GROWTH OF *Inga vera* WILLD. SUBSP. *Affinis* UNDER RIZOBIA INOCULATION

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ABSTRACT

Nitrogen, in general, is the largest limiting plant growth nutrient in the tropics and is required as a synthetic fertilizer to improve plants productivity. Therefore, studies aiming in understanding and using nitrogen fixation by leguminous trees have been done as a low-cost alternative for chemical fertilizer. Native legume trees such as *Inga vera* have been recommended in the rehabilitation of degraded areas due their ability to establish symbiosis with nitrogen fixation organisms replacing nitrogen fertilization. These species are able to increase soil organic matter, nitrogen and phosphorus availability. Thus, the present study aims to assess the inoculation effects of native rhizobia strains on nodulation, dry matter production, nitrogen and phosphorus leaf incorporation in *I. vera* seedlings. With this purpose, four strains were obtained from inga nodules and tested in a greenhouse. The inoculation of *I. vera* seedlings with native rhizobium strains promoted an increase in shoot dry mass as well as in leaf nitrogen content. According to symbiotic efficiency equation, this approach ranged from 50 to 80% indicating that the nitrogen fertilization for this species can be partially replaced by rhizobia inoculation.

Keywords: nitrogen, fertilizer, symbiotic efficiency

INTRODUCTION

Nitrogen, besides being an essential element for plant growth, is found in the soil mostly as nitrate, a form which can be easily leached out. For this reason it is considered the most limiting plant growth nutrient in the tropics (Souza and Silva, 1996) and due its limitation in these soils, the use of synthetic fertilizers is required to improve agricultural productivity. About 100 million tons of the expensive nitrogen chemical fertilizers are annually used in agriculture (Heffer and Prud'Homme, 2008) and in spite of these facts, the biological nitrogen fixation (BNF) can be considered as an alternative with low costs to input nitrogen for ecosystems maintenance and for agriculture exploitation around the world (Bohlool *et al*., 1992; Amanuel *et al*., 2000).

Many bacteria genders (Jordan, 1938), collectively referred by rhizobia, are capable of using dinitrogen and forming an important interaction with leguminous plants called symbiosis (Allen and Allen, 1981; Townsend *et al*., 2006). The effects of rhizobia inoculation are already well understood for several crop species like herbaceous forage legumes and grains, such as soybeans (*Glycine max*), which

had nitrogen fertilization completely replaced by rhizobia inoculation with the Bradyrhizobium genus (CNPSO, 2008; Alves *et al.*, 2003). However, only in recent decades the interest increased in expanding knowledge and use of rhizobia inoculation in woody legume trees. The growth effect of rhizobia inoculation on some tree species like Acacia ariculiformes, *Acacia mangium*, *Centrolobium tomentosum*, *Dalbergia nigra*, *Inga oerstediana*, and others has already been tested and the results were very satisfactory (Dela-Cruz *et al.*, 1988; Marques *et al*, 1997; Gonçalves *et al.,* 1995; Santiago *et al*., 2002; Grossman *et al*., 2006). Several authors showed that it is possible to improve, under greenhouse conditions, the growth of leguminous trees by inoculation with effective rhizobia (Badji *et al.* 1988; Wolde-Meskel and Sinclair 1998; Bogino *et al*., 2006), and for some of them the inoculation with rhizobia replaced nitrogen fertilizer at the approximately 60% - 80% and promoted higher increase in shoot dry matter.

Despite the fact that the Gallery forests in Brazil are located into permanent protection areas by law, they are also included in an unsustainable exploitation context that amend the soil, leading to large organic matter and biodiversity losses, basic conditions for sustainable development. Revegetation strategies, aiming to restore soil chemical, physical and biological characteristics are extremely relevant and plants inoculated with rhizobia have proven to be very effective in assisting degraded areas restoration by maximizing natural processes (Gonçalves *et al.*, 2000; Santiago *et al*., 2002; Scotti and Correa, 2004, Duarte *et al*., 2006), reducing carbon/nitrogen relationship in the soil through the BNF (Danso *et al*.. 1992) and improving the natural organic fertilization. Thus, the use of atmospheric nitrogen fixing species configures as an alternative for faster soil and vegetation recovery with reduced fertilizers use, especially in tree species (Marques *et al*., 2001; Santiago *et al*., 2002; Scotti and Correa, 2004).

