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"ATENUAÇÃO, PELA ASSOCIAÇÃO MICORRÍZICA ARBUSCULAR, DO ESTRESSE CAUSADO POR CÁDMIO EM PLANTAS"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular na área de Microbiologia.

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A meu filho Daniel

e

a Tere, minha mãe.

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RESUMO

A acumulação de Cd pelas plantas e a crescente presença desse metal no sistema edáfico é, atualmente, de grande preocupação ambiental pois pode acarretar problemas para a saúde humana e causar efeitos deletérios no ecossistema edáfico. A micorriza arbuscular (MA), considerada uma simbiose quase que universal entre plantas superiores e fungos da ordem Glomales, constitui uma ponte entre o solo e a planta tendo influência direta na absorção de nutrientes e de elementos potencialmente tóxicos. Nesse contexto, o presente trabalho estudou a influência da associação micorrízica arbuscular em plantas potencialmente remediadoras de solos contaminados, feijão de porco, milho e girassol, na absorção e acúmulo de Cd, e na possível atenuação do estresse causado pelo excesso de Cd nas plantas hospedeiras. Com esse propósito, foram avaliados parâmetros de crescimento, a distribuição de Cd na parte aérea, raízes e no micélio extrarradicular dos fungos micorrízicos arbusculares (FMAs) inoculados e associados às plantas hospedeiras. A atividade da guaiacol peroxidase nas raízes, umas das enzimas chave no sistema antioxidante da planta, a absorção de nutrientes e a atividade da fosfatase em folhas foram utilizadas na avaliação do estresse e da possível tolerância das plantas ao Cd. De forma geral, as plantas beneficiaram-se da associação com FMAs, tanto na ausência como na presença de Cd. No entanto, mostraram respostas diferentes em termos de acúmulo de Cd. O feijão de porco, quando associado

a *Glomus etunicatum*, e o girassol colonizado por *G. intraradices* absorveram maiores quantidades de Cd do que plantas não micorrizadas (NM). Já o milho colonizado por *G. macrocarpum* absorveu menores quantidades de Cd do que os homólogos NM. O girassol acumulou concentrações de Cd superiores a 200 mg kg^{-1} na parte aérea, podendo ser considerada uma planta hiperacumuladora de Cd, sendo que absorveu e translocou ainda maiores concentrações de Cd quando associado a *G. intraradices*. A micorrização do milho promoveu o seu crescimento, melhorando a nutrição mineral e atenuando o estresse da planta em condições de excesso de Cd. A atividade da enzima guaiacol peroxidase mostrou-se um indicador sensível do estresse oxidativo causado pelo Cd nas raízes, sendo induzida na presença do metal e influenciada pela micorrização das plantas.

ABSTRACT

Cd accumulation by plants and the increasing presence of this metal in the soil system is nowadays of great environmental concern. Cd contamination can bring human health problems and harmful effects to the soil ecossystem. Arbuscular mycorrhiza (AM) is considered a universal symbiosis between higher plants and fungi from the order Glomales. This symbiosis is a direct link between soil and plant roots, with a direct influence in nutrient and potentially toxic elements uptake. In this context, the present research studied the influence of the arbuscular mycorrhizal association in potencial remediator plants of contaminated soil, jackbean, maize and sunflower, on Cd uptake and accumulation patterns and evaluated the possible alleviation of the stress caused by excess Cd confered to the host plants. With this intention, plant growth parameters and Cd distribution in shoots, roots and in the extraradical mycelia of the arbuscular mycorrhizal fungi (AMF) inoculated were evaluated. Guaiacol peroxidase activity in plant roots, as one of the key enzymes of the antioxidant system in plants, nutrient uptake and phosphatase activity in leaves were used to evaluatethe stress caused by Cd and the possible phytotoxicity alleviation in plants due to mycorrhiza. In a general way, plants benefited from the AM association, both in the ausence and in the presence of Cd. However, plant response in terms of Cd accumulation differed. Jackbean plants associated to *G. etunicatum* and sunflower plants colonized by *G. intraradices* absorved higher amounts of Cd than non-mycorrhizal (NM) plants.

Sunflower plants colonized by *G. intraradices* showed a shoot Cd concentration higher than 200 mg kg^{-1} and can be considered a Cd hyperaccumulator plant. In addition, sunflower plants absorved and translocated still higher Cd amounts when in association with *G. intraradices*. Maize mycorrhization promoted plant growth, ameliorated mineral nutrition and attenuated plant stress in excess Cd conditions. Guaiacol peroxidase activity showed to be a sensitive biomarker of the oxidative stress caused by Cd in plant roots. The enzime was induced in the presence of the metal and influenced by plant mycorrhal status.

1. INTRODUÇÃO

1.1 O CÁDMIO E SUA FITOTOXICIDADE

Entre os metais comumente chamados metais pesados (MPs), os quais referem-se aqueles com massa específica maior que 5 g cm^{-3} e com capacidade de formar sulfetos (Adriano, 1986), encontra-se o cádmio. O Cd (densidade = 8,6 g cm^{-3}) situa-se no sétimo lugar na lista das 20 substâncias mais tóxicas existentes na natureza (Al-Kedhairy et al., 2001). Esse metal é um elemento traço de grande ubiqüidade nos solos, mas são as atividades antropogênicas que têm liberado quantidades preocupantes ao meio ambiente. Entre essas atividades, destacam-se a mineração e atividades metalúrgicas, queima de combustíveis fósseis, incineração de lixo, adição de pesticidas e fertilizantes fosfatados, uso do lodo de esgoto ou a produção, uso e dejeto de baterias (Wagner, 1993). Estima-se que as emissões antropogênicas de Cd se situam em torno de 30.000 toneladas por ano, sendo que solos considerados não poluídos apresentam concentrações de 0,1 a 0,5 mg kg^{-1} de Cd e solos com concentrações maiores são considerados poluídos (Sanità di Toppi e Gabbrielli, 1999).

Esse elemento, não essencial para as plantas, pode ser absorvido pelas raízes de muitas espécies de plantas e ser acumulado nos tecidos vegetais. A absorção de ions Cd^{+2} parece competir por transportadores transmembrânicos com nutrientes como o K^{+}, Ca^{+2}, Mg^{+2}, Fe^{+2}, Mn^{+2}, Cu^{+2}, Zn^{+2} e Ni^{+2} (Rivetta et al., 1997). O Cd entra na raiz através do tecido cortical e chega ao xilema pelo espaço apoplástico, complexado por vários tipos de ligantes (Cataldo et al., 1988). O Cd, uma vez no interior da planta, é um elemento relativamente móvel e pode influenciar a sua nutrição mineral, sendo que os principais sintomas de toxicidade são clorose e necrose da raiz e das folhas, o que

acarreta a diminuição do crescimento (Hernandez e Cooke, 1997). Além disso, pode influenciar o balanço hídrico e danificar o aparato fotossintético em vários níveis: fixação de CO_2, condutância estomática, síntese de clorofila, transporte de elétrons e enzimas do ciclo de Calvin (Barcelo e Poschenrieder, 1990; Ernst, 1980). A principal base da toxicidade do Cd nos sistemas biológicos relaciona-se a sua forte afinidade por ligantes contendo radicais sulfidrilo (SH), particularmente politióis, modificando a estrutura e atividade de várias enzimas, os fosfolipídeos das membranas e a fosforilização oxidativa (Wagner, 1993).

O Cd, como um dos metais mais agressivos, causa estresse oxidativo e modifica a atividade de várias enzimas antioxidantes em diferentes espécies de plantas e condições ambientais (Vitoria et al., 2001; Sanità di Toppi e Gabrielli, 1999). Estresse oxidativo é o conjunto dos efeitos causados pelas formas reativas do oxigênio como o radical superóxido ($O_2^{\cdot-}$), peróxido de hidrogênio (H_2O_2), radical hidroxila ($OH^{\cdot}$) e o oxigênio "singlet" ($^1O_2$) (Gratão et al., 2005a). A indução de enzimas antioxidantes, portanto, é um sinal do estresse oxidativo causado pelo Cd no nível celular (Ferreira et al., 2002).

1.2 A MICORRIZA ARBUSCULAR E SUA INTERAÇÃO COM O CÁDMIO

1.2.1 A simbiose micorrízica

A micorriza arbuscular (MA) é a associação micorrízica mais comum na natureza, a qual é formada pelas raízes da maioria das plantas superiores e fungos Zygomicetes da ordem Glomales (Harrier, 2001). Calcula-se que mais de 80% de todas as plantas terrestres formem esse tipo de associação, incluindo-se muitas plantas de

importância na agricultura e horticultura (Smith e Read, 1997). A MA é uma simbiose antiga e já foram encontrados hifas e arbúsculos em fósseis de *Aglaophyton* que comprovam a existência desse tipo de associação desde o começo do período Devoniano (Taylor at al., 1995). A datação molecular, baseada na divergência da seqüência de nucleotídeos do DNA ribosomal 18S, sugere que os Glomales originaram-se entre 350 e 460 milhões de anos atrás e que a simbiose foi decisiva para a colonização do meio terrestre pelas plantas (Simon et al., 1992). Os fungos micorrízicos arbusculares (FMAs) compreendem seis gêneros dentro da ordem Glomales e existem cerca de 150 espécies classificadas (Morton e Benny, 1990).

O ciclo vital dos FMAs inicia-se quando os propágulos fúngicos (esporos, hifas intra e extrarradicais) começam a crescer. Durante seu limitado crescimento, de forma independente, mobilizam-se triglicéridos e glicogênio, principais reservas de C, que impulsionam o desenvolvimento do chamado tubo germinativo. O crescimento assimbiótico se mantém por uma ou duas semanas, durante as quais o tubo germinativo pode alcançar vários centímetros. Se a simbiose não se estabelece nesse tempo, o FMA detém seu crescimento. No entanto, quando o fungo crescendo de forma assimbiótica contata a raiz hospedeira desencadeia-se uma série de sinais entre os parceiros que levam a raiz a dar o "aceite", como simbionte, ao FMA (Lambais, 2000). Posteriormente, o FMA se desenvolve extensivamente entre e no interior das células da raiz, formando estruturas intrarradicais que incluem arbúsculos e vesículas. Os arbúsculos e outras estruturas fúngicas não penetram nas membranas celulares do hospedeiro, mas as invaginam. Assim, os arbúsculos aumentam de forma considerável a superfície de contato entre planta e fungo, sendo que na interface periarbuscular

acontece a transferência bidirecional de nutrientes. A colonização da raiz acompanha o desenvolvimento do micélio extrarradical, que inclui estruturas ramificadas características envolvidas na absorção de nutrientes. Finalmente, esporos externos se desenvolvem em algumas dessas estruturas ramificadas completando o ciclo vital desses fungos.

Portanto, os FMAs são biótrofos obrigatórios que formam simbiose do tipo mutalística com plantas superiores pela qual o fungo obtém o carbono da planta (Bago et al., 2000) e transfere fosfato e outros elementos minerais à planta. As plantas com raízes colonizadas por FMA beneficiam-se, na maioria dos casos, da associação tendo efeitos positivos sobre o seu desenvolvimento devido, principalmente, à maior aquisição de nutrientes. Essa é resultante do maior volume de solo explorado pelas raízes micorrizadas (M) (Smith e Read, 1997), já que as hifas extrarradicais podem se afastar vários centímetros das raízes e absorver tanto nutrientes como MPs. Não obstante, a eficiência micorrízica, em termos de aquisição de nutrientes, difere significativamente entre genótipos de FMA e da planta hospedeira (Marschner, 1995). Sabe-se que a colonização também aumenta a resistência da planta a estresses de tipo biótico, como os causados por agentes fitopatogênicos, e abiótico, como a seca, a salinidade e/ou o excesso de metais (Leyval et al., 2002).

1.2.2 Efeito dos FMAs na transferência de Cd à planta hospedeira

O efeito dos FMAs na absorção de metais pelas plantas é controvertido. Quando as concentrações de metais no solo são altas, alguns trabalhos mostram que há maior absorção deles pelas plantas M (Gildon e Tinker, 1983; Killham e Fierestone, 1983; Weisenhorn e Leyval, 1995). No entanto, outros autores observaram concentrações

menores de MPs na planta ou na parte aérea devido à colonização micorrízica (Leyval et al., 1991; Trindade et al., 1996; Andrade et al., 2004). Weinsenhorn et al. (1995) observaram que em solo contaminado com MPs, a micorrização do milho diminuiu as concentrações de Cd na parte aérea e raízes ou não teve efeito na absorção de metais, e nem mesmo no crescimento da planta, sugerindo que a reposta depende da densidade de raiz, condições de crescimento da planta e da espécie de FMA. Raízes M de *Avena sativa*, apesar de apresentarem maior absorção de Cu, Zn e Cd, tiveram a sua translocação à parte aérea reduzida em comparação com plantas NM (Loth e Höfner, 1995). A maior parte desses estudos foi realizada em vasos, onde não é possível separar o efeito do fungo e o da planta hospedeira na mobilização e absorção dos MPs. Os experimentos com vasos compartimentalizados, nos quais é possível separar raízes de hifas extrarradicais, têm se mostrado de muita utilidade (Joner e Leyval, 1997). Assim, Guo et al. (1996) atribuíram a absorção de até 37% do Cd presente na parte aérea de *Phaseolus vulgaris* à transferência micorrízica, via hifas, a partir do compartimento sem raízes. Joner e Leyval (1997) pesquisaram a contribuição do FMA *Glomus mosseae* à absorção do isótopo ^{109}Cd em *Trifolium subterraneum* e verificaram que as hifas extrarradicais do fungo podem absorver e transferir o Cd presente no solo às plantas, mas que essa transferência estaria restrita devido à imobilização fúngica do metal, bem por adsorção na parede celular do fungo ou no interior do micélio. Turnau (1998) observou um elevado conteúdo de Zn, associado a matériais ricos em fosfato, no micélio intrarradical de *Euphorbia cyparisias*.

1.2.3 FMA em solos contaminados

Gildon e Tinker (1981) isolaram ecotipos de FMAs tolerantes ao Cd. A tolerância a metais tem sido avaliada em relação à capacidade de germinação de esporos em areia com solução de Cd e em solos contaminados com MPs (Weinsenhorn et al., 1993, 1995). Assim, *G. mosseae* P2 (BEG 69) isolado de solo poluído com Cd, foi mais tolerante a esse metal do que o isolado procedente de solo não poluído. Nesse estudo, observou-se que a germinação dos esporos foi mais sensível ao Cd do que o crescimento da hifa. Isso também é observado em fungos não micorrízicos, nos quais a esporulação e a germinação de esporos são mais afetadas pelo Cd do que o desenvolvimento das hifas (Babich e Stotzky, 1977; Ross, 1982), sugerindo que as concentrações que inibem a germinação dos esporos são diferentes das que inibem o crescimento fúngico. Como os FMAs não podem ser cultivados sem a planta hospedeira, a tolerância a metais não foi avaliada em relação ao crescimento fúngico em meio axênico. Utilizando vasos compartimentalizados, Joner e Leyval (1997) mostraram que o comprimento das hifas não foi afetado pelo Cd adicionado ao solo (100 mg kg^{-1}) observando-se, inclusive, esporos em formação.

1.3 RESPOSTA DAS PLANTAS AO ESTRESSE POR CÁDMIO: MECANISMOS DE TOLERÂNCIA

As respostas das plantas ao excesso de Cd e outros metais essenciais e não-essenciais são diversas, sendo que alguns dos mecanismos ao nível celular podem estar envolvidos na desintoxicação e, portanto, na tolerância ao estresse causado pelo excesso de metais (Hall, 2002). Entende-se por tolerância das plantas aos MPs como a

capacidade de sobreviver em um ambiente que é tóxico para outras plantas, manifestando-se pela interação entre o genótipo e o seu ambiente (Macnair et al., 2000). Entretanto, o mesmo termo é utilizado com freqüência para caracterizar mudanças fisiológicas que podem ser observadas experimentalmente na resposta à sensibilidade aos MPs (Hall, 2002).

O objetivo principal desses mecanismos é, primeiramente, evitar a acumulação de concentrações tóxicas em locais sensíveis do interior celular. Extracelularmente à parede celular, os exudatos extracelulares e as micorrizas podem ter um papel relevante (Hall, 2002). Mesmo assim, micorrizas não são incluídas normalmente na lista de mecanismos de tolerância das plantas aos MPs, apesar de seus efeitos na diminuição da fitotoxicidade dos metais estarem bem documentados (Marschner, 1995). Sanità di Toppi e Gabbrielli (1999) enumeraram vários mecanismos de defesa da planta ao excesso de Cd, tais como: imobilização, exclusão, síntese de fitoquelatinas, compartimentação, síntese de metalotioneínas, síntese de proteínas de estresse e produção de etileno. A possível contribuição da MA em cada um dos mecanismos de tolerância ou de defesa contemplados a seguir é incluída quando possível, sendo a própria MA considerada como um desses mecanismos.

1.3.1 Imobilização

A imobilização é a primeira barreira contra o estresse por MPs, atuando principalmente nas raízes por meio da imobilização na parede celular e de carboidratos extracelulares (Verkleij e Schat, 1990; Wagner, 1993). Os carboidratos da parede celular da raiz podem adsorver ions metálicos, de forma limitada e, portanto, este mecanismo possui efeito limitado na tolerância ao excesso de metais (Ernst et al.,

1992). As hifas de FMA também podem adsorver quantidades significativas de Zn e Cd (Joner et al., 2000). Sabe-se que os componentes da parede celular de fungos, entre os quais encontram-se aminoácidos livres, grupos hidroxilos, carboxilos entre outros, são ótimos ligantes de metais (Zhou, 1999). Assim, os valores da capacidade de retenção de cátions das hifas de FMA variam entre 100 e 3000 μmol g^{-1}, faixa significativamente superior àquela observada em raízes, 100-700 μmol g^{-1} (Marschner et al., 1998). Portanto, os FMAs podem atuar como um filtro durante a absorção de ions (Joner et al., 2000) e, dessa forma, limitar o seu transporte à planta.

Recentemente, foi abordado um possível mecanismo que poderia aumentar a tolerância das plantas M ao excesso de MPs. Uma glicoproteína produzida extracelularmente e de forma abundante por FMAs, a glomalina, mostrou-se capaz de estabilizar elementos como o Cu e Cd, reduzindo a sua disponibilidade e toxicidade para as plantas. Dessa forma, a capacidade dos FMAs de acumular e seqüestrar elementos tóxicos pode melhorar o desenvolvimento das plantas em áreas contaminadas (Gonzalez-Chaves et al., 2004).

1.3.2 Exclusão

A exclusão de íons metálicos por meio da ação da membrana plasmática poderia representar, teoricamente, o melhor mecanismo de defesa da planta, evitando a entrada de ions potencialmente tóxicos e os danos por eles causados. Esse mecanismo envolve canais de Ca^{2+} no interior da membrana (Rivetta et al., 1997). Mesmo assim, o número de exemplos de exclusão em plantas superiores é bastante reduzido. A presença de

mecanismos específicos de absorção restringindo a entrada de íons tóxicos seria outra forma de exclusão (Hall, 2002).

