Universidade do Minho
Escola de Ciências

Inês Isabel de Sousa Conceição

Potential trade-off of weak rhizobial mutualism in the protection against root-knot nematodes

outubro de 2024

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Trabalho efetuado sob a orientação de

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Resumo

As leguminosas são amplamente utilizadas na agricultura para produção ou como culturas de cobertura que, através de mutualismos com rizóbios, enriquecem os solos com azoto. Os rizóbios são bactérias do solo que se associam às raízes de leguminosas, induzindo por mecanismos fisiológicos a formação de nódulos. Estas estruturas têm uma morfogénese semelhante às das galhas induzidas por nemátodes fitoparasitas do género *Meloidogyne* (nemátodes-das-galhas-radiculares). Os nemátodes deste género causam perdas significativas na agricultura a nível mundial, sendo considerados os parasitas edáficos de maior relevância nos sistemas de produção, e de muito difícil gestão. Estudos anteriores sugerem que poderá haver um potencial papel dos rizóbios na redução de danos provocados por estes nemátodes, que partilham alguns mecanismos de comunicação e infeção das raízes com estas bactérias.

Através de ensaios *in vitro* e em solo em condições controladas, foram testados diferentes isolados de rizóbios em combinação com *Meloidogyne javanica* na leguminosa modelo *Medicago truncatula*. A capacidade mutualista dos rizóbios foi avaliada segundo o número de nódulos e a biomassa da planta e os efeitos dos nemátodes avaliados tendo em conta o número de galhas, massas de ovos e ovos formados. Os resultados mostraram efeitos significativos na capacidade de alguns rizóbios mutualistas mais fracos protegerem a planta contra o ataque dos nemátodes-das-galhas-radiculares, diminuindo o número de galhas nas raízes e sua deposição de ovos. O efeito observado variou conforme a identidade dos rizóbios testados, no entanto, independente do seu local de origem. Os resultados obtidos sugerem que os rizóbios poderão apresentar diversidade funcional independentemente da sua capacidade de fixar azoto. O potencial de utilização de leguminosas inoculadas com rizóbios como cultura de cobertura permanece incerto, tornando-se necessário investigar os mecanismos específicos envolvidos e descobrir as combinações mais eficientes de rizóbio-planta-nemátode para o controlo de nemátodes-das-galhas-radiculares em sistemas agrícolas.

Palavras-chave: fixação de azoto, interações ecológicas, *Medicago truncatula*, *Meloidogyne,* mutualismo, parasitismo

Abstract

Legumes are widely used in agriculture for production or as cover crops that, through mutualisms with rhizobia, enrich soils with nitrogen. Rhizobia are soil bacteria that associate with legume roots, inducing the formation of nodules through physiological mechanisms. These structures have a morphogenesis similar to that of galls induced by phytoparasitic nematodes of the genus *Meloidogyne* (root-knot nematodes). The nematodes of this genus cause significant losses in agriculture worldwide, being considered the most relevant soil parasite in production systems, and very difficult to manage. Previous studies suggest that there may be a potential role for rhizobia in reducing damage caused by these nematodes, which share some root communication and infection mechanisms with these bacteria.

Through *in vitro* and soil assays under controlled conditions, different rhizobial isolates were tested in combination with *Meloidogyne javanica* in the model legume *Medicago truncatula*. The mutualistic capacity of rhizobia was assessed according to the number of nodules and plant biomass and the nematode effects were assessed counting the number of galls, egg masses and eggs formed. The results showed significant effects on the ability of some weaker mutualistic rhizobia to protect the plant against nematode attack, reducing the number of galls and egg deposition in the roots. The observed effects were dependent on rhizobia identity, however, with no observed pattern according to locality of origin. The results obtained suggest that rhizobia may present functional diversity independently of their ability to fix nitrogen. The potential for using legumes inoculated with rhizobia as a cover crop remains uncertain, making it necessary to investigate the specific mechanisms involved and discover the most efficient rhizobia-plant-nematode combinations for controlling root-knot nematodes in agricultural systems.

Keywords: ecological interactions, nitrogen fixation, *Medicago truncatula*, *Meloidogyne*, mutualism, parasitism

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1. Introduction

1. Introduction

1.1 Legumes in agriculture

Agriculture is facing a range of interconnected and complex challenges that need problem-solving strategies involving both public health and environmental safety (Worku et al., 2024). Among them, sustainably overcoming nutrient limitation and pest and disease infestations in soils, that account for major crop losses around the world, is paramount (Sanginga et al., 2003).

Nitrogen is a plant macronutrient required for many processes and yet with limited availability in soil (Sanginga et al., 2003). Given its relevance in numerous nitrogenous biomolecules i.e., amino acids, nucleic acids, plant enzymes, and hormones, plants require nitrogen for growth and development (Marschner, 1995). If nitrogen is scarce, plants become incapacitated to synthesize the biomolecules for their biological processes, leading to pronounced adverse effects in their growth and performance, and reflecting in poor crop yields (Vos et al., 2005; Uribelarrea et al., 2009). Elemental nitrogen cannot be metabolized by eukaryotes, only becoming biologically active when it is fixed or bound, i.e., incorporated into other reduced forms ammonium (NH_4+) and nitrate (NO_3–) (Ohyama et al., 2010; Oh et al., 2008). In agricultural systems, these forms of nitrogen can be conveyed by the addition of external ammonia/nitrate fertilizers, and organic fertilizers that undergo mineralization during organic matter decomposition; atmospheric nitrogen (N_2), the prevalent gas in the atmosphere, can be fixed into organic molecules by biological nitrogen fixation (Vance, 2001) and to a lesser extent by lightning (Scott, 2023).

Synthetic fertilizers can be a fast-response solution for limited soil nitrogen but their arbitrary use can cause severe environmental and public health concerns (Sutton et al., 2011). Exceeding nitrates and ammonia can leach, volatilise, and runoff from the soil surface with detrimental effects on terrestrial and aquatic ecosystems such as eutrophication and algae blooms in water bodies (Cameron, 2013), as well as accumulate in groundwater (Follet et al., 1991), and spread gaseous emissions by denitrification and volatilization (Jenkinson, 1990; Hauck, 1990). The production of synthetic fertilizers itself releases greenhouse gases resulting from burning fossil fuels (Vitousek, 1997), adding to its high economic costs compared to only 50% of efficiency (Mondal et al., 2021). To meet these challenges, the Farm to Fork Strategy under the- European Green Deal programme, focuses on the agriculture and food sectors, setting regulatory measures for harmful fertilizers and pesticides, promoting the transition to more sustainable alternative methods, by 2030 (EC, 2020).

Biological nitrogen fixation is estimated to contribute about 200 million tonnes of naturally fixed N per year through vast ecosystems (Galloway et al., 2004). One of the most relevant biological nitrogen fixation inputs in agricultural systems is performed by leguminous plants in symbiotic associations with nitrogen-fixing bacteria – rhizobia (Postgate, 1982; Boyd and Peters, 2013). Rhizobia fix available N_2 from the atmosphere and convert into a form the plant can assimilate in exchange for carbon resources (Boyd and Peters, 2013; Mathesius, 2022). Comparatively to other non-legume species, the seeds and foliage of legumes have higher protein content due to the additional nitrogen (Iannetta et al., 2016). Acquired nitrogen can be transferred from plant root exudates into the soil, becoming available to neighbouring non-legume plants (Stern 1993; Johansen and Jensen 1996), through root senescence (Watt, 2006) and through decomposition of the nitrogen-rich plant litter, making nitrogen available for subsequent crops (Gzryb et al., 2021). Although possibly increasing herbivore populations (Simson et al., 2014), increased cultivation of legumes as crop rotations, intercrops or green manure is being encouraged as they can deliver multiple services, e.g., the improvement of crop performance and yield by improving the nitrogen, phosphorus and carbon status of the soil (Sinclair, 2002; Stagnari et al., 2017), restoring soil fertility and soil water retention (Ghosh et al., 2007) and simultaneously breaking the cycles of pests and diseases affecting crops (Kebede, 2020a).

1.2 Rhizobia and nodulation

The nitrogen-fixing bacteria that form symbiotic relationships with legumes are commonly called rhizobia, because of their largest and first described genus, Rhizobium (Sprent, 2008). Currently, this group of bacteria is divided into 18 genera (Mousavi, 2016; De Lajudie et al., 2019). They can live and survive free in soil or in associations with legumes inside specialized root structures called nodules (Oldroyd and Downie, 2008). Rhizobia-legume symbioses are the most researched plant-microbe interactions and consequently the best understood, through extensive biochemical and molecular tools (Ledermann et al., 2021).

When nitrogen availability is limited, the interaction between a certain legume species and its corresponding rhizobial species begins with the legume host exudating a mixture of specific chemo-attractants, such as flavonoids, in the rhizosphere, that have several roles in the rhizobium-legume symbiosis (Munoz et al., 1988; Dharmatilake and Bauer, 1992). Free-living rhizobia perceive these chemo-attractants and respond activating (or downregulating depending on environmental factors) the production of nodulation genes and related proteins (Fig.1) (Peters et al., 1986; Redmond et al., 1986).

Rhizobia express a group of the genes called nod genes that encode enzymes that biosynthesise a signalling lipo-chitooligosaccharide molecule – the Nod factor (Heidstra and Bisseling, 1996; Kistner and Parniske, 2002). Nod factors mediate the interaction specificity and induce nodule development, being recognized by corresponding host plant receptors (Oldroyd and Downie, 2008). Symbiosis-associated nitrogen fixation takes place inside specialized structures to avoid nitrogenase inhibition (enzyme responsible for nitrogen fixation) through the exposure to oxygen (Robson & Postgate, 1980). Nitrogenase is protected from oxygen inside organelle-like structures called symbiosomes by the presence of the oxygen-binding protein leghemoglobin, as well as by an oxygen diffusion barrier (Ott et al., 2005; Minchin et al., 2008). Rhizobia also produce exopolysaccharides linked to the depression of host defence responses in early root invasion, where mutants typically infect the host poorly and form white, ineffective nodules (Jones et al., 2008). They further produce quorum sensing molecules AHLs (acetyl homoserine lactones) that allow rhizobia communication within its own and across bacterial species, that then adapt their behaviour in a population-density manner (Hartmann et al., 2014; Sanchez-Contreras et al., 2007).

In most legumes, after plant host and symbiotic partner recognition in the rhizosphere, rhizobial Nod factor molecules induce infection thread formation and nodule development (van Spronsen et al., 1994). The infection thread is a cellulose-lined tube formed from cell wall invaginations of the host root hairs that grows towards the inside of the root (Masson-Boivin et al., 2009) to release rhizobia into the cortical cells that are already dividing, differentiating, and proliferating to shape the nodule (Murray 2011; Gage 2004; Downie and Walker, 1999). Inside the nodule, one or several rhizobia reside inside symbiosomes (Oldroyd and Downie 2008) and change morphology, differentiating into bacteroides that can fix atmospheric nitrogen into ammonium, that is itself converted into amino acids and exported to the plant (Long, 1996). In exchange, rhizobia receive and metabolize in the nodules a large amount of carbon sources from photosynthesis (in the form of sucrose) via the phloem to fuel nitrogen fixation (Udvardi and Poole, 2013). The plant also provides rhizobia with oxygen (tightly bound by leghaemoglobin) for cellular respiration (Ott al., 2005).

When nodules senesce, surviving rhizobia are released into the soil where they can possibly maintain resident populations, depending on their viability (Hirsch, 1996). Rhizobia inoculants are being commercialized worldwide as seed coating or in direct soil application, for their low cost of acquisition (Herridge et al., 2008) and for legume improvement in crops in combination with non-legumes, leading to significant contributions to the productivity of food cultivation in agricultural systems (Volpiano et al., 2019).

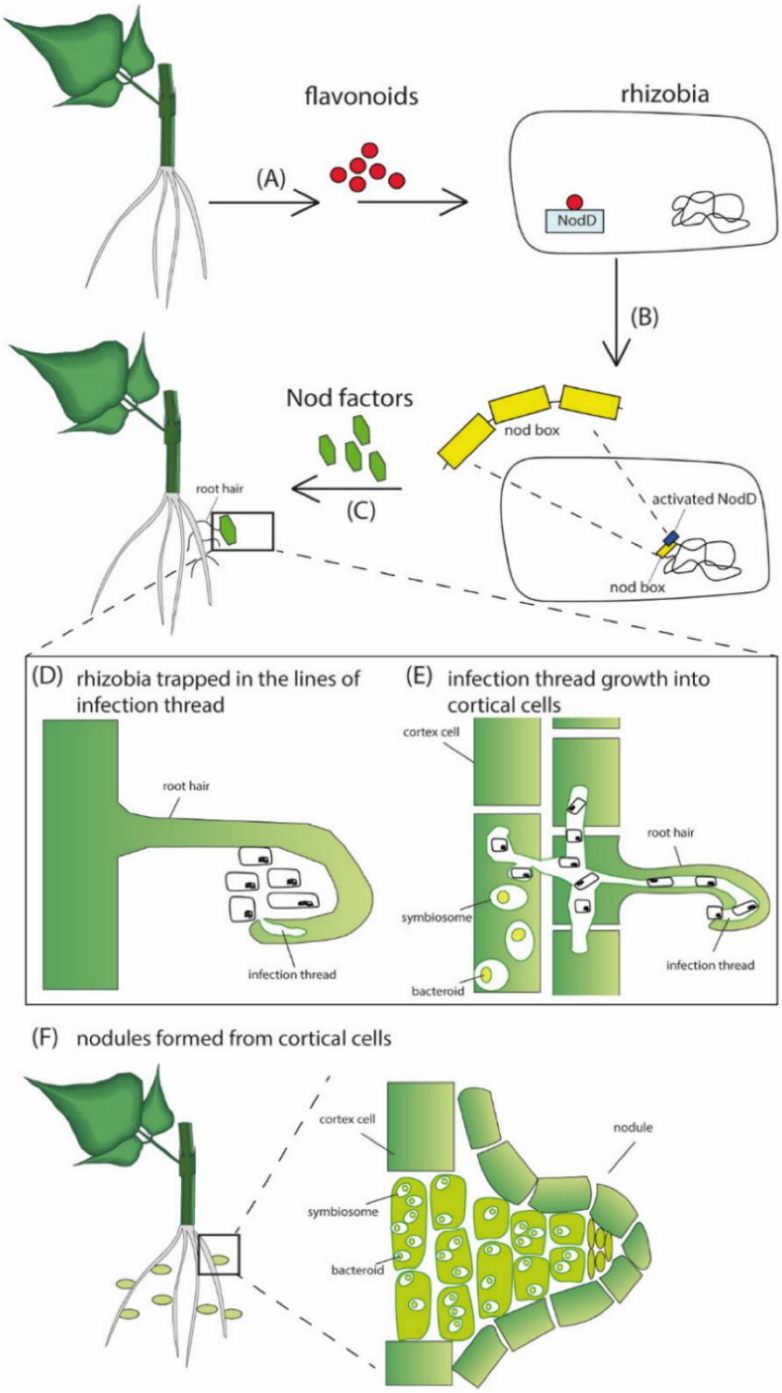


Figure 1. Schematic representation of legume host-rhizobia communication in the rhizosphere and nodulation process. **(A)** Host plant releases chemo-attractants into the rhizosphere that are perceived by specific free-living rhizobia in the soil; **(B), (C)** The chemo-attractants (flavonoids) induce the expression of Nod factors recognized by the plant, allowing the symbiosis to occur; **(D)** The accumulation of rhizobia outside the infection zone, induces the curling of root hairs, and the formation of an infection thread; **(E)** Rhizobia are encapsulated by curling root hairs, lead through the infection thread and released into the cortical cells. Rhizobia differentiate into bacteroids inside symbiosomes where nitrogen fixation takes place. (Wekesa et al., 2022)

1.3 Rhizobia functional diversity and host sanctioning

Legumes can be colonized by multiple rhizobial strains, inside the same root system (and even in the same nodule), that vary greatly in symbiotic properties (Burdon et al., 1999), potentially covering the entire range from mutualists to parasites, with different nitrogen fixation efficiencies provided to their hosts (Ballard et al., 2002; Denton et al., 2000; Poole et al., 2018).

