

ANNA ABRAHÃO

**“Estratégias de forrageamento de plantas em campos rupestres da
Cadeia do Espinhaço, MG, Brasil: aquisição e uso de nitrogênio e
fósforo”**

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INSTITUTO DE BIOLOGIA

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RUPESTRES DA CADEIA DO ESPINHAÇO, MG, BRASIL: AQUISIÇÃO E USO
DE NITROGÊNIO E FÓSFORO”**

Orientador: Prof. Dr. Rafael Silva Oliveira

**“PLANT FORAGING STRATEGIES IN RUPESTRIAN FIELDS FROM THE
ESPINHAÇO RANGE, MINAS GERAIS, BRAZIL: NITROGEN AND
PHOSPHORUS ACQUISITION AND USE”**

**Dissertação de Mestrado apresentada ao Programa de Pós Graduação em Ecologia do
Instituto de Biologia da Universidade Estadual de Campinas para obtenção do título
de Mestre em Ecologia.**

**Master's thesis presented to the Ecology Postgraduation Programme of the School of
Medical Sciences of the University of Campinas to obtain the Master's grade in
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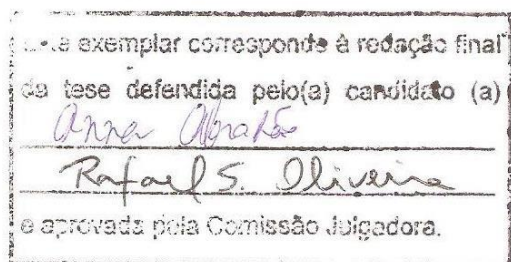
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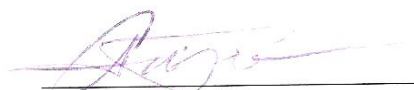
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BANCA EXAMINADORA

Prof. Dr. Rafael Silva Oliveira (Orientador)


Assinatura

Prof. Dr. Ricardo Augusto Gorne Viani


Assinatura

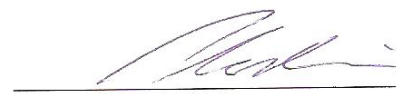
Prof. Dr. Alexandre de Siqueira Pinto

Assinatura

Prof. Dr. Fernando Roberto Martins

Assinatura

Prof. Dr. Pedro Henrique Santin Brancalion


Assinatura

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RESUMO

Apesar da grande diversidade de espécies de plantas nativas de solos tropicais e pobres em nutrientes, pouco se sabe sobre a diversidade de mecanismos de aquisição e uso de nutrientes nesses ambientes. Nesse contexto, o objetivo desse estudo foi entender como ocorre a aquisição e o uso de nitrogênio (N) e fósforo (P) em fisionomias rupestres de cerrado na Cadeia do Espinhaço, em Minas Gerais, sudeste do Brasil, em ambiente muito pobre em nutrientes.

No primeiro capítulo, nós descrevemos e investigamos o status micorrízico e o papel funcional da especialização radicular de *Discocactus placentiformis* (Cactaceae), uma espécie abundante de campos rupestres na Cadeia do Espinhaço. As raízes possuem pelos abundantes que aderem grãos de areia. A nossa hipótese foi que as raízes de *D. placentiformis* não possuem colonização por micorrizas, e funcionam de forma similar a *cluster roots*, aumentando a liberação de ácidos orgânicos quando submetidas à deficiência de P. Nós não observamos colonização por micorrizas nas raízes de *D. placentiformis*. Ao coletar e analisar os exsudatos radiculares, identificamos ácido oxálico, málico, cítrico, láctico, succínico, fumárico e malônico, em ordem decrescente de concentração. Como esperado, a exsudação total aumentou com a deficiência de P. O papel ecofisiológico dessa nova especialização radicular parece ser semelhante ao de raízes do tipo *cluster roots*. Esses resultados sugerem uma convergência funcional entre estratégias nutricionais de plantas em habitats pobres em nutrientes.

No segundo capítulo, em nível de comunidade, comparamos as assinaturas isotópicas de N ($\delta^{15}\text{N}$) e a colonização por micorrizas dos representantes mais abundantes de três fisionomias rupestres de cerrado, com disponibilidades distintas de N no solo (campos rupestres, campos úmidos e cerrados ralos). Nós testamos a hipótese de que o $\delta^{15}\text{N}$ foliar aumenta com o N total no solo e que as fisionomias mais pobres em N apresentam um maior intervalo de valores de $\delta^{15}\text{N}$ foliar, possuindo então maior diversidade de estratégias de aquisição de N. Em todas as fisionomias nós observamos uma disponibilidade de nitrogênio muito baixa, principalmente nos campos rupestres. Ao contrário do esperado, nós observamos uma relação negativa entre os valores de $\delta^{15}\text{N}$ foliar e o N total no solo. Apesar das diferenças de nitrogênio no solo, os valores de N foliar foram similares entre as fisionomias. Os resultados também mostram que o maior intervalo

de valores de $\delta^{15}\text{N}$ foliar não está associado com o ambiente com menor valor de N total no solo. No entanto, como esperado, a grande variação nos valores de $\delta^{15}\text{N}$ foliar aponta a diversidade de mecanismos de aquisição de nitrogênio.

A eficiência na aquisição de fósforo por meio de especializações radiculares e a diversidade de mecanismos de absorção de nitrogênio podem ajudar a manter a grande diversidade de espécies nesses ambientes rupestres. Além disso, parece haver uma convergência funcional destes ambientes pobres em nutrientes da América do Sul com ambientes pobres em nutrientes de outras partes do mundo.

ABSTRACT

Despite the great plant species richness in tropical and nutrient-poor soils, little is known about nutrient-acquisition strategies in these environments. In this context, the aim of this work was to understand the acquisition and use of nutrients in a nutrient-impooverished site in Brazil. Therefore, we studied nitrogen and phosphorus acquisition and use in rupestrian physiognomies at the Espinhaço mountain range, in Minas Gerais, southeastern Brazil.

In the first chapter, we described and investigated a sand-binding root specialization of *Discocactus placentiformis* (Cactaceae), a common species in rupestrian grasslands white sands. We hypothesized that *D. placentiformis* is non-micorrhizal and increases organic acid release when submitted to P defficiency. We collected and analyzed root exudates from plants subjected to different phosphorus supplies. We identified oxalic, malic, citric, lactic, succinic fumaric and malonic acids, in decreasing order of concentration. As expected, total exudation decreased with P supply. These results show that the ecophysiological role of this root specialization seems to be similar to cluster roots, pointing towards a functional global convergence in nutrient-acquisition strategies between plants from nutrient-poor habitats.

In the second chapter, at the community level, we compared N isotopic signatures ($\delta^{15}\text{N}$), from the most abundant species in three rupestrian cerrado communities (rupestrian fields, humid fields and shrublands). We tested the hypothesis that foliar $\delta^{15}\text{N}$ values increases with N availability, and that physiognomies with lower N availability present a higher range of foliar $\delta^{15}\text{N}$ values. In all the physiognomies, we observed a very low N availability, especially in the rupestrian grasslands. Contrary to expectations, we observed a negative relation between foliar $\delta^{15}\text{N}$ values and total soil N. Despite the differences in soil N, total foliar N values were similar among physiognomies. These results show that higher foliar $\delta^{15}\text{N}$ do not always indicate higher soil N availability and that the widest range of foliar $\delta^{15}\text{N}$ is not always associated with lowest total soil N. However, as expected, we observed a high range in foliar $\delta^{15}\text{N}$, indicating diversity in N-acquisition strategies.

The efficiency in phosphorus acquisition by root specializations and the diversity in nitrogen-acquisition mechanisms may help maintaining the high species diversity in these

habitats. Additionally, there seems to be a functional convergence between rupestrian communities and other nutrient-poor sites in the world.

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Introdução geral

A disponibilidade de nutrientes no solo é um dos fatores seletivos, ou filtros ambientais, que influenciam a distribuição das plantas em mosaicos ou gradientes, e afetam a biodiversidade em ecossistemas naturais (Lambers *et al.* 2010). Em ambientes oligotróficos, ou seja, depauperados em nutrientes no solo, a aquisição de nutrientes se torna um desafio ainda maior para as plantas. Os nutrientes mais limitantes para o crescimento vegetal são o nitrogênio (N) e o fósforo (P) (Chapin 1980). O nitrogênio é pouco abundante em paisagens jovens, férteis e com alta frequência de distúrbios (YODFELs – *Young, often disturbed, fertile landscapes, sensu* Hopper (2009)), uma vez que é pouco disponível no estrato rochoso, mas se acumula por deposição atmosférica ou fixação biológica, no entanto, ele é perdido por lixiviação pelos processos pedológicos ou volatilização, tornando-se escasso em solos muito intemperizados. Já o fósforo é abundante em paisagens jovens em estágios precoces de desenvolvimento do ecossistema, uma vez que se origina do material parental do solo, e se torna disponível por processos de intemperismo em rochas ricas nesse elemento, mas também é perdido por lixiviação com o envelhecimento dos solos (Lambers *et al.* 2008b; Peltzer *et al.* 2010). Assim, em solos muito antigos, tanto o N quanto o P são escassos. Em solos de ambientes antigos, inférteis e com clima tamponado, que não sofreu glaciações, ou grandes distúrbios climáticos recentes (OCBILs – *Old, climatically buffered, infertile landscapes sensu* Hopper 2009), o crescimento vegetal é limitado por P, mas também ocorre limitação por N (Pekin *et al.* 2012). Esses ambientes antigos estão associados com alta diversidade de espécies, possivelmente devido à especialização de estratégias de aquisição de nutrientes, ou partição de nichos (McKane *et al.* 2002; Bustamante *et al.* 2004; Lambers *et al.* 2010; Pekin *et al.* 2012). Entender a aquisição de nutrientes pode auxiliar explicar a diversidade de espécies nesses ambientes.

Nos ambientes oligotróficos, é comum a ocorrência de simbioses com fungos chamadas micorrizas (Lambers *et al.* 2008b, 2010; Brundrett 2009). As hifas dos fungos crescem cobrindo um volume de solo muito maior do que as adaptações radiculares, e realizam uma exploração extensiva do solo (Lambers *et al.* 2008b), mas possuem um alto custo de manutenção, já que em troca dos nutrientes obtidos pelo fungo, a planta lhe fornece o carbono (Redman *et al.* 2001; van der Heijden *et al.* 2008). Acredita-se que 90%

das espécies de plantas possuam associações do tipo micorriza (Fitter *et al.* 2000). No entanto, em ambientes muito pobres em nutrientes, o custo em carbono para o fungo pode se tornar muito alto em comparação com o retorno (Lynch & Ho 2005; Smith & Read 2008). Nos ambientes mais distróficos como os OCBILs, são mais comuns especializações radiculares chamadas *cluster roots*, como as raízes proteóides (descritas inicialmente em Proteaceae), raízes dauciformes (Cyperaceae) e as capilaróides (Restionaceae) (Neumann & Martinoia 2002; Lambers *et al.* 2008b). Raízes proteóides são concentrações de raízes secundárias ao longo de um eixo, de crescimento limitado (Purnell 1960), e as raízes dauciformes e capilaróides possuem ramificações (raízes laterais) de crescimento determinado com longos pelos radiculares muito abundantes (Lambers *et al.* 2006; Shane *et al.* 2006). Essas especializações radiculares permitem uma exploração intensiva *in situ* do solo (Lynch & Ho 2005). Elas possuem uma alta eficiência na aquisição de fósforo por meio da liberação em pulsos de exsudatos radiculares localizados que facilitam a aquisição de P mesmo quando esse é pouco disponível ou existente em formas previamente indisponíveis (Watt & Evans 2003).

Os exsudatos radiculares englobam uma grande variedade de compostos como aminoácidos, ácidos orgânicos, compostos fenólicos, açúcares, vitaminas, nucleotídeos, enzimas, íons inorgânicos e moléculas gasosas (Dakora & Phillips 2002), no entanto, nem todos estão envolvidos na aquisição de nutrientes. Nos OCBILs, como a disponibilidade de P é muito baixa, mecanismos que possibilitem o acesso a esse nutriente fornecem uma vantagem adaptativa. Os exsudatos radiculares envolvidos na aquisição de P são enzimas extracelulares como fosfatases ácida e alcalina, apirases e fitases que hidrolisam e mobilizam P inorgânico a partir de fosfatos orgânicos do solo (Duff *et al.* 1994). Compostos orgânicos como os fenóis podem solubilizar P de fontes pouco disponíveis (Dakora & Phillips 2002), flavonóides solubilizam P ligado a ferro e limitam a mineralização de ácidos orgânicos pela microbiota do solo (Tomasi *et al.* 2008). Por fim, os ácidos orgânicos ou carboxilatos promovem a troca direta de ligantes, pela qual o seu ânion orgânico substitui o P em superfícies de troca de ligantes (*e.g.* superfície de óxidos); ou ainda geram a dissolução mineral promovida por ligantes. Esse processo ocorre por meio da adsorção do ligante e posterior destacamento do complexo metal-ligante (Johnson & Loeppert 2006; Lambers *et al.* 2008a; Oburger *et al.* 2011). O grau de complexação do

carboxilato depende do ácido orgânico envolvido (número e proximidade de grupos carboxílicos), da concentração, do tipo de metal e do pH da solução do solo (Jones 1998). O citrato (ânion trivalente), o malato (ânion bivalente) e o oxalato (ânion bivalente) possuem mais grupos carboxílicos, portanto, mais afinidade com cátions trivalentes como Fe^{3+} e Al^{3+} do que o lactato (ânion monovalente) (Oburger *et al.* 2009). Uma vez no solo, ácidos orgânicos como o citrato são capazes de induzir um aumento de cinco a 13 vezes na mobilização de P com relação ao controle (KCl) (Oburger *et al.* 2011), mostrando sua importância para as plantas nesses ambientes.

As províncias florísticas inicialmente definidas como OCBILs foram os Pantepuis Venezuelanos, a região do Cabo e a vegetação suculenta Karoo na África do Sul, e a região florística do sudoeste da Austrália (SWAFR – *Southwest Australian Floristic Region*) (Hopper, 2009). No entanto, Hopper (2009) reconhece que partes do Brasil podem se enquadrar nessa categoria. Os campos rupestres de cerrado possuem várias características comuns aos OCBILs e, assim como estes, são, apesar dos solos pobres em nutrientes, reconhecidamente muito ricos em espécies, possuindo altas taxas de endemismo (*Gondwanan Heritage Hypothesis*) (Alves & Kolbek 1994; Romero & Nakajima 1999; Benites *et al.* 2003; Echternacht *et al.* 2011). As barreiras geográficas entre áreas de campos rupestres, assim como as de OCBILs, levaram ao isolamento e geraram maiores taxas de endemismo, principalmente em espécies com dispersão limitada (Alves & Kolbek 1994). Da mesma forma que ocorre em OCBILs, apesar do isolamento e das populações serem reduzidas, a diversidade genética parece ser alta como, por exemplo, em *Bulbophyllum* (Orchidaceae) (*James Effect*) (Azevedo *et al.* 2007). Os indivíduos podem ser muito antigos, assim como em OCBILs (*Ultimate Self Hypothesis*), como é o caso de *Vellozia*, de crescimento muito lento, podendo levar até 100 anos para atingir a idade adulta (Alves & Kolbek 1994). Além disso, a ausência de distúrbios climáticos ou geológicos recentes como glaciações ou erupções vulcânicas no Brasil central favorece a manutenção de linhagens antigas (Salgado-Labouriau *et al.* 1998; Ledru *et al.* 1998; Hopper 2009). Especializações nutricionais como a carnivoría e a protocarnivoría já foram descritas (Pereira *et al.* 2012; Nishi *et al.* 2012) e outras estão em descrição (Candido *et al.*, Campos *et al.*, Abrahão *et al.* em preparação). Além de especializações nutricionais, as espécies de campos rupestres apresentam atributos relacionados com a resistência ao calor extremo ou à

seca semelhantes às de OCBILs, como tricomas, bainhas foliares persistentes, suculência, esclerofilia e tolerância à dessecação como, por exemplo, as plantas de ressurreição (Gaff 1987; Messias *et al.* 2011). Por fim, os solos são extremamente susceptíveis à erosão (Minas Gerais 2004). Portanto, os campos rupestres de cerrado parecem se encaixar na descrição de OCBILs.