1.3.3 Síntese de fitoquelatinas

Uma vez que o Cd entra no citosol, um sistema relacionado com o metabolismo do enxofre é ativado, produzindo importantes agentes complexantes chamados fitoquelatinas (FQs), os quais são decisivos na desintoxicação celular e na tolerância ao íon metálico (Inouhe, 2005). As FQs são uma família de peptídeos complexantes de metais com uma estrutura geral $[\gamma\text{-Glu-Cys}]_n$–Gly, onde n varia entre 2 e 11. As FQs formam vários complexos com o Cd, com massas moleculares entre 2500 e 3600 daltons, devido aos grupos tiólicos da cisteína, os quais quelam o Cd^{2+}, evitando a circulação de íons Cd^{+2} livres no citoplasma (Inouhe, 2005). Além do papel das FQs na desintoxicação do Cd, outras funções também estão relacionadas a esses peptídeos, tais como a homeostase de MPs, o metabolismo do enxofre e a ação antioxidante (Rauser, 1999; Dietz et al., 1999; Cobbett, 2000). Esses peptídeos são metabólitos secundários produzidos enzimaticamente no citoplasma a partir da glutationa (GSH), rapidamente induzidos na presença de Cd^{2+}, e já foram descritos em alguns fungos e em plantas superiores (Steffens, 1990). Uma vez sintetizadas, as FQs se ligam ao metal e o complexo entra no vacúolo.

Há poucos estudos que confirmem a síntese ou a indução de FQ por FMA. Um deles é o de Galli et al. (1995), os quais observaram que raízes de milho micorrizadas, cultivadas em areia com excesso de Cu, tiveram um aumento no conteúdo de Cys, γ-EC (γ-glutamilcisteina) e GSH (glutationa), compostos intermediários da síntese de FQs. O

aumento desses compostos foi relacionado à condição micorrízica. Como não foi possível separar o fungo da raiz, não foi esclarecido até que ponto o aumento no conteúdo de tióis relacionou-se às hifas fúngicas, vesículas ou arbúsculos, ou às células da planta infectada. Esse foi o único trabalho encontrado em que se prova que raízes colonizadas por FMA contêm esse tipo de peptídeos quelantes. Anteriormente, Turnau et al. (1993) sugeriram que as altas concentrações de N, S e Cd nos vacúolos de um FMA em raízes de *Pteridium aquilinum*, coletadas de área contaminada com Cd, indicariam, de forma indireta, a existência de tióis quelantes de metais nesse tipo de fungo.

1.3.4 Ácidos orgânicos e aminoácidos

Ácidos carboxílicos, como o cítrico e o málico, e aminoácidos como a histidina, são quelantes potenciais de MPs. Numerosas espécies vegetais podem liberar ácidos orgânicos nas raízes como reposta ao excesso de MPs (Mariano et al., 2005), sendo que esses ligantes orgânicos podem desenvolver um papel na desintoxicação e tolerância ao excesso de metais (Hall, 2002).

1.3.5 Compartimentação

A compartimentação do Cd no vacúolo é um mecanismo de relevância na desintoxicação e tolerância das células vegetais a esse metal. Dessa forma, o Cd se mantém fora de circulação e em uma área limitada. A acumulação do Cd no vacúolo, na forma livre Cd^{+2} ou complexado a FQ, requer um gasto energético, sendo transportado contra um gradiente de concentração através do tonoplasto por sistemas de antiporte $Cd^{+2}/2H^{+}$ ou de carregadores específicos, no caso das FQs. Devido ao baixo pH do interior vacuolar, os complexos Cd-FQ se dissociam e o Cd pode ser complexado por

ácidos orgânicos como citrato, malato ou oxalato (Krotz et al., 1989) e possivelmente também por aminoácidos (Sanità di Toppi e Gabbrielli, 1999). Posteriormente, as apo-FQs, FQs já desligadas do metal e no citoplasma, poderiam ser degradadas por hidrolases vacuolares ou ainda retornarem ao citoplasma onde continuariam com sua função de agente quelante.

1.3.6 Síntese de metalotioneínas

Em plantas, as metalotioneínas, proteínas de baixa massa molecular e ricas em cisteína, são induzidas pelo Cu^{2+} e possuem grande afinidade por esse metal (Cobbett e Goldsbrough, 2002). As metalotioneínas atuam como agentes quelantes diminuindo a circulação de ions metálicos livres pelo citoplasma e evitando danos às moleculas celulares. Já foram observadas metalotioneínas em *Vicia faba* e *Arabidopsis thaliana*, em condições de excesso de Cd, concluindo-se que as metalotioneínas ligam-se ao metal no citoplasma da célula e não no interior vacuolar como no caso das FQs (Lee et al., 2004).

1.3.7 Síntese de proteínas de estresse

Em resposta a altas temperaturas, as células vegetais mostram um aumento na expressão de proteínas do choque térmico (HSPs). Mais recentemente foi observado que esse tipo de proteínas é também expresso em outras condições de estresse como salinidade, seca ou excesso de MPs. Foi demonstrado que o DNA de células submetidas a excesso de Cd produz transcritos de mRNA específicos e que regulam a síntese de HSPs. Por meio de localização com anticorpos, foi encontrado este tipo de proteínas no núcleo, citoplasma e na membrana citoplasmática, o que sugere uma função de proteção e reparação das membranas (Neumann et al., 1994).

1.3.8 Produção de etileno

O estímulo na produção de etileno induzido pelo Cd foi observado em *Phaseolus vulgaris* (Mehlhorn, 1990). Tal produção de etileno pode estar relacionada a um mecanismo de defesa da planta, que desencadeia reações metabólicas relacionadas à desintoxicação de peróxido de hidrogênio.

1.3.9 Associação com FMA

Sabe-se que as micorrizas produzem substâncias estimuladoras do crescimento, tais como fitohormônios, melhoram a nutrição da planta, incrementando a produção de biomassa vegetal. O maior fornecimento de nutrientes à planta hospedeira pelo fungo poderia diminuir o estresse fisiológico causado pelo Cd (Meharg e Cairney, 2000). A proteção ao estresse causado por excesso de MPs e a maior capacidade para absorver nutrientes resultam em uma maior produção de biomassa, requisito fundamental para uma remediação com êxito de áreas contaminadas por MPs.

1.3.10 Sistema antioxidante

Atualmente, considera-se que as espécies reativas de oxigênio tenham um papel importante no sistema de defesa da planta contra patógenos, na formação de elementos traqueários, lignificação, processos de formação da parede celular e que atuam como sinais moleculares que regulam a expressão de certos genes. Por essas múltiplas funções, é necessário que as células controlem os níveis dessas moléculas de oxigênio, mas sem serem eliminadas completamente (Schützendubel e Polle, 2002). Para combater o estresse oxidativo, ou seja, excesso de produção de espécies reativas de oxigênio, as plantas possuem um sistema de defesa que compreende enzimas como catalases, peroxidases e dismutases do superóxido, e também metabólitos não

enzimáticos como o ascorbato, glutationa e carotenóides e outros pigmentos que neutralizam as formas reativas do oxigênio (Gratão et al., 2005a). A atividade desse grupo de enzimas é de grande importância na estratégia de defesa celular contra espécies reativas do oxigênio formadas na célula devido à presença de concentrações tóxicas de metais (Chaoui et al., 1997). Apesar de o Cd não pertencer aos metais do grupo de transição, os quais produzem diretamente, por autooxidação, espécies reativas de oxigênio (via reações de Fenton e/ou Haber-Weiss), causa danos oxidativos em plantas e modifica a atividade de várias enzimas antioxidantes dependendo da planta e das condições ambientais (Clijsters et al., 1999; Sanità di Toppi e Gabbrielli, 1999). A indução da atividade de enzimas antioxidantes como a catalase, dismutase do superóxido e redutase da glutationa pelo Cd está bem documentada em diferentes tecidos e espécies vegetais (Ferreira et al., 2002; Pereira et al., 2002; Vitoria et al., 2001). A indução da atividade da redutase da glutationa já foi relacionada a processos de desintoxicação, via o ciclo da glutationa-ascorbato, ou à síntese de glutationa reduzida para posteriormente fazer parte na síntese de FQs (Ferreira et al., 2002).

1.4 APLICAÇÃO PRÁTICA: FITORREMEDIAÇÃO DE SOLOS CONTAMINADOS COM Cd

A fitorremediação é definida como o uso de plantas para retirar poluentes do meio ambiente. Entre as opções de fitorremediação de áreas contaminadas com metais destacam-se: a fitoextração, a rizofiltração e a fitoestabilização (Gratão et al., 2005b). O objetivo da primeira é usar plantas acumuladoras de metais que produzam suficiente biomassa para remover os metais do solo. A rizofiltração utiliza as raízes das plantas

para retirar metais de efluentes e água, e a fitoestabilização consiste em promover o crescimento da planta com a finalidade de reduzir ou eliminar a disponibilidade de poluentes, minimizar as erosões causadas pela água e o vento, melhorar a qualidade do solo e reduzir a lixiviação de metais. Nesse caso, é necessário o fornecimento adequado de nutrientes utilizando fertilizantes e corretivos e/ou o emprego de espécies de plantas tolerantes a excesso de metais.

A fitoestabilização é uma solução temporária pois os metais não são eliminados e existe o risco da mobilização do metal na rizosfera e da sua transferência das plantas aos animais. Por essas razões, as plantas fitoestabilizadoras devem também imobilizar os metais na raiz e ter baixa acumulação na parte aérea. Plantas micorrizadas seriam, portanto, de grande interesse já que, além de promover o crescimento da planta e melhorar a sua nutrição mineral, podem reter metais e limitar sua translocação à parte aérea. Por esses motivos vem crescendo o interesse pelos fungos simbióticos em diferentes setores das biotecnologias agro-ambientais. De fato, atuando como biofertilizadores e fitoestimuladores permitem práticas de manejo mais naturais, com menor distúrbio do meio ambiente (Perotto e Martino, 2001; Vosatka, 2001).

Pelo fato de que absorção e tolerância a MPs dependem tanto da planta quanto dos fatores do solo, incluindo-se os microrganismos nele presentes, é necessária maiores informações sobre as interações entre a raiz e os seus simbiontes, tais como FMAs e microrganismos fixadores de N_2. A presença de associações simbióticas em plantas que colonizam solos contaminados com MPs supõe uma vantagem seletiva dessas plantas como espécies pioneiras, garantindo o sucesso de revegetação de tais habitats. O uso de fungos micorrízicos como agentes de biorremediação foi sugerido por Donnelly e

Fletcher (1994). No entanto, para a utilização de FMA em fitorremediação é necessário mais conhecimento sobre os mecanismos de proteção exercidos por tais fungos ao excesso de metais.

1.5 OBJETIVOS E HIPÓTESES

As respostas da simbiose micorrízica ao excesso de metais variam em função das espécies de planta e fungo envolvidas na associação, dos metais e das condições ambientais, o que pode dificultar a interpretação dos resultados. Portanto, há necessidade de maiores informações sobre a possível contribuição desses simbiontes na absorção e acúmulo de metais, assim como dos mecanismos pelos quais esses fungos podem proteger seus hospedeiros dos efeitos nocivos do excesso de metais. As principais hipóteses de trabalho foram:

- A planta beneficia-se da associação micorrízica quando é cultivada na presença de alta concentração de Cd.
- A micorrização confere tolerância ao excesso de Cd no meio de crescimento.
- Plantas micorrizadas apresentam melhores condições nutricionais na presença de excesso do metal.
- Plantas micorrizadas retêm maiores quantidades de Cd na raiz, devido à presença de hifas intra e extrarradicais, atuando como barreira e evitando a translocação do Cd à parte aérea.

O presente estudo foi composto de três experimentos independentes mas com objetivos comuns. Com o estudo, pretendeu-se entender e avaliar o efeito da micorrização em três espécies de plantas, com potencial para remediação de solos

contaminados com MPs, na presença do metal cádmio. Também foram estudados os padrões de absorção, acumulação e distribuição do Cd em plantas micorrizadas e não micorrizadas e o efeito do metal no desenvolvimento e nutrição mineral das plantas. Além disso, foi avaliada a influência do metal na colonização intra e extraradical do FMA associado às plantas. As espécies de plantas utilizadas foram: uma leguminosa, feijão de porco (*Canavalia ensiformis*); uma gramínea, milho (*Zea mays*); e uma oleaginosa, girassol (*Helianthus annuus*).

A estratégia seguida foi a avaliação das respostas em termos de crescimento da planta, conteúdo de clorofila e concentração e conteúdos totais de nutrientes e Cd na parte aérea e raiz. O estudo comparativo da atividade da peroxidase na raiz foi de utilidade na avaliação do estado de estresse da planta em condições de excesso de Cd.

2. Experimento com feijão de porco

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CADMIUM EFFECT ON THE ASSOCIATION OF JACKBEAN (*Canavalia ensiformis* L.) AND ARBUSCULAR MYCORRHIZAL FUNGI

CADMIUM EFFECT ON THE ASSOCIATION OF JACKBEAN (*Canavalia ensiformis*) AND ARBUSCULAR MYCORRHIZAL FUNGI

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ABSTRACT: The effect of cadmium (Cd) on mycorrhizal association and on shoot and root Cd concentration was investigated in jackbean plants under hydroponic conditions. The treatments consisted of the inoculation of three different species of arbuscular mycorrhizal fungi (AMF), *Glomus etunicatum, G. intraradices* and *G. macrocarpum*, and a non-inoculated control, two Cd (0 and 5 μmol L^{-1}) and two P (1 and 10 mg L^{-1}) levels in the nutrient solution. Mycorrhizal colonization, length of AMF extraradical mycelium, guaiacol peroxidase activity in roots, plant growth and root and shoot Cd and P concentrations were determined. Mycorrhizal status did not promote jackbean growth but in most of the cases mycorrhization increased root and shoot Cd concentrations. Cd ions were accumulated mainly in roots and only small amounts were translocated to the shoot. Cd addition did not affect root colonization by AMF but the AM extraradical mycelium (ERM) was sensitive to the added Cd. ERM length was reduced by 25% in the presence of Cd. This reduction was more pronounced under conditions of low P concentration. Also at this P concentration, Cd addition decreased guaiacol peroxidase activity in non-mycorrhizal roots and in roots colonized by *G. macrocarpum*. However, mycorrhizal roots maintained lower values of peroxidase activity. *G. etunicatum* showed the best performance when associated to jackbean plants and it could be a promising association for phytoremediation of Cd-contaminated soil.
Key words: peroxidase, Cd accumulation, extraradical mycelium, phytoremediation, hydroponics

EFEITO DO CADMIO NA ASSOCIAÇÃO DE FEIJÃO DE PORCO (*Canavalia ensiformis*) E FUNGOS MICORRÍZICOS ARBUSCULARES

RESUMO: O efeito do cádmio na associação micorrízica e no teor e acúmulo de Cd na raiz e parte aérea de feijão de porco foi avaliado em condição de hidroponia. Os tratamentos consistiram da inoculação ou não de três espécies de fungos micorrízicos arbusculares (FMAs), *Glomus etunicatum*, *G. intraradices* e *G. macrocarpum*, e uma testemunha (ausência de FMA), duas concentrações de Cd (0 e 5 μmol L^{-1}) e de P (1 e 10 mg L^{-1}) na solução nutritiva. Foram determinados a colonização micorrízica, o comprimento do micélio extraradical, atividade da peroxidase nas raízes, crescimento das plantas e teor e acúmulo de Cd e P na raiz e na parte aérea das plantas. A associação micorrízica não promoveu crescimento das plantas mas aumentou a concentração foliar e radicular de Cd. O metal pesado foi acumulado principalmente nas raízes e somente uma pequena quantidade foi translocada para a parte aérea. A colonização micorrízica não foi influenciada pelo Cd adicionado, mas o micélio extrarradicular mostrou-se sensível ao metal, tendo sido reduzido em 25%, principalmente na menor concentração de P adicionado. Nesta mesma concentração de P, a adição de Cd reduziu a atividade da peroxidase nas raízes das plantas não colonizadas e nas colonizadas por *G. macrocarpum.* Entretanto, as raízes micorrizadas mostraram valores menores de atividade da enzima. Melhor desempenho da associação micorrízica foi constatado nas plantas colonizadas por *G. etunicatum,* o qual mostrou-se promissor na fitorremediação de solos contaminados por Cd quando em associação ao feijão de porco.
Palavras-chave: peroxidase, acúmulo de Cádmio, micélio extrarradicular, fitorremediação, hidroponia

INTRODUCTION

Cadmium accumulation in biotic systems due to anthropogenic activities is becoming a growing environmental problem. Atmospheric deposition of industrial emissions, sludge, phosphate fertilizers and mining are some of the sources of Cd in soils (Jackson & Alloway, 1992). The main symptoms of Cd toxicity in plants are leaf chlorosis, leaf and root necrosis and general decrease in growth (Hernandez & Cooke, 1997).

Little is known about the interactions between Cd and arbuscular mycorrhiza. Some reports showed that

arbuscular mycorrhizal fungi (AMF) decreased or did not affect foliar Cd concentrations in soils with high amounts of this metal (Gildon & Tinker, 1983; Heggo et al., 1990; Tonin et al., 2001). However, AMF attenuated the negative effects of Cd in *Pisum sativum*, in spite of higher, or similar, concentrations of Cd in mycorrhizal than in non-mycorrhizal plants (Rivera-Becerrril et al., 2002). Some authors suggest that negatively charged surfaces on AMF mycelium adsorbed Cd decreasing its transfer to root cells (Joner et al., 2000), or that AMF hyphae do in fact transfer Cd from the soil to the roots but with restricted translocation to the shoot (Joner & Leyval, 1997). Thus, the interaction between plants, AMF and Cd involves a variety of arranged factors, which influence plant and fungal development and Cd availability. The enhanced nutrient supply, mainly phosphorus, to the host plant by the AMF may attenuate the effect of physiological stress caused by Cd (Meharg & Cairney, 2000). Some scarce data suggest that the antioxidative system is affected by mycorrhizal fungi in conditions of high metal concentrations (Schutzendubel & Polle, 2002).

The aim of this study was to investigate the effects of Cd on the association of jackbean and different species of AMF, the participation of AMF in Cd uptake and the relation between the activity of root guaiacol peroxidase and mycorrhization.

MATERIAL AND METHODS

Experimental design

A pot experiment was carried out under greenhouse conditions in Campinas, SP, Brazil, from April to May 2002, using a hydroponic sand culture. Greenhouse conditions were 28/16°C day/night temperature, about 1200 µmol m^{-2} s^{-1} light intensity and 12h photoperiod. The experiment consisted of a $2 \times 2 \times 4$ factorial scheme and completely randomised design, with five replications. Treatments were two Cd concentrations, 0 and 5 µmol L^{-1}, two P concentrations, 1 and 10 mg L^{-1} in the nutrient solution and the inoculation or not of three AMFs.

