University of Trás-os-Montes and Alto Douro

Characterization and selection of microbial symbionts of faba bean (*Vicia faba* L.) and cowpea (*Vigna unguiculata* (L.) Walp.) for development of inoculants

PhD thesis

Agricultural Production Chains - From Fork to Farm

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To my sons Rodrigo and Rafael,

To my husband Fernando,

To my parents Adélia and Alfredo,

To my family…

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RESUMO

A agricultura global deve duplicar a produção de alimentos até 2050 por forma a alimentar a crescente população mundial. Neste sentido, alimentos com um elevado valor nutritivo, como por exemplo as leguminosas (proteína, minerais, vitaminas e compostos bioativos) aparecem como uma resposta a esta necessidade. É, no entanto, essencial aumentar a sua produtividade. Ao mesmo tempo, é também necessário reduzir a aplicação de fertilizantes inorgânicos, devido ao elevado impacto negativo que estes têm para o ambiente. Para atingir estes objetivos, é essencial tirar proveito das múltiplas interações benéficas que ocorrem entre as plantas e os microrganismos.

Os microrganismos benéficos presentes no solo, nomeadamente os rizóbios e os fungos micorrízicos arbusculares, em simbiose com plantas leguminosas, resultam numa simbiose tripartida e podem ser uma ferramenta biológica para melhorar a produção das culturas, através da fixação biológica de azoto e da absorção de fósforo do solo. Esta simbiose também aumenta a resistência das culturas à seca e às altas temperaturas, melhora a produtividade e a qualidade das culturas e a fertilidade do solo e diminui a incidência de ervas daninhas, doenças e pragas, sem os impactos negativos para o ambiente provocados pela aplicação de fertilizantes químicos.

Neste sentido, os objetivos deste trabalho foram selecionar estirpes melhoradas de *Rhizobium leguminosarum* e *Bradyrhizobium* spp. para melhorar a fixação biológica de azoto e o desempenho das cultivares de fava e feijão-frade, e efetuar a caracterização fenotípica e genotípica dos simbiontes microbianos usando uma abordagem polifásica baseada em propriedades fenotípicas e na análise molecular.

No presente trabalho, foi assim efetuada a identificação molecular dos rizóbios presentes em plantas de feijão-frade e fava recolhidas em diversas regiões de Portugal com diferentes condições climáticas e diferentes tipos de solo, utilizando uma abordagem de "Multilocus Sequence typing" (MLST) com 9 genes ("housekeeping" e simbióticos), a fim de obter informações ao nível da espécie e da simbiovar, uma vez que a amplificação da região 16SrDNA isoladamente não providenciou poder de resolução suficiente. Após a identificação molecular, foram realizados estudos *in vitro* para verificar a capacidade infectiva dos isolados (postulados de Koch) e para selecionar os melhores inóculos para cada cultura, os quais foram depois testados em condições de estufa, com o objetivo de avaliar os efeitos das inoculações simples e das co-inoculações com os microrganismos selecionados no crescimento, produtividade e conteúdo em proteína da respetiva leguminosa.

Foi identificada uma elevada diversidade de rizóbios nos diferentes campos e regiões. Para o feijão-frade, foram selecionadas duas estirpes de rizóbios, *Bradyrhizobium* sp. e *Bradyrhizobium elkanii*. Para a faveira, foram selecionadas as bactérias *Rhizobium laguerreae*

e *Burkholderia* sp.. Relativamente aos inóculos micorrízicos, uma mistura de *Rhizophagus irregularis* BEG140, *Funneliformis geosporum* BEG199 e *Claroideoglomus claroideum* BEG210 (1: 1: 1) foi desenvolvida e preparada pela Symbiom (Sázava, República Checa) para a cultura da faveira. Para o feijão-frade, o fungo micorrrízico (*Claroideoglomus claroideum* BEG210) foi cedido pelo Dr. Rui Oliveira, da Universidade de Coimbra, Portugal.

No trabalho realizado em estufa com inoculação e co-inoculação com *Rhizobium laguerreae* e AMF, as plantas de faveira inoculadas com a bactéria mostraram um aumento significativo no número de folhas, área foliar, massa foliar por área e razão de área foliar, bem como em todos os parâmetros de produtividade avaliados. A inoculação simples dessas plantas com AMF também aumentou significativamente os parâmetros de produtividade. A co-inoculação mostrou melhorias significativas na proporção da área foliar e em todos os parâmetros de produtividade quando comparado com o controlo, mas não foi significativamente diferente das inoculações individuais.

Nos estudos com feijão-frade, em condições de estufa, usando solo não esterilizado, a co-inoculação das plantas com *Rhizobium* sp. e AMF, *Bradyrhizobium elkanii* e AMF e *Bradyrhizobium* sp. e AMF aumentaram o teor de proteína das sementes em plantas sujeitas a déficite hídrico (25% da capacidade de campo) em 13, 17 e 30%, respetivamente.

Considerando todas as análises realizadas neste trabalho em ambas as culturas, é possível concluir que a inoculação simples e combinada de plantas leguminosas com os microrganismos selecionados mostrou ter um grande potencial como ferramenta biológica para melhorar o crescimento e a produtividade das plantas leguminosas sujeitas a stress abiótico, mitigando os efeitos das alterações climáticas e reduzindo a necessidade de aplicação de fertilizantes de síntese.

ABSTRACT

Global agriculture has to double food production by 2050 in order to feed the world's growing population. In this sense, food with a high nutritional value, such as the leguminous plants (protein, minerals, vitamins and bioactive compounds) appear as an answer to this need. However, it is crucial to increase its productivity. At the same time, it is also necessary to reduce the application of inorganic fertilizers, due to the high negative impact they have on the environment. To achieve these goals, it is essential to take advantages from the multiple beneficial interactions that occur between plants and microorganisms.

Beneficial microorganisms present in the soil, namely rhizobia and arbuscular mycorrhizal fungi, in symbiosis with leguminous plants, results in a tripartite symbiosis and can be a biological tool to enhance crop production, through biological nitrogen fixation and phosphorus uptake from soil. This symbiosis also increases the resistance of crops to drought stress and high temperatures, improves crop productivity and quality and soil fertility and decreases the incidence of weeds, diseases and pests, without the negative impacts in the environment provoked by chemical fertilizer inputs.

In this sense, the objectives of this work were to select improved strains of *Rhizobium leguminosarum* and *Bradyrhizobium* spp. for enhanced biological nitrogen fixation and field performance on cultivars of faba beans and cowpeas, and to perform the phenotypic and genotypic characterization of microbial symbionts using a polyphasic approach based on phenotypic properties and molecular analysis.

In the present work, the molecular identification of rhizobial bacteria present in cowpea and faba bean plants collected from several regions of Portugal with different climatic conditions and different types of soil was performed using a Multilocus Sequence typing" (MLST) approach with 9 genes (housekeeping and symbiotic genes), to obtain information at species and symbiovars level, since the amplification of 16SrDNA region alone did not provide enough resolution power. After the molecular identification, *in vitro* studies were performed to check the ability of the isolates to nodulate other plants (Koch's postulates) and to select the best inoculants for each crop, which were after tested under greenhouse conditions, with the purpose of evaluating the effects of single and co-inoculation with the selected microorganisms on the growth, yield and protein content of the respective leguminous plants.

High diversity of rhizobial bacteria was identified in different fields and regions. For cowpea plants, were selected two rhizobial strains, *Bradyrhizobium* sp. and *Bradyrhizobium elkanii*. For faba bean, were selected *Rhizobium laguerreae* and *Burkholderia* sp.. Regarding the mycorrhizal inoculants, a mix of *Rhizophagus irregularis* BEG140, *Funneliformis geosporum* BEG199 and *Claroideoglomus claroideum* BEG210 (1 : 1 : 1) was developed and prepared by Symbiom (Sázava, Czech Republic) for the faba bean crop. For cowpea, the

mycorrhizal fungi (*Claroideoglomus claroideum* BEG210) was provided by Dr. Rui Oliveira, from de University of Coimbra, Portugal.

In the greenhouse work developed with inoculation and co-inoculation with *Rhizobium laguerreae* and AMF, faba bean plants single inoculated with the bacteria showed a significant increase in the number of leaves, leaf area, leaf mass per area and leaf area ratio, as well as in all evaluated yield parameters. Single inoculation of these plants with AMF also significantly increased the yield parameters. Co-inoculation showed significant improvement in leaf area ratio and in all productivity parameters when compared with the control, but it was not significantly different from the individual inoculations.

In the studies with cowpea, under greenhouse conditions, using non-sterilized soil, the co-inoculation of plants with *Rhizobium* sp. and AMF, *Bradyrhizobium elkanii* and AMF and *Bradyrhizobium* sp. and AMF increased the crude protein content of the seeds in plants under drought stress (25% of field capacity) in 13, 17 and 30%, respectively.

Considering all analyses performed in this work in both crops, it is possible to conclude that single and combined inoculation of leguminous plants with selected microorganisms showed great potential as a biological tool to improve the growth and yield of leguminous plant under abiotic stress, mitigating the effects of climate change and reducing the need for chemical fertilizer inputs.

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LIST OF ABBREVIATIONS AND SYMBOLS

µg- Microgram

µL- Microlitre

A- Net CO_2 assimilation rate

A/gs- Intrinsic water-use efficiency

AMF- Arbuscular mycorrhizal fungi

BLASTn- Basic Local Alignment Search Tool (by nucleotides)

BTB- Bromothymol blue

CFU- Colony-forming unit

Chl a- Chlorophyll a

Chl b- Chlorophyll b

Ci- Intercellular CO_2 concentration

cm^2- Square centimeter

CR- Congo red

CTAB- Cetyltrimethylammonium bromide

DNA- Deoxyribonucleic Acid

E- Transpiration rate

EDTA - Ethylenediaminetetraacetic acid

Fig.- Figure

g- Gram

gs- Stomatal conductance

ha- Hectare

kg- Kilogram

KOH- Potassium hydroxide

L- Litre

LAR- Leaf area ratio

LMA- Leaf mass per area

mg- Milligram

Min- Minute

mL- Millilitre

N- Nitrogen

NaCIO- Sodium hypochlorite

NaCl- Sodium chloride

ng- Nanogram

ºC- Degrees Celsius

P - Significance value of probability

PCA- Principal component analysis

PCR - Polymerase chain reaction

PPFD- Photosynthetic photon flux density

RNA - Ribonucleic acid

rpm - Rotations per minute

rRNA - Ribosomal RNA

s – Second

TBE - Tris-borate-EDTA

TE - Tris-EDTA buffer

Tris-HCl - Tris-Hydrochloride buffer

USA- United States of America

UV- Ultraviolet

YMA- Yeast mannitol agar

CHAPTER I

GENERAL INTRODUCTION AND OBJECTIVES

Chapter I- General Introduction and Objectives

In the recent years, the global demand for food and agricultural crops is increasing, due to the rapid increase in global population. Moreover, until 2050, food demand is expected to increase anywhere between 59% to 98% (Valin *et al*., 2014). This growth causes dietary changes such as eating more protein and meat (Valin *et al*., 2014). The high nutritional value (protein, minerals, vitamins and bioactive compounds) of leguminous plants make them a promising alternative to help to solve this problem. As some legume species can be grown to produce high quality protein in a short growth cycle, two cropping seasons can be produced in a year.

Legumes are relatively low demanding crops with a low production art and well adapted to a wide range of agricultural production systems. The biodiversity of legume crops around the world, its soil and environment adaptability and their multiuse as food products, represent a great opportunity to improve food production, and particularly vegetable protein, under a more sustainable cropping system.

Apart from a broad human and animal consumption, legumes have also important advantages to the soil, since in symbiosis with rhizobial bacteria, they can fix atmospheric nitrogen, thus reducing the need of nitrogen fertilizer inputs, with positive effects in the environment and production costs, on soil fertility improvement and a decrease in the incidence of weeds, diseases and pests (Peoples *et al*., 1995).

Legume crops are also better adapted to climate changes, being considered as a good strategy of mitigation. Indeed, it is consensual that climate change, in particular, water scarcity, rising global temperatures and extreme weather, will have severe long-term effects on crop yields (Vadez *et al*., 2012). In this sense, the symbiosis between leguminous plants, rhizobia and arbuscular mycorrhizal fungi can improve crop production, by the increase of plant resistance to high temperatures and water deficit (Oliveira *et al*., 2017).

Although the effects of single and co-inoculation with beneficial microorganisms have been widely evaluated, there are just few studies on cowpea and faba bean, particularly in Portugal. In fact, this Ph.D. thesis includes the study that represents the first analysis on the phylogenetic diversity of indigenous cowpea- and faba bean-nodulating rhizobia using Multilocus Sequence Analysis (MLSA). Moreover, this study is of extreme importance in Portugal because almost 90% of the consumed dried leguminous are imported, and the yield increase can lead to savings of around 10 million euros per year (Rosa, pers. comm.).

Within leguminous plants, cowpea and faba bean crops were studied in this work, due to their symbiotic relationship establishment with different genera of rhizobia.

As these symbioses are host-specific, to optimize the biological nitrogen fixation, it is necessary to select bacteria well adapted to the plant genotype, as well as to the particular edapho-climatic conditions. Faba bean is one of the most efficient nitrogen (N)-fixing legumes that can meet all of their N needs through biological nitrogen fixation (BNF) and this crop usually establishes symbiosis with fast-growing rhizobia of the species *Rhizobium leguminosarum* sv*. viciae*, *R. fabae*, *R. laguerreae*, *R. etli* and *Agrobacterium tumefaciens* (Youseif *et al.*, 2017). On the other hand, in this respect, cowpea is a promiscuous legume, able to establish efficient symbiosis with diverse bacteria, mainly slow-growing rhizobial species belonging to the genus *Bradyrhizobium* (Jaiswal and Dakora, 2019). Moreover, it is predicted the increase of protein crops for around 46% until 2030 (FAO). Since cowpea and faba bean are very widely consumed in Portugal and well adapted to portuguese soil and environmental conditions, further studies that can improve our knowledge on their production and yield increase and adaptability to climate changes, are obviously quite relevant.

The activities conducted in this work were part of the Work Package 3 of the "*European project EUROLEGUME- Enhancing of legumes growing in Europe through sustainable cropping for protein supply for food and feed*", which aimed the sustainable production of legumes by ensuring improved varieties, better microbial inoculants to support nitrogen fixation and plant growth, and developed innovative foods and feeds, turning EU more competitive. In addition, this work was also part of the National Project "*PTDC/AGR-TEC/1140/2014- Legume seed coating with beneficial microorganisms for increased productivity and resilience under climate change conditions*", funded by FCT-Portuguese Foundation for Science and Technology. Simultaneously, the work was also included in the International PhD program "*Agricultural Production Chains- From Fork to Farm (AgriChains)*", since these biological technologies can improve directly the productivity of leguminous plants, and indirectly the productivity of other crops, contributing to the improvement of the production chains, in sustainable agriculture.

The main objective of this work was to collect, identify and select beneficial microorganisms to improve the biological nitrogen fixation and phosphorus uptake of two main legume crops of high relevance for the agricultural systems worldwide, namely cowpea and faba bean, through a synergetic effect of both rhizobia and arbuscular mycorrhizal fungi (AMF).

To achieve this main objective, specific tasks were designed and developed as following:

- Collection of rhizobial bacteria from cowpea and faba bean root nodules, in several regions of Portugal with different edapho-climatic conditions;

- Phenotypic and genotypic characterization of bacteria using a polyphasic approach based on phenotypic properties and molecular analysis. The aim of this task was to identify the collected bacteria at species level to understand the biodiversity of rhizobial bacteria existing over Portugal;
- Selection of rhizobial strains and AMF for enhanced biological nitrogen fixation, and consequently legume growth and yield. The aim of this task was the selection of the best strains of *Rhizobium leguminosarum*, *Bradyrhizobium* spp. to improve biological nitrogen fixation, legume growth and yield under field conditions on cultivars of faba bean and cowpea, from existing collections and field surveys.
- Evaluation of the effects of single and co-inoculation with selected rhizobial bacteria and arbuscular mycorrhizal fungi in the growth and yield of cowpea and faba bean plants (pot studies in the greenhouse).

Following the work developed according to the main objective and tasks, this PhD thesis is divided in eight chapters:

- **Chapter I**- General introduction and objectives;
- **Chapter II**- State-of-the-art;
- **Chapter III**- Phylogenetic diversity of rhizobial bacteria associated with cowpea (*Vigna unguiculata* (L.) Walp.), in Portugal;
- **Chapter IV**- Co-inoculation with rhizobia and mycorrhizal fungi increases yield and protein content of cowpea (*Vigna unguiculata* (L.) Walp.) under drought stress;
- **Chapter V**- Phylogenetic diversity of rhizobial bacteria associated with faba bean (*Vicia faba* L.) in Portugal;
- **Chapter VI**- Improvement of some growth and yield parameters of faba bean (*Vicia faba* L.) by inoculation with *Rhizobium laguerreae* and arbuscular mycorrhizal fungi;
- **Chapter VII**- General discussion;
- **Chapter VIII**- Concluding remarks and future prospects.

In the current chapter (**Chapter I**), is addressed the framework and the relevance of the work and the respective objectives. In this chapter it is also presented an overview of the structure and organization of this thesis.

The **chapter II** corresponds to the state-of-the-art, which covers all the topics of the thesis and a critical review about the scientific information and knowledge, showing the needs for further studies.

The phylogenetic analysis of rhizobial bacteria present in cowpea and faba bean plants collected from different soils in Portugal were performed, using a Multilocus Sequence Analysis (MLSA) and the results are presented in **Chapters III and V**, respectively.

Following the phylogenetic analysis, *in vitro* and pot studies were performed to select the best inoculants for each crop, which were then evaluated in greenhouse experiments. The **Chapter IV** addresses a greenhouse experiment in cowpea plants under two water stress levels (25 and 75% of field capacity), which were single and co-inoculated with the selected bacteria and arbuscular mycorrhizal fungi. In this work, growth and yield parameters and protein content in the seeds were evaluated. The **Chapter VI** corresponds to a study performed in faba bean plants single and co-inoculated with the selected rhizobial bacteria for this crop and arbuscular mycorrhizal fungi, in order to evaluate growth and yield parameters.

The **Chapter VII** corresponds to the General discussion and intends to interconnect all the results obtained in the previous chapters.

Finally, the **Chapter VIII** includes the Concluding remarks and the Future prospects. In this chapter, the main achievements of this work are highlighted and it is also referred the future work that can be performed taking into account the results obtained.

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CHAPTER II

STATE-OF-THE-ART

Chapter II- State-of-the-art

1. The legumes

Legumes are plants from the family Fabaceae (or Leguminosae), the third largest family of flowering plants, with around 800 genera and 18 000 to 19 000 species (Morel *et al*., 2012). Legumes and legume-based foods are an important and sustainable source of nutrients such as protein and carbohydrates for human diet (Table 1) and constitute almost 25% of the world's primary crop production (Vioque *et al.,* 2012; Summo *et al.,* 2016). Grain legumes can be also used to produce animal feeds or as whole-crop forage (Watson *et al*., 2017). The consumption of legumes and their derived products present several human health benefits. Apart from the high protein contents in their seeds, legumes provide many other important components, such as slowly digestible starch, soluble sugars, fibre, minerals and vitamins, as well as secondary metabolites (isoflavonoids), which play a major nutritional role in the prevention of cancer, obesity and other health-promoting effects (Arnoldi *et al.,* 2015).

In Table 1 are provided the major features regarding the nutritional composition of the most consumed legume seeds.

Table 1. Nutritional composition of the main leguminous seeds (USDA, 2019).

	Cowpea	Faba bean	Lupins	Chickpea	Lentils	Black bean	Soybean	Pea	Pigeon pea
Water (g)	11.05	10.98	10.44	7.68	8.26	11.02	8.54	8.69	10.59
Energy (kJ)	1435	1425	1554	1581	1473	1425	1866	1521	1435
Protein (g)	23.85	26.12	36.17	20.47	24.63	21.6	36.49	23.12	21.7
Total lipid (fat) (g)	2.07	1.53	9.74	6.04	1.06	1.42	19.94	3.89	1.49
Ash (g)	3.39	3.08	3.28	2.85	2.71	3.6	4.87	2.67	3.45
Carbohydrate (g)	59.64	58.29	40.37	62.95	63.35	62.36	30.16	61.63	62.78
Fiber (g)	10.7	25	18.9	12.2	10.7	15.5	9.3	22.2	15
Calcium (mg)	85	103	176	57	35	123	277	46	130
Iron (mg)	9.95	6.7	4.36	4.31	6.51	5.02	15.7	4.73	5.23
Magnesium (mg)	333	192	198	79	47	171	280	63	183
Phosphorus (mg)	438	421	440	252	281	352	704	334	367
Potassium (mg)	1375	1062	1013	718	677	1483	1797	852	1392
Sodium (mg)	58	13	15	24	6	5	2	5	17
Zinc (mg)	6.11	3.14	4.75	2.76	3.27	3.65	4.89	3.49	2.76
Copper (mg)	1.059	0.824	1.022	0,656	0.754	0.841	1.658	0.809	1.057
Manganese (mg)	1.544	1.626	2.382	21.306	1.393	1.06	2.517	1.19	1.791
Selenium (µg)	9.1	8.2	8.2	0	0.1	3.2	17.8	10.7	8.2
Vitamin C, ascorbic acid (mg)	1.5	1.4	4.8	4	4.5	0	6	1.8	0
Thiamin (mg)	0.68	0.555	0.64	0.477	0.873	0.9	0.874	0.719	0.643
Riboflavin (mg)	0.17	0.333	0.22	0.212	0.211	0.193	0.87	0.244	0.187
Niacin (mg)	2.795	2.832	2.19	1.541	2.605	1.955	1.623	3.608	2.965
Pantothenic acid (mg)	1.511	0.976	0.75	1.588	2.14	0.899	0.793	0.962	1.266
Vitamin B-6 (mg)	0.361	0.366	0.357	0.535	0.54	0.286	0.377	0.14	0.283
Vitamin A, ERA (µg)	2	3	0	3	2	0	1	7	1
Vitamin A (IU)	33	53	0	67	39	17	22	149	28
Fatty acids, saturated (g)	0.542	0.254	1.156	0.603	0.154	0.366	2.884	0.408	0.33
Fatty acids, monounsaturated (g)	0.173	0.303	3.94	0.603	0.193	0.123	4.404	0.615	0.012
Fatty acids, polyunsaturated (g)	0.889	0.627	2.439	2.731	0.526	0.61	11.255	1.022	0.814
Tryptophan (g)	0.294	0.247	0.289	0.2	0.221	0.256	0.591	0.159	0.212
Threonine(g)	0.908	0.928	1.331	0.766	0.882	0.909	1.766	0.813	0.767
Isoleucine (g)	0.969	1.053	1.615	0.882	1.065	0.954	1.971	0.983	0.785
Leucine (g)	1.828	1.964	2.743	1.465	1.786	1.725	3.309	1.68	1.549
Lysine (g)	1.614	1.671	1.933	1.377	1.72	1.483	2.706	1.771	1.521
Methionine (g)	0.34	0.213	0.255	0.27	0.21	0.325	0.547	0.195	0.243
Cystine (g)	0.263	0.334	0.446	0.279	0.322	0.235	0.655	0.273	0.25
Phenylalanine (g)	1.393	1.103	1.435	1.103	1.215	1.168	2.122	1.151	1.858
Tyrosine (g)	0.771	0.827	1.36	0.512	0.658	0.608	1.539	0.518	0.538
Valine (g)	1.137	1.161	1.51	0.865	1.223	1.13	2.029	1.035	0.937
Arginine (g)	1.652	2.411	3.877	1.939	1.903	1.337	3.153	1.902	1.299
Histidine (g)	0.74	0.664	1.03	0.566	0.693	0.601	1.097	0.586	0.774
Alanine (g)	1.088	1.07	1.296	0.882	1.029	0.905	1.915	1.049	0.972
Aspartic acid (g)	2.881	2.916	3.877	2.422	2.725	2.613	5.112	2.549	2.146
Glutamic acid (g)	4.518	4.437	8.686	3.603	3.819	3.294	7.874	3.871	5.031
Glycine (g)	0.985	1.095	1.539	0.857	1.002	0.843	1.88	1.012	0.802
Proline (g)	1.072	1.099	1.476	0.849	1.029	0.916	2.379	1.035	0.955
Serine (g)	1.194	1.195	1.476	1.036	1.136	1.175	2.357	1.069	1.028

Additionally to human and animal consumption, leguminous plants are also used as pulp for paper production, fuel-woods, timber, oil production, sources of chemicals and medicines, and are also cultivated as ornamental, used as living fences and firebreaks, among others (Lewis *et al*., 2005).

These crops are also recognized to have several benefits to the soil, being used as cover crops, in intercropping with cereals and other staple foods. In symbiosis with rhizobial

bacteria presented in the soil, they are responsible for a considerable part of the global flux of nitrogen (N) from atmospheric N_2 to fixed forms (Ferguson *et al.*, 2010; Hameren *et al.*, 2013). Furthermore, it is a current practice in agriculture to inoculate legumes with superior inoculant strains to increase nitrogen fixation and yield (Herridge *et al.*, 2008). In fact, symbiosis between legume plants and soil microbes contribute at least with 70 million tons of N per year, with half originating from zones with cool and warm temperature and the remainder from the tropics (Brockwell *et al.*, 1995). This symbiosis allows to increase the soil organic matter, improve soil porosity and structure, recycle nutrients, decrease soil pH, reduce soil compaction, diversify microorganisms and mitigate disease problems (U.S Department of Agriculture [USDA], 1998).

Despite the high nutritional value of grain legumes provided for both humans and livestock, the cultivation of these crops in Europe, and particularly in Portugal, has been constantly decreasing over the last 40 years (Table 2). Indeed, almost 90% of dried leguminous consumed in Portugal and 70% of those consumed in Europe are imported. In general, Portugal has followed the European trend and became a net importer of grain legumes, although it holds highly potential genetic resources and scientific expertise to reverse this trend (Patto and Araújo, 2016), since the increase production of only pea, cowpea and faba bean can improve Europe's and Portuguese's autonomy and result in savings of around 10 million euros in the trade balance (Rosa, pers. comm.).

Table 2. Trend for continent harvested area (%) during the 40-year period (1974-2014) for legume crops included in FAOSTAT; for comparison, the major three cereal crops are also reported (Stagnari *et al.*, 2017).

	Δ harvested area 1974–2014 (%)					
	Africa	**Northern America**	**South America**	**Asia**	**Europe**	**Oceania**
Legume crops						
Bambara bean	+612	–	–	–	–	–
Dry bean	+207	+16	−20	+25	−84	+1778
Faba bean	+7	Disappeared	−53	−59	−54	+75,085
Chickpea	+30	Appeared	+1	+37	−35	–
Cowpea	+168	Appeared	Appeared	+402	+153	–
Groundnut	+69	−10	−22	+6	+16	−39
Lentil	−20	+3376	−75	+72	−45	Appeared
Lupin	−82	–	+577	−89	−64	+315
Pea	+49	+1119	+7	−21	−63	+578
Pigeon pea	+226	–	−83	+108	–	–
Soybean	+642	+71	+882	+116	+291	−10
French bean	Appeared[a]	−39[a]	+129[a]	+66[a]	−18[a]	+122[a]
Vetch	+109	–	–	−73	−80	+4757
Pulses, nes	+20	–	−69	−15	+73	+7648
Vegetables, leguminous nes	+180[a]	Appeared[a]	+118[a]	+23[a]	−31[a]	−52[a]
Major cereal crops						
Wheat	+11	−20	+16	+39	−33	+51
Maize	+98	+29	+45	+76	+21	+31
Rice (paddy)	+185	+15	−16	+16	−28	+2

In 2017, the main European producers of leguminous plants were Spain (59 210 tons), Italy (45 304 tons) and Poland (19 069), followed by Portugal, which with an area harvested of only 1 195 ha could produce 16 412 tons (Table 3) (FAOSTAT, 2019). According to the European Parliament, the European Union devotes only 3% of its arable land to protein crops and imports approximately 70% of its protein-rich animal feed, mainly from Brazil, Argentina and the United States. In Portugal, in 2018, 77 731 tons of dry bean legumes were imported, whilst the export was 21 872 tons, with a negative trade balance of 55 859 tons (INE).

Table 3. Area harvested (ha), yield (hg/ha) and production (tons) of leguminous plants in European countries, in 2017 (FAOSTAT, 2017).

	Harvested area (ha)	Yield (hg/ha)	Production (tons)
Spain	6 774	87 408	59 210
Italy	7 553	59 981	45 304
Poland	2 228	85 588	19 069
Portugal	1 195	137 285	16 412
Greece	5 700	28 246	16 100
France	1 248	82 980	10 354
United Kingdom	2 134	42 830	9 140
Germany	583	60 412	3 522
Netherlands	404	79 208	3 200
Malta	847	31 525	2 669
Bulgaria	376	58 171	2 187
Romania	192	38 157	731
Austria	822	6 740	554
Montenegro	100	50 008	500
Ukraine	65	72 574	475
Czechia	78	48 729	380
Russian Federation	255	12 633	322
Slovakia	47	39 360	186
Albania	18	49 590	88
Switzerland	31	22 258	69

Within the huge diversity of leguminous plants, cowpea (*Vigna unguiculata* (L.) Walp.) and faba bean (*Vicia faba* L.) studies have a great importance in Europe, and in Portugal in particular. The study of these two crops is essential, because legume species differ greatly in

their specificity for rhizobial symbionts, and these crops in particular are nodulated by different genera of rhizobial bacteria. Additionally, these are very consumed pulses in Portugal, this country having the adequate soil and climatic conditions for their production.

1.1. Cowpea

The genus *Vigna* belongs to the family Fabaceae (Table 4) and comprises more than 200 species scattered throughout the tropics (Fery, 2002).

Table 4. Taxonomy hierarchy of cowpea (*Vigna unguiculata* (L.) Walp.).

Taxonomy Hierarchy of cowpea (*Vigna unguiculata* (L.) Walp.)	
Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae
Genus	*Vigna*
Species	*Vigna unguiculata* (L.) Walp.

Cowpea (*Vigna unguiculata* (L.) Walp.) (Fig. 1) is an annual legume crop native of Africa and is the most widely cultivated seed-legume in arid and semi-arid areas (Alkama *et al.*, 2009; Johnson *et al.*, 2013). Indeed, cowpea is one of the most drought-tolerant legumes and it is deeply rooted and may have reduced leaf size with thickened cuticles to reduce water loss (Graham and Vance, 2003).

Figure 1. Cowpea plants, pods and seeds (this work).

This legume was introduced from Northern Africa into Southern Europe being, nowadays, widely distributed around the world. In fact, this culture can grow under relatively poor and acid soils, low water availability and high temperatures (Santos *et al.,* 2008; Bejarano *et al.*, 2014).

It is difficult to obtain consistent data on cowpea cultivated area and production as this crop is grown in mixture with other crops (Ngalamu *et al*., 2014). However, it could be estimated that, in 2017, the world area harvested was over 12.5 million ha (Table 5), with an annual production of around 7 million tons worldwide (FAO, 2017). Despite its wide distribution, Africa amounts to around 98% of the total area cultivated with cowpea in the world.

Table 5. Area harvested (ha), yield (hg/ha) and production (tons) of cowpea in the world.

	Harvested area (ha)	Yield (hg/ha)	Production (tons)
World	12 577 845	5 890	7 407 924
Africa	12 332 372	5 763	7 107 334
Asia	166 605	12 227	203 714
America	70 319	9 965	70 076
Europe	8 550	31 347	26 801
Oceania	-	-	-

The leading cowpea producing countries are: Nigeria, Niger, Mali, Burkina Faso, Senegal, Ghana, Togo, Benin, Cameroon, and Chad in Central and West Africa; Sudan, South Sudan, Somalia, Kenya, Malawi, Uganda, Tanzania, Zambia, Zimbabwe, Botswana and Mozambique in East and Southern Africa; India, Bangladesh, Nepal, Myanmar, Sri Lanka, Indonesia, China and Philippines in Asia; Cuba, Haiti, and West Indies in Central America; Brazil in South America and USA in North America (Ngalamu *et al*., 2014).

Cowpea is adapted to high temperatures between 20 to 35 ºC. Regarding the precipitation, the optimal annual rainfall of some cowpea varieties is 188 mm, however for forage purpose, rainfall of 750 to 1100 mm is preferable. The growth period of cowpea ranges between 90-240 days, depending on the climatic conditions and the maturity period of the cultivar. The best seeds are produced when the crop is grown under the optimum temperature range. Regarding to soil requirements, cowpea grows well in a wide range of soil textures, from heavy clay, if well drained, to varying proportions of clay and sand (Ngalamu *et al*., 2014).

All the parts of cowpea used for food (fresh leaves, flowers, immature pods and grains) are nutritious, providing protein, carbohydrate, vitamins (B1 and B2) and minerals (Ngalamu *et al*., 2014) that can supply essential aminoacid needs when combined with cereals (Iqbal *et*

al., 2006). Indeed, cowpea seeds provide a rich source of proteins (23%), carbohydrates (56%), fibre (4%) and calories, as well as minerals and vitamins (Table 1). These high protein and carbohydrate levels are relevant features for its use as a nutritional food *de per se* or in mixtures to produce other food products (Imungi and Porter, 1983). On the other hand, these seeds have very low-fat content. Mature cowpea seeds contain a low amount of free amino acids compared to the immature ones. This is mainly a result of the utilization of free amino acids in protein synthesis during the seed development process (Jayathilake *et al*., 2018). Cowpea leaves are a significant source of β-carotene and ascorbic acid (Ngalamu *et al*., 2014) and they can be also used to generate household income (Muli and Saha, 2000).

Additionally to human consumption, cowpea also provides high quality feed for animals, such as cattle, sheep and goats. This crop can be also used as a cover crop, suppressing the growth of weeds, providing protection against soil erosion and reducing soil temperature. After harvest, root, stem and haulm residues provide organic matter and the contained nutrients to the soil (Ngalamu *et al*., 2014).

Another attribute of cowpea is its contribution to soil nitrogen improvement, through the symbiosis between plant roots and a soil bacteria called rhizobia (Figure 2), which improves soil fertility and reduces fertilization needs (Martins *et al.*, 2003). Indeed, the nitrogen content of the soil increases for around 40-80 kg/ha after a cowpea crop (Ngalamu *et al*., 2014).

Figure 2. Cowpea root nodules formed in the symbiosis with rhizobial bacteria (this work).

A heterogeneous group of slow-growing rhizobia known as “cowpea-miscellany”, belonging to the genus *Bradyrhizobium,* have the ability to nodulate cowpea (Allen and Allen, 1981; Appunu *et al.*, 2009). In some works, carried out in Africa, China and Brazil, bradyrhizobia were identified as *Bradyrhizobium elkanii*, *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, unnamed *Bradyrhizobium* genospecies, or as novel *Bradyrhizobium* lineages (Appunu *et al.*, 2009). Although less abundant, fast-growing rhizobia have also been isolated

from cowpea nodules and classified in the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Lindete *et al.*, 1997; Germano *et al.*, 2006; Yokoyama *et al.*, 2006; Zhang *et al.*, 2007; Zhang *et al.*, 2008). Additionally, cowpea forage has a relatively low C:N ratio and N is rapidly mineralized, making it a valuable green manure, which provides readily available N for subsequent crops (Tarawali *et al.*, 1997). Several cowpea genotypes are tolerant to phosphorus (P) deficiency and aluminium toxicity in tropical and acid soils (Kolawole *et al.*, 2000; Sanginga, 2003), and the best adapted genotypes can increase in 50% the P availability in the soil, after a culture-cycle (Ankomah *et al.*, 1995; Rajput and Singh, 1996).

When cowpea is grown mixed with cereals (maize, sorghum or millets), there is an increase in the yield of cereal crops and it can also be grown in rotation with rice to replenish the soil fertility for the next crop (Ngalamu *et al*., 2014).

1.2. Faba bean

Faba bean (*Vicia faba* L.) (Fig. 3), also known as fava or broad beans, is a cool-season grain legume, which belongs to the kingdom Plantae and to the family Fabaceae (Table 6); it is originated from the Near East and Mediterranean basin in the prehistoric times and is an important winter crop in warm temperate and subtropical areas (Zohary and Hopf, 2000; Jensen *et al.*, 2010).

Figure 3. Faba bean plants in the field (this work).

Table 6. Taxonomy hierarchy of faba bean (*Vicia faba* L.).

Taxonomy Hierarchy of faba bean (*Vicia faba* L.)	
Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae
Genus	*Vicia* L.
Species	*Vicia faba* L.

Faba bean can grow on a wide range of soils with different textures (Kopke and Nemecek, 2010), however deep and well-structured clayey soils and fine-textured soils are preferable. In optimum growing conditions, germination of faba bean seeds takes 10-14 days (Etemadi *et al.*, 2015), the maturity period ranges from 90 to 220 days, depending upon the cultivars and climatic conditions (Bond *et al*., 1985), and plants can grow 90-130 cm tall, depending on the genotype (Etemadi *et al.*, 2019). The ideal pH to faba bean growth is ≥ 7 (Jensen *et al*., 2010; Kopke and Nemecek, 2010) and the ideal temperatures range from 18 to 27 ºC, but heat during flowering and pod-filling hampers yields (Muehlbauer *et al.,* 1997; Matthews *et al.*, 2003). This culture can be cultivated where annual rainfall is between 700 mm and 1000 mm (Muehlbauer *et al*., 1997). In the tropics and subtropics, faba bean can be grown above 1200 m and up to an altitude of 2500 m (Ecocrop, 2014).

In recent years, this crop has been growing worldwide in a diverse cropping system as a grain and green-manure legume and it is now widespread in Europe, North Africa, Central Asia, China, South America, the USA, Canada and Australia (Table 7). In fact, in 2017, the total world area cultivated with faba bean was around 2.4 million ha, with most of production located in China, Ethiopia and Australia (FAOSTAT, 2019).

Table 7. Area harvested (ha), yield (hg/ha) and production (tons) of faba bean in the world, and in each continent (FAOSTAT, 2019).

	Harvested area (ha)	Yield (hg/ha)	Production (tons)
World	2 463 966	19 643	4 840 090
Asia	946 929	20 089	1 902 277
Africa	776 655	17 524	1 361 044
America	179 077	11 869	212 547
Europe	333 283	29 723	990 617
Oceania	228 021	16 385	373 605

Within Europe in particular (Table 8), the main faba bean producers of immature seeds are UK, France, Italy and Germany, Europe contributing with 16.8% to the world faba bean production (Jensen *et al.*, 2010). Since 1960s occurred a decline of 56% of the faba bean area sown, despite its high nutritional value (Crépon *et al.*, 2010), due to the replacement of traditional cropping systems by industrialized cereal-based systems (Jensen *et al*., 2010; McVicar *et al*., 2013). However, the average yield almost doubled during this period, allowing a decrease of only 20% in the total production (Jensen *et al.*, 2010). In the EU, faba bean ranks 2nd after field peas for legume seed production and is mostly used for animal feeding (FAO, 2014).

Table 8. Area harvested (ha), yield (hg/ha) and production (tons) of faba bean in European countries (FAOSTAT, 2019).

	Harvested area (ha)	Yield (hg/ha)	Production (tons)
United Kingdom	79 010	38 282	302 468
Germany	46 400	40 690	188 800
France	62 582	29 990	187 681
Sweden	30 490	35 881	109 400
Italy	51 135	18 142	92 767
Spain	36 574	13 252	48 468
Austria	10 296	22 302	22 962
Ukraine	3 500	23029	8 060
Russian Federation	3 817	19 437	7 419
Greece	2 256	17 866	4 031
Belgium	853	40 270	3 435
Portugal	319	105 460	3 362
Switzerland	1 039	29 105	3 024
Czechia	1 927	12 244	2 359
Lithuania	1 355	17 383	2 356
Netherlands	325	50 803	1 649
Malta	270	28 801	777
Slovakia	533	13 433	716
Hungary	150	20 000	300
Poland	200	14 028	281
Albania	166	12 480	207
Luxembourg	76	11 577	88
Bulgaria	11	7 273	8

Faba bean varieties fall into two categories: tannin varieties and low or near zero tannin varieties. The first ones have coloured flowers or white flowers with a black spot, tan seed coats, and seeds are often larger and grown especially for human consumption, as fresh or dry; on the other hand, the low or near zero tannin varieties have white flowers, greyish-white seed coats and are usually grown for livestock feed industry. According to its size, faba bean can also be classified as *Vicia faba* var. *major* (broad beans), which produces large seeds (650-850 g/1000 seeds) and is cultivated mainly for human consumption, and *Vicia fab*a var.

minor (horse beans, field beans), which produces smaller seeds (250-350 g/1000 seeds) and is used mainly for livestock feeding (Smith *et al*., 2013).

Due to its superior nutritional values including protein, carbohydrates, B group vitamins and minerals (Table 1), faba bean is considered as one of the most important pulse crops in the world. Also, faba bean seeds are low in fats and sodium and are cholesterol-free (Adamu *et al*., 2015). Additionally, its interest is also related to the fact that its germination can tolerate cold soil temperature better than the other seed legumes (Etemadi *et al*., 2019).

Like cowpea, faba bean plants also have the ability to fix nitrogen through a symbiotic relationship with rhizobia, particularly with *Rhizobium leguminosarum* bv. *viciae* (Jensen *et al.*, 2010). Nevertheless, faba bean presents an advantage when compared with other legume plants since can continue with N fixation rates in the presence of high quantities of available N in the soil, which can be related to its low rooting density and depth in comparison with other legumes (Kopke and Nemecek, 2010). Furthermore, the N fixation capacity of faba bean is the highest among the cool season legumes (50-330 kg N/ha) (Galloway *et al*., 2004; Mekkei, 2014; Etemadi *et al*., 2018). Indeed, faba bean can meet all of its N requirements through biological nitrogen fixation, being considered as an effective N fixer (N´Dayegamiye *et al*., 2015). In the development and function of symbiotic nodules, a high P requirement is observed (Ribet and Drevon, 1996), thus the symbiosis with arbuscular mycorrhizal fungi is also very important in faba bean, since this interaction improves phosphorus uptake from deeper soil (Jensen *et al.*, 2010).

This leguminous plant plays an important role in the maintenance of soil fertility, because, additionally to the BNF, it can solubilize insoluble P in soil, improving the soil physical environment and, consequently, increasing soil microbial activity (Rashid *et al*., 2016). Moreover, faba bean also contributes to the sustainability of cropping system through diversification of systems which leads to a decrease of the disease, pest and weed, and potentially to the increase of biodiversity and to the reduction of fossil energy consumption in plant production (Duke, 1981; Jensen *et al.*, 2010).

2. Metabolic responses to nutrients

Plants require at least 16 macro and micro nutrients for their growth and development (Marschner, 1995). Nitrogen (N) and phosphorus (P) are among the most important nutrients for ecosystem structure, processes and function, since their unavailability limits plant biomass and growth (Hu and Schmidhalter, 2005). Thus, N and P have been the key elements in the study of nutrient limitations. According to previous works, the combined application of these nutrients can increase root surface area, root length, and root-shoot mass (Song *et al*., 2010).

Nitrogen plays a very important role in plant metabolism being incorporated in the structure of some important primary and secondary plant metabolites (Marschner, 1995); it also plays a particular role for the optimal photosynthesis and vegetative growth (Parsons *et al.*, 1991). Indeed, the nitrogen cycle is one of the most important nutrient cycles, in which nitrogen is converted between its various chemical forms, thought out several processes such as fixation, ammonification, nitrification and denitrification (Fig. 4).