In controlled experiments, legumes were found to discriminate among partners, "sanctioning" the nodules colonized by rhizobia with less cooperative behaviour, based in the criteria of fixing nitrogen poorly or not at all (Kiers and Denison, 2008; Van de Velde et al., 2010). The host sanction possibly decreases carbon and oxygen supply, reducing the nodule growth and leading to early nodule death (Westhoek et al., 2021). The mechanism of sanctioning nodules by reducing carbon allocation is not known but it is presumed that sanctioning might be an adaptive response of the plant containing some inhibitory flavonoids that limit the growth of incompatible but not compatible rhizobia, that gained the ability to metabolize it (Parniske et al., 1991). This allows the plant to supress the spread of ineffective strains but also of pathogens, including root-knot nematodes (RKN, genus *Meloidogyne*), whilst allowing more efficient symbionts (Parniske et al., 1991). In adverse conditions, rhizobia form symbiotic associations with legumes whether they are good nitrogen fixers or not (Mathesius, 2022). In contrast, under high soil nitrogen availability, the plant can also prevent nodulation by good nitrogen-fixers, preferring uptake of nitrogen from the soil, reducing biomass allocation to nodules and nitrogen fixation (Nishida and Suzaki, 2018, Chen and Markham, 2021).

Given the costs involved in nodulation and nitrogen fixation, it may be expected that natural selection would weed out weaker mutualists, but not-so compatible associations are still common in the field (Burdon et al., 1999; Kiers and Denison, 2008; Singleton and Tavares, 1986; Bronstein, 2001; Westhoek et al., 2021), leading to an ecological paradox (Wood et al., 2018). Some hypotheses suggested that legumes may not recognize the potential of the symbiotic partner at first instance, only sanctioning post-infection and post-symbiosis via 'quality assessment' (Westhoek et al., 2017). Sanctions may also not be effective in all legumes; or rhizobia that contribute even small amounts of nitrogen fixation could escape the strongest sanctions (Kiers et al., 2006). Other empirical studies have questioned the allegedly "ineffective mutualistic partners" (Ferriere et al., 2002). While the primary role of rhizobia symbiosis is often associated with nitrogen fixation, some rhizobia strains have already been found to enhance plant resistance to certain stresses such as pathogens (Mourad et al., 2009; Tu, 1978), reportedly acting as biocontrol agents of diseases caused by parasitic nematodes (Reitz et al., 2000; Siddiqui et al., 2007).

The persistence of poor-nitrogen fixers in legume-rhizobia symbiosis, might be explained by the fact that these not-so compatible strains can display host-compatibility in other functions, instead of being functionally redundant (Taylor et al., 2020). Resistance against RKN can be an underlying contribution of the allegedly weakest mutualists to the plant host, aiding to solve this ecologic paradox, as suggested by Costa et al. (2021).

1.4 Root-knot-nematodes and galling

With legume root exudates being publicly broadcast in the rhizosphere, other microorganisms perceive and interact with its chemo-attractants, with beneficial or harmful effects to the plant (Hassan and Mathesius, 2012). Root-knot-nematodes are a group of sedentary endoparasitic nematodes, that exploit similar mechanisms of host-interaction as rhizobia, but the effects to the plant can be extremely damaging, causing losses in yield quantity and quality to crops worldwide, with an economic cost of an estimated US$100 billion annually (Nicol et al., 2011). Although nitrogen-fixing nodules are only formed in legumes, RKN have a wide host range, collectively parasitising virtually all vascular plants (Wesemael et al., 2024) and are thought to be the most destructive plant parasites (Jones et al., 2013). The most relevant species of RKN regarding occurrence and damage to crops *include Meloidogyne arenaria, M. hapla, M. incognita* and *M. javanica* (Wesemael et al., 2024; Trudgill and Blok, 2001).

Like rhizobia, RKN are attracted by root exudates, infect the root, and form peculiar structures in them, named galls (Čepulytė et al., 2018; Perry, 1997). These obligate parasites depend on the host root system to complete their life cycle (Fig. 2). Only the juveniles in the second stage (J2), the infective stage, roam freely in the rhizosphere, in water films around soil particles (Jones and Jones, 1964). The infection starts with J2s perceiving root exudates and following their gradients to the root tips. Similarly to rhizobia, RKN produce and exude species-specific, pheromone-like signalling molecules called ascarosides that control nematode behaviours e.g., the direct movement and aggregation (or dispersal) in the rhizosphere (Choe et al., 2012; Manosalva et al., 2015). Outside the root, J2 forge their entrance in an enzymatic and mechanical way using their stylet to pierce through root tip cells (Goulart, 2007) or at the site of an emerging lateral root (Wyss et al., 1992). The physical act of RKN entrance in the host roots causes little to no injury to the plant. Once inside, RKN migrate intercellularly via the stele of the root to the zone of differentiation, where the females become sedentary and settle a permanent feeding site – commonly called giant cells (Sijmons et al., 1991; Lohar and Bird, 2003). Root-knot nematodes penetrate individual cells with their stylet secreting a large number of cell wall–degrading enzymes and effectors that control

plant defence responses, cell-wall modification, and giant cell formation (Mejias et al., 2019). Parenchyma cells differentiate into giant cells, while pericycle and cortical cells divide to form a gall (Bird and Kaloshian, 2003). The female matures inside the gall (that can contain one or more females) and lay numerous eggs in a gelatinous matrix outside of the root (Jones et al. 2013a; Bird and Kaloshian, 2003). Once hatched, J2 can infect new roots or survive in the soil for a long period of time by feeding on their stored lipid reserves, acting as dauer larvae (Bird and Kaloshian, 2003).

While rhizobia reside inside symbiosomes in nodule cells, RKN-induced giant cells are directly connected to phloem and xylem, where the products of photosynthesis are hijacked and mobilized to the giant cells (Wesemael et al., 2024). The greater damage is done with allocation of host resources to the giant cells, reducing host nutrient and water access, compromising photosynthesis, causing yellowing and wilting of the leaves, possibly debilitating the whole plant. These non-specific aboveground symptoms can be mistaken for water stress in the crops (Abad et al., 2003; Wyss et al., 1992; Lohar and Bird, 2003).

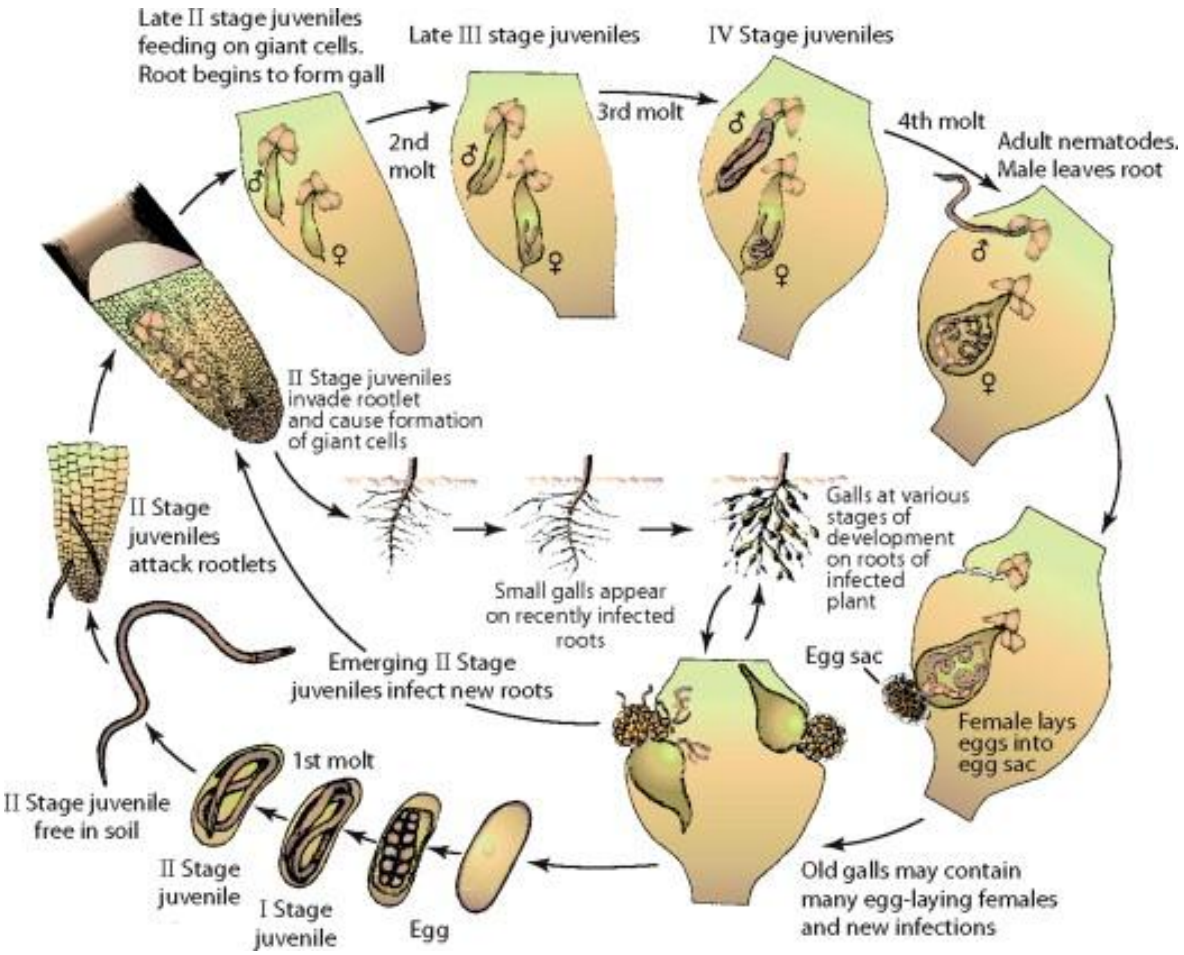


Figure 2. Root-knot-nematode (genus *Meloidogyne*) lifecycle. (Agrios, 2005).

Severeness of damage can be directly linked to the number of galls formed in the root system that, besides capturing nutrients, change root architecture and reduce water and nutrient uptake efficiency (Dropkin 1972; Hussey, 1987). Root-knot nematode infection also increases host susceptibility to various other secondary infections by other pathogens (Williamson and Hussey, 1996; Back et al., 2002). Since RKN inhabit the belowground part of the plant, their management becomes difficult because damaging populations are only detected when in high infestation levels (Stirling, 2011). Infestations by these nematodes are conventionally managed by chemical nematicide application, but their extended usage can abruptly affect other animal and plant communities, harming public health and the environment (Forget, 1993; Igbedioh, 1991), including non-target nematodes in the soil food web, and nematode antagonists (Wesemael et al., 2024). As a result, chemical nematicides are being put through regulatory pressure, with many products banned or withdrawn from the market (PAN, 2017). Since the ban on the most efficient fumigant nematicides, RKN problems have been increasing in Europe (Seid et al., 2012).

1.5 Interaction between rhizobia and root-knot nematodes on the same host

Root-knot nematodes and rhizobia are well-distributed microorganisms around the world, so their concurrent legume infection is common in nature (Costa et al., 2021). Many ecologists and evolutionary biologists pointed out that adaptations to parasitism can change the host susceptibility to mutualism and vice-versa (Strauss and Irwin, 2004), and even further when the same mechanisms and biochemical pathways are being exploited by both mutualist and parasite (Ballhorn et al., 2014). Co-evolving with RKN and rhizobia for millions of years, legumes must have evolved defence mechanisms to mediate parasitism that interfere with their susceptibility to mutualism and adaptations to mutualism that favoured - or not - the invasion of RKN through overlapping pathways (Wood et al., 2018).

Over the years, research studies have been conducted to understand the type of RKN and rhizobia interactions on the same host, both in the rhizosphere and in the legume-root system. When a plant is infected with both rhizobia and root-knot nematodes, the overall effect can be complex and context-dependent, depending on the identity and diversity (Costa et al., 2021) of the interactive partners as well as environmental factors (Dowling and Broughton, 1986). Nodulation and galling have been associated with different RKN genotypes (also at the subspecific level) (Wood et al., 2018), plant mutants (Costa et al., 2020), and rhizobial strains (Duponnois et al., 1999); representing a contributing factor to explain the biochemical and molecular aspects of interaction that shape different outcomes.

Rhizobia and RKN can interact indirectly by inducing a mixed systemic resistance in the plant, in theory attributable to: i) simultaneous rhizosphere signalling; ii) overcoming the overlap of plant host defences; iii) overcoming the overlap of development signals; iv) changes in the root architecture and v) directly through nutrient competition. Rhizobia and RKN produce signalling molecules perceived and reacted upon by plants and microorganisms (Werner, 2004; Hartley et al., 2019; Klessig et al., 2019). Plant perception of those diffuse signals can modulate their defence responses, which may have indirect effects on the host tolerance for further infections e.g., rhizobia can induce flavonoids that are inhibitory for other organisms (Mapope and Dakora, 2013). Rhizobia can also metabolize these attractants, leading to their structural conversion and breakdown in the soil, possibly altering its role in rhizobia and nematode activity and movement towards the root (Wuyts et al., 2006a; Wuyts et al., 2006b), nod gene expression (Mapope and Dakora, 2013) and signalling (Pérez-Montaño et al., 2011). Rhizobial metabolites (so far unidentified) can also affect the hatching process of *M. hapla*, changing viable nematode numbers in the soil (Khan et al., 2018). Resulting in an overlap of both infections, induced plant defence responses are

typically related to cell wall chemical and physical barriers, i.e. cell wall reinforcement, production of metabolites and enzymes against invading organisms (Gourion et al., 2015; Goverse and Smant, 2014; Zipfel and Oldroyd, 2017). Rhizobia and RKN were seen to develop strategies to overcome those responses associated to damage (damage-associated molecular patterns) DAMP) and pathogen-associated molecular patterns (PAMP) in order to perform nodulation and giant cell formation (Damiani et al., 2012; Sato et al. 2019). The plant produces biotic stress signalling-related hormones that can be downregulated by RKN and rhizobia to favour their invasion, e.g. ethylene, jasmonic and salicyclic acid (Doyle and Lambert, 2003). Ethylene plays a negative role in nodulation (Miyata et al., 2013; Penmetsa and Cook, 1997) while strongly repelling certain nematodes (Čepulytė et al., 2018), however Costa et al. (2020) found evidence of ethylene positive role on RKN parasitism. Salicylic acid and jasmonic acid pathways were seen being inhibitory for nodulation (Ferguson and Mathesius, 2014), altering nematode motility (Sikder and Vestergaard, 2020) and coordinating plant resistance and susceptibility to root-knot-nematodes (Bhattarai et al., 2008; Fujimoto et al., 2011; Hamamouch et al., 2011). Nodules and galls also share some similarities in the organogenesis process, with the invading organism initiating new cell divisions and differentiation upon invasion, by putatively manipulating similar plant biochemical pathways. So, there is an extensive overlap of genes expressed in the plant for both development of root organs, encoding signalling, host and microorganism perceptions and corresponding receptors, cell cycles and plant hormone regulations (Costa et al., 2021), providing strong evidence for the idea that genes involved in aspects of root development have been modulated by RKN and rhizobia during evolution (Liu and Bisseling, 2020). Targeting these essential genes to control RKN infection, would putatively simultaneously target rhizobia, with major effects on nodulation.