AMF inoculum

The mycorrhizal fungi were *Glomus etunicatum* (Becker & Gerdemann) (IAC-42), *G. intraradices* (Schenck & Smith) (IAC-43) and *G. macrocarpum* (Tul. & Tul.) (IAC-50). Spores used as inoculum arose from a non-contaminated soil. AMF inoculum was obtained from stock -cultures with *Brachiaria brizantha* Stapf and belong to the collection maintained by IAC, in Campinas, Brazil. The inoculum consisted of sand-soil mixture containing spores, mycelium and colonized root fragments, with approximately 1,500 spores cm^{-3} of soil-inoculum. Non-mycorrhizal treatments received washings (20 mL) of the soil-inoculum mixture filtered through Whatman n°42 filter paper.

Pot culture experiment

Two litters of sterilised quartz sand (0.045 to 1 mm of size) were used to fill in 2.79 L plastic pots. Jackbean (*Canavalia ensiformis* L. D.C) seeds were disinfected with 1:3 (v/v) of 2.5% sodium hypochlorite solution for 10 minutes and then rinsed several times with sterilized water. Three seeds were sown per pot and after emergence thinned to one plant per pot. Jackbeans were cultivated in hydroponics system with sand, and irrigated with complete nutrient solution (N-NO_3 154.6; N-NH_4 19.5; S-SO_4 18.7; Ca 151.2; K 70.9; Mg 18.8; B 0.53; Fe 1.99; Mn 0.97; Cu 0.076; Zn 0.3; Mo 0.15 mg L^{-1}) (Furlani & Furlani, 1988), with P and Cd concentrations adjusted for each treatment. Cd was supplied as $Cd(NO_3)_2$. Cd-nutrient solutions had a pH of 5.58. Speciation calculations using Visual MINTEQ ver. 2.23 (Gustafsson, 2003) indicated that 92 and 90% of the Cd in solution was free Cd^{2+} ion, for solutions with 1 and 10 mg L^{-1} of phosphorus, respectively. The total volume added to each pot during the experiment was of 5 L corresponding to 0.554 mg of Cd added cumulatively. The plants were harvested after 45 days at the flowering stage.

Analytical methods

Shoots and roots were separated at harvest. The shoots were washed in distilled water, dried at 60°C, weighed and the leaves ground. Root subsamples were: 1) washed, dried and ground, 2) stored in ethanol 50% in order to evaluate mycorrhizal colonization and 3) stored in liquid nitrogen until enzyme analysis. Plant Cd and P concentrations were determined after dry-digesting with HNO_3 and $HClO_4$ by ICP-AES (Inductively-Coupled Plasma Atomic Emission Spectrometry). Mycorrhizal colonization was evaluated by the grid-line intersected method (Giovannetti & Mosse, 1980) by first clearing the roots with 25 g L^{-1} KOH, followed by root acidification in 1% HCl and staining with 0.05% trypan blue. The length of the extraradical mycelium (ERM) of AMF in the substrate was estimated according to Melloni & Cardoso (1999). The ERM was extracted by wet-sieving from 10 g of substrate, which was mixed in a blender with 1.5 L of tap water. A subsample of 11 mL was vacuum-filtered onto nitro-cellulose membrane (0.40 µm pore size). The extracted ERM was stained by 0.05% trypan blue in lactoglycerol and the total length of ERM assessed under compound microscope (125x magnification). Sixty four fields were counted and the results expressed as cm of mycelium in 1g of dry soil.

Guaiacol peroxidase (EC 1.11.1.7) (GPX) was assayed spectrophotometrically using a diode array spectophotometer (8452.A, Hewlett Packard, USA), according to Boscolo et al. (2003), based on the method of Calmak & Horst (1991). Liquid nitrogen frozen roots were washed three times in deionized water and about 0.05 g were homogenized in a phosphate buffer (KH_2PO_4/

K_2HPO_4, pH 6.8). The homogenates were centrifuged at 10,000 x *g* for 8 minutes and in the supernatant was immediately determined the peroxidase activity. The reaction mixture contained 500 mmol L^{-1} phosphate buffer, 8 mmol L^{-1} guaiacol, 8 mmol L^{-1} H_2O_2 and protein extract. The increase in absorbance due to tetraguaicol formation was recorded at 470 nm (ε = extinction coefficient of 26.6 mmol L^{-1} cm^{-1}). Proteins in each extract were assayed according to Bradford (1976). Determinations of enzyme activity were performed with three parallels in three replicates per treatment.

All data were processed by analysis of variance and Tukey test at 5% for mean comparisons. Data expressed as percentage were arcsin-square root transformed prior to statistical analysis.

RESULTS AND DISCUSSION

Cadmium addition to the nutrient solution did not affect jackbean growth and no chlorosis symptoms were observed in plants. As expected, high P concentration in the nutrient solution (10 mg L^{-1}) promoted plant growth (Figure 1). AMF did not influence plant growth (Figure 1), because in sand culture with salts as nutrient supply, the mycorrhizal growth effect, which is usually related to an enhanced nutrient absorption, is less relevant than in the soil (Weissenhorn & Leyval, 1995). The similar growth between mycorrhizal an non-mycorrhizal plants allowed to confront plants with comparable biomass production, avoiding dilution or concentration effects on nutrients and Cd contents (Jarrell & Beverly, 1981).

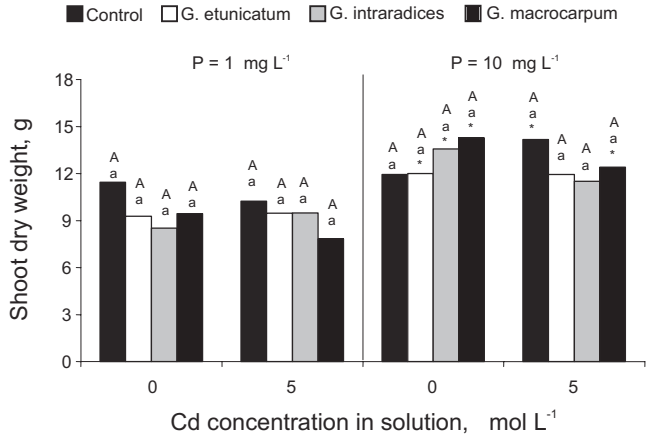

Figure 1 - Shoot dry weight of *Canavalia ensiformis* in association or not with the arbuscular mycorhizal fungi *Glomus etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A, B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

Although the amount of Cd added cumulatively during the experiment (0.554 mg pot^{-1}) was not very high, it can be considered of environmental relevance. The added Cd concentration may simulate a chronic Cd stress condition (Sanita di Toppi & Gabbrielli, 1999) since soil solutions with moderate level of Cd pollution have concentrations varying from 0.32 to 1 µmol Cd L^{-1} (Wagner, 1993). Most of the publications regarding Cd toxicity in plants are related to acute stress with non-realistic concentrations of Cd (Sanita di Toppi & Gabbrielli, 1999).

Cd was taken up by jackbean roots and translocated to leaves, however its concentration was more than 20 times higher in roots than in leaves (Figure 2). On the average, 96% of the absorbed Cd was retained in the roots, which was already observed for bean, pea and maize plants (Weigel & Jäger, 1980; Rivera-Becerril et al., 2002; Rauser, 2000). Mycorrhizal roots accumulated higher amounts of Cd (16% more) than non-mycorrhizal

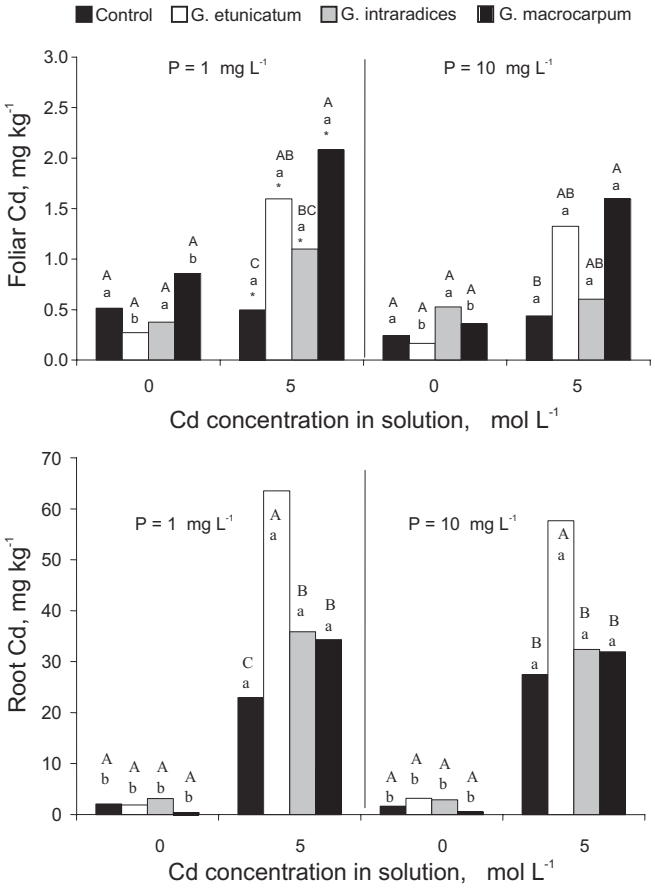

Figure 2 - Foliar and root Cd concentrations of *Canavalia ensiformis* in association or not with the arbuscular mycorhizal fungi *G. etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A, B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

roots. The association of *C. ensiformis* and *G. etunicatum* lead to higher Cd accumulation in both, roots and leaves, and in addition, only this AMF promoted a higher foliar P concentration (Figure 3). Therefore, the association *C. ensiformis-G. etunicatum* might be a promising symbiosis for Cd polluted soils.

Root AMF colonization did not change by Cd addition, but P concentration in the nutrient solution had an inhibitory effect, being 24% lower in plants cultivated under 10 mg P L^{-1} (Figure 4). Several authors showed that high heavy metal concentrations in soils can reduce mycorrhizal colonization (Gildon & Tinker, 1983; Liao et al., 2003; Andrade et al., 2004) and some reported even an elimination of root colonization due to the presence of Cd in the nutrient solution (Weissenhorn & Leyval, 1995), in spite of these concentrations being higher than that added in the present study. The colonization rates were different among the inoculated AMF. The highest rate of root colonization was observed for *G. etunicatum*, followed by *G. intraradices* and *G. macrocarpum* (Figure 4). However, the amount of ERM was reduced by Cd and was also influenced by P concentration in the nutrient solution. In general, the ERM length was 25% lower in the presence of Cd and the reduction was more pronounced at low P concentration (Figure 4). However, the length of ERM was about 1.5 time higher at low P than at high P concentration (Figure 2). *G. intraradices* presented the highest amounts of external mycelia followed by *G. etunicatum* and *G. macrocarpum* which had low amounts of ERM (Figure 2). Root colonization and amount of ERM were highly correlated (R^2=0.908, $P < 0.001$) and both were positively correlated with root P concentration, possibly due to phosphorus transfer to the roots by AMF hyphae and P accumulation inside intraradical hyphae. Despite the importance of the ERM on mycorrhizal symbiosis functioning, studies on this subject have been usually neglected.

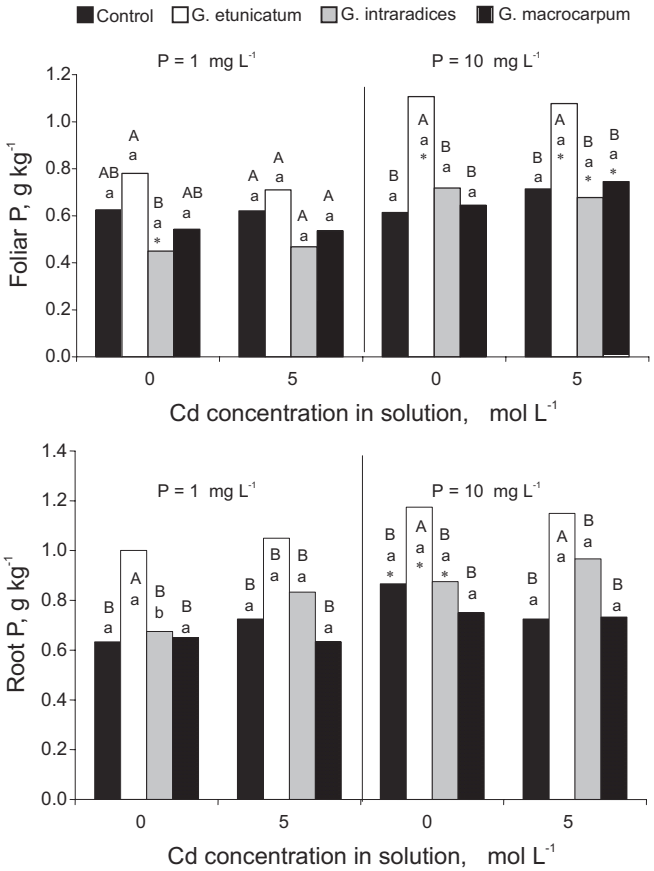

Figure 3 - Foliar and root P concentrations of *Canavalia ensiformis* in association or not with the arbuscular mycorhizal fungi *G. etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A,B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

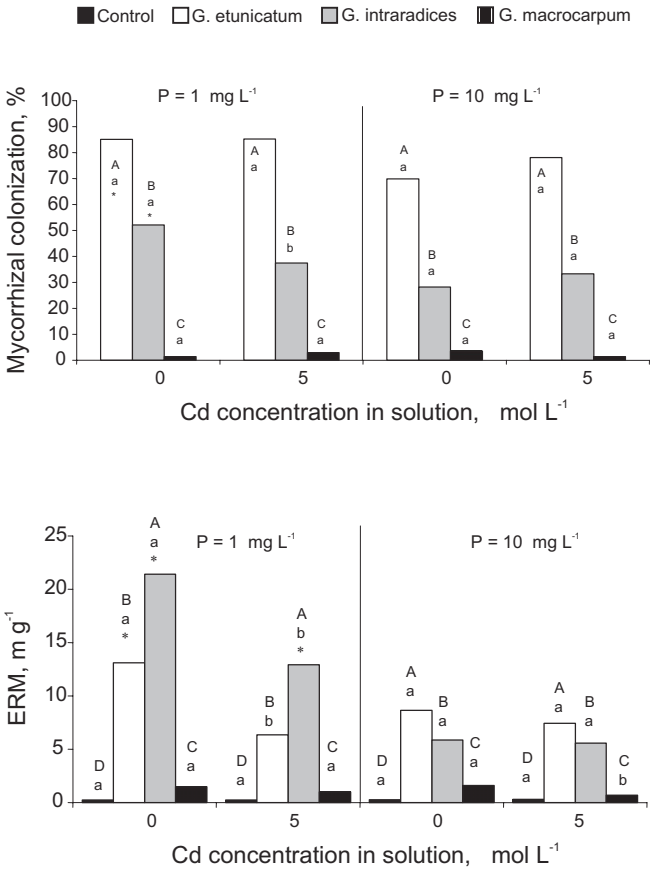

Figure 4 - Mycorrhizal colonization and length of extraradical mycelium (ERM) of *Glomus etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A,B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

Table 1 - Guaiacol peroxidase (GPX) activity in roots of *Canavalia ensiformis* grown hydroponically and associated or not to arbuscular mycorrhizal fungi (AMF) in different Cd (0 and 5 µmol L^{-1}) and P concentrations (1 and 10 mg L^{-1}) in the nutrient solution. (U = unit of enzymatic activity)

Treatment		Control	*G. etunicatum*	*G. intraradices*	*G. macrocarpum*
Cd	P				
µmol L^{-1}	mg L^{-1}	U mg protein^{-1}			
0	1	44.93 A	16.76 A	23.47 A	39.28 A
5	1	31.14 B	10.70 A	20.57 A	19.90 B
Average		38.03 a	13.68 a	22.02 a	29.59 a
0	10	28.36 A	12.38 A	16.00 A	25.13 A
5	10	27.03 A	11.76 A	15.59 A	18.89 A
Average		27.70 b	12.07 a	15.79 b	22.02 b

A,B compare between Cd rates in each of the P and AMF treatments and a, b compare between P concentrations independently of Cd concentrations by the Tukey test. Means with the same letter are not different ($P \leq 0.05$)

Extraradical hyphae of mycorrhizal fungi are able to transport Cd from the soil solution to the plant and even restrict metal transfer to the shoot due to fungal immobilization in the roots (Joner & Leyval, 1997; Joner et al., 2000).

Among the antioxidant enzymes, which play an important role in the cellular defence against oxidative stress, peroxidases can transform peroxides into non-reactive species (Chaoui et al., 1997). An induction in the activity of antioxidant enzymes is expected as a strategy to overcome oxidative stress due to excess of Cd. However, the addition of 5 µmol Cd L^{-1} reduced guaiacol peroxidase (GPX) activity only in non-mycorrhizal roots and in roots colonized by *G. macrocarpum* (Table 1), which had a very low infection rates (Figure 4), at low P condition. Since increase and decrease of antioxidant activity in response to Cd have been reported in different plant species and Cd concentrations, it is possible that, besides the detoxification function, enzyme molecules may also be sensitive targets of Cd toxicity in plant roots (Gallego et al., 1996). P concentration in the nutrient solution modified GPX activity in roots, which was 25% lower in plants at high P concentration (Table 1). This suggests that higher P concentration in the solution could be buffering the effect of added Cd.

The response of AM symbiosis under heavy metal stress conditions depends on plant and fungi species. In the present experiment, *G. etunicatum* associated to jackbean plants had the best performance and, so, this could be a promising association for phytoremediation of Cd-contaminated soils.

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3. EXPERIMENTO COM MILHO

Mycorrhiza influence on Cd accumulation and on Cd stress alleviation in maize

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Abstract

The role of the arbuscular mycorrhizal fungus (AMF) *Glomus macorcarpum* on Cd accumulation and distribution and on the possible attenuation of Cd stress in maize (*Zea mays* L. var. Exceller) plants was studied. Plants inoculated or not with *G. macrocarpum* were exposed to Cd (20 μmol L^{-1}), at two P levels (1 and 5 mg L^{-1}), in the nutrient solution. The experiment was conducted in a hydroponic system, in a randomized 2x2x2 factorial design. The mycorrhiza-Cd interaction on plant growth, nutrients and Cd accumulation in plant was evaluated. Cd addition effects on AMF root colonization and extraradical mycelium were also investigated. As expected, mycorrhiza promoted plant growth and Cd addition affected both shoots and root biomass production. No differences in plant Cd concentrations were found between mycorrhizal (M) and non-mycorrhizal (NM) plants and they accumulated Cd mainly in roots. In general, roots showed

slightly higher Cd concentration in the cell wall than in the cytoplasmic fraction, showing. M roots 26% more Cd accumulated in the cell wall fraction than NM roots. M plants showed higher P/Cd, N/Cd and S/Cd ratios than NM plants. Mycorrhizal root colonization and the length of extraradical mycelium diminished by Cd addition, being the reduction more pronounced under high P supply. In general, Cd addition induced GPX activity in roots. However, M plants, in addition to the higher root protein contents, showed no induction of GPX activity in the presence of Cd, suggesting their higher tolerance to the presence of Cd. It can be concluded that Cd addition affected mycorrhizal symbiosis decreasing root colonization and the development of the extraradical mycelium. Nevertheless higher growth and nutrients/Cd ratios observed in M plants indicated an efficient symbiosis that was able to alleviate nutritional and Cd stress.

