universidad de león

Chemical, Environmental and Bioprocess Engineering Group

PhD THESIS

Development of formulations for the application of biofertilisers based on autochthonous microorganisms for high added–value crops

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"En cuanto al futuro, no se trata de preverlo sino de hacerlo posible"

Antoine de Saint-Exupéry (1900 – 1944)

A mi familia,

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Figures and Tables index

List of abbreviations

Abbreviations of bacterial strains

In the summary, and in the article Pastor-Bueis et al. (2021)

A	Type strain *Azotobacter chroococcum* Beijerinck 1901 (ATCC 9043^T)
P	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* (RVPB 2-2)
R	*Rhizobium leguminosarum* bv. *phaseoli* (LCS 0306)
B	*Bacillus siamensis* (SCFB 3-1)

In the article Pastor-Bueis et al. (2019)

Re CFN42^T	*Rhizobium etli*, strain CFN42^T
Rlp LCS 0306	*Rhizobium leguminosarum* bv. *phaseoli*, strain LCS 0306
Rlv UPM 791	*Rhizobium leguminosarum* bv. *viciae*, strain UPM 791
Rp ATCC 14482^T	*Rhizobium phaseoli*, strain ATCC 14482^T

Other abbreviations through the document

%Ndfa	Nitrogen derived from the fixation of atmospheric N_2 (%)
^{15}N	^{15}N isotope
16S rRNA	16S ribosomal ribonucleic acid
ACC	1-aminocyclopropane-1-carboxylate deaminase
AD	Anaerobic Digestate
AD-m	Anaerobic Digestate and molasses before sterilization
AD-m-ST	Anaerobic Digestate and molasses after sterilization
AMF	Arbuscular Mycorrhizal Fungi
ANI	Average Nucleotide Identity
ANOVA	Analysis of Variance
BF	Biofertiliser
BLAST	Basic Local Alignment Search Tool
BMP	Bioestimulante Microbiano de Plantas
BNF	Biological Nitrogen Fixation
BRIG	BLAST Ring Image Generator
BSL	Biological Safety Levels
C:N	Carbon : Nitrogen
CLSM	Confocal laser scanning microscopy

Ca	Calcium
CAP	Common Agricultural Policy
CC	Carbocompost
cfu	Colony-forming unit
Co	Compost
CO_2	Carbon dioxide
COG	Clusters of Orthologous Group
CSTR	Anaerobic Continuously Stirred Tank Reactor
DA	Digestato Anaerobio
DAS	Days After Sowing
DNA	Deoxyribonucleic acid
DNW	Dry nodule weight
EIAF	Escuela de Ingeniería Agraria y Forestal
EPS	Exopolysaccharide
FAO	Food and Agriculture Organization of the United Nations
FVW	Fruit and Vegetable Wastes
g	Gram
GFP	Green Fluorescent Protein
GHG	Greenhouse Gasses
GI	Germination Index
GLM	General Linear Model
GPI	Geographical Protected Indication
HI	Harvest Index
IAA	Indole-3-acetic acid
ICEs	Integrative and Conjugative Elements
K	Potassium
KAAS	KEGG Automatic Annotation Server
l	Litre
LSD	Least Significant Difference test
Mg	Magnesium
mg	Milligram
ml	Millilitre
MPB	Microbial Plant Biostimulant
NN	Number of nodules
NPK	Nitrogen Phosphorous and Potassium

NRT	Nutrient Recovery Technologies
NUE	Nitrogen Use Efficiency
OH	Hydroxyle radical
PB	Perlite plus Biochar
Pe	Perlite
PGAP	Prokaryotic Genome Annotation Pipeline
PGI	Protected Geographic Indication
PGP	Plant Growth Promoter
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
pH	Potential of Hydrogen
PNB	Partial Nutrient Balance
PPM	Plant Probiotic Microorganism
RCB	Randomized complete block
rDNA	Ribosomal Deoxyribonucleic acid
RFP	Red fluorescent protein
RGP	Relative Germination Percentage
RPAD	Random Amplification of Polymorphic DNA
RRG	Relative Radicle Growth
RSM	Response Surface Methodology
SIDI	Servicio Interdepartamental De Investigación
SPSS	Software platform of statistical analysis
TCA	Tricarboxylic acid
TS	Total Solids
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume
WG	Water-dispersable Granules
WHO	World Health Organization
YM	Yeast Mannitol
YMA	Yeast Mannitol Agar
YMB	Yeast Mannitol Broth

General abstract

To achieve sustainability in agriculture, new strategies are based on the use of biotechnologies to reduce the dependence on chemical products. After proper selection and adequate formulation, the microorganisms pertaining to the plant holobiont are useful to stimulate plants' natural nutrition processes (named microbial plant biostimulants [MPBs]); they can also be used as bio-controllers, replacing conventional chemical inputs and avoiding their associated environmental issues. The objectives of this work were: i) to design, produce, formulate, and evaluate in the field microbial inoculants to be used as MPBs in common bean (*Phaseolus vulgaris* L.) and pepper (*Capsicum annuum* L.) crops in a protected geographical indication; and ii) to search for explanations for the superior performance in the field of the designed inoculants to establish strategies for the future development of MPBs. To fulfil these objectives, the experimental design involved the following symbionts and crops: i) rhizobial endosymbionts (*Rhizobium leguminosarum* bv. *phaseoli* strain LCS 0306, subsequently designated as R) in a legume crop (common bean); ii) non-rhizobial endophytes of root nodules in the common bean (*Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain RVPB 2-2, subsequently designated as P); and iii) non-rhizobial endophytes (*Bacillus siamensis* strain SCFB 3-1, subsequently designated as B) in a non-legume crop (pepper). R was tested in four field trials and produced an average 36% yield increase with the uninoculated and unfertilised-with-nitrogen control; the increase in the nitrogen derived from fixation (Ndfa) was 10%; all the increases were statistically significant. The autochthonous R produced a similar yield to that with nitrogen fertilisation and significantly higher (24% and 22%, respectively) than the allochthonous strains *Rhizobium phaseoli* (ATCC 14482^T) and *Rhizobium etli* (CFN 42^T), both of which belong to the sv *phaseoli*. The Ndfa (%) was also significantly higher when inoculated with R. Three formulations, prepared using residues according to the principles of a circular economy, were tested with R; the formulation based on a carrier consisting of 25% perlite and 75% pine bark biochar was the best option. The superior performance of R was due to its high nitrogen-fixing ability and competitiveness, both of which were explained by the genome mining. On the one hand, R contains genes that facilitate efficient symbiosis with *Phaseolus* (the symbiotic repertoire of *R. etli* CFN42^T). On the other hand, R contains genes that explain its competitiveness, namely a large repertoire of secretion systems and other genes related to exploitative competition (chemotaxis and transport systems) and interference competition (bacteriocin-like compounds). The autochthonous endophyte P in combination with R increased the yield by 17% compared with single inoculation with R. Confocal laser scanning microscopy revealed that while P and R both colonise the interior of the nodules, R is

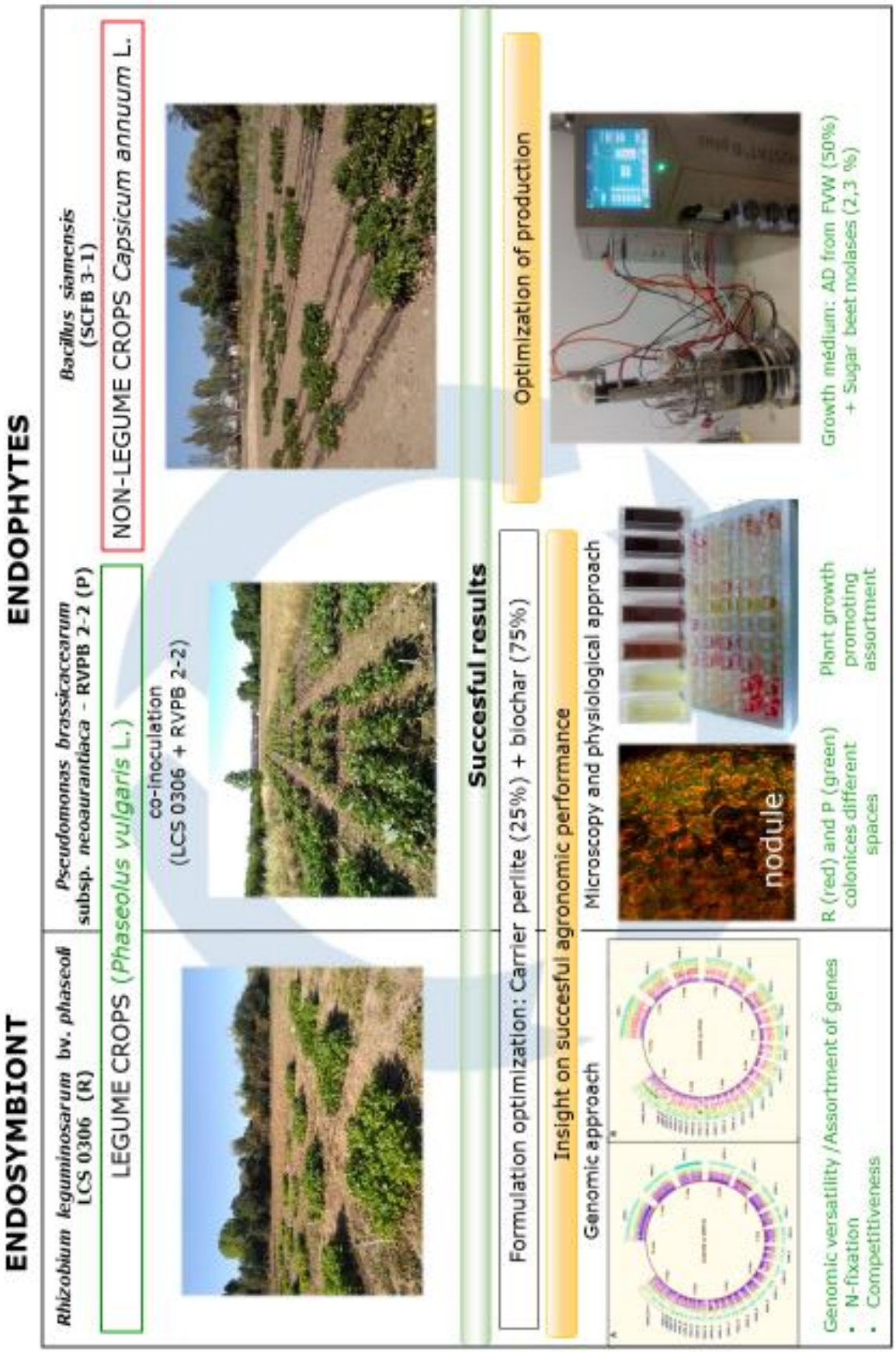
located intracellularly, and P is located intercellularly. This difference prevents the strains from competing with each other and could be one of the reasons for the observed crop yield increase. Moreover, P improved plant growth promoting properties, which can also contribute to explain the yield increase. The field trials with pepper inoculated with the endophyte B and fertilised with decreased mineral nitrogen (80% of the expected plant extractions) produced a significantly higher yield compared with the uninoculated control fertilised with reduced nitrogen (31% increase) and the uninoculated control fertilised with full nitrogen (34% increase). The formulation and growth medium to produce B were developed considering the guidelines of the European Commission Circular Economy Action Plan and comprised two residues: anaerobic digestate (AD) – from food and vegetable wastes – and sugar beet molasses. The concentration of both was optimised with the response surface methodology; determined values were 50% (v:v) AD and 2.3% (v:v) sugar beet molasses.

Resumen

Para lograr la sostenibilidad en la agricultura, las nuevas estrategias se basan en el uso de biotecnologías que reduzcan la dependencia de los productos químicos. Tras una adecuada selección y formulación, los microorganismos pertenecientes al holobionte de la planta son útiles para estimular los procesos naturales de nutrición de las plantas (denominados Bioestimulantes Microbianos de la planta, [BMP]); también pueden ser utilizados como bio-controladores, sustituyendo los insumos químicos convencionales y evitando sus problemas ambientales asociados. Los objetivos de este trabajo fueron: i) diseñar, producir, formular y evaluar en campo, inoculantes microbianos para ser usados como BMP en cultivos de alubia (Phaseolus vulgaris L.) y pimiento (Capsicum annuum L.) de Indicación Geográfica Protegida; ii) buscar explicación sobre el superior rendimiento en campo de los inoculantes diseñados, con el fin de establecer estrategias para el futuro desarrollo de BMP. Para cumplir con dichos objetivos, el diseño experimental incluyó los siguientes simbiontes y cultivos: i) endosimbiontes rizobiales (Rhizobium leguminosarum bv. phaseoli cepa LCS 0306, en adelante R) en un cultivo leguminoso (alubia blanca); ii) endófitos no rizobios de nódulos de raíces de alubia (Pseudomonas brassicacearum subsp. neoaurantiaca cepa RVPB 2-2, en adelante P), y iii) endófitos no rizobios (Bacillus siamensis cepa SCFB 3-1, en adelante B) en cultivos no leguminosos (pimiento). R se experimentó en cuatro ensayos de campo y produjo un aumento medio del rendimiento del 36% comparado con el control no inoculado y no fertilizado con Nitrógeno; el incremento del Nitrógeno derivado de la fijación (Ndfa) fue del 10%; todos los aumentos fueron estadísticamente significativos. La cepa autóctona R produjo un rendimiento similar al de la fertilización nitrogenada, y significativamente superior (24% y 22% respectivamente) que las cepas alóctonas Rhizobium phaseoli (ATCC 14482T) y Rhizobium etli (CFN 42T), ambas pertenecientes al sv. phaseoli. El Ndfa (%) también fue significativamente mayor con la cepa autóctona. Se probaron tres formulaciones diferentes, preparadas usando residuos de acuerdo con los principios de la Economía Circular, con la cepa R; y la formulación basada en un soporte compuesto por un 25% de perlita y un 75% de biochar de corteza de pino fue la mejor opción. El rendimiento superior de la cepa R se debió a

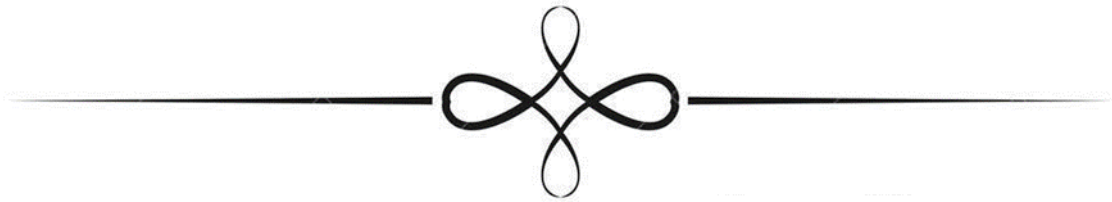
una alta capacidad de fijación de nitrógeno y una alta capacidad de competencia, ambas han sido explicadas desde el punto de vista de su genoma. Por un lado, la cepa R contiene los genes implicados en establecer una eficiente simbiosis con Phaseolus (el repertorio simbiótico de R. etli CFN42T). Por otro lado, R contiene genes que explican su capacidad de competencia, esto es, un amplio repertorio de sistemas de secreción y otros genes relacionados con la competencia de explotación (quimiotaxis y sistemas de transporte) y con la competencia de interferencia (compuestos bacterianos como bacteriocinas). El endófito autóctono P en consorcio con R aumentó el rendimiento en un 17% en comparación con la inoculación única con R. El microscopio confocal de barrido láser reveló que mientras que P y R colonizan el interior de los nódulos, R se localiza intracelularmente y P intercelularmente. Esta diferencia impide que las cepas compitan entre sí y podría ser una de las razones del aumento observado en el rendimiento de los cultivos. Además, la cepa P mejoró las propiedades promotoras del crecimiento de las plantas lo que también puede contribuir a explicar el aumento del rendimiento. Los ensayos de campo con pimiento inoculados con el endófito B y fertilizados con nitrógeno mineral reducido (80% de las extracciones esperadas de la planta), produjeron un rendimiento significativamente mayor que el control no inoculado fertilizado con nitrógeno reducido (31% de aumento) y que el control no inoculado fertilizado con nitrógeno completo (34% de aumento). La formulación y el medio de cultivo para producir B se desarrollaron teniendo en cuenta las directrices del Plan de Acción sobre Economía Circular de la Comisión Europea y consistió en dos residuos: Digestato Anaerobio (DA) de residuos de alimentos y vegetales, y melaza de remolacha azucarera. La concentración óptima de ambos fue optimizada con la metodología de la superficie de respuesta y consistió en un 50% de DA (v:v) y un 2,3% de melaza (v:v).

Graphical abstract



Chapter 1

General introduction



1.1. Agriculture in 20th century: the first Green Revolution

Agriculture has followed a marked transformation in the 20th century. A major concern about how to solve food shortage because of the Second World War promoted the modernisation of agriculture. This transformation was named the 'Green Revolution' and involved the introduction of new technologies including modern molecular biology, which was rapidly applied to plant research and induced the use of new crop varieties genetically resistant to pest and diseases (Liu et al., 2020). Besides, the use of first-generation pesticides and chemical products, including mineral fertilisers, and the use of heavy machinery, have made it possible to increase crop production rapidly (Pellegrini & Fernández, 2018).

Due to the Green Revolution, the ancient model of agriculture based on self-sufficiency was quickly replaced by an industrial model focussed on maximising crop production for agricultural marketing. Shortly thereafter, the industrial model was consolidated in industrialised and developed countries, a phenomenon that changed the thinking of farmers (Tyagi, 2016). Traditional agriculture was environmentally friendly, and the use of natural resources and traditional methods like crop rotation or organic fertilisers comprised the basic principles of that production system (Kumar & Singh, 2019). However, in modern agriculture, more food is produced in less time and space, changes that have involved intensive exploitation of non-renewable resources (Martin-Guay et al., 2018).

In Europe, the Common Agricultural Policy (CAP) released in the 1960s (Parsons, 1962) was the main factor that boosted the transition to an industrialised agriculture model (Frison, 2016). The CAP is one of the world's largest agricultural policies; it was originally focussed mostly on improving production (Pe'er et al., 2019), increasing yields, and ensuring a food supply (Harwood, 2019) at reasonable prices.

In 19th century, agriculture in Spain showed very low productivity compared with the production levels achieved in other European countries such as England and the Netherlands (Clar et al., 2015). With the predominance of a Mediterranean climate and the presence of mountains in a large part of the territory, some experts like Clark (1957) considered Spain to be the European

country with the lowest agricultural potential in Europe. From the 1960s onwards, this situation has changed: Spain's agricultural policies gradually converged with the CAP policies (Clar et al., 2018), which culminated in 1986 when Spain joined the European Union.

1.2. Environmental consequences of agricultural intensification

The type of intensive agriculture in which the quantity produced prevails over the quality and environmental conservation has resulted in the emergence of a large number of environmental issues (Maxwell et al., 2016) as well as a loss of biodiversity (de Graaff et al., 2019). However, these consequences have been ignored until recently, causing among other problems a loss of soil fertility, a decline in agronomical biodiversity, and an increase of the pollution of soil and water bodies by chemical products as fertilisers or pesticides (Weldeslassie et al., 2018). In addition, there have been other negative global consequences such as impacts on atmospheric constituents and climate change (Chang et al., 2019).

1.2.1. Loss of soil fertility

Soil fertility is defined as 'the quality of a soil that enables it to provide nutrients in adequate amounts and in proper balance for the growth of specified plants or crops' (Soil Science Society of America, 1997). Thus, soil fertility is a complex concept encompassing physical, chemical, and biological properties, all of which are linked to crop productivity and food security (Kim & Bevis, 2019). Natural ecosystems may self-maintain high productivity through the conservation of nutrients, water, and soil organic matter. However, intensive cropping systems could deplete soil fertility (Nair, 2019), a phenomenon that compromises the crop quality and future crop yields (Bonanomi et al., 2020).

Although there are different reasons why soil loses nutrients (mainly nitrogen, phosphorus, potassium, calcium, and magnesium), the most important causes are soil erosion, crop consumption, and nutrient leaching. First, soil erosion has been considered the major cause of global soil degradation, which threatens land, fresh water, and oceans (Borrelli et al., 2020). According to Montanarella (2015), it is estimated that every year 75 billion tonnes of soil

suitable for cropping are lost from agricultural systems worldwide, due to erosion by wind and water, degrading the environment and the arable lands. Equally important, when the essential plant nutrients are continuously removed from soil by an intensive crop production, the natural soil fertility also decreases (Amanullah et al., 2019).

Leaching is another well-known natural process (Tukey, 1970) consisting of the removal of soluble substances from the soil by the effect of the percolation of aqueous solutions. Nutrient leakage must be kept to a minimum with appropriate agronomic practices. However, this natural process cannot be completely avoided, and thus leached nutrients need to be replaced in the soil in the form of fertilisers to compensate for the leakage.

Moreover, it is estimated that around 50% of inorganic fertilisers applied to crops are not used by plants and are retained in the soil or are displaced to other ecosystems (Foley et al., 2011). The accumulation of excess chemicals in the soil results in loss of fertility (Singh et al., 2020) – as a consequence of soil acidification (Pan et al., 2020), increased osmotic potential, and decreased organic matter – that in turns inhibits microbial communities (Bruulsema, 2018). Fertilisers as well as other chemicals such as herbicides, insecticides, and fungicides, among others, can accumulate in the soil and reduce fertility (e.g., reduce the cation exchange capacity; Graversgaard et al., 2018).

Finally, it is well known that organic fertilisation, besides providing nutrients, improves soil physical properties such as porosity, aeration (Cercioglu, 2017), and water-holding capacity (Zong & Lu, 2020), all of which are linked to soil fertility. After the arrival of the Green Revolution, organic fertilisers were displaced by mineral fertilisers, thus reducing soil organic matter.

1.2.2. Loss of biodiversity

Agrobiodiversity is defined by the Food and Agriculture Organization (FAO, 2018a) as the diversity of animals, plants, and microorganisms that are used directly or indirectly for agricultural production, including crops, livestock, forestry, and fisheries. Maintaining biodiversity is essential for global food and nutrition security because it provides valuable ecosystem services and

functions for agricultural production (Chaudhary et al., 2019). It also includes genetic differences within plant species that determine the uniqueness of each one (Convention of Biological Diversity, 2020).

Modern agriculture has focussed on the cultivation and production of a few major staple crops, which are grown as large monocultures of genetically uniform individuals (Jacobsen et al., 2015). As a consequence, the genetic diversity of crop plants has gradually decreased (Besset-Manzoni et al., 2018). Moreover, diversity is important for ecosystem functions, and the abundance of different species is essential for providing food in many indigenous nations and also as a part of their cultural heritage, so it needs to be preserved (Roe, 2019).

1.2.3. Water pollution

Water is a scarce and valuable resource that can be easily contaminated. Although three quarters of Earth are covered by water, only 1% of such water is considered safe to meet the daily needs of humans (Ghaly & Ramakrishnan, 2015). Water availability is essential for agriculture, which accounts for about 70% of global freshwater withdrawal (Huang et al., 2019) and it is key to increase crop yields (Lu et al., 2015). Water pollution comprises the presence of external chemical, physical, or biological components that impair water bodies, change its natural quality (Schweitzer & Noblet, 2018), and may damage health of humans, animals, and plants (FAO, 2018b).

In crop production, one of the major agricultural contributions to water and soil pollution occurs when nutrients from fertilisers and manure, pesticides, or other chemical products are applied inefficiently or in excess to the soil and they end up in water bodies (Graversgaard et al., 2018). In the case of nitrogen fertilisers, when the excess enters the food chain it is a major threat to food safety (FAO & Intergovernmental Technical Panel on Soils, 2015). Ammonium (NH_4^+) is incorporated in the soil by means of several mineral nitrogen fertilisers. It can be strongly adsorbed on soil and can undergo the nitrification process, by which it is biologically oxidised into nitrate (NO_3^-). Unlike NH_4^+, NO_3^- is susceptible to leaching because it is not adsorbed to soil particles and moves readily with water in the soil (Ayars et al., 2017). Thus,

NO_3^-, either directly incorporated via fertilisers or indirectly after the nitrification process, is a potential source of water contamination.

1.2.4. Air contamination

Industrial activity, deforestation, and large-scale agriculture have become important sources of the emission of greenhouse gasses (GHGs) into the atmosphere (Mersin et al., 2019). According to Dorich et al. (2020) agriculture contributes approximately 20% of global GHG emissions (United Nations, 2015).

Some nitrogen species are among the main agricultural pollutants in the air: ammonia (NH_3), nitrous oxide (N_2O), and nitrogen oxides (NO_x = NO + NO_2) (Bray et al., 2020). NH_3 sources have been mainly associated with agricultural farming and livestock production (Bouwman et al., 1997). In agricultural soils, NH_3 is generated by natural volatilisation of nitrogen-NH_4^+ fertilisers (Sun et al., 2020). Within the nitrogen cycle, the biological processes of nitrification and denitrification originated by soil bacteria are considered to be the predominant sources of N_2O and NO_x in agricultural soils (Castellano-Hinojosa et al., 2020). Regarding N_2O, human activity delivers approximately 30% of the N_2O in the atmosphere, mainly originating from agricultural sources (Velthof & Rietra, 2020). N_2O is a powerful GHG that can absorb 298 times more ultraviolet (UV) radiation than carbon dioxide (CO_2; Forster et al., 2008). Furthermore, N_2O undergoes photolysis, which converts it into nitric acid and contributes to acid deposition (Saxena et al., 2019). Finally, agriculture is other important source of NO_x, with the largest soil emissions from regions with heavy nitrogen fertiliser applications (Almaraz et al., 2018). NO_x emissions are key components in tropospheric oxidation chemistry, affecting air quality by triggering the production of ground-level ozone and acid rain (Wang et al., 2020).

1.3. Nitrogen fixation and its contribution to sustainable agriculture

Although molecular nitrogen (N_2) accounts for 78% of atmospheric gas, it is not a useful form for plants (Gao et al., 2019). The 'nitrogen demand' to

support the natural turnover of nitrogen in terrestrial ecosystems can be satisfied in various ways, including, apart from biomass turnover, biological nitrogen fixation (BNF, the dominant pathway under natural conditions), lightning-induced abiotic nitrogen fixation, nitrogen uptake from sedimentary substrates, and nitrogen deposition from natural and anthropogenic sources (Xu-Ri & Prentice, 2017). The NO_x emitted into the atmosphere from soil by natural causes (including fires) is subjected to dry or wet deposition in other ecosystems; in addition, natural NH_x emissions are subjected to the same process (Galloway et al., 1995). However, in our industrialised world, the amount of nitrogen deposited from the NO_x and NH_3 emissions coming from human activities is much larger than the natural nitrogen deposition rate (Dentener et al., 2006). However, enhanced atmospheric nitrogen deposition is concentrated near populous industrialised regions (Cleveland et al., 2013).

BNF is performed by microorganisms and plays a critical role in terrestrial nutrient cycling (Zheng et al., 2019). Indeed, more than 60% of the fixed nitrogen on Earth results from BNF (Soumare et al., 2020). In this microbial process, atmospheric N_2 is reduced to NH_4^+ and it is incorporated into soil and plants (Dynarski et al., 2019). The involved microorganisms are prokaryotes, namely archaea and bacteria. The bacterial groups include free-living bacteria belonging to genera such as *Azotobacter*, *Azospirillum*, *Bacillus*, and *Clostridium*; symbiotic bacteria like *Rhizobium* associated with legumes; *Frankia* associated with actinorhizal plants; and cyanobacteria associated with cycads (Ininbergs et al., 2011; Ravikumar et al., 2007). For archaea, nitrogen fixation is restricted to methanogens (Welte, 2018).

Symbiotic nitrogen fixation (SNF) has begun to gain relevance for sustainable agriculture (Carvalho et al., 2019), because it is the major route of fixing atmospheric N_2 (Geurts et al., 2012). By recovering a part of the nitrogen contained in the atmosphere, this process allows a reduction in the use of nitrogen fertilisers, with consequent savings in energy consumption and reduced environmental risks associated with the production and use of mineral fertilisers (Harchaoui & Chatzimpiros, 2019).

1.4. The Green Biorevolution in the 21st Century

In the current century, meeting global food demands continues to be one of the main objectives for agriculture. However, humankind is now very conscious that agriculture overexploitation generates negative environmental consequences. Thus, it has become necessary to search for alternatives for practices that are aggressive towards the environment while optimising the yield to achieve sustainability (Michel-Villarreal et al., 2019). It is now time for the Green Biorevolution – an evolution of the Green Revolution – that will allow us to improve the quality and yield of crops while using the lowest possible levels of chemical inputs and water. The ongoing Green Biorevolution involves searching for alternatives to chemicals to be used in crop production systems (Besset-Manzoni et al., 2018).

One of the chief strategies to achieve sustainability in agriculture inside the frame of the Green Biorevolution is **the substitution of the most contaminating conventional inputs by microorganism-based inoculants** (Timmusk et al., 2017). The phytomicrobiome, which comprises microorganisms associated with plants, is considered to be the main source of microbial strains that may be used as inoculants in agriculture (Bettenfeld et al., 2020; Orozco-Mosqueda et al., 2018). The combination of a plant host and its associated phytomicrobiome is called the 'holobiont' (Rosenberg & Zilber-Rosenberg, 2016).

Healthy soil can host up to 10 billion bacterial cells per gram of rhizospheric soil (de Vrieze, 2015). Plants have developed the capacity to take advantage of beneficial soil microbes. Indeed, plants use their roots and their own secretions to select from the microbial communities that live around their roots, especially those with a beneficial effect on plant defence and nutrient availability (Bennett et al., 2020). Interactions between plants and soil microorganisms occur in different ways and at different levels. In most cases, those interactions generate benefits for both: on the one hand, plants serve as habitats where microorganisms release compounds that attract and feed other associated microbes; on the other hand, the microbes may secrete metabolites and other compounds that favour plant growth and health (Schirawski & Perlin, 2018). Such a mutually beneficial relationship, in either

a symbiotic or free-living association, has been the consequence of a co-evolution between plants and their associated beneficial microorganisms (Porto de Souza et al., 2017).

The beneficial soil microbes associated with plants act as plant probiotics and are called 'plant probiotic microorganisms' (PPMs) because they produce probiotic effects in plants (Gonzalez-Fontes et al., 2010). These effects including improving plant nutrition, reducing negative responses to plant stress, and increasing agricultural productivity (Walker et al., 2020).

1.5. Development of bacterial inoculants for agriculture

The first bacterial inoculant for agriculture, was registered for inoculating leguminous plants with *Rhizobium* sp. (Nobbe & Hiltner, 1896). The specific plant–*Rhizobium* interaction has been one of the most exploited from the commercial point of view due to the capacity of fixing all the nitrogen needed by the legume crops (Friel & Friesen, 2019). Furthermore, there are many other groups of rhizospheric and endophytic bacteria that also colonise roots to promote plant growth directly or indirectly (Lugtenberg & Kamilova, 2009). Those bacteria, called plant growth promoting rhizobacteria (PGPR), have been used more recently as inoculants to increase the yield of different crops due to their different modes of action (Kloepper et al., 1986).

Microbial inoculants have evolved considerably in the last decades. The 'first generation inoculants' were based exclusively on rhizobia strains for legume crops and focussed on SNF that occurs inside the nodules. The 'second generation inoculants' comprise products formulated with PGPR strains for legume and non-legume crops (Mulas et al., 2013) and those with arbuscular mycorrhizal fungi (AMF; Barea et al., 2008).

The generalised sale of products based on microorganisms for agriculture started in the 1980s but almost disappeared shortly thereafter (Stamenković et al., 2018). In general, those initial products presented poor quality and tenuous effectiveness in the field due, in part, to the absence of an adequate legislation that guarantees product quality. This fact led to a lack of farmers' confidence (Bashan et al., 2014) and it was the reason for the disappearance

from the market. However, in recent years, microbial inoculants have come back on the market. According to Keswani et al. (2019) and Sanches Santos et al. (2019), farmers and other agriculture stakeholders are now more receptive to the use of microbial inoculants because legislation is continuously tightening, limiting, and even forbidding the use of synthetic fertilisers and pesticides. Therefore, farmers need sustainable alternatives that allow maintenance or even increase yield crops reducing production costs.

The Green Biorevolution is the context in which microbial inoculants can find their second and probably last opportunity to burst definitively onto the agricultural inputs market. However, to achieve consistency in field performance, it is necessary to provide stringent regulation to ensure quality. In 2017, Spain established a new regulation about the use of microorganisms that can be part of a fertiliser product (R.D. 999/2017, 2017). In 2019, the European Union passed a new regulation establishing rules on the registration and use of microorganisms as fertilisers in agriculture (Regulation (EU) 2019/1009, 2019). Both regulations recognise that microorganisms that stimulate plant growth in different ways – namely improving their nutrient use efficiency, tolerance to abiotic stress, quality properties, or increasing the availability of confined nutrients in the soil or rhizosphere – are, by nature, more similar to fertilising products than to other categories of plant protection products. For this reason, they must be included in the legislation of fertilisers (Figure 1).

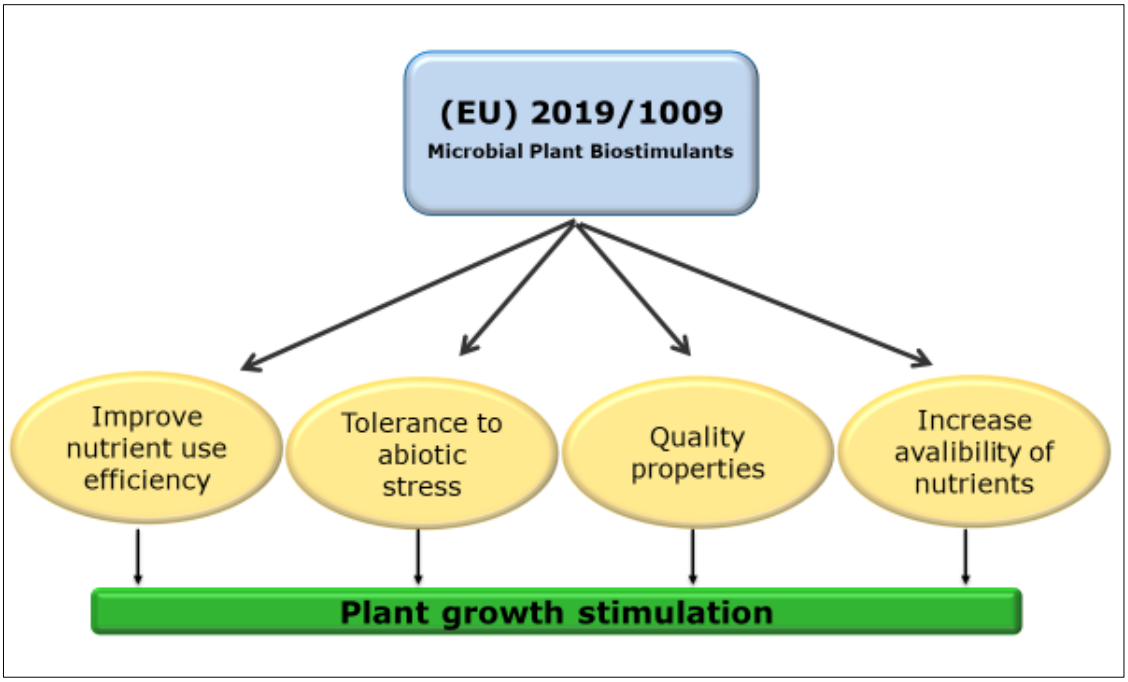


Figure 1. Modes of action of the microbial inoculants considered plant biostimulants (MPBs) regulated by fertiliser regulations (R.D. 999/2017 in Spain and Regulation EU 2019/1009 in Europe)

Before the European Union regulation, several terms were used to denominate those microbial inoculants aimed to improve plant growth by mechanisms different from biocontrol. They were frequently termed 'biofertilisers' (Bhardwaj et al., 2014), 'bioproducts' (Berg et al., 2013), 'plant probiotics' (Flores-Félix et al., 2015), or biostimulants (Barquero et al., 2019). The term biofertiliser has been the most frequently used; however, there have been discrepancies within the scientific community regarding what is considered a biofertiliser. Some scientists have used the term biofertiliser for any type of organic fertiliser with an uncontrolled microbial population – for example, compost and digestates (Diacono et al., 2019). Nevertheless, many others have defined a biofertiliser as a substance that contains a selected population of live microorganisms that under particular conditions do not directly supply any nutrients to crops but can accelerate certain microbial processes in the soil that improve the availability of nutrients in plants (Timmusk et al., 2017).

To avoid confusion to farmers, the European Union regulation does not use the term biofertiliser for microbial products based on selected microorganisms and covered by the regulation 1009/2019. Instead, the term used for such kind of products is 'microbial plant biostimulants' (MPBs); this description

focusses mainly on the demonstration of their effect in the field (Ricci et al., 2019).

1.6. Prospective challenges of microbial plant biostimulants

Due to the growing interest in microbial inputs for agriculture, many companies around the world are increasing investments in MPBs (Bulgari et al., 2019). However, there is still a lack of consistency between the results obtained in the lab and those in the greenhouse and in the field. While several reasons underlie these inconsistences, insufficient research on formulation and application methods could be most important, in addition to not selecting locally adapted strains (Pastor-Bueis et al., 2019). Although the basic research on MPBs is relevant and the technology of bioinoculants holds a promising future, achieving a good formulation with a reliable and consistent effect under field conditions is still a bottleneck that hinders their development (Backer et al., 2018). Therefore, the development of an adequate and effective technology for field application is still essential to achieve commercial development of inoculants. The technological challenges needed to improve the field performance of MPBs are listed and analysed below.

1.6.1. Strain selection for MPB

To achieve effectiveness in field conditions, it is necessary to identify and isolate the bacterial strains most suitable for each crop in each geographical region. Rhizospheres, phyllospheres, and endospheres are the natural sources for isolating plant growth promoting bacteria (PGPB; Singh et al., 2020). To characterise genotypically the complex microbial communities of the rhizospheric and endophytic microbiome, it is very common to perform massive sequencing techniques using the 16S ribosomal DNA gene (16S rDNA), a highly conserved region for bacteria in which bacterial taxonomy is based (Hawkes et al., 2007). However, there is a serious limitation when trying to use the phytomicrobiome as a source of MPBs; it is estimated that from the 1,200 known bacterial taxa associated with the rhizosphere (Kumar et al., 2016), only a maximum of 5% of them could be cultivated under *in vitro* conditions (Mendes et al., 2013), even though they could have a

significant plant growth promoting effect. Despite the existing microbiological diversity, the technology is, for the moment, quite limited in selecting suitable PGPB strains from the phytomicrobiome.

A second limitation to select MPBs from the phytomicrobiome is that strains with proven plant growth promoting qualities may sometimes display poor performance when tested in the field. This is a common situation that usually occurs when the selected microorganisms lack an optimal adaptation to the environment in which they are expected to work (Macouzet, 2016). It has been proved that native strains play a key role in the inoculum success (Koskey et al., 2017); the best MPB products generally comprise autochthonous microbial isolates (Araujo et al., 2020a). Indeed, native strains show a better capacity to interact with abiotic and biotic factors in the soil, due to their physiological and genetic adaptation to the local conditions. Thus, they can be more competitive than allochthonous strains (Martínez et al., 2016).

Last but not least, biosafety for the environment and humans is an essential issue (Selvakumar et al., 2014). Microorganisms applied in sustainable agriculture should guarantee the protection and the sustainability of agri-systems; thus, it is necessary to ensure that they do not have harmful effects. Therefore, agricultural scientists, plant pathologists, and commercial companies involved in inoculant development must collaborate to share knowledge and infrastructures for the characterisation and development of safe MPB (Keswani et al., 2019).

Biological safety levels (BSLs) are specific safeguards designed to protect the environment, animals, and humans from dangerous biological agents. Although the classification of risk in microbial agents may be based of different factors such as the pathogenicity of the organism, modes of transmission, or the microorganism's host range (Selvakumar et al., 2014), there is a guide provided by the World Health Organization (WHO) that proposes classification with four risk groups of infectious organisms. This system indicates the security measures to be carried out according to the risk group to which a microorganism belongs (WHO, 2004). The normative from the European Union restricts the use of microorganisms for agricultural

purposes to those taxa identified as risk level 1, that is, microorganisms that are unlikely to cause human or animal disease.

1.6.2. Inoculant formulation

The formulation of the inoculant is one of the most important factors to ensure the effectiveness of an MPB. However, studies on inoculants have been mainly focussed on obtaining optimal PGPB strains in controlled conditions, neglecting the production process and formulation procedures that would ensure the viability of the inoculum (Barquero et al., 2019). This could be the consequence of the lack of interest from scientific and academic journals, a factor that has discouraged researchers from tackling this type of work (Vassilev et al., 2015), creating a knowledge gap that must be filled (Araujo et al., 2020a).

For a microbial inoculant to be marketable, it must have a suitable shelf life and consistent effectiveness in the field; both depend on a very relevant rate from an adequate formulation (Pastor-Bueis et al., 2019). In field conditions, there are many external factors that are impossible to control; these factors compromise the survival probabilities of the inoculant. Thus, the formulation should preserve the survival of the microorganisms and is key for the dispersion in the volume of soil near the root system (Malusá et al., 2011). In this way, in addition to enabling long-term storage of the final product, the formulation provides easy handling and acts as a delivery vehicle to release live microorganisms that counteract with the plant in optimal conditions (Shaikh & Sayyed, 2015).

