive agricultural exploitation.

To achieve this goal, glyphosate tolerance testing methods were developed and applied on different plant species with commercial value. Then, phenotypic and genotypic characterization were made on isolated glyphosate degrading and tolerant bacterial strains. Two of the most promising strains were selected for whole genome sequencing. Additionally, soil and rhizosphere samples from which bacteria have been obtained were also characterized based on its physicochemical and microbial community properties. Finally, different plant-microorganism interaction experiments were conducted, together with a microcosms assay, where different plant-bacteria combinations were exposed to an agronomic dose of glyphosate in soil.

Among all tested plant species, *L. corniculatus* showed the most suitable tolerance to glyphosate, since it was able to grow in a maximum bioavailability medium with 5.0 mg Kg^{-1} of a commercial glyphosate product, and to endure a typical product application of 700 g ha^{-1}. Besides, methods such as measurement of shikimate and the impact of the analysis using a visual scoring should also be considered for future applications in field trials for their simplicity, costs and the provided information.

A culture collection was obtained from different sample types, and physicochemical and community structure characterization was also performed. When explaining the observed differences in the microbial communities, it was found that the rhizosphere effect is more important than the soil characteristics or the periodically glyphosate use in the plot.

Almost all the isolated microorganisms corresponded to the class *Alphaproteobacteria*. These microorganisms fulfilled the requirements of having a greater glyphosate tolerance in several orders of magnitude, to the maximum reported glyphosate concentration in agronomic soils, the capability of using glyphosate as the sole source of phosphorus, which is crucial in soils with very low contents in bioavailable phosphorus, and different *in vitro* plant growth promotion abilities, showing all the strains no less than three positive results on each test.

The whole genome sequencing of two of the most promising strains, *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXI was performed. The use of different methodologies of *in silico* genome-to-genome comparison was used to obtain complementary information regarding the genome structure of *Rhizobium* sp. P44RR-XXIV. At the same time, *Ochrobactrum* sp. P6BS-III genome contributed with a large number of unique genes to the genus, many of them having no assigned function.

Finally, plant-microorganism interaction studies were carried out using glyphosate tolerant and degrading bacteria strains together with *L. corniculatus*, which consisted of inoculation tests on vertical agar plates, imaging studies using different microscopy methods and a microcosm assay. In vertical agar plates assays, strains showed a high growth when inoculated with the plant, without causing any sign of damage on them. The microscopy images obtained showed the different colonization patterns of *Ochrobactrum* sp. P6BS-III and *Rhizobium*

sp. P44RR-XXIV in roots. The microcosm assay revealed that *L. corniculatus* inoculated with *Ochrobactrum* sp. P6BS-III caused the degradation of almost all the amount of 5 mg Kg^{-1} of glyphosate in soil after 20 days post application, considering this time adequate for the proposed remedial system.

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This is for you

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Chapter 1 - Background

1.1 The Agricultural landscape of Argentina

Argentina is one of the world's four most eligible countries to offer and expand its food production. In a context where the demand for food and other agricultural products is expected to increase globally by 50% between 2012 and 2050 (Liu, 2017), a high level of technological competitiveness and a strong representation in the world's economy, the efforts of the country are focused on the intensification of the use of lands through the adoption of new and more efficient technologies where the use of crop protection inputs shall be more reasonable then has been applied in the past and oriented to minimize the environmental impact (Ministerio de Agroindustria, 2017).

1.1.1 The Humid Pampa Region

The Humid Pampa Region is the main agricultural territory in Argentina and one of the most important agricultural and livestock regions in South America. It covers approximately 52 million hectares, an area mostly covered by cereal and grain crops (Viglizzo et al., 2001). It comprises the provinces of Buenos Aires, the Center and South of Santa Fe, the South East of Córdoba and the East of La Pampa (Figure 1.1). The South of the province of Entre Ríos is sometimes included as well. The climate is mild; the average summer temperatures range from 20 to 25 °C and average winter temperatures range from 5 to 12 °C. The annual average is 17 °C. Based on the geographical patterns, the soil, productive and climate characteristics, the Humid Pampas is subdivided into the following sub-regions (Matteucci, 2015): Rolling Pampas, Mesopotamian Pampas, flooding Pampas, inland Pampas and southern Pampas.

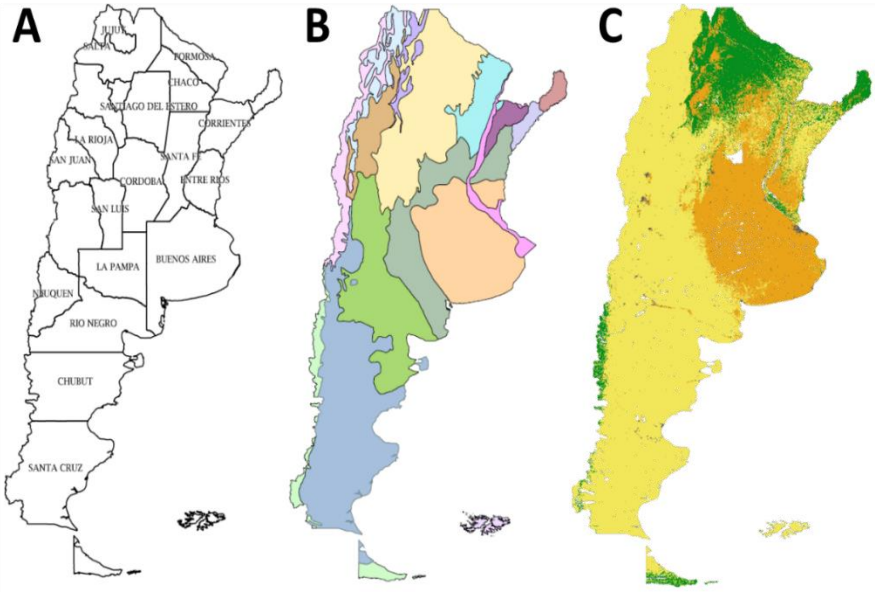

Figure 1.1. (A) Provinces of Argentina. (B) Eco-regions of Argentina. Each color represents a different Eco-region. The Pampean Region is signaled in pale orange. (C) Different soil managements reported during 2007-2012. Orange represents farmlands and pastures. Source: Portal de datos abiertos del Ministerio de Agroindustria de la Nación (https://www.agroindustria.gob.ar/datosabiertos/).

1.1.2 Historical development of the Argentine Humid Pampas

The establishment of the Humid Pampas as the agricultural and livestock region of Argentina was a gradual process, the path of which has been marked by the tone of the central countries, since their needs and geopolitical strategy established the trading manner and conditions of the products produced in the area as well as the degree of industrialization obtained. It is possible to identify five historical periods to understand the technological advances in the Humid Pampa (Lombardo, 2014):

The first period, called "*colonial and transition period*", took place from century XVI until 1860 and consisted in a scarce economic development based on primary regional economies oriented to self-sufficiency and not to export products. The first farms as productive and administrative units were formed, the breeding system (herds) was adapted with the incorporation of new breeds of

cattle, wire fences were installed, and the jerky meat industry was developed since it was necessary for the export of meat products. The grain production continued to be oriented towards the national market (Ferrer, 2004).

The second period laid the foundations of the Republic as a worldwide food producer and supplier. It was called the "*agricultural products exporter period*" and took place from 1860 until 1930. The industrial revolution in Europe meant the consolidation of the role of Argentina as food exporter: storage and transport of cereals and oilseeds, corn, wheat, linseed, meats and wools exports. The improvements for the agricultural sector were based on the implementation of the crop-livestock rotation, the incorporation of imported agricultural machinery (tractors, plows, mowers, etc.), and the skilled workforce coming from the European immigration. The yield increase was based on the increase of cultivated lands and on mechanization.

The third period took place between 1930 and 1960 and is known as "standstill of agriculture in the Pampas". As its name suggests, it was a period marked by the fall of exports and the agricultural gross product due, in first place, to the 30´s economic crisis and, in second place, to the development and the consequences of the Second World War. This period was marked by a change in the economic policy, which turned from the agricultural exporter model to the so called "imports substitution". As a consequence, in the 50´s, a new series of measures oriented to boost the agricultural sector were taken, such as the manufacture of agricultural machinery, the establishment of subsidiaries of foreign companies and the creation of the National Institute of Agricultural Technology (INTA, by its acronym in Spanish), as well as the appearance of the private industry for the production of seeds and agrochemicals and the Argentine Association of Regional Consortiums for Agricultural Experimentation (AACREA, by its acronym in Spanish).

The fourth period is the consequence of the productive restructuring of the end of the previous period and took Argentina back to the record levels of export of primary products. It is called the "modernization period" and took place from 1960 to 1990. This was a period of great technological transformations. Obschatko (1988) defines those most important as: the specialized production of the five crops that would contribute **90% of the agricultural production** (wheat, corn, sorghum, soybean and sunflower) with the technological innovation

corresponding to each crop, the complete mechanization of tasks, the **high degree of usage of herbicides** (pre- and post-emergent) and new application methods, the incorporation of wheat fertilizers, the improvement of grain storage and drying facilities, the improvement and specialization in the management of an agricultural company and the appearance of new conducts and forms of business organization. During these years, the value of production tripled, the land productivity doubled, and the workforce productivity tripled as well. The coup d'état of 1976 put an end to this dynamic tending to install the neoliberal model and meant the end of the development of the sector and the interruption of the process of incorporation of new technologies. In the 80´s, the **soybean** became established as the most important crop. This crop required the incorporation of a technology package mostly based on the application of agrochemicals, especially the post-emergent ones, which were produced abroad. No-till farming was also incorporated at such stage. Such practice requires the use of a large number of agrochemicals. It is during this period that factors such as monocultures (Roccatagliata, 1992), the development of new agrochemicals and the adoption in our country as part of a the management strategy of the farm, the adoption of no-tillage practice and a renewed global demand for grains, substantially increases the use of agrochemicals.

The fifth and last period, called "intensification of agriculturalization" took place from the 90´s until now, and was based on the **incorporation of the transgenic soybean since 1996**, the no-tillage management and the subsequent use of agrochemicals. With the incorporation of the transgenic glyphosate-resistant soybean, agrochemicals, mainly due **to glyphosate, reached their maximum level of use** (Mikkelsen, 2008; Reboratti, 2010).

At the beginning of this century, the farm management model of crop rotation with grazing pastures suffered an intensification. In this intensification, animals are submitted to a fattening process (called Feedlot) while agriculture tends to take over the full year in the plot, wheat in winter and soybean in summer (Pengue, 2014). In order to sustain the changes in productivity and specialization, more inputs are needed and more residues and waste that harm the environment are generated (nutrients, wastewater, pesticides, antibiotics, etc.). In the last 20 years, the Argentine agriculture in general –and the Pampas agriculture in particular– has expanded within a modern technology matrix defined by

transgenic crops, no-tillage farming, further use of fertilizers and pesticides and, to a lesser extent, by precision farming related practices (Satorre, 2005).

1.1.3 Consequences of the intensification of agriculture

The intensification of the agriculture towards the end of the last century and which continues nowadays, brought about complex socio-economic, environmental, and technological changes.

1.1.3.1 Socio-economic changes of the intensification of agriculture.

The attitude towards the adoption of technologies in the sector (the use of transgenic seeds, no-tillage farming and grain storage), which has historically been conservative, appears as a positive aspect. Yield increase and the domestic crop prices led agriculture to a high global competitivity level (Reboratti, 2010). According to the National Institute of Statistics and Census, the agriculture-livestock sector represents 57,6% of total exports during the year 2016. The oilseed and cereal exports represented 31.4% and 13.2% respectively and sales of soybean itself represented 30% of the exports, amounting to **17,310 million dollars**.

The social concerns are concentrated on three topics: (i) The concentration of lands. Farming management represents a big scale economy, implying that the unit cost of production is reduced if cultivated land increases. This motivates a higher demand for larger territories than those used in traditional crops and farming methods. (ii) The economic revenue the technology package offered, produces the shift of traditional crops and livestock activities in the region. Therefore, the cropland frontier area increases provoking deforestation and the loss of natural areas and it biodiversity. (iii) The food sovereignty jeopardizing due to the dependency on inputs manufacturers and importers have (which are generally from offshore capitals). The sovereign nations are entitled to decide how and who will produce food, in this way controlling its own food and agriculture system.

1.1.3.2 Changes in the quality of soils

The changes in the **quality of the soil** are mainly given as a consequence of a decreased fertility and an increase in **soil erosion** (Robertson & Vitousek,

2009). Crop rotation and the implementation of resting periods (non-sowing periods) were strategies tending to minimize these effects. However, in the last few years, the economic revenues and renting costs hindered those strategies (Reboratti, 2010). Hence, even when soil fertilization is usual, it does not compensate the nutrient loss. That is how fertilization only replaces 43% of nitrogen, 70% of phosphorus (Figure 1.2), 50% of sulfur and 2% of potassium exported in soybean (García & González, 2015).

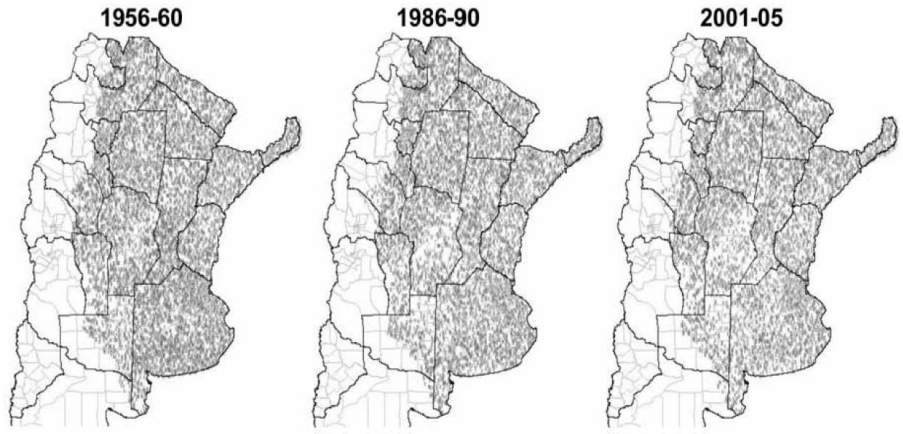

Figure 1.2. Evolution of the available phosphorus stock in the cultivable areas in the indicated years. Being the intensity of the grey dots in each place in the map a representation of the amount of available phosphorus, it is possible to observe a decrease over the years. The image was generously supplied by Viglizzo and Jobbágy (2010)

The analysis of the consequences of the increase in the use of agrochemicals encompasses also **loss of biodiversity**, increased weed resistance and increased risk of contamination. As mentioned above, the intensification of agriculture brought about a progressive increase in the use of agrochemicals. Table 1.1 shows the total amount of agrochemicals used per year in our country and, separately, the total percentage corresponding to herbicides.

Table 1.1. Evolution of the phytosanitary market in Argentina, with special emphasis

	Year						
	1991	**1993**	**1995**	**1997**	**1999**	**2001**	**2003**
Total phytosanitaries (millons of kg or L)	39.2	50.3	72.4	123.8	127.5	142.3	198.5
Herbicides over total (%)	50.1	52.1	57.9	60.9	76.4	78.5	80.1
	Year						
	2005	**2007**	**2009**	**2011**	**2013**	**2015**	**2016**
Total phytosanitaries (millons of kg or L)	234.2	254.1	260.5	335.9	281.7	274.4	310.0
Herbicides over total (%)	77.3	78.1	77.0	75.0	72.0	86.8	87.0

1.1.3.3 Effects on Plants Species and Herbicide Resistant Weeds

The significant increase in the use of herbicides, especially glyphosate, changed the typical weed profile of the region, mainly reducing the amount of annual dicotyledonous (de la Fuente et al., 2006). An increase in the propagation of woody species related to no-tillage management was also observed (Ghersa et al., 2002). As a result of the agricultural intensification, the non-selectivity action of glyphosate and the use of transgenic germplasm, the natural vegetation in plot borders and biological niches were reduced.

In the last two decades, in our country, in Latin America and worldwide, an increase of herbicide resistant weeds was observed. This trend causes concerns both in the public and the private sector (Powles et al., 2008; Vila-Aiub et al., 2008; Valverde, 2010). Among the species reported in our country by the *Survey of Resistant Weeds Network of the No-Tillage Management Users (AAPRESID* by its acronym in Spanish), the following are widely reported in literature: the hairy fleabane (*Conyza bonaerensis*), the johnsongrass (*Sorghum halepense*), two ryegrass species (*Lolium multiflorum* and *Lolium rígidum*) as well as the junglerice (*Echinochloa colona*), the goosegrass (*Eleusine indica*), knotgrass (*Cynodon hirsutus*) and the green amaranth (*Amaranthus quitensis* and *Amarathus palmeri*), all of them resistant to glyphosate, the wild oat (*Avena fatua*) resistant

to arylphenoxypropionic herbicides and the wild radish (*Raphanus sativus*) resistant to ALS inhibitors.

As part of the global trend, in the last 50 years our country suffered a gradual change that consisted in the replacement of agrochemicals for those less toxic and less persistent. This implied a strong decrease in the risk of contamination towards the end of the last century. This trend has reversed with the intensification of agriculture model, in which the risk of contamination with pesticides grew along with the increase of cultivated areas, the relative proportion of soybean and other transgenic crops (Viglizzo & Jobbágy, 2010).

Figure 1.3 shows the above mentioned. The equation used to obtain the value in relative units indicates the risk of contamination of aquifers subject as a function of physicochemical characteristics of the agrochemicals used, its toxicity, persistence and leaching as well. The formulation of each agrochemical and the involved farming management were also considered. From this analysis, it also emerges that soybean is the crop having more associated risk.

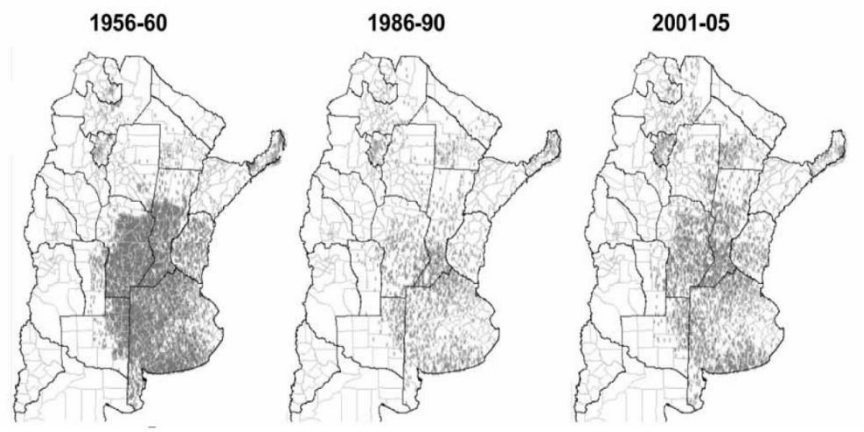

Figure 1.3. Estimated risk of contamination by pesticides in cultivable areas in the indicated years. The image was generously supplied by Viglizzo and Jobbágy (2010)

In the Humid Pampa, we are positioned at the interface of a historically, economically and socially relevant area of the country which is ready to undergo a productive expansion and transformation. In this context, there is a real need to include solutions that can help mitigate the environmental impact caused by the current practices of the agriculture industry. The controversy in the use of agrochemicals and its consequences on the environment and human health shall be addressed providing biotechnological tools which, once incorporated in the plot, allow the farmers to continue developing its activities without sacrificing earnings while, at the same time, being successful in the mitigation of the impact caused by the intensive use these products. Glyphosate is among the most used agrochemicals, exceeding the 60% of the total phytosanitary products used in the country.

1.2 Glyphosate

Glyphosate, also known under the commercial name of Roundup® is now widely used as an herbicide, but this was initially different. It took 20 years between the first synthesis of the compound, which served other purposes, and the recognition of its herbicidal actions. How did it develop into this well-known herbicide? Furthermore, how plants and microorganisms are affected when exposed to glyphosate? Which are the toxicological and environmental concerns regarding its use, especially in Argentina? The aim of this section is to review these topics specifically related to the history, management and consequences of glyphosate use.

1.2.1 Synthesis, formulations and physicochemical properties

The N-(phosphonomethyl)-glycine molecule was synthesized for the first time in 1950 by Dr. Henri Martin at the labs of the Swiss pharmaceutical company Cilag. The company was sold to Johnson & Johnson in 1959 and after a short overtake by Aldrich Chemical Company, the product glyphosate was transferred to the American Monsanto Company. At that time, the interest of the company was in developing chelating molecules based on phosphonates similar to aminomethylphosphonic acid (AMPA) that could work as water softeners. Afterwards, Monsanto began to test these molecules in relation to their potential herbicidal effects and that was when, for the first time, its herbicidal activity against perennial weeds was reported. The compound was resynthesized and then tested in greenhouse experiments. Towards 1971, Monsanto patented the compound (US patent 3799758) and towards 1974 it began to commercialize the Roundup®, the first and most successful glyphosate formulation (Dill et al., 2010).

Formulation is a word used to refer to the different commercial presentations of the product. They formed by glyphosate (active ingredient), surfactants and water. Glyphosate can be found conjugated with different cations (isopropylamine, ammonium, diammonium, dimethylammonium, potassium, sesquisodium and trimesium), and is incorporated in different proportions depending on the commercial product; formulas from 13.5 % to 95 % can be found. The most commonly used concentrations are 39.2 %, 48.0 % and 75.7 %.

Surfactants are tensioactive molecules, involved in favour the absorption of the herbicide in contact with the weeds. Generally, represent up to 15% of the formulation. The most used nonionic surfactant is the polyethoxylated tallow amine (and referred to in literature by its acronym, POEA), which is a formula derived from animal fatty acids mixed with long chain polyethoxylated alkylamines. The POEA acts in synergy with glyphosate increasing its toxicity with respect to the active principle alone. This fact of great importance has been taken into account in several ecotoxicological studies and is also taken into account in this thesis (Tsui & Chu, 2003).

Glyphosate effectiveness as herbicide can be confirmed in a wide range of species, according to Monsanto technical data sheet (Monsanto Company, 2004): 111 broadleaf weeds and annual grasses, including 62 perennial and 70 tree species.

According to its chemical structure, glyphosate is a weak acid consisting of a molecule formed by amino acid glycine, and to such amino group a phosphonomethyl group is joined (Figure 1.4). In its pure form, it is a crystalline, white, colorless and odorless solid. It is commonly synthesized as sodium, ammonium or isopropylamine salt.

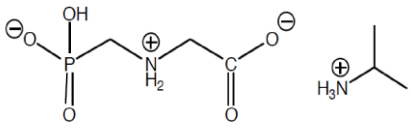

Figure 1.4. Chemical structure of the isopropylamine salt of N-(phosphonomethyl)-glycine.

It has an amphoteric nature, existing in 5 different zwitterionic structures with pKa values for its functional groups of <2, 2,6, 5,6 and 10,6, being stable to hydrolysis at pH range from 3 to 9 at 5 °C and 35 °C (FAO, 2001). Most of the environmental consequences produced by this molecule are related to its physicochemical properties, which are summarized in table 1.2. Glyphosate is barely soluble in organic solvents and highly soluble in water. The acid form is the least soluble. It has very low Henry's constant and vapor pressure values, consequently, volatilization has little relevance and is set aside in environmental

impact analyses (Franz et al., 1997). The logarithm of the octanol-water partition coefficient (log K_{ow}) shows, apart from its low hydrophobicity, a low theoretical bioaccumulation, and so all formulations necessarily require a surfactant as additive. The organic carbon adsorption coefficients (K_{oc}), that is, the measure of compound susceptibility to be adsorbed in the soil, present high variability and consequently, high differences regarding its mobility in soil.

Table 1.2. Physicochemical characteristics of different salts of glyphosate

Property	Molecule		
	Glyphosate (acid)	Glyphosate (isopropylamine salt)	Glyphosate (ammonium salt)
Melting point (°C)	184.5 (Decomposition begins at 187°C)	-	-
Density (g cm^{-3} at 20 °C)	1.704	-	-
Vapor pressure (mPa at 25 °C)	$1.31.10^{-2}$	$2.1.10^{-3}$	9.10^{-3}
Molecular weight (g mol^{-1})	169.07	228,19	186.11
Water solubility (mg L^{-1} and ~100% purity)	11600	786000	144000
K_H (atm m^3 mol^{-1})	$4.08.10^{-19}$	$6.27.10^{-27}$	$1,5.10^{-13}$
log Kow	<-3.2	-5.4	-5,32
Molar extinction coefficient (295 nm)	0.086 L $mol^{-1}cm^{-1}$	-	-
pH (1% solution in H_20)	2.5	-	-
K_{oc} (mL g^{-1})		300 - 20,100	

1.2.2 Effects of glyphosate in plants and microorganisms

1.2.2.1 Glyphosate mode of action in plants

Once glyphosate is absorbed by the aerial parts of the plant, it is translocated within the phloem to meristematic tissues, exerting it effects over structures that generally are not directly exposed to application, such as, rhizomes, stolons and bulbs. Once in the meristems, glyphosate inhibits the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Funke et al., 2006b) by blocking the substrate binding site of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EC 2.5.1.19). EPSP synthase catalyzes the reaction from shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate, the intermediate for the aromatic amino acids. The enzyme is part of the metabolic shikimate pathway (Maeda & Dudareva, 2012).

Glyphosate shows a large structural similarity with the natural substrate of the enzyme, the phosphoenolpyruvate derivative developed at the time of the transition state during the catalysis of the enzyme, the phosphenol pyruvate (PEP) oxonium (Schönbrunn et al., 2001), besides, the binding sites of

phosphoenolpyruvate and glyphosate overlap. However, and although the theory of the transition state analogue is the most accepted one, new evidence obtained would indicate that glyphosate could act as noncompetitive inhibitor with 5-enolpyruvylshikimate-3-phosphate, a fact that contradicts such hypothesis (Funke et al., 2006a). The inhibition of EPSP synthase as its sole mechanism of action, encouraged the development of tolerant enzyme through genetic engineering and transgenesis of crops.

1.2.2.2 Glyphosate tolerance in plant species

According to the Weed Science Society of America, (WSSA), the term "tolerance" is the inherent ability of a species to survive and reproduce after herbicide treatment, while "resistance" is the inherited ability of a plant to survive and reproduce following the exposure to a dose of herbicide which is normally lethal to the wild type plant. The term "resistance" is therefore reserved to processes that involve any kind of genetic selection or manipulation in a certain cultivar. In this chapter, the term "tolerance" will be adopted to refer to any mechanism providing a differential response to glyphosate, since the specialized bibliography usually reserves the term "resistance" to transgenic plants or to a genetic breeding selection along several generations (Nandula, 2010).

Mechanisms conferring glyphosate tolerance may be grouped in two general categories: tolerance related to the glyphosate binding site and tolerance not related to the glyphosate binding site.

Mechanisms related to the binding site respond to modifications that can be suffered by the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS, EC 2.5.1.19), the glyphosate molecular target, and include mutations that modify the enzyme affinity towards the herbicide and the increase of expression levels of the enzyme, either due to the increase in the number of gene copies or the overexpression of the single-copy (Dun et al., 2007; Healy-Fried et al., 2007b; Gaines et al., 2010; Gao et al., 2014).

The mechanisms not linked to the binding site are related to absorption, differential herbicide translocation, sequestration and exclusion processes in the different plant tissues (Sammons & Gaines, 2014).

When investigations of glyphosate tolerance in plants take place, the usual situation is that where certain population of a species survive a

recommended field herbicide spraying to which it is generally sensitive. It is also common to come across tolerance reports or studies in species that, throughout the years, show a predominance in areas where glyphosate is not directly applied, such as channels, ditches, borders, etc. (Vila-Aiub et al., 2011).

Under this traditional approach there are a large number of works that assess the glyphosate tolerance in weeds, conveniently classified by Shaner (2010). In this classification, it is possible to find different types of experiments, among which are: greenhouse and field assays; discriminatory doses assays; germination assays; assays based on the plant metabolism; *In vitro* assays; assays based on DNA studies of the plant species and, lastly, absorption and translocation in plant species using isotopically labeled glyphosate (^{14}C-glyphosate).

Even though that the previously presented experimentation is usually the order followed by authors, not all the experiments are performed in whole in a single work (Shaner, 2010). In general, reports on glyphosate-tolerant weeds begin with the acquisition of the germplasm and are followed by the performance of greenhouse assays using the plants in its vegetative stage. Greenhouse experiments consist in the spraying of one or several doses of herbicide under controlled conditions and then one or several response variables such as fresh weight, dry weight, visual damage, death rate, are measured and the values are compared with those obtained in the wild ecotype or reference cultivars. Afterwards, one or several metabolic assays are usually carried out, among which the widely prevails the measurement of the accumulation of shikimate in plant tissue as a response of the spraying. Shikimate is accumulated as a result of the inhibition of the enzyme EPSP synthase by glyphosate. Comments on this methodology can be found on sections 3.2 and 3.3 of this chapter. Lastly, subject to the lab resources and the hypothesis regarding the tolerance mechanism involved, the following assays can be performed: determination of the activity of enzyme EPSPS, DNA or mRNA extraction for later analysis of EPSPS sequence, or determination of ^{14}C-glyphosate percentage absorbed and/or translocated after the spraying of one drop of formulation in a leave of the plant to be assessed. The latter is usually considered the most complex in experimental terms both in design and in interpretation.

1.2.2.3 Transgenesis in plants

Although glyphosate was rapidly adopted as herbicide due to its effectiveness and versatility, by the end of the 90's, its demand increased drastically.

Based on the mode of action, transgenesis was adopted to breed *Glyphosate Resistant* (GR) crops. The first GR crop was Monsanto's Roundup Ready® (RR) soybean patented in 1996 in the United States of America and authorized in our country on March 25 of the same year (Padgette et al., 1996). This event was followed by the GR cotton, GR corn, GR rapeseed, GR alfalfa and GR sugar beet (Green, 2016).

Strategies for developing GR crops

The genetic engineering approach used two strategies for the development of GR crops: an insensitive EPSP synthase expression or the inactivation of glyphosate. The first strategy consists in the obtention of mutated EPSP synthases in order to attain a loss of affinity in the union of glyphosate to the enzyme (increase the inhibition constant, Ki) without modifying the union to phosphoenolpyruvate (Km constant). Although some mutations managed to achieve this goal, the isolation of a glyphosate tolerant *Agrobacterium* sp. CP4 from the effluent of an herbicide manufacturing company, turned out to be considerably better than any mutant developed so far. The first transgenic commercial event consisted in the insensitive CP4 EPSP synthase enzyme expression in soybean, which nowadays is part of different GR cultivars. However, there exist other available alternatives such as mEPSPS and 2mEPSPS, which are mutant EPSPS of *Zea mays* (Yadava et al., 2017).

The second strategy followed to obtain GR crops was carried out through the inactivation of glyphosate in the plant tissue, expressing glyphosate oxidoreductase (GOX) found in species of *Ochrobractrum* genus turning glyphosate into AMPA (Barry et al., 1992) or expressing the glyphosate acetyltransferase (GAT) (Castle et al., 2004), turning glyphosate into N-acetyl glyphosate. However, none of these strategies are commercially used *per se* (there exists a variety of rapeseed with two expression cassettes, one with the *cp4 epsps* gene and the other one with *gox* gene) (Que et al., 2010).

1.2.2.4 Glyphosate degradation by microorganisms

EPSP synthase is also present in bacteria and fungi. The information related to bacterial EPSP synthase inhibition and tolerance will be further discussed during chapter 5 of this thesis.

Glyphosate degradation is caused mainly by soil and water microorganisms. The rest of the typical degradation pathways for xenobiotics (such as photolysis or hydrolysis) have little relevance. Figure 1.5 shows the glyphosate degradation pathways in soil and water including the respective metabolites. The glyphosate decomposition by microorganisms is carried out via two main pathways according to which chemical group, the carboxylic acid or the phosphonate is hydrolyzed in the first time (Schuette, 1998; Székács & Darvas, 2012; Sviridov et al., 2015). The first is catalyzed by oxidoreductases and the second by by C-P lyases. As a consequence of the first pathway, AMPA metabolite is obtained, while as a consequence of the second, sarcosine and phosphate metabolites are obtained. These two events are simultaneous and happen both in soils and in water. The soil microbial activity and, therefore, the glyphosate degradation, shall depend on parameters such as temperature, relative moisture, aeration and microbial communities present (Stenrod et al., 2006). Chapters 4 and 5 of this thesis shall discuss the microbial degradation of glyphosate.

1.2.3 Glyphosate residues in soil and water

Glyphosate can reach the ground directly from the application by the spraying machine, as part of the water draining and drips from applied crops and weed, or within the plant tissue once the weed is controlled. Once in the ground, it moves to waterbodies. Glyphosate can also reach waterbodies when farmers wash the agricultural machinery or when improperly wash the containers.

1.2.3.1 Adsorption

Glyphosate adsorption in soil matrices mainly occurs in the mineral component. The clay and oxides content and the cation exchange capacity play a fundamental role in the dynamics of the herbicide in the matrix. The phosphonate group present in the molecule interacts with different minerals, forming complexes (Gimsing et al., 2007). The presence of cations Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+},

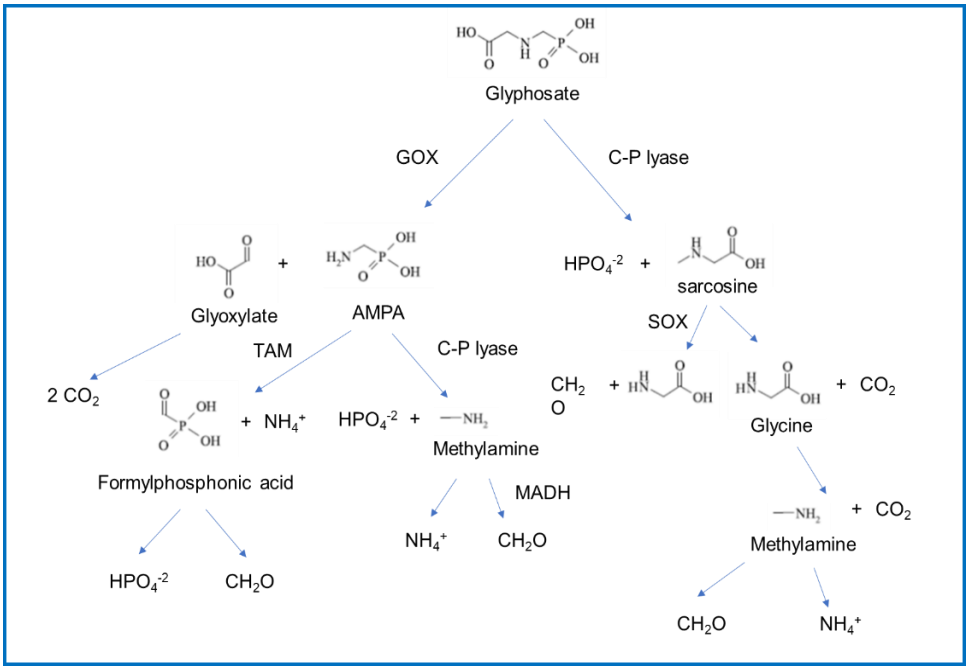

Figure 1.5. Glyphosate degradation pathways in soil and water. GOX: Glyphosate oxidoreductase, AMPA: Aminomethylphosphonic acid, SOX: Sarcosine oxidase, TAM: Transaminase, MADH: Methylamine dehydrogenase (amicyanin).

Fe^{3+} and Al^{3+} in clays like bentonite or montmorillonite, increase the capacity of glyphosate to form these complexes. The metals Fe^{3+} and Al^{3+} are the most relevant (Vereecken, 2005). The presence of amorphous aluminum and iron oxides has an even greater impact on clays adsorption, according to Gimsing and Borggaard (2002). In general, soils contain clays of different origins and different iron and aluminum oxides, so the final adsorption will be the result of the quantity and composition of those components; therefore, this characterization as a more appropriate indicator than the K_{oc} constant, a typical parameter used in molecule adsorption studies. The soil pH is also important, since it mediates the net charge of both glyphosate and oxides in soil; glyphosate adsorption is higher when the pH is low since it presents more favorable electrokinetic conditions (Day et al., 1997). Another two abiotic soil components usually discussed by the bibliography are the humic acids and the phosphates. Glyphosate can also be adsorbed to humic acids, being the macromolecular structure relevant (Piccolo et al., 1996). Phosphates compete with glyphosate for the same soil binding sites, and both the available phosphorus concentrations as well as the background on the use of

phosphates-based fertilizers should also be considered since they would reduce its adsorption (Gimsing et al., 2007).

1.2.3.2 Mobilization

The studies about glyphosate mobility in the soil try to explain under what conditions do leaching and runoff phenomena happen. These are the water movements that transport the agrochemical from the soil to underground and surface water sources, respectively. They are, therefore, studies of major environmental relevance. Vereecken (2005) also discusses this aspect, focusing on studies performed on soil columns and lysimeters at field scale. He highlights the importance of rains (in amount and time) and the formation of macroporous structures, cracks, heterogeneities and "funnel effects" in the soil profile since they cause preferential flows increasing the transport. These preferential flows, having a descending direction, are more relevant regarding runoff movement than leaching, and directly depend on the relative moisture of the plot. Studies show that there are no significant differences in terms of soil mobility and tillage management (Fomsgaard et al., 2003; Okada et al., 2016). As it was mentioned, glyphosate can interact with the soluble fraction of humic acids, a fact that has a strong impact on its mobility.

1.2.3.3 Persistence

The glyphosate persistence is given by the balance between the soil adsorption, the soil structure and the preferential flows which can provide further mobility and influence microbial activity. The combination of different causes, all of them complex to analyze *per se*, determine that the average life of glyphosate in different soils may vary from some days up to more than two years (Székács & Darvas, 2012).

1.2.3.4 Environmental fate of glyphosate and AMPA in waters and soil in Argentina

Adsorption studies of different pampean and extra pampean soils have been carried out in our country. Pessagno et al. (2008) carried out adsorption isotherms on aqueous suspensions of three different soils of the province of Santa Cruz. The results obtained indicate that glyphosate formed superficial complexes with different minerals (goethite, kaolinite, illite, montmorillonite); pH was

relevant since the adsorption was favored under low values. Maitre and collaborators (2008) performed adsorption studies in soils of Santa Fe (typical argiudol) and Entre Ríos (acuic argiudoll) provinces. In these studies, the authors report values of K_{oc} 516 for typical argiudoll and 631 for acuic argiudoll. These results would imply certain mobility of the molecule. A rapid herbicide adsorption in both soils is also reported, having differences between isopropylamine salt adsorption with respect to the acid form. On a study using ^{14}C-glyphosate, Rampoldi and collaborators (2014) studied the adsorption and desorption of glyphosate, its degradation and the influence of the plot management in three different soils of the province of Córdoba (typical argiudoll, typical haplustoll and entic haplustoll). Changes in the distribution of the adsorbed and soluble forms were observed depending on the soil, causing changes in the mineralization patterns (degradation). It was also observed that the degree of the extension of glyphosate adsorption and the desorption hysteresis was higher in soils under crop rotation than soils with monocultures. Okada and collaborators (2016) studied the adsorption and mobility of glyphosate under different tillage methods in three different soils, entic haplustoll in the province of Córdoba, acuic argiudoll in the province of Entre Ríos and typical argiudoll in the province of Buenos Aires. A strong soil adsorption was observed, depending on the clay content, the cation exchange capacity, the phosphorus concentration and the pH. The results of the study on vertical columns, showed that total leached glyphosate was 0.24%, with no differences among the different tillage methods.

Several glyphosate and AMPA monitoring studies were carried out in different locations, mainly in the Humid Pampa and driven by the increase in the use of this pesticide and changes in agricultural practices, in order to obtain relevant information to be used by environmental agencies. Studies were oriented to investigate three types of matrices: soil, surface waters and sediments and focused on the different mobility phenomena: leaching and runoff.

Probably the first report published correspond to Peruzzo et al. (2008). The presence of glyphosate in soil and in the water streams adjacent to a field sown with transgenic soybean was measured. Samples of surface water and sediments, both next to the field, 150 meters and 1500 meters downstream were analyzed. The sampling moments were performed during relevant events such as applications and rainfalls. The glyphosate concentrations measured in surface

waters varied between 0.10 and 0.70 mg L^{-1} and the higher values were obtained after rainfall; authors attribute the increase in glyphosate concentration to runoff phenomena. With respect to sediments, the greater concentrations were obtained after glyphosate application in water samples taken adjacent to the plot and 150 meters downstream. With rainfall, the concentration decreases, according to the authors, as a result of the increase in the flow. In soils, the values obtained were from 0.45 mg kg^{-1} (after the greatest rainfall event) to 4.45 mg kg^{-1} (after the application of 1.5 kg Ha^{-1} in the plot). In the same study, there were also measured samples of surface waters and sediments of other streams and rivers of the region, the Areco stream, the Arrecifes river, the Ramallo river and the *arroyo del Medio* stream.

A study performed by Aparicio et al. (2013), measured glyphosate and AMPA concentrations in 44 water courses and 16 agricultural production plots in 9 districts of the province of Buenos Aires. In this case, authors differentiated three different types of samples in water: the surface water, the sediments and the suspended particulate matter. In soils, glyphosate was detected in concentrations ranging from 35 to 1502 µg kg^{-1} and AMPA was detected in concentrations ranged from 299 to 2256 µg kg^{-1}. Sampling period ranged from 1 to 188 days following the application of the herbicide. The authors highlight the presence of glyphosate and AMPA in soils without any application (used as control). This may be given by drifts in the application and/or by the movement of soil particles from one field to another. With regard to analysis of water samples, glyphosate was detected in 35 % and AMPA in 33% in surface waters samples, with values ranging from 0.5 to 4.0 µg L^{-1} and 0.5 to 2.3 µg L^{-1} respectively. In suspended particulate matter samples, glyphosate was detected in 53% of the samples and AMPA in 16% with values ranging from 2.2 to 562.8 µg kg^{-1} and 4.0 to 118.7 µg kg^{--1} respectively. In sediments, glyphosate was detected in 66% of the samples and AMPA in 89% with values ranging from 5.7 to 221.2 µg kg^{-1} and 5.1 to 235.0 µg kg^{-1} respectively. This research concludes glyphosate is transported in the particulate matter through runoff: the plots that use glyphosate are the one that contribute it although there is no direct link between the plots and the aquifers.

A field experiment simulating rainfall 1, 5 and 8 days after the application of a glyphosate dose to plots with different treatments (combined or not with a

phosphorus fertilization) was designed to determine the amount of glyphosate and AMPA in the runoff water and the effect that the combined application of phosphorus could cause in the herbicide mobility (Sasal et al., 2015). An increase in glyphosate mobility was observed when glyphosate application was combined with phosphorus fertilization. It was also observed a minor amount of glyphosate and AMPA in runoff waters after repeated rainfall simulations. Glyphosate and AMPA were positive in control plots (with no application) and in the plot where only phosphorus was applied (a maximum of 74.1 μg L^{-1} for glyphosate and a maximum of 199.9 μg L^{-1} for AMPA), since plots used were subject to a glyphosate application 2 month prior to the test. Authors observed that 28 % of the glyphosate is lost in runoff due after a rainfall of 60 mm, 1 day after application when fertilization with phosphorus is also performed.

In a very complete report, 17 establishments were monitored by measuring glyphosate and AMPA in soils, in surface waters of adjacent streams (including suspended particulate matter and sediments), together with aquifers waters (Primost et al., 2017). Results show that, in soil, glyphosate and AMPA are positive in the 100% of the samples, with average values of 2,299 μg kg^{-1} and 4,204 μg kg^{-1} respectively. In aquifers, no analytes were detected. In surface waters, glyphosate was detected in 27% and AMPA in 55% of samples, with average values of 0.73 μg L^{-1} and 0.65 μg L^{-1} respectively, while in suspended matter, the detection considerably increased and reached 100% of samples, with average concentrations of 340.2 μg kg^{-1} for glyphosate and 223.2 μg kg^{-1} for AMPA. Lastly, the occurrence of analytes in sediments was also ubiquitous, with average values of 1,126 μg kg^{-1} for glyphosate and 2,260 μg kg^{-1} for AMPA.

Perhaps one of the most complex samplings procedure was performed by Ronco and collaborators (2016). Authors monitored glyphosate and AMPA concentrations during two seasons (2011 and 2012) in surface waters, sediments and particulate matter along the course of the Paraná river. Paraná river has a basin of 1,500,000 km^2, an average flow of 17,000 $m^3\ s^{-1}$, and transports the 118.7 million tons of suspended matter a year. Samples in 23 sites were collected, 20 of which corresponded to the confluences of the Paraná river with their main tributaries and to 3 areas of the main river course. Over the course of the two seasons, 15 % of the samples were positive for glyphosate in surface water, with an average value of 60.0 μg L^{-1}. AMPA was not detected in any of the cases. In

suspended particulate matter, the positive samples for glyphosate were 39 % and 9% for AMPA, with an average value of 0.1 and 0.04 μg L^{-1} respectively. The sediments analysis showed an average of 37 % of positive samples for glyphosate and 17 % for AMPA, with an average value of 742 and 521 μg kg^{-1} respectively. In the confluence with the Luján river, the highest concentrations were detected: 3,000 μg kg^{-1} for glyphosate and 521 μg kg^{-1} for AMPA. The study shows that the detection of analytes occurs in the mid and low part of the Paraná river, where outflows of the rivers coming from the Pampa region are most influent.

In the flooding Pampa region, Pérez et al (2017) performed studies in the El Crespo stream during the season 2014-2015. The authors collected samples in two sites over the course of the stream. One, within the area of influence of agricultural exploitation, with a 45 % of the crops were soybean, 17% sunflower, 15 % wheat, 10 % corn and the remaining 13 % other minor crops. The other, 45 km downstream, located in grasslands where no agricultural activity is being developed. From the 30 pesticides detected, glyphosate and AMPA were the most prevalent. The detection frequency was of 85 % in surface waters for both molecules, with an average value of 0.78 μg L^{-1} and a maximum of 2.9 μg L^{-1} for glyphosate, and an average value of 0.32 μg L^{-1} and a maximum of 2.0 μg L^{-1} for AMPA. In sediments, glyphosate reached 79 % positives and AMPA, 96 %, with an average value of 3.85 μg kg^{-1} and a maximum of 18.50 μg kg^{-1}, and an average value of 6.18 μg kg^{-1} and a maximum of 475.0 μg kg^{-1} respectively.

Table 1.3 and Figure 1.6 show a summary of the above-mentioned studies and other relevant studies in the country. Although monitoring studies have been performed in the whole Pampean region, the articles available regarding glyphosate and AMPA concentration in environmental samples are scarce considering our country. **The authors of these studies agree that runoff is the main cause of mobility of glyphosate from the plot to aquifers** being of considerable relevance the time elapsed between the application and the first rainfall. Analytes were more frequently detected in sediments and in suspended matter than in surface water.

Table 1.3. Glyphosate and AMPA monitoring studies carried out in Argentina.

