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Inoculation of *Bradyrhizobium japonicum* (Kirchner, 1896) Jordan (1982) (*Bradyrhizobiaceae*) on *Inga edulis* Mart. (Leguminosae, Mimosoideae)

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ABSTRACT

Legumes that have the ability to fix atmospheric nitrogen through symbiosis with bacteria known as rhizobia, have great importance from an economic and ecological perspective, because in agricultural crops and reforestation, may waive the use of nitrogenous fertilizers and provide more resistant seedlings, minimizing impacts in nature and low cost. *Bradyrhizobium japonicum* (Kirchner, 1896) Jordan (1982) (Bradyrhizobiaceae) is a rhizobium which has association with several species of the subfamily Papilionoideae. This study aimed to analyze the initial growth of *Inga edulis* Mart (Leguminosae, Mimosoideae) inoculated with *B. japonicum* to test interaction in the subfamily Mimosoideae. We used the parameters: height, stem diameter and number of expanded leaves; data analysis consisted of the Shapiro-Wilk normality test. The inoculated seedlings showed a significant difference for height and number of leaves, but no significant difference for stem diameter. From the results, it is inferred that *I. edulis* presents positive interaction with *B. japonicum*, i.e., the interaction of rhizobia extends to Mimosoideae, which allows to infer that the interaction of rhizobia can be shown as homologous synapomorphy of the clade Mimosoideae-Papilonoideae, which is supported by molecular phylogenies. The results are compared with other studies and the implications in the evolution of the clades of the Leguminosae are discussed in phylogenetic studies. To corroborate the hypothesis, further studies of species of different subfamilies of these clades should be tested in order to verify that indeed this interaction is a homologous synapomorphy of the clade or an apomorphy for Mimosoideae, Leguminosae and interaction with *B. japonicum* has arisen more than once in the evolution scale of the family.

RESUMO (ABSTRACT IN PORTUGUESE)

Leguminosas tem a capacidade de fixar nitrogênio atmosférico através da simbiose com bactérias, conhecidas como rizóbios, tem grande importância sob ponto de vista econômico e ecológico, pois em cultivos agrícolas e reflorestamentos, podem dispensar o uso de fertilizantes nitrogenados e propiciar mudas mais resistentes, o que minimiza os impactos desses insumos na natureza e diminui os custos. *Bradyrhizobium japonicum* (Kirchner, 1896) Jordan (1982) (Bradyrhizobiaceae) é um rizóbio com associação com diversas leguminosas de Papilionoideae. O presente trabalho teve por objetivo analisar o crescimento inicial de indivíduos de *Inga edulis* (Leguminosae, Mimosoideae) submetidas à inoculação do rizóbio a fim de testar a interação deste em Mimosoideae, filogeticamente, grupo irmão de Papilionoideae. Foram utilizados os parâmetros altura, diâmetro do caule e número de folhas; a análise dos dados consistiu no teste de normalidade de Shapiro-Wilk. A partir dos resultados, é inferido que *I. edulis* apresenta interação positiva com *B. japonicum*. Os resultados são confrontados com outros trabalhos e as implicações na evolução dos clados de Leguminosae são discutidos com estudos filogenéticos.

Palavras-chave: Fabaceae, rizóbios, leguminosa, cultivo agrícola.

INTRODUCTION

Nitrogen-fixing bacteria, known as rhizobia (e.g. *Bradyrhizobium* e *Rhizobium*) are species that live in a symbiotic relationship with plants, mainly legumes. The legume-rhizobium symbiosis is the source of fixed nitrogen that supply this element to the plant and in return receive carbon from the host plant (Taiz and Zeiger, 2004; Souchie *et al.,* 2005). The rhizobia species can improve the development of seedlings in nurseries and tillage (Barnett and Baker, 1999; Moreira, 1994), increases disease tolerance (Green *et al*., 1999), maximizing the ability to establish these seedlings in the field, which assists in reforestation (Souchie *et al.,* 2005).

