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Identification and characterization of beneficial microorganisms associated with chickpea (*Cicer arietinum L.*) for increased productivity and drought tolerance

Treball final de grau

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Resum

En vista de l'impacte de l'escassetat d'aigua a la productivitat, és important proporcionar a les plantes eines que les facin més tolerants. Aquest desenvolupament es pot aconseguir mitjançant l'explotació de microorganismes beneficiosos per augmentar la productivitat i la resistència en condicions climàtiques. A més, els problemes econòmics i ambientals dels fertilitzants minerals fomenten activament el desenvolupament d'alternatives que siguin amigables amb el medi ambient, rendibles i satisfagin les demandes dels productors i consumidors.

Per tant, l'ús de microorganismes beneficiosos com a biofertilitzants s'ha tornat important en el sector de l'agricultura a causa del seu paper productiu i sostenible. Els microorganismes del sòl, Plant Growth Promoting Rhizobacteria (PGPR), inclosos els rizobios, són substituts prometedors dels fertilitzants químics a causa dels seus mecanismes biològics que milloren el rendiment de la planta i augmenten la resistència als patògens fúngics i l'estrès abiòtic. Prendre consciència d'aquest potencial, requereix una comprensió del seu paper en la producció de cultius i el desenvolupament de inoculantes eficients i disponibles per als agricultors.

El present estudi té com a objectiu identificar el millor inoculant del la rizosfera del cigró. Això s'ha dut a terme amb els següents procediments: Cultius trampa per a la recol·lecció dels bacteris, Avaluació in vitro per provar que els bacteris són iguals al primer aïllament, identificació molecular i anàlisi filogenètica de les seqüències. Els arbres filogenètics són prou clars, però és difícil extreure conclusions sense estudis addicionals.

Resumen

En vista del impacto de la escasez de agua en la productividad, es importante proporcionar a las plantas herramientas que las hagan más tolerantes. Este desarrollo puede lograrse mediante la explotación de microorganismos beneficiosos para aumentar la productividad y la resistencia en condiciones climáticas. Además, los problemas económicos y ambientales de los fertilizantes minerales fomentan activamente el desarrollo de alternativas que son amigables con el medio ambiente, rentables y satisfaciendo las demandas de los productores y consumidores.

Por lo tanto, el uso de microorganismos beneficiosos como biofertilizantes se ha vuelto importante en el sector de la agricultura debido a su papel productivo y sostenible.Los microorganismos del suelo, PGPR, incluidos los rizobios, son sustitutos prometedores de los fertilizantes químicos debido a sus mecanismos biológicos que mejoran el rendimiento de la planta y aumentan la resistencia a los patógenos fúngicos y al estrés abiótico. Tomar conciencia de este potencial, requiere una comprensión de su papel en la producción de cultivos y el desarrollo de inoculantes eficientes disponibles para los agricultores.

El presente estudio tiene como objetivo identificar al mejor inoculante del la rizosfera del garbanzo. Esto se ha llevado a cabo con los siguientes procedimientos: Cultivos trampa para la recolección de las bacterias, Evaluación in vitro para probar que las bacterias son iguales al primer aislamiento, identificación molecular y análisis filogenético de las secuencias. Los árboles filogenéticos son bastante claros, pero es difícil extraer conclusiones sin estudios adicionales.

Abstract

In view of the impact of water scarcity on productivity, it is important to provide plants with tools that make them more tolerant. Such development can be attained by exploiting beneficial microorganisms for increased productivity and resilience under climate conditions. Furthermore,

the economic and environmental constrains of mineral fertilizers actively encourage alternative sources which are environment-friendly, cost effective and ensure the demands and constrains of producers and consumers.

Therefore, the use of beneficial microorganisms as biofertilizers has become important in agriculture sector because of their potential role in food safety and sustainable production. Among soil microorganisms, PGPR, including rhizobia, are promising substitutes of chemical fertilizers because of their biological mechanisms that improve plant performance and enhance the resistance to fungal pathogens and abiotic stress. Becoming conscious of this potential, requires an understanding of their role in crop production and the development of efficient inoculants available for farmers.

The present study has the objective of identified the best inoculant of chickpea rizhosphera microorganism. This has been carried out with the following procedures: Trap culture collection of the bacterias, In-vitro assessment to prove that the bacterias are the same as the first isolation, molecular identification, and phylogenetic analysis of the sequences. The phylogenetic trees are quite clear but is difficult to extract conclusions with out further studies.

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Contents

List of Figures

List of Tables

Acronyms

AMF Arbuscular Mycorrhizal Fungi

BNF Biological Nitrogen Fixation

PGPR Plant Growth Promoting Rhizobacteria

DNA DeoxyriboNucleic Acid

CTAB Cetyl trimethylammonium Bromide

PCR Polymerase Chain Reaction

NCIBI National Center for Integrative Biomedical Informatics

IAA Indole-3-Acetic Acid

HCN Hydrogen Cyanide

P Phosphorus

Introduction

Chickpea *(Cicer arietinum L.)* is the second most important legume crop consumed worldwide, especially in North Africa, South-East Asia, the Middle East, southern Europe, America and Australia.

Chickpea has been considered an important source of proteins, carbohydrates, minerals, vitamins and health-promoting fatty acids in the human diet [Jukanti et al., 2012]. As a cheaper source of protein it is particularly important for low-income consumers around the world and in developing countries. Chickpea is usually cultivated in regions where climate variability, drought and limited use of fertilizers significantly reduce productivity. However, being a leguminous crop, it exhibits the important characteristic of fixing atmospheric nitrogen (N_2) through its symbiosis with rhizobia, enabling cultivation in many N-poor soils with acceptable yields. Chickpea rhizobia are included in the *Mesorhizobium* genus and can fix up to 140 kg N/ha per annum, depending on soil and climatic conditions [Gaur et al., 2010]. Biological Nitrogen Fixation (BNF) is the key environmental benefit of legumes under a broad spectrum of environment conditions and crop rotations resulting in reduced mineral N fertilizer use in agriculture. BNF is thus a tool to diminishing the load of environment pollution in a sustainable way reducing freshwater pollution and reducing CO_2 emissions.

Legume plants also associates with Arbuscular Mycorrhizal Fungi (AMF) and several studies have shown that the tripartite symbiosis of legume-rhizobia-AMF leads to increases in nodulation, nitrogen-fixation, P concentration and plant growth [Ossler et al., 2015]. AMF have the capacity to improve uptake of water and nutrients and reduce the effects of environmental stresses, such as drought [Augé et al., 2015] which may contribute to improved crop yield under adverse environmental conditions. Even though rhizobia and AMF species are widely distributed, there are several soils where appropriate strains for specific species are absent, or the population density is low, leading to the need for commercial inoculants [Brockwell et al., 1995]. Inoculants are commercial formulations which contain selected microorganisms to be applied to the seeds or to the soil during planting with the aim to reduce inorganic fertilizer inputs.

The global market of biofertilizers is rising at an estimated rate of approximately 12,9 per annum, during the forecast period of 2017 to 2025, valued at USdollars 1,254 million in 2016 and is estimated that should reach USdollars 4.092 million by the end of 2025. This market is gaining traction from a number of factors such as growing awareness regarding its health and environmental benefits, gradual shift from chemical-based farming techniques to organic practices, growing adoption of biofertilizers in soil fertility management activities and rise in the cost of chemical fertilizers and pesticides.

