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Tesis Doctoral

Integrative approaches for the analysis of abiotic stress responses in the legume-*Rhizobium* symbiosis: from shoots to roots

Memoria presentada por Dña. María Isabel Rubia Galiano para optar al grado de Doctor con Mención de Doctor Internacional

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HACEN CONSTAR:

Que el trabajo titulado "**Integrative approaches for the analysis of abiotic stress responses in the legume-*Rhizobium* symbiosis: from shoots to roots**" que presenta Dña. María Isabel Rubia Galiano para optar al título de Doctor Internacional por la Universidad Pública de Navarra, ha sido desarrollado bajo su dirección en el grupo de Fisiología Vegetal y Agrobiología en el Institute for Multidisciplinary Research in Applied Biology de la Universidad Pública de Navarra y que reúne todos los requisitos necesarios para su defensa, por lo que:

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- ¿Y cómo me ves tú a mí?
- Como un misterio.
- Ése es el cumplido más raro que me han hecho nunca.
- No es un cumplido. Es una amenaza.
- ¿Y eso?
- Los misterios hay que resolverlos, averiguar qué esconden.
- A lo mejor te decepcionas al ver lo que hay dentro.
- A lo mejor me sorprendo. Y tú también.

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

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Abbreviations

6PGDH	6-phosphogluconate dehydrogenase
AAT	Aspartate aminotransferase
ABA	Abscisic acid
ABC	ATP-binding cassette
AGO	Argonaute
AHAS	Acetohydroxyacid synthase
AI	Alkaline invertase
AlaAT	Alanine aminotransferase
ANA	Apparent nitrogenase activity
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
AS	Asparagine synthase
BCAA	Branched-chain amino acid
BNF	Biological nitrogen fixation
C	Carbon
CFU	Colony-forming units
CGS	Cysthationine gamma-synthase
CPCA	1,1-cyclopropanedicarboxylic acid
cps	Counts per second
DHQS	3-dehydroquinate synthase
dpi	Days post inoculation
EA-IRMS	Elemental analysis-isotope ratio mass spectrometry
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
FBN	Fijación biológica de nitrógeno
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
GOGAT	Glutamate synthase

GS	Glutamine synthetase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRAC	Herbicide Resistance Action Committee
ICDH	Isocitrate dehydrogenase
KARI	Ketolacid reducto-isomerase
Lb	Leghemoglobine
LC	Liquid chromatography
LDW	Leaf dry weight
LEA	Late embryogenesis abundant
MDH	Malate dehydrogenase
MPa	Megapascal
MS	Mass spectrometry
Mt	Millions of tons
N	Nitrogen
NDW	Nodule dry weight
OAA	Oxalacetate
OD600	Optical density at 600 nm
ODB	Oxygen diffusion barrier
P5C	Pyrroline-5-carboxylate
P5CDH	Delta-1-pyrroline-5-carboxylate dehydrogenase
PBS	Peribacteroid space
PCA	Principal component analysis
PEP(C)	Phospho*enol*pyruvate (carboxilase)
PGPR	Plant growth-promoting rhizobacteria
ProDH	Proline dehydrogenase
PRs	Pathogenesis-related proteins
QS	Quorum-sensing
RLU	Relative luminescence units
ROS	Reactive oxygen species

SE	Standard error
SuSy	Sucrose synthase
Tc	Tetracycline
TCA	Tricarboxylic acid
TS	Threonine synthase
U	Uniformly or Uniformemente
UMA	Universal minimal agar
UMS	Universal minimal salts
v/v	Volume for volume
w/v	Weight for volume
$\delta^{13}C$	Carbon isotopic composition
Ψ	Water potential

Summary

The current world population together with the predictions of further growth suggest that it is necessary to increase crop yields worldwide. Legumes are the second most important food crop after cereals, and thanks to their ability to establish a symbiotic relationship with soil bacteria, the impact of the use of nitrogen fertilizers on the environment is reduced. This symbiosis gives rise to the process known as biological nitrogen fixation (BNF), which consists in the reduction of molecular nitrogen to ammonium, from which plants synthesize organic nitrogenous products essential for their nutrition. Unfortunately, BNF is a very sensitive process to biotic and abiotic stresses such as salinity, drought, or nutrient limitation, among others. The general aim of this work was to gain further insights in the regulation of BNF and the physiological and biochemical mechanisms that plant activate in response to abiotic stresses.

In order to counteract the negative effects of osmotic stresses, plant and bacteria are able to synthesise osmoprotectant compounds to maintain cell viability, e.g. the amino acid proline. A real-time monitoring of proline utilisation in both plant and bacterial systems is a first key step towards understanding the multiple roles of this molecule under osmotic stress situations. Our results in chapter one showed that, in bacteroids, proline accumulation does not occur during the stress phase, but during recovery, once optimal plant growth conditions are re-established.

In chapter two, a proteomic and metabolic study was performed to gain further insights about amino acid metabolism in pea nodules. In the classical model of nutrient exchange between symbionts, plant supplies energy in the form of dicarboxylates to the N_2-fixing bacteroids in exchange for ammonium. However, this classic model was challenged upon the observation that mutations in the general ABC amino-acid transporters AapJQMP and BraDEFGC in *Rhizobium leguminosarum* resulted in N starvation symptoms in both pea and bean plants. The uptake of branched-chain amino acids (BCAAs) from the plant by the bacteroid was found to be essential for an effective BNF at least in *R. leguminosarum* species. Another experimental approach to further understand the role of amino acid metabolism in nodules is the application of compounds that inhibit the biosynthesis of BCAAs in plant cells such as group B herbicides. These approaches allowed us to verify how the blockage of BCAA transport between symbionts had a greater effect on nodule metabolism than the inhibition of BCAA biosynthesis. In fact, BCAA biosynthesis was also inhibited due to the *aap/bra* double mutation. In chapter two, we also evaluate the effect of water deficit on nodule proteome, since among the strategies that plants use

in response to abiotic stresses there are several related to amino acid metabolism. This study highlights the relevance of low abundant amino acids, such as methionine, aromatic amino acids or γ-aminobutyric acid, in the response to water deficit.

Finally, until now no attempt has been made to carry out an integral approach in which possible changes caused by drought in carbon (C) allocation, and in addition, the effect on the consumption or accumulation of metabolites in all plant organs be analysed. For this purpose, in chapter three, the effect of drought on both the [U-^{13}C]-sucrose distribution and ureides, organic acids and carbohydrates content were analysed. We found that drought decreased ^{13}C transport to sink tissues and changed the priority of C allocation between sink organs.

Resumen

La actual población mundial junto con las predicciones de un mayor crecimiento sugieren que es necesario incrementar el rendimiento de los cultivos a nivel mundial. Las leguminosas son el segundo cultivo más importante para alimentación después de los cereales, y gracias a su capacidad de establecer una relación simbiótica con bacterias del suelo, se reduce el impacto del uso de fertilizantes nitrogenados sobre el medio ambiente. Esta simbiosis da lugar al proceso conocido como fijación biológica de nitrógeno (FBN), que consiste en la reducción de nitrógeno molecular a amonio, a partir del cual, las plantas sintetizan compuestos orgánicos nitrogenados esenciales para su nutrición. Desafortunadamente, la FBN es un proceso muy sensible a estreses bióticos y abióticos tales como salinidad, sequía o limitación de nutrientes, entre otros. El objetivo general de este trabajo es ampliar los conocimientos sobre la regulación de la FBN y los mecanismos fisiológicos y bioquímicos que activan las plantas en respuesta a estreses abióticos.

Para contrarrestar los efectos negativos de estreses osmóticos, las plantas y las bacterias son capaces de sintetizar compuestos osmoprotectores para mantener la viabilidad de las células, por ejemplo, el aminoácido prolina. El primer paso clave para entender las múltiples funciones de esta molécula bajo situaciones de estrés osmótico es una monitorización del uso de prolina a tiempo real. En el capítulo uno nuestros resultados mostraron que, en bacteroides, la acumulación de prolina no ocurre durante la fase de estrés, si no durante la recuperación, una vez las condiciones óptimas para el crecimiento de la planta se han reestablecido.

En el capítulo dos, se llevó a cabo un estudio proteómico y metabólico dirigido para ampliar el conocimiento sobre el metabolismo de aminoácidos en nódulos de guisante. En el modelo clásico de intercambio de nutrientes entre simbiontes, la planta suministra energía en forma de dicarboxilatos a los bacteroides fijadores de nitrógeno a cambio de amonio. Sin embargo, este modelo clásico fue cuestionado por la observación de que las mutaciones en los transportadores de aminoácidos ABC, AapJQMP and BraDEFGC, en *Rhizobium leguminosarum* dieron lugar a síntomas de falta de nitrógeno en plantas tanto de guisante como de alubia. Se encontró que era esencial la absorción de aminoácidos de cadena ramificada (AACRs) para una efectiva FBN, al menos en especies de *R. leguminosarum.* Otro enfoque experimental para comprender mejor el papel del metabolismo de los aminoácidos en los nódulos es la aplicación de compuestos que inhiben la biosíntesis de AACRs en las células de las plantas tales como los herbicidas del grupo B. Estos enfoques nos permitieron

verificar como la inhibición del transporte de AACRs entre simbiontes tuvo un mayor efecto en el metabolismo nodular que la inhibición de la biosíntesis de AACRs. De hecho, la biosíntesis de AACRs fue también inhibida debido a la doble mutación de *aap/bra*. En el capítulo dos, también evaluamos el efecto del estrés hídrico sobre el proteoma nodular, ya que entre las estrategias que usan las plantas en respuesta a estreses abióticos hay varias relacionadas con el metabolismo de aminoácidos. Este estudio destaca la relevancia de aminoácidos poco abundantes, como metionina, aminoácidos aromáticos o el ácido γ-aminobutírico, en la respuesta al estrés hídrico.

Finalmente, hasta ahora no se ha intentado llevar a cabo un enfoque integral en el que se analicen los posibles cambios causados por sequía en la distribución de carbono (C) y, además, se analice el efecto sobre el consumo o la acumulación de metabolitos en todos los órganos de la planta. Con este propósito, en el capítulo tres, se analizó el efecto de la sequía tanto en la distribución de [U-^{13}C]-sacarosa como en el contenido ureidos, ácidos orgánicos y carbohidratos. Descubrimos que la sequía disminuyó el transporte de ^{13}C a los tejidos sumidero y cambió la prioridad en la distribución de C entre los órganos sumideros.

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General Introduction

A. Relevance of legumes

The Leguminosae is the third largest angiosperm family in terms of number of species after Asteraceae and Orchidaceae (Azani et al., 2017), and the second most important food crop after cereals (Liew et al., 2014). It is currently divided into three subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) and contains close to 770 genera and over 19,500 species (Bruneau et al., 2013; Lewis et al., 2013). Recently, it has been described a new subfamily classification, which includes six subfamilies: Caesalpinioideae, Cercidoideae, Detarioideae, Dialioideae, Duparquetioideae and Papilionoideae (Azani et al., 2017). Legumes are especially relevant as a source of protein and micronutrients, particularly in developing countries, but also as fodder and green manure. The manufacture of varnishes and paints are also benefited from the cultivation of legumes, as well in medicine and in the horticultural trade (Azani et al., 2017).

One of the best-known ecological features of the family is the ability to establish a symbiotic relationship with microorganisms, generically called rhizobia. This symbiosis allows the process known as biological nitrogen fixation (BNF), which consists in the reduction of nitrogen to ammonium by the bacterial nitrogenase complex. This ability allows legume plants to colonize soils with low or no nitrogen availability. This fact has favoured their cosmopolitan distribution, being able to find them even in the most extreme habitats (Schrire et al., 2005).

From the origins of agriculture, the importance of legumes for both soil health and productivity have been known, implementing agricultural techniques such as intercropping and crop rotation. Synthetic nitrogen fertilizers are responsible for around 12% of the annual average of CO_2-equivalent greenhouse gas emissions calculated to be associated with agriculture over the 5-year period 2010-14 (Peoples et al., 2019). Thus, agricultural techniques based on the use of legumes help to alleviate the impact of nitrogen fertilizers on environment. Although the rotational benefits of legumes has only been related to an increased supply of plant-available forms of N in soil, there is other factor that increase the economic value of the boost in yield of crops grown following legumes, i.e. increased availability of nutrients other than N, changes in soil structure and chemistry, a reduced incidence of pests and pathogens, or increased abundance of beneficial soil biology, among others (Peoples et al., 2019).

Legumes can be divided into two groups depending on the use and destination of production: (i) forage legumes, which leaves and stems are used for animal feed, such as clover (*Trifolium spp.*), alfalfa (*Medicago sativa*) or barrel medic (*Medicago truncatula*); and (ii) grain legumes, which main interest is their dry seeds for both animal and human feed, such as soybean (*Glycine max*), pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), peanut (*Arachis hypogaea*) or faba bean (*Vicia faba*). Other classification divide legumes depending on the climatic requirements for its development in (i) tropical, such as soybean, common bean or cowpea (*Vigna unguiculata*) and (ii) temperate, such as pea, faba bean or chickpea (*Cicer arientinum*).

Regarding forage legumes, alfalfa constitutes around 30% of the total legume-based pastures around the world (Peoples et al., 2019). In Europe, the cultivation of forage legume mono-cultures have markedly decreased during the last 50 years, but thanks to the interest in grass-legumes mixtures and the contribution of white clover (*Trifolium repens*) and red clover (*T. pratense*) the forage production is improving in recent years (Peoples et al., 2019).

Grain legumes contribute with around 33% of the dietary protein nitrogen needs of humans (Vance, 2000). Around 230 million ha of legume crops grown globally for commercial trade each year providing around 400 million tons of grain (Peoples et al., 2019). In 2013, grain legumes are grown on only 1.6% of arable lands in the European Union, which was 4.7% in 1961 (Reckling et al., 2016). The European decline in grain legume´s production has been related to several causes such as (i) specialization in cereal crop production; (ii) low and unstable yields; (iii) low gross margins; (iv) low and unpredictable policy support; (v) inability to recognize or evaluate the long-term benefits of legumes within cropping systems; or (vi) inadequate development of supply chains and markets (Reckling et al., 2016, and references therein). However, in the Mediterranean countries (Cyprus, Greece, Italy, Malta, Portugal and Spain), the decline has been lower due to the role of food legumes in the regional diet (Zander et al., 2016).

A.1 Glycine max

This tropical legume is native of East Asia (Lee et al., 2011) and it is one of the most important grain crops worldwide, although it is well below of the major cereals (wheat, maize, and rice) in global production terms. Thanks to its high-quality protein content, it is a crop very appreciated all over the world, both for human and animal nutrition, with a well-developed global market. Soybean provides around 70% of the total legume grain produced (Peoples et al.,

2019). Soybean areas in Europe are limited by climatic constraints, but the development of new varieties better adapted to cool weather are also promising (Zimmer et al., 2016). Between 2013 and 2017, soybean production increased in Spain above European and global values (70, 44 and 21% increase, respectively) (FAOStat, 2019). Similar trend was observed regarding the harvested area (70, 43 and 10% increase, respectively; FAOStat, 2019). The economic importance of soybean has made it one of the most studied legumes, considering it a model organism for agronomic, physiological and molecular studies.

A.2 Pisum sativum

This temperate legume is the most commonly grown grain legume in Europe, due to its higher yield potential and great flexibility of utilization (Pecetti et al., 2019). Although its production is mainly for animal feed as dry seed (Zander et al., 2016). Between 2013 and 2017, global and European production of pea increased, for both dry and green seed (31 or 58% for dry seed and 16 or 8% for green seed, respectively; FAOStat, 2019). In Spain, both the production and harvested area of pea is around 100 times higher than soybean, and, although an increase in total production (28%) and harvested area (9%) was observed during this 5 years period, this was well below those observed in soybean (FAOStat, 2019).

B. The rhizosphere

The current world population together with the predictions of further growth suggests that it is necessary to increase crop yields worldwide. This causes greater pressure to be exerted on the crops and soil that sustain them, so an improved soil and crop management is needed. Both soil dynamics and plant growth and development are closely related with microorganisms that live in association with plants, either in the rhizosphere, or within plant tissues, or even as epiphytes attached to aboveground plant tissues (Vorholt, 2012). The rhizosphere is the nutrient rich zone between soil and plant roots where many microorganism lives influenced by plants through the production of root exudates, mucilage and sloughed cells (Turner et al., 2013). A myriad of organisms, like fungi, bacteria, nematodes, arthropod herbivores, archaea or viruses live in the rhizosphere (Figure 1). They are attracted towards this rich soil zone and feed on rhizo-deposits (i.e. nutrients, exudates, border cells and mucilage released by the plant root) (Philippot et al., 2013). Moreover, these organisms may interact with the plant positively (increasing their growth),

negatively (reducing their growth and development) or in a neutral way. Therefore, understanding the interactions between plant and organism in the rhizosphere can help to improve plant resistant to both biotic and abiotic stresses, which will ultimately translate to an increase in crop yield (Massalha et al., 2017).

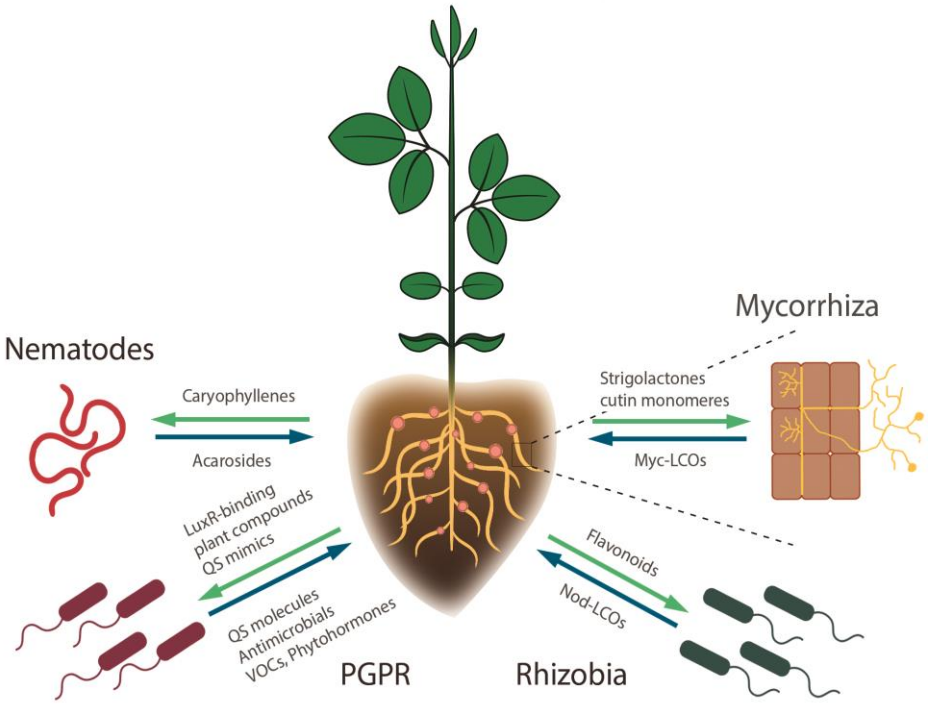

Figure 1 Main signals interchanged between plants and microorganism in the rhizosphere. Microorganisms produce a diversity of signals, among them quorum-sensing (QS) molecules such as N-acyl homoserine lactones, volatile organic compounds (VOCs), and, in the case of nematodes, specific pheromones (acarosides). Recruitment of plant-beneficial rhizosphere microorganisms results in the intimate symbioses undergone by mycorrhiza and rhizobia, initiated by the exchange of specific plant signals (strigolactones and flavonoids, respectively) and microbial signals (Myc and Nod factors, respectively), and other mutualisms such as those involving plant growth-promoting rhizobacteria (PGPR), and entomopathogenic nematodes. In addition, plant also uses confusion and inhibition strategies (e.g., QS mimicry and quenching) to ward off harmful microorganisms. Adapted from Venturi & Keel (2016).

B.1 Root exudates

Plants secrete a large array of primary and secondary plant metabolites to promote the complex interactions that occur in the rhizosphere (Fig. 1; Bais et al., 2006). The release of root exudates have a high energy cost for the plant

since may contain between 5%–21% of their photosynthetically fixed carbon (Huang et al., 2014, and references therein) and 15% of total plant nitrogen (Venturi & Keel, 2016). Root exudates can be divided in two groups depending on their weight: (i) low molecular weight compounds (< 1000 Da; amino acids, organic acids, sugars, among others); or (ii) high molecular weight compounds (> 1000 Da; mucilage, proteins flavonoids, enzymes, terpenoids, among others; Rasmann & Turlings, 2016).

Different mechanisms of root exudation, mediated by passive or active transport, have been described: (i) diffusion through the plasma membrane, depending on membrane permeability, polarity of the exuded compounds, and cytosolic pH (passive); (ii) secretion or re-uptake, depending on different membrane-bound proteins (e.g., ATP-binding cassette (ABC) transporters, the multidrug and toxic compound extrusion (MATE) family, and the aluminium-activated malate transporter family (active); (iii) exocytosis release of exudates when vesicles fuse with the plasma membrane (active) (Huang et al., 2014; Oburger & Jones, 2018, and references therein).

The importance of root exudates lies in the number of aspects over which they influence such as: (i) soil structure; (ii) nutrient mobilization; (iii) chemical signal exchange; (iv) the release of defence compounds; (v) regulation of size, composition and activity of the microbiota; or, (vi) greenhouse gas emissions (Bais et al., 2006; Oburger & Jones, 2018, and references therein). Moreover, root exudation depends on several aspects such as cultivar, type of soil, plant species, plant development stage, mode of photosynthesis of the plant (C3 or C4), nutrient availability, pH, temperature, water status or biotic factors (Bais et al., 2006; Gargallo-Garriga et al., 2018; Huang et al., 2014). Some of these aspects can be modulate by the plant and have a strong impact in both structure and microbiota present in the rhizosphere, since a greater or lesser amount of a certain compound can alter the interactions that may take place there or affect the survival of a certain species.

B.2 Interactions mediated by root exudates

B.2.1 Plant-Plant interactions

Allelopathy is the phenomenon by which plants produce and release some chemicals compounds named phytotoxins that are involved in positive and negative interactions between plants. Phytotoxins include different chemical classes such as benzene-derived compounds, phenolics, hydroxamic acids, and terpenes (reviewed by Massalha et al., 2017). Microbiota living in the

rhizosphere could be affected by phytotoxins and, in turn, the degradation of this compound could also be affected by microbiota (Massalha et al., 2017). The positive effects involve the induction of herbivore resistance and defence, or increased nutrient availability in the rhizosphere in neighbouring plants (reviewed by Bais et al., 2006). As for negative interactions, phytotoxins affect factors such as: (i) metabolite production; (ii) photosynthesis; (iii) respiration; (iv) membrane transport; (v) germination; among others, reducing competition and increasing resource availability (Bais et al., 2006).

B.2.2 Plant-Microbe interactions

Root exudates contain a wide variety of chemicals compounds that promote positive or negative interactions with soil microorganisms. Between the most studied beneficial plant-microbe interactions are legume-*Rhizobium* interactions, mycorrhizal associations and the interaction with plant growth–promoting bacteria (PGPR). Regarding legume-*Rhizobium* symbiosis, the exchange of signals begins with the release by the plant of a substance belonging to a class of compounds named flavonoids, causing the release of lipo-chitooligosaccharides (LCOs, also known as Nod factors) by the rhizobia, ultimately leading to nodule formation (reviewed by Oldroyd, 2013). In mycorrhizal associations, interestingly, LCO (named Myc-LCOs) released by the fungus are also involved, and in response, plants secrete strigolactone as signals for initiating mycorrhizal symbiosis (Venturi & Keel, 2016). Some chemical compounds of root exudates, such as carbohydrates, amino acids (tryptophan) or benzoxazinoids, also influence chemotaxis and flagellar motility in some PGPR bacteria that produce phytostimulators, antimicrobial and antifungal metabolites making a favourable environment for plant development (Bais et al., 2006; Rasmann & Turlings, 2016). Other signals exchanged by plants and bacteria in the rhizosphere include mechanism of regulation as quorum sensing (QS). QS is a regulatory system that allows bacteria to regulate gene expression in response to cell density. It has been described that many plant-associated bacteria require QS for colonization of the rhizosphere (Venturi & Keel, 2016). An interesting type of chemical released by root exudates are volatile organic compounds (VOCs), these are involved, for example, in the attraction of nematodes as a result of insect herbivores, e.g. caryophyllenes.

The presence of pathogenic fungi and viruses, arthropods, nematodes and some classes of bacteria can result in negatives interactions with plants. In some cases, QS is related to the production and secretion of virulence factor, which in

turn cause the plant defence response, releasing a variety of phytoalexins, defence proteins, and other unknown chemicals (Bais et al., 2006).

B.3 Studying root exudates: techniques and constraints

The analysis of root exudates is highly challenging due to different factors, for example, the diverse chemical nature of organic compounds released, the many interactions that occur in the rhizosphere (Oburger & Jones, 2018) or other factors that were discussed above. Therefore, an adequate initial design of the study is crucial. There are different systems for plant growth (soil, hydroponic or aeroponic), and each one has its own limitations and aspects to consider. For example, metabolites may be adsorbed by particles in the growth substrate. The time invested in the collection of the sample is also important, since long sampling times are not desired due to the degradation of the compounds or undesirable chemical reactions (Oburger & Jones, 2018). It is also necessary to consider whether the exudates will be obtained from the entire radical system or only from one part (root tip or elongation zones), since the quantity and composition of the exudates is different along the root. The most used techniques aimed at the identification of a specific compound include spectrophotometry, ion chromatography, high pressure liquid chromatography (HPLC), gas or liquid chromatography coupled with tandem mass spectrometry (GC-MS/MS, LC-MS/MS). Alternatively, techniques based on microscopy and acquisition of images use bacterial biosensor to study the effect of individual compound on the rhizosphere through the emission of light (bioluminescence, i.e. *lux* genes) or expression of green-fluorescent proteins. As an advantage, these techniques allow the *in vivo* observation of the spatial and temporal distribution of exudates. Nevertheless, they rely on survival and competitiveness of the applied marker organisms (Oburger & Jones, 2018).

C. Biological nitrogen fixation

Nitrogen (N) is an essential element for all life forms since it is part of organic molecules as important as amino acids and nucleotides, the building blocks of proteins and nucleic acids, respectively. It is also the most abundant element in the air, since 78% of the atmosphere is composed of N_2. Nevertheless, N is not directly assimilable by living organisms due to the stable triple bond that form N_2 that requires an enormous amount of energy to break it. So despite being an abundant element, it is a limiting nutrient for plant development and productivity. In agriculture, N deficiencies are replaced by the use of fertilizers,

which implies a high energy, economic and environmental cost. Fertilizers constitutes a risk for health and environment, firstly, due to nitrates not being retained strongly to the soil so they present high mobility through the flow of water and being able to reach surface and underground waters. And secondly, the application of nitrogen fertilizers increases the emission of nitrogenous gases, especially, nitrous oxide (N_2O), which contributes to the greenhouse effect, the destruction of the ozone layer and acid rain. It has been estimated that 275 millions of tons (Mt) of N_2 are fixed every year, of which 30 Mt are due to natural sources (storms and volcanic eruptions), 70 Mt due to industrial synthesis through Haber-Bosch process and 175 Mt due to BNF for both free living (35 Mt) or symbiotic (140 Mt) organisms (de Felipe, 2006).

C.1 N_2-fixing organism

BNF is carried out by a group of prokaryotic microorganisms called diazotrophs which have the ability to reduce the N_2 to ammonium thanks to the reaction catalysed by the nitrogenase enzyme complex (see section C.3). Therefore, BNF represents an economical and ecologically alternative to nitrogen fertilizers for the development of sustainable agricultural systems. The ability to fix molecular nitrogen is a widespread characteristic distributed among various groups of bacteria including some archaea (Table 1). This fact highlights that BNF developed early in the evolution of microbial life on Earth (Boyd & Peters, 2013). Diazotrophs are present almost in any habitat and include both free-living and symbiotic bacteria.

Table 1 Free-living nitrogen fixers (adapted from Aparicio-Tejo et al., 2008; Kneip et al., 2007; Leigh, 2000).

	Phylum	Species
Archaea	Methanococcales	*Methanococcus* sp.
	Methanosarcinales	*Methanosarcina* sp.
	Methanobacteriales	*Methanothermobacter* sp. *Methanobacterium* sp.
Bacteria	Cyanobacteria	*Nostoc* sp.; *Anabaena* sp.; *Oscillatoria* sp.; *Gloeothece* sp.; *Trichodesmium* sp.
	Firmicutes (Clostridia)	*Clostridium* sp.
	Bacteroidetes/Chlorobiales	*Chlorobium* sp.
	Chloroflexi	*Dehalococcoides* sp.
	Proteobacteria	*Klebsiella* sp.; *Azospirillum* sp.; *Azotobacter* sp.; *Beijerinckia* sp.; *Rhodospirillum* sp.; *Chromatium* sp.; *Citrobacter* sp.; *Xanthobacter* sp.

Free-living diazotrophs have a wide metabolic diversity. In archaea, BNF operates similarly than in bacteria and is restricted to methanogens (Euryarcheota) within the orders Methanococcales, Methanobacteriales, Methanosarcinales (Table 1). In these organisms, most nitrogenases are probably of the molybdenum type (Leigh, 2000). Regarding bacteria, free-living N fixers has been described for members of cyanobacteria, proteobacteria, clostridiales, chlorobiales, or Chloroflexi, among others (Table 1, 2). Within cyanobacteria, there are species that carry out BNF both in free-living or in association with plants or fungi, e.g. *Nostoc* or *Anabaena* sp. These species fix N_2 thanks to the differentiation of part of their cells in heterocysts, which express the enzyme nitrogenase. Other interesting cyanobacteria are *Oscillatoria* and *Gloeothece* sp. which temporarily separate N_2 fixation and photosynthesis in a day/night cycle (Gallon et al., 1988; Stal & Krumbein, 1987). Nitrogen fixation is also important in oceans, where *Trichodesmium* sp. is one of the most important marine cyanobacterium (Hutchins et al., 2015). Other free-living diazotrophs with different metabolisms are: anaerobic bacteria (*Clostridium* sp., *Dehalococcoides* sp.), facultative anaerobic (*Klebsiella* sp., *Citrobacter*), microaerobic (*Azospirillum, Xanthobacter*), strict aerobic (*Azotobacter* sp., *Beijerinckia* sp.) and cyanobacteria, among others. Azotobacter is an interesting type of diazotroph since, despite the sensitivity of the nitrogenase complex to oxygen

(O_2), it is capable of carrying out nitrogen fixation in the presence of O_2 (Aparicio-Tejo et al., 2008).

Table 2 summarizes the main genera and some examples of species described for symbiotic N fixers and their host. They have been classified as belonging to α- or β-proteobacteria, actinobacteria or cyanobacteria group. Although, interestingly, it has been described a *Pseudomonas* sp. belonging to the γ-proteobacteria that can nodulate *Robinia pseudoacacia* (Shiraishi et al., 2010). For more detailed analysis readers are referred to the taxonomy of rhizobia revised by Peix et al. (2015) and Shamseldin et al. (2017). Within α-proteobacteria the classical (*Azorhizobium*, *Bradyrhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium* and *Rhizobium*) are included and the new rhizobia described in the last years thanks to new taxonomic approaches, including morphological and biochemical characteristics, genetic fingerprinting, fatty-acid methyl ester analysis, and 16S rRNA gene-sequence analysis (Graham, 2008, and references therein; references cited in the Table 2).

Until very recently, rhizobia were described as belonging exclusively to the α-proteobacteria group, but Moulin et al. (2001) found two rhizobia belonging to the *Burkholderia* genus from β-proteobacteria group. It has been described that *Burkholderia* sp. are better colonizer of *Mimosa sp*. than other rhizobia (Gyaneshwar et al., 2011), although they are also able to nodulate species from the genus *Acacia*, *Piptadenia* (Bournaud et al., 2013), *Aspalathus* (Hassen et al., 2012) or *Phaseolus* and *Sorghum* (Gyaneshwar et al., 2011; Wong-Villarreal & Caballero-Mellado, 2010). Among the group of symbiotic nitrogen fixers we can find *Frankia*, an actinomycete able to establish an association with 200 species belonging to eight non-legume angiosperm families, generically called actinorhizal plants (e.g. *Alnus, Casuarina*). These associations have a great importance in forest areas and for the regeneration of N poor soils. Root nodules of actinorhizal plants show some similarities but differ significantly in many respects of legumes nodules, as for example, *Frankia* strains fix nitrogen in vesicles within infected cells, with the exception of nodules of *Casuarina* and *Allocasuarina* (van Nguyen & Pawlowski, 2017).

Table 2 Examples of symbiotic nitrogen fixers (based on (Aparicio-Tejo et al., 2008; Nazaret et al., 1991; Peix et al., 2015; Valverde et al., 2003).

Genus	Selected Species	Selected host
α-proteobacteria		
Aminobacter	*A. anthyllidis*	*Anthyllis vulneraria*
Azorhizobium	*A. dobereinereae*	*Sesbania virgata*
	A. caulinodans	*Sesbania rostrata*
Bradyrhizobium	*B. diazoefficiens; B. elkanii*	*Glycine max*
Devosia	*D. neptuniae*	*Neptunia natans*
Ensifer	*E. medicae*	*Medicago truncatula*
	E. meliloti	*Medicago sativa*
Mesorhizobium	*M. loti*	*Lotus* sp.
	M. mediterraneum	*Cicer arietinum*
Methylobacterim	*M. nodulans*	*Crotalaria* sp.
Microvirga	*M. lupini*	*Lupinus texensis*
	M. vignae	*Vigna unguiculata*
Ochrobactrum	*O. lupini*	*Lupinus albus*
	O. cytisi	*Cytisus scoparius*
Phyllobacterium	*P. trifolii*	*Trifolium pratense*
Rhizobium	*R. leguminosarum* bv. *viciae*	*Pisum sativum*
	R. etli bv. phaseoli	*Phaseolus vulgaris*
Shinella	*S. kummerowiae*	*Kummerowia stipulacea*
β-proteobacteria		
Burkholderia	*B. mimosarum*	*Mimosa* sp.
	B. phymatum	*Phaseolus vulgaris*
Cupriavidus	*C. necator*	*Phaseolus vulgaris*
	C. taiwanensis	*Mimosa* sp.
Herbaspirillum	*H. lusitanum*	*Phaseolus vulgaris*
Actinobacteria		
Frankia	*F. alni*	*Alnus* sp.
Cyanobacteria		
Anabaena	*Anabaena* sp.	*Azolla* sp.
Nostoc	*Nostoc* sp.	*Blasia* sp.*; Gunnera* sp.

C.2 The legume-*Rhizobium* symbiosis

Due to its importance in the agriculture and environment, the most studied N_2-fixing symbioses are the ones established between the classical rhizobia (*Azorhizobium*, *Bradyrhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium* and *Rhizobium*) and certain legumes (*Leguminosae*) and non-legumes plants (e.g. *Parasponia*, *Ulmaceae*) (Table 2). This beneficial association has been known for more than a century when, in 1888, Beijerink obtained the first pure bacterial culture from *Vicia faba*, naming them *Bacillus radicicola*. In 1889, Frank named this bacteria *Rhizobium leguminosarum* and from this date the bacteria with the ability to form nodules were named "rhizobia". As mentioned above, these bacteria are gram-negative soil bacteria, belonging to the α-proteobacteria group. The establishment of the symbiosis occurs through the formation, in roots and stems (*Azorhizobium* sp.*, Sesbania* sp. symbiosis), of a new organelle called nodule, where the process of BNF takes place.

C.2.1 Infection and organogenesis in legumes

The rhizosphere is populated by a large variety of microorganisms. Therefore, the first step in the symbiotic process is the approximation of rhizobia to the root of the host plant. Under low nitrogen conditions, legume roots exude certain phenolic compounds (mainly flavonoids, isoflavonoids and betaines) inducing the chemotaxis of rhizobia to roots (Fig. 1, 2) (Perret et al., 2000). The perception of flavonoid signals by the rhizobia also activate *nodA*, *nodB*, and *nodC* genes, which encode for the enzymes required for the synthesis and secretion of bacterial Nod factors. The Nod factors are recognised by membrane receptor-like kinases present in the roots, activating the symbiosis signalling pathway, inducing root-hair deformation, activating nodule development and initiating the first steps of infection (reviewed in Downie, 2014). The specificity in the signalling between symbionts is due to the chemical structure of Nod factors. Nod factors possess an oligosaccharide backbone of β-1,4-linked N-acetyl-D-glucosamine that can be decorated with a variety of substituents on the N-acetyl-D-glucosamine subunits, such as methyl, fucosyl, acetyl and sulphate groups (Oldroyd, 2013). These facts, together with the variability in the length and degree of saturation of the N-acyl group, are primarily responsible for the compatibility between the host plant and particular species of rhizobia.

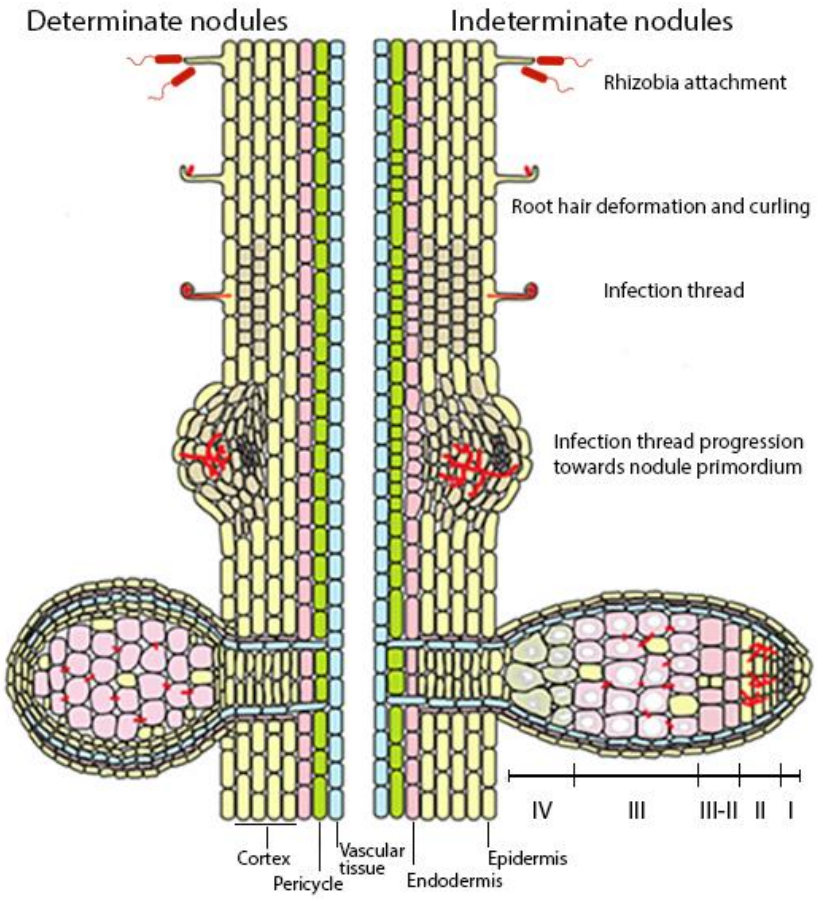

Fig. 2 Development of determinate and indeterminate root nodules. The exudation of flavonoids by root hairs attracts compatible rhizobia and stimulates them to produce nod factors. The root hair deforms and forms a curl trapping rhizobia. Infection thread structures (red) initiate in the curl enabling the rhizobia to enter the plant. Later cell divisions lead to the formation of the nodule primordium. The infection threads progress towards this primordium and release the rhizobia into membrane-bound compartments inside the cells of the nodule, in which they differentiate into nitrogen-fixing bacteroids. In indeterminate nodules, the development of a nodule primordium is accompanied by the presence of a persistent meristem leading to successive zones: the meristem (zone I); the invasion zone (zone II); the interzone (zone II-III); the nitrogen-fixing zone (zone III), and the senescent zone (zone IV). In contrast, determinate nodules do not develop a persistent meristem and hence their invaded cells are all at a similar developmental phase. Adapted from Popp & Ott (2011) and Ferguson et al. (2010).

Bacteria get into the plant through some root hair deformations and curling induced by Nod factors (Fig. 2). At the site of root hair curls, the plant cell wall is degraded by the action of enzymes from both the plant itself and the rhizobia, i.e. cellulases (Oldroyd et al., 2011). The infection process continues with the formation and development of the infection threads by invagination of the

plasma membrane, which grows inwardly through several layers of cortical cells of the root, at the same time as the rhizobia advance and multiply. Simultaneously to the infection process, induction of cell division occurs in the root cortex, forming the so-called nodule primordium (Fig. 2). When the infection thread reaches nodule primordium, rhizobia are released by endocytosis of the plant membrane, called the peribacteroid membrane or symbiosome membrane. This membrane is essential for a stable symbiosis, since all exchanges and communication must take place across this membrane (Udvardi & Day, 1997). The membrane and the bacteria within are together forming a structure called symbiosome (Coba de la Peña et al., 2018; Whitehead & Day, 1997). Rhizobia continue to grow and divide along with the symbiosome membrane to finally differentiate into nitrogen-fixing bacteroids, by morphological, metabolic and genetic changes as the induction of genes related with nitrogen fixation process (i.e. *nif* and *fix* genes) (Udvardi & Poole, 2013). In indeterminate nodules, the cortex cells that form the nodule primordium continue dividing in a perpendicular way to the root.

C.2.2 Types of nodules

The mature nodule is formed by a central tissue with infected and uninfected cells, granules of poly-β-hydroxybutyrate, all surrounded by non-infected tissue that connects with the root vascular tissue (Udvardi & Poole, 2013). Nodules can be classified in two groups according to their pattern of meristem development (Fig. 2): determinate and indeterminate. In general, tropical legumes, as soybean or common bean, have determinate nodules with a characteristic spherical shape, and export N compounds in form of ureides, mainly allantoin and allantoic acid. Determinate nodules do not have a persistent meristem and this is derived from outer cortical cells. All the cells in determinate nodule are in the same stage of differentiation and they are relatively homogenous at any given time point. The growth of this type of nodule depends on the expansion in cell size rather than in cell number (Gibson et al., 2008). Usually, the symbiosome of determinate nodules may contain several bacteroids, while in indeterminate nodules contain a single bacteroid (Oldroyd et al., 2011). Temperate legumes, as pea or faba bean, develop indeterminate nodules with a cylindrical shape and export N compounds in form of amides, mainly asparagine and glutamine. In indeterminate nodules, there is a persistent meristem that continuously generates new cells and cortical divisions begin in the internal cortex. Therefore, different developmental stages are observed within the same nodule (Fig. 3): the meristem, the invasion zone,

the interzone, the nitrogen-fixing zone, and the senescent zone (Gibson et al., 2008).

C.3 Nitrogenase enzyme complex

Biological nitrogen fixation, i.e. the production of ammonia from molecular nitrogen, is catalysed by the nitrogenase enzyme complex (Fig.3) by the following reaction:

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$$

This complex can be separated into two proteins:

A. The dinitrogenase reductase, also named iron protein (Fe-protein), consists in two identical subunits that vary in mass from 30 to 72 kDa, depending on the bacterial species. Each subunit contains an iron-sulfur cluster. It is encoded by the *nifH* gene.

B. The dinitrogenase, also named the iron-molybdenum protein (FeMo-protein), consists in four subunits that vary in mass from 180 to 235 kDa each, depending on the bacterial species. Each subunit has two Mo-Fe-S clusters. It is encoded by the *nifDK* genes.

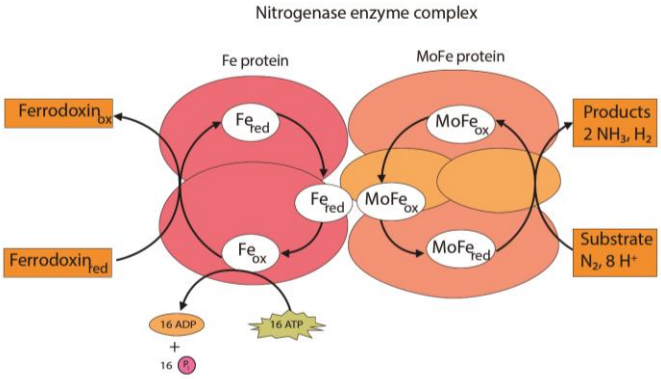

Fig. 3 Reaction catalysed by the nitrogenase enzyme complex. Adapted from Taiz et al. (2015).

There are also alternatives nitrogenases that are homologous to this system, in which molybdenum is substituted by vanadium or iron (Eady, 1996).

The mechanism of nitrogenase involves four steps: (1) formation of a complex between the reduced Fe-protein with two bound ATP molecules and the MoFe-protein; (2), electron transfer between the two proteins coupled to the

hydrolysis of ATP; (3), dissociation of the Fe-protein accompanied by re-reduction and exchange of ATP for ADP; and (4), repetition of this cycle eight times until sufficient numbers of electrons (and protons) have been accumulated, since eight electrons are needed for the reduction of a N_2 molecule (Rees & Howard, 2000).

The nitrogenase complex is inactivated in the presence of O_2, but thanks to the presence of a leghemoglobin (Lb) and a variable O_2 diffusion barrier (ODB), it is possible to maintain a very low O_2 concentration within the infected cells (Arrese-Igor et al., 2011).

It is estimated that plants generate 9.3 moles of CO_2 per mole of N_2 fixed (Taiz et al., 2015). But the production of ammonia (NH_3) is limited due to the slow operation of the nitrogenase complex. To compensate this, the bacteroid synthesizes large amount of nitrogenase. Moreover, in rhizobia, 30 to 60% of the energy supplied to nitrogenase may be lost as H_2, diminishing the efficiency of BNF. Although some bacteria have an H_2-uptake hydrogenase (*hup*) able to split the H_2 formed and generate electrons for N_2 reduction, improving the efficiency of BNF (Ruiz-Argüeso et al., 2001).

D. Carbon and nitrogen metabolism in nodules

D.1 Carbon metabolism

The reaction catalysed by the nitrogenase enzymatic complex is a high energy demanding process. Plant cells must supply the bacteroids enough C compounds for the production of the ATP needed for the reaction. Labelling experiments showed that sucrose is the main C compound supplied to the nodule through phloem (Fig. 4) (Gordon et al., 1985; Streeter, 1980). Besides of the energy requirements for BNF, sucrose is also needed for the maintenance of normal host-cell cytosol metabolism and as C skeleton for the synthesis and transport of the nitrogen compounds produced in the nodule (Rawsthorne et al., 1980). Once in the nodule cytosol, sucrose can be hydrolysed by two enzymes: sucrose synthase (SuSy, reaction 1) or alkaline invertase (AI, reaction 2) (Sturm & Tang, 1999, and references therein).

(1) Sucrose + UDP + H^+ ↔ Fructose + UDP-glucose

(2) Sucrose → Glucose + Fructose

In sink organs such as nodules, SuSy operates in the direction of sucrose cleavage to generate fructose and UDP-glucose (reaction 1). UDP-glucose is a

molecule with a high energy content (Sturm & Tang, 1999), which can react further with pyrophosphate to produce glucose-1-phosphate (G1P) and UDP, with a low consumption of ATP. This ability represents an advantage due to the high demand of ATP and the low rates of O_2 in nodules (Kuzma et al., 1999; de Lima et al., 1994). Studies carry out with SuSy pea mutants (*rug4*), which lack SuSy enzymatic activity, have determined that the presence of SuSy in nodules is essential for BNF, since these mutants are not able to produce nitrogen-fixing nodules (Gordon et al., 1999). In these mutants, AI could not provide the energy needed to support BNF. In *Lotus japonicus*, however, it has been proposed that AI is the main sucrose cleavage enzyme in the first stages of nodule development and it is important in releasing hexoses and starch production in *L. japonicus* nodules (Flemetakis et al., 2006).

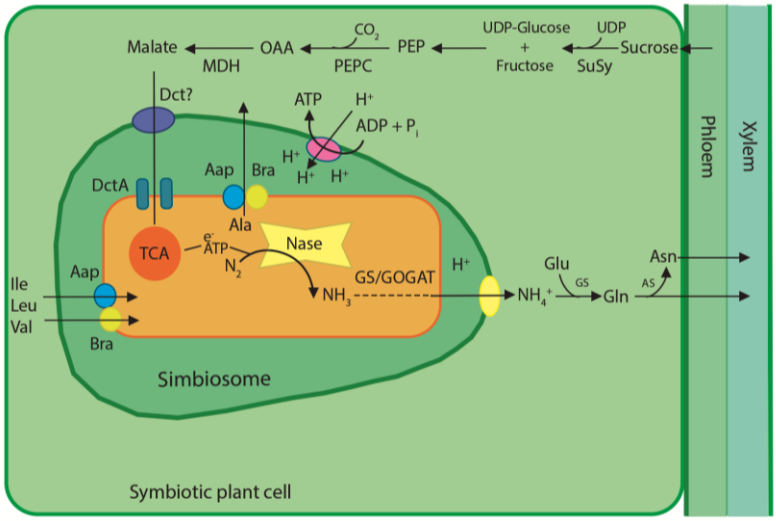

Fig. 4 Schematic illustration of C and N metabolism in an indeterminate nodule. SuSy, sucrose synthase; PEP(C), phospho*enol*pyruvate (carboxylase); OAA, oxaloacetate; MDH, malate dehydrogenase; Ala, alanine; Gln, glutamine; GS, Gln synthetase; Glu, glutamate; GOGAT, glutamate synthase; Asn, asparagine; AS, Asn synthetase; Ile, isoleucine; Leu, leucine; Val, valine; Nase, nitrogenase enzymatic complex. Adapted from Oldroyd et al. (2011) and Larrainzar et al. (2009).

The glycolytic pathway begins with the phosphorylation of fructose and UDP-glucose to fructose-6-phosphate and G1P by the action of a fructokinase and UDP-glucose pyrophosphorylase (UDPGPP), respectively (Fig. 4). Then, these products are hydrolysed to phospho*enol*pyruvate (PEP) and further converted to oxalacetate (OAA) by PEP carboxylase (PEPC) (Fig. 4). Finally, OAA is reduced to malate thanks to the enzymatic activity of malate dehydrogenase (MDH) (Fig. 4). Moreover, OAA can also be metabolized

through the TCA cycle. At this stage, malate can follow two major pathways: it can be used as main C and energy source for bacteroid respiration or as C skeleton for the biosynthesis of N-containing transport compounds via the ammonium assimilation cycle glutamine synthetase/NADH-dependent glutamate synthase (Schubert, 1986).

It is generally accepted that C_4-dicarboxylates, most precisely malate, are the main products of sucrose degradation supplied to bacteroids to support nitrogen fixation in most legumes (Udvardi & Day, 1997, and references therein). Although it has been detected some passive transport of sugars and amino acids through the peribacteroid membrane, these compounds alone would not be able to support nitrogenase activity (Udvardi et al., 1990). The dicarboxylate transport (Dct) system is responsible for importing C_4-dicarboxylates to bacteroids. Dct system consists of three proteins, a dicarboxylate carrier protein, DctA (Fig. 4), and a two-component kinase regulatory system, DctB/DctD (Yurgel & Kahn, 2004).

D.2 Nitrogen metabolism

NH_3 is the principal export product of nitrogen fixation from bacteroids to the peribacteroid space (PBS), although it has also been described the secretion of amino acids, such as alanine and aspartate (Fig. 4) (Allaway et al., 2000; Li et al., 2002). Bacteroids lack the AmtB transporter required for NH_3 uptake, which is expressed only under nitrogen limitation. Therefore, bacteroids assimilate very little of the fixed ammonia, being mainly exported to the host plant by simple diffusion (Patriarca et al., 2002). Because of the acidic environment found in the PBS, at this stage NH_3 is protonated to ammonium (Day et al., 2001).

Ammonium assimilation occurs through the GS/GOGAT systems (Fig. 4). Glutamine is the primary amino compound synthesized from ammonium in the plant fraction by GS through the ATP-dependent incorporation of ammonia to glutamate (Cullimore & Bennett, 1988). GS can be found both in the cytosol and plastids (Carvalho et al., 2000). GOGAT catalyses the transfer of the amide group from glutamine to α-ketoglutarate leading to the production of two molecules of glutamate and it is localized in plastids. Another important enzyme involved in the assimilation of ammonium is aspartate amino transferase (AAT) which catalyses the transamination of glutamate to generate aspartate, which could be exported to the host plant or used by asparagine synthetase (AS) as a substrate for the synthesis of asparagine (Fig. 4) (Day et al., 2001).

Fixed N is further transferred from glutamine to either asparagine or to purine derivates known as ureides. N-fixing legumes can be classified into amide- or ureide-exporters according to the compounds used for the translocation of fixed N. Temperate legumes, such as pea, chickpea or lentil, export fixed N in form of amides, mainly the amino acids glutamine and asparagine (Vance, 2000). Tropical legumes, such as soybean, common bean or peanut, export fixed N in form of ureides, namely allantoic acid, allantoin and citrulline. Ureide production in nodules is a complex compartmentalized process between organelles in infected cells and uninfected cells (Tajima et al., 2004). Glutamine, together with other amino acids, is used by both plastids and mitochondria to form purines. Then, purines are oxidized within the cytosol of infected cells to urate that is transferred to uninfected cells and further oxidized to allantoin and allantoic acid (Smith & Atkins, 2002). Ureide-exporting legumes can be considered more efficient as the C:N ratio of ureides is more favourable (4:4) when compared with glutamine (5:2) and asparagine (4:2) (González et al., 2015).