Sampling date	Map reference	Region	Description of the sampling site	Matrix	Concentration		References
					Glyphosate	AMPA	
From 11-2003 to 03-2004		Humid Pampa - Rolling Pampas	Areco-Arrecifes rivers,in the province of Buenos Aires. Samples taken adjacent and downsetram to a transgenic soybean field	Surface water	100 - 700 µg L^{-1}		*Peruzzo et al., 2008*
				Sediments	<LD - 4,900 µg Kg^{-1}		
				Soil	530 - 4,450 µg Kg^{-1}		
			Arrecifes river - Buenos Aires Province	Surface water	<LD - 280 µg L^{-1}		
				Sediments	<LD - 1,380 µg Kg^{-1}		
			Ramallo River - Buenos Aires Province	Surface water	160- 560 µg L^{-1}		
				Sediments	<LD - 1,850 µg Kg^{-1}		
			Arroyo Areco stream- Buenos Aires Province	Surface water	230 - 330 µg L^{-1}		
				Sediments	ND		
			Arroyo del Medio stream - Buenos Aires Province	Surface water	100 - 370 µg L^{-1}		
				Sediments	ND		
From 07-2011 to 07-2012		Mesopotamic region - Paraná Basin	23 sample sites in the Paraná river	Surface water	0.6 µg L^{-1}	ND	*Ronco et al., 2016*
				Sediments	742 µg Kg^{-1}	521 µg Kg^{-1}	
				SPM	0.1 µg L^{-1}	0.04 µg L^{-1}	
From 02-2012 to 03-2012		Humid Pampa - Mesopotamic Pampas	17 farms in the province of Entre Ríos	Surface water	0.73 µg L^{-1}	0.65 µg L^{-1}	*Primost et al., 2017*
				Sediments	1,126 µg kg^{-1}	2,260 µg kg^{-1}	
				SPM	340.2 µg kg^{-1}	223.2 µg kg^{-1}	
				Soil	2,299 µg kg^{-1}	4,204 µg kg^{-1}	
				Underground water	ND	ND	
From 04-2012 to 09-2012		Humid Pampa - Southern Pampas	South-east counties in the Buenos Aires province(Tres Arroyos, Adolfo Gonzales Chaves, San Cayetano, Necochea, Lobería, General Alvarado, General Pueyrredon, Balcarce y Mar Chiquita)	Surface water	0.5 - 4.0 µg L^{-1}	0.5 - 2.3 µg L^{-1}	*Aparicio et al., 2013*
				Sediments	5.7 - 221.2 µg kg^{-1}	5.1 - 235.0 µg kg^{-1}	
				MSP	2.2 - 562.8 µg kg^{-1}	4.0 - 118.7 µg kg^{-1}	
				Soil	35 - 1,502 µg kg^{-1}	299 - 2,256 µg kg^{-1}	
11-2012		Humid Pampa - Mesopotamic Pampas	Paraná Experimental farm in the Entre Rios Province	Runoff water 1 DPA (GL)	767.2 µg L^{-1}	561.5 µg L^{-1}	*Sasal et al., 2015*
				Runoff water 1 DPA (GL+P)	1417.6 µg L^{-1}	453.7 µg L^{-1}	
				Runoff water 5 DPA (GL)	168.8 µg L^{-1}	127.7 µg L^{-1}	
				Runoff water 5 DPA (GL+P)	277.9 µg L^{-1}	478.4 µg L^{-1}	
				Runoff water 8 DPA (GL)	65.5 µg L^{-1}	262.2 µg L^{-1}	
				Runoff water 8 DPA (GL+P)	133.1 µg L^{-1}	543,3 µg L^{-1}	
From 06-201 to 01-2013		Humid Pampa - Southern Pampas	Four sites next to a farm using glyphosate.Quequén river, Buenos Aires province	Surface water	<LC	0.5 µg L^{-1}	*Lupi et al., 2017*
				Sediments	3 – 14 µg kg^{-1}	2 – 13 µg kg^{-1}	
				Soil	6.5 – 245.9 µg kg^{-1}	5.6 – 291.2 µg kg^{-1}	
From 10-2014 to 10-2015		Humid Pampa - Flooding Pampas	El Crespo stream in the province of Buenos Aires	Surface water	0.78 µg L^{-1}	0.32 µg L^{-1}	*Pérez et al., 2017*
				Sediments	3.85 µg kg^{-1}	6.18 µg kg-1	
11-2015		Humid Pampa - Inland Pampas	A farm belonging to University of La Pampa Agriculture School, in La Pampa province	Soil	1 – 3 µg kg^{-1}	80 – 150 µg kg^{-1}	*Mendez et al., 2017*
				Breathable fraction particles of soil	11.0 – 19.5 µg kg^{-1}	520 – 750 µg kg^{-1}	
From 08-2015 to 01-2016		Humid Pampa - Rolling Pampas	5 sites in Carnaval stream, Buenos Aires Province	Sediments 1rst sampling period	11.0 – 98.9 µg kg^{-1}	18.0 – 54.2 µg kg^{-1}	*Mac Loughlin et al., 2017*
				Sediments 2nd sampling period	211.5 – 404.4 µg kg^{-1}	86.2–224.9 µg kg-1	

ND: Not determined SPM: Solid particulate matter

Figure 1.6. Glyphosate and AMPA monitoring studies carried out in Argentina. Each color corresponds to a reference indicated in table 1.4.

1.2.4 Glyphosate effects on fauna and human health

1.2.4.1 Micro, meso- and macrofauna

In waterbodies, glyphosate exercises toxic effects over different organisms. Since glyphosate is an herbicide, the most sensitive aquatic species are algae and aquatic plants. For instance, microalgae treated with the glyphosate (acid form) , and a formulation, showed sensibilities ranging from EC_{50} of 0.68 mg L^{-1} to 600 mg L^{-1} (Pérez et al., 2007). Micro and macrocosms studies showed significant effects using doses between 10 µg L^{-1} and 8,000 µg L^{-1}, both in the number of species as in the relative abundance of phytoplankton (Pérez et al., 2007; Pesce et al., 2009; Stachowski-Haberkorn et al., 2008; Vera et al., 2010). The macrophyte *Lemma minor* also showed sensibility to formulations (Cedergreen & Streibig, 2005). Cyanobacteria have shown resistance to glyphosate due to the effect of eutrophication produced by the contribution of phosphorus provided by the pesticide (Annett et al., 2014). It has been demonstrated than bacteria, protozoa and invertebrates and vertebrates are affected as well (Bonnet et al., 2007; Cauble & Wagner, 2005; Contardo-Jara et al., 2009; Everett & Dickerson, 2003; Pérez et al., 2007). In general, it is also observed that formulations are more toxic than the active ingredient *per se*. The toxic effects (either lethality or growth inhibition) exercised by the different formulations are related in many cases to the concentration levels in waterbodies and runoff waters in our country. Organisms are generally studied in an in micro or mesocosms, where no sediments are present.

In soil, glyphosate also showed toxicity at different trophic levels. The impact on bacteria will be discussed further in chapters 4 and 5 of this thesis, where the study will be addressed at two levels: working with bacterial communities and working with isolated strains. There are reports where it is observed that the use of glyphosate stimulates the development of certain fungi species (some of them pathogenic) like *Fusarium solani, Pithyum ultimum, Corynespora cassicola and Sclerotinia sclerotiorum* (Kawate et al., 1997; Johal & Huber, 2009), as well as other species studied at a community level (Anza et al., 2016). A negative effect in growth was observed in arbuscular mycorrhiza exposed to glyphosate (Druille et al., 2013). Certain alterations in growth development were reported in the nematodes *Caenorhabditis elegans,*

Eisenia fétida, *Lumbricus terrestris* and *Lumbriculus variegatus* (Contardo-Jara et al., 2009b; Gaupp-Berghausen et al., 2015; McVey et al., 2016; Verrell & Van Buskirk, 2004; Wang et al., 2017) and the arthropods *Alpaida veniliae*, *Pardosa milvina, Chrysoperla externa, Eriopis connexa and Apis mellifera* (Schneider et al., 2009; Benamú et al., 2010; Mirande et al., 2010; Griesinger et al., 2011; Helmer et al., 2015).

1.2.4.2 Impact of glyphosate on human health

There is a wide range of literature reports regarding toxicity tests in mammals, epidemiological studies and monitoring in humans aimed to understand the toxicity glyphosate has in humans.

The lethal dose 50 values (LD50) in rat (4,373 mg Kg^{-1}) and rabbit (4,373 mg Kg^{-1}) administered orally (Denton, 2007) indicate a low risk level of acute toxicity. A studied performed in Sri Lanka between 2002 and 2007 (Roberts et al., 2010), analyzed 601 patients who had ingested different types of formulations and quantities of glyphosate. Among them, 166 were poisoned but reported no symptoms (average intake of 50 mL), 383 had minor symptoms such as nausea, vomits, abdominal pain and sedation (average intake of 58 mL), 33 suffered moderate to severe symptoms with signs of high blood pressure, oliguria, respiratory and/or cardiac arrest, ventricular dysrhythmias, sedation or coma and seizures (average intake of 53 mL) and finally, 19 individuals suffered a fatal poisoning (average intake of 200 mL). It was found a weak correlation between mortality and age, and the intake and the glyphosate concentration in plasma at the arriving time in the hospital (> 734 µg mL^{-1}).

In a review published by Meyers et al. (2016), whose colleagues are experts in environmental and health sciences, the most relevant associations between chronic exposure to glyphosate and different pathologies are mentioned. Among the most relevant chronic adverse effects are: glyphosate causes oxidative damage in liver and kidney in rat since it alters the mitochondrial metabolism at the exposure levels currently considered acceptable for regulatory agencies. An increased incidence of acute chronic liver disease in rural workers from regions where glyphosate is widely used is also pointed out. Glyphosate and its formulations work as in-vitro endocrine disruptor, including several steroid hormones which are relevant in vertebrates (Benachour et al., 2007; Romano et

al., 2010). In rat, a sublethal dose of a formulation implied an impairment of the reproductive development. Chronic intake can alter the gastrointestinal microbiome in vertebrates. The soybean used as food for poultry and pigs, which has high residual glyphosate values, is associated with gastrointestinal and congenital problems. Finally, the use of formulations is related to an increase incidence of Hodgkin lymphoma.

The evidence of occurrence of Hodgkin lymphoma and the use of glyphosate formulations led to a reassessment of its carcinogenicity potential. Consequently, it acquired a "2A" category which means that it is a probably carcinogenic to humans (International Agency for Research on Cancer, 2014; Guyton et al., 2015)

1.3 Bioremediation

1.3.1 Strategies for remediation of soils polluted by agrochemicals

Pollutants arrive to the environment as a result of different anthropogenic activities, whether as a consequence of accidents (leaks during transport, improper storage of products, container leaks, etc.) or as an implicit consequence of the activity (industrial production, effluents discharge, etc.). In all of these cases, there is a need to recover the polluted soil in order to avoid the risks that pollutants can cause in the human health and in the environment. Several remediation strategies have been developed in response to these pollution events. These strategies consider the nature and origin of the pollutant, the extension of the pollution, the urgency in removing the pollutant, the environmental regulations, the costs and the possibility of performing *in-situ* or *ex-situ* processes. Depending on the mechanism involved in the xenobiotic removal practice, several methods either physical, chemical and biological or their combination can be distinguished (Lim et al., 2016).

Among the physical and chemical methods there are: thermal desorption, incineration, microwave thermal treatment, solvent and surfactant extraction, vapor extraction, ultrasonic treatment, chemical and electrokinetic oxidations, etc. These treatments are carried out both *in-situ* and *ex-situ* and have been proved to be successful against several xenobiotics. However, they have serious disadvantages, which are the destruction of the soil structure together with the native microbial communities and fauna. In many cases, pollutants remains deeply adsorbed, and soils can´t be completely (Gan et al., 2009; Concetta Tomei & Daugulis, 2013).

Biological methods developed for *in-situ* removal of xenobiotics in soil are based on the use of microorganisms (bacteria and/or fungi), or the use of vascular plants.

Solving the problem of soil pollution caused by pesticides in the Argentine Republic implies an inapplicability of physical and chemical methods, either due to the extension of the pollution, the costs associated to it applications, the continuous use of pollutants and the protection of the resource itself (soil). That is why we will focus on the biological remediation methods.

1.3.2 Bioremediation

Bioremediation is the process by which the toxicity, the mobility or the concentration of a pollutant in a matrix is reduced using biological systems, mainly microorganisms (Ritter & Scarborough, 1995; Chen et al., 2015; Singh et al., 2016). In this case, the microorganisms will breakdown the contaminant using different metabolic pathways. This is possible either by aerobic or anaerobic microorganisms.

The efficiency of these processes depends on the properties of the microorganisms within the polluted environment and on the characteristics of the pollutant to be remedied. The process will be more efficient if the microorganisms present contain a suitable gene supplement, if they are metabolically dynamic and resilient, if they can uptake the xenobiotic, if they are adequately tolerant and if they have properties that facilitate biodegradation, such as the production of surfactants that makes the pollutant more bioavailable, and chemotaxis, that helps the approach to the contaminant. The bioavailability of the pollutant in the matrix will depend on the adsorption/absorption to the solid phase. At the same time, environmental factors like pH, moisture, oxygen pressure, hydraulic conditions and oxide-reduction potential will highly influence the metabolic capacity of the microorganisms and the interactions between the pollutant and the soil (Gkorezis et al., 2016).

1.3.2.1 Bioremediation strategies

The *in-situ* reported bioremediation processes consist in three types of approaches: natural attenuation, bioaugmentation and biostimulation.

The natural attenuation refer to all physical, chemical or biological processes that, under favorable conditions, work with no human intervention to reduce the concentration of pollutants and turn them into less toxic or innocuous products, whether in soils and in surface waters (Khan et al., 2004). The approach consists in the monitoring of the contaminated place. It is valid if: i) the pollutant breakdown is carried out mainly by the microbial communities present in contaminated site; ii) the site does not require to be urgently cleaned; iii) the natural attenuation take place in an acceptable period of time and iv) the extent of the contamination has been accurately determined. Natural attenuation has

been applied in long chain aliphatic hydrocarbons and aromatic hydrocarbons remediation (ICCS, 2006), while is not the best option for persistent organic pollutants (Megharaj & Naidu, 2017).

A bioaugmentation process implies the introduction of specific degrading microorganisms in the polluted site. This is necessary when the native microorganisms present in the contaminated place are unable to degrade the pollutant, at least in the desired period of time. The microorganisms used are previously isolated and cultivated and possess the enzymatic metabolic pathways involved in the degradation of the contaminant. The selected strain or consortium (culture composed by two or more microbial species) will ideally have a high pollutant degradation rate, a fast grow and easy cultivation, will be tolerant to the observed contaminant concentrations will be capable of adapting and subsisting once incorporated into the contaminated site (Min et al., 2017). The strain can be obtained from the contaminated site itself or from a different site; however, the succeed in survival and proliferation of exogenous microorganisms is generally difficult. That is why most bioaugmentation strategies are performed with autochthonous microorganisms, combining the degradation capacity with the adaptation to the environment. Bioaugmentation was attempted to clean-up soils contaminated with chlorinated volatile compounds, diesel and heavy metals, DDT, lindane, endosulfan, pentachlorophenol (PCP), polyaromatic hydrocarbons and total petroleum hydrocarbons (Alisi et al., 2009; Abhilash et al., 2011; Saez et al., 2014; Wang et al., 2014; Chen et al., 2015; Kuppusamy et al., 2016).

Biostimulation comprises all the processes aimed at generating a propitious environment through the addition of additives, in order to stimulate the metabolic activity towards the pollutant in the microbial communities present in polluted sites. In this case, the native microbial community has an intrinsic degrading capacity, but natural degradation does not happen, or occurs at a very low speed. Biostimulation includes the addition of essential nutrients such as nitrogen, phosphorus and potassium, as well as other animal fertilizers, rice straw, domestic wastewater, carbon and crop residues. Surfactants and electron acceptors (O_2 and Fe (III)) are considered as well (Ruberto et al., 2009; Gkorezis et al., 2016). Examples of these processes are the reduction of perchlorate (Waller et al., 2004) and several petroleum-based hydrocarbons such as diesel, pyrene and phenanthrene (Gkorezis et al., 2016).

The main advantages of bioremediation arise when this type of process is compared to the conventional physical and chemical methods. This is given, partly because their application is generally *in-situ* and partly because they require less logistics and inputs complexity. Bioremediation is considered environment-friendly since it maintains the soil properties according to the method proposed. In theory, it is applicable to a large variety of pollutants, among them pesticides. However, many of such works have not been performed at field scale (Chen et al., 2015; Lalevic et al., 2016; Lim et al., 2016; Singh et al., 2016; Sun et al., 2018).

Among its limitations, it can be mentioned that, since it is a biological process, the pollutant may not be degraded completely, and metabolites can end up being more toxic than the initial compound under certain conditions. They are generally slower than the physical and chemical remediation processes and sometimes, they are not an option if the contaminated site needs to be urgently intervened. Site-specific and therefore each process will be, to a greater or lesser extent, optimized in terms of the matrix, the weather and other diverse factors corresponding to the site to be remediated.

1.3.2 Phytoremediation

Phytoremediation is the use of plants and its associated microorganisms for the removal, degradation or stabilization of pollutants in water, soils and sediments. It consists in a set of technologies that use natural processes by which plants are capable of degrading, transforming, sequestering, or immobilizing the pollutant, which can have organic or inorganic nature.

Phytoremediation is a booming technology that is being used by public and private sectors because it is an innovating, environment-friendly and affordable alternative of remediation compared to other soil remediation technologies.

Among the main advantages of these technologies are:

- It is applicable to a large extension of polluted environments.
- It can be used in parallel to other technologies.
- It is widely accepted since it is environmentally friendly and gives aesthetic value.

- It is simple and easy to apply.
- Its *in-situ* implementation helps reducing the costs and risks of exposure to the pollutant.
- Since plants generally have to be implanted, it improves the soil properties such as structure, fertility, organic matter and reduces water and wind erosion.
- It does not require large operating costs.

- Among its disadvantages are:
- Processes with longer treatment periods when compared to physical and chemical methods.
- The depth reached by the root will be the limiting of the extent of the phytoremediation process.
- The soil properties and weather must also be considered when choosing the proper plant species.
- The process is usually limited to the fraction of bioavailable pollutant.
- There is a possibility of contamination of the food chain.
- It is used for low pollution concentration levels and where pollution is superficial.

1.3.2.1 Phytoremediation strategies

A proper phytoremediation process must consider a large number of variables. The physicochemical properties of the pollutant, the persistence in the environmental matrix, the weather, the different land management and the xenobiotic concentrations have a major relevance. It is, certainly, of high importance the choice of a plant species and the further characterization in terms of the chosen phytoremediation method.

Overall, five different methods of phytoremediation processes can be distinguished; the classification is proposed according to the fate of the contaminant and to the compartment in which the process take place, being: Phytotransformation, Phytostabilization, Phytovolatilization, Phytoextraction and Rhizoremediation.

Phytotransformation

During phytotransformation, the plant uptake the pollutant of organic nature and metabolizes it due to specific enzymatic pathways. The enzymatic machinery or, as it was called by Sandermann (1992) the "green liver", can partially or completely degrade the molecule. If degradation is partial, the resulting metabolites will remain associated to the plant tissue, either sequestered in vacuoles or in the cell wall.

Once implantation of the plant species is assured, the pollutant needs to be uptake and then metabolized. The hydrophobic substances would be adsorbed to the organic fraction of the soil. Therefore, the uptake of the xenobiotic will depend on its bioavailability (related to the chemical structure and to the matrix), as well as on the root extension and structure. The anthropogenic nature of the organic compounds implies that, as a general rule, there are no specific transporters in the cell wall and consequently the uptake is carried out by diffusion through the membrane. The pollutant hydrophobicity, based on the octanol-water (K_{ow}) coefficient, largely determine the entrance and further transport via xylem. According to Reichenhauer & Germida (2008) the substances with a $\log K_{ow}$ between 0.5 and 3.0 are the ones with the better uptake. Those which are very hydrophilic, with a $\log K_{ow}$ lower tan 0.5, can be transported within the plant but cannot cross the plasmatic lipid bilayer. Highly hydrophobic substances with $\log K_{ow}$ higher than 3.0 are kept within the plasmatic membrane and become less available to the enzymes of the cell.

Once in the plant cell, the metabolization of the contaminant takes place in three phases: Phase I or transformation phase where oxidations, reductions and hydrolysis occur, leading the molecule to become more polar and, therefore, more hydrophilic. Enzymes like the superfamily of hemoprotein cytochromes P450, dehalogenases, peroxidases, carboxylesterases, etc. take part in the process. Afterwards, follows phase II or conjugation reactions: most of the metabolites are conjugated with glutathione molecules, UDP-glucose and other activated dinucleotides, amino acids or sugars. In phase III or compartmentalization phase, the molecule is stored in plant cell vacuoles or apoplasts or incorporated into the cell wall through lignification.

Several phytotransformation processes have been carried out during herbicide remediation through the use of highly adaptable cultivars or plants coming from the original ecosystem. Among the most used plants are *Cucurbita*

pepo, Zea mays, Lolium multiflorum, Plantago major, Typha latifolia and lemna minor, among others.

Phytoextraction

Phytoextraction uses the ability of certain plant species to accumulate one or several metals within its tissue by extracting them from the soil through the root (Maric et al., 2013). Plants must be harvested once the growing stage is completed, since the pollutant (mainly heavy metals and radionuclides) cannot be transformed nor degraded. The following traits should be taken into account when selected a plant species for a phytoextraction process: i) Must tolerate the concentrations of the contaminant in the contaminated site ii) Present the highest growth rate, maintaining a high degree of efficiency in the accumulation of the contaminant among the process iii) The contaminant should be accumulated in the aerial parts and iv) Must be an easy to harvest species, since it will be necessarily harvested after the process (Ghori et al., 2015; Truu et al., 2015).

Phytostabilization

Phytostabilization implies the stabilization of the xenobiotic reducing its bioavailability through the use of plant species and, when the case needs it, additives -amendments- of different chemical nature (Vangronsveld et al., 2009). The pollutants treated in these processes are from inorganic origin. Additives that enable the stabilization of the different contaminant are from different nature: hydroxyapatite, phosphates, trace elements (Fe and Mn), organic materials such as biosolids, sludge or compost, zeolites, ashes, etc. (Adriano et al., 2004). By establishing a plant coverage in the contaminated soil, the mobilization of the pollutant through leaching or runoff, or the mere wind action is avoided. Afterwards, the field can be treated *in tandem* with another remediation process to remove the pollutant metal.

Phytovolatilization

Phytovolatilization is the uptake of the pollutant by the plant species and later released into the atmosphere by evapotranspiration. In the atmosphere, contaminants are rapidly degraded by the action of UV radiation and atmospheric

oxygen, or simply diluted. Contaminants can be released in the same form as it was removed from the contaminated place or can be partially metabolized. This strategy is used when remediation of volatile organic pollutants is intended. Phytovolatilization is also involved in increasing the transport of the contaminant from deep to surface layers of the soil as a result of the water pumping by the roots (Limmer & Burken, 2016).

Rhizoremediation

Rhizoremediation is the degradation of the pollutant through microorganisms present in the rhizosphere. The rhizosphere is the soil volume under the influence of the plant root system where there is an increase of the number of microorganisms of several orders of magnitude when compared to the bulk soil. This phenomenon, known as "rhizosphere effect" (Hartmann et al., 2008; Hussain et al., 2009), is caused by the physical and chemical stimulation produced by the plant in the root-influence zone. The most important factors related to this effect are:

- An increase in oxygen pressure, porosity and permeability in the soil near the roots, ensuring the occurrence of aerobic reactions.
- Release of root exudates which are a set of organic compounds derived from the photosynthesis of aerial parts among which can be mentioned: carbohydrates (glucose, fructose, sucrose, maltose and oligosaccharides); amino acids (glycine, glutamic acid, serine, lysine and arginine); aromatic compounds (phenols and isoprenoids; organic acids such as acetic, propionic, citric, butyric and malic acid); volatile compounds (ethanol, methanol, formaldehyde, acetone, acetaldehyde and propionaldehyde); vitamins (thiamin, biotin, niacin, riboflavin, pyridoxine and pantothenic acid); and enzymes (phosphatases, dehydrogenases, peroxidases, dehalogenases, nitro-reductases, laccases and nitrilases). The exudates, together with the mucigel of the roots and the cell debris, generate an environment with a large availability of nutrients for the heterotrophic microbial communities inhabiting the rhizosphere causing an increase of metabolic activity as well (Schnoor, 2002; Gerhardt et al., 2009; Glick, 2010; Segura & Ramos, 2013).
- Among the released molecules are secondary metabolites which are capable of inducing enzymatic pathways related to the metabolization of the pollutant

in the microorganisms, or directly capable of stimulating metabolic pathways acting as cometabolites (Segura et al., 2009).

- In turn, microbial communities can have a beneficial effect on the plant, producing a series of compounds such as enzymes, hormones, organic acids and molecules that produce the solubilization of different nutrients. The result is the increase of the root area, an improvement in the bioavailability of nutrients and protection against phytopathogenic microorganisms.

The rhizoremediation process is a combination of bioremediation and phytoremediation in which the plant species act as a platform for the microorganisms responsible for the degradation of the contaminant. This may occur naturally or through a process by which the degrading bacteria and/or fungi are firstly isolated and then inoculated in the plant. Literature related to pesticides rhizoremediation processes can be found (Eevers et al., 2017).

1.3.3 A glyphosate rhizoremediation strategy suitable of being used in the Argentinean Humid Pampa

When taking into consideration the vast extension of the Humid Pampa, the need to preserve soil texture and the quality of the cropland, phytoremediation technologies arise as possible solutions to be explored, given that they are of in-situ implementation, low cost of application and maintenance, socially well accepted, easy to manage by agricultural producers and could bring a series of additional benefits to the farm plot.

Given the physical and chemical glyphosate characteristics, which rend it to be retained in soil clay; and given the fact that its barely metabolized by plants (and that in addition it does not tend to pass through cell walls), **rhizoremediation strategies are the most suitable to assess as a possible application**.

The prevalent agricultural production model in the Humid Pampa region is currently based on the employment of crops that require glyphosate for their management. Prairie´s reigning geography make it possible to distinguish, small knolls with little slopes and their corresponding plain button areas, where rainfall waters run off and surface water bodies, such as channels, streams and rivers are located.

This landscape, although it is often imperceptible, is certainly significant, since the fertility of the bottom plains is lower due to the presence of hydromorphism, high proportion of soil sodium ions, and salinity phenomena, which directly affect the implantation and yield of the crop. That is why the farmer, often circumscribes the crop sowing to knolls, assigning the use of the bottoms to livestock.

The need of not interfering with the main agricultural usage means that the glyphosate mitigation strategy limits the employment of the rhizoremediation system to, at least, **two places within the agricultural plot**.

The one, between the knoll and the bottom plain, is proposed as **a barrier against the runoff** coming from the knoll, ideally preventing the pesticide carried in it reaches the surface water bodies.

The second, in the knoll, proposes the application of the remediation system through **inter-row sowing** together with the main crop. In this way, applying the tolerant plant after chemical fallow, would cause the remnant glyphosate in soil being stabilized in the root-influence zone, enhancing the degradation and avoiding vertical and horizontal migration of the molecule.

These two approaches will be considered as strategies when thinking about the use of the rhizoremediation system to be developed throughout this thesis work.

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Chapter 2 – Objectives, rationale and thesis outline

2.1 Objectives, rationale and thesis outline

Phytoremediation has been studied since the lasts decades of the twentieth century and received increasing attention over the last years. Triggered by the large-scale glyphosate contamination problem in Argentina, the know-how of environmental remediation both Institutions have, the focus of this work was on the applicability of rhizoremediation to degrade glyphosate in soils. This thesis aimed at clarifying the opportunities where rhizoremediation technologies can help to solve the challenges of glyphosate pollution in Humid Pampa soils in Argentina under intensive agricultural exploitation as discussed in the background.

To optimize the installation of phytoremediation in the field and remove the contaminant from soils, it is of utmost importance that the best candidate phytoremediation plant species is selected. The plant must present a high tolerance to the herbicide and allows a dense colonization by degrading microorganisms in the rhizosphere. In addition, the plant is preferably of productive value for the farmers in order to not interfere with revenues of the estate. In **Chapter 3**, a systematic search for different crop species was performed and assessed for their glyphosate tolerance. It has been hypothesized that inoculation of the rhizosphere with beneficial glyphosate tolerant microbiota will enhance the plant's phytoremediation potential. In **Chapter 4**, the isolation of glyphosate tolerant and degrading bacteria was performed, besides the physicochemical and microbiological characterization of the sampling sites. In **Chapter 5** the phenotypic and genotypic characterization of the isolated tolerant and glyphosate degrading bacteria was performed. The strains were assessed for their plant growth promoting (PGP) capacities and colonization of the roots of the candidate plant species. Following from the previous, to maximize the pesticide's degradation and go straightforward to the field application, it is convenient to rely on one or several sequenced genomes of glyphosate tolerant bacteria, capable of establishing themselves in the root of the tolerant plant. The availability of the genetic potential of these microorganisms will enforce the strategy's probability of success. The genome sequencing of strains which arose as most interesting were sequenced using Ion Torrent® platform and posterior bioinformatic analysis was performed. Up till now only lab tests were performed to degrade glyphosate and strains showing *in vitro* PGP potential. To demonstrate its efficacy, the

experiments need to be upscaled using agricultural soil from the field. **Chapter 6** was focused on the exploration of different experimental systems as the initial steps in understanding the plant-microbe associations and the suitability of the rhizoremediation method. In **Chapter 7**, a general discussion on the combined work, together with some future perspectives for further research is presented.

As it was proposed for the realization of this project, the following specific objectives were accomplished:

1. Systematic search of plant species of productive value to be assessed in regards to their glyphosate tolerance.
2. Development and implementation of methodologies to evaluate root and leaf level tolerance in the plant species.
3. Optimization of an analytical method to measure shikimic acid in vegetal tissues, as a glyphosate exposure biomarker.
4. Isolation and phenotypic and genotypic characterization of glyphosate tolerant and degrading bacteria.
5. Assessment of plant growth promoting (PGP) abilities of the microorganisms obtained in point 4.
6. Assessment of the isolated degrading strains' capacities to colonize the roots of the candidate plant species.
7. Genome sequencing of the strain or strains which arise as most interesting with the Ion Torrent® platform and posterior bioinformatic analysis.
8. Evaluation of biodegradation efficiency of different rhizoremediation systems proposed by microcosm assays.

Chapter 3 - Glyphosate tolerance assessment in plant species of productive value

3.1 Introduction

When considering the most relevant factors to carry out glyphosate rhizoremediation strategies in the Humid Pampa, the following arises as the most relevant:

The plant species to be chosen shall not interfere with the main productive activity of the plot and therefore it shall not occupy productive soils in case of plots engaged in agricultural activity. It shall represent a proper and attractive option for cattle grazing in case of plots engaged in mixed activities (farming and livestock production). The most logical choice are grazing pastures, which are capable of being planted in intercropping sowings or in plot borders (when they have main crops) as well. In this way, there is no interference with the main economic activities and the species can be an additional source of economic return since the implanted pasture might potentially be a nutritional source for cattle either directly (through cattle grazing) or indirectly (through hay).

For such purposes, there shall be used preferably fodders which are commercially available for the Humid Pampas since this would sort out, in logistical terms, a large obstacle in the planning of the rhizoremediation strategy in such a large territory, since it would require a large availability, storage and proper distribution of the germplasm. The introduction of exotic plant species is therefore not taken into account, since it requires the compliance with a large number of regulations and due to the unwanted ecosystem effects, that they usually cause at the time of establishment.

The plant species shall promote the presence and establishment of the degrading microorganism, consortium or biosystem within the environment of its rhizosphere through the secretion of proper root exudates, being variable according to the plant species. It shall present a large root biomass covering horizons 0 and A, since it has been mostly determined that glyphosate is mainly retained, due to its adsorption to organic matter and clay particles present in these layers. It shall also have a good coverage, which means a good amount of green matter for cattle grazing and shall also imply a better root density.

Besides, plant selection process must consider those requirements proposed by the United States Environmental Pollution Agency (EPA, 2000). It must be a species previously reported as being capable of metabolizing or tolerating the pollutant to be remedied, easy to implant, with a large production

of biomass (specially root biomass) and are easy to handle and reproduce. The use of native species, preferably those with productive value, such as crops or fodder, which are commercially available and in a sufficient amount to comply with the requirements of the remediation protocol to be implemented.

As it was mentioned in the above, grazing pastures were considered among plant species as those suitable to carry out the rhizoremediation strategy and, therefore, to be assessed as to their tolerance to glyphosate. Four cultivars were tested: *Lotus corniculatus*, *Lotus tenuis*, *Medicago sativa* and *Lolium multiflorum.* All of them are used in the Argentine Humid Pampas fields and all of them have an excellent fodder quality.

Lotus species are member of the *Fabaceae* family, represented by 100 to 130 species. Five species have productive character and two of them are commercially available in the Humid Pampas: *Lotus corniculatus* L. (*birdsfoot trefoil*) and *Lotus tenuis* Waldst. et Kit. (=Lotus glaber Mill., *narrowleaf trefoil*). As a legume, it has a high digestibility and crude protein value, as well as greater homogeneity in in performance as compared to grasses. They can establish symbiotic associations with microorganisms, which enable them to fix nitrogen and thus increase its levels in soil and, besides, they have low requirement of phosphorus; these two features can increase the productivity of the areas where they are implanted, especially in the bottoms of the field. That is why *Lotus* species are key for the management of the livestock systems in the region (Cambareri et al., 2012). As far as production is concerned, there have been reported all-time highs yields of *L. corniculatus* from 8,000 to 10,000 Kg of dry matter ha^{-1} $year^{-1}$, productivity levels who can compete with other legume pastures of such as alfalfa and clover. *L. tenuis* has lower productivity levels. In South America, a total of 1,85 million hectares are sowed with genus Lotus species (Escaray et al., 2011). *L. tenuis* compensates yield differences showing more adaptability than *L. corniculatus* in regions where saline stress and floods prevail. Within the topography of the Flooding Pampas, the *L. tenuis* crops are usually established in mid-knoll and bottom areas, while the *L. corniculatus* can be placed in knolls, mid-knolls and, to a lower extent, part of the floodplain areas (Figure 3.1). Due to the above mentioned features, both species have been widely used in soil recovering, renewing and replanting and they have worldwide large acceptability as far as environmental processes are concerned (Beuselinck, 1999).

Finally, and as a highlight, there are records of a *L. corniculatus* cultivars with differential tolerance to glyphosate (Boerboom et al., 1990;Boerboom et al., 1991).

M. sativa. Alfalfa, also called Lucerne, is also another member of the *Fabaceae* family, broadly distributed worldwide and generally referred to as the most widely used legume among grazing pastures. It has a high nutritional value required by any cattle grazing system and, in turn, they contribute to the improvement and recovering of fertility and edaphic stability due to its perennial quality, its plasticity and its capacity to symbiotically fix the atmospheric nitrogen. Its sown area worldwide is esteemed to be of 32 million hectares (Bouton, 2012). It has high resistance to droughts and is sensitive to flooding stress (ASA, 2004), making this species being frequently found in knolls (Figure 3.1). Alfalfa has shown resistance and capacity of detoxification of several pollutants, therefore it is considered as a plant species with a great potential for phytoremediation processes (Cuadrado, 2009). It has been used in several phytoremediation assays (mostly in rhizoremediation and phytovolatilization processes), for organic compounds such as crude oil derivatives, polycyclic aromatic hydrocarbons (PAHs) and pesticides, among others (Singh et al., 2009).

L. multiflorum, known as Ryegrass, annual Ryegrass or Italian Ryegrass in Argentina, belongs to the family of grasses *Poaceae*. It has rapid germination and vigorous growth adapted to subtropical temperatures and is easy to handle. It has an important energetic potential, high palatability and digestibility as distinctives grazing pasture features. (Hannaway et al., 1999). Yields of 3000 Kg of dry matter ha^{-1} $year^{-1}$ have been reported in the Salado River basin in the province of Buenos Aires (Hagen, 2012). It adapts well to both acid and alkaline soils (in a range of pH 5,0 to 7,8), and, although its best production is given in well drained soils, it is one of the grazing pastures that can tolerate floods (Figure 3.1). *L. multiflorum* together with other *Lolium* species are among the most glyphosate tolerant reported plant species (Perez and Kogan, 2003; Perez-Jones et al., 2007). Reports have been performed in several countries, including Australia, The United States, Brazil, Chile and Argentina; countries that widely use the RR crop technology (RoundUp Ready Crops).

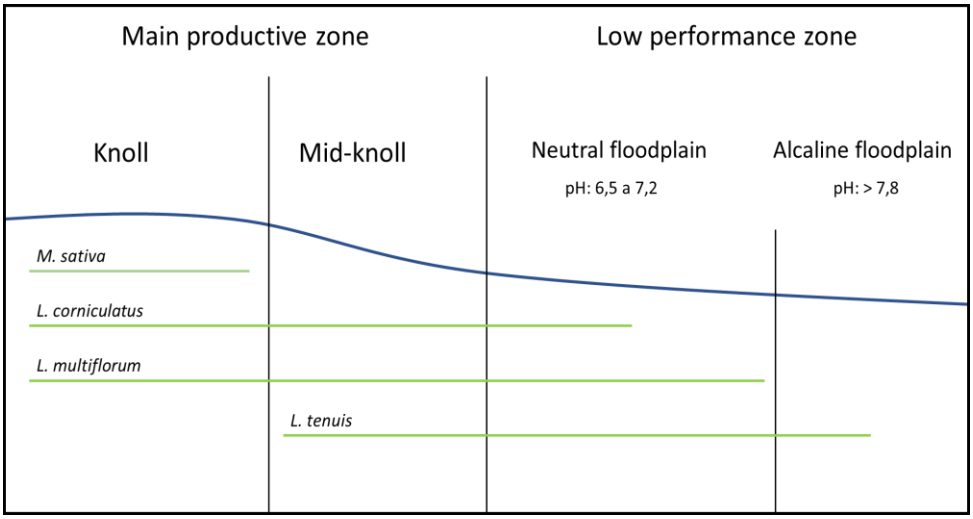

Figure 3.1. Implantation site of proposed plant species to perform the rhizoremediation

In this chapter, tolerance to glyphosate was assessed proposing different experiments mentioned in chapter 1. Field concentrations and route of expositions have been taken into consideration. We furthermore analyzed specific and non-specific metabolites related to glyphosate exposure, together with a genetic characterization of the selected tolerant plants.

This chapter presents the results of the comparative evaluation of 4 different cultivars to be considered for phytoremediation of glyphosate trials. Based on results obtained we could successfully select the most promising cultivar.

The following specific objectives were met during this stage:

1. Systematic search of plant species of productive value to be assessed in regard to their glyphosate tolerance.
2. Development and implementation of methodologies to evaluate root and leaf level tolerance in the plant species.
3. Optimization of an analytical method to measure shikimic acid in vegetal tissues, as a glyphosate exposure biomarker.

3.2 Materials and Methods

3.2.1 Origin of germplasm

The plant species used in the assays are: *(i) Lotus corniculatus* (Gladiador cultivar)*, (ii) Lotus tenuis, (iii) Medicago sativa* (Express cultivar*), (iv) Lolium multiflorum* (Bill Max cultivar). All these cultivars were kindly provided by the company Gentos, from Pergamino, province of Buenos Aires. *L. tenuis* seeds were collected from a plot in the flooding Pampa (geographic locations, PhD Analía Sannazzaro) that presented a population of this plant that escaped to a conventional spraying of glyphosate. This means an exceptional, and possible unique seed source, and was therefore decided to be included to assess the tolerance to herbicide as well.

3.2.2 Seeds surface sterilization

Seed surface sterilization was optimized taking into account the need of scarification of the seed coat required by these species in order to germinate. Each species with different seed size, and hull structure and properties, need different strengths of sanitizing solutions. The two main traits taken onto account to design the protocols were the origin of the plant species (legume or grass) and the structure and characteristics of the episperm. A sandpaper of 200 μm grain size was used for evaluation of scarification. The concentrations of NaClO, Ethanol and $HgCl_2$ solutions were tested to ensure the surface sterilization.

To assess the effectiveness of each surface sterilization process, the seeds were sowed in Petri dishes containing Murashige-Skoog (MS) medium (S3.1.1) (Murashige and Skoog, 1962) with 10 g L^{-1} sucrose and 4 g L^{-1} agar, then incubated at 24 ± 1 °C during 14 days. Each step in the optimization of the sterilization process was assessed using batches of 50 seeds, which were sowed in groups of 10 in Petri containing MS medium. This medium can evaluate simultaneously the germination rate together with the surface sterilization, since its allow the growth of microorganism.

3.2.3 Glyphosate tolerance assay in semi-solid agar

For each species, a set of glass flasks of 360 mL containing 50.0 mL of MS medium with 0.8% of agar, with increasing concentrations of glyphosate (Roundup Ultramax®, Monsanto 74.7% of ammonium salt) was prepared. Five levels were tested, each with final concentrations of: 0.5, 1.0, 5.0, 20.0 and 50.0 mg Kg^{-1}, corresponding to a dose of 1 Kg ha^{-1} of product distributed among the first 10.0, 5.0, 1.0, 0.25 and 0.01 cm soil deeps respectively, assuming a soil density of 1.5 g mL^{-1} (Figure 3.2).

The choice of the assessed concentration ranges was based on a literature review about studies performed in the Humid Pampas and other world regions where glyphosate had been quantified in soils under agricultural activity (Veiga et al., 2001; Scribner et al., 2007; Peruzzo et al., 2008; Aparicio et al., 2013).

Flasks containing the MS medium were sterilized using the autoclave (20 minutes, 1 atm). A mother solution of glyphosate was sterilized separately through filtration (pore size 0.20 µm) and later added to the flasks in different volumes in order to achieve the concentrations corresponding to the different levels. For each level, 5 replications were made, sowing 10 seeds of each plant species in each of them. Following, flasks were sealed in the upper part using polyethylene film and cultivated at 24 ± 1 °C, 50 ± 5 % of relative humidity and 16 hours of photoperiod (400 µM $cm^{-2}sec^{-1}$ of light intensity).

The number of germinations per flask and the biomass and shikimic acid concentration in whole plant at end time (28 days) was recorded. According to Tong (Tong et al., 2009), and taking into account that the concentration of shikimic acid in tissues is an accurate indicator of glyphosate exposure in plants (Singh and Shaner, 1998), the tolerance index (Ti) and the exposure index (Ei) were calculated as follows:

$$Ti = \frac{Bt}{Bc}$$

Where *Bt* is the average plant biomass in the treatment and *Bc* is the average plant biomass in the control (without glyphosate spraying).

$$Ei = \frac{Ct}{Cc}$$

Where *Ct* is the average shikimic acid concentration in the treatment and *Cc* is the average shikimic acid concentration in the control (without glyphosate spraying).

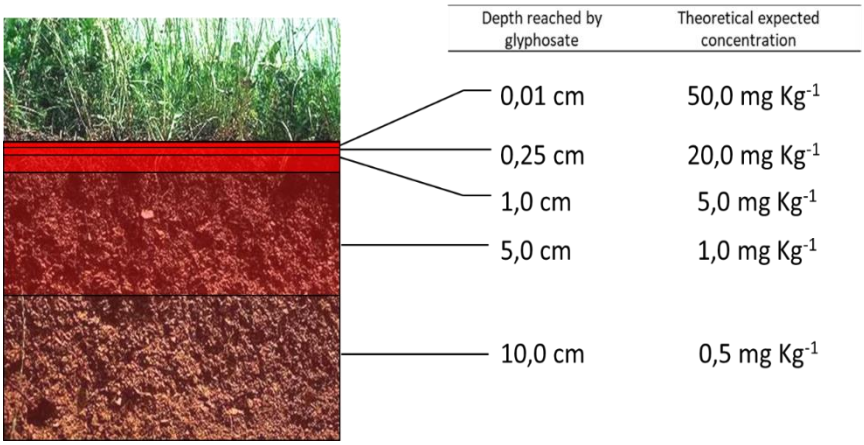

Figure 3.2. Soil theoretical concentration obtained after adding a glyphosate dose (747 g ha^{-1} of monoammonium salt), and the corresponding depth that represents.

3.2.4 Foliar spraying assay

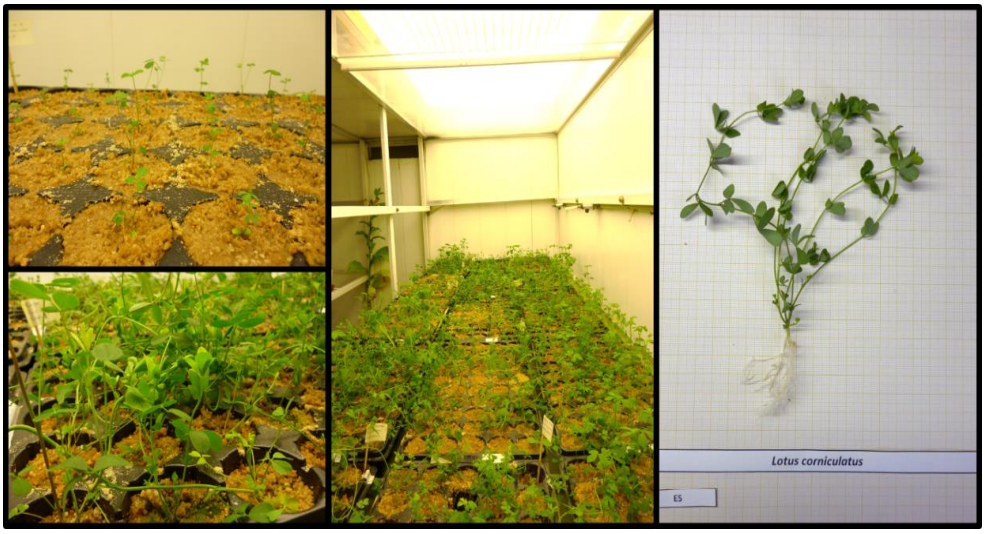

Figure 3.3. Set up (left and center) and experimental unit picture (right) of the foliar application assay.

L. corniculatus, *L. tenuis* and *M. sativa* seeds were surface sterilized according to the scheme described, and sowed in Petri dishes with filter paper soaked with Hoagland solution (S3.1.2) (Hoagland & Arnon, 1950). After root emergence, 25 seedlings of each species were manually transplanted to nurseries

with 100 cm^3 dishes and sand-pearlite substrate 1:1. The nurseries were watered with half strength Hoagland solution during the assay period. The experiment was carried out in a growth chamber at 24 ± 1 °C, relative humidity de 50 ± 5 % and 16 h de photoperiod (400 µM $cm^{-2}seg^{-1}$ of light intensity). Plants having 5 to 7 leaves were sprayed with glyphosate (74,7% ammonium salt) (Roundup Ultramax®, Monsanto) at product dose of 0; 700; 1,400; 2,800 and 5,600 g ha^{-1} (Figure 3.3). The sprayer used had a flat nozzle with an output volume equivalent to 200 L ha-^{1} (previously calibrated).

The doses of the commercial product used that range between 1,400 and 2,800 g ha^{-1} correspond to typical field spraying of the product in several crops, according to the manufacturer recommendations. A dose of 700 g ha^{-1} represents 70% of the minimum spraying recommended (1,000 g ha^{-1}) and can be considered as a strong spraying drift.

Fourteen days after the spraying, each plant's biomass and a visual impact *scoring* was determined (Table 3.1). The period was considered according to the growth of the assessed plants. After this period, the specimen (in average) root begins to grow outside the dish, while its aerial parts lose stiffness falling on others and causing a "*shading effect*" that impedes the homogenous exposure to the spraying.

Table 3.1. Visual score used to assess the herbicide impact on different plant species

Phytotoxicity symptoms	Assigned score (U)
yellowing	5
Chlorosis	5
Necrosis	20
Defoliation/Extended necrosis	70

3.2.5 shikimic acid and chlorophyll content in the plant

To measure of shikimic acid accumulation kinetics in plant tissue, *L. corniculatus and L. tenuis* were cultivated in a sand-perlite mixture 1:1 in culture chamber at 24 ± 1 °C, 50 ± 5 % relative humidity and 16 h of photoperiod (400 $\mu M^{-2}seg^{-1}$ de light intensity). Plants with 5-7 leaves were sprayed with glyphosate (74.7% ammonium salt) (Roundup Ultramax®, Monsanto) at 400 and 1,400 g ha^{-1} doses as described above. Eight plants per treatment were sampled on days 0,

1, 3, 5, 7, 14 and 21 post spraying, and chlorophyll and shikimic acid concentrations were measured. Each plant represents an individual experimental unit on which a non-destructive measurement is performed first (amount of total chlorophyll on leaves) and a destructive measurement is performed in second place (shikimic acid concentration).

The content of chlorophyll was recorded using a portable chlorophyll meter (Minolta, Plainfield, USA). The average chlorophyll value per plant was obtained measuring three full developed leaves per specimen, with eight determinations on every experimental unit by level and by sampling time.

The shikimate extraction and analysis was performed under the original method of Cromartie and Polge with minor changes suggested by Shaner (2005) (S3.1.3). Briefly, each seedling was frozen on liquid nitrogen and then grinded with mortar until complete homogeneity. 100 mg of tissue was placed on 1.5 ml microtubes and 1.0 ml of HCl 0,25 M was added. Then, the samples were mixed in vortex for one minute, then centrifuged at 25,000 x g for 15 minutes. 250 µL of supernatant was taken and mixed with 250 µL a periodic acid 0.25%/(meta) sodium periodate 0.25% solution. The reaction mixture was incubated at 24 ± 1 °C for 30 minutes. Following, 500 µL of a NaOH 0.6 N/Na_2SO_3 0,22N solution was added and the absorbance at 382 nm was measured within 10 minutes. The concentration values were obtained interpolating sample results into a shikimic acid standard curve (Sigma-Aldrich, Buenos Aires, Argentina).

3.2.6 Partial 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase cDNA sequence analyses

Three *L. corniculatus* and *L. tenuis* plants were cultivated in culture chamber at 21 ± 1 ◦C, with a light intensity of 100 $\mu M^{-2} seg^{-1}$ and 16 h of photoperiod. Watering was performed every day with a half strength Hoagland solution. When the plants reached 5-7 leaves they were sprayed with a glyphosate solution (Roundup Ultramax®, Monsanto) equivalent to a dose of 400 g ha^{-1} in order to induce the transcription of mRNA from the EPSP synthase. The plants were harvested 48 hours after the treatment. For RNA extraction, the aerial parts were frozen in liquid nitrogen and immediately stored at -70 ± 1 °C. Between 70 and 90 mg of plant tissue were weighted and then ZR Plant RNA MiniPrep Zimo Research Corporation, Irvine, USA) commercial kit was used to perform RNA

extraction, according to the manufacturer recommendations. RNA was incubated with DNase I (Ambion, Austin, USA) in order to eliminate any possible contamination with genomic DNA. The synthesis of the first cDNA strand was performed using 2.5 µg of total RNA and the Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT) (Invitrogen, Carlsbad, USA) combined with oligo (dT) between 12 and 18 nucleotides length (Invitrogen, Carlsbad, USA) according to the manufacturer recommendations.

