UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM AGROBIOLOGIA

EFEITO DO ZINCO EM PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS DE *Pfaffia glomerata* (SPRENG.) PEDERSEN

DISSERTAÇÃO DE MESTRADO

Katieli Bernardy

Santa Maria, RS, Brasil
2015

EFEITO DO ZINCO EM PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS DE *Pfaffia glomerata* (SPRENG.) PEDERSEN

Katieli Bernardy

Dissertação apresentada ao Programa de Pós-Graduação em Agrobiologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Agrobiologia**

Orientador: Prof. Fernando Teixeira Nicoloso

Santa Maria, RS, Brasil
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A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

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Elaborada por
Katieli Bernardy

como requisito parcial para obtenção do grau de
Mestre em Agrobiologia

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Santa Maria, 6 de março de 2015

DEDICATÓRIA

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"O que vale na vida não é o ponto
de partida e sim a caminhada.
Caminhando e semeando, no fim terás o
que colher."

Cora Coralina

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Agrobiologia
Universidade Federal de Santa Maria

EFEITO DO ZINCO EM PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS DE
Pfaffia glomerata (SPRENG.) PEDERSEN

AUTORA: KATIELI BERNARDY
ORIENTADOR: FERNANDO TEIXEIRA NICOLOSO
Data e Local da Defesa: Santa Maria, 6 de março de 2015.

Atividades industriais e de mineração combinadas com o uso inadequado de fertilizantes e pesticidas tem contribuído para o aumento dos níveis de zinco (Zn) no solo. Vários distúrbios fisiológicos e bioquímicos em plantas estão associados com esse aumento dos teores de metais pesados como o Zn nos solos e a *Pfaffia glomerata* (Spreng.) Pedersen vêm sendo utilizada como espécie modelo em estudos relacionados sobre o efeito de diferentes níveis de metais pesados e já foi verificado que a mesma apresenta tolerância intermediaria à alta para diversos metais, bem como diferentes respostas entre os acessos. Assim, o presente estudo teve como objetivo caracterizar os efeitos de diferentes concentrações de Zn sobre parâmetros fisiológicos e bioquímicos de acessos de *P. glomerata*. Para o primeiro experimento foram utilizados quatro níveis de Zn (2, 100, 200 e 300 µM) e três acessos de *P. glomerata* (BRA, GD e JB) a fim de avaliar a produção de matéria seca, morfologia radicular e o nível de tolerância entre os acessos. Os acessos JB e GD apresentaram redução na produção de matéria seca tanto da parte aérea quanto radicular, bem como redução da área, comprimento e volume radicular, no entanto o acesso GD apresentou incremento para a dose 100 µM de Zn. Já o acesso BRA mostrou as menores variações para esses mesmos parâmetros, no entanto, isso se deve ao seu baixo crescimento em relação aos demais acessos. Como base nos resultados da produção de matéria seca e parâmetros radiculares dos acessos, os mesmos apresentaram a seguinte classificação em relação à tolerância ao Zn: GD> JB> BRA. No segundo experimento foram utilizados três níveis de Zn (2, 100 e 200 mg kg^{-1}) e os dois acessos de *P. glomerata* mais tolerantes selecionados no experimento anterior (GD e JB) e avaliadas a produção de matéria seca, parâmetros fotossintéticos e acúmulo do Zn aos 34 e 74 dias. Para a maioria dos parâmetros avaliados as plantas responderam positivamente para a dose de 100 mg de Zn, sendo a dose de 200 mg prejudicial. A máxima fotossíntese, transpiração e condutância estomática foram observadas para as plantas do acesso GD mediante a adição de 100 mg kg^{-1} de Zn ao solo aos 34 dias de cultivo. O acesso GD apresentou aumento no índice estomático com a adição de 200 mg Zn, já o JB não mostrou qualquer alteração. O aumento da concentração de Zn no solo resultou em aumento na concentração do metal nos tecidos das plantas. O acesso GD mostrou maior translocação de Zn para as folhas e menor acúmulo de Zn nas raízes aos 74 dias,

enquanto o JB não apresentou diferenças para os níveis de Zn testados No terceiro experimento avaliou-se a influência do excesso de Zn sobre a resposta antioxidante e produção de β-ecdisona em plantas de *P. glomerata* cultivadas em dois ambientes distintos. Foram utilizados os mesmos dois acessos de *P. glomerata* (JB e GD) e três níveis de Zn (2, 100 e 200 µM) para cultivo em hidroponia com avaliações aos 7 e 14 dias, e os mesmos acessos de *P. glomerata* submetidos à três níveis de Zn (2, 100 e 200 mg kg^{-1}) para o experimento em solo com avaliações aos 34 e 74 dias. Os efeitos do excesso de Zn sobre os acessos de *P. glomerata* dependeram do sistema de cultivo e também do período de avaliação. O excesso de Zn em ambos os sistemas de crescimento testados causou aumento na peroxidação lipídica (concentração MDA) e aumento da atividade das enzimas superóxido dismutase (SOD) e guaiacol peroxidase (GPX) em ambos os acessos de *P. glomerata*, no entanto, no experimento em solo os acessos apresentaram maior diferença entre ambos em relação à enzima GPX, tendo o GD apresentado os maiores valores. A concentração de antocianinas nas folhas e β-ecdisona em raízes foram significativamente alteradas após a adição de Zn no substrato de cultivo. O acesso JB mostrou redução no conteúdo de β-ecdisona com a adição de Zn no solo, já para o GD foi observado o oposto, no entanto, em condições ideais o acesso JB mostrou um conteúdo mais elevado de β-ecdisona. A concentração de antocianinas variou de acordo com o sistema de cultivo, onde as plantas cultivadas em hidroponia exibiram maiores alterações em ambos os períodos de avaliações.

Palavras chave: metais pesados, toxidez de Zn, ginseng brasileiro, crescimento, sistema antioxidante.

ABSTRACT

Master Dissertation
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EFFECT OF ZINC IN BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS OF *Pfaffia glomerata* (Spreng.) PEDERSEN

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Date and Place of Presentation: Santa Maria, March 6 2015.

Industrial and mining activities combined with the inadequate use of fertilizers and pesticides have contributed to increase of zinc (Zn) levels in soil. Several physiological and biochemical disorders in plants are frequently associated with the increase of heavy metals levels such Zn in the soils and the *Pffafia glomerata* (Spreng.) Pedersen have been used as model species in studies associated about the effect of different levels of heavy metals and already was verified that the same presented intermediate to high tolerance to various metals, as well as different responses between accessions. Thus, the present study was aimed at characterizing the effects of different concentrations of Zn on physiological and biochemical parameters of *P. glomerata* accessions. For the first experiment were used four levels of Zn (2, 100, 200 and 300 μM) and three accessions of *P. glomerata* (BRA, GD and JB) to evaluate the production of dry matter, root morphology and the level of tolerance between accessions. The JB and GD accessions showed reduction in dry matter production, both in shoots as roots, as well as reduction of area, length and root volume, however the GD accession showed increase around of level of 100 μM Zn. Already the BRA accession showed smaller variations to those same variables, however, this is due to its low growth in relation to other accessions. As base in the results of dry matter production and root parameters of accessions, the same presented the following classification in relation to tolerance to Zn: GD> JB> BRA. In the second experiment were used three levels of Zn (2, 100 and 200 mg kg^{-1}) and the two *P. glomerata* accessions selected in the previous experiment (GD and JB) and evaluated the production of dry matter, photosynthetic parameters and Zn accumulation to 34 and 74 days. For most parameters evaluated the plants responded positively to dose of 100 mg Zn, and the dose of 200 mg detrimental. The maximum photosynthesis, stomatal conductance and transpiration were observed for GD accession plants by the addition of 100 mg kg^{-1} of Zn in soil to 34 days of cultivation. The GD accession presented increase in stomatal index with the addition of 200 mg Zn, whereas the JB did not show any alteration. The increase of Zn concentration in soil resulted in an increase in the metal concentration in plant tissues. The accession GD showed increase in Zn translocation to the leaves and reduction in the Zn accumulation in roots to 74 days, while the JB presented not differences to the Zn levels tested. In the third experiment was evaluated the influence of

excess zinc about the antioxidant response and production of β-ecdysone in *P. glomerata* plants cultivated in two different environments. Were used the same two accessions of *P. glomerata* (JB and GD) and three levels of zinc (2, 100 and 200 μM) for cultivation in hydroponics with evaluations to 7 and 14 days, and the same accessions submitted to three Zn levels (2, 100 and 200 mg kg^{-1}) for the experiment in soil with evaluated to 34 and 74 days. The effects of excess Zn on *P. glomerata* accessions were dependent of culture system and also evaluation period. The excess of Zn in both systems growth tested caused increase in lipid peroxidation (MDA concentration) and increase of enzyme superoxide dismutase (SOD) and guaiacol peroxidase (GPX) in both *P. glomerata* accessions, however, in soil experiment the accessions presented higher difference between them in relation to GPX enzyme, GD having presented the highest values. The concentration of anthocyanin in leaves and β-ecdysone in roots were significantly altered after the addition of Zn in the growth substrate. JB accession showed reduction in β-ecdysone content with the addition of Zn in soil, already for the GD was observed the opposite, however under ideal conditions the JB accession showed higher β-ecdysone content. The anthocyanin concentration varied according with the cultivation system, where the plants grown in hydroponic exhibit higher changes in both evaluation periods.

Keywords: Heavy metals, Zn toxicity, Brazilian ginseng, growth, antioxidant system.

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO GERAL

A utilização de plantas como fonte de medicamentos para o tratamento de enfermidades é relatada desde o início da civilização. Atualmente o uso de espécies medicinais está em expansão em todo o planeta, inclusive no Brasil, pois as plantas representam uma fonte valiosa de metabólitos secundários, os quais são muito utilizados pela indústria farmacêutica na produção de medicamentos fitoterápicos, além de servirem de modelos para a síntese de fármacos (FLORES, 2006; BARBOZA, 2007).

O emprego de medicamentos fitoterápicos vem ganhando cada vez mais espaço no tratamento de patologias e por isso a impotância de estudos relacionados a espécies medicinais. Entre as plantas brasileiras com potencial medicinal pode-se citar a *Pfaffia glomerata* (Spreng.) Pedersen, conhecida popularmente como ginseng brasileiro. A espécie é considerada de grande importância em razão de apresentar uma longa lista de indicações medicinais. A *P. glomerata* apresenta efeito adaptógeno, tônico e afrodisíaco, além de ser utilizada para o tratamento de diversas enfermidades (MAGALHÃES, 2000; ZIMMER et al., 2006).

As raízes, caules e folhas da *P. glomerata* são ricas em saponinas triterpênicas e ecdisteróides, sendo que vários compostos já foram isolados, como o ácido gloméricolo, ácido oleanólico, ácido pfamérico, rubresterona e ecdisterona (SHIOBARA et al., 1993). Os teores dos metabólitos podem variar conforme o local da coleta, devido as diferentes condições ambientais e variabilidade genética existente, e ainda conforme a parte da planta coletada (raiz, caule ou folhas), pois existem diferenças de concentrações entre elas (FIGUEIREDO et al., 2004). As condições impostas durante o cultivo das plantas (como por exemplo, déficit hidríco, radiação UV, deficiência ou excesso de nutrientes, entre outras) também apresentam influência direta na biossíntese e/ou acúmulo de diferentes metabólitos (TOMÁS et al., 1993; GOBBO-NETO; LOPEZ, 2007).

Os metais pesados são encontrados naturalmente no solo em baixas concentrações, como resultado do intemperismo e de outros processos pedogenéticos (KEDE et al., 2008). Alguns desses elementos são essenciais para várias funções fisiológicas nos seres vivos, porém requeridos em baixas concentrações, enquanto outros não apresentam funções biológicas conhecidas (BAKER et al., 1994). No entanto, devido à intensificação das atividades agrícolas,

industriais e urbanização, a poluição do solo com metais pesados vem se tornando relativamente frequente (MARSCHNER, 1995; PINTO et al., 2009).

Dentre esses metais pesados o zinco (Zn) é essencial para o crescimento das plantas, pois é importante no metabolismo de carboidratos e proteínas, bem como para a síntese de RNA. O elemento também é importante para a ativação enzimática, requerido na biossíntese de clorofila, formação da estrutura de auxinas, multiplicação celular e fertilidade dos grãos de pólen (BORKET, 1989; MALAVOLTA et al., 1991; MARSCHNER, 1995; BROADLEY et al., 2007; PRADO, 2008). No entanto, altas concentrações desse metal podem torná-lo altamente tóxico e quando acumulado em excesso nos tecidos vegetais pode provocar várias alterações tanto no metabolismo primário quanto no secundário em plantas (MARCHESE, 1999; STOYANOVA; DONCHEVA, 2002; BROADLEY et al., 2007).

O excesso do Zn no solo pode causar a inibição na germinação de sementes (MICHAEL; KRISHNASWAMY, 2011), atraso ou retardo no crescimento das plantas (ANDRADE et al., 2009), danos em raízes, indução à cloroses foliares (BROADLEY et al., 2007; WANG et al., 2009), alteração da atividade mitótica (ROUT; DAS, 2003), redução da taxa fotossintética devido a inibição da biossíntese de clorofila e diminuição da assimilação de carbono (CHEN et al., 2008; DHIR et al., 2008). Além disso, níveis elevados de Zn induzem a efeitos negativos sobre a integridade da membrana (De MAGALHÃES et al., 2004), interferem na absorção de solutos, transporte e regulação osmótica (CHERIF et al., 2010), aumentam a produção de espécies reativas de oxigênio (EROs) e inibem ou incrementam a atividade enzimática (GRATÃO et al., 2005; LIN et al., 2005).

Tendo em vista que nem todas as espécies vegetais desenvolvem-se em ambientes contaminados por metais pesados e que podem ocorrer diferentes níveis de tolerância entre genótipos ou acessos de uma mesma espécie, torna-se necessário o conhecimento sobre o comportamento de uma espécie quando exposta a um determinado contaminate e também o nível de tolerância entre genótipos e acessos dessa mesma espécie (MARQUES et al., 2011). Essa varredura pode ser feita por meio da avaliação da capacidade de crescimento, produção de biomassa e acúmulo do elemento nos tecidos na presença de concentrações crescentes do elemento no solo (WATANABE, 1997; MARQUES et al., 2006; ROSA, 2006).

Pesquisas realizadas com *P. glomerata* demostram que a espécie apresenta tolerância intermediária a alta para vários metais como alumínio, cádmio, mercúrio, chumbo, arsênio e cobre, bem como diferenças nas respostas entre acessos da espécie (MALDANER, 2008;

SKREBSKY et al., 2008; CALGAROTO, 2009; GUPTA et al., 2013; NEIS, 2013). Kamada et al. (2009) ressaltam que a espécie apresenta grande diversidade genética entre as suas populações naturais, o que pode resultar em uma significativa variabilidade e, portanto diferentes níveis de tolerância a metais pesados. Em relação ao metabolismo secundário da espécie, variações no conteúdo de β-ecdisona entre diferentes acessos da espécie também foram observados, comprovando assim a variabilidade genética existente, bem como a influência de diferentes níveis de alguns nutrientes na concentração do composto, comprovando assim a influência da nutrição sobre o metabolismo secundário (NEIS, 2013).

O aumento nos níveis de Zn no solo ocorre por meio de processos industriais como mineração e fundição ou por meio de técnicas agrícolas como aplicação de biossólidos, fertilizantes e pesticidas (REICHMAN, 2002; WUANA; OKIEIMEN, 2011). Tendo em vista o aumento das contaminações do ambiente pelo excesso de Zn, torna-se de extrema importância o estudo do efeito do excesso desse elemento sobre os processos fisiológicos das espécies vegetais, bem como os possíveis mecanismos adotados pelas mesmas para contornar essa situação de estresse. Sendo a *P. glomerata* utilizada como planta modelo para estudos sobre o efeito de metais pesados e já relatado o comportamento da espécie em relação a alguns metais pesados, torna-se importante também conhecer o efeito de diferentes níveis de Zn sobre a espécie.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Caracterizar os efeitos de níveis crescentes de Zn sobre parâmetros fisiológicos e bioquímicos de acessos de *Pfaffia glomerata* (Spreng.) Pedersen.

1.1.2 Objetivos específicos

I) Distinguir três acessos de *P. glomerata* quanto ao nível de tolerância ao Zn;
II) Caracterizar aspectos bioquímico-fisiológicos da toxidez de Zn em dois acessos de *P. glomerata*;

III) Avaliar a produção de β-ecdisona em raízes de dois acessos de *P. glomerata* em condições de exposição a diferentes concentrações de Zn.

2 REVISÃO DE LITERATURA

2.1 Características gerais sobre a *Pfaffia glomerata* (Spreng.) Pedersen

A família Amaranthaceae apresenta cerca de 170 gêneros e 2000 espécies, sendo encontradas no Brasil cerca de 20 gêneros e aproximadamente 100 espécies nativas (SOUZA; LORENZI, 2005). O gênero *Pfaffia* possui 27 espécies distribuídas no Brasil, sendo essas caracterizadas como ervas ou subarbustos eretos ou semiprostrados que ocorrem em cerrados, campos rupestres ou limpos, orlas de matas e beiras de rios (MARCHIORETTO et al., 2009).

Dentro do gênero *Pfaffia*, espécies como a *P. glomerata*, *P. iresinoides* (Kunth) Spreng e *P. paniculata* (Mart.) Kuntze são muito utilizadas como fitoterápicos em substituição da espécie coreana *Panax Ginseng* C.A. Meyer, devido as suas semelhantes composições químicas e efeitos (SHIOBARA et al., 1992). *P. glomerata* também é conhecida como fáfia, para-tudo, caxixe, ou ginseng brasileiro (SMITH; DOWNS, 1972) e apresenta raízes com formatos variados, destacando-se as típicas formas humanoides, das quais deriva o nome "ginseng" que significa "imagem do homem", em chinês (SCHENKEL et al., 2002).

A espécie pode ser encontrada naturalmente em ambientes de mata ciliar, campos inundáveis à beira de rios e em orlas de matas de galeria, e no Brasil ocorre principalmente no estado do Paraná e Mato Grosso do Sul (PACHECO et al., 2012). A *P. glomerata* caracteriza-se por ser uma espécie higrófila e heliófila, que apresenta grande adaptabilidade e rápido crescimento, no entanto se desenvolvendo melhor em regiões com temperatura elevada, sendo bastante sensível à geadas (MAGALHÃES, 2000; VIGO et al., 2004).

As raízes da *P. glomerata* são conhecidas por apresentarem efeitos tônicos, adaptógenos e afrodisíacos (MARQUES et al., 2004; FREITAS et al., 2004), além de apresentarem componentes que atuam na regeneração de células, purificação do sangue e inibição do crescimento de células cancerígenas (NISHIMOTO et al., 1986; NISHIMOTO et al., 1987; SHIOBARA et al., 1993; GUEREIRO, 2006). Outros autores ainda relataram que

folhas e raízes da espécie possuem propriedades antioxidantes, tranquilizantes, anti-inflamatórias, analgésicas, febrífugas, cicatrizantes, anti-hemorroidas, melhoradoras da visão e da memória e antidiabéticas (NETO et al., 2005; SILVA JÚNIOR; OSAIDA, 2005; SOUZA et al., 2005; LEITE et al., 2008).

Muitos estudos clínicos já foram realizados com *P. glomerata* para comprovar os seus efeitos. Extratos de raízes de *P. glomerata* favoreceram a aprendizagem e memória de cobaias (MARQUES et al., 2004), mostraram-se ativos contra alguns protozoários como *Leishmania brasilienses* (NETO et al., 2004), apresentaram atividade antimicrobiana (ALCÂNTARA et al., 1994), bem como efeito protetor da mucosa gástrica de ratos contra úlceras (FREITAS et al., 2004). Neto et al. (2005) observaram que o extrato hidroalcoólico das raízes apresentou atividades analgésicas e anti-inflamatórias. Nicolodi et al. (2002) comprovaram a atividade adaptógena descrita para a espécie. Em idosos foi observado o aumento da capacidade aeróbica e melhora na memória de curto prazo (MARQUES et al., 1998; ACKEL et al., 1999).

2.2. Características gerais sobre metabólitos secundários em *P. glomerata*

Os vegetais produzem grande variedade de compostos químicos, denominados metabólitos secundários, que têm função principal de defesa contra condições adversas, e constituem-se peças fundamentais no processo adaptativo dos vegetais. Os metabólitos secundários são derivados biossinteticamente do metabolismo primário, portanto estas rotas apresentam uma estreita relação. Existem três principais grupos de metabólitos secundários, de acordo com sua biossíntese: os terpenos, os compostos fenólicos e os compostos contendo nitrogênio (TAIZ; ZEIGER, 2013).

