Chapter 5
The Potential of Nitrogen-Fixing Bacteria in the Sustainability of Agro-Forestry Ecosystems

Isabel Videira e Castro, Márcia de Castro Silva, Concepción Fernandez, Belén Colavolpe, and Helena Machado

Abstract Microorganisms are critical for the maintenance of soil functions in natural and managed agro-forestry ecosystems. This explains the importance of evaluating a particular group of microorganisms, such as legume root nodule bacteria. Legumes and their root nodule bacteria are considered powerful management tools for improving pasture yield in the *montado* ecosystem, which is an agro-forestry system associated with the exploitation of cork and holm oaks. In Portugal, the widespread mortality registered in cork and holm oaks in recent decades has been attributed to infections by *Phytophthora cinnamomi*. In addition to nitrogen fixation, evaluated by the size and symbiotic effectiveness of the rhizobial population, other important functions were also investigated, such as mineral phosphate solubilization and cellulase activity, as well as the antagonistic activity against *P. cinnamomi*. This work showed the important role that root nodule bacteria can play in the sustainability and recovery of these ecosystems by promoting biological nitrogen fixation, especially in low fertility soils, through the establishment of pastures with legumes using pre-selected and characterized rhizobia as biofertilizers. The *Phytophthora* antagonistic activity from some of these bacteria, as well as their ability to degrade cellulose, an important component of *Phytophthora* cell walls, indicate that they may be used as potential biocontrol agents against this disease.

Keywords Cork and holm oaks · Rhizobia · Cellulose degradation · *Phytophthora cinnamomi* · Pastures · Biological nitrogen fixation (BNF)

I. Videira e Castro (✉) · M. de Castro Silva · C. Fernandez · H. Machado
Soil Microbiology Laboratory, National Institute of Agricultural and Veterinary Research (INIAV, I.P.), Oeiras, Portugal
e-mail: isabel.castro@iniav.pt

B. Colavolpe
Soil Microbiology Laboratory, National Institute of Agricultural and Veterinary Research (INIAV, I.P.), Oeiras, Portugal

Instituto Tecnológico de Chascomús (INTECH) (UNSAM-CONICET), Chascomús, Argentina

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5.1 Introduction

Microorganisms play a key role in soil by performing processes that maintain its structure and fertility. In agro-forestry ecosystems a determinant factor for sustainability are natural or introduced biodiverse pastures with legumes. Their associated microbial diversity (mainly nitrogen-fixing bacteria), plays an important role towards increasing and improving soil quality (Crespo 2006). Biological nitrogen fixation (BNF) is a major process providing nitrogen to soil, hence its importance in ecosystems such as the *montado* (or "*dehesa*" in Spain) (Olea and San Miguel-Ayanz 2006; Ferreira et al. 2010; Soares et al. 2014; Castro et al. 2016). In fact, pasture legumes are among the most efficient leguminous plants in terms of nitrogen fixation and, depending on adequate management and on the establishment of effective symbioses with rhizobia, they may contribute to high input rates of fixed nitrogen into the soil (Materon 1988). Therefore, increasing the contribution of BNF represents a significant challenge for more sustainable agro-forestry ecosystems. Different species of rhizobia form nodules with a range of hosts, determined by their nodulation genes. Nodulation and N_2 fixation in these symbioses require host and microorganisms compatibility. Also, soil environment must be appropriate for the exchange of signals that precede infection. Soil abiotic factors, such as temperature, water content, desiccation (drought), salinity and pH (Graham and Vance 2000; Sadowsky 2005; Arrese-Igor et al. 2011) are also critical in the ecology of rhizobia. Symbioses success results in fact from the interaction among legumes, rhizobia, and environmental factors (Fierer and Jackson 2006). Thus, as environmental factors influence all aspects of nodulation and symbiotic N_2 fixation, in some extreme cases they can reduce rhizobial survival and diversity in soil. In other cases, they may affect nodulation, nitrogen fixation and the growth of host plants.