Primers used to amplify the cDNA of EPSP synthase sequence were designed based on the available sequences of taxonomically closely-related plant species, *Lotus japonicus* (GenBank accession number AP009741.1) and *Medicago truncatula* (GenBank accession number XM 003605091.1). There were found several conserved regions throughout nucleotide sequences and, thereafter, Primer3 program (Untergasser et al., 2012) was used to obtain primers that showed suitable GC percentage and absence of self-complementarity. From the admissible primer sets obtained, those showed on table 3.2 were the ones synthesized. The choice was also based on *in-silico* tests performed on other DNA enzyme sequences belonging to others related genus. Length

Table 3.2. Primers designed for the partial EPSP synthase

Primer pair	Sequence (5' -> 3')		Expected length (pb)
1	ACGGCTATTCGGGTGTGTTT	(forward)	719
	TCCAAAGCGCTCCATCAACT	(reverse)	
2	TTATTGGAAATTGTGATGTGGG	(forward)	639
	CTGCCATAAGCAAAGCAGTC	(reverse)	
3	CCAGGTGAGCAGAATCCACA	(forward)	614
	GGGGAACCCCGTCAAGTATG	(reverse)	

PCR was performed in a 25 µL reaction volume containing: 1X PCR buffer, $MgCl_2$ 2 mM, 0.2 µM of primers, dNTPs 0.2 mM, 1 unit of DNA polymerase Taq (Thermo Scientific, MA, USA) and 1.5 µL of cDNA. The cycling conditions were: 3 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 30 seconds at 72°C, followed by a final extension step of 10 minutes at 72°C. Products of the PCR reaction were checked in a 1.5% agarose gel electrophoresis. After confirmation of the amplicon's size, DNA was sequenced at Macrogen

(Macrogen, Seoul, South Korea). The sequences were analyzed using the MEGA software v5.2 (Tamura et al., 2011).

3.2.7 Statistical analysis

The statistical analysis of all results was performed using the GraphPad Prism Statistics software version 5.01 for windows. The media and standard deviation parameters were obtained. The homogeneity of variances was tested and afterwards significant differences between treatments were found through ANOVA one-way test. The post-hoc comparisons were performed using the DUNNET test with a 95% confidence interval.

3.3 Results and discussion

3.3.1 Seed surface sterilization

A satisfactory seed surface sterilization technique shall be the one that manages to inhibit the development of microorganisms associated to the seed with no interference of its typical germination rate. The final surface sterilization protocols are shown in figure 3.4. No growth was observed in MS agar after 15 days of cultivation, and a proper rate of germination was achieved.

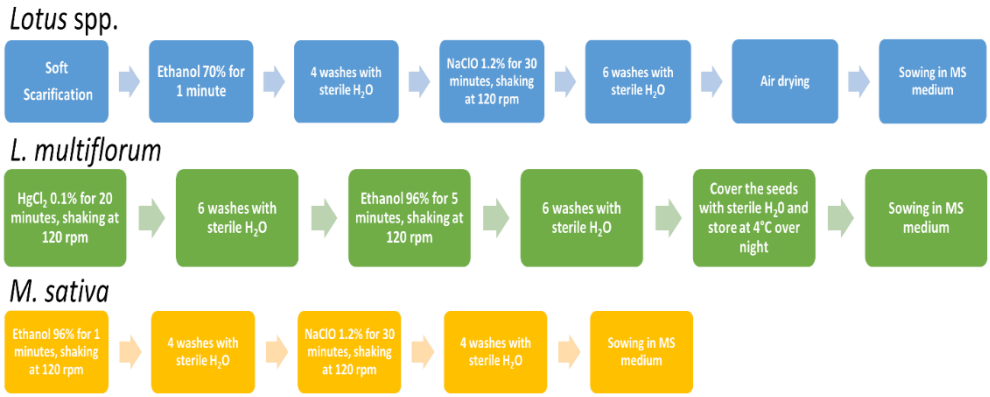

Figure 3.4. Different protocols established for seed surface sterilization. (A) *Lotus spp.* (B) *L. multiflorum.* (C) *M. sativa.*

3.3.2 Glyphosate tolerance assay in semisolid agar medium

L. multiflorum showed significant lower germination rate at glyphosate concentrations of 20.0 mg kg^{-1} and 50.0 mg kg^{-1} compared to the rest of species (p value < 0,001 and confidence interval of 95%, Figure 3.5). There were no significant differences for the other assessed species in germination compared to the non-exposed control. Some authors reported a decrease in the germination rate in dose ranges from 10 to 160 mg L^{-1} (Perez-Jones et al., 2007) and of 12.5 to 400 mg L^{-1} (Perez and Kogan, 2003; Yanniccari et al., 2012a) related to plant species with agronomic value. Based on this, germination rates at concentrations of 20.0 and 50.0 mg L^{-1} could indicate tolerance. The analysis requires the inclusion of more response variables that relate germination rates with plant endurance.

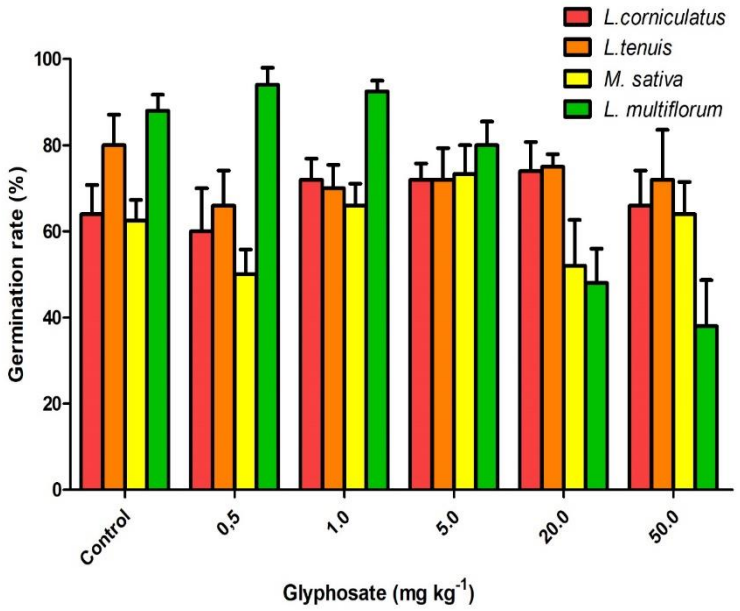

Figure 3.5. Germination rate of each plant species at different glyphosate concentrations in the agar tolerance assay.

Results of the rest of response variables are shown in table 3.3 and 3.4, and plants are shown in figure 3.6. *M. sativa* showed the most sensitive response to glyphosate, with a tolerance index (Ti) of 0.55, followed by *L. multiflorum*, with 0.92, both at minimum concentration of 0.5 mg Kg^{-1}. By contrast, *L. tenuis* and *L. corniculatus* increased their biomass after being exposed to low doses of glyphosate, reaching Ti values of 1.32 and 1.53 to 1.0 mg Kg^{-1}, respectively. This paradoxical effect is possible attributed to hormesis, a phenomenon previously reported by other authors who worked with glyphosate tolerance in plants (Pline et al., 2002a; Petersen et al., 2007; Cedergreen and Olesen, 2010). That is how *L. corniculatus* increases 32% and *L. tenuis* 53% of their total biomass under a glyphosate concentration of 1.0 mg Kg^{-1} without affecting at all their average survival rate or their morphology (Figure 3.6).

Of all parameters determined during the assay, the biomass was the one that showed better correlation with the observed damage and survival of each species. *L. corniculatus* showed minimum foliar changes (reduction of leaf area) at 5.0 mg Kg^{-1} dose of glyphosate, while, at the same concentration, the rest of

the species were severely affected, showing chlorotic leaves and abnormal growth of roots. At such concentration, *L. tenuis* also showed an increase in the number of meristems, in the same way as *L. corniculatus* did at a concentration of 20.0 mg kg^{-1} (Figure 3.6). The concentration limit of 5.0 mg kg^{-1} is critical since it corresponds to the maximum concentrations determined in soils under agricultural use and, therefore, it is desirable that the selected plant species be able to tolerate such concentration. It is worth noting that the experimental system under which the assay was carried out guaranteed the maximum availability of xenobiotic, representing the worst-case scenario the plant could deal with. It can be assumed that, in soils, the tolerance limits of this species may be extended.

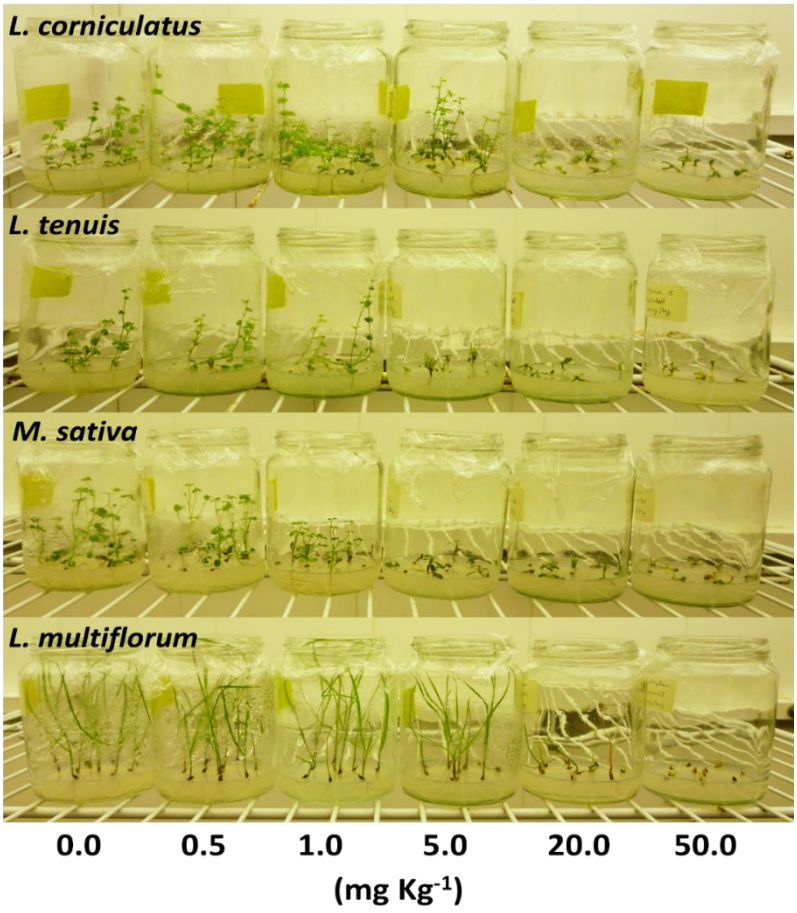

Figure 3.6. Glyphosate tolerance assay in semisolid agar

Table 3.3. Results of the glyphosate tolerance assay in semisolid agar medium assay for *L. corniculatus* and *L. tenuis*.

Glpyhosate dose (mg kg^{-1})	*L.corniculatus*				*L.tenuis*			
	Biomass (mg fresh wt)	Tolerance index (Ti)	Shikimate (µg g fresh wt^{-1})	Exposure index (Ei)	Biomass (mg fresh wt)	Tolerance index (Ti)	Shikimate (µg g fresh wt^{-1})	Exposure index (Ei)
0.0	42 ± 17	1.00	60 ± 17	1.00	31 ± 15	1.00	70 ± 18	1.00
0.5	51 ± 22	1.18	61 ± 24	1.02	44 ± 23 *	1.39	101 ± 73	1.43
1.0	57 ± 33	1.32	87 ± 32	1.45	48 ± 25	1.53	153 ± 142	2.17
5.0	47 ± 23	1.11	278 ± 140	4.63	25 ± 11	0.80	627 ± 305	8.91
20.0	27 ± 11	0.63	678 ± 292 *	11.30	20 ± 7	0.64	2262 ± 1553 *	32.16
50.0	25 ± 8	0.58	1290 ± 447 *	21.50	14 ± 7 *	0.45	8357 ± 3924 *	118.82

*Indicates significant differences compared to control, with a significance of α = 0.05, and using the Dunnett

Table 3.4. Results of the glyphosate tolerance assay in semisolid agar medium assay for *M. sativa* and *L. multiflorum*.

Glpyhosate dose (mg kg^{-1})	*M.sativa*				*L.multiflorum*			
	Biomass (mg fresh wt)	Tolerance index (Ti)	Shikimate (µg g fresh wt^{-1})	Exposure index (Ei)	Biomass (mg fresh wt)	Tolerance index (Ti)	Shikimate (µg g fresh wt^{-1})	Exposure index (Ei)
0.0	90 ± 58	1.00	41 ± 15	1.00	47 ± 19	1.00	61 ± 31	1.00
0.5	49 ± 38 *	0.55	66 ± 38	1.62	43 ± 13	0.92	86 ± 33	1.40
1.0	34 ± 23 *	0.38	101 ± 51	2.48	44 ± 15	0.92	48 ± 35	0.78
5.0	23 ± 10 *	0.26	531 ± 289	13.08	34 ± 11 *	0.71	490 ± 138	8.01
20.0	19 ± 6 *	0.21	996 ± 343 *	24.55	21 ± 5 *	0.43	1609 ± 607 *	26.32
50.0	22 ± 9 *	0.24	2117 ± 1369 *	52.16	13 ± 3 *	0.27	1294 ± 1431 *	21.17

*Indicates significant differences compared to control, with a significance of α = 0.05, and using the Dunnett

All the plant species increased their shikimate concentration as they were exposed to higher concentrations of glyphosate. In this trend, *L. corniculatus* shows a lower initial increase of such concentrations. *M. sativa and L. tenuis* were the plant species with higher increase in exposure index (Ei), while *L. multiflorum* occupies an intermediate position, since it shows low values for some concentration levels of glyphosate (the Ei for 1.0 mg Kg^{-1} stands out) and high values such as that observed at a 5.0 mg Kg^{-1} glyphosate level.

As far as the relationship between shikimate accumulation, the biomass production and the visual appearance concerned, it is a very species-dependent relationship. For instance, a concentration of 1.0 mg kg^{-1} glyphosate, gives 2.48 Ei in *M. sativa, which* was related to a significant decrease of its biomass and to strong visual damage, while in *L. tenuis*, a 2.17 Ei did not cause a significant difference in biomass compared to control. Moreover, a 4.63 Ei in *L. corniculatus* exposed to a 5.0 mg kg^{-1} of glyphosate only caused a change in the foliar area of such species.

A hypothesis for the observed differences in concentration of shikimate is that the lower concentration observed in *L. corniculatus* could be related to a difference in the root uptake; such hypothesis could be confirmed in future experiments using isotopically labeled glyphosate.

As a consequence of such observations and taking into account the particular response of each species to the same glyphosate concentration, special care should be taken when the concentration of shikimate is used as criterion to assess the impact of the herbicide in future phytoremediation assays. It is essential to know in advance the basal values for each plant species, and the correlation between the value of this exposure marker and the damage produced.

The results observed suggest that *L. corniculatus* is, among the assessed species, the best candidate to be implanted since its root system tolerate the maximum concentrations of glyphosate reported in agricultural soils. The possibility of using commercial seeds instead of seedlings is a significant agro-technological advantage and substantially reduces the implementation costs. Other interesting point to be considered is the hormesis phenomenon observed in this species when exposed to low concentrations of glyphosate; this factor can contribute to improve the average survival rate after germination and could represents an environmental advantage if improves its germination capacity

compared to other species in the plot. This is a key aspect for farmers who wishes to work with *Lotus*.

Observations made during this assay show that multiple response variables shall be considered at the time of performing tolerance assays. During this assay, the biomass results, the visual assessment and the concentration of shikimate in the plant tissue were complementary and enabled a more comprehensive interpretation of the experiment.

3.3.3 Foliar spraying assay.

The response of the plant species to foliar spraying was assessed, as this is the usual herbicide spraying method. The information obtained in this assay allows, on the one hand, a comparison of the response of the assessed species with the response published in other research works where glyphosate tolerance was also studied, and, on the other hand, an estimation of a drift level could be tolerated if used as barrier within a plot under agricultural exploitation.

Based on the results obtained from the agar tolerance assay, it was decided to determine the response to different foliar sprayings of a glyphosate commercial formulation in two *Lotus* species. Alfalfa was also included (as control) since it presented the highest sensibility to all measured parameters and, therefore, it is expected to show higher sensibility in this case too.

After the assay time elapsed, it was observed that *L. corniculatus* and *L. tenuis* showed no significant differences in fresh and dry weight with respect to control when a dose of 700 g ha^{-1} of glyphosate was sprayed, while the differences were significant for the rest of the levels for fresh weight (p value < 0.05 and confidence interval of 95%). *M. sativa* showed significant differences with respect to the control for all doses, and it was clearly affected after treatment. In spite of alfalfa presented the biggest biomass when no glyphosate was applied, an spraying of glyphosate that is below the dose the manufacturer recommends (700 g ha^{-1}), reduced its total weight in 68% based on fresh weight (Figure 3.7A and 3.7B).

In order to provide the assay with an interpretation in terms of the agricultural use and management, the biomass determinations were validated through visual scoring, by which each specimen obtained a score of damage according to table 3.1 of section 3.2.4. Results are shown in table 3.5.

Visual scoring shows a very good correlation with the differences in biomass for all species. When a sub-recommended dose of 700 g ha^{-1} of glyphosate was applied, both *Lotus* species showed no damage over the cut-off point (15 points), suggesting these species can tolerate such dose, while, at recommended doses, they are controlled by the spraying.

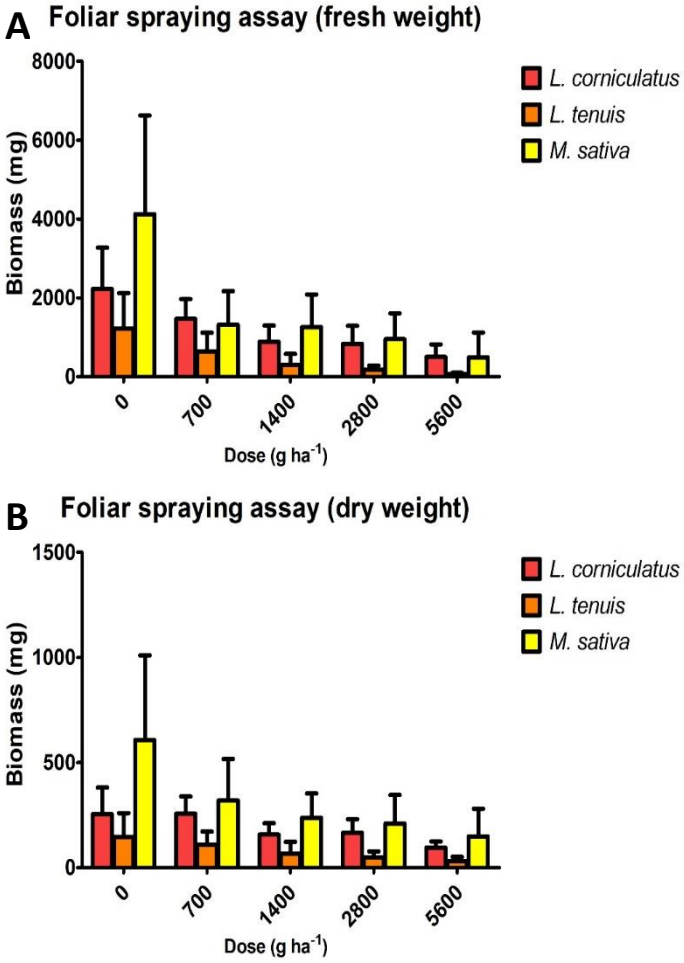

Figure 3.7. Foliar spraying assay. A. Fresh weight in the three species. B. Dry weight in the three species

As part of the standard procedure to assess the effectiveness of herbicides on weeds and the side effects on crops, it is frequently included a visual impact *scoring*. Most visual assessment protocols are based on the experience of

professionals and on the context of agricultural spraying, but there are also standardized protocols or guides, such as that of the European and Mediterranean Plant Protection Organization (EPPO, 2011). Therefore, a visual *scoring* chart was designed to assess the impact during the assay. Such scale is based on considering phytotoxicity specific signs for legumes and giving it a semi-quantitative value, establishing a hierarchy according to the type of damage. To prepare the chart, we were assisted by the Agricultural Engineer Jorgelina C. Montoya, a well-known professional of National Institute of Agricultural Technology (INTA) specialized in weed control and herbicide management. The scores were established in relative units, according to table 3.1. Each experimental unit may have a minimum of 0 and a maximum of 100 units. Plants with a score higher than 15 were classified as "irreversibly damaged".

Visual scoring shows a very good correlation with the differences in biomass for all species. When a sub-recommended dose of 700 g ha^{-1} of glyphosate was applied, both *Lotus* species showed no damage over the cut-off point (15 points), suggesting these species can tolerate such dose, while, at recommended doses, they are controlled by the spraying.

Table 3.5. Visual Scoring obtained for each plant species assessed in the foliar application assay.

Dose	Plant species		
(g ha^{-1})	*L. corniculatus*	*L. tenuis*	*M. sativa*
Control	0	0	0
700	7	8	35
1,400	15	26	35
2,800	28	62	56
5,600	54	86	78

3.3.4 shikimic acid and chlorophyll content quantification in entire plant

The concentration of shikimate in plant tissues has been widely used as an indicator of exposure to glyphosate (Singh and Shaner, 1998; Pline et al., 2002b; Henry et al., 2007). Glyphosate exerts its herbicidal activity through the inhibition of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. This enzyme catalyzes the transfer of an enolpyruvate moiety of phosphoenolpyruvate (PEP) molecule to a shikimate-3-phosphate molecule resulting in the formation of

5-enolpyruvylshikimate-3-phosphate. Activity of this enzyme represents is a key step in aromatic amino acids, hormones and other relevant metabolites synthesis pathway (Schmid & Amrhein, 1995; Maeda & Dudareva, 2012). As a consequence of such enzymatic inhibition, the concentration of shikimate in plant tissues is increased.

In parallel, total chlorophyll content in leaves was also determined on each experimental unit. Such determination is frequently used as an indicator of the general physiological status in plants, since it provides information on the movement of proteins and the content of nitrogen of each plant (Maxwell and Johnson, 2000; Carter and Knapp, 2001). In this sense, it complements the measurement of shikimate as specific indicator of exposure to glyphosate.

From the results obtained after the two tolerance assays, where *L. corniculatus* is seen as the most suitable species to be used in a future remediation assay, it was decided to investigate the kinetics of shikimate accumulation in its tissue.

This assay was also accompanied by the measurement of total chlorophyll content on leaves in order to contrast the shikimate concentration values with a regularly measured biochemical parameter, giving account of the general physiological status of the plant.

Two different doses of glyphosate: a sub-lethal one of 400 g ha^{-1} and a lethal one (or one that can be considered as such) of 1,400 g ha^{-1} were sprayed to *L. corniculatus* plants in a vegetative stage, having from 5 to 7 leaves. Afterwards, the concentration of shikimate of entire plant and the chlorophyll in leaves were measured over time (See section 3.2.5).

After a glyphosate spraying of 400 g ha^{-1}, *L. corniculatus* showed shikimate concentration values similar to those reported for other tolerant species (Dinelli et al., 2006; Perez-Jones et al., 2007) (Figure 3.8). These shikimate concentrations were found in plant species which tolerance is related to deficit in the absorption and/or translocation of the herbicide, which evolves into a reduction in the accumulation in meristematic tissues. This mechanism of tolerance would contribute to preserve root meristematic tissue after an spraying and may ensure a continuous root elongation. However, further assays should be performed to confirm the above.

The highest increase in shikimate concentration was obtained between the fourth and seventh day after spraying. As from such day, the levels of shikimate decreased and did not return to the basal levels during the term of the assay. This may be given by the fact that there may not be enough time to restore the EPSP synthase pool. It was also observed that the increase in concentration of shikimate anticipates the occurrence of visual damages, since they were observed as from the 10th day following the spraying.

The spraying of 1,400 g ha^{-1} showed a profile similar to that of the spraying of 400 g ha^{-1}, however, the amount of accumulated shikimate was significantly higher for every sampling time (Figure 3.8). Although the shikimate levels decreased as from the fifth day, as in the spraying of 400 g ha^{-1}, in this case the plant did not survive. Based on this, both the maximum point of accumulation of shikimate (on the fifth day) and the accumulation of shikimate (area below the curve), could be associated with a threshold value from which the plant species does not recover the EPSP synthase pool and therefore dies.

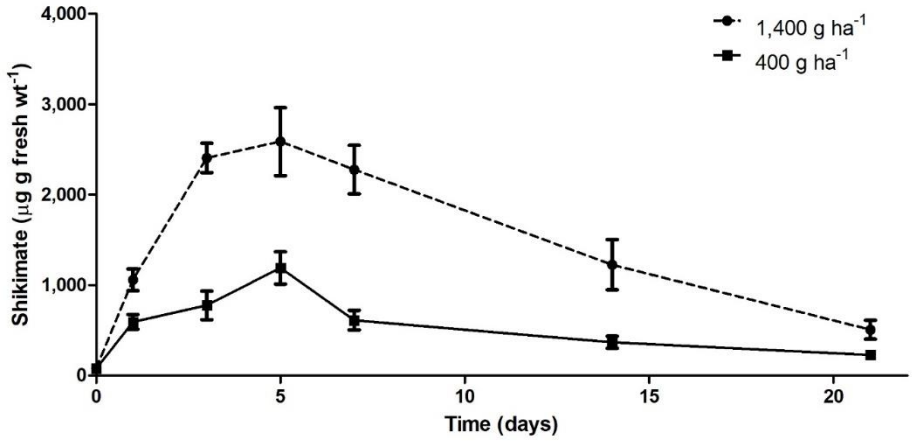

Figure 3.8. Shikimate accumulation in *L. corniculatus* after a sub-lethal dose (400 g ha^{-1}) and a lethal dose (1,400 g ha^{-1}) of glyphosate.

Due to the fact that it is a low cost spectrophotometric determination, the quantification of shikimate could be very useful in the future to monitor the exposure to glyphosate in *L. corniculatus* during field rhizoremediation assays.

At the same time, results obtained showed an initial reduction in chlorophyll content and then a gradual increment over time. This behavior was repeated in two sprayings (Figure 3.9). Chlorophyll measurement was also sensitive to glyphosate, but it showed a different profile from that reported by the literature where the total chlorophyll decreases over time (Yanniccari et al., 2012b; Gao et al., 2014). In this case, no significant differences were observed among doses for each sampling points.

This chlorophyll concentrations profile is possibly caused by an initial stress associated with the spraying, followed by the specific effects in the meristematic tissue, where the inhibition in development of new leaves would lead to an accumulation of chlorophyll in the leaves in lower parts of the plant. The re-mobilization of nutrients from the lower to the higher parts is a common process during the plant development, possibly interrupted in this case by the action of glyphosate in the meristematic tissue (Avice et al., 1996).

It is worth mentioning that the implementation of the shikimate measurement and the visual scoring protocol were not only thought as response variables for *in-vitro* and greenhouse assays, but they should also be considered for future use in field assays at the time of assessing the impact of glyphosate in *L. corniculatus*, due to its simplicity, costs and informative techniques.

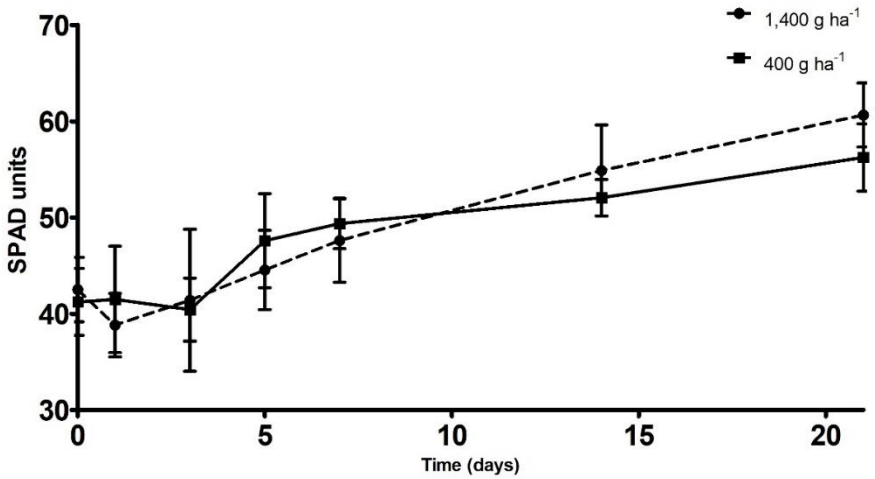

Figure 3.9. Total chlorophyll (SPAD units) in *L. corniculatus* after a sub-lethal dose (400 g ha^{-1}) and a lethal dose (1,400 g ha^{-1}) of glyphosate.

3.3.5 Analysis of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase cDNA partial sequence

The mechanism of glyphosate tolerance in weeds most frequently reported is the presence of a single nucleotide polymorphism at amino acid position 106 of the enzyme enolpyruvyl-shikimate-3-phosphatesynthase (EPSP synthase) sequence, resulting in a substitution of the amino acid Proline to Serine, Alanine or Threonine (Christoffers & Varanasi, 2010). The replacement of such position modifies the special orientation of other two close amino acids related to glyphosate binding site. The conformational change caused by this substitution produces a decrease in the enzyme affinity to glyphosate, resulting in a resistant plant species (Healy-Fried et al., 2007).

Successful primer design allows to acquisition of the partial cDNA sequence of the enzyme, which was deposited at GenBank under the access number KM076642.4. As the three sets of primers resulted in the amplification of a single amplicon with expected molecular weight (Table 3.2), it was decided to sequence the first of them since is the longest one (Figure 3.11). After sequencing the cDNA in both senses and editing the information provided by the electropherograms, it was obtained the consensus sequence used for this analysis.

The results show a unique sequence that match to EPSP synthase gene. The substitution reported on position 106 of the enzyme (Figure 3.10) was not found, which indicates that the level of tolerance to glyphosate observed in this cultivar is not given by the typically reported mutation.

These results are consistent with those observed in the accumulation of shikimate induced by the foliar spraying of herbicide and suggest that the mechanisms of tolerance are indeed related to a differential uptake or translocation of shikimate by the plant.

Figure 3.10. Partial alignment of the EPSP synthase cDNA sequence and the inferred amino acids sequences of *L. corniculatus and A. thaliana*. The codon in the box corresponds to the position of amino acid 106, based on the sequence of *A. thaliana*.

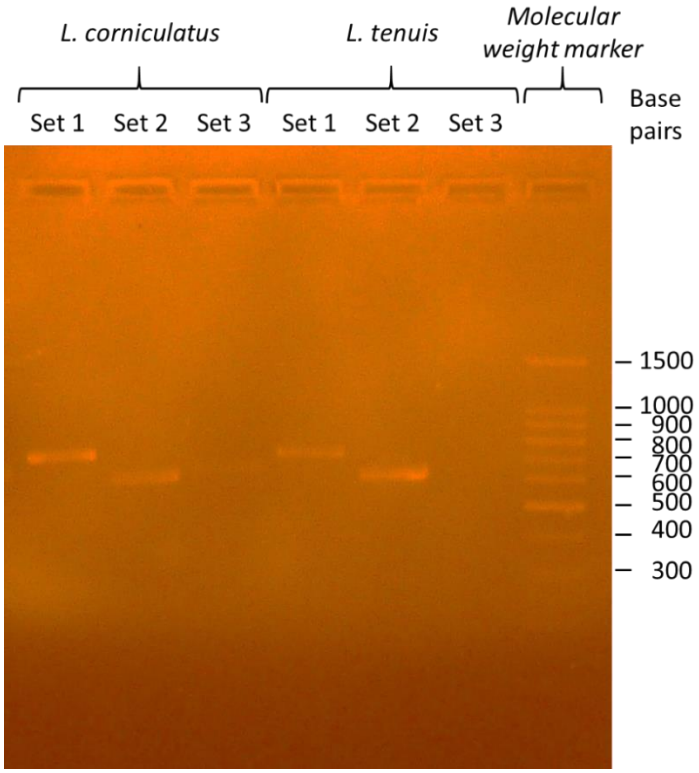

Figure 3.11. Agarose electrophoresis gel of PCR products obtained using the 3 primer sets designed for the amplification of the EPSP synthase cDNA sequence in *L. corniculatus and L. tenuis*. In this case, the amplification in *L. tenuis* was also performed to verify the inter-species versatility of the primers. The primers pair 1 and 2 work on both species while primer

3.4 Conclusions

L. corniculatus was selected as the most tolerant plant species feasible to develop a glyphosate rhizoremediation strategy.

In this sense, *L. corniculatus* is capable of tolerating 5.0 mg Kg^{-1} of the herbicide in a maximum bioavailability medium (which possibly implies tolerances to higher concentrations when tested on a complex matrix like the Humid Pampa soils), Besides, *L. corniculatus* has also proven to resist a 700 g ha^{-1} dose of the commercial product delivered. In the usual application conditions.

All this adds to very good agricultural properties that imply not only an excellent nutritional value for cattle breeding but also its contribution to the soil quality improvement and its versatility with respect to the location within the plot area.

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S3.1 Supplementary data

S3.1.1 Murashige-Skoog (MS) medium

Reagent	**Amount (g L^{-1})**
NH_4NO_3	1.650
KNO_3	1.900
$CaCl_2.2H_2O$	0.440
$MgSO_4.7H_2O$	0.370
KH_2PO_4	0.170
Na_2-EDTA	0.0373
$FeSO_4.7H_2O$	0.0278
H_3BO_3	6.2 mg
$MnSO_4.4H_2O$	22.3 mg
$ZnSO_4.4H_2O$	8,6 mg
KI	0.83 mg
$Na_2MoO4.2H_2O$	0.036 mg
$CuSO_4.5H_2O$	0.025 mg
$CoCl_2.5H_2O$	0,025 mg
Sucrose	30.0
Glycine	2.0 mg
3-Indolacetic Acid	15.0 mg
myo-Inositol	100.0 mg
Pyridoxine. HCl	0.5 mg
Thiamine.HCL	0.1 mg
Nicotinic Acid	0.5 mg
Agar "plant tissue" grade	10.0
pH: 5.7-5.8	

Reference:

Murashige T, Skoog F (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol. Plant. 15, 473–497. doi:10.1111/j.1399-3054.1962.tb08052.x.

S3.1.2 Hoagland solution

Reagent	**Amount (g L^{-1})**
KNO_3	101.1
$Ca(NO_3)_2.4H_2O$	236.16
$(NH_4)\ H_2PO_4$	114.93
$MgSO_4.7H_2O$	246.25
$MnCl_2.4H_2O$	1.78
H_3BO_3	2.84
$Zn\ SO_4.7H_2O$	0.05
$CuSO_4.5H_2O$	0.08
$MoO_4Na_2.2H_2O$	0.025
FeNa EDTA	37.0
pH: 6.5-7.5	

Reference:

Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without soil. doi:citeulike-article-id:9455435.

S3.1.3 Shikimate concentration measurement

Vegetal tissue sampling

1- Take an estimated amount of vegetal tissue greater than 50 mg.
2- Weight the vegetal tissue in scale (±1 mg).

Shikimate extraction

1- Add the vegetal tissue into a 1,5 mL microtube and close the lid.
2- introduce the microtube in liquid nitrogen until sample is completely frozen.
3- Use a microtube pestle to homogenize the vegetal tissue
4- Add to the tube 1,0 mL of HCl 0,25 N.
5- Vortex the tube for 10 minutes.
6- Freeze the tube at -20 °C.
7- Thaw and keep at room temperature for at least 30 minutes.

Quantification

1- Centrifuge at 25,000 g for 15 minutes.
2- Take 250 µL of supernatant and place it in a new 1.5 mL microtube.
3- Add 250 µL of a HIO_4 0,25% $NaIO_4$ 0,25% solution.
4- Incubate at 37 °C for 30 minutes.
5- Add 500 µL of a NaOH 0,6 N/Na_2SO_3 0,22 M solution.
6- Measure in spectrophotometer at 382 nm wavelength.
7- Interpolate results in calibration curve and inform the shikimate concentration.

Calibration curve

1- Make Shikimate standard solutions of: 0, 4, 8, 12, 20 y 28 µM.
2- Take 250 µL of each standard solution and add them into a 1,5 mL microtube separately.
3- Make at least 3 replicates for each standard concentration.
4- Add 250 µL of a HIO_4 0,25% $NaIO_4$ 0,25% into the microtuetube.
5- Incubate at 37 °C for 30 minutes.
6- Add 500 µL of NaOH 0,6 N/Na_2SO_3 0,22 M into the tube.
7- Measure in spectrophotometer at 382 nm wavelength.
8- Make a calibration curve of absorbance as a function of concentration.

Reference:

Shaner, D. L., Nadler-Hassar, T., Henry, W. B., and Koger, C. H. (2005). A rapid in vivo shikimate accumulation assay with excised leaf discs. *Weed Sci.* 53, 769–774. doi:10.1614/WS-05-009R.1.

Chapter 4 - Studies on a glyphosate chronically contaminated farm and isolation of tolerant microorganisms to perform rhizoremediation processes

4.1 Introduction

During chapter 3*, L. corniculatus* "*Gladiador*" was properly characterized as a glyphosate tolerant plant species, and will be used to design the rhizoremediation tool, together with the tolerant microorganisms obtained in this chapter.

It remains to define the way the xenobiotic degradation by the microorganisms will occur. As explained in previous chapters, there are no relevant glyphosate degradation mechanisms in vegetable species (Sammons and Gaines, 2014). At the same time, glyphosate presents a negative log K_{ow} value, causing transport from the soil to metabolic active tissues in the roots to be minority. Therefore, a given rhizoremediation method considers the vegetable species responsible of distributing and securing the viability of soil microorganisms, who will be at time truly in charge of glyphosate degradation. Hence, for any granted microorganism assisted phytoremediation process (Gerhardt et al., 2009), there are different approaches to ensure a significantly high number of degradative microorganisms in the rhizosphere environment, which may be classified as: Bioaugmentation, Biostimulation and the use of transgenic plants (Thijs & Vangronsveld, 2015).

Bioaugmentation consists in the inoculation of plant roots with microorganisms (either a single strain or a consortium), whose metabolic efficiency for the contaminant has been already been properly characterized. A vast number of microorganisms with a variety of degradation capacities and/or tolerant to a wide range of pollutants have been isolated from an immense number of environments. Several attempts to apply degrading microorganisms directly on contaminated soils have been made, mostly of which have been unsuccessful or presented controversial results regarding the variables on which their success depend on (Thompson et al., 2005; Tyagi et al., 2011).

The inoculation of plant species with strains or a consortium, bring back the idea of the utilization of degrading microorganisms isolated in the laboratory, as it hopes to make use of the intricate relationship between plant and bacteria. That is how a plant-microorganism association is seek, that will benefit the plant as much as the microorganism. Certain microorganisms possess one or more abilities to promote vegetal growth. These are variety of cell mechanisms destined to positively favors, changes in structure and root extension, nutrient accessibility

and defense against various pathogens. The acquisition of such microorganisms will provide the plant with adaptive advantages over the other vegetable species present in the field (Figueiredo et al., 2010). On the other hand, plant roots deposit a great amount of photosynthates in soil, phenomena called rhizodeposition. Through these depositions, a wide variety of organic compounds, such as organic acids, carbohydrates and amino acids are released in form of exudates, secretions, mucilage and cellular lysates, greatly stimulating microorganism growth in the zones close to the root. As mentioned in chapter 1, this stimulus increases in several fold the number of microorganisms compared to those in bulk soils and is called "rhizosphere effect". Consequently, the establishment of the microorganism in the root will secure access to diverse nutritional sources. To date, there are numerous studies where specific interactions of microorganisms (among them bacteria and fungus) and different types of vegetable species were explored focused on rhizoremediation (Vangronsveld et al., 2009; Gerhardt et al., 2017; Jambon et al., 2018).

The main goal in this chapter is to get pure microorganisms available, which will not only possess an adequate tolerance to glyphosate and degradation capabilities, but also provide at least one plant growth promotion ability trait. The assessment of these features is thoroughly presented in chapter 5. The center of the current chapter is to depict the isolation proceedings and the characterization of soil and extract samples.

The following specific objectives were met during this stage:

4. Isolation of glyphosate tolerant and degrading bacteria.

4.2 Materials and Methods

4.2.1 Sampling

In order to carry out the isolation of microorganisms, samples were taken at Manantiales Experimental Farm, dependent of the National Institute of Agricultural Technology (INTA), in Chascomús county, Buenos Aires province, Argentina (35°34'30"S 58°00'32"W) (Figure 4.1).

Figure 4.1. Map of Manantiales Experimental Farm. The four plots sampled during this thesis are indicated with yellow borders.

Among its projects, the experimental farm carries out a study about *Lotus tenuis* "promotion" in soils of the Flooding Pampa. Two plots, marked as L1 and L3, are sprayed twice a year with high doses of glyphosate, approximately 3.5 litres ha^{-1}. The aim of the promotion proceeding is to avoid other vegetal species from competing with *L. tenuis*, so that the *L. tenuis* may grow and have a good yield over the summer season. Thus, it's essential for the plant to achieve great germination rate and coverage, which is fostered by the cleansing of the plot in winter time by applying glyphosate. The predominance of *L. tenuis* is attained by controlling the remaining plant species, which possess higher rates of germination. Without the selection pressure that promotion method exerts, the

plot would result in mixed pastures (for instance: Lotus, ryegrass and white clover).

Throughout the years and systematically, L. tenuis seed population has been increased over the other species within the plots. Another two plots, L2 y L4, present 'natural' pastures, where the land was let to evolve without any sort of treatment resulting in a different vegetal profile from those in L1 and L3. As shown in figure 4.1, plots L1 is located next to plot L2, as is L3 from L4. This is because L1 and L3 have different soil characteristics, leaving the promotion treatment as the only variable. Therefore, plot L1 has plot L2 as control and plot L3 has plot L4 as control.

Plots L1 and L3, consider as chronically exposed to glyphosate, will be used to obtain degrading and tolerant microorganisms from samples of rhizosphere and soil. Plots L2 and L4 will be used as control samples when studies of microbial communities and cultivable microorganism comparisons are carry out.

These plots offer excellent conditions for the isolation of microorganisms, as we can count on closely related plant species to the one we described as tolerant and which is located in soils chronically exposed to glyphosate, applied in high regular doses. This may imply that the microorganisms from these plots are under a double selection mechanism: The first carried out by L. tenuis rhizosphere and the second by the presence of glyphosate.

The sampling procedure was carried out on August 19th, 2014, with the invaluable cooperation of Dr. Ing. Matias Bailleres, in charge of the technical aspects of the L. tenuis promotion proceeding.

For sampling, a transect was drawn on each plot, avoiding terrain features such as small depressions or elevations that may present different edaphic characteristics. Nine samples per plot were taken, achieving a total of thirty-six samples for all four plots. Each of the thirty-six samples consisted of 0 and A horizon portions with approximately 30 cm side and 30 cm deep and included at least one *L. tenuis* specimen. Samples were taken with using a shovel and then placed in a double polyethylene bag properly labelled to be sent to the laboratory. Geolocation of each sample was also registered (Figure 4.2).

Figure 4.2. Pictures taken during sampling proceeding. On the left picture, the transect is indicated by the bags containing the samples already taken. On the right, one sample next to the record log and the GPS used to indicate de position of each single sample.

4.2.2 Physicochemical analysis of soils

Soil samples were sent to Anguil Experimental Agricultural Station, in La Pampa province, Argentina. Samples corresponding to plots L1 and L2 were homogenized into one sample, as L3 and L4 as well according to Ing Bailleres recommendations, based on soil genesis of the plots. Both resulting samples (1+2 and 3+4) were homogenised via quartering method.

Dr. Nanci S. Kloster carried out the analysis of physicochemical proprieties, following internal work protocols. The involved methods and bibliographical references, when informed, are listed below:

- Available Phosphorus (Bray and Kurtz, 1945)
- Total Nitrogen (Chemical Methods Soil Science Society of America Book Series, 1996)
- Organic matter (Walkley and Black, 1934)
- Texture (Bouyoucos, 1962)
- pH (soil – water ratio 1:2,5)
- Cationic exchange capacity (Chemical Methods Soil Science Society of America Book Series, 1996)
- Calcium and Magnesium (via Atomic Absorption Spectrophotometry)
- Sodium and Potassium (via Flame Spectrophotometty)

The moisture percentage of each soil was calculated through gravimetric method. For this purpose, an aluminum tray (P1) was weighed. Afterwards, approximately 5 grams of soil were added and the tray, now containing the soil (P2) was weighted. It was then placed in the stove at 105°C for 24 hours. Then, the tray with the soil was removed from the stove and let to cool in a desiccator. Once the tray reached room temperature, the tray was weighted again (P3). Calculations were performed as follows:

$$Moisture\ \% = 100 - (100 * \left(\frac{P2 - P3}{P2 - P1}\right))$$

4.2.3 Soil processing for biological analyses

4.2.3.1 Rhizosphere soil

Once in the laboratory, the Lotus specimens on every sample were carefully removed from the soil. Roots were gently shaken to remove the soil weakly attached to them. Finally, aerial parts were removed, roots placed in an aluminum foil tray and labelled as 'rhizosphere-rhizoplane sample'.

Each of these samples were weighed and placed in a 250 mL Erlenmeyer flask, with 50 mL of a 0.9% NaCl solution and then shaken for 30 minutes at 200 rpm. After this time, the 50 mL (now containing the root washing) was removed and stored separately, and then another 50 mL of a second solution NaCl 0.9% Tween 80 0.01% was added to each Erlenmeyer. Another 30 minutes of agitation at 200 rpm was carried out. Subsequently, the volume of the second solution was also removed and added to the same container where the first 50 mL solution was stored. In the end, approximately 100 mL of solution was collected during the two subsequent washings. The first washing was aimed at extracting the remainder soil attached to the roots and microorganisms weekly attached and associated to soil particles. While the second washing, with the detergent, was aimed to extracting microorganisms strongly attached and located in the rhizoplane.

The volume of three samples belonging to the same plot were put together to create composite samples. The mixing of the composite samples from individual samples was randomly done. Three ´composite rhizosphere-rhizoplane samples´ were obtained for each plot. Under laminar flow, aliquots were taken to perform enrichment cultures, conservation at -80°C in 2.0 mL microtubes, and heterotrophs microorganisms cell counts on plates.

4.2.3.2 Bulk soil

On the other hand, the remaining soil present in each sample, now lacking Lotus roots, was also separated, ensuring not to incorporate soil touching the polyethylene bag where it was transported. Approximately 100 grams were sieved (mesh size 2.0 mm) and stored in an aluminum paper tray. These samples were labelled as ´soil samples´.

Three individual soil samples were put together by quartering, in order to achieve proper homogenization. Altogether, a sample of about 50 grams was obtained and labelled as ´composite soil sample'. The composition of each composite samples followed the same random assignment as the rhizosphere-rhizoplane composite samples. Under laminar flow of each composite sample, aliquots were taken to perform enrichment cultures, conservation at -80°C in polystyrene bags, physicochemical analysis and heterotrophs microorganisms cell counts on plates.

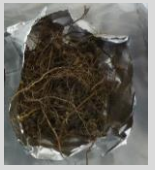
4.2.3.3 Conservation of composite samples

Aliquots of every composite soil sample were stored by placing approximately 30 grams of soil into polystyrene bags, which were stored at -80 ± 1 °C. Aliquots of all samples were preserved.

For storage of composite rhizosphere-rhizoplane samples, approximately 1.5 ml of each sample was introduced into microtubes with 2.0 ml capacity. Five microtubes per sample were stored in the freezer at -80 ± 1 °C. Aliquots of all samples were preserved.

Table 4.1 presents the workflow carried out for each experimental unit and the nomenclature assigned to each composite sample. In total, twenty-four composite samples were obtained, twelve of them corresponding to rhizosphere-rhizoplane and twelve corresponding to soil samples.

Table 4.1. Diagram showing treatment performed to each of the thirty-six samples taken in the field. Of all samples, twenty-four composite samples were made.

