UNIVERSIDADE DE ÉVORA

SCHOOL OF SCIENCES AND TECHNOLOGY
DEPARTMENT OF BIOLOGY

Anupoma Niloya Troyee

Supervisors| Ana Alexandre
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Impact of soil treatments in the conservation of native rhizobia populations

Masters in Conservation Biology

Dissertation

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Glossary

DMSO -dimethyl sulfoxide

BSA- Bovine serum albumin

DNA- deoxyribonucleic acid

min-minutes

Kb- kilobase

NJ- Neighbor-joining

RNA- ribonucleic acid

rRNA- ribosomal ribonucleic acid

16S rRNA- 16S ribosomal RNA subunit

U- unit

UV- Ultraviolate

g- gravity acceleration

PCR- Polymerase Chain Reaction

GFP- Green fluorescent protein

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Summary

Sustainable agriculture aims to achieve high crop production, reducing the use of chemical fertilizers and herbicides. Rhizobia-legume symbioses are important N_2-fixing systems that can improve the productivity of soils. The aim of this work was to evaluate the diversity of rhizobia on nodules of chickpea plants grown in soil treated with a glyphosate, nitrate and *Mesorhizobium* inoculation. The impact of glyphosate and nitrate on mesorhizobia growth and on the early stages of infection was also evaluated. The phylogenetic analysis of the 16S rRNA gene sequences showed a low diversity of rhizobia nodulating chickpea, regardless of the treatments applied. The detection of *Mesorhizobium muleiense* as predominant species may be related to the high competitiveness of this species. Smaller root hairs and less curling was observed for the highest concentrations of nitrate tested. This work has contributed to clarify the impact of chemical fertilizers and herbicide application on the legume-rhizobia symbiosis.

Impacto de tratamentos do solo na conservação das populações nativas de rizóbio

Sumário

A agricultura sustentável tem como objetivo atingir elevadas produções, reduzindo o uso de fertilizantes químicos e herbicidas. As simbioses rizóbio-leguminosa são importantes sistemas de fixação de N_2, que podem melhorar a produtividade dos solos. O objetivo deste trabalho foi avaliar a diversidade de rizóbios nos nódulos de grão-de-bico plantado em solo tratado com glifosato, nitrato e inoculação de *Mesorhizobium*. Um outro objetivo foi a avaliação do impacto do glifosato e nitrato no crescimento de rizóbios e nas fases iniciais da infeção. A análise filogenética das sequências do gene 16S rRNA mostrou uma baixa diversidade de rizóbios em nódulos de grão-de-bico, independentemente do tratamento. A deteção de *Mesorhizobium muleiense* como espécie predominante poderá estar relacionada com elevada competitividade desta espécie. Pêlos radiculares mais pequenos e menos "curling" foram observados para as concentrações mais elevadas de nitrato testadas. Este trabalho contribuiu para clarificar o impacto de fertilizantes e herbicidas nesta simbiose.

State of Art

Finding a common ground between conservation biology and agriculture

Since both natural ecossytems and highly productive agriculture are important to sustain the growing population, these two disciplines need to be integrated, recognizing their different goals. Approaches that combine benefits of conservation biology and agriculture are a trajectory to move to sustainable agriculture. Results of empirical and theoretical work such as landscape perspective, economics, external chemical product use need to be complied in an inclusive way that could be applied at the interface of agriculture and conservation biology research (Kristjanson et al., 2009; Rudd et al., 2011).

The world, as it progresses, is seeing more necessity for sustainability and agriculture is no difference. Unlike conventional farming, the sustainable farming enforces on the health of soils, ecosystem and people. Sustainable practices rely more on the efficient use of the natural resources, ecological processes and biodiversity, than on the use of external inputs with adverse effects.

Sustainable agriculture could be achieved in many ways, for example, reducing application of synthetic pesticides, herbicides and fertilizers in production of food. In other way, increasing yields and protection against diseases and pests relying on natural biodiversity is a possible approach towards sustainable farming. Sustainable agriculture practices need more attention because they can both profit farmers, economies, food banks and it also co-exist with landscape management (Brussaard, de Ruiter, & Brown, 2007).

In conservation agriculture, the emphasis is higher on sustainable agriculture because it can provide high yields without compromising the integrity of environment. Conservation agriculture is a concept that holds tremendous potential for agro-ecological farms and its adoption can combine profitable agricultural production with environmental concern and sustainability. Food and Agriculture Organization (FAO,2009) defines it as a practice to achieve sustainable and profitable agriculture and subsequently aims on improved livelihoods of farmers by the application of the three conservation agriculture principles. These principles were catagorized like minimal soil disturbance, permanent soil cover and crop rotations. One subject of interest here is Plant Growth-Promoting Bacteria (PGPB) in sustainable agriculture industry, which has helped agriculture in a certain way by increasing the crop production with a significant reduction of synthetic chemical fertilizers.

Biodiversity refers to all species of plants, animals and micro-organisms existing and interacting within ecosystems (Vandermeer J., 1995). In global biodiversity, considerable genetic diversity of traditional varieties (landraces) of crops is most useful and economically valuable (Wood & Lenne´, 1997).

In broader sense, agrobiodiversity comprises the whole diversity of living organisms in agricultural landscapes that includes functioning soil biota. The definition refers to two terms, one is the planned agrobiodiversity, meaning the biodiversity of crops and livestock chosen by

farmers, and the other is called associated agrobiodiversity, which refers to the biota ,e.g. , soil microbes and fauna, weeds, herbivores, carnivores etc. that colonize the agroecosystem and subsist according to the local management and environment (Vandermeer J., 1995).

The relevance of an agroecological approach to food provision was recently highlighted by the United Nations Special Rapporteur on the right to food where an emphasize has been given on reorientation of agricultural systems towards modes of production that are highly productive, highly sustainable and that contribute to the progressive realization of the human right to adequate food (UN, 2010). Although as a matter of fact, over the years, less environment friendly methods, like chemical usage and fertilizers, have been researched over more than integrating and natural methods in agriculture, which could lead to more sustainable agricultural practice(Rigby & Cáceres, 2001).

In agriculture, different crops, especially leguminous crops have significance for its nutritive value. Wild legumes (herbs, shrubs or trees) are economically and environmentally helpful species because of their ability to establish nitrogen-fixing symbiosis. . Natural nodulation is a process that depends on many factors like genetic diversity, the dynamics of soil fertility and it has vital role in natural ecosystems and agriculture (Altieri, 1999; Peoples, Herridge, & Ladha, 1995).

Microbial activity has the principal role in biogeochemical cycle of different elements of soil(Roldán, García-Orenes, & Lax, 1994). Hence, microorganisms work as the chief protagonist for maintaining soil quality and its structural stability(Bastida, Moreno, Hernández, & García, 2007; Roldán, Salinas-García, Alguacil, Diaz, & Caravaca, 2005). It has been reported that N fixation in soils treated with agrichemical and pesticides can lead to disruption in natural chemical communication between plant and Rhizobia which are important for Symbiotic Nitrogen Fixation (SNF) (Fox, Gulledge, Engelhaupt, Burow, & McLachlan, 2007). There has been evident environmental consequences of synthetic chemicals that compromise symbiotic nitrogen fixation and escalate the dependency on synthetic nitrogenous fertilizer, reduce soil fertility, and pave the way of unsustainable long-term crop yields (Fox et al., 2007).

For example, chickpea (*Cicer arietinum L*.), which an annual legume, is one of the most important crops that has widespread production and represents an important source of protein in many countries. Chickpea has a sustainable role in cropping systems because of its symbiotic association with nitrogen fixing bacteria. From the nutritive point, it is one highly important source of protein, hence a significant alternative to animal protein (Hossain, Ford, McNeil, Pittock, & Panozzo, 2010).

Biological Nitrogen fixation (BNF) is an alternative to the chemical N and its a microbiological process that converts atmospheric nitrogen into a form that plants can use. Nitrogen-fixing not only mandates to a economically attractive and ecologically sound means of reducing external inputs and not only expand internal resources but also in has potencial advantages on the natural environment. The signifcant benefits are mostly seen in acidification of soil , different processes of rhizosphere and CO2 fixation (Bohlool, Ladha, Garrity, & George, 1992; Jensen & Hauggaard-Nielsen, 2003). Symbiotic system such as legumes and rhizobia can either alone or in combination may offer a better solution to supply nitrogen to the cropping systems of the

future and rhizobia possess the potential ability to deal with the environmental stresses or changes in farming systems(Bohlool et al., 1992). Legume-rhizobia symbiosis leads to the formation of special structures, usually in the plant roots, designated by nodules where the bacteria convert atmospheric nitrogen to ammonia. The ammonia produced in the bacteroids is transferred to the plant, where it do assimilation into glutamine or asparagine. BNF is an environment-friendly to supply N to agro-system compared to the N fertilizers that mostly rely on fossil energy for their production. The nitrogen resulting from this BNF is less prone to leaching than the chemical fertilizers used in agriculture. Fertilizers use can also cause soil acidification and eutrophication (Hauggaard-Nielsen, Ambus, & Jensen, 2003). In the global setting of agriculture and conservation of environment using natural ways to provide nitrogen to the soil and crops is very important and that is the reason why much attention has been given on this symbiotic system that deals with leguminous plants and rhizobia. Around the world in many types of land this symbiotic system has worked on different way. For example, in degraded lands a novel and suitable wild legumes-rhizobia association helped in vegetation cover (Jha, Nair, Gopinathan, & Babu, 1995).

The role of beneficial soil microorganisms in agriculture

Rhizosphere is the soil region where the microorganisms mediate processes and that is specifically influenced by the root system (R. d. Souza, Ambrosini, & Passaglia, 2015). Bacterial populations are found in larger number in rhizospheric soil than in bulk soil, mainly because of the greater availability of nutrient in rhizosphere. The rhizosphere zone is usually rich in nutrients in comparison with the rest of bulk soil and the reason behind it is the accumulation of different plant exudates (e.g. amino acids, sugars) that imparts energy and nutrients for bacteria (Gray EJ, 2005). The rhizoshere area is normally consists of the few millimeters soil that is connected to the plant roots and it is considered as vital environment for the plant and microbes interactions (Gray & Smith, 2005; Lynch, 1990). Different microorganisms live around of roots and colonize them,depending on factors like soil environment, soil nutrient status and plant defense system (Badri & Vivanco, 2009; Lynch, 1990).

Soil microorganisms are essential for the maintenance of soil quality and also important for cycling of both inorganic and organic nutrients in the soil. Soil micro-organisms are considered to be present in a large number and ‘crucial to life’. Beneficial soil microorganisms may have a significant role in plant life especially by facilitating the acquirement of nutrients and/or by promoting plant growth under biotic and abiotic stresses (Yang, Kloepper, & Ryu, 2009). The accessibility of nutrients to plants is influenced by the microbial activity in rhizosphere (Jeffries, Gianinazzi, Perotto, Turnau, & Barea, 2003). These beneficial soil microorganisms can play a bigger role nowadays, since more emphasize is been given on environment-friendly agricultural practice.

As the emphasis on environmentally friendly and sustainable agriculture increases, the interest of utilizing soil microbial communities in agriculture practice are rising. ‘Ecosystem services’ is a term concerning the benefits (monetary) provided by ecosystems that contribute to human

well-being (Norris et al., 2011). Utilization of microbial activity in agricultural practice instead of external chemical and fertilizers, can be considered as one of the ecosystem services as well. To amplify the benefits of microbial activity on agriculture, such as reducing nitrogen fertilizer by using natural nitrogen fixation bacteria and beneficial soil microbe inoculation etc. need to be studied more and thoroughly. The soil microorganisms shape the soil structure and the presence of different organisms are influenced by environmental factors like soil type, nutrient status, pH, moisture as well as plant factors (e.g. species, age). Presence of organic matter has great effect on the microbial population and microbial growth in soil is usually carbon limited (Lynch, 1990; Wardle, 1992).

Microorganisms have one of the major roles in maintaining soil processes, hence overall functioning of ecosystems. As portion-wise soil micro-organisms are significant in biodiversity and they hold significant biomass of soil . For boosting the soil microbial capacity in agriculture, deeper and detailed understanding of microbial diversity and activity is paramount.

Chemical pollutants vs natural contributor

From the beginning of agricultural practices, the use of synthetic chemical fertilizers, weed-killers, pesticides caused environmental damages as well as posed potential threat to human health. In recent days, for agriculture production, an extensive and large-scale use of chemical fertilizers has become very common (Adesemoye, Torbert, & Kloepper, 2009) and essential to provide plant nutrients like nitrogen, phosphorus and potassium. But unforeseen and shocking negative environmental impacts have become noticeable due to the overuse of these fertilizers(Adesemoye et al., 2009; Shenoy & Kalagudi, 2005). One of the examples of such chemical pollutant is Nitrous oxide (N_2O), which is used excessively as nitrogen fertilizer and considered a major source of greenhouse gases causing global warming. In 2013, the largest single source of total U.S. N_2O emissions (74% of total) was reported by agricultural soil management ("Draft U.S. Greenhouse Gas Inventory Report:1990–2014,"). Another consequence of applying high concentration of nitrogen fertilizers by farmers is the reduction of the natural biological nitrogen fixation in the soil. As most N-fertilizers use ammonium nitrate as the main source of nitrogen provided to the crops, the given influx of ammonium leads to diminishing of atmospheric nitrogen fixation by symbiotic microbes (Vejan, 2016) . Additionally, excess ammonium is utilized by nitrifying bacteria to produce nitrate and this high amount of nitrate then utilized by denitrifying bacteria to produce N_2O. In addition, excess nitrate can easily leach into the groundwater (Galloway & Seitzinger, 2008).

Plant Growth-Promoting Bacteria

The plant growth-promoting bacteria (PGPB) are widely known as heterogeneous group of microorganisms usually inhabit in the rhizosphere, on the root surface or associated to it. These group are capable of enhancing the growth of plants and/or protecting them from diseases or several abiotic stresses (B.R. Glick, 2012; Grover, Ali, Sandhya, Rasul, & Venkateswarlu, 2011) . In rhizophere different types of soil bacteria interactions were observed and different traits and function of PGPB are evident (fig. 1). PGPB work as a stimulator of plant growth with mechanisms that include, for example, obtainability of nutrients generating from processes like biological nitrogen fixation, stress lessening through the modulation of ACC deaminase expression, and production of phytohormones and siderophores. As a matter of fact, only 1 to 2% bacteria promote plant growth in rhizosphere (Antoun & Kloepper, 2001).

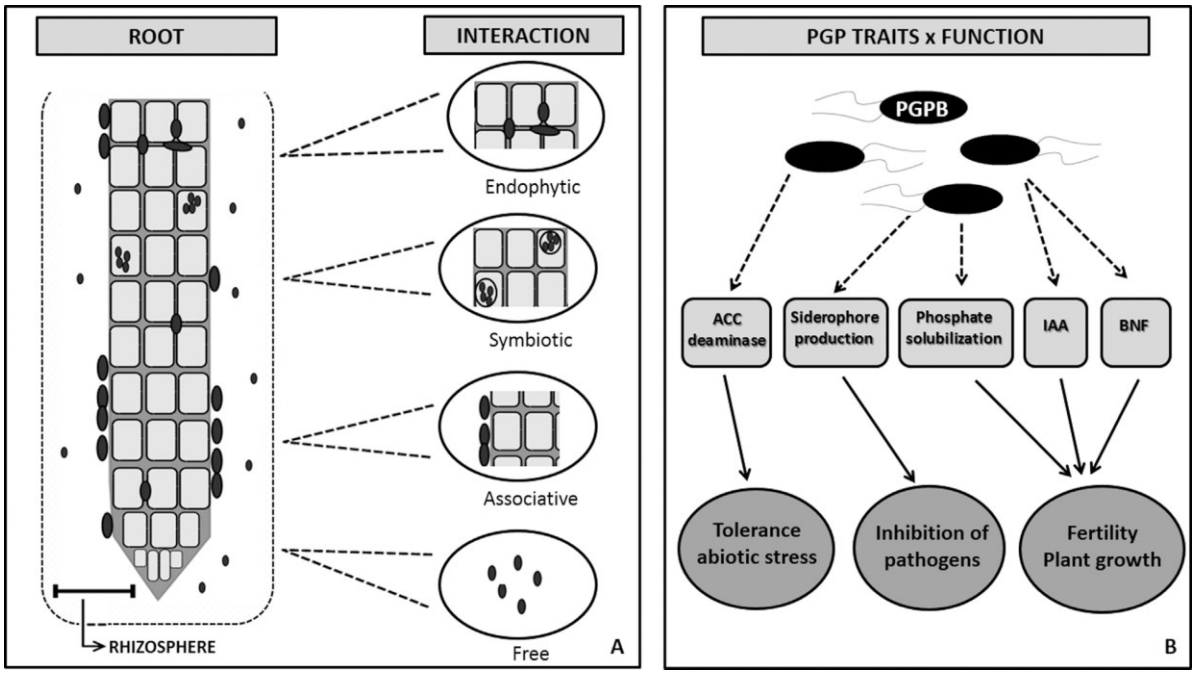

Figure 1: Rhizosphere- root/bacteria interactions. A) Different types of association, namely endophytic, symbiotic, associative are seen between plant roots and beneficial soil bacteria, which may also be found as free-living; B) PGPB may benefit the plant by enhancing (i) tolerance toward abiotic stress through action of ACC deaminase; (ii) defense against pathogens by the presence of competitive traits such as siderophore production; (iii) fertility and plant growth through biological nitrogen fixation (BNF), IAA (indole-3-acetic acid) production, and phosphate solubilization. (adapted from R. d. Souza et al., 2015)

As inoculant candidate, endophytic PGPB are good because they can colonize roots and also maintains a helpful environment for functioning and development of nodules. On other hand, intercellular space of plant tissues containing high levels of amino acids, carbohydrates and inorganic sugars, are normally the place occupied by non-symbiotic endophytes (Bacon &

Hinton, 2006).

There are two ways by which the PGPB affect plant growth which are known as indirect and direct. The indirect promotion of plant growth occurs when PGPB reduce or prevent the damaging or detrimental effects of one or more phytopathogenic organisms , generally by producing antagonistic substances or by inducing resistance to pathogens (Bernard R Glick, 1995). On the other hand, the direct promotion of plant growth refers to PGPB that provide the plant with a compound such as phytohormones, or enable the uptake of certain nutrients (B.R. Glick, 2012). PGPR can actually act as biocontrol agents through various mechanisms not only because of its role in direct growth promotion, but also different known roles like production of auxin phytohormone (Patten & Glick, 2002), or nitrogen fixing associated with roots (Dobereiner, 1992).

Leguminous plants have the attribution to colonise environments with low-nitrogen soils because of the unique capacity of symbiotic association with N_2-fixing rhizobia (Spencer, James, Ellis, Shaw, & Sprent, 1994). The current rhizobial taxonomy includes species from the *Alphaproteobactria* and the *Betaproteobacteira* classes and most of which were described in the last decade using rhizobia isolated from tropical legume species (Bala, Murphy, & Giller, 2001). Although there is a relatively high turnover of rhizobial groups, still orders of true assessment of the diversity of tropical rhizobia are yet to be done (Bala et al., 2001).

The 16S rRNA gene is the most common genetic marker used for both bacterial phylogeny and taxonomy. 16S ribosomal RNA (or 16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. There are several main reasons to the ubiquitous use 16S rRNA for bacterial identification. These reasons include (i) its presence in all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene shows high conservation and slow rates of evolution; its sequence shows hypervariable regions that may provide species-specific signature sequences and (iii) the 16S rRNA gene (approximately 1,500 bp) is large enough for informatics purposes (Janda & Abbott, 2007). The 16S rRNA gene is commonly used in reconstructing phylogenies, identifying bacteria and subsequently found to be capable of reclassifying bacteria into completely new species or even genera. It has also been used to describe new species that have never been successfully cultured (Woo, Lau, Teng, Tse, & Yuen, 2008).

So, the 16S ribosomal RNA gene sequence analysis has been most conventional tool to study bacterial phylogeny and taxonomy because of its characteristics of being highly conserved. Methods to estimate microbial diversity have developed rapidly to understand the distribution and diversity of microorganisms in natural environments. For bacterial communities, the 16S rRNA gene is the phylogenetic marker gene of choice, but most studies select only a specific region of the 16S rRNA to estimate bacterial diversity (Case et al., 2007; Dunbar, Ticknor, & Kuske, 2000).

Use of Plant growth promoting bacteria(PGPB) in leguminous plants

Plant Growth-Promoting Rhizobia (PGPR) from the group of PGPB, as a whole has a synergistic and antagonistic interactions with microorganisms within rhizosphere and beyond in bulk soil that can directly or indirectly improve plant growth rate and be a potential application in agriculture.

For exemple, rhizobia inoculation in different chickpea cultivars with highly effective strains resulted in an increased number of nodules and shoot dry weight (Romdhane, Tajini, Trabelsi, Aouani, & Mhamdi, 2007). Some rhizobia are capable of promoting chickpea growth and multiple strains are proved effective in providing nitrogen to this host legume (Alexandre, Brígido, Laranjo, Rodrigues, & Oliveira, 2009; İçgen, Özcengiz, & Alaeddinoglu, 2002). Before the widespread practice of agriculture, biological fixation of atmospheric nitrogen annually is estimated to be responsible for 90% of the 100 to 140 Tg of nitrogen (1 Tg is 10^{12} g [106 metric tons]) in terrestrial environment and the rest of 10% was fixed by mainly by lightening. (Gage, 2004). But the present age is different and the human activity has changed these numbers through more ammonium production and increasing the amount of leguminous plants that are cultivated due to agriculture.

Rhizobia inoculation also signifies an advantage as biocontrol of plant parasites for chickpea crops using plant growth promoting bacteria. (Siddiqui & Akhtar, 2009). A study in India showed the positive effects of chickpea field co-inoculation with *Mesorhizobium* sp. and *Pseudomonas aeruginosa*, which reported escalation of 32% in grain yield, in comparison with uninoculated control (Verma, Yadav, Tiwari, & Kumar, 2013). In restricting disease development, rhizobia also play a part by activating the chickpea genes involved with the production of higher levels of protective compounds (e.g. phenolic compounds/phytoalexins etc.) (Arfaoui et al., 2007).

To move towards sustainable agriculture, using of soil microorganism instead of chemically synthetised productsis a key option to be considered.The use of efficient inoculants can be a key strategy for sustainable management along with the reduction of environmental hazards by cutting the use of chemical fertilizers(Adesemoye et al., 2009; Balasubramanian et al., 2004; Hungria, Nogueira, & Araujo, 2013)

Despite their potential, PGPR utilization in agriculture is not yet a common practice used worldwide. (Bashan, de-Bashan, Prabhu, & Hernandez, 2014).Different species of PGPB have been commercially used, namely *Bacillus pumilus,* Pseudomonas *sp.* ,Variovorax *paradoxus, Serratia proteamaculans etc.* (B.R. Glick, 2012). PGPR and their interactions with plants are exploited commercially (Podile & Kishore, 2007) and they represent a huge promise for sustainable agriculture. Applications of these associations have been mostly studied in maize, wheat, oat, barley, peas, canola, soy, potatoes, tomatoes, lentils, radicchio and cucumber (Gray & Smith, 2005).

However, there are few challenges behind utilization of PGPR that may account for the limited use of these tools. There are two major reasons pointed out that limit the use of PGPB use in

agriculture.First, the beneficial effects of inoculated PGPR depend on their survival in different soils and some inconsistency may result from that and influence the expected crop production. The success of PGPR utilization also depends on few other factors like compatibility with crop, interaction ability with other indigenous microorganisms of soil and other environmental factors (Martínez-Viveros, Jorquera, Crowley, Gajardo, & Mora, 2010). Another challenge is the mode of action of PGPR, which is still unknown in few cases (Dey, Pal, Bhatt, & Chauhan, 2004) (Choudhary, Sharma, & Gaur, 2011). An ideal PGPR need to have some characteristics like high rhizosphere competence, having the quality to enhance plant growth capabilities, have a broad spectrum of action, be compatible with other rhizobacteria, be safe for the environment, and have resistance to common stress factors as UV radiation, oxidizing agent and heat .(Vejan, 2016). Recognizing the potential of PGPR now allows us to, in the future, use technologies like nano-encapsulation that can revolutionize today's PGPR biofertilizers' formulation and already there is a successful enhance in growth of mung bean (*Vigna radiata*) and chickpea (*Cicer arietinum*) seedlings at low concentrations using zinc-oxide nanoparticles. (Duhan, Kumar, Kumar, Kaur, & Nehra, 2017).

Biological Nitrogen Fixation

Although nitrogen can be found widely in atmosphere, plants can only uptake nitrogen in nitrate or ammonia form. There are some diazotrophic prokaryotes that are able to convert atmospheric nitrogen (N_2) to the accessible form in which non-fixing organisms can uptake (NH_3). The biological nitrogen fixation (BNF) is a process catalyzed by the nitrogenase enzyme complex in bacteria and Archaea (encoded by *nif* genes), which comprises a dinitrogenase reductase (Fe protein) and a dinitrogenase (usually a Mo-Fe protein). The dinitrogenase produce two molecules of ammonia and one molecule of hydrogen using ATP as an electron donor by reducing the triple bond of the nitrogen atoms of atmospheric N_2. (Taiz, Zeiger, Møller, & Murphy, 2015). In this process, rhizobia release ammonia that needs to be immediately converted into organic forms in the root nodules before it get transported to shoot via xylem, otherwise it can cause toxicity (Taiz et al., 2015).

The most studied example of symbiotic diazotrophs is rhizobia, yet there are other relations between eukaryotess and diazotrophic bacteria in nature, as for example actinorhizal plants and *Frankia*. For non-legume crops, rhizobia may act as non-symbiotic Plant growth promoting bacteria (PGPB) that are proven economically beneficial endophytes for crops like rice or wheat (Biswas, Ladha, Dazzo, Yanni, & Rolfe, 2000).