1.6.2.1 Carriers

The carrier is quantitatively the main component of the inoculant (by volume or weight) and the most important factor in providing a suitable microenvironment for the microorganism to assure survival and functioning (Thirumal et al., 2017). A good carrier must be considered safe for the environment (Bulgari et al., 2019) and possess a broad spectrum of specific properties (Herrmann & Lesueur, 2013):

a) have adequate physiochemical characteristics such as good moisture, a high water holding and absorption capacity, a good pH-buffering capacity, and an easily adjustable pH;
b) be easy to process and ensure stability, be sterile or easy to sterilise, be amenable to mixing with other compounds (nutrients, adjuvants), be easy to handle and process, and be suitable for as many bacterial or fungal species and strains as possible;
c) optimise the conditions for storage and inoculation to assure a long shelf life; and
d) be economically and environmentally sustainable using low-cost, biodegradable, non-polluting, and non-toxic materials to minimise environmental risks.

Many organic, inorganic, or synthetic substances may be potential solid or liquid carriers. Regarding solid carriers, peat has been widely used to formulate PGPR due to its richness in organic matter (Albareda et al., 2008). However, some countries lack natural peat deposits and the exploitation of peat bogs has caused serious environmental impacts, so its extraction is subject to limitations (Santos et al., 2019). Due to the difficulty using peat as a carrier, other more sustainable and available alternatives have been used as solid carriers, such is perlite (Albareda et al., 2008), vermiculite (Malusá et al., 2011), clay (Vassilev et al., 2015), sugarcane bagasse (Khavazi et al., 2007), and biochar (Araujo et al., 2020a). With regard to liquid carriers, several agro-industrial wastes such as filter mud, wastewater, fly ash (Ben Rebah et al., 2007), and anaerobic digestate (AD; Pastor-Bueis et al., 2017) have been proposed and proven as carriers.

1.6.3. Application methods

Releasing PGPB to field crops entails a limited array of application methods because farmers are not willing to buy specialised equipment for applying microbe-based products. Thus, formulated inoculants must be adapted to the farmers' equipment so that they can be applied using standard farming machinery with simple application methods (Berninger et al., 2018).

Moreover, the inoculant application methods must be adapted to the type of crop. In the case of annual crops, the inoculant is usually disseminated along with seeds, either separately or by coating the seed. The inoculant can be integrated in the seed coating – to generate pre-inoculated seeds – or the mix can be made by the farmers *in situ*. Whilst some authors, such as Atieno et al. (2018), have stated that in the particular case of legume crops pre-inoculation of seeds is the most convenient delivery system, many strains die rapidly after seed coating and drying and the shelf-life rarely is of more than three weeks (Santos et al., 2020). However, survival for more than a year is easily achieved in a solid or liquid inoculant, and for this reason, the best option is *in situ* inoculation by farmers. With this method, the inoculant can be stored and transported separately from the seeds, and it is easier to keep the cold chain because of the reduced space needed.

Another important factor that is necessary to consider to ensure the success of the inoculant application is instructing farmers with the necessary knowledge about the proper use of the product in the field. This will avoid misuse and inconsistent results (Malusá & Vassilev, 2014).

1.6.4. Growth media for bacteria production using a circular economy approach

Industrialisation has induced the accumulation of a large quantity of agro-industrial wastes, which have become one of the major environmental problems faced by the world today; this problem has continued to increase (Anwar & Qamar, 2003). However, bio-residues open new opportunities in a circular economy approach (Rouphael & Colla, 2020), which aims to minimise or eliminate input materials from non-renewable sources in a production system and maximise the reuse of these materials within the same system (Korhonen et al., 2018). Recently, the European Commission (Sustainable Development Goals, 2020) published the 'New Circular Economy Action Plan for a Cleaner and More Competitive Europe'. This proposal calls for an economic model to reach sustainable development. There is increased awareness in Europe that the European Union needs to accelerate the transition towards a regenerative growth model and advance towards maintaining sustainable resource consumption. In the case of agriculture, the

circular economy concept implies the re-design of existing production systems by the promotion of reducing, reusing, and recycling agricultural and agro-industrial wastes (Kirchherr et al., 2017).

Agro-industrial wastes can contain an adequate nutrient composition for bacterial growth. A bacterial growth medium must include a carbon source, water, different salts, amino acids, and nitrogen, all in specific concentrations adapted to the particular developmental necessities of each microorganism (Madigan & Martinko, 2006; Pastor-Bueis et al., 2017). There are several examples of agro-industrial wastes being successfully used as bacterial growth media for commercial production of inoculants, including whey (Caballero et al., 2020), brewer's yeast, bagasse, waste water sludge (Ben Rebah et al., 2007), vegetal wastes (Boraste et al., 2009), and animal sewage (Onyia et al., 2020).

Considering the important economic costs associated with the growth medium in inoculant production (Flores-Félix et al., 2013; Pastor Bueis et al., 2017), the use of recycled materials may be a key point to keep production costs as low as possible because the cost of a residue-based growth media is cheaper than the cost of synthetic media. From the environmental point of view, the re-use of agro-food wastes connects with the principles of the widespread transition to a circular economy (Diacono et al., 2019). Araujo et al. (2020b), using a life-cycle analysis approach, demonstrated that the use of residues for the bacteria production contributes to reduce the carbon footprint and energy demand of MPBs.

1.7. Crop selection for the present research on the optimisation of MPB

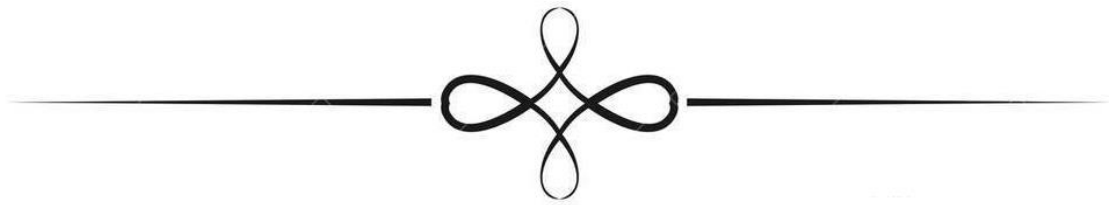
The sweet pepper (*Capsicum annuum* L.) and the common bean (*Phaseolus vulgaris*) are two important global commercial and nutritive crops. On the one hand, the sweet pepper has an important place among vegetable crops because its production has constantly increased in Europe over the last 20 years, reaching in 2018 around 107,000 ha and more than 3 million tonnes harvested, of which almost 40% were produced in Spain (Food and Agriculture Organization Statistics, 2020). On the other hand, the common

bean – with 30.5 million tonnes harvested in 2018, is one of the most important and oldest cultivated crops in the world. Indeed, it is a globally important source of dietary protein (Broughton et al., 2003) and the most consumed pulse in human diets (Baptista et al., 2017).

The sweet pepper and the common bean are two traditional crops in the province of León (Spain). The sweet pepper variety 'morrón' ecotype 'de Fresno' and the common bean variety 'Riñón' belong to the protected geographical indication (PGI) 'Pimiento de Fresno-Benavente' and 'Alubia de la Bañeza-León', respectively. They are two high value-added crops that require research to improve their quality and environmental performance because they have great impact on the agriculture and economy of the region. The present thesis has addressed the agronomic improvement of those two products with the development of effective MPB inoculant formulations, using native endophytic and endosymbiont bacteria specially selected for those crops (Pastor-Bueis et al., 2017, 2019, 2021) with the aim of improving economic profitability to the farmer and the environmental performance of the crop.

Chapter 2

Objectives



The general objective of this work was to design and to test in the field inoculant formulations based on selected autochthonous bacteria, for high value-added legume and non-legume crops, using as a transversal strategy the principles of a circular economy.

For this purpose, the following **specific objectives** were proposed:

1. To demonstrate using 'on farm' conditions with the common bean that an adequate formulation of an autochthonous rhizobium elite strain allows a total replacement of the mineral nitrogen fertilisation by BNF to attain technical and economic viability;
2. To explore, from the genomic point of view, the genetic adaptations of the autochthonous rhizobium strain LCS0306 (*Rhizobium leguminosarum* bv. *phaseoli*, subsequently designated as R) that make it an elite strain for the common bean using 'on farm' conditions;
3. To improve the effectiveness of the inoculant for the common bean based on R using the strategy of co-inoculating with R plus other endophytes or rhizospheric non-rhizobial strains;
4. To explain the superior yield and the superior nitrogen fixation of the common bean co-inoculated with R and the autochthonous endophyte *Pseudomonas brassicacearum* subsp. *neoaurantiaca* (subsequently designated as P), compared with single inoculation with R, using microscopy and analysis of physiological characteristics of the non-rhizobial partner;
5. To assess using 'on farm' conditions the effectiveness of an MPB, based on the autochthonous strain SCFB 3-1 of *Bacillus siamensis* (subsequently designated as B) for the sweet pepper, combined with a reduced nitrogen fertilisation dose; and
6. To analyse the potential of agro-industrial wastes as a growth medium for agricultural bacteria-based inoculant production, according to the principles of a circular economy.

To meet these objectives, the work has been structured in three parts, as shown in the graphical abstract. Two types of MPBs have been used in this work: one endosymbiont and two endophytes. Furthermore, two types of

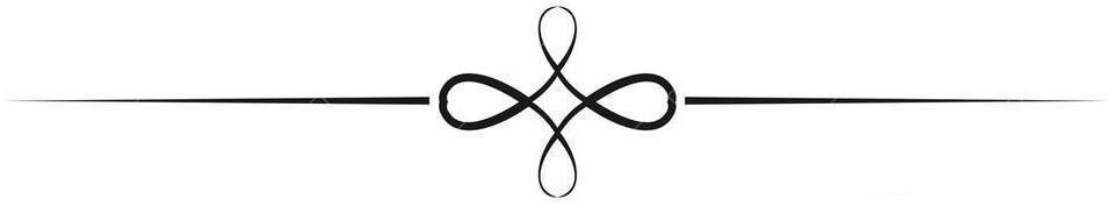
crops have been analysed, legume and non-legume. Table 1 summarises the structure of the present work. It explains the correspondence between each inoculant bacteria, the crop in which it has been used, and the article in which results have been published.

Table 1 Genesis of the three parts of the present work based on the combination of three MPBs belonging to two different types, and two crops.

Inoculant		Crop	Article
Type of MPB	Bacteria name		
Endosymbiont	*Rhizobium leguminosarum* bv. *phaseoli* LCS0306	Legume crops (common bean)	Pastor-Bueis et al. (2019)
Endophytes	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* RVPB2-2		Pastor-Bueis et al. (2021)
	Bacillus siamensis SCFB3-1	Non-legume crops (sweet pepper)	Pastor-Bueis et al. (2017)

Chapter 3

Compendium of materials and methods



This thesis has been developed in the form of compendium of publications. Hence, the materials and methods are specified in detail in the 'Materials and Methods' section of each article (presented in chapters 4 and 5). Nevertheless, the most relevant materials and methods used are indicated below.

3.1 Materials

3.1.1 Bacterial strains

The bacterial strains used in this thesis were the following:

- The authochtonous strain LCS0306 from *Rhizobium leguminosarum* bv. *phaseoli*, which belongs to the IQUIMAB bacterial collection from the University of León (used in Pastor-Bueis et al., 2019, 2021);
- The autochthonous strain RVPB2-2 from *Pseudomonas brassicacearum* subsp. *neoaurantiaca* (P), which belongs to the IQUIMAB bacterial collection (used in Pastor-Bueis et al., 2021);
- The autochtonous strain SCFB3-1 from *Bacillus siamensis*, which belongs to the IQUIMAB bacterial collection (used in Pastor-Bueis et al., 2017);
- *Rhizobium leguminosarum* bv. *viciae* strain UPM791 (used in Pastor-Bueis et al., 2019);
- *Rhizobium phaseoli* ATCC 14482^T (used in Pastor-Bueis et al., 2019);
- *Rhizobium etli* CFN42^T (used in Pastor-Bueis et al., 2019); and
- *Azotobacter chroococcum* Beijerinck 1901 ATCC 9043^T (used in Pastor-Bueis et al., 2021).

3.1.2 Plant material

The common bean cultivar used in Pastor-Bueis et al. (2019, 2021) was 'Riñón', also known as 'Riñón de León'. It is considered the most important cultivar in the cropping area in the PGI 'Alubia de La Bañeza – León'.

The sweet pepper cultivar used in Pastor Bueis et al. (2017) was Maor from FITÓ.

In addition, seeds of lettuce (*Lactuca sativa* L.), radish (*Raphanus sativus* L.), cress (*Lepidium sativum* L.), and tomato (*Solanum lycopersicum* L.) were

utilised to carry out the phytotoxicity test reported in Pastor-Bueis et al. (2017).

3.1.3 Carriers and growth media to produce the bacterial inoculant.

The design of carriers and growth media has been driven by the principles of a circular economy. As such, the individual components of the carriers and growth media were residues or by-products.

Individual components for carriers:

- **Compost** derived from de-alcoholised grape pomace with vinasses of lees and lignocellulosic plant material (Supplementary Table S1 in Pastor-Bueis et al., 2019; p. 127)
- **Biochar** obtained from pine bark by slow pyrolysis in a pilot plant in a semi-continuous, electrically heated reactor as described by Rosas et al. (2015)

Moreover, perlite of non-residual origin was used as the control to compare the rest of the carriers, and it was also used as a component for one of the carriers used in Pastor-Bueis et al. (2019).

The three individual components were combined and four 'combined carriers' were analysed to select the best one (Pastor-Bueis et al., 2019). In Pastor-Bueis et al. (2021), the most successful was used; it comprises 75% pine bark biochar and 25% perlite.

Individual components for growth media

- AD from food and vegetable waste (FVW; Table 1 in Pastor-Bueis et al., 2017; p. 69)
- Sugar beet molasses (Table 1 in Pastor-Bueis et al., 2017; p. 69)

The optimal concentration of the two individual components in the growth media was estimated using response surface methodology (RSM; Pastor-Bueis et al., 2017).

3.2 Methods

3.2.1 Inoculum production and design of inoculant formulations

For small-scale bacterial production, each bacterium was produced in the optimal synthetic medium, the contents of which can be obtained from the corresponding article.

To produce the **inoculant broth** for the pilot field trials, we used a pilot fermenter (Sartorius BIOSTAT Bplus-MO, 5l). The conditions were 28ºC, 10% dissolved oxygen, and a fermentation time from 2 to 5 days, depending on the strain. At the end of the fermentation process, a minimum bacterial concentration of 10^9 colony-forming units (cfu) ml^{-1} was achieved in all cases. For the pilot study, the growth media consisted of residual materials: sugar beet molasses 2.3% v:v and, depending on the strain, another source of nitrogen and other minerals. Details can be obtained from the corresponding article.

To prepare the **solid inoculants**, all the carrier materials were ground with an 80 µm sieve, autoclaved at 120ºC, and aseptically mixed with the inoculant broth and other additives. The carrier: inoculant broth ratio varied according to the moisture retention capacity of each carrier. The specific details of the procedure are explained in Pastor-Bueis et al. (2019, 2021).

In the case of the **liquid inoculant** for sweet peppers, the only processing required for the inoculant broth was to add carrageenan (1% w/v) at the end of the fermentation as an additive to improve the bacterial shelf-life and the inoculant performance in the field (Pastor-Bueis et al., 2017).

3.2.2 Molecular methods

3.2.2.1. Whole genome sequencing

The details about the genome sequencing of *R. leguminosarum* bv. *phaseoli* are presented in Pastor-Bueis et al. (2019).

3.2.2.2. Random amplified polymorphic DNA to calculate the recovery rate of the inoculated strains from the root endosphere and nodules

Random amplified polymorphic DNA (RAPD) is an established polymerase chain reaction (PCR)-based technique, that, despite its obsolescence, is useful for the detection of the presence of specific bacterial strains because it can produce band profiles that are strain dependent. We used it to check the presence of the inoculated strain in the root endosphere and inside nodules in the three papers.

In summary, a representative sample of roots or nodules (as appropriate) was collected (for details, see Pastor-Bueis et al., 2017, 2019, 2021), surface sterilised, and crushed in sterile saline solution or distilled water. Appropriate dilutions were plated onto Petri dishes with the corresponding solid nutritive growth medium, and the plates were incubated at 28ºC. Following this, individual colonies with the typical morphology of the strains *R. leguminosarum* bv. *phaseoli* LCS0306 (Pastor-Bueis et al., 2019, 2021), *P. brassicacearum* subs. *neoaurantiaca* RVPB2-2 (Pastor-Bueis et al., 2021), and *B. siamensis* SCFB 3-1 (Pastor-Bueis et al., 2017), as appropriate, were selected for DNA extraction and RAPD profiling with the M13 primer (5'-GAGGGTGGCGGTTCT-3') following the procedure described by Rivas et al. (2006). The obtained RAPD profiles were compared with those of the pure strains.

3.2.2.3. 16S rRNA sequencing for taxonomic identification

This methodology was applied for the taxonomic identification of *P. brassicacearum* subs. *neoaurantiaca* RVPB2-2. The details about the application of this methodology and about the identification process can be obtained from Pastor-Bueis et al. (2021).

3.2.3 Tests involving plants at different scales

3.2.3.1. Plant assays under controlled conditions: germination and plant growth tests

We carried out two tests with plants under controlled conditions (i.e., growth chamber) during the work; the details are presented in Pastor-Bueis et al. (2017).

First, the potential phytotoxicity of the growth media before sterilisation (AD-m), the growth media after sterilisation (AD-m-ST), and biofertiliser (BF) was analysed using the Zucconi test (Zucconi et al., 1981), which involves germination tests in Petri dishes.

Second, the effect of AD-m, AD-m-ST, and BF on the growth of sweet pepper plants was also tested. They were grown in multi-cell thermoformed seedling trays filled with professional substratum, in a growth chamber under controlled light and temperature conditions. For more details, see Pastor-Bueis et al. (2017).

3.2.3.2. Field experiments

Six different field trials were carried out, a design that gives this work a clear agronomic character. The distribution of the field trials and the correspondence with the objectives of the research are summarised in Table 2.

Table 2. Distribution of the field trials according to the crop, and the research objective (either endosymbionts or endophytes)

Crop	Research objective	Total no. of environments	Years of experiment	Article
Common bean	Endosymbiont	4	2017, 2018, 2019	Pastor-Bueis et al. (2019, 2021)
Common bean	Endophytes	2	2019	Pastor-Bueis et al. (2021)
Sweet pepper	Endophytes	2	2015	Pastor-Bueis et al. (2017)

The design for each experiment followed a statistical pattern of a randomised complete block (RCB) with three replicates. Before establishing the experiment, each plot was fertilised with phosphorus and potassium, with the same dose for all the treatments and controls inside the same plot. The dose

for each plot was calculated taking into account the soil content in each element; in addition, the pH (in the case of phosphorus) and the soil texture (in the case of potassium) were also considered, according to Urbano Terron (2008). Conversely, the nitrogen fertilisation varied according to the different treatments and controls, as described in detail in the corresponding article.

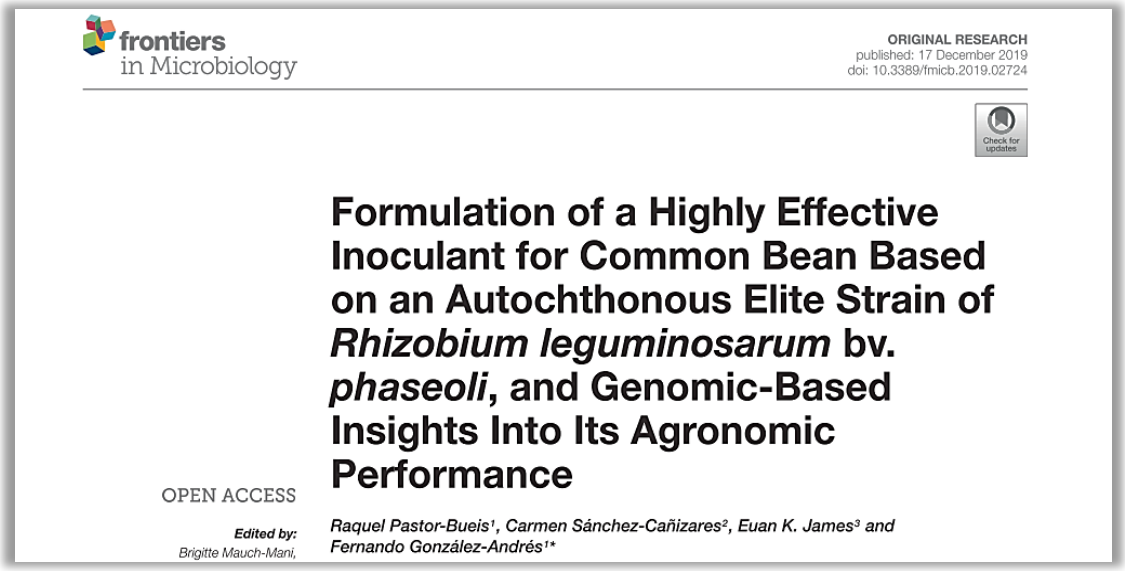
The dependent variables estimated were, in the case of sweet pepper, related to the fruit yield and yield components, and with the physical and chemical characteristics of the fruit, the last of which is related to their quality. In the case of the common bean, we analysed two parameters:

- dependent variables related to the nodule formation and functioning: the number of nodules, nodule biomass, and the nitrogen fixed estimated with the methodology of the natural abundance of ^{15}N and
- dependent variables related to the yield and the yield components.

We statistically analysed the results with analysis of variance (ANOVA), with the specifics explained in each article.

Chapter 4

MPB based on rhizobial endosymbionts for common bean: Field testing of autochthonous strains, formulation optimization and genome mining to explain superiority in field



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Formulation of a Highly Effective Inoculant for Common Bean Based on an Autochthonous Elite Strain of *Rhizobium leguminosarum* bv. *phaseoli*, and Genomic-Based Insights Into Its Agronomic Performance

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Common bean is a poor symbiotic N-fixer, with a low response to inoculation owing to its promiscuous nodulation with competitive but inefficient resident rhizobia. Consequently, farmers prefer to fertilize them rather than rely on their capacity for Biological Nitrogen Fixation (BNF). However, when rhizobial inoculants are based on autochthonous strains, they often have superior BNF performance in the field due to their genetic adaptations to the local environment. Nevertheless, there is scant information at the genomic level explaining their superiority or on how their genomes may influence the inoculant performance. This information is especially important in technologically advanced agri-systems like Europe, where environmental concerns and increasingly stringent fertilizer regulations are encouraging a return to the use of rhizobial inoculants, but based upon strains that have been thoroughly characterized in terms of their symbiotic performance and their genetics. The aim of this study was to design an inoculant formulation based on a superior autochthonous strain, *Rhizobium leguminosarum* bv. *phaseoli* LCS0306, to assess its performance in the field, and to determine the genomic features contributing to the high effectiveness of its symbiosis with common bean. Plants inoculated with the autochthonous strain LCS0306 fixed significantly more nitrogen than those with the allochthonous strains *R. phaseoli* ATCC 14482^T and *R. etli* CFN42^T, and had grain yield similar to the nitrogen-fertilized controls. Inoculation with LCS0306 was particularly efficacious when formulated with a carrier based upon a mixture of perlite and biochar. Whole genome comparisons revealed no differences in the classical symbiotic genes of strain LCS0306 within the symbiovar phaseoli. However, its symbiotic superior performance might be due to its

genomic versatility, as it harbors a large assortment of genes contributing to fitness and competitiveness. It is concluded that inoculation with elite rhizobia formulated with perlite-biochar carriers might constitute a step-change in the sustainable cultivation of common bean in Spanish soils.

Keywords: common bean, Biological Nitrogen Fixation, inoculant biofertilizers, *Rhizobium leguminosarum* bv. *phaseoli*, inoculant carrier, biochar, formulation

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an outstanding pulse crop with more than 35 million ha cultivated per year worldwide (Mulas et al., 2011; FAOSTAT, 2019) and is a globally important source of dietary protein to millions of people (Broughton et al., 2003). Like many legume species, common bean forms root nodules in symbiosis with rhizobia belonging to different genera and species in the Alpha- and Betaproteobacteria (Peix et al., 2015). Within the Alphaproteobacteria, the species nodulating common bean mostly belong to the genus *Rhizobium* but also to other closely-related genera like *Ensifer* (*Sinorhizobium*) and *Pararhizobium* (Mousavi et al., 2015), as well as more distantly related genera like *Bradyrhizobium* (Andrews and Andrews, 2017; Mwenda et al., 2018). Dall'Agnol et al. (2013) has recently reported at least 27 species of common bean-nodulating rhizobia; these include both nitrogen-fixing and non-nitrogen-fixing strains. It has long been known that symbiotic genes, encompassing genes for plant nodulation (*nod*) and nitrogen fixation (*nif*, *fix*), are plasmid-borne in *Rhizobium* (López-Guerrero et al., 2012). Based on the phylogeny of their symbiotic genes, rhizobial strains belonging to the same species in terms of their "core" genomes are defined as symbiotic variants (symbiovars) (Rogel et al., 2014). In order to explain the multiplicity of symbiovars for a single species, it has been proposed that symbiotic genes are transferred between strains by Horizontal Gene Transfer (Andrews et al., 2018) or by mobile integrative and conjugative elements (ICEs) (Haskett et al., 2016). In the case of common bean, several nodulating symbiovars have been reported, namely phaseoli, gallicum, tropici and giardini, linked to *Rhizobium* and *Pararhizobium*, and mediterranense linked to *Ensifer* (Rouhrazi et al., 2016). Due to the numerous rhizobial partners, common bean is considered as a promiscuous legume host under field conditions (Andrews and Andrews, 2017). As a consequence of this promiscuity, common bean is often nodulated by very competitive but inefficient indigenous rhizobia (Graham, 1981; Hardarson, 1993), resulting in poor BNF, which is considered the lowest amongst the most widely grown grain legumes (Martínez-Romero, 2003).

Another consequence of the promiscuous nodulation of common bean is the inefficiency of the inoculants based on allochthonous elite strains, even when they were selected based on their reputation as good nitrogen fixers (Daza et al., 2000; Rodriguez-Navarro et al., 2000). These allochthonous strains are not successful in competition with overall inefficient native rhizobia, potentially due to their lack of adaptation to the local environment (Martínez et al., 2016). To avoid the failure of common bean inoculants, the current trend is the selection of naturally evolved locally sourced rhizobia (Díaz-Alcántara et al., 2014; Martínez et al., 2016; Koskey et al., 2017). These autochthonous symbionts show superior characteristics of competitiveness in nodule infection and occupancy due to their better adaptation to the local agro-climatic conditions (Meghvansi et al., 2010) and to their positive interaction with the resident microbial populations (Tena et al., 2016). Thus, rhizobial strains isolated under local field conditions usually result in successful inoculants, as already reported for various crops (Dall'Agnol et al., 2013), including *P. vulgaris* (Mulas et al., 2011, 2015; Yanni et al., 2016; Zhou et al., 2017).

Rhizobium leguminosarum bv. *phaseoli* LCS0306 (Rlp LCS0306) is indigenous to the Protected Geographic Indication (PGI) "Alubia de La Bañeza-León," which is the region with the most ancient tradition of common bean cultivation and has the largest common bean-cropped area in Spain. Isolated from a root nodule of common bean, it was selected for its high N-fixation effectiveness under hydroponic conditions (Mulas et al., 2011). It was classified as *R. leguminosarum* on the basis of sequences of its *recA* and *atpD* genes (GenBank references JF792210 and JF792197, respectively), belongs to the symbiovar phaseoli and carries the *nodC* γ-allele present in *R. etli* Viking 1 (Mulas et al., 2011). Small-scale field trials in the PGI "Alubia de La Bañeza-León" showed that inoculation with Rlp LCS0306 produced the same grain yield as uninoculated plants given mineral Nitrogen fertilization, confirming that it was adequate for common bean inoculation (Mulas et al., 2011, 2015).

Although an adequately performing strain is an essential prerequisite in the development of successful inoculants for common bean, the non-biological components of formulations are still key bottlenecks in the commercial development of inoculants (Bashan et al., 2014). The use of pre-inoculated seeds is the most convenient delivery system, but while rhizobia survive well in inoculant formulations, many species die rapidly after seed-coating owing to desiccation (Atieno et al., 2018). Currently, the most widespread formulation consists of peat as the rhizobia carrier, plus other additives such as bacterial protectors and adhesives (Bashan et al., 2014; Atieno et al., 2018). However, the lack of natural peat deposits in several countries or their location in preserved areas, taken together with peat being a dwindling non-renewable resource, is driving the search for alternative carriers (Benrebah et al., 2007; Albareda et al., 2008). Perlite was proposed by

Albareda et al. (2008) as an optimal carrier alternative to peat, as well as other mineral or organic carriers (Benrebah et al., 2007; Lugtenberg and Kamilova, 2009; Malusá et al., 2012; Bashan et al., 2014). Among them, biochar (Khavazi et al., 2007; Egamberdieva et al., 2017), and compost (Kumar and Singh, 2001; Arif et al., 2017) have been proposed as outstanding options.

Given the global importance of common bean as a crop, the improvement of its BNF capacity would be advantageous both to the environment and the economy. Currently, in the PGI "Alubia de La Bañeza-León" the BNF ability of common bean is under-used as farmers prefer to fertilize it with ammonium nitrate, which constitutes a significant financial cost (>100 Euros ha^{-1}). Therefore, the general aim of this study was to design a successful inoculant for common bean based on an elite autochthonous strain with an adequate formulation which can result in grain yields that are at least equal to those obtained through current fertilization practices. The study involved first the design of the formulation based on the elite local strain *R. leguminosarum* bv. *phaseoli* LCS0306 (Rlp LCS0306) and bio-based carriers. The agronomic performance of these innovative inoculants was tested in two field trials to appraise the superiority of the inoculant containing the autochthonous Rlp LCS0306 compared to the inoculants based on the type strains of *Rhizobium etli* (Re CFN42^T) and *Rhizobium phaseoli* (Rp ATCC 14482^T), which are allochthonous. As the formulation based on the elite local strain Rlp LCS0306 performed better than the type strains, we then attempted to explain its superiority from a genomic perspective.

MATERIALS AND METHODS

Common Bean Cultivar, *Rhizobium* Strains Used, and Verification of Their Nodulation Ability

Four strains were used in this study: (1) the autochthonous strain Rlp LCS0306 isolated from Sueros de Cepeda located in the PGI "Alubia de La Bañeza-León," as described by Mulas et al. (2011), (2) *R. leguminosarum* bv. *viciae* (Rlv UPM791) (Ruiz-Argüeso et al., 1978), (3) *R. phaseoli* ATCC 14482^T (Rp ATCC 14482^T) and (4) *R. etli* CFN42^T (Re CFN42^T). Rlv UPM791 was included because it showed the highest similarity with Rlp LCS0306 in a genome BLAST comparison, and the other two strains were included as allochthonous controls, because they both belong to sv. phaseoli, a symbiovar that only nodulates legumes in the genus *Phaseolus* (and it is not currently possible to find strains recommended for common bean inoculation in Spain).

The common bean cultivar used was "Riñón" also known as "Riñón de León," the most important cultivar in cropping area in the PGI "Alubia de La Bañeza – León."

Nodulation tests were assessed in a hydroponic experiment under axenic conditions. Five plants were used per strain in independent 1 L pots, filled with sterile washed vermiculite and irrigated from a bottom reservoir with sterile N-free solution (Rigaud and Puppo, 1975). Each plant was inoculated with 1 ml of a suspension of 10^9 cfu ml^{-1} of the corresponding strain. Five additional plants with no rhizobial suspension added were grown as uninoculated controls. The plants were grown in a growth chamber under controlled conditions (16 h light at 24°C and 8 h darkness at 18°C) for 4 weeks.

Inoculum Production and Design of the Inoculant Formulations

The growth medium was Yeast Mannitol Agar (YMA) or broth (YMB) (Fred et al., 1928; Vincent, 1970) for the four *Rhizobium* strains. The liquid inoculum was produced in a pilot fermenter (Sartorius BIOSTAT Bplus-MO; 5 l) at 28°C and with 10% dissolved oxygen for 5 days to achieve a concentration $> 1 \times 10^9$ cfu ml^{-1}. Following centrifugation at 8,000 *g*, the cfu ml^{-1} concentration was increased by one order of magnitude.

The individual components for the carriers were perlite, compost and biochar from pyrolysis. The compost was derived from de-alcoholized grape pomace together with vinasses of lees and lignocellulosic plant material (**Supplementary Table S1**). The biochar was obtained from pine bark by slow pyrolysis in a pilot plant in a semi-continuous, electrically heated reactor. The system for biochar production had an auger furnace (1,400 mm in length × 290 mm inner diameter) with three electric resistances, as described by Rosas et al. (2015). The carriers were the following: perlite (Pe) as control; compost (Co); 94% compost plus 6% biochar, denoted carbo-compost (CC); 25% perlite plus 75% biochar (PB).

To prepare the inoculum, all the carrier materials were ground, passed through an 80 μm sieve, and autoclaved in pots at 120°C for 20 min, except for the carriers with compost, which were autoclaved for 40 min. The inoculum obtained as indicated above was combined with a cell protector, consisting of 1% locust bean plus 1% trehalose (weight:volume) (unpublished data). The cellular suspension was uniformly and aseptically mixed with the carrier, according to the moisture retention characteristic curves of each carrier (data not shown). The final moisture was selected to allow a maximum volume of bacterial culture in the inoculant but providing an adequate consistency in the final mix as follows: 50% for Pe and 33% for Co, CC and PB. Therefore, the theoretical concentration of viable cells per g of inoculant after inoculation was 5×10^9 cfu for Pe and 3×10^9 cfu for the other formulations. After preparation, the inoculants with an available carbon source (Co and CC) were incubated for 15 days at 28°C, and then stored at 4–6°C until the sampling time, whereas those of mineral origin (Pe) or with short-time unavailable carbon sources (PB) were immediately transferred to 4–6°C.

Determination of Bacterial Survival in the Inoculants (Shelf-Life Assessment)

The survival of the strain Rlp LCS0306 was assessed for each formulation at different time intervals (0, 60, 120, 180, 270, and 365 days after inoculum preparation). The obtained information served for a pre-selection of carriers, allowing to reject those which did not have adequate compatibility with the strain. At each sampling date, three samples for each formulation were used

for the inoculum survival analysis. Viable bacteria were estimated by plating 10-fold serial dilutions on YMA plates supplemented with Congo red in duplicate for each sample. The mean values of the viable number of rhizobia per g of inoculant were then calculated for the different times and plotted on a logarithmic scale. One-way ANOVA was used to analyze the effect of the carrier in the bacterial survival at each sampling date, and Tukey test was used for *post hoc* means comparisons.

Field Experiment

Experimental Design

Two field experiments within the demarcation of the PGI "Alubia de la Bañeza-León," were conducted one in 2017 and one in 2018, in two different plots in order to preserve the principles of the existing crop rotation. The plots were more than 45 km away from the place where the strain Rlp LCS0306 was isolated. In the statistical analysis, the field experiment 2017 and 2018 respectively, were considered as the environment. The coordinates of each field, as well as the Edapho-climatic conditions and count of nodulating rhizobia based in the Most Probable Number (Beck et al., 1993), are shown in **Supplementary Table S2**. The sowing and harvesting dates were 16th June – 27th September, respectively, for 2017 and 12th July – 25th October 25th, respectively, for 2018, due to the abnormally high rainfall during June 2018 (**Supplementary Table S2**).

The experimental design followed a statistical pattern of randomized complete blocks with three replications. The experimental unit was a 49 m^2 (7 × 7) plot, with rows 0.5 m apart and a space between plants of 0.15 m. Experimental units were spaced 2 m apart to prevent spread of rhizobia in the soil solution. The six treatments were the following six inoculants: Rlp LCS0306 formulated with the carriers Pe, P-B, Co and CC; Re CFN42^T formulated with Pe, and Rp ATCC14482^T formulated with Pe. Two uninoculated controls were also included, one fertilized with mineral nitrogen (N) and one without. Prior to sowing, seeds were dried in the shade and the appropriate quantity of seeds was then mixed with 2% by weight of the inoculant, plus 1% (weight:volume) of gum arabic solution (40% weight of gum arabic in water) as binder.

Agronomic Practices

Before establishing the experiment, each plot was fertilized with phosphorus (P) and potassium (K), taking into account the soil texture, the soil content of P and K, respectively, and for P, the soil pH, and in accordance with Urbano Terron (2008). The fertilizer rates were calculated for theoretical yields of 3,500 kg ha^{-1}. Hence the P rates, expressed as kg P ha^{-1} were 21 kg ha^{-1} for 2017 and 24 kg ha^{-1} for 2018. All applications were as triple superphosphate (46% P_2O_5, that is 20% P). With regard to K, the plots received 126 kg ha^{-1} in 2017 and 131 kg ha^{-1} in 2018 as KCl (60% K_2O, that is 50% K). The N-fertilized control plot received 170 kg N ha^{-1} which corresponds to the expected total N extraction (Urbano Terron, 2008). Nitrogen-fertilizer was applied as ammonium nitrate (27% N). Half of this amount was applied 5 days before sowing and the other half at the beginning of flowering. The fields were irrigated when necessary, according to the soil moisture content at the time using drip irrigation. The soil was kept free from weeds by mechanical systems. In 2018 the plot received lamda cyalothrin 2.5% WG (20 Days After Sowing, DAS) to control an infection of *Helicoverpa armigera*.

Sampling to Assess Nodulation and Recovery Rate of the Inoculated Strain From the Nodules

At the phenological stage of early pod set, R3 (one pod at maximum length), five central plants from the third row of each treatment and replication were randomly collected for nodulation assessment to appraise the number of nodules per plant and the dry nodule biomass per plant (g). In order to check the presence of the inoculated strain in each of the five plants, one random nodule was surface-sterilized, crushed in sterile distilled water, plated onto YMA and incubated at 28°C for 72 h. Following this, five isolated colonies with the morphology of strain Rlp LCS0306 were selected for DNA extraction and RAPD profiling with the M13 primer (Rivas et al., 2006), which is strain-dependent. The RAPD profile of each colony was compared with the RAPD profile of the pure strain Rlp LCS0306 (Araujo et al., unpublished).

Sampling to Assess Nitrogen Fixation

At the phenological stage of physiological maturity, R7, eight central plants from the fifth row of each replicate plot were randomly collected and oven-dried at 70°C for 48 h. The aerial dry biomass of the common bean plants was expressed as kg ha^{-1}. A sub-sample consisting on a proportional basis of the above ground biomass components was ground at 0.85 mm for ^{15}N isotopic analysis. Non-legume weed species (*Sinapis arvensis* L., *Chenopodirum album* L. and *Oxalis corniculata* L.) from within the plots were collected, processed in the same way, and used as reference plants as a proxy for the ^{15}N natural abundance of plant-available soil mineral N. Isotopic analysis was performed at SIDI (Universidad Autónoma de Madrid). The isotopic composition of plant samples was expressed as $\delta^{15}N_{AIR}$ (‰). Raw data from $\delta^{15}N_{AIR}$ (‰) are in **Supplementary Table S3**. The percent N derived from the fixation of atmospheric N_2 (% Ndfa) by the common bean plants was calculated from the ^{15}N abundance of the legume species and that of the non-fixing reference plant as indicated by Shearer and Kohl (1986) and Unkovich and Baldock (2008). The *B* value was determined as proposed by Pacheco et al. (2017); in the case of cv. Riñón at R7 stage this was found to be −1.97‰.

The N content in the common bean aerial biomass was calculated as: Aerial biomass N (kg N ha^{-1}) = aerial dry biomass (kg ha^{-1}) × N content in the aerial biomass (%); the last was determined using the Kjeldahl method. The amount of N-fixed was calculated as: N-fixed (kg N ha^{-1}) = %Ndfa × Aerial biomass N (kg N ha^{-1}) (Maskey et al., 2001). The soil uptake (kg N ha^{-1}) was calculated as the difference between aerial biomass N (kg N ha^{-1}) and N-fixed.

Sampling to Assess Yield and Yield Components

Sampling at harvesting was carried out in the rows that remained complete after the intermediate samplings described above, leaving at least one untouched row at each edge as a border. The six central meters of the 7th to 13th rows were hand-harvested

at the harvest maturity stage. The yield was recorded as weight of air-dried beans which corresponds to the commercial grain, and then corrected to absolute dry weight after drying the seeds at 80°C to a constant weight. The dry matter of air-dried beans was 88.43%. The yield was calculated from each corresponding 21 m^2 plot, and finally expressed as kg ha^{-1}. The following yield components were also recorded for each plant: (i) number of pods per plant; (ii) number of seeds per pod; and (iii) 100-seeds dry weight in g. Finally, the harvest index (HI) was calculated on the basis of dry matter.

Data Analysis

Analysis of the inoculation treatment

The treatments considered for this analysis were the inoculation with the strains Rlp LCS0306, Re CFN42^T and Rp ATCC 14482^T, formulated with perlite, plus the two uninoculated controls. The year of the experiment was considered the environment. The dependent variables were the parameters about nodulation and N fixation (**Table 1** and **Figure 2**), yield and yield components (**Table 2** and **Figure 3**).

The treatment factor was subjected to Analysis of variance (ANOVA) appropriate to a randomized complete block design for all the dependent variables, considering the year of the experiment as the environment. For those parameters in which the ANOVA detected significant differences, the mean values were compared using the LSD test. A pair-wise correlation analysis between the dependent variables was carried out using the Pearson coefficient.