Alguns resultados preliminares de análise de resposta à adição de fertilizantes mostraram que as espécies do cerrado respondem tanto ao incremento em N quanto ao P, indicando uma colimitação simultânea (Bucci *et al.* 2006; Kozovits *et al.* 2007). Outros trabalhos indicam forte limitação apenas por P (Nardoto *et al.* 2006) ou apenas por N (Bustamante *et al.* 2006). Em outro trabalho ainda, os autores não observaram efeito em crescimento ou sobrevivência com o incremento de nutrientes de duas das três espécies estudadas (Viani *et al.* 2011). No entanto, efeitos de toxicidade são observados com um incremento excessivo de N ou P, especialmente em espécies de plantas adaptadas a ambientes tidos como pobres (Grundon 1972; Aerts & Chapin 2000), indicando uma resposta à seleção por oligotrofia, associada à distribuição relativamente restrita. Para evitar a interferência no sistema pela adição de fertilizantes, é possível estudar limitação nutricional ao longo de gradientes naturais de disponibilidade de nutrientes, como ocorre nas fitofisionomias de cerrado (Goodland & Pollard 1973), ou ainda a partir dos isótopos estáveis do nitrogênio (^{14}N e ^{15}N).

O ^{15}N é o isótopo pesado do nitrogênio, e sua abundância natural é menor que a do isótopo leve, o ^{14}N (Robinson 2001). Se as abundâncias naturais de ^{15}N de todas as fontes de nitrogênio forem conhecidas, é possível estimar a contribuição de cada fonte usando modelos de mistura. No entanto, como a assinatura isotópica não é um rastreador conservativo, se as fontes se misturam, variam ao longo do tempo, ou sofrem fracionamento, a assinatura perde o seu significado (Högberg 1997). O fracionamento isotópico ocorre em processos físicos, enzimáticos e biológicos que discriminam contra o ^{15}N (isótopo pesado) em favor do ^{14}N (isótopo leve) quando ligações químicas são quebradas (Pardo & Nadelhoffer 2010). Ele ocorre porque algumas reações requerem menos energia para formar ou quebrar ligações químicas com ^{14}N do que com ^{15}N , e nesse caso, o ^{14}N reage mais rapidamente (Bigeleisen 1965). Esse fenômeno gera diferenças entre

a razão $^{15}\text{N}/^{14}\text{N}$ entre os reagentes e os produtos da reação. Se a reação for incompleta, o produto será menos enriquecido em ^{15}N do que o reagente (Högberg 1997).

O valor de $\delta^{15}\text{N}$ (medida da razão $^{15}\text{N}/^{14}\text{N}$) aumenta com o enriquecimento em ^{15}N de uma amostra. Cada etapa do ciclo do nitrogênio possui um fator de fracionamento diferente que depende dos fatores abióticos e da direção da reação (Högberg 1997). Exemplos de processos no ciclo do nitrogênio que sofrem fracionamento incluem volatilização, nitrificação, denitrificação, amonificação, desaminação e transaminação (Robinson 2001). Entre os fatores que influenciam a ciclagem estão o clima e a microbiota, que interagem um com o outro produzindo padrões complexos de valores de $\delta^{15}\text{N}$ (Pardo & Nadelhoffer 2010).

A meta-análise realizada em gradientes climáticos apresentada por Craine *et al.* (2009) aponta para as influências do clima nos valores de $\delta^{15}\text{N}$. Nesse estudo, os valores de $\delta^{15}\text{N}$ foliar aumentaram com a diminuição da precipitação anual média. Com relação à temperatura, os valores de $\delta^{15}\text{N}$ foliar aumentaram com o aumento da temperatura anual média em locais com temperatura anual média maior do que $-0,5^{\circ}\text{C}$, mas não variaram em locais com temperatura anual média menor do que $-0,5^{\circ}\text{C}$ (Craine *et al.* 2009).

Ao contrário de outros recursos que tem *inputs* abióticos, a fonte de nitrogênio primária é a fixação biológica. Uma vez o nitrogênio fixado, o ecossistema é particularmente susceptível à sua perda por lixiviação e volatilização (LeBauer & Treseder 2008). Os processos de ciclagem de nitrogênio mediados por microrganismos como a hidrólise enzimática, amonificação, nitrificação ou denitrificação contribuem para a perda de nitrogênio que gera a diferenciação da assinatura isotópica do nitrogênio entre os horizontes do solo. Por exemplo, a discriminação microbiana durante a mineralização produz amônio empobrecido em ^{15}N e causa o enriquecimento em ^{15}N do solo orgânico residual (Högberg 1997). A nitrificação produz nitrato empobrecido em ^{15}N e amônio enriquecido em ^{15}N (Handley & Raven 1992). Outros processos que fracionam muito são a denitrificação e a volatilização de amônia. Quando o produto empobrecido em ^{15}N é removido por lixiviação, perda gasosa, etc., os reservatórios de N restantes (solo, vegetação, e pool de N inorgânico – NH_4^+ e NO_3^-) se tornam enriquecidos em ^{15}N . Por exemplo, após a nitrificação, se o amônio enriquecido em ^{15}N é retido no solo e o nitrato

empobrecido em ^{15}N é lixiviado do ecossistema, então o efeito bruto da nitrificação é o enriquecimento do solo em ^{15}N (Pardo & Nadelhoffer 2010).

Os valores de $\delta^{15}\text{N}$ das plantas e do solo são interligados. As etapas de aquisição e liberação de nitrogênio pelas plantas modificam o $\delta^{15}\text{N}$ do solo. Assim sendo, o $\delta^{15}\text{N}$ da camada de solo na qual as plantas absorvem o nitrogênio será diferente das outras, pois geralmente ocorre discriminação contra o ^{15}N no processo de absorção, deixando o solo localmente enriquecido nesse isótopo (Högberg 1997). Adicionalmente, a contribuição da serapilheira (pobre em ^{15}N por causa da discriminação na aquisição) empobrece os horizontes superiores do solo. Consequentemente, é comum ocorrer um enriquecimento em ^{15}N do solo com a profundidade. No entanto, pode ocorrer mistura de solo entre diferentes horizontes por bioturbação, ação eólica ou outros processos mecânicos (Hobbie & Ouimette 2009).

Como a maior parte das plantas adquire o nitrogênio a partir do solo ou através de micorrizas (exceto carnívoras, epífitas e plantas associadas com microrganismos fixadores de nitrogênio) (Lambers *et al.* 2010), sua assinatura isotópica será dependente da assinatura do solo. Cada espécie possui uma assinatura diferente, pois existem diferenças na forma de nitrogênio adquirida, as taxas relativas de ciclagem e perda de N, a extensão e o tipo de associação com micorrizas, a profundidade do enraizamento, e as transformações e perdas de N dentro das plantas (Pardo & Nadelhoffer 2010).

Quando a aquisição de N é intermediada por micorrizas, o padrão geral é de enriquecimento em ^{15}N do fungo e empobrecimento em ^{15}N na planta hospedeira (Adams & Grierson 2001). A associação com diferentes micorrizas implica em diferentes assinaturas isotópicas do N. Plantas associadas com ectomicorrizas possuem folhas mais empobrecidas em ^{15}N do que as associadas com micorrizas arbusculares, que por sua vez possuem folhas mais empobrecidas em ^{15}N do que as plantas sem associação com micorrizas (Schmidt & Stewart 2003). Se a aquisição é feita por meio de bactérias endofíticas ou produtoras de nódulos que fixam nitrogênio atmosférico, espera-se que haja pouco fracionamento, resultando em assinaturas próxima do $\delta^{15}\text{N}$ atmosférico (entre 0‰ e 2‰).

O $\delta^{15}\text{N}$ final nos tecidos vegetais será então dependente da espécie, das variações no $\delta^{15}\text{N}$ da(s) fonte(s) ao longo do tempo, da profundidade na qual o N é adquirido do solo,

bem como dos mecanismos envolvidos na aquisição, transporte e perda de N (Evans 2001). Entre as formas possíveis de perda de N estão a senescência de partes da planta, a volatilização de N das folhas para a atmosfera e a perda de N solúvel pelas raízes para a rizosfera (Robinson *et al.* 2000).

Em ambientes com maior disponibilidade de N, as plantas tendem a apresentar maiores valores de $\delta^{15}\text{N}$, pois é mais provável que as perdas ocorram sob a forma de ^{15}N (Craine *et al.* 2009). Além disso, em ambientes pobres em nitrogênio, é mais provável que as plantas dependam de micorrizas para a obtenção de nitrogênio, obtendo assim N depauperado em ^{15}N (Hobbie & Colpaert 2003).

Os valores de $\delta^{15}\text{N}$ foliares e do solo nos permitem integrar várias etapas do ciclo do nitrogênio, permitindo inferências que nenhuma outra medida nos permite. As sínteses com grandes quantidades de dados de $\delta^{15}\text{N}$ nos permitem entender os padrões subjacentes ao $\delta^{15}\text{N}$, que por sua vez, permite uma maior compreensão dos padrões e controles da ciclagem do N nos ecossistemas (Pardo & Nadelhoffer 2010).

Nesse contexto, o objetivo do presente trabalho é investigar mecanismos de uso e aquisição de N e P em ambientes rupestres. Estudos preliminares (Cândido 2012; de Campos 2012) investigaram especializações radiculares nas mesmas áreas em que ocorreu o presente estudo e os resultados sugerem que espécies com especializações radiculares semelhantes a *cluster roots* ocorram nessas áreas. No entanto, não foi encontrado na literatura trabalho que investigasse os mecanismos de aquisição do fósforo por essas plantas.

No capítulo I, para compreender como ocorre a aquisição de fósforo em uma espécie, nós selecionamos entre as espécies mencionadas nos trabalhos preliminares (Cândido 2012; de Campos 2012), *Discocactus placentiformis* (Cactaceae), uma espécie comum em areias brancas de campos rupestres. Visando entender o papel funcional dessas raízes, verificamos a presença de exsudação ácida, e analisamos os carboxilatos liberados em plantas submetidas a concentrações diferentes de fósforo. Além disso, analisamos raízes para identificar a presença de micorrizas. Neste estudo, nossas hipóteses foram que em ambientes com maiores concentrações de P no solo, essa espécie reduz a liberação de ácidos orgânicos, uma vez que o suprimento de fósforo é adequado; e que essa espécie não se associa com micorrizas.

Outra questão importante para atingir nosso objetivo diz respeito à compreensão do uso do nitrogênio nessas comunidades. Embora tenhamos encontrado trabalhos que avaliaram o uso de fósforo em nível de comunidade, bem como a estequiometria entre o nitrogênio e o fósforo, o uso de nitrogênio não foi relatado na literatura. No capítulo II, de modo a investigar os padrões de ciclagem e diversidade no uso de nitrogênio em comunidades rupestres pobres em nutrientes, nós comparamos o conteúdo total de N e os valores de $\delta^{15}\text{N}$ foliares e do solo, além da colonização por micorrizas, em 60 espécies de seis comunidades em três fisionomias diferentes na Cadeia do Espinhaço, Minas Gerais. As fisionomias estudadas foram o campo rupestre, o campo sujo úmido e o cerrado ralo. Nossa hipótese foi que com o aumento da disponibilidade de nitrogênio no solo, as plantas apresentam maiores valores de $\delta^{15}\text{N}$. Além disso, esperamos que em menores disponibilidade de nitrogênio, as espécies apresentam maior variação no $\delta^{15}\text{N}$ foliar.

Esperamos então, com esse trabalho, somar informações na classificação dos campos rupestres de cerrado como OCBILs e compreender melhor os mecanismos de forrageamento das plantas nesses ambientes, por meio da abordagem do uso e da aquisição dos macronutrientes essenciais nitrogênio e fósforo.

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Capítulo I. Especialização radicular e exsudação ácida em *Discocactus placentiformis* (Cactaceae), espécie nativa de campo rupestre de cerrado.

Chapter I. Specialized root morphology and acid exudation in *Discocactus placentiformis* (Cactaceae), native species from cerrado rupestrian grasslands.

Abstract

The cerrado savannas occur over the Brazilian shield, an ancient domain characterized by acidic and nutrient-poor soils. Despite its recognized species richness, very little is known about the diversity of nutrient-acquisition strategies of rupestrian grassland plants. Here we describe a novel sand-binding root specialization in *Discocactus placentiformis*, a common species in cerrado vegetation over white sands. To evaluate the functional role of these roots in P uptake, we collected adult plants from the field and checked for acid exudation with the pH change indicator, the bromocresol purple. We also collected root exudates from plants submitted to four phosphorus concentrations from 0 to 100 $\mu\text{mol l}^{-1}$. The roots are morphologically similar to early stages of capillaroid roots of Restionaceae, but they differ anatomically, representing a new root specialization. We observed acid exudation in the bromocresol experiment and UPLC-MS analysis allowed us to identify oxalic, malic, citric, lactic, succinic, fumaric and malonic acids in decreasing order of concentrations. Overall exudation was reduced with increasing phosphorus supply. Oxalic acid was the major exudate released and is the main cause for this exudation reduction with P supply because it was the only exudate affected by phosphorus supply. The ecophysiological role of this new root specialization seems to be similar to that of cluster roots, which are known to enhance nutrient uptake, especially in nutrient-poor habitats. These results may indicate that plants from nutrient-impooverished habitats around the world function similarly.

Resumo

O Cerrado ocorre na zona de escudo brasileira, um domínio antigo caracterizado por solos ácidos e pobres em nutrientes. Apesar da reconhecida riqueza de espécies, pouco se sabe sobre a diversidade de estratégias de aquisição de nutrientes de plantas de campos rupestres de cerrado. Nesse trabalho, nós descrevemos uma nova especialização radicular que adere fortemente os grãos de areia em *Discocactus placentiformis*, uma espécie comum no cerrado em areias brancas. As raízes são morfológicamente semelhantes aos estágios iniciais de raízes capilaróides de Restionaceae, mas são anatomicamente diferentes, representando uma nova especialização radicular. De modo a entender o papel funcional dessas raízes, nós coletamos plantas adultas no campo e verificamos a presença de exsudação ácida com bromocresol púrpura, um indicador de pH. Nós também coletamos os exsudatos e preparamos padrões de ácido cítrico, málico, oxálico, succínico, láctico, maleico, tartárico, DL-isocitrônico, malônico e fumárico, que estão envolvidos na mobilização de fósforo. Nós observamos exsudação ácida no experimento com bromocresol e a análise em UPLC-MS nos permitiu identificar ácido oxálico, málico, cítrico, láctico, succínico, fumárico, malônico em ordem decrescente de concentração. A exsudação total foi reduzida com o suprimento de fósforo e o ácido oxálico foi o responsável por esse padrão, pois além de ser o ácido liberado em maior concentração, foi o único cujo padrão de exsudação foi afetado pelo suprimento de fósforo. O papel ecofisiológico dessa nova especialização radicular parece ser semelhante ao de raízes do tipo *cluster roots*, que são conhecidas por aumentar a absorção de nutrientes. Esses resultados podem apontar para uma convergência funcional global entre estratégias nutricionais de plantas em habitats pobres em nutrientes.

