AIRON JOSÉ DA SILVA

SILÍCIO NA AMENIZAÇÃO DA FITOTOXICIDADE DE CÁDMIO E ARSÊNIO AVALIADA POR FLUORESCÊNCIA DA CLOROFILA

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Tese apresentada ao Programa de Pós-Graduação em Ciência do Solo da Universidade Federal Rural de Pernambuco como parte dos requisitos para obtenção do título de Doutor em Ciência do Solo.

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AIRON JOSÉ DA SILVA

Tese "**SILÍCIO NA AMENIZAÇÃO DA FITOTOXICIDADE DE CÁDMIO E ARSÊNIO AVALIADA POR FLUORESCÊNCIA DA CLOROFILA**", apresentada ao Programa de Pós-Graduação em Ciência do Solo da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Doutor, e aprovada em 26 de julho de 2013.

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Dr. Clístenes Williams Araújo do Nascimento

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Examinador

“Ninguém ignora tudo. Ninguém sabe tudo. Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa. Por isso aprendemos sempre.”

Paulo Freire

Ofereço

Aos meus pais, Biatriz e Abdias, por todo apoio e incentivo que me deram para que eu estudasse e fosse alguém na vida;
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À minhas sobrinhas, Marta e Ana Beatriz, pelo ideal de futuro;
À minha namorada, Andresa, pela atenção e carinho.

Dedico

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BIOGRAFIA DO AUTOR

Airon José da Silva, filho de Biatriz Maria da Silva e Abdias José da Silva, nasceu no dia cinco de maio de 1982, natural de Caruaru - PE, morou toda infância até os 17 anos com seus pais no Sítio Gavião, zona rural do munícipio de Cumaru - PE. No ano de 1990 iniciou sua vida estudantil, em uma escola rural (multi seriada), localizada na zona rural do município de Cumaru - PE, onde fez até a quarta série do ensino fundamental. Estudou os últimos quatro anos do ensino fundamental do município de Riacho das Almas - PE, na Escola Municipal Santos Cardoso, localizada na Vila Pinhões. No período de 2000 a 2002, fez o ensino médio concomitante com o curso Técnico em Agropecuária pela Escola Agrotécnica Federal de Belo Jardim – atual IFPE, Campus Belo Jardim - PE. Nesta instituição, teve o primeiro contato com o estudo de solos, na disciplina de Agricultura Geral, sendo incentivado pelos professores para prestar vestibular para a UFRPE, onde foi aprovado no ano de 2003 para o curso de Engenharia Agronômica. No início da graduação, logo no primeiro período do curso iniciou atividades como estagiário voluntário da Área de Solos da UFRPE, colaborando em trabalhos na linha de pesquisa de Salinidade do Solo; posteriormente, trabalhou com Fertilidade do Solo, no Laboratório de Fertilidade do Solo. Inicialmente desenvolveu trabalhos na linha de pesquisa de fertilidade do solo e posteriormente colaborou em trabalhos de dissertações e teses sobre poluição ambiental de solos por metais pesados, linha de pesquisa que vem atuando até o presente momento. Durante a graduação foi bolsista do Programa de Educação Tutorial (PET), desenvolveu trabalhos de pesquisa também na área de Economia Rural e Comercialização de Produtos Agrícolas. Ingressou no ano de 2006 no curso de graduação em Licenciatura em Ciências Agrícolas da UFRPE. Em 2009.1 concluiu os cursos de graduação, obtendo o grau de Engenheiro Agrônomo e Licenciatura em Ciências Agrícolas. No segundo semestre de 2009 iniciou suas atividade como discente do Programa de Pós-Graduação em Ciência do Solo da UFRPE, sendo posteriormente solicitado transição para o curso de doutorado, tendo como linha de pesquisa o estudo da toxidez de metais pesados em plantas e a amenização da toxidez de metais pesados por silício.

SUMÁRIO

LISTA DE SÍMBOLOS

F685 - Comprimento de onda da região do vermelho.

F690 - Comprimento de onda da região do vermelho.

F735 - Comprimento de onda na região do vermelho distante.

FFr – Comprimento de onda na região do vermelho distante.

Fm – Fluorescência máxima com a filha adaptada ao escuro.

Fo – Fluorescência mínima com a folha adaptada ao escuro.

Fr – Comprimento de onda da região do vermelho.

Fr/FFr – razão entre os comprimentos de onda na região do vermelho e do vermelho distante.

Fv – Variação de fluorescência com a folha adaptada ao escuro.

Fv/Fm – Eficiência quântica máxima da fotoquímica no fotossistema II.

Fv/Fo – Máximo rendimento primário da fotoquímica no fotossistema II.

qP - Coeficiente quântico da fotoquímica.

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RESUMO

Cádmio e arsênio são elementos que estão comumente associados à contaminação e/ou poluição ambiental, além de oferecer risco à saúde humana. O silício tem se destacado na amenização de estresse causado por elementos tóxicos em plantas. A técnica de análise da fitotoxidez por fluorescência da clorofila é um ferramenta que tem demonstrado ser bastante promissora no monitoramento e na detecção de estresse. O objetivo deste trabalho foi investigar os efeitos do silício na amenização do estresse causado por cádmio e arsênio em plantas de milho cultivadas em solução nutritiva e avaliar o potencial dos parâmetros de emissão espectral e da razão Fr/FFr, além da determinação da dose tóxica de cádmio e arsênio em plantas de milho cultivadas em solução nutritiva. Para isso foram montados quatro experimentos em solução nutritiva com doses crescentes de cádmio e arsênio, e em solução nutritiva contaminada com doses tóxicas de cádmio (30 µmol L^{-1}) e arsênio (68 µmol L^{-1}) e seis doses crescentes de Si (0; 0,25; 0,5; 1; 1,5 e 2 mmol L^{-1}), sendo avaliados a produção de matéria seca, teores de cádmio, arsénio, silício e pigmentos fotossintéticos. Medidas de fluorescência da clorofila foram tomadas ao longo do cultivo. O silício promove efeitos positivos na amenização do estresse causado por cádmio e arsênio em plantas de milho, comprovado pelo incremento de pigmentos fotossintéticos, e pela redução na translocação deste elemento para a parte aérea das plantas, cada cádmio e arsênio, respectivamente. A aplicação de silício proporcionou maiores teores de As no tecido vegetal e a sua utilização em estudos de técnicas de fitorremediação de solos contaminados pode ser promissora. A análise de fluorescência da clorofila demonstrou ser uma ferramenta sensível na detecção precoce da toxidez causada por cádmio e arsênio em plantas de milho e pode ser empregada também com sucesso no estudo dos efeitos amenizantes do silício na proteção de plantas, sendo a razão Fr/FFr a variável recomendada na identificação de alterações temporais em plantas.

Palavras chaves: toxidez, metais pesados, metaloide, pigmentos fotossintéticos, fotossistema II.

ABSTRACT

Cadmium and arsenic are elements that are commonly associated with contamination and / or environmental pollution, besides offering risk to human health. Silicon has excelled in alleviating stress caused by toxic elements in plants. The technical analysis of phytotoxicity by chlorophyll fluorescence is a tool that has proven to be very promising in monitoring and detection of stress. The aim of this study was to investigate the effects of silicon in alleviating the stress caused by cadmium and arsenic in maize plants grown in nutrient solution and evaluate the potential of emission spectral parameters and the ratio Fr/FFr, besides determining the toxic dose of cadmium and arsenic in maize plants grown in nutrient solution. For that were mounted four experiments in nutrient solution with increasing doses of cadmium and arsenic, and nutrient solution contaminated with toxic doses of cadmium (30 mmol L^{-1}) and arsenic (68 mmol L^{-1}) and six increasing doses of Si (0; 0,25; 0,5; 1; 1;5 and 2 mmol L^{-1}), were evaluated for the production of dry matter levels of cadmium, arsenic, silicon and photosynthetic pigments. Chlorophyll fluorescence measurements were made during the cultivation. Silicon promotes positive effects in alleviating the stress caused by cadmium and arsenic in maize plants as evidenced by the increase of photosynthetic pigments, and the reduction in the translocation of this element to the shoot, each cadmium and arsenic, respectively. The application of silicon resulted in higher levels of As in plant tissue and its use in studies of techniques for phytoremediation of contaminated soils may be promising. The analysis of chlorophyll fluorescence proved to be a sensitive tool to detect early toxicity caused by cadmium and arsenic in maize plants and can also be used successfully in the study of the effects amenizantes silicon in plant protection, the ratio Fr/FFr recommended in the variable identifying temporal changes in plants.

Keywords: toxicity, heavy metal, metalloid, photosynthetic pigments, photosystem II.

INTRODUÇÃO GERAL

Toxicidade de Cd e As em plantas

O aumento da população mundial e o crescimento das atividades industriais têm aumentado a degradação ambiental. O solo é um ambiente muito impactado, por ser o destino final de resíduos e rejeitos das atividades antrópicas. Neste contexto, tem ocorrido aumento considerável da contaminação dos solos por várias fontes de poluição, incluindo a presença de metais pesados. Dentre estes, os mais comumente associados com contaminação ambiental são As, Cd, Co, Cr, Cu, Pb, Hg, Mo, Ni, Se e Zn (NELLESSEN & FLETCHER, 1993).

O Cd é altamente tóxico, mesmo em baixas concentrações, estando presente em solos agrícolas devido a aplicação de fertilizantes fosfatados, lodo de esgoto, esterco e calagem (GONÇALVES et al., 2008; FREITAS et al., 2009). A utilização intensiva de agroquímicos ricos em As também pode promover o aumento da disponibilidade deste elemento às plantas, potencializando o risco de sua entrada na cadeia trófica (MENDES et al., 2010).