Rhizobia-legume specificity

In soil, the diversity and size of native rhizobia populationsis influenced by the host presence. There were study that reported that an increase in the size of common bean-nodulating rhizobia population was associated with the continuous host crop(Andrade, Murphy, & Giller, 2002). Other studies on soybean rhizobia, reported a reduced rhizobia diversity in the presence of the host (Coutinho, Oliveira, Lovato, Maia, & Manfio, 1999).

There are legume species that can establish nitrogen-fixing symbioses with rhizobia from different genera, while other legume species are limited for nodulation and only accept as microsymbionts a reduced number of species. Common bean (*Phaseolus vulgaris*) is considered a unrestricted host, as it is efficiently nodulated by strains of at least three rhizobia genera (*Bradyrhizobium*, *Rhizobium*, and *Ensifer*). Yet the host range depends on the legume cultivar used and conditions tested (Martinez-Romero, 2003). Legume host could have an influence on the growth of specific rhizobia, and competition among rhizobia in the rhizosphere (Denison, 2000). Furthermore, biotic and abiotic factors can influence rhizobial species relative abundance, and genetic diversity within species. For example, Bradyrhizobia populations associated with soybean crop in Brazil mainly result from the frequent inoculation of this crop with two *Bradyrhizobium* species. However, studies found that soil pH, soil organic matter and clay content as main factors influencing Bradyrhizobia diversity (Giongo et al., 2008).

Rhizobia and nodulation process

Legume roots, as a part of their usual defense mechanism releases exudates, which include flavonoids and isoflavonoids compounds that were found to be involved in the legume-rhizobia symbiosis (Dakora & Phillips, 1996). Flavonoids can act as both *nod* genes inducer and inhibitor (Djordjevic, Redmond, Batley, & Rolfe, 1987) . NodD protein interaction with specific legume flavonoids is the first level of host recognition and then NodD activate the *nod* genes for the synthesis of Nod factors.

Nod factors are at the second level of host specificity recognition because of its structural modification and amount of Nod factors released, which regulate the nodulation initialization and these are usually lipochitooligosaccharides (LCO) (Perret, Staehelin, & Broughton, 2000). Nod factors are responsible for some fundamental actions to initiate nodule formation like induction of cell division in the root cortex and pericycle.

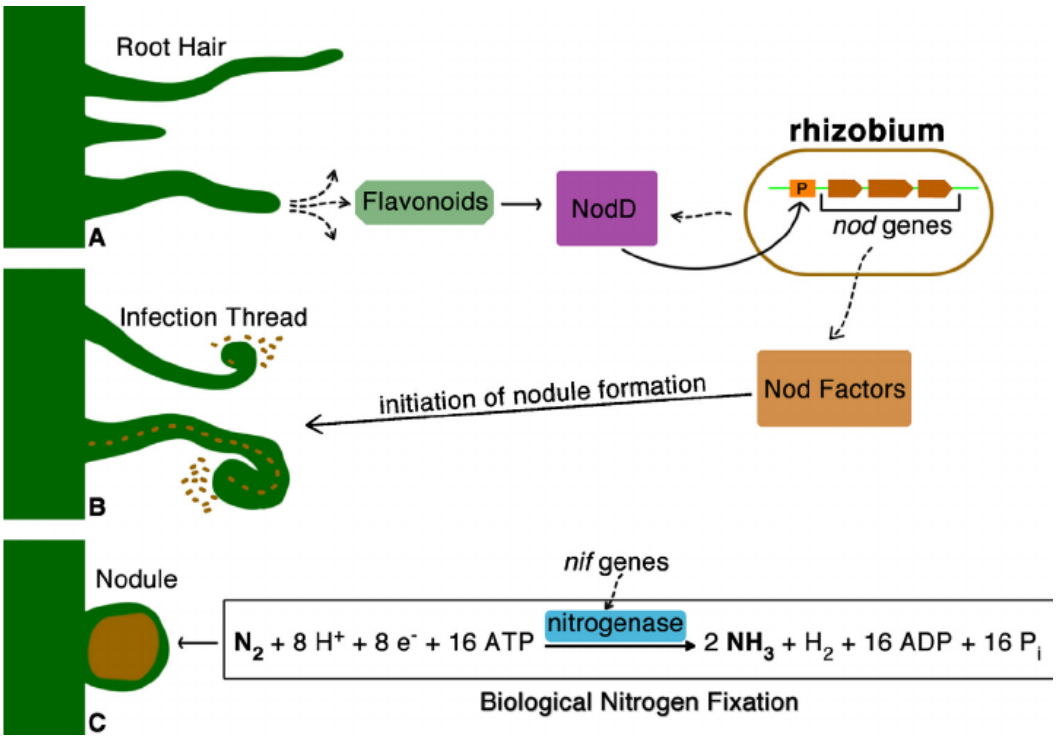

Figure 2: illustrating the biological nitrogen fixation and nodulation process (adapted from Laranjo, Alexandre, & Oliveira, 2014) .Legumes interact with their bacterial symbionts, which have the capacity to fix atmospheric nitrogen via a process called biological nitrogen fixation.

Rhizobia are able to process the atmospheric nitrogen in a specialized structure called nodules and convert it into nitrogen compounds accessible to the plant. By doing so rhizobia also benefit from carbon substrates that are obtained from plant photosynthesis. Rhizobia-legumes symbioses can work as an advantage for other subsequent crops as well, as the levels of avaible nitrogen in the soil is increased after legume crops (Lupwayi, Clayton, Hanson, Rice, & Biederbeck, 2004).

Bacterial migration towards the roots of plant is a chemotactic response to chemical attractants like flavonoids released by roots. As the figure 2 (Laranjo et al., 2014) shows, the flavonoids and other chemical attractants stimulate the activation and tightly binding to the rhizobial NodD protein. Interestingly each strain of rhizobia identifies only a limited number of flavonoids structures, and each species of legumes produces its own set of flavonoids and some of those are specific for inducing the transcription of other *nod* genes (F. X. Nascimento, Brígido, Glick, & Rossi, 2016; Taiz et al., 2015).
Along with the process, simultaneously rhizobia get entrapped and produce lipochitooligosaccharide Nod factors which induce extreme curling of root hair cells (Taiz et al., 2015). After binding to a legume root receptor, mitotic cell division in root cortical cells lead to the formation of the nodule primordium (Cooper, 2007; Oldroyd, 2013). Infection thread which

is basically an internal tubular extension of the plasma membrane buildup by the fusion of Golgi-derived membrane vesicles at the site of infection, forms after the development of nodule primordium (Taiz et al., 2015). Root hairs are one of the best studied plant cells that elongate through the process of tip growth (Carol & Dolan, 2002; Smith, 2003). Infection threads are thought to be tip-growing structures that grow from growing root hairs and most likely elongate by using at least some machinery that was supporting root hair growth before the infection occurred.

The infection thread is generally filled with proliferating rhizobia, develops and continuously grows until reaching the nodule primordium that finally differentiate into a specialized symbiotic organelle-like: the nodule (Cooper, 2007). *Lotus japonicus* and *Medicago truncatula* are two model legume species well studied for their legume-rhizobium interaction. Nodules belong to the determinate or to the indeterminate nodule type and these two plants represent these two major types (Pawlowski & Bisseling, 1996; Stougaard, 2001). The underlying mechanisms for the formation of nodules of these two plants are very similar, but they show different morphology and ontology. As *M. truncatula*, chickpea nodules belong to the indeterminate type.

The determination of the type of nodule is done by the host plant. As shown in the figure 3 by Ferguson et al., 2010, there are morphologic differences between two nodule types. The site of the first internal cell divisions, meristematic region maintenance and formation of mature nodules are some points where the dissimilarity can be observed (Newcomb, Sippell, & Peterson, 1979; Rolfe & Gresshoff, 1988). Indeterminate nodules have a more persistent meristem that results in a cylindrical shape (e.g. alfalfa, clover, chickpea etc.) unlike determinant nodule which are spherical (Newcomb et al., 1979). Matured indeterminate nodules comprise a heterogenous population of nitrogen-fixing bacteroids due to continued cell division activity that ultimately develops a gradient of developmental states in the elongated nodule. (figure 3).
The formation of nodule requires the reprogramming of differentiated root cells to form a primordium from where the nodule is able to develop. Changes in three root tissues, namely epidermis, cortex, and pericycle are important steps in order to form the nodules.

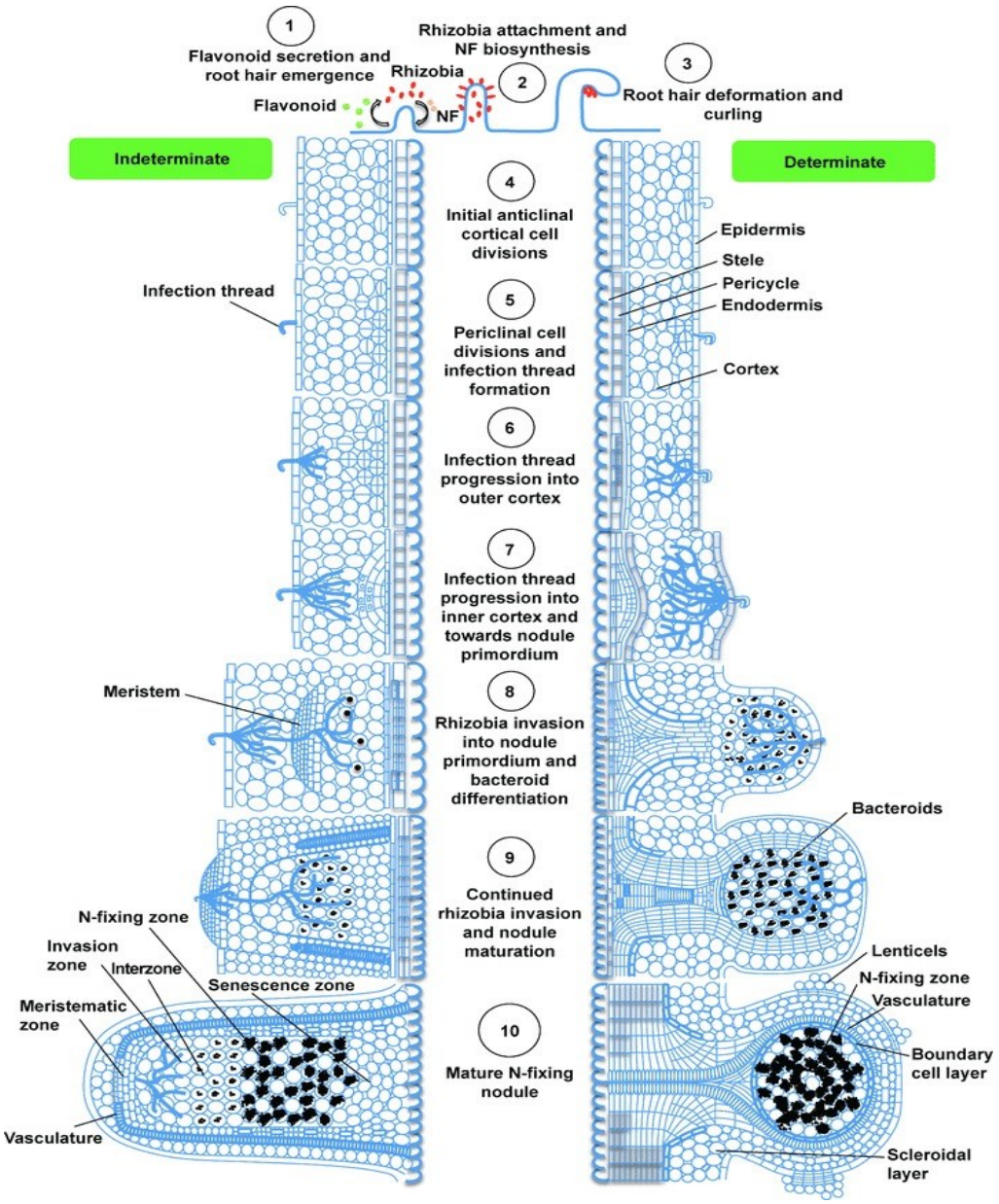

Figure 3 : The illustration shows the development stages of indeterminate and determinate legume nodules. The left side of the figure indicates the developmental stages of pea (indeterminate) while the right side of the figure shows soybean nodule development (determinate) (adapted from Ferguson et al., 2010).

The chickpea-rhizobia symbiosis

The chickpea (*Cicer arietinum*) is a legume of the family Fabaceae, subfamily Faboideae. It has usually been known as traditional low-input crop in the farming systems of Indian subcontinent and the Near-East and there it is considered as one of the basic daily diets. Chickpeas are one of the most popular vegetarian foods in the Indian subcontinent and frequently used in many other European countries such as Portugal, Spain and Italy. Chickpea is also common crop in Ethiopian Highlands and Central and South America and then it has become popular in Australia, Canada and the USA (Saxena & Singh, 1987).
According to data available from FAO (Food and Agriculture Organization), India is by far the major chickpea producer with approximately 7.7 millions of tonnes produced in 2008 and data from 2014 showed that Asia alone produces 87% where Europe, Africa, Ocenia produce the rest (http://faostat.fao.org/). In Semiarid tropical (SAT) countries it occupies approximately 92% of the area and 89% of the total grain-legume production (Ahlawat, 2000; Khan, Zaidi, & Aamil, 2004). In Portugal, chickpea production decreased significantly in the 80's and it is now reduced to 650 tones (FAO data available for 2008), which means that Portugal imports almost all the chickpea consumed (Duarte Maçãs, 2003). Previously, spring was the time when the chickpea in Portugal were sown but now with the development of new cultivar chickpea is sown on Autumn/Winter and this also helped to maximize the atmospheric nitrogen fixation carried out by rhizobia (Duarte Maçãs, 2003).

Unlike model plants such as *Medicago truncatula* or *Lotus japonicus*, chickpea (*Cicer arietinum*) -rhizobia symbiosis is rather recently studied. Although chickpea stands as the second major pulse crop in terms of area cultivated (first is common bean), many aspect of its symbiosis with rhizobia started to get investigated in the 90's. Chickpea rhizobia species were first described as *Rhizobium ciceri* (1994b) and *Rhizobium mediterraneum* (1995) by Nour et. al. (1994a)(Nour, Fernandez, Normand, & Cleyet-Marel, 1994). After few years, these two species were transferred to the genus *Mesorhizobium* (Jarvis et al., 1997). For chickpea (*Cicer arietinum* L.), the predominant symbiotic nitrogen-fixing bacteria associated with it in Europe and in India have been classified as members of the genus *Mesorhizobium*, including *Mesorhizobium ciceri, Mesorhizobium mediterraneum, Mesorhizobium amorphae, Mesorhizobium loti, Mesorhizobium muleiense* and *Mesorhizobium tianshanense* but still a higher magnitude of mesorhizobial diversity is yet to be discovered (Alexandre et al., 2009; Laranjo, Machado, Young, & Oliveira, 2004; Nour et al., 1994). Rhizobia have been involved in promoting plant growth through mechanisms other than nitrogen fixation (B.R. Glick, 2012). For example, the presence of ACC deaminase activity in some rhizobia strains promotes plant growth through lowering of plant ethylene levels(Duan, Müller, Charles, Vesely, & Glick, 2009) Successful colonization of the host root by free-living rhizobia can also assist to deal with adverse conditions in the soil like stresses. Additionally, there are also reports on rhizobia help to provide increased resistance against plant pathogens (Avis, Gravel, Antoun, & Tweddell, 2008). Still diversity of rhizobia to nodulate chickpea and also the effect of agricultural products on

microbes are insufficiently studied over all.

Effects of agricultural treatments on soil microbes

Several studies have shown in vivo evidence of a subset of organochlorine pesticides, agrichemicals, and environmental contaminants causing inhibition or delayed recruitment of rhizobia to host plant roots, which caused fewer number of root nodules, lower rates of nitrogenase activity and a reduction in overall plant yield at time of harvest (Fox et al., 2007). Besides this declining effect on the yield of crops,the increasing use of synthetic chemicals is also responsible for environmental consequences like reducing soil fertility and in this way, leading to unsustainable long -term crop yields (Fox et al., 2007).

Nitrogen application on agriculture

Nitrogen is a fundamental nutrient for plant growth and development but is unobtainable in its most prevalent form as atmospheric nitrogen. Plants depend upon combined or fixed forms of nitrogen like ammonia and nitrate. In the cropping systems, nitrogen is usually provided in form of industrially produced nitrogenous fertilizers.

Using higher amount of fertilizers has consequently led to worldwide ecological problems like formation of coastal dead zones from agricultural waste such as phosphorus and nitrogen, and excessive amounts of nitrogen lead to eutrophication. A study on soybean showed that higher rates of KNO_3 and NH_4Cl (128 kg N/ha) significantly depressed nodulation and specific nitrogenase activity, but slightly decreased the plant dry matter (Abdel-Wahab & Abd-Alla, 1995). Biological nitrogen fixation represents an important alternative to chemical N-fertilizers, since it can be used to naturally offer nitrogen for plants.

Absence of suitable strains, small population size and poor survival of rhizobia can cause difficulties in forming nodules (Kantar et al., 2007). Suitable bacteria in the soil may provide an effective utilization of atmospheric nitrogen and eventually suppress the need for N-fertilizers. To ascertain the nodulation in crops, inoculation of seeds may be required in soil where nodulating is poor or mostly ineffective.

For example, rhizobial inoculation can assist to significantly increase the nitrogen fixing potential of chickpea genotypes (Khattak, Zeb, Bibi, Khalil, & Khattak, 2007). From different studies conducted, it was found that competitive rhizobia inoculation not only enhance chickpea yield but also was proven to be economically promising to increase the chickpea production (Romdhane et al., 2007).

Glyphosate application on agriculture

Glyphosate [N-(phosphonomethyl)-glycine] is a broad-spectrum, non-selective, post-emergence herbicide that is widely used in agriculture (fig. 4). The IUPAC name is 2 (phosphonomethylamino) acetic acid. In agriculture, horticulture and at amenity sites glyphosate has been widely used as an active ingredient of unwanted weed killing products.

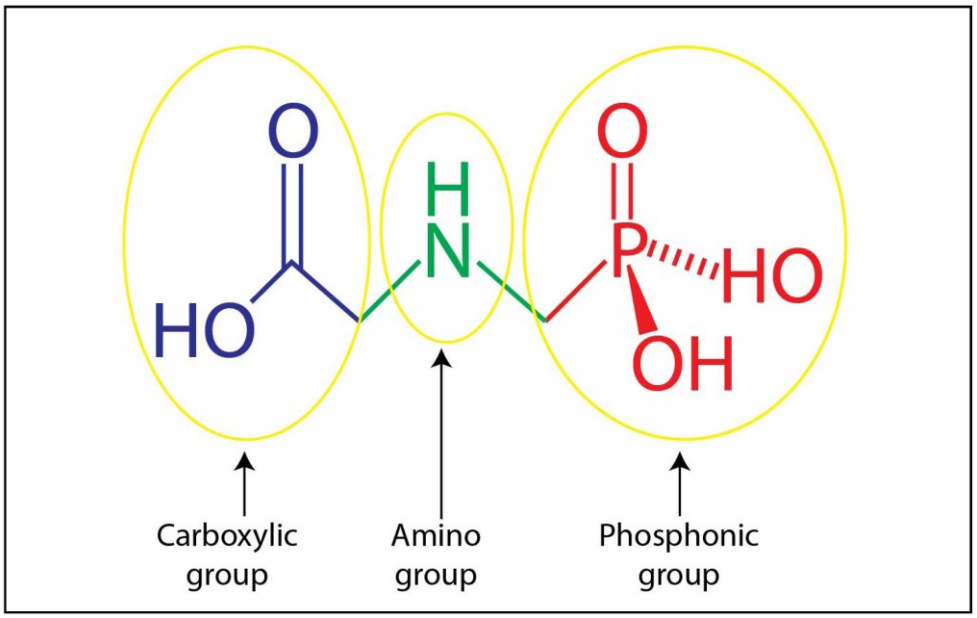

Figure 4 : Chemical and 3D structure of glyphosate (adapted from Jayasumana, Gunatilake, & Senanayake, 2014)

The amount of glyphosate applied worldwide over the last decade is about 6.1 billion Kilograms (Benbrook, 2016). Monsanto company first released a glyphosate herbicide for agricultural use in early seventies (Woodburn, 2000). Glyphosate is generally used as broad spectrum systemic herbicide that destroys weeds, especially annual broadleaf weeds and grasses that invade growing area of crops.

An increasing volume and escalated application of glyphosate treatment are introduced worldwide. For a long time plantations, orchards, vineyards and industries used glyphosate, but now it is highly associated with conventional crop agricultural system. The introduction of an increasing number of genetically glyphosate-tolerant crop varieties are one of reasons for the prevalence of glyphosate. As glyphosate-tolerant crops allowed the farmers to kill the weeds without affecting the crops, the use has become more prevalent. There is a upward reliance on the broad-spectrum herbicide glyphosate which has set off the spread of tolerant and resistant weeds in the U.S. and globally (Duke, 2015),(Cerdeira, Gazziero, Duke, & Matallo, 2010). To combat weeds less sensitive to glyphosate, farmers typically increase glyphosate application rates and spray more often (Owen, Beckie, Leeson, Norsworthy, & Steckel, 2015; Powles, 2008).

Globally the use of glyphosate has risen almost 15-fold since 1996 after the introduction of genetically engineered glyphosate-tolerant “Roundup Ready” crops(Benbrook, 2016). In 2007, glyphosate was the most used herbicide in agricultural sectors of the United States and also, the second-most utilized to home and garden, government and industry, and commerce according to United States EPA report on 2007. The average rate of glyphosate applications per hectare per crop year during 2014 fell in the range of 1.5–2.0 kg/hectare (Benbrook, 2016). At these rates of application, the total volume of glyphosate applied in 2014 was sufficient to treat between 22 and 30 % of globally cultivated cropland. No pesticide in history has been sprayed so widely.

Glyphosate has been used in an extensive manner all around the world and get the tag of most used herbicide of the world (Myers, Antoniou, Blumberg, Carroll, Colborn, Everett, Hansen, Landrigan, Lanphear, & Mesnage, 2016) . Despite it has its potential downside by being classified as “probably carcinogenic to humans”, following a review of evidence from human exposure studies and in research on laboratory animals, by the International Agency for Research on Cancer in 2015, which belongs to the World Health Organization ((WHO), 2015)), with all the studies conducted later the obligation of researching on the safety of glyphosate based herbicides have become more important.

Due to the above mentioned questions raised by the WHO further investigations were carried out regarding the safety of glyphosate-based herbicides and calls for biomonitoring, toxicology and epidemiological studies have been made (Myers, Antoniou, Blumberg, Carroll, Colborn, Everett, Hansen, Landrigan, Lanphear, Mesnage, et al., 2016).

Along with the concerns on human health due to the use of glyphosate, one more unanswered question has come into light and that is our insufficient information, inadequate studies on the ecological safety of glyphosate and on its working mechanism in natural environment. In particular, the knowledge on glyphosate’s interaction with living beings and its degradation pathways were quite unidentified (Sviridov et al., 2015).

Previously, glyphosate has been considered to be safe based on the assumption of quick inactivation after spraying and it was considered to have rapid sorption onto particles in soil and also it was supposed to be degraded by the microbes faster. (Hagner et al., 2015). Now, it has become important to understand the effect of standardized amount of glyphosate on plant growth, nodulation and soil micro organism specially rhizobia.

Mechanism of action of glyphosate

The mechanism by which glyphosate kills plants is inhibiting the shikimate pathway of the synthesis of aromatic amino acids such as phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). Taking into account the unique mechanism of inhibiting shikimate pathway, glyphosate was also shown to affect the growth of some micro-organisms, including bacteria, algae and fungi; yet, it was theoretically considered as non-threatening to mammals, since the shikimate pathway is not found in animals.(Gaupp-Berghausen, Hofer, Rewald, & Zaller, 2015; Tanney & Hutchison, 2010). Nonetheless, later on evidence from several studies showed that glyphosate-based herbicides, via multiple mechanisms, can adversely affect the biology of mammals and and that lead to the classification of glyphosate as 'probably carcinogenic to humans ((WHO), 2015; Myers, Antoniou, Blumberg, Carroll, Colborn, Everett, Hansen, Landrigan, Lanphear, & Mesnage, 2016).

The concerning factor is the half-life of glyphosate which is an indication of its persistence in the soil and water and which is believed to be longer than presumed (Myers, Antoniou, Blumberg, Carroll, Colborn, Everett, Hansen, Landrigan, Lanphear, & Mesnage, 2016). Few recent reports suggested that the herbicides persisted longer with the return of crop residues containing glyphosate to the soil. (Mamy, Barriuso, & Gabrielle, 2016).

In the shikimate pathway, aromatic amino acids are synthesized and consequently produce chorismate, from which Trp, Tyr, and Phe are produced through branched pathways. The production of Phe is an important portal for downstream biosynthesis because it work as the starting point for the synthesis of phenylpropanoids. The primary step to synthesis chorismate is catalyzed by the chloroplastic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that converts shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvyl-shikimate- 3-phosphate (Vivancos et al., 2011). This EPSPS is specifically mentioned to be inhibited by glyphosate.(Powles, 2008).