Legumes and root nodule bacteria are amenable to management to improve pasture yield in the *montado* ecosystem. This is an agro-silvo-pastoral ecosystem dominated by cork and holm oaks, that combines the exploitation of cork with livestock husbandry, pastures and other uses. *Quercus suber* L. and *Q. ilex* ssp. *rotundifolia* (syn. *Q. rotundifolia*) are typically endemic to southern European and North African regions, where the climate is characterized by hot and dry summers, and rainy winters with mild temperatures. *Montado* is one of the richest biodiversity ecosystems in Europe and one of the most valuable in Portugal, due to the exploitation (and export) of cork. Portugal is the world's largest producer of cork, accounting for 53% of its production, and cork is one of the Portuguese products with highest impact in the international markets and therefore in the Portuguese economy. Unfortunately, in the last decades there has been a tree decline of Mediterranean *Quercus* species resulting from simultaneous interactions among environmental factors, such as poor soils and climate changes, the action of pests and pathogens, and the negative effects of some anthropic actions. In some cases the pathogenic oomycete *Phytophthora cinnamomi* can be considered as the main factor in the widespread mortality of *Quercus* spp. observed (Brasier et al. 1993; Brasier 1996; Camilo-Alves et al. 2013).

In addition to nitrogen fixation, bacteria root nodules may also directly promote the growth of plants through the solubilization of minerals such as phosphorus or, indirectly, as biological control agents inhibiting the growth of pathogens. Most soil phosphorus (present or applied as fertilizer) is immobilized as insoluble or very poorly soluble forms, thus being unavailable to plants. Phosphate solubilizing bacteria can transform the insoluble mineral phosphates into soluble forms, mainly through the production of organic acids and acidification of the surrounding micro-environment, thus increasing its availability to plants (Dobbelaere et al. 2003). The ability of rhizobia to solubilize inorganic phosphate and to promote plants growth has been extensively reported (Antoun et al. 1998; Alikhani et al. 2006; Sridevi and Mallaiah 2009; Vargas et al. 2009).

The enzymatic activities related to cellulolytic enzymes may also have an important role in controlling the spread and growth of phytopathogenic agents. Downer et al. (2001) showed that the addition of 10–25 U ml^{-1} of cellulase to *Phytophthora* sp. cultures in soil extract impaired zoospore and chlamydospore development. Concentrations higher than 25 U ml^{-1} disrupted the mycelium. Hydrolytic enzymes, including cellulases, were suggested to play an important role as a mechanism of biological control (Schrempf 1995; Tarabily et al. 1996; Richter et al. 2011) since the cell walls of *Phytophthora* spp. consist largely of cellulose (Bartnicki-Garcia and Wang 1983).

This study aimed at characterizing and selecting root nodule bacteria isolated from subterranean clover plants. The latter were grown in soils proceeding from cork and holm oaks from two *montado* areas, in different phytosanitary and vegetative conditions. Main objective was to evaluate the potential of the root nodule bacteria as plant growth promoters and their contribution to the sustainability of these ecosystems. *Trifolium subterraneum* L. was used as host plant because it is an annual legume, commonly used at the onset of upland pastures in the *montado* ecosystems. *Trifolium* is one of the largest genera within the *Fabacea* family and is very specific in its association with rhizobia. It interacts efficiently with *Rhizobium leguminosarum* bv. *trifolii* strains, with a variable symbiotic effectiveness (Ferreira and Marques 1992; Slattery and Coventry 1995; Drew et al. 2011; Carranca et al. 2015). Besides the evaluation of the size and nitrogen fixation capacity of the rhizobial populations, other important functions were also searched, such as the solubilization of mineral phosphate, cellulase activity and antagonism against *P. cinnamomi*.

5.2 Materials and Methods

Soil samples were collected in a cork oak *montado* in Grândola (G) and in an holm oak *montado* in Barrancos (B), both located in the south of Portugal. The soil samples were collected from different plots with dominant trees showing distinct crown defoliation levels. Four classes of crown defoliation were recorded, according to Cadahia et al. (1991): C0 – no defoliation, C1 – slight defoliation (≤ 25%), C2 – moderate defoliation (26–60%) and C3 – severe defoliation (> 60%). Additional symptoms related to the loss of tree vigor, such as wounds and exudations, were noted. From each site, composite soil samples were collected aseptically at 0–20 cm depth. Bulked samples were stored in sealed plastic bags under cooled conditions (6 °C) until microbial analysis and processing.