Key words: arbuscular mycorrhizal fungi, Cd distribution, heavy metals, peroxidase, extraradical mycelium

Introduction

Cadmium is a potential toxic metal and thus, its transfer from plants to humans is of great concern. This heavy metal is ranked number seven among the top toxins, mainly due to its negative influence on cell's enzymatic systems (ATSDR, 1999), and it has been estimated that 70% of the Cd intake by humans originates from plant foods (Wagner, 1993). The critical Cd level in nutrient solution for conventional crop plants is reported to be 8 μmol L^{-1} (Yang et al., 1995). Nevertheless, Inouhe et al. (1994) showed that cereals as maize, rice and barley tolerate Cd as much as 100 μmol L^{-1}. In Cd enriched soils, plants may accumulate 20 mg kg^{-1} of Cd in shoots. In the case of maize, 23 mg kg^{-1} of Cd in shoot dry weight is considered the phytotoxicity

limit relevant for production (Klein et al., 1981). However, some studies reported the ability of maize plants to accumulate metal ions and showed its relative tolerance to excessive soil metal concentrations (Salt et al., 1998). Cd is taken up through the roots and accumulated mainly in roots, but it can be also translocated to shoots, grains or fruits (Page et al., 1981). Maize roots show a great ability to retain Cd (Nocito et al. 2002, Florijn and Van Beusichem, 1993), which is a feature largely desirable in the context of food chain, avoiding, in this way, translocation of high Cd quantities to the aerial part. In plants, Cd may cause a variety of toxic effects: from morphological and physiological disturbances to disorders at enzymatic and molecular levels (Benavides et al., 2005). It may particularly damage photosynthetic apparatus (Vassilev et al., 2002) and influence mineral nutrition causing nutritional disturbances (Siedlecka et al., 1997; Greger and Lindberg, 1987) that may interfere with other metabolic processes. Cd also induces the production of oxygen reactive species affecting important macromolecules and as result alters the activity of enzymes related to the antioxidant defense system. Among the enzymes participating, gluthathione reductase, catalases and peroxidases play and important role in the control of oxigen reactive species levels in cells (Gratão et al., 2005).

Environmental and health problems caused by excessive accumulation of metals in soils are leading to search for new technologies for soil remediation (Salt et al., 1998). Look for heavy metals tolerant plants that are able to accumulate metals in their tissues is nowadays of a great scientific concerning. The effect of arbuscular mycorrhizal fungi (AMF) on metal stress alleviation in plants on different growth conditions has been frequently reported (Li and Christie, 2001; Riveira-Becerril et al., 2002). Immobilization of metals in the AMF biomass is proposed as a mechanism by which these fungi may increase plant tolerance to heavy metals (Joner et al., 2000). It has been observed a reduced metal transfer, showing mycorrhizal plants enhanced root/shoot Cd ratios, showing that mycorrhizal roots acts as a barrier against metal transport

(Joner et al., 2000; Turnau et al., 1993). This is attributed to adsorption on hyphal walls, since chitin has an important metal-binding capacity (Joner et al., 2000). Recently has been suggested that glomalin, a glycoprotein produced by AMF, may have a metal chelating function diminishing metal availability for plants (Gonzalez-Chavez et al., 2004). Another possible mechanism includes dilution effects of metal ions in tissues due to AMF plant growth promotion (Jarrell and Beverly, 1981). AMF associated with metal-tolerant plants may contribute to the accumulation of heavy metals in plant roots in a non-toxic form inside hyphal cell walls or complexed to phosphate materials inside the cells (Galli et al., 1995). It has been reported the ability of different plant species to accumulate metal ions (Nedelkoska and Doran, 2000). Among them maize, a universal host for AMF and a high biomass producer plant, which shows relative tolerance to excess of metals in soils, features that could make this plant a good option for soil remediation (Jiang et al., 2001; Jurkiewicz et al., 2004).

The objective of this study was to investigate mycorrhiza effects on Cd accumulation and distribution in maize plants. It is also investigated the AMF contribution to Cd stress alleviation in maize using growth and nutritional parameters as well as an enzyme involved in the antioxidative defense, the guaiacol peroxidase.

Materials and methods

Experimental design

A pot experiment was conducted under greenhouse conditions in the Instituto Agronômico, SP, Brazil, using hydroponic culture. During this period, the day and night temperatures ranged between 29^{o}C and 16^{o}C respectively; with a photoperiod of 12h and about 1200 μm m^{-2} s^{-1} light intensity. The experimental design was completely randomised in a 2x2x2

factorial scheme, with seven replications. The treatments were composed of two Cd (0 and 20 μmol L^{-1}) and two P (5 and 10 mg L^{-1}) concentrations in the nutrient solution, and the inoculation or not of the AMF.

AMF inoculum

The mycorrhizal fungi used was *Glomus macrocarpum* (Tul. and Tul.) (IAC-50). Original spores of the fungus arose from a non-contaminated soil. The inoculum was obtained from stock cultures with *Brachiaria brizantha* Stapf for a six months period. The inoculum consisted of sand-soil mixture containing spores, mycelium and colonized root fragments, with approximately 1,500 spores cm^{-3} of soil-inoculum. The non-mycorrhizal treatments received washings of a mixture of the AMF inoculum filtered through Whatman n°42 filter paper.

Pot culture experiment

Maize (*Zea mays* L. var. Exceller) seeds were disinfected with 1:3 (v/v) of 2.5% sodium hypochlorite solution for 10 minutes and then rinsed several times with sterilized water. Seed plants were germinated in trays containing ground silica with and without AMF inoculum. Two litters of sterilized ground quartz silica (2-3 mm) were put in 2.79 L plastic pots. After 20 days in the trays, two plants per pot were transplanted, and a week later, one plant was left in each pot. Maize plants were cultivated in hydroponic system with silica and irrigated every second day with complete nutrient solution (N-NO_3 154.6; N-NH_4 19.5; S-SO_4 18.7; Ca 151.2; K 70.9; Mg 18.8; B 0.53; Fe 1.99; Mn 0.97; Cu 0.076; Zn 0.3; Mo 0.15 mg L^{-1}) (Furlani and Furlani, 1988), with P and Cd concentrations adjusted for each treatment. Cd was supplied as $Cd(NO_3)_2$ and P as KH_2PO_4. Speciation calculations using Visual MINTEQ ver. 2.23 (Gustafsson, 2003) indicated that 82 and 75% of the Cd in solution was free Cd^{2+} ion, for solutions with 5 and 10 mg L^{-1} of phosphorus, respectively. The total volume of nutrient solution added to each pot during the

experiment was of 2.850 L corresponding to 6.406 mg of Cd added cumulatively per pot, resulting in a concentration which is considered of environmental relevance and in the range found in Cd-polluted soils (Sanità di Toppi and Gabbrielli, 1999). Plants were harvested after 70 days at the flowering stage.

Analytical methods

Shoots and roots were separated at harvest. Shoots were washed in distilled water, dried (60^{o}C for 76 h), weighted and ground for chemical analysis. Some of the root was kept for mycorrhizal colonization determination, some was stored in liquid nitrogen until use in enzymatic assay and some dried and ground. Cd concentrations in plant tissues were determined by inductively coupled plasma - optical emission spectroscopy (ICP-OES). Concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, and Cd in plant shoots and roots were determined after dry-digestion with HNO_3 and $HClO_4$ by ICP-OES and total-N in the sulphuric-digest determined by Kjeldahl analysis.

Acid phosphatase (APase) activity in leaf was determined "*in vivo*" according to Besford (1980). 0.1g of fresh tissue was taken from youngest fully expanded leaves at 60 days after transplanting. Leaves sampled were cut in 3 mm stripes and incubated in p-nitrophenylphosphate, as enzyme substrate, in 0.1 mol L^{-1} sodium acetate buffer (pH 4.0), for 20 minutes at 30^{o}C. Nitrofenol formed was measured spectrofophometricaly in the extracts at 410 nm length wave. APase activity was expressed in μg p-nitrofenolphosphate g^{-1} fresh tissue h^{-1}.

Mycorrhizal root colonization was evaluated by the grid-line intersected method (Giovannetti and Mosse, 1983) by first clearing the roots with 25 mg L^{-1} KOH, followed by root acidification in 1% HCl and staining with 0.05% trypan blue. The length of the extraradical mycelium (ERM) of AMF in the substrate was estimated according to Boddington et al. 1999 and

Melloni and Cardoso, 1999. The ERM was extracted from 20 g of substrate by flotation in 1000 mL of tap water, blended, sieved (45 μm) and filtered onto a nitrocelullose membrane filter (0.45 μm). The extracted ERM was stained by 0.05% Trypan blue in lactoglycerol and the total length of ERM assessed under compound microscope using an ocular at 125x magnification. Sixty-four fields were counted and the results expressed as m of mycelium in 1g of dry substrate.

Fractionation of root into cytoplasm and cell wall for determination of Cd content in each fraction was performed according to Inouhe et al. (1994) with modifications. Liquid nitrogen frozen roots (0.5-1.0 g fresh weight) were homogenized by mortar and pestle with 20 mmol L^{-1} Tris-HCl buffer (pH 7.8). The homogenates were centrifuged twice, at 1,500 x g for 10 min and at 10,000 x g for further 10 min. The supernatants were collected and used directly as cytoplasmic fractions. The precipitates in the tubes were collected, dried at 37-40 ºC and considered as cell wall fraction. Cd concentration in the cytoplasmic fraction was determined directly by flame atomic absorption spectrophotometry (F-AAS). Dry up cell wall fractions were hydrolized with 2 mL of HNO_3 for 20 min at 100ºC. In the hydrolizates four drops of $HClO_4$ were added and again hydrolized for 15 min at 100ºC. After this process, three drops of 30% H_2O_2 were added and maintained at 100ºC for 15 min and finally diluted to 5 mL with deionized water. Cd content was determined by F-AAS in this extract.

Guaiacol peroxidase (EC 1.11.1.7) (GPX) activity was assayed spectrophotometrically using a diode array spectophotometer according to Boscolo et al. (2003). Liquid nitrogen frozen roots (0.1-0.05 g) were washed three times in deionized water and homogenized in phosphate buffer (KH_2PO_4/K_2HPO_4, pH 6.8). The homogenates were centrifuged at 10,000x g for 8 minutes and peroxidase activity immediately determined in the supernatant. The reaction mixture contained 500 μmol L^{-1} phosphate buffer, 8 mmol L^{-1} guaiacol, 8 mmol L^{-1} H_2O_2 and protein

extract. The increase in absorbance was recorded at 470 nm (extinction coefficient, ε= 26.6 mmol L^{-1} cm^{-1}). Total soluble proteins in each extract were determined using the Bio-Rad protein assay.

All variables were processed by analysis of one-way ANOVA and Tukey test at 5% for means comparison; correlation was performed by simple regression analysis. Data expressed as percentage were arcsin-square root transformed prior to statistical analysis. Cd translocation index (TI) was calculate as the percentage of the total Cd absorved that was translocated to the shoots and Cd transfer factor ($TF_{SOL\text{-}SHOOTS}$) calculated as the ratio of Cd concentration in plant shoots to that in the solution.

Results

Cd addition decreased both shoots and root biomass production in relation to control plants (Figure 1). By contrast, AMF inoculation and the higher P supply to the nutrient solution favoured shoot and root growth (Table 1). Mycorrhizal (M) plants showed 6.5% and 22% higher shoot dry mass and root fresh mass than non-mycorrhizal (NM) plants, respectively. Plants under high P solution had 1.6 and 1.3 times more shoots dry mass and root fresh mass, respectively, than plants under low P solution (Table 1). Root colonization and AMF extraradical mycelium (ERM) length decreased in treatments with Cd addition (Table 1). P supply affected maize roots colonization and the ERM development (Table 1).

Table 1. Shoot dry weight (SDW), root fresh weight (RFW), mycorrhizal colonization (Colon.) and AMF extraradical mycelium lenght (ERM) in maize plants inoculated (Myc) or not (Non-myc) with *G. macrocarpum* and treated with 0 and 20 μM L^{-1} of Cd in two P concentrations in the nutrient solution.

Treatments		**SDW** g		**RFW** g		**Colon** %	**ERM**
Cd μmol L^{-1}	mg L^{-1}	Non-myc	Myc	Non-myc	Myc	Myc	Myc
0	**5**	7.3 aA	7.7 aA	25.8 aA	27.8 aA	44.5 A	5.11 A *
0	**10**	12.3 bA *	13.4 aA *	31.6 bA *	35.2 aA *	42.8 A	2.27 A
20	**5**	6.2 aB	6.4 aB	18.5 bB	22.3 aB	26.6 B *	2.23 B
20	**10**	9.3 bB *	10.3 aB *	27.3 aB *	30.5 aB *	13.1 B	1.89 A

Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. a, b compare between mycorrhizal and non-mycorrhizal plants in each Cd and P concentrations. A, B compare between Cd treatments in each P concentration and mycorrhyzal condition.

Table 2. Cd concentration in shoots and roots, total Cd absorbed, Cd translocation index (TI) and transfer factor ($TF_{sol\text{-}shoot}$) in maize plants inoculated (Myc) or not (Non-myc) with *G. macrocarpum*, and treated with 0 and 20 μM L^{-1} of Cd in two P concentrations in the nutrient solution.

Treatments		**Cd shoots** mg kg^{-1}		**Cd roots** mg kg^{-1}		**Total Cd** mg kg^{-1}		**TI**		**$TF_{SOL\text{-}SHOOT}$**	
Cd μm L^{-1}	P mg L^{-1}	Non-myc	Myc	Non-myc	Myc	Non-myc	Myc	Non-myc	Myc	Non-myc	Myc
0	**5**	0.14 a	0.14 a	3.3 a	3.02 a	3.4 a	3.1 a	--	--	--	--
0	**10**	0.24 a	0.24 a	1.78 a	1.76 a	2.0 a	2.0 a	--	--	--	--
20	**5**	29.5 a	30.5 a	369 a	355 a	399 a	385 a	7.1	7.2	11.3	10.5
20	**10**	20.1 a	16.9 a	275 a	253 a	295 a	269 b				

Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. a, b compare between Myc and Non-myc plants in each Cd and P concentrations. (TI = 100 x Cd concentration in shoots / Cd concentration in shoots + Cd roots; $TF_{SOL\text{-}SHOOT}$ = Cd concentration in plant shoots / Cd concentration in the solution).

No significant differences in shoots and roots Cd concentrations between M and NM plants were found (Table 2). Cell wall fraction accumulated, in average, 56% of Cd, thus 44% was in the cytoplasmic fraction (Figure 1). In general, M roots accumulated 26% more Cd in the cell wall fraction than NM roots, reaching 32% more at low P conditions (Figure 1). P concentration in the solution did not influence Cd distribution in root cells. Cd retained in each of the root fractions showed high correlation with total Cd concentration in shoots and roots and negative correlation with biomass production (Table 5).

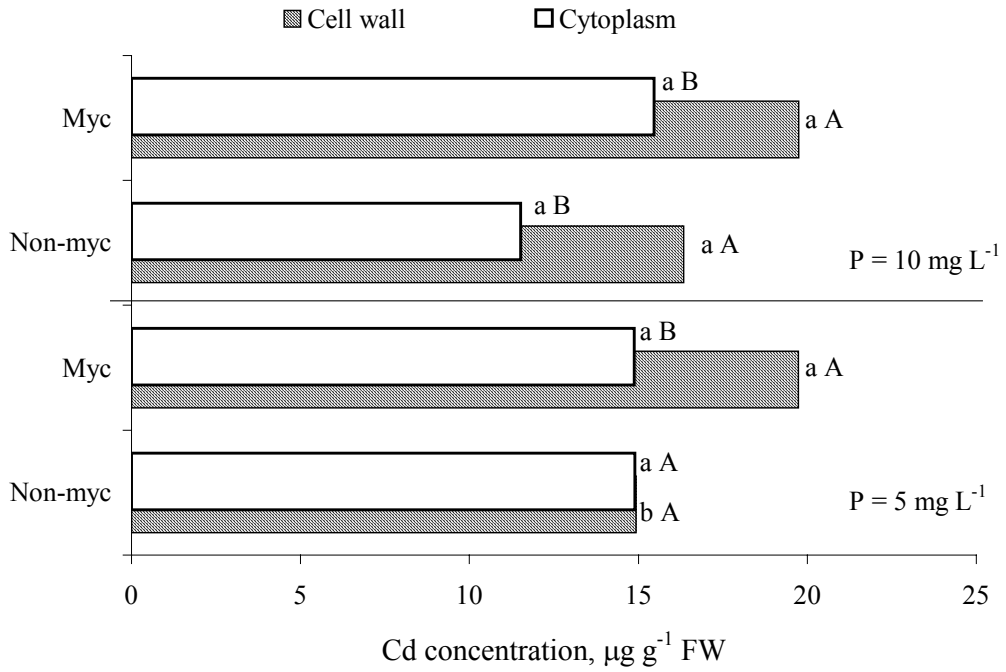

Figure 1. Cd distribution in cytoplasm and cell wall fractions of mycorrhizal (Myc) and non-mycorrhizal (Non-myc) maize roots treated with 20 μmol L^{-1} of Cd, in two P concentrations in the nutrient solution. Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. a, b compare between Myc and Non-myc treatments in each cell fraction and P concentration. A, B compare Cd concentration between cell fractions in each mycorrhizal status and in each P concentration. (Data are mean of triplicated samples).

Table 3. Effect of Cd and P levels in the nutrient solution on shoot and root mineral concentrations and shoot mineral contents in mycorrhizal (Myc) and non.mycorrhizal (Non-myc) maize plants.