D.2.1 Symbiotic auxotrophy

The classical model of nutrient exchange between symbionts implies that the plant supplies dicarboxylates to the bacteroids in exchange for ammonium. But, in 2003, Lodwig et al. showed that a more complex amino acid cycle was essential for an effective BNF by *Rhizobium* in pea nodules. *R. leguminosarum* bv. *viciae* has two broad-specificity amino-acid transporters, AapJQMP and BraDEFGC, which belong to the ABC family (Fig. 4) (Hosie et al., 2002; Walshaw & Poole, 1996). Both ABC uptake systems contain a periplasmic solute-binding proteins (AapJ and BraC), an integral membrane proteins (AapQM and BraDE), and ATP-binding cassettes (AapP and BraFG) (Hosie et al., 2002; Walshaw & Poole, 1996). When both uptake systems are mutated in *R. leguminosarum*, nodulated pea plants present symptoms of N starvation while presenting pink nodules. Results also showed the blocking of the uptake of a broad range of amino acids, including glutamate, aspartate and leucine in the *aap/bra* transport mutant. Moreover the N-fixation rates measured were around 30% of wild-type rates, although the nitrogenase activity per bacteroid was equal or exceeded the wild-type rates (Lodwig et al., 2003). Carbon metabolism was also affected due to the blocking of amino acid movement in bacteroids, observing an accumulation of PHB in nodules. Taken together, a new model of amino acid cycling was described, in which, plant provides an amino acid, such as glutamate, or a derivative, to the bacteroid, in addition to dicarboxylates. Via Aap/Bra, glutamate would enter to transaminate oxaloacetate or pyruvate to

produce aspartate or alanine. Finally, these would be secreted to the plant enabling asparagine synthesis (Lodwig et al., 2003).

To better understand this amino acid cycle it was necessary to determine which amino acids were transported via Aap/Bra, and whether uptake and/or efflux was required (White et al., 2007). Later studies by Prell et al. (2009a) with the *aap/bra* transport mutant showed that only the uptake of BCAAs were essential for an effective BNF, at least in peas (Fig. 4). Then, the idea of amino acids cycle was discarded because amino acids did not need to cycle back to the plant. Given that this phenomenon only occurs in symbiosis with the plant, it was named symbiotic auxotrophy. Consequently, a symbiotic auxotrophy requires that BCAAs are supplied by the plant, due to a down regulation of BCAA biosynthesis in bacteroids (Prell et al., 2009a). Similar results were found in the common bean-*R. leguminosarum* bv. *phaseoli* symbiosis (Prell et al., 2010). In contrast, alfalfa plants inoculated with *S. meliloti* mutated in Aap/Bra uptake systems did not show symptoms of N starvation. But, in pea bacteroids it was described that a low rate of BCAAs are required to prevent amino acid starvation. Then, the contrasting results with *S. meliloti* may be due to several causes: (i) low rate of BCAA transport necessary for bacteroids development in that symbiosis; (ii) the existence of other transport systems in *S. meliloti*, which provide enough BCAAs; (iii) the delay or less impact in the reduction in transcription of the genes for BCAA biosynthesis in *S. meliloti* (Prell et al., 2010).

E. Long distance transport in plants

E.1 Water movements in plants

E.1.1 Water absorption by roots

Water absorption by roots requires contact between the surface of the root and the soil. For a better absorption plants increase the number of root hairs to maximize the contact area. The most permeable parts of the root are close to the root tip, since mature regions have developed a modified epidermal layer containing hydrophobic materials in its walls (Frensch et al., 1996). Once inside the root, water can flow through three different pathways: (i) apoplastic, in which water moves without crossing any membranes through cell walls and extracellular spaces as it travels across the root cortex; (ii) symplastic, in which water moves through the plasmodesmata across the root cortex; and (iii)

transmembrane, in which water crosses the plasma membrane of each cell in its path twice (for a review see Kim et al. (2018). The three routes can be intermixed for a same molecule of water depending on both the forces that drive flows and on the water permeability of components of the pathway (Ranathunge et al., 2003).

E.1.2 Transport through the xylem

Xylem tissues are mainly formed by two types of water-transporting cells: tracheids and vessel elements. Water is transport through the xylem thanks to the cohesive properties of water, which sustain large tension in the xylem water column. Thanks to that, water transport through the xylem is more efficient than transport through living cells, since it requires a lower pressure gradient. Transpiration in the leaves is responsible of the negative pressure in the xylem needed to pull water from the soil. Bulk flow of water is responsible for long distance transport of water in the xylem and it is independent of solute concentrations gradients (reviewed by Brodersen et al., 2019). N-fixing compounds produced in nodules are also transported through the xylem to aerial organs.

E.2 Translocation in the phloem

Photoassimilates are translocated from source to sink tissues through the sieve elements, in addition of companion cells and parenchyma cells (Taiz et al., 2015). Source tissues include mature leaves which are able to produce photosynthates in excess of their own needs. Sink tissues include organs that depend on photosynthate for their growth or development (i.e. young leaves, stem, roots, fruits or seeds). Phloem transport is driven by the turgor difference created by source tissues loading solutes into the translocation stream, increasing turgor, and sink tissues withdraw solutes, therefore, lowering turgor (Thompson, 2006). An increase in the photosynthetic rates result in an increase in the translocation rate from the source. Translocation patterns are also affected by the sink strength, which depends on the size and activity of the sink tissue. There are several mechanisms for phloem loading: apoplastic loading, symplastic loading with polymer trapping, and passive symplastic loading. Because sink organs are diverse in structure and function, there is no single mechanism of phloem unloading, which also varies depending on the stage of sink development (Taiz et al., 2015). In all stages of plant development there are a series of sources and sinks, then, several sinks can compete for the available photoassimilates. According to Wardlaw (1990), in early developmental stages,

roots and young leaves are major sinks, whereas in the reproductive stages tubers, fruit and seeds become major sinks. Moreover, biotic and abiotic factors could alter the source-sink relationships.

F. Environmental constraints for plants

World agricultural production is largely affected by different environmental constraints, such as water stress, heat, light, salinity or temperature. Thus, abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels (Cramer et al., 2011). It has been estimated that crop production can be limited by as much as 70% by abiotic stresses (Boyer, 1982). Moreover, in 2007, FAO reported that only 3.5% of the global land area is not affected by some environmental constraint. As complex organisms, plant responses to abiotic stresses are dynamic and complex, and they depends on the tissue or organ affected by the stress (Cramer et al., 2011). Moreover, plants are able to identify abiotic stresses and respond with various strategies to survive.

F.1 Salinity

One of the major constraints affecting plant growth and productivity is salt stress. It was estimated that soil salinity affects around 800 million hectares of arable lands in the world (Munns & Tester, 2008). Salinity problems have worsened over the last 20 years due to the increase in irrigation requirements in arid and semi-arid regions such as those found in the Mediterranean area (Acosta-Motos et al., 2017, and references therein). Salt stress is first perceived by the below-ground tissues due to the increased salt concentration in the rhizosphere, inducing a lower soil water potential around the roots. Coupled with the reduced water availability, ions enter the plant cells inducing a solute imbalance in the cytosol, causing ion toxicity (Araújo et al., 2015). Therefore, salinity effects on plants are related to osmotic stress and ion toxicity, both associated in turn to excessive Cl^- and Na^+ uptake, leading to Ca^{2+} and K^+ deficiency and to other nutrient imbalances, and oxidative stress mediated by reactive oxygen species (ROS) (Acosta-Motos et al., 2017, and references therein).

Plants respond to salt stress at three different levels, i.e., cellular, tissue and whole plant level (Díaz et al., 2005). Plants activate different physiological and biochemical mechanisms for salt tolerance, such as cell-based mechanisms of ion homeostasis, synthesis of osmoprotectants, changes in morphology, anatomy,

water relations, photosynthesis, the hormonal profile, toxic ion distribution and biochemical adaptation (such as the antioxidative metabolism response) (reviewed in Acosta-Motos et al., 2017).

In the case of *Rhizobium*, as it is in other microorganisms, the response to salt stress is mediated by modulating their cytoplasmic osmolality. To do so, they have developed different mechanisms to adjust the levels of compatible solutes needed to maintain cellular functions under salinity. They are able to modulate both the biosynthesis and catabolism of compatible solutes and the uptake or efflux of osmoprotectants, which once inside the cell can turn into compatible solutes (Miller & Wood, 1996). Compatibles solutes include for example, proline, glutamate, potassium, sodium, glycine betaine, glucose, fructose or trehalose. In general, it is considered that rhizobia are more tolerant than their respective plant host to stress (Zahran, 1999).

The *Rhizobium*-legume symbiosis is affected by salt stress from the beginning, in its establishment, to the export of N-compounds formed in the nodules. It has been shown an alteration in the root hair infection process and, therefore, a reduction in the number of nodules under salt stress (Zahran & Sprent, 1986). Moreover, nodule respiration rates are reduced due to the inhibition of O_2 uptake by bacteroids (Delgado et al., 1994). N and C nodule metabolism are also affected by salt stress. Cordovilla et al. (1994) showed a reduced activity of GS/GOGAT pathway in *Vicia faba*, leading to altered ammonium assimilation. SuSy activity decline under salt stress, in addition to other environmental stresses, causing an accumulation of sucrose in nodules, and, in turn a depletion of C compounds for bacteroids (Arrese-Igor et al., 1999). Other C metabolism enzymes have different responses depending on the legume tolerance to salinity. For instance, a decline in PEPC and MDH activities have been detected in common bean (Ferri et al., 2000), but just the contrary in pea nodules (Delgado et al., 1993). In chickpea, the activity of PEPC and 6PGDH increased in the nodule cytosol, but MDH and ICDH activities decreased (Soussi et al., 2001). These alterations in the metabolism lead to a decline in the efficiency of BNF under salt stress. For example, Delgado et al. (1993) showed a decline of the acetylene reduction activity (ARA) and Lb content in pea nodules under salt stress.

F.2 Herbicides

Control of weeds is an important factor to improve worldwide crop production. Crop resources may be comprised by the presence of weeds competing for water, nutrients, light or space. Moreover, weeds can serve as a

carrier of pests, affecting crop yield and development. Herbicides use is the most important and widespread method to control weeds, since other methods such as tillage or flooding are decreasing in order to conserve natural resources: soil, water and energy. The problem of extended herbicide application, however, is the rapid evolution of resistant weeds, reducing its effectiveness (Duke, 2012).

Herbicides target specific plant enzymes belonging to essential metabolic pathways for the plant. Thus, when a herbicide inhibit a specific pathway, the inhibition of growth and plant death could be caused by a direct depletion of the end products, from the depletion of intermediates of the pathway for some critical processes, or from a build-up of a toxic substrate (Royuela et al., 2000).

Herbicides have been shown to negatively impact the establishment of the *Rhizobium*-legume symbiosis and the BNF process (Drew et al., 2007; González et al., 1996; Vieira et al., 2007; Zaidi et al., 2005). Four possible mechanisms have been described: (i) the damaging effect on the host plant; (ii) in the establishment of the symbiosis and nodule formation due to the effect on growth and survival of the rhizobia; (iii) reducing the efficacy of the rhizobia to nodulate and form an effective symbiosis; (iv) ultimately, herbicides can reduce BNF by enzymatic inhibition or interruption of biochemical pathways in the bacteroids (Drew et al., 2007, and references therein).

The Herbicide Resistance Action Committee (HRAC) classifies herbicides in groups according to their chemical structure. There are three groups that inhibit the biosynthesis of amino acids (named B, G and H). Herbicides belonging to the group B, interfere with the biosynthesis of BCAAs by inhibiting acetohydroxyacid synthase (AHAS) enzyme (also referred as to acetolactate synthase). Group G contains herbicides belonging to the chemical family of glycine interfere with the biosynthesis of aromatic amino acid by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). And in the group H, glufosinate inhibit the enzyme GS in the glutamine synthesis (Tan et al., 2006).

F.2.1 Branched-chain amino acid biosynthesis-inhibiting herbicides

The BCAAs are among the 10 essential amino acids that are not synthesized in mammals. As such, it is necessary to include them in our diet. The synthesis of Ile begins with the reaction catalysed by threonine deaminase giving place to 2-ketobutyrate (Fig. 5). In this points a parallel pathway for the synthesis of Ile and Val takes place with a single set of four enzymes (AHAS; ketolacid reducto-

isomerase (KARI); dihydroxyacid dehydratase and branched-chain aminotransferase) that utilize different substrates (Singh & Shaner, 1995). Leu biosynthesis is catalysed by isopropylmalate synthase from 3-methyl-2-oxobutanoate, the last intermediate in the pathway that produces Val or Leu. In the Ile biosynthesis pathway, AHAS catalyses the condensation of pyruvate and 2-oxobutanoate to form CO_2 and 2-aceto-2-hydroxybutanoate (Singh & Shaner, 1995). In the Val and Leu biosynthesis pathway, AHAS also catalyses the condensation of two pyruvate molecules to produce CO_2 and 2-acetolactate (Singh & Shaner, 1995).

According to the HRAC classification, commercialized BCAA biosynthesis-inhibiting herbicides belong to five chemical classes: sulfonylureas, imidazolinones, pyrimidinyl (thio) benzoates, triazolopyrimidines and sulfonylamino-carbonyl-triazolinones. Moreover, there are 56 active ingredients classified as AHAS inhibitors. In the 1980s the imidazolinones chemical family was introduced by the American Cyanamid company (now BASF), becoming one of the most used herbicides worldwide. There are six available active ingredients belonging to the imidazolinone group (imazamethabenz-methyl, imazapic, imazapyr, imazethapyr, imazamox and imazaquin). They control a wide spectrum of grass and broadleaf weeds, are quickly absorbed by roots and leaves, are highly selective, are effective at low application rates, can be applied at pre-sowing, pre- and post-emergence, and present low mammalian toxicity (Ahrens, 1994). In Spain, however, only the use of imazamox is allowed (Regulation (EC) Nº 1107/2009). The persistence of imazamox on soils ranging from 20 to 90 days, depending on initial concentration, temperature, soil moisture and soil type (Vischetti et al., 2002).

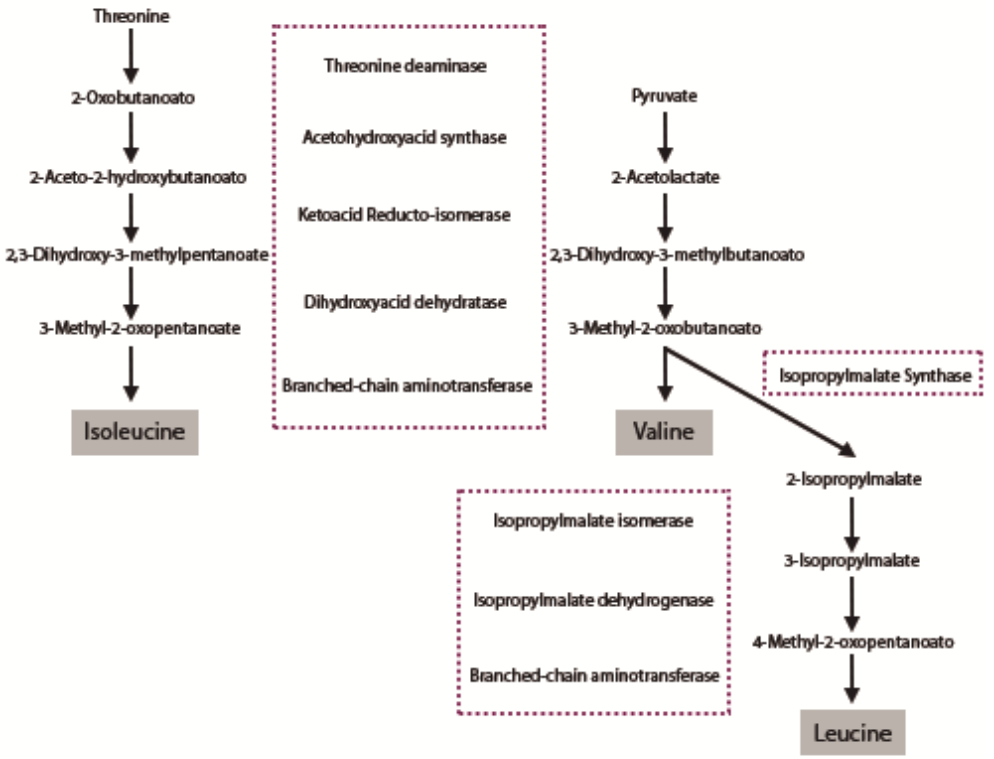

Fig. 5 Pathway of branched-chain amino acid biosynthesis in plants.

Moreover, imazamox can be easily leached, causing environmental problems in groundwater (Sakaliene et al., 2007). It can also affect microbial biomass in the soil, resulting in decreases of up to 25% compared to untreated soils (Vischetti et al., 2002). One of the most important aspects to keep in mind is that its use can be lethal for sensitive rotational crops, for example legumes (O'Sullivan et al., 1998). Imazamox and other AHAS inhibitors affect the development of an effective *Rhizobium*-legume symbiosis (García-Garijo et al., 2014; González et al., 1996; Royuela et al., 1998). In common and faba bean, imazamox has been reported to cause a decrease in nodule dry weight and total nitrogen content, without altering the levels of BCAAs (García-Garijo et al., 2014).

F.3 Drought

Global climate change has become a research priority in the past decade (Shanker et al., 2014). Although several scenarios have been proposed, general predictions include more frequent droughts and floods, increase in atmospheric CO_2 and temperature, giving place to increased surface aridity and a reduction in freshwater availability. In the 21st century, the prediction of the precipitation distribution shows an increase in the amount of precipitation over most af Eurasia, tropical Africa, and extra-tropical North America, but to decrease in the subtropical regions, including areas around the Mediterranean Sea, south-western North America, southern Africa, most of Australia, and parts of South America (Dai et al., 2018).

With the expected 40% increase in world population, understanding the consequences of climate change on crop production it is a challenge to ensure an increase in food supplies between 70% or even 100% by 2050 (Bruinsma, 2009; Shanker et al., 2014). In this sense, water stress is one of the most limiting resources that affect plant growth and crops yield in agricultural systems (Boyer, 1982). Daryanto et al. (2015) showed a positively relationship in the amount of water deficit and yield reduction in legumes crops between 1980 and 2014. Plant responses to water deficit depend of the plant phenological state and involves adaptative changes and/or deleterious effects (Chaves et al., 2002). During drought stress water potential becomes more negative giving place to a greater number of cellular processes affected. According to Taiz et al. (2015), the most negatively affected process by water deficit is cell expansion. As a consequence, shoot growth and leaf expansion is inhibited, although it is usually observed a root elongation (Geiger et al., 1996). Water stress induces the accumulation of abscisic acid (ABA) causing the stomatal closure, and, in turn, gas exchange and photosynthesis are reduced (Cai et al., 2017). One strategy to cope with water stress is the production of compatible solutes (e.g. proline, raffinose, and trehalose) to sustain physiological processes, stabilize proteins and cellular structures and/or to maintain cell turgor by osmotic adjustment (Krasensky & Jonak, 2012). In addition, these compatible solutes are involved in redox metabolism removing the excess of ROS production under water stress. The main physiological and biochemical effects of water deficit on plants are summarized in Table 3.

Table 3 Effects of water deficit on plants (adapted from Taiz et al., 2015).

Primary effects	Secondary effects
Water potential (Ψ) reduction	Reduced cell/leaf expansion
Cell dehydration	Reduced cellular and metabolic activities
Hydraulic resistance	Stomatal closure
	Photosynthetic inhibition
	Leaf abscission
	Altered carbon partitioning
	Cytorrhysis
	Cavitation
	Membrane and protein destabilization
	ROS production
	Ion cytotoxicity
	Cell death

F.3.1 Regulation of BNF under drought

The *Rhizobium*-legume symbiosis is a process very sensitive to environmental stresses. Thus, for the establishment of an effective symbiosis, an optimal physiological state of both partners is needed. Although it is generally accepted that rhizobia are more tolerant to water deficit that their respective host plant, in the case of plant limitation it is not expected that bacteria express their full potential for nitrogen fixation (Zahran, 1999). Water deficit affects the symbiosis from the first steps of its establishment, since bacterial growth and movement are reduced in dried soils. Moreover, root hair infection and the number of infection threads formed are also reduced under water deficit (Serraj et al., 1999a). As a consequence, the development of nodules and the BNF process are widely compromised. The reduction of BNF due to drought stress has been described for several legumes, including soybean, pea, common bean, alfalfa or barrel medic (Gil-Quintana et al., 2013a, b; González et al., 1995, 1998; Larrainzar et al., 2009; Naya et al., 2007; Ramos et al., 1999). Despite the sensitivity of BNF to drought conditions, several studies suggest that N_2-fixing plants can be more tolerant under mild drought stress than nitrate-reducing plants, at least under controlled conditions (Arrese-Igor et al., 2011; Staudinger et al., 2016). Although the molecular mechanisms responsible of such inhibition are not yet fully understood, several hypotheses have been described to explain

the decline in BNF during drought, namely, oxygen limitation, carbon shortage, and regulation by nitrogen metabolism.

F.3.1.1 Regulation by O_2 supply

Nodules require high rates of respiration needed to supply enough energy for BNF, but most nitrogenases are very sensitive to O_2 (Arrese-Igor et al., 2011). This situation is solved thanks to the presence of Lb and ODB (see C.3.). The ODB, located in the nodule inner cortex, changes its thickness in response to environmental constraints, increasing the nodular O_2 diffusion resistance, resulting in an O_2 limitation of nitrogenase-linked respiration (Diaz del Castillo et al., 1994; Minchin, 1997). However, it is unlikely that this change in ODB permeability may be responsible of the BNF inhibition since an increase in O_2 concentration around the rhizosphere cannot restore such inhibition (Diaz del Castillo et al., 1994). In other words, even in the presence of sufficient O_2, nodules of water-stressed plants no longer support optimal levels of BNF (Arrese-Igor et al., 2011). Thus, changes in ODB permeability may be the effect and not the cause of drought-induced BNF inhibition. Regarding Lb, Guerin et al. (1990) and Irigoyen et al. (1992) showed a decrease in the Lb content due to drought stress, giving place to a reduction in the supply of O_2 to the bacteroid. In this sense, ABA application induced a decreased in BNF related with a lower Lb content since photosynthesis and other nodular enzymes remain actives (González et al., 2001a). Thus, two different situations can be considered depending on the stress severity (González et al., 2001b; Marino et al., 2006): (i) under severe drought, BNF inhibition could be due to a limitation in the supply of O_2 to bacteroid, related, among other factors, with a lower Lb content; (ii) under mild stress, BNF inhibition would be rather caused by a reduction in SuSy activity.

F.3.1.2 BNF and N-feedback inhibition

The flux of water through phloem to nodules influences the export from nodules via the xylem (Walsh, 1995). Then, environmental constraints that decrease the water supply to nodules through the phloem lead to an accumulation of N compounds due to the reduced water availability for export (Purcell, 2009). This accumulation of N compounds in nodules has been related with a feedback inhibition of BNF. Several compounds have been suggested to play a role in this inhibition, including glutamine (Neo & Layzell, 1997), ureides (Atkins et al., 1992; Serraj et al., 1999b; Vadez et al., 2000), asparagine (Bacanamwo & Harper, 1997), ureides/asparagine (Vadez et al., 2000) or aspartic

acid/asparagine (Lima & Sodek, 2003). In soybean, it has been shown that an increase in the concentration of leaf ureides under drought is related with the BNF inhibition (Serraj et al., 1999b; Vadez et al., 2000). Furthermore, drought tolerance in soybean has been associated with increased shoot ureides, with sensitive lines showing a higher accumulation compared to tolerant lines (Purcell et al., 1998; Serraj & Sinclair, 1996). King & Purcell (2005) showed that total free amino acids in leaves and nodules increased during water deficits and coincided with a decline in N_2 fixation. However, the concentration of ureides and asparagine remain high after stress even when BNF rates have been recovered, that results indicate that leaf ureides and nodule asparagine do not feedback inhibit BNF (King & Purcell, 2005). Later studies by Ladrera et al. (2007), showed that in the early stages of water deficit, an accumulation of ureides in leaves of tolerant and sensitive soybean lines could not be detected despite the fact that BNF was already inhibited. They concluded that at least two mechanisms are involved in the drought-induced inhibition of symbiotic nitrogen fixation (SNF) in soybean nodules: an accumulation of ureides in nodules and an impairment of metabolic C flux in nodules (Ladrera et al., 2007). Recently, Aldasoro et al. (2019) analyzed the effects of changes in transpiration rates in the regulation of BNF through the application of anti-transpirants to pea plants subjected to a progressive water deficit. This work shows a fast BNF inhibition after anti-transpirants treatment which not coincide with the accumulation of either amino acids or soluble carbohydrates observed at later drought stages in nodules. Thus, these results could not be explained by the hypothesis of a N-feedback regulation of symbiotic nitrogen fixation.

F.3.1.3 Involvement of carbon metabolism in nodules

Traditionally, the BNF inhibition observed under drought had been related with the reduction in photosynthetic rates. However, later studies corroborated that BNF declines previous any effect on photosynthesis (Djekoun & Planchon, 1991; Durand et al., 1987). As earlier mentioned, sucrose that reaches the nodule may be hydrolysed by either SuSy or AI. Under drought, SuSy has been shown to be the first nodule enzyme activity that declines in both tropical legumes such as soybean or common bean (González et al., 1995; Gordon et al., 1997; Ramos et al., 1999) and temperate legumes such as pea (Gálvez et al., 2005; González et al., 1998), leading to an accumulation of sucrose and a depletion of organic acids, mostly malate, in nodules. Thus, C shortage of substrates for bacteroid respiration could induce an impairment of bacteroid functioning, including BNF (Gálvez et al., 2005; González et al., 2001b). Additional evidence for the involvement of C metabolism within nodules in the response to drought

comes from the fact that lines showing contrasting tolerance also display different responses in the SuSy level, in both soybean (Ladrera et al., 2007) and common bean (Sassi et al., 2008). Nevertheless, in pasture legumes such as alfalfa (Naya et al., 2007) and medic barrel (Ladrera PhD Thesis, 2007) subjected to drought, it was observed significant declines in the activity levels of SuSy only after the inhibition of SNF, in parallel to an accumulation of malate. This fact has been related with the larger tolerance of these legumes to water stress and suggest that C availability in nodules was not the limiting factor responsible of the inhibition of BNF, at least, in these plants.

General aim

The *Rhizobium*-legume symbiosis is a process very sensitive to environmental stresses, such as drought and salinity stress. For the establishment of an effective symbiosis, an optimal physiological state of both partners is needed. Thus, plant and bacteria are able to synthesise osmoprotectant compounds, to maintain cell viability. In general, the analysis of these compounds involve the breakage of plant tissue, so it would be interesting to monitor these compounds during the stress period in a non-destructive manner. Although the molecular mechanisms responsible for BNF inhibition are not yet fully understood, several hypotheses have been described to explain the decline in BNF during stress, namely, oxygen limitation, carbon shortage, and regulation by nitrogen metabolism. Important aspects in FBN regulation are the C/N exchanges in the nodules, as well as the source-sink relationships and long-distance transport effects. The general aim of this work was to gain further insights in the regulation of BNF and the physiological and biochemical mechanisms that plant activate in response to abiotic stresses.

Within this general objective, the following specific objectives were defined:

1. To monitor proline levels in a non-destructive manner to understand the dynamics of this molecule under osmotic stress situations in the *Rhizobium leguminosarum* bv. *viciae*-pea symbiosis analysing the release of proline by the plant and the use by the rhizobia.
2. To investigate the effect of the impairment of the amino acid metabolism in pea nodules.
    - 2.1 Part 1. To analyse the response of both plant and bacteroids to the blockage of amino acids transport and the inhibition of BCAA biosynthetic pathway by a proteomic and targeted metabolic approach.
    - 2.2 Part 2. To investigate the effect of water deficit on nodules based on a physiological and proteomic approach, analysing the response of plant and bacteroid proteins, paying attention on amino acids metabolism.
3. To analyse the effect of drought stress in the dynamics of carbon allocation and metabolite distribution across tissues in soybean plants.

Chapter 1

A novel biosensor to monitor proline in pea root exudates and nodules under osmotic stress and recovery

1.1 Introduction

Drought and salinity stress are some of the environmental factors most affecting plant growth and crop yield worldwide. In order to counteract the negative effects of osmotic stresses, plant and bacteria are able to synthesise osmoprotectant compounds to maintain cell viability. The amino acid proline, being highly soluble in water and a scavenger of ROS, has been thought to provide protection under salt and water-deficit stresses (Aspinall & Paleg, 1981; Hasegawa et al., 2000; Szabados & Savouré, 2010; Verdoy et al., 2006). In plants, proline catabolism is mediated by two enzymes; proline dehydrogenase (ProDH) producing pyrroline-5-carboxylate (P5C) from proline, and delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH), which converts P5C to glutamate (Szabados & Savouré, 2010). In bacteria, however, both steps are catalysed by a single polypeptide encoded by the gene *putA,* whose expression is regulated by *putR* and is induced in response to proline (Jimenez-Zurdo et al., 1997; Keuntje et al., 1995; Kohl et al. 1988). Besides its role as an osmoprotectant, proline catabolism, has been also suggested to serve as an energy, carbon and nitrogen source under environmental stress conditions (Lee et al. 2009; van Overbeek and van Elsas 1995; Tanner 2008; Vives-Peris et al. 2018). Additionally, proline exudation has been shown to have a chemotactic effect in alfalfa roots (Bais et al., 2006; Webb et al., 2014). Monitoring proline utilisation in both plant and bacterial systems is a first key step towards understanding the multiple roles of this molecule under osmotic stress situations.

The rhizosphere is the nutrient-rich zone of soil in close proximity with the plant root system where microbial communities depend on the release of root exudates (Turner et al., 2013). Plant root exudates are composed of a great variety of primary and secondary metabolites, including low-molecular weight compounds such as sugars, amino acids and organic acids, as well as high-molecular weight molecules such as mucilage and proteins (Bais et al., 2006; Oburger & Jones, 2018). The different molecules present in root exudates can mediate both positive and negative interactions in the rhizosphere (Huang et al., 2014; Olanrewaju et al., 2019). Focusing on the former, the symbiosis established between plants of the *Leguminosae* family and a group of alpha-proteobacteria named rhizobium has been widely studied. During this interaction, rhizobia are able to infect root cells through a complex signal exchange process, which requires the transcriptional reprogramming of roots cells to develop an organ specialized in nitrogen fixation named the nodule. The symbiosis between pea (*Pisum sativum*) plants and *Rhizobium leguminosarum* bv. *viciae* bacteria is a well-established model system to understand this plant-microbe interaction

(Oldroyd et al. 2011; Udvardi and Poole 2013) and has been effectively used to analyse the effect of root exudates in bacterial gene expression (Ramachandran et al., 2011). This transcriptional analysis led to the identification of a number of bacterial genes specifically induced in response to certain solutes. Cloning the promoter regions of such genes upstream of the *lux* operon, Pini et al. (2017) generated a suite of luminescence-based bacterial bioreporters for the specific detection of metabolites in the rhizosphere. These biosensors allow real time monitoring of the release of a number of compounds including sugars, polyols, organic acids and amino acids in a non-destructive semi-quantitative manner, avoiding possible artefacts associated with other methodologies (Oburger & Jones, 2018; Rilling et al., 2019, and references therein). Plants are grown on plates and, upon inoculation of the specific biosensor, the presence and the abundance of a specific compound can be monitored over time using a photon counting CCD camera.

In the current work, we extend this elite set of biosensors by describing a new *lux* biosensor for the detection of the amino acid proline. The construct relies on the expression of the *lux* reporter driven by the promoter of the pRL120553, a gene located in the proximity to the gene *putA*, responsible for proline catabolism in gram-negative bacteria (Jiménez-Zurdo et al., 1995; Liu et al., 2017). We monitored the levels of luminescence of the biosensor in pea roots and during nodulation both under optimal growth conditions and upon the application of water-deficit or salt stress. Our results show that, in bacteroids, proline accumulation does not occur during the stress phase, but during recovery, once optimal plant growth conditions are re-established.

1.2 Material and methods

1.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1.1 *R. leguminosarum* bv. *viciae* 3841 strains were grown at 28°C in tryptone yeast agar or broth (Beringer, 1974) or universal minimal agar supplemented with 30 mM pyruvate and 10 mM ammonium chloride as the carbon and nitrogen sources, respectively. Universal minimal salts (UMS) is based on the acid minimal salts (AMS; Poole et al., 1994) with the addition of EDTA-Na_2 (1 μM), $CoCl_2O{\cdot}6H_2O$ (4.2 μM), $FeSO_4O{\cdot}7H_2O$ (0.04 mM), and $CaCl_2O{\cdot}2H_2O$ (0.51 mM).

16 g L^{-1} agar was used for solid medium. Antibiotics were added to the cultures at the following concentrations (μg mL^{-1}): streptomycin, 500; tetracycline, 2.

For the construction of the proline biosensor, the promoter region of pRL120553 (605 bp, including the complete upstream regulator pRL120552, *putR*; see gene map in Fig. S1.1a) was amplified using the primers listed in supporting Table 1.1 with Phusion High-Fidelity DNA Polymerase (Thermo Fisher). Fragments were purified and double digested with *Kpn*I (at the 5' end) and *Bam*HI (at the 3' end). Restriction fragments were cloned in front of the *luxCDABE* operon in the lux biosensor vector pIJ11268 (Frederix et al., 2014) to generate the proline biosensor plasmid pOPS0238 (Fig. S1.1b). As a positive control, the neomycin promoter producing constitutive luminescence was cloned upstream of *lux* genes into the same plasmid, generating pIJ11282. The promoterless lux vector pIJ11268 was used as a negative control. Plasmids were transferred into wild-type *R. leguminosarum* bv. *viciae* 3841 by triparental mating as previously described (Pini et al., 2017). All plasmids are available from Addgene (https://www.addgene.org).

1.2.2 Free-living assays

Bacterial strains were grown for 3 days on an UMA (30 mM sodium pyruvate and 10 mM ammonium chloride) slope, resuspended in UMS without carbon or nitrogen and washed three times. Then cells were grown in UMS with 30 mM sodium pyruvate and 10 mM ammonium chloride with antibiotics for 16 h. These cultures were then used as inocula for subsequent induction experiments during 3 h. In these experiments, cells were grown in 10 mL UMS with different supplements (as specified in the corresponding figure). Luminescence (expressed as relative luminescence units, RLU) and OD_{600} were measured using the GloMax-Multi+ Detection System (Promega). Specific luminescence was calculated as RLU per OD_{600}.

Table 1.1 Bacterial strains and plasmids used in this work. Tc, tetracycline.

R. leguminosarum bv. *viciae* 3841 strain (plasmid)	Description of the strain	Description of the plasmid	Resistance	Source
LMB542 (pIJ11268)	Strain with no *lux* expression used as a negative control.	Plasmid derived from pJP2, containing the *lux* operon with no promoter	Tc	Frederix et al. 2014
D5250 (pIJ11282)	Strain with constitutive *lux* expression used as a positive control.	pIJ11268 with the promoter region of *nptII* cloned upstream of the *luxCDABE* operon	Tc	Frederix et al. 2014
OPS0650 (pOPS0238)	Proline biosensor strain	pIJ11268 with the promoter region of *putA* (pRL120554), including the upstream gene pRL120553 and the divergent gene pRL120552 (*putR*) cloned upstream of the *luxCDABE* operon	Tc	This work

1.2.3 Plant growth conditions

Pea (*Pisum sativum* var. Avola) seeds were surface sterilized and germinated on distilled water agar (0.8%, w/v) plates for 5 days in the dark at room temperature. Seedlings were then transferred to 13-cm square Petri plates containing Fahräeus agar (Somasegaran & Hoben, 1994) covered with sterile filter paper (one seedling per plate) as previously described (Pini et al., 2017). Seedlings were then inoculated with the corresponding bacterial strains by pouring liquid inoculum to the roots, adjusted to an OD_{600} equivalent to 2 x 10^7 CFU/per root. Plates were closed with the lid and covered with aluminium foil to prevent exposure of roots to light. Plants were grown in a growth chamber under controlled environmental conditions (23°C temperature, 16-h/8-h day/night cycle).

1.2.4 Application and physiological characterisation of water deficit and salt stress

Seven days after inoculation, plants were transferred to fresh Fahräeus agar plates. For control plants, plates were replaced every three days to keep adequate moisture and nutrient levels. To generate water-deficit conditions, plants were maintained in the same plates for seven days so that water was progressively depleted. For the salt stress treatment, plants were transferred to Fahräeus plates containing 150 mM NaCl at 15 dpi for 24h. In both cases, plants were transferred to fresh Fahräeus plates at 16 dpi and further grown for 5 days (recovery). Plates were analysed at 4, 7, 10, 13, 15, and then daily until 21 days post inoculation (dpi).

To establish the effect of water-deficit and salt stress on plants, stomatal conductance, leaf water potential (Ψ_{leaf}) and net photosynthesis were measured 2 h after the beginning of photoperiod in the youngest fully expanded leaf. Stomatal conductance and net photosynthesis were measured with a portable open system mode (model LCpro+; ADC BioScientific Ltd., Great Amwell, UK) using an ADC PLC-7504 leaf chamber. Ψ_{leaf} was measured using a pressure chamber (Scholander et al., 1965). Symbiotic nitrogen fixation was measured as apparent nitrogenase activity (ANA) according to the method described by Witty & Minchin (1998). H_2 evolution of intact plants was measured in an open flow-through system under N_2/O_2 (79%/21%) using an electrochemical H_2 sensor (Qubit Systems). The H_2 sensor was calibrated with high purity gases (Praxair, Pamplona, Spain) using a gas mixer (Air Liquide, Madrid, Spain) flowing at the same rate as the sampling system (500 mL min^{-1}).

1.2.5 Image acquisition

Plates were photographed using a NightOWL camera (Berthold Technologies) as previously described (Pini et al. 2017). Briefly, CCD images (1,024 by 1,024 pixels) of light output were exposed for 120 s and analysed with the imaging software IndiGO (Berthold Technologies). Data are expressed as counts per second (cps) or as the ratio of luminescence to surface (cps mm^{-2}).

1.3 Results

1.3.1 The proline biosensor OPS0650 is able to detect concentrations of proline in the nanomolar range

The gene pRL120553 (hypothetical protein) and pRL120554 (*putA*) was shown to be induced 16-fold and 2-fold respectively in *R. leguminosarum* bv. *viciae* 3841 when grown in the presence of proline (Ramachandran et al., 2011). The gene *putA* encodes the putative multifunctional proline utilization enzyme A (PutA), which combines ProDH and P5CDH activities, responsible for proline catabolisation in gram-negative bacteria (Liu et al., 2017). pRL120553 codes for a protein of unknown function and protein-BLAST of the predicted protein analysis do not show significant homology with previously described proteins. The gene *putR*, a putative AsnC family transcriptional regulator (pRL120552) is located upstream of pRL120553. These genes are located very close to one another, with intergenic regions around 50 nt each (Fig. S1.1a). Thus, to generate a proline biosensor strain, a 605-bp region upstream of pRL120553 from *R. leguminosarum* bv. *viciae* 3841 was cloned into the lux vector pIJ11268 in front of the *lux* operon (Fig. S1.1b). The fragment cloned included the putative transcriptional regulator *putR*, since, from our experience, including the divergent regulator has been shown to improve the sensitivity of the promoter fusions (Pini et al., 2017). Transformation of *R. leguminosarum* bv. *viciae* 3841 with this plasmid generated the proline biosensor strain OPS0650 (Table 1.1).

To determine the specificity of the induction of *lux* expression in the biosensor, bacterial cells were grown in UMS supplemented with different compounds and luminescence was measured after 3 hours of incubation (Fig. 1.1). Specific luminescence data are represented as relative luminescence units per optical density at 600 nm (RLU/OD_{600}) to account for the differences in

bacterial growth. In all tests, strain LMB542 containing the empty vector pIJ11268 and the strain D5250 with constitutive *lux* expression were included as negative and positive controls, respectively. The proline biosensor showed luminescence values 86-fold higher in the presence of 500 μM of proline than those to cells grown in control medium. Cells grown in media supplemented with 10 mM of L-4-hydroxyproline, a closely related non-proteinogenic amino acid, presented luminescence values comparable to those of a culture grown in the absence of proline, suggesting that the luminescence recorded is specific for proline. When pyruvate was eliminated from the media, the luminescence showed a decline, yet was still 17-fold higher than that of cells grown in the absence of the amino acid (Fig. 1.1).

To define the limit of detection of the biosensor, the strain was incubated in UMS media supplemented with different proline concentrations ranging from 1nM to 500 μM (Fig. 1.1). The biosensor was able to detect concentrations of proline as low as 300 nM, showing a significant specific luminescence of around $4.42 \times 10^5 \pm 0.82 \times 10^5$ RLU/OD600. Increases in the concentration of proline over 50 μM did not produce significant increments in luminescence emission, maintaining luminescence values of approximately $5.63 \times 10^6 \pm 8.22 \times 10^5$ RLU/OD600.

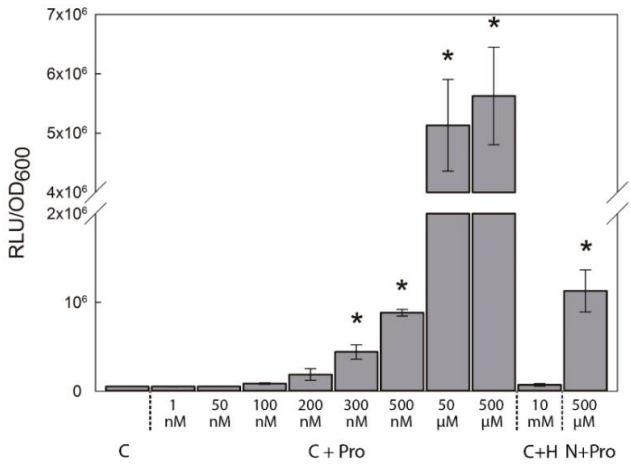

Fig. 1.1 Specificity and sensitivity of the proline biosensor strain OPS0650. Luminescence values are expressed as relative luminescence units (RLU) per optical density at 600 nm (OD600). Cells were grown using different combinations of universal minimal salts (UMS) media. C stands for control growth media containing UMS + 30 mM pyruvate + 10 mM ammonium chloride (NH4Cl); C+Pro, control medium supplemented with various concentrations of proline as stated; C+H, control medium +10 mM L-4-hydroxyproline; N+Pro, UMS medium + 10 mM NH4Cl + 500 μM Pro. Values represent mean ± SE from two independent experiments in the case of nM proline concentrations and three independent experiments in the other measurements. An asterisk (*) indicates significant differences from C (Student´s *t*-test at $p \leq 0.05$).

1.3.2 *In vivo* monitorisation of proline in root exudates and nodules

We then analysed the spatial and temporal expression of the *lux* reporter in plants inoculated with the biosensor strain during root growth and nodule development. Pea plants were inoculated with either the biosensor strain OPS0650 or the negative and positive control strains LMB542 and D5250, respectively. Luminescence was measured at different time points from 4 to 20 dpi (Fig. 1.2). We noted that the hypocotyl showed background luminescence most likely due to the presence of chlorophyll in the upper part of the root in proximity to the light. Thus, to measure luminescence in roots we selected the area of the root corresponding to a length of 2-3 cm per root width, located aprox. 2 cm from the hypocotyl to prevent the above-mentioned background luminescence. To calculate luminescence in nodules we used the area corresponding to single nodules. No proline was added to the agar plates and the light output measured in plant roots inoculated with the negative control strain showed average values of 2.90 ± 0.18 cps mm^{-2} across the experiment (Figs. S1.2, S1.3). Therefore, the differences in luminescence detected can be attributed to either the proline present in root exudates or the proline detected by the differentiated nitrogen-fixing form of rhizobium, the bacteroids, within the infected nodule cells. Four days after inoculation, luminescence was induced mainly on primary roots, particularly in the upper half section of the main root (Fig. 1.2a). As lateral roots developed, luminescence could also be observed in the oldest lateral roots, although at relatively lower levels (Fig. 1.2b-f). These discrepancies may be related to the inoculation procedure, which was carried out when plants presented only a main root. At 10 dpi luminescence was detected in nodules (Fig. 1.2c), reaching maximum intensity values at 15 dpi (Fig. 1.2d).

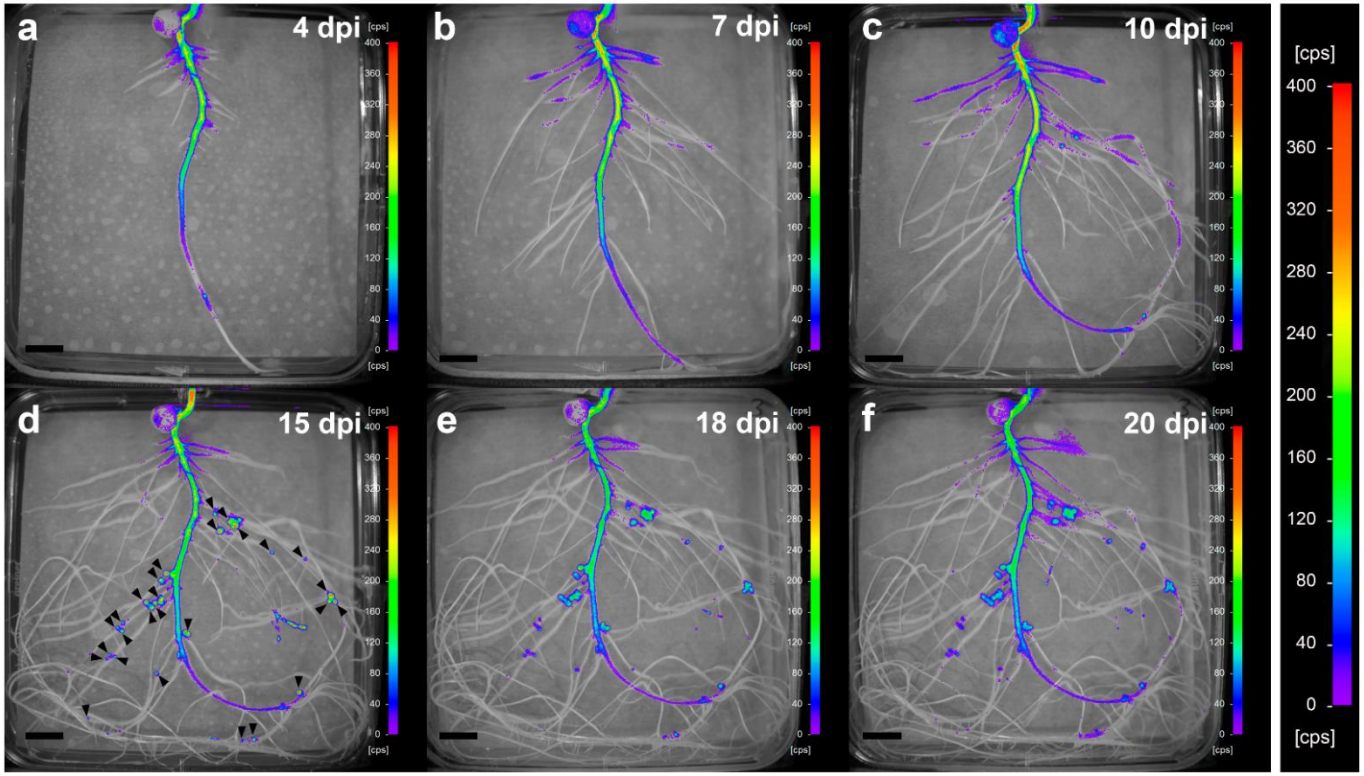

Fig. 1.2 *In vivo* temporal and spatial expression of the proline biosensor strain in pea roots and nodules. Images are representative of plates corresponding to 5 biological replicates and were acquired at 4 **(a)**, 7 **(b)**, 10 **(c)**, 15 **(d)**, 18 **(e)**, and 20 **(f)** dpi. Nodules were visible to the naked eye at around 10 dpi. Arrowheads were added in image **(d)** to show nodules position. Note that the same scale has been used in all images to facilitate comparisons. Scale bar, 1 cm.

1.3.3 Induction of the proline biosensor occurs after a water-deficit and osmotic stress period

We then employed the luminescence-based proline biosensor strain to analyse the effect of gradual water loss and short-term salt stress on nodulated plants. Water-deficit conditions were created by growing the plants on the same plates for seven days. Doing so, the water content of the agar medium, and therefore, the water available for the plant, was progressively depleted. To monitor the level of the water-deficit stress imposed, we checked the following physiological parameters: leaf water potential (Ψ_{leaf}), stomatal conductance, net photosynthesis and symbiotic nitrogen fixation (Table 1.2). At this stage (16 dpi), Ψ_{leaf} showed a significant decline, with values around -0.49 ± 0.1 MPa, while control plants maintained at optimal water conditions showed Ψ_{leaf} values of -0.24 ± 0.05 MPa. Similarly, water deficit caused a 66% decline in stomatal conductance and a 36% reduction in photosynthetic rates compared to control plants (Table 1.2). Regarding nitrogen fixation, water deficit and salt stress caused a 52 and 67% reduction in the rates of nitrogen fixation, respectively, compared to plants under optimal conditions (Table 1.2).

Table 1.2 Effect of water deficit and osmotic stress on leaf water potential, stomatal conductance, photosynthesis an ANA of pea plants. Values represent the mean ± SE ($7 \leq n \leq 10$ biological replicates, except in ANA with n = 4). An asterisk (*) indicates significant differences compared to control plants. (Student´s *t*-test at $p \leq 0.05$)

Parameter (Units)	Control	Water deficit	Salinity
Stomatal conductance (mol m^{-2} s^{-1})	0.09 ± 0.01	0.03 ± 0.01*	0.04 ± 0.01*
Leaf water potential (MPa)	- 0.24 ± 0.05	- 0.49 ± 0.1*	- 0.39 ± 0.03*
Photosynthesis (µmol CO_2 m^{-2} s^{-1})	7.07 ± 0.38	4.52 ± 0.47*	4.73 ± 0.26*
ANA (µmol H_2 g NDW^{-1} min^{-1})	0.54 ± 0.06	0.26 ± 0.01*	0.23 ± 0.07*

In regard to the effects of the different stress treatments on plants inoculated with the proline biosensor, initial experiments showed that the main changes in luminescence were observed in nodules, not in the root exudates. Therefore, analyses of the effect of the stresses are mostly focused on the luminescence of nodules alone. Nevertheless, we also analyzed the variations in

luminescence in roots, selecting a region of the main root to facilitate comparisons across treatments. Plants inoculated with the proline biosensor OPS0650 showed significant changes in luminescence when compared with the negative control strain LMB542 (Fig. S1.3). However, as earlier observed, with the exception of samples at day 16 (24 h after the stress treatments), roots inoculated with biosensor strain did not show significant changes across the experiment.

Regarding the effect of the stresses on nodules, during this period of gradual water loss, the luminescence of nodules was maintained at relatively constant values, suggesting that proline levels did not show significant changes in bacteroids (Figs. 1.3a and S1.4). At 16 dpi, plants were transferred to fresh plates (i.e., optimal water availability) as a recovery treatment. Recovery provoked a rapid increase in the luminescence observed in nodules within the first 24 h, showing a gradual reduction in the following days (Figs. 1.3a, 1.3c, S1.4), almost reaching the levels of *lux* expression in nodules of control plants (Figs. 1.3a and b).

Salt stress was generated by incubating plants on plates containing 150 mM NaCl for one day. Similarly, to the water-deficit experiment, recovery was carried out by transferring the plants to fresh plates under optimal growth conditions, corresponding to the time point 16 dpi. Salinity also had a negative impact on the physiology of the plants, which showed Ψ_{leaf} values of -0.39 ± 0.03 MPa, a 55% decline in stomatal conductance and a 35% reduction in net photosynthesis (Table 1.2). 24 h after the application of the salt stress there were no significant changes in the level of luminescence of the proline biosensor compared to the luminescence recorded before the onset of the treatment (Figs. 1.3a, S1.5). At 17 dpi, however, luminescence started to increase, reaching its maximum at 18 dpi (Figs. 1.3a, 1.3d and S1.5). Subsequently, the levels of luminescence progressively declined, yet were significantly higher than those of control nodules at the end of the experiment (Fig. 1.3a).

Nodules of plants inoculated with the negative control strain LMB542 showed negligible luminescence levels (Figs. 1.3e, S1.2), while nodules of plants inoculated with D5250, the strain carrying the constitutive *lux* expression plasmid, showed the maximum levels of luminescence (Figs. 1.3f, S1.6).

