

Minireview

Legume–rhizobium dance: an agricultural tool that could be improved?

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Summary

The specific interaction between rhizobia and legume roots leads to the development of a highly regulated process called nodulation, by which the atmospheric nitrogen is converted into an assimilable plant nutrient. This capacity is the basis for the use of bacterial inoculants for field crop cultivation. Legume plants have acquired tools that allow the entry of compatible bacteria. Likewise, plants can impose sanctions against the maintenance of nodules occupied by rhizobia with low nitrogen-fixing capacity. At the same time, bacteria must overcome different obstacles posed first by the environment and then by the legume. The present review describes the mechanisms involved in the regulation of the entire legume–rhizobium symbiotic process and the strategies and tools of bacteria for reaching the nitrogen-fixing state inside the nodule. Also, we revised different approaches to improve the nodulation process for a better crop yield.

Introduction

Legumes (soybeans, clover, lotus, pea, bean and chick-pea bean) are a fundamental source for human food and livestock feed. The selection of legume varieties with better growth relies on the application of traditional

agronomic techniques. In the last years, transgenic legume plants with a desirable trait have been obtained using molecular biotechnology tools. Such is the case of glyphosate-resistant transgenic soybean, an herbicide used to eliminate weed growth. In the 2018–2019 season, 364 million tons of soybean have been commercialized (FAO, 2019), giving an idea of the importance of this crop in the world economy.

Nitrogen is one of the dominant rate-limiting nutrients in natural systems (Ferguson *et al.*, 2010; Guignard *et al.*, 2017). Legumes have developed a particular ability to establish a symbiotic relationship with certain bacteria from the soil, whereby they can utilize the atmospheric nitrogen (Sprent, 2008). Thus, the application of nitrogen fertilizers can be avoided by means of this symbiosis and the consequent biological nitrogen fixation (BNF) (Ferguson *et al.*, 2010). This is auspicious because chemical fertilizers generate a negative effect on the ecosystem and involve the use of non-renewable fossil energies (Ferguson *et al.*, 2010; Galloway *et al.*, 2013). The term rhizobia refers to bacterial species that can interact with the roots of legumes and induce the formation of structures called nodules, where gaseous di-nitrogen is transformed into ammonium (BNF) and can thus be assimilated by the plant (Lindström and Mousavi, 2019). Most of the rhizobial species belong to families of the alpha-proteobacteria class, including *Rhizobiaceae* (*Rhizobium*, *Sinorhizobium*, *Allorhizobium*, *Pararhizobium*, *Neorhizobium* and *Shinella*), *Phyllobacteriaceae* (*Mesorhizobium*, *Aminobacter*, *Phyllobacterium*), *Brucellaceae* (*Ochrobactrum*), *Methylobacteriaceae* (*Methylobacterium*, *Microvirga*), *Bradyrhizobiaceae* (*Bradyrhizobium*), *Xanthobacteraceae* (*Azorhizobium*) and *Hyphomicrobiaceae* (*Devosia*) (Lindström and Mousavi, 2019). Some species of the *Burkholderiaceae* family (*Paraburkholderia*, *Cupriavidus*) of the beta-proteobacteria class can also induce an active nodulation process on legumes (Lindström and Mousavi, 2019). The nodulation process ranges from the interaction of bacteria with the root hairs to the formation of root nodules, where bacteria inside organelles called symbiosomes differentiate into nitrogen-fixing bacteroids. While the plant benefits from the nitrogen supply, bacteria get

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carbon compounds provided by the plant. This is a specific process by which certain species of rhizobia induce the formation of nodules on particular legumes. However, some legumes such as *Glycine max* and *Phaseolus vulgaris* may be nodulated by more than one species of rhizobia (Ji *et al.*, 2017; Andrews and Andrews, 2017) and some rhizobia like *Rhizobium* sp. NGR234 can nodulate different legume genera (Pueppke and Broughton, 1999). Using *in vitro* culture methods and more recently by applying metagenomic approaches, it has been found that symbiotic rhizobia cohabit in the nodules with other non-nodulating rhizobia and non-rhizobial species (Busby *et al.*, 2016; Lu *et al.*, 2017), which may affect process performance (Peix *et al.*, 2015; Gano-Cohen *et al.*, 2016; Lu *et al.*, 2017).

The nodulation process is controlled primarily by the plant (Sachs *et al.*, 2018). Legumes benefit from and have evolved to allow this symbiosis, but such energy-demanding process also needs to be regulated (Sachs *et al.*, 2018). The plant has developed mechanisms to enable and prevent the entry of compatible and incompatible bacteria respectively (Clúa *et al.*, 2018; Wang *et al.*, 2018). Additionally, legumes can display sanctions against the maintenance of nodules occupied by rhizobia with low nitrogen-fixing capacity (Kiers *et al.*, 2003). Bacteria must overcome different obstacles posed first by the environment and then by the legume. In this context, rhizobia often adopt tools to survive under certain adverse soil conditions and have also developed mechanisms and strategies to facilitate the nodulation of a given legume (Soto *et al.*, 2006; Clúa *et al.*, 2018; Syska *et al.*, 2019). Below we will discuss the different pathways and mechanisms behind the limitations of the nodulation process and the tools by which bacteria could adapt to and/or overcome them.

Mechanisms behind the limitations of the nodulation process and bacterial tools to overcome them

Bacterial survival in the rhizosphere

The diversity of soil bacterial communities decreases in close proximity to the root. Root exudation modulates the microbial community composition in the rhizosphere, *that is*, the soil in contact to plant roots (Lundberg *et al.*, 2012; Bulgarelli *et al.*, 2013; Philippot *et al.*, 2013; Eng *et al.*, 2020; Vives-Peris *et al.*, 2020). The maintenance of a bacterial population in the rhizosphere community partly depends on its chemotaxis towards certain root-secreted components and its ability to use them as substrates (Bais *et al.*, 2006; Badri *et al.*, 2009; Chaparro *et al.*, 2013). Flagellar function and bacterial motility are required to reach the root and can also influence the effectiveness of nodulation (López García *et al.*, 2009). Competitive and cooperative microbe–microbe

interactions also contribute to shape the overall community structure in the rhizosphere (Hassani *et al.*, 2018; Han *et al.*, 2020). In microbe–microbe interactions, the competitive effect depends on the growth rate, the ability to capture limiting nutrients such as iron or the presence of toxic compounds (Hassani *et al.*, 2018; Eng *et al.*, 2020). The example of iron limitation is very illustrative. Bacteria secrete siderophores to capture ferric iron, exhibiting membrane proteins for the uptake of the ferric-siderophore complexes. Several rhizobial species secrete siderophores and may present receptors for both their own siderophores and heterologous ones (Geetha and Joshi, 2013). Competition for iron will depend on the quality and quantity of siderophores and receptors displayed by each strain (Geetha and Joshi, 2013; Eng *et al.*, 2020).

Numerous bacterial species have information to produce toxins, which are used to restrict the growth of competitors. The structures, the mechanisms of action and the genomic organization of toxin-encoding loci are highly diverse (Zhang *et al.*, 2012; Jamet and Nassif, 2015; Scholl, 2017). Polymorphic toxins are multidomain proteins that can be secreted by different protein secretion systems (Zhang *et al.*, 2012). Some bacteria translocate polymorphic toxins directly into target bacteria through the Type VI secretion system (T6SS) (Zhang *et al.*, 2012; Ho *et al.*, 2014). Bacteria possessing this toxic activity, which may have the function of DNase, RNase, phospholipase, amidase or muramidase, also code for the corresponding immunity protein (antitoxin) (Russell *et al.*, 2011; Russell *et al.*, 2013; Ma *et al.*, 2014; Whitney *et al.*, 2014). Interbacterial competition activity mediated by T6SS-secreted toxins has been described for some soil bacteria as *Agrobacterium tumefaciens* or *Pseudomonas putida* (Bondage *et al.*, 2016; Bernal *et al.*, 2017). Despite the available information on T6SS and related toxin-immunity protein pairs in different rhizobial strains (Bondage *et al.*, 2016; Bernal *et al.*, 2018; Salinero-Lanzarote *et al.*, 2019; Zalguizuri *et al.*, 2019), a role for rhizobial putative T6SS toxins in interbacterial competition has not been demonstrated thus far (Lin *et al.*, 2018). Another type of interbacterial toxins is bacteriocins (Holtsmark *et al.*, 2008). The secretion of trifolitoxin, a bacteriocin-type peptide with antibiotic activity, has been described in *Rhizobium leguminosarum* *bv. trifolii* strain T25 (Triplett and Barta, 1987). This peptide inhibits the growth of most species in the alpha-proteobacteria class.