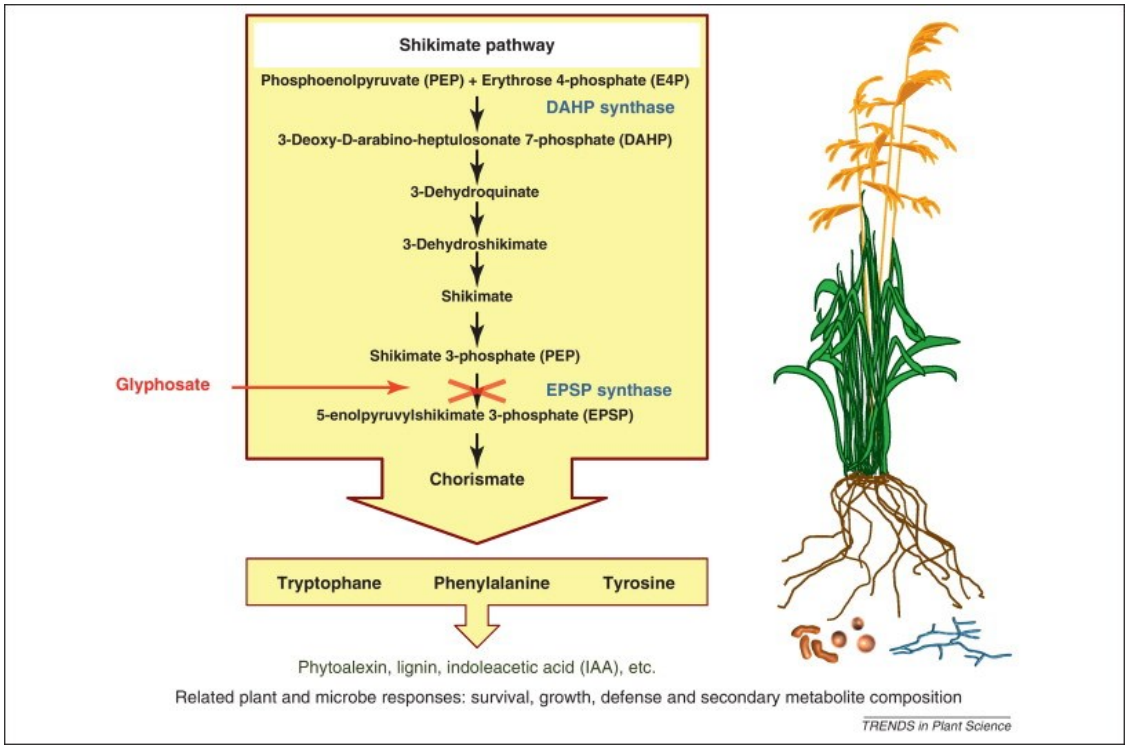

Figure 5: Main steps of the shikimate pathway, which is present in plants and microbes. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate acid pathway, which in consequence interferes with the synthesis of proteins and other compounds that need tryptophan, phenylalanine or tyrosine as precursors, as for example plant hormones (Helander, Saloniemi, & Saikkonen). Adapted from Helander et. al. (2012).

There have been two mechanisms that were identified responsible for glyphosate resistance in weeds. To elaborate on the trait of glyphosate-resistant crops, it can be said that constitutive overexpression of highly efficient glyphosate-resistant form of EPSPS has ultimately advanced to the evolution of resistance in weedy species that threats the continued success of transgenic glyphosate-resistant crops. (Powles, 2008). There are three reasons found for herbicide resistance which are alterations of the target site, changes in sequestration and/or translocation of the herbicide and changes in rates of metabolism of the herbicide. Currently, two of these causes have been identified as responsible for glyphosate resistance in weeds. Alterations of the target site via a mutation in the EPSPS gene so that it is no longer inhibited by glyphosate or overexpression of EPSPS have been documented in goosegrass [*Eleusine indica* (L.) Gaertn.], rigid ryegrass (*Lolium rigidum* Gaudin).

In a recent paper, a new mechanism was described about a large amplification of the EPSPS gene on multiple chromosomes involved in glyphosate resistance (Gaines et al., 2010). Although, extensive use of glyphosate in agriculture, horticulture and forestry has been done, the precise mechanisms through which glyphosate kills the plants still has some uncertainties. The characterization of interaction between amino acid metabolism through the shikimate

pathway was done on glyphosate-resistant Roundup Ready Soybean (RRS) plants which resulted in data showing that RRS plants accumulate much higher level of glyphosate than the sensitive line and this was linked with enhanced cellular oxidation and specific enhancement of proteins associated with photorespiration which is in line with the previous results (Ireland, Percival, & Baker, 1986) that emphasized on the inhibitory effect of glyphosate on photosynthetic C02 assimilation and chlorophyll fluorescence emission (Vivancos et al., 2011).

Glyphosate penetrates crops through leaves and eventually, then it will be transported to growing points. Therefore, it can only influence on actively growing plants and not be efficient as pre-emergence herbicide. (Myers, Antoniou, Blumberg, Carroll, Colborn, Everett, Hansen, Landrigan, Lanphear, Mesnage, et al., 2016)

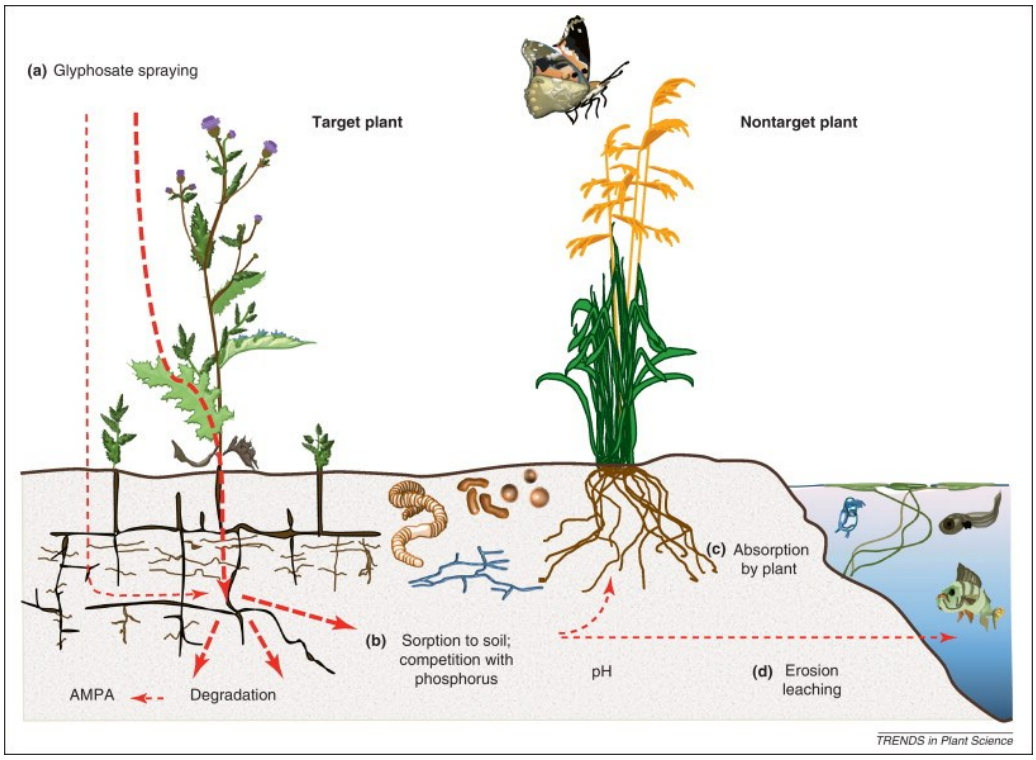

Figure 6: Illustration of glyphosate accumulation and transport in the field. Transportation of glyphosate is indicated with the red arrows. (adapted from Helander et al.2012)

In soil surface, from foliage glyphosate translocate throughout the all part of plant including roots and shoots via phloem. The applied glyphosate partly could end up in soil surface. (b) Glyphosate can be released to soil or can come from roots to contact animals living in the soil (e.g., earthworms) feeding on dead plant material, degraded by soil microbes, or adsorbed to the soil particles. Some part of glyphosate can be reduced to 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA; the main metabolite of glyphosate). (c) Non target plant growth can be influenced by the residues of glyphosate and it can turn pathogens and herbivores resistant. Possibility of glyphosate residue ending up as animal feed or finally find its way into human consumption. (d) By erosion glyphosate can be transported to aquatic system.

Effect of glyphosate on environment

Currently, the intensive use of herbicides has been increasingly in a very fast rate and thus it is a matter of environmental concern. The concerns have become more serious because of the harmful effect of these chemicals on soil microorganisms, which as mentioned previously, play an important role in decomposition of organic matter, mineralization and other biochemical processes that are fundamental to the ecosystem and ultimately contribute to the services provided by the soil. Glyphosate represents an example of an herbicide which has been hugely successful commercially due to its high effectiveness.

Microbial degradation is considered as the most notable transformation process that actually ascertain the persistence of herbicides in soil (A. Souza, Ferreira, Silva, Cardoso, & Ruiz, 1999). Glyphosate mineralization depends of both the activity and biomass of soil organisms. (von Wirén-Lehr, Komoßa, Gläßgen, Sandermann, & Scheunert, 1997). Glyphosate somehow compromises the ability of plants to defend against pathogens that inhabit the rhizosphere and glyphosate blocks the shikimic acid pathway which is reliable pathway for so many of plants for defences(Johal & Huber, 2009).

There are few recent studies which have suggested the indirect effect of glyphosate on the soil microbial community. These studies have also mentioned the necessity of assessing glyphosate effect on the microbial community structure in cultivable land ecosystems. Different reports evaluated the impact of commercial glyphosate (as RoundUp) on the soil bacterial communities and found changes in composition of bacterial communities and proliferation of protists (a varied group of single celled organisms) (Imparato, Santos, Johansen, Geisen, & Winding, 2016). One of the reasons behind these changes is the escalating availability of easily degradable carbon compounds from the roots of plants killed by glyphosate application (Imparato et al., 2016). Glyphosate use has been found to have different effect on different soil organisms and several studies have examined it. For example, in response to glyphosate treatment, *Proteobacteria* (particularly the class *Gammaproteobacteria*) increased in relative abundance for corn and soya crops, whereas relative abundance of *Acidobacteria* decreased (Newman et al., 2016). Another interesting observation was about Acidobacteria, which are highly involvement in biogeochemical processes like cellulose degradation, and the decline in these bacteria after glyphosate treatment may lead to the soil's long-term impaired ability to perform certain biogeochemical reactions (Newman et al., 2016).

Arbuscular mycorrhizal fungi (AMF) improve water access and soil minerals for plants, improve drought tolerance and help with resistance against pathogens(Yang et al., 2009). There are many recent studies showing a reduction of spore viability and decline the root colonization of AMF following glyphosate application and that consequently decrease on diversity (Druille, Cabello, Omacini, & Golluscio, 2013). A recent study found that the application of Roundup in soils caused a decline of 40% on mycorrhization (Zaller, Heigl, Ruess, & Grabmaier, 2014).

To understand more precisely about the impact of these widely used agricultural practices

further research needs to be carried out on different soil treatments discussed on a particular leguminous plant (chickpea), answers were sought to know the impact of glyphosate and nitrogen source on soil microorganisms.

Objective and Motivation

In the present context of growing human population and increasing need of food production, protection of environment and conservation of ecosystems are challenging. To address this issue sustainable agriculture practices are needed to be developed as well as widespread.
Rhizobia are plant beneficial bacteria that establishes symbiosis with leguminous plants, such as bean, soybean, and chickpea, among others. Within the nodules, rhizobia convert atmospheric nitrogen into a form that plants can uptake and thus providing this macronutrient to crops. These symbiosis contribute to improve agricultural sustainability in food production, since an optimal use of these beneficial bacteria can reduce the use of environmentally and economically detrimental chemical N-fertilizers.
The proposed work of this Master's thesis mainly focuses on the impact of soil treatments that farmers usually use in the field in the conservation on the native populations of rhizobia. My work has emphasized on the effect of standardized amount of glyphosate (Roundup Ultramax) and nitrate on the chickpea plant growth, nodulation and rhizobia diversity found in the nodules. This work is planned on chickpea which is one of the most important foods and feed grain crops in the world. The specific objectives of the present work are:

-To evaluate the impact of herbicide (glyphosate), N-chemical fertilizer (nitrate) and rhizobia inoculation treatments in the rhizobia diversity found in chickpea nodules
-To determine the effect of the same treatments in the chickpea plant growth
-To analyse the effect of chemical nitrogen source on rhizobia-chickpea early infection process

Scientific Article

Impact of soil treatments in the conservation of native rhizobia populations

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Keywords

rhizobia, biodiversity, legume, nitrogen fixation, chickpea, nitrogen fertilizer, glyphosate

Abstract

The need of sustainable agriculture for keeping the balance in food production and ecology is one of the most challenging undertaking that the world is facing now with the growing human population. Different sustainable agricultural practices like the optimization of rhizobia-legumes symbiosis for biological nitrogen fixation requires more studies addressing the influence of commercial agricultural treatments used on soil microbial communities. Particularly the soil treatments which are used widely all over the world (e.g. application of glyphosate, nitrate fertilizer) need to be taken under extensive research to understand its impact on native rhizobia population. The present study used soil from Herdade da Tramagueira, Beja, Portugal to observe the impact of herbicide (glyphosate), N-chemical fertilizer and rhizobia inoculation treatments in the in the diversity of rhizobia able to nodulate chickpea. The analysis of partial 16S rRNA gene sequence revealed that all the nodules collected from the four treatments had predominance of one *Mesorhizobium* species, since all sequences grouped in a single cluster with the type strain *Mesorhizobium muleiense CCBAU* 83963. Interestingly LMS-1 inoculation treatment did not show any 16S rRNA sequences compatible with LMS-1. Overall, these results showed a low diversity of rhizobia on chickpea nodules that was not affected by any of the treatments. Nevertheless, the nodule sections analysed under bright field microscopy showed less bacteriod occupation for the glyphosate and nitrate source treatments, compared to the control and LMS-1 inoculation ones. In terms of shoot dry weight (SDW), it was found that plants from pots where glyphosate was applied had 21% lower SDW compared to control plants.

The analysis of the effects of higher concentration of chemical nitrogen source on rhizobia-

chickpea infection process via confocal microscopy showed that these treatments had a visible delay in root growth and less curling than control. Further investigation on the effects commercial treatments on native rhizobia will certainly contribute to understand the influence of different factors in soil microbial community and will help to develope sustainable practice in agriculture.

Introduction

Although conservation of environment and agriculture are rarely found on the same page, both have remained equally significant for humankind and civilization. Modern agriculture has often implied simplification of the ecosystems, as monoculture systems are the most common. From a farmer's perspective conservation biology could be seemed overly idealistic (Banks, 2004). Taking into account the growing human population, escalating pollution and increasing environmental hazards, it has become a question of survival to maintain the equilibrium between sufficient food production and sustainable use of natural resources. For better ecological balance, reduction of man-made influences over natural environment are encouraged. The important challenge lying here is to balance the conservation of ecosystems and maintaining production of agricultural products to feed the human population. Food production frequently seems to have opposing goals and methodologies against preservation of biological biodiversity, yet there are some common ground of concerns. Some potential benefits are found in integrating both agricultural production and conservation biology worldwide, like excutation of sustainable agricultural practice that assist biological conservation efforts is one of those examples (Banks, 2004).

On one hand, agriculture has often relied on using fertilizers and pesticides in order to attaining more yield and increasing crop production. On the other hand, conservation suggests environment safety and supports biological conservation, which commonly limits the use of any sort of added artificial chemical products like fertilizer, weed killer or pesticides (Banks, 2004). Recognizing different impacts of agriculture on environment is a topic that has been introduced quite recently and which needs to be spread out since approaching worldwide sustainability in agriculture is vital (http://www.fao.org/sustainability/background/en/). The evaluation of the effects of different agricultural practices such as the use of fertilizers, pesticides and other hazardous chemical products are some of the significant issues that need to be addressed and ensuing implication needs to be done globally. "Green Revolution" that significantly escalate the agricultural output due to widely spread use of synthetic nitrogenous fertilizers, pesticides and irrigation, with time resulted as unsustainable for environment. To demote the dependency on nitrogenous fertilizers, a strategy could be the optimized use of leguminous crops that fix atmospheric nitrogen via symbiosis with N-fixing bacteria in rotation with non-leguminous crops (Fox et al., 2007). In addition, it has been stated that synthetic chemicals compromised symbiotic nitrogen fixation (Fox et al., 2007).

It is high time to integrate the two disciplines of natural conservation and agronomy to optimize food production while preserving the ecosystems. There are different ways to move towards sustainable agricultural practices and understanding the recent ways of agricultural practice and knowing the advantages and shortcoming of it can help us in improving the practice. Agriculture and Conservation Biology are often considered as the two sides of the same coin because of their different requirements and goals.

Two important external products used in agricultural fields for high yield in crops, are nitrogen fertilizer and herbicides, yet their effects on the soil microbes population remain poorly studied.

The main aim of this study was to evaluate the impact of different treatments commonly used by famers in agricultural practice (e.g. herbicide, chemical fertilizer) as well as that of rhizobia inoculation, on rhizobia able to nodulate chickpea. In addition, the impact of different both nitrate and herbicide on bacterial growth kinetics and on the earlier steps of chickpea roots infection was evaluated.

Materials and Method

Bacterial Strains

Rhizobial strains used in this study were as follows: LMS-1, PMI-6-Portimão and V-15b-Viseu (from the Laboratório de Microbiologia do Solo-ICAAM collection)(Alexandre et al., 2009; F. Nascimento, Brígido, Glick, Oliveira, & Alho, 2012). Since the publication of information on these chickpea mesorhizobia strains, many *Mesorhizobium* new species were decribed, so a new phylogenetic analysis, based on the 16S rRNA gene sequence was performed (details of this analysis are shown below on the "Phylogeny Based on the 16S rRNA gene partial sequence" section). For confocal analysis, *Mesorhizobium* strains LMS-1 (Rodrigo, unpublished work), PMI-6 and V15b previously tagged with green fluorescence protein (GFP) were used (Brígido, Robledo, Menéndez, Mateos, & Oliveira, 2012)

All strains were grown in tryptone yeast (TY) (REF Beringer, 1974) at 28°C for routine use and preserved in 30% (v/v) glycerol at -80°C; for the strains harboring pMRGFP 50 µg.ml-1 kanamycin was added to the culture medium(García-Fraile et al., 2012)

Bacterial Growth Kinetics Assay

The commercially used weed killer Glyphosate Roundup Ultramax and a source of nitrogen (KNO_3) were added to the TY medium. The formulation of Roundup UltraMax is *SL with 360 g / l or 28.85% (w / w) glyphosate (as potassium); contains ethoxylated ether. AV 0261.* Three different concentration of glyphosate source (Roundup) were used: 15.5µl/100mL, which corresponds to the standard 2L/hectare field application recommended by the manufacturer; 31µl/100mL and 46.5µl/100mL. Different nitrogen source (KNO_3) concentrations were also used

to see the effect on bacterial growth, namely 1.37 mM , 5 mM and 7 mM. 1.37mM KNO_3 corresponds to the standard amount of 50kg/hectare nitrogen fertilizer application and all the calculation was done using a volume of soil of 2600 tons/hectare , taking into account of 10,000m^2, 0.2m height, 1.3m density. Each strain was also grown in plain TY liquid medium, considered as controls conditions (no herbicide or extra nitrogen source added).
The effect of different levels of herbicide and nitrogen source on bacterial growth were evaluated on three different chickpea mesorhizobia strains: LMS-1, PMI-6-Portimão and V-15b-Viseu. Three replicates were performed for all the strains, in each treatment. Growth curves were generated based on optical density (O.D.) readings at 540 nm using a spectrophotometer (Cecil CE 1021 high Specification UV/Visible Spectrophotometer 200-1100nm by Cecil Instruments Limited).

Analysis of the effect of chemical nitrogen source on rhizobia-chickpea infection process

Confocal microscopy was used to analyze the early stages of rhizobium-legume interaction, namely rhizobial infection in chickpea pre-germinated roots. The aim of this analysis is to observe the effect of nitrogen sources on the infection process.
The *Mesorhizobium* strains LMS-1, PMI-6 and V15b previously tagged with green fluorescence protein (GFP) were used.
Surface-sterilized seeds of *Cicer arietinum* (Elixir variety, provided by the Estação Nacional de Melhoramento de Plantas) were germinated on water agar (Alexandre et al., 2009). Chickpea seeds were surface sterilized first using calcium hypochlorite solution (14% active chlorine) for 25 minutes. Seeds were then washed 10 times with sterile distilled water and incubated for 1 hour in sterile distilled water at 4°C. Finally, the seeds were then washed 3 times with sterile distilled water and transferred to 0.75% water-agar plates that were incubated at 28°C for 48 hours.
Pre-germinated seedlings were transferred to new water agar plates (1.5%) with different concentration of KNO_3. The same three levels of KNO_3 concentration (1.37mM, 5mM, 7mM) was used in the experiment which previously was used for bacterial growth evaluation.
For each strain of rhizobia 3 replicates per treatment with done. A set of plates with only water agar media and bacteria inoculum was used as control plates.
Each plate contained 3 chickpea seedlings positioned on top of a filter paper. Each seedling was inoculated with 400 µL of rhizobia with an OD at 540 nm of 0.5. Then another filter paper was used to cover the seedlings, and finally the plates were covered with brown paper up to the shoot level, so that the roots could grow protected from light. All plates were kept in the plant growth chamber under controlled temperature, humidity and light (16 h-light and 8 h-dark cycle with 24°C-day and 18°C-night temperature at a relative humidity of 65%) and several time points were analyzed, namely on day 3 , 4 and 5 after inoculation.
The analysis of root hairs was performed using a Confocal Laser Scanning microscope (Leica TCS SPE) equipped with solid-state laser, allowing visualization of GFP (488 nm), and propidium iodide (532 nm). Roots and root hairs were stained with 8 µM propidium iodide (Sigma-

Aldrich). Projections were made from adjusted individual channels in the image stacks using Leica LAX software.

Plant growth assay for measuring plant growth parameters

Soil samples from Herdade da Tramagueira, Beja, were collected (personal contact Mário de Carvalho, University of Évora, Portugal) and used to grow chickpea in pots under greenhouse conditions. The soil used here taken from a cultivable area that has been previously used to grow chickpea.

To evaluate the effects of the herbicide (glyphosate) and nitrate fertilizer applications and rhizobia inoculation on the plant growth parameters and also on the chickpea rhizobia diversity on nodules, a pot assay was carried out. A total of 6 pots per treatment, containing 1 kg of soil were planted with two chickpea pre-germinated seeds each. All the pots were previously autoclaved and the seeds were disinfected according to the protocol described above. The pots used were o.013m in height and 0.075m in radius with a surface area of 0.018m^2. Glyphosate (Roundup Ultramax) and nitrate (KNO_3) treatments were added as pre-emergent to moist soil 24h before planting the seeds of chickpea in the soil. Glyphosate was applied in the soil at the recommended dose (2L/hectare diluted with water in 1:200 ratio) as well as the nitrogen fertilizer (50kg/hectare). Mesorhizobium LMS-1 was taken from freshly cultured plate and grown overnight on 28° C; cell density was adjusted to OD 1 to inoculate with 1 ml on each seedling during the plantation.

Pots were watered with sterile distilled water every two days. Plants from the four treatments were collected after 8 weeks on the greenhouse chamber and several parameters were measured, such as shoot dry weight, and number of nodules.

Nodules histological analysis

Nodules were collected from 8-week-old chickpea plants from the four soil treatments described above. Nodule samples were sectioned using Microtec 4055 microtome and processed for light microscopy. Approximately 8 representative nodules from each sample plants of every treatment were fixed in 4% formaldehyde dehydrated in an increasing ethanol series and xylene was used as a clarifying agent. The nodules were embedded in paraffin at a melting temperature of 54-56°C. 0.01% Toluidine blue-stained sections (8 µm) of embedded nodules were examined by bright field microscopy, using a Leica CTR6000 microscope.

Collection of nodules for diversity analysis

For each of the four treatments, 20 nodules (with diverse size) were collected per replicate (3 replicates per treatment). Nodules were disinfected in 96% ethanol for 30 seconds, followed by 0.1% $HgCl_2$ for 4 minutes and finally washed with sterile distilled water 7 times (Vincent, 1970). Nodules from all treatment were kept at -80^{o}C until the DNA extraction.

Extraction of bacteroid DNA from nodules

A rapid DNA extraction method was used to obtain DNA from bacteroids based on (Rivas, Velázquez, Valverde, Mateos, & Martínez-Molina, 2001) as briefly described below. The nodules were crushed (in 1 ml of sterile water) using a sterile pestle. A centrifugation at 800 g for 5 min was performed to pellet the nodule debris. After recovering the supernatant a new centrifugation was done at 12000 g for 5 min to pellet the bacteroids. The supernatant was discarded and the pellet was resuspended in 200 µl of Sarkosyl 0.1 % and a new centrifugation at 12000 rpm, 2 min was carried out. The pellet was resuspended in 100 µl of NaOH 0.05 M and this suspension was boiled for 4 min and immediately cooled on ice. 600 µl of nuclease free water was added to each sample and centrifuged at 4000 rpm, 3 min. A total of 400 µl of the supernatant containing the DNA from bacteroids was transferred to a new tube and stored at -20 °C.

PCR amplification of the 16S rRNA gene

After DNA extraction from nodules, amplification of a partial fragment of the 16S rRNA gene was performed by PCR. Amplification reactions were prepared in a total volume of 50 µl, containing 1X green Go Taq® buffer, 0.2 mM dNTP, 0.6 pmol/µl of 27F upstream (5'-AGAGTTTGATCCTGGCTCAG-3') (Turner, Pryer, Miao, & Palmer, 1999) and IntR downstream (5'-TTTACRGCGTGGACTACC-3') primers (Laranjo et al., 2004) 0,25 µl of Go Taq® DNA polymerase (5u/µl) and 2µl of template DNA. The PCR was carried out using the following program: initial denaturation for 2min at 95^{o}C, 38 cycles of 1 min s at 95^{o}C, 1min at 54^{o}C, 1 min at 72^{o}C and a final elongation of 5 min at 72^{o}C. For some samples the conditions of PCR amplifications required optimization, namely the amount of template DNA, number of cycle and addition of Bovine Serum Albumin (0.001% BSA) and Dimethyl sulfoxide (5% DMSO). PCR products were analyzed by 1.0 % agarose gel electrophoresis and visualized after ethidium bromide staining.