The leguminosae are flowering plants with significant importance on component of forests in terms of richness and density, especially in biomes such as the Amazon, Cerrado and Atlantic Rainforest (BFG, 2015). The family has interaction with various rhizobial species (Jesus *et al*., 2005; Perret *et al*., 2000), most often the subfamilies Papilionoideae and Mimosoideae, uncommon in Caesalpinoideae (Souza *et al.,* 1994; Moreira, 1994; Barberi *et al*., 1998). Molecular data support that the family is monophyletic, as well as the Mimosoideae and Papilionoideae subfamilies, but Caesalpinioideae is paraphyletic (Wojciechowski, 2003; Wojciechowski *et al*., 2004). In the present work, we use Caesalpinioideae term to refer this basal grade. For legumes there are records of interaction with several bacteria families: Xanthobacteraceae, Hyphomicrobiaceae, Phyllobacteriaceae, Methylobacteriaceae, Brucellaceae, Burkolderiaceae, Rhizobiaceae and Bradyrhizobiaceae (Moreira, 2008; Gyaneshwar *et al*., 2011).

Bradyrhizobium japonicum (Kirchner, 1896) Jordan (1982) (Bradyrhizobiaceae) is a important rhizobium species which has symbiotic interaction with many Papilionoideae (Leguminosae) species (Lírio *et al*., 2012) and is widely used to improve the production of *Glycine max* (L.) Merr, popularly known as “soy” in agricultural systems (Ruiz-Díez *et al*., 2012). However, no tests have been carried out with species of other legume subfamilies.

A typical legume species of South America is *Inga edulis* Mart. (subfamily Mimosoideae), found along the Atlantic Rainforest, Caatinga and Amazon Rainforest (Pennington, 1997; BFG, 2015) very much utilized by communities as food source, timber and medicine, especially by Andean countries (Pennington, 1997). Furthermore, is used in the Amazon in agroforestry systems to improve soil fertility and to shade in perennial crops as coffee and cacao (Lojka *et al*., 2010).

Once *B. japonicum* is capable of interacting with Papilionoideae species, and nodulation is considered by some authors an important taxonomic character (Faria and Sprent, 1994) the aim of this article was to evaluate whether the interaction of *B. japonicum* extends to Mimosoideae, the Papilionoideae sister group. In this context, we evaluated the initial development of *I. edulis* inoculated with *B. japonicum*.

MATERIAL AND METHODS

Study Area

The work was performed in the experimental field of the São Francisco de Assis School, in the municipality of Santa Teresa, Espírito Santo state, Brazil (19°56’10" S; 40°36’06" W). The elevation of the region is 650 m (Mendes and Padovan, 2000). The *I. edulis* seeds were collected in a private property localized in the municipality of Afonso Cláudio, Espírito Santo state, Brazil (20° 04' 26" S; 41°07' 32 W).

Experimental Design

A total of 200 seeds was used, 50% were inoculated and the rest used as control that were placed in tillage on soil, and after sowing, kept in full sun. We obtained *B. japonicum* (strain 5080) on peat substrate (Nitrosuper F45). To inoculation we diluted the bacteria in distilled water as carried out in Lírio *et al*. (2012). The dilution consisted of 1 mL of solution bacteria and 3 mL of distilled water for 5 g of seeds and they were protected from sunlight at the time of sowing. Height, stem diameter and number of expanded leaves parameters were measured two months after seeding.

Statistical Analysis

Statistical analysis was performed using the PAST software (Hammer *et al*., 2001). We used the Shapiro-Wilk (Shapiro and Wilk, 1965) test for normality. The *t*-test was used for the comparison of data obtained for the inoculated and control samples, with a significance level of 0.05%.