5.2.1 Size and Nitrogen Fixing Capacity of Rhizobial Populations

For each soil sample, the rhizobial natural population was estimated by the most probable number (MPN) plant infection method using subterranean clover cv. Clare as trap host and a ten-fold dilution series (Somasegaran and Hoben 1994). Clover seeds were rinsed with ethanol, surface sterilized using a freshly prepared 5% solution of calcium hypochlorite for 10 min, washed extensively with sterile distilled water, left in sterile water to hydrate for 2 h and placed in water-agar (0.8%) plates, at 28 °C, in the dark, for 1–2 days. The pre-germinated seeds were transferred to flasks (one seed per flask) containing 50 ml agar slants of Jensen's medium (Jensen 1941). Four replicates were inoculated with 1 ml suspension of each soil dilution. Non-inoculated (T0) and nitrogen (TN) controls were also included, by adding 1 ml liquid Jensen medium (¼ diluted) and 1 ml 1.75% KNO_3, respectively. Plants were grown during 6–8 weeks in a controlled-environment chamber with 16 h light/8 h dark cycle at 23 °C (day)/18 °C (night). After this period, plants were harvested and examined for nodulation. The results were expressed as $\log_{10}$ of rhizobia number per g of dried soil (Somasegraran and Hoben, 1994).

Plants shoots of the first two soil dilutions of each soil sample and also from non-inoculated (T0) and nitrogen (TN) controls, were dried in an oven at 80 °C for 2 days. Shoots dry weight was used to calculate the index of effectiveness (Es) of the rhizobial population according to Ferreira and Marques (1992):

$$\mathrm{Es}(\%) = \left(\mathrm{X_s} - \mathrm{X_{T0}}\right) / \left(\mathrm{X_{TN}} - \mathrm{X_{T0}}\right) \bullet 100$$

where X_s represents the mean dry weight of soil inoculated plant shoots, X_{TN} the mean dry weight of plants from nitrogen control and X_{T0} the mean dry weight of non-inoculated plants.

5.2.2 Isolation of Bacterial Strains

A total of 200 root nodule bacteria (RNB) were isolated from *T. subterraneum* plants nodules inoculated with the soil dilutions of the previous assay, following the methods described in Vincent (1970). Nodules were surface sterilized using a 5% solution of calcium hypochlorite and repeatedly washed with sterilized water. Each nodule was individually crushed and a containing droplet was used for cultivation in yeast-manitol agar media (YMA) with Congo red dye. Isolates were incubated at 27 °C in the dark, until complete growth. Colony purity was checked based on the morphology and Congo red absorption. Each pure isolate was stored at 4 °C.

5.2.3 Other *In Vitro* Activities Related to Plant Growth Promotion

In vitro tests for phosphate-solubilization and cellulase activity were conducted qualitatively. The ability to solubilize mineral phosphate was assayed on yeast extract dextrose (YED) agar medium supplemented with 5 g l^{-1} of $Ca_3(PO_4)_2$ (Peix et al. 2001). Rhizobia strains were inoculated and incubated for 4 days at 27–30 °C. After this period, the colonies showing a surrounding halo were considered as solubilizers.

Cellulase activity was evaluated on TY media supplemented with 0.2% w/v carboxymethylcellulose (CMC), as described by Verma et al. (2001). After inoculation and incubation during 3 days at 27 °C, plates were covered with Congo red solution (1 mg ml^{-1}) for 30 min to facilitate the distinction of an orange halo around the bacterial colonies, associated with the production of the lytic enzyme.

5.2.4 Antagonist Activity Against *Phytophthora cinnamomi*

Phytophthora cinnamomi was isolated from cork oak in Grândola and holm oak in Barrancos, Portugal. Tests for the antagonistic activity against *P. cinnamomi* were performed in Potato Dextrose Agar (PDA) and in YMA media. *Phytophthora cinnamomi* was inoculated in the center of Petri dishes. After 2 days of incubation at 27 °C in the dark, 3 μl of each bacterial culture (grown for 48 h in TY liquid medium) were placed in the periphery of the plate and incubated for 10 days at 27 °C in the dark.

5.3 Results and Discussion

From both cork and holm oak *montado* areas (G and B, respectively) the size of the rhizobial population, estimated by MPN, appeared highly variable among samples (Fig. 5.1). In areas in which trees showed no or slight crown defoliation (C0 and C1) the bacterial densities were close to or above 10^4 cells g^{-1} of soil. These numbers could be considered enough for an efficient nodulation and indicative of a biological nitrogen fixation and an established symbioses contributing to soil fertility. These results agree with those reported by Ferreira et al. (2010) in which the size of the rhizobial population in similar ecosystems was around $5 \cdot 10^4$ bacteria g^{-1} soil.