PCR purification

The PCR products from were purified using Zymo research DNA Clean & Concentrator™-5 for further DNA ligation, cloning and sequencing. Following the manufacturer's instructions, PCR products were mixed with the binding buffer in a 5:1 ration (binding buffer:sample) and then transferred into Zymo-Spin™ Column with collection tube; after centrifuging for 30 seconds at 13000g, 15 µl of Elution buffer was used to elute the purified DNA from the column. Purified PCR amplified DNA stored at -20 °C.

Library construction using the cloning vector pNZY28

After DNA quantification with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific), the ligation reaction was prepared with a 1: 3 ratio of vector: insert, using 50 ng of pNZY28 and 1-3ng of purified PCR product 1 U of T4 DNA, 1x of binding buffer, and left to incubate overnight at 4 ° C

An aliquot of 100 µl of competent E. coli DH5α (NZYtech) was thawed on ice and transferred into pre-chilled microtube adding 10µL of ligation mix directly into the cells. After stirring gently an incubation for 30 min on ice was carried out. A heat shock at 42 °C for exactly 40 seconds was performed on water bath. Then cells were placed on ice for 2 minutes. 900 µL of SOC medium was added and cells were incubated for 1 hour in the incubator at 37 ° C with shaking at 225 rpm. 100 µL of cells were spread on LB agar plates containing 100 µg/mL ampicillin,40 µg/mL X-gal and 0.1 mM IPTG. The remaining cells were pellet and plated onto another LB agar plates containing same components as before.

Isolation of plasmid DNA and sequencing

In order to sequence different clones from each replicate, the extraction of Plasmid DNA was performed using the Thermo Scientific GeneJET Plasmid Miniprep Kit according to the recommended protocol. Cultures of *E. coli* DH5-alpha were grown overnight in 3.5 ml of LB medium using 100 µg/mL ampicillin. Bacterial culture was harvested by centrifugation at 8000 rpm (6800 x g) for 2 minutes and resuspended in 250 µL of the Resuspension Solution by gently vortexing. 250 µL of the Lysis Solution was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. Then 350 µL of the Neutralization Solution was added and mixed immediately by inverting the tube 4-6 times. Cell debris and chromosomal DNA were pelleted and the supernatant was transferred to the supplied GeneJET spin column by pipetting without disturbing the white precipitate. The column was washed twice by adding 500 µL of Wash Solution I and finally the GeneJET spin column was transferred into a new tube and plasmid DNA was eluted by adding 50 µL of the Elution Buffer to the center of the column membrane. The eluted plasmid DNA was stored at -20 ° C. The size of the cloning vector was confirmed by electrophoresis and positive clones were sent for sequencing using the M13 universal primer (Stabvida).

Phylogeny Based on the 16S rRNA gene partial sequence

Sequencing results were analyzed and edited using BioEdit Sequence Alignment Editor (version 7.2.6.1)(Hall, 1999). Preliminary analysis of edited sequences was performed with BLAST 15 (Altschul, Gish, Miller, Myers, & Lipman, 1990). Alignments were generated using Clustal W (Larkin et al., 2007) using the obtained 16S rRNA gene sequences together with those of the type strains of the following species: *Mesorhizobium albiziae* (DQ100066), *M. amorphae* (AF041442), *M. chacoense* (AJ278249), *M. ciceri* (DQ444456), *M. huakuii* (FJ491264), *M. loti* (X67229), *M. mediterraneum* (AM181745), *M. plurifarium*(Y14158), *M. septentrionale* (AF508207), *M. Temperatum* (AF508208), *M thiogangeticum* (AJ864462), *M. tianshanense* (AF041447), *M. muleiense* (HQ316710), *M. robiniae* (EU849582).
Rhizobium leguminosarum bv. viciae (U29386), *Sinorhizobium meliloti* (X67222) and *Bradyrhizobium japonicum* (U69638) were included as outgroup. MEGA (Molecular Evolutionary Genetics Analysis, version7.0.24) (Tamura, Dudley, Nei, & Kumar, 2007) software was used to infer the molecular phylogeny by the neighbor-joining method based on a distance matrix with the distance correction calculated by Kimura's two parameter model(Kimura, 1980), with 1,000 resamplings in the bootstrap analysis.

Statistical analysis

The data obtained from the chickpea plant growth assay was characterized by analysis of variance, and means were compared by One-way ANOVA, using the SPSS Statistics v.22 software (SPSS Inc., IBM Company). For growth kinetics average value and standard deviation of the three replicas were calculated and time points in stationary phases of all replicates were compared using by T-test, using the SPSS Statistics of the above-mentioned version.

Results

Different assays were conducted in order to evaluate the effect on growth and diversity of chickpea rhizobia of glyphosate and chemical nitrogen sources.

Evaluation of the effects of glyphosate and nitrogen sources on the growth kinetic of different mesorhizobia

The three chickpea mesorhizobia strains selected for evaluation of the effects of glyphosate and nitrogen sources on growth and early host plant interaction were previously described (Alexandre et al., 2009; F. Nascimento et al., 2012). Nevertheless, a new phylogenetic analysis of the three strains LMS-1, PMI-6-Portimão and V-15b-Viseu was performed because 31 new *Mesorhizobium* species have been described (LPSN; http://www.bacterio.net)(Parte, 2017), since 2009. A dendrogram was generated by the neighbour-joining method (NJ) from a 1271 bp long alignment (257 variable sites) of the 16S rRNA gene sequence. According to the 16S rRNA gene molecular phylogeny, PMI-6-Portimão and V-15b-Viseu both group together and with 100% similarity to the *Mesorhizobium japonicum* MAFF 303099 and *Mesorhizobium erdmanii* type strains which were reported to be isolated from nodules of *Lotus corniculatus* (Fig. 7). These two chickpea isolates were previously described as closely related to *Mesorhizobium* huakuii, *Mesorhizobium plurifarium* , *Mesorhizobium amorphae* , and *Mesorhizobium septentrionale* (Alexandre et al., 2009). On the other hand, LMS-1 which was previously described as *M. ciceri*, kept it 100% similarity with *Mesorhizobium ciceri* UPM-Ca7, also isolated from chickpea nodules (F. Nascimento et al., 2012), however a second species is currently grouping in the same cluster also with 100% similarity, namely *Mesorhizobium cantuariense* , isolated from *Sophora microphylla* root nodules (De Meyer, Tan, Heenan, Andrews, & Willems, 2015). Phylogenetic analysis using maximum likelihood methods revealed an identical topology.

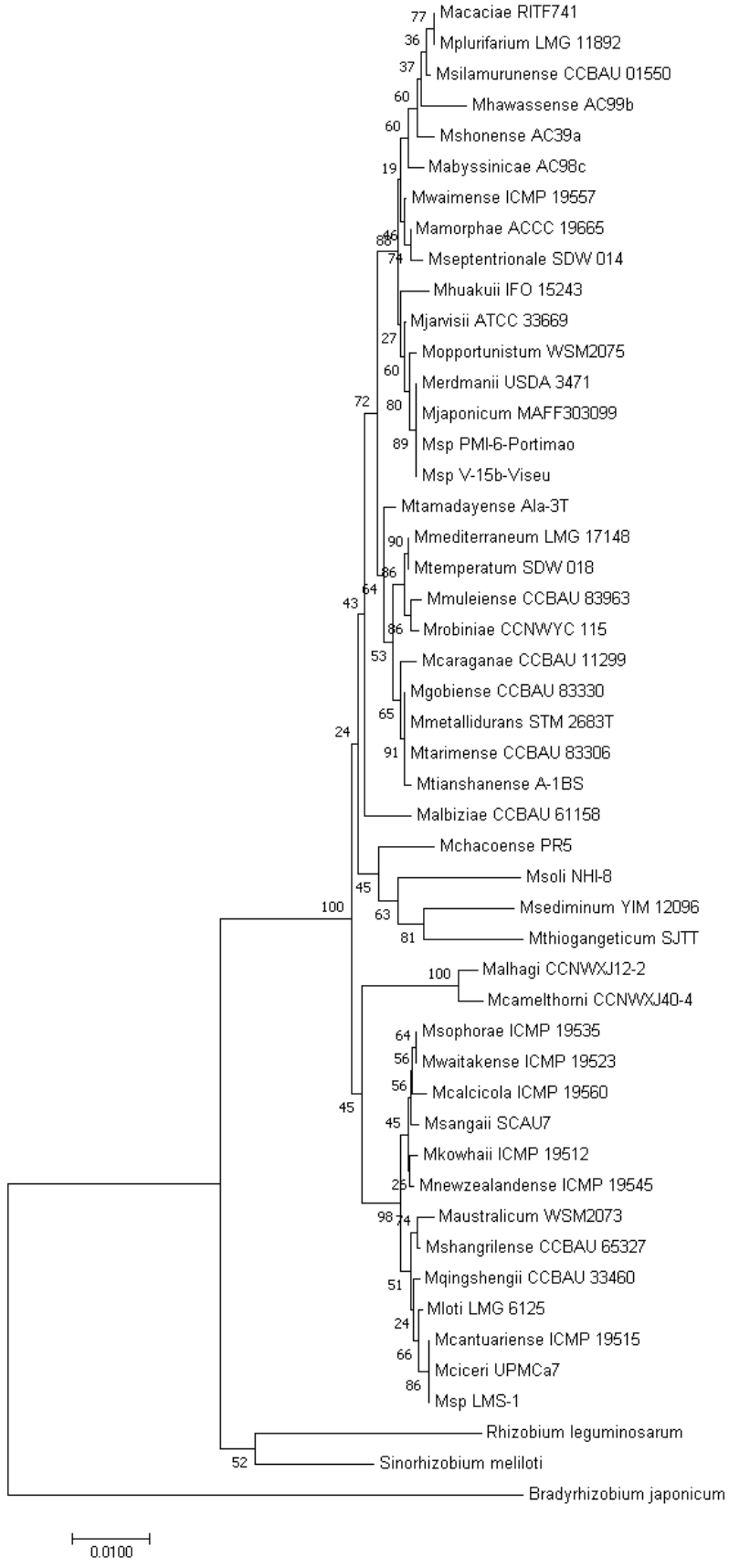

Figure 7: Phylogenetic analysis of chickpea rhizobia isolates and Mesorhizobium type strains, based on 16S rRNA gene sequence (alignment length 1271 bp). Neighbour-joining method was used. Bootstrap values are listed at the nodes. The scale bar indicates 1% substitutions per site.

To assess the effect widely used herbicide and chemical fertilizer in agriculture field, glyphosate and nitrogen sources application were done on rhizobial free-living growth. Three different strain of mesorhizobia were used: LMS-1, PMI-6-Portimão and V-15b-Viseu. Different concentrations of Roundup as Glyphosate source (15.5µL/100mL, 31µL/100mL, 61µL/100mL) as well as different concentrations of KNO_3 (1.37mM, 5mM, 7mM) were used to assess the bacteria capacity to tolerate and/or utilize these compounds.

Figure 8 shows that the different concentrations of glyphosate and nitrogen sources used have no detrimental effect on the growth of LMS-1 in liquid culture. In fact, the growth rate on the exponential phase is faster than control, for all the other treatments tested. Upon stationary phase, after 54 hour, the two lower glyphosate concentrations (15.5µL/100mL, 31µL/100mL) and all nitrate concentrations showed significant ($p < 0.05$) higher OD value than control, in the following three time points. The exception was the highest concentration of glyphosate source, for which the growth curve is very similar to the control conditions.

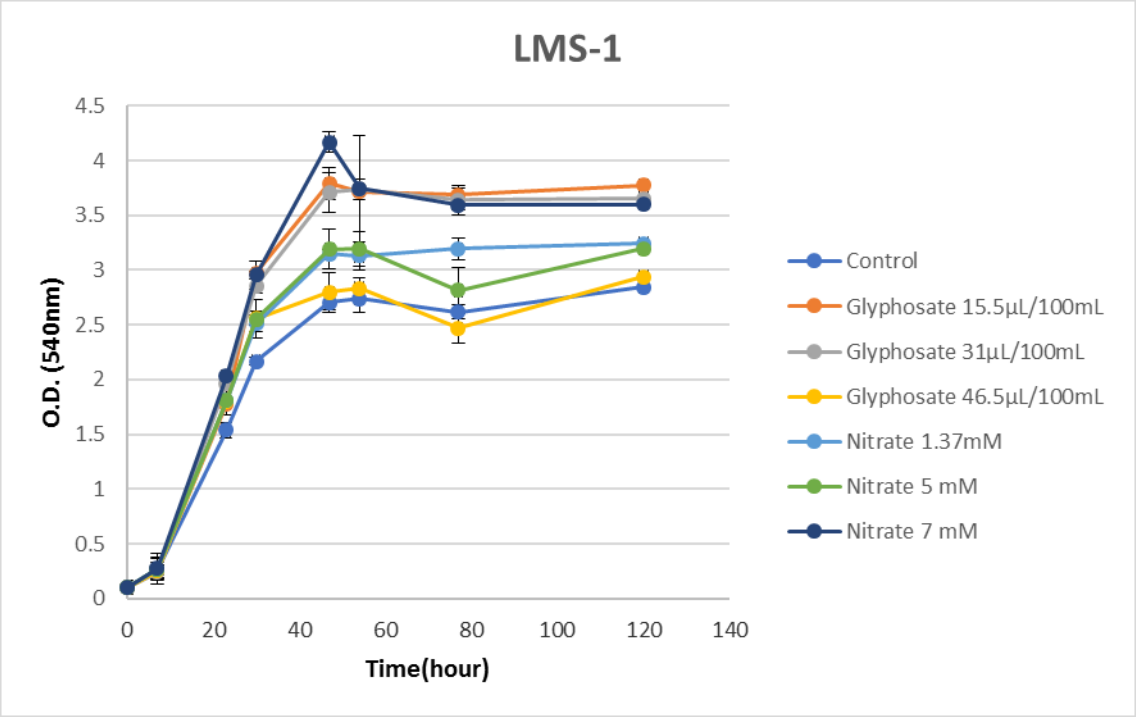

Figure 8: Growth curve of Mesorhizobium ciceri LMS-1 using different glyphosate source (Roundup Ultra) and nitrogen source (KNO_3) concentrations. Bars indicate the standard deviation.

Another mesorhizobium strain, V15-b was grown in liquid culture with the same conditions but unlike LMS-1, the different concentrations of glyphosate and nitrogen sources used have no significantly different effect on the growth of V15-b in liquid culture (Fig.9). In fact, the OD values for both logaritmic phase and stationary phase, for all the treatments tested, showed no significant ($p < 0.05$) difference from control in most of the time points.

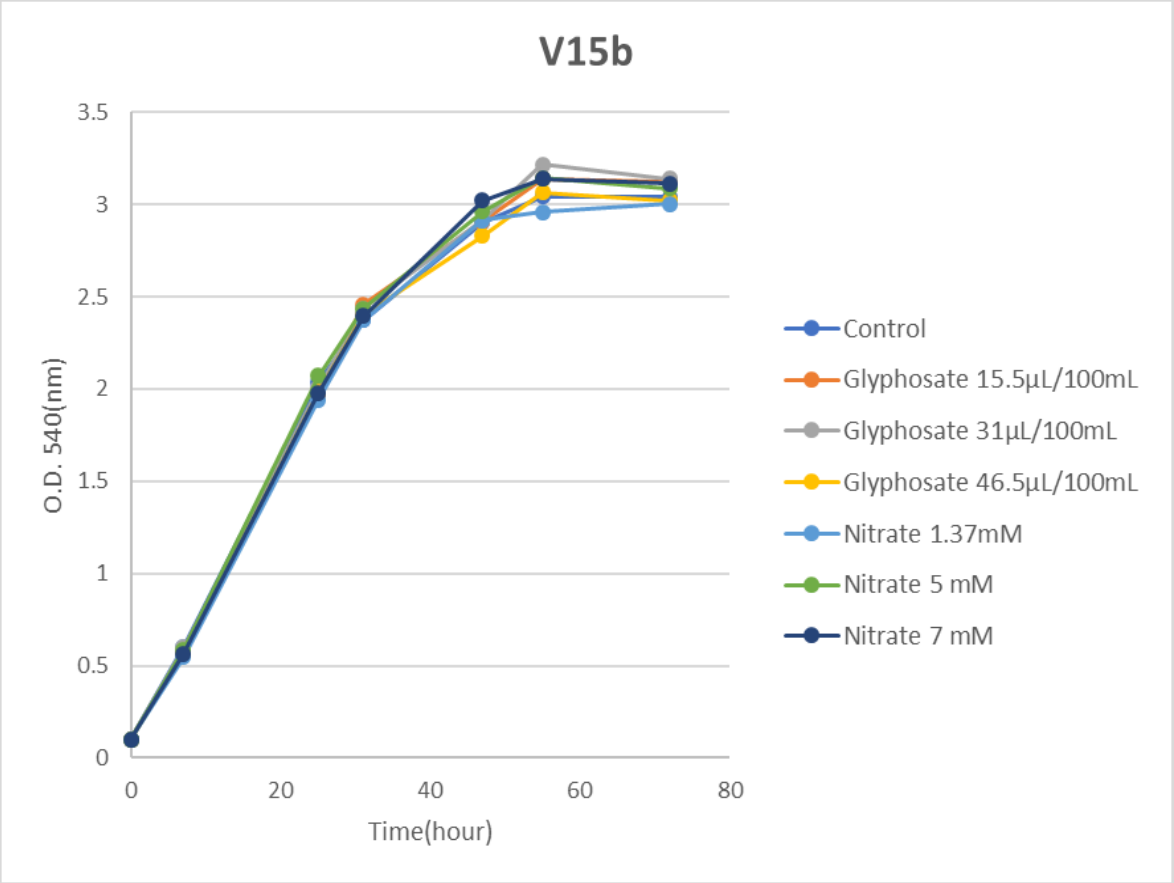

Figure 9: Growth curve of *Mesorhizobium* V15-b using different glyphosate source (Roundup Ultra) and nitrogen source (KNO_3) concentrations**.** *Bars indicate the standard deviation.*

Figure 10 shows that the different concentrations of glyphosate and nitrogen sources used have no detrimental effect on the growth of PMI-6 in liquid culture. In fact, the OD values on the exponential phase and stationary phase have no significant difference ($p < 0.05$) from control.

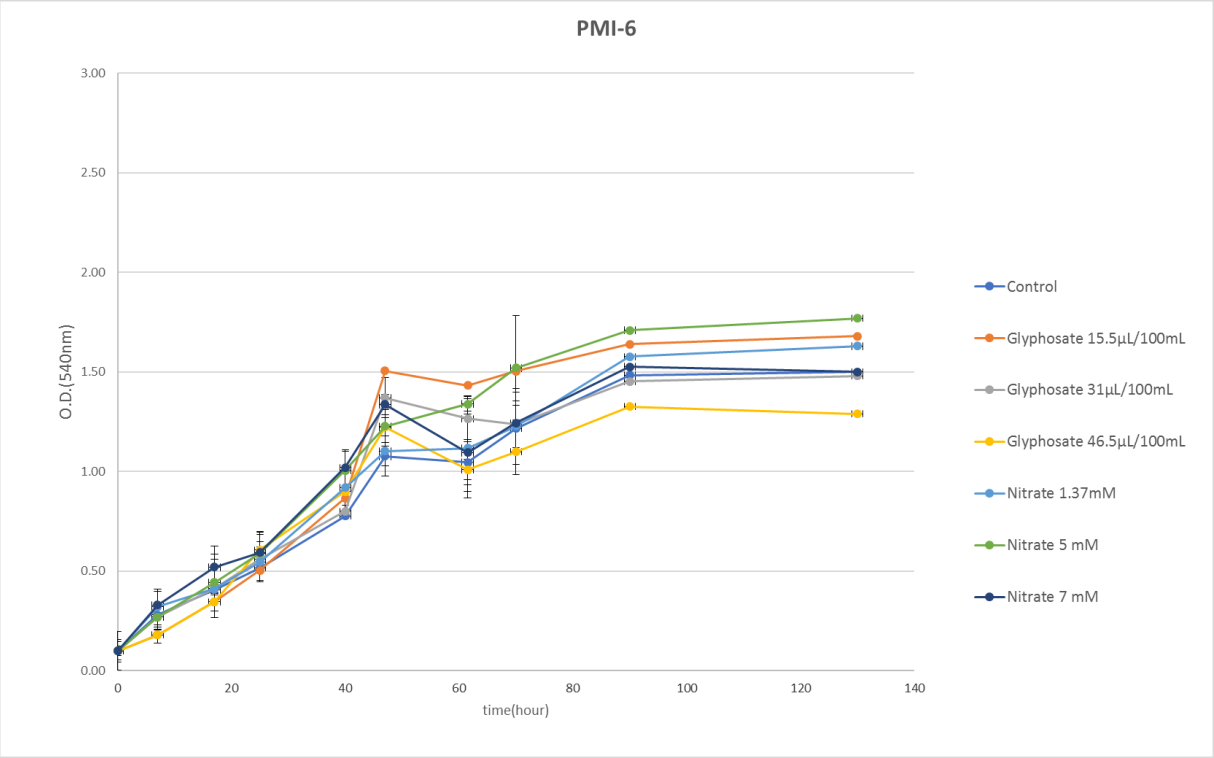

Figure 10: *Growth curve of PMI-6 using different glyphosate source (Roundup Ultra) and nitrogen source (KNO_3) concentrations. Bars indicate the standard deviation.*

Overall, the growth curves of the three mesorhizobia strains showed that all the tested amounts of nitrate and glyphosate sources did not cause any growth arrested or a slower growth rate. The OD values for strain LMS-1 were even higher than control on the stationary phase in the presence of glyphosate and additional nitrogen sources.

Analysis of the effect of different concentrations of chemical nitrogen source on inoculated chickpea root development

To study the effect of nitrogen sources on the early steps of the infection of chickpea (*Cicer* arietinum) roots, confocal microscopy analysis was performed using three different *Mesorhizobium* strains, namely LMS-1, PMI-6 and V15-b. Different concentrations of Potassium Nitrate (nitrogen chemical source) were used on plates, using replicates per treatment.

Different concentrations of KNO_3 were applied on chickpea roots along with GFP-tagged *Mesorhizobium* PMI-6 and evaluated on 5th day after inoculation. Inoculated roots where 1.37mM of nitrogen applied source showed, secondary roots and root hairs are bigger than other concentration applied . When the nitrogen source amount was increased to 5mM the roots structure seemed to have stunted growth and very less secondary roots with root hair, in comparison with the control conditions (fig. 11). To take the nitrogen effect study further, application of 7mM of nitrigen source in the media was done. For this treatment, the root hairs showed no curling or infection; only very few root hairs were observed.

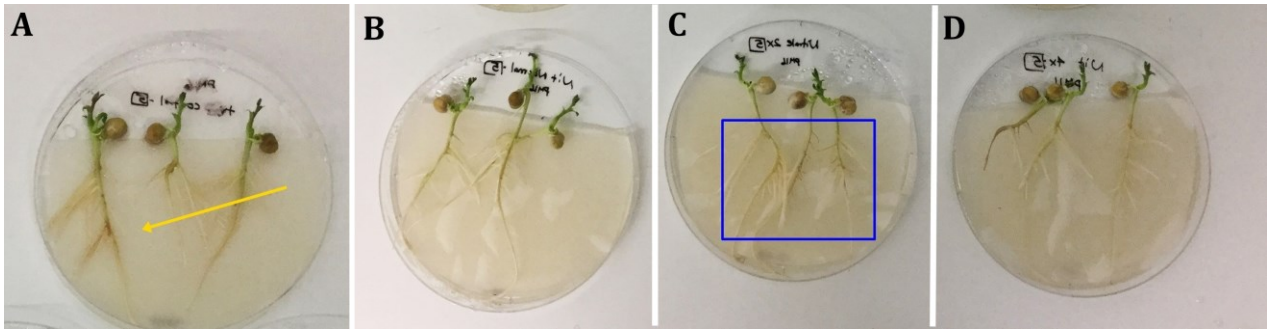

Figure 11: Chickpe seedlings growth after inoculating with GFP-tagged PMI-6. GFP-tagged PMI-6 with (A) with no treatment , used as control. Root growth (*Yellow arrow)* (B) 1.37mM **KNO_3**, (C) 5mM **KNO_3** Secondary. Root growth (Blue rectangle) (D) 7mM **KNO_3**.

Effect of different concentration of nitrogen source (KNO_3) on Infection process

The effects of different levels of nitrogen source (KNO_3) on the early steps of host infection were analyzed. Observations were made on 3rd, 4th and 5th day after inoculation of the three different rhizobial strains previously modified with a plasmid encoding the *gfp* gene (LMS-1, PMI-6 and v15-b). In the control condition, curling of root hairs were observed as well as GFP-tagged mesorhizobia initiating the infection thread, namely LMS-1 (Fig 12 A), PMI-6 (Fig 12 B) and V15b (Fig 12 C).

The treatment with 1.37mM nitrogen source (KNO_3) that corresponds to 50kg/hectare, application showed apparently better growth of root hair, and bacteria were seen to start the process of curling (fig 12 D,E and F). Upon 5 mM KNO_3 application, less root hairs were observed, but the bacteria could be still seen attached to the root hairs (Fig. 12 G, H, I). For PMI-6, 5 mM of nitrogen source had a more detrimental effect than on other two strains (fig. 12 H). When higher amount of nitrate was applied, less GFP-tagged bacteria were detected inside the root hairs and less secondary roots were observed (Fig.12 J, K, L). But different strains, were differently affected: V15-b seemed to be perform worse than the other strains on 7mM nitrate application (fig 12 L); roots were populated with PMI-6, but there were less amount of infection threads (fig. 12 K).