Essas substâncias geralmente possuem estruturas complexas, com baixo peso molecular e apresentam-se em concentrações baixas em determinados grupos de plantas (BERG; LUBERT, 2008). Muitos são de grande importância comercial não apenas na área farmacêutica, mas também nas áreas alimentar, agronômica e perfumaria, devido à imensa atividade farmacológica que possuem (SIMÕES et al., 2007).

Apesar da influência ambiental na produção desses compostos, o controle de sua biossíntese é realizado por genes que codificam enzimas específicas, responsáveis pela formação e regulação das vias biossintéticas. Assim, o controle do metabolismo secundário

depende da capacidade genética da planta em responder a estímulos internos ou externos e da existência desses estímulos no momento apropriado (VIEIRA, 2000).

A produção dessas substâncias é geralmente baixa (menos de 1% de peso seco) e depende do estado fisiológico e fase de desenvolvimento das plantas, sendo a maioria desses compostos armazenados nos vacúolos de células de tecidos específicos da planta (OKSMAN-CALDENTEY; INZÉ, 2004). Como possuem uma grande diversidade química, técnicas cromatográficas e espectroscópicas têm permitido um grande avanço na separação e elucidação estrutural de diferentes compostos, provenientes de diversos grupos de plantas, otimizando sua produção (GIL et al., 2005).

Shiobara et al. (1993) identificaram várias substâncias presentes em extratos de *P. glomerata*, tais como: *β*-ecdisona, rubrosterona, ácido oleanólico, *β*-glucopiranosil oleanolato, ácido glomérico (triterpenóide) e ácido pfamérico (nortriterpenóide). O ecdisteróide β-ecdisona presente na *P. glomerata* pode ser encontrado em diversos órgãos da planta, como em flores, folhas, caules e raízes (NISHIMOTO et al., 1987; FESTUCCI-BUSELLI et al., 2008), no entanto sua extração comercial ocorre principalmente nas raízes (TANAKA et al.,1995).

Freitas et al. (2004) confirmaram a presença de quantidades significativas de β-ecdisona em raízes desta planta. A β-ecdisona é o mais importante ecdisteróide, sendo muito empregada em formulações cosméticas, pois é capaz de fortalecer a barreira hídrica da pele, proporcionando função hidratante e impedindo a perda excessiva de água da epiderme (CORTEZ et al., 1998). Além disso, têm-se extraído β-ecdisona para diversos usos: atividade analgésica, feromônio no controle de insetos e como inibidor ao desenvolvimento de microrganismos (OLIVEIRA, 2012).

Análises realizadas com raízes de diferentes acessos da espécie mostraram teores de β-ecdisona variando de 0,15 a 0,75 % do peso seco, mostrando a variabilidade em relação ao acúmulo deste metabólito pela espécie (MONTANARI JÚNIOR et al., 1999; FREITAS et al., 2004; ZIMMER et al., 2006). Dentre todos os fatores que podem interferir na concentração dos princípios ativos das plantas, a nutrição é um dos que requerem maior atenção, pois o excesso ou a deficiência de nutrientes está diretamente correlacionado à variação na produção dessas substâncias (MARTINS et al.,1995). No entanto, ainda existem poucos estudos relacionando às condições de nutrição das plantas e possíveis alterações em seu metabolismo secundário.

2.3. Efeitos de metais em *P. glomerata*

A contaminação do solo por metais pesados tem origem principalmente através de atividades antrópicas (McBRIDE, 1994) como mineração, atividades industriais, fertilizantes, pesticidas e resíduos urbanos (KÄRELAMPI et al., 2000; REICHMAN, 2002). No solo, esses rejeitos sofrem transformações químicas que podem liberar metais pesados para a solução do solo e causar toxidez às plantas e organismos; ou ainda serem adsorvidos às argilas ou complexados à matéria orgânica representando uma fonte poluidora potencial (CUNNINGHAM et al., 1996).

A presença de metais pesados no solo é perigosa porque, além dos desequilíbrios ambientais causados, os contaminantes podem entrar na cadeia alimentar e colocar em risco à saúde humana (ANGELONI; BINI, 1992). Carneiro et al. (2002) avaliaram o conteúdo de metais pesados presente nos tecidos de uma espécie não identificada pertencente ao gênero *Pfaffia*, encontrada em uma área contaminada por minério de calamina em Minas Gerais. Os mesmos constataram que as plantas acumularam quantidades significativas de Cd e Zn na parte aérea, o que pode indicar um potencial genético à tolerância a metais pesados.

Alguns estudos mais recentes demonstraram o efeito de diferentes metais em acessos de *P. glomerata*. Maldaner (2008) observou que a concentração de Al nos tecidos das raízes e parte aérea aumentou com o incremento de Al no substrato de cultivo, porém a concentração foi maior nas raízes. Calgaroto (2009) observou que a concentração de Hg também foi maior nas raízes do que na parte aérea das plantas expostas ao Hg. Gomes et al. (2013) observaram que o conteúdo Cd na raiz e parte aérea de plantas de *P. glomerata* aumentou à medida que houve o incremento das doses de Cd na solução nutritiva.

Maldaner (2008) ainda observou que em condições de cultivo *in vitro* e hidroponia, as concentrações de Al acima de 100 mg L^{-1} afetaram negativamente a maioria dos parâmetros de crescimento de acessos de *P. glomerata* (JB e BRA). Neis (2013) observou que a máxima produção de massa seca para esses mesmos acessos ocorreu nas doses de 40 mg kg^{-1} de Cu e 10 mg kg^{-1} de P, sendo que a massa seca do sistema radicular do acesso JB foi significativamente maior quando comparada ao acesso BRA. Calgaroto (2009) observou redução na massa fresca e seca das raízes e da parte aérea de plantas expostas ao Hg. Gomes et al. (2013) relatou que embora a produção de matéria seca não foi afetada pelos mais altos níveis de Cd, as plantas apresentaram sintomas de fitotoxicidade.

Em relação à peroxidação lipídica, Maldaner (2008) observou que o Al promoveu maior peroxidação lipídica em plantas do acesso BRA em compração com as do acesso JB. A concentração de H_2O_2 nas raízes do acesso JB diminuiu na presença de 150 mg L^{-1} de Al, por outro lado, houve aumento de H_2O_2 na parte aérea dos dois acessos. Calgaroto (2009) observou aumento na peroxidação lipídica nos tecidos das plantas quando expostos a 50 μM Hg, indicando assim que houve dano aos tecidos. A mesma autora ainda relata que plantas expostas ao Zn (50 μM), apesar de apresentarem diminuição na massa fresca e seca, não mostraram alterações na peroxidação lipídica.

Em relação ao sistema antioxidante, Maldaner (2008) observou que o Al provocou maior alteração na atividade da catalase (CAT) nas raízes do que na parte aérea. Em geral, tanto em raízes como na parte aérea, a atividade da ascorbato peroxidase (APX) foi mais alta no acesso BRA do que no JB. Por outro lado, o decréscimo na atividade da superóxido dismutase (SOD) pela presença de Al foi maior no acesso BRA do que no JB. Calgaroto (2009) observou que a atividade das enzimas antioxidantes e a concentração de antioxidantes não enzimáticos aumentaram com a presença de Hg e com a adição de Zn. O sistema antioxidante de *P. glomerata* foi importante na regulação do dano oxidativo, no entanto, em 50 μM de Hg, estes mecanismos, mesmo com a adição de Zn na solução nutritiva, não foram suficientes para reverter o dano causado pelo elemento.

Neis (2013) ainda avaliou a produção de *β*-ecdisona de acessos de fáfia submetidos a diferentes níveis de P e Cu. As concentrações de *β*-ecdisona detectadas nas raízes de *P. glomerata* do acesso JB foram maiores quando comparadas ao acesso BRA. O conteúdo de β-ecdisona variou entre 0,03 a 0,38 % (g $raiz^{-1}$) para o acesso JB e de 0,08 a 0,14 % (g $raiz^{-1}$) para o acesso BRA. O maior teor de β-ecdisona nas raízes do acesso JB ocorreu na ausência da adição de Cu e 10 mg kg^{-1} de P, enquanto que nas plantas do acesso BRA a maior concentração foi encontrada no tratamento com 40 mg kg^{-1} de Cu e 10 mg kg^{-1} de P.

2.4 Toxidez de Zinco - respostas fisiológicas e bioquímicas em plantas

O Zinco (Zn) está presente nos solos em concentração entre 25 e 68 mg kg^{-1} (ALLOWAY, 1995). Esse elemento é absorvido na forma de Zn^{2+}, podendo também ser na forma de Zn^- quelato. O aumento do pH ou a presença de ânions levam o Zn a precipitar podendo reduzir sua disponibilidade na solução do solo (FRAIGE et al., 2007). Alguns autores

consideram o Zn altamente móvel, enquanto outros atribuem a ele uma mobilidade intermediária (CARNEIRO et al., 2002, LI et al., 2011). Sua disponibilidade para as plantas é governada por vários fatores como pH, teor de matéria orgânica, adsorção pelas argilas e teor de fósforo (TISDALE et al., 1985; BROADLEY et al., 2007).

Dentre esses fatores que afetam a disponibilidade do Zn o mais importante é o pH do solo, e quanto maior seu valor, menor será a disponibilidade do Zn. Além do pH, altas doses de fertilizante fosfatados podem induzir ou aumentar a deficiência de Zn, particularmente em condições de baixa disponibilidade do mesmo. Solos com alto teor de matéria orgânica podem fixar o Zn, ou a microbiota pode imobilizá-lo temporariamente. Já em solos argilosos parte do Zn permanece adsorvido as argilas diminuindo assim sua disponibilidade (PRADO, 2008).

O Zn é essencial para o crescimento das plantas porque participa de vários processos metabólicos, tais como, ativação enzimática, síntese de proteínas e metabolismo de carboidratos, lipídeos e ácidos nucléicos. Além disso, o Zn ainda faz parte dos fatores que controlam a multiplicação e diferenciação celular (LIN et al., 2005). No entanto, altas concentrações deste metal no solo, nas formas disponíveis, tornando-o altamente tóxico (MARSCHNER, 1995; BROADLEY et al., 2007).

O Zn é um metal muito utilizado, especialmente como cobertura protetora para outros metais como o ferro e o aço, ligas de bronze e latão, baterias e em componentes elétricos (SMITH et al., 1995), e sua contaminação está geralmente ligada à mineração e uso inadequado de fertilizantes e pesticidas (REICHMAN, 2002). Outras fontes de contaminação são o aço galvanizado, o lodo de esgoto e os dejetos suínos (ALLOWAY; AYRES, 1997).

Quando o Zn é acumulado em excesso pelas plantas, pode provocar inibição no crescimento vegetal, acarretando em diminuição tanto da produção de matéria seca da parte aérea, quanto da biomassa radicular, além de redução na taxa fotossintética e conteúdo de clorofila, inibição de várias enzimas, alteração da atividade mitótica e da integridade das membranas (CARNEIRO et al., 2002; STOYANOVA; DONCHEVA, 2002; LIN et al., 2005; LI et al., 2011; LI et al., 2013).

O excesso de Zn pode ainda induzir à formação de espécies reativas de oxigênio (EROs), que são considerados os principais agentes causadores de injúrias em tecidos depois da exposição das plantas à metais pesados (MALLICK, 2004). O Zn não é capaz de produzir EROs diretamente pela Reação de Fenton, pois é um metal não-redox. No entanto a geração de

estresse oxidativo se dá pela interferência no sistema de defesa antioxidante das plantas, devido ao imbalanço entre respostas antioxidantes e o aumento das EROs (GRATÃO et al., 2005).

As EROs possuem potencial para interagir de forma não específica com muitos componentes celulares, desencadeando reações peroxidativas e causando danos em membranas e outras macromoléculas, como pigmentos fotossintéticos, proteínas, ácidos nucléicos e lipídios (FOYER et al., 1994). Com o rompimento das membranas, ocorre também a perda de fluidos e do gradiente osmótico nas células (LIN et al., 2005). Para controlar os nível de EROs e proteger as células, as plantas possuem um sistema de defesa antioxidante enzimático e não enzimático (GRATÃO et al., 2005).

Os antioxidantes enzimáticos incluem a superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT) e peroxidases (POD), já os antioxidantes não enzimáticos incluem a glutationa (GSH), ácido ascórbico (ASA), carotenóides e tocoferóis que atuam na eliminação das EROs (MITTLER et al., 2004; GRATÃO et al., 2005; SHING et al., 2008). A atividade da enzima SOD é essencial para transformar o radical O_2^- em H_2O_2, enquanto as enzimas POD atuam na remoção do excesso de H_2O_2 (CHOUDHARY et al., 2007; TANYOLAC et al., 2007). A atividade destas enzimas está entre os principais parâmetros bioquímicos afetados pelo estresse oxidativo (YANG et al., 2011).

MANUSCRITOS

O presente estudo foi dividido em três tópicos, que em síntese resultaram em três manuscritos. O primeiro manuscrito foi escrito seguindo as normas da Revista Ciência Rural e os demais foras escritos seguindo as normas da revista Australian Journal of Crop Science.

1 - Changes in root morphology and dry matter production in different *Pfaffia glomerata* accessions in response to excess Zn.

2 - Effect of zinc on biomass, Zn concentration and parameters of stomata and photosynthesis of two accessions of *Pfaffia glomerata*

3 - Antioxidant activity and β-ecdysone production of accessions of *Pfaffia glomerata* in response to excess zinc in nutrient solution and soil.

Changes in root morphology and dry matter production in different *Pfaffia glomerata* accessions in response to excess Zn

ABSTRACT

Zinc (Zn) participates of numerous metabolic processes in plants, however may become toxic under supra-optimal concentrations, but plants can have different strategies to cope with high toxic levels of Zn in their environment. The aim of the present work was to evaluate the effect of excess Zn on many root morphological parameters of **Pfaffia glomerata**. A 4 x 3 factorial design was employed in a completely randomized scheme with 3 replicates. The treatments consisted of four levels of Zn (2, 100, 200 and 300μM) and three accessions of **P. glomerata** (BRA, GD and JB) grown in a hydroponic system for 7 and 14 days. Differences in root morphology and dry matter production were observed among the three accessions in response to excess Zn. Some growth parameters of GD accession increased upon the addition of Zn ranging from 36 to 79μM. However, the GD and JB accessions presented reduction in the dry matter production, root area, length and volume with increasing Zn levels. The BRA accession, which had the lowest growth among accessions, showed chlrotic leaves. The shoot/root dry matter ratio and root diameter increased linearly independently of the accessions at 7 days. Based on the evaluated parameters the following order of Zn tolerance in **P. glomerata** accessions was observed: GD> JB> BRA.

Key-words: root morphology, heavy metal, Brazilian ginseng, growth, zinc.

1. INTRODUCTION

The contamination by heavy metals and other toxic elements in agricultural areas is one of the big concerns in the last few decades due to risks to humans and ecosystem (WUANA & OKIEIMEN, 2011). There are many studies describing the negative effects of non-essential and toxic elements like cadmium (Cd), lead (Pb) and arsenic (As) on the anatomy, morphology, and metabolic reactions for many plant species (MEHARG & HARTLEY-WHITAKER, 2002; ZHAO et al., 2009; LUX et al., 2011; POURRUT et al.,

2011). In addition, plant micronutrients such as zinc (Zn), copper (Cu) and iron (Fe) have received attention due to their crescent inputs in the agricultural environment and consequently increase in cases of toxicity to plants. Although, Zn participates in numerous metabolic processes such as activation of enzymes, protein synthesis and metabolism of carbohydrates, lipids and nucleic acids, this element may become toxic to plants under supra-optimal concentrations (LIN et al., 2005; BROADLEY et al., 2007; MARSCHNER, 2012).

For most soils, Zn is encountered in the adequate concentration range (around 60 mg kg^{-1}) for plant growth, but its content can increase significantly by anthropogenic activities through industrial processes such as mining and smelting or through agricultural techniques, such as addition of biosolid fertilizes, mineral fertilization and pesticides (WUANA & OKIEIMEN, 2011). When present in high concentrations in plant tissues (100 to 300µg Zn g^{-1} leaf dry weight), this micronutrient can promote severe physiological and morphological changes, including the inhibition of root elongation (BROADLEY et al., 2007; DISANTE et al., 2010).

Plants have shown different strategies to cope with high (toxic) levels of metals such as Zn in their growth environment (RICACHENEVSKY et al., 2015). These tolerance strategies mainly include detoxification processes that are based on complexation by organic chelators and sequestering of Zn ions in the vacuoles (MARSCHNER, 2012; RICACHENEVSKY et al., 2015). The knowledge on Zn homeostasis of land plants is largely based on the model plant *Arabidopsis thaliana*, for which our molecular understanding is most developed at present (SHANMUGAN et al., 2012; SOFO et al., 2013; RICACHENEVSKY et al., 2015). SOFO et al. (2013) observed that the Cd/Cu/Zn-induced changes in root morphology of *Arabidopsis* were caused by a hormonal unbalance, mainly governed by the auxin/cytokinin ratio. It has been indicated that Zn toxicity depends on plant species and growth stage, and that growth inhibition and biomass reduction are the general responses of plants to Zn excess (LI et al., 2009; LI et al., 2012; MARQUES & NASCIMENTO, 2014; ALONSO-BLÁZQUEZ et al., 2015).

Pfaffia glomerata (Spreng.) Pedersen has been used as a model plant for studies of the effect of heavy metals, in which it was found that young plants showed moderate tolerance to Hg, As, Cu and Cd (CALGAROTO et al., 2009; GUPTA et al., 2013; NEIS, 2013). KAMADA et al. (2009) have reported that this species presented great genetic diversity among its natural populations, which can result in significant variability that can promote different levels of

tolerance to heavy metals. Therefore, the use of different accessions of this species can help to understand how plants cope with high levels of Zn in the environment. Based on this, the aim of the present work was to evaluate the influence of Zn levels on many root morphological parameters that can be used to distinguish accessions of *P. glomerata* according to their tolerance level to excess Zn.

2. MATERIALS AND METHODS

Three accessions (BRA, JB and GD) of *P. glomerata* were used in this study. The accession BRA belongs to Germplasm Bank of Embrapa Recursos Genéticos e Biotecnologia (Cernagem), the JB belongs to collection of the Botanical Garden of Universidade Federal de Santa Maria (UFSM) and the GD belongs to collection of medicinal plants of the Universidade Federal da Grande Dourados (UFGD).

Plants used in the experiment were obtained by *in vitro* culture from nodal segments (1.0 cm long and without leaves) of the middle portion of micropropagated plantlets cultivated on MS (MURASHIGE & SKOOG, 1962) medium, which was supplemented with 6g L^{-1} agar, 30g L^{-1} sucrose, 0.1g L^{-1} myo-inositol, and in the absence of growth regulators.

At 25 days of *in vitro* growth, plants were transferred to plastic containers with a capacity of 17 liters of nutrient solution with the following composition (in μM): 6090.5 N; 974.3 Mg; 4986.76 Cl; 2679.2 K; 2436.2 Ca; 359.9 S; 243.592 P; 0.47 Cu; 2.00 Mn; 1.99 Zn; 0.17 Ni; 24.97 B; 0.52 Mo; 47.99 Fe ($FeSO_4$/Na EDTA). After seven days of acclimatization, the treatments of Zn levels were added as a solution of $ZnSO_4.7H_2O$. The treatments were arranged in a completely randomized design with 4 x 3 factorial scheme, being four levels of Zn (2, 100, 200 and 300μM) and three accessions (BRA, JB and GD) of *P. glomerata*, with three replicates for each treatment containing 30 plants each. The nutrient solution pH was adjusted daily to 5.5±0.2 using HCl or NaOH solution of 0.1mol L^{-1}. The nutrient solution was changed every 7 days of cultivation.

Seven and 14-day-old plants were collected for evaluation of dry matter and parameters of root morphology. For dry matter evaluation, five plants per replication were collected and separated into shoot and root; subsequently roots were gently washed twice with distillated water and after were dried at 65°C, until a constant dry weight was obtained. For root

morphology, five plants for replication were collected, gently washed twice (3 min each) with distilled water, and after frozen in paper bags for 7 days at -20ºC. Roots were thawed at room temperature for 30 minutes and digitalized with the aid of a scanner Epson 11000 XL. Root morphology analysis was performed with the aid of WinRhizo Pro Software, which determines the total length, surface area, total volume, average diameter, and the number of root tips.

All the data were tested by the assumptions of the mathematical model (normality and homogeneity of variance). The analysis of variance of the experimental data was performed using the F-test. The quantitative factor, when significant ($P<0.05$), was subjected to polynomial regression analysis, by testing the linear and quadratic models. Pearson's correlations between dry weight and root morphological parameters were performed using SigmaPlot software version 12.3 ($P<0.05$).