Analysis of the inoculant formulation

The treatment considered for this analysis was the formulation of the strain LCS0306, with four levels, corresponding to the four different carriers, Pe, Co, CC, and PB. The data were subjected to ANOVA and the means comparison was performed with the Dunnet test, using the formulation Pe as reference for comparison. All the statistical analyses were carried out with IBM-SPSS v.24.

Rlp LCS0306 Genome Sequencing, Annotation and Comparative Genomics

Genome sequencing was performed by MicrobesNG (Birmingham) by Illumina NGS with a coverage of 30%. The reads were trimmed using Trimmomatic (Bolger et al., 2014) and *de novo* assembly was performed using SPAdes 3.7 (Bankevich et al., 2012). Annotation was undertaken using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v4.10 (Tatusova et al., 2016).

Average Nucleotide Identity (ANI) using MUMmer (Delcher et al., 2003) as the alignment algorithm (ANIm) or BLAST (ANIb) was calculated using the JSpeciesWS package (Richter and Rosselló-Móra, 2009). Pairwise comparisons were made between the genome sequences of the strains Re CFN42^T (accessions CP000133.1–CP000138.1, U80928.5), Rp ATCC 14482^T (accessions RJJV01000001–RJJV01000081) and Rlv UPM791 (accessions CP025505.1–CP025510.1) using a custom BLAST database on Geneious 10.0.9 (Biomatters). Clusters of orthologous groups (COGs) of proteins were predicted using the WebMGA server (Wu et al., 2011) and KAAS (KEGG Automatic Annotation Server) for the functional annotation of genes (Moriya et al., 2007). BLAST Ring Image Generator (BRIG) software was used to display circular genome comparisons (Alikhan et al., 2011).

The draft of this whole-genome shotgun project has been deposited in GenBank under the provisional accession no WNKD00000000.

RESULTS

Pre-selection of the Carrier by Compatibility With the Strain Rlp LCS0306 Based on the Bacterial Survival in the Inoculant (Shelf-Life Assessment)

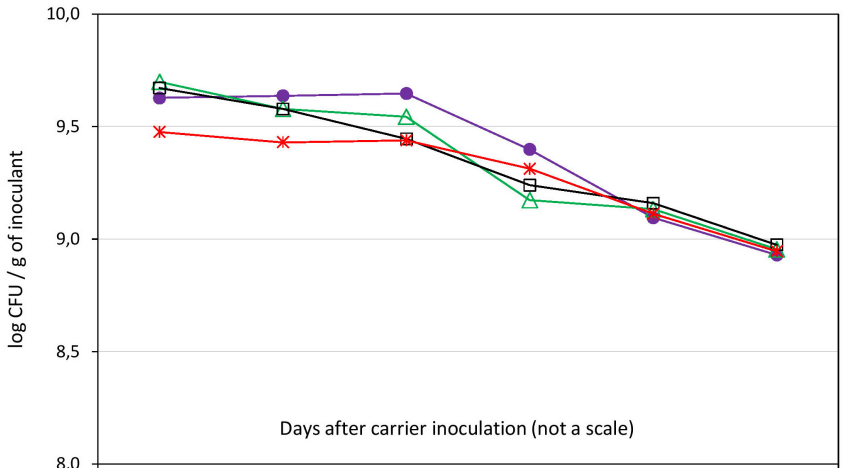
The survival of strain Rlp LCS0306 was evaluated in four different carriers: Pe as control, Co, CC and PB. The initial load of cfu g^{-1} of the inoculant was slightly but significantly higher in Pe and CC than in Co. PB showed the significantly lowest load compared to the rest of the carriers, with 0.22 logarithmic units less than Pe which had the highest value, due to the preparation process (see Inoculum Production and Design of the Inoculant Formulations) (**Figure 1**). All the carriers showed very similar capacities to maintain adequate survival of Rlp LCS0306, with a total loss of viability of 0.75 logarithmic units in the control (Pe), 0.70 in Co and CC, and 0.53 in PB during the whole 365 days period. At 60 days after inoculation, PB showed significantly lower load than the rest, and 120 days after inoculation, three groups were observed: The lowest load was for CC and PB, the intermediate for the control Pe, and the highest for Co. However, from 180 days onward, there were no statistically significant differences in survival in the four carriers. PB formulation showed the most stable values throughout the whole period analyzed.

Effect of the Inoculation With Rlp LCS0306, *R. phaseoli* ATCC 14482^T and *R. etli* CFN42^T on Nodulation, Nitrogen Fixation, Yield and Biomass Production in the Field

Nodulation and Nitrogen Fixation Parameters

The initial nodulation assay under hydroponic conditions showed that the strain Re CFN42^T produced an average of 31.6 nodules per plant, significantly lower than the average number of nodules produced by Rlp LCS0306 (65.6) and Rp ATCC 14482^T (78.6). The latter two strains did not significantly differ in this respect (one-way ANOVA, LSD test $p < 0.05$). These strains were then evaluated under field conditions to compare their symbiotic performance according to various parameters.

In the field, the parameters evaluated were the average number of nodules, dry nodule biomass per plant and symbiotic nitrogen fixation, evaluated by the ^{15}N natural abundance method. The combined ANOVA for all the parameters (year, replication and treatment) is shown in **Supplementary Table S4**. The treatment produced significant differences for all the evaluated parameters



	0	60	120	180	270	365
Compost (Co)	9.63 b	9.64 b	9.65 c	9.40 a	9.09 a	8.93 a
Perlite (Pe)	9.70 c	9.58 b	9.54 b	9.17 a	9.13 a	8.95 a
Carbocompost 6 % (CC)	9.67 c	9.58 b	9.45 a	9.24 a	9.16 a	8.97 a
Perlite 25% - biochar 75% (PB)	9.48 a	9.43 a	9.44 a	9.31 a	9.11 a	8.95 a
F value	159.061***	17.166***	28.609***	3.068 ns	0.420 ns	0.749 ns

FIGURE 1 | Evolution in time of the survival of Rlp LCS0306 in different carrier materials at 4–6°C. Each point represents decimal logarithmic of viable cells g inoculant^{-1} and it is the mean value of three replicas (with two independents counts per replicate). One-way ANOVA has been performed within each sampling date, thus within each column in de data table, and the *F* and significance values (***$p \leq 0.001$, ns not significant) are provided; the values followed by the same letter, within each column, are not significantly different at $p < 0.05$ in the Tukey test.

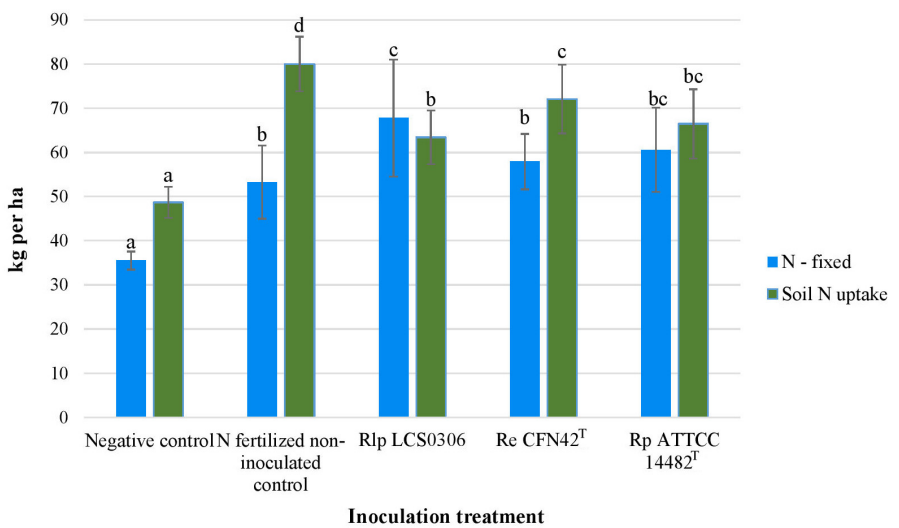


FIGURE 2 | N-fixed and Soil N-uptake of the different inoculant treatments in the field trial. The figure shows the average values from the combined analysis of 2017 and 2018. Data followed by the same letter did not significantly differ at $p < 0.05$ in the LSD test.

($p \leq 0.01$ or $p \leq 0.001$, depending on parameter), except for the number of nodules per plant. There was no significant interaction between the treatment and the environment.

Although unfertilized uninoculated plants were nodulated and fixed some of their N requirements, all the inoculation treatments significantly increased the amount of N fixed, particularly in those plants inoculated with strain Rlp LCS0306 which fixed nearly double the amount of N (**Figure 2**). The nodule biomass was significantly higher in all the inoculated treatments, although the number of nodules per plant did not show any significant difference between treatments and uninoculated controls (**Table 1**). The treatment inoculated with the autochthonous strain Rlp LCS0306, showed the highest values for nodule biomass (1.230 g plant^{-1}), Ndfa (50%)

(**Table 1**) and N-fixed (67.8 kg ha^{-1}) (**Figure 2**), although these values did not statistically differ from those obtained after inoculation with Rp ATCC 14482^T. However, Rlp LCS0306 produced significantly higher values than Re CFN42^T for the three parameters tested, even when Re CFN42^T and Rp ATCC 14482^T did not significantly differ between each other (**Table 1** and **Figure 2**). As expected, the negative controls (uninoculated and non-N-fertilized) produced the significantly lowest values for the amount of N fixed (35.5 kg/ha^{-1}) and the soil N uptake (48.7 kg ha^{-1}), compared to the other treatments (**Figure 2**). The positive control (N-fertilized, uninoculated) showed the lowest Ndfa value (39.2%), differing from neither the negative control (41.1%) nor the treatment inoculated with Re CFN42^T (43.3%) (**Table 1**).

The nodule occupancy was tested in 25 independent nodules per treatment each year. Rlp LCS0306 and Rp ATCC 14482^T showed a high nodule occupancy both in 2017 and 2018. Rlp LCS0306 had a recovery rate from nodules of 84% in 2017 and 72% in 2018, whereas Rp ATCC 14482^T had a recovery of 80% in 2017 and 72% in 2018. However, for Re CFN42^T recovery was lower at 60% in 2017 and 44% in 2018. The bacteria isolated from the root nodules which were not the inoculated strain could be either rhizobia which actively induced nodule formation, or other endophytic bacteria which entered the nodule. In order to elucidate the identity of the other strains isolated, it would be necessary to sequence of specific genes of the unknown isolates, which is out of the scope of the present work.

Yield and Yield Components

The yield and yield components were evaluated at harvest maturity (**Supplementary Table S4**, **Table 2**, and **Figure 3**). The combined ANOVA for all the parameters (year, replication and treatment) showed that the inoculation treatment resulted in significant increase in the grain yield, the number of pods per plant and the number of seeds per pod ($p \leq 0.001$), but not in the 100-seeds weight. The interaction between the inoculation treatment and the year was significantly higher for the yield and all the yield components ($p \leq 0.001$ or $p \leq 0.01$) (**Supplementary Table S4**). Such interactions were due to an exceptionally good performance of Rlp LCS0306 in 2018, compared to 2017 (data not shown). Overall, inoculation with Rlp LCS0306 produced the significantly highest grain yield (3,166 kg ha^{-1}), compared to the inoculation with Re CFN42^T (2,551 kg ha^{-1}) or Rp ATCC 14482^T (2,604 kg ha^{-1}), which did not differ from each other (**Figure 3**). Moreover, the grain yield value obtained for Rlp LCS0306 was similar to that from the N-fertilized positive control (3,050 kg ha^{-1}) and was more than 1200 kg ha^{-1} greater than the uninoculated negative control plants (**Figure 3**). These significantly greater grain yields compared to uninoculated unfertilized plants were a consequence of a higher number of pods per plant and seeds per pod in all the treatments (**Table 2**).

Correlation Analysis

The parameters to estimate nodulation (i.e., nodule biomass and number of nodules) did not show any significant correlation between them (**Table 3**); although the number of nodules was unaffected by the inoculant treatment, the nodule biomass was. Nodule biomass was positively and significantly correlated with the %Ndfa (*R* value 0.7, $p \leq 0.001$) and the N-fixed (*R* value 0.6, $p \leq 0.001$), and weakly correlated with the aerial biomass (*R* value 0.53 in 2017, $p \leq 0.01$ and 0.43 in 2018, $p \leq 0.05$) and the grain yield (*R* value 0.50 in 2017 and 0.42 in 2018, $p \leq 0.05$) (**Table 3**).

TABLE 1 | Nodulation and nitrogen symbiotic fixation indicators for the combined analysis of 2017 and 2018 and inoculant treatments in field trial.

Inoculation treatment	Number of nodules per plant	Nodule biomass (dry) (g per plant)	Aerial biomass (dry) (kg ha^{-1})	Aerial biomass N (%)	Ndfa (%)
Negative control	36.8 a	0.647 a	3428 a	2.45 a	41.3 ab
Re CFN42^T (perlite)	37.7 a	1.002 b	5131 bc	2.50 a	43.3 ab
Rp ATCC 14482^T (perlite)	29.0 a	1.154 bc	4911 b	2.53 a	46.6 bc
N fertilized non-inoculated control	34.0 a	0.512 a	5328 bc	2.35 a	39.2 a
Rlp LCS0306 (perlite)	38.3 a	1.230 c	5592 c	2.58 a	50.0 c

The table contains the mean values for the following treatments: inoculation with the autochthonous strain Rhizobium leguminosarum bv. phaseoli LCS0306, the type strains of Rhizobium etli (Re CFN42^T) and Rhizobium phaseoli (Rp ATCC 14482^T), and the two non-inoculated controls (non-fertilized and N-fertilized). Data followed by the same letter did not significantly differ at $p < 0.05$ in the LSD test.

TABLE 2 | Yield components and HI for the combined analysis of 2017 and 2018 and inoculant treatments in field trial.

Inoculation treatment	Pods per plant	Seeds per pod	100-seeds weight (dry) (g)	HI (dry basis)
Negative control	9.31 a	3.90 a	37.6 a	52.2 bc
Re CFN42^T (perlite)	11.32 b	4.30 b	37.6 a	47.6 a
Rp ATCC 14482^T (perlite)	12.54 c	4.27 b	37.8 a	50.7 ab
N fertilized non-inoculated control	13.20 c	4.43 b	38.9 a	54.9 bc
Rlp LCS0306 (perlite)	13.40 c	4.51 b	40.8 a	59.7 c

The table contains the mean values of the yield obtained for the following treatments: inoculation with the autochthonous strain Rhizobium leguminosarum bv. phaseoli LCS0306, the type strains of Rhizobium etli (Re CFN42^T) and Rhizobium phaseoli (Rp ATCC 14482^T) and the two non-inoculated controls (non-fertilized and N-fertilized). Data followed by the same letter did not significantly differ at $p < 0.05$ in the LSD test.

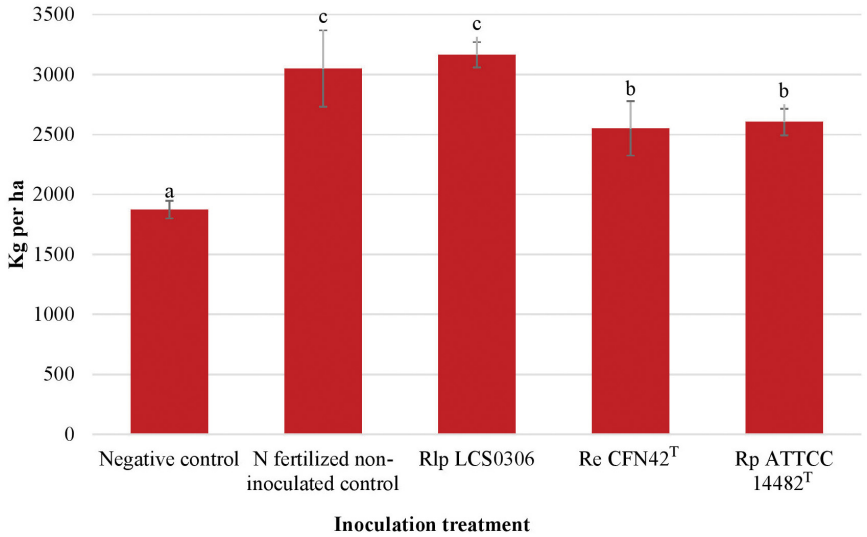


FIGURE 3 | Grain yield (air dried which corresponds to 88.43% dry matter) of the different inoculant treatments in the field trial. The figure shows the average values from the combined analysis of 2017 and 2018. Data followed by the same letter did not significantly differ at $p < 0.05$ in the LSD test.

The correlation coefficients (**Table 3**) showed that, apart from the expected positive correlation between %Ndfa and N–fixed, %Ndfa showed a weak but significant correlation with the aerial biomass (*R* value 0.47 in 2017 and 0.43 in 2018, $p \leq 0.05$) and the grain yield, although in this case, only in 2017 (*R* value 0.51, $p \leq 0.05$). N-fixed showed a significant correlation with the soil N uptake (*R* value 0.54, $p \leq 0.01$ in 2017 and 0.62 in 2018, $p \leq 0.001$), the aerial biomass (*R* value 0.88 in 2017 and 0.80 in 2018, $p \leq 0.001$) and the grain yield (*R* value 0.83 in 2017 and 0.70 in 2018, $p \leq 0.001$).

TABLE 3 | Correlation (R) among: Nodule biomass, symbiotic performance, aerial plant biomass and grain yield.

Parameters		*R* value and significance level	
		Year 2017	Year 2018
Dry nodule biomass (mg/plant)	Number of nodules per plant	0.342 ns	0.222 ns
	Ndfa (%)	0.753***	0.645***
	N – fixed (kg/ha)	0.619***	0.627***
	Soil N uptake (kg/ha)	−0.024 ns	0.190 ns
	Dry aerial biomass (kg/ha)	0.534**	0.433*
	Grain yield (air-dried)[1] (kg/ha)	0.499*	0.420*
Ndfa (%)	N – fixed (kg/ha)	0.730***	0.809***
	Soil N uptake (kg/ha)	−0.163 ns	0.052 ns
	Dry aerial biomass (kg/ha)	0.473*	0.432*
	Grain yield (air-dried)[1] (kg/ha)	0.506*	0.363 ns
N – fixed (kg/ha)	Soil N uptake (kg/ha)	0.541**	0.622***
	Dry aerial biomass (kg/ha)	0.877***	0.800***
	Grain yield (air-dried)[1] (kg/ha)	0.833***	0.701***

*Significance levels: ***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *$0.01 < p \leq 0.05$; ns, not significant.*

Analysis of the Draft Genome of Rlp LCS0306

In order to correlate the agronomic traits and superior performance of the autochthonous strain Rlp LCS0306 with its genetic background, its genome was sequenced and analyzed. The draft genome of Rlp LCS0306 comprises 135 contigs, 7,395,396 bp and 60.72% GC content (**Supplementary Table S5**). For genospecies classification, we compared the LCS0306 genome to the representative strains of *R. etli, R. phaseoli* and the closely related strain, Rlv UPM791. The highest ANI scores were obtained against Rlv UPM791 (**Table 4**, ANIm 98.19%, ANIb 97.39%). As genomes that belong to the same species show genomic ANI values above 95%, the obtained ANI values indicated that Rlp LCS0306 and Rlv UPM791 were members of the same genospecies.

The Cluster of Orthologous Groups (COG) analysis reflected a large number of protein families involved in metabolism (**Supplementary Table S6**). The metabolic network of LCS0306 was constructed by the KEGG automatic annotation server KAAS, confirming that LCS0306 resembles Rlv UPM791, Re CFN42^T and Rp ATCC 14482^T in terms of central metabolism. All the strains harbor the genes encoding the enzymes of the tricarboxylic acid (TCA) cycle, required for aerobic respiration and energy production; the pentose phosphate pathway, required for the oxidation of glucose and the synthesis of nucleotides, and the Entner–Doudoroff pathway,

TABLE 4 | Average nucleotide identity (ANI) comparison.

	Rlp LCS0306	Rlv UPM791	Rp ATCC 14482^T	Re CFN42^T
ANIm				
Rlp LCS0306	*	98.19% [87.14%]	89.06% [65.03%]	88.78% [63.86%]
Rlv UPM791	98.19% [82.87%]	*	88.27% [57.89%]	87.96% [56.97%]
Rp ATCC 14482^T	89.06% [72.60%]	88.27% [67.75%]	*	90.42% [75.80%]
Re CFN42^T	88.78% [72.97%]	87.96% [67.98%]	90.41% [77.59%]	*
ANIb				
Rlp LCS0306	*	97.62% [86.96%]	86.80% [66.73%]	86.45% [66.57%]
Rlv UPM791	97.39% [82.72%]	*	85.64% [60.59%]	85.17% [60.77%]
Rp ATCC 14482^T	87.32% [73.05%]	86.28% [69.09%]	*	88.86% [76.26%]
Re CFN42^T	87.07% [73.53%]	85.87% [70.48%]	89.01% [77.64%]	*
TETRA				
Rlp LCS0306	*	0.99956	0.99728	0.99725
Rlv UPM791	0.99956	*	0.99631	0.9969
Rp ATCC 14482^T	0.99728	0.99631	*	0.99904
Re CFN42^T	0.99725	0.9969	0.99904	*

Values represent the aligned percentage and correlation indexes of their pairwise comparisons: ANIm (ANI using MUMmer), ANIb (ANI algorithm using BLAST), and Tetra-nucleotide signatures. ANI scores obtained from JSpeciesWS with the following genomes: R. etli CFN42^T (Re CFN42^T) (6,530,093 bp; 7 molecules); R. phaseoli ATCC14482^T (Rp ATCC 14482^T) (6,652,103 bp; 81 contigs); R. leguminosarum bv. vicieae UPM791 (Rlv UPM791) (7,837,567 pb; 6 molecules); R. leguminosarum bv. phaseoli LCS0306 (Rlp LCS0306) (7,395,396 pb; 135 contigs).

for the catabolism of glucose to pyruvate. These similarities were reflected in the growth pattern with different carbon sources (**Supplementary Table S7**). A noticeable difference in this assay was the assimilation of a higher number of both carbon and nitrogen sources in the case of LCS0306, which combined the metabolic abilities of Re CFN42^T, Rp ATCC 14482^T and of the strain of *R. leguminosarum* tested, USDA 2370^T, thus highlighting the metabolic versatility of the autochthonous strain.

The ability to persist in the soil and outcompete local rhizobia populations is based on many different parameters. At the genomic level, different traits have been described as having a role in competitiveness, such as motility and chemotaxis, exopolysaccharide (EPS) production, ABC transporters or secretion systems among others. Some of these traits have been analyzed in Rlp LCS0306 to give an overview of its genomic potential in terms of competition (**Supplementary Table S8**) and symbiosis (**Supplementary Table S9**).

Given the proposed role of secretion systems in rhizosphere colonization ability, the secretion systems of the strains were also analyzed in order to explain their competitiveness (**Supplementary Table S8**). Strain Rlp LCS0306 contains a large repertoire of secretion systems that combines those of Re CFN42^T, Rp ATCC 14482^T and Rlv UPM791. For instance, Rlp LCS0306 contains the T1SSd proteins orthologous to the PrsD and PrsE proteins required for biofilm formation. The Rlp LCS0306 genome also harbors a putative T4SS-pili (*virB1–virB11*) system homologous to the cluster in pRlvA, involved in colonization of surfaces in Gram-negative bacteria. Strains Rlp ATCC14482^T and Rlp LCS0306 harbor syntenic *imp* (*tss*) and *hcp* clusters encoding a putative T6SS. As with Re CFN42^T, Rlp LCS0306 contains a homologous T3SS cluster that might also play a role in symbiosis, and the T4SS *traGDCAFBHMR* genes involved in conjugal transfer, followed by the *nocQMT* nopaline transporter, a signal involved in DNA transfer.

In *Rhizobium*, the symbiotic genes, i.e., the genes involved in nodulation and nitrogen fixation are plasmid-borne. When the Rlp LCS0306 genome was aligned against the genome of Rlv UPM791 (**Figure 4A**), the Rlp LCS0306 contigs exhibited not only a high degree of synteny with the chromosomal sequence, but also with the chromid pRlvA and plasmids pRlvB and pRlvE, whereas pRlvD was absent and pRlvC showed a very low sequence conservation. The Rlp LCS0306 genome sequence was then compared against that of Re CFN42^T (**Figure 4B**); the Rlp LCS0306 contigs showed homology to the symbiotic plasmid of Re CFN42^T (p42d), indicating that Rlp LCS0306 contained a putative symbiotic plasmid belonging to the symbiovar phaseoli. As expected from its high efficiency in N-fixation, Rlp LCS0306 harbors all the nodulation and nitrogen fixation genes required to establish a successful symbiotic relationship with *Phaseolus* (**Supplementary Table S9**).

Effect of the Formulation of the Inoculant Containing Strain Rlp LCS0306 on Nodulation, Nitrogen Fixation, Yield and Yield Components of Field-Grown Common Bean

In consideration of previous inoculant formulation designs and the symbiotic performance parameters of Rlp LCS0306, the formulation of the LCS0306-containing inoculant was tested under agronomically realistic conditions in the field. The combined ANOVA for all the parameters combined (year, replication and formulation treatment) is shown in **Supplementary Table S4**. The replication parameter did not produce any significant difference in the dependent variables, and the interaction between replication and treatment was weakly

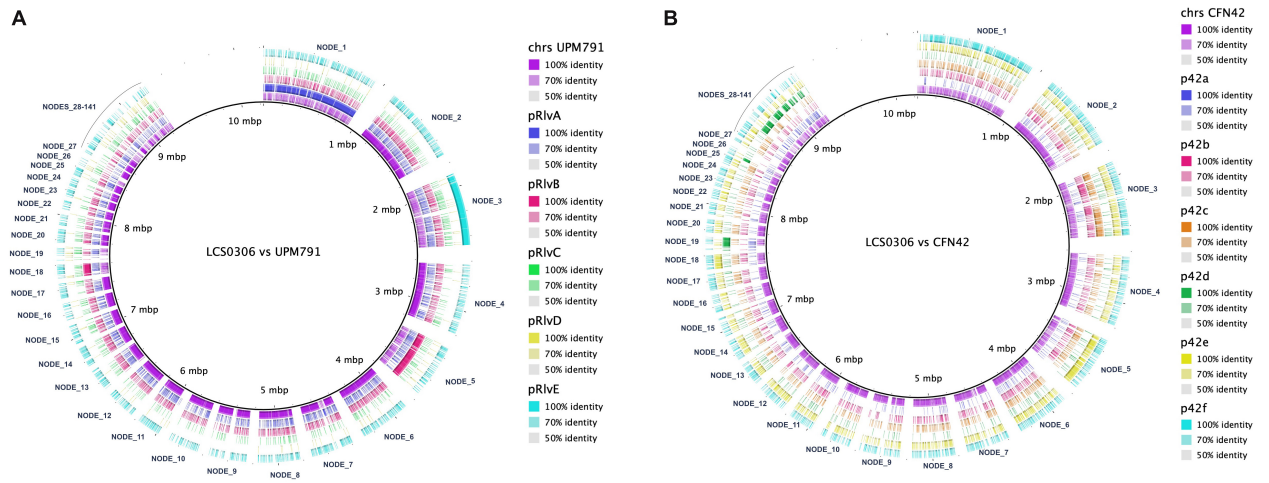


FIGURE 4 | Genome comparison between Rlp LCS0306 and Rlv UPM791 and Re CFN42^T. The figure shows the global synteny between the chromosome and plasmids from the fully closed genomes of Rlv UPM791 **(A)** and Re CFN42^T **(B)** against the contigs of Rlp LCS0306, labeled in the alignment.

significant only for two parameters. Interestingly, the formulation produced significant differences for the amount of N-fixed, soil N uptake ($p < 0.05$ in both cases), grain yield, pods per plant and 100-seeds weight ($p < 0.01$). There was a significant interaction between the formulation and the year for 100-seeds weight ($p < 0.01$) due to the fact that better results were achieved in 2017 for CC formulation and in 2018 for PB formulation.

The formulations with biochar produced significantly higher values for the following parameters, compared to the control (Pe) (**Table 5**): PB formulation produced significantly higher values for soil N uptake, grain yield and pods per plant ($p < 0.01$ in the Dunnet test). Moreover, CC formulation produced significantly higher number of pods per plant and 100-seeds weight ($p < 0.05$). The best performance of the Rlp LCS0306 inoculant was obtained with the PB formulation, with a 15% higher yield compared to the perlite control.

DISCUSSION

Despite the advantages of BNF and other microbial processes in agriculture, the use of bacterial inoculants to provide nutrients to crops or to promote their nutrient use efficiency, tolerance to abiotic stress, or crop quality, is increasing but still not a common practice (Bhardwaj et al., 2014). Especially in developed countries, the easy availability of N-containing mineral fertilizers and the perceived erratic field performance of inoculants, have discouraged farmers from using them (Stamenković et al., 2018; Barquero et al., 2019). In the case of Europe, new rules about fertilizers have been recently approved, such as Regulation (EU) 2019/1009 which pays special attention to the regulation of the use of microbial inoculants targeted to improve crop nutrition. Thus, a surge into the market of this kind of products is to be expected. To avoid the aforementioned erratic performance of inoculants which could generate a significant failure, it is necessary to develop elite inoculants capable of satisfying the increased demand by markets in developed countries. For nitrogen-fixing rhizobia, research must focus on three main aspects: the intrinsic characteristics of the strain, the formulation, and the optimization of the production process by industrial fermentation (Herridge et al., 2008; Herrmann and Lesueur, 2013; Bashan et al., 2014; Checcucci et al., 2017). The present work has focused on the analysis of the determinant genetic factors of the strain, its symbiotic performance and the optimal inoculant formulation, with the aim of designing a successful inoculant for common bean based on autochthonous strains with an adequate formulation explained from agronomic and genomic perspectives.

Regarding the strain, one of the main challenges is the selection of superior rhizobial strains by inferring a high performance only based on their genetic features (Checcucci et al., 2017; Aguilar et al., 2018). It is necessary to separately consider colonization and nodulation abilities from symbiotic nitrogen fixation abilities (Checcucci et al., 2017), as rhizobial strains highly competitive for nodule occupancy do not necessarily fix nitrogen efficiently (Westhoek et al., 2017). Thus, elite strains must combine the two aforementioned capabilities, i.e., effectively compete with the native rhizobia for nodule occupancy and effectively provide the plant with fixed nitrogen (Checcucci et al., 2017; Onishchuk et al., 2017).

The strain Rlp LCS0306 was pre-selected among several isolates on the basis of its N-fixation effectiveness in axenic conditions (Mulas et al., 2011), which optimizes the interaction between the bacterial and plant genotypes in terms of N-fixation (Sessitsch et al., 2002). In the present study, strain Rlp LCS0306 has proven to be a superior strain in the field trial, as shown in terms of symbiotic efficiency (Ndfa, total N-fixed) and most importantly, in grain yield, which was increased by more than 1200 kg ha^{-1} above uninoculated plants. As symbiotic N-fixation abilities are uncoupled from colonization and nodulation abilities (Westhoek et al., 2017), the high

TABLE 5 | Field evaluation of different formulations of the autochthonous strain Rlp LCS0306 from *Rhizobium leguminosarum*.

Formulation of the inoculant	Nodule number per plant	Nodule biomass (dry) (g per plant)	Aerial biomass (dry) (kg/ha)	Ndfa (%)	N fixed (kg/ha)	Soil N uptake (kg/ha)	Grain yield (air dried)[1] (kg/ha)	Pods per plant	Seeds per pod	100-seeds weight (g)	HI (dry basis)
Control (Pe)	38.3	1.230	5592	50.0	67.8	63.4	3165	13.40	4.51	40.8	54.6
CC	31.0 ns	1.099 ns	5509 ns	48.5 ns	67.2 ns	68.4 ns	3281 ns	14.22*	4.40 ns	41.8*	57.2 ns
Co	33.5 ns	1.081 ns	5588 ns	45.5 ns	63.5 ns	73.3 ns	3185 ns	13.76 ns	4.52 ns	39.9 ns	55.0 ns
PB	34.5 ns	1.189 ns	6093 ns	49.3 ns	81.0 ns	79.9**	3640**	14.48**	4.65 ns	41.5 ns	57.3 ns

The table includes the mean values of the parameters related with nodulation, nitrogen fixation, yield, yield components and HI for the combination of years and treatments. ANOVA values are in ***Supplementary Table S4****. The formulation differed in the carrier, with the following options: Control consisting on perlite (Pe); compost (Co); compost 94% in weight plus biochar 6% in weight named as carbocompost (CC); perlite 25% in weight plus biochar 75% in weight (PB). For each dependent variable, mean values were compared to the control formulation with the Dunnet test, with the following significance levels: **0.001 < p ≤ 0.01; *0.01 < p ≤ 0.05; ns not significant. [1]Corresponds to the commercial beans (11.57% dry matter).*

performance of Rlp LCS0306 in the field must be due to the fact that, in addition to being a good N-fixer, it is also competitive. This ability has indeed been confirmed by the high recovery of the inoculant from the sampled nodules compared to the soil-borne rhizobia.

Competing Ability of the Strains Used as Inoculant Treatments

Rhizobia are ubiquitous in the soil, their ability to form nodules in the presence of other strains determine their nodulation competitiveness (Onishchuk et al., 2017). In our field experiment, the resident rhizobia were capable of forming root nodules, which is a common situation for this promiscuous crop (Andrews and Andrews, 2017). Interestingly the number of nodules per plant was similar in the un-inoculated controls and in the inoculated treatments regardless of the strain used as inoculant treatment, which is consistent with the tight control which the plant exerts on the number of nodules (Ferguson et al., 2019). However, the nodule biomass differed between treatments and controls, being significantly higher in the inoculated treatments compared to the uninoculated controls. Moreover, the recovery of the inoculated strains reached the highest value for Rlp LCS0306 followed by Rp ATCC 14482^T. The inoculated strain was recovered in over 75% of the nodules, whereas Re CFN42^T was recovered in only 52% of the nodules of the treatment inoculated with this strain. Indeed, the three strains tested overcame in competitiveness the soil resident rhizobia to varying degrees. Therefore, even if nodule characteristics such as size and biomass partitioning are strongly influenced by the common bean genotype (Rodiño et al., 2011), the rhizobial strain plays an important role in the nodule characteristics, as plants can sanction nodules that are inefficient at fixing nitrogen (Kiers et al., 2003), resulting in inefficient nodules of smaller size (Westhoek et al., 2017). In our experiment, soil-borne strains, which are less efficient than the inoculated ones, have produced smaller nodules, resulting in the observed differences in nodule biomass. Thus, in order to evaluate the nodulation success of a given strain, the number of nodules alone is not a definitive parameter and should be considered along with the nodule biomass and the retrieval of the inoculated strain in the nodules produced by the legume host.

In order to explain competitiveness from a genomic perspective, the presence of secretion systems in Plant Growth Promoting Rhizobacteria (PGPRs) and rhizobial strains has been proposed to play a role in their rhizosphere colonization ability (Gupta et al., 2014). The T3SS, T4SS, and T6SS are generally used to inject effector proteins directly into eukaryotic host cells or into other bacteria, which can mediate compatibility with the host in rhizobia (Nelson and Sadowsky, 2015). Accordingly, Rlp LCS0306 contains a large repertoire of secretion systems, which could explain its competitiveness in field conditions. Strains Rlp LCS0306, Rp ATCC 14482^T, Rl Norway and Rlv 3841 harbor syntenic *imp (tss)* and *hcp* clusters encoding a T6SS (Liang et al., 2018; Sánchez-Cañizares et al., 2018). Impaired T6SS mutants in *R. etli* Mim1 have been shown to generate small and white nodules in *P. vulgaris*, although with similar

nitrogenase activity. The authors suggested a positive role for T6SS in high competition with other soil bacteria, as it was active at high cell density and in the presence of plant exudates (Salinero-Lanzarote et al., 2019). Rlp LCS0306 also harbors the T4SS-pili present in Rlv UPM791 and a putative T3SS, absent in the reference strains Rlv UPM791 and ATCC14482^T. Rhizobia with a functional T3SS (*R. etli* CFN42^T, *S. fredii* HH103, *B. diazoefficiens* USDA110, or *Rhizobium* sp. NGR234) secrete nodulation outer proteins (Nops) in the presence of flavonoids, inducing the transcription of nodulation genes (Jiménez-Guerrero et al., 2017).

Competition has also been discussed from two different perspectives by Onishchuk et al. (2017): exploitative (indirect), involving more effectively utilizing a common limiting nutrient, or by interference (direct), preventing other cells from growing and surviving in the environment. Regarding exploitative competition, bacterial chemotaxis toward exuded compounds is an important trait for root colonization and plant-driven selection of microorganisms (Bais et al., 2006). In particular, the major chemotaxis gene cluster of *R. leguminosarum* bv. *viciae,* Che1, present in Rlp LCS0306, has shown to be essential for competitive nodulation (Miller et al., 2007). The diversity and variety of transport systems in rhizobia reflects the nutritional complexity of the rhizosphere environment (Prell and Poole, 2006) and are, therefore, important for growth and exploitative competition. Accordingly, the Rlp LCS0306 genome contains 183 genes involved in putative ABC transporters, such as the ABC-type broad specificity amino-acid transporter *aapJQMP,* upregulated in bacteroids of both dwarf bean (*P. vulgaris*) cv. Tendergreen (Green et al., 2019); *teuBAC1C2*, required for utilization of root exudates (Rosenblueth et al., 1998), or *nocQMT* and its regulator *nocR*, an uptake ABC transporter for nopaline, which may confer competitive ability (Oger et al., 1997).

In terms of interference competition, one of the strategies is the production of antibacterial compounds, such as bacteriocins (Onishchuk et al., 2017). Production of small bacteriocin appears to be a typical character of all fast-growing rhizobia (*R. leguminosarum*, *R. trifolii* and *R. phaseoli*) (Wijffelman et al., 1983). The quorum sensing system *cinRIS,* responsible for its production (Schripsema et al., 1996) and present in *R. leguminosarum* strains (e.g., 3841, UPM791) and Rp CFN42^T (Wisniewski-Dyé and Downie, 2002) is also conserved in Rlp LCS0306.

Genomic Features Related to the Superior Field Performance of Rlp LCS0306

Although all of the inoculated strains produced higher yields than the native ones, the particularly high N-fixing ability of Rlp LCS0306 in the field has been demonstrated, i.e., Ndfa of 50%, compared to 40% for native rhizobia, and an almost doubling of total N-fixed. Moreover, the inoculation with the strain Rlp LCS0306 produced the same aerial biomass and grain yield as the N-fertilized control. Thus, this autochthonous strain produced considerably higher aerial biomass and yield than the resident strains, and the yield was even higher than in the treatments inoculated with the other strains, hence confirming the agronomic potential of Rlp LCS0306. According to the Observatory of prices of agriculture and livestock products of Castille and León (Spain), the medium sale price of dry beans in the PGI "Alubia de La Bañeza-León" was 100.54 eur 100 kg^{-1} for 2017 and 2018. Thus, in our field trials the increase in the gross income due to the inoculation would have been 1,245 eur in 2017 and 1,352 eur in 2018. Therefore, the present study has indicated that rhizobial inoculation with elite strains like Rlp LCS0306, if applied, could constitute a step-change in the sustainable cultivation of common bean in Spanish soils.

However, the improvement of the grain yield or the aerial biomass produced by the crop as a consequence of inoculation, was only partially explained on the basis of the Ndfa (%) or the Nodule biomass, i.e., the correlation between aerial biomass or the grain yield with the Ndfa or the Nodule biomass was, at the most, weakly significant and not in all the cases there was statistical significance. The aforementioned results indicate that even if the functional link between plant growth and symbiotic functioning proposed by other authors (Belane and Dakora, 2010; Qureshi et al., 2013; Mohale et al., 2014) has been reflected in our experiment, it is not enough to fully explain the positive effect of Rlp LCS0306 inoculation on the crop yield. Indeed, the Ndfa value of 50% indicates that even with a superior strain like Rlp LCS0306 the plant still relies on the soil N-pool for half of its N-requirements. The Ndfa value obtained in our work is similar to that obtained for common bean by other authors, and can be considered low compared to other legumes (Guinet et al., 2018). These latter authors assigned the Ndfa values to the different ability among legume crop species to take up inorganic N from the soil. In the case of *P. vulgaris*, a high inorganic N uptake combined with relatively low values of %Ndfa maximizes N use efficiency (NUE) in soils with relatively high N-levels (a legacy owing to applications of fertilizer to previous seasons non-legume crops), which in turn reduces the risk of nitrogen leaching from the soil.