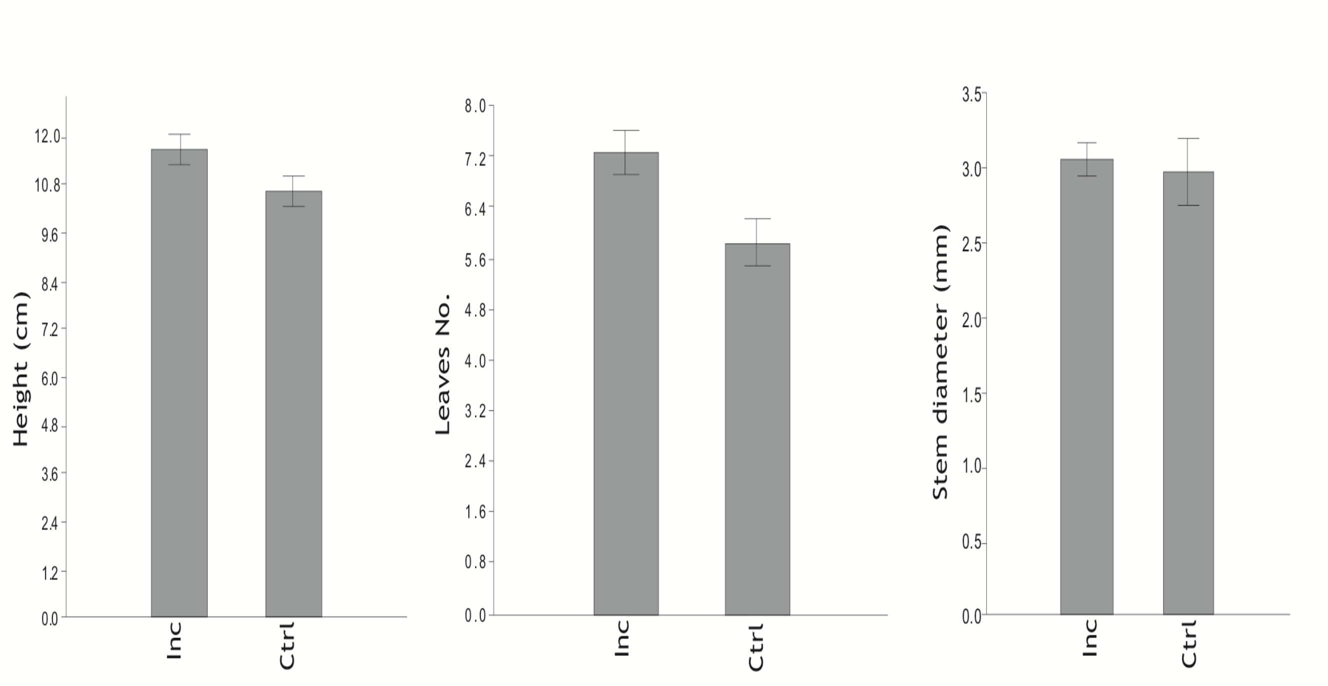
RESULTS AND DISCUSSION

The data are normally distributed in the comparison of all parameters, which justified the use of the t-Student test (tab. 1). The treatment of *I. edulis* presents significant difference in the parameters height and number of sheets and did not show significant difference to stem diameter (fig. 1). The results suggest that the interaction of *B. japonicum* occur in *I. edulis*, included in the subfamily Mimosoideae.

Table 1: Results of the normality test Shapiro-Wilk, N=20 (“hgt” = height, “lvn” = leaves number, “stmd” = stem diameter, “i = inoculated and “c” = control)

	hgt_i	lvn_i	stmd_i	hgt_c	lvn_c	stmd_c
Shapiro-Wilk W	0.9788	0.9073	0.9694	0.9544	0.9309	0.9671
p (normal)	0.9175	0.05667	0.7425	0.4385	0.1604	0.693

Figure 1: Barchat showing the mean and standard



error for height (n=20; t=2.338; p=0.03046), leaves number (n=20; t= 2.833; p= 0.01063) and stem diameter (n=20; t=0.3451; p= 0.7338), respectively. “Inc” = inoculated sample and “Ctrl” = control sample

Leblanc *et al*. (2005) isolated four *Bradyrhizobium* strains of *I. edulis*, and through BLAST analysis came to the conclusion that these strains are closely related (with molecular data) to *B. japonicum* (96% similar), however they consider that there was insufficient information of *B. japonicum* affiliation for strong conclusions.