Original Sample	Individual Samples	Individual Samples Processing	Composite Samples	Composite Samples Processing	Name of Composite Sample
	 Soil	1º Sieving (mesh size 2.0 mm) 2º Mass measurement	 Soil conformed by three individual samples from the same plot	- Enrichment culture - Heterotrophs microorganisms cell count on plates - Storage - Physicochemical analysis	L1G -278RR - L1G-278BS L1G-935RR - L1G-935BS L1G-146RR - L1G-146BS L2T-297RR - L2T-297BS L2T-368RR - L2T-368BS L2T-154RR - L2T-154BS
	 Rhizosphere-Rhizoplane	1º Mass meassurement 2ºSequential isolation in Erlenmeyer flasks	 Extraction solution obtained mixing 3 individual samples from the same plot	- Enrichment cultures - Heterotrophs microorganisms cell count on plates - Storage	L3G-418RR - L3G-418BS L3G-765RR - L3G-765BS L3G-293RR - L3G-293BS L4T-286RR - L4-286BS L4T-974RR - L4T-974BS L4T-513RR - L4T-513BS

4.2.4 Enrichment cultures to obtain glyphosate degrading and tolerant microorganisms

4.2.4.1 Enrichment cultures

In order to obtain pure strains of glyphosate degrading and tolerant microorganisms, enrichment cultures were made to composite rhizosphere-rhizoplane samples and composite soil samples obtained through the proceedings detailed in section 4.2.1. To fulfil this task, a basal salt medium proposed by Radosevich (Radosevich et al., 1995) was used, in which modifications where made to fit the purposes of this specific study. The formulation of the basal salt medium with added glyphosate (BSM-Gly) consists in inorganic salts of macro and micronutrients, glucose (1 g L^{-1}) as carbon and energy sources and glyphosate (technical grade of the acid, >95% purity, 500 mg L^{-1}) as the only phosphorus source (for details of the medium composition refer to section S4.1.1). During the first three subcultures, antimycotic cycloheximide (0.100 g L^{-1}) was added to prevent fungal growth.

During the BSM-Gly preparation, macronutrients and micronutrients solutions were separately sterilized to avoid the precipitate formation during the process. Stock solutions of glyphosate and cycloheximide were prepared and sterilized by filtering (0.20 µm), and later added in a suitable amount to achieve the proper concentration in the medium. Inside each 250 mL Erlenmeyer flask, 50 ml of BSM were made, which were inoculated immediately after with 5.000 grams of composite soil sample or 5.00 mL of composite rhizosphere-rhizoplane sample, accordingly. The entire process, that involved transference of the different solutions and the inoculation, was made under laminar flow. The enrichments were incubated at 25 ± 1 °C under 200 rpm agitation for six days. A total of six subcultures were carried out for each composite sample, where each subculture was inoculated with 5.00 mL of the previous culture.

4.2.4.2 Isolation of microorganisms

After the sixth subculture, the isolation process of degrading and tolerant microorganisms was performed. The culture medium used was the enrichment medium (BSM-Gly) added with agar 15 g L^{-1} in Petri dishes. From the last subculture, an aliquot portion of 0.1 mL was taken to make serial dilutions with

base 10. Afterwards, these dilutions were pipetted in the Petri dishes containing BSM-Gly medium and incubated at 25 ± 1 °C for five days.

After the incubation time, and observing the microbiological growth, the isolation process was carried out based on the morphology of each colony. Single colonies were selected by its morphotype, a unique code was assigned to each of them for identification purposes and then streaked again on Petri dishes with fresh BSM-Gly. All Petri dishes where incubated again at 25 ± 1 °C for five days. After re-growth, single colonies where selected again and streaked on Petri dishes with fresh BSM-Gly and R2A medium (Reasoner and Geldreich, 1985) (S4.1.2) in order to corroborate its purity.

4.2.4.3 Conservation of microorganisms

Pure colonies conservation was made through two different methods. Each colony was reseeded in liquid BSM-Gly medium to obtain an adequate biomass amount. As soon the culture reached its maximum Optic density value, a part of it was transferred to a sterile tube and then centrifuged at 10,000 rpm at 10 ± 1 °C for 20 minutes. Supernatant was discarded, and the pellet was resuspended in 3 mL of BSM solution and 40% glycerol, to be later transferred to three different cryotubes. The cryotubes were stored at -80 ± 1 °C. The remaining volume in the cell culture was centrifuged in the same manner as the later. Supernatant was discarded, and the resulting pellet was resuspended in a 10% milk powder solution and then transferred equally into three different cryotubes, concealed with cotton swabs, frozen at -80 ± 1 °C and later lyophilized.

Lastly, a sample was taken from each culture made for strain conservation for purity check in solid BSM-Gly and R2A mediums. Petri dishes were incubated at 25 ± 1 °C for twenty days and then stored in cold room for ninety days.

4.2.5 Heterotrophs microorganisms cell counts on plates

In order to estimate the number of aerobic heterotroph microorganisms present in each composite sample, 1.000 gram of soil (or 1.00 mL of rhizosphere-rhizoplane sample) was taken and placed in a sterile 15 mL plastic tube, with 9 mL of a 0.09% NaCl Tween 80 0.01% solution and then put under constant agitation for 30 minutes. An aliquot of 0.1 mL of the suspension was taken and

then a series of dilutions with base 10 was made in microtubes. Volumes of 0.1 mL from each dilution were sown in Petri dishes with R2A medium by triplicate. The dishes were incubated for five days at 25 ± 1 °C.

The counting was carried out in those plates showing colony forming units (CFU) between 30 and 300. In the case of soil samples, the number of dilutions and relative humidity were taken into consideration when estimating the results, informing the number of CFU per gram in dry soil (UFC g^{-1}). On the contrary, when expressing the results in the case of rhizosphere-rhizoplane samples, the number of dilutions and the root mass used for extraction were taken into consideration, informing the number of UFC per gram of root (UFC g^{-1}).

4.2.6 Total DNA isolation

The total DNA from soil samples was isolated by using commercial kit MO BIO PowerSoil® DNA Isolation (MO BIO Laboratories, Carlsbad, USA). As rhizosphere-rhizoplane samples are liquid, total DNA isolation was carried out in tandem by using Qiagen DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) followed by a MO BIO PowerSoil® DNA Isolation. This was necessary since liquid extract samples showed high amount of humic matter and a low content of soil particles. Both purification processes were done as specified by the manufacturer.

4.2.7 Microbial community analysis in soil and rhizosphere-rhizoplane samples

ARISA was performed on all extracted DNA samples. The PCR of the ISR region, was carried out using primers set forward: TGCGGCTGGATCCCCTCCTT - reverse: CCGGGTTTCCCCATTCGG specific for bacteria (Sillen et al., 2015). PCR was performed in a 25 µL reaction volume containing: 1X PCR buffer, $MgCl_2$ 2 mM, 0.1 µM of primers, dNTPs 0.5 mM, 0.12 µL of DNA polymerase Platinum Taq High Fidelity polymerase (Thermo Fisher Scientific, Waltham, USA) and 1.0 µL of DNA. The cycling conditions were: 3 minutes at 94°C, 25 cycles of 60 seconds at 94°C, 30 seconds at 56°C and 60 seconds at 72°C, followed by a final extension step of 5 minutes at 72°C. Products were checked in a 1 % agarose gel electrophoresis.

PCR products were loaded in an Agilent DNA 1000 chips according to manufacturer specifications and then automatic capillary electrophoresis based on DNA fragment detection by fluorescence was carried out in a 2100 Bioanalyzer® (Agilent Technologies, Diegem, Belgium). Electropherograms and densitometric profiles for each run were acquired from Bioanalyzer® software and then analyzed using StatFingerprints package, on R software v2.13 (http://cran.at.r-project.org). Statistical analysis and plots were made with PAleontological STatistics (PAST) v3.14 (Hammer et al., 2001) and GraphPad v5.1 programs.

4.3 Results and discussion

4.3.1 Physicochemical properties of soils

Manantiales Experimental Farm provided the details of the soil taxonomic criteria which, according to the USDA (United States Department of Agriculture), are specified as: order Mollisol, suborder Aquoll, group Natraquoll, subgroup Typic. The texture and physicochemical characterization of both plot pairs (L1+L2 and L3+L4) was performed and the results are shown in Table 4.2.

Table 4.2. Physicochemical properties of soils

Property		Soil	
		L1 + L2	L3 + L4
Texture (%)	Clay	23	18
	Silt	46	50
	Sand	31	32
Element	P $mg\ Kg^{-1}$	6.36	8.6
	N %	0.256	0.2
	K $mEq\ 100g^{-1}$	1.1	1.1
	Ca $mEq\ 100g^{-1}$	20	7.6
	Na $mEq\ 100g^{-1}$	8.6	7.0
	Mg $mEq\ 100g^{-1}$	0.4	8.6
pH		8.3	6.8
Organic Matter (%)		5.9	4.5
CEC ($mEq\ 100g^{-1}$)		6.7	7.4

The results show that both soils possess a loamy texture with low levels of phosphorus, high sodium content and low cation exchange capacity, all properties frecuently found in the Flooding Pampa . These soils also present adequate amount of organic matter and C/N ratio values presumed as normal. Soil samples from plots L1 and L2 are slightly alkaline, while soil samples from plots L3 and L4 presented neutral pH. It's possible that lower phosphorus levels in soils L1 and L2 are related to high levels of Ca cations which, in presence of

alkaline pH, could make phosphorus less bioavailable (favoring the the formation of the insoluble $Ca_3(PO_4)_2$).

The moderate clay content and the presence of divalent metals in these soils (either Ca in L1 and L2 or Ca and Mg in L3 and L4) could be play a relevant role in glyphosate absortion capacity, as the phosphonate group in the molecule can interact with the divalent ions, either those in free form as well as those that make up the different minerals of the clay.

As opposed to what was previously discussed, the neutral to alkaline pH present in these soils, together with the high concentrations of humic matter found, could cause a higher leaching and run-off rates of the molecule.

The decision to search for glyphosate tolerant and degrading microorganisms in these soils was not based solely on the promotion treatment carried out there, but also on the fact that the contents of soluble phosphorus in the Flooding Pampa region are usually very low, being able to favor the presence of microorganisms capable of metabolizing phosphonate groups. Thus, the studied plots also had low phosphorus levels.

The degree in which the regularly applied glyphosate is adsorbed on these soils is ultimately defined by the integration of all complex phenomena involved, some of them mentioned above. Phosphorus as an external factor (fertiliser) was particularly studied and as discussed in the introduction of this thesis, there is evidence indicating that the phosphonate group of the glyphosate competes with the soluble phosphates for the same binding sites present in the matrix. Given the characteristic low content of phosphorus in these soils, the adsortion (and consequently, the retention) of glyphosate could be highly favoured. This analysis is particularly important from the environmental perspective, since it helps to reduce the mobility of a molecule that does not present high levels of residuality in soil, but it does contaminate the water courses that receive the runoff from the different cultivation areas.

During the removal of soil and root sample from the original sample, a higher number of Lotus specimens was observed in treated plots L1 and L3, over control plots L2 and L4, which was reflected in a higher amount of root biomass (Table 4.3. Differences are statistically significant between L3 and L4) in coherence with the promotion with glyphosate used in those plots (Table 4.4). The observed differences in the soil moisture of the plots (humidity is higher in

the control plots than in treated plots) could be related to the previous observation, since the larger presence of Lotus specimens lowers the plant coverage in the plots, possibly resulting in a loss of humidity.

Considering the wide soil diversity in the Argentinian humid Pampa, the analysis of soil's physicochemical properties constitutes a main factor when proposing a rhizoremediation strategy as the one intended here. The implantation process and survival of the plant species will have, as main variable, the effect the edaphic properties and terrain conditions exert on them. Rhizoremediation field tests should include physicochemical measurements usually carried out during the characterisation of any agricultural plot, results which will serve to compare to those here obtained.

Table 4.3. Root biomass per plot plot

Plot	Root weight (g)
L1	2.9 ± 1.6
L2	2.4 ± 1.1
L3	4.3 ± 2.6
L4	0.35 ± 0.14*

*Differences are significant with respect to plot L3 (n = 22, p<0,05)

Table 4.4. Average moisture per plot

Plot	Soil moisture (%)
L1	28.0 ± 7.0
L2	33.0 ± 8.5
L3	14.3 ± 0.7
L4	17.3 ± 1.9

4.3.2 Bacteria isolation

The twenty-four enrichment cultures, resulting from the six subcultures per composite sample in BSM-Gly, presented turbidity with maximum cell development between days 2 and 4. The cultures that were originally inoculated with rhizosphere-rhizoplane composite samples, had an average higher cell density than those that were inoculated with composite soil samples (Figure 4.3).

All cultures were plated BSM-Gly medium to obtain single colonies which may be feasible to be purify using classic microbiology techniques.

After the colonies growth, it was observed that, apart from a few plates, only one type of morphology was distinguishable. Each one of these colonies was purified, its purity corroborated in various solid media and then inoculated again in liquid medium. Of the total of colonies originally purified, twenty-four strains were able to grow again and develop a considerable cell density. These twenty-

four strains were preserved as details in section 4.2.4.3, to be characterized once verifying its survival after three months of conservation. In chapter 5, the biochemical and molecular characterization of these microorganisms is explained in detail.

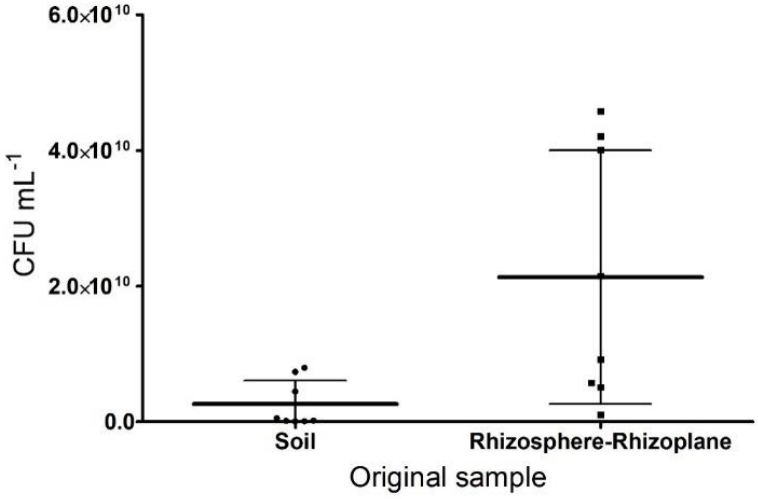


Figure 4.3. Values of CFU mL^{-1} found in enrichment cultures according to the origin of the samples. The differences between the origin of the sample are significant with $p<0.05$ (T-test, n = 8). The thick bars represent the mean, while the thin bars represent the standard deviation.

4.3.3 Heterotroph microorganisms count of the composite samples

Simultaneously to the proceedings described above, heterotrophs microorganisms' counting was carried out in all composite samples (figures 4.4A and figure 4.4B).

The average of CFU per gram of root obtained was $1{,}54.10^7$, and no significant differences between the plots were found (Figure 4.4A).

The number of the cultibable microorganisms obtained during an isolation procedure correspond basicly to 3 factors, which are: microorganisms present in the sample, the isolation method and the medium used for growing the microorganisms.

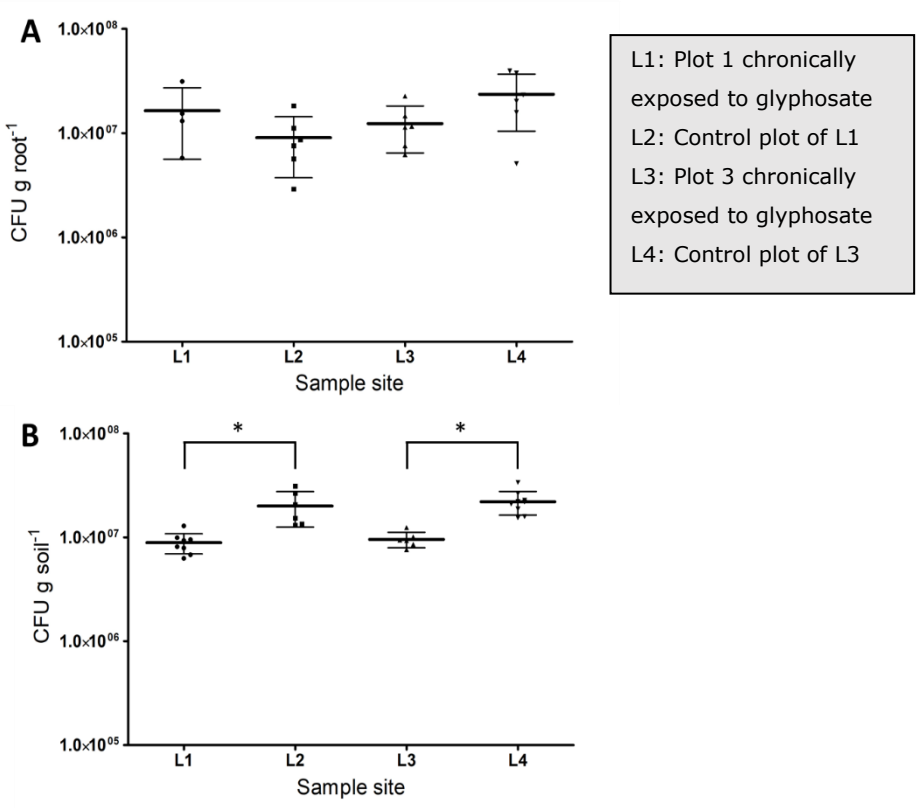


Figure 4.4.A. Number of CFU per root gram. There are no significant differences between sample sites. B. Number of CFU per gram of dry soil. Samples showed significant differences between plots L1 and L2 ($p<0.01$), and between plots L3 and L4 ($p<0.01$) (T-test, n = 8).

Due to the already mentioned 'rhizosphere effect', the abundance of microorganisms in the surroundings area of the root in any vegetal species exceed by many orders of magnitude to those found in the soil with no roots.

The isolation method for rhizosphere soils depends on the rhizosphere definition being considered (Hartmann et al., 2008) and the importance given to the rhizoplane (the root area in contact with soil) as source of relevant biological resources. Therefore, in some isolation proceedings, the soil softly attached to the root is removed by vigorous mechanical shaking (HUANG, 2018; Park et al., 2005) (bulk soil), followed by a second stronger shaking or by manually removing the root. The recollection of the detached soil in this second step (rhizosphere soil)

constitutes the sample used to carry out the isolation. In other studies, after the first shaking to remove the weakly attached soil, roots are transferred into a tube or reservoir with the extraction solution, with the purpose of separating from roots the strongly attached soil and root attached bacteria (Barillot et al., 2013; Hammami et al., 2013).

This latter approach was chosen to accomplish the particular objective, as it was considered that rhizoplane microorganisms were also of importance to the proposed biotechnological strategy. Thus, and with the intention of eliminating errors during root handling and weighing, it was decided that the result would be expressed in terms of fresh root weight. This way, even though there's no data to compare 'bulk soil gram to rhizosphere soil gram', it was possible to have an idea of the reproducibility of the microorganisms' extraction method quite well. This is rather relevant when dealing with 'in house' proceedings. Because there weren't any significant differences between the different composite rhizosphere-rhizoplane samples, it's possible to assume that the extracting method would not be a potential source of bias in future analysis.

Regarding the culture medium used for the recount, the selected medium was chosen due to its complex nutritional composition (Balestra and Misaghi, 1997; Vieira and Nahas, 2005) and for being indicated for the growth of slow growers microorganisms, as is the case of R2A medium (Reasoner and Geldreich, 1985) (S4.1.2)

When analyzing the CFU per gram of composite soil samples, significant differences were observed on the two treated plots when comparing to corresponding control plots. The treatment (or its consequences, like dissimilarities in coverage between treated and control plot) could be responsible of this difference in cultivable colonies number. The number of microorganisms per gram of soil lies among the expected values (Whitman et al., 1998).

4.3.4 Analysis of microbial communities

When proposing a rhizorremediation strategy based on bioaugmentation, the exploration of microbial communities makes sense and becomes relevant, since it is important to know if changes in soil managements or physicochemical properties have any ifluence on them.

The Bioanalizer® system delivered electropherograms showing fluorescence profiles having different intensity peaks positioned according to the molecular weight (expressed in base pairs). Each electropherogram was converted into an intensity signal to molecular weight matrix, which was then exported and analyzed by the StatFingerprints software. Using this software, each profile was processed to carry out statistical analysis. Figure 4.5 illustrates these profiles.

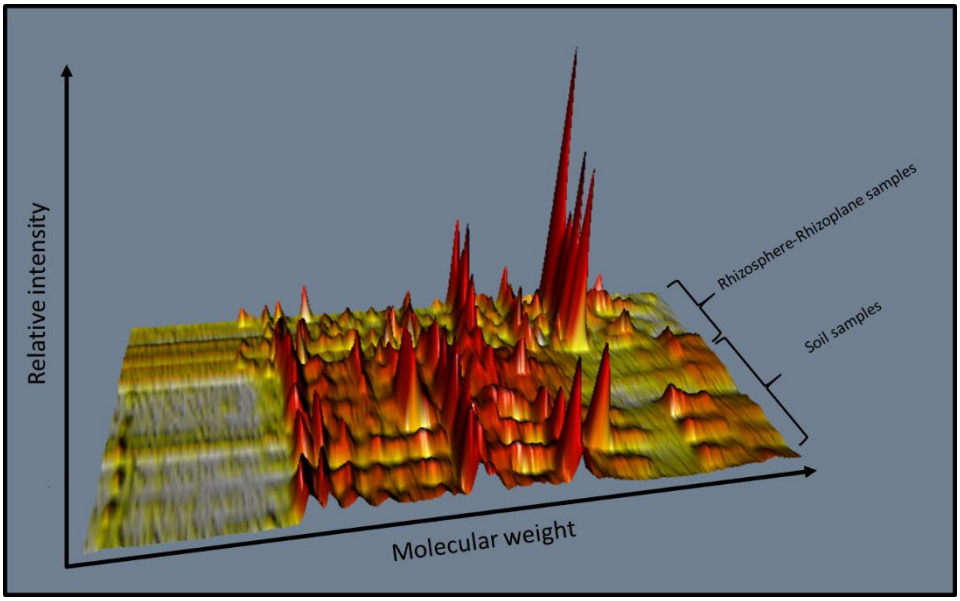


Figure 4.5. 3D ARISA profiles of all composite samples after being processed using StatFingerprints for analysis.

In order to understand the similarity between profiles obtained for each sample, *clustering* analyses with Bray-Curtis similarity index, whose criterion considers abundances, was carried out in combination with Ward's algorithm to compute the hierarchical clustering of the heatmap. Results are shown in Figure 4.6.

According to the results, a marked separation is observed between soil samples and rhizosphere-rhizoplane samples, represented in the two main clades. It is also possible to observe that samples from plots L1 and L2 are grouped together in the same clade and samples corresponding to L3 and L4 are grouped together in another clade inside the soil clade.

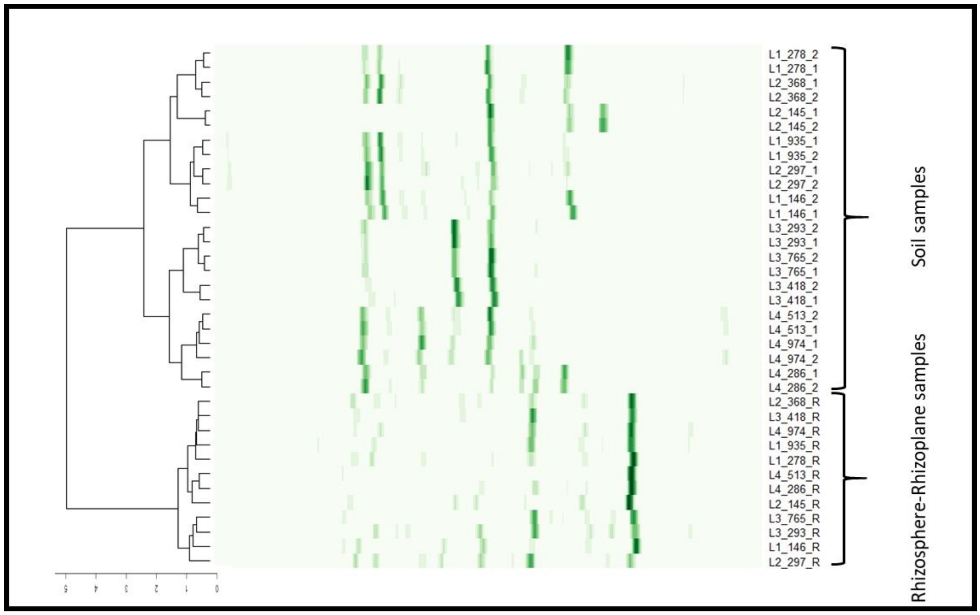


Figure 4.6. Cluster analysis of composite samples. The hierarchical clustering is shown in the left, and 2D ARISA heatmap is shown in the center. Bray-Curtis similarity index was used for clustering, in combination with Ward's algorithm to compute the hierarchical clustering of the heatmap.

The analysis proceeded through comparing the different alpha diversity indices. Three were taken into consideration: number of peaks (as richness index), Shannon index and inverse Simpson index.

Results shown no -significant differences bewteen composite soil samples nor between rhizosphere-rhizoplane samples for any of the analysed parameters (ANOVA and Tukey *post hoc* test, with a significance level of $\alpha = 0.05$), apart from rhizosphere-rhizoplane sample corresponding to plot L4 (control for plot L3) which showed significant differences with all soil samples (Shannon and 1-Simpson) and rhizosphere-rhizoplane sample from plot L3 (1-Simpson) (Figure 4.7).

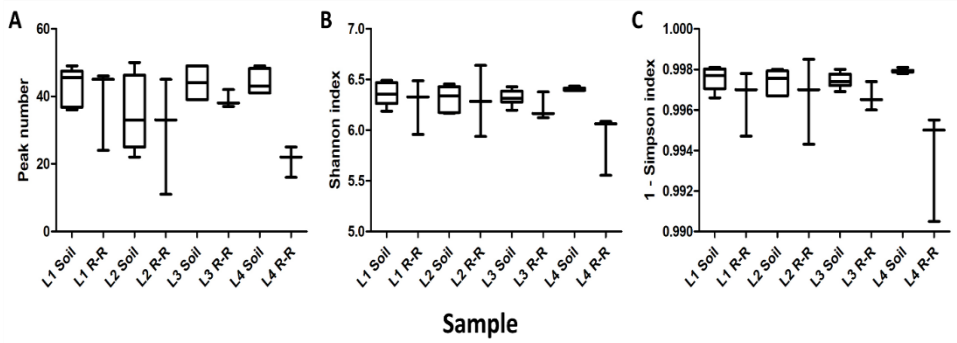


Figure 4.7. Alpha diversity indices of samples. A. Number of peaks. B. Shannon index C. 1-Simpson index. The mean and standard deviations are represented for each sample (n = 6 for soil samples, n = 3 for rhizosphere-rhizoplane samples)

The non-metric multidimensional scaling multivariate analysis (nMDS) with the Bray-Curtis distance metric for the ARISA fingerprint was employed to compare the structure of communities of all composite samples. Each sample is represented in a space of two dimensions, where the proximity between objects corresponds to a greater similarity between them (Ramette, 2007). The analysis of similarities ANOSIM, with 9999 permutations, was used to assess the differences between the various groups of samples. Figure 4.8 shows the different nMDS plots, indicating the different comparisons made. Likewise, the associated stress to each generation, the statistic test result R and p (significance), referred to each analysis related to colored ellipses is shown on the left side of every graph.

There is a significant difference between soil samples and rhizosphere-rhizoplane samples (R: 0.97, p: 0.001 ANOSIM) (Figure 4.8A). Additionally, by a global analysis of all the treated samples compared to all control samples, it's also possible to conclude that the promotion treatment would not cause significant differences in bacterial community structure (Figure 4.8B).

When soil samples are studied separately, a slight separation appears between the different types of soil (Figure 4.8C), indicating that communities structures could be affected by soil properties; a conclusion already reported in literature by means of other *fingerprinting* methods (Kuramae et al., 2012; Bajsa et al., 2013).

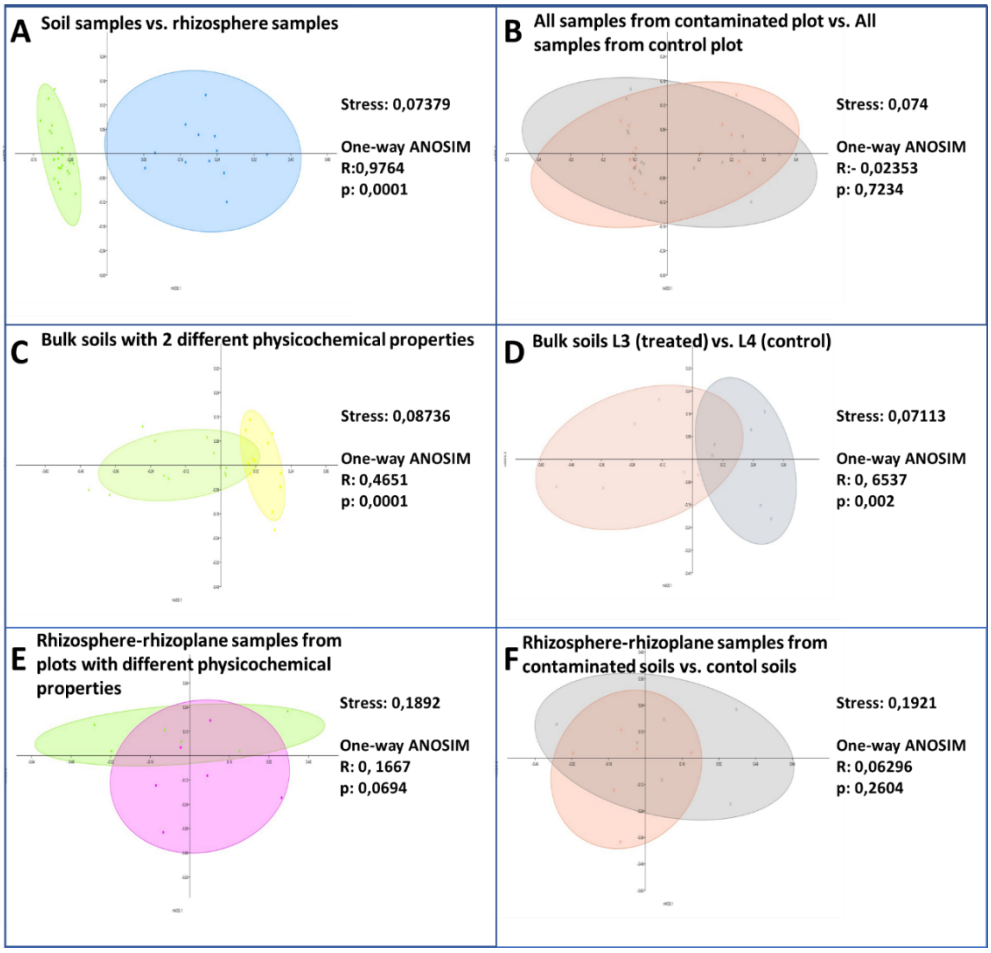


Figure 4.8. nMDS plots analysis. Group means are shown with a probability ellipse (standard deviation, p=0.95) in left plots. Group means are shown with a probability ellipse (standard deviation, p=0.68) in left plots

A- Comparison between soil samples (in green) and rhizosphere-rhizoplane samples (in blue).

B- Comparison between all samples corresponding to treated plots (L1 and L3, in pink) and all samples corresponding control plots (L2 and L4, in grey).

C- Comparison between soil samples from plots L1 and L2 (in green) and soil samples from plots L3 and L4 (in yellow).

D- Comparison between soil samples from plot L3 (treated, in pink) and from plot L4 (control, in grey).

E- Comparison between rhizosphere-rhizoplane samples from plots L1 and L2 (in green) and soil samples from L3 and L4 (in violet).

F- Comparison between rhizosphere-rhizoplane samples from treated plots (L1 and L3, in pink) and rhizosphere-rhizoplane samples form control plots (L2 and L4, in grey).

When observing the distribution of each soil sample, soils from L3 and L4, which face treated soil versus control soil, were also compared (Figure 4.8D). In this case, the statistical test result shows a more significant separation between groups and less overlapping, with a better significance (R: 0.6537, p: 0.002). Other studies carried out in agricultural soils, where different soil management associated with the use of glyphosate take place, also show changes both in soil and rhizosphere community structures (Ratcliff et al., 2006; Kremer & Means, 2009; Vera et al., 2010; Lane et al., 2012; Newman et al., 2016b). When samples from plot L1 and L2 compared, neither significant difference were observed (data not provided).

Lastly, rhizosphere-rhizoplane samples showed no significant differences between them, both from comparing plots having different soil properties (L1 and L2 versus plots L3 and L4) (Figure 4.8E) and soils having different treatments (treated plots L1 and L3 versus control plots L2 and L4) (Figure 4.8F). This would indicate that the so-called rhizosphere effect is more relevant than the soil properties or the pasture promotion method using glyphosate when explaining the differences among microbial communities.

Rhizosphere-rhizoplane samples, as already mentioned, presented well differenciated communities to those present in soil samples. But there were no significant difference on them in terms of plot of origin nor treatment. This observation provides the opportunity for future studies on the influence this legume may have on the communities of the rhizosphere and the resilience when faced with the various agronomic practices involving the use of agrochemicals. As far as the rhizoremediation strategy, such a phenomenon could be much appreciated when carrying out inoculations with different microorganisms, since working with microorganisms belonging to this community structure modulated by the root may provide guarantee for stability.

4.4 Conclusions

Based on the work carried out during the sampling process, it was possible to obtain a culture collection of pure strains resulting from different types of samples. The isolation process designed for rhizosphere-rhizoplane microorganisms yielded around 1.10^7 UFC g^{-1} microorganisms per gram of root, sufficient microorganism numbers to perform the subsequent enrichment and isolation.

The physicochemical characterization and the study of the community structure of soils made possible to answer questions that had been part of the sampling planification. It was decided that samples would be collected from soil without Lotus' roots and rhizosphere-rhizoplane soil, assuming a difference between microbial communities in the matrices. This difference was later corroborated by the used fingerprinting method.

In regards to the influence by treatment of Lotus promotion using glyhposate, quantitative and qualitative effectes were observed: the total number of heterotroph microorganisms in treated soils was higher than in control soils (quantitative effect) and when communities structures were compared, there was a weak difference between plots L3 and L4 (qualitative effect).

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S3.1 Supplementary data

S4.1.1 Basal Salt medium with glyphosate (BSM-Gly)

Reagent	**Amount (g L^{-1})**
Glucose	2.000
$MgSO_4$	0.244
$(NH_4)_2SO_4$	0.500
$NaHCO_3$	0.175
$FeCl_3.6H_2O$	0.015
$CaCl_2$	8.6 mg
$MnCl_2$	0.16 mg
$ZnSO_4$	0.018 mg
GLyphosate	0.500
Cycloheximide	0.100

pH: 6.8

Reference:

Radosevich, M., Traina, S. J., Hao, Y. L., and Tuovinen, O. H. (1995). Degradation and mineralization of atrazine by a soil bacterial isolate. Appl. Environ. Microbiol. 61, 297–302.

S4.1.2 R2A Medium

Reagent	**Amount (g L^{-1})**
Yeast extract	0.5
Peptone	0.5
Casein hydrolysate	0.5
Glucose	0.5
Starch	0.5
$K_2H(PO_4)$	0.3
$MgSO_4$	0.024
Sodium Pyruvate	0.3
Agar	15.0

pH: 7.2 ± 0.2

Reference:

Reasoner, D. J., and Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7.

Chapter 5 - Identification, characterization and full genome sequencing of glyphosate-tolerant microorganisms

5.1 Introduction

Rhizoremediation (or microorganism-assisted phytoremediation) implies xenobiotic degradation by native microorganisms associated to the root in a reasonable time; this process will succeed depending on the competence these microorganisms present for such purpose. When glyphosate rhizoremediation is attempted, this is particularly important for two reasons mentioned before in this thesis, but which are worth highlighting again: (1) Glyphosate is not degraded in plant tissue, and (2) It has low hydrophobicity ($\log K_{ow}$ <-3.2), which makes the diffusion through root tissue difficult. As it was discussed in chapter 4, Although *L. corniculatus* "Gladiador" may be able to create a rhizosphere environment capable of degrading glyphosate, the seed inoculation with a tolerant and degrading bacteria capable of settling in the rhizosphere and, therefore, of occupying the root influence area, would increase the possibility of establishing a successful rhizoremediation system (Kuiper et al., 2004; Gerhardt et al., 2017). The search for the most rhizocompetent strain should not only be based on the appropriate glyphosate degradation and tolerance capacity, but also on the ability to settle in the long term in the root of *L. corniculatus*. For such purpose, the exploration of direct plant growth promotion abilities tests was included during the characterization of isolated the microorganisms, intending these favorable traits shall constitute a future benefit for plant species. The characterization of the possible plant-microorganism interaction allows to widen the strategy in the future since, once the growth promotion mechanisms are identified and recognized, the inoculation can be performed on other plant species.

Finally, the availability of a culture collection of glyphosate tolerant and degrading microorganisms further represents a biotechnological advantage, since it would allow their use in different bioremediation processes. In this sense, the whole genome sequence of the promising microorganisms, would allow to understand future scopes and to evaluate other related traits, such as the production of specific enzymes or biomolecules of commercial interest.

5.1.1 Glyphosate-tolerant bacteria

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC2.5.1.19), also called aroA, is the glyphosate only molecular target.

Ever since Steinrucken and Amrhein discovered in 1980 that EPSPS is the molecular target of glyphosate (Steinrücken & Amrhein, 1980), many structural and functional studies have been carried out on this enzyme. Based on the inherent sensibility present on EPSPS organisms, it has been divided into two types or "Classes". Class I enzymes are those present in plants and in many Gram-negative bacteria and represents the most sensitive molecules. This class is generally represented by the EPSPS of *E. coli*. Class II enzymes can be found in tolerant bacteria, such as *Agrobacterium* sp.CP4, and they exhibit a high catalytic efficiency, even when exposed to high glyphosate concentrations. These enzymes also show important differences in the peptide sequence when compared to Class I enzymes (Pollegioni et al., 2011).

Point mutations in the enzyme sequence, such as the P101S substitution (*S. typhimurium*) or G96A (*K. pneumoniae*), were described for conferring herbicide tolerance. Mutations in G96 cause a loss of affinity to the natural substrate, phosphoenolpyruvate, while P101 mutations results in a lower tolerance than those in G96 but allows an adequate catalytic efficiency. Enzymes with two mutation sites were also reported: the EPSPS G101A/G137D and G101A/P158S present in the plant species *Petunia hybrida,* the EPSPS G96A/A183T present in *E. coli* and the T102I/P106S EPSPS present in *Zea mays*. T102I/P106S EPSPS, named TIPS, has a Class I enzyme peptide sequence, but holds a Class II-like kinetic and tolerance characteristics, being used in transgenic corn events.

Apart from the ones mentioned above, some examples of Class I enzymes are those present in *Aeromonas salmoncida* and *Arabidopsis thaliana*, while some examples of Class II enzymes are those present in *Pseudomonas sp. strain PG2982, Bacillus subtilis, Ochrobactrum anthropi* and *Staphylococcus aureus* (Yi et al., 2016).

In recent years, an acetyltransferase with acceptable affinity to N-(phosphonomethyl)-glycine molecule, the glyphosate acetyltransferase or GAT, was described. The substrate of this enzyme is transformed into N-acetilglyphosate. Once acetylated, it is deprived of its inhibitory activity towards EPSPS synthase. For this reason (and because it does not degrade glyphosate, such as the enzymes involved in the following section), it is considered an alternative resistance mechanism. Three variants of the enzyme were found in

Bacillus licheniformis (Castle, 2004; Siehl et al., 2007), and ongoing research strives to improve their kinetic properties in order to use them as a glyphosate detoxification mechanism in double transgenic events (Guo et al., 2015).

5.1.2 Glyphosate degrading bacteria

Glyphosate is a member of a large family of compounds, the phosphonic acids or phosphonates, which possess the chemically stable carbon-phosphorus (C-P) bond. Its degradation by bacteria occurs by two different metabolic pathways which are the AMPA (aminomethylphosphonic acid) pathway and sarcosine pathway. The first one involves the action of an oxidoreductase (glyphosate oxidoreductase or GOX) or also a glycine oxidase, yielding AMPA and glyoxylate as degradation products. The second one involves specific phosphonates, C-P lyases, yielding sarcosine and inorganic phosphate as degradation products.

The oxidoreductase GOX (EC 1.14.14.1) was first reported on *Ochrobactrum antropi* GPK 3 (Ermakova et al., 2008). Once the glyphosate molecule is oxidized by this enzyme, the glyoxylate is easily incorporated into the cell metabolism, while AMPA (a phosphonate) can be transported outside the cell or metabolized by specific C-P lyases to produce methylamine and phosphate. In other cases, AMPA can suffer transamination via a pyruvate-dependent transaminase forming formyl phosphonic acid and ammonium. The enzyme glycine oxidase (EC 1.4.3.19) has also showed the ability to metabolize glyphosate to produce AMPA, although the mechanism seems to be different than that of the GOX (Zhan et al., 2013). Some bacteria reported as capable of metabolizing glyphosate through the AMPA pathway are: *Achromobacter* sp., *Athrobacter atrocyaneous*, *Flavobacterium* sp., *Geobacillus caldoxylosilyticus*, *O. antrhropi*, *Pseudomonas pseudomallei* (Sviridov et al., 2015).

The use of glyphosate as a source of phosphorus implies that the microorganism has the enzymatic capacity to cleave the C-P bond in the compound. This function is performed by the enzyme C-P lyase, widely distributed among bacteria (Villarreal-Chiu et al., 2012), and its activity requires the expression of a total of 14 polypeptides found in the operon *phn, phnCDEFGHIJKLMNOP*. Table 5.1 describes the function of each of the peptide components. The component *phnCDE* is an ABC-type phosphonate transporter,

while component *phnFGHIJKLMNOP* is responsible for the transformation of the phosphorus in the phosphonate group to 5'-phosphoribosyl 1'-diphosphate (PRPP) and subsequent mineralization (Hove-Jensen et al., 2014).

Among the organisms that use glyphosate as source of phosphorus we can find: *Sinorhizobium meliloti, Ochrobactrum anthropi, Agrobacterium radiobacter, Burkholderia pseudomallei, Nostoc* sp. PCC7120, *Achromobacter* MPS 12A, *Alcaligenes* sp. GL, *Pseudomonas* sp.ASW, *Pseudomonas* sp. GLC11, *Pseudomonas* sp. PG2982, *Rhizobium meliloti* 1021, *Streptomyces* sp. StC.

Table 5.1. *Phn* operon polypeptides. The NCBI Access number shows the sequence used in the search of equivalents in the sequenced genomes.

Phn polypeptide	Activity and/or function	Query Accession Number
PhnD	ABC transport system, periplasmic-binding protein	NP_418529.1
PhnE	ABC transport system, membrane-binding protein	NC_000913.3
PhnF	Regulatory protein	NP_418526.1
PhnG	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	NP_418525.1
PhnH	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	NP_418524.1
PhnI	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	NP_418523.1
PhnJ	C-P lyase, component of PhnGHIJK	NP_418522.1
PhnK	Component of PhnGHIJK, accessory protein,nucleotide-binding protein of an ABC transport system	YP_026282.1
PhnL	Purine ribonucleoside triphosphate phosphonylase component or nucleotide-binding protein of an ABC transport system	NP_418520.1
PhnM	5-Triphosphoribosyl 1-phosphonate diphosphohydrolase	NP_418519.1
PhnN	Ribosyl bisphosphate phosphokinase	NP_418518.1
PhnO	Aminoalkylphosphonate N-acetyltransferase	NP_418517.1
PhnP	Phosphoribosyl cyclic phosphodiesterase	NP_418516.1

5.1.3 Bacteria with plant growth promotion abilities

Plant growth promotion abilities expressed by the microorganisms may be direct, when they facilitate the availability of a certain nutrient or modulate the concentration of plant hormones, or indirect, when they inhibit the action of pathogens or sensibility to different types of stress (Glick, 2012).

The direct mechanisms comprise a series of molecular processes in the rhizosphere and the endosphere, aimed at facilitating the uptake of different nutrients by the plant species. Such nutrients are frequently found in suboptimal concentrations, are not bioavailable, or the polluted environment prevents the uptake.

Perhaps the most widely explored trait is the nitrogen biological fixation. Plants are capable of uptake nitrogen in the form of ammonium (NH_4^+) or nitrate (NO_3^-), but not from atmospheric nitrogen N_2. A certain group of microorganisms, called diazotrophs, can fix the atmospheric nitrogen and transform it into

ammonium, so in this way it may be incorporated to the plant. This process is carried out by nitrogenases coded in the gene complex *nif*, a group of seven different operons coding 20 proteins in a 20–24 kb locus. The diazotroph bacteria can make symbiotic associations in specific organs (nodules) formed by the bacteria and plants where the nitrogen fixation occurs or can be found in free form in the rhizosphere. Among the fixing bacteria we can find the following genera: *Rhizobium, Azospirillum, Sinorhizobium, Mesorhizobium, Azotobacter, Bradyrhizobium, Azorhizobium* and *Allorhizobium*.

Phosphorus in soil, which is an essential nutrient for plant development, is mostly found in insoluble forms, hence not bioavailable. That is why the ability certain microorganisms possess to solubilize phosphorus therefore rendering it bioavailable for the plant is of great interest. Phosphorus can be found in its inorganic form, generally as apatite or calcium phosphates, or several organic forms, such as inositol phosphate (phytates) and phosphate esters.

Bacteria solubilize inorganic phosphate by a decrease in pH, the secretion of chelating molecules, the formation of soluble metal complexes, displacement of those insoluble complexes (with calcium, aluminum, iron, etc.) and the secretion of low molecular weight organic acids such as gluconic acid and 2-ketogluconic acid. In regards to the mechanisms involved in organic phosphates solubilization, we can find enzymes such acid phosphatases, phytases and C-P lyases (Sharma et al., 2013; Oteino et al., 2015). Among the bacterial species commonly related to this activity, the following can be mentioned: *Alcaligenes* sp., *Aerobactor aerogenes*, *Achromobacter* sp., *Actinomadura oligospora*, *Agrobacterium* sp., *Azospirillum brasilense*, different species of *Bacillus*, *Bradyrhizobium* sp., *Brevibacterium* sp., *Citrobacter* sp., different species of *Pseudomonas*, *Erwinia* sp., *Micrococcus* sp., *Enterobacter* asburiae, *Serratia* phosphoticum, *Nitrobacter* sp., *Thiobacillus ferroxidans*, T. *thioxidans*, *Rhizobium* meliloti, *Xanthomonas* sp. (Sharma et al., 2013).

Iron is another element which, even though it is quite abundant in the pedosphere, it presents low bioavailability both for microorganisms and plants. That is why certain bacteria have developed the ability to produce low molecular weight molecules, called siderophores, which, together with adequate transporters, contribute to produce an increase in iron cell acquisition. The plant also benefits from the secretion of these molecules, uptaking the mineral through

the root. There are a vast number of siderophores but not all of them have been identified (Hider and Kong, 2010). In general, they can be classified into 4 groups, in terms of the type of ligand that binds the iron: hydroxamates, catecolates, α-hydroxycarboxylates and mixed ligands (Zawadzka et al., 2006; Barry and Challis, 2009).

The modulation of phytohormone levels is another growth promoting effect that microorganisms typically produce in the different plant species. Phytohormones are crucial in the development of the root structure. That is why, by locally increasing or decreasing their concentrations, the microorganisms can transform the root architecture. A great variety of plants-associated bacteria are capable of synthesizing phytohormones such as auxins, abscisic acid, cytokinins and gibberellins, while producing volatile organic compounds (VOC) and enzymatically modulating ethylene concentration.

The auxin by excellence is the indoleacetic acid (IAA). The IAA affects cell division, tissue enlargement and differentiation, it increases the xylem and root growth rate, controls the vegetal growth, it is involved in the first stage of development of lateral and adventitious roots, it mediates the response to light and photosynthesis, pigment formation, the biosynthesis of several metabolites and the development of nodulation, among other functions. The growth and action on each tissue is modulated by specific concentrations of the hormone. The result between the exogenous production derived from microorganisms and the endogenous production shall be responsible for the effects in the root (Spaepen and Vanderleyden, 2011; Duca et al., 2014).

The volatile compounds, in particular the 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol are stress and root development modulators. The main mechanism by which they are involved in plant growth promotion is the role as regulators in the synthesis of other phytohormones like cytokinins and ethylene. This is why may be cause of growth inhibition under certain conditions as well (Bailly and Weisskopf, 2012; Tahir et al., 2017). The metabolic pathways for the acetoin and 2,3-butanediol biosynthesis include the *Embden-Meyerhof-Parnas* (EMP) pathway, where two molecules of pyruvate form one of α-acetolactate that is subsequently decarboxylated to form acetoin, which can reversibly be converted into 2,3-butanediol.

Ethylene is also a volatile hormone, known for its capacity to induce fruit ripening and flower senescence. In the root, it promotes initiation development stages, inhibits primary and lateral root elongation, activates the synthesis of other phytohormones, inhibits nodulation, plant-mycorrhizal interaction and is involved in the response to stress (Dugardeyn and Van Der Straeten, 2008). The response to stress is the most widely known function of this compound, increasing its production under extreme temperatures, high light exposure, floods and droughts, toxic compounds, radiation, mechanic damage, insects, salinity and pathogens like virus, fungi and bacteria. Ethylene is synthesized from S-adenosyl methionine, which turns to 1-aminocyclopropanecarboxylic acid (ACC) through the ACC-synthase. The secretion of the enzyme ACC-deaminase by the plant growth promoting microorganisms cleaves the precursor. The depletion of this molecule therefore results in the inhibition of the distinctive inhibiting effect of this hormone (Glick, 2003, 2014). Among the bacterial species bearing this enzymatic activity are: *Alcaligenes* spp., *Bacillus* spp., *Bacillus pumilus*, *Burkholderia* sp., *Burkholderia phytofirmans, Enterobacter* spp*., Enterobacter cancerogenus, Enterobacter clocae, Erwinia herbicola, Klebsiella* sp*., Kibdelosporangium phytohabitans, Micrococcus* sp*., Methylobacterium oryzae, Pantoea agglomerans, Pseudoalteromonas maricaloris, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas thivervalensi, Rhodococcus* sp*., Serratia marcesens , Variovorax paradoxus* among others (Ahemad and Kibret, 2014).