Root architecture can be indirectly altered with the emergence of new lateral roots induced by nematode and rhizobia infection, creating more entry points for infection (Bhuvaneswari et al., 1981; Gheysen and Mitchum, 2011) and direct alterations, hijacking each other's structures within the plant in competition for host nutrients. Nodules can be formed on galls (Taha and Kassab, 1980; Taha and Raski, 1969) and galls can be formed on nodules (Desaeger et al., 2005), although some RKN can display preference for nodules, maybe because of their high export of amino acids that may benefit RKN nutrition (Taha and Raski, 1969), nematode-infected-nodules can become structurally altered, senesce more rapidly and nitrogen fixation can be reduced due to changes in the access to plant resources (Ali et al., 1981; Barker and Hussey, 1976; Taha and Raski, 1969).

1.6 Putative trade-off effect in maintaining associations with weakly mutualistic rhizobia

Resulting from both direct and indirect interactions, negative to neutral to positive results were found for both nodulation and galling, suggesting an idiosyncratic relationship (Siddiqui et al., 2013; Duponnois et al., 1997; Duponnois et al., 1999). Nodulation cannot be proportionally linked to nitrogen fixation values and was found to protect, but not always, plant hosts from RKN infection and vice versa - nematode parasitism often, but not always, reduced nodulation and neither can be directly linked with nematode infection numbers (Wood et al.,2018; Costa et al., 2021). However, there is an overall trend for reduced nodule and gall numbers resulting from these interactions (summarized in Costa et al., 2021).

As mentioned in 1.3, instead of being functionally redundant, poor nitrogen-fixing rhizobia may present variability in the rhizobia-plant-nematode interaction, inducing different outcomes. Genes that affect interactions with mutualists are often also used in defence against parasites (Sachs et al., 2011; Damiani et al., 2012). Kelly et al. (2018) compared the induction of strong defence responses in less-compatible rhizobia to a certain host, with the ones induced by compatible rhizobia that showed no induction of any of the host typical defence responses, likely through repression of such responses by Nod factor signalling. Infection by root-knot-nematodes might benefit or be compromised from these changes in root defence during either weaker or stronger mutualist interaction with legumes (Mathesius, 2022). The plant could experience a trade-off between recruiting rhizobia with poor-nitrogen fixation ability, that could however, reduce infection by RKN, as an unanticipated function. To date, there are no published studies that support this hypothesis, but it becomes important to have a deep understanding of plant-host trade-offs and their underlying mechanisms, both to deepen mechanistic knowledge of these interactions, and for application in agricultural systems. In these, such trade-off effects could be exploited by combining a species-specific strategy for legume-rhizobia potential effective biocontrol of root-knot-nematodes.

1.7 Objectives

The overall aim of this project is to assess the effects on root nodulation and galling of concurrent legume-rhizobia associations with RKN on the same host, and particularly involving weak mutualists, to infer whether there is a trade-off in maintaining weak mutualisms if they grant protection from nematodes. Specific objectives were: i) to understand whether legumes promote associations with rhizobia when they are challenged by RKN, by assessing nodulation of a range of indigenous and well-known range of

rhizobia, including weak to strong nodulators and nitrogen-fixers, with or without concurrent RKN interactions; and ii) to determine whether associating with rhizobia grants protection against RKN galling, by assessing galling by RKN in legumes, with or without concurrent interactions with a range of rhizobia, differing in their nodulation and nitrogen fixation ability; and iii) to further interpret mechanisms behind observed effects, by assessing plant performance and nutrition, as well as RKN fitness on legume hosts resulting from single or combined inoculations of mutualists and parasites. The study of these interactions was relevant to better comprehend rhizobia and RKN ecological interactions and may uncover their contribution to field application in agriculture.

2. Materials and Methods

2. Materials and Methods

This project was conducted in two different laboratories: the Nematology Laboratory, in the University of Minho, Portugal and the Mathesius Laboratory, in the Australian National University, Australia. To ascertain whether weak rhizobial mutualists negatively affect root-knot-nematode parasitism, plant-nematode-rhizobia interactions were assessed through *in vitro* and pot experiments in the two laboratories. The *in vitro* assays allowed the rapid and controlled assessment of RKN-plant-rhizobia interactions, whereas the pot assays were used to estimate the application of different nodulation and nitrogen fixation efficiencies in conditions closer to real agroecosystems.

2.1 Biological materials

2.1.1 Plants

Medicago truncatula was the legume used in this study, with international recognition as a model system for legume biology, due do its already sequenced, relatively small genome and extensive phenotypical, biochemical and molecular characterization (Young et al., 2011; Roy et al., 2020). The seeds of wild type (WT) *Medicago truncatula* Gaertn. cv. Jemalong A17 were purchased from the South Australian Research and Development Institute (SARDI), Adelaide, Australia.

2.1.2 Nematodes

In the Nematology Laboratory, the *Meloidogyne javanica* P018 population was collected by picking egg masses from infected roots of susceptible tomato Solanum lycopersicum cv. Tiny Tim, from available laboratory cultures at the University of Minho. These plants were grown in 300 ml pots with autoclaved substrate composed of vermiculite, sand, and peat moss (1:1.5:1.5), in controlled conditions (24 °C temperature and 16-8 h photoperiod). The *M. javanica* population was maintained by inoculating tomato plants at the two pairs of true leaves stage with up to 10 egg masses, every ca. 60 days. In the Mathesius Laboratory, the *M. javanica* population was maintained by picking egg masses from infected roots of *Medicago truncatula*, growing *in vitro* in Fåhraeus Medium (5 mM $MgSO_4 \cdot H_2O$, 7 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 50 μM Fe-EDTA, 1 μg/liter $MnSO_4$, 1 μg/liter $CuSO_4$, 1 μg/liter $ZnSO_4$, 1 μg/liter H_3BO_3, 1 μg/liter Na_2MoO_4 and Milli-Q-purified H2O up to 1 liter (Fåhraeus, 1957)) in a controlled temperature room at 25 °C with 16 h light and 8 h dark cycle with 150 μE light intensity. The nematode

population was maintained by inoculating 1 week-old *M. truncatula* seedlings with ca. 5 egg masses every 3 weeks.

2.1.3 Reference rhizobial strains

The rhizobia used in this study came from two sources: i) a collection of reference rhizobia available from the Mathesius Laboratory, that were well-characterized and included some modified rhizobial isolates, providing a basis for further deepening the underlying physiological or genetic mechanisms (Table 1); and ii) a collection of wild strains isolated from soil samples (section 2.1.4). The typified *Sinorhizobium meliloti* and *S. medicae* are *M. truncatula* symbionts (Cook, 1999), chosen because of their different range of compatibility in communication with the legume host, formation of nodules and nitrogen fixation ability (Terpolilli et al., 2008).

Table 1. Species, strains and biological characteristics (regarding nodulation and nitrogen fixation) of reference rhizobia isolates from the Mathesius Laboratory research collection.

Species	Strain	Characteristics	Reference
Mesorhizobium ciceri	CC1192	Non-specific of *Medicago truncatula*; nodulates with the genus *Cicer*	Kamboj et al., 2010
Sinorhizobium meliloti	*exoY-*	Mutant with exoY gene deletion; makes defective exopolysaccharides; poor capacity of infecting and fixing nitrogen	Cheng and Yao, 2004
	nodC-	Mutant with nodC gene deletion; makes no Nod factors, forming very occasional nodules; poor N_2 fixation	Southwick et al., 2002
	Sm1021	Wildtype, symbiont of *Medicago sativa*, forms smaller nodules and has poor capacity of N_2 fixation in *Medicago truncatula*	Young et al., 2005; Terpolilli et al., 2008
	Sm1021 'E65'	Constitutively makes Nod factors from the E65 plasmid; fairly good nodulator	Barnett et al., 2004
	WSM1022	Wildtype, effective nitrogen fixing symbiont; good nodulator	Terpolilli et al., 2008
Sinorhizobium medicae	WSM419	Highly effective nitrogen fixing symbiotic strain with a broad range of hosts; good nodulator, usually makes fewer but bigger nodules	Reeve et al., 2010

2.1.4 Indigenous rhizobia isolates

Sample origin

Two sites with actively growing legumes were selected for soil sampling: 1) a field site growing the perennial legume lucerne, *Medicago sativa* (Luc), located in Barcelos at the coordinates 41°28'28.1" N, 8°35'02.1" W; and 2) an uncultivated meadow colonized by Trifolium spp. among other herbaceous species at the University of Minho, Gualtar Campus (IB-S) at the coordinates 41°33'39.8"N, 8°23'57.7" W. Soil samples were collected at a depth of 0–20 cm with a hori hori knife, and ca. 10 samples mixed

to obtain a composite sample, transferred into labeled plastic bags and kept refrigerated at 4 °C for a few days before use. To ascertain whether legumes were potentially challenged by root-knot nematodes *Meloidogyne* spp. in the sampled sites, a 100 ml soil subsample was used to extract nematodes using the tray method (Whitehead and Hemming, 1965) for 72 hours. The obtained suspension was concentrated by sieving through a 20 µm pore sieve, and nematodes observed using an inverted microscope at up to 200x amplification. Root-knot nematode second stage juveniles (J2) were identified morphologically (to genus level) using simple keys and illustrations (Mai and Mullin, 1996), and quantified. To isolate and identify RKN to species level, a 200 ml subsample of soil was transferred to a pot with a single plantlet of tomato cv. Tiny Tim, maintained as described above. After 60 days, the plant was harvested, and the roots washed with tap water to remove adhering soil particles. The roots were observed using a stereomicroscope and young females excised from galls. These were crushed in 5 µl of buffer solution (20% sucrose and 1% Triton X-100) in non-heparinized hematocrit tubes. A total of 9 hematocrit tubes were prepared (3 tubes, each one with 1 female; 3, each with 3 females; and 3 with 5 females each), which were stored at -20 °C. Before native polyacrylamide gel electrophoresis, samples were centrifuged at 10,000 g for 15 minutes to separate the protein from lipid and cuticle fractions. Proteins in the extracts were then separated by electrophoresis, esterase bands stained through their activity, and mobility used to ascertain species patterns according to the protocol presented in the annex I (Esbenshade and Triantaphyllou, 1985).

Isolation of rhizobia from soil samples

Rhizobia were baited from each soil (Luc and IB-S), by growing legume hosts in pots containing soil samples. Seeds of *M. truncatula* were lightly scarified using fine sandpaper, surface sterilized with 6% sodium hypochlorite for 5 minutes and rinsed with sterile distilled water (adaptation of Sauer and Burroughs, 1986). Surface sterilized seeds were then transferred to 500 ml pots containing sampled soil (three pots for each soil). One negative control with autoclaved soil was used to check for environmental or cross-contamination of rhizobia among pots. The plants were watered every day with distilled water and kept in a controlled environment cabinet with 16 h-8 h photoperiod, at 25 °C.

Two months later, four individual nodules from each pot were collected, cutting about 3 mm of root on each side of the nodule. The nodules were surface sterilized in a laminar flow cabinet by immersion in 3% sodium hypochlorite for 1 minute and then rinsed in sterilized water five times. Each nodule was crushed separately using a flame-sterilized scalpel and forceps in a drop of sterile water on a

petri dish. A loopful of the suspension was streaked onto petri dishes containing Yeast Mannitol Agar (YMA) (15 g Technical Agar LP0012 (Oxoid, 2024); 10 g Mannitol; 0.5 g K_2HPO_4; 0.2 g $MgSO_4$ $7H_2O$; 0.1 g NaCl; 0.5 g Yeast Extract and 1000 ml of distilled water (Vincent, 1970)) and labeled. Round, whitish and translucent bacterial colonies that visually resembled rhizobia were selected, re-isolated and propagated in petri dishes containing YMA in the dark at room temperature. For long-term preservation, all rhizobial isolates were grown overnight in Yeast Mannitol Broth (YMB: 10 g Mannitol; 0.5 g K_2HPO_4; 0.2 g $MgSO_4$ $7H_2O$; 0.1 g NaCl; 0.5 g Yeast Extract and 1000 ml of distilled water) (Subba Rao, 1977) mixed with sterile 60% glycerol (1:1), snap frozen in liquid nitrogen in a cryotube and stored in a -80 °C freezer.

2.2 Fingerprinting of rhizobial isolates

Rhizobial isolates were compared through molecular fingerprinting using the primer BOX-A1R (5'CTACGGCAAGGCGACGCTGACG 3') (Versalovic, 1994). From fresh cultures with 2-3 days of growth in YMA, a colony of each rhizobial isolate was touched with a sterilized micropipette tip, that was transferred to a microtube containing 50 µl of distilled water. The samples went through a thermal shock of 95 °C for 10 minutes in a thermocycler (Perkin Elmer GeneAmp PCR system 2400), to disrupt cells and release DNA, then kept on ice and used immediately.

To amplify the DNA, a mixture of 8 µl DNA Taq polymerase (Green Master Mix, Supreme NZYTech II), 2 µl of 10 µM primer BOX A1R and 10 µl of DNA template was added to PCR microtubes. As the PCR negative controls, the DNA template was replaced for 10 µl of MilliQ sterile water. The DNA went through an initial denaturating step at 95 °C-for 7 minutes, followed by 35 cycles of denaturing at 94 °C for 1 minute, annealing at 53 °C for 1 minute, and extension at 72 °C for 4 minutes, followed by a final extension at 72 °C for 10 minutes. Microtubes containing the PCR products were kept refrigerated until further processing.

Agarose gel (1%) electrophoresis was used to separate the PCR products in Tris-Acetate-EDTA buffer 1X (400 mM Tris, 200 mM acetic acid and 10 mM EDTA) at 100 V. To visualize DNA bands, 4 µl of the fluorescent dye GreenSafe (NZYTech) was added to the agarose gel. Two molecular weight ladders Hyperladder I (200 bp to 1 Kbp) were also applied to the gel (5 µl) for reference. After running for ca. 1:30 h, the DNA bands were visualized under ultraviolet light and images of the gels stored in digital support for subsequent analysis. The SynGene – GeneTools program was used to calculate band position (number of base pairs) in each lane, with band patterns forming the fingerprints of each sample. The

molecular weights of each lane were organized in groups of 200 by 200 and subjected to cluster analysis via the unweighted pair group method with arithmetic mean (UPGMA) algorithm, with Bray-Curtis similarity index in PAST version 4.16 (Hammer et al., 2001).