Chickpea, *(Cicer arietinum L.)*, taxonomy and botany

The genus *Cicer* belongs to family Leguminosae, sub-family Papilionoideae. It is considered at his own tribe, the Cicerea [Van der Maesen, 1987]. It has been recognised 43 species the generous *Cicer*, but chickpea, is the only cultivated one. There are two different groups of cultivars, Desi and Kabuli, Figure: 1. Desi has small leaves, pods and seeds, the flowering has a hight amplitud range of colours. While Kabuli cultivars has bigger leaves, pods and seeds, and the flowering colours use to be in the spectrum of whites [Hawtin et al., 1980].

The botany of chickpeas has been reviewed from Purseglove (1974), Summerfield and Roberts (1985), Malhotra and Singh (1985), Cubero (1987) and is as follows: The plant is an erect or spreading, much branched, annual herb, about 25-50 cm tall. It has a well developed tap root and numerous lateral roots with large nodules. Practically all the nodules occur in the top 30 cm of soil with 90 % within the first 15 cm from the surface. Its root system is more extensive in late

maturing. Leaves are pinnate about 5 cm long and leaflets ovate, elliptic or obovate and serrate. The average weight of 100 seeds is in between 17-27g in Desi cultivars and 15-75g in Kabuli. It is a self pollinated spice, and the germinations is hypogea.

Figura 1: The different chickpea cultivars: Kabuli, Desi, Green desi and Black desi

The scene of chickpea crop in the world

The origin of chickpea is undefined, it has been reported presence in india since 2000 BC. Also recipes were found in some Roman manuscripts. The origin area is located between Asia and eastern Mediterranean. From this centres the chickpea was spread all around the Mediterranean coast, south east Asia and after in the americas.

Globally, it is one of the most cultivated pulses in terms of world production, with a total production of 14.2 million tons and an average yield of 0.96 t/ha [FAO, 2018]. As we can see in the Figure: 2 India is the first world producer with 6,3 millions of tones per year, followed by Turkey and Pakistan that produce around 0,5 millions of tones per year.

Talking about trades, Australia is the fist exporter in the world with almost 300k tones per year. India is just the fourth exporter with the quantity of 70k tones per year, Figure: 3. In the other hand, Imports, Indias is the first one consuming 200k tones per year. Spain is the fourth importer in the world, Figure: 3, being the unique Europe's country entering in global chickpea trades.

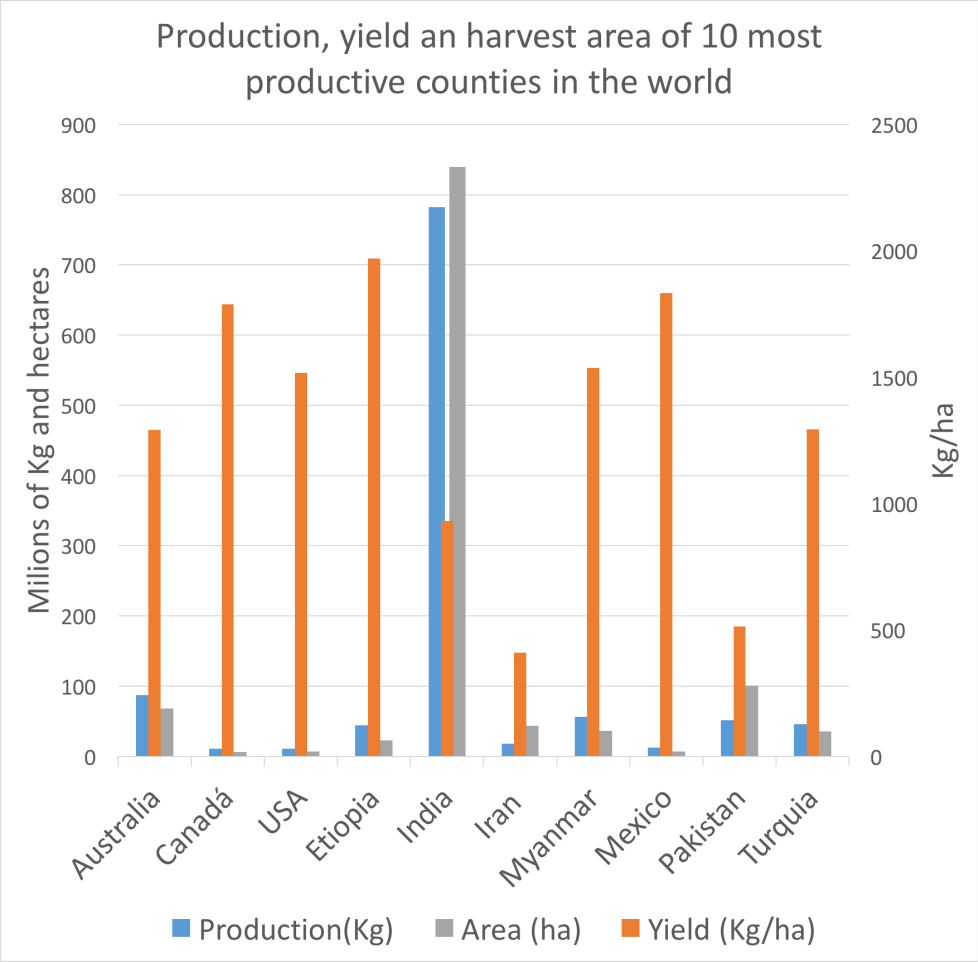

Figura 2: Grafic with production, yield and harvest area of 10 most producctive chickpea counties in the world. Blue bars are indicating the production in tones, orange bars the yeild in hectograms per hectare and grey bars indicate the cultivation area in hectare [FAO, 2018].

Figura 3: Grafic with top five countries exporters and importers. Blue bars indicate the exports in tones and orange bars the imports in tones [FAO, 2018]

Biological nitrogen fixation (BNF)

The biological nitrogen fixation is a process that just some of the prokaryotes can accomplish, transforming atmospheric N_2 into NH_3 form. There are three ways of nitrogen fixation, symbiotic, non-symbiotic but associative, and free living fixers. In agriculture production the most profitable ones are de symbiotic and the associative. The plants are taking profit of this process after the bacteria die and release the the nitrogen into the soil. More strong association is happening between legumes and few other plants, with rhizobium establishing the mutuality association in root nodules.

Biological nitrogen fixation is highly energy consuming process by bacteria, is why the legumes in symbiotic association are providing carbohydrates and other nutrient to the rhizosfera and the nodules. Nitrogen molecules is reduced to NH_3 under consumption of ATP and redox equivalents, the equation of the nitrogenase reaction to fix N_2 into NH_3 [Rogers et al., 2009], (Figure: 4).

$$N_2 + 8\,e^- + 8\,H^+ \xrightarrow{\text{nitrogenase}} 2\,NH_3 + H_2$$
$$16\,\text{MgATP} + 16\,H_2O \longrightarrow 16\,\text{MgADP} + 16\,\text{Pi}$$

Figura 4: Nitrogen fixation equation

The organisms that are carring out the nitrogen fixation are some aquatic cyanobacteria, *Azotobacter* as free-living, *Azospirillum, Bacillus, Burkholderia, Frankia* that have relationship with plants and the most important groups are *Rhizobium* that establish the symbioses with legumes, in Figure: 5 we found the information summarised.

