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# Symbiosis between *Chamaecrista fasciculata* and nitrogen-fixing bacteria: a review

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by

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## ABSTRACT

Nitrogen-fixing bacteria reside in nodules on roots of legumes. Within these nodules, they convert nitrogen gas to ammonia, a bioavailable form of nitrogen that the plant can use for nutrition. Although the vast majority of legumes form nodules, members of the Caesalpinioideae subfamily generally do not nodulate and are instead characterized by bacteria that remain in the infection thread during nitrogen fixation. As one of the few genera within this subfamily capable of nodulation, *Chamaecrista* presents a rare opportunity to study the symbiosis between primitive legumes and nitrogen fixing bacteria. Observing nitrogen fixation in *Chamaecrista* allows for a greater understanding of less advanced mechanisms of nitrogen fixation which can potentially be conferred to plants that are not legumes. Previous literature regarding legume rhizobium symbiosis, however, is primarily focused on Papilionidae legumes. Therefore, this review aims to synthesize literature on nodulation in primitive legumes to further characterize the process of nitrogen fixation in *Chamaecrista*.

## Introduction

As a major component of compounds involved in cell structure and metabolism, nitrogen is one of the most essential nutrients for plant growth. Deemed a macronutrient, nitrogen is found at concentrations of parts per million or billion within plant tissues where it is assimilated into many important biomolecules such as proteins, nucleic acids, hormones, coenzymes, and chlorophyll (Novoa and Loomis, 1981). Nitrogen deficiencies, therefore, significantly impact plant development and often result in the reduction of foliage, root length, and shoot length (Zhao et al. 2005). Despite its abundance in the air, atmospheric nitrogen is not bioavailable due to its stable triple bond and must be converted to other forms such as ammonium, nitrites, and nitrates in order for plants to access the nitrogen. This process of nitrogen fixation is extremely energy intensive and can be achieved through lightning, nitrogen fixing bacteria, and the Haber Bosch method. However, nitrogen bioavailability is still reduced by processes that remove nitrogen from the soil such as denitrification, ammonia volatilisation, and leaching (Cameron et al. 2013).

Consequently, crop yield and plant growth in general is often limited by nitrogen. To produce one ton of grain, crops such as wheat, corn, and rice need around 20-40 kg of soil N ha<sup>-1</sup> over a period of 3-5 months (Peoples and Craswell, 1992). To satisfy the nitrogen requirement of these crops, farmers usually employ the use of nitrogen fertilizers or microorganisms to improve soil quality. However, synthetic nitrogen fertilizers, while effective, can increase the risk of ammonia volatilisation and further harm the environment through runoff (Cameron et al. 2013). In contrast, soil bacteria known as *Rhizobium* fix nitrogen just as efficiently with far less

environmental damage. In fact, the majority of biologically fixed nitrogen on Earth is produced by these nitrogen fixing bacteria (Halbleib and Ludden, 2002).

Nitrogen fixing bacteria (also known as diazotrophic bacteria) are therefore extremely useful to plants, especially in nitrogen poor soils. These bacteria employ the use of the nitrogenase enzyme complex which is comprised of two protein components: dinitrogenase and dinitrogenase reductase. Both proteins are the product of nitrogen fixation (*nif*) genes, with dinitrogenase coming from the *nifD* and *nifK* genes and dinitrogen reductase coming from the *nifH* gene (Huergo et al. 2005). Together, these proteins help convert nitrogen gas to ammonia, a bioavailable form of nitrogen that plants can use for nutrition. This process however is extremely energy intensive, coming at the cost of 16 ATP (Igarashi and Seefeldt, 2003). Engaging in a symbiotic relationship with plants greatly helps with this cost.

The ability of diazotrophic bacteria to fix nitrogen is highly reliant on this symbiotic relationship as free living *Rhizobium* are not able to fix nitrogen. These bacteria reside within structures called nodules that grow on the roots of legumes. Here they fix nitrogen in exchange for housing and food provided by the plant. The photosynthates/carbohydrates provided are used to generate the ATP that is then used to reduce nitrogen. The nodule environment is also critical for the process of nitrogen fixation. Dinitrogen reductase does not function well in the presence of oxygen. However, legumes produce leghemoglobin which functions in a similar way to hemoglobin by binding oxygen within the plant and consequently, maintaining an anaerobic environment so that dinitrogen reductase is stable enough to work. The oxygen is then carried to the bacteria so that oxidative phosphorylation can still occur and drive nitrogen fixation (Igarashi

and Seefeldt, 2003). In this way, the carefully maintained environment within the nodule increases the efficiency of nitrogen fixation.