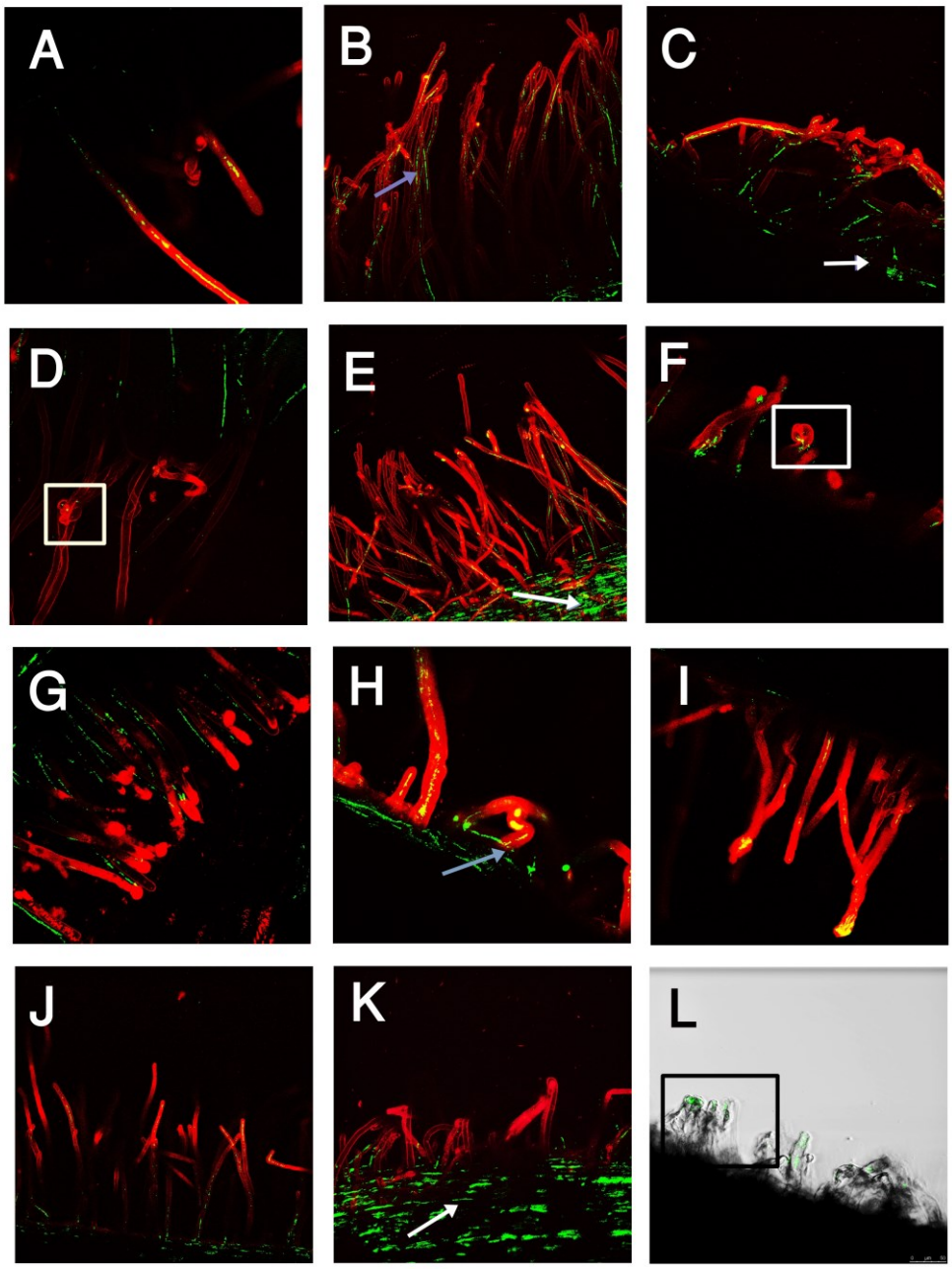

Figure 12: Effect of different concentration of nitrogen chemical source (KNO3) on the mesorhizobia infection process of chickpea. Infection thread development visualized by confocal microscopy after inoculation of chickpea seedlings with mesorhizobia strains (LMS-1, PMI-6-Portimão and V-15b-Viseu) tagged with GFP. Roots were stained with propidium iodide. Control condition inoculated with (A) GFP-tagged LMS-1 (B) GFP-tagged PMI-6-Portimão (C) GFP-tagged V-15b-Viseu. 1.37 mM Nitrate source applied (KNO3) applied on chickpea roots inoculated with (D) GFP-tagged LMS-1 (E) GFP-tagged PMI-6-Portimão (F) GFP-tagged V-15b-Viseu. 5mM Nitrate source (KNO3) applied on chickpea roots inoculated with (G) GFP-tagged

LMS-1 (H) GFP-tagged PMI-6-Portimão (I) GFP-tagged V-15b-Viseu. 7mM nitrogen source (KNO_3) applied on chickpea roots inoculated with (J) GFP-tagged LMS-1 (K) GFP-tagged PMI-6-Portimão (L) GFP-tagged V-15b-Viseu. Infection threads on root hair (blue arrows) Caps on root hair tips (white square box), rhizobial attachment on root (white arrow), roots with stunted growth and rhizobial attachment (black box) are indicated. Scale bars: A,C,D,I; 100 µm: B,E,G: 130 µm; F,H,I,J: 75 µm; K,L: 50 µm

Plant growth assay to evaluate shoot weight, nodule number and histology

In order to the evaluate the effects of glyphosate and nitrogen sources application as well as rhizobial inoculation in plant growth, 6 replicates from each of the four treatments were collected after 8 weeks of growth on the greenhouse. Upon visual inspection, there were no clear differences among plants from different treatments (Fig.13).

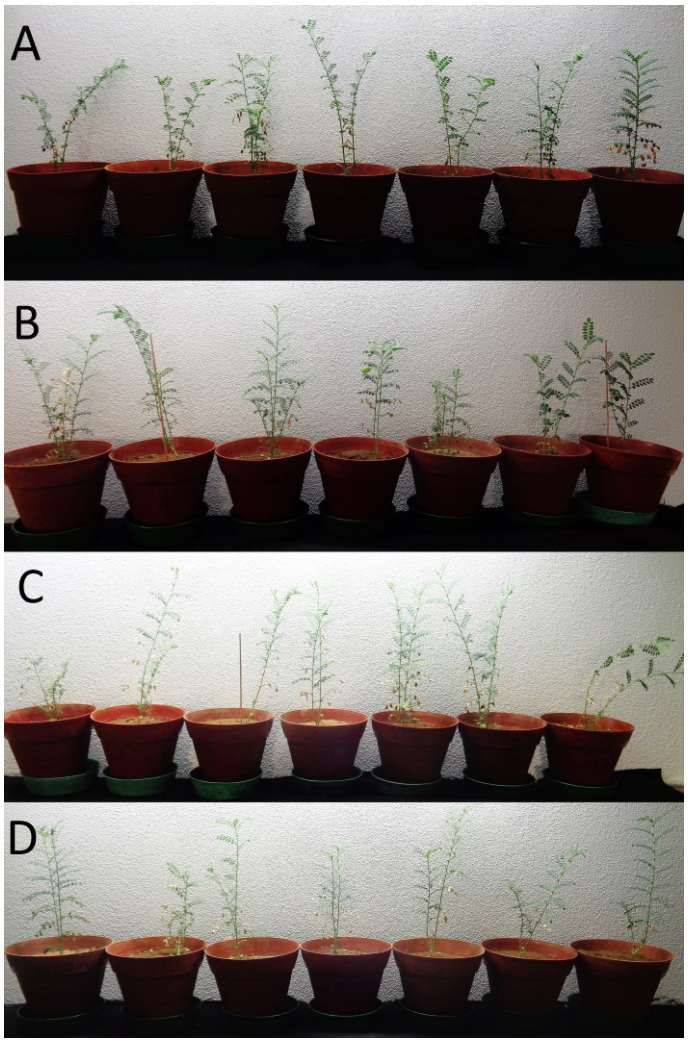

Figure 13: Chickpea trial on the 7th week. A) Control B) LMS-1 inoculation. C) KNO_3 as Nitrogen source (50kg/hectare). D) Roundup Ultramax as Glyphosate source (2L/hectare).

Several plant parameters were evaluated as shoot dry weight (SDW) (Fig. 14) and number of nodules (Fig. 15). There were significant differences observed in SDW between glyphosate and nitrate treated plants ($p < 0.05$). Plants from the Glyphosate application treatment showed the lowest SDW, while plants from the nitrate application treatment showed the highest SDW. Nevertheless, none of the treatments were statistically different from the control conditions.

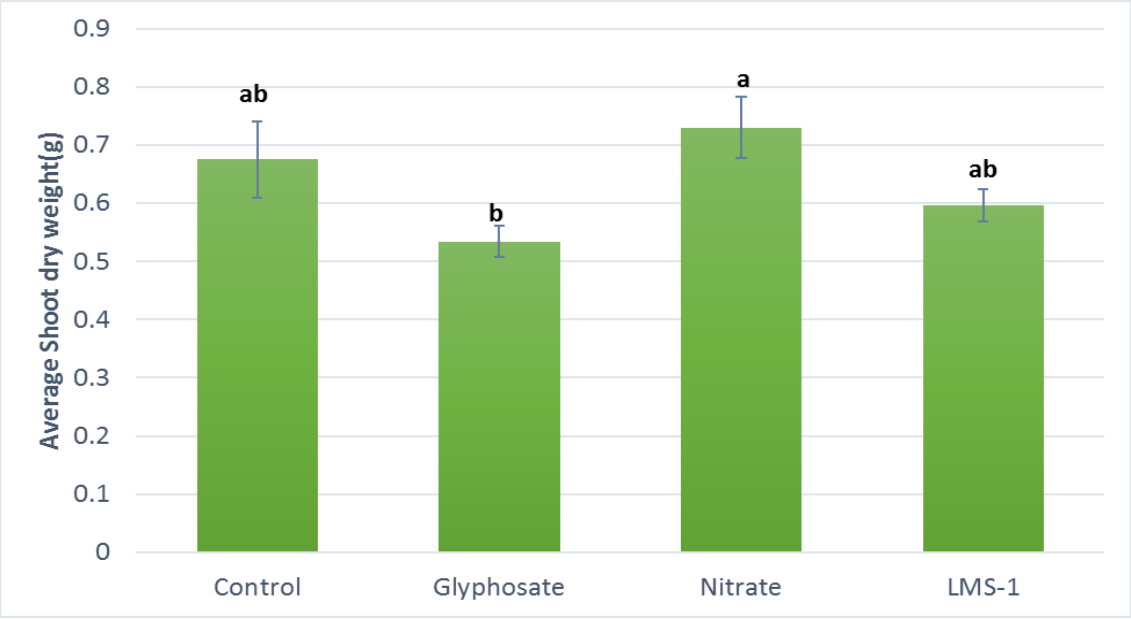

Figure 14: Average shoot dry weight (SDW) of 8-week-old chickpea plants grown with different soil treatments: control conditions, Glyphosate application, Nitrate application and inoculation of LMS-1. Means and standard error result from 6 replicates for each treatment. The letters(a,b) denote statistical differences for $p < 0.05$, detected using ANOVA and the post hoc Tukey test, performed in SPSS V.21 software (SPP Inc., Chicago, U.S.A). Bars indicate standard error.

In terms of the number of nodules observed for the plants from the same treatments, there were no significant differences detected ($p < 0.05$). Nevertheless, the chickpea plants from the nitrate application treatment showed lower number of nodules, compared to the remaining treatments (fig 15).

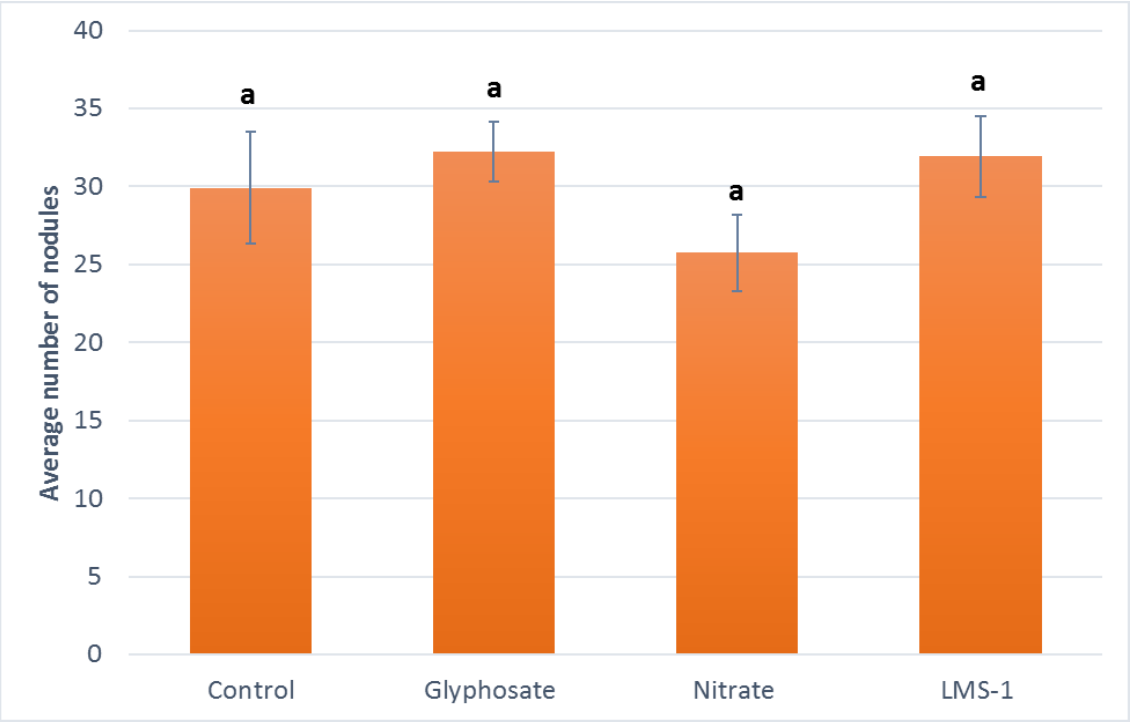

Figure 15: Nodule number of chickpea plants of 8-week-old chickpea plants grown with different soil treatments: control conditions, Glyphosate application, Nitrate application and inoculation of LMS-1. Means and standard error result from 6 replicates for each treatment. ANOVA and the post hoc Tukey test were performed in SPSS V.21 software (SPP Inc., Chicago, U.S.A), letter(a) indicate no statistical difference ($p < 0.05$). Bars indicate standard error.

Histological analysis of nodules

Nodules were collected from chickpea plants grown in soil, from the four different treatments used to evaluate plant growth (as described above). To investigate potential differences in the nodule formation and development induced by these treatments, nodules were collected from 8-week-old chickpea plants. Light microscope photos showed sequential zones of development in nodules from all treatments (Fig. 16).

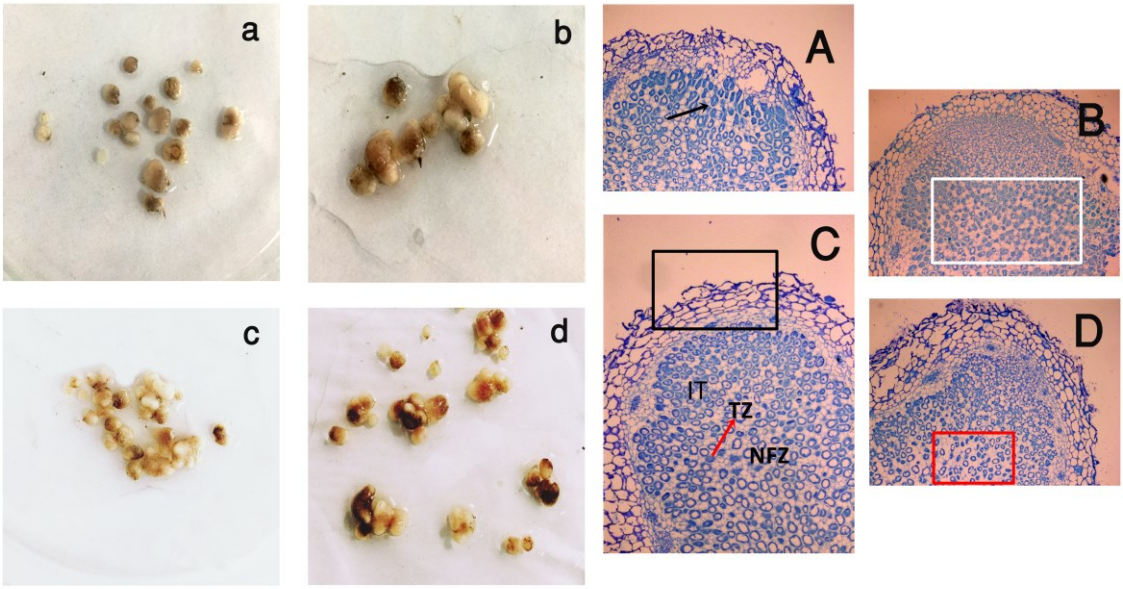

Figure 16: Nodules from chickpea collected from 8-week-old plants a) control conditions. b) LMS-1 inoculation. c) nitrogen source (50kg/hectare). d) glyphosate source (2L/hectare).Nodule zonation are indicated by the capital A-D. A) Infection Zone (black arrow) on nodules taken from chickpea grown in control condition. B) nodules from the LMS-1 inoculated treatment; infection zone is indicated by white rectangle box C) nodules from nitrogen source application treatment, black rectangle indicates the Meristem, IT= Infection zone. Red arrow is zone transition between infection and fixation zone. D) nodules from the Glyphosate source application treatment. Red rectangle indicates the area with many non infected cells.

Histological sections of several nodules from each treatment were compared using bright field microscopy. All the nodules observed showed the expected structure of indeterminate nodules, namely meristematic, infection, fixation and senescent zones. The main differences observed between the nodules were in the fixation zone. In control conditions, the fixation zone of nodules as well as bacteroid organization within the cortical cells was well defined and infected cortical cell and bacteria released from

the infection threads into the root cortical cells were evident (fig. 17 A). Nodules from the treatment that included LMS-1 inoculation were similar to the control ones (fig. 17 B). For nodules collected from soil treated with a nitrogen source (KNO_3 was applied in concentration of 50kg/hectare), many cells in the fixation zone had differentiated bacteroids, which are actively fixing nitrogen, but in less number than control conditions (fig.17 C.) For the glyphosate source treatment (Roundup Ultra at an amount of 2L/hectare), a higher number of uninfected cells was observed, compared to other treatments. These qualitative microscopy results confirm that the area occupied by bacteriods in the infected cells is smaller in nodules from the glyphosate source treatment than in any of the other three treatments.

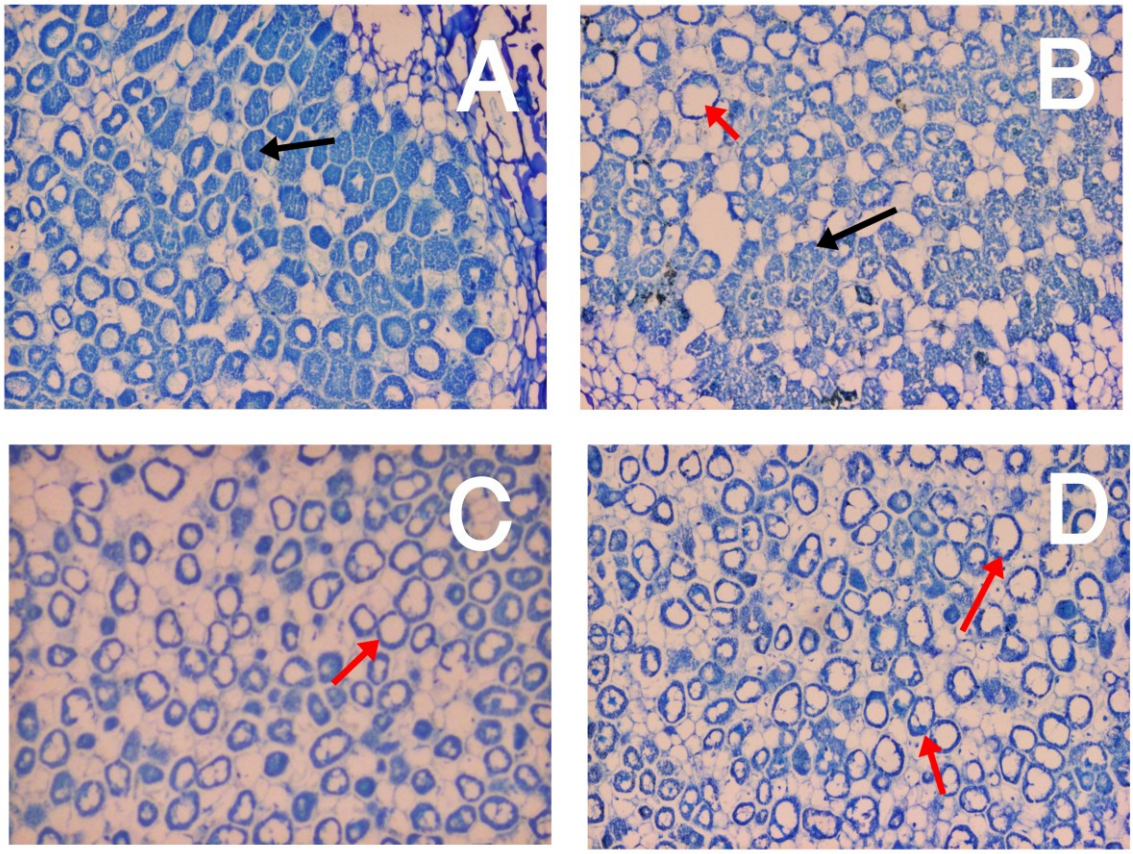

Figure 17: Bright field micrographs of nodule sections from chickpea plants grown in soil with different treatments. The fixation zone is shown in nodules stained with Toluidine Blue. (A) Nodules from plants grown in control conditions. (B) Nodules collected from plants treated with LMS-1 inoculation.(C) Nodule collected from soil treated with KNO_3 (50kg/hectare). Bacteroid differentiation (red arrow) (D) Nodules from soil treated with 2L/hectare of glyphosate source (Roundup Ultra) Cells infected with bacteroids (black arrows) and uninfected cells (red arrows)´. Scale bars: A,B,D: 200 µm; C: 50 µm

Analysis of rhizobia diversity from chickpea nodules

In order to evaluate the effect of rhizobia inoculation and of application of glyphosate and nitrate sources on the diversity of rhizobia in chickpea nodules, 16S rRNA gene sequences were obtained from the nodules of chickpea plants grown in the four different soil treatments mentioned before. The phylogenetic analysis of partial 16S rRNA sequence showed that all the sequences grouped within the genus *Mezorhizobium*. This phylogenetic analysis included the obtained 77 sequences and 43 known *Mesorhizobium* species to assess the diversity of mesorhizobia nodulating chickpea (Parte, 2017). The phylogeny based on the 16S rRNA gene (Fig 18) shows that all the sequences amplified from chickpea nodules group in a single cluster, which includes the type strain *Mesorhizobium muleiense CCBAU 83963*. This low diversity found among all the sequences amplified indicates that no effect on the diversity of the mesorhizobia nodulating chickpea was detected for the treatments analyzed. In addition, contrary to what was expected, no sequence compatible with the strain LMS-1 was obtained from nodules from the treatment where this strain was inoculated. All sequences showed a high similarity with type strain of *M. muleiense*, which was originally isolated from chickpea (Zhang et al., 2012).

Other closely related species to the cluster containing the nodules sequences is *Mesorhizobium robiniae* CCNWYC 115, which was originally isolated from *Robinia pseudoacacia*, a tree originally from North America. Despite this low diversity found in nodules from all the treatments, few isolates (e.g. C1-13, C3-8, N1-1) showed 100% identical to *Mesorhizobium muleiense* CCBAU 83963. Phylogenetic analysis using maximum likelihood methods also revealed an identical topology (data not shown).