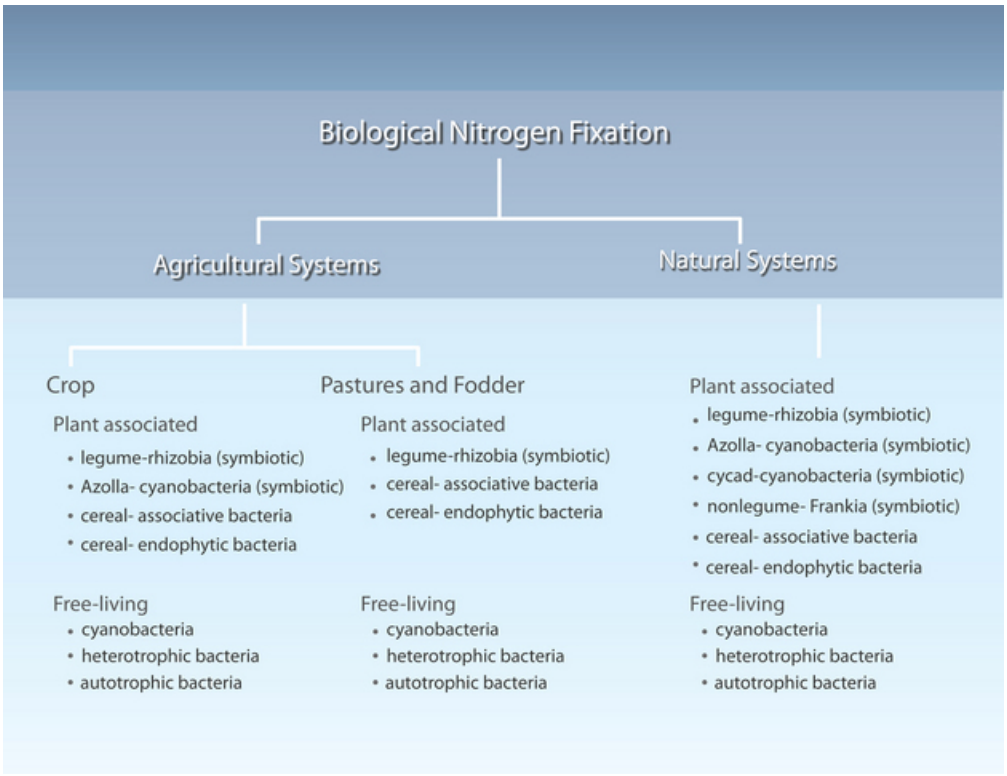

Figura 5: Nitrogen-fixing organisms found in agricultural and natural systems [Nat, 2016]

Rhizobia

Rhizobia is a huge group of soil and rhizosfera microorganisms.They belong to the subclass *Proteobacteria*. They are split in six genera: *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*,*Bradyrhizobium*, *Azorhizobium* and *Allorhizobium*[Lindström et al., 1995]. Also know as root-nodule bacteria, with the main characteristic that are able to establish mutually beneficial association with legume plants to fix atmospheric nitrogen, Figure: 6. This faculty give to the legume crops an independent soil fertilisation needs, increasing the productivity and soil nitrogen content. This association is happening in a specific structure called root nodules. The mutually exchange is going by the absorption of soluble short chain carbohydrate by the bacterias and in return their fix the nitrogen atmospheric to be used by the plant. Despite rhizobia are very similar ones to each other they are very genetically divers. There are more than 90 species and all this with different capacity of nodulation and nitrogen fixation. Every year are upcoming more than 20 new species of rhizobia because the scientist study directly the association with legumes founding new possible inoculants for the agriculture. Although probably underestimated, it is well established that many rhizobia are able to nodulate different legume genera [Johnston et al., 1978], and that many legumes can be nodulated by several rhizobial species, but not all the symbiosis are establish in mutualistic relationship. This is why its important to study and select the proper inoculants for every crop we are managing, in the Table: 1 we can see some of the common comercial inoculants.

Cuadro 1: Principal commercial bacterial inoculants for legume association

Legumes	**Bacteria nodulation**
Perennial clovers Most annual clovers	*Rhizobium leguminosarum bv. trifolii*
Field peas and vetch Faba beans and lenti	*Rhizobium leguminosarum bv. viciae*
Chickpea	*Mesorhizobium ciceri*
Soybeans	*Bradyrhizobium japonicum*
Peanuts Cowpeas, Mungbeans Pigeon peas	*Bradyrhizobium spp.*

Legume nodule formation

The process begin when the rhizobia are attracted by the flavonoids released by the legume roots. This flavonoids are released when the plant feel the nitrogen soil content is insufficient. After the rhizobia send some chemicals called Nod factors that cause the a deformation in the root hairs [Banfalvi and Kondorosi, 1989]. Then rhizobia create a structure called tubular infection thread for permitting the entrance to the root cells in the root hair. At the time the bacteria are inside the cell roots, the cells start a untypical division of the cortical cells crating the nodule, Figure: 6.

After the nodules are formatted, starts the symbiosis exchanges. The plant contribute to the bacteroids with some photo-assimilates like sugars and other nutrition factors. All this is transform by the bacteria into leg-hemoglobin, nitogenase and additional enzymes required for the N_2 atmospheric transformation in ammonia or ammonium, that is the asimilable form of nitrogen by plants, [Rolfe and Gresshoff, 1988]. Leg-hemoglobin making a binding with oxygen, and providing it to the nodule bacteria tissues, but keeping the O_2 pressure in super low levels, because the nitrogenase is denatured by the oxygen. Depending on the ambient, the plant species and bacteria the nodules are well formatted and eye visible after 2 weeks of germination. If the leg-hemoglobin contents are enough for the nitrogen fixation the nodule is showing a pink color while is cut in half. If not the nodule will appear green, grey or withe means that the nitrogen fixation is not happening or is not supplying the levels that the plant is demanding, so we have and inefficient nodule and kind of parasitic symbiosis.

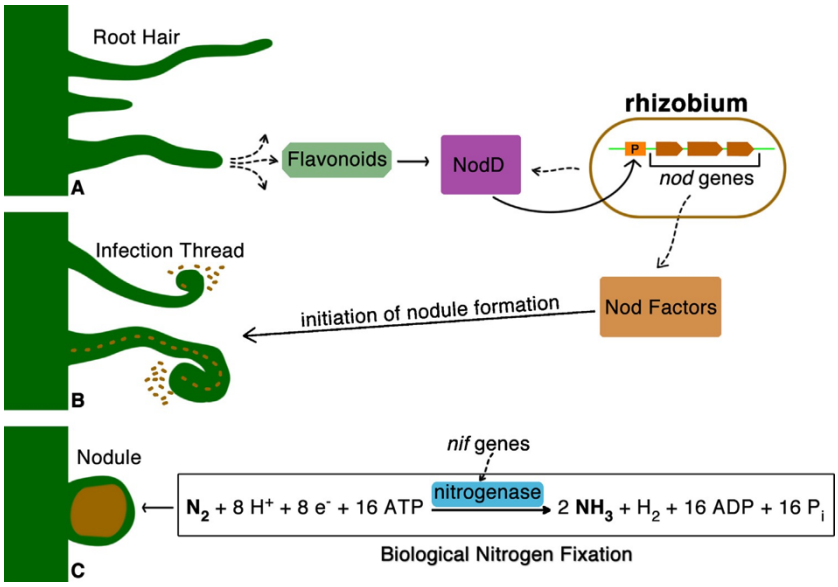

Figura 6: Schematic nodulation process and biological nitrogen fixation [Laranjo et al., 2014]