We then hypothesized that the superior effect of Rlp LCS0306 on the grain yield could be explained by its gene assortment, as it contains a large repertoire of secretion systems as well as the genes involved in an efficient symbiosis with *Phaseolus*. Apart from characterizing the N-fixing genetic machinery of Rlp LCS0306, the genomic analysis has also revealed other data of interest that taken together help to explain its superiority compared to strains Re CFN42^T and Rp ATCC 14482^T. Despite having a genomic backbone homologous to the biovar viciae, Rlp LCS0306 contains the symbiotic repertoire of Re CFN42^T. Rhizobial genomes are extremely variable (MacLean et al., 2007), with large replicons called chromids that appear to contain genus-specific genes in *Rhizobium*, *Ensifer* and *Agrobacterium* (Harrison et al., 2010) and secondary replicons, like symbiotic plasmids, that are generally more genetically diverse between strains than the primary chromosome (Galardini et al., 2013). Indeed, the largest contig in LCS0306 (NODE_1) shows a high degree of synteny compared to pRlvA chromid in Rlv UPM791 (as shown in **Figure 4**), which was in turn highly similar to

pRL12 in Rlv 3841 (Sánchez-Cañizares et al., 2018). In general, the Rlp LCS0306 genome sequence showed ANI values above 95% when compared with Rlv UPM791 (**Table 4**), indicating that they share most of their genome content. For example, as with Rlv UPM791, LCS0306 only harbors an ortholog of the type I PHB synthase, *phbC1*, required for free-living poly-β-hydroxybutyrate (PHB) biosynthesis, a carbon polymer that seems to play a role during root infection and invasion (Trainer and Charles, 2006). Strain Rlp LCS0306 also harbors the two *fnrN* copies controlling the expression of the *fixNOQP* genes present in Re CFN42^T and Rlv UPM791 (Colombo et al., 2000; Lopez et al., 2001). Strain Rlp LCS0306 has three *nodD* copies, as has the p42d symbiotic plasmid from Re CFN42^T, again highlighting the commonalities with the *R. etli* symbiotic plasmid. *NodD* regulates the expression of the *nodABCFE* cluster and, therefore, it is involved in Nod factor production. Similarly, five *nodD* reiterations were found in *R. tropici* CIAT899, necessary to engage the symbiont in nodulation with different legume species (Del Cerro et al., 2015). These *nodD* reiterations were also present in various different N-fixing rhizobial strains from *P. vulgaris,* suggesting a potential role in host range (Peralta et al., 2016). Sequence heterogeneity within p42d already suggested extensive genomic rearrangements, recombination rates, lateral transfer, and relaxation or intensification of selective pressures (González et al., 2003). This might have been the case in Rlp LCS0306 at the genome level, incorporating all those features that might impact positively on its competitiveness and symbiotic performance, thus resulting in a strain with outstanding agronomic properties.

Effect of the Formulation

Once the superior behavior of Rlp LCS0306 was reinforced by its genomic potential, in order to design an inoculant based on this elite strain, the next step was to determine the optimal formulation to be applied as an inoculant under field conditions. Compared to the control formulation based on perlite, the PB formulation based on perlite and biochar produced a significantly higher number of pods per plant (14.48 versus 13.40 in the control) and also a significantly higher grain yield (3640 kg ha^{-1} versus 3165 kg ha^{-1} in the control). Interestingly, this was not accompanied with an increase either in the Ndfa (%), N-fixed in kg ha^{-1}, nodule number, or nodule biomass compared to the other formulations used in the experiment. Thus, the improvement in field performance of the PB formulation suggests that it is related to the plant growth promoting effect assigned to biochar (Yang et al., 2019), rather than to a direct effect on the strain performance as a consequence of the formulation. The plant growth promoting effect of biochar can be explained in terms of hormone analogs contained within it (Graber et al., 2010), inducing the expression of certain genes related to plant growth (Huang et al., 2015; Mehari et al., 2015; Yang et al., 2019).

CONCLUSION

The results obtained in this study explained the success under field conditions of an outstanding inoculant for common bean based on the autochthonous strain Rlp LCS0306 when it has been appropriately formulated, in terms of its symbiotic performance, genomic features and agronomic traits. Overall, the superior performance of strain Rlp LCS0306 appears to be due to the combination of different modes of action, which together produced a significantly higher grain yield and a high rate of recovery from nodules, compared to the resident rhizobia and to other common bean-nodulating strains like Re CFN42^T and Rp ATCC 14482^T. From an agronomic perspective, Rlp LCS0306 is both a highly efficient N-fixer, which is competitive against the native rhizobia, as well as providing common bean with a stimulus to enhance its NUE. From the genomic perspective, the competitive behavior could be explained by its broad metabolic capacities and the large variety of its secretion systems. On the other hand, the enhanced yield obtained in the field with Rlp LCS0306 could be partially explained to some extent in terms of Ndfa (%) and nodule biomass. However, there may be other factors contributing to the superiority of LCS0306 in field trials, potentially derived from its gene assortment, as the strain harbors a *R. leguminosarum* scaffold with a symbiotic plasmid characteristic of strains nodulating *Phaseolus*, containing the genes required for an efficient symbiosis. Rlp LCS0306 has evolved in the local conditions of northern central plateau in Spain, and it has gathered several genomic characteristics as enumerated above, putatively involved in its adaptation to such local conditions. The ensemble of its diverse genomic characteristics, rather than a specific characteristic itself, seems to contribute to the superior performance of Rlp LCS0306 in the field. To date, cultivation of common bean with the available commercial inoculants has resulted in a suboptimal nodulation and BNF, as this crop is not native to Europe. This study constitutes the first evidence of a native inoculant enhancing BNF and grain yield in common bean in Spain, stressing its economic value for the future sustainable cultivation of this important crop.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI BioProject no. PRJNA552714.

AUTHOR CONTRIBUTIONS

RP-B executed the field trial, collected the data and wrote the manuscript. CS-C analyzed the genomic data and wrote the manuscript. EJ worked on the general structure and the integration of the different parts. FG-A designed the field trial, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02724/full#supplementary-material

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Chapter 5

MPB based on non-rhizobial endophytes for legume and non-legume crops: Field testing of autochthonous strains, microscopy insight on nodule colonization (legume) and inoculant production (non-legume)


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Yield response of common bean to co-inoculation with *Rhizobium* and *Pseudomonas* endophytes and microscopic evidence of different colonised spaces inside the nodule

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Innovative liquid formulation of digestates for producing a biofertilizer based on *Bacillus siamensis*: Field testing on sweet pepper

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ABSTRACT

Microbial inoculants are gaining prominence in technologically advanced agri-systems due to the need for alternatives to the most pollutant agricultural inputs. The objective of this work was to improve the agronomic performance of the rhizobial inoculants for common bean, based on the superior native strain Rlp-LCS0306 of *Rhizobium leguminosarum* bv. *phaseoli* (R), through co-inoculation with non-rhizobial partners, namely the autochthonous isolate RVPB2-2 from *Pseudomonas brassicacearum* subsp. *neoaurantiaca* (P) and the type strain of *Azotobacter chroococcum*. It has been reported that co-inoculation improves nodulation, nodule functions and plant growth, although there is a lack of field testing in technologically advanced agri-systems. This work bridges this gap. In the field trial which was carried out in two different environments, the consortium R + P was the most successful, because it increased the N_2 fixation by 51.7 kg ha^{-1} (87 %) and the yield by 1337 kg ha^{-1} (59 %), compared with the uninoculated and unfertilised control. In addition, the increased yield observed following inoculation with the above indicated consortium was 16.7 %, compared with the single rhizobia inoculation, and this increase was also superior to that observed with other consortia. The superiority of the R + P consortium could partially be explained because in this study, there was an increased tendency for improved nodule biomass and function following co-inoculation. While this increase was not deemed to be statistically significant, it is noteworthy that nodule biomass increased by 25 % in average and N-fixed by more than 20 %, which, in turn, could be explained by the indole-3-acetic acid (IAA) production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity of the P strain. However, further delineation of the system is required in order to explain the yield improvement exerted by the consortium. Here, we observed, i) the strong plant growth-promoting potential displayed by the P strain; ii) the colonisation of the nodules by the P strain; and iii) the strategy of colonisation of complementary spaces inside the nodules by P (intercellular) and the rhizobia (intracellular), by confocal microscopy.

1. Introduction

Sustainability is increasingly essential in agroecosystems worldwide due to the need to feed a population that is continuously growing (FAO, 2019). The Green Revolution of the 20th century enabled unprecedented gains in agricultural production but led to an uncontrolled increase in the use of some of the technological advances associated with a high environmental costs; this is the case for pesticides, herbicides and mineral fertilisers (Backer et al., 2018). In order to meet the sustainability criteria, a second green revolution is currently underway; it aims to maintain improved crop yields while reducing the levels of chemical inputs and substituting them with biological inputs (Besset-Manzoni et al., 2018). For this reason, the new green revolution is labelled the bio-revolution, and it is based, at least in part, on the rational exploitation of the phytomicrobiome (Timmusk et al., 2017), which is already enabling a partial substitution of synthetic products by microbial

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inoculants (Backer et al., 2018; Bhardwaj et al., 2014).

From all the associations between crops and their phytomicrobiome, the best understood and characterised, as well as the best exploited in agriculture, is the symbiosis between legumes and nitrogen-fixing rhizobia (Herridge et al., 2008). This symbiosis provides legume crops with significant amounts of the N required by the crop, allowing important savings of mineral N fertilisers (Saikia et al., 2017), and thus legumes are considered beneficial for the mitigation of climate change (Jensen et al., 2012). Moreover, legumes are high-protein plant foods, and they have great protein delivery energy efficiency, therefore legume-based diets are considered a sustainable option in the present scenario of a growing population (Sabaté and Soret, 2014). Pulses represent 27 % of the world crop production, and common beans (*Phaseolus vulgaris* L.) are the most consumed pulses in human diets worldwide (Baptista et al., 2017), with almost 35 million ha and more than 30 million t of grains produced (FAOSTAT, 2020). The common bean is an excellent and sustainable source of protein, essential amino acids (mainly lysine) (Kan et al., 2018), dietary fibre, minerals, vitamins and phytochemicals (Dwivedi et al., 2015; White and Broadley, 2009).

Common beans form root nodules with at least 27 nodulating rhizobia belonging to several genera and species of Alpha- and Beta-proteobacteria (Peix et al., 2015); moreover, such rhizobia belong to at least five different symbiovars (Rouhrazi et al., 2016). The nodulating rhizobia include nitrogen-fixing strains, but also several non-nitrogen-fixing or very inefficient nitrogen-fixing strains (Dall'Agnol et al., 2013). Because of this, the crop is often nodulated by very competitive but inefficient native rhizobia (Andrews and Andrews, 2017), which frequently results in poor biological nitrogen fixation (BNF) (Martínez-Romero, 2003). Thus, inoculation with selected effective strains is generally necessary in order to achieve a significant BNF. However, the inefficiency of inoculants based on allochthonous elite strains, due to their failure in competition with the native inefficient rhizobia and as a consequence of their lack of adaptation to the environment, has also been reported (diCenzo et al., 2019; Martínez et al., 2016). Fortunately, it has been proved that the use of native-naturalised rhizobia, selected for their high N fixation efficiency, results in successful inoculants as long as they are adequately formulated (Araujo et al., 2020a; Koskey et al., 2017). The advantage of autochthonous rhizobia lies in their better competitive ability for nodule occupancy (Irisarri et al., 2019). An inoculant formulation for common bean was previously developed and optimised for the Protected Geographic Indication (PGI) "Alubia de la Bañeza-León" (León, Spain), using the elite strain LCS0306, which was identified as *Rhizobium leguminosarum* bv. *phaseoli* (R) (Mulas et al., 2011; Pastor-Bueis et al., 2019). This strain was isolated from a root nodule of the common bean, in a field located at the mentioned PGI (Sueros de Cepeda, León, Spain). This strain was selected, among other isolates, for its high N fixation efficiency by Mulas et al. (2011). Later, Pastor-Bueis et al. (2019) demonstrated that its effects towards a successful agronomic performance were not only due to efficient N fixation, but also due to genomic versatility, as a consequence of harbouring a large assortment of genes contributing to fitness and competitiveness.

Currently, inoculants are bursting onto the agriculture inputs market worldwide (Keswani et al., 2019). Especially significant is the interest aroused by inoculants in technologically advanced agri-systems like Europe, where regulation of the inoculants market has been recently laid down by Regulation (EU) 2019/1009, which ensures the safety and quality of the products. To satisfy the demanding European market for agrarian inputs, microbial inoculants must be engineered to optimise their efficiency. In addition to the selection of efficient autochthonous rhizobia and the use of an adequate formulation (Araujo et al., 2020a), an interesting strategy to improve the effectiveness of rhizobial inoculants for legumes is to add a non-rhizobial bacterial co-inoculant (Menendez and Paço, 2020). Legume root nodules are an optimal habitat not only for N-fixing rhizobia but also for many other non-N-fixing microbial residents that are able to occupy different spaces in the nodule structure (Martínez-Hidalgo and Hirsch, 2017). It has been demonstrated that the simultaneous infection with rhizobia and other bacteria also present in nodules can enhance plant growth in a wide variety of legumes (Benito et al., 2017).

Starting from the hypothesis that co-inoculation of legumes with rhizobial plus non-rhizobial strains could improve nodule performance compared with single rhizobial inoculation, the general objective of this work was to explore, from an agronomic point of view, the effectiveness of common-bean inoculants consisting of a consortium of rhizobial plus non-rhizobial bacteria. The specific objective was to improve the effectiveness of the rhizobial inoculant based on the elite strain LCS0306, searching for the optimal combination of this strain with non-rhizobial strains, namely a *Pseudomonas* strain (which is cited in this work for the first time) and an *Azotobacter* strain. The agronomic performance of the strain combinations was compared with that of a single rhizobia inoculant and also of the uninoculated controls, with and without N fertilisation. All field trials were carried out using an appropriately formulated inoculant. The parameters used to evaluate the agronomic performance were those related to nodulation, N fixation and crop yield. Following this, the reasons for the agronomic superiority of the best bacteria combination (in terms of crop yield) were analysed, and using confocal microscopy, the location of the consortium partners within the nodules were identified.

2. Materials and methods

2.1. Common bean cultivar, bacterial strains used, bacterial culture media and characterisation of the novel strain

The common bean cultivar was "Riñón" also known as "Riñón de León". It is one of the four authorised local varieties in the PGI "Alubia de La Bañeza-León" (Spain). It shows an upright growth habit, with the weight of 100 seeds ranging between 32 and 45 g.

The strains selected for this work were: (1) rhizobial strain LCS0306 (R) (see introduction section for more details); this strain belongs to the IQUIMAB bacterial collection (University of León, Spain); (2) the type strain from *Azotobacter chroococcum* Beijerinck 1901 (ATCC 9043^T, Skerman et al., 1980) (A), purchased from the Spanish Type Culture Collection (accession no. CECT 4103); (3) the strain RVPB2-2 from *Pseudomonas brassicacearum* subsp. *neoaurantiaca* (P), which is a root endophyte isolated from common bean at the PGI "Alubia de La Bañeza-León" (Riego de la Vega, León, Spain) and is reported here for the first time; this strain belongs to the IQUIMAB bacterial collection. The culture media used to grow the strains were the following: Yeast Mannitol (YM) (Vincent, 1970) for R; Triptic Soy (TS) (Millipore reference number 22092) for P; and Ashby's Glucose (Rao, 1977) for A. Incubations were at 28 °C for five days (R and A), and for two days (P).

For the taxonomic identification of strain P (described here for the first time), the 16S rRNA gene was sequenced. Amplification and sequencing of the 16S rRNA gene was performed by Macrogen (The Netherlands), using the primers and conditions previously described by Marcano et al. (2016). The obtained sequences were processed, deposited at GenBank (Benson et al., 2013) (accession no. MT212725) and compared with those from EzBioCloud database, which contains the type strains of all described bacterial species; then the phylogenetic tree with the closest type strains was inferred. Four plant growth promoting activities, namely phosphate solubilisation, siderophore production, ACC-deaminase activity and IAA production were measured as indicated by Marcano et al. (2016) in strains A and P.

2.2. Field trial

2.2.1. Inoculant production

The liquid inoculum was produced in a pilot fermenter (Sartorius BIOSTAT Bplus-MO 5 L) at 28 °C with 10 % dissolved oxygen. The growth media consisted of 2.3 % vol.:vol. of sugar beet molasses with a

composition described by Pastor-Bueis et al. (2017). The medium for the rhizobial strain R had 1.5 % vol.:vol. of beer vinasse, in addition to sugar beet molasses. The primary inoculum was 0.5 % vol.:vol. of a 1×10^9 cfu mL^{-1} bacterial suspension produced in YM (for R), TS (for P) and Ashby's Glucose (for A) broth, as indicated in Section 2.1. The incubation time was five days at 28for R and A and two days for P, to achieve a concentration $> 1 \times 10^9$ cfu mL^{-1} by the end of the fermentation process. Following centrifugation at 8000 *g*, the cfu mL^{-1} was increased by one order of magnitude. The exact cfu mL^{-1} obtained in the liquid inoculum after centrifugation was measured with decimal dilutions and plated onto Petri dishes containing the solid media required for each bacterial strain (YM-agar, TS-agar and Ashby's glucose-agar). The concentrations were adjusted by dilution with sterile distilled water to the following: for single inoculation, 3×10^9 cfu mL^{-1}; for double co-inoculation, 6×10^9 cfu mL^{-1} and for triple co-inoculation, 9×10^9 cfu mL^{-1}. The resulting bacterial suspensions received two cells protectors, a polysaccharide and a disaccharide, as indicated by Araujo et al. (2020b).

The final inoculant was prepared by mixing 33 % of the liquid inoculant with 66 % of the solid carrier (volume:weight). In the cases of co-inoculation, the volume of the liquid inoculant for each strain was identical for all of them and altogether formed the mentioned 33 %. The solid carrier consisted of 25 % perlite plus 75 % pine bark biochar, as described by Pastor-Bueis et al. (2019), and the procedure for the inoculant preparation is described in the same study. In this way, the final concentration of each individual strain, either in single inoculation or co-inoculation, was always 1×10^9 cfu g^{-1}.

2.2.2. Field experimental design

There were two experimental environments established in 2019 in fields located within the demarcation of the PGI "Alubia de la Bañeza-León", namely the experimental fields located at the Agrarian School (EIAF) (University of León, Spain) and Armunia municipality (León, Spain), respectively. The climatic conditions of the experimental fields are shown in Table 1, and the coordinates of each field, as well as the soil data and the count of nodulating rhizobia based on the most probable number, are shown in Table 2. The sowing and harvesting dates were 22nd May–3rd September for the EIAF field and 24th May–6th September for the Armunia field.

The experimental design for each of the two environments followed a statistical pattern of a randomised complete block (RCB) design, with three replicates. The analysed factor was the inoculation treatment with one of the following eight options: i) inoculation with R; ii) co-inoculation with R and P (R + P); iii) co-inoculation with R and A (R + A); iv) co-inoculation with R, P and A (R + P + A); v) co-inoculation with P and A (P + A) vi) uninoculated and full N-fertilised control; vii) uninoculated and 80 % N-fertilised control viii) uninoculated and non-N-fertilised control. All the inoculated or co-inoculated treatments did not receive any mineral N fertilisation.

Table 1
Climatic data during the field experiments, recorded at the León - Virgen del Camino provincial meteo station.

Date	Temperatures (°C)[a]					Monthly rainfall (mm)
	Hmax (°C)	Havg (°C)	Tavg (°C)	Lavg (°C)	Lmin (°C)	
May 2019	29.5	21.1	13.1	5.2	1.0	8.5
June 2019	35.7	23.3	16.3	9.3	1.2	32.1
July 2019	34.5	28.6	20.9	13.2	9.9	23.7
August 2019	31.6	27.6	19.9	6.7	12.2	8.0
September 2019	29.2	23.7	16.7	9.7	4.0	31.4
October 2019	26.9	18.1	12.7	7.3	2.8	58.6

[a] Hmax: maximum high temperature) (°C); Havg: average high temperature (°C); Tavg: average mean temperature (°C); Lavg: average low temperature (°C); Lmin: minimum low temperature (°C).

Table 2
Soil data corresponding to the experimental fields. Samples were taken in April 2019.

Parameter	Units	Location	
		EIAF	Armunia
Latitude	–	42°34′55″N	42°36′51″N
Longitude	–	5°35′27″W	5°36′01″W
Texture	Sand (%)	40	25
	Silt (%)	38	45
	Clay (%)	22	30
pH 1:2 (soil: water)	–	7.43	6.90
Electric conductivity	(dS/m)	0.04	0.14
Organic matter	(%)	2.75	3.05
Total nitrogen[a]	(%)	0.20	0.31
Ratio C/N	–	7.15	9.55
Lime	(%)	negligible	negligible
P - Olsen	(mg kg^{-1})	13.94	20.1
K	(cmol (+) kg^{-1})	0.15	0.51
Ca	(cmol (+) kg^{-1})	13.02	15.27
Mg	(cmol (+) kg^{-1})	1.46	3.38
Na	(cmol (+) kg^{-1})	0.04	0.21
Nodulating rhizobia count (MPN)[b]	Rhizobia g $soil^{-1}$	1.7×10^4	1×10^3

[a] Total N: organic + nitric + ammonia nitrogen.
[b] Most Probable Number.

The mineral fertilisation was as follows: Before establishing the experiment, the corresponding plot was fertilised with phosphorus (P) and potassium (K). The fertiliser rates were calculated for a theoretical expected yield of 3500 kg ha^{-1} following the methodology indicated by Urbano Terron (2008) which also considers the soil characteristics shown in Table 2; hence the plots received a dose equivalent to 74 kg ha^{-1} (EIAF) and 54 kg ha^{-1} of P (Armunia), in the form of triple superphosphate (46 % P_2O_5, therefore 20 % P). Regarding the K, the plots received a dose of 140 (EIAF) kg ha^{-1} and 117 kg ha^{-1} (Armunia) in the form of KCl (60 % K_2O, therefore 50 % K). The non-inoculated and full N-fertilised control plot received a dose equivalent to 187 kg N ha^{-1}, which corresponds to the expected total N extraction for a theoretical yield of 3500 kg ha^{-1} (Urbano Terron, 2008). N fertiliser was applied as ammonium nitrate (27 % N). Half of this amount was applied five days before sowing and half at the beginning of flowering. The non-inoculated and 80 % N-fertilised control plot received 80 % of the aforementioned amount. The non-inoculated, non-N-fertilised control plot and the inoculated treatments did not receive any dose of N.

The experimental unit was a 25 m^2 (5 × 5 m) plot, with rows 0.5 m apart and a space between plants of 0.15 m. The experimental units were spaced 1.5 m apart to prevent spread of the inoculated bacteria in the soil water.

Prior to sowing the seeds, the appropriate quantity of seeds was mixed with 2% by weight of the inoculant plus 1% (volume:weight) of arabic gum solution as a binder. Seeds were dried in the shade and then manually sown.

The soil moisture content was assessed daily, and irrigation was applied when necessary, using a drip irrigation system. After the flowering stage, the two fields suffered an infection with *Tetranychus urticae*; therefore, each plot received the dose equivalent to 1.5 L ha^{-1} of Fenpyroximate 5.12 % in order to control the infection. The soil was kept free from weeds by mechanical methods.

2.2.3. Sampling to assess nodulation and recovery rate of the inoculated strain from the nodules

At the phenological stage of early pod set, R3 (one pod at maximum length), four central plants from the second row of each replicate plot from each treatment were randomly collected from a depth of 2–15 cm and washed with distilled water to assess the number of nodules (NN) and dry nodule weight (DNW) of each plant (g).

In order to check for the presence of the inoculated strains R and P

inside the nodules, a sample of nodules was collected with a piece of root from the four plants, in one replicate per treatment at the EIAF location; next, they were surface sterilised. Then 1 g of nodules was separated from the piece of root and crushed in 10 mL of sterile saline solution. Following that, a dilution series was prepared with sterile saline solution. Aliquots of 100 mL from the dilutions of 10^{-1} to 10^{-8} were plated onto Petri dishes with YMA medium for the treatments inoculated with R; in addition, the treatments inoculated with P were also plated onto Petri dishes with TSA medium—in all the cases in duplicate—and supplemented with 1 mg L^{-1} cycloheximide to prevent fungal growth. Plates were incubated at 28 °C for 72 h. In dilutions that showed between 30–60 individual colonies, the number of colonies with the typical morphological aspect of the inoculated strain, as well as the total number of colonies, was recorded; the colonies with the morphological aspect of the inoculated strain were purified, and the RAPD profile with the M13 primer, which is strain-dependent, was obtained and compared with that of the pure strain inoculated to verify its identity as the inoculated strain. The complete process is detailed in Pastor-Bueis et al. (2017). *Azotobacter chroococcum* was assumed to be rhizospheric, not endophytic (Ambesh et al., 2017; Singh et al., 2016; Wani et al., 2016), and thus its presence inside the nodule was not assessed.

2.2.4. Determination of the N fixation

The aerial biomass of eight central plants from the fourth row of each replicate plot of each treatment was randomly collected 15 days before final harvest, when the plant was at the phenological stage of physiological maturity (stage R7). The aerial biomass was oven-dried at 60 °C for 48 h and a representative sub-sample, in weight, of the contribution of each organ (leaves, stems and fruits) to the total aerial biomass, was ground to 0.85 mm for ^{15}N isotopic analysis. Further details are in Supplementary Material 2.

2.2.5. Sampling to assess yield and yield components

At the harvest maturity stage (R8), the two central metres of rows numbered six and seven (corresponding to 2 m^2) of each replicate plot from each treatment were fully harvested by hand for the analysis of the yield and the yield components. The number of plants was counted, as well as the total number of pods, to estimate the mean number of plants per m^2 at harvest and the mean number of pods per plant. Moreover, the mean number of seeds per pod was estimated in 25 randomly selected pods. The grain yield obtained in the 2 m^2 subplot was expressed in kg ha^{-1} of air-dried beans, which corresponds to the commercial grain. Following that, the grains and the rest of the aerial biomass were dried at 70 °C for 48 h to obtain dry matter values. With dry matter contents, the harvest index (HI) and the 100-seeds dry weight (g) were also calculated.

2.2.6. Statistical analysis

To analyse the obtained data, the experimental site was considered the environment and the environment and replicate were included in the statistical model as random factors. The fertilisation strategy was considered to be a fixed factor. Analysis of variance (ANOVA) appropriate to a complete randomised block design was performed using the univariate procedure of SPSS Statistics v. 21.0. The normality of standardised residuals was checked with Kolmogorov-Smirnov's test and the homoscedasticity with Levene's test. In order to meet both criteria, the dependent variables NN, DNW, plants m^{-2} and seeds per pod were subjected to logarithmic transformation before the statistical analysis, and backtransformed to show results. A Tukey's post-hoc test was used to compare mean values.

2.3. Microscopy evidence of nodule colonisation by R and P strains

The most successful bacterial consortium in field conditions in terms of yeld was subjected to a microscopic analysis to visualise the nodule colonisation strategies followed by the two consortium's partners. With this purpose, strains R and P were respectively labelled with different fluorescent proteins, co-inoculated in common bean cv. Riñón plants and observed with Confocal Laser Scanning Microscopy (CLSM).

2.3.1. Bacterial labelling

The bacterial labelling was performed following the instructions described by Jiménez-Gómez et al. (2018), with the following modifications: strain R was labelled with the plasmid pBMRmRFP. This plasmid was introduced into the strain by triparental mating using *E. coli* DH5α-pBMRmRFP as a donor strain and *E. coli* DH5α-pRK2013 as a helper strain. Fresh cultures of donor, helper and recipient strains were mixed on YMA plates and incubated overnight at 28°C. The selection of transconjugant colonies were carried out on minimal medium plates (O'Gara and Shanmugam, 1976) supplemented with tetracycline (10 μg/mL). R-RFP was obtained.

P was labelled by biparental mating using *E. coli* S17.1-phC60 as a donor strain containing the plasmid phC60 (Cheng and Walker, 1998). The protocol followed was similar; fresh cultures of donor and recipient strains were mixed on TSA plates and incubated overnight at 28 °C. The selection of transconjugant colonies was also carried out on plates supplemented with tetracycline (10 μg/mL). P-GFP was obtained.

The RFP and GFP marked strains were checked by fluorescence microscopy using a NIKON eclipse 8Oi fluorescence microscope. The recombinant strains were routinely grown at 28 °C in YMA or TSA medium supplemented with the antibiotic.

2.3.2. Growth of common bean cv. Riñón plants and inoculation with transformed R-RFP and P-GFP

In order to assess the natural disposition of both strains inside the nodules, a test was performed in hydroponic conditions with the transformed strains. For this purpose, bean seeds were surface-sterilised with a solution of ethanol (70 %) for 1 min, followed by 7 min in NaClO (5% solution) and several washes with sterilised distilled water. They were sown in plastic pots, 1 L in capacity, containing 300 mL of vermiculite previously sterilised by autoclaving. The pots were irrigated alternatively with 100 mL of nitrogen-free nutrient solution (Rigaud and Puppo, 1975), followed by two irrigations with sterile distilled water (100 mL each). Plants were grown in a growth chamber at 23 °C during 16 h of light and at 16 °C during 8 h of dark, at 60 % relative humidity. After emergence, one plant was left in the pot and inoculated with 1 mL of each of the two transformed strains.

2.3.3. Visualisation of the nodule colonisation by bacteria using confocal laser scanning microscopy (CLSM)

Plants were grown in the above conditions for 21 days after inoculation, and thereafter, the nodules were cleaned from vermiculite particles. The nodules of three different plants were used for the microscopy analysis, using a confocal laser scanning microscope Leica SP5 (Leica Microsystems, Wetzlar, Germany). GFP-tagged and RFP-tagged cells were visualised under the microscope at an excitation wavelength of 488 nm and 561 nm, respectively. Root segments of 2 cm were observed individually under the CLSM. At the same time, roots and mature nodules were cut transversally by hand into cross-sections with a doubled-edged razor blade and directly observed under CLSM. Roots inoculated with *Pseudomonas* were stained with 10 μM of propidium iodide (Sigma) before visualisation under the microscope.

3. Results

3.1. Identification and characterisation of the strain P

The 16S rRNA gene of the strain P showed 99.92 % similarity with respect to the type strain of *Pseudomonas brassicacearum* subsp. *neoaurantiaca* (strain ATCC4954^T). That similarity was the highest compared with all the 16S rRNA gene sequences of type strains held in EzBioCloud (Yoon et al., 2017). The phylogenetic analysis based on the

16S rRNA gene sequence of P and that of the closer type strains (Supplementary Material 3, Figure S1) confirmed that the strain P belongs to *Pseudomonas brassicacearum* and more specifically, to the subsp. *neoaurantiaca*, that belongs to the *Pseudomonas fluorescens* group, which comprises other *Pseudomonas* species considered plant growth promoting rhizobacteria (PGPR) such as *P. brassicacearum*, *P. salomonii* and *P. kilonensis*, but also plant pathogens such as *P. corrugata* (Supplementary Material 3, Fig. S1).

The main *in vitro* PGP characteristics of P and A are in Table 3. The strain P showed relevant PGP activity in the four parameters analysed. In three out of the four parameters, it was clearly superior to the strain A, with the exception of IAA production, in which the *Azotobacter* strain outperformed the *Pseudomonas* strain.

3.2. Field trial

3.2.1. Nodulation and N fixation parameters

As expected, the nodule biomass was significantly higher in the treatments inoculated with R (alone or in co-inoculation) than in the non-inoculated controls (either fertilised with N or not fertilised with N) and also significantly higher than in the treatment inoculated with non-rhizobia strains (A + P) (Table 4). However, interestingly, the number of nodules was not significantly affected by inoculation, as there were no significant differences between the treatments and controls (Table 4). Thus, the correlation value between the nodule biomass and the number of nodules was the lowest of all the correlations (0.427), which were significant (Table 5).

Because of the spontaneous nodulation with the soil native rhizobia, the uninoculated controls fixed some of their N requirements. However, as expected, the treatments inoculated with the autochthonous rhizobia strain R, either alone or in co-inoculation, showed a significantly higher percentage of N derived from the fixation of atmospheric N_2 (Ndfa %) than the uninoculated controls (Table 4), (for raw data from $\delta^{15}N_{AIR}$ (‰) values obtained, Supplementary Material 1, Table S1); as a consequence, the N-fixed in kg ha^{-1} was between 1.7 times and more than two times higher in the treatments inoculated with R than in the uninoculated and unfertilised control (Table 4).

Regarding the Ndfa in the treatment inoculated with the *Azotobacter* strain without rhizobia, it was 51.9 %, an intermediate value between the uninoculated and unfertilised control (43.5 %), and the treatments inoculated or co-inoculated with rhizobia (56.4 % on average) and the N-fixed followed the same pattern (Table 4).

Conversely, the soil N uptake was maximum for the uninoculated and full N-fertilised control, which statistically differed from the rest of the treatments. It was followed by the uninoculated and 80 % N-fertilised control, but it did not significantly differ from the inoculated treatments (Table 4).

In general, the combination of R with other strains increased all the nodulation and nitrogen fixation parameters for each combination compared to single inoculation with R (Table 4). The highest DNW, Ndfa and N-fixed was achieved with the consortium R + P, but it did not statistically differ from the rest of the treatments with rhizobia, either single inoculation or co-inoculation, not even from the treatment inoculated with the *Azotobacter* strain (A) without rhizobia. However, in order to deepen the statistical analysis of the effect of co-inoculation, two different orthogonal contrasts were performed (Fig. 1). In the first orthogonal contrast, we compared the average values obtained for all the R-based consortia with the values obtained in the single inoculation with R. Even if the consortia always produced higher values than the single inoculation, the differences were not statistically significant for any of the variables (Fig. 1). In the second orthogonal contrast, we compared the values obtained with the most successful consortium (R + P) with the average values of the other consortia, which include R (namely R + A and R + A + P), and the differences were not statistically significant either (Fig. 1).

The results of the test to assess the nodule occupancy by the inoculated strains R and P are presented in Supplementary Material 1, Table S2. The test is a verification that the inoculated strains were capable of colonising the nodule, and it also gives information about their colonisation ability. In the treatments inoculated with R, the presence of this strain ranged from 76.8%–79.2%, indicating a good competitiveness of the inoculated strain. The strain P was retrieved from inside the nodule, which is a result itself because this strain was isolated as a roots endophyte but not from inside the nodules. This strain appeared inside the nodule at a relevant percentage, near 50 %; it has to be considered that the TSA medium is very generalist, and a broad range of soil resident bacteria that have colonised the nodule are able to grow in it. The nodule occupancy by A was not investigated because *Azotobacter* is considered a rhizospheric not endophytic strain.

3.2.2. Yield, yield components and harvest index

Inoculation with R, either alone or in consortium with other bacteria, significantly improved the yield and the number of pods per plant compared with the uninoculated and unfertilised control, with an average yield increase of almost 1000 kg ha^{-1}, which is more than 40 % (Table 6). The uninoculated and full N-fertilised control statistically produced the same yield and number of pods per plant as the treatments inoculated with R, alone or in consortium, although the yield obtained with the consortia exceeded the yield of the uninoculated and full N-fertilised control by between 5 % and 17 % (Table 6). The final plant density, the 100-seed weight and the harvest index were not significantly affected by the treatments (Table 6).

Co-inoculation improved, in general terms, the yield and the yield components compared to single inoculation with R. The corresponding orthogonal contrast (Fig. 2) shows that the best consortium (R + P) significantly outperformed the two other consortia that include R. Specifically, the yield of the best consortium was 455 kg ha^{-1} higher, which is 14 %, the pods per plant was 12 % higher, and the seeds per pod was 8% higher. Conversely, and as an expected consequence of the increase in the number of seeds per pod, the 100-seed weight was 9% lower in the R + P consortium. In all the cases, the differences were statistically significant.

In spite of this, the orthogonal contrast performed to compare the average values obtained for all the R based consortia with the values obtained in the single inoculation with R (Fig. 2) did not show significant differences for any of the parameters evaluated. This was a consequence of the decrease in the average values exerted by the least successful consortia.

3.2.3. Correlation analysis between nodulation and N fixation parameters and growth and yield parameters

The DNW was significantly and positively correlated (Pearson R value 0.7 or above) with the Ndfa (%), N-fixed, total aerial biomass and yield (Table 5). Conversely, the DNW was weakly although significantly correlated with the NN per plant, as explained in Section 3.2.1; however, it was not correlated with the soil N uptake. The Ndfa (%) was significantly and negatively correlated with the soil N uptake ($p \leq 0.05$) and

Table 3
Plant Growth Promotion (PGP) properties measured *in vitro*, corresponding to the strain P (strain RVPB2-2 from *Pseudomonas brassicacearum* subsp. *neoaurantiaca*) and the strain A (type strain fro d from *Azotobacter chroococcum,* Azc-ATCC 9043^{T})).).

PGP property	Bacterial strain P	A
IAA production (μg mL^{-1})	8.9	28.5
Ca3PO4 solubilizing index*	2.1	1.0
Siderophore production index*	3.0	1.29
ACC deaminase activity (μM α-Ketobutyrate mg of protein^{-1} h^{-1})	259.2	3.84

* The index was calculated as (diameter of the colony + halo) / diameter of the colony. Values of 1 mean absence of halo.

Table 4
Mean values and standard errors related to nodulation and nitrogen symbiotic fixation, obtained in two field trials and carried out in two different locations during the year 2019. In the corresponding ANOVA, the location was considered a random factor (significance level *** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; ns not significant). The number of degrees of freedom was 7 for all parameters. A Tukey's test was used to compare mean values; the means followed by the same letter did not significantly differ for $p \leq 0.05$.

Treatment[a]	Number of nodules (NN)		Dry nodule weight (DNW) (mg plant-1)		Aerial biomass (dry) (kg ha-1)		Total N (kg ha-1)		Ndfa (%)[b]		N-fixed (kg ha-1)		Soil N uptake (kg ha-1)	
Control 0 N – non-inoculated	39.2 (±5.3)	a	770.5 (±132.8)	a	4070 (±429)	a	106.1 (±10.7)	a	43.5 (±2.4)	ab	46.9 (±6.4)	ab	59.2 (±5.2)	a
Control 100 % N – non-inoculated	39.5 (±9.6)	a	786.5 (±181.4)	a	5704 (±499)	b	155.2 (±15.8)	ab	34.7 (±1.5)	a	54.7 (±7.8)	abc	100.5 (±8.3)	b
Control 80 % N – non-inoculated	44.8 (±8.2)	a	792.3 (±124.1)	a	4485 (±338)	ab	114.9 (±9.6)	a	36.8 (±1.7)	a	43.0 (±5.3)	a	71.9 (±4.6)	a
R+P	42.2 (±5.5)	a	2097.2 (±170.9)	b	5752 (±97)	b	166.1 (±6.1)	b	59.2 (±1.5)	c	98.6 (±5.6)	d	67.5 (±1.9)	a
R	31.3 (±4.2)	a	1645.3 (±180.5)	B	5176 (±321)	ab	143.5 (±11.6)	ab	55.2 (±2.6)	c	80.5 (±9.7)	bcd	63.0 (±2.8)	a
R+A	33.5 (±3.8)	a	1795.8 (±218.8)	b	5579 (±381)	ab	156.3 (±14.3)	ab	55.2 (±2.6)	c	87.6 (±10.8)	cd	68.7 (±4.9)	a
R+A+P	38.3 (±5.1)	a	1805.8 (±174.1)	b	5501 (±292)	ab	152.4 (±8.9)	ab	55.9 (±2.6)	c	86.0 (±8.3)	cd	66.4 (±2.5)	a
A+P	36.0 (±4.3)	a	806.0 (±182.8)	a	4669 (±340)	ab	126.4 (±10.3)	ab	51.9 (±3.4)	bc	67.0 (±9.0)	abcd	59.4 (±3.4)	a
ANOVA Mean Square	116.9		1972306.5		2407178.0		2834.3		529.5		2570.9		1053.4	
F value and singificance	0.532	ns	10.986	***	3.190	**	3.716	**	15.626	***	6.548	***	8.205	***

[a] R: *Rhizobium leguminosarum* bv. *phaseoli* strain LCS0306; P: *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain RVPB2-2; A: *Azotobacter chroococcum* Beijerinck 1901 (ATCC 9043^T).
[b] Ndfa (%) Percent of N derived from the fixation of atmospheric N_2.

Table 5
Correlation (R-value) among: Nodule biomass, symbiotic performance, aerial plant biomass and grain yield.

Parameters		R value and significance level	
Dry nodule weight (DNW) (mg plant^{-1})	Number of nodules per plant	0.427	**
	Ndfa (%)	0.818	***
	N-fixed (kg ha)	0.919	***
	Soil N uptake (kg ha)	0.045	ns
	Aerial biomass (dry) (kg ha)	0.680	***
	Grain Yield (commerical) 87 % dry matter (kg ha)	0.749	***
Ndfa (%)	N-fixed (kg ha)	0.879	***
	Soil N uptake (kg ha)	−0.352	*
	Aerial biomass (dry) (kg ha)	0.473	**
	Grain Yield (commerical) 87 % dry matter (kg ha)	0.487	***
N-fixed (kg ha)	Soil N uptake (kg ha)	0.101	ns
	Aerial biomass (dry) (kg ha)	0.796	***
	Grain Yield (commerical) 87 % dry matter (kg ha)	0.751	***

significantly and positively correlated with the rest of the parameters, namely N-fixed, total aerial biomass and yield. Finally, the N-fixed was not correlated with the soil N uptake, and it was significantly and positively correlated with the total aerial biomass and yield.

3.3. Microscopy observation of the nodule colonisation by the two partners of the most successful consortium, R and P

Through confocal microscopy, we have been able to determine the different locations of the inoculants, both on the surface (Fig. 3A, C) and inside the root (Fig. 3B) and nodule (Fig. 3D). The images of the surface (Fig. 3A) and the interior of the roots (Fig. 3B) inoculated only with *Pseudomonas* (green) show that *Pseudomonas* colonises the root surface very well and is also capable of penetrating inside it, colonising the epidermis and cortex intercellularly. This behaviour is similar in the co-inoculation (Fig. 3C, D) of *Pseudomonas* (green) and *Rhizobium* (red), except that *Rhizobium*-induced root nodules now appear (Fig. 3C). When we analyse the interior of the nodules (Fig. 3D), we observe that the location of *Pseudomonas* (green) is intercellular while the location of *Rhizobium* (red) is intracellular. These results suggest the possibility that there is no contact between the inoculants once inside the nodules.