The process of nodule formation starts with the accumulation and release of flavonoids by legume roots. This attracts nearby *Rhizobium* which then express nodulation genes and subsequently, create Nod Factor (Franssen et al. 1992). Upon recognizing this Nod factor, the plant begins the process of nodule formation. Infection begins when the bacteria attach to the root hair where Nod factor causes the root hair to curl in a way where the Rhizobia become trapped (root hair curling is called a shepherd's crook). The process of attachment is aided by exopolysaccharides which enhance bacterial adhesion and survival (Backer et al. 2018). The root hair invaginates and along the epidermal and cortical cells, an infection thread is formed in which the bacteria multiply. The infection thread then grows from the root hair to the developing nodule and once it reaches the nodule, the bacteria are released into the host cell cytoplasm within a symbiosome, where they continue to develop into the rhizobium symbiotic form known as the bacteroid.

Nodule formation is a characteristic of many legumes, giving these plants an evolutionary advantage in terms of nitrogen stress. However, not all legumes can nodulate. The Leguminosae family consists of three sub-families: Caesalpinioideae, Mimosoideae and Papilionoideae. Legumes within the Caesalpinioideae subfamily are considered to be evolutionarily primitive and many of them are not known to nodulate. However, 23% of the Caesalpinioideae legumes are known to nodulate (Pueppke and Broughton 1999). Caesalpinioideae legumes that are able to do so generally fall within the Caesalpiniae tribe, although the *Chamaecrista* genus (which lies in the Cassiae tribe) is also known to form nodules. Thus, it is suggested that the nodulation

independently evolved in *Chamaecrista* (Doyle 1998). These nodulating, Caesalpinioideae legumes are distinguished by “primitive” nodules in which infection occurs through intercellular spread in a “fixation thread” rather than by an infection thread (Naisbitt et al. 1992)). It is proposed that the infection thread evolved in infected cells and then expanded to earlier stages of infection such as root hair entry (de Faria et al. 1987). Additionally, the rhizobium symbiotic form of bacteria remain within these infection thread like structures during nitrogen fixation (Naisbitt et al. 1992). This differs from the nodulation process in more advanced legumes where the *Rhizobium* are released into the host cell cytoplasm in symbiosomes where they then develop into the bacteroid form.

It is suggested that nitrogen fixing bacteria co-evolved alongside legumes to establish the symbiotic relationship that exists in most legumes today (Martinez-Romero, 2009). Several hypotheses involve the replacement of *Bradyrhizobium* (which is thought of by many as the ancestral symbiont) by *Rhizobium* and the replacement of  $\alpha$  bacteria with  $\beta$  bacteria in South American legumes (Martinez-Romero, 2009). A deeper investigation into the symbiotic relationship between nitrogen fixing bacteria and *Chamaecrista* may reveal how the process evolved in more advanced symbioses and allow for a better understanding of less advanced mechanisms of nitrogen fixation that may potentially be conferred to non legume plants. However, most literature primarily analyzes the symbiosis in Papilionoideae legumes which includes more advanced genera such as peas and beans. Therefore, this review aims to synthesize previous research on nodulation in primitive legumes to further characterize the process of nitrogen fixation in *Chamaecrista*.

### **Nodulation of *Chamaecrista* Species**

Nodules in herbaceous *Chamaecrista* are more similar than other to nodules of other Caesalpinioideae legumes in that the bacteroids are located within actual symbiosomes as opposed to a fixation thread (dos Santos et al., 2017). Because *Chamaecrista* displays an absence of polyploidy, it is suggested that polyploidy is a necessary requirement for nodulation and that polyploidy may have occurred after the evolution of nodulation. Additionally, research involving nodulation in *P. andersonii*, a non legume, suggest that a Myc factor receptor served as a Nod factor receptor and that the Myc and Nod factor are structurally similar. Because *Chamaecrista* predates the polyploidy seen in other legumes, it was concluded then that one receptor (CfNFP) controls mycorrhization and *Rhizobium* symbiosis (den Camp et al., 2011). Furthermore, studies have found that non symbiotic hemoglobin evolved into leghemoglobin in the Caesalpinioideae family. Therefore, the hemoglobin found in *C. fasciculata* fall somewhere between non symbiotic hemoglobins and leghemoglobins, making it somewhat of an ancestral leghemoglobin (Gopalasubramaniam et al., 2008).