The *Nod* genes

The *Nod* genes are induced by the plant flavonoids propitiating the Nod factors which unleash the nodule formation. The type and the amount of the Nod factors are what determining the host specificity. Despite, rhizhobia have different *Nod* genes and produce different Nod factors can nodulated the same plant with and efficient mutual symbiosis. This is a highly conserved DeoxyriboNucleic Acid (DNA) sequence called *nod box* [Hong et al., 1987]. *NodC* gene that is the one we are going to study, it is involved in the flavonoid binding [Shearman et al., 1986]. This means that that different rhizobium have different *NodC* that respond to different flavonoids to nodulated certain types of legumes. The phylogenetic classification by the *Nod* genes presents some incongruences whit the trees based on the 16s rDNA region but shows some correlation with the host plant range [Laguerre et al., 2001]. This could be explained by the hypothesis of lateral gene transference of the symbiotic gene between the bacterias.

The *nifH* gene

The *nifH* gen that code the enzymes for atmospheric nitrogen fixation into ammonia or ammonium. The primary enzyme that encode the *nifH* sequence is the dinitrogenase reductase, more common known as nitrogenase, some other proteins involved in this process are also regulated by it. It is a gen found in the free-living bacteria and the symbiotic ones and it is well conserved in the genoma. The gen expression is being induced when the medium has low concentrations of nitrogen, also hypoxia conditions are needed, because nitrogenase is denatured by O_2.

In rhizobia the *nifH* phylogenesis is not matching with the 16s rDNA results, but has many similarities with the *nod* phylogenesis. This fact could be understand by the fact that this two genes are close in the genoma sequence, and in this location there is possible plasmid transmission or transposon in the region [Laguerre et al., 2001].

Objectives

The propose of this experiment was to study, select, and understand the functions of the microorganisms in symbiosis with the chickpea plant. Their nodulation activity, biofilm formation, root colonisation were observed and characterised.

Therefore, the primary objectives of this bachelor dissertation were to 1) Collect the samples of the further study via trap culture 2) Phylogenetic analysis and bacteria identification 3) Choose the samples that have significant interest for further studies.

The need for sustainable agricultural practices is revitalising the interest in biological nitrogen fixation and rhizobia-legumes symbioses, particularly those involving economically important legume crops in terms of food and forage. All this is with the aims to replace the pesticides, chemical fertilisers and other synthetic inputs by microorganisms. The final objective of the project is to develop field inoculum, with plant grow promoting bacteria, for the chickpea crop. After this previous laboratorial study the inoculum it will be tested in the field.

Material and Methos

Trap culture method, collection of nodulating bacterias

Soil for chickpea cultivation were collected from different fields, table: 2, preferable fields where chickpea or other legumes used to be cultivated. The samples were a big range of different characteristic soils, from the acidic to calcareous and different granulometry composition. The samples were homogenised to produce a single composite sample. The homogenisation was made mixing perlite 50 % (v/v) with the soil samples, to give better structure for the plants. After that chickpea seeds were planted in the different soils samples. 5L containers were used to to grow between 5 to 7 seeds. Plants were growing 4 to 6 weeks in greenhouse during the March and April period, Figure: 7 . In this fase the objective was to promote the nodulation of the plants to capture the bacterias involved in.

Cuadro 2: Origin of soil samples and crop precedent

Samples	**Origin**	**Crop precedence**
1	Elvas	Chickpea
2	Elvas	Chickpea
3	Elvas	Chickpea
4	Cabanões	Chickpea
5	UTAD	Chickpea
6	UTAD	Chickpea
7	UTAD	Chickpea
8	Barbacena	Chickpea
9	Aronches	Treacherous beans
10	Aronches	Black beans
11	Aranchoes	Crab beans
12	Barbacena	Treacherous beans
13	UTAD	Black beans
14	Mirandela	Black beans
15	Mirandela	Crab beans

Figura 7: Trap Cultures, soil samples from different origins, with chickpea growing

After to pulled up the plant from the soil, the roots were cleaned with water for removing the earth leftovers. Follow in order, nodules we searched and the plants were classified in two big groups the nodulated ones and the non nodulated. Adjacent the nodules of the nodulated plants were examined. One nodule of each plant was cut splitting the nodule in two parts. If the inside of the nodule becomes green it means that is an inefficient nitrogen fixation. The nodules that become pink, red or brown are efficient in nitrogen fixation Figure: 8, this color comes from the protein leg-hemoblobine. The leg-hemoglobine is the responsible to maintain the N-fixation, fixation of atmospheric nitrogen is accomplished by the enzyme nitrogenase, Figure: 6 . Nitrogenase i highly desnaturalized by the oxygen, but at the same time rhizobium bacteria are depending of oxygen to produce the nitrogenase. Leg-hemoglobin make an oxygen-binding that supply the bacterias the oxygen requirements but maintain the oxygen pressure inside the nodule in super low levels [Wittenberg et al., 1974].

Figura 8: Efficient nodule, with the pink/red color meaning the leg-hemoglobine is present

Isolation an cultivation of rhizobia from nodules

We isolated the nodules from the root by cutting about 0.5cm of root on each side of the nodule. After that, working in the laminar flow hood, the nodules were surface washed by immersing in a solution of 70 % of ethanol for 1 minute. Then the nodules were transferred in a solution of 3 % (v/v) sodium hypochlorite for 1 minute subsequent the nodules were washed in sterile distilled water in three different recipients. Following, the nodules were crushed in a petri plate with sterile glass rod, with the glass rod we lapful the milky substance that came out of the nodules in a petri dish with yeast mannitol agar (YMA) plate having Congo red and incubated at 28°Cin dark for three four days.

Nodulation and nitrogen fixation in-vitro assessment

The previous isolated bacteria were tested for the capacity of nodulation and nitrogen fixation [Evans, 1976]. This was conducted in vitro. Recipes were prepared with sterile 1:1 (v/v) of perlite and vermiculite and seedling broth. Chickpea seeds were surface sterile by of 10 min in 95 % ethanol followed by 3 min in 0.2 % $HgCl_2$-0.5 % HC_1. After exposure to the sterilising agent, seed was rinsed four times by decantation with sterile distilled water [Caetano-Anolles et al., 1990], and placed individually on the recipes for germination. The seeds were grown till the radices were 0.5-1.0cm. Next in line the inoculation solution was prepared inside of the laminar flow hod, 1ml of 0.8 % NaCl solution with half of the colony of the petri plates isolated where mixed on a eppendorf Figure: 9 and after dropped into the plants already germinated Figure: 10.