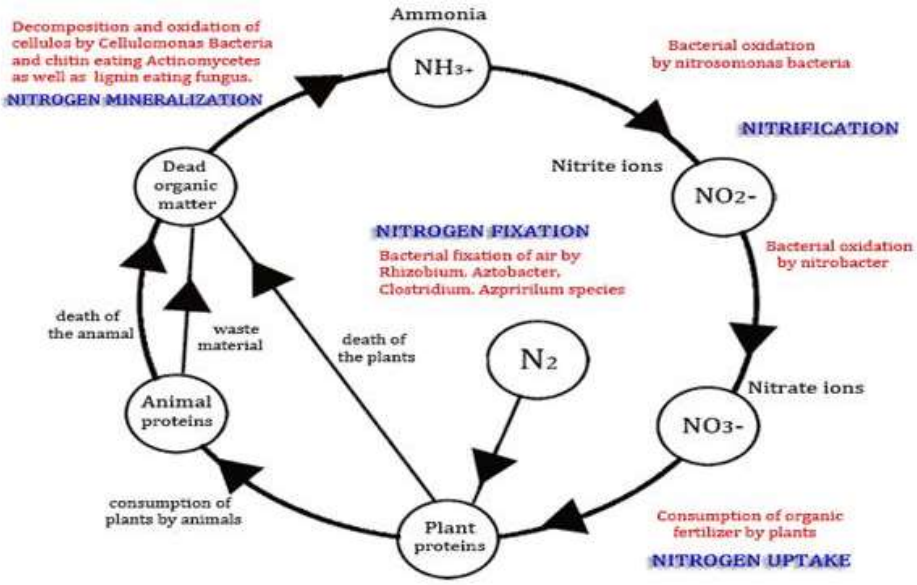
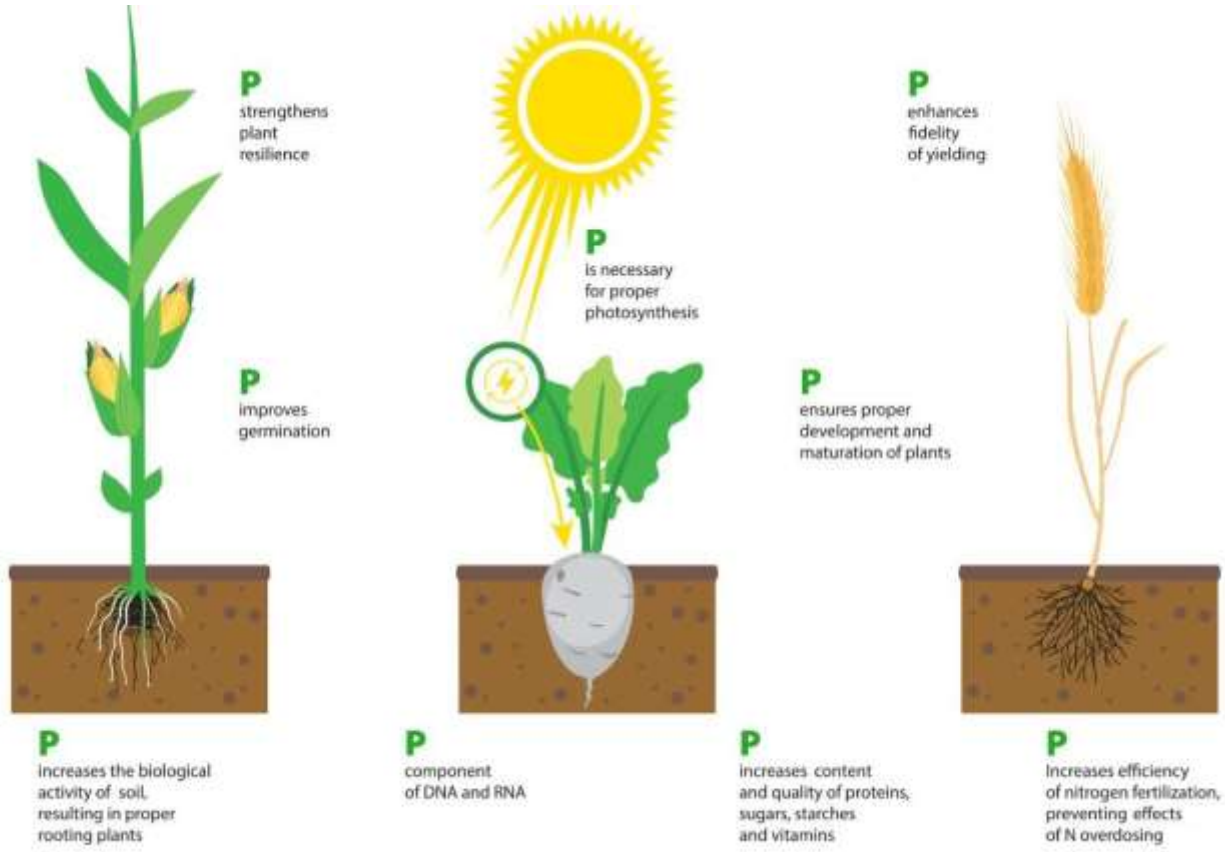


Figure 4. Nitrogen cycle (http://www.kingstonmillerav.de/nitrogen-cycle-diagram.html)

The Earth´s atmosphere contains about 10^{15} tons of N_2 gas; however, it is simultaneously a limiting element for the growth of most plants due to its unavailability (Smil, 1999; Socolow, 1999; Graham and Vance, 2000). In fact, nitrogen is required in the largest quantities, and its availability and internal concentration affect the partitioning of biomass between roots and shoots (Bown *et al*., 2010). In more detail, N deficiency leads to changes in root formation, photosynthesis, production and translocation of photoassimilates and plant growth rate (Shridhar, 2012). Plants can acquire N from two principal sources. The first one is from the soil as ammonium or nitrate (Crawford and Glass, 1998; Rodrigues *et al.*, 2013), through commercial fertilizers, manure and mineralization of organic matter. However, as nitrogen is a mobile element in soil, hence due to humid conditions is susceptible to leaching, an appropriate fertilization is necessary under such conditions. On the other hand, under arid and semi-arid conditions, water deficiency can limit the use of inorganic N by plants (Miransari, 2011). The second principal source is from the atmosphere through biological nitrogen fixation, in symbiosis with rhizobia (Vance, 2001).

Phosphorus (P) is the second most limiting nutrient for plant growth, immediately after nitrogen (Bieleski, 1973; Vance *et al.*, 2000) and is needed to sustain optimum plant growth and quality, being very important in root development and nodulation, nitrogen fixation, and formation of glycolate phosphate involved in photosynthesis (Kubure *et al*., 2016). In more detail, phosphorus is responsible for the stimulation of root development, increase of stalk and stem strength, improvement of flower formation and seed production, more uniform and earlier



crop maturity, increase of nitrogen N-fixing capacity of legumes, improvements in crop quality,

increased resistance to plant diseases, and it supports development throughout entire life cycle (Fig. 5).

Figure 5. The role of phosphorus in plant development.

This element is also essential for cell division, reproduction, plant metabolism and acquisition, storage and use of energy (Epstein and Bloom, 2004). Phosphorus is present in small quantities in the lithosphere (0.1%), with two major forms in soil, the organic and inorganic P, from which the inorganic mono/divalent phosphate ion, $H_2PO_4^-$ and HPO_4^{2}, are taken up by plants (Alkama *et al.*, 2009). Soil P have two different origins: the legacy P, as result of past applications of fertilizers and manures, and native P, which results from

geological processes that convert the P bound in rocks, minerals and large oceanic in phosphate ions into the soil, where it can be absorbed by plant roots (Ruttenberg, 2003). High P availability can increase plant growth and share more carbon sources to roots and nodules, resulting in a larger root system or higher nodule formation, and consequently higher N_2 fixation. Indeed, it is evident that the addition of P results in an increase in many parameters, such as nodule number and weight, nitrogenase activity and N_2 fixation in numerous legumes (Mei *et al.*, 2012). However, plant-available phosphorus concentrations in the soil solution are inherently low (Marschner, 1995), because P rapidly forms insoluble complexes with cations and is incorporated into organic matter by microbes (Vance, 2001). In the absence of available P from inorganic fertilizers, plants must use several strategies to acquire soil inorganic (Pi) and organic (Po) quickly and effectively to guaranteeing an appropriate supply of P during the growing season (Richardson and Simpson, 2011). Soil P is converted to the plant-available phosphate ion through many mechanisms: dissolution/precipitation (mineral equilibria), sorption/desorption (interactions between P and mineral surfaces) and mineralization/immobilization (transformation of Po to Pi by biological transformations) (Owen *et al.*, 2015).

3. Food demands and climate changes

According to The United Nations Food and Agriculture Organization (FAO), the total demands for agricultural products will be 60% higher in 2050 than now and more than 85% of this additional demand will come from developing countries (Abd-Alla *et al.*, 2014). Moreover, 90% of the growth in crop production globally should come from higher yields and increased cropping intensity (FAO, 2009). In addition, the superimposition of drought in several areas of the world is predicted for the next years, due to the decrease in precipitation events and the increase in global temperatures (prediction of 3-4 °C until 2100, depending on the gas emissions) (Fig. 6) (IPCC Climate Change, 2018).

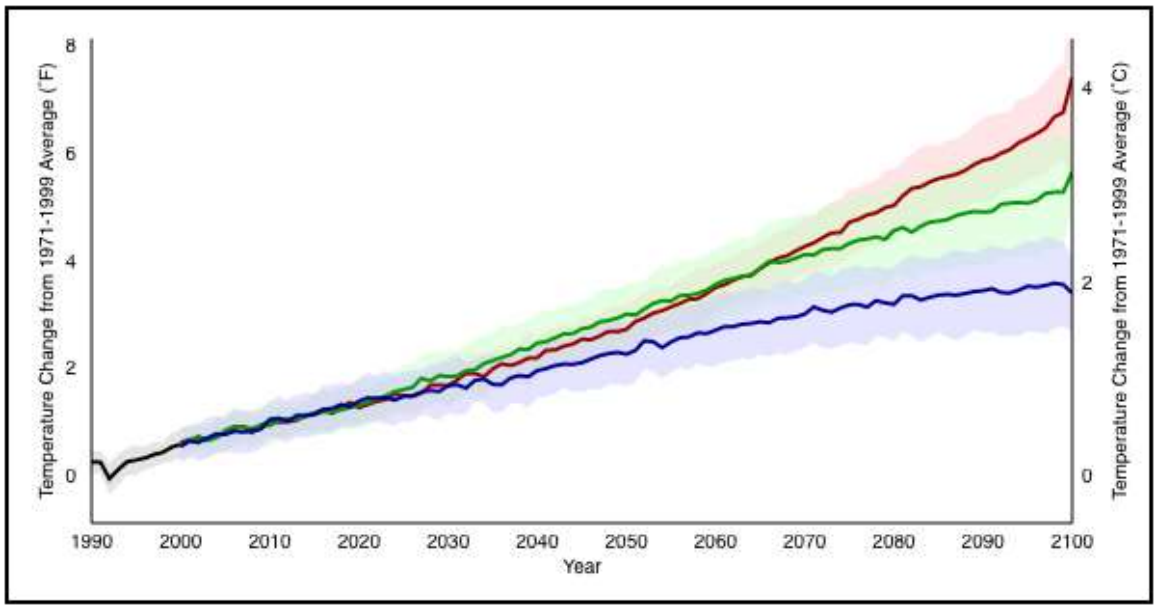


Figure 6. Predictions of global warming until 2100, based on a range of emissions scenarios. Blue line assumes that humans worldwide will make more sustainable development choices by using a greater range of, and more efficient, technologies for producing energy. On the other hand, red line assumes humans will continue to accelerate the rate at which we emit carbon dioxide (Herring, 2012- https://www.climate.gov/).

Moreover, beyond the high cost of industrial fertilizers, their use to supply the plants with the adequate levels of nitrogen can cause serious environmental and human health problems. To solve all these concerns, alternative sources which are cost effective and environment-friendly have been explored (Iantcheva *et al.*, 2013; Rodrigues *et al.*, 2013; Abd-Alla *et al.*, 2014; Janczarek *et al.*, 2015). Thus, biofertilizers, especially rhizobia and arbuscular mycorrhizal fungi in legume symbiosis, is a promising technology as an alternative source to reduce N and P fertilizer inputs (Abd-Alla *et al.*, 2014). They improve plant performance under different environmental conditions by recycling nutrients and making them available, play a key role in natural ecosystems and influence plant productivity and nutrition and enhance the inhibition of fungal plant pathogens (Demir and Akkopru, 2007; Wehner *et al.*, 2010; Abohatem *et al.*, 2011). Furthermore, the symbiosis between rhizobia and legumes is a cheaper and usually more effective agronomic practice to ensure an adequate supply of N for legumes and to reduce the emission of the greenhouse gases carbon dioxide and nitrous oxide (CO_2 and NO_2), in comparison to nitrogen-fertilizer crops (Zahran, 1999).

4. Rhizobia and Biological Nitrogen Fixation

Soil is a complex and dynamic system that supports plant growth and development which in turn are influenced by several biotic (plant pathogens and pests) and abiotic stressors. The abiotic stresses include salinity, drought, flooding, heavy metals, temperature, gases and nutrient deficiency or excess and are considered the central source of yield reduction (Nadeem *et al.*, 2014). All plant-associated microenvironments, especially the rhizosphere, are colonized in high abundances by microbes (Berg *et al.*, 2005). Of all different microbial populations existing in the rhizosphere, bacteria are the most abundant microorganisms.

Rhizobia is the common name given to a group of small, rod-shaped and Gram-negative soil bacteria that have the ability to fix nitrogen inside root nodules formed on many legume species, including more than 100 agriculturally important plants (Sprent, 2007; Herridge *et al.*, 2008; Masson-Boivin *et al.*, 2009; Abd-Alla *et al.*, 2014). This occurs through a process called biological nitrogen fixation (BNF), responsible for the conversion of atmospheric N_2 into ammonium, an available form to the plants (Janczarek *et al.*, 2015). The amount of nitrogen fixed by rhizobia is similar to that from synthetic ammonia production (Gruber and Galloway, 2008). Zander *et al*. (2016) referred that the supply of N to the soils by leguminous plants is estimated to between 130 and 153 kg N/ha. Rhizobial species are divided into four different families: *Rhizobiaceae*, *Phyllobacteriaceae*, *Hyphomicrobiaceae* and *Bradyrhizobiaceae* (Madigan *et al.*, 2000), according to their genetic characteristics. During several years, it was believed that only a limited number of genera within these families, have the ability to fix nitrogen in a symbiosis with leguminous plants (De Lajudie *et al.*, 1998), belonging to the group of alphaproteobacteria. Indeed, symbiosis is not obligate for either partner: some rhizobia may grow endophytically in non-legumes and non-symbiotic rhizobia occasionally exceed symbiotic genotypes in soil (Segovia *et al.*, 1991; Ji *et al.*, 2010). However, nowadays, other alphaproteobacterial genera, such as *Ochrobactrum* (Trujillo *et al*., 2005), *Methylobacterium* (Sy *et al*., 2001), *Microvirga* (Ardley *et al*., 2012; Radl *et al*., 2014), *Devosia* (Rivas *et al*., 2003) and *Phyllobacterium* (Zakhia *et al*., 2006) have also been considered as nitrogen fixing root nodule bacteria. Recently, betaproteobacteria from the genera *Burkholderia* and *Cupriavidus* were also described as betarhizobia (Meyer *et al*., 2013a, b, 2014). Despite the genetic diversity, the bacteria that are able to form the symbiosis with legumes have many genetic and biochemical characteristics in common, namely the capacity to recognize specific signal molecules, flavonoids, from the host plants and to produce special signal molecules, nod factors (NF) (Fig. 7), which apparently are not produced by other related genera (Spaink, 2000).

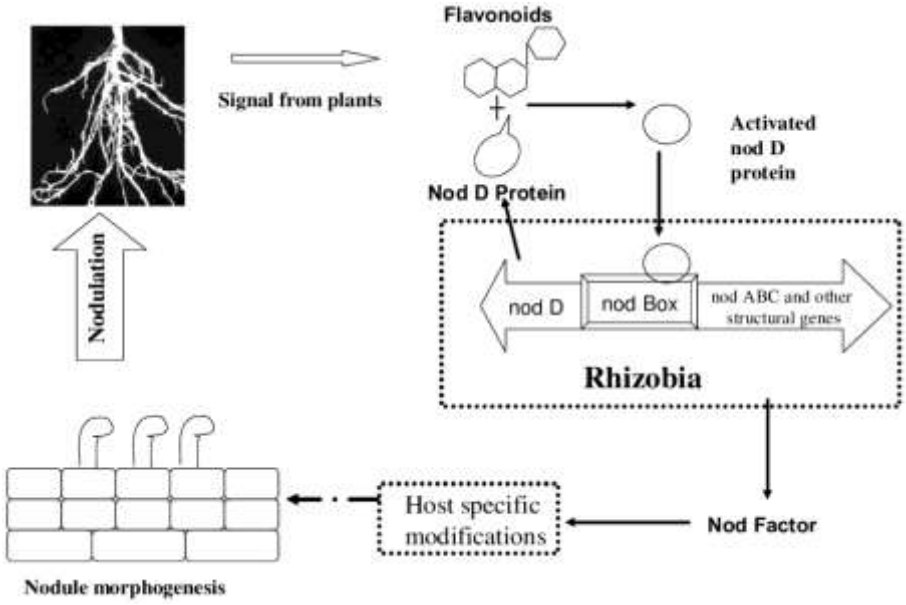


Figure 7. Nodulation process in Rhizobium-legume symbiosis of initial stages of nodulation (Kamboj *et al.*, 2008).

Nod factors are bacterial lipochitooligosaccharide (LCOs) signals, consisting of a chitin backbone, four to five N-acetyl-D-glucosamine units in length, with the fatty acyl group always attached to the nitrogen of the non-reducing saccharide (Spaink, 2000; Abd-Alla *et al.*, 2014). However, this basic structure has some modifications that are dependent on each strain or species and determine the host-specificity (Perret *et al.*, 2000; Pacios-Bras *et al.*, 2002). Nod factors are produced by rhizobia and secreted in the rhizosphere, to initiate the infection process (Gourion *et al.*, 2014). It initiates many developmental changes in the host plant, namely root hair deformation, membrane depolarization, intracellular calcium oscillations, and the initiation of cell division in the root cortex, which establishes a meristem and nodule primordium (Abd-Alla *et al.*, 2014). However, in a study carried out by Roux *et al.* (2014), they demonstrated that rhizobial genes responsible for NF are not only actively transcribed before the infection but also in the nitrogen fixation zone of nodules. Nod genes encode about 25 proteins required for the bacterial synthesis and export of Nod factor. Furthermore, several proteins encoded by the *nod*, *nol* and *noe* genes have been demonstrated to have an important role in the biosynthesis of LCOs (Spaink, 2000).

When a host plant is present, some rhizobia infect its roots and nodules are formed (Denison and Kiers, 2011). In most legumes, the rhizobia enter the plant through the root hairs. The invagination of the plasma membrane leads to the formation of an infection thread (IT) that contains the multiplying bacteria and grows towards the root cortex (Fig. 8). On the other hand, in certain legumes, a less frequency and ancient mode of infection occurs via cracks on

the root surface (Maróti and Kondorosi, 2014). Successful rhizobial invasion is indispensable for further nodule development.

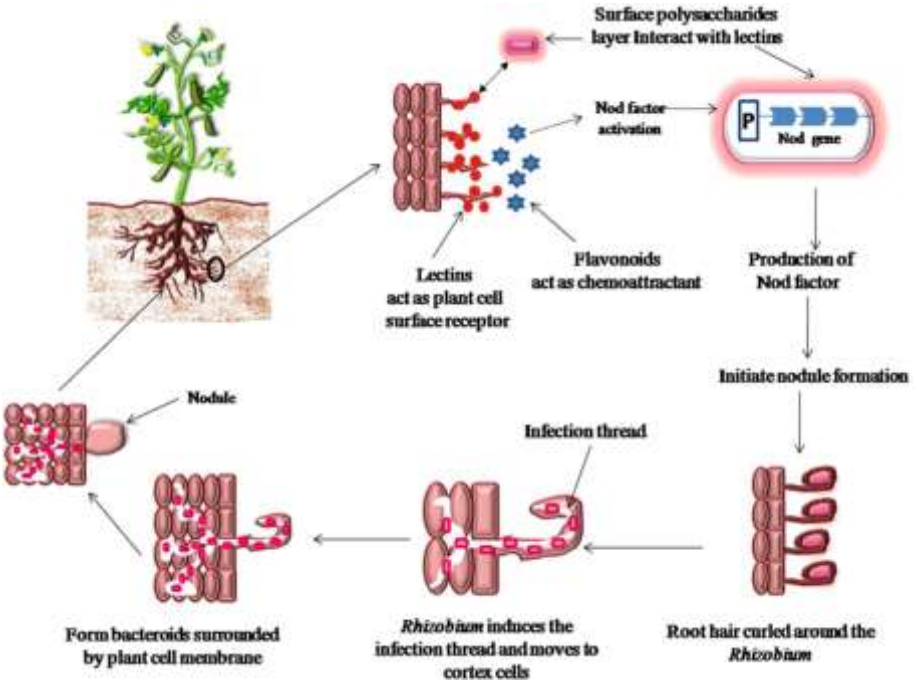


Figure 8. Host plant recognition by rhizobial bacteria and nodule formation (Singh *et al.,* 2019).

The main function of the nodule is the production of an appropriate environment to biological nitrogen fixation, imposing limitations on the free flow of oxygen, since that nitrogenase, the enzyme responsible for BFN is irreversibly inactivated by oxygen (Postgate, 1982; Dixon and Wheeler*,* 1986; Oldroyd, 2013). Leghemoglobin is the protein responsible for oxygen binding in symbiotic root nodules of nitrogen-fixing plants and play a role in the effective diffusion of oxygen and their autoxidation results in the production of O_2^- and H_2O_2 (Puppo *et al.*, 1981; Appleby, 1984; Christensen *et al.*, 1991). Inside the nodule, some bacteria differentiate into bacteroids that can convert atmospheric N_2 into available forms to the host plant, which, in turn, supplies the bacteria with several nutrients (Kahn *et al.*, 1998; Denison and Kiers, 2011) and carbohydrates, mainly as sucrose, derived from leaves, transported by phloem and released in the roots (Fig. 9) by the action of an enzyme present in carbon flux regulation in root nodules called sucrose synthase (Ben Salah *et al.*, 2011; Shridhar, 2012). It is converted in hexoses, which are oxidized in bacteroids as an energy source during BNF (Larrainzar *et al.*, 2009; Shridhar, 2012).

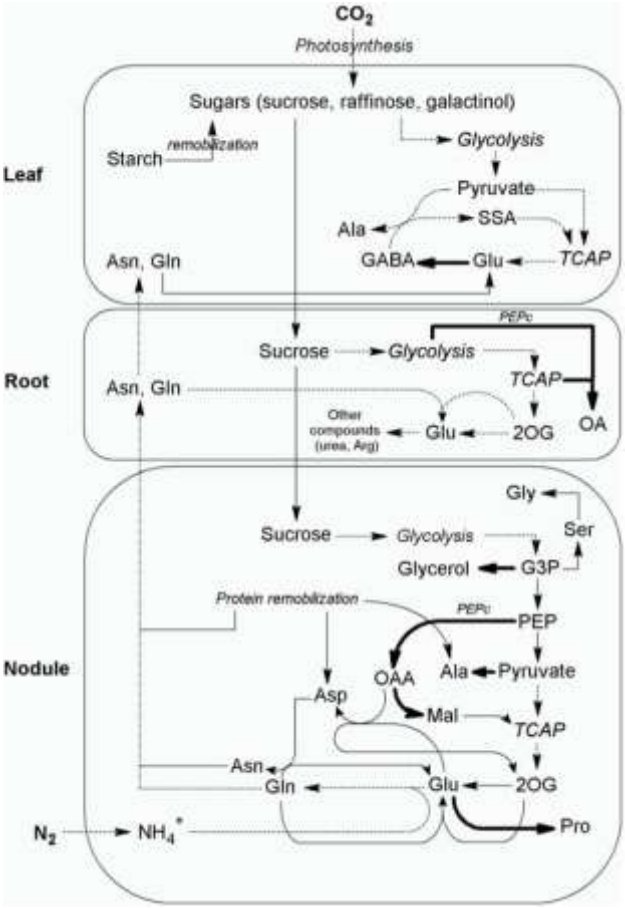


Figure 9. Most visible changes in carbon primary metabolism of leaves, roots and nodules (Aranjuelo *et al.*, 2013).

The first nodules appear about two weeks after seed germination. However, the highest nitrogen fixation rates are only observed after flowering, because of the strong active sinks for assimilates and fixed nitrogen of the pods and seeds (Vinther and Dahlmann-Hansen, 2005; Kopke and Nemecek, 2010). When the nodule senescence occurs, some of the bacteria present inside the nodule escape to the soil, thus increasing soil populations (Denison and Kiers, 2011), as reported by Brockwell *et al.* (1987). However, this higher bacteria concentration in soil tends to decrease in a few months without host plants, due to the predation by protozoa (Danso *et al.*, 1975; Ramirez and Alexander, 1980) as well as due to the abiotic factors (Hirsch, 2010). However, after this initial period, rhizobial population remains relatively constant for years, even without host plants, as reported by some authors (e.g., Kucey and Hynes, 1989; Hirsch, 1996). Nitrogen fixed by rhizobia is supplied to the soil, through decomposition of roots and other crop residues after the death of the plant, and to some extent through leakage of N into the soil also from the living nodules and roots (Olsson, 2017). In addition to nitrogen fixation, rhizobia give other benefits to the soil environment: stimulation, amplification and diversification of the microflora; breaking disease cycles

inseparable from monocultures; provision of organic nitrogen which interacts with soil organic carbon to enhance soil structural stability (Brockwell *et al.*, 1995). In fact, many works showed an induction of plant defenses after the inoculation with rhizobia (Kouchi *et al.*, 2004; Lohar *et al.*, 2006; Libault *et al.*, 2010; Lopez-Gomez *et al.*, 2012). The rhizobia-legume symbiosis is extremely related to the physiological state of the plant. Indeed, some factors, e.g. salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, extreme temperatures, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases and grazing, limit the vigor of the host plant and, consequently, a competitive and persistent rhizobial strain may not express its full capacity for N fixation (Brockwell *et al.*, 1995; Peoples *et al.*, 1995; Thies *et al.*, 1995). Nevertheless, some alphaproteobacteria (*Aminobacter*, *Ochobactrum*, *Methylobacterium* and *Phyllobacterium*), betaproteobacteria (*Herbaspirillum* and *Shinella*) and gammaproteobacteria (*Pantoea*, *Enterobacter* and *Pseudomonas*) have been described as non-rhizobial endophytes (NRE) presented in legume nodules along with rhizobia (Lin *et al*., 2008; Ibáñez *et al*., 2009; Shiraishi *et al*., 2010; Aserse *et al*., 2013). Most of these bacteria are not able to form root nodules, but they can enter infection threads when leguminous plants are also inoculated with rhizobial strains (Leite *et al*., 2017). NRE can also have beneficial effects on the host plants, such as growth promotion, nitrogen fixation, siderophore production, increase of stress tolerance and biological control of plant pathogens (Rajendran *et al*., 2008; Ibáñez *et al*., 2009; Andrews *et al*., 2010; El-Tarabily *et al*., 2010; Tariq *et al*., 2014). In fact, Martínez-Hidalgo and Hirsch (2017) suggest that rhizobia and NRE can work together inside root nodules, in order to improve plant growth and yield, mainly under environmental stress conditions.

5. Arbuscular mycorrhizal fungi

Additionally to bacterial population, fungi also represent a significant part of soil rhizosphere microflora that influences plant growth (Nadeem *et al.*, 2014). The term ‘mycorrhiza’ is derived from the Greek *myco* (fungus) and *rhiza* (root) (Owen *et al.*, 2015). Mycorrhizas are categorized into seven main groups: arbuscular (AM), ericoid, arbutoid, monotropoid and orchid, which are endomycorrhizas, and ecto- and ectendomycorrhizas, which are ectomycorrhizas (Table 9) (Smith and Read, 2008).

Table 9. Summary of main characteristics of the seven types of mycorrhizas (adapted from Harley, 1991; Smith and Read, 1997).

		Fungi septate	Fungi aseptate	Intracellular colonization	Fungal sheath	Hartig net	Vesicles	Plant host chlorophyllous	Fungal taxa	Plant taxa
Endomycorrhizas	**AM**	x	√	√	x	x	√ or x	√ or x	Glomeromycota	Bryo/Pterido/Gymno/Angio
	Ericoid	√	x	√	x	x	x	√	Ascomycota	Ericales/Bryo
	Arbutoid	√	x	√	√ or x	√	x	√	Basidiomycota	Ericales
	Monotropoid	√	x	√	√	√	x	x	Basidiomycota	Monotropaceae
	Orchid	√	x	√	x	x	x	x	Basidiomycota	Orchidaceae
Ectomycorrhizas	**Ecto-**	√	x	x	√	√	x	√	Basidio/Ascomycota	Gymno/Angio
	Ectendo-	√	x	√	√ or x	√	x	√	Basiodio/Asco/Glomeromycota	Gymno/Angio

In the past, arbuscular mycorrhizal fungi were classified as zygomycetes and their spore morphological characteristics were used as taxonomic markers (Morton and Benny, 1990). However, more recently, a new phylum, the Glomeromycota, was created based on analyses of the small subunit rRNA sequences (Schuβler *et al.*, 2001).

The arbuscular mycorrhizal fungi symbiosis, formed between plant roots and fungi, is one of the most widespread symbiotic associations in plants (Harrison, 1998). In fact, about 70-90% of plant species are involved in mycorrhizal symbiosis (Parniske, 2008). Klironomos (2000) indicates that more than 2000 species of arbuscular mycorrhizal fungi are able to colonize the roots of over 300 000 species of plants in ecosystems around the world. The AMF consists of an internal phase inside the root and an external phase, also called extraradical mycelium phase, which can form an extensive network within the soil (Gosling *et al.*, 2006). This association is a non-specific, highly compatible and long-lasting mutualism whereby both partners (fungi and plant) have advantages (Harrison, 1998; Abdel-Fattah *et al.*, 2011). The fungi enable the host plant to absorb water and nutrients more efficiently, because mycelium from mycorrhizal plant roots can grow up to 100 times more than root hairs and proliferate in the surrounding soil, allowing the access to a greater volume of soil. On the other hand, plant supplies the fungus with a direct and constant access to carbohydrates (Ezawa *et al.*, 2002; Smith and Read, 2008; Nadeem *et al.*, 2014). AMF are obligate symbionts that completely depend on a plant host for obtaining carbon and, consequently, for the growth and reproduction (Parniske, 2008; Denison and Kiers, 2011). The access to a greater volume of soil is of particular importance for both partners: to the fungi, because it provides a means to constantly search for new hosts (Denison and Kiers, 2011) and to the plant, due to the higher absorption and transport of low diffusing and mobility nutrients, such as phosphorus (Franzini *et al.*, 2010). In fact, the available phosphorus concentration in soil is limiting for the plant growth. AMF have an important role in the inorganic P acquisition from insoluble P sources in soils and its transfer to the host plants (Read and Perez-Moreno, 2003; Cappellazzo *et al.*, 2008). However, it has also been shown that high levels of available P have a suppressive effect on fungal colonization, leading to malformed arbuscules with reduced branching (Denison and Kiers, 2011).

The uptake of nitrogen can also be influenced by the AMF symbiosis. A research work carried out by McFarland *et al.* (2010) indicated that more than 50% of plant N requirement was supplied by mycorrhizal association. It is also reported that the fungus can contribute to the formation of soil structure (Gianinazzi *et al.*, 2010), improve the plant's resistance to invading pathogens and abiotic stresses (Evelin *et al.*, 2009; Miransari, 2010; Oyewole *et al*., 2017), increase tolerance to salinity and heavy metals (Mohammad *et al.*, 2003) and excrete proteases that break down organic matter and

subsequently capture nitrogen-containing compounds, thus providing a direct path from organically bound nitrogen in the soil to plant (Schimel and Bennett, 2004). A study carried out by Harrier and Watson (2004) showed that different AMF species are effective in reducing plant diseases caused by pathogens on different host species. In other works carried out with the application of *Glomus mosseae*, *Glomus intraradices, Glomus clarum*, *Gigaspora gigantean* and *Gigaspora margarita* on several crops (Abdel-Fattah *et al.*, 2011), it was described that AMF have an important role in the improvement of plant growth, nutrition, water relations and resistance without any recorded side effects (Guenoune *et al.*, 2001; Abdel-Fattah and Shabana, 2002; Chandanie *et al.*, 2005). Hacisalihoglu *et al.* (2005) also demonstrated that the application of *G. intraradices* on different bean leads to an increase on plant growth and production. In other experiments carried out by Wright *et al.* (1998 a,b) it was found that, despite their similar N and P status, AM-infected plant has higher photosynthetic rates than non-mycorrhizal plants, suggesting that the additional photosynthetic products had been transferred to the fungal symbiont. Additionally, symbiotic interactions of faba bean with the mycorrhizal fungus *Glomus fasciolatum* in other study resulted in improved crop growth and yield parameters, with the expression of a leghemoglobin gene induced by both microsymbionts (Fruhling *et al*., 1997). Although several works describe substantial yield increases with mycorrhizal inoculation, this technology is still far from being routinely applied in agricultural practices (Johnson *et al.*, 2013). However, AMF can be, in fact, an eco-friendly and cost-effective strategy to increase crop yields and reduce fertilizer application (Gosling *et al.*, 2006; Abdel-Fattah *et al.*, 2011).

When not associated with a plant, AMF exist in the soil as spores which in some species are large enough to be visible with the naked eye (Harrison, 2005). Some spores can also be observed inside the root cortex. The development of spores is an important reproductive strategy of AMF that allows its propagation, recovery from disturbance and survival in the absence of a host, for more than 10 years (Giovannetti and Sbrana, 2010). The life cycle of mycorrhizal fungi begins when the spores germinate and the external hyphae grow and penetrate on the inner root cortex of the cells to form dichotomously branched structures called arbuscules or coils (Harrison, 2005; Javot *et al.*, 2007; Denison and Kiers, 2011). These structures create a specific interface, where nutrient exchange occurs, but they are short-lived, being active for only 4 or 5 days (Genre *et al.*, 2005, 2008; Denison and Kiers, 2011). In addition to internal growth within the root, the fungus also maintains external hyphae into the soil. These external structures colonize the soil and allow the uptake of nutrients such as phosphate (Gianinazzi-Pearson, 1996; Harrison, 1997, 1998; Denison and Kiers, 2011).

Vesicles are also important AMF structures in some families, such as Glomeraceae (Denison and Kiers, 2011). Whereas a high arbuscule frequency indicates efficient nutrient exchange in both directions, high vesicular colonization is an indicator of fungal resource hoarding (Denison and Kiers, 2011). Indeed, the carbon derived from the host plant is transferred to the fungi, and stored in vesicles to support vegetative growth or spores (Bonfante and Genre, 2010). Soils from low-input farming systems have a greatly enhanced capacity to initiate the mycorrhizal symbiosis (Ezawa *et al.*, 2000). In fact, many studies showed a limited AMF colonization in sites with high intensity of fertilizer input (Johnson and Pfleger, 1992).

6. Legumes co-inoculation with both rhizobia and AMF

Dual inoculation with both rhizobia and AMF (Fig. 10) results in a tripartite mutualistic symbiosis and usually improves N_2 fixation and the uptake of nutrients and water, leading to an increase of many legumes growth and yield to a greater extent than inoculation with only one microorganism (Chalk *et al.*, 2006; Marulanda *et al.*, 2006).

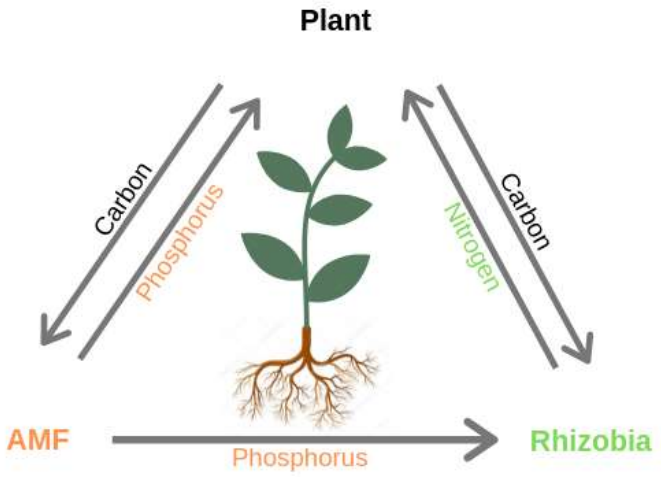


Figure 10. Tripartite symbiosis between plant, rhizobia and AMF (adapted from Chang *et al*., 2017).

In fact, additive and occasionally synergistic effects on legume performance are frequently observed when both rhizobia and AMF are present (Sanginga *et al.*, 1999; Gloss and de Varennes, 2002). Jia *et al.* (2004) demonstrated that faba bean involved in tripartite symbiotic association had higher elemental P to N ratios compared with non-symbiotic plants, and this ratio was an important factor to determine the plant productivity levels. According to Tajini and collaborators (2012), the symbiosis with the both microorganisms under P limitation can enhance biological nitrogen fixation in leguminous plants, being a friendly technique to the environment and well appreciated by the

consumers. Other work carried out with pot experiments using sterilized soil showed positive effects of AMF inoculation, including those where dual inoculation with *Rhizobium* resulted in improved crop growth and yield parameters (El-Wakeil and El-Sebai, 2007). Many other studies also showed that the combined inoculation with rhizobia and AMF promotes establishment and increases the biomass production of native species (Marques *et al.*, 2001; Santiago *et al.*, 2002; Scotti and Corrêa, 2004; Duarte *et al.*, 2006). Despite all these works involving the both microorganisms, there is still a lack of genotypic evaluation as well as of effectiveness of particular strains in BNF in diverse agro-ecological conditions.

7. Inoculant production

Although rhizobia and AMF species are widely distributed, there are several soils where appropriate strains for specific species are absent, or where the population density is low, leading to the need of inoculation (Brockwell *et al.*, 1995). Inoculants are commercial formulations which contain selected microorganisms to be applied to the seeds or to the soil during plantation (Brockwell and Bottomley, 1995) with the aim to reduce inorganic fertilizer inputs (Owen *et al.*, 2015). Bioinoculants improve the health of plants by enhancing their defense system by the mechanism of Induced Systematic Resistance (ISR) (Pieterse *et al*., 2014). There are already many microbial inoculants and its global market is rising at an estimated rate of approximately 10% per year (Berg, 2009), valued at $440 million in 2012 and should reach $1.295 million until 2020 (Transparency Market Research, 2014).

This microbial inoculant popularity increased substantially, due to extensive and systematic research that has improved their effectiveness and consistency (Thakore, 2006). In fact, nowadays, about 2000 tons of inoculants of different organisms are produced annually around the world, which is enough to inoculate approximately 20 million ha of legumes (Rebah *et al.*, 2007). The plant growth-promoting microorganisms (PGPM) most used in bioinoculants belong to two main groups: bacteria and fungi (Owen *et al.*, 2015), which include rhizobia and AMF, respectively (Fig. 11).

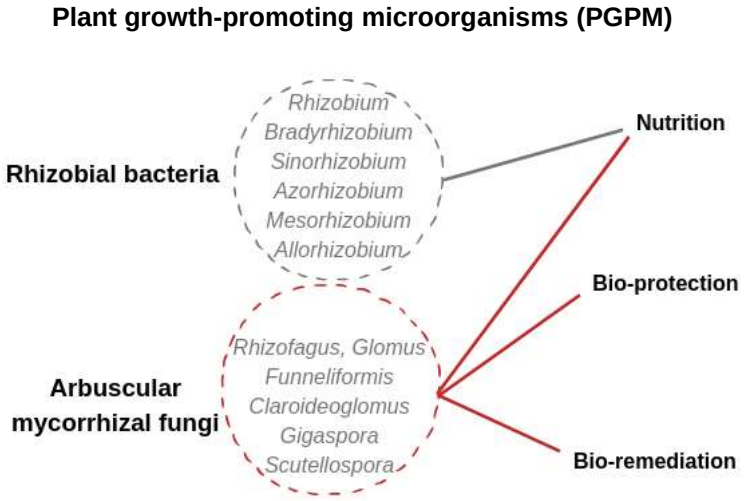


Figure 11. Main plant growth-promoting microorganisms used in commercial bioinoculants and the various mechanisms each employ to promote plant growth (adapted from Owen *et al.*, 2015).

Generally, inoculants are commercialized as solid inoculants, in powder from peat or in granular forms, or as liquid inoculants, in broth formulations (Stephens and Rask, 2000). The method of inoculation depends on the inoculant type. Powdered and liquid products are usually used for inoculation and direct application on seeds. However, liquid inoculants can be also applied in the furrow during plantation. On the other hand, granular products are normally applied directly to the soil, in the furrow, deep banded below the seed or side banded, preferably deeper than the seed (Stephens and Rask, 2000; Rebah *et al.*, 2007). To the inoculant preparation, it is required the use of sterile or non-sterile peat carriers. However, according to Date and Roughley (1977), the inoculants using sterile peat can contain 100-fold more rhizobia and it can have much longer shelf lives than the inoculants with non-sterilized peat. This difference can increase during the storage process, because the mortality of rhizobia is greater in non-sterile than in sterile peat carriers (Roughley and Vincent, 1967; Date and Roughley, 1977). The most used sterilization method has been gamma irradiation, nonetheless it is an expensive and slow technique. Autoclaving is another sterilization method that has been broadly used in laboratorial works but it has some disadvantages: it is laborious, costly and time consuming and in a commercial context can compromise the inoculant quality by the production of toxins during the sterilization process (Hari and Perumal, 2010). More recently, a new sterilization method, called electron acceleration, appeared. This is a non-nuclear method and depends on the exploitation of a series of acceleration cavities, which result in an electron beam. The major benefit of this sterilization method is the turn-around time, since the pre-packaged peat flour is exposed to the sterilization

process for only few seconds, whereas the gamma irradiation process takes some hours (Stephens and Rask, 2000).

Microbial inoculants present several advantages, in comparison with chemical or synthetized pesticides and fertilizers, such as: they are more safe, present reduced environmental impact and potentially smaller risk for human health, show much more targeted activity, are effective in small quantities, multiply themselves but are controlled by the plant and indigenous microbial populations, resistance development is reduced and can be also used in organic, conventional and integrated pest management systems (Berg, 2009). The commercial bioinoculants should lead to an economic gain, improved yields or reduced inorganic fertilizer application, or both (Owen *et al.*, 2015).

However, each inoculant can have a variable response because the bacteria needs to compete with persistent and well-adapted indigenous microorganisms and survive in variable environmental conditions (Argaw and Mnalku, 2017). Because of this, Ruiz-Díez *et al*. (2012) reinforced the requirement of the selection of symbiotically efficient strains for every cultivated legume in each specific area.

7.1. Rhizobia inoculant production

In 2012, rhizobia bioinoculants were the most produced, constituting almost 79% of the global demand (Owen *et al.*, 2015). The development of commercial rhizobial inoculants require some features which should be previously confirmed (Brockwell *et al.*, 1995; Stephens and Rask, 2000):

- Capacity to form nodules and fix N on the target legume;
- Capacity to compete in nodule formation with populations of rhizobia already present in the soil;
- Capacity to fix N across a range of environmental conditions;
- Capacity to form nodules and fix N in the presence of soil nitrate;
- Capacity to grow well in artificial media, in inoculant carrier and in the soil;
- Capacity to persist in soil, particularly for annually regenerating legumes;
- Capacity to migrate from the initial site of inoculation;
- Capacity to colonize the soil in the absence of a legume host;
- Capacity to tolerate environmental stresses;
- Capacity to fix N with a wide range of host genotypes;
- Genetic stability;
- Compatibility with agrochemicals;
- Wide host range;
- Low mortality on inoculated seed;

- Capacity to colonize the rhizosphere of the host plant;
- No or minimal contamination by microorganisms not detrimental to rhizobia or pathogen to plants and humans.

Indeed, the major areas of research are related to increase rhizobial populations per unit weight or volume of product, organism efficacy and product durability (Stephens and Rask, 2000; Rebah *et al.*, 2007).