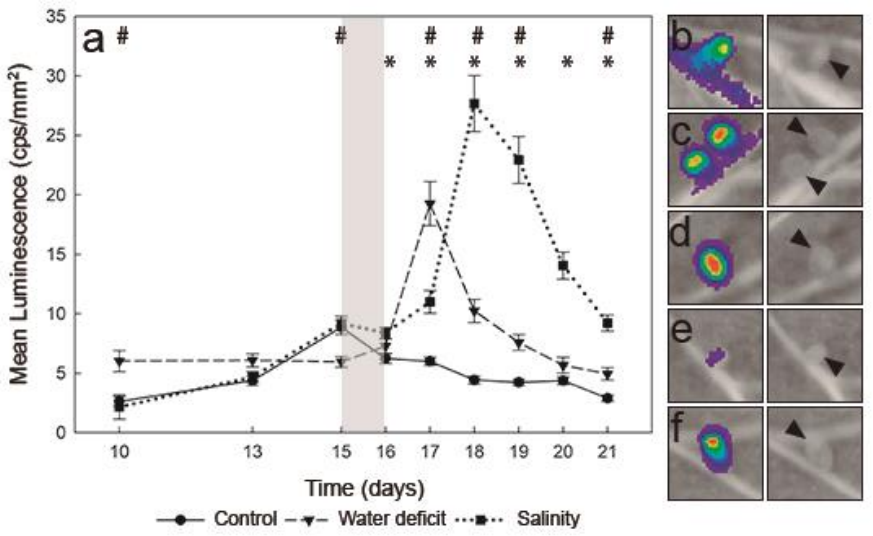

Fig. 1.3 (a) *In vivo* monitoring of proline in pea nodules of plants inoculated with the biosensor *R. leguminosarum* bv. *viciae* strain OPS650. Values represent mean luminescence [counts per second (cps) mm^{-2}] from nodules of pea plants inoculated with the proline biosensor. Stress was induced at 15 dpi for 24h (grey area). For recovery, plants were transferred to fresh plates (optimal growth conditions). Values represent the mean ± SE calculated from 5 biological replicates using the luminescence values of all the nodules present in a plant. An asterisk or a hash sign (* or #) indicate significant differences between salt-stressed or water-deficit plants and control plants, respectively (ANOVA at $p < 0.05$, Dunnett T3 post hoc test). **(b)** to **(f)** representative images from nodules of pea plants under different conditions, and their corresponding luminescence image. **(b)** nodules of plants inoculated with the proline biosensor strain OPS0650 under control conditions (17 dpi, scale 0-400 cps); **(c)** nodules from water-deficit plants at the maximum level of luminescence of the proline biosensor during recovery (17 dpi, scale 0-1100 cps); **(d)** nodules from salt-stressed plants at the maximum luminescence of the proline biosensor during recovery (18 dpi, scale 0-3000 cps); **(e)** nodules from plants inoculated with the negative control strain LMB542 (no *lux* expression, 17 dpi, scale 0-400 cps); **(f)** nodules from plants inoculated with the positive control strain D5250 (constitutive *lux* expression, 17 dpi, scale 1200-25200 cps). Arrowheads were added in images **(b)** to **(f)** to show nodules position.

1.4 Discussion

This work describes a novel *lux* biosensor for the detection of proline. The biosensor takes advantage of the proline-specific promoter of the gene pRL120553, a gene in close proximity to the gene pRL120554 (*putA*) of *Rhizobium leguminosarum* bv. *viciae* 3841, which were highly expressed during growth in proline. The induction assay showed that this biosensor is very sensitive as it can sense proline at concentrations as low as 300 nM. Also, testing

with L-4-hydroxyproline, a close relative of proline showed that this biosensor is very specific to proline. Currently, this biosensor is housed in *Rhizobium leguminosarum* bv. *viciae* 3841, although it should work in other related rhizobia. This novel construct allows the semi-quantitative estimation of the levels of proline both secreted by roots in the rhizosphere as well as the proline accessible for bacteroids inside nodules. The measurement is carried out in a non-invasive manner, maintaining tissue integrity, and thus, avoiding possible artefacts or degradation issues faced when collecting root exudates or isolating symbiosomes. Additionally, it allows the monitoring of proline levels across time using a simple plate growth assay. Although *lux*-based systems come with their own limitations (i.e., dependence on oxygen, ATP or reducing power; Brodl et al., 2018), this type of *lux*-based biosensor has been successfully used for the in vivo monitoring of a number of metabolites including sugars, polyols and organic acids (Pini et al., 2017), as well as signalling compounds such as rhizopines (Geddes et al., 2019).

In terms of root secretion, proline was detected mainly on the primary root and, at later stages, with lateral roots and nodules (Figs. 1.2, S1.2 and S1.3). This luminescence was shown to be specific of the presence of proline since plants inoculated with the negative control strain showed negligible light output (Figs. S1.2 and S1.3). Interestingly, these regions do not correspond to root zones that are preferentially colonised by rhizobia (i.e., the elongation zone), neither co-localise with the secretion of other metabolites such as the amino acid phenylalanine or carbohydrates like sucrose (Pini et al., 2017). This suggests that the composition of the root exudates varies depending on the root zones, an observation that could not be made using classical root exudate extraction approaches.

Symbiotic root nodules represent a strong sink tissue for the plant, requiring the transport of high levels of sucrose to fuel nitrogenase activity and the biosynthesis of a large number of proteins and other biomolecules to maintain the high rates of metabolic activities. Once inside nodules, the major energy source provided by the plant to the bacteroids is in the form of malate (Driscoll & Finan, 1993; Ronson et al., 1981; Udvardi et al., 1988). However, the peribacteroid membrane also allows the transport of amino acids, including proline (Udvardi et al., 1990; Zhu et al., 1992). This fact, along with the observation of increased proline degradation activity under drought and salt stress conditions (Kohl et al., 1988), has led to suggest that besides its osmoprotectant role, proline can also be used as a carbon, nitrogen and energy source for bacteroids (Curtis et al., 2004; Kohl et al., 1994). Indeed, exogenous application of proline or inoculation with a strain overexpressing *putA* has been

shown to improve nitrogen fixation under drought stress conditions (Kohl et al., 1994; van Dillewijn et al., 2001; Zhu et al., 1992).

The current work shows that proline levels increased within bacteroids during nodule development, reaching a maximum level at 15 dpi. It is also interesting to note that, based on the luminescence detected, proline levels in nodules are significantly higher than those detected in root exudates. However, in contrast to the classical stress-induced accumulation of proline in nodules (Fougere et al., 1991; Gil-Quintana et al., 2013a; Larrainzar et al., 2009), proline levels in pea bacteroids did not show significant variations during the stress period, but during the recovery phase. Although there are several factors that could account for these discrepancies such as the duration or the intensity of the stress treatment, it is noteworthy that this is the first time that proline levels are measured in bacteroids within intact nodules (i.e., without disrupting the tissue). Thus, the increase in proline previously reported may account, at least partially, to the proline accumulation in the plant fraction of nodules. In this scenario, recuperation of optimal growth conditions during recovery may lead to a reactivation of bacteroid proline catabolism and/or increased import of proline from the cytosol to the symbiosomes. This activation of proline catabolism upon recovery has, to our knowledge, been so far only described in plants, where the expression of proline dehydrogenase is suppressed during osmotic stress but induced again upon the relief of the stress (Mani et al., 2002; Satoh et al., 2002). Proline uptake has been shown to occur through a diffusive process in bacteroids from alfalfa and soybean nodules (Pedersen et al., 1996; Trinchant et al., 1998; Udvardi et al., 1990). Thus, one possibility is that at least a fraction of the proline accumulated during the stress in the plant fraction catabolised by the bacteroid with a two-fold benefit: to facilitate restoring pre-stress proline levels in the cytosol of infected cells, and to provide an additional source of energy, carbon or nitrogen for bacteroid metabolism. In this regard, in experiments with bacteroids isolated from *Vicia faba* nodules, salt stress produced an accumulation of proline in the peribacteroid space, suggesting that symbiosomes may behave as osmometers to accommodate the osmotic changes occurring in infected cells (Trinchant et al., 1998). Based on results presented here, this could also be the situation in nodules subjected to water deficit. It would be of great interest to combine multiple biosensors driving the expression of, for instance, different fluorescence proteins so that the levels of key metabolites can be simultaneously monitored in vivo.

1.5 Conclusions

The construction of a proline biosensor allows to monitor the levels of proline during pea root development and nodulation in a non-invasive manner. In roots, proline is detected mainly on the primary root and, at later stages, in lateral roots, suggesting that the composition of the root exudates varies depending on the root zones. In nodules, proline levels are significantly higher than those detected in roots. This methodology also allows to monitor the levels of luminescence of the biosensor under abiotic stresses, such as water-deficit or salt stress. Our results show that, in bacteroids, proline accumulation occur after stress, when optimal plant growth conditions are re-established. Thus, bacteroid could use proline as a source of energy and, additionally, the levels of proline in the cytosol of infected cells are restored.

1.6 Supplementary material

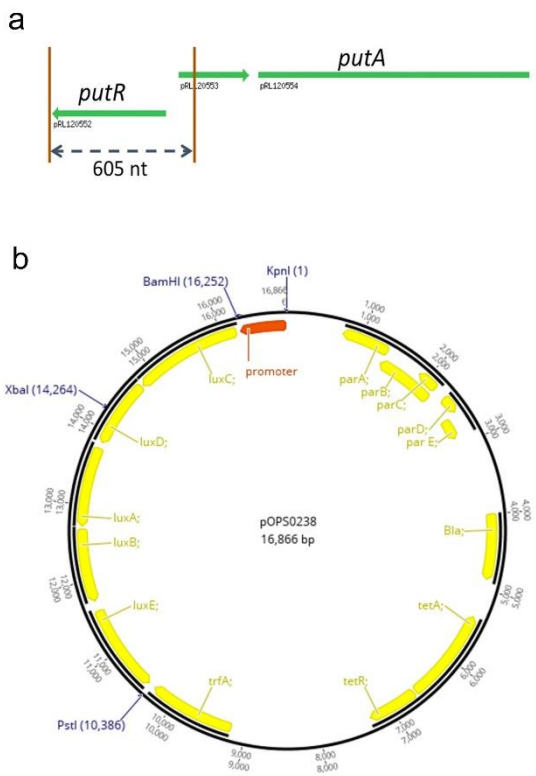

Fig. S1.1 Gene **(a)** and plasmid map **(b)** indicating the 605-bp region cloned to construct the lux-based proline biosensor strain OPS0650 in *R. leguminosarum* bv. *viciae* 3841.

Table S1.1 Primers used in this work.

Primer	Sequence	Description	Restriction site	Source	Biosensor
oxp0648	TTTTGGTACCCGTCATAGCCTCCCACGGAT	Sense primer for region upstream of pRL120553 including divergent gene pRL120552	*Kpn*I	This work	Proline
oxp0649	TTTTGGATCCCTATCATAATCACAAATCGCAATCTCA	Antisense primer for region upstream of pRL120553 including divergent gene pRL120552	*Bam*HI	This work	Proline

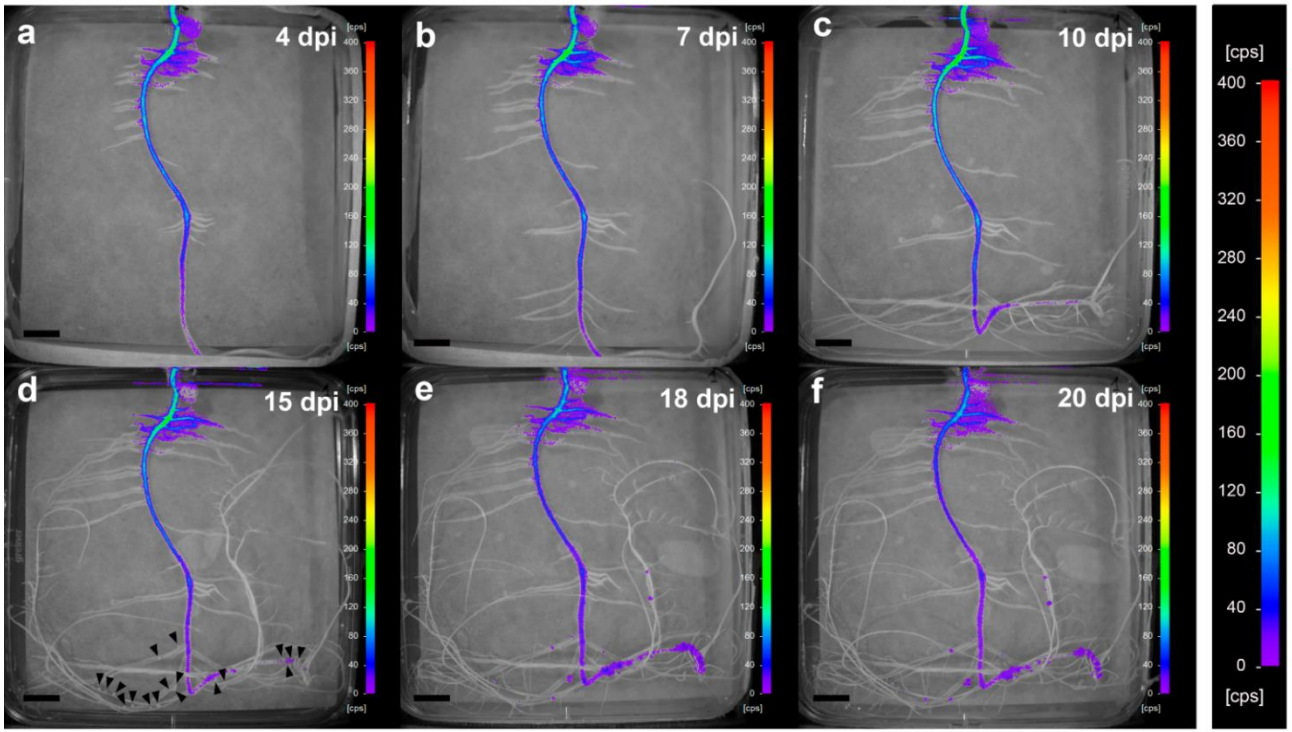

Fig. S1.2 *In vivo* temporal and spatial expression of negative control strain LMB542 in roots and nodules of pea plants. Luminescence is expressed in counts per second (cps). Images were acquired at 4 **(a)**, 7 **(b)**, 10 **(c)**, 15 **(d)**, 18 **(e)**, and 20 **(f)** dpi. Arrowheads were added in image **(d)** to show nodules position. Note that the same scale has been used in all images to facilitate comparisons. Representative images of plates belonging to a time series experiment (n=5 biological replicates). Scale bar, 1 cm.

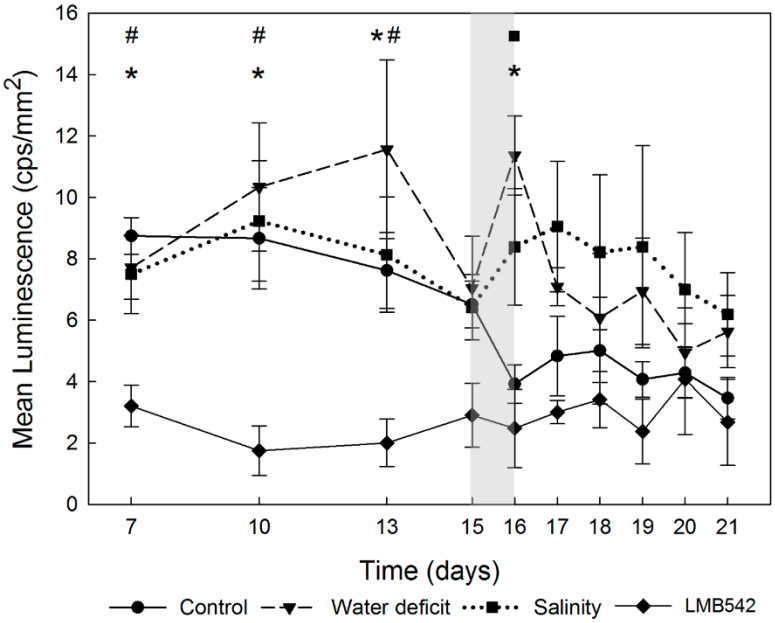

Fig. S1.3 *In vivo* monitoring of proline in roots of plants inoculated with the biosensor *R. leguminosarum* bv. *viciae* OPS650 or LMB542 strains. Values represent mean luminescence (cps mm^{-2}) from roots of pea plants inoculated with the proline biosensor and the negative control. Stress was induced at 15 dpi for 24h (grey area). Values represent the mean ± SE calculated from 4 biological replicates using the luminescence values of a section of the main root (section of 2-3 log per root width, 2 cm away from hypocotyl). An asterisk (*) indicate significant differences between water and salt-stressed plants from negative control strain; a hash (#) indicate significant differences between control plants and negative control; a square (▪) indicate significant differences between salt-stressed and water-deficit plants compared to control plants (ANOVA at $p < 0.05$, LSD post hoc test).

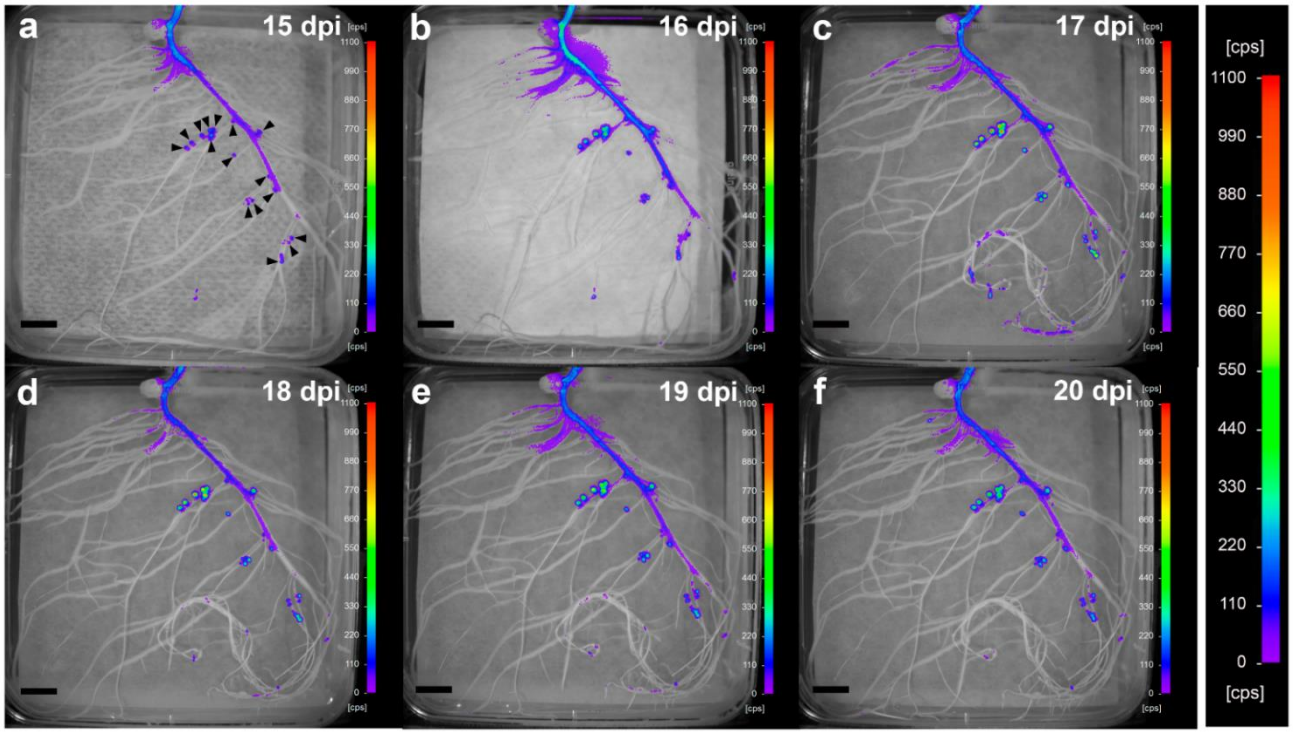

Fig. S1.4 *In vivo* temporal and spatial expression of the proline biosensor strain in roots and nodules of water-stressed pea plants. Luminescence is expressed in counts per second (cps). Images were acquired at 15 **(a)**, 16 **(b)**, 17 **(c)**, 18 **(d)**, 19 **(e)**, and 20 **(f)** dpi. Arrowheads were added in image **(a)** to show nodules position. Note that the same scale has been used in all images to facilitate comparisons. Representative images of plates belonging to a time series experiment (n=5 biological replicates). Scale bars, 1 cm.

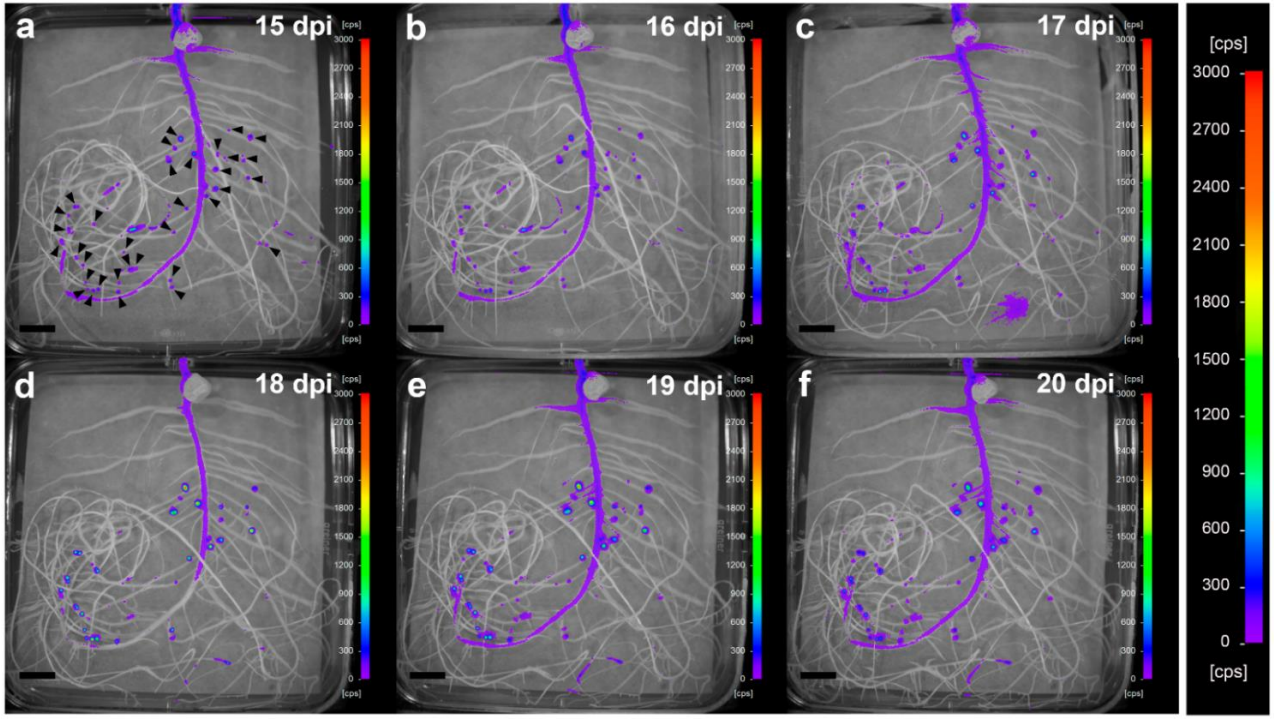

Fig. S1.5 *In vivo* temporal and spatial expression of the proline biosensor strain in roots and nodules of salt-stressed pea plants. Luminescence is expressed in counts per second (cps). Images were acquired at 15 **(a)**, 16 **(b)**, 17 **(c)**, 18 **(d)**, 19 **(e)**, and 20 **(f)** dpi. Arrowheads were added in image **(a)** to show nodules position. Note that the same scale has been used in all images to facilitate comparisons. Representative images of plates belonging to a time series experiment (n=5 biological replicates). Scale bar, 1 cm.

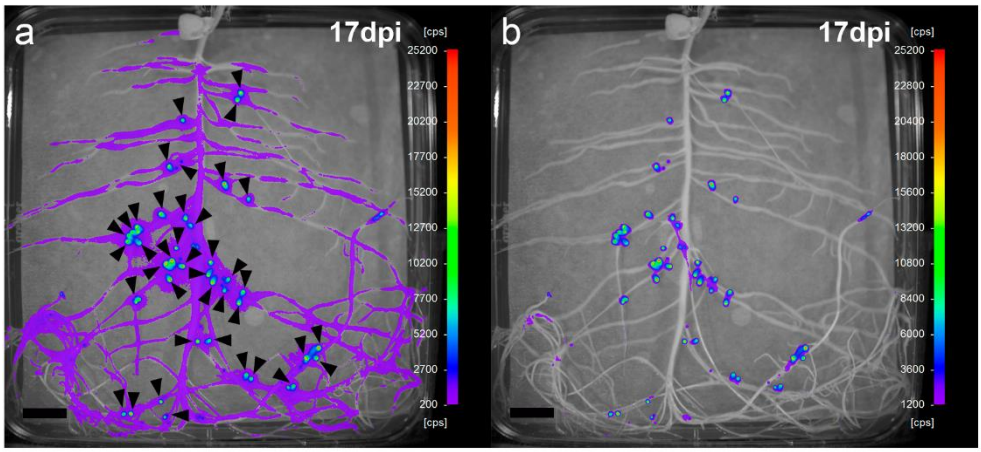

Fig. S1.6 *In vivo* spatial expression of the positive control strain D5250, constitutively expressing the *lux* construct in roots and nodules of pea plants. Luminescence is expressed in counts per second (cps). Panels **(a)** and **(b)** show the same plant analyzed using two different detection thresholds: 200 and 1200 cps, respectively. Images were acquired at 17 dpi. Arrowheads were added in image **(a)** to show nodule position. Representative images of plates belonging to a time series experiment (n=5 biological replicates). Scale bar, 1 cm.

Chapter 2

Proteomic and targeted metabolic analysis provide further insights about the role of symbiotic amino acid transport in pea nodules

Part 1

2.1.1 Introduction

Legume crops are important for productivity and soil maintenance in traditional farming systems thanks to their ability to carry out the process of biological nitrogen fixation (BNF) as the result of the establishment of a symbiotic relationship with bacteria, generically called rhizobia. The symbiosis occurs through the development, in roots or stems, of a new organelle called nodule, where BNF takes place. In the classical model of nutrient exchange between symbionts, plant supplies energy in the form of dicarboxylates in exchange for ammonium to the N_2-fixing bacteroids, the differentiated form of bacteria inside nodules (Udvardi & Poole, 2013). However, this classic model was challenged upon the observation that mutations in the general ABC amino-acid transporters AapJQMP and BraDEFGC in *Rhizobium leguminosarum* resulted in N starvation symptoms in both pea and bean plants (Lodwig et al., 2003; Prell et al., 2010). More specifically, the uptake of BCAAs (Val, Ile and Leu) from the plant by the bacteroid was found to be essential for an effective BNF in these legume species (Prell et al., 2010). These observations describe a situation of symbiotic auxotrophy in *R. leguminosarum*, in which bacteroids reduce the expression (Karunakaran et al., 2009) and activity of enzymes related to the biosynthesis of BCAAs, and this group of amino acids must be then supplied by the plant (Prell et al., 2009a). The hypothesis of symbiotic auxotrophy was further supported by the fact that bacterial mutants in the ammonium assimilatory enzymes GOGAT (*gltB)* and alanine dehydrogenase (*aldA*) are able to fix N_2 on pea plants only when carrying suppressor mutations that increase bacteroid amino acid uptake (Mulley et al., 2011). Thus, at least in *R. leguminosarum* species, bacteroids depend on the plant supply of BCAAs within nodules for the establishment of an effective BNF.

Another experimental approach to further understand the role of amino acid metabolism in nodules is the application of compounds that inhibit the biosynthesis of BCAAs in plant cells such as group B herbicides. Herbicides belonging to this group inhibit the action of the enzyme acetohydroxy acid synthase (AHAS), also known as acetolactate synthase, which is the first common enzyme in the BCAA biosynthesis pathway (Singh & Shaner, 1995). AHAS-inhibitors herbicides do not affect the growth of free-living bacteria at recommended doses, although they may have a detrimental effect on the

establishment of the symbiosis and therefore on BNF, depending on the plant species under study (Anderson et al., 2004; Farquharson PhD Thesis, 2009; González et al., 1996; Royuela et al., 1998; Zawoznik & Tomaro, 2005). Early work by Royuela et al. (1998) analysed the effect of imazethapyr, an AHAS-inhibitors herbicide, on nodulated peas. AHAS activity was around 6 times higher in bacteroids than in the plant fraction and around 20 times higher than in pea leaves. Upon the application of the herbicide, bacteroid AHAS was less sensitive than leaf AHAS. This fact, together with the higher AHAS activity found in nodules, was associated to the observed greater tolerance of nodulated plants to AHAS-inhibitors herbicides (García-Garijo et al., 2014; Royuela et al., 1998; Zawoznik & Tomaro, 2005).

In the present work, based on the current bacteroid amino-acid auxotrophy model, we hypothesized that blocking the plant BCAA biosynthetic pathway would have a negative effect on nodule functioning and/or N_2-fixing capacity of the bacteroids. To test this hypothesis, we applied the AHAS-inhibitors herbicide imazamox to pea plants nodulated with either a wild type *R. leguminosarum* bv. *viciae* strain 3841 or the double mutant *aap/bra* strain RU1722. Proteomic and targeted metabolic profiling of nodule samples allowed us to verify how the blockage of BCAA transport between symbionts had a greater effect on nodule metabolism than the inhibition of plant BCAA biosynthesis. We also analysed how different mechanisms could be responsible for the observed inhibition of BNF including the activation of plant defence mechanisms in both treatments.

2.1.2 Materials and methods

2.1.2.1 Bacterial strains and growth conditions

Strains *R. leguminosarum* bv. *viciae* 3841 (wild type, Str^r) (Johnston & Behringer, 1975) and RU1722 (*aapJQM*::ΩSp *braEF*::ΩTc) (Lodwig et al., 2003) were grown at 28°C in tryptone yeast (TY) agar or broth (Beringer, 1974). Antibiotics were added to the cultures at the following concentrations (μg ml^{-1}): streptomycin (Str), 500; spectinomycin (Sp), 100; tetracycline (Tc), 5.

2.1.2.2 Plant growth conditions

Pea (*Pisum sativum* var. Avola) seeds were surfaced sterilized in 70% EtOH (v/v) for 1 min, washed with deionized water and subsequently incubated in 2%

NaClO (v/v) for 5 min. Seeds were germinated on distilled water agar (0.8%, w/v) plates during 5 days in the dark at room temperature. Seedlings were then sown in 600 mL pots containing autoclaved vermiculite:perlite (2:5, v/v) and inoculated with the corresponding bacterial strains ($2{\cdot}10^7$ CFU/per seedling). Plants were grown during 1 month under controlled conditions (22 °C/18 °C day/night temperature, 60%/70% day/night relative humidity, and 12 h photoperiod), and watered to field capacity two times a week with sterile nitrogen-free nutrient solution (Rigaud & Puppo, 1975).

2.1.2.3 Microscopy

For microscopy observations, nodules were harvested and immediately embedded in 4% (w/v) agarose (Sigma-Aldrich, Spain). Longitudinal 100 µm-sections were cut with a vibratome (Lancer Series 1000). Sections were transferred to glass slides, kept under a cover slip in distilled water, and visualised using an Olympus BX40 optical microscope. Photographs were taken with an Olympus camera LC20 (Münster, Germany). Lugol staining was done by incubating sections for 3 min in 0.4% (v/v) lugol (Panreac Química SA, Spain).

2.1.2.4 Experimental design and physiological characterisation of herbicide treatment

Plants inoculated with *R. leguminosarum* 3841 or RU1722 strains were separated into two groups; ones were treated with the herbicide and the others were maintained under control conditions. One month-old plants were treated with the herbicide imazamox (Pulsar®40, BASF Española SA, Spain) added to the nutrient solution. Imazamox was applied at a final concentration of 100 µM. After four days of treatment plants were harvested.

To establish the effect of imazamox on plants, various parameters were measured 2 h after the beginning of photoperiod: stomatal conductance, leaf and nodule water potential, net photosynthesis, and chlorophyll content were measured in the youngest fully expanded leaf, while apparent nitrogenase activity (ANA) was measured in nodules.

Stomatal conductance was measured using an AP4 porometer (Delta-T Devices, UK). Ψ_{leaf} was measured using a pressure chamber (Scholander et al., 1965). Ψ_{nodule} was analysed using C52 sample chambers coupled to a Wescor HR-33T Dew Point Microvoltmeter (Wescor, USA). Net photosynthesis was measured in a LI-COR 6400 gas exchange portable photosynthesis system (LI-

COR, Lincoln, USA). Determinations were carried out at 25 °C. Photosynthetic assimilation (A) was estimated at a saturating photosynthetic photon flux density of 1200 μmolm^{-2} s^{-1} using equations developed by von Caemmerer & Farquhar (1981). Symbiotic nitrogen fixation was measured as in section 1.2.4.

2.1.2.5 Determination of *in vivo* AHAS activity

In vivo AHAS activity was measured as previously described Gaston et al. (2002). Pea leaves were excised, and petioles were immersed in deionized water or 1,1-cyclopropanedicarboxylic acid (CPCA). Leaves where then placed under light for 4 h allowing the absorption of CPCA via xylem (Lee & Owen, 2000). CPCA is an inhibitor of the KARI enzyme, reason why the incubation with CPCA allows the accumulation of acetolactate due to the AHAS activity. Deionized water was use to obtain the basal levels of acetolactate in leaves (Gerwick et al., 1993). Leaf samples were then weighed and frozen at -80 °C until acetolactate determination.

Acetolactate was extracted by washing the plant material with deionized water (1.5 mL/0.2 g fresh weigh) at 80 °C for 1 h. 120 μl of the sample was used for acetolactate determination by its conversion to acetoin by incubation with 0.4 N H_2SO_4 for 30 min at 60°C. Acetoin was determined according to Westerfeld (1945) adapted to a microplate reader. Napthol (25 mg/l) and creatine (5 mg/l) were dissolved in 2 N NaOH solution in the dark; 125 μl of this solution was added to each sample an incubated at 60 °C for 15 min. After centrifugation, absorbance was measured at 525 nm to determine acetoin content.

2.1.2.6 Integrative protein and metabolite extraction

Nodules (0.1 g fresh weight) were homogenized to a fine powder under liquid N_2 using a mortar and pestle. Aliquots were extracted twice in the presence of 400 μl of water:chloroform:methanol (3:5:12, v/v) and the two supernatants were combined. Chloroform (200 μl) and water (300 μl) were added, and the resulting mixture was centrifuged again. The upper water-methanol phase, containing metabolites, was collected, dried by vacuum concentration (Savant SPD111V Speed Vac®), and stored at -20 °C (adapted from Hacham et al., 2002) for further determinations of anions, organic acids and amino acids. Protein pellets were subjected to phenol extraction according to Wienkoop et al. (2008). Pellets were dissolved in 400 μl of protein extraction buffer (50 mM HEPES-KOH, 40% sucrose (w/v), 0.07% β-mercaptoethanol, pH

7.5). 3 volumes of TE-buffer (10 mM Tris, 1 mM EDTA-Na_2)-equilibrated phenol (Sigma-Aldrich, Spain) were added and shaken for 30 min at 4 °C. After centrifugation at 4,000 g and 4 °C for 8 min, the soluble proteins were dissolved in the upper phenolic phase. The phenolic phase was collected and proteins were precipitated overnight in 5 volumes of ice-cold acetone. After centrifugation at 4,000 g and 4 °C for 8 min, the pellets were washed 3 times with ice-cold acetone, air dried and finally dissolved in 6M Urea, Tris 100mM (pH 7.8). Resuspended proteins were stored at -80 °C until used for digestion and LC-MS/MS analyses. Protein quantitation was performed with the Bradford assay kit according to the manufacturer's instructions (Bio-Rad, USA).

2.1.2.7 In-gel protein digestion

20 µg of protein extract from each sample was diluted in Laemmli sample buffer and loaded into a 0.75 mm-thick polyacrylamide gel with a 4% stacking gel casted over a 12.5% resolving gel. The run was stopped as soon as the front entered 3 mm into the resolving gel so that the whole proteome became concentrated in the stacking/resolving gel interface. Bands were stained with Coomassie Brilliant Blue and excised from the gel. Protein enzymatic cleavage was carried out with trypsin (Promega, Spain; 1:20, w/w) at 37 °C for 16 h as previously described (Shevchenko et al., 2006). Purification and concentration of peptides was performed using C18 Zip Tip Solid Phase Extraction (Millipore, Spain).

2.1.2.8 Mass spectrometry analysis

Peptides mixtures were separated by reverse phase chromatography using an Eksigent nanoLC ultra 2D pump fitted with a 75 µm ID column (Eksigent 0.075 x 150). Samples were first loaded for desalting and concentration into a 0.5 cm length 300 µm ID precolumn packed with the same chemistry as the separating column. Mobile phases were 100% water 0.1% formic acid (FA) (buffer A) and 100% Acetonitrile 0.1% FA (buffer B). Column gradient was developed in a 200 min gradient from 2% B to 40% B in 180 min. Column was equilibrated in 95% B for 9 min and 2% B for 10 min. During all the process, the precolumn was in line with the column and flow was maintained all along the gradient at 300 nl min^{-1}. Eluting peptides from the column were analysed using a Sciex 5600 Triple-TOF+ system. Information data acquisition was acquired upon a survey scan performed in a mass range from 350 m/z up to 1250 m/z in a scan time of 250 ms. Top 35 peaks were selected for fragmentation. Minimum

accumulation time for MS/MS was set to 100 ms giving a total cycle time of 3.8 s. Product ions were scanned in a mass range from 230 m/z up to 1500 m/z and excluded for further fragmentation during 15 s.

2.1.2.9 Peptide identification and quantification

MS/MS data acquisition was performed using Analyst 1.7 (Sciex) and spectra files were processed through Protein Pilot™ Software (v.4.0.8085-Sciex) using Paragon™ Algorithm (v.4.0.0.0) (Shilov et al., 2007) for database search, Progroup™ for data grouping, and searched against the concatenated target-decoy database provided by Dr. Stefanie Wienkoop from University of Vienna. False discovery rate was performed using a non-lineal fitting method (Tang et al., 2008) and displayed results were those reporting a 1% Global false discovery rate or better.

The peptide quantification was performed using the Progenesis LC–MS software (ver. 4.0, Waters). Using the accurate mass measurements from full survey scans in the TOF detector and the observed retention times, runs were aligned to compensate for between-run variations in our nanoLC separation system. To this end, all runs were aligned to a reference run automatically chosen by the software, and a master list of features considering m/z values and retention times was generated. The quality of these alignments was manually supervised with the help of quality scores provided by the software. The peptide identifications were exported from Protein Pilot and imported in Progenesis LC–MS where they were matched to the respective features.

Output data files were managed using Perseus software (version 1.6.5.0) (Tyanova et al., 2016) for subsequent statistical analyses and data visualization. Identifications from the reverse database, common contaminants and proteins only identified through a modification peptide were removed. Label-free intensities were then logarithmized (base 2) and the samples were then grouped according to the replicates defined in the experimental design. At least three valid values across the four replicates were required for each identified protein. Following the Perseus analysis pipeline, empty values were imputed with random numbers from a normal distribution to simulate low abundance values below the detection limit of the instrument. A multiple sample test (ANOVA, $p < 0.05$) was then applied to identify specific hits over experimental conditions. Statistically different proteins were then transformed with Z-score normalization in order to represent heatmaps and cluster aggrupations. Venn diagrams were generated using Venny 2.1 software

(http://bioinfogp.cnb.csic.es/tools/venny/). AgriGO tool was used to get an overview of Gene Ontology (GO) functional term enrichment of differentially accumulated plant proteins in pea nodules (Du et al., 2010). To do this, we first had to convert *Pisum sativum* accessions into Uniprot accessions of *Glycine max*.

2.1.2.10 Anions and organic acid determination

Anions and organic acids were determined by ion-exchange chromatography in a 940 Professional IC Vario (Metrohm AG, Switzerland) by gradient separation with a Metrosep A Supp16 150/4.0 column. Samples were eluted with solvent A (deionized water) and solvent B (20 mM Na_2CO_3 + 2.5 mM NaOH) at 55 °C according to the manufacturer's instructions.

2.1.2.11 Amino acid determination

For free amino acid determination samples were derivatised and analysed using a MDQ Plus capillary electrophoresis system (Ab Sciex, Spain) coupled to laser-induced fluorescence detection at 488 nm, as previously described (Gil-Quintana et al., 2013a). Analyses were performed at 20ºC and at a voltage of 30 kV. Total free amino acids were calculated as the summation of single amino acids for each sample, with the exception of cysteine and proline, which could not be quantified using this protocol.

2.1.2.12 Statistical analysis

Physiological measurements and metabolite content data are presented as the mean ± standard error of at least 4 independent biological replicates. Results were analysed using one-way ANOVA tests after checking the normal distribution of the samples via the Shapiro-Wilk test and the homogeneity of variances via Levene's test, and applying a log transformation of the data in the case of no homogeneity. Comparisons between each treatment were performed using least significant difference (LSD) or Dunnet T3 tests. Differences were considered to be significant at $p \leq 0.05$.

2.1.3 Results

2.1.3.1 Physiological characterization of plants inoculated with the *aap/bra* double mutant and treated with imazamox

Pea plants inoculated with *R. leguminosarum* bv. *viciae* RU1722 (*aapJ braEF*) presented lower shoot dry weights and chlorophyll content (Table 2.1.1) compared to plants inoculated with the wild-type strain 3841 (Fig. 2.1.1A). Likewise, they presented a greater number of smaller and pale pink nodules than plants inoculated with the wild type (Figs. 2.1.1B-C). Despite not present apparent differences in infection (Figs. 2.1.1D-E), lugol-staining showed a large accumulation of starch in RU1722 nodules compared to wild-type nodules (Figs. 2.1.1F-I). Plants inoculated with RU1722 also showed a greater stomatal closure that those inoculated with 3841 (0.38 ± 0.03 vs. 0.83 ± 0.04 cm s^{-1}, respectively) (Table 2.1.1). Additionally, plants presented a decline in net photosynthesis in peas inoculated with RU1722 strain compared to 3841 (4.64 ± 0.44 vs. 14.97 ± 0.53 μmol m^2 s^{-1}, respectively) (Table 2.1.1). Although RU1722 nodules showed the classical indeterminate nodule structure as observed in plants inoculated with the wild type, the *aap/bra* double mutation had a great impact on BNF rates, leading to an 81% decline on ANA, compared to wild-type nodules (Table 2.1.1).

To further understand how nodule metabolism and BNF were affected, on the one hand, by the blockage of amino acid transport between symbionts, and on the other, by the inhibition of BCAA biosynthesis, peas inoculated with both strains were subjected to imazamox treatment. Based on preliminary experiments with different doses and exposure times to the herbicide, we decided to treat the plants with a dose 10 times higher than the recommended dose for alfalfa, and an exposure time of four days. Since imazamox relies on the inhibition of AHAS enzymatic activity, we tested the effectiveness of the herbicide treatment by measuring AHAS activity in pea leaves. Results showed significant declines in AHAS activity in leaves of herbicide-treated peas inoculated with either 3841 or RU1722 strains (-96 and -82%, respectively) (Table 2.1.1). Interestingly, plants grown with the symbiotic *aap/bra* double mutant showed lower AHAS activity compared to plants inoculated with the wild-type strain (79.38 ± 12.58 vs. 24.93 ± 4.36 nmol acetolactate g^{-1} h^{-1}) (Table 2.1.1). Moreover, the herbicide treatment caused a reduction in shoot dry weight in plants inoculated with 3841 (-40%), while this parameter was not

affected in plants inoculated with RU1722 (Table 2.1.1). Nodule dry weight was only affected in herbicide-treated RU1722 nodules (Table 2.1.1). Regardless of the microsymbiont, imazamox caused a significant decrease in chlorophyll content, stomatal conductance and net photosynthesis (Table 2.1.1). The herbicide had a negative impact in the nitrogen fixation levels of plants inoculated with the wild-type strain, but there was no effect in plants inoculated with the double mutant, already showing relatively low ANA values (Table 2.1.1).

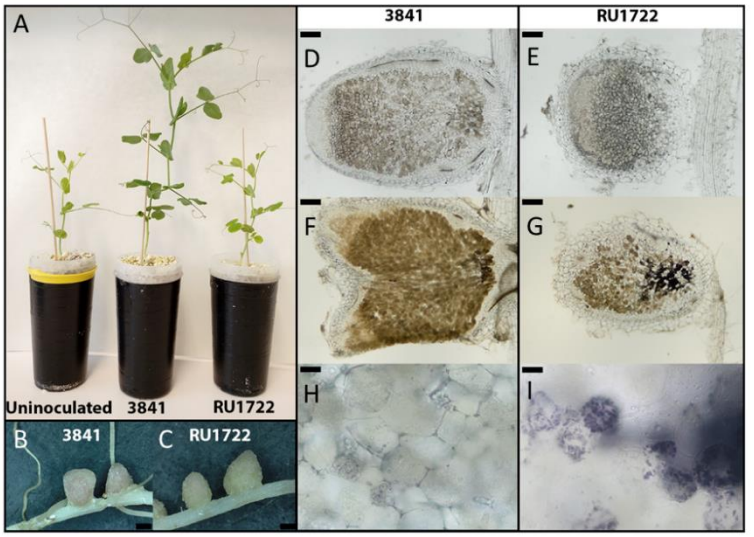

Fig. 2.1.1 Effect of the mutation of the Aap/Bra transport systems in *R. leguminosarum* bv. *viciae* on growth and nodulation of pea plants. **(A)** Plants (*Pisum sativum* var. Avola) were inoculated with *R. leguminosarum* bv. *viciae* strains 3841 (wild type) (centre) or RU1722 (*aap/bra*) (right), or uninoculated (left), and grown for 1 month. **(B-C)** One month-old 3841 and RU1722 nodules, respectively. **(D-E)** 100-µm vibratome sections of 3841 and RU1722 nodules, respectively. **(F-I)** lugol stained sections of 3841 and RU1722 nodules, respectively. **(B-G)** scale bars, 200 µm. **(H-I)** scale bars, 20 µm.

Table 2.1.1 Plant physiological responses of pea plants inoculated with *R. leguminosarum* bv. *viciae* strains 3841 (wild type) or RU1722 (*aap/bra*) upon herbicide treatment. Values represent mean ± SE (n ≥ 4 biological replicates). For each parameter, numbers followed by a different letter are significantly different at p ≤ 0.05 in a LSD/T3 Dunnet test. NDW, nodule dry weight. LFW, leaf fresh weight.

	3841		RU1722	
Parameter (units)	**Control**	**Imazamox**	**Control**	**Imazamox**
Shoot dry weight (g)	0.44 ± 0.02 a	0.26 ± 0.05 b	0.26 ± 0.03 b	0.20 ± 0.01 b
Nodule dry weight (g)	0.05 ± 0.01 a	0.04 ± 0.01 ab	0.05 ± 0.01 a	0.03 ± 0.01 b
Chlorophyll content (SPAD)	43.98 ± 0.63 a	38.76 ± 1.47 b	31.00 ± 0.65 c	25.72 ± 2.00 d
Stomatal conductance (cm s^{-1})	0.83 ± 0.04 a	0.60 ± 0.07 b	0.38 ± 0.03 c	0.12 ± 0.03 d
Photosynthesis (µmol m^{-2} s^{-1})	14.97 ± 0.53 a	5.44 ± 0.76 b	4.64 ± 0.44 b	2.00 ± 0.42 c
ANA (µmol H_2 g NDW^{-1} min^{-1})	1.13 ± 0.16 a	0.26 ± 0.08 b	0.21 ± 0.02 b	0.26 ± 0.02 b
AHAS activity (nmol acetolactate g LFW^{-1} h^{-1})	79.38 ± 12.58 a	3.31 ± 0.20 c	24.93 ± 4.36 b	4.37 ± 0.73 c

2.1.3.2 Changes in amino acid metabolism modified the proteome profile of pea nodules

To get an overview of the impact of both the *aap/bra* double mutation and the inhibition of plant BCAA biosynthesis, nodules were analysed by LC-MS/MS proteomics. From the 2,525 proteins identified, 1,197 proteins were used for the quantitative analysis (corresponding to 1,149 proteins from plant fraction and 48 from bacteroid; Table S2, see attached CD). GO enrichment analysis for quantified proteins of nodule plant fraction highlighted the importance in nodules metabolism of biological process such as nodulation, nitrogen fixation, flavonoid biosynthetic process, organic acid metabolic process or generation of precursor metabolites and energy, among others (Fig. S2.1.2). Firstly, we performed a principal component analysis (PCA) including all quantified proteins (Fig. 2.1.2A). PCA differentiated four groups within the data, two groups corresponding to control samples, and the two other groups corresponding to the herbicide treatment (Fig. 2.1.2A). Then, hierarchical cluster analysis was used to group the quantified proteins based on similar accumulation patterns. The hierarchical clustering yielded six major groups of proteins (Fig. 2.1.2B). Results highlight the major effect of *aap/bra* double mutation over nodule proteome since two of the clusters correspond to proteins with greater or lower abundance in RU1722 nodules compared to 3841 nodules regardless the herbicide treatment [417 (turquoise cluster) and 116 proteins (light blue cluster), respectively] (Fig. 2.1.2B). The second major cluster (green), which shows the effect of the herbicide, includes 187 proteins with greater abundance in imazamox-treated nodules (Fig. 2.1.2B).

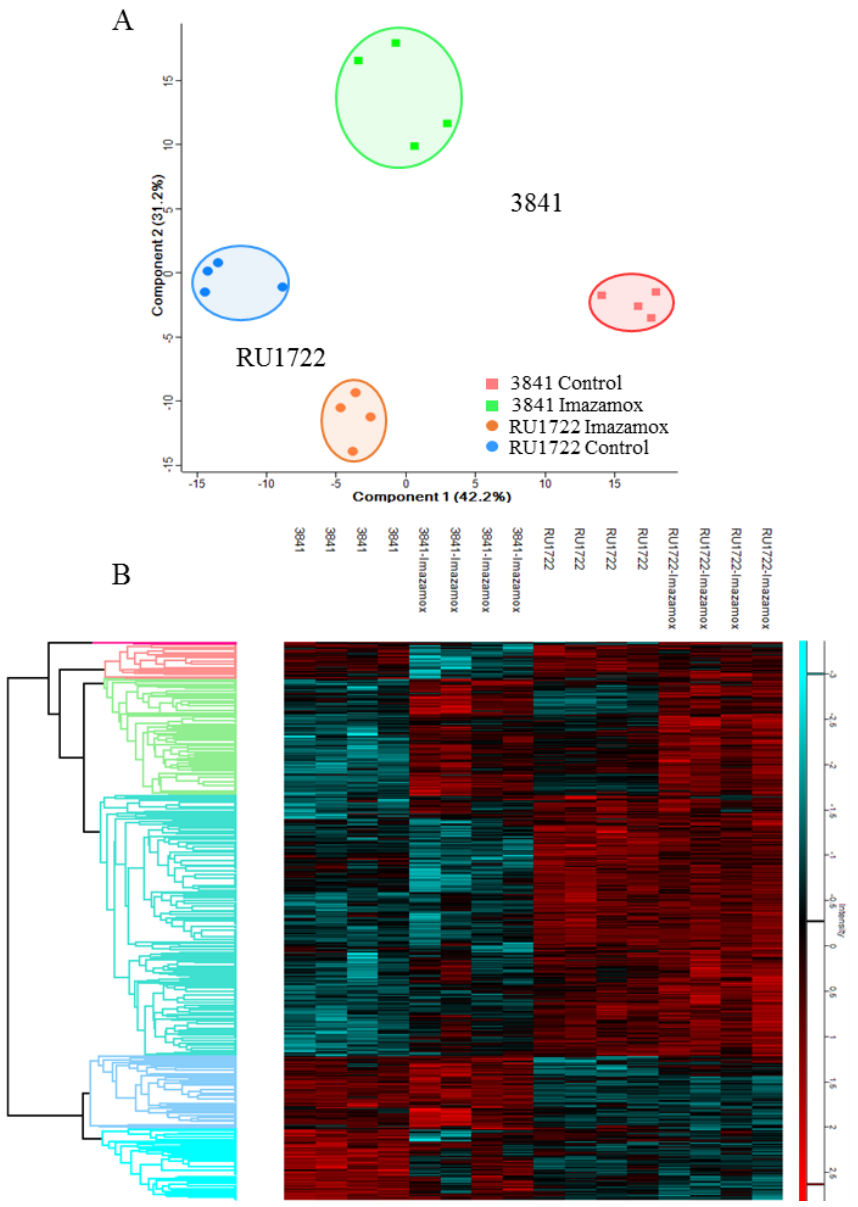

Fig. 2.1.2 Analysis of the quantitative changes in the nodule proteome of pea plants inoculated with *R. leguminosarum* bv. *viciae* strains 3841 (wild type) and RU1722 (*aap/bra*) and subjected to imazamox treatment. **(A)** Principal Component Analysis (PCA) of all the quantified proteins. Components 1 and 2 represented 74% of the total variance observed. Control 3841 nodules: pink squares; imazamox treated 3841 nodules: green squares; control RU1722 nodules: orange circles; imazamox treated RU1722 nodules: blue circles. **(B)** Heatmap representing 892 proteins found to significantly change in abundance in pea nodules with *R. leguminosarum* bv. *viciae* 3841 and RU1722 with or without imazamox treatment (ANOVA, $p < 0.05$).