Vários estudos tem apresentado a toxidez de Cd como sendo um fator limitante no desenvolvimento das plantas devido a alterações causadas no transporte de elétrons e efeitos negativos nos processos fotossintéticos, na divisão celular e na absorção de água e nutrientes (KABATA-PENDIAS & PENDIAS, 1984). Plantas de *Artemisia annua* cultivadas sob doses crescentes de Cd apresentaram redução dos pigmentos fotossintéticos com o incremento das doses deste metal (LI et al., 2012), sendo clorose, necrose e escurecimento radicular os sintomas típicos da fitotoxidez de Cd (COSTA et al., 2012).

O As é um metalóide pertencente à classe I (quais os outros) dos contaminantes carcinogênicos, podendo causar diversos danos à saúde humana, sendo uma das causas clássicas de câncer de pele (CHEN et al., 2009; MELKONIAN et al., 2011; AHSAN & STEINMAUS, 2013). Sintomas de fitotoxidez podem ser observados próximos as áreas de mineração, pela aplicação excessiva de pesticidas ricos em As, pelo uso de resíduos contaminados e pela água de irrigação contendo As (KABATA-PENDIAS,

2010), sendo uma preocupação quando espécies de importância alimentar e medicinal crescem em condições de contaminação (VACULÍK et al., 2013).

Os sintomas associados à toxidez de As em plantas de arroz são murcha das folhas, redução da taxa de transpiração e da taxa fotossintética, tendo como consequência restrição do crescimento e redução da produção de matéria seca (STOEVA et al., 2004; HOFFMAM & SCHENK, 2011). A redução da produção de matéria seca e o menor crescimento das plantas são as consequências mais observadas na toxidez deste elemento em plantas (MELO et al., 2009; LI et al., 2011).

A redução da produção de matéria seca de plantas cultivadas sob condição de estresse é bastante utilizada na determinação da toxidez. Porém, ainda existem diferenças nos percentuais de redução da produção de matéria seca que correspondam à dose tóxica. Os países europeus determinam como toxidez a redução de 25% da produção de matéria seca (SAEFL, 1998), enquanto nos Estados Unidos este valor é de 50% (KING, 1996).

Silício na amenização da fitotoxidez de cádmio e arsênio

O silício é o segundo elemento mais abundante na crosta da terra, ficando atrás apenas do oxigênio. No solo, o teor de Si varia de < 1 a 45 dag kg^{-1}, sendo as formas mais ativas representadas pelo ácido monossilícico, ácido polissilícico e compostos orgânicos (MATICHENKOV & CALVERT, 2002). Em plantas, Si se acumula nos tecidos de todas as espécies vegetais, sendo seus teores observados entre 0,1 a 10% da matéria seca (KORNDÖRFER et al., 2004). A alta variação na capacidade das plantas absorverem e acumularem Si no tecido vegetal permite classificar as plantas em acumuladoras, quando o teor de Si for maior que 1% e a relação Si/Ca for > 1; intermediárias, quando o teor de Si for de 0,5-1% e relação Si/Ca < 1; e por fim, e não acumuladoras, quando o teor de Si for menor que 0,5% (MA et al., 2001).

A absorção de Si pelas plantas depende da concentração deste elemento na solução do solo e da espécie vegetal, podendo ser absorvido de forma passiva e ativa (LIANG et al., 2006), predominando a absorção ativa nas plantas acumuladoras e passiva nas plantas não acumuladoras.

A adição de Si ao meio de cultivo favorece a formação de dupla camada de sílica-cutícula e sílica-celulose no tecido vegetal, favorecendo o acúmulo

destas substâncias na parede celular da epiderme das folhas, conferindo resistência a vários tipos de estresse de natureza biótica e abiótica, possibilitando o cultivo agrícola mais saudável e produtivo de diversas culturas, estando seu efeito mais evidente quando associado ao cultivo de plantas sobre condições de estresse (MA & YAMJI, 2006). A deposição de Si nos tecidos dos vegetais ocorre na forma de sílica amorfa, principalmente nas paredes celulares, interagindo com compostos fenólicos e pectinas, dando maior rigidez, o que melhora a estruturação das plantas (CURRIE & PERRY, 2007).

De forma semelhante ao íon carbonato (CO_3^{2-}), os ânions SiO_3^{2-} combinam-se com íons H^+ na solução do solo, diminuindo a atividade química dos metais por precipitação na forma de hidróxidos e silicatos (PAIM et al., 2006).

O Si tem efeitos direto de amenização da fitotoxidez causada por metais pesados nas plantas, independente da precipitação ou não dos metais no solo, mas através da elevação do pH (LIANG, et al., 2005; CUNHA et al., 2008). A ação do Si na amenização de metais pesados é bem conhecida, principalmente quando se refere à amenização destes poluentes no solo e nas plantas (LIANG et al., 2005). Os principais mecanismos internos de redução do estresse de plantas mediados pelo Si são o estímulo ao sistema antioxidante, complexação ou co-precipitação de íons metálicos tóxicos com Si, imobilização de íons metálicos livres, alterações nos processos de absorção e compartimentação de íons metálicos nos vacúolos (LIANG et al., 2007).

Fluorescência da clorofila no monitoramento de alterações fotossintéticas no fotossistema II

A presença de elementos tóxicos no meio de cultivo das plantas pode vir a causar danos ao aparato fotossintético. Cd pode alterar a biossíntese de clorofila, inibindo a redutase e o transporte de elétrons localizado no fotossistema II (LAGRIFFOUL et al., 1998). O cultivo de plantas em local contaminado por Cd provoca danos ao sistema fotossintético das plantas, causando redução dos teores de clorofila e carotenoides (SANTOS et al., 2011). O fotossistema II é formado por um complexo de pigmentos proteico supramolecular presente no cloroplasto, o qual catalisa a luz induzida na

transferência de elétrons a partir de água em um processo que envolve o oxigênio (GIARDI et al., 2001).

As plantas respondem rapidamente a qualquer alteração na condição de cultivo, quando os processos fotossintéticos são alterados. A modificação na fotoquímica pode ser importante na identificação de mudanças na emissão de luz na forma de fluorescência emitida pela clorofila das plantas (KALAJI & GUO, 2008). Sua aplicação torna-se possível, devido à redução da assimilação do CO_2 no ciclo de Calvin ser a principal via de utilização da energia luminosa, e que durante períodos desfavoráveis da assimilação, as plantas respondem a excitação luminosa no aparato fotossintético, modificando as rotas de conversão da energia luminosa, refletindo diretamente pelo fotossistema II (MATTOS, 2006).

A análise espectral da fluorescência da clorofila induzida por LED utilizando o sistema espectroscópico convencional é constituído por uma fonte de luz na região UV-Visível, um espectrômetro, um fotodetector, fibras ópticas e um computador (Figura 1).

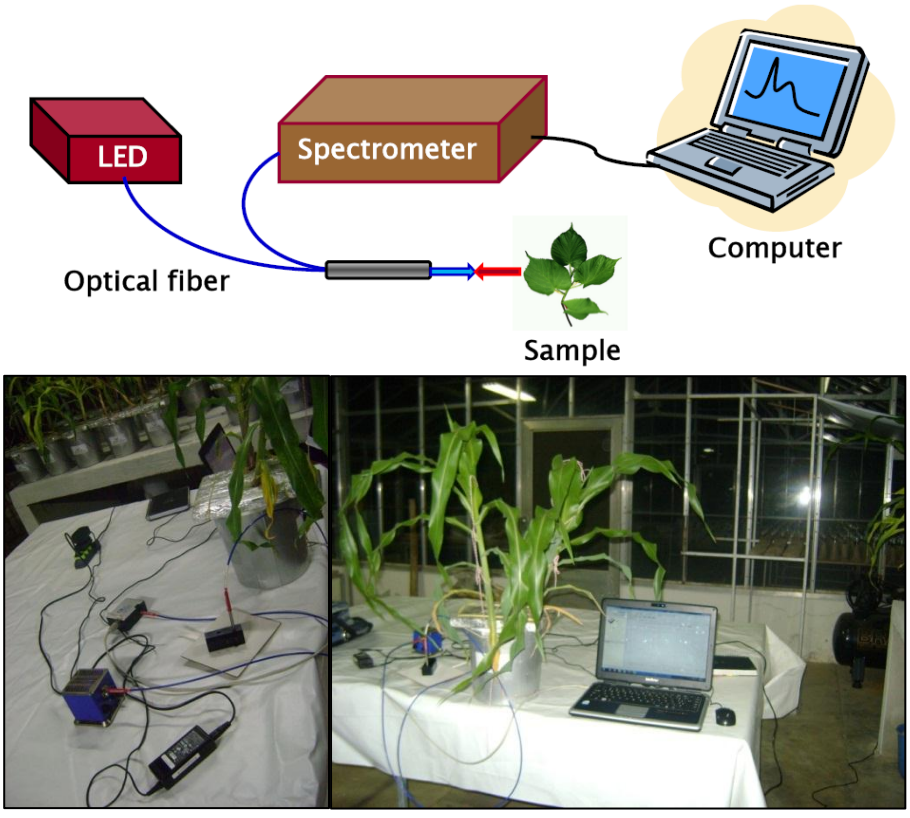

Figura 1. Esquema do espectrômetro de fluorescência (**Fonte:** Gouveia Neto, 2012). Fotos do aparato experimental utilizado em casa de vegetação.

Esta técnica permite estudar indiretamente diversas alterações a nível foliar, que são possíveis de serem aplicadas na identificação de estresse, que estejam associadas a alterações nos pigmentos fotossintéticos, reações primárias da luz, no transporte de elétrons nos tilacóides, reações enzimáticas que ocorrem no estroma e processos lentos de regulação (KALAJI & GUO, 2008).