The standard culture medium for rhizobia growth, yeast mannitol agar (YMA), includes mannitol as carbon source, yeast extract as nitrogen source, growth factors and mineral salts and has been broadly used for laboratory scale production of rhizobia inoculants, though its industrial use is limited due to its high cost (Rebah *et al.*, 2007).

7.2. AMF inoculant production

The production of AMF is generally performed by the cultivation of plants and associated symbionts in a substrate, like soil or sand (IJdo *et al.*, 2010). The process is often conducted in greenhouses or growth chambers, under controlled or semicontrolled conditions, for the easy handling and control of some parameters such as humidity, temperature and light. However, according to the host plant and climate conditions, large-scale production is occasionally conducted in open air and at a reduced frequency on field plots (IJdo *et al.*, 2010). Low-nutrient availability, mainly P, can favor AMF colonization (Smith and Read, 2008). AMF bioinoculants contain preparations of spores propagated in pot cultures mixed with an inert carrier (Gentili and Jumpponen, 2006). The AMF inoculant production usually starts from isolated spores or a mixture of spores and mycorrhizal roots (Gaur and Adholeya, 2000). In fact, whereas spores persist longer in the soil, they are slow to colonize host plants compared to fragments, thus inoculants usually consist of both (Marin, 2006). To obtain a mixed inoculum, roots have to be dried and chopped into small pieces. On the other hand, wet sieving and decanting methods are often used to obtain isolated spores (IJdo *et al.*, 2010). The direct inoculation of plants with isolated spores or mixed inoculum can be performed, but additionally plantlets can also be precolonized before their transplantation into the containers (IJdo *et al.*, 2010). AMF pure isolates can also be obtained by trap cultures. The International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM), The International Bank for the Glomeromycota (BEG) and The Glomales In Vitro Collection (GINCO) are some examples of international culture collections that can ensure the delivery of well-identified monospecies and provide a clear traceability of the organism by a repository identification code (IJdo *et al.*, 2010).

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CHAPTER III

BIODIVERSITY OF RHIZOBIA ASSOCIATED WITH COWPEA PLANTS

CHAPTER III- BIODIVERSITY OF RHIZOBIA ASSOCIATED WITH COWPEA PLANTS

BRIEFING NOTE

This chapter includes the morphological and molecular characterization of the rhizobial isolates associated with cowpea plants (*Vigna unguiculata* (L.) Walp.). Bacteria were isolated from fresh surface sterilized nodules present in the roots collected from cowpea plants in regions with different edaphoclimatic conditions in Portugal.

The 16S rDNA gene sequences have been widely used for taxonomic classification of bacteria. However, as this genomic region did not provide enough resolving power in discriminating closely related species in the studied genera, the analysis using other housekeeping and accessory genes, such as those involved in nodulation of the host plant (*nod*, *nif)*, were performed for optimal species-level differentiation. The results of multilocus sequence analysis (MLSA) showed a high diversity of rhizobia strains including putative new species and symbiovars.

The authors contribution for the article converted in the present chapter was: Sandra Pereira, Lav Sharma and Ângela Mucha were responsible for the DNA extraction, amplifications, and phylogenetic analysis. Sandra Pereira was also responsible for data interpretation and manuscript writing. Eduardo Rosa and Guilhermina Marques were responsible for the study conception and design of the experiment and critical revision of the article. All authors reviewed and approved the final manuscript.

Biodiversity of rhizobial bacteria associated with cowpea (*Vigna unguiculata* (L.) Walp.) in portuguese soils

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Abstract

Cowpea (*Vigna unguiculata* (L.) Walp.) is a grain legume which establish efficient symbiosis with a high diversity of bacteria. Most of these bacteria are not rhizobia, able to form root nodules, but they can enter infection threads when leguminous plant are colonized with rhizobial strains, being described as non-rhizobial endophytes (NRE).

Many works have been performed on cowpea-nodulating bacteria in several countries around the world. However, little is known about the genetic and symbiotic diversity of indigenous cowpea rhizobia in Europe. The aim of this study was to describe the biodiversity of bacterial communities associated with cowpea root nodules. Thirty-five bacteria were isolated from plants collected in several regions of Portugal with different edapho-climatic conditions. A multilocus sequence analysis (MLSA) based on 16S rDNA region, two symbiotic genes (*nodA* and *nodC*) and six housekeeping genes (*recA*, *gyrB*, *SMc00019, thrA*, *atpD* and *truA)* were performed.

Rhizobium was the most abundant genus of the detected genera. Furthermore, we found a high bacterial diversity associated to cowpea root nodules, namely from *Bradyrhizobium* and *Caulobacter* (α-proteobacteria), *Burkholderia* and *Herbaspirillum* (β-proteobacteria) and *Kosakonia* and *Enterobacter* (γ-proteobacteria) genera. Although all the strains isolated were able to nodulate *in vitro* cowpea plants, no *nodA* and *nodC* genes were amplified in some of them. Some of these rhizobial strains are promising candidates for biofertilizers formulation for the improvement of the productivity of cowpea plants.

Keywords: Leguminous plants, rhizobia diversity, MLSA, molecular identification

1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is considered a multifunctional crop due to its use as vegetable, fodder and textile resource (Vanderborght and Budoin, 2001). Due to its high amount of protein, carbohydrates and fibre, cowpea seeds are important nutritional food in the human diet (Iqbal *et al.*, 2006). Additionally, cowpea contributes to the sustainability of cropping systems through symbiotic nitrogen (N) fixation. Its tolerance to different soil pH, high temperatures and drought stress compared to other legumes, make cowpea as one of the most important grain legume crops (Hall *et al.*, 2002; Oliveira *et al*., 2017). Even so, low yield of cowpea plants is frequently attributed to several environmental limitations, namely drought, low fertility of soil and low symbiotic efficiency of indigenous rhizobia (Dakora and Keya, 1997).

Cowpea is one of the most widely cultivated seed-legume in arid and semi-arid areas (Johnson *et al.*, 2013), providing a cheap source of protein for human consumption (Ehlers and Hall, 1997; Timko and Singh, 2008). It is difficult to obtain consistent data on cowpea cultivated area and production as this crop is grown in mixture with other crops (Ngalamu *et al*., 2014). However, it could be estimated that, in 2018 the world area harvested was over 12.5 million ha, with an annual production of around 7.2 million tons worldwide (FAOSTAT, 2018). Despite its wide distribution, Africa amounts to around 96% of the total area cultivated with cowpea in the world (FAOSTAT, 2018). Around 70% of world cowpea production comes also from the West and Central Africa (Alkama *et al.*, 2009; Oliveira *et al*., 2017), however, Asia, Central and South America, and southern and south-eastern Europe are also large producers (Singh *et al*., 2002).

One of the main concerns regarding leguminous plants in general and cowpea in particular is its low productivity in some countries. In this sense, legume inoculation with efficient rhizobial bacteria can be an eco-friendly strategy to increase cowpea productivity and at the same time to improve seed nutritional value, in particular protein content in the grain (Oliveira *et al*., 2017). Annually, symbiotic relationship between rhizobial strains and legumes produces about 60% of the total biological nitrogen fixation inputs in world agriculture (Herridge *et al*., 2008).

For decades, rhizobia were thought to be the only nitrogen-fixing inhabitants of legume nodules (Leite *et al*., 2017; Martínez-Hidalgo, 2017) and this group of bacteria included the genera *Rhizobium, Bradyrhizobium, Azorhizobium*, *Ensifer* and *Mesorhizobium* (Sawada *et al*., 2003). Within these genera, a heterogeneous group of slow-growing rhizobia belonging to the genus *Bradyrhizobium* and known as “cowpea-miscellany” has the ability to nodulate cowpea (Allen and Allen, 1981; Appunu *et al.*, 2009), being *Bradyrhizobium elkanii*, *B. yuanmingense* and *B. japonicum* the main rhizobial species associated with this culture (Zhang *et al*., 2008). Fast-growing rhizobia have also been reported to nodulate this species (Chidebe

et al., 2018). Recently, other α-proteobacterial genera, such as *Ochrobactrum* (Trujillo *et al*., 2005), *Methylobacterium* (Sy *et al*., 2001), *Microvirga* (Ardley *et al*., 2012; Radl *et al*., 2014), *Devosia* (Rivas *et al*., 2003) and *Phyllobacterium* (Zakhia *et al*., 2006) have also been considered as nitrogen fixing root nodule bacteria of leguminous plants. β-proteobacteria from the genera *Burkholderia* and *Cupriavidus* were also described as β-rhizobia (Moulin *et al*., 2001; De Meyer *et al*., 2014). However, some α-proteobacteria (*Aminobacter*, *Ochobactrum*, *Methylobacterium* and *Phyllobacterium*), β-proteobacteria (*Herbaspirillum* and *Shinella*) and γ-proteobacteria (*Pantoea*, *Enterobacter* and *Pseudomonas*) has been described as non-rhizobial endophytes (NRE) presented in legume nodules along with rhizobia (Valverde *et al*., 2003; Benhizia *et al*., 2004; Lin *et al*., 2008; Ibáñez *et al*., 2009; Shiraishi *et al*., 2010; Aserse *et al*., 2013). Most of these bacteria are not able to form root nodules, but they can enter infection threads when leguminous plants are colonized with rhizobial strains (Leite *et al*., 2017). NRE can have beneficial effects on the host plants, such as growth promotion, nitrogen fixation, siderophore mediated interactions, increased promotion of stress tolerance and biological control of plant pathogens (Rajendran *et al*., 2008; Ibáñez *et al*., 2009; Andrews *et al*., 2010; El-Tarabily *et al*., 2010; Tariq *et al*., 2014). Rhizobia and NRE can work together inside root nodules, in order to improve plant growth and yield, mainly under environmental stress conditions (Martínez-Hidalgo and Hirsch, 2017).

In recent years, the interest in deepening the knowledge about the bacteria existing inside the legume root nodules has increased, in order to select more efficient strains and to reduce the chemical fertilizer inputs, in a more sustainable agriculture. In this sense, the aim of this work was to identify the bacteria presented in cowpea root nodules collected from several regions of Portugal with different edaphoclimatic conditions.

The sequencing of 16S rDNA region has been widely used for a preliminary identification of the isolates. However, it did not provide enough resolving power in discriminating closely related species, since it is extremely conserved in *Rhizobium* and *Bradyrhizobium* genera. So, in order to improve the bacterial identification, other genomic regions, including housekeeping genes (located on chromosomes) and nodulation (*nod*) genes presented in mobile extra-chromosomal plasmids or symbiotic islands have been included in the phylogenetic analysis (Peix *et al*., 2015; Zahran, 2017). *Nod* genes are involved in the formation of nodules and determination of host specificity (Perret *et al*., 2000; Wang *et al*., 2013). When *nod* genes are expressed, extracellular bacterial compounds called *Nod* factors are also synthetized (van Rhijn and Vanderleyden, 1995). These factors act as signals and are responsible for the first symptoms of nodule formation, such as deformation of root hairs, formation of infection threads and cell division in the root cortex (Lerouge *et al*., 1990). The mobile elements presented in *nod* genes can be laterally transferred between organisms, even

disparate evolutionary lineages, in a process called horizontal gene transfer (HGT). This process occurs through several mechanisms, namely transformation, transduction and conjugation (Davison, 1999). Most of the studies involving HGT in rhizobia are focused on closely related species, normally within the same genus (Ling *et al*., 2016). It means that the occurrence of HGT from alpha to beta-proteobacteria and vice versa remains poorly studied.

To the best of our knowledge, there are no previous reports on the biodiversity of bacteria present in root nodules of cowpea plants in Portugal. So, we propose to access the diversity of root-nodulating bacteria associated with cowpea in Portugal, using multilocus sequence analysis (MLSA).

2. Material and methods

Nodule collection and bacteria isolation

Nodules were excised from cowpea plant roots, collected in several regions of Portugal with different edaphoclimatic conditions. Details of sampling (host plant, collection site and coordinates) of the 35 isolates are shown in Table 1.

Root nodules were surface sterilized (1.5% sodium hypochlorite (NaClO) (v/v) washing for 1 min, 70% ethanol washing for 1 min and several washes with sterilized distilled water), crushed aseptically and bacteria was streaked on Yeast Mannitol Agar (YMA) medium (1 g L^{-1} of yeast extract, 10 g L^{-1} of mannitol, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.1 g L^{-1} NaCl and 15 g L^{-1} agar) supplemented with 0.025 g L^{-1} congo red (CR). After an incubation of 3-5 days, a single colonie was peaked to plates with same medium supplemented with 0.1 g L^{-1} bromothymol blue (BTB). This process was repeted until pure cultures were obtained.

For authentication tests, in order to test infection ability in cowpea plants (Kock's postulates), all purified isolates were inoculated in cowpea plants. This experiment was performed with surface-sterilized cowpea seeds cultivar Fradel. Sterilization was performed with 1.5% sodium hypochlorite (NaClO) (v/v) washing for 2 min, 70% ethanol washing for 1 min and several washes with sterilized distilled water. Seeds were pre-germinated and transferred to a sterilized glass bottle filled with a semi solid sterile nutrient solution (1 g L^{-1} $CaHPO_4$, 0.2 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.2 g L^{-1} NaCl, 0.1 g L^{-1} $FeCl_3.6H_2O$, 1.0 mL L^{-1} micronutrients (0.5% B; 0.05%Mn; 0.005% Zn; 0.005% Mo and 0.002% Cu) and 9.0 g L^{-1} agar) (Jensen, 1942). One bacterial strain was inoculated in each bottle and uninoculated plants were used as negative control. Plants were uprooted, 4 weeks after inoculation, and assayed for the presence of nodules, which were re-isolated and grown in the same culture media.

Table 1- Bacterial isolates collected from cowpea plants in several regions of Portugal.

Isolate	Coordinates	Molecular identification	GenBank acession number
R16	41°17'13.10''N 7°44'13.34''W	*Rhizobium* sp.	MT425985
R17	39°27'52''N 8°02'00''4W	*Rhizobium* sp.	MT425991
R18	39°27'05''N 8°00'21''W	*Rhizobium* sp.	MT425993
R19	39°23'57''N 7°53'40''W	*Rhizobium* sp.	MT425996
R22	39°27'05''N 8°00'21''W	*Rhizobium* sp.	MT425995
R24	39°27'05''N 8°00'21''W	*Rhizobium* sp.	MT425989
R25	39°27'52''N 8°02'00''4W	*Kosakonia* sp.	MT426004
R30	41°17'08.35''N 7°44'28.83''W	*Rhizobium* sp.	MT425997
R31	41°11'47.48''N 7°45'14.35''W	*Rhizobium* sp.	MT425994
R32	39°23'57''N 7°53'40''W	*Rhizobium* sp.	MT425988
R33	38°53'17''N 7°08'37''W	*Rhizobium* sp.	MT425984
R34	41°25'55.70''N 8°23'03.15''W	*Rhizobium* sp.	MT425983
R35	37°47'09''N 7°43'10''W	*Rhizobium* sp.	MT425987
R36	39°27'05''4N 8°00'21''W	*Rhizobium* sp.	MT425992
R37	39°27'52''N 8°02'00''4W	*Rhizobium* sp.	MT425998
R43	39°23'57''N 7°53'40''W	*Rhizobium* sp.	MT425999
R44	39°27'05''N 8°00'21''W	*Burkholderia* sp.	MT426011
R45	41°25'55.70''N 8°23'03.15''W	*Rhizobium* sp.	MT425990
R50	38°53´17´´N 7°08'37''W	*Enterobacter* sp.	MT426010
R51	41°25'55.70''N 8°23'03.15''W	*Rhizobium* sp.	MT425986
R53	39°27'05''N 8°00'21''W	*Bradyrhizobium elkanii*	MT426001
R57	39°23'57''N 7°53'40''W	*Bradyrhizobium* sp.	MG973287
R59	39°23'57''N 7°53'40''W	*Caulobacter* sp.	MT426000
R62	41°25'55.70''N 8°23'03.15''W	*Burkholderia fungorum*	MT426012
R63	41°17'13.10''N 7°44'13.34''W	*Bradyrhizobium elkanii*	MG973286
R121	41°20'09''N 6°42'09''W	*Enterobacter* sp.	MT426005
R122	41°19'28''N 6°56'15''W	*Enterobacter* sp.	MT426006
R123	41°17'54''N 6°42'46''W	*Enterobacter* sp.	MT426007
R124	41°20'09''N 6°42'09''W	*Enterobacter* sp.	MT426008
R125	41°18'21''N 6°40'37''W	*Enterobacter* sp.	MT426009
R133	41°18'21''N 6°40'37''W	*Bradyrhizobium* sp.	MT426002
R141	41°20'09''N 6°42'09''W	*Herbaspirillum* sp.	MT426014
R142	41°17'54''N 6°42'46''W	*Herbaspirillum* sp.	MT426013
BF9b	41°16'53.86''N 7°44'43.09''W	*Bradyrhizobium* sp.	MT426003
BF10	41°16'53.86''N 7°44'43.09''W	*Bradyrhizobium elkanii*	MG973288

DNA extraction, PCR amplification and sequencing

DNA extraction for PCR amplification was done from re-isolated bacteria and according to the method used by Laguerre *et al*. (1996), with some modifications. In this process, cell lysis was performed with CTAB lysis buffer (cetyltrimethylammonium bromide) and also using mechanical lysis, through the FastPrep-24 equipment (MP Biomedicals). The concentration of obtained DNA was estimated by spectrophotometer or electrophoresis.

Amplification of 16S rDNA region was performed with the universal primers fD1 and rD1 (Table 2). Furthermore, for multilocus sequence analysis (MLSA) and in order to identify the isolates at species level, this analysis was complemented with 6 housekeeping genes: *recA* (DNA

recombination protein), *gyrB* (DNA gyrase B), *SMc00019* (conserved hypothetical protein), *thrA* (homoserine dehydrogenase), *atpD* (atpD synthase β-subunit) and *truA* (RNA pseudouridine synthase A). Taxonomic position at symbiovar level was determined by the inferred phylogenies based on the symbiotic genes of nodulation: *nodA* (N-acyltransferase nodulation protein A) and *nodC* (N-acetylglucosaminyltransferase). Primers used are presented in Table 2.

PCR mixtures were performed with 7.5 µl of master mix (MyTaq HS Mix, 2x of Bioline), 1 µl of each forward and reverse primer and 5.5 µl of DNA template, with 15 µl of final volume. Amplified samples were sequenced (Stabvida, Portugal), using the same primer set described for PCR amplification.

Table 2- List of primers used in this work.

Primers	Sequence (5'-3')	Reference
fD1	AGA GTT TGA TCC TGG CTC AG	Weisburg *et al*., 1991
rD1	AAG GAG GTG ATC CAG CC	
thrAB-F	TGC TTC GTC GAR YTG ATG G	Zhang *et al*., 2012
thrAB-R	ACR CCC ATC ACC TGY GCR ATC	
thrAMRS-F	TAA TAC GAC TCA CTA TAG GGG CNG GBG GYA TYC CSG TBA TCA AG	modified by Tampakaki from Zhang *et al*., 2012
thrAMRS-R	GAT TTA GGT GAC ACT ATA GCG YTC GAT NCG RAT SAC YTG SGG	
SMc00019B-F	CAT TCV KCS GAR GGV GCS ATG GGY ATC	Zhang *et al*., 2012
SMc00019B-R	GCG TGB CCB GCS KCG TTS GAV AGC AT	
SMc00019MRS-F	TAA TAC GAC TCA CTA TAG GGC ADT TCC TBA THG CCA TGC C	modified by Tampakaki from Zhang *et al*., 2012
SMc00019MRS-R	GCV GGR CAN KTS AGC CAD CCR TT	Zhang *et al*., 2012
truAB-F	TAA TAC GAC TCA CTA TAG GGC GCT ACA AGC TCA YYA TCG A	modified by Tampakaki from Zhang *et al*., 2012
truAB-R	CCS ACC ATS GAG CGB ACC TG	Zhang *et al*., 2012
truAR-F	TGA CCG TSG AAT ATG ACG G	
truAR-R	ACA TCS AGY CGG TCV AGS GT	
truAMS-F	TAA TAC GAC TCA CTA TAG GGC AGG TSG CDC ATS TCG AYC T	modified by Tampakaki from Zhang *et al*., 2012
truAMS-R	GAD CGB AYC TGG TTR TGM AG	Zhang *et al*., 2012
gyrB340F-T7	TAA TAC GAC TCA CTA TAG GGT TCG ACC ARA AYT CYT ACA AGG	modified by Tampakaki from Zhang *et al*., 2012
gyrB1057R-SP6	GAT TTA GGT GAC ACT ATA GCC AAY TTR TCC TTG GTC TGC G	
gyrB-F	ACC GGT CTG CAY CAC CTC GT	Spilker *et al*., 2009
gyrB-R	YTC GTT GWA RCT GTC GTT CCA CTG C	
recA6F	CGK CTS GTA GAG GAY AAA TCG GTG GA	Gaunt *et al*., 2001
recA555R	CGR ATC TGG TTG ATG AAG ATC ACC AT	
atpD273F	SCT GGG SCG YAT CMT GAA CGT	Gaunt *et al*., 2001
atpD-294F	TAA TAC GAC TCA CTA TAG GGA TCG GCG AGC CGG TCG ACG A	modified from Gaunt *et al*., 2001
atpD771R	GCC GAC ACT TCC GAA CCN GCC TG	Gaunt *et al*., 2001
nodA-1	TGC RGT GGA ARN TRN NCT GGG AAA	Haukka *et al*., 1998
nodA-2	GGN CCG TCR TCR AAW GTC ARG TA	
nodCF	AYG THG TYG AYG ACG GTT C	Laguerre *et al*., 2001
nodCFu	AYG THG TYG AYG ACG GIT C	
nodCI	CGY GAC AGC CAN TCK CTA TTG	

Data analysis

Nucleotide sequences were corrected using BioEdit version 6.0 software and homology searches were performed at the National Center for Biotechnology Information (NCBI) server using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). Corrected sequences

were submitted in GenBank database with the accession numbers MT425983-MT426014 and MG973286-MG873288. For phylogenetic analysis, sequences were aligned with the most similar sequences retrieved from NCBI database using MAFFT software version 7 (Katoh and Standley, 2013). Maximum Likelihood (ML) phylogenetic trees were constructed in MEGA 6.06 (Tamura *et al*., 2013), using GTR+G (5 categories) substitution model and considering all sites in final datasets. Robustness of tree topologies was estimated using 500 bootstrap replicates. Trees were drawn to scale, with branch lengths in same units as those of the evolutionary distances used to infer phylogenetic tree. Evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site.

Concatenation of all genes was performed using Geneious 9.1.6 (Biomatters Ltd, New Zealand) and network analysis was done using NeighborNet analysis in SplitsTree 4.0 (Huson and Bryant, 2006). Concatenated tree was made with RAxML 8.2 (Stamatakis, 2014) using GTR+G+I model. Editing of trees was done in MEGA 6.06.

3. Results and discussion

Isolation of root nodule bacteria

A total of 35 bacterial isolates were obtained from the root nodules of cowpea plants collected in several regions of Portugal, with different edaphoclimatic conditions. All strains were able to form effective pink-red coloured nodules on their host of origin in the authentication tests. The negative control did not develop any nodules, confirming aseptic experimental conditions. The effectiveness of the strains was shown by the pink colour inside the nodules and the dark green colour of leaves compared to negative controls. These authenticated rhizobial isolates were then genetically analysed using various molecular tools.

16S analysis of cowpea isolates

The analysis of 16S rDNA sequences involved 106 nucleotide sequences, with 1111 positions in the final dataset.

Phylogenetic tree built with 16S rDNA gene sequences of cowpea nodules (Supplementary material 1) split the strains into 3 well-supported separate clades (100%): alpha (α-PB), beta (β-PB) and gamma (γ-PB) proteobacteria. In the first group (α-PB), it was possible to observe 3 subgroups- *Rhizobium*, *Bradyrhizobium* and *Caulobacter*. Most of the isolated species (*N*=17) were from *Rhizobium* genus, followed by *Bradyrhizobium* (*N*=6) and *Caulobacter* (N=1) genera. Second group (β-PB) was split in 2 subgroups: *Herbaspirillum*

(*N*=2) and *Burkholderia* (N=2) and the subgroup of γ-PB was subdivided in *Kosakonia* (N=1) and *Enterobacter* (*N*=6).

Most of the studied sequences in this work were placed in *Rhizobium* and *Bradyrhizobium* clades, which strongly indicates that, as expected (Pule-Meulenberg *et al.,* 2010), *Rhizobium* and *Bradyrhizobium* species were the major rhizobial symbionts of cowpea, irrespectively of plant genotype and soil type.

However, the use of 16S rDNA gene as a single molecular marker has been censured and nowadays multilocus sequence analysis (MLSA) is a more reliable classification method than methodology based on solely ribosomal sequences, due to several reasons. Firstly, several unlinked genes dispersed in the core genome better represent the true genealogy of the organism than just one single sequence or sequences from a locus that might show within strain variation (Young and Haukka, 1996). Secondly, especially in rhizobia, ribosomal sequences show mosaicism as a consequence of homologous recombination, which interferes with phylogenetic tree construction (Terefework *et al*., 1998; Van Berkum *et al*., 2003; Eardly *et al*., 2005). Thirdly, 16S rRNA genes of rhizobia often display low polymorphism in comparison with other taxonomic markers. They are thus often unreliable for species delineation (Li *et al.,* 2009). In fact, MLSA is capable of yielding sequence clusters at a wide range of taxonomic levels, from intraspecific through the species level to clusters at higher levels (Gevers *et al.,* 2005).

Analysis of housekeeping genes in cowpea isolates

A MLSA approach is widely used where the housekeeping and the nodulation genes are also considered, along with 16S rDNA, for rhizobial taxonomy and phylogeny. Bacterial genes encoding for the proteins recombinase A (*recA*), β-subunit of ATP synthase F1 (*atpD*) and DNA gyrase B subunit (*gyrB*) are some of the examples of such housekeeping genes. Genes necessary for the nodulation process, for e.g., biosynthesis of nod factors (N-acyltransferase) (*nodA*) and biosynthesis of nod factors (N-acetylglucosaminyltransferase) (*nodC*) are also used. Recently, three different markers, namely, a conserved hypothetical protein (*SMc00019*), homoserine dehydrogenase (*thrA*), and RNA pseudouridine synthase A (*truA*) were described for their abilities for a congruent and robust rhizobia phylogeny (Zhang *et al*., 2012).

Sequences of the corresponding housekeeping genes from type and reference strains were retrieved from the Genbank and were trimmed appropriately. The sequence availability in this database determined the number of type strains/taxa included in the analysis as well as the number of positions, i.e., the length of the alignments in the final dataset. Some nucleotide

sequences are missing in each phylogenetic tree, due to difficulties in PCR amplification and/or bad sequence results.

The individual ML phylogenetic tree of *atpD* (Supplementary material 2) involved 87 nucleotide sequences, with 1427 positions in the final dataset, while the analysis of *recA* phylogeny (Supplementary material 3) involved 79 nucleotide sequences, with 1621 positions in the final dataset.

In these individual trees, all the amplified isolates were placed in α-PB clade. In fact, in *atpD* tree, 13 isolates were placed in *Rhizobium* subgroup and 5 in *Bradyrhizobium* ones. The R63_MG973286 and R142_ MT426013 isolates were placed in *Rhizobium* subgroup in *atpD* tree, however in 16S tree, they were classified as *Bradyrhizobium* and *Herbaspirillum*, respectively.

In *recA* phylogenetic tree, 29 isolates were located in the *Rhizobium* subgroup, and the remaining 6 in *Bradyrhizobium* one. Indeed, this region is not adequate to discriminate these isolates. In *recA* tree, *Rhizobium* subgroup included samples that were classified as *Bradyrhizobium* (R53_MT426001), *Burkholderia* (R62_MT426012), *Enterobacter* (R50_MT426010, R121_MT426005, R122_MT426006 and R123_MT426007), *Kosakonia* (R25_MT426004), *Herbaspirillum* (R142_MT426013) and Caulobacter (R59_ MT426000) in 16S tree.

Taking in account the individual tree of *gyrB* gene (Supplementary material 4), which was constructed with 97 nucleotide sequences and 833 positions in the final dataset, four isolates were placed in *Herbaspirillum* clade, although the R122_MT426006 and R123_MT426007 isolates, in 16S individual tree, are located in *Enterobacter* clade. Similar to 16S classification, the isolate R44_MT426011 was placed in *Burkholderia* clade. For this gene, the remaining amplified isolates were placed, like in 16S tree, in α-PB clade, in particular in *Rhizobium* (N=9), *Ochrobactrum* (N=1), *Bradyrhizobium* (N=5) and *Caulobacter* (N=1) subgroups.

The distribution of isolates using the recently designed primer *SMc00019* was performed using 83 nucleotide sequences and 593 positions in the final dataset. This phylogenetic tree (Supplementary material 5) was in accordance with 16S tree for all the isolates, with exceptions of isolates R53_MT426001, R50_MT426010 and R124_MT426008 that were placed in *Rhizobium* clade in this gene and in *Bradyrhizobium* and *Enterobacter* clade by the 16S region and the isolate R125_MT426009 that was placed in *Burkholderia* clade using this new set of primers, but it was classified as *Enterobacter* in 16S tree.

The *thrA* tree (Supplementary material 6) was performed using 50 nucleotide sequences and with 952 positions and the *truA* tree (Supplementary material 7) was made with 42 nucleotide sequences and using 543 positions in the final dataset. Although the

amplification success was lower using *thrA* and *truA* genes, all the isolates were placed in *Rhizobium* and *Bradyrhizobium* clade, once sequences from the other genera are not yet available in the database.

Slight differences in the tree topologies of the individual ML trees were observed. Incongruence of phylogenetic relationships for housekeeping genes in some species has also been reported in previous studies, which may be the result of recombination, migration or horizontal gene transfer (HGT) (Vinuesa *et al*., 2005; Islam *et al*., 2008; Rivas *et al*., 2009).

Phylogenetic analysis of nodulation genes

The nodulation and nitrogen fixation capacity are characters usually studied in rhizobia research, since they give an idea of symbiotic potential and host specificity (Moulin *et al*., 2004; Perret *et al*., 2000). Currently, the similarities of *nod* sequences together with the host spectrum are used to define symbiovars in rhizobia (Roche *et al*., 1996; Rogel *et al*., 2011).

In the present study, both *nodA*- (Supplementary material 8) and *nodC*-based phylogenies (Supplementary material 9) placed the isolates in two distinct well-supported clusters: *Rhizobium* and *Bradyrhizobium*.

Incongruence between the phylogenies of symbiosis (*nod* gene) and those of chromosomal genes have been reported in several studies on rhizobia and this has been inferred as an indication of horizontal inheritance of the symbiosis genes (Chen *et al*., 2003; Moulin *et al*., 2004; Huang and Gogarten, 2006; Liu *et al*., 2012; Aoki *et al*., 2013). According to Kumar *et al*. (2015), strains with closely similar core genomes could have very different *nod* genes, while genetically distant strains could share similar *nod* genes, due to HGT between different genospecies. In our work, the conflicting phylogenetic relationships between the *nodA* and the 16S rDNA and *recA* gene trees suggest different evolutionary histories of the chromosomal and extra-chromosomal genes, possibly due to HGT of nodulation genes within and among the different genera. Furthermore, despite α-and β-rhizobia are evolutionary divergent, their symbiotic genes are highly similar suggesting lateral transfer (Bontemps *et al*., 2010; Chen *et al*., 2003; De Meyer *et al*., 2016; Moulin *et al*., 2001).

Other works also referred non-rhizobial endophytes (NRE) isolates from legume root nodules that present *nod* genes similarity with those of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Burkholderia* species (Martínez-Hidalgo, 2017).

MLSA of the isolates

Based on the analysis of the concatenated tree (Supplementary material 10) and network (Supplementary material 11), the bacterial isolates from cowpea plant root nodules clustered into three main groups: α-, β- and Ɣ-proteobacteria. Within α-proteobacteria, the

isolates were distributed into three groups: *Rhizobium*, *Bradyrhizobium* and *Caulobacter*. The *Rhizobium* group included 17 isolates and the type strains of *Rhizobium laguerreae*, *R. pisi*, *R. fabae*, *R. phaseoli*, *R. pusense*, *R. grahamii*, *R. skierniewicense* and *Agrobacterium tumefaciens*. The *Bradyrhizobium* clade included, as expected according to the 16S phylogenetic tree, the isolates R53_MT426001, R57_MG973287, R63_MG973286, R133_MT426002, BF9B_MT426003 and BF10_MG973288 and the type strains of *Bradyrhizobium elkanii*, *B. japonicum*, *B. ferriligni* and *B. tropiciagri*. The last group within α-proteobacteria, *Caulobacter*, included just one isolate (R59_MT426000) and the type strains of *Caulobacter segnis* and *C. henricii*. Within the β-proteobacteria, the bacterial isolates were divided in two groups: *Burkholderia* and *Herbaspirillum*. *Burkholderia* clade included two isolates from cowpea plants: R44_MT426011 and R62_MT426012. In the first branch, it was observed the sample R44_MT426011 with the type strains of *Burkholderia ambifaria* and *B. vietnamiensis* and in the second branch the isolate R62_MT426012 and the strains *B. fungorum*, *B. xenovorans* and *B. phenazinium. Herbaspirillum* group contained the isolates R141_MT426014 and R142_MT426013 and also the type strains of *Herbaspirillum rubrisubalbicans* and *H. seropedicae.* Ɣ-proteobacteria formed only one group that included seven isolates from our work (R25_MT426004, R50_MT426010, R121_MT426005, R122_MT426006, R123_MT426007, R124_MT426008 and R125_MT426009) and the type strains of *Kosakonia sacchari* and K. *pseudosacchari*, *Enterobacter hormaechei*, *E. cloacae* and *E. ludwigii*.

Some evidences of HGT occurred in the individual genes, however the placement of all the isolates in the concatenated tree and network is in agreement with the placement of the isolates in the individual 16S tree (Supplementary material 1).

In the rhizosphere, legumes can harbour rhizobial and non-rhizobial strains in the same nodule (Shiraishi *et al*., 2010). Additionally, cowpea have been emphasized as being promiscuous in relation to their rhizobial symbionts under field conditions (Andrews and Andrews, 2017). In some works carried out in Africa, China and Brazil, bradyrhizobia were identified as *Bradyrhizobium elkanii*, *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, and several unnamed *Bradyrhizobium* spp. (Appunu *et al.*, 2009). In the present work, out of 35 isolates collected from cowpea root nodules, only 6 belong to *Bradyrhizobium* clade. Inside this clade, we can differentiate *Bradyrhizobium* spp. (R57_MG973287, R133_ MT426002 and BF9B_ MT426003) and *Bradyrhizobium elkanii* (R53_ MT426001, R63_MG973286 and BF10_MG973288).

Although less abundant, fast-growing rhizobia have also been isolated from cowpea nodules in other studies and classified in the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Lindete *et al.*, 1997; Germano *et al.*, 2006; Yokoyama *et al.*, 2006; Zhang *et*

al., 2008). In our work, 21 isolates of *Rhizobium* genus but no isolates of *Sinorhizobium* or *Mesorhizobium* genera were isolated.

According to Andrews and Andrews (2017), cowpea is also nodulated by rhizobia from different genera across the β-proteobacteria, in particular *Burkholderia* and *Cupriavidus,* which are able to form functional nodules on specific legumes*.* Moulin *et al*. (2001) also reported the symbiotic nodulation ability of *Burkholderia* species. Furthermore, they suggest that the presence of *nod* genes in both α- and β-rhizobia probably occurred through HGT. In our work, some evidences of HGT also occurred in both *nodA* and *nodC* genes, which are responsible for the synthesis of the core structure of the *Nod* factors that act as signalling molecules for nodulating specific legume hosts (Moulin *et al*., 2001).

In the study of Rönkkö *et al*. (1993), other bacteria from several genera, including *Enterobacter*, were referred to live on plant roots as associative nitrogen fixers. In our study, 6 *Enterobacter* sp. strains (R50_ MT426010, R121_ MT426005, R122_ MT426006, R123_ MT426007, R124_ MT426008 and R125_ MT426009) were isolated for cowpea root nodules. Within these 6 isolates, R121_ MT426005 and R123_ MT426007 presented *nodA* gene, responsible for the biosynthesis of *nod* factors (N-acyltransferase), which may indicate that these two isolates are able to form root nodules, without the presence of rhizobial bacteria. In fact, the presence of *nod* genes, i.e., *nodA* and *nodC* in isolates belonging to β-proteobacteria*,* i.e., *Burkholderia* and *Herbaspirillum* spp. suggests that they can nodulate legumes. Valverde *et al*. (2003) referred that almost all *Herbaspirillum* species are nitrogen-fixing bacteria able to establish close associations with plants and Moulin *et al*. (2001) also considered *Herbaspirillum*, a β-proteobacteria, as a nitrogen-fixing bacteria. In our work, 2 *Herbaspirillum* isolates (R141_ MT426014 and R142_ MT426013) were found in the nodules collected from cowpea plant roots.

These non-rhizobial endophytes (NRE) are not able to form nodules in association with cowpea, although some can fix nitrogen and could possibly contribute to the N supply of the plants (Leite *et al*., 2017).

In Brazil, where cowpea is an introduced species, a higher diversity of bacteria associated with root nodules has been also described (Leite *et al*., 2009). In particular, for the Amazonian region, a number of strains classified as *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Paenibacillus* were isolated from cowpea nodules (Jaramillo *et al*., 2013; Oliveira-Longatti *et al*., 2014).

A diverse bacterial community associated to cowpea nodules was also observed by other authors in previous studies (De Meyer *et al*., 2015; Leite *et al*., 2017), namely *Pseudomonas*, *Bacillus*, *Paenibacillus* and *Enterobacter*. Recent studies gave a hint on the

possible role of NRE in the reduction of oxidative stress in cowpea nodules, leading to a delay of the process of nodule senescence (Rodrigues *et al*., 2013).

4. Conclusion

Phylogenetic analysis showed that cowpea plants were able to form nodules with different rhizobial species and investigation of their symbiotic performance requires further attention for selection of highly effective strains when developing inoculants.

Despite the surface disinfection of root nodules, it cannot be completely excluded that, besides true endophytes, also some bacteria tightly attached to the surface of the nodules remains stable after the surface sterilization procedure.