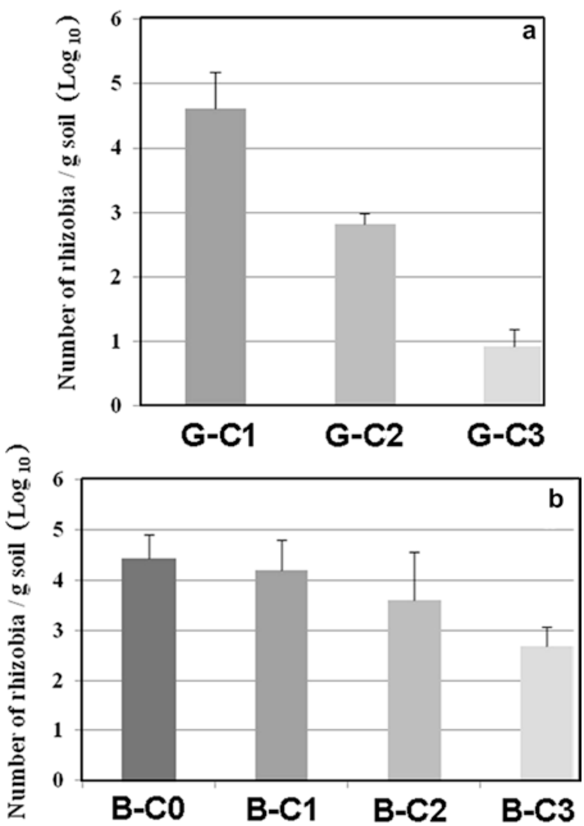


Fig. 5.1 Rhizobial population size ($\log_{10}$ of rhizobia bacteria number per g of dried soil) in soils from *montado* ecosystems, using *Trifolium subterraneum* as host plant. Crown defoliation classes are: C0 – no defoliation; C1 – ≤ 25%; C2 – 26–60%; C3 – >60% of crown defoliation. Soils from Grândola (**a**) and Barrancos (**b**)

In soils with trees showing a severe crown defoliation (C3), the rhizobial population had the lowest values with only 10 bacteria g^{-1} of soil (G-C3, Fig. 5.1a). Intermediate values around 10^3 bacteria g^{-1} of soil, were obtained in soils with trees with a moderate crown defoliation (C2).

Data obtained from dry weight evaluation of sub-clover plants inoculated with dilutions of soil samples collected in the *montado* areas showed that nitrogen fixing capacity was variable (Fig. 5.2). In general, values similar to nitrogen controls were observed only in few soil samples, corresponding to soils with holm oak trees in the C0 (data not shown) and C1 defoliation classes (G-C1, Fig. 5.2a and B-C1, Fig. 5.2b). In soils with trees with severe crown defoliation (C3), the dry weight values were the lowest and lower than controls with nitrogen. In some soil samples (G-C3, Fig. 5.2a), the dry weights were even lower than the control (T0), indicative of a failure in nitrogen fixing population for annual clovers.

The effectiveness index data (Fig. 5.2c) confirmed the results mentioned above for dry weight. The effectiveness was higher in soils from trees with slight crown defoliation, reaching 89% in soils from holm oak (B-C1), followed by a decrease in soils from trees showing a moderate defoliation. Lowest values were found in soils proceeding from trees with highest crown defoliation class. Even for soils of cork