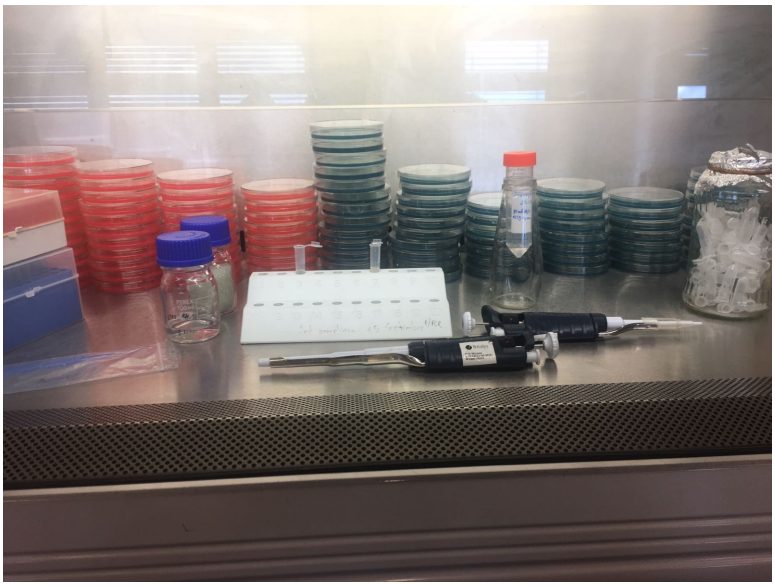

Figura 9: Laminar flow hod with the ingredients and the material for preparing the inoculation solution.

Figura 10: Plants growing on the in-vitro contents after the microorganism inoculation, some of them are bigger than the other because the date of plantation was different.

Molecular Characterization

The nodulating bacterias were phylogenetic characterized by applying different molecular methods:

Bacteria DNA extraction

A suspension of the bacteria was made in 1,5ml Eppendorf, with 250µL Cetyl trimethylammonium Bromide (CTAB) buffer plus 50µL of NaCl 5M and 1mm of crystal spheres. 5µL of lisozim was added and wait 20min at ambient temperature. We shaked the tubes in the FastPrep, 6.5m/s velocity, 40s, 3 times. The tubes were introduced in the hot water bath at 65°C30min. After 300µL of chloroform was added plus isoamilic alchool and shake the tubes. Centrifugation at 13000rpm for 15min was made. The supernatant was removed. 1µL of RNase was added, and 30 min of wait. 200µL of isopropanol was added and the tubes was keep overnight at -20°C. Then it was centrifugate at 13000rpm, 20min, 4°C. Supernatant was removed. 20µL of ice ethanol at 70 % was added to wash. Another centrifugation was made at 7000rpm, 5min, 4°C. Supernatant was removed and the pellet was obtained, Figure: 11. 20µL of ultra pure sterile water was added.

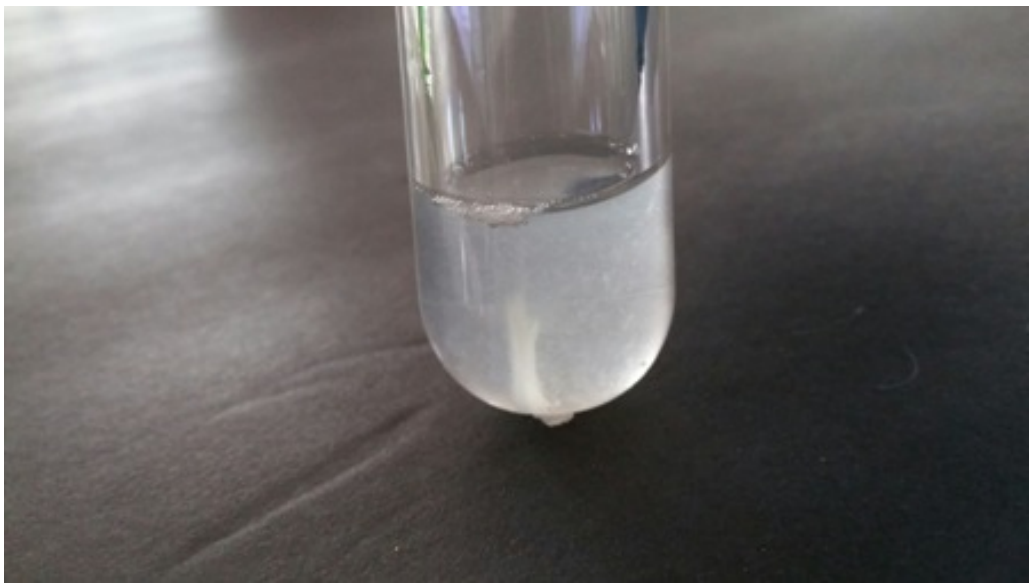

Figura 11: DNA pellet, after the centrifugation, ready for the PCR.

Pimers for PCR Amplification

The Polymerase Chain Reaction (PCR) was used to amplify gene fragments for DNA. Three genes were studied: 16s rDNA, N−acetylglucosaminyl transferase nodulation protein C *(nodC)* and nitrogenase iron protein *(nifH)*. The primers for amplification are shown in the table

Cuadro 3: Information of the primers used

Gene	Primer	Sequence	Reference
16S rRNA	F27	AGA GTT TGA TCM TGG CTC AG	[Weisburg et al., 1991]
	R1494	CTA CGG YTA CCT TGT TAC GAC	[Weisburg et al., 1991]
nodC	nodCMesoF	5'-CGA(CT)CG(AG)AG(AG)TTCAA(CT)TTC-3'	[Rivas-Martínez et al., 2002]
	nodCMesoR	5' -CT(CT)AATGTACACA(AG)(GC)GC-3'	[Rivas-Martínez et al., 2002]
nifH	nifHF genbank	5'-TACGGNAARGGSGGNATCGGCAA-3'	[Laguerre et al., 2001]
	nifHI genbank	5'-AGCATGTCYTCSAGYTCNTCCA-3'	[Laguerre et al., 2001]

PCR conditions

To execute the PCR amplifications we used a master mix following in the table 4. The PCR conditions were taken from the same publications of the primers, in the table 5. The reactions were made in the BIO-RAD T100 thermo circulator, the products were analysed in agarose gel 1 % using GelRed to see the DNA fragments.

Cuadro 4: Components of PCR master mix

Master mix	µL/tube
2x My Taq HS Mix (Bioline company)	7.5
Primer forward	1
primer reverse	1
DNA	5.5
Total	15

Cuadro 5: PCR conditions

PCR	**Temperature(°C)**	**Duration**	**Cycle**
16s rRNA	95	3 min	1x
	95	30 sec	35x
	65	30 sec	
	72	30 sec	
	72	7 min	1x
	4		1x
nodC	95	2 min	1x
	95	30 sec	34x
	49	30 sec	
	72	1 min	
	72	10 min	1x
	4		1x
nifH	95	3 min	1x
	94	1 min	30x
	55	1 min	
	72	2 min	
	72	5 min	1x
	4		1x

DNA Sequencing

The samples that reach the expected size on the electrophoresis test were send to the STABVIDA company that sequenced it. DNA sequence data were obtained via BLAST servidor (NCBI database) and edited and assembled using MEGA6 and Chomas.