Among shrub and tree species, members of *Inga* genus are the most widely used in gallery forests re-vegetation programs, since they comprise a large and representative group of wet forest species (Pennington, 1997) *Inga vera* seedlings and trees can tolerate floods (Lieberg and Joly, 1993; Lawrence *et al*., 1995) and serve as an important source of resources for the local fauna (Oliveira-Filho *et al*., 1994; Pott and Pott, 1994; Melo *et al*., 1999).

The aim of our work was to assess the inoculation effects of four selected rhizobia strains on growth, nodulation, dry matter production, nitrogen and phosphorus leaf incorporation in *Inga vera* seedlings targeting BNF efficient strains selection in a greenhouse to produce seedlings with reduced fertilizers use.

MATERIALS AND METHODS

Seedlings

The *I. vera* seeds were collected at the UFMG campus (Federal University of Minas Gerais) and then stored at 10°C for few days. The seeds surfaces were disinfected with the following treatment: briefly dipped in 70% ethanol (1 min) and bichloride of mercury for 30 seconds, and then washed eight times in sterile distilled water. Then, the seeds were incubated to germinate under germination paper and in a gerbox. The gerbox were placed inside a germination chamber adjusted at 25°C and at 12 hours photoperiod. After radicle protrusion (radicle with 5-10mm), the seedlings were transplanted into plastic bags (2 kg of soil capacity) and

transferred to a greenhouse environment. The used substrate was the soil from impacted area collected at 20cm deep.

Inoculants

Four strains were isolated from *I. vera* nodules and screened for their effectiveness under greenhouse conditions. They were called BHICB-Ig1, BHICB-Ig2, BHICB-Ig3 and BHICB-Ig4, respectively. The rhizobia cultures were grown and stored on Yeast Manitol Agar medium (Fred and Waksman, 1928). The stock cells of each strain were placed in Erlenmeyer flasks containing 20 mL of YMB (liquid medium) and grown in shaker (100 rpm), at 29°C for 5 days. The bacterial inocula were provided at 2 mL per pot (10^8 cfu mL^{-1}), according Somasegaran and Hoben (1985). The inoculation with rhizobia was performed seven days after transplanting the seedlings and the growth analysis after a 90-day period.

Experimental design and treatments

The experiment was conducted in a greenhouse at UFMG, Belo Horizonte, Brazil, and the experimental design was a completely randomized block. Eight replicates of each of seven treatments were used as follows: (1) Fertilization without nitrogen + strain BHICB-Ig1; (2) Fertilization without nitrogen + strain BHICB-Ig2; (3) Fertilization without nitrogen + strain BHICB-Ig3; (4) Fertilization without nitrogen + strain BHICB-Ig4; (5) Without fertilization: control and; (6) Complete chemical fertilization. Based on the widely used method to produce seedlings aiming on restoration procedures, a treatment with organic fertilizer (1V/2V) was included, treatment 7. Treatments with chemical fertilization were made according to Somasegaran and Hoben (1985) as

follows: KH_2PO_4: 468 mg pot^{-1}; KCl: 404.2 mg pot^{-1}; $MgSO_4$ x 7 H_2O: 53.3 mg pot^{-1}; $ZnSO_4$ x 7 H_2O: 49.5 mg pot^{-1}; $(NH_4)_6$ Mo_7O_{24} x H_2O: 1.95 mg pot^{-1}; $CO(NH_2)_2$: 219 mg pot^{-1}.

