Fixação de nitrogênio e micorrização em leguminosas de mata ciliar

CAMILA MAISTRO PATREZE

Dissertação apresentada ao Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Rio Claro, para a obtenção do título de Mestre em Ciências Biológicas (Área de Concentração: Biologia Vegetal).

Rio Claro
Estado de São Paulo – Brasil
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*Aos meus pais **João** e **Ângela***
*e minhas irmãs **Flávia** e **Larissa**,*
- minha família querida -
dedico este trabalho.

Metade (Oswaldo Montenegro)

Que a força do medo que tenho não me impeça de ver o que anseio.
Que a morte de tudo que acredito não me tape os ouvidos e a boca.
Porque metade de mim é o que eu grito,
mas a outra metade é silêncio.
Que a música que eu ouço ao longe seja linda, ainda que triste.
Que a mulher que eu amo seja sempre amada, mesmo que distante.
Porque metade de mim é partida e a outra metade é saudade.
Que as palavras que eu falo não sejam ouvidas como prece nem repetidas com fervor,
Apenas respeitadas como a única coisa que resta a um homem inundado de sentimento.
Porque metade de mim é o que eu ouço,
mas a outra metade é o que calo.
Que essa minha vontade de ir embora se transforme na calma e na paz que eu mereço,
Que essa tensão que me corrói por dentro seja um dia recompensada.

Porque metade de mim é o que eu penso,
e a outra metade é um vulcão.
Que o medo da solidão se afaste, que o convívio comigo mesmo se torne ao menos suportável
Que o espelho reflita em meu rosto o doce sorriso que eu me lembro de ter dado na infância.
Porque metade de mim é a lembrança do que fui,
a outra metade eu não sei...
Que não seja preciso mais do que uma simples alegria para me fazer aquietar o espírito.
E que o teu silêncio me fale cada vez mais.
Porque metade de mim é abrigo, mas a outra metade é cansaço.
Que a arte nos aponte uma resposta, mesmo que ela não saiba, e que ninguém a tente
Complicar porque é preciso simplicidade para fazê-la florescer.
Porque metade de mim é a platéia e a outra metade, a canção.
E que minha loucura seja perdoada.
Porque metade de mim é amor e a outra metade... também.

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RESUMO

Algumas leguminosas formam simbiose mutualística com bactérias fixadoras de nitrogênio e com fungos micorrízicos arbusculares. Conseqüentemente, estas plantas podem crescer mais rapidamente, além de enriquecer o solo com nitrogênio, fósforo e outros nutrientes. O presente trabalho analisou a nodulação, colonização micorrízica, crescimento e desenvolvimento iniciais de *Anadenanthera colubrina* (Vell. Conc.) Brenan. (angico-branco); *Mimosa bimucronata* (DC.) Kuntze (espinho-de-maricá); *Parapiptadenia rigida* (Benth.) Brenan (angico-vermelho); *Enterolobium contortisiliquum* (Vell. Conc.) Morong (tamboril); *Inga laurina* (Sw.) Willd. (ingá); *Platypodium elegans* Vogel (jacarandá-banana) e *Lonchocarpus muehlbergianus* Hassl (embira-de-sapo), usando solo de mata ciliar não esterilizado, adubação mineral e inoculação de rizóbio e micorriza, em viveiro. Todas as espécies foram colonizadas por fungos micorrízicos (inoculados e nativos) e nodularam. Somente *P. rigida* e *P. elegans* não apresentaram nodulação espontânea. A adição de nitrogênio mineral inibiu o número e a massa seca de nódulos, a atividade da nitrogenase e o teor de leg-hemoglobina em todas as espécies. A adição de fósforo mineral diminuiu a colonização micorrízica somente em *A. colubrina* e *M. bimucronata.* A inoculação dos fungos não afetou o crescimento das plantas e não favoreceu a absorção de fósforo; entretanto, a inoculação de rizóbio favoreceu a nodulação de *A. colubrina* e a nodulação e crescimento de *M .bimucronata, L. muehlbergianus e E.*

contortisiliquum em tratamentos inoculados apenas com rizóbio ou conjuntamente com fungo micorrízico.

Nitrogen fixation and mycorrhizal in legumes from riparian forest

Camila Maistro Patreze
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ABSTRACT

Some legumes develop mutualistic symbiosis with nitrogen-fixing bacteria, as rhizobia and arbuscular mycorrhizal fungus. As a result, these plants can grow more rapidly and also to amend the soil with nitrogen, phosphorus and others nutrients. The present study examined the nodulation, mycorrhizal colonization, initial growth and development of seven species: *Anadenanthera colubrina* (Vell. Conc.) Brenan. (angico-branco); *Mimosa bimucronata* (DC.) Kuntze (espinho-de-maricá); *Parapiptadenia rigida* (Benth.) Brenan (angico-vermelho); *Enterolobium contortisiliquum* (Vell. Conc.) Morong (tamboril); *Inga laurina* (Sw.) Willd. (ingá); *Platypodium elegans* Vogel (jacarandá-banana) and *Lonchocarpus muehlbergianus* Hassl (embira-de-sapo), using riparian forest soil non-sterilized, mineral fertilization and inoculation of rhizobia and mycorrhiza, in the nursery. All species were colonized by mycorrhizal fungus (inoculated and native) and were nodulated. Only *P. rigida* and *P. elegans* did not show spontaneous nodulation. The mineral nitrogen added inhibited nodules number and dry weight, nitrogenase activity and leghemoglobin content in all species. The mineral phosphorus added diminished the mycorrhizal colonization only in *A. colubrina* and *M. bimucronata.* Fungi inoculation did not affect the plants growth and neither support the P uptake; however, rhizobial inoculation supported the nodulation of *A. colubrina* and the nodulation and growth of *M. bimucronata, L. muehlbergianus* and *E. contortisiliquum* in inoculated treatments only with rhizobia or associated with mycorrhizal fungusinoculation.

1. INTRODUÇÃO

1.1. Considerações sobre as matas ciliares e as leguminosas

As matas ciliares ou florestas ripárias são importantes facilitadoras dos fluxos biológicos, reduzem a erosão, estabilizam as margens dos rios e exercem função protetora sobre os recursos naturais bióticos e abióticos. Esta vegetação apresenta ampla distribuição pelo território nacional e grande heterogeneidade florística (Rodrigues & Nave 2000). No entanto, apesar das matas ciliares terem sido incluídas como áreas de preservação permanente (Lei n. 4771/65 do Código Florestal Brasileiro), elas vem sendo degradadas e/ou destruídas por interesses que, muitas vezes, desrespeitam o ambiente e as relações ecológicas.

A família Leguminosae representa uma parcela significativa na composição florística de vários ecossistemas, inclusive nas matas ciliares. Gibbs & Leitão Filho (1978) encontraram 10 representantes desta família em uma área de floresta de galeria próxima de Mogi Guaçu - SP, tendo sido esta a família que apresentou maior número de espécies. Bernacci *et al*. (1998) constataram uma segunda maior riqueza de espécies pertencentes à subfamília Fabaceae em 15 fragmentos florestais ripários da Bacia do Jacaré- Pepira – SP

e, se considerarmos também Mimosaceae e Caesalpiniaceae como subfamílias da família Leguminosae (Polhill & Raven, 1981), esta família apresentará maior riqueza. Segundo Leitão Filho (1982), o estrato superior das matas ciliares apresenta uma clara dominância de espécies da família Leguminosae.

Plantas dessa família podem crescer mais rapidamente e ter importante papel na recuperação de áreas florestais, uma vez que elas são capazes de se associar a bactérias conhecidas como rizóbios e também a fungos micorrízicos, relacionados ao processo de fixação do nitrogênio atmosférico e à maior absorção de fósforo e alguns nutrientes, respectivamente. Estas associações garantem um suprimento adequado de nutrientes para a planta, além de aumentar o potencial de enriquecimento do solo.

1.2. A simbiose rizóbio-leguminosa

A associação entre rizóbios e leguminosas traz benefícios para ambos os simbiontes e é visualizada comumente pela presença de nódulos na superfície das raízes. A nodulação, quer seja expontânea ou através da inoculação de estirpes do rizóbio, pode apresentar diferentes graus de eficiência na fixação biológica de nitrogênio (FBN). A FBN é a incorporação de dinitrogênio (N_2) à planta por meio de sua transformação em amônia realizada por diversos seres vivos, entre os quais, o rizóbio. A nodulação não parece freqüente na subfamília Caesalpinioideae (23%), mas é muito freqüente em Mimosoideae (90%) e Papilionoideae (97% das espécies estudadas) (Roggy & Prévost, 1999). Apesar disso, 40% dos 643 gêneros de Leguminosae ainda não foram estudados quanto à capacidade de nodular e fixar nitrogênio (Sprent, 2001).

A eficiência do processo de fixação de nitrogênio pode ser analisada observando-se os efeitos dessa associação na planta hospedeira, tais como no crescimento da parte aérea e

do sistema radicular, no aumento da área foliar, teor de nitrogênio, do número de nódulos e na disposição destes no sistema radicular, entre outros. Além disso, o teor de leg-hemoglobina (Lb), coloração dos nódulos e atividade da enzima nitrogenase podem evidenciar o potencial de fixação.

A enzima nitrogenase é responsável pela catálise do nitrogênio atmosférico (N_2) e sua transformação em amônia (NH_3), que corresponde à fixação do nitrogênio propriamente dita. Essa atividade é regulada por nodulinas, como a Lb, produzidas pela planta em associação. A Lb tem por função transportar oxigênio para o bacteróide na zona central do nódulo, devido à alta sensibilidade que a enzima nitrogenase apresenta em relação ao oxigênio, apesar do rizóbio ser aeróbico. Na região central do nódulo a concentração de oxigênio é baixa, e conseqüentemente não inativa o complexo nitrogenase (Bruijn *et al*., 1994).

A associação com rizóbio ocorre na natureza geralmente quando há deficiência de nitrogênio no solo, pois o processo de fixação biológica é altamente energético. A aplicação de nitrogênio combinado na forma de nitrato ou uréia pode inibir a infecção das raízes por rizóbio ou inibir a nodulação e também a atividade da enzima nitrogenase. O nitrato, na forma KNO_3, foi inibitório para o crescimento e nodulação de *Anadenanthera colubrina* e *Anadenanthera peregrina* nos experimentos conduzidos por Mendonça & Schiavinato (1996).

Entretanto alguns pesquisadores enfatizam que a aplicação de nitrogênio combinado é requerida para maximizar a produção ou ainda que, em concentrações baixas, doses iniciais de nitrogênio combinado podem estimular a fixação de nitrogênio (Becker *et al.* 1991), principalmente em espécies anuais. Estes autores observaram em *Sesbania rostrata* que o aumento na quantidade de N mineral inibiu a nitrogenase (ARA) e diminuiu o número de nódulos nas raízes, mas não afetou em nódulos caulinares e, no campo, uma

dose inicial de 30 Kg ha^{-1} estimulou a FBN, o crescimento e a acumulação de nitrogênio. Desta maneira, a inibição ou o estímulo do processo de FBN pela presença de nitrogênio no solo dependem do requerimento nutricional da planta, da concentração e formas de nitrogênio disponíveis no solo.

Há uma certa especificidade entre o rizóbio e a planta hospedeira que depende de propriedades intrínsecas dos simbiontes, compatibilidade genética e da influência ambiental. Os principais fatores ambientais que ocorrem nos trópicos e influenciam a FBN são alta temperatura, déficit hídrico e acidez do solo, a qual influencia no balanço nutricional do mesmo, favorecendo o aumento na concentração ou a deficiência de determinados nutrientes. Assim, a toxidez por Al provocada pela acidez do solo pode provocar a deficiência de P e Mo e o aumento do pH aumenta a disponibilidade de Ca para a planta e para a bactéria (Hungria & Vargas, 2000).

1.3. Micorrizas vesículo-arbusculares (MVA)

Micorriza é um termo geral para designar as associações mutualísticas entre certos fungos do solo e as raízes da maioria das espécies vegetais. Existem vários tipos de micorrizas, sendo as MVA as de maior importância ecológica (Smith & Read, 1997). Nessa associação morfológica e fisiológica, a planta beneficia-se pelo aumento da absorção de água e nutrientes, principalmente o fósforo, proporcionado pelas hifas fúngicas, que funcionam como uma extensão do sistema radicular (Lopes *et al*. 1983), enquanto fornece ao fungo fotoassimilados permitindo que ele complete seu ciclo, o que só ocorre na presença da planta hospedeira (Smith & Read, 1997). Além disso, estas associações são conhecidas por aumentar a resistência das plantas a estresses ambientais e a patógenos (Hirsh & Kapulnik, 1998).

O fungo funciona como um "competidor" por produtos elaborados pela planta, pois ele aumenta a respiração das raízes e causa acúmulo de carbono no citoplasma das células corticais. Para compensar essas perdas, plantas colonizadas exibem maiores taxas fotossintéticas. O balanço entre o efeito benéfico do fungo na maior absorção de nutrientes e a utilização dos produtos da planta geralmente resulta em aumento no crescimento das plantas colonizadas, mas pode também resultar em redução no caso de plantas crescendo em altos níveis de P disponível (Lopes *et al.* 1983). Entretanto, a fertilização com N ou P pode aumentar o crescimento dos fungos micorrízicos onde há uma limitação inicial de nutrientes, diminuir o crescimento micorrízico onde as plantas são limitadas em termos nutricionais mas os fungos não o são e pode não ter efeito no crescimento dos fungos quando nenhum organismo é limitado em termos nutricionais (Treseder & Allen, 2002).

Os fungos MVA são atualmente classificados na ordem Glomales, nos gêneros *Glomus, Acaulospora, Scutellospora, Gigaspora, Paraglomus* e *Archaeospora* (Morton & Redecker, 2001) e são conhecidos por ocorrerem na maioria das plantas tropicais, sendo uma parte integral do sistema radicular de quase todas as plantas nos trópicos, não somente sob condições naturais mas também em condições de cultivo (Michelsen, 1992) e apresentar, geralmente, baixa especificidade (Zhu *et al.* 2000).

Os fungos são simbiontes obrigatórios que na ausência de uma planta hospedeira estão presentes no solo como esporos multinucleados envolvidos por uma parede celular ou como fragmentos de hifas em raízes mortas ou secas. A germinação do esporo, proliferação e ramificação das hifas são estimuladas por sinais moleculares sintetizados e secretados pela raiz. Essas moléculas são geralmente flavonóides, várias das quais também servem como sinais nas simbioses rizóbio-leguminosas (Hirsch & Kapulnik, 1998).

Embora seja possível quantificar a colonização micorrízica, é necessário levar em consideração as interações fisiológicas entre os fungos e as plantas hospedeiras e também

medidas da viabilidade dos fungos uma vez que a biomassa nem sempre reflete a eficiência das associações simbióticas em termos de aumento de crescimento da planta hospedeira (Zhao *et al.* 1997)

1.4. As relações rizóbio-micorriza-leguminosas

Os fungos MVA também podem aumentar a performance de leguminosas inoculadas por rizóbio (Barea *et al*. 1992, Abd-Alla *et al.* 2000), sendo portanto benéfica a dupla inoculação de rizóbio e esses fungos. A dupla inoculação melhora a aquisição de nutrientes, ajuda as plantas a se estabelecerem no campo e confere resistência ao estresse hídrico (Herrera *et al.*, 1993). A presença de fungos micorrízicos e o aumento na fixação de N_2 também podem estar relacionados ao fato de que plantas micorrizadas podem ter altas concentrações de P, Zn, e Cu, os quais influenciam a nodulação e fixação de N_2. (Redente & Reeves, 1981).

Existem muitos estudos envolvendo a dupla inoculação em plantas de interesse agronômico (Ross & Harper, 1970; Mosse, 1977; Abbott & Robson, 1977; Redente & Reeves, 1981; Barea & Azcon-Aguilar, 1983, Ames & Bethlenfalvay (1987), Vejsadová *et al.*, 1992, Abd-Alla, 2000; Goss & Varennes, 2002), sendo que a maior parte deles envolve a soja. Os trabalhos de dupla inoculação em espécies arbóreas florestais são geralmente conduzidos em viveiro, enfocando a posterior utilização das plantas em sistemas agroflorestais ou objetivando a recuperação de áreas degradadas.

No campo, Bakarr *et al.* (1996), ao examinar as raízes de espécies arbóreas em uma floresta plantada e um sítio de reflorestamento na África, encontraram 7, de 11 espécies de Mimosoideae e 2, de 3 espécies de Papilionoideae com dupla infecção por rizóbio e micorriza. Marques *et al.* (2001) trabalhando com *Centrolobium tomentosum* (araribá)

cultivado em mata ciliar observaram que a dupla inoculação com estirpes selecionadas de rizóbio e fungos micorrízicos melhoraram o crescimento dessa leguminosa nativa da Floresta Atlântica Brasileira.

Gonçalves (2000) estudou o crescimento de *Dalbergia nigra* (jacarandá-da-bahia) inoculada com rizóbio e fungos micorrízicos em área ciliar e constatou que os níveis de nitrogênio foliar da espécie estudada consolidaram a efetiva contribuição dos inoculantes para o enriquecimento do solo, especialmente com nitrogênio, favorecendo o crescimento das espécies consorciadas. Entretanto, Santiago *et al*. (2002) observaram que a co-inoculação de uma estirpe de rizóbio e fungo micorrízico não aumentaram o peso seco desta mesma espécie vegetal, conteúdo de nitrogênio e fósforo ou colonização micorrízica em solo de floresta Atlântica e solo de eucalipto. O tempo de análise dos dados pode não ter sido suficiente para a observação dos efeitos dos simbiontes no crescimento do jacarandá-da-bahia, uma vez que essa espécie apresenta crescimento lento e os dados analisados por esses autores foram em plantas de até 12 meses de idade.

Em condições de viveiro, Rocha (1995) observou em plantas de *Acacia mangium* que a nodulação foi favorecida pela pré-colonização com fungos micorrízicos, independentemente das doses de P testadas, apesar das plantas não terem sido inoculadas com rizóbio. Outras espécies de *Acacia* foram estudadas por De La Cruz *et al.* (1988) Khan & Uniyal (1999) e Munro *et al.* (1999). Khan & Uniyal (1999) observaram maior crescimento em termos de biomassa seca nos tratamentos com dupla inoculação seguido da inoculação simples com micorriza em *A. nilotica*, e, Munro *et al.* (1999), verificaram que a nodulação foi baixa em todos os tratamentos de *A. tortilis*, embora tenham sido inoculados com rizóbio, mas a micorrização melhorou a infecção micorrízica e o crescimento das plântulas em solo não esterilizado. Esses últimos autores sugerem que os métodos de inoculação por eles aplicados podem ser adaptados a condições de viveiro a baixo custo.