The vast majority of studies examining symbionts of native *Chamaecrista* species have suggested that they are preferentially nodulated by *Bradyrhizobium*. However, the gene sequences (both core and symbiotic) of *Chamaecrista* symbionts vary between regions, suggesting that divergent evolution occurred. *Bradyrhizobium* symbionts in Brazil were not genetically similar to bradyrhizobia in other caesalpinoid legumes, suggesting that these strains were more specific to *Chamaecrista* (dos Santos et al. 2017). In contrast, *Chamaecrista* symbionts in India displayed a similarity to rhizobia capable of nodulating other local legumes (Rathi et al. 2018). It is suggested however that environmental factors such as soil pH impact the rhizobium legume symbiotic relationship because many of the bacteria found in nodules of the



native legumes such as *Chamaecrista* were acid tolerant and included bacteria such as *Bradyrhizobium* and *Burkholderia* (dos Santos et al. 2017)

It was found in lab that *Rhizobium* sp. Strain NGR234 and *Rhizobium fredii* USDA257, both model bacteria that have an exceptionally broad host range, are also able to nodulate some legumes in the Caesalpinioideae subfamily such as *Delonix regia* and *Chamaecrista fasciculata* (Pueppke and Broughton 1999). Most of the nodules formed on the Caesalpinioideae legumes were not nitrogen fixing. The exception to this was *Chamaecrista fasciculata* inoculated with NGR234 which displayed effective nodules. Other species of *Chamaecrista* tested in this study were not able to form nodules when inoculated with either bacteria.

This ability of *Chamaecrista fasciculata* to be nodulated by *Rhizobium* species is explored in other studies. Previously, it has been shown that *C. fasciculata* formed nodules when inoculated with *Rhizobium tropici* Strain UMR 1899 (Pennington and Fujishige 2017). These nodules while pink, indicating that they were nitrogen fixing, were not completely effective as the plants still showed symptoms of nitrogen deficiency (Fig 1). *Chamaecrista fasciculata* inoculated with *Burkholderia tuberum* produced small white bumps known as pseudonodules and plants showed signs of severe nitrogen deficiency, both of which point to a lack of nitrogen fixation (Fig 2). Interestingly, when co-inoculated with both *R. tropici* and *B. tuberum*, the plants formed healthy pink nodules and were very green (Fig. 3). This data suggests that  $\alpha$  bacteria (*R. tropici*) and  $\beta$  bacteria (*B. tuberum*) cannot individually produce effective nitrogen fixing nodules, but are able to do so together. While it is unknown whether *R. tropici* or *B. tuberum* is responsible for nitrogen fixation during this process, *Chamaecrista* inoculated with an

*R. tropici* mutant in which the *nifH* gene was knocked out showed more severe symptoms of nitrogen deficiency than plants inoculated with the wild type.

### **Conclusion**

Therefore, future experiments would observe which bacteria species (*R. tropici* or *B. tuberum*) expressed the *nifH* gene by creating an *R. tropici* mutant in which this gene was knocked out and then observing if plants co-inoculated with the *R. tropici* mutant and *B. tuberum* still showed signs of nitrogen deficiency and/or pink nodules. This experiment would be repeated with a *B. tuberum* mutant to further characterize the *nifH* expression of these two bacteria in *Chamaecrista fasciculata*. By performing such experiments, it is possible to further understand the process of co-evolution that occurred in plants and rhizobacteria that allowed for a successful symbiosis.

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**Figure 1:** *Chamaecrista* inoculated with only *R. tropici* displayed small nodules and signs of nitrogen deficiency. (Penner and Fujishige, 2017)



**Figure 2:** *Chamaecrista* inoculated with only *B. tuberum* displayed pseudonodules as well as signs of severe nitrogen deficiency. (Penner and Fujishige, 2017)



**Figure 3:** *Chamaecrista* co-inoculated with *R. tropici* and *B. tuberum* showed large pink nodules and were very healthy.