The rhizosphere community structure is influenced by cooperative effects in microbe–microbe interactions, probably due to nutritional interdependences, biofilm formation and molecular communication through microbial compounds (Hassani *et al.*, 2018). The arbuscular mycorrhizal mycelium can release energy-rich organic

compounds, modifying the composition of plant exudates and the ensuing growth of other soil microbes in the rhizosphere (Barea *et al.*, 2005).

The environment may offer adverse conditions such as watering, dryness and salinity, or present harmful components. Bacterial multiplication in these soils will rely on the capacity to tolerate the stress conditions and the presence of specific enzymes to eliminate the harmful components. For instance, the ability of rhizobia to accumulate trehalose promotes their growth and nodulation capacity in saline or dry soils (Reina-Bueno *et al.*, 2012; Moussaid *et al.*, 2015), whereas glyphosate tolerance is a good example of tolerance to harmful compounds. Glyphosate inhibits the activity of 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS). This enzyme is found in plants, bacteria and fungi and is required for the biosynthesis of aromatic amino acids, which are fundamental for growth (Jaworski, 1972). Some microorganisms have a glyphosate-resistant EPSPS enzyme (Barry *et al.*, 1992). While soybean EPSPS is naturally sensitive to glyphosate, transgenic soybeans carry the EPSPS gene from *Agrobacterium* sp. CP4, which codes for a resistant enzyme (Padgett *et al.*, 1995). Thus, glyphosate can be used as an herbicide in transgenic soybean crops. The presence of a glyphosate-resistant enzyme in rhizobia would favour their survival in soils treated with this compound.

Factors affecting bacterial survival in the rhizosphere are schematized in Fig. 1.

Specific recognition for initiation of the nodulation process

Flavonoids and isoflavonoids are components of the plant root exudates. Rhizobia express a transcriptional factor called NodD, which is activated by the specific flavonoid secreted by the compatible legume (Broughton *et al.*, 2000; Chen *et al.*, 2005; Peck *et al.*, 2006). Different rhizobial species respond to different flavonoids. Often, rhizobia contain multiple *nodD* copies that encode different NodD isoforms (Ferguson *et al.*, 2020). These isoforms may perform divergent roles, may be involved in distinct stages during symbiotic infection, or may be required under different environmental conditions, and have been described to enhance nodulation competitiveness or to extend symbiotic host range through perception of different plant signalling molecules (Demont *et al.*, 1994; del Cerro *et al.*, 2015, 2017, 2020; Kelly *et al.*, 2018; Acosta-Jurado *et al.*, 2019; Ferguson *et al.*, 2020). Once activated, NodD induces the expression of bacterial genes involved in the synthesis of the so-called Nod factors (Broughton *et al.*, 2000). Nod factors are lipochito-oligosaccharides with different substituents that constitute a characteristic molecule which will be recognized by specific receptors (LysM receptor-like kinases) on the root of the compatible legume (D’Haeze and Holsters, 2002; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Smit *et al.*, 2007). Once the legume recognizes the specific Nod factors, a cascade of signals is initiated in the plant tissue, promoting the physiological,

Bacterial survival in the rhizosphere

Microbe-microbe interactions

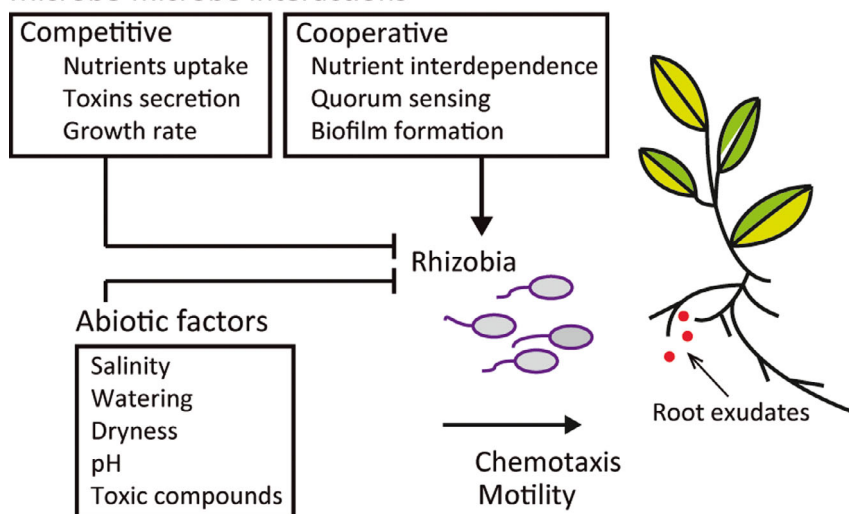


Fig. 1. Factors affecting bacterial survival in the rhizosphere. Competitive and cooperative microbe–microbe interactions, along with the environmental conditions and root-secreted compounds, contribute to shape the overall microbial community structure in the rhizosphere.

morphological and molecular changes that characterize the nodulation process. The molecular signal cascade induced in the plant by the specific Nod factor recognition has been extensively revised (Ferguson *et al.*, 2010; Oldroyd *et al.*, 2011; Liu *et al.*, 2018; Tang and Capela, 2020).

Morphological changes start with curling of the root hair tips, cell multiplication and differentiation in the root cortex, leading to the formation of the nodule primordium (Gage, 2004). Simultaneously, bacteria are entrapped in the curled root hair, proliferate and infect it through a tube-like structure made of the plant cell wall, known as the infection thread (Gage, 2004). Infection threads grow towards the base of the root hair where rhizobia are released into the nodule primordium by endocytosis of the plasma membrane (Patriarca *et al.*, 2002). Once inside the nodule cell, bacteria are surrounded by a membrane of plant origin called peribacteroid membrane, and differentiate into nitrogen-fixing bacteroids (Brewin, 1998; Geurts and Bisseling, 2002; Gage, 2004). Depending on the nodulated legume, two types of nodules have been described, determinate and indeterminate. Determinate nodules have no persistent meristem, originate by multiplication of cells in the outer cortex and are round-shaped (Crespi and Gálvez, 2000; Patriarca *et al.*, 2002). Within them, bacteria differentiate reversibly to a bacteroid state. Determinate nodules are observed in soybean, lotus and bean. Indeterminate nodules derive from the multiplication of cells of the inner cortex; they are elongated, present persistent apical meristem and different areas corresponding to different states of development. In these nodules, bacterial differentiation to the bacteroid state is irreversible. Such nodules are formed in alfalfa, pea and clover, among others. Albeit to a lesser extent, another nodulation mechanism occurs through a crack-entry infection process of plant tissues (Oldroyd and Downie, 2008; Markmann *et al.*, 2012).