Our results provide the first analysis on the phylogenetic diversity of indigenous root-nodulating bacteria in cowpea, in Portugal, and further confirm the promiscuity of cowpea and extend our knowledge regarding the diversity, distribution and evolution of these bacteria in European soils. Molecular identification of indigenous bacteria from fields without inoculation are very important for selecting novel strains adapted to the local environmental conditions. Such strains often exhibit a better performance in similar habitats and thus they are more preferable for inoculant formulations. The putative novel lineages isolated in the present study and their close phylogenetic relationships with strains used as inoculants render them worthy for further investigation as inoculants in fields with similar edapho-climatic conditions.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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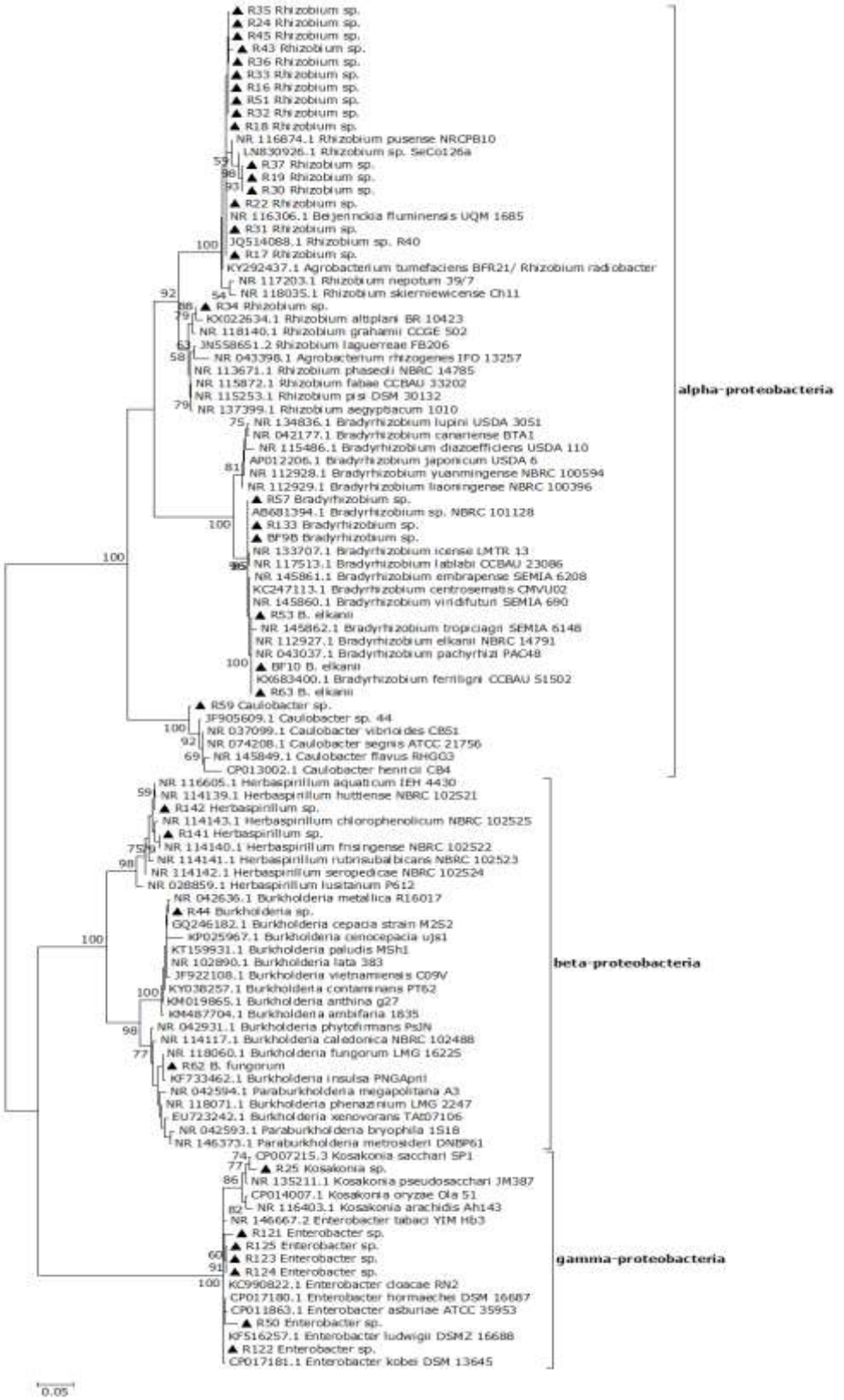
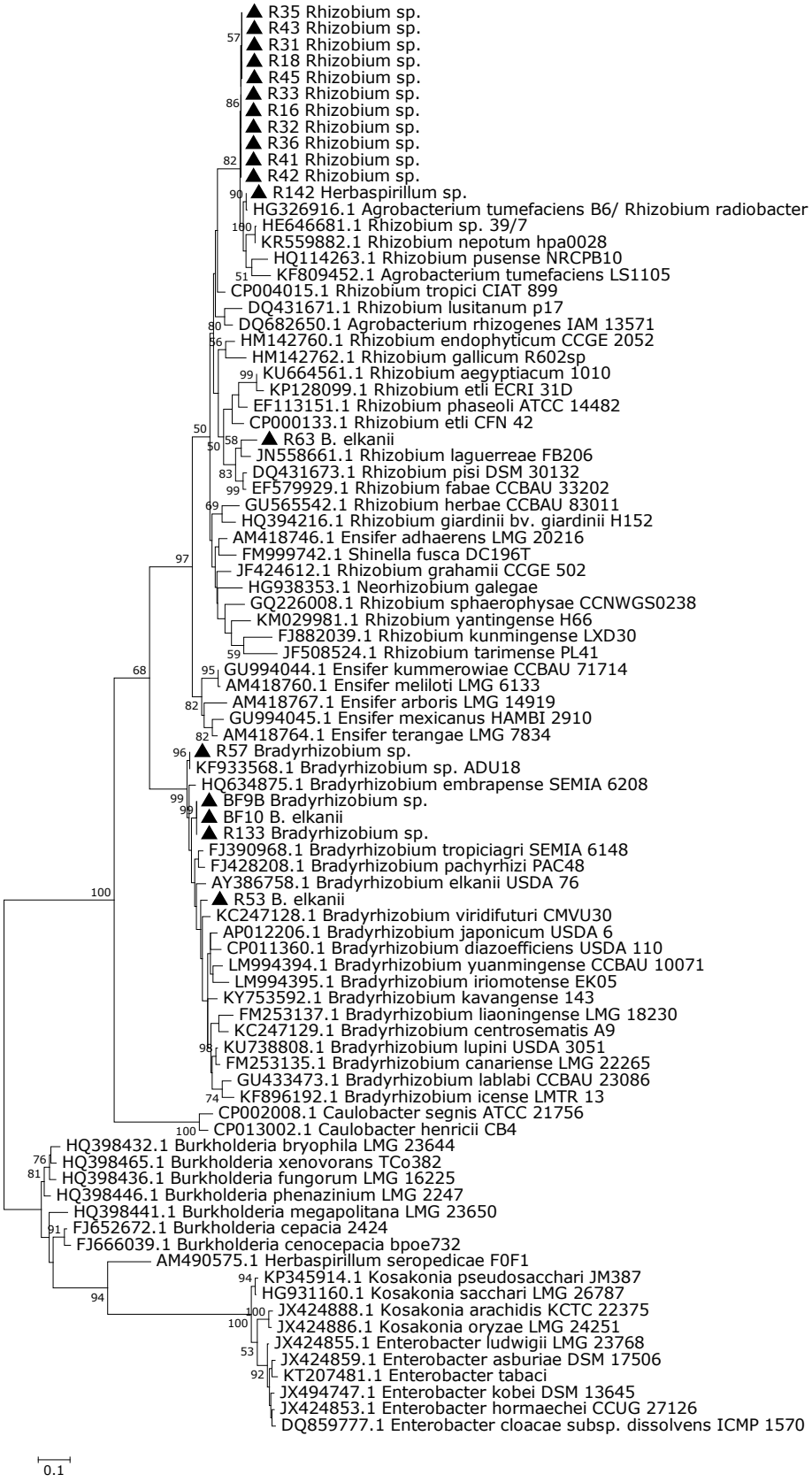
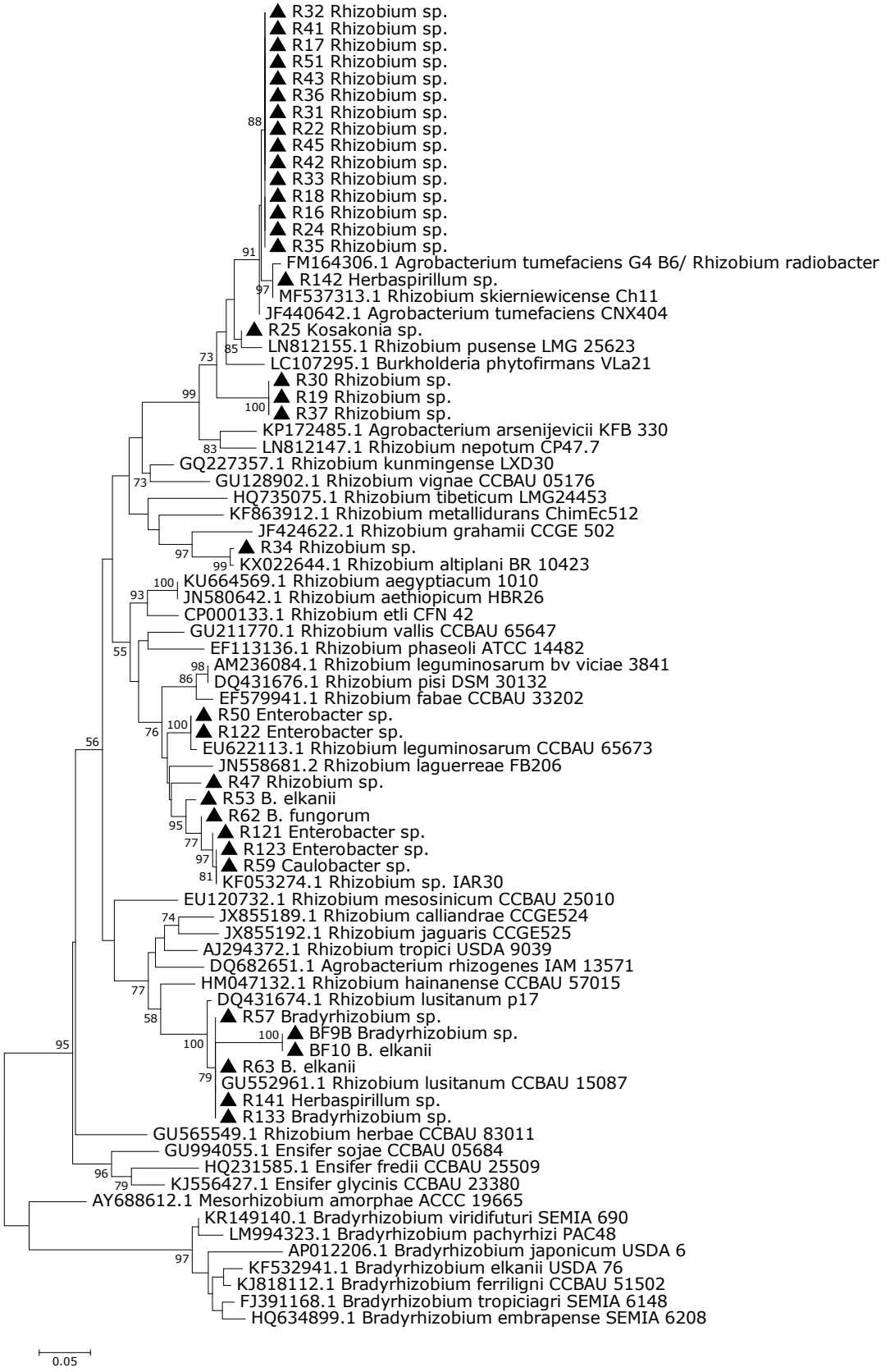
Supplementary material



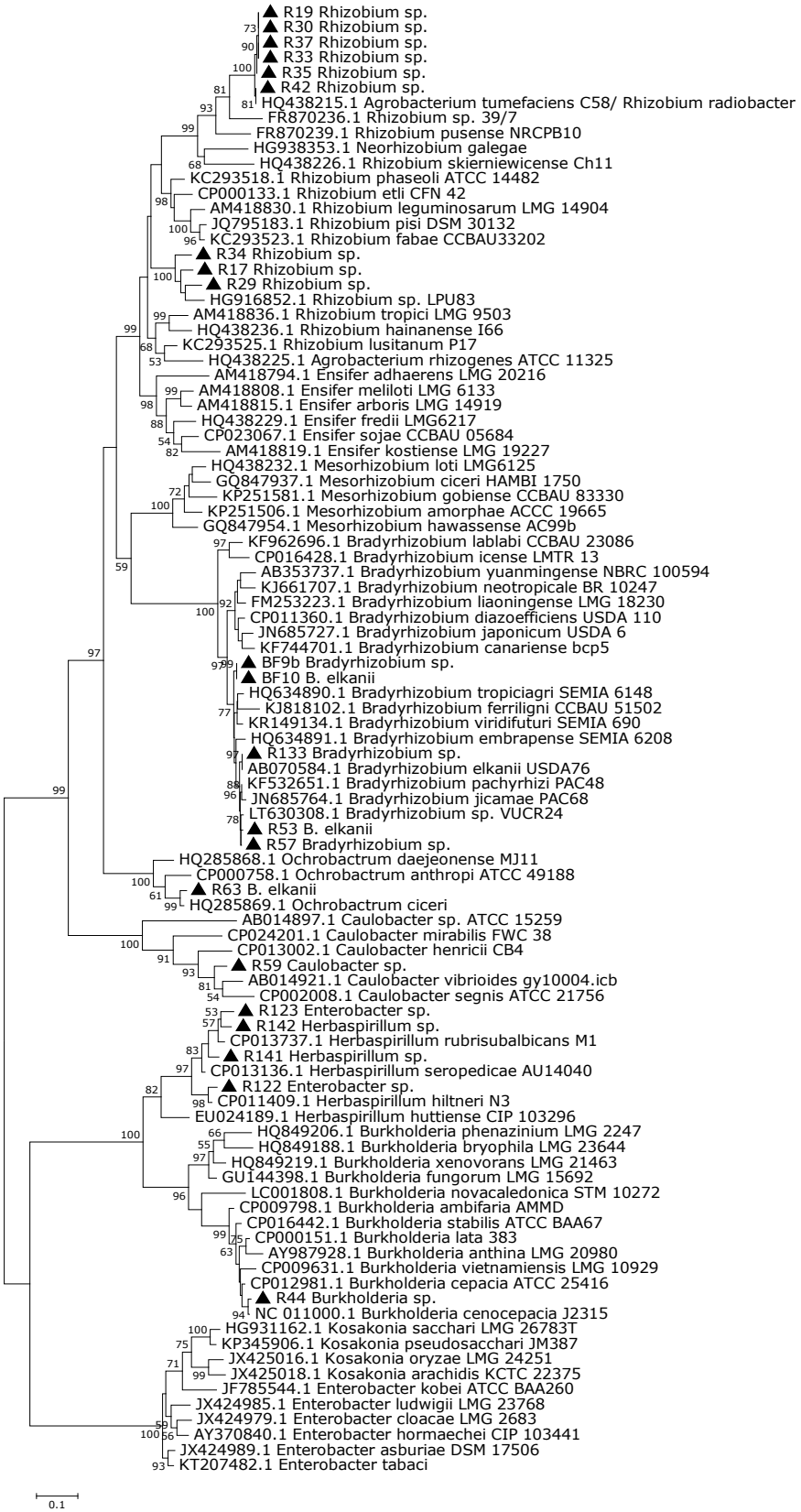
SM 1- Maximum likelihood tree of the 16S rRNA of cowpea's rhizobial isolates. Individual tree was made with 1111 positions in the final dataset and 106 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



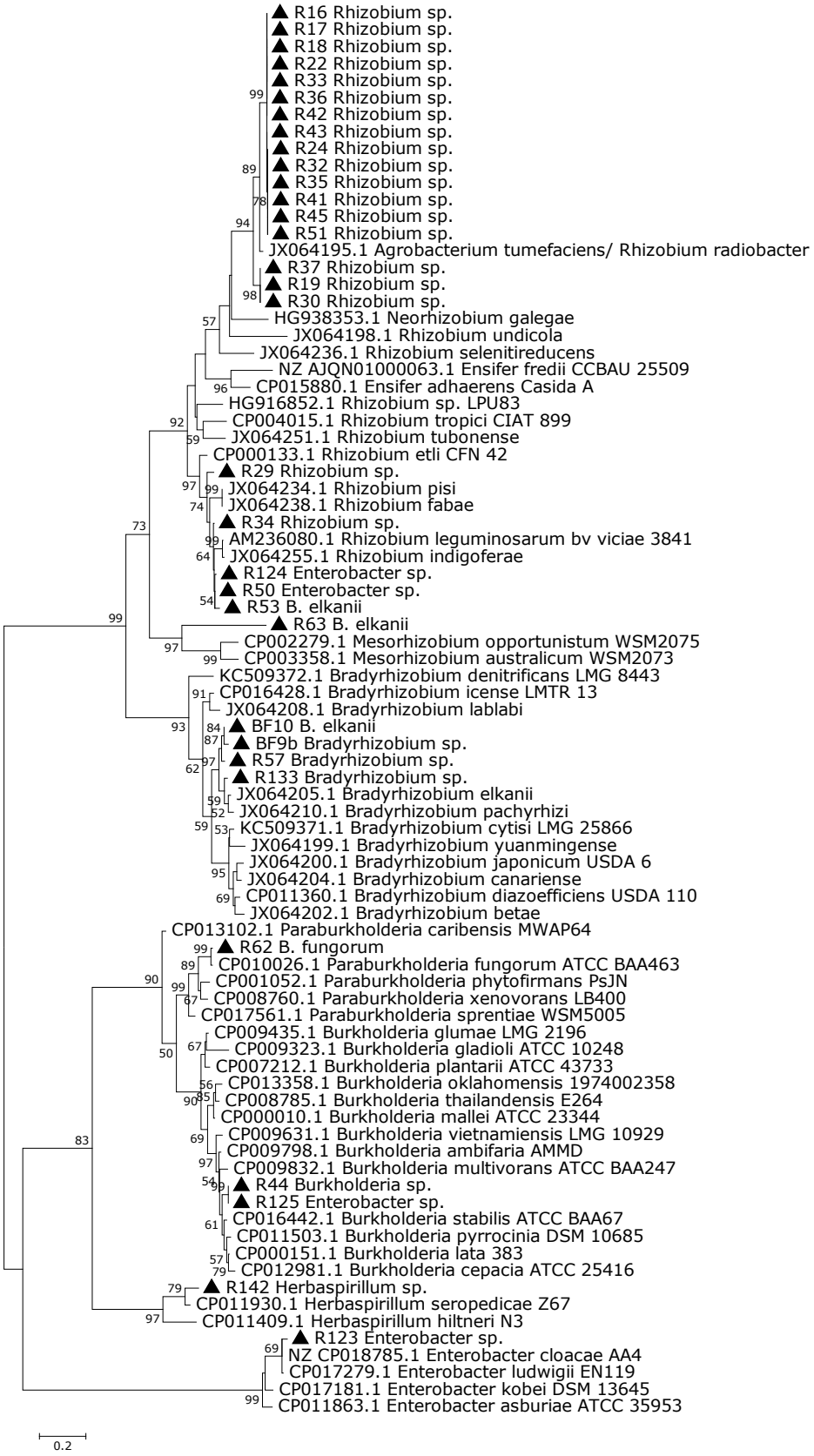
SM 2- Maximum likelihood tree of the *atpD* of cowpea's rhizobial isolates. Individual tree was made with 1427 positions in the final dataset and 87 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



SM 3- Maximum likelihood tree of the *recA* of cowpea's rhizobial isolates. Individual tree was made with 1621 positions in the final dataset and 79 nucleotide sequences**.** The identification of the isolates was made according to their position in the concatenated tree and network.

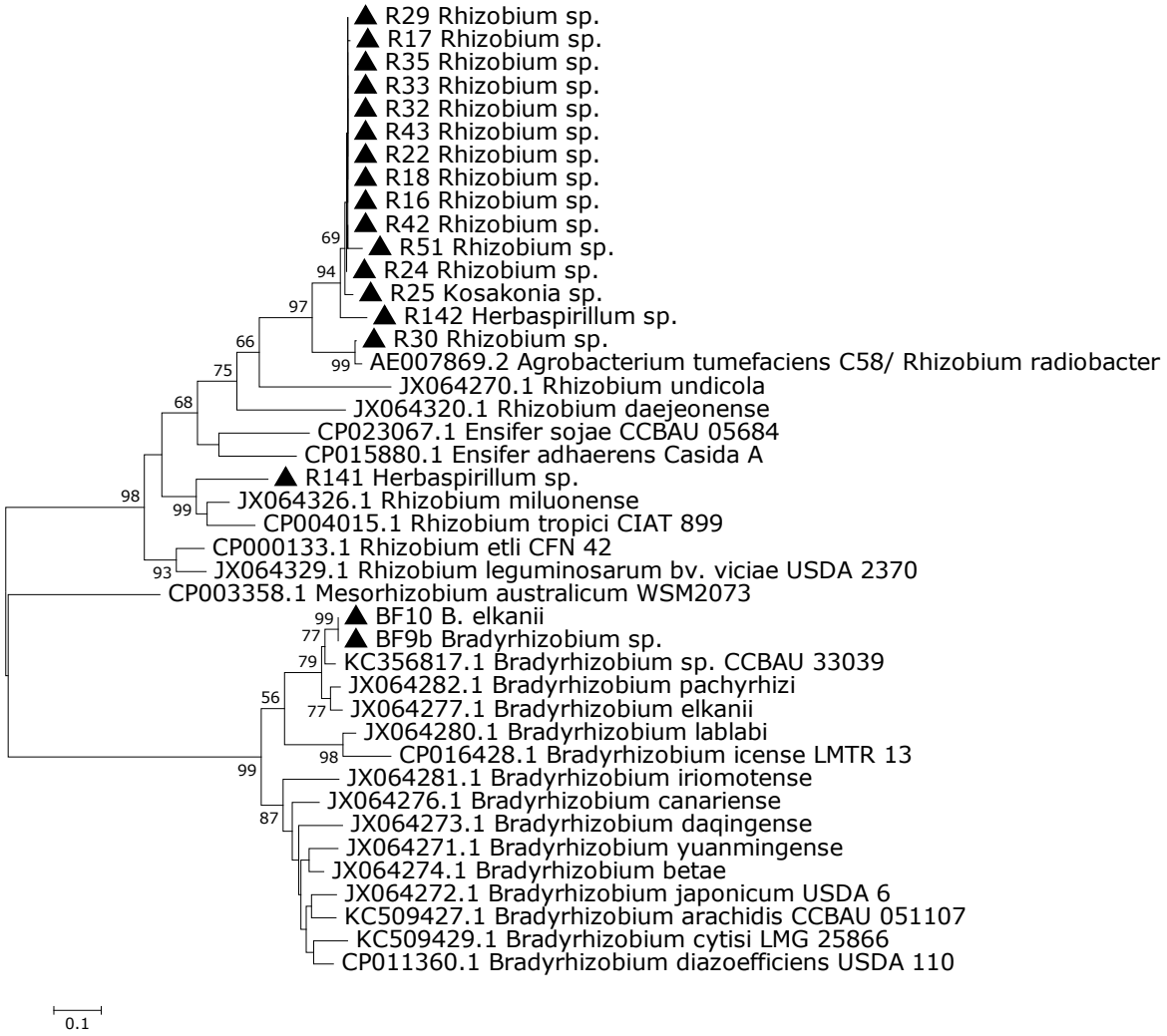


SM 4- Maximum likelihood tree of the *gyrB* of cowpea's rhizobial isolates. Individual tree was made with 833 positions in the final dataset and 97 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.

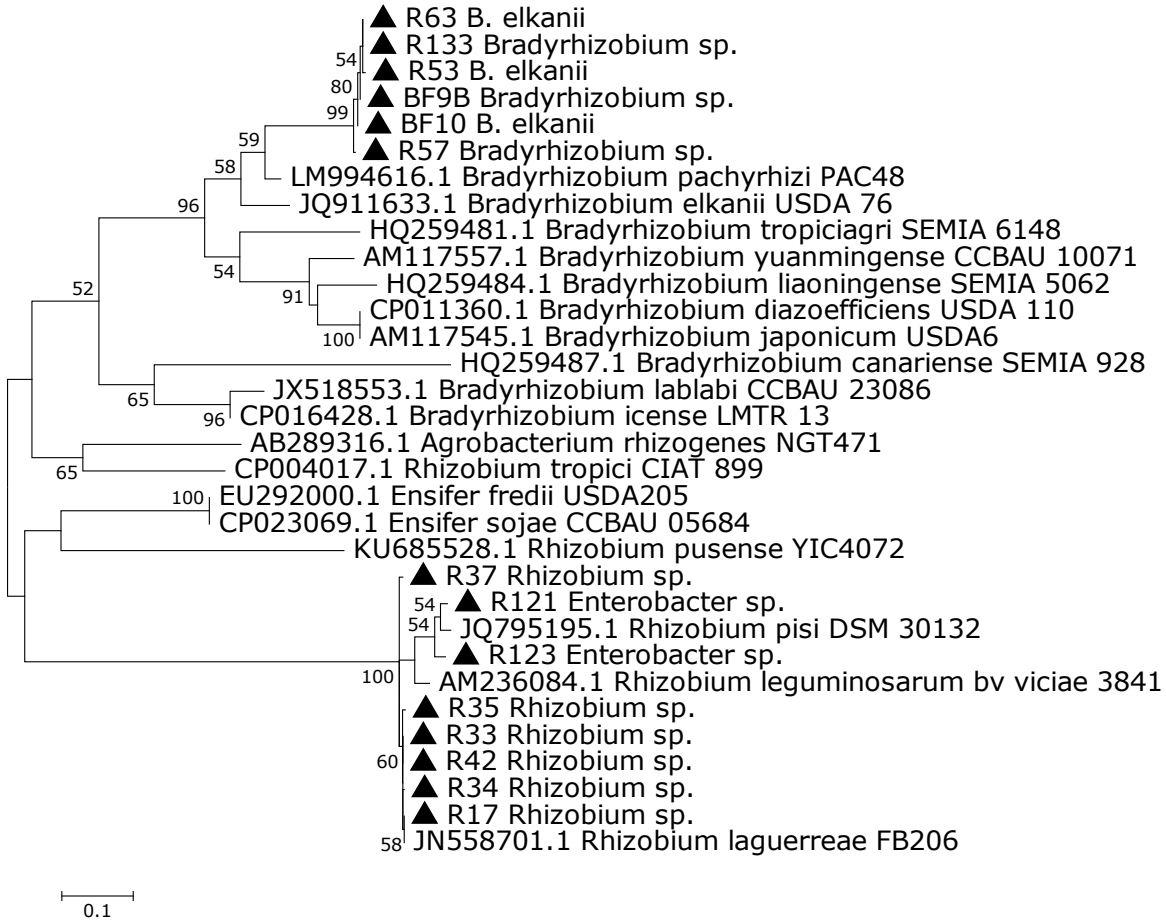


SM 5- Maximum likelihood tree of the *SMc00019* of cowpea's rhizobial isolates. Individual tree was made with 593 positions in the final dataset and 83 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.

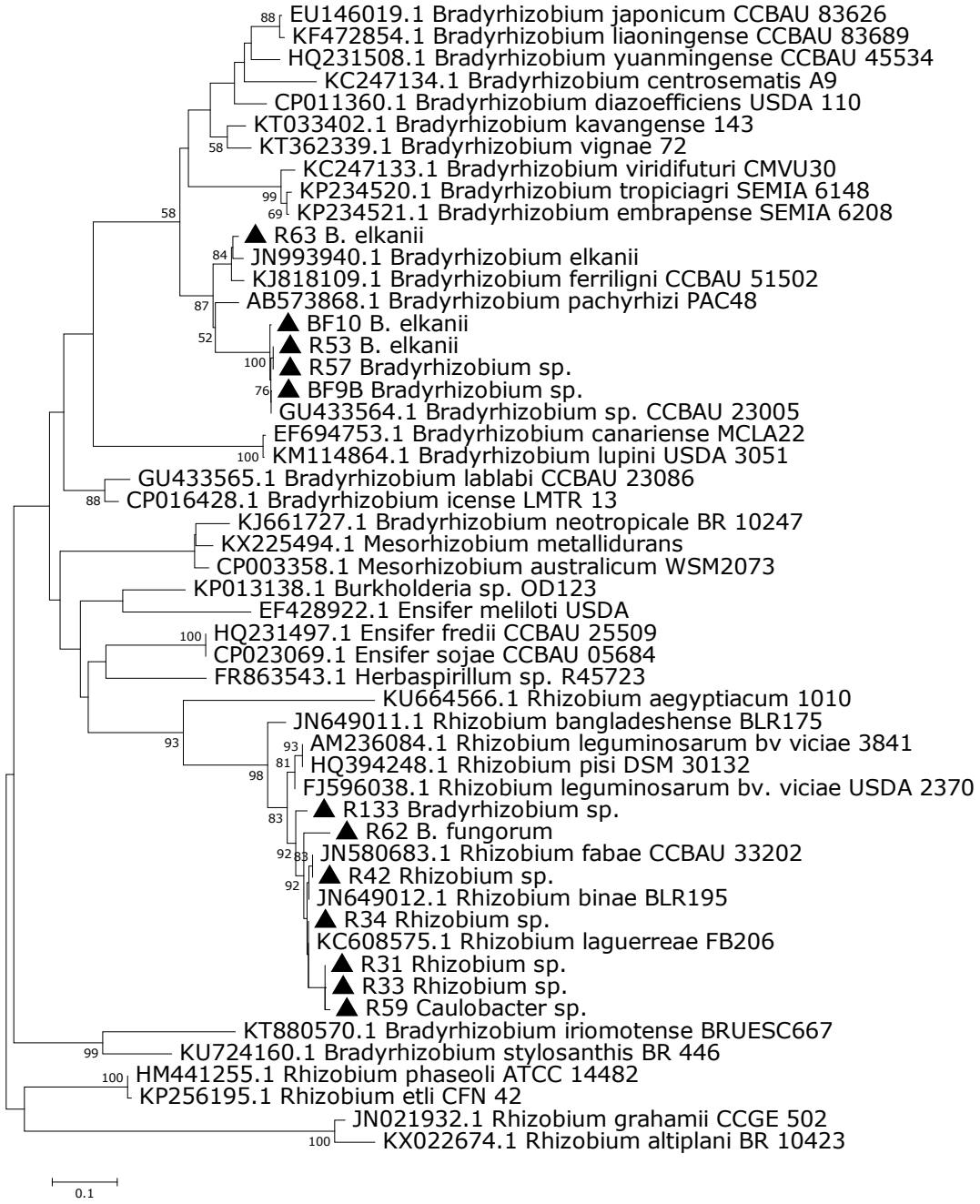
SM 6- Maximum likelihood tree of the *thrA* of cowpea's rhizobial isolates. Individual tree was made with 952 positions in the final dataset and 50 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



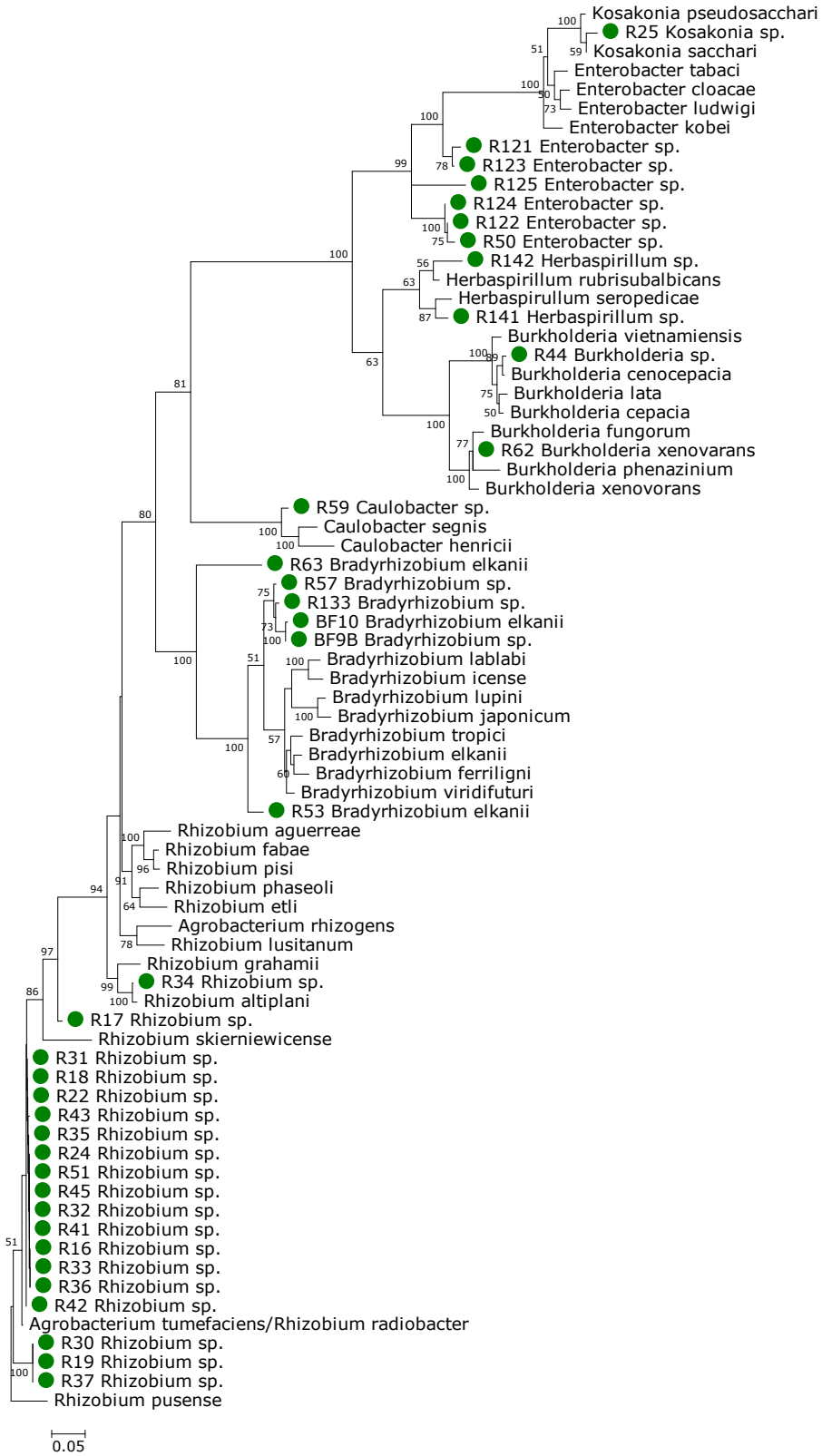
SM 7- Maximum likelihood tree of the *truA* of cowpea's rhizobial isolates. Individual tree was made with 543 positions in the final dataset and 42 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



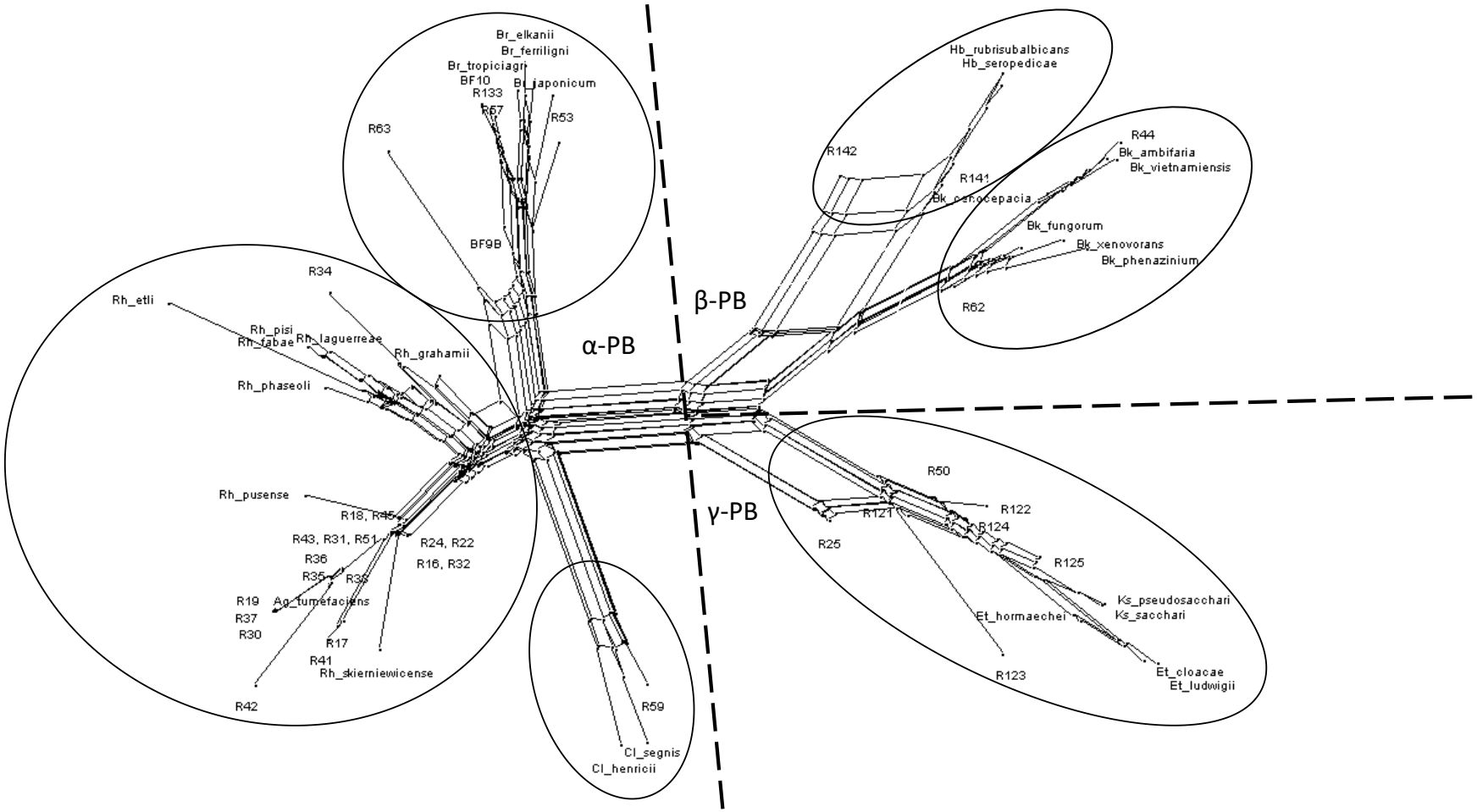
SM 8- Maximum likelihood tree of the *nodA* of cowpea's rhizobial isolates. Individual tree was made with 733 positions in the final dataset and 32 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



SM 9- Maximum likelihood tree of the *nodC* of cowpea's rhizobial isolates. Individual tree was made with 910 positions in the final dataset and 52 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



SM 10- Concatenated tree based on seven core genes 16S rRNA, *atpD*, *gyrB*, *recA*, *SMc*, *thrA* and *truA* of cowpea rhizobial isolates. The RAxML tree was made using 2530 positions in the final dataset and the 74 nucleotide sequences of the rhizobial strains from cowpea plants. The bootstrap support values less than 50 were not displayed.



SM 11- Concatenated network based on seven core genes 16S rRNA, *atpD*, *gyrB*, *recA*, *SMc*, *thrA* and *truA* of cowpea rhizobial isolates. The network was made using SplitsTree 4.0. The final dataset has 65 nucleotide sequences and 2530 positions. The isolates were clustered in six main groups: α-proteobacteria- *Rhizobium*, *Bradyrhizobium* and *Caulobacter*, β-proteobacteria- *Herbaspirillum* and *Burkholderia* and ϒ-proteobacteria- *Kosakonia* and *Enterobacter*.

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CHAPTER IV

CO-INOCULATION WITH RHIZOBIA AND MYCORRHIZAL FUNGI INCREASES YIELD AND CRUDE PROTEIN CONTENT OF COWPEA (*VIGNA UNGUICULATA* (L.) WALP.) UNDER DROUGHT STRESS

CHAPTER IV- CO-INOCULATION WITH RHIZOBIA AND MYCORRHIZAL FUNGI INCREASES YIELD AND CRUDE PROTEIN CONTENT OF COWPEA (*VIGNA UNGUICULATA* (L.) WALP.) UNDER DROUGHT STRESS

BRIEFING NOTE

This chapter envisages to answer the objectives focused on the selection of improved rhizobial strains and AMF for enhanced biological nitrogen fixation, and consequently legume growth and yield and evaluation of the effects of singe and co-inoculation with these selected microorganisms in cowpea plants. Following the molecular identification of the collected bacteria in Chapter III, this chapter covers a greenhouse experiment in which is evaluated the effect of a mix of arbuscular mycorrhizal fungi (AMF) and three previously selected rhizobial bacteria. Thus, a single and dual inoculation with *Rhizobium* sp., *Bradyrhizobium elkanii* or *Bradyrhizobium* sp. and an AMF was performed in cowpea plants grown in non-sterilized soil. Several parameters were evaluated at harvesting stage. All the bacteria collected from cowpea root nodules across several regions in Portugal were identified through a multilocus sequence analysis (data presented in Chapter III). After testing all these isolates *in vitro* and in a pot experiment, the ones which showed a better performance were selected for the experiments of the present work. This chapter is an adaptation of a research paper accepted in “Landbauforschung – Journal of Sustainable and Organic Agricultural Systems”.

The authors contribution to the present chapter was as follows: Sandra Pereira and Shweta Singh were responsible for establishment and maintenance of the experiment, collection of the data in the greenhouse and performance of the laboratory analysis. Sandra Pereira was also responsible for data analysis and manuscript writing. Rui S. Oliveira was responsible for the supply of the arbuscular mycorrhizal fungi used in the present work. Luis Ferreira performed the protein content in the grains in his laboratory. Finally, Eduardo Rosa and Guilhermina Marques were responsible for the design of the experiment and for the critical review of the article. Guilhermina Marques also monitored and helped in the practical work.

Co-inoculation with rhizobia and mycorrhizal fungi increases yield and crude protein content of cowpea (*Vigna unguiculata* (L.) Walp.) under drought stress

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Highlights

- Cowpea is one of the most consumed legumes worldwide, due to its high seed protein content;
- Rhizobial bacteria and arbuscular mycorrhizal fungi can improve growth and yield of leguminous plants;
- The selection of appropriate microorganisms is essential to the success of symbiosis;
- Co-inoculation with selected beneficial microorganisms increased crude protein content in the grain of plants under drought stress;
- This eco-friendly strategy can be a good tool to mitigate climate changes, in a more sustainable agriculture;

Abstract

Recent trends in sustainable agricultural production seek improved bioinoculants that can benefit crop adaptation and production and reduce external inputs of pesticides and synthetic fertilizers, particularly under abiotic and biotic stress conditions. Drought is within of the critical and more often conditions which can drastically reduce plant biomass and yield. The use of bioinoculants are even more relevant to mitigate climate changes and to reduce the water needs of plants. Leguminous plants are very important to improve sustainable cropping systems, because they can form effective symbiotic associations with both nitrogen fixing bacteria and arbuscular mycorrhizal fungi. These microorganisms can act as an

alternative source of nitrogen and phosphorus fertilizers. Cowpea is a multipurpose crop of recognized interest under abiotic stress. This study aimed to test the effect of three previously selected rhizobial bacteria (*Rhizobium* sp.- B1, *Bradyrhizobium elkanii*- B2 and *Bradyrhizobium* sp.-B3) and AMF (*Claroideoglomus claroideum* BEG210) on the yield and crude protein content of cowpea, under drought conditions and also to compare the competitiveness of the inoculated bacteria with native rhizobial bacteria naturally present in the soil. The combined inoculation with each bacteria and arbuscular mycorrhizal fungi *Claroideoglomus claroideum* BEG210 was shown to increase the crude protein content of cowpea seeds in plants under drought stress (25% of field capacity) in 13, 17 and 30%, respectively. This study indicated that the used microorganisms are potentially resistant to drought and can be used as a biotechnological tool for sustainable agriculture under drought conditions.

Keywords: AMF, drought, rhizobia, tripartite symbiosis, *Vigna unguiculata* (L.) Walp.

1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an annual legume crop native of Africa and is the most widely cultivated seed-legume in arid and semi-arid areas (Alkama *et al.*, 2009; Johnson *et al.*, 2013; Lazaridi *et al*., 2017). It is adapted to high temperatures (20-35ºC) and can grow well in a wide range of soil textures and with only 188 mm of annual rainfall. Its growth period can range between 90 to 240 days, depending on the climatic conditions and the maturity period of the cultivar (Ngalamu *et al*., 2014; Carvalho *et al*., 2017).

It could be estimated that the total cultivated area has increased in the last years, from approximately 2.4 Mha in 1961 to around 12.5 Mha in 2017 (FAOSTAT, 2017). Despite the wide distribution of cowpea, around 98% of the world production is located in Africa (12.3 Mha) (Alkama *et al*., 2009; Oliveira *et al*., 2017).

Cowpea seeds provide a rich source of proteins (23%), carbohydrates (56%), fiber (4%) and calories, as well as minerals and vitamins, being called as “poor man’s meat” (Iqbal *et al*., 2006). Additionally, cowpea can also provide an alternative protein source for people that suffer from allergies to soybean protein (Ravelombola *et al*., 2016).

Nowadays, the increasing food demand, the rising global temperatures and the global water scarcity lead to a need to produce more food with less water (Oliveira *et al*., 2017). The water scarcity is highly responsible for the reduction in agricultural productivity, because it can lead to anatomical, morphological, physiological and biochemical modifications that affect the plant growth and development (Bezerra *et al*., 2003). In fact, according to Bastos *et al.* (2011), well-watered cowpea plants can produce more than 1 000 kg grain ha^{-1}, but the water scarcity can reduce this potential to approximately 360 kg ha^{-1}. In this sense, the

understanding of the physiological, biochemical and agromorphological mechanisms that can explain the resistance of cowpea varieties to drought is of extreme importance (Cruz de Carvalho *et al*., 1998). The physiological mechanisms include the closing of the stomata when the water in the soil is not sufficient and the decrease in the transpiration and photosynthetic rates. The biochemical mechanisms involve the osmotic adjustment which is characterized by the accumulation of organic solutes to maintain the cell turgor and the agromorphological processes include the turning of the leaves upwards to protect them from excessive temperatures and the reduction in the root volume (Krouma, 2010; Hall, 2012; Halilou *et al*., 2015). Despite the inherent resistance of cowpea plants to the drought, the inoculation of cowpea and other legumes with beneficial and drought-resistant microorganisms, such as rhizobial bacteria and arbuscular mycorrhizal fungi (AMF), also has a great potential to reduce the negative effects of water scarcity and global warming in cowpea plants. Within rhizobial bacteria, a heterogeneous group of slow-growing rhizobia belonging to the genus *Bradyrhizobium* and known as “cowpea-miscellany” has the ability to nodulate cowpea (Allen and Allen, 1981; Appunu *et al*., 2009), increasing plant resistance to high temperatures and water deficit and reducing the need for chemical fertilizer inputs. *Bradyrhizobium elkanii*, *B. yuanmingense* and *B. japonicum* are among the main rhizobial species associated with cowpea (Zhang *et al*., 2008).

The association with AMF is a non-specific, highly compatible and long-lasting mutualism whereby both partners have advantages (Abdel-Fattah *et al*., 2011; Harrison, 1998). AMF can be applied to increase the growth potential and reduce water and fertilizer inputs. Indeed, in this symbiosis, the fungal hyphae (thread-like structures) spread through the soil, taking up nutrients such as phosphorus and absorbing water, and transporting them to the plant root, and in return the fungi receive sugars from the plant. This association between AMF and plants can increase drought tolerance (Augé *et al*., 2001; Oliveira *et al*., 2017) and consequently improve cowpea yield under adverse environmental conditions.

Co-inoculation with both rhizobia and AMF in legumes results in a mutualistic tripartite symbiosis (Antunes and Goss, 2005) that usually leads to a highest increase of growth and yield than single inoculation with one microorganism (Chalk *et al*., 2006; Marulanda *et al*., 2006). In fact, in this kind of symbiosis, the presence of one microorganism can affect the activity of the other and, consequently, the interaction of both has normally a positive effect in the host plant (Vejsadova *et al*., 1993; Xie *et al*., 1995).

The objective of the present work was to evaluate the effect of single and co-inoculation with several rhizobial bacteria (*Rhizobium* sp., *Bradyrhizobium elkanii* and *Bradyrhizobium* sp.) and an AMF (*Claroideoglomus claroideum* BEG210) on the growth,

yield and protein content of cowpea seeds under drought conditions and compare the competitiveness of the inoculated bacteria with those naturally present in the soil.