4. Discussion

A high number of studies have already demonstrated that the co-inoculation of legumes with rhizobia and non-rhizobia bacteria improves nodule functions and plant growth (Santos et al., 2019), but there are a lack of field trials using adequately formulated products (Menéndez and Paço, 2020). This work covers this gap, and is, to the best of our knowledge, the first field trial on common bean co-inoculation in Western Europe. However more field experiments should follow this current study (which is based in two environments), before making financial decisions on the replacement of the inoculation technology in technologically advanced agri-systems. To develop inoculants which are suitable for Western Europe, the research must be performed in agri-systems that are representative of this region in terms of agronomical management - an extrapolation of the research carried out in other parts of the world is not valid (Mulas et al., 2015)-. For instance, a characteristic of Western European agri-systems is a very high soil N-pool, as a consequence of intensive mineral fertilisation since the mid-20th century. This can provide a significant amount of common bean N-requirements (Guinet et al., 2018); in our work, for the treatments inoculated with rhizobia, the soil N-pool was capable of providing 40–43 % of the crop N requirements, thus the BNF only needed to cover the remaining 60 %. Moreover, in our work, we used a well-adapted European *Rhizobium leguminosarum* strain. Other field trials of common bean co-inoculation reported in the literature were carried out in South America, Asia or Africa. In South America, in tropical climate regions or near to the tropics, trials were carried out with South American native rhizobia species (*R. tropici* and *R. etli*), and included technologically advanced agri-systems in Brazil (Barbosa de Souza and de Brito Ferreira, 2017; Hungria et al., 2013) and others in less advanced regions (Remans et al., 2008). In Asia, the field trials were carried out in Anatolia (Turkey) (Elkoca et al., 2010) and in the Himalayan region (Kumar et al., 2016), both using a R.leguminosarum strain; and in Iran, with a *R. phaseoli* strain (Yadegari and Rahmani, 2010). In Africa, field

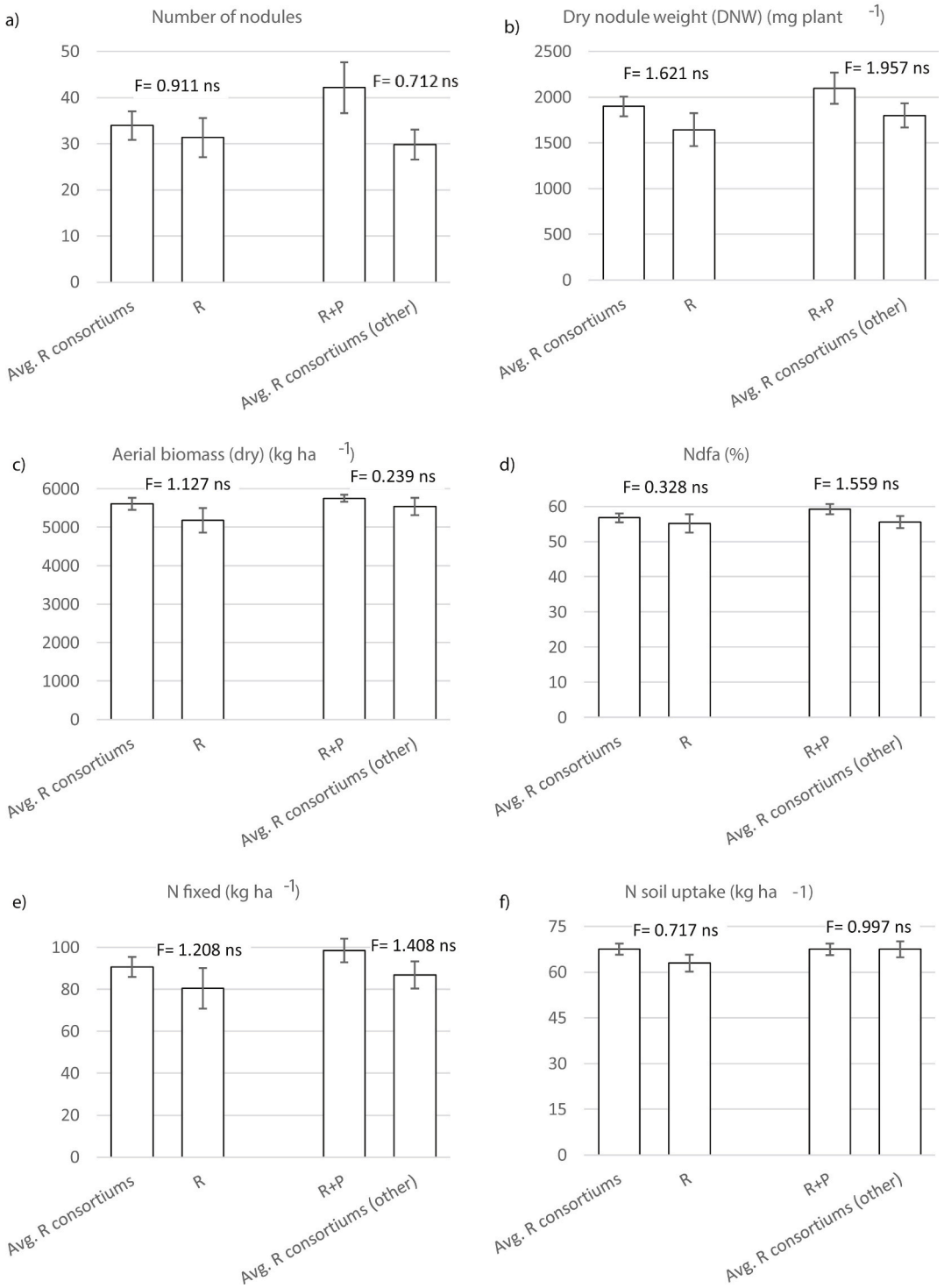

Fig. 1. Orthogonal contrasts performed for nodulation and N fixation parameters to evaluate the effect of bacterial consortia. On the left-hand side of each graphic, the contrast between the average values obtained for all the R-based consortia and the values obtained for the single inoculation with R is presented. On the right hand side of each graphic, the contrast between the most successful consortium (R + P) and the average values of the other consortia, which include R (namely R + A and R + A + P), is represented. For the ANOVA performed, the location (either EIAF or Armunia) was considered a random factor. (ns not significant). R: *Rhizobium leguminosarum* bv. *phaseoli* strain LCS0306; P: *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain RVPB2-2; A: *Azotobacter chroococcum* Beijerinck 1901 (ATCC 9043^T).

trials were carried out in Egypt (Massoud et al., 2009). Consequently, this work provides important information for the optimisation of common bean inoculants for use in Western Europe.

4.1. The agronomic performance of R strain in soils with native low efficiency nodulating bacteria

All the treatments and controls formed nodules, even the uninoculated controls, due to the nodulation capability of the resident rhizobia,

Table 6
Mean values and standard errors related to yield, yield components and harvest index, obtained in two field trials carried out in two different locations during the year 2019. In the corresponding ANOVA, the location was considered a random factor (significance level *** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$). The number of degrees of freedom was 7 for all parameters. A Tukey's test was used to compare mean values; the means followed by the same letter did not significantly differ for $p \leq 0.05$.

Treatment[b]	Plants m^{-2}		Pods per plant		Sedes per pod		100-seeds weight (dry) (g)		Harvest Index (%)		Yield commercial (87 % dry matter) (kg ha^{-1})	
Control 0 N – non-inoculated	13.0 (±0.2)	a	10.9 (±0.7)	a	3.30 (±0.07)	a	40.4 (±0.7)	a	49.8 (±2.6)	a	2270 (±140)	a
Control 100 % N – non-inoculated	12.5 (±0.3)	a	15.0 (±0.6)	bcd	3.60 (±0.05)	ab	39.5 (±1.5)	a	47.6 (±1.8)	a	3076 (±145)	bcd
Control 80 % N – non-inoculated	13.0 (±0.2)	a	13.4 (±0.7)	abc	3.68 (±0.12)	ab	37.4 (±1.4)	a	53.3 (±2.5)	a	2716 (±160)	abc
R+P	13.2 (±0.2)	a	17.4 (±0.4)	d	3.80 (±0.06)	b	35.8 (±1.1)	a	54.6 (±0.6)	a	360 7(±79)	d
R	13.2 (±0.2)	a	15.1 (±0.6)	bcd	3.73 (±0.11)	ab	36.5 (±1.0)	a	52.3 (±1.4)	a	3091 (±148)	bcd
R+A	13.3 (±0.0)	a	15.6 (±0.7)	cd	3.63 (±0.10)	ab	38.2 (±1.0)	a	49.8 (±1.8)	a	3163 (±145)	cd
R+A+P	12.8 (±0.2)	a	15.4 (±0.3)	cd	3.42 (±0.11)	ab	40.5 (±1.4)	a	50.3 (±2.7)	a	314 1(±80)	bcd
A+P	13.0 (±0.2)	a	12.5 (±0.6)	ab	3.39 (±0.17)	ab	41.2 (±2.2)	a	48.3 (±1.6)	a	2563 (±122)	ab
ANOVA Mean Square	0.381		25.012		0.193		24.067		35.002		1042567.551	
F-value and significance	1.524	ns	12.047	***	2.905	*	2.192	ns	1.457	ns	10.171	***

[b] R: *Rhizobium leguminosarum* bv. *phaseoli* strain LCS0306; P: *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain RVPB2-2; A: *Azotobacter chroococcum* Beijerinck 1901 (ATCC 9043^T).

which is a common situation for a promiscuous crop like common bean (Andrews and Andrews, 2017). However, inoculation with R significantly increased the DNW (113 %), the Ndfa (27 %), the N-fixed (85 %), the yield (36 %) and the pods per plant (38 %) compared to the uninoculated and unfertilised control, but conversely, it did not significantly affect the NN, which was also observed by Pastor-Bueis et al. (2019) for the same common bean cv. In other works with different legume crops, the inoculation produced not only an increase in the DNW and the crop yield but also in the NN (Barbosa de Souza and de Brito Ferreira, 2017; Htwe et al., 2018; Kumar et al., 2016). It has been proposed that the NN is controlled by the plant, as legumes possess a systemic negative feedback regulatory system called 'autoregulation of nodulation' (Oka-Kira and Kawaguchi, 2006). In our case, the infective but not effective native rhizobia induced a high nodulation, and we hypothesise that the maximum nodulation capability of the cultivar in the environmental conditions of the trial was probably expressed, resulting in a small whitish-inside and few effective nodules. However, fortunately, the strain R was more competitive for nodule occupancy than the native rhizobia as was already demonstrated by Pastor-Bueis et al. (2019); interestingly, in our work, the treatment inoculated with the strain R produced a similar number of nodules but ones that were larger in size and with clear evidence of the presence of leghaemoglobin inside. As a consequence, the DNW was significantly higher in the inoculated treatment, as were the parameters indicative of nodules' effectiveness, such as the Ndfa and the N-fixed.

4.2. The agronomic effect of the R-based consortia and the superior performance of P in terms of its PGP activities

The consortium of R + P was the most successful in terms of nodulation, nitrogen fixation and crop yield. P was locally isolated from the root endosphere but not from inside the nodule. The nodule is considered a noteworthy source of PGPR that are potentially useful as inoculants in agriculture (Velázquez et al., 2017), but our results indicate that not only the nodule but also the roots' endosphere can be a source of non-rhizobial strains that are capable of colonising the nodule and improving their functions.

The combination R + P increased the NN by more than 30 %, the DNW by more than 25 % and the N-fixed by more than 20 % compared to the single inoculation with R, but this increase in numerical terms was not statistically significant. The lack of statistical significance for such parameters in field trials that compare rhizobial inoculation to co-inoculation is common due to the high data dispersion typical of the mentioned parameters (*e.g.*, Htwe et al., 2018), but the observed tendencies are worthy of being analysed. The increase that we observed in NN and DNW was similar to that observed by Barbosa de Souza and de Brito Ferreira (2017) and Hungria et al. (2013) who co-inoculated common bean with rhizobia and *Azospirillum* and compared it to a single inoculation with rhizobia. Several authors have demonstrated that co-inoculation with rhizobia and *Azospirillum* improves nodulation and yield in other legume crops (Puente et al., 2019; Vicario et al., 2015). Interestingly, it has been suggested that the benefits of *Azospirillum* could be related with several PGPR properties and not only the increased N fixation, which would not even be the most important action mode (Hungria et al., 2013). For instance, the IAA produced by *Azospirillum* has been reported to enhance the secretion by the crops roots of nod-gene-inducing flavonoids, improving nodulation by rhizobia (Dardanelli et al., 2008; Okon et al., 2015; Puente et al., 2019; Vicario et al., 2015). Moreover, it has been proved that the PGPRs with ACC-deaminase activity can improve the nodulation by rhizobia, and this has been attributed to the reduction of the endogenous level of ethylene's precursor called ACC and consequently also of ethylene in the plant roots (Chaudhary and Sindhu, 2016; Sepúlveda-Caamaño et al., 2018; Subramanian et al., 2015). Ethylene inhibits the early stages of nodulation by regulating the threshold concentration of Nod factor required for nodule initiation (Nascimento et al., 2012; Oldroyd et al., 2001), and accordingly, the reduction of endogenous ethylene levels improves the nodulation. Consistent with this, the strain P has a wide range of PGP mechanisms, and this could explain its good performance in the field. In particular, it showed a high level of ACC-deaminase activity and a medium-to-high level of IAA production, compared for instance with the values indicated by Marcano et al. (2016). When we investigated the effects of nodulation resulting from co-inoculation with R + P, compared to a single inoculation with R, we did not find a significant difference. It is worth mentioning that while these results were statistically insignificant, we did observe a 30 % increase in number of nodules and a 25 % in nodules biomass in the R + P group, which we suspect was caused by the two PGP properties of strain P (ACC-deaminase activity and IAA production)

The consortium R + P significantly increased the yield (14 %) and some yield components, namely, pods per plant, seeds per pod and 100-seed weight, compared with the consortium of R and the N-fixing *Azotobacter* (A). A shows some PGP activity besides diazotroph activity, particularly in terms of its IAA production, in which it is superior to P. On the other hand, P was shown to have greater PGP capabilities overall and we have proved that it is a nodule endophyte that would avoid competition with rhizospheric bacteria. We hypothesise that the assortment of PGP activities associated with P, encompassing not only IAA and ACC deaminase production, but also mineral P solubilisation and siderophore production, altogether exerted a more important effect on the crop yield than the N directly fixed by A. In fact, the subsp. *neoaurantiaca* from *Pseudomonas brassicacearum* has been previously described as a PGPR for crops by Seo and Song (2013); the authors assigned this effect to drought stress alleviation as a consequence of its

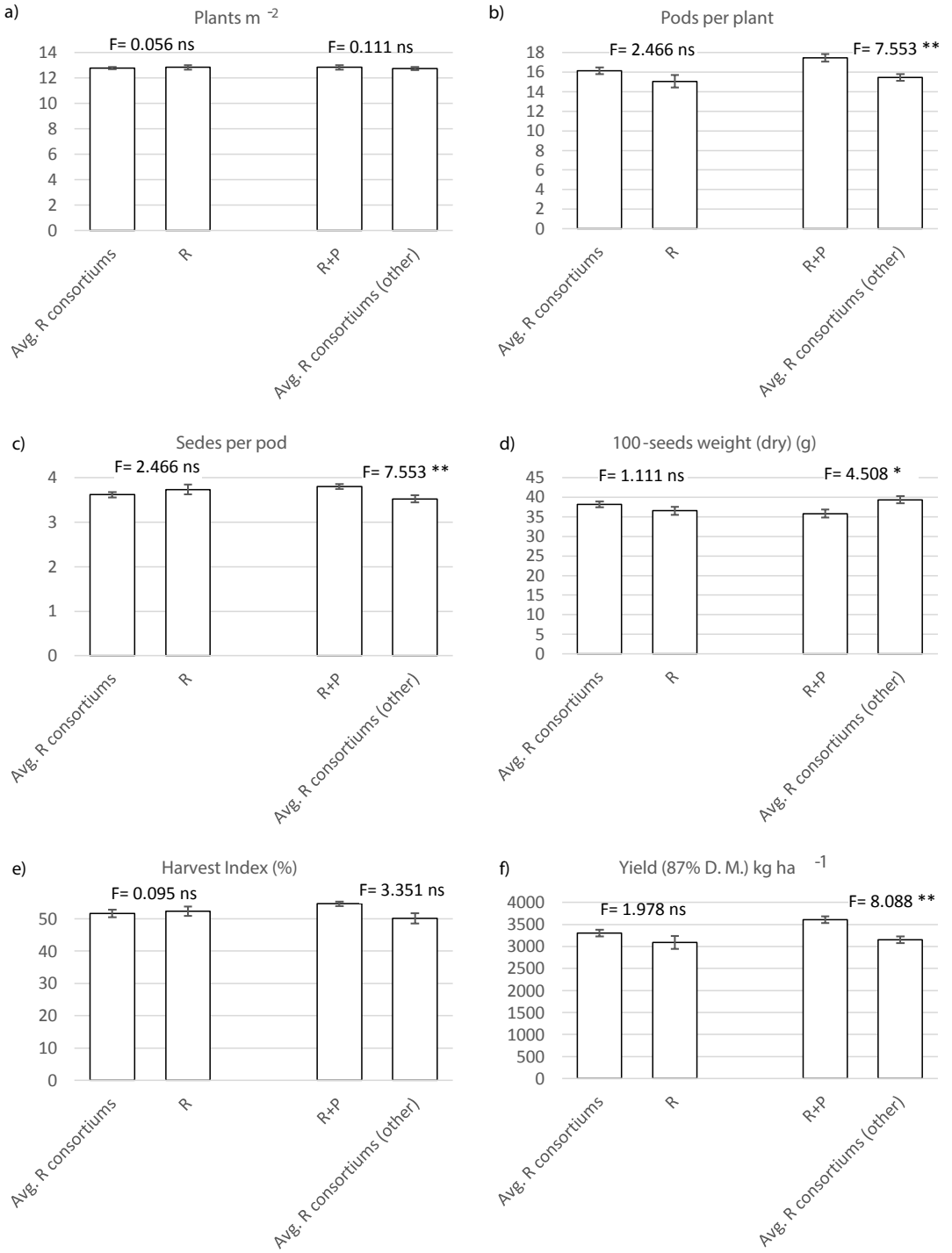

Fig. 2. Orthogonal contrasts performed for yield, yield components and harvest index to evaluate the effect of bacterial consortia. On the left-hand side of each graphic, the contrast between the average values obtained for all the R-based consortia and the values obtained for the single inoculation with R is presented. On the right-hand side of each graphic, the contrast between the most successful consortium (R + P) and the average values of the other consortia, which include R (namely R + A and R + A + P), is represented. For the ANOVA performed, the location (either EIAF or Armunia) was considered a random factor. (significance level ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; ns not significant). R: *Rhizobium leguminosarum* bv. *phaseoli* strain LCS0306; P: *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain RVPB2-2; A: *Azotobacter chroococcum* Beijerinck 1901 (ATCC 9043^T).

ACC-deaminase activity (estimated at 20.26 μM α-Ketobutyrate mg of protein^{-1} h^{-1}). In this study, strain P showed a high ACC-deaminase activity, producing up to ten times more α-ketobutyrate than the strain from the above-mentioned authors.

Interestingly, the most successful consortium in field conditions was the binary consortium with the two autochthonous strains, the rhizobia and the pseudomonad, whereas the introduction of the *Azotobacter* strain as a third partner reduced either the nodulation and N-fixing parameters or the yield. Elkoca et al. (2010), working with common bean, also observed that triple inoculation performed worse than double inoculation and assigned this result to a probable interspecies competition and/or interaction.

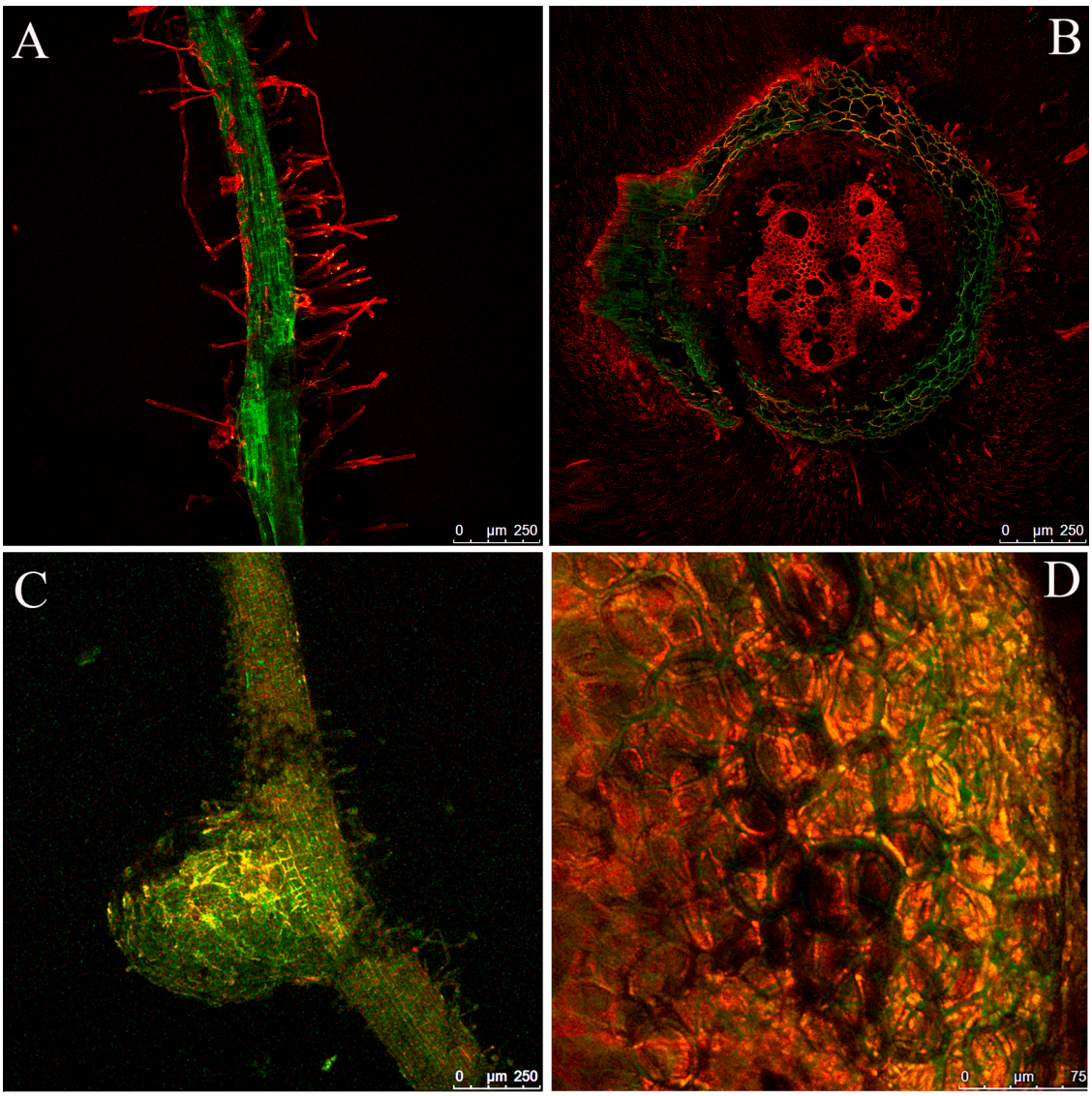

Fig. 3. Roots and nodules colonisation by R-RFP (red) and P-GFP (green), observed with confocal laser scanning microscopy (CLSM). **A.** Whole bean root showing its surface inoculated with *Pseudomonas* (green) and stained with propidium iodide (red); **B**. Cross section of a bean root showing its interior inoculated with *Pseudomonas* (green) and stained with propidium iodide (red); **C**. Whole bean root showing its surface co-inoculated with *Pseudomonas* (green) and *Rhizobium* (red); **D**. Cross section of a bean nodule showing its interior co-inoculated with *Pseudomonas* (green) and *Rhizobium* (red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4.3. Colonisation of root nodules by the partners of the most effective consortia and implications for the design of successful consortia

Confocal microscopy has emerged as a very useful technique for *in vivo* studies of the interactions of plants with other organisms, both at the molecular and cellular level. By applying this technique, we have been able to identify the colonisation pattern of the co-inoculants as well as the possible implications of this colonisation on the crop growth in field conditions. The confocal microscopy results revealed that the two partners of the most effective consortia (R + P) have the ability to colonise the interior of the nodules. However, we observed that the location of the two partners was quite different; in the case of Rhizobium, as expected, their location was intracellular, whereas Pseudomonas was observed in an intercellular location. These different locations of both partners inside the nodule suggest the possibility that they avoid competition with each other.

By this time, the development of effective co-inoculants is mainly based on the empirical test of numerous rhizobia-PGPR combinations, but this approach must be refined to design a more specific strategy of co-inoculant development in the future. To achieve this, it is necessary to learn more about biochemical pathways, microbe–microbe interactions, and plant–microbe interactions (Martínez-Hidalgo and Hirsch, 2017). Regarding the latter, with the results obtained in this work, we hypothesise that the ability of both partners to colonise the interior of the nodule and locate themselves in separate niches is probably one of the causes of the increase in crop yield, although no other hypothesis can be ruled out.

4.4. Economic implications of inoculation with the most successful co-inoculant R + P

The purchase availability of inoculants for agriculture is low in Europe, while most of the revenues are globally recorded in the Latin American market, particularly in Argentina and Brazil (Keswani et al., 2019). According to Brisk Insights (2016), the inoculants market is growing globally by 14 % annually, and worldwide values of USD 1.88 billion by the end of 2020 and USD 1.95 billion by 2022 are expected (Keswani et al., 2019). However, in Europe, to the best of our

knowledge, there is no inoculant for common bean available on the market, and therefore, there are no prices either. In South America, the cost of inoculating 1 ha of soybeans, the most popular inoculated crop, ranges between approximately USD 3.2 and USD 6.4 (Agroclick, 2020). In Europe (León, Spain), F. González-Andrés (unpublished) estimated the price of inoculating 1 ha of common bean at 15.5 euro using an inoculant with one bacterial strain and 17.8 euro using a co-inoculant with two strains. These figures were obtained for the formulation used in this work (see Section 2.2.1) and for the following prices of the formula's components: the price of the carrier plus the cell protectors and the package, which was estimated at 5.5 euro ha^{-1}, and the price of the bacterial broth, which was estimated at 2.3 euro ha^{-1} per bacteria strain included in the formulation (J.-L. Barredo, pers. comm.). On the other hand, the price of mineral N fertilisation with 187 kg N ha^{-1} in the form of calcium ammonium nitrate (27 % in N) is approximately 140 euro ha^{-1}. Moreover, the price paid to the farmer for the common bean cv. "Riñón" commercialised under the PGI is 0.95 euro kg^{-1}. Extrapolating the average yield increase obtained in the two environments of our field trial to the whole PGI "Alubia de La Bañeza-León", mineral fertilisation would result in a yield increase of 806 kg ha^{-1} and 766 euro ha^{-1}, compared with the uninoculated and unfertilised control. The gross margin increase was 626 euro ha^{-1}. Inoculation with R resulted in an average yield increase of 821 kg ha^{-1} and 780 euro ha^{-1} compared with the uninoculated and unfertilised control; the gross margin increase was 764 euro ha^{-1}. Finally, co-inoculation with R and P resulted in an average yield increase of 1337 kg ha^{-1} and 1270 euro ha^{-1} compared with the uninoculated and unfertilised control; the gross margin increase was 1252 euro ha^{-1}. Thus, compared with the N-fertilised and uninoculated control, the single inoculation produced a gross margin increase of 138 euro ha^{-1} and the co-inoculation, one of 626 euro ha^{-1}. Moreover, the co-inoculation produced a gross margin increase of 488 euro ha^{-1}compared with the single inoculation.

5. Conclusions

The preliminary results obtained from two environments located in technologically advanced agri-systems in north west Spain, indicate that inoculation with the autochthonous *Rhizobium leguminosarum* strain LCS0306 could replace fertilisation with mineral N, to produce similar crop yields. Compared to the uninoculated and unfertilised control, the inoculation with rhizobia and the co-inoculation with R + P significantly increased the crop yield by 36 % and 59 %, respectively. However, even with co-inoculation, the crop's dependence on soil N is still 40 % of its total N requirements. Therefore, some authors have suggested that the replacement of mineral fertilisation by inoculation might not be viable in soils where the concentration of N is very low (Santos et al., 2019). Therefore, our conclusions could be applicable to agricultural soils with a sufficient N reservoir, such as most Western European soils, where the use of mineral fertilisers in the non-legume crops of the crop rotation has been high or even excessive over the last 70 years, and is expected to continue (perhaps more moderately) in the future.

The success of the co-inoculant formulation R + P may be due to a complex combination of several factors, namely i) the competing ability of the rhizobial partner, demonstrated by the nodule occupancy; ii) the large assortment of PGPR characteristics of the Pseudomonas strain; iii) the capability of the Pseudomonas strain to colonise the interior of the nodule, locating itself in separate niche from the rhizobia, enabling the exertion of its PGPR activity without competition.

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CRediT authorship contribution statement

Raquel Pastor-Bueis: Investigation, Formal analysis, Writing - original draft. **Alejandro Jiménez-Gómez:** Investigation. **Marcia Barquero:** Resources, Investigation. **Pedro F. Mateos:** Methodology, Writing - original draft, Supervision. **Fernando González-Andrés:** Conceptualization, Formal analysis, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eja.2020.126187.

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Innovative liquid formulation of digestates for producing a biofertilizer based on *Bacillus siamensis*: Field testing on sweet pepper

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Abstract

A biofertilizer (BF) based on the plant growth promoting rhizobacterium (PGPR) *Bacillus siamensis* was produced using anaerobic digestate (AD) as the main ingredient of the growth medium, alongside a carbon source from residual origin. The use of residues for the growth of PGPR reduces the production costs of biofertilizers, but makes an assessment of the possible toxicity of residues for the bacteria or plants necessary. Therefore, the growth medium of PGPR was first optimized using the response surface methodology (RSM), followed by phytotoxicity tests and a field trial of the BF in a sweet pepper (*Capsicum annuum* L.) crop at two different locations. AD at 50% dilution, supplemented with 2.3% sugar beet molasses, was the optimum growth medium for producing the BF, with a bacterial concentration of 10^9 cfu mL^{-1}. In the field trial, the treatments inoculated with BF and fertilized with decreased mineral N (80%) produced significantly better yields per ha than the controls with decreased N (80%) and full N (100%) without BF. This indicates improved efficiency of N use by the crop, as a consequence of the use of BF.

Key words: anaerobic digestate / *Capsicum annuum* / molasses / plant probiotic microorganism

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1 Introduction

Products based on plant growth promoting rhizobacteria (PGPR) for use in agriculture have received widespread attention in recent years. The proper use of these products may lead to an increase in crop yields and enhanced plant natural defense mechanisms against pathogens, resulting in a reduced need for chemical inputs (*Bhardwaj* et al., 2014). The intensive use of mineral fertilizers, pesticides, and other supplements has led to several issues such as high cost, pollution, and loss of soil carbon (*Good* and *Beatty*, 2011; *Nkoa*, 2014). Thus, products based on PGPR offer an attractive method for reducing the input of chemical products, partially or even completely, while achieving similar crop yields as with conventional methods (*Bhattacharyya* and *Jha*, 2012). Products based on PGPR for agriculture are called bioprotectants, biofertilizers, or biostimulants, depending on the intended effect (*Martínez-Viveros* et al., 2010; *Prashar* et al., 2014).

There are multiple mechanisms by which these PGPR influence plant growth, such as stimulation of hormonal regulators (*Papenfus* et al., 2015), improved nutrition through better nutrient uptake, N_2 fixation, and better solubilization, or mineralization of phosphate, potassium, and iron. Other features are improvements in the control of diseases by several mechanisms, a reduction in ethylene levels (responsible for the transmission of stress signals in plants) and an increase in chlorophyll concentration and photosynthetic activity (*Adesemoye* et al., 2009; *Singh* et al., 2011a; *Glick*, 2014; *Vafadar* et al., 2014). However, there have been some inconsistencies in the performance of these inoculants at the field scale (*Morrissey* et al., 2004). In order to address this issue, several studies have focused on the design of an optimal formulation for supporting bacterial growth and attaining a sufficient number of viable cells to trigger the plant response (*Herrmann* and *Lesueur*, 2013; *Bashan* et al., 2014).

To produce a biofertilizer at an industrial scale, a growth medium with an adequate nutrient composition for PGPR growth is needed based on inexpensive and easily available sources. For this reason, several agro-industrial wastes, such as filter mud, wastewater, and fly ash, have been proposed as growth media (*Ben Rebah* et al., 2007; *Singh* et al., 2011b; *Singh* et al., 2013). As a result, the use of biofertilizers may be an efficient and low-cost alternative (*Flores-Felix* et al., 2013).

Anaerobic digestion is considered an environmentally friendly technology for bio-energy production, organic biodegradable waste valorization, and potential recovery of nutrients from the digestate (*Vaneeckhaute* et al., 2017). Although the digestates can be returned to agricultural land in their crude unprocessed form, there are controversial reports on their agronomic potential and serious concerns about negative effects on the environment and human health, including biological contamination (*Nkoa*, 2014). As a consequence, post-AD-treatment is generally required prior to using AD in agriculture (*Münch*, 2009; *Nkoa*, 2014). This is also necessary to adapt AD to the stringent quality standards for certification (*Siebert* et al., 2008). The proposed post-treatments consist of pasteurization, steaming, or sterilization (*Alburquerque* et al., 2012), curing (*Drennan* and *DiStefano*, 2010), or composting (*Smet*

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et al., 1999). Such treatments increase the final cost and energy consumption adding little or no value. Alternatively, it has been proposed that nutrient recovery technologies (NRT) be used for the digestate in order (1) to create an end-product with a higher nutrient concentration than the crude digestate or (2) to separate the envisaged nutrients from the digestate to produce an end-product that is fit for use in the chemical or fertilizer industry (*Vaneeckhaute* et al., 2017).

In this study, we propose an alternative method of valorization of anaerobic digestate (AD) obtained from fruit and vegetable wastes (FVW) (*Bouallagui* et al., 2005). The objective was to use such AD to produce a biofertilizer (BF) based on *Bacillus siamensis* for sweet pepper crop (*Capsicum annuum* L.). The experimental approach consisted of several sequential phases: (1) to optimize the proportions of AD and molasses (as the carbon source) in the growth medium for the PGPR strain in order to achieve a count of at least 10^9 cfu mL^{-1} in the pilot fermenter, which is the biofertilizer (BF), (2) to assess the possible phytotoxic effects of the BF, but also of the mix of AD and molasses used as the growth medium for the PGPR strain, both before sterilization (AD-m) and after sterilization (AD-m-ST), and (3) to test the BF in sweet pepper crops under field conditions with decreased N fertilization.

2 Material and methods

2.1 PGPR strain and preparation of the primary inoculum

The *Bacillus siamensis* strain used for this work was SCFB3-1, isolated from the rhizosphere of a sweet pepper (*Capsicum annuum* L.) crop at San Cristobal de Entreviñas (León, Spain), and identified by comparison of the 16S rRNA sequence (> 1400 bp) with sequences deposited in EzTaxon-e server (*Kim* et al., 2012), as described by *Barquero* (2014). To obtain the primary inoculum, the strain (stored at –80°C in 30% glycerol) was suspended in tryptic soy broth (TSB; Sigma, Catalogue No. T8907) and incubated for 3 d at 28°C to reach a bacterial concentration of 10^9 cfu mL^{-1}.

2.2 Plant material

The sweet pepper cultivar used for all the experiments was Maor from FITÓ.

2.3 Production and characterization of the anaerobic digestate (AD)

The AD was obtained from a 3 L anaerobic continuously stirred tank reactor (CSTR) treating residues from a local industry dedicated to the production of fourth range vegetables and fruits (ready-to-eat fresh-cut vegetables and fruits packaged in protective atmosphere). The average composition of the feed consisted of pineapple peels (40.2%), pineapple fleshy axis (20.2%), pumpkin peels (19.6%), and apple peels and cores (10.6%); the remaining (9.4%) was a mix of residues from mango, sweet pepper ,and cauliflower. The material was crushed and homogenized to attain a particle size of less than 1 cm. The reactor worked under semi-continuous operation at 35°C. The reactor was supplemented with NH_4Cl and KH_2PO_4 with a weekly addition of these compounds dissolved in a solution containing micronutrients with the composition proposed by *Gonzalez-Gil* et al. (1999). The final composition of the AD is shown in Tab. 1. The AD, as obtained from the reactor, was homogenized and ground to further reduce the particle size to obtain a liquid stream with solid particles less than 3 mm in size. This mixture was used for further experiments.

2.4 Optimization of the composition of the AD-based-medium for *Bacillus siamensis* growth

A preliminary assay was carried out to assess whether *B. siamensis* can grow in AD as the sole medium component, or if the AD medium needs supplementation with an additional carbon source. Briefly, 50 mL of AD and mixtures of AD with different carbon sources (sucrose, lactose, and glucose at a sugar concentration of 10 g L^{-1}) were used to test the growth of *B. siamensis*. The media thus prepared were autoclaved in 100-mL bottles (121°C for 20 min). Afterwards, the bottles were inoculated with 0.5% (v/v) of the primary inoculum obtained as described above. After 8 d of incubation at 28°C, the bacterial concentration was measured by serial dilutions, plated onto TSA medium, and incubated at the same temperature. The experiment was carried out in duplicate.

The results obtained from this first set of experiments indicated the need for a source of assimilable carbon (see section 3.1). Therefore, the following experimental phase was carried out using sucrose in the form of molasses, which was obtained from the sugar beet-processing facility Azucarera Española Ebro (Toro, Zamora, Spain). The molasses had a density of 1.33 g mL^{-1} and a sucrose concentration of 328 mg g^{-1}. The chemical characteristics are shown in Tab. 1.

The optimum conditions for using the AD medium for the growth of *B. siamensis* were assessed in a shaking flask experiment. Response surface methodology (RSM), using a second order polynomial function [Eq. (1)], was applied to evaluate the results of bacterial growth. The total number of experiments performed was based on a factorial design with a 2k factorial nucleus (four replications of the central point) and 2k axial points, with k being the number of factors. The selected factors were the concentration of total solids in AD (X1) and the concentration of sucrose (X2) added in the form of molasses. The experimental set-up is presented in Tab. 2. The response was the bacterial concentration measured by serial dilutions plated onto TSB medium and incubated at 28°C. This assay was carried out in duplicate.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2. \quad (1)$$

Fermentation tests were carried out using 500-mL flasks with a working volume of 250 mL at 28°C for 8 d with continuous

Table 1: Analysis of anaerobic digestate and molasses used for the growth medium of *Bacillus siamensis.*

Parameter	Anaerobic Digestate		Molasses	
Organic Matter	% of dry matter	58.87	% of dry matter	62.1
Total Nitrogen	% of dry matter	4.43	% of dry matter	1.23
$N\text{-}NH_4^+$	% of total N	23.7		nd
C : N Ratio		7.72		39.1
Total phosphorus	% of dry matter	0.743	mg L^{-1}	204
Potassium	% of dry matter	4.04	mg L^{-1}	41600
Calcium	% of dry matter	0.808	mg L^{-1}	3000
Magnesium	% of dry matter	0.334	mg L^{-1}	0.2
Sodium	% of dry matter	0.048	mg L^{-1}	1000
Manganese	mg kg^{-1} of dry matter	233.40	mg L^{-1}	55800
Iron	mg kg^{-1} of dry matter	1580.76	mg L^{-1}	47.6
Copper	mg kg^{-1} of dry matter	46.20	mg L^{-1}	0.89
Zinc	mg kg^{-1} of dry matter	145.97	mg L^{-1}	4.66
Total solids	g L^{-1}	5.49	g L^{-1}	693
pH		7.24		7.89
Conductivity	dS m^{-1}	3.91	dS m^{-1}	2.84

Table 2: Response surface analysis to determine the most suitable proportion of the anaerobic digestate (AD; total solids 5.49 g L^{-1}) and molasses (328 mg sucrose g^{-1}). The volume of AD was calculated on the basis of the final concentration of total solids (TS) in g L^{-1}, and the volume of molasses on the basis of the final concentration of glucose (g L^{-1}). Data are the means of two replicates, with the exception of T9, which is the mean of four replicates.