A energia luminosa que é absorvida pela clorofila no sistema fotossintético presente nas folhas das plantas podem sofrer três destinos, podendo ser usada nos processos da fotossíntese (fotoquímica), dissipada na forma de calor e ser reemitida como fluorescência vermelha. Alteração na eficiência de um destes processos resultará numa diminuição do rendimento dos outros dois. Deste modo, a análise da fluorescência da clorofila fornece informações de alterações ocorridas no aparato fotossintético das plantas (MISRA et al., 2012).

A identificação e o monitoramento da toxidez de Cd em *Thalassiosira weissflogii* por fluorescência da clorofila é eficiente, uma vez que alterações na emissão da fluorescência da clorofila foram observadas quando as plantas foram submetidas a condições de estresse, isto é possível porque sob condições de estresse ocorre um aumento no tempo de emissão de fluorescência da clorofila, devido à inibição na taxa de transporte de elétrons (ZENG et al., 2012). A avaliação da toxidez de Cd em plantas de milho, utilizando a técnica de análise da fluorescência da clorofila causou variações nos picos de emissão de fluorescência e consequentemente nas suas respectivas razões (MAURYA et al., 2008).

É importante lembrar que os parâmetros mensurados pela intensidade de fluorescência emitida nem sempre expressam uma variação do que esta acontecendo, sendo os parâmetros obtidos pelos cálculos mais sensíveis na identificação do estresse (MATTOS, 2006). Segundo Cherif et al (2010) a relação entre o comprimento de onda no vermelho e vermelho distante F690/F735 obtida na análise da fluorescência da clorofila é sensível na identificação de estresse de plantas.

A toxidez de As em *Hydrilla verticillata* provocou uma redução nos teores dos pigmentos fotossintéticos, seguidos de alterações nos parâmetros da fluorescência da clorofila, após 96 horas de exposição a este metalóide (SRIVASTAVA et al., 2012). Este elemento causou decréscimo das razões

Fv/Fo e Fv/Fm em plantas de milho, indicando que As causa redução na função da atividade do fotossistema II (STOEVA et al., 2003).

Por ser uma técnica não invasiva e não destrutiva a análise da fluorescência da clorofila permite sua aplicação ao longo do tempo. Além disso, é uma técnica altamente sensível e de simples utilização, permitindo a obtenção de informações qualitativas e quantitativas sobre a condição fisiológica do aparato fotossintético das plantas (BAKER & ROSENQVIST, 2004; FALQUETO et al., 2007), trazendo informações importantes referente a atividade fotoquímica do fotossistema II, transporte de elétrons e ajustes nos processos de regulação do aparato fotossintético de plantas (STIRBET & GOVINDJEE, 2011).

O objetivo geral deste trabalho foi investigar o potencial do Si a amenização da toxidez de Cd e As em plantas de milho, avaliar a técnica da fluorescência da clorofila na detecção e no monitoramento da toxidez de Cd e As em plantas de milho e avaliar também o efeito amenizante proporcionado por Si a plantas de milho cultivadas sob estresse de Cd e As e determinar as doses tóxicas de Cd e As para plantas de milho cultivadas em solução nutritiva sob doses crescentes destes elementos.

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CAPÍTULO I

LED-Induced Chlorophyll Fluorescence Spectral Analysis for the Early Detection and Monitoring of Cadmium Toxicity in Maize Plants

LED-Induced Chlorophyll Fluorescence Spectral Analysis for the Early Detection and Monitoring of Cadmium Toxicity in Maize Plants

RESUMO

A análise de fluorescência de clorofila permite a identificação de alterações em pigmentos fotossintéticos mediante indução por laser. Esta técnica permite avaliar o nível de estresse ao qual uma planta está submetida através dos danos fisiológicos causados à fotossíntese, especialmente aqueles relacionados ao fotossistema II. O objetivo do trabalho foi estudar a toxicidade de Cd em plantas de milho via análise de fluorescência de clorofila. Esta análise não invasiva e não destrutiva foi utilizada para monitorar as alterações na produção de matéria seca, teores de clorofila e acumulação de Cd. As plantas apresentaram redução na produção de matéria seca e nos teores de clorofila, a medida que as doses de Cd aumentaram em solução. A análise de fluorescência mostrou ser sensível a essas alterações provocadas pelo Cd as plantas de milho, diferenciando os tratamentos antes mesmo de sintomas visuais serem observados. A técnica apresenta praticidade de aplicação e rapidez na obtenção dos resultados, podendo ser usada na avaliação de estresses provocados por Cd em plantas cultivadas e no monitoramento de áreas contaminadas pelo elemento.

Palavras-chave: metais pesados, estresse, toxidez, solo contaminado, poluição do solo, solução nutritiva.

ABSTRACT

Chlorophyll fluorescence spectral analysis permits detection, monitoring, and evaluation of abiotic stresses upon healthy plants using illumination of a light source in the UV–VIS spectral range. This technique indirectly assesses the amount of physiological stress caused by photosynthetic damage, specifically damage to photosystem II, in plants. The objective of this study was to detect the toxicity of cadmium in maize plants via spectral analysis of chlorophyll fluorescence. The analysis is noninvasive and nondestructive and is used to follow the temporal evolution of changes in the chlorophyll content and physiological state of *Zea mays* L. seedlings under cadmium stress. Conventional techniques were also used to evaluate the dry matter production and Cd accumulation in plant leaves. Plants exhibited a notable reduction in dry matter production and chlorophyll levels with the administration of increasing doses of Cd in the nutrient solution. The fluorescence analysis was sensitive to changes caused by Cd in maize plants, detecting damage caused by different treatments before visual symptoms were observed. This technique has a practical application and produces rapid results that can be used in the evaluation of Cd-induced stress in plants and the detection of areas contaminated by this element.

Keywords: Heavy metals, Stress, Toxic, Soil contamination, Soil pollution, Nutrient solution

INTRODUCTION

Cadmium is toxic to plants, even at low concentrations. The increasing levels of Cd in the food chain as a result of environmental pollution have become increasingly disturbing. In general, due to its solubility in soil, plants have the capacity to accumulate Cd in their tissues (HART et al., 1998), and this element is easily transported to the edible parts, which represents serious problems for the health of consumers (LÓPEZ-MILLÁN et al., 2009).

Several studies have shown that the phytotoxicity of Cd limits the development of plants as a result of disruptions in electron transfer reactions, which negatively affects processes such as photosynthesis, mitosis, and water absorption (KABATA-PENDIAS and PENDIAS, 1984). Therefore, changes in photosynthesis and photosynthetic pigments might indicate potential Cd toxicity in plants (KUMMEROVA et al., 2006; LÓPEZ-MILLÁN et al., 2009). Symptoms of chlorosis in maize plants, such as a reduction in the number of chloroplasts per cell and changes in cell size, might directly reflect the influence of Cd on the enzymes responsible for the biosynthesis of chlorophyll, with photosystem II (PS II) being the most affected (KURDZIEL et al., 2004).

The growing importance of Cd as an element with a high toxicity requires the development of rapid and practical analytical methods to monitor and identify Cd contamination, owing to the fact that current techniques are expensive and time consuming (GIARDI et al., 2001; TALIO et al., 2009). The detection of metal induced stress in plants by the spectral analysis of chlorophyll fluorescence (LICHTENTHALER et al., 1990; BUSCHMANN, 2007) is a sensitive technique that infers the degree of plant stress from the ratio of the emission intensities of chlorophyll fluorescence (CHERIF et al., 2010). Mishra and Gopal (2008) applied this technique in a study to detect the toxicity of heavy metals in plants grown in soil during the early stages of plant development.

Changes in fluorescence are primarily observed in PS II because this process seems to be more sensitive to environmental stresses. The most common parameter used in this analysis is the ratio of fluorescence intensities peaked around 685 and 735 nm, corresponding to emissions from PS II and PS I, respectively. The fluorescence ratio (F685/F735) provides information about the physiological status and chlorophyll content of plants (BUSCHMANN, 2007).

This relationship is used to quantify the maximum efficiency of PS II (LU et al., 2000; GORINOVA et al., 2007; MECHRIA et al., 2011), as there is a decreased efficiency of the photosystem with decreasing levels of chlorophyll in leaves. In the analysis of chlorophyll content, the fluorescence ratio is a good indicator of Cd-induced stress in plants (MAURYA et al., 2008).

The objective of this study was to monitor Cd toxicity in maize plants using the spectral analysis of chlorophyll fluorescence induced by light emitting diode (LED). This noninvasive and nondestructive analysis was used to monitor and track temporal changes in dry matter production, chlorophyll content, and the accumulation of Cd in plants for the early detection of metal-induced stress *in plants* before the manifestation of visible symptoms of toxicity. Additionally, we tried to relate these changes to the tissue concentration of Cd (phytotoxicity threshold) at which plants displayed visual symptoms.

MATERIAL AND METHODS

Plant Material and Growth Conditions

Maize seeds (*Zea mays* L., variety São José) were germinated between sheets of moist paper towel; the lower towel was immersed in a solution of 0.67 mmol L^{-1} Ca as $Ca(NO_3)_2 \cdot 4H_2O$ (VILELA and ANGHINONI, 1984). Seven days after sowing, two seedlings were transferred to plastic pots containing 6 L of a modified Hoagland nutrient solution (HOAGLAND and ARNON, 1950), containing the following nutrients: 105.05 mg L^{-1} N, 15.5 mg L^{-1} P, 117.3 mg L^{-1} K,100.2 mg L^{-1} Ca, 24.3 mg L^{-1} Mg, 32.1 mg L^{-1} S, 0.325 mg L^{-1} Cl, 0.25 mg L^{-1} Mn, 0.025 mg L^{-1} Zn, 0.01 mg L^{-1} Cu, 0.25 mg L^{-1} B, 0.005 mg L^{-1} Mo, and 7.53 mg L^{-1} Fe. The nutrient solution was replaced weekly and/or each time the electrical conductivity reached a value of 0.4 dS m^{-1} during the experiment. Deionized water was added daily to the pots to replace the water lost by evapotranspiration. The pH was maintained at approximately 5.50±0.2 and adjusted with 1 mmol L^{-1} solutions of H_2SO_4 or NaOH. After an 8-day adaptation period, doses of 0, 0.5, 1.5, 3.0, and 6.0 mg L^{-1} Cd (Cd_2Cl) were added to the pots, and the seeds were cultivated for an additional 20-day period.