Burity *et al*. (2000) verificaram que mudas de *Mimosa caesalpiniifolia* com dupla inoculação apresentaram valores significativos no crescimento, área foliar, atividade da enzima nitrogenase e porcentagem de colonização radicular, independentemente do nível de P usado. Khan *et al.* (2001) investigaram o crescimento de *Dalbergia sissoo* e verificaram que a inoculação apenas com micorriza pode reduzir a dependência de *D. sissoo* da fertilização. Entretanto, todos os tratamentos com combinações entre rizóbio e micorriza não diferiram dos três tratamentos com diferentes doses do fertilizante testado. Ingleby et al (2001) concluíram que a inoculação micorrízica de *Calliandra calothyrsus* tem o potencial de aumentar o crescimento e a nodulação das plântulas em casa de vegetação.

Além destes estudos, há as revisões de Hirsch & Kapulnik (1998), sobre a transmissão de sinais em associações micorrízicas em comparação com a simbiose *Rhizobium* – leguminosas, onde é discutido que alguns sinais moleculares como flavonóides produzidos pela raiz são conservados em ambas as simbioses e de Provorov *et al.* (2002), sobre desenvolvimento da genética e evolução de estruturas simbióticas em nódulos fixadores de nitrogênio e micorrizas arbusculares.

Pode-se contudo perceber que ainda existem muitas espécies a serem testadas quanto a dupla inoculação, o que requer uma "simbiose" entre os estudos de taxonomia, anatomia, fisiologia, genética, evolução e ecologia desses microrganismos e das relações destes com o sistema radicular das plantas hospedeiras nas mais diversas condições ambientais.

2. OBJETIVOS

2.1 Objetivos gerais

Estudar o potencial de fixação de nitrogênio e micorrização de 7 espécies de leguminosas que ocorrem em matas ciliares brasileiras e fornecer subsídios para utilização dessas espécies em plantios de recuperação de áreas alteradas, sendo elas:

Mimosoideae (Mimosae): *Anadenanthera colubrina* (Vell.Conc.) Brenan. (angico-branco); *Mimosa bimucronata* (DC.) Kuntze (espinho-de-maricá); *Parapiptadenia rigida* (Benth.) Brenan (angico-vermelho);

Mimosoideae (Ingeae): *Enterolobium contortisiliquum* (Vell. Conc.) Morong (tamboril); *Inga laurina* (Sw.) Willd. (ingá);

Papilionoideae (Tephrosieae): *Platypodium elegans* Vogel (jacarandá-banana); *Lonchocarpus muehlbergianus* Hassl (embira-de-sapo).

2.2 Objetivos específicos

1- Verificar se a fertilização com adição de N mineral inibe a nodulação das plantas;

2- Verificar se a fertilização com P inibe a colonização micorrízica;

3- Verificar a ocorrência de nodulação e colonização micorrízica em tratamentos não inoculados com rizóbio ou micorriza;

4- Verificar se a colonização micorrízica é favorecida pelas inoculações simples ou dupla de rizóbio e fungos MVA;

5- Verificar o crescimento das plantas no tratamento com deficiência em N e inoculação com rizóbio ou com rizóbio e micorriza, comparado ao crescimento das plantas do mesmo tratamento, porém não inoculadas;

6- Verificar o crescimento das plantas no tratamento com deficiência em P e inoculação com micorriza ou com rizóbio e micorriza, comparado ao crescimento das plantas do mesmo tratamento, porém não inoculadas;

7- Verificar se a nodulação, atividade da nitrogenase e os teores de leg-hemoglobina são aumentados em tratamentos com inoculação de rizóbio.

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CAPÍTULO 1

Nitrogen-fixing and vesicular-arbuscular mycorrhizal symbioses in tropical tree of tribe Mimoseae (Leguminosae-Mimosoideae)

(Trabalho a ser enviado para a revista *Plant and Soil*)

Symbioses in tropical tree of Mimoseae

Nitrogen-fixing and vesicular-arbuscular mycorrhizal symbioses in tropical tree of tribe Mimoseae (Leguminosae-Mimosoideae)

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Abstract

Response to mineral fertilization and dual inoculation with rhizobia and arbuscular mycorrhiza fungi (AMF) were studied in nursery conditions in *Anadenanthera colubrina*, *Mimosa bimucronata* and *Parapiptadenia rigida* legume native trees from Brazilian riparian forests. Each species was submitted to seven treatments, being fertilization with N and P added, with P without N and with N without P and inoculated or not with rhizobia (r), mycorrhiza (m) or both (rm), respectively: NP, P, P+r, P+rm, N, N+m and N+rm. Results showed that P uptake by symbionts was not sufficient to sustain good growth plants of three species. Native fungi infected these three hosts and AMF inoculations not enhanced the mycorrhizal colonization. Also, the P treatments affected negatively the AMF colonization in *A. colubrina* and *M. bimucronata*, but not in *P. rigida*. The absence of mineral N in *A. colubrina* and *P. rigida* plants not fertilized with this element limited the growth them. This probably mineral nitrogen deficiency in *M. bimucronata* was supplied by biological nitrogen fixation. Spontaneous nodulation occurred in *A. colubrina* and *M. bimucronata.* In N treatments, this element inhibited the nodulation. *A. colubrina* and *M. bimucronata* plants of P+r treatment and P+r and P+rm treatments, respectively, had increased in nodules number, nitrogenase activity and leghemoglobin content, being that the growth and development of *M. bimucronata* seedlings were positively influenced by nodulation. Thus, extents of rhizobial and mycorrhizal symbiosis in this specie upon nursery conditions can amend post-planting success.

Key words

Biological nitrogen fixation, Mimoseae, mycorrhiza, rhizobia, riparian forest, symbiosis.

Introduction

The Mimoseae tribe (Leguminosae- Mimosoideae) comprises several genera as *Anadenanthera, Mimosa* and *Parapiptadenia* which are common in lowland tropical rainforests, especially near rivers and lakes (Polhill et al., 1981). *Anadenanthera colubrina* (Vell.) Brenam, *Mimosa bimucronata* (DC) O. Kuntze and *Parapiptadenia rigida* (Benth) Brenan are legume trees which have been sampled in various floristic surveys of riparian forests in Brazil. *A. colubrina* was sampled by Nilsson (1989), Silva et al. (1992), Vilela et al. (1995), Santarelli (1996); Bernacci et al. (1998), Dias et al. (1998) and Sampaio et al. (2000). *M. bimucronata* was founded in floristic studies of Santarelli (1996) and Metzger el al. (1998) and *P. rigida* was sampled by Nilsson (1989) and Dias et al. (1998).

The most of Mimoseae plants, including the species cited above, are capable of fixing dinitrogen in association with root nodule bacteria (rhizobia) and increases phosphorus contents and others nutrients by association with arbuscular mycorrhizal fungi (AMF). Nodulation is very frequent in the subfamily Mimosoideae (90% of studied species) (Sprent, 1995; according to the taxonomy of Polhill et al., 1981) and the AMF are of widespread occurrence (Gerdemann, 1968; Trufem, 1990) beyond to represent the natural status of most plant species (Siqueira et al., 1998). Also, AMF symbiosis may improve defense herbivores, enhance the acquisition of water (Herrera et al., 1993) and increase a plant resistance to pathogens (Moora and Zobel, 1998).

Many previous studies, mainly on cultivated plants, have shown that dual inoculation of legumes with rhizobia and AMF increases plant growth (Ross and Harper, 1970; Mosse et al., 1976; Redente and Reeves, 1981; Abd-Alla, 2000). In West Africa

were found dual infections involving AMF and bacterial nodules in seven forest and plantation of tropical legume trees of Mimosoideae (Bakarr et al., 1996). In Brazil, Franco and Faria (1997) have leaded studies of nodulated and mycorrhizal legume trees to revegetate poor or depleted soils with the goal to restarte their fertility. Little information is available about symbiotic relationships of dual inoculation in Brazilian legume native tree whereas we require ecophysiological knowing of these species in nursery conditions. Burity et al. (2000) recorded that the plants growth and nodulation of *M. caesalphiniifolia* were improved by mycorrhiza.

Information on nodulation or only reporting the capacity of nodulation in *A. colubrina* (Mendonça and Schiavinato, 1996), *M. bimucronata* (Campello, 1996) and *P. rigida* (Lechtova-Trnka, 1931; Rothschild, 1970; Corby, 1988) contributes to studies of both rhizobia and AMF inoculations, which were not reported for these species yet. In addition, they contribute with important information about the use of these species as revegetation on drastically disturbed lands.

The aim of our study was to investigate nitrogen-fixing and arbuscular mycorrhizal symbioses of three Mimoseae species that occur in Brazilian riparian forest. We submitted *A. colubrina, M. bimucronata* and *P. rigida* to treatments of mineral fertilization and inoculation with rhizobia and AMF in nursery conditions in order to aid the choice of species to recover riparian forests.

Materials and methods

Rhizobia inoculum

Rhizobia isolates were obtained from root nodules collected from ***Anadenanthera peregrina*** (L.) Speg and ***Mimosa bimucronata*** plants growing in the field of Unesp

(Universidade Estadual Paulista "Júlio de Mesquita Filho")- Rio Claro, SP, Brazil (22º44'S and 47º33'W, 610 m a.s.l.) and riparian forest of Corumbataí, SP, Brazil (22º 20'S and 47º40'W, 604 m a.s.l.), respectively. The isolates were grown on yeast extract mannitol (YEM) agar at 28ºC and stored in the Rhizobial Bank of Unesp Rio Claro, SP Brazil with numbers IBRC 199 and 200 for *A. peregrina* and IBRC 201 and 204 for *M. bimucronata* isolates. Aerial parts of these plants were pressed and catalogued in the Bioscience Institute Herbarium –UNESP as a number HRCB-34330 and 34501, respectively.

Source and germination of seeds

Seeds of *A. colubrina* were supplied by IPEF (Instituto de Pesquisas e Estudos Florestais) da Esalq/USP (Escola Superior de Agricultura Luiz de Queiroz/Universidade de São Paulo) and seeds of *P. rigida,* supplied by IF (Instituto Florestal de São Paulo). Seeds of *M. bimucronata* and the soil used as substrate were collected in the riparian forest of Corumbataí, SP, Brazil (22º 20'S and 47º40'W, 604 m a.s.l.). All seeds had their surface sterilized and were germinated in 4L plastic pots containing a non-sterilized mixture (2:1) of soil (Table 1) and vermiculite. *M. bimucronata* required dormancy break treatment with imbibitions in boiling water for 30 seconds.

Table 1 Chemical analysis of substrate used in the experiment before the applications of mineral nutrients and inoculations.

N	P Resina	M.O.	pH	K	Ca	Mg	H+Al	Al	SB	CTC	V	B	Cu	Fe	Mn	Zn	S
ppm	$mg.dm^{-3}$	$g.dm^{3}$	$CaCl_2$				$mmol_c.dm^{-3}$				%			$mg.dm^{-3}$			
1000	7	6	5.5	1	12	4	10	7	17	27	63	0.01	0.7	23	8.7	0.4	9

Plant growth

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) and three fertilizations treatments: a full mineral fertilization contained N (40) and P_2O_5 (80) (NP); fertilization with P_2O_5 (80) without N (P) and fertilization with N (40) without P (N). These treatments varied in relation to inoculation of microsymbionts: inoculated or not with rhizobia (r), mycorrhiza (m) or both (rm). In this way, the experiment comprised seven treatments with ten replicates: NP, P, P+r, P+rm, N, N+m and N+rm. For plants from P treatments, we added a start dose of 3.8 m*M* of N. All plants were grown in a greenhouse under natural daylight in randomized blocks. Additional nutrients (10 mL of solution) were added to the surface of each pot every 30 days, according to fertilization treatments above cited.

For rhizobial treatments, seeds were left to soak in a turbid suspension (100 mL) of mixture of isolates IBRC 199 and 200 to inoculate *A. colubrina* and *P. rigida* and IBRC 201 and 204 to *M. bimucronata* during 1 hour. Reinoculation (10 mL of the same mixture) was made near the roots of seedlings 30 days later. For the AMF inoculation, pieces of *A. peregrina* var. *falcata* roots with ±1cm of length collected from Corumbataí cerrado reserve (22º15'S and 47º00'W, 810 m a.s.l.) were mixed on each pot (0.4g) surface nearly seedlings at twenty-eight days of sowing.

Measurements

Height growth of plants was recorded each fifteen days, starting at 30 days of sowing, being measured ten plants per treatment until 120 days and five plants per treatment from 120 days until the end of the experiment, at 255 days of sowing.

Five plants per treatment were harvested at 120 and 255 days of sowing. Leaf area was measured by CI-202 Area Meter (CID, Inc). Roots, stems and leaves were separated, dried to constant weight at 70ºC, weighed and analyzed the nutrient content of the shoot and substrate. Root nodules were weighted, counted and sieved with sieves of mesh to

separate: larger than 4mm, between 2-4mm and smaller than 2mm of diameter size. Nitrogenase activity of root nodules of two plants per treatment was assayed by acetylene reduction activity (ARA) according to Hardy et al. (1968) and nodules were stored in the refrigerator at 10ºC to leghemoglobin content analysis, following Becana et al. (1986). The nodules morphology was classified according to Corby (1981). Standard methods were followed for staining of roots (Philips and Hayman 1970), and the quantification of AMF infection in roots was estimated using the gridline intersect method (Giovannetti and Mosse, 1980) under a stereomicroscope (40x).

Statistical analysis

Data were analyzed separately using ANOVA. Dunn's test was applied to compare means at $P \leq 0.05$ using the program BioEstat 2.0 (Ayres et al., 2000). Principal component analysis (PCA) was performed for all data by PCOrd program, version 4.0 for Windows. Variables were log transformed or arc square root transformed in order to equalize variance. Coefficients correlation $r < 0,05$ in the first axis were eliminated of analysis.

Results

Growth of plants and AMF colonization

Although seedlings were grown under similar nursery conditions, their growth indicated specific responses. *M. bimucronata* presented the best growth, as measured by height, dry biomass and leaf area (Table 2), mainly at 255 days of sowing. The growth parameters were positively affected by full mineral fertilization (NP) in the three species (Table 2). Plants not fertilized with P and N had their growth limited, except *M.*

bimucronata in the P treatments, which mineral nitrogen deficiency was probably supplied by biological fixation with rhizobia.

Table 2: Response of *A. colubrina, M. bimucronata* and *P. rigida* to mineral fertilization and inoculation treatments at 120 and 255 days of sowing. Mean of five plants per treatment. N and P content were measured in the shoot (leaf plus stem).

Treatments	At 120 days						At 255 days					
	Area leaf (cm^2)	Height (cm)[a]	Biomass (g)	AMF (%)[b]	N ($mg.g^{-1}$)	P ($mg.g^{-1}$)	Area leaf (cm^2)	Height (cm)	Biomass (g)	AMF (%)[b]	N ($mg.g^{-1}$)	P ($mg.g^{-1}$)
					Anadenanthera colubrina							
NP	208.4 aA	54.9 aA	7.4 aA	1.7	23.0	2	259.2 aA	94 aB	35.3 aA	0.0	19	2.9
P	41.9 bcA	41.9 abA	4.1 abA	1.3	14.0	2.8	25.3 bcB	51.6 cB	5.7 bB	1.5	17.5	5.6
P+r	33.4 bcA	26.7 bcA	1.5 bcA	2.8	20.0	3.8	59.5 bcB	54.8 bcB	7.1 bB	4.5	15.5	4.2
P+rm	25.1 cAB	15.2 dB	0.5 cB	3.6	20.0	5.4	18.8 cB	55.8 bcB	5.4 bB	2.0	14.5	3.8
N	38.5 bcA	20.2 cdA	0.8 cB	8.1	25.0	1.2	192.1 aA	71.4 abB	11.2 abA	13.5	18	0.8
N+m	28.7 bcB	24.3 cB	0.8 cB	1.4	26.5	0.9	128.4 abA	71 abcB	5.6 bB	14.0	22	0.7
N+rm	66.3 abA	27.8 bA	1.3 bcA	12.4	29.0	1	195.2 aA	76.2 abB	7.2 bB	10.8	18.5	0.7
					Mimosa bimucronata							
NP	195.3 aA	64.6 aA	11.8 aA	32.2	39.5	1.9	530.9 aA	138.2 abA	46.7 aA	46.6	15.5	1.9
P	139.7 aA	53.5 abA	6.8 abA	22.9	11.5	2.1	295.4 abA	161.8 aA	40.8 aA	23.2	10.5	1.6
P+r	78.6 aA	33.6 cdA	2.5 bcA	46.3	21.0	2.8	452.1 aA	143.2 abA	55.6 aA	12.8	17	1.6
P+rm	70.7 aA	28.8 dA	2.4 bcA	43.8	22.0	2.7	225.2 abA	127.4 abcA	38.2 abA	17.3	16.5	1.5
N	74 aA	29.1 dA	2.9 bcA	50.5	14.5	1.0	158.7 abcA	100.8 cA	15.1 cA	70.3	17	0.5
N+m	102.9 aA	39.5 bcA	2.8 bcA	1.9	22.0	0.7	131.3 bA	127.4 abcA	17.4 bcA	68.4	15	0.4
N+rm	67.3 aA	31.5 cdA	1.6 cA	9.6	27.5	1.1	61 cB	114.8 bcA	15.0 cA	70.3	16.5	0.4
					Parapiptadenia rigida							
NP	94.8 aA	12.6 abB	1.6 aB	21.6	22.5	1.8	197.1 aA	40.6 aC	18.02 aB	25.4	23	2.6
P	32.9 bcA	14.3 abB	0.5 aB	12.1	22.5	3.6	53.5 aB	28.4 abB	3.7 cdB	47.0	19	6
P+r	18.6 bcA	8.8 cdB	0.5 aA	13.1	14.5	3.7	53.2 aB	24.2 bcC	2.1 dC	45.6	19.5	4.4
P+rm	16.2 cB	7.2 dC	0.3 aB	35.0	[c]	4.6	29.8 aB	24 bcC	2.5 dB	58.6	22	6.5
N	55.7 abA	11.7 bcB	0.6 aB	10.7	28.0	1.8	142.6 aA	37.2 abC	8.5 bA	20.9	23.5	0.9
N+m	120.8 aA	16 aC	1.8 aAB	21.2	25.5	1.1	71.8 aA	23.2 bcC	4.3 cdB	22.4	27	0.8
N+rm	31.2 bcA	11.2 bcB	0.6 aA	5.0	26.5	0.7	72.9 bA	34.8 abC	6.1 bcB	32.0	23.5	0.8

[a] Mean of ten plants per treatment.

[b] Mean of two plants per treatment.

Means followed by the same letter are not significantly different by ANOVA, test Dunn at $P<0.05$ level of significance. Lower case letters compare treatments within of the one species and upper case letters compare species for the same treatments. No such analyses were indicated for AMF, N and P of shoots.