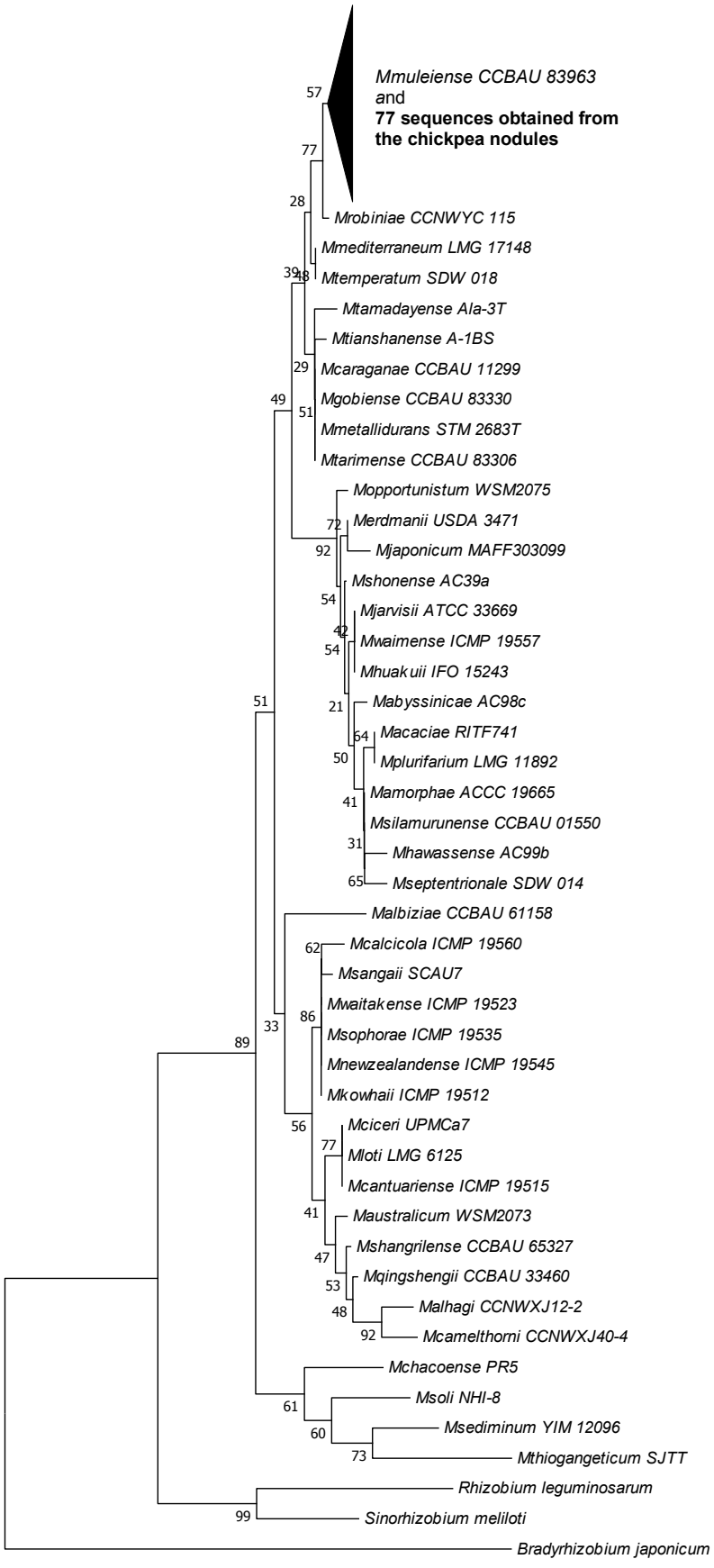

Fig 18: Phylogenetic analysis, based on partial 16S rRNA gene sequences obtained from chickpea nodules (alignment length 712 bp). The neighbor-joining tree is based on a distance matrix with the distance correction calculated by Kimura's two-parameter nucleotide substitution model (Kimura, 1980), with a discrete Gamma distribution. Bootstraps values are listed at the nodes. The 77 sequences indicated in the black triangle were obtained from four different treatments, namely 21 sequences from Control, 18 sequences from LMS-1 inoculation, 21 se-quences from nitrogen source application, 17 sequences from glyphosate source application. The scale bar indicates 1% substitutions per site.

Discussion

To understand the influence of widely used agricultural products and to assess its impact on native mesorhizobia population several experiments were conducted. The application of nitrogen fertilizers is a standard agricultural practice, since this macro-nutrient is often a limited factor of plant growth. Another common application in agriculture is the spread of herbicides, namely glyphosate, which is a broad-spectrum systemic herbicide used to control weed. Despite the generalized used of these agricultural practices, their influence on rhizobia-legume symbiosis still requires further studies.

In order to evaluate the effect of the presence of glyphosate and nitrogen sources on rhizobial growth, bacterial growth kinetics assays were carried out using mesorhizobia strains LMS-1, PMI-6-Portimão and V-15b-Viseu and showed that different strains may respond differently to the presence of both glyphosate and nitrate sources. For example, LMS-1 reached significantly higher OD values at stationary phase for all the concentration of KNO_3 used as well as for the two lowest concentration of glyphosate source tested, when compared to control conditions. Even the highest concentration of glyphosate source used was not detrimental for LMS-1 growth (which was similar to the control conditions). These results suggest that LMS-1 is able to degrade glyphosate and use it, which is in agreement with previous findings that reported the short term effects on growth which might be due to a rapid metabolization of glyphosate (Mijangos, Becerril, Albizu, Epelde, & Garbisu, 2009; Ratcliff, Busse, & Shestak, 2006). For the other two chickpea mesorhizobia strains tested (PMI-6 and V-15b), no effect on bacterial growth curve was detected. Contrary to these findings, other studies observed a reduction in bacterial growth in root exudates from glyphosate-treated soybean plants (Kremer, Means, & Kim, 2005). However, there were reports that did not find any negative effects on numbers of bacteria, fungi and actinomycetes, when glyphosate was applied at recommended rate (Stratton & Stewart, 1992). Further studies are required to validate that the currently recommended amounts of glyphosate and nitrate for field application are not hazardous for environment, particularly under changing soil and climatic conditions.

To assess the effects of same concentration of nitrogen source treatments on the early stages of the infection process, chickpea roots inoculated inoculated with GFP-tagged mesorhizobia were analysed by confocal microscopy on 3^{rd},4^{th} and 5^{th} day after inoculation. The results from this analysis suggested that the ability of rhizobia to initiate the infection was affected by the treatments and concentration of nitrogen fertilizer used. The impaired in attachment gets higher in frequency. For the three GFP-tagged rhizobial strains used, the treatment with nitrogen source (KNO_3), application of 1.37mM that corresponds to 50kg/hectare showed apparently better growth of root hair, and bacteria were seen to start the process of curling in three strains tested, PMI-6 root hairs were more and bacteria poputated the roots, some started the process of initiating infection thread. Upon 5 mM KNO_3 application, although PMI-6 root hairs got affected higher than other two, less root hairs were observed overall for all, but

the bacteria could be still seen attached to the root hairs. For PMI-6. With 7mM which was the highest amount of nitrogen source applied, less GFP-tagged bacteria were detected inside the root hairs and v-15b showed worst performance and detrimental effect on secondary roots were observed. However, with the higher concentration of KNO_3 used, different strains responded differently. Nitrate in small amount can be beneficiary to rhizobia infection, but higher amount use is seen to be deleterious for nature and also for rhizobia symbiosis. So for inoculation of rhizobia on agricultural fields, study of the effect of applied nitrogen on specific strains can bring out a balanced solution in nitrogen fertilizer application.

In order to evaluate the impact of different treatments commonly used by farmers on the plant biomass, the present study used soil from an agricultural field of Portugal named Herdade da Tramagueira, in Beja where chickpeas were grown previously and four treatments were performed: control, glyphosate source application, potassium nitrate application and LMS-1 inoculation. Chickpea pre-germinated were sown on pot kept for 8 weeks on the greenhouse. The analysis of the average of shoot dry weight (SDW) of chickpea plants grown in these conditions showed that the SDW is higher for the chickpea plants from the nitrate source treatment, where the soil was supplemented with 50kg nitrate/hectare. Plants likely benefited from the supplemented nitrate, in addition to the nitrogen provided by rhizobia symbiosis. Significant inhibition of nodulation and nodule senescence was reported to be occurred at 3–10 mM nitrate in clover, but in this work 1.37 mM nitrate were applied on chickpea which did not had significant impact on nodulation inhibition (Carroll & Gresshoff, 1983). On the other hand, the lowest of shoot dry weight obtained corresponds to chickpea plants grown in the presence of glyphosate source and, furthermore the difference between the average SDW of nitrate and glyphosate treatments is statistically significant ($p<0.05$) (SDW was 21% lower in glyphosate treatment compared to control and 27% lower compared to nitrogen treatment). One possible explanation for this is that the presence of glyphosate could cause reduced activity of chickpea rhizobia due to this herbicide. The SDW values obtained in control conditions were very similar to the ones corresponding to LMS-1 inoculation.

Number of nodules formed in chickpea plants from different treatments showed no significant differences, showing that the lower shoot dry weight obtained for the treatment where glyphosate was applied was not due to a lower number of nodules. This supports our hypothesis that the lower plant biomass obtained in the glyphosate treatment was probably a result of a less efficient nitrogen fixation, as suggested by a less dense plant cell colonization observed in the histological analysis of nodules from the glyphosate treatment, compared to control.

From the histological nodule sections analysis, it seems that the treatments like glyphosate and nitrate application have affected the nodule fixation zone and there are more uninfected cells than in control and LMS-1 inoculation treatment. This analysis also showed that the area occupied by bacteriods is smaller in glyphosate treated nodules than all the three other treatments.

Previous studies have also reported a negative effect of glyphosate application in legume crops. In glyphosate susceptible soybean (*Glycine max* [L.] Merr.) varieties, a single application of 0.28

kg/ha glyphosate reduced chlorophyll content (49%), and shoot and root dry weight (50 and 57%, respectively) at 2 weeks after treatment (Reddy, Hoagland, & Zablotowicz, 2001). Using soybean plants resistant to glyphosate, application of 1.12 kg/ha of glyphosate, followed by sequential applications at 0.56 or 1.12 kg/ha, did not affect plant growth and chlorophyll content, but 2.24 kg/ha of glyphosate reduced these parameters in three of five trials (Reddy et al., 2001). In relation to rhizobia, negative effects of glyphosate application on soybean nodulation and N_2 fixation has been previously reported. Application of glyphosate generally delayed N_2 fixation and decreased biomass and N accumulation in the cultivar Terral TV5866RR (TV5866RR) harvested at 19 d after emergence (DAE), but plants had recovered by 40 DAE (King, Purcell, & Vories, 2001).

The evaluation of rhizobia diversity on chickpea nodules from plants grown on soil, using the treatments mentioned above, was carried out using a 16S rRNA gene sequence analysis. A culture-independent approach was used, since PCR amplification was performed using as template the DNA from nodules. The phylogenetic analysis showed that all sequences obtained from nodules grouped with *Mesorhizobium muleiense,* which is a species previously isolated from chickpea (*Cicer arietinum* L.)(Zhang et al., 2012). This result indicates, not only that no effect from nitrate or glyphosate source application was detected on the diversity of chickpea rhizobia able to induce nodule formation, but also that LMS-1 inoculation was not successful in terms of nodulation. Despite inoculating the chickpea seeds with LMS-1, the 18 clones analysed from this treatment grouped with *M. muleiense* CCBAU 83963. In the 16S rRNA-based phylogeny, LMS-1 groups closer to *M. ciceri* and *M. cantuariense*, which would allow its clear distinction from *M. muleiense*. LMS-1 inoculation seems to have been mostly ineffective, i. e., LMS-1 induced the formation of few or even no nodules and this probably accounts for the fact that no difference in terms of plant growth was detected between control and LMS-1 inoculation treatments. The fact that no sequences representing LMS-1 were retrieved from its corresponding inoculation treatment, may be due to higher competitiveness of *Mesorhizobium muleiense* strains. These results also suggest the higher competitiveness of *M. muleiense* over other chickpea rhizobia eventually present in the soil. Previous studies reported that *M. muleiense* was more competitive than *M. mediterraneum* or *M. ciceri* in non-sterilized soils. *M. muleiense* was the predominant nodule occupier (Zhang et al., 2014). *Mesorhizobium muleiense* CCBAU 83963 was described to have the ability to nodulate chickpea only and not other legumes, after a cross-nodulation test that was done on other legumes like *Medicago truncatula, Trifoliium pretense, Pisum sativum, Vicia faba, Phaseolus vulgaris, Astragalus propinquus, Glycine max or Vigna aconitifolia* (Zhang et al., 2012). Another study reported that the predominant genotypes of *M. muleiense* had changed significantly, so natural or adapting evolution of *M. muleiense* was occurring in fields subjected to changing environmental factors (Zhang et al., 2014). In addition, the biogeography and symbiotic associations of rhizobia with their host legumes were also influenced by biological factors in the soil, such as indigenous rhizobia and other organisms and its capability of competitive nodulation against the other two exotic species (Zhang et al., 2012). In the mixed inoculations of all three strains, *M. muleiense* occupied 100% of the nodules in most of the treatments, except in the one soil sample (Zhang

et al., 2014).

The symbiotic genes *nodC* and *nifH* from *M. muleiense* CCBAU 83963 share a high similarity with those from *M. mediterraneum* UPM-Ca36 and *M. ciceri* UPM-Ca7, which were also isolated from chickpea nodules, but they have different geographic origins (Laranjo et al., 2008). *M. muleiense* has been isolated and found only in alkaline soils of Xinjiang, China, whereas the other two strains, *M. mediterraneum* and *M. ciceri* have been found in the Mediterranean and India (Nour et al., 1994; Zhang et al., 2012)

The diversity found in the present study probably does not reflect the diversity in the soil, so only rhizobia that successfully invaded and colonized the plant host will be represented in our approach, i. e., only the strains from the soil population that are able to accomplish a successful symbiosis with chickpea plants. The low diversity of chickpea rhizobia found in nodules may also be related to a history of chickpea cultivation on the sampled sites, as shown by several studies that suggest a decrease in rhizobia diversity associated with the presence of the host plant (Coutinho et al., 1999). A previous national survey using soils mostly with no history of chickpea cultivation suggested a high diversity of chickpea rhizobia species in Portugal, yet the two *Mesorhizobium* species closely related to the sequences obtained in the present study (*Mesorhizobium muleiense CCBAU 83963 and Mesorhizobium robiniae* CCNWYC 115) were not were not described at that time (Alexandre et al., 2009). A previous study suggested that the non-existence of chickpea wild relatives in Portugal can be another reason for the high diversity rhizobia found in the soil (Talavera & Castroviejo, 2000).

Furthermore, there are no records of the use of commercial inoculants that could reduce the natural chickpea rhizobia diversity (Alexandre et al., 2009). Interestingly, isolates collected from the single site where chickpea has been cultivated (Elvas) grouped with *M. ciceri* or *M. mediterraneum (Alexandre et al., 2009)* showing lower diversity than other sites sampled in the same study. Description of new species might change isolates affiliation based on the 16S rRNA gene sequence analysis, as there is probably a large magnitude of rhizobia species yet to be described.

To study the impact of agricultural practices on plant symbionts is essential for understanding the factors that modulate rhizobia populations diversity and effectiveness. Previous studies were performed to evaluate the influence of glyphosate, on the viability of arbuscular mycorrhizal fungi (AMF), rhizobium and other ecosystem traits in native grasslands (Druille, Cabello, Parisi, Golluscio, & Omacini, 2015). These studies have found that four year late-summer glyphosate application in pampean grassland reduced viability of root-symbiont propagules which are mentioned as 10-fold reduction of rhizobium density and reduction of arbuscular mycorrhizal fungal species, compared to untreated soils (Druille et al., 2015). The worldwide use of glyphosate and nitrogen fertilizer requires extensive and conclusive research to understand the effects observed from the usage of different soil treatments that modulate plant community productivity and diversity. These studies will help understanding the effects of glyphosate on non-target species and designing sustainable land management systems.

Conclusion

This study sought to explore the impact of common agricultural practices, as fertilizer and herbicide application, on rhizobia-legume symbiosis. Experiments conducted in both greenhouse condition and in laboratory had provided some data on how different concentrations of glyphosate and nitrate source can influence nodule occupation and biomass of chickpea plants. In terms of diversity, all the 77 sequences collected from different treatments showed the presence of same *M. muleiense*-affiliated mesorhizobia. Surprisingly, the finding was same for the 18 sequences resulting from nodules collected from the LMS-1 inoculation treatment and no single 16S rRNA gene sequence corresponding to LMS-1 was recovered from that treatment (which would be *M. ciceri*/*M. cantuariense*-affiliated). This phenomena indicates the importance of the competitiveness of native mesorhizobia that will overcome any other mesorhizobia species in terms of nodulation. Nevertheless, the nodules histological analysis showed effects of glyphosate and nitrate application on nodules, with less bacteriod occupation and more non-infected cells observed for these treatment, when compared with control and LMS-1 inoculation. This seems to give an indication of how using soil treatments can influence the nitrogen-fixation capacity. The infection thread initiation has been tested with several different concentrations of nitrogen sources that have put perspective of how the higher concentration negatively affect the root hair growth and curling on different rhiozobial strains. On the other hand, in the liquid culture LMS-1 showed significantly better growth than control even with the higher glyphosate and nitrate concentrations. In the soil, the interaction between native rhizobia and its compatible host depends on many biotic and abiotic factors. Further studies to understand the different impact of commercial agricultural products on native soil microorganisms are fundamental to ensure sustainability.

General Conclusion

The evaluation of the diversity of chickpea rhizobia in nodules samples from several treatments using soil from a field used to cultivate chickpea (Herdade da Tramagueira -Beja) showed the predominance of sequences that grouped with the chickpea nodulating species *Mesorhizobium muleiense*. 77 sequences of mesorhizobia from nodules collected from chickpea plants grown with different soil treatments, grouped with *Mesorhizobium muleiense CCBAU 83963,* a species previously described as specific for chickpea. Other closely related species was *Mesorhizobium robiniae*. The detection of *M. muleiense* as the predominant species inside nodules, regardless of the treatment, could be due to this species high competitiveness. Despite LMS-1 inoculation, no LMS-1 similar sequence was recovered, rather the same *M. muleiense* was predominant, which shows its competitiveness in nodule formation in the given soil. The evaluation of the impact of glyphosate on chickpea suggests a decrease in biomass (Shoot Dry Weight) by 21% comparing to the control. The rhizobia-legume symbiosis could be affected by the glyphosate use, as suggested by the histological analysis of nodules, where nodules from plants treated with standard amount of glyphosate (roundup) and nitrate showed more uninfected cells and less bacteroids than the nodules collected from control soil. Evaluation of the effects of glyphosate and nitrogen sources on the growth of different rhizobial strain showed that LMS-1 is probably able to metabolize glyphosate and nitrate, since higher OD values on the stationary phase were detected for most of these treatments. The initiation of the infection process was seen to be affected by higher amount of nitrate but varied effects were seen in different strains. Root hair growth stunted, delayed curling and less amount of rhizobial occupancy on nodules cells were observed when higher amount of nitrogen source was applied. All the different soil treatments resulted in same low diversity of mesorhizobia found on chickpea root nodules. From nodule histological analysis assay, it could be seen that glyphosate and nitrate treated nodules had less bacteroids invasion. In terms of biomass (shoot dry weight), significant differences were only detected between glyphosate and nitrogen source treatments. This work has contributed for a better knowledge of the effects of soil treatments on rhizobia-legume symbiosis found in agro-ecosystems.

References

"Draft U.S. Greenhouse Gas Inventory Report:1990–2014." Retrieved 2 May, 2017, from https://www3.epa.gov/climatechange/ghgemissions/usinventoryreport.html.

(2013). "Global research team decodes genome sequence of 90 chickpea lines." International Crops Research Institute for the Semi-Arid Tropics Retrieved 21 June, 2017, from http://www.icrisat.org/newsroom/news-releases/icrisat-pr-2013-media2.htm.

(WHO), W. H. O. (2015). "IARC Monographs Volume 112: evaluation of

five organophosphate insecticides and herbicides." Retrieved 10/5/17, 2017, from https://www.iarc.fr/en/media-centre/iarcnews/pdf/MonographVolume112.pdf.

Abdel-Wahab, A. and M. Abd-Alla (1995). Effect of different rates of N-fertilizers on nodulation, nodule activities and growth of two field grown cvs. of soybean. Fertilizers and Environment, Springer**:** 89-93.

Abdel-Ghaffar, A. (1989). "Aspects of microbial activities and dinitrogen fixation in Egyptian desert soils." Arid Land Research and Management **3**(2): 281-294.

Adesemoye, A., H. Torbert and J. Kloepper (2009). "Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers." Microbial ecology **58**(4): 921-929.

Ahlawat, I. (2000). "Chickpea." Rathore, PS: Technique and Management of Field Crop Production. Agrobios Publications, Jodhpur, Rajasthan, India: 317-335.

Alexandre, A., C. Brígido, M. Laranjo, S. Rodrigues and S. Oliveira (2009). "Survey of Chickpea Rhizobia Diversity in Portugal Reveals the Predominance of Species Distinct from Mesorhizobium ciceri and Mesorhizobium mediterraneum." Microbial Ecology **58**(4): 930-941.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic local alignment search tool." Journal of molecular biology **215**(3): 403-410.

Andrade, D., P. Murphy and K. Giller (2002). "Effects of liming and legume/cereal cropping on populations of indigenous rhizobia in an acid Brazilian Oxisol." Soil Biology and Biochemistry **34**(4): 477-485.

Antoun, H. and J. Kloepper (2001). Plant growth promoting rhizobacteria. Encyclopedia of Genetics.(Brenner, S. and Miller, JF, Eds.), Academic Press, New York.

Arfaoui, A., A. El Hadrami, Y. Mabrouk, B. Sifi, A. Boudabous, I. El Hadrami, F. Daayf and M. Chérif (2007). "Treatment of chickpea with Rhizobium isolates enhances the expression of phenylpropanoid defense-related genes in response to infection by Fusarium oxysporum f. sp. ciceris." Plant Physiology and Biochemistry **45**(6): 470-479.

Bacon, C. and D. Hinton (2006). "Bacterial endophytes: the endophytic niche, its occupants, and its utility." Plant-associated bacteria: 155-194.

Badri, D. V. and J. M. Vivanco (2009). "Regulation and function of root exudates." Plant, Cell & Environment **32**(6): 666-681.

Bala, A., P. Murphy and K. E. Giller (2001). "Genetic diversity of rhizobia from natural populations varies with the soil dilution sampled." Soil Biology and Biochemistry **33**(6): 841-843.

Balasubramanian, V., B. Alves, M. Aulakh, M. Bekunda, Z. Cai, L. Drinkwater, D. Mugendi, C. van Kessel and O. Oenema (2004). "Crop, environmental, and management factors affecting nitrogen use efficiency." Agriculture and the Nitrogen Cycle, SCOPE **65**: 19-33.

Banks, J. E. (2004). "Divided culture: integrating agriculture and conservation biology." Frontiers in Ecology and the Environment **2**(10): 537–545.

Bashan, Y., L. E. de-Bashan, S. Prabhu and J.-P. Hernandez (2014). "Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013)." Plant and Soil **378**(1-2): 1-33.

Bastida, F., J. Moreno, T. Hernández and C. García (2007). "The long-term effects of the management of a forest soil on its carbon content, microbial biomass and activity under a semi-arid climate." Applied Soil Ecology **37**(1): 53-62.

Batenburg, F., R. Jonker and J. Kijne (1986). "Rhizobium induces marked root hair curling by redirection of tip growth: a computer simulation." Physiologia plantarum **66**(3): 476-480.

Benbrook, C. (2012). "Impacts of genetically engineered crops on pesticide use in the U.S. -- the first sixteen years." Environ Sci Eur **24**.

Benbrook, C. M. (2016). "Trends in glyphosate herbicide use in the United States and globally." Environmental Sciences Europe **28**(1): 3.

Biswas, J. C., J. K. Ladha, F. B. Dazzo, Y. G. Yanni and B. G. Rolfe (2000). "Rhizobial Inoculation Influences Seedling Vigor and Yield of Rice." Agronomy Journal **92**: 880-886.

Brewin, N. J. (1998). Tissue and cell invasion by Rhizobium: the structure and development of infection threads and symbiosomes. The rhizobiaceae, Springer**:** 417-429.

Calvo, P. N., L.M.; Kloepper, J.W. (2014). "Agricultural uses of plant biostimulants." Plant Soil **383**: 3–41.

Carol, R. J. and L. Dolan (2002). "Building a hair: tip growth in Arabidopsis thaliana root hairs." Philosophical Transactions of the Royal Society of London B: Biological Sciences **357**(1422): 815-821.

Cerdeira, A. L., D. L. Gazziero, S. O. Duke and M. B. Matallo (2010). "Agricultural impacts of glyphosate-resistant soybean cultivation in South America." Journal of agricultural and food chemistry **59**(11): 5799-5807.

Choudhary, D. K., K. P. Sharma and R. K. Gaur (2011). "Biotechnological perspectives of microbes in agro-ecosystems." Biotechnology letters **33**(10): 1905-1910.

Cooper, J. (2007). "Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue." Journal of applied microbiology **103**(5): 1355-1365.

Coutinho, H. L., V. M. Oliveira, A. Lovato, A. H. Maia and G. P. Manfio (1999). "Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans." Applied Soil Ecology **13**(2): 159-167.

Dakora, F. and D. Phillips (1996). "Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins." Physiological and Molecular Plant Pathology **49**(1): 1-20.

Dey, R., K. Pal, D. Bhatt and S. Chauhan (2004). "Growth promotion and yield enhancement of peanut (Arachis hypogaea L.) by application of plant growth-promoting rhizobacteria." Microbiological research **159**(4): 371-394.

Djordjevic, M. A., J. W. Redmond, M. Batley and B. G. Rolfe (1987). "Clovers secrete specific phenolic compounds which either stimulate or repress nod gene expression in Rhizobium trifolii." The EMBO journal **6**(5): 1173.

Dobereiner, J. (1992). "History and new perspectives of diazotrophs in association with non-leguminous plants." Symbiosis.

Druille, M., M. N. Cabello, M. Omacini and R. A. Golluscio (2013). "Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi." Applied Soil Ecology **64**: 99-103.

Druille, M., M. N. Cabello, P. G. Parisi, R. Golluscio and M. Omacini (2015). "Glyphosate vulnerability explains changes in root-symbionts propagules viability in pampean grasslands." Agriculture, Ecosystems & Environment **202**: 48-55.

Duarte Maçãs, I. (2003). "Selecção de linhas de grão de bico (Cicer arietinum L.) adaptadas ao ambiente Mediterrânico-critérios morfológicos e fisiológicos." Universidade de Évora, Évora **171**.

Duhan, J. S., R. Kumar, N. Kumar, P. Kaur and K. Nehra (2017). "Nanotechnology: The new perspective in precision agriculture." Biotechnology Reports.

Duke, S. O. (2015). "Perspectives on transgenic, herbicide-resistant crops in the United States almost 20 years after introduction." Pest Manag Sci **71**.

Ferguson, B. J., A. Indrasumunar, S. Hayashi, M. H. Lin, Y. H. Lin, D. E. Reid and P. M. Gresshoff (2010). "Molecular analysis of legume nodule development and autoregulation." Journal of Integrative Plant Biology **52**(1): 61-76.