3. RESULTS AND DISCUSSION

Interestingly, at 7 days, GD accession showed increase in root, shoot and total plant dry mass, respectively at about 50, 36 and 40μM Zn (Figure 1). However, at 14 days, root dry mass linearly decreased upon addition of Zn levels, whereas shoot and total dry mass increased at about 79 and 70μM Zn, respectively (Figure 1). The increase in dry matter observed for GD accession is due to the fact that the accession presented higher growth rate and, consequently greater demand for Zn. On the other hand, the accession JB showed linear decrease to all growth parameters based on dry matter with increasing levels of Zn (Figure 1). FAGERIA (2000) reported significant decrease in shoot biomass of rice, corn, soybeans and wheat upon addition of high levels of Zn. However, the response varied accordingly to the species, in which most species showed increase in shoot dry matter upon addition of low levels of Zn and this probably reflects the requirement of this element. The threshold of Zn toxicity sharply varies among plant species, time of exposure and composition of the nutrient solution. Significant decrease in total dry matter of ***Popolus deltoids*** and ***Populus nigra*** developed after additions of 100μM and 1000μM Zn, respectively (DI BACCIO et al., 2003).
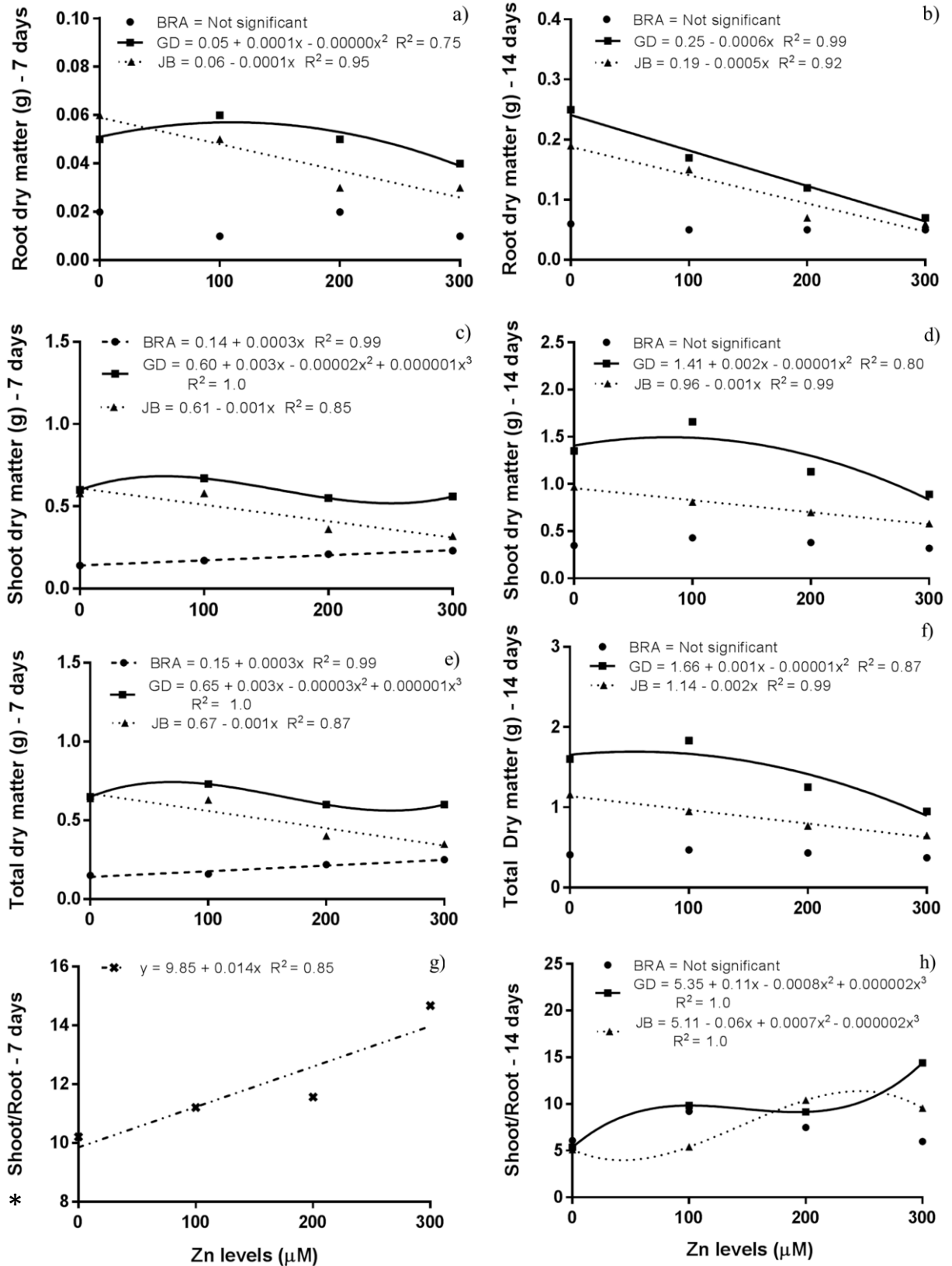
Figure 1. Effect of zinc levels on dry matter production of roots (a and b), shoot (c and d) and whole plant (e and f), as well as shoot/root dry matter ratio (g and h) of three accessions (BRA, GD and JB) of *P. glomerata* cultivated in hydroponics for 7 and 14 days.
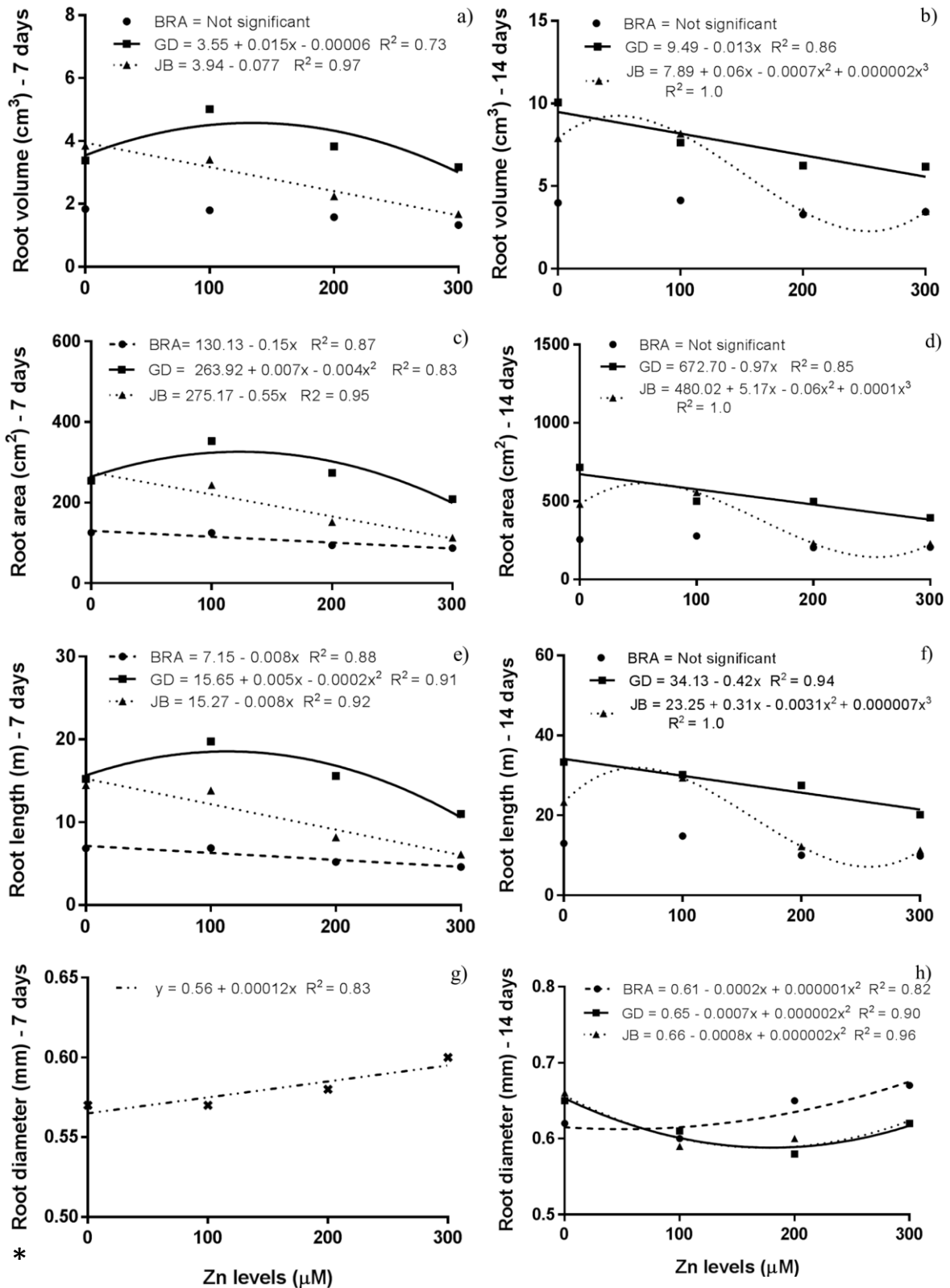
Figure 2. Effect of zinc levels on the volume (a and b), surface area (c and d), length (e and f) and average diameter (g and h) of roots of three accessions (BRA, GD and JB) of *P. glomerata* grown in hydroponics at 7 and 14 days.

In general, all *P. glomerata* accessions showed some reduction in growth based on dry mass with increasing levels of Zn in nutrient solution, but this response was genotype dependent (Figure 1). The accession BRA showed either no alteration in dry mass or a small linear increase in shoot and total plant dry mass with increasing levels of Zn. However, independently of the Zn level tested, this accession showed chlorotic leaves (data not shown) and low growth during the whole evaluation period, indicating that it was not adapted to the hydroponic system used, when compared to the other accessions. The observed typical symptoms of inhibition of growth of *P. glomerata* by excess Zn have also been reported for other species such as *Sedum alfredii* (LI et al., 2009), *Ricinus communis* (MARQUES & NASCIMENTO, 2014), *Triticum aestivum* (LI et al., 2012) and *Zea mays* (ALONSO-BLÁZQUEZ et al., 2015).

The shoot/root dry mass ratio linearly increased independently of the *P. glomerata* accessions with increasing Zn levels at 7 days (Figure 1g), whereas at 14 days the accessions GD and JB showed a cubical response (Figure 1h). Differences between root and shoot growth under Zn excess were also observed for *Holcus lanatus* by RENGEL (2000) and in *Phyllostachys pubescens* by LIU et al. (2014). DISANTE et al. (2010) reported reduction in leaf biomass of woody species in response to excess Zn, as well as increase in root biomass production under relatively low levels of Zn.

The accession JB presented strong positive correlation between the yield of root dry mass and shoot and total dry mass (ρ = 0.92 and 0.96, respectively) (Table 1). The accession GD also showed a positive correlation between these variables, however the correlation was not so straight (ρ = 0.63 and 0.75, respectively). All accessions presented strong positive correlation between shoot and total dry mass (ρ above 0.9) (Table 1). These results indicated that the production of shoot and total dry mass for these two accessions is proportional and dependent of the root dry mass, which means that an alteration in growth of roots will affect the performance of the shoot.

In general, the volume (Figure 2a and 2b), surface area (Figure 2c and 2d) and length (Figure 2e and 2f) of roots to all accessions of *P. glomerata* decreased with increasing Zn levels in nutrient solution. Root length is a parameter which affects plant ability to uptake nutrients and water from its surroundings. The length, surface area, diameter and volume of roots may be valuable parameters to compare the response of different genotypes under excess heavy metals (LI et al., 2009). LI et al. (2012) observed that the loss of cell viability and the

significant increases of lignification in response to excess Zn may be associated with the remarkable reduction of root growth in ***Triticum aestivum*** seedlings.

Nevertheless, at 7 days of exposure to Zn, GD accession showed higher volume, surface area and length of roots than the other accessions (Figure 2). In addition, this response was quadratic, where the dose of maximum yields observed to volume (Figure 2a), surface area (Figure 2c) and length of roots (Figure 2e) were 134.5, 122.7 and 11.3μM Zn, respectively. On the other hand, the other two accessions showed a negative linear response; except for root volume of BRA that was not altered (Figure 2a, 2c and 2e). Meanwhile, at 14 days, the accession GD presented a negative linear response, and the JB accession showed a cubic response, with similar volume (Figure 2b), surface area (Figure 2d) and length (Figure 2f) of roots up to the concentration of about 100μM Zn for both accessions.

Several environmental factors were reported to influence root morphology such as metal stress (LI et al., 2005), soil moisture conditions (DAVIES & BACON, 2003), soil temperature and nutrient availability (ZHANG et al., 2003). The inhibition of root elongation is one of the major responses of plants to excess metals and may occur faster than other physiological responses. Zinc toxicity symptoms are striking in the root system, mainly on root elongation and cell division (BROADLEY et al., 2007). SHARIFIANPOUR et al. (2014) observed that at 30mg L^{-1} of Zn there were significant decreases in surface area, number, volume and length of root rice, whereas at lower levels of Zn caused an increase on these parameters. LIU et al. (2014) observed that root morphological parameters were initially increased by low levels of Zn, but on the other hand at 400μM Zn, the length, surface area and volume of roots decreased 51, 24, and 57%, respectively.

Among the three accessions of ***P. glomerata***, only the GD showed a positive correlation between root, shoot and total dry mass with root length; however, this correlation was weak (ρ above 0.3) (Table 1). This result indicates that the production of biomass is partially dependent of the root length, since higher root length promotes higher uptake of water and nutrients. LI et al. (2005) reported that the length, surface area and volume of roots of the hyperaccumulator ecotype of ***Sedum alfredii*** increased with high levels of Zn, whereas in the non-hyperaccumulator these parameters were significantly decreased.

Several growth parameters analyzed showed correlation with the number of root tips (Table 1). The accessions JB and GD showed moderate positive correlation between root dry mass and number of root tips (ρ above 0.4), as well as between root length and number of root

tips (ρ above 0.6) (Table 1). In addition, the accession JB showed a weak positive correlation between shoot and total dry mass, and root diameter with the number of root tips (ρ above 0.33). However, the accession GD presented negative correlation between the root diameter and the number of root tips (ρ = - 0.34), being the same observed for the BRA (ρ = - 0.42), which on the other hand showed a positive correlation between root length and number of root tips (ρ = 0.88) (Table 1). It has been reported that under Zn excess, the DNA synthesis and mitotic activity of root tips may be inhibited, which subsequently results in inhibition of root growth (JAIN et al. 2010).

Table 1. Coefficient of Pearson correlation (ρ) between the dry matter (DW) production of roots, shoot and whole plant, as well as the length, average diameter of roots and numbers of root tips of three accessions (JB, GD and BRA) of *P. glomerata* grown in hydroponics with increasing levels of Zn.

JB	Shoot DW	Total DW	Length	Av. Diameter	Root Tips
Root DW	0,922*	0,958*	0,0466	0,058	0,460*
Shoot DW	xx	0,995*	0,205	0,205	0,350*
Total DW	xx	xx	-0,009	0,168	0,385*
Length	xx	xx	xx	0,607*	0,634*
Av. Diameter	xx	xx	xx	xx	0,325*

GD	Shoot DW	Total DW	Length	Av. Diameter	Root Tips
Root DW	0,627*	0,745*	0,364*	0,132	0,431*
Shoot DW	xx	0,987*	0,302*	-0,033	-0,084
Total DW	xx	xx	0,335*	-0,001	0,017
Length	xx	xx	xx	-0,597*	0,609*
Av. Diameter	xx	xx	xx	xx	-0,336*

BRA	Shoot DW	Total DW	Length	Av. Diameter	Root Tips
Root DW	0,085	0,199	0,016	-0,088	-0,041
Shoot DW	xx	0,993*	-0,103	0,685*	-0,138
Total DW	xx	xx	-0,099	0,664*	-0,141
Length	xx	xx	xx	-0,253	0,879*
Av. Diameter	xx	xx	xx	xx	-0,421*

Significant to level of * $P < 0.05$

The accession GD showed a moderate negative correlation for between root length and root diameter (ρ = -0.60) (Table 1). In addition, the BRA accession presented moderate positive

correlation between shoot and total dry mass with root diameter (ρ above 0.6) (Table 1). There were no difference among accession in relation the root diameter, and this parameter showed a significant linear increase with increasing Zn levels at 7 days (Figure 2g). However, at 14 days, the accessions GD and JB showed a quadratic response, where the dose of minimum yield observed to root diameter was about 200μM Zn (Figure 2h). The increase in root diameter probably was a response related to decrease in cellular division in the apical portion of roots (JAIN et al. 2010).

It is desirable that plant species used for phytoremediation purposes show a higher rate of growth (LAMEGO and VIDAL, 2007). In the present study, although the accession GD and JB of *P. glomerata* showed symptoms of Zn toxicity, these genotypes may be used in environments with high levels of Zn, because their growth rates are much higher when compared to the accession BRA.

CONCLUSIONS

There were differences in root morphology and dry mass production among the three accessions of *P. glomerata* plants grown under Zn excess. The accession GD showed increase in dry mass production, root volume, root surface area and root length at Zn levels below 70μM. Based on the evaluated parameters the following order of Zn tolerance in *P. glomerata* accessions was observed: GD> JB> BRA.

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Effect of zinc on biomass, Zn concentration and parameters of stomata and photosynthesis of two accessions of *Pfaffia glomerata*

ABSTRACT

Pfaffia glomerata (Spreng.) Pedersen known as Brazilian ginseng is a species widely used in popular medicine due to its phytotherapics properties and has been used as model species in studies related to the effect of heavy metals in plant species. Some of these studies indicate that this species presents moderate tolerance to many heavy metals. Industrial and mining activities, combined with inadequate use of fertilizers and pesticides has contributed to the increase of zinc (Zn) levels in the soil, what can cause physiological changes in plants. Thus, the objective of this study was to evaluate the influence of excess Zn on growth, photosynthetic parameters and Zn concentration in plants of *P. glomerata* after 34 and 74 days of cultivation. The treatments consisted of two accessions of *P. glomerata* (JB and GD) and three levels of Zn (2, 100 e 200 mg kg^{-1} of soil). Tissue Zn concentration increased with increasing Zn levels in the soil. The Zn translocation to leaves was higher in accession GD at 74 days, which also showed a lower accumulation of Zn in the roots. For most growth parameters evaluated there were increase upon addition of 100 mg Zn kg^{-1} soil, but at 200 mg Zn kg^{-1} soil the opposite was observed. Photosynthesis, transpiration and stomatal conductance increased at 100 mg Zn kg^{-1} soil in GD accession. The same accession also presented increase in stomatal index upon addition of 200 mg Zn kg^{-1} soil, whereas the JB did not show any alteration.

Keywords: Heavy metal, photosynthesis, Brasilian ginseng, chlorophyll, Zn accumulation.

1. INTRODUCTION

Zinc (Zn) is an important micronutrient for plant growth and development. However, this element can easily become toxic when present in high concentrations in tissues. The Zn is involved in many metabolic processes such as enzyme activation, protein synthesis, metabolism of carbohydrates, reduction of nitrate, maintenance of photosynthetic rate and

chlorophyll content (Lin et al., 2005; Prado, 2008; Brunes et al., 2011) and requested in the synthesis of tryptophan, which is precursor of the biosynthesis of indoleacetic acid (Taiz and Zeiger, 2013).

Heavy metals are found naturally in the most soils in low concentrations as result of weathering and other pedogenic processes (Baker et al., 1994). However, due to increasing industrial, agricultural and urbanization, soil pollution with metals and Zn is becoming relatively common, which is worrying because heavy metals are extremely toxic and can cause pollution of soil and water (Reichman, 2002; Cunha et al., 2008).

When the Zn reaches a concentration that exceeds the optimum levels for plants may occur inhibition of metabolic functions, which result in reduction on growth, root and shoot dry matter production, photosynthetic rate, chlorophyll content in leaves, changes in enzymes activity and can also induce plant senescence (Carneiro et al., 2002; Stoyanova et al., 2002; Lin et al., 2005; Jin, 2008; Li et al., 2011). There are several reports about the effect of Zn excess on different plants, however it is known that the response varies among species as well as among genotypes.

Pfaffia glomerata (Spreng.) Pedersen is a Brazilian species with medicinal properties and have been widely used by the pharmaceutical industry in the production of phytotherapic medicines (Vigo et al., 2004; Zimmer et al., 2006). Popularly known as Brazilian ginseng, this species has stood out due to the numerous properties and compounds that have been discovered and studied, mainly triterpene saponins and ecdysteroids (Magalhães, 2000).

The species features high genetic diversity in natural populations, resulting in significant variability, which can promote different levels of tolerance to heavy metals such as Zn (Kamada et al., 2009). Studies conducted with *P. glomerata* shown intermediate and high tolerance to metals as aluminum, mercury, cadmium and lead (Maldaner, 2008; Skrebsky et al., 2008; Calgaroto, 2009), which may be related to Zn tolerance.