The molecular consequence of the plant signalling cascade induced by the Nod factors is a transcriptional and translational reprogramming that involves the activity of different transcriptional factors, micro RNAs, enzymes and phytohormones (Libault *et al.*, 2010; Breakspear *et al.*, 2014; Dalla Via *et al.*, 2015; Boivin *et al.*, 2016; Liu *et al.*, 2018; Huang *et al.*, 2019; Traubenik *et al.*, 2020). More recently, another bacterial component involved in the specific recognition process, the exopolysaccharide (EPS), has been described (Kawaharada *et al.*, 2015). Different bacterial species and even different strains from the same species differ in the structure of their EPS (Skorupska *et al.*, 2006). The role of EPS in the infection process has been extensively studied. Mutants affected in EPS biosynthesis show defects in their ability to initiate or elongate infection threads (Breedveld *et al.*, 1993; Cheng and Walker, 1998).

Interestingly, a specific *Mesorhizobium japonicum* R7A EPS receptor called EPR3 (LysM receptor-like kinase) was described in *Lotus japonicus* Gifu. This receptor distinguishes between EPS variants, acting positively towards compatible EPS and negatively towards incompatible EPS by restricting the entry of incompatible strains into epidermal cells of the root (Kawaharada *et al.*, 2015; Kawaharada *et al.*, 2017).

Recently, rhizobial transfer RNA (tRNA)-derived small RNA fragments (tRFs) were described as positive regulators of nodulation through silencing of legume host genes engaged in a negative regulation of this process (Ren *et al.*, 2019). Host gene repression is achieved through mRNA base-pairing at the so-called target sites with the rhizobial tRFs and their subsequent cleavage (Ren *et al.*, 2019). Silencing of tRFs or overexpressing their target host genes inhibits nodule formation, suggesting a new mechanism by which bacteria can regulate nodulation through tRFs (Ren *et al.*, 2019). This mechanism was described for *B. japonicum*–*Glycine max* interaction, but tRFs were also identified in other rhizobia (Ren *et al.*, 2019). Further research is needed to determine the relevance of this host gene regulation in other rhizobium–legume interactions.

Plant defence response

In response to pathogen attack, plants trigger a first defence response through the recognition of general elicitors known as pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs) (Wanke *et al.*, 2021). Flagella, elongation factor thermo unstable (EF-Tu), different polysaccharides as well as different bacterial surface proteins can be recognized as PAMPs by the plant (Chisholm *et al.*, 2006; Wanke *et al.*, 2021). The same as pathogens, rhizobia that present MAMPs will induce a defence response in the plant. Unlike pathogens, rhizobial flagella are not recognized as MAMPs (López-Gómez *et al.*, 2011). The recognition of PAMPs occurs through plant-specific receptors (LysM receptor-like kinases); this first defence response is called PAMP-triggered immunity (PTI). Bacteria have acquired type three secretion system (T3SS) through either horizontal gene transfer or adaptation of the flagellar apparatus, by which they translocate effector proteins into the plant cells and thus suppress the PTI defence (Mudgett, 2005; Chisholm *et al.*, 2006). In turn, the plant encodes for resistance proteins that recognize these effectors (or molecules modified by them) and induce a hypersensitivity response (HR) called effector-triggered immunity (ETI) (Mudgett, 2005; Chisholm *et al.*, 2006). It has been reported that bacterial T3SS secretes additional effectors that would suppress HR (Chisholm *et al.*, 2006). Several rhizobia also code for a T3SS (Viprey *et al.*, 1998;

Krause *et al.*, 2002; Krishnan *et al.*, 2003; Hubber *et al.*, 2004; Vinardell *et al.*, 2004; Sánchez *et al.*, 2009). In addition to the induction of *nod* genes expression, flavonoid-activated NodD also upregulates the expression of TtsI transcriptional factor, which in turn induces transcription of components and effectors of the T3SS (Krause *et al.*, 2002; Marie *et al.*, 2004). A role in suppressing the defence response has been described for some rhizobial T3SS effectors (Bartsev *et al.*, 2004; Xin *et al.*, 2012). Accordingly, mutant strains deficient in the synthesis or secretion of T3SS effectors have shown reduced symbiotic properties on specific host plants (Skorpil *et al.*, 2005; Kambara *et al.*, 2009; Sánchez *et al.*, 2012). On the other hand, direct or indirect recognition of T3SS effectors by plant cells expressing specific resistance (R) proteins can result in effector-triggered defence responses that negatively affect rhizobial infection (Stahelin and Krishnan, 2015; Sugawara *et al.*, 2018). Thus, T3SS participates in the specific recognition that determines which strain can nodulate a given legume. The determination of compatibility by T3SS effectors could occur at the infection and post-infection stages (Kusakabe *et al.*, 2020). The presence of specific R genes in the plant implies a restriction towards rhizobia displaying specific effectors (Yasuda *et al.*, 2016; Fan *et al.*, 2017) which may create a host-range limitation for a given rhizobium strain as a result of T3SS functionality (Hubber *et al.*, 2004; Jiménez-Guerrero *et al.*, 2020). For example, *M. japonicum* MAFF303099 does not form effective nodules in *Leucaena leucocephala* unless its T3SS or effectors are mutated (Hubber *et al.*, 2004). Examples of altered compatibility have also been reported for other legume–rhizobium partnerships (Jiménez-Guerrero *et al.*, 2021). Rhizobial strains carrying a mutation in the T3SS or in a specific T3SS effector exhibited an extension of their host range to other legumes (Jiménez-Guerrero *et al.*, 2021). Thus, both positive and negative effects on symbiotic performance have been attributed to T3SS effectors. The combination of these effects will determine whether the T3SS acts positively, negatively, or has no effect on nodule formation in a particular legume (Skorpil *et al.*, 2005; Kambara *et al.*, 2009; Sánchez *et al.*, 2012).

In addition to the T3SS, other bacterial protein secretion systems have been described as having a role in the symbiotic process: type IV and type VI secretion system (T4SS and T6SS respectively) (Nelson and Sadowsky, 2015; Lin *et al.*, 2018; Salinero-Lanzarote *et al.*, 2019). In *M. japonicum* R7A, T4SS has an analogous role to that attributed to T3SS in *M. japonicum* MAFF303099 (Hubber *et al.*, 2004).

Bacterial polysaccharides also modulate the plant defence response (Battisti *et al.*, 1992; Aslam *et al.*, 2008; Jones *et al.*, 2008; Wanke *et al.*, 2021). It was

hypothesized that the EPS receptor 3 (EPR3)-compatible EPS recognition step in *M. japonicum* R7A–*Lotus* symbiosis could be the signal for suppressing the plant defence response and the subsequent sustained infection (Kawaharada *et al.*, 2015). Another polysaccharide involved in the nodulation process is cyclic glucan (Geremía *et al.*, 1987; D'Antuono *et al.*, 2005). It has been determined that cyclic glucan had a role in the suppression of the plant defence response in the plant pathogen *Xanthomonas* (Rigano *et al.*, 2007). In *Nicotiana benthamiana*, inoculation of a *Xanthomonas* mutant strain affected in cyclic glucan biosynthesis induced an observable defence response by the increase in callose deposition, while prior infiltration of the leaves with purified cyclic glucan suppressed such defence response (Rigano *et al.*, 2007). Cyclic glucan was shown to be essential for the onset of infection in rhizobia–legume interactions; however, the pleiotropic effect of its mutation, affecting membrane stability, must be considered (D'Antuono *et al.*, 2005). This would explain why cyclic glucan mutants cannot penetrate infection threads. Empty pseudo-nodules formed after inoculation with cyclic glucan mutants had a higher level of phenolic compounds, indicating an increase in the defence response (D'Antuono *et al.*, 2005). However, the increased defence response may be due to the absence of bacteria and not to the specific absence of glucan. It has been reported that bacterial cyclic glucan competes with a hepta-beta-glucose for its binding to a solubilized membrane fraction of soybean roots, suggesting the presence of glucan receptors in the plant (Bhagwat *et al.*, 1999). So far, there is no clear evidence of its role as a defence suppressor in rhizobia–legume symbiosis.