[c] not determined

M. bimucronata exhibited the highest values for root colonization by AMF (Table 2), which ranged 70.3% of infected roots. *A. colubrina* and *P. rigida* had percentages ranging 14% and 58.6%, respectively. Typical hyphae and vesicles were observed in the preparations of all species. Native-born fungi were capable to infect these three host plants. When we compare the results in the same fertilization treatments, AMF inoculated treatments promoted similar values of infection of roots than those uninoculated. These results indicate that the AMF inoculation not enhanced the mycorrhizal colonization.

Treatments with P added in *A. colubrina* and *M. bimucronata*, regardless of fungi inoculation, presented smaller AMF percents than N treatments, without P added. In *P. rigida* occurred the inverse situation.

Biomass allocation

A variation in resource allocation patterns was observed among species, as well as within species through time, but the treatments did not influence the biomass allocation in the three species studied. *A. colubrina* and *M. bimuconata* plants had more biomass of stem and leaves (Figure 1) while *P. rigida* produced more roots at 120 days of sowing. At 255 days, *A. colubrina* and *P. rigida* increased biomass allocation to roots and *M. bimucronata* remained allocating stem and leaves biomass.

Nodulation

Morphological characteristics of the *A. colubrina, M. bimucronata and P. rigida* nodules result in its being classified as indeterminate and astragaloid, similarly to observations of Corby (1981) and Sprent (2001).

Spontaneous nodulation from native soil born rhizobia occurred in *A. colubrina* and *M. bimucronata* but not in *P. rigida* (Table 3). In *A. colubrina* and *P. rigida* plants from all

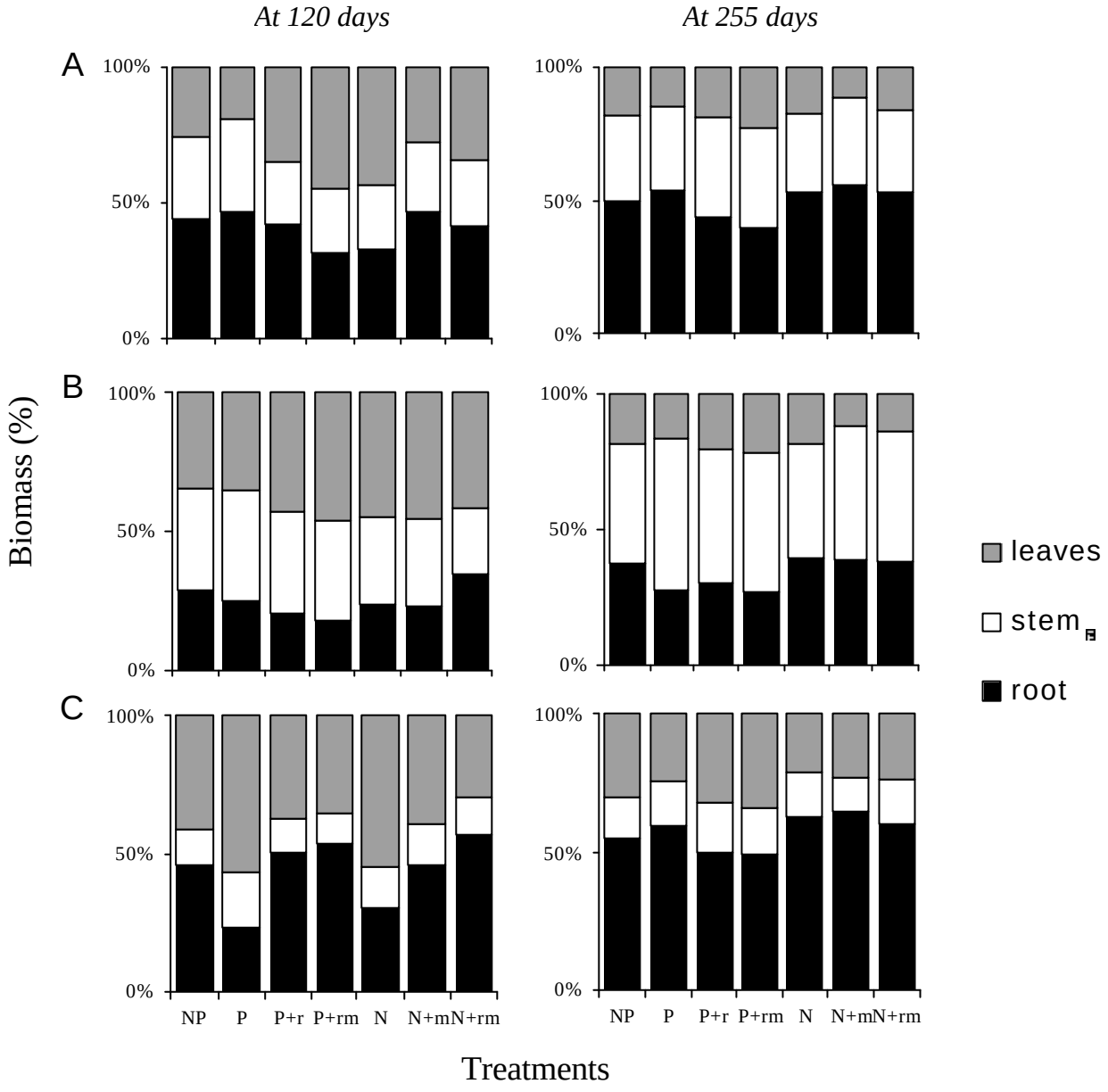

Figure 1: Biomass production average (%) of roots, stems and leaves in *A. colubrina* (A), *M. bimucronata* (B) and *P. rigida* (C) plants cultiveted upon treatments of fertilization (NP, P and N) and inoculation of rhizobia (r), mycorrhiza (m) and the both (rm) at 120 and at 255 days of sowing.

Table 3: Nodule number per diameter size larger than 4, between 2 and 4 and smaller than 2 milimeters at 120 and 255 days of sowing and nodule dry weight at 255 days of *A. colubrina, M. bimucronata* and *P. rigida*. Mean of five replicates, followed by the same letter in the colunn are not significantly different by ANOVA, test Dunn at $p<0.05$ level of significance.

	Mean number of nodule								Dry weight of nodule (g)			
	At 120 days				At 255 days							
Treatment	>4	2 a 4	<2	Total	>4	2 a 4	<2	Total	>4	2 a 4	<2	Total
					A. colubrina							
NP	0.8	3.6	1	5.4 b	3	7.2	9.6	19.8 b	0.08	0.04	0.01	0.13 a
P	2.2	3.4	0.6	6.2 b	5.6	9.2	25.2	40 b	0.16	0.03	0.01	0.20 a
P+r	2.6	42.8	63.6	109 a	12.8	36	75.8	124.8 a	0.19	0.18	0.05	0.42 a
P+rm	0.8	4	7.4	12.2 b	8.8	12	9	29.6 b	0.23	0.09	0	0.32 a
					M. bimucronata							
NP	33	118	99.4	250.6 a	99.2	311	279	688.4 b	0.92	0.90	0.25	2.07 b
P	73.4	117	64.4	254.8 a	149	246	155	550.2 b	2.95	1.07	0.18	4.20 a
P+r	17.2	66.8	47	131 ab	146	473	836	1455.4 a	2.35	1.94	0.76	5.05 a
P+rm	18	41.4	12.6	72 abc	41	367	1844	2252.2 a	1.06	1.35	1.61	4.02 a
N	1	7.2	18.4	26.6 bcd	2	30	161	193 bc	0	0.05	0.11	0.15 c
N+m	0	0.8	2	2.8 d	0.2	7.8	133	141.4 c	0	0.02	0.03	0.04 c
N+rm	0	0	7.8	7.8 cd	0	0.8	37	37.8 c	0	0	0.01	0.01 c
					P. rigida							
P+r	0.8	2.2	5.6	8.6 a	0	0.6	5.2	5.8 a	0	0	0	0a
P+rm	0	0.6	1.4	2 b	1	2.2	10	13.2 a	0.01	0.00	0.01	0.02 a

treatments with mineral N added, regardless of rhizobia inoculation, did not produced nodules. For *M. bimucronata* there were nodules in these treatments, but the nodules occurred in lower number (Table 3). Dual inoculation (rm) and single inoculation of rhizobia (r), associated with low N and P available in the P treatments improved the nodules number in *M. bimucronata*, P+r and P+rm treatments were statistically differ to the others, at 255 days of sowing (Table 3). The nitrogenase activity (ARA) and leghemoglobin contents in *M. bimucronata* nodules also were higher in these inoculated treatments (Figure 2). Nevertheless, dual inoculation (rm) did not increase nodules number (Table 3) in *A. colubrina* and *P. rigida*. Furthermore, *A. colubrina* roots presented significantly higher nodules number in the P+r than P+rm treatment. For this species, ARA presented similar response and leghemoglobin contents was similar between P treatments.

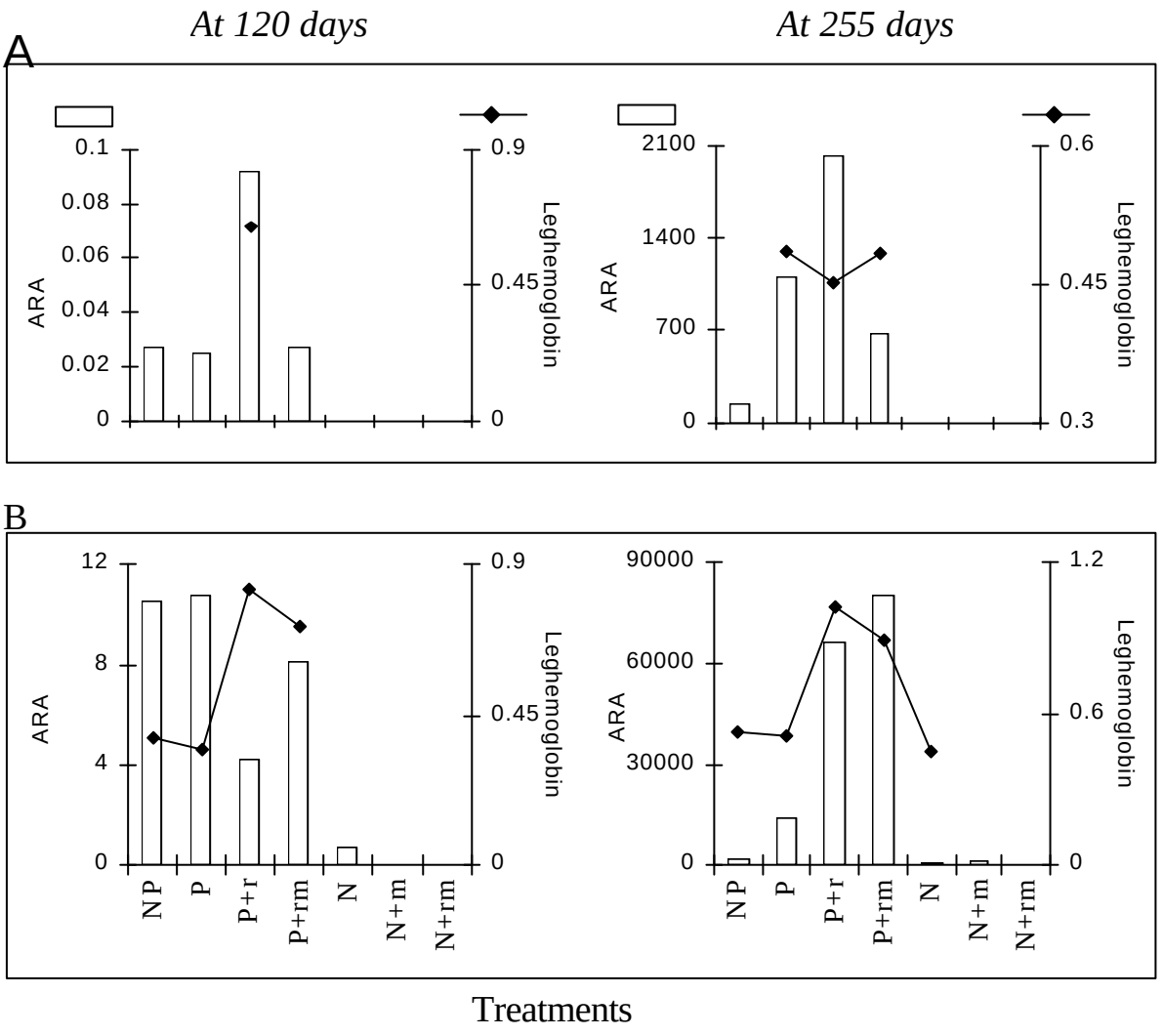

***Figure 2*:** Acetylene reduction activity (ARA) and leghemoglobin content averages of *A colubrina* (A) and *M. bimucronata* (B) nodules upon fertilization treatments (NP, P and N) with inoculation of rhizobia (r), mycorrhiza (m) and the both (rm) at 120 and 255 days of sowing. Bar graph corresponds to ARA (in µmol C_2H_4 h^{-1} g^{-1} nodule fresh), line graph corresponds to leghemoglobin content (in mg Hb g^{-1} nodule fresh).

The nodule dry weight of *A. colubrina* plants did not differ between the treatments. This probably occurred in spite of dry weight of nodules higher than 4mm (Table 3). For *M. bimucronata* nodules dry weight, had not difference between those formed with native rhizobia (P treatment) and inoculated treatments (P+r and P+rm). The ARA was relatively low in *P. rigida*, being measured at 255 days of sowing 128,58 and 292,23 (µmol of ethylene per g of fresh nodule per hour) at P+r and P+rm treatments, respectively. Leghemoglobin contents were not assessed of this specie because there were insufficient samples of nodule.

Results from P contents in shoot (Table 2) and chemical analysis of the substrate at the last harvest (Table 4) showed low values of P in N treatments, which not had P added. There was apparent difference between the treatments on the N contents in leaves and in the substrate of the pots.

Table 4: Chemical analysis of substrate of pots in relation to nitrogen (%), phosphorous ($mg.dm^{-3}$) and K ($mmol_c.dm^{-3}$) of *A. colubrina, M. bimucronata* and *P. rigida* at 255 days of sowing, cultived upon treatments of fertilization (NP, P and N) and inoculation of rhizobia (r), mycorrhiza (m) and the both (rm).

	A colubrina			*M. bimucronata*			*P. rigida*		
Treatments	N	P	K	N	P	K	N	P	K
NP	0.05	56	1.9	0.07	51	2.5	0.05	34	1.5
P	0.07	108	1.4	0.05	85	2.2	0.05	64	1.6
P+r	0.05	84	0.9	0.07	54	1.3	0.05	73	1.4
P+rm	0.07	88	1.3	0.07	60	1	0.05	97	2.5
N	0.05	4	1.2	0.07	3	2.1	0.07	4	2.4
N+m	0.07	2	1.6	0.07	1	2.4	0.05	1	2.8
N+rm	0.07	3	2.1	0.07	1	2.2	0.07	1	2.9

Nutrient contents and PCA analysis

We performed the PCA analysis of treatments and all data studied in order to see roughly, if the treatments would form groups and which factor(s) might explain the grouping. The PCA analysis simplifies the interpretation of complex results. Results of

nutrient contents of shoots and substrate were included in this analysis and each species was treated separately. The PCA plots showed that PC1 + PC2 axis explained 77,59; 88,47 and 77,79 percent of total for *A. colubrina, M. bimucronata* and *P. rigida*, respectively (Figure 3).

Mineral fertilization and nodulation influenced grouping in the three species. P treatments were grouping in the left and N treatments formed a group of the right of the axis. PC 1 can be seen as representing the nitrogen fixation capacity of *A. colubrina* and *M. bimucronata*. In *P. rigida* the axis PC 1 can be attributed to mineral fertilization, since that the nodulation was negligent. Because the vectors representing data nodulation pointed to the left of orig., the treatments on the left responded more positively to rhizobial inoculation. Vectors representing AMF colonization and K level pointed to the right of orig. for *A. colubrina* and *M. bimucronata* plants. This fact indicates that these features were increased in plants growth in N treatment (without P). In contrast, AMF percents pointed to P treatment (left) in *P. rigida*.

Mn and B were positively correlated with mycorrhizal treatments in *A. colubrina*; Zn in *M. bimucronata* and S, Cu and Fe in *P. rigida* (Figure 3).