Measurement of Chlorophyll Fluorescence

Five measurements of chlorophyll fluorescence (ChlF) were conducted during the experiment; the first and last measurements were taken before the addition of Cd and 1 day before the collection of plants, respectively. The measurements were performed at night to ensure the deactivation of photosynthetic electron transport. The ChlF experimental apparatus consisted of a fiber-integrated LED source, spectrometer, and light detector (Ocean Optics USB2000). The detection system had an overall operating spectral resolution of ~1.0 nm. The excitation source was directed to the leaf surface by means of a 200-μm diameter fiber cable which possessed a mechanical system at the fiber cable output extremity in order to prevent any ambient light of reaching the leaf surface during the measurements.

Moreover, as the fiber itself was in contact with the leaf surface, it effectively shadowed away any leakage of ambient light. All spectra presented in this study were handled employing appropriate (Ocean Optics-Spectra Suite) software of the spectrometer. A blue emission LED with a wavelength of 405 nm, spectral width of 10 nm, and maximum output power of about 2 Mw was used as the fluorescence excitation light source. The measurements were conducted with samples that had been placed in an environment without illumination for a minimum of 20 min. Measurements were obtained from three different positions on two leaves from each plant. The spectra were obtained and stored using the

Ocean Optics-Spectra Suite instrument software and adjusted to two Gaussian curves corresponding to red (685 ηm) and far-red (735 ηm). The fluorescence intensity ratio (F685/F735) was calculated from the fitted curve for each of the doses of Cd in solution and used to infer the effect of this element on the biosynthesis of chlorophyll and photosystem II using commercial software (Origin version 6.0).

Estimation of Chlorophyll Contents

Leaf samples were collected for the determination of chlorophylls a and b (ARNON, 1949), from the middle third region of the same leaves used for the analysis of chlorophyll fluorescence. The chlorophyll contents were determined in 80 % acetone extract on a Biospectro UV-SP-220 spectrophotometer using equations of Arnon for calculations, and the results used to compare with the ones obtained by the ChlF ratio technique.

Biomass and Cd Determination

The roots and aerial plant parts were washed with tap water, followed by three washes with distilled water. This material was packaged into paper bags. Subsequently, the samples were kept in an incubator with forced air circulation at 65°C until constant weight. Then the dry weight of roots and aerial parts was obtained and added to determine the total weight of the dry matter. The samples were ground in a Willey type mill. The dried matter was packaged in plastic bags. The plant material was digested in nitric and hydrochloric acids in a microwave oven (Mars Xpress) according to the method USEPA 3051A (United States Environmental Protection Agency 1998). Atomic absorption spectrophotometry (AAnalyst 800) was used to measure the Cd content from digestion extracts. The data were subjected to ANOVA and regression analysis.

RESULTS AND DISCUSSION

Production of Dry Material and Absorption of Cd

The dry matter production of plants decreased significantly with the addition of increasing doses of Cd to the nutrient solution (Fig. 2). The highest dose resulted in a 69 % reduction of dry matter production compared with the control. A reduction of approximately 50 % of aerial part dry matter was achieved with an estimated dose of 3.4 mg L^{-1} of Cd. The tissue concentration corresponding to 50% reduction in growth was selected as the phytotoxicity threshold (PT50) by the US Environmental Protection Agency (KING, 1996) and is equivalent to

the dose in our experiment at which the plants exhibited visual symptoms of toxicity.

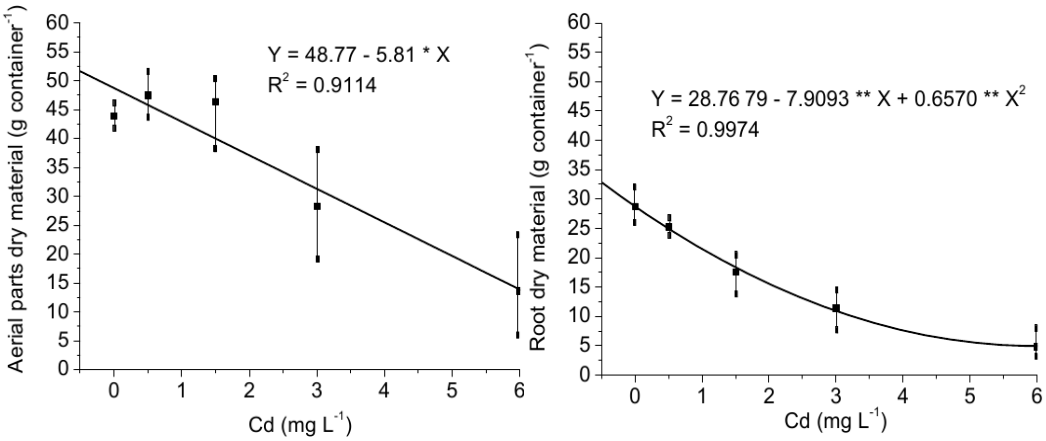

Fig. 2 Production of dry matter in the roots and aerial parts of maize plants grown in the presence of increasing doses of Cd in the nutrient solution. (Single asterisk, double asterisks significance at 5 and 1 % probabilities, respectively).

The accumulation of Cd in the roots and aerial parts of maize plants increased significantly with increasing doses of Cd (Table 1). The higher accumulation of Cd in the roots indicates a mechanism for decreasing the translocation of the metal to the aerial parts, which is a strategy for Cd toxicity mitigation via metal retention in the root system to prevent deleterious effects on the aerial parts (LÓPEZ-MILLÁN et al., 2009). The maize plants showed browning of the roots at the highest doses of Cd, indicating necrosis caused by the toxicity of the element. Notably, the retention of Cd in the root system is limited by the concentration of metal in the solution. At Cd concentrations higher than the PT50 (3.4 mg L^{-1}), the physiological barriers for the retention of Cd in the roots are broken, and the accumulation of the element in the aerial parts increases.

Table 1 Concentrations of Cd in plant tissues from maize grown in nutrient solution with increasing doses of Cadmium

Dose	Concentration	
Cd	Root	Aerial parts
mg L^{-1}	----------mg kg^{-1} ----------	
0.0	0.96	0.00
0.5	527.24	11.85
1.5	995.34	28.57
3.0	1494.32	49.27
6.0	2733.16	338.75

Chlorophyll Levels in Plants

The concentrations of photosynthetic pigments (chlorophylls a, b, and total) decreased with increasing Cd concentrations in the nutrient solution (Fig. 3). This reduction tended to stabilize at the highest doses and was similar for both types of chlorophyll. For total chlorophyll, the reduction was 35% at the highest dose of the metal, while for chlorophylls a and b the reductions were 34% and 37%, respectively.

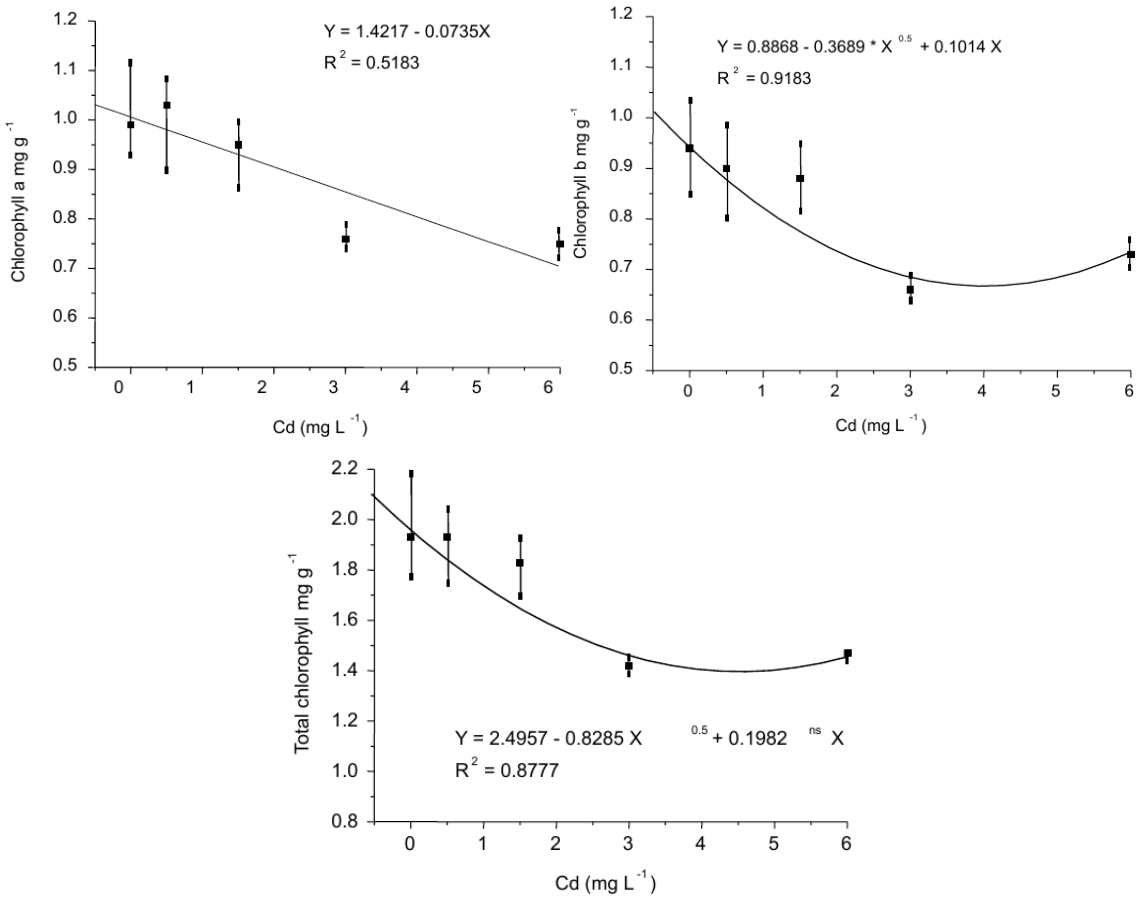

Fig. 3 Content of chlorophylls a and b and total chlorophyll in the leaves of maize grown under increasing doses of Cd in the nutrient solution. (Asterisk significance at 5% probability).