Lipopolysaccharide (LPS) structure was relevant for bacterial symbiotic capacity in some rhizobia–legume interactions (Noel *et al.*, 2000; D'Antuono *et al.*, 2005; Di Lorenzo *et al.*, 2020). LPSs have been attributed a role in protecting against (D'Antuono *et al.*, 2005) and suppressing (Perotto *et al.*, 1994; Scheidle *et al.*, 2005; Tellström *et al.*, 2007) the plant defence response. It has been recently demonstrated that Nod factors possess the ability to suppress the PAMP-induced plant defence in both legumes and non-legumes (Liang *et al.*, 2013). Plant transcriptome analysis revealed many induced and repressed defence genes throughout the nodulation process (Kouchi *et al.*, 2004; Lohar *et al.*, 2006). Comparative analyses of the transcriptional profile of legume genes upon inoculation with wild-type and mutant strains affected in different bacterial components allowed to explore their involvement in plant defence response regulation (Kouchi *et al.*, 2004; D'Antuono *et al.*, 2008; Dalla Via *et al.*, 2015). Defence response induction results in callose deposition, production of phenolic compounds and phytoalexins, increased levels of reactive

oxygen species (ROS) and induction of phytohormones such as salicylic acid and ethylene (Soto *et al.*, 2009; Katagiri and Tsuda, 2010; Liu *et al.*, 2018; Syska *et al.*, 2019). Besides the mechanisms for suppression of the plant defence response, bacteria have also developed tools to tolerate and eliminate ROS through the activity of ROS-scavenging enzymes such as catalase, superoxide dismutase and peroxidase, or by accumulating antioxidant compounds such as glutathione (Ma *et al.*, 2002; Soto *et al.*, 2006; Becana *et al.*, 2010; Syska *et al.*, 2019). Some bacteria produce rhizobitoxine, an ethylene synthesis inhibitor, to decrease the plant ethylene levels locally generated, while others possess the enzyme ACC deaminase which degrades 1-aminocyclopropane-1-carboxylate (ACC), the substrate for ethylene biosynthesis (Ma *et al.*, 2002).

Nodule-cysteine-rich (NCR) peptides

Host–rhizobium compatibility is defined also at the late stage of the nodulation process, that is, during bacterial differentiation into bacteroids and in the determination of bacteroids viability. A diversity of peptides is expressed in the nodules formed in inverted repeat-lacking clade (IRLC) legumes such as *Medicago* spp. (alfalfa), that is, indeterminate nodules where bacteria are irreversibly differentiated into bacteroids. These peptides are known as NCR peptides due to the presence of various cysteine residues (Mergaert *et al.*, 2003; Alunni *et al.*, 2007; Lima *et al.*, 2020). Several of these peptides present *in vitro* antimicrobial activity (Van de Velde *et al.*, 2010) that may affect membrane permeability and cause cell elongation, DNA duplication and, eventually, bacterial death. The NCR peptides have evolved from defensins, which form part of the innate immunity of plants (Sathoff and Samac, 2018). The activity of NCR peptides in the nodule does not necessarily lead to the death of bacteria, but rather to irreversible terminal bacteroid differentiation (the nitrogen fixation state), characterized by increased membrane permeability, polyploidy, larger size and alteration of the cell shape (Kondorosi *et al.*, 2013). The NCR peptides enter the bacteria through membrane protein BacA (Wehmeier *et al.*, 2010; Guefrachi *et al.*, 2015). The fact that lethal effects were not observed inside the nodule has been attributed to different factors, such as the presence of sublethal concentrations of NCR peptides or the transport function of BacA, which prevents the accumulation of NCR peptides in the membrane and therefore their negative effect on it (Van de Velde *et al.*, 2010; Haag *et al.*, 2011). Some strains of *S. meliloti* have plasmids that code for a metalloprotease that degrades NCRs (Hrrp protease). This protease, while promoting bacterial viability, has a negative effect on nitrogen fixation (Price *et al.*, 2015). The outcome of

the action of Hrrp protease depends on both the host variety and the bacterial strain (Price *et al.*, 2015). Besides, NCR peptides also affect bacteroid viability. Their involvement in the recognition and subsequent elimination of incompatible bacteria has been described (Yang *et al.*, 2017). In *M. truncatula*, NCRs encoded by NSF1 and NSF2 genes recognize and degrade bacteroids of the *Sinorhizobium meliloti* Rm R41 strain, giving the Fix[−] phenotype (formation of non-fixing nodules) and advancing senescence. On the other hand, they do not affect bacteroids of the A145 strain (Yang *et al.*, 2017). In conclusion, the dialogue between NCRs in nodules, the extent of NCR antibacterial activity, the sensitivity of the rhizobial strain and the mechanisms it has developed to tolerate or eliminate these NCR peptides will collectively determine the nitrogen fixation phenotype of the formed nodule (Price *et al.*, 2015; Lindström and Mousavi, 2019; Syska *et al.*, 2019). Given the multiple bacterial targets of NCRs, the development of resistance against them is unlikely (Lima *et al.*, 2020). In non-ICRL legumes such as soybean, lotus and bean, determinate nodules are induced and bacteria are reversibly differentiated into bacteroids. These legumes do not code for NCR peptides (Mergaert *et al.*, 2003). While the existence of incompatibility manifested at the nitrogen fixation step has been described for certain combinations of non-ICRL legumes–bacterial strains (Parniske *et al.*, 1994), the underlying mechanism has not yet been elucidated (Wang *et al.*, 2018).

Autoregulation of nodule number

Legumes have a systemic mechanism for nodule number regulation that prevents from unnecessary waste of energy (Caetano-Anollés and Gresshoff, 1991). Autoregulation of nodulation (AON) is a negative feedback system mediated by long-distance signalling between shoots and roots. Small peptides (12–13 aminoacids) that constitute the signal generated when the nodulation process begins at roots are involved in AON (Mortier *et al.*, 2012a). The CLAVATA3/embryo surrounding region-related (CLE) small peptides named LjCLE-RS1 and LjCLE-RS2 are nodule-specific and were first described in *L. japonicus* (Okamoto *et al.*, 2009). Orthologues of these peptides have also been described in other legumes (Reid *et al.*, 2011; Mortier *et al.*, 2012a; Hastwell *et al.*, 2017). The negative effect of these CLE peptides on nodulation involves specific kinase receptors in the shoot (Liu *et al.*, 2018). It was suggested that CLE peptides travel through the xylem from the roots to the shoot where they bind to their specific receptor (Okamoto *et al.*, 2013). The CLE-specific receptor in *L. japonicus* is LjHAR1 (Okamoto *et al.*, 2009), and orthologues of this receptor have been reported in other leguminous

species (Krussell *et al.*, 2002; Searle *et al.*, 2003; Schnabel *et al.*, 2005). The perception of shoot CLE peptides induces the biosynthesis of a shoot-derived inhibitor (SDI) for further nodule development (Sasaki *et al.*, 2014). In *Lotus japonicus*, it was proposed that cytokinin hormone could act as a SDI (Sasaki *et al.*, 2014). The signal transduction cascade induced by Nod factors that will give rise to nodule formation will also induce the AON mechanism (Mortier *et al.*, 2012b). Besides, it has been described that Nod factor signalling induces the expression of other factors which will negatively regulate the AON mechanism (Lei *et al.*, 2019). This suggests that nodule number regulation is the result of a delicate balance involving different signal pathways induced by rhizobia (Lei *et al.*, 2019). Specific CLE peptides also participate in the mechanism of inhibition of nodule formation in soils with high nitrate levels (Reid *et al.*, 2011).