Sampling and analysis

After 90 days, the seedlings were collected and analyzed using the following parameters: (1) Dry weight of the shoot; (2) Dry weight of the roots and nodules; (3) Symbiotic effectiveness; (4) Total nitrogen leaf (Oliveira, 1986); and (5) Total phosphorus leaf (Sarruge and Haag, 1974). Data were subjected to several distribution tests to evaluate their normality, submitted to ANOVA and the averages were compared with the Duncan test at the level of 1% of probability. For shoot biomass analysis, eight plants per treatment were removed and their roots washed carefully with water to prevent nodules detachment. The shoot, root and nodules were separated and dried at 60°C until constant weight. Dry weight was recorded. Symbiotic effectiveness (SE) was estimated by using the equation: SE = 100 x Dry weight of inoculated plants/Dry weight of uninoculated plants. The SE values less than 35% indicated non effective strains, 35-50% low effectiveness, 50-80% effective and over 80% high effectiveness. The symbiotic efficiency values were calculated in relation to the complete chemical fertilization (Treatment 6) and organic fertilizer (Treatment 7). Eight seedlings shoot parts per treatment were individually ground to fine powder and samples of 0.1 g were acid digested for total nitrogen determination by Nessler's reagent method (Oliveira, 1986) and total phosphorus leaf determination by spectrophotometry using the ascorbic acid method modified by Braga and Defelipo (1974).

RESULTS

Nodule dry weight

Only the inoculated plants showed nodule development and the nodules presented a wide range of forms and size. Nodule dry weights tended to be higher in the plants inoculated with BHICB-Ig2 and BHICB-Ig3 when compared with the other inoculated plants, but not significant, P ≤ 0.01 (Table 1) which reinforce the influence of the strains effectiveness in the biomass and nitrogen improvements.

Shoot dry weight, nitrogen content and root dry weight

The results presented in Table 1 showed that the dry matter production of inoculated plants (BHICB-Ig1, BHICB-Ig2, BHICB-Ig3 and BHICB-Ig4) did not show statistical differences when compared with the nitrogen fertilized plants ($P \leq 0.01$). However, the best results in dry matter production were found, respectively, for plants treated with organic matter, inoculated plants and plants supplied with nitrogen chemical fertilization. The control plants (no inoculated and no fertilized) showed the smallest amount of dry shoots and nitrogen content in leaves when compared with all other treatments.

The higher nitrogen content was found in plants treated with organic matter followed by the decreasing order: Organic matter > BHICB-Ig4 > BHICB-Ig3 = Chemical fertization > BHICB-Ig1 = BHICB-Ig2 > control (Table 1). The root dry weight did not statistically differ between the treatments, $P \leq 0.01$ (Table 2) and was not considered a good parameter to evaluate the rhizobium strains effectiveness in *I. vera* seedlings.

Symbiotic effectiveness

In a global terms, the symbiotic efficiency ranged for 50 to 80% (Figure 1). Plants inoculated with the strains BHICB-Ig1, BHICB-Ig2, BHICB-Ig3 and BHICB-Ig4 achieved symbiotic efficiency values of 72, 69, 80 and 74% in relation to the chemically fertilized plants and 52, 50, 58 and 53% in relation to the organic matter treated plants, respectively (Figure 1).

Table 1. Effect of rhizobia strains inoculation on nodule and shoot dry weight and on nitrogen content of *Inga vera*.

Treatments	Nodule dry weight[NS] (mg)	Shoot dry weight* (g)	Nitrogen content* (µg g^{-1})
(1) BHICB-Ig1	32.6 ± 12.01	0.85 ± 0.18^{bc}	45.62 ± 0.35^{d}
(2) BHICB-Ig2	47.0 ± 21.13	0.81 ± 0.31^{bc}	45.87 ± 0.35^{d}
(3) BHICB-Ig3	44.8 ± 35.24	0.94 ± 0.253^{bc}	47.75 ± 0.27^{c}
(4) BHICB-Ig4	13.4 ± 14.29	0.87 ± 0.25^{bc}	62.56 ± 0.42^{b}
(5) Without fertilization: control	-	0.77 ± 0.41^{c}	43.25 ± 0.60^{e}
(6) Complete chemical fertilization	-	1.18 ± 0.36^{b}	38.0 ± 0.53^{f}
(7) Organic fertilizer (1V/2V)	-	1.62 ± 0.57^{a}	64.12 ± 0.44^{a}

*Averages in columns with different letters are significantly different as determined by Duncan test at 1% of probability ($P \leq 0.01$). NS: No significantly different. Treatments: (1) Fertilization without nitrogen + strain BHICB-Ig1; (2) Fertilization without nitrogen + strain BHICB-Ig2; (3) Fertilization without nitrogen + strain BHICB-Ig3; (4) Fertilization without nitrogen + strain BHICB-Ig4; (5) Without fertilization: control; (6) Complete chemical fertilization; and (7) Organic fertilizer (1V/2V).