Discussion

The results showed that *M. bimucronata* plants grew better than *A. colubrina* and *P. rigida* and also exhibited the highest values for root colonization by AMF. Also, *M. bimucronata* was very nodulated, with rhizobia inoculation increasing the rhizobial efficiency. Native rhizobia were capable to promote high levels of infection in this plant

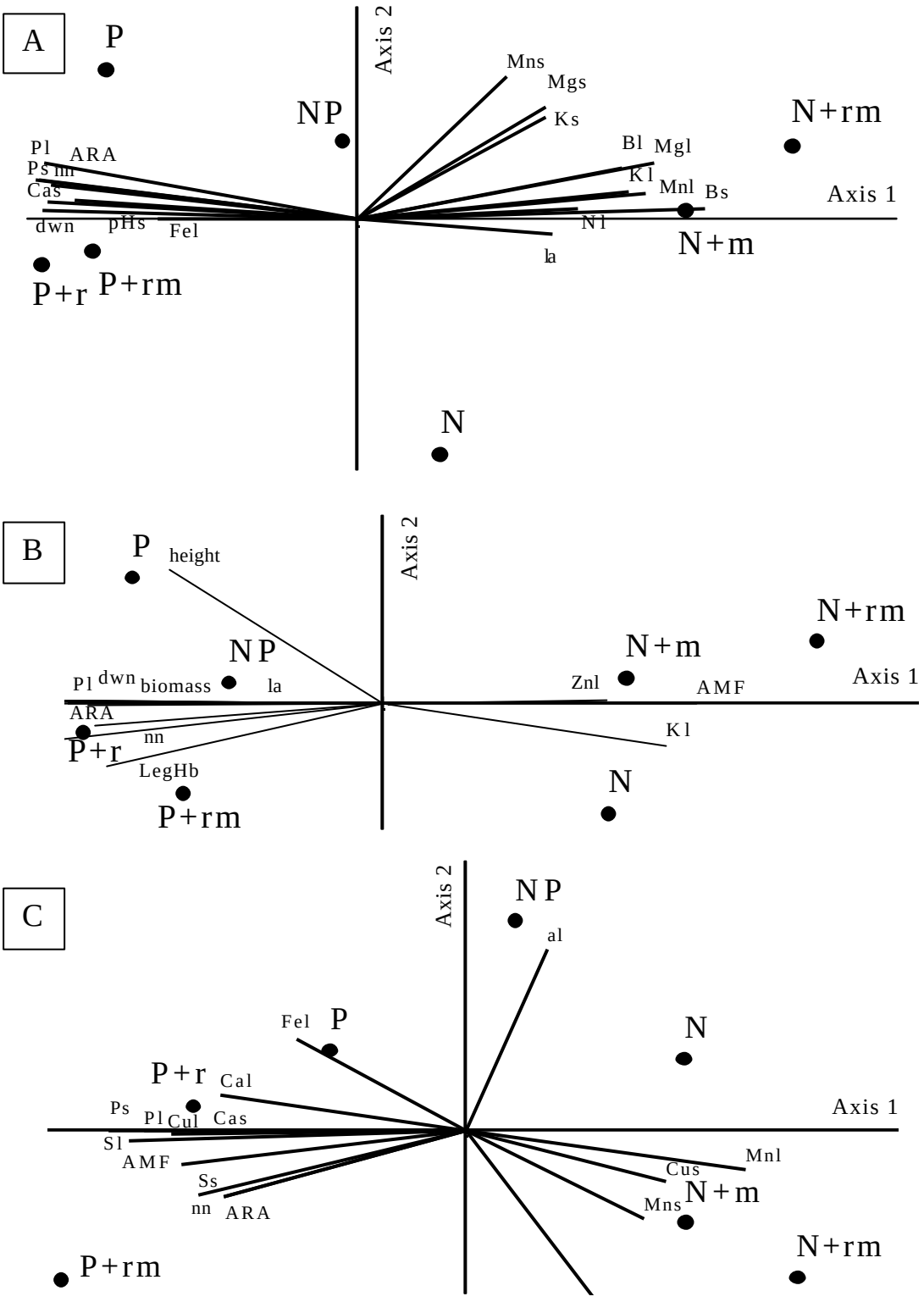

Figure 3: Principal component analysis (PCA) to mineral fertilization treatments (NP, P and N) with inoculation of rhizobia (r), mycorrhiza (m) or the both (rm). Leaf area (la), height, biomass, nodule number (nn), dry weight nodules (dwn), acetylene reduction activity (ARA), leghemoglobin content (LegHb) and mycorrhization percentage (AMF) were performed for (A) *A. colubrina*, (B) *M. bimucronata* and (C) *P. rigida*. s: substrate; l: leaf, macroelements: N, P, K, Ca, S and microelemnts: B, Mn, Zn, Cu and Fe.

probably because the soil used in the experiment was collected nearly to *M. bimucronata* nodulated plants population on the riparian forest of Corumbatai. This species is typically pioneer and produce small seeds, about 105.000 seeds Kg^{-1} (Lorenzi, 1998). Some authors reports that small-seeded species are seedlings that had high relative growth rate (Gross and Smith, 1991, Siqueira et al., 1998). These former authors recorded that under nutrient stress conditions, mycorrhizal effects on initial seedling growth are greater in small seeded species. The small seeds and therefore the low cotyledon reserves can have influenced the initial biomass allocation of seedlings to leaves and stems than roots in this species. *A. colubrina* and *P. rigida* present larger seeds in comparison to *M. bimucronata*, about 15.600 and 38.600 seeds.kg^{-1} (Lorenzi, 1992), respectively.

In this experiment we did not observe effect of inoculation of AMF in the root colonization and P uptake. Probably the fertilization treatments influenced both inoculated and native fungi. Both *A. colubrina* and *M. bimucronata* plants presented low AMF colonization in P treatments. This result is according to currently accepted literature that nutritionally adequade or high P supply tends to reduce colonization (Smith and Read 1997, Siqueira et al., 1998), with the magnitude of the effect varying between plant species and also sensitive to change in environmental parameters (Smith and Read 1997). In opposition, Paron et al. 1997 observed benefic growth responses in *Trema micrantha* (L.) Blume inoculated by AMF and with 100 $mg.kg^{-1}$ of P added. Similar response seems to have occurred in *P. rigida* in our experiment, whereas that higher percentage of mycorrhizal colonization in P treatments was noted. The substrate P level, in P treatments, for this species resulted in about 64 to 97 $mg.dm^{-3}$ (see Table 4). Burity et al. (2000) noted that addition of P level enhanced AMF root colonization in *M. caesalpiniifolia* in both 20 and 40 $Kg.ha^{-1}$ of P_2O_5. Also, P addition (50 to 200 $mg.dm^{-3}$) did not affect the colonization of *Dalbergia nigra* (Chaves et al., 1995). P level of 20 $mg.kg^{-1}$ in the substrate

was not inhibitory in 26 tree species studied by Carneiro et al. (1996). These variable responses to P level in different species leads to requirements of more experiments on legume trees to recognize the optimal P level to fungi colonization for each species of fungi and host plant.

In *A. colubrina,* the AMF colonization was relatively low (0-14%). Carneiro et al., (1998) founded 20-49% for *A. falcata* and 1-19% for *A. peregrina* in nursery conditions. A part of nutritional factors, this low infection can be associated to specificity between host plant and fungi. In the other hand, our values of mycorrizal colonization of *M. bimucronata* (12.8 to70.3 %) and *P. rigida* (20.9 to 58.6 %) were similar to reported by Siqueira (1998), who founded AMF colonization in 8 pioneer species being generally high (mean of 60%), and by Frioni et al. (1999) in roots segments of *Mimosa spp*. (60%) and *P. rigida* (50%) in the field. However, the uptake of P by AMF in N treatments (without P added) was not sufficient to sustain a good growth plants.

Information on the association of host plant with specific AMF is ambiguous: while some species of AMF have a wide distribution among host plants, others have been founded in rhizospheres of a single host plant (Carrenho et al., 2002). In *P. rigida*, for instance, Trufem (1990) observed the occurrence of only two *Glomus* species.

Nutrient contents by PCA analysis showed positive correlation between K, Mn, B, S, Zn, Cu and Fe and AMF percentage. Carneiro et al. (1996) observed higher contents of S and Mn in *Cassia rosa* plants mycorrhized. According to Redente and Reeves (1981), plants colonized by mycorrhiza can have higher concentrations of Zn and Cu.

A. colubrina and *P. rigida* were inoculated with rhizobia isolated from *A. peregrina*. This can have limited the infection or the satisfactory development of this symbiosis, mainly in *P. rigida*, which nodulation was negligent. Poor nodulation in the nursery condition due host specificity was reported in *P. rigida* by Frioni et al. (1998).

The high capacity of nodulation in *A. colubrina* and *M. bimucronata* plants of P treatments can be related to absence of N, once that its presence has a negative influence on the nodulation and nitrogen fixation, or P nutrition enough, an essential element to enhance biological nitrogen fixation. The increasing of mineral nitrogen contents diminished the nodule number and inhibited the nitrogenase, as measured by ARA, in *Sesbania rostrata*, but the start of 30 Kg (N).ha^{-1} affected positively the biological nitrogen fixation (Becker et al. 1991). Mendonça and Schiavinato (1996) founded higher dry mass of *Anadenanthera colubrina* e *A. peregrina* in soil with 20 mg of $(NH_4)_2SO_4$ than treatments without N. To found the adequate N dose to encourage the nodulation without stimulating its inhibition are others findings that we need to research in ours legume tree.

Uliassi and Ruess (2002) observed that P fertilization increased total nodule dry biomass in *Alnus teniufolia*. Also, P and K contents increased the biological nitrogen fixation and nitrogenase activity (ARA) in *Sesbania rostrata* (Becker et al. 1991). Frioni et al. (1998) reported that environmental constrains, such as P deficiency could explain the failure in nodulation of *P. rigida*. Nitrogenase activity (ARA) for this species was 24.200 µmol of ethylene h^{-1} g^{-1} fresh nodule h^{-1} by Frioni et al. (1998), who considered low activity compared with those of crop legumes, as already recorded by many authors. In opposition, *M. bimucronata* presented here about $8.0.10^4$ µmol of ethylene h^{-1} g^{-1} fresh nodule h^{-1} at 255 days of sowing. Although low values of ARA that we founded to *P. rigida* and *A. colubrina* can be due to many factors, such as P deficiency, ineffective rhizobia, we believe that high rates of N_2 fixation are less essential for perennial than annual species, according to Sprent (1994). We also need consider the size and morphology of nodules when are discussing the activity and potential efficiency of them. In spite of have been founded some nodules higher than 4 mm of size, with elevate values of dry weight in uninoculated treatments of *A. colubrina* and *M. bimucronata*, these

treatments not presented high ARA or leghemoglobin content in the occasion of measurement.

When trees are being grown in nurseries, the methods of cultivation and extent of rhizobial and mycorrhizal symbiosis can affect post-planting success, particularly when the trees are destined for disturbed lands or regeneration of riparian forest. In this way, we suggest that *M. bimucronata* may be more adapted to N-limited environments, mainly when dual inoculation (rhizobia and mycorrhiza) are performed in nursery. Although physiological aspects of seedling growth requirements still remain as a great gap in our knowledge and inoculation of rhizobia and mycorrhiza technique still not have been a practice commom, we can indicate the dual inoculation in this species in nursery and its use to reforestation and to recover riparian forests.

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CAPÍTULO 2

Nodulation, mycorrhizal colonization and growth of *Enterolobium contortisiliquum* and *Inga laurina* seedlings in the nursery

(Trabalho a ser enviado para a revista *Forest Ecology and Management*)

Nodulation, mycorrhizal colonization and growth of *Enterolobium contortisiliquum* and *Inga laurina* seedlings in the nursery

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Abstract

Effects of dual inoculation with rhizobia and arbuscular mycorrhiza fungi (AMF) on nodulation, mycorrhizal colonization and initial growth of *E. contortisiliquum* and *I. laurina* were examined in unsterile soil and fertilized with addition of N, P or both NP, inoculated or not by rhizobia (r), mycorrhiza (m) or dual inoculation (rm), following seven treatments: NP, P, P+r, P+rm, N, N+m and N+rm. Growth and nodulation of *E. contortisiliquum* and *I. laurina* plants were affected by P deficiency. On the other hand, P level added not diminished the mycorrhizal colonization. Seedlings of almost all treatments were colonized by mycorrhizal fungus. P deficiency in N treatments influenced the size of nodules, which were in general smaller than 4 mm and nodules activity, measured by acetylene reduction activity (ARA) and leghemoglobin content also were inhibited. Our results showed that dual inoculation increased the nodulation and mycorrhizal colonization of these plants in nursery with extension of the growth of *E. contortisiliquum* host, when associated to P fertilization. This suggest that the capacity of *E. contortisiliquum* to associate with two soil microorganisms (rhizobia and mycorrhiza) can be a strategy for their establishment in soils with low nitrogen levels.

Keywords – Nodulation; Rhizobia; Mycorrhiza; *Enterolobium contortisiliquum*; *Inga laurina*

1. Introduction

Enterolobium contortisiliquum (Vell.) Morong and *Inga laurina* (Sw.) Willd are woody leguminous trees with potential for reforestation on drastically disturbed lands, due to their ability to develop symbiotic associations with rhizobia and/or arbuscular mycorrhizal fungus (AMF). These plants differ in relation to the characteristics of succession. *E. contortisiliquum*, a species with 20-30 m tall at maturity (Engel & Parrota 2001), is classified as pioneer or initial secondary, according to Resolution of SMA-Secretaria do Meio Ambiente nº21, 21/11/2001 and it has been used as enrichment planting, due to the great survival rate and growth height (Montagnini *et al.* 1997), and in the agroforestry systems for soil improvement (Eibl *et al.*, 2000) and it is a tolerant species in heavy metal contaminated soil (Trannin *et al.*, 2001). This tree species has been sampled in several Brazilian riparian forests (Catharino, 1989; Nilsson, 1989 and Bernacci *et al.*, 1998); its nodulation ability is well known (Stefan, 1906; Lechtova-Trnka, 1931; Rothschild, 1970; Corby, 1988; Oliveira, 1997; Milnitsky *et al.*, 1997; Barberi *et al.*, 1998; Frioni *et al.*, 1998, Eibl *et al.*, 2000) and AMF colonization also was already reported (Frioni *et al*.1999).

I. laurina is classified as latest secondary or climax, according to Resolution of SMA- Secretaria do Meio Ambiente nº21, 21/11/2001. This species is generally founded in riparian forests primarily in seasonally flooded areas (Romagnolo & Souza 2000), but also has long been used as a shade tree and green manure in coffee and cacao plantations with good potential as agroforestry species (Tilki & Fisher, 1998). *I. laurina* was previously reported to be a N_2 fixing tree (Halliday & Nakao, 1982) and *I. fagifolia* (L.) Benth., currently accepted as nomenclatural synonym, was reported to nodulation by the first time by Faria *et al.* (1987); however these studies were not on the physiology of nodulation to

this species. AMF colonization in *I. laurina* has not been reported previously, although some *Inga* species had been studied in this aspect. In this way, our work also aims the capacity of *I. laurina* plants to develop mycorrhizal association (AMF) in nursery conditions.

The importance of associations with rhizobia and mycorrhiza in forests is well recognized, but the effectiveness of these associations change according to plant, rhizobia and fungus species involved and the environmental conditions. Thus, different plant species exhibit varied ability to establish mycorrhizal associations (Siqueira *et al.*, 1998). Relatively few leguminous trees have been tested for their nodulation or nitrogen fixation ability (Sprent, 2001) and few is known about how we can manage symbiotic fungal and rhizobial associations more effectively (Marques *et al.*, 2001).

The purpose of the present study was to evaluate the effects of dual inoculation with rhizobia and mycorrhizal fungi on nodulation, mycorrhizal colonization and growth, of *E. contortisiliquum* and *I. laurina* under nursery conditions.

2. Materials and methods

Seeds of *E. contortisiliquum* were supplied by IPEF (Instituto de Pesquisas e Estudos Florestais) – Esalq/USP (Escola Superior de Agricultura Luiz de Queiroz/ Universidade de São Paulo). Seeds of *I. laurina* were collected in the field of Unesp (Universidade Estadual Paulista "Júlio de Mesquita Filho") - Rio Claro, SP, Brazil (22º44'S and 47º33'W, 610 m a.s.l.). *E. contortisiliquum* seeds were scarified to dormancy break prior to planting. *I. laurina* seeds were storage in trays with vermiculite and ABA solution (10^{-4}M) in the proportion 2:1 in volume in the cold chamber with 10±1ºC and

85%±5% of relative humidity (Barbedo & Cicero, 2000) during twenty-four days, since the lost viability of *Inga* species occur rapidly under normal environmental conditions.

Two rhizobia strains were previously isolated from nodules of *E. contortisiliquum* plants, collected from Corumbataí region (22º 08'S and 47º40'W, 684 m a.s.l), and two strains were isolated from nodules of *Inga* spp., collected in the riparian forest of Corumbataí, SP, Brazil (22º 20'S and 47º40'W, 604 m a.s.l.). The rhizobia cultures were grown on YEM (yeast extract mannitol agar) at 28ºC and were stored in the Rhizobial Bank of Unesp Rio Claro, SP Brazil (IBRC). For rhizobia inoculated treatments, seeds of *E. contortisiliquum* were left to soak in a turbid suspension (100 mL) of a mixture of two strains, IBRC-206 and 207 from *E. contortisiliquum*, during 1 h before the planting and pre-germinated seedlings of *I. laurina* received 10 mL of a mixture of two strains, IBRC-195 and 197 from *Inga* spp., directly on the surface of pots, one week after planting. To ensure the rhizobia infection, plants of both species were subsequently re-inoculated 30 days after sowing. For the AMF inoculation, pieces of *A. peregrina* var. *falcata* roots with ±1cm of length collected from Corumbataí cerrado reserve (22º15'S and 47º00'W, 810 m a.s.l.) were mixed on each pot (0.4g) surface nearly seedlings at twenty-eight days of sowing.

Soil and vermiculite (2:1) not sterilized were performed as substrate of plastic pots of 4 L. The soil from riparian forest of Corumbataí, SP, Brazil (22º 20'S and 47º40'W, 604 m a.s.l.) contained (in $mmol_c.dm^{-3}$): K, 1; Ca, 12; Mg, 4; H+Al, 10; Al, 7; P, $7mg.dm^{-3}$ and N, 1000ppm. Micronutrients (in $mg.dm^{-3}$): B, 0.01; Cu, 0.07; Fe, 23; Mn, 8.7; Zn, 0.4 and S, 9, with pH 5.5 (Centro de Ciências Agrárias, Depto. de Recursos Naturais e Proteção Ambiental, Universidade Federal de São Carlos). Basal nutrients were added in the substrate 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). Beyond of these basal nutrients, were added

nitrogen and phosphorus, according to three different lots: NP, fertilization with addition of N (40) and P_2O_5 (80); P, with phosphorus P_2O_5 (80) and a start dose of 3.8 m*M* of N; and N, with nitrogen N (40) but without phosphorus. So, these lots were submitted to inoculations with rhizobia (r), mycorrhiza (m) and both (rm), resulting in seven treatments: NP; P; P+r; P+rm; N; N+m; N+ rm. Ten plants per treatment were grown in a greenhouse under natural daylight in randomised blocks. After 120 days of sowing, plants were fertilized with additional nutrients (10 mL of solution) every 30 days, following initial fertilization treatments.

The height of the plants was recorded each two weeks, starting at 30 days of sowing, in an average of ten plants per treatment until 120 days, thereafter they were averages of five plants. Five plants per treatment were harvested at 120 and 255 days, with intact root system for evaluation of nodulation and nitrogenase activity. Leaf area was measured by CI-202 Area Meter (CID, Inc). Roots, stems and leaves were separated, dried to constant weight at 70ºC and weighted. Chemical analyses of shoots (roots plus stems) were performed in both harvests, and chemical analyses of substrate were determined in the beginning and end of experiment (Centro de Ciências Agrárias, Depto. de Recursos Naturais e Proteção Ambiental, Universidade Federal de São Carlos).

Nodules were weighted, counted, sieved with sieves mesh and they were classified per size: larger than 4 mm, between 2-4 mm and smaller than 2 mm. The nodules morphology was classified according to Corby (1981). Nitrogenase activity was assayed by acetylene reduction activity (ARA) according to Hardy *et al*. (1968) in root nodules of two plants per treatment. Nodules of these same pots were stored in the refrigerator at 10ºC to leghemoglobin content analysis, following Becana *et al*. (1986) with absortion at 540nm.

For AMF colonization, roots were stained (Philips & Hayman, 1970) and the percentage of infected roots was estimated using the gridline intersect method (Giovannetti & Mosse, 1980) under a stereomicroscope (40x).

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).

3. Results

In general, *E. contortisiliquum* plants fertilized with N, but not with P had the lowest growth parameters in both the harvests (Table 1). Plants of treatment P+r grew similarly to the NP treatment plants and also presented similar values of leaf, stem and root dry mass, but it was significantly differ to the others treatments (Table 1). The highest root/shoot ratios were in the last harvest of the N without P treatments (Table 1).

I. laurina plants fertilized with NP were significantly taller than the others (Table 2), which were not statistically differ among them. More differences, although not statistically significant, can be observed between the treatments at 255 days than 120 days of sowing.