Sanctions for poor nitrogen fixation

Nodules containing nitrogen-fixing rhizobia have been shown to develop normally, while ineffective nodules tend to remain small (Sachs *et al.*, 2010; Oono *et al.*, 2011; Regus *et al.*, 2015). The capacity of legume hosts to target ineffective and less-effective rhizobia and reduce their fitness relative to beneficial genotypes is termed sanctions (Kiers *et al.*, 2003; Oono *et al.*, 2011). This has been observed in both determinate and indeterminate nodules. The plant can sanction ineffective bacteria in the nodule by inducing a cellular senescence process (Regus *et al.*, 2017). Nodule senescence is the nodule ageing process, also defined as the nodule organ break down (Thomas, 2013). Nodules containing both effective and ineffective rhizobia may include different sectors with different bacterial genotypes. The senescence characteristics are located only in cells occupied by ineffective rhizobia (Regus *et al.*, 2017). The mechanism by which the lack of nitrogen fixation results in accelerated senescence is not yet known. Despite the plant ability to eliminate uncooperative strains, nodules occupied by less-effective rhizobia are observed in nature, probably because of the existence of a variation in the degree of control exerted by different host genotypic variants on these rhizobia (Wendlandt *et al.*, 2019).

Figure 2 presents a scheme of the different mechanisms developed by legumes to regulate or limit the nodulation process, together with the bacterial tools or strategies to overcome them.

Improve the nodulation process to improve legume crop yield

Evolutionarily, the legume–rhizobium interaction has been going in the direction of mutual benefits. Thus, the

plant obtains assimilable nitrogen and bacteria obtain the necessary nutrients for their development and a niche without competition to multiply. Taking advantage of this beneficial interaction, bacterial inoculants can be used on legume crops to overcome the problem of nitrogen limitation in soils. However, it is often observed that the inoculated strain does not predominantly occupy the nodules of the cultivated plant (Rodriguez Blanco *et al.*, 2010). The symbiotic process depends on many variables that will determine whether a strain can compete efficiently and will lead to an effective nitrogen fixation process. The aim of the inoculant industry is to use those bacteria that allow the best crop yield. In search of this higher performance, it looks for.

Improved competitiveness

Inoculated bacteria must compete with native soil rhizobia (van Dillewijn *et al.*, 2001; Rodriguez Blanco *et al.*, 2010; Geetha and Joshi, 2013; Irisarri *et al.*, 2019). Nodulation competitiveness refers to the ability of the inoculated bacteria to induce more nodules on the roots of the compatible legume than the native bacteria from the rhizosphere (Brewin *et al.*, 1983). Nodulation competitiveness could be the consequence of a better performance of the inoculated bacteria at some or several steps of the process. At first, a higher competitive capacity could arrive from a better soil survival capacity. Therefore, the relevance of enzymatic functions and activities for survival in a particular soil condition or within the microbial community or favouring a chemotactic response or a higher multiplication in the rhizosphere or plant surface by an increased capacity to utilize plant exudates should be considered. For instance, proline dehydrogenase is required to metabolize the proline found in roots exudates. Its mutation in rhizobia negatively affects colonization and therefore nodulation competitiveness of *S. meliloti* on alfalfa roots (Jiménez-Zurdo *et al.*, 1995).

If we focus on the competitive capacity for the nodulation process itself, a better performance in either of the subsequent stages (initiation of infection, multiplication in the infection threads, symbiosome formation and transformation to bacteroids) will contribute to a better nodulation competitiveness. In this respect, the alteration of bacterial components involved in some of these steps may affect competitiveness. For instance, modifications in the Nod factors (Lamrabet *et al.*, 1999; Madinabeitia *et al.*, 2002) or the EPS (Kawaharada *et al.*, 2015), involved in the initial recognition step, negatively affected nodulation competitiveness. Similar results were described for a modification in the LPS structure that provokes bacterial accumulation in infection threads (D'Antuono *et al.*, 2005) and for a mutation in a bacterial

