Nodulation, arbuscular mycorrhizal colonization and growth of some legumes native from Brazil[1]

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RESUMO – (Nodulação, colonização micorrízica arbuscular e crescimento de algumas leguminosas nativas do Brasil). Foram examinados os efeitos da inoculação com rizóbio e micorriza, da fertilização com nitrogênio e fósforo na nodulação, na colonização micorrízica e no crescimento inicial das leguminosas arbóreas brasileiras *Enterolobium contortisiliquum* (Vell. Conc.) Morong, *Inga laurina* (Sw.) Willd., *Lonchocarpus muehlbergianus* Hassl e *Platypodium elegans* Vogel. O experimento foi conduzido em casa de vegetação usando sacos plásticos contendo uma mistura de solo arenoso de mata ciliar e vermiculita (2:1) fertilizados com nutriente basais incluindo NP, P e N inoculados ou não com rizóbio (r), micorriza (m) ou ambos (rm), totalizando sete tratamentos: NP, P, P+r, P+rm, N, N+m e N+rm, com dez repetições cada. As plantas foram analisadas aos 120 e 255 dias após o plantio. Tratamentos com deficiência de P afetaram negativamente o crescimento e nodulação de todas as espécies. Fungos micorrízicos arbusculares (FMAs) autóctones colonizaram as raízes do hospedeiro e as inoculações com fungos não aumentaram a colonização micorrízica, a qual foi favorecida pelo nível de P adicionado. A nodulação foi relativamente alta em *E. contortisiliquum* e *L. muehlbergianus*, principalmente em tratamentos contendo P, inoculados com rizóbio (P+r). Plantas deste tratamento desenvolveram-se melhor que as outras e, apesar dos rizóbios inoculados não apresentarem relações sinergísticas com os FMAs inoculados, também apresentaram as melhores porcentagens de colonização micorrízica. Além disso, estas duas espécies apresentaram maiores taxas de redução de acetileno e maior conteúdo de leghemoglobina. Estes resultados sugerem que *E. contortisiliquum* e *L. muehlbergianus* podem ter vantagens para se estabelecer em solos com baixos níveis de nitrogênio.

Palavras-chave: rizóbio, dupla inoculação, *Enterolobium contortisiliquum*, *Inga laurina*, *Lonchocarpus muehlbergianus*, *Platypodium elegans*

ABSTRACT – (Nodulation, arbuscular mycorrhizal colonization and growth of some legumes native from Brazil). The effects of rhizobial and mycorrhizal inoculation, nitrogen and phosphorus fertilization on nodulation, mycorrhizal colonization and initial growth were examined in Brazilian native plants, *Enterolobium contortisiliquum* (Vell. Conc.) Morong, *Inga laurina* (Sw.) Willd., *Lonchocarpus muehlbergianus* Hassl and *Platypodium elegans* Vogel. The experiment was carried out in a glasshouse using plastic bags filled with a mixture of sandy soil from riparian forest and vermiculite (2:1) amended with basal nutrients including NP, P and N and infected with rhizobia (r), mycorrhiza (m) or both (rm), amounting seven treatments: NP, P, P+r, P+rm, N, N+m and N+rm, with ten replications each. The plants were analyzed at 120 and 255 days after sowing. P deficiency negatively affected growth and nodulation of all species. Autochton arbuscular mycorrhizal fungi (AMF) colonized host roots and fungal inoculations did not enhance mycorrhizal colonization, which was also favored by added P. Nodulation was relatively higher in *E. contortisiliquum* and *L. muehlbergianus*, mainly in treatments containing P, and with rhizobial inoculation (P+r). Plants from these treatments developed better than others and, despite the rhizobia inoculated had no synergistic relationship with inoculated AMF, they also showed the best percentages of mycorrhizal colonization. Moreover, these two species showed highest rates of acetylene reduction and highest leghemoglobin content. These results suggest that *E. contortisiliquum* and *L. muehlbergianus* can have advantages for establishment in soils with low nitrogen levels.

Key words: rhizobia, dual inoculation, *Enterolobium contortisiliquum*, *Inga laurina*, *Lonchocarpus muehlbergianus*, *Platypodium elegans*

Introduction

Dual inoculation with rhizobia and mycorrhizal fungi is currently being suggested as a possible solution to reforestation and amendment of soil fertility. The effectiveness of rhizobia-mycorrhiza-plant interactions varies with host species, rhizobial strain, fungus species and soil conditions. In contrast to taxonomically restricted N_2-fixing rhizobia, arbuscular mycorrhizas are extremely widespread (Brundrett 2002), besides showing low specificity (Mosse 1975).

Relatively few leguminous trees have been tested for their nodulation or nitrogen fixation ability (Sprent 2001) and little is known about how we can manage

symbiotic fungal and rhizobial associations more effectively (Marques *et al.* 2001). Some woody legume species, for instance *Albizia lebbeck* (L.) Benth (Faria *et al.* 1995), *Dalbergia nigra* Allem. ex Benth (Santiago *et al.* 2002), *Centrolobium tomentosum* Guill. ex Benth (Marques *et al.* 2001) and *Mimosa caesalpiniifolia* Benth (Burity *et al.* 2000) have been studied in this way. Positive impacts of dual inoculation have been demonstrated in forest tree species in Philippines (De La Cruz *et al.* 1988), India (Khan & Uniyal 1999) and Kenya (Munro *et al.* 1999).