The following specific objectives were met during this stage:

4. Phenotypic and genotypic characterization of glyphosate tolerant and degrading bacteria.
5. Assessment of plant growth promoting (PGP) abilities of the microorganisms obtained in specific objective 4.
7. Genome sequencing of the strain or strains which arise as most interesting with the Ion Torrent® platform and posterior bioinformatic analysis.

5.2 Materials and Methods

5.2.1 Viability control of the stored strains and indirect determination of the use of glyphosate as sole phosphorus source

Three months after isolation, purification and cryopreservation of the tolerant strains, survival and purity control were performed as part of the necessary procedures to establish a culture collection. For this purpose, all the strains were re-cultivated in solid and liquid media: LB (Bertani, 1951), R2A and BSM-Gly.

Strains that showed growth in agar were periodically checked for two weeks to detect possible contaminations. At the same time, it was corroborated that the morphology of the colonies was similar to that of the colonies obtained in in section 4.2.4.2.

In order to control that the only source of phosphorus in the BSM-Gly broth came from glyphosate and not from possible spurious sources, different preventive measures were taken before the characterization of strains. Firstly, the glyphosate reagent used as substrate during the enrichment cultures was replaced for one of maximum purity (>99%, Sigma Aldrich, Buenos Aires, Argentina). Next, 5 cultures were randomly chosen and subcultivated repeatedly in BSM broth without glyphosate or any additional source of phosphorus, in order to observe if the strains were able to growth in absence of the proposed source of phosphorus. Finally, the concentration of phosphate available in the culture medium was measured using the methodology suggested by Murphy and Ripley (Murphy J & Riley JP, 1962).

5.2.2 Genotypic characterization of isolated bacteria

Bacteria cultures were performed in BSM-Gly until the stationary phase was reached. Subsequently, 4 mL of culture were centrifuged, and the biomass obtained in the pellet was re-suspended in 250 µL of TEG (Tris-EDTA-Glucose) buffer solution, with 5 µL of lysozyme (300 mg mL^{-1}), 5 µL of RNase (20 mg mL^{-1}) and 20 µL of protease (7,500 U mL^{-1}). The solution was incubated during 30 minutes at 37 °C ± 1 °C. The total genomic DNA was isolated using the commercial kit Highway DNA PuriPrep-S (Inbio Highway®, Tandil, Argentina)

according to the manufacturer´s recommendations. The amplification of the gene of 16s rRNA through PCR was performed using the universal bacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Concentrations of reagents in the raction tube were: Reaction buffer 1X, $MgCl_2$ 1.5 mM, dNTP-mix 200 µM, primers (fwd and rev) 0.3 µM, polymerase T-Plus 1.5 U. For a 50 µL reaction, 1 µL of DNA was used. The PCR program was: 3 minutes at 94 °C for DNA denaturation, 35 cycles of 30 seconds at 94 °C, 60 seconds at 55 °C and 120 seconds at 72 °C, followed by a final extension of 10 minutes at 72 °C. The products were checked in a 1% gel agarose electrophoresis and subsequently purified with the Highway DNA PuriPrep-GP kit (Inbio Highway®, Tandil, Argentina) according to the manufacturer´s recommendations. The fragments sequencing was performed at the Genomics Research Unit of the Institute for Biotechnology CNIA-INTA (Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina) using an automated capillary sequencer model ABI3130XL (Applied Biosystems®, California, USA).

The most probable taxonomic affiliation was performed through the comparison of the obtained partial sequences of the 16S rRNA gene (an average of 1350 base pairs, representing approximately 95% of the gene coverage) versus homologous sequences of prokaryote strains using the EzTaxon server of the EzBioCloud (Kim et al., 2007; Yoon et al., 2017) database and the Ribosomal Database Project server (RDP) (Cole et al., 2014).

5.2.3 *In-vitro* studies of plant growth promotion abilities

5.2.3.1 ACC-deaminase activity

The method developed by Belimov et al. (2005) was used to assess the production of ACC deaminase. The cultures were performed in 24 well plates containing 1.0 mL of liquid medium 869 (S5.1.1) and incubated during 72 hours at 25 ± 1 °C with no agitation. Then, a centrifugation under 3,000 rpm was performed for 20 minutes and the supernatant was removed. The cell pellets were washed twice with 1.0 mL of $MgSO_4$ 10 mM and then resuspended in 0.5 mL of 10 mM $MgSO_4$. Subsequently, 250 µL of each cell suspension was inoculated in 1.2 mL of ACC deaminase salt medium (S5.1.2), with ACC 5 mM as sole source of nitrogen. The cultures were incubated during 72 hours at 25 ± 1 °C under 100

rpm agitation, centrifuged at 4,000 rpm for 15 minutes and the supernatants were removed. The pellets were suspended again in 100 µL of buffer Tris-HCl 0,1 M (pH 8.5) and a cell lysis was carried out adding 3 µL of toluene followed by vortexing for 10 minutes. Then, 10 µL of ACC 0.5 M and 100 µL of Tris-HCl 0.1 M (pH 8.5) buffer were added, followed by an incubation of 30 minutes at 30 ± 1 °C under 150 rpm agitation. Following, 900 µL of HCl 0.56 N and 150 µL of 2,4-dinitrophenylhydrazine reactive at 0.2 % were added to the cell suspensions. The reaction was performed during 30 minutes at 30 ± 1 °C, and then stopped adding 700 µL of NaOH 2N. One culture of each strain with no addition of ACC was used as control. *Pantoea ananatis* GB1 (Gkorezis et al., 2016) was used as positive control. The presence of α-ketobutyrate indicates ACC deaminase activity, since it is generated by ACC hydrolysis. The absorbance of the compound was visually determined, with the appearance of a yellow color (540 nm).

5.2.3.2 Indoleacetic acid production.

A qualitative assay to assess the production of indoleacetic acid (IAA) by the strains was performed according to that suggested by Patten and Glick (2002). Strains were cultivated in 24 well plates containing 1.0 mL of a 1:10 diluted 869 medium added with 500 mg L^{-1} of tryptophan, at 25 ± 1 °C for 4 days and under 150 rpm agitation. Subsequently, the culture was centrifuged at 3,000 rpm for 30 minutes, and 0.5 mL of the supernatant were carefully removed, avoiding not to touch the pellet. The supernatant was placed in a new well, then 1.0 ml of modified a Salkowski reagent was added (98 ml of perchloric acid 35% and 2 mL $FeCl_3$0.5 M), letting the reaction to develop at room temperature for 20 minutes. The IAA producing bacteria were identified by the development of a pink color (535 nm) in the solution. The strain *Pantoea ananatis* GB1 (previously reported as producer of IAA) was used as positive control. Reaction medium without bacteria was used as negative control.

5.2.3.3 Acetoin production

The production of acetoin by strains was studied using the Voges-Proskauer test, according to modifications made by Romick and Fleming (1998). The strains were cultivated in 869 medium during 24 hours at 25 ± 1 °C in a 24 well plate. Afterwards, 20 µL of each culture were transferred to a new 24 well

plate; 1.0 mL MR-VP medium (S5.1.3). After an incubation of 24 hours at 37 ± 1 °C and 180 rpm agitation, 10 µL of L-arginine (10 mg mL^{-1}), 10 µL of alpha naphthol (50 mg mL^{-1} dissolved in ethanol), and 25 µL of KOH 40% p/v were sequentially added to each well. Then, samples were exposed to air for 20 minutes. The developing of a pink to red color was considered positive, while no change of color or the developing of a light-yellow color was considered negative.

5.2.3.4 Inorganic phosphorus solubilization

The assay was performed following the protocol described by Nautiyal (1999). Cultures of each strain were performed in 869 broth until reaching the late exponential stage, then they were centrifuged at 4,000 rpm for 15 minutes at room temperature. Afterwards, pellets were washed twice with a $MgSO_4$ 10 mM solution. Then, 50 µL of resuspended cells were inoculated in agar with the National Botanical Research Institute's phosphate growth medium (NBRIP) (S5.1.4) which contains 5 g L^{-1} of insoluble $Ca_3(PO_4)_2$. The plates were incubated at 25 ± 1 °C for 2 weeks. The development of a clear halo around the colonies indicated the solubilization of phosphate. The strain *Pantoea ananatis* GB1 (previously reported as a strain capable of solubilizing phosphate) was used as positive control.

5.2.3.5 Phytase activity

The ability of mineralizing phytates by strains was studied according to the method suggested by Jorquera and collaborators (2008). Cultures of each strain were made in 869 broth until reaching the late exponential stage. Subsequently, they were centrifuged at 4,000 rpm for 15 minutes at room temperature and then the pellet was washed twice with $MgSO_4$ 10 mM solution. Next, 50 µL of resuspended cells were inoculated in Petri dishes with phytate-screening medium (PSM) (S5.1.5) containing 4 g L^{-1} of sodium phytate. These were incubated for 10 days at 25 ± 1 °C. The development of clear halos around the colonies was observed, indicating the solubilization of phytates. The strain *Pantoea ananatis* GB1 (previously reported as a strain capable of mineralizing phytates) was used as positive control.

5.2.3.6 Organic acids production

The procedure was carried out following the method suggested by Cunningham and Kuiack (Cunningham & Kuiack, 1992). The strains were incubated in a 24 well plate containing 2.0 mL of 869 medium at 25 ± 1 °C for 48 hours under agitation. The plate was centrifuged at 2,000 rpm for 30 minutes at room temperature. The supernatant was removed, and the pellet was washed twice in $MgSO_4$ 10 mM solution. Next, 20 µL of cell suspension were added to 800 µL of tryptone sucrose medium (TS) (S5.1.6) in a new plate and incubated for 5 days at 25 ± 1 °C and 200 rpm agitation. Finally, 100 µL of Alizarin Red S at 0,1% were added. A change in color from red to yellow was considered positive. *Pantoea ananatis* GB1 (previously reported as a strain capable of producing organic acids) was used as positive control and the TS medium was used as negative control. The Alizarin Red S is a pH indicator that turns from red to yellow in an acid environment. The bacteria capable of producing organic acids shall cause an acidification of the medium and, as a result, it will turn yellow.

5.2.3.7 Siderophore production

The qualitative assessment of siderophores production is based on the method described by Schwyn and Neilands (1987). Each strain was incubated in 96 well plates for 5 days at 25 ± 1 °C in three different 284 (S5.1.7) broths, containing 0 µM, 0.25 µM and 3.00 µM iron (III) citrate. After incubation, 100 µL of CAS solution were added to each well. This solution contains Chrome azurol S (CAS) 60.5 mg L^{-1}, hexadecyltrimethylammonium bromide (HDTMA) 72.9 mg L^{-1}, piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g L^{-1} and $FeCl_3.6H_2O$ 1 mM (in 10 mL HCl 10 mM). After 4 hours, color change from blue to purple (catechol-type siderophores) or from blue to orange/yellow (hydroxamate-type siderophores) was observed on positive wells.

5.2.4 Estimation of the glyphosate minimal inhibitory concentration in agar plates

With the purpose of investigating the maximum concentration of glyphosate that each strain can tolerate, the method proposed by The Clinical and Laboratory Standards Institute, which was originally used to test the response to antibiotics, was followed (Cockerill et al., 2012). For this purpose, Petri dishes

with BSM-Gly in agar with different concentrations of glyphosate (>99%, Sigma Aldrich, Buenos Aires, Argentina) were prepared. The concentrations assayed were: 0, 100, 500, 1,000, 2,000, 3,000, 5,000, 7,500 and 10,000 mg Kg-1; all of them prepared on the same day. The strains were cultivated until they reached cell densities between 1.10^8 CFU mL^{-1} to 2.10^8 CFU mL^{-1}. Once this cell density was reached, a 1:10 dilution in NaCl 0.9% sterile solution was performed, and 2 µL of suspension were added with micropipette in the agar surface, with an estimated number of 1.10^4 CFU. Five replicates of each strain were performed for each concentration. Plates were incubated at 25 ± 1 °C for 6 days and the end points were determined as the concentration under which the growth of the colonies was inhibited in more than 50% compared to the colonies in control plate (BSM without glyphosate). The strain *E. coli* was used as control.

5.2.5 Complementary studies on *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV.

5.2.5.1 Bacterial cell cultures

Aerobic cultures in BSM-Gly broth at 25 ± 1 °C and 200 rpm of both strains were carried out, 50 mg Kg^{-1} glyphosate. In total, 6 independent cultures of each strain were studied, taking samples alternatively, according to the requirements of each measurement. The following assessment were performed:

1. Optical density at 600 nm. The measurement was made spectrophotometrically.
2. Colony forming units per milliliter (CFU mL^{-1}). Through microdroplet technique: 10 µL of each of the 1:10 serial dilutions were dropped and then inoculated in Petri dishes containing 869 rich medium for 3 days at 25 ± 1 °C. The counting was performed on dilutions that showed 3 to 30 CFU.
3. Remaining glyphosate concentration in culture medium. The measurement of glyphosate was performed separately in two laboratories.
   Supernatant of cell cultures were taken at initial time (0 hours) and at ending time (216 hours) by triplicate, storing it at -20°C. Subsequently, subsamples of each supernatant were sent to the laboratory of Doctor Bojana Spirovic Trifunovic from the School of Agriculture of the University of Belgrade, Serbia. Glyphosate was measured by UPLC tandem-mass spectrometry. The protocol

used is briefly described: an aliquot of 100 µL of the sample was transferred to a centrifuge tube of 15.0 mL with 9.0 mL of water and 1.0 mL of KOH 0.1 M, vortexing for 1 minute. Following, 1.0 mL of the mixture was transferred to a new 15.0 mL plastic tube, adding 1.0 mL of H_3BO_3 pH: 9 buffer solution and 0.5 mL of FMOC (Fluorenylmethyloxycarbonyl chloride, 10 mM in acetonitrile) solution. The sample was vortexed for 1 minute and left in the dark overnight. The derivatization reaction was stopped by the addition of 100 µL of H_3PO_4 2 % and 100 µL of EDTA 0.1 M. Finally, the sample was filtered using a cellulose filter and then placed in a vial. The sample was measured in a HPLC system of the Agilent 1260 Infinity (Agilent Technologies, Santa Clara, USA) series coupled with a mass spectrometer 6460 TripleQuad (Agilent Technologies, Santa Clara, USA) equipped with an electrospray ionization interface set up in negative polarity. The chromatographic column used was an Agilent Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 µm), and the mobile phase consisted of two solvents used in gradient by the analyst (Solvent A: 90% 5 mM of NH_4Ac inH_20 / 10% 5 mM of NH_4Ac in methanol solution; Solvent B: 90% 5 mM of NH_4Ac in methanol/ 10% 5 mM of NH_4Ac in H_20 solution). The chromatographic conditions, as well as the mass spectrometer parameters, are reserved by the laboratory.

On the other hand, another 1.0 mL aliquot of the same samples was sent to the company Groen Agro Control (Delft, Netherlands) by duplicate, where glyphosate was also measured through liquid chromatography tandem-mass spectrometry, and under a protocol reserved by the laboratory.

5.2.5.2 Biochemical metabolic profile

The biochemical metabolic profile of the strains *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV was carried out using GN2 MicroPlate™ (Biolog, Hayward, USA) plates. Each plate provides 95 different biochemical tests for Gram negative microorganisms.

Cell suspensions of both strains were prepared, which were inoculated in early exponential phase (DO_{600} of approx. 0.05) into each plate well. Subsequently, the plates were incubated at 30 ± 1 °C, with no agitation and examined daily. Once no further positive reactions were observed, the absorbance of the plates was measured with a FLUOstar Omega (BMG Labtech, Isogen Life

Sciences®, Temse, Belgium) microplate reader. The shift of the medium from colorless to purple indicates the occurrence of respiration in the well and, therefore, the ability of the strain to metabolize or use the substrate present.

5.2.5.3 Complete genome sequencing through the Ion Torrent® platform

According to Edwards and Holt (Edwards & Holt, 2013), the workflow involved in whole genome analysis consisted of 5 steps which are mentioned below:

a- DNA extraction with a maximum purity quality

b- DNA sequencing and *contigs* assembling

c- *Contigs* reordering

d- Genome annotation in different platforms

e- Particular studies on the genome and complementary analysis

The total DNA extraction was carried out from cultures in early exponential growth phase, in order to obtain the highest amount of plasmid copies. The cells were lysed using an enzymatic lysis buffer containing Tris-EDTA, Triton X-100 and lysozyme (pH 8.0) for 30 minutes at 37 ± 1 °C. Subsequently, the DNA was isolated using the DNeasy Blood & Tissue (Qiagen®, Hilden, Germany) kit according to the specifications of the manufacturer. A final DNA concentration of 82.3 ng μl^{-1} for *Ochrobactrum* sp. P6BS-III and 30.9 ng μl^{-1} for *Rhizobium* sp. P44RR-XXIV was obtained. The spectrophotometric quality standard indexes for DNA (260/280 nm and 260 /230 nm ratios) were adequate for both samples. The concentration values and the quality standard indicators were obtained using the UV-Vis Nanodrop 1000 (Thermo Fisher Scientific®, Wilmington, USA) spectrophotometer.

Additionally, the presence of different genomic structures was studied using the protocol adapted from Ramírez-Bahena and collaborators (2012), which consisted in mixing a pellet of each cell culture with 20 µL of a lysis solution (lysozyme 500 U mL^{-1}, RNAase 3.15 U mL^{-1} and 13% of sucrose in TBE buffer) and immediately loading them in the wells of a 0.8% agarose gel with 0.75% sodium dodecyl sulfate (SDS). The samples were run in sextuplicate during 12 hours at 80 V.

Genome sequencing took place at Thomson Rivers University, in Canada.

The sequencing adapters and the bar codes were added to the RNA-free DNA using the Ion Xpress Plus Fragment Library Kit (Life Technologies® Inc., Burlington, Canada). The DNA fragments ligated to adaptors were selected by size (480 pb) on 2% agarose gel E-Gel SizeSelect, and Agencourt MAPure XP (Beckman Coulter®, Mississauga, Canada) spheres were used for purification. The library dilution factor was determined using the Ion Library Quantitation (Life Technologies Inc., Carlsbad, USA) kit previous amplification and enrichment with an Ion PGM Hi-Q OT2 400 kit, using the Ion OneTouch 2 (Life Technologies® Inc., Carlsbad, USA) system. The enriched spheres were quantified using the Ion Sphere Quality Control Kit, and the sequencing was performed in a 316v2 chip, with an Ion PGM Hi-Q View sequencing kit in an Ion Torrent PGM (Life Technologies Inc., Carlsbad, USA) sequencer.

Each of the fragments obtained was assembled using the SPAdes algorithm, version 3.8.2 (Bankevich et al., 2012) (uniform coverage mode; kmers 21, 33, 55, 77, 99). The Mauve software (Rissman et al., 2009) was used to reorder the *contigs* obtained and, in turn, for further studies of comparative genomics.

Genome annotation was performed using the Rapid Annotation using Subsystem Technology (RAST) annotation system (Aziz et al., 2008; Overbeek et al., 2014), NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAP)(Tatusova et al., 2016) and the MicroScope platform using the Magnifying Genomes tool (MaGe) (Vallenet et al., 2006).

The Clusters of Orthologous Genes (COG) and the reconstruction of the metabolic pathways were performed using the MaGe KEGG, MetaCyc and BioCyc tools (Caspi et al., 2016). The metabolic pathways of interest, such as that related to the genes conferring tolerance and which enable the degradation of glyphosate, genes related to the metabolism of phosphate, as well as those genes involved in plant growth promotion abilities, were found in this manner. For each query, homologous of genes were defined according to a cut-off *e-value* of <0.0001, >20% of *query* coverage and >50% sequence similarity. The genome features were also described according to the different properties assignable to the set of annotated genes: Genes in internal clusters were assigned using CD-HIT Suite v4.6.8 (Huang et al., 2010), genes with function prediction were assigned with EggNog v4.5.1 (Huerta-Strains et al., 2016), the *Pfam* domain assignation was

performed using HMMER server (Finn et al., 2015), genes containing signal peptide were assigned using SignalP v4.1 server (Petersen et al., 2011) and finally, prediction of genes with transmembrane helices was performed using the TMHMM Server v2.0 (Krogh et al., 2001).

The comparative genomics studies were performed using the JSpecies web server (Richter et al., 2015) and the Genome-to-genome distance calculator (Meier-Kolthoff et al., 2013). The Tetra Correlation Search algorithm provided by JSpecies was used to carry out a rapid search of the most similar genomes, and subsequently the ANIm, ANIb and GGDC 2.1 methods were executed to determine if such genomes corresponded to the strains in study at a species level.

The overall genomic analysis of the 24 species of the genus *Ochrobactrum* available in the prokaryote genomes database of the NCBI until 06-10-2017 (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/) was performed using the Bacterial Pan Genome Analysis software (BPGA) (Chaudhari et al., 2016) that uses the Usearch clustering algorithm (Edgar, 2010) with an identity cut-off of 50% for sequence clustering. The functional annotation of the unique sequences obtained was performed with EggNog v4.5.1. The Blast Ring Image Generator (BRIG) (Alikhan et al., 2011) was used to generate the genome circular map that includes all of the ORFs with COG, rRNA and tRNAs functional assignments, G+C content and GC skew, and to examine the genotypical differences between *Ochrobactrum* sp. P6BS-III and the representative members of the genus *Ochrobactrum*.

The PHASTER software (Arndt et al., 2016) was used for searching phages and prophages on genome sequences.

5.3 Results and discussion

5.3.1 Identification and characterization of the microoganisms with plant growth promotion abilities

The selection of the glyphosate tolerant and potential degrading microorganisms from soil and rhizosphere samples is part of an integrative and complex technology as is rhizoremediation, in which such microorganisms are not only expected to have the above mentioned abilities, but are also expected to gather a series of traits that make them manageable and applicable for use both in lab-scale and in large-scale applications. Among those traits is the stability under storage and conservation. That is why, before starting with the different strain characterization studies, the survival rate of each of the isolated strains was assessed after three months of glycerol and lyophilized conservation methods. Although subcultures on agar were continued for the originally isolated 24 pure strains, it was decided to set up the culture collection with those strains that had been successfully survived in at least one of the two methods of preservation performed.

From the 24 strains originally isolated, 16 of them showed growth once again when cultivated. It was verified that, in liquid medium, the cultures developed a similar growth to that recorded before storage, while in different agar media, the morphology similarity of colonies was verified in comparison with plates kept in cold chamber and images taken during isolation. The 16 cultures matched in such characteristics, therefore the work continued with the entirety of the strains.

Next, the growth in BSM liquid medium without glyphosate or any source of phosphorus was assessed in five strains randomly selected, and it was compared to the same cultures in BSM-Gly. Figure 5.1 shows the growth curves for the five strains selected after the third subculture.

After 24 hours of starting the cultures, those performed in BSM-Gly abandon the *lag* phase and begin the exponential phase to reach the highest growth about at 96 hours, while the cultures performed in BSM without any source of phosphorus do not enter the exponential phase, showing a significant inferior growth compared to that reached by those supplemented with glyphosate.

During the first and second culture a considerable growth was observed in the strains in BSM without glyphosate (data not shown). This is probably due to the fact that the strains retain a stock of intracellular phosphorus, which must be depleted before effectively assessing the influence of the phosphorus availability. The intracellular phosphorus pool depletion strategy was used by Sushkova and collaborators (2012) in order to store cell biomass with active degrading capacity to be used in bioremediation experiments. Apart from that, strains that can store glyphosate in the intracellular space have been reported (Liu et al., 1991).

In order to corroborate the absence of soluble phosphorus, it was spectrophotometrically measured in the culture broth. No result obtained was above the quantification limit of the method (0.05 mg Kg^{-1}) The growth observed in BSM-Gly could not be stoichiometrically granted at these phosphorus concentrations; considering a typical bacterial elemental formula as C:N:P 45:9:1 (mol to mol) (Heldal et al., 1997) and the values of C and N provided by the BSM.

Having replaced the glyphosate used in the enrichment process for one of higher quality (>99% purity), it can be assumed that there are no alternative sources of phosphorus stemming from the culture medium (traces deriving from the salts that comprise it), nor part of the glyphosate used (sub products deriving from the synthesis, such as AMPA and other phosphonates).

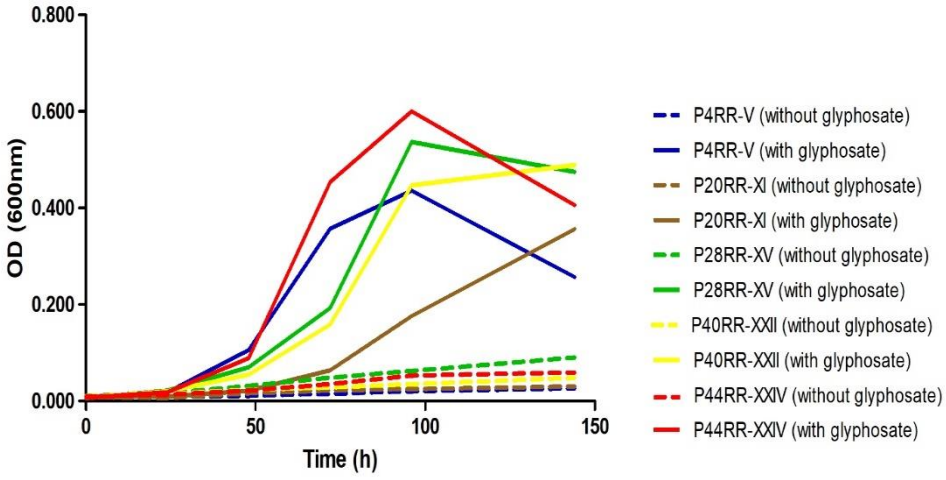

Figure 5.1. Growth cultures of degrading microorganisms in absence of glyphosate (dashed lines) and with glyphosate (continuous lines).

The identification of strains of was performed through the partial amplification, sequencing and bioinformatic analysis of the 16s rRNA gene, and the results are showed in table 5.2.

Table 5.2. Cultivation traits, Gram staining, genotyping results and GenBank access number of glyphosate tolerant and degrading microorganisms.

Source	Strain	Colony morphology	Gram	Most likelyhood neighbor (type strain)	Identity	Coverage	GenBank Accession Number
L.tenuis rhizosphere-rhizoplane	P8RR - IV	Mucoid, bright-white	-	*Rhizobium vallis* CCBAU 65647	99.56	96.4	MG786743
	P4RR - V	Mucoid, bright-white	-	*Rhizobium mayense* CCGE526	100.0	68.3	MG786744
	P12RR - VI	Mucoid, bright-white	-	*Rhizobium vallis* CCBAU 65647	99.41	96.4	MG786745
	P16RR - IX	Mucoid, bright-white	-	*Rhizobium vallis* CCBAU 65647	99.56	96.2	MG786746
	P20RR - XI	Mucoid, bright-white	-	*Rhizobium vallis* CCBAU 65647	99.52	88.9	MG786747
	P20RR - XII	Mucoid, pale - white	-	*Ochrobactrum anthropi ATCC 49188*	100.0	95.5	MG786748
	P28RR - XV	Mucoid, bright-white	-	*Rhizobium miluonense HAMBI 2971*	100.0	96.4	MG786749
	P32RR - XVIII	Mucoid, bright-white	-	*Rhizobium vallis* CCBAU 65647	99.56	96.2	MG786750
	P40RR - XXII	Mucoid, pale -white and irregular margins	-	*Rhizobium miluonense* HAMBI 2971	100	95.2	MG786751
	P44RR - XXIV	Mucoid, bright-white and umbonate	-	*Rhizobium lusitanum* P1-7	99.85	96.2	MG786752
Soil	P6BS - III	Mucoid, bright-white	-	*Ochrobactrum haematophilum* CCUG 38531	99.78	96.4	MG786722
	P14BS - VII	Mucoid, bright-white	-	*Rhizobium miluonense* CCBAU 41251	100.0	96.2	MG786723
	P26BS - XIV	Moist, translucent-white and irregular margins	-	*Pedobacter nutrimenti J22*	98.65	92.5	MG786724
	P30BS - XVII	Mucoid, translucent-white	-	*Phyllobacterium myrsinacearum* IAM 13584	99.01	93.9	MG786725
	P38BS - XIX	Mucoid, bright-white	-	*Rhizobium freirei PRF 81*	98.89	96.2	MG786726
	P38BS - XX	Mucoid, pale -white and irregular margins	-	*Ochrobactrum anthropi ATCC 49188*	100.0	96.4	MG786727

The selectivity achieved during the isolation process undoubtedly draws attention. While during other isolations on salt media, strains from a diverse phylogenetic origin are usually obtained (Cuadrado, 2009), here, almost all of the strains, except for the *Sphingobacteriia Pedobacter* sp. P26BS-XIV, belong to class *Alphaproteobacteria*. If we take into account that a simple carbon and energy source such as glucose was used, a 25°C temperature was set (to obtain mesophyll microorganisms), and samples were obtained from different origins (soils and roots), it could possible to endow the observed selectivity to the use of glyphosate as sole source of phosphorus.

As it has been previously discussed, bacteria with glyphosate tolerance are among different taxonomic groups. Nevertheless, the ability to use glyphosate as source of phosphorus, that is, exhibit a specific C-P lyase for this phosphonate, would seem to be an uncommon resource in microorganisms. The above agree with the statements of Hove-Jensen and collaborators in their review (Hove-Jensen et al., 2014). In fact, in the case of sarcosine pathway, the authors mention *O. anthropi, S. meliloti, A. radiobacter, B. pseudomallei* and *Nostoc* sp. *S* as degrading species, microorganisms which are taxonomically close to those isolated. It also stands out that, neither *Pseudomonas* nor *Burkholderia*, genera frequently observed in soil and rhizosphere isolations and which have been reported as glyphosate degraders via the sarcosine pathway, have not been isolated.

During a glyphosate dissipation test performed in communities (Forlani et al., 1999), it was observed that the preferential degradation pathway of glyphosate in soil occurs through the AMPA pathway preferably to sarcosine pathway. The C-P bond of phosphonate presents a higher stability than the C-O bond, however, the preference of one pathway over the other cannot be explained by this, given that the C-P lyase complex is widely distributed in bacteria, but can be explained by the fact that glyphosate is a synthetic phosphonate (Ternan et al., 1998) which makes its breakdown much more specific. In future studies on microbial communities exposed to glyphosate, studies over the specificity of C-P lyase operon will result in meaningful information.

One dominating colony morphotype was mostly found during the procedure, except for the isolations labeled as P20RR and P38BS, where two

colonies of different appearance was distinguished. For both cases, the combinations consisted of *Rhizobium* spp. and *Ochrobactrum spp.*.

In rhizosphere-rhizoplane isolations, all species (except for the P20RR combination previously mentioned) correspond to the genus *Rhizobium*, while in soil isolations it is possible to identify a higher number of species, although the number of microorganisms obtained for every sample type prevents us to make any conclusion regarding the diversity in this case.

Historically, the order *Rhizobiales* has been widely and thoroughly studied since it presents species capable of performing nitrogen biological fixation through specific symbiosis with legumes, as well as different plant growth promotion abilities. A direct relationship between the formation nodule ability and the and productivity of the inoculated plant species was demonstrated. That is how the first registered inoculant (bio-inoculant) was formulated with a species from this order (Bashan, 1998). A broad spectrum of rhizobiums can establish a symbiosis with different plant species (Cooper et al, 2008). This would increase the boundaries of the rhizoremediation strategy in the future, being possible to perform plant-bacteria interaction assays on different legumes. Among all the *Rhizobium* species isolated, 5 of them have as the closest neighbor the strain type *R. vallis* CCBAU 65647, although stains were obtained from diverse samples (different agronomic management, different locations).

This may be given by the fact that the plant species could exert selectivity in regard to the *Rhizobium* species colonizing the root. It may as well be considered that *R. vallis* has a higher tolerance or has a degrading ability which makes it stand out over the rest of the *Rhizobium* species.

Previously, *Ochrobactrum* species have been characterized both as tolerant as well as degraders. This genus, less known than *Rhizobium*, has been isolated in different habitats, among which are roots (Aujoulat et al., 2014). In recent years, *Ochrobactrum* has been investigated due to its plant growth promotion abilities and its degrading capabilities on different xenobiotics (Faisal, 2013; De Las Nieves Rodríguez Mendoza et al., 2013; Abraham and Silambarasan, 2016).

Phyllobacterium is a genus closely related to rhizosphere environments, including that of *L. corniculatus* (Valverde et al., 2005; Mantelin et al., 2006; Sánchez et al., 2014).

On the other hand, *Pedobacter* is a genus associated to cold environments. *P. nutrimenti* was first described in 2014 isolated from frozen food, during a study of microorganisms adapted to cold and their relationship with food contamination (Derichs et al., 2014).

After the identification, the study of the plant growth promotion abilities in all strains was carried out using *in-vitro* tests. Results showed a great number of positive tests, were the production of Indole Acetic Acid, the two different phosphate solubilization studied (mineral and phytate) and the production of siderophores, stood out (Table 5.3 and Figure 5.2). The low levels of phosphorus registered in the soils (and the Flooding Pampa in general) could explain the ubiquity phosphorus solubilization traits. The analysis of different strains of *R. vallis* shows phenotypical differences in some of them. For instance, P16RR-IX is the only producer of acetoin and siderophores, while P12RR-VI has a higher $Ca_3(PO_4)_2$ solubilization ability with respect to the others *R. vallis*. These observations have been previously reported (Duan et al., 2009; Dinesh et al., 2015; Karnwal, 2017). In many studies in which the microorganisms with plant growth promotion activity are investigated, the strains are not phylogenetically identified; being simply named by the isolation code. These observations motivate to perform the genotypic characterization of microorganisms, in order to enrich the phenotypical profile of the species.

Table 5.3. Results of the plant growth promotion ability assays performed on the isolated stains.

Source	Strain	*In Vitro* Plant Growth Promotion Abilities						
		ACC-desaminase	IAA	Acetoine	$Ca_3(PO_4)_2$ Solubilization	Phytate Mineralization	Organic Acids Production	Siderophores Production
L.tenuis rhizosphere-rhizoplane	P8RR - IV	−	+	−	+	++	−	−
	P4RR - V	+	−	−	+	++	−	+
	P12RR - VI	−	+	−	+++	+++	−	−
	P16RR - IX	−	+	+	+	+	−	+
	P20RR - XI	−	+	−	+	+	−	−
	P20RR - XII	+	+	−	++	+	+	+
	P28RR - XV	+	−	+	+	++	−	+
	P32RR - XVIII	−	+	−	+	+	−	−
	P40RR - XXII	+	+	−	+	+	−	+
	P44RR - XXIV	+	+	+	−	++	+	+
Soil	P6BS - III	−	+	−	++	+++	+	+
	P14BS - VII	−	−	−	+	++	−	+
	P26BS - XIV	+	−	+	+	++	−	+
	P30BS - XVII	−	+	+	+++	+++	+	+
	P38BS - XIX	−	+	−	-	++	+	+
	P38BS - XX	+	+	+	-	-	+	+

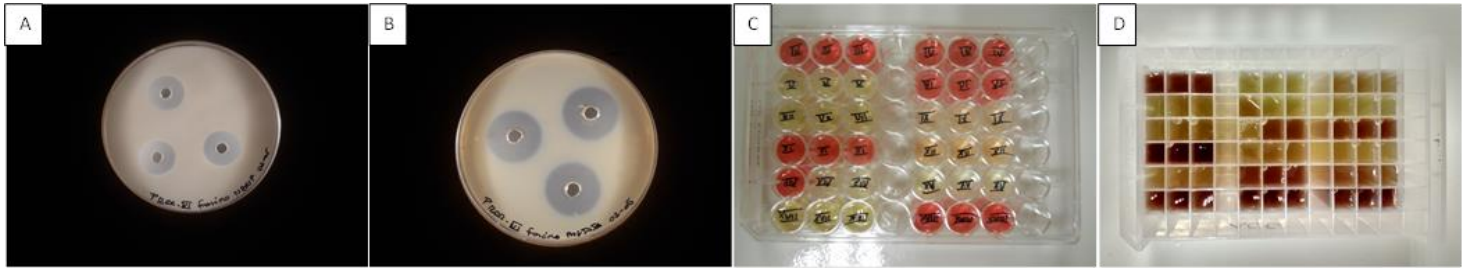

Figure 5.2. Images of different plant growth promotion tests. A: solubilization of $Ca_3(PO4)_2$. B: Solubilization of phytates. C: Plate where the AIA production test was performed (the pink wells indicate a positive reaction). D: Plate where the ACC-deaminase activity test was performed (the yellow wells indicate a positive reaction).

5.3.2 Estimation of the minimum inhibitory concentration of glyphosate in agar plates

With the purpose of characterizing the highest level of tolerance to glyphosate in the strains, an agar plate assay was performed where the growth of the isolated colonies submitted to increasing concentrations of glyphosate was observed.

In literature, when the tolerance glyphosate level of a certain strain is mentioned, it generally refers to the concentration of glyphosate present in the culture medium used for isolation, or to subsequent studies in pure cultures, assessing the inhibition of growth under specific doses. (Pipke and Amrhein, 1988; Liu et al., 1991; Fan et al., 2012). It is possible to find experiments that use different doses of glyphosate where the catalytic efficiency of the enzyme EPSP synthase obtained from a certain microorganism is examined. Although there is a correlation between this catalytic efficiency of the enzyme and the tolerance of the carrier species (since these tolerant enzymes are found during the isolation of tolerant microorganisms), the proper characterization of tolerance for a rhizoremediation process must involve the complete cell because, even though different EPSPS forms confer different grades of tolerance, there are several mechanisms apart from this enzyme that can be complemented to incrase or decrease such tolerance. The sum of all the attributes will result in a differential cell growth. For instance, the ability of microorganisms of using glyphosate as a phosphorus and/or carbon source could also result in an increase of tolerance.

In this assay, the range of concentrations allowed by the glyphosate solubility in BSM was explored. Glyphosate solubility in BSM is slightly lower than in distilled water (approximately 11.6 g L^{-1}). Concentration values between these ranges are the ones logically found in different isolation processes (Shushkova et al., 2012; Kryuchkova et al., 2014). As a control, an EPSPS Class I carrier microorganism was selected; *E. coli*, since this enzyme is sensitive, and no tolerance and/or degradation mechanisms in this species in wild type have been reported.

All the culture collection was analyzed. From all the strains, 6 of them tolerated the highest glyphosate concentration assessed (10,000 mg kg^{-1}). Among them are all of those belonging to the genus *Ochrobactrum*, *Phyllobacterium* sp. P30BS-XVII and two *Rhizobium*, P16RR-IX and P44RR-XXIV. Another 6 strains

were inhibited by a concentration of 7,500 mg kg^{-1} (all of them belonging to the genus *Rhizobium*), while the remaining 4 were inhibited by lower concentrations. The results agrees with the reports in literature, since the genus *Ochrobactrum* has, by nature, a glyphosate-tolerant enzyme which, additionally, has an adequate catalytic ability (Tian et al., 2010). The genus *Rhizobium* showed a variable tolerance, for instance, P8RR-IV was tolerant to the concentration used during the isolation (500 mg kg^{-1}) but sensitive to higher doses. *Phyllobacterium* sp. P30BS-XVI presented growth at the highest assessed concentrations and, to our knowledge, there are no reports in literature of its glyphosate-tolerance. Figure 5.3 show these results, while the supplementary figure S5.1.8 shows the strain morphology of each strain under different glyphosate concentrations. *E. coli* presented growth under doses of 100 mg kg^{-1}, indicating that the concentration chosen to perform the isolation was appropriate to obtain tolerant microorganisms.

5.3.3. Complementary studies on *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV strains.

From all the studied strains, *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV were selected to carry out a deeper characterization. *Ochrobactrum* sp. P6BS-III, possibly *Ochrobactrum haematophilum* (Kampfer et al., 2007), has not been profoundly studied and its genome has not been previously fully sequenced. This strain presents different *in vitro* plant growth promotion abilities, such as production of AIA, several phosphorus solubilization and production of siderophores. At the same time, the genus was previously reported with the ability to degrade different xenobiotics (Kiliç, 2009; Abraham & Silambarasan, 2016; Chudasama & Thaker, 2017) and with different plant growth promotion abilities (Verma et al., 2004; Chakraborty et al., 2009; Khan et al., 2009).

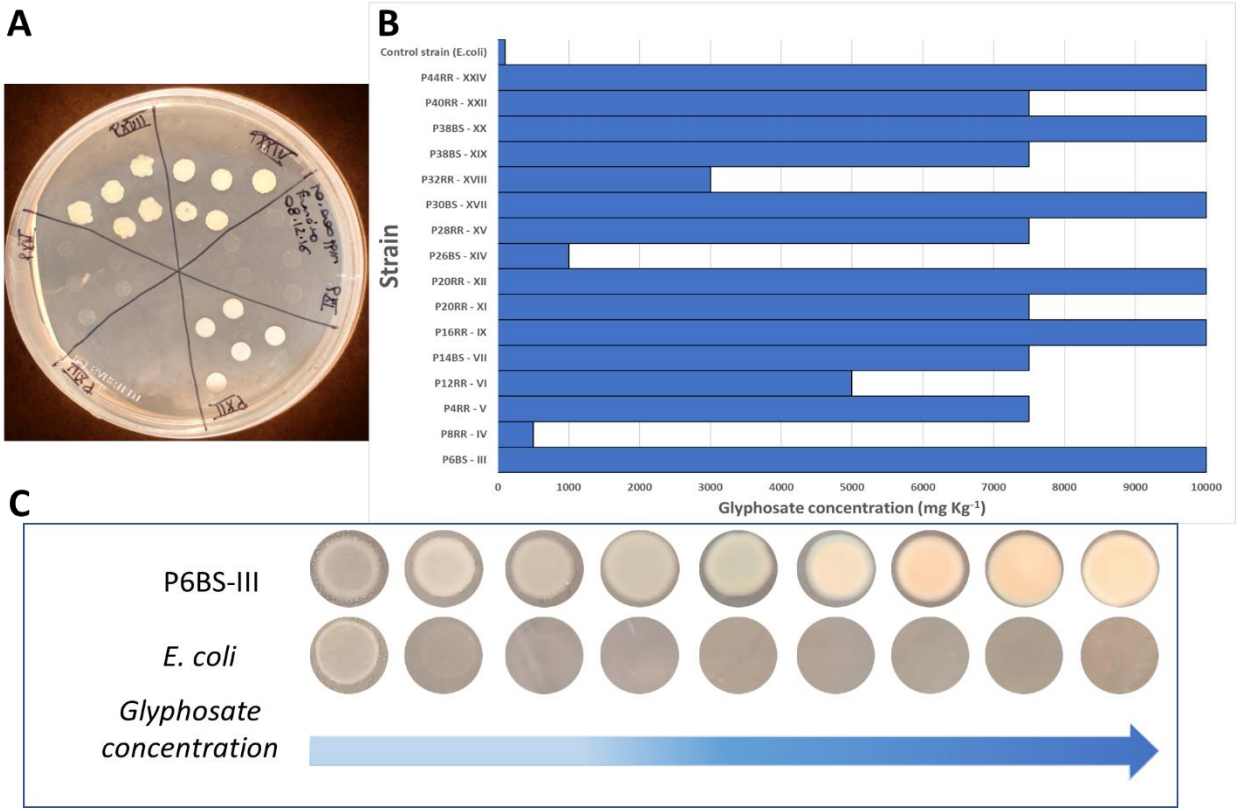

Figure 5.3. Glyphosate tolerance assay in agar plates. A. Agar Petri dish used in the assay (10,000 mg kg^{-1} glyphosate). Inhibition caused by this dose in 3 of the 6 strains assessed can be observed. B. Results showing, strain by strain, the minimum inhibitory concentration obtained. C. Morphology of strains P6BS-III and control (*E. coli*) under different doses.

Rhizobium sp. P44RR-XXIV, identified using the 16s rRNA gene as *Rhizobium lusitanum* (Valverde et al., 2006) presents a very good growth rate and an excellent grade of glyphosate tolerance (> 10,000 mg kg^{-1}) compared to the rest of the strains of the genus, as well as a wide range of in vitro plant growth promotion abilities (in particular, the ability to produce AIA, acetoin and ACC deaminase activity). All these traits result in the strain arising as an interesting candidate for inoculation assays and subsequent studies under a glyphosate application (see chapter 6). Having acquired the strain from rhizosphere isolation increases a priori the possibility to obtain a beneficial interaction with the plant species.

5.3.3.1 Bacterial cell cultures

In order to perform bacterial growth studies on BSM-Gly broth in which the number of CFU mL^{-1}, the OD_{600} and the end point of the glyphosate concentration were determined, two initial subcultures in the same medium were performed to remove any possible source of phosphorus that gycerol or any other spurious source could provide, and therefore, consider glyphosate as the sole source of phosphorus.

Ochrobactrum sp. P6BS-III reached the maximum growth on day 2, showing approximately 2.10^{11} CFU mL^{-1}. From this maximum peak, the OD_{600} remained constant until the fourth day, while the CFU number decreased. After the fourth day, both parameters decreased (figure 5.4A). *Rhizobium* sp. P44RR-XXIV showed a similar profile that reached a maximum of $2.7.10^{10}$ CFU mL^{-1} on the second day and maintained a maximum OD_{600} until the sixth day (figure 5.4B).

Some aspects of these experiments deserve to be highlighted: the number of cells obtained in both growing experiments is significantly high, considering that the growth was developed in a minimum medium with 2 g L^{-1} glucose as carbon source and glyphosate as sole source of phosphorus. From the biotechnological point of view, these growth conditions (minimum composition formulation, low culture temperatures 25 ± 1 °C and maximum growth between the second and third day) would imply a low scaling cost during the production of these strains as inoculants.

It is also important to point out the relevance of measuring the CFU mL^{-1} throughout the experiment, since the maximum number of viable cells only

correlates to the maximum OD_{600} between the second and third day of the experiment.

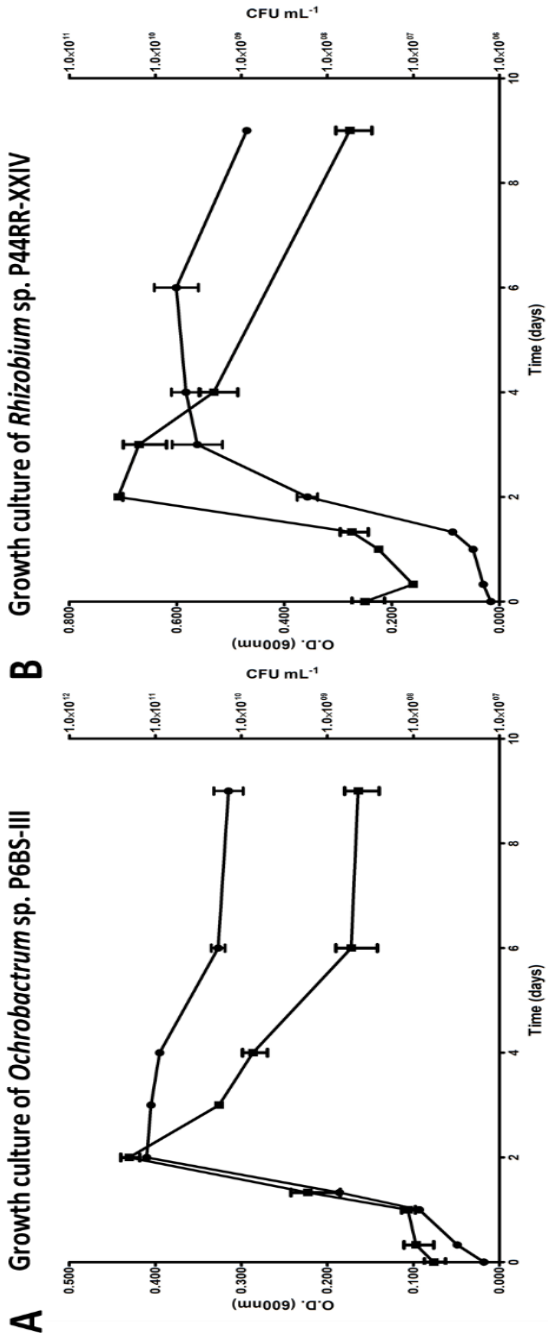

Figure 5.4. Growth cultures for *Ochrobactrum* sp. P6BS-III (A) and *Rhizobium* sp. P44RR-XXIV (B). The curves with circles indicate OD_{600}, while the curves with rectangles indicate CFU mL^{-1} count.