There are few studies relating the toxicity of Zn and its effect on plant growth and photosynthetic parameters in medicinal species, as well as none for *P. glomerata*. A characterization of these parameters is of utmost importance, as well as metal accumulation and possible implications for human health. In this view, the aim of this study was to evaluate the influence of Zn excess on plant growth, photosynthetic parameters and Zn accumulation in two accessions of *P. glomerata* cultivated in soil with three levels of Zn.

2. MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The experiments were developed by the Research Group on Physiology of Plants with Agrobiological Interest at the Biology Department of Universidade Federal de Santa Maria. Two accessions of *P. glomerata* (JB and GD) were used for the experiment. The accession JB is belonging to the collection of the Botanical Garden of the Federal University of Santa Maria (UFSM) and the accession GD was obtained from the collection of medicinal plants from the Federal University of Grande Dourados (UFGD).

The plants used in the experiment were obtained by *in vitro* culture from nodal segments (1.0 cm long) without leaf of the middle portion of micropropagated plantlets cultivated in MS (Murashige and Skoog, 1962) medium supplemented with 6 g L^{-1} agar, 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol, and in the absence of growth regulators as previously described for Nicoloso et al. (2001).

After 21 days, homogeneous plants were selected and acclimated in plastic boxes containing 20 kg of sand in a hydroponic system according to Bandinelli et al. (2013). The plants were submitted at the following nutrient solution (in μM) described by Tabaldi et al. (2007): 6090.5 N; 974.3 Mg; 4986.76 Cl; 2679.2 K; 2436.2 Ca; 359.9 S; 243.592 P; 0.47 Cu; Mn 2.00; 1.99 Zn; Ni 0.17; 24.97 B; 0.52 Mo; 47.99 Fe ($FeSO_4$ / Na EDTA) through three irrigations daily for a period of 14 days. In order to minimize the stress caused by solar radiation, the plants were protected as a screen of shading with 50% radiation interference in the first five days.

At the end of acclimatization period the accessions were transplanted into pots containing 5 kg of soil. The soil was collected from the A horizon (0-20 cm depth) of a Typic Hapludalf in Santa Maria, RS. The pots were lined internally with plastic bags to prevent loss of nutrients and water by drainage. Over the cultivation under greenhouse conditions the water evapotranspired was replacement by daily irrigation with distilled water, and kept at field capacity in 80%, performed by daily weighing the pots. Two plants were planted per pot and after a period of seven days were left only one plant per pot.

2.2 Soil characteristics

The collected soil presented the following characteristics: 18% of clay, 3.2% organic matter, pH 4.5, 30.6% Al, 26.5% bases saturation, 6.2 cmol dm^{-3} CTC, 2.8 cmol Ca dm^{-3}, 1.4 cmol Mg dm^{-3}, 2 mg P kg^{-1}, 2 mg Zn kg^{-1} and 55 mg K kg^{-1}. The SMP was 6.4, ranging from 5.4 to 5.6 after pH adjustment and addition of Zn concentrations: without addition, 100, and 200 mg kg^{-1}, in the form of zinc sulphate. In addition, it was added to the soil 60 mg kg^{-1} of phosphorus (P) as triple superphosphate, 40 mg kg^{-1} potassium (K) as potassium chloride and 20 mg kg^{-1} of nitrogen (N) as ammonium nitrate based on the results of soil analysis and previus data observed for biomass production of *P. glomerata* (Neis 2013). At 20 and 40 days of cultivation, 20 mg kg^{-1} N was added.

2.3 Production of dry matter and tissue Zn concentration

Roots, stems and leaves were collected and placed in paper bags, and dried at 65°C until constant weight to determine the dry matter (DM).

Samples for determining the concentration of Zn in the tissues were subjected to an acid digestion procedure in an open system with block digester DK Model (Velp Scientifica, Italy). The samples were pre weighed (170 mg) and transferred to vials of decomposition (borossilicato vials). It swas added 5 mL of HNO$_3$ (14 mol L^{-1}) in each tube. The procedure consisted in heating the mixture at 80°C for 30 minutes and then at 130°C for 90 minutes. After the digestion step, samples were transferred to polypropylene vials and the volume was completed to 30 mL with previously distilled or deionized and purified on a Milli-Q water system. For the determinations of the concentration of Zn was used optical emission spectrometer with inductively coupled plasma (ICP OES, PerkinElmer Optima 4300 DV).

The translocation index of Zn to the leaves was defined using the following equation: $[Zn]_{root}$ x DM_{root} / ($[Zn]_{root}$ x DM_{root}) + ($[Zn]_{leaves}$ x DM_{leaves}), and the accumulation of Zn in roots was defined using the following equation: $[Zn]_{leaves}$ x DM_{leaves} / $[Zn]_{root}$ x DM_{root}.

2.4 Plant height, leaf area and stomata parameters measurements

Plant height was measured with a ruler in which the plant height was measured from the stem base to apex of the main stem. For the determination of leaf area, scanning of the

leaves with the help of a scanner Epson 11000 XLwas performed and after the analysis with the aid of WinRhizo Pro Software was carried out. The count of epidermal cells and number of stomata was performed by capturing microscopic fields by means of a camera attached to the microscope, with subsequent counting of these under computer with the Paint software and counting of five fields (1 mm^2) in three leaves of each treatment. The laminas for the counting were prepared without cutting the leaves or detachment of the epidermis.

With the aid of the fingers, the leaf face under an investigation was pressed on a glass lamina containing a drop of glue (Super Bonder) for 30 seconds, subsequently the leaf sheet is removed and the printing of leaf epidermis remains on the glass lamina. To determine the stomatal index and the relation of the stomata number of a given area (1mm^2), it was used an adaptation of the Salisbury´s formula (Wilkinson, 1979). To determine the ratio between the number of stomata of the abaxial and adaxial epidermis (ab/ad), the number of stomata on both epidermis were divided by each other.

2.5 Photosynthetic parameters

The stomatal conductance (g_s), photosynthesis (A), transpiration (E), internal CO_2 concentration (c_i) and the efficiency of water use (WUE), obtained by the ratio between the amount of CO_2 fixed in photosynthesis and amount of water transpired, were performed using a Li-Cor 6400XT portable infrared gas analyzer. These measurements were always made in the median region of a completely expanded leaf, totally exposed to solar radiation, starting at 9h in the morning. Three plants were analyzed per treatment, one leaf per plant (the fourth leaf from the apex of the main stem).

2.6 Chlorophyll and carotenoids concentrations

Leaf disks ($\pm$ 0.1 g) were weighed and used to determine chlorophyll and carotenoids concentration. Chlorophyll and carotenoids were extracted following the method of Hiscox and Israeslstam (1979) and calculated with the aid of Lichtenthaler's formula (Lichtenthaler, 1987). Fresh samples of the leaves were incubated at 65 °C in dimethylsulfoxide (DMSO) until the pigments are completely removed. For the determination of chlorophyll a, b and carotenoids,

the absorbance of the solution was measured at 663 and 645 nm and 470 nm, respectively, using a spectrophotometer (CELM E-205D).

2.7 Statistical analysis

Data were subjected to analysis of variance and the means of treatments were compared by Tukey test at 5% probability of error, using the statistical program SISVAR (Ferreira, 2000).

3   RESULTS AND DISCUSSION

Plants from both accessions cultivated at highest level of Zn in soil (200 mg kg^{-1}) showed a significant reduction in root, shoot and total dry matter production during both evaluation periods (Figure 1). However, there was an increase in dry matter production of shoots and total to accession GD after 34 days of cultivation at 100 mg Zn kg^{-1} (Figure 1C and E). This accession also had a higher dry matter production when compared to JB at the same evaluation period (Figure 1A, C and E). In general, the symptoms of Zn toxicity are associated with growth inhibition base on biomass (Cambrollé et al., 2011). However, several authors also related increase in dry matter production in lower Zn levels, as observed in this experiment. This increase probably reflects the requirement of this element on carbohydrate metabolism, protein synthesis, regulation and expression of genes, ribosome structure and its role as cofactor in a large number of enzymes (Clemens et al., 2002; Broadley, 2007).

When compared to 34-days-old plants, 74-days-old plants showed increase in the root, shoot and total dry matter production for both accessions (Figure 1B, D and F). The accession JB did not showed differences for root dry matter production between control and intermediate level of Zn (Figure 1B) and for shoot dry matter presented reduction for intermediate level of Zn (Figure 1D) to 74 days. There was a higher difference between the ratio of shoot/root at 34 days (Figure 1G) and lower to 74 days (Figure 1H). In both evaluation periods, the JB accession showed higher difference between shoot/root dry matter at 200 mg kg^{-1} of Zn in comparison with the other levels of Zn. On the other hand, this response was not observed for the GD accession, which maintained similar ratio for the Zn levels (Figure 1G, H).
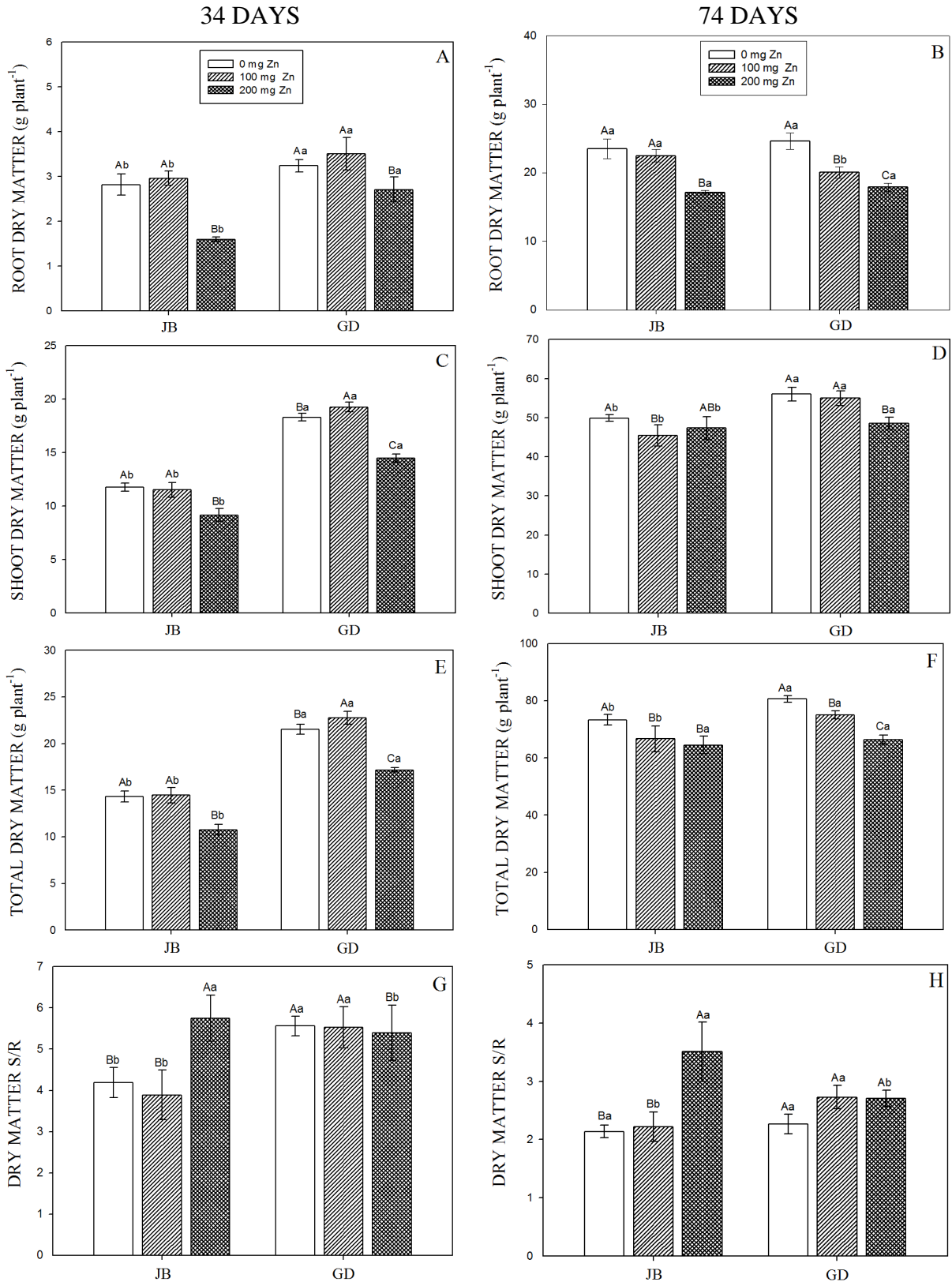
Figure 1. Effect of Zn levels on the dry matter production of roots at 34 (A) and 74 days (B), shoots at 34 (C) and 74 days (D), total plant at 34 (E) and 74 days (F), and ratio of dry weight of Shoot/Root at 34 (G) and 74 days (H) of growth of *P. glomerata* accessions. The data represent the mean ± S.D. four different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatments.

Several authors have reported the effect of high levels of Zn on the growth of many plant species. Marques and Nascimento (2014) observed linear decrease on dry matter of castor bean with increasing levels of Zn in the nutrient solution, however, up to the level of 100 µM no alteration was observed. Soares et al. (2001) reported that increasing levels of Zn negatively affected the growth of *Eucalyptus maculata* and *Eucalyptus urophylla*. Di Baccio et al. (2009) also showed that Poplar plants grown at 5 mM Zn for 5 weeks had low decrease in total dry weight, however, the shoot/root dry weight ratio decreased dramatically at 10 mM Zn.

The increase of Zn levels in the soil caused a significant increase in Zn concentration in the roots and leaves (Table 1). It was observed a higher concentration of Zn in roots during the first evaluation period (34 days), as compared to the second evaluation period (74 days) (Table 1). Overall, the *P. glomerata* acessions had similar Zn concentration in leaf tissues for both evaluation periods under the same Zn treatment and there was an increase in tissue Zn concentration with increasing Zn levels in the soil (Table 1).

Table 1. Zn concentration in tissues of leaves and roots at 34 and 74 days of cultivation of *P. glomerata* plants.

Concentration of Zn in the roots (µg g^{-1} DW)				
Zn levels (mg kg^{-1})	34 days		74 days	
	JB	GD	JB	GD
0	72.7 Ca	67.4 Ca	38.55 Ca	32.7 Ba
100	835 Ba	785.5 Ba	278.5 Ba	224.5 ABa
200	1424.5 Aa	1537.5 Aa	641 Aa	426 Ab
Concentration of Zn in the leaves (µg g^{-1} DW)				
Zn levels (mg kg^{-1})	34 days		74 days	
	JB	GD	JB	GD
0	76.25 Ca	74.4 Ca	109 Ca	62.5 Ca
100	1316 Ba	1238.5 Ba	1144 Bb	1311.5 Ba
200	1816 Aa	1930.5 Aa	1537 Ab	1,861 Aa

The data represent the mean ± S.D. from three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within Zn treatments.

Interestingly, in contrast with that observed for most plant species, where roots showed higher Zn concentration than shoots (Soares et al., 2001; Azzarello et al., 2012; Sharifianpour et al., 2014), in the present study *P. glomerata* plants showed higher Zn

concentration in the leaves, independently of accessions, Zn levels in the soil and period of evaluation. Higher zinc concentration in shoot was also reported by Frey et al. (2000) in *Thlaspi caerulescens* plants and by Malik et al. (2011) in *Amaranthus sp.*

        The accession JB showed no differences in Zn translocation to the leaves and accumulation in roots at 34 and 74 days of cultivation when exposed to Zn levels in the soil (Table 2). Interestingly, the GD accession showed increase in Zn translocation to the leaves and reduction in Zn accumulation in roots with increasing Zn levels in the soil at both evaluation periods (Table 2), which suggests significant differences between these accessions in relation to the regulations of the mechanisms for Zn absorption, transport and compartmentalization.

Table 2. Zn translocation to the leaves and accumulation in roots at 34 and 74 days of cultivation of *P. glomerata* plants grown on soil.

Zn levels	Zn translocation index to leaves			
	34 days		74 days	
(mg kg^{-1})	JB	GD	JB	GD
0	2.28 Aa	2.33 Ba	1.81 Aa	1.02 Ba
100	3.01 Aa	3.08 ABa	2.7 Aa	4.02 Aa
200	3.08 Aa	3.92 Aa	1.96 Ab	3.38 Aa

Zn levels	Zn accumulation index in roots			
	34 days		74 days *	
(mg kg^{-1})	JB	GD	JB	GD
0	0.31 Aa	0.31 Aa	0.36 Ab	0.5 Aa
100	0.26 Aa	0.25 ABa	0.28 Aa	0.20 Ba
200	0.25 Aa	0.21 Ba	0.35 Aa	0.25 Bb

The data represent the mean ± S.D. from three different replicates. * Significant interaction between doses and accessions. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within Zn treatments.

        The accession GD showed interesting features as higher Zn translocation to the leaves, lower accumulation in roots and also higher total dry matter production upon the 200 mg kg^{-1} Zn. However, since *P. glomerata* is a medicinal plant and the root has been used as the main biomass source for phytoterapic medicines, it is of paramount importance that the Zn concentration in the roots does not exceed the levels allowed for human consumption.

        Marques and Nascimento (2014) reported that the increase of Zn levels in the nutrient solution was accompanied by accumulation of this nutrient in the leaves, stems and roots of

castor bean plants, and the highest level was found in the roots at 200 µM Zn. These authors considered that the accumulation of metals in the roots as an important mechanism that may contribute to metal tolerance in plants.

Although the root dry matter production was similar for both accessions (Figure 1A and B), the plant height differed greatly between accessions (Figure 2C and D). The accession GD showed higher plant height in both evaluation periods, however at 34 days there was a gradual decrease in plant height with increasing Zn levels in the soil (Figure 2C) and at 74 days the higher plant height was observed at 100 mg kg^{-1} Zn (Figure 2D).
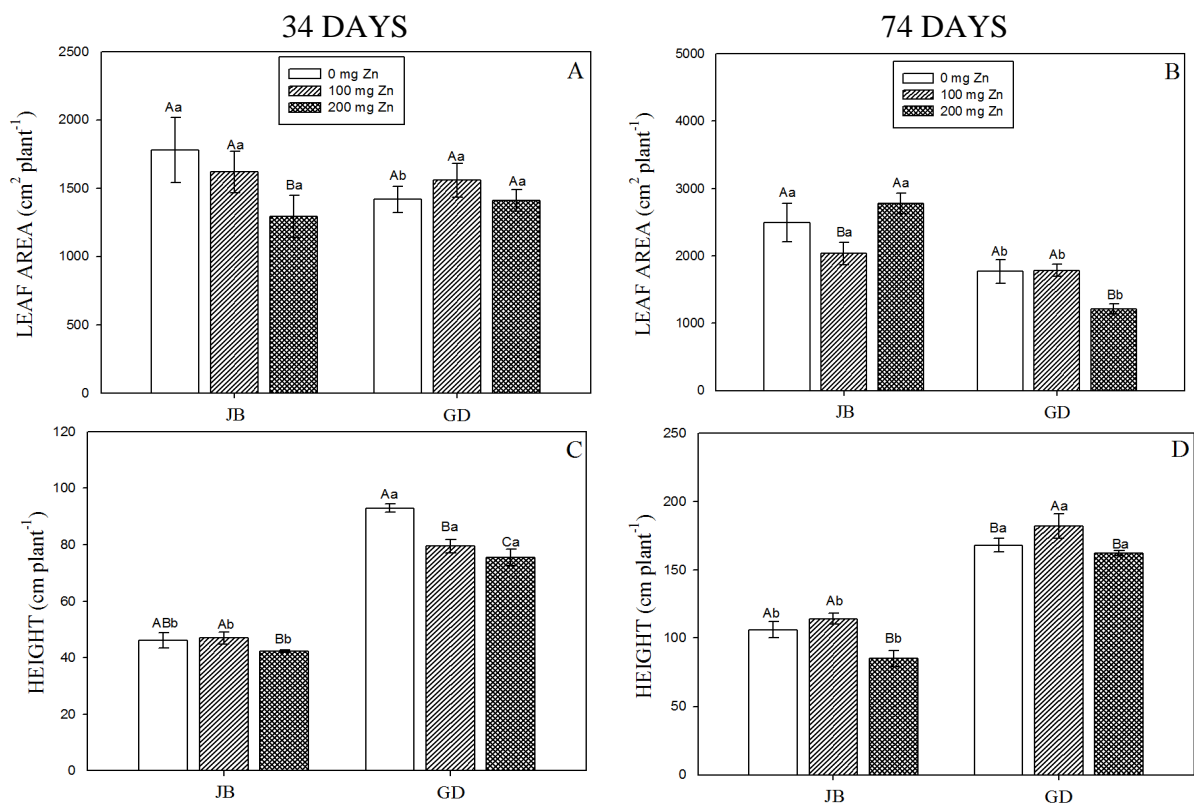
Figure 2. Effect of zinc on the leaf area (cm^2 plant^{-1}) at 34 (A) and 74 days (B), plant height (cm plant^{-1}) at 34 (C) and 74 days (D) of cultivation of two accessions (JB and GD) of *P. glomerata* plants. The data represent the mean ± S.D. four different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within Zn treatments.