2. Material and methods

2.1. Bacterial inoculant and arbuscular mycorrhizal fungi inoculant

The bacterial strains used in this work were isolated from fresh surface sterilized root nodules of cowpea plants and previously selected among others according to its performance in *in vitro* experiments. Bacteria B1 and B2 were collected in Elvas, Portugal (39'23'59.72''N, 7'53'25.99''W), in July 2014 and bacteria B3 was collected in Vila Real, Portugal (41'28.54''N, 7'74.14''W), in September 2014. The bacteria identification was performed by amplification of 16S rDNA using the universal primers fD1 and rD1 (Weisburg *et al*., 1991). Furthermore, for multilocus sequence analysis (MLSA) and in order to identify the isolates at species level, this analysis was complemented with 6 housekeeping genes: *recA* (DNA recombination protein), *gyrB* (DNA gyrase B), *SMc00019* (conserved hypothetical protein), *thrA* (homoserine dehydrogenase), *atpD* (atpD synthase β-subunit) and *truA* (RNA pseudouridine synthase A). Taxonomic position at symbiovar level was determined by the inferred phylogenies based on the symbiotic genes of nodulation: *nodA* (N-acyltransferase nodulation protein A) and *nodC* (N-acetylglucosaminyltransferase) (Table 1). PCR mixtures were performed with 7.5 µl of master mix (MyTaq HS Mix, 2x of Bioline), 1 µl of each forward and reverse primer and 5.5 µl of DNA template, with 15 µl of final volume. Amplified samples were sequenced in Stabvida, Portugal. Nucleotide sequences were corrected using BioEdit software and homology searches were performed at the National Center for Biotechnology Information (NCBI) server using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990).

Bacteria B1, B2 and B3 were identified, respectively, as *Rhizobium* sp., *Bradyrhizobium elkanii* and *Bradyrhizobium* sp. and the obtained sequences for 16S ribosomal RNA region were deposited in Genbank database with the accession numbers MH938299- MH938301.

For the inoculum preparation, each bacteria was grown in six plates of Yeast Mannitol Agar media (1 g/L of yeast extract, 10 g/L of mannitol, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L NaCl and 15 g/L agar) supplemented with 0.1 g/L bromothymol blue. After 3-5 days of growing, bacterial inoculant was suspended in sterilized 0.8% NaCl and then transferred to a sterilized mix of peat and vermiculite (1:1).

The AMF isolate *Claroideoglomus claroideum* BEG210 was grown for 8 months in a multi-spore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as the host plant.

Primers	Sequence (5'-3')	Reference
fD1	AGA GTT TGA TCC TGG CTC AG	Weisburg et al. 1991
rD1	AAG GAG GTG ATC CAG CC	
thrAB-F	TGC TTC GTC GAR YTG ATG G	Zhang et al., 2012
thrAB-R	ACR CCC ATC ACC TGY GCR ATC	
thrAMRS-F	TAA TAC GAC TCA CTA TAG GGG CNG GBG GYA TYC CSG TBA TCA AG	modified by Tampakaki from Zhang et al., 2012
thrAMRS-R	GAT TTA GGT GAC ACT ATA GCG YTC GAT NCG RAT SAC YTG SGG	
SMc00019B-F	CAT TCV KCS GAR GGV GCS ATG GGY ATC	Zhang et al., 2012
SMc00019B-R	GCG TGB CCB GCS KCG TTS GAV AGC AT	
SMc00019MRS-F	TAA TAC GAC TCA CTA TAG GGC ADT TCC TBA THG CCA TGC C	modified by Tampakaki from Zhang et al., 2012
SMc00019MRS-R	GCV GGR CAN KTS AGC CAD CCR TT	Zhang et al., 2012
truAB-F	TAA TAC GAC TCA CTA TAG GGC GCT ACA AGC TCA YYA TCG A	modified by Tampakaki from Zhang et al., 2012
truAB-R	CCS ACC ATS GAG CGB ACC TG	Zhang et al., 2012
truAR-F	TGA CCG TSG AAT ATG ACG G	
truAR-R	ACA TCS AGY CGG TCV AGS GT	
truAMS-F	TAA TAC GAC TCA CTA TAG GGC AGG TSG CDC ATS TCG AYC T	modified by Tampakaki from Zhang et al., 2012
truAMS-R	GAD CGB AYC TGG TTR TGM AG	Zhang et al., 2012
gyrB340F-T7	TAA TAC GAC TCA CTA TAG GGT TCG ACC ARA AYT CYT ACA AGG	modified by Tampakaki from Zhang et al., 2012
gyrB1057R-SP6	GAT TTA GGT GAC ACT ATA GCC AAY TTR TCC TTG GTC TGC G	
gyrB-F	ACC GGT CTG CAY CAC CTC GT	Spilker et al., 2009
gyrB-R	YTC GTT GWA RCT GTC GTT CCA CTG C	
recA6F	CGK CTS GTA GAG GAY AAA TCG GTG GA	Gaunt et al., 2001
recA555R	CGR ATC TGG TTG ATG AAG ATC ACC AT	
atpD273F	SCT GGG SCG YAT CMT GAA CGT	Gaunt et al., 2001
atpD-294F	TAA TAC GAC TCA CTA TAG GGA TCG GCG AGC CGG TCG ACG A	modified from Gaunt et al., 2001
atpD771R	GCC GAC ACT TCC GAA CCN GCC TG	Gaunt et al., 2001
nodA-1	TGC RGT GGA ARN TRN NCT GGG AAA	Haukka et al., 1998
nodA-2	GGN CCG TCR TCR AAW GTC ARG TA	
nodCF	AYG THG TYG AYG ACG GTT C	Laguerre et al., 2001
nodCFu	AYG THG TYG AYG ACG GIT C	
nodCI	CGY GAC AGC CAN TCK CTA TTG	

Table 1. List of primers used in this work for the molecular identification of collected rhizobial bacteria.

2.2. Plant culture and experimental design

Cowpea seeds were surface-sterilized with 0.5% (v/v) sodium hypochlorite (NaCIO) for 20 min, followed by serial washes with sterilized distilled water. Seeds were from the cv. Fradel, the only cowpea cultivar registered at the Portuguese National Catalog for commercial use (CNV, 2019). After germination, 3 seedlings of similar size were kept in each plastic pot (6 liters), containing a mixture of soil, vermiculite, sand and peat (1:1:1:1, w/w). No-sterilized soil was used in this work. Chemical analyses of soil mixture revealed the following values: 8.10% organic matter, pH (1:2.5 w/v water) 5.0, 51 mg P/kg and 132 mg K/kg (method of Égner-Riehm). Each pot was inoculated with approximately 1 g of mix with the selected bacteria or AMF inoculant, according to the different treatments. All pots from

the non-bacterial treatments received the same amount of autoclaved peat and vermiculite and sterilized 0.8% NaCl and every pot from non-mycorrhizal treatments received same amount of AMF inoculum autoclaved twice (121 °C, for 30 min) on 2 consecutive days.

The study was conducted in a greenhouse at the University of Trás-os-Montes e Alto Douro, Vila Real, Portugal, during growing season of cowpea (May-September 2015), under natural conditions of light, temperature and humidity. Pots were occasionally rotated to different places to minimize the effect of the location in the greenhouse.

For each treatment, twelve pots were prepared and distributed equally for the two water regimes used in the experiment (25% and 75% of field water capacity- FC), in a total of 6 pots (biological replicates) per treatment and water regime. Field water capacity of the soil in the pots was determined according to Grewal *et al*. (1990). The water regime of 25% FC was used to simulate the drought stress and 75% FC was used to simulate well-watered plants. After inoculation and during 4 weeks, all the pots were kept at 75% FC by weighting and watering the pots every 2 days. The drought stress was initiated 4 weeks after plant emergence, and it lasted 2 months, until the flowering stage. During this period, the plants were weighted and watered accordingly, in order to ensure the amount of required water.

2.3. Nodule number and biomass and assessment of AMF colonization

After a growth period of three months, at full maturation stage, plants were harvested and the number and weight of root nodules were determined.

After counting and weighting the nodules, root systems were used for estimation of the extent of root colonization by AMF. For this purpose, roots were cleared in potassium hydroxide (KOH) 2.5%, at 80 °C, for 40 min, followed by rinsing with water. Roots were immersed in staining solution containing 5% blue ink in vinegar, and kept at 80 °C, for 5 min (Vierheilig *et al*., 1998). After washing away the staining solution, roots were de-stained with tap water containing some drops of vinegar and examined under a compound microscope for quantitative colonization assessment by magnified-intersection method according to McGonigle *et al*. (1990).

2.4. Biomass production, seed yield and protein determination

At harvest, shoots and roots were separated for the evaluation of dry weight. The number of seeds and the weight of 100 seeds was also determined.

Dry samples were analysed for ash (942.05) and for total N (954.01) as Kjeldahl N following the methods of the Association of Official Analytical Chemists (AOAC). Total nitrogen was converted to crude protein by the formula N x 6.25.

2.5. Statistical analysis

Statistical analysis was performed using Software SPSS V.25 (SPSS-IBM, Orchard Road-Armonk, New York, NY). Statistical differences were evaluated by one-way and two-way of analysis of variance (ANOVA), followed by the post hoc Duncan' s multiple range test ($P < 0.05$), establishing treatments and water regime effects. One-way of ANOVA establishing treatment effect within each water regime was also performed.

3. Results

3.1. Cowpea growth

Taking in account the single application of beneficial microorganisms, a significant increase was observed in the shoot weight (Fig. 1A) of plants under drought stress (25% of field capacity-FC) and inoculated with the bacteria *B. elkanii* B2, the bacteria *Bradyrhizobium* sp. B3 and the AMF comparing to the control (1.77, 1.96 and 2.06 of fold increase, respectively). Under this water regime, plants single inoculated with the bacteria B2 and B3 also presented significantly higher shoot weight than plants co-inoculated with the respective bacteria and fungi (B2+AMF and B3 + AMF).

No effect was observed in the shoot weight by co-inoculation with rhizobial bacteria and AMF. On the other hand, comparisons between water regimes showed that, with the exception of single inoculation with the bacteria *B. elkanii* B2 that presented similar shoot weight in both water regimes, all of the other treatments presented higher shoot weight in well-watered plants (75% of FC) than in plants under drought stress (25% of FC). In fact, shoot weight was affected by the water regime *($P<0.001$)* and the interaction between the treatment and the water regime *($P<0.001$).*

Similarly, root weight was also affected by the water regime *($P<0.001$)* and the interaction between the treatment and the water regime *($P<0.05$).* Root weight (Fig. 1B) of well-watered plants (75% of FC) was not affected by microbial inoculation (either with single or in combination). However, under drought stress (25% of FC), simple inoculation with fungi benefited cowpea plants, since root weight was significantly higher in these plants than in control, with a 1.69 fold increase. In general, this parameter was higher in well-watered plants (75% of FC) than in plants under drought (25% of FC), with the exception of pants inoculated with AMF, which presented similar root weight in both water regimes.

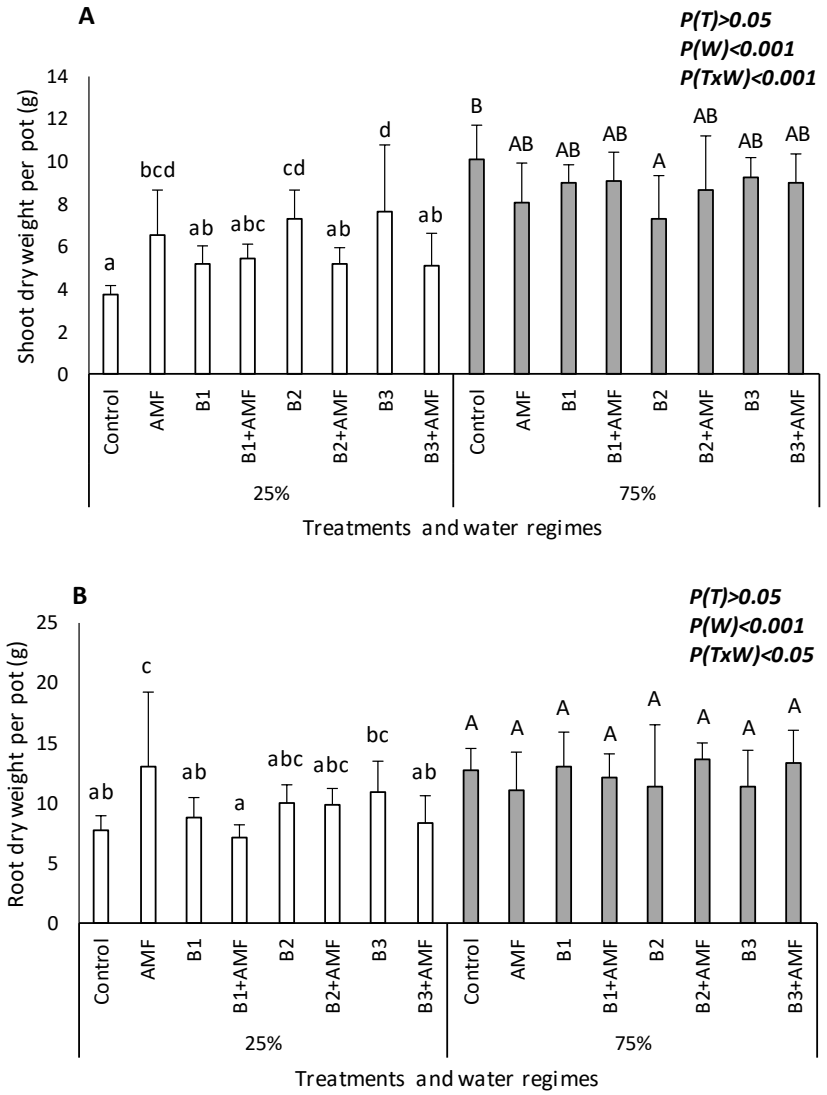

Figure 1. Shoot weight (A) and root weight (B) of cowpea plants uninoculated (Control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. 32- B1, *Bradyrhizobium elkanii* 57- B2 and *Bradyrhizobium* sp. 63- B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75% of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (*P<0.05*) among treatments, within plants under drought stress (25% of field capacity) and uppercase letters indicate significant differences (*P<0.05*) among treatments, within well-watered plants (75% of field capacity), according to Duncan´s test.

3.2. Cowpea seed yield

The number of seeds was affected by the water regime *(P<0.001)* and the interaction between the treatment and the water regime *(P<0.05).* The number of seeds (Fig. 2A) of well-watered plants (75% of FC) was positively affected by single inoculation with AMF comparing with control group, with a fold increase of 1.53.

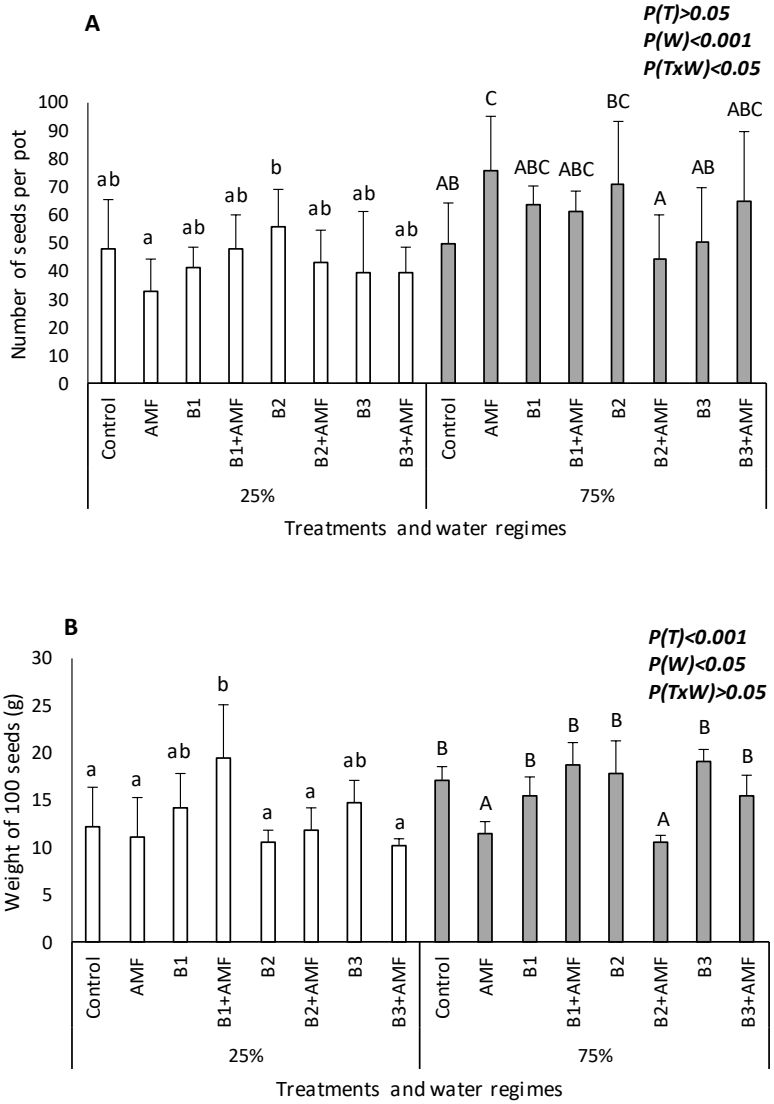

Figure 2. Number of seeds (A) and weight of 100 seeds (B) of cowpea plants uninoculated (Control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. 32- B1, *Bradyrhizobium elkanii* 57- B2 and *Bradyrhizobium* sp. 63- B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75% of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (*P<0.05*) among treatments, within plants under drought stress (25% of field capacity) and uppercase letters indicate significant differences (*P<0.05*) among treatments, within well-watered plants (75% of field capacity), according to Duncan´s test.

There was no effect of co-inoculations in both water regimes. In general, this parameter was higher in well-watered plants (75% of FC) than in plants under drought (25% of FC), with the exception of pants co-inoculated with the bacteria *B. elkanii* B2 and AMF. The weight of 100 seeds was affected by the treatment *(P<0.001)* and the water regime

(P<0.05). Despite no significant differences were observed by single inoculations in the weight of 100 seeds (Fig. 2B), the co-inoculation of plants under drought stress (25% of FC) with the bacteria *Rhizobium* sp. B1 and AMF presented significantly heavier seeds than control (1.59 of fold increase). In well-watered plants (75% of FC), single inoculation with fungi and co-inoculation with bacteria *B. elkanii* B2 and fungi significantly decreased the weight of seeds comparing with all the other treatments. In general, seeds were slightly heavier in well-watered plants (75% of FC) than in plants under drought (25% of FC).

3.3. Cowpea seed crude protein

Crude protein content was affected by the treatment *(P<0.001),* the water regime *(P<0.001) and* the interaction between the treatment and the water regime *(P<0.001)*.

All plants under drought stress (25% of FC) and co-inoculated with one bacteria and fungi presented significantly higher (*P*<0.05) crude protein content in the seeds (Fig. 3), with a 1.2, 1.3 and 1.3 fold increase following the co-inoculation with *Rhizobium* sp. B1 and AMF, *Bradyrhizobium elkanii* B2 and AMF and *Bradyrhizobium* sp. B3 and AMF, respectively, when compared to the control.

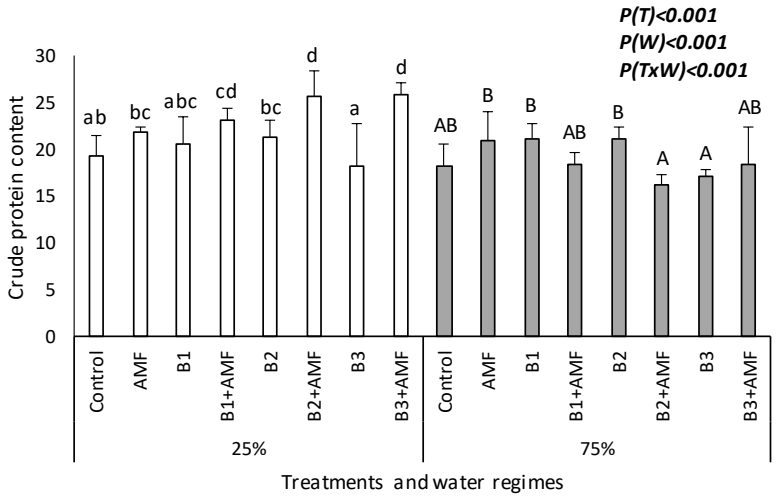

Figure 3. Crude protein content in the grains of cowpea plants uninoculated (Control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. 32- B1, *Bradyrhizobium elkanii* 57- B2 and *Bradyrhizobium* sp. 63- B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75% of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (*P<0.05*) among treatments, within plants under drought stress (25% of field capacity) and uppercase letters indicate significant differences (*P<0.05*) among treatments, within well-watered plants (75% of field capacity), according to Duncan´s test.

A positive effect was observed by the addition of AMF to *B. elkanii* B2 and *Bradyrhizobium* sp. B3, since plants co-inoculated with one of these bacteria and fungi

presented significantly higher crude protein in the seeds than plants single inoculated with either each bacteria or with fungi. In well-watered plants (75% of FC), crude protein content in the seeds was significantly higher in plants single inoculated with fungi and with *B. elkanii* B2 than in plants co-inoculated with both microorganisms together, with a 1.29 fold increase for each. Comparing single inoculation with all the bacteria, *Rhizobium* sp. B1 and *B. elkanii* B2 presented significantly higher crude protein in the seeds than single inoculation with bacteria *Bradyrhizobium* sp. B3 (1.22 fold increase for each).

Taking in account the crude protein yield per pot (Fig. 4), calculated taking in account the number of seeds and its weight and the crude protein percentage per treatment, under water stress, only plants co-inoculated with the bacteria *Rhizobium* sp. B1 plus the AMF presented significantly higher crude protein yield than the control plants. On the other hand, the well-watered plants inoculated with the bacteria *Bradyrhizobium elkanii* B2 presented a significantly higher crude protein yield than control plants, plants co-inoculated with the same bacteria and AMF and plants single inoculated with the bacteria *Bradyrhizobium* sp. B3. Similar to crude protein content in the grain, crude protein yield per pot was also affected by the treatment *(P<0.001),* the water regime *(P<0.001)* and the interaction between the treatment and the water regime *(P<0.001).*

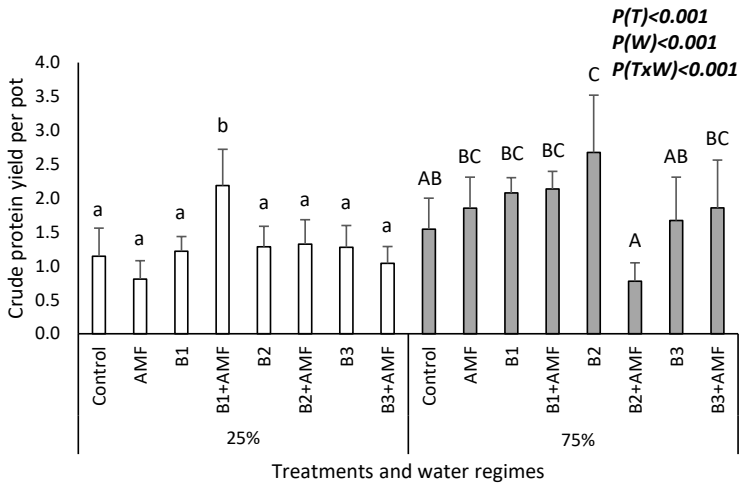

Figure 4. Crude protein yield per pot of cowpea plants uninoculated (Control) and inoculated with three rhizobial bacteria (Rhizobium sp. 32- B1, Bradyrhizobium elkanii 57- B2 and Bradyrhizobium sp. 63- B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75% of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (P<0.05) among treatments, within plants under drought stress (25% of field capacity) and uppercase letters indicate significant differences (P<0.05) among treatments, within well-watered plants (75% of field capacity), according to Duncan´s test.

3.4. Microbial performance

The number of nodules was only affected by the treatment (*P<0.05*). Although a higher number of nodules (Fig. 5A) was observed in all inoculated plants under drought stress (25% of FC), a significant increase was only observed in plants inoculated with the *Bradyrhizobium* sp. B3 when compared to control plants. On the other hand, in well-watered plants (75% of FC), the number of nodules was positively affected by single inoculation with the bacteria B2 and the bacteria B3 and co-inoculation with *Rhizobium* sp. B1 or *Bradyrhizobium* sp. B3 and fungi, comparing with control and with plants inoculated only with fungi. A positive correlation was observed between the number and weight of nodules (*r*=0.444).

The weight of nodules was affected by the treatment (*P<0.05*), the water regime (*P<0.001*) and the interaction between treatment and water regime (*P<0.05).* Well-watered plants (75% of FC) single and co-inoculated with each bacteria and AMF presented significantly heavier nodules (Fig. 5B) than control and plants single inoculated with AMF. Despite the similar number of nodules observed in both water regimes, they were heavier in well-watered plants (75% of FC), in all the performed treatments.

Under drought stress (25% of FC), mycorrhizal colonization rate (Fig. 5C) was positively affected by single inoculation with fungi and co-inoculation with *Bradyrhizobium* sp. B3 and AMF, with a fold increase of 1.41 and 1.44 to control, respectively. Despite no significant differences were observed, co-inoculation with bacteria *Rhizobium* sp. B1 or *B. elkanii* B2 and AMF also increased the mycorrhizal colonization of plants under drought stress (25% of FC). In well-watered plants (75% of FC), co-inoculation with *B. elkanii* B2 and AMF was the unique treatment that increased significantly mycorrhizal colonization rate comparing with control, with a fold increase of 1.47. Mycorrhization rate followed the same profile within each water regime. Indeed, this parameter was only affected by the treatment (*P<0.05*).

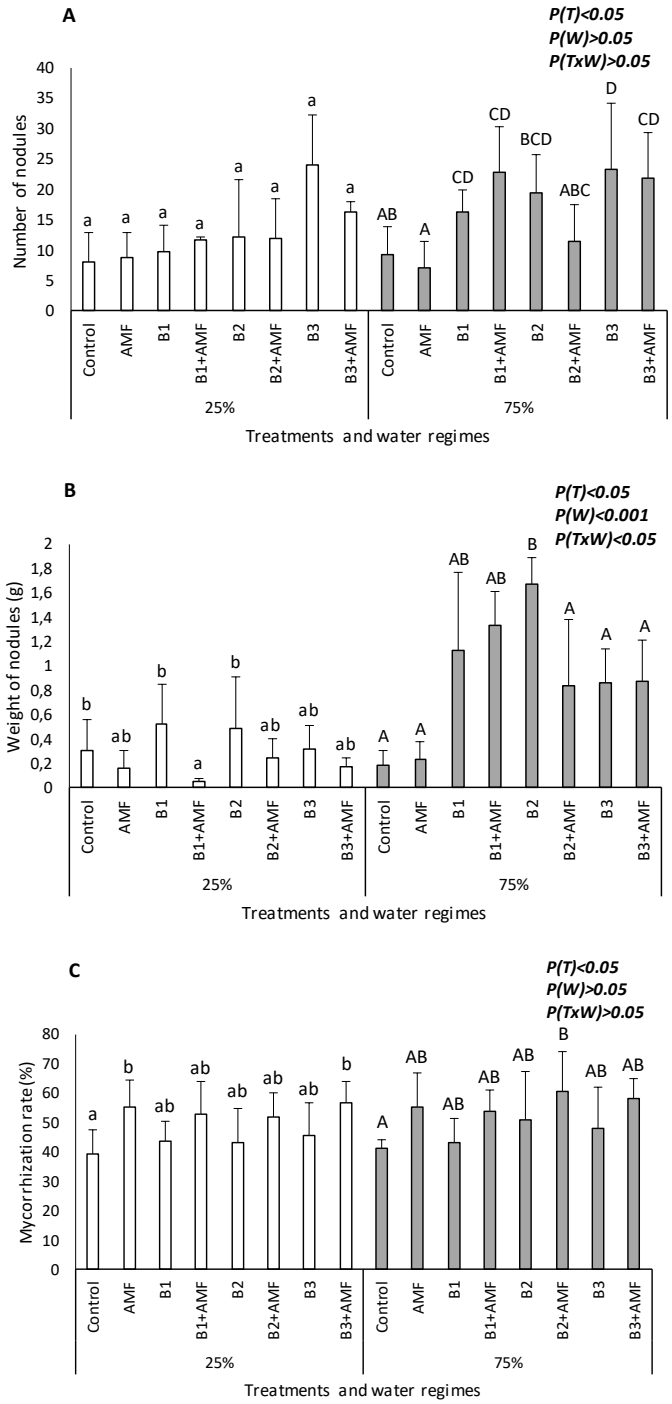

Figure 5. Number of nodules (A), weight of nodules (B) and mycorrhization rate (C) of cowpea plants uninoculated (Control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. 32- B1, *Bradyrhizobium elkanii* 57- B2 and *Bradyrhizobium* sp. 63- B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75% of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (*P<0.05*) among treatments, within plants under drought stress (25% of field capacity) and uppercase letters indicate significant differences (*P<0.05*) among treatments, within well-watered plants (75% of field capacity), according to Duncan´s test.

4. Discussion

Although cowpea has been referred as a well-adapted plant to abiotic stress, drought is one of the main concerns in its production. Thus, inoculation with selected rhizobial bacteria and AMF has great potential to reduce the impact of water scarcity (Oliveira *et al*., 2017). Though, the selection of appropriate combinations of specific AMF and rhizobia is very important to improve the yield of cowpea, since the response of a legume host to a given set of AMF-*Rhizobium* partners may or may not be favorable for plant growth depending on the interaction of symbionts (Xavier and Germida, 2003). In fact, Ahmad (1995) demonstrated that symbiotic effectiveness depends on combination of AMF species, *Rhizobium* strain and also host plant.

In our work, the inoculation and co-inoculation with the studied microorganisms influence the plant performance mainly in drought stress. In well-watered plants the beneficial effects of the inoculation are less evident. This can be due to the presence of other native bacteria and fungi in the soil that will also interact with plants giving them the advantages of symbiosis, even in control plants. However, under drought stress it is possible to observe some differences between control and inoculated plants, suggesting that the native microorganisms present in the soil were not so resistant to drought as the inoculated strains. As shown in other studies, drought, among other stresses, affects the ability to grow and even the basic survival of native microorganisms (Haruta and Kanno, 2015; Goufo *et al.*, 2017).

In general, in plants under drought, single inoculation with the studied microorganisms did not improve the plant responses, however, when both microorganisms were inoculated together, an improvement in the general plants' performance was observed. This can be due to the simultaneous improvement in the nitrogen fixation provided by the bacteria (Hardarson and Atkins, 2003) and the improvement in water and other minerals provided by the fungi (Nadeem *et al*., 2014). According to previous studies, in general, co-inoculation with rhizobial bacteria and AMF (tripartite symbiosis) improve plants water and nutritional status in a bigger scale that single inoculation with one microorganism, since as the nodulation process by rhizobia requires a high amount of P, the association with AMF help in the development and function of symbiotic nodules (Ribet and Drevon, 1996). As described in some studies, this symbiosis ameliorates plant photosynthetic efficiency (Jia *et al*., 2004, Kaschuk *et al*., 2009) and consequently increases photoassimilates production, which can be used by the plants to improve the growth, productivity and/or quality. Indeed, the impact that the microbial symbionts had on photosynthetic rates appeared to be mediated by their effects on the plant N:P ratio (Jia *et al*., 2004).

In the present study, co-inoculation did not affect the growth of plants, taking in account the absence of significant differences in the shoot and root weight between control

and co-inoculated plants. In line, Diallo *et al*. (2001) found no benefits in plant root and shoot biomass with AMF inoculation. The authors attributed this lack of effect to the fact that the production of fungal mycelium is much more cost-effective in terms of organic carbon (C) than the production of equivalent root length. Consequently, plants adjust belowground C allocation contributing to the formation of a shorter mycorrhizal root system, relying on the fungal mycelium for nutrient uptake (Smith *et al*., 2000).

Moreover, in the present study, co-inoculations also did not influence the productivity parameters, since the number and weight of seeds was not affected, except for the mix B1 and AMF that presented heavier seeds than control.

It was observed a significant increase in the crude protein content (derived from the nitrogen level by the Kjeldahl method) in the seeds of plants under drought stress (25% of FC) and co-inoculated with one bacteria and AMF, when compared to the control plants, which suggest that these plants have the ability to mobilize the photoassimilates to the seed, a sink of protein production, in detriment of growth and yield. Despite the increase in nitrogen observed in co-inoculated plants under water stress, through this method it is not possible to distinguish between protein nitrogen and non-protein nitrogen and therefore it cannot be ruled out that this increase occurred in the non-protein fraction of nitrogen.

In a meta-analysis with 12 legume species performed in a previous study, it was also observed that inoculation with rhizobia in the field and with AMF in pots increased seed protein content (Kaschuk *et al*., 2010). In fact, according to Dubova *et al*. (2015), protein accumulation in the seeds depends not only on plant biosynthetic activity but can also be affected by microbial symbionts. From the results of this study, it can be concluded that under drought stress (25% of FC), the microorganisms used in this study were efficient and competitive, benefiting more the plants than the native microbiota present in the soil (control plants). In previous studies, it was also shown that these beneficial microorganisms can increase plant resistance to high temperatures and water deficit and that their application can reduce the needs of chemical fertilizer inputs in agriculture (Peoples *et al*., 1995; Oliveira *et al*., 2017), as soil microbes are critical for sustainable functioning of natural and managed ecosystems (Sharma *et al*., 2018). Additionally to the treatment influence, the crude protein content was also affected by the water regime, being higher in plants under drought stress.

This can be explained by the increase in nitrogenous compounds, such as the proline amino acid usually synthesized in large amounts in plants under stress, previously described by da Costa *et al*. (2011). In fact, the proline amino acid has a high sensitivity of response to stress conditions (Ashraf *et al*., 2011), increasing up to 100 times its concentration, compared to that observed in plants grown under normal conditions (Verbruggen and Hermans, 2008). This increase can occur by “de novo” synthesis or by inhibiting the proline

oxidation process. The accumulation, in vacuole or cytosol, of proline and other compatible solutes (glycine betaine, trehalose, sucrose, polyamines, mannitol, pinitol, among others) contributes to the maintenance of water balance and the preservation of the integrity of proteins, enzymes and cell membranes (Marijuan and Bosch, 2013). These solutes also have an osmoprotective function against toxic by-products of metabolism, resulting from water stress. This accumulation is not harmful to cell metabolism and, by increasing the osmotic pressure inside the cells, maintains the water absorption and the turgor pressure of the cells, which allows the continuity of physiological processes, even at lower levels (Marijuan and Bosch, 2013). Considerable accumulation of proline is a feature in the response of plants under water stress (Fukutoku and Yamada 1981, Levy 1983). Furthermore, water stress induces a net loss of leaf protein since its synthesis is inhibited and its degradation is stimulated, leading to an accumulation of free amino acids (Cooke et al. 1979, 1980, Dungey and Davies 1982). Thus, a relationship between proline accumulation and protein metabolism has been described, since protein may be a source of nitrogen for proline synthesis during water stress. In these conditions, as reported by Fukutoku and Yamada (1984), a loss of leaf protein-^{15}N occurs, which is balanced by a gain in ^{15}N in the free amino acids, namely proline and asparagine.

The use of non-sterilized soil makes this work very useful because we can extrapolate the results obtained in pots to the field, in real conditions. However, it is important to note that the potential of the microorganisms used in this work, especially the fungi, could be underestimated, due to the confined space of the pot, which does not allow the maximum development of the root. According to the results obtained in this work, it is possible to extrapolate that the studied bacteria should have same strategies to cope with stressful conditions, which can be the formation of cysts and spores, changes in cellular membranes, expression of repair enzymes for damage, synthesis of molecules for relieving stresses, among others (Storz and Hengge, 2011). These strategies make them potentially resistant to drought, which can be used as an improved biotechnological tool for sustainable agriculture in drought situations. Indeed, climate change will seriously impact food security and nutrition, making crucial to support a transition toward smart and sustainable food systems that take climate into account (FAO, 2008). With this eco-friendly approach it is possible to increase the nutritional and commercial value of leguminous plants by the increase in crude protein content, a cheap and alternative source of protein for human consumption, without chemical fertilizer applications and genetic improvements.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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CHAPTER V

BIODIVERSITY OF RHIZOBIA ASSOCIATED WITH FABA BEAN PLANTS

CHAPTER V- BIODIVERSITY OF RHIZOBIA ASSOCIATED WITH FABA BEAN PLANTS

BRIEFING NOTE

This chapter includes the morphological and molecular characterization of the rhizobial isolates associated with faba bean plants (*Vicia faba* L.). Rhizobial strains analysed in this work were isolated from fresh surface sterilized nodules present in the roots collected from faba bean plants in regions with different edaphoclimatic conditions in Portugal.

As 16S rRNA analysis did not provide enough resolving power in discriminating closely related species, this analysis was complemented with other genes, such as nodulation genes (*nodA*, *nodC*) and housekeeping genes (*atpD*, *gyrB*, *thrA*, *truA*, *SMc*, *recA*)- Multilocus sequence analysis (MLSA). The results of this work showed a high abundance of bacteria from *Rhizobium* genus in faba bean root nodules. However, other bacteria, such as, *Burkhoderia* sp. And *Burkhoderia lata* were also identified.

The authors contribution for the article converted in the present chapter was: Sandra Pereira, Lav Sharma and Ângela Mucha were responsible for the DNA extraction, amplifications, sequence edition and phylogenetic analysis. Sandra Pereira was also responsible for data interpretation and manuscript writing. Eduardo Rosa and Guilhermina Marques were responsible for study conception and design of the experiment and critical revision of the article. All the authors reviewed and approved the final manuscript.

Biodiversity of rhizobial bacteria associated with faba bean (*Vicia faba* L.) in portuguese soils

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Abstract

In legume-rhizobium symbiosis, the composition of the nodulating population varies, which is mostly explained by differences in soil condition, climate and plant variety. Faba bean is considered as a selective host plant that is commonly nodulated by *Rhizobium leguminosarum* symbiovars *viciae*, *trifolii* and *phaseoli*, *R. etli*, *R. fabae*, *R. laguerreae* and *Agrobacterium* spp. Despite all the works that had been performed on faba bean-nodulating bacteria in several countries around the world, little is known about the genetic and symbiotic diversity of indigenous faba bean rhizobia in Europe, and in particular in Portugal. The aim of this study was to describe the biodiversity of bacterial communities associated with faba bean root nodules. Thirty-four faba bean-nodulating bacteria were isolated from plants collected in several regions of Portugal with different edapho-climatic conditions. Their symbiotic effectiveness, genetic diversity and phylogeny were assessed. The phylogenetic analysis was based on 16S rDNA region, two symbiotic genes (nodA and nodC) and six housekeeping genes (*recA*, *gyrB*, *SMc00019, thrA*, *atpD* and *truA*).

Rhizobium was the most abundant genus detected in faba bean root nodules. In fact, 20 isolates were identified as *Rhizobium leguminosarum* bv. *viciae*, 10 were identified as *Rhizobium* sp. and one isolate was identified as *Rhizobium laguerreae*. Additionally, some β-proteobacteria were also identified: *Burkholderia* sp. (N=2) and *Burkholderia lata* (N=1). According to some authors, β-proteobacteria from the genera *Burkholderia* were also described as β-rhizobia.

The phylogeny of housekeeping genes and symbiotic genes was not congruent for all the isolates, implying that the strains had been shaped by vertical evolution of the housekeeping genes and lateral evolution of the symbiotic genes.

Keywords: Leguminous plants, MLSA, molecular identification, *Vicia faba* L.

1. Introduction

Faba bean (*Vicia faba* L.) is considered as a multipurpose crop since it can be used for human consumption, either by its dry and fresh seeds and immature pods, and for animal feed. Its dry seeds have been also used to extract protein and to produce flour (Van Berkum *et al*., 1995; Xu *et al.*, 2015).

In recent years, this crop has been growing worldwide in a diverse cropping system as a grain and green-manure legume and it is now widespread in Europe, North Africa, Central Asia, China, South America, the USA, Canada and Australia. In fact, in 2017, the total world area cultivated with faba bean was around 2.4 million ha, with most of production located in China, Ethiopia and Australia (FAOSTAT, 2019).

The rhizobium-legume symbioses vary in specificity for both the breadth of host range and the diversity of bacterial species nodulating a given host plant, being that the symbiosis between rhizobia and faba bean provides one of the highest amounts of fixed N, reaching up to 45-300 kg N ha^{-1} per year (Smil *et al*., 1999).

In the past, the symbiosis between *Vicieae* (*Vicia*, *Pisum*, *Lens* and *Lathyrus*) and strains of *Rhizobium leguminosarum* bv. *viciae* was considered to be one of the most specific (Tian *et al*., 2010). This species was divided into three symbiovars, based on the host plant specificity (Jordan, 1984): *viciae* (pea and vetch), *trifolii* (clover), and *phaseoli* (beans) (Laguerre *et al*., 2001; Rogel *et al*., 2011). Rhizobial strains nodulated faba bean plants were assumed to be classified as *R. leguminosarum* bv. *viciae*, due to cross-infection of pea (Van Berkum *et al*., 1995). Nevertheless, other symbionts could also nodulate faba bean plants (Van Berkum *et al*., 1995; Tian *et al*., 2007). In 2013, Saidi *et al*. found that some rhizobial strains, able to nodulate faba bean plants, have 16S rDNA sequences similar to *Rhizobium leguminosarum*. Nevertheless, *recA* and *atpD* sequences were phylogenetically distant from that species. So being, they classified these bacteria, distinguishable by the housekeeping genes, as a novel species: *Rhizobium laguerreae* (Saidi *et al.*, 2013).

During recent years, the taxonomy and phylogeny of rhizobia have undergone several changes due to the increased availability of phylogenetic and polyphasic data, which helped the description of new taxa (Young *et al*., 1996). Compiling this new information, a growing number of new rhizobia have been isolated and characterized, especially from zones where diversity is poorly documented.

Most of the currently known rhizobia are in the Rhizobiales order, in the class α-Proteobacteria, including the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Ensifer*

(*Sinorhizobium)*, *Mesorhizobium*, *Phyllobacterium* (Zakhia *et al*., 2006), *Methylobacterium* (Sy *et al*., 2001), *Microvirga* (Ardley *et al*., 2012; Radl *et al*., 2014) and *Devosia* (Rivas *et al*., 2003). The *Rhizobium* genus was united with *Agrobacterium* and *Allorhizobium*, because of their close relation (Young *et al*., 2001), but some studies support the revival of the *Allorhizobium* genus within the Rhizobiaceae and additional new genera have been proposed (Ormeño-Orrilo *et al*., 2015). There are also rhizobial species in the β-proteobacteria, from genera *Burkholderia* and *Cupriavidus* (*Ralstonia*), in the Burkholderiales order (Berrada *et al*., 2014). Due to the increasing importance of these bacteria, currently, the classification of their taxonomy remains a pertinent issue (Vieira *et al*., 2010). In addition to strains that can form nodules, several other bacterial species, called non-rhizobial endophytes (NRE), can enter infection threads when leguminous plant are colonized with rhizobial strains, having beneficial effects on the host plant (De Meyer *et al*., 2015; Leite *et al*., 2017). This group include some α-proteobacteria (*Aminobacter*, *Ochobactrum*, *Methylobacterium* and *Phyllobacterium*), β-proteobacteria (*Herbaspirillum* and *Shinella*) and γ-proteobacteria (*Pantoea*, *Enterobacter* and *Pseudomonas*) (Valverde *et al*., 2003; Benhizia *et al*., 2004; Lin *et al*., 2008; Ibáñez *et al*., 2009; Shiraishi *et al*., 2010; Aserse *et al*., 2013).

The need to select more efficient strains and reduce the chemical fertilizer inputs, in a more sustainable agriculture, has led to a greater interest in deepening the knowledge about the bacteria existing inside the root nodules. In this sense, the aim of this work was to identify the bacteria presented in faba bean root nodules collected from several regions of Portugal with different edaphoclimatic conditions. The 16S rDNA gene sequencing was used to obtain a preliminary identification of the isolates. Therefore, the analysis using other housekeeping and accessory genes, such as those involved in nodulation of the host plant *(nod*), was also performed for optimal species-level differentiation. Gene flow, including recombination and horizontal gene transfer (HGT), has been demonstrated to play an important role in the evolution of *Bradyrhizobium* spp., *Sinorhizobium* spp., *Rhizobium gallicum* and other bacteria (Silva *et al*., 2005; Vinuesa *et al*., 2005; Bailly *et al*., 2007; Maiden, 2006). However, most of the studies involving these phenomena in rhizobia are focused on closely related species, normally within the same genus (Ling *et al*., 2016) and occurrence of HGT from α- to β-proteobacteria and vice versa therefore remains poorly studied.