The phylogenetic relationship of Leguminosae subfamilies is supported by molecular studies, which Papilionoideae and Mimosoideae are sister groups (Wojciechowski, 2003; Wojciechowski *et al*., 2004). Analyzing the interaction of legumes with rhizobia, other studies corroborate this pattern , where it is proposed that there are a gradual trend of interaction: Papilionoideae with the highest number of species with interaction, followed by Mimosoideae and Caesalpinioideae, with the lowest number of species (Souza *et al*., 1994; Barbieri *et al*., 1998). Possibly the smallest interaction with Caesalpinioideae is due the fact that the group is more basal in the evolution scale of Leguminosae, and that the interaction of the family with rhizobia arose in the period that Caesalpinioideae has been established (Faria *et al*., 1984; Barbieri *et al*., 1998). It is possible that the ability to interact with rhizobia has arisen more than once in the scale of evolution of the family, as has been proposed (Sprent, 2001; Doyle and Luckow, 2003), however, the specificity by rhizobia with different clades or species (Perret *et al*., 2000; Miller *et al*., 2007; Wang *et al*., 2012), permits the inference that the interaction of several species of rhizobia can be seen to be homologous synapomorphies of Leguminosae clades as inferred for *B. japonicum* in Mimosoideae + Papiloinoideae. In Papilionoideae 11 species nodulate with *B. japonicum,* which belong to the tribes Phaseoleae, Genisteae and Loteae (Lírio *et al*., 2012). Furthermore, Perret *et al*. (2000) cite three tribes as non-host of *B. japonicum*: Vicieae, Cicereae and Trifolieae. These absences of interaction can be explained as a single reversal, since phylogenetic analysis indicated that these tribes form a monophyletic clade called by Steele and Wojciechowski (2003) as "vicioid clade". Based on parsimony, is possible to infer that the nodulation of *B. japonicum* with Leguminosae may have arisen in the common ancestor of Papilionoideae and Mimosoideae.

CONCLUSIONS

According to the results, *B. japonicum* has positive interactions with *I. edulis*. This interaction may be a synapomorphy of the clade Papilionoideae + Mimosoideae. To corroborate our hypothesis that *B. japonicum* interacts with Papilionoideae and Momosoideae, but not with Caesalpinioideae, other studies with species of different subfamilies clades should be evaluated. Particularly between Mimosoideae and Caesalpinioideae to verify if this interaction is a homologous synapomorphy of Papilionoideae + Mimosoideae clade, or should be an apomorphy for Mimosoideae, and the interaction of *B. japonicum* group has arisen more than once in the scale of Leguminosae evolution.

Also we propose studies with native rhizobia species which can be isolated from other legumes, not only by the potential to elucidate evolutionary interactions between rhizobia and Leguminosae, but also by the economic and ecological importance that can make in agricultural crops and reforestation. Another aim to be evaluated is the effects caused by introduction of exotic rhizobia to local biodiversity. The studies should be performed by isolation of rhizobia in native legume species from adjacent crops areas, mainly of soy, once *B. japonicum* strains that have been inoculated in large scale (Ruiz-Díez *et al*. 2012). Furthermore, studies on competition with native rhizobia or negative interactions with local biodiversity are needed.

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COMPETING INTEREST

The authors declare that they have no competing interest.

AUTHOR’S CONTRIBUTION

JF and AMZ conducted the experiments. VBSJ performed the statistical tests. JF, AMZ, EJL, BN and FB organized and discussed the results. SAH performed the study design and supervised the work. All authors read and approved the final manuscript.

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