All nodules were classified as indeterminate (Corby, 1988), being *E. contortisiliquum* astragaloid and *I. laurina* muconoid (Sprent, 2001). In general, plants of two species were well nodulated, mainly in the second harvest (Figure 1). For the two species the most part of nodules were smaller than 2 mm after 120 days and between 2 and 4 mm of diameter size 255 days.

Table 1 Growth of *E. contortisiliquum* in response to mineral fertilization (NP, P and N) and inoculation treatments with rhizobia (r), mycorrhiza (m) or both (rm) at 120 and 255 days of sowing.

Growth of	Treatments of fertilization and rhizobia (r) and mycorrhiza (m) inoculations						
E. contortisiliquum	NP	P	P+r	P+rm	N	N+m	N+rm
After 120 days							
Height (cm)	33.6 a	20.3 b	20.9 b	15.0 c	12.5 c	14.6 c	14 c
Leaf area (cm^2)	261.1 ab	164 bc	284.4 a	141.9 bc	51.1 c	92 c	43.4 c
Leaf dry mass (g)	3.30 a	1.68 b	2.34 a	1.72 b	0.49 c	0.64 c	0.33 c
Stem dry mass (g)	4 a	1.47 bc	1.71 b	1.18 b	0.3 c	0.41 c	0.28 c
Root dry mass (g)	2.85 a	1.56 ab	1.51 ab	1.1 ab	0.37 b	0.51 b	0.34 b
Root/shoot ratio	0.39	0.49	0.37	0.38	0.47	0.49	0.56
After 255 days							
Height (cm)	143.6 a	114.2 ab	146.4 a	87.6 b	36.6 c	35.8 c	32.2 c
Leaf area (cm^2)	338.6 b	727.1 ab	1397.2 a	1121.7 a	204 b	266.3 b	116.4 b
Leaf dry mass (g)	13.78 ab	9.41 b	20.15 a	12.44 b	1.96 c	1.97 c	1.48 c
Stem dry mass (g)	33.68 a	15.71 b	34.47 a	18.14 b	3.03 c	2.53 c	1.91 c
Root dry mass (g)	16.09 a	12.01 b	24.43 a	16.03 ab	3.81 b	5.01 b	2.71 b
Root/shoot ratio (g)	0.34	0.48	0.45	0.52	0.76	1.11	0.80

[a] Mean of five plants per treatment in all parameters, except to height after 120 days (ten replicates). Mean in lines with different letters are significantly different as determined by Duncan test at 5% significance level ($P<0.05$).

Table 2. Growth of *I. laurina* in response to mineral fertilization (NP, P and N) and inoculation treatments with rhizobia (r), mycorrhiza (m) or both (rm) at 120 and 255 days of sowing.

Growth of	Treatments of fertilization and rhizobia (r) and mycorrhiza (m) inoculations [a]						
I. laurina	NP	P	P+r	P+rm	N	N+m	N+rm
After 120 days							
Height (cm)	17.1 a	11.5 b	6.9 c	6.15 c	8.3 c	9.2 bc	8.05 c
Leaf area (cm^2)	145.9 a	62.3 b	36.2 b	8.9 b	36.5 b	51.5 b	42.7 b
Leaf dry mass (g)	1.41a	0.72 b	0.43 b	0.12 b	0.43 b	0.57 b	0.43 b
Stem dry mass (g)	0.36 a	0.21 b	0.09 bc	0.03 c	0.11 bc	0.15 bc	0.10 bc
Root dry mass (g)	0.70 a	0.53 ab	0.31 ab	0.05 b	0.29 ab	0.3 ab	0.26 ab
Root/shoot ratio	0.40	0.58	0.60	0.36	0.54	0.41	0.50
After 255 days							
Height (cm)	78.2 a	39.6 b	26.4 cd	21 d	31 bcd	35.2 bc	29.6 bcd
Leaf area (cm^2)	503.4 a	132.6 b	139.9 b	182.9 b	379.4 ab	377.3 ab	280.6 ab
Leaf dry mass (g)	15.30 a	5.10 b	2.31 c	2.01 c	4.18 b	4.84 b	4.10 b
Stem dry mass (g)	9.81 a	2.16 b	0.85 cd	0.64 d	1.46 bcd	1.85 bc	1.97 b
Root dry mass (g)	15.63 a	4.19 b	1.57 bc	0.87 c	4.05 bc	3.91 bc	3.63 bc
Root/shoot ratio (g)	0.62	0.58	0.50	0.33	0.72	0.58	0.60

[a] Mean of five plants per treatment in all parameters, except to height after 120 days (ten replicates). Mean in lines with different letters are significantly different as determined by Duncan test at 5% significance level ($P<0.05$).

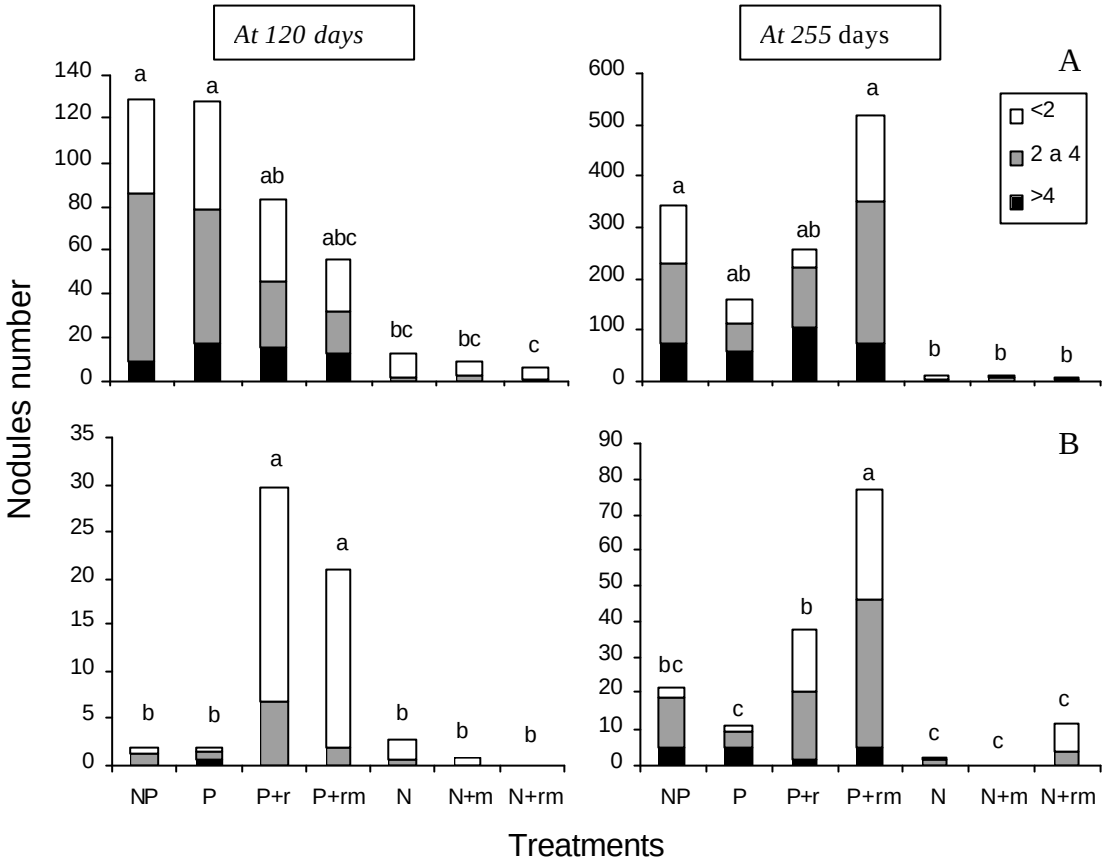

Fig.1. Nodule number average per diameter size (larger than 4 mm, between 2 to 4 mm and smaller than 2 mm) of A, *E. contortisiliquum* and B, *I. laurina* plants in the substrate fertilized with NP, P and N and inoculated by rhizobia (r), mycorrhiza (m) or both (rm), at 120 and 255 days of sowing. Values are means of five plants per treatment. Nodules number total followed by the same letter are not significantly different by ANOVA, test Duncan at P<0,05 level of significance.

Regardless of harvest, the number (Figure 2) and dry weight (Table 3) of nodules from two species were affected by P deficiency in N treatments. Also, the most of the nodules in these treatments were smaller than 2 mm of size (Figure 2) and the nodules activity, as measured by acetylene reduction activity (ARA) and leghemoglobin content (Figure 3) were inhibited. Nodules of *E. contortisiliquum* of the P+r treatment presented higher ARA at 255 days (Figure 3A). ARA of *I. laurina* nodules were not detected in the first harvest, despite of nodule presence, and leghemoglobin content were not shown because of not achieving of replicates. In the second harvest, the ARA was higher in uninoculated treatments (NP and P) and leghemoglobin was assayed on the NP, P, P+r and P+rm treatments (Figure 3B).

Table 3

Dry weight average of nodules (g) of A, *E. contortisiliquum* and B, *I. laurina* plants in the substrate fertilized with NP, P and N and inoculated by rhizobia (r), mycorrhiza (m) or both (rm), at 255 days of sowing. Mean of five plants per treatment, followed by the same letter are not significantly different by ANOVA, test Duncan at $p<0.05$.

	Nodule dry weight (g)			
	>4 mm	2 a 4 mm	<2 mm	Total
Treatments	*E. contortisiliquum*			
NP	4.407 ab	0.811 ab	0.098 ab	5.317
P	2.207 bc	0.213 b	0.018 b	2.440
P+r	6.807 a	0.730 ab	0.038 ab	7.577
P+rm	3.533 abc	1.562 a	0.215 a	5.311
N	0.011 c	0.007 b		0.019
N+m	0.007 c	0.002 b		0.010
N+rm	0.001 c	0.001 b		0.004
	Inga laurina			
NP	0.102 b	0.034 b	0.001 b	0.139
P	0.350 a	0.021 b		0.373
P+r	0.016 b	0.052 b	0.006 ab	0.075
P+rm	0.085 b	0.100 a	0.010 a	0.197
N		0.004 b		0.005
N+m				0.000
N+rm	0.001 b	0.012 b		0.015

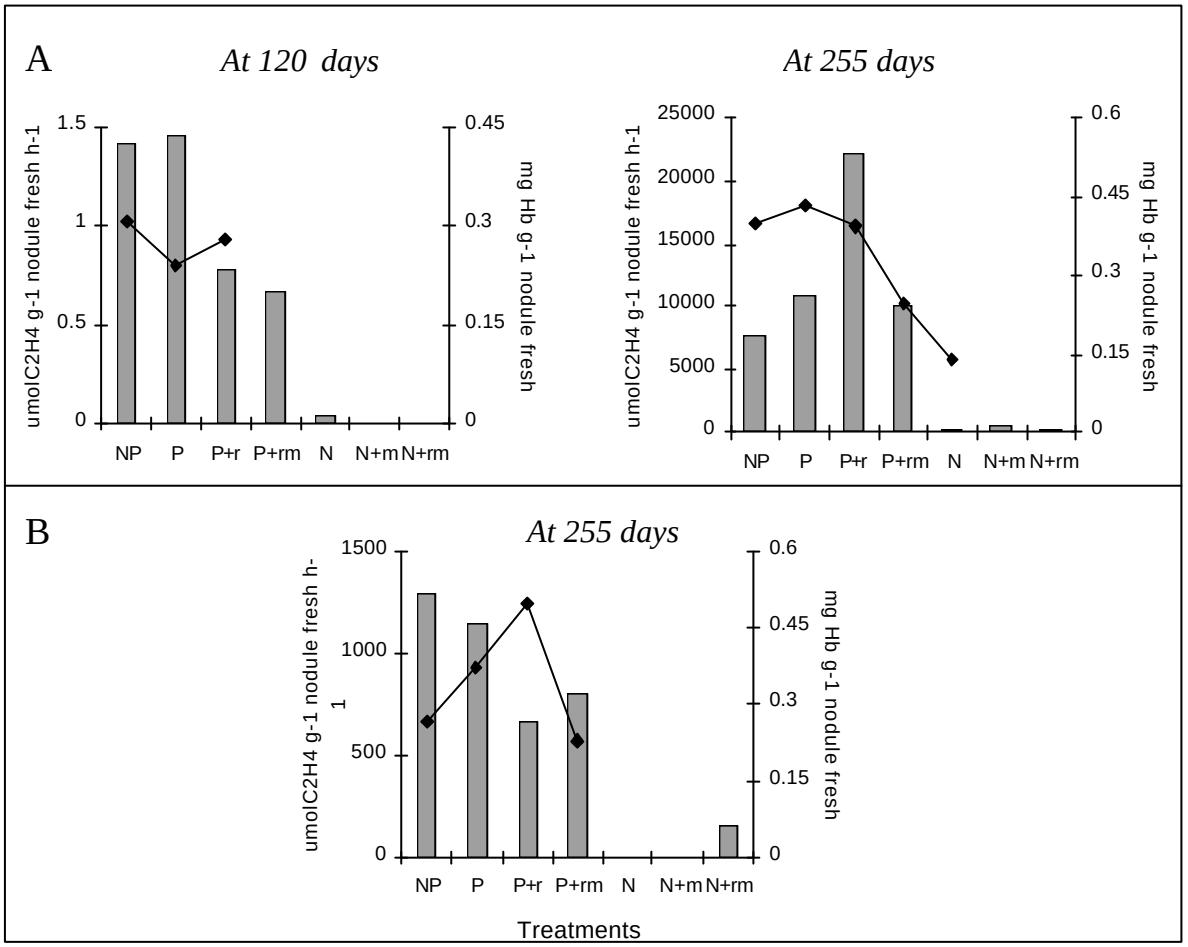

Fig. 2. Acethylene reduction activity (µmol C_2H_4 g^{-1} nodule fresh h^{-1}) and leghemoglobin content (mg Hb g^{-1} nodule fresh) averages of A, *E. contortisiliquum* plants, at 120 and 255 days of sowing and B, *I. laurina* plants at 255 days, fertilized with NP, P or N and inoculated with rhizobia (r), mycorrhiza (m) and both (rm).

Although the plants of the two species had been nodulated with native rhizobia, which were actives, demonstrated by ARA e leghemoglobin content mainly at 255 days (Figure 2), the nodulation of the two hosts were benefited by inoculation of rhizobia strains and mycorrhizal fungi, as can be seen in nodule number of P+rm treatment of Figure 1. Thus, dual inoculation associated with P fertilization increased nodulation in these two species.

Despite of the N content of plants not have been tested statistically, we could note that treatments not fertilized with nitrogen (P treatments) presented N content similar those

plants of NP treatments, although for *E. contortisiliquum* these treatments had been lower than those fertilized only with N (Figure 3A). For *I. laurina* this difference were not apparently observed (Figure 3B).

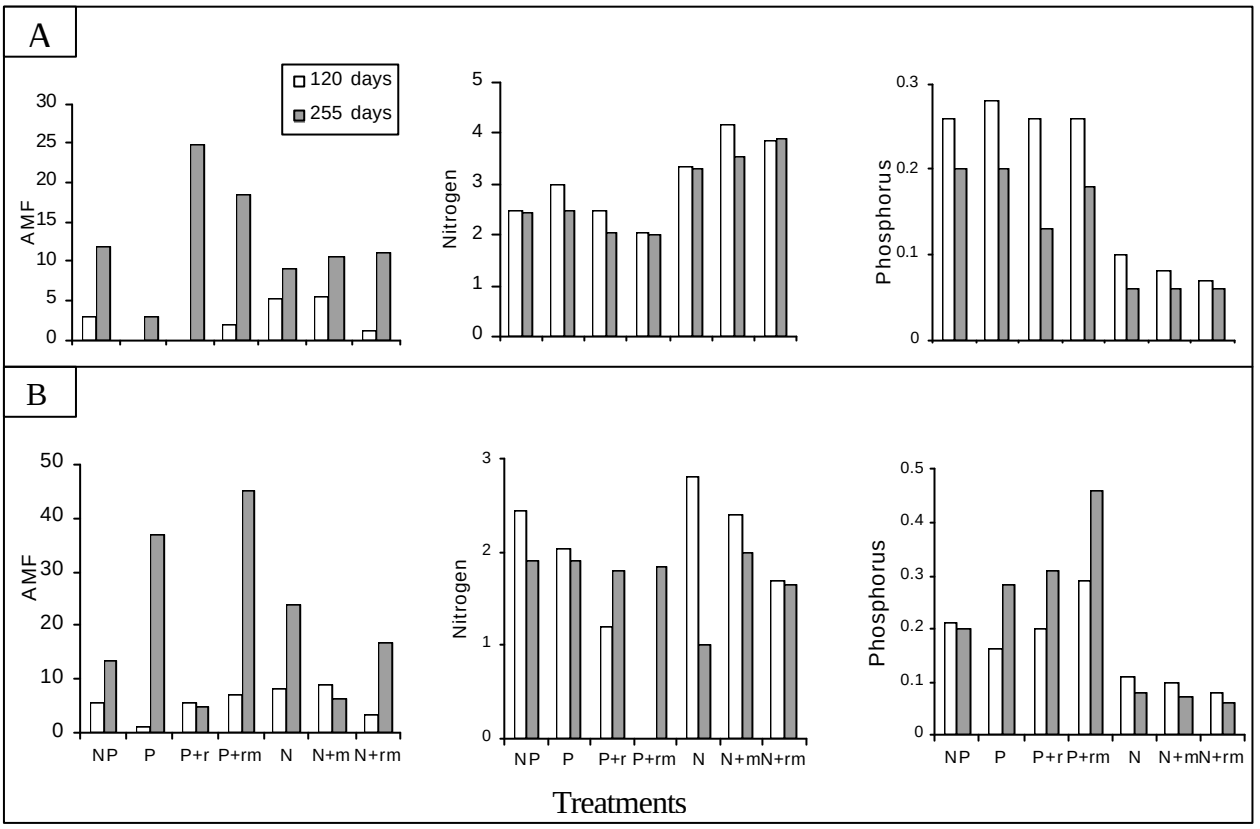

Fig.3. Percentage (%) of colonization by arbuscular mycorrhiza fungi (AMF), N and P contents (%) in shoots of A, *E. contortisi*liquum and B, *I. laurina* plants on the fertilization (NP, P and N) and inoculation with rhizobia (r), mycorrhiza (m) and both (rm) treatments, at 120 and 255 days after sowing. Mean of two plants for AMF colonization and a mixture of five plants per treatment for chemical analysis of shoots.

I. laurina was colonized either by inoculated as native mycorrhizal fungi, demonstrating its mycorrhizal infection potential, still not reported in the literature. However, in general, mycorrhizal infections were low in both species (Figure 3).