Phylogenetic analyses

All the sequences obtained were certify using Chromas Pro software (version 1.5). Sequences obtained were compared from those from GenBnak using BLAST servidor [Altschul et al., 1997]. We include in all gens, (16s, *NodC* and *nifH*), dataset sequences from the National Center for Integrative Biomedical Informatics (NCIBI). We analysed the gens separately. Using another software call MEGA6 we assembled and many alignments were performed using CLUSTAL W [Tamura et al., 2013]. Phylogenetic analysis was inferred by using the neighbour-joining method [Saitou and Nei, 1987] using the kimura s-2 parameter model [Posada and Crandall, 1998] implemented in the MEGA 6 software package. Tree robustness was calculated with the 1000 replication of bootstrap.

Results and discussion

Trap culture collection

On the following table: 6 we found the trap culture results. All the plants present nodules.If the inside of nodule was red means that the leg-hemoglobin was present, so were nitrogen fixation efficient. On the other side the nodules that present white or green inside were not efficient.

Cuadro 6: Trap culture results, indicating the nodule presence and the efficiency of it

Samples	Origin	Crop precedence	Nodulation	Inside color of nodule
1	Elvas	Chickpea	Yes	Red
2	Elvas	Chickpea	Yes	White
3	Elvas	Chickpea	Yes	Red
4	Cabanões	Chickpea	Yes	Red
5	UTAD	Chickpea	Yes	Red
6	UTAD	Chickpea	Yes	Green/Red
7	UTAD	Chickpea	Yes	Red
8	Barbacena	Chickpea	Yes	White
9	Aronches	Treacherous beans	Yes	Green/Red
10	Aronches	Black beans	Yes	Red
11	Aranchoes	Crab beans	Yes	Red
12	Barbacena	Treacherous beans	Yes	Red
13	UTAD	Black beans	Yes	Red
14	Mirandela	Black beans	Yes	Red
15	Mirandela	Crab beans	Yes	Red

In-vitro results

The in-vitro assessment was conducted to prove the Koch's postulates, [Evans, 1976]. As Helne Kock said, the firts agent isolated has to be inoculated and reisolated to accept that was the one that was provoking the first symptoms. In the following table (7) we found the parameters that were evaluated on the in-vitro study. The parameters are the incoming, Weeks of growing, Height of the plants, Number of nodules per plant and The inside color of the nodules of each plant.

The weeks of growing, height and number of nodules could had some correlation between it. But we were not focus on this relation.

Some of the samples (2, 8, 9) had absence of leg-hemoglobine meaning no efficient nitrogen fixation. The 11 sample that in the first collect appears as red nodules now after the re-inoculation is green.

Cuadro 7: In-vitro results

Samples	Weeks of growing	Height (cm)	Number of nodules	Inside color of nodule
1	5	37	5	Red
2	5	33	7	Green
3	6	43	4	Red
4	6	44	5	Red
5	4	28	3	Green
6	5	35	3	Red
7	5	42	5	Red
8	5	39	6	Green
9	4	36	10	Green
10	5	46	10	Red
11	6	34	7	Green
12	6	43	5	Red
13	6	39	4	Red
14	4	37	3	Red
15	5	45	8	Red

Phylogenetic results of the three DNA sections, 16s, *nodC* and *nifH*

All the sequences from the bacterias isolated in trap culture study were clustered in the software MEGA6. Also the sample sequences were clustered with the most close sequences found in the NCIBI. An alignment of the sequences was made to find the variation of the sequences. This permit us to estimate the relation of the sequences.

All the phylogenetic trees (Figure: 12, 13 and 14)are made of branches, the branches length represents genetic distance, also meaning a mesure of time of divergence of the species. The scale bar under each tree represent percentage of mutation of the nucleotides of homologous sequence positions. The numbers on the branches are the level of statistical confidence that we have in the shape of the tree. The percentages 50 % or higher of 1000 bootstrap trials that support each topological element are indicated beside the nodes.[Schloss and Handelsman, 2005].

On the table: 8 we found the closest identify organism related to our samples, taking over as most probable identification for our samples.

16s region phylogenetic tree

On the 16s genetic region tree (Figure: 12) we can differentiate 5 different clusters. All of them with more than 50 % of tree robustness. The main groups are: *Rhizobium/Sinorhizobium*, *Mesorhizobium*, *Bacillus*, *Enterobacter*, *Burkholderia*. *Burkholderia* is the closest group to the common ancestral, but also the one farther to the others. The *Rhizobium/Sinorhizobium* and *Mesorhizobium* are the major groups, with 8 of the samples of the study on it. The other seven samples are distributed on the remaining clusters.

NodC phylogenetic tree

The *nodC* phylogenetic tree (Figure: 13) is a very strange tree, with no clusters very differentiated. We only see one big group of identified organism, *Mesorhizobium* group. Inside this group we find two *Sinorhizobium sp.* organism and many type of subspecies of *Mesorhizobium*. Our samples

are not really clear classified or closest to the NCIBI identified organism. If we take a look on the scale of the tree is really low. Indicating that just 0,02 per 10 nucleotides are different, helping us to explain that in this tree is really difficult to find differences between the species. Also if we reefer the level of confidence, the robustness of the shape, we find some branches with less than 50 % of bootstrapping. Accepting that this branches are not statistical well draw.

NifH phylogenetic tree

Phylogenetic tree of the *nifH* DNA section (Figure: 14) is conformed with two clear clusters. The major one with all the *Mesorhizobium* subspecies and 7 of the studied samples. On the other hand there is the *Rhizobium* group with 3 of the samples clearly on it, and 4 samples with no certain membership to any group. Looking at the length branches all of them are quite short indicating really short differences on the sequences of the members. In addition the statistical level of confidence of some of the branches is too low (50 %) to accept that are suitably drawn.

Cuadro 8: Identification of the samples by the nearest organism on the phylogenetic trees

Sample	**Identify Organism 16s region**	**Identify Organism nodC**	**Identify Organism nifH**
1	*Agrobacterium tumefaciens*	*Indeterminated*	*Indeterminated*
2	*Rhizobium mesosinicum*	*Mesorhizobium*	*Indeterminated*
3	*Sinorhizobium sp.*	*Indeterminated*	*Rhizobium*
4	*Bacillus altitudinis*	*Mesorhizobium*	*Indeterminated*
5	*Burkholderia sp*	*Mesorhizobium*	*Indeterminated*
6	*Mesorhizobium sp.*	*Mesorhizobium*	*Mesorhizobium ciceri*
7	*Bacillus altitudinis*	*Mesorhizobium*	*Mesorhizobium*
8	*Mesorhizobium sp.*	*Mesorhizobium*	*Mesorhizobium ciceri*
9	*Rhizobium sp.*	*Mesorhizobium*	*Mesorhizobium*
10	*Burkholderia sp.*	*Indeterminated*	*Rhizobium*
11	*Burkholderia sp.*	*Mesorhizobium*	*Rhizobium*
12	*Mesorhizobium sp.*	*Mesorhizobium*	*Mesorhizobium ciceri*
13	*Burkholderia sp.*	*Mesorhizobium*	*Mesorhizobium ciceri*
14	*Enterobacter sp.*	*Mesorhizobium*	*Indeterminated*
15	*Mesorhizobium sp.*	*Mesorhizobium*	*Mesorhizobium ciceri*