Introduction

Nutrient availability is one of the main environmental filters influencing plant distribution (Lambers *et al.* 2008a). Old landscapes tend to be phosphorus-limited rather than nitrogen-limited because phosphorus (P) is originally present in the soil parent material and is lost with soil leaching, while nitrogen is scarce in the parent material and is gradually accumulated by atmospheric deposition and biological fixation (Lambers *et al.* 2010). Therefore, unless soils happen to be rejuvenated, ecosystem aging is usually accompanied by nutrient impoverishment (Lambers *et al.* 2008b), especially P, and also by increasing species diversity (Pekin *et al.* 2012). This diversity is also reflected in plant-nutritional strategies (Bustamante *et al.* 2004; Lambers *et al.* 2010; Viani *et al.* 2011). (Hopper 2009) called these environments OCBILs (Old, Climatically Buffered, Infertile Landscapes) and based his OCBIL theory on three floristic provinces: the succulent Karoo vegetation from South Africa, the Southwest Australian Floristic Region (SWAFR), and the Venezuelan Pantepuis, but he also suggested that other parts of South America function as OCBILs, however did not name them.

A set of nutritional and other biological specializations have been associated with these old, P-impoverished habitats (Lambers *et al.* 2008b, 2010). In extremely nutrient-impoverished soils, plants are expected to be nonmycorrhizal and rather present root specializations (Lambers *et al.* 2008b; Lambers & Teste 2013). The carbon cost of maintaining mycorrhizae associations may become too high when compared to profits (Lynch & Ho 2005; Smith & Read 2008). Alternative strategies are nutritional specializations. Among the nutritional specializations, such environments present plants that invest in root proliferation in shallow soil horizons (Lynch & Brown 2001), increase abundance and length of root hairs (Zhang *et al.* 2003, Bates & Lynch 2001), increase root biomass (Kirschbaum *et al.* 1992), and length (Steingrobe 2001). Plants also present non-mycorrhizal root specializations, such as cluster roots (Miller 2005; Lambers *et al.* 2010). These root specializations are known to be involved in organic nitrogen acquisition (Turnbull *et al.* 1996), but they are especially recognized for their ability to acquire sparingly soluble forms of phosphorus (Watt & Evans 2003; Shane & Lambers 2005;

Lambers *et al.* 2006; Pearse *et al.* 2007), not only by increasing surface area, but by releasing a high amount of localized root exudates, such as organic acids and phosphatases (Raghothama 1999) in a small soil volume (Dinkelaker *et al.* 1995).

Organic acids (or carboxylates) are important exudates released by the roots that participate in nutrient acquisition. In the cytosolic pH (around 7.4), they are usually present in the dissociated form, and are therefore called carboxylates or organic anions. Some species increase carboxylate exudation in response to P, K or Fe deficiency (Hoffland *et al.* 1992; Hoffland 1992; Jones 1998; Neumann & Römheld 1999; Playsted *et al.* 2006). The mobilization of P can occur by two main mechanisms. The first is by direct ligand exchange, where the organic anion replaces P on ligand exchange surfaces (oxide surfaces). The second mechanism is ligand-promoted mineral dissolution, by ligand adsorption followed by subsequent detachment of the metal-ligand complex (Johnson & Loeppert 2006; Lambers *et al.* 2008a; Oburger, Jones, & Wenzel 2011). Citrate (trivalent anion), malate (bivalent anion), and oxalate (bivalent anion) have more carboxyl groups, therefore have more affinity with trivalent metal cations such as Fe^{3+} and Al^{3+} . Other organic compounds such as lactate (monovalent) have little complexing ability (Oburger *et al.* 2009).

Carboxylates are involved in metabolic processes, but also in stress-relief. The release of carboxylates is localized at the root tips and is mainly diurnal (Neumann & Römheld 2001). It is mediated by anion channels in the plasma membrane with a concomitant release of protons or K^+ , mediated by plasmalemma ATPase, or K^+ channels (Zeng *et al.*). They cross the plasma membrane through the electrochemical gradient without expending energy (Ryan *et al.* 2001). Many carboxylates are intermediates of the tricarboxylic acid (TCA) cycle, the main respiratory pathway involved in the oxidation of pyruvate (Ryan *et al.* 2001). They are accumulated in the root cells by transport from shoot to root (Hoffland 1992), and by local biosynthesis (Theodorou & Plaxton 1993; Massonneau *et al.* 2001). The rate of exudation is regulated by the transport-rate, rather than by the synthesis rate (Watt & Evans 1999). They also play an essential role in cell metabolism, being involved with the assimilation of carbon and nitrogen, the regulation of cytosolic pH and osmotic potential, the charge balance during excess cation uptake, and the

supply of energy to symbiotic bacteria (Ryan *et al.* 2001). Their release participate in Al detoxification (Ryan, Delhaize, & Randall 1995) but also in relieving phytotoxicity caused by lactic acid accumulation in the fermentation pathway under anoxic stress (Neumann & Römheld 2001).

In Brazil, old and nutrient-impoverished soils are found in the mountain ranges of Cadeia do Espinhaço, especially in the rupestrian grasslands within the Cerrado domain (Neves, Abreu, & Fraga 2005). Resin P concentrations at the collection site were under detection limit (De Campos 2012), and total N concentrations were about 53 mg kg⁻¹ (Cândido 2012). As there are few published studies on nutritional strategies of rupestrian grasslands, and these are mostly focused on carnivorous plants (Pereira *et al.* 2012; Nishi *et al.* 2012), our aim was to study mineral nutrition of a non-carnivorous species that occurs in these environments. Therefore, we studied root morphology, anatomy and exudation of *Discocatus placentiformis* in solution culture under different phosphorus supply. Based on observations on severely P-impoverished habitats in Australia (Lambers & Shane 2007) and South Africa (Lamont 1982), we hypothesized that *D. placentiformis* roots would be nonmycorrhizal, and have alternative root adaptations. These alternative strategies are hypothesized to be similar in function to cluster roots or dauciform roots, and the roots to exhibit increased carboxylate exudation under P deficiency.

Material and methods

Study site and species selection

Rupestrian grasslands present extremely nutrient-impoverished soils (Benites *et al.* 2003) with high porosity and permeability (Neves *et al.* 2005). The soils in these environments are rather heterogeneous, ranging from thin soil layers between rock outcrops, to deep sandy layers depositions in valleys and depressions. These fields are dominated mainly by herbaceous and subshrubs species within Poaceae, Cyperaceae, Xyridaceae, Velloziaceae, Cactaceae, Eriocaulaceae, Euphorbiaceae, Lamiaceae, Melastomataceae, Myrtaceae, and Orchidaceae (Alves & Kolbek 1994; Rapini *et al.* 2008).

The Espinhaço mountain range is marked by discontinuity, and is usually divided in southern and northern Espinhaço mountain range (Saadi 1995). The southern Espinhaço

range, where the study site is located, is inserted in the sub warm zone, and is strongly influenced by the orographic factor, because the range has a mean altitude of 1250 m above sea-level (IBGE 1997). The climate within the range is characterized as Cwb in the classification of Köppen. Mean annual precipitation varies from 1250 to 1550 mm, varying from 225 mm per month during the summer to 8 mm per month during the winter. Mean annual temperature ranges between 18° and 19°C (minimum is 4°C in July and maximum is 35°C from October to March). Mean annual insolation is elevated (2203 h year⁻¹), generating an important mean annual potential evapotranspiration (776 mm year⁻¹) (Neves *et al.* 2005). Together with the sandy soil, the climatic attributes create an environment with high insolation, seasonally dry nutrient-poor soils.

The southern Espinhaço range was originated during the pre-Cambrian. The study site is located within the Espinhaço super-group, which is characterized mainly by quartzites forming a rigid cover that is densely fractured. Most of the rocks are constituted by metasandstones, which later formed the white sandy soils (Saadi 1995; Neves *et al.* 2005).

Discocactus placentiformis (Lehm.) K. Schum. (Cactaceae) is a globose cactus species that occurs only in sandstone rocks, quartz sand and gravel in cerrado rupestrian fields (Taylor 2011). It is in a vulnerable state of conservation by IUCN since most subpopulations are small and isolated from each other in Bahia and Minas Gerais states of Brazil (Martin & James 2009). It is especially affected by unsustainable exploitation for the production of cactus candy, when located close to towns and villages (Taylor 2011). In the preliminary studies, we observed in the field that the roots of *D. placentiformis* formed cluster-like roots that were strongly adhered to sand and could be compared to other sand-binding roots described for Restionaceae and Cyperaceae (Shane *et al.* 2009, 2011; Smith, Hopper, & Shane 2011). This is why we chose this species.

Plants were collected in a sandy grassland in Parque Estadual da Serra do Cabral, in the municipality of Joaquim Felício, Minas Gerais State, southeastern Brazil (S17°42' W44°11', 1030 m altitude) in January of 2010 and 2011. We collected 23 young plants and transplanted into 10 cm diameter, 30 cm high PVC pots (2.4 l), keeping the whole root system in their native soil. Plants were transported to the greenhouse of the Laboratory of

Plant Functional Ecology, Campinas State University, southeastern Brazil, where they were acclimated for at least six months until the beginning of the experiment.

Root morphology, anatomy and mycorrhizal colonization

We collected root samples in the field during the growing season (wet summer). We collected the whole root system and fine, ramified roots (< 5mm) from four individuals. The roots were preserved in ethanol 70% (v/v) and their percentage of arbuscular mycorrhizal colonization was determined at the Laboratory of Soil Microbiology – "Luiz de Queiroz" College of Agriculture (ESALQ), Piracicaba-SP, following the protocols of Giovanetti and Mosse (1980) and Vierheilig et al. (1998). These protocols start with warming 1 g of washed fine roots in 10% (w/v) KOH solutions. The roots were then autoclaved and removed from KOH, washed, and transferred to 1% (v/v) HCl. The staining procedure comprises washing the roots and transferring them to Trypan blue in 0.05% (v/v) lactophenol. Colonization rate was obtained by observing the roots under a stereomicroscope.

We selected both roots with primary and secondary growth structures for anatomical procedures. Root fragments were cut and fixed in FAA 50 (formalin–acetic acid–alcohol, 1:1:18 v/v/v) during 24 h following Johansen (1940). The roots were dehydrated by submersing them in an increasing sequence of ethanol concentrations (70%, 96% and 100% v/v), for two hours each time. To pre-infiltrate the samples, ethanol 100% was used with infiltration resin (HistoResin – Leica, Cambridge, UK), 1:1, for two hours. The same solution was used with the activating powder to infiltrate the samples, where the material remained for 24 h. Finally, for polymerization, a polymerization solution was used (HistoResin – Leica, Cambridge, UK) in polyethylene casts. The root samples were organized in order to obtain both longitudinal and transversal sections. The casts were dried at 40°C. Blocks were cut with a microtome into 10-20 µm sections. Sections were stained in toluidine blue, and observed under an Olympus BX51 microscope (New York, USA) equipped with an Olympus® DP71 camera (Tokyo, Japan). The root hairs were measured using the camera software. For morphological descriptions, plants were removed from the soil and their roots gently washed to remove adhering sand. Individual roots were

photographed with a digital camera mounted on a stereoscopic microscope Leica® MSV266 using Leica Application Suite (LAS) version 3.8.0 (Heerbrugg, Switzerland) for focus stacking.

Root exudation

Acid exudation

Prior to the hydroponic experiment, we removed four plants from the pots, gently washed their roots with distilled water and placed young root fragments onto agar plates with 1% bromocresol purple, a color pH change indicator (Neumann *et al.* 2000). This pH indicator switches from purple to yellow at pHs below 5. We chose young but fully developed root sections, as they are usually the active period of exudate release (Shane *et al.* 2004). The agar was left to dry in ambient temperature and the plates were photographed the following day.

P effects on carboxylate exudation

We carried out an experiment in hydroponics, growing plants in four P availability treatments (0, 10, 50 and 100 $\mu\text{mol l}^{-1}$ of P), n=5, 8, 5, 5, respectively, for eight months. Each plant was cultivated in an individual plastic bag filled with 2 l of nutrient solution as follows (in $\mu\text{mol l}^{-1}$): 400 NO_3^{2-} , 200 Ca^{2+} , 200 K^+ , 154 SO_4^{2-} , 54 Mg^{2+} , 20 Cl^- , 2,0 Fe-EDTA, 0.24 Mn^{2+} , 0.10 Zn^{2+} , 0.02 Cu^{2+} , 2.4 H_3BO_3 , and 0.3 Mo^{4+} prepared in deionized water (pH= 5.8). Plastic bags were installed in PVC pots inside a polystyrene box to provide thermal isolation. The polystyrene box was covered in a black polypropylene lid perforated to allow root installation. Shoots were supported in the center of a foam disc that made a light-tight seal. Each plant was provided with individual aeration tube with an aquarium stone at the end of the tube to facilitate oxygen dissolution in the nutrient solution. From August 2011 to January 2012, we changed nutrient solution three times a week. From January to March, we changed nutrient solution once a week, and replenished with deionized water twice a week to replace evaporated water as we realized the nutrient supply could be in excess by changing it three times a week. Although hydroponic systems provide different conditions from soil ones, and may cause different root formation patterns, these systems provide useful approaches to visualize root growth and control nutrient concentrations (Jones 1998).

Exudate collection and analyses

In order to identify the root organic acid exudates, we collected three 1-2 cm of fresh root tips per plant and gently shook it in 0.3 or 0.6 ml of 0.1% Mili-Q water-solved formic acid. After five minutes, we removed the root tips and froze the samples until analysis. We weighed each root tip with an analytic scale in order to correct acid concentration. We prepared working standards of citric, malic, oxalic, succinic, lactic, maleic, tartaric, DL-isocitric, malonic, and fumaric acid, which are known to be involved in P mobilization (Roelofs *et al.* 2001).

We analyzed our samples using an ultra-high performance liquid chromatography system with a triple quadrupole mass spectrometer (UPLC-MS) and an electrospray ionization source, Acquity UPLC-TQD (Waters, Milford, MA, USA). The compounds were separated with a Waters Acquity BEH C₁₈ column (2.1 x 50 mm) with 1.7 µm particle size at a temperature of 30 °C. The mobile phase consisted of Mili-Q water containing 0.1 % (v/v) of formic acid (phase A), and methanol (phase B) at a flow-rate of 0.2 ml min⁻¹ and the injected volume was 10 µl. Elution was carried out using a gradient starting with 99% A maintained until 2.5 min, then ramping to 50% A by 3 min, maintaining this concentration till 4 min, then returning to the initial conditions and re-equilibrating the system until 6 min. All data were acquired and processed with MassLynxV4 software (Waters) MS detection in the negative ion mode and Selected Ion Mode was used for the specific detection of analytes. Source temperature was 150 °C, and solvation temperature was 350°C. Curtain gas was 10 psi, and source voltage was -2.8 kV capillary and -25 V cone. Peak areas below 100 were excluded from analyses, as they can be considered errors. When we observed a very large variation in concentrations (malic, citric and oxalic acids), we used two calibration curves, as the relationship between peak area and concentration is not linear in a broad range of concentrations.