Treatment	Codified values		Real values				Responses
	Concentration of TS from the AD (g L^{-1})	Concentration of sucrose (g L^{-1})	AD		Molasses		Cfu mL^{-1}
			Concentration of TS (g L^{-1})	Volume of AD (mL L^{-1})	Concentration of sucrose (g L^{-1})	Volume of molasses (mL L^{-1})	
T1	–1	1	4.34	789	3.35	7.7	3.75×10^1
T2	–1	–1	1.16	211	3.35	7.7	3.40×10^7
T3	1	1	4.34	789	17.15	39.2	2.08×10^1
T4	1	–1	1.16	211	17.15	39.2	1.60×10^5
T5	0	1.414	5.00	909	10.25	23.5	1.65×10^2
T6	0	–1.414	0.50	91	10.25	23.5	2.07×10^7
T7	1.414	0	2.75	500	20.00	45.8	7.15×10^7
T8	–1.414	0	2.75	500	0.50	1.14	8.00×10^1
T9[a]	0	0	2.75	500	10.25	23.5	4.04×10^8

[a]Repeated four times.

shaking. Prior to fermentation, the shaking flasks were sterilized by autoclaving at 121°C for 20 min. Inoculation was performed by adding 0.5% (v/v) of the primary inoculum.

2.5 Production of BF for the upcoming tests

Once the composition of the growth medium was optimized at the lab scale, subsequent production of the BF product was conducted in a pilot fermenter (Sartorius BIOSTAT Bplus-MO 5 l) at 28°C and 10% dissolved oxygen for 48 h to achieve 10^9 cfu of *B. siamensis* per mL. Carrageenan was

added at 1% w/v at the end of the fermentation as an additive, with the purpose of improving bacterial survival (R. Mulas, R. Pastor, and F. González-Andrés, unpublished).

2.6 Germination test

The phytotoxicity of the products AD-m (the mix of AD and molasses used as the growth medium for the PGPR strain, before sterilization), AD-m-ST (AD-m after sterilization, which is a necessary step before inoculation), and BF (the biofertilizer produced using AD-m-ST as growth medium, with 10^9 cfu mL^{-1} of *B. siamensis*) was evaluated with the Zucconi test (*Zucconi* et al., 1981), modified by *Varnero* et al. (2006). Briefly, seeds of lettuce (*Lactuca sativa* L.), radish (*Raphanus sativus* L.), cress (*Lepidium sativum* L.), tomato (*Solanum lycopersicum* L.), and sweet pepper (*Capsicum annuum* L.) were surface-sterilized by soaking for 20 min in 2% (v/v) sodium hypochlorite, then rinsed in sterile distilled water. Five 10-seed replicates for germination tests were carried out with three different concentrations of the products to be tested: the product as obtained (100%) and two levels of dilution with sterile distilled water (20% and 10%). A control treatment was also performed using sterile distilled water. The seeds of each species were placed on filter paper (Prat Dumas medium flow) in 9 cm Petri dishes containing 5 mL of the solution to be tested. The Petri dishes were hermetically sealed and kept in a growth chamber at 25°C in the dark. Seeds were considered germinated when the radicle had extended at least 2 mm. The number of seeds germinated was recorded daily until the control reached 100% of germination. The germination index (GI), expressed as a percentage, was calculated as the product of the relative germination percentage (RGP) and relative radicle growth (RRG) (*Zucconi* et al., 1981).

2.7 Plant growth test in the growth chamber

The effect of AD-m, AD-m-ST, and BF on sweet pepper seedlings was assessed in a growth chamber under controlled conditions at 23°C for 16 h in light and 16°C for 8 h in dark, with 65% relative humidity. Two seeds per cell were sown using multi-cell thermoformed seedling trays (100 mL capacity). Each cell was filled with professional substratum (70% blonde peat, 30% black peat; NPK 12–14–24, pH 6, 350 kg m^{-3}) from Pindstrup Mosebrug SAE (Ryomgaard, Denmark). After nascence, one single seedling was allowed per cell. The statistical design within the growth chamber was a split plot in a complete randomized design with four repetitions, which included two experimental factors: treatment with the products AD-m, AD-m-ST or BF (the BF product containing 10^9 cfu of *B. siamensis* per mL; see section 2.5); and the doses: 1.5, 1.0, 0.5, and 0.1 mL per seedling. The number of plants per each treatment and each dose was 40. The corresponding treatment was applied to the substrate, close to the plant neck, 3 weeks after nascence. The plants were cultivated under the same conditions for 9 more weeks. Sampling was conducted at the end of the experiment to measure dry (60°C in oven to constant weight) aerial and root biomass. The effects of the treatment (T), dose (D), and the interaction between T × D were analyzed with ANOVA using the general linear model (GLM) procedure in SPSS v. 21.0.

2.8 BF tests in field trials

The trial was performed during 2015 at two locations: León (42°35'03"N, 5°35'22"W, 818 m asl) and Oteruelo (42°34'32"N, 5°36'13"W, 846 m asl). At each location, the experimental design was a randomized complete block with three blocks. The elementary plot was 3 m^2, with four rows 0.50 m apart and a space between plants of 0.15 m, for a total of 40 plants per plot. The treatments and controls were the following:

- BF 2 mL $plant^{-1}$ for N 80%: received 2 ml $plant^{-1}$ of BF, one in the nursery and one at the beginning of flowering, in plants fertilized with 144 kg N ha^{-1} in the form of ammonium nitrate 27% N, which corresponds to 80% of the recommended N dose for sweet pepper in Mediterranean-type agriculture, for an expected yield of 45 t ha^{-1}, according to *Urbano* (2008).
- BF 1.5 m L^{-1} plant for N 80%: received 1.5 mL $plant^{-1}$, 0.75 mL in the nursery and 0.75 mL at the beginning of flowering in plants fertilized with 80% of the recommended N dose.
- Non-N-fertilized control: not fertilized with N, nor inoculated.
- N 80% control: fertilized with 80% of the recommended N dose (144 kg N ha^{-1} in the form of ammonium nitrate 27% N).
- N 100% control: fertilized with 100% of the recommended N dose (180 kg N ha^{-1} in the form of ammonium nitrate 27% N) for an expected yield of 45 t ha^{-1}.

BF contained 10^9 cfu of *B. siamensis* per mL (see section 2.5). The controls received (instead of BF) 2 mL of AD-m-ST (which is the culture medium for PGPR after sterilization), once in the nursery and once at the beginning of flowering.

The plants were produced as indicated in section 2.7 and transplanted in Oteruelo at five true leaves stage on May 25 in 2015 and in León on June 7 in 2015. The central area of each plot was 1.2 m^2, where 16 plants were transplanted, and was then harvested. Fruit harvesting started in Oteruelo on September 22 in 2015 and in León on October 6 in 2015, and lasted until October 21 in Oteruelo and November 03 in León. The dependent variables analyzed were the yield and the yield components, and the following characteristics of the fruit: morphology, pH and total soluble solids, and the N, P, K, Ca and Mg concentrations. The effects of the location (L), the repetition (R), the treatment (T), and the interactions of L × T and R × T were analyzed using ANOVA.

2.9 Counts of the strain SCFB3-1 of *B. siamensis* in the root endosphere at the end of the field experiment

After the last harvest, the roots of five plants per plot were collected to a depth of 3–10 cm, washed with water to eliminate soil particles, and air-dried. For each plot, a representative sample of 20 g composed of secondary roots and taproots was surface-sterilized (70% ethanol for 1 min and 6% NaClO for 6 min following three successive washings with sterile distilled water), and crushed in 180 mL of sterile saline solution. This was then filtered and a dilution series was prepared with

saline solution. Aliquots of 100 μL from dilutions of 10^{-1} to 10^{-8} were plated onto Petri dishes with tryptic soy agar (TSA) medium supplemented with 1 mg L^{-1} cycloheximide to prevent fungal growth. Individual colonies with the typical morphological aspect of the strain SCFB 3-1 were purified, and the RAPD profile was obtained and compared with that of the pure strain SCFB 3-1 to verify its identity as the inoculated strain. The bacterial DNA isolated according to *Álvarez-Martínez* et al. (2009), was used to obtain the RAPD patterns following the procedure described by *Rivas* et al. (2006) using primer M13 (5-GAGGGTGGCGGTTCT-3').

3 Results and discussion

3.1 Optimization of the AD-based-medium for *Bacillus siamensis* growth

Bacillus siamensis showed weak growth when cultured with AD as the sole growth medium. Conversely, the final concentrations were 3×10^7, 3.9×10^8, and 1.5×10^8 cfu mL^{-1} when AD was supplemented with lactose, glucose, and sucrose, respectively. Due to the scarce difference obtained in the final count of viable cells between the experiments supplemented with glucose and sucrose, we decided to supplement the AD with a residue rich in sucrose, *i.e*., molasses.

The response surface methodology (RSM) used a second order polynomial function to optimize the composition of the growth medium based on AD and molasses. The response was adjusted to the following second order polynomial equation:

$$Y = 4.05\times10^8 + 8.41\times10^6 X_1 - 7.93\times10^6 X_2 + 8.46\times10^6 X_1 X_2 - 1.88\times10^8 X_1^2 - 2.01\times10^8 X_2^2. \quad (2)$$

The ANOVA results for the calculated model are presented in Tab. 3. The results indicate that only quadratic effects were significant and that linear and interaction effects presented no significance. The response presented a decrease in values when moving further from the center point in any direction (graph not shown). The maximum point obtained (from derivation of the polynomial function and equating to zero) was coincident with the center point. The evolution of the response indicated that factors evaluated at their lower levels presented insufficient growth of the microorganism either due to a lack of sufficient assimilable carbon and/or essential elements and compounds in the AD. Conversely, when factors were evaluated at their higher levels, the effect on the response may have been associated with the prevalence of inhibitory conditions due to the excessive concentration of complex molecules that may be present in AD and an excessive increase in the osmotic potential of the bacterial growth medium due to higher levels of sucrose.

Table 3: ANOVA results obtained for the response surface methodology (RSM) of the calculated model of the anaerobic digestate (AD) and molasses.

Parameter	Error ($\times 10^7$)	t value	Prob > |t|
β0	3.11	12.98	1.28×10^{-5}
β1	2.20	0.38	0.72
β2	2.20	–0.356	0.73
β12	3.11	0.27	0.80
β11	2.46	–7.63	2.64×10^{-4}
β22	2.46	–8.15	1.84×10^{-4}
R2	0.972		
R2 adj	0.900		

3.2 Germination test with AD-m, AD-m-ST, and BF

The assessment of phytotoxicity is necessary prior to the use of any kind of residue in agriculture (*Panuccio* et al., 2016), and also in the development of a biofertilizer (*Kantha* et al., 2015). The tests show that pure products were highly phytotoxic and prevented the germination of the seeds in most cases (Tab. 4). Conversely, at the 10% dilution, all tested products stimulated the growth of all plant species when compared with the control. At the 20% dilution, the BF was a stimulant for all the species; the AD-m produced a moderate phytotoxic effect in radish and cress, while it was a stimulant in the other species. AD-m-ST was highly phytotoxic for tomato and cress, but was a stimulant for the other species.

Such results are in accordance with those reported by other authors (*Alburquerque* et al., 2012; *Pivato* et al., 2015; *Stefaniuk* et al., 2015; *Tigini* et al., 2016), who also observed that as a general trend, the application of low concentrations of AD acted as a stimulant giving higher values of GI and stimulating seedling growth. Such an improvement in germination could be related not only to the nutrients provided by the digestate solutions, but also to a phytohormone-like effect (*Emino* and *Warman*, 2004; *Moldes* et al., 2006). However, the phytotoxicity and stimulant effects of the AD presented

Table 4: Germination index (%) according to *Zucconi* et al. (1981) to estimate the phytotoxicity of the indicated products in the used plants. A germination index below 50% corresponds to highly phytotoxic materials, between 50% and 80% to moderately phytotoxic materials, and above 80% to non-phytotoxic materials.

Plant	AD-m			AD-m-ST			BF		
	Pure	20%	10%	Pure	20%	10%	Pure	20%	10%
Letttuce	37	341	275	0	155	270	0	160	111
Tomato	0	184	186	0	37	171	0	112	111
Radish	3	87	146	0	230	198	0	129	130
Cress	13	204	226	0	49	409	20	458	544
Sweet pepper	0	72	193	0	232	275	0	141	247

high variability with a great dependence on the origin of the input feedstock. Compared with other AD, which were also tested at a broad range of dilutions, the AD from FVW in the present work showed a high stimulant effect, as we observed GI values from 146% to 341% for AD-m and from 155% to 409% for AD-m-ST (Tab. 4), while *Pivato* et al. (2015) observed maximum GI values of nearly 140%, and *Alburquerque* et al. (2012) a maximum GI of 150% in the standardized tests of phytotoxicity. The AD from this work showed a low EC, a neutral pH, and a low N-NH_4^+ (Tab. 1). High pH, EC (*McLachlan* et al., 2004; *Stefaniuk* et al., 2015), and NH_4^+ (*Tam* and *Tiquia*, 1994) have been recognized as potential sources of phytotoxicity, as well as heavy metals (*Stefaniuk* et al., 2015). The origin of the feedstock (FVW lacking recalcitrant substances) may have been the main reason for the positive effect reported here in the phytotoxicity test. FVW is an important source of material for anaerobic digestion or co-digestion (*Bouallagui* et al., 2005), and it is therefore possible to find available AD from FVW.

The changes to the AD as a consequence of the autoclaving process affected the GI values obtained from the different plant species in different ways. This was a consequence of the interaction between two phenomena. The first one was related to the chemical transformations that occurred during the thermal process, which generated new and complex substances; these frequently affect living organisms in a negative way (*Khavazi* et al., 2007; *Wang* et al., 2015). The second phenomenon is the differential response of each plant species used in the phytotoxicity test to such substances (*Panuccio* et al., 2016). Apparently, some of the substances generated in the sterilization process were toxic for tomato and cress at the 20% dilution (Tab. 4), but this was a stimulant for the other plant species.

Interestingly, the growth of *B. siamensis* counteracted possible phytotoxicity and, consequently, tests with BF produced GI values above 100% for all species in the two dilutions tested. Similarly, *Kantha* et al. (2015) also observed that the presence of bacteria counteracted the phytotoxic effect initially presented by a solid carrier intended for use as an inoculant for rice crops.

3.3 Seedling growth test with AD-m, AD-m-ST, and BF

The treatments led to significant differences in the evaluated parameters, *i.e.*, dry shoot and root biomass ($P < 5\%$), whereas the dose did not lead to any significant differences, and no interaction was found between the factors treatment and dose for any of the evaluated parameters (data not shown). The BF treatment significantly increased the dry shoot and root biomass, compared to the AD-m treatment, and the dry aerial biomass compared to the AD-m-ST treatment (Tab. 5). The AD-m product was rich in microorganisms from the digestion process, which had not been optimized by its interaction with the plants. The AD-m-ST product was free of living microorganisms and the BF product had 10^9 cfu mL^{-1} of the selected strain *B. siamensis*. From these results, it can be seen that PGPR *B. siamensis* is responsible for the growth improvement observed in the BF treatment. For the range of doses used in the experiment, which exerted no differences, the concentration of PGPR ranged between 10^8 and 1.5×10^9 cfu $plant^{-1}$, a typical concentration of PGPR needed to trigger a response in the plant (*Zhao* et al., 2011). For the field trial, we selected the highest dose of the range and one higher, because of the conditions of greater competitiveness among microorganisms, and harder environmental conditions in 'real' soil.

3.4 Field trial

3.4.1 Fruit characteristics

The location produced significant differences regarding length, maximum circumference of the fruits, °Brix of the juice, and K concentration; these values were higher in Oteruelo than in León (data not shown). Transplanting at an earlier date could have been the main reason for the greater length, circumference, and °Brix in Oteruelo because, as a consequence of earlier transplanting, the flowering occurred earlier in the season. Thus, the sum of degrees on days above 6°C from transplanting to the last harvest was higher in Oteruelo (1682°C) than in León (1609°C).

The treatment caused significant differences regarding the maximum circumference ($P < 0.1\%$), the thickness ($P < 0.1\%$), and the P concentration ($P < 1\%$). There was no significant interaction between location and treatment, or between repetition and treatment (data not shown). The fresh mass per single fruit was significantly ($P < 1\%$) correlated with length, maximum circumference, and thickness of the fruits (Pearson correlation coefficients were 0.44, 0.82, and 0.44, respectively), indicating that the final weight of the fruit depended on three parameters, although the diameter of the fruit, estimated here by the circumference, had a greater contribution. The diameter of fruits has previously been used as an indicator of the effect of PGPR in pepper crops (*García* et al., 2004).

The treatment did not significantly affect pH or °Brix. When considering differences between the BF treatments and the N 80% and N 100% controls, the concentrations of N, K, Ca, and Mg in the fruit were not significantly affected by the treatments, nor was the concentration of P (Tab. 6). This result is in contrast to the observations of *Rocha* et al. (2006), who in a previous work on pepper cultivation, reported a significantly lower concentration of P in the biofertilized treatment and an improvement in the efficiency in the use of P because of biofertilization. The significantly higher concentration of P in the

Table 5: Average values obtained for seedling growth in the growth chamber. The values presented are mean values of the different doses tested for each treatment. Values followed by the same letter do not significantly differ (LSD test $P < 5\%$).

Source of variation	Dry shoot biomass (mg $plant^{-1}$)	Dry root biomass (mg $plant^{-1}$)
AD-m	360[a]	237[a]
AD-m-ST	407[a]	258[ab]
BF	458[b]	276[b]

Table 6: Average values of the dependent variables related to the physical and chemical characteristics of the fruits, and for the yield and its components in the field experiments at Oteruelo and León. Values followed by the same letter do not significantly differ (LSD test P < 5%).

Treament	Fruit morphology			Fruit juice		Elemental concentration					Yield per plant (fresh fruits)	Yield components			Yield per ha (fresh fruit)	Aerial biomass per plant (dry matter)	HI
	Length	Maximum Circunference	Thickness	pH	°Brix	N	P	K	Ca	Mg		Average number of fruits per plant	Average fresh mass per single fruit	Plants per square m			
	(cm)	(cm)	(mm)			(%)	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(g)		(g)		(kg ha^{-1})	(g)	
Non-N-control*	9.7^{a}	23.4^{a}	0.577^{a}	5.04^{a}	6.25^{a}	1.286^{a}	2850^{b}	18678^{a}	762^{a}	854^{a}	224^{a}	1.49^{a}	142^{a}	12.9^{b}	29043^{a}	26^{a}	0.619^{a}
N 80% control*	9.2^{a}	23.2^{a}	0.617^{a}	5.01^{a}	6.36^{a}	1.384^{a}	2587^{a}	18147^{a}	781^{a}	862^{a}	332^{b}	2.06^{b}	152^{a}	12.6^{b}	41804^{b}	36^{b}	0.626^{a}
N 100% control*	9.5^{a}	24.3^{ab}	0.615^{a}	4.98^{a}	6.33^{a}	1.408^{a}	2418^{a}	18162^{a}	801^{a}	839^{a}	392^{bc}	2.39^{bc}	163^{ab}	10.7^{a}	40703^{b}	45^{c}	0.664^{a}
BF 2 ml/plant for N 80 %$^{\#}$	10.2^{a}	25.1^{bc}	0.723^{b}	5.02^{a}	6.36^{a}	1.367^{a}	2485^{a}	17799^{a}	875^{a}	861^{a}	424^{c}	2.39^{bc}	180^{bc}	12.9^{b}	54621^{c}	47^{c}	0.662^{a}
BF 1.5 ml/plant for N 80 %§	9.8^{a}	26.1^{c}	0.724^{b}	5.01^{a}	6.40^{a}	1.407^{a}	2542^{a}	17120^{a}	875^{a}	867^{a}	433^{c}	2.53^{c}	190^{c}	12.4^{b}	53464^{c}	49^{c}	0.664^{a}

*The controls received, instead of the biofertilizer (BF), 2 mL of AD-m-ST (which is the culture medium for the PGPR after sterilization);
$^{\#}$2 x 10^{9} cfu PGPR plant^{-1};
§1.5 x 10^{9} cfu plant^{-1}.

non-N control compared to the rest of the treatments and controls (Tab. 6) was the consequence of the lower use efficiency of this element when N fertilization was insufficient.

3.4.2 Yield, yield components, harvest index, and nutrient use efficiency

Significant differences in the yield of fresh fruits per ha and the total dry biomass per plant were found between the locations of the experiment. Both parameters were higher in Oteruelo than in León (data not shown). As in the case of fruit characteristics, these results were probably due to the transplanting date rather than to edaphological differences.

There were significant differences based on the treatment for all evaluated parameters, except for the harvest index (Tab. 6). There was no significant interaction between location and treatment, or between repetition and treatment (data not shown). Regarding the treatments, the non-N control, not inoculated, produced the lowest yield per plant and yield per ha, significantly different from the rest of the treatments and controls. This result was the consequence of the combination of a significantly lower number of fruits per plant and a tendency toward a lower average mass per single fruit. The total aerial biomass per plant was also significantly lower in the non-N control, not inoculated (Tab. 6).

On the other hand, the yield per plant and the yield per ha were significantly higher in the BF treatments (fertilized with 80% of the recommended N dose) than in the N 80% control (Tab. 6), with an increase in yields of around 30% for both variables. The observed differences were mainly due to a combination of a significantly higher mass per single fruit and a tendency toward a higher number of fruits per plant (Tab. 6). This result is similar to that obtained by *Constantino* et al. (2008) with *Capsicum chinense* Jacquin. In their experiment, the highest yields per plant were obtained with a combination of reduced fertilization and inoculation with a PGPR from the genus *Azotobacter*. Moreover, the yield per ha was significantly higher in the BF treatments than that obtained from the N 100% control (Tab. 6). This result may be associated with the higher number of plants per m^{-2} in the BF treatments, while in the N 100% control trials, several plants died due to blight caused by *Phytophthora capsici*. This disease appeared spontaneously in both locations, but mostly in León, and only affected plants in the N 100% control experiment, with a higher quantity of mineral N.

The observed effects can be attributed to the action of the living PGPR, and not to the action of the components of the growth medium for the PGPR (AD-m-ST) used in the controls, which exerted a negligible effect compared to BF at the doses used. The modes of action of the PGPR involve direct effects on plant growth and/or yield, but also indirect effects as a consequence of a reduction in disease incidence by direct interactions with the pathogen or improved plant defense (*Prashar* et al., 2014). In our case, we can discard the control of blight as the reason for the yield increase in the BF treatments, because this disease only affected the N 100% control as a result of the higher incidence of blight under conditions of high availability of mineral N (*Liu* et al., 2008). The BF treatments were fertilized with 80% of the recommended N dose. For such a level of N, the incidence of the disease was irrelevant, as was observed in the N 80% control.

In the present work, the most probable mode of action of *B. siamensis* was the synthesis and release of phytohormones, as well improved tolerance to environmental stressors through the production of phytohormones and ACC deaminase. The strain used in this work was selected by *Barquero* (2014) on the basis of a combination of high IAA production and ACC deaminase activity. From the taxonomic viewpoint, *Bacillus siamensis* (*Sumpavapol* et al., 2010) belongs to the *Bacillus amyloliquefaciens* group, which includes several strains known as biocontrollers but also strains with growth-promoting activity by mechanisms other than biocontrol (*Wu* et al., 2015; *Chung* et al., 2015), as was observed in our study.

The BF improved the nutrient use efficiency, which was estimated by the partial nutrient balance (PNB) for N and P (Tab. 7). The PNB was calculated as the ratio between the nutrient content of the harvested portion of the crop and the amount of nutrient applied (*Dobermann*, 2007; *Drechsel* et al., 2015). The PNB was higher in Oteruelo than in León, due

Table 7: Nitrogen and P use efficiency of the various treatments in the field experiments (León and Oteruelo). The nutrient use efficiency was estimated by the partial nutrient balance (see the text for details).

	Partial N balance		Partial P balance	
	León	Oteruelo	León	Oteruelo
Non-N control[a]	Not applicable	Not applicable	8	17
N 80% control[a]	25	34	13	19
N 100% control[a]	18	29	11	19
BF 2 ml plant^{-1} for N 80%[b]	31	45	16	26
BF 1.5 ml plant^{-1} for N 80%[c]	34	42	18	23

[a]The controls received (instead of the biofertilizer) 2 mL of AD-m-ST (which is the culture medium for the PGPR after sterilization);
[b]2×10^9 cfu PGPR plant^{-1};
[c]1.5×10^9 cfu plant^{-1}.

to the better general performance of the crop in Oteruelo as discussed above. In general, the PNB for N showed the lowest values for the control that received the highest N dose (N 100% control), and improved for the control fertilized with 80% of the N recommended dose (N 80% control). The BF treatments, regardless of the dose used, increased the PNB compared with the N 80% control from 25% to 31–34% in León and from 34% to 42–45% in Oteruelo. In the case of P, all the treatments received the same P fertilization, and there was also a slight increase of the PNB as a consequence of the BF.

3.4.3 Reisolation of the inoculated bacteria at the end of the field experiment

Different techniques have been used to identify the inoculated strain in the endosphere or rhizosphere of the crop; for example, the use of a GFP-tagged strain (*Zhao* et al., 2011; *Bernabeu* et al., 2015) or the use of spontaneous rifampicin-resistant strains (*Hassen* and *Labuschagne*, 2010). The RAPD analysis used in the present study presents the advantage that there is no need for either genetic transformation of the strain or using antibiotic-resistant strains. RAPD allowed simple and unambiguous identification because the RAPD profile is strain-dependent (*Rivas* et al., 2006).

The successful reisolation of the SCFB 3-1 strain from the crop roots after the last harvest confirms the colonization and survival of the inoculated strain during the growth season. The counts of the inoculated strain showed the following average values expressed in log cfu g^{-1} of roots: for the plants inoculated with the lower dose of BF at 1.5 mL $plant^{-1}$: Oteruelo 3.01, and León 2.91; for the plants inoculated with the higher dose of 2.0 mL $plant^{-1}$: Oteruelo 3.12 and León 3.07. There were no statistical differences between the average counts with the lower (1.5 mL $plant^{-1}$) and the higher (2.0 mL $plant^{-1}$) inoculation doses (t test: $P < 5\%$). Moreover, there were no significant differences in the counts between Oteruelo and León (t test: $P < 5\%$). The observed number of *Bacillus siamensis* SCFB 3-1 was in the order of 10^3 cfu g^{-1} of roots (0.8–1.3 10^3 cfu g^{-1} of roots), which can be considered low when compared to other works, which reported 10^4–10^7 cfu g^{-1} of roots, although these studies were performed in pots rather than in field plots (*Zhao* et al., 2011; *Hassen* and *Labuschagne*, 2010).

3.5 Future implications for using a biofertilizer based on AD from FVW

The use of AD from FVW for producing a microbial biofertilizer has been demonstrated to be a technically viable option, which can become a revenue source as an alternative to the direct return of the AD to the agricultural lands. Our approach aligns with the European politics of Circular Economy, which, through the action plan of December 2015 from the European Commission, encourages the recycling of materials in fertilizers. However, the production of microbial biofertilizers using AD is not included in the so-called nutrient recovery technologies (NRT) from digestates (*Vaneeckhaute* et al., 2017) and as such it can be considered an innovative use of the digestate. The amounts of nutrients provided by the biofertilizer are negligible and the most important action is the improvement of the efficiency of the agricultural system triggered by the bacteria. Besides the yield increase, this type of improvement has advantages from the environmental point of view. The higher use of soil N by the crop when biofertilized reduces the available soil N for leaching processes.

4 Conclusions

The AD obtained from food and vegetable waste is adequate for the production of a microbial biofertilizer based on *B. siamensis* (BF). The AD at a 50% dilution and supplemented with 2.3% of sugar beet molasses was the optimum growth medium for these bacteria. In the field, the treatments inoculated with BF and fertilized with reduced mineral N produced the best yield per ha, significantly higher than the control with reduced mineral N, and even higher than the control with full N. Therefore, BF improved the efficiency of N use by the crop, with a positive effect from the environmental perspective.

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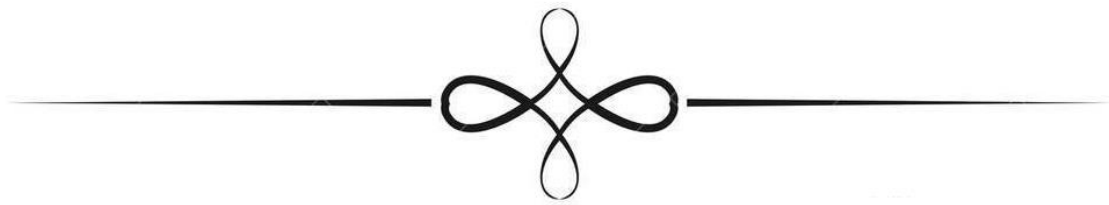
Chapter 6

Compendium of results and general discussion



From the point of view of the plant–bacteria interaction, MPBs can be divided into endosymbionts and endophytes. The term endosymbionts refers to bacteria that produce, in symbiosis with plants, specific structures in which the symbiotic functions occur; this is the case for rhizobia, which are endosymbionts of legumes (Peix et al., 2015). Endophytes are microorganisms that enter plant tissues and can live inside them, either within or among host cells (Harrison & Griffin, 2020). Endophytes and plants form a symbiotic relationship (Dang et al., 2020). This work encompasses the study of endosymbionts and endophytes, and it has generated relevant knowledge about both MPB types for the agricultural market. The work covers the value chain of such kinds of products from the lab to the farm.

More specifically, this work tackles the agronomical exploitation of the interaction between plant roots and bacteria from a triple perspective: i) rhizobial endosymbionts in legume crops, as in the case of the common bean (Pastor-Bueis et al., 2019); ii) non-rhizobial endophytes in non-legume crops, as in the case of the sweet pepper (Pastor-Bueis et al., 2017); and iii) non-rhizobial endophytes of root nodules in the common bean (Pastor-Bueis et al., 2021).

A relevant aspect of the obtained results is that they cover knowledge gaps in the development of MPBs. The first of the mentioned gaps is the lack of publicly available knowledge about formulations. The second gap is about the field performance of MPBs, because most of the research published in scientific papers has not reached beyond the microcosm level (Barquero et al., 2019; Menéndez & Paço, 2020). The third gap is the limited knowledge about plant–endophyte interactions from the molecular and cellular points of view, a deficiency that hinders the efficient design of endophyte-based products to be used in more effective and sustainable agriculture (Papik et al., 2020).

The field testing at a relevant scale in terms of plot size has been one of the most significant aspects covered by this work. Indeed, all the MPBs used for the non-legume crop (pepper) and for the common bean (the rhizobia and the pseudomonad co-inoculant) have been tested in the field using a fully formulated product. The development of a successful formulation has also been a relevant part of this work because as previously highlighted by

Herrmann and Lesueur (2013), inoculating plants with unprotected bacterial strains usually results in a failure at the field level, which renders the conclusions from the experimental works spurious with regard to unformulated inoculants (Barquero et al., 2019).

The approach followed in this thesis is, in our opinion, the most adequate regarding MPB research when the aim is, as in this case, to develop knowledge that is directly transferable to the companies involved in the production and commercialisation of MPBs. In this sense, the research must be driven by field trials because only microbial genotypes successful in the field should be subjected to further research and development. Thus, the starting point of this work was three elite microbial strains that provided promising results in small-scale field tests in previous works: *B. siamensis* SCFB3-1 (subsequently designated as B) and *P. brassicacearum* subsp. *neoaurantiaca* RVPB2-2 (subsequently designated as P) were isolated, selected, and pre-evaluated in the field by Barquero (2014) and *R. leguminosarum* LCS0306 (subsequently designated as R) by Mulas et al. (2011).

This compendium of results is organised in two main sections. The first one is about the advances achieved in common bean production inoculated with the endosymbiont strain R. The second one is about the advances achieved in the crops inoculated with endophytes; it comprises two subsections: i) sweet pepper production after inoculation with B and ii) common bean production after co-inoculation with P and R. Each section and subsection starts with a summary of the results from the field experiment to demonstrate the agronomic success achieved by each of the three strains, namely R, P, or B, for each intended agricultural use. Subsequently, the obtained results are summarised and discussed, and further research – optimising the formulation (with R), optimising the inoculant production with a fermentative process (with B), or understanding the superior agronomic performance, based on the analysis of the interaction between the microbes and the plant (with R and P) – is described.

6.1. Advances in the contributions of the inoculation of legumes with endosymbiont rhizobia to agriculture sustainability

As stated in the introduction, the common bean is the most consumed pulse in human diets around the world (Baptista et al., 2017), but unfortunately it is the poorest nitrogen fixer amongst the most widely grown grain legumes (Martínez-Romero, 2003). Thus, an improvement in the BNF would be very relevant worldwide to improve the economic and environmental sustainability of common bean production.

6.1.1. Superiority in the field trials of the autochthonous rhizobial strain R versus other allochthonous strains and the native soil bacteria

This thesis gathers four field trials with the common bean inoculated with the elite strain R (Pastor-Bueis et al., 2019, 2021). The overall number of trials is sufficient to affirm definitively that R is effective as an inoculant in the agrosystem in which it was isolated (the PGI 'Alubia de La Bañeza-León'). In brief, inoculation with R increased the yield by 36% on average compared with the uninoculated and unfertilised-with-nitrogen control (Pastor-Bueis et al., 2019, 2021); the increase was statistically significant.

It is worth mentioning that the native soil bacteria produce nodules with the common bean due to the promiscuous behaviour of the crop (Andrews & Andrews, 2017). Indeed, in Pastor-Bueis et al. (2019, 2021), the number of nodules produced by the uninoculated control was similar to the number of nodules of the inoculated treatments. However, the nodule biomass was significantly greater in the inoculated treatments. Moreover, the nodules formed with R were much more efficient in nitrogen fixation than the nodules formed with the native soil bacteria. Indeed, the Ndfa (%) in the uninoculated treatment ranged from 41.3% (Table 1 in Pastor-Bueis et al., 2019; p. 41) and 43.5% (Table 4 in Pastor-Bueis et al., 2021; p. 58), whilst with the R treatment it ranged between 50% (Table 1 in Pastor-Bueis et al., 2019; p. 41) and 55.2% (Table 4 in Pastor-Bueis et al., 2021; p. 58). In all the cases, the difference between the uninoculated and inoculated crops was statistically significant.

Our results confirm the promiscuity of the common bean: it is able to form nodules with several genera and species of *Alphaproteobacteria* and *Betaproteobacteria* (Peix et al., 2015), belonging to at least five different symbiovars (Rouhrazi et al., 2016). Our results also confirm the ineffectiveness or very low effectiveness, in terms of nitrogen fixation, of many of the associations between the common bean and resident soil bacteria (Dall'Agnol et al., 2013).

Besides, compared with the nitrogen fertilised and non-inoculated control, inoculation with R produced a slightly higher yield. However, the increase was not statistically significant: it was < 4% in Pastor Bueis et al. (2019) and < 1% in Pastor-Bueis et al. (2021).

Moreover, we compared, at the field scale, the effect in the crop yield between the autochthonous strain R and the two other allochthonous strains: i) the type strain of *R. phaseoli* (ATCC 14482^T) and the type strain of *R. etli* (CFN 42^T), both belonging to the symbiovar *phaseoli*, which only nodulates legumes in the genus *Phaseolus* (Pastor Bueis et al., 2019). R outcompeted, in terms of crop yield, the allochthonous strains. The yield obtained with R was 24% higher than the one obtained with *R. etli* and 22% higher than the one obtained with *R. phaseoli*; the differences were statistically significant (Figure 3 in Pastor Bueis et al., 2019; p. 42). In addition, the Ndfa% was significantly higher after inoculation with R compared with *R. etli* (Table 1 in Pastor Bueis et al., 2019; p. 41). However, both *R. phaseoli* and *R. etli* were effective nitrogen fixers compared with the uninoculated and unfertilised-with-nitrogen control, although the yield was far below (~15%) the nitrogen-fertilised control. The results obtained with the allochthonous strains confirm those of Daza et al. (2000) and Rodriguez-Navarro et al. (2000); these findings have saddled the common bean with a reputation as a bad nitrogen fixer (Martínez-Romero, 2003). However, our work has definitively confirmed the hypothesis raised in other previous works, namely that the use of native-naturalised rhizobia selected for their high nitrogen fixation effectiveness usually results in successful inoculants as long as they are adequately formulated (Araujo, et al., 2020a; Koskey et al., 2017).

6.1.2. Advances in inoculant formulation: carrier selection

An important part of the research effort of Pastor Bueis et al. (2019) has been dedicated to the design of a formulation based on the principles of a circular economy. Considering the environmental impact derived from the overexploitation of peat, which is the traditional carrier, Albareda et al. (2008) proposed perlite as the best alternative in terms of bacterial survival during storage (i.e., a long shelf-life) and in terms of effectiveness of the inoculant formulation (which is evaluated by the crop yield). However, perlite is obtained from a volcanic siliceous rock after an industrial process that requires temperatures ≥ 1000°C (Angelopoulos et al., 2014). Hence, when perlite is used as a carrier, the carrier alone accounts for most of the environmental impact of the finished inoculant, as demonstrated by Araujo et al. (2020b) in a life cycle analysis study; specifically, perlite accounted for 67% of the global warming potential and for 54% of the energy demand.

In our work, we tested alternatives to perlite, namely three carriers based on sterilised bio-residues following the principles of a circular economy: compost Co; carbocompost CC (compost with 6% w/w pine bark biochar); and perlite-biochar PB (25% perlite and 75% pine bark biochar). Perlite alone (Pe) served as the control. The four carriers showed a good capacity to maintain adequate survival during the 365-day shelf-life experiment, although PB showed the most stable survival values during the experiment (Figure 1 in Pastor-Bueis et al., 2019; p. 40).

In the field trial, the grain yield obtained with the bio-based carriers was similar to the control Pe, but PB significantly increased (15%) the grain yield compared with the control Pe. Interestingly, the crop yield improvement was not accompanied by a significant increase in the parameters related to symbiosis: the nitrogen derived from atmospheric fixation (Ndfa %), nodule number, and nodule biomass (Table 5 in Pastor-Bueis et al., 2019; p. 45) did not differ between carriers. Thus, we hypothesise that the yield improvement with the formulation based on biochar was due to the recognised plant growth promoting effect of the carbon obtained from pyrolysis (Yang et al., 2019), rather than to a direct effect in the strain performance as a consequence of the formulation.

6.1.3. Genome mining to explain the superiority of the rhizobial autochthonous strain R in the field

Our subsequent research in Pastor Bueis et al. (2019) focussed on unravelling the reasons for the superior performance of R for the farm conditions. The approach was based on a comparative genomic analysis of the draft genome of R with the complete genomes of the allochthonous strains used in the field tests (*R. phaseoli* ATCC 14482^T and *R. etli* CFN 42^T) and with that of the closest related strain according to the Average Nucleotide Identity (ANI) values, namely *R. leguminosarum* bv. *viciae* (UPM791) (ANIm 98.19% and ANIb 97.39%). Based on the ANI values, we considered R and UPM791 to belong to the same genospecies.

In a previous work, Mulas et al. (2011) demonstrated that R has the greatest symbiotic nitrogen fixation ability from a collection of autochthonous isolates from the PGI 'Alubia de la Bañeza-León'. Those authors reported its superiority in hydroponic and axenic tests compared with uninoculated controls, one with mineral nitrogen added and the other without any nitrogen input. As expected, we found that the R draft genome harbours all the nodulation and nitrogen fixation genes, which are required to establish a successful symbiotic relationship with *Phaseolus* (Supplementary Table S9 in Pastor-Bueis et al., 2019; p. 136).

However, for a strain to be effective in the field, it must show high colonisation and nodulation abilities, in addition to the mentioned symbiotic nitrogen fixation, because both abilities are uncoupled between them in bacteria (Westhoek et al., 2017). Thus, the success observed in the field trials for R can only be explained if it is competitive for colonisation and nodulation with respect to the resident soil bacteria. We demonstrated the competitiveness of R in the field; when the crop was inoculated with R, the recovery rate of the inoculated strain from the interior of the nodules was between 72% and 84% (Pastor Bueis et al., 2019). These values were higher than the recovery rate of *R. phaseoli* or *R. etli* when the crop was inoculated with these strains; for *R. etli*, the recovery rate was very low (40%–64%).

Our genome mining focussed on the search for genes that explain the ability of R to compete (Supplementary Table S8 in Pastor-Bueis et al. (2019); p.

134). In brief, R contains a large repertoire of secretion systems, which combine with the secretion systems of the two allochthonous strains used in the field trials. Secretion systems have been proposed to play an important role in the ability to colonise the rhizosphere (Gupta et al., 2014). R and *R. phaseoli* ATCC 14482^T harbour gene clusters encoding a type VI secretion system (T6SS). Liang et al. (2018) and Sánchez-Cañizares et al. (2018) suggested that T6SS increases competitiveness with respect to other soil bacteria. However, R also harbours type IV secretion system (T4SS) pili, which are absent in the allochthonous strains. Moreover, R harbours a putative type III secretion system (T3SS), which is absent in *R. phaseoli* ATCC 14482^T but present in *R. etli* CFN42^T, in which it is functional. T3SS reportedly induces the transcription of nodulation genes in the presence of flavonoids (Jiménez-Guerrero et al., 2017).

Moreover, the competitive ability has also been discussed from two other perspectives: exploitation (more efficient utilisation of a common limiting nutrient) and interference (preventing another cell from growing and surviving). Regarding exploitation, bacterial chemotaxis towards the root exudates is important for competition. In this sense, R has the important Che1 gene cluster from *R. leguminosarum* bv. *viciae*; this cluster is essential for competitive nodulation (Miller et al., 2007). The transport systems are important for growth and exploitative competition, given the complexity of the rhizosphere environment (Prell & Poole, 2006). In this sense, R contains 183 genes involved in putative ATP-binding cassette (ABC) transporters.