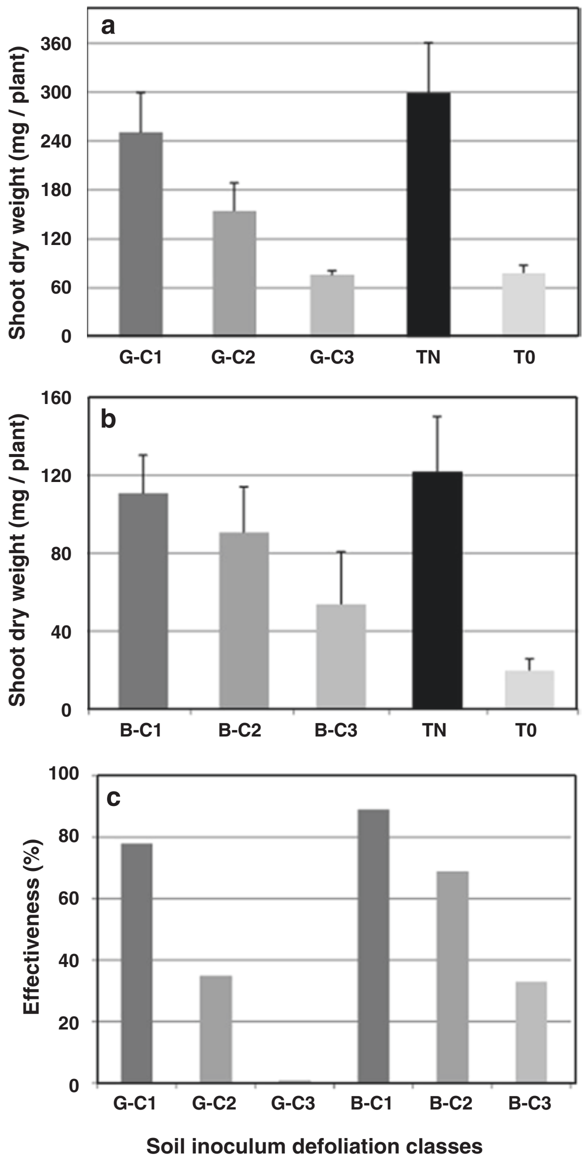


Fig. 5.2 Shoot dry weight values (**a** and **b**) and corresponding effectiveness index (**c**) of *Trifolium subterraneum* plants inoculated with different soil samples from trees with different crown defoliation classes: C1 – ≤ 25%; C2 – 26–60%; C3 – >60% of total crown defoliation. Also included TN (plants only with nitrogen) and T0 (non-inoculated plants and without mineral N) controls. Soils from Grândola (**a**) and Barrancos (**b**). Effectiveness index (%) of rhizobial population in both soils

oak, the effectiveness of rhizobial natural population was about 1%, indicative of a completely ineffective population (G-C3).

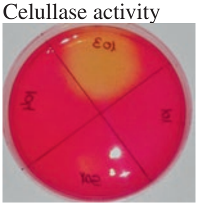
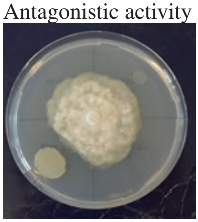
The results obtained for both rhizobial counts and nitrogen fixing capacity confirmed a relationship between trees health and rhizobial populations development. The lowest abundance of rhizobia was in fact generally found in soils with trees showing a severe defoliation. These had also a non-effective (cork oak, *montado* G) or less effective (holm oak, *montado* B) rhizobial populations. On the contrary, in soils with the healthiest trees, the number of rhizobia was the highest and these populations were the most effective in nitrogen fixation. These results indicate that these parameters could be good indicators of soil quality in these ecosystems, providing valuable informations about the establishment or maintenance of pasture. As soil microorganisms are critical for the maintenance of soil functions in both natural and managed agricultural ecosystems, it appears of paramount importance to highlight the effect of a particular group of microorganisms, such as the legume root nodule bacteria.

In a second assay, one hundred RNB isolated from each soil were analyzed for *in vitro* activities related to plant growth promoting and antagonism against *P. cinnamomi* (Table 5.1). Results showed that cellulase activity was present in more than half (62%) of the isolates from Grândola (G) with 12% being capable of solubilizing mineral phosphate. On the other hand, 17% of the isolates from Barrancos (B) showed cellulase activity and only 6% were capable of solubilizing mineral phosphate. Moreover, results obtained in the *in vitro* antagonism tests against *P. cinnamomi* showed that 14 and 4% of the isolates from these sites, respectively, could inhibit the growth of the phytopathogen.

Cellulase has a direct impact on *Phytophthora* spp. cell walls, due to the fact that their cell wall is essentially constituted by cellulose (Bartnicki-Garcia and Wang 1983). Consequently, isolates with this activity may contribute to the degradation of the *Phytophthora* propagules, originating by this way a suppressive environment limiting the development of this phytopathogenic agent. The ***in vitro*** results showed that almost all RNB with cellulase activity were isolated from clover plants grown in soils with low crown defoliation (Table 5.2).

Phosphate-solubilizing activity was also found in some isolates from both areas. In the *montado* ecosystem desertification is a common situation due to inconsistent rainfalls, resulting in the progressive degradation of the vegetation cover and the erosion of surface soil. As a consequence, soils are generally poor, deficient in phosphorus and calcium, and contain low levels of organic matter, making arable and intensive farming not sustainable (Tóth et al. 2014). As a possible remedy to this condition, the application of some isolates could solubilize immobilized phosphorus,

Table 5.1 Number (%) of root nodule bacteria (RNB) showing cellulase activity, phosphate solubilization, and antagonistic activity

Isolate group[a]	Celullase activity 	Phosphate solubilization 	Antagonistic activity 
Grândola	62	12	14
Barrancos	17	6	4

[a]One hundred RNB from two sampled *montado* soil

Table 5.2 Frequency of root nodule bacteria (Grândola and Barrancos isolates) with *in vitro* cellulase activity, originating from soils of trees showing different crown defoliation classes

Crown defoliation classes	Cellulase activity (isolates %)
C0 + C1	95.7
C2	69.0
C3	34.6

providing higher amounts of this important macronutrient to plants, contributing by his way to sustain soil fertility in the *montado* ecosystem.