Enterolobium contortisiliquum (Vell. Conc.) Morong plants have been used in enrichment planting and in agroforestry systems (Montagnini *et al.* 1997; Eibl *et al.* 2000). This species is tolerant to heavy metal contaminated soil (Trannin *et al.* 2001). It has been sampled in several Brazilian riparian forests (Catharino 1989; Nilsson 1989; Bernacci *et al.* 1998) and its nodulation has been known since 1906 (Allen & Allen 1981). Arbuscular mycorrhizal fungi (AMF) colonization was reported by Frioni *et al.* (1999).

Inga laurina (Sw.) Willd. is generally found in riparian forests primarily in seasonally flooded areas (Romagnolo & Souza 2000), but it has also long been used as a shade tree and green manure in coffee and cacao plantations and has good potential as an agroforestry species (Tilki & Fisher 1998). It was listed as a nitrogen-fixing tree by Halliday & Nakao (1982) and under its currently accepted synonym *I. fagifolia*, (L.) Benth. was reported to be nodulated (Faria *et al.* 1987). However these studies did not include any information on nodule physiology. AMF colonization in *I. laurina* has not been reported previously, although other *Inga* species are known to form such symbioses.

Lonchocarpus muehlbergianus Hassl has been used in carpentry and as an ornamental species (Lorenzi 1992). The genus includes 130 species of shrubs or trees from tropical and subtropical America and one species in Africa. *L. muehlbergianus* is one of 21 species from this genus with known nodulation capacity (Sprent 2001). Faria *et al.* (1987) were the first to observe its nodulation. This species can be founded in riparian forest (Silva *et al.* 1992; Bernacci *et al.* 1998; Nilsson 1989). Frioni *et al.* (1999) observed colonization by AMF in *L. nitidus*.

Platypodium elegans Vogel is used for wood and as an ornamental, mainly along the streets and avenues (Lorenzi 1994). This species was reported as nodulated by Halliday & Nakao (1982). It was sampled in 23.9% of studies involving floristic survey of riparian forest of Brazil (Rodrigues & Nave 2000). Carneiro *et al.*

(1998) characterized the occurrence of AMF of *P. elegans* in a nursery.

The aim of this work was to investigate the effects of inoculation with rhizobia and/or arbuscular mycorrhizal fungi on nodulation, mycorrhizal colonization and growth of *E. contortisiliquum*, *I. laurina*, *L. muehlbergianus* and *P. elegans* under greenhouse conditions and different fertilization treatments.

Materials and methods

Seeds of all species were surface sterilized. *E. contortisiliquum* and *P. elegans* seeds were scarified to break dormancy prior to planting. *E. contortisiliquum* seeds were smoothed and *P. elegans* seeds were left to soak in 50% sulphuric acid during 45 min and washed nine times prior to planting. *I. laurina* seeds were firstly surface sterilized and stored in trays with vermiculite and abscisic acid solution (10^{-4}M) in the proportion 2:1 v/v in a cold chamber at 10 ± 1 ºC and 85%$\pm$5% of relative humidity (Barbedo & Cicero 2000) during twenty-four days, since seeds of *Inga* species rapidly lose viability under normal environmental conditions. After that, the seeds of these species and *L. muehlbergianus* were germinated directly on substrate.

Aerial parts of collected species were pressed and catalogued at Bioscience Institute Herbarium - UNESP (Universidade Estadual Paulista "Júlio de Mesquita Filho") under the numbers HRCB-34503 (*E. contortisiliquum*), HRCB- 5021 (*I. laurina*), HRCB-34500 (*L. muehlbergianus*) and HRCB-34502 (*P. elegans*).

Seeds of all species, except *I. laurina*, were soaked in a turbid suspension (100 mL) of a mixture of two rhizobia strains for one hour before sowing. *E. contortisiliquum* seeds were inoculated with a mixture of strains IBRC-206 and 207, isolated from *E. contortisiliquum* nodules. *L. muehlbergianus* and *P. elegans* seeds were inoculated with IBRC-208 and 209 rhizobia strains isolated from nodules of *L. muehlbergianus*. Pre-germinated seedlings of *I. laurina* received 10 mL of a mixture of IBRC- 195 and 197 strains isolated from *Inga* spp., directly on surface of pots, one week after sowing. To ensure rhizobia infection, seedlings of all species were re-inoculated 30 days after sowing, with 10 mL of rhizobia suspension containing the same strains.

For mycorrhizal inoculation, pieces of *Anadenathera peregrina* (L.) Speg. var. *falcata* (Benth.) Altschul roots

±1 cm in length, collected from Corumbataí Cerrado (22°15'S and 47°00'W, 810 m a.s.l.) were added on each pot surface (0.4 g) near seedlings stem twenty-eight days after sowing as described by Gross *et al.* (2004). The roots of *A. peregrina* var. *falcata* (used as inoculum) had been previously characterized by Gross *et al.* (2004) that identified spores of *Glomus microaggregatum* Koske, *Glomus etunicatum* Becker & Gerd., *Glomus* sp., *Enthrophospora* sp., *Gigaspora* sp. and *Acaulospora*. Our preliminary tests showing hyphae and spores of mycorrhiza inside pieces of *A. peregrina* var. *falcata* roots proved its potential as inocule.

Aerial parts of this species were catalogued in the Bioscience Institute Herbarium - UNESP under the number HRCB-34330.