Cd	P	AMF	N	P	K	S	Ca	Mg	Cu	Fe	Mn	Zn
µmol L^{-1}	mg L^{-1}		g kg^{-1}						mg kg^{-1}			
	Shoot											
0	5	Non-myc	16.92	0.62	18.63	2.76	5.34	2.50	6.70	107	48.2	21.1
0	5	Myc	16.24	0.58	15.40	3.68	6.0	3.12	4.46	130	39.8	21.6
0	10	Non-myc	15.38	0.7	12.60	4.06	4.24	2.88	4.02	119	36.0	18.7
0	10	Myc	14.92	0.7	12.80	4.66	4.86	3.06	3.18	120	31.4	16.2
20	5	Non-myc	13.88	0.66	17.65	5.62	5.08	2.32	3.16	129	44.2	21.7
20	5	Myc	14.47	0.66	16.18	6.78	5.04	2.64	3.10	132	28.4	25.8
20	10	Non-myc	14.28	0.74	14.20	6.70	4.56	2.68	3.42	103	40.4	20.4
20	10	Myc	14.46	0.78	13.60	6.50	4.62	3.04	3.50	129	25.2	19.2
Significance												
Cd			0.0003	0.033	N.S	0.00001	N.S	N.S	0.001	N.S	0.02	0.07
P			0.09	0.001	0.0001	0.00001	0.01	0.04	0.03	N.S	001	0.004
AMF			N.S	N.S	0.09	0.00009	N.S	0.009	0.052	N.S	0.00002	N.S
Cd x AMF			N.S	N.S	N.S	N.S	N.S	N.S	0.03	N.S	0.02	N.S
Cd x P			0.033	N.S	N.S	0.005	N.S	N.S	0.003	N.S	0.07	N.S
P x AMF			N.S	N.S	N.S	0.002	N.S	N.S	N.S	N.S	N.S	N.S
Cd x AMF x P			N.S	N.S	N.S	0.04	N.S	N.S	N.S	N.S	N.S	N.S
	Root											
0	5	Non-myc	17.4	0.46	8.32	2.60	7.08	2.27	15.7	481	90.4	35.8
0	5	Myc	18.9	0.66	7.10	3.18	10.22	1.83	18.2	832	54.2	31.8
0	10	Non-myc	15.5	0.56	5.50	2.40	8.23	2.42	10.0	572	53.2	21.1
0	10	Myc	16.4	0.62	3.64	2.64	7.69	3.20	10.9	628	37.2	19.3
20	5	Non-myc	13.4	0.50	8.90	3.22	7.58	1.76	12.0	777	166	14.7
20	5	Myc	15.4	0.56	8.72	3.44	9.18	1.66	14.5	710	54.4	18.5
20	10	Non-myc	14.4	0.58	6.30	2.92	11.23	2.84	11.8	619	93.2	15.5
20	10	Myc	16.0	0.58	4.50	3.04	7.94	2.84	13.1	595	25.6	15.8
Significance												
Cd			0,0007	N.S	0.004	0.1	N.S	0.005	N.S	N.S	0.006	0.00001
AMF			N.S	N.S	0.00001	0.04	N.S	0.00001	0.002	0.006	0.0002	0.0003
P			0.01	0.03	0.0006	0.09*	N.S	N.S	N.S	0.001	0.00001	N.S
Cd x AMF			N.S	N.S	N.S	N.S	N.S	0.004	N.S	0.02	0.001	N.S
Cd x P			0,01	N.S	N.S	N.S	N.S	0.009	0.01	N.S	N.S	0.001
P x AMF			N.S	N.S	0.08*	N.S	0.002	0.001	N.S	0.01	0.07*	N.S
Cd x AMF x P			N.S	N.S	N.S	N.S	N.S	N.S	N.S	0.06*	N.S	N.S

Continue

Continue

Cd	P	AMF	N	P	K	S	Ca	Mg	Cd	Cu	Fe	Mn	Zn
$\mu M\ L^{-1}$	$mg\ L^{-1}$					(mg $plant^{-1}$)					($\mu g\ plant^{-1}$)		
Shoot content													
0	5	Non-myc	120.5	4.44	131	19.6	37.8	17.8	0.99	38.4	768	316	150
0	5	Myc	128.5	4.78	124	29.1	47.4	24.5	1.12	35.3	1031	383	171
0	10	Non-myc	188.5	8.26	153	49.9	59.3	35.5	3.04	46.6	1475	443	228
0	10	Myc	199.1	9.34	171	62.2	64.6	40.9	3.17	42.5	1600	418	236
20	5	Non-myc	85.75	3.92	109	34.8	31.3	14.3	197	18.9	717	274	134
20	5	Myc	88.94	4.16	110	41.8	31.1	16.3	183	19.1	812	212	158
20	10	Non-myc	137.1	6.60	135	60.1	43.6	25.5	193	33.1	978	393	192
20	10	Myc	153.2	8.28	143	68.8	48.8	32.1	178	37.0	1377	267	203
Significance													
Cd			0,00001	0,0009	0.001	0.00001	0.00001	0.00001	0.00001	0.00001	0.004	0.0004	0.009
P			0,00001	0,00001	0.00004	0.00001	0.00001	0.00001	N.S	0.00001	0.00001	0.002	0.00001
AMF			0,01	0,002	N.S	0.00001	0.017	0.0003	N.S	N.S	0.01	0.007	0.08
Cd x AMF			N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
Cd x P			N.S	N.S	N.S	0.03	N.S	N.S	N.S	0.02	0.07	N.S	N.S
P x AMF			N.S	0,03	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
Cd x AMF x P			N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	0.08	N.S	N.S

Cd addition reduced shoots N, P, K, Ca, Mg, Cu, Fe, Mn and Fe contents and increased shoot and root S concentrations (Table 3). M plants showed higher contents of N, P, Ca, Mg and S in shoots than NM plants, but they had a lower K concentration in shoots (Table 3). Nutrient concentrations in roots did not differ significantly due to mycorrhizal condition (Table 3). M plants showed higher P/Cd, N/Cd and S/Cd ratios, in shoots and roots, than the NM counterparts (Table 4).

Table 4. Phosphorus, sulphur and nitrogen to cadmium ratios in shoots and roots of Cd-treated mycorrhizal (Myc) and non-mycorrhizal (Non-myc) maize plants in two P concentrations in the nutrient solution. (a, b compare between Myc and non-myc plants in each P concentration; x, y compare between P treatments in each mycorrhizal status).

Treatment		Nutrient/Cd ratio in shoots			Nutrient/Cd ratio in roots		
AMF	P (mg L^{-1})	P/Cd	S/Cd	N/Cd	P/Cd	S/Cd	N/Cd
Non-Myc	5	21.6 a y	193 b y	478 a y	1.3 b y	8.63 a x	34 b y
Myc	5	23.8 a y	246 a y	510 a y	1.56 a y	9.65 a y	44 a y
Non-Myc	10	33.8 b x	312 b x	718 b x	2.06 b x	10.32 b x	53 b x
Myc	10	46.9 a x	391 a x	870 a x	2.30 a x	12.34 a x	73 a x

The APase (EC3.1.3.2) activity in leaves was influenced by mycorrhizal condition. In this case, NM plants showed significantly higher enzyme activity than in M plants (Figure 2). Moreover, plants under low P solution (5 mg L^{-1}) showed higher APase activity than plants under high P solution. Cd addition increased the foliar APase activity only in plants under low P solution (Figure 2).

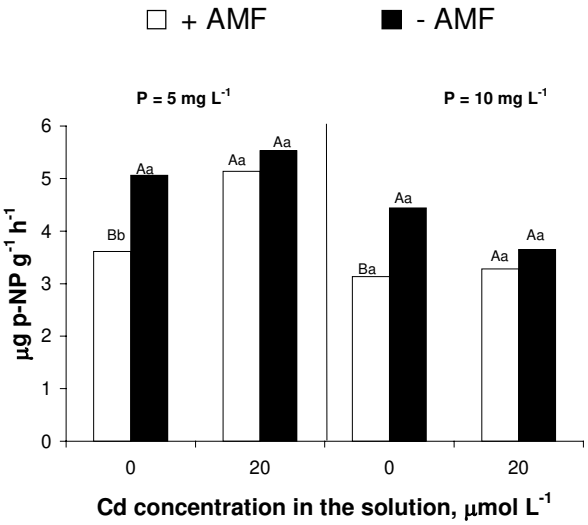

Figure 2. Acid phosphatase activity in leaves of maize plants, inoculated (My) or not (Non-myc) with *G. macrocarpum*, and in two Cd and P phosphorus concentrations in the nutrient solution. Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. A, B compare between Myc and Non-myc plants in each Cd and P concentration; a, b compare between Cd treatments in each of the P concentrations and mycorrhizal status.

In general, Cd addition increased by 13.5% GPX activity in roots in relation to non-Cd-treated plants (Figure 3). However, the increase in GPX activity was statistically significant only in NM roots and at low P concentration, increasing 53% in relation to M plants due to Cd addition (Figure 3). In the presence of Cd and at high P concentration, M and NM plants showed different GPX activity pattern, being 30% higher in M plants. GPX activity was positively correlated with Cd concentration in roots and shoots and also with root Cd concentration in the cytoplasm and cell wall fractions (Table 5).

The amount of total soluble proteins in M roots decreased 24% due to Cd addition under high P solution (Figure 3). In general, M roots showed 30% higher protein concentration than NM plants. P concentration in the solution also influenced root protein concentrations, occurring a general reduction of 14% in plants under high P solution (Figure 3). Protein concentration was negatively correlated with root Cd concentration and positively with root P concentration, AMF colonization and with mycorrhizal ERM amount (Table 5).

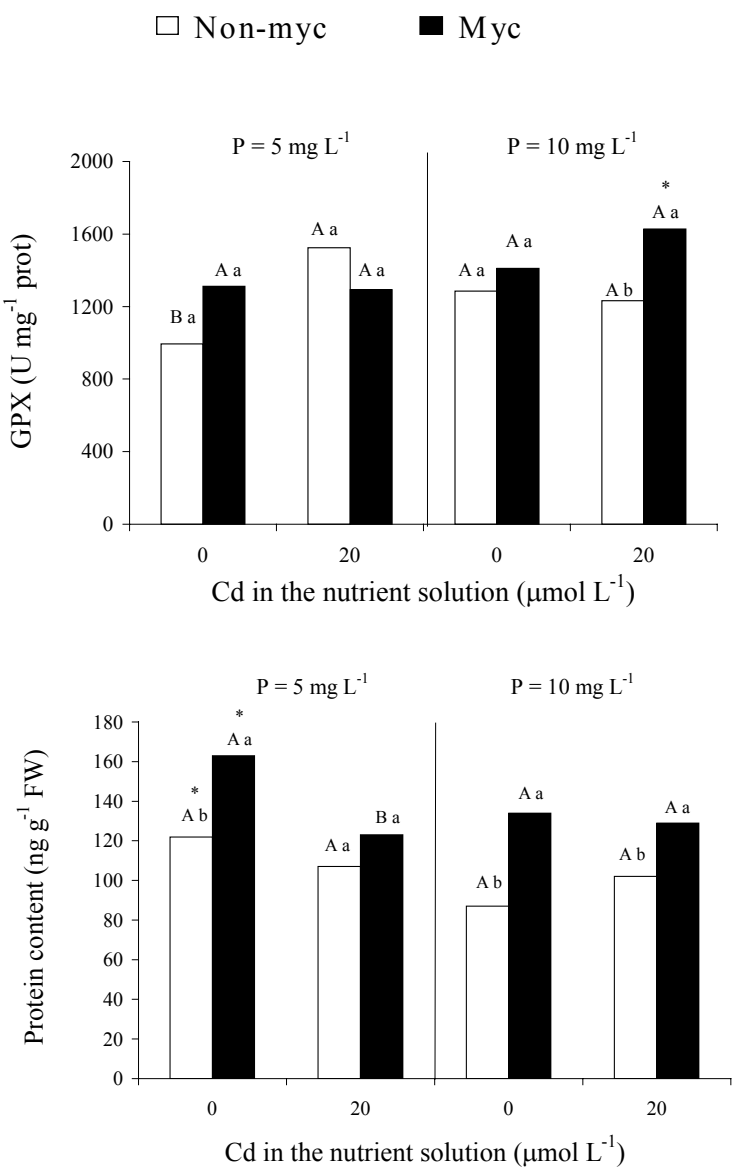

Figure 3. Guaiacol peroxidase (GPX) activity and total soluble protein contents in mycorrhizal (Myc) and non-mycorrhizal (Non-myc) maize roots in two Cd and P levels in the nutrient solution. Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. a, b compare Myc and Non-myc in each Cd and P concentrations; A, B compare Cd treatments in each P level and mycorrhizal status; * Significant difference between P levels in each Cd and mycorrhizal status.

Table 5. Correlation coefficients and significant levels between shoot dry weight (SDW), AMF root colonization (%Col.), AMF extraradical mycelium (ERM), guaiacol peroxidase activity (GPX), total soluble proteins in roots (Protein), Cd concentration in roots, shoot and in roots cytoplasm and cell wall fractions, P concentration and contents in shoots and P concentration in roots. (ns- non-significant)

	% Col.	ERM	GPX	Protein	Cd roots	Cd shoots	Cd Cytopl.	Cd cell wall	P shoots	P content	P root
SDW	ns	ns	ns	ns	-0.477 **	-0.525***	-0.402**	-0.349*	0.461*	0.931***	ns
% Col.	1.000	0.791***	ns	0.440*	ns	ns	ns	ns	ns	ns	0.581**
ERM		1.000	ns	0.510**	ns	ns	ns	ns	ns	ns	0.359*
GPX			1.000	ns	0.626**	0.671**	0.601**	0.652**	ns	ns	ns
Protein				1.000	-0.423*	ns	ns	ns	ns	ns	0.402*
Cd roots					1.000	0.950***	0.890***	0.880***	ns	ns	ns
Cd shoots						1.000	0.871***	0.872***	ns	-0.402*	ns
Cd Cytopl							1.000	0.945***	ns	ns	ns
Cd Cell wall								1.000	ns	ns	ns
P shoots									1.000	0.621**	ns
P content										1.000	ns

p< 0.05, ** p< 0.01, *** p<0.001

Discussion

Cd is a relative mobile metal in plants that may cause a general decrease in plant growth (Hernandez and Cooke, 1997) due to various effects on plant metabolism as photosynthesis reduction and CO_2 fixation among others. In contrast, mycorrhiza promoted maize plants growth even in the presence of Cd, confirming the importance of AMF in phytoremediation practices (Table 1) in which higher plant growth is a key factor for its success (Jurkiewicz et al., 2004). The stimulatory effect of the AMF inoculation on the development of plants treated with metals was also observed for maize, soybean, pea and sunflowers plants (Jurkiewicz et al., 2004; Andrade et al., 2004; Riveira-Becerril et al., 2002; Davies et al., 2002).

Several works showed that high concentrations of heavy metals might reduce mycorrhizal colonization (Citterio et al., 2005; Andrade et al, 2004; Liao et al. 2003). In the present study this was confirmed, being maize root colonization severely reduced by Cd addition, especially in those roots with high P supply (Table 1). Mycorrhizal ERM decreased also due to Cd addition, confirming the deleterious effect of Cd on mycorrhizal development. As observed in the present study (Table 1), high P supply usually reduce root colonization by AMF (Boddington et al., 1999) and thus possibly the development of ERM, being that root colonization and extraradical mycelium amount are frequently correlated (Olsson et al., 1997). In fact, we found a positive correlation (Table 5) between them, showing the relation between AMF intraradical colonization rate and extraradical development of AMF hyphae.

The ability of maize roots to retain Cd has been already documented by several authors (Nocito et al. 2002, Rauser and Meuwly, 1995; Florijn and Van Beusichem, 1993). In the present

study maize plants accumulated Cd mainly in root but less than 10% of the total Cd was translocated to the shoots, showing a high Cd-retention capability in both M and NM roots (Table 2). This feature is of interest from the point of view of food chain and undesirable for phytoextraction practices, which pretend higher metal concentration in plant shoots than in roots.

Cohen et al. (1998) observed that plant roots may accumulate Cd in the apoplast by ionic interactions with cell wall components. Maize roots accumulated similar Cd^{2+} concentrations in cell wall and cytoplasm fractions, independently of the mycorrhizal condition, with a slightly higher proportion in the cell wall fraction. These results are comparable to that observed by Inouhe et al. (1994), which reported that cereal roots accumulated Cd almost equally in both fractions. However, it is suggested that AMF can modify Cd uptake and accumulation, since AMF hyphae are able to transport Cd from soil to the host (Guo et al., 1996). In this study, M maize roots showed, in general, higher Cd amounts bound to the cell wall fraction (Figure 1) and this Cd-binding in the cell wall components may be seen as a barrier mechanism whereby plants may reduce Cd concentration in shoots (Lozano-Rodriguez et al., 1997). In addition, some authors observed that M plants increased root/shoot Cd ratios protecting shoots from excessive concentrations of metals (Joner et al., 2000; Turnau et al., 1993), suggesting that AMF hyphae may act as a barrier in metal transport by adsorption of the metal on hyphal walls. However, in the present research, M plants had a lower Cd transfer factor value from solution to the shoots and a lower total Cd concentration in plant (Table 2), indicating that AMF could be somehow efficient in avoiding Cd uptake from the solution. Cd immobilisation in AMF mycelia has been proposed to occur, acting as a "filter" during metal uptake (Joner et al., 2000). Nevertheless, since there was no differences in shoot Cd concentrations between M and NM plants, it could

indicate that extraradical mycelium of *G. macrocarpum* did not avoid the plant from a lesser Cd translocation (TI) to the shoots.

Cd reduced altered mineral nutrition in maize (Table 3), corroborating the findings of Greger and Lindberg (1987) who found that excessive Cd concentrations could induce deficiencies and imbalances of plant mineral nutrients. It is worth noting that sulphur concentration in shoots and roots of Cd-treated plants was much higher, 69% and 17%, respectively, than that measured in control plants. Nocito et al. (2002) observed an increase in sulphate uptake as direct effect of Cd accumulation in roots, and they suggested that the higher sulphate influx in Cd-treated roots could be an adaptive response to support S demand for phytochelatins biosynthesis, an important group of Cd-chelating peptides involved in cell Cd detoxification.

M plants showed some differences in shoots nutrient concentrations and contents than NM plants. However, mineral concentrations in roots did not differ significantly by the mycorrhizal condition. Differences in biomass production can explain, in some cases, higher and lower acquisition of minerals due to the dilution or concentration effects (Jarrell and Beverly, 1981). However, there are other mycorrhizal effects, such those on root physiology, that must be considered in interpreting nutritional results (Marschner and Dell, 1994). Thus, not all changes in plant mineral acquisition are caused by changes in biomass production (Clark and Zeto, 1996). On the other hand, AMF inoculation had a great influence in the ratio of P, N and S to Cd, in shoots and roots of Cd-treated plants, showing M plants between 20% and 30% higher P/Cd, N/Cd and S/Cd ratios, in shoots and roots, respectively, than NM plants (Table 4). Higher ratios P/metal in M plants were also observed for other authors in other plant species (Andrade et al.,

2004; Shetty et al., 1995) suggesting that the higher P status of these plants may alleviate metal stress by complexation of P with metal ions inside the cells, forming phosphate-metal rich materials (Shetty et al., 1995). The higher contents of N and S on M shoot may also indicate a higher production of thiol-rich proteins that, in addition to P complexation, play an important role in Cd detoxification in vascular plants. Turnau et al. (1993) observed a high N and S concentrations, together with Cd, in the vacuoles of an AM fungus associated with *Pteridium aquilinum* collected from Cd contaminated soil, suggesting the existence of thiols-binding peptides in this class of fungi. Galli et al. (1995) verified the existence of Cu-binding peptides in M maize roots showing an increased concentration of thiols (cysteine, γ-glutamylcystein and glutathione) in M roots.