The spectral profile of the chlorophyll fluorescence in plants grown under different concentrations of Cd was investigated (Fig. 4). The results clearly show significant variations in the spectral profile for the stressed plants as compared with the control sample. It is also noteworthy the increase in the fluorescence ratios (F685/F735) as the Cd concentration increases. Chlorophyll levels are important parameters for identifying stress in plants and are associated with other important variables that affect plant development (DAI et al., 2009). Several studies have reported a reduction in photosynthetic pigments and chlorophyll in plant leaves under Cd-induced stress (GORINOVA et al., 2007; MAURYA et al., 2008; LÓPEZ-MILLÁN et al., 2009; LIU et al., 2011). The reduction results in a decreased photosynthetic activity efficiency by the inhibition of chlorophyll biosynthesis and a decreased carbon assimilation, which ultimately leads to a decrease in biomass production.

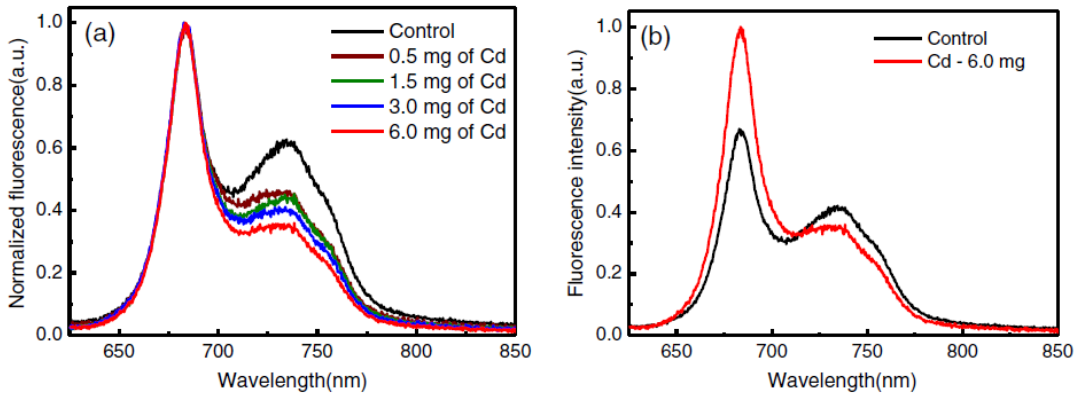

Fig. 4 Average spectra of chlorophyll fluorescence at 19 days of experiment a normalized to the 685-ηm emission intensity and b emission intensity, in maize plants grown under increasing doses of Cd added in nutrient solution.

Spectra of Chlorophyll Fluorescence

At 19 days of cultivation, the difference in the fluorescence ratios between the highest Cd dosage and the control was 37% (Fig. 4), which was similar to the reduction of total chlorophyll (35%). This result demonstrates that the technique of in vivo fluorescence analysis was effective in assessing the behavior of photosynthetic pigments in plants under Cd-induced stress. Thus, the Cd-induced stress in maize plants is directly associated with decreased levels of chlorophyll and changes in PS II. It is clear in Fig. 4b the decrease in the 685-ηm emission of the control sample owing to the higher chlorophyll levels when compared to the non-absorbed emission of the highly stressed sample. It is also clear that the 735-ηm emission presents a moderate intensity variation in the process. Several authors have demonstrated the effectiveness of this technique to assess metal-induced changes in photosynthetic systems for case studies with moderate metal concentrations (LU et al., 2000; GORINOVA et al., 2007; DAI et al., 2009; MECHRIA et al., 2011).

Measurements were carried out over a period of 20 days to monitor the temporal evolution of Cd- induced stress (Fig. 5). It should be noted that this study of temporal evolution is technically not feasible using conventional and destructive techniques for the analysis of metal-induced stress in vitro, owing to the very high number of samples required to perform such investigation.

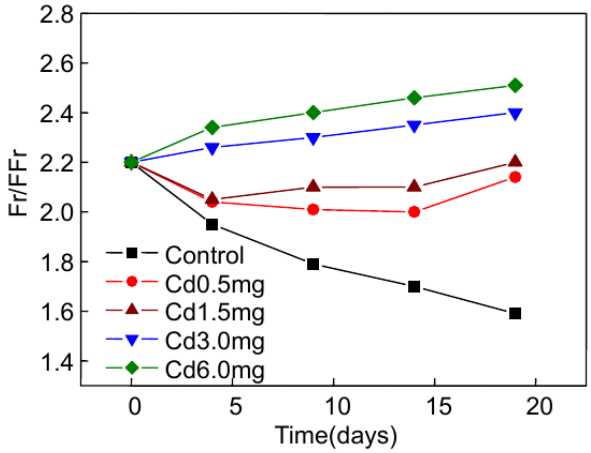

Fig. 5 Ratio of the chlorophyll fluorescence spectra as a function of cultivation time of maize plants grown under increasing doses of Cd in the nutrient solution using measurements of red and far-red (Fr/FFr) light, which correspond to fluorescence wavelengths of 685 and 735 ηm, respectively.

The chlorophyll fluorescence ratio (Fig. 5) indicates that prior to the addition of Cd, all plants had similar fluorescence spectrum. In healthy plants, the natural increase in chlorophyll concentration is indicated by an increased reabsorption of fluorescence at approximately 685 nm and a decrease in the F685/F735 ratio (Fig. 5). However, in plants under Cd stress, a decrease in fluorescence reabsorption due to a decrease in chlorophyll levels (Fig. 3) and, therefore, a decreased reabsorption of the fluorescence at 685 nm are accompanied by an increase in the F685/F735 ratio.

At 4 days after the addition of Cd, we have observed differences in the fluorescence ratio, even in the absence of visually detectable symptoms of toxicity in the leaves. Symptoms of toxicity were visible at 9 days after exposure to Cd and were manifested by a chlorosis of the young leaves and a necrosis of the older leaves, in addition to reduced plant growth under the two highest doses of Cd. To ensure the health of ecosystems, the assessment of contaminated sites requires speed and practicality to identify toxicity caused by heavy metals (TALIO et al., 2009). The use of simple and inexpensive techniques for the identification of harmful pollutants present in aquatic environments and in the soil is fundamental for this monitoring (GIARDI et al., 2001). The results of the fluorescence analysis allowed us to detect differences in the synthesis of photosynthetic pigments when plants were subjected to Cd-induced stress, even at low concentrations and only 4 days after the addition of

metal to the nutrient solution. Importantly, the results were obtained before the plant presented any visible symptoms of toxicity.

CONCLUSIONS

The phytotoxicity threshold (PT50) of 3.4 mg kg^{-1} of Cd in shoots was related to the tissue concentration of the metal at which plants displayed visual symptoms. The concentrations of photosynthetic pigments were also highly related to the PT50. They decreased with increasing Cd concentrations in shoots up to the PT50 from where there is no further decrease.

The chlorophyll fluorescence technique is satisfactory and effective for the early detection and monitoring of Cd-induced changes and could be used in studies to identify contaminated environments and monitor risk areas through the analysis of plants. Therefore, monitoring Cd toxicity using chlorophyll fluorescence analysis is promising for the evaluation of environments contaminated by the metal. The technique is a simple, applicable, and rapid detection method with the added advantage of being noninvasive and nondestructive. Moreover, chlorophyll fluorescence analysis is sensitive to changes in plant tissue, even at low pollutant concentrations.

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CAPÍTULO II

Temporal monitoring of Cd phytotoxicity alleviation by Si using chlorophyll fluorescence

Temporal monitoring of Cd phytotoxicity alleviation by Si using chlorophyll fluorescence

RESUMO

A toxidez de cádmio (Cd) em plantas pode ser amenizada pelo Si por vários mecanismos. Entre estes, destacam-se a redução da translocação de Cd para a parte aérea e a redução da atividade de Cd^{2+} no simplasma. O objetivo deste trabalho foi avaliar os efeitos da amenização da toxidez de Cd em plantas de milho e investigar a emissão espectral e a razão Fr/FFr obtidas na análise da fluorescência da clorofila como parâmetros de estudo da amenização do estresse. Foi montado um experimento em solução nutritiva com uma dose tóxica de Cd (30 µmol L^{-1}) e seis doses de Si (0; 0,25; 0,5; 1; 1,5 e 2 mmol L^{-1}) no qual foram avaliados produção da matéria seca, teores de Cd e Si, e pigmentos fotossintéticos. Também foram feitas medidas da fluorescência da clorofila ao longo do cultivo das plantas. A adição de Si reduziu a absorção e a translocação de Cd para a parte aérea das plantas de milho. Os pigmentos fotossintéticos não apresentaram variação com a adição de doses crescentes de Si. Os parâmetros determinados na análise da fluorescência da clorofila foram sensíveis na avaliação da amenização da toxidez de Cd por Si, possibilitando a determinação de uma dose ótima para a amenização. A utilização de Si pode se tornar uma ferramenta importante em programas de fitoestabilização de solos contaminados por Cd e na promoção da segurança alimentar.