To identify the main biological processes involved in these responses, we carried out a comparative proteomic approach. From the total set of quantified proteins, 466 were found to significantly change in abundance due to the *aap/bra* double mutation (fold change of < 0.77 or > 1.3, Student's *t*-test, $p < 0.05$), which was the treatment with the highest impact on the protein profiles of the samples (Figs. 2.1.3A and S2.1.1A). Go enrichment analysis of proteins differentially accumulated in 3841 vs. RU1722 nodules revealed the relevance of processes such as: flavonoid biosynthetic process, organic acid metabolic process, or interspecies interaction between organisms, highlighting nodulation subcategory (Fig. S2.1.3). Moreover, functional analysis of the data showed that *aap/bra* double mutation caused the accumulation of 321 proteins, most of them with functions related to cellular processes, response to stress and regulation of gene expression (Figs. 2.1.3B and S2.1.1A). Some of the proteins showing the largest changes in abundance included an L-allo-threonine aldolase-like protein (34.1-fold increase), as well as a defensin-like and a disease resistance response proteins (Fig. 2.1.3B, Table 2.1.2). Similarly, transport was one of the most representative categories overrepresented, including a mitochondrial import inner membrane translocase, two ferritins, a non-specific lipid-transfer protein, a metal-nicotianamine transporter or a sugar transport protein (Fig. 2.1.3B, Table 2.1.2). The *aap/bra* double mutation also caused an accumulation of proteins related to proteolysis such as a thiol protease aleurain or senescence-specific cysteine protease, related in turn to hormone responses (Fig. 2.1.3B, Table 2.1.2). On the other hand, the double mutant caused a reduction in the abundance of 145 proteins compared with wild-type nodules (Figs. 2.1.3B and S2.1.1A). A late embryogenesis abundant protein, which is involved in stress responses, was the protein showing the largest decline in abundance (0.07-fold) in RU1722 compared to wild-type nodules (Fig. 2.1.3B, Table 2.1.2). The levels of GOGAT and sucrose synthase (SuSy), essential proteins in carbohydrate and nitrogen nodule metabolism, respectively, were also reduced in *aap/bra* nodules (Fig. 2.1.3B, Table 2.1.2). Regarding changes in the bacteroid proteome, the abundance of glutamine synthetase (GS), one of the key enzymes for ammonium assimilation, was increased in *aap/bra* bacteroids (Fig. 2.1.3B, Table 2.1.2). In contrast, several proteins related to nitrogen fixation and bacteroid metabolism were reduced due to the *aap/bra* double mutation, including a ferredoxin-like protein and FixC, citrate synthase, C4-dicarboxylate transport protein or alanine dehydrogenase (Fig. 2.1.3B, Table 2.1.2).

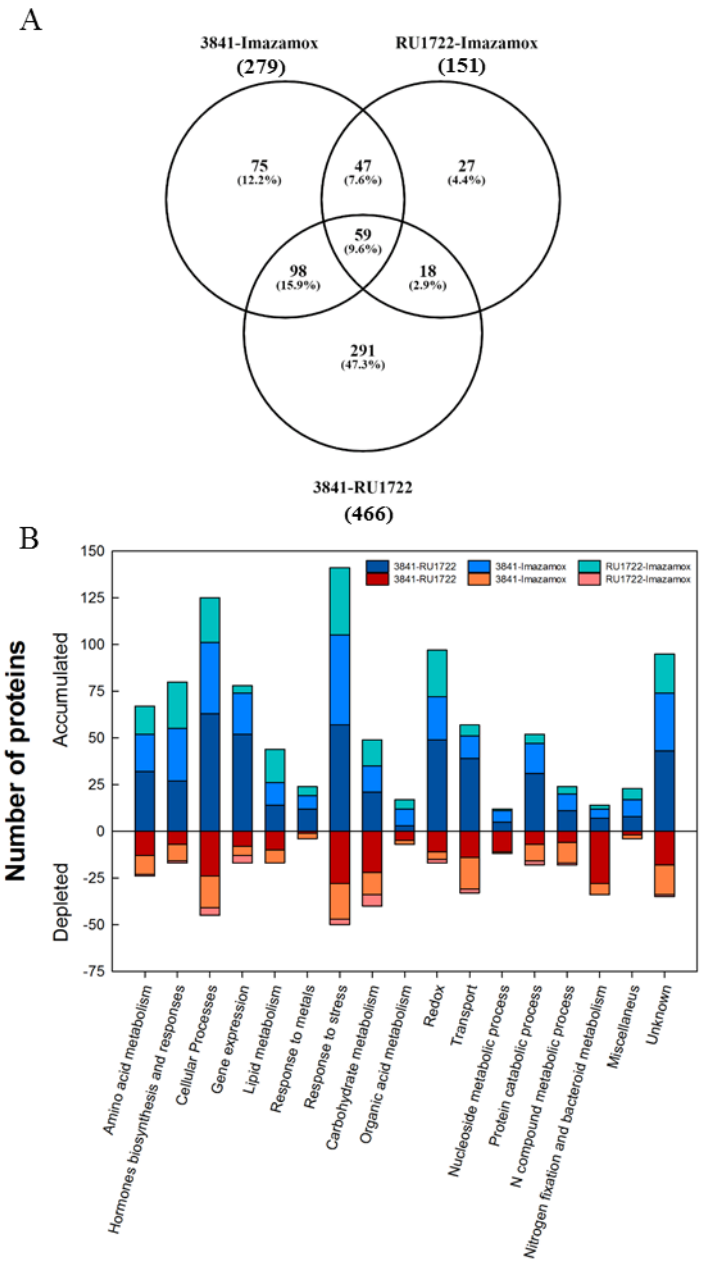

Fig. 2.1.3 (A) Venn diagram including proteins of pea nodules with *R. leguminosarum* bv. *viciae* strains 3841 (wild type) and RU1722 (*aap/bra*) and subjected to imazamox treatment with a fold change of < 0.77 or > 1.3 and a *t*-test p < 0.05 on a linear scale. **(B)** Functional classification of the differentially accumulated nodular proteins based on the Gene Ontology Annotation Database (*t*-test p < 0.05, fold change of < 0.77 or > 1.3) and number of proteins within each comparison.

Table 2.1.2 Differentially accumulated proteins in pea nodules inoculated with *R. leguminosarum* bv. *viciae* RU1722 (*aap/bra*) compared to 3841 nodules (wild type). Proteins from bacteroids in bold. Proteins with a fold change of ≤ 0.5 or > 3 and a *t*-test p < 0.05 on a linear scale.

Description	Uniprot Code	Fold Change	p-value
Increased abundance			
L-allo-threonine aldolase-like protein	G7I6S7	34.10	2.54E-03
Embryo-specific protein	I3SJT1	28.83	1.20E-02
Thiol protease aleurain-like	Q8RWQ9	19.87	1.89E-03
Somatic embryogenesis receptor kinase	Q94AG2	13.19	1.46E-02
Cysteine proteinase inhibitor	Q06445	8.21	3.92E-04
Mitochondrial import inner membrane translocase	Q6NKU9	6.79	3.75E-03
Cellulose synthase-like protein	Q570S7	6.50	7.31E-03
Defensin-like protein	Q9FFP8	5.93	7.09E-04
Glutamine synthetase	A8VID1	5.81	2.38E-06
Disease resistance response protein	P13240	5.25	5.75E-06
Endo-1,3;1,4-beta-D-glucanase	Q9ZT66	5.03	2.20E-06
Ferritin	A0A1S2XL47	4.58	1.58E-08
Senescence-specific cysteine protease SAG39	A2XQE8	4.19	2.18E-03
Non-specific lipid-transfer protein	A0A1S2XFK0	4.05	1.51E-02
S1/P1 nuclease family protein	G7ID90	3.95	7.53E-05
Ferritin-1	P19976	3.90	1.24E-07
Glycine-rich protein	Q41652	3.81	1.03E-04
Probable metal-nicotianamine transporter	Q6R3K6	3.70	6.82E-04
Ribonuclease	P42815	3.57	3.43E-02
Sugar transport protein	Q94AZ2	3.46	1.83E-04
2-methylene-furan-3-one reductase	Q84V25	3.30	2.53E-03
Glutathione S-transferase	Q1RSI2	3.21	1.31E-02
Plant cysteine oxidase 5	Q9LXT4	3.20	9.22E-04
60S ribosomal protein L7-4	Q9LHP1	3.08	1.72E-04
60S ribosomal protein L35a-3	I3T9M0	3.05	3.15E-04
UBP1-associated protein 2C	Q9LK44	3.02	5.20E-03
Reduced abundance			
Copper transport protein	Q94BT9	0.50	3.07E-02
RNA polymerase sigma factor RpoH	Q3S3D9	0.50	8.94E-06
Alanine dehydrogenase	Q9RLB2	0.49	3.53E-05
Fatty acid amide hydrolase	Q7XJJ7	0.49	2.00E-02

Protein FixC	P09821	0.49	2.57E-05
Succinyl-CoA synthetase beta sub-unit	O33526	0.49	1.99E-05
Inactive protein RESTRICTED TEV MOVEMENT	D9UC01	0.48	5.22E-05
C4-dicarboxylate transport protein	Q01857	0.47	8.14E-06
Probable inactive ATP-dependent zinc metalloprotease	A8MPR5	0.47	2.76E-03
Annexin-like protein	P51074	0.46	1.91E-02
Citrate synthase	Q14SJ8	0.46	5.03E-05
Tripeptidyl-peptidase	F4JVN6	0.46	4.11E-05
Probable carboxylesterase	Q9FG13	0.46	1.42E-03
ER membrane protein complex subunit	A0A1S2XSE8	0.45	9.01E-04
Allergenic isoflavone reductase-like protein Bet protein	I3SC37	0.44	7.76E-07
Ferredoxin-like protein	Q52776	0.40	3.24E-05
Thioredoxin-like protein	G7J1S6	0.33	4.17E-03
Ornithine aminotransferase	B1A0U3	0.32	1.72E-02
Protein TONSOKU	Q6Q4D0	0.29	3.58E-05
UPF0160 protein C27H6.8	A0A1S2YLJ2	0.28	9.20E-07
Sucrose synthase	Q9T0M9	0.28	4.58E-08
Glutamate synthase	Q03460	0.27	5.21E-07
Thiosulfate sulfurtransferase	Q9FKW8	0.18	2.47E-07
Late embryogenesis abundant protein	G7L6Q3	0.07	2.78E-02

Regarding the response to the application of imazamox, 279 proteins changed abundance in response to the herbicide in 3841 nodules (fold change of < 0.77 or > 1.3, Student's *t*-test, $p < 0.05$) (Figs. S2.1.1B and 2.1.3A). Nodules treated with imazamox were enriched in proteins related to secondary metabolic or flavonoid biosynthetic processes, among others (Figs. S2.1.4 and S2.1.5). Imazamox increased the abundance of 186 proteins, mainly related to stress response, cellular processes and hormone biosynthesis and responses (Figs. S2.1.1B and 2.1.3B). These include several pathogenesis related proteins (PRs), as detoxification-related proteins such as cytochrome P450, three glutathione S-transferases, and a putative 1-aminocyclopropane-1-carboxylate oxidase, among others (Table 2.1.3). An anthranilate N-methyltransferase, which is involved in anthranilate catabolism, was the protein with the highest fold change (63.8-fold; Table 2.1.3). Proteins with a reduced abundance due to the imazamox treatment (93) mainly belong to response to stress, cellular processes and transport functional categories (Figs. S2.1.1B and 2.1.3B). A Lb, responsible of preventing

the inactivation of nitrogenase by O_2, was the protein most negatively affected by the herbicide (0.09-fold) (Table 2.1.3). Imazamox also reduced the abundance of other proteins related to the symbiotic process including proteins involved in flavonoid biosynthesis, an early nodulin-16 as well as the bacteroid protein NifA, with a key regulatory role for the expression of genes related to nitrogen fixation (*fix* and *nif* genes) (Table 2.1.3).

Table 2.1.3 Differentially accumulated proteins in pea nodules inoculated with *R. leguminosarum* bv. *viciae* 3841 under imazamox treatment. Proteins from bacteroids in bold. Proteins with a fold change of ≤ 0.5 or > 3 and a *t*-test $p < 0.05$ on a linear scale.

Description	Uniprot Code	Fold Change	p-value
Increased abundance			
Anthranilate N-methyltransferase	A9X7L0	63.76	7.27E-05
Pathogenesis-related protein STH-2	P17642	48.18	2.49E-05
Cysteine proteinase inhibitor	Q06445	35.83	3.68E-05
Embryo-specific protein	I3SJT1	32.68	1.12E-02
Disease resistance response protein	P13240	21.99	1.64E-07
Senescence-specific cysteine protease SAG39	A2XQE8	21.34	6.26E-06
Cytochrome P450 family protein	G7L8J9	13.40	1.69E-04
Thaumatin	A0A089WUI5	11.71	9.01E-05
Glucan endo-1,3-beta-glucosidase	Q03467	11.09	7.61E-04
Glutathione S-transferase	Q1RSI2	9.34	1.41E-03
1-aminocyclopropane-1-carboxylate oxidase	P31239	9.21	3.93E-07
Isoflavone reductase	P52576	9.11	2.36E-05
Phenylcoumaran benzylic ether reductase-like protein	B7FHV0	8.99	2.58E-05
Isoliquiritigenin 2'-O-methyltransferase	P93324	7.74	3.27E-03
Class-10 pathogenesis-related protein	P93333	6.24	4.05E-04
Pathogenesis-related protein bet V I family protein	G7JLL1	5.47	2.67E-05
Thiol protease aleurain-like	Q8RWQ9	5.24	3.24E-02
Salicylic acid-binding protein	Q6RYA0	5.09	2.10E-04
Vestitone reductase	Q40316	4.38	1.00E-06
Glutathione S-transferase	B7FN12	4.06	1.03E-03
DNA-damage-repair/toleration protein	Q00874	3.68	7.87E-03
Non-symbiotic hemoglobin	Q9FVL0	3.65	4.39E-04
Seed linoleate 9S-lipoxygenase	P24095	3.64	1.21E-03
Chalcone--flavonone isomerase	P28012	3.62	2.15E-04

12-oxophytodienoate reductase-like protein	G7K3S2	3.56	3.77E-04
Endochitinase	P21226	3.48	7.33E-04
Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase	P81898	3.34	5.78E-03
Mitochondrial import inner membrane translocase	Q6NKU9	3.29	2.93E-02
Senescence-specific cysteine protease SAG39	A2XQE8	3.18	2.13E-05
Phenazine biosynthesis PhzC/PhzF family protein	B7FJ84	3.11	2.36E-05
Glutathione S-transferase zeta class	P57108	3.11	1.66E-03
Reduced abundance			
Glucose-1-phosphate adenylyltransferase	Q43816	0.50	4.59E-04
Brefeldin A-inhibited guanine nucleotide-exchange protein	F4IXW2	0.50	3.37E-02
Methyl-CpG-binding domain-containing protein	Q9XI36	0.49	5.36E-03
Thiamine thiazole synthase	A0A072W0W5	0.48	4.44E-04
NifA regulatory protein	Q99329	0.47	1.80E-03
Probable sulfate transporter	Q94LW6	0.45	1.61E-03
Aspartic proteinase nepenthesin-like protein	G7J557	0.43	3.48E-04
DNA topoisomerase 3-alpha	Q9LVP1	0.41	2.17E-02
Granule-bound starch synthase	Q00775	0.40	1.52E-03
Protein argonaute	Q9ZVD5	0.39	1.27E-05
STS14 protein	Q41495	0.36	8.58E-03
Probable thimet oligopeptidase	F4HTQ1	0.35	6.25E-03
Early nodulin-16	P93328	0.35	5.35E-03
ATP sulfurylase	Q9LIK9	0.32	1.43E-02
Probable polygalacturonase	A7PZL3	0.28	5.76E-03
Amine oxidase	Q9SXW5	0.27	2.17E-05
Leghemoglobin	P93848	0.09	1.93E-02

In contrast to the impact of the herbicide on wild-type nodules, the proteomic profile of RU1722 nodules was less affected by the imazamox treatment with fewer proteins changing in relative abundance (151; fold change of < 0.77 or > 1.3, Student's *t*-test, p < 0.05) (Figs. 2.1.3A and S2.1.1C). Interestingly, in contrast to 3841 treated nodules, there was no enrichment in nitrogen fixation or nodulation categories in RU1722 nodules under imazamox (Figs. S2.1.4 and S2.1.5). Similarly to wild-type nodules, imazamox increased the abundance of proteins related to stress response, including PRs, redox and hormone biosynthesis and responses (Figs. 2.1.3B and S2.1.1C, Table 2.1.4). Imazamox also increased the abundance of proteins involved in the oxylipin biosynthesis such as a seed linoleate 9S-lipoxygenase and an alpha-dioxygenase (Table 2.1.4). Interestingly, an ornithine aminotransferase, involved in arginine and proline biosynthesis, accumulated due to herbicide in RU1722 nodules, but not in 3841 nodules (Tables 2.1.3 and 2.1.4). Proteins showing a reduction in relative abundance in imazamox-treated nodules mainly belong to carbohydrate metabolism, cellular processes and gene expression functional categories (Figs. 2.1.3B and S2.1.1C). A somatic embryogenesis receptor kinase and a cysteine oxidase were found with a fold change greater than 0.5 (Table 2.1.4). Interestingly, the former was also accumulated in RU1722 nodules compared to wild type nodules (Table 2.1.2).

Table 2.1.4 Differentially accumulated proteins in pea nodules inoculated with *R. leguminosarum* bv. *viciae* RU1722 under imazamox treatment. Proteins with a fold change of ≤ 0.5 or > 3 and a *t*-test p < 0.05 on a linear scale.

Description	Uniprot Code	Fold Change	p-value
Increased abundance			
Pathogenesis-related protein STH-2	P17642	26.43	4.98E-03
Anthranilate N-methyltransferase	A9X7L0	18.14	7.30E-06
Senescence-specific cysteine protease SAG39	A2XQE8	10.36	1.30E-04
Glucan endo-1,3-beta-glucosidase	Q03467	9.02	3.26E-04
Seed linoleate 9S-lipoxygenase	P24095	7.23	9.26E-05
Cytochrome P450 family protein	G7L8J9	6.96	6.12E-05
Thaumatin	A0A089WUI5	5.74	3.50E-06
1-aminocyclopropane-1-carboxylate oxidase	P31239	5.43	8.90E-06
Cysteine proteinase inhibitor	Q06445	4.70	3.48E-04
Disease resistance response protein	P13240	4.35	8.58E-05
Isoflavone reductase	P52576	4.34	6.83E-05
Alpha-dioxygenase	Q9SGH6	4.24	1.33E-04
Class-10 pathogenesis-related protein	P93333	4.18	7.98E-05
Ornithine aminotransferase	B1A0U3	4.17	3.33E-03
Annexin-like protein	P51074	4.02	1.01E-04
Non-symbiotic hemoglobin	Q9FVL0	3.36	5.18E-05
Flavonoid glucosyltransferase	A0A072UML8	3.29	1.23E-04
3-hydroxy-3-methylglutaryl coenzyme A synthase	G7KER8	3.22	1.40E-06
Soyasapogenol B glucuronide galactosyltransferase	D4Q9Z4	3.17	8.83E-06
Beta-amylase	O64407	3.16	5.81E-05
Glutathione S-transferase	Q1RSI2	3.13	1.13E-03
Embryo-specific protein	I3SJT1	3.10	3.63E-04
Phenylcoumaran benzylic ether reductase-like protein	B7FHV0	3.03	4.60E-04
Reduced abundance			
Plant cysteine oxidase	Q9LXT4	0.46	1.34E-02
Somatic embryogenesis receptor kinase	Q94AG2	0.41	5.00E-02

2.1.3.3 The *aap/bra* double mutation reduced the level of free amino acids while an increase was observed due to the imazamox treatment

Free amino acid content was measured in 3841 and RU1722 nodules in order to know the effect of mutation of the two broad specificity amino acid ABC transporters, AapJQMP and BraDEFG. The *aap/bra* double mutation caused a 73% decrease in total free amino acid content compared to wild type nodules (280.95 vs. 76.23 μmol g NDW^{-1}) (Fig 2.1.4A). Asparagine (Asn), the most abundant amino acid, decreased by 82% due to the *aap/bra* double mutation (Fig 2.1.4B). Regarding the other amino acids involved in primary N assimilation, the concentration of aspartic acid (Asp) and glutamine (Gln) decreased by 78.5 and 41% in RU1722 nodules compared with 3841 nodules, respectively (Figs. 2.1.4C, D). Glutamic acid (Glu) content was 27.25 μmol g NDW^{-1} in 3841 nodules, decreasing to 12.34 μmol g NDW^{-1} in RU1722 nodules (Fig. 2.1.4E). Regarding BCAAs, the *aap/bra* double mutation caused a 51% decline in isoleucine (Ile) and valine (Val), while leucine (Leu) showed a 1.6-fold increase compared to 3841 nodules (Figs. 2.1.4F-H). In the case of aromatic amino acids, tyrosine (Tyr) and tryptophan (Trp) content increased by 2.1 and 2.7-fold in RU1722 nodules, while phenylalanine (Phe) decreased by 20% in RU1722 compared to 3841 nodules (Figs. 2.1.4I-K). Other amino acids that were also affected by the *aap/bra* double mutation were threonine (Thr), alanine (Ala), serine-glycine (Ser-Gly), histidine (His), and γ-aminobutyric acid (GABA), which experienced a 55, 44.5, 33, 70.5, and 29.5% decline, respectively, compared to wild-type nodules (Fig. S2.1.6). In contrast, there was no effect on metionine (Met), lysine (Lys) and arginine (Arg) (Fig. S2.1.6).

Regarding the inhibition of BCAA biosynthesis, the imazamox treatment caused a 63 and 54% increase in total free amino acid content in 3841 and RU1722 nodules, respectively (Fig. 2.1.4A). There was also an increase in the concentration of amino acids involved in primary N assimilation, both in group amide (Asn, Gln) and acidic (Asp, Glu), highlighting the 3.4-fold increase in Gln content in 3841 nodules (Figs. 2.1.4B-E). The effect of imazamox on BCAAs was greater in 3841 nodules where it was observed a 4.5, 5.4 and 3.4-fold increase in Ile, Leu and Val content compared to its control (Figs. 2.1.4F-H). Although smaller, the herbicide also produced an increase in Ile and Leu content (2.18 and 1.81-fold, respectively) in RU1722 nodules (Figs. 2.1.4F-H). Imazamox also caused the accumulation of aromatic amino acids in 3841 nodules leading to a 2.3-fold increase in Tyr, and 3.2-fold increase in Phe and Trp (Figs. 2.1.4I-K). In

RU1722 nodules, Phe increase 1.73-fold due to imazamox treatment, but, interestingly, there was no effect of herbicide on Tyr and Trp content in RU1722 nodules, observing a similar effect of herbicide and *aap/bra* double mutation on these amino acids (Figs. 2.1.4I-K). Regarding the other amino acids determined, imazamox caused its accumulation in 3841 nodules (Fig. S2.1.6). The same effect was observed in RU1722 nodules, although to a lesser extent than in 3841 nodules, except for Met, Lys and Arg, which were not affected by the herbicide (Fig. S2.1.6).

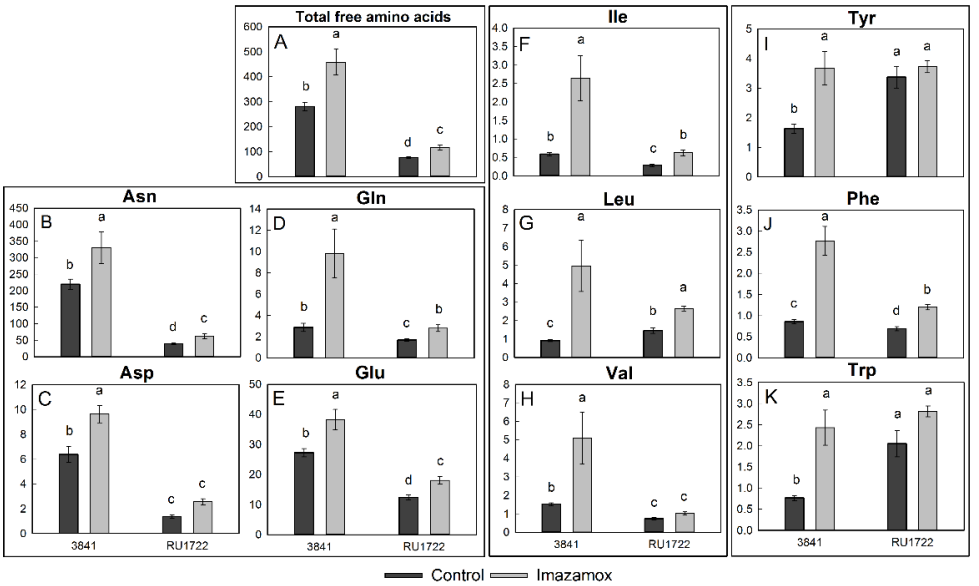

Figure 2.1.4 Effect of the *aap/bra* double mutation and imazamox treatment on the levels of **(A)** total free amino acids, **(B-E)** amide and acidic amino acids, **(F-H)** branched-chain and **(I-J)** aromatic amino acids content in nodules of pea plants inoculated with either *R. leguminosarum* bv. *viciae* 3841 or RU1722 strains. Values are expressed as μmol g NDW^{-1} and represent the mean ± SE ($6 \leq n \leq 8$ biological replicates). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

2.1.3.4 Effects of *aap/bra* double mutation and imazamox on anions and organic acids content in pea nodules

In order to understand the effects of *aap/bra* double mutation and imazamox on anions and organic acids, the levels of phosphate and sulphate, as well as, acetate, lactate, pyruvate, malate, succinate, α-ketoglutarate and citrate were measured in pea nodules. The *aap/bra* double mutation reduced the levels of malate in nodules from 126.93 ± 8.37 μmol g NDW^{-1} to 72.77 ± 9.72 μmol g NDW^{-1} (Fig. 2.1.5A). Interestingly, malate content increased under imazamox

treatment up to 115.99 ± 9.95 µmol g NDW^{-1} in RU1722 nodules, reaching values close to those obtained in 3841 nodules (Fig. 2.1.5A). Under imazamox treatment, α-ketoglutarate was affected, decreasing by 35% in 3841 nodules, while it did not show significant changes in RU1722 nodules (Fig. 2.1.5B). Similarly, there was no effect on acetate, lactate, pyruvate, succinate or citrate content in the different treatments (Table S2.1.4). Regarding anions, phosphate and sulphate content increased by 77 and 69% in RU1722 compared with 3841 nodules, respectively (Figs. 2.1.5C and D). While the herbicide did not caused any change in phosphate concentration (Fig. 2.1.5C), the concentration of sulphate decreased by 31 and 48% due to imazamox in RU1722 and 3841 nodules, respectively (Fig. 2.1.5D).

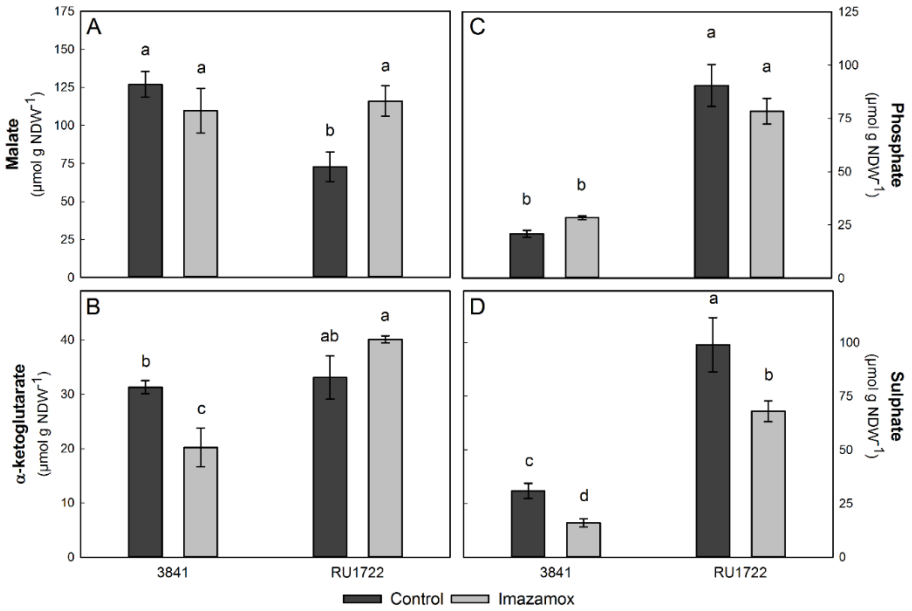

Figure 2.1.5 Effect of the *aap/bra* double mutation and imazamox treatment on the content of **(A)** malate, **(B)** α-ketoglutarate, **(C)** phosphate, and **(D)** sulphate in pea nodules. Values represent mean ± SE (n = 4 biological replicates). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

2.1.4 Discussion

2.1.4.1 Proteomics reveals differential BNF regulation caused by both *aap/bra* double mutation and imazamox treatment leading to a nitrogen-starved plant phenotype

In this work, we employed two approaches to gain deeper insights about the role of amino acids on the whole nodule metabolism, the blockage of BCAA transport from the plant to the microsymbiont and the inhibition of BCAA biosynthesis in the plant partner. Previous works studying the effect of the *aap/bra* double mutation and the imazamox treatment independently showed a reduction of shoot and nodule dry weights (García-Garijo et al., 2014; Lodwig et al., 2003). In the current work, however, no differences were observed in terms of nodule dry weights, with the exception of the double mutant upon herbicide treatment. This discrepancy could be explained by experimental differences such as earlier harvest time or different herbicide application conditions. Nevertheless, the effect of the herbicide was clear, with a significant decline in AHAS activity in leaves of plants inoculated with either 3841 or RU1722 strains. Growth arrest is a well-known indicator of physiological stress, and here both stresses caused a decline in shoot dry weights. This growth arrest could be explained by the nitrogen-starved phenotype observed due to either the *aap/bra* double mutation or the imazamox treatment. Nitrogen deficiency could be caused by the inhibition of BNF or by a decrease in nitrate uptake by roots (in the case of the herbicide treatment) (Shaner, 1991; Zabalza et al., 2006). But, in this work, nitrogen deficiency was caused by the inhibition of BNF (observed in both stresses) since we used nitrogen-free nutrient solution. As shown in the current study, nitrogen deficiency induced a decline in leaf chlorophyll content and in stomatal closure, which can in turn produce a decline in photosynthetic rates and nutrient translocation (Zabalza et al., 2006; Zhao et al., 2005).

Several proteins involved in the onset of BNF were identified in the quantitative proteomic analysis. The beginning of BNF requires the activation of the bacterial *fix* and *nif* genes which are mediated by a low-O_2 signal through the two-component FixL-FixJ system in *Rhizobium* bacteria (Green et al., 2009). At low O_2 levels, FixL is first autophosphorylated and later it phosphorylates FixJ, switching on transcriptional regulators such as *nifA* and *fixK* (Terpolilli et al., 2012). Nonetheless, in *R. leguminosarum* bv. *viciae*, the regulation of nitrogen fixation by O_2 is not evident, since no gene encoding for FixJ has been

found (Terpolilli et al., 2012). Instead, there is another transcriptional regulator, FnrN, which together with FixK, is essential for BNF (Terpolilli et al., 2012). Moreover, under symbiotic conditions, *nifA* expression occurs mainly from a σ^{54}-dependent promoter, which is positively autoregulated by NifA, and therefore its regulation is independent of both FixL and FixK (Martínez et al., 2004; Terpolilli et al., 2012). In the absence of this σ^{54}-dependent promoter and NifA, basal *nifA* expression is observed due to other uncharacterized promoter of *nifA* (Terpolilli et al., 2012). The expression of *nifHDK* or *fixABC* genes are regulated by NifA, and low levels of *nifA* expression has been related to less efficient BNF process (Martínez et al., 2004). In this work, the proteomic analysis showed an accumulation of NifA in RU1722 bacteroids, while proteins belonging to FixABCX (in particular the FixA and FixC subunits), NifHDK (NifH, the dinitrogenase reductase component of nitrogenase) or the nitrogenase protein itself were found less abundant (Table S2.1.1, see attached CD). In contrast, in imazamox-treated 3841 bacteroids the abundance of NifA was reduced along with FixC and NifH subunits (Table S2.1.2, see attached CD). This suggests that the inhibition of BNF found in this study due to the imazamox treatment could be related to the reduced abundance of NifA leading to a lower *nifA* expression. In contrast, although NifA positively regulates *nifA* expression, its accumulation could suggest a feedback inhibition of *nifA* expression in the *aap/bra* double mutant, although further investigations are needed to understand why an increase of NifA led to a decline in other *fix* and *nif* genes.

2.1.4.2 Plants inoculated with the double mutant *aap/bra* or imazamox-treated show protein profiles related to altered O_2 levels in nodules

Under low O_2 concentration, FnrN initiates the transcription of *fixNOQP* and *fixGHIS* which encode for a cytochrome *cbb*$_3$-type oxidase, and for a protein required for Cu transport into the cytochrome *cbb*$_3$-type oxidase, respectively (Preisig et al., 1993; Thöny-Meyer, 1997). In bacteroids, the levels of proteins encoded by the FixNOQP operon were reduced in both treatments (Tables S2.1.1 and S2.1.2, attached CD), suggesting the occurrence of an imbalance of O_2 levels in nodules. In RU1722 bacteroids, a reduction in the protein content of FixN, FixO, FixP and FixH subunits was observed, while imazamox treatment only reduced FixP content in 3841 bacteroids.

On the plant side, we observed changes in the levels of both leghemoglobins (Lbs) and nonsymbiotic hemoglobins. We found six family

members among leghemoglobin category, set of proteins responsible of maintaining low O_2 concentrations in nodules (recently reviewed in Larrainzar et al., 2020). The relatively more abundant leghemoglobins decreased in both *aap/bra* nodules and upon imazamox treatment. In contrast, the relative amount of other leghemoglobin family member (P93848) increased in RU1722 nodules while opposite trend was observed under imazamox treatment. Interestingly, the total Lb content was no affected in soybean nodules by chlorimuron-ethyl, other AHAS-inhibitors herbicide, although it was found to decline in lupin nodules treated with glyphosate, an herbicide involve in aromatic amino acid biosynthesis (de María et al., 2005; Zawoznik & Tomaro, 2005). Regarding the non-symbiotic hemoglobin category there was an accumulation in RU1722 nodules and nodules treated with imazamox. Non-symbiotic hemoglobins are not directly involved in the regulation of BNF, although they are required for the infection process as part of the nitrosative stress defense (Fukudome et al., 2016). Moreover, their expression has been induced under different stresses, including herbicides treatment (Dordas, 2009; Gil-Monreal et al., 2019). Finally, several proteins involved in O_2 detoxification were also accumulated such as a cytochrome P450, peroxidases or alpha-dioxygenases, among others.

2.1.4.3 The *aap/bra* double mutation and imazamox differentially alter amino acid metabolism in nodules

Both the inhibition of amino acid transport between symbionts and the BCAA biosynthesis had an impact in the abundance of the main enzymes involve in ammonium assimilation in nodules (Tables S2.1.1, S2.1.2 and S2.1.3, see attached CD). In the plant fraction, asparagine synthetase (AS), GS and GOGAT declined in RU1722 as well as in imazamox-treated 3841 nodules. A decrease in GS and GOGAT activities was also found in *Vicia faba* shoots treated with an AHAS-inhibitor herbicide (Scarponi et al., 1997). Interestingly, imazamox did not cause any significant effect on the levels of AS, GOGAT and GS in RU1722 nodules, probably due the preexisting stress in nodules infected with the double mutant strain. An aspartate aminotransferase (AAT), other important protein in ammonium assimilation, was accumulated due to the imazamox treatment, while no effect of the *aap/bra* double mutation was observed. In rhizobia, three GS isoenzymes encoded by the *glnA*, *glnII* and *glnT* genes have been described (Dunn, 2015). In both free-living and symbiotic conditions, *glnII* expression is activated under nitrogen deficiency and requires an upstream activating sequence (Patriarca et al., 2002). In this work, the levels of GSII were accumulated in *aap/bra* bacteroids compared to wild-type

bacteroids, suggesting a more limited nitrogen availability due to the double mutation. Although, in other works, no GSII protein had been found in bacteroids probably due to the action of a glutamine synthetase translational inhibitor protein (GstI) (Dunn, 2015). Moreover, proteins related to amino acid catabolism were also accumulated, such as glutamate decarboxylase (GAD) in both treatment, and an argininosuccinate synthase in RU1722 nodules. These results partly explain the variations observed in the content of amide and acidic amino acids content in RU1722 nodules or in imazamox-treated nodules, respectively. It is generally accepted that AHAS-inhibiting herbicides lead to an accumulation of free amino acid content by an increase in cellular proteolytic activity (Rhodes et al., 1987; Zulet et al., 2013). In agreement with this model, the proteomic data obtained here showed an increase in the abundance of several proteases such as a senescence-specific cysteine protease, a thiol protease aleurain or a cysteine proteinase in both RU1722 and imazamox-treated nodules.

Regarding the set of BCAAs, the synthesis of Ile begins through Thr with the reaction catalysed by threonine deaminase (see section F.2.1, Fig. 5). In this points, a parallel pathway for the synthesis of Ile and Val takes place with a single set of four enzymes (AHAS; KARI; dihydroxyacid dehydratase and branched-chain aminotransferase), and Leu is synthetized from the last intermediate in this pathway (Singh & Shaner, 1995). The *aap/bra* double mutation led to a decline in the abundance of the dihydroxyacid dehydratase, while a 3-isopropylmalate dehydrogenase and 3-isopropylmalate dehydratase, two proteins involved in the synthesis of Leu, increased. These results support the decline observed in Ile and Val content due to the *aap/bra* double mutation, and also the increase in Leu content. It should be noted that Leu levels were higher in the plant fraction of the double mutant nodules, since Leu transport to bacteroids is blocked in this mutant. Moreover, the observed decline in Ile could also be explained by the lower Thr content detected, in turn, which could be related to the increased abundance of a L-allo-threonine aldolase-like protein, a protein involved in Thr catabolism (Broeckling et al., 2005; Joshi et al., 2010), as found in the proteomic profile. AHAS, the target enzyme for imazamox, was not be quantified in this study, most likely due to the relative low abundance of this protein. However, we could measure AHAS activity in leaves and, indeed, the aap/bra double mutation had a significant impact, with plants inoculated with RU1722 strain presenting a reduction of 70% AHAS activity compared to plants inoculated with the wild-type strain. Despite the negative effect of imazamox found on a branched-chain aminotransferase in wild-type nodules, the content of BCAAs increased, probably due to proteolysis, in contrast to results found in *Phaseolus vulgaris* or *Vicia sativa* nodules (García-Garijo et al., 2014).

In terms of the biosynthesis of aromatic amino acids, the first step involves the synthesis of chorismate through the shikimate pathway, from which the pathways to Trp, Phe and Tyr branch (Maeda & Dudareva, 2012). 3-dehydroquinate synthase (DHQS) and chorismate synthase, two proteins involved in the chorismate biosynthesis, were found to accumulate in RU1722 nodules. In addition, the levels of 4-hydroxyphenylpyruvate dioxygenase, an enzyme involved in the catabolism of Tyr, showed a decline in the double mutant. These observations could explain the increase in Tyr and Trp content in these nodules compared with the wild type. In wild-type nodules, imazamox treatment led to an increase in the abundance of phospho-2-dehydro-3-deoxyheptonate aldolase (DAHPS) and DHQS, which catalyse the first steps of the shikimate pathway, and a bifunctional aspartate aminotransferase and a glutamate/aspartate-prephenate aminotransferase-like, which catalyse the first steps of the arogenate pathway. These last two proteins were also accumulated in imazamox-treated RU1722 nodules. These results support the existence of a possible link between different amino acid pathways, in which variations in the levels of key amino acids could act as a signal to regulate the expression of genes involved in the aromatic amino acid biosynthetic pathway (Orcaray et al., 2010, and references therein). This may also explain the similar effect that the *aap/bra* double mutation and imazamox had on Tyr and Trp content.

The content of Ala, a product of BNF in bacteroids (Allaway et al., 2000; Li et al., 2002), was reduced in RU1722 nodules. This reduction could be caused by the inhibition of BNF as it was observed under darkness (Tsikou et al., 2013), but also, by the reduced abundance of alanine aminotransferase (AlaAT), and Glu content found in the double mutant nodules. In contrast, imazamox led to an increase in Ala despite the inhibition of BNF. The accumulation of Ala has been related to an increase in the AlaAT activity during hypoxia (Rocha et al., 2010; Zabalza et al., 2011) and to the maintainance of energy homeostasis or increased carbon use efficiency under several abiotic stresses (Rocha et al., 2010).

GABA is a non-proteogenic amino acid related to an upregulation of nodule activity and the reduction of ROS under stress thanks to the GABA shunt (Bouché et al., 2003; Sulieman & Schulze, 2010). In this work, GABA content declined in RU1722 nodules. In fact, two proteins involved in degradation and biosynthesis of GABA, a succinate-semialdehyde dehydrogenase and GAD, respectively, were found to accumulate in RU1722 nodules. The imazamox treatment had, however, the opposite effect; the levels of GABA increased, as also observed in other plant tissues such as shoots and roots treated with an

AHAS inhibitor (Royuela et al., 2000; Zabalza et al., 2011) in parallel to an accumulation of GAD at the protein level.

2.1.4.4 *aap/bra* nodules show impaired carbon metabolism and increased defence responses

It is generally accepted that C4-dicarboxylates, most precisely malate, are the main products of sucrose degradation supplied to bacteroids to support BNF (Udvardi & Day, 1997). In this work, the *aap/bra* double mutation led to a decline in the abundance of SuSy, phospho*enol*pyruvate carboxylase (PEPC), and two isoforms of malate dehydrogenase (MDH, O81609 and A0A072VMC4), in addition to the a C4-dicarboxylate transport protein (Table S2.1.1, attached CD). These observations, in addition to the decline in malate content measured in RU1722 nodules (Fig. 2.1.5.A), suggest that RU1722 bacteroids were not only BCAA starved but also carbon starved. Indeed, another indication of a reduced carbon use favouring carbon accumulation is the increased levels of several proteins related to starch biosynthesis in nodules (Table S2.1.1), as well as, the abundance of starch granules observed in RU1722 nodules compared to the wild type (Figs. 2.1.1F-I), as previously reported in other symbiotic mutant strains (Kim, 2002; Vance et al., 1980).

Regarding the effect of the imazamox treatment on carbon metabolism, PEPC declined in 3841 while no effect was observed in RU1722 nodules (Tables S2.1.3 and S2.1.4, see attached CD). In this work, we found several MDH isoforms (Table S2.1.1). The relative content of the most abundant MDH isoform, a nodule-enhanced MDH, decreased in wild-type nodules while no effect of the herbicide was observed in RU1722 nodules. In contrast, other MDH isoform (P37228), relatively less abundant, was accumulate in response to imazamox treatment (Tables S2.1.2 and S2.1.3). This fact could explain the increase in malate content observed in imazamox-treated RU1722 nodules, but not the results in imazamox-treated 3841 nodules, which suggest a lower utilization rate. Although, in pea roots, imazethapyr did not affect malate content either (Zabalza et al., 2006).

Response to stress was the most representative functional group affected by both *aap/bra* double mutation and imazamox treatment (Tables S2.1.1, S2.1.2 and S2.1.3). Among those proteins with a higher fold-change, a defensin-like protein was accumulated in RU1722 nodules. This protein is related to defence response, but it is also important in the interactions between symbionts (Nallu et al., 2013).There was a common response in proteins belonging to PRs group.

These proteins are induced in response to infection by diverse pathogens, as well as under stress condition, so they are important in the plant defence response (Linthorst, 1991). An accumulation of PRs has been described under different abiotic stresses such as water deficit (Castillejo et al., 2016) or salinity (Manchanda & Garg, 2008), but also under herbicide treatment, for instance, glyphosate (Vivancos et al., 2011) or flumetsulam, an AHAS inhibitor (Holmes et al., 2006). Moreover, an induction on PRs was observed under boron deficiency in nodules, suggesting that plants could recognized the rhizobia as a pathogen (Reguera et al., 2010). This may also be the case under nitrogen deficiency caused by an impaired BNF. Interestingly, stress-related proteins reduced by the *aap/bra* double mutation or imazamox were different. A late embryogenesis abundant (LEA) protein was the most reduced protein due to the *aap/bra* double mutation. LEA proteins not only have been given a protective role during water deficit in different tissues (Battaglia & Covarrubias, 2013) but also a possible role during infection (Porcel et al., 2005). In imazamox-treated nodules, there was a decrease in an argonaute (AGO) protein. AGO proteins are important in the regulation of legume-rhizobium symbiosis, as well as in plant defence, so that decrease suggest an impaired mutualism due to the herbicide (Valdés-López et al., 2019).

2.1.5 Conclusions

This work demonstrates the importance of amino acid metabolism in pea nodules, highlighting the effect of the blockage of BCAA transport between symbionts, which was able to buffered the effect of the herbicide. Both the *aap/bra* double mutation and the imazamox treatment cause an inhibition of BNF inducing a nitrogen deficiency in pea plants.

The *aap/bra* double mutation and the imazamox treatment led to a deregulation of ammonium assimilation, which, in turn, triggered the decline in amino acid content in RU1722 nodules. Both stresses caused a shutdown of BCAA, except in RU1722 nodules where Leu biosynthesis was promoted. The induction of the biosynthesis of aromatic amino acids support the existence of a possible link between different amino acid pathways, where a decrease in certain amino acids increase the expression of genes involved in the biosynthesis of aromatic amino acids.

In addition, carbon metabolism was also impaired due to both stresses but only the *aap/bra* double mutation declined malate content, leading to carbon-starved bacteroids. Finally, the differential accumulation of stress-related proteins also suggests impairment in the legume-rhizobium symbiosis caused by both stresses.

2.1.6 Supplementary material

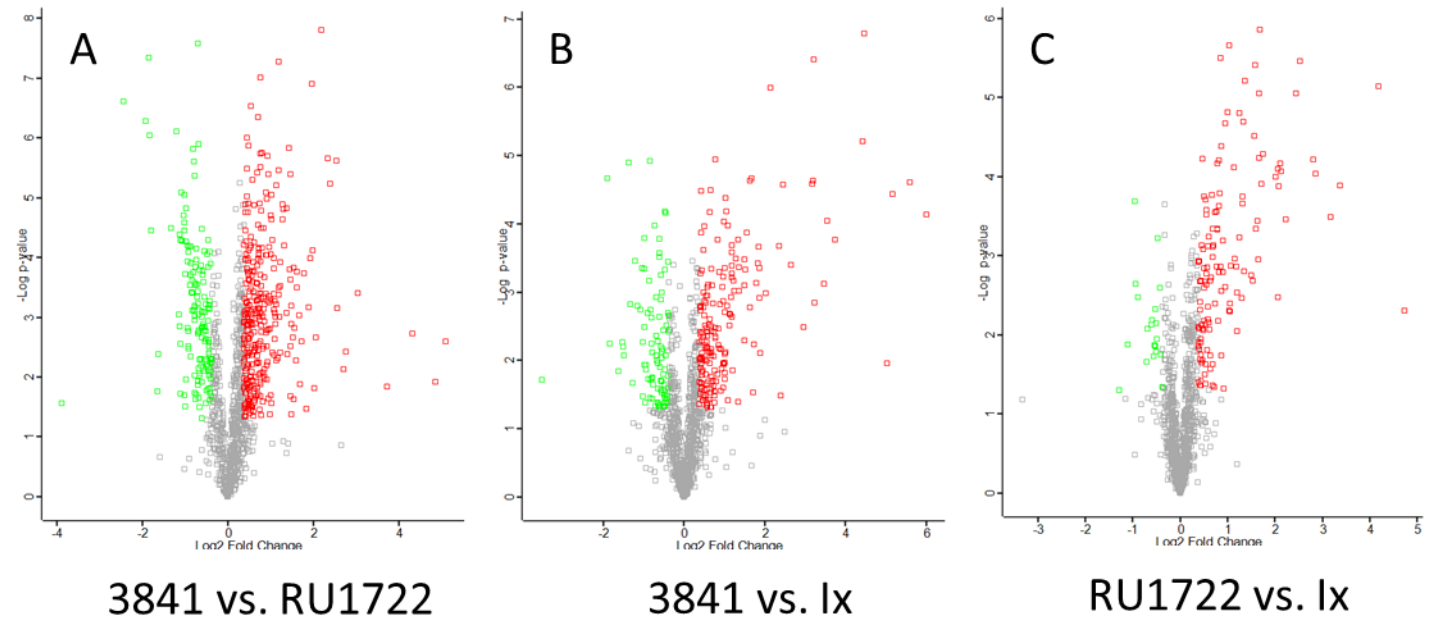

Fig. S2.1.1 Volcano plots representing the fold-change of the total number of identified nodular proteins from the pair-wise quantitative comparisons of pea plants inoculated with *R. leguminosarum* bv. viciae 3841 and RU1722 strains upon imazamox (Ix) treatment. Proteins that presented a p-value smaller than 0.05 and a fold change smaller than 0.77 or larger than 1.3 on a linear scale were selected. In green, proteins that were found more abundant under control conditions; in red, proteins more abundant in RU1722 or in samples treated with imazamox.

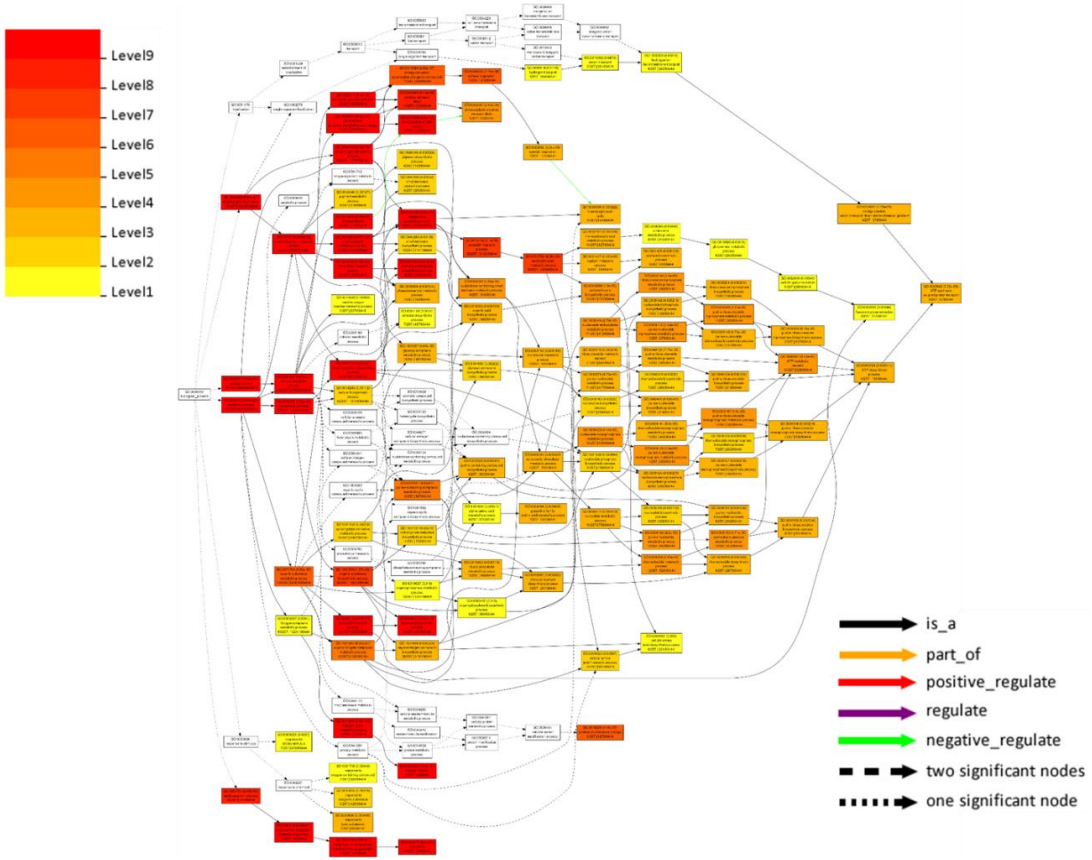

Fig. S2.1.2 GO enrichment analysis of the biological processes of quantified plant proteins of pea nodules inoculated with either *R. leguminosarum* bv. *viciae* 3841 or RU1722 strains treated with imazamox.