Other indicative parameter of plant productivity is its leaf area, since the photosynthetic process depends on the interception of sunlight and its conversion into chemical energy (Favarin et al., 2002). In the present study, the leaf area of JB accession was decreased

upon the addition of Zn levels of 200 and 100 mg kg^{-1}, respectively at 34 and 74 days of cultivation, but the GD accession only showed decrease in the leaf area upon the addition of 200 mg kg^{-1} Zn at 74 days (Figure 2). This response of JB accession under intermediate Zn level may have affected the stomatal conductance and consequently transpiration and photosynthesis for the same period of evaluation (Figure 4A, 3D, 3B). High Zn concentrations in the nutrient solution caused reduction in leaf area of *Beta vulgaris* (Sagardoy et al., 2010). Casierra-Posada and Poveda (2005) reported reduction of 31% in leaf area of strawberry plants upon the adition of 350 mg kg^{-1} of Zn.

In the present study, the chlorophyll A and B concentration decreased for the GD accession upon the addition of 100 mg kg^{-1} Zn at 74 and 34 days of cultivation, respectively. On the other hand the JB accession showed increased chlorophyll B concentration at 200 mg kg^{-1} Zn at 74 days, when compared to the control treatment (Table 3). These data had direct effect on the concentration of total chlorophyll and on chlorophyll A/B ratio, then the total chlorophyll concentration was decreased only in GD accession at 100 mg kg^{-1} Zn at 74 days of cultivation, and the chlorophyll A/B ratio decreased in JB upon addition of 200 mg kg^{-1} Zn at 74 days (Table 3).

Table 3. Effect of Zn levels on the concentration of chlorophyll A, B, ratio A/B and total (mg/g FW) in leaves of *P. glomerata* plants cultivated in soil at 34 and 74 days.

Zn levels	Chlorophyll A				Chlorophyll B			
	34 days		74 days		34 days *		74 days *	
(mg Kg^{-1})	JB	GD	JB	GD	JB	GD	JB	GD
0	1,51 Ab	1,84 Aa	0.7 Aa	0.69 Aa	0,39 Ab	1,11 Aa	0.13 Bb	0.19 Aa
100	1,77 Aa	1,77 Aa	0.62 Aa	0.56 Ba	0,63 Aa	0,64 Ba	0.14 ABa	0.15 Aa
200	1,67 Aa	1,75 Aa	0.68 Aa	0.63 ABa	0,47 Aa	0,76 ABa	0.19 Aa	0.15 Ab

Zn levels	Chlorophyll A/B				Chlorophyll total			
	34 days *		74 days *		34 days *		74 days	
(mg Kg^{-1})	JB	GD	JB	GD	JB	GD	JB	GD
0	3.87 Aa	1.66 Ab	5.38 Aa	3.63 Ab	1,9 Ab	2,95 Aa	0.83 Aa	0.88 Aa
100	2.80 Aa	2.77 Aa	4.43 ABa	3.73 Aa	2,4 Aa	2,41 Aa	0.76 Aa	0.71 Ba
200	3.55 Aa	2.30 Ab	3.58 Ba	4.2 Aa	2,14 Aa	2,51 Aa	0.89 Aa	0.78 Aba

Table 2. The data represent the mean ± S.D. from three different replicates. * Significant interaction between dose and accessions. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatments.

Besides the reduction in the content of chlorophylls, the low tolerance of plants to toxicity of trace elements still can reduce the content of carotenoids (Joshi and Mohanty, 2004; Maurya et al, 2008; Shi and Cai, 2009). In the present study, it was observed differences only between accessions at 34 days of cultivation (0.39 and 0.47 mg g^{-1} FW, for JB and GD respectively), and at 74 days of cultivation no difference on carotenoids concentration was either noticed in relation to Zn treatments or accessions (data not shown).

Vijayarengan and Mahalakshmi (2013) reported reduction of photosynthetic pigments in tomato plants grown under Zn excess, which they related to the interference of excess Zn on the chlorophyll synthesis. Treatments with increasing levels of Zn affected the chlorophyll content, both in young leaves as in older leaves of Populus (Di Baccio et al., 2009). The content of chlorophyll a, b and carotenoids of tomato leaves increased in lower concentrations (50-100 mg kg^{-1}) of Zn and decreased with increasing Zn levels (150-250 mg kg^{-1}) (Vijayarengan and Mahalakshmi 2013). Li et al. (2013) reported that the reduction in the content of chlorophyll b of wheat plants in response to Zn excess was more pronounced than that of chlorophyll a.

In *P. glomerata* leaves it was observed stomata on both abaxial and adaxial epidermis. The JB accession showed increase in the ratio between the number of stomata of the abaxial and adaxial epidermis (ab/ad) with increasing Zn levels in soil. Already the GD accession showed increase in the ab/ad stomata ratio for the intermediate Zn level (100 mg kg^{-1}) and decrease upon the addition of 200 mg kg^{-1} Zn (Figure 3A).
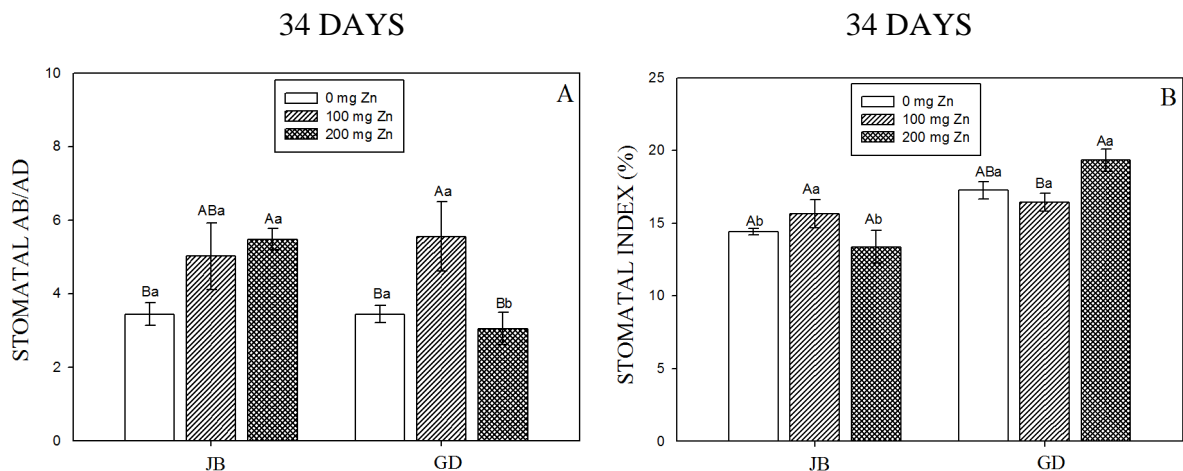

Figure 3. Effect of zinc levels on the stomatal relation ab/ad (A) and stomatal index (%) (B). The data represent the mean ± S.D. from five different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession. Different small letters indicate significant differences among accession within the same Zn treatments.

Solar irradiation or shading are factors that can affect the frequency stomata or number of epidermal cells of leaves, however, it is believed that the stomatal index is an intrinsic feature of the plant, remaining unchanged under the influence of these factors (Pompelli et al., 2010). In the present study, it was observed that the GD accession presented an increase in stomatal index upon the addition of 200 mg Zn (Figure 3B), whereas the JB accession did not show any alteration. This increase in stomatal index has been described for species under environmental stresses as an possibly change in leaf anatomy in order try to maintain the concentration of CO_2 for that the photosynthesis is not affected, since under stress there is a decrease in the opening of the pore stomatal.

Beta vulgaris plants stressed by excess Zn presented lower stomatal frequency and size of stomata (Sagardoy et al. 2010). Similar data were also found in *Phaseolus vulgaris* grown in high levels of Zn (Van Assche et al. 1980). Also in *Phaseolus vulgaris* Vassilev et al. (2011) found reduction in the stomatal frequency at 500 µM of Zn. Sagardoy et al. (2010) reported that stomata showed the shape more rounded and in many cases, the opening seemed apparently sealed with substances similar to wax when plants were grown at 300 µM of Zn.

Stomatal density and size are sensitive to heavy metal toxicity. Kastori et al. (1992) showed a significant increase in stomatal density on both adaxial and abaxial leaf surfaces under heavy metal toxicity, in particular Cd, Cu and Zn. Additionally, the stomatal size also decreased as a result of heavy metals stress. Anjana et al. (2006) and Gostin (2009) related that the Cd caused reduction in the size of stomata and their frequency on adaxial and abaxial sides of leaves. Reduction of the size of stomata and their frequency can lead to negative impact on transpiration, photosynthesis and gas exchange, because in the presence of metal the stomata can be closed.

In the present study the higher photosynthesis, transpiration and stomatal conductance occurred in GD plants upon the addition of 100 mg kg^{-1} Zn at 34 days of cultivation (Figure 4A, C and 5A), already for the level of 200 mg kg^{-1} Zn there was decrease in the photosynthesis and stomatal conductance. At 74 days, the same accession had the transpiration increased with increasing Zn levels in the soil (Figure 4D), but the photosynthesis and stomatal conductance were negatively affected in both accessions (Figure 4B and 5B), due to physiological state of the plants. During the development of plants, the photosynthetic by leaf area increase with leaf age until its maximum expansion, decreasing until that occurs senescence (Santos and Carlesso, 1998), as observed for plants in this experiment (Figure 6B).
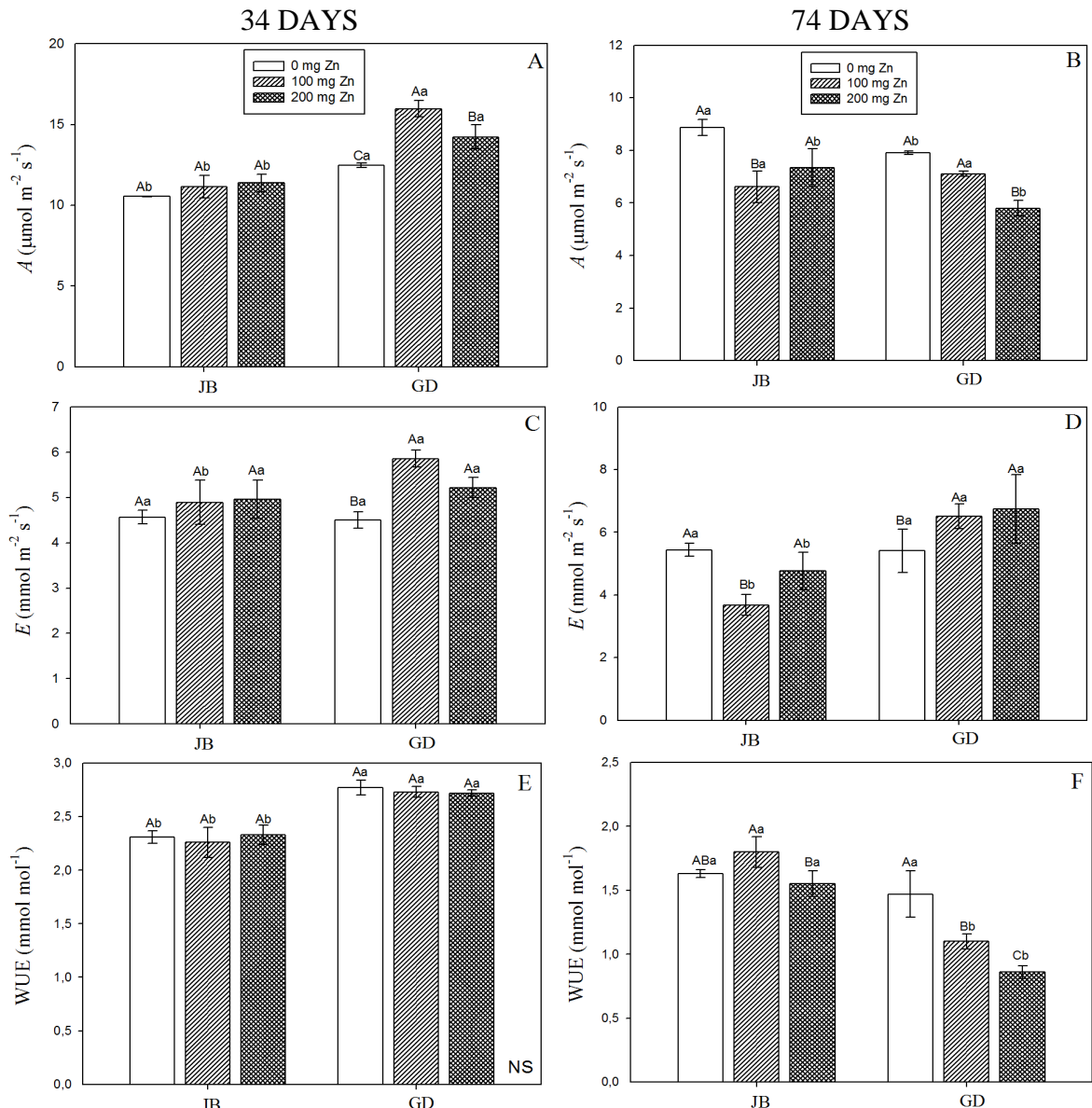
Figure 4. Effect of zinc levels on the photosynthetic rate at 34 (A) and 74 days (B), transpiration rate at 34 (C) and 74 days (D) and water use efficient at 34 (E) and 74 days (F) of growth of *P. glomerata* accessions. The data represent the mean ± S.D. from three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatments. NS = No significant interaction between accession and Zn levels.

Seedlings of *Quercus suber* L. exposed to 150 µM Zn showed lower stomatal conductance and transpiration rate when grown under lower doses of Zn (Disante et al., 2011). The authors also point out that the increase in Zn accumulation resulted in a decrease stomatal conductance and a tendency to lower CO_2 assimilation. Vijayarengan and Mahalakshmi (2013) observed that levels of 50 and 100 mg kg^{-1} of Zn in the soil were beneficial to the photosynthetic responses of tomato plants, but Zn levels above 150

mg kg^{-1} promoted toxic effects. Sagardoy et al. (2010) related reduction of 70% in the stomatal conductance in *Beta vulgaris* submitted to Zn excess. Cambrollé et al. (2013) observed that Zn levels above 90 µM were responsible for the decline in photosynthetic activity, resulting in decrease of pigments contents in *Limoniastrum monopetalum* (L.).

Heavy metals as Zn can inhibit the photosynthesis at various stages and through different mechanisms, as increased of stomatal resistance, chlorophyll degradation or impairment of PS II activity (Bazzaz and Govindjee, 1974; Baszynski et al., 1989; Li et al., 2013). High levels of Zn may yet decrease the activity of enzymes involved in carbon fixation as Rubisco, through of substitution of Mg^{2+} (Ralph and Burchett, 1998; Mysliwa-Kurdziel et al., 2004; Cambrollé et al., 2013) and also affect stomatal dynamics by inhibiting water channels (Yang et al., 2004) and reduction in K^+ uptake, thus altering the ratio between K^+ and other ions that controlling stomata movement (Tsalandzono et al., 1994; Vaillant et al., 2005).
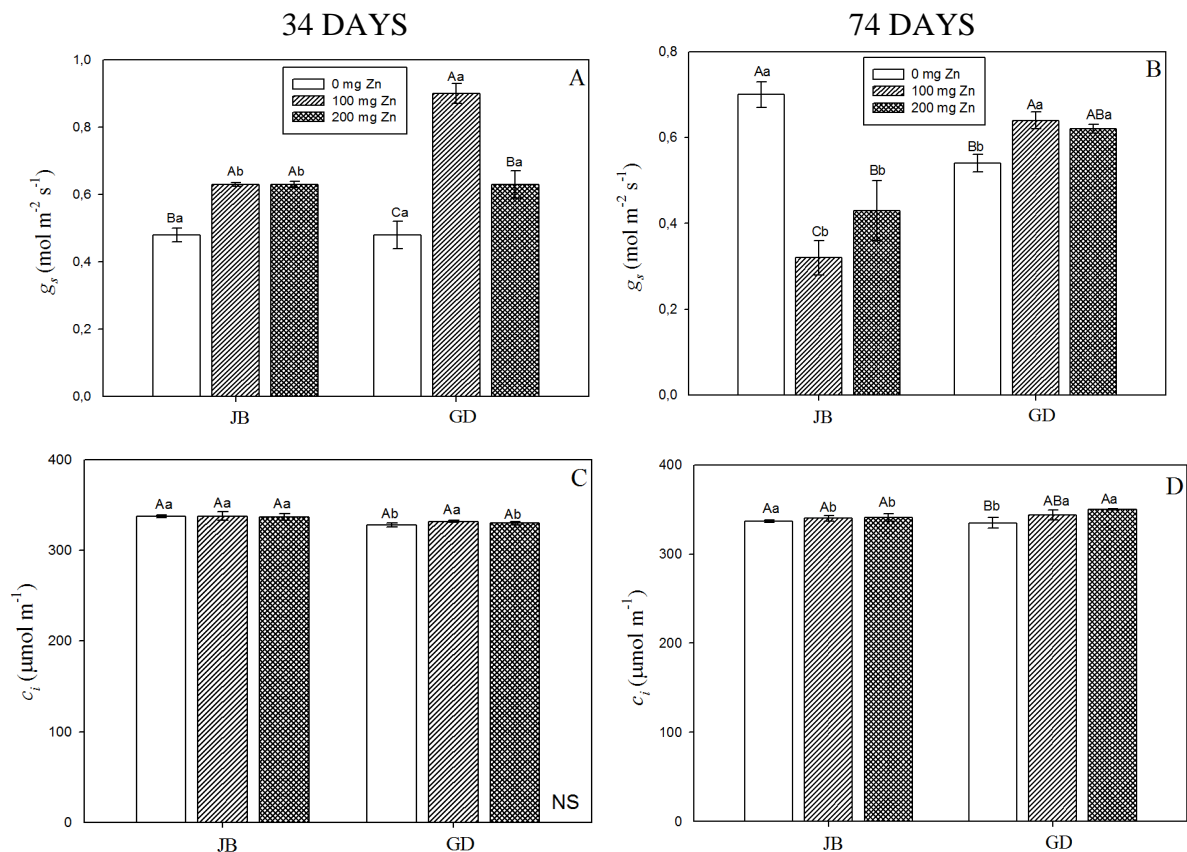
Figure 5. Effect of zinc levels on the stomatal conductance at 34 (A) and 74 days (B) and internal CO_2 concentration at 34 (C) and 74 days (D) of growth of *P. glomerata*. The data represent the mean ± S.D. from three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession. Different small letters indicate significant differences among accession within the same Zn treatments. NS = No significant interaction between accession and Zn levels.

In relation to internal CO_2 concentration, after 34 days of cultivation it was observed only differences between accessions. After 74 days of cultivation, it was observed an increase in the internal CO_2 concentration for GD accession (Figure 5D). On the other hand, the H_2O use efficiency after 74 days of cultivation was lower as compared to 34 days for both acessions (Figure 4F). The accession JB showed less efficiency as compared to the GD accession, which increased its efficiency under 100 mg Zn (Figure 4F).

After 10 days of cultivation of plants in soil containing 200 mg kg^{-1} of Zn it was observed small white spots on the older leaves of both accessions (Figure 6C). The excess of Zn combined with good supply of nutrients and the restrictions imposed by the pot size both accessions showed flowering at about 40 days (Figure 6A). After 65 days of cultivation it was observed the early initiation of senescence of older leaves in all treatments, but this was more pronounced for plants exposed to higher level of Zn (200 mg kg^{-1}) (Figure 6B). Leaf senescence is a common feature of this species next to the maturation phase (Santos and Carlesso, 1998) and, probably occourred due to increased ethylene production.
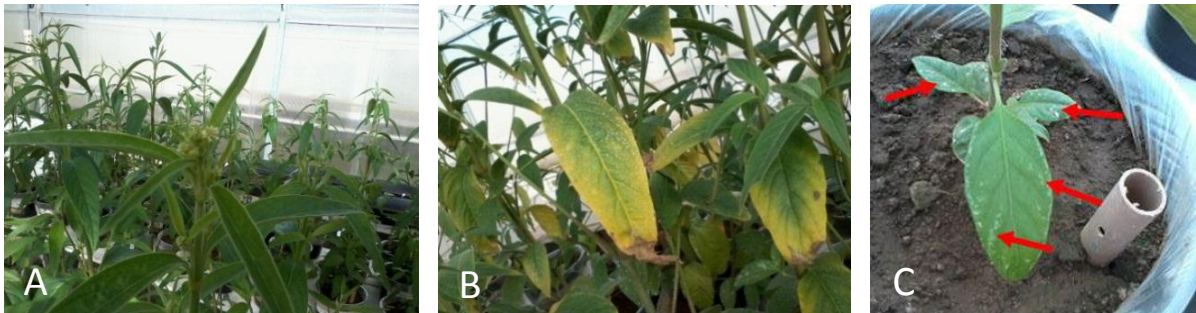
Figure 6. *P. glomerata* plants in flowering at 40 days (A), and at 65 days some sign of senescence in the older leaves were present (B), and presence of small white spots on the older leaves at 200 mg kg^{-1} Zn (C).