N treatments of *E. contortisiliquum* host had relatively low AMF infection and low P content in both shoot (figure 3A) and substrate (Figure 4A). For *I. laurina*, the treatment with P added and dual inoculated (P+rm) presented the highest value of P content in the shoot of plants (Figure 3B). The level of this nutrient on the substrate was equally affected by treatments, being higher in P+r and P+rm treatments (Figure 4B).

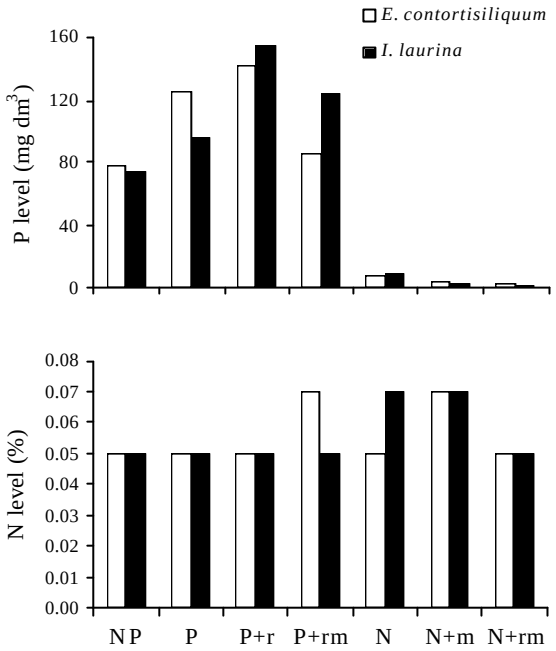

Fig.4. Nitrogen (%) and phosphorous (mg dm^3) contents of substrate of pots of *E. contortisiliquum* and *I. laurina* plants on the treatments of fertilization (NP, P and N) and inoculation with rhizobia (r), mycorrhiza (m) and both (rm).

4. Discussion

Phosphorus nutrition appeared to be the major factor influencing growth and nodulation of *E. contortisiliquum* and *I. laurina* plants. 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), in part due relation with their N_2 fixing capability (Frioni *et al.* 1999). Similar P effects had been reported in leucena by Brandon et al (1997).

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 is according to our result, which *I. laurina*, a latest secondary or climax species was less responsive to the treatments than *E. contortisiliquum* a pioneer tree. 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* are smaller (3.600 seeds Kg^{-1}, according to Lorenzi, 1992) than *I. laurina* seeds (530 seeds Kg^{-1}, according to Lorenzi, 1998). *E. contortisiliquum* grew about 120 cm in the best treatments and *I. laurina* 80 cm. In opposition, Moreira (1997) observed positive correlation between seed size and height growth in 49 species native from Amazonian ecosystems in nursery conditions. Muthukumar & Udaiyan (2000) showed that seed reserves are not only important for seedling growth, but also for mycorrhizal formation and nodulation. They observed that nodule number in *Leucena*, which was related positively to plant biomass and mycorrhizal infection levels, was reduced when one or both cotyledons were severed.

The increasing in the concentration of soluble phosphate in the soil normally decreases the percentage infection of roots by AMF (Amijee *et al.*, 1989). In our experiment, the mycorrhizal colonization was low independently of P level added. However, the seedlings of almost all treatments were colonized by mycorrhizal fungus. 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. Frioni *et al.* 1999, in the field, recorded that *E. contortisiliquum* and *I. uruguensis* had levels of colonization by AM fungi of 60 and 47%, respectively. Bereau *et al.* (1997) recorded 68 and 30% of

endomycorrhizas in *Inga sp*. in two of 12 sites the forest of French Guiana. In our experiment the maximum rates of colonization were 25 and 45% at 255 days for *E. contortisiliquum* and *I. laurina* respectively.

Usually mycorrhizal effects on legume nodulation, nitrogen fixation and plant growth are similar to the effects of adding phosphate (Hayman, 1982). Ours results showed the benefic effect of AMF colonization on the increasing of nodule number of both species. Although nodule mass had been greatly reduced by P deficiency it is uncertain whether nitrogenase activity per weight of nodule is decreased or not by P deficiency (Vadez *et al.* 1997). P deficiency limits nodulation indirectly by reducing legume growth rather by direct action on the infection process (Bordeleau & Prévost, 1994).

The N_2 fixing capacity of *E. contortisiliquum* and *I. laurina* species was confirmed by the ARA assay. Some values of ARA were reported in the literature for species belonging to the same genera, in µmol ethylene g^{-1} fresh nodule h^{-1}: *I. jinicuil,* 1-18.3 (Sutherland & Sprent 1993); *I. uraguensis,* 2.800 (Frioni et al 1998); and, in µmol ethylene nodule h^{1}, *E. gummiferum, E. mongollo, I. capitata, I. luschnathiana, I. nuda, I. sessilis* showed, respectively, 0.098, 0.18, 0.09, 0.06, 0.1 and 0.016 (Faria *et al*. 1984). It's difficult to compare these data firstly because they are different species and because ARA can be influenced by season pattern, soil temperature 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 it's conditions themselves cause declines in nitrogenase activity (via acetylene-induced declines, carbohydrate limitations, drought stress, physical disturbances, etc), but they 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.

In general, the nodulation in our experiment was high, considering the observations of Moreira (1997) where with *Inga* species showed from 0.4 to 2.6 nodules number per plant (in mean), with 2 to 7 mg of dry weight and *E. maximum* plants had 51.6 nodules number with 147 mg of nodule dry weight. *I. laurina* in our experiment was about fourty times more nodulated (approximately 80 nodules in average), with 195 mg of dry weight on P+rm treatment and *E. contortisiliquum* plants were about ten times more nodulated (mean of 500 nodules), with 5310 mg of dry weight on P+rm treatment. But, the use of rhizobial inoculants may be crucial in order to exploit nitrogen capacity of species here studied, similar to *Acacia* and *Prosopis* trees reported by Rasanen *et al.* (2001).

Our results showed that dual inoculation increased the nodulation and mycorrhizal colonization of these plants in nursery with extension of the growth and development of *E. contortisiliquum* host, mainly associated to P fertilization. But, best results can be reached with the use of selected symbionts. This research can encourage researchers to evaluate the effects of dual inoculation of these plants under field conditions, considering that it can enhance plant survival.

Acknowledgements

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CAPÍTULO 3

Inoculation of *Lonchocarpus muehlbergianus* and *Platypodium elegans* with rhizobia and/or mycorrhizal fungi

(Trabalho a ser enviado para a revista *Acta Botânica Brasílica*)

Inoculation of *Lonchocarpus muehlbergianus* and *Platypodium elegans* with rhizobia and/or mycorrhizal fungi[1]

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RESUMO – (Inoculação de *Lonchocarpus muehlbergianus* e *Platypodium elegans* com rizóbio e/ou fungo micorrízico). Foi verificado o efeito das inoculações de rizóbio e micorriza e do suprimento de nitrogênio e fósforo na nodulação, colonização micorrízica e crescimento de *Lonchocarpus muehlbergianus* e *Platypodium elegans* em viveiro. Os tratamentos foram: fertilização com N, P e ambos NP com inoculação de rizóbio (r), micorriza (m) ou ambos (rm), totalizando sete tratamentos: NP, P, P+r, P+rm, N, N+m e N+rm. Plantas de *P. elegans* foram afetadas negativamente pelos tratamentos com deficiência mineral e as inoculações não foram capazes de melhorar a nodulação, colonização micorrízica e crescimento. O tratamento P+r de *L. muehlbergianus* cresceu similarmente ao tratamento NP e exibiu altas taxas de nodulação, redução de acetileno e teor de leg-hemoglobina. Nestes dois últimos parâmetros, a dupla inoculação no tratamento P+rm também apresentou resultados similares. A inoculação por fungos micorrízicos não aumentou a colonização micorrízica e os fungos nativos provavelmente infectaram as raízes dos hospedeiros. A inoculação com somente rizóbio favoreceu o crescimento e desenvolvimento de plântulas de *L. muehlbergianus*. No entanto, é necessário maior conhecimento sobre a dupla inoculação em árvores nativas como *L. muehlbergianus* and *P. elegans* necessário para sua utilização satisfatória na restauração florestal em solos degradados.

Palavras-chave: Dupla inoculação, *Lonchocarpus muehlbergianus*, micorriza, *Platypodium elegans*, rizóbio.

ABSTRACT – (Inoculation of *Lonchocarpus muehlbergianus* and *Platypodium elegans* with rhizobia and/or mycorrhizal fungi). It was examined the effect of rhizobia and mycorrhiza inoculations and of nitrogen and phosphorus supply on the nodulation,

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mycorrhizal colonization and growth of *Lonchocarpus muehlbergianus* and *Platypodium elegans* in nursery. The treatments were: fertilization with N, P or the both, NP with rhizobia (r), mycorrhiza (m) or the both (rm) inoculations, totalizing seven treatments: NP, P, P+r, P+rm, N, N+m and N+rm. *P. elegans* plants were negatively affected by treatments with mineral deficiency and the inoculations were not capable to improve the nodulation, mycorrhizal colonization and growth in this species. P+r treatment of *L. muehlbergianus* grew similarly to NP treatment and exhibited higher nodulation rates, acethylene reduction and leghemoglobin content. In this two last parameters, dual inoculation in P+rm also exhibited similar results. Mycorrhizal fungi inoculations not enhanced the mycorrhizal colonization and native fungi probably infected roots hosts. The inoculation with only rhizobia aided the growth and development of *L. muehlbergianus* plants. However, it is necessary major knowledge about dual inoculation in native trees as *L. muehlbergianus* and *P. elegans* to their use in the forest restoration at degraded soils.

Key words - Dual inoculation, *Lonchocarpus muehlbergianus*, mycorrhiza, *Platypodium elegans*, rhizobia.

Introduction

Some nitrogen fixing bacteria and mycorrhizal fungi are among the most widespread and ecologically important plant symbionts. The symbiosis formed by legumes and rhizobia is the best system of plant-microbe interaction studied (Provorov *et al.* 2002).

The highest proportion of species detected for nodulation occurs in the subfamily Papilionoideae, with about ninety-seven per cent of species examined (Faria *et al*. 1989), including *Lonchocarpus muehlbergianus* Hassl, observed for the first time to be nodulated by Faria *et al* (1987) and *Platypodium elegans*, reported for this feature by Halliday & Nakao (1982), Faria *et al*. (1984) and Barberi *et al.* (1998). In contrast to the taxonomically restricted N_2-fixing nodules, arbuscular mycorrhiza is extremely widespread (Brundrett 2002), besides to show low specificity (Mosse 1975).

Dual inoculation with rhizobia and mycorrhizal fungi is currently being suggested as a possible solution to the reforestation and amendment in the soil fertility. Some woody legumes species, for instance, *Albizia lebbeck* (Faria *et al*. 1995), *Centrolobium tomentosum* (Marques *et al*. 2001) and *Mimosa caesalpiniifolia* (Burity *et al.* 2000) were studied in this regarding and these symbionts were capable to increase the growth of these

plants. In India, positive impact of dual inoculation has been demontrated in many forest tree species (Khan & Uniyal 1999). In addition, studies about genetic and molecular mechanisms in nitrogen-fixing nodules and arbuscular mycorrhiza are being carried out (Provorov *et al*. 2002).

The genera *Lonchocarpus* include 130 species of shrub or tree from tropical and subtropical America with one species in Africa (Sprent 2001). *L. muehlbergianus* is one of 21 species with known nodulation capacity. Besides, this species is usually founded in riparian forest (Silva *et al*. 1992, Bernacci *et al*. 1998, Nilsson 1989, Gisler 2000).

Platypodium include one or two species from Central and South America (Sprent, 2001). *P. elegans* was sampled in 23,9% of studies involving floristic survey of riparian forest of Brazil (Rodrigues & Nave 2000).

Mycorrhizal fungi and nodulating bacteria are important for tree seedling establishment in degraded areas in the tropics. Thus, the aim of the present study was to investigate the effects of dual inoculation with rhizobia and mycorrhizal fungi on nodulation, mycorrhizal colonization and growth of *L. muehlbergianus* and *P. elegans* cultivated upon different treatments of mineral fertilization and inoculation of these symbionts in the nursery conditions. This study may contribute with information for post-planting success of these species.

Material and Methods

Seeds of *L. muehlbergianus* were collected in the Constantine Peruchi (SP-316) highway, between Cordeirópolis and Santa Gertrudes, Km 166 and *P. elegans* seeds were collected in the Mogi Mirim – Limeira – Piracicaba (SP-147) highway, between Limeira and Mogi Mirim, Km 92. Aerial parts of these species were pressed and catalogued in the Bioscience Institute's Herbarium –UNESP (Universidade Estadual Paulista "Júlio de Mesquita Filho") as a number HRCB-34500 and 34502, respectively. *P. elegans* seeds were left to soak in 50% sulphuric acid during 45 min prior the sowing to break of dormancy.

Two rhizobia strains, previously isolated from nodules of *L. muehlbergianus* plants from field of Unesp Rio Claro, SP, Brazil (22º44'S and 47º33'W, 610 m a.s.l.) were grown on YEM (yeast extract mannitol agar) at 28ºC (Vincent, 1970) and they were stored in the Rhizobial Bank of Unesp Rio Claro, SP Brazil as a number IBRC-208 and 209.

Seeds of treatments inoculated with rhizobia were left to soak in a turbid suspension (100 mL) of a mixture of these two strains during 1 h before the planting. To ensure the rhizobia infection, seedlings were re-inoculated 30 days after planting, with 10 mL of rhizobia suspension containing the same strains. For the AMF inoculation, pieces of *A. peregrina* var. *falcata* roots with ±1cm of length collected from Corumbataí cerrado reserve (22º15'S and 47º00'W, 810 m a.s.l.) were mixed on each pot (0.4g) surface nearly seedlings at twenty-eight days of sowing.

All seeds were germinated in plastic pots of 4 L containing soil and vermiculite (2:1) not sterilized. The soil was collected on the riparian forest of Corumbataí, SP, Brazil (22º 20'S and 47º40'W, 604 m a.s.l.) and fertilized with (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). Beyond these basal nutrients, were added nitrogen and phosphorus, according to three different lots: NP, fertilization with addition of N (40) and P_2O_5 (80); P, with phosphorus P_2O_5 (80) and 3.8 m*M* of N as a start dose; and N, with nitrogen N (40) but without phosphorus. These lots varied in function of inoculations of rhizobia (r), mycorrhiza (m) or both rhizobia and mycorrhiza (rm). So, our experiment followed seven treatments, with ten replicates: NP; P; P+r; P+rm; N; N+m; N+ rm. Seedlings were fertilized with 10 mL of nutritive solution every 30 days, beginning at 120 days of sowing. Plants were grown in a greenhouse under natural daylight in randomised blocks and the height of plants was recorded each fifteen days, starting at 30 days of sowing and being measured ten plants per treatment until 120 days and five plants per treatment from 120 days until the end of the experiment, at 255 days of sowing.

Plants were harvested at 120 and 255 days and leaf area (CI-202 Area Meter, CID, Inc), dry weighed of roots, stems and leaves and chemical analyses of shoots (roots plus stems) and substrate per treatment were evaluated.

Nodulation parameters measured were: number, size and dry weight of nodules. Nodules were sieved with sieves mesh and separated: larger than 4 mm, between 2-4 mm and smaller than 2 mm of diameter. Nitrogenase activity, by acetylene reduction activity (ARA), (Hardy *et al*. 1968) and leghemoglobin content (Becana *et al*. 1986) at 540 nm from nodules of two plants per treatment were evaluated in both harvests. The nodule morphology was classified according to Corby (1981).

For AMF colonization, roots were stained (Philips & Hayman 1970) and the percentage of infected roots of two plants per treatment was estimated using the gridline intersect method (Giovannetti & Mosse 1980) under a stereomicroscope (40x).

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).

Results

Treatments without P and treatments without N added grew poorly in relation to NP treatments in both species (Fig. 1). These last treatments had a significantly ($P<0.05$) greater height than the other treatments, at 120 and 255 days of sowing (Fig. 1).

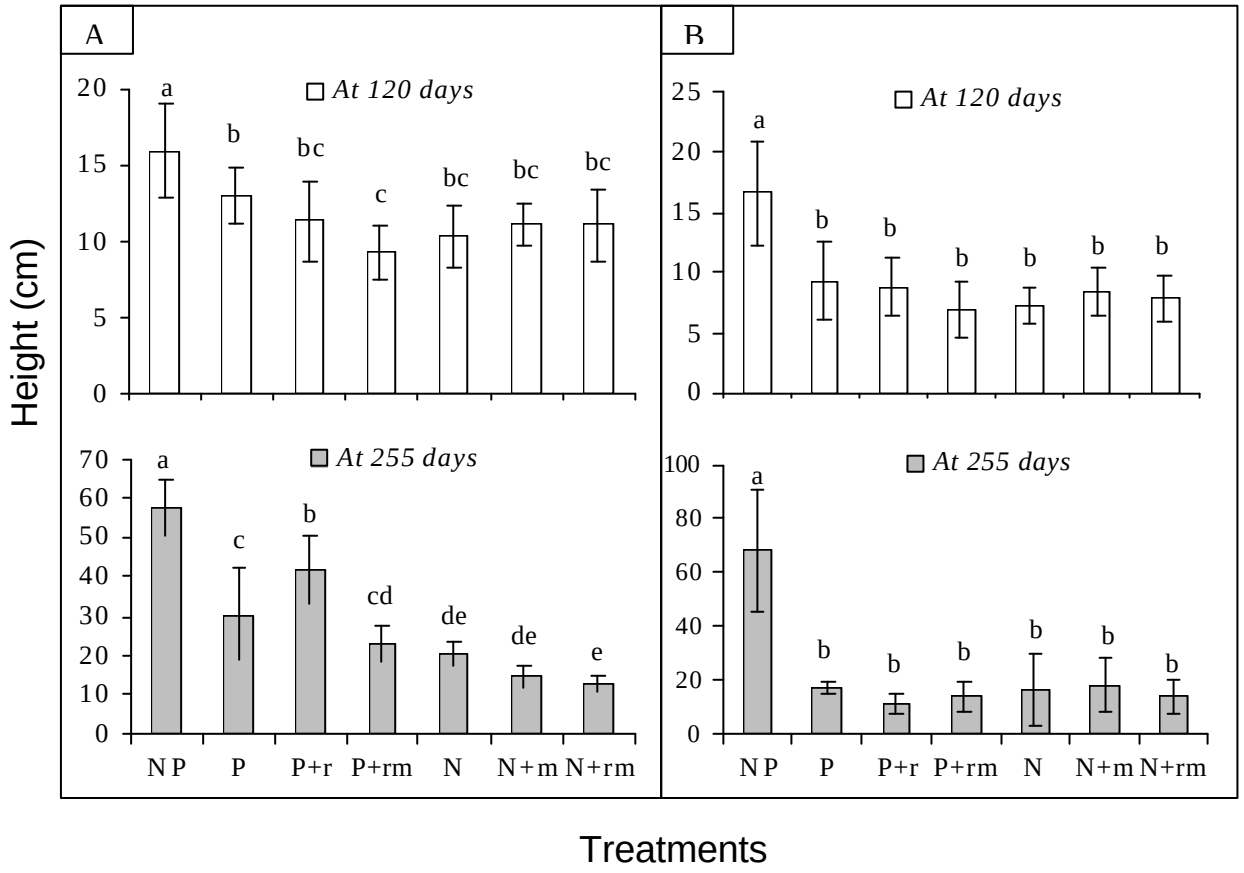

Figure 1. Height average of *L. muehbergianus* (A) and *P. elegans* (B) at 120 and 255 days of sowing cultived upon treatments with NP, N and P, inoculated by rhizobia (r), mycorrhiza (m) or both (rm). Different letters indicates statistic differences, determined by Duncan test at the 5% confidence level.