Fox, J. E., J. Gulledge, E. Engelhaupt, M. E. Burow and J. A. McLachlan (2007). "Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants." Proceedings of the National Academy of Sciences **104**(24): 10282-10287.

Gage, D. J. (2004). "Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes." Microbiology and Molecular Biology Reviews **68**(2): 280-300.

Gaines, T. A., W. Zhang, D. Wang, B. Bukun, S. T. Chisholm, D. L. Shaner, S. J. Nissen, W. L. Patzoldt, P. J. Tranel and A. S. Culpepper (2010). "Gene amplification confers glyphosate resistance in Amaranthus palmeri." Proceedings of the National Academy of Sciences **107**(3): 1029-1034.

Galloway, J. N. T., A.R.; Erisman, J.W.; Bekunda, M.; Cai, Z.; Freney, J.R.; Martinelli, L.A.; and S. P. S. Seitzinger, M.A. (2008). "Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions." Science **320**: 889–892.

García-Fraile, P., L. Carro, M. Robledo, M.-H. Ramírez-Bahena, J.-D. Flores-Félix, M. T. Fernández, P. F. Mateos, R. Rivas, J. M. Igual and E. Martínez-Molina (2012). "Rhizobium promotes non-legumes growth and quality in several production steps: towards a biofertilization of edible raw vegetables healthy for humans." PLoS One **7**(5): e38122.

Gaupp-Berghausen, M., M. Hofer, B. Rewald and J. G. Zaller (2015). "Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations." Scientific reports **5**.

Giongo, A., A. Ambrosini, L. Vargas, J. Freire, M. Bodanese-Zanettini and L. Passaglia (2008). "Evaluation of genetic diversity of bradyrhizobia strains nodulating soybean [Glycine max (L.) Merrill] isolated from South Brazilian fields." applied soil ecology **38**(3): 261-269.

Glick, B. R. (1995). "The enhancement of plant growth by free-living bacteria." Canadian Journal of Microbiology **41**(2): 109-117.

Glick, B. R. (2012). "Plant Growth-Promoting Bacteria: Mechanisms and Applications." Hindawi Publishing Corporation Scientifica: 15.

Graham, P., M. Sadowsky, H. Keyser, Y. Barnet, R. Bradley, J. Cooper, D. De Ley, B. Jarvis, E. Roslycky and B. Strijdom (1991). "Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria." International Journal of Systematic and Evolutionary Microbiology **41**(4): 582-587.

Graham, P. H. and C. P. Vance (2003). "Legumes: importance and constraints to greater use." Plant physiology **131**(3): 872-877.

Gray, E. and D. Smith (2005). "Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes." Soil Biology and Biochemistry **37**(3): 395-412.

Gray EJ, S. D. (2005). "Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes." Soil Biol Biochem. **37**: 395–412.

Grover, M., S. Z. Ali, V. Sandhya, A. Rasul and B. Venkateswarlu (2011). "Role of microorganisms in adaptation of agriculture crops to abiotic stresses." World Journal of Microbiology and Biotechnology **27**(5): 1231-1240.

Hagner, M., S. Hallman, L. Jauhiainen, R. Kemppainen, S. Rämö, K. Tiilikkala and H. Setälä (2015). "Birch (Betula spp.) wood biochar is a potential soil amendment to reduce glyphosate leaching in agricultural soils." Journal of environmental management **164**: 46-52.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series, [London]: Information Retrieval Ltd., c1979-c2000.

Hauggaard-Nielsen, H., P. Ambus and E. S. Jensen (2003). "The comparison of nitrogen use and leaching in sole cropped versus intercropped pea and barley." Nutrient Cycling in Agroecosystems **65**(3): 289-300.

Heidstra, R., R. Geurts, H. Franssen, H. P. Spaink, A. Van Kammen and T. Bisseling (1994). "Root hair deformation activity of nodulation factors and their fate on Vicia sativa." Plant Physiology **105**(3): 787-797.

Helander, M., I. Saloniemi and K. Saikkonen "Glyphosate in northern ecosystems." Trends in Plant Science **17**(10): 569-574.

Hepler, P. K., L. Vidali and A. Y. Cheung (2001). "Polarized cell growth in higher plants." Annual review of cell and developmental biology **17**(1): 159-187.

Herridge, D. F., M. B. Peoples and R. M. Boddey (2008). "Global inputs of biological nitrogen fixation in agricultural systems." Plant and Soil **311**(1-2): 1-18.

Hossain, S., R. Ford, D. McNeil, C. Pittock and J. Panozzo (2010). "Inheritance of Seed Size in Chickpea ('Cicer arietinum'L.) and Identification of QTL Based on 100-seed Weight and Seed Size Index." Australian Journal of Crop Science **4**(2): 126.

Hungria, M., M. A. Nogueira and R. S. Araujo (2013). "Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability." Biology and Fertility of Soils **49**(7): 791-801.

İçgen, B., G. Özcengiz and N. G. Alaeddinoglu (2002). "Evaluation of symbiotic effectiveness of various Rhizobium cicer strains." Research in Microbiology **153**(6): 369-372.

Imparato, V., S. S. Santos, A. Johansen, S. Geisen and A. Winding (2016). "Stimulation of bacteria and protists in rhizosphere of glyphosate-treated barley." Applied Soil Ecology **98**: 47-55.

Ireland, C., M. Percival and N. Baker (1986). "Modification of the induction of photosynthesis in wheat by glyphosate, an inhibitor of amino acid metabolism." Journal of experimental botany **37**(3): 299-308.

Janda, J. M. and S. L. Abbott (2007). "16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls." Journal of clinical microbiology **45**(9): 2761-2764.

Jarvis, B., P. Van Berkum, W. Chen, S. Nour, M. Fernandez, J. Cleyet-Marel and M. Gillis (1997). "Transfer of Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum, and Rhizobium tianshanense to Mesorhizobium gen. nov." International Journal of Systematic and Evolutionary Microbiology **47**(3): 895-898.

Jeffries, P., S. Gianinazzi, S. Perotto, K. Turnau and J.-M. Barea (2003). "The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility." Biology and Fertility of Soils **37**(1): 1-16.

Jensen, E. S., M. B. Peoples, R. M. Boddey, P. M. Gresshoff, H. Hauggaard-Nielsen, B. J.R. Alves and M. J. Morrison (2012). "Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review." Agronomy for Sustainable Development **32**(2): 329-364.

Jha, P. K., S. Nair, M. Gopinathan and C. Babu (1995). "Suitability of rhizobia-inoculated wild legumes Argyrolobium flaccidum, Astragalus graveolens, Indigofera gangetica and Lespedeza stenocarpa in providing a vegetational cover in an unreclaimed limestone quarry." Plant and Soil **177**(2): 139-149.

Kantar, F., F. Hafeez, B. Shivkumar, S. Sundaram, N. Tejera, A. Aslam, A. Bano and P. Raja (2007). "Chickpea: Rhizobium management and nitrogen fixation." Chickpea Breeding and Management: 179-192.

Ketelaar, T. and A. M. C. Emons (2001). "The cytoskeleton in plant cell growth: lessons from root hairs." New Phytologist **152**(3): 409-418.

Khan, M. S., A. Zaidi and M. Aamil (2004). "Influence of herbicides on Chickpea-Mesorhizobium symbiosis." Agronomie **24**(3): 123-127.

Khattak, A. B., A. Zeb, N. Bibi, S. A. Khalil and M. S. Khattak (2007). "Influence of germination techniques on phytic acid and polyphenols content of chickpea (Cicer arietinum L.) sprouts." Food chemistry **104**(3): 1074-1079.

King, C. A., L. C. Purcell and E. D. Vories (2001). "Plant Growth and Nitrogenase Activity of Glyphosate-Tolerant Soybean in Response to Foliar Glyphosate Applications This paper is published with the approval of the director of the Arkansas Agricultural Experiment Station (manuscript no. 00001)." Agronomy Journal **93**(1): 179-186.

Laranjo, M., A. Alexandre and S. Oliveira (2014). "Legume growth-promoting rhizobia: an overview on the Mesorhizobium genus." Microbiological research **169**(1): 2-17.

Laranjo, M., J. Machado, J. P. W. Young and S. Oliveira (2004). "High diversity of chickpea Mesorhizobium species isolated in a Portuguese agricultural region." FEMS microbiology ecology **48**(1): 101-107.

Larkin, M. A., G. Blackshields, N. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm and R. Lopez (2007). "Clustal W and Clustal X version 2.0." bioinformatics **23**(21): 2947-2948.

Lupwayi, N. Z., G. W. Clayton, K. G. Hanson, W. A. Rice and V. O. Biederbeck (2004). "Endophytic rhizobia in barley, wheat and canola roots." Canadian Journal of Plant Science **84**(1): 37-45.

Lynch, J. M. (1990). "The rhizosphere." Wiley Series in Ecological & Applied Microbiology. John Wiley: somerset, New Jersey & Chichester, England **458**: 40056575-40056576.

Mamy, L., E. Barriuso and B. Gabrielle (2016). "Glyphosate fate in soils when arriving in plant residues." Chemosphere **154**: 425-433.

Martinez-Romero, E. (2003). "Diversity of Rhizobium-Phaseolus vulgaris symbiosis: overview and perspectives." Plant and Soil **252**(1): 11-23.

Martínez-Viveros, O., M. Jorquera, D. Crowley, G. Gajardo and M. Mora (2010). "Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria." Journal of soil science and plant nutrition **10**(3): 293-319.

Myers, J. P., M. N. Antoniou, B. Blumberg, L. Carroll, T. Colborn, L. G. Everett, M. Hansen, P. J. Landrigan, B. P. Lanphear and R. Mesnage (2016). "Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement." Environmental Health **15**(1): 19.

Myers, J. P., M. N. Antoniou, B. Blumberg, L. Carroll, T. Colborn, L. G. Everett, M. Hansen, P. J. Landrigan, B. P. Lanphear, R. Mesnage, L. N. Vandenberg, F. S. vom Saal, W. V. Welshons and C. M. Benbrook (2016). "Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement." Environmental Health **15**(1): 19.

Nascimento, F. X., C. Brígido, B. R. Glick and M. J. Rossi (2016). "The Role of Rhizobial ACC Deaminase in the Nodulation Process of Leguminous Plants." International Journal of Agronomy **2016**.

Newcomb, W., D. Sippell and R. Peterson (1979). "The early morphogenesis of Glycine max and Pisum sativum root nodules." Canadian Journal of Botany **57**(23): 2603-2616.

Newman, M. M., N. Hoilett, N. Lorenz, R. P. Dick, M. R. Liles, C. Ramsier and J. W. Kloepper (2016). "Glyphosate effects on soil rhizosphere-associated bacterial communities." Science of The Total Environment **543**: 155-160.

Norris, K., M. Bailey, A. Keith, L. Maskell, C. Reading, S. Turner, A. Vanbergen and A. Watt (2011). "Biodiversity in the context of ecosystem services [chapter 4]."

Nour, S. M., M. P. Fernandez, P. Normand and J.-C. Cleyet-Marel (1994). "Rhizobium ciceri sp. nov., consisting of strains that nodulate chickpeas (Cicer arietinum L.)." International Journal of Systematic and Evolutionary Microbiology **44**(3): 511-522.

Oldroyd, G. E. D. (2013). "Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants." Nat Rev Micro **11**(4): 252-263.

Owen, M. D., H. J. Beckie, J. Y. Leeson, J. K. Norsworthy and L. E. Steckel (2015). "Integrated pest management and weed management in the United States and Canada." Pest management science **71**(3): 357-376.

Parte, A. C. (2017). Retrieved 6 September, 2017, from http://www.bacterio.net/.

Patten, C. L. and B. R. Glick (2002). "Role of Pseudomonas putida indoleacetic acid in development of the host plant root system." Appl Environ Microbiol **68**(8): 3795-3801.

Pawlowski, K. and T. Bisseling (1996). "Rhizobial and actinorhizal symbioses: what are the shared features?" The Plant Cell **8**(10): 1899.

Perret, X., C. Staehelin and W. J. Broughton (2000). "Molecular basis of symbiotic promiscuity." Microbiology and Molecular Biology Reviews **64**(1): 180-201.

Podile, A. R. and G. K. Kishore (2007). Plant growth-promoting rhizobacteria. Plant-associated bacteria, Springer**:** 195-230.

Powles, S. B. (2008). "Evolved glyphosate-resistant weeds around the world: lessons to be learnt." Pest management science **64**(4): 360-365.

Reddy, K. N., R. E. Hoagland and R. M. Zablotowicz (2001). "Effect of Glyphosate on Growth, Chlorophyll, and Nodulation in Glyphosate-Resistant and Susceptible Soybean (Glycine max) Varieties." Journal of New Seeds **2**(3): 37-52.

Rivas, R., E. Velázquez, A. Valverde, P. F. Mateos and E. Martínez-Molina (2001). "A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species." Electrophoresis **22**(6): 1086-1089.

Roldán, A., F. García-Orenes and A. Lax (1994). "An incubation experiment to determine factors involving aggregation changes in an arid soil receiving urban refuse." Soil Biology and Biochemistry **26**(12): 1699-1707.

Roldán, A., J. Salinas-García, M. Alguacil, E. Diaz and F. Caravaca (2005). "Soil enzyme activities suggest advantages of conservation tillage practices in sorghum cultivation under subtropical conditions." Geoderma **129**(3): 178-185.

Rolfe, B. G. and P. Gresshoff (1988). "Genetic analysis of legume nodule initiation." Annual Review of Plant Physiology and Plant Molecular Biology **39**(1): 297-319.

Romdhane, S. B., F. Tajini, M. Trabelsi, M. E. Aouani and R. Mhamdi (2007). "Competition for nodule formation between introduced strains of Mesorhizobium

ciceri and the native populations of rhizobia nodulating chickpea (Cicer arietinum) in Tunisia." World Journal of Microbiology and Biotechnology **23**(9): 1195-1201.

Saxena, M. C. and K. Singh (1987). The chickpea, Commonwealth Agricultural Bureaux International.

Shaw, S. L., J. Dumais and S. R. Long (2000). "Cell Surface Expansion in Polarly Growing Root Hairs ofMedicago truncatula." Plant Physiology **124**(3): 959-970.

Shenoy, V. and G. Kalagudi (2005). "Enhancing plant phosphorus use efficiency for sustainable cropping." Biotechnology advances **23**(7): 501-513.

Siddiqui, Z. A. and M. S. Akhtar (2009). "Effect of plant growth promoting rhizobacteria, nematode parasitic fungi and root-nodule bacterium on root-knot nematodes Meloidogyne javanica and growth of chickpea." Biocontrol Science and Technology **19**(5): 511-521.

Sieberer, B. and A. M. C. Emons (2000). "Cytoarchitecture and pattern of cytoplasmic streaming in root hairs ofMedicago truncatula during development and deformation by nodulation factors." Protoplasma **214**(1): 118-127.

Smit, G., J. Kijne and B. Lugtenberg (1987). "Involvement of both cellulose fibrils and a Ca2+-dependent adhesin in the attachment of Rhizobium leguminosarum to pea root hair tips." Journal of Bacteriology **169**(9): 4294-4301.

Smit, G., J. Kijne and B. Lugtenberg (1989). "Roles of flagella, lipopolysaccharide, and a Ca2+-dependent cell surface protein in attachment of Rhizobium leguminosarum biovar viciae to pea root hair tips." Journal of bacteriology **171**(1): 569-572.

Smith, L. G. (2003). "Cytoskeletal control of plant cell shape: getting the fine points." Current opinion in plant biology **6**(1): 63-73.

Souza, A., F. Ferreira, A. Silva, A. Cardoso and H. Ruiz (1999). "Respiração microbiana do solo sob doses de glyphosate e de imazapyr." Planta Daninha **17**(3): 387-398.

Souza, R. d., A. Ambrosini and L. M. P. Passaglia (2015). "Plant growth-promoting bacteria as inoculants in agricultural soils." Genetics and Molecular Biology **38**: 401-419.

Spencer, D., E. James, G. Ellis, J. Shaw and J. Sprent (1994). "Interaction between rhizobia and potato tissues10." Journal of Experimental Botany **45**(10): 1475-1482.

Spurr, A. R. (1969). "A low-viscosity epoxy resin embedding medium for electron microscopy." Journal of ultrastructure research **26**(1-2): 31-43.

Stougaard, J. (2001). "Genetics and genomics of root symbiosis." Current opinion in plant biology **4**(4): 328-335.

Sviridov, A., T. Shushkova, I. Ermakova, E. Ivanova, D. Epiktetov and A. Leontievsky (2015). "Microbial degradation of glyphosate herbicides (Review)." Applied Biochemistry and Microbiology **51**(2): 188.

Taiz, L., E. Zeiger, I. M. Møller and A. Murphy (2015). Plant physiology and development, Sinauer Associates, Incorporated.

Tamura, K., J. Dudley, M. Nei and S. Kumar (2007). "MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0." Molecular biology and evolution **24**(8): 1596-1599.

Tanney, J. B. and L. J. Hutchison (2010). "The effects of glyphosate on the in vitro linear growth of selected microfungi from a boreal forest soil." Canadian journal of microbiology **56**(2): 138-144.

UN (2010). Report submitted by the Special Rapporteur in the right to food, Oliver de Schutter, to the UN General Assembly Human Rights Council, Sixteenth Session, A/HRC/16/49., United Nations

Vandermeer J., P. I. (1995). Breakfast of Biodiversity: The Truth about Rainforest Destruction. Oakland, California, USA, The Institute for Food and Development Policy.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Nasrulhaq Boyce, A. (2016). "Role of plant growth promoting rhizobacteria in agricultural sustainability—a review." Molecules **21**(5).

Verma, J. P., J. Yadav, K. N. Tiwari and A. Kumar (2013). "Effect of indigenous Mesorhizobium spp. and plant growth promoting rhizobacteria on yields and nutrients uptake of chickpea (Cicer arietinum L.) under sustainable agriculture." Ecological Engineering **51**: 282-286.

Vincent, J. M. (1970). "A manual for the practical study of the root-nodule bacteria." A manual for the practical study of the root-nodule bacteria.

Vivancos, P. D., S. P. Driscoll, C. A. Bulman, L. Ying, K. Emami, A. Treumann, C. Mauve, G. Noctor and C. H. Foyer (2011). "Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration." Plant Physiol **157**(1): 256-268.

von Wirén-Lehr, S., D. Komoßa, W. E. Gläßgen, H. Sandermann and I. Scheunert (1997). "Mineralization of [14C] glyphosate and its plant-associated residues in arable soils originating from different farming systems." Pest Management Science **51**(4): 436-442.

Wardle, D. (1992). "A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil." Biological reviews **67**(3): 321-358.

Woo, P., S. Lau, J. Teng, H. Tse and K. Y. Yuen (2008). "Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories." Clinical Microbiology and Infection **14**(10): 908-934.

Wood, D. and J. M. Lenne´ (1997). "The conservation of agrobiodiversity on-farm: questioning the emerging paradigm." Biodiversity & Conservation **6**(1): 109-129.

Woodburn, A. T. (2000). "Glyphosate: production, pricing and use worldwide." Pest Management Science **56**(4): 309-312.

Zaller, J. G., F. Heigl, L. Ruess and A. Grabmaier (2014). "Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem." Scientific reports **4**.

Zhang, J. J., T. Y. Liu, W. F. Chen, E. T. Wang, X. H. Sui, X. X. Zhang, Y. Li, Y. Li and W. X. Chen (2012). "Mesorhizobium muleiense sp. nov., nodulating with Cicer arietinum L." International Journal of Systematic and Evolutionary Microbiology **62**(11): 2737-2742.

Zhang, J. J., T. Yu, K. Lou, P. H. Mao, E. T. Wang, W. F. Chen and W. X. Chen (2014). "Genotypic alteration and competitive nodulation of Mesorhizobium muleiense against exotic chickpea rhizobia in alkaline soils." Systematic and Applied Microbiology **37**(7): 520-524.

Hossain, S; Ford, R; McNeil, D; Pittock, C and Panozzo, JF. Inheritance of Seed Size in Chickpea ('Cicer arietinum' L.) and Identification of QTL Based on 100-seed Weight and Seed Size Index [online]. Australian Journal of Crop Science, Vol. 4, No. 2, 2010: 126-135.

Availability: <http://search.informit.com.au/documentSummary;dn=982616406639670;res=IELHSS>ISSN: 1835-2693. [cited 21 Feb 17].

Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M.,, Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W.V., Benbrook, M.C.(2016) Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement, Environmental Health, 15:19 DOI 10.1186/s12940- 016-0117-0.

World Health Organisation (WHO) (2015) International Agency for Research on Cancer (IARC) Monographs, Volume 112: Evaluation of five organophosphate insecticides and herbicides, 20th March 2015 available at https://www.iarc.fr/en/mediacentre/iarcnews/pdf/MonographVolume112.pdf accessed 4/3/16

UK National Ecosystem Assessment (2011) The UK National Ecosystem Assessment: Technical Report. UNEP-WCMC, Cambridge. Chapter 4: Biodiversity in the Context of Ecosystem Services. pp. 80. Available at accessed 4/3/16

Primary Sources

Secondary Sources

Uncategorized References

(WHO), W. H. O. (2015). IARC Monographs Volume 112: evaluation of

five organophosphate insecticides and herbicides. Retrieved 10/5/17, 2017, from https://www.iarc.fr/en/media-centre/iarcnews/pdf/MonographVolume112.pdf

Abdel-Wahab, A., & Abd-Alla, M. (1995). Effect of different rates of N-fertilizers on nodulation, nodule activities and growth of two field grown cvs. of soybean *Fertilizers and Environment* (pp. 89-93): Springer.

Adesemoye, A., Torbert, H., & Kloepper, J. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microbial Ecology, 58*(4), 921-929.

Ahlawat, I. (2000). Chickpea. *Rathore, PS: Technique and Management of Field Crop Production. Agrobios Publications, Jodhpur, Rajasthan, India*, 317-335.

Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., & Oliveira, S. (2009). Survey of Chickpea Rhizobia Diversity in Portugal Reveals the Predominance of Species Distinct from Mesorhizobium ciceri and Mesorhizobium mediterraneum. *Microbial Ecology, 58*(4), 930-941. doi: 10.1007/s00248-009-9536-6

Altieri, M. A. (1999). The ecological role of biodiversity in agroecosystems. *Agriculture, Ecosystems & Environment, 74*(1), 19-31. doi: https://doi.org/10.1016/S0167-8809(99)00028-6

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology, 215*(3), 403-410.

Andrade, D., Murphy, P., & Giller, K. (2002). Effects of liming and legume/cereal cropping on populations of indigenous rhizobia in an acid Brazilian Oxisol. *Soil Biology and Biochemistry, 34*(4), 477-485.

Antoun, H., & Kloepper, J. (2001). Plant growth promoting rhizobacteria. Encyclopedia of Genetics.(Brenner, S. and Miller, JF, Eds.): Academic Press, New York.

Arfaoui, A., El Hadrami, A., Mabrouk, Y., Sifi, B., Boudabous, A., El Hadrami, I., . . . Chérif, M. (2007). Treatment of chickpea with Rhizobium isolates enhances the expression of phenylpropanoid defense-related genes in response to infection by Fusarium oxysporum f. sp. ciceris. *Plant Physiology and Biochemistry, 45*(6), 470-479.

Avis, T. J., Gravel, V., Antoun, H., & Tweddell, R. J. (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. *Soil Biology and Biochemistry, 40*(7), 1733-1740.

Bacon, C., & Hinton, D. (2006). Bacterial endophytes: the endophytic niche, its occupants, and its utility. *Plant-associated bacteria*, 155-194.

Badri, D. V., & Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant, Cell & Environment, 32*(6), 666-681.

Bala, A., Murphy, P., & Giller, K. E. (2001). Genetic diversity of rhizobia from natural populations varies with the soil dilution sampled. *Soil Biology and Biochemistry, 33*(6), 841-843.

Balasubramanian, V., Alves, B., Aulakh, M., Bekunda, M., Cai, Z., Drinkwater, L., . . . Oenema, O. (2004). Crop, environmental, and management factors affecting nitrogen use efficiency. *Agriculture and the Nitrogen Cycle, SCOPE, 65*, 19-33.

Banks, J. E. (2004). Divided culture: integrating agriculture and conservation biology. *Frontiers in Ecology and the Environment, 2*(10), 537–545. doi: 10.1890/1540-9295(2004)002[0537:DCIAAC]2.0.CO;2

Bashan, Y., de-Bashan, L. E., Prabhu, S., & Hernandez, J.-P. (2014). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil, 378*(1-2), 1-33.

Bastida, F., Moreno, J., Hernández, T., & García, C. (2007). The long-term effects of the management of a forest soil on its carbon content, microbial biomass and activity under a semi-arid climate. *Applied Soil Ecology, 37*(1), 53-62.

Benbrook, C. M. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe, 28*(1), 3.

Biswas, J. C., Ladha, J. K., Dazzo, F. B., Yanni, Y. G., & Rolfe, B. G. (2000). Rhizobial Inoculation Influences Seedling Vigor and Yield of Rice. *Agronomy Journal, 92*, 880-886. doi: 10.2134/agronj2000.925880x

Bohlool, B. B., Ladha, J. K., Garrity, D. P., & George, T. (1992). Biological nitrogen fixation for sustainable agriculture: A perspective. In J. K. Ladha, T. George & B. B. Bohlool (Eds.), *Biological Nitrogen Fixation for Sustainable Agriculture: Extended versions of papers presented in the Symposium, Role of Biological Nitrogen Fixation in Sustainable Agriculture at the 13th Congress of Soil Science, Kyoto, Japan, 1990* (pp. 1-11). Dordrecht: Springer Netherlands.

Brígido, C., Robledo, M., Menéndez, E., Mateos, P. F., & Oliveira, S. (2012). A ClpB chaperone knockout mutant of Mesorhizobium ciceri shows a delay in the root nodulation of chickpea plants. *Molecular Plant-Microbe Interactions, 25*(12), 1594-1604.