2.3 Plant-nematode-rhizobia interactions *in vitro*

This part of the project was conducted in the Mathesius Laboratory. Two plate trials with pre germinated seedlings were used to assess nematode-plant-rhizobia interactions in Fåhraeus medium. One assay assessed effects of rhizobia isolates from IBS and Luc soils on nodulation and galling, with 10 replicates per treatment and had 4 weeks duration; another assay was set up with 6 weeks duration elucidating such effects using the typified rhizobia isolates from the Mathesius Laboratory with 20 replicates per treatment. To assess the phenotypic response of *M. truncatula*, a multi-factor experiment for both trials was designed: i) inoculation with only rhizobia strains and isolates; ii) inoculation with only RKN *Meloidogyne javanica*; iii) co-inoculation of rhizobia isolates and RKN; and iv) a further treatment that did not receive RKN nor rhizobia inoculation. The plates were covered with black paper and placed in a semi-vertical position in a plastic tray. Plants were maintained in a controlled temperature room at 25 °C with 16 h light and 8 h dark cycle.

2.3.1 Nematode inoculum

Axenic cultures of *M. javanica* were multiplied and maintained on *M. truncatula* A17 in enriched Fåhraeus medium. Egg masses were collected from infected roots using a pair of forceps and surface-sterilized with 0.06% (v/v) sodium hypochlorite for 4 min with intermittent shaking. The eggs were centrifuged at 1,467 g for 5 min and rinsed thoroughly with 1 ml sterile MilliQ water five times, then transferred into 100 mL drops of sterile tap water in petri dishes sealed with parafilm. Three days later, the hatched J2s were counted in 5 mL aliquots, and 10 to 15 J2s were inoculated per root by directly pipetting onto the root tip of 1-week pre-germinated seedlings.

2.3.2 Seeds sterilization and pre germination

Medicago truncatula legume seeds were lightly scarified using fine sandpaper, sterilized at 6% (w/v) solution of sodium hypochlorite for 5 minutes and then washed five times in sterile distilled water. The plates were kept in the dark at 4 °C for 24 h and incubated at 25 °C until the radicles were 0.5 to 1.0 cm long. The pre-germinated seedlings with ~0.5–1 cm radicle length were transferred aseptically with forceps onto the surface of 15 cm diameter round plates (Corning, USA) containing Fåhraeus medium (2.1.2).

2.3.3 Rhizobial inoculum

To obtain active rhizobia, a colony of each rhizobial isolate was transferred with a loopful to a 15-ml sterile Falcon tube containing 10 ml of Bergersen's Modified Medium BMM medium ($NaH_2PO_4 \cdot 2H_2O$ 179 mg, $MgSO_4 \cdot 7H_2O$ 80 mg, $FeCl_3$ 3 mg, $CaCl_2 \cdot 2H_2O$ 40 mg, thiamin 2 mg, biotin 0.2 mg, sodium glutamate 0.5 g, yeast extract 0.5 g, mannitol 3 g, H3BO3 3 mg, $MnSO_4 \cdot 4H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 3 mg, $CuSO_4 \cdot 5H_2O$ 0.25 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg,$CoCl_2 \cdot 6H_2O$ 0.25 mg and 1000 of distilled water (Rolfe et al., 1980)) and incubated at 28 °C and 150 rpm overnight on an orbital shaker. The rhizobial isolates were streaked into fresh BMM petri dishes to be kept active before inoculation. The optical density of the resulting suspension was adjusted to 0.1 (at 600 nm) with sterile water and 10 µl of the rhizobia solution was pipetted to each side of the root of 1-week-old seedlings in plates.

2.3.4 Harvesting and observations

After four and six weeks, corresponding to each trial duration, early enough to see visible structures but also to prevent plant stress from lack of space in the petri dishes, nodules and galls of each trial were directly counted per root, using a stereomicroscope, and recorded according to the treatment.

2.4 Plant-nematode-rhizobia interactions in soil

The pre-germinated *M. truncatula* seedlings (described in 2.3.2) with ~0.5–1 cm radicle length were transferred onto 60 ml plastic pots, with a mixture of sieved (through 2.8 mm pore sieve) and autoclaved vermiculite, sand, and peat moss (1:1.5:1.5). Two weeks after emergence, the plantlets were thinned to one per pot. Rhizobium inoculum preparation was the same as described in 2.3.3, but isolates were maintained in YMB. Inoculation was done by pipetting directly into soils with 1 ml suspension of each rhizobial isolate at 0.1 optical density (2.3.3), and a suspension of 180 RKN juveniles at the J2 stage (in sterile water). This represents a moderate infestation level of three J2 per g of soil. Each treatment was replicated five times.

The plants were watered daily with distilled water and maintained in a controlled environment cabinet at 25 °C with a 16-8 h photoperiod for six weeks.

2.4.1 Nematode inoculum

The *M. javanica* eggs from tomato cv. Tiny Tim cultures were collected by cutting the infected roots, dipping them in 0.5% (v/v) solution of sodium hypochlorite with constant shaking for 2 minutes (Hussey and Barker, 1973). The resulting suspension was washed through a stack of two sieves (from top to bottom) of 75 μm and 20 μm aperture. The eggs were collected on the 20 μm sieve and transferred into a small system: a glass dish with a minimum amount of water with a small mesh and a Kleenex tissue wrapped on top to retain the eggs and organic debris, but simultaneously allowing the hatched J2s to move into the water. The system was covered with a plastic lid and kept at room temperature to allow hatching and collection of large numbers of freshly hatched J2. Hatched J2s were counted in aliquots to estimate suspension volumes needed to inoculate plants.

2.4.2 Harvesting and data collection

After six weeks, the plants were harvested, and roots were washed carefully with tap water to remove adhering soil. The aboveground part was cut from the roots and its length was measured. Fresh weight of both shoots and roots was recorded and shoots were then dried at 60 °C for 48 h to determine their dry weight.

The nodules, galls, egg masses and eggs per root were counted using a stereomicroscope and registered in a table according to the treatment. During the counting the roots were kept in petri dishes with distilled water. To better detect RKN egg masses, the roots were immersed for 15 minutes in a solution of Phloxine B 0.0015% in water. After staining for 15 minutes, the roots were rinsed with water, to remove excess dye (Hartman, 1983).

The average number of eggs produced per egg mass was determined by first extracting total eggs by the sodium hypochlorite method (described in 2.4.1). To produce an estimate of the total number in the suspension, the eggs in five replicates of 0.5 ml were counted. The total number of eggs per root was divided by the respective number of egg masses to obtain an estimate of female fecundity (eggs per egg mass).

2.5 Assessment of the effects of nitrogen enrichment on *Medicago truncatula*

To analyze the influence of nitrogen inputs on the plants, a pot experiment was carried out with different levels of nitrogen added to the substrate. Seeds of *M. truncatula* were pre-germinated on agar plates and transferred into 60 ml pots containing an autoclaved mixture of vermiculite, sand and peat moss (1:1.5:1.5). Two weeks after germination, 2 ml of six different molarity levels of KNO_3 in distilled water were applied onto the soils: 0 mM; 0.0625 mM; 0.250 mM; 0.625 mM; 1.25 mM and 2.5 mM. Each level had five replicates. Plants were watered daily and kept in a controlled environment cabinet at 25 °C with 16 h light and 8 h dark cycle, for six weeks. Root and shoot parameters were recorded as described above (2.4.5).

2.6 Data analysis

Results on the numbers of nodules, galls, egg masses and eggs, shoot measurements, shoot and root fresh weight, were analyzed statistically in SPSS for Windows v. 29 using Generalized Linear Models (GZLM) considering the effects of main factors ‘nematode’ and ‘rhizobial isolate’ and their interaction. Significant effects were further assessed by pairwise comparisons using LSD ($p<0.05$). Pearson correlations were calculated to better describe the data, comparing numbers of galls, eggs, egg masses and nodules formed in roots, and the putative relationship between levels of nematode infection and mutualist efficiency.

3. Results

3. Results

3.1 Characterization of organisms in field samples

Nematodes

Root-knot nematodes were detected in soil in IB-S samples, but not in Luc samples. The RKN recovered from roots of tomato cv. Tiny Tim grown in the IB-S soils were identified via esterase band patterns analysis as *Meloidogyne hapla* (Fig. 3, Pais et al., 1998).

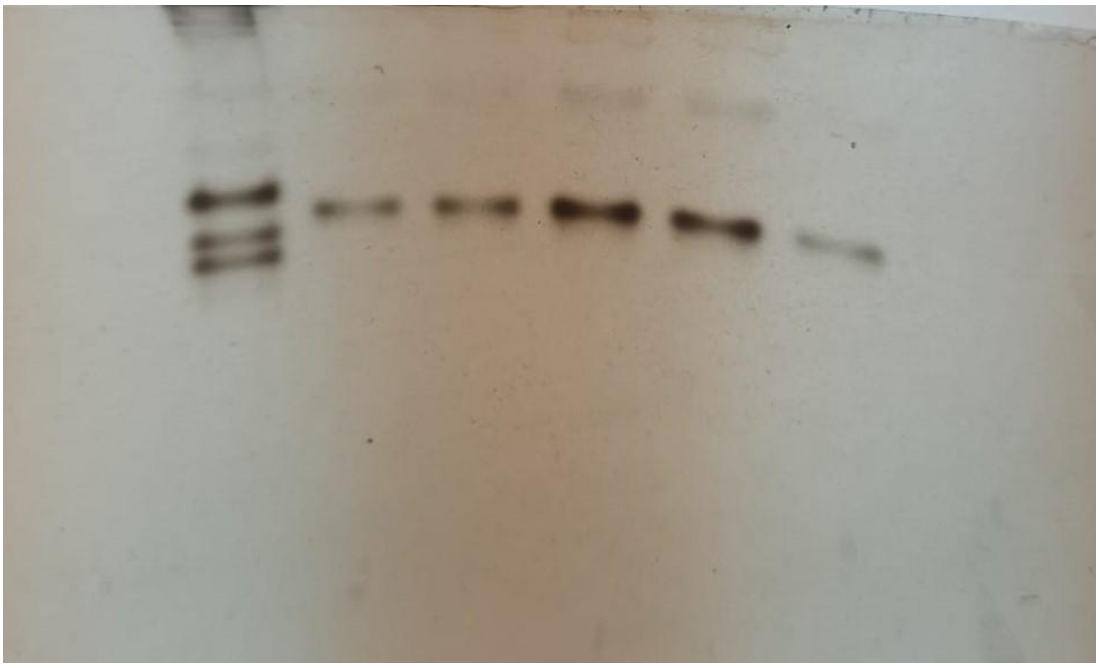


Figure 3. Polycrilamide gel with esterase activity represented by dark bands, after native gel eletrophoresis of *Meloidogyne* female extracts. Left lane contained *Meloidogyne javanica*, population P018 used as standard, with three bands of relative mobility between 0.42 and 0.54. Five lanes on the right had extracts of the IB-S *Meloidogyne* population, all with a single band of relative mobility 0.46, corresponing to an *M. hapla* phenotype.

Rhizobia

Rhizobia isolated from crushed nodules of *M. truncatula* roots growing on the two Portuguese sampled soils originated a total of 18 isolates: 10 from Luc and 8 from IB-S (Table 2). After amplification with primer BOX A1R, these revealed genetic heterogeneity (Figure 3.1). Cluster analysis allowed the interpretation of rhizobial isolate fingerprints. All 18 isolates from the sampled soils used in this study appeared to be genetically different from each other. Although three main clusters were formed (dividing isolates L1, L2 and S2; and isolate L5 from the remaining isolates), fingerprints do not seem to cluster

according to isolate origin/host plant or any recognizable pattern. The DNA fragment profiles are displayed in Annex II.

Table 2. Rhizobia isolates isolated from Portuguese soils. "Luc" - Lucerne soil isolates. C and S and - IB-S soil isolates.

Soil samples	
Lucerne (Luc)	IB-S
L1	S1
L2	S2
L3	S4
L4	S6
L5	S7
L7	C2
L8	C6
L9	C10
L11	
L12	

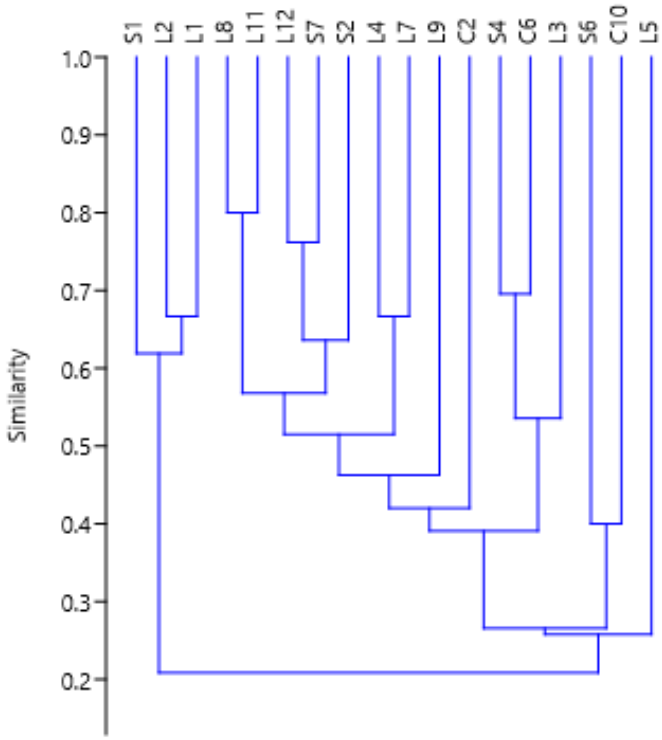


Figure 4. Clustering of DNA fingerprints of 18 rhizobia strains from *Medicago truncatula* roots growing on sampled soils from Lucerne (L) and IBS (C, S) sites.

3.2 Rhizobial nodulation and root-knot nematode galling of *Medicago truncatula* roots *in vitro*

Nodulation

Numbers of nodules formed on roots differed according to rhizobia strains and isolates and the duration of the experiment (4 or 6 weeks). Not all isolates successfully formed nodules in both experiments, and the ones that did differed quantitatively and significantly in their ability to nodulate. Nodulation was affected by RKN inoculation, with positive to negative outcomes, depending on the rhizobia isolate.

Comparing nodulation amongst isolates, indigenous rhizobia collected at Luc seemed to outperform those from IB-S and the *M. truncatula* compatible symbiont *S. meliloti* WSM1022 (Sm1022) ($p<0.001$, Fig5A). Maximum nodulation was recorded for Luc isolates with 1 to 23 nodules per plant, followed by Sm1022, and minimum nodulation was recorded for S4 and C2, both IB-S isolates ($p<0.001$, Fig5A). As expected, *Sinorhizobium meliloti* exopolysaccharide-deficient mutant (exoY-) formed white ineffective nodules on the host roots along with nodC-, the only tested-strains displaying this pattern. The *M. truncatula*-compatible symbiont Sm1022 was tested in both experiments as a reference, and nodulated significantly less at six weeks than at the 4-weeks experiment ($p<0.001$).