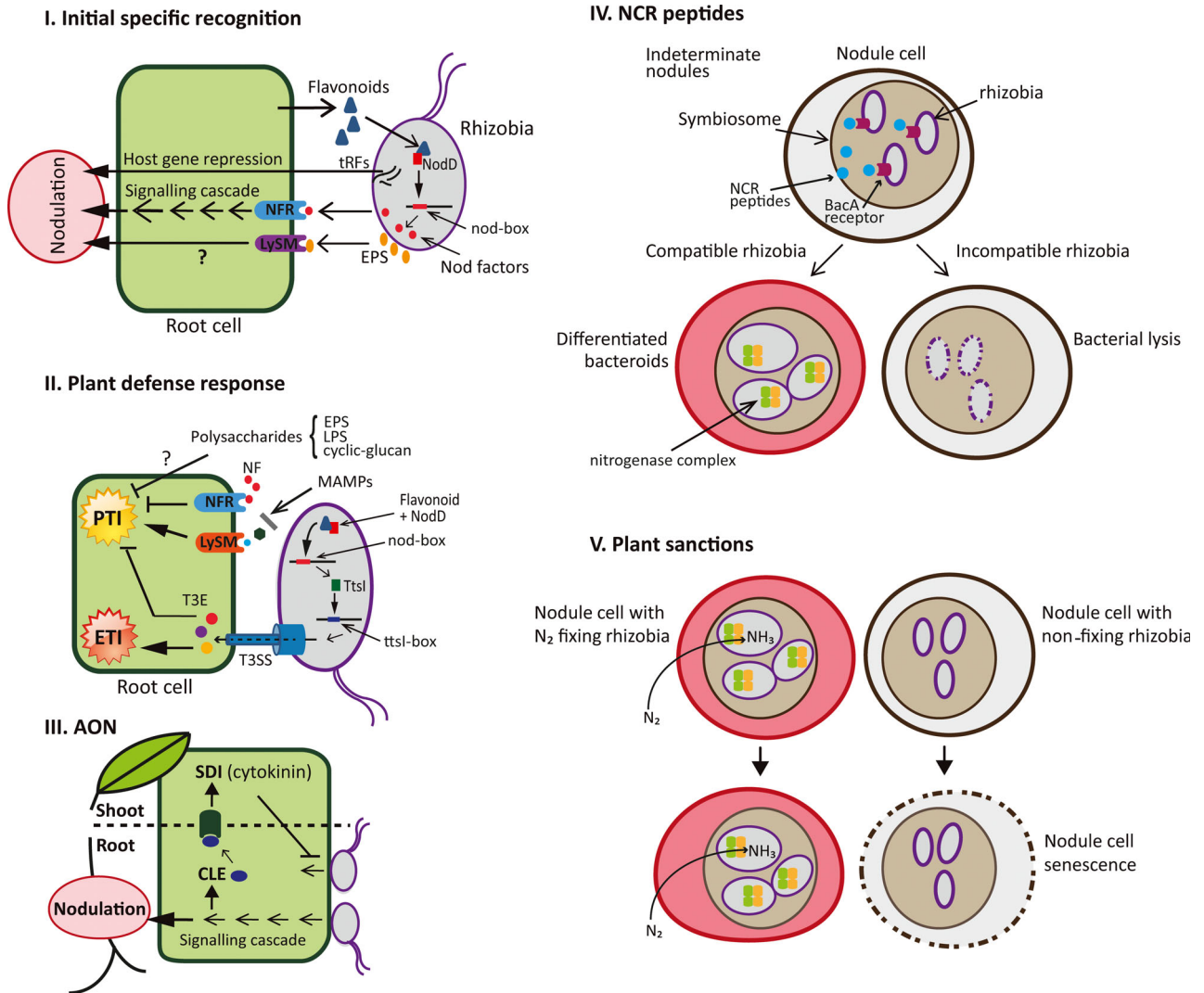


Fig. 2. Mechanisms behind the limitations of nodulation and bacterial tools for overcoming them. (I) Flavonoids exuded by the legume induce, through the activation of the transcriptional factor NodD, the production of Nod factors in compatible bacteria. Nod factors are recognized by specific legume receptors and induce a signal cascade in the plant tissue responsible for the first physiological, biochemical and transcriptional changes that lead to the initiation of the nodulation process. EPS is also recognized by root-specific receptors, being involved in the initial specific recognition. Rhizobial transfer RNA (tRNA)-derived small RNA fragments (tRFs) are also involved in the regulation of the nodulation process through silencing of legume host genes. (II) Following MAMPs recognition, a first defence response is induced in the plant (PTI). Bacterial T3SS and its effectors, induced through NodD activation by plant flavonoids, participate in the suppression of the plant defence response. Resistance proteins recognize T3SS effectors (or molecules modified by them) and induce a plant defence response (ETI). T3SS effectors would also participate in the suppression of ETI (not drawn). A role for polysaccharides and Nod factors (NF) in the suppression of the plant defence response has also been proposed. (III) Autoregulation mechanism of nodule number. The initial signalling cascade induced by Nod factors leads to the induction of CLE peptide biosynthesis on roots. These peptides are specifically recognized by shoot cell receptors, inducing the production of a shoot-derived inhibitor (SDI) of further nodule development. It was proposed that cytokinin hormone would act as an SDI. (IV) In indeterminate nodules, NCR peptides are involved in the differentiation of compatible bacteria to bacteroids. In addition, a role for NCR peptides in the recognition of incompatible bacteria and their subsequent elimination has been described. Transport of NCR through BacA avoids NCR accumulation at bacteroid membranes. (V) The plant can sanction ineffective bacteria present in the nodule by inducing a senescence process in the cells containing them.

peptidase that protects against NCR peptides (Arnold *et al.*, 2017). There are also examples in which an altered bacterial function results in improved competitiveness. In *M. japonicum* MAFF303099, mutants in T3SS effectors showed higher competitiveness on *Lotus tenuis*

cv. Esmeralda than the wild-type strain (Sánchez *et al.*, 2012). Disruption of *S. medicae nolR*, a negatively acting transcriptional regulator of Nod factor biosynthesis, led to increased competitiveness on *Medicago truncatula* (Sugawara and Sadowsky, 2014). Rhizobial strains

overexpressing its own ACC deaminase (Conforte *et al.*, 2010) or expressing an exogenous one (Ma *et al.*, 2004) were more competitive in nodulation.

Higher number of fixing nodules per root

Several mechanisms regulate nodule number. Local regulation is provided by the level of different phytohormones (Mortier *et al.*, 2012b; Ferguson and Mathesius, 2014). Increased ethylene levels generated after Nod factors perception (Reid *et al.*, 2018) negatively regulate nodule number (Nukui *et al.*, 2000; Nascimento *et al.*, 2018). Rhizobia can degrade the substrate for ethylene biosynthesis through ACC deaminase activity, resulting in decreased ethylene local levels and increased number of nodules (Ma *et al.*, 2004; Nascimento *et al.*, 2018). As previously mentioned, the number of fixing nodules per root is also determined by the plant systemic AON mechanism, which relies on the induction of CLE peptides in the plant (Liu *et al.*, 2018). While this induction is already observed in the nodule primordium, the strength and range of the signal will depend on the number, activity and state of development of the nodules (Mortier *et al.*, 2012b). Mature nitrogen-fixing nodules display greater inhibition for further nodulation than less effective ones (Li *et al.*, 2009). This could be the reason why an increase in nodule number is usually observed in mutants that induce non-fixing nodules, as is the case with mutants in cyclic glucan (D'Antuono *et al.*, 2005) or the Rieske iron/sulphur protein (a component of the respiratory electron transport chain required for nitrogen fixation inside the nodule) (Pickering *et al.*, 2012; Basile *et al.*, 2018).

Improved nitrogen fixation efficiency

Efficiency during the nitrogen fixation step is concerned with bacterial nitrogenase activity. The nitrogenase complex, coded by the *nifHDK* genes, is functional under microoxic conditions. These conditions are provided by the barrier that constitutes the external wall of the nodule and by plant leghaemoglobin (Ott *et al.*, 2005). The presence of more efficient nitrogenases could increase the amount of fixed nitrogen and, therefore, crop yield. Likewise, a greater nitrogenase expression would also contribute to an improvement in performance. Regulation of the nitrogen fixation process is under *nifA*, *fixLJ* and *fixK* genes (Lindström and Mousavi, 2019). Besides the microoxic conditions required, nitrogenase expression can be controlled through the action of several inducers and suppressors (Soberón *et al.*, 2001; Mesa *et al.*, 2008; Torres-Quesada *et al.*, 2010; Quelas *et al.*, 2016). For example, it has been suggested that the mutation on PhaR in *B. diazoefficiens* could have a positive effect on

the amount of nitrogen fixed, since it has been described as a negative regulator of the FixK2 transcriptional factor (Quelas *et al.*, 2016).

The release of hydrogen in the nitrogen fixation process implies loss of energy (Schubert and Evans, 1976). It was proposed that the hydrogenase enzyme involved in H₂ recycling and present in some rhizobial strains (Baginsky *et al.*, 2002) would help improve fixation efficiency (Schubert *et al.*, 1978; Albrecht *et al.*, 1979).

In the nodule, nitric oxide (NO) levels increase (Baudouin *et al.*, 2006) due to the utilization of nitrite instead of O₂ as the final electron acceptor in the mitochondrial electron transport chain under microoxic conditions (Igamberdiev and Hill, 2009). Nodule NO levels have also been observed to increase under stress conditions (Fancy *et al.*, 2017; Syska *et al.*, 2019). Nitric oxide not only participates in signal transduction in the plant–symbiont interaction (Ferrarini *et al.*, 2008; Boscari *et al.*, 2013), but also has a toxic effect. It has been described that NO inhibits nitrogenase activity (Trinchant and Rigaud, 1982; Kato *et al.*, 2010) as well as nitrogen-fixing activity through repression of the nitrogenase genes (Sánchez *et al.*, 2010). The plant has mechanisms for NO detoxification (Berger *et al.*, 2019) and bacteria also possess NO-degrading enzymes (Cam *et al.*, 2012; Berger *et al.*, 2019; Syska *et al.*, 2019). Both symbiotic partners would contribute to balance different signalling, metabolic and toxic effects, and to maintain symbiotic N₂ fixation (Berger *et al.*, 2019).

An exhaustive revision about the different processes involved in the effectiveness of nitrogen fixation in rhizobia can be found in Lindström and Mousavi (2019).

Increased duration of the nitrogen fixation stage

A longer nitrogen fixation stage implies maintaining bacteroid viability and/or delaying senescence. Senescence is part of the normal nodule development; it depends on plant regulation and is the least studied stage of the nodulation process. When the plant needs to direct the carbon source to seed development, it no longer supports the costly process of nitrogen fixation in the nodule. Senescence is followed by the release of rhizobia (differentiated bacteroids from indeterminate nodules and undifferentiated cells from determinate nodules) to the soil (Denison and Kiers, 2004). Besides, senescence can be induced early when the plant is under stress or by ineffective nitrogen fixation (Regus *et al.*, 2017). A role for NCR peptides in early nodule senescence has been described in *Medicago truncatula* (Yang *et al.*, 2017). High levels of NO, such as those induced by mutants in NO-scavenging enzymes, also induced premature senescence in nodules (Cam *et al.*, 2012). In contrast, overexpression of these enzymes led to a

delay in nodule senescence (Cam *et al.*, 2012). The activity of ROS-scavenging enzymes has also been shown to delay senescence (Redondo *et al.*, 2009).