For *P. elegans*, all treatments differed significantly from the NP treatment in terms of leaf area (Fig. 2) and dry weight of leaves, stems and roots (Fig. 3), and these treatments not were different between them, in both harvests. In opposition *L. muehlbergianus* plants growing upon treatments with P added with only a start dose of nitrogen, uninoculated (P) or rhizobia inoculated (P+r) treatments, were not statistically different of NP treatments for leaf area (Fig. 2), leaf and root dry weight (Fig. 3) at 255 days of sowing. For stem dry weight, only P+r treatment didn't differ of NP treatment. In general, treatments with P added showed the best results in respect to growth plants when compared to N treatments.

The P deficiency in N treatments also affected the nodulation. Within P added treatments, those inoculated with rhizobia presented more nodule number on the *L. muehlbergianus* roots, in the two harvests (Tab. 1).

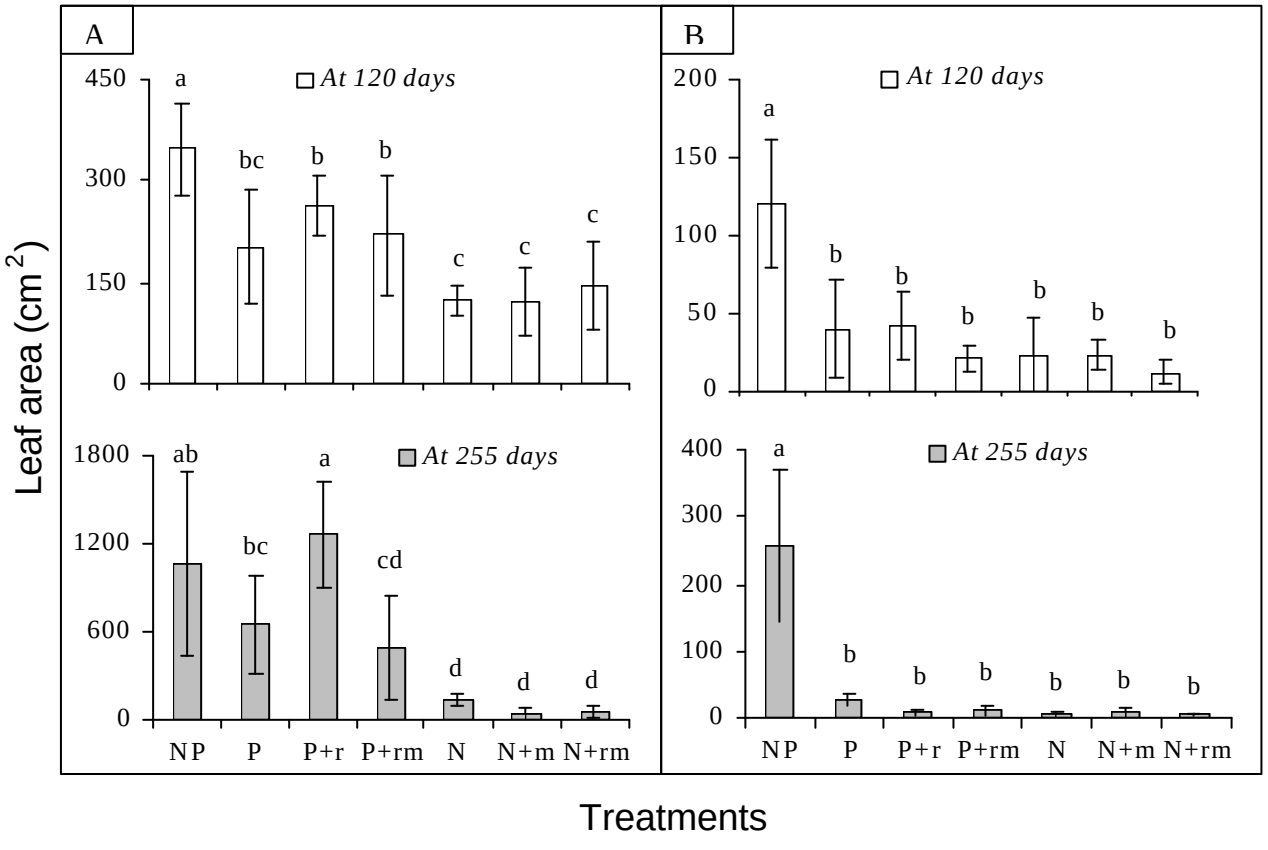

Fig. 2. Leaf area average of *L. muehbergianus* (A) and *P. elegans* (B) at 120 and 255 days of sowing cultived upon treatments with NP, N and P, inoculated by rhizobia (r), mycorrhiza (m) or both (rm). Different letters indicates statistic differences, determined by Duncan test at the 5% confidence level.

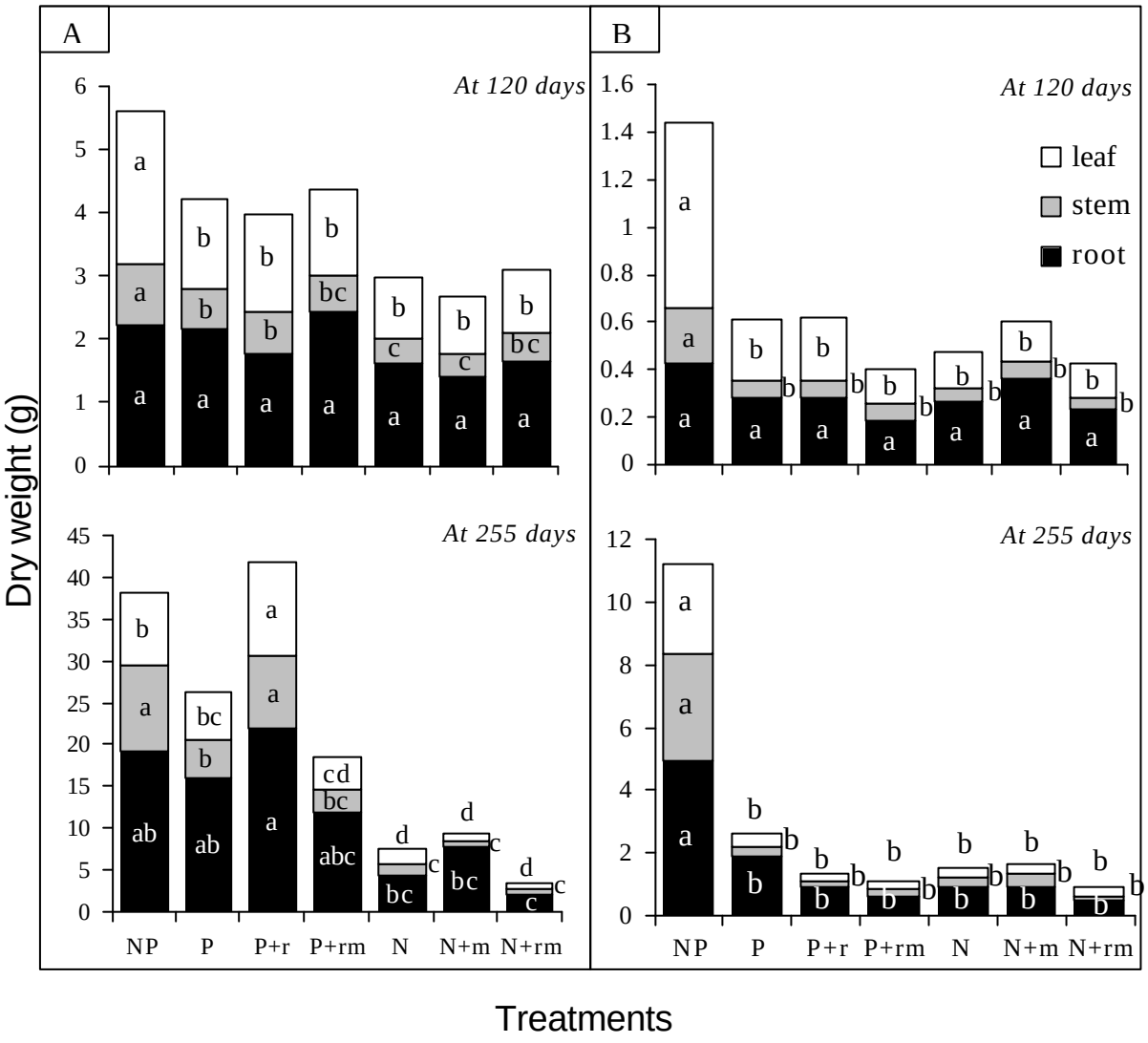

Figure 3. Dry weight average of leaves, stems and roots of *L.muehlbergianus* (A) and *P. elegans* (B) at 120 and 255 days of sowing cultived upon treatments with NP, N and P, inoculated by rhizobia (r), mycorrhiza (m) or both (rm). Different letters indicates statistic differences between the compartments separately, determined by Duncan test at the 5% confidence level.

Nodulation was low in *P. elegans* and nodules smaller than 2 mm of diameter prevailed. The number and dry weigh of nodules on this species didn't differ between the treatments. Due to the low occurrence and small size of nodules, we were unable to obtain accurate data on nodule mass, number, nitrogenase activity and leghemoglobin contents for this species. However, nodules from *L. muehlbergianus* were actives, with higher acethylene reduction in P+r and P+rm treatments, i.e, fertilized with P and inoculated (Fig. 4). NP treatments also presented high values of ARA and leghemoglobin content at 255 days of sowing.

Tab. 1. Nodule number average larger than 4 mm, between 2 to 4 mm and smaller than 2 mm of diameter and nodule dry weight (g) of *L. muehlbergianus* plants cultivated upon treatments with NP, N and P, inoculated by rhizobia (r), mycorrhiza (m) or both (rm). Different letters indicates statistic differences between the treatments (column) determined by Duncan test at the 5% confidence level.

	Nodule number						Nodule dry weight (at 255 days)		
	At 120 days			At 255 days					
Treatments	>4 mm	2 a 4 mm	<2 mm	>4 mm	2 a 4 mm	<2 mm	>4 mm	2 a 4 mm	<2 mm
NP	3 b	12.2 b	2 b	29 b	51.2 b	46.6 b	1.895 a	0.407 a	0.019 ab
P	6.2 ab	4.2 b	0.2 b	22.2 b	19.6 b	4 b	1.186 ab	0.070 a	0.001 b
P+r	10.2 a	52.6 a	18.8 a	46.8 a	147.8 a	107.4 a	1.376 ab	0.418 a	0.066 a
P+rm	8.6 ab	41.2 a	17.6 a	26.2 b	80.2 ab	33 b	0.697 ab	0.257 a	0.030 ab
N	0.2 c	6.2 bc	7.6 ab	2.2 c	5.2 b	3 b	0.01 b	0.006 a	-
N+m	-	-	-	-	-	-	-	-	-
N+rm	-	0.2 c	-	-	-	-	-	-	-

Percentage of AMF colonization of roots reached 39.48% and 15.35% on the P treatment from *L.muehlbergianus* and *P. elegans* at 255 days, respectively (Tab. 2). For the both species, seedlings from treatments that didn't receive P had the level of this nutrient in the shoot lower than the others, except for *P. elegans* plants (N treatment) at 255 days. This treatment also was relatively well colonized by AMF (Tab. 2). The N content apparently wasn't affect by the treatments in the *P. elegans* shoot. For *L. muehlbergianus*, the treatments without addition of this element had values comparable to the NP treatment. Results of P and N contents on the substrate responded like their contents in shoot.

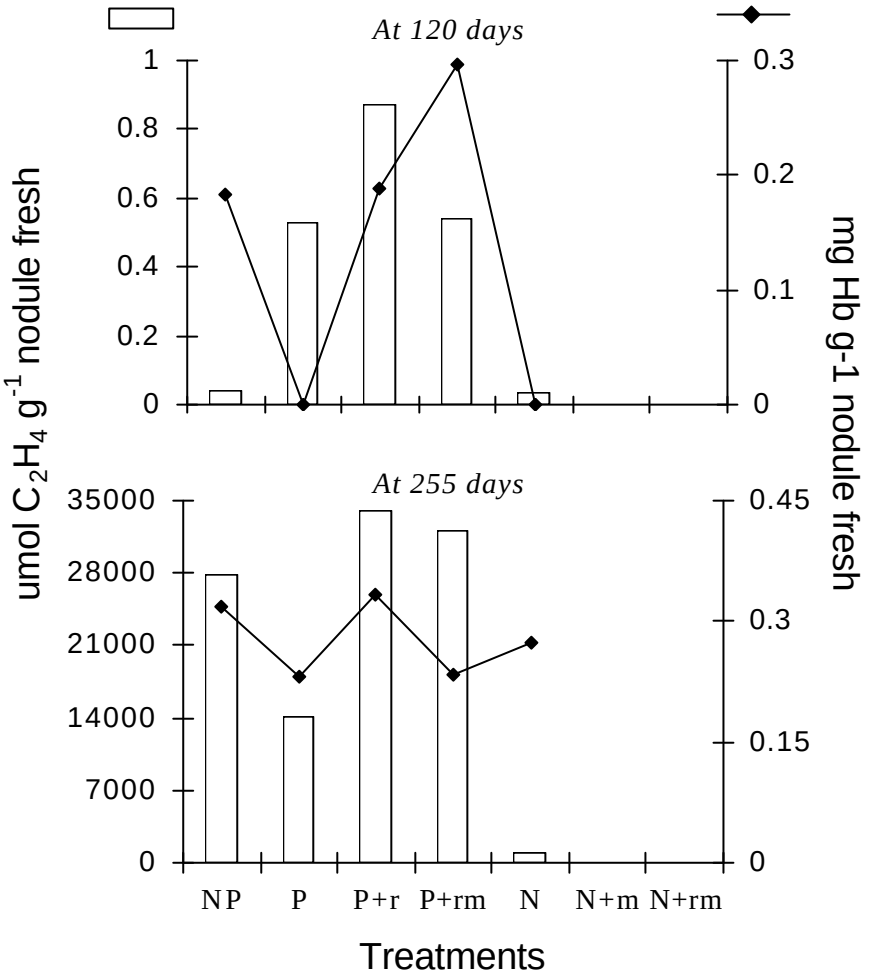

Figure 4. Acethylene reduction activity average in µmol C_2H_4 g^{-1} nodule fresh h^{-1} (bars) and leghemoglobin content average (lines) of *L. muehlbergianus* at 120 and 255 days of sowing fertilized with nitrogen and phosphorous (NP, N and P treatments) and inoculated by rhizobia (r), mycorrhiza (m) or both (rm).

Tab. 2. Percentage of mycorrhizal colonization average (AMF%), P and N content in the shoots of *L. muehlbergianus* and *P. elegans* cultivated upon treatments with NP, N and P, inoculated by rhizobia (r), mycorrhiza (m) or both (rm) at 120 and 255 days of sowing.

Treatments	*At 120 days*			*At 255 days*		
	AMF%	P (shoot)	N (shoot)	AMF%	P (shoot)	N (shoot)
			Lonchocarpus muehlbergianus			
NP	0.79	0.27	3.05	14.96	0.24	2.50
P	31.99	0.25	2.30	39.49	0.17	2.60
P+r	7.29	0.22	2.40	12.37	0.21	2.75
P+rm	1.01	0.21	2.55	9.22	0.20	3.35
N	4.58	0.09	2.70	5.68	0.09	3.05
N+m	4.30	0.10	3.75	16.56	0.08	4.25
N+rm	16.25	0.10	3.80	12.34	0.06	4.55
			Platypodium elegans			
NP	1.64	0.30	3.35	9.03	0.35	3.00
P	11.43	0.30	2.40	15.36	0.68	2.45
P+r	0.89	0.44	2.40	0.00	0.46	2.15
P+rm	5.56	0.34	*	0.00	0.07	2.10
N	0.00	0.15	*	12.43	0.76	2.60
N+m	0.91	0.10	*	1.82	0.11	2.85
N+rm	0.00	0.08	*	3.47	0.11	3.25

* not determinated.

Discussion

Although the probably deficiency of P and N in the treatments without addition of these nutrients had affected negatively the growth and development of two plant species, both grew less in the absence of P added than in the absence of N, exhibiting great response to mineral phosphorous. These results suggest that the growth of *L. muehlbergianus* plants in the P treatments was in response to N uptake via biological fixation by rhizobia, so this element hadn't been added, but it was present in the shoot of plants. In addition, this species was well nodulated in the P treatments, showing 302 nodules per plant in the P+r treatment at 255 days of sowing. Cordeiro *et al*. (1996) examined some developmental and structural aspects of nodules of *L. muelhbergianus* and founded a maximum value of 158 nodules per plant, when the seedlings were inoculated with a strain isolated from *L. muehlbergianus*.

Nodules morphology of *L. muehlbergianus* founded here can be classified as astragaloid although Faria *et al*. (1987) classified as mucunoid. *P. elegans* nodules were aeschynomenoid, as related by Faria *et al*. (1984). This last species had low nodulation in

all treatments. Probably, the nutritional deficiency affected negatively the growth and nodulation in this species. This low nodulation can be associated to specificity between host plant and rhizobia, since that the strains inoculated were isolated from *L. muehlbergianus*. On the other hand, the nodulation and acetylene reduction activity of 9.2 µmol C_2H_4 per nodule h^{-1} for *P. elegans* were detected in plants nodulated with native strains of rhizobia, by Goi *et al.* (1984). Faria *et al*. (1984) founded ARA of 180 µm C_2H_4 per nodule h^{-1} in seedlings of this species inoculated by a mixture of 222 strains of rhizobia.

The relatively high ARA and leghemoglobin content founded in NP treatment of *L. muehlberginanus* may be resultant of appropriate mineral nutrition. Bordeleau & Prévost (1994) reported that P deficiency limits nodulation indirectly by reducing legume growth.