The glyphosate degradation at the ending point determined through UPLC-MS are showed on table 5.4.

Table 5.4. Glyphosate degradation percentages in the strains obtained from the two methodologies used: At the laboratory of the Faculty of Agriculture of the University of Belgrade (A) and at Groen Agro Control (B).

Method	Glyphosate degradation (%)	
	Ochrobactrum sp. P6BS-III	*Rhizobium* sp. P44RR-XXIV
A	42 ± 14	49.2 ± 9.3
B	39.9 ± 2.9	20 ± 12

5.3.3.2 Studies on microbial metabolism using the GN2 MicroPlate™ system

The study of the different metabolic activities was carried out according to the specifications of the manufacturer. The results are shown on table 5.5. *Ochrobactrum* sp. P6BS-III was found to be capable of assimilating a wider spectrum of the compounds than *Rhizobium* sp. P44RR-XXIV, including different carbohydrates, amino acids and carboxylic acids. The obtained metabolic profiles show that both strains may use different nutritional sources beyond glucose, fact that should be considered previous to the formulation of the culture medium when scaling up the production of the inoculant, since this versatility suggests that carbon and other energy sources, cheaper than glucose, could be assayed. Another alternative that may be proposed is the microorganism growth in media formulated directly from glyphosate contaminated effluents, coming from agrochemical factories or acutely contaminated regions.

5.3.3.3 Studies on the genome of Ochrobactrum sp. P6BS-III

The strain was deposited in the culture collection of the Hasselt University, under identification UHS000020 as well as in the Weeds and Herbicides Laboratory culture collection, under identification LMH00001. The genome sequencing project was deposited in the *DNA Database of Japan* (DDBJ), *Eurpoean Nucletide Archive*

(ENA) and *GenBank* (NCBI) under access number MPPJ00000000.1 and *BioProject* PRJNA354074.

Table 5.5. Metabolic profile of strains obtained through GN2 MicroPlate™ system

Biochemical test	*Ochrobactrum* sp. P6BS-III	*Rhizobium* sp. P44RR-XXIV	Biochemical test	*Ochrobactrum* sp. P6BS-III	*Rhizobium* sp. P44RR-XXIV
Carbohydrates			**Amino acids**		
N-Acetyl-D-galactosamine	+	+	D-Alanine	+	-
N-Acetyl-D-glucosamine	+	+	L-Alanine	+	-
Adonitol	+	+	L-Alanyl-glycine	-	+
L-Arabinose	+	-	L-Asparagine	+	+
D-Arabitol	+	+	L-Aspartic acid	+	+
D-cellobiose	+	-	L-Glutamic acid	+	+
i-Erythritol	-	-	Glycyl-L-Aspartic acid	+	-
D-Fructose	+	+	Glycyl-L-Glutamic acid	+	-
L-Fucose	+	+	L-Histidine	+	-
D-Galactose	+	+	Hydroxy-L-Proline	+	-
Gentiobiose	+	+	L-Leucine	+	-
α-D-glucose	+	+	L-Ornithine	+	-
m-inositol	+	+	L-Phenylalanine	-	-
α-D-Lactose	-	+	L-Proline	+	+
Lactulose	-	-	L-pyroglutamic acid	-	-
Maltose	+	+	D-Serine	-	-
D-Mannitol	+	+	L-Serine	+	+
D-mannose	+	+	L-Threonine	+	-
D-Melibiose	-	-	D,L-Carnitine	+	-
β-Methyl-D-Glucoside	+	-	γ-Aminobutyric acid	+	-
D-Psicose	+	+			
D-Raffinose	-	-	**Amines**		
L-Rhamnose	+	-	Phenyethylamine	+	-
D-Sorbitol	+	+	Putrescine	-	-
Sucrose	+	-	2-aminoethanol	+	-
D-Trehalose	+	+			
Turanose	+	+	**Aromatic chemicals**		
Xylitol	+	-	Urocanic Acid	-	-
			Inosine	+	+
Carboxilic acids			Uridine	-	-
Acetic acid	+	+	Thymidine	-	-
Cis-Aconitic Acid	+	+			
Citric Acid	-	+	**Amides**		
Formic acid	-	+	Succinamic Acid	+	+
D-Galactonic Acid Lactone	-	-	Glucuronamide	+	-
D-Galacturonic acid	+	-	L-Alaninamide	+	-
D-Glucuronic acid	+	-			
D-Glucosaminic acid	-	-	**Phosphorylated chemicals**		
D-Gluconic acid	+	-	D,L-α-Glycerol phosphate	+	-
α-Hydroxybutyric acid	-	-	Glucose-1-phosphate	+	-
β-Hydroxybutyric acid	+	-	Glucose-6-phosphate	-	-
γ-Hydroxybutyric acid	+	-			
P-Hydroxyphenylacetic acid	-	-	**Alcohols**		
Itaconic Acid	+	-	2.3-Butanediol	+	-
α-Ketobutyric acid	+	-	Glycerol	+	+
α-Ketoglutaric acid	+	-			
α-Ketovaleric acid	+	-	**Esters**		
D,L-Lactic acid	+	-	Methyl Pyruvate	+	-
Malonic acid	-	-	Mono-methyl-succinate	-	-
Propionic acid	+	-			
Quinic acid	+	-	**Polymers**		
D-Saccharic acid	-	-	α-Cyclodextrin	-	-
Sebacic acid	-	-	Dextrin	+	+
Succinic acid	+	+	Glycogen	-	-
			Tween 40	+	-
Brominated chemicals			Tween 80	+	-
Bromosuccinic acid	+	+			

The sequencing resulted in 1.1 million *reads* (average length: 308 bp) that generated 345 million bases (Mb) of data (324 Mb ≥ Q20) in Torrent Suite

5.0.4. These were assembled using SPAdes 3.8.2 (uniform coverage mode; kmers 21, 33, 55, 77, 99) in a total of 65 *contigs* greater than 1000 base pairs (bp), giving a consensus length of 5,253,115 bp and an average coverage of 61.7 times (GC content 56.63%; largest *contig*: 445,701 bp; N50 = 242,240 bp). *Contig* reordering was performed using the Mauve software employing *Ochrobactrum anthropi* ATCC 49188 (Accession number GenBank: GCA_000017405.1) which is the closest related genome to P6BS-III. Subsequently, annotation was carried out in the different web-based platforms, according to what was described in section 5.2.7. Table 5.6 shows a summary of the genome statistics and figure 5.5 shows the P6BS-III circular genome as ring-based graphic complement

Table 5.6. General features of the *Ochrobactrum* sp. P6BS-III genome.

Attribute	Value	% of Total
Genome size (bp)	5,253,115	100
Coding DNA (bp)	4,639,026	88.31
DNA G+C (bp)	2,974,839	56.63
DNA *contigs*	65	100
Total genes	5,526	100
Protein coding genes	5,291	95.75
RNA genes	57	1.03
Pseudo genes	231	4.37
Genes in internal clusters	81	1.53
Genes with function prediction	5,204	94.17
Genes with COG assignment	4,587	86.69
Genes with Pfam domains	4,973	93.99
Genes with signal peptides	443	8.37
Genes with transmembrane helices	1,201	22.7

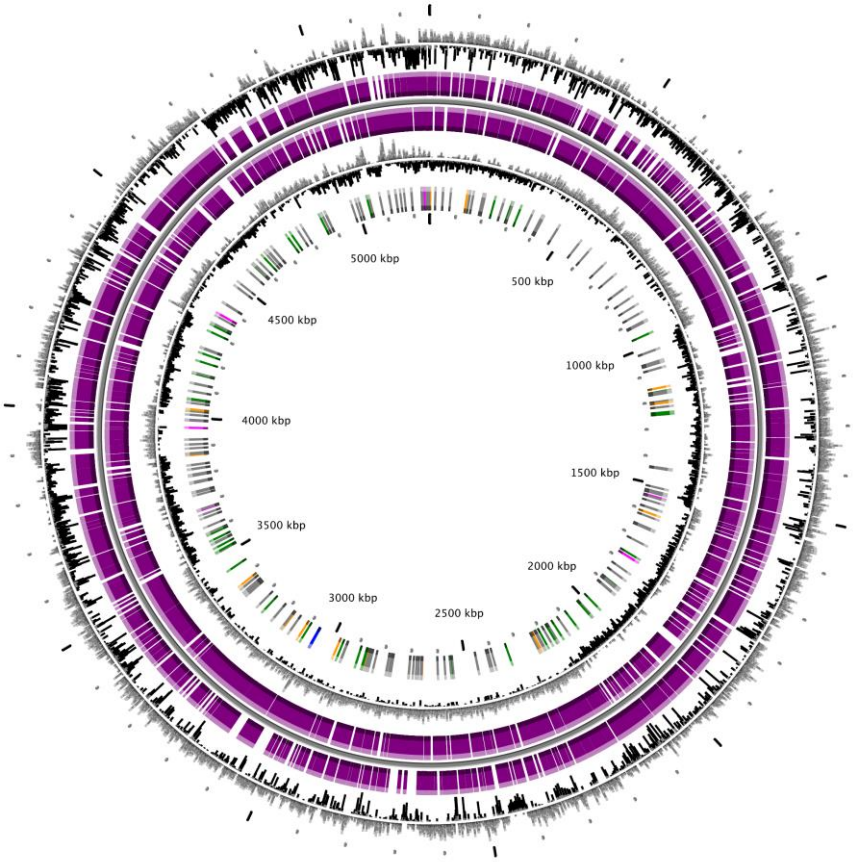

Figure 5.5. Circular genome of *Ochrobactrum* sp. P6BS-III. The circles show (from the outside to the inside): Percentage of GC deviation; clockwise predicted coding sequences; counterclockwise predicted coding sequences; GC *skew*; and in the inner circle, rRNA (blue), tRNA (green), miscRNA (orange), transposon related elements (pink) and pseudogenes (grey).

The interpretation of agarose gel electrophoresis shows the presence of 4 genomic elements of different molecular size, those near the wells being possible chromosomes or mega plasmids (Figure 5.6A). Although a reference genome of the genus could not be run in parallel to P6BS-III genome, the agarose gel provides relevant information about the genomic organization, if complete reference genome sequences are available. The genus *Ochrobactrum* has two chromosomes, as well as *Brucella*. *O. anthropi* ATCC 49188 chromosomes have a molecular weight of 2.89 Mb and 1.9 Mb. Even though this difference in molecular

weight between both chromosomes seems to be excessive for the separation within the two heaviest bands, it can be found for instance that, in *Ochrobactrum* sp. A44 (GCA_002278035.1), the chromosomes have a size of 2.59 and 2.01 Mb, a more similar situation to the one observed in the gel. ATCC 49188 strain has 4 plasmids of size between 0.17 and 0.06 Mb, and *O. anthropi* OAB (GCA_000742955.1) possess two plasmids of 0.16 and 0.11 Mb. From this, it could be proposed that P6BS-III has two chromosomes and two plasmids. The Bandage software assembled a fragment of approximately 1.3 Mb (Figure 5.6B) that roughly match with the result obtained using PlasmidSpades of a 1.2 Mb megaplasmid. Running BLAST using the PlasmidSpades output assemblies, revealed coincidences with plasmids pOANT01 and pOANT02 of ATCC 49188 and plasmid 2 of OAB. The differences between megaplasmids, chromosomes of similar molecular weight or linear chromosomes cannot be discriminate without running a reference genome, but the information provided by the gel will be essential in the future when attempting to close the genome and present a complete version.

The PHASTER software additionally identified a region corresponding to a prophage of 58 kb (Figure 5.6C).

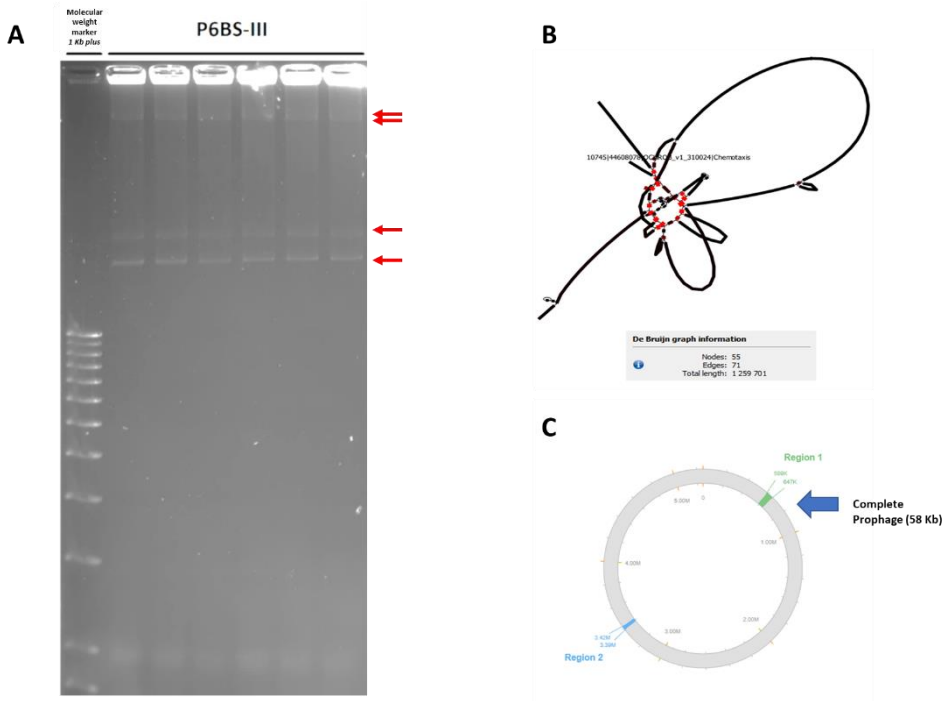

Figure 5.6. Studies on the genome structure of *Ochrobactrum* P6BS-III. A. Electrophoresis gel of the complete P6BS-III genome, where two elements of high molecular weight and two of medium molecular weight can be distinguished (red arrows). B. Structure of 1.3 Mb generated by Bandage. C. Phaster analysis of the complete genome where two bands can be seen, one corresponding to the incomplete prophage sequence and the other (marked with an arrow) corresponding to a complete

Study of the traits associated to glyphosate tolerance and degradation.

In regard to tolerance, genome analysis revealed a single copy of a EPSP synthase Class II sequence. A phylogenetic analysis of this sequence was performed using the MEGA software (Kumar et al., 2016), showing that P6BS-III EPSP synthase is closely related to that present in *Agrobacterium* sp. CP4 and in other members of the genus *Ochrobactrum* (Figure 5.7), where two conserved regions of the enzymes Class II, RXHTE and NPTR are present (Yi et al., 2016). The multiple alignment indicates the presence of the substitution P101L, which has been reported as the cause of structural changes in the active site that influence the spatial orientation of G96 and T97 with respect to the glyphosate

binding site. These results are consistent with the tolerance observed in section 5.3.2.

Information related to the degradation of glyphosate was also found. Operon *phn* homologous sequences organized in the possible transcriptional unit *phnGHIJKLMNCDEE* corresponding to the function C-P lyase (Seweryn et al., 2015), as well as the region *phnM-DUF1045* of unknown function, separated by 160,000 bp and previously reported in the genus *Ochrobactrum* (Hove-Jensen et al., 2014) are described in figure 5.8. The Reading frames are shown in different vertical levels.

A homologue of the glycine oxidase gene (*ThiO*) (EC 1.4.3.19) was found, enzyme involved in the degradation of glyphosate (Zhan et al., 2013). Although this enzyme is capable of oxidizing glyphosate, the affinity for this substrate is low compared to the physiological substrates (primary and secondary amines). This activity could not be proved experimentally because AMPA was not detected in the culture medium; however, its potential could be assessed increasing the glyphosate concentration in future assays.

As to plant growth promotion abilities, the *Ochrobactrum* sp. P6BS-III genome presents gene homologues coding for nine high affinity phosphate transporters, PHO regulon genes, 26 individual genes related to the phosphate metabolism, three homologues related to the use of polyphosphate and 17 to the use of alkylphosphonates (including the operon *phn*). In addition, gene homologues related to the solubilization of calcium phosphate and phytate were found, including 4-phytase and the operon *pqqABCDE* involved in the synthesis of gluconic acid (An and Moe, 2016).

The iron uptake is possibly carried out through several transporters, such as ABC pitA, pitD, pitC and the inducible system at low pH. Homologous of genes involved in the synthesis of siderophore *Aerobactin* were found, although not all the homologues of the pathway are present, being the L-lysine-N-(6)-monooxygenase (NADPH) and N6-hydroxylysine-O-acetyltransferase absents. The indole acetic acid synthesis probably occurs through tryptophan dependent biosynthesis, given the fact that homologues implied in the synthesis of aromatic amino acids aldehyde dehydrogenase and *aldA*, responsible for the oxidation of indole-3-acetaldehyde to indole-3-acetic acid were found.

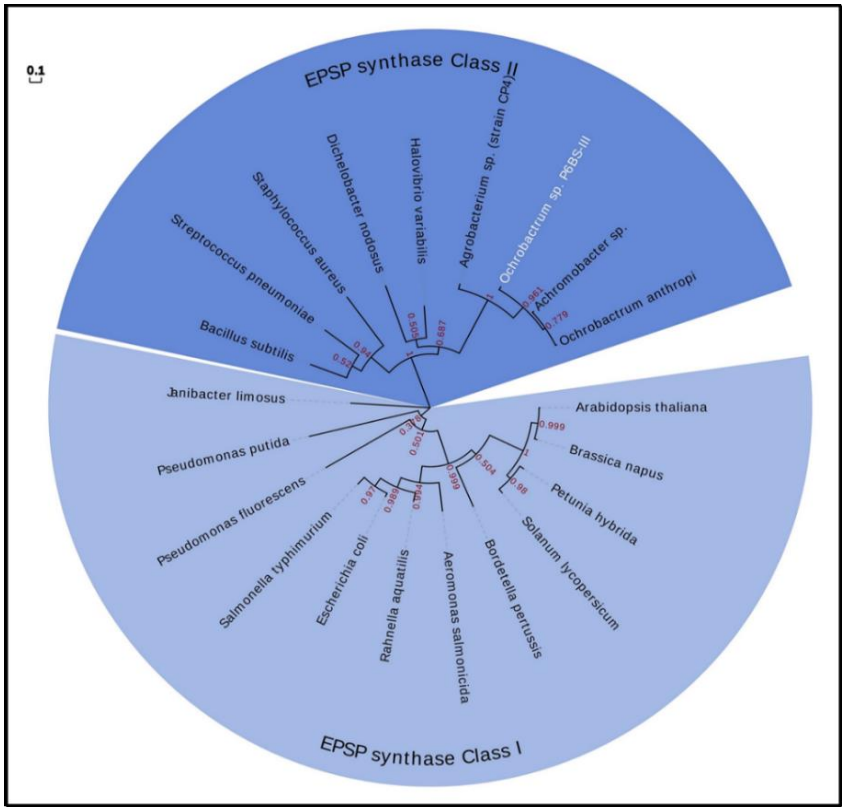

Figure 5.7. Phylogenetic tree built with amino acid sequences of EPSP synthase. Class I sequences were obtained from: *Arabidopsis thaliana* (P05466), *Brassica napus* (P17688) *Petunia hybrida* (P11043) *Solanum lycopersicum* (P10748), *Bordetella pertussis* (P12421), *Aeromonas salmonicida* (Q03321), *Rahnella aquatilis* (C9EGX2), *Escherichia coli* (P0A6D3), *Salmonella typhimurium* (P07637), *Pseudomonas fluorescens* (A1Z0H6), *Pseudomonas putida* (Q683S1*) Janibacter limosus* (L7PA93). Class II sequences were obtained from*: Bacillus subtilis* (P20691), *Streptococcus pneumoniae* (Q9S400), *Staphylococcus aureus* (Q05615), *Dichelobacter nodosus* (Q46550), *Halovibrio variabilis* (Q1H624), *Agrobacterium sp.* CP4 (Q9R4E4), *Achromobacter sp.* LBAA (P0A2Y5), *Ochrobactrum anthropi* (A6WUV8). P6BS-III is shown in white letters. The multiple alignment of amino acid sequences was performed using the *Muscle* algorithm and the phylogenetic tree was obtained through the *Maximum Likelihood* method with 1000 iterations (bootstrap) in MEGA software version 7.0

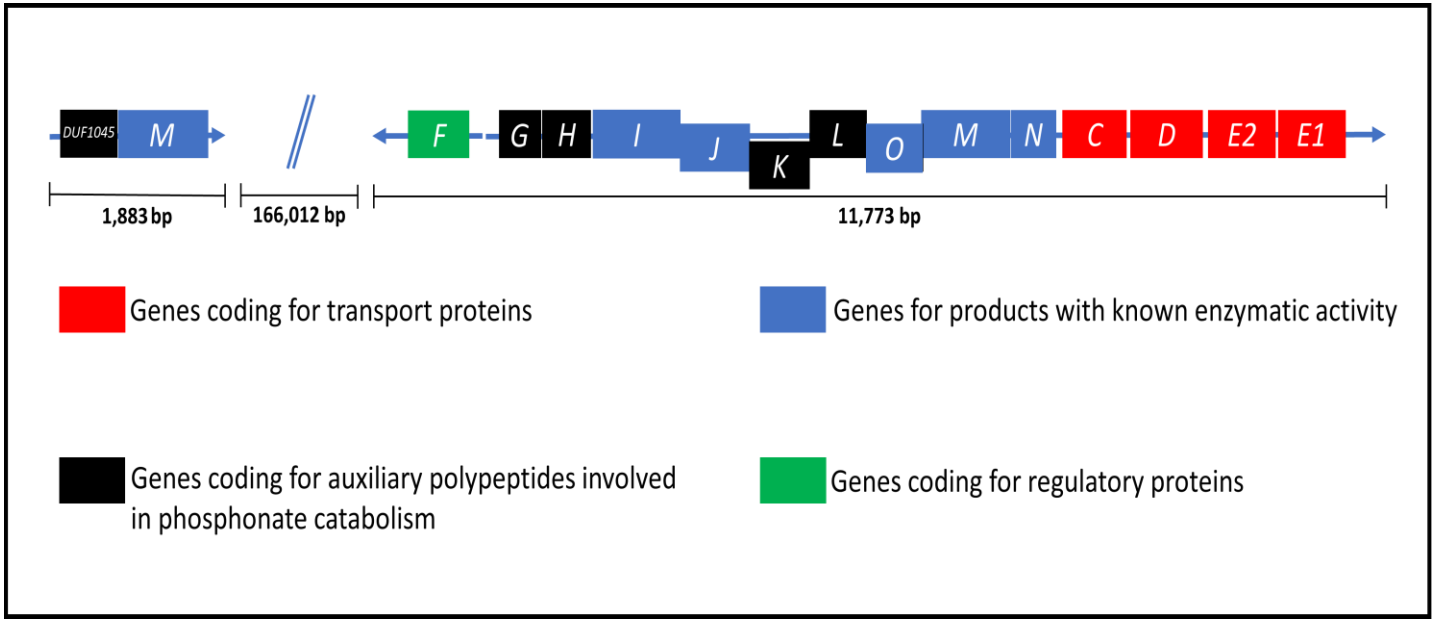

Figure 5.8. Structure of the probable operon *phn* of *Ochrobactrum* sp. P6BS-III

As mentioned above, the P6BS-III genotypic identification indicated that the closest neighbor is *O. haematophilum* CCUG 38531, with an identity of 99.8. Since there are no fully sequenced genomes corresponding to the species *O. haematophilum*, *genome-to-genome* comparative studies with other of the GenBank available genomes were performed. The algorithm *Tetra Correlation Search* was used to to obtain a list of closest reference genomes to P6BS-III and, subsequently, the algorithms ANIm, ANIb and GGDC 2.1 with the closest 14 genomes were executed. The results obtained showed that, P6BS-III has no match at species level (Table 5.7)

Table 5.7. Comparison of *Ochrobactrum* sp. P6BS-III genome with other genomes using different *genome to genome* comparison methods.

Strain name	BioProject	Size (Mb)	GC %	ANIb (%)	ANIm (%)	GGDC (%)
O. anthropi OAB	PRJNA244571	4.9	56.1	80.05	85.9	24.7
O. anthropi ATCC 49188	PRJNA19485	5.21	56.2	80.03	85.94	24.7
Ochrobactrum sp. UNC390CL2Tsu3S39	PRJNA234825	4.62	56	80.18	85.82	24.6
O. anthropi ML7	PRJNA274439	4.9	56	80.19	85.81	24.7
O. anthropi CTS-325	PRJNA85133	4.73	56	80.17	85.82	24.7
O. anthropi 60a	PRJNA169004	4.59	56.1	80.46	86.15	25.7
O. anthropi W13P3	PRJNA238403	5.28	56.3	80.05	85.86	24.7
Ochrobactrum sp. EGD-AQ16	PRJNA215965	4.83	57.6	80.39	85.75	25
O. intermedium 2745-2	PRJNA233556	4.8	57.6	80.31	85.55	24.8
O. intermedium LMG 3301	PRJNA37725	4.73	57.7	80.27	85.58	24.8
O. intermedium M86	PRJNA178498	5.19	57.9	80.17	85.58	24.7
B. inopinata BO1	PRJNA41855	3.37	57.1	80.16	85.03	24.3
B. suis bv. 4 str.40	PRJNA34745	3.31	57.2	80.27	85.04	24
Brucella sp. BO2	PRJNA41857	3.31	57.2	80.21	84.99	24.1

Afterwards, and since P6BS-III genome had never been described, a pan-genome analysis of *Ochrobactrum spp.* was performed, using all the available strains in the NCBI database up to 06-10-2017, with the intention of describing the core genome (gene family shared by all the members of the genus) and the unique genes with which P6BS-III contributes. For this purpose, the *Bacterial Pan Genome Analysis* (BPGA) program was implemented, using the Usearch clustering algorithm (Edgar, 2010) with identity *cut-off* for the sequences of 50%. Once the genes of each category had been obtained, graphs were made using gnuplot 4.6.6 (Williams and Kelley, 2011). The results are shown in Figure 5.9.

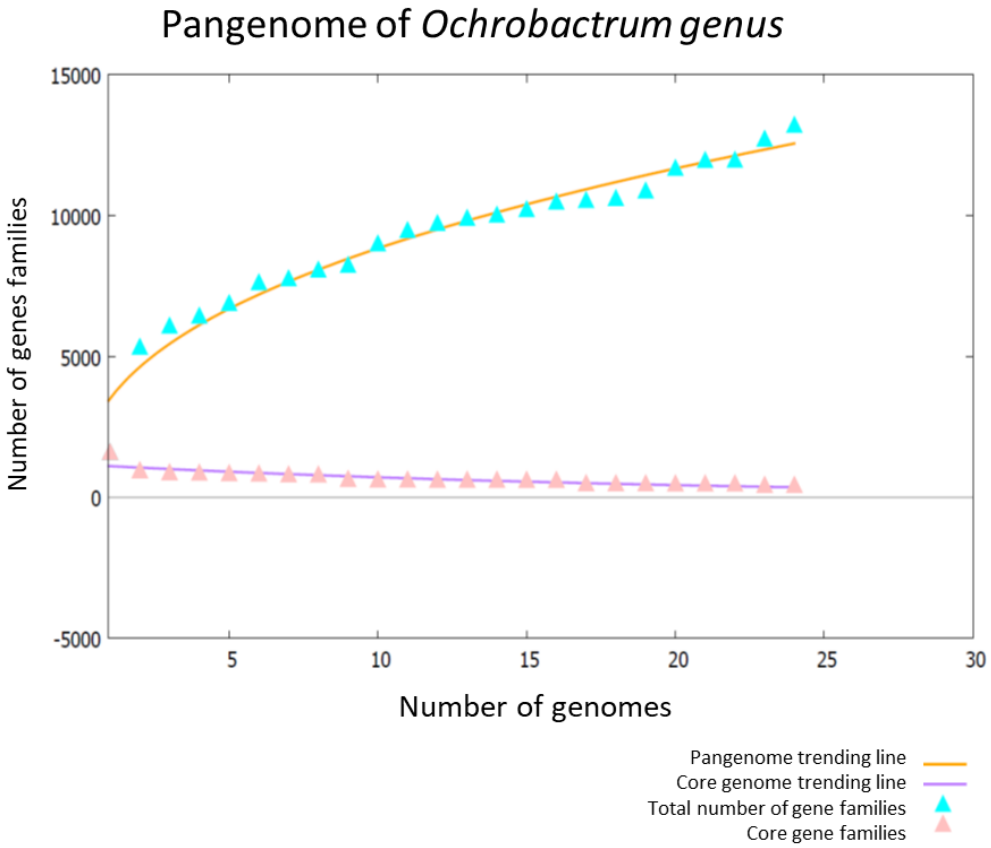

Figure 5.9. Pan-genome analysis of the *Ochrobactrum* genus.

Results indicate that the pan-genome of the 24 *Ochrobactrum* species deposited in NCBI consists in 13,194 families of orthologous genes, while the core genome is formed by 429 gene families. The trend line that defines the evolution of pan-genome and core genome does not clearly reach a plateau, indicating that future sequencing projects of this genus will significantly contribute to the characterization, which is to be expected since the genus has a few reported genomes. P6BS-III contributes with 744 unique genes to the pan-genome, a very significant contribution which, in turn, may indirectly suggests that it is a different species from *O. antrhopi*, which is over-represented in the database. From the unique genes, 158 have been assigned to at least one function prediction (checked against the COG and KEGG databases), while the rest have no known function.

A visual complementary analysis to the pan-genome study was also performed, using the BRIG software. In this study the complete genome of *Ochrobactrum* P6BS-III was compared with that of the *type strain* of *O. anthropi* and other relevant species of the genus (Figure 5.10). The plot assigns a color bar when it finds a homologue that match in the genome used as a template (in this case, P6BS-III). Every ring, with a particular color, belongs to a different genome. That is how "high homology regions" and "low homology regions" are created, given the look of a colored line or forming a discontinuous line, correspondingly. These regions can be associated with the presence of plasmids or gene isles where the unique genes of the strain could be found.

A region with a low synteny region matches with the prophage found with the Phaster program.

From the analysis of figure 5.10, it could be suggested that the unique genes found in P6BS-III genome through the analysis of the pan-genome correspond to the empty spaces or regions of no homology with respect to the genomes of the type strains. This complementary analysis supports even more the importance of the contribution of this genome to the genus.

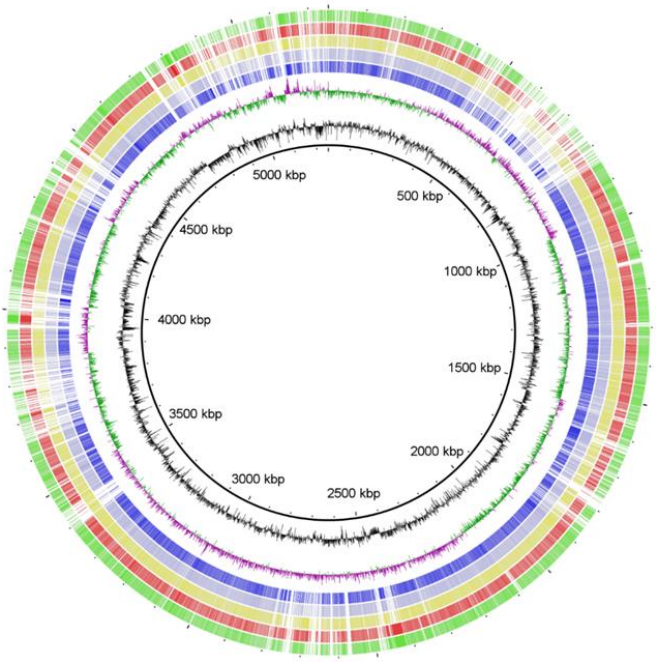

Figure 5.10. Comparison of *Ochrobactrum*. sp. P6BS-III genome with representative genomes of the genus through BRIG software. From inside out, the rings represent: GC content, GC Skew, comparison with *O. anthropi* ATTC 49188 (dark blue), *O. anthropi* OAB (light blue), *O. intermedium* LMG 3301 (yellow), *O. pituitosum* AA2 (red), and *O. pseudogrignonense* K8 (green). All the genomes include plasmids.

5.3.3.4 Studies on the genome of Rhizobium sp. P44RR-XXIV

The strain was deposited in the Hasselt University culture collection under identification UHS000021 as well as in the Weeds and Herbicides Laboratory culture collection under identification LMH00002. The genome sequencing project was deposited in the *DNA Database of Japan* (DDBJ), *European Nucleotide Archive* (ENA) and the *GenBank* (NCBI) under access number MPVZ00000000.1 and *BioProject* PRJNA354620.

For P44RR-XXIV, a total amount of 1.65 million *reads* (average length: 296 bp) generated 488 Mb of data (458 Mb ≥ Q20) in Torrent Suite 5.0.4. The *reads* were assembled using SPAdes 3.8.2 (uniform coverage mode; k-mer 21, 33, 55, 77, 99) in a total of 119 *contigs* greater than 1000 bp, giving a consensus length of 7,446,815 bp and an average genome coverage of 61.5% (largest *contig* 750,306 bp; N50 = 280,104 bp).

The sequencing was later repeated in a Nanopore 1-D run (Thermo Fisher Scientific, Whaltham, USA), where a decrease of the quantity of contigs was achieved, reaching 29 contigs. The genome statistics are shown on Table 5.8.

Table 5.8. General features of *Rhizobium* sp. P44RR-XXIV genome

Attribute	First seq. project		Second seq. project	
	Value	% of Total	Value	% of Total
Genome size (bp)	7,458,574	100	7,408,308	100
Coding DNA (bp)	6,589,650	88.35	-	-
DNA G+C (bp)	4,436,360	59.48	4,370,902	59.5
DNA *contigs*	119	100	29	100
Total genes	7,133	100	7,445	100
Protein coding genes	6,579	95.75	7,229	98.04
RNA genes	65	1.03	58	0.78
Pseudo genes	489	4.37	-	-
Genes in internal clusters	-	-	80	1.07
Genes with function prediction	-	-	6,263	84.12
Genes with COG assignment	-	-	6,225	83.61
Genes with Pfam domains	-	-	6,126	82.28
Genes with signal peptides	-	-	653	8.77
Genes with transmembrane helices	-	-	1,749	23.49

The total genomic DNA analyzed through agarose gel revealed one or maybe two molecules of high molecular weight (not confirmed, since not all the replicates showed two bands), followed by several bands of lower molecular weight possibly attributable to plasmids or shredded DNA (Figure 5.11A). Even though no *Rhizobium* genome was available at the time of running the gel, in this case, as opposed to P6BS-III, phylogenetically species level-related microorganisms are available (both by 16s sequence and by genome to genome comparison). Therefore, the analysis can be complemented using Mauve.

The reordering of *contigs* was performed using the closest *Rhizobium* reference genome completely sequenced, *Rhizobium tropici* CIAT 899

(GCA_000330885.1). The genome of P44RR-XXIV was also compared to two other genomes, one corresponding to the closest strain match according to the information of the 16s rRNA gene, and the other one corresponding to the closest strain match according to results obtained using the ANIm, ANIb and GGDC analysis (Table 5.9). These are *R. lusitanum* P7 (NZ_FMAF00000000.1) and *Rhizobium* sp. AC27/96 (NZ_LXKN00000000.1), respectively (Figure 5.11B).

The chromosome and the three plasmids of CIAT 899 genome are shown framed in red on the top of Figure 5.11B. In this first ordering of *contigs* between CIAT 899 and P44RR-XXIV, it was intended to align the highest number of homologous sequence regions between both genomes by using CIAT 899 as reference genome. It can be seen that the chromosome of CIAT 899 shows a large number of blocks (LCBs or "*Locally Colinear Blocks*") present in 6 *contigs* with a high molecular weight of P44RR-XXIV. LCBs are sequence segments which would be exempt from participating in arrangements within the genome. Thus, the sequences within these blocks can be considered as those highly conserved (syntenic regions) for both compared genomes. It can be suggested, therefore, that the chromosome of P44RR-XXIV is formed by at least those 6 *contigs*.

Within those aligned contigs with CIAT 899 chromosome, an inversion of large extension (yellow block), and an insertion and deletion (blank spaces within the blocks) can be observed. The plasmids of CIAT 899 do not seem to be present in the genome of P44RR-XXIV (a series of blocks with homologous regions within the plasmid pRtcCIAT899c can be observed, but in a very incomplete way).

The *contigs* that show a high degree of sequence homology in P44RR-XXIV are marked with a green bar below them, since the following comparisons had to be done necessarily from *draft* genome (incomplete), to *draft* genome. This helps locate those loci in the genomes to be compared that would correspond to the possible chromosome of P44RR-XXIV. Even though it cannot be confirmed that the chromosome of P44RR-XXIV is formed only by these 6 *contigs*, it is very useful to mark them since they can be used as reference to locate those *contigs* associated to chromosomes in other *Rhizobium* and to observe rearrangements and the presence of possible plasmids shared among strains.

The second ordering, showed in the center of figure 5.11B, corresponds to P44RR-XXIV and *R. lusitanum* P7 (this time with P44RR-XXIV on the top, since it is considered as the reference genome by the program). The comparison reveals

that the sequences corresponding to the *contigs* related to the chromosome of CIAT 899 are also present in the genome of P7, this time without the inversion observed between CIAT 899 and P44RR-XXIV and including a larger number of insertions and deletions. The order of *contigs* in P7 is, in this case, difficult to predict since it draft presents 79 *contigs*. In this genome the largest *contig* in P44RR-XXIV (*contig* 1, 1,976,189 bp, located in the right end), has also a partial representation in P7, although it is incomplete. The rest of the *contigs* of P44RR-XXIV, possibly attributed to plasmids or repetitive regions, have no sequence homology in P7 genome.

Lastly, the comparison between P44RR-XXIV and *R.* sp. AC27/96 was performed, which can be seen in the bottom part of figure 5.11B. There, it can be observed that, apart from matching the *contigs* corresponding to the chromosome of CIAT 899, there is also coincidence between the largest *contig* in P44RR-XXIV and the remaining large size *contigs* (*contig* 3 partially, and *contig* 9 and *contig* 10 completely, including approximately an additional 700,000 bp). This similarity between both genomes is interpreted by the ANIm algorithm as a species level coincidence (%ANIm>95), while other methods consider that it does not belong to the same species, despite presenting, in all of them, the highest comparative value with respect to other species (Table 5.9).

In this case, the comparison of genome of P44RR-XXIV with the three genomes reveals a strategy to fully complete the chromosome sequence, through the search of CDS shared between two different fragments.

Both *genome-to-genome* comparisons and the Mauve analysis indicate that *R.* sp. AC27/96 is closest related available genome in the databases (GenBank and JGI). A considerable number of bases (around 90 kb which correspond to two *contigs* in the genome of P44RR-XXIV) are not represented at all in AC27/96. Therefore, together with the low homology region within *contig* 3 (central part of the figure), this may determine that ANIb and GGDC result in non-correspondence at species level.

The PHASTER software did not identify, in this case, complete phages or prophages (Figure 5.11C).

Table 5.9. Comparison between *Rhizobium* sp. P6BS-III genome with other genomes using different *genome-to-genome* comparison methods.

Strain name	BioProject	Size (Mb)	GC %	ANIb (%)	ANIm (%)	GGDC (%)
R. freirei PRF 81	PRJNA13459	7.08	59.96	83.48	86.62	29.2
Rhizobium sp. AC27/96	PRJNA319063	7.29	59.7	94.57	95.13	60.6
Rhizobium sp. CF142	PRJNA83051	7.46	60.2	75.16	84.23	21.5
Rhizobium sp. YK2	PRJNA324744	7.16	59.7	82.14	85.98	27.4
R. tropici CIAT 899	PRJNA42391	6.69	59.51	82.09	86.09	27.5
R. rhizogenes NBRC 13257	PRJNA224116	7.04	59.91	80.39	85.60	25.6
R. rhizogenes ATCC 15834	PRJNA240998	7.06	60	80.44	85.59	26.7
R. lusitanum P7	PRJEB14985	7.92	59.6	84.61	87.74	31.5
A. radiobacter K84	PRJNA13402	7.27	59.87	80.41	85.59	25.6
Rhizobium sp. AP16	PRJNA83035	6.50	60.21	80.45	85.57	25.6

Study of the traits associated to glyphosate tolerance and degradation.

The genome of P44RR-XXIV reveals a single copy of the EPSP synthase sequence, of 431 amino acid residues. The phylogenetic analysis of the sequences of different EPSP synthases grouped the EPSP synthase of P44RR-XXIV with those enzyme sequences belonging to other members of *Rhizobium* genus and in a separate cluster from those Class I sequences where the EPSP synthase of *E. coli* and different plant species are found (Figure 5.12).

In the same analysis, the typical Class II EPSP synthase sequences clustering can be seen, such as *Agrobacterium* sp. CP4, *Ochrobactrum anthropi* and *Staphylococcus aureus*. For further analysis of the P44RR-XXIV EPSP synthase, the amino acid sequence was compared to those of *E. coli*, *Agrobacterium* sp. CP4 and other members of the same cluster through alignment of amino acid sequences (Figure 5.13).

The exploration of EPSP synthase variants obtained, either from *wild type* tolerant microorganisms or by means of different mutagenesis methods, is an approach generally used to design new versions of transgenic plant species, capable of having a catalytic efficiency similar to that of the *wild type* enzyme and, at the same time, resistance to high doses of glyphosate (Funke et al., 2009; Pollegioni et al., 2011; Tian et al., 2011). Even though the purpose of this work is not to assess the suitability of the enzyme to be used in a transgenic event, these studies shed light on the regions of the enzyme found that would be relevant, both for their tolerance to glyphosate and their catalytic ability.

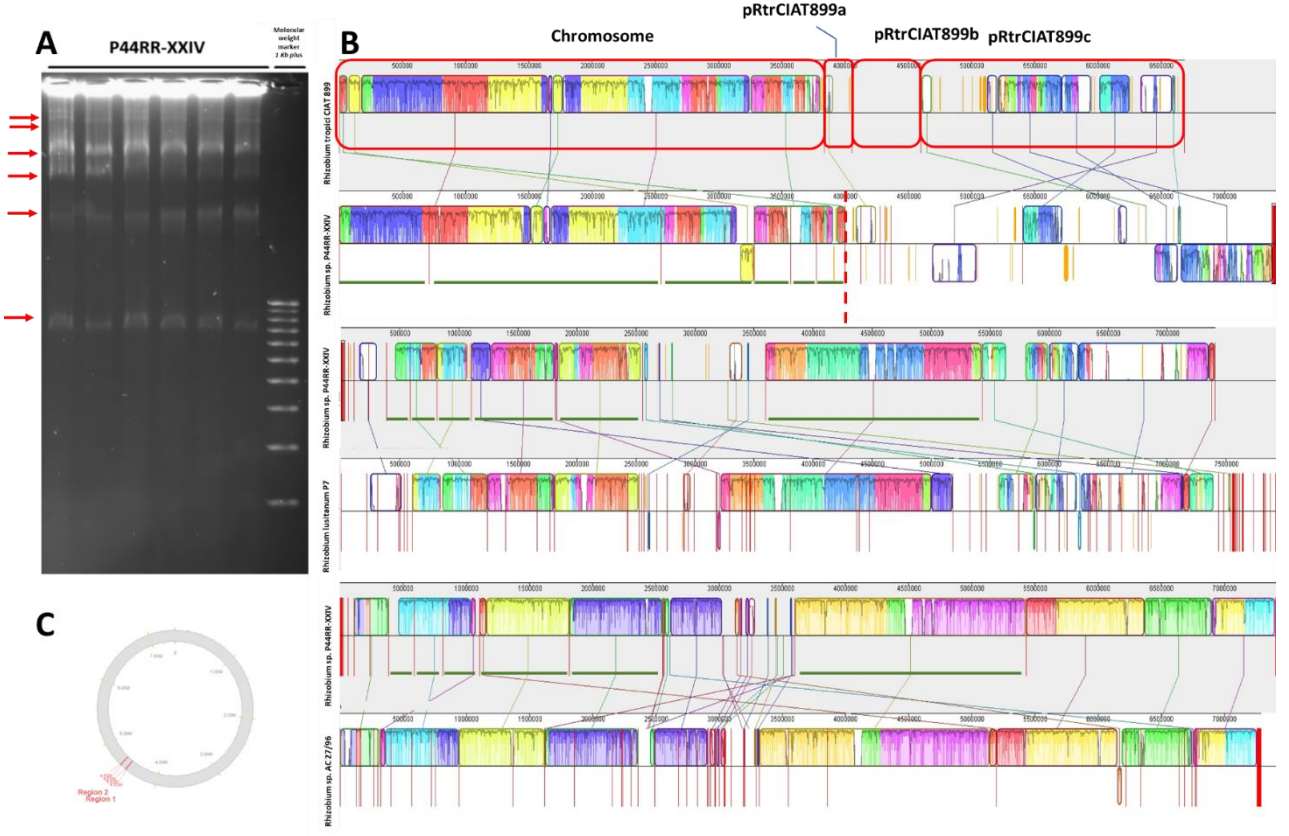

Figure 5.11. Studies of the genomic structure of *Rhizobium* sp. P44RR-XXIV. Electrophoresis in agarose gel of *Rhizobium* sp. P44RR-XXIV showing genomic elements and possible DNA shreading (red arrows) (A). Alignment and reordering of *contigs* of P44RR-XXIV genome with the most relevant reference genomes (B). Result of the search of phages and prophages using Phaster (C)

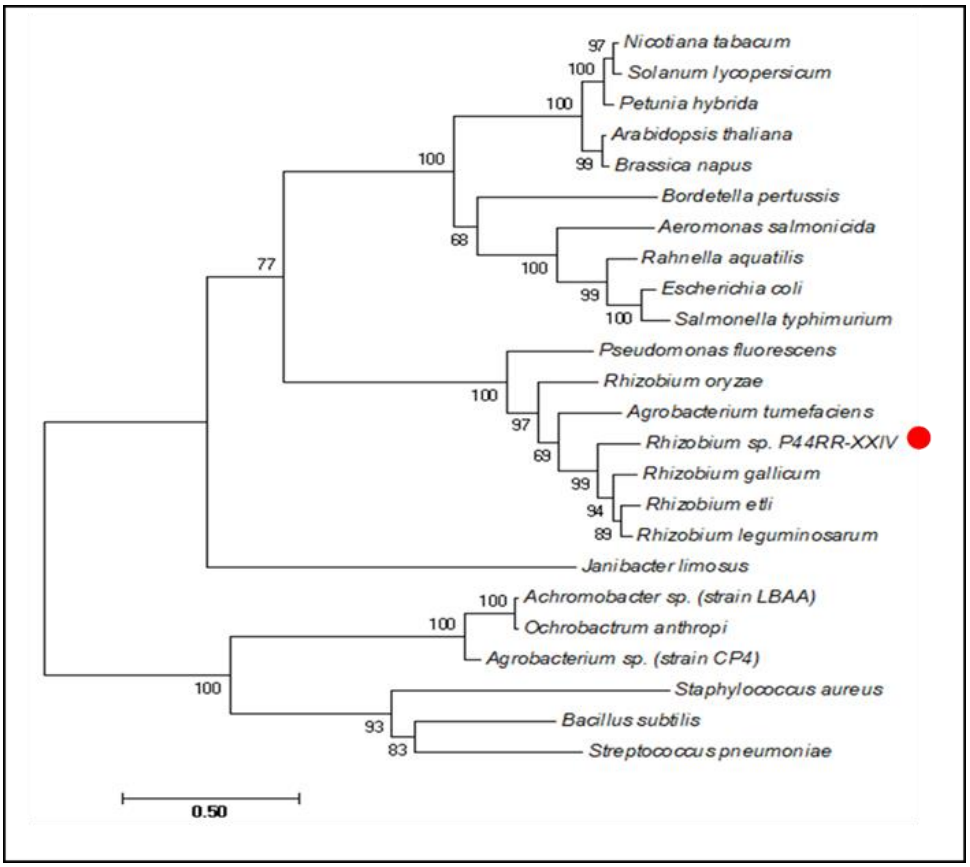

Figure 5.12. Phylogenetic tree built with amino acid sequences of EPSP synthase. Class I sequences were obtained from: *Arabidopsis thaliana* (P05466), *Brassica napus* (P17688) *Petunia hybrida* (P11043) *Solanum lycopersicum* (P10748), *Nicotiana tabacum Bordetella pertussis* (P12421), *Aeromonas salmonicida* (Q03321), *Rahnella aquatilis* (C9EGX2), *Escherichia coli* (P0A6D3), *Salmonella typhimurium* (P07637), *Pseudomonas fluorescens* (A1Z0H6), *Pseudomonas putida* (Q683S1*) Janibacter limosus* (L7PA93). Class II sequences were obtained from*: Bacillus subtilis* (P20691), *Streptococcus pneumoniae* (Q9S400), *Staphylococcus aureus* (Q05615), *Dichelobacter nodosus* (Q46550), *Halovibrio variabilis* (Q1H624), *Agrobacterium sp.* CP4 (Q9R4E4), *Achromobacter sp.* LBAA (P0A2Y5), *Ochrobactrum anthropi* (A6WUV8). P6BS-III is marked with a red dot. The multiple alignment of amino acid sequences was performed using the *Muscle* algorithm and the phylogenetic tree was obtained through the *Maximum Likelihood* method with 1000 iterations (bootstrap) in MEGA software version 7.0.