4   CONCLUSIONS

For most of parameters (dry matter production, photosynthesis, transpiration and stomatal conductance) evaluated the plants responded positively up to 100 mg kg^{-1} of Zn, but the level of 200 mg kg^{-1} Zn was detrimental.

The increase of Zn levels in the soil resulted in increase of Zn concentration in roots and leaves in both evaluated periods.

The accession JB showed no differences between Zn levels in relation to Zn translocation to the leaves and accumulation in roots in both evaluation periods. Already the accession GD showed increase in Zn translocation to the leaves and reduction in Zn accumulation in roots.

The accession GD also presented increase in stomatal index upon the addition of highest level of Zn, whereas the JB accession did not show any alteration.

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Antioxidant activity and β-ecdysone production of accessions of *Pfaffia glomerata* in response to excess zinc in nutrient solution and soil

ABSTRACT

Zinc (Zn) is an essential element for plant growth and development, acting in many metabolic processes. However, industrial, mining and agricultural activities have contributed to the increase of this element in the soil and in excess this element can induce to alterations in plants. *Pfaffia glomerata* is one of the Brazilian medicinal species that have aroused great interest from researchers due to its phytotherapics properties and also for its heavy metal tolerance. Thus, the objective of this study was to evaluate the effect of excess Zn on the antioxidant system and β-ecdysone production of accessions of *P. glomerata* grown in hydroponic floating system and soil. Were used two accessions of *P. glomerata* (JB and GD) and three levels of zinc (2, 100 and 200 µM) for cultivation in hydroponics with evaluations to 7 and 14 days, and the same accessions were submitted to three Zn levels (2, 100 and 200 mg kg^{-1}) for the experiment in soil with evaluated to 34 and 74 days. The effects of excess Zn on *P. glomerata* accessions were dependent of culture system and also evaluation period. The excess of Zn in both systems growth tested caused increase in MDA concentration (lipid peroxidation) and consequently increase of enzyme superoxide dismutase (SOD) activity and guaiacol peroxidase (GPX) in both accessions of *P. glomerata*. The concentration of anthocyanin in leaves and β-ecdysone in roots were significantly altered with the addition of Zn in growth substrate. The GD accession presented increase in β-ecdysone content and the JB presented decrease with the increase of Zn levels in soil, however, under ideal conditions the JB accession showed higher β-ecdysone content. The highest levels of anthocyanin were observed for JB plants grown in hydroponic, already, for soil the values of anthocyanin concentration were similar between accessions.

Keywords: Antioxidant enzymes, Brazilian ginseng, secondary metabolites, growth systems.

1 INTRODUCTION

Zinc (Zn) is an essential element to plants because it is involved with carbohydrate metabolism, regulation of gene expression, structural integrity of ribosomes, cell multiplication and metabolism of phosphate and nitrogen (Shier, 1994; Broadley et al., 2007). This micronutrient is constituent of metalloenzymes, as well as cofactor of enzymes such superoxide dismutase, dehydrogenase, protease, peptidase and fosfohidrolase (Hewitt, 1983; Cherif et al., 2011; Kabata-Pendias, 2011).

Despite the importance of Zn for plant growth and development, this micronutrient can easily become toxic when its concentration reaches high levels in the plant tissues (Marschner, 1995). Thus, several physiological and biochemical disorders in plants are often associated with the increase of Zn contents (Cambrollé et al., 2012; Naranjo et al., 2013). Toxic levels of Zn in the environment occurs mainly due to industrial and mining activities, inadequate use of fertilizers, pesticides and urban residues applications (Soares et al., 2001; Cunha et al., 2008).

The excessive uptake of Zn by plants can cause oxidative stress due to an imbalance between the antioxidant responses and increased of reactive oxygen species (ROS) production such as superoxide (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2), and when interacting with cellular components promote oxidative damage to nucleic acids, proteins, sugars, lipids and under extreme conditions, cell death (Gratão et al., 2005; Gadjev et al., 2008; Girotto et al., 2013).

To control the level of ROS and protect the cells, plants possess antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidases (POD), that may be involved in the detoxification of heavy metals (Gratão et al., 2005). Different antioxidant responses to excess Zn have been observed to be dependent of plant organ, Zn levels, exposure time, plant species or ecotypes (Cuypers et al., 2002). There are also many reports in the literature about the beneficial effects of secondary metabolites to protect plants against adverse conditions such hydric stress, high or low temperatures, ultraviolet radiation, nutritional deficiency and exposure to heavy metals (Marchese, 1999, Ling-Peng et al., 2006).

Plants may exhibit different responses to excess metals in substrates. *Pfaffia glomerata* plants, popularly known as Brazilian ginseng, had shown moderate to high tolerance to metals and metalloids such as Al, As, Cd, Cu, Pb and Hg (Maldaner, 2008; Skrebsky et al., 2008; Calgaroto, 2009; Gupta et al., 2013; Neis, 2013). This species has great genetic diversity

among its natural populations, which results in significant variability, which can promote different levels of tolerance to heavy metals (Kamada et al., 2009).

The content of secondary metabolites in plants might be related with heavy metals toxicity (Shützendübel and Polle, 2002), but differences among different species and accessions have also been observed. Neis (2013) observed that for the BRA accession of *P. glomerata*, independently of the availability of P in the soil, the highest content of β-ecdysone was found upon addition of 40 mg Cu kg^{-1} of soil. Variations in β-ecdysone content also were found in different accessions of *P. glomerata*, thus proving the existence of genetic variability among the accessions.

There are few studies relating the toxicity of heavy metal and its effect on the antioxidant system and β-ecdysone production in medicinal species, so it is of utmost importance of characterize the behavior of species in relation to high concentrations of Zn in different growth systems. Therefore, the aim of this study was to evaluate the effect of excess Zn on the antioxidant system and β-ecdysone production of two accessions of *P. glomerata* (JB and GD) grown in hydroponic floating system and soil.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The experiments were developed by the Research Group on Physiology of Plants with Agrobiological Interest at the Biology Department of Universidade Federal de Santa Maria. Two accessions of *Pfaffia glomerata* (JB and GD) were used. The JB accession belongs to the Botanical Garden collection of the Universidade Federal de Santa Maria (UFSM) and the GD accession was obtained from the medicinal plants collection of Universidade Federal da Grande Dourados (UFGD).

The plants used in the experiments were obtained by *in vitro* culture, from which nodal segments (1.0 cm long) without leaf were used.to micropropagate new plants on MS (Murashige and Skoog, 1962) medium supplemented with 6 g L^{-1} agar, 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol, and the absence of growth regulators, as previously described for Nicoloso et al. (2001).

2.1.1 Experiment 1 - Cultivation in hydroponic floating system

After 21 days of *in vitro* growth, the plants were transferred to plastic containers with a capacity of 17 liters of nutrient solution. The nutrient solution used was that one established by Tabaldi et al. (2007) with following composition (in µM): 6090.5 N; 974.3 Mg; 4986.76 Cl; 2679.2 K; 2436.2 Ca; 359.9 S; 243.592 P; 0.47 Cu; Mn 2.00; 1.99 Zn; Ni 0.17; 24.97 B; 0.52 Mo; 47.99 Fe (FeSO$_4$/Na EDTA). After seven days of acclimatization, Zn levels were added as ZnSO$_4$.7H$_2$O to the new nutrient solution.

The treatments were arranged in a completely randomized design with 3x2 factorial scheme, being three levels of Zn (without addition, 100 and 200 µM) and two accessions of *P. glomerata* (JB and GD), with three replicates for each treatment containing 30 plants each. The pH was adjusted daily to 5.5 ± 0.2 using HCl or NaOH solution of 0.1 M. The nutrient solution was changed every 7 days of cultivation. At seven and 14 days of cultivation 10 plants were collected for evaluations.

2.1.2 Experiment 2 - Cultivation in pots with soil

After 21 days of *in vitro* culture, homogeneous plants were selected and acclimated in plastic boxes containing 20 kg of sand in a hydroponic system according to Bandinelli et al. (2013). The hydroponic system was supplied with a nutrient solution described by Tabaldi et al. (2007), and three irrigations (15 min each) were carried out for 14 days. In order to minimize the stress caused by excess solar radiation, a shading screen (50%) was place over plants for the first five days.

Acclimatized plants were transplanted into pots containing 5 kg of a Typic Hapludalf soil, The soil was collected from the A horizon (0-20cm depth) in Santa Maria, RS. The soil chemical and physical data before the cultivation were: 18% of clay, 3.2% organic matter, pH 4.5, 30.6% Al, 26.5% bases saturation, 6.2 cmol dm^{-3} CTC, 2.8 cmol Ca dm^{-3}, 1.4 cmol Mg dm^{-3}, 2 mg P kg^{-1}, 2 mg Zn kg^{-1} and 55 mg K kg^{-1}. The SMP was 6.4, ranging from 5.4 to 5.6 after pH adjustment and addition of Zn concentrations: without addition, 100, and 200 mg kg^{-1}, in the form of zinc sulphate. It was added to the soil 60 mg kg^{-1} of phosphorus as triple superphosphate, 40 mg kg^{-1} potassium as potassium chloride and 20 mg kg^{-1} of nitrogen (N) as

ammonium nitrate based on results of soil analysis and previus data observed for biomass of *P. glomerata* (Neis 2013). At 20 and 40 days of cultivation, 20 mg kg^{-1} N was added.

The experimental unit consisted of a pot that contained 4 kg of air-dried soil (2 mm mesh) and one plant. Additionally, the pot was internally lined with a plastic bag to prevent both the loss of nutrients and water drainage. The experimental design consisted of randomized treatments with four replications. The experiment was conducted in a greenhouse that had no automatic temperature control. However, ventilation and mist was used to partially control the temperature on warmer days. Additionally, the pots received periodic rotations in the greenhouse to avoid the effects of a single location. Using distilled water, daily irrigations were performed soil samples. The soil moisture content was measured daily (by weighing) and maintained between 60 and 80% of field capacity. At 34 and 74 days of cultivation three plants were collected for evaluations.

2.2   Estimation of lipid peroxidation

The level of lipid peroxidation products was estimated according to the method of El-Moshaty et al. (1993), by determining the concentration of malondialdehyde (MDA) as the product of lipid peroxidation by reaction with thiobarbituric acid. A correction for non-specific turbidity was made by subtracting the absorbance value obtained at 600 nm. Lipid peroxidation was expressed as nmoles of MDA (mg fresh weight^{-1}).

2.3   Enzyme activities of antioxidant system

One gram of frozen tissue homogenized in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8) including 1 mM EDTA and 1% (w/v) Triton X was used for each assay (Zhu et al., 2004). The supernatant was used for the enzymes Superoxide dismutase (SOD) and Guaiacol peroxidase (GPX) activity assays. The SOD activity was assayed according to Misra and Fridovich (1972) and GPX was determined according Zeraik et al. (2008), and calculated according Chance and Maehly (1955).

2.4   Anthocyanin concentration

Approximately 0.1 g FW of plant tissue was homogenized in 5 ml methanol- 1% HCl overnight. Total anthocyanins were determined as the difference between absorbance at 536 and 600 nm (to correct for pheophytin absorbance; Hodges and Nozzolillo 1996). Results were expressed as cyanidin 3-glucoside equivalents (mg g FW^{-1}) and represent the mean of three samples.

2.5 β-ecdysone content

The chromatographic conditions utilized in this study were adapted from the methodology proposed by Magalhães (2000) and Figueiredo et al. (2004). Dried samples (1.0 g) were subjected to the extraction process with 125 ml of methanol in soxhlet for 4 hours, and further concentrated to 100 ml. Subsequently, the solutions were filtered through a membrane filter of 0.45μm and injected into the chromatographic system.

Analyses were performed on high performance liquid chromatography (HPLC-PAD). The mobile phase used was methanol (100%) and the time of standard retention was 2.7 minutes. The sample peaks were identified by comparison with the time of retention of the standard solution and the absorption spectrum at a wavelength of 245 nm.

2.6 Statistical analysis

Data were submitted to analysis of variance and the means of treatments were compared by Tukey test at 5% probability of error, using the statistical program SISVAR (Ferreira, 2000).

3   RESULTS AND DISCUSSION

The MDA concentration was higher in the roots as compared to shoots tissues of both accessions of *P. glomerata* plants cultivated in hydroponic system at 7 and 14 days (Figure 1). The higher MDA concentration was observed at higher Zn level (200 μM) in both accessions cultivated in hydroponic (Figure 1), except for the roots of GD accession at 14 days (Figure 1B). Several researchers have reported increase in MDA concentration in plants subjected to

metal stresses. In a research with *Hydrilla verticillata* exposed to 15, 75 and 150 µM of Zn for 7 days, Xu et al. (2013) observed gradual increase in tissue MDA levels with increasing Zn levels. Additionally, at 150 µM Zn the MDA content was 40% higher as compared with the control plants. Gomes et al. (2013) also observed that MDA concentrations in leaves of *Myracrodruon urundeuva* increased in presence of high levels of Zn.

Figure 1. Effect of zinc levels on the MDA concentration (mmol/g FW^{-1}) in root and shoot of *P. glomerata* accessions cultivated in hydroponics at 7 days (A and C) and 14 days (B and D). The data represent the mean ± S.D. of three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession. Different small letters indicate significant differences among accession within the same Zn treatment. NS = No significant interaction between accession and Zn levels.

In plants cultivated on soil, shoot MDA concentration of both accessions increased with increasing Zn levels in soil at 34 days, however the JB accession showed higher alteration in MDA concentration in the same period (Figure 2A). At later growth stage (74 days), the accession GD presented higher MDA concentration in the shoot upon the addition of 200 mg kg^{-1} Zn. On the other hand, the accession JB showed no differences in MDA concentration with

increasing Zn levels (Figure 2B). The data from the soil experiment indicates that possibly the shoot of GD accession was more sensitive than the JB accession to excess Zn in soil (Figure 2B), when plants were older.
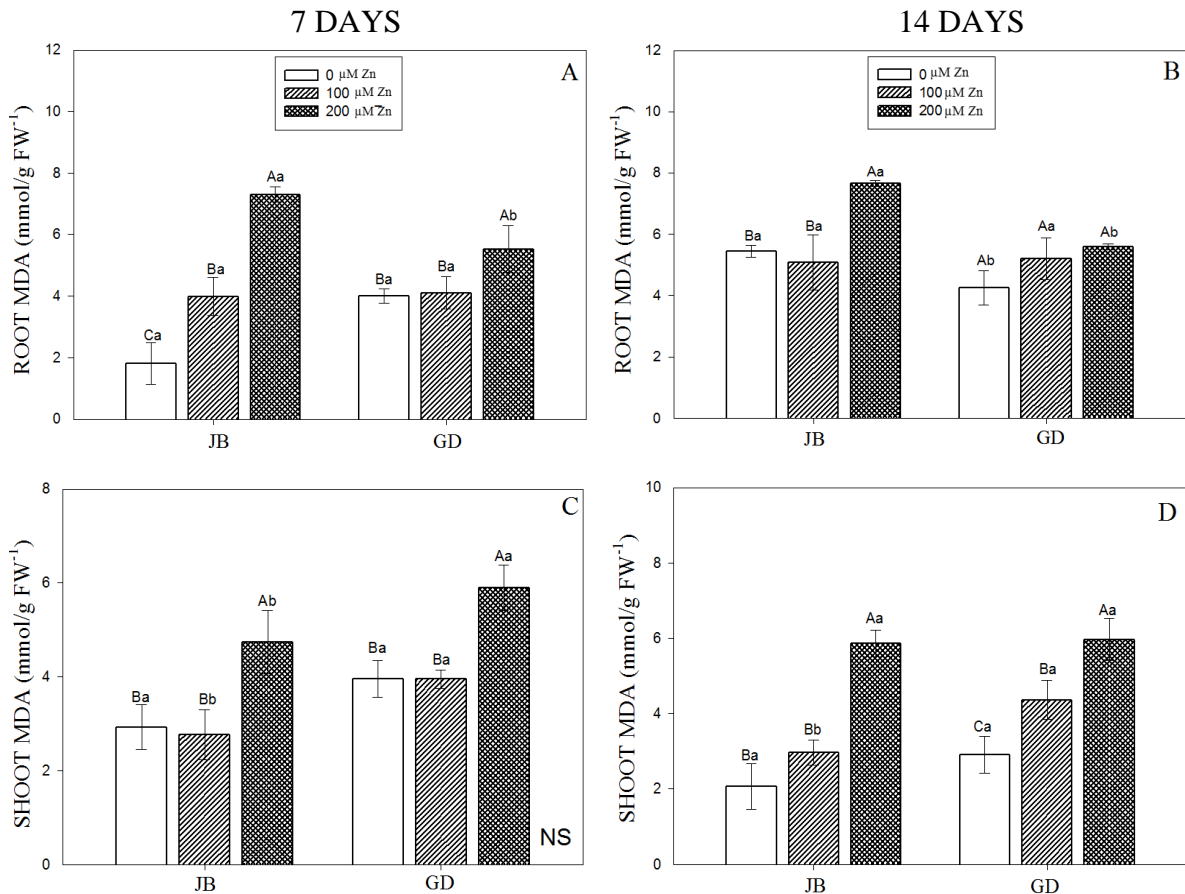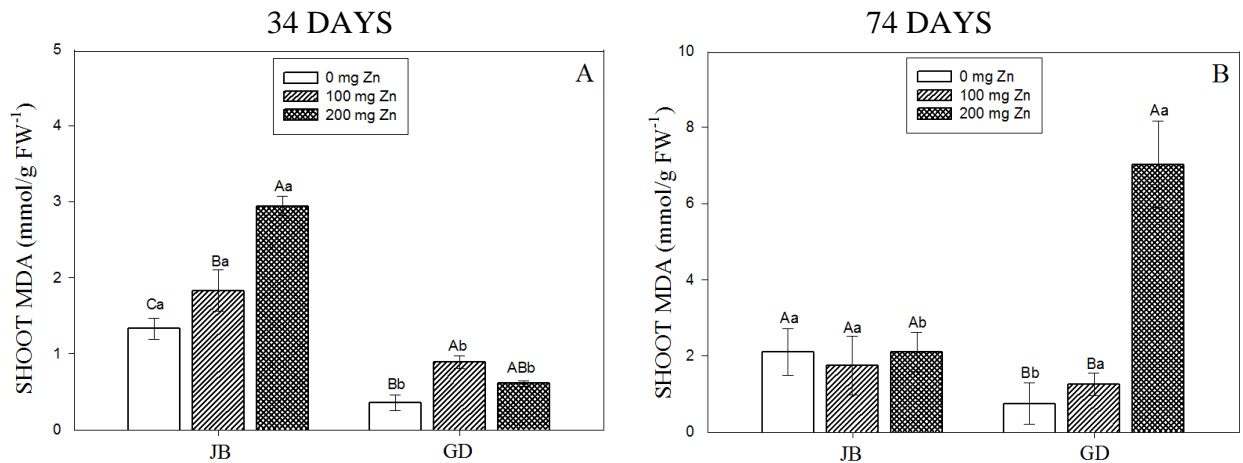

Figure 2. Effect of zinc levels on the MDA concentration (mmol/g FW^{-1}) in shoots of *P. glomerata* accessions cultivated on soil for 34 days (A) and 74 days (B). The data represent the mean ± S.D. of three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatment.

Alteration in lipid peroxidation of plants exposed to toxic Zn levels has been attributed to the increase of lipoxygenase activity, which is known to oxidize polyunsaturated fatty acids and produce free radicals (Chaoui et al., 1997). The lipid peroxidation also promotes changes in the membrane permeability, diminishing the sulphydryl content in the proteins of the plasma membrane (Tripathi and Gaur, 2004).