To the best of our knowledge, there are no previous studies on the biodiversity of bacteria from root nodules of faba bean plants in Portugal. So, we propose to access the diversity of root-

nodulating bacteria associated with faba bean plants in Portugal, using multilocus sequence analysis (MLSA).

2. Material and methods

Nodule collection and bacterial isolation

Rhizobial strains were isolated from faba bean root nodules collected in several regions of Portugal with different edaphoclimatic conditions. Details of sampling (host plant, collection site and coordinates) of the 34 isolates are shown in the Table 1.

Surface sterilized nodules (1.5% sodium hypochlorite (NaCIO) (v/v) washing for 1 min, 70% ethanol washing for 1 min and several washes with sterilized distilled water) were crushed aseptically and streaked on Yeast Mannitol Agar (YMA) medium (1 g L^{-1} of yeast extract, 10 g L^{-1} of mannitol, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.1 g L^{-1} NaCl and 15 g L^{-1} agar) supplemented with 0.025 g L^{-1} congo red (CR). After 2-3 days, a single colony was streaked to plates with the same medium supplemented with 0.1 g L^{-1} bromothimol blue (BTB). This process was repeted until pure cultures were obtained.

A growth chamber (Panasonic MIR-162-PE) experiment was performed to check the ability of isolates to infect other faba bean plants *in vitro* (Kock's postulates). The sterilization of faba bean seeds used in this experiment was performed with 1.5% sodium hypochlorite (NaCIO) (v/v) washing for 2 min, 70% ethanol washing for 1 min and several washes with sterilized distilled water. After the pre-germination, seeds were transferred to a sterilized glass bottle with a semi solid sterile nutrient solution (1 g L^{-1} $CaHPO_4$, 0.2 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.2 g L^{-1} NaCl, 0.1 g L^{-1} $FeCl_3.6H_2O$, 1.0 mL L^{-1} micronutrients (0.5% B; 0.05%Mn; 0.005% Zn; 0.005% Mo and 0.002% Cu) and 9.0 g L^{-1} agar) (Jensen, 1942). Each seed was inoculated with a different bacteria and uninoculated plants were used as negative control. Four weeks after inoculation, plants were uprooted and the presence of nodules was evaluated. The existing nodules were re-isolated and grown again in YMA media.

Table 1- Bacterial isolates collected from faba bean root nodules

Isolate	Collection site	Coordinates	Molecular identification
R20	Famalicão, Portugal	41°25'55.70''N 8°23'03.15''W	*R. leguminosarum bv. viciae*
R65	Vila Real, Portugal	41°16'53.86''N 7°44'43.09''W	*R. leguminosarum bv. viciae*
R66	Famalicão, Portugal	41°25'55.70''N 8°23'03.15''W	*R. leguminosarum bv. viciae*
R67	Famalicão, Portugal	41°25'55.70''N 8°23'03.15''W	*R. leguminosarum bv. viciae*
R68	Vila Real, Portugal	41°16'53.86''N 7°44'43.09''W	*R. leguminosarum bv. viciae*
R69	Vila Real, Portugal	41°16'53.86''N 7°44'43.09''W	*R. leguminosarum bv. viciae*
R70	Famalicão, Portugal	41°25'55.70''N 8°23'03.15''W	*R. leguminosarum bv. viciae*
R71	Famalicão, Portugal	41°25'55.70''N 8°23'03.15''W	*R. leguminosarum bv. viciae*
R72	Famalicão, Portugal	41°25'55.70''N 8°23'03.15''W	*R. leguminosarum bv. viciae*
R73	Vila Real, Portugal	41°16'53.86''N 7°44'43.09''W	*R. leguminosarum bv. viciae*
R74	Vila Real, Portugal	41°16'53.86''N 7°44'43.09''W	*R. leguminosarum bv. viciae*
R77	Nogueira, Portugal	41°13'36.21''N 7°44'01.62''W	*R. leguminosarum bv. viciae*
R80	Nogueira, Portugal	41°13'36.21''N 7°44'01.62''W	*R. leguminosarum bv. viciae*
R83	Nogueira, Portugal	41°13'36.21''N 7°44'01.62''W	*R. leguminosarum bv. viciae*
R84	Nogueira, Portugal	41°13'36.21''N 7°44'01.62''W	*R. leguminosarum bv. viciae*
R85	Nogueira, Portugal	41°13'36.21''N 7°44'01.62''W	*R. leguminosarum bv. viciae*
R86	Nogueira, Portugal	41°13'36.21''N 7°44'01.62''W	*R. leguminosarum bv. viciae*
R88	Campo Maior, Portugal	39°01'27''N 7°03'55''W	*Burkholderia* sp.
R89	Caia, Portugal	38°52'59.70''N 7°01'59.73''W	*Burkholderia lata*
R90	Vila Boim (Elvas), Portugal	38°51'22''N 7°17'51''W	*Rhizobium* sp.
R92	Caia, Portugal	38°52'59.70''N 7°01'59.73''W	*R. leguminosarum bv. viciae*
R94	Varche (Elvas), Portugal	38°51'46''N 7°12'40''W	*Rhizobium* sp.
R96	Caia, Portugal	38°52'59.70''N 7°01'59.73''W	*Rhizobium* sp.
R97	Caia, Portugal	38°52'59.70''N 7°01'59.73''W	*Rhizobium* sp.
R98	Herdade da Comenda, Portugal	38°53'--N 7°02'--W	*R. leguminosarum bv. viciae*
R99	Cabeça do Carneiro, Portugal	38°31'52.68''N 7°23'27.82''W	*Rhizobium* sp.
R101	Cabeça do Carneiro, Portugal	38°31'52.68''N 7°23'27.82''W	*Rhizobium* sp.
R102	Reguengos de Monsaraz, Portugal	38°23'44.19''N 7°32'50.79''W	*Rhizobium* sp.
R103	S. Julião- Portalegre, Portugal	39°19'--N 7°19'--W	*Rhizobium* sp.
R104	Alegrete- Portalegre, Portugal	39°16'15''N 7°17'--W	*Rhizobium* sp.
R106	Fonte do Freixo, Portugal	38°48'45''N 7°27'35''W	*Rhizobium laguerreae*
R107	Reguengos de Monsaraz, Portugal	38°23'44.19''N 7°32'50.79''W	*Rhizobium* sp.
R109	Reguengos de Monsaraz, Portugal	38°23'44.19''N 7°32'50.79''W	*Rhizobium* sp.
R110	Montes Novos (Estremoz), Portugal	38°50'06''N 7°39'52''W	*R. leguminosarum bv. viciae*

PCR amplification, sequencing, and phylogenetic analysis

DNA extraction for PCR amplification was performed from re-isolated bacteria and according to the method used by Laguerre *et al*. (1996), with some modifications.

Cell lysis was performed with CTAB lysis buffer (cetyltrimethylammonium bromide) and also using mechanical lysis, through the FastPrep-24 equipment (MP Biomedicals). A chloroform and isoamyl alcohol solution was used to denature proteins. Precipitation of DNA was performed

and after washing, DNA was eluted with sterilised ultra-pure water.The concentration of obtained DNA was estimated by spectrophotometer or eletrophoresis.

Primers fD1 and rD1 (table 2) were used to amplify the 16S rDNA region. Additionally and in order to identify the different isolates at species level, the 16S analysis was complemented with six housekeeping genes: *recA* (DNA recombination protein), *gyrB* (DNA gyrase B), *SMc00019* (conserved hypothetical protein), *thrA* (homoserine dehydrogenase), *atpD* (atpD synthase β-subunit) and *truA* (RNA pseudouridine synthase A) and two nodulation genes: *nodA* (N-acyltransferase nodulation protein A) and *nodC* (N-acetylglucosaminyltranferase) to determine the taxonomic position at symbiovar level. PCR mixtures were performed with 7.5 µl of master mix (MyTaq HS Mix, 2x of Bioline), 1 µl of each forward and reverse primer and 5.5 µl of DNA template, with 15 µl of final volume. Amplified samples were sequenced (Stabvida, Portugal), using the same primer set described for PCR amplification.

Data analysis

Nucleotide sequences were corrected using BioEdit software and homology searches were performed at the National Center for Biotechnology Information (NCBI) server using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). For phylogenetic analysis, sequences of the isolates and the most similar sequences retrieved from the NCBI database, were aligned using MAFFT software version 7 (Katoh and Standley, 2013). Maximum Likelihood (ML) phylogenetic trees were constructed in MEGA 6.06 (Tamura *et al*., 2013), using GTR+G (5 categories) substitution model and considering all sites in the final datasets. Robustness of the tree topologies was estimated using 500 bootstrap replicates. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site.

Concatenation of all housekeeping genes was performed using Geneious 9.1.6 (Biomatters Ltd, New Zealand) and network analysis was done using NeighborNet analysis in SplitsTree 4.0 (Huson and Bryant, 2006). Concatenated tree was made with RAxML 8.2 (Stamatakis, 2014) using GTR+G+I model. Editing of trees was done in MEGA 6.06.

Table 2- List of primers used in this work.

Primers	Sequence (5'-3')	Reference
fD1	AGA GTT TGA TCC TGG CTC AG	Weisburg *et al*., 1991
rD1	AAG GAG GTG ATC CAG CC	
thrAB-F	TGC TTC GTC GAR YTG ATG G	Zhang *et al*., 2012
thrAB-R	ACR CCC ATC ACC TGY GCR ATC	
thrAMRS-F	TAA TAC GAC TCA CTA TAG GGG CNG GBG GYA TYC CSG TBA TCA AG	modified by Tampakaki from Zhang *et al*., 2012
thrAMRS-R	GAT TTA GGT GAC ACT ATA GCG YTC GAT NCG RAT SAC YTG SGG	
SMc00019B-F	CAT TCV KCS GAR GGV GCS ATG GGY ATC	Zhang *et al*., 2012
SMc00019B-R	GCG TGB CCB GCS KCG TTS GAV AGC AT	
SMc00019MRS-F	TAA TAC GAC TCA CTA TAG GGC ADT TCC TBA THG CCA TGC C	modified by Tampakaki from Zhang *et al*., 2012
SMc00019MRS-R	GCV GGR CAN KTS AGC CAD CCR TT	Zhang *et al*., 2012
truAB-F	TAA TAC GAC TCA CTA TAG GGC GCT ACA AGC TCA YYA TCG A	modified by Tampakaki from Zhang *et al*., 2012
truAB-R	CCS ACC ATS GAG CGB ACC TG	Zhang *et al*., 2012
truAR-F	TGA CCG TSG AAT ATG ACG G	
truAR-R	ACA TCS AGY CGG TCV AGS GT	
truAMS-F	TAA TAC GAC TCA CTA TAG GGC AGG TSG CDC ATS TCG AYC T	modified by Tampakaki from Zhang *et al*., 2012
truAMS-R	GAD CGB AYC TGG TTR TGM AG	Zhang *et al*., 2012
gyrB340F-T7	TAA TAC GAC TCA CTA TAG GGT TCG ACC ARA AYT CYT ACA AGG	modified by Tampakaki from Zhang *et al*., 2012
gyrB1057R-SP6	GAT TTA GGT GAC ACT ATA GCC AAY TTR TCC TTG GTC TGC G	
gyrB-F	ACC GGT CTG CAY CAC CTC GT	Spilker *et al*., 2009
gyrB-R	YTC GTT GWA RCT GTC GTT CCA CTG C	
recA6F	CGK CTS GTA GAG GAY AAA TCG GTG GA	Gaunt *et al*., 2001
recA555R	CGR ATC TGG TTG ATG AAG ATC ACC AT	
atpD273F	SCT GGG SCG YAT CMT GAA CGT	Gaunt *et al*., 2001
atpD-294F	TAA TAC GAC TCA CTA TAG GGA TCG GCG AGC CGG TCG ACG A	modified from Gaunt *et al*., 2001
atpD771R	GCC GAC ACT TCC GAA CCN GCC TG	Gaunt *et al*., 2001
nodA-1	TGC RGT GGA ARN TRN NCT GGG AAA	Haukka *et al*., 1998
nodA-2	GGN CCG TCR TCR AAW GTC ARG TA	
nodCF	AYG THG TYG AYG ACG GTT C	Laguerre *et al*., 2001
nodCFu	AYG THG TYG AYG ACG GIT C	
nodCI	CGY GAC AGC CAN TCK CTA TTG	

3. Results and discussion

Isolation of root nodule bacteria

A total of 34 bacterial isolates were obtained from the root nodules of faba bean plants collected in several places of Portugal, with different edaphoclimatic conditions. This number was higher but, in this work, just the isolates with at least 5 amplified genes were considered. The negative control did not develop any nodules, confirming aseptic conditions of the experiment. The effectiveness of the strains was shown by the pink colour inside the nodules and the dark green colour of leaves compared to negative controls. These authenticated rhizobial isolates were then genetically analysed using a Multilocus sequence analysis.

16S analysis of faba bean isolates

Phylogenetic tree built with 16S rDNA gene sequences of faba bean nodules (SM1) was performed with 82 nucleotide sequences, including 34 strains isolated in this work, and split the strains into 3 well-supported separate clades: alpha (α-PB), beta (β-PB) and gamma (γ-PB) proteobacteria. Most of the isolates belonged to the genus *Rhizobium* (*N*=31) and were placed in the first clade. *Rhizobium leguminosarum* bv. *viciae* (*N*=20) was the most dominant species among all faba bean root nodules colonising bacteria, followed by Rhizobium sp. (N=10). One isolate of *Rhizobium laguerreae* was also observed. Moreover, 3 isolates were clustered in the second clade of β-PB and were identified as *Burkholderia* sp. (N=2) and *Burkholderia lata* (N=1). The presence of these bacteria strengthens the claim that β-PB are common legume symbionts. Moreover, no isolates were observed in the γ-PB clade.

However, the use of 16S rRNA gene as a single molecular marker has been censured due to several reasons, such as (a) generally it is present in multiple copies in a genome of bacteria, which lead to sequence heterogeneity sometimes, (b) it is susceptible to genetic recombination and horizontal gene transfer and, (c) its low phylogenetic power among closely related species. Therefore, a multilocus sequence analysis (MLSA) approach is widely used where the housekeeping and the nodulation genes are also considered, along with 16S rRNA, for rhizobial taxonomy and phylogeny. Bacterial genes encoding for the proteins recombinase A (*recA*), β-subunit of ATP synthase F1 (*atpD*) and DNA gyrase B subunit (*gyrB*) are some of the examples of such housekeeping genes. Genes necessary for the nodulation process, for e.g., biosynthesis of nod factors (N-acyltransferase) (*nodA*) and biosynthesis of nod factors (N-acetylglucosaminyltransferase) (*nodC*) are also utilized. Recently, three different markers, i.e., a conserved hypothetical protein (*SMc00019*), homoserine dehydrogenase (*thrA*), and RNA pseudouridine synthase A (*truA*) were described for their abilities for a congruent and robust rhizobia phylogeny (Zhang *et al*., 2012). The present study incorporated a nine gene approach to understand the population structure of the rhizobia isolated from faba bean plants.

Analysis of housekeeping genes in faba bean isolates

Sequences of the corresponding housekeeping genes from type and reference strains were retrieved from the Genbank and were trimmed appropriately. The sequence availability in this database determined the number of type strains/taxa included in the analysis as well as the number of positions, i.e., the length of the alignments in the final dataset. Some nucleotide

sequences are missing in each phylogenetic tree, due to difficulties in PCR amplification and/or bad sequence results.

The molecular identification of the isolates according to the *atpD* gene is present in SM2. This tree is formed by 67 nucleotide sequences, including 31 isolates from this work. Additionally, the results of *recA* gene are present in SM3, with a total of 58 nucleotide sequences, including 32 sequences amplified in this work. In these individual trees, all the amplified isolates were placed in α-PB clade, even the isolates R88, R89 and R103, which were placed in β-PB clade, in 16S tree. A particularity is the fact that the isolate R106 perfectly clustered with the sequence JN558681.2 *Rhizobium laguerreae* FB206. In fact, Saidi *et al.* (2013) showed that several fast-growing rhizobial strains able to nodulate faba bean have 16S rRNA sequences similar to *Rhizobium leguminosarum*; however, their recA and atpD sequences were phylogenetically distant from that species. Therefore, this group of bacteria, distinguishable by its housekeeping genes, was classified as a novel species called *Rhizobium laguerreae* (Saidi *et al.,* 2013).

The results of *gyrB* gene amplifications are shown in SM4, which was performed with 53 nucleotide sequences, including 21 isolates of this work. In this individual tree, all the isolates were clustered in α-PB clade, with the exception of the isolate R88, which was placed in β-PB clade, similarly to 16S tree.

Taking in account the *SMc* individual tree, which was constructed with 79 sequences, including 33 isolates from this work, it is possible to observe that this individual tree is in agreement with 16S tree, except to the isolate R103, which clustered in β-PB clade in 16S tree and in α-PB clade in *SMc* tree.

With the recently described primers *thrA* and *truA*, it was possible to amplify respectively 26 and 25 isolates, in a total of 62 and 50 nucleotide sequences (SM6 and SM7). Although the amplification success was lower using these genes, all the isolates were placed in α-PB clade.

According to some authors, faba bean can be effectively nodulated by *Rhizobium leguminosarum* bv. *viciae*, *Rhizobium fabae*, *Rhizobium laguerreae*, *Rhizobium etli* and *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) (Youseif *et al.,* 2017). *R. leguminosarum* bv. *viciae* specifically nodulates the legume tribe Vicieae which comprises the genera *Lathyrus*, *Lens*, *Pisum* and *Vicia*. *Rhizobium leguminosarum* is a complex species and many different rhizobial species can group between this species (Mousavi *et al.,* 2015).

Protein coding genes showed improved resolutions within bacteria with closely related 16S rRNA gene. Although some evidence of HGT of the core genes were evident in each of these

genetic markers, such reports are not new in *R. leguminosarum* (Kumar *et al*., 2015) and specifically in *R. leguminosarum* bv. *viciae* (Tian *et al*., 2010; Xu *et al*., 2015).

The presence of *Burkholderia* spp. in the root nodules of faba bean suggest that β-proteobacteria can nodulate legume plants. Previous reports about the microbial diversity from the root nodules suggest the same (De Meyer *et al*., 2015). Moreover, Bontemps *et al*. (2010) reports *Burkholderia* as the ancient symbionts of legume plants.

Slight differences in the tree topologies of the individual ML trees were observed. In bacteria, this kind of phylogenetic incongruence can occur when chromosomal DNA is transferred between members of the same species by conjugation, transduction or transformation, and part of the incoming DNA replaces existing sequences by homologous recombination. Such a recombination can serve as a cohesion mechanism that maintains the identity of a species by preventing the isolation of clonal lineages, but it depends on a high degree of sequence similarity, so that allelic replacement is much less frequent between more distantly related organisms (Thomas and Nielsen, 2005).

Analysis of nodulation genes in faba bean isolates

In the present study, both *nodA*- (SM8) and *nodC*-based phylogenies (SM9) placed the isolates in the α-PB clade. The presence of *nod* genes in isolates belonging to β-PB suggests that they are self-sufficient in fixing nitrogen. In the present work, the isolate R89 identified as *Burkholderia lata* presented both *nodA* and *nodC* genes. According to Kumar *et al*. (2015), strains with closely similar core genomes could have very different *nod* genes, while genetically distant strains could share similar *nod* genes, due to HGT between different genospecies. Furthermore, despite α-and β-rhizobia are evolutionary divergent, their symbiotic genes are highly similar suggesting lateral transfer (Bontemps *et al*., 2010; Chen *et al*., 2003; De Meyer *et al*., 2016; Moulin *et al*., 2001).

MLSA of the isolates

Based on the concatenated tree (SM10) and network analysis (SM11), the isolates from this work clustered into three groups encompassing α and β-proteobacteria. Most of the isolates belonged to α- proteobacteria genus *Rhizobium*. Within this genus, there were two groups which represent the isolates belonging to *R. leguminosarum* group and *Rhizobium* sp. In relation to the isolates identified as β-PB, R88 and R89 clustered, in both concatenated tree and network, in β-PB clade, while the isolate R103 clustered in α-PB clade. The concatenated tree was almost

congruent with the 16S rRNA tree, with the exception of the isolate R103. This isolate clustered as *Burkholderia* sp. in 16S tree, however according to the other genes and the concatenated tree and network, its correct identification is *Rhizobium* sp. This finding shows the great importance of multilocus sequence analysis in the molecular identification of rhizobial bacteria.

4. Conclusion

Phylogenetic analysis showed that the cowpea plants were able to form nodules with different rhizobial species and investigation of their symbiotic performance requires further attention for selection of highly effective strains when developing inoculants.

The 16S rDNA gene sequencing did not provide sufficient resolving power in discriminating closely related species in the studied genera and analysis using other markers, such the 16S-23S intergenic spacer (ITS) and several housekeeping genes, are needed for optimal species-level differentiation.

It is possible to conclude from the phylogenetic trees that strains with closely similar core genomes could have very different *nod* genes, while genetically distant strains could share similar *nod* genes, due horizontal gene transfers (HGT) between different genospecies, as discussed by several authors (Kumar *et al*., 2015).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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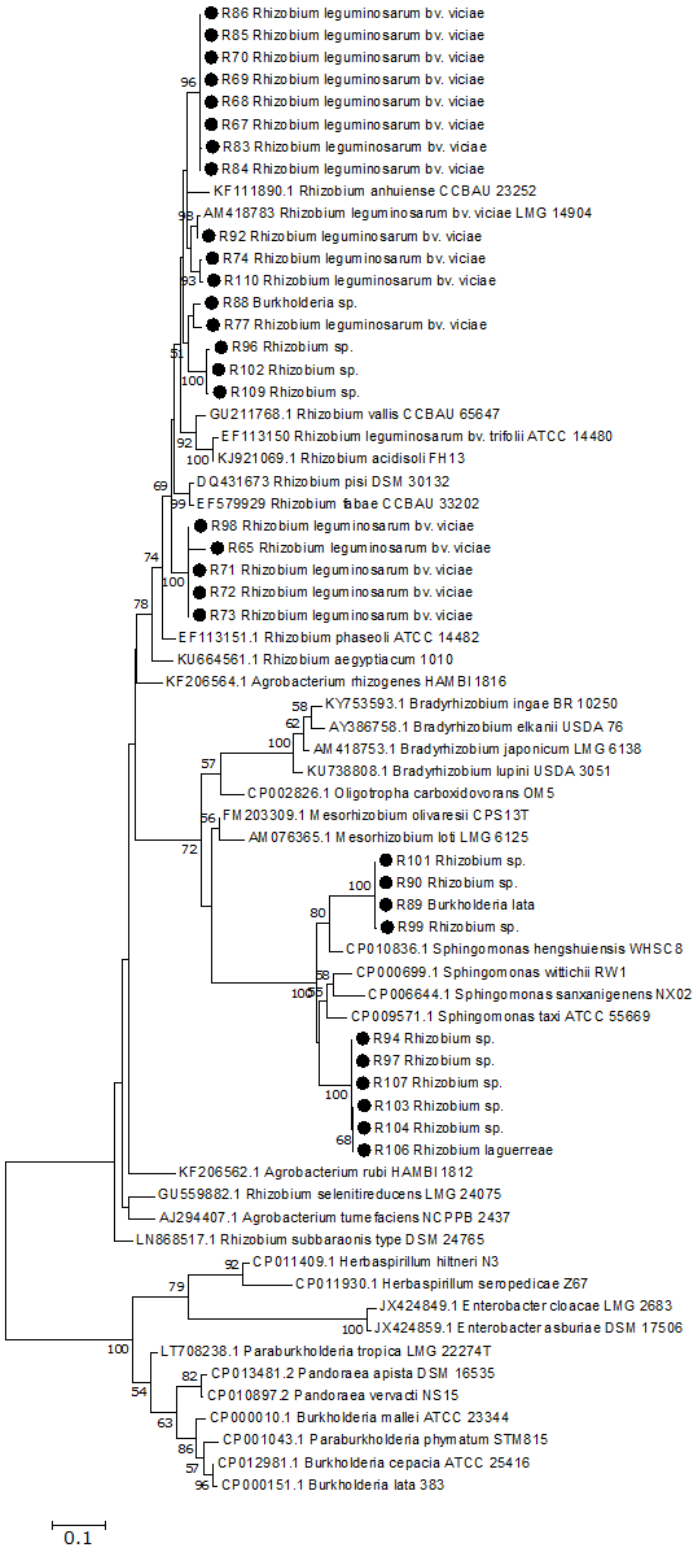
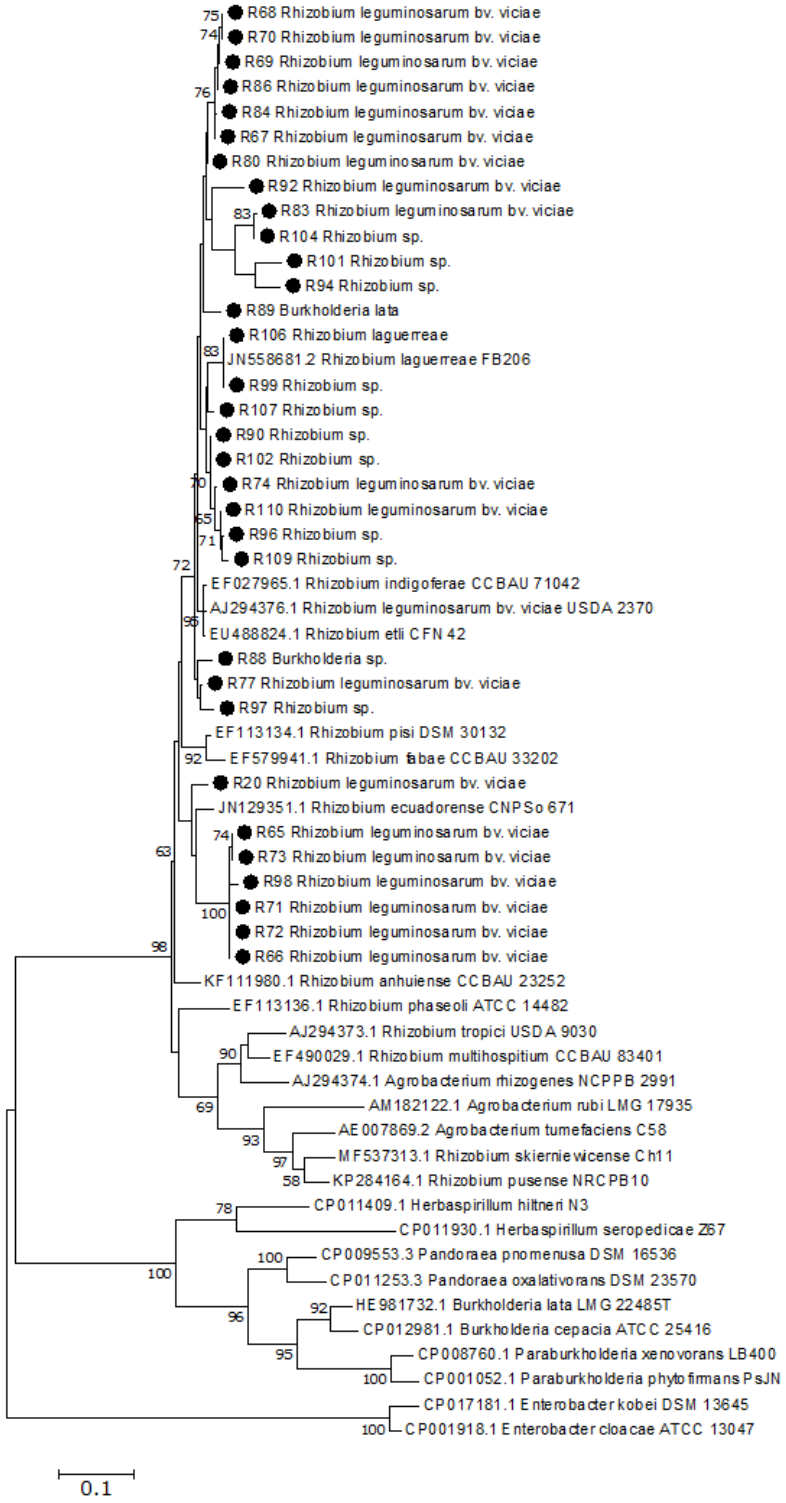
Supplementary material

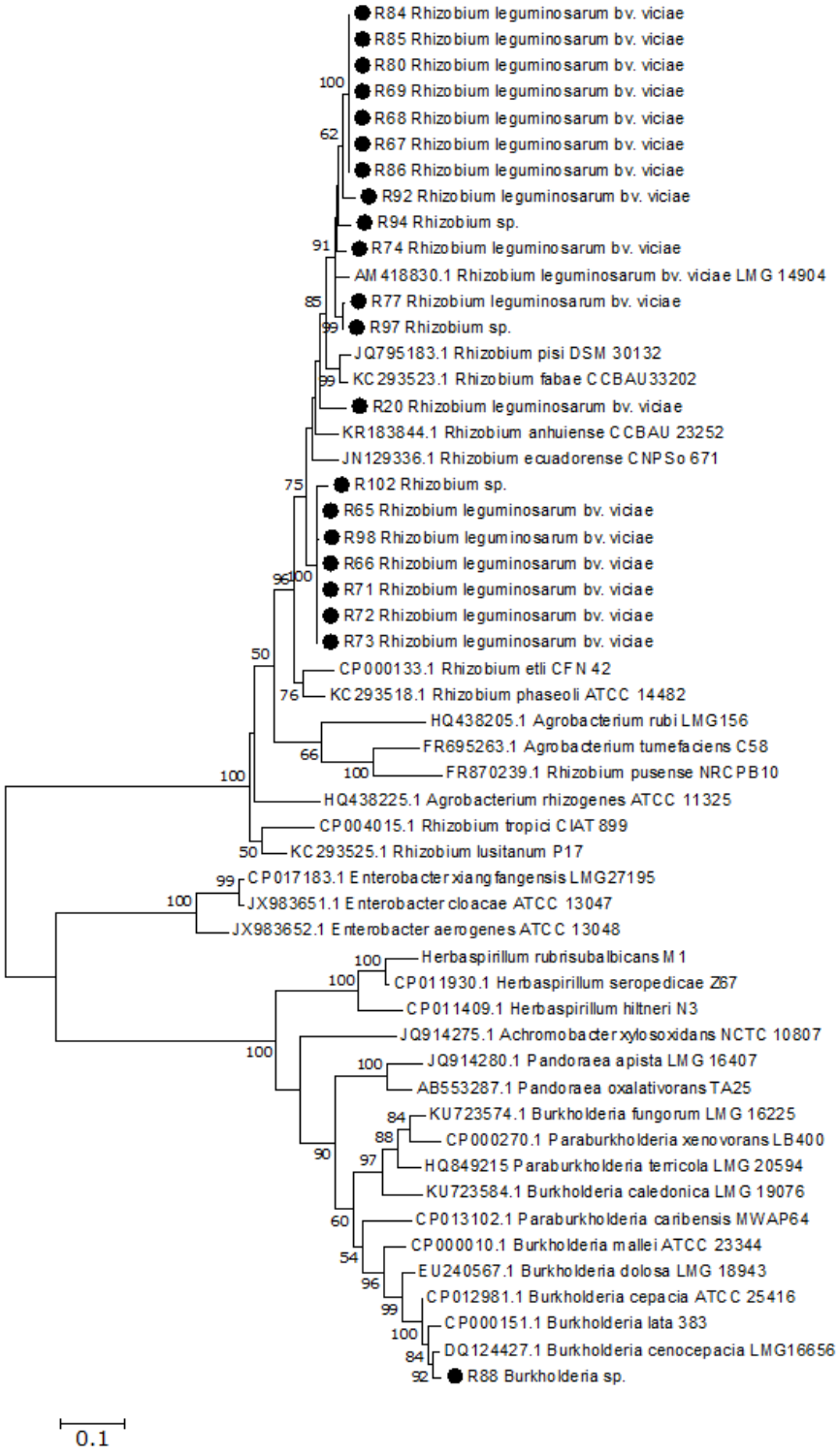
SM 1- Maximum likelihood tree of the 16S rRNA of faba bean rhizobial isolates. Individual tree was made with 875 positions in the final dataset and 82 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



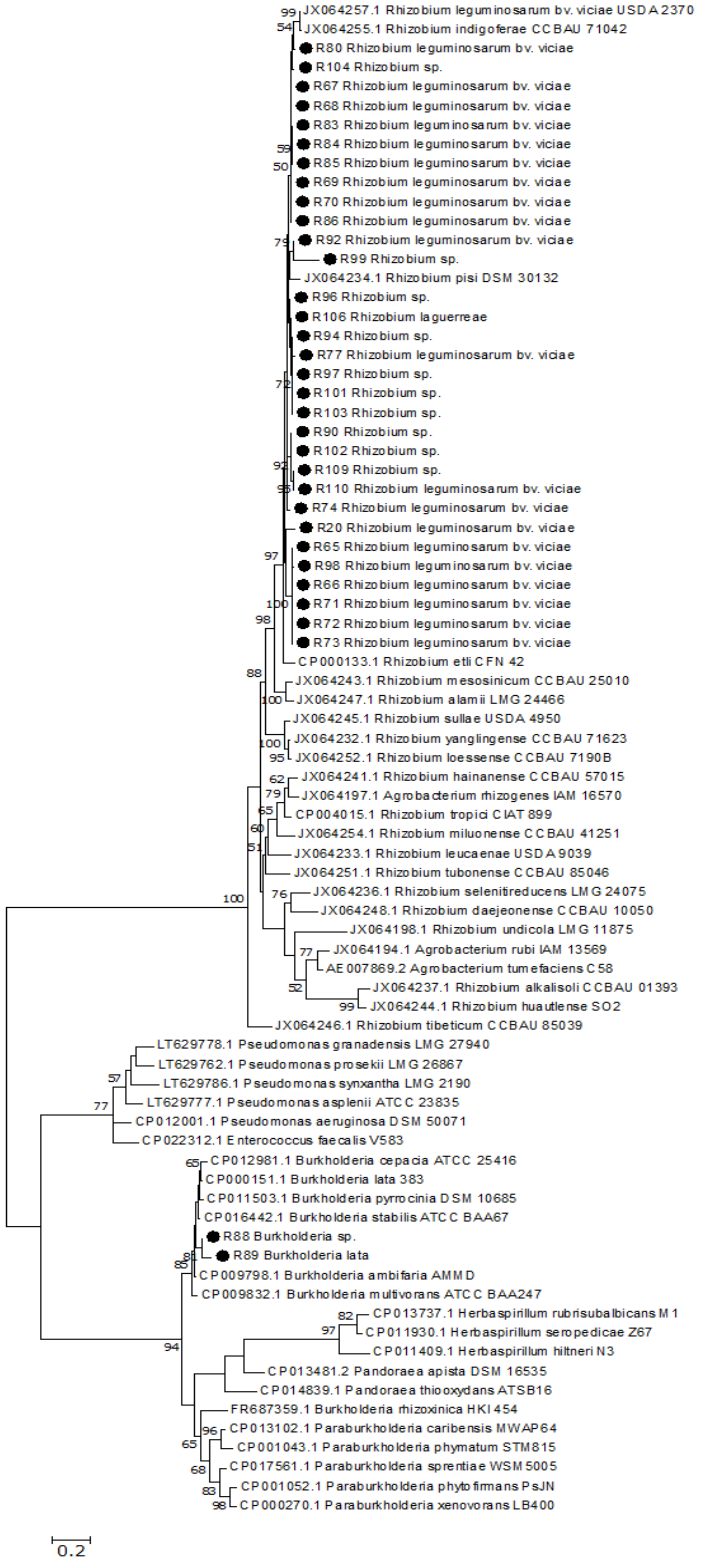
SM 2- Maximum likelihood tree of the *atpD* of faba bean rhizobial isolates. Individual tree was made with 455 positions in the final dataset and 67 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



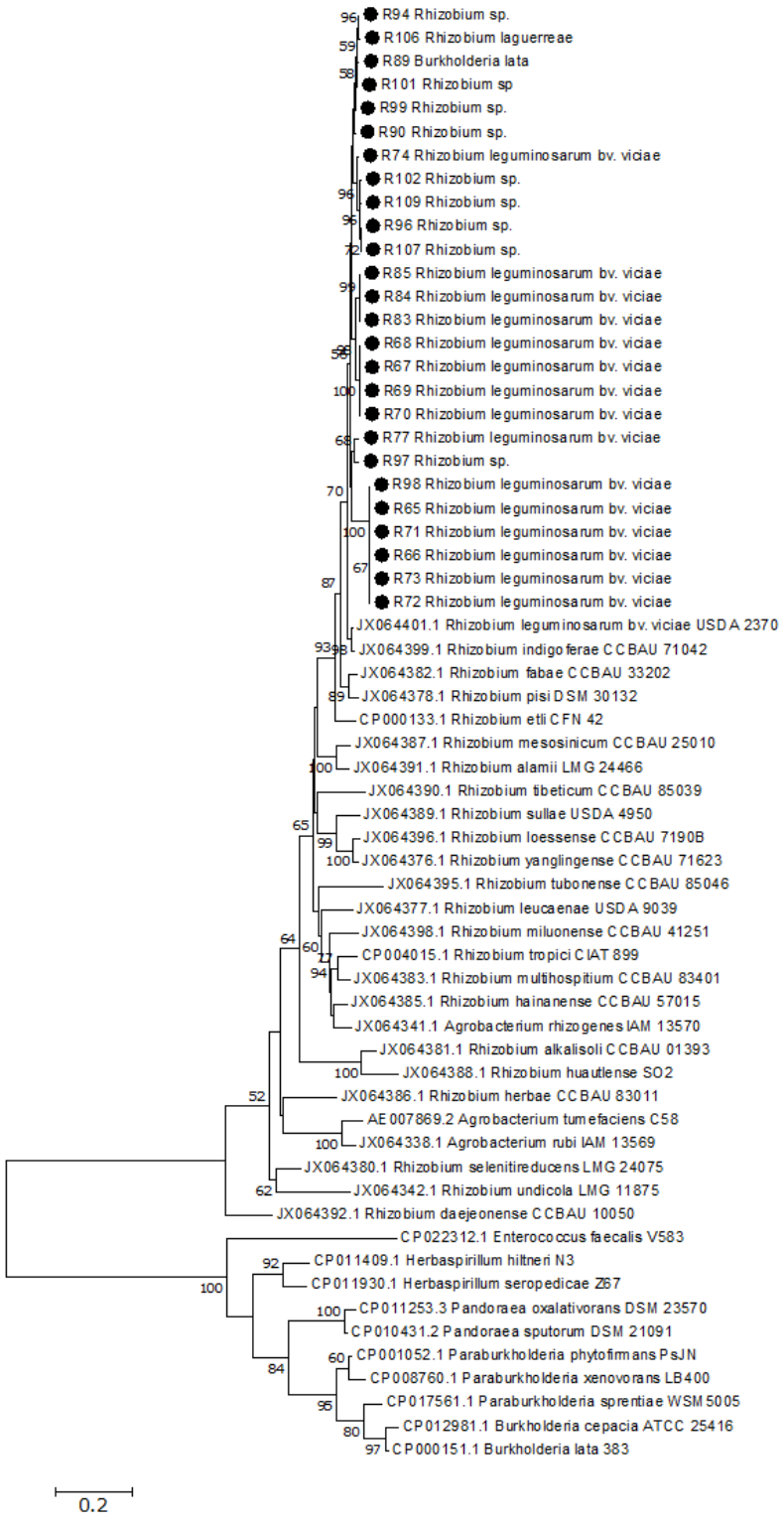
SM 3- Maximum likelihood tree of the *recA* of faba bean rhizobial isolates. Individual tree was made with 505 positions in the final dataset and 58 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



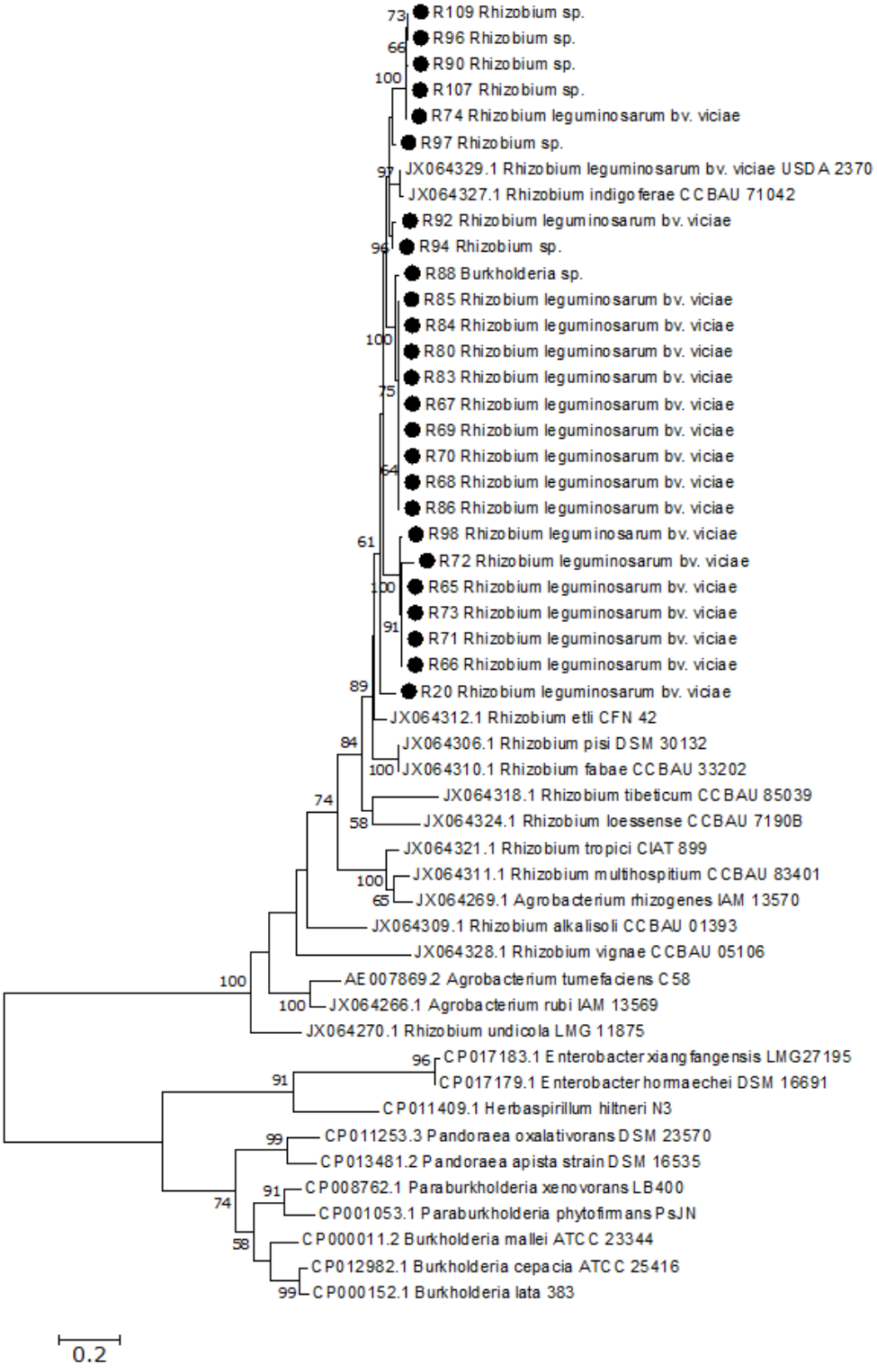
SM 4 Maximum likelihood tree of the *gyrB* of faba bean rhizobial isolates. Individual tree was made with 670 positions in the final dataset and 53 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