The exploration of EPSP synthase variants obtained, either from *wild type* tolerant microorganisms or by means of different mutagenesis methods, is an approach generally used to design new versions of transgenic plant species, capable of having a catalytic efficiency similar to that of the *wild type* enzyme and, at the same time, resistance to high doses of glyphosate (Funke et al., 2009; Pollegioni et al., 2011; Tian et al., 2011). Even though the purpose of this work is not to assess the suitability of the enzyme to be used in a transgenic event, these studies shed light on the regions of the enzyme found that would be relevant, both for their tolerance to glyphosate and their catalytic ability.

The sequence comparison revealed the substitution P101F (position 131 in the alignment, marked in a red box), which is not typical as those generally found in Class I enzymes, such as P101S, P101T or P101A. All the enzymes analyzed clustered have the same substitution.

The CP4 Class II enzyme, with a P101L substitution, has other residues related to tolerance and catalytic efficiency, marked in yellow boxes in the figure 5.13. Studying these regions is necessary when there is an important sequence variation with respect to Class I enzymes, since variation of EPSP synthase sequences in bacteria is not observed in plant species, where the mayor strategy for the investigation of the tolerance relays in studying the mutations in position 101, as it was exposed on chapter 3.

In the first yellow box (following the alignment position) the sequence LGNAGTAXRXL is highlighted (where X represents any amino acid). This region is highly conserved and critical for the attachment of phosphoenolpyruvate to the enzyme (Yi et al., 2016). The tolerant EPSP synthase of P44RR-XXIV has more sequence similarity to the typical EPSP synthase Class I of *E. coli* than to the Class II of *A. sp.* CP4. Having a previous knowledge of the tolerance of this strain, this could suggest that the catalytic efficiency could be conserved with respect to those of Class I. In the second and third yellow boxes, residues present in Class II enzymes RXHXE and NXTR are highlighted. These amino acids are involved in the destabilization of the glyphosate binding (Li et al., 2009; Tian et al., 2010). The sequence RXHXE in P44RR-XXIV differs from typical sequences in both classes and is similar to those sequences present in *Rhizobiaceae* included in the analysis. The NXTR sequence is not present in P44RR-XXIV, as well as the rest of the Type I enzymes.

The individual residues involved in the attachment of phosphoenolpyruvate and shikimate-3-phosphate (highlighted in the figure with green and red arrows, respectively) are conserved in all sequences, with one exception in position 203, where the substitution F203Y is observed in P44RR-XXIV (one aromatic amino acid for another). This does not happen in *A. sp.* CP4, where the substitution F203Q results in the replacement of one aromatic amino acid for one aliphatic amino acid.

It could be suggested that the substitution of region RXHXE to LGYID and the single mutation P101L contribute to the glyphosate tolerance of EPSP synthase of P44RR-XXIV. Even though there is abundant literature supporting this conclusion, it might be premature to assume this statement without studying the protein structure, since many differences are observed in EPSP synthase of P44RR-XXIV sequence and those of Class I and Class II. However, this represents adequate information to continue the analysis of this cluster of enzymes in the future.

The focus of the alignment was based on previously characterized regions of the enzyme, being clear that the rest of the members of the *Rhizobium* genus assessed show the same amino acid residues present in P44RR-XXIV EPSP synthase for these regions.

There were several culturable strains of *Rhizobium* species obtained that showed different level of tolerance to the herbicide (see section 5.3.2). Even though the sequences of these enzymes are not included in the analysis, the conservation observed in the regions analyzed within the genus, leads to suggest two possible alternatives with respect to the tolerance achieved specifically in P44RR-XXIV: 1- the differences in amino acid sequence of *Rhizobium* sp. P44RR-XXIV with respect to the rest of the genus are responsible of conferring the high amount of tolerance, or 2- other tolerance mechanisms, such as degradation, compartmentalization or transformation of the glyphosate molecule are relevant in the analysis of global tolerance (less probable than the previous one, due to the growth rate of the strain compared to the degradation rate of the herbicide).

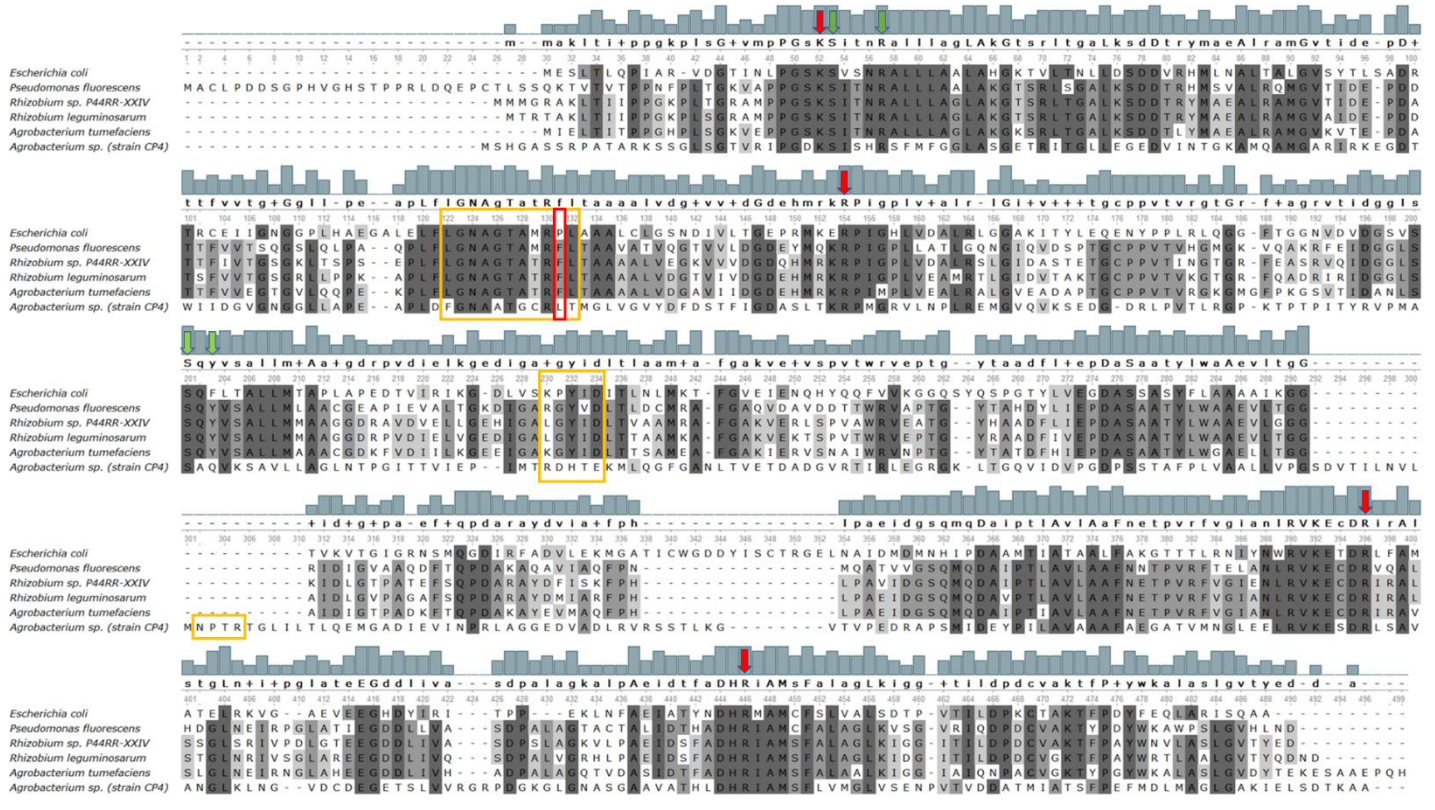

Figure 5.13 Multiple alignment of amino acid sequences of EPSP synthases, including sequences of the cluster where EPSP synthase of P44RR-XXIV was included, together with typical Class I *E. coli* and Class II *A*. sp. CP4, performed using the T-COFFEE algorithm. The alignment visualization was made using Unipro UGENE v1.26.1. The red box shows the critical substitution involved in conferring tolerance to plant and bacteria species. The regions involved in the attachment of phosphoenolpyruvate and the binding stability of glyphosate are shown in yellow boxes. The red and green arrows show the individual residues implied in the phosphoenol pyruvate and shikimate-3-phosphate, respectively.

Considering the structural differences of this enzyme with Class I and II enzymes, while it is similar in sequence to the rest of the enzymes within its genus, it would be interesting to perform structural and biochemical studies on this particular EPSP synthase in the future. This study aims to explain both tolerance and the catalytic efficiency in presence and absence of glyphosate in order to compare them to other commercially available enzymes. In a study performed by Han and contributors (2014), the EPSP synthase of *Rhizobium leguminosarum* is characterized with this purpose, although the sequence corresponds to a Class II enzyme.

In regards to the degradation of glyphosate, there are homologous sequences corresponding to the operon *phn* organized in the probable transcriptional unit *phnGHIJKLOCDEEMN*, and is similar to that present in members of the *Rhizobiaceae* family, such as *A. radiobacter, R. leguimnosarum* WSM1325 and *R. etli* CFN42 (Hove-Jensen et al., 2014).

At the same time, the operon *phnGHIJKLM* was found in the largest *contig* (*contig* 1) (Figure 5.11). This operon has a questionable *per se* phosphonatase activity, since it lacks the aminoalkylphosphonate *N-acetyltransferase* activity provided by *phnO*. *Mesorhizobium loti* and *Sinorhizobium meliloti* have plasmids containing *phn* operons which differ in structure with respect to the operon *phnGHIJKLM* (Kaneko et al., 2000; Finan et al., 2001). So far, this is the first record of homologues for the second *phn* operon in the *Rhizobium*/*Agrobacterium* genus. The structures of these operons are showed in figure 5.14.

Apart from the presence of two operons *phn* in P44RR-XXIV, an additional difference found between this strain and *Ochrobactrum* sp. P6BS-III operon is that these two loci have genes that coding for hypothetical peptides within the operon sequence and which at first, would not be related to its activity. The characterization of those proteins will be, in the future, of great interest, since they may be involved in the high specificity observed in microorganisms to different molecules presenting phosphonate groups (White & Metcalf, 1943; Wackett et al., 1987; Parker et al., 1999; Fox & Mendz, 2006).

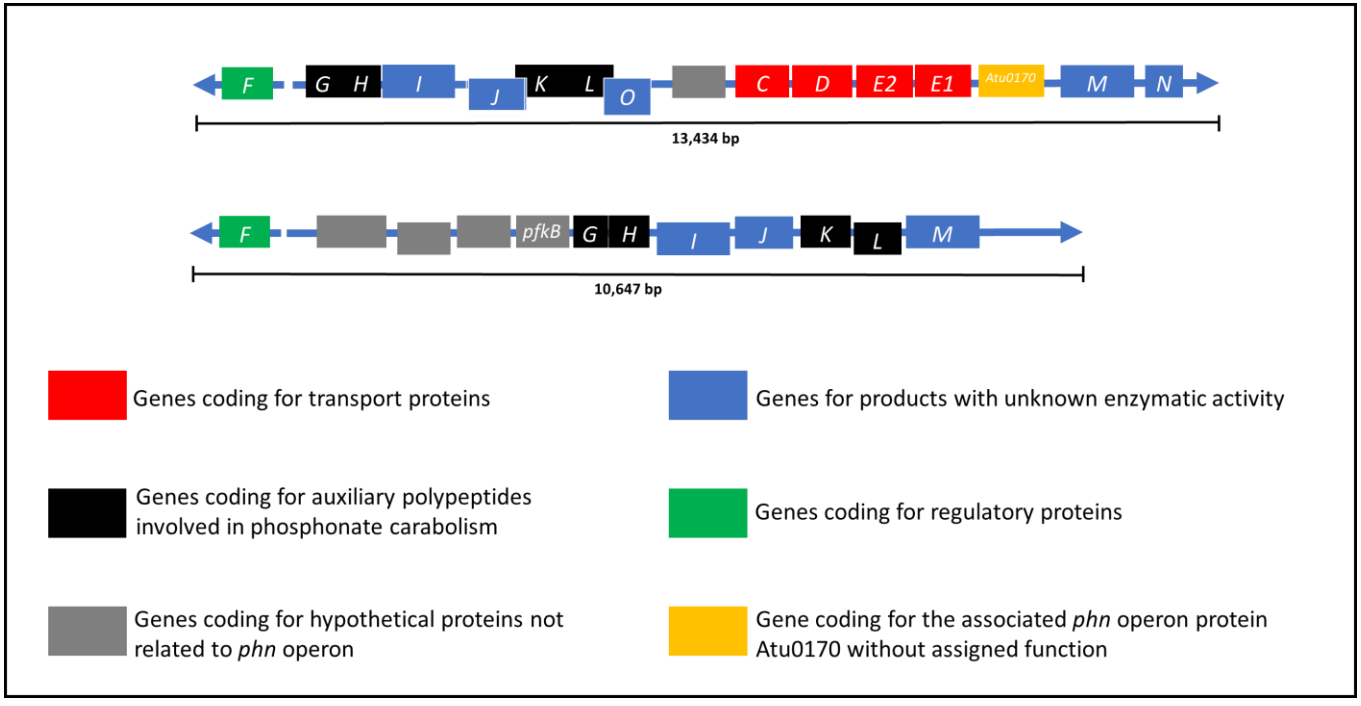

Figure 5.14. *Phn* sequence homologues found in *Rhizobium* sp. P44RR-XXIV, organized in two posible operons. It stands out the presence in the structure of genes not related to the C-P lyase activity.

Rhizobium sp. P44RR-XXIV has a single copy of the gene *AcdS* that codes for the enzyme ACC deaminase (1-aminocyclopropane-1-carboxylate deaminase, EC 3.5.99.7). Next to *AcdS* sequence, a homologue of the *AcdR* protein regulator was found in region 5`. The regulation of the ACC deaminase synthesis does not only depend on this regulator gene and it is the first time found in this genus. In another close member of the Rhizobiaceae, Mesorhizobium spp., the regulation mechanism proposed involves the genes nifA1 and nifA2 , while *AcdR* is not present (Singh et al., 2015).

The presence of genes involved in different Indole Acetic Acid (IAA) synthesis pathways was studied. Homologues of the aldehyde dehydrogenase gene, *AldA* (indole-3-pyruvate pathway), the nitrile and nitrile hydratase genes, *nit4* and *nthAB* (indole-3-acetonitrile/ indole-3-acetamide pathways), and a possible homologue of indoleacetamide hydrolase, *iaaH* (indole-3-acetamide pathway) were found.

Regarding to phosphorus uptake and metabolism, results of RAST annotation platform showed a total of 87 genes involved. These are related to the high affinity phosphate transporter and the PHO regulon, the different phosphates metabolism (including homologues of alkaline phosphatase), metabolism of polyphosphates and of alkylphosphonates (including the genes of the two *phn* operons).

The iron uptake is carried out through several transporters, such as the ABC transporters pitA, pitD, pitC and the iron-B12-siderophore-hemin system. Even though there were found genes related to the synthesis of siderophore *Aerobactin*, like in P6BS-III, the complete number of genes necessary for the biosynthesis of this molecule are not present, apparently.

Surprisingly, *Rhizobum* sp. P44RR-XXIV does not have homologous genes related to the nitrogen fixation (*nif* genes), nor the NOD nodulation factors (*nod* genes). This may indicate that the strain is not capable of forming a symbiotic association with *Lotus* through nodulation. This finding has already been documented recently in *Rhizobiaceae*, and is of great interest for the scientific community since the ecological role these saprophytes play in the rhizosphere remains unknown (Giraud et al., 2007; Jones et al., 2016; Okazaki et al., 2016). Another possibility is that, during the enrichment process, the pressure of the

selection exercised by the medium may have caused the loss of the symbiosis (*Sym*) plasmid (López-Guerrero et al., 2012).

Even though this finding does not hinder the remediation strategy for which the use of the microorganism was thought, due to the fact that P44RR-XXIV belongs to the *Rhizobium* genus and since it is phylogenetically close to the phytopathogen *Rhizobium rhizogenes* (Ream, 2009), additional studies were performed before the assessment of plant-microorganism interactions. Previously, pathogenic members of *Rhizobium* were grouped under the genus *Agrobacterium*, while those members related to benefic traits were part of the *Rhizobium* genus. Nowadays, the taxonomy classification tends to unify these two groups under the sole genus *Rhizobium* naming pathogens as part of the "*Agrobacterium group*".

There is no publication showing how *Rhizobium* sp. AC27/96, the closest genome to P44RR-XXIV, has been isolated and identified. Although, it has been considered as not phytopathogen according to Davis II and contributors (2016) in a bioinformatic analysis carried out specifically to investigate this trait. In the study, a bioinformatic tool for genomes was developed in order to investigate a tumor disease called "*gall disease*", which is cause, among other microorganisms, by *Agrobacterium tumefaciens*. AC27/96 has possibly been included in the study since *Rhizobium rhizogenes* NBRC 13257 it the closest genome.

The phylogenetic analysis through the complete fragment of the gene of rRNA 16s, together with the identification through complete genome (Figure 5.15A and Table 5.9) confirm that the closest *Agrobacterium* to P44RR-XXIV is effectively *Rhizobium rhizogenes* (previously known as *Agrobacterium rhizogenes*), a microorganism that causes the "*hairy root disease*". The ability to cause the disease lies in possession of the Ri-plasmid (Hodges et al., 2006).

The *R. rhizogenes* K599 (complete reference genome) was then compared to *R.* sp. P44RR-XXIV using Mauve, as it was done previously done with closest related microorganisms (Figure 5.11B). The group *Agrobacterium* is known for having two chromosomes (one linear and one circular). It can be expected to find a large number of regions in the genome of P44RR-XXIV represented in the two chromosomes of *R. rhizogenes* if P44RR-XXIV is close to *Agrobacterium* group. In the comparison (Figure 5.15B) it is observed that there are several regions that present synteny in chromosome I of K599, while presents a lower extension and quantity to those genomes studied in figure 5.11B. A higher amount of inversions

is also seen. This clearly indicates that P44RR-XXIV has more similarity to the chromosome of *Rhizobium* than to chromosome I of *Agrobacterium.* As far as chromosome II is concerned, a low synteny between chromosome and the different *contigs* of P44RR-XXIV is observed. The above leads us to conclude that, in terms of chromosome sequences, P44RR-XXIV presents more similarity to *Rhizobium* group.

In order to study the presence of Ri-plasmid, queries of genes present in pRi1724 plasmid belonging to *R. rhizogenes* MAFF301724 (Moriguchi et al., 2001) were searched using blast in the genome of P44RR-XXIV. The results are shown in figure 5.15C. There is no presence of sequences related to T-DNA, the opine synthesis "*ocp_h2*" and genes *virA*, *virH*, *virF*, and part of *VirD* homologues.

Homologues of genes present in three different regions of the plasmid were found, which are identified in figure 5.15C as Region 1, present in *contig* 10 (contig size: 124.257 pb) and corresponding to the syntenic region observed for pRi2659 plasmid in figure 5.15B, Region 2, located in *contig* 9 (contig size: 150.008 pb) and which also includes a homologue of *virD4* and, Region 3, present in contig 1 (contig size: 1.976.191 pb) and where the homologues of *agaG*, *agaF*, *riorf32* and *riorf33* are associated to a locus of biogenic amine synthesis, and separated to the rest of the genes of opine synthesis.

The absence of homologues of *rol* genes together with the absence of those necessary for the complete synthesis of any particular type of opine: genes *virD1*, *virD2* or *virE* group genes, plus the fact that the regions that present homology correspond to different contigs within the genome of P44RR-XXIV, would indicate the absence of an Ri-plasmid in P44RR-XXIV. (Ream, 2009).

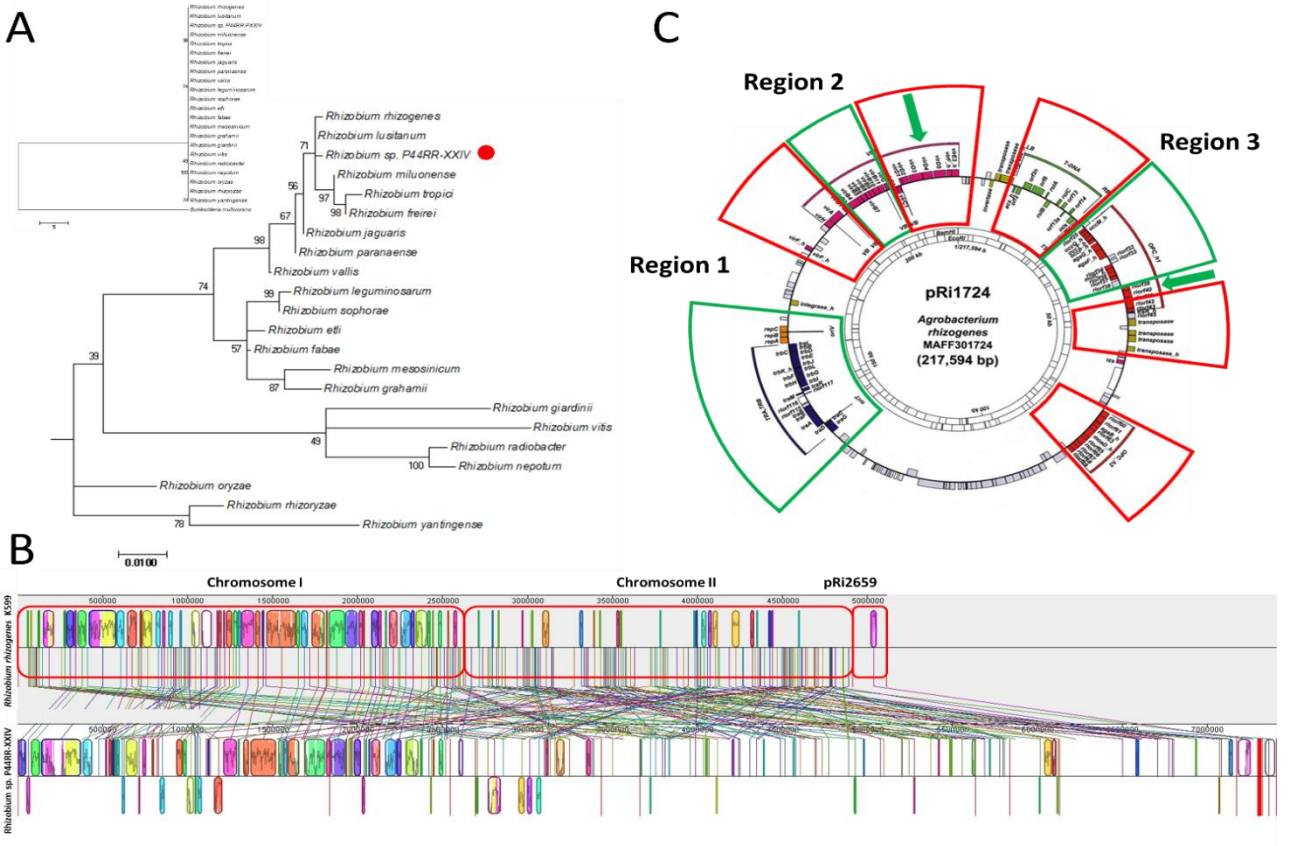

Figure 5.15. Comparative study between *Rhizobium* sp. P44RR-XXIV and *R. rhizogenes* K599. Phylogenetic tree made with the *type strains* of *Rhizobium genus* (A). The multiple alignment of amino acid sequences was performed usinf the *Muscle* algorithm, and the phylogenetic tree was obtained through the *Maximum Likelihood* method with 1000 iterations (bootstrap) MEGA software version 7.0. Alignment of genome of P44RR-XXIV against K599 using Mauve (B). Results of BLAST of peptides present in Ri-plasmid in genome of P44RR-XXIV (C), where the positive results are shown in red and positive results are shown in green (for more information, refer to the text).

5.4 Conclusions

A set of pure microorganisms was isolated from root and soil samples during chapter 4. In this chapter, a culture collection of those isolations was established. Strains were assessed according to its glyphosate tolerance and degradation capabilities and different plant growth promotion abilities. Besides, properties related to the industrial scale-up and use during a phytoremediation process were taken into account. Thus, specific objective 4, related to the genotypical and phenotypical characterization of these microorganisms and the specific objective 5, related to the assessment of plant growth promotion abilities were accomplished.

Throughout the characterization process, attention was given to traits such as conservation, use of simple energy sources and biomass and CFU mL^{-1} production. This perspective, referring to the use and application of microorganisms in different processes in the agroindustry, is generally left aside in literature regarding on isolation of glyphosate-tolerant microorganisms, diverting the focus of experimental work from any kind of technological application purpose.

Almost all the strains belong to the order of *Rhizobiales*, a promising result, since there are countless reports on plant beneficial microorganisms belonging to this group. In addition, all of the strains comply with the necessary precondition to be selected to perform further experimentation to select the most suitable plant-bacteria combination for a glyphosate rhizoremediation strategy in soils of the Humid Pampa, which are:

- A tolerance higher in several orders of magnitude to the maximum reported glyphosate concentration in soil. This would guarantee the survival of the strain in case of acute exposures to xenobiotic. According to phylogenetic origin of strains it is also possible to suggest inoculation assays in plant species which are directly located in the productive soil (i.e. *Glicine max*, *Medicago sativa*, the most relevant oilseed and pasture in the country). From results acquired during tolerance assay in agar, it is also observed that the genus *Rhizobium* shows a high variable sensibility to glyphosate (ranging from 500 to 10.000 mg kg^{-1}). Therefore, there is a need to study the sequences of EPSP synthase belonging to *Rhizobium* strains, since structural differences of

the enzyme within the genus may possibly contribute with information about regions involved in the conservation of catalytic activity and the glyphosate tolerance. That is how the sequence of P44RR-XXIV is highly tolerant, although it belongs to Type I enzymes. Future findings could break the traditional paradigm which groups Class I and Class II enzymes as sensitive and tolerant, respectively.

- The use of glyphosate as sole source of phosphorus, significant in soils having low concentration of phosphorus, where the availability of any alternative source may result in an advantage in those soils subjected to chronic exposure.
- A considerable number of *in vitro* plant growth promotion abilities. All strains have no less than three positive results in the different *in vitro* tests studied, where phosphorus solubilization stands out and it is probably correlated to the measured available levels of phosphorus. Having strains with more than one benefical trait encourages us to continue performing *in vivo* interaction studies using plant species.

Finally, the whole genome sequencing of the two strains that showed excellent results in all the assays performed was carried out, being these strains *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV, allowing the group to acquire new capabilities in the field of genomics. This experimentation fulfilled the specific objective number 7.

The acquisition of new genomic data arising from microorganisms which genome has not been sequenced or studied in depth, contributes significantly with the comprehension of the genus, as it happened with these two selected microorganisms. *Ochrobactrum* sp. P6BS-III, probably *Ochrobactrum heamatophilum*, has not been previously sequenced, and *Rhizobium* sp. P44RR-XXIV, probably *Rhizobium lusitanum* or AC 27/96 has been poorly described.

Full genome sequencing is now an essential tool to be considered in future plant-microorganism interaction assays, where endless possibilities could determine a positive interaction and a subsequent establishment of the microorganism in the rhizosphere. Even though relevant plant growth promotion tests have been explored, there are several positive traits that have not been

phenotypically tested. New experimentation can be suggested if homologous sequences responsible of those benefic characteristics are found in the genome.

The use of different methods for *in silico* genome to genome comparison led to obtain supplementary information regarding the structure of the genome of *Rhizobium* sp. P44RR-XXIV giving not only the idea of global nucleotides similarity, but also enabled to identify those regions with high sequence homology and those with low sequence homology, and the possible genomic element to which those regions belong. At the same time, the genome of *Ochrobactrum* sp. P6BS-III contributed with a large number of unique genes to the genus having many of them unknown functions.

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S5.1 Supplementary data

S5.1.1 Nutrient rich 869 medium

It is used in liquid and solid form

Reagent	**Amount (g L^{-1})**
Tryptone	10.0
Yeast extract	5.0
NaCl	5.0
D-(+) -glucose.H2O	1.0
$CaCl_2.2H_2O$	0.345
Bacteriological agar	15.0
pH: 7.0	

Reference:

Mergeay M, Nies D, Schlegel HG, Gerits M, Charles P, Van Gijesegem F (1985) Alcaligens eutrophus CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. Journal of Bacteriology 162: 328-334.

S5.1.2 ACC-deaminase salt medium

Reagent	Amount (g L^{-1})
KH_2PO_4	0.4
K_2HPO_4	2.0
$MgSO_4$	0.2
$CaCl_2$	0.1
Glucose	1.0
Sucrose	1.0
Sodium acetate	1.0
Sodium citrate	1.0
Malic acid	1.0
Mannitol	1.0
$FeSO_4$	0.005
H_3BO_3	0.002
$ZnSO_4$	0.005
Na_2MoO_4	0.001
$MnSO_4$	0.003
$CoSO_4$	0.001
$CuSO_4$	0.001
$NiSO_4$	0.001
1-Aminocyclopropanecarboxylic Acid	0.500
pH: 6.6	

Reference:

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (Brassica juncea L. Czern.). Soil Biology & Biochemistry 37: 241-250. doi: 10.1016/j.soilbio.2004.07.033.

S5.1.3 MR-VP medium (acetoin production test)

Reagent	Amount (g L^{-1})
Peptone	7.0
Glucose	5.0
K_2HPO_4	5.0
pH: 6.9	

Reference:

Voges O & Proskauer B, (1898) Beitrage zur Ernahrungsphysiologie und zur differential diagnose der Bakterien der hemmorrhagischen septicamie. Zeit. fur Hyg., 28: 20-32.

S5.1.4 NBRIP growth medium (calcium phosphate solubilization test)

Reagent	Amount (g L^{-1})
Glucose	10.0
$Ca_3(PO_4)_2$	5.0
$MgCl_2.6H_2O$	5.0
$MgSO_4.7H_2O$	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1
Bacteriological agar	15.0
pH: 7.0	

Reference:

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiology Letters 170(1): 265-270.

S5.1.5 PSM medium (phytate mineralization test)

Reagent	Amount (g L^{-1})
Glucose	10.0
Sodium phytate	4.0
$CaCl_2$	2.0
KCl	0.5
$(NH_4)_2NO_3$	5.0
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	0.01
$MnSO_4.H_2O$	0.01
Bacteriological agar	15.0
pH:7.0	

Reference:

Jorquera MA, Hernandez MT, Rengel Z, Marschner P, Mora MD (2008) Isolation of culturable phosphobacteria with both phytate-mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. Biology and Fertility of Soils 44: 1025-1034. doi: 10.1007/s00374-008-0288-0.

S5.1.6 ST medium (organic acids production test)

Reagent	Amount (g L^{-1})
Sucrose	20.0
Tryptone	5.0
$Na_2MoO_4.2H_2O$	0.0002
H_3BO_3	0.002
$CuSO_4.5H_2O$	0.0002
$FeCl_3$	0.001
$MnCl_2.4H_2O$	0.0002
$ZnCl_2$	0.0028
pH:7.0	

Reference:

Cunningham JE, Kuiack C (1992) Production of Citric and Oxalic Acids and Solubilization of Calcium-Phosphate by Penicillium-BILAII. Applied and Environmental Microbiology 58: 1451-1458.

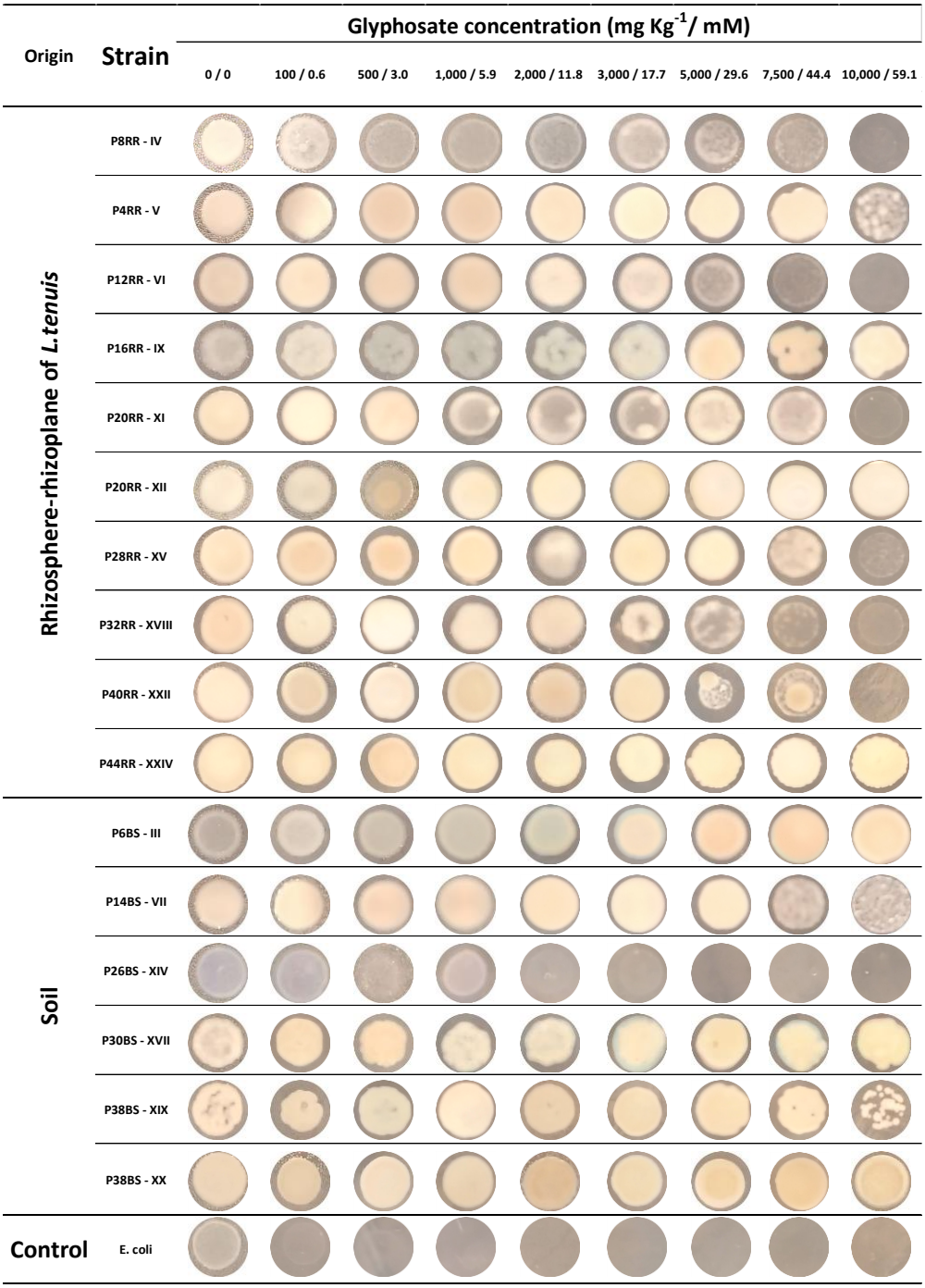
S5.1.7 284 medium (Siderophores production test)

Reagent		**Amount (g L^{-1})**
Tris-HCl		6.06
NaCl		4.68
KCl		1.49
NH_4Cl		1.07
Na_2SO_4		0.43
$MgCl_2.6H_2O$		0.2
$CaCl_2.2H_2O$		0.03
$Na_2HPO_4.2H_2O$		0.04
$ZnSO_4.7H_2O$		0.144 mg
$MnCl_2.4H_2O$		0.100 mg
H_3BO_3		0.062 mg
$CoCl_2.6H_2O$		0.190 mg
$CuCl_2.2H_2O$		0.017 mg
$NiCl_2.6H_2O$		0.024 mg
$Na_2MoO_4.2H_2O$		0.036 mg
Glucose		0.52
Sodium gluconate		0.66
Fructose		0.54
Sodium succinate		0.81
Lactic acid		0.7mL
Iron (III) citrate	0.066 mg (0.25 μM)	0.797 mg (3.00 μM)
pH: 7.0		

Reference:

Schwyn B, Neilands JB (1987) UNIVERSAL CHEMICAL-ASSAY FOR THE DETECTION AND DETERMINATION OF SIDEROPHORES. Analytical Biochemistry 160: 47-56. doi: 10.1016/0003-2697(87)90612-9

S5.1.8 Determination of minimum inhibitory concentration of glyphosate in agar. Individual results.



Chapter 6 - Approaches for the study of plant-microorganism interactions

6.1 Introduction

In order to know the true potential of the association of a microorganism to a plant species, it is necessary to understand, as thoroughly as possible, the influence of the different variables in this complex system, which is formed by the bacteria and the plant, but also by the substrate, the different environmental conditions, the presence of pollutants and different soil managements.

The first step in this long path of knowledge is to start studying whether interactions take place between the candidate bacteria and the plant species, whether these interactions are stable through time and whether the purpose of the association is fulfilled association (Kuiper et al., 2004). Once a positive effect is observed, the object of study will be the *molecular* mechanisms involved in the association.

By having available both a glyphosate tolerant plant species and a degrading and tolerant bacteria culture collection, complying with all the initially set requirements in the objectives of this thesis for a rhizoremediation strategy, the pursued goal was to evaluate the interactions between the two elements.

Firstly, the bacteria and the plant species were placed in axenic systems where it was possible to assess the influence the microorganisms, which *in vitro* expressed different plant growth promotion abilities, have on *L. corniculatus.*

Secondly, molecular tools like fluorescent tagging, result of the expression of fluorescent proteins by the strains of interest, were used to observe the colonization and spatial distribution of these strains in the root system. This is a highly important tool in microbial ecology (Errampalli et al., 1999; Ramos et al., 2013). The colonization patterns and the spatial distribution of the microorganisms on the surface of the root were complemented with scanning electron microscopy (SEM) imaging.

Finally, to test if the rhizoremediation system works, a microcosm experiment was carried out, which is a controlled laboratory system that simulated the field conditions. From the results obtained from the microcosm systems, critical factors may be identified for future field trials (Ruberto et al., 2013).

The following specific objectives were met during this stage:

6. Assessment of the isolated degrading strains' capacities to colonize the roots of the candidate plant species.

8. Evaluation of biodegradation efficiency of different rhizoremediation systems proposed by microcosm assays.

6.2 Materials and Methods

6.2.1 Inoculation tests in vertical agar plates

Plant-microorganisms *In vitro* interaction studies were conducted to study possible plant growth promoting effects on *Lotus corniculatus* by strains previously typified in chapter 5. This interaction was assessed through bacteria inoculations and subsequent growth in vertical agar plates (VAPs). The used protocol was originally designed for *Arabidopsis thaliana* (Remans et al., 2006) and adapted for the first time to a *L. corniculatus* cultivar. *Lotus* seeds were obtained from the Vreeken´s Zaden company, The Netherlands.

The method consisted of inoculating recently germinated seedlings (with approximately 1 cm root) with the strain of interest, and then observing the growth rate and development, and comparing the growth to a control seedling (not inoculated).

For this purpose, Gamborg B5 culture medium is solidified (Gamborg et al., 1968) (S6.1.1) in square plates with 120 mm sides. A 10 mm agar strip is then removed from one of the edges. The bacterial strain is streaked on the agar in a concentration of approximately 1.10^5 CFU mL^{-1}. The seedling, superficially sterilized beforehand (according to the protocol described in chapter 3), is set in the space where the removed agar strip used to be, ensuring to place the root in intimate contact with the inoculated agar. Next, the plate is placed vertically and taken to the vegetal growth chamber (conditions: 12 hours of photoperiod, 65% of relative humidity, day/night temperatures of 22°C/18°C and PAR of 170 µmol m^{-2} s^{-1}), where growth will be visually assessed for 10 days, time in which the control primary root of the seedlings contacts the base of the plate. Five replicates of plates per strain were made, each one containing 5 seedlings, giving a total of 25 seedlings per condition. On the tenth day, pictures of each plate were taken, the total root length, the number and length of the primary root, the number and length of the secondary roots were measured using the image analysis software RootNav V 1.8.1 (Pound et al., 2013). Afterwards, the seedlings were harvested to determine the total fresh weight and root.

Based on the results obtained in chapter 5, the following 8 strains were selected and analyzed through three independent experiments: *Ochrobactrum*

P6BS - III, *Rhizobium* P8RR - IV, *Rhizobium* P4RR - V, *Rhizobium* P14BS - VII, *Rhizobium* P16RR - IX, *Phyllobacterium* P30BS - XVII, *Ochrobactrum* P38BS - XX and *Rhizobium* P44RR - XXIV.

6.2.2 Microscopy studies

6.2.2.1 Conjugation of bacterial strains

In order to visualize the location of the microbial strains in *Lotus* roots using laser confocal microscopy, the pMP4655 or pMP7604 plasmids, carrying the gene for green fluorescent protein (GFP) and the gene for resistance to the antibiotic tetracycline (Bloemberg et al., 2000) or the gene for red fluorescent protein (RFP) and the gene for tetracycline resistance (Lagendijk et al., 2010) were transferred respectively to *Ochrobactrum sp.* P6BS-III and *Rhizobium* sp. P44RR-XXIV by conjugation. These procedures involve the use of three strains: the donor strain, *E. coli* dH5a carrying plasmid pMP4655 with the gene that codes for green fluorescent protein (egfp) or plasmid pMP7604 with gene that codes for red fluorescent protein (erfp); the helper strain, *E. coli* dH5a carrying plasmid pRK2013; and the recipient strains, P6BS-III or P44RR-XXIV, according to the conjugation.

Conjugation protocols consisted of cultivating in 869 medium for one day the strains P6BS-III y P44RR-XXIV and, at the same time, the donor strain (D) and helper strain (H). On the next day, 100 µL of D strain culture were transferred to a fresh 869 medium with tetracycline (20 or 15 µg mL^{-1} according to plasmid resistance) while 100 µL of the remaining strains where transferred to fresh 869 medium. D and H strains were cultivated at 37°C until reaching an OD_{600} of 0.300-0.400, while strains P6BS-III and P44RR-XXIV were kept at 30°C until reaching an OD_{600} of approximately 0.700. All 869 broths were centrifuged at 3,000 rpm for 10 minutes, and the supernatant was discarded, and the pellets were resuspended in fresh 869 medium once again. At the same time, a Petri dish containing solid 869 medium was divided in two halves; one half was covered with a sterile filter made of cellulose acetate Isopore (Millipore, Billerica, USA), while the other half was divided into three equivalent parts, each one corresponding to one of the strains and labelled accordingly: recipient strain (P P6BS-III o P44RR-XXIV), strain D and strain H. One plate was reserved for each conjugation. Then,

50 µL of each cell suspension was taken, placing a droplet in the corresponding part of the plate and the rest in the cellulose acetate filter. All plates were incubated at 30°C overnight. After incubation, the cellulose filter was removed, placed in a sterile tube containing $MgSO_4$ 10mM and shaken to release all cells from the filter. From the resulting suspension, 100 µL and a 1/10 dilution of it were streaked in plates with selective medium, containing tetracycline (20 or 15 µg mL^{-1}) and glyphosate (500 mg Kg^{-1}) and the plates were incubated for 3 days at 30°C. Once the development of colonies was observed, five of each plate were removed and streaked again in a selective medium, in order to perform the conjugation controls to each of these isolations afterwards.

The success of transconjugation was corroborated with the following set of experiments:

1. Presence of fluorescence in a fluorescence microscope (excitation of 480/40 nm with emission of 510 nm for GFP and excitation 542/27 with emission of 610 nm for RFP).
2. BOX-PCR, using the protocol suggested by Weyens and collaborators (2009).
3. Sequencing of the 16S ribosomal RNA gene in the fluorescent strain.
4. The stability of the strains was corroborated by carrying out subcultures and repeating the monitoring under microscope.

6.2.2.2 Confocal microscopy studies

L. corniculatus was grown in vertical plates, according to proceedings described in section 6.2.1, but this time seeds were sterilized and then inoculated with the transconjugant strains obtained in section 6.2.2.1. Additionally, since strains expressing GFP and RFP were available, the co-inoculation of *Ochrobactrum sp* P6BS-III and *Rhizobium sp.* P44RR-XXIV was also tested. After a week in the plant growth chamber, the plates containing the inoculated seedlings were removed and washed thoroughly with sterile PBS solution, then placed once more in plates with fresh medium and transported to the Central Core Facility for Biomedical Microscopic Imaging & Laboratory of Cell Biology and Histology, University of Antwerp. Once there, the roots were washed one more time with sterile PBS solution and then placed on a glass slide to obtain images under the microscope Ultra VIEW VoX (Perkin Elmer, Zaventem, Belgium) under the operation and supervision of PhD Isabel Pintelon.

An excitation wavelength of 561 nm was used for the strain labelled with RFP, 475 nm for the strain labelled with GFP and 405 (DAPI) for the cell wall and plant structure contrast. The lenses used for image acquisition included a 40X CFI Plan Fluor lens (numerical opening of 0.75; work distance of 0.72 mm) and a 20X CFI Plan Apochromat VC lens (numerical opening of 0.75; work distance of 0.72 mm). The images were taken using a Hamamatsu C9100-50 camera (Hamamatsu Photonics K.K., Hamamatsu, Japan) and analyzed with the ImageJ software (https://imagej.nih.gov/ij/index.html).

6.2.2.3 Scan electron microscopy studies

To obtain the images by scan electron microscopy (SEM), samples consisting of inoculated roots obtained as described in section 6.2.2.2 were fixated using 4% glutaraldehyde overnight. Samples where then dehydrated using ethanol in an increasing gradient, which consisted of placing the root in 30%, 50%, 70%, 95% and 100% solutions each for 15 minutes. Finally, the samples were frozen in tertbutyl ethanol at -20°C, to be sublimated later. The samples were analyzed at IMO-IMOMEC, the Institute for Material Research of Hasselt University, Belgium, where images were taken in a Quanta 200 FEG-SEM apparatus (FEI Company, Eindhoven, Netherlands).

6.2.3 Microcosm studies

A microcosm assay was conducted in order to assess efficiency in glyphosate removal of the proposed plant-microbe associations. The bioaugmentation with *Ochrobactrum sp* P6BS-III and *Rhizobium sp* P44RR-XXIV was also tested in soil. Table 6.1 shows the study design.

Table 6.1. Microcosm assay set up design

Inoculation	Soil	Soil + *Lotus corniculatus*
Not inoculated	3 replicates	6 replicates
Ochrobactrum sp. P6BS-III	3 replicates	9 replicates
Rhizobium sp. P44RR-XXIV	3 replicates	9 replicates

Soil from Plot L4 was used in the assay (control plot of treated plot L3, see chapter 4), which was again sieved and homogenized by quartering and then. Two hundred grams of soil were loaded into glass flasks of 360 mL capacity, covered and then subjected to 3 autoclave cycles of 40 minutes at 1 atm of overpressure. Soil moisture was adjusted to approximately 38%.

L. corniculatus where surface sterilized seeds and grown in Murashige-Skoog medium (S3.1.1) for sterility check. Seedlings with approximately 1 cm root were selected to be part of the study.

Strains corresponding to *Ochrobactrum sp* P6BS-III and *Rhizobium sp* P44RR-XXIV were grown in BSM-Gly (S4.1.1) with 200 rpm agitation and 25 ± 1 °C until reaching late exponential stage. Cell culture was then centrifugated at 3000 rpm for 10 minutes and the cell pellet was resuspended to reach a concentration of approximately 1.10^8 UFC mL^{-1}.

At the same time, the inoculation carrier solution was prepared according to the protocol suggested by Sridhar (Sridhar et al., 2004) (S6.1.2). Bacteria suspensions were then incorporated to this inoculation solution, reaching a final cell density of approximate 1.10^7 UFC mL^{-1}. Roots where then inoculated. Bulk soils where also inoculated, reaching a final concentration of approximately 1.10^7 UFC mL^{-1}. Non-inoculated experimental units spiked with the sterile carrier solution in the same proportion as experimental units with bacterial presence.

Seedlings were planted in the microcosm soils and after 30 days, having reached a stage of 7 true leaves, commercial glyphosate (Roundup Ultramax®, Monsanto 74.7% ammonium salt) was spiked to soils attaining 5 mg Kg^{-1} final concentration in all experimental units.

Sampling was carried out at three different moments: after glyphosate application, at day 6 and day 20. The procedure for obtaining each sample was the following:

From experimental units without plants, a soil aliquot of approximately 1.00 gram was taken to perform a total heterotroph microorganism count, according to proceedings described chapter 4; three aliquots of approximately 1.00 gram were stored in cryotubes. Total DNA was isolated and ARISA (see chapter 4) was used to monitor the strain's presence in soil. Another soil aliquot of approximately 5.00 grams was destined to the Environmental Research Centre

of the University of La Plata, Argentina and analyzed by PhD Damián Marino, who measured glyphosate residues.