In many plant species heavy metals have been reported to cause oxidative damage due to the reactive oxygen species (ROS) production (Shah et al., 2001; Bazzo et al., 2011; Xu et al., 2013; Malar et al., 2014). As Zn is not a redox metal, it is not able to generate ROS directly through the Fenton reaction; however it can generate oxidative stress by interfering with the antioxidant defense system of plants (Gratão et al., 2005). ROS may cause plant injuries, as damages in membrane lipids, proteins, pigments and nucleic acids and in extreme cases resulting in plant death (Miller et al., 2008). To cope with oxidative damage, antioxidant enzymes and certain metabolites present in plants play an important role leading to plant adaptation and survival during periods of stress (Foyer et al., 1997; Malecka et al., 2001).

Plants possess several potential cellular mechanisms that may be involved in the detoxification of heavy metals. To control the level of ROS and protect the cells, plants possess an antioxidant defense system that can be enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidases (POD) and the non-enzymatic antioxidants are glutathione (GSH), ascorbic acid (ASA) carotenoids and tocopherols (lipid soluble) that scavenge the ROS (Mittler et al., 2004; Gratão et al., 2005; Shing et al., 2008).

In this context, the activity of enzyme superoxide dismutase (SOD) is essential to transform the O_2^- radical in H_2O_2, while the enzymes peroxidases remove the excess of H_2O_2 (Choudhary et al., 2007; Tanyolac et al., 2007). The activity of these enzymes is among the major biochemical parameters affected by oxidative stress (Yang et al., 2011), thus excess Zn can increase significantly the activity of these enzymes, as indirect effect generated by increased of MDA concentrations and increase in activity of SOD enzyme (isoform dependent of Cu/Zn), which would be a defense mechanism against oxidative stress processes (Cuypers et al., 2001).

In the present study, the SOD activity in roots and shoots of *P. glomerata* plants increased with increasing Zn levels in the nutrient solution, being the higher enzyme activity observed for the highest Zn level tested (200 µM) (Figure 3), except for the shoots of JB accession at 14 days (Figure 3D). In addition, the accession GD showed lower root SOD activity upon the addition of 100µM Zn at 7 days of exposure (Figure 3A), however has not been verified decrease in MDA production of the same level of Zn (Figure 1A).

In the experiment with soil, the shoot SOD activity in younger plants (34 days) was similar for the intermediate and higher Zn level tested in JB accession, occurring increase in relation to control level (Figure 4A), while for GD accession there was increase only for the higher Zn level. On the other hand, for older plants (74 days) the SOD activity was observed the higher activity for the intermediate level of Zn and GD accession showed no differences in enzyme activity for the tested Zn levels.

Cherif et al. (2011) reported that in Zn-treated tomato plants the SOD activity increased with increasing Zn concentration, but at the highest Zn level tested (150 mM) there was a decrease in the activity. Islam et al. (2014) observed significant increase in SOD activity upto 40, 23 and 32% in shoot while 36, 21 and 27% in root of *Zea mays* plants stressed with Zn, respectively at 10, 20 and 30 days of growth.

Figure 3. Effect of zinc levels on the SOD activity (Un. Enzyme/ml) in roots and shoots of *P. glomerata* accessions cultivated in hydroponics at 7 (A and C) and 14 days (B and D). The data represent the mean ± S.D. three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatment.
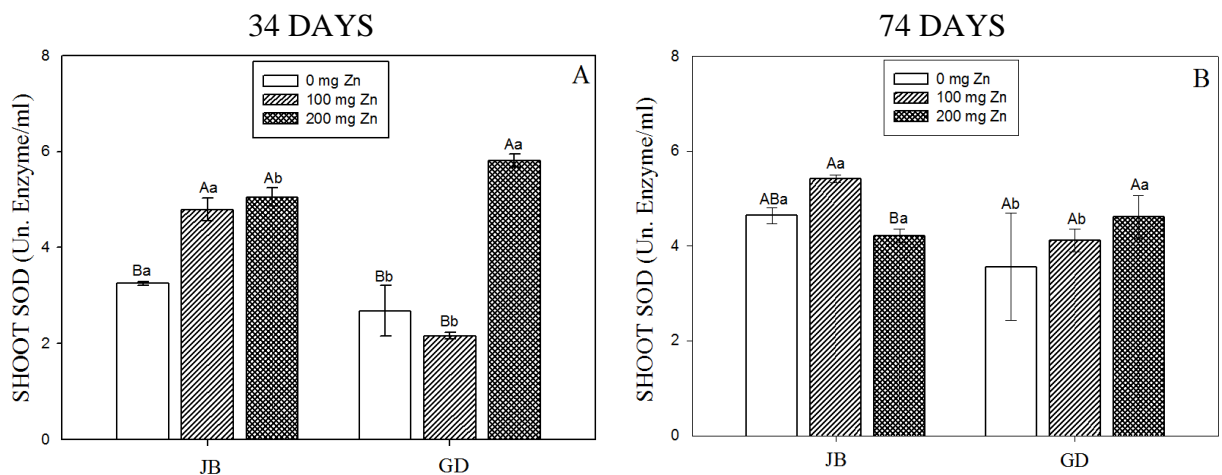
Figure 4. Effect of zinc levels on the SOD activity (Un. Enzyme/ml) in shoots of *P. glomerata* accessions cultivated on soil at 34 days (A) and 74 days (B). The data represent the mean ± S.D. three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatment.

The peroxidases (POD) represent an important role in cellular detoxification because they are capable of catalyzing a great number of oxidative reactions in plants using hydrogen peroxide as substrate or in some cases, oxygen as acceptor of hydrogen (Hiraga et al., 2001). The enzymes guaiacol peroxidase and ascorbate peroxidase participate in the direct elimination of H_2O_2, where the first acts in the cytosol, vacuole and cell wall and the second in organelles (Noctor and Foyer, 1998).

In the present study, there were no differences in root and shoot GPX activities of the accession GD at both evaluation periods in hydroponic system (Figure 5), possibly other enzyme peroxidase is acting, since there was increase in enzyme activity SOD and is necessary that occur removal of excess H_2O_2. On the other hand, JB accession showed increase in GPX activity for level of 200 μM Zn in roots to 7 days ando shoots to 14 days (Figure 5A and D).
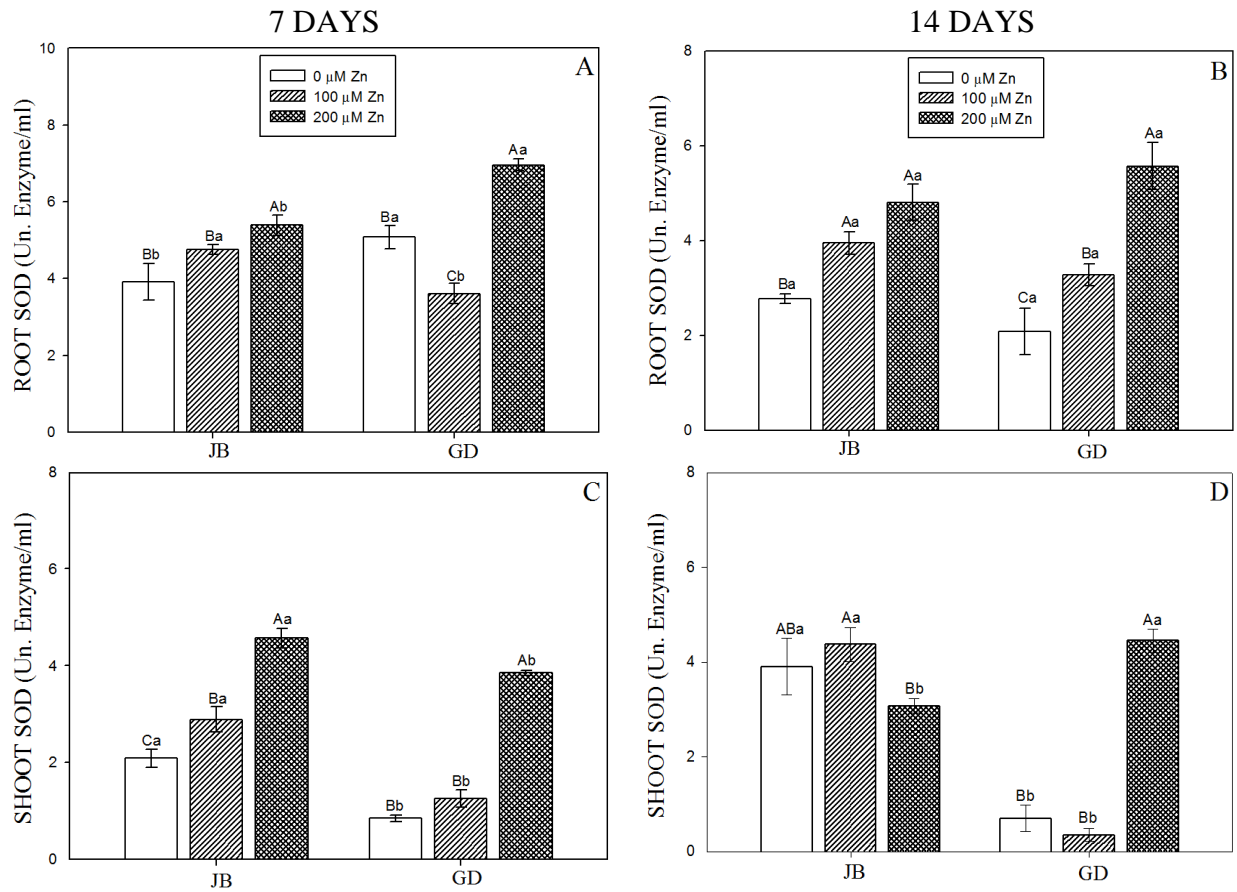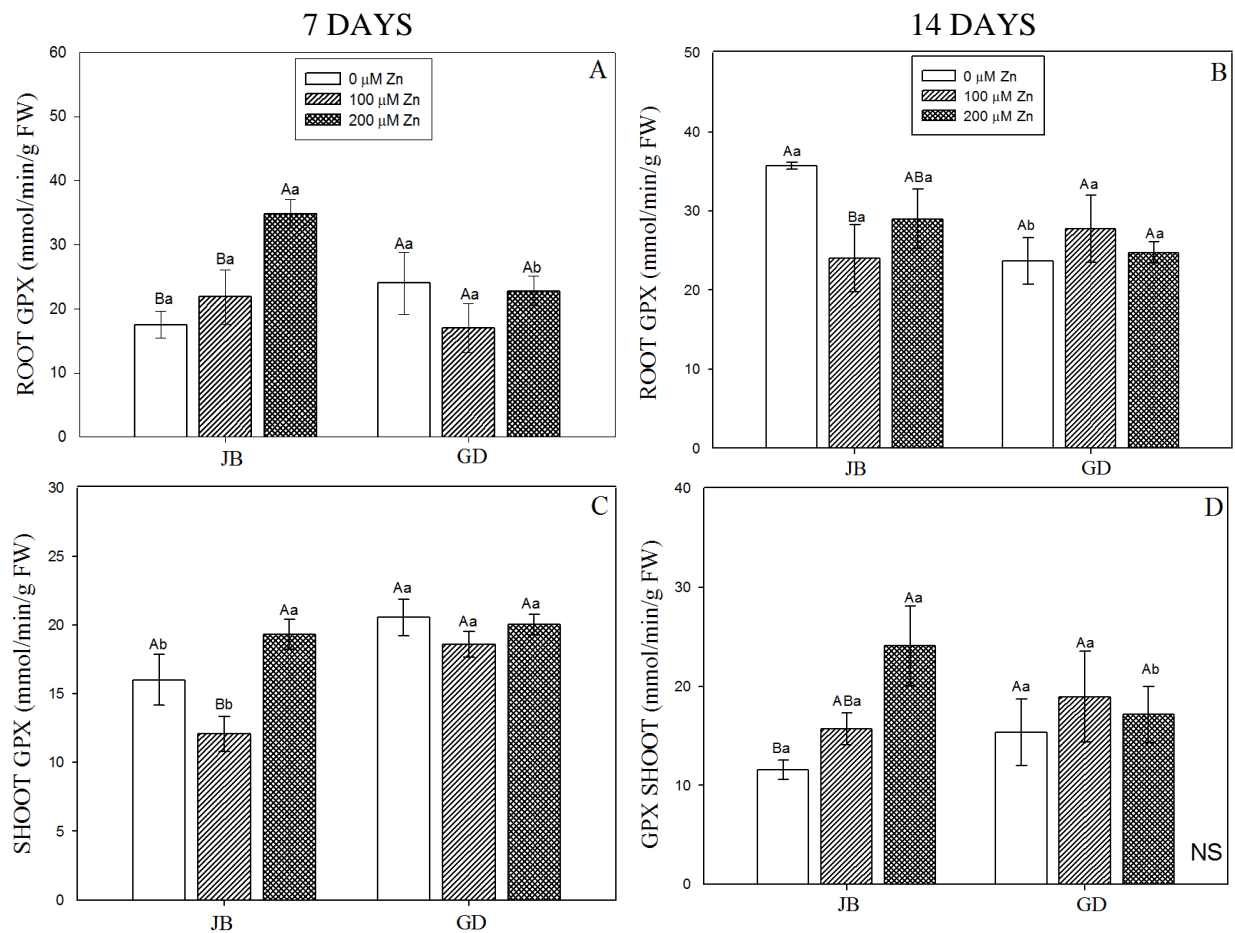

Figure 5. Effect of zinc levels on the GPX activity (mmol/min/g FW) in roots and shoots in *P. glomerata* accessions cultivated in hydroponics at 7 (A and C) and 14 days (B and D). The data represent the mean ± S.D. three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatments. NS = No significant interaction between accession and Zn levels.

It was observed a higher GPX activity in roots as compared with the shoots (Figure 5), which may be related with the higher enzyme SOD activity also observed for roots (Figure 3). Interestingly, the accession JB seems to present a more fast response based on GPX activity than GD accession. On the other hand, for *P. glomerata* plants grown on soil, the shoot GPX activity was higher in GD accession when compared to the JB accession at both evaluation periods (Figure 6A, B).
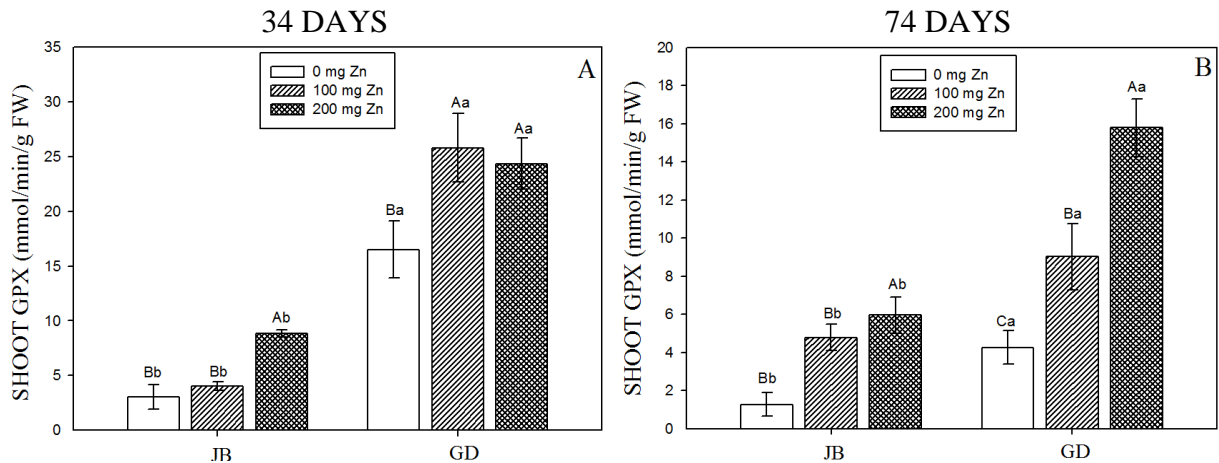

Figure 6. Effect of zinc levels on the shoot GPX activity (mmol/min/g FW) of two accessions of *P. glomerata* cultivated on soil at 34 days (A) and 74 days (B). The data represent the mean ± S.D. three different replicates. Different capital letters indicate significant differences ($P < 0.05$) among Zn treatments within the same accession. Different small letters indicate significant differences among accession within the same Zn treatment.

Previous studies have also shown that enzyme activity was correlated with changes in physiological processes such as respiration, photosynthesis and transpiration and therefore represents a commitment indicator of these metabolic activities (Aravind and Prasad, 2005). The induction of POD activity has been reported in many plant species, when exposed to zinc stress or others heavy metals (Prasad et al., 1999; McGeer et al., 2000). Verma and Dubey (2003) reported that GPX activity increased in roots and shoot of rice upon lead excess.

There are also several reports in the literature about the beneficial effects of secondary metabolites to plants under stress situations (Marchese, 1999, Ling-Peng et al., 2006). Anthocyanins are natural pigments that belong to a subclass within the group of flavonoids (Eibond et al., 2004). These metabolites are responsible for most of colors red, purple and blue observed in different parts of the plant tissues and may also protect leaves from excess light or UV light, nutrient deficiency, water deficit and heavy metal stress (Nogués et al., 1998; Pinto et al., 1999; Warren et al., 2003; Ling-Peng et al., 2006). Anthocyanins attract pollinators and seed dispersers, protect plant tissues from photoinhibition and oxidation from photosynthesis

and serve as scavengers of reactive oxygen intermediates (Sherwin and Farrant, 1998; Yamasaki, 1997).

In the present study, the concentration of anthocyanin in leaves of *P. glomerata* plants cultivated in hydroponics was higher for the accession JB at both evaluation periods (Figure 7). However, the anthocyanin concentration increased with increasing Zn levels for JB accession at 7 days and in both accessions at 14 days of cultivation (Figure 7B). For the soil experiment (Figure 8), the anthocyanin concentration was lower in JB accession at 100 mg kg^{-1} Zn at 34 days of cultivation (Figure 8A), however has remained unchanged at 74 days of cultivation (Figure 8B). Differently from JB accession, GD showed a similar response in both evaluation periods, where the addition of Zn increased the anthocyanin concentration (Figure 8).

Figure 7. Effect of zinc levels on the leaf anthocyanin concentration (µM g^{-1} FW) of two accessions of *P. glomerata* cultivated in hydroponics at 7 (A) and 14 days (B). The data represent the mean ± S.D. three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatment.

In general, it is believed that the increase in anthocyanins content increase the antioxidant response of plants, in order to uphold the regular physiological status in tissues directly or indirectly affected by biotic or abiotic stressors (Neill et al., 2002). Gülçin et al. (2005) still reported that total anthocyanin of *Perilla pankinensis* had strong antioxidant activity, scavenging the superoxide anion and hydrogen peroxide. Park et al. (2012) reported that the anthocyanin content of *Arabidopsis* Col-0 ecotypes increased significantly under excess Zn, however, the response was exposure time dependent. These authors still observed that there was no significant difference between anthocyanin accumulations in two ecotypes in relation to Cd, Pb and Co treatments.
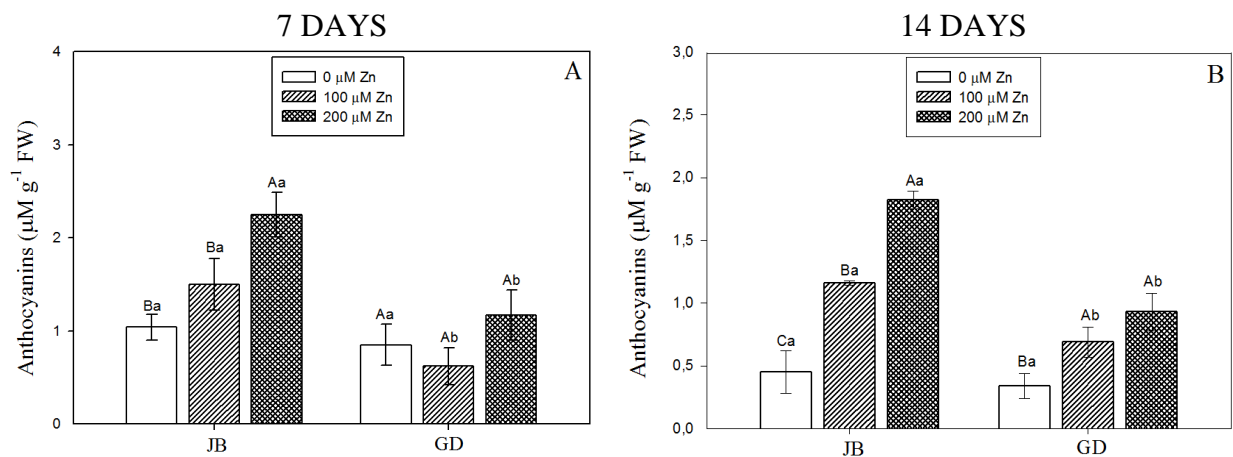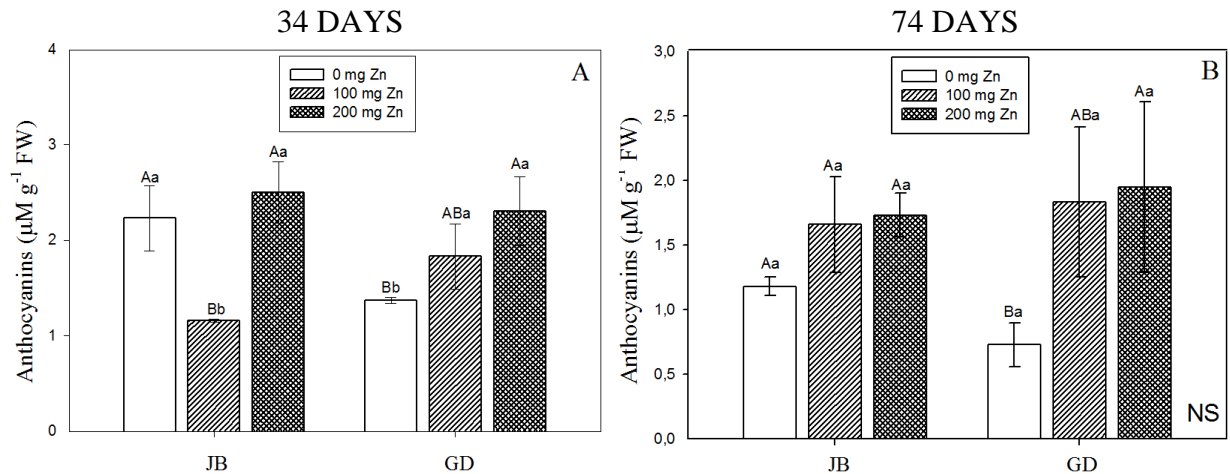
Figure 8. Effect of zinc levels on the leaf anthocyanins concentration (μM g^{-1} FW) of two accessions of *P. glomerata* accessions cultivated on soil at 34 (A) and 74 days (B). The data represent the mean $\pm$ S.D. three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession within the same Zn treatment. NS = No significant interaction between accession and Zn levels.