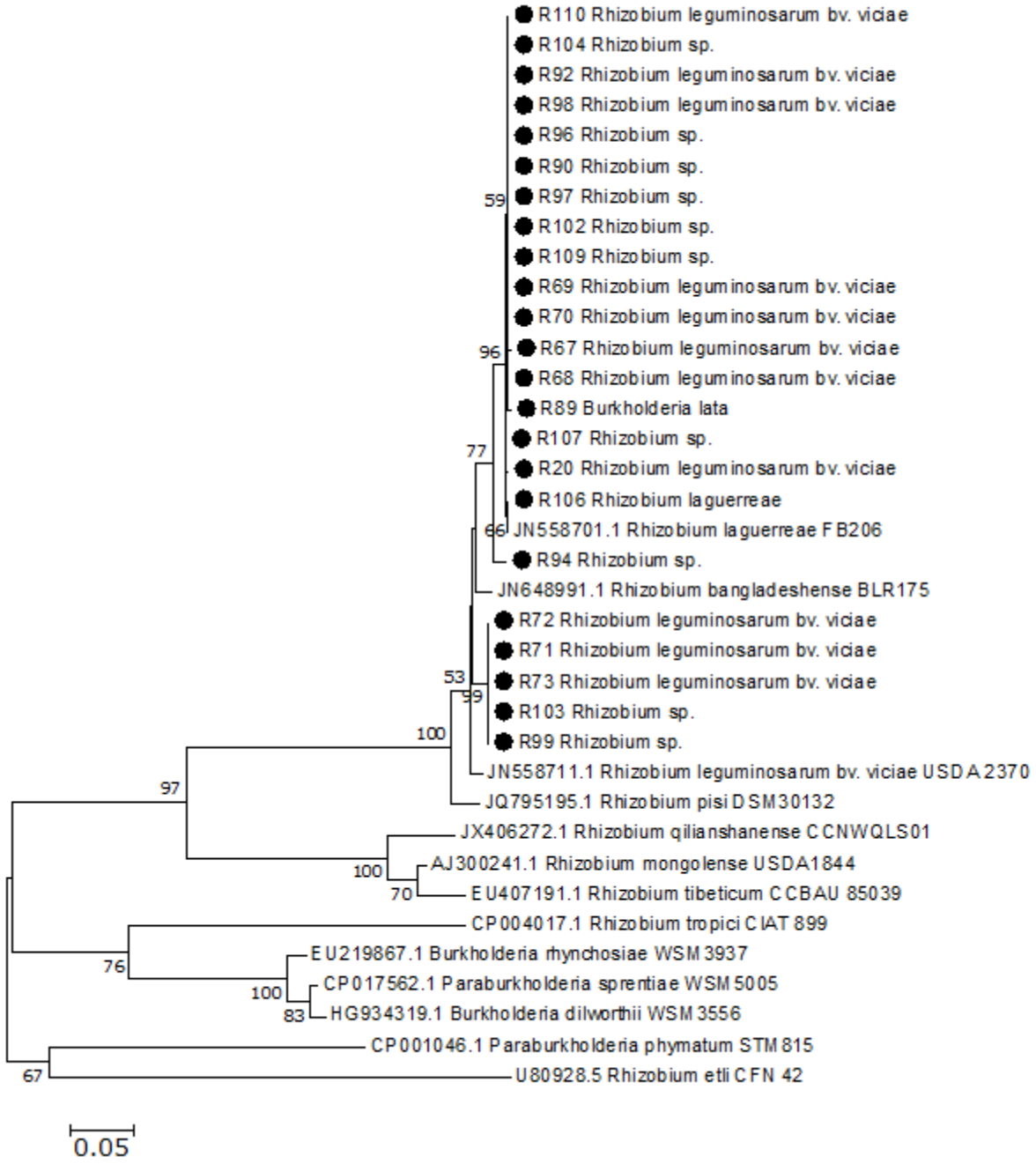
SM 5- Maximum likelihood tree of the *SMc00019* of faba bean rhizobial isolates. Individual tree was made with 400 positions in the final dataset and 79 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



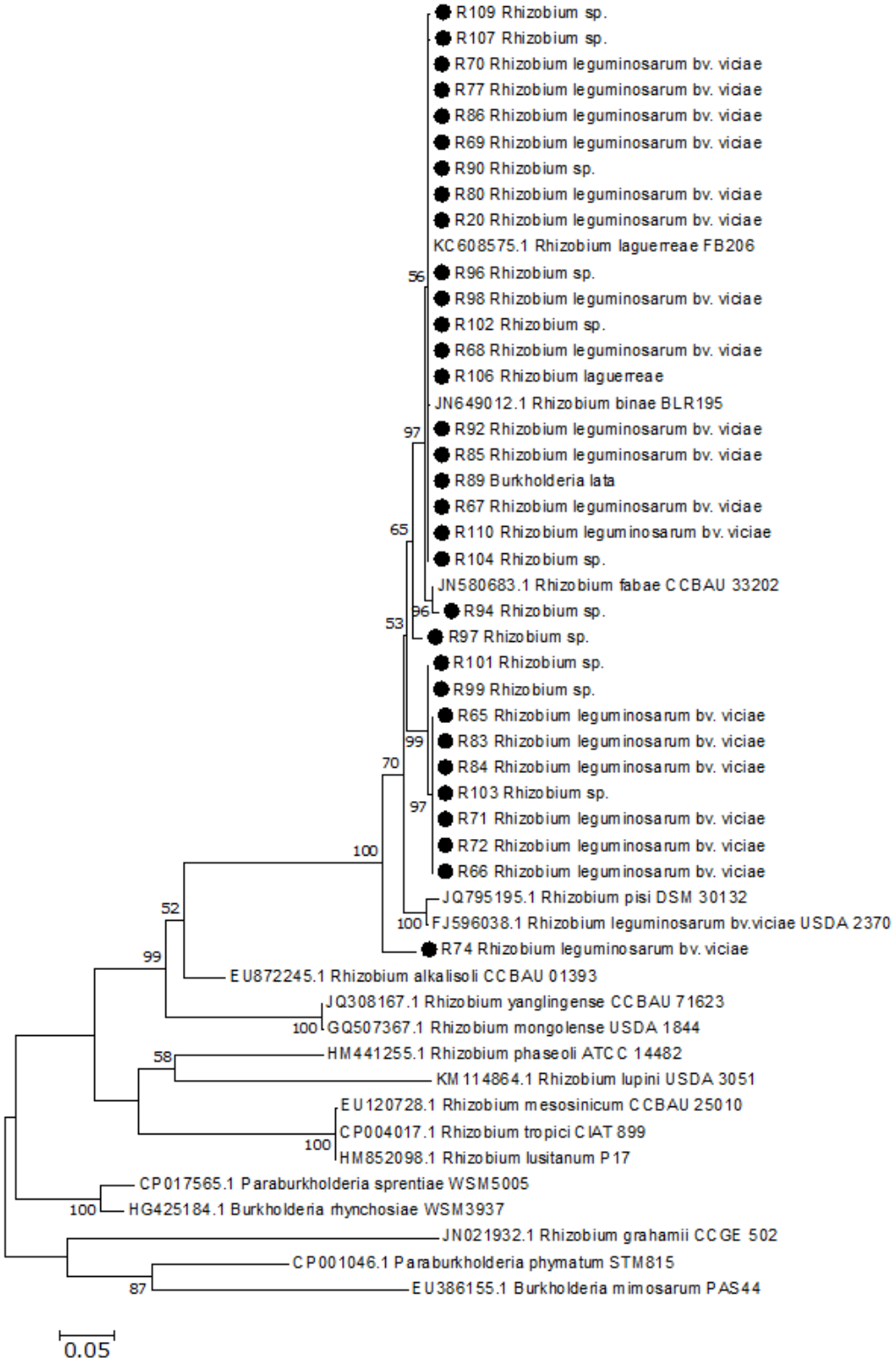
SM 6- Maximum likelihood tree of the *thrA* of faba bean rhizobial isolates. Individual tree was made with 722 positions in the final dataset and 62 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



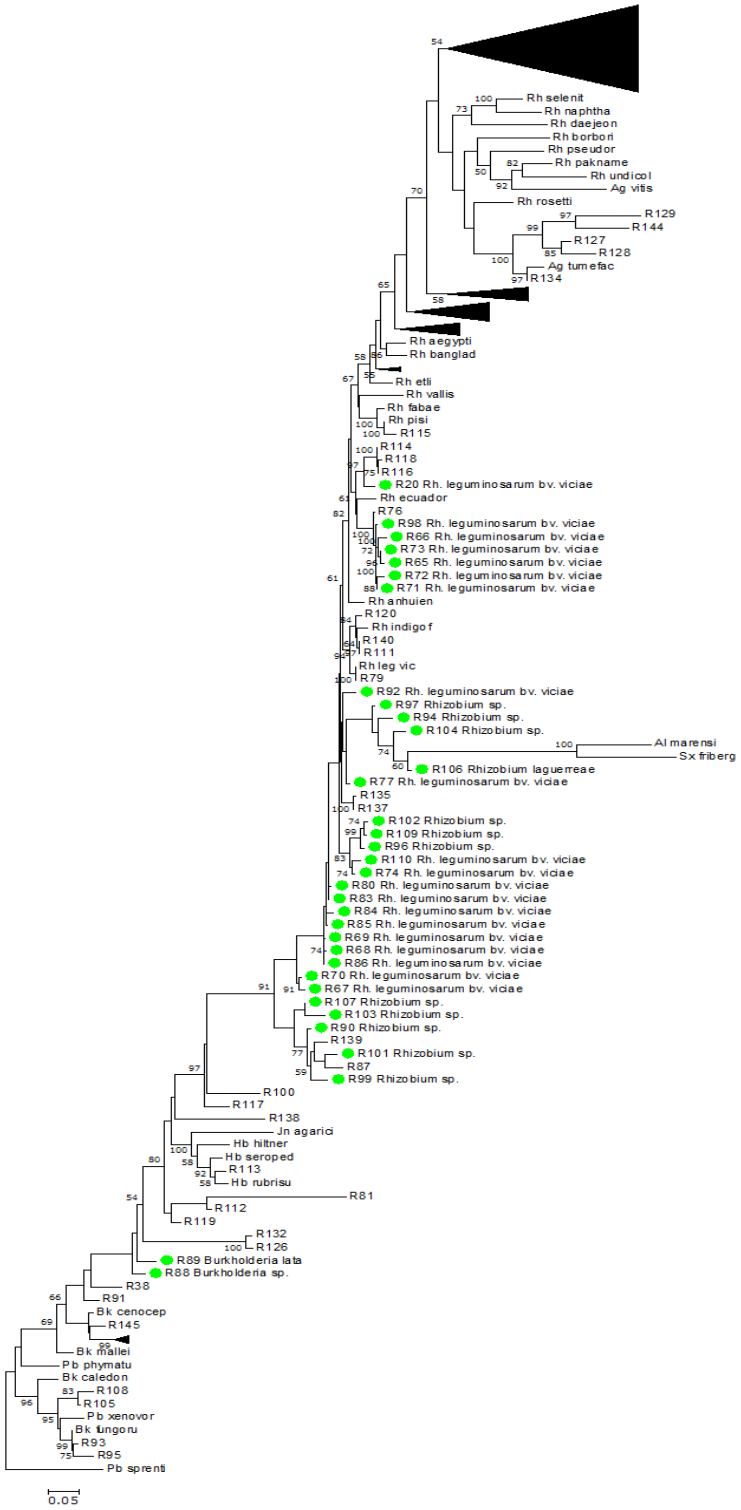
SM 7- Maximum likelihood tree of the *truA* of faba bean rhizobial isolates. Individual tree was made with 392 positions in the final dataset and 50 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



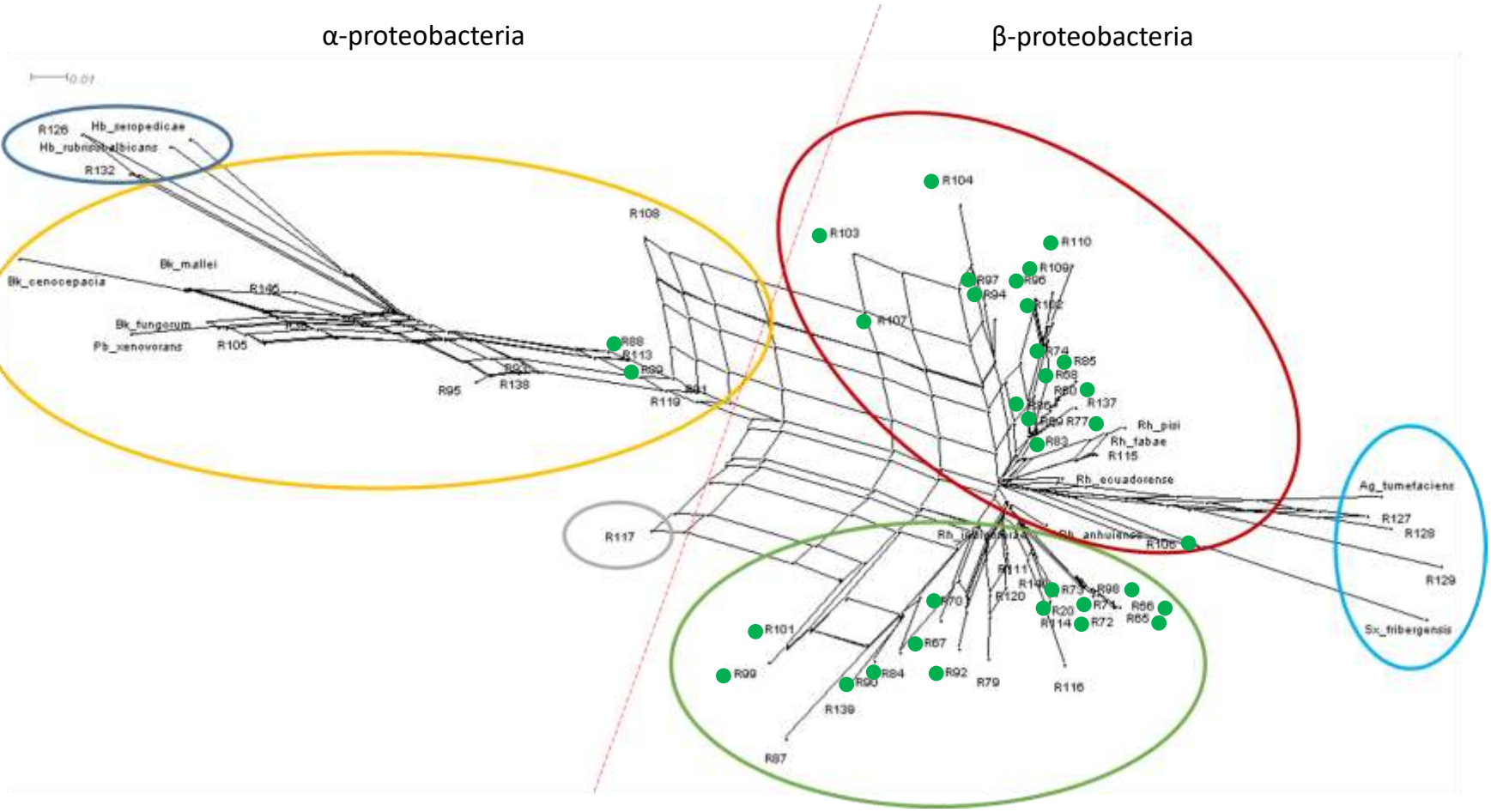
SM 8- Maximum likelihood tree of the *nodA* of faba bean rhizobial isolates. Individual tree was made with 501 positions in the final dataset and 37 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



SM 9- Maximum likelihood tree of the *nodC* of faba bean rhizobial isolates. Individual tree was made with 829 positions in the final dataset and 50 nucleotide sequences. The identification of the isolates was made according to their position in the concatenated tree and network.



SM 10- Concatenated tree based on seven core genes 16S rRNA, *atpD*, *gyrB*, *recA*, *SMc*, *thrA* and *truA* of faba bean rhizobial isolates. The RAxML tree was made using 3617 positions in the final dataset. The bootstrap support values less than 50 were not displayed. Green circles represent the isolates from this work.



SM 11- Concatenated network based on seven core genes 16S rRNA, *atpD*, *gyrB*, *recA*, *SMc*, *thrA* and *truA* of faba bean rhizobial isolates. The network was made using SplitsTree 4.0. The final dataset has 73 nucleotide sequences and 3617 positions. Five main groups were highlighted with different colours: α-proteobacteria, belong to *Rhizobium* clade, namely *Agrobacterium* (light blue), *Rhizobium* sp. (red) and *Rhizobium leguminosarum* group (green) and β-proteobacteria- *Herbaspirillum* (dark blue) and *Burkholderia* (orange). Green circles represent the isolates of this work.

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CHAPTER VI

IMPROVEMENT OF SOME GROWTH AND YIELD PARAMETERS OF FABA BEAN (*VICIA FABA* L.) BY INOCULATION WITH *RHIZOBIUM LAGUERREAE* AND ARBUSCULAR MYCORRHIZAL FUNGI

CHAPTER VI- IMPROVEMENT OF SOME GROWTH AND YIELD PARAMETERS OF FABA BEAN (*VICIA FABA* L.) BY INOCULATION WITH *RHIZOBIUM LAGUERREAE* AND ARBUSCULAR MYCORRHIZAL FUNGI

BRIEFING NOTE

Following, in the previous chapter, the molecular identification of the collected bacteria from faba bean nodules, we pursued the objective of selecting improved rhizobial strains and AMF and evaluating the effects of single and co-inoculation with these selected microorganisms in faba bean plants. Thus, this chapter covers a greenhouse experiment in which is evaluated the influence of the selected bacteria collected from faba bean root nodules, across several regions in Portugal. A single and dual inoculation with the recently described rhizobial bacteria *Rhizobium laguerreae* and a mix of AMF was performed in faba bean plants grown in sterilized soil. Several parameters were evaluated at flowering (growth parameters, microbial performance and leaf gas-exchange measurements) and harvesting (photosynthetic pigments and productivity parameters) stages.

This chapter is an adaptation of a research paper entitled “Improvement of some growth and yield parameters of faba bean (*Vicia faba* L.) by inoculation with *Rhizobium laguerreae* and arbuscular mycorrhizal fungi” published in “Crop and Pasture Science”, 70(7):595-605, in 2019.

The authors contribution to the published paper, which was converted into the present chapter was, as follows: Sandra Pereira and Ângela Mucha were responsible for establishing and maintaining the experiment, collecting data in the greenhouse, and performing the laboratory analyses. Sandra Pereira was also responsible for the data analysis and manuscript writing. Berta Gonçalves and Eunice Bacelar were responsible for the collection and analysis of gas exchange data. Ales Latr was responsible for the arbuscular mycorrhizal fungi used in the present work. Helena Ferreira helped in the photosynthetic pigment determination and Irene Oliveira was the responsible for carrying out the PCA analysis. Eduardo Rosa and Guilhermina Marques were responsible for the design of the experiment and for the critical review of the article. Guilhermina Marques also monitored and helped in all the practical work.

Improvement of some growth and yield parameters of faba bean (*Vicia faba* L.) by inoculation with *Rhizobium laguerreae* and arbuscular mycorrhizal fungi

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Abstract

The use of improved biofertilisers such as rhizobia and arbuscular mycorrhizal fungi (AMF) in legume crops is a promising technology that can be an alternative source of nitrogen and phosphorus. A common problem when growing faba bean (*Vicia faba* L.) and other leguminous plants is the low efficiency of native rhizobial strains. Consequently, there is a need to search for efficient nitrogen-fixing inoculant strains able to increase crop productivity. This study aimed to test the effects of single and dual inoculation with *Rhizobium laguerreae* and AMF on the growth and yield of faba bean plants. Several parameters were evaluated at flowering stage (number of flowers, stems and leaves, shoot and root biomass, leaf area, leaf mass per area and leaf area ratio, and gas-exchange parameters) and at harvesting stage (number and weight of pods and seeds). Plants receiving single inoculation with *Rhizobium laguerreae* showed a significant increase in number of leaves, leaf area, leaf mass per area and leaf area ratio, as well as in all yield parameters. Single inoculation with AMF also significantly increased the yield parameters of faba bean plants. Co-inoculation presented significant improvements in leaf area ratio and in all productivity parameters compared with the control, but co-inoculation was not significantly different from the individual inoculations.

Keywords: broad beans, tripartite symbiosis

1. Introduction

Faba bean (*Vicia faba* L.) is native to the Near East and Mediterranean Basin and is an important winter crop in warm temperate and subtropical areas (Zohary and Hopf, 2000; Jensen *et al.,* 2010), although it can grow in a broad spectrum of soils and temperatures (Köpke and Nemecek, 2010). This leguminous plant is of great economic and agronomic interest owing to the high nutritional value of its seeds, which are rich in protein and starch (Xu *et al.,* 2015). It is considered as multipurpose crop because it can be used for human consumption of its dry and fresh seeds and immature pods, for animal feed, as well as for industrial processing of dry seeds to extract protein and produce flour (Van Berkum *et al.,* 1995; Xu *et al*., 2015).

Despite the great importance of this crop, the total area sown with faba beans has declined to less than half since 1960 (Crépon *et al.,* 2010). In 2016, the total global production of faba bean was 4.5 Mt, with China representing >36% (FAOSTAT, 2018). In Europe, total faba bean production is 0.87 Mt, with the UK (33%), France (23%) and Germany (18%) being the main producers (FAOSTAT, 2018).

During the past 50 years, the widespread use of chemical fertilisers to supply nitrogen (N) and phosphorus (P) has had considerable impact on food quality and security, and has become a major input in crops around the world, with implications for the costs of production and consequent reduction in competitiveness and quality. Moreover, a significant amount of the applied nutrients is lost through different processes (Ladha *et al.,* 1998). In addition, with global warming, heat waves are becoming more frequent and pronounced and there is less annual precipitation and longer periods without rain. Consequently, in Mediterranean countries, crop productivity will decrease and water requirements will increase by ~37% (Carvalho *et al.,* 2014).

Adaptation measures are needed to avoid negative impacts of climate change and chemical fertiliser inputs. Single inoculation and co-inoculation of leguminous plants with rhizobia and arbuscular mycorrhizal fungi (AMF) is a cost-effective and environmentally friendly technology to overcome these problems (Abd-Alla *et al.,* 2014). Indeed, these beneficial microorganisms can increase plant resistance to high temperatures and water deficit, and their application can reduce the need for traditional fertiliser inputs in agriculture (Peoples *et al.,* 1995; Oliveira *et al.,* 2017). Soil microbes are critical for sustainable functioning of natural and managed ecosystems (Sharma *et al.,* 2018a; 2018b).

The symbiosis between faba bean plants and rhizobia provides large quantities of fixed N, up to 200 kg N/ha. year (Hardarson and Atkins, 2003). Moreover, this relationship

can benefit non-leguminous plants, because intercropping of faba bean with other crops increases yield and P-use efficiency (Mei *et al.,* 2012).

Several studies have shown that faba bean plants can establish symbiotic relationships with fast-growing rhizobia previously classified as *Rhizobium leguminosarum* (Van Berkum *et al.,* 1995). This species was divided into three symbiovars, based on host-plant specificity for infection and nodulation (Jordan and Genus, 1984): *viciae* (pea and vetch), *trifolii* (clover), and *phaseoli* (beans) (Laguerre *et al.,* 2001; Rogel *et al.,* 2011). Strains for faba bean were assumed to be classified as *R. leguminosarum* bv. *viciae*, probably because of cross-infection of pea (Van Berkum *et al.,* 1995). However, other symbionts could also nodulate faba bean (Van Berkum *et al.,* 1995; Tian *et al.,* 2007). Saidi *et al.* (2013) showed that several fast-growing rhizobial strains able to nodulate faba bean have 16S rRNA sequences similar to *Rhizobium leguminosarum*; however, their recA and atpD sequences were phylogenetically distant from that species. Therefore, this group of bacteria, distinguishable by its housekeeping genes, was classified as a novel species called *Rhizobium laguerreae* (Saidi *et al.,* 2013).

The association with AMF is a non-specific symbiosis that occurs with >80% of plant species around the world (Jensen, 1942; Smith and Read, 2008). These fungi have beneficial effects on the host plant through improvement of absorption of water and nutrients from the soil, especially immobile P; mycelium from mycorrhizal plant roots can increase root size, allowing the access to a greater volume of soil (Smith and Read, 2008; Nadeem *et al.,* 2014). In a tripartite mutualistic symbiosis, because the nodulation process by rhizobia requires a large amount of P, the association with AMF also helps in the development and function of symbiotic nodules (Ribet and Drevon, 1996). AMF can contribute to improving soil structure (Gianinazzi *et al.,* 2010), the plant's systematic resistance responses against pathogens and abiotic stresses (Pozo *et al.,* 2009; Li *et al.,* 2013; Oyewole *et al.,* 2017; Omomowo *et al.,* 2018), tolerance to salinity and heavy metals (Mohammad *et al.,* 2003), and the assimilation of N-containing organic compounds following the excretion of proteases that break down organic matter (Schimel and Bennett, 2004).

Although rhizobia seem to be as widely distributed as AMF, many soils used for legume cultivation do not have adequate numbers of native rhizobia, or they can be ineffective for enhancing biological N_2 fixation (Denton *et al.,* 2013). Only a few studies have evaluated the effects on faba bean plants of co-inoculation with rhizobia and AMF (Jia *et al.,* 2004; Abd-Alla *et al.,* 2014; Ismaiel *et al.,* 2014; Dubova *et al.,* 2015), and all of them presented beneficial effects on the analysed parameters of faba bean plants by inoculation and co-inoculation. Little is known regarding the impact of inoculation and co-inoculation with

rhizobia and AMF on faba bean leaf gas exchange and morphological characteristics including leaf mass per area (LMA) and leaf area ratio (LAR).

The aim of this work was to investigate the effects of single and dual inoculation with *Rhizobium laguerreae* and a mix of AMF on nodulation, photosynthetic pigments, leaf gas exchange, morphological characteristics, growth and yield of faba bean plants under greenhouse conditions.

2. Material and methods

Bacterial inoculant

The bacterial strain used in this work was isolated from fresh surface-sterilised nodules present in the roots of faba bean plants collected during May 2015 in Fonte do Freixo, Portugal (38°48'45"N, 7°27'35"W). The bacterial isolate, selected from a collection of native rhizobial strains on the basis of its ability to improve the growth of faba bean plants *in vitro*, was identified by multilocus sequence analysis (Youseif *et al.,* 2014) as *Rhizobium laguerreae*. Obtained sequences were deposited in GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers MH628649 and LC413689–LC413694.

For inoculant preparation, the bacteria were grown on yeast mannitol agar media (per L: 1 g yeast extract, 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 15 g agar) supplemented with 0.1 g/L of Bromothymol Blue. After 3–5 days of growing, bacterial inoculant was suspended in sterilised 0.8% NaCl and then transferred to a sterilised mix of peat and vermiculite. Colony-forming units (CFU) were adjusted to 109/g.

Arbuscular mycorrhizal fungi inoculant

The AMF inoculant used in this study was produced by Symbiom (Sázava, Czech Republic) and was a mix of *Rhizophagus irregularis* BEG140, *Funneliformis geosporum* BEG199 and *Claroideoglomus claroideum* BEG210 (1 : 1 : 1) grown for 8 months in pot cultures containing a 1 : 2 (v/v) mixture of clinoptilolite and expanded clay, with red clover (*Trifolium pratense* L.) and maize (*Zea mays* L.) as host plants. The inoculum contained 60 viable spores/g final mycorrhizal blend. Each AM fungus was cultivated separately in a mother pot.

Plant culture, inoculation and experimental conditions

Seeds of faba bean (cv. Favel) were surface-sterilised by washing in 1.5% sodium hypochlorite (NaClO) (v/v) for 2 min and 70% ethanol for 1 min, followed by serial washes

with sterilised distilled water. After germination, three seedlings of similar size were kept in each 5-L plastic pot containing an autoclaved mixture of soil, peat, sand and perlite (3 : 3 : 2 : 2). Chemical analysis of the soil mixture revealed the following values: 6.7% organic matter, pH (1 : 2.5 w/v water) 5.1, 56 mg P_2O_5/kg and 612 mg K_2O/kg.

There were four treatments: non-inoculated plants (control), plants inoculated with *Rhizobium laguerreae*, plants inoculated with a mix of AMF, and plants co-inoculated with both microorganisms. Inoculated treatments received 1 g bacterial and/or 1 g AMF inoculant in each pot. Each pot from non-bacterial treatments received the same amount of autoclaved mix with peat and vermiculite and sterilised 0.8% NaCl, and each pot from non-mycorrhizal treatments received the same amount of AMF inoculum autoclaved twice (121°C, for 30 min) on two consecutive days. Within each treatment, eight replicates (pots) were performed, giving 32 pots in total.

Experiment was conducted in a greenhouse at the University of Trás-os-Montes and Alto Douro during the 2015–16 growing season of *Vicia faba* (November–April), under natural conditions of light, temperature and humidity. The average minimum temperature was 8.1°C and the average maximum temperature 17.6°C. During the growth period, pots were irrigated with tap water as required. Pots of different treatments were occasionally rotated to different places to minimise the effect of location in the greenhouse.

Plant measurements and analyses

Growth parameters and microbial performance

Initial harvest was performed ~4 months after inoculation, using four pots per treatment, with three plants each. Flowers, stems and leaves were separated, and the number and fresh weight of each plant fraction determined. These parameters have been widely used for the assessment of plant yield in several studies, and they allow us to ascertain whether an increase of the number of flowers, leaves and stems translates to a greater number of pods and seeds at harvesting stage. Shoot biomass was calculated by using the dry weight of flowers, stems and leaves. Leaf area was assessed by using a LI-3100 area meter (LI-COR, Lincoln, NE, USA). The dry weight of each fraction was determined after 48 h at 60°C. The root system was gently washed to remove adhered soil, and the existing nodules were counted and weighted. A fresh sample (0.2 g) of roots from each plant was collected for estimation of extent of root colonisation by AMF. These samples were cleared in 2.5% KOH at 80°C for 40 min, followed by rinsing with distilled water. Roots were then immersed in staining solution containing 5% blue ink in vinegar, for 5 min at 80°C (Vierheilig *et al.,* 1998). After washing away the staining solution, roots were de-stained with distilled water containing drops of vinegar and examined under a compound microscope for

quantitative colonisation assessment by the magnified-intersection method according to McGonigle *et al.* (1990). The remaining root system was weighed and its dry weight after 48 h at 60°C was determined in order to calculate root biomass. LMA was determined as the dry weight : leaf area ratio, and LAR as the total leaf area : total dry weight ratio of the entire plant.

Gas exchange

Leaf gas-exchange measurements were performed on four pots per treatment by using a portable LCpro+ infrared gas analyser system (ADC BioScientific, Hoddesdon, UK) with a 2.5-cm^2 leaf chamber, operating in the open mode, on four well-exposed leaves during the morning (09:30–11:00) and the afternoon (14:00–15:30) of 5 and 26 April. The first day of measurements corresponded to the flowering stage and the second to pre-harvesting stage.

Net CO_2 assimilation rate (A), stomatal conductance (*gs*), transpiration rate (E) and intercellular CO_2 concentration (Ci) were estimated from gas-exchange measurements, using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water-use efficiency was calculated as *A/gs*.

Photosynthetic pigments and productivity parameters

Before the final harvest, fully expanded leaves were collected, immediately frozen in liquid nitrogen, and stored at –80°C for determination of the photosynthetic pigments. Leaf discs of area 1.57 cm^2 each were used for extraction of chlorophylls *a* (Chl *a*) and *b* (Chl *b*), and carotenoids. Chl *a* and Chl *b* were extracted in 80% acetone and quantified spectrophotometrically (Sesták, 1971). Total carotenoids were extracted with chlorophylls and determined via the equations of Lichtenthaler (1987).

At harvesting time, the remaining four pots of each treatment were harvested, and productivity parameters, i.e. number and weight of pods and seeds, were evaluated.

Statistical analyses

Normality and homogeneity of variances were confirmed, and data were analysed by one-way analysis of variance (ANOVA). When a significant F-value was obtained ($P < 0.05$), treatment means were compared by Duncan's multiple range test. All statistical analyses were performed with the SPSS 22.0 software package (IBM, Armonk, NY, USA).

A principal component analysis (PCA) using all of the observations was performed to obtain the interrelationships between the variables studied. First dimension scores were evaluated and pairwise comparisons between treatments were performed with Tukey's honest significance tests (Tukey HSD), using the package 'multcomp' and adjusted P-values,

of the R statistical package version 3.3.2 and RStudio 1.0.136 (R Foundation for Statistical Computing, Vienna).

3. Results

Microbial performance

Bacterial nodules were present in plants inoculated and co-inoculated with *Rhizobium laguerreae* and were not detected in either control plants or in plants inoculated only with AMF (Table 1). Comparing the two groups of nodulated plants, the nodule number was significantly higher in plants single-inoculated with the bacteria, showing an increase of 94.3% over co-inoculated plants.

There was no AMF colonisation in control plants, or in those inoculated only with *Rhizobium laguerreae*. AMF colonisation occurred only in plants with single and dual inoculation with fungi. Comparing these two treatments, co-inoculated plants showed a higher AMF colonisation rate than plants those inoculated only with AMF, with an increase of 23.7%.

Table 1- Number of nodules and AMF infection rate (%) of faba bean plants that were uninoculated (control), inoculated with *Rhizobium laguerreae* (Bacteria) or arbuscular mycorrhizal fungi (AMF), and co-inoculated with both microorganisms (Bact+AMF).

	Number of nodules	AMF colonization (%)
Control	0 a	0 a
Bacteria	239 ± 116 c	0 a
AMF	0 a	33.8 ± 4.0 b
Bact + AMF	123 ± 58 b	41.8 ± 6.2 c

Data are expressed as mean±SD and different letters indicate significant differences among treatments (P <0.05), according to Duncan test

Faba bean growth analysis

Although no significant differences were found between treatments in the statistical analysis of total number of flowers per pot, all of the inoculated treatments recorded higher values than the control; in fact, single inoculation with AMF presented an apparent increase of 412.5% over the control, closely followed by single inoculation with *Rhizobium laguerreae* (Fig. 1a). The absence of significant differences among treatments could be due to the variability of the data within each treatment.

No significant differences were observed for number of stems per pot between control plants and those from any inoculation treatment (Fig. 1b). Number of leaves per pot was

significantly higher in plants inoculated with *Rhizobium laguerreae* than in control plants and those inoculated with AMF (increase of 66.1% and 64.7%, respectively) (Fig. 1c).

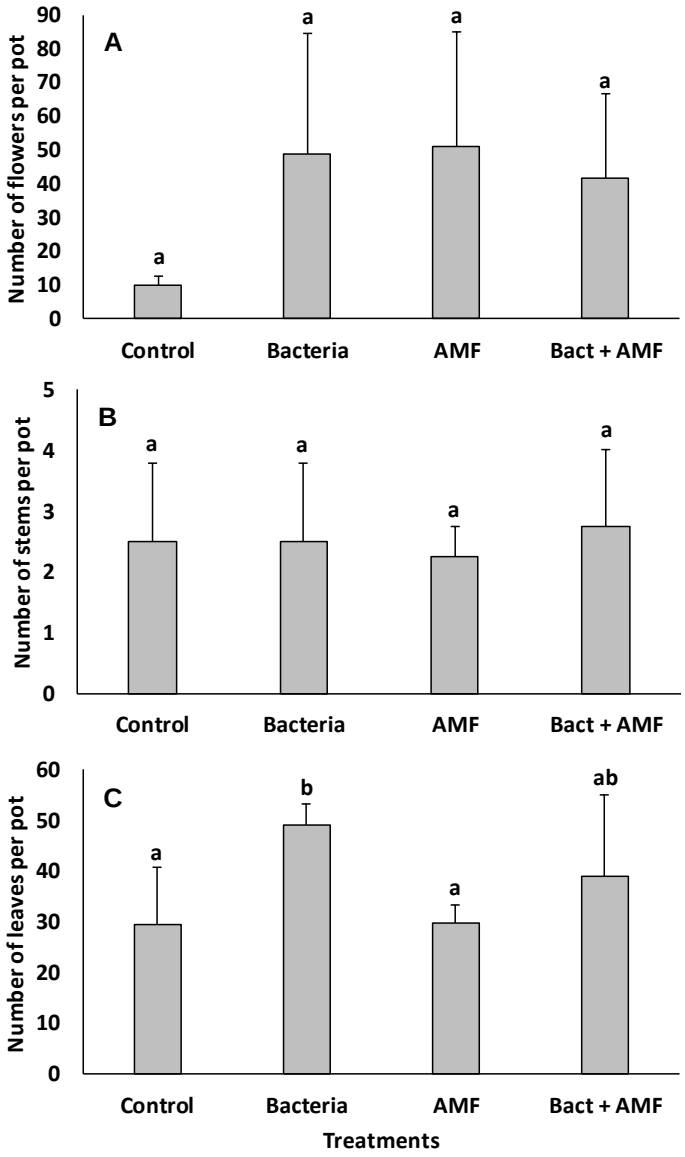

Figure 1. Number of flowers (A), stems (B), and leaves (C) per pot of faba bean plants that were uninoculated (control), inoculated with rhizobial bacterium *Rhizobium laguerreae* (Bacteria), inoculated with a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with *Rhizobium laguerreae* and AMF (Bact+AMF). Capped lines are standard deviations. For each parameter, treatments with the same letter are not significantly different ($P > 0.05$), according to Duncan's test.

Although plants inoculated with *Rhizobium laguerreae* presented the highest shoot and root biomass, no significant differences were observed among treatments (Fig. 2).

Leaf area was positively affected by inoculation and co-inoculation of plants with both microorganisms (Fig. 3a); however, only plants single-inoculated with *Rhizobium laguerreae* had leaf-area values significantly higher than control plants, with an increase of 144.4%.

Plants single-inoculated with *Rhizobium laguerreae* presented LMA values significantly higher than in all the other treatments, with increases of 23.1% over the control plants, 30.9% over plants single-inoculated with AMF, and 16.1% over co-inoculated plants (Fig. 3b). LAR followed the same profile as LMA, and it was positively affected by single inoculation with *Rhizobium laguerreae* compared with the control and inoculation with AMF, with increases of 32.1% and 46.3%, respectively (Fig. 3c). Co-inoculation of plants with *Rhizobium laguerreae* and AMF also significantly increased LAR compared with control and AMF treatments, with increases of 22.8% and 36.0%, respectively.

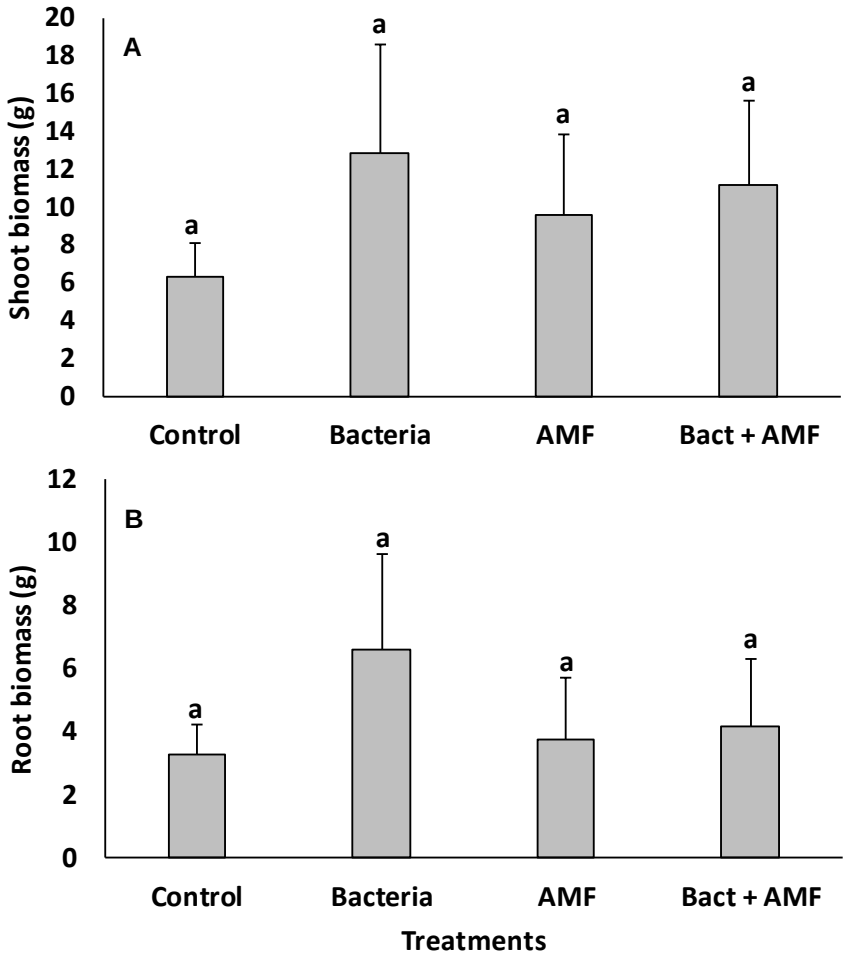

Figure 2. Shoot (A) and root (B) biomass per pot of faba bean plants that were uninoculated (control), inoculated with rhizobial bacterium *Rhizobium laguerreae* (Bacteria), inoculated with a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with *Rhizobium laguerreae* and AMF (Bact+AMF). Capped lines are standard deviations. For each parameter, treatments with the same letter are not significantly different ($P > 0.05$), according to Duncan's test.

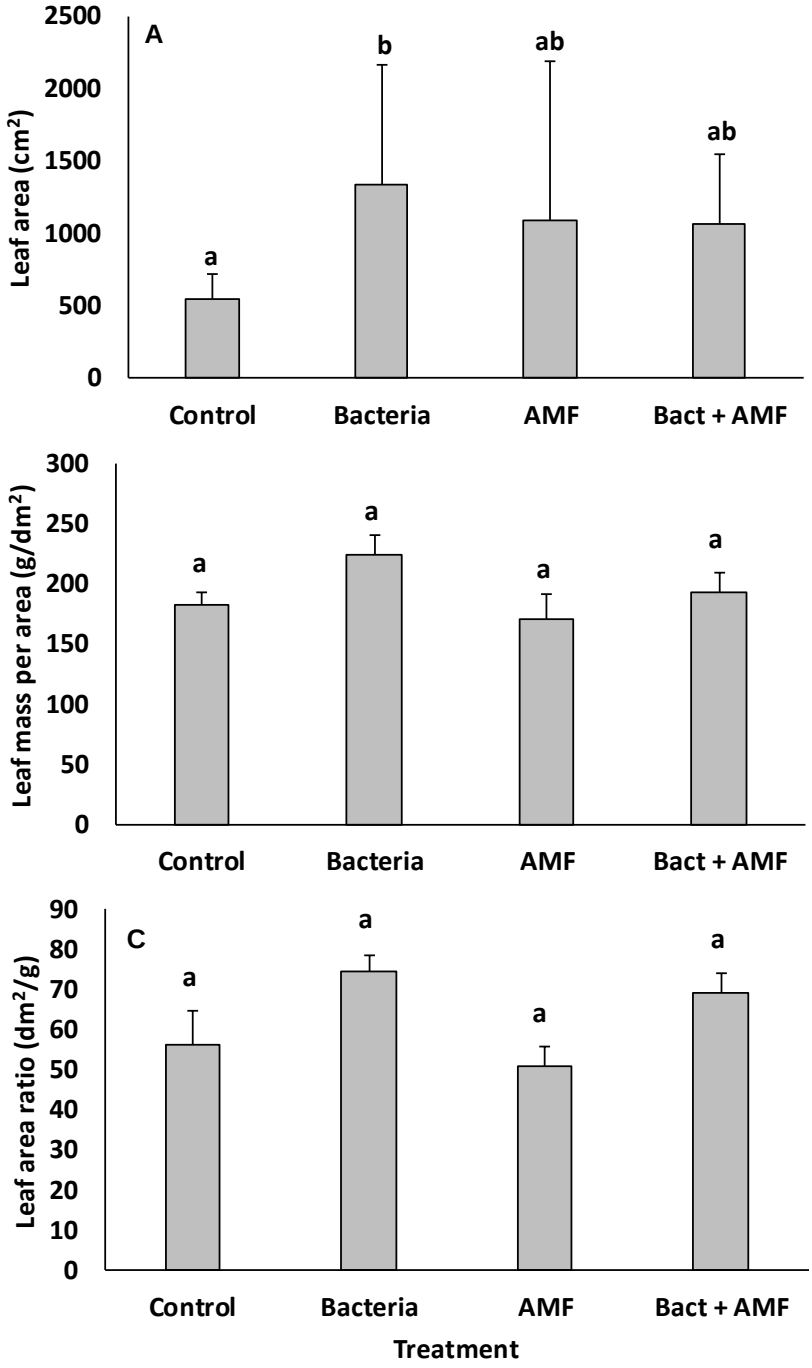

Figure 3. Leaf area (A), leaf mass per area (B), and leaf area ratio (C) per pot of faba bean plants that were uninoculated (control), inoculated with rhizobial bacterium *Rhizobium laguerreae* (Bacteria), inoculated with a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with *Rhizobium laguerreae* and AMF (Bact+AMF). Capped lines are standard deviations. For each parameter, treatments with the same letter are not significantly different ($P > 0.05$), according to Duncan's test.