Acid posphatases (APases) are related to the intracellular hydrolysis of phosphate reserves and its expression is regulated by several environmental and physiological factors (Duff et al., 1994). Thus, APase activity can be considered an adequate indicator to evaluate plant P-nutricional status, since its activity increases with P deficiency level (Besford, 1980; McLachlan et al., 1987). In addition, its activity may be related to P use efficiency in the shoot (Duff et al., 1994). Increased APase activity has been also related to leaf senescence (Snapp and Lynch, 1996). In the present study, leaves APase activity was decreased by M condition, showing NM higher enzyme activity (Figure 2), possibly reflecting the higher P supply in M plants. A expected, P supply also influenced APase activity, being its activity 36% higher in plants under low P solution. Interestingly, Cd addition affected foliar APase activity, increasing 23% in relation to non Cd-treated plants and under low P solution (Figure 2). Dube et al. (2004) observed that excessive amounts of Cr increased APase activity in leaves of radish, while Olivares (2003)

found that leaves of mexican sunflower plants under Pb excess conditions had increased activity of APase acivity. Hence, it is suggested that Cd may be interfering in P uptake and inducing APase activity to counteract a possible extra P demand in the intracellular environment to form a non-toxic metal complexe with P material (Shetty et al., 1995).

Cd stress can interfere with the activity of the antioxidant defence system (Balestrasse et al., 2001). Therefore, changes in enzyme activities, as peroxidases, in response to Cd presence in the growth solution may be useful in detecting metabolic stress signs in plants. The root system is particularly affected by excess metal concentrations and by mycorrhiza, as the plant organ where the mycorrhizal association occurs and in direct contact with excessive metal concentrations solution. Thus, maize roots GPX activity was influenced by Cd addition, reflected in the positive correlation found with Cd concentration in plant tissues and root cellular fractions, and also by mycorrhiza, since M and NM plants showed different activity patterns (Figure 2). GPX ativity was induced by Cd addition indicating that is involved in the defence system against antioxidative stress caused by Cd ions in maize roots. During stress, oxygen reactive species accumulate in cells and this leads to the induction of enzymes such peroxidases, superoxide dismutases and catalases (Gratão et al., 2005). But an increase in peroxidase activity is also a usual response in other type of stresses (Lamport, 1986). It is known that peroxidases participate in lignification processes and some authors suggest that this lignin biosynthesis may build-up a physical barrier against heavy metal toxicity (Degenhardt and Gimmler, 2000). Some plants develop cell wall thickenings in different root tissues, or even higher amount of lignin in the cell walls, in response to high levels of heavy metals (Degenhardt and Gimmler, 2000).

Protein content in M roots was higher than in NM roots, confirming the results of other authors (Gianinazzi-Pearson and Gianinazzi, 1995), which observed an increased amount of

proteins in roots colonized by AMF. Root protein content was positively correlated with mycorrhizal colonization and with the amount of mycorrhizal ERM, confirming the influence of mycorrhiza in root physiology. Root protein content correlated also positively with root P concentration and the later correlated with intra and extraradical AMF colonization (Table 5). A marked alteration on gene expression occurs in M plants (Wyss et al., 1990) and a new class of soluble proteins inside mycorrhizal roots, denominated as endomycorrhizins (Dumas-Gaudot et al. 1994) has been found. These and other expressed proteins may be contributing to the higher protein contents found in M maize roots. The lower protein content observed in roots of plants under high P solution was, possibly, related to the dilution effect caused by higher growth of these roots due to the higher P availability. Souza et al. (2002) suggested that an increased protein expression in roots might be a plant reaction to Al toxicity, and so, it could be proposed a similar reaction in the case of Cd stress in maize roots. In this case, M plants, in addition to the higher root protein contents showed no induction of GPX activity in the presence of Cd. These findings could suggest higher tolerance to the metal in M plants. The higher proportion of Cd in root cell wall fraction of M plants may also have contributed to a higher Cd retention in roots and consequently reduce its translocation to the aerial part and decrease Cd transfer to the food chain.

Conclusion

The results evidence the different physiological response of M and NM plants in response to excess Cd in the medium. Cd addition seriously affected mycorrhizal symbiosis diminishing intra and extraradical development of the AMF. Nevertheless the higher growth and nutrients/Cd ratios observed in M plants indicate a efficient symbiosis that alleviated nutritional and Cd stress.

Possibly, the higher protein contents and the lack of GPX induction in the presence of Cd in M plants show a higher tolerance to Cd in the specific conditions of the study.

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4. EXPERIMENTO COM GIRASSOL

Alleviation of cadmium stress in mycorrhizal sunflower (*Helianthus annuus* L.) plants

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Abstract

In order to investigate the possible alleviation of Cd-stress by mycorrhization and compare Cd accumulation patterns, sunflower plants (*Helianthus annuus* L. cv. IAC Uruguai) were grown in the absence and presence of 20 μmol L^{-1} of Cd, and inoculated or not with the arbuscular mycorrhizal fungus *Glomus intraradices*. Although, no visual symptoms of Cd phytotoxicity were observed, Cd-treated plants showed reduced development and they were influenced positively by mycorrhizal condition. Sunflower roots accumulated most of the Cd absorbed and Cd^{2+} was preferentially accumulated in the cell wall fraction. However, one fourth of the total Cd absorbed was translocated to the shoots. In spite of the greater absortion of Cd, mycorrhizal (M) plants showed higher growth, concentrations of photosynthetic pigments and shoot P contents. The presence of Cd influenced mineral nutrition, decreasing Ca and Cu shoot concentrations, N, Fe and Cu shoot contents, and increasing, by contrast, S and K shoot concentrations. The translocation index of N, K, P and Ca was generally higher in M plants. Guaiacol peroxidase activity in roots was induced in M and non-mycorrhizal (NM) plants in response to Cd addition, but its increase was much more accentuated in NM roots. In conclusion, sunflower plants when associated with *G. intraradices* were less sensitive to Cd stress than non-associated plants. M sunflowers had enhanced Cd accumulation and tolerance to excessive concentrations of Cd in plant tissues, behaving as a promising Cd phytoextraction system.

Key words: cadmium accumulation; heavy metals; mycorrhiza; nutrient uptake; peroxidase; phytoextraction

Introduction

Cadmium (Cd) is a widespread trace element found in many natural and agricultural environments mainly due to human activities (Wagner, 1993). This non-essential metal is readily taken-up by the root system and may be accumulated in plant tissues, inhibiting plant growth and development (Hernadez and Cooke, 1997). Cd is usually retained in the roots and small amounts of the metal ions are translocated to the shoots (Benavides et al., 2005). It is known that Cd damages the photosynthetic apparatus and may affect chlorophyll contents (Krupa and Baszynski, 1995). Another toxic effect caused by excessive concentrations of Cd is the oxidative stress, which is related to lipid peroxidation of cellular membranes (Verma and Dubey, 2003). Excess Cd may trigger an increased production of the reactive oxygen species (ROS) that affect important biomolecules (Benavides et al., 2005). To combat the oxidative damage, plants have an antioxidant defence system comprising of enzymes catalases, peroxidases, superoxide dismutases and also of non-enzymatic metabolites which neutralize and scavenge the ROS (Gratão et al., 2005).

However, there are significant differences in plant tolerance to Cd and some species are able to tolerate higher concentrations of Cd in their tissues than others. Plant mechanisms to avoid Cd toxicity, such as immobilization, exclusion and synthesis of phytochelatin, among others, are well reviewed by Sanità di Toppi and Gabrielli (1999). Arbuscular mycorrhiza (AM) is not frequently considered in the list of plant metal tolerance mechanisms but its role in ameliorating the effects of heavy metal toxicity in the host plant has been frequently reported (Rivera-Becerril et al., 2002; Davies et al., 2002; Andrade et al., 2003; 2004; 2005). Depending on growth conditions, fungi and plant species involved in the mycorrhizal symbiosis, increases or decreases of metal content in the host plant have been observed (Joner and Leyval., 1997;

Weinsenhorn and Leyval, 1995). Being the possibility of increasing metal accumulation in plant tissues, shoot particularly, very interesting for phytoextraction purposes (Citterio et al., 2005) Arbuscular mycorrizal fungi (AMF) can alleviate metal phytotoxicity by enhancing nutrient supply, improving water relations (Meharg and Cairney, 2000) or by metal sequestration in fungal structures, such as intra and extraradical hyphae (Joner et al., 2000). Another possible metal-tolerance mechanisms in the AM symbiosis include metal dilution due to increased root or shoot growth, exclusion by precipitation of polyphosphate granules, and compartmentalization into plastids (Kaldorf et al., 1999). Some scarce data also suggests that mycorrhizal (M) plants have significant differences in the response of the antioxidative system to heavy metal toxicity (Schützendübel and Polle, 2002). In relation to another mechanism of metal tolerance, phytochelatin production, Turnau et al. (1993) observed high N, S and Cd concentrations in the vacuoles of an AMF associated with *Pteridium aquilinum* collected from Cd contaminated soil, suggesting the existence of thiol-binding peptides in this class of fungi. This presence of metal-binding peptides in AM plants was verified by Galli et al. (1995), who found an increase in thiol compounds related to the M status of maize in response to Cu stress.

The aim of this study was to evaluate how the mycorrhization of sunflower plants by the AMF G*lomus intraradices* alleviates the effect of Cd-stress, and compare the Cd accumulation patterns in M and non mycorrhizal (NM) plants.

Materials and methods

Experimental design

A greenhouse experiment was conducted in a completely randomised design and in 2x2 factorial scheme, with ten replications. The treatments were composed of two Cd concentrations,

0 and 20 μmol L^{-1} in the nutrient solution, and the inoculation or not of the AMF *Glomus intraradices* (Schenk & Smith). Day and night temperatures ranged between 29°C and 16°C respectively; with a photoperiod of 12 h.

AMF inoculum

The AMF used was *G. intraradices* propagated on stock cultures with *Brachiaria brizantha* Stapf for six months. Colonized root fragments, mycelium and sand-soil mixture containing spores were used as inoculum. Each pot received approximately 2700 spores, at the time of sowing. The NM treatments received washings of the soil-inoculum mixture filtered through Whatman n° 42 filter paper.

Pot culture experiment

Two litters of sterilized ground silica (2-3 mm) were put in 2.79 L plastic pots. Sunflower (*Helianthus annuus* L. cv. IAC Uruguai) seeds were surface-sterilized with 1:3 (v/v) of 2.5% sodium hypochlorite solution for 10 minutes. Six seeds were sown per pot and after emergence were thinned to one plant per pot. Plants were cultivated in a hydroponic system with silica and irrigated with complete nutrient solution (N-NO_3 154.6; N-NH_4 19.5; S-SO_4 18.7; Ca 151.2; K 70.9; Mg 18.8; P 10; B 0.53; Fe 1.99; Mn 0.97; Cu 0.076; Zn 0.3; Mo 0.15 mg L^{-1}) (Furlani and Furlani, 1988), and with distilled water in alternating days. Plants treated with Cd received 20 μmol L^{-1} of Cd supplied as $Cd(NO_3)_2$ in the nutrient solution. Speciation calculations using Visual MINTEQ ver. 2.23 (Gustafsson, 2003) indicated that 86% of the Cd in solution was free Cd^{2+} ion. The total volume of nutrient solution added to each pot during the experiment was 3.62 L, which corresponded to 8.137 mg of Cd added cumulatively per plant. The plants were allowed to grow for 8 weeks till harvest at the beginning of the flowering stage.

Analytical methods

At harvest, leaves, stems and roots were separated. Leaf area was measured immediately with LiCor 3100 area meter. Shoots and roots were washed in distilled water. Root fresh weight was recorded, and subsamples were: 1) stored in 50% ethanol for mycorrhizal colonization determination, 2) stored in liquid nitrogen until use in enzymatic assays, or 3) dried and ground. After drying at 60ºC shoots were weighed and ground. The contents of P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, and Cd in shoots and roots were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Jobin Yvon, JY50P) after HNO_3-$HClO_4$ digestion. Total-N in the sulphuric digest was determined by Kjeldahl analysis.

Chlorophyll (Chl.) a, b and carotenoids were extracted with 100% dimethylsulphoxide from the last total expanded leaf a week before the harvest and measured spectrophotometrically (Hitachi U-2000) (Lichtenhaler and Welburn, 1983) in the extracts. Root mycorrhizal colonization was evaluated by the grid-line intersect method (Giovannetti and Mosse, 1980) after staining with Trypan blue. The length of the extraradical mycelium (ERM) of AMF in the substrate was estimated according to Melloni and Cardoso (1999). The ERM was extracted by wet sieving of 20 g of substrate. The extracted ERM was stained with 0.05% Trypan blue in lactoglycerol and its total length assessed under compound microscope (125x magnification) in 64 fields. The results expressed as m g^{-1} of dry substrate. T Substrate with and without AMF treatment was submitted to the procedure of mycelium extraction and only hyphae with characteristics and morphology of AMF were considered. The absence of AMF hyphae was verified in non-inoculated treatment.

In order to quantify Cd adsorved on the AMF extraradical mycelia, a hyphal extraction was perfomed by flotation based on Joner et al. (2000). At harvest, 2 L of the substrate of each

pot, with AMF inoculation, was washed several times in a large bucket with running water and the washing suspension passed through a 0.45 mm mesh and collected in amber flasks. The mycelial suspension was cleaned by flotation and observed its purity under dissecting microscope. The cleaned suspension was vacuum filtered in a nitrocelullose filter, collected, dried at 40ºC for 36 h and weighed. Dry mycelium was hydrolyzed with 2 mL of HNO_3 and four drops of $HClO_4$ and Cd concentration in the digest determined by flame atomic absorption spectrometry (F-AAS; Perkin Elmer, 5000).

Fractionation of root cytoplasm and cell wall for determination of Cd content in each fraction was performed, with modifications, according to Inouhe et al. (1994). Liquid nitrogen frozen roots (0.5-1.0 g fresh weight) were homogenized by mortar and pestle with 20 mmol L^{-1} Tris-HCl buffer (pH 7.8). The homogenates were centrifuged twice, at 1,500 x g for 10 min and at 10,000 x g for another 10 min. The resulting supernatants were collected and used as cytoplasmic fractions. The precipitates in the tubes were collected, dried at 37-40 ºC and considered as cell wall fraction. Cd concentration in the cytoplasmic fraction was determined directly by F-AAS. Dried cell wall fractions were hydrolized with 2 mL of HNO_3 for 20 min at 100ºC. Four drops of $HClO_4$ were added to the hydrolizates and again hydrolized for 15 min at 100ºC. After this process, three drops of 30% H_2O_2 were added and maintained at 100ºC for 15 min and finally diluted to 5 mL with deionized water. Cd content was determined by F-AAS.

Guaiacol peroxidase (GPX) (EC 1.11.1.7) activity was assayed spectrophotometrically using a diode array spectophotometer (Hewlett Packard, 8452 A) according to Boscolo, Menossi and Jorge (2003). Liquid nitrogen frozen roots (0.1-0.05 g) were washed three times in deionized water and homogenized in phosphate buffer (KH_2PO_4/K_2HPO_4, pH 6.8). Homogenates were centrifuged at 10,000g for 8 minutes and immediately determined the peroxidase activity in the

supernatant. The reaction mixture contained 500 μmol L^{-1} phosphate buffer, 8 mmol L^{-1} guaiacol, 8 mmol L^{-1} H_2O_2 and protein extract. The increase in absorbance was recorded at 470 nm (extinction coefficient, ε = 26.6 mmol L^{-1} cm^{-1}). Catalase (CAT) (EC 1.11.1.6) activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm (ε = 39.4 mmol L^{-1} cm^{-1}). Total soluble proteins in each extract were determined using the Bio-Rad protein assay. Determinations of enzyme activity were performed in duplicate in five roots per treatment.

The nutrient and Cd translocation indexes were calculated as the percentage of the total amount absorbed that was translocated to the shoots. Cd transfer index (TF) was calculated as the ratio of Cd concentration in the plant tissues (shoot or root) to that in the solution. All data were processed by analysis of variance. Means from significant treatment effects were compared by Tukey test (α= 0.05).

Results

The effects of Cd and AMF inoculation on growth on shoot dry weight (SDW), leaf area and root fresh weight (RFW) are shown in Table 1. No visual symptoms of Cd phytotoxicity were observed. Nevertheless, the presence of Cd decreased SDW and leaf area of NM plants 22% and 11%, respectively, relative to M plants (Table 1). M plants showed 10% higher SDW and 17% higher leaf area than NM plants. Root growth was not influenced by Cd addition or AMF inoculation (Table 1). Inoculated plants had around 33% of root length colonized and no mycorrhizal structures were found in non-inoculated plants. Not significative effect of Cd

addition was observed neither on mycorrhizal colonization nor on the amount of mycorrhizal ERM (Table 1).

Table 1. Shoot dry weight (SDW), leaf area (LA), root fresh weight (RFW), mycorrhizal colonization (Myc. Colon.) of sunflower plants and mycorrhizal extraradical mycelium length (ERM), as affected by *G. intraradices* and Cd addition (0 and 20 μmol L^{-1}).

Treatment		**SDW**	**LA**	**RFW**	**Myc. Colon.**	**ERM**
AMF	Cd	g	cm^2	g	%	m g^{-1}
-	0	3.85 Ba	367 Ba	13.16 Aa	-- 0 --	-- 0 --
+	0	4.05 Aa	464 Aa	12.34 Aa	35.88 a	0.98 a
-	20	3.01 Bb	345 Ba	13.56 Aa	-- 0 --	-- 0 --
+	20	3.49 Ab	395 Ab	12.72 Aa	30.8 a	0.92 a

Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. A, B compare between different mycorrhizal status in each Cd concentration; a, b compare between Cd treatments in each of the mycorrhizal status.

Both, Cd addition and AMF inoculation influenced the contents of photosynthetic pigments in sunflower leaves (Table 2). In general, Cd reduced the contents of Chl a, b and carotenoids only in NM plants. Cd or mycorrhizae did not influence Chl a/ b ratio (Table2). M plants showed higher chlorophyll contents only in the presence of Cd compared to their homologues NM. In M plants, the percentage reduction in Chl. a contents from 0 to 20 μM Cd was approximately 12% whereas in NM plants the decrease reached 26% (Table 2).

Table 2. Chlorophyll (Chl) a, Chl b, Chl a+b and xantophyll and carotenoids (C x+c) contents in mycorrhizal and non-mycorrhizal sunflowers leaves, at two different Cd (0 and 20 μmol L^{-1}) concentrations in the nutrient solution.

Treatment		**Chl a**	**Chl b**	**Chl a + b**	**C x + c**	**R a/b**
AMF	Cd			μg mL^{-1} extract		
-	0	11.46 aA	2.41 aA	13.88 aA	3.23 aA	4.72 Aa
+	0	12.24 aA	2.55 aA	14.80 aA	3.47 aA	4.79 Aa
-	20	8.44 bB	1.78 bB	10.22 bB	2.47 bB	4.77 Aa
+	20	10.62 aA	2.15 aA	12.78 aA	3.02 aA	4.99 Aa

Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. A, B compare between mycorrhizal status in each Cd concentration; a, b compare between Cd treatments in each mycorrhizal status.