Plants were grown in plastic pots of 4 L containing non-sterile soil and vermiculite (2:1), in a greenhouse, under natural daylight, in seven randomized blocks. The soil was collected from riparian forest of Corumbataí, SP, Brazil (22°20'S and 47°40'W, 604 m a.s.l.) and contained in $mmol_c.dm^{-3}$: K (1), Ca (12), Mg (4), H+Al (10), Al (7); in $mg.dm^{-3}$: B (0.01), Cu (0.07), Fe (23), Mn (8.7), Zn (0.4), S (9), P (7) and in ppm: N (1000) with pH 5.5. All pots received the following basal nutrients prior to sowing (in $mg. kg^{-1}$ substrate): K (60), $CaCO_3$ (80), $MgCO_3$ (40), S (30), B (1), Zn (2), Cu (2), Fe (4), Mn (20), Mo (4). Nitrogen and phosphorus were added at three different levels: NP, with N (40) and P_2O_5 (80); P, with P_2O_5 (80) and 3.8 mM of N as a start dose; and N, with N (40), using NH_4NO_3. These lots varied in function of inoculations with rhizobia (r), mycorrhiza (m) or both (rm). So, our experiment had seven treatments, with ten replicates each: NP; P; P+r; P+rm; N; N+m; N+rm. Additional nutrients (10 mL of solution) were added to the surface of each pot every 30 days, according to fertilization treatments above.

The plant height was measured at two weeks intervals from 30 days after sowing until the end of the experiment (255 days after sowing). Five plants per treatment were harvested 120 days after sowing and fresh nodules were sieved and counted those larger than 4 mm, between 2-4 mm and less than 2 mm of diameter. The other five plants per treatment were harvested 255 days after sowing, for leaf area measurement (CI-202 Area Meter, CID, Inc), dry weight of roots, stems and leaves, AMF colonization and nitrogen and phosphorus content of shoot tissues and substrate.

Nitrogenase activity and leghemoglobin content were evaluated in the nodules of two plants per treatment. Nitrogenase activity was measured by the acetylene reduction activity (ARA; Hardy *et al.* 1968) using the whole root system, and leghemoglobin content by the method of Becana *et al.* (1986) at 540 nm, using three samples of 0.5 g each. After that, fresh nodules were sieved to separate those larger than 4 mm, between 2 and 4 mm and less than 2 mm of diameter. They were then counted and dried. Nodule morphology was classified according to Sprent (2001).

Roots were stained (Philips & Hayman 1970) and percentage of AMF infected roots of two plants per treatment was estimated using the gridline intersects method (Giovannetti & Mosse 1980) under a stereomicroscope (40x) at 255 days after sowing.

Data were analyzed separately by ANOVA and means were compared by Duncan's test, at $P \leq 0.05$, using the Statistica for Windows (StatSoft, Inc. 2000), except for P and N contents that were analyzed on combined samples (per treatment), AMF colonization, ARA and leghemoglobin content that were evaluated in two plants per treatment.

Results

Plants that received NP were the tallest ($P<0.05$) in relation to other treatments, mainly N treatments (phosphorous deficiency) (Fig. 1). *E. contortisiliquum* plants cultured on P and P+r treatments were as tall as the NP plants at 255 days (Fig. 1). Plants of this species receiving P+r treatment had highest leaf area. The dry mass of leaf, stem and root were also high, but did not differ of NP ones (Tab. 1). For *I. laurina*, N, N+m and N+rm treated plants had leaf area similar to NP. For *P. elegans*, NP treatment differed significantly from the others in all growth parameters. P-fertilized treatments showed the best results of plant growth at 255 days in *E. contortisiliquum* and *L. muehlbergianus* (Fig. 1). Rhizobia inoculation in P-fertilized plants increased leaf area, leaf, stem and root dry mass of *L. muehlbergianus* at 255 days after sowing (Tab. 1).

Mycorrhizal colonization was relatively low in all species (Tab. 1). Percentage of AMF colonization of roots reached 24.72; 45.25; 39.48 and 15.35% in *E. contortisiliquum*, *I. laurina*, *L. muehlbergianus* and *P. elegans*, respectively (Tab. 1). However, the highest values of AMF colonization occurred in P treatments inoculated with rhizobia, therefore non-inoculated with AMF in *E. contortisiliquum* and *L. muehlbergianus*. This indicates higher potential of infection by native fungi in the soil used as substrate and it becomes evident that the P fertilization is important for the

establishment of AMF. For *I. laurina*, AMF colonization occurred with both the inoculated and native fungi and it is the first register of AMF colonization in this species.