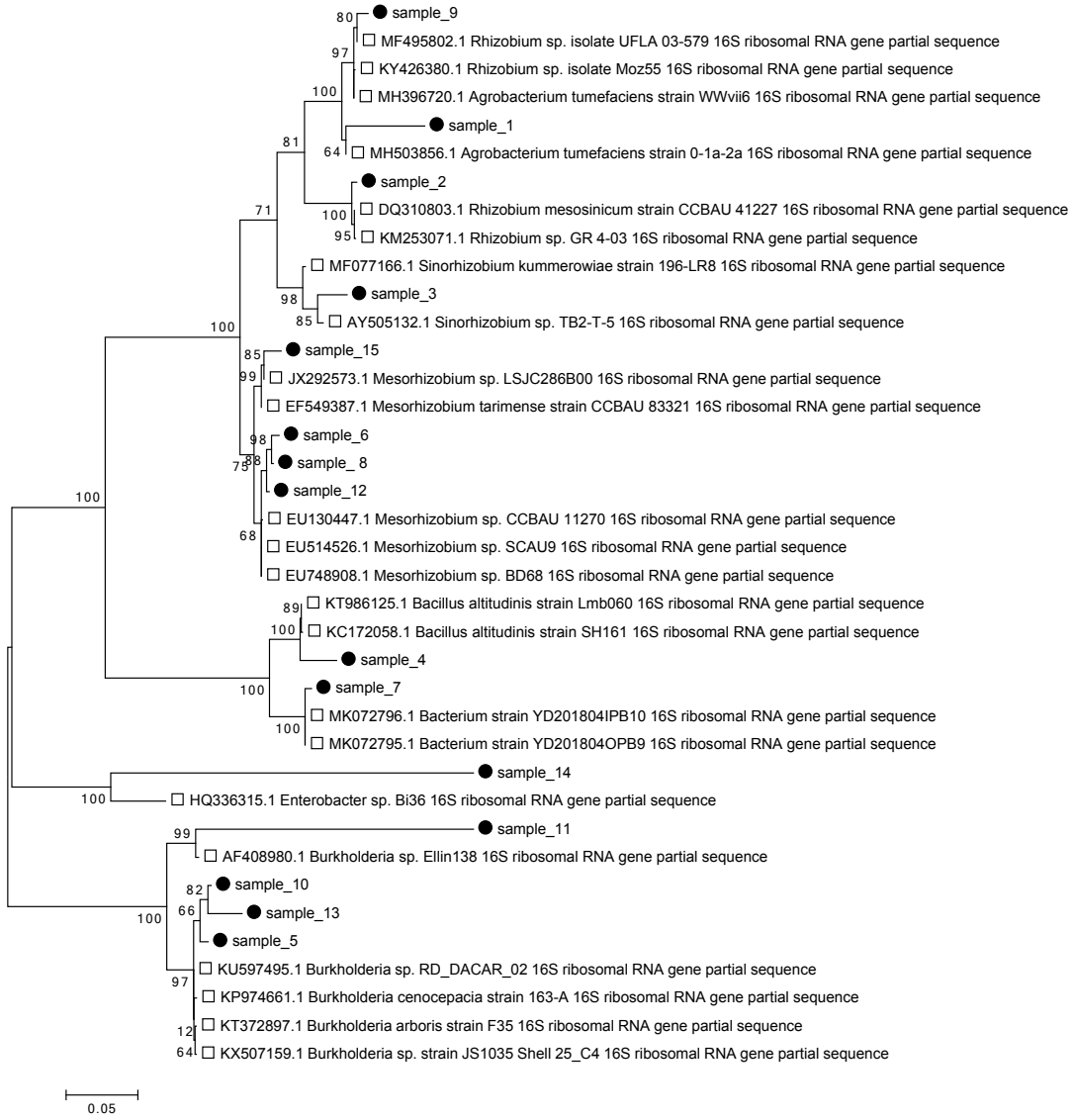

Figura 12: 16s phylogenetic tree gene of bacterial isolated from the trap culture. • indicate the samples strains focused on in current study. □ indicate the data set sequences obtained in NCIBI after the use of BLAST algorithm. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-8065.7100) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 38 nucleotide sequences. All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1101 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

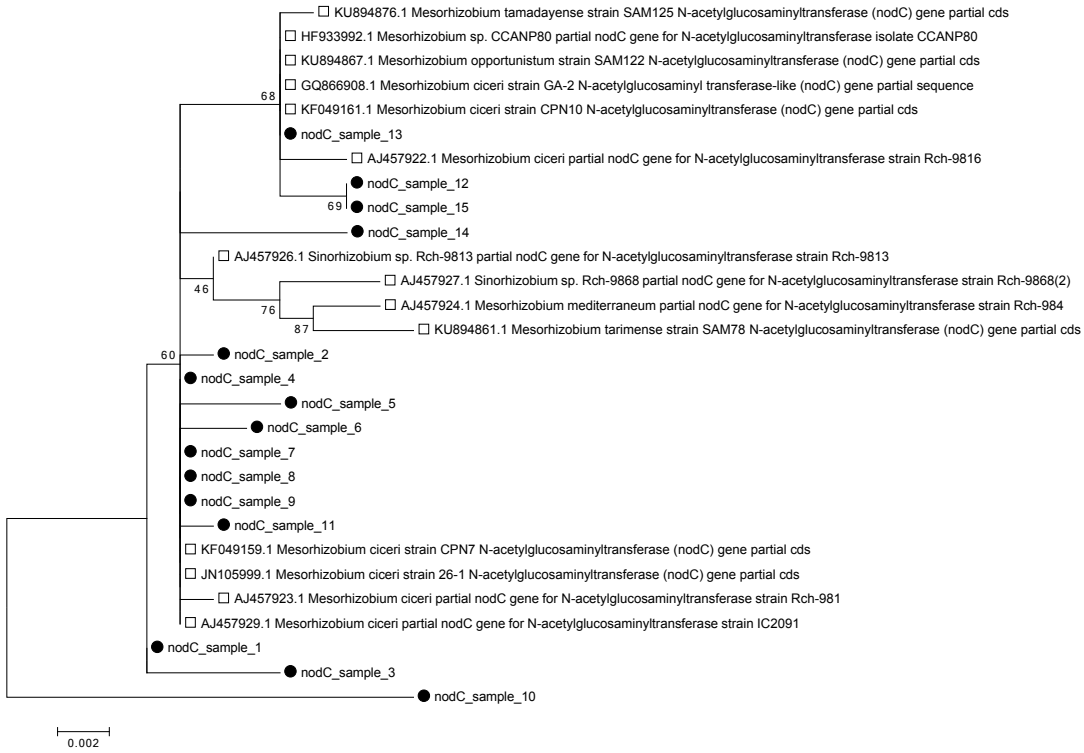

Figura 13: *NodC* phylogenetic tree gene of bacterial isolated from the trap culture. • indicate the samples strains focused on in current study. □ indicate the data set sequences obtained in NCIBI after the use of BLAST algorithm. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1455.9027) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 779 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