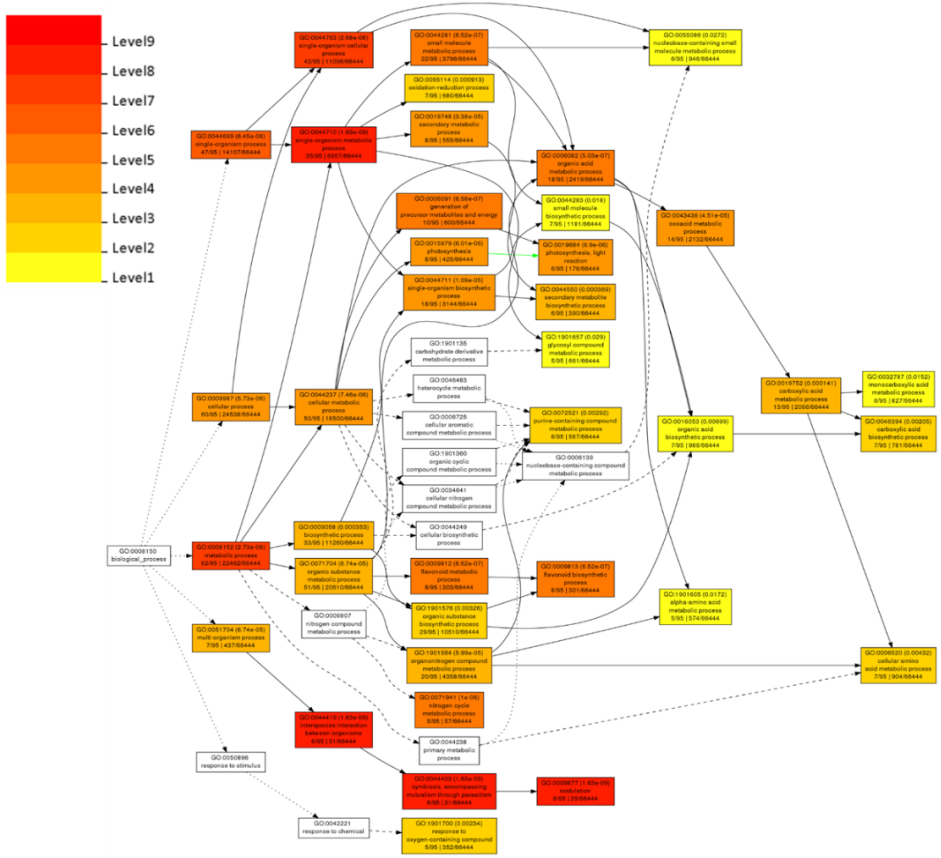

Fig. S2.1.3 GO enrichment analysis of the biological processes of differentially accumulated plant proteins of pea nodules inoculated with R. leguminosarum bv. *viciae* 3841 and RU1722 under control conditions. Arrow legend in Fig. S2.1.2.

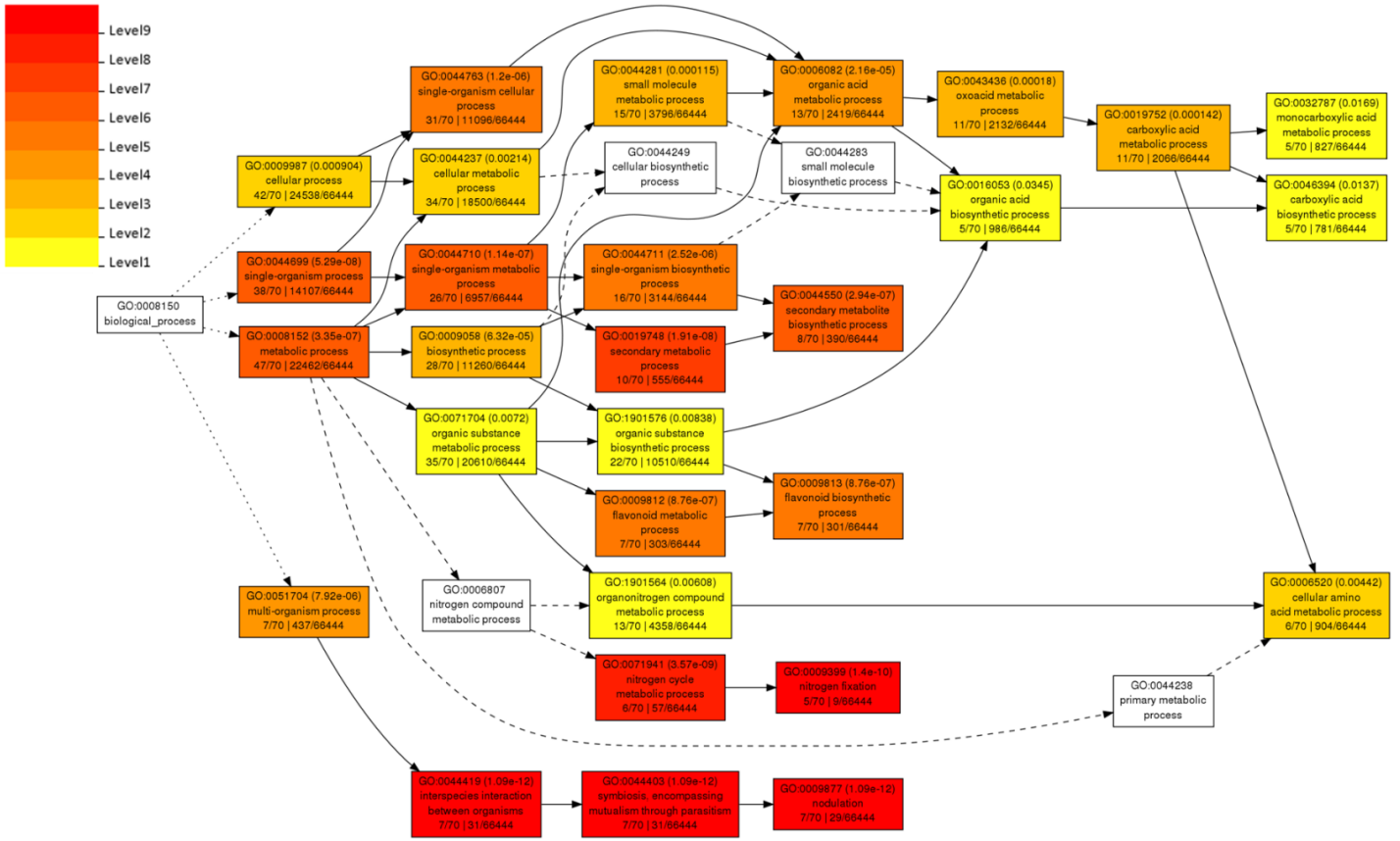

Fig. S2.1.4 GO enrichment analysis of the biological processes of differentially accumulated plant proteins of pea nodules inoculated with *R. leguminosarum* bv. *viciae* 3841 under imazamox treatment. Arrow legend in Fig. S2.1.2.

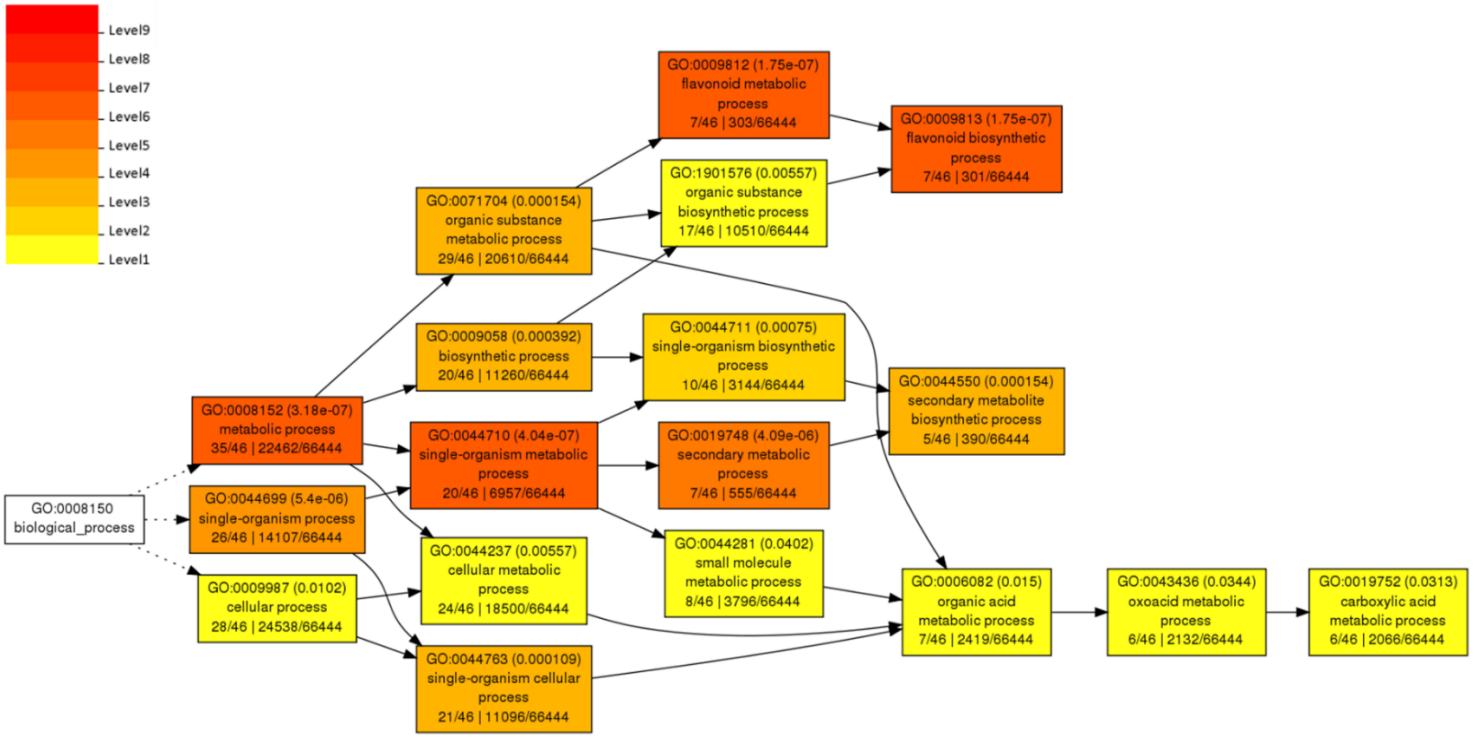

Fig. S2.1.5 GO enrichment analysis of the biological processes of differentially accumulated plant proteins of pea nodules inoculated with *R. leguminosarum* bv. *viciae* RU1722 under imazamox treatment. Arrow legend in Fig. S2.1.2.

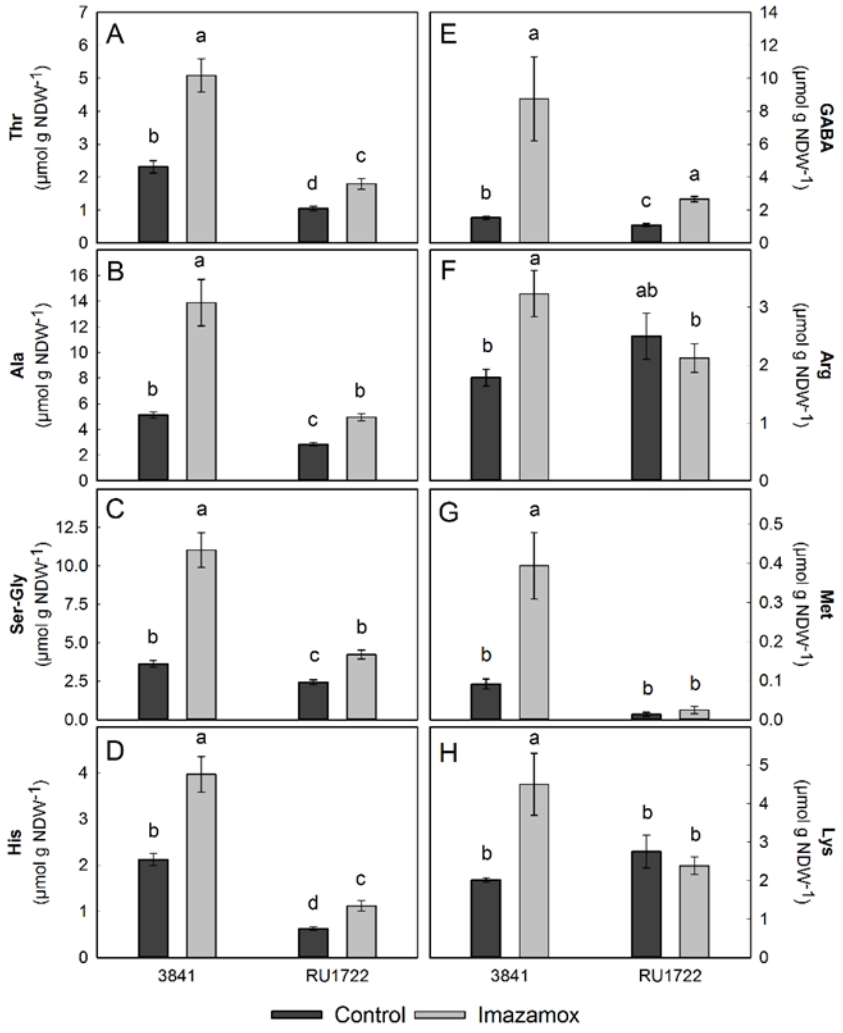

Fig. S2.1.6 Effect of the *aap/bra* double mutation and imazamox treatment on the levels of amino acids in nodules of pea plants inoculated with *R. leguminosarum* bv. viciae 3841 (wild type) and RU1722 (double mutant) strains. Values represent mean ± SE ($6 \leq n \leq 8$ biological replicates). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

Table S2.1.4 Effect of the *aap/bra* double mutation and imazamox treatment on the content of organic acids in pea nodules. Values in µmol g NDW^{-1} represent mean ± SE (n = 4 biological replicates). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

Organics acids	3841	RU1722	3841+Ix	RU1722+IX
Acetate	4.23 ± 0.49 a	5.49 ± 1.5 a	3.69 ± 0.97 a	6.1 ± 1.52 a
Citrate	12.49 ± 1.03 a	14.91 ± 2 a	27.04 ± 5.01 a	14.78 ± 0.62 a
Lactate	3.67 ± 0.89 a	6.73 ± 0.53 a	6.44 ± 1.77 a	7.32 ± 0.81 a
Pyruvate	1.98 ± 0.12 a	1.93 ± 0.24 a	1.45 ± 0.05 a	1.63 ± 0.31 a
Succinate	29.59 ± 3.77 a	24.48 ± 3.31 a	23.09 ± 2.89 a	25.88 ± 1.53 a

Part 2

2.2.1 Introduction

In a global climate change context, an increase in arid surface and a reduction in the availability of freshwater are expected to have a clear impact on crop production worldwide. Given the importance of legume crops in human nutrition, ensuring adequate levels of production in a sustainable manner becomes imperative. Plants posses several strategies to minimize the impact of abiotic stresses. Focusing on those based on amino acid metabolism we can cite at least three: (i) osmoregulation (Irigoyen et al., 1992; Larrainzar et al., 2009), (ii) synthesis of secondary metabolites and signalling molecules (e.g. polyamines from Arg, ethylene from Met or auxin from Trp), or (iii) organic nitrogen storage. The role of proline or other abundant amino acids in response to water deficit has been widely described. However, less attention has been given to those amino acids that are in smaller proportion, even though their content increases relatively more than other more abundant amino acids (Batista-Silva et al., 2019; Gil-Quintana et al., 2013a, b; Hildebrandt, 2018; Joshi et al., 2010; Rai, 2002; Sulieman & Tran, 2013).

Water deficit affects the *Rhizobium*-legume symbiosis from the first steps of its establishment, impairing nodule development, and in consequence, inhibiting the BNF process (Serraj et al., 1999a). Several hypotheses have been described to explain the decline in BNF during water deficit, namely, oxygen limitation, carbon shortage, and regulation by nitrogen metabolism (Arrese-Igor et al., 2011). A lower supply of water to nodules reduces the transport of N compounds to shoots leading to their accumulation (Purcell, 2009), which has been related with a feedback inhibition of BNF. Several compounds have been suggested to play a role in this inhibition, including glutamine (Neo & Layzell, 1997), ureides (Atkins et al., 1992; Serraj et al., 1999b; Vadez et al., 2000), asparagine (Bacanamwo & Harper, 1997), or the ratios ureides/asparagine (Vadez et al., 2000) or aspartic acid/asparagine (Lima & Sodek, 2003). However, subsequent research has challenged these hypotheses (Aldasoro et al., 2019; King & Purcell, 2005; Ladrera et al., 2007, among others). For instance, in amide-exporter legumes, a relationship between amino acid accumulation and N-feedback inhibition could not be clearly established (Gil-Quintana et al., 2013a; Larrainzar et al., 2009).

To shed light on the involvement of amino acid accumulation and metabolism in the regulation of BNF during drought, we took advantage of the availability of rhizobium bacteria with altered amino acid transport such as the strain *Rhizobium leguminosarum* bv. *viciae* RU1722, which contains a double mutation on the ABC transporters Aap and Bra (*aap/bra*) (Lodwig et al., 2003). The aim of this work is to test the N feedback regulation hypothesis comparing the response to drought of pea plants grown in symbiosis with the wild-type *R. leguminosarum* bv. *viciae* 3841 strain and the RU1722 *aap/bra* double mutant using a combination of physiological measurements and shotgun proteomics.

2.2.2 Materials and methods

Materials and methods are shared with the first part of this chapter with the exception of the experimental design, which is explained below. Bacterial and plant growth conditions are included in the sections 2.1.2.1 and 2.1.2.2. The protocol followed to carry out the proteomic analysis is explained from the section 2.1.2.6 to the section 2.1.2.9. Metabolite determination is described in the sections 2.1.2.10 and 2.1.2.11. Finally, statistical analysis is defined in section 2.1.2.12.

2.2.2.1 Experimental design and physiological characterisation of water deficit

Water deficit was gradually imposed in plants inoculated with *R. leguminosarum* bv. *viciae* 3841 or RU1722 strains by water withholding. Plants were separated into two groups depending on their values of leaf water potential (Ψ_{leaf}). The groups kept under optimal water conditions maintained a Ψ_{leaf} value around -0.3 MPa (thereafter named control), and the group subjected to water restriction reached a Ψ_{leaf} value around -1 MPa (severe drought). To establish the effect of water deficit and herbicide on plants, various parameters were measured: stomatal conductance, leaf and nodule water potential, and apparent nitrogenase activity (ANA). Stomatal conductance and Ψ_{leaf} were measured 2 h after the beginning of photoperiod in the youngest fully expanded leaf. Methodology explained in the section 2.1.2.4.

2.2.3 Results

2.2.3.1 Water-deficit effects on the physiological response of pea plants

Peas inoculated with either *R. leguminosarum* bv. *viciae* 3841 or RU1722 strains were subjected to water withholding to know the effect of the interruption of amino acid transport across symbionts on drought-stressed nodules. The physiological response of the plants was very similar regardless of the strain used for inoculation. Drought stress provoked a decline in stomatal conductance, reaching values close to 0.07 ± 0.01 cm s^{-1} in average, while a gradual decline in both Ψ_{leaf} and Ψ_{nodule} was observed, reaching values around -1 MPa (Table 2.2.1). Instead, plants under optimal water availability maintained a Ψ_{leaf} of -0.25 ± 0.04 and -0.32 ± 0.05 MPa, and a Ψ_{nodule} -0.13 ± 0.03 and -0.28 ± 0.07 MPa in plants inoculated with 3841 or RU1722, respectively (Table 2.2.1). Similarly, drought stress decreased nodule water content around 8% in 3841 and RU1722 nodules (Table 2.2.1). The effect was most notable for roots, with around 30 % decline in both 3841 and RU1722 nodules (Table 2.2.1). In shoot, water content declined around 4.5% under water deficit (Table 2.2.1). Regarding nitrogen fixation rates, drought caused a 73% decline in plants inoculated with 3841, while there was no effect of drought in plants inoculated with RU1722, most like due to the fact that nitrogen fixation was already severely impaired in plants inoculated with the *aap/bra* strain (Table 2.2.1).

Table 2.2.1 Effects of water deficit on stomatal conductance, leaf and nodule water potential, photosynthesis, chlorophyll content and apparent nitrogenase activity (ANA) of pea plants inoculated with *R. leguminosarum* bv. *viciae* strains 3841 and RU1722. Values represent mean ± SE ($4 \leq n \leq 6$ biological replicates). For each parameter, numbers followed by a different letter are significantly different at $p \leq 0.05$ in a LSD test. NDW, nodule dry weight.

	3841		RU1722	
Parameter (Units)	**Control**	**Water deficit**	**Control**	**Water deficit**
Stomatal conductance (cm s^{-1})	0.83 ± 0.04 a	0.06 ± 0.01 c	0.38 ± 0.03 b	0.08 ± 0.01 c
Leaf water potential (MPa)	-0.25 ± 0.04 a	-1.08 ± 0.11 b	-0.32 ± 0.05 a	-1.03 ± 0.05 b
Nodule water potential (MPa)	-0.13 ± 0.03 a	-1.01 ± 0.04 b	-0.28 ± 0.07 a	-1.04 ± 0.13 b
Nodule water content (%)	89.16 ± 0.47 a	81.57 ± 1.26 c	86.4 ± 0.82 b	80.08 ± 1.32 c
Root water content (%)	92.74 ± 0.4 a	62.93 ± 2.29 b	91.00 ± 0.53 a	66.46 ± 1.84 b
Shoot water content (%)	88.14 ± 0.41 a	83.8 ± 0.43 b	84.67 ± 0.62 b	81.11 ± 0.71 c
ANA (μmol H_2 g NDW^{-1} min^{-1})	1.13 ± 0.16 a	0.31 ± 0.07 b	0.21 ± 0.02 bc	0.15 ± 0.01 c

2.2.3.2 Changes in the pea nodule proteome under water deficit

Nodules of plants inoculated with 3841 or RU1722 strains and subjected to water deficit were analysed using proteomics to obtain an overview of the main changes taking place at the protein level. As mentioned in previous sections (section 2.1.3.2), of the 2,525 proteins identified, 1,197 proteins were finally quantified (1,149 from plant fraction and 48 from bacteroid) (Table S2, see attached CD). A PCA, including all quantified proteins, showed clear differentiation in protein abundances between control and drought-stressed nodules of both strains (Fig. 2.2.1A). Water deficit caused changes in abundance (fold change of < 0.77 or > 1.3, Student's *t*-test, $p < 0.05$) on 90 and 122 proteins in 3841 and RU1722 nodules, respectively (Fig. S2.2.1, Tables S2.2.1 and S2.2.2, see attatched CD). Those proteins were represented in a Venn diagram to illustrate relationships between treatments and genotypes (Fig. 2.2.1B). In 3841 nodules, 69 (36.1%) proteins changed exclusively in response to water deficit, while in RU1722 nodules the levels of 101 proteins (52.9%) were found significantly affected, showing a greater impact of water deficit on RU1722 nodules (Fig. 2.2.1B). Moreover, 21 (11%) proteins were affected in a similar way in both 3841 and RU1722 nodules (Fig. 2.2.1B).

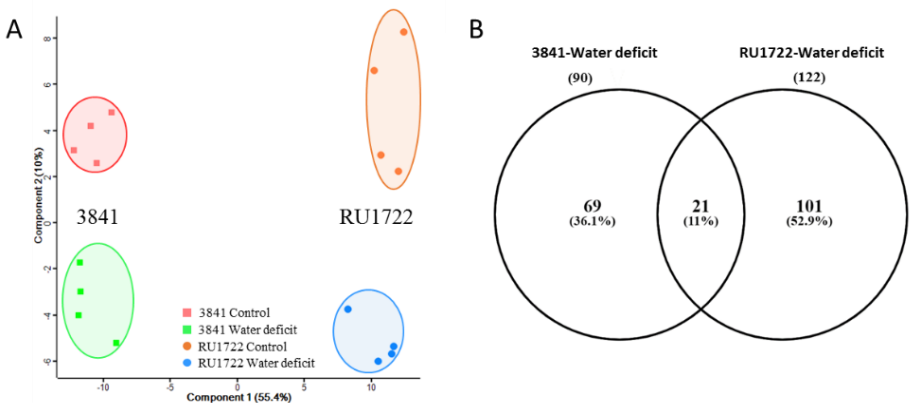

Fig. 2.2.1 Analysis of the quantitative proteome of nodules of pea plants inoculated with *R. leguminosarum* bv. *viciae* strains 3841 (wild type) and RU1722 (*aap/bra*) under drought. **(A)** PCA of quantified proteins. Components 1 and 2 represented 65.4% of the total variance observed. Control 3841 nodules: pink squares; 3841 nodules under drought: green squares; control RU1722 nodules: orange circles; RU1722 nodules under drought: blue circles. **(B)** Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/) representing significantly changed proteins. Venn diagram include all proteins with a fold change of < 0.77 or > 1.3 and a *t*-test $p < 0.05$ on a linear scale.

GO enrichment analysis of the plant proteins of drought-stressed 3841 nodules highlighted the relevance of single-organism metabolic and cellular processes, such as carboxylic acid or secondary metabolite biosynthetic processes (Fig. S2.2.2). Water deficit caused the accumulation of 35 proteins in wild-type nodules (Fig. S2.2.1A), being a cysteine proteinase inhibitor protein accumulating the most (4-fold increase; Table 2.2.2). Similarly, the content of proteins related to response to stress was found to increase, including two thaumatins, a late embryogenesis abundant protein and an annexin D1, among others (Fig. 2.2.2). There was also a greater abundance in proteins such as calcium-dependent protein kinases or a rhicadhesin receptor, which are likely to be involved in the early stages of the establishment of symbiosis. Proteins related to carbohydrate metabolism were also more abundant under water deficit, including an endochitinase or a myo-inositol 1-phosphate synthase. The only bacteroid protein accumulated under water deficit was NifB, a protein involved in the biosynthesis of the iron-molybdenum cofactor of the nitrogenase complex (Table S2.2.1). In contrast, water deficit reduced the abundance of 55 proteins in 3841 nodules, mainly related to cellular processes, stress response, and hormone biosynthesis and responses (Figs. S2.2.1A and 2.2.2). The most reduced proteins were an ATP sulfurylase (0.3-fold) involve in selenium and sulfur uptake and assimilation, and a plant cysteine oxidases which carry out the oxidation of cysteine residues (Table 2.2.2). A granule-bound starch synthase, involve in the biosynthesis of starch, was also reduced under water deficit. Among the group of proteins showing the strongest reduction in relative content, we can find proteins related to stress response such as a brefeldin A-inhibited guanine nucleotide-exchange protein, involved in the activation of auxin response factor, a thiamine thiazole synthase, or a acyl-[acyl-carrier-protein] desaturase, related in turn with lipid metabolism (Table 2.2.2).

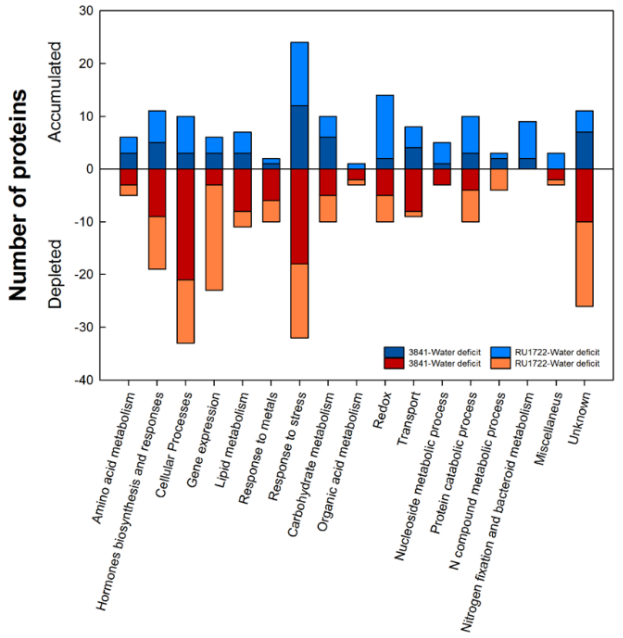

Fig. 2.2.2 Functional classification of differentially accumulated proteins based on Gene Ontology Annotation Database (*t*-test $p < 0.05$, fold change of < 0.77 or > 1.3) and number of proteins within each comparison.

Table 2.2.2 Differentially accumulated proteins in pea nodules inoculated with *R. leguminosarum* bv. *viciae* strains 3841 (wild type) under drought. Proteins with a fold change of ≤ 0.6 or ≥ 1.5 and a *t*-test $p < 0.05$ on a linear scale.

Description	Uniprot Code	Fold Change	p-value
Increased abundance			
Cysteine proteinase inhibitor	Q06445	4.01	8.78E-03
Glucan endo-1,3-beta-glucosidase	P27666	3.94	1.89E-03
Thaumatin	A0A089WUI5	2.49	2.13E-03
Thaumatin-like protein	P31110	2.44	1.86E-03
Late embryogenesis abundant protein	G7L6Q3	2.33	2.86E-03
Endochitinase	P21226	2.20	8.89E-04
Glucan endo-1,3-beta-glucosidase	Q03467	2.20	1.93E-02
Non-symbiotic hemoglobin	Q9FVL0	2.01	7.72E-03
Phospholipase D	A0A1S2XED8	1.99	2.24E-02
Cysteine proteinase 15A	P25804	1.95	1.00E-04
Annexin D1	Q9SYT0	1.70	8.03E-03

Calcium-dependent protein kinase	Q42479	1.66	7.64E-03
Salicylic acid-binding protein	Q6RYA0	1.59	4.05E-02
Rhicadhesin receptor	Q9S8P4	1.58	2.35E-03
Myo-inositol 1-phosphate synthase	G7J4B5	1.55	2.78E-02
AIG2-like (Avirulence induced protein) family protein	G7J2V1	1.52	8.52E-03
Reduced abundance			
Acyl-[acyl-carrier-protein] desaturase	Q4U601	0.59	5.56E-03
Probable calcium-binding protein	Q94AZ4	0.58	9.76E-03
Importin subunit alpha-1a	Q71VM4	0.58	8.13E-03
Uroporphyrinogen decarboxylase	Q42967	0.57	9.70E-03
Importin subunit alpha-2	F4JL11	0.56	5.92E-04
Probable phosphoribosylformylglycinamidine synthase	Q9M8D3	0.56	8.16E-03
Phospholipase A1-IIgamma-like isoform X1	A0A1S2YI44	0.54	1.27E-02
Brefeldin A-inhibited guanine nucleotide-exchange protein	F4IXW2	0.54	2.35E-04
Granule-bound starch synthase	Q00775	0.50	6.40E-03
Thiamine thiazole synthase	A0A072W0W5	0.48	7.79E-05
Keratin, type I cytoskeletal 10-like	A0A1S2XIR6	0.44	1.31E-04
Plant cysteine oxidase 5	Q9LXT4	0.43	7.52E-03
ATP sulfurylase	Q9LIK9	0.30	9.76E-03

Water deficit had also a significant impact in the nodule proteome of plants inoculated with RU1722. GO enrichment analysis including plant proteins of drought-stressed RU1722 nodules highlighted proteins related to nitrogen fixation, nodulation and secondary metabolite biosynthetic processes, among others (Fig. S2.2.3). Water deficit caused the accumulation of 55 proteins (Fig. S2.2.1B), mainly related to response to stress, redox and nitrogen fixation and bacteroid metabolism functional groups (Fig. 2.2.2). A thaumatin-like protein was the protein showing the highest accumulation (7-fold), together with other stress-related proteins such as a subunit of the COP9 signalosome complex, a class-10 pathogenesis-related protein or a disease resistance response protein (Table 2.2.3). Proteins involved in transport were also more abundant in drought-stressed plants as a copper transport protein or two proteins related to O_2 transport, a leghemoglobin annotated as LB5-10 and a non-symbiotic hemoglobin (Table 2.2.3). Among the most abundant proteins we could find several redox associated proteins, such as a FQR1-like NAD(P)H dehydrogenase,

a 9-divinyl ether synthase, an isoflavone reductase, or a 1-aminocyclopropane-1-carboxylate oxidase-like protein (Table 2.2.3). Interestingly, several bacteroid proteins were also accumulated due to water deficit, such as DNA gyrase subunit beta, NifH, NifA, FixN and FixC (Tables 2.2.3 and S2.2.2). As in 3841 nodules, a glucan endo-1,3-beta-glucosidase and an endochitinase were more abundant under water deficit in RU1722 nodules (Tables 2.2.2 and 2.2.3). In contrast, 67 proteins were less abundant in RU1722 nodules due to water deficit (Fig. S2.2.1B). Proteins showing reduced content mainly belonged to gene expression, response to stress and cellular processes functional groups (Fig. 2.2.2; Table S2.2.2). There are several proteins related to gene expression in this group, including a ribonuclease, the small subunit A of the splicing factor U2af, a protein belonging to the high mobility group (HMG-I/Y), a 40S ribosomal protein S3a or a subunit putatively acting as a mediator of RNA polymerase II transcription (Table 2.2.3). Cell wall related proteins were also reduced under water deficit, such as pectin acetylesterase or glucurokinase (Table 2.2.3). As in 3841 nodules, a keratin, type I cytoskeletal 10-like and an ATP sulfurylase were less abundant in RU1722 nodules under water deficit (Table 2.2.3).

Table 2.2.3 Differentially accumulated proteins in pea nodules inoculated with *R. leguminosarum* bv. *viciae* RU1722 under drought. Proteins from bacteroids in bold. Proteins with a fold change of ≤ 0.6 or ≥ 1.5 and a *t*-test $p < 0.05$ on a linear scale.

Description	Uniprot Code	Fold Change	p-value
Increased abundance			
Thaumatin-like protein	P31110	7.74	9.16E-05
Glucan endo-1,3-beta-glucosidase	P27666	4.30	5.16E-06
Copper transport protein	Q94BT9	2.49	7.87E-03
Aldehyde dehydrogenase family 2 member B4	Q9SU63	2.32	3.71E-02
Nodule lectin	Q40987	2.04	9.01E-03
COP9 signalosome complex subunit 1	P45432	2.01	1.91E-02
DNA gyrase subunit beta	A0A060PN51	1.95	1.40E-02
Probable NAD(P)H dehydrogenase (quinone) FQR1-like	Q6NQE2	1.93	1.20E-03
Isoliquiritigenin 2'-O-methyltransferase	P93324	1.82	3.32E-02
Senescence-specific cysteine protease SAG39	A2XQE8	1.80	1.20E-02
Leghemoglobin Lb5-10	O48668	1.67	4.10E-03
9-divinyl ether synthase	Q0PHS9	1.63	8.08E-04

Isoflavone reductase	P52576	1.63	1.41E-02
1-aminocyclopropane-1-carboxylate oxidase-like protein	G8A030	1.60	8.58E-04
Peptide methionine sulfoxide reductase-like	A0A1S2YA98	1.58	3.30E-03
Endochitinase	P21226	1.56	2.13E-02
NifH	C3UR98	1.56	5.32E-03
Non-symbiotic hemoglobin	Q9FVL0	1.55	1.45E-03
Class-10 pathogenesis-related protein	P93333	1.55	3.43E-02
Zinc-metallopeptidase	O22941	1.54	2.29E-02
Basic 7S globulin	P13917	1.50	1.94E-03
Disease resistance response protein	P13240	1.50	3.97E-02
Reduced abundance			
Mediator of RNA polymerase II transcription subunit 36a	Q94AH9	0.58	1.15E-03
Glucuronokinase	Q93ZC9	0.57	5.66E-03
ATP-citrate synthase alpha chain protein	G7IT44	0.55	2.01E-02
40S ribosomal protein S3a	Q8GTE3	0.55	2.27E-03
1-aminocyclopropane-1-carboxylate oxidase 1-like protein	A0A2K3PPE5	0.55	1.17E-03
Pectin acetylesterase	Q6DBP4	0.55	3.12E-02
Keratin, type I cytoskeletal 10-like	A0A1S2XIR6	0.54	1.38E-03
HMG-I/Y	Q43877	0.54	4.70E-02
Splicing factor U2af small subunit A	Q9ZQW8	0.50	1.12E-02
FAM10 family protein At4g22670	Q93YR3	0.49	6.90E-03
Nascent protein associated complex alpha chain	G7L4T7	0.48	1.70E-04
ATP sulfurylase	Q9LIK9	0.48	1.72E-02
Uncharacterized protein At5g48480-like	A0A1S2XNZ5	0.45	2.33E-02
Disease resistance response protein 206-like/Dirigent protein	A0A1S2YWX7	0.41	2.31E-04
Ribonuclease	P42815	0.04	2.96E-02

2.2.3.3 Effects of water deprivation on the content of amino acids and organic acids in pea nodules

To better characterize the effect of the *aap/bra* double mutation in the amino acid profile of nodules, we measured the free amino acid content in nodules of pea plants inoculated with the wild type *R. leguminosarum* bv. *viciae* 3841 and the mutant strain RU1722 under a progressive water deficit. As earlier described, the *aap/bra* double mutation led to a decrease in total free amino acid content in RU1722 nodules, mainly due to a decrease in Asn content, but also in the levels of Met and Asp (Figs. 2.2.3 and S2.2.4). In contrast, there was a relative increase in the content of Lys, Leu, Trp and Tyr, compared to 3841 nodules (Fig. 2.2.3).

Water deficit led to an accumulation in total free amino acids regardless the strain used (Fig. 2.2.3). The highest accumulation was observed for Met, GABA, BCAAs and aromatic aminoacids in 3841 nodules, while in RU1722 nodules again GABA and also Ala experienced the highest increases (Figs. 2.2.3 and S2.2.4). Despite this general accumulation trend, water deficit also caused a decrease in some amino acids, for instance, Asp, which decreased in both 3841 and RU1722 nodules (Figs. 2.2.3 and S2.2.4).

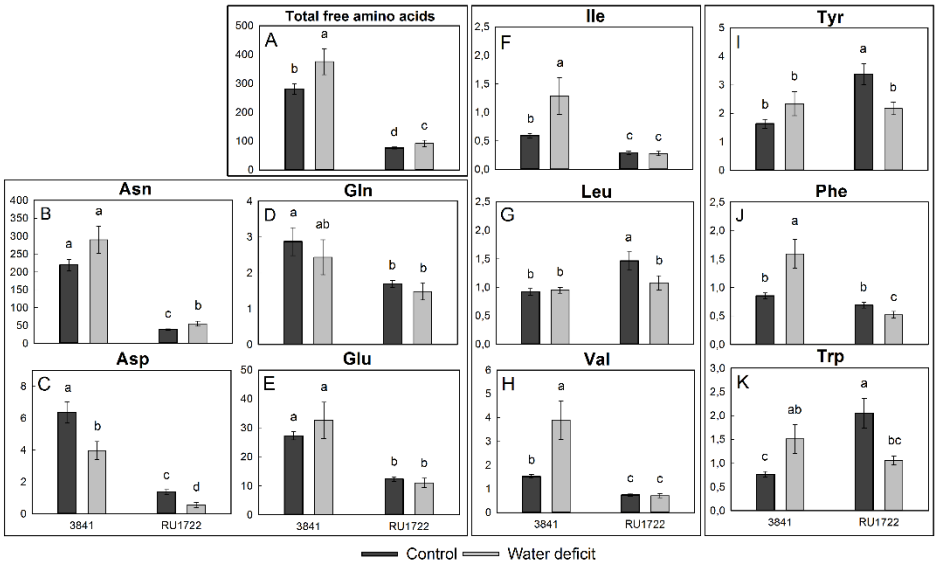

Fig. 2.2.3 Effects of drought on **(A)** total free, **(B-E)** amide and acidic, **(F-H)** branched-chain, and **(I-J)** aromatic amino acids content in nodules of pea plants inoculated with *R. leguminosarum* bv. *viciae* 3841 and RU1722 strains. Values in µmol g NDW^{-1} represent mean ± SE ($6 \leq n \leq 8$

biological replicates). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

Monitoring the levels of organic acids in nodules provides an estimation of the carbon and energy status of the infected cells, since malate is the main carbon source fueling bacteroid metabolic activity. The effect of the *aap/bra* double mutation on the content of anions and organic acids is described in section 2.1.3.4. Here we will focus on the effect of drought stress on these metabolites. The most significant change observed was the decline in the levels of acetate in RU1722 nodules (-70%), while no significant effect was observed in wild-type nodules (Fig. 2.2.4A). Just the opposite was observed for malate, whose content decreased in the wild type but not in nodules of the double mutant (Fig. 2.2.4B). In contrast, drought stress caused a decrease of citrate and succinate in both types of nodules (Figs. 2.2.4C, D). Water deprivation did not cause a significant effect on phosphate, sulphate, α-ketoglutarate, lactate and pyruvate content (Table S2.2.3).

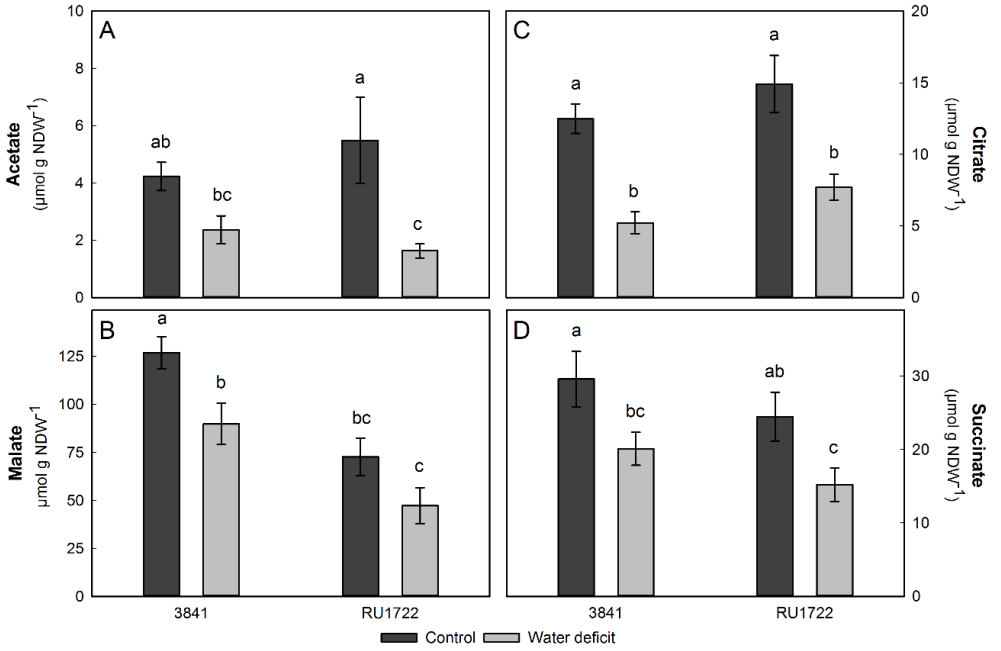

Fig. 2.2.4 Effects of water deficit on (**A**) acetate, (**B**) malate, (**C**) citrate, (**D**) succinate content in nodules of pea plants inoculated with *R. leguminosarum* bv. *viciae* 3841 and RU1722 strains. Values represent mean ± SE (n biological replicates = 4). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD test). NDW, nodule dry weight.

2.2.4 Discussion

2.2.4.1 Water deficit was equally perceived in plants inoculated with either 3841 or RU1722 strains

In the present study, we tested the hypothesis of N feedback regulation of nitrogenase activity during drought stress using the bacterial mutant *aap/bra* with blocked transport of BCAAs at the bacteroid membrane. At the physiological level, both plants inoculated with the wild type strain 3841 and the double mutant RU1722 responded similarly to the water deficit imposed. This similar response was observed both at the level of stomatal conductance, showing a clear decline, as well as leaf and nodule water potential, in agreement with previous works (Aldasoro et al., 2019; Flexas & Medrano, 2002; Gálvez et al., 2005; González et al., 1998, 2001b). Highlights that plants inoculated with RU1722, due to its smaller growth, needed more days to reach values of water potential similar to plants inoculated with 3841.

2.2.4.2 Plants inoculated with wild-type bacteria show a higher decline in BNF under drought stress

In relative terms, nitrogen fixation, measured as ANA, was more severely affected by water deficit in plants inoculated with the wild-type strain 3841 than those inoculated with the double mutant, the latter presenting already low ANA rates under control conditions. It is interesting to note that the levels of nitrogen fixation measured do not correlate well with the content of proteins related to the nitrogenase complex, such as NifB, a protein required for the biosynthesis of the iron-molybdenum cofactor (Christiansen et al., 1998; Shah et al., 1994), NifH, which encodes the iron protein, or the regulatory protein NifA (Martínez et al., 2004). These observed discrepancies indicate that additional regulatory mechanisms modulate the nitrogen fixing rates of symbiotic bacteroids under drought stress conditions.

2.2.4.3 Drought provokes the alteration in the levels of proteins related to O_2 status, carbon metabolism and antioxidant defence

It has been widely described that high O_2 levels in nodules inactivate nitrogenase enzyme complex. One of the strategies used to combine high respiratory rates and low oxygen concentrations in nodules is the presence of

specific high affinity cytochrome *cbb*$_3$-type oxidases encoded by the *fixNOQP* operon in bacteroids. As mentioned in the section 2.1.4.2, low O_2 concentration is required for the transcription of genes within the *fixNOQP* operon. Interestingly, the protein content of FixN, a subunit belonging to this operon, was found to decrease under drought stress in RU1722 bacteroids, suggesting a possible imbalance in the levels of O_2 in the double mutant under stress. In contrast, we did not observe significant changes in the levels of FixP or FixO, as previously reported in *R. etli* bacteroids (Talbi et al., 2012). Regarding the plant protein profile, the content of one of the Lb proteins, group of proteins responsible to maintain suitable O_2 levels in nodules, decreased in 3841 nodules under water deficit, in agreement with observations in *Medicago truncatula* nodules (Larrainzar et al., 2007, 2009). In RU1722 nodules, however, out of the two Lb isoforms detected, the levels of the most abundant one (O48668) increased while the other one (P93848) decreased its content. Furthermore, both in wild-type nodules and in those generated by the double mutant strain, the levels of a non-symbiotic hemoglobin increased under drought stress conditions. In agreement with our work, the accumulation of non-symbiotic hemoglobins has been previously observed under osmotic stress, and it has been related to the regulation of nitric oxide levels (Fukudome et al., 2016).

Earlier studies showed that both the activity and the content of Susy, one of the main enzymes involved in sucrose cleavage in nodules, decline under drought, leading to a decrease in malate content in soybean and pea nodules (González et al., 1995, 1998, 2001b). In 3841 nodules, we found a correlation between the levels of malate and SuSy protein content, thus supporting previous works. The accumulation of GABA previously discussed could be responsible of the lower succinate content, since is the end-product of the pathway known as the GABA-shunt (Shelp et al., 2006). Moreover, in RU1722 nodules, an isocitrate dehydrogenase was accumulated, while an ATP-citrate synthase alpha chain was reduced due to water stress. A higher activity of isocitrate dehydrogenase was found in nodules under both drought (Gálvez et al., 2005) and oxidative stress (Marino et al., 2007b), and it is suggested to be caused to balance carbon and nitrogen metabolism or to improve the antioxidant defence (Gálvez et al., 2005).

Several proteins belong to response to stress functional group were accumulated under water stress in both 3841 and RU1722 nodules. A common response was observed in some proteins, such as thaumathin-like protein, glucan endo-1,3-beta-glucosidase or disease resistance response protein. Thaumatin-like protein is involved in the control of nodulation specificity (Tang et al., 2016). The accumulation of glucan endo-1,3-beta-glucosidase has been also observed in

pea leaves in response to fungal infection (Desalegn et al., 2016), and in the root of a supernodulating variety of soybean (Salavati et al., 2012). Apart from these, in RU1722 nodules, an AGO protein, involved in the regulation of legume-rhizobium symbiosis, as well as in plant defense was accumulated (Valdés-López et al., 2019). In 3841 nodules, a LEA protein was also accumulated, supporting its role in protection during water deficit in different tissues (Battaglia & Covarrubias, 2013). In contrast, the abundance of thiamine thiazole synthase, involved in biosynthesis of the thiamine precursor thiazole, was reduced in both 3841 and RU1722 nodules. The synthesis of thiamine has been related to nodule growth (Nagae et al., 2016). Taken together, our results suggest an activation of plant defense response in pea nodules under drought stress.

2.2.4.4 The *aap/bra* double mutation caused a different regulation of amino acid metabolism under water deficit

In previous works, the accumulation of amino acids involved in the primary N assimilation during stress condition has been related with a possible N-feedback inhibition of BNF (Bacanamwo & Harper, 1997; Neo & Layzell, 1997; Vadez et al., 2000). However, more recent studies in our laboratory indicate that the decline in BNF observed occurs before a significant accumulation os such compounds (Aldasoro et al., 2019; Gil-Quintana et al., 2013a; Ladrera et al., 2007; Larrainzar et al., 2009). In the current work, we observed a drought-induced increase in the content of total free amino acid content in both wild type and double mutant nodules (Fig. 2.2.3). However, the levels of Asn, Gln and Glu, key candidates for this N-feedback regulation, were not found significantly affected by the water deficit imposed, despite BNF being severely reduced in 3841 nodules. Similarly, the proteomic analysis did not reveal any major effect on the content of the main proteins related to N assimilation (i.e. AS, GS, GOGAT or AAT) in 3841 nodules, while AS levels increased in RU1722 nodules (Tables S2.2.1 and S2.2.2). These results are contrast with previous studies in which the content of proteins related to N assimilation was reduced under more severe drought stress conditions (Gil-Quintana et al., 2015; Larrainzar et al., 2007, 2009).

It is interesting to note that Asp content decreased in both 3841 and RU1722 nodules (Fig. 2.2.3). The Asp metabolic pathway gives rise to other amino acids such as Lys, Met, Thr and Ile (Azevedo et al., 1997). An induction of this pathway could explain the observed increase in Lys, Met, Thr and Ile content in drought-stressed 3841 nodules, although this was not the case in plants inoculated with the double mutant. This fact, together with the increase

in the relative content of AS suggest that, in RU1722 nodules, Asp catabolism was through AS to the synthesis of Asn, which levels increased due to water deficit.

Regarding BCAAs, in 3841 nodules, water deficit caused an increase in Ile and Val content, while no effect on Leu was observed (Fig. 2.2.3). The accumulation of these amino acids was also described for nodules under water deficit (Gil-Quintana et al., 2013a, b; Larrainzar et al., 2009; Nasr Esfahani et al., 2014). Studies carried out in non-legume plants have suggested that protein degradation was the main reason for the accumulation of BCAAs in stressed tissues (Huang & Jander, 2017; Hildebrandt, 2018). Although in this work, proteins related to proteolysis were found to accumulate in 3841 nodules, studies in which the proteolytic activity in nodules was measured did not support this hypothesis (Gil-Quintana et al., 2013a). Alternatively, the observed accumulation in the current work could provide substrate for the synthesis of stress-induced proteins, alternative respiration, or act as signalling molecules (Hildebrandt, 2018; Joshi et al., 2010; Pires et al., 2016). Thr, a precursor in the Ile biosynthesis pathway, was also accumulated. But, in this work, the mentioned decrease in TS abundance does not correlate with an accumulation of Thr in wild-type modules. In contrast, in RU1722 nodules, although TS abundance also decreased, only Leu content declined, while Ile, Val and Thr did not show significant variations. Moreover, other proteins involved in BCAA biosynthesis were not affected by water deficit in either 3841 or RU1722 nodules. These suggests that the variations of BCAA are not associated to reduce biosynthetic activity.

In the case of aromatic amino acid content, opposite trends were observed in both 3841 and RU1722 nodules. In wild-type nodules, an accumulation was measured, which could be related with the induction of the synthesis of secondary metabolites related with the response to abiotic stresses, such as, glucosinolates or phenylpropanoids (Batista-Silva et al., 2019). Regarding RU1722 nodules, the decline observed could be related with the reduced abundance in the protein chorismate mutase, involved in the synthesis of Tyr and Phe.

Lastly, the content of GABA was found to largely increase under drought stress in both 3841 and RU1722 nodules, as it was previously observed in *Medicago truncatula* and *Glycine max* nodules (Gil-Quintana et al., 2013a; Serraj et al., 1998). GABA is one of the amino acids actively metabolized in bacteroids, although not strictly required BNF at least in pea (Prell et al., 2009b). The role of GABA in nodules has been mainly related to energy supply to bacteroids or part

of an amino acid cycle between bacteroids and the cytosol (Prell et al., 2009b; Sulieman & Schulze, 2010). GABA can be taken up by Bra ABC transport system, therefore, the higher increase observed in RU1722 nodules compared to 3841 nodules may be due to the inhibition of its transport to bacteroids, where 3841 bacteroids can be degrading it to mitigate stress. It would be interesting to determine in which nodule fraction, plant vs. bacteroid, is GABA actually accumulating.

2.2.5 Conclusions

Water deficit inhibited BNF in plants inoculated with the wild-type strain. In contrast, there is no effect of water deficit on BNF rates in plants inoculated with the RU1722 strain, probably because BNF was already inhibited in plants inoculated with the double mutant. Although an increased abundance of proteins related to BNF is observed in RU1722 bacteroids, additional metabolic factors are clearly limiting BNF in these plants.

This study highlights the relevance of low abundant amino acids, such as Met, aromatic amino acids or GABA, in the response to water deficit at the nodule level. In 3841 nodules, water deficit led to an induction of amino acid pathways related to the biosynthesis of stress-associated related compounds. In RU1722 nodules these alterations in amino acid metabolism were attenuated. A similar effect was observed in another key group of metabolites, organic acids. Interestingly, our proteomic work shows the activation of a plant defence response under drought stress both in wild type nodules and in those inoculated with the double mutant.