In experimental units with plants, the roots were removed and, softly shaken to remove soil particles and then placed in tubes of 50 mL containing 40 mL of the extraction solution (NaCl 0.9% Tween 80 0.01%) previous removal of aerial parts. Tube was set in the orbital shaker at 200 rpm for 30 minutes. The suspension was centrifuged at 10,000 rpm at 4 ± 1 °C. The pellet was preserved, in a to a cryotube. Total DNA was isolated and ARISA (see chapter 4) was used to monitor the strain's presence in the root. Similarly, soil aliquots of approximately 1.00 gram were taken from the zone near the root to perform a total heterotroph microorganism count and another soil aliquot of approximately 5.00 grams was used to measure glyphosate.

6.3 Results and discussion

6.3.1 Inoculation assays in vertical agar plates

The complete set of results of all measured biometric parameters during the assays are shown in table 6.2. As tests were conducted in batches, each tested strain corresponds had it control.

Table 6.2. Vertical agar plates assay

Strain	Biomass		Root length			
	Total (mg)	Root (mg)	Total (mm)	Primary (mm)	Average of lateral roots (mm)	Number of lateral roots
Control*	42.5 ± 5.8	18.5 ± 3.8	177.1 ± 39.0	100.4 ± 14.5	21.1 ± 9.5	3.9 ± 1.7
Control**	39.5 ± 6.9	16.9 ± 3,8	170.4 ± 46.1	108.5 ± 31.6	19.0 ± 12.8	3.0 ± 1.8
Control***	41.3 ± 6.5	17.0 ± 3.7	179.7 ± 61.4	112.6 ± 23.4	12.2 ± 6.5	5.1 ± 2.1
P6BS-III	39.9 ± 6.6	18.8 ± 4.6	162.0 ± 39.5	111.5 ± 25.1	14.9 ± 10.2	3.5 ± 1.4
P8RR-IV	36.7 ± 6.1	15.9 ± 3.6	161.2 ± 42.1	113.0 ± 28.0	18.0 ± 12.7	2.9 ± 1.4
P4RR-V	43.4 ± 9.8	18.6 ± 6.4	167.9 ± 45.6	108.4 ± 16.1	11.1 ± 5.3	4.8 ± 2.6
P14BS-VII	39.1 ± 6.3	17.5 ± 3.2	192.1 ± 44.2	109.4 ± 23.7	28.3 ± 15.0	3.1 ± 1.5
P16RR-IX	35.2 ± 5.9	16.8 ± 3.8	182.3 ± 46.8	112.6 ± 36.5	20.9 ± 12.2	3.3 ± 1.9
P30BS-XVII	43.2 ± 6.8	19.0 ± 5.2	175.7 ± 53.9	105.4 ± 21.3	17.1 ± 9.3	3.6 ± 1.8
P38BS-XX	39.1 ± 7.9	17.2 ± 4.5	176.0 ± 39.3	115.1 ± 31.0	17.3 ± 10.7	3.8 ± 2.0
P44RR-XXIV	39.4 ± 8.0	19.8 ± 6.5	169.7 ± 71.7	105.5 ± 21.9	18.7 ± 12.8	3.1 ± 1.1

* Control for strains P6BS-III and P44RR-XXIV

** Control for strains P8RR-IV, P14BS-VII, P16RR-IX and P38BS-XX

*** Control for strains P4RR-V and P30BS-XXVII

None of the tested strains showed significant differences when compared to control for all parameters assayed (ANOVA test, Dunnet post-hoc test, α: 0.05). These results may be due to the differences in rate growth of the root between *A. thaliana* y *L. cornicualtus*. Even though the development of Lotus plants showed no signs of alteration not stress, a different size of the root, growth rate and development could have masked the positive effects caused by the microorganisms. That is how, primary roots of non-inoculated Arabidopsis´ seedlings reach the bottom of the plate after two weeks, showing an important number of secondary roots and, therefore, a large radicular system. In contrast,

Lotus reaches the bottom with its primary root at day 7 to 10, and many times without developing secondary roots.

Nevertheless, despite of the above mentioned, both *Ochrobactrum* P6BS - III and *Rhizobium* P44RR – XXIV exhibited significant growth in plate without any negative effect on the plant species. The presence of hairy roots was observed too as shown in figure 6.1 (although observations are not statistically significant).

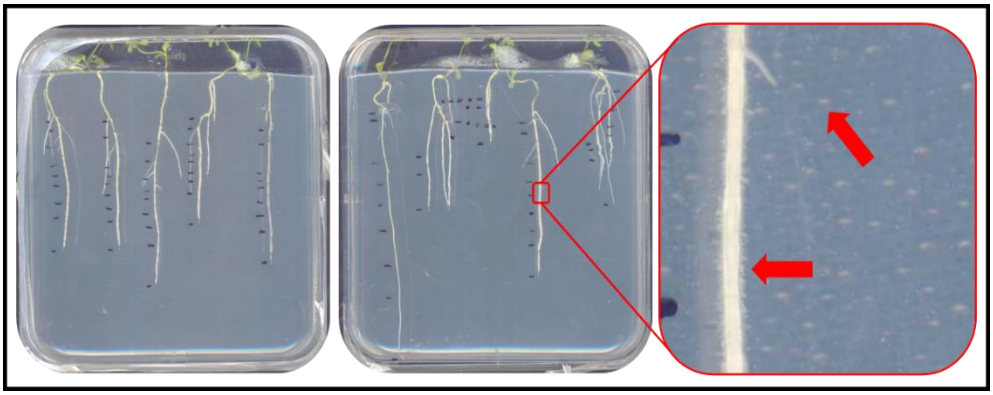


Figure 6.1. Vertical agar plates assay. On the left side a plate with non-inoculated seedlings can be seen (control). The central figure shows a plate with plants inoculated with *Ochrobactrum sp*. P6BS-III, and on the right side figure an amplification of one of the roots from the central plate can be seen, where the red arrows indicate on one hand the root villi, and on the other hand, the great microbial growth on the plate, appearing as whitish homogenous colonies.

6.3.2 Microscopy studies

Three different transconjugant microorganisms were obtained after conjugation: *Ochrobactrum* P6BS-III GFP+, *Ochrobactrum* P6BS-III RFP+ and *Rhizobium* P44RR-XXIV GFP+. These three strains complied with every control step. In Figure 6.2, positive fluorescence is displayed by *Rhizobium* P44RR-XXIV GFP+ and *Ochrobactrum* P6BS-III RFP+, in a co-inoculated *L. corniculatus*. All strains were individually checked in plate at first. The band patterns generated by PCR BOX is also shown in the figure. Each band pattern is characteristic in each strain and, consequently, it is very useful to differentiate the recipient bacteria from the donor, the helper, or any contamination. Thus, it was expected that the band pattern corresponding to *wild-type* strains would be similar to the one from transconjugant strains. This happened in three of the four studied cases.

Transconjugant strains were regrown once more. Once the maximum value of OD was reached, each strain was stored in a cryotube in $MgSO_4$ 10 mM and 20% glycerol solution at -40°C. Simultaneously, a second DNA extraction was conducted, to sequence and confirm the identity from the 16S ribosomal RNA gene.

Images taken during confocal laser microscopy studies confirm that both bacterial strains remained attached to the root even after sequential rinsing. In

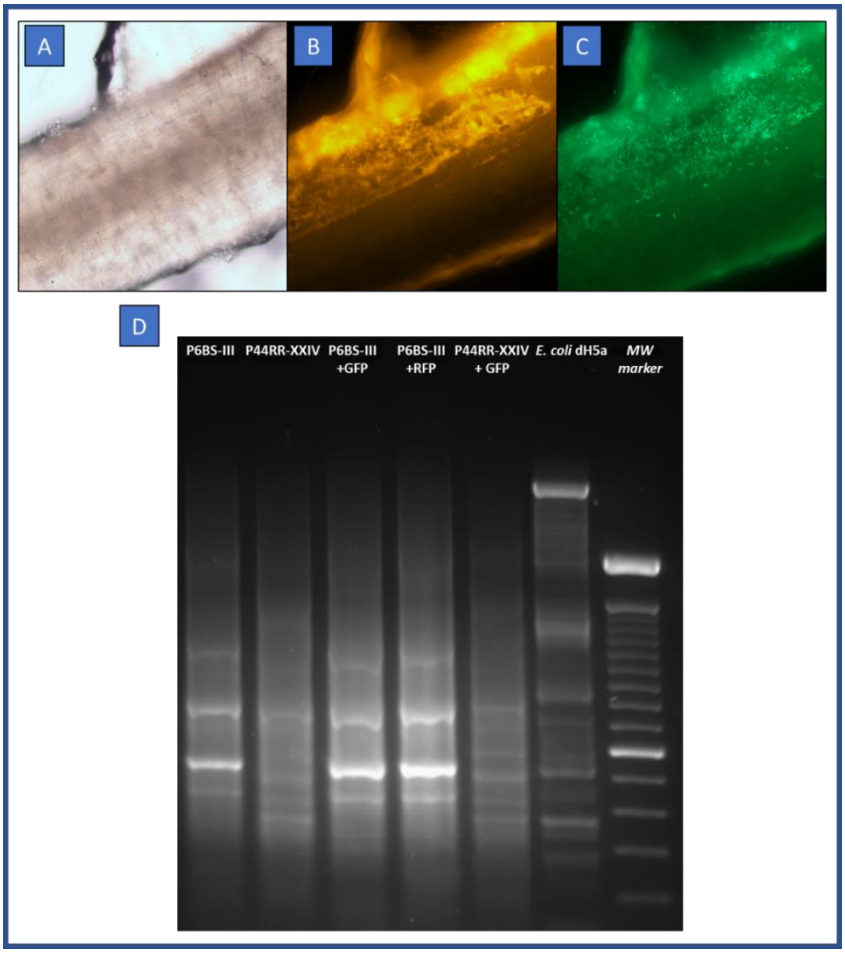


Figure 6.2. A. Microscopical image of a root section of *L. corniculatus* co-inoculated with *Rhizobium sp.* P44RR-XXIV GFP+ and *Ochrobactrum sp.* P6BS-III RFP+ without excitation. B. Same field, with excitation light 542/27, where *Ochrobactrum sp.* P6BS-III RFP+ cells attached to the root can be seen. C. Same field, with excitation light 480/40, where *Rhizobium sp.* P44RR-XXIV GFP+ cells attached to the root can be seen. D. BOX-PCR showing the different band patterns produced by the *wild-type* strains and

all analyzed images of roots of *L. corniculatus*, bacterial strains were present in the root surface. P6BS-III can be observed associated in small and dense cell groups next to the cell wall edges of the root P44RR-XXIV seems to be uniformly distributed possibly associated to hairy roots, mainly as individual cells, and in a lower number than P6BS-III. Figures 6.3, 6.4 and 6.5 reveal images of *L. corniculatus* inoculated with P6BS-III, with P44RR-XXIV and co-inoculated with both strains, respectively.

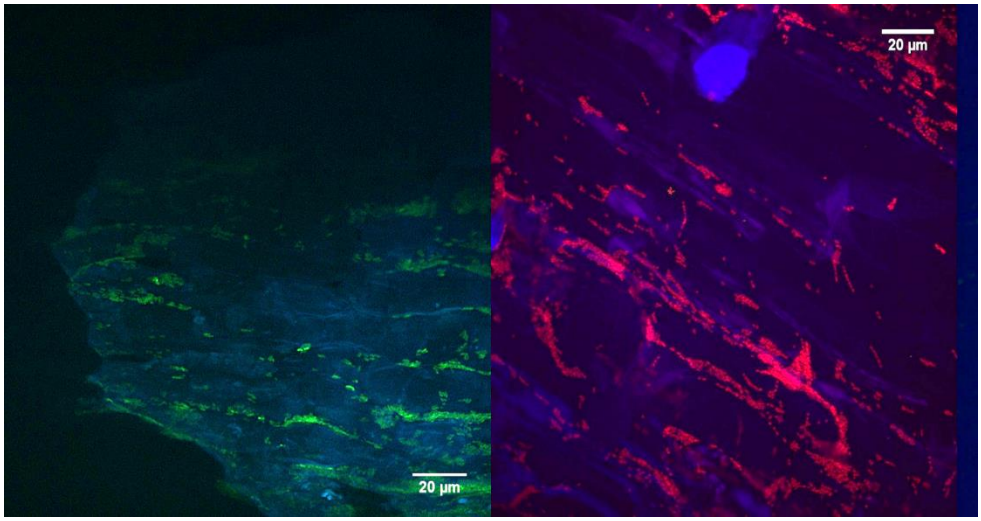


Figure 6.3. Confocal laser microscopy images of *L. corniculatus* roots inoculated with *Ochrobactrum* sp. P6BS-III. On the left, the tip of the main root and *Ochrobactrum* sp. And P6BS-III GFP+ are shown. On the right, another the main root in an intermediate zone, inoculated with *Ochrobactrum* sp. P6BS-III RFP+.

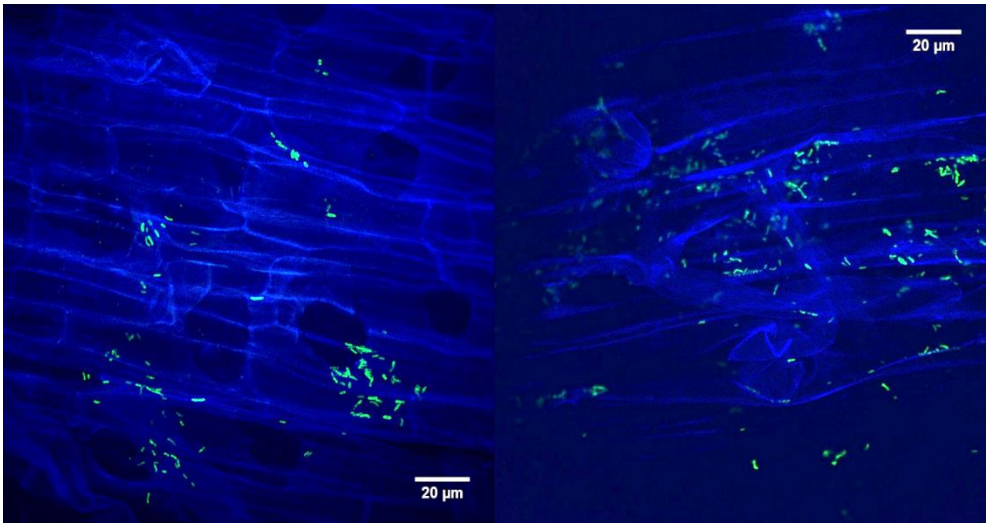


Figure 6.4. Confocal laser microscopy images of *L. corniculatus* roots inoculated with *Rhizobium* sp. P44RR-XXIV GFP+. The left figure shows that the bacteria are in a different plane in regard to that of the root cell wall, possibly associated to hairy roots. In the figure on the right, the bacteria are found in a low number and in an irregular distribution.

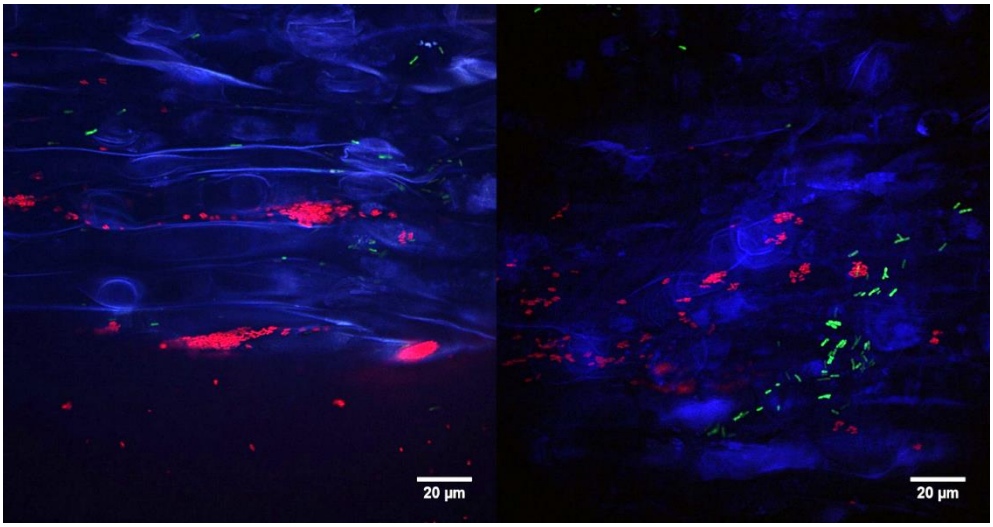


Figure 6.5. Confocal laser microscopy images of *L. corniculatus* roots inoculated with *Rhizobium* sp. P44RR-XXIV GFP+ and *Ochrobactrum.* sp. P6BS-III RFP+. In both figures the different cell locations in the root surface can be observed.

Images taken during SEM microscopy studies shows the presence of *Rhizobium* sp. P44RR-XXIV and *Ochrobactrum* sp. P6BS-III on the roots of *L. corniculatus* (Figure 6.6 and figure 6.7), which may indicate some kind of association, taking into account the difficulties of the cell fixation process.

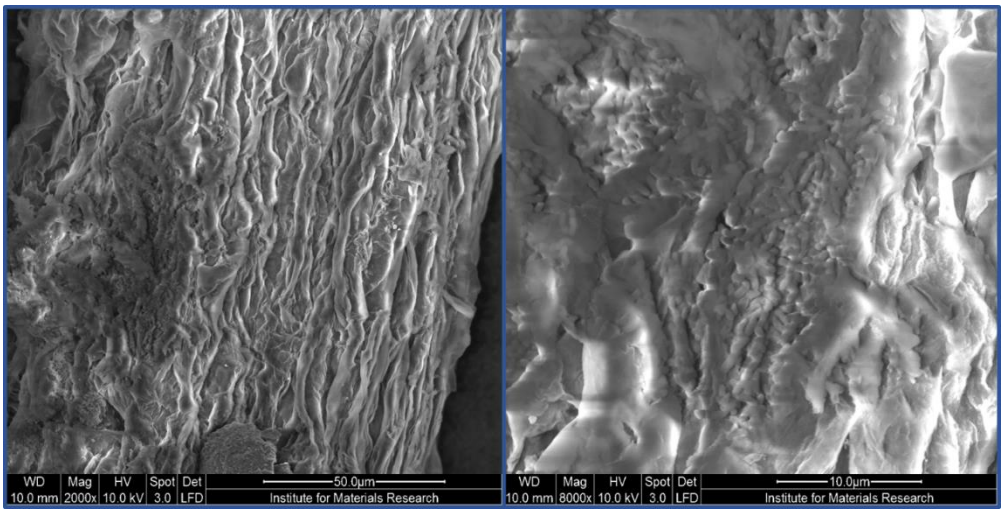


Figure 6.6. Scan electron microscopy images of *L. corniculatus* roots inoculated with *O.* sp. P6BS-III and later fixated. The figure on the left has been magnified 2000 times, whereas the one on the right has been magnified 8000 times.

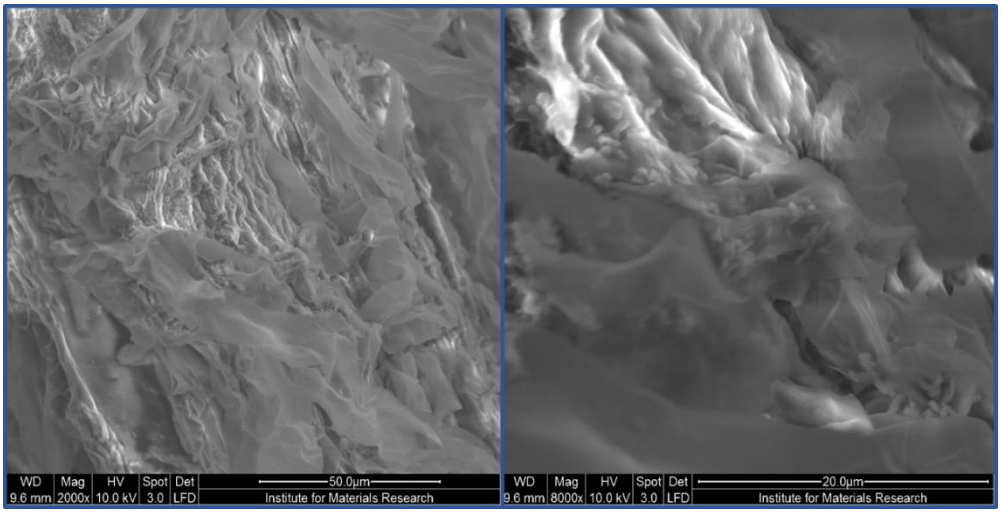


Figura 6.7. Scan electron microscope images of *L. corniculatus* roots inoculated with *R.* sp. P44RR-XXIV and later fixated. The figure on the left has been magnified 2000 times, whereas the one on the right has been magnified 8000 times.

6.3.3 Microcosm studies

As mentioned in section 6.2.3, the microcosm study was aimed to, on one hand, to assess the evolution of the strains inoculated throughout the study, and in the other hand, to verify glyphosate mitigation in the experimental units (Figure 6.8)

According to previously obtained results in other experimental systems during chapter 3, it was possible to corroborate that *L. corniculatus* tolerated the application of an estimated dose of 5.0 mg kg $^{-1}$ of a commercial glyphosate without showing any sign of damage throughout the study.

Figure 6.8. Microcosm assay. On the left side of the picture, experimental units having bulk soil are shown, while on the right the experimental units with *L. corniculatus* are shown.

As generally suggested for soils presenting these physicochemical and texture characteristics, before initiating the assay a total heterotroph count was carried out in non-inoculated experimental units, to assess the success of the sterilization process (Trevors, 1996). values around 1.10^8 CFU g^{-1} of dry soil were obtained. These results clearly show that the three autoclaving cycles, were not enough to make soil samples show an undetectable number of microorganisms, illustrating the resilience of these soils' microflora and how can complex could be the experimental handling of microcosm systems.

Even though it is not absolutely necessary to start from sterile soils, since the purpose of microcosm systems is to bring the study closer to real-life implementation conditions, the attempt to reduce the number of total microorganisms in soils was aimed to minimize the influence of other microorganisms that may participate in glyphosate degradation. Additionally, by reducing the total number of viable microorganisms the visualization of the band corresponding to the traced strain in the ARISA profiles can be facilitated, since both isolated strains present weak intensity bands that are not visible in band profiles from the sample form which they were originally isolated (Figure S6.1.3). Thus, even though it is clear that the employed method does not amplify DNA from viable microorganisms, but from total bacterial DNA, the large amount of biomass added plus the lack of competence seek to facilitate the tracing of strains by molecular means.

Figure 6.9 shows the results obtained in non-inoculated experimental units used as controls. Both in ARISA profiles obtained from soil samples, as well as from rhizosphere-rhizoplane soil samples. The presence/absence of the strain specific band throughout the assay can be seen. In soil samples (Figure 6.9A), the band pattern is similar at times 6 and 20 for replicates. This may be the result of the survived bacterial community after sterilization process, having already achieved a certain degree of stability even before the sampling.

Regarding the measured glyphosate (both with and without *L. corniculatus* present in the sample), a great variation concentration values is observed at all times, a phenomenon repeated in all measurements (Figure 6.9C). It is well known that the glyphosate measurement in soil samples is, in analytical terms, extremely difficult (Ibanez et al., 2005; Druart et al., 2011; Arkan, 2015) due to its chemical structure and due to the various interactions the molecule could have in matrix, as was appropriately discussed in chapter 1. Variations in adsorption combined with changes in humidity (caused by irrigation or evaporation) may have modified the efficiency in the extraction of the molecule on each sample. Considering such dispersion, there were no significant differences between values at the beginning of the experiment and values at ending time.

Results from microcosm samples containing *Ochrobactrum* sp. P6BS-III are shown in figure 6.10. In ARISA soil profiles, bands with similar molecular weight to P6BS-III can be distinguished, with minor differences in the position

(Figure 6.10A). In this case a decreasing tendency of glyphosate in time exists, even though differences between values at the beginning of the experiment and other times are not statistically significant (Figure 6.10C). Although there is no significant differences on each sample time, it is possible to see a clear trend in the reduction of glyphosate concentration in microcosms having inoculated roots with P6BS-III (Figure 6.10C). In the same experimental units, a band with similar molecular weight to P6BS-III can be observed in at least one profile in all times assayed (Figure 6.10B).

ARISA patterns in soil microcosms of *Rhizobium* sp. P44RR-XXIV revealed the presence of a band of similar molecular weight in two of the three replicas, decreasing its intensity over time (Figure 6.11A). When ARISA patterns are analyzed in rhizosphere-rhizoplane samples, it is not possible to find a band with a similar molecular weight to P44RR-XXIV (Figure 6.11B). Even though differences among the various concentrations of glyphosate in time are not significant, it is also possible in this case to notice a trend in the reduction in rhizosphere-rhizoplane samples (Figure 6.11C).

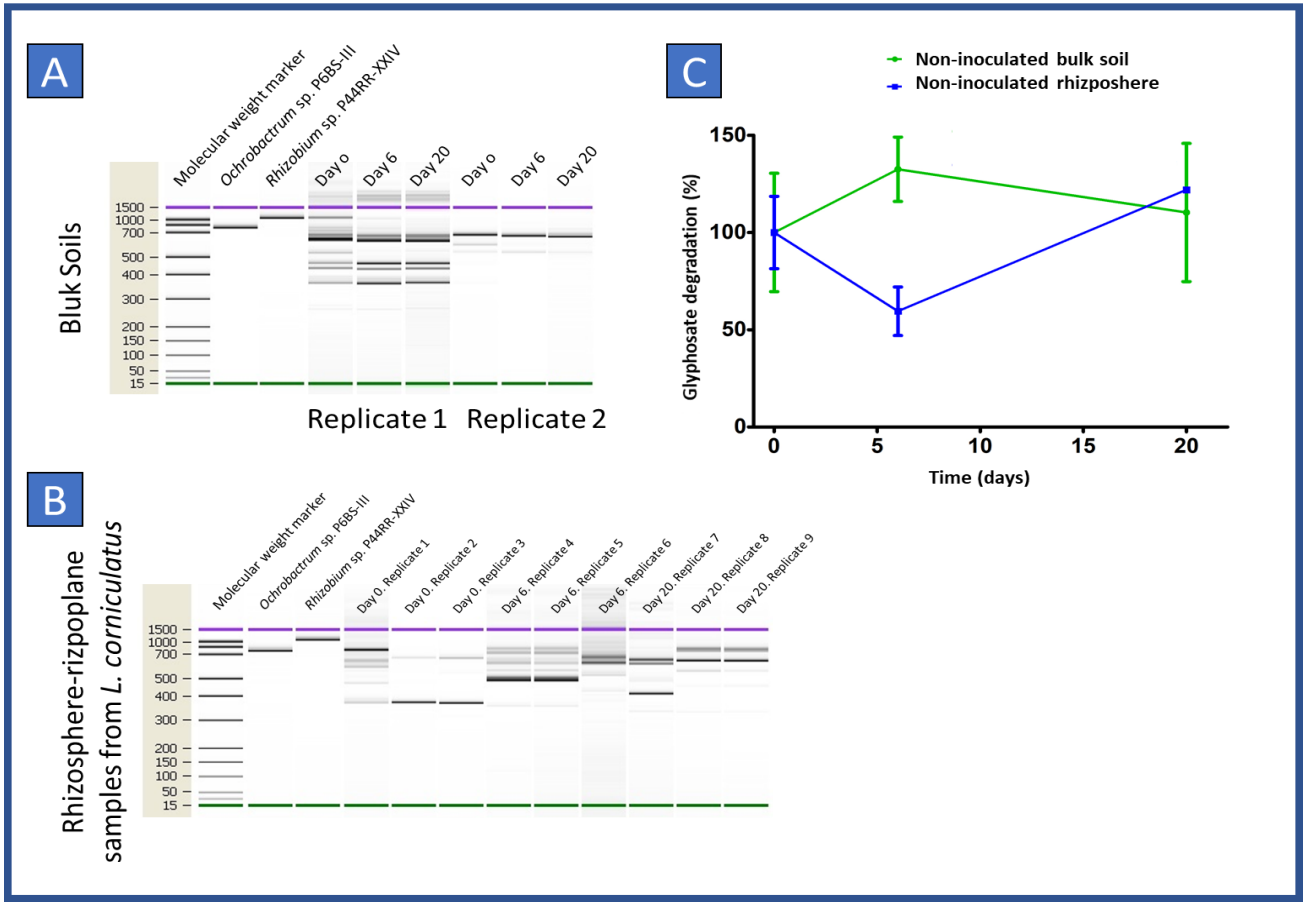


Figure 6.9. A. ARISA profiles of non-inoculated soils. B. ARISA profiles of non-inoculated rhizosphere soils. C. Glyphosate quantification in the experimental units of the non-inoculated microcosm.

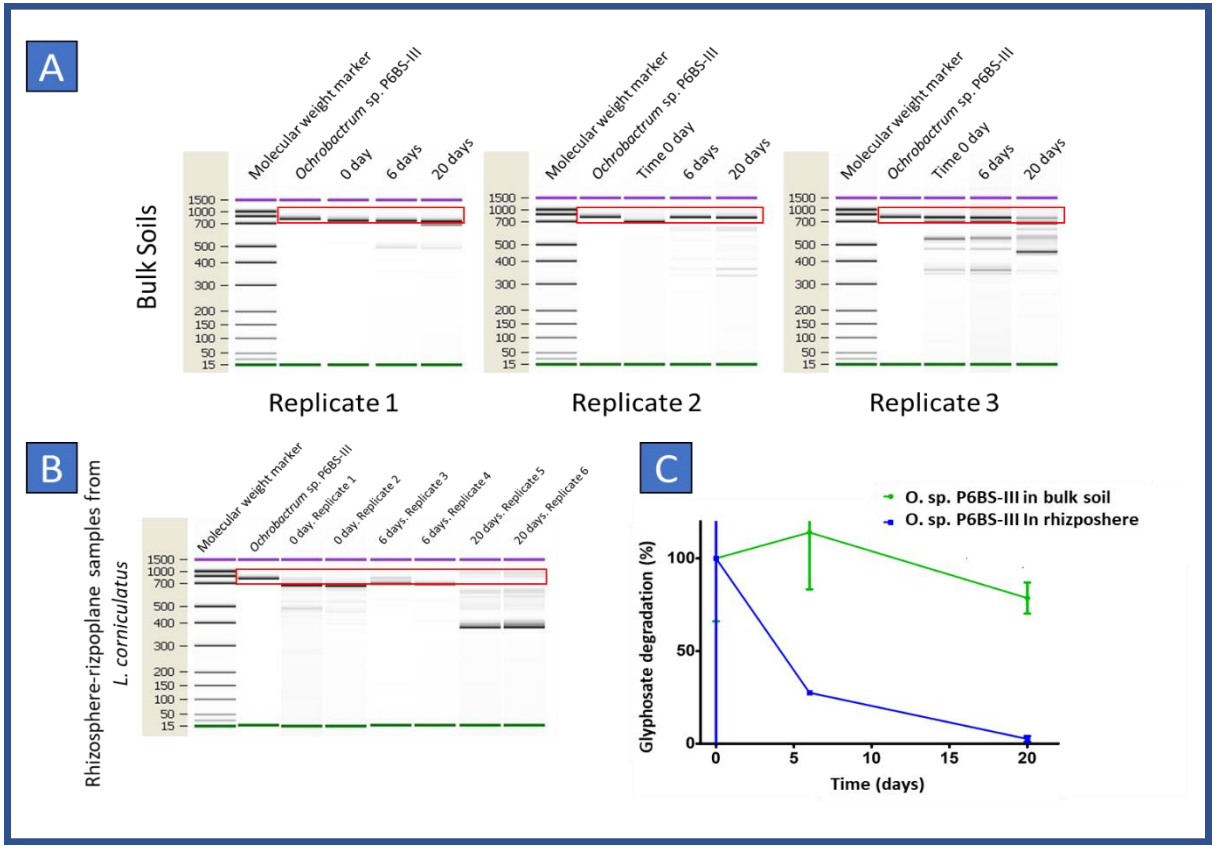


Figure 6.10. A. ARISA profiles of soils inoculated with *Ochrobactrum sp.* P6BS-III. B. ARISA profiles of rhizosphere-rhizoplane soils inoculated with the same strain. C. Glyphosate quantification in experimental units of the microcosm inoculated with P6BS-III.

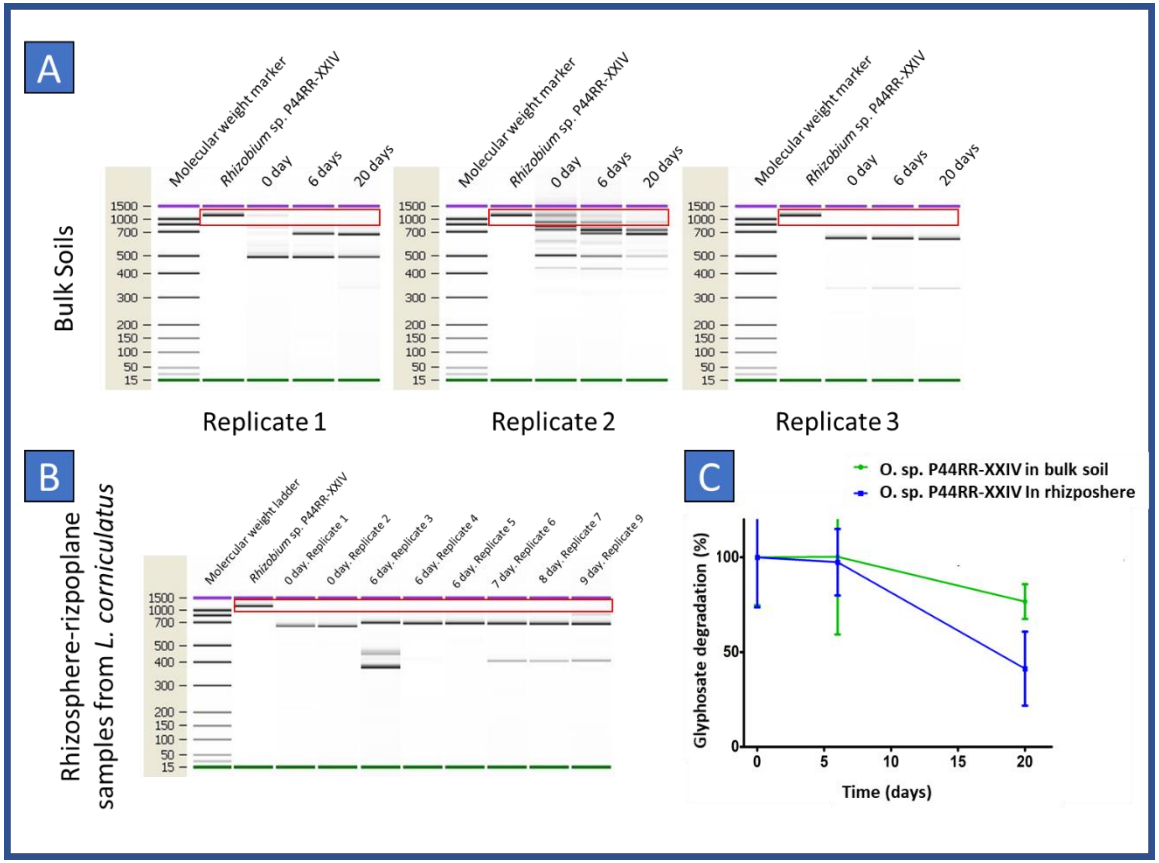


Figure 6.11. A. ARISA profiles of soils inoculated with *Rhizobium sp.* P44RR-XXIV. B. ARISA profiles of rhizosphere-rhizoplane soils inoculated with the same strain. C. Glyphosate quantification in the experimental units of the microcosm inoculated with P44RR-XXIV.

6.4 Conclusions

Over the course of this chapter, relevant aspects of the plant-microorganism interaction were considered between degradative bacterial strains and selected tolerant plant species.

Vertical agar plates assays, although did not show significant differences for the analyzed parameters between inoculated seedlings and control seedlings, revealed that the strains of interest were able to co-exist with the plant species without causing any signs of damage. This may mean that different alternatives for the transportation of the associated microorganism into the seed prior to implantation could be explored, enhancing the possibilities of application these technologies. The high growth rate of the main root and the scarce aerial development in the proposed time encourage to try different experimental systems that may allow to obtain more prolonged observations.

On another note, the obtained images from microscopy allowed to visualize the location of the candidate strains on the root. *Ochrobactrum sp*. P6BS-III showed association patterns compatible with positive adherence to the root due. From these observations, and relying on the completely sequenced genome, doors open up to explore in greater depth the capacities related to adhesion, *quorum sensing* and biofilm formation, among others.

In the case of *Rhizobium sp*. P44RR-XXIV, the observed pattern in the root remains unclear and, despite the fact that the infection process for nodule formation starts in the hairy roots, P44RR-XXIV genome does not present symbiosis genes (nor was observed a nodule formation in any of the experiments carried out). Therefore, it remains unclear whether the strain's distribution is random (and a product of washing in PBS) or if there is a preference to inhabit these hairy roots over other parts of the root structure. Conversely, electron microscopy images show the strain present in the root, which is no minor information, since the chemical astringency involved in the washing and fixing process requires the strain to be strongly attached to the root.

Based on these studies, conundrums appear to continue the examination of these possible plant-bacteria associations in the future.

The microcosm study revealed that *L. corniculatus* inoculated with *Ochrobactrum* sp. P6BS-III presents an almost complete degradation of the initial 5 mg Kg^{-1} of glyphosate soil after a 20-day period, a very encouraging time for

the proposed remediation system. Even though the microcosm study posed experimental difficulties typical to those in field-scale experiments, such as the difficulties in soil sterilization or the complex analytical recovery, degradation results and bands in ARISA profiles, might indicate that this association may result in a successful rhizoremediation system. At the same time, despite the non-significant differences, the soil degradation assay also shows a degradation tendency. These results are beyond promising and encourage us to continue working in the future with different trials that involve this plant-bacteria association in a greater experimental scale.

6.5 References

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S6.1 Supplementary data

S6.1.1 Gamborg B5 medium

Reagent	Amount (mg L^{-1})
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	3.00
KI	0.75
$MnSO_4.H_2O$	10.00
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	2.00
$CaCl_2$	113.23
KNO_3	2500.00
$MgSO_4$	121.56
NaH_2PO_4	130.44
$(NH_4)_2SO_4$	134.00
Myo inositol	100.00
Nicotinic Acid	1.00
Pyridoxine.HCl	1.00
Thiamine.HCl	10.00
Agar:	10 g
pH: 3.5-4.5	

Reference

Gamborg, O. L., Miller, R. A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158. doi:10.1016/0014-4827(68)90403-5.

S6.1.2 Medio de inoculación Líquido

Compound	**Amount (g L^{-1})**
Peptone	5.0
Beef extract	3.0
Glucose	5.0
NaCl	5.0
Polyvinyl pyrrolidone (PVP)	20.0
$CaCl_2$	0.14
$FeCl_3$	8.4 mg
NH_4Cl	0.6
NaOH	0.16
Glycerol	12 mL

pH: 6.8

Reference:

Sridhar, V., Brahmaprakash, G. P., and Hegde, S. V (2004). Development of a Liquid Inoculant Using Osmoprotectants for Phosphate Solubilizing Bacterium (Bacillus megaterium). 17, 251–257.

S6.1.3 Comparison of ARISA profiles of the strains used in the microcosm assay and the ARISA profiles corresponding to the original samples from which the strains were isolated.

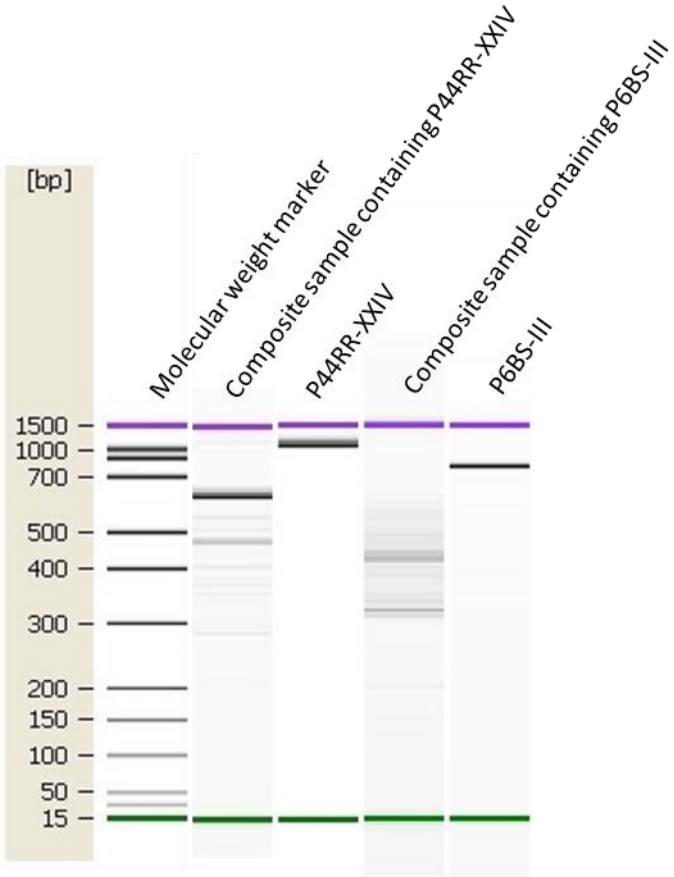

Figure S6.1.3. Comparison of ARISA profiles of the strains used in the microcosm assay and the ARISA profiles corresponding to the original samples from which the strains were isolated.

Chapter 7 - Conclusions and future perspectives

Currently, glyphosate is among the major environmental concerns in Argentina, especially in the Humid Pampa region. This region not only represents the main agricultural zone of the country, but also concentrates the highest density of population. Being the glyphosate and its associated products the core technology in the prevailing agricultural model, it is unlikely that its use will decrease in the future.

The topics involving the glyphosate use, understood from a historical, economic and structural point of view of the agricultural sector, are far from the understanding of the general public. However, there are many voices that rise in favor or against the use of this technological package.

Visions focused on organic and family agriculture propose a shift of the current agricultural production paradigm, focusing on the development of small-scale economies and promoting strong social and cultural transformations. The improvement on the rural economies, the protection of the biodiversity and the generation of minimum environmental liabilities are among the benefits proposed by organic agriculture. Its practice, however, trends like these do not consider the economic consequences of this change, since our country heavily depends on grains and oilseeds exports. Besides, partakers who are not directly involved in the production plot are not being considered either.

On the other hand, the voices in favor of the current agricultural model, focus on the globalized economy model that positions the country as a leader in the field of agricultural biotechnology, with well-defined and organized social and economic structures, establishing the driving force of the current economic development. However, they dismiss the pressures that the market has on pesticide selling, the lack of regulations and poor education regarding the use of agrochemicals in general, causing great environmental damage, among all the other consequences discussed in chapter 1 of this thesis.

Beyond taking any position, there is an undeniable reality, which is that glyphosate is contaminating the surface water bodies present in the Humid Pampa, and consequences are still unknown.

This work developed during this thesis aims to develop the knowledge to design a biotechnological tool for the rhizoremediation of glyphosate residues in agricultural soils able to operate in a completely integrated way to the mode of exploitation prevailing in the region, attempting to take the first step towards

generating progressive changes that result in an improvement of the agroecosystem through the reduction of environmental liabilities.

The incorporation of the rhizoremediation system prevents the glyphosate residues in soil from reaching the water bodies without disturbing the main economic exploitation and even increasing its profitability in a more sustainable way.

As a starting point, the systematic search of commercially available plant species able to tolerate the highest levels of glyphosate reported in agronomic soils was conducted. This means that the incorporation of this technology does not only imply the concept of economic benefit for the agricultural producer, but also ensures the access to germplasm so that it can be used in the large area comprised by the Humid Pampa. Results obtained in chapter 3 showed that *Lotus corniculatus* cultivar Gladiator tolerated a dose of 5 mg Kg^{-1} of a commercial glyphosate product in a medium with the maximum bioavailability showing no significant differences, exceeding the highest residual concentrations measured in agricultural soils. Further studies determined it can tolerate a dose equivalent to a strong application drift, since its permanent implantation next to the crops would make the impact by application drifts very likely. Given the fact that *L. corniculatus* is a legume, it presents several extra benefits to the plot. It improves the biological nitrogen fixation by symbiosis with microorganisms, thus fertilizing the soil, it can grow in plot areas where the main crops do not grow, and has a good technological development due to its extensive use throughout the European continent, America and Asia.

The sample sites where glyphosate tolerant and degrading microorganisms were obtained presented outstanding conditions compared to other possible sites where Lotus species could be found within the Humid Pampa. Firstly, the management method of promoting this pasture using glyphosate carried out there, gave us the possibility of obtaining roots of a plant species closely related to *L. corniculatus*, implanted in soils chronically exposed to glyphosate. Secondly, the sampled plots are located in the Flooding Pampa region, where the low fertility and high waterlogging rates mimic the proposed implantation zones according to the suggested strategy. Thus, the conditions where Lotus samples were obtained are similar with those expected during the application of the rhizoremediation system. Finally, the chance of working in

collaboration with the Manantiales Experimental Farm, is an excellent opportunity, since more than 20 assays are carried out annually, and professionals there are always well prepared. All this, together with the farm equipment and the invaluable advice provided there, makes this establishment an ideal place to carry out future field trials of rhizoremediation strategies.

The isolation of glyphosate tolerant microorganisms from Lotus rhizosphere-rhizoplane and bulk soils subjected to different conditions, together with the physicochemical and microbiological characterization of those samples, was described in chapter 4. Studies using a culture-independent method showed the influence of the rhizosphere effect on the microbial communities over the agronomic management (which includes the use of glyphosate) and the edaphic properties. However, when treated and control soils were compared, it was found that two plots having similar soil properties presented weak differences regarding the structure of their communities. This suggests that, once the presence of the strain is assured in experiments under controlled conditions, the strategy of vehiculation of microorganisms by inoculation could be successful in the field, since the rhizosphere effect could ensure its continuity over time. This conclusion however, should be taken as guidelines when proposing new studies, since it would be wrong to assume that this effect will be imposed in any agronomic condition, having studied soils with only two different managements and properties. In the future, we expect to study the consequences of the implantation of the same Lotus cultivar in soils with a greater diversity of management, climates and soil properties. In this way, microbial ecology can strongly complement soil remediation studies, improving the chances of success.

A culture collection of glyphosate tolerant and degrading bacteria was appropriately characterized during chapter 5. The almost total predominance of *Alphaproetobacteria* class was a remarkable find among the results obtained. Studies on sample communities using amplicon sequencing, for instance could define whether this predominance was based on the large initial contribution of this class or whether the procedure of isolation which includes the use of glyphosate as a source of phosphorus, was the cause for this finding. Although C-P-lyase is present in phylogenetically diverse organisms, the specific ability to degrade glyphosate could be associated to this *Alphapreptobacteria* class. Future

experiments in the C-P lyase complex related to glyphosate degradation are proposed, in order to carry out biotechnological processes.

The strains were investigated both for their ability to tolerate and degrade glyphosate, and for their *in vitro* plant growth promotion ability. The presence of multiple plant growth promotion abilities in each strain was observed, together with a very diverse response to glyphosate tolerance. There is no apparent association between traits. These results together with culture properties of each strain allowed us to define suitable microorganisms to carry out further characterizations and eventually, to continue with plant-microorganism interaction assays. At this point, the whole genome sequencing of *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV allowed to obtain vast information related to previously reported properties tested using biochemical and classical microbiology methods. At the same time, genomes are important resources eventually, when testing any phenotypic trait and/or interaction with other organisms. The whole genomes were also studied with respect to the genus of which they are part, using comparative genomic methods. Findings revealed *Ochrobactrum* sp. P6BS-III is the first sequenced genome of its kind, providing the genus with a large number of unique genes, and *Rhizobium* sp. P44RR-XXIV does not possess the symbiotic plasmid required for nitrogen fixation.

In Chapter 6, plant-microorganism interaction studies were performed using selected strains. Inoculation tests in vertical agar plates showed a normal Lotus development and a great growth of the strain for some microorganisms, among which *Ochrobactrum* sp. P6BS-III and *Rhizobium* sp. P44RR-XXIV are included. These findings, together with the rest of the traits that each of these two strains presented, motivated us to continue with studies of greater complexity, which involved molecular biology and different microscopy techniques.

Finally, a microcosm study was carried out using soils from the Flooding Pampa, were the proposed plant-microorganism systems had a positive effect on the degradation of the herbicide compared to the controls, although it is necessary to improve the inoculation method, as well as glyphosate quantification. It is then proposed to deepen in plant-microorganism studies to understand the real extent of the effect of the inoculated strain, as well as to carry out greenhouse trials with a greater number of samples, in the future.

Finally, this thesis has always taken into account the challenges of the field scale application of the phyto/rhizoremediation strategies in order to be considered of interest by the people in the Government and private sector who believe that changes in the agricultural system of our country are possible.