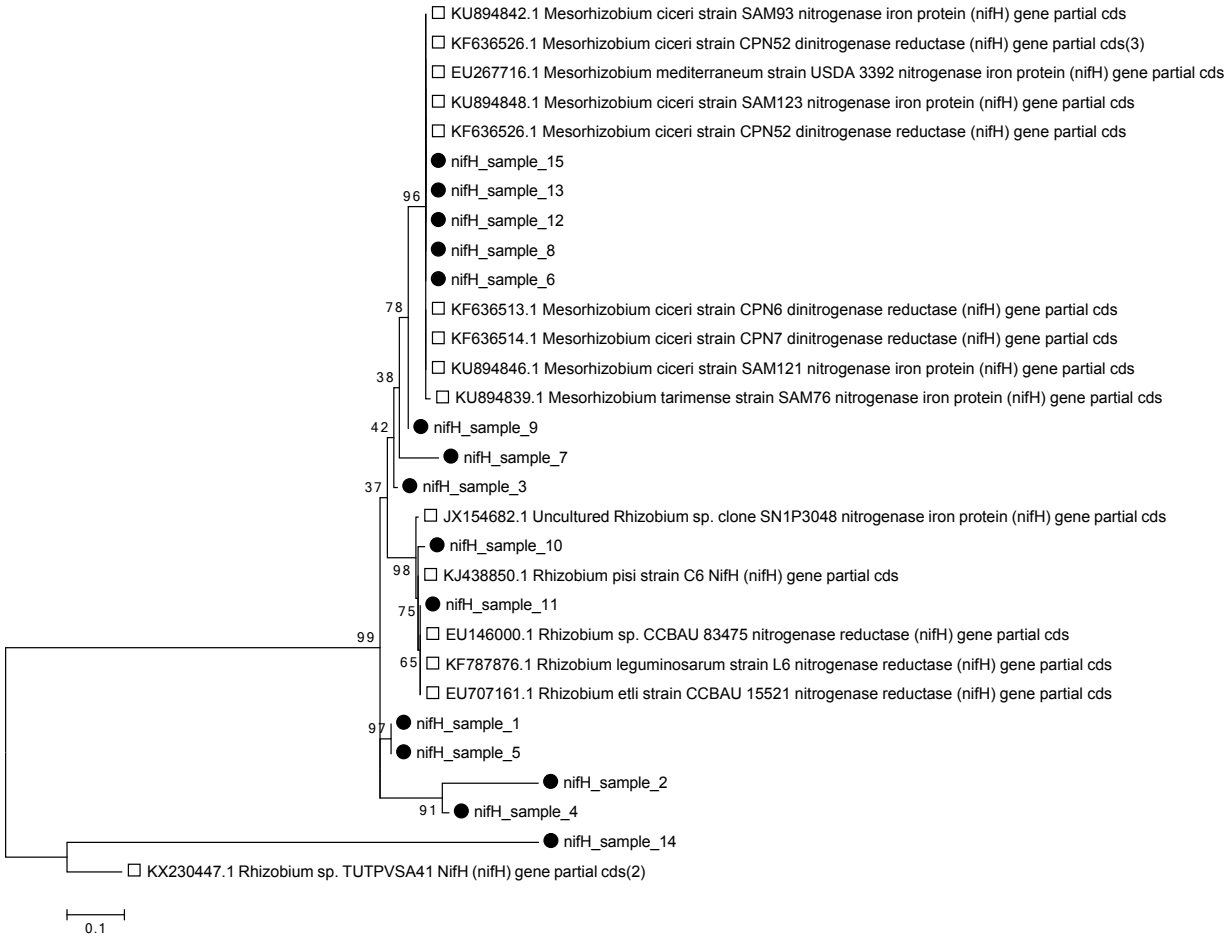

Figura 14: *NifH* phylogenetic tree gene of bacterial isolated from the trap culture. • indicate the samples strains focused on in current study. □ indicate the data set sequences obtained in NCIBI after the use of BLAST algorithm.The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1669.3046) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 266 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Discussion

Is well known that many rhizobia are able to nodulate different legume genera, and that legumes also can be nodulated by a huge range of rhizobial species. Host specificity is determinate by the Nod factor, the amount and the type. However, rhizobia can nodulate the same plant having

different *nod* genes and producing different Nod factors, [Laguerre et al., 2001].Is already prove that different genetical micro-symbiont can be isolated from the same legume species or genus. Examples include *Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* associated with soybean [Zotov et al., 2012] and *Mesorhizobium ciceri*, *M. tianshanense*, *M. amorphae* isolated from *Cicer arietinum* [Rivas et al., 2007].

On the present study, across the sequences analyses of the 16s rRNA gene, *nodC* gene and *nifH* gene, we were able to identify and characterise four samples (6, 8, 12 and 15) as a *Mesorhizobium* strains, because in all studied regions were appearing as it. In addition there are five samples (7, 9, 11, 13 and 14) that are well classified by three regions but at least showing differences in one of the regions. Incongruences between the phylogenetic identification via 16s region and *nodC* were usualy happening [Laguerre et al., 2001]. This phenomenon could be explained by the horizontal gene transfer between the bacterias, horizontal transfer has been occurred on major *rhizobial* groups studied by [Sullivan et al., 1995]. Referring to *nifH* gene was also not matching in some samples (3, 7, 9, 10, 11, 13) with the 16s gene. But it present concordance with the identification via *nodC* (7, 9, 13), explained by the [Laguerre et al., 2001], saying this two genes are close in the genoma and some transmission, transport or exchange between it, could be happened. In all cases this bacterias are classified as *Mesorhizobium* at list in one of the regions. The other reminding six samples (1, 2, 3, 4, 5 and 10) were not well identify on one region or more. [Fisher et al., 1985] suggest that *nod* and *nif* genes are identical or close linked in rhizobium species, provoking a difficult lecture of them. This cause of this indetermination we found is unclear, because any difficult were happened during PCR with the selected primers. But always the material and methods could be revised and proper optimised.

Conclusions

To conclude and summarise all this work we are going to review if the main objectives were accomplish. The collection of the samples via trap culture it has shown a success in all the samples studied. Accepting that is an useful procedure to keep and know for similar projects.

The phylogenetic analysis and the samples identification was the most important objective of the study. In more than 50 % of the samples the identification and the position on the phylogenetic trees are clear but in some cases were unclear, explained and talk about in the discussion. The procedures were well executed but always extra repetitions of the experiments had to be taken.

The samples we choose in conclusion of the study are, also are the ones with more interest for further studies, (6, 7, 11, 12, 13, 14, 15), the parameters we use to select the samples were: positive in the the in-vitro assessment as green nodule and identification on the three DNA regions. Although with this present results we can not choose any sample as the best one for our propose we can say that probably some of the *Mesorhizobium* strain is going to accomplish all the demands that we had.

The study has to go further to select the best bacterial inoculant for the chickpea. The samples we had selected might be test to find out other promoting growth proprieties as Indole-3-Acetic Acid (IAA) Hydrogen Cyanide (HCN) or Phosphorus (P) solubilization, siderophore, [Etesami et al., 2009] and [Ahmad et al., 2008]. The most interesting samples are going to pass to the next step study. The project might be end with a field study [Antoun et al., 1998] and pot study [Peix et al., 2001] in simultaneous. The select bacterias will be inoculate. Also the water input will be regulated for each inoculant replica at least three different doses(insufficient or inexistent, livelihood and field capacity). Growth parameters of the chickpea plant will be take [Chabot et al., 1996]. Replicas will be compared and conclusion will be extracted.

Also the new genetic technologies as CRISPR should be use to develop new bacterias or organism inoculants for replace the chemical fertilisers [Wang et al., 2017]. The future is unknown but the tools for the future are ready.

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