Statistical analyses were performed using R (R Core Team 2012) and Statistica 8.0 (StatSoft 2007). Comparisons of acids concentration within plants, regardless of the treatment, were made using Friedman ANOVA (non-parametric two-way ANOVA). Comparisons of fractions of total exudation from each acid were performed with Kruskal-Wallis test (non-parametric one way ANOVA). Comparisons of organic acid exudation

between treatments were done by comparing a mixed linear model using P concentration as a predictor variable and a mixed linear model without a predictor variable with the lme4 package (Bates *et al.* 2012). Linear mixed model approach allows us to include hierarchy in the factors. In the first model, P treatment was used as fixed effect, and in the second model, no fixed effect was used. Random effects in both models include plants and repetitions within plants. We verified likelihood using a χ^2 distribution to evaluate the models. Values with Cook's distance higher than one were considered outliers and were removed from the analyses using the influenceME package to calculate Cook's distance (Nieuwenhuis *et al.* 2012). Fumaric and malonic acids were observed in fewer samples, and therefore we used linear regression, avoiding outlier exclusion to reduce the loss of degrees of freedom.

Results

Morphology, anatomy and mycorrhizal status

The root system of *D. placentiformis* consists of a main branched tap root with several ramified lateral roots (Figure 1a). In the field, a small cactus (around 8 cm diameter) had roots up to 40-50 cm long. In primary growth, these lateral roots present white color and very dense root hairs along the whole axis. In secondary growth, the root hairs are lost and the roots turn to a yellow or brown color (Figure 1b). Anatomical sections showed that the root hairs are extensions of epidermal cells (unicellular trichomes), and measurements showed that root hairs from roots with 200 μ m diameter are more than 1 mm long (Figure 1c-e). No mycorrhizal colonization was observed on any individual of this species.

Root exudates

We observed *D. placentiformis* root acid exudation in the bromocresol purple experiment, as shown by the yellow aura around the roots (Figure 2). The UPLC-MS analysis showed a high variability in carboxylate concentrations between samples. With this analysis, we identified oxalic, malic, citric, lactic, succinic, fumaric and malonic acids in decreasing order of concentration (Friedman ANOVA χ^2 (N = 24, df = 6) = 112.1429 p < 0.0001). Maleic and isocitric acids were observed, but could not be quantified because to concentration were too low.

Overall exudation (the sum of all acids) was reduced with increasing P supply ($\chi^2=4.2512$; $p=0.0392$, Figure 3) but P supply did not influence individual acid exudation, except for oxalic acid. Both the quantity and composition of carboxylates released by *D. placentiformis* roots also varied according to P supply (Figure 4). Oxalic acid exudation decreased with increasing P concentration ($y=30.837-0.185x$, $\chi^2=4.461$; $p=0.035$). However, P treatment did not influence individual exudation of citric ($\chi^2=3.095$; $p=0.079$), succinic ($\chi^2=0.292$; $p=0.589$), lactic ($\chi^2=0.015$; $p=0.901$), malic ($\chi^2=3.098$; $p=0.078$), fumaric ($F=1.232$, $p=0.2846$), or malonic acid ($F=0.1783$, $p=0.6798$).

Discussion

Similar to what is known for dominant plant species on severely P-impooverished soils in Australia and South Africa (Lamont 1982), *D. placentiformis* was found to be nonmycorrhizal, and showed a new specialized root morphology. This specialized root morphology was also associated with the release of carboxylates, especially when plants were subjected to P deficiency.

The abundance of fine root hair and the release of organic acids suggest that *Discocactus placentiformis* roots are functionally similar to cluster roots, and might enhance phosphorus uptake in the nutrient-poor soils where they occur. *D. placentiformis* roots are structurally different from proteoid roots, since they do not form cluster roots. However, the release of carboxylates by the very long root hairs from the ramified root system of *D. placentiformis* is very similar to cluster root functioning. The exuded carboxylates are the same released by cluster roots in Proteaceae (see Roelofs *et al.* 2001) and the ability to acidify the rhizosphere as shown by the pH indicator bromocresol purple was also observed in cluster-rooted species (Neumann & Römheld 1999; Neumann *et al.* 2000). In view of nutrient limitation in sandy soils of rupestrian grasslands, these specialized root structures are comparable to other strategies found in OCBILs, such as cluster and dauciform roots (Lambers *et al.* 2008b).

The morphology of the root system of *D. placentiformis* is similar to that of early stages of capillaroid roots of Restionaceae ((Shane *et al.* 2005; Lambers *et al.* 2006a; Lambers & Shane 2007). The main difference between both root systems is that *D.*

placentiformis loses the root hairs with secondary growth, while capillaroid root hairs get lignified with secondary growth and are persistent (Shane *et al.* 2011).

Root hairs are a common root structure, and abundant long root hairs promote water absorption by capillarity (Gullan 1975 *apud* Lamont 1982). Root hairs also function as anchors to enhance root penetration in the soil (Bengough *et al.* 2011), and to avoid soil erosion, especially in sandy soils (Moreno-Espíndola *et al.* 2007). Although we did not measure root hair density or length in every treatment, we observed that root hair formation is not suppressed by phosphorus supply. In P-poor environments, root hair abundance and length provides competitive advantage (Bates & Lynch 2001). As the increased surface area releases a high amount of localized exudates that promote nutrient desorption, it also facilitates nutrient absorption (Lambers *et al.* 2006).

Overall organic acid exudation was increased under P deficiency, as observed for white lupin (Gardner, Barber, & Parbery 1983; Keerthisinghe *et al.* 1998; Shane *et al.* 2003b) and other wild plants within Cyperaceae, Proteaceae and Brassicaceae families (Dinkelaker *et al.* 1995; Playsted *et al.* 2006). Chen *et al.* (2013) reviews papers on cultivated and wild plants that increase organic acid exudation under P deficiency in order to increase soil P dissolution. However, Wouterlood *et al.* (2004) found that carboxylate exudation in chickpea was related to the developmental stage rather than to P status, and Pearse *et al.* (2006) found no variation in total exudation, but in citrate: malate ratio, showing that this response varies among species.

Shane *et al.* (2004) already showed that cluster roots start releasing exudates when they reach maturity, increase their exudation rate until they reach a peak in exudation, and exudation decreases as roots suffer turn-over. Although we selected as homogeneous root tips as possible, there might be differences in the developmental stage between root tips, causing the large differences between samples. Additionally, Roelofs *et al.* (2001) mentioned that the exudation rates reported in their work were higher than those previously presented. Here, we present exudation rates one order of magnitude higher than those reported by Roelofs *et al.* (2001). This result might be due to differences in the analytic process, because UPLC is more sensitive than HPLC (Nordström *et al.* 2006; Spácil *et al.* 2008) or to differences in exudate collection methodologies. Our method might have

caused spill of carboxylates from inside the cells when excising the root tips, increasing the concentration in the collection solution, while Roelofs *et al.* (2001) prevented it by keeping the roots intact and collecting only the released exudates.

Oxalate was the carboxylate released in higher quantities in *D. placentiformis*. Rice and soybean also release mainly oxalate under P deficiency (Dong *et al.* 2004; Hoffland *et al.* 2006). However, citrate is the main carboxylate released by cluster roots of Cyperaceae and Proteaceae under P deficiency (Shane *et al.* 2003a; Playsted *et al.* 2006). Oxalate-release by the root tips can be related to stress caused by Al (Zheng *et al.* 1998) and relieve of Pb toxicity (Yang *et al.* 2000), but also to P deficiency (Hoffland *et al.* 2006). It binds to toxic metals, relieving their effects (Ma *et al.* 1998) and increase P solubilization, even if exuded in low continuous amounts (Fox & Comerford 1992). Cerrado soils present very high Al content, and this is also true for the soils from the present study (Cândido 2012). Oxalate can be an important strategy for Al detoxification of *D. placentiformis*.

Oxalate releases P from soil particles less efficiently than citrate, because citrate is a stronger acid (tricarboxylic) than oxalate (dicarboxylic) (Bolan *et al.* 1994), nevertheless the release of oxalate can be more physiologically efficient because oxalate costs less carbon and energy when exuded, and is not a metabolic intermediate like citrate and malate (Dong *et al.* 2004). Additionally, oxalate was released from *D. placentiformis* in much higher concentration than citrate from cluster roots, which could compensate for the efficiency of oxalate when compared to citrate.

The composition of carboxylates depends on the species, age of the plant and the tissue type, but also on the metabolic pathway (C₃, C₄ and CAM) (López-Bucio *et al.* 2000). Nobel & Hartsock (1986) reported that members of the Cactoidae subfamily (Cactaceae family) utilize CAM fixation pathway, and fix CO₂ during the night. As *D. placentiformis* belongs to the Cactoidae subfamily, the pattern of relative contributions of the measured carboxylates released could be related to the CAM metabolism. CAM plants are especially efficient conserving water because they fix carbon at night, when the weather is cooler, allowing them to grow in dryer areas such as semideserts or to possess an epiphytic habit that does not access water easily even in tropical forests (West-Eberhard *et*

al. 2011). Together, nutritional and hydraulic specializations allow successful plant establishment in environments such as rupestrian grasslands.

Conclusion

The retention of sand grains by the root hairs indicate the presence of root exudates, usually involved in mineral nutrition and water absorption (Shane & Lambers 2005; Moreno-Espíndola *et al.* 2007). In our study, we observed the exudation of carboxylates by the root tips of *D. placentiformis*. Root exudation increased with P deficiency, a similar response to cluster roots found in other parts of the world. These results indicate that there might be a global functional convergence in the nutrition strategies of nutrient-poor habitats

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Figure 1. Sand-binding root of *Discocactus placentiformis*. **a.** General view of the root system. Note that the ramification pattern is similar to capillaroid roots. Scale bar = 4 cm. **b.** Washed root system of *Discocactus placentiformis* (p) in primary growth and covered with thin root hairs, (s) in secondary growth without root hairs. Scale bar = 0.5 cm. **c.** Root

hairs from the root system of *Discocactus placentiformis* in primary growth. Scale bar = 500 μm . **d.** Cross-section from the *Discocactus placentiformis* root system in primary growth. Root hairs (h) are extensions of epidermal cells, being characterized as unicellular trichomes. Scale bar = 100 μm . **e.** Cross-section from the *Discocactus placentiformis* root system in secondary growth. Note the absence of root hairs. Scale bar = 50 μm

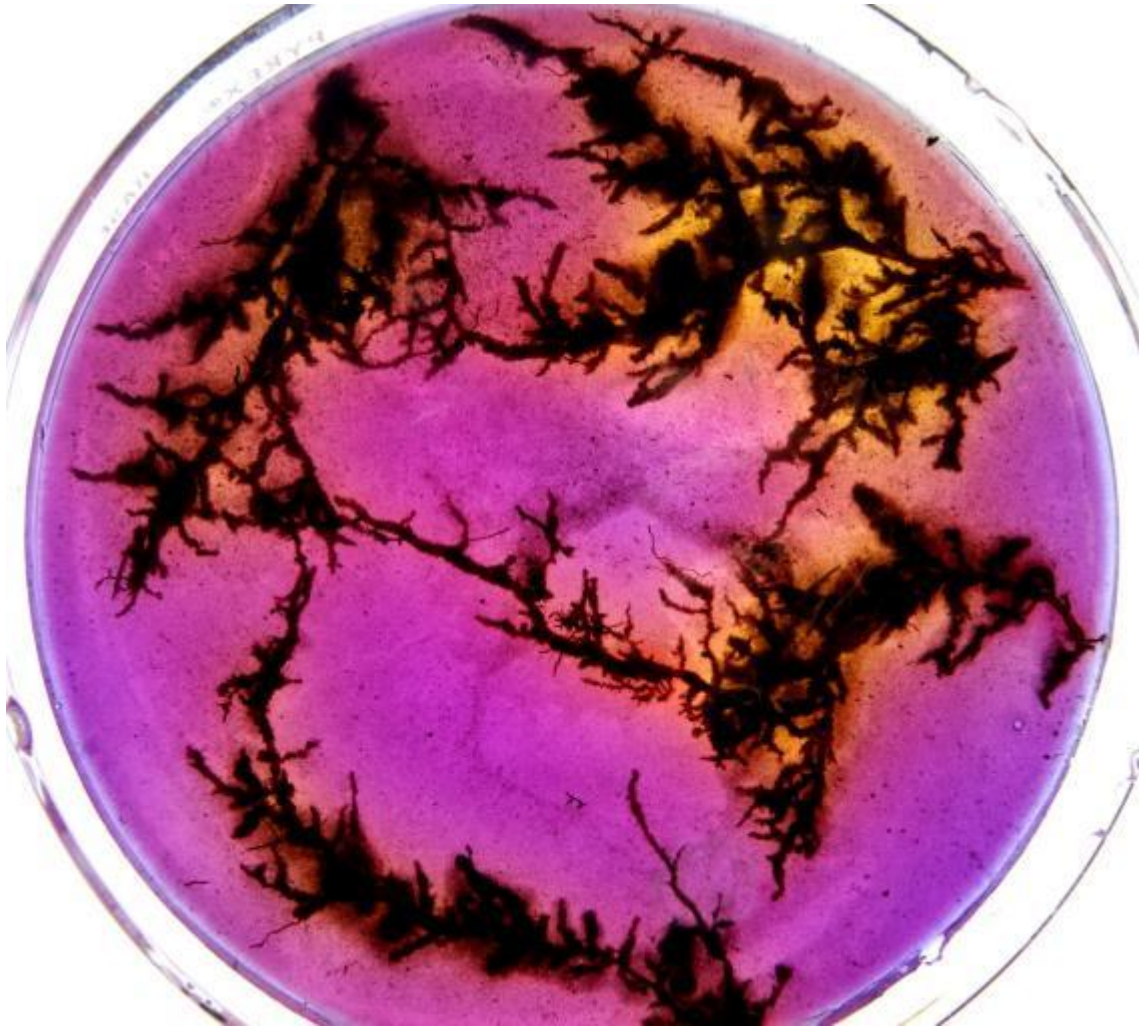


Figure 2. *Discocactus placentiformis* roots in agar plates with 1% bromocresol purple that switches from purple to yellow at pH<5. This experiment shows that this species releases root acid exudatesinvolved in P mobilization.