2.2.6 Supplementary material

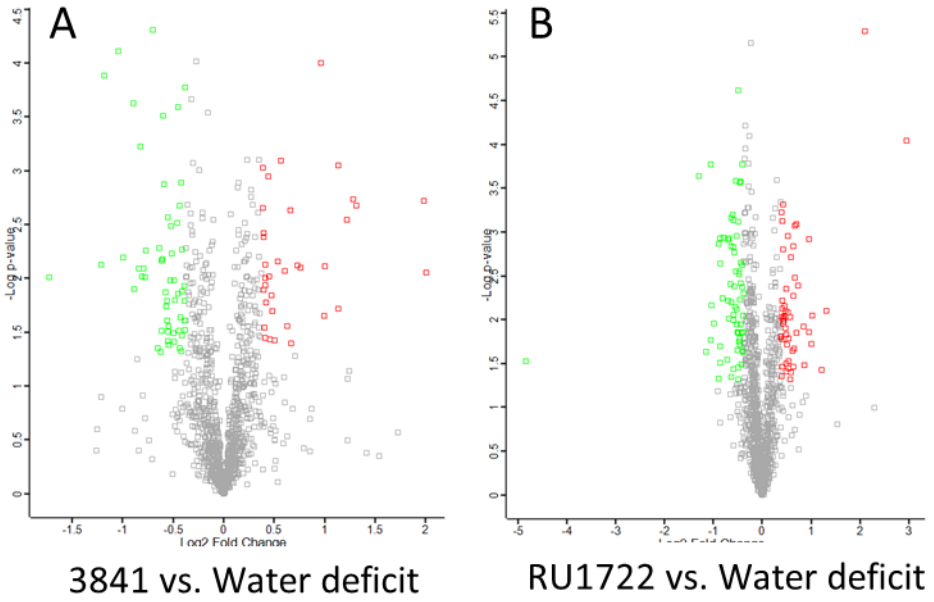

Fig. S2.2.1. Volcano plots representing the fold-change of identified proteins with associated p values from the pair-wise quantitative comparisons of pea nodules inoculated with *R. leguminosarum* bv. *viciae* 3841 and RU1722 strains under drought. In red, proteins more abundant under control conditions, in green proteins more abundant under water deficit. $p < 0.05$, and a fold change of < 0.77 or > 1.3 on a linear scale.

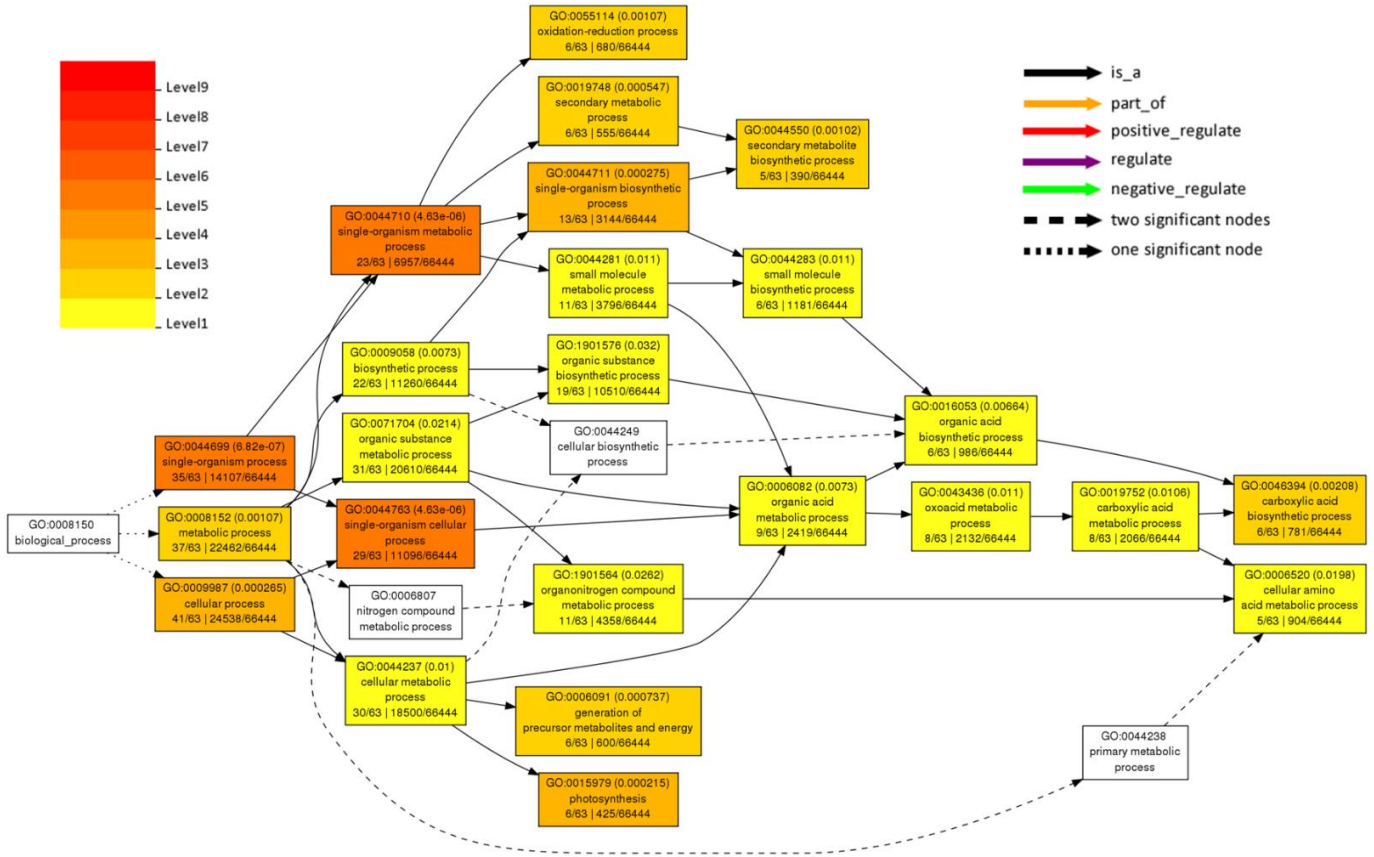

Fig. S2.2.2. GO enrichment analysis of the biological process of differentially accumulated plant proteins of pea nodules inoculated with *R. leguminosarum* bv. *viciae* 3841 under water deficit.

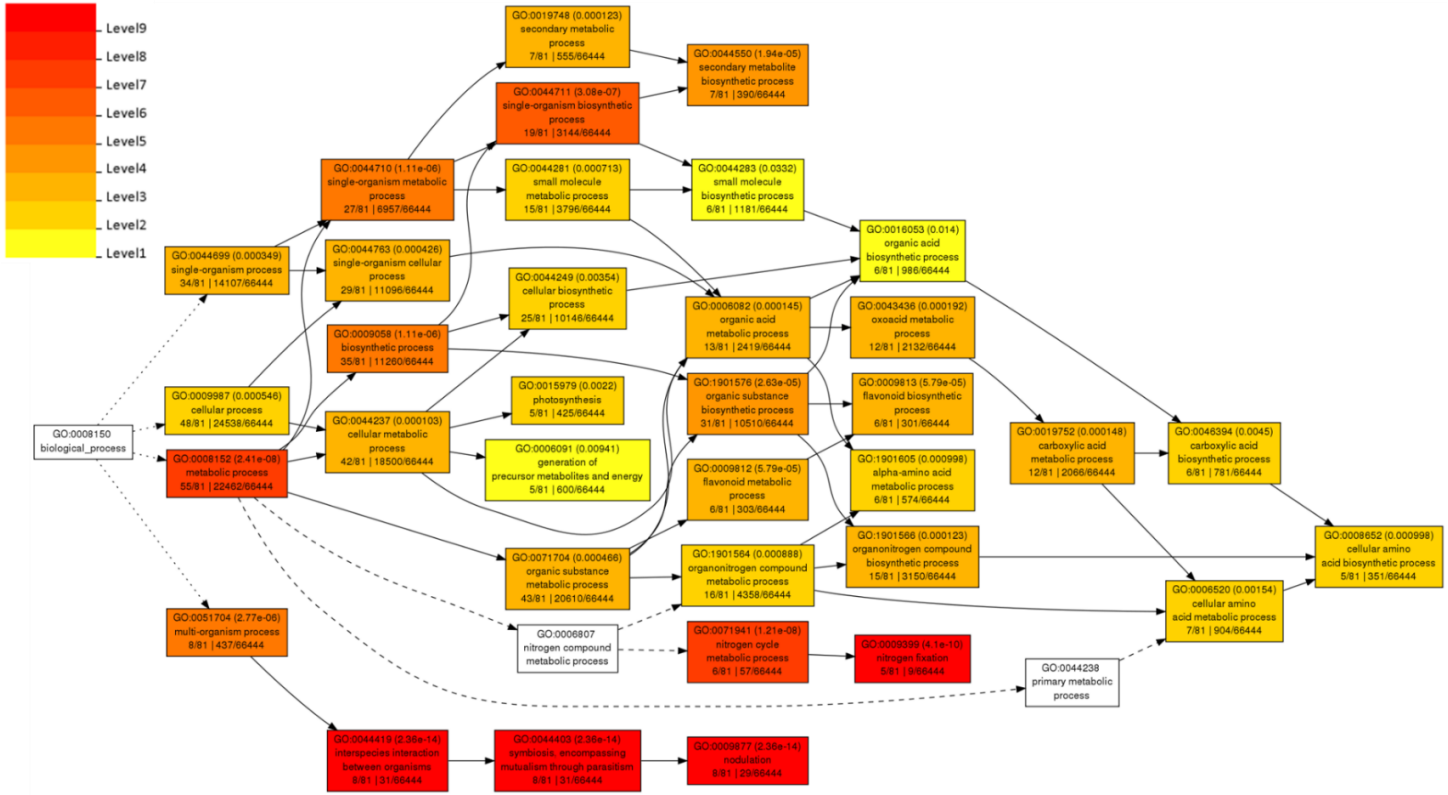

Fig. S2.2.3 GO enrichment analysis of the biological process of differentially accumulated plant proteins of pea nodules inoculated with *R. leguminosarum* bv. *viciae* RU1722 under water deficit. Arrow legend in Fig. S2.2.2.

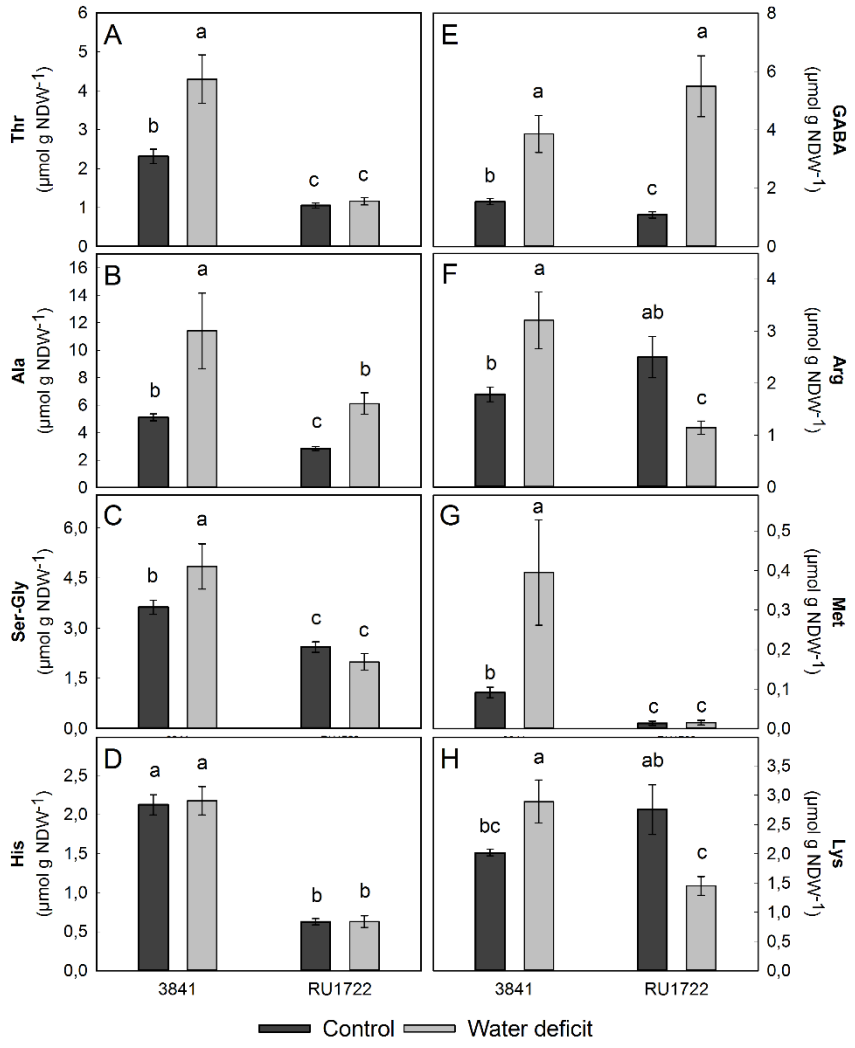

Fig. S2.2.4 Effect of water deficit on amino acids content in nodules of pea plants inoculated with *R. leguminosarum* bv. *viciae* 3841 and RU1722 strains. Values represent mean ± SE ($6 \leq n \leq 8$ biological replicates). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

Table S2.2.3 Effect of water deficit (WD) on organic acid content in nodules of pea plants inoculated with *R. leguminosarum* bv. *viciae* 3841 and RU1722 strains. Values in µmol g NDW^{-1} represent mean ± SE (n biological replicates = 4). Different letters indicate significant differences between treatments and/or strains ($p \leq 0.05$, LSD/T3 Dunnet test). NDW, nodule dry weight.

Organic Acids	3841	RU1722	3841+WD	RU1722+WD
α-ketoglutarate	31.29 ± 1.22 a	33.06 ± 3.97 a	23.05 ± 3.56 a	25.85 ± 3.54 a
Lactate	3.67 ± 0.89 b	6.73 ± 0.53 ab	5.99 ± 1.03 ab	8.86 ± 1.49 a
Pyruvate	1.98 ± 0.12 a	1.93 ± 0.24 a	2.04 ± 0.43 a	1.17 ± 0.29 a

Chapter 3

Drought causes a source-sink imbalance in the soybean-*Bradyrhizobium diazoefficiens* symbiosis

3.1 Introduction

One of the best-known ecological features of legumes is the ability to establish a symbiotic relationship with microorganisms, generically called rhizobia. This symbiosis gives rise to the process known as symbiotic nitrogen fixation (SNF), which consists in the reduction of molecular nitrogen to ammonium. Using ammonium, plants synthesize organic nitrogenous products essential for their nutrition, reducing the impact of use of nitrogen fertilizers on the environment. This fact has favoured their cosmopolitan distribution, being able to find them even in the most extreme habitats (Schrire et al., 2005).

However, BNF is a very sensitive process to environmental stresses such as extreme temperatures, defoliation, salinity, drought, acidity, nutrient limitation, among others (reviewed by Araújo et al., 2015). Therefore, research lines aimed at improving BNF, have a considerable interest from the ecological and agronomical point of view. Among the different stresses, water deficit is one of the natural hazards that affect most significantly plant growth and crop yield in agricultural systems (Bray, 1997). Considering current climate change scenarios and the need to provide food for a growing population, it is necessary to know and anticipate the consequences of drought stress in agriculture (Shanker et al., 2014). Under drought stress one of the physiological processes that is first affected in nodulated legumes is BNF (Arrese-Igor et al., 2011; Kunert et al., 2016). This fact has been mainly related to three factors: i) oxygen limitation in nodules (reviewed in Aranjuelo et al., 2014; Arrese-Igor et al., 2011; Minchin, 1997); ii) feedback regulation of nitrogenase by nitrogen compounds (Aranjuelo et al., 2014; Arrese-Igor et al., 1999; Murray et al., 2016; Serraj et al., 1999b); iii) a reduction in the flux of carbon within nodules due to a decrease in sucrose synthase activity (González et al., 1995). Focusing on the role of carbon shortage, previous results of our group suggested the existence of a local rather than a systemic regulation of these responses (Gil-Quintana et al., 2013b; González et al., 2015; Marino et al., 2007a). However, the role of the sink strength and whether drought alters the source-sink relations in the plant has not been studied in detail. Likewise, the physiological mechanisms in which drought impacts the plant C flux remains largely unknown.

One of the classical approaches for the study of source-sink relationships in plants is the use of labelling techniques. Several methodologies can be applied: i) assimilation chambers (Bach et al., 1958; Geiger & Fondy, 1979); ii) perfusion techniques (Long et al., 1971); or iii) by small wounds made on the leaf cuticle (Fondy & Geiger, 1977). Moreover, C fluxes can be analysed using either CO_2 or

carbohydrates (sucrose, fructose or glucose) enriched with different carbon isotopic forms (Bach et al., 1958; Housley et al., 1977; Nelson & Gorham, 1957; Vernon & Aronoff, 1952). Nevertheless, labelled-CO_2 approaches have several shortcomings for drought stress studies. In natural conditions, the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) discriminates against ^{13}C due to its lower reactivity (Farquhar et al., 1989; O'Leary, 1981). However, under drought, $^{13}CO_2$ fixation by Rubisco increases (Brüggemann et al., 2011; Farquhar et al., 1989). Under situations of lower stomatal aperture the incorporation of labelled CO_2 is decreased thus affecting the accumulation of $^{14}CO_2$ (Zolkevich et al., 1958). Additionally, it has also been described that application of CO_2 levels beyond natural concentration may alter plant metabolism (Dersch et al., 2016). Therefore, for drought stress studies, foliar application of ^{13}C-labelled sucrose is a suitable alternative.

In the current work we tested the hypothesis that drought stress may have an impact in the source-sink relationships of nodulated legumes. To test it, we employed soybean plants (Glycine max (L.) Merr. var. Sumatra) grown under symbiotic conditions and subjected them to progressive water deficit. To monitor the C flux across the plant, the youngest fully expanded leaf was labelled with [U-^{13}C]-sucrose. Tissue samples were taken hours after the labelling to monitor sucrose distribution over time. In parallel, a set of key metabolites was measured in the different tissue sampled. Results show that drought stress decreased ^{13}C transport to sink tissues and changed the priority of C allocation between sinks organs. We also found that drought affected C and N metabolism in different waysly in source orand sink organs.

3.2 Materials and methods

3.2.1 Plant growth conditions

Soybean seeds (*Glycine max* (L.) Merr. var. Sumatra) were surfaced sterilized by placing them in 1% NaClO (v/v) and 0.01% SDS (w/v) for 40 minutes and after washing them with deionized water, they were placed in 0.01N HCL for 10 minutes (Labhilili et al., 1995). Then, seeds were germinated and sown in 1 L-pots containing a mixture of vermiculite:perlite (2:5, v/v). Seedlings were then inoculated with 1 mL of the *hup Bradyrhizobium diazoefficiens* strain UPM792 at day 0 and 3 days after sowing. Plants were grown during 1 month under controlled conditions (24°C/18°C day/night temperature, 60%/70% day/night relative humidity, and 16 h photoperiod), and

watered to field capacity two times a week with nitrogen free-nutrient solution (Rigaud & Puppo, 1975).

3.2.2 Experimental design and stable isotope labelling method

Drought stress was gradually imposed in one month-old soybean plants by withholding water. Plants were separated in three groups depending on the leaf water potential (Ψ_{leaf}) reached. The group kept under optimal water conditions maintained a Ψ_{leaf} value (mean ± standard error) of -0.24 ± 0.05 MPa (control), the two others groups subjected to water restriction reached a Ψ_{leaf} value of -0.44 ± 0.03 MPa (mild drought) or -0.85 ± 0.08 MPa (severe drought). Each group was separated, in turn, in three subsets and samples were collected at 2, 5 and 8 h after the beginning of photoperiod. Due to low weight of sink leaf, it was necessary 4 biological replicates of this tissue for the metabolite extraction, and, for this reason, make a pool for the measurements.

In order to know the natural abundance of ^{13}C and for physiological characterization of soybean plants, only half of them were labelled with [U-^{13}C]-sucrose (Sercon Ltd., Crewe, United Kingdom). The labelled half was used to know the effect of drought on carbon allocation. Once drought stress was reached, control and stressed plants were labelled 2 h after the beginning of photoperiod (time 0 h) as described Fondy & Geiger (1977). At that moment, the upper surface of the youngest fully expanded leaf was gently abraded with a fine grain sandpaper. Then, 25 µL solution containing 20 mM [U-^{13}C]-sucrose buffered in a 5 mM K-phosphate buffer (pH 6.5) was applied to the abraded area (Fondy & Geiger, 1977). Then, the sugar solution uptake was allowed to continue during 3 or 6 h. At these times the plants were separated into labelled leaf, sink leaf (around 26% of full lamina length), other leaves (source leaves), stem, roots and nodules. These samples were separately weighed. One set of aliquots were immediately frozen in liquid nitrogen and stored at -80 °C for analytical determinations. Other set was dried at 70 °C during 72 h, grounded to fine powder and weighed into tin capsules for elemental analysis-isotope ratio mass spectrometry (EA-IRMS) and for dry weight (DW) measurements. The same procedure was carried out for non-labelled plants.

3.2.3 Physiological characterization

To establish the effect of drought stress on plants, various parameters were measured: stomatal conductance, net photosynthesis, leaf water potential and

apparent nitrogenase activity (ANA). These parameters were measured 2 h after the beginning of photoperiod in the youngest fully expanded leaf.

Stomatal conductance and net photosynthesis were measured with a portable open system mode (model LCpro+; ADC BioScientific Ltd., Great Amwell, UK) using an ADC PLC-7504 leaf chamber. Ψ_{leaf} was measured using a pressure chamber (Scholander et al., 1965). Symbiotic nitrogen fixation was measured as in section 1.2.4.

3.2.4 Carbon isotope analysis

The carbon percentage and $\delta^{13}C$ value were determined by EA-IRMS in a DeltaPlus (ThermoQuest, Bremen, Germany) coupled to an elemental analyser NC2500 (CarloErba, Milan, Italy). The results of the carbon isotope ratio analyses are expressed in delta notation. According to Farquhar et al. (1982), the carbon isotope composition with respect to VPDB (Vienna Pee Dee Belemnite) is: $\delta^{13}C$ (‰) = $[(R_s/R_{VPDB}) - 1] \times 1000$, where R is the $^{13}C/^{12}C$ ratio of the sample and the standard, respectively. These results were used to obtain the estimation of the transported carbon from the labelled source leaf. The total ^{13}C presented in different tissues was determined by subtracting the $\delta^{13}C$ of the tissues from labelled plants from that of the unlabelled plants, taking into account, in addition, the carbon percentage and the biomass of each tissue.

3.2.5 Metabolites extraction and determination

For the determination of carbohydrates, aliquots (0.1 g) of fresh tissue (sink leaf, source leaf, stem, roots and nodules) were extracted as described by Marino et al. (2006). Carbohydrates were determined by ion-exchange chromatography in a 940 Professional IC Vario with amperometric detector (Metrohm AG, Herisau, Switzerland) equipped with Metrosep Carb 2 Guard/4.0 + Metrosep Carb 2 - 150/4.0 columns. Samples were eluted with 300 mM NaOH/1mM $C_2H_3NaO_2$ at 30°C.

To determine the organic acid content, aliquots (0.1 g) of fresh tissue (sink leaf, source leaf, stem, roots and nodules) were extracted as described by Gálvez et al. (2005). Organics acids were determined as described in section 2.1.2.10.

Finally, the same extract that for carbohydrates analysis was used for the determination of nitrogen compounds. Ureides and asparagine were determined by high performance capillary electrophoresis (P/ACE 5500; Beckman Coulter Instruments, Fullerton, CA, USA) by the method described in Sato et al. (1998).

A solution of 0.1 M $Na_2B_4O_7 \cdot 10H_2O$ (pH 9.2) in 25 mL L^{-1} OFM-Anion BT (Waters Corp., Milford, MA, USA) was used as electrolyte in a fused-silica capillary tube 60 cm length. Ureides and asparagine were detected by optical density at 190 nm.

3.2.6 Statistical analysis

All data are presented as mean ± standard error of n ≥ 3 biological replicates. Significant differences in physiological parameters between control and stress treatments were determined by using Student´s *t*-test at $p \leq 0.01$. In the case of ^{13}C distribution, data were expressed in percentages, then, for the statistical analysis data were transformed according to the following formula: $arcsin\sqrt{x/100}$. Significant differences between treatments or times were determined by using Student´s *t*-test at $p \leq 0.05$. Results of $\delta^{13}C$, carbohydrates, organics acids and nitrogen compounds were analysed by one-way ANOVA after checking the normal distribution of the samples via the Shapiro-Wilk test and the homogeneity of variances via Levene's test, applying log transformation in the case of no homogeneity. Comparisons between each treatment and times were performed using least significant difference (LSD) or Dunnet T3 test. Differences were considered to be significant at $p \leq 0.05$. The Perseus software (version 1.6.5.0) (Tyanova et al., 2016) was used to represent the heatmap and cluster aggrupations. To that, metabolite content was logarithmized (base 2), and a multiple sample test (ANOVA, $p < 0.05$) was applied followed by Z-score normalization.

3.3 Results

3.3.1 Drought effects on the physiological status of soybean plants

To analyze the effects of water withholding on physiological status of soybean plants, the following physiological parameter were measured: stomatal conductance, net photosynthesis, leaf water potential (Ψ_{leaf}) and apparent nitrogenase activity (ANA). Drought led to a gradual decline in Ψ_{leaf}, reaching values of -0.44 ± 0.03 and -0.85 ± 0.08 MPa under mild and severe drought, respectively, compared with control plants which maintained a Ψ_{leaf} of -0.24 ± 0.05 MPa (Table 3.1). Over the course of the experiment, stomatal conductance remained constant in control plants (0.31 ± 0.01 mol m^{-2} s^{-1}), while drought stress provoked an 81% and 93.2% decline under mild and severe drought,

respectively (Table 3.1). Related to stomatal closure is net photosynthesis decline, which declined 59% under mild drought conditions, while under more severe conditions there was a 91.5% decline (Table 3.1). The effect of drought stress on BNF was measured as ANA in intact soybean plants. Under mild drought stress, ANA was more negatively affected than photosynthesis or Ψ_{leaf}, which only decline around 50% while ANA decline 71.4% (Table 3.1), compared to well-watered plants. Under severe drought stress, there was an almost complete inhibition of BNF with an 88.8 % decline (Table 3.1).

Table 3.1 Drought effects on several parameters (stomatal conductance, leaf water potential, photosynthesis, apparent nitrogenase activity (ANA)) of control and drought-stressed plants before labelling (0 hours). Values represent mean ± SE (n biological replicates ≥ 4). An asterisk (*) within a row indicate significant differences from control plants. (Student´s *t*-test at $p \leq 0.01$). NDW, nodule dry weight.

Parameter (Units)	Control	Drought	
		Mild	Severe
Stomatal conductance (mol m^{-2} s^{-1})	0.31 ± 0.01	0.06 ± 0.02*	0.02 ± 0.00*
Leaf water potential (MPa)	-0.24 ± 0.05	-0.44 ± 0.03*	-0.85 ± 0.08*
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	7.91 ± 0.29	3.24 ± 0.54*	0.67 ± 0.20*
ANA (μmol H_2 g NDW^{-1} min^{-1})	1.16 ± 0.02	0.33 ± 0.04*	0.13 ± 0.02*

3.3.2 Drought stress reduces natural carbon isotopic composition

$\delta^{13}C$ was determined in source leaves, sink leaf, stem, roots and nodules of soybean plants under different water status. Measures were carried out at three times on the morning, 2, 5 or 8 h after the beginning of photoperiod (referred to as time 0, 3 and 6 h, respectively). In well-watered plants, source leaves had a lower $\delta^{13}C$ than sink leaf, stem, nodules or root samples, indicating a ^{13}C enrichment in non-photosynthetic tissues (Figure 3.1). Water withholding affected carbon isotopic composition, resulting in less negative $\delta^{13}C$ (Fig. 3.1). In source leaves, there was no significant variation in $\delta^{13}C$ throughout the 6 h of experiment under drought stress. In contrast, in control plants $\delta^{13}C$ changed towards more negatives values, indicating a ^{13}C loss in this tissue (Fig. 3.1A). Drought effect over $\delta^{13}C$ was more significant in sink than in source leaves. Moreover, $\delta^{13}C$ of sink leaves under severe drought became less negative over time, unlike the observed trend in severe stressed source leaves (Figs. 3.1A, B). In root samples, $\delta^{13}C$ of drought-stressed plants remained constant over the

course of the experiment, while $\delta^{13}C$ of control root declined over time (Fig. 3.1D). In contrast, there was no significant effect of drought on $\delta^{13}C$ of stem and nodules (Figs. 3.1C, E).

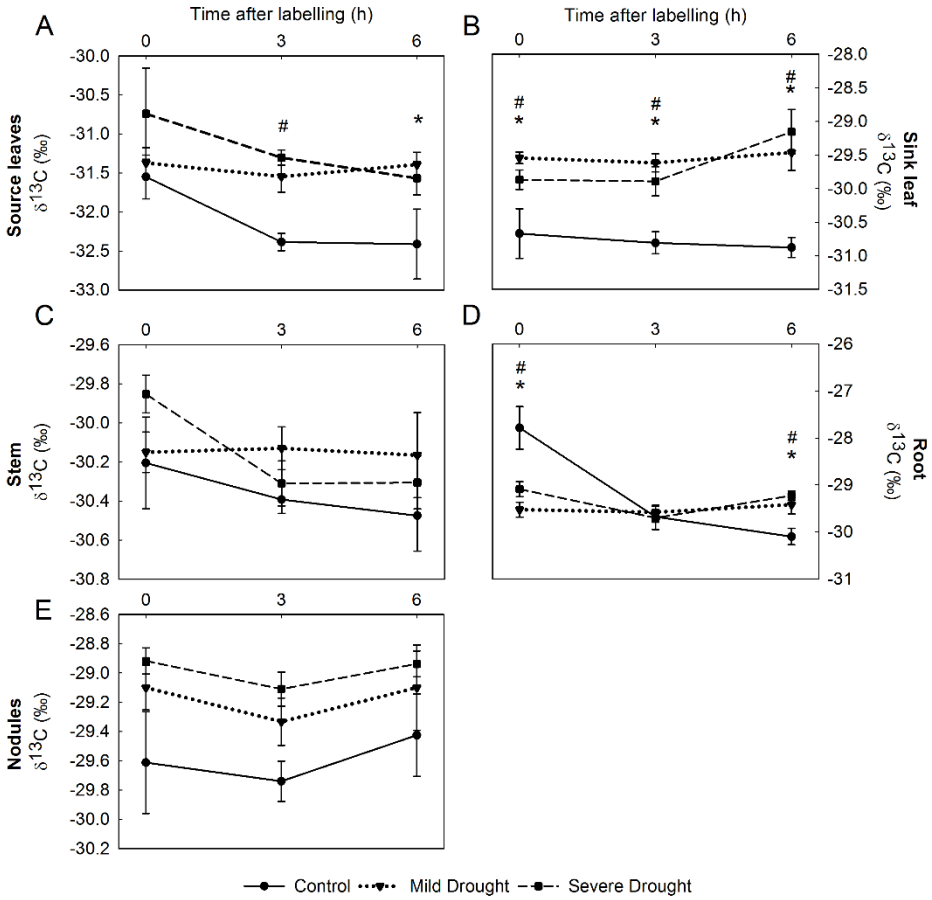

Fig. 3.1 Changes in $\delta^{13}C$ values in (A) source and (B) sink leaves, (C) stem, (D) root an (E) nodules of unlabelled soybean plants under two levels of drought. Data points correspond to samples taken 2, 5 and 8 h after the beginning of photoperiod (named as: time 0, 3 and 6 h, respectively, in order to compare with labelling time). Values represent mean ± SE (n biological replicates ≥ 3). An asterisk or a hash sign (* or #) indicate significant differences between mild or severe drought and control plants, respectively (one-way ANOVA with posthoc LSD test; $p \leq 0.05$).

3.3.3 Drought changes carbon allocation between source and sink tissues

To test the effect of water deficit on C allocation, the youngest source leaf was labelled with [U-^{13}C]-sucrose, and the distribution of ^{13}C was monitored.

Drought stress did not produce significant changes in the total amount of ^{13}C (from [U-^{13}C]-sucrose), compared with well-watered plants (Fig. 3.2). However, the total amount of ^{13}C decreased by ca. 35% in both control and mild-droughted plants at the end of the experiment. Note that the total amount of ^{13}C in each tissue is expressed as a proportion of total amount of ^{13}C found in soybean plants at each time (Fig. 3.3).

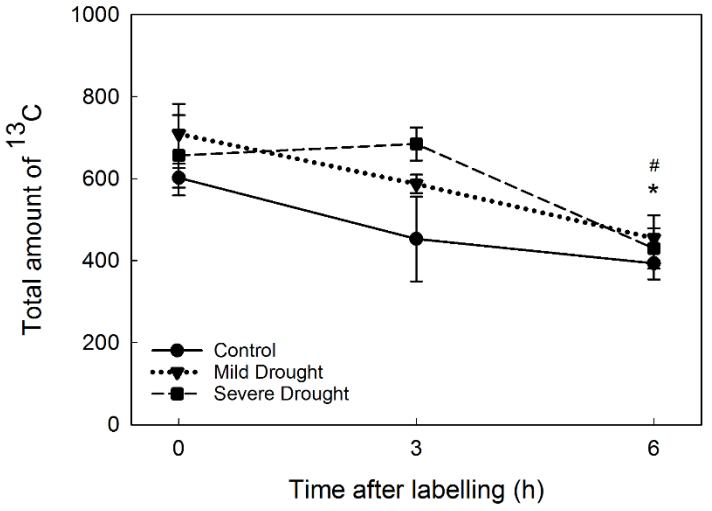

Fig. 3.2 Amount of ^{13}C found in labelled soybean plants under three different water regimes, well-watered plants (control) and drought-stressed plants (mild or severe drought). Plants were labelled with [U-^{13}C]-sucrose 2 h after the beginning of the photoperiod (time 0 h) and for 3 and 6 h. Values represent mean ± SE (n biological replicates ≥ 3). Statistical differences with respect to initial time (0 h) (One-way ANOVA with posthoc LSD test; $p \leq 0.05$) are represented with an asterisk or a hash sign (* or #) in control and mild drought plants, respectively.

In control plants, after 3 h of labelling, 83.3% of total ^{13}C was retained in the labelled leaf, this percentage dropped to 78.9% at the end of the experiment (Fig. 3.3A). Therefore, around 16.7 or 21.1% of ^{13}C was transported to other tissues after 3 or 6 h of labelling time, respectively. In the first 3 h, the major fate of ^{13}C transported was roots (5.8%) following by sink leaf (5.4%); third and fourth place was to nodule (4%) and stem (1.9%), respectively (Fig. 3.3A). After 6 h of labelling, there was a change in the previous trend, taking priority ^{13}C transport to sink leaf (14.9%), decreasing the ^{13}C in the remaining tissues (Fig. 3.3A).

Mild drought stress caused around 60% decline in the amount of ^{13}C transported (Fig. 3.3B). Under this stress situation, the main fate of ^{13}C transported was roots following by the stem, nodule and, finally, sink leaf (Fig.

3.3B). The same trend was observed in both 3 and 6 h after labelling. When compared with control plants, the largest decline in ^{13}C was observed in sink leaves going from 5.4 or 14.9% to 0.1 or 0.3% after 3 or 6 h after labelling, respectively (Figs. 3.3A, B). In nodules, mild stress caused a delay in C input, showing a ^{13}C decrease after 3 hours of labelling, but reaching values close to control plants at the end of the experiment (Figs. 3.3A, B).

Severe drought led to a further decline in the ^{13}C transported (Fig. 3.3C). As in mild drought, ^{13}C transport towards sink leaf was almost inhibited when compare with control plants (Figs. 3.3A, C). In roots and stem, water deficit caused a delay in the arrival of ^{13}C (Fig. 3.3C). Finally, in nodules, ^{13}C decreased by ca. 85% over the course of the experiment, comparing with control plants (Figs. 3.3A, C).

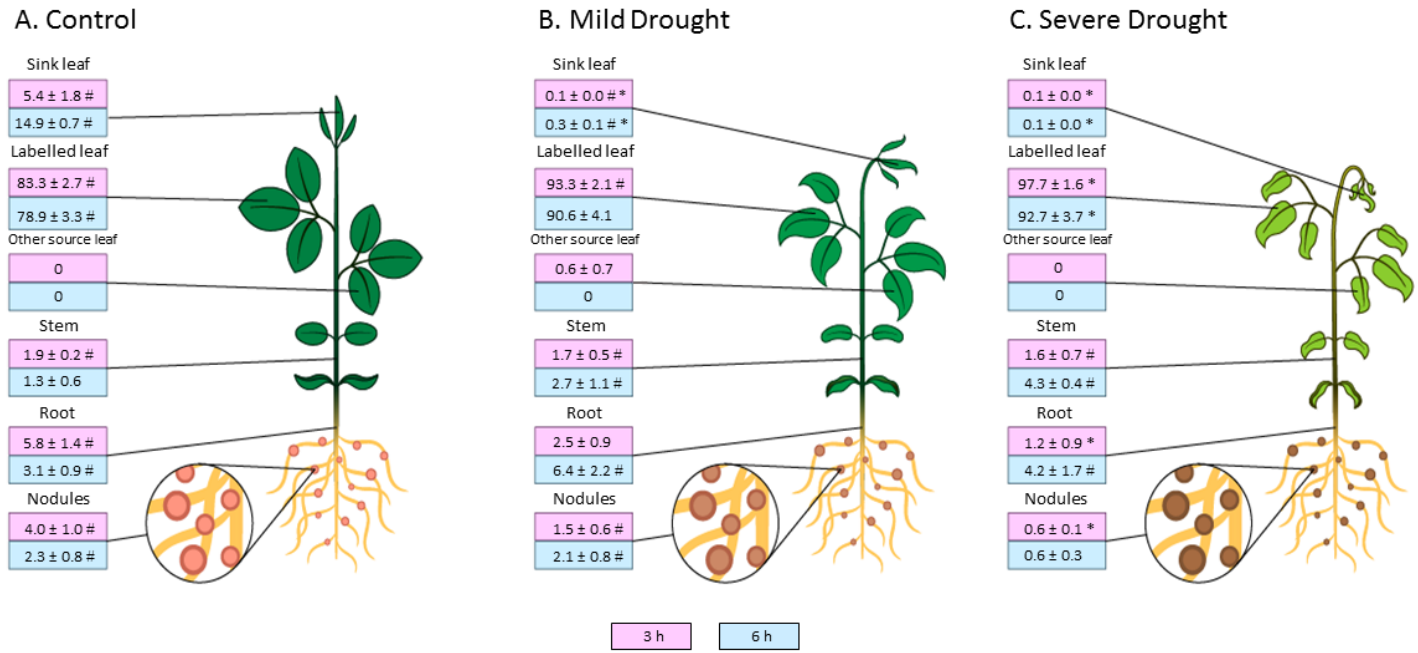

Fig. 3.3 Drought effects on distribution of ^{13}C from labelled leaf to other plant tissues after 3 (pink) or 6 (blue) hours of labelling with [U-^{13}C]-sucrose. At the beginning of experiment (0 h) 100% of ^{13}C was in labelled leaf, and therefore, 0% in the other tissues. Percentages represent mean ± SE ($n \geq 3$). An asterisk (*) indicate significant differences from the control plants. A hash (#) indicate significant differences from time 0 h within the same water status (Student´s *t*-test at $p \leq 0.05$).

3.3.4 Plant metabolite content and distribution were affected by drought

To obtain an overview of the metabolic responses of soybean under our experimental conditions, carbohydrates, organic acids and the key N compounds asparagine and ureides were measured in the different plant tissues.

Drought caused a general increase in carbohydrates detected in source leaves (Table 3.2). In the case of sucrose, its content remained stable under mild drought but increased under more severe stress (235.4 ± μmol g DW^{-1}). In contrast, starch concentration was higher under mild than in severe drought stress. Regarding nitrogen compounds, drought caused a decrease in ureides concentration, being not detected under severe drought (Table 3.2). Instead, asparagine (Asn) concentration increased due to drought stress, reaching values of 9.5 ± 1.3 μmol g DW^{-1} under severe stress. The organic acids content decreased, being malonate the most negatively affected, which concentration decreased around 85 or 70 % under mild or severe stress, respectively (Table 3.2). Regarding polyols, water deficit increased its concentration (Table 3.2). The most significant increase was observed in myo-inositol content, which increased until 44.8 ± 3.8 μmol g DW^{-1} under severe stressed (Table 3.2).

In sink leaves, drought also caused a general increase in all carbohydrates detected (Table 3.3). Under mild drought was glucose which experienced the biggest increase (8.1-fold), instead, under severe drought was sucrose (7.2-fold) (Table 3.3). In contrast, the concentration of starch was found to be almost negligible. The concentration of ureides decreased due to mild drought, but, under a severe stress, there was no significant effect of water status. Asn concentration increased as the drought stress increased (Table 3.3). Unlike the trend observed in source leaves, drought increased malate and malonate content in sink leaves. Moreover, drought also induced the accumulation of polyols. The greatest increase was observed in myo-inositol concentration under both levels of drought (4.7 and 6.3-fold, respectively) (Table 3.3).

Table 3.2 Changes in the concentration of carbohydrates, nitrogen compounds, organic acids and polyols (μmol g DW^{-1}) in source leaves from either, well-watered plants (control) or drought-stressed plants (mild or severe drought). Data set correspond to samples taken 2 h after the beginning of photoperiod. Values represent mean ± SE (n ≥ 3 biological replicates). An asterisk (*) within a row indicates significant differences from the control plants. (One-way ANOVA with posthoc LSD/T3 Dunnet test; $p \leq 0.05$). ND, not detected.

Source leaves	Control	Drought	
		Mild	Severe
Carbohydrates			
Fructose	40.81 ± 9.24	92.68 ± 25.24*	180.78 ± 26.32*
Glucose	24.17 ± 5.82	50.90 ± 15.16	95.24 ± 16.88*
Starch	1083.15 ± 188.39	1827.02 ± 116.95*	1409.24 ± 127.53
Sucrose	82.00 ± 8.80	75.57 ± 4.56	235.36 ± 20.37*
N compounds			
Allantoic Acid	57.26 ± 15.91	1.16 ± 0.58*	ND
Allantoin	1.96 ± 0.77	0.18 ± 0.12	ND
Asparagine	0.77 ± 0.45	2.81 ± 0.90	9.49 ± 1.26*
Organic Acids			
Citrate	14.44 ± 2.84	11.15 ± 1.12	12.72 ± 3.43
Malate	19.98 ± 2.54	10.82 ± 3.00	12.03 ± 4.94
Malonate	111.13 ± 19.57	17.02 ± 4.68*	36.09 ± 4.73*
Oxalate	51.37 ± 4.47	36.92 ± 4.94	44.61 ± 0.92
Polyols			
Mannitol	0.85 ± 0.03	1.13 ± 0.14	1.45 ± 0.22*
Myo-Inositol	20.35 ± 3.15	28.88 ± 5.76	44.76 ± 3.82*
Pinitol	71.94 ± 2.06	77.71 ± 8.55	81.59 ± 2.03
Sorbitol	1.17 ± 0.12	0.90 ± 0.05	1.61 ± 0.07*

Table 3.3 Changes in the concentration of carbohydrates, nitrogen compounds, organic acids and polyols (μmol g DW^{-1}) in sink leaves from either, well-watered plants (control) or drought-stressed plants (mild or severe drought). Data set correspond to samples taken 2 h after the beginning of photoperiod. Values represent mean ± SE (n ≥ 3 biological replicates). An asterisk (*) within a row indicates significant differences from the control plants. (One-way ANOVA with posthoc LSD/T3 Dunnet test; $p \leq 0.05$).

Sink leaves	Control	Drought	
		Mild	Severe
Carbohydrates			
Fructose	13.24 ± 0.03	69.76 ± 1.47*	37.31 ± 0.73*
Glucose	14.52 ± 0.45	117.72 ± 2.82*	63.96 ± 2.12*
Starch	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
Sucrose	29.23 ± 0.64	198.53 ± 5.38*	211.39 ± 6.46*
N compounds			
Allantoic Acid	38.90 ± 1.51	19.03 ± 10*	35.80 ± 2.64
Allantoin	6.90 ± 0.32	4.55 ± 0.22*	6.56 ± 0.40
Asparagine	5.30 ± 0.40	6.82 ± 0.37*	10.28 ± 0.13*
Organic Acids			
Acetate	10.27 ± 1.94	9.47 ± 0.16	4.96 ± 0.18*
Citrate	29.06 ± 1.60	25.86 ± 0.44	15.31 ± 1.76*
Malate	65.37 ± 4.15	106.82 ± 0.64*	84.08 ± 2.81*
Malonate	29.12 ± 1.53	41.70 ± 0.39*	51.13 ± 1.01*
Oxalate	48.94 ± 3.74	49.20 ± 1.46	46.41 ± 7.90
Polyols			
Mannitol	2.58 ± 0.07	4.13 ± 0.12*	2.77 ± 0.06
Myo-Inositol	8.09 ± 0.10	37.89 ± 1.38*	51.48 ± 1.54*
Pinitol	144.50 ± 1.45	226.82 ± 9.24*	198.00 ± 6.07*
Sorbitol	4.16 ± 0.09	5.08 ± 0.14	2.40 ± 0.03

In stem, hexoses concentration increased similarly under mild and severe drought stress (around 2 and 4-fold, respectively), while sucrose was only accumulated under severe stress, reaching 98.8 ± 3.1 μmol g DW^{-1} (Table 3.4). Allantoic acid increased under mild drought but remained stable under severe drought, in contrast, allantoin was not affected by drought (Table 3.4). Interestingly, Asn concentration increased around 5-fold under both levels of drought stress (Table 3.4). Between detected polyols, malate content decreased a 71 and 41% under mild or severe stress, respectively (Table 3.4). Regarding polyols, myo-inositol content increased 2.4 or 1.7-fold under mild or severe stress, respectively (Table 3.4).

In roots, the concentration of carbohydrates increased due to drought (Table 3.5). The greatest increase was detected in fructose with a 5.5 or 11.6-fold increase under mild or severe stress, respectively. Regarding N compounds, its concentration was almost negligible in control roots, but it could be seen a large increase under drought (Table 3.5). Different trend was observed in organic acids, while water deficit increased malate, malonate and oxalate content, the citrate and lactate content were reduced (Table 3.5). By last, drought increased myo-inositol and pinitol concentration (Table 3.5).

Table 3.4 Changes in the concentration of carbohydrates, nitrogen compounds, organic acids and polyols (μmol g DW^{-1}) in stem from either, well-watered plants (control) or drought-stressed plants (mild or severe drought). Data set correspond to samples taken 2 h after the beginning of photoperiod. Values represent mean ± SE (n ≥ 3 biological replicates). An asterisk (*) within a row indicates significant differences from the control plants. (One-way ANOVA with posthoc LSD/T3 Dunnet test; $p \leq 0.05$). ND, not detected.

Stem	Control	Drought	
		Mild	Severe
Carbohydrates			
Fructose	31.19 ± 8.93	60.69 ± 4.24*	136.19 ± 8.92*
Glucose	28.11 ± 8.00	54.26 ± 5.25*	118.00 ± 6.81*
Raffinose	3.37 ± 0.90	5.77 ± 1.43	4.16 ± 0.52
Starch	460.34 ± 129.66	818.98 ± 172.08*	659.90 ± 119.08
Sucrose	50.34 ± 4.56	62.13 ± 6.93	98.80 ± 3.14*
N Compounds			
Allantoic Acid	26.26 ± 5.31	48.77 ± 4.50*	26.96 ± 6.65
Allantoin	2.90 ± 0.48	5.40 ± 0.73	3.48 ± 1.33
Asparagine	4.85 ± 0.26	26.88 ± 4.50*	25.37 ± 3.72*
Organic Acids			
Acetate	5.87 ± 0.49	ND	5.10 ± 0.35
Citrate	6.55 ± 1.86	4.43 ± 0.63	4.77 ± 0.88
Malate	14.71 ± 1.78	4.26 ± 0.73*	8.66 ± 1.53*
Malonate	19.37 ± 2.97	8.64 ± 3.80	16.12 ± 2.51
Oxalate	33.66 ± 3.39	27.06 ± 1.15	25.30 ± 1.76
Polyols			
Mannitol	1.06 ± 0.43	1.34 ± 0.21	1.18 ± 0.33
Myo-Inositol	7.03 ± 0.78	16.89 ± 0.87*	11.96 ± 1.72*
Pinitol	38.72 ± 1.52	43.95 ± 1.63	42.17 ± 2.01

Table 3.5 Changes in the concentration of carbohydrates, nitrogen compounds, organic acids and polyols (μmol g DW^{-1}) in roots from either, well-watered plants (control) or drought-stressed plants (mild or severe drought). Data set correspond to samples taken 2 h after the beginning of photoperiod. Values represent mean ± SE (n ≥ 3 biological replicates). An asterisk (*) within a row indicates significant differences from the control plants. (One-way ANOVA with posthoc LSD/T3 Dunnet test; p ≤ 0.05).

Root	Control	Drought	
		Mild	Severe
Carbohydrates			
Fructose	5.35 ± 0.42	29.53 ± 4.22*	62.09 ± 11.02*
Glucose	8.91 ± 0.60	29.30 ± 4.85*	52.93 ± 8.09*
Starch	12.48 ± 2.31	12.71 ± 3.38	24.00 ± 3.86*
Sucrose	27.70 ± 0.43	60.65 ± 12.92	73.97 ± 5.66*
N Compounds			
Allantoic acids	1.02 ± 0.51	10.11 ± 2.66*	17.87 ± 1.94*
Allantoin	0.17 ± 0.10	8.51 ± 3.17*	14.28 ± 3.00*
Asparagine	1.39 ± 0.16	8.28 ± 1.77	12.76 ± 1.24*
Organic Acids			
Citrate	7.94 ± 0.43	3.37 ± 0.74*	3.15 ± 0.43*
Lactate	17.89 ± 2.38	4.63 ± 1.29*	2.45 ± 0.25*
Malate	4.77 ± 0.65	6.60 ± 1.22	5.68 ± 0.80
Malonate	13.94 ± 3.18	26.69 ± 4.85*	28.88 ± 2.43*
Oxalate	40.01 ± 2.36	54.15 ± 6.64	40.53 ± 7.58
Polyols			
Mannitol	0.83 ± 0.13	0.64 ± 0.14	0.66 ± 0.16
Myo-Inositol	1.09 ± 0.10	3.51 ± 0.91*	5.22 ± 0.80*
Pinitol	10.57 ± 0.42	16.21 ± 3.44	18.68 ± 0.95*

In general, drought increased the concentration of detected carbohydrates in nodules (Table 3.6). Interestingly, trehalose, that was only detected in nodules, accumulated around 2.4-fold under both level of drought. The concentration of ureides increased as the drought stress increased, with a greater effect on allantoin concentration with 2.1 or 4.7-fold increase (Table 3.6). Asn content was bigger than in the other tissues, and also increased due to water deficit (Table 3.6). Regarding organic acids, drought stress caused a general decrease, being more significant the effect on malate content, which decreased a 50 and 58% under mild or severe stress, respectively (Table 3.6). As to polyols, drought increased 2.4-fold the pinitol content, while sorbitol content decreased (Table 3.6).

To get a general overview of the main variations in metabolite content due to drought stress and between the different plant tissues, we performed a heatmap to group metabolites sharing similar variation patterns (Fig. 3.4). The hierarchical cluster analysis yielded two major groups. The first one included those metabolites whose distribution depends to a greater extent on plant tissue tested, such as organic acids. The second one includes metabolites showing changes induced by the drought stress, such as sucrose, Asn or myo-inositol, among others.

Table 3.6 Changes in the concentration of carbohydrates, nitrogen compounds, organics acids and polyols (μmol g DW^{-1}) in nodules from either, well-watered plants (control) or drought-stressed plants (mild or severe drought). Data set correspond to samples taken 2 h after the beginning of photoperiod. Values represent mean ± SE (n ≥ 3 biological replicates). An asterisk (*) within a row indicates significant differences from the control plants. (One-way ANOVA with posthoc LSD/T3 Dunnet test; p ≤ 0.05). ND, not detected.