The toxin–antitoxin (TA) system has been described to participate in some bacterial symbiotic processes. This system is involved in the regulation of bacterial metabolism in response to environmental stress (Goeders and Van Melder, 2014; Lobato-Márquez *et al.*, 2016; Syska *et al.*, 2019). While toxins can affect protein synthesis, DNA replication, cell wall synthesis and DNA or RNA integrity, among other functions (Lobato-Márquez *et al.*, 2016), antitoxins inhibit this activity. There are different types of TA systems based on the nature (RNA or protein) and mode of action of the antitoxin (Page and Peti, 2016). In type II TA system, the antitoxin is a protein which counteracts the effect of the toxin by forming a complex with it (Yamaguchi and Inouye, 2011). Since the antitoxin is more unstable than the toxin, it is eliminated more quickly by protease degradation under a stress situation (Gerdes *et al.*, 2005; Lobato-Márquez *et al.*, 2016). Once the toxin is free of its antitoxin, it acts on bacterial metabolism, resulting in the arrest of bacterial growth and persistence or cell death (Hayes and Kędzierska, 2014). In *S. meliloti*, several TA modules have been described. One of them is *vapBC-5*, corresponding to the type II TA system (Lipuma *et al.*, 2014). Deletion of the complete TA *vapBC-5* module had no effect on the nodulation and nitrogen fixation phenotype of *M. sativa*. The antitoxin mutation produced less efficient nodules for nitrogen fixation and lower plant yields. In contrast, the toxin mutation produced higher nitrogen fixation efficiency and plant performance compared with the wild-type strain (Lipuma *et al.*, 2014). Besides, analysis of the expression of markers of active nitrogen-fixing zones showed that the mutation in the toxin delayed the senescence phenotype (Lipuma *et al.*, 2014). The role of other TA modules in *S. meliloti* has also been studied (Oláh *et al.*, 2001). Inside the nodule, bacteria must adapt to stress conditions given by acidic pH, microoxia, ROS and antimicrobial peptides. It was proposed that the activity of different bacterial TA modules would play a role in the adaptation to these stresses by limiting the symbiotic interaction and contributing to the appearance of the senescence stage (Syska *et al.*, 2019). It was also suggested that the inactivation of toxin genes in *S. meliloti* could constitute a strategy for improving alfalfa production (Lipuma *et al.*, 2014).

Strategies to improve inoculants

In addition to the leguminous specie to be nodulated, soil conditions should be considered when choosing a suitable inoculant. The carrier used and the way the inoculation is applied are among the variables that

determine the inoculant efficiency. We will not discuss these topics here; they can be revised in (Hungria *et al.*, 2005; Albareda *et al.*, 2008; Ruiz-Valdiviezo *et al.*, 2015; O'Callaghan, 2016; Sanches Santos *et al.*, 2019). The inoculant potential will depend mainly on the microbial strain (or strains) used. The strategy is to develop an inoculant based on the use of rhizobial strains that can survive in the soil conditions that are competitive over the native strains that properly complete the whole process and have an optimal nitrogen fixation capacity. The inoculant may also contain non-nodulating strains that promote the process, such as plant growth-promoting rhizobacteria (PGPR) or mycorrhizas. The different strategies that could be used for the development of an appropriate inoculant are discussed below.

Selection of native strains that are favoured by the mentioned characteristics

Numerous studies have tried to find highly competitive and highly efficient nitrogen-fixing native soil strains. One strategy consists of isolating the different strains found in the nodules, selecting highly efficient nitrogen-fixing strains and, finally, selecting more competitive strains than at least the commercial strain through competitiveness assays performed under laboratory and field conditions (Melchiorre *et al.*, 2011; Irisarri *et al.*, 2019). Competitiveness between different strains over a given legume may vary depending on soil conditions (Ji *et al.*, 2017). Therefore, the optimal strain for each crop in different types of soil may not be the same. The selection of native strains ensures the best adaptation to a particular soil. For instance, in Ethiopia, a local strain isolated from acidic soils, highly adapted to those conditions, produced enhanced soybean yields (Muleta *et al.*, 2017). New strains of *Mesorhizobium* have been identified in nodules of *Lotus tenuis* grown in flooded soils (Estrella *et al.*, 2020) and saline–alkaline soils (Sannazzaro *et al.*, 2018) in Argentina. A possible strategy would be the isolation of native glyphosate-tolerant *Bradyrhizobium* strains from glyphosate-treated soils to be used as inoculant (Kuykendall, 2012). In soils with a history of legume crops, native strains lacking the ability to nodulate and/or fix nitrogen can acquire it from inoculated strains through horizontal gene transfer. This can give rise to new strains with greater adaptability to the soil, together with the ability to nodulate. Searching for these strains may be a good source of inoculants with a better capacity to inhabit the nodules (Melchiorre *et al.*, 2011).

Co-inoculation with PGPR and mycorrhizas

Both PGPR and mycorrhizas improve plant growth. The former are soil bacteria with the ability to either be part

of the rhizosphere or colonize the root; some can even penetrate and grow between root cells (endophytes). The name of these bacteria is derived from their role in favouring plant growth by producing phytohormones as indole acetic acid (IAA), by fixing N_2 in free life, and by producing siderophores (Bhattacharyya and Jha, 2012; Sanches Santos *et al.*, 2019). They can also prevent the multiplication of plant pathogens through spatial or nutrient competition or via the secretion of toxic compounds or the induction of plant defence responses (Vacheron *et al.*, 2013; Peix *et al.*, 2015). Arbuscular mycorrhiza (root-associated fungi) can solubilize the insoluble phosphate present in soil (Baslam *et al.*, 2014) and increase surface absorption through their association with roots (Barea *et al.*, 2017). The co-inoculation of PGPR and mycorrhizas has a synergic effect on both root colonization by mycorrhizas and bacterial growth, which results in the stimulation of plant growth (Artursson *et al.*, 2006). In some systems, rhizobial co-inoculation with PGPR, mycorrhizas or both elicited a positive effect on nodulation process efficacy and plant performance (Hungria *et al.*, 2015; Korir *et al.*, 2017; Nascimento *et al.*, 2018; Raklami *et al.*, 2019; Alemneh *et al.*, 2020; Swarnalashmi *et al.*, 2020). For instance, rhizobial co-inoculation with endophytes having ACC deaminase activity increased the nodulation abilities of both alpha- and beta-rhizobia, resulting in increased leguminous plant growth (Nascimento *et al.*, 2019). The dual inoculation of *Rhizobium* with either *Pseudomonas putida*, *P. fluorescens* or *Bacillus cereus* on pigeon pea significantly increased plant growth and nodulation (Tilak *et al.*, 2006). Co-inoculation of *Rhizobium tropici* and *Azospirillum brasilense* strains on common bean enhanced crop yields (Souza and Ferreira, 2017). *Rhizobium etli* nodulation efficiency on *Phaseolus vulgaris* was improved by co-inoculation with *Rhizobium fabae*, showing that the quorum-sensing signals from the commensal bacteria were involved in this better performance (Miao *et al.*, 2018). Dual inoculation of *R. leguminosarum* and a mixture of arbuscular mycorrhizal fungi on fava bean legumes grown on alkaline soils significantly increased nodule mass and number and dry weight of roots and shoots compared with individual inoculation (Abd-Alla *et al.*, 2014). Currently, numerous inoculants are designed as rhizobial combinations with PGPR and/or mycorrhiza (Bashan *et al.*, 2014; Vejan *et al.*, 2016; Sanches Santos *et al.*, 2019).

Genetic engineering in bacteria

Knowledge of the molecular basis of the rhizobium–legume interaction enables the design of new bacterial strains with increased or added capacities that could improve some steps of the nodulation process, leading

to successful nodulation. This is usually intended to modify a single function in strains used as inoculants. Several studies have reported the construction of such strains as well as improvements in nodulation performance and plant yields in controlled laboratory experiments (Robleto *et al.*, 1997; Geetha and Joshi, 2013). Yet, examples of field experiments are scarce (Scupham *et al.*, 1996; Dillewijn *et al.*, 2001; Hirsch, 2005). The modification of a strain with the traits sought could be an appropriate strategy, despite restrictions to accept genetically engineered strains as inoculants (Geetha and Joshi, 2013). Modifications for nodulation improvement might consist in the heterologous expression of a new function (Robleto *et al.*, 1997), the expression of an endogenous function at an increased level (Conforte *et al.*, 2010) or the deletion of a specific function (Hubber *et al.*, 2004). The incorporation of a new or an increased function should be achieved via chromosomal integration instead of being carried on a plasmid to avoid its transfer to other soil bacteria or loss in the absence of selection pressure (Conforte *et al.*, 2010). In case, the modification involves genes present in the symbiotic island, the fact that these regions may also be transferred to another bacterium in the rhizosphere should be considered (Bamba *et al.*, 2019). The use of molecular methodologies that enable the desired modification without the need to incorporate a permanent selection marker such as an antibiotic resistance gene is required. For instance, point mutagenesis (Quelas *et al.*, 2021) is a useful tool for achieving modifications on rhizobial endogenous information.