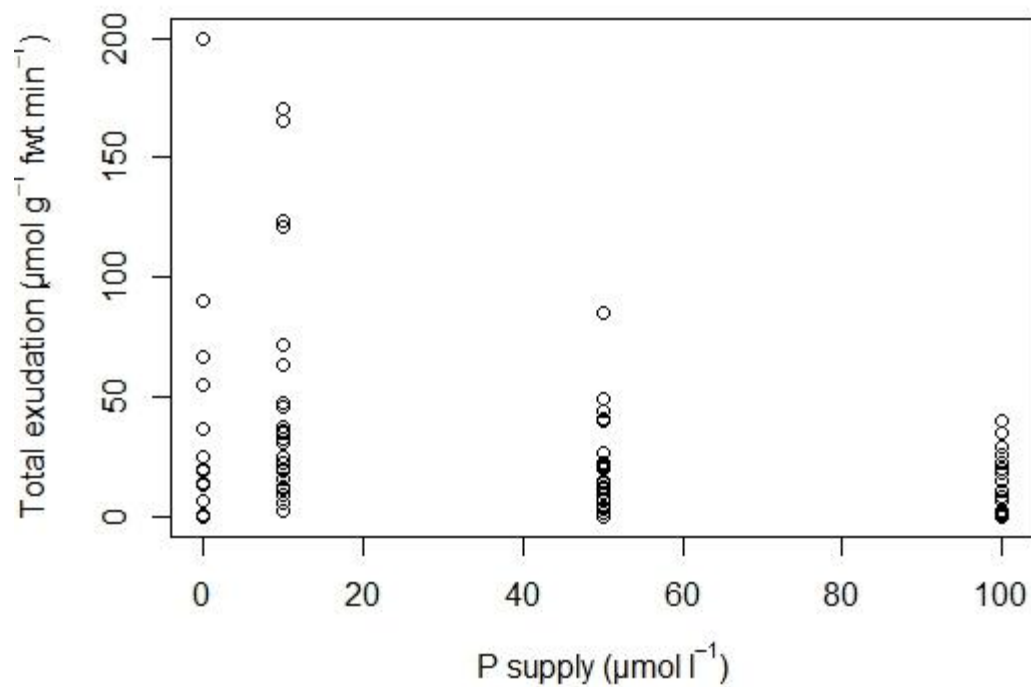


Figure 3. Total exudation from *Discocactus placentiformis* root tips submitted to different concentrations of phosphorus in water culture (Each circle represents a root tip, N= 5 plants per treatment, 3 roots tips per plant, $\chi^2 = 4.2512$; $p = 0.0392$) Outliers (Cook's Distance > 1) were excluded.

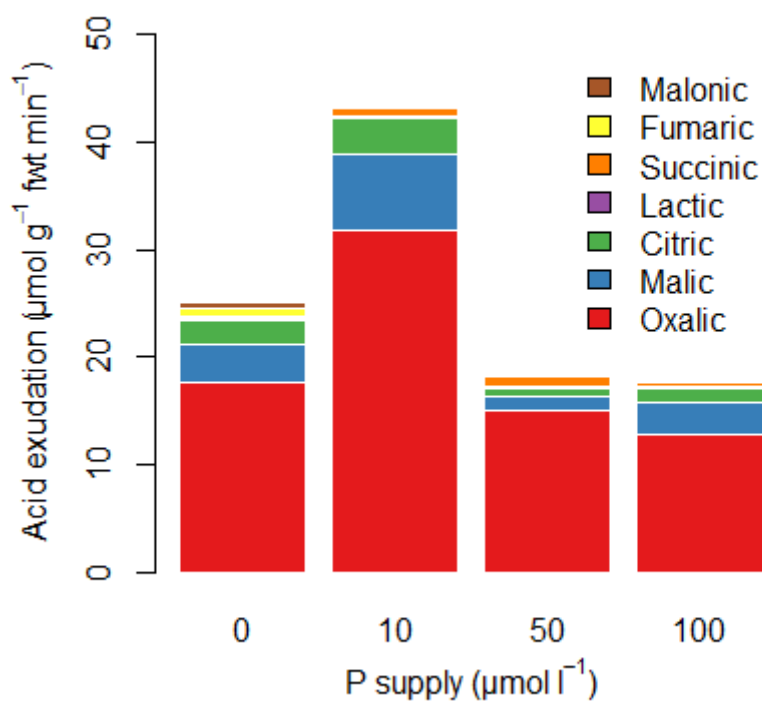


Figure 4. Median exudation of oxalic, malic, citric, lactic, succinic and fumaric acids from root tips of *Discocactus placentiformis* submitted to different concentrations of phosphorus in water culture (N= 5 plants per treatment, 3 roots tips per plant).

Capítulo II: Abundância natural de ^{15}N em três fisionomias rupestres de cerrado na Cadeia do Espinhaço, Minas Gerais, Brasil

Chapter II: Natural abundance of ^{15}N in three rupestrian cerrado physiognomies at the Espinhaço range, Minas Gerais, Brazil

Abstract

Plants have evolved a great diversity of nutrient acquisition-strategies in nutrient-poor environments. Foliar and soil $\delta^{15}\text{N}$ provide information about nitrogen dynamics and availability and the range of foliar nitrogen (N) values indicate the diversity in N-use strategies. To investigate the patterns of nitrogen cycling and diversity in N use in nutrient-impovertised communities, we compared soil and foliar N, $\delta^{15}\text{N}$, $\Delta\delta^{15}\text{N}$ and mycorrhizal colonization of 60 species from six communities of three different physiognomies (rupestrian grasslands, wet grasslands and scrublands) at the Espinhaço mountain range in Minas Gerais State, southeastern Brazil. We observed very low total soil N in all the communities, especially in rupestrian grasslands. Altogether, foliar $\delta^{15}\text{N}$ values varied between -6.41 and 8.27‰. Rupestrian grasslands had the lowest N availability and foliar $\delta^{15}\text{N}$ values equal to wet grasslands. Consequently, contrary to expectations, foliar $\delta^{15}\text{N}$ was negatively related to total soil N. However, foliar $\delta^{15}\text{N}$ was positively related to $\Delta\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{\text{foliar}} - \delta^{15}\text{N}_{\text{soil}}$), showing that foliar ^{15}N -enrichment is related to soil ^{15}N -enrichment. Despite the differences in soil N availability, mean foliar total N was similar between physiognomies. When we compared seasons, we observed no differences in total soil N, foliar $\delta^{15}\text{N}$ or soil $\delta^{15}\text{N}$. Additionally, total soil N did not vary between soil depths, but $\delta^{15}\text{N}$ values increased with soil depth. When we looked at specific nutrient-acquisition strategies, we observed that legume species had higher total foliar N, and $\delta^{15}\text{N}$ values between -2.99 and 1.12‰, a higher range than expected. Contrary to expectations, mycorrhizal colonization rate was weakly, but positively related to foliar $\delta^{15}\text{N}$. Our results show that higher foliar $\delta^{15}\text{N}$ values do not always indicate higher N availability. However, as expected, we observed high variability in foliar $\delta^{15}\text{N}$ values, indicating diverse nitrogen-

acquisition strategies. This diversity in nutrition mechanisms could allow the maintenance of high observed species diversity in these sites.

Resumo

As plantas evoluíram uma grande diversidade de estratégias de aquisição de nutrientes em ambientes pobres em nutrientes. Os valores de $\delta^{15}\text{N}$ foliar e do solo fornecem informações sobre a dinâmica e a disponibilidade de nitrogênio, e o intervalo de valores de $\delta^{15}\text{N}$ indica diversidade nas estratégias de uso desse nutriente. De modo a investigar os padrões de ciclagem e diversidade no uso de nitrogênio em comunidades pobres em nutrientes, nós comparamos o conteúdo total de N e os valores de $\delta^{15}\text{N}$ foliares e do solo, além da colonização por micorrizas em 60 espécies de seis comunidades em três fisionomias vegetais diferentes (campos rupestres, campo sujo úmido e cerrado ralo) na Cadeia do Espinhaço, Minas Gerais, Brasil. Em todas as fisionomias nós observamos uma disponibilidade de nitrogênio (N total) muito baixa, principalmente nos campos rupestres, e os valores de $\delta^{15}\text{N}$ incluindo todas as comunidades variaram de -6.41 a 8.27‰. Os campos rupestres apresentaram a menor disponibilidade de nitrogênio, e altos valores de $\delta^{15}\text{N}$ foliar (iguais aos dos campos sujos úmidos). Consequentemente, ao contrário das expectativas, o $\delta^{15}\text{N}$ foliar foi negativamente relacionado com o N total do solo. No entanto, os valores de $\delta^{15}\text{N}$ foliar foram positivamente relacionados com o $\Delta\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{\text{foliar}} - \delta^{15}\text{N}_{\text{solo}}$), mostrando que o enriquecimento em ^{15}N foliar está relacionado com o enriquecimento do substrato. Apesar das diferenças na disponibilidade de nitrogênio, o conteúdo médio de N total foliar foi similar entre as fisionomias. Quando nós comparamos as estações, não observamos diferenças nos valores de N total foliar, $\delta^{15}\text{N}$ foliar e N total do solo. Além disso, o nitrogênio total do solo não variou entre diferentes profundidades, mas o $\delta^{15}\text{N}$ do solo aumentou com a profundidade. Ao olhar para estratégias específicas de aquisição de nutrientes, observamos que as leguminosas apresentaram maior N foliar e valores de $\delta^{15}\text{N}$ entre -2.99 e 1.12‰, um intervalo maior do que o esperado. Também ao contrário das expectativas, a taxa de colonização por micorrizas foi positivamente relacionada com $\delta^{15}\text{N}$ foliar. Os nossos resultados mostram que altos valores de $\delta^{15}\text{N}$ foliar nem sempre indicam alta disponibilidade de N. No entanto, como esperado, a grande variação nos valores de $\delta^{15}\text{N}$ foliar aponta a diversidade de mecanismos de aquisição de nitrogênio. Essa

diversidade de mecanismos de absorção de nitrogênio pode ser um dos fatores responsáveis pela grande diversidade de espécies nesses ambientes.

Introduction

Nutrient availability is an important abiotic filter influencing plant community composition (Lambers, *et al.* 2008a). As ecosystem ages, nutrients, specially P, tend to be lost with pedogenic processes (Walker & Syers 1976). However, in the oldest soils, such as in OCBILs (old, climatically buffered infertile landscapes *sensu* Hopper 2009), even most of the nitrogen has been lost by weathering (Aerts & Chapin 2000). Under severe nutrient limitation, plants tend to diversify nutrient-acquisition strategies (Schulze, Chapin, & Gebauer 1994; Lambers *et al.* 2010). Among these strategies, plants can associate with other organisms (Aerts & Chapin 2000; van der Heijden *et al.* 2008) or invest in root specializations (Lambers *et al.* 2008b) in order to absorb nutrients they cannot access otherwise. This resource partitioning allows the coexistence of more rare plant species in a relatively small area (Tilman *et al.* 1987 *apud* Bustamante *et al.* 2004, McKane *et al.* 2002, Pekin *et al.* 2012)

Foliar and soil $\delta^{15}\text{N}$ provide information about nitrogen dynamics and availability and the range of foliar nitrogen (N) values indicate the diversity in N-use strategies, the rate of photosynthesis and respiration, and leaf lifespan (Robinson 2001; Wright *et al.* 2004). The final $\delta^{15}\text{N}$ in plant tissues depends on the $\delta^{15}\text{N}$ of the source over time and the physiological mechanisms involved in acquisition, use and losses of nitrogen (Evans 2001). If nitrogen acquisition is mediated by nodulating or endophytic bacteria that fix atmospheric nitrogen, for example, little fractionation occurs and as atmospheric nitrogen isotopic signature is 0‰, the range of isotopic signatures is expected to be $0‰ < \delta^{15}\text{N} < 2‰$ (Craine *et al.* 2009). During direct nitrate and ammonium uptake, for example, plants increase discrimination against ^{15}N with N concentration, but decrease discrimination with plant age (Evans 2001). When N supply is higher than demand, the discrimination is expected to occur, whereas in N-limited soils, almost all the available inorganic N is taken up, resulting in little fractionation (Evans 2001).

According to global-level studies, foliar $\delta^{15}\text{N}$ increases with increasing N availability (Craine *et al.* 2009). Foliar $\delta^{15}\text{N}$ also increases with increasing foliar N concentrations, because plants from N-rich sites tend to have higher total foliar N (Vitousek, Turner, & Kitayama 1995; Aerts & Chapin 2000; Hobbie & Gough 2002). Additionally, a few studies have shown that foliar $\delta^{15}\text{N}$ increases with decreasing foliar phosphorus (P) concentrations, suggesting that $\delta^{15}\text{N}$ enrichment indicates low water availability (Austin & Vitousek 1998; Handley *et al.* 1999), but also low P availability (Craine *et al.* 2009; Pekin *et al.* 2012). Furthermore, a between-site correction ($\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{leaf}} - \delta^{15}\text{N}_{\text{soil}}$) can be introduced to evaluate the foliar $\delta^{15}\text{N}$ signatures regardless of soil $\delta^{15}\text{N}$ (Amundson *et al.* 2003). Lower (more negative) $\Delta\delta^{15}\text{N}$ may indicate mineral N acquisition rather than organic N (Robinson 2001; Kahmen *et al.* 2008).

The Cerrado vegetation complex shows a wide range of vegetation physiognomies (Eiten 1972; Ribeiro & Walter 2008). It originally occupied 23% of the Brazilian territory (Furley & Ratter 1988). The Cerrado savannas have a fertility gradient from grasslands to woodlands (Goodland & Pollard 1973). Agents such as fire frequency, seasonal water availability and soil nutrient content influence native vegetation cover (Eiten 1972; Hoffmann *et al.* 2012; Murphy & Bowman 2012). Cerrado soils tend to be acidic, very weathered, with low cation exchange capacity, and high aluminum saturation (Goedert 1983; Furley & Ratter 1988; Haridasan 2008).

Over the mountain ranges within the Cerrado domain, there are rupestrian cerrados that are extremely species-rich ecosystems with high endemism rates (Alves & Kolbek 1994). High soil porosity and permeability in sandy soils of the rupestrian grasslands associated with very low nutrient content restricts species occurrence, and only species with efficient nutritional and water-saving attributes are able to survive in these environments (Menezes & Giuliatti 2000). Therefore, rupestrian physiognomies within the Cerrado domain can be characterized as OCBILs (*sensu* Hopper 2009). Even presenting high N:P ratios (Cândido 2012), the nutrient availability is very low in rupestrian grasslands and these environments are not only limited by phosphorus, but also by nitrogen (Güsewell 2004). For this reason, it is important to investigate both P and N-nutrition strategies.

In this work, we evaluated soil and foliar $\delta^{15}\text{N}$, but also $\Delta\delta^{15}\text{N}$ from three cerrado physiognomies that differed in soil type and species composition. Cerrado rupestrian grasslands (*campos rupestres sensu* Ribeiro & Walter 2008) are usually nutrient-poor sandy soils patches between rocky outcrops (similar to arenosols), wet grasslands (*campos sujos úmidos sensu* Ribeiro & Walter 2008) are marked by wet soils near valley-side marshes (similar to histosols) and scrublands (*cerrado ralo sensu* Ribeiro & Walter 2008) are characterized by higher clay content than the other vegetation types, with high Fe and Al content (similar to oxisols). In this context, we hypothesized that physiognomies with higher total soil N would have higher foliar $\delta^{15}\text{N}$, and sites with lower soil N would have a higher variation in foliar $\delta^{15}\text{N}$, showing higher diversity in N-nutrition strategies.

Material and methods

Study sites

In order to study a fertility gradient, especially a phosphorus (P) gradient, we chose three protected areas in the Southern Espinhaço range in Minas Gerais State, Southeastern Brazil. The Espinhaço range is part of the Brazilian Shield and was originated during the Precambrian (Neves, Abreu, & Fraga 2005). Our sites are situated within the Espinhaço super group which is mainly covered by sandstones (Saadi 1995). Most of the super group rocks are constituted by metasandstones, which are very resistant to weathering and therefore usually form shallow soils (Neves *et al.* 2005).