Cd ions accumulated mainly in roots and only 22% of the total Cd absorbed was translocated to the shoots (IT) (Table 3). Sunflowers plants that received Cd contained an average of 228 mg kg^{-1} of Cd SDW. Although the ratio of Cd in shoots to roots was similar in both M and NM plants, the total absorbed Cd was 23% higher in M plants. The concentration of Cd found in root and shoots of M plants were 23% and 34% higher, respectively, than in NM plants. The distribution of Cd was about 80% and 20% in the root cell wall fraction and in the cytoplasm fraction, respectively (Table 3). The Cd concentration found in ERM of the AMF was of 728 mg kg^{-1} dry weight.

Cd addition did not affect P concentration but reduced significantly N content in NM shoots by 22% relative to controls (Table 4). In general, shoot P concentration was 18% higher in

M plants than in NM plants, and P content in M plants was 24% higher even in the presence of Cd (Table 4). P concentration in roots of M and NM plants was not different (Table 4). Shoot S concentration was 20% higher in Cd-treated plants (Table 4). However, root S concentration was not influenced by AMF inoculation or Cd addition (Table 4). Shoot K concentration increased 9% in the presence of Cd. M plants had 19% higher shoot K contents than NM plants (Table 4). In roots, K concentration was similar in M and NM plants (Table 4). Shoot and root Zn concentrations were higher in NM plants and showed a different response to Cd addition in M and NM plants. Shoots Zn contents were reduced in M plants and increased in the NM.

Table 3. Cd concentration in shoots and roots, total Cd absorbed, shoot Cd content, translocation index (TI), shoot to root Cd concentration ratio and Cd transfer index from nutrient solution to the shoot (TF_{shoot}) and to the roots (TF_{root}) in sunflowers plants (*Helianthus annuus* var. IAC Uruguai) inoculated or not with the AMF *G. intraradices*.

Treatment		**Cd shoot**	**Cd root**	**Cd absorbed**	**Cd content**	**Cd TI**	**Cd shoot/ root**	**TF_{shoot}**	**TF_{root}**	**Cd cell wall**	**Cd cytoplasm**
Cd μmol L^{-1}	**AMF**	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	μg $plant^{-1}$					mg g^{-1} FW	
0	**-**	10	15	-	31	-	-	-	-	-	-
0	**+**	7.8	14	-	32	-	-	-	-	-	-
20	**-**	204 b	714 b	918 b	674 b	22.1 a	0.28 a	90 b	318 b	22.5 a	5.13 a
20	**+**	252 a	885 a	1137 a	905 a	22.2 a	0.28 a	114 a	394 a	17.9 a	4.88 a

Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. a, b compare between mycorrhizal and non-mycorrhizal plants. (TI = 100 x Cd concentration in shoots / Cd concentration in shoots + Cd roots; $TF_{SOL\text{-}SHOOT}$ = Cd concentration in plant shoots / Cd concentration in the solution).

Table 4. Shoot nutrient concentrations and contents and root nutrient concentrations in mycorrhizal and non-mycorrhizal sunflower (*Helianthus annuus* var. IAC Uruguai*)* plants treated or not with 20 μmol L^{-1} of Cd.

Cd, μmol L^{-1}	AMF	N	P	K	S	Ca	Mg	Cu	Fe	Mn	Zn
Shoot					(g kg^{-1})				(mg kg^{-1})		
0	-	24.0	0.7	15.4	2.0	27.0	4.58	8.88	67	323	56
0	+	23.9	1.0	15.0	2.2	31.2	5.16	9.62	79	228	55
20	-	21.0	0.8	16.0	2.5	25.4	5.26	4.90	67	331	78
20	+	23.7	0.9	17.1	2.5	23.3	4.96	5.12	71	288	50
Significance											
AMF		NS	0.007	NS	NS	NS	NS	NS	NS	0.03	0.0005
Cd		NS	NS	0.02	0.001	0.002	NS	0.00002	NS	NS	0.01
AMF x Cd		NS	NS	NS	NS	0.02	0.004	NS	NS	NS	0.001
LSD		3.19	0.14	1.60	0.31	3.91	0.40	1.67	15.7		9.55
Shoot					(mg $plant^{-1}$)				(μg $plant^{-1}$)		
0	-	89.8	2.9	59.3	7.8	101	4.5	34.1	254	1145	217
0	+	91.3	3.8	57.9	8.3	113	5.1	33.4	302	825	210
20	-	69.3	2.9	62.7	8.7	90	5.2	16.1	222	1091	257
20	+	82.2	3.4	52.7	8.4	82	4.9	18.0	271	978	178
Significance											
AMF		NS	0.0006	NS	NS	NS	NS	NS	0.004	0.03	0.0007
Cd		0.007	NS	NS	NS	0.003	NS	0.00001	0.04	NS	NS
AMF x Cd		NS	NS	0.02	NS	NS	0.004	NS	NS	NS	0.002
LSD		14.6	0.46	6.89	1.18	18.5	2.33	6.06	43.6	280	29.14
Root					(g kg^{-1})				(mg kg^{-1})		
0	-	14.8	1.26	8.6	1.9	8.9	2.5	17	376	125	42
0	+	12.9	1.18	5.4	1.6	6.7	1.8	15	1102	112	46
20	-	10.8	1.04	8.0	1.5	7.0	1.8	14	527	109	52
20	+	12.9	1.22	6.2	1.8	6.9	2.0	16	1394	132	32
Significance											
AMF		NS	NS	0.00001	NS	NS	NS	NS	0.0001	NS	0.01
Cd		0.02	NS	NS	NS	NS	NS	NS	NS	NS	NS
AMF x Cd		0.03	NS	0.01	0.0504	NS	0.02	NS	NS	NS	0.001
LSD		2.55	0.26	0.88	0.35	1.76	0.52	3.53	391	45.3	9.05

In the presence of Cd, NM roots showed 24% higher GPX activity than M roots and 96% higher activity than non Cd- treated roots (Figure 1). Increase in GPX activity was 37% in M roots in relation to non Cd- treated plants. GPX activities of M and NM roots were similar in the absence of Cd (Figure 1). CAT activity was not observed in sunflower roots (data non shown). Total soluble protein in M roots was higher than in NM roots, which had a reduction in protein content in the presence of Cd (Figure 1)

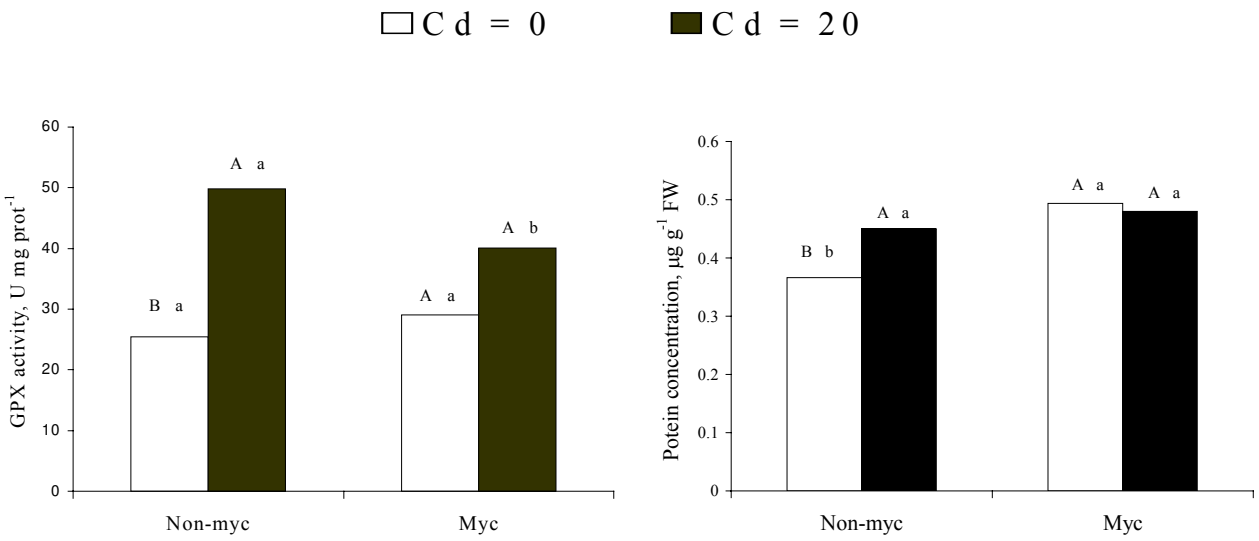

Figure 1. Guaiacol peroxidase (GPX) ativity and total protein concentrations in mycorrhizal (Myc) and non-mycorrhizal (Non-myc) sunflowers roots at two Cd (0 and 20 μmol L^{-1}) concentrations in the nutrient solution. (Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. a, b compare between mycorrhizal and non-mycorrhizal plants in each Cd concentration; A, B compare between Cd treatments in each mycorrhizal status).

Discussion

The amount of Cd added to each plant was 8.14 mg, considered of environmental relevance and in the range found in Cd-polluted soils (Sanità di Toppi and Gabbrielli, 1999). Plant growth, as result of co-ordination of many physiological processes, especially reflects the stress that heavy metals may cause in plants. Although, no visual symptoms of Cd phytotoxicity were observed, Cd-treated plants showed reduced development (Table 1). By contrast, it is known that plant physiology is strongly influenced by AMF and plant growth promotion by these fungi has been frequently observed in conditions of excess metal concentrations (Rivera-Becerril et al., 2002; Andrade et al., 2003; 2004, Greipsson and Hovsepyan, 2004). As expected, mycorrhizal condition positively influenced plant development yielding higher shoot dry weights, leaf area and higher photosynthetic pigments contents (Table 1). The fact that mycorrhizal fungus colonization and the amount of ERM produced by the AMF were not affected by Cd addition to the solution (Table 1), allowed us to compare the effects of Cd in plants with very similar colonization rates and amounts of ERM. Thus, we propose that plant growth reduction in mycorrhizal Cd-treated plants was due to a direct toxic effect on plant physiology and not to reduced growth promotion due to lower colonization rates. Other authors (Rivera-Becerril et al., 2002) also reported a lack of effect of relevant Cd levels on mycorrhizal formation.

The observations of Cd accumulation indicate that sunflower plants may attain levels of 228 mg Cd kg^{-1} SDW and may therefore be regarded as Cd hyperaccumulating plant, defined as a plant able to accumulate more than 100 mg kg^{-1} of Cd in the SDW (Baker et al., 2000). Although sunflower roots acted as a barrier against Cd

translocation, retaining most of the Cd absorbed, a fourth of the total absorbed metal was translocated to the shoots (Table 3). Davies et al. (2002) already observed the capacity of sunflower plants for chromium phytoextraction. AMF may either reduce or increase metal absorption depending on the plant and AMF species, metal concentration and growth conditions (Weinserhorn and Leyval, 1995). Even though in this study the Cd shoots:roots accumulation ratio was similar in M and NM plants, the total absorbed Cd was higher in M plants (Table 3). We calculated the transfer factor (TF), in order to quantify the plant ability to accumulate Cd with respect to the concentration of this metal in the solution, according to other authors (Elkhatib et al., 2001). The TF indicated that sunflower plants had a great ability to accumulate Cd in the shoots, as previously reported by Elkhatib et al. (2001). M sunflowers had a greater TF than NM plants, 114 versus 90, respectively, indicating that M plants had greater Cd accumulating capacity (Table 3). Enhanced Cd absorption in M plants was also observed for other plant and AMF species (Rivera-Becerril et al, 2002; Weinserhorn et al. 1995). It is known that roots can accumulate Cd in the apoplast by ionic interactions with components of the cell wall, and part of the metal can be complexed by phytochelatins and sequestered in the cytoplasm into the vacuole (Cohen et al., 1998). In this study sunflower roots, independent of their mycorrhizal condition, accumulated Cd^{2+} preferentially in the cell wall fraction (80%), results comparable to that observed by Inouhe et al (1994). Cd-binding to components of the roots cell walls is one of the mechanisms whereby plants may reduce Cd concentration in the shoots. In addition, the immobilization of metals in the AMF mycelium has been proposed as a “filter” during metal uptake (Joner et al., 2000). The Cd concentration found in AMF mycelium (728

mg kg^{-1}) was similar to that in roots and confirms the high adsorption capability of the AMF mycelia for Cd (Joner et al., 2000). Nonetheless, in this study M plants had greater root and shoot Cd concentrations, and no differences in the Cd content of cell wall fraction was observed between M and NM roots, indicating that AMF was not particularly efficient in avoiding Cd translocation to the shoots and did not especially prevent excess Cd in aboveground tissues.

Cd addition to the nutrient solution reduced contents of photosynthetic pigments in sunflower leaves (Table 2). By contrast, Chl a/b ratio remained constant in the absence and presence of the metal, indicating that neither of the chlorophyll was preferentially affected by Cd addition (Table 2). Chlorophyll have shown to be one of the targets of Cd toxicity, and reduction in these pigment contents has been observed by other authors (Vassilev et al 2002). This reduction is due to clhorophyll biosynthesis inhibition by Cd ions, and has been proposed, in part, as responsible for growth reduction caused by this metal (Bazzaz et al., 1992). Nevertheless, M plants showed higher photosynthetic pigments contents only in the presence of Cd, being in general less affected by Cd ions than NM plants (Table 2).

Disturbances in the uptake and distribution of nutrients in plants have been also correlated to Cd toxicity. Cd may affect the contents of polyvalent cations through antagonism processes mediated by metal competition for binding sites or transporters (Gussarson et al., 1996). In this study, Cd addition decreased Ca and Cu shoot concentration and N, Fe and Cu shoot contents (Table 4). Cd caused a reduction in N and Mg root concentration and an increase in Mg shoot content in NM plants (Table 4).

By contrast, S and K shoot concentrations increased in the presence of the metal (Table 4). The increase in S content in plant tissues in Cd-treated plants has already been observed (Nocito et al., 2002). An increase in sulphate uptake as direct effect of Cd accumulation has been proposed as an adaptive response to support S demand for phytochelatins biosynthesis (Nocito et al., 2002). AMF inoculation influenced plant mineral contents in sunflower plants (Tables 4). M plants showed higher shoot P concentrations and contents in the presence and absence of Cd. The translocation index of N, K, P and Ca was generally higher in M plants even in the presence of Cd (Table 5), and the higher TI for nutrient found in M sunflowers plants may be related to the higher development (Table 1).

Table 5. Translocation index of nutrients in mycorrhizal and non-mycorrhizal sunflower (*Helianthus annuus* var. IAC Uruguai) plants in the absence and in the presence of 20 μmol L^{-1} of Cd in the nutrient solution.

Cd μmol L^{-1}	**AMF**	**N**	**K**	**P**	**Ca**	**Mg**	**Cu**	**Fe**	**Mn**	**Zn**	**S**
0	**-**	59 aB	64 aB	38 bB	75aB	64 bB	30 aB	14 aA	72 aA	55 aA	50 bB
20	**-**	63 aA	66 aA	44 aA	78 aA	74 aA	24 aA	11 aA	75 aA	60 aA	62 aA
0	**+**	66 aA	74 aA	46 aA	82 aA	73 aA	38 aA	7 aB	67 aA	56 aA	57 aA
20	+	65 aA	73 aA	47 aA	77 bA	71 aA	24 bA	5 aB	68 aB	61 aA	60 aA

Means with the same letter are not significantly different ($p \leq 0.05$) by the Tukey test. A, B compare between mycorrhizal (+) and non-mycorrhizal (-) plants in each Cd concentration a, b compare between Cd treatments.

GPX, together with superoxide dismutase and glutathione reductase, is considered as one of the key enzymes involved in cell protection against ROS.

Although Cd does not generate ROS directly, it generates oxidative stress which interferes with the antioxidant defence system (Gratão et al., 2005). In this study, GPX activity in M and NM roots was similar in the absence of Cd (Figure 1). However, GPX activity was induced in M and NM plants as response to Cd addition to the nutrient solution, suggesting that this enzyme, and possibly other important antioxidant enzymes, acted as a defence tool to resist to Cd-induced oxidative damage in sunflower roots. The increase in GPX activity due to Cd addition was much more accentuated in NM than in the M roots (Figure 1). By these results, it can be inferred that M plants were less stressed than NM plants, managing better under stressful levels of Cd. CAT activity could not be detected in sunflower roots, indicating the absence of significant amounts of this enzyme, an observation also reported for barley roots (Hegedüs et al., 2001). In the present investigation, the total soluble protein content was higher in M roots, maintaining similar protein content in the presence and absence of Cd (Figure 1). Meanwhile, the content of soluble proteins of NM roots increased due to Cd addition (Figure 1). This increase may be related to a higher synthesis of phytochelatins since it was observed that Cd induced progressively the synthesis of water-soluble proteins with an aminoacid composition very similar to that found in phytochelatins (Leita et al., 1993). Gianinazzi-Pearson and Gianinazzi (1995) observed a higher protein concentration in extract of M roots, most of them with unknown functions.

In conclusion, this study showed the alleviation of Cd stress by mycorrhization in sunflower plants, which, in spite of their higher Cd accumulation, exhibited better growth than NM plants, having tolerance to excess concentrations of Cd in plant tissues. This was reflected by higher levels of photosynthetic pigments, higher P levels in shoots

and the lower activity of guaiacol peroxidase in roots. Therefore, sunflower when associated with this AMF was less sensitive to Cd stress than non- AMF associated plants. In addition, sunflower associated to *G. intraradices* appears to be a promising Cd-phytoextraction system, and also, its high biomass production and tolerance to excess concentrations of Cd makes it an excellent candidate for phytoextraction programs. Ongoing research will help to elucidate the role of mycorrhiza on the metal sequestration and alleviation of heavy metal stresses.

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5. DISCUSSÃO

Nos três experimentos realizados, o Cd alterou de modo diferente a fisiologia das plantas estudadas, embora as três plantas tenham apresentado certa tolerância ao metal e não tenham sido observados sintomas visuais típicos da sua toxicidade, indicados freqüentemente por necrose foliar, descoloração avermelhada das bordas das folhas e enegrecimento das raízes (Hernandez e Cooke, 1997). Na presença de Cd, as plantas de milho, para as quais uma maior quantidade de Cd foi adicionada na solução nutritiva (Tabela 1), apresentaram uma diminuição na produção de biomassa, além de menor área foliar e menor concentração de clorofilas, no caso do girassol (Tabela 1). No entanto, somente plantas de girassol não micorrizadas (NM) apresentaram diminuição significativa na produção de biomassa (Tabela 1). Pode-se dizer, portanto, que no geral o Cd mostrou-se, nas concentrações adicionadas, um metal com efeito negativo sobre o desenvolvimento do milho e do girassol e que a associação dessas plantas com os FMAs inoculados favoreceu o seu crescimento (Tabela 1), confirmando assim resultados anteriormente observados (Rivera-Becerril et al., 2002). Esse efeito benéfico da associação micorrízica no milho e no girassol pode estar relacionado à melhor nutrição mineral das plantas, já que esta foi significativamente influenciada pelo estabelecimento da simbiose. Particularmente no experimento com milho, foi evidente a influência da micorriza na nutrição da planta, inclusive na presença do Cd, sendo que as plantas M apresentaram maiores relações de N, P, S com Cd tanto na parte aérea quanto nas raízes (Capítulo 3). A absorção de nutrientes aparece, portanto, como um dos benefícios mais influentes da micorrização das plantas.