No significant differences were observed for photosynthetic pigments compared with control plants (Table 2); however, plants single-inoculated with *Rhizobium laguerreae* presented significantly higher contents of Chl *a*, Chl *b*, Chl a+*b* and carotenoids than plants single-inoculated with fungi.

Table 2. Concentrations (mg/dm^2) of photosynthetic pigments of faba bean plants that were uninoculated (control), inoculated with *Rhizobium laguerreae* (Bacteria) or arbuscular mycorrhizal fungi (AMF), and co-inoculated with both microorganisms (Bact+AMF).

	Chl_a (mg/dm^2)	Chl_b (mg/dm^2)	$Chl_{(a+b)}$ (mg/dm^2)	Car (mg/dm^2)
Control	2.76±0.42 ab	1.09±0.14 ab	3.85±0.52 ab	0.76±0.17 ab
Bacteria	2.99±0.28 b	1.20±0.13 b	4.19±0.41 b	0.88±0.06 b
AMF	2.27±0.52 a	0.88±0.18 a	3.15±0.69 a	0.62±0.10 a
Bact + AMF	2.76±0.23 ab	1.05±0.09 ab	3.81±0.31 ab	0.79±0.16 ab

Data are expressed as mean±SD and different letters indicate significant differences among treatments (P <0.05), according to Duncan test

Gas exchange

At flowering stage, in the afternoon, no differences were observed for net CO_2 assimilation rate (*A*); however, in the morning, *A* was significantly smaller in faba bean plants inoculated with *Rhizobium laguerreae* (decreases of 106.6%, 131.6% and 116.8% relative to control, AMF and co-inoculated plants, respectively) (Fig. 4a).

At flowering stage, morning transpiration rate (*E*) was significantly increased in plants inoculated with AMF or co-inoculated compared with control plants, with increases of 64.7% and 36.2%, respectively (Fig. 4b). At pre-harvesting stage in the morning, co-inoculated plants presented the highest *E*, especially relative to the control, with an increase of 69.1%. At pre-harvesting stage in the afternoon, plants inoculated with AMF presented smaller *E* than those in other treatments, with decreases of 112.1% relative to control plants, 81.3% relative to plants inoculated with *Rhizobium laguerreae* and 69.2% relative to co-inoculated plants. At flowering stage, *E* increased in the afternoon relative to the morning, whereas at pre-harvesting stage, the trend was reversed.

No significant differences were observed for intrinsic water-use efficiency (*A*/*gs*) in the measurements at pre-harvesting stage (Fig. 4d). However, at flowering stage, plants inoculated with AMF and co-inoculated with both microorganisms presented the highest values.

At flowering stage, morning measurements of intracellular CO_2 concentration (*Ci*) were significantly higher in control plants and those inoculated with *Rhizobium laguerreae* than in co-inoculated plants, with increases of 43.7% and 26.7%, respectively (Fig. 4e). At

flowering stage in the afternoon, only inoculation with *Rhizobium laguerreae* induced a significant increase in the *Ci* compared with inoculation with AMF and co-inoculation with *Rhizobium laguerreae* and AMF, with increases of 18.0% and 14.4%, respectively. At pre-harvesting stage, *Ci* was similar in all analysed treatments, and consequently, no significant differences were observed.

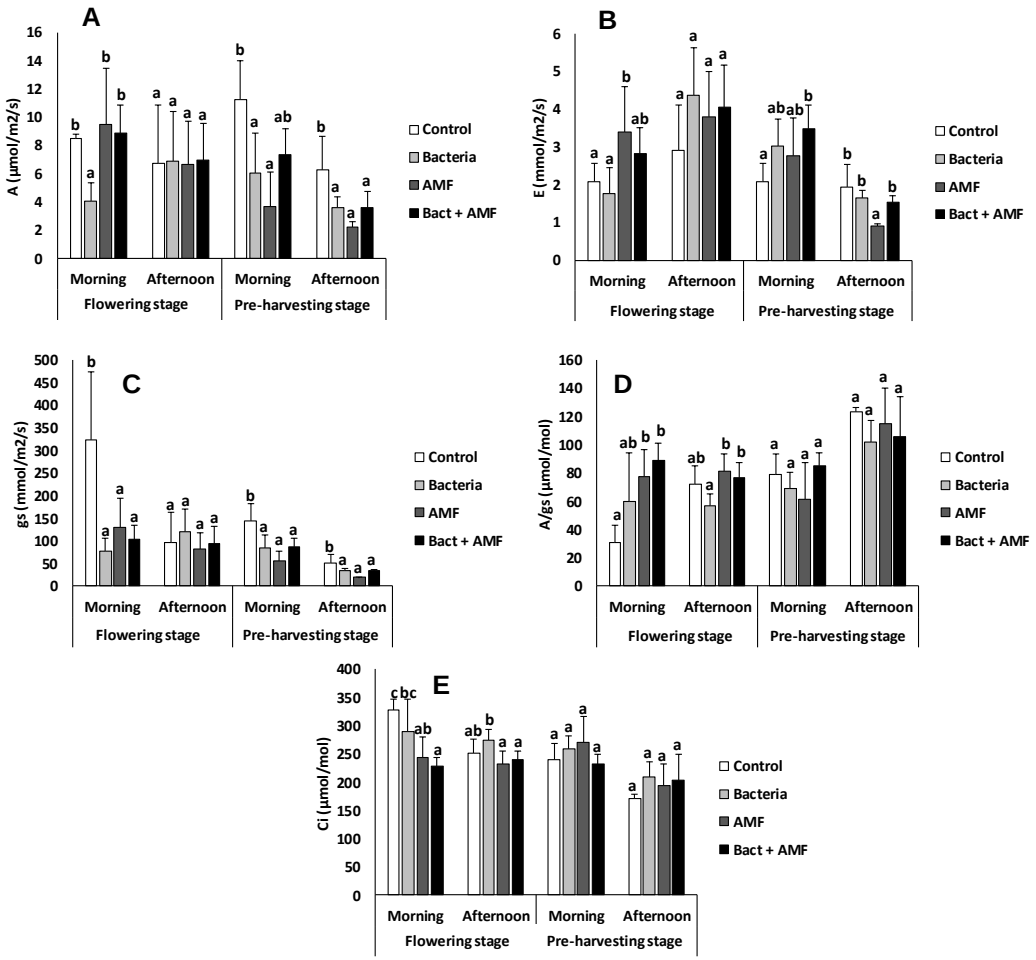

Figure 4. Net CO_2 assimilation rate (A), transpiration rate (B), stomatal conductance (C), intrinsic water-use efficiency (D) and intracellular CO_2 concentration (E) of faba bean plants that were uninoculated (control), inoculated with rhizobial bacterium *Rhizobium laguerreae* (Bacteria), inoculated with a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with *Rhizobium laguerreae* and AMF (Bact+AMF). Capped lines are standard deviations. For each parameter, treatments with the same letter are not significantly different ($P > 0.05$), according to Duncan's test.

Faba bean grain yield parameters

Single and dual inoculation with *Rhizobium laguerreae* and/or AMF positively affected all of the analysed productivity parameters. Regarding the number of pods per pot, plants

inoculated with *Rhizobium laguerreae* or AMF and those co-inoculated with both presented significantly higher values than control plants (increases of 144.4%, 144.4% and 150.0%, respectively) (Fig. 5a). Weight of pods per pot followed the same trend (Fig. 5b); indeed, single and dual inoculation with *Rhizobium laguerreae* and AMF significantly increased the weight of pods by 339.2%, 283.8% and 377.2%, respectively compared with the control.

Number of seeds per pot was positively affected by single or combined inoculation with *Rhizobium laguerreae*, with increases of 152.1% and 145.8%, respectively, compared with the control (Fig. 5c). Likewise, regarding weight of seeds per pot, inoculation with *Rhizobium laguerreae* and co-inoculation resulted in significantly higher values than the control (increases of 321.8% and 265.5%, respectively) (Fig. 5d). Although the number of seeds per pot with AMF inoculation was not significantly different from any other treatment, it tended to be greater than the control, and the weight of seeds per pot was significantly higher than in the control, with an increase of 242.7%.

Multivariate analysis

In the PCA, the first component (PC1) explained 53.4% of the variance, whereas PC2 accounted for only 21.7% (Fig. 6). Together the two components explained >75% of the variance. There was a separation of treatments along both PC axes. PC1 discriminated between control and plants inoculated with *Rhizobium laguerreae*, whereas PC2 allowed discrimination between these two treatments and the remaining two (inoculation with AMF and co-inoculation with *Rhizobium laguerreae* and AMF). Indeed, the control samples presented negative values for both PC1 and PC2. However, plants single-inoculated with *Rhizobium laguerreae* presented negative values in PC2 and positive values in PC1, being more influenced by LAR, LMA, number of leaves, stems and nodules, and shoot and root biomass, which had a negative effect. Plants inoculated with *Rhizobium laguerreae* and co-inoculated with both microorganisms presented positive values in PC2 and were more influenced by mycorrhization rate and number and weight of pods and seeds, which had a positive effect. Pairwise comparisons between treatments showed that PC1 presented significant differences between control plants and those inoculated with *Rhizobium laguerreae* ($P = 0.014$) and between control and co-inoculated plants ($P = 0.045$). Regarding PC2, highly significant differences were observed between plants single-inoculated with AMF and control plants ($P = 0.007$) and plants single-inoculated with *Rhizobium laguerreae* ($P = 0.005$). Significant differences were also observed between plants single-inoculated with *Rhizobium laguerreae* and co-inoculated plants ($P = 0.031$).

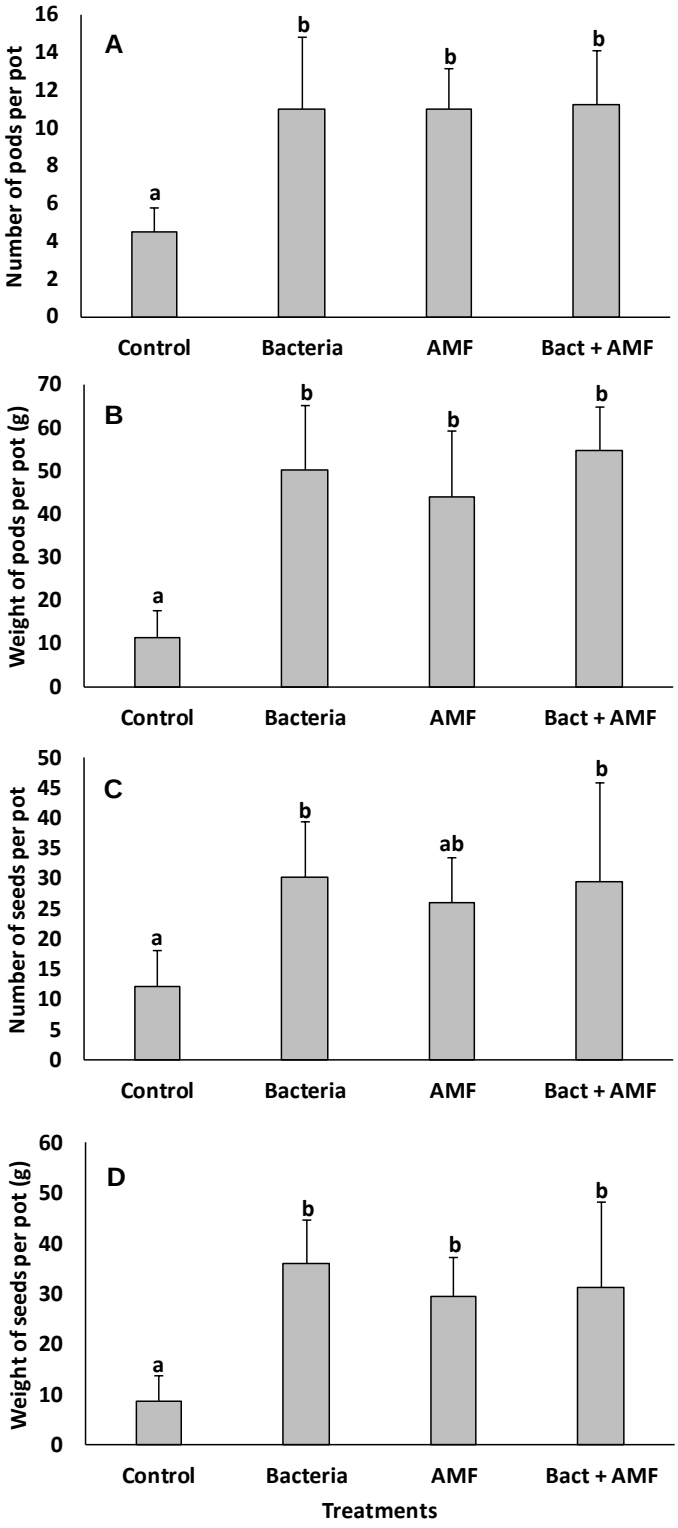

Figure 5. Number (A) and weight (B) of pods per pot, and number (C) and weight (D) of seeds per pot of faba bean plants that were uninoculated (control), inoculated with rhizobial bacterium *Rhizobium laguerreae* (Bacteria), inoculated with a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with *Rhizobium laguerreae* and AMF (Bact+AMF). Capped lines are standard deviations.

For each parameter, treatments with the same letter are not significantly different ($P > 0.05$), according to Duncan's test.

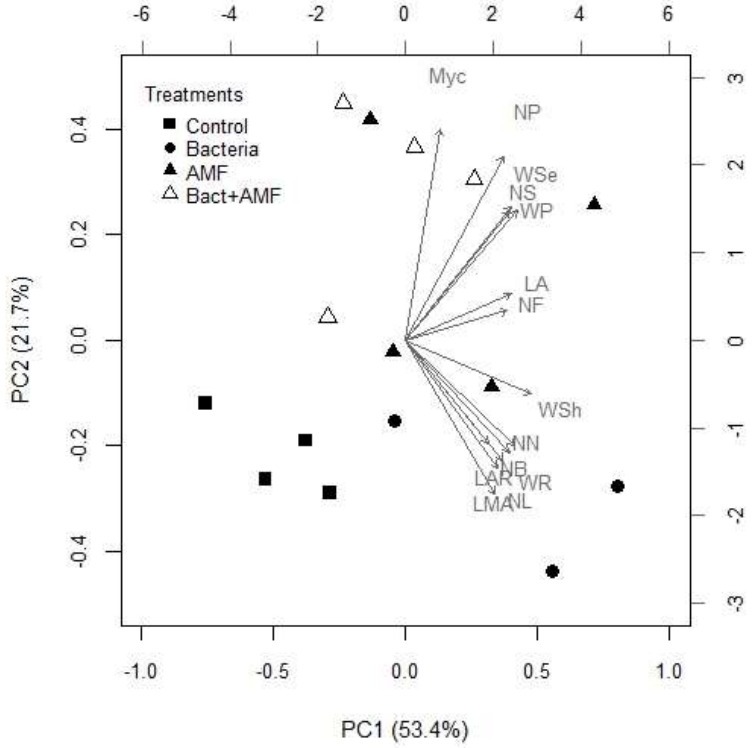

Figure 6. Principal component analysis using the whole dataset of faba bean plants that were uninoculated (control), inoculated with rhizobial bacterium *Rhizobium laguerreae* (Bacteria), inoculated with a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with *Rhizobium laguerreae* and AMF (Bact+AMF). Analysed parameters were: Myc, mycorrhization rate; NP, number of pods; WSe, weight of seeds; NS, number of seeds; WP, weight of pods; LA, leaf area; NF, number of flowers, WSh, weight of shoot, NN, number of nodules, NB, number of branches; WR, weight of root; NL, number of leaves; LAR, leaf area ratio; LMA, leaf mass per area.

4. Discussion

Effective symbiosis between legume plants and rhizobia and/or AMF is characterised by the number and weight of nodules and/or the mycorrhizal colonisation rate on host-plant roots (Dubova *et al.,* 2015). The increase in number of nodules observed in plants single-inoculated with *Rhizobium laguerreae* compared with co-inoculated plants agrees with previous studies with faba bean, which support that co-inoculation has a negative effect on rhizobial bacteria, nodulation being higher with bacteria inoculated independently, and nodulation decreasing with co-inoculation (Jia *et al.,* 2004; Ismaiel *et al.,* 2014). Bethlenfalvay *et al.* (1982) also reported an inhibition of nodule formation in co-inoculated *Phaseolus vulgaris* L., indicating that the factors causing inhibition in host plant and bacterial

endophyte are the same, but that they affect the micro-symbiont more severely. By contrast, Abd-Alla *et al.* (2014) found that the number of nodules was higher in mycorrhizal than in non-mycorrhizal faba bean plants grown under alkalinity stress, owing to the synergistic effect between the two microorganisms. Furthermore, Scheublin and van der Heijden (2006) suggested that it is not yet clear whether the presence of AMF influences nodule functioning. On the other hand, dual inoculation with *Rhizobium laguerreae* and a mixture of AMF significantly increased mycorrhizal colonisation compared with single inoculation with AMF. Xie *et al.* (1995) attributed this stimulatory effect to Nod factors, the specific signal molecules of rhizobia that trigger the colonisation and development of AMF via the so-called 'increased nod genes induction response'.

Regarding shoot and root biomass, no significant differences were observed among treatments despite higher values observed in inoculated plants, especially those inoculated with *Rhizobium laguerreae*. In previous studies performed in faba bean, shoot and root biomass was increased by single inoculation with rhizobia or AMF (Dubova *et al.,* 2015; Youseif *et al.,* 2017) or by all inoculated and co-inoculated treatments (Abd-Alla *et al.,* 2014). Other studies of different crop plants (pepper (*Capsicum annuum* L.) and maize) also showed that AMF association increased the fitness of the host plant by enhancing its biomass (Kaya *et al.,* 2009; Sheng *et al.,* 2009; Abiala *et al.,* 2013). In the present study, although inoculation did not lead to an increase in plant biomass, it contributed to improved grain yield.

In most previous studies of co-inoculation with rhizobia and AMF in faba bean, the numbers of flowers, stems and leaves were not evaluated. However, these are important parameters that should be analysed to provide a complete evaluation of the plant status. In the work of Youseif *et al.* (2017), inoculation of faba bean with different rhizobial strains did not influence the number of stems. However, in work developed by Ravikumar (2012), greater numbers of leaves were observed in black gram (*Vigna mungo* (L.) Hepper) and mung bean (*Vigna radiata* (L.) R.Wilczek) inoculated with *Rhizobium* than in the respective controls. In the present study, an increase in the number of leaves and in leaf area was observed in plants inoculated with *Rhizobium laguerreae*. The increase in leaf area leads to an increase in whole-plant photosynthesis, and consequently to an increase in net assimilated C available to growth, allowing an improvement in overall plant growth. According to Bacelar *et al.* (2004), large leaves of inoculated plants transpire more water and may be susceptible to desiccation, especially because these large leaves were associated with high vegetative growth.

Leaf mass per area and LAR are fundamental leaf traits for ecosystem functioning, related to important processes such as carbon gain or litter decomposability. LMA is a

morphological trait widely used in plant ecology, agronomy, forestry and plant physiology as a good indicator of plant functions including photosynthetic and respiratory rates, chemical composition and resistance to herbivory, among others (Poorter *et al.,* 2009; Lopez-Iglesias *et al.,* 2014; Reich, 2014). Changes in LMA can be caused by variations in internal anatomy and leaf-tissue density and are not simply a consequence of changes in leaf thickness (Witkowski and Lamont, 1991). On the other hand, LAR is defined as a measure of photosynthetic machinery per unit of plant biomass (Amanullah *et al.,* 2007). In the present work, inoculation with *Rhizobium laguerreae* increased the LMA, meaning that plants inoculated with these bacteria presented higher density and/or high thickness of foliar tissue. LAR was positively affected by single and dual-inoculation with bacteria.

Faba bean plants inoculated with *Rhizobium laguerreae* presented the highest values of Chl *a*, Chl *b*, Chl *a*+*b* and carotenoids. This increase in chlorophyll content in inoculated plants occurs to meet carbon requirements from their host plants (Sivaprasad and Rai, 1987; Lalitha and Santhaguru, 2012), and can be due to an increase in stomatal conductance, photosynthesis, transpiration and enhanced plant growth (Rajasekaran *et al.,* 2006). The lower levels of photosynthetic pigments observed in the other treatments may indicate lower leaf N content, because the majority of leaf N is contained in chlorophyll molecules (Netto *et al.,* 2005). In the work of Ismaiel *et al.* (2014), single and dual inoculation of faba bean with rhizobia and AMF increased the photosynthetic capacity by increasing Chl *a* and Chl *b* content. Similar results were observed in other leguminous plants such as cowpea (*Vigna unguiculata* (L.) Walp.) and chickpea (*Cicer arietinum* L.) (Oliveira *et al.,* 2005; Bejandi *et al.,* 2011). In general, at flowering stage of faba bean plants, *Rhizobium laguerreae* alone or co-inoculated with AMF promoted plant growth, and this may be due to the higher accumulation of N per plants (Rodelas *et al.,* 1999).

Gas-exchange measurements were performed in morning and afternoon at two plant-developmental stages. The second stage (pre-harvesting) was hotter (average temperature 34.5°C) and with higher light intensity (*photosynthetic photon flux* density, PPFD, 1393 µmol/m2.s) than the first stage (flowering: average temperature 31.3°C and PPFD 1111 µmol/m2.s). At pre-harvesting stage, with increased light intensity and higher temperatures, control plants showed higher net CO_2 assimilation rate (*A*) in both periods, followed by co-inoculated plants. Similarly, stomatal conductance (*gs*) was significantly higher in the control than in the other treatments for all analysed periods, in close association with *A*. Thus, stomatal closure may be one of the factors responsible for reduction in *A* in inoculated plants (Bacelar *et al.,* 2007b).

Transpiration rate (*E*) presented opposite trends in the two days of measurements. At flowering stage, *E* was higher in the afternoon, whereas at pre-harvesting stage, it was

higher in the morning, decreasing in the afternoon, in a closer association with decreased *gs* (Bacelar *et al.,* 2009).

Although having the highest photosynthetic pigments, plants inoculated with *Rhizobium laguerreae* exhibited the lowest *A* and *gs*, and the highest intracellular CO_2 concentration (*Ci*) at flowering stage. These responses suggest that, beyond the greater stomatal adjustment to avoid excessive water loss in inoculated plants, non-stomatal limitations such as biochemical changes also contributed to the reduction in *A* in these plants (Schultz, 1996; Medrano *et al.*, 2002; Moutinho-Pereira *et al.,* 2004). At pre-harvesting stage, *Ci* and intrinsic water use efficiency (*A/gs*) values did not differ significantly among treatments; therefore, the decrease in *A* in inoculated plants was mostly attributed to stomatal closure (Moutinho-Pereira *et al.,* 2007). However, under environmental stress conditions, the *Ci* calculated from gas-exchange measurements can be overestimated and lead to wrong conclusions about non-stomatal limitation of photosynthesis (Downton *et al.,* 1988).

Gas-exchange measurements were not in agreement with the other analyses, but this can be explained by the higher number of leaves and pods and the thicker leaves of plants inoculated with *Rhizobium laguerreae*, which may justify the lower photosynthetic rate in each individual leaf. Another reason could be the momentary nature of gas-exchange measurements.

In the present study, grain yield (in terms of number of pods and seeds produced, and total weight of pods and seeds per pot) was positively affected by single and combined application of both beneficial microorganisms. However, comparing co-inoculated and single-inoculated plants, no significant differences were observed for grain yield parameters, showing that, at least in these experimental conditions, inoculation just with one microorganism was sufficient to improve grain yield. Improvements in grain yield, namely in the number of pods and seeds and in the weight of seeds, were reported in other studies with rhizobial inoculation in faba bean (Denton *et al.,* 2013; Youseif *et al.,* 2017) and in other leguminous plants (Ali *et al.,* 2000; Malik *et al.,* 2006; Ravikumar, 2012; Oliveira *et al.,* 2017). All of the performed analyses are important; however, it is the productivity parameters that are fundamental in agriculture, because plants with more and/or heavier seeds lead to a positive economic impact.

The interrelationships between characteristics observed in PCA showed that plants inoculated with *Rhizobium laguerreae* and co-inoculated with *Rhizobium laguerreae* and AMF presented higher rates of mycorrhization and number and weight of pods and seeds than plants in the other treatments. PC1 also revealed that control plants presented lower LAR, LMA, numbers of leaves, branches and nodules, and shoot and root biomass.

Considering these results and the individual analyses, we can conclude that inoculation of faba bean with *Rhizobium laguerreae* significantly increased growth and yield parameters and photosynthetic pigments, as well as improved morphological characteristics, which supports their use in the development of commercial faba bean inoculants targeted to better crop yields with reduced usage of N fertilisation.

Inoculation with fungi also improved productivity parameters such as number of pods and weight of pods and seeds. Although co-inoculation with bacteria and fungi also presented higher values for all of the analysed productivity parameters than the control, the values were similar to those found in plants receiving single inoculation with only one microorganism.

In conclusion, considering all analyses performed in this work, single inoculation of faba bean plants with the bacterium *Rhizobium laguerreae* provided the best results, showing great potential as a biological tool to improve the growth and yield of this leguminous plant, and reducing the need for chemical fertiliser inputs.

Conflicts of interest

The authors declare no conflicts of interest.

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CHAPTER VII

GENERAL DISCUSSION

GENERAL DISCUSSION

The improvement on the potential adaptability and productivity of leguminous crops, by exploring the symbiosis with beneficial microorganisms, is a great contribution to the Sustainable Development Goal 2 of the United Nations Development Programme, aiming to achieve zero hunger by 2030.

On the other hand, the increase on protein and nutritional value of pulses fostered by the symbiosis is also important to human health. For this reason, several studies addressed the effects of inoculation and co-inoculation with beneficial microorganisms in the growth and yield of legume plants (Gloss and de Varennes, 2002; Jia *et al*., 2004; Oliveira *et al*., 2017). In general, these studies revealed positive effects on legume performance by single inoculation with one microorganism and generally synergistic effects by co-inoculation with both rhizobia and AMF. However, some authors defend that it is not yet clear whether the presence of AMF influences nodule functioning (Scheublin and van der Heijden, 2006) and some authors even reported an inhibition of nodule formation in co-inoculated plants (Bethlenfalvay *et al.*,1982; Jia *et al.,* 2004; Ismaiel *et al.,* 2014). Despite all these works involving beneficial microorganisms, there is still a lack of genotypic evaluation as well as of effectiveness of particular strains in BNF in diverse agro-ecological conditions.

Additionally, although rhizobia seem to be as widely distributed as AMF, many soils used for legume cultivation do not have adequate numbers of native rhizobia, or they can be ineffective for enhancing biological N_2 fixation, which translates into extremely low legume productivity in some countries.

Thus, in this work, following *in vitro* studies to verify the ability of bacteria to nodulate other plants and to select the best inoculants, it was performed a molecular identification of rhizobial bacteria collected from several regions of Portugal with different edaphoclimatic conditions. The selected inoculants were then tested alone and in co-inoculation with AMF, in larger scale greenhouse trials.

Our results provide the first analysis on the phylogenetic diversity of indigenous root-nodulating bacteria from cowpea and faba bean plants, in Portugal.

Within the bacteria isolated from cowpea plants, *Rhizobium* (N=17) was the most abundant genus of the detected genera. It was also found a high bacterial diversity associated to cowpea root nodules, namely from *Bradyrhizobium* (N=6) and *Caulobacter* (N=1) (α-proteobacteria), *Burkholderia* (N=2) and *Herbaspirillum* (N=2) (β-proteobacteria) and *Kosakonia* (N=1) and *Enterobacter* (N=6) (γ-proteobacteria) genera. This work allowed to confirm the promiscuity of cowpea, since this culture could establish symbiotic relationships with different genera of bacteria.

Within the bacteria isolated from faba bean plants, *Rhizobium* was the most abundant genus: *Rhizobium leguminosarum* bv. *viciae* (N=20), *Rhizobium* sp. (N=11) and *Rhizobium laguerreae* (N=1). Additionally, few isolates were identified as β-proteobacteria*: Burkholderia* sp. (N=1) and *Burkholderia lata* (N=1).

According to Moulin *et al*. (2001) and Andrews and Andrews (2017), rhizobia from different genera across the β-proteobacteria, in particular *Burkholderia,* are able to form functional nodules, having consequently a symbiotic nodulation ability. Additionally, some α-proteobacteria (*Aminobacter*, *Ochobactrum*, *Methylobacterium* and *Phyllobacterium*), β-proteobacteria (*Herbaspirillum* and *Shinella*) and γ-proteobacteria (*Pantoea*, *Enterobacter* and *Pseudomonas*) have been described as non-rhizobial endophytes (NRE) presented in legume nodules along with rhizobia (Valverde *et al*., 2003; Benhizia *et al*., 2004; Lin *et al*., 2008; Ibáñez *et al*., 2009; Shiraishi *et al*., 2010; Aserse *et al*., 2013). Usually these bacteria are not able to form root nodules, but they can enter infection threads when leguminous plant are colonized with rhizobial strains (Leite *et al*., 2017), giving some advantages to the pants. Other works also referred NRE isolates from legume root nodules that present *nod* genes similarity with those of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Burkholderia* species (Martínez-Hidalgo, 2017). This was also observed in the present work. Indeed, 2 cowpea isolates identified as *Enterobacter* sp. presented *nodA* gene and 2 isolates identified as Caulobacter sp. and *Burkholderia fungorum* presented *nodC* gene. In relation to faba bean symbionts, 2 isolates identified as *Burkholderia* sp. and *Burkholderia lata* presented both nodulation genes.

For rhizobia from both crop cultures, slight differences in the tree topologies of the individual ML trees were observed. Incongruence of phylogenetic relationships for housekeeping genes in some species has also been reported in previous studies, which may be, according to the authors, the result of recombination, migration or horizontal gene transfer (HGT) (Vinuesa *et al*., 2005; Islam *et al*., 2008; Rivas *et al*., 2009). Furthermore, incongruences between the phylogenies of symbiosis (*nod* gene) and those of chromosomal genes have been reported in several studies on rhizobia and this has been inferred as an indication of horizontal inheritance of the symbiosis genes (Chen *et al*., 2003; Moulin *et al*., 2004; Huang and Gogarten, 2006; Liu *et al*., 2012; Aoki *et al*., 2013). According to Kumar *et al*. (2015), strains with closely similar core genomes could have very different *nod* genes, while genetically distant strains could share similar *nod* genes, due to HGT between different genospecies. Indeed, despite α-and β-rhizobia are evolutionary divergent, their symbiotic genes are highly similar suggesting lateral transfer (Bontemps *et al*., 2010; Chen *et al*., 2003; De Meyer *et al*., 2016; Moulin *et al*., 2001).

For faba bean crop, *Rhizobium laguerreae* was tested, alone and in combination with a mix of arbuscular mycorrhizal fungi. The results showed the efficiency of the selected bacteria and the AMF in the overall plant performance, under sterile and slightly acidic soil (pH=5.1). Indeed, single inoculation of faba bean with *Rhizobium laguerreae* significantly increased the number of nodules, the number of leaves and leaf area, the LMA, the LAR, all the photosynthetic pigments and the gain yield. In relation to the increased number of nodules, our results are in agreement with previous studies in faba bean, which support that co-inoculation has a negative effect on rhizobial bacteria (Bethlenfalvay *et al.*, 1982; Jia *et al.,* 2004; Ismaiel *et al.,* 2014). However, bibliography shows controversial results and some authors defend that the number of nodules is higher in mycorrhizal than in non-mycorrhizal plants (Abd-Alla *et al.*, 2014).

The increase in the number of leaves observed in the present work by single inoculation with rhizobial bacteria was corroborated by previous studies carried out in other leguminous plants: black gram (*Vigna mungo* (L.) Hepper) and mung bean (*Vigna radiata* (L.) R.Wilczek) also inoculated with *Rhizobium* (Ravikumar, 2012).

The higher leaf area of plants inoculated with *Rhizobium laguerreae* can be justified by the improvement in overall plant growth, due to an increase in whole-plant photosynthesis, and consequently to an increase in net assimilated C available to growth.

Photosynthetic pigments were also increased in plants inoculated with the bacteria. This occurs to answer to the carbon requirements of host plants (Sivaprasad and Rai, 1987; Lalitha and Santhaguru, 2012), and can be due to an increase in stomatal conductance, photosynthesis, transpiration and enhanced plant growth (Rajasekaran *et al.,* 2006). Although having the highest photosynthetic pigments, plants inoculated with *Rhizobium laguerreae* exhibited the lowest *A* and *gs*, and the highest intracellular CO_2 concentration (*Ci*) at flowering stage. These responses suggest that, beyond the greater stomatal adjustment to avoid excessive water loss in inoculated plants, non-stomatal limitations such as biochemical changes also contributed to the reduction in *A* in these plants (Schultz, 1996; Medrano *et al.*, 2002; Moutinho-Pereira *et al.,* 2004). The lower photosynthetic rate in each individual leaf observed in plants inoculated with *Rhizobium laguerreae* can be explained by the higher number of leaves and pods and the thicker leaves of these plants. This thickness of foliar tissue was also corroborated by the increased LMA observed in plants inoculated with the bacteria.

Inoculation with fungi just improved productivity parameters such as number of pods and weight of pods and seeds.

In fact, in this experiment, the productivity parameters were improved in all the inoculated and co-inoculated plants. Improvements in grain yield, namely in the number

of pods and seeds and in the weight of seeds, were reported in other studies with rhizobial inoculation in faba bean (Denton *et al.,* 2013; Youseif *et al.,* 2017) and in other leguminous plants (Ali *et al.,* 2000; Malik *et al.,* 2006; Ravikumar, 2012; Oliveira *et al.,* 2017). Despite this increase in productivity in co-inoculated plants, the observed values were similar to those found in plants receiving single inoculation with only one microorganism. This means that, at least in this experimental conditions, single inoculations were sufficient to improve grain yield.

The study in cowpea plants included the inoculation and co-inoculation with three rhizobial bacteria (*Rhizobium* sp., *Bradyrhizobium elkanii* and *Bradyrhizobium* sp.) and a mix of AMF. These plants were subjected to two different water regimes: 25 % of field capacity (plants under drought stress) and 75% of field capacity (well-watered plants), because although cowpea has been referred as a well-adapted plant to abiotic stress, drought is one of the main concerns in its production.

In well-watered plants, the effects of the inoculation and co-inoculation are not very evident. This can be due to the presence of other native microorganisms in the soil, even in control plants, once the soil was not sterilized. However, under water stress, an increase in the crude protein content of seeds of plants co-inoculated with each rhizobial bacteria and AMF was observed, when compared to the control plants. Thus, inoculation with selected rhizobial bacteria and AMF has great potential to reduce the impact of water scarcity (Oliveira *et al*., 2017). This can be due to the simultaneous improvement in the nitrogen fixation provided by the bacteria (Hardarson and Atkins, 2003) and the improvement in water and other minerals provided by the fungi (Nadeem *et al*., 2014). Furthermore, in this work, co-inoculated plants could mobilize the photoassimilates to the seed, a sink of protein production, in detriment of growth and yield.

The use of non-sterilized soil in this experiment makes this work very useful because it is possible to extrapolate the results obtained to the field, in real conditions. Additionally, the use of non-sterilized soil also allows to compare the competitiveness of these microorganisms and the native ones present in the soil. In fact, the improvements obtained in co-inoculated plants under drought stress compared to control allow to conclude that the selected microorganisms in this work can be more resistant to drought than the native microorganisms of the soil, since this effect was not observed in well-watered plants.

Taking in account both of the greenhouse experiments, it is possible to conclude that the microorganisms inoculated in faba bean, especially the bacteria, improved the growth and yield of the plants, while the microorganisms inoculated in cowpea plants improved the crude protein content of the seeds. Moreover, the benefits of co-inoculation comparing to single inoculation were more visible in cowpea, especially in plants under

drought stress, since, in faba bean, single inoculated plants presented generally similar or better results.

In conclusion, considering the molecular identification of the isolates, a high diversity of bacteria belonging to α-, β- and γ-proteobacteria can be found inside the root nodules of leguminous plants. Furthermore, single and combined inoculation of cowpea and faba bean plants with the selected microorganisms improved the growth, yield and crude protein content, showing its great potential to be used in the development of commercial inoculants, to improve the growth and yield of leguminous plant and reduce the need for chemical fertiliser inputs. The selected inoculants for cowpea have been shown to be able to increase the plant tolerance to climate changes, which are responsible for the increasingly frequent episodes of dryness in the Mediterranean region.

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CHAPTER VIII

CONCLUDING REMARKS AND FUTURE PROSPECTS

CONCLUDING REMARKS

The objectives of this thesis have been achieved and the results have definitely contributed to the advancement of scientific knowledge in the rhizobia biodiversity associated with cowpea and faba bean in Portugal, as well as, in the selection of inoculants that bring advantages to the leguminous plants, through the following outcomes:

1. A better understanding about the biodiversity of rhizobial bacteria associated with cowpea plants. To the best of our knowledge, there are no previous reports using a multilocus sequence analysis approach to evaluate the biodiversity of rhizobia present in root nodules of cowpea plants in Portugal. Although rhizobia are widely distributed, the absence of effective rhizobia is the main reason to the failure of leguminous crops. 35 isolates were collected from cowpea root nodules and identified as *Rhizobium* sp., *Bradyrhizobium* sp., *Bradyrhizobium elkanii*, *Burkholderia* sp., *Enterobacter* sp., *Burkholderia fungorum*, *Herbaspirillum* sp., *Kosakonia* sp. and *Caulobacter* sp., being that *Rhizobium* sp. was the most common bacteria (N=17), followed by *Bradyrhizobium* sp.;

2. Further knowledge about the biodiversity of rhizobial bacteria associated with faba bean root nodules in Portugal. From this crop, 34 isolates were collected and identified as *Rhizobium leguminosarum* bv. *viciae*, *Rhizobium* sp., *Rhizobium laguerreae, Burkholderia* sp. and *Burkholderia lata*, being that *Rhizobium leguminosarum* bv. *viciae* (N=20) and *Rhizobium* sp. (N=11) were the most common;

3. The performed studies also increased the knowledge about the effects of inoculation and co-inoculation with beneficial microorganisms in cowpea plants, particularly the ability to improve the crude protein content in the seeds of plants under drought stress. The use of non-sterilized soil in this work allows a more real extrapolation of the behaviour of these bacteria in the field. With this work, it was also possible to conclude that our inoculants were more resistant to drought stress than the native microorganisms present in that soil, since, under drought stress, co-inoculations increased the crude protein content in the seeds when compared to the control plants. This means that the microorganisms used in this study were efficient and competitive, benefiting more the plants than the native microbiota present in the soil. With this eco-friendly approach it is possible to increase the nutritional and commercial value of leguminous plants by the increase in crude protein content, a cheap alternative for human consumption, without chemical fertilizer applications and genetic improvements;

4. The greenhouse experiment carried out with faba bean showed the efficiency of the bacteria *Rhizobium laguerreae* to increase the photosynthetic pigments and the growth and yield parameters of plants, which supports their use in the development of commercial faba bean inoculants targeted to better crop yields with reduced usage of N fertilisation. Despite the good results obtained by the co-inoculation with both *Rhizobium laguerreae* and AMF, there was no gain to justify this investment, since the obtained results for the analysed parameters were similar or inferior to those obtained with the single inoculation with the bacteria;

5. The selected microorganisms have a great potential to the development of commercial inoculants to improve the growth, productivity and/or crude protein content of leguminous plants, reducing the chemical fertilisers inputs. This eco-friendly tool allows to reduce the environment pollution and simultaneously to benefit our health as consumers.

6. By increasing the production of leguminous plants at national level, through its inoculation with beneficial microorganisms, it will be possible to improve the trade balance, since a high amount (80-90%) of the dried legumes consumed in Portugal are imported. This biological tool can be also very useful in countries with poor soils, where the productivity of leguminous plants is very low.

FUTURE PROSPECTS

The research presented in this thesis have raised some interesting questions awaiting further investigation. Hence, were identified several lines of research which should be pursued:

1. The study with cowpea plants was performed in non-sterilized soil, but under greenhouse conditions. It was possible to conclude that our inoculants were more resistant to drought stress than the native microorganisms present in that soil, and co-inoculation with each selected bacteria and AMF increased the crude protein content in the seeds of plants under drought stress. However, it is still necessary to test these inoculants under field conditions to check the true potential of the microorganisms, especially the arbuscular mycorrhizal fungi. Indeed, the symbiosis with AMF improve the surface absorbing capability of host roots, allowing the access to a great volume of soil and improving consequently the uptake of water and immobile nutrients such as phosphorus. In the pots, these advantages of fungi can be undervalued, since the root growth and development were limited. Still, the positive effects observed make the results very promising, encouraging to continue to the field experiments;
2. Although we observed beneficial effects in the growth and productivity parameters of faba bean plants single and co-inoculated with the selected microorganisms, the study was performed under greenhouse conditions and using sterilized soil. Thus, it is important to confirm the efficiency of our symbionts under field conditions, as well as, their competitiveness in relation to the native microorganisms present in the soil;
3. The promising results obtained with the selected beneficial microorganisms also encourages to evaluate its effects in other leguminous plants, despite rhizobia-legume symbiosis be a highly specific interaction, due to the changes in Nod factors, the bacterial lipochitooligosaccharide (LCOs) signals that determine the host-specificity. Thus, urge the necessity to check if these inoculants can establish a symbiotic relationship with other legume crops and if the symbiosis can efficiently improve biological nitrogen fixation and consequenty plant growth and yield. Moreover, it might be interesting to test their effects and efficiency in other locations with different edaphoclimatic conditions, such as soils with different pH and composition and places with other climatic conditions, especially in countries where leguminous plant productivity is very low;
4. In this thesis, to identified the collected rhizobial bacteria, the amplification of 16S rDNA region was complemented with 6 housekeeping genes (*recA*, *gyrB, SMc00019*, *thrA*, *atpD* and *truA*) and 2 symbiotic genes of nodulation (*nodA* and *nodC*). In future

works, more regions can be amplified to make the identification more robust, such as IGS region (16S-23S rDNA intergenic space) and other symbiotic genes (*nifH* and *rhcRST*). IGS region contains greater variability than 16S rDNA and is suitable in order to examine chromosomally encoded genetic variations at the intra-species level (Pongsilp, 2012);

5. Surface polysaccharides are the second key molecules in legume infection by rhizobia and can be exocellular (EPS), capsular (KPS) and lipopolysaccharides (LPS). The analysis of lipopolysaccharide profile can be an interesting tool, to be used in future works, not only to help in the identification of the rhizobial strains (Kutkowska *et al.,* 2017), but also to study the specificity between rhizobia and the host plant;

6. The identification, selection and confirmation of efficiency of these inoculants is of utmost importance to the future development of commercial inoculants in order to contribute to a more sustainable agriculture, less based on synthetic chemical fertilizers.

References

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