Palavras-chave: pigmentos fotossintéticos, metais pesados, toxidez.

ABSTRACT

Cadmium (Cd) toxicity in plants can be alleviated by silicon (Si) via several mechanisms. Of these mechanisms, the reduced translocation of Cd to aerial parts of the plant and the reduced activity of Cd^{2+} in the symplasm are highlighted. The aim of this study was to evaluate the alleviation of Cd toxicity in maize plants and to investigate the use of *emission spectra* and the red to far-red (Fr/FFr) ratio obtained in the chlorophyll fluorescence analysis as parameters for the study of stress alleviation. An experiment was developed using a nutrient solution with a toxic dose of Cd (30 µmol L^{-1}) and six doses of Si (0; 0.25; 0.5; 1; 1.5 and 2 mmol L^{-1}); the dry matter production, Cd and Si levels and levels of photosynthetic pigments of the plants were evaluated. In addition, chlorophyll fluorescence was measured over the course of plant cultivation. The addition of Si resulted in the reduced uptake and translocation of Cd to the aerial parts of the maize plants. The levels of photosynthetic pigments did not vary with increasing doses of Si. The parameters determined in the chlorophyll fluorescence analysis were sensitive to the alleviation of Cd toxicity by Si, thus enabling the determination of an optimum dose for Cd detoxification. The use of Si represents an important tool for programs geared towards the phytostabilization of Cd-contaminated soils and the promotion of food safety.

Keywords: photosynthetic pigments, Heavy metals, toxicity.

INTRODUCTION

The contamination of the environment is of great concern, and the contamination of soils by heavy metals represents a potential risk for water, plant and animals. The entry of Cd into agricultural soils proceeds from the application of phosphate fertilizers, sewage sludge, manure and liming (GONÇALVES et al., 2008; FREITAS et al., 2009). Cd can alter the biosynthesis of chlorophyll in plants by inhibiting the reductase and the electron transport located in photosystem II (LAGRIFFOUL et al., 1998) and by causing nutritional imbalances in the plant (NASCIMENTO et al., 1998; COSTA et al., 2012; LI et al., 2012). Kabata-Pendias (2010) states that Cd levels ranging from 5 to 20 mg kg^{-1} in the dry matter of aerial plant parts are considered toxic.

Fertilizing maize plants with Si results in an increase in plant metabolism and consequently in crop yields, which, in turn, further reduces abiotic stresses (KIM et al., 2011; SURIYAPRABHA et al., 2012). High concentrations of Si in the plant tissue can promote Si-metal co-precipitation (GU et al., 2011), which makes this element more useful for soil acidity correction than other elements. Zn, Mn, Cu and Ni levels are highly correlated with Si levels in the trichomes, indicating a possible co-precipitation effect; the presence of SiO_2 in intercellular spaces further provides evidence of the stabilization of metal silicates when Si is used to alleviate the toxicity of these metals. Si was also associated with the alleviation of toxicity caused by Cd, Zn and Mn in *Picris divaricata* (BROADHURST et al., 2013).

The main mechanisms of Si-mediated stress reduction in plants are the stimulation of the antioxidant system, complexation or co-precipitation of toxic metal ions with Si, the immobilization of free metal ions, changes in the processes of absorption and the compartmentalization of metal ions in the vacuoles (LIANG et al., 2007).

Because of the changes induced by Cd in the photosynthetic apparatus of plants, chlorophyll fluorescence analysis can be used to detect stress induced by Cd toxicity (SILVA et al., 2012). This technique is also sensitive to the beneficial effects produced by Si during the alleviation of heavy metal toxicity (FENG et al., 2010); it represents a very important tool for evaluating changes in levels of photosynthetic pigments, as it provides information on the photochemical activity of photosystem II, changes in the photosynthetic

pigments, primary light reactions, electron transport in the thylakoids, enzymatic reactions that occur in the stroma and the slow processes of metabolic regulation in plants (KALAJI & GUO, 2008).

Silicon has positive effects on the photosynthesis of plants under stress; for example, Si increased the quantum yield and maximum effective quantum yield in cucumber plants grown in the presence of Cd (FENG et al., 2010). Si was also able to alleviate Cr stress, increasing the concentrations of the photosynthetic pigments and the efficiency of the chlorophyll fluorescence parameters (ALI et al., 2013). The application of Si increased the Fv/Fm and q_P parameters of rice plants grown under Cd stress (NWUGO & HUERTA, 2008).

Several studies have shown that the ratio between the emission peaks at the red (F685) and far red (F735) regions is sensitive to stress caused by metals in plants (CHERIF et al., 2010; CHERIF et al., 2012; SILVA et al., 2012). The measurements needed to characterize stress using the chlorophyll fluorescence tool can be obtained quickly and easily; furthermore, this technique is non-invasive, non-destructive and simple to use (KALAJI & GUO, 2008; WOO et al., 2008).

The objective of this study was to determine the ability of Si to alleviate Cd toxicity in maize plants grown in a Cd-contaminated nutrient solution and to investigate the emission spectrum and the Fr/FFr ratio obtained from the chlorophyll fluorescence analysis.

MATERIALS AND METHODS

Maize seeds (Zea mays L., cv. São José) were germinated on layers of paper towels with the bottom layer immersed in a solution containing 0.67 mmol L^{-1} Ca in the form of $Ca(NO_3)_2$ $4H_2O$ (VILELA & ANGHINONI, 1984). Seven days after sowing, two seedlings were transferred to a plastic pot containing six liters of a modified nutrient solution described by Hoagland and Arnon (1950) containing 105.05, 15.5, 117.3, 100.2, 24.3, 32.1, 0.325, 0.25, 0.025, 0.01, 0.25, 0.005 and 7.53 mg L^{-1} of N, P, K, Ca, Mg, S, Cl, Mn, Zn, Cu, B, Mo and Fe, respectively. The nutrient solution was replaced once a week and/or when the electrical conductivity reached 0.4 dS m^{-1}. Deionized water was added to the pots to replace the water lost by evapotranspiration. The pH was maintained

close to 5.50 (+/- 0.2) and adjusted with 1 mmol L^{-1} solutions of H_2SO_4 or NaOH.

After a period of eight days during which the plants were allowed to adapt to the nutrient solution, the toxic dose of Cd used in a previous experiment (30 µmol L^{-1}) was added to the solution; doses of 0, 0.25, 0.5, 1, 1.5 and 2 mmol L^{-1} Si (K_2SiO_3) were also added to the solution and the maize plants were allowed to grow for 21 days.

Five chlorophyll fluorescence measurements were performed throughout the experiment. The first measurement was performed before the addition of Cd and the last one was performed one day before the plants were harvested. Measurements were performed at night to ensure that electron transport in the photosynthetic apparatus was deactivated. The analysis of *in vivo* chlorophyll fluorescence was performed using an ultraviolet light emitting diode (LED) as an excitation source. The spectral fluorescence peaks at wavelengths of 685 nm and 735 nm (Ocean Optics, USB 2000) were analyzed. The spectra were obtained using the Ocean Optics-Spectra Suite software and fitted to two Gaussian curves corresponding to red (685 ηm) and far-red (735 ηm). The F684/F735 fluorescence intensity ratio and the peak height were calculated from the fitted curve for each dose of Si in solution; these data were used to infer the effect of the element on photosystem II using the Origin software version 6.0.

Leaves were sampled when the plants were harvested to determine the levels of chlorophyll a and b, which, together, provide the total chlorophyll content (ARNON, 1949); the samples were obtained from the middle third of the leaf used for the chlorophyll fluorescence analysis.

Leaves, stems and roots were rinsed once in tap water and three times in distilled water. Subsequently, the samples were kept in a forced air circulation oven at 65ºC until they reached a constant weight. The dry matter of the leaves, stems and roots was obtained and added together to obtain the total dry matter.

The digestion of the plant material was performed in nitric acid and hydrochloric acid in a microwave oven (Mars Xpress) according to the 3051A method (USEPA, 1998). The Cd content of the plant material was determined in the digestion extract using an atomic absorption spectrophotometer (AAnalyst 800 Perkin Elmer). The digestion of Si in the plant tissue was performed with hydrogen peroxide (H_2O_2) and sodium hydroxide (NaOH) in an autoclave. The

measurement was determined in a photocolorimeter using ammonium molybdate as a complexing agent (KORNDÖRFER et al., 2004). The data were submitted to analysis of variance (ANOVA) and regression analyses.

RESULTS AND DISCUSSION

Dry matter production

The addition of Si to the nutrient solution did not significantly increase the dry matter production of the plants (Table 2); however, the dry matter production was higher in all plants treated with Si than in the controls that received only Cd. Silicon was effective at alleviating Cd toxicity in cucumber plants, resulting in increased dry matter production in the roots (KHODARAHMI et al., 2012). Si also increased the dry matter production of wheat plants grown in soil contaminated with Cd (RIZWAN et al., 2012). The highest Si dose (2 mmol L^{-1}) caused a reduction in the dry matter of the plants, indicating that even though Si is a beneficial element, the application of high doses of Si may cause a nutritional imbalance and may therefore reduce the dry matter production of the plants (ARAÚJO et al., 2011).

Table 2. Dry matter production of maize plants under Cd stress and exposed to Silicon

Si Doses	Dry matter production (g pot^{-1})			
mmol L^{-1}	Leaf ^{ns}	stem ^{ns}	Root ^{ns}	Total ^{ns}
0.00	12.41	8.50	6.49	28.04
0.25	14.83	10.29	7.48	32.61
0.50	14.96	9.99	7.37	32.32
1.00	14.26	10.25	7.13	31.63
1.50	15.73	10.49	7.38	33.60
2.00	12.80	9.13	7.15	28.45
C.V. (%)	11.41	13.41	8.16	10.92

^{ns}- not significant.