When plants were concurrently inoculated with rhizobia and with RKN, the rhizobia-legume interaction was affected, as revealed by changes in numbers of nodules formed. A summary table of all the outcomes regarding *in vitro* and in soil co-inoculated experiments with all tested rhizobia and RKN is presented in Annex IV. Inoculation with RKN stimulated nodulation in cases where nodulation was low, including some IB-S isolates (in the 4 weeks experiment), Sm1021 and Sm1022 (in the 6 weeks experiment), although a significant increase in nodule numbers was only detected for S4 and Sm1021 ($p<0.001$). On the other hand, the nodulation was supressed in co-inoculation of RKN with better nodulators S2 and WSM419 (in the six weeks experiment) ($p<0.001$, Fig.5A and 6A).

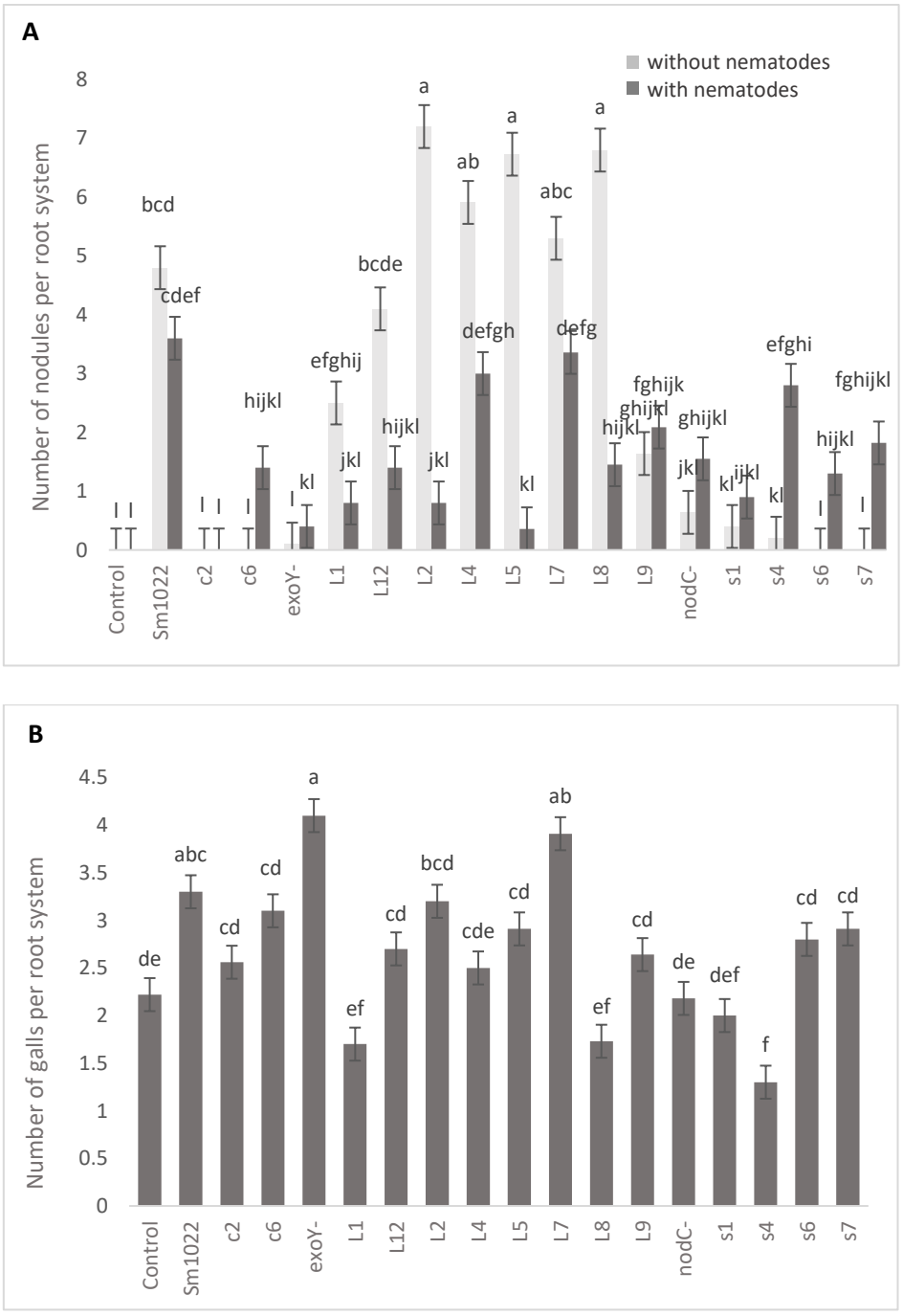


Figure 5. Root organs formed by root-knot nematodes *Meloidogyne javanica* and rhizobial isolates in the legume host *Medicago truncatula in vitro*, four weeks post-inoculation. A- Numbers of nodules formed per root system; B- Number of galls formed per root system (root-knot nematode-inoculated treatments represented). Values are mean +/- standard error of 20 replicates per treatment. Values with the same letter are not significantly different according to GLZM-LSD ($p<0.05$).

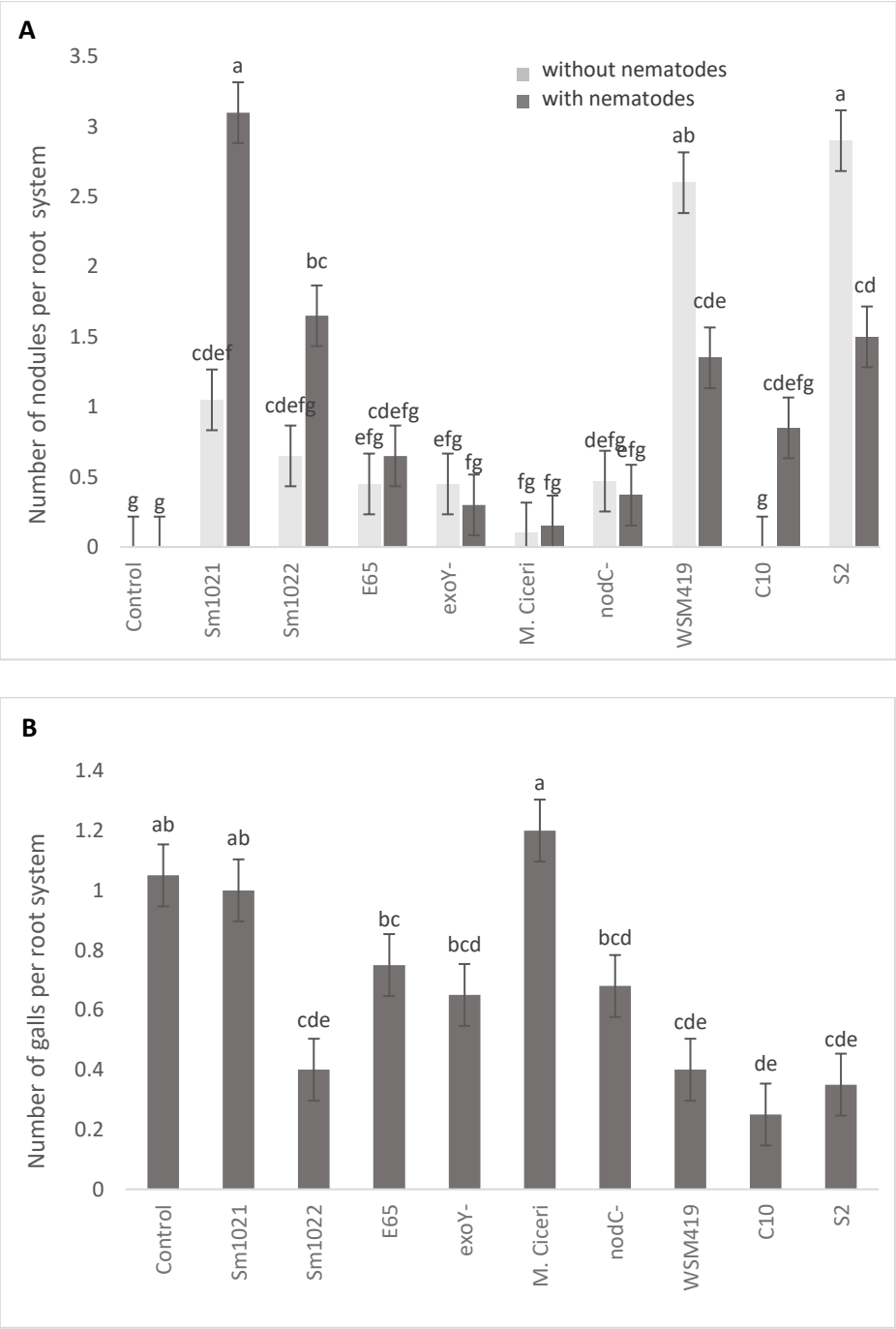


Figure 6. Root organs formed by root-knot nematodes *Meloidogyne javanica* and rhizobial isolates in the legume host *Medicago truncatula in vitro*, six weeks post-inoculation. A- Numbers of nodules formed per root system; B- Number of galls formed per root system (root-knot nematode-inoculated treatments represented). Values are mean +/- standard error of 20 replicates per treatment. Values with the same letter are not significantly different according to GLZM-LSD ($p<0.05$).

Galling

Root galling was observed in *M. javanica* inoculated roots, and either positively or negatively affected by rhizobial strains; a consistent pattern could not be detected between rhizobial origin and their effect in gall formation (Fig.5B).

In the earlier stages of interaction (4 weeks), plant inoculation with one of the rhizobial isolates reduced gall formation – S4, a relatively poor nodulator) ($p<0.001$). In fact, co-inoculation of S4 and RKN resulted simultaneously in the stimulation of nodulation and the reduction of galling. However, root galling was significantly increased in co-inoculations with good nodulators L7 and Sm1022, but also with ineffective nodulator exoY- mutant ($p<0.001$, Figure.5B). The increased root galling only negatively affected root nodulation with L7 rhizobia ($p<0.001$).

Rhizobial inoculants more successfully reduced RKN galling in later stages of interaction, as detected in the six-week experiment (Fig.6B). Four of the tested strains reduced gall numbers irrespectively of their ability to nodulate: C10 (relatively poor nodulator) and relatively good nodulators - S2 and *M. truncatula* compatible symbionts WSM419 and Sm1022 ($p<0.001$). The consequences for nodulation emerging from gall reduction could follow a pattern for nodulation being significantly decreased for good nodulators ($p<0.001$) and stimulated for low nodulators (although there were no significant results), as previously detected for S4.

3.3 Rhizobial nodulation and root-knot nematode galling of *Medicago truncatula* roots in soil

Nodulation

In *M. truncatula* grown in pots, nodule numbers had a wide range, from 1 to 103 per root system. Similar to *in vitro* results, nodulation varied according to rhizobia isolate and also with RKN inoculation. A clear trend could not be detected on effects according to the origin of rhizobial isolates. Overall, although RKN stimulated or supressed nodulation of a few rhizobia, most RKN-rhizobia combinations resulted in neutral effects for nodulation.

Numbers of nodules in plant roots obtained in pot experiments differed from those obtained in *in vitro* experiments, with IB-S and Luc isolates producing so few nodules that nodulation was not significantly different from that in the non-inoculated controls. Shoot dry and fresh weights were smaller in plants inoculated with IB-S isolates than in uninoculated controls, either with or without nematode

inoculation ($p<0.001$, Fig.8E and F). The most successful indigenous nodulators were IB-S isolate C10, with 7 to 26 nodules being formed per plant ($p<0.001$), and three Luc isolates - L4, L8 and L9 (Fig.8A and B). Maximum nodulation did not match maximum plant biomass, but overall Luc isolates associated with shoot length and weights higher than the non-inoculated control ($p<0.01$, Annex III), with further stimulation of plant biomass when inoculated with nematodes in L3, L11 and L12.

The typified compatible rhizobia highly outperformed the indigenous rhizobia in nodulation. Maximal nodulation was achieved with the *M. truncatula* compatible symbiont Sm1022, either inoculated alone or in co-inoculation with *M. javanica*, with 2 to 103 nodules per plant root formed ($p<0.001$, Fig.7A), followed by WSM419. Higher nodulation was matched by significant increase of shoot fresh and dry weights as a result of inoculation with these strong mutualists ($p<0.001$). Although exoY- stimulated shoot length, inoculation with this strain caused overall reduced number of leaves, in contrast with WSM419 that resulted in higher density of leaves with regular size. Interestingly, both mutants and wildtype Sm1021 formed few nodules, and did not affect shoot fresh and dry weights, but resulted in significantly lower root fresh weight ($p<0.001$).

Inoculation with RKN affected nodulation in combinations with indigenous rhizobia only (Luc or IB-S). Negative effects of RKN on rhizobial nodulation were observed in combination with C10 (fairly good nodulator in soil), reducing nodule numbers completely ($p<0.001$) and resulting also in smaller shoot length ($p<0,01$). On the other hand, nematode interaction with rhizobia resulted in higher nodule numbers for S2 ($p<0.001$), L3 and L11 ($p<0.01$).

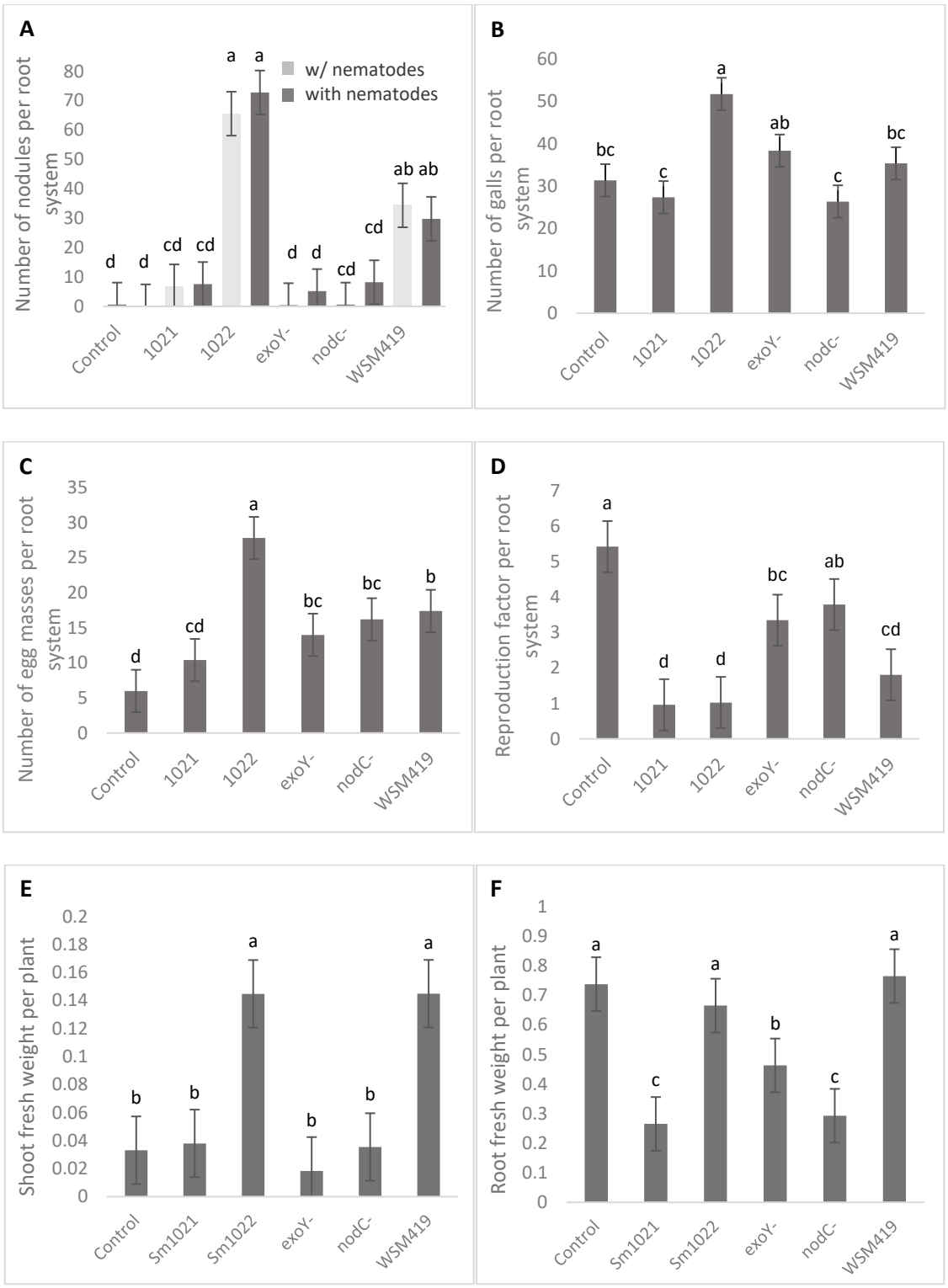


Figure 7. Root organs formed by root-knot nematodes *Meloidogyne javanica* and rhizobial isolates in the legume host *Medicago truncatula* in soil, with typified strains from the Mathesius Laboratory. A- Numbers of nodules formed per root system; B- Number of galls formed per root system (root-knot nematode-inoculated treatments represented); C- Number of egg masses formed per root system (root-knot nematode-inoculated treatments represented); D- Nematode reproduction factor per root system; E, F- Shoot and root fresh weight per plant. Values are mean +/- standard error of 20 replicates per treatment. Values with the same letter are not significantly different according to GLZM-LSD ($p<0.05$).