All nodulated species and nodules were identified as indeterminate (Corby 1988; Sprent 2001). We consider *E. contortisiliquum* and *L. muehlbergianus* as astragaloids (or caesalpinoids), *I. laurina* and *P. elegans* as mucunoid and aeschynomenoid respectively. All species, except *P. elegans*, were well nodulated and the nodules had a reddish internal colour (Fig. 2 D-F). Most part of nodules was between two
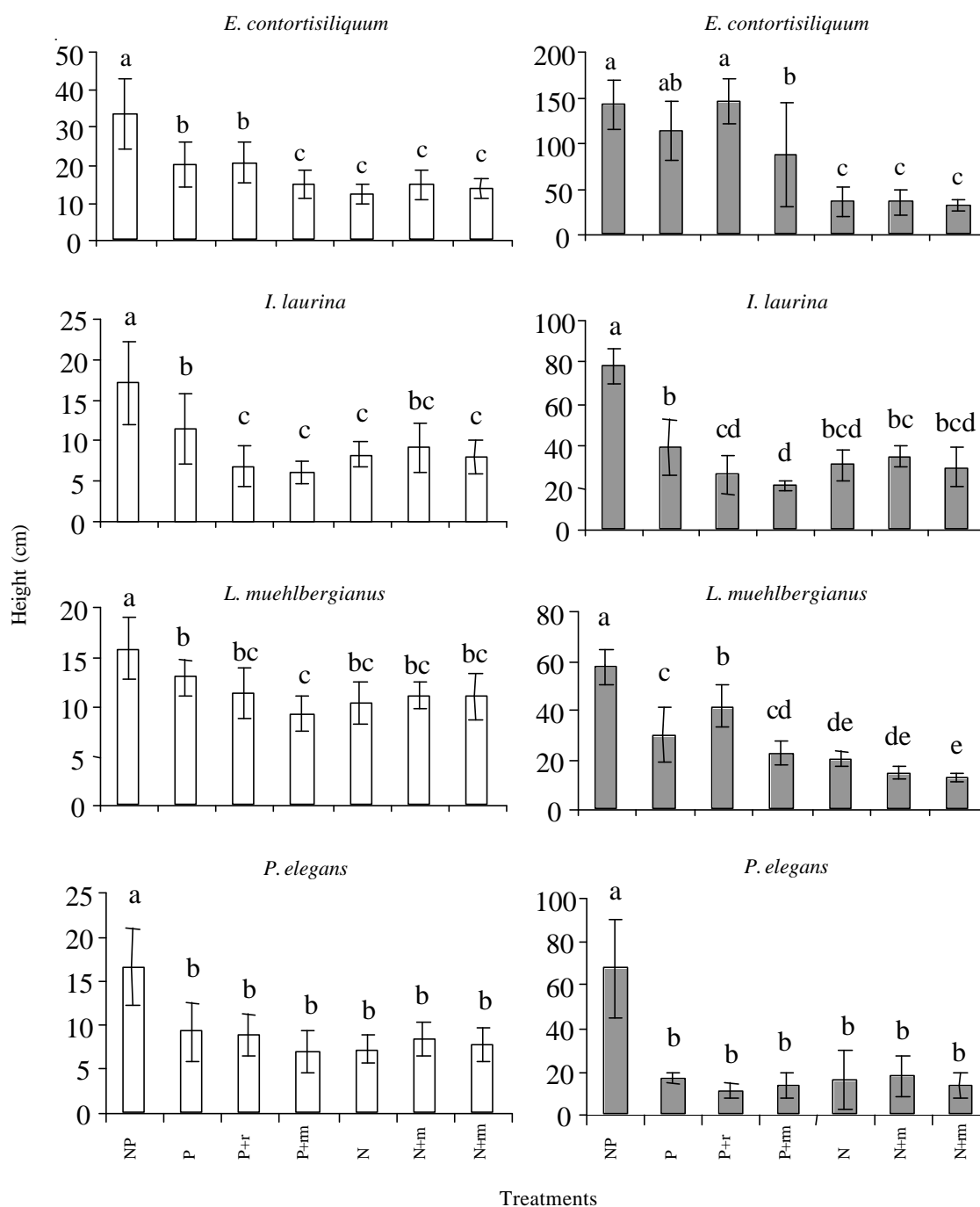

Figure 1. Average height of *Enterolobium contortisiliquum* (Vell. Conc.) Morong, *Inga laurina* (Sw.) Willd.*, Lonchocarpus muehlbergianus* Hassl and *Platypodium elegans* Vogel. seedlings at 120 and 255 days after sowing and growth under greenhouse conditions, fertilized with NP, P, N and inoculated by rhizobia (r), mycorrhiza (m) or both (rm). Values followed by the same letter(s) are not significantly different at 5% level using the Duncan test. □ = 120 days; ■ = 255 days.

Table 1. Response of *Enterolobium contortisiliquum* (Vell. Conc.) Morong, *Inga laurina* (Sw.) Willd, *Lonchocarpus muehlbergianus* Hassl and *Platypodium elegans* Vogel seedlings to treatments of mineral fertilization (NP, P, N,) and inoculation with rhizobia (r), mycorrhiza (m) or both (rm) at 255 days.