The foliar application of Si to rice plants grown in soil contaminated with Cd increased the dry matter production of the grains and the aerial plant parts, reducing the concentration of the metal in the grains (LIU et al., 2009). This reduction of Cd in grains was possibly due to metal *sequestration* in the cells of the roots and shoots, thus limiting the translocation of Cd to the grains and ensuring safer food.

Silicon and Cadmium accumulation in plants

The addition of Si to the nutrient solution influenced the Si content of the leaves, stems and roots (Figure 6). The accumulation of Si in the shoots confirms the classification of this grass species as a Si accumulator (MA et al., 2001); in this case, the absorption most likely occurred through the active form via specific transporters (Lsi1 and Lsi2) (CHEN et al., 2012). Si accumulation in leaves occurs due to the loss of water through the stomata; this water loss favors Si concentration, as Si polymerizes in the apoplast of leaves, forming an important barrier that protects the plants from various types of stress (MITANI et al., 2005) or causes the co-precipitation of toxic elements in plant tissues (GU et al., 2011).

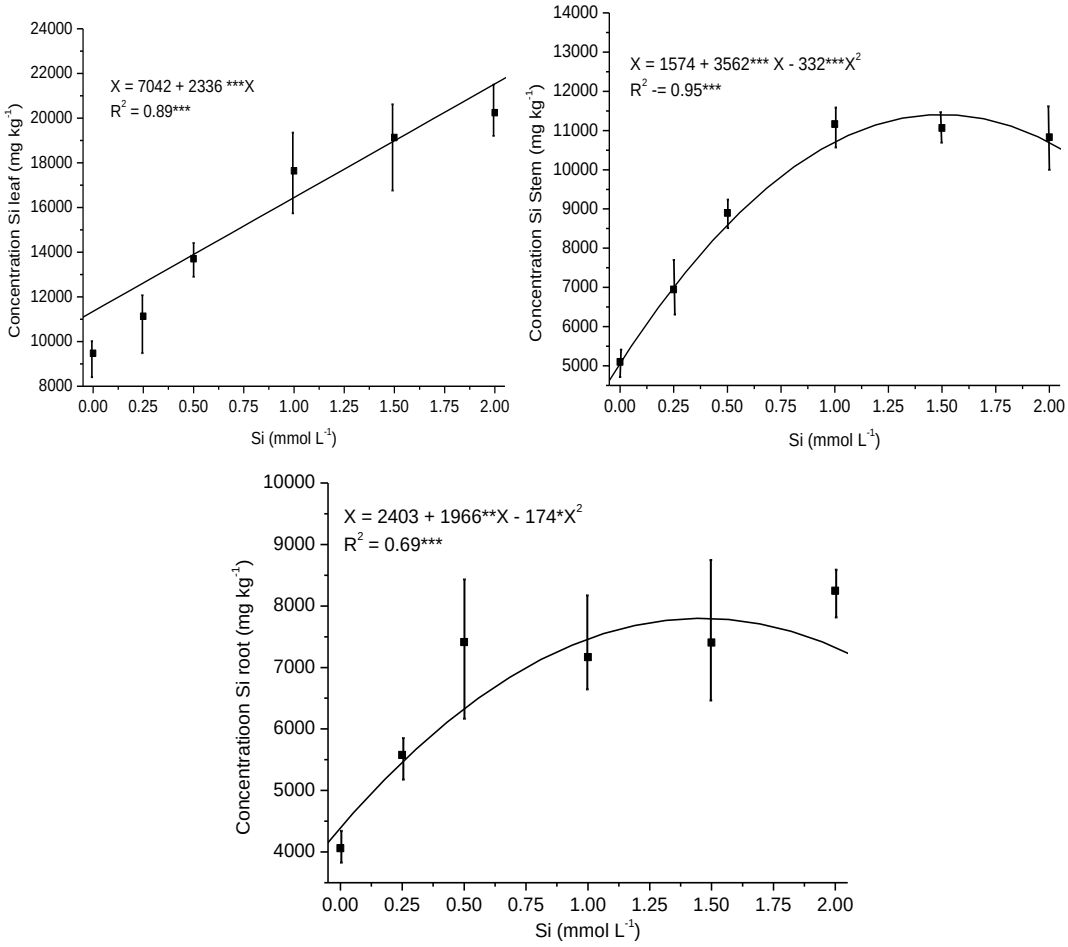

Figure 6. Silicon levels in the leaves, stems and roots of maize plants grown in a nutrient solution contaminated with Cd with increasing doses of Si. *, **, *** - significant at 5, 1 and 0.1% probability levels, respectively.

Silicon was able to reduce the Cd content in the leaves and stems of maize plants in the presence of a toxic dose of this metal (Table 3). The reduction of Cd levels in the aerial parts of the plant indicates that Si interfered with the processes of absorption and translocation of Cd to the aerial parts. The addition of silicon reduced the toxicity of Cd and significantly decreased the absorption and translocation of this metal from the roots to the aerial parts of *Brassica chinensis* plants grown in a nutrient solution (SONG et al., 2009; LIU et al., 2013). Si reduces the availability of Cd in the soil, making the sequestration of Cd in plant roots more efficient and thus limiting the translocation of this element to the aerial parts of the plant (RIZWAN et al., 2012). The increase in the ratio of Cd in the leaves to Cd in the stems (Table 3) indicates that Si promoted the reduction of Cd concentrations in the aerial plant parts; the 1 mmol L^{-1} dose of Si promoted the highest increase in this ratio.

Table 3. Cd levels in the leaves, stems and roots of maize plants grown in nutrient solutions with silicon

Si Doses	Cd content			Ratio [Cd]	
	Leaf*	Stem*	Root^{ns}	Root/leaf**	Root/Stem**
µmol L^{-1}	----------mg kg^{-1}----------			----------	
0.00	137.33	129.83	1652.42	12.03	12.73
0.25	130.83	106.00	2040.68	15.60	19.25
0.50	135.12	118.52	1634.68	12.10	13.79
1.00	94.07	94.67	2138.19	22.73	22.86
1.50	110.24	94.42	1632.39	14.81	17.29
2.00	104.08	78.35	1191.82	11.45	15.21
C.V. (%)	2.47	14.76	22.19	19.05	21.85

*,** significant at 5 and 1% probability levels, respectively. ^{ns} – not significant.

Silicon in solution caused an incremental decrease in Cd levels in the roots of *Arabidopsis thaliana;* the observed reductions in Cd levels in the shoots of this species are due to a reduced translocation of this metal (CABOT et al., 2013). The higher dose of applied Si caused a reduction in Cd levels in the roots of maize plants (Table 3). Silicon accumulation in maize plants reduces the concentration of Cd in the roots and the translocation of Cd to aerial parts of the plant. The Si-mediated alleviation of Cd stress may vary by plant species and even between plants of the same species (KULIKOVÁ & LUX, 2010). Silicon increases the proportion of Cd in the apoplast and reduces the amount of Cd in the symplasm, which reinforces the theory that Si enhances the binding of Cd to the cell walls and limits the transport of Cd through the apoplastic pathway (YE et al., 2012).

The reduction in the translocation of Cd to the aerial parts of the plant reflects the importance of Si in protecting plant tissue from the various possible effects of Cd. Furthermore, the application of Si proved to be an important tool for reducing Cd concentrations in the aerial parts of the plant, making its application in agricultural crops a promising alternative for enhancing food safety.

Photosynthetic pigment production

The levels of photosynthetic pigments did not significantly change with the addition of Si to the nutrient solution (Table 4). A trend towards increasing levels of chlorophyll a and b was observed when doses of 1 and 2 mmol L^{-1} of Si were used, suggesting that Si alleviates the effect of Cd, even in the absence of an increase in the levels of photosynthetic pigments. The levels of photosynthetic pigments do not always change with the application of Si in maize plants (LUKACOVÁ et al., 2013). An increase in the levels of photosynthetic pigments was observed in cucumber plants grown under Cd stress and supplied with Si (FENG et al., 2010). The levels of photosynthetic pigments reflect the Cd content found in the leaves (Table 3), as these levels tended to be higher when the Cd content was lower.

Table 4. Photosynthetic pigment levels as a function of Si doses in maize plants grown in a nutrient solution enriched with cadmium

Si Doses	Chlorophyll content		
	A ^{ns}	B ^{ns}	Total ^{ns}
mmol L^{-1}	----------mg g^{-1}----------		
0.00	0.61	0.40	1.01
0.25	0.66	0.33	0.99
0.50	0.52	0.31	0.82
1.00	0.62	0.49	1.11
1.50	0.46	0.39	0.85
2.00	0.71	0.51	1.22
C.V. (%)	27.65	23.63	19.66

^{ns}- not significant.

Monitoring by chlorophyll fluorescence

The spectral emission obtained in the chlorophyll fluorescence analysis was very important for differentiating the effects of the Si doses on the alleviation of Cd toxicity in plants (Figure 7). The results of the analysis of emission intensity of chlorophyll fluorescence illustrate the role of Si in alleviating the stress caused by Cd and the importance of this technique for the

study of Cd detoxification by Si. According to the spectral emission data, the 1 mmol L^{-1} dose of Si provided the best Cd toxicity alleviation in plants.