Finally, one of the chief strategies of interference competition is the production of bacteriocin-like compounds (Onishchuk et al., 2017). R contains the *quorum sensing* system cinRIS, which is responsible for the production of small bacteriocins, which are typical of fast-growing rhizobia (Wijffelman et al., 1983).

6.1.4. An explanation for the field performance of R, beyond nitrogen fixation

The improved grain yield produced by R was only partially explained on the basis of Ndfa% or nodule biomass – that is, the correlation between grain yield and Ndfa% or nodule biomass was, at best, weakly significant (Table 3

in Pastor-Bueis et al., 2019; p. 42), although in our later publication, the correlation was stronger (see Table 5 in Pastor-Bueis et al., 2021; p. 58). Thus, symbiotic functioning can only partially explain the superior yield increase observed in the crop inoculated with R compared with the allochthonous strains *R. phaseoli* and *R. etli*. We hypothesised that other PGPR activities exerted by R are the reason for the superiority; in a future work, we will analyse the PGPR genes in the R genome.

We also emphasised the genetic versatility of R, revealed by genome mining, to explain its superiority (Pastor Bueis et al., 2019). Such versatility is due to the gene assortment: R contains the genes required for efficient symbiosis with *Phaseolus* spp. as well as a large repertoire of secretion systems. R combines 'the best' of several known rhizobial strains; this combination helps to explain its superiority over other strains. It has a genetic backbone homologous to the biovar *viciae* from *R. leguminosarum* strain UPM791, with the symbiotic repertoire of *R. etli* CFN42T. Moreover, R shows genetic evidence of having incorporated features that might positively impact its competitiveness and symbiotic performance; this is demonstrated by the heterogeneity within the symbiotic plasmid (p42d), which shows extensive genomic rearrangements, recombination rates, lateral transfer, and relaxation or intensification of selective pressures (González et al., 2003).

6.2. Advances in the study of the contribution of endophytes to sustainable agriculture

This thesis includes the analysis of two different endophytes: P was used as co-inoculant for the common bean together with R (Pastor-Bueis et al., 2021) and B was used as inoculant for the sweet pepper (Pastor-Bueis et al., 2017). In both cases, the field trial demonstrated that the endophytes were agronomically effective. The focus of the subsequent research was different in the two works.

6.2.1. Endophytes as co-inoculants of legume crops: the cases of *P. brassicacearum* in co-inoculation with an endosymbiont rhizobium in the common bean

Numerous scientific publications have demonstrated the improved nodule performance in legume crops co-inoculated with an endosymbiont rhizobium and a non-rhizobial strain (Barbosa de Souza & De Brito Ferreira, 2017; Elkoca et al., 2010; Hungria et al., 2013; Kumar et al., 2016; Remans et al., 2008; Santos et al., 2019). We addressed the development of a co-inoculant for the common bean in the PGI 'Alubia de La Bañeza-León' and tested it in field conditions (Pastor-Bueis et al., 2021). However, we went beyond the mere development of a consortium; moreover, we investigated the reasons for the success in the field trials of the best rhizobia – the PGPR combination – in terms of crop yield. We analysed the nodule spaces that are occupied by the microbial members of the consortium as well as the physiological characteristics of the non-rhizobial partner. This information is very relevant to outline the criteria required to select adequate partners in the design of co-inoculants; according to Martínez-Hidalgo and Hirsch (2017), in the future, the selection of strains that can be used as co-inoculants will be based on the knowledge about involved biochemical pathways and microbe–microbe and plant–microbe interactions.

The endosymbiont used as the co-inoculant was R, whilst the partners were: i) the autochthonous strain P, which is an endophyte isolated from common bean roots (not nodules), and ii) the type strain of *A. chroococcum* Beijerinck 1901 (ATCC9043^T) (subsequently designated as A), which is a rhizospheric strain (Ambesh et al., 2017; Chaudhary & Sindhu, 2016). The tested consortia were R+P, R+A, and R+P+A.

The field trial for this work is, to the best of our knowledge, the first to co-inoculate the common bean in Western Europe. Agri-systems in Western Europe are technologically advanced; due to the intensive use of mineral nitrogen fertilisers, the soil has a large nitrogen reservoir. Hence, the common bean crop uptakes nearly 60% of its nitrogen requirements from the soil reservoir, although the dependence on the soil nitrogen fell to ≤ 50% when successful symbiosis took place (Pastor-Bueis et al., 2019, 2021). For

this reason, the inoculation of legume crops has been traditionally neglected in Europe. Due to this particularity of European agri-systems, the field tests intended to make agronomic and business decisions must be conducted locally because extrapolating from remote agri-systems is not valid (Mulas et al., 2013).

6.2.1.1. A search for explanations for the superior field performance of R+P

The field trial indicated that the most successful partner for the endosymbiont R was the endophyte P. We reported that the R+P consortium performed better than inoculation with R alone and better than the R+A and R+A+P consortia in terms of crop yield (Pastor-Bueis et al., 2021). Specifically, the yield obtained with R+P was 17% higher compared with single inoculation, 12% higher compared with R+A, and 15% higher compared with R+A+P (Table 6 in Pastor-Bueis et al., 2021; p. 60). To determine if the R+P consortium performed statistically better than the other two consortia, we performed an orthogonal contrast analysis. In it, we compared the R+P consortium against the other consortia (Figure 2f in Pastor-Bueis et al., 2021; p. 59). The R+P consortium produced a significantly higher yield than the other R-based consortia. We hypothesised that the explanation for the superiority of R+P could be one of the following, or most probably a combination of them: i) the autochthonous origin of the P strain provides it with better adaptation; ii) the endophytic colonisation of P compared with the rhizospheric colonisation of A; iii) R and P colonise different spaces inside the nodule, a phenomenon that avoids competition; and iv) the plant growth promoting activity of P.

First, as already discussed, locally isolated bacteria usually perform better in the field than allochthonous ones, due to better adaptation to the local agro-climatic conditions (Meghvansi et al., 2010) and the resident microbial populations (Tena et al., 2016). However, in this work we evaluated one strain of *P. brassicacearum* subsp. *neoaurantiaca*, and thus the hypothesis of the better performance of a local strain compared with an allochthonous one has not been tested.

Second, the endophytic nature of P provides it with a protected environment where the mutual benefit of the plant–bacteria association can be better

expressed (Santoyo et al., 2016). We assumed P was endophytic because it was isolated from surface-sterilised common bean roots (Barquero, 2014); we subsequently confirmed this hypothesis (Pastor-Bueis et al., 2021) using confocal laser scanning microscopy (CLSM; Figure 3B in Pastor-Bueis et al., 2021; p. 62). We observed that P enters the root cortex.

Third, using CLSM to observe root nodules (Figure 3D in Pastor-Bueis et al., 2021; p. 62), we confirmed that P colonises the interior of the nodules. Interestingly, R and P are localised in separate niches: whilst R is intracellular, P is intercellular. The results suggest that there is no contact between the inoculants once inside the nodules, and we hypothesise that this separation prevents their competition with each other and could be one of the causes of the observed crop yield increase. Moreover, we confirmed the P colonisation of the nodule that we observed microscopically with transformed bacteria in hydroponic conditions in field conditions, with the re-isolation of this strain from surface-sterilised root nodules. Fifty per cent of the endophytes from nodules that grew in TSA medium corresponded to the inoculated P strain.

Fourth, P showed relevant plant growth promoting characteristics, which could be also responsible for the improved performance of R+P. Some authors (e.g. Hungria et al., 2013) have indicated that, the improved nodule functioning and crop yield observed after co-inoculation with rhizobia and a different PGPR partner is due to the plant growth promoting properties of the PGPR. Specifically, those authors co-inoculated legumes with a specific rhizobium plus a nitrogen-fixing *Azospirillum* strain and concluded that the increased nitrogen fixation in the co-inoculated treatment was due to other plant growth promoting properties from the *Azospirillum* strain, rather than to the nitrogen fixation exerted by *Azospirillum*. In our research, P exceeded A on three of the four plant growth promoting properties we analysed (Table 3 in Pastor-Bueis et al., 2021; p. 57), namely insoluble phosphate solubilisation, siderophore production, and ACC deaminase activity. Interestingly, the R+P consortium increased the number of nodules per plant by more than 30%, the dry nodule weight by more than 25%, and the fixed nitrogen by more than 20% compared with the single inoculation with R. By contrast, co-inoculation with the R+A consortium produced a smaller increase in those aforementioned variables (Table 4 in Pastor-Bueis et al., 2021; p.

58). This difference was not statistically significant due to the data dispersion typical of the mentioned parameters in field trials (e.g., Htwe et al., 2018), but the observed tendencies are worth analysing. The improved nodulation could be due to P's high ACC deaminase activity, because it has been proved that PGPR with ACC deaminase activity improve the nodulation by rhizobia due to the reduction of the endogenous level of ethylene in the plant roots (Chaudhary & Sindhu, 2016; Sepúlveda-Caamaño et al., 2018; Subramanian et al., 2015). Ethylene inhibits the early stages of nodulation (see section 4.2 in Pastor-Bueis et al., 2021; p. 60 of this summary provides further details on the effects of ethylene in nodulation), and thus the reduction of ethylene levels improves nodulation (Nascimento et al., 2012).

Besides, P produces a medium-to-high level of IAA. Research has shown that the IAA produced by non-rhizobial bacteria associated with plants enhances the secretion of nod-gene-inducing flavonoids from the crops' roots, a phenomenon that improves nodulation by rhizobia (Dardanelli et al., 2008; Okon et al., 2015; Puente et al., 2019; Vicario et al., 2015). Although the IAA produced by P is only one third of that produced by A, we hypothesise that the combination in P of a relevant (medium-to-high) rate of IAA production and exceptionally high ACC deaminase activity confers P with the ability to improve nodulation by the rhizobial strain R. While the differences in nodulation are not statistically significant, there is strong evidence for the influence of P in the nodulation process.

Moreover, P was superior to A in other plant growth promoting activities such as phosphate solubilisation and siderophore production. Altogether, the superior plant growth promoting properties of P could explain the 14% increase in crop yield after inoculating with the R+P compared with the R+A consortium. Again, the difference was not statistically significant, but the numerical tendencies are worth mentioning, even if from the strictly academic point of view they must be analysed with caution.

In summary, it seems that the superiority of P compared with A is due to a combination of characteristics, namely A is rhizospheric and P is endophytic, a characteristic that would avoid competition with other rhizospheric bacteria, and P shows a superior assortment of plant growth promoting activities

compared with A, which altogether exerted a more important effect on the crop yield than the nitrogen fixed by A.

6.2.2. Endophytes for non-legume crops: the case of *B. siamensis* in the sweet pepper

6.2.2.1. Effect of the inoculant in the field trials

The results of the two field trials showed that the higher dose of inoculant (2.0 ml per plant divided into two applications, the first in the nursery and the second at the beginning of flowering), in combination with reduced nitrogen fertilisation (80% of the theoretical crop extraction), produced a statistically significant increase in the crop yield. The increase was 31% compared with the uninoculated control fertilised with the reduced (80%) nitrogen dose and 34% compared with the uninoculated control fertilised with the complete (100%) nitrogen dose (Table 6 in Pastor-Bueis et al., 2017; p. 79). Interestingly, for the uninoculated treatments, the yield with the complete nitrogen dose was smaller than with the reduced dose, due to the higher incidence of *Phytophthora capsici* associated with a higher nitrogen dose.

Equally important is the improvement in the nutrient use efficiency because of inoculation combined with the reduced nitrogen dose (Table 7 in Pastor-Bueis et al., 2017; p. 74). The efficiency was estimated using the partial nutrient balance (PNB), which corresponds to the percentage of the nutrient applied with the fertiliser that is recovered in the crop aerial biomass. The PNB for nitrogen was, on average, 8.5% higher in the inoculated treatments with the reduced nitrogen dose compared with the uninoculated control with the complete nitrogen dose. Interestingly, even if the phosphorus dose was the same in treatments and controls, the inoculation improved the PNB for phosphorus by 4.8% due to the biomass increase because of inoculation, which involved an increase in the phosphorus quantity extracted from the soil. The inoculated strain B was re-isolated at the end of the field trial from inside the root, at a rate of 1×10^3 cfu g^{-1} root.

6.2.2.2. Inoculant production based on the principles of a circular economy and previous tests

Our subsequent research in Pastor-Bueis et al. (2017) focussed on the optimisation of growth media for bacteria production in a fermentative process. The approach involved the use of agro-industrial wastes for the growth media, following the guidelines of the 2015 European Commission Circular Economy Action Plan, which has been revised in 2020 (COM, 2020). Such an action plan encourages the recycling of materials in fertilisers.

In the present work, our starting hypothesis was that a sterilised AD obtained from FVW can be used as growth media for the production of the inoculant with B. To evaluate the suitability of the AD, we tested this product alone and in combination with different carbon sources (namely glucose, lactose, and sucrose) at a very small scale (50 ml growth media in 100 ml flasks). As expected, growth with AD as the solely growth media ingredient was very weak, and a supplementary carbon source was necessary. Glucose and sucrose produced the best growth, with very similar growth rates; thus, we selected sugar beet molasses as the carbon sources because, as a by-product, it fulfils the principles of a circular economy and it is rich in sucrose.

The next step was to optimise the rates of AD and sugar beet molasses on the growth medium for B, based on RSM, which used a second order polynomial function (Equation 2 in Pastor-Bueis et al., 2017; p. 71). The ANOVA results of the calculated model (Table 3 in Pastor-Bueis et al., 2017; p. 71) indicated that only quadratic effects were significant and that linear and interaction effects presented no significance. The response showed a decrease in values when moving further from the centre in any direction (Figure 2) – that is, at the lower and higher levels, there was insufficient bacterial growth. At the lower levels, this could be due to the lack of sufficient assimilable carbon and/or essential elements and compounds from AD. At the higher levels, there could be an inhibitory effect due to the excessive concentration of complex molecules present in the AD and to an excessive increase in the osmotic potential due to high levels of sucrose. The optimal composition of the growth media was 50% (v:v) AD and 2.3% (v:v) sugar beet molasses.

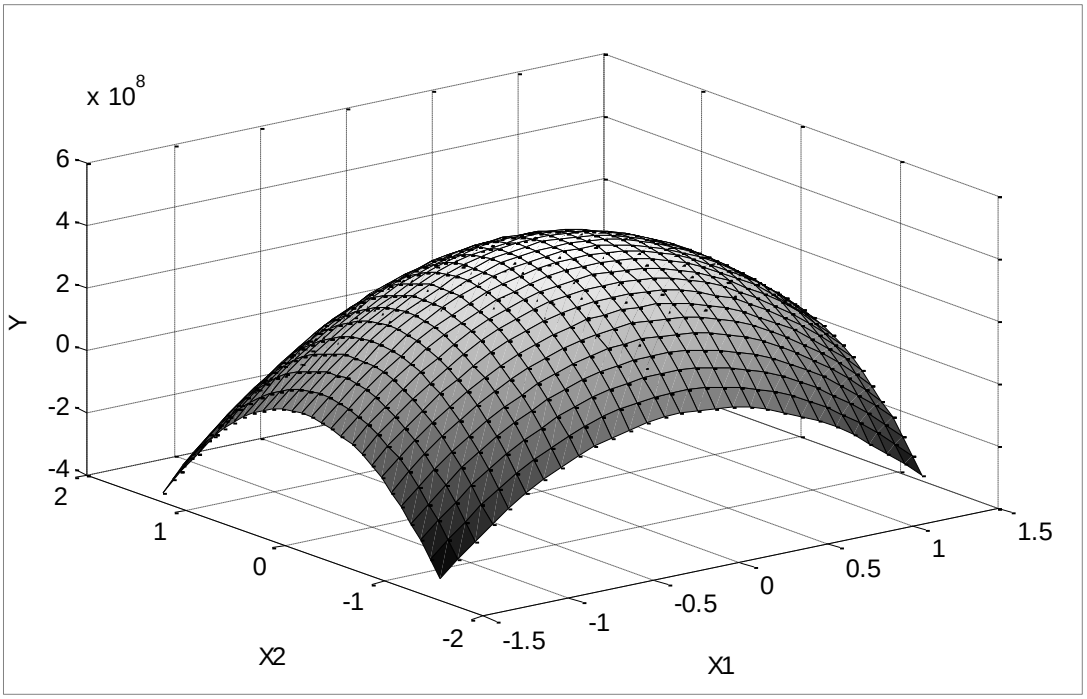


Figure 2. Graphic corresponding to response surface methodology (RSM). The selected factors were the concentration of total solids in the anaerobic digestate (AD) (X1) and the concentration of sucrose (X2) added in the form of sugar beet molasses. The response (Y) was the measured bacterial concentration

The next step was to analyse the possible phytotoxic/phytostimulant effect of the inoculant. Because we published our results (Pastor-Bueis et al., 2017) before the European regulation about microorganisms as fertilisers (Regulation (EU) 2019/1009, 2019), we used the now obsolete term 'biofertiliser' (BF) to refer to the MPB based on B (see chapter 1, section 1.5 for further discussion on this matter). We carried out the phytotoxicity/phytostimulation tests with i) AD-m, ii) AD-m-ST, or iii) BF. We performed two tests, namely a modification of the Zucconi phytotoxicity test and a seedling growth test in nursery conditions.

The Zucconi phytotoxicity test revealed that the pure products were highly phototoxic, but at a 10% dilution all were phytostimulant (Table 4 in Pastor-Bueis et al., 2017; p. 71). Interestingly, the phytostimulant effect we observed for the AD-m (germination index [GI] from 146% to 341%) and AD-m-ST (GI from 155% to 409%) was higher than that observed in other works: Pivato et al. (2015) observed a maximum GI of 140% and Alburquerque et al. (2012) observed a maximum GI of 150%. We hypothesised that the origin of the AD (i.e., FVW) could be the reason for the

good results we obtained. Due to its origin, the AD was free from recalcitrant substances, showed a neutral pH, had low electric conductivity, and had a low N-NH_4^+ content. The sterilisation process increased the phytotoxicity for some plant species and decreased it in others, indicating that the new and complex substances generated during the sterilisation process (Khavazi et al., 2007; Wang et al., 2015) differentially affects the species included in the phytotoxicity test. Interestingly, the growth of B counteracted the phytotoxicity of the products in the AD, as BF showed a phytostimulant effect for all the plant species not at the 10% and 20% dilutions.

Finally, we tested AD-m, AD-m-ST, and BF in the sweet pepper plant production process in the nursery. Compared with AD-m and AD-m-ST, the BF significantly increased plant biomass production (Table 5 in Pastor-Bueis et al., 2017; p. 72).

6.3. Transfer of the generated knowledge and prospective research

The results achieved in this work are relevant for the agriculture sector. In summary, the single inoculation of the common bean with the selected rhizobial endosymbiont strain (R) enables a complete replacement of the mineral nitrogen fertiliser; the crop yield is even slightly superior with the inoculation than with the mineral fertilisation. Moreover, the co-inoculation with the endosymbiont R and the endophyte P improves the crop yield 17% compared with the control fertilised with mineral nitrogen (not statistically significant). Although from the academic viewpoint non-statistically significant tendencies must be analysed with caution, for the registration of a microbial inoculant, the Spanish regulation about microorganisms as fertilisers (R.D. 999/2017, 2017) accepts a sufficient number of field trials

that show a consistent increase in the crop yield (or other quality parameters), even if the differences are not significant.[1]

The main drawback for the introduction of the inoculation technology in farms is the shortage of commercial rhizobial inoculants for legume crops. Unlike what happens in South America, where the use of rhizobial inoculants for legume crops is generalised (Keswani et al., 2019), in Europe only a few of them are currently commercialised for crops like soya (*Glycine max* (L.) Merr.) or other legume grasses;[2] however, to the best of our knowledge, in Europe there are no specific inoculants for the common bean. In Spain, there are no rhizobial inoculants for legume crops registered as microbial fertiliser products in the register at the Spanish Ministry of agriculture.[3] The lack of entrepreneurial interest in the commercialisation of rhizobia for legumes could be related to the specificity of the products – that is, each crop requires a specific strain for each agroclimatic regions to attain optimal field performance, as has been demonstrated. Unlike what happens with soya in South America, the number of cultivated hectares in Europe is much smaller, and the diversity of legume crops is higher; that factor reduces the demand for each single product.

However, we have demonstrated (Pastor-Bueis et al., 2021) that the inoculation of common bean in the PGI 'Alubia de La Bañeza-León' could be very profitable for farmers. Our estimation is based on the actual prices of common beans to farmers, the costs of the fertilisers, and the estimated cost of the inoculant based on real productive costs from local companies. The estimated gross margin increase due to replacing the mineral nitrogen

[1] Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente. Comunicación de la Dirección General de Producciones y Mercados Agrarios por la que se publican los criterios para demostrar la eficiencia agronómica de un producto fertilizante del subgrupo 4.4. Productos especiales basados en microorganismos del anexo 1: Protocolo de ensayos https://www.mapa.gob.es/es/agricultura/temas/medios-de-produccion/report_protocolo_tcm30-435697.pdf (Accessed 10/12/2020)

[2] For products commercialised in Europe, see https://legumetechnology.co.uk/ or, from transnational companies, https://agriculture.basf.com/ar/es/proteccion-de-cultivos-y-semillas/productos/histick-plus.html. These are just examples and are not meant to be exhaustive

[3] https://www.mapa.gob.es/app/consultafertilizante/consultafertilizante.aspx

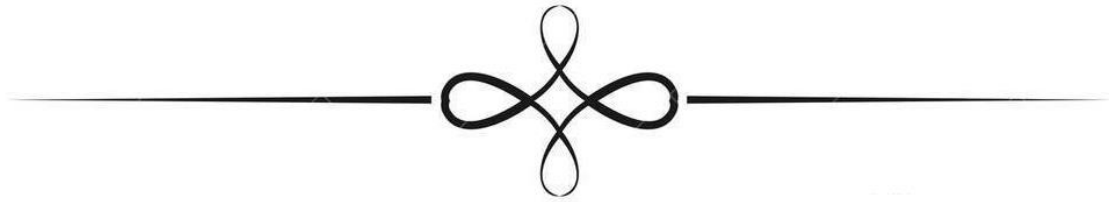
fertilisation with R inoculation is 138 euro ha^{-1}; the increase with R+P co-inoculation is 626 euro ha^{-1}.

MPBs have an unforeseeable but promising future. The investment of public funds for research on the beneficial plant–microorganism interactions must be accompanied by interest from the entrepreneurial sector. As of 2020, stakeholders have shown an increasing interest in MPBs (Barquero et al., 2019). Indeed, the most important European companies in agricultural enterprises are currently creating production lines for microbial products, and several small- or medium-sized companies are also entering into the business. The prospective research must be focussed on:

- The design of strategies to achieve a more efficient selection of effective strains in local conditions. Such strategies must be based on molecular, microscopic, and physiological advances to explain plant–microorganism interactions.
- The development of methodologies for tracking microorganisms in the field after inoculant application. Even if at this moment the regulations do not require this assessment, it is of great interest to know how the populations change in soils and survive across the different growth stages of the crop (Barquero et al., 2019). This information is needed to identify and distinguish the inoculated strain from other resident microorganisms, even from the same species (Reddy et al., 2016). This identification system must be inexpensive and effective. Because the price of genome sequencing is decreasing, in the future it will be more feasible to find distinctive sequences, based on the analysis of the full genome.
- The development of formulations that enable an improved shelf-life and a better establishment of the symbiosis in field conditions.

Chapter 7

General conclusions



1. Regarding the study of the innovative inoculant formulation based on the rhizobial endosymbiont of the common bean, we found the following:

    1.1 In field conditions, an adequate formulation of the autochthonous elite strain R produced a similar yield compared with the un-inoculated and nitrogen fertilised control, and a significantly higher yield (36%) compared with the un-inoculated and unfertilised-with-nitrogen control.

    1.2 From all the formulations designed according to the principles of a circular economy, the best one in terms of shelf-life and crop yield comprised a carrier with 25% perlite and 75% pine bark biochar.

    1.3 In field conditions, the autochthonous strain R performed significantly better in terms of yield and nitrogen fixation than other allochthonous strains from *R. etli* and *R. phaseoli*.

    1.4 Genome mining contributed to explain the superiority of the autochthonous strain R compared with the allochthonous one from two perspectives: a high nitrogen-fixing ability and high competitiveness.

    1.5 The genome analysis explains the high nitrogen-fixing ability because R contains the genes involved in an efficient symbiosis with *Phaseolus* (the symbiotic repertoire of *R. etli* CFN42^T).

    1.6 The genome analysis explains the competitive ability against the native strains because R contains a large repertoire of secretion systems and genes related to exploitative competition (chemotaxis and transport systems) and interference competition (bacteriocin-like compounds).

2. Regarding the use of the autochthonous endophyte P to co-inoculate the common bean together with the rhizobial endosymbiont, we found the following:

    2.1 The co-inoculation of the common bean with the R+P consortium increased the yield by 17% compared with single inoculation with R, and the yield obtained with the R+P consortium was significantly higher than obtained with other R-based consortia.

2.2 Microscopy revealed that P colonises the interior of the nodules, but whilst R is intracellular, P is intercellular, a spatial difference that prevents competition between the strains. This could be one of the causes of the observed increased crop yield.

2.3 The co-inoculation of the common bean with the R+P consortium increased the number of nodules per plant by more than 30% and the dry nodule weight by more than 25% compared with the single inoculation with R. Due to the high dispersion typical of these data, these differences were not statistically significant.

2.4 P significantly improves the rates of four plant growth promoting properties, a phenomenon that could help explain the observed yield increase because of co-inoculation with the R+P consortium. IAA production and ACC deaminase activity could explain the observed tendency towards improved nodulation.

2.5 The nitrogen derived from fixation was 40% in the un-inoculated control due to the nodulation with native soil bacteria, 50% when inoculated with R, and 60% when inoculated with the R+P consortium.

2.6 Inoculation with R and the R+P consortium allows suppression of nitrogen fertilisation, but in the best case the crop's dependence on soil nitrogen is still 40%. Therefore, our findings are only applicable to agricultural soils with a sufficient nitrogen reservoir, such as most Western European soils.

2.7 The gross margin of common bean farmers of the PGI 'Alubia de La Bañeza-León' would increase by 138 euro ha^{-1} (R inoculation) or 626 euro ha^{-1} (R+P consortium inoculation).

3. Regarding the inoculation of the sweet pepper with the autochthonous endophyte B, we found the following:

3.1 The sweet pepper inoculated with the endophyte B and fertilised with decreased mineral nitrogen produced significantly higher yield than the un-inoculated control fertilised with reduced nitrogen (31% increase) and even higher than the un-inoculated control fertilised with full nitrogen (34% increase).

3.2 The inoculation of sweet pepper with the endophyte B fertilised with reduced mineral nitrogen improved the nitrogen use efficiency (estimated with the partial nutrient balance) by 8.5% and the phosphorus use efficiency by 4.8%.

3.3 The optimal growth medium to produce the innovative inoculant formulation of B for sweet pepper comprised 50% (v:v) AD (from FVW) and 2.3% (v:v) sugar beet molasses.

1. Teniendo en cuenta el estudio sobre la formulación innovadora del inoculante basado en el rizobio endosimbionte de alubia, podemos asumir que:

1.1 En condiciones de campo, una adecuada formulación de la cepa autóctona *Rhizobium leguminosarum* bv. *phaseoli* LCS 0306 (R) produjo un rendimiento similar al control no inoculado y fertilizado con Nitrógeno, y un rendimiento significativamente superior (36%) que el control sin inocular y sin fertilizar con Nitrógeno.

1.2 De todas las formulaciones diseñadas de acuerdo con los principios de Economía Circular, la mejor en términos de vida útil y rendimiento de cultivo consistió en un soporte compuesto con un 25% de perlita y un 75% de biochar de corteza de pino.

1.3 En condiciones de campo, la cepa autóctona R se desenvolvió significativamente mejor en términos de rendimiento y fijación de Nitrógeno que las otras cepas alóctonas *R. etli* y *R. phaseoli.*

1.4 El estudio del genoma ha contribuido a explicar la superioridad de la cepa autóctona R frente a la alóctona desde dos perspectivas: una elevada capacidad de fijación de nitrógeno y competitividad.

1.5 El análisis del genoma explica la alta capacidad de fijación de Nitrógeno de la cepa R, ya que contiene los genes involucrados en una simbiosis eficiente con *Phaseolus* (el repertorio simbiótico de *R. etli* CFN42^{T}).

1.6 El análisis del genoma explica la capacidad de competencia de las cepas nativas, ya que la cepa R contiene un amplio repertorio de sistemas de secreción y de genes relacionados con la competencia por explotación (quimiotaxis y sistemas de transporte) y con la competencia por interferencia (bacteriocinas de tipo compuesto).

2. En cuanto al uso del endófito autóctono P para co-inocular alubia junto con el rizobio endosimbionte, podemos asumir que:

2.1 La co-inoculación de la alubia con R+P incrementó el rendimiento del cultivo en un 17% en comparación con la inoculación única con R, y el rendimiento obtenido con el consorcio R+P fue

significativamente mayor que el obtenido con otros consorcios basados en R.

2.2 El estudio de microscopía reveló que la cepa P coloniza el interior de los nódulos; mientras R se ubica intracelularmente, P lo hace intercelularmente, una diferencia de espacios que evita la competencia entre ellos. Esta podría ser una de las causas que explican del aumento observado en el rendimiento de los cultivos.

2.3 La co-inoculación de la alubia con el consorcio R+P incrementó el número de nódulos por planta en más del 30% y el peso seco del nódulo en más del 25% comparado con la inoculación única con R. Debido a la alta dispersión típica de estos datos, las diferencias no fueron significativas.

2.4 La cepa P mejora significativamente las características de cuatro propiedades promotoras del crecimiento vegetativo, esto puede contribuir a explicar el aumento del rendimiento observado como consecuencia de la co-inoculación con R+P. La producción de IAA y la actividad de la deaminasa ACC podrían explicar las tendencias observadas en la mejora de la nodulación.

2.5 El nitrógeno derivado de la fijación fue del 40% en el control no inoculado debido a la nodulación producida por las bacterias nativas del suelo, del 50% inoculando con R y del 60% co-inoculando con el consorcio R+P.

2.6 La inoculación con R y la co-inoculación con R+P permiten la supresión de la fertilización con nitrógeno, sin embargo, en el mejor de los casos la dependencia del cultivo del nitrógeno del suelo sigue siendo del 40%. Por tanto, nuestras conclusiones pueden ser aplicables a suelos agrícolas que tengan un reservorio de nitrógeno suficiente, como es el caso de la mayoría de los suelos de Europa occidental.

2.7 El margen bruto de los agricultores pertenecientes a la IGP "Alubia de La Bañeza-León" podría aumentar en 138 euros ha^{-1}(en la inoculación con R) y en 626 euros ha^{-1} (en la co-inoculación con el consorcio R+P).

3. En cuanto a la inoculación del cultivo de pimiento con el endófito autóctono B, podemos concluir que:

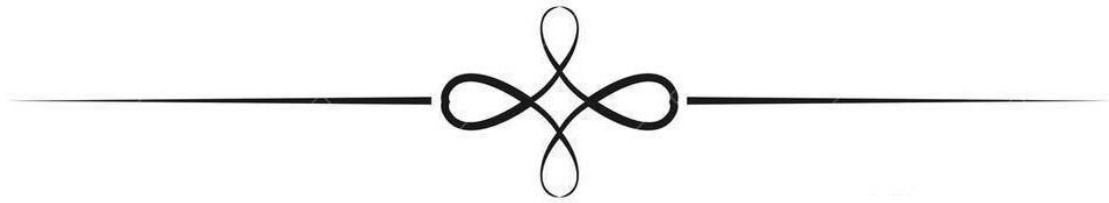
3.1 Los pimientos inoculados con el endófito B y fertilizados con la dosis reducida de Nitrógeno mineral, presentaron un rendimiento significativamente superior que el control no inoculado fertilizado también con un contenido reducido en Nitrógeno (aumento del 31%) e incluso fue mayor que el control no inoculado fertilizado con la totalidad de Nitrógeno (aumento del 34%).

3.2 La inoculación de pimiento con el endófito B fertilizado con Nitrógeno mineral reducido mejoró la eficiencia de uso de Nitrógeno (estimada con el balance parcial de nutrientes) en un 8,5% y la eficiencia de uso de Fósforo en un 4,8%.

3.3 El medio de cultivo óptimo para producir la formulación innovadora de inoculante de B para el cultivo de pimiento consiste en un 50% (v:v) de digestato anaerobio de desechos de alimentos y vegetales, y un 2,3% (v:v) de melaza procedentes de remolacha azucarera.

Chapter 8

Bibliography



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Supplementary material

Supplementary material (Pastor-Bueis et al., 2019)

Supplementary material for section 2. Materials and Methods

Complementary material to section 3.2.3.2. Field experimental design

Table S1

Composition of the compost used as carrier

Parameter	Value
Ca (mg/kg)	19.95
Cd (mg/kg)	0.48
Cr (mg/kg)	21.81
Cu (mg/kg)	0.23
Fe (mg/kg)	21.65
Hg (mg/kg)	0.11
K (mg/kg)	12.13
Mg (mg/kg)	18.34
Mn (mg/kg)	42.08
N Kjeldahl (%)	1.80
Na (mg/kg)	15.26
Ni (mg/kg)	10.32
P (mg/kg)	14.95
Pb (mg/kg)	6.08
Zn (mg/kg)	28.29
Oxidizable organic carbon (%)	21.49
Organic Matter (%)	48.01
pH (soil:water)	6.91
C/N Ratio	15.49
Electrical Conductivity (ds/m)	2.07

Table S2

Climatic and edaphic conditions corresponding to the field experiments during 2017 and 2018. The climatic data were recorded at the León - Virgen del Camino provincial meteo station.

Date	**Temperatures (°C) ***					**Monthly rainfall (mm)**
2017	Hmax (ºC)	Havg (ºC)	Tavg (ºC)	Lavg (ºC)	Lmin (ºC)	
May	24.1	21.1	14.8	8.5	-2.0	69.3
June	35.4	27.0	20.0	12.9	7.0	23.0
July	33.6	28.4	20.3	12.2	6.6	2.1
August	34.7	27.8	20.1	12.3	5.5	9.2
September	29.9	23.5	16.0	8.5	3.3	3.3
October	30.5	22.7	15.1	7.6	2.5	14.6
2018						
May	24.1	19.0	12.7	6.5	-1.5	72.7
June	32.9	23.6	17.5	11.4	6.3	111.3
July	29.8	25.8	19.2	12.5	9.5	57.9
August	35.3	29.3	20.7	12.1	6.6	2
September	31.2	27.1	19.6	12.0	5.8	14.1
October	27.1	18.1	11.8	5.5	-0.4	27.1

Year		**2017**	**2018**
Location		León EIAF	Oteruelo
Latitude		42°34'59.3"N	42°34'56"N
Longitude		5°35'32.0"W	5°36'40"W
Texture (%)	Sand	24	22
	Silt	42	34
	Clay	34	44
pH 1:2	(soil : water)	6.94	7.15
Electric conductivity	(dS/m)	0.16	0.14
Organic matter (%)		3.56	2.34
Total	nitrogen** (%)	0.22	0.14
Ratio C/N		9.22	9.96
Lime	(%)	negligible	negligible
P -Olsen	(mg kg^{-1})	17.28	21.13
K	(cmol (+) kg^{-1})	0.23	0.30
Ca	(cmol (+) kg^{-1})	17.47	15.85
Mg	(cmol (+) kg^{-1})	2.46	3.18
Na	(cmol (+) kg^{-1})	0.09	0.17
Nodulating rhizobia count (MPN***)	Rhizobia g $soil^{-1}$	5.8 x 10^3	1 x 10^4

* Hmax: maximum high temperature) (ºC); Havg: average high temperature (ºC); Tavg: average mean temperature (ºC); Lavg: average low temperature (ºC); Lmin: minimum low temperature (ºC); R: monthly precipitation (mm)

**Total N: organic + nitric + ammonia nitrogen.

*** Most Probable Number

Table S3

Raw data from $\delta^{15}N_{AIR}$ (‰) values obtained for the common bean plants and the reference plants (*Sinapis arvensis* L., *Chenopodium album* L. and *Oxalis corniculata* L., one third in weight from each).

Treatment	Block	Year 2017		Year 2018	
		$d^{15}N_{common\ bean}$	$d^{15}N_{reference\ plants}$	$d^{15}N_{common\ bean}$	$d^{15}N_{reference\ plants}$
Negative control	1	3.4	9.7	5.1	8.1
Negative control	2	4.3	9.7	4.9	8.1
Negative control	3	3.8	9.7	4.7	8.1
N fertilised non-inoculated control	1	4.1	9.7	4.8	8.1
N fertilised non-inoculated control	2	3.8	9.7	5.0	8.1
N fertilised non-inoculated control	3	4.0	9.7	5.8	8.1
Rlp LCS0306 (Co)	1	3.4	9.7	5.1	8.1
Rlp LCS0306 (Co)	2	3.1	9.7	4.1	8.1
Rlp LCS0306 (Co)	3	3.2	9.7	4.1	8.1
Rlp LCS0306 (CC)	1	2.9	9.7	4.6	8.1
Rlp LCS0306 (CC)	2	2.6	9.7	4.0	8.1
Rlp LCS0306 (CC)	3	2.7	9.7	4.5	8.1
Rlp LCS0306 (PB)	1	2.5	9.7	4.7	8.1
Rlp LCS0306 (PB)	2	3.0	9.7	3.9	8.1
Rlp LCS0306 (PB)	3	2.6	9.7	4.1	8.1
Rlp LCS0306 (perlite)	1	2.2	9.7	4.6	8.1
Rlp LCS0306 (perlite)	2	2.5	9.7	4.4	8.1
Rlp LCS0306 (perlite)	3	2.8	9.7	3.8	8.1
Re CFN42T (perlite)	1	3.3	9.7	5.1	8.1
Re CFN42T (perlite)	2	3.6	9.7	5.0	8.1
Re CFN42T (perlite)	3	3.5	9.7	4.2	8.1
Rp ATCC 14482T (perlite)	1	2.8	9.7	4.7	8.1
Rp ATCC 14482T (perlite)	2	3.2	9.7	4.5	8.1
Rp ATCC 14482T (perlite)	3	3.3	9.7	4.1	8.1

Table S4

Mean squares corresponding to the combined ANOVA of the dependent variables related to nodulation, nitrogen fixation, yield, yield components and harvest index, collected in the field trial. Two different ANOVA were carried out, the first analysing the effect of the **inoculation** with different rhizobia strains (*R. leguminosarum* bv. *phaseoli* LCS0306, *R. phaseoli* ATCC 14482^T and *R. etli* CFN 42^T) plus two uninoculated controls, one of them fertilised with mineral nitrogen. The second ANOVA analyses the effect of the **formulation** of the *R. leguminosarum* bv. *phaseoli* LCS0306 strain, using perlite as control (see text for more details). Significance levels: *** p≤0.001; ** 0.001<p≤0.01; *0.01<p≤0.05; ns not significant.

Treatments analysed	Source of variation	DF	Number of nodules per plant	Dry nodule biomass (g per plant)	Dry aerial biomass (kg/ha)	Aerial biomass N content (%)	Ndfa (%)	N fixed (kg/ha)	Soil N uptake (kg/ha)	Grain yield (air dried)[1] (kg/ha)	Pods per plant	Seeds per pod	100-seeds weight (dry) (g)	Harvest index
Inoculation treatment (rhizobia strain plus controls)	Analysis of the repetition (R)	2	42.146 ns	0.014 ns	172902.437 ns	0.092 ns	5.896 ns	58.557 ns	95.106 ns	69220.23 ns	1.446 ns	0.039 ns	7.833 ns	1.192 ns
	Analysis of the treatment													
	Treatment (T)	4	63.211ns	0.421***	3808183.48 ***	0.046 ns	92.116 **	1034.93 ***	615.937***	1560018 ***	17.086 ***	0.324 ***	11.147 ns	54.522 *
	T x Year	5	8.021 ns	0.007 ns	100139.655 ns	0.053 ns	3.283 ns	99.616 ns	35.231 ns	593239.9** *	4.379**	0.355 **	65.791 ***	123.941 ***
	T x R	8	75.765 ns	0.026 ns	63948.271 ns	0.064 ns	7.348 ns	55.184 ns	45.77 ns	4668.15 ns	0.402 ns	0.01 ns	3.442 ns	5.781 ns
Formulation for the LCS0306 strain (Formulation with Perlite was the control)	Analysis of the repetition (R)	2	8.667 ns	0.001 ns	118374 ns	0.053 ns	15.167 ns	32.596 ns	90.791 ns	87751.5 ns	0.353 ns	0.062 ns	4.314 ns	5.399 ns
	Analysis of the treatment													
	Treatment (T)	3	55.667 ns	0.031 ns	429316.819 ns	0.054 ns	23.667 ns	350.339 *	297.946 *	292425.042 **	1.373 **	0.061 ns	4.095 **	12.332 ns
	T x Year	3	8.944 ns	0.007 ns	162426.264 ns	0.035 ns	2.5 ns	44.112 ns	8.092 ns	13856.931 ns	0.076 ns	0.089 ns	5.338 **	6.563 ns
	T x R	6	129.667 *	0.044 ns	116453.444 ns	0.024 ns	3.167 ns	42.927 ns	23.648 ns	33450.167 ns	0.284 ns	0.028 ns	2.144 *	23.569 ns

[1] Corresponds to the commercial beans (11.57 % dry matter)

Table S5

Rlp LCS0306 genome statistics.