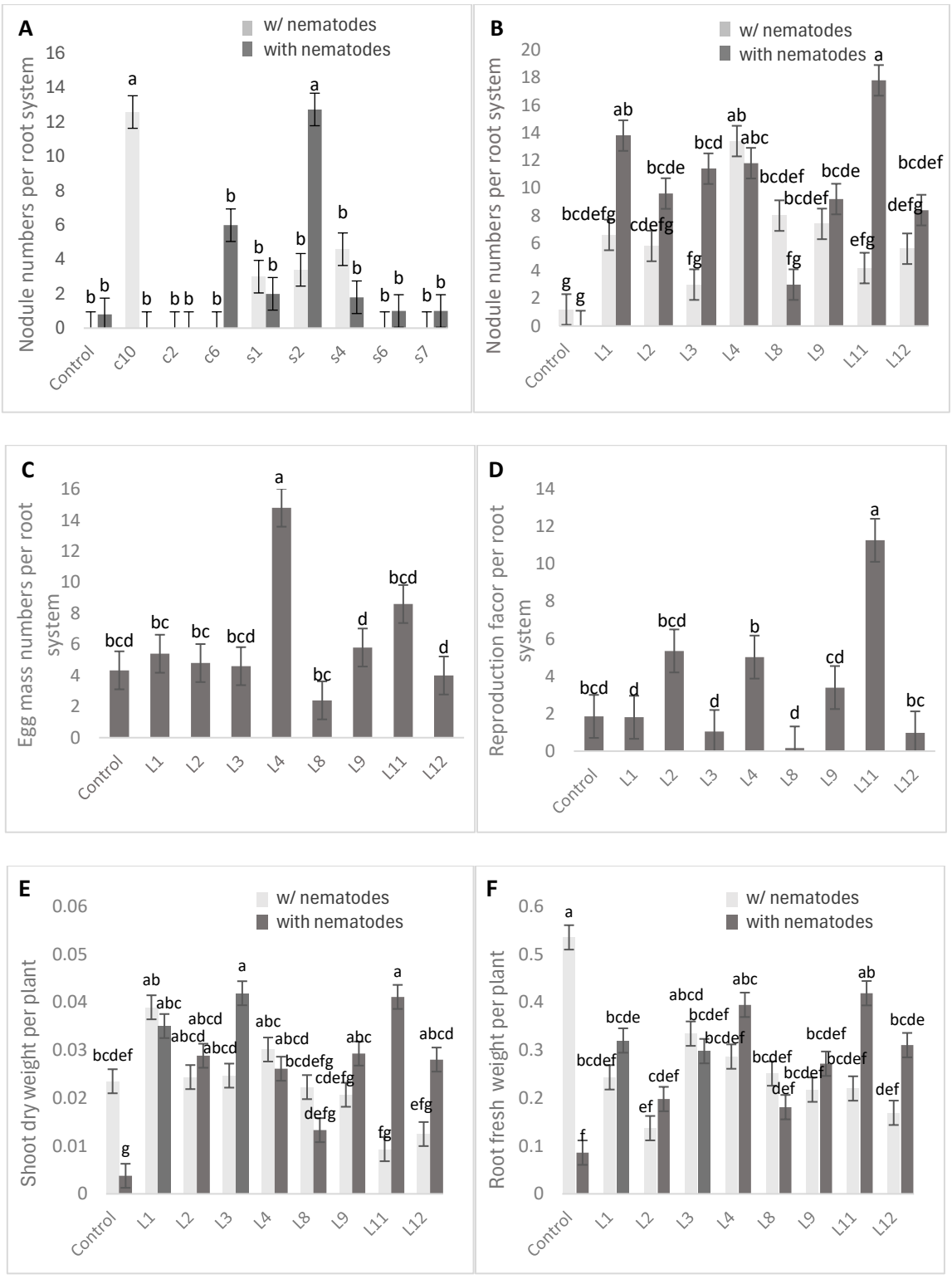


Figure 8. Root organs formed by root-knot nematodes *Meloidogyne javanica* and rhizobial isolates in the legume host *Medicago truncatula* in soil, with indigenous rhizobia. A- Numbers of nodules formed per root system with IB-S isolates; B- Number of nodules formed per root system, with Lucerne isolates; C- Number of egg masses formed per root system (root-knot nematode-inoculated treatments represented), with Lucerne isolates; D- Nematode reproduction factor per root system with Lucerne isolates; E, F- Lucerne shoot and root fresh weight per plant. Values are mean +/- standard error of 20 replicates per treatment. Values with the same letter are not significantly different according to GLZM-LSD ($p<0.05$).

Galling and nematode reproduction

Neutral, negative and/or positive effects were found on RKN galling depending on the rhizobial isolate, sharing some similarities to previous results found *in vitro*. Some combinations promoted nematode infection in pot experiments, assessed as galling, egg mass production and eggs laid. But, none of the tested rhizobial strains reduced gall or egg mass formation, with results obtained in pot experiments differing from those obtained *in vitro*. Nematode suppression as an effect of rhizobial inoculation was only detected in the total number of eggs produced per root (nematode reproduction). Concomitant inoculation with IB-S isolates did not affect any nematode parameters significantly (Annex III). Correlation matrices of RKN co-inoculation with all the tested rhizobia in this experiment are shown in Annex V, providing insight into how nematode infection, plant growth, and root nodulation vary in the experiment.

Gall numbers increased in co-inoculation of nematodes with Sm1022 ($p<0.01$, Fig.7B) and four rhizobial isolates increased numbers of egg masses: Sm1022, WSM419, exoY- and Luc isolate L4 ($p<0.01$). There was a negative but not significant correlation with Sm1022 nodule and gall numbers and a positive but not significant correlation with nodule and egg mass numbers in Sm1022, WSM419 and L4. Larger gall and egg mass numbers were not matched by nematode fecundity; on the contrary, this was either significantly decreased or not affected and ranged from 0.0068 to 1,857 eggs per egg mass. The only nematode fecundity increase in response to rhizobia inoculation was detected for isolate L11 ($p<0.001$), that had no significant effect on galling or egg mass formation.

A negative effect of rhizobia inoculation on nematodes was detected for some rhizobia that resulted in increased egg mass formation, but simultaneously, decreased total eggs produced. Maximum reduction relative to control was found under Sm1021 (82.26%), followed by Sm1022 (81.08%), WSM419 (66.66%) and exoY- mutant (38.28%) ($p<0.001$). Although its reproduction factors were significantly low comparing to the nematode-only infected control (Fig.8F), the nematode population slightly decreased with Sm1021 only (Rf= 0.9628). Major effects on nematode fecundity were irrespective of the rhizobial ability to nodulate; and their ability to nodulate was unaffected by coinoculation with nematodes.

In strains associated with decreased nematode fertility, the only correlations found for egg numbers were egg mass parameters, either positive (Sm1022) or negative (exoY-). A pattern of correlations for the protective effect of rhizobia towards nematodes could not be detected, neither a

pattern according to rhizobia origin. There were no significative correlations between nodules and galls, except for L8 (positive). Although, there's groups of rhizobia that show an inverse relationship between nodules and galls (L1, L2, S1, S4, Sm1021, Sm1022) and a positive one (L9, L11, S2, S6, nodC-), these are not significative. Increases in plant health were only associated with nodule numbers in L3 and with egg mass numbers in WSM419 (both positive). Some rhizobia - L8, L9, S6, C6 and nodC- – have nodulation, nematode and biomass parameters all correlating positively within the same root system.

3.4 Effects of nitrogen enrichment in *Medicago truncatula* biomass

The effect of the six different levels of KNO_3 inputs onto *M. trunctatula* plants did not result in consistent relationships between nitrogen concentration and shoot/root weights. None of the concentrations tested contributed to increase plant biomass (Annex VI). In fact, at the highest concentration tested, shoot length and weight were both significantly lower compared to the control ($p<0.01$ and $p<0.001$, respectively).

4. Discussion

4. Discussion

The aim of this study was to evaluate the impact of weak rhizobia mutualists in the plant-nematode interaction. Results obtained revealed a range of weak to strong mutualists in the tested rhizobia, according to nodulation capacity and effects on plant biomass. We found neutral, positive and negative mutualistic effects of the tested rhizobia alone, differing within origin and substrates. When co-inoculated with RKN *M. javanica*, some weak rhizobial mutualists compromised their ability to establish feeding sites and produce eggs. This supports the hypothesis that weak mutualisms may be maintained among plants and rhizobia when they are able to bring other benefits to the plant besides nitrogen fixation. However, this was an idiosyncratic result, dependent on the identity of rhizobia tested and not directly attributable to any of the observed parameters.

The various nodulation and galling outcomes for *M. truncatula* roots resulting from the interaction with different rhizobia strains and isolates, support the fact that rhizobia identity plays an important role in determining plant-nematode interactions. A few isolates, both good and poor nodulators, indigenous or typified rhizobia, contributed to deter RKN infection, making this potential rhizobial trait independent of origin or nodulation and nitrogen fixing capacity. Within rhizobial strains and isolates, we found markedly different effects in rhizobia exerting a range of negative to neutral to positive influences on nematode galling, likely linked to their genetic diversity (Costa et al., 2021) and different conditions amongst assays (Siddiqui et al., 1999; Desaeger et al., 2005).

Under nematode attack, depending on the conditions and rhizobia available, different mechanisms could have been applied by the plant host as a result of the different interactions: i) recruitment of rhizobia that enhance plant health (L3) by allocating its resources to nodulation, with no negative impacts on RKN; ii) lessen nodulation with compatible rhizobia strains, negatively affecting nematode infection as well (Sm1022); iii) and favour some poor nodulators (S4 and C10) that are less compatible with the plant in fixing nitrogen, but negatively affect nematode infection.

Only 11% of the general outcomes for rhizobia-plant-nematode interaction corroborate findings by Wood et al. (2018) indicating that rhizobia infection was directly linked to the attractiveness of nematodes. The authors described this interaction as a genetic conflict between attracting a mutualist and simultaneously resisting antagonistic nematodes, resulting in a trade-off mediated by the plant. Molecular evidence suggests that genetic conflict between legume responses to mutualistic rhizobia and parasitic nematodes is extensive (Goverse and Smant, 2014; Wood et al., 2018) and might impact root signalling

and exudation, resource allocation trade-offs and changes in host defence status, contributing to determine the different outcomes (De Román et al., 2011), that could possibly explain the mechanism suggested in ii). In our work the outcomes tended to affect either rhizobia phenotypes or nematode phenotypes only, suggesting that genetic conflict may be observed depending on the identity of the interacting partners

The full range of mutualistic-parasitic spectrum of rhizobia from ineffective to less compatible, low and good nodulators resulted in different galling phenotypes, according to rhizobial identity and experimental conditions. Our results show not only good mutualists, but also some poor and ineffective nodulators acting in order to supress RKN infection. Nitrogen fixation is an energetically expensive process, consuming resources that rhizobia could otherwise use for their own growth and multiplication (Hoffman et al., 2014). IB-S low nodulators S4 and C10 supressed gall formation *in vitro*, accompanied by the allocation of the host resources for nodulation. Increasing nodulation with typically low nodulators could be an evolutionary strategy that may lead to increased resistance to RKN attack, our proposed outcome iii). The exoY- mutant for instance, with the known incapacity of fixing nitrogen, supressed egg production by nematodes in pot experiments, but not galling. Plant interaction with these rhizobia could have not prevent initial interactions that led to root infection and feeding site establishment, but may have then induced plant defence, or inhibited nutrient acquisition by the nematodes. According to Becker and Pühler (1998), rhizobia exopolysaccharides supress plant defence responses, pointing to a potential positive role of rhizobia exopolysaccharide structures in successful *M. javanica* parasitism. This exoY- mutant was previously reported to induce plant defence responses in *M. truncatula* A17 and *M. sativa* (Jones et al., 2008; Niehaus et al., 1993). Although, the absence of exoY- structures benefits the defence against RKN, the signalling process of rhizobia with the plant-host must be assured. Costa et al. 2020 found that nodulation signalling is required for rhizobia's protective effect against RKN, which is confirmed by our results showing no role of nodC- mutant supressing RKN, in either substrate, and actually leading to increased egg mass numbers in pot experiments. The authors also found a positive role of ethylene signalling in the susceptibility to nematode infection, reporting suppression of gall and egg formation in an ethylene insensitive, hypernodulating mutant, rather explained by a putative resource competition between galls and nodules (Costa et., al 2020). Exopolysaccharide structures are also host-specific, which may be associated with the fact that Sm1021 - a not-so compatible strain of *M. truncatula*, poorly matched for N_2 fixation, reached maximum levels of egg production suppression as well by 82.26% in pot experiments. Although inducing a large number of nodules, less bacteroides reside in Sm1021 nodule cells in *M. truncatula*, compared to the compatible symbionts, thus fixing less nitrogen (Simsek et al.,

2007). The plant host could sanction Sm1021 nodules for lack of nitrogen fixation, but still maintain them in its roots (Terpolilli et al., 2008). We propose maintaining this mutualism may be due to its ability to deter RKN infection. The higher resistance to RKN associated with low nodulators could be due to opposing expression of genes of both microorganisms (Plett et al., 2016), with a symbiosis less likely to favour nematode infection, when different mechanisms and biochemical pathways are being explored. We cannot exclude that the plant manages a potential trade-off between the costs of associating with poor nitrogen-fixing rhizobia that, on the other hand, in some way supresses RKN.