The properties and strategies that can potentially improve inoculant performance are summarized in Table 1.

Table 1. Properties and strategies that can potentially improve inoculant performance.

Inoculants: desired properties
Improved competitiveness
Soil survival, Initial recognition steps, Multiplication in infection threads, Symbiosome formation, Transformation to bacteroid
Higher number of fixing nodules per root
Inhibition of phytohormones production
Improved nitrogen fixation efficiency
Regulation of nitrogenase activity, Hydrogenase and NO-degrading enzymes
Increased duration of the nitrogen fixation stage
NO and ROS-scavenging enzymes, TA modules
Strategies to improve inoculant performance
Native strain isolation
Strain selection based on competitive and nitrogen efficiency assays
Carrier and application mode
Co-inoculation with PGPR and mycorrhizas
Genetic modification of bacterial functions
Chromosomal integration, avoiding of antibiotic selection marker

We will give below examples of genetically engineered strains with positive effects on the nodulation process with reference to competitiveness, nitrogen fixation and plant yield. A very complete list of rhizobial genetic modifications and the corresponding effects on nodulation was recently published (Goyal *et al.*, 2021). As mentioned above, competitiveness may be given by increased survival or proliferation and niche occupation in the rhizosphere at the expense of other competing bacteria. So, a good strategy could be the release of toxic compounds by rhizobia. Such is the case of *R. etli* production of the antibiotic trifolixin. Isogenic strains that differed only in their antibiotic-producing capacity were generated and co-inoculated on *Phaseolus vulgaris* under agricultural conditions (Robledo *et al.*, 1997). It was found that the antibiotic-producing strain showed increased nodule occupation (Robledo *et al.*, 1997). The ability to metabolize the proline present in root exudates was also used to obtain more competitive strains. An engineered strain that constitutively expressed proline dehydrogenase (encoded by the *putA* gene) presented greater competitiveness for the nodulation process (van Dillewijn *et al.*, 2001). This is one of the few examples in which experiments were conducted in microcosms with non-sterile soil and controlled field conditions. A higher nitrogen fixation has been achieved through the introduction of a chimeric copy of the nitrogenase operon (*nifHDK*) under the activity of a strong promoter into *R. etli* (Peralta *et al.*, 2004). This construction was added to the two endogenous copies that are naturally encoded under the regulation of low-activity promoters, resulting in increased nitrogen activity and plant weight (Peralta *et al.*, 2004). Recently, heterologous hydrogenase was expressed in rhizobial strains lacking endogenous hydrogenase activity and used as commercial inoculants of *Phaseolus vulgaris* L. The resulting strains presented increased nodule efficiency and seed nitrogen content as compared with the corresponding wild-type strains (Ribeiro Torres *et al.*, 2020).

The effect of ethylene on the initiation of nodule formation and the ability of some bacterial species to decrease ethylene levels is highly studied. As stated above, this is achieved through the activity of ACC deaminase, which degrades the substrate for ethylene biosynthesis (Nascimento *et al.*, 2018). The heterologous ACC deaminase expression of *R. leguminosarum* in *S. meliloti*, which does not naturally possess this activity, produced more competitive strains (Ma *et al.*, 2004). In *M. japonicum* MAFF303099, ACC deaminase expression was induced under microoxic conditions (Uchiumi *et al.*, 2004; Nukui *et al.*, 2006). Its constitutive expression led to an increase in competitiveness for nodulation (Conforte *et al.*, 2010).

Overexpression of bacterial adhesin RapI in *R. leguminosarum* promoted its nodulation on red clover roots

(Mongiardini *et al.*, 2009). The hydroxamate-type siderophore receptor of *E. coli* (Fhu) was expressed in two *Rhizobium* spp. strains which do not naturally code for this receptor. The heterologous expression led to greater nodule number and fresh weight of *Pigeon pea* plants (Geetha *et al.*, 2009). The engineered strains presented higher nodule occupation than the original ones, even in non-sterile soils (Geetha *et al.*, 2009). In *Sinorhizobium meliloti*, overexpression of flavodoxin, a protein involved in the oxidative stress response, delayed nodule senescence (Redondo *et al.*, 2009) and provided enhanced tolerance to salinity stress in the nodules (Redondo *et al.*, 2012). With regards to glyphosate tolerance, *Bradyrhizobium diazoefficiens* USDA 110 is naturally sensitive to this herbicide (Zablotowicz and Reddy, 2004). A glyphosate-resistant engineered *Bradyrhizobium* strain expressing the EPSPS from *A. tumefaciens* strain CP4 has already been patented (King and Purcell, 2005). The strain thus engineered displayed tolerance to the compound in *in vitro* assays. However, it would be unstable for the information incorporated considering that it carries two copies of the EPSPS gene (endogenous and heterologous). Recently, our research group obtained a glyphosate-tolerant strain by changing only two nucleotides in the active site of the EPSPS enzyme of *B. diazoefficiens* USDA110 (Quelas *et al.*, 2021). This strain does not contain any exogenous information and proved to be more competitive than the wild-type strain in laboratory co-inoculation trials performed in the presence of glyphosate (Quelas *et al.*, 2021).

Conclusions

Based on the hypothesis that the rhizobia–legume symbiotic process has evolved towards the selection of strains that best interact and induce efficient nodules for nitrogen fixation, is there a way to improve it? Despite inoculants composed of highly efficient nitrogen-fixing rhizobia were used, the populations found in plant nodules were often diverse, with a high proportion of native strains. Native strains are certainly better adapted to the environment than those making up the inoculant. Their competitiveness for nodulation may be greater, equal, or less than that of the inoculant and may be therefore very, little or not efficient at all in nitrogen fixation. At the beginning of the nodulation process, the plant cannot discriminate rhizobia with respect to their nitrogen fixation efficiency. Once inside the nodule, some plants have the capacity to sanction non-fixing bacteria, without preventing them from still being in the rhizosphere and competing for nodulation. In relation to the question raised, we believe that the symbiotic process between legumes and rhizobia can still be improved by seeking strains able to adapt to the environment, more

competitive in the nodulation process than native strains and, at the same time, having a better capacity to fix nitrogen. This can be achieved by selecting strains with good nitrogen fixation efficiency from the most competitive ones, and then finding the appropriate inoculation conditions to increase their presence in the proximity of the root. Also, by accompanying efficient nitrogen-fixing strains with other PGPR strains or mycorrhizas that favour proliferation in the rhizosphere and root colonization, or by modifying some functions through genetic engineering of rhizobia. The genetic modification may be targeted to improving bacterial adaptation to a particular soil condition, improving one of the multiple stages involved in the nodulation process itself or increasing the nitrogen fixation capacity. However, overexpression or mutation of a function may have pleiotropic effects that should be avoided. The resulting mechanism of action of different bacterial components may be dual and depend on the variety of the nodulated legume. As shown in various field campaigns, different varieties of the same legume species may be grown and the genetic modifications on these bacterial components may not be desirable. Accordingly, a thorough knowledge of the molecular mechanisms involved in the different stages of the nodulation process is required for the selection of strains by genetic engineering.

Conflict of interest

None declared.

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