Nodules	Control	Drought	
		Mild	Severe
Carbohydrates			
Fructose	3.73 ± 0.25	13.14 ± 2.75*	22.20 ± 3.38*
Glucose	7.65 ± 0.75	15.20 ± 2.20*	22.06 ± 2.49*
Raffinose	2.59 ± 0.24	ND	ND
Starch	150.35 ± 12.60	240.22 ± 58.51	209.14 ± 50.15
Sucrose	40.28 ± 3.43	126.12 ± 29.55	206.72 ± 21.82*
Trehalose	11.19 ± 1.29	27.86 ± 4.70*	26.21 ± 1.44*
N compounds			
Allantoic Acid	19.31 ± 1.33	28.52 ± 2.58*	39.98 ± 1.42*
Allantoin	3.08 ± 0.29	6.39 ± 0.92*	14.54 ± 0.61*
Asparagine	17.30 ± 4.13	36.60 ± 2.61*	35.35 ± 4.68*
Organic Acids			
Acetate	7.83 ± 0.26	5.10 ± 0.47*	4.19 ± 0.43*
Citrate	17.35 ± 2.62	12.88 ± 0.94	14.59 ± 0.93
Lactate	8.16 ± 0.57	4.82 ± 0.28*	5.06 ± 0.43*
Malate	61.42 ± 5.19	30.80 ± 2.96*	25.93 ± 0.92*
Malonate	37.42 ± 1.51	28.71 ± 1.92*	29.47 ± 2.21*
Oxalate	33.13 ± 2.23	27.05 ± 2.28	25.18 ± 3.04*
Polyols			
Mannitol	0.90 ± 0.13	0.76 ± 0.03	0.75 ± 0.15
Myo-Inositol	46.94 ± 0.52	55.03 ± 8.00	53.28 ± 4.38
Pinitol	40.14 ± 2.18	68.24 ± 13.31*	96.62 ± 4.84*
Sorbitol	2.36 ± 0.05	1.79 ± 0.16*	1.55 ± 0.08*

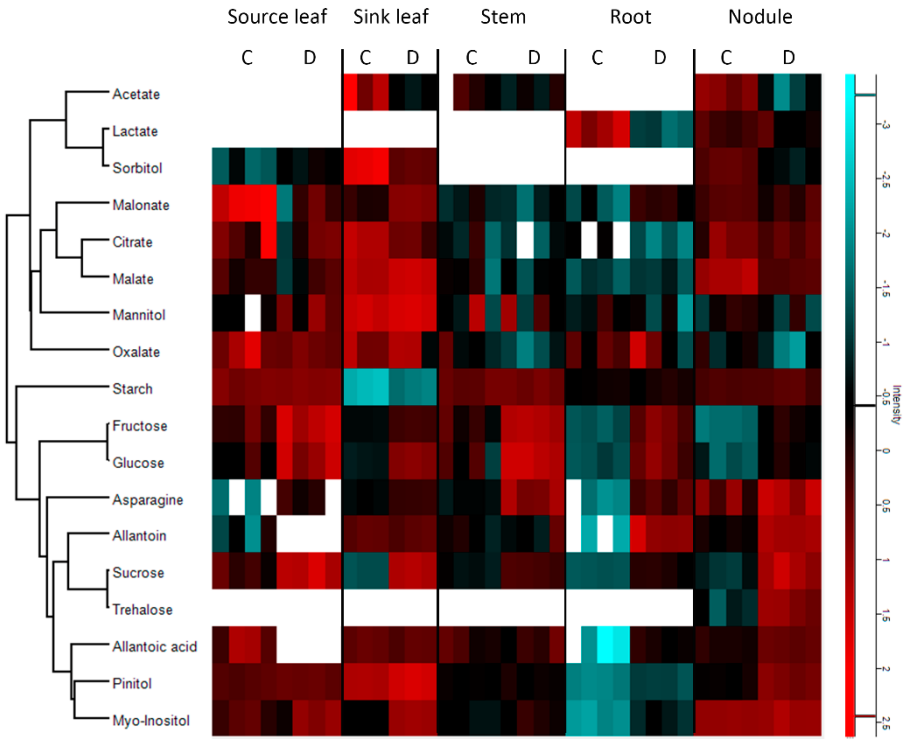

Fig. 3.4 Heatmap representing plant metabolites measured in source and sink leaves, stem, root and nodules of soybean plants inoculated with *B. diazoefficiens* under control and water-deficit conditions (ANOVA, $p < 0.05$). Intensity of red or cyan indicates higher or lower abundance regarding the mean, respectively. Data was logarithmized (base 2) followed by Z-score normalization. C, control; D, drought.

3.4 Discussion

The implication of long distance transport on BNF regulation has remained largely unexplored. In the present work, we use [U-^{13}C]-sucrose to know the source-sink relationships in soybean plants subjected to mild and severe drought. In the present work, one of the first physiological responses observed in plants subjected to progressive drought is stomatal closure. Moreover, it was observed prior decline in photosynthesis, as previously described (Flexas & Medrano, 2002). The effect of mild stress was higher in ANA than in photosynthesis or Ψ_{leaf}, as previously described (Djekoun & Planchon, 1991; Durand et al., 1987). This previous decline of ANA, highlights the sensitivity of BNF to water deficit, and also suggest that this fact is not due to a lack of carbon sources, since, an accumulation of sugars in nodules was also observed under

drought (Table 3.6). Similar results were reported by González et al. (1995), showing an accumulation of sucrose content in nodules relate to a decline in sucrose synthase activity under drought.

To understand the effect of drought in the source-sink relationships, we analyse first the natural $\delta^{13}C$ values obtained in well-watered plants. Results showed a ^{13}C enrichment in non-photosynthetic and sink tissues (Fig. 3.1). There is probably not a single cause and this effect is derived from the interplay of various combinations, such as: (1) CO_2 fixation carried out by phosphoenolpyruvate carboxylase; (2) ^{13}C discrimination due to respiration processes; (3) isotopics differences in biochemical composition between organs; (4) effect of daily or seasonal and developmental variation in photosynthetic ^{13}C discrimination; (5) ^{13}C discrimination due to translocation processes between source and sink tissues (Cernusak et al., 2009, and references therein). Moreover, different causes can act in a different ways depending on the tissue considered (Bathellier et al., 2009). Regarding to ^{13}C translocation study in control plants (Fig. 3.3A), it could be seen that in the first 3 h of labelling, sink leaf, root and nodule were three competing sinks for carbohydrates, but after 6 hours, sink leaf became the strongest sink receiving 15% of total ^{13}C translocated. This suggests that under optimal environmental conditions, shoot growth was prevalent compared with below-ground tissues.

Several environmental factors also affect C isotopic composition like drought stress, salinity, light, air pollution (Farquhar et al., 1989), temperature (O´Leary, 1995) or physiological characteristics such as nodulation (Kumarasinghe et al., 1992; Yoneyama & Ohtani, 1983). Moreover, interpretations mentioned above can also be applied to droughted plants, although our interest was focused on analysing the effect of drought on carbon fluxes from sources to sinks tissues. Results from [U-^{13}C]-sucrose translocation, showed that much ^{13}C remained in labelled leaf (Fig. 3.3) under drought, this suggests that carbon flux was slowed down. Similar results were obtained by Purcell et al. (1997) in labelled leaf. In case of sink leaf, less negatives δ^{13} values (Fig. 3.1B) under drought cannot be explained by an increase in ^{13}C translocation since as seen in Fig. 3.3, translocation towards this tissue was almost completely inhibited. Then, other factors such as ^{13}C discrimination due to respiration or photosynthetic processes, become relevant in this tissue. Under a severe stress, ^{13}C allocation was mainly towards stem and roots (Fig. 3.3), probably because limited growth of leaves causes more C to be available for roots and stem growth (Arndt & Wanek, 2002; Palta & Gregory, 1997). Arndt & Wanek (2002) also shown that drought stress affected translocation by increasing carbon allocation

to nodules, but, in this work, no differences in δ^{13} values were found in nodules due to drought (Fig. 3.1D). Moreover, when the ^{13}C allocation towards nodules was analysed, we observed that, under mild drought, ^{13}C reached control values after 6 h of labelling, but under severe drought ^{13}C translocation fell down (Fig. 3.3). Similar results were obtained by Purcell et al. (1997), who related a higher tolerance to drought with a continued allocation of photosynthate to nodules. In this study, despite the inhibition of ^{13}C translocation from labelled leaf, a sugar accumulation in nodules was observed under drought. Therefore, this accumulation could be due to the described decline in sucrose synthase activity under drought (González et al., 1995).

Throughout the 6 h of labelling a decrease of ca. 35% in the total amount of ^{13}C was observed in both control and mild drought plants (Fig. 3.2). This decline could be explained by losses due to respiration or through root exudates, but, in this study, possible ^{13}C losses by both processes were not measured. Regarding to root exudates, the ^{13}C amount found in soil was relatively small, and under drought stress even smaller (Derrien et al., 2004; Holz et al., 2018; Palta & Gregory, 1997). As to ^{13}C discrimination due to respiration processes, several authors have shown that dark respiration causes a ^{13}C enrichment in CO_2 respired by leaves, but not by roots (Cernusak et al., 2009, for a review). It is not clear if this effect is altered by environmental factors, for instance Palta & Gregory (1997) found in wheat an increase in $^{13}CO_2$ respired by root under drought stress; Duranceau et al. (1999) working with *Phaseolus vulgaris*, observed changes in $^{13}CO_2$ respired by leaves related with water status, but Ghashghaie et al. (2001), working with *Nicotiana sylvestris* and *Helianthus annuus* found opposite results. Regarding nodules, it has been shown tracer losses by nodules respiration in well-watered plants (Gordon et al., 1985; Thorpe et al., 1998). Therefore, this 35% decrease in the total amount of ^{13}C, could be mainly explained by losses related to respiration processes.

Plant growth limitation imposed by drought could cause a decrease in sugar sink demand, leading to the accumulation of photoassimilates in source tissues (Lemoine et al., 2013; Schubert et al., 1995). Moreover, in sink leaves, plant growth could be negatively affected earlier and more intensively than photosynthesis and metabolism, leading also to an accumulation of sugars (Muller et al., 2011). Moreover, osmotic stress promote sucrose instead of starch biosynthesis in sink tissues, explaining the observed trend in starch content in this study (Lemoine et al., 2013). The reduction in shoot growth lead to a sugars accumulation in stem and roots, promoting root growth to improve the acquisition of water (Geiger et al., 1996). Thus, in this work, the carbon

allocation towards stem and roots increased under drought (Fig. 3.3; Table 3.5). As mentioned above, the increase in sucrose concentration in nodules has been related with an early decline in sucrose synthase activity under drought stress (Gálvez et al., 2005; González et al., 1995). Moreover, under mild drought stress, carbon allocation towards nodules was maintained at similar rates than in control plants, although with a slight time delay (Fig. 3.3). That could explain the increase of carbohydrates concentration at mild drought stress and maintaining of these levels as stress increases (Table 3.6).

The accumulation of N compounds in nodules has been related with a feedback inhibition of BNF. Several compounds have been suggested to play a role in this inhibition, including ureides (Atkins et al., 1992; Serraj et al., 1999b; Vadez et al., 2000), and Asn (Bacanamwo & Harper, 1997). In this work, drought caused an accumulation of ureides and Asn in roots and nodules (Tables 3.5 and 3.6). Instead, in source leaves, the concentration of ureides declined until they were not detectable under severe stress, but Asn content increased (Table 3.2). These results suggest that the inhibition of BNF are not related with an accumulation of ureides in leaves as previously described (King & Purcell, 2005; Ladrera et al., 2007). The role of Asn in the inhibition of BNF has also been questioned, since a general increase of single amino acids content observed in both nodules and leaves suggest a more complex nitrogen signal regulation (Gil-Quintana et al., 2013a). Interestingly, in sink leaf, unlike source leaves, the ureides content remain stable under severe stress (Table 3.3). These results suggest that different ureides catabolism pathways are acting in the different tissues analysed more than an impairment of long-distance transport from nodules to shoots occurs. Thus, Gil-Quintana et al. (2013b) shown that ureides catabolism was more negatively affected than the de novo synthesis under stress.

Organic acids are synthesized in plants due to the incomplete oxidation of photosynthetic products, their role is provide redox equilibrium, support ionic gradients on membranes, and acidification of the extracellular medium (for a review, see Igamberdiev & Eprintsev (2016). In leaves, malate accumulation under drought has been related to stomatal closure (Patonnier et al., 1999), therefore, its accumulation may reflect control on leaf transpiration (Charlton et al., 2008), although in this work, malate only accumulated in sink leaves (Table 3.3). The decline observed in malate content in droughted nodules has been attributed to a decline in sucrose synthase content and activity, leading to an impairment in bacteroid functioning (Gálvez et al., 2005; González et al., 1995). Regarding malonate, Li & Copeland (2000) suggested that its metabolism are not related to abiotic stress, but rather a consequential effect related to malate

metabolism, as it seems to happen in this study with a similar trend in both organic acids. Moreover, despite being an abundant organic acid in nodules, it is a poor carbon source for bacteroids (Karunakaran et al., 2013). In the case of oxalate, Trinchant & Rigaud (1996) suggested that its oxidation is important in nodules, preventing the production of ROS nearby bacteroids, this could explain the decline in oxalate content in drought-stressed nodules (Table 3.6).

Finally, early studies highlighted the role of polyols in osmotic regulation under different stresses such as temperature, salinity, light or drought (Fougere et al., 1991; Loescher, 1987; Noiraud et al., 2001; Streeter et al., 2001; Tsikou et al., 2013, for a review). Polyols synthesis occurs mainly in source leaves, although sorbitol synthesis has also been described in nodules (Colebatch et al., 2004), thus, its occurrence in sink tissues indicate its translocation (Noiraud et al., 2001, Streeter et al., 2001). In this study, it could be observed how drought affect polyols metabolism in a different way depending of the tissue analysed. For instance, the concentration of mannitol and sorbitol in sink leaf showed an effect of drought prior to source leaves, indicating both a lower assimilation in sink leaf and a transport impairment under severe stress (Tables 3.2 and 3.3). The accumulation of myo-inositol and pinitol under abiotic stress has been previously described (Aranjuelo et al., 2014; Dumschott et al., 2019). The biosynthesis of pinitol from glucose-6-phophate, include myo-inositol biosynthesis (Dumschott et al., 2019). In this work, in source leaves and roots there was an accumulation of myo-inositol but no effect on pinitol, while in nodules there was no effect on myo-inositol, although pinitol content increased. This fact suggest than pinitol synthesis was impaired due to drought or that its translocation to sink tissues increased.

3.5 Conclusions

Results presented here highlight the complexity of the source-sink relationships under drought stress. The applied methodology, with [U-^{13}C]-sucrose labelling along with the data of natural ^{13}C abundance in all plant tissues, provide valuable information about the differential C allocation in drought-stressed plants, avoiding assimilation problems related to stomatal closure caused by water stress conditions.

Under optimal environmental conditions, it has been observed how C allocation was directed mainly to shoot growth, but under water deficit, C allocation was greater towards root, promoting its growth and thus the access to water. Nodules, being a strong sink under well-watered conditions, cannot

compete with roots for photoassimilates under drought. Although nodules are not carbon limited, the main energy source for bacteroids decline.

The differential content of nitrogen compounds in the different plant tissues support that the inhibition of BNF in soybean is not related with an accumulation of ureides in leaves, and suggest that different ureides catabolism pathways are acting in the different tissues analysed. Biosynthesis and long-distance transport of organic acids and polyols were also altered by drought.

General conclusions

General conclusions

1. The construction of a lux-based proline biosensor allows measuring proline levels in bacteroids within intact pea nodules for the first time, in both optimal plant growth conditions and during stress.
2. The levels of proline detected in pea roots suggest that the composition of the root exudates varies depending on the root zones. In addition, inside nodules, proline levels are significantly higher than those detected on the root surface.
3. In bacteroids, proline accumulation occurs after the stress phase, a stage at which optimal plant growth conditions have been re-established.
4. Both the blockage of amino acid transport across symbionts and the inhibition of BCAA biosynthesis have been shown to affect BNF in pea. The *aap/bra* double mutation led to the accumulation of NifA, along with a decline in FixA, FixC and NifH, suggesting a feedback inhibition of *nifA* expression in bacteroids. In contrast, under imazamox treatment, the lower levels of NifA could explain, in turn, the observed FixC and NifH decline.
5. Both the *aap/bra* double mutation and the imazamox treatment cause a deregulation of ammonium assimilation metabolism in pea nodules. This leads to a decline in amide and acidic amino acids in RU1722 nodules, while higher proteolysis could be responsible of the increase observed in imazamox-treated nodules. Both stresses caused a shutdown of BCAA biosynthesis and an induction of the biosynthesis of aromatic amino acids supporting the existence of a possible link between the different amino acid pathways.
6. Both the *aap/bra* double mutation and the imazamox treatment cause an impairment of C metabolism but only the blockage of amino acid transport between symbionts caused a decline in the content of malate, main energy source for bacteroids.
7. There was not any significant effect of water deficit on BNF rates in plants inoculated with the *aap/bra* double mutant. In contrast, it was observed an increased abundance of proteins related to BNF in RU1722 bacteroids, then, additional metabolic factors are clearly limiting BNF in these plants.

8. The combination of proteomic and targeted metabolic analysis on pea nodules highlights the relevance of low abundant amino acids, such as Met, aromatic amino acids or GABA, in the response to water deficit.

9. The labelling with [U-^{13}C]-sucrose along with the data of natural ^{13}C abundance in soybean plant tissues, provide valuable information about the carbon allocation in drought-stressed plants, avoiding assimilation problems related to stomatal closure.

10. In soybean, the allocation of C under drought is mainly directed towards roots, most likely to maintain growth in this organ so as to improve the plant water absorption capacity. Nodules, being a strong carbon sink under optimal growth conditions, cannot compete with roots for photoassimilates under drought.

11. The differential content of N compounds in the different tissues of soybean plant support the hypothesis that the inhibition of BNF in soybean is not related with an accumulation of ureides in leaves, and suggest that several ureide catabolism pathways are acting in the tissues analysed. Biosynthesis and long-distance transport of organic acids and polyols are also altered by drought.

Conclusiones generales

Conclusiones generales

1. La construcción de un biosensor de prolina basado en la expresión de los genes *lux* permite medir los niveles de prolina en bacteroides dentro de nódulos intactos de guisante por primera vez, tanto en condiciones de crecimiento óptimo de la planta como durante estrés.
2. Los niveles de prolina detectados en raíces de guisante sugieren que la composición de los exudados radiculares varía dependiendo de la zona de la raíz. Además, en nódulos, los niveles de prolina son significativamente mayores que los detectados en la superficie de la raíz.
3. En bacteroides, la acumulación de prolina ocurre tras la fase de estrés, una vez se han restablecido las condiciones óptimas de crecimiento de la planta.
4. Tanto el bloqueo del transporte de aminoácidos entre simbiontes como la inhibición de la biosíntesis de AACRs afectan a la regulación de la FBN. La doble mutación da lugar a una acumulación de NifA, junto con un descenso en FixA y FixC, sugiriendo una inhibición de la expresión de *nifA*. Por el contrario, bajo el tratamiento con imazamox, los niveles más bajos de NifA explican, a su vez, el descenso observado en FixC y NifH.
5. Tanto la doble mutación *aap/bra* como el tratamiento con imazamox causan una desregulación del metabolismo de asimilación de amonio en nódulos de guisante. Esto da lugar a un descenso en aminoácidos ácidos y amidas en los nódulos inoculados con la cepa RU1722, mientras que un incremento en la proteólisis puede ser responsable del incremento observado en nódulos tratados con imazamox. Ambos estreses causaron un descenso en la biosíntesis de AACRs y a una inducción de la biosíntesis de aminoácidos aromáticos respaldando la existencia de una posible conexión entre diferentes rutas de aminoácidos.
6. Tanto la doble mutación *aap/bra* como el tratamiento con imazamox causaron una disfunción del metabolismo del carbono pero solo el bloqueo del transporte de aminoácidos entre simbiontes redujo el contenido en malato, fuente principal de energía para los bacteroides.
7. No se observó ningún efecto significativo del déficit hídrico sobre la FBN en plantas inoculadas con la doble mutante *aap/bra*. En cambio, se observó una mayor abundancia de proteínas relacionadas con la FBN en bacteroides de la cepa RU1722, por tanto, otros factores metabólicos están claramente limitando la FBN en esas plantas.

8. La combinación de análisis proteómico y metabólico dirigido en nódulos de guisante destaca la relevania de aminoácidos menos abundantes, como Met, aminoácidos aromáticos o GABA, en la respuesta a estrés hídrico.

9. El marcado con [U-^{13}C]-sacarosa junto los datos de abundacia natural de ^{13}C obtenidos en los tejidos de soja, proporciona información valiosa sobre la distribución de carbono en plantas estresadas por sequía, evitando problemas de asimilación relacionados con el cierre estomático.

10. En soja, la distribución de carbono en sequía se dirige principalmente hacia las raíces probablemente para mantener el crecimiento de este órgano y mejorar la capacidad de absorción de agua. Los nódulos, siendo un fuerte sumidero de carbono en condiciones óptimas de crecimiento, no puede competir con las raíces por los fotoasimilados bajo sequía.

11. El diferente contenido de compuestos nitrogenados en los distintos tejidos de soja apoya la hipótesis de que la inhibición de la FBN en soja no está relacionada con la acumulación de ureidos en hojas, y sugiere que varias rutas de catabolismo de ureidos están actuando en los tejidos analizados. La biosíntesis y el transporte a larga distancia de ácidos orgánicos y polioles también se alteran por sequía.

Bibliography

Bibliography

Acosta-Motos, J. R., Ortuño, M. F., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M. J., & Hernandez, J. A. (2017). Plant responses to salt stress: Adaptive mechanisms. *Agronomy*, *7*(1), 18. https://doi.org/10.3390/agronomy7010018

Ahrens, W. H. (Ed.) (1994). *Herbicide Handbook* , 7th ed. Weed Science Society of America. Champaign, IL.

Aldasoro, J., Larrainzar, E., & Arrese-Igor, C. (2019). Application of anti-transpirants temporarily alleviates the inhibition of symbiotic nitrogen fixation in drought-stressed pea plants. *Agricultural Water Management*, *213*, 193–199. https://doi.org/10.1016/J.AGWAT.2018.10.014

Allaway, D., Lodwig, E. M., Crompton, L. A., Wood, M., Parsons, R., Wheeler, T. R., & Poole, P. S. (2000). Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. *Molecular Microbiology*, *36*(2), 508–515. https://doi.org/10.1046/j.1365-2958.2000.01884.x

Anderson, A., Baldock, J. A., Rogers, S. L., Bellotti, W., & Gill, G. (2004). Influence of chlorsulfuron on rhizobial growth, nodule formation, and nitrogen fixation with chickpea. *Australian Journal of Agricultural Research*, *55*(10), 1059. https://doi.org/10.1071/AR03057

Aparicio-Tejo, PM, Arrese-Igor, C, Becana, M. (2008). Fijación biológica de nitrógeno. In M. T. J Azcon-Bieto (Ed.), *Fundamentos de Fisiología Vegetal* (pp. 305–322). McGraw-Hill/Interamericana de España.

Aranjuelo, I., Arrese-Igor, C., & Molero, G. (2014). Nodule performance within a changing environmental context. *Journal of Plant Physiology*, *171*(12), 1076–1090. https://doi.org/10.1016/J.JPLPH.2014.04.002

Araújo, S. S., Beebe, S., Crespi, M., Delbreil, B., González, E. M., Gruber, V., Lejeune-Henaut, I., Link, W., Monteros, M. J., Prats, E., Rao, I., Vadez, V., & Patto, M. C. V. (2015). Abiotic stress responses in legumes: strategies used to cope with environmental challenges. *Critical Reviews in Plant Sciences*, *34*(1–3), 237–280. https://doi.org/10.1080/07352689.2014.898450

Arndt, S. K., & Wanek, W. (2002). Use of decreasing foliar carbon isotope discrimination during water limitation as a carbon tracer to study whole plant carbon allocation. *Plant, Cell and Environment*, *25*(5), 609–616. https://doi.org/10.1046/j.1365-3040.2002.00838.x

Arrese-Igor, C, González, E. M., Marino, D., Ladrera, R., Larrainzar, E., & Gil-Quintana, E. (2011). Physiological responses of legume nodules to drought. *Plant Stress*, *5*(Special issue 1), 24–31.

Arrese-Igor, Cesar, González, E. M., Gordon, A. J., Minchin, F. R., Galvez, L., Royuela, M., Cabrerizo, P. M., & Aparicio-Tejo, P. M. (1999). Sycrose synthase and nodule nitrogen fixation under drought and other environmental stresses. *Symbiosis*, *27*(3–4), 189–212.

Aspinall, D., & Paleg, L. (1981). Proline accumulation: physiological aspects. In L. Paleg & D. Aspinall (Eds.), *Physiology and biochemistry of drought resistance in plants* (pp. 205–240). Academic Press.

Atkins, C. A., Fernando, M., Hunt, S., & Layzell, D. B. (1992). A metabolic connection between nitrogenase activity and the synthesis of ureides in nodulated soybean. *Physiologia Plantarum*, *84*(3), 441–447. https://doi.org/10.1111/j.1399-3054.1992.tb04688.x

Azani, N., Babineau, M., Bailey, C. D., Banks, H., Barbosa, A., Pinto, R. B., Boatwright, J., Borges, L., Brown, G., Bruneau, A., Candido, E., Cardoso, D., Chung, K.-F., Clark, R., Conceição, A. deS., Crisp, M., Cubas, P., Delgado-Salinas, A., Dexter, K., … Zimmerman, E. (2017). A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny – The Legume Phylogeny Working Group (LPWG). *Taxon*, *66*(1), 44–77. https://doi.org/10.12705/661.3

Azevedo, R. A., Arruda, P., Turner, W. L., & Lea, P. J. (1997). The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. *Phytochemistry*, *46*(3), 395-419. https://doi.org/10.1016/S0031-9422(97)00319-1

Bacanamwo, M., & Harper, J. E. (1997). The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. *Physiologia Plantarum*, *100*(2), 371–377. https://doi.org/10.1111/j.1399-3054.1997.tb04795.x

Bach, M. K., Magee, W. E., & Burris, R. H. (1958). Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation. *Plant Physiology*, *33*(2), 118–124. https://doi.org/10.1104/pp.33.2.118

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., & Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology*, *57*(1), 233–266. https://doi.org/10.1146/annurev.arplant.57.032905.105159

Bathellier, C., Tcherkez, G., Bligny, R., Gout, E., Cornic, G., & Ghashghaie, J. (2009). Metabolic origin of the $\delta^{13}C$ of respired CO_2 in roots of *Phaseolus vulgaris*. *New Phytologist*, *181*(2), 387–399. https://doi.org/10.1111/j.1469-8137.2008.02679.x

Batista-Silva, W., Heinemann, B., Rugen, N., Nunes-Nesi, A., Araújo, W. L., Braun, H., & Hildebrandt, T. M. (2019). The role of amino acid metabolism during abiotic stress release. *Plant, Cell & Environment*, *42*(5), 1630–1644. https://doi.org/10.1111/pce.13518

Battaglia, M., & Covarrubias, A. A. (2013). Late Embryogenesis Abundant (LEA) proteins in legumes. *Frontiers in Plant Science*, *4*. https://doi.org/10.3389/fpls.2013.00190

Beijerink, M. W. (1888). Cultur des *Bacillus radicicola* aus den Knöllchen. *Bot Ztg*, *46*, 740–750.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Microbiology*, *84*(1), 188–198. https://doi.org/10.1099/00221287-84-1-188

Bournaud, C., de Faria, S. M., dos Santos, J. M. F., Tisseyre, P., Silva, M., Chaintreuil, C., Gross, E., James, E. K., Prin, Y., & Moulin, L. (2013). *Burkholderia* species are the most common and preferred nodulating symbionts of the Piptadenia group (Tribe Mimoseae). *PLoS ONE*, *8*(5), e63478. https://doi.org/10.1371/journal.pone.0063478

Boyd, E. S., & Peters, J. W. (2013). New insights into the evolutionary history of biological nitrogen fixation. *Frontiers in Microbiology*, *4*, 201. https://doi.org/10.3389/fmicb.2013.00201

Boyer, J. S. (1982). Plant productivity and environment. *Science*, *218*(4571), 443–448. https://doi.org/10.1126/science.218.4571.443

Bray, E. A. (1997). Plant responses to water deficit. *Trends in Plant Science*, *2*(2), 48–54. https://doi.org/10.1016/S1360-1385(97)82562-9

Brodersen, C. R., Roddy, A. B., Wason, J. W., & McElrone, A. J. (2019). Functional status of xylem through time. *Annual Review of Plant Biology*, *70*(1), 407–433.

https://doi.org/10.1146/annurev-arplant-050718-100455

Brodl, E., Winkler, A., & Macheroux, P. (2018). Molecular mechanisms of bacterial bioluminescence. *Computational and structural biotechnology journal*, *16*, 551-564. https://doi.org/10.1016/j.csbj.2018.11.003

Broeckling, C. D., Huhman, D. V, Farag, M. A., Smith, J. T., May, G. D., Mendes, P., Dixon, R. A., & Sumner, L. W. (2005). Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. *Journal of Experimental Botany*, *56*(410), 323–336. https://doi.org/10.1093/jxb/eri058

Brüggemann, N., Brüggemann, B., Gessler, A., Kayler, Z., Keel, S. G., Badeck, F., Barthel, M., Boeckx, P., Buchmann, N., Brugnoli, E., Esperschütz, J., Esperschütz, E., Gavrichkova, O., Ghashghaie, J., Gomez-Casanovas, N., Keitel, C., Knohl, A., Kuptz, D., Palacio, S., … Bahn, M. (2011). Carbon allocation and carbon isotope fluxes in the plant-soil-atmosphere continuum: a review. *Biogeosciences*, *8*, 3457–3489. https://doi.org/10.5194/bg-8-3457-2011

Bruneau, A., Doyle, J. J., Herendeen, P., Hughes, C., Kenicer, G., Lewis, G., Mackinder, B., Pennington, R. T., Sanderson8, M. J., & Wojciechowski, M. F. (2013). Legume phylogeny and classification in the 21st century: Progress, prospects and lessons for other species-rich clades. The Legume Phylogeny Working Group. *Taxon* 62(2), 217-248. https://doi.org/10.12705/622.8

Cai, S., Chen, G., Wang, Y., Huang, Y., Marchant, D. B., Wang, Y., Yang, Q., Dai, F., Hills, A., Franks, P. J., Nevo, E., Soltis, D. E., Soltis, P. S., Sessa, E., Wolf, P. G., Xue, D., Zhang, G., Pogson, B. J., Blatt, M. R., & Chen, Z. H. (2017). Evolutionary conservation of ABA signaling for stomatal closure. *Plant Physiology*, *174*(2), 732–747. https://doi.org/10.1104/pp.16.01848

Carvalho, H., Lescure, N., de Billy, F., Chabaud, M., Lima, L., Salema, R., & Cullimore, J. (2000). Cellular expression and regulation of the *Medicago truncatula* cytosolic glutamine synthetase genes in root nodules. *Plant Molecular Biology*, *42*(5), 741–756. https://doi.org/10.1023/A:1006304003770

Castillejo, M.-Á., Iglesias-García, R., Wienkoop, S., & Rubiales, D. (2016). Label-free quantitative proteomic analysis of tolerance to drought in *Pisum sativum*. *Proteomics*. https://doi.org/10.1002/pmic.201600156

Cernusak, L. A., Tcherkez, G., Keitel, C., Cornwell, W. K., Santiago, L. S., Knohl, A., Barbour, M. M., Williams, D. G., Reich, P. B., Ellsworth, D. S., Dawson, T. E., Griffiths, H. G., Farquhar, G. D., & Wright, I. J. (2009). Why are non-photosynthetic tissues generally ^{13}C enriched compared with leaves in C3 plants? Review and synthesis of current hypotheses. *Functional Plant Biology*, *36*(3), 199. https://doi.org/10.1071/FP08216

Charlton, A. J., Donarski, J. A., Harrison, M., Jones, S. A., Godward, J., Oehlschlager, S., Arques, J. L., Ambrose, M., Chinoy, C., Mullineaux, P. M., & Domoney, C. (2008). Responses of the pea (*Pisum sativum* L.) leaf metabolome to drought stress assessed by nuclear magnetic resonance spectroscopy. *Metabolomics*, *4*(4), 312–327. https://doi.org/10.1007/s11306-008-0128-0

Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osório, M. L., Carvalho, I., Faria, T., & Pinheiro, C. (2002). How plants cope with water stress in the field. Photosynthesis and growth. *Annals of Botany*, *89*(SPEC. ISS.), 907–916. https://doi.org/10.1093/aob/mcf105

Coba de la Peña, T., Fedorova, E., Pueyo, J. J., & Lucas, M. M. (2018). The symbiosome: Legume and Rhizobia co-evolution toward a nitrogen-fixing organelle? *Frontiers in Plant Science*, *8*, 2229. https://doi.org/10.3389/fpls.2017.02229

Colebatch, G., Desbrosses, G., Ott, T., Krusell, L., Montanari, O., Kloska, S., Kopka, J., & Udvardi, M. K. (2004). Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *The Plant Journal*, *39*(4), 487–512. https://doi.org/10.1111/j.1365-313X.2004.02150.x

Cordovilla, M. P., Ligero, F., & Lluch, C. (1994). The effect of salinity on N fixation and assimilation in *Vicia faba*. *Journal of Experimental Botany*, *45*(10), 1483–1488. https://doi.org/10.1093/jxb/45.10.1483

Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M., & Shinozaki, K. (2011). Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biology*, *11*(1), 163. https://doi.org/10.1186/1471-2229-11-163

Cullimore, J. V., & Bennett, M. J. (1988). The molecular biology and biochemistry of plant glutamine synthetase from root nodules of *Phaseolus vulgaris* L. and other legumes. *Journal of Plant Physiology*, *132*(4), 387–393. https://doi.org/10.1016/S0176-1617(88)80050-6

Curtis, J., Shearer, G., & Kohl, D. H. (2004). Bacteroid proline catabolism affects N_2 fixation rate of drought-stressed soybeans. *Plant Physiology*, *136*(2), 3313–3318. https://doi.org/10.1104/pp.104.044024

Daryanto, S., Wang, L., & Jacinthe, P.-A. (2015). Global synthesis of drought effects on food legume production. *PLoS ONE*, *10*(6), e0127401. https://doi.org/10.1371/journal.pone.0127401

Day, D. A., Poole, P. S., Tyerman, S. D., & Rosendahl, L. (2001). Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. *Cellular and Molecular Life Sciences*, *58*(1), 61–71. https://doi.org/10.1007/PL00000778

de Felipe, M. R. (2006). Fijación biológica de dinitrógeno atmosférico en vida libre. In E. J. Bedmar, J González, C Lluch & B. Rodelas (Eds.), *Fijación de Nitrógeno: Fundamentos y Aplicaciones.* (pp. 9–16). Sociedad Española de Fijación de Nitrógeno.

de María, N., de Felipe, M. R., & Fernández-Pascual, M. (2005). Alterations induced by glyphosate on lupin photosynthetic apparatus and nodule ultrastructure and some oxygen diffusion related proteins. *Plant Physiology and Biochemistry*, *43*(10–11), 985–996. https://doi.org/10.1016/j.plaphy.2005.09.001

Delgado, M. J., Garrido, J. M., Ligero, F., & Lluch, C. (1993). Nitrogen fixation and carbon metabolism by nodules and bacteroids of pea plants under sodium chloride stress. *Physiologia Plantarum*, *89*(4), 824–829. https://doi.org/10.1111/j.1399-3054.1993.tb05291.x

Delgado, M. J., Ligero, F., & Lluch, C. (1994). Effects of salt stress on growth and nitrogen fixation by pea, faba-bean, common bean and soybean plants. *Soil Biology and Biochemistry*, *26*(3), 371–376. https://doi.org/10.1016/0038-0717(94)90286-0

Derrien, D., Marol, C., & Balesdent, J. (2004). The dynamics of neutral sugars in the rhizosphere of wheat. An approach by ^{13}C pulse-labelling and GC/C/IRMS. *Plant and Soil*, *267*(1–2), 243–253. https://doi.org/10.1007/s11104-005-5348-8

Dersch, L. M., Beckers, V., Rasch, D., Melzer, G., Bolten, C., Kiep, K., Becker, H., Bläsing, O. E.,

Fuchs, R., Ehrhardt, T., & Wittmann, C. (2016). Novel approach for high-throughput metabolic screening of whole plants by stable isotopes. *Plant Physiology*, *171*(1), 25–41. https://doi.org/10.1104/pp.15.01217

Desalegn, G., Turetschek, R., Kaul, H.-P., & Wienkoop, S. (2016). Microbial symbionts affect *Pisum sativum* proteome and metabolome under *Didymella pinodes* infection. *Journal of Proteomics*, *143*, 173–187. https://doi.org/10.1016/j.jprot.2016.03.018

Diaz del Castillo, L., Hunt, S., & Layzell, D. B. (1994). The role of oxygen in the regulation of nitrogenase activity in drought-stressed soybean nodules. *Plant Physiology*, *106*(3), 949–955. https://doi.org/10.1104/pp.106.3.949

Díaz, P., Monza, J., & Márquez, A. (2005). Drought and saline stress. In Márquez A. J. (eds.), *Lotus japonicus Handbook* (pp. 39–50). Springer-Verlag. https://doi.org/10.1007/1-4020-3735-X_3

Djekoun, A., & Planchon, C. (1991). Water status effect on dinitrogen fixation and photosynthesis in soybean. *Agronomy Journal*, *83*(2), 316–322. https://doi.org/10.2134/agronj1991.00021962008300020011x

Dordas, C. (2009). Nonsymbiotic hemoglobins and stress tolerance in plants. *Plant Science*, *176*(4), 433-440. https://doi.org/10.1016/j.plantsci.2009.01.003

Downie, J. A. (2014). Legume nodulation. *Current Biology*, *24*(5), R184–R190. https://doi.org/10.1016/j.cub.2014.01.028

Drew, E. A., Gupta, V. V. S. R., & Roget, D. K. (2007). Herbicide use, productivity, and nitrogen fixation in field pea (*Pisum sativum*). *Australian Journal of Agricultural Research*, *58*(12), 1204. https://doi.org/10.1071/AR06394

Driscoll, B. T., & Finan, T. M. (1993). NAD^+-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. *Molecular Microbiology*, *7*(6), 865–873. https://doi.org/10.1111/j.1365-2958.1993.tb01177.x

Du, Z., Zhou, X., Ling, Y., Zhang, Z., & Su, Z. (2010). agriGO: A GO analysis toolkit for the agricultural community. *Nucleic Acids Research*, *38*(SUPPL. 2). https://doi.org/10.1093/nar/gkq310

Duke, S. O. (2012). Why have no new herbicide modes of action appeared in recent years? *Pest Management Science*, *68*(4), 505–512. https://doi.org/10.1002/ps.2333

Dumschott, K., Dechorgnat, J., & Merchant, A. (2019). Water deficit elicits a transcriptional response of genes governing D-pinitol biosynthesis in soybean (*Glycine max*). *International Journal of Molecular Sciences*, *20*(10), 2411. https://doi.org/10.3390/ijms20102411

Dunn, M. F. (2015). Key roles of microsymbiont amino acid metabolism in rhizobia-legume interactions. *Critical Reviews in Microbiology*, *41*(4), 411–451. https://doi.org/10.3109/1040841X.2013.856854

Duranceau, M., Ghashghaie, J., Badeck, F., Deleens, E., & Cornic, G. (1999). $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell & Environment*, *22*(5), 515–523. https://doi.org/10.1046/j.1365-3040.1999.00420.x

Durand, J. L., Sheehy, J. E., & Minchin, F. R. (1987). Nitrogenase activity, photosynthesis and nodule water potential in soyabean plants experiencing water deprivation. *Journal of Experimental Botany*, *38*(2), 311–321. https://doi.org/10.1093/jxb/38.2.311

Eady, R. R. (1996). Structure–function relationships of alternative nitrogenases. *Chemical Reviews*, *96*(7), 3013–3030. https://doi.org/10.1021/cr950057h

FAOStat, 2019. Food and Agriculture Organization of the United Nations, Statistics Division. http://www.fao.org/faostat/en/#data. Accessed 7 June 2019

Farquhar, G. D., Ehleringer, J. R., & Hubick, K. T. (1989). Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, *40*(1), 503–537. https://doi.org/10.1146/annurev.pp.40.060189.002443

Farquharson, R. L. (2009). *The impact of acetohydroxyacid synthase inhibiting herbicides on symbiotic nitrogen fixation of grain and pasture legumes*. University of Adelaide

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D. E., & Gresshoff, P. M. (2010). Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology*, *52*(1), 61–76. https://doi.org/10.1111/j.1744-7909.2010.00899.x

Ferri, A., Lluch, C., & Ocaña, A. (2000). Effect of salt stress on carbon metabolism and bacteroid respiration in root nodules of common bean (*Phaseolus vulgaris* L.). *Plant Biology*, *2*(4), 396–402. https://doi.org/10.1055/s-2000-5956

Flemetakis, E., Efrose, R. C., Ott, T., Stedel, C., Aivalakis, G., Udvardi, M. K., & Katinakis, P. (2006). Spatial and temporal organization of sucrose metabolism in *Lotus japonicus* nitrogen-fixing nodules suggests a role for the elusive alkaline/neutral invertase. *Plant Molecular Biology*, *62*(1–2), 53–69. https://doi.org/10.1007/s11103-006-9003-4

Flexas, J., & Medrano, H. (2002). Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. *Annals of Botany*, *89*(2), 183–189. https://doi.org/10.1093/aob/mcf027

Fondy, B. R., & Geiger, D. R. (1977). Sugar selectivity and other characteristics of phloem loading in *Beta vulgaris* L. *Plant Physiology*, *59*(5), 953–960. https://doi.org/10.1104/pp.59.5.953

Fougere, F., Le Rudulier, D., & Streeter, J. G. (1991). Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.). *Plant Physiol*, *96*(4), 1228–1236. https://doi.org/10.1104/pp.96.4.1228

Frank, B. (1889). Ueber die pilzsymbiose der Leguminosen. *Bet. Dtsch. Bot. Ges.*, *7*, 332–346.

Frederix, M., Edwards, A., Swiderska, A., Stanger, A., Karunakaran, R., Williams, A., Abbruscato, P., Sanchez-Contreras, M., Poole, P. S., & Downie, J. A. (2014). Mutation of *praR* in *Rhizobium leguminosarum* enhances root biofilms, improving nodulation competitiveness by increased expression of attachment proteins. *Molecular Microbiology*, *93*(3), 464–478. https://doi.org/10.1111/mmi.12670

Frensch, J., Hsiao, T. C., & Steudle, E. (1996). Water and solute transport along developing maize roots. *Planta*, *198*(3), 348–355. https://doi.org/10.1007/BF00620050

Fukudome, M., Calvo-Begueria, L., Kado, T., Osuki, K. I., Rubio, M. C., Murakami, E. I., Nagata, M., Kucho, K. I., Sandal, N., Stougaard, J., Becana, M., & Uchiumi, T. (2016). Hemoglobin LjGlb1-1 is involved in nodulation and regulates the level of nitric oxide in *the Lotus japonicus-Mesorhizobium loti* symbiosis. *Journal of Experimental Botany*, *67*(17), 5275–5283. https://doi.org/10.1093/jxb/erw290

Gallon, J. R., Perry, S. M., Rajab, T. M. A., Flayeh, K. A. M., Yunes, J. S., & Chaplin, A. E. (1988). Metabolic changes associated with the diurnal pattern of N_2 fixation in *Gloeothece*.

Microbiology, *134*(12), 3079–3087. https://doi.org/10.1099/00221287-134-12-3079

Gálvez, L., González, E. M., & Arrese-Igor, C. (2005). Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. *Journal of Experimental Botany*, *56*(419), 2551–2561. https://doi.org/10.1093/jxb/eri249

García-Garijo, A., Tejera, N. A., Lluch, C., & Palma, F. (2014). Metabolic responses in root nodules of *Phaseolus vulgaris* and *Vicia sativa* exposed to the imazamox herbicide. *Pesticide Biochemistry and Physiology*, *111*(1), 19–23. https://doi.org/10.1016/j.pestbp.2014.04.005

Gargallo-Garriga, A., Preece, C., Sardans, J., Oravec, M., Urban, O., & Peñuelas, J. (2018). Root exudate metabolomes change under drought and show limited capacity for recovery. *Scientific Reports*, *8*(1), 12696. https://doi.org/10.1038/s41598-018-30150-0

Gastón, S., Zabalza, A., González, E. M., Arrese-Igor, C., Aparicio-Tejo, P. M., & Royuela, M. (2002). Imazethapyr, an inhibitor of the branched-chain amino acid biosynthesis, induces aerobic fermentation in pea plants. *Physiologia Plantarum*, *114*(4), 524–532. https://doi.org/10.1034/j.1399-3054.2002.1140404.x

Geddes, B. A., Paramasivan, P., Joffrin, A., Thompson, A. L., Christensen, K., Jorrin, B., Brett, P., Conway, S. J., Oldroyd, G. E. D., & Poole, P. S. (2019). Engineering transkingdom signalling in plants to control gene expression in rhizosphere bacteria. *Nature Communications*, *10*(1), 3430. https://doi.org/10.1038/s41467-019-10882-x

Geiger, D R, & Fondy, B. R. (1979). A method for continuous measurement of export from a leaf. *Plant Physiology*, *64*(3), 361–365. https://doi.org/10.1104/pp.64.3.361

Geiger, Donald R, Koch, K. E., & Shieh, W.-J. (1996). Effect of environmental factors on whole plant assimilate partitioning and associated gene expression. *Journal of Experimental Botany*, *47*, 1229–1238.

Gerwick, B. C., Mireles, L. C., & Eilers, R. J. (1993). Rapid diagnosis of ALS/AHAS-resistant weeds. *Weed Technology*, *7*(2), 519–524. https://doi.org/10.1017/s0890037x00027986

Ghashghaie, J., Duranceau, M., Badeck, F.-W., Cornic, G., Adeline, M.-T., & Deleens, E. (2001). δ ^{13}C of CO_2 respired in the dark in relation to δ ^{13}C of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell and Environment*, *24*(5), 505–515. https://doi.org/10.1046/j.1365-3040.2001.00699.x

Gibson, K. E., Kobayashi, H., & Walker, G. C. (2008). Molecular determinants of a symbiotic chronic infection. *Annual Review of Genetics*, *42*(1), 413–441. https://doi.org/10.1146/annurev.genet.42.110807.091427

Gil-Monreal, M., Giuntoli, B., Zabalza, A., Licausi, F., Royuela, M., & Wilson, Z. (2019). ERF-VII transcription factors induce ethanol fermentation in response to amino acid biosynthesis-inhibiting herbicides. *Journal of Experimental Botany*, *70*(20), 5839–5851. https://doi.org/10.1093/jxb/erz355

Gil-Quintana, E., Larrainzar, E., Arrese-Igor, C., & González, E. M. (2013a). Is N-feedback involved in the inhibition of nitrogen fixation in drought-stressed *Medicago truncatula*? *Journal of Experimental Botany*, *64*(1), 281–292. https://doi.org/10.1093/jxb/ers334

Gil-Quintana, E., Larrainzar, E., Seminario, A., Díaz-Leal, J. L., Alamillo, J. M., Pineda, M., Arrese-Igor, C., Wienkoop, S., & González, E. M. (2013b). Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean. *Journal of Experimental Botany*, *64*(8),

2171–2182. https://doi.org/10.1093/jxb/ert074

Gil-Quintana, E., Lyon, D., Staudinger, C., Wienkoop, S., & González, E. M. (2015). Medicago truncatula and Glycine max : different drought tolerance and similar local response of the root nodule proteome. *Journal of Proteome Research*, *14*(12), 5240–5251. https://doi.org/10.1021/acs.jproteome.5b00617

González, A., González-Murua, C., & Royuela, M. (1996). Influence of imazethapyr on *Rhizobium* growth and its symbiosis with pea (*Pisum sativum*). *Weed Science*, *44*, 31–37. https://doi.org/10.2307/4045779

González, E. M., Aparicio-Tejo, P. M., Gordon, A. J., Minchin, F. R., Royuela, M., & Arrese-Igor, C. (1998). Water-deficit effects on carbon and nitrogen metabolism of pea nodules. *Journal of Experimental Botany*, *49*(327), 1705–1714. https://doi.org/10.1093/jxb/49.327.1705

González, E. M., Gordon, A. J., James, C. L., & Arrese-lgor, C. (1995). The role of sucrose synthase in the response of soybean nodules to drought. *Journal of Experimental Botany*, *46*(10), 1515–1523. https://doi.org/10.1093/jxb/46.10.1515

González, E. M., Gálvez, L., & Arrese-Igor, C. (2001a). Abscisic acid induces a decline in nitrogen fixation that involves leghaemoglobin, but is independent of sucrose synthase activity. *Journal of Experimental Botany*, *52*(355), 285–293. https://doi.org/10.1093/jxb/52.355.285

González, E. M., Gálvez, L., Royuela, M., Aparicio-Tejo, P. M., & Arrese-Igor, C. (2001b). Insights into the regulation of nitrogen fixation in pea nodules: lessons from drought, abscisic acid and increased photoassimilate availability. *Agronomie*, *21*(6–7), 607–613. https://doi.org/10.1051/agro:2001151

González, E. M., Larrainzar, E., Marino, D., Wienkoop, S., Gil-Quintana, E., & Arrese-Igor, C. (2015). Physiological responses of N_2-fixing legumes to water limitation. In S. Sulieman & L. S. Tran (eds), *Legume Nitrogen Fixation in a Changing Environment: Achievements and Challenges* (pp. 5–33). Springer International Publishing. https://doi.org/10.1007/978-3-319-06212-9_2

Gordon, A. J., Minchin, F. R., Skot, L., & James, C. L. (1997). Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. *Plant Physiology*, *114*(3), 937–946. https://doi.org/10.1104/pp.114.3.937

Gordon, A. J., Ryle, G. J. a, Mitchell, D. F., & Powell, D. C. E. (1985). The flux of ^{14}C-labelled photosynthate through soyabean root nodules during N_2 fixation. *Journal of Experimental Botany*, *36*(5), 756–769. https://doi.org/10.1093/jxb/36.5.756

Gordon, A. J., Minchin, F. R., James, C. L., & Komina, O. (1999). Sucrose synthase in legume nodules is essential for nitrogen fixation. *Plant Physiology*, *120*(3), 867–878. https://doi.org/10.1104/pp.120.3.867

Graham, P. H. (2008). Ecology of the root-nodule bacteria of legumes. In M. J. Dilworth, E. K. James, J. I. Sprent, & W. E. Newton (eds.), *Nitrogen-fixing leguminous symbioses* (Vol 7, pp. 23–58). Springer Netherlands. https://doi.org/10.1007/978-1-4020-3548-7_2

Green, J., Crack, J. C., Thomson, A. J., & LeBrun, N. E. (2009). Bacterial sensors of oxygen. *Current opinion in microbiology*, *12*(2), 145-151. https://doi.org/10.1016/j.mib.2009.01.008

Guerin, V., Trinchant, J. C., & Rigaud, J. (1990). Nitrogen fixation (C_2H_2 Reduction) by broad bean (*Vicia faba* L.) nodules and bacteroids under water-restricted conditions. *Plant Physiology*,

92(3), 595–601. https://doi.org/10.1104/pp.92.3.595

Gyaneshwar, P., Hirsch, A. M., Moulin, L., Chen, W.-M., Elliott, G. N., Bontemps, C., Estrada-de Los Santos, P., Gross, E., Dos Reis, F. B., Sprent, J. I., Young, J. P. W., & James, E. K. (2011). Legume-nodulating betaproteobacteria: diversity, host range, and future prospects. *Molecular Plant-Microbe Interactions*, *24*(11), 1276–1288. https://doi.org/10.1094/MPMI-06-11-0172

Hacham, Y., Avraham, T., & Amir, R. (2002). The N-terminal region of *Arabidopsis* cystathionine γ-synthase plays an important regulatory role in methionine metabolism. *Plant Physiology*, *128*(2), 454–462. https://doi.org/10.1104/pp.010819

Hasegawa, P. M., Bressan, R. A., Zhu, J.-K., & Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology*, *51*(1), 463–499. https://doi.org/10.1146/annurev.arplant.51.1.463

Hassen, A. I., Bopape, F. L., Habig, J., & Lamprecht, S. C. (2012). Nodulation of rooibos (*Aspalathus linearis* Burm. f.), an indigenous South African legume, by members of both the α-Proteobacteria and β-Proteobacteria. *Biology and Fertility of Soils*, *48*(3), 295–303. https://doi.org/10.1007/s00374-011-0628-3

Hildebrandt, T. M. (2018). Synthesis versus degradation: directions of amino acid metabolism during *Arabidopsis* abiotic stress response. *Plant Molecular Biology*, *98*(1–2), 121–135. https://doi.org/10.1007/s11103-018-0767-0

Holmes, P., Farquharson, R., Hall, P. J., & Rolfe, B. G. (2006). Proteomic analysis of root meristems and the effects of acetohydroxyacid synthase-inhibiting herbicides in the root of *Medicago Truncatula*. *Journal of Proteome Research*, *5*(9), 2309–2316. https://doi.org/10.1021/pr0600677

Holz, M., Zarebanadkouki, M., Kaestner, A., Kuzyakov, Y., & Carminati, A. (2018). Rhizodeposition under drought is controlled by root growth rate and rhizosphere water content. *Plant and Soil*, *423*(1–2), 429–442. https://doi.org/10.1007/s11104-017-3522-4

Hosie, A. H. F., Allaway, D., Galloway, C. S., Dunsby, H. A., & Poole, P. S. (2002). *Rhizobium leguminosarum* has a second general amino acid permease with unusually broad substrate specificity and high similarity to branched-chain amino acid transporters (Bra/LIV) of the ABC family. *Journal of Bacteriology*, *184*(15), 4071–4080. https://doi.org/10.1128/JB.184.15.4071-4080.2002

Housley, T. L., Peterson, D. M., & Schrader, L. E. (1977). Long distance translocation of sucrose, serine, leucine, lysine, and carbon dioxide assimilates: I. Soybean. *Plant Physiology*, *59*(2), 217–220. https://doi.org/10.1104/pp.59.2.217

Huang, T., & Jander, G. (2017). Abscisic acid-regulated protein degradation causes osmotic stress-induced accumulation of branched-chain amino acids in *Arabidopsis thaliana*. *Planta*, *246*(4), 737–747. https://doi.org/10.1007/s00425-017-2727-3

Huang, X.-F., Chaparro, J. M., Reardon, K. F., Zhang, R., Shen, Q., & Vivanco, J. M. (2014). Rhizosphere interactions: root exudates, microbes, and microbial communities. *Botany*, *92*(4), 267–275. https://doi.org/10.1139/cjb-2013-0225

Hutchins, D. A., Walworth, N. G., Webb, E. A., Saito, M. A., Moran, D., McIlvin, M. R., Gale, J., & Fu, F.-X. (2015). Irreversibly increased nitrogen fixation in *Trichodesmium* experimentally adapted to elevated carbon dioxide. *Nature Communications*, *6*(1), 8155.

https://doi.org/10.1038/ncomms9155

Igamberdiev, A. U., & Eprintsev, A. T. (2016). Organic acids: The pools of fixed carbon involved in redox regulation and energy balance in higher plants. *Frontiers in Plant Science*, *7*, 1042. https://doi.org/10.3389/fpls.2016.01042

Irigoyen, J. J., Einerich, D. W., & Sanchez-Díaz, M. (1992). Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum*, *84*(1), 55–60. https://doi.org/10.1111/j.1399-3054.1992.tb08764.x

Jiménez-Zurdo, J. I., García-Rodríguez, F. M., & Toro, N. (1997). The *Rhizobium meliloti putA* gene: Its role in the establishment of the symbiotic interaction with alfalfa. *Molecular Microbiology*, *23*(1), 85–93. https://doi.org/10.1046/j.1365-2958.1997.1861555.x

Jiménez-Zurdo, J. I., van Dillewijn, P., Soto, M. J., de Felipe, M. R., Olivares, J., & Toro, N. (1995). Characterization of a *Rhizobium meliloti* proline dehydrogenase mutant altered in nodulation efficiency and competitiveness on alfalfa roots. *Molecular Plant-Microbe Interactions : MPMI*, *8*(4), 492–498. https://doi.org/10.1094/mpmi-8-0492

Johnston, A. W. B., & Behringer, J. E. (1975). Identification of the Rhizobium strains in pea root nodules using genetic markers. *Journal of General Microbiology*, *87*(2), 343–350. https://doi.org/10.1099/00221287-87-2-343

Joshi, V., Joung, J.-G., Fei, Z., & Jander, G. (2010a). Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress. *Amino Acids*, *39*(4), 933–947. https://doi.org/10.1007/s00726-010-0505-7

Karunakaran, R., East, A. K., & Poole, P. S. (2013). Malonate catabolism does not drive N_2 fixation in legume nodules. *Applied and Environmental Microbiology*, *79*(14), 4496–4498. https://doi.org/10.1128/AEM.00919-13

Karunakaran, R., Ramachandran, V. K., Seaman, J. C., East, A. K., Mouhsine, B., Mauchline, T. H., Prell, J., Skeffington, A., & Poole, P. S. (2009). Transcriptomic analysis of *Rhizobium leguminosarum* biovar *viciae* in symbiosis with host plants *Pisum sativum* and *Vicia cracca*. *Journal of Bacteriology*, *191*(12), 4002–4014. https://doi.org/10.1128/JB.00165-09

Keuntje, B., Masepohl, B., & Klipp, W. (1995). Expression of the *putA* gene encoding proline dehydrogenase from *Rhodobacter capsulatus* is independent of NtrC regulation but requires an Lrp-like activator protein. *Journal of Bacteriology*, *177*(22), 6432–6439. https://doi.org/10.1128/JB.177.22.6432-6439.1995

Kim, Y. S. (2002). Malonate metabolism: biochemistry, molecular biology, physiology, and industrial application. *Journal of Biochemistry and Molecular Biology*, *35*(5), 443–451. https://doi.org/10.5483/bmbrep.2002.35.5.443

Kim, Y. X., Ranathunge, K., Lee, S., Lee, Y., Lee, D., & Sung, J. (2018). Composite transport model and water and solute transport across plant roots: An Update. *Frontiers in Plant Science*, *9*. https://doi.org/10.3389/fpls.2018.00193

King, C. A., & Purcell, L. C. (2005). Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. *Plant Physiology*, *137*(4), 1389–1396. https://doi.org/10.1104/pp.104.056317

Kneip, C., Lockhart, P., Voß, C., & Maier, U.-G. (2007). Nitrogen fixation in eukaryotes – New

models for symbiosis. *BMC Evolutionary Biology*, *7*(1), 55. https://doi.org/10.1186/1471-2148-7-55

Kohl, D. H., Schubert, K. R., Carter, M. B., Hagedorn, C. H., & Shearer, G. (1988). Proline metabolism in N_2-fixing root nodules: energy transfer and regulation of purine synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(7), 2036–2040. https://doi.org/10.1073/pnas.85.7.2036

Kohl, D. H., Straub, P. F., & Shearer, G. (1994). Does proline play a special role in bacteroid metabolism? *Plant, Cell & Environment*, *17*(12), 1257–1262. https://doi.org/10.1111/j.1365-3040.1994.tb00527.x

Krasensky, J., & Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany*, *63*(4), 1593–1608. https://doi.org/10.1093/jxb/err460

Kumarasinghe, K. S., Kirda, C., Mohamed, A. R. A. G., Zapata, F., & Danso, S. K. A. (1992). ^{13}C isotope discrimination correlates with biological nitrogen fixation in soybean (*Glycine max* (L.) Merrill). *Plant and Soil*, *139*(1), 145–147. https://doi.org/10.1007/BF00012852

Kunert, K. J., Vorster, B. J., Fenta, B. A., Kibido, T., Dionisio, G., & Foyer, C. H. (2016). Drought stress responses in soybean roots and nodules. *Frontiers in Plant Science*, *7*, 1015. https://doi.org/10.3389/fpls.2016.01015

Kuzma, M. M., Winter, H., Storer, P., Oresnik, I., Atkins, C. A., & Layzell, D. B. (1999). The site of oxygen limitation in soybean nodules. *Plant Physiology*, *119*(2), 399–408. https://doi.org/10.1104/PP.119.2.399

Labhilili, M., Joudrier, P., & Gautier, M. F. (1995). Characterization of cDNAs encoding *Triticum durum* dehydrins and their expression patterns in cultivars that differ in drought tolerance. *Plant Science*, *112*(2), 219–230. https://doi.org/10.1016/0168-9452(95)04267-9

Ladrera, R. (2007). *Modelos de regulación de la fijación de nitrógeno en respuesta a sequía: soja y Medicago*. Universidad Pública de Navarra.