| Treatment	Leaf area	Dry mass (g)*			AMF	N (%)		P (%)	
	$(cm^2)^{-1}$	Leaf	SteM	Root	(%)	Tissue	Substrate	Tissue	Substrate
E. contortisiliquum									
NP	338.6 b	13.78 ab	33.68 a	16.09 a	11.78	2.45	0.05	0.20	0.0078
P	727.1 ab	9.41 b	15.71 b	12.01 b	2.94	2.50	0.05	0.20	0.0126
P+r	1397.2 a	20.15 a	34.47 a	24.43 a	24.72	2.05	0.05	0.13	0.0142
P+rm	1121.7 a	12.44 b	18.14 b	16.03 ab	18.27	2.00	0.07	0.18	0.0085
N	204.0 b	1.96 c	3.03 c	3.81 b	9.04	3.30	0.05	0.06	0.0008
N+m	266.3 b	1.97 c	2.53 c	5.01 b	10.59	3.55	0.07	0.06	0.0004
N+rm	116.4 b	1.48 c	1.91 c	2.71 b	11.03	3.90	0.05	0.06	0.0003
I. laurina									
NP	503.4 a	15.30 a	9.81 a	15.63 a	13.41	1.90	0.05	0.20	0.0074
P	132.6 b	5.10 b	2.16 b	4.19 b	37.04	1.90	0.05	0.28	0.0096
P+r	139.9 b	2.31 c	0.85 cd	1.57 bc	4.85	1.80	0.05	0.31	0.0155
P+rm	182.9 b	2.01 c	0.64 d	0.87 c	45.25	1.85	0.05	0.46	0.0124
N	379.4 ab	4.18 b	1.46 bcd	4.05 bc	23.68	1.00	0.07	0.08	0.0009
N+m	377.3 ab	4.84 b	1.85 bc	3.91 bc	6.26	2.00	0.07	0.07	0.0002
N+rm	280.6 ab	4.10 b	1.97 b	3.63 bc	16.78	1.65	0.05	0.06	0.0001
L. muehlberianus									
NP	1063.7 ab	8.79 b	10.09 a	19.34 ab	1496	2.50	0.04	0.24	0.0064
P	646.0 bc	5.70 bc	4.66 b	16.03 ab	39.48	2.60	0.05	1.17	0.0079
P+r	1257.1 a	11.30 a	8.55 a	22.07 a	12.37	2.75	0.05	1.21	0.0111
P+rm	489.4 cd	3.88 cd	2.71 bc	11.97 abc	9.21	3.35	0.06	0.20	0.0134
N	132.9 d	1.67 d	1.48 c	4.31 bc	5.68	3.05	0.05	0.09	0.0018
N+m	42.8 d	0.93 d	0.71 c	7.78 bc	16.55	4.25	0.05	0.08	0.0003
N+rm	50.6 d	0.77 d	0.54 c	2.16 c	12.34	4.55	0.06	0.06	0.0001
P. elegans									
NP	257.4 a	2.86 a	3.37 a	4.99 a	9.03	3.00	0.05	0.35	0.0104
P	27.1 b	0.43 b	0.25 b	1.91 b	15.35	2.45	0.05	0.68	0.0086
P+r	7.7 b	0.23 b	0.16 b	0.95 b	0	2.15	0.05	0.46	0.0063
P+rm	10.4 b	0.27 b	0.24 b	0.62 b	0	2.10	0.05	0.07	0.0056
N	4.5 b	0.31 b	0.31 b	0.93 b	12.42	2.60	0.05	0.76	0.0006
N+m	7.4 b	0.37 b	0.37 b	0.93 b	1.81	2.85	0.06	0.11	0.0004
N+rm	4.5 b	0.14 b	0.14 b	0.49 b	3.46	3.25	0.07	0.11	0.0003

*Values followed by the same letter(s) in the columns are not statistically different at 5% level determined by Duncan test.

and four mm in diameter (Fig. 2 and 3). Nodulation was suppressed by N-fertilizer and/or P deficiency (N, N+m and N+rm). The highest numbers and dry weight of nodules were observed in *E. contortisiliquum*, *I. laurina* and *L. muehlbergianus* plants grown in P, P+r and P+rm treatments (Fig. 3 and Tab. 2), showing the success of rhizobia inoculation in these plants.

Nitrogenase activity (ARA) and leghemoglobin content were higher in *E. contortisiliquum* and *L. muehlbergianus* than *I. laurina* and *P. elegans* (Tab. 2). Due to the low numbers and small size of nodules from *P. elegans*, we were unable to obtain accurate data on nodule mass, nitrogenase activity and leghemoglobin content.