The AMF colonization was low in our experiment, compared with the results of Carneiro *et al*. (1998) who characterized the occurrence of AMF of *P. elegans* in the nursery as an average of 20 to 49% of infected roots. Although low values, it was observed the highest P content in the shoot of *P. elegans* in the treatments that had more AMF colonization. This fact is in agreement with literature about the increase of P acquisition by plants associated with mycorrhizal fungi (Colebatch *et al*. 2002), also because mycorrhizal fungi are more efficient scavengers for nutrients from the soil than are plant roots (Treseder & Allen 2002).

Equally to observations of *Acacia mangium, Senna macranthera, S. multijuga and Anadenanthera peregrina* plants evaluated by Pereira *et al.* (1996), plants from the treatments supplied with P (P treatments) probably didn't have the nodulation stimulated by AMF, but due the P availability. The amount of P applied by Dekkers & van der Werff (2001) in their experiment was inversely related to the mycorrhizal colonization. Another way, we didn't observe relation between AMF colonization and N-nutrition like reported by these same authors.

As we have observed and according to Roldan-Fajardo (1994) and Marques *et al.* (2001), the extent of root colonization is not an indication of AMF's ability to enhance plant growth. An effective mycorrhizal infection may affect nodule weight, leghemoglobin content and nitrogen fixing activity. Because the plant growth may be modified by changing legume variety, either the rhizobia or AMF, more studies involving native tree species need to be carry out.

The growth and development of seedlings in the nursery conditions with the use of non-sterilized soil can to require only rhizobia inoculation, like observed in *L. muehlbergianus* plants, depending of presence of mycorrhiza in the soil. The same could be extended for *P. elegans* in experiments with inoculation of specific strains of rhizobia.

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4. CONSIDERAÇÕES FINAIS

Informações sobre a capacidade das espécies em formar as simbioses com rizóbio e com fungos micorrízicos são de fundamental importância para o sucesso das práticas de reflorestamento, podendo determinar a necessidade ou não de inoculação das plantas na fase de muda, uma vez que as áreas destinadas ao reflorestamento têm geralmente baixa fertilidade e baixo potencial de inóculos, tanto de rizóbios como de fungos micorrízicos. Entretanto, para que tal prática seja exercida, são necessários maiores detalhes sobre alguns aspectos básicos do crescimento das plantas arbóreas nativas em viveiro. Encontramos relativamente poucas informações sobre as características de germinação, requerimentos nutricionais e crescimento de algumas espécies usadas neste estudo.

As diferenças de respostas no crescimento das espécies pesquisadas, mesmo dentro da mesma tribo botânica demonstram a especificidade das associações e o cuidado que se deve ter nas generalizações, como a do fósforo agindo como inibidor da micorrização radicular**.** Esta informação mostrou-se coincidente para as espécies *A. colubrina* e *M. bimucronata*, uma vez que o nível de fósforo no solo parece ter inibido a colonização micorrízica em suas raízes, mas não provocou o mesmo resultado em *P. rigida*, *E.*

*contortisiliquum, I. laurina, L. muehlbergianus e P. elegan*s. Estes resultados levam-nos a sugerir experimentos para testar a colonização micorrízica em plantas submetidas a diferentes doses de fósforo mineral, especialmente para aquelas com características de tolerância a determinadas situações de degradação ambiental. Assim, uma vez detectada a concentração ótima de fósforo para a colonização micorrízica, pode-se associá-la à inoculação com rizóbios de alta eficiência na fixação biológica de nitrogênio. Neste sentido, também podemos propor o estudo do nível ótimo de nitrogênio para o desenvolvimento da dupla associação. Extrapolando um pouco mais, isso poderia ser feito com outros nutrientes conhecidos por interferir nessas simbioses, como o K, Ca, S, Zn, entre outros.

A colonização micorrízica foi baixa para a maioria das espécies, excetuando-se *M. bimucronata* e *P. rigida*, que apresentaram mais que 50% de raízes infectadas. Mesmo para estas espécies, a colonização micorrízica não afetou o teor fósforo e o crescimento das plantas.

A ausência de nodulação espontânea em *P. rigida* e *P. elegans* sugere que estas espécies apresentam maior especificidade com rizóbio em comparação às demais espécies estudadas. Projetos envolvendo o estudo da nodulação espontânea destas espécies, utilizando solos de diferentes regiões podem fornecer informações sobre suas especificidades.

As espécies *M. bimucronata, E. contortisiliquum* e *L. muehlbergianus* podem ser indicadas como promissoras para plantios em solos com deficiência nutricional, uma vez que apresentaram um crescimento satisfatório em substrato com deficiência de nitrogênio, quando inoculadas por rizóbio. A utilização de fungos micorrízicos mais eficientes poderão favorecer ainda mais o vigor das mudas dessas espécies, quando noduladas.

5. APÊNDICE

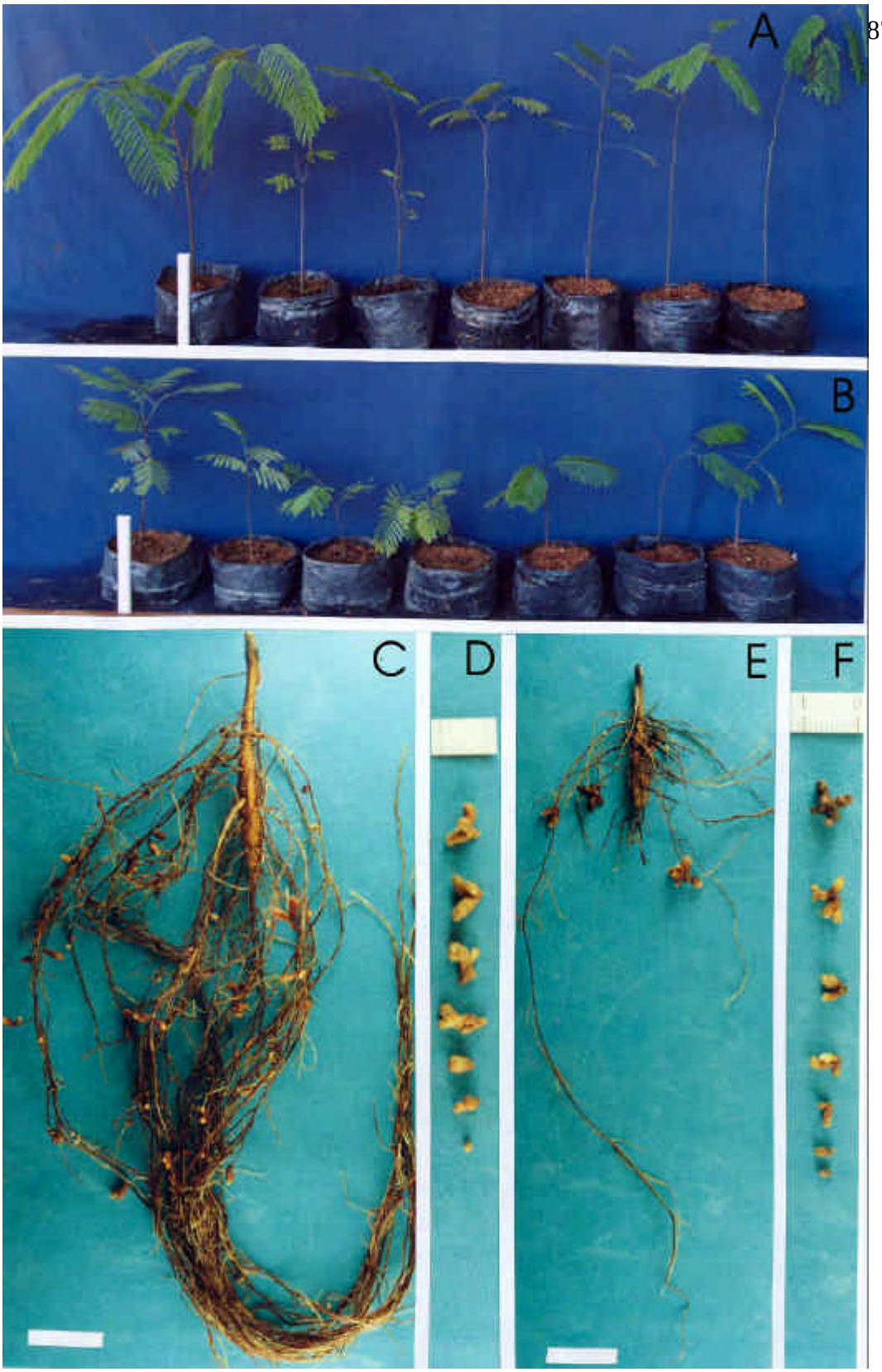

Fig. 1: Aspecto geral de *Anadenathera colubrina* e *Parapiptadenia rigida*. A, plantas *de A. colubrina* em solo de mata ciliar não esterilizado, fertilizadas nos tratamentos do estudo, sendo da esquerda para a direita: NP, P, P+r, P+rm, N, N+m, N+rm (Régua= 20 cm), aos 255 dias; B, plantas de *P. rigida* nas mesmas condições; C, raiz com nódulos de *A. colubrina* (Barra= 2 cm) aos 120 dias; D, nódulos em diferentes estádios de desenvolvimento aos 120 dias; E, raiz com nódulos de *P. rigida* (Barra= 2 cm) aos 120 dias; D, nódulos de *P. rigida* em diferentes estádios de desenvolvimento aos 120 dias.

Fig. 2: Aspecto geral de *Mimosa bimucronata* inoculada com rizóbio. A, planta em solo de mata ciliar não esterilizado, fertilizada e inoculada com rizóbio aos 255 dias (Régua= 20 cm); B: raiz com nódulos aos 120 dias (Barra= 2 cm); C: nódulos em diferentes estádios de desenvolvimento aos 120 dias; D: nódulos cortados mostrando a sua coloração interna avermelhada devido a leg-hemoglobina aos 120 dias.

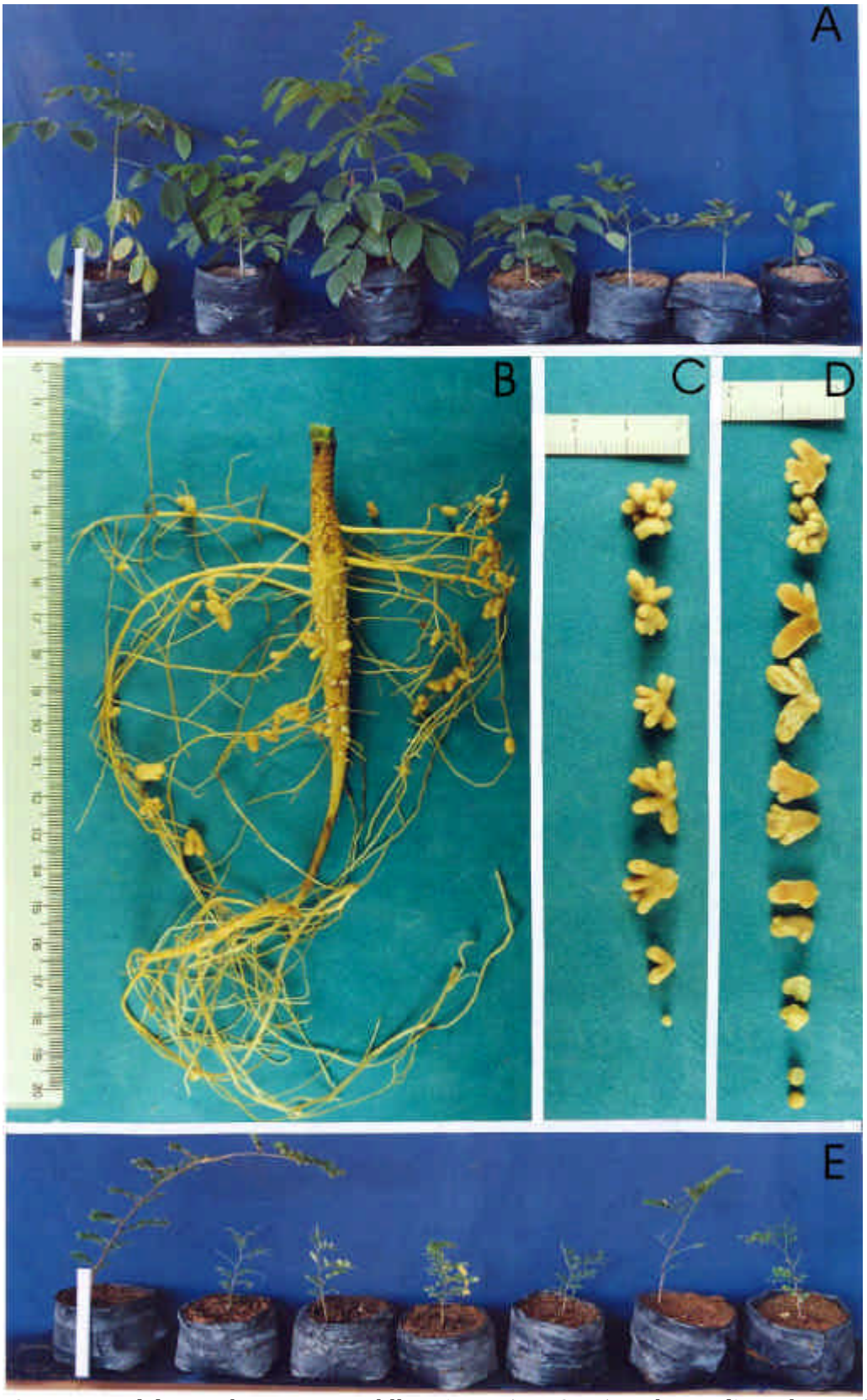

Fig. 3: Aspecto geral de *Lonchocarpus muehlbergianus* (Fig. A–D) e *Platypodium elegans* (Fig. E). A, plantas de *L. muehlbergianus* em solo de mata ciliar não esterilizado, fertilizadas nos tratamentos do estudo, sendo da esquerda para a direita: NP, P, P+r, P+rm, N, N+m, N+rm (Régua= 20 cm) aos 255 dias; B, raiz com nódulos aos 120 dias; C: nódulos em diferentes estádios de desenvolvimento aos 120 dias; D: nódulos cortados mostrando a sua coloração interna aos 120 dias; E, plantas de *P. elegans* nas mesmas condições que *L. muelhergianus*, aos 255 dias.

Fig. 4: Aspecto geral de *E. contortisiliquum* inoculado com rizóbio. A, planta em solo de mata ciliar não esterilizado, fertilizada e inoculada com rizóbio aos 255 dias (Régua= 20 cm); B: raiz com nódulos aos 120 dias (Barra= 2 cm); C: nódulos em diferentes estádios de desenvolvimento aos 120 dias; D: nódulos cortados mostrando a sua coloração interna avermelhada devido a leg-hemoglobina aos 120 dias.

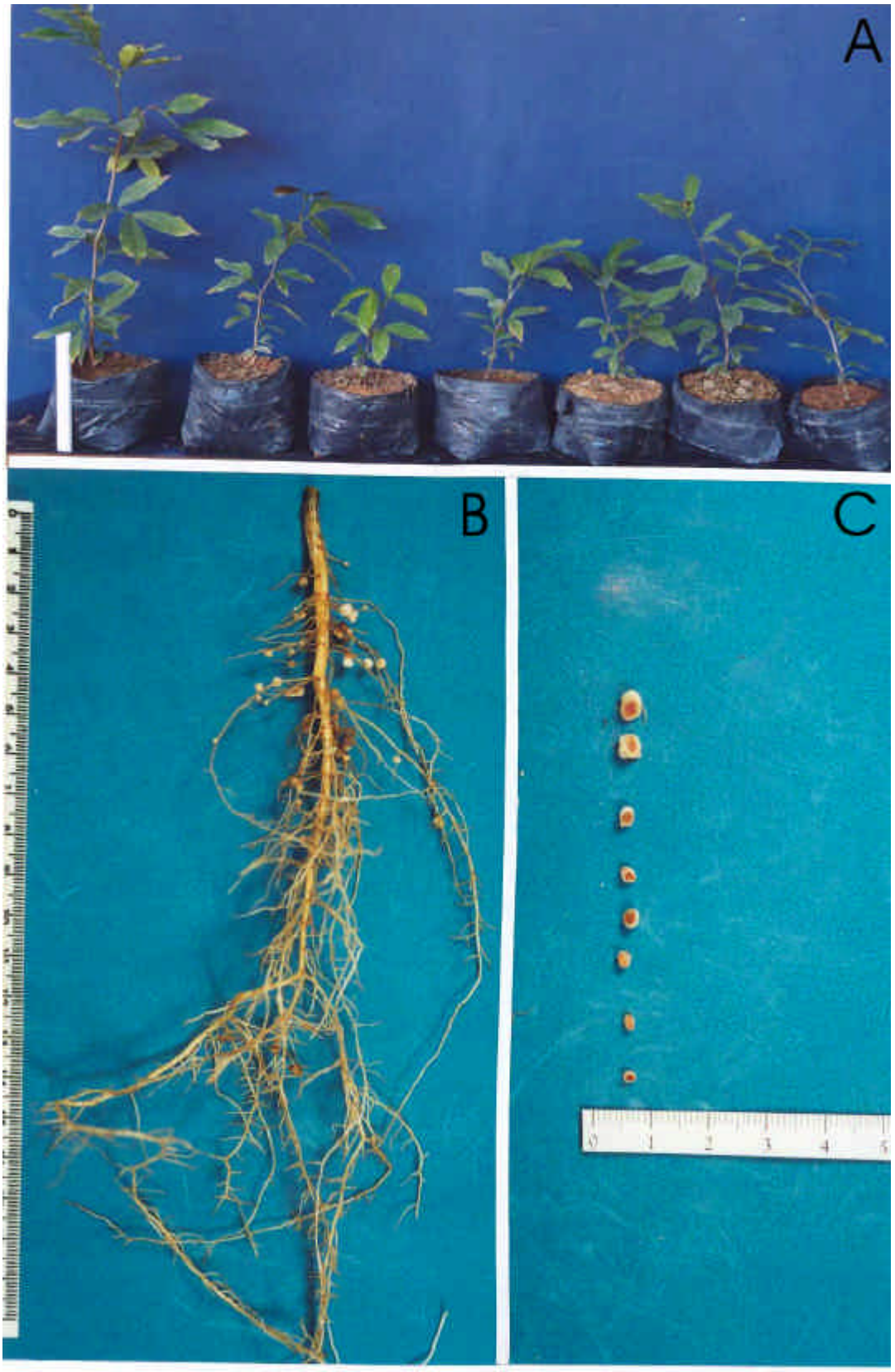

Fig. 5: Aspecto geral de *I. laurina*. A, plantas em solo de mata ciliar não esterilizado, fertilizadas nos tratamentos do estudo, sendo da esquerda para a direita: NP, P, P+r, P+rm, N, N+m, N+rm (Régua= 20 cm) aos 255 dias; B, raiz com nódulos aos 120 dias; C, nódulos cortados em diferentes estádios de desenvolvimento aos 120 dias, mostrando a sua coloração interna avermelhada devido a leg-hemoglobina.