The Southern Espinhaço range has a mesothermic, Cwb Köppen's climate type, strongly influenced by the orographic factor as the mean altitude of the range is 1250 m above sea level (IBGE 1997). Mean annual temperature is between 18 and 19°C with warm and humid summers easily reaching 35°C from October to April, and fresh dry winters that can reach 4°C from June to August. Mean annual precipitation ranges from 1250 to 1550 mm, varying from 225 mm per month during the summer to 8 mm per month during the winter. High insolation is observed, approximately 2203 h per year, which allows high mean annual potential evapotranspiration (776 mm) (Neves *et al.* 2005).

The three protected areas within the Espinhaço range we studied are *Parque Estadual Do Rio Preto* (PERP), *Parque Estadual da Serra do Cabral* (PESC) and *Parque*

Nacional da Serra do Cipó (PNSC). Three vegetation physiognomies were chosen to compose the gradient. The first one is the most P-impooverished one, rupestrian grasslands (*campo rupestre*) over sandy entisols, the second physiognomy was the wet grassland (*campo úmido*), over histosols and finally a scrubland (cerrado ralo) over inceptisols or oxisols. We had two collection sites for each physiognomy. The rupestrian grasslands were located at the PERP (18°05'20"S, 43°20'40"W, 849 m) (*Areião do Deco* in Cândido 2012) and PESC (17°42'28"S, 44°11'35"W, 1029 m) (*Areião do Cabral* in Cândido 2012), the scrublands at the PNSC (19°16'13"S, 43°39'38"W, 787 m, and 19°16'28"S, 43°40'33"W, 871 m) (Cipó I and Cipó II, respectively in Cândido 2012) and the wet grasslands at the PERP (18°05'28"S, 43°20'30"W, 830 m) (Campo úmido Rio Preto in Cândido 2012) and PESC (17°42'29"S, 44°11'30"W, 1024 m) (Campo úmido Cabral in Cândido 2012).

Leaf, root and soil sampling and analysis

In each site, we sampled five 5 x 5 m plots, 15 m apart from each other. We surveyed all species in each plot in order to select the ten most abundant species of each site. Leaves were collected from three individuals per species selected in each site per season. We collected leaf samples during the winter (July 2009 – dry season) and the summer (March 2010 - wet season) in order to compare leaf attributes between seasons. We collected at least three leaves per individual, depending on leaf size and prepared one compound sample per plant. We selected only fully-expanded, pathogen-free leaves. Leaves were oven-dried at 60°C for at least 48 h. Table 1 presents the list of species collected at each site. *Gaylussacia virgata* replaced *Anacardium humile* during the summer season.

We collected root samples only during the growing season (wet summer). We collected the whole root system when possible (herbs and a few shrubs) and fine, ramified roots (< 5mm) from tree species from the same individuals we collected leaf samples. The roots were preserved in ethanol 70% and their percentage of arbuscular mycorrhizal colonization was determined at the Laboratory of Soil Microbiology – "Luiz de Queiroz" College of Agriculture (ESALQ), Piracicaba-SP, following the protocols of Giovanetti & Mosse (1980) and Vierheilig *et al.* (1998). These protocols start with warming 1 g of washed fine roots in 10% KOH solutions. The roots were then autoclaved and removed

from KOH, washed, and transferred to 1% HCl. Dying procedure consists in washing the roots and transferring them to Tripa blue in 0.05% lactophene. Colonization rate was obtained by observing the roots in stereoscopic microscope. We selected roots with colonization rates higher than 1% for the statistical analyses.

We collected two soil samples from three different depths (0-10, 11-20 and 21-30 cm) in all the areas in both seasons. Soil samples were transported in polyethylene bags and oven-dried at 60°C for at least five days. The samples were sieved (2 mm sieve) to remove roots and leaves and analyzed for P content at the Laboratory of Chemical Analyses at the department of Soil Science at ESALQ.

In order to determine C and N content, as well as $\delta^{15}\text{N}$, leaf samples were individually ground in a mill (MA-048, Marconi, Brazil and M. Micro, MR Manesco & Ranieri LTDA, Brazil). C, N and $\delta^{15}\text{N}$ were obtained with a mass spectrometer ThermoQuest-Finnigan Delta Plus (Finnigan-MAT; CA, USA) coupled with an Elemental Analyzer (Carla Erba model 1110; Milan, Italy) at the Nuclear Energy Center for Agriculture - CENA/USP, Brazil. We placed 1.5-2 mg of leaf samples and 15-30 mg of soil in tin capsules for elemental analysis. Isotope ratios are relative to international standards, internal reference material was included in each analytical run. We used delta notations to record isotopic values in parts per thousand where $\delta\text{‰} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1,000$, and R is the molar ratio between the rare and the abundant isotope ($^{15}\text{N}/^{14}\text{N}$). Nitrogen standard sample was atmospheric air.

Statistical analyses

We compared total N and $\delta^{15}\text{N}$ values among physiognomies with the Kruskal-Wallis test and between seasons with the Mann-Whitney test because of the non-normal distribution of the data. In order to compare species that are shared between sites, we used T-tests. These tests were made using Statistica 8.0 (StatSoft 2007). Finally, linear regressions were performed with the R software (R Core Team 2012).

Results

Total soil N, P and soil $\delta^{15}\text{N}$

The three areas had contrasting soils characteristics (see Cândido 2012 for complete soil analyses). Scrublands and wet grasslands had higher total N (Figure 1a) and P content (Figure 2) than rupestrian grasslands.

Mean soil $\delta^{15}\text{N}$ values per physiognomy were always positive and varied from 4.8‰ to 9.6‰ (Figure 1b). At the rupestrian grasslands they varied from 5.1‰ to 9.6‰, at the wet grasslands from 4.8 to 6.3‰ and at the scrublands from 5.3 to 5.7‰. When we considered different depths, values extended their range from 2.5 to 14.5‰. At the rupestrian grasslands they varied from 2.5 to 14.5‰, at the wet grasslands, from 2.7 to 7.9‰ and at the scrublands from 2.9 to 7.1‰. Regardless of the physiognomy, soil $\delta^{15}\text{N}$ did not differ between the wet and the dry season (Mann-Whitney, $U = 11.00$, $n_{\text{dry}} = n_{\text{wet}} = 6$; $p = 0.26$). Regardless of the season, soil $\delta^{15}\text{N}$ was higher at the rupestrian grasslands than at the scrublands ($H(2, N = 29) = 8.3854$; $p = 0.015$, Figure 1b). Soil $\delta^{15}\text{N}$ values increased with soil depth when all the sites were put together ($R(N=84) = 0.64$, $p < 0.0001$), but no similar trend was observed for total soil N ($R(N=84) = -0.16$, $p = 0.15$).

Total leaf N and leaf $\delta^{15}\text{N}$

Unlike soil values, leaf $\delta^{15}\text{N}$ values showed both positive and negative values and varied from -6.5 to 8.3‰. Mean signatures per species varied from -3.9 to 6.6‰. Regardless of the area, we did not find differences between wet and dry season samples ($t(362) = -0.62$ $p = 0.53$). Rupestrian grasslands and wet grasslands presented higher leaf $\delta^{15}\text{N}$ values than the scrublands ($H(2, N = 61) = 14.982$ $p = 0.0006$) (Figure 1d). The same pattern was observed when we used the between-site correction $\Delta\delta^{15}\text{N}$ (leaf $\delta^{15}\text{N}$ - soil $\delta^{15}\text{N}$), where scrublands presented the lowest values ($H(2, N = 356) = 39.53$, $p < 0.0001$).

Using all the samples, in the rupestrian grasslands, mean leaf $\delta^{15}\text{N}$ was 2.25‰, ranging from -3.8‰ (*Kielmeyera rubriflora*, Clusiaceae) to 7.2‰ (Cyperaceae 1) (range of 11‰). In the wet grassland, mean leaf $\delta^{15}\text{N}$ was 1.1‰, ranging from -6.4‰ (*Lavoisiera imbricata*, Melastomataceae) to 8.3‰ (Eriocaulaceae 3) (range of 14.7‰). In the scrubland mean leaf $\delta^{15}\text{N}$ was -1.2‰, ranging from -5.6‰ (*Peixotoa* sp., Malpighiaceae) to 3.2‰ (*Myrcia* cf. *lasiantha*, Myrtaceae) (range of 8.8‰).

Shared species did not show the same leaf $\delta^{15}\text{N}$ values in both communities where they were sampled (Figure 3). Also, the same species exhibited different leaf N concentrations in different communities. However, when we introduced between-site corrections ($\Delta\delta^{15}\text{N}$), we noticed no differences between species (Figure 4).

Total leaf N concentrations from all samples varied from 0.36% (Iridaceae 2) to 3.35% (*Philcoxia minensis*, Plantaginaceae), and mean total leaf N per species varied from 0.54 to 2.61%. When we compared communities, they all had similar leaf N concentrations, $F(2, 58)=1.3368$, $p= 0.27066$ (Figure 1c). Legumes presented higher total leaf N ($U=1741.00$, $p<0.0001$). Legumes also presented lower leaf $\delta^{15}\text{N}$ values than non-legumes ($U= 3210$, $p= 0.0082$) and foliar $\delta^{15}\text{N}$ from -3.0 to 1.1‰.

A negative relation was found between foliar $\delta^{15}\text{N}$ and soil N concentration ($r^2= 0.22$, $F(1, 59)= 16.9$, $p= 0.0001$) (Figure 5b) and we found a positive relation between foliar and soil $\delta^{15}\text{N}$ ($r^2= 0.17$, $F(1, 59)= 12.37$, $p= 0.0008$) (Figure 5a). When we applied between site correction, leaf $\delta^{15}\text{N}$ was positively related with $\Delta\delta^{15}\text{N}$ (leaf $\delta^{15}\text{N} - \text{soil } \delta^{15}\text{N}$) ($r^2= 0.85$, $F(1, 59)= 325.3$, $p< 0.0001$) (Figure 5c). No relation was found between leaf $\delta^{15}\text{N}$ and leaf N concentration ($r^2= 0.03$, $F(1, 59)= 1.577$, $p= 0.2141$) (Figure 5d) or between total leaf N and total soil N ($r^2= 0.02$, $F(1, 59)= 1.425$, $p= 0.2374$) (Figure 5e).

Most of the plants did not present mycorrhizal colonization. The highest colonization rate was 33.7% in Eriocaulaceae. The wet grasslands sites had the highest colonization rates ($H(2, N= 161) = 40.617$, $p< 0.0001$). Families such as Xyridaceae and Asteraceae also presented mycorrhizal colonization. A positive but weak relation was found between arbuscular mycorrhiza root colonization rate and leaf $\delta^{15}\text{N}$ ($r^2= 0.23$, $F(1, 54)= 16.38$, $p= 0.0002$) (Figure 5f) and between arbuscular mycorrhiza root colonization rate and $\Delta\delta^{15}\text{N}$ ($r^2= 0.38$, $F(1, 54)= 32.72$, $p< 0.0001$) but no relation was found between root colonization rate and leaf N content ($r^2= 0.01$, $F(1, 157)= 2.294$, $p= 0.1$), or root colonization rate and total soil N ($r^2= 0.002$, $F(1, 105)= 0.2008$, $p= 0.66$).

Discussion

Our results showed that all the field sites presented very low total soil N ($< 0.1\%$) and P ($< 1 \text{ mg kg}^{-1}$), comparable to other ancient landscapes (Lambers *et al.* 2010).

Associated with strong seasonality in precipitation, these characteristics represent an extremely harsh environment for most plants. However, the high diversity indices in cerrado physiognomies, specially the rupestrian physiognomies (Alves & Kolbek 1994; Myers *et al.* 2000) reveal that plants in these environments are somehow able to deal with these constraints. Diverse nutrient acquisition strategies may facilitate the establishment of rich communities (Lambers *et al.* 2010), by nutrient niche partitioning (McKane *et al.* 2002). In the present study, we observed highly variable leaf $\delta^{15}\text{N}$ and total leaf N in each community, which reflects variable N-nutrition strategies (Bustamante *et al.* 2004). Among the strategies, rupestrian grassland species may have mycorrhizal associations, root specializations, carnivory, and nitrogen fixation (Cândido *et al.* in preparation, Campo *et al.* in preparation, Pereira *et al.* 2012, Abrahão *et al.* in this volume).

Soil analyses

Cerrado *sensu lato* soils are usually dystrophic with low pH and high aluminum content (Goedert 1983; Haridasan *et al.* 1996). In fact, in our sites, there is very low nutrient concentrations and very acidic pHs (Cândido 2012). When we compared between physiognomies, total soil N was lower in the rupestrian grasslands, which is consistent with the soil type. Higher clay content at the scrubland (Cândido 2012) is related to higher field capacity and cation exchange capacity, making nutrients more available (Marimon & Haridasan 2005). In all the field sites, total soil N was lower than the mean values registered for the cerrado physiognomies (Goodland & Pollard 1973; Haridasan *et al.* 1987; Silva & Haridasan 2007). Rupestrian grasslands were the most N-impooverished soils. P, K and Al were also present in the lowest concentrations at the rupestrian grasslands (Cândido 2012). Although we did not measure biomass, extreme soil nutrient impoverishment in this environment is reflected in lower productivity in rupestrian grasslands than in wet grasslands and scrublands.

As expected for tropical environments, soil $\delta^{15}\text{N}$ values were all positive (Martinelli *et al.* 1999). Positive values of $\delta^{15}\text{N}$ do not provide evidence of high N availability or open cycles, but rather indicates nitrogen losses via fractionating pathways (Martinelli *et al.* 1999; Houlton *et al.* 2006). When compared to cerrado savannas (Bustamante *et al.* 2004), soil $\delta^{15}\text{N}$ values from the studied sites occurred within the cerrado range of values. As in

other studies (Högberg 1997; Bustamante *et al.* 2004; Viani *et al.* 2011), soil $\delta^{15}\text{N}$ increased with depth, probably due to fractionation during mineralization and subsequent uptake of ^{15}N -depleted N by plants, leaving ^{15}N -enriched soils, but also to litter contribution of the ^{15}N -depleted plant material to the upper soil layers (Högberg 1997).

Despite the lowest total soil N, soil $\delta^{15}\text{N}$ values were higher in rupestrian grasslands than in scrublands. The highly ^{15}N -enrichment of rupestrian grasslands could be caused by lower water availability in the sandy soils when compared to more clayey soils of wet grasslands and scrublands. Austin & Vitousek (1998) and other authors (Handley *et al.* 1999; Robinson 2001) have shown that lower $\delta^{15}\text{N}$ values are associated with greater water availability. ^{15}N -enrichment can be also associated with P-poor soils (Pekin *et al.* 2012), as it occurs in rupestrian grasslands. $\delta^{15}\text{N}$ values of carnivorous *Philcoxia* *minensis* did not contribute to soil ^{15}N -enrichment because it presented low $\delta^{15}\text{N}$ values when compared to other carnivorous plants (Pereira *et al.* 2012). Finally, open vegetation types are more susceptible to nutrient losses than woody ecosystems. Losses occur through volatilization and transport of particulate matter after surface fires (Kauffman *et al.* 1994). As the N_2O emissions are negligible, and NO emissions represent a small loss in the cerrado physiognomies, the outputs of nitrogen in the absence of fire are very low (Bustamante *et al.* 2006).