Table 2. Effect of rhizobia strains inoculation on root dry weight, Shoot/Root relation and on phosphorus content of *Inga vera* seedlings.

Treatments	Root dry weight[NS] (g)	Root/Shoot*	Phosphorus content* (mg kg^{-1})
(1) BHICB-Ig1	0.56 ± 0.17	0.66 ± 0.11[a]	0.11 ± 0.01 [c]
(2) BHICB-Ig2	0.37 ± 0.19	0.44 ± 0.12[b]	0.13 ± 0.01 [b]
(3) BHICB-Ig3	0.39 ± 0.14	0.41 ± 0.08[b]	0.13 ± 0.005 [b]
(4) BHICB-Ig4	0.38 ± 0.09	0.44 ± 0.07[b]	0.10 ± 0.005 [cd]
(5) Without fertilization: control	0.43 ± 0.25	0.57 ± 0.13[a]	0.10 ± 0.01 [d]
(6) Complete chemical fertilization	0.45 ± 0.18	0.38 ± 0.13[b]	0.13 ± 0.01 [b]
(7) Organic fertilizer (1V/2V)	0.60 ± 0.14	0.39 ± 0.07[b]	0.18 ± 0.01[a]

*Averages in columns with different letters are significantly different as determined by Duncan test at the level of 1% of probability ($P \leq 0.01$). NS: No significantly different. Treatments: (1) Fertilization without nitrogen + strain BHICB-Ig1; (2) Fertilization without nitrogen + strain BHICB-Ig2; (3) Fertilization without nitrogen + strain BHICB-Ig3; (4) Fertilization without nitrogen + strain BHICB-Ig4; (5) Without fertilization: control; (6) Complete chemical fertilization; and (7) Organic fertilizer (1V/2V).
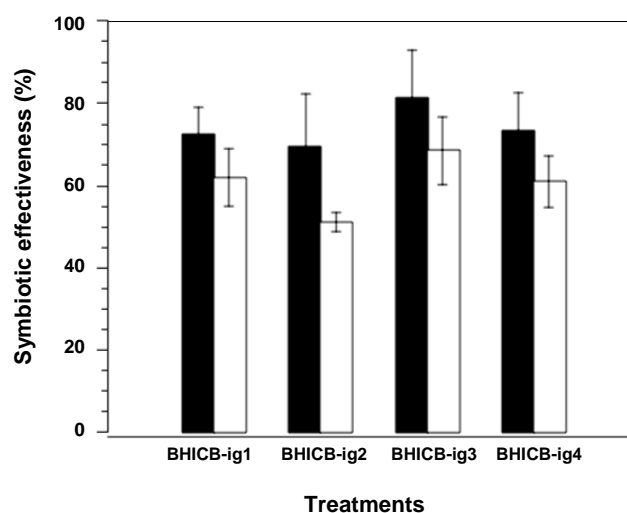
Figure 1. Symbiotic effectiveness of four rhizobia strains in relation to plants treated with chemical fertization, black bars (■), and organic matter, white bars (□). Bars represent standard error.

Root/shoot relation

The *I. vera* root/shoot (R/S) relation observed for the inoculated plants with the strains BHICB-Ig1, BHICB-Ig2, BHICB-Ig3, BHICB-Ig4 and for the plants treated with chemical fertilization, organic fertilization and without fertilizer are showed in Table 2. The *I. vera* root/shoot relation did not differ

statistically ($P \leq 0.01$) between the seedlings inoculated with the strains BHICB-Ig2, BHICB-Ig3, BHICB-Ig4 and the seedlings treated with complete chemical fertilizer or organic matter (Treatments 2, 3, 4, 6, and 7). In contrary, this ratio was bigger in seedlings inoculated with the strain BHICB-Ig1 and in control plants (Treatments 1 and 5) (Table 2).