The excess Zn in soil increased the root β-ecdysone concentration in both accessions of *P. glomerata* at 74 days of cultivation, but this increase was higher for GD accession (Figure 9A). However, there was an effect of concentration due to the decrease in the production of root dry matter. In the present study, the lower β-ecdysone content (0.16%) was observed for accession GD in the control treatment and the highest content (0.23%) was also for the JB accession in the control treatment (Figure 9B).
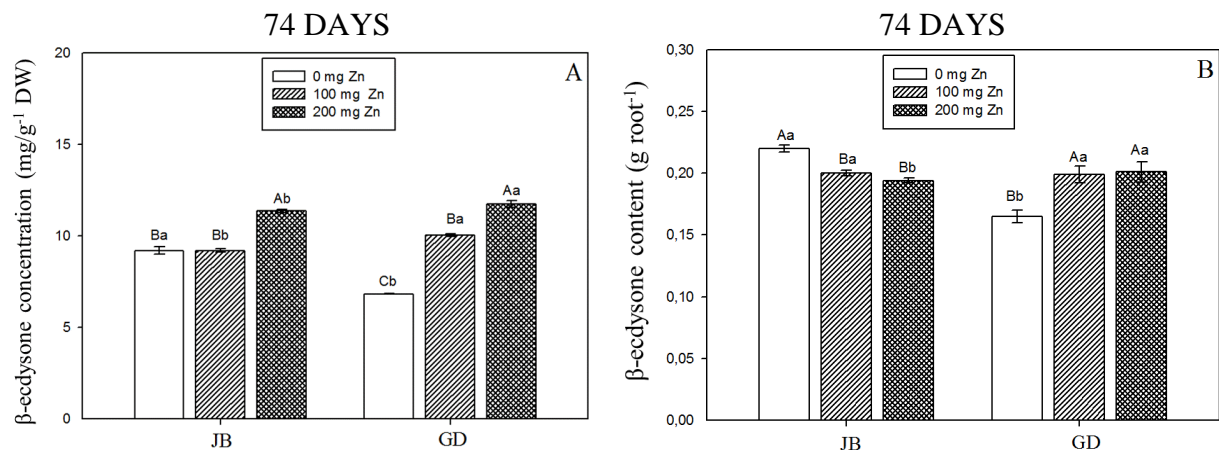
Figure 9. Effect of zinc levels on β-ecdysone concentration (mg/g^{-1} DW) (A) and content (g root^{-1}) (B) in roots of two accessions of *P. glomerata* cultivated in soil at 74 days. The data represent the mean $\pm$ S.D. of three different replicates. Different capital letters indicate significant differences (P <0.05) among Zn treatments within the same accession and different small letters indicate significant differences among accession with the same Zn treatment.

Changes in β-ecdysone content are often observed between different accessions of *P. glomerata* even under controlled conditions, which according to Magalhães (2000) and Figueiredo et al. (2004) are based mainly on genetic factors. The JB accession showed reduction in β-ecdysone content with increasing levels of Zn in soil, whereas for the accession GD was observed the opposite, where root β-ecdysone content increased. Other difference between accessesions observed was that under control conditions the JB accession showed higher β-ecdysone content that the GD (Figure B).

Some studies conducted with *P. glomerata* showed contents of β-ecdysone in roots ranging from 0.15 to 0.75%, indicating that the species has a wide variation in relation to the accumulation of this compound (Figueiredo et al., 2004; Freitas et al., 2004; Zimmer et al, 2006). Flores et al. (2010) observed lower β-ecdysone contents in the root tissues of BRA (0.42%) and JB (0.28%) accessions after 2 years of cultivation, these higher values found reflect the longest time of plants cultivation and in this present experiment were only 74 days. In addition, Neis (2013) reported that the highest β-ecdysone concentration (66 μg g^{-1} dry weight) in the roots of JB accession was found in the lower availability of P and Cu. On the other hand, the BRA accession had the highest β-ecdysone concentration (60 μg g^{-1} dry weight) in the presence of 40 mg Cu kg^{-1}, regardless of the P availability in the soil.

4 CONCLUSIONS

The effects of Zn are dependent on the cultivation system and evaluation period.

Excess Zn in both growth systems tested caused increase in lipid peroxidation (MDA concentration) and increase in SOD and GPX activities of *P. glomerata* plants. However, plants growth on soils showed higher alteration in GPX activity.

The concentration of anthocyanins in leaves and β-ecdysone in roots were significantly altered upon the addition of Zn in the growth substrate. JB accession showed higher concentrations of anthocyanins in hydroponic culture in both time periods.

The same accession showed reduction in β-ecdysone content with the addition of Zn in soil, already for the GD was observed the opposite, however under ideal conditions the JB accession showed higher β-ecdysone content that the GD accession.

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DISCUSSÃO GERAL

O primeiro experimento em hidroponia foi realizado afim de avaliar os efeitos do Zn sobre a morfologia radicular e produção de biomassa de três accesos de *Pfaffia glomerata* e distinguir ambos de acordo com seu nível de tolerância. Após, foi realizado um segundo experimento em solo com maior duração afim de avaliar o efeito de concentrações do elemento sobre a eficiência fotossintética, alterações estomáticas, produção de biomassa, acumulação de Zn nas raízes e a translocação para as folhas dos dois acessos de *P. glomerata* selecionados no experimento anterior. Por fim, realizou-se um terceiro experimento, utilizando os acessos JB e GD em duas condições de cultivo distintas, afim de avaliar as respostas do sistema antioxidante e a produção de β-ecdisona dos acessos.

Nos três experimentos, os acessos apresentaram sintomas típicos de plantas sob stresse de metais pesados como: redução no crescimento, alterações na morfologia radicular, redução significativa na produção de biomassa, redução da taxa fotossintética e conteúdo de clorofila, alteração no índice estomático, aumento da peroxidação lipídica, incremento na atividade de enzimas antioxidantes, bem como surgimento de cloroses e senescência foliar. Também foi observado que para diversos parâmetros avaliados ambos acessos apresentaram incremento para as menores concentrações de Zn no substrato de cultivo. Vários autores relacionam esse incremento com a grande exigência do elemento para o metabolismo de carboidratos, síntese de proteínas, regulação e expressão de genes, estrutura de ribossomos e o seu papel como cofator em um grande número de enzimas (CLEMENS et al., 2002; BROADLEY, 2007).

No primeiro experimento, as raízes dos acessos de *P. glomerata* foram mais afetados pelo excesso de Zn do que a parte aérea. Possivelmente isso ocorreu porque as raízes estão em contato direto com a solução nutritiva e são o principal local de entrada do elemento na planta (MARSCHNER, 1995), bem como em virtude de ser um experimento de curto prazo, a planta pode não ter tido tempo suficiente para a translocação do Zn, já em um experimento de longo prazo possivelmente a parte aérea seria mais afetada. O aumento no diâmetro radicular foi provavelmente uma resposta relacionada à diminuição da divisão celular na porção apical das raízes, com a diminuição da atividade meristemática a tendência é que ocorra redução do comprimento radicular e aumento do diâmetro.

Os resultados da correlação de Pearson mostraram que as produções de biomassa da parte aérea e total total da planta foram proporcionais e dependentes da produção de biomassa

de raízes. Também foi observada que a produção de biomassa é dependente do comprimento radicular. Maior comprimento radicular promove maior absorção de água e nutrientes e, consequentemente, aumento da produção de biomassa (WEI e ZHOU, 2006). O número de pontas das raízes influenciou o diâmetro, o comprimento e a produção de biomassa seca das mesmas, no entanto, o oposto também foi observado.

Vários fatores ambientais podem influenciar a morfologia radicular. Esses fatores incluem estresse por metais pesados (LI et al., 2005), condições de umidade do solo (DAVIES e BACON, 2003), temperatura e disponibilidade de nutrientes (ZHANG et al., 2003). Sintomas de toxicidade de zinco são marcantes no sistema radicular, principalmente no comprimento das raízes, uma vez que o excesso de Zn afeta a síntese de DNA, atividade mitótica e alongamento (ROUT e DAS, 2003; JAIN et al., 2010).

É desejável que espécies tolerantes demonstrem maior crescimento, bem como produção de biomassa e maior comprimento radicular quanto expostas a presença de metais pesados (LAMEGO e VIDAL, 2007). Assim, dentre os três acessos estudados de *P. glomerata*, o GD apresentou maior tolerância ao Zn, seguido pelo JB e por último o BRA. Apesar do BRA manter pequena alteração nos parâmetros avaliados, independente do nível de Zn, houve pequeno crescimento das plantas, bem como o surgimento de folhas cloróticas, resposta que sugere que esse acesso não se adaptou as condições de cultivo em hidroponia. Portanto, os acessos GD e JB foram selecionados para os experimentos subsequentes.

O aumento dos níveis de Zn no solo promoveu aumento da concentração do metal nos tecidos das raizes e folhas. Observou-se maior concentração de Zn nas raízes durante o primeiro período de avaliação (34 dias), e diminuição para o segundo período de avaliação (74 dias). As plantas do acesso JB não apresentaram diferenças entre os níveis de Zn em relação a translocação do elemento para as folhas e acúmulo nas raízes. Já o acesso GD apresentou maior translocação do elemento para as folhas e menor acúmulo no sistema radicular, e por isso possivelmente apresente mecanismos específicos relacionados com a absorção, transporte e compartimentalização.

O acesso GD mostrou características interessantes como maior translocação de Zn para as folhas, menor acúmulo nas raízes e maior produção de biomassa. No entanto, como se trata de uma espécie medicinal, e a raiz é o principal órgão de interesse da espécie, é de suma importância que não ocorra o acúmulo do elemento nas raízes em níveis tóxicos para o consumo humano.

Interessantemente, o incremento dos níveis de Zn no solo alterou significativante o conteúdo de β-ecdisona produzido pelos acessos. Em condições ideais o acesso JB apresentou maior conteúdo do composto em comparação com o acesso GD, sendo inclusive o maior conteúdo observado entre os tratamentos (0,23%) e o menor conteúdo foi de 0,16%, observado para o tratamento testemunha de Zn do acesso GD. Foram observadas respostas contrárias entre os acessos com o incremento de Zn no solo, o acesso JB mostrou redução no conteúdo do composto, já o acesso GD mostrou incremento.

O ecdisteróide β-ecdisona é o composto mais importante presente na *P. glomerata,* sendo também utilizado como marcador químico de qualidade das raízes (MAGALHÃES, 2000; ZIMMER et al., 2006). Alterações no conteúdo β-ecdisona são frequentemente observadas entre diferentes acessos de *P. glomerata* (MAGALHÃES, 2000; FIGUEIREDO et al., 2004) que ocorrem principalmente devido à variação genética existente entre os acessos (KAMADA et al., 2000). As condições impostas durante o cultivo das plantas também apresentam influência direta na biossíntese e/ou acúmulo de metabólitos (TOMÁS et al., 1993). De acordo com o presente estudo foram comprovadas diferenças na concentração entre os acessos, bem como o efeito do Zn, que promoveu aumento na produção do composto.

O acesso GD apresentou um aumento no índice estomático com a adição de mais alto nível de Zn ao solo, enquanto o acesso JB não mostrou qualquer alteração. Esse aumento no índice estomático tem sido descrito para espécies submetidas a estresses ambientais e possivelmente seja uma modificação na anatomia foliar afim de tentar promover uma maior captação de CO_2 para que a fotossíntese não seja afetada, já que sob estresse ocorre uma diminuição na abertura do poro estomático. A maior fotossíntese, transpiração e condutância estomática ocorreu em plantas do acesso GD com a adição de 100 mg kg^{-1} de Zn ao solo aos 34 dias de cultivo. Aos 74 dias, o acesso apresentou aumento na transpiração, mas a fotossíntese e a condutância estomática foram afetados negativamente para ambos os acessos.

Vários autores relatam que metais pesados como Zn podem inibir a fotossíntese em várias fases e através de diferentes mecanismos, devido ao aumento da resistência estomática, degradação da clorofila ou menor atividade do PS II (BAZZAZ; GOVINDJEE, 1974; BASZYNSKI et al., 1989; LI et al., 2013). Altos níveis de Zn podem ainda diminuir a atividade de enzimas envolvidas na fixação de C como a Rubisco, através da substituição dos íons Mg^{2+} (MYSLIWA-KURDZIEL et al., 2004; CAMBROLLÉ et al., 2013), bem como afetar a

dinâmica estomática através da inibição dos canais de água e redução da absorção do K^+ (TSALANDZONO et al., 1994; VAILLANT et al., 2005).

Em muitas espécies de plantas o excesso de metais pesados foi responsável por causar danos oxidativos às moléculas orgânicas devido ao aumento da produção de espécies reativas de oxigênio (EROs) (BAZZO et al., 2011; XU et al., 2013; MALAR et al., 2014). O Zn é um metal não redox e por isso não é capaz de gerar EROs diretamente através da reação de Fenton, no entanto o mesmo pode gerar estresse oxidativo através da interferência no sistema de defesa antioxidante das plantas, devido ao desequilíbrio entre as respostas antioxidantes e produção de EROs (GRATÃO et al., 2005).

As EROs podem causar danos em lípidos e proteínas da membrana plasmática, pigmentos e ácidos nucleicos e em situações severas morte da planta (MILLER et al., 2008). A peroxidação lipídica é um evento primário que inicia uma sequência de lesões citotóxicas nas células e alterações na permeabilidade da membrana, que levam a alterações no fluxo de íons e outras substâncias, o que resulta na perda da seletividade para a entrada ou saída de nutrientes e substâncias tóxicas na célula (BARBER; HARRIS, 1994; LIMA; ABDALLA, 2001). O malondialdeído (MDA) é o produto citotóxico da peroxidação lipídica e indicador da produção de radicais livres e lesões nos tecidos (OHKAWA et al., 1979).

O excesso de Zn no solo e na solução hidropônica levou ao aumento da peroxidação lipídica, induzindo assim o aumento na atividade das enzimas antioxidantes em plantas de *P. glomerata*. A indução nas atividades de enzimas antioxidantes é uma estratégia geral adotada pelas plantas para superar o estresse oxidativo imposto por estresses ambientais (ZAMAN; ZEREEN, 1998; VERMA; DUBEY, 2003).

O aumento na peroxidação lipídica em plantas expostas a níveis tóxicos de Zn é atribuído ao aumento da atividade da enzima lipoxigenase, que é conhecida por oxidar os ácidos graxos poliinsaturados das membranas e produzir radicais livres (CHAOUI et al., 1997). Ernst (1998) também relatou que as proteínas, especialmente os grupos SH também podem ser afetados pelo excesso de Zn, causando assim danos para a estabilidade da membrana.

Para controlar os nível de EROs e proteger as células, as plantas possuem um sistema de defesa antioxidante enzimático e não enzimático. Os antioxidantes enzimáticos incluem a superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT) e peroxidases (POD), já os antioxidantes não enzimáticos incluem a glutationa (GSH), ácido ascórbico

(ASA), carotenóides e tocoferóis que atuam na eliminação das EROs (MITTLER et al., 2004; GRATÃO et al., 2005; SHING et al., 2008).

A atividade da enzima SOD é essencial para transformar o radical O_2^- em H_2O_2, enquanto as enzimas peroxidases atuam na remoção do excesso de H_2O_2 (CHOUDHARY et al., 2007; TANYOLAC et al., 2007). A atividade destas enzimas está entre os principais parâmetros bioquímicos afetados pelo estresse oxidativo (YANG et al., 2011). O Zn pode aumentar significativamente a atividade destas enzimas como efeito indireto gerado pelo aumento das concentrações de MDA e aumentar a atividade da enzima SOD pelo aumento das isoformas da enzima SOD dependente de Zn (Cu/Zn), o que seria um mecanismo de defesa contra o estresse oxidativo (CUYPERS et al., 2001).

No experimento em hidroponia foi observada maior atividade da enzima SOD e GPX nas raízes e menor na parte aérea. A enzima GPX é importante para quebrar o H_2O_2 nas raízes, uma vez que está localizada na parede celular (BAZZO et al., 2011), o que foi confirmado pelo experimento. Outro detalhe verificado foi que o acesso JB parece apresentar uma resposta mais rápida em relação ao aumento da atividade da enzima GPX, diferente do acesso GD.

Estudos demonstram que a atividade das enzimas peroxidases está relacionada com mudanças nos processos fisiológicos, tais como a respiração, fotossíntese e transpiração e representa um indicador do comprometimento destas atividades metabólicas (ARAVIND; PRASAD, 2005). A indução da atividade das peroxidases tem sido relatada em muitas espécies expostas a metais pesados (PRASAD et al., 1999; McGEER et al., 2000).

Ainda em relação à proteção das plantas às condições adversas, existem vários relatos que o aumento no teor de antocianinas nas folhas seja importante para a resposta antioxidante das plantas (GÜLÇIN et al., 2005). No experimento em hidroponia o acesso JB apresentou as maiores concentrações de antocianinas em ambos os períodos de avaliação, no entanto o mesmo não foi observado para o experimento em solo.

CONCLUSÕES

Nos três experimentos os acessos apresentaram sintomas típicos de plantas sob stresse de metais pesados, bem como incremento em diversos parâmetros para níveis baixos de Zn.

A tolerância das plantas de *P. glomerata* para o excesso de zinco diferiu entre os acessos. Em relação aos parâmetros de produção de biomassa e radiculares, conclui-se que o acesso GD mostrou-se mais tolerante ao elemento, seguido pelo acesso JB e BRA.

O aumento dos níveis de Zn no solo resultou em aumento da concentração do elemento no tecido de raízes e folhas. O acesso GD apresentou menor acúmulo de Zn nas raízes e maior translocação para as folhas, já para o acesso JB não foram observadas diferenças para os níveis de Zn testados.

A máxima fotossíntese, transpiração e condutância estomática ocorreu em plantas do acesso GD com a adição de 100 mg kg^{-1} de Zn ao solo aos 34 dias de cultivo.

O acesso GD apresentou aumento no índice estomático com a adição de 200 mg Zn, já o acesso JB não mostrou qualquer alteração.

Os efeitos do excesso de Zn sobre os acessos de *P. glomerata* dependeram do sistema de cultivo e também do período de avaliação.

O excesso de Zn em ambos os sistemas de crescimento testados causou aumento na peroxidação lipídica (concentração MDA) e aumento da atividade das enzimas SOD e GPX em plantas de *P. glomerata*.

A produção de antocianinas em folhas e conteúdo de β-ecdisona em raízes foram significativamente alteradas após a adição de Zn no substrato de crescimento em ambos os acessos de *P. glomerata*.

O acesso JB apresentou as maiores concentrações de antocianinas no experimento em hidroponia, bem como também maiores diferenças entre os níveis de Zn testados. Já em relação ao conteúdo de β-ecdisona o mesmo apresentou decréscimo na produção com o incremento de Zn no solo, sendo observado o oposto para o acesso GD.

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