Discussion

Phosphorus nutrition appeared to be the major factor influencing growth and nodulation of these species. Low phosphorus availability is the limiting factor for plant growth in more than 60% of tropical soils and this affects particularly symbiotic legumes, which usually have higher P requirements than non-symbiotic plants (Vadez *et al.* 1997), partly associated with their N_2 fixing capability (Frioni *et al.* 1999). Similar P effects have been reported in *Leucaena leucocephala* (Lam.) de Wit. by Brandon *et al.* (1997). In our experiments, *E. contortisiliquum* and *L. muehlbergianus* grew well in the presence of
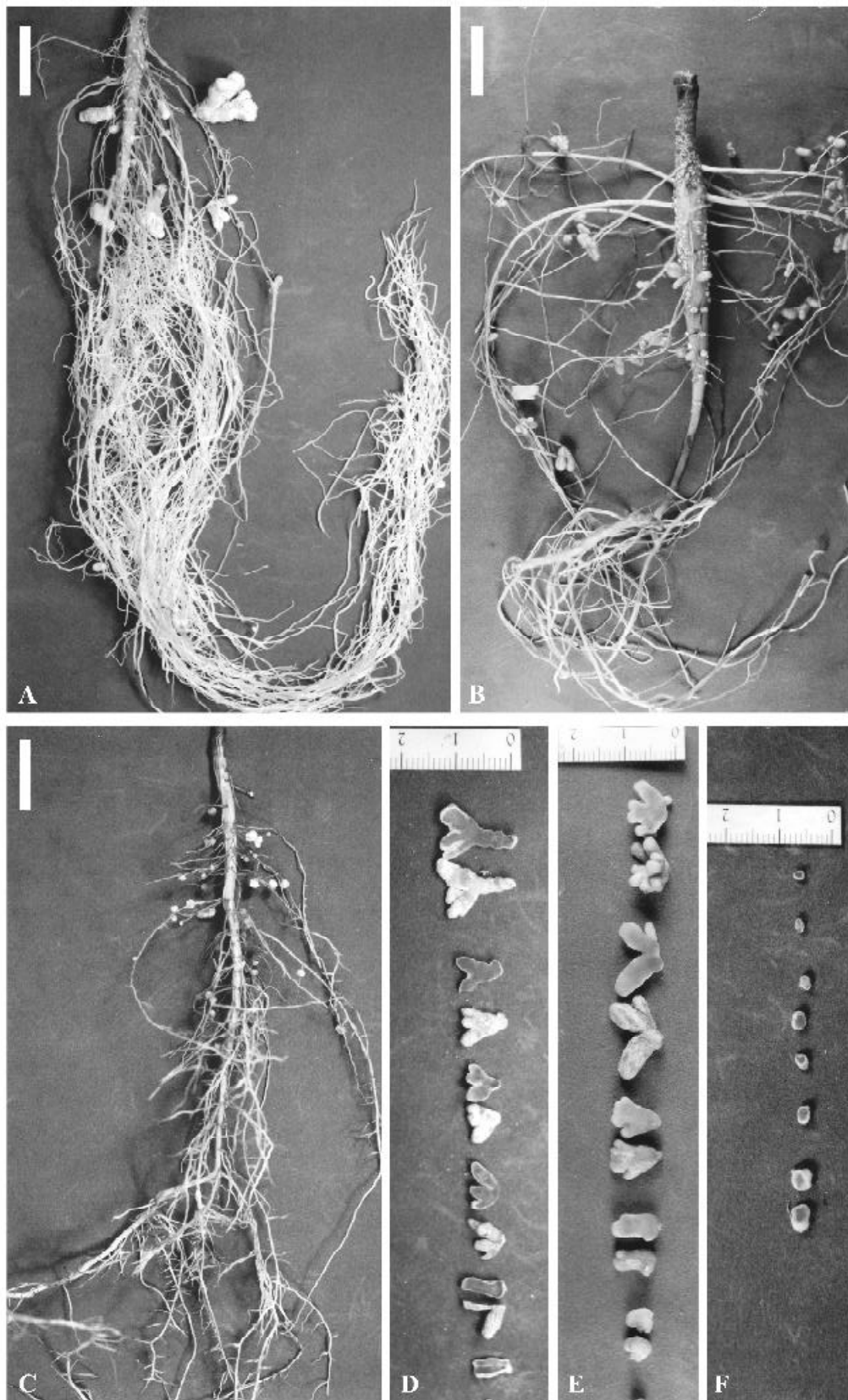
Figures 2A-C. Nodulated roots at 120 days. A. *Enterolobium contortisiliquum* (Vell. Conc.) Morong (bar = 2 cm). B. *Lonchocarpus muehlbergianus* Hassl (bar = 2 cm). C. *Inga laurina* (Sw.) Willd. (bar = 2 cm). D-F. Excised nodules evidencing internal reddish hue due to leghemoglobin content. D. *E. contortisiliquum.* E. *L. muehlbergianus.* F. *I. laurina.*

P-fertilizer treatment when dependent on biological nitrogen fixation by rhizobia (no N added), since N content in shoot tissue was high.

As demonstrated by Siqueira *et al.* (1998) in a study comprising 28 native species in woodland fragments in the state of Minas Gerais, Brazil, the response to treatments of P fertilization and mycorrhiza inoculation diminish with advance in succession. This agrees with our results, where *I. laurina* and *P. elegans*, late secondary or climax species (SMA,

2001), were less responsive to the P treatments than the pioneer trees *E. contortisiliquum* and *L. muehlbergianus*. In addition, under nutrient stress conditions mycorrhizal effect on initial seedlings growth is greater in small seeded species (Siqueira *et al.* 1998). Seeds of *E. contortisiliquum* and *L. muehlbergianus* are smaller (3.600 and 1.160 seeds kg^{-1}, respectively, according to Lorenzi, 1992) than *I. laurina* and *P. elegans* seeds (530 and 770 seeds kg^{-1}, respectively, according to Lorenzi 1992; 1994).
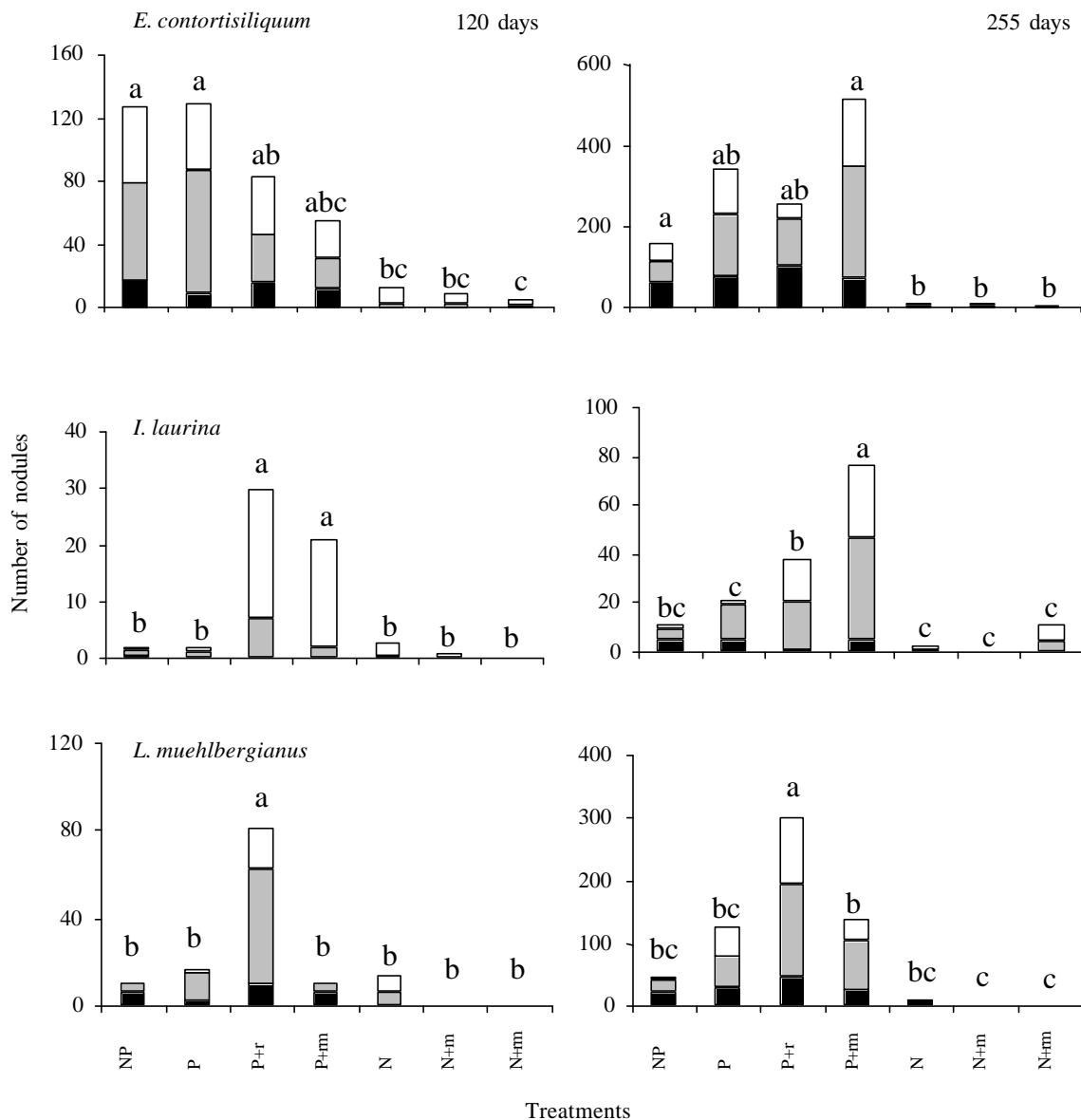