Interestingly, the same rhizobial strain can simultaneously positively and negatively affect different nematode parameters, on the same root, perhaps by different mechanisms being played at the same time with different effects on nodulation. In some cases of low egg production, our results show gall and egg mass formation being significantly promoted by plant interactions with rhizobia and yet compromise nematode fitness (fecundity), with no changes in nodulation. The observed effects could have changed according to rhizobia identity along with many other combined factors, such as plant resistance to infection (ability to prevent pathogen multiplication, Clarke, 1986), tolerance (ability to overcome damage, Råberg et al., 2014), hypersensitivity in recognizing nematodes (Bagivora, 2007) and nematode virulence (Bobardt et al., 2020). The initial increase in gall and egg mass numbers could be associated with *M. javanica* strain P018 virulence to *M. truncatula* in the combination of certain rhizobia, since our results show overall increase of nematode population (reproduction factor greater than one), except in the combined presence of Sm1021. The larger the nematode numbers infecting the roots, the higher is the nutrient limitation and corresponding stronger competition effects, leading to lower overall fitness and smaller number off -eggs laid (Costa, 2024).

Changes in nematode parameters on the same root could also be related to pre- and post-infection plant resistance mechanisms upon invasion. Pre-infection resistance prevents nematodes to enter the plants roots due to the presence of toxic or antagonistic chemicals in root cells (Haynes and Jones, 1976; Bendezu and Starr, 2003). In post-infection resistance mechanisms the nematodes are able to penetrate roots but fail to develop, often associated with an early Hypersensitive Reaction (HR)-mediated cell death, where localized necrosis in root tissue around the nematode prevents the formation of a developed feeding site (Dropkin, 1969; Williamson, 1999). However, two cases with no evidence of early HR, but a delayed resistance against RKN have been reported (Powell, 1962; Das et al., 2008), matching our findings related to high gall/egg mass - low fecundity rates. For example, Das et al. (2008) found out that in the presence of resistant cowpea plants, infective nematode juveniles were able to enter

the root, establish feeding sites and develop into females, until development was arrested before egg-laying, with giant cells deteriorating 2 to 3 weeks after inoculation. The control of nematode females and egg production does not necessarily prevent root invasion and plant damage by the nematodes, but their multiplication can be reduced (Lamovšek et al., 2013). Some rhizobia might also indirectly induce systemic resistance (IRS), phenotypically similar to pathogen-induced systemic acquired resistance (van Loon et al., 1998). Although, induced resistance responses would decrease nematode ability to penetrate the roots, but, with no effect on nematode attraction and only a slight effect on development inside the roots (Lamovšek et al., 2013).

Another possible explanation includes the production of antimicrobial and toxic compounds by rhizobia (Breil and Triplett, 1996; Chakraborty and Purkayastha, 1984), that could specifically target the reproductive nematode stages more effectively than the juvenile stages responsible for gall formation. The mechanism of action causes membrane damages by inhibiting the synthesis of pathogen cell walls, which consequently influence the cell membrane structures (Jeyanthi and Kanimozhi, 2018). However, research on rhizobial toxic compounds is not as extensive as for other antagonists like certain fungi or bacteria (Lamovšek et al., 2013). Targeting plant defence responses might be a more rapid, long-term effect in the protection against different plant pathogens (reviewed in Hallmann et al., 2009), that could be acting on later nematode stages, allowing the initial process of infection. The lack of a correlation pattern for our plant-rhizobia-nematode parameters could mount to the possibility of different explanations being considered for the different mechanisms being played with different rhizobia. The later stages of the *M. javanica* lifecycle were not recorded in *in vitro* experiments, as their main purpose was to rapidly collect data on the earlier stages of the interaction. But, it would be interesting to observe in the future if rhizobia "localized" effects on egg suppression were consistent to plate conditions.

It's important to note that RKN suppression often differed between *in vitro* and in soil conditions. The differences in the common tested rhizobia between four and six weeks *in vitro* suggests that the protective nature of rhizobia may not act in the earlier stages of the interaction but later on the symbiosis, observed with Sm1022 and exoY- rhizobia strains. The protective nature of rhizobia in pot experiments were only detected on later stages of nematode development (numbers of eggs produced) and producing results that were inconsistent with those obtained *in vitro*. Our results show IB-S poor nodulators S4 with the ability to induce RKN negative effects on the earlier stages of infection *in vitro* and C10 and good nodulator S2, significantly suppressing gall formation *in vitro*, but not in pot experiments. The rhizobia isolated from the field growing lucerne (Luc) outperformed nodulation *in vitro* in comparison to the

compatible symbionts, however, with less success in pot experiments and with no results of RKN antagonism. *In vitro* conditions were more favourable to indigenous rhizobia nodulation than soil conditions. IB-S rhizobia being extracted from a field co-living with *Meloidogyne hapla* and both Luc and IB-S isolates growing with a probably more compatible host than *M. truncatula*, might have led to direct or indirect impacts on RKN, perhaps with a more notable difficulty in soil. The effects on nodulation and RKN phenotypes could have been modulated by changes in rhizosphere signalling since the microorganisms were directly pipetted to the root tips *in vitro*. Also, the substrate in pot experiments maintains its physical 3-dimensional structure, in contrast to the limited spaces on the plates, allowing for a more natural root architecture with extensive growth and a more conducive environment. Therefore, rhizobia can colonize roots effectively without severe nutrient competition and less impact on galling (Jones et al., 2013b; Nishida et al., 2023). In nature, there are thousands of other species of organisms and mixed populations of rhizobia and RKN constantly interacting, changing the host microbiome, supporting and challenging their interactions and effect on the host defence responses (Costa 2024, Mathesius and Costa 2021, Zgadzaj et al., 2016). It is important to have in mind that the absence of the natural microbiome assembly on our assays, cannot replicate the same conditions that challenge rhizobia in the field and could modulate observed outcomes.

In summary, the trade-offs mediated by the plant involved balancing the benefits of symbiotic relationships with rhizobia against the costs of nematode infection, including nutrient loss, root damage, and impaired growth. The plant could mediate recruiting rhizobia genetically linked to attractiveness of nematodes, but also accept the cost of recruiting weak mutualists for the nitrogen fixing trait, that supress nematodes. Our results add to the theory that antagonizing nematodes could be an unanticipated function of "weak mutualistic rhizobia", instead of being functionally redundant, presenting variability in the outcomes of rhizobia-plant-nematode interactions. This would explain the widespread associations in the field, with weak mutualists not being successively outcompeted by other strains (Quigley et al., 1997). Although contributing to a different notion of rhizobia mutualistic efficiency in ecological interactions, the results must be interpreted with some caution when considering field application.

Conclusions and Future Perspectives

This work allowed us to address the initial objectives of evaluating rhizobial mutualistic capacity and the performance of weak rhizobial mutualists in the protection against root-knot-nematodes. Here,

we showed that the nematode suppression trait in rhizobia depends on their identity that per se, is independent of local of origin and nitrogen fixing capacity. Some poor symbiotic performance of some rhizobia in nodulating with a specific host, can bring negative consequences for nematode establishment of feeding sites and fecundity on the same root system. The mechanisms behind these outcomes remain unknown, so further genetic and biochemical research will be meaningful to contribute to the understanding of plant-microbe interactions. Not all weak mutualistic induced the same outcome, highlighting the importance of studying specific plant-rhizobia-nematode interactions for an accurate application of rhizobia benefits in nematode control in agricultural systems. That could be accomplished by assessing the genetic and chemical nature of the rhizobia-RKN in different scenarios, biochemical pathways and gene expression overlapping, and any early plant-rhizobia-nematode signalling occurring through the analyses of root exudates.

Some legumes, including lucerne (*Medicago sativa*), have been shown to promote nodule development in the absence of rhizobia, through spontaneous nodule formation (Joshi et al., 1991), so it would be important to understand to which extent nodules in our experiments are actually colonized by rhizobial bacteroides and whether the plant host resorts to this mechanism in the presence of RKN. Although we have employed some well-characterised rhizobial strains and used nodulation and plant biomass as proxies of mutualistic efficiency, the quantification of fixed N by each rhizobial strain and isolate in single and combined inoculation with RKN would be highly informative.

Applying rhizobia in real agricultural systems to reduce RKN pressure on crops, still needs substantial research since many plant-RKN-rhizobia combinations actually stimulate nematode infection whilst reducing nodulation. Therefore, the potential of using legume-inoculated rhizobia as cover crops has to be carefully evaluated because of the risk of rhizobia favouring the increase in RKN populations. It is important to understand which rhizobia characteristics are responsible for the plant protection against RKN trait and how it is affecting mutualism establishment. In our work we used *M. truncatula* to mediate the interactions, but since rhizobia are host-specific, the outcomes must be carefully tested and interpreted according to plant-rhizobia-nematode compatibility. It therefore seems important to further invest in deepening the emerging sustainable ways of RKN control in modern agriculture, while reducing negative impacts to environment and non-target organisms.

5. References

5. References

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Annex I

Root-knot nematode electrophoresis and identification

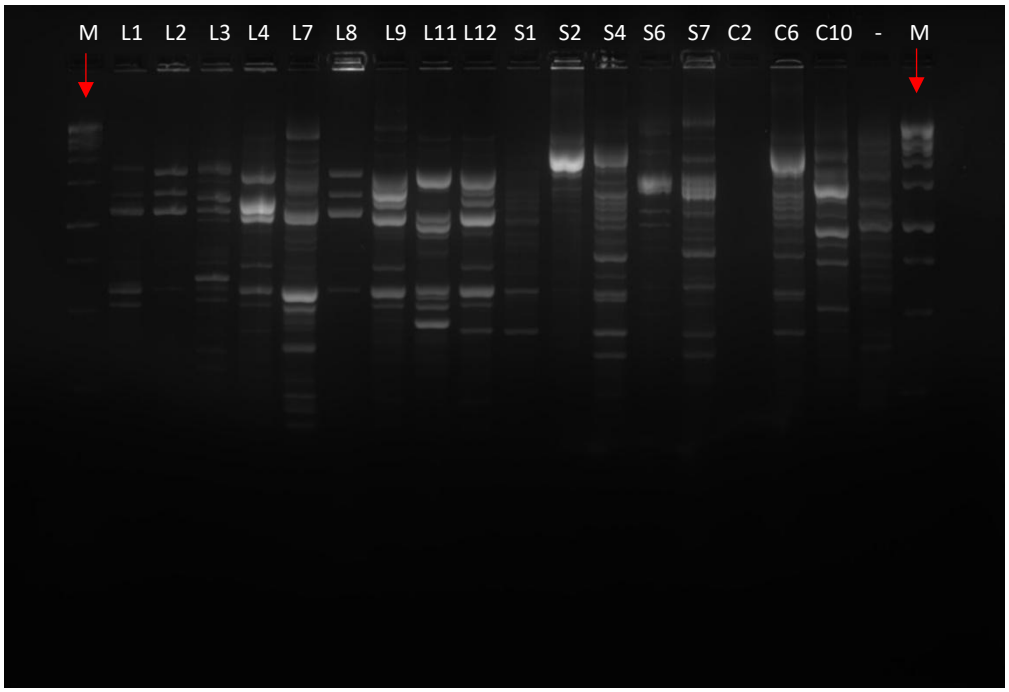
The macerated material from the females was thawed and centrifuged at 10,000 g for 15 minutes, at room temperature, and then aspirated with a micro-syringe the protein material located between the pellet and the surface lipid layer and mixing it with a solution of bromophenol blue (constituted by 0.05 g of bromophenol blue dissolved in 100 mL of distilled water).

The samples were then applied and concentrated in wells of a gel 3% (v/v) polyacrylamide in Tris-HCl buffer (pH 6.8) and separated on a 7% (v/v) gel in Tris-HCl buffer (pH 8.8). The gels were subjected to a constant current of 6 mA/gel for 10 minutes for the samples to penetrate the separation gel. At this point the constant current was increased to 20 mA/gel and electrophoresis was allowed to run for 45-55 min, until the bromophenol blue line approaches the edge of the gel.

Finally, the gels were placed in a Fast Blue RR staining solution (0.1 mg of Fast Blue RR salt in 10 mL of distilled water) in sodium phosphate buffer (pH 7.2), acetone and α-naphthyl-acetate, in an oven at 37 °C, until bands were visible (about 30 minutes) (Esbenshade and Triantaphyllou, 1985).

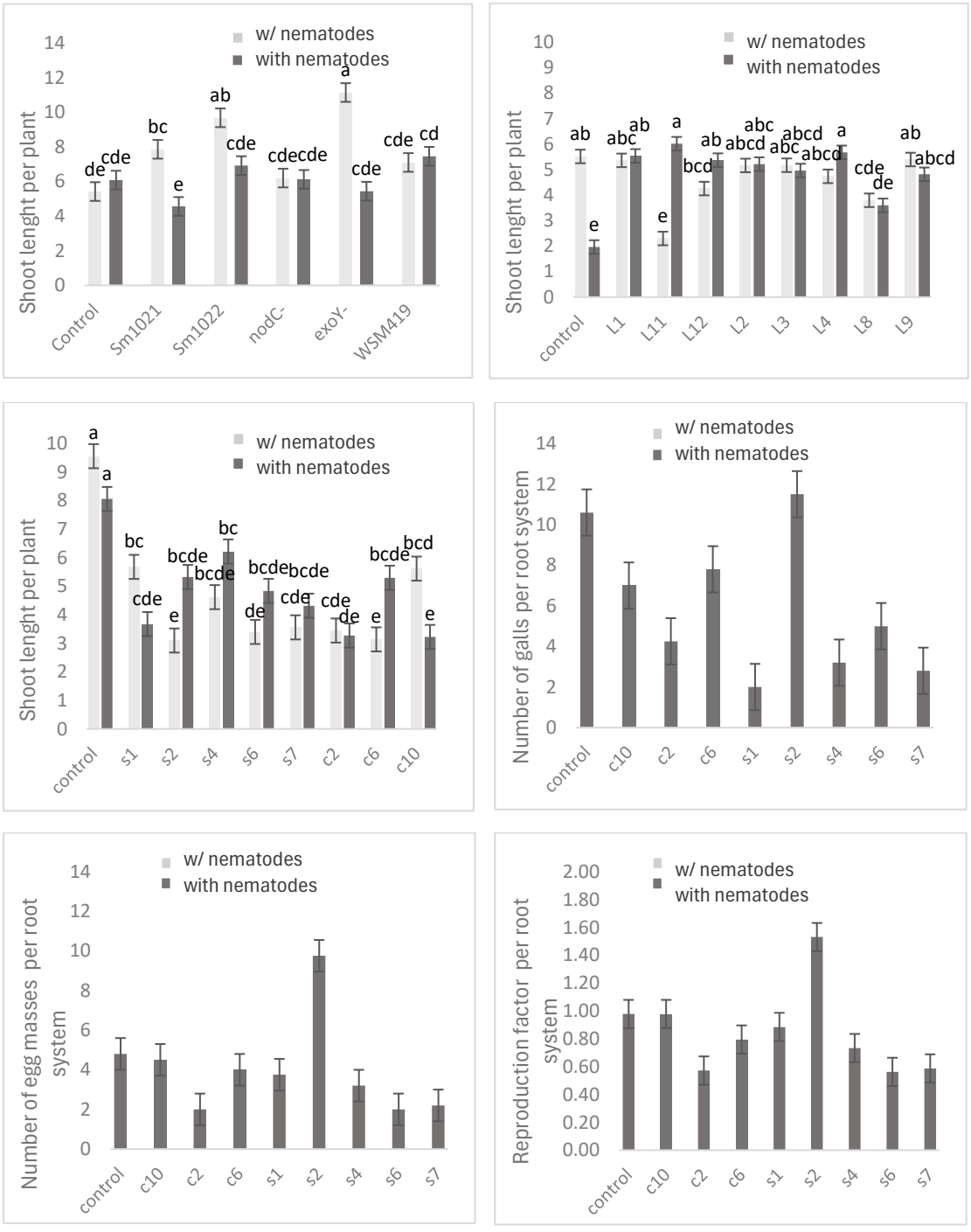
The species identification of the analyzed populations was done by measuring the relative mobility of bands in comparison to those of the standard sample population of M. javanica, P018 (Esbenshade and Triantaphyllou, 1985; Pais & Abrantes, 1989).