Genome ID	**Rlp LCS0306**
Total length (bp)	7,395,396
GC (%)	60.72
N50	340,266
N75	161,667
L50	7
L75	14
# N's	0.00
# N's per 100 kbp	0.00
# contigs (>= 0 bp)	135
# contigs (>= 1000 bp)	58
Total length (>= 0 bp)	7,395,396
Total length (>= 1000 bp)	7,360,538
# contigs	71
Largest contig	929,560
Genes (total)	7,172
CDSs (total)	7,115
Genes (coding)	6,906
Genes (RNA)	57
rRNAs	2, 1, 4 (5S, 16S, 23S)
tRNAs	46
ncRNAs	4
Pseudo Genes (total)	209

Table S6

LCS0306 search for Cluster of Orthologous Groups (COG). The values reflect the number of protein families, coverage, and abundance in LCS0306 genome as a result of the comparison of LCS0306 functional annotation to protein sequences encoded in complete genomes from the COG protein database (WebMGA server).

#Class	No families	Coverage	Abundance	Description
J	245	0.0082	0.1242	Translation, ribosomal structure, and biogenesis
A	25	0	0	RNA processing and modification
K	231	0.0087	0.0567	Transcription
L	238	0.0126	0.2313	Replication, recombination and repair
B	19	0	0	Chromatin structure and dynamics
D	72	0	0	Cell cycle control, cell division, chromosome partitioning
Y	2	0	0	Nuclear structure
V	46	0	0	Defence mechanisms
T	152	0	0	Signal transduction mechanisms
M	188	0	0	Cell wall/membrane/envelope biogenesis
N	96	0	0	Cell motility
Z	12	0	0	Cytoskeleton
W	1	0	0	Extracellular structures
U	158	0	0	Intracellular trafficking, secretion, and vesicular transport
O	203	0.0049	0.0298	Posttranslational modification, protein turnover, chaperones
C	258	0.0155	0.2372	Energy production and conversion
G	230	0.0087	0.0966	Carbohydrate transport and metabolism
E	270	0.0074	0.0420	Amino acid transport and metabolism
F	95	0.0105	0.0341	Nucleotide transport and metabolism
H	179	0	0	Coenzyme transport and metabolism
I	94	0	0	Lipid transport and metabolism
P	212	0.0047	0.0210	Inorganic ion transport and metabolism
Q	88	0	0	Secondary metabolites biosynthesis, transport, and catabolism
R	702	0.0028	0.1419	General function prediction only
S	1347	0.0007	0.0210	Function unknown
TOTAL	5163	0.0848	1.0358	

Table S7

Use of carbon and nitrogen sources by reference *Rhizobium* strains and the autochthonous strain *R. leguminosarum* bv. *phaseoli* LCS0306. Characterisation and selection of rhizobia nodulanting common bean (*Phaseolus vulgaris* L.) in the P.G.I. "*Alubia de la Bañeza-León*". Unpublished data from Mulas, D. 2010. PhD. Dissertation. University of León. Spain. 191 pp.

Carbon sources	***R. phaseoli* ATCC 14482^T**	***R. leguminosarum* USDA 2370^T**	***R etli* CFN42^T**	***R. leguminsarum* bv. *phaseoli* LCS0306**
D-raffinose	+	+	+	+
Galactose	+	+	+	+
Maltose	+	+	+	+
D-cellobiose	+	+	+	+
Melibiose	+	+	+	+
D-sucrose	+	+	+	+
D-salicin	-	-	-	+
D-trehalose	+	+	+	+
L-rhamnose	+	+	+	+
L-sorbose	+	+	+	+
D-mannose	+	+	+	+
Fructose	+	+	+	+
Xylose	+	+	+	+
Melezitose	-	+	+	+
Xylitol	+	+	+	+
D-sorbitol	+	+	+	+
Meso-erythritol	+	+	-	+
Inositol	+	+	+	+
Mannitol	-	+	+	+
Na Pyruvate	+	+	+	+
Na Glucuronate	-	-	-	-
Na Propionate	+	-	-	+
Na Gluconate	-	-	-	-
Na Citrate	-	+	+	+
Nitrogen sources				
L-serine	-	-	+	+
DL-valine	-	-	+	+
L-alanine	-	+	+	+
L-proline	+	+	+	+
Betaine	+	+	+	+
L-arginine	-	-	-	-
L-lysine	-	-	-	-
L-histidine	+	-	+	+
Sarcosine	+	+	+	+
Aspartate Mg	+	+	+	+
Glutamate Na	+	-	+	+

Table S8

Distribution of genes linked to competitiveness in Rlp LCS0306 genome compared to reference *Rhizobium* strains. Homologue genes are indicated in the corresponding column.

GENE FUNCTION	GENE NAME	LOCUS_TAG	CONTIG	*R. LEGUMINOSARUM* UMP791	*R. PHASEOLI* ATCC14482^T	*R.ETLI* CFN42^T
ABC TRANSPORTERS	*aapJQMP* *teuBAC1C2* *nocR, nocQCT*	FML87_20425-FML87_20440 FML87_29775 - FML87_29760 FML87_29495 FML87_29500-FML87_29510	Node 9 Node 19* Node 19*	RLV_4523-RLV_4520		 RHE_PD00128-RHE_PD00131 RHE_PD00181 RHE_PD00180-RHE_PD00178
MOTILITY	*motA motB* *flg* and *fli* genes	FML87_25710 FML87_25610 FML87_25750-FML87_25545	Node 13 Node 13	RLV_3097 RLV_3117 RLV_3089-RLV_3131		
CHEMOTAXIS	*cheAWRBYD* (Che1 cluster)	FML87_25785- FML87_25760	Node 13	RLV_3082-RLV_3087		
EPS BIOSYNTHESIS AND TRANSPORT	*pssSRMLKJIFCDE* *pssTONP* *prsED*	FML87_17650-FML87_17705 FML87_17755-FML87_17740 FML87_17715-FML87_17720	Node 7 Node 7 Node 7	RLV_5915-RLV_5925 RLV_5932-RLV_5935 RLV_5927 RLV_5928		
PEPTIDOGLYCAN BIOSYNTHESIS	*ftsI murEF mraY murD ftsW murGBC,*	FML87_12315-FML87_12275	Node 4	RLV_5562-RLV_5560	EFD56_29325-EFD56_29285	
RSI BACTERIAL INVASION SWITCH	*pckA chvIG hprK manX npr*	FML87_28000-FML87_27975	Node 16	RLV_7039-RLV_7044		

GENE FUNCTION	GENE NAME	LOCUS_TAG	CONTIG	*R. LEGUMINOSARUM* UMP791	*R. PHASEOLI* ATCC14482^T	*R.ETLI* CFN42^T
PHB	*phbC1*	FML87_20625	Node 9	RLV_4485		
TYPE III SECRETION SYSTEM (T3SS)	*rhcJ, rhcLNQRSTU, hrpW, rhcVD, rhC1O*	FML87_34175-FML87_34265	Node 33			RHE_PD00051-RHE_PD00067
TYPE IV SECRETION SYSTEM - PILUS (T4SS)	*virB1-virB11*	FML87_29685 – FML87_29510	Node 19	RLV_0329-RLV_0340		
TYPE IV SECRETION SYSTEM – *TRA* CONJUGAL SYSTEM (T4SS)	*traCDG* *traA-traFBHMR*	FML87_29555 - FML87_29565 FML87_29550 - FML87_29525	Node 19*			RHE_PD00167-RHE_PD00175
TYPE VI SECRETION SYSTEM (T6SS)	*tssABC Hcp tssEFGI* *tssKLM*	FML87_29395 - FML87_29430 FML87_29375 - FML87_29360	Node 19		EFD56_30825-EFD56_30795	
QUORUM SENSING (BACTERIOCIN PRODUCTION)	*cinRIS*	FML87_12665-FML87_12675	Node 4	RLV_5631-RLV_5632		

*Note that Node_19 aligns with the symbiotic plasmid of *R. etli* CFN42^T (p42d)

Table S9

Distribution of genes contributing to symbiosis in Rlp LCS0306 genome compared to reference *Rhizobium* strains. Homologue genes are indicated in the corresponding column.

GENE FUNCTION	***GENE NAME***	**LOCUS_TAG**	**CONTIG**	**R. LEGUMINOSARUM UMP791**	**R. PHASEOLI ATCC14482^T**	**R. ETLI CFN42^T**
NITROGENASE	*rpoN-nifUSW* *nifAB* *fdxN* *nifZT* *nifDKEX* *fdxB*	FML87_33610 – FML87_33625 FML87_33650 - FML87_33670 FML87_34560 – FML87_34585	Node 30 Node 30 Node 36		EFD56_29645 - EFD56_29620 EFD56_29595 - EFD56_29575	RHE_PD00218- RHE_PD00222 RHE_PD00228- RHE_PD00231 RHE_PD00307- RHE_PD00302
N FIXATION	*fixNOQP GHIS* (FIX1 region) *fixABCX* (FIX2 region)	FML87_34615- FML87_34655 FML87_33630 – FML87_33645	Node 36 Node 30	RLV_1827- RLV_1834		RHE_PD00296- RHE_PD00289 RHE_PD00224- RHE_PD00227
NODULATION	*nodA* *nodBCSUIJ* *nodD1* *nodD2* *nodD3*	FML87_35315 FML87_34775- FML87_34745 FML87_34730 FML87_34950 FML87_34945	Node 47 Node 37 Node 37 Node 39 Node 39			RHE_PD00310 RHE_PD00282- RHE_PD00277 RHE_PD00275 RHE_PD00316 RHE_PD00318
REGULATION	*fnrN1* *fnrN2* *rosR*	FML87_28695 FML87_33600 FML87_24335	Node 17 Node 30 Node 12	RLV_5077 RLV_1980 RLV_3788		RHE_CH02479 RHE_PD00216 RHE_CH01249

Supplementary material (Pastor-Bueis et al., 2021)

Supplementary material for section 2. Materials and Methods

Complementary material to section 3.2.3.2. Field experimental design

Agronomic practices

Before establishing the experiment, the corresponding plot was fertilised with phosphorus (P) and potassium (K). The fertiliser rates were calculated for a theoretical expected yield of 3,500 kg ha^{-1} following the methodology indicated by Urbano Terrón (2008) which also considers the soil characteristics shown in Supplementary Material 1 (Table S2); hence the plots received a dose equivalent to 74 kg ha^{-1} (EIAF) and 54 kg ha^{-1} of P (Armunia), in the form of triple superphosphate (46% P_2O_5, therefore 20% P). Regarding the K, the plots received a dose of 140 (EIAF) kg ha^{-1} and 117 kg ha^{-1} (Armunia) in the form of KCl (60% K_2O, therefore 50% K).
The non-inoculated and full N-fertilised control plot received a dose equivalent to 187 kg N ha^{-1}, which corresponds to the expected total N extraction for a theoretical yield of 3,500 kg ha^{-1} (Urbano Terron, 2008). N fertiliser was applied as ammonium nitrate (27% N). Half of this amount was applied five days before sowing and half at the beginning of flowering. The non-inoculated and 80% N-fertilised control plot received 80% of the aforementioned amount. The non-inoculated, non-N-fertilised control plot and the inoculated treatments did not receive any dose of N.

The soil moisture content was assessed daily, and irrigation was applied when necessary, using a drip irrigation system. After the flowering stage, the two fields suffered an infection with *Tetranychus urticae*; therefore, each plot received the dose equivalent to 1.5 l ha^{-1} of Fenpyroximate 5.12% in order to control the infection. The soil was kept free from weeds by mechanical methods.

Complementary information to section 3.2.3.4. Determination of the N fixation

Isotopic determination of N fixation

The following non-legume weeds, which were naturally growing within the plots, were collected, and processed in the same way as the rest of the samples and used as reference plants, as a proxy for the ^{15}N natural abundance of plant-available soil mineral N: at the EIAF plot, *Sinapis arvensis* and *Lactuca serriola* and at the Armunia plot, *Sinapis arvensis* and *Chenopodium album*. Isotopic analysis was performed at SIDI (Universidad Autónoma de Madrid). The isotopic composition of the plant samples was expressed as $\delta^{15}N_{AIR}$ (‰). Raw data from $\delta^{15}N_{AIR}$ (‰) are in Supplementary Material 1 (Table S1). The percent N derived from the fixation of atmospheric N_2 (% Ndfa) by the common bean crop was calculated from the ^{15}N abundance of the legume species and that of the non-fixing reference plants, as indicated by Shearer and Kohl (1986) and Unkovich and Baldock (2008). The B value was determined as proposed by Pacheco et al. (2017) and it corresponded to -1.97 ‰.

The N content in the aerial biomass of the common bean was calculated as Aerial biomass N (kg N ha^{-1}) = aerial dry biomass (kg ha^{-1}) × N content in the aerial biomass, determined using the Kjeldahl method (%). The amount of N-fixed was calculated as N-fixed (kg N ha^{-1}) = %Ndfa × Aerial biomass N (kg N ha^{-1}) (Maskey et al., 2001). The soil uptake (kg N ha^{-1}) was calculated as Aerial biomass N - N-fixed.

Table S1

Raw data from $\delta^{15}N_{AIR}$ (‰) values obtained for the common bean plants and the reference plants (*Sinapis arvensis* and *Lactuca serriola* at EIAF plot, and *Sinapis arvensis* and *Chenopodium album* at Armunia plot, one half in weight from each at each location).

Treatment	Block	EIAF		Armunia municipality	
		$d^{15}N_{common\ bean}$	$d^{15}N_{reference\ plants}$	$d^{15}N_{common\ bean}$	$d^{15}N_{reference\ plants}$
Control 0 N – non-inoculated	1	3.80	9.5	4.44	8.9
	2	4.50	9.5	4.77	8.9
	3	3.60	9.5	4.82	8.9
Control 100 % N – non-inoculated	1	5.31	9.5	5.75	8.9
	2	5.60	9.5	4.99	8.9
	3	4.91	9.5	5.35	8.9
Control 80% N – non-inoculated	1	4.92	9.5	4.99	8.9
	2	4.53	9.5	5.42	8.9
	3	5.30	9.5	5.30	8.9
Rlp-LCS0306+Pbn-RVPB2-2	1	2.29	9.5	2.60	8.9
	2	2.77	9.5	2.38	8.9
	3	2.34	9.5	3.13	8.9
Rlp-LCS0306	1	2.29	9.5	3.03	8.9
	2	2.87	9.5	3.79	8.9
	3	2.58	9.5	3.57	8.9
Rlp-LCS0306+Azc-ATCC 9043^T	1	2.19	9.5	2.92	8.9
	2	3.36	9.5	3.90	8.9
	3	2.51	9.5	3.25	8.9
Rlp-LCS0306+Azc-ATCC 9043^T + Pbn-RVPB2-2	1	2.19	9.5	2.60	8.9
	2	3.26	9.5	3.79	8.9
	3	2.50	9.5	3.36	8.9
Azc-ATCC 9043^T + Pbn-RVPB2-2	1	2.19	9.5	4.23	8.9
	2	3.36	9.5	3.68	8.9
	3	2.69	9.5	4.12	8.9

Table S2

Average percentage of recovery of the inoculated strains inside the nodules. The percentages correspond to the number of colonies, with a RAPD profile corresponding to the inoculated strain, versus the total number of colonies growing in the indicated culture medium. Data were obtained from one block at the location of EIAF and are the average of duplicate plating of each sample.

Inoculation Treatment	**% of the colonies growing on YMA medium with the RAPD profile of Rlp-LCS0306**	**% of the colonies growing on TSA medium with the RAPD profile of Pbn-RVPB2-2**
Rlp-LCS0306+Pbn-RVPB2-2	79.2 ± 7.4	47.6 ± 7.2
Rlp-LCS0306	76.8 ± 6.0	-
Rlp-LCS0306+Azc-ATCC 9043T	77.8 ± 7.8	-
Rlp-LCS0306+Azc-ATCC 9043T + Pbn-RVPB2-2	78.4 ± 4.0	40.5 ± 7.2

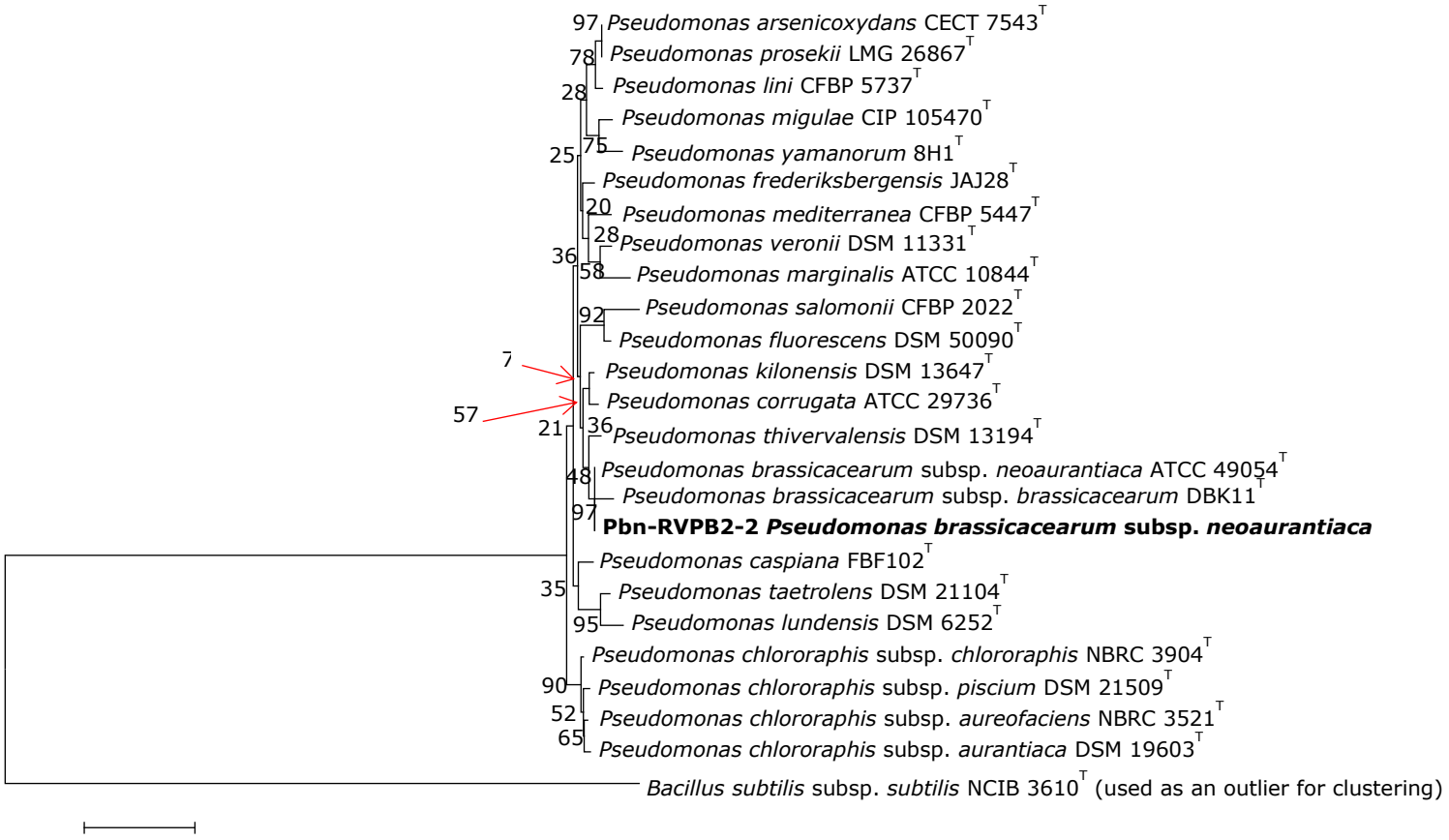


Figure S1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of the *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain Pbn-RVPB2-2 included in this study which were isolated from *Phaseolus vulgaris* L. root endosphere (no in nodules). Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt.

Supplementary material (Pastor-Bueis et al., 2017)

Supplementary material for section 2. Materials and Methods

Complementary material to section 2.8. BF tests in field trials

Agronomic practices

All the plots of all the treatments and controls were fertilized with mineral phosphorus and potassium five days before transplant, following the methodology of Urbano Terron (2008), which calculates the dose depending on the expected yield (45 tons/ha) and the soil content in the corresponding nutrient. Thus, in the soil of León, all the plots received 53 kg P/ha in the form of superphosphate (18% P2O5) and 180 kg K/ha in the form of potassium chloride (60% K2O), while the soil of Oteruelo received 45 kg P/ha in the form of superphosphate (18% P2O5) and no K because of the high content of this nutrient in the soil. The different treatments and controls received differential nitrogen fertilization as specified in the main text. Half of the N fertilization was provided five days before transplanting to the field, and half at the beginning of flowering.

The crop was drip irrigated, kept free from weeds, and observed daily in order to detect the possible appearance of pests or diseases.

Dependent variables analyzed

Several variables related to the yield and yield components were analyzed. These included the yield of fresh fruits per plant, and its components, i.e., the number of fruits per plant and the average fresh mass per single fruit, the number of plants per square m and the yield of fresh fruits per hectare, the total aerial dry biomass (fruits and vegetative parts) per ha and the harvest index (HI). The data referring to dry biomass were corrected to absolute dry weight on the basis of two random plants and 10 pepper fruits per plot, that were dried in oven at 80ºC to a constant weight.

For each pepper fruit, the following morphological characters were measured: length, maximum circumference and mean thickness of the fruit wall measured with a calliper in four points opposed two by two in the equatorial

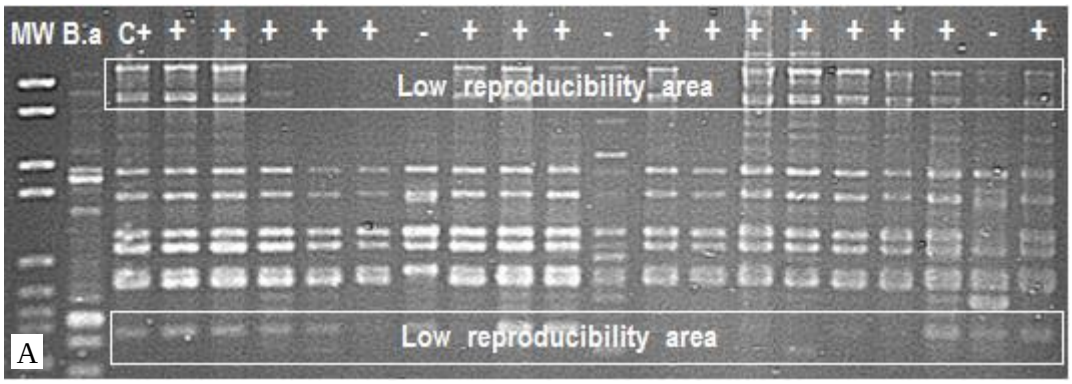
axis. Moreover, in four random fruits per elementary plot, the stem and the seeds were eliminated, the rest of the fruit was mechanically crushed, and the obtained paste was used to measure the pH. This was then filtered through a quantitative analysis Prat Dumas medium flow filter and the filtrate was used to measure the sugar content by the determination of total soluble solids (Brix). Another four random fruits per elementary plot were oven dried at 60°C until a constant weight, ground to particles < 2 µm in diameter, and used to determine the nitrogen content by the Kjeldhal method and the content of P, K, Ca, Mg by ICP-AES after acid digestion.

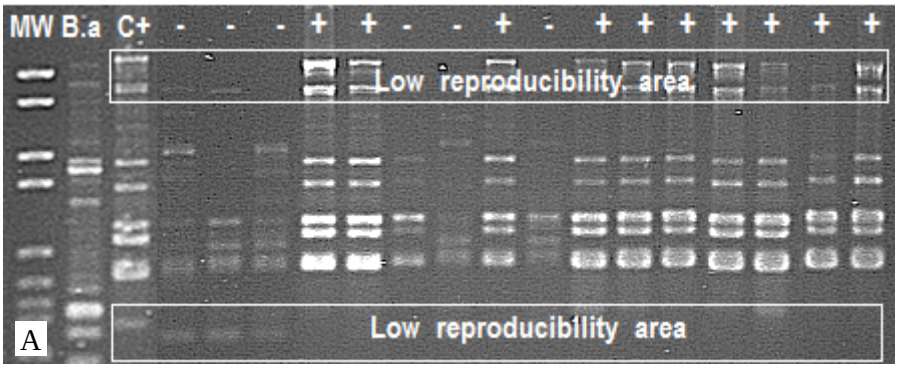
Supplementary material for section 2.9. Counts of the strain SCFB3-1 of *B. siamensis* in the root endosphere at the end of the field experiment

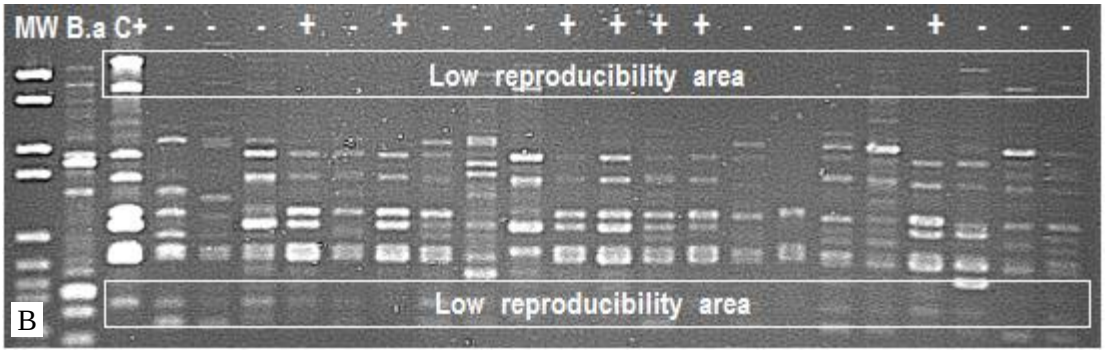
Material and methods of the RAPD analysis

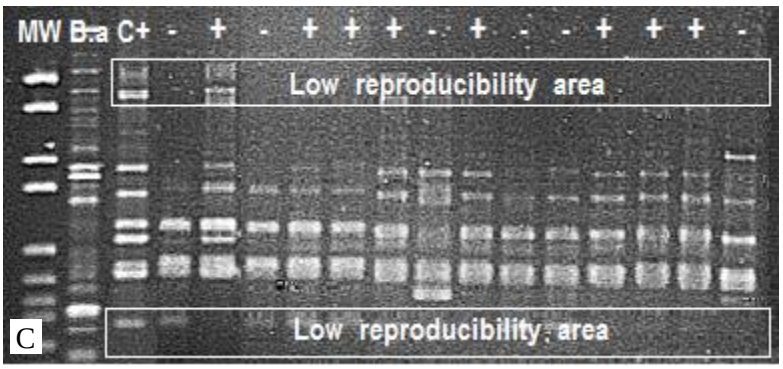
For RAPD analysis, the bacterial DNA isolated according to Álvarez-Martínez et al. (2009), was used to obtain the RAPD patterns following the procedure described by Rivas et al. (2006) using primer M13 (5'– GAGGGTGGCGGTTCT –3') (2 mM final concentration) purchased from ISOGEN and Dream Taq Green PCR Master Mix from Thermo Fisher Scientific, USA. PCR conditions were; preheating at 95°C for 9 min, 35 cycles of denaturing at 95°C for 1 min, annealing at 45°C for 1 min and extension at 75°C for 2 min and a final extension at 72°C for 7 min. Seventeen microliters of each PCR product were used in electrophoresis on a 1.5% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 0.5 g/ml ethidium bromide, and photographed under UV light. Standard VI (Roche, USA) was used as a size marker.

Sample of the obtained results









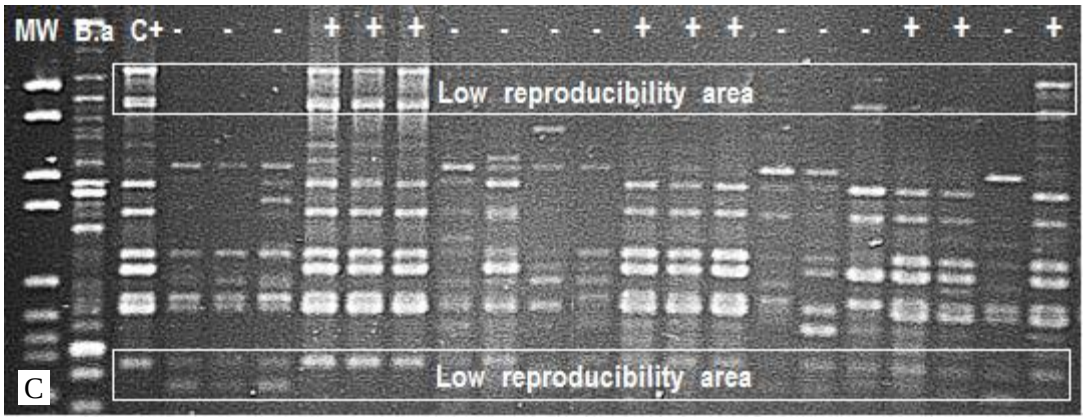


Figure S1. The figure corresponds to a sample of the RAPD profiles that were obtained in the test for the verification of the identity of the strains reisolated from the endosphere of sweet pepper crop at the end of the field experiment. The sample corresponds to the Oteruelo trial, and the treatment with the dose at 1.5 ml/plant. MW molecular weight marker; B.a. corresponds to the negative control, i.e. the RAPD profile of a pure culture of a strain of *Bacillus aerophilus*; C+ corresponds the positive control, i.e. the RAPD profile of the pure strain SCFB 3-1; + means positive match with SCFB 3-1 and – a different strain. The low reproducibility areas were demonstrated after several replications and were excluded from the analysis. A. Corresponds to strains isolated from the block 1 of the field experiment; B the same from the block 2; C the same from the block 3.

Edapho-climatic conditions of the field plots

Table S1. Edapho-climatic conditions of soils from the sites in which the field experiment was established

A. Edaphic characteristics

	Texture (%)			pH 1:2 (soil:water)	Electric conducitivity (dS/m)	Organic matter (%)	Total nitrogen* (%)	Ratio C/N	Lime (%)	Phosphorus (Olsen) (mg kg-1)	Potassium (cmol(+) kg-1)	Calcium (cmol(+) kg-1)	Magnesium (cmol(+) kg-1)	Sodium (cmol(+) kg-1)
	Sand	Silt	Clay											
León	60	24	15	7.5	0.07	2.1	0.17	7.9	0.6	13.9	110	4849	407	67
Oteruelo	36	40	24	8.0	0.12	1.9	0.14	8.1	11.3	16.2	360	5627	608	53

B. Climatic characteristics

	Temperatures (°C)					R (mm)	I (%)	D<10 ºC
Month	Hmax	Havg	Tavg	Lavg	Lmin			
May	29.2	21.3	13.1	5.5	-0.2	19	11.4	7
June	34.7	26.6	17.9	10.3	5.1	44	11.6	0
July	35	30	20.7	12.6	7.9	14	12.5	0
August	32.3	26.9	18.6	11.3	5.6	12	10.7	0
September	26.7	22	14.3	7.5	3.3	54	9.2	0
October	22.1	16.3	10.7	5.8	-0.6	104	5.4	9
November	20	13.5	8.3	3.7	-4.1	33	5.5	15

*Total N: organic + nitric + ammonia nitrogen. Hmax: maximum high temperature (ºC); Havg: average high temperature (ºC); Tavg: average mean temperature (ºC); Lavg: average low temperature (ºC); Lmin: minimum low temperature (ºC); R: monthly precipitation (mm); PET: potential evapotranspiration (mm); I: average daily solar radiation (hours/day); D<10ºC: number of days with average mean temperature under 10 ºC. ¥The climatic data were recorded at the León meteorological stations.

Detailed data obtained from the ANOVA for the field experiment

Table S2. Mean squares and significance level for the combined analysis of variance of the physical and chemical characteristics of the fruits in the field experiment.

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Source of variation		Fruit morphology			Fruit chemical characters			Elemental content					
	df	Length (cm)	Maximum circunference (cm)	Thickness (mm)	df	pH of the fruit juice	°Brix of the fruit juice	df	N (%)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)
Analysis of the environment													
Location (L)	1	52.751***	60.974**	0.029ns	1	0.71ns	19.453***	1	0.150ns	29510.154ns	13240935.59**	54522.804ns	22887.268ns
Repetition (R)	2	5.599ns	8.676ns	0.035ns	2	0.09ns	0.408ns	2	0.016ns	36317.366ns	1630079.122ns	43482.758ns	1046.898ns
Analysis of the treatment													
Treatment (T)	4	3.490ns	34.774***	0.111***	4	0.009ns	0.169ns	4	0.015ns	164940.385**	1981420.356ns	16998.501ns	730.996ns
L x T	4	0.319ns	1.998ns	0.016ns	4	0.026ns	0.156ns	4	0.010ns	14911.579ns	3195094.312ns	15855.733ns	474.340ns
R x T	8	3.103ns	6.238ns	0.010ns	8	0.034ns	0.201ns	8	0.017ns	25020.847ns	1134753.213ns	28121.764ns	3360.420ns

Table S3. Values of the dependent variables related to the physical and chemical characteristics of the fruits in the field experiment. The comparison of means was performed within columns. Mean values followed by the same letter do not significantly differ (LSD test $p<0.05$).

Fertilisation treatment	Fruit morphology																		Elemental content											
	Length (cm)			Maximum circunference (cm)			Thickness (mm)			pH of the fruit juice			°Brix of the fruit juice			N (%)			P (mg/kg)			K (mg/kg)			Ca (mg/kg)			Mg (mg/kg)		
	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean
Non-N-fertilised	9.0^{a}	10.3^{a}	9.7^{a}	22.1^{ab}	23.9^{a}	23.4^{a}	0.577^{a}	0.577^{a}	0.577^{a}	5.11^{a}	4.97^{a}	5.04^{a}	5.85^{a}	6.5^{a}	6.25^{a}	1.280^{a}	1.292^{a}	1.286^{a}	2857^{a}	2844^{b}	2850^{b}	19131^{a}	18225^{a}	18678^{a}	783^{a}	741^{a}	762^{a}	812^{a}	896^{a}	854^{a}
N 80% control	8.6^{a}	9.6^{a}	9.2^{a}	22.6^{a}	24.2^{ab}	23.2^{a}	0.571^{a}	0.657^{ab}	0.617^{a}	5.04^{a}	4.98^{a}	5.01^{a}	5.94^{a}	6.7^{a}	6.36^{a}	1.302^{a}	1.466^{a}	1.384^{a}	2606^{a}	2568^{a}	2587^{a}	19544^{a}	16751^{a}	18147^{a}	710^{a}	852^{a}	781^{a}	841^{a}	883^{a}	862^{a}
N 100% control	8.7^{a}	10.0^{a}	9.5^{a}	23.8^{abc}	24.5^{ab}	24.3^{ab}	0.639^{ab}	0.600^{a}	0.615^{a}	5.04^{a}	4.92^{a}	4.98^{a}	5.71^{a}	6.7^{a}	6.33^{a}	1.348^{a}	1.468^{a}	1.408^{a}	2374^{a}	2462^{a}	2418^{a}	19608^{a}	16716^{a}	18162^{a}	698^{a}	903^{a}	801^{a}	816^{a}	862^{a}	839^{a}
BF 2 ml/plant for N 80 %[a]	9.3^{a}	10.9^{a}	10.2^{a}	25.5^{c}	26.0^{bc}	25.1^{bc}	0.686^{b}	0.756^{c}	0.723^{b}	5.04^{a}	5.00^{a}	5.02^{a}	6.05^{a}	6.6^{a}	6.36^{a}	1.271^{a}	1.463^{a}	1.367^{a}	2377^{a}	2592^{a}	2485^{a}	17601^{a}	17998^{a}	17799^{a}	876^{a}	875^{a}	875^{a}	831^{a}	891^{a}	861^{a}
BF 1.5 ml/plant for N 80 %[b]	9.0^{a}	10.6^{a}	9.8^{a}	24.2^{bc}	26.7^{c}	26.1^{c}	0.702^{b}	0.745^{bc}	0.724^{b}	4.99^{a}	5.03^{a}	5.01^{a}	5.91^{a}	6.9^{a}	6.40^{a}	1.298^{a}	1.516^{a}	1.407^{a}	2511^{a}	2573^{a}	2542^{a}	17344^{a}	16895^{a}	17120^{a}	814^{a}	937^{a}	875^{a}	846^{a}	889^{a}	867^{a}
Mean per column	8.9	10.2	9.7	23.6	25.0	24.3	0.635	0.663	0.650	5.04	4.99	5.01	5.90	6.68	6.34	1.300	1.441	1.370	2545	2608	2576	18646	17317	17981	776	862	819	829	884	857
df	4	4		4	4		4	4		4	4		4	4		4	4		4	4		4	4		4	4		4	4	
Mean squares	0.867ns	2.883ns		19.482**	20.098**		0.041**	0.096***		0.020ns	0.014ns		0.163ns	0.182ns		0.003ns	0.022ns		120x10^{3}ns	60x10^{3}ns		357x10^{4}ns	161x10^{4}ns		16x10^{3}ns	17x10^{3}ns		657ns	548ns	

[a] 2x10^{9} cfu of the PGPR bacteria per plant [b] 1.5x10^{9} cfu per plant

Table S4. Mean squares and significance levels for the combined analysis of variance of the fruit yield. The yield components and the biomass production in the field experiment are shown.

Source of variation	df	Yield per plant (fresh fruits) (g)	Fruits per plant	Average fresh mass per single fruit (g)	Plants per square m	Yield per ha (fresh fruit) (kg/ha)	Total aerial biomass per plant (dry matter) (g)	Harvest index
Analysis of the environment								
Location (L)	1	13602.953	0.102	4858.612	0.576	4.118×10^{8}**	594.212*	6.023×10^{-5}
Repetition (R)	2	2045.233	0.028	1506.525	2.856	1.834×10^{7}	62.253	0.001
Analysis of the treatment								
Treatment (T)	4	44581.476**	1.054**	9206.053***	5.159*	6.626×10^{8}**	572.861**	0.003
L x T	4	3510.286	0.118	921.901	2.303	5.948×10^{7}	33.629	0.006
R x T	8	1528.452	0.026	344.929	2.395	1.561×10^{7}	20.422	0.000

Table S5. Values of the dependent variables related to fruit yield, yield components and biomass production in the field experiment. The comparison of means was performed within columns. Mean values followed by the same letter do not significantly differ (LSD test $p<0.05$).

Treatment	Yield per plant (fresh fruits) (g)			Yield components									Yield per ha (fresh fruit) (kg/ha)			Aerial biomass and Harvest Index (HI)					
				Average number of fruits per plant			Average fresh mass per single fruit (g)			Plants per square m						Aerial biomass per plant (dry matter) (g)			HI		
	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean	León	Oter.	Mean
Non-N-fertilised	174^{a}	275^{a}	224^{a}	1.24^{a}	1.75^{a}	1.49^{a}	141^{ab}	143^{a}	142^{a}	13.1^{b}	12.8^{a}	12.9^{b}	22625^{a}	35460^{a}	29043^{a}	21^{a}	30^{a}	26^{a}	0.593^{a}	0.644^{a}	0.619^{a}
N 80% control	308^{b}	356^{b}	332^{b}	2.02^{b}	2.10^{b}	2.06^{b}	138^{a}	164^{ab}	152^{a}	12.8^{b}	12.5^{a}	12.6^{b}	39336^{b}	44272^{b}	41804^{b}	29^{ab}	42^{b}	36^{b}	0.597^{a}	0.655^{a}	0.626^{a}
N 100% control	393^{c}	391^{bc}	392^{bc}	2.38^{cd}	2.41^{c}	2.39^{bc}	164^{bc}	162^{ab}	163^{ab}	9.4^{a}	11.9^{a}	10.7^{a}	34348^{b}	47058^{bc}	40703^{b}	45^{c}	46^{b}	45^{c}	0.678^{c}	0.650^{a}	0.664^{a}
BF 2 ml/plant for N 80 %^{a}	386^{bc}	462^{d}	424^{c}	2.28^{bc}	2.50^{c}	2.39^{bc}	169^{c}	189^{bc}	180^{bc}	13.1^{b}	12.8^{a}	12.9^{b}	50219^{c}	59024^{d}	54621^{c}	40^{bc}	53^{c}	47^{c}	0.664^{ac}	0.660^{a}	0.662^{a}
BF 1.5 ml/plant for N 80 %^{b}	439^{c}	428^{cd}	433^{c}	2.66^{d}	2.41^{c}	2.53^{c}	181^{c}	198^{c}	190^{c}	12.5^{b}	12.2^{a}	12.4^{b}	54581^{c}	52346^{cd}	53464^{c}	44^{c}	54^{c}	49^{c}	0.710^{c}	0.618^{a}	0.664^{a}
Mean per column	340	382	361	2.12	2.23	2.17	158	170	164	12.2	12.4	12.3^{b}	40221	47632	43927	36	45	41	0.649	0.646	0.647
df	4	4		4	4		4	4		4	4		4	4							
mean squares	32487**	15604***		0.882***	0.289**		3893.884**	6931.829***		7.071*	0.392		4.88x10^{8}***	2.34x10^{8}**		329.400*	277.090***		0.008**	0.001	

^{a} 2x10^{9} cfu of the PGPR bacteria per plant ^{b} 1.5x10^{9} cfu per plant

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