Brussaard, L., de Ruiter, P. C., & Brown, G. G. (2007). Soil biodiversity for agricultural sustainability. *Agriculture, Ecosystems & Environment, 121*(3), 233-244. doi: https://doi.org/10.1016/j.agee.2006.12.013

Carol, R. J., & Dolan, L. (2002). Building a hair: tip growth in Arabidopsis thaliana root hairs. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 357*(1422), 815-821.

Carroll, B. J., & Gresshoff, P. M. (1983). Nitrate inhibition of nodulation and nitrogen fixation in white clover. *Zeitschrift für Pflanzenphysiologie, 110*(1), 77-88.

Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., & Kjelleberg, S. (2007). Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Appl Environ Microbiol, 73*(1), 278-288.

Cerdeira, A. L., Gazziero, D. L., Duke, S. O., & Matallo, M. B. (2010). Agricultural impacts of glyphosate-resistant soybean cultivation in South America. *Journal of agricultural and food chemistry, 59*(11), 5799-5807.

Choudhary, D. K., Sharma, K. P., & Gaur, R. K. (2011). Biotechnological perspectives of microbes in agro-ecosystems. *Biotechnology letters, 33*(10), 1905-1910.

Cooper, J. (2007). Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *Journal of applied microbiology, 103*(5), 1355-1365.

Coutinho, H. L., Oliveira, V. M., Lovato, A., Maia, A. H., & Manfio, G. P. (1999). Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans. *Applied Soil Ecology, 13*(2), 159-167.

Dakora, F., & Phillips, D. (1996). Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. *Physiological and Molecular Plant Pathology, 49*(1), 1-20.

De Meyer, S. E., Tan, H. W., Heenan, P. B., Andrews, M., & Willems, A. (2015). Mesorhizobium waimense sp. nov. isolated from Sophora longicarinata root nodules and

Mesorhizobium cantuariense sp. nov. isolated from Sophora microphylla root nodules. *International Journal of Systematic and Evolutionary Microbiology, 65*(10), 3419-3426.

Denison, R. F. (2000). Legume Sanctions and the Evolution of Symbiotic Cooperation by Rhizobia. *The American Naturalist, 156*(6), 567-576. doi: 10.1086/316994

Dey, R., Pal, K., Bhatt, D., & Chauhan, S. (2004). Growth promotion and yield enhancement of peanut (Arachis hypogaea L.) by application of plant growth-promoting rhizobacteria. *Microbiological research, 159*(4), 371-394.

Djordjevic, M. A., Redmond, J. W., Batley, M., & Rolfe, B. G. (1987). Clovers secrete specific phenolic compounds which either stimulate or repress nod gene expression in Rhizobium trifolii. *The EMBO journal, 6*(5), 1173.

Dobereiner, J. (1992). History and new perspectives of diazotrophs in association with non-leguminous plants. *Symbiosis*.

Draft U.S. Greenhouse Gas Inventory Report:1990–2014. Retrieved 2 May, 2017, from https://www3.epa.gov/climatechange/ghgemissions/usinventoryreport.html

Druille, M., Cabello, M. N., Omacini, M., & Golluscio, R. A. (2013). Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi. *Applied Soil Ecology, 64*, 99-103.

Druille, M., Cabello, M. N., Parisi, P. G., Golluscio, R., & Omacini, M. (2015). Glyphosate vulnerability explains changes in root-symbionts propagules viability in pampean grasslands. *Agriculture, Ecosystems & Environment, 202*, 48-55.

Duan, J., Müller, K. M., Charles, T. C., Vesely, S., & Glick, B. R. (2009). 1-aminocyclopropane-1-carboxylate (ACC) deaminase genes in rhizobia from southern Saskatchewan. *Microbial Ecology, 57*(3), 423-436.

Duarte Maçãs, I. (2003). Selecção de linhas de grão de bico (Cicer arietinum L.) adaptadas ao ambiente Mediterrânico-critérios morfológicos e fisiológicos. *Universidade de Évora, Évora, 171*.

Duhan, J. S., Kumar, R., Kumar, N., Kaur, P., & Nehra, K. (2017). Nanotechnology: The new perspective in precision agriculture. *Biotechnology Reports*.

Duke, S. O. (2015). Perspectives on transgenic, herbicide-resistant crops in the United States almost 20 years after introduction. *Pest Manag Sci, 71*. doi: 10.1002/ps.3863

Dunbar, J., Ticknor, L. O., & Kuske, C. R. (2000). Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl Environ Microbiol, 66*(7), 2943-2950.

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M. H., Lin, Y. H., Reid, D. E., & Gresshoff, P. M. (2010). Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology, 52*(1), 61-76.

Fox, J. E., Gulledge, J., Engelhaupt, E., Burow, M. E., & McLachlan, J. A. (2007). Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants. *Proceedings of the National Academy of Sciences, 104*(24), 10282-10287.

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews, 68*(2), 280-300.

Gaines, T. A., Zhang, W., Wang, D., Bukun, B., Chisholm, S. T., Shaner, D. L., . . . Culpepper, A. S. (2010). Gene amplification confers glyphosate resistance in Amaranthus palmeri. *Proceedings of the National Academy of Sciences, 107*(3), 1029-1034.

Galloway, J. N. T., A.R.; Erisman, J.W.; Bekunda, M.; Cai, Z.; Freney, J.R.; Martinelli, L.A.;, & Seitzinger, S. P. S., M.A. . (2008). Transformation of the nitrogen cycle: Recent trends, questions, and potential

solutions. *Science, 320*, 889–892.

García-Fraile, P., Carro, L., Robledo, M., Ramírez-Bahena, M.-H., Flores-Félix, J.-D., Fernández, M. T., . . . Martínez-Molina, E. (2012). Rhizobium promotes non-legumes growth and quality in several production steps: towards a biofertilization of edible raw vegetables healthy for humans. *PLoS One, 7*(5), e38122.

Gaupp-Berghausen, M., Hofer, M., Rewald, B., & Zaller, J. G. (2015). Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. *Scientific reports, 5*. doi: 10.1038/srep12886

Giongo, A., Ambrosini, A., Vargas, L., Freire, J., Bodanese-Zanettini, M., & Passaglia, L. (2008). Evaluation of genetic diversity of bradyrhizobia strains nodulating soybean [Glycine max (L.) Merrill] isolated from South Brazilian fields. *Applied Soil Ecology, 38*(3), 261-269.

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology, 41*(2), 109-117.

Glick, B. R. (2012). Plant Growth-Promoting Bacteria: Mechanisms and Applications. *Hindawi Publishing Corporation Scientifica*, 15. doi: 10.6064/2012/963401

Gray, E., & Smith, D. (2005). Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil Biology and Biochemistry, 37*(3), 395-412.

Gray EJ, S. D. (2005). Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biol Biochem., 37*, 395–412.

Grover, M., Ali, S. Z., Sandhya, V., Rasul, A., & Venkateswarlu, B. (2011). Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World Journal of Microbiology and Biotechnology, 27*(5), 1231-1240.

Hagner, M., Hallman, S., Jauhiainen, L., Kemppainen, R., Rämö, S., Tiilikkala, K., & Setälä, H. (2015). Birch (Betula spp.) wood biochar is a potential soil amendment to reduce glyphosate leaching in agricultural soils. *Journal of environmental management, 164*, 46-52.

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* Paper presented at the Nucleic acids symposium series.

Hauggaard-Nielsen, H., Ambus, P., & Jensen, E. S. (2003). The comparison of nitrogen use and leaching in sole cropped versus intercropped pea and barley. *Nutrient Cycling in Agroecosystems, 65*(3), 289-300.

Helander, M., Saloniemi, I., & Saikkonen, K. (2012) Glyphosate in northern ecosystems. *Trends in Plant Science, 17*(10), 569-574. doi: 10.1016/j.tplants.2012.05.008

Hossain, S., Ford, R., McNeil, D., Pittock, C., & Panozzo, J. (2010). Inheritance of Seed Size in Chickpea ('Cicer arietinum'L.) and Identification of QTL Based on 100-seed Weight and Seed Size Index. *Australian Journal of Crop Science, 4*(2), 126.

Hungria, M., Nogueira, M. A., & Araujo, R. S. (2013). Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. *Biology and Fertility of Soils, 49*(7), 791-801.

İçgen, B., Özcengiz, G., & Alaeddinoglu, N. G. (2002). Evaluation of symbiotic effectiveness of various Rhizobium cicer strains. *Research in Microbiology, 153*(6), 369-372. doi: http://dx.doi.org/10.1016/S0923-2508(02)01329-3

Imparato, V., Santos, S. S., Johansen, A., Geisen, S., & Winding, A. (2016). Stimulation of bacteria and protists in rhizosphere of glyphosate-treated barley. *Applied Soil Ecology, 98*, 47-55.

Ireland, C., Percival, M., & Baker, N. (1986). Modification of the induction of photosynthesis in wheat by glyphosate, an inhibitor of amino acid metabolism. *Journal of Experimental Botany, 37*(3), 299-308.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology, 45*(9), 2761-2764.

Jarvis, B., Van Berkum, P., Chen, W., Nour, S., Fernandez, M., Cleyet-Marel, J., & Gillis, M. (1997). Transfer of Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum, and Rhizobium tianshanense to Mesorhizobium gen. nov. *International Journal of Systematic and Evolutionary Microbiology, 47*(3), 895-898.

Jayasumana, C., Gunatilake, S., & Senanayake, P. (2014). Glyphosate, hard water and nephrotoxic metals: are they the culprits behind the epidemic of chronic kidney disease of unknown etiology in Sri Lanka? *Int J Environ Res Public Health, 11*. doi: 10.3390/ijerph110202125

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., & Barea, J.-M. (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils, 37*(1), 1-16. doi: 10.1007/s00374-002-0546-5

Jensen, E. S., & Hauggaard-Nielsen, H. (2003). How can increased use of biological N2 fixation in agriculture benefit the environment? *Plant and Soil, 252*(1), 177-186. doi: 10.1023/a:1024189029226

Jha, P. K., Nair, S., Gopinathan, M., & Babu, C. (1995). Suitability of rhizobia-inoculated wild legumes Argyrolobium flaccidum, Astragalus graveolens, Indigofera gangetica and Lespedeza stenocarpa in providing a vegetational cover in an unreclaimed limestone quarry. *Plant and Soil, 177*(2), 139-149.

Johal, G. S., & Huber, D. M. (2009). Glyphosate effects on diseases in plants. *Eur J Agronomy, 31*. doi: 10.1016/j.eja.2009.04.004

Kantar, F., Hafeez, F., Shivkumar, B., Sundaram, S., Tejera, N., Aslam, A., . . . Raja, P. (2007). Chickpea: Rhizobium management and nitrogen fixation. *Chickpea Breeding and Management*, 179-192.

Khan, M. S., Zaidi, A., & Aamil, M. (2004). Influence of herbicides on Chickpea-Mesorhizobium symbiosis. *Agronomie, 24*(3), 123-127.

Khattak, A. B., Zeb, A., Bibi, N., Khalil, S. A., & Khattak, M. S. (2007). Influence of germination techniques on phytic acid and polyphenols content of chickpea (Cicer arietinum L.) sprouts. *Food chemistry, 104*(3), 1074-1079.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution, 16*(2), 111-120.

King, C. A., Purcell, L. C., & Vories, E. D. (2001). Plant Growth and Nitrogenase Activity of Glyphosate-Tolerant Soybean in Response to Foliar Glyphosate Applications This paper is published with the approval of the director of the Arkansas Agricultural Experiment

Station (manuscript no. 00001). *Agronomy Journal, 93*(1), 179-186. doi: 10.2134/agronj2001.931179x

Kremer, R., Means, N., & Kim, S. (2005). Glyphosate affects soybean root exudation and rhizosphere micro-organisms. *International Journal of Environmental Analytical Chemistry, 85*(15), 1165-1174.

Kristjanson, P., Reid, R. S., Dickson, N., Clark, W. C., Romney, D., Puskur, R., . . . Grace, D. (2009). Linking international agricultural research knowledge with action for sustainable development. *Proceedings of the National Academy of Sciences, 106*(13), 5047-5052.

Laranjo, M., Alexandre, A., & Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the Mesorhizobium genus. *Microbiological research, 169*(1), 2-17.

Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W., & Oliveira, S. (2008). Chickpea rhizobia symbiosis genes are highly conserved across multiple Mesorhizobium species. *FEMS microbiology ecology, 66*(2), 391-400.

Laranjo, M., Machado, J., Young, J. P. W., & Oliveira, S. (2004). High diversity of chickpea Mesorhizobium species isolated in a Portuguese agricultural region. *FEMS microbiology ecology, 48*(1), 101-107.

Larkin, M. A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P. A., McWilliam, H., . . . Lopez, R. (2007). Clustal W and Clustal X version 2.0. *bioinformatics, 23*(21), 2947-2948.

Lupwayi, N. Z., Clayton, G. W., Hanson, K. G., Rice, W. A., & Biederbeck, V. O. (2004). Endophytic rhizobia in barley, wheat and canola roots. *Canadian Journal of Plant Science, 84*(1), 37-45. doi: 10.4141/P03-087

Lynch, J. M. (1990). The rhizosphere. *Wiley Series in Ecological & Applied Microbiology. John Wiley: somerset, New Jersey & Chichester, England, 458*, 40056575-40056576.

Mamy, L., Barriuso, E., & Gabrielle, B. (2016). Glyphosate fate in soils when arriving in plant residues. *Chemosphere, 154*, 425-433.

Martinez-Romero, E. (2003). Diversity of Rhizobium-Phaseolus vulgaris symbiosis: overview and perspectives. *Plant and Soil, 252*(1), 11-23.

Martínez-Viveros, O., Jorquera, M., Crowley, D., Gajardo, G., & Mora, M. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of soil science and plant nutrition, 10*(3), 293-319.

Mijangos, I., Becerril, J. M., Albizu, I., Epelde, L., & Garbisu, C. (2009). Effects of glyphosate on rhizosphere soil microbial communities under two different plant compositions by cultivation-dependent and-independent methodologies. *Soil Biology and Biochemistry, 41*(3), 505-513.

Myers, J. P., Antoniou, M. N., Blumberg, B., Carroll, L., Colborn, T., Everett, L. G., . . . Mesnage, R. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environmental Health, 15*(1), 19.

Myers, J. P., Antoniou, M. N., Blumberg, B., Carroll, L., Colborn, T., Everett, L. G., . . . Benbrook, C. M. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environmental Health, 15*(1), 19. doi: 10.1186/s12940-016-0117-0

Nascimento, F., Brígido, C., Glick, B., Oliveira, S., & Alho, L. (2012). Mesorhizobium ciceri LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. *Letters in applied microbiology, 55*(1), 15-21.

Nascimento, F. X., Brígido, C., Glick, B. R., & Rossi, M. J. (2016). The Role of Rhizobial ACC Deaminase in the Nodulation Process of Leguminous Plants. *International Journal of Agronomy, 2016*.

Newcomb, W., Sippell, D., & Peterson, R. (1979). The early morphogenesis of Glycine max and Pisum sativum root nodules. *Canadian Journal of Botany, 57*(23), 2603-2616.

Newman, M. M., Hoilett, N., Lorenz, N., Dick, R. P., Liles, M. R., Ramsier, C., & Kloepper, J. W. (2016). Glyphosate effects on soil rhizosphere-associated bacterial communities. *Science of The Total Environment, 543*, 155-160.

Norris, K., Bailey, M., Keith, A., Maskell, L., Reading, C., Turner, S., . . . Watt, A. (2011). Biodiversity in the context of ecosystem services [chapter 4].

Nour, S. M., Fernandez, M. P., Normand, P., & Cleyet-Marel, J.-C. (1994). Rhizobium ciceri sp. nov., consisting of strains that nodulate chickpeas (Cicer arietinum L.). *International Journal of Systematic and Evolutionary Microbiology, 44*(3), 511-522.

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat Rev Micro, 11*(4), 252-263.

Owen, M. D., Beckie, H. J., Leeson, J. Y., Norsworthy, J. K., & Steckel, L. E. (2015). Integrated pest management and weed management in the United States and Canada. *Pest Management Science, 71*(3), 357-376.

Parte, A. C. (2017). Retrieved 6 September, 2017, from http://www.bacterio.net/

Patten, C. L., & Glick, B. R. (2002). Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Appl Environ Microbiol, 68*(8), 3795-3801.

Pawlowski, K., & Bisseling, T. (1996). Rhizobial and actinorhizal symbioses: what are the shared features? *The Plant Cell, 8*(10), 1899.

Peoples, M. B., Herridge, D. F., & Ladha, J. K. (1995). Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil, 174*(1), 3-28. doi: 10.1007/bf00032239

Perret, X., Staehelin, C., & Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews, 64*(1), 180-201.

Podile, A. R., & Kishore, G. K. (2007). Plant growth-promoting rhizobacteria *Plant-associated bacteria* (pp. 195-230): Springer.

Powles, S. B. (2008). Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest Management Science, 64*(4), 360-365.

Ratcliff, A. W., Busse, M. D., & Shestak, C. J. (2006). Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. *Applied Soil Ecology, 34*(2), 114-124.

Reddy, K. N., Hoagland, R. E., & Zablotowicz, R. M. (2001). Effect of Glyphosate on Growth, Chlorophyll, and Nodulation in Glyphosate-Resistant and Susceptible Soybean (Glycine max) Varieties. *Journal of New Seeds, 2*(3), 37-52. doi: 10.1300/J153v02n03_03

Rigby, D., & Cáceres, D. (2001). Organic farming and the sustainability of agricultural systems. *Agricultural Systems, 68*(1), 21-40. doi: https://doi.org/10.1016/S0308-521X(00)00060-3

Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F., & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis, 22*(6), 1086-1089.

Roldán, A., García-Orenes, F., & Lax, A. (1994). An incubation experiment to determine factors involving aggregation changes in an arid soil receiving urban refuse. *Soil Biology and Biochemistry, 26*(12), 1699-1707.

Roldán, A., Salinas-García, J., Alguacil, M., Diaz, E., & Caravaca, F. (2005). Soil enzyme activities suggest advantages of conservation tillage practices in sorghum cultivation under subtropical conditions. *Geoderma, 129*(3), 178-185.

Rolfe, B. G., & Gresshoff, P. (1988). Genetic analysis of legume nodule initiation. *Annual Review of Plant Physiology and Plant Molecular Biology, 39*(1), 297-319.

Romdhane, S. B., Tajini, F., Trabelsi, M., Aouani, M. E., & Mhamdi, R. (2007). Competition for nodule formation between introduced strains of Mesorhizobium ciceri and the native populations of rhizobia nodulating chickpea (Cicer arietinum) in Tunisia. *World Journal of Microbiology and Biotechnology, 23*(9), 1195-1201.

Rudd, M. A., Beazley, K. F., Cooke, S. J., Fleishman, E., Lane, D. E., Mascia, M. B., . . . Bellefontaine, T. (2011). Generation of priority research questions to inform conservation policy and management at a national level. *Conservation Biology, 25*(3), 476-484.

Saxena, M. C., & Singh, K. (1987). *The chickpea*: Commonwealth Agricultural Bureaux International.

Shenoy, V., & Kalagudi, G. (2005). Enhancing plant phosphorus use efficiency for sustainable cropping. *Biotechnology advances, 23*(7), 501-513.

Siddiqui, Z. A., & Akhtar, M. S. (2009). Effect of plant growth promoting rhizobacteria, nematode parasitic fungi and root-nodule bacterium on root-knot nematodes Meloidogyne javanica and growth of chickpea. *Biocontrol Science and Technology, 19*(5), 511-521. doi: 10.1080/09583150902887792

Smith, L. G. (2003). Cytoskeletal control of plant cell shape: getting the fine points. *Current opinion in plant biology, 6*(1), 63-73.

Souza, A., Ferreira, F., Silva, A., Cardoso, A., & Ruiz, H. (1999). Respiração microbiana do solo sob doses de glyphosate e de imazapyr. *Planta Daninha, 17*(3), 387-398.

Souza, R. d., Ambrosini, A., & Passaglia, L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and Molecular Biology, 38*, 401-419.

Spencer, D., James, E., Ellis, G., Shaw, J., & Sprent, J. (1994). Interaction between rhizobia and potato tissues10. *Journal of Experimental Botany, 45*(10), 1475-1482.

Stougaard, J. (2001). Genetics and genomics of root symbiosis. *Current opinion in plant biology, 4*(4), 328-335.

Stratton, G. W., & Stewart, K. E. (1992). Glyphosate effects on microbial biomass in a coniferous forest soil. *Environmental Toxicology, 7*(3), 223-236.

Sviridov, A., Shushkova, T., Ermakova, I., Ivanova, E., Epiktetov, D., & Leontievsky, A. (2015). Microbial degradation of glyphosate herbicides (Review). *Applied Biochemistry and Microbiology, 51*(2), 188.

Taiz, L., Zeiger, E., Møller, I. M., & Murphy, A. (2015). *Plant physiology and development*: Sinauer Associates, Incorporated.

Talavera, S., & Castroviejo, S. (2000). *Flora ibérica: plantas vasculares de la Península Ibérica e Islas Baleares. Leguminosae (partim)*: Editorial CSIC-CSIC Press.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular biology and evolution, 24*(8), 1596-1599.

Tanney, J. B., & Hutchison, L. J. (2010). The effects of glyphosate on the in vitro linear growth of selected microfungi from a boreal forest soil. *Canadian Journal of Microbiology, 56*(2), 138-144.

Turner, S., Pryer, K. M., Miao, V. P., & Palmer, J. D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *Journal of Eukaryotic Microbiology, 46*(4), 327-338.

UN. (2010). Report submitted by the Special Rapporteur in the right to food, Oliver de Schutter, to the UN General Assembly Human Rights Council, Sixteenth Session, A/HRC/16/49.: United Nations

Vandermeer J., P. I. (1995). *Breakfast of Biodiversity: The Truth about Rainforest Destruction*. Oakland, California, USA: The Institute for Food and Development Policy.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Nasrulhaq Boyce, A. . (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules, 21*(5).

Verma, J. P., Yadav, J., Tiwari, K. N., & Kumar, A. (2013). Effect of indigenous Mesorhizobium spp. and plant growth promoting rhizobacteria on yields and nutrients uptake of chickpea (Cicer arietinum L.) under sustainable agriculture. *Ecological Engineering, 51*, 282-286.

Vincent, J. M. (1970). A manual for the practical study of the root-nodule bacteria. *A manual for the practical study of the root-nodule bacteria.*

Vivancos, P. D., Driscoll, S. P., Bulman, C. A., Ying, L., Emami, K., Treumann, A., . . . Foyer, C. H. (2011). Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. *Plant Physiol, 157*(1), 256-268. doi: 10.1104/pp.111.181024

von Wirén-Lehr, S., Komoßa, D., Gläßgen, W. E., Sandermann, H., & Scheunert, I. (1997). Mineralization of [14C] glyphosate and its plant-associated residues in arable soils originating from different farming systems. *Pest Management Science, 51*(4), 436-442.

Wardle, D. (1992). A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil. *Biological reviews, 67*(3), 321-358.

Woo, P., Lau, S., Teng, J., Tse, H., & Yuen, K. Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection, 14*(10), 908-934.

Wood, D., & Lenne´, J. M. (1997). The conservation of agrobiodiversity on-farm: questioning the emerging paradigm. *Biodiversity & Conservation, 6*(1), 109-129. doi: 10.1023/a:1018331800939

Woodburn, A. T. (2000). Glyphosate: production, pricing and use worldwide. *Pest Management Science, 56*(4), 309-312.

Yang, J., Kloepper, J. W., & Ryu, C.-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science, 14*(1), 1-4. doi: https://doi.org/10.1016/j.tplants.2008.10.004

Zaller, J. G., Heigl, F., Ruess, L., & Grabmaier, A. (2014). Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Scientific reports, 4*. doi: 10.1038/srep05634

Zhang, J. J., Liu, T. Y., Chen, W. F., Wang, E. T., Sui, X. H., Zhang, X. X., . . . Chen, W. X. (2012). Mesorhizobium muleiense sp. nov., nodulating with Cicer arietinum L. *International Journal of Systematic and Evolutionary Microbiology, 62*(11), 2737-2742. doi: doi:10.1099/ijs.0.038265-0

Zhang, J. J., Yu, T., Lou, K., Mao, P. H., Wang, E. T., Chen, W. F., & Chen, W. X. (2014). Genotypic alteration and competitive nodulation of Mesorhizobium muleiense against exotic

chickpea rhizobia in alkaline soils. *Systematic and applied microbiology, 37*(7), 520-524. doi: http://dx.doi.org/10.1016/j.syapm.2014.07.004

Hossain, S; Ford, R; McNeil, D; Pittock, C and Panozzo, JF. Inheritance of Seed Size in Chickpea ('Cicer arietinum' L.) and Identification of QTL Based on 100-seed Weight and Seed Size Index [online]. Australian Journal of Crop Science, Vol. 4, No. 2, 2010: 126-135.
Availability: <http://search.informit.com.au/documentSummary;dn=982616406639670;res=IELHSS>ISSN: 1835-2693. [cited 21 Feb 17].

Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M.,, Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W.V., Benbrook, M.C.(2016) Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement, Environmental Health, 15:19 DOI 10.1186/s12940- 016-0117-0.

World Health Organisation (WHO) (2015) International Agency for Research on Cancer (IARC) Monographs, Volume 112: Evaluation of five organophosphate insecticides and herbicides, 20th March 2015 available at https://www.iarc.fr/en/mediacentre/iarcnews/pdf/MonographVolume112.pdf accessed 4/3/16

UK National Ecosystem Assessment (2011) The UK National Ecosystem Assessment: Technical Report. UNEP-WCMC, Cambridge. Chapter 4: Biodiversity in the Context of Ecosystem Services. pp. 80. Available at accessed 4/3/16

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