Phosphorus content

The organic fertilized plants showed the highest content of leaf phosphorus, 0.18 mg kg-1, followed by the plants fertilized with complete chemical fertilization (0.129) and the plants inoculated with the strains BHICB-Ig2, BHICB-Ig3; 0.134; 0.129 mg kg^{-1}, respectively) which did not differ statistically by the Duncan's test by setting the *P*-value at ≤ 0.01. The control treatment plants showed the lowest values of leaf phosphorus content (0.10 mg kg^{-1}) followed by the plants inoculated with the strain BHICB-Ig4, BHICB-Ig1 (Table 2).

DISCUSSION

The forest species inoculation with native rhizobia strains can increase nitrogen content and dry biomass production in the field or in greenhouse experiments (Marques *et al*., 2001; Santiago *et al*., 2002, Scotti and Correa, 2004). The data obtained for *I. vera* showed a positive effect of the rizobia inoculation on dry matter production indicating that all the tested strains have high efficiency in nitrogen fixation since the nitrogen fixation have a positive relationship with growth (Table 1). The organic fertilizer treated plants presented higher leaf nitrogen content then the other plants, corroborating the obtained data for the leaf biomass production. However, why

the dry matter production not improved the same way in the inoculated plants? This finding suggests that the fixed nitrogen was not completely converted into biomass. Thus, even showing higher nitrogen contents in relation to the other inoculated plants, the shoot dry matter of the BHICB-Ig4 treated plants did not differ from the other inoculated plants (Table 1). These results indicate that the biological N_2 fixation process occurred, but the fixed nitrogen until the third month is still in other forms like amides and ureides and was not incorporated as plant protein.

Experiments with *Inga oerstediana* indicated that inoculation with selected strains of rhizobia does not confer any advantage to young *I. oerstediana* seedlings (90 Days after planting - DAP). However, in older seedlings (150 DAP), statistical differences in shoot biomass and nodulation between inoculated treatments and fertilized controls can be noted (Grossman *et al*., 2006). Native plants, generally, require an average of four months to establish total symbiotic efficiency, thereby delaying the start of the BNF process (Goi *et al*., 1992; Jacob-Neto *et al*., 1998). *I. vera* has intermediary growth and therefore it can present in the rhizobia interaction a relative slow nodulation and BNF processes.

It is known that some leguminous trees take 20 to 30 days to form primordial radical nodule and these observations reinforce the hypothesis that for inga seedlings the fixed nitrogen is allocated in biomass after a growth period higher than the assessed in the present study (90 days). In comparison with the other inoculated plants, the high nitrogen content found in the BHICB-Ig4 treatment indicates that these plants would also achieve, in a long term scale, superior biomass values confirming this strain BNF high efficiency.

The symbiotic efficiency ranged from 50 to 80% (Figure 1) indicating that these inoculants can partially replace artificial fertilization. The inoculation procedures with strain BHICB-Ig3 could replace 80% chemical fertilization and 60% of organic matter nitrogen while BHICB-Ig4 replaces 74% and 53% of the chemical fertilizers and organic matter nitrogen, respectively.

Indigenous tree species are very dependent to symbiotic associations as shown in literature (Dela-Cruz *et al*., 1988; Marques *et al*., 2001) and these data are in agreement with the *I. vera* obtained data. Ndoye *et al*. (1995) showed a great variability among various species in terms of symbiotic nitrogen fixation and several authors have demonstrated that there is a strong relationship between the genetic origin of woody legumes and their capacity to grow, nodulate, and fix atmospheric nitrogen in symbiosis with rhizobia (Nasr *et al*., 1999; Lesueur and Diouf, 2001).

Between the inoculated plants, the higher phosphorus levels was found for plants treated with the strains BHICB-Ig2 and BHICB-Ig3 and it may be related to the rhizosphere effect promoted by the inoculation. This effect results in an increase of phosphate solubilizing, microorganisms and mycorrizal population (Herrera *et al*., 1993; Fernandes *et al*., 2005).