Figure 3. Average number of nodules per diameter size class (larger than 4 mm, between 2 and 4 mm and smaller than 2 mm) of *Enterolobium contortisiliquum* (Vell. Conc.) Morong, *Inga laurina* (Sw.) Willd. and *Lonchocarpus muehlbergianus* Hassl seedlings in response to treatments of mineral fertilization (NP, P, N) and inoculation with rhizobia (r), mycorrhiza (m) or both (rm) at 120 and 255 days of sowing. Total number of nodules followed by the same letter is not significantly different by ANOVA, followed by Duncan's test at *P<0.05*. □ = < 2; ▨ = 2 a 4; ■ > 4.

Table 2. Nodulation parameters of *Enterolobium contortisiliquum* (Vell. Conc.) Morong, *Inga laurina* (Sw.) Willd, *Lonchocarpus muehlbergianus* Hassl and *Platypodium elegans* Vogel seedlings in response to treatments of mineral fertilization (NP, P, N) and inoculation with rhizobia (r), mycorrhiza (m) or both (rm) at 255 days. Legend: (nd) - not determined.

Treatments	Nodule dry weight (g)[1]				ARA[2]	LegHb[3]
	>4 mm	2 a 4 mm	<2 mm	Total	255 days	255 days
E. contortisiliquum						
NP	4.407 ab	0.811 ab	0.098 ab	5.317 a	77.61	0.396
P	2.207 bc	0.213 b	0.018 b	2.439 ab	108.71	0.431
P+r	6.807 a	0.730 ab	0.038 ab	7.577 a	223.03	0.392
P+rm	3.533 abc	1.562 a	0.215 a	5.311 ab	101.23	0.244
N	0.011 c	0.007 b	0	0.018 b	1.98	nd
N+m	0.007 c	0.002 b	0	0.010 b	4.67	nd
N+rm	0.001 c	0.001 b	0	0.003 b	1.87	nd
I. laurina						
NP	0.102 b	0.034 b	0.001 b	0.138 b	12.88	0.264
P	0.350 a	0.021 b	0	0.373 a	11.40	0.372
P+r	0.016 b	0.052 b	0.006 ab	0.075 b	6.64	0.496
P+rm	0.085 b	0.100 a	0.010 a	0.196 b	8.04	0.227
N	0	0.004 b	0	0.005 b	0	nd
N+m	0	0	0	0	nd	nd
N+rm	0.001 b	0.012 b	0	0.015 b	1.47	nd
L. muehlbergians						
NP	1.895 a	0.407 a	0.019 ab	2.321 a	278.13	0.319
P	1.186 ab	0.070 a	0.001 b	1.257 ab	142.07	0.232
P+r	1.376 ab	0.418 a	0.066 a	1.86 a	341.22	0.333
P+rm	0.697 ab	0.257 a	0.030 ab	0.984 ab	321.48	0.234
N	0.01 b	0.006 a	0	0.016 b	9.33	0.273
N+m	0	0	0	0	nd	nd
N+rm	0	0	0	0	nd	nd
P. elegans						
NP	0	0	0	0	nd	nd
P	0	0.00126	0	0.00126	29.92	nd
P+r	0	0.0001	0	0.00016	nd	nd

[1]Values followed by the same letter(s) are not statistically different at 5% level determined by Duncan test; [2]ARA - Acetylene reduction activity in ìmol C_2H_4.plant[-1].h[-1].10[-2]; [3]LegHb - leghemoglobin content in mg Hb.g[-1] fresh nodule.