Ladrera, R., Marino, D., Larrainzar, E., González, E. M., & Arrese-Igor, C. (2007). Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiology*, *145*(2), 539–546. https://doi.org/10.1104/pp.107.102491

Larrainzar, E., Villar, I., Rubio, M. C., Pérez-Rontomé, C., Huertas, R., Sato, S., Mun, J., & Becana, M. (2020). Hemoglobins in the legume-rhizobium symbiosis. *New Phytologist*. https://doi.org/10.1111/nph.16673

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., Ladrera, R., Arrese-Igor, C., Weckwerth, W., & González, E. M. (2009). Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. *Molecular Plant-Microbe Interactions*, *22*(12), 1565–1576. https://doi.org/10.1094/MPMI-22-12-1565

Larrainzar, E., Wienkoop, S., Weckwerth, W., Ladrera, R., Arrese-Igor, C., & González, E. M. (2007). *Medicago truncatula* root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. *Plant Physiology*, *144*(3), 1495–1507. https://doi.org/10.1104/pp.107.101618

Lee, B., Jin, Y. L., Avice, J., Cliquet, J., Ourry, A., & Kim, T. (2009). Increased proline loading to phloem and its effects on nitrogen uptake and assimilation in water-stressed white clover (*Trifolium repens*). *New Phytologist*, *182*(3), 654–663. https://doi.org/10.1111/j.1469-8137.2009.02795.x

Lee, G.-A., Crawford, G. W., Liu, L., Sasaki, Y., & Chen, X. (2011). Archaeological soybean (*Glycine max*) in East Asia: Does size matter? *PLoS ONE*, *6*(11), e26720. https://doi.org/10.1371/journal.pone.0026720

Lee, J. M., & Owen, M. D. K. (2000). Comparison of acetolactate synthase enzyme inhibition among resistant and susceptible *Xanthium strumarium* biotypes. *Weed Science*, *48*(3), 286–290. https://doi.org/10.1614/0043-1745(2000)048[0286:coasei]2.0.co;2

Leigh, J. A. (2000). Nitrogen fixation in methanogens: the archaeal perspective. *Current issues in molecular biology*, *2*(4), 125-131.

Lemoine, R., Camera, S. La, Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., Bonnemain, J.-L., Laloi, M., Coutos-Thévenot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J., & Durand, M. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science*, *4*, 272. https://doi.org/10.3389/fpls.2013.00272

Lewis, G. P., Schrire, B. D., Mackinder, B. A., Rico, L., & Clark, R. (2013). A 2013 linear sequence of legume genera set in a phylogenetic context — A tool for collections management and taxon sampling. *South African Journal of Botany*, *89*, 76–84. https://doi.org/10.1016/J.SAJB.2013.06.005

Li, J., & Copeland, L. (2000). Role of malonate in chickpeas. *Phytochemistry*, *54*(6), 585–589. https://doi.org/10.1016/S0031-9422(00)00162-X

Li, Y., Parsons, R., Day, D. A., & Bergersen, F. J. (2002). Reassessment of major products of N_2 fixation by bacteroids from soybean root nodules. *Microbiology*, *148*(6), 1959–1966. https://doi.org/10.1099/00221287-148-6-1959

Liew, L. C., Singh, M. B., & Bhalla, P. L. (2014). Unique and conserved features of floral evocation in legumes. *Journal of Integrative Plant Biology*, *56*(8), 714–728. https://doi.org/10.1111/jipb.12187

Lima, J. D., & Sodek, L. (2003). N-stress alters aspartate and asparagine levels of xylem sap in soybean. *Plant Science*, *165*(3), 649–656. https://doi.org/10.1016/S0168-9452(03)00251-6

Lima, M. L., Oresnik, I. J., Fernando, S. M., Hunt, S., Smith, R., Turpin, D. H., & Layzell, D. B. (1994). The relationship between nodule adenylates and the regulation of nitrogenase activity by O_2 in soybean. *Physiologia Plantarum*, *91*(4), 687–695. https://doi.org/10.1111/j.1399-3054.1994.tb03006.x

Linthorst, H. J. M. (1991). Pathogenesis-related proteins of plants. *Critical Reviews in Plant Sciences*, *10*(2), 123–150. https://doi.org/10.1080/07352689109382309

Liu, L.-K., Becker, D. F., & Tanner, J. J. (2017). Structure, function, and mechanism of proline utilization A (PutA). *Archives of Biochemistry and Biophysics*, *632*, 142–157. https://doi.org/10.1016/J.ABB.2017.07.005

Lodwig, E. M., Hosie, A. H. F., Bourdès, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J. A., & Poole, P. S. (2003). Amino-acid cycling drives nitrogen fixation in the legume-

Rhizobium symbiosis. *Nature*, *422*(April 2003), 722–726. https://doi.org/10.1038/nature01549.1.

Loescher, W. H. (1987). Physiology and metabolism of sugar alcohols in higher plants. *Physiologia Plantarum*, *70*(3), 553–557. https://doi.org/10.1111/j.1399-3054.1987.tb02857.x

Long, J. W., Basler, E., & Reisbeck, S. (1971). Translocation of stem-injected glucose-^{3}H and sucrose-^{14}C in bean seedlings as affected by 2,4,5-trichlorophenoxyacetic acid treatment. *Proceedings of Oklahoma Academy of Sciences*, *51*, 52–55.

Maeda, H., & Dudareva, N. (2012). The shikimate pathway and aromatic amino acid biosynthesis in plants. *Annual Review of Plant Biology*, *63*(1), 73–105. https://doi.org/10.1146/annurev-arplant-042811-105439

Manchanda, G., & Garg, N. (2008). Salinity and its effects on the functional biology of legumes. *Acta Physiologiae Plantarum*, *30*(5), 595–618. https://doi.org/10.1007/s11738-008-0173-3

Mani, S., Van De Cotte, B., Van Montagu, M., & Verbruggen, N. (2002). Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. *Plant Physiology*, *128*(1), 73–83. https://doi.org/10.1104/PP.010572

Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., & González, E. M. (2007a). Nitrogen fixation control under drought stress. Localized or systemic? *Plant Physiology*, *143*(4), 1968–1974. https://doi.org/10.1104/pp.106.097139

Marino, D., González, E. M., & Arrese-Igor, C. (2006). Drought effects on carbon and nitrogen metabolism of pea nodules can be mimicked by paraquat: Evidence for the occurrence of two regulation pathways under oxidative stresses. *Journal of Experimental Botany*, *57*(3), 665–673. https://doi.org/10.1093/jxb/erj056

Marino, D., González, E. M., Frendo, P., Puppo, A., & Arrese-Igor, C. (2007b). NADPH recycling systems in oxidative stressed pea nodules : a key role for the NADP^{+}-dependent isocitrate dehydrogenase. *Planta*, *225*(2), 413–421. https://doi.org/10.1007/s00425-006-0354-5

Martínez, M., Palacios, J. M., Imperial, J., & Ruiz-Argüeso, T. (2004). Symbiotic autoregulation of *nifA* expression in *Rhizobium leguminosarum* bv. *viciae*. *Journal of Bacteriology*, *186*(19), 6586–6594. https://doi.org/10.1128/JB.186.19.6586-6594.2004

Massalha, H., Korenblum, E., Tholl, D., & Aharoni, A. (2017). Small molecules below-ground: the role of specialized metabolites in the rhizosphere. *The Plant Journal*, *90*(4), 788–807. https://doi.org/10.1111/tpj.13543

Miller, K. J., & Wood, J. M. (1996). Osmoadaptation by rhizosphere bacteria. *Annual Review of Microbiology*, *50*(1), 101–136. https://doi.org/10.1146/annurev.micro.50.1.101

Minchin, F. R. (1997). Regulation of oxygen diffusion in legume nodules. *Soil Biology and Biochemistry*, *29*(5–6), 881–888. https://doi.org/10.1016/S0038-0717(96)00204-0

Moulin, L., Munive, A., Dreyfus, B., & Boivin-Masson, C. (2001). Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature*, *411*(6840), 948–950. https://doi.org/10.1038/35082070

Muller, B., Pantin, F., Génard, M., Turc, O., Freixes, S., Piques, M., & Gibon, Y. (2011). Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. *Journal of Experimental Botany*, *62*(6), 1715–1729. https://doi.org/10.1093/jxb/erq438

Mulley, G., White, J. P., Karunakaran, R., Prell, J., Bourdes, A., Bunnewell, S., Hill, L., & Poole, P. S. (2011). Mutation of GOGAT prevents pea bacteroid formation and N_2 fixation by globally downregulating transport of organic nitrogen sources. *Molecular Microbiology*, *80*(1), 149–167. https://doi.org/10.1111/j.1365-2958.2011.07565.x

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*(1), 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Murray, J. D., Liu, C.-W., Chen, Y., & Miller, A. J. (2016). Nitrogen sensing in legumes. *Journal of Experimental Botany*, erw405. https://doi.org/10.1093/jxb/erw405

Nagae, M., Parniske, M., Kawaguchi, M., & Takeda, N. (2016). The thiamine biosynthesis gene *THI1* promotes nodule growth and seed maturation. *Plant Physiology*, *172*(3), 2033–2043. https://doi.org/10.1104/pp.16.01254

Nallu, S., Silverstein, K. A. T., Samac, D. A., Bucciarelli, B., Vance, C. P., & VandenBosch, K. A. (2013). Regulatory patterns of a large family of defensin-like genes expressed in nodules of *Medicago truncatula*. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0060355

Nasr Esfahani, M., Sulieman, S., Schulze, J., Yamaguchi-Shinozaki, K., Shinozaki, K., & Tran, L.-S. P. (2014). Mechanisms of physiological adjustment of N_2 fixation in *Cicer arietinum* L. (chickpea) during early stages of water deficit: single or multi-factor controls. *The Plant Journal*, *79*(6), 964–980. https://doi.org/10.1111/tpj.12599

Naya, L., Ladrera, R., Ramos, J., González, E. M., Arrese-Igor, C., Minchin, F. R., & Becana, M. (2007). The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. *Plant physiology*, *144*(2), 1104–1114. https://doi.org/10.1104/pp.107.099648

Nazaret, S., Cournoyer, B., Normand, P., & Simonet, P. (1991). Phylogenetic relationships among *Frankia* genomic species determined by use of amplified 16S rDNA sequences. *Journal of Bacteriology*, *173*(13), 4072–4078. https://doi.org/10.1128/jb.173.13.4072-4078.1991

Nelson, C. D., & Gorham, P. R. (1957). Uptake and translocation of C^{14}-labelled sugars applied to primary leaves of soybean seedlings. *Canadian Journal of Botany*, *35*(3), 339–347. https://doi.org/10.1139/b57-031

Neo, H. H., & Layzell, D. B. (1997). Phloem glutamine and the regulation of O_2 diffusion in legume nodules. *Plant Physiology*, *113*(1), 259–267. https://doi.org/10.1104/pp.113.1.259

Noiraud, N., Maurousset, L., & Lemoine, R. (2001). Transport of polyols in higher plants. *Plant Physiology and Biochemistry*, *39*(9), 717–728. https://doi.org/10.1016/S0981-9428(01)01292-X

O'Leary, M. H. (1981). Carbon isotope fractionation in plants. *Phytochemistry*, *20*(4), 553–567. https://doi.org/10.1016/0031-9422(81)85134-5

O'Leary, M. H. (1995). Environmental effects on carbon isotope fractionation in terrestrial plants. In E. Wada, T. Yoneyama, M. Minagawa, T. Ando, & B. Fry (Eds.), *Stable isotopes in the biosphere* (pp. 78–91). Kyoto University Press.

O'Sullivan, J., Thomas, R. J., & Bouw, W. J. (1998). Effect of imazethapyr and imazamox soil residues on several vegetable crops grown in Ontario. *Canadian Journal of Plant Science*, *78*(4), 647–651. https://doi.org/10.4141/P97-127

Oburger, E., & Jones, D. L. (2018). Sampling root exudates – Mission impossible? *Rhizosphere*, *6*,

116–133. https://doi.org/10.1016/j.rhisph.2018.06.004

Olanrewaju, O. S., Ayangbenro, A. S., Glick, B. R., & Babalola, O. O. (2019). Plant health: feedback effect of root exudates-rhizobiome interactions. *Applied Microbiology and Biotechnology*, *103*(3), 1155–1166. https://doi.org/10.1007/s00253-018-9556-6

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology*, *11*(4), 252–263. https://doi.org/10.1038/nrmicro2990

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., & Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Review of Genetics*, *45*(1), 119–144. https://doi.org/10.1146/annurev-genet-110410-132549

Orcaray, L., Igal, M., Marino, D., Zabalza, A., & Royuela, M. (2010). The possible role of quinate in the mode of action of glyphosate and acetolactate synthase inhibitors. *Pest Management Science*, *66*(3), 262–269. https://doi.org/10.1002/ps.1868

Palta, J. A., & Gregory, P. J. (1997). Drought affects the fluxes of carbon to roots and soil in ^{13}C pulse-labelled plants of wheat. *Soil Biology and Biochemistry*, *29*(9–10), 1395–1403. https://doi.org/10.1016/S0038-0717(97)00050-3

Patonnier, M. P., Peltier, J. P., & Marigo, G. (1999). Drought-induced increase in xylem malate and mannitol concentrations and closure of *Fraxinus excelsior* L. stomata. *Journal of Experimental Botany*, *50*(336), 1223-1229. https://doi.org/10.1093/jxb/50.336.1223

Patriarca, E. J., Tatè, R., & Iaccarino, M. (2002). Key Role of Bacterial NH_4 Metabolism in *Rhizobium*-Plant Symbiosis. *Microbiology and Molecular Biology Reviews*, *66*(2), 203–222. https://doi.org/10.1128/MMBR.66.2.203

Pecetti, L., Marcotrigiano, A. R., Russi, L., Romani, M., & Annicchiarico, P. (2019). Adaptation of field pea varieties to organic farming across different environments of Italy. *Crop and Pasture Science*, *70*(4), 327. https://doi.org/10.1071/CP18216

Pedersen, A. L., Feldner, H. C., & Rosendahl, L. (1996). Effect of proline on nitrogenase activity in symbiosomes from root nodules of soybean (*Glycine max* L.) subjected to drought stress. *Journal of Experimental Botany*, *47*(10), 1533–1539. https://doi.org/10.1093/jxb/47.10.1533

Peix, A., Ramírez-Bahena, M. H., Velázquez, E., & Bedmar, E. J. (2015). Bacterial associations with legumes. *Critical Reviews in Plant Sciences*, *34*(1–3), 17–42. https://doi.org/10.1080/07352689.2014.897899

Peoples, M. B., Hauggaard-Nielsen, H., Huguenin-Elie, O., Jensen, E. S., Justes, E., & Williams, M. (2019). The contributions of legumes to reducing the environmental risk of agricultural production. *Agroecosystem Diversity*, 123–143. https://doi.org/10.1016/B978-0-12-811050-8.00008-X

Perret, X., Staehelin, C., & Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews*, *64*(1), 180–201. https://doi.org/10.1128/MMBR.64.1.180-201.2000

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, *11*(11), 789–799. https://doi.org/10.1038/nrmicro3109

Pini, F., East, A. K., Appia-Ayme, C., Tomek, J., Karunakaran, R., Mendoza-Suárez, M., Edwards,

A., Terpolilli, J. J., Roworth, J., Downie, J. A., & Poole, P. S. (2017). Bacterial biosensors for in vivo spatiotemporal mapping of root secretion. *Plant Physiology*, *174*(3), 1289–1306. https://doi.org/10.1104/pp.16.01302

Poole, P. S., Schofiel, N. A., Reid, C. J., Drew, E. M., & Walshaw, D. L. (1994). Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology*, *140*(10), 2797–2809. https://doi.org/10.1099/00221287-140-10-2797

Popp, C., & Ott, T. (2011). Regulation of signal transduction and bacterial infection during root nodule symbiosis. *Current Opinion in Plant Biology*, *14*(4), 458–467. https://doi.org/10.1016/J.PBI.2011.03.016

Porcel, R., Azcón, R., & Ruiz-Lozano, J. M. (2005). Evaluation of the role of genes encoding for dehydrin proteins (LEA D-11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. *Journal of Experimental Botany*, *56*(417), 1933–1942. https://doi.org/10.1093/jxb/eri188

Preisig, O., Anthamatten, D., & Hennecke, H. (1993). Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(8), 3309–3313. https://doi.org/10.1073/pnas.90.8.3309

Prell, J, White, J. P., Bourdes, A., Bunnewell, S., Bongaerts, R. J., & Poole, P. S. (2009a). Legumes regulate *Rhizobium* bacteroid development and persistence by the supply of branched-chain amino acids. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(30), 12477–12482. https://doi.org/10.1073/pnas.0903653106

Prell, Jurgen, Bourdès, A., Karunakaran, R., López-Gómez, M., & Poole, P. (2009b). Pathway of gamma-aminobutyrate metabolism in *Rhizobium leguminosarum* 3841 and its role in symbiosis. *Journal of Bacteriology*, *191*(7), 2177–2186. https://doi.org/10.1128/JB.01714-08

Prell, Jurgen, Bourdès, A., Kumar, S., Lodwig, E., Hosie, A., Kinghorn, S., White, J., & Poole, P. (2010). Role of symbiotic auxotrophy in the *Rhizobium*-legume symbioses. *PLoS ONE*, *5*(11), e13933. https://doi.org/10.1371/journal.pone.0013933

Purcell, L. C. (2009). Physiological responses of N fixation to drought and selecting genotypes for improved N fixation. In D. W. Emerich & H. B. Krishnan (Eds.), *Nitrogen Fixation in Crop Production* (pp. 211–238). John Wiley & Sons, Ltd. https://doi.org/10.2134/agronmonogr52.c8

Purcell, L. C., de Silva, M., King, C. A., & Han Kim, W. (1997). Biomass accumulation and allocation in soybean associated with genotypic differences in tolerance of nitrogen fixation to water deficits. *Plant and Soil*, *196*(1), 101–113. https://doi.org/10.1023/A:1004289609466

Purcell, L. C., Serraj, R., de Silva, M., Sinclair, T. R., & Bona, S. (1998). Ureide concentration of field-grown soybean in response to drought and the relationship to nitrogen fixation. *Journal of Plant Nutrition*, *21*(5), 949–966. https://doi.org/10.1080/01904169809365456

Ramachandran, V. K., East, A. K., Karunakaran, R., Downie, J. A., & Poole, P. S. (2011). Adaptation of *Rhizobium leguminosarum* to pea, alfalfa and sugar beet rhizospheres investigated by comparative transcriptomics. *Genome Biology*, *12*(10), R106. https://doi.org/10.1186/gb-2011-12-10-r106

Ramos, M. L. G., Gordon, A. J., Minchin, F. R., Sprent, J. I., & Parsons, R. (1999). Effect of water

stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris* L.). *Annals of Botany*, *83*(1), 57–63. https://doi.org/10.1006/anbo.1998.0792

Ranathunge, K., Steudle, E., & Lafitte, R. (2003). Control of water uptake by rice (*Oryza sativa* L.): Role of the outer part of the root. *Planta*, *217*(2), 193–205. https://doi.org/10.1007/s00425-003-0984-9

Rasmann, S., & Turlings, T. C. (2016). Root signals that mediate mutualistic interactions in the rhizosphere. *Current Opinion in Plant Biology*, *32*, 62–68. https://doi.org/10.1016/J.PBI.2016.06.017

Rawsthorne, S., Minchin, F. R., Summerfield, R. J., Cookson, C., & Coombs, J. (1980). Carbon and nitrogen metabolism in legume root nodules. *Phytochemistry*, *19*(3), 341–355. https://doi.org/10.1016/0031-9422(80)83181-5

Reckling, M., Hecker, J.-M., Bergkvist, G., Watson, C. A., Zander, P., Schläfke, N., Stoddard, F. L., Eory, V., Topp, C. F. E., Maire, J., & Bachinger, J. (2016). A cropping system assessment framework—Evaluating effects of introducing legumes into crop rotations. *European Journal of Agronomy*, *76*, 186–197. https://doi.org/10.1016/J.EJA.2015.11.005

Rees, D. C., & Howard, J. B. (2000). Nitrogenase: standing at the crossroads. *Current Opinion in Chemical Biology*, *4*(5), 559–566. https://doi.org/10.1016/S1367-5931(00)00132-0

Reguera, M., Bonilla, I., & Bolaños, L. (2010). Boron deficiency results in induction of pathogenesis-related proteins from the PR-10 family during the legume-rhizobia interaction. *Journal of Plant Physiology*, *167*(8), 625–632. https://doi.org/10.1016/j.jplph.2009.11.017

Rhodes, D., Hogan, A. L., Deal, L., Jamieson, G. C., & Haworth, P. (1987). Amino acid metabolism of *Lemna minor* L. *Plant Physiology*, *84*(3), 775–780. https://doi.org/10.1104/pp.84.3.775

Rigaud, J., & Puppo, A. (1975). Indole-3-acetic Acid Catabolism by Soybean Bacteroids. *Journal of General Microbiology*, *88*(2), 223–228. https://doi.org/10.1099/00221287-88-2-223

Rilling, J. I., Acuña, J. J., Nannipieri, P., Cassan, F., Maruyama, F., & Jorquera, M. A. (2019). Current opinion and perspectives on the methods for tracking and monitoring plant growth–promoting bacteria. *Soil Biology and Biochemistry*, *130*, 205–219. https://doi.org/10.1016/J.SOILBIO.2018.12.012

Rocha, M., Sodek, L., Licausi, F., Hameed, M. W., Dornelas, M. C., & Van Dongen, J. T. (2010). Analysis of alanine aminotransferase in various organs of soybean (*Glycine max*) and in dependence of different nitrogen fertilisers during hypoxic stress. *Amino Acids*, *39*(4), 1043–1053. https://doi.org/10.1007/s00726-010-0596-1

Ronson, C. W., Lyttleton, P., & Robertson, J. G. (1981). C4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proceedings of the National Academy of Sciences*, *78*(7), 4284–4288. https://doi.org/10.1073/pnas.78.7.4284

Royuela, M., González, A., Arrese-Igor, C., Aparicio-Tejo, P. M., & González-Murua, C. (1998). Imazethapyr inhibition of acetolactate synthase in *Rhizobium* and its symbiosis with pea. *Pesticide Science*, *52*(4), 372–380. https://doi.org/10.1002/(SICI)1096-9063(199804)52:4<372::AID-PS730>3.0.CO;2-P

Royuela, M., González, A., González, E. M., Arrese-Igor, C., Aparicio-Tejo, P. M., & González-

Murua, C. (2000). Physiological consequences of continuous, sublethal imazethapyr supply to pea plants. *Journal of Plant Physiology*, *157*(3), 345–354. https://doi.org/10.1016/S0176-1617(00)80057-7

Ruiz-Argüeso, T., Palacios, J. M., & Imperial, J. (2001). Regulation of the hydrogenase system in *Rhizobium leguminosarum*. *Plant and Soil*, *230*(1), 49–57. https://doi.org/10.1023/A:1004578324977

Sakaliene, O., Papiernik, S. K., Koskinen, W. C., & Spokas, K. A. (2007). Sorption and predicted mobility of herbicides in Baltic soils. *Journal of Environmental Science and Health, Part B*, *42*(6), 641–647. https://doi.org/10.1080/03601230701465601

Salavati, A., Bushehri, A. A. S., Taleei, A., Hiraga, S., & Komatsu, S. (2012). A comparative proteomic analysis of the early response to compatible symbiotic bacteria in the roots of a supernodulating soybean variety. *Journal of Proteomics*, *75*(3), 819–832. https://doi.org/10.1016/j.jprot.2011.09.022

Sassi, S., González, E. M., Aydi, S., Arrese-Igor, C., & Abdelly, C. (2008). Tolerance of common bean to long-term osmotic stress is related to nodule carbon flux and antioxidant defenses: Evidence from two cultivars with contrasting tolerance. *Plant and Soil*, *312*(1–2), 39–48. https://doi.org/10.1007/s11104-008-9613-5

Sato, T., Yashima, H., Ohtake, N., Sueyoshi, K., Akao, S., Harper, J. E., & Ohyama, T. (1998). Determination of leghemoglobin components and xylem sap composition by capillary electrophoresis in hypernodulation soybean mutants cultivated in the field. *Soil Science and Plant Nutrition*, *44*(4), 635–645. https://doi.org/10.1080/00380768.1998.10414487

Satoh, R., Nakashima, K., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2002). ACTCAT, a novel cis-acting element for proline- and hypoosmolarity-responsive expression of the *proDH* gene encoding proline dehydrogenase in *Arabidopsis*. *Plant Physiology*, *130*(2), 709–719. https://doi.org/10.1104/PP.009993

Scarponi, L., Younis, M. E., Standardi, A., Hassan, N. M., & Martinetti, L. (1997). Effects of chlorimuron-ethyl, imazethapyr, and propachlor on free amino acids and protein formation in *Vicia faba* L. *Journal of Agricultural and Food Chemistry*, *45*(9), 3652–3658. https://doi.org/10.1021/jf960771f

Scholander, P. F., Bradstreet, E. D., Hemmingsen, E. A., & Hammel, H. T. (1965). Sap pressure in vascular plants: negative hydrostatic pressure can be measured in plants. *Science*, *148*(3668), 339–346. https://doi.org/10.1126/science.148.3668.339

Schrire, B., Lavin, M., & Lewis, G. P. (2005). Global distribution patterns of the Leguminosae: insights from recent phylogenies. In I. Friis & H. Balslev (eds.), *Plant Diversity and Complexity Patterns: Local, Regional and Global Dimensions* (pp. 375-422.). Royal Danish Academy of Sciences and Letters.

Schubert, K. R. (1986). Products of biological nitrogen fixation in higher plants: Synthesis, transport, and metabolism. *Annual Review of Plant Physiology*, *37*(1), 539-574.

Schubert, S., Serraj, R., Plies-Balzer, E., & Mengel, K. (1995). Effect of drought stress on growth, sugar concentrations and amino acid accumulation in N_2-fixing alfalfa (*Medicago sativa*). *Journal of Plant Physiology*, *146*(4), 541–546. https://doi.org/10.1016/S0176-1617(11)82021-3

Serraj, R., Sinclair, T. R., & Purcell, L. C. (1999a). Symbiotic N_2 fixation response to drought.

Journal of Experimental Botany, *50*(331), 143–155. https://doi.org/10.1093/jxb/50.331.143

Serraj, Rachid, Shelp, B. J., & Sinclair, T. R. (1998). Accumulation of γ-aminobutyric acid in nodulated soybean in response to drought stress. *Physiologia Plantarum*, *102*(1), 79–86. https://doi.org/10.1034/j.1399-3054.1998.1020111.x

Serraj, Rachid, & Sinclair, T. R. (1996). Processes contributing to N_2-fixation intensitivity to drought in the soybean cultivar Jackson. *Crop Science*, *36*(4), 961. https://doi.org/10.2135/cropsci1996.0011183X003600040024x

Serraj, Rachid, Vadez, V., Denison, R. F., & Sinclair, T. R. (1999b). Involvement of ureides in nitrogen fixation inhibition in soybean. *Plant Physiology*, *119*(1), 289–296. https://doi.org/10.1104/pp.119.1.289

Shamseldin, A., Abdelkhalek, A., & Sadowsky, M. J. (2017). Recent changes to the classification of symbiotic, nitrogen-fixing, legume-associating bacteria: a review. *Symbiosis*, *71*(2), 91–109. https://doi.org/10.1007/s13199-016-0462-3

Shaner, D. L. (1991). Physiological effects of the imidazolinone herbicides. In D. L. Shaner & S. L. O´Connor (Eds.), *The imidazolinone herbicides* (pp. 129–137). CRC Press.

Shanker, A. K., Maheswari, M., Yadav, S. K., Desai, S., Bhanu, D., Attal, N. B., & Venkateswarlu, B. (2014). Drought stress responses in crops. *Functional & Integrative Genomics*, *14*(1), 11–22. https://doi.org/10.1007/s10142-013-0356-x

Shelp, B. J., Bown, A. W., & Faure, D. (2006). Extracellular γ-aminobutyrate mediates communication between plants and other organisms. *Plant Physiology*, *142*(4), 1350–1352. https://doi.org/10.1104/pp.106.088955

Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V., & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols*, *1*(6), 2856–2860. https://doi.org/10.1038/nprot.2006.468

Shilov, I. V., Seymourt, S. L., Patel, A. A., Loboda, A., Tang, W. H., Keating, S. P., Hunter, C. L., Nuwaysir, L. M., & Schaeffer, D. A. (2007). The paragon algorithm, a next generation search engine that uses sequence temperature values sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Molecular and Cellular Proteomics*, *6*(9), 1638–1655. https://doi.org/10.1074/mcp.T600050-MCP200

Shiraishi, A., Matsushita, N., & Hougetsu, T. (2010). Nodulation in black locust by the Gammaproteobacteria *Pseudomonas* sp. and the Betaproteobacteria *Burkholderia* sp. *Systematic and Applied Microbiology*, *33*(5), 269–274. https://doi.org/10.1016/J.SYAPM.2010.04.005

Singh, B. K., & Shaner, D. L. (1995). Biosynthesis of branched chain amino acids: From test tube to field. *The Plant Cell*, *7*(7), 935–944. https://doi.org/10.1105/tpc.7.7.935

Smith, P. M. C., & Atkins, C. A. (2002). Purine biosynthesis. Big in cell division, even bigger in nitrogen assimilation. *Plant Physiology*, *128*(3), 793–802. https://doi.org/10.1104/pp.010912

Somasegaran, P., & Hoben, H. J. (Eds.) (1994). *Handbook for rhizobia: Methods in legume-Rhizobium Technology*. Springer New York. https://doi.org/10.1007/978-1-4613-8375-8

Soussi, M., Khadri, M., Lluch, C., & Ocaña, A. (2001). Carbon metabolism and bacteroid respiration in nodules of chick-pea (*Cicer arietinum* L.) plants grown under saline conditions. *Plant Biosystems*, *135*(2), 157–164.

https://doi.org/10.1080/11263500112331350770

Stal, L. J., & Krumbein, W. E. (1987). Temporal separation of nitrogen fixation and photosynthesis in the filamentous, non-heterocystous cyanobacterium *Oscillatoria* sp. *Archives of Microbiology*, *149*(1), 76–80. https://doi.org/10.1007/BF00423140

Staudinger, C., Mehmeti-Tershani, V., Gil-Quintana, E., González, E. M., Hofhansl, F., Bachmann, G., & Wienkoop, S. (2016). Evidence for a rhizobia-induced drought stress response strategy in *Medicago truncatula*. *Journal of Proteomics*, *136*, 202–213. https://doi.org/10.1016/j.jprot.2016.01.006

Streeter, J. G. (1980). Carbohydrates in Soybean Nodules: II. Distribution of compounds in seedlings during the onset of nitrogen fixation. *Plant Physiology*, *66*(3), 471–476. https://doi.org/10.1104/pp.66.3.471

Streeter, J. G., Lohnes, D. G., & Fioritto, R. J. (2001). Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. *Plant, Cell and Environment*, *24*(4), 429–438. https://doi.org/10.1046/j.1365-3040.2001.00690.x

Sturm, A., & Tang, G. Q. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science*, *4*(10), 401–407. https://doi.org/10.1016/S1360-1385(99)01470-3

Sulieman, S., & Schulze, J. (2010). Phloem-derived γ-aminobutyric acid (GABA) is involved in upregulating nodule N_2 fixation efficiency in the model legume *Medicago truncatula*. *Plant, Cell and Environment*, *33*(12), 2162–2172. https://doi.org/10.1111/j.1365-3040.2010.02214.x

Szabados, L., & Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science*, 15(2), 89-97 https://doi.org/10.1016/j.tplants.2009.11.009

Taiz, L., Zeiger, E., Moller, I. M., & Murphy, A. (2015). *Plant Physiology and Development* (6th ed.). Sinauer Associates. Inc., Sunderland.

Tajima, S., Nomura, M., & Kouchi, H. (2004). Ureide biosynthesis in legume nodules. *Frontiers in Bioscience*, *9*, 1374-1381. https://doi.org/10.2741/1345

Talbi, C., Sánchez, C., Hidalgo-García, A., González, E. M., Arrese-Igor, C., Girard, L., Bedmar, E. J., & Delgado, M. J. (2012). Enhanced expression of *Rhizobium etli cbb*$_3$ oxidase improves drought tolerance of common bean symbiotic nitrogen fixation. *Journal of Experimental Botany*, *63*(14), 5035–5043. https://doi.org/http://dx.doi.org/10.1093/jxb/ers101

Tan, S., Evans, R., & Singh, B. (2006). Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. *Amino Acids*, *30*(2), 195–204. https://doi.org/10.1007/s00726-005-0254-1

Tang, F., Yang, S., Liu, J., & Zhu, H. (2016). *Rj4* , a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. *Plant Physiology*, *170*(1), 26–32. https://doi.org/10.1104/pp.15.01661

Tang, W. H., Shilov, I. V., & Seymour, S. L. (2008). Nonlinear fitting method for determining local false discovery rates from decoy database searches. *Journal of Proteome Research*, *7*(9), 3661–3667. https://doi.org/10.1021/pr070492f

Tanner, J. J. (2008). Structural biology of proline catabolism. *Amino Acids*, *35*(4), 719–730. https://doi.org/10.1007/s00726-008-0062-5

Terpolilli, J. J., Hood, G. A., & Poole, P. S. (2012). What determines the efficiency of N_2-fixing *Rhizobium*-legume symbioses? In *Advances in microbial physiology* (Vol. 60, pp. 325-389). Elsevier Ltd. https://doi.org/10.1016/B978-0-12-398264-3.00005-X

Thompson, M. V. (2006). Phloem: the long and the short of it. *Trends in Plant Science*, *11*(1), 26–32. https://doi.org/10.1016/J.TPLANTS.2005.11.009

Thöny-Meyer, L. (1997). Biogenesis of respiratory cytochromes in bacteria. *Microbiology and Molecular Biology Reviews*, *61*(3), 337–376. https://doi.org/10.1128/.61.3.337-376.1997

Thorpe, M. R., Walsh, K. B., & Minchin, P. E. H. (1998). Photoassimilate partitioning in nodulated soybean I. ^{11}C methodology. *Journal of Experimental Botany*, *49*(328), 1805–1815. https://doi.org/10.1093/jxb/49.328.1805

Trinchant, J.-C., & Rigaud, J. (1996). Bacteroid oxalate oxidase and soluble oxalate in nodules of faba beans (*Vicia faba* L.) submitted to water restricted conditions: possible involvement in nitrogen fixation. *Journal of Experimental Botany*, *47*(12), 1865–1870. https://doi.org/10.1093/jxb/47.12.1865

Trinchant, J. C., Yang, Y. S., & Rigaud, J. (1998). Proline accumulation inside symbiosomes of faba bean nodules under salt stress. *Physiologia Plantarum*, *104*(1), 38–49. https://doi.org/10.1034/j.1399-3054.1998.1040106.x

Tsikou, D., Kalloniati, C., Fotelli, M. N., Nikolopoulos, D., Katinakis, P., Udvardi, M. K., Rennenberg, H., & Flemetakis, E. (2013). Cessation of photosynthesis in *Lotus japonicus* leaves leads to reprogramming of nodule metabolism. *Journal of Experimental Botany*, *64*(5), 1317–1332. https://doi.org/10.1093/jxb/ert015

Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. *Genome Biology*, *14*(6), 209. https://doi.org/10.1186/gb-2013-14-6-209

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., & Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods*, *13*(9), 731–740. https://doi.org/10.1038/nmeth.3901

Udvardi, M. K., & Day, D. A. (1997). Metabolite transport across symbiotic membranes of legume nodules. *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*, 493–523. https://doi.org/10.1146/annurev.arplant.48.1.493

Udvardi, M. K., Ou Yang, L.-J., Young, S., & Day, D. A. (1990). Sugar and amino acid transport across symbiotic membranes from soybean nodules. *Molecular Plant-Microbe Interactions*, *3*(5), 334–340. https://doi.org/10.1094/MPMI-3-334

Udvardi, M. K., Price, G. D., Gresshoff, P. M., & Day, D. A. (1988). A dicarboxylate transporter on the peribacteroid membrane of soybean nodules. *FEBS Letters*, *231*(1), 36–40. https://doi.org/10.1016/0014-5793(88)80697-5

Udvardi, M., & Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annual Review of Plant Biology*, *64*, 781–805. https://doi.org/10.1146/annurev-arplant-050312-120235

Vadez, V., Sinclair, T. R., & Serraj, R. (2000). Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N_2 fixation in soybean. *Physiologia Plantarum*, *110*(2), 215–223. https://doi.org/10.1034/j.1399-3054.2000.110211.x

Valdés-López, O., Formey, D., Isidra-Arellano, M. C., Reyero-Saavedra, M. del R., Fernández-

Göbel, T. F., & Sánchez-Correa, M. del S. (2019). Argonaute proteins: Why are they so important for the legume–rhizobia symbiosis? *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.01177

Valverde, A., Velázquez, E., Gutiérrez, C., Cervantes, E., Ventosa, A., & Igual, J. M. (2003). *Herbaspirillium lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. *International Journal of Systematic and Evolutionary Microbiology*, *53*(6), 1979–1983. https://doi.org/10.1099/ijs.0.02677-0

van Dillewijn, P., Soto, M. J., Villadas, P. J., & Toro, N. (2001). Construction and environmental release of a *Sinorhizobium meliloti* strain genetically modified to be more competitive for alfalfa nodulation. *Applied and Environmental Microbiology*, *67*(9), 3860–3865. https://doi.org/10.1128/aem.67.9.3860-3865.2001

van Nguyen T., Pawlowski K. (2017) *Frankia* and actinorhizal plants: symbiotic nitrogen fixation. In: Mehnaz S. (eds), *Rhizotrophs: Plant Growth Promotion to Bioremediation. Microorganisms for Sustainability*, (vol 2., pp. 237-261) Springer, Singapore. https://doi.org/10.1007/978-981-10-4862-3_12

van Overbeek, L. S., & van Elsas, J. D. (1995). Root exudate-induced promoter activity in *Pseudomonas fluorescens* mutants in the wheat rhizosphere. *Applied and Environmental Microbiology*, *61*(3), 890–898.

Vance, C. P. (2000). Amide biosynthesis in root nodules of temperate legumes. In E. W. Triplett (Ed.), *Prokaryotic nitrogen fixation: a model system for the analysis of a biological process.* (pp. 589–607). Horizon Scientific Press.

Vance, C. P., Johnson, L. E. B., & Hardarson, G. (1980). Histological comparisons of plant and *Rhizobium* induced ineffective nodules in alfalfa. *Physiological Plant Pathology*, *17*(2), 167–173. https://doi.org/10.1016/0048-4059(80)90049-1

Venturi, V., & Keel, C. (2016). Signaling in the rhizosphere. *Trends in Plant Science*, *21*(3), 187–198. https://doi.org/10.1016/J.TPLANTS.2016.01.005

Verdoy, D., Coba de la Peña, T., Redondo, F. J., Lucas, M. M., & Pueyo, J. J. (2006). Transgenic *Medicago truncatula* plants that accumulate proline display nitrogen-fixing activity with enhanced tolerance to osmotic stress. *Plant, Cell and Environment*, *29*(10), 1913–1923. https://doi.org/10.1111/j.1365-3040.2006.01567.x

Vernon, L. P., & Aronoff, S. (1952). Metabolism of soybean leaves. IV. Translocation from soybean leaves. *Archives of Biochemistry and Biophysics*, *36*(2), 383–398. https://doi.org/10.1016/0003-9861(52)90424-4

Vieira, R. F., Silva, C. M. M. S., & Silveira, A. P. D. (2007). Soil microbial biomass C and symbiotic processes associated with soybean after sulfentrazone herbicide application. *Plant and Soil*, *300*(1–2), 95–103. https://doi.org/10.1007/s11104-007-9392-4

Vischetti, C., Casucci, C., & Perucci, P. (2002). Relationship between changes of soil microbial biomass content and imazamox and benfluralin degradation. *Biology and Fertility of Soils*, *35*(1), 13–17. https://doi.org/10.1007/s00374-001-0433-5

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

photorespiration. *Plant Physiology*, *157*(1), 256–268. https://doi.org/10.1104/pp.111.181024

Vives-Peris, V., Molina, L., Segura, A., Gómez-Cadenas, A., & Pérez-Clemente, R. M. (2018). Root exudates from citrus plants subjected to abiotic stress conditions have a positive effect on rhizobacteria. *Journal of Plant Physiology*, *228*, 208–217. https://doi.org/10.1016/J.JPLPH.2018.06.003

von Caemmerer, S., & Farquhar, G. D. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*, *153*(4), 376-387. https://doi.org/10.2307/23375285

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nature Reviews Microbiology*, *10*(12), 828–840. https://doi.org/10.1038/nrmicro2910

Walsh, K. B. (1995). Physiology of the legume nodule and its response to stress. *Soil Biology and Biochemistry*, *27*(4–5), 637–655. https://doi.org/10.1016/0038-0717(95)98644-4

Walshaw, D. L., & Poole, P. S. (1996). The general L-amino acid permease of *Rhizobium leguminosarum* is an ABC uptake system that also influences efflux of solutes. *Molecular Microbiology*, *21*(6), 1239–1252. https://doi.org/10.1046/j.1365-2958.1996.00078.x

Wardlaw, I. F. (1990). Tansley Review No. 27 The control of carbon partitioning in plants. *New Phytologist*, *116*(3), 341–381. https://doi.org/10.1111/j.1469-8137.1990.tb00524.x

Webb, B. A., Hildreth, S., Helm, R. F., & Scharf, B. E. (2014). *Sinorhizobium meliloti* chemoreceptor McpU mediates chemotaxis toward host plant exudates through direct proline sensing. *Applied and Environmental Microbiology*, *80*(11), 3404–3415. https://doi.org/10.1128/AEM.00115-14

Westerfeld, W. W. (1945). A colorimetric determination of blood acetoin. *The Journal of Biological Chemistry*, *161*, 495–502.

White, J., Prell, J., James, E. K., & Poole, P. (2007). Nutrient sharing between symbionts. *Plant Physiology*, *144*(2), 604–614. https://doi.org/10.1104/pp.107.097741

Whitehead, L. F., & Day, D. A. (1997). The peribacteroid membrane. *Physiologia Plantarum*, *100*(1), 30–44. https://doi.org/10.1111/j.1399-3054.1997.tb03452.x

Wienkoop, S., Morgenthal, K., Wolschin, F., Scholz, M., Selbig, J., & Weckwerth, W. (2008). Integration of metabolomic and proteomic phenotypes. *Molecular & Cellular Proteomics*, *7*(9), 1725–1736. https://doi.org/10.1074/MCP.M700273-MCP200

Witty, J. F., & Minchin, F. R. (1998). Methods for the continuous measurement of O_2 consumption and H_2 production by nodulated legume root systems. *Journal of Experimental Botany*, *49*(323), 1041–1047. https://doi.org/10.1093/jxb/49.323.1041

Wong-Villarreal, A., & Caballero-Mellado, J. (2010). Rapid identification of nitrogen-fixing and legume-nodulating *Burkholderia* species based on PCR 16S rRNA species-specific oligonucleotides. *Systematic and Applied Microbiology*, *33*(1), 35–43. https://doi.org/10.1016/j.syapm.2009.10.004

Yoneyama, T., & Ohtani, T. (1983). Variations of natural ^{13}C abundances in leguminous plants. *Plant and Cell Physiology*, *24*(6), 971–977. https://doi.org/10.1093/oxfordjournals.pcp.a076627

Yurgel, S. N., & Kahn, M. L. (2004). Dicarboxylate transport by rhizobia. *FEMS Microbiology*

Reviews, *28*(4), 489–501. https://doi.org/10.1016/j.femsre.2004.04.002

Zabalza, A., Gastón, S., Ribas-Carbó, M., Orcaray, L., Igal, M., & Royuela, M. (2006). Nitrogen assimilation studies using ^{15}N in soybean plants treated with imazethapyr, an inhibitor of branched-chain amino acid biosynthesis. *Journal of Agricultural and Food Chemistry*, *54*(23), 8818–8823. https://doi.org/10.1021/jf0618224

Zabalza, A., Orcaray, L., Igal, M., Schauer, N., Fernie, A. R., Geigenberger, P., van Dongen, J. T., & Royuela, M. (2011). Unraveling the role of fermentation in the mode of action of acetolactate synthase inhibitors by metabolic profiling. *Journal of Plant Physiology*, *168*(13), 1568–1575. https://doi.org/10.1016/j.jplph.2011.02.015

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews : MMBR*, *63*(4), 968–989. https://doi.org/10.1128/MMBR.63.4.968-989.1999

Zahran, H. H., & Sprent, J. I. (1986). Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. *Planta*, *167*(3), 303–309. https://doi.org/10.1007/BF00391332

Zaidi, A., Khan, M. S., & Rizvi, P. Q. (2005). Effect of herbicides on growth, nodulation and nitrogen content of greengram. *Agronomy for Sustainable Development*, *25*(4), 497–504. https://doi.org/10.1051/agro:2005050

Zander, P., Amjath-Babu, T. S., Preissel, S., Reckling, M., Bues, A., Schläfke, N., Kuhlman, T., Bachinger, J., Uthes, S., Stoddard, F., Murphy-Bokern, D., & Watson, C. (2016). Grain legume decline and potential recovery in European agriculture: a review. *Agronomy for Sustainable Development*, *36*(2), 26. https://doi.org/10.1007/s13593-016-0365-y

Zawoznik, M. S., & Tomaro, M. L. (2005). Effect of chlorimuron-ethyl on *Bradyrhizobium japonicum* and its symbiosis with soybean. *Pest Management Science*, *61*(10), 1003–1008. https://doi.org/10.1002/ps.1077

Zhao, D., Reddy, K. R., Kakani, V. G., & Reddy, V. R. (2005). Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. *European Journal of Agronomy*, *22*(4), 391–403. https://doi.org/10.1016/j.eja.2004.06.005

Zhu, Y., Shearer, G., & Kohl, D. H. (1992). Proline fed to intact soybean plants influences acetylene reducing activity and content and metabolism of proline in bacteroids. *Plant Physiology*, *98*(3), 1020–1028. https://doi.org/10.1104/PP.98.3.1020

Zimmer, S., Messmer, M., Haase, T., Piepho, H.-P., Mindermann, A., Schulz, H., Habekuß, A., Ordon, F., Wilbois, K.-P., & Heß, J. (2016). Effects of soybean variety and *Bradyrhizobium* strains on yield, protein content and biological nitrogen fixation under cool growing conditions in Germany. *European Journal of Agronomy*, *72*, 38–46. https://doi.org/10.1016/J.EJA.2015.09.008

Zolkevich, V., L, P., & L, L. (1958). Translocation of assimilates and respiration of conductive tissues in relation to soil moisture. *Fiziologiya Rastenii*, *5*, 337–344.

Zulet, A., Gil-Monreal, M., Villamor, J. G., Zabalza, A., van der Hoorn, R. A. L., & Royuela, M. (2013). Proteolytic pathways induced by herbicides that inhibit amino acid biosynthesis. *PLoS ONE*, *8*(9), e73847. https://doi.org/10.1371/journal.pone.0073847.