Tabela 1. Resumo das condições, características e efeitos principais do Cd e da micorrização nos experimentos realizados.

Característica	***Experimento I***	***Experimento II***	***Experimento III***
Planta	*Canavalia ensiformis*	*Zea mays*	*Helianthus annuus*
FMA	*Glomus intraradices* *G. macrocarpum* *G. etunicatum*	*G. macrocarpum*	*G. intraradices*
Substrato	areia (0,045 to 1 mm)	sílica moída (2-3 mm)	sílica moída (2-3 mm)
Tempo de cultivo (dias)	45	70	56
Fase fenológica colheita	Florescimento	Florescimento	Florescimento
P solução (mg L^{-1})	1 e 10	5 e 10	10
Cd solução (μmol L^{-1})	5	20	20
Cd adicionado (mg/vaso)	0,554	6,4	8,13
Cd P.A (mg kg^{-1})			
Média	1,154	24,2	228
Plantas M	1,46 (*G. etunicatum*)	23,7	252
Plantas NM	0,46	24,8	204
Cd raiz (mg kg^{-1})			
Média (% total absorvido)	38,25 (96%)	313 (92%)	789 (77%)
Plantas M	60,6 (*G. etunicatum*)	304	885
Plantas NM	25,2	322	714
Cd absorvido (P.A + raiz)	mg $planta^{-1}$		
Média	39.4	337.2	1017
Plantas M	62.06 (*G. etunicatum*	327.7	1137
Plantas NM	25.66	346.8	918
Cd micélioFMA (mg kg^{-1})	n.d.	720	728
I T Cd			
Média	2,9	7,2	22,4
Plantas M	2,4	7,2	22,1
Plantas NM	1,8	7,1	22,2
F T_{SOL-PA} Cd			
Média	2	11	101
Plantas M	2,5	10,5	114
Plantas NM	0,8	11,3	90
Efeito no Crescimento			
Cd	n.s	-	plantas NM – e M- n.s
FMA	n.s	+	+
Efeito do Cd			
Colonização	n.s	-	n.s
MET	-	-	n.s
Principal efeito da micorriza na atenuação do estresse por Cd	- Maior concentração de P na P.A.	- Menor concentração Cd total absorvido - Maior [Cd] retido na parede celular da raiz - Maiores relações P, N, S/Cd em P.A e raiz - Melhor nutrição em P - Menor atividade GPX na raiz na presença de Cd	- Maior crescimento - Maior concentração de clorofila - Maior conteúdo P na P.A - Maior IT nutrientes - Menor atividade GPX na raiz na presença de Cd

M- micorrizada, NM- não micorrizada, P.A- parte aérea, IT- índice de translocação, FT_{SOL-PA}- fator de transferência de Cd da solução à parte aérea, [Cd]- concentração de cádmio, n.d- não determinado, n.s não significativo, - efeito negativo, + efeito positivo. (IT = 100 x concentração de Cd na P.A / concentração de Cd P.A + Cd raiz; $FT_{SOL-P.A}$ = concentração Cd na P.A / concentração Cd na solução).

Uma das possíveis razões para as diferentes respostas fisiológicas ao excesso de Cd está relacionada à sua distribuição e acumulação nos diferentes tecidos e órgãos vegetais (Vassilev et al., 1998). Os resultados mostraram que a concentração de Cd nas raízes foi sempre maior do que na parte aérea das plantas (Tabela 1). De 77 a 96% do Cd absorvido foi retido nas raízes, nas três plantas estudadas (Tabela 1). O conteúdo de Cd na parte aérea do milho e girassol foi, em média, 25 e 228 mg kg^{-1}, enquanto que nas raízes foi 313 e 789 mg kg^{-1} no milho e no girassol, respectivamente. Portanto, de fato, as raízes dessas plantas atuaram como uma barreira para a translocação de íons Cd à parte aérea. Pode se dizer que a resposta diferiu para cada associação FMA-hospedeiro no que se refere à absorção e acumulação de Cd. O feijão de porco, o qual foi colonizado por diferentes espécies de FMA, mostrou respostas diferentes dependendo do fungo associado (Capítulo 2). Assim, o feijão de porco colonizado por *G. etunicatum* apresentou significativamente maior concentração de Cd do que plantas NM (Tabela 1). Já a absorção de Cd foi menor ou não significativa quando o feijão de porco foi colonizado pelas outras espécies de FMA (Capítulo 2). De forma similar, o girassol associado a *G. intraradices* acumulou maiores concentrações de Cd na parte aérea e raiz do que plantas não associadas com FMA (Tabela 1). No entanto, a influência da micorrização na absorção de Cd pelo milho não foi significativa, apresentando plantas M concentrações de Cd similares ou menores do que plantas NM. A observação de alguns autores de que plantas M aumentam a relação Cd raiz/Cd parte aérea, protegendo a parte aérea de concentrações excessivas de metais (Joner et al., 2000; Turnau et al., 1993) não foi constatada neste estudo. Portanto, a hipótese de que plantas M retêm maiores quantidades de Cd na raiz, evitando a translocação do Cd à parte aérea, não

pode ser aceita neste caso, já que plantas M e NM apresentaram índices de translocação de Cd similares (Tabela 1).

O índice de translocação (IT) de Cd foi cerca de três vezes maior em plantas de girassol do que nas de milho e, em ambas, o IT de Cd foi similar para plantas M e NM indicando que da concentração total de Cd absorvida, quantidades similares de Cd foram translocadas para a parte aérea (Tabela 1). No entanto, no girassol o fator de transferência (FT) de Cd, que indica a capacidade da planta de acumular Cd em relação a concentração de Cd na solução, foi maior nas plantas M sugerindo que estas têm maior capacidade de absorção de Cd, acumulando maiores quantidades na parte aérea e nas raízes (Tabela 1).

Do ponto de vista da fitoextração, observou-se que o girassol foi a planta que maior quantidade de Cd translocou à parte aérea e a que apresentou maior capacidade de bioconcentração de Cd (FT) (Tabela 1), sendo os valores acumulados (228 mg kg^{-1}) comparáveis aos observados em espécies hiperacumuladoras. Já no milho, o FT foi menor em plantas M do que nas NM indicando menor absorção de Cd pela planta. Esses dados sugerem que no milho M, o FMA atuou como uma barreira, podendo ter retido o Cd no micélio extraradicular, já que, de fato, este acumulou duas vezes mais Cd do que as próprias raízes da planta (Tabela 1). Assim, o efeito da micorrização na absorção de metais, mais uma vez, demonstrou estar relacionado com a espécie de FMA e o hospedeiro, como já constatado por outros autores (Killham e Fierestone, 1983; Weissenhorn e Leyval, 1995).

A hipótese de que a micorrização confere certa tolerância ao excesso de Cd no meio de crescimento foi verificada, ou ao menos, que as plantas associadas com FMA

apresentaram melhores condições fisiológicas em situações de estresse por excesso de Cd. Este efeito benéfico da associação micorrízica foi fundamentalmente devido ao melhor estado nutricional apresentado por plantas M. Tanto o feijão de porco quanto o milho e o girassol tiveram maiores conteúdos de P e de outros nutrientes na presença de Cd. Também foi interessante a relação P, N e S com o Cd que foi maior nas plantas M, o que se refletiu em maior produção de biomassa no caso do milho e do girassol. Cabe destacar também a maior absorção de S, acompanhada de uma maior relação S/Cd na parte aérea e raízes de milho M em relação às plantas NM (capítulo 3.1). Essa maior absorção de S pode estar relacionada com a maior demanda de glutationa, composto rico em S, como conseqüência da indução da síntese de FQs (Nocito et al., 2002). Galli et al. (1995) constataram que raízes de milho micorrizadas apresentaram incremento nos seus conteúdos de thiois (cisteina, γ-glutamilcisteina e glutationa) em meio com adição de cobre, mesmo que a micorriza não tenha evitado a absorção excessiva do metal pela planta.

Raízes M de milho e girassol apresentaram, no geral, maiores quantidades de proteínas solúveis totais indicando diferente condição metabólica em resposta a micorrização (Capítulos 3 e 4). Mesmo que esse resultado não esclareça o tipo de proteínas presentes, nem a quantidade em que cada uma foi expressa, há uma indicação de que, de fato, raízes M e NM são fisiologicamente diferentes. Raízes de girassol tratadas com Cd mantiveram significativamente maiores quantidades de proteínas solúveis totais quando micorrizadas do que plantas NM (Capítulo 4), sendo que essa maior concentração de proteínas pode estar relacionada com a atenuação do estresse causado pelas altas concentrações de Cd no meio. Repetto et al. (2003), utilizando

estudos do proteoma de plantas associadas à FMAs, observaram que a expressão de proteínas induzidas na presença de Cd são reguladas pela simbiose MA. Assim, foi constatado que enquanto em raízes NM de *Pisum sativum* o Cd induziu a expressão da proteína *Sad* A, uma álcool desidrogenase de cadeia curta, as raízes M tiveram repressão da sua expressão (Repetto et al., 2003). As proteínas *Sad* estão envolvidas no metabolismo de fitoesteroides (Broshé e Strid, 1999) e estes induzem uma série de processos celulares, como alongamento do talo, inibição do crescimento radicular ou do fluxo de prótons ou a regulação da expressão gênica, que são, por sua vez, afetados pelo Cd (Sanità di Toppi e Gabbrielli, 1999). Por outro lado, substâncias similares às giberelinas e ao ácido abscísico tem sido relatadas em FMA assim como também mudanças nos níveis hormonais de raízes M (Ludwig-Müller, 2000). Com base nestas observações, Repetto et al. (2003) sugeriram que a síntese ou a indução da produção hormonal em plantas M sob estresse por Cd, diminuiria os níveis de expressão de proteínas *Sad*, reduzindo-se os efeitos negativos do Cd sobre planta.

Sabe-se que o sistema antioxidante de plantas M difere significativamente daquele de plantas não colonizadas por FMA (Lambais et al., 2003). No entanto, a maioria dos estudos observando diferenças nas atividades antioxidativas em plantas M centra-se nas mudanças que acontecem no processo da colonização micorrízica ou ainda em resposta a fatores de tipo biótico, como atenuação da ativação do sistema de defesa da planta (Lambais et al., 2003; Lambais e Mehdy, 1998). Em relação ao efeito do estresse por MPs no sistema antioxidante da MA, os dados ainda são escassos, havendo alguns trabalhos com fungos ectomicorrízicos como revisto por Schutzendübel e Polle (2002). A atividade de peroxidases pode ser utilizada como marcador bioquímico da

resposta específica da raiz ao estresse causado pelo Cd ou ainda à presença do simbionte. No presente estudo, a atividade GPX das raízes foi sensível à presença do FMA, no feijão de porco e girassol, bem como à presença de Cd na solução nutritiva. A resposta da atividade GPX à adição de Cd variou com a planta; no feijão de porco a resposta foi diferente da esperada, pois a atividade diminuiu em raízes tratadas com Cd, enquanto que no girassol e no milho, a atividade foi induzida na presença do metal. Assim, a enzima foi um indicador sensível do estresse oxidativo causado pelo Cd. Além disso, houve interação significativa do Cd e FMA na resposta da atividade GPX das raízes, indicando que plantas M e NM tem atividade peroxidase diferente em situação de excesso de Cd.

Joner et al. (2000) observaram que a competição por sítios de adsorção nas hifas de FMA entre Cd, Ca e Zn favorecia o Cd, sendo que as hifas fúngicas apresentaram maior capacidade de adsorção deste metal do que os demais íons. O micélio externo dos FMAs que colonizaram plantas de milho e de girassol apresentaram alta capacidade de retenção de ions Cd (Tabela 1). A concentração de Cd foi duas vezes maior nas hifas extraradiculares do FMA do que nas raízes de milho e muito semelhantes as observadas em raízes de girassol, cerca de 720 μg Cd g^{-1}. Esses resultados são similares aos observados por Joner et al. (2000), os quais encontraram concentrações de Cd nas hifas de FMA 2 a 4 vezes maiores que as encontradas nas raízes de trevo e centeio. A tolerância a metais em um organismo pode estar associada com um maior seqüestro intracelular do metal e uma menor absorção (Gadd, 1993). A menor absorção, por sua vez, pode estar relacionada com transporte seletivo de ions ou impermeabilidade das membranas, mas a adsorção de metais em paredes celulares, pigmentos ou ainda em

metabólitos extacelulares, pode constituir um mecanismo relevante na tolerância de fungos e sua sobrevivência em ambiente com níveis excessivos de metais (Gadd, 1993). Sendo que os fungos micorrízicos pertecem ao único grupo de microrganismos capazes de transferir elementos minerais do solo, ou do meio de crescimento, às plantas, a possibilidade de atuarem como um filtro durante a absorção de metais pelas plantas torna-os de relevância na tolerância e proteção contra os elementos potencialmente tóxicos. No presente estudo, o FMA somente atuo como filtro na absorção de Cd em plantas de milho, pois tanto o FT quanto a quantidade total de Cd absorvida (parte aérea + raiz) foram menores nas plantas M. No entanto, o FMA não protegeu a planta da absorção de concentrações excessivas do metal, retendo na parte aérea concentrações de 24 mg kg^{-1} de Cd na matéria seca

As raízes de várias espécies vegetais atuam como barreira na restrição da translocação de metais à parte aérea, sendo esse mecanismo considerado como uma das estratégias de tolerância a metais (Lozano-Rodriguez et al., 1997). Além disso, a adsorção de metais na parede celular dos tecidos da raiz pode reduzir também as concentrações intracelulares de metais em níveis fisiologicamente menos prejudiciais (Rivera-Becerril et al., 2002). No presente estudo, tanto em raízes de milho como nas de girassol, o Cd ficou principalmente retido na parede celular sendo que no caso de girassol não houve diferença entre raízes M e NM, mas nas raízes de milho M o Cd foi retido em maior quantidade na parede celular do que nas raízes NM.

Além dos efeitos deletérios dos MPs nas plantas, os microorganismos do solo e sua atividade são também afetados pelo excesso de metais (Andrade e Silveira, 2004), tendo particular importância a consideração de tais efeitos naqueles microorganismos

que formam simbioses com as plantas. Sabe-se que os MPs podem reduzir a colonização intraradical do FMA (Citterio et al., 2005; Andrade et al., 2004;) e que processos como germinação de esporos e crescimento de hifas podem também ser inibidos por MPs (Weissenhorn et al., 1993), causando atraso na colonização radicular. Mesmo assim, há casos em que MPs em concentrações excessivas não afetaram a colonização radicular do FMA (Riveira-Becerril et al., 2002). Neste trabalho, o Cd somente afetou a colonização das raízes do milho pelo FMA *G. intraradices*. Apesar disso, a eficiência da simbiose micorrízica não está diretamente relacionada com o comprimento de raiz colonizada, pois plantas com menor colonização micorrízica podem estar se beneficiando ainda da associação com o FMA. Isso foi verificado no caso do milho, que apesar do Cd reduzir a colonização da raiz pelo FMA, o fungo promoveu o seu crescimento e teve maior fornecimento de P e outros nutrientes mesmo na presença do metal (Tabela 1, Capítulo 3). A coloração da raiz com azul de tripano, para observação de estruturas micorrízicas, não discrimina estruturas vivas e ativas das mortas ou inativas e portanto, não se pode relacionar a colonização avaliada dessa forma com a funcionalidade da simbiose. Corantes vitais como aqueles que revelam as atividades da succinato desidrogenase ou fosfatase alcalina seriam de maior utilidade na avaliação da redução e perda de eficiência da colonização intraradical do FMA (Smith e Gianinazzi-Pearson, 1990; Tisserant et al., 1993) pelo Cd.

Plantas capazes de absorver e tolerar altos níveis de metais oferecem uma alternativa para remediação de solos contaminados com metais. As plantas possuem mecanismos constitutivos, presentes no seu fenótipo, e adaptativos, presentes somente naqueles fenótipos mais tolerantes, para o acúmulo e tolerância a metais. Um desses

mecanismos adaptativos que as plantas adquirem no meio é a formação de micorriza arbuscular. A micorriza arbuscular proporciona, normalmente, uma nutrição mineral mais eficiente conferindo à planta certa vantagem seletiva sobre outras plantas em locais contaminados com metais (Khan et al., 2000). A maior biomassa e a maior capacidade de absorver P e outros nutrientes, requisitos importantes para o sucesso de remediação de solos, tornam a micorriza um interessante sistema com potencial no uso de remediação de solos contaminados. O uso de biotecnologias que utilizem plantas associadas à FMA na remediação, bem como revegetação de áreas contaminadas ou visando a fitoextração de metais, é uma ferramenta ecologicamente correta e com potencial que deveria ser mais bem abordado e considerado de forma mais efetiva nos programas e pesquisa sobre fitorremediação.

6. CONCLUSÕES

- As plantas estudadas, feijão de porco, milho e girassol, beneficiam-se da associação com fungos micorrízicos, tanto na ausência como na presença de Cd.
- O girassol e o feijão de porco associados à FMAs mostraram maior capacidade de retenção de Cd nas raízes do que plantas não associadas a esse tipo de fungos devido, possivelmente, à presença das hifas intrarradicais do FMA. No entanto, as hifas dos FMAs não evitaram a translocação de quantidades excessivas de Cd à parte aérea.
- As plantas estudadas associadas com os diferentes FMA mostraram respostas diferentes em termos de acúmulo de Cd. O feijão de porco, quando associado a *G. etunicatum*, e o girassol absorveram maiores quantidades de Cd; já o milho micorrizado absorveu menores concentrações de Cd do que homólogos não micorrizados.
- O girassol foi um extrator eficiente de Cd e pode ser considerada uma planta hiperacumuladora de Cd, acumulando na parte aérea concentrações de Cd superiores a 100 mg kg^{-1} de massa seca.
- A micorriza arbuscular formada pelo girassol e o FMA *G. intraradices* mostrou-se um eficiente sistema de fitoextração de Cd, transferindo maiores quantidades de Cd da solução à parte aérea da planta e conferindo maior tolerância à planta ao excesso do metal na solução.

- O girassol micorrizado absorveu e translocou quantidades de Cd maiores do que plantas não colonizadas pelo FMA, mostrando ainda melhor desenvolvimento.
- O FMA *G. macrocarpum* atenuou o estresse do milho ao excesso de Cd no meio promovendo o crescimento da planta e melhorando sua nutrição mineral.
- A enzima guaiacol peroxidase foi um indicador sensível do estresse oxidativo causado pelo Cd nas raízes, sendo induzida na presença do metal e influenciada pela micorrização das plantas.

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