The increasing concentration of soluble phosphate in the soil normally decreases the percentage colonization of roots by AMF (Smith & Read 1997). In our experiment, the mycorrhizal colonization was relatively low, but the highest values were found in the treatments with higher P level. Moreover, mycorrhizal fungi colonized the seedlings of almost all treatments. This confirms that AMF are an integral part of the root system of almost all plants in the tropics, not only under natural conditions, but also in cultivation. Bereau *et al.* (1997) recorded 68 and 30% of endomycorrhizas in *Inga sp.* in two of 12 sites of the forest of French Guiana. Carneiro *et al.* (1998) characterized the occurrence of AMF of *P. elegans* in the greenhouse as an average of 20 to 49% of infected roots. Frioni *et al.* (1999) reported that *E. contortisiliquum*, *I. uruguensis* and *L. nitidus* had levels of colonization

by AM fungi of 60, 47 and 69%, respectively, in the field.

Due to the low AMF colonization in our experiment, we could not observe the effects of the increase of P acquisition by plants associated with mycorrhizal fungi, as discussed by Colebatch *et al.* (2002). Treseder & Allen (2002) proposed a model where fungi are more efficient scavengers for soil nutrients than plant roots. Nevertheless the time of observation could be extended for some years, once the species studied are perennial trees and 255 days could be short time to observe the AMF infection, colonization and efficiency in the root system.

In our experiment nodulation was high, except for *P. elegans*, where average numbers of nodules were 4.33 and 0.66 for P and P+r treatment, respectively, at the second harvest (data not shown). According to

observations of Moreira (1997), *Inga* species showed averages of 0.4 to 2.6 nodules per plant; with two to seven mg of dry weight and *E. maximum* plants had 51.6 nodules weighing 147 mg. In our experiment, *I. laurina* had about forty times more nodules (approximately 80 nodules per plant), weighing 195 mg (dry) and *E. contortisiliquum* plants about ten times more nodules (mean 500 per plant), weighing 5.310 in the P+rm treatment, compared with Moreira (1997) datas. In addition, *L. muelhbergianus* showed on average 302 nodules per plant in the P+r treatment at 255 days from sowing. Cordeiro *et al.* (1996) examining some developmental and structural aspects of nodules of this species, found a maximum value of 158 nodules per plant.

The N_2 fixing capacity of *E. contortisiliquum*, *I. laurina* and *L. muehlbergianus* was confirmed by the ARA assay. Some values of ARA were reported in the literature for species belonging to the same genera: 2.800 (in µmol ethylene.g^{-1} fresh nodule.h^{-1}) for *I. uraguensis* Hook & Arn. (Frioni *et al.* 1998) and 0.098, 0.18, 0.09, 0.06, 0.1 and 0.016 (in µmol ethylene nodule.h^{-1}) for *E. gummiferum* (Mart.) Macb., *E. mongollo* Mart., *I. capitata* Desv., *I. luschnathiana* Benth., *I. nuda* Salzm. ex Benth., *I. sessilis* (Vell. Conc.) Martius, respectively (Faria *et al.* 1984). The units are different on each case: in g of nodules and per nodule. It is difficult to compare these data firstly because they are from different species and secondly because ARA can be influenced by season, soil temperature drought and photoperiod (Moro *et al.* 1992). Furthermore, acetylene reduction assay can only indicate relative differences in nitrogenase activity; the assay does not measure total nitrogenase activity because the assay itself causes a decline. Nevertheless it can be useful in measuring relative differences in N_2 fixation potential with low levels of technology (Vessey 1994), as used in our experiment to compare the fertilization and inoculation treatments or to compare species, as long as the nodules must have the same type of growth.

Although in our experiments nodulation in *P. elegans* was low, this species is known to fix nitrogen (Tab. 2). Goi *et al.* (1984) detected acetylene reduction activity of 9.2 µmol C_2H_4 per nodule.h^{-1} and Faria *et al.* (1984) obtained 180 µmol C_2H_4 per nodule.h^{-1} in seedlings inoculated by a mixture of 222 strains of rhizobia.

The low nodulation in *P. elegans* may be associated with lack of specificity between host plant and rhizobia, since the strains inoculated were isolated from *L. muehlbergianus*. It could also be related to P deficiency, which can limit nodulation indirectly by reducing legume growth, rather than by direct action on the infection process (Bordeleau & Prévost 1994). Thus, the slow growth of *P. elegans* and the low values of AMF colonization and nodulation (number of nodules, ARA and leghemoglobin content) show that this species must be more studied in relation to mineral nutrition.

The extent of root colonization cannot be an indication of AMF's ability to enhance plant growth as observed by and according to Roldan-Fajardo (1994) and Marques *et al.* (2001). An effective mycorrhizal colonization may affect nodule weight, leghemoglobin content and nitrogen fixing activity. The growth and development of seedlings in the greenhouse conditions with the use of non-sterilized soil may require only rhizobial inoculation, as found here for *E. contortisiliquum*, *P. elegans* and *L. muehlbergianus* plants that can form mycorrhizas with soil fungi. However, better results might be obtained with the use of selected mycorrhizal fungi. Our results support the evaluation of the effects of dual inoculation of these legume tree species under field conditions, as a way of enhancing plant survival.

Acknowledgements

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