Phytophothora cinnamomi appears to act as a predisposing stress factor in combination with other features, such as soil compaction, shallow soil, drought or water excess events, and other diseases (Camilo-Alves et al. 2013). Effective and long-term rehabilitation of areas infected by *P. cinnamomi* is difficult. Moreover, the use of chemicals to control the pathogen is impractical and environmentally unfriendly. The presence of root nodule bacteria with antagonistic activity against this pathogen may contribute to develop adequate conditions that may help trees to overcome the disease progression. Therefore, an alternative and promising method is the integration of management systems with biological control, through the manipulation of the environment and the introduction of specific microorganisms. The results obtained in this study allowed us to select strains that were highly effective in nitrogen fixation and also capable of solubilizing phosphorus. The production of cellulase also indicates a potential as antagonists against *Phytophothora*. These isolates could be hence considered as biofertilizers/biocontrol agents to be used in the *montado* areas, by inoculating legume seeds before planting.

In summary, and considering the results obtained, the contribution of root nodule bacteria/rhizobia to the sustainability of the *montado* ecosystem, as biofertilizers or/and biocontrol agents of *Phytophothora* is drawn in Fig. 5.3. The conjugation of nitrogen fixation with other important activities, such as phosphate-solubilization and siderophore production (not evaluated in this work) should be considered, as well the production of the lytic enzyme cellulase and the antagonistic activity, which seem also essential for this process.

5.4 Conclusion

Rhizobial population size and nitrogen fixation capacity could be used as a possible bioindicator of the soil quality/fertility in the *montado* ecosystems due to their relationship with the vegetative status of cork and holm oaks, evaluated by crown defoliation. The protection of more biodiverse pastures may help to overcome diseases and, together with biological nitrogen fixation, should contribute to the recovery of soil fertility in the *montado* ecosystem.

Tests in controlled and field conditions should be performed using several plant species naturally occurring in *montado* undercover, inoculated with selected strains

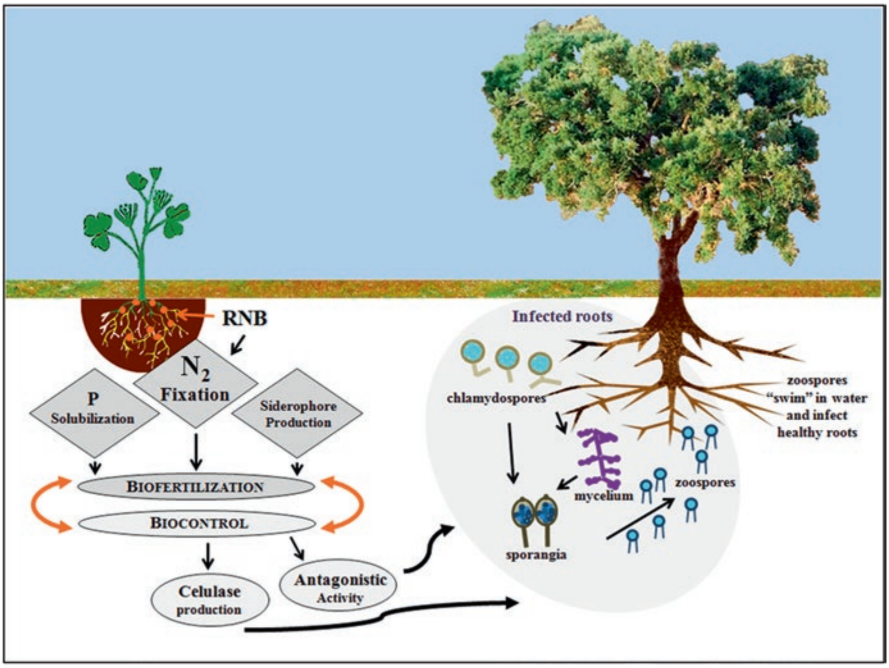


Fig. 5.3 Contribution of root nodule bacteria (RNB) to the sustainability of the *montado* ecosystem, and their interactions with *Phytophothora cinnamomi* life-cycle

with antagonistic activity against *Phythophora* to identify the best, highly-performant "bacteria-plant" associations.

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