Leaf analyses

Leaf N concentrations did not follow soil N concentrations, being similar in all the physiognomies, despite the differences in soil N availability. A similar result was obtained by Araújo & Haridasan (1988). According to Ernst and Tolsma (1989) *apud* Haridasan (2001), leaf nutrient concentrations do not necessarily reflect the nutrient levels in the soil because of species-specific uptake. Also, it is difficult to correlate leaf concentrations with nutrients available on the soil surface layers because these correlations do not establish cause and effect relations. Usually the vegetation influences soil nutrient availability through litter fall, not the other way around (Haridasan 2001).

Several nutrient acquisition strategies were described for the same rupestrian grasslands we studied in Pereira *et al.* (2012), Campos *et al.* and Cândido *et al.* (in preparation). Among them, arbuscular mycorrhizas and sand-binding roots are the most common

strategies. For example, *Discocactus placentiformis* presented sand granules strongly bound to the root hairs (Chapter 1 of this thesis). Dauciform roots were observed within the Cyperaceae family (Cândido 2012) and five species within Eriocaulaceae and species of Xyridaceae presented sand-binding roots (Campos *et al.*, in preparation). Other interesting unknown root morphologies were found in Asteraceae, Velloziaceae and *Anacardium humile* (Anacardiaceae). At the PESC rupestrian grassland site, *Philcoxia minensis* is found, a carnivorous species (Pereira *et al.*, 2012) that presents one of the highest leaf N concentrations of this study probably due to the extra nitrogen supply. According to Ellison (2006), carnivorous plants are in energetic disadvantage compared to non-carnivorous plants, however, our data shows higher content of nitrogen in this carnivorous species, indicating that it may be in advantage in this extremely-impoverished physiognomy. Members of Asteraceae family also present high leaf N values.

This diversity in N-acquisition strategies reflects in foliar $\delta^{15}\text{N}$ values. Foliar $\delta^{15}\text{N}$ values integrates the isotope ratio of the external N source (organic N, NH_4^+ or NO_3^-), the availability of each N source, and the physiological mechanisms within the plant, such as reabsorption and reallocation of N (Evans 2001). As expected, we observed a wide range of foliar $\delta^{15}\text{N}$ values (Martinelli *et al.* 1999; Bustamante *et al.* 2004; Coletta *et al.* 2009; Viani *et al.* 2011). Rupestrian grasslands presented the highest $\delta^{15}\text{N}$ signatures. However, contrary to expectations, the physiognomy with lowest soil N concentrations did not present the widest variation in foliar $\delta^{15}\text{N}$ values, or the lowest total leaf N. The physiognomy with the widest range of foliar $\delta^{15}\text{N}$ values were the wet grasslands, and the highest $\delta^{15}\text{N}$ signatures were found in rupestrian grasslands and wet grasslands. The widest range of $\delta^{15}\text{N}$ signatures in wet grasslands may be due to more diverse sources of N contributions from the surroundings, because wet grasslands collect water from leaching due to its lower position, therefore plants can acquire more diverse forms of N, contributing to the wider range of $\delta^{15}\text{N}$ signatures.

When we compared $\Delta\delta^{15}\text{N}$, however, we noticed that foliar ^{15}N -enrichment in rupestrian grasslands was due to soil ^{15}N -enrichment because $\Delta\delta^{15}\text{N}$ values from rupestrian grasslands and wet grasslands were the same. $\Delta\delta^{15}\text{N}$ values of wet grasslands were higher than scrublands, and rupestrian grasslands presented intermediate values. We also noticed

that all $\Delta\delta^{15}\text{N}$ values were very negative when compared to global-level studies (Craine *et al.* 2009). This result may indicate mineral N acquisition rather than organic N (Robinson 2001; Kahmen *et al.* 2008).

Long-lived leaves are subjected to N losses via fractionating pathways longer than short-lived leaves, this contributes to ^{15}N -enrichment of the ecosystem (Evans 2001). As long leaf life-spans are found in nutrient-poor habitats (Chapin 1980), longer leaf life spans in rupestrian grasslands and wet grasslands can explain higher $\delta^{15}\text{N}$ in leaves, but it can also be due to low P-supply (Pekin *et al.* 2012).

Members of the Fabaceae family presented $\delta^{15}\text{N}$ signatures close to zero, but with higher variability than expected, showing that they do not rely only on N-fixation by symbionts, but also acquire other forms of N, such as soil mineral N. As previously shown, legumes present higher total leaf N than other families because of high acquisition-efficiency and high N-demand (Vitousek *et al.* 2002; Bustamante *et al.* 2004; Coletta *et al.* 2009). Most of the species that co-occurred in different sites presented different $\delta^{15}\text{N}$ signatures, but the same $\Delta\delta^{15}\text{N}$, therefore, the difference in $\delta^{15}\text{N}$ signatures were due to substrate differences (Viani *et al.* 2011). *Davilla elliptica*, however, exhibited differences in $\Delta\delta^{15}\text{N}$ in different sites of the same physiognomy, showing that it may rely on different N forms in different sites.

Root colonization rate by arbuscular mycorrhizae was usually low in most studied species (<1%) and it was not related with total foliar N, but was positively related with foliar $\delta^{15}\text{N}$. Plants that acquire N through mycorrhizae usually present ^{15}N -impoverished signatures because mycorrhizae retains ^{15}N -enriched nitrogen and transfers ^{15}N -impoverished nitrogen to the host plant (Hobbie & Colpaert 2003; Craine *et al.* 2009).

Conclusion

Rupestrian cerrado physiognomies present very high species diversity (Alves & Kolbek 1994; Myers *et al.* 2000), and also exhibit a wide range of $\delta^{15}\text{N}$ signatures, indicating a diverse suite of nitrogen-use strategies. This diversity in N-use strategies in rupestrian physiognomies should contribute to the maintenance of the high plant species-diversity in these ecosystems. This study also showed that high foliar $\delta^{15}\text{N}$ signatures do

not always indicate higher N availability. We also showed that rupestrian physiognomies presented very negative $\Delta\delta^{15}\text{N}$ values.

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Table 1. Total leaf nitrogen, foliar $\delta^{15}\text{N}$ and arbuscular mycorrhizal (AM) colonization from all the species analyzed for each site. Values represent the mean from six samples per species (three plants per season).

Species	Family	Total leaf N (%)	Leaf $\delta^{15}\text{N}$ (‰)	AM colonization (%)
Cabral Rupestrian Grassland				
<i>Discocactus placentiformis</i> (Lehm.) Shumann	Cactaceae	1.61	2.31	0.04
<i>Kielmeyera rubriflora</i> Cambess.	Clusiaceae	1.11	-3.31	12.43
Cyperaceae 2	Cyperaceae	1.35	5.17	0.17
Cyperaceae 3	Cyperaceae	0.79	5.16	0.23
<i>Gaylussacia reticulata</i> Mart. ex Meisn.	Ericaceae	0.94	-1.21	0.13
<i>Syngonanthus bisulcatus</i> (Koern) Ruhland	Eriocaulaceae	1.06	-0.68	0.15
<i>Mimosa misera</i> Benth.	Fabaceae	1.78	0.51	0.67
<i>Philcoxia minensis</i> V.C. Souza & Giul.	Plantaginaceae	2.61	2.59	2.58
<i>Vellozia albiflora</i> Pohl	Velloziaceae	1.30	0.74	0.00
<i>Xyris obcordata</i> Kral & Wanderley	Xyridaceae	1.14	2.38	1.58
Rio Preto Rupestrian Grassland				
<i>Anacardium humile</i> A. St.-Hil.	Acardiaceae	1.63	3.78	-
<i>Vernonia</i> cf. <i>rufogrisea</i> A. St.-Hil.	Asteraceae	2.41	5.63	0.16
<i>Discocactus placentiformis</i> (Lehm.) Shumann	Cactaceae	1.50	3.18	0.00
<i>Kielmeyera rubriflora</i> Cambess.	Clusiaceae	1.38	-0.76	3.37
Cyperaceae 1	Cyperaceae	1.16	0.31	0.71
<i>Gaylussacia virgata</i> var. <i>hilaireana</i> Sleum.	Ericaceae	0.74	5.57	1.87
Eriocaulaceae 1	Eriocaulaceae	1.14	4.77	11.23
<i>Mimosa misera</i> Benth.	Fabaceae	1.75	-3.83	2.30
Poaceae 1	Poaceae	0.71	2.69	2.53
<i>Vellozia resinosa</i> Mart.	Velloziaceae	1.29	4.15	2.00
<i>Vellozia</i> sp.	Velloziaceae	1.51	3.22	-

Cabral wet grassland

<i>Trichogonia</i> sp.	Asteraceae	2.61	-0.45	0.00
<i>Paepalanthus</i> sp.	Eriocaulaceae	1.29	-1.76	0.12
<i>Syngonathus</i> sp.	Eriocaulaceae	1.03	0.02	9.91
Iridaceae 1	Iridaceae	0.77	-3.08	1.35
<i>Lavoisiera imbricata</i> DC.	Melastomataceae	1.18	-0.56	0.70
<i>Microlicia fulva</i> (Spreng.) Cham.	Melastomataceae	1.62	-3.87	-
Poaceae 4	Poaceae	0.66	0.55	0.35
Poaceae 5	Poaceae	0.56	1.37	0.08
<i>Declieuxia</i> cf. <i>cordigera</i> Müll. Arg.	Rubiaceae	1.60	3.56	0.73
Xyridaceae 1	Xyridaceae	0.74	3.81	12.18

Rio Preto wet grassland

<i>Mikania</i> sp.	Asteraceae	1.65	-0.65	0.33
Eriocaulaceae 2	Eriocaulaceae	1.22	6.58	23.22
Eriocaulaceae 3	Eriocaulaceae	0.94	-0.75	3.90
<i>Hyptis</i> sp.	Lamiaceae	1.12	-0.77	3.53
<i>Lavoisiera imbricata</i> DC.	Melastomataceae	1.08	0.42	1.17
<i>Microlicia fulva</i> (Spreng.) Cham.	Melastomataceae	1.22	5.38	26.30
Poaceae 2	Poaceae	0.54	-1.56	8.67
Poaceae 3	Poaceae	1.31	-1.73	3.20
<i>Psyllocarpus laricoides</i> Mart. ex Mart. & Zucc.	Rubiaceae	1.38	-0.38	9.15
Xyridaceae 1	Xyridaceae	0.99	4.71	16.52

Cipó I scrubland

<i>Baccharis</i> sp.	Asteraceae	1.15	-2.02	0.93
<i>Davilla elliptica</i> A. St.-Hil.	Dilleniaceae	0.90	-0.36	0.03
<i>Mimosa foliolosa</i> Benth.	Fabaceae	1.75	-3.13	0.00
Iridaceae 2	Iridaceae	0.66	-2.37	0.27
<i>Myrcia</i> cf. <i>lasiantha</i> DC.	Myrtaceae	0.95	0.95	0.00
<i>Axonopus</i> sp.	Poaceae	0.88	0.65	0.00

<i>Echinolaena</i> sp.	Poaceae	1.42	2.41	0.11
<i>Schizachyrium</i> sp.	Poaceae	0.71	-2.28	0.08
<i>Qualea grandiflora</i> Mart.	Vochysiaceae	1.24	4.33	0.00
<i>Vochysia elliptica</i> Mart.	Vochysiaceae	0.96	-2.68	1.37
Cipó II scrubland				
<i>Eremanthus</i> sp.	Asteraceae	0.84	-2.92	2.29
<i>Davilla elliptica</i> A. St.-Hil.	Dilleniaceae	1.07	1.02	0.09
<i>Chamaecrista</i> cf. <i>trachycarpa</i> (Voguel) H. S. Irwin & Barneby	Fabaceae	1.38	0.82	5.57
<i>Galactia martii</i> DC.	Fabaceae	1.51	-0.68	0.38
<i>Byrsonima</i> sp.	Malpighiaceae	1.06	0.72	2.98
<i>Peixotoa</i> sp.	Malpighiaceae	2.00	-2.43	0.88
<i>Echinolaena</i> sp.	Poaceae	1.72	-1.35	3.17
Poaceae 6	Poaceae	0.84	-2.84	3.17
<i>Palicourea rigida</i> Kunth	Rubiaceae	1.47	5.59	1.23
<i>Vochysia elliptica</i> Mart.	Vochysiaceae	1.12	-1.87	1.30

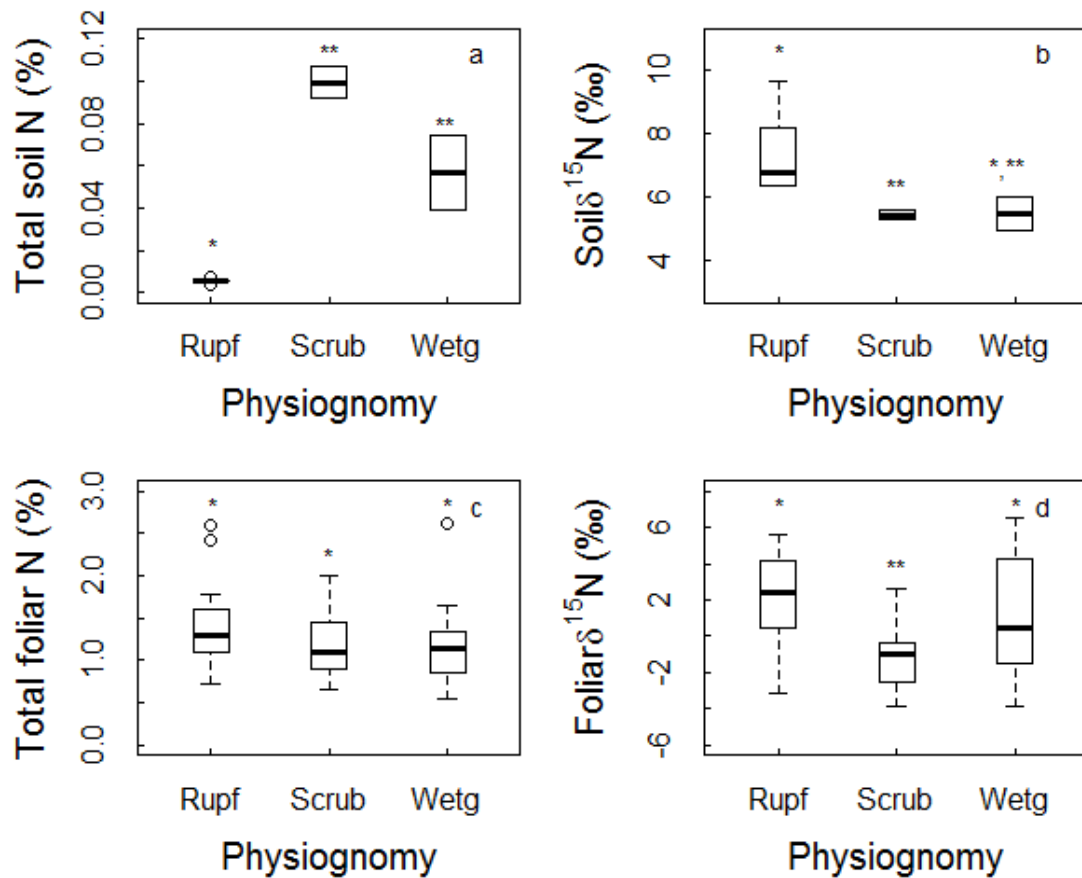


Figure 1. **a.** Total soil N (%) (N=2 per physiognomy), **b.** soil $\delta^{15}\text{N}$ (N=2 per physiognomy), **c.** total foliar N (%) (N= 20 species per physiognomy) and **d.** foliar $\delta^{15}\text{N}$ (N= 20 species per physiognomy) from rupestrian grasslands (Rupf), scrublands (Scrub) and wet grasslands (Wetg) from the Espinhaço range in Minas Gerais, Brazil. Different signs (*,**) represent significant differences ($p < 0.05$, Kruskal-Wallis test). Bars represent minimum and maximum observations, box limits represent lower and upper quartiles, and bold bar represents median.