Besides the results obtained, the root/shoot relation (R/S) was analyzed. R/S is a correlation of development, expressing the fact that the root growth can affect the shoot so its relation reflects growth and dry matter accumulation between roots and shoot (Goss, 1973; Lioert *et al*., 1999). The R/S is affected by nutrient availability (Liang, 1996; Marsh and Pierzynski, 1998) and an excessive low R/S indicates poor root growth, resulting in insufficient water and nutrients for shoot growth. An extremely

high R/S may lead to root redundancy, which reduces shoot growth, yield, and water and nutrient use efficiencies (Zhang, 1995). Therefore, it is important to coordinate root and shoot relations and maximize dry matter accumulation and water and nutrient use efficiencies (Tomar *et al*., 1997; Kahn and Schroeder, 1999).

The *I. vera* root/shoot relation did not differ statistically between the seedlings inoculated with the strains BHICB-Ig2, BHICB-Ig3, BHICB-Ig4 and the seedlings treated with complete chemical fertilizer or organic matter (Treatments 2, 3, 4, 6, and 7), suggesting that these plants invested in shoot growth since they have access to the nutrients. In contrast, this ratio (R/S) was bigger in seedlings inoculated with the strain BHICB-Ig1 and for control plants (Treatments 1 and 5) (Table 2). This fact explains the investment on root growth to nutrient seeking. Observing Tables 1 and 2 it can be noted that the missing nutrient that most influenced R/S was phosphorus, since its absence was definitive to increase this relationship. Plants treated with complete chemical fertilizer reinforce this hypothesis. Even though presenting low nitrogen content, these plants kept their R/S low due the phosphorus availability. A higher R/S relation can be a mechanism of resistance to drought and nutritional deficiency. According to Leyser and Fitter (1998), nitrate and phosphate have a positive effect on lateral roots growth. When nitrate is supplied in excess, the elongation stimulus on these roots is blocked. The R/S relation observed in the treatments 1 and 5 do not indicate superiority of the same. Although the other treatments R/S relation values were lower, they were balanced and therefore they did not compromised the seedlings quality. The growth pattern and architecture of the plant root system under normal conditions, culture, competitive or

poor environments, have a key role in determining the water and nutrients absorption efficiency (Diem and Skene, 2001; McCully, 1995).

Finally, in this study we demonstrated that the selected rhizobium strains for *I. vera* inoculation had no effect on root dry weight, and it may be attributed to the fact that these strains did not need to invest in roots growth. These plants showed a high symbiotic efficiency and available nitrogen to invest in shoot growth similarly to those which have nutrient access from organic matter or chemical fertilizer. Similar to the data found to *I. vera*, studies with *Acacia nilotica* demonstrated no significant differences in root dry weight of plants between the different inoculation methods in relation to the non-inoculated control plants (Lesueur *et al.*, 2005).

Other studies with woody legume tree species also have showed divergent results for root dry weight. Forestier *et al.* (2001), noticed a significant difference between root dry weight of the rhizobia inoculated plants versus the non-inoculated plants in their experiments. However, the inoculation of *Acacia mangium* with Bradyrhizobium resulted in a significant increase in shoot dry weight when compared to non-inoculated controls, but it had no significant effect on the root dry weight for this specie (Weber *et al.*, 2005). In contrast, a study with *Calliandra calothyrsus* showed that there are distinct responses for different rhizobium strains and host plant response may differ significantly in relation to root biomass acquisition.

Specimens of *C. calothyrsus* inoculated with KCC13 strain obtained significant increase in root dry weight, while plants inoculated with the KCC17 strain no presented significant difference for this approach (Odee *et al.*, 2002).

CONCLUSIONS

The greenhouse experiment showed that the inoculation of *I. vera* seedlings with rhizobia native strains promoted increase in shoot dry weight and nitrogen content. The more efficient BNF strain was BHICB-Ig4. This study indicates that the *I. vera* subsp. affinis seedlings production aiming forest gallery recovery may be made with chemical fertilizer partially replaced by native rhizobia inoculation.

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