Annex II



DNA fingerprints of rhizobia isolates obtained from growing *Medicago truncatula* on sampled soils from Lucerne and IBS sites. Marker used was 1kb DNA Ladder. The marker is placed under the two red arrows in the picture.

Annex III



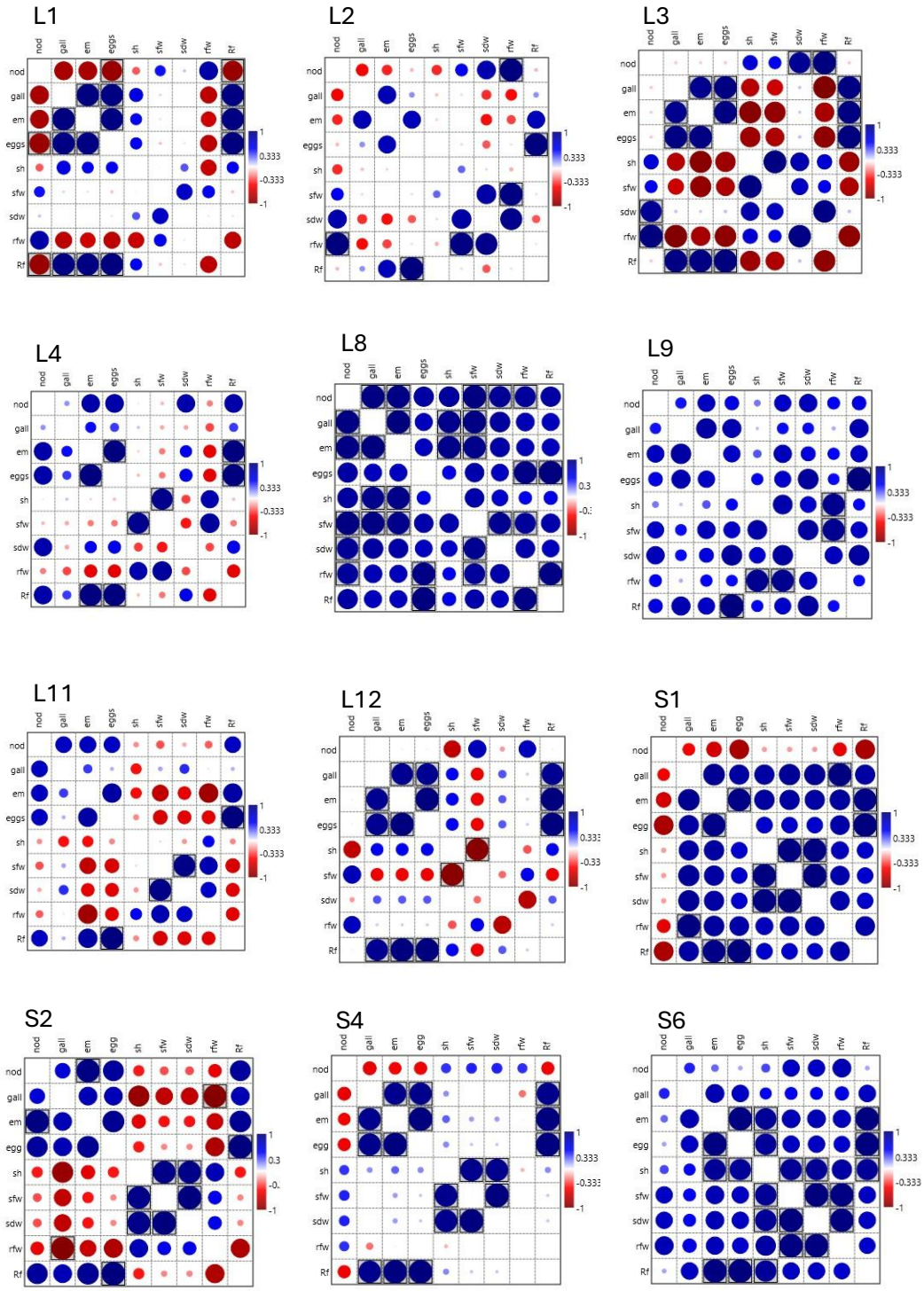
Root organs formed by root-knot nematodes *Meloidogyne javanica* and rhizobial isolates in the legume host *Medicago truncatula* in soil, six weeks post-inoculation. A, B,C – Shoot length per plant from typified strains, Lucerne and IB-S isolates, respectively. D- Number of galls formed per root system (root-knot nematode-inoculated treatments represented) with IB-S isolates ; E- Number of egg masses formed per root system (root-knot nematode-inoculated treatments represented) with IB-S isolates; F- Nematode reproduction factor per root system with IB-S isolates;Values are mean +/- standard error of 20 replicates per treatment. Values with the same letter are not significantly different according to GLZM-LSD ($p<0.05$). The values in D, E and F were non statistically significant.

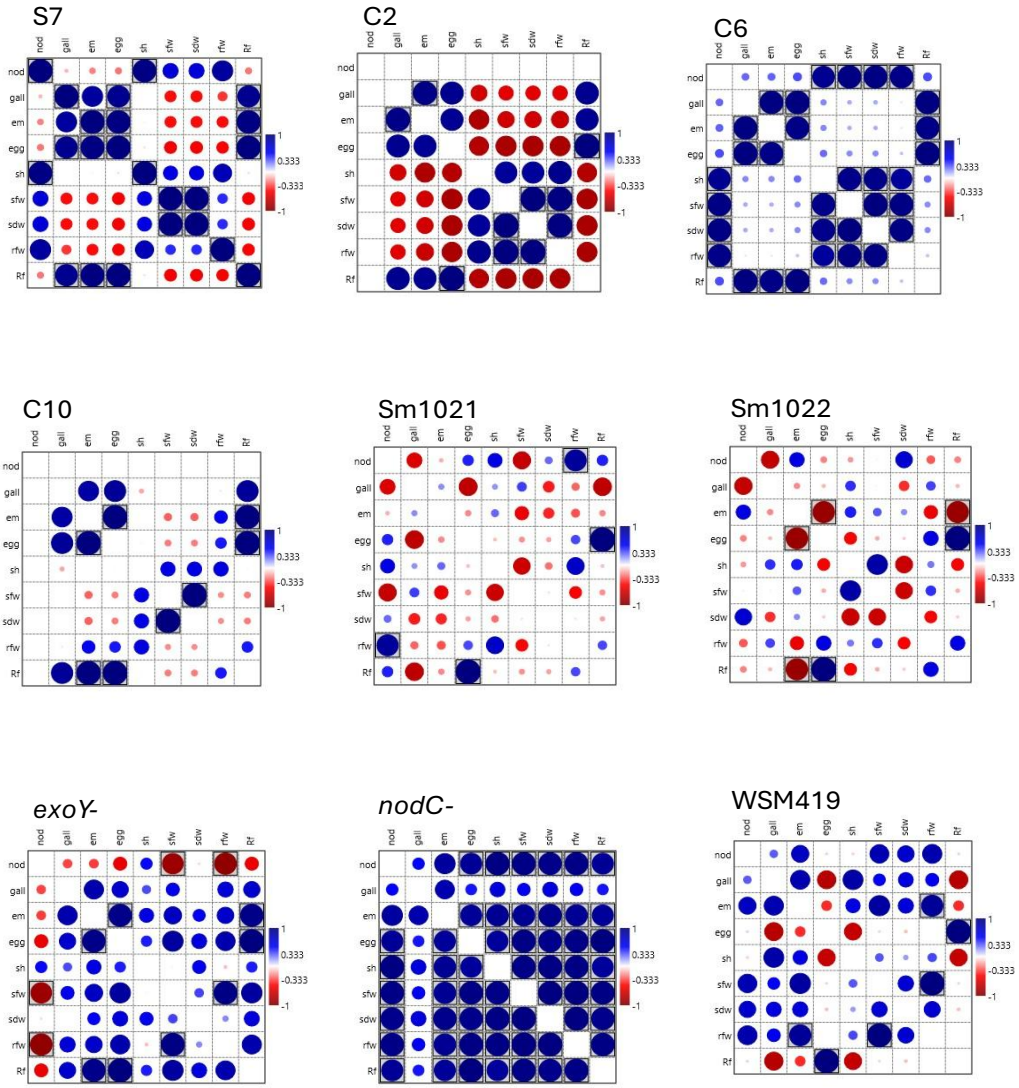
Annex IV

Combination of the effects on rhizobial nodulation and root-knot nematode galling in coinoculation in *Medicago truncatula* roots in soil and *in vitro*. "+"- Positive effects in the number of nodules, galls, masses , egg masses of the reproduction factor compared to the controls;"-"- Negative effects. "0"- Neutral effects;"N/A"- not applicable; NS – not significative.

In vitro			**In soil**				
Rhizobia	No. nodules	No. galls	No. nodules	No. galls	No. egg masses	No. eggs	Rf
4 weeks			**6 weeks**				
L1	0	0	0	NS	0	0	0
L2	-	0	0	NS	0	0	0
L3	N/A	N/A	+	NS	0	0	0
L4	-	0	0	NS	+	0	0
L5	-	0	N/A	N/A	N/A	N/A	N/A
L7	-	+	N/A	N/A	N/A	N/A	N/A
L8	-	0	0	NS	0	0	0
L9	0	0	0	NS	0	0	0
L11	N/A	N/A	+	NS	0	+	0
L12	-	0	0	NS	0	0	0
S1	0	0	0	NS	NS	NS	NS
S4	+	-	0	NS	NS	NS	NS
S6	0	0	0	NS	NS	NS	NS
S7	0	0	0	NS	NS	NS	NS
C2	0	0	0	NS	NS	NS	NS
C6	0	0	0	NS	NS	NS	NS
6 weeks							
S2	-	-	+	NS	NS	NS	NS
C10	+	-	-	NS	NS	NS	NS
Sm1021	+	0	0	0	0	-	-
Sm1022	0	-	0	+	+	-	-
exoY-	0	0	0	0	+	-	-
nodC-	0	0	0	0	+	0	0
WSM 419	0	-	0	0	+	-	-
E65	-	0	N/A	N/A	N/A	N/A	N/A
M. ciceri	0	0	N/A	N/A	N/A	N/A	N/A

Annex V

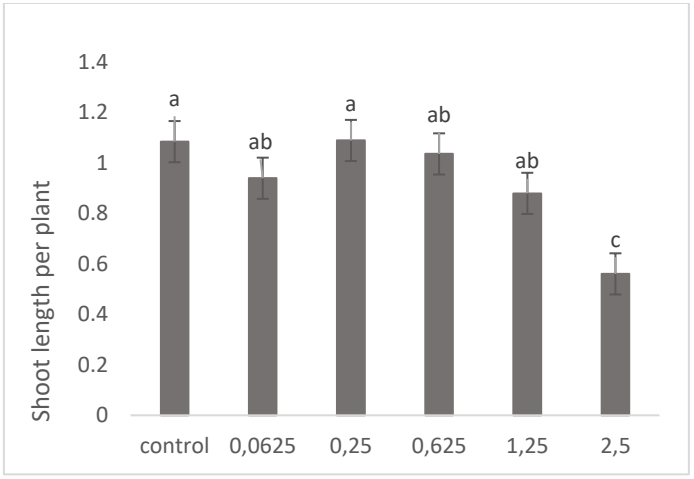


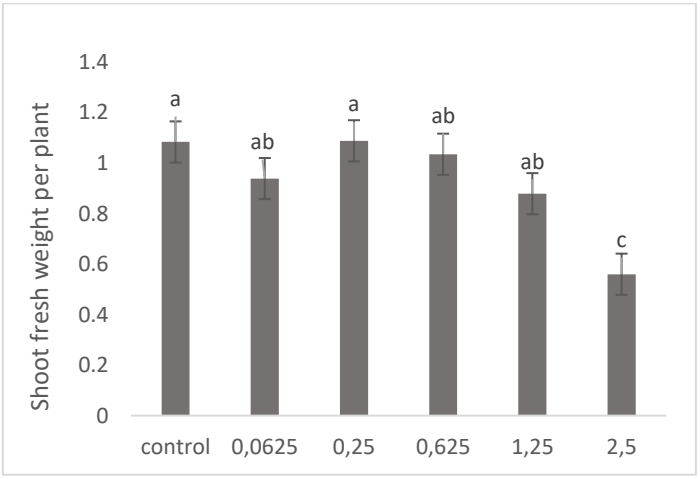


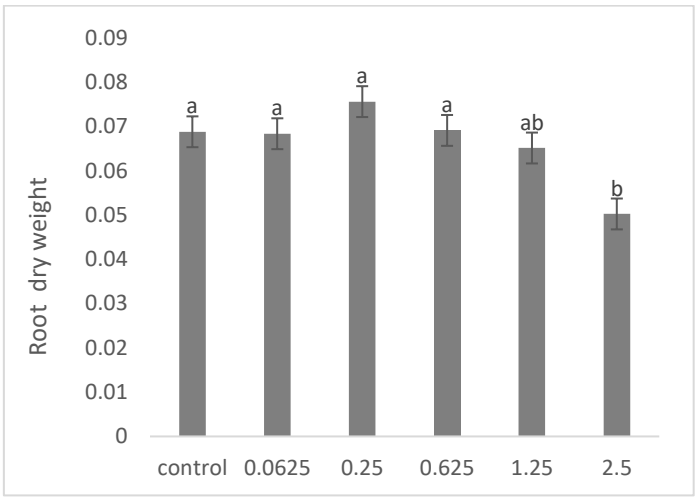
Correlation between numbers of nodules, galls and egg masses, shoot and root fresh and dry weights and nematode reproduction factor obtained per root system from co-inoculation with root-knot nematode *Meloidogyne javanica* and the typified rhizobial strains in pot experiments. The circles represent Pearson correlation r coefficients. The intensity of the color and circle sizes indicates the strength of the correlation (darker colors for stronger correlations; bigger circles for stronger connections). Boxed circles represent significant correlations ($p < 0.05$).

em: egg masses; sh: shoot length; sfw: shoot fresh weight; sdw: shoot dry weight; rfw: root fresh weight; Rf: nematode reproduction factor (final/initial population).

Annex VI







Nitrogen (KNO_3) addition effects on *Medicago truncatula*. A - shoot length; B - shoot fresh weight and B- root dry weight. Values are means ± standard deviation of 5 replicates per treatment. Columns followed by different letters that represent different levels of significancy.