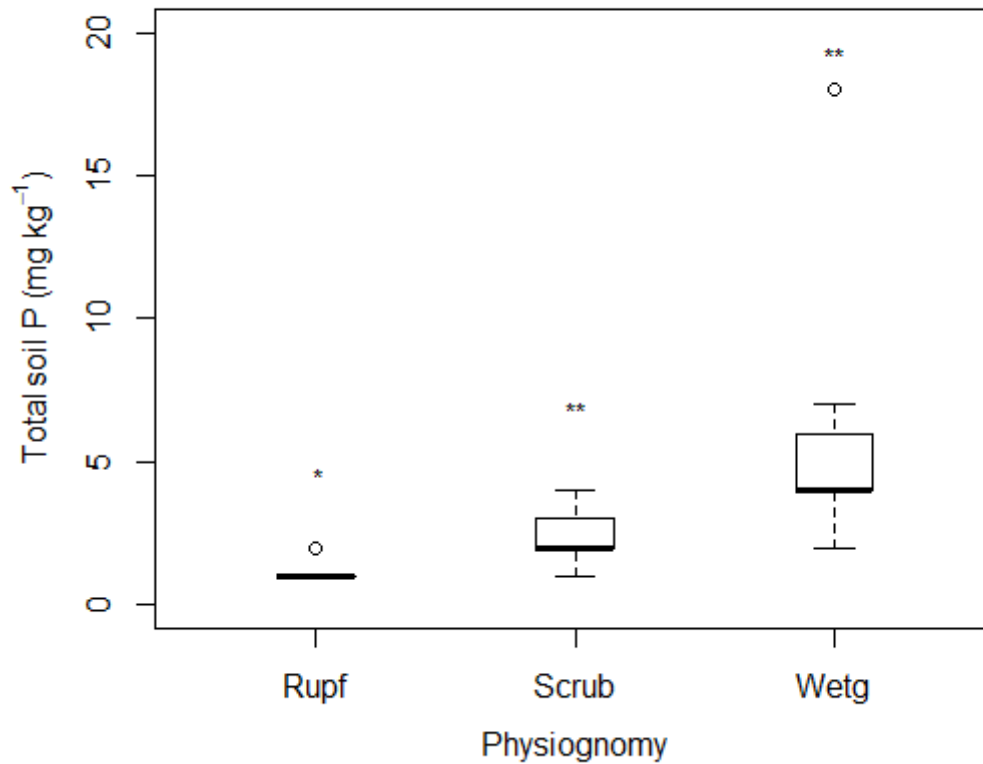


Figure 2. Total soil phosphorus (P) from 0-30 cm cores from rupestrian grasslands (Rupf), scrublands (Scrub) and wet grasslands (Wetg) from the Espinhaço range in Minas Gerais, Brazil (N= 12 per physiognomy). Different signs (*,**) represent significant differences ($p < 0.05$, Kruskal-Wallis test). Bars represent minimum and maximum observations, box limits represent lower and upper quartiles, and bold bar represents median.

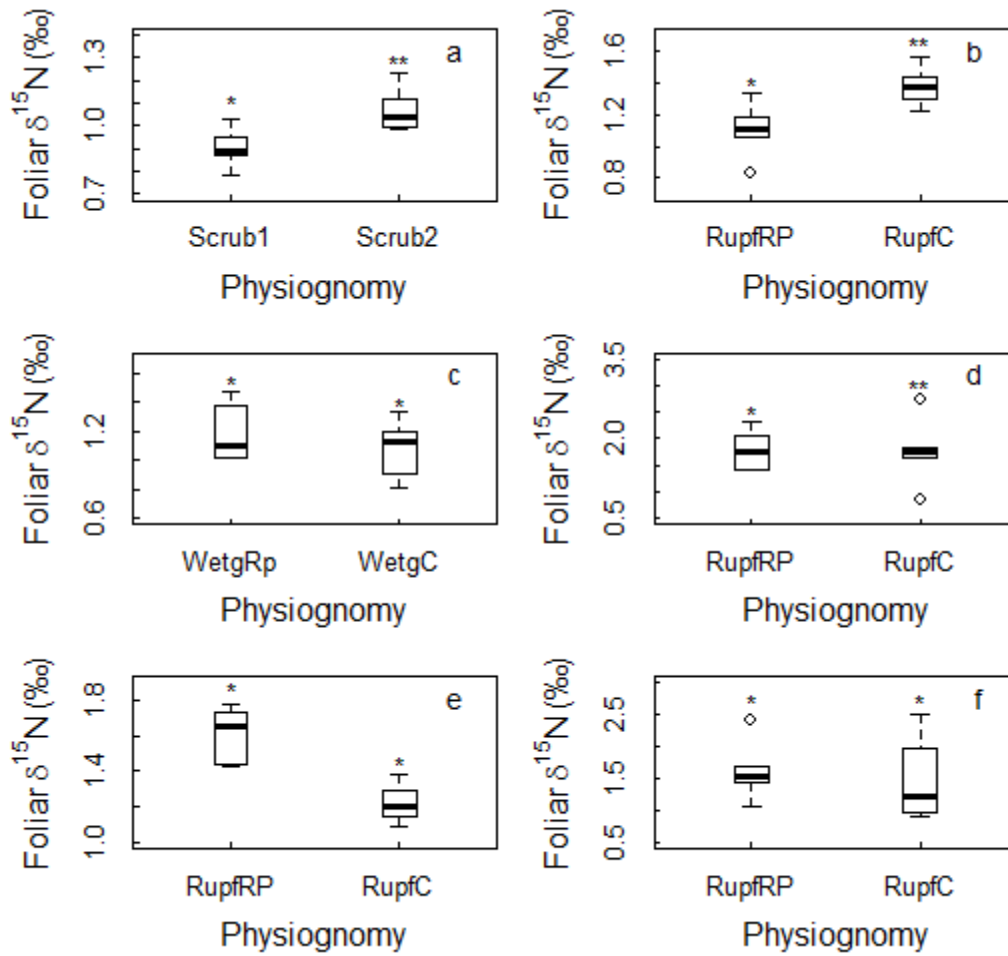


Figure 3. Foliar $\delta^{15}\text{N}$ (‰) from **a.** *Davilla elliptica*, **b.** *Kielmeyera rubra*, **c.** *Lavoisiera imbricata*, **d.** *Mimosa misera*, **e.** *Microlicia fulva* and **f.** *Discocactus placentiformis* from rupes from Parque Estadual da Serra do Cabral (RupfC) and Parque Estadual da Serra do Rio Preto (RupfRP), scrublands from Parque Nacional da Serra do Cipó (Scrub 1 and 2) and wet grasslands from Parque Estadual da Serra do Cabral (WetgC) and Parque Estadual da Serra do Rio Preto (WetgRP) that belong to the Espinhaço range in Minas Gerais, Brazil. N= 10 species per physiognomy. Different signs (*,**) represent significant differences ($p < 0.05$, Mann-Whitney test). Bars represent minimum and maximum observations, box limits represent lower and upper quartiles, and bold bar represents median.

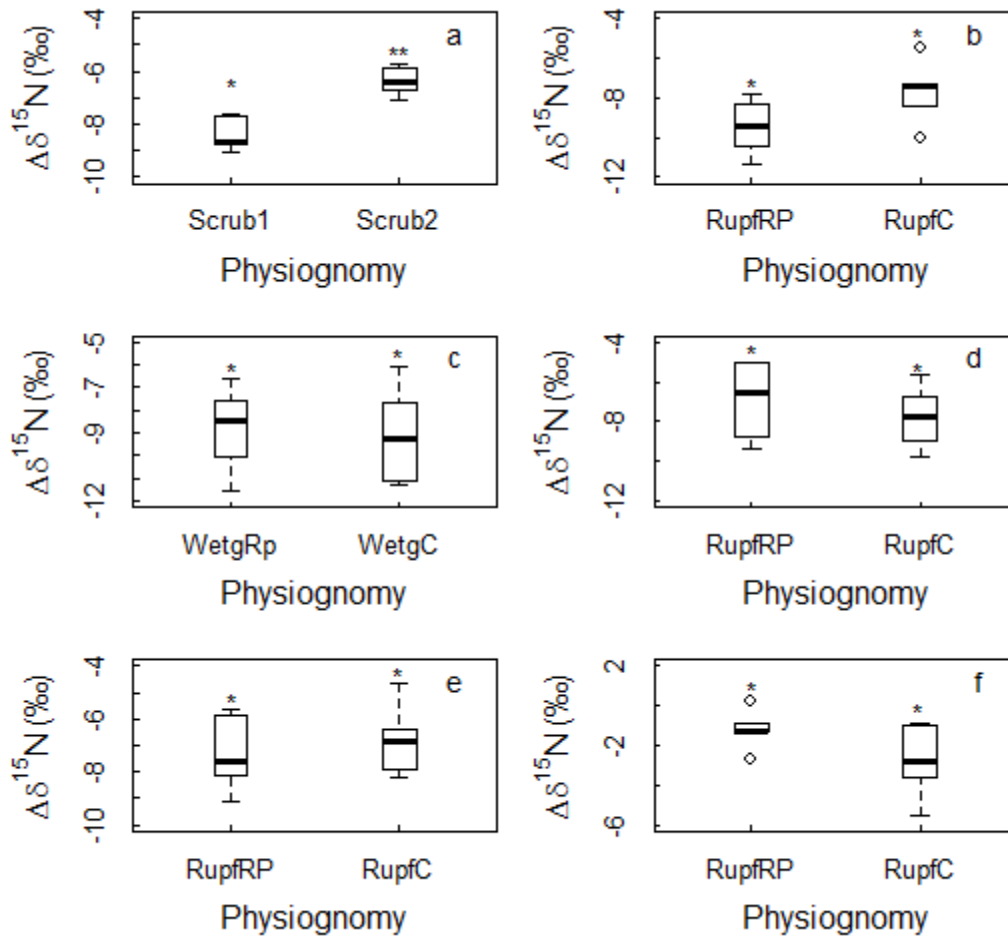


Figure 4. $\Delta\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{\text{foliar}} - \delta^{15}\text{N}_{\text{soil}}$) (‰) from **a.** *Davilla elliptica*, **b.** *Kielmeyera rubra*, **c.** *Lavoisiera imbricata*, **d.** *Mimosa misera*, **e.** *Microlicia fulva* and **f.** *Discocactus placentiformis* from rupestrian grasslands from Parque Estadual da Serra do Cabral (RupfC) and Parque Estadual da Serra do Rio Preto (RupfRP), scrublands from Parque Nacional da Serra do Cipó (Scrub 1 and 2) and wet grasslands from Parque Estadual da Serra do Cabral (WetgC) and Parque Estadual da Serra do Rio Preto (WetgRP) that belong to the Espinhaço range in Minas Gerais, Brazil. N= 10 species per physiognomy. Different signs (*,**) represent significant differences ($p < 0.05$). Bars represent minimum and maximum observations, box limits represent lower and upper quartiles, and bold bar represents median.

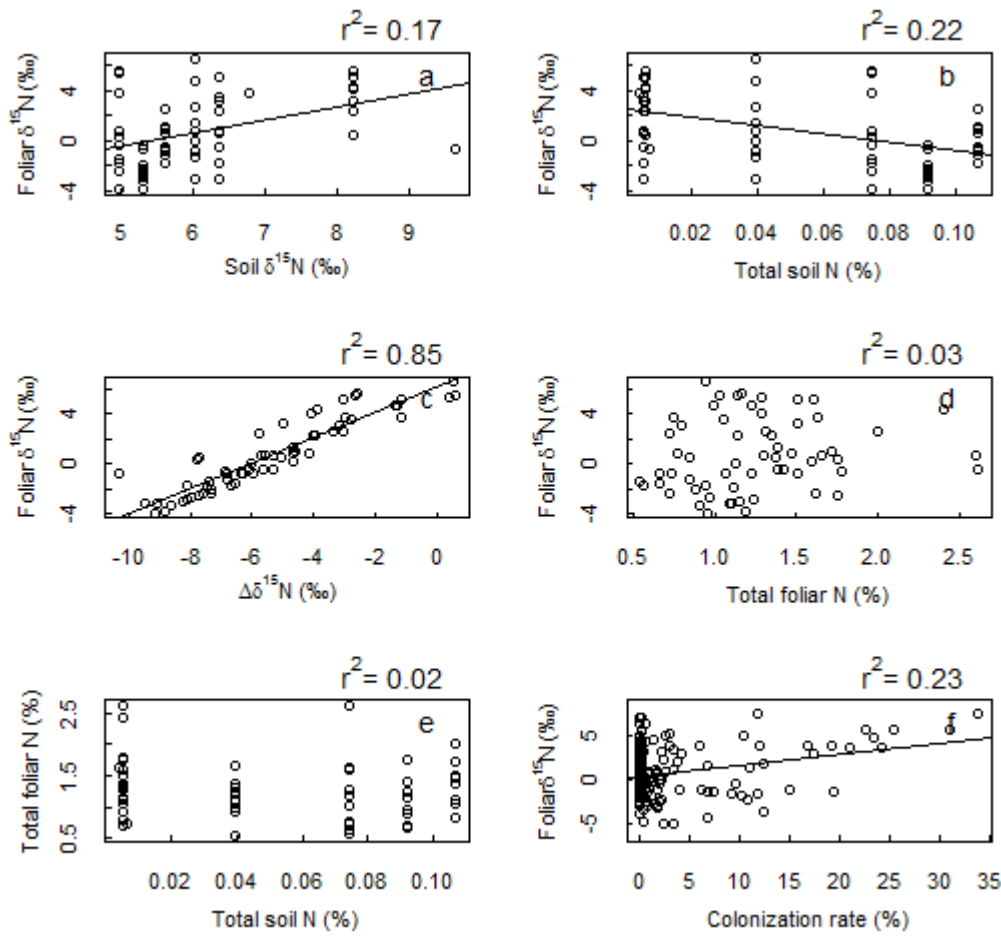


Figure 5. Linear regressions between **a.** foliar and soil $\delta^{15}\text{N}$ (‰), **b.** foliar $\delta^{15}\text{N}$ (‰) and total soil nitrogen, **c.** foliar $\delta^{15}\text{N}$ (‰) and $\Delta\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{\text{foliar}} - \delta^{15}\text{N}_{\text{soil}}$) (‰), **d.** foliar $\delta^{15}\text{N}$ (‰) and total foliar nitrogen (N (%)), **e.** total foliar nitrogen (N (%)) and total soil nitrogen (N (%)) and **f.** foliar $\delta^{15}\text{N}$ (‰) and mycorrhizal colonization rate (%) from three physiognomies in the Espinhaço range in Minas Gerais, Brazil. N= 60 species. Regression lines are shown for $p < 0.05$.