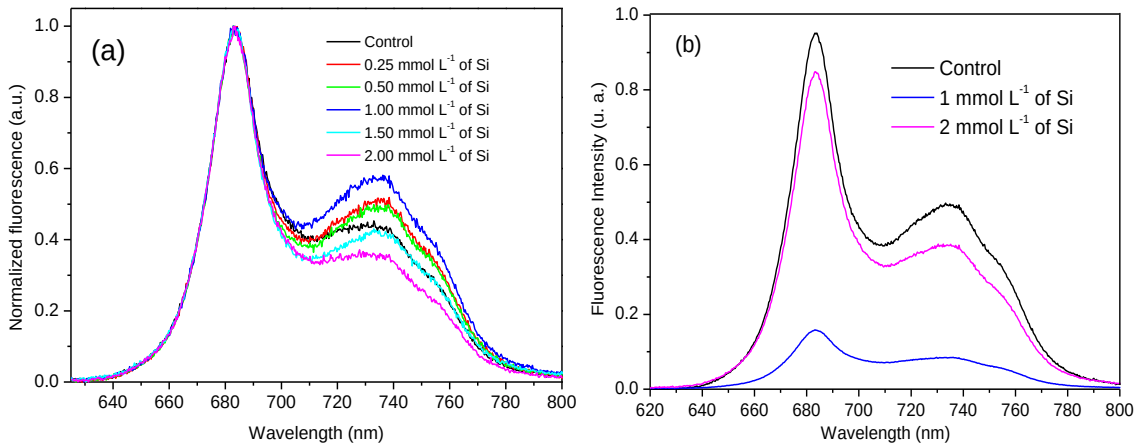

Figure 7. (a) Chlorophyll fluorescence spectra from maize plants grown under different doses of Si in a nutrient solution contaminated with Cd. (b) Maximum intensity of the chlorophyll fluorescence for the highest doses of Si in the nutrient solution.

The Fr/FFr ratio obtained from chlorophyll fluorescence spectra over time also indicates that 1 mmol L^{-1} of Si was the optimal dose for the Si-mediated alleviation of Cd stress in plants (Figure 8), confirming the results observed in the spectral emission (Figure 7) and the reduction of the Cd content in the aerial parts of the plant (Table 3).

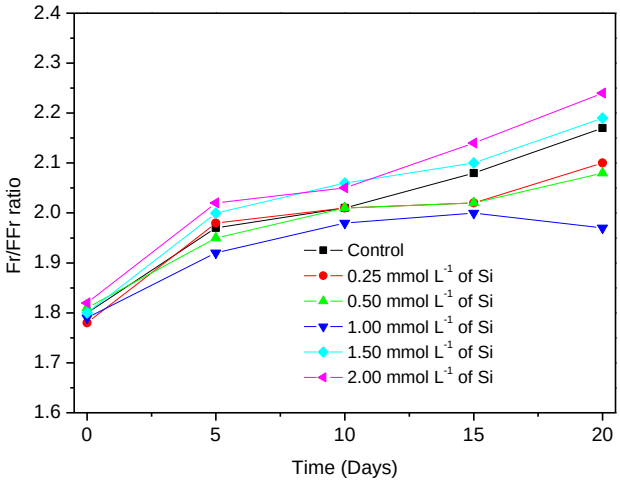

Figure 8. Ratio of chlorophyll fluorescence spectra as a function of cultivation time of maize plants grown under increasing Si doses in a nutrient solution

contaminated with Cd; this ratio refers to the red and far-red (Fr/FFr) readings in the wavelengths of F685 and F735 ηm, respectively.

The alleviation of the toxic effect of Cd in maize plants depends on the concentrations of Cd and Si in solution. Silicon promotes Cd retention in the plant roots, reducing the translocation of Cd to the aerial parts of the plant (LUKACOVÁ et al., 2013). The reduced Fr/FFr ratio shows that, after 15 days, the 1 mmol L^{-1} Si dose improved the photosynthetic apparatus of the plants. All other doses resulted in increased ratios.

Si can exert a positive effect on the growth, photosynthesis and chlorophyll fluorescence parameters of plants under Cd stress; indeed, the addition of Si increases the quantum yield and the maximum photochemical efficiency of photosystem II (FENG et al., 2010). The chlorophyll fluorescence analysis also demonstrated that Si alleviated Cd toxicity in rice plants by reducing Fo and increasing the Fv/Fm ratio and qP value (NWUGO & HUERTA, 2008).

CONCLUSIONS

The chlorophyll fluorescence analysis demonstrated that Si alleviated Cd toxicity in maize plants grown in a nutrient solution, even when significant changes in dry matter production and levels of photosynthetic pigments were not observed. The spectral emission and the Fr/FFr ratio were sensitive to the effects of Si and represent promising tools for environmental studies. Silicon caused a reduction in the translocation of Cd to the aerial parts of the plant and can be used in the phytoremediation or phytostabilization of contaminated soils.

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CAPÍTULO III

The monitoring of As toxicity in maize plants using chlorophyll fluorescence spectra analysis

The monitoring of As toxicity in maize plants using chlorophyll fluorescence spectra analysis

RESUMO

O arsênio (As) é um metaloide muito tóxico para plantas, mesmo em baixas concentrações. As técnicas tradicionais de monitoramento da toxidez de elementos químicos em plantas são geralmente caras e demoradas, o que tem tornado a análise da fluorescência da clorofila uma técnica interessante na detecção desse tipo de estresse. O objetivo desse trabalho foi investigar os efeitos da toxidez de As em plantas de milho cultivadas em solução nutritiva e avaliar o potencial dos parâmetros de emissão espectral e da razão Fr/FFr obtidos na análise da fluorescência da clorofila na determinação de estresse causado por As em plantas de milho. O ensaio foi conduzido em solução nutritiva com plantas de milho submetidas a doses crescentes de As (0, 10, 20, 40 80 e 120 µmol L^{-1} durante 21 dias). A produção de matéria seca das plantas, os teores de As e pigmentos fotossintéticos e a emissão espectral da fluorescência da clorofila foram medidos. As foi muito tóxico para as plantas, causando redução na produção de matéria seca das folhas, colmos e raízes, sendo a dose de 68 µmol L^{-1} a responsável pela redução de 50% da produção de matéria seca total. As doses de As proporcionaram incremento do teor do elemento nas diferentes partes das plantas e redução dos teores de pigmentos fotossintéticos. A detecção do estresse de As por fluorescência da clorofila mostrou ser uma técnica sensível na identificação de alterações causadas por este metaloide ao aparato fotossintético das plantas de milho, sendo o espectro de emissão da fluorescência da clorofila e as razões Fr/FFr indicados como parâmetros sensíveis para o monitoramento do estresse de As em plantas de milho.

Palavras-chave: metais pesados, metalóides**,** pigmentos fotossintéticos.

ABSTRACT

Arsenic (As) is a metalloid that is very toxic to plants, even at low concentrations. Traditional techniques for monitoring the toxicity of chemicals in plants are often expensive and time-consuming; as a result, chlorophyll fluorescence analysis is an interesting technique for detecting stress in plants due to toxicity. The aim of this study was to investigate the effects of As toxicity in maize plants grown in a contaminated nutrient solution and to evaluate the potential of the emission spectra and the Fr/FFr ratio parameters obtained from chlorophyll fluorescence analysis to determine As stress in maize plants. The maize plants were grown in a nutrient solution with increasing doses of As (0, 10, 20, 40, 80 and 120 µmol L^{-1}) for 21 days. The dry matter production of the plants, the As and photosynthetic pigments levels and the chlorophyll fluorescence emission spectrum were measured. Arsenic was very toxic to plants, causing a reduction in leaf, stem and root dry matter production, and an As dose of 68 µmol L^{-1} was responsible for a 50% reduction in total dry matter production. Arsenic doses led to an increase in the As levels in the different parts of the plants and a reduction in the levels of photosynthetic pigments. The use of chlorophyll fluorescence to detect As stress was effective at identifying changes caused by this metalloid in the photosynthetic apparatus of maize plants; in addition, the chlorophyll fluorescence emission spectrum and the Fr/FFr ratio were sensitive to As stress in maize plants.

Keywords: Heavy metals, metalloid, photosynthetic pigments.

INTRODUCTION

Arsenic (As) is a class I carcinogenic metalloid that has several harmful effects on human health and is one of the common causes of skin cancer (CHEN et al., 2009; MALKONIAN et al., 2010; AHSAN & STEINMAUS, 2013). The presence of As in the environment may cause toxicity in plants. The symptoms of toxicity can be observed in plants grown near mining areas where the excessive application of As-rich pesticides and the use of contaminated waste and of As-contaminated irrigation water is common (KABATA-PENDIAS, 2010). High levels of As can be found in plants that grow spontaneously near contaminated areas. The concern is greater when species that are important for food and medicinal uses grow under these conditions (VACULÍK et al., 2013).

Arsenic toxicity causes wilting of leaves and a reduction in the transpiration rate and photosynthetic rate of rice plants, resulting in impaired growth and reduced dry matter production (STOEVA et al., 2004; HOFFMAM & SCHENK, 2011). Controlled studies of plants exposed to As have demonstrated the high toxicity of this element; the reduction in dry matter production is one of the most severe consequences of this toxicity (MELO et al., 2009; LI et al., 2011). Changes in the photochemical efficiency of plants caused by the presence of As in the growth solution causes a decrease in photoassimilate production and therefore a reduction in dry matter production. This reduction is also associated with changes in gas exchange in plants. Significant changes in the rate of photosynthesis and respiration of oat plants subjected to stress caused by As were observed, as illustrated by a trend towards a reduction in stomatal conductance of plants (STOEVA & BINEVA, 2003).

While the production of photosynthetic pigments is affected by stress, other symptoms of toxicity are not always easily visualized. This makes the use of equipment sensitive to changes in the levels of photosynthetic pigments more attractive, especially due to their ability to detect stress early, even under conditions of low stress. Thus, changes in the photosynthetic apparatus can be investigated by chlorophyll fluorescence analysis, especially in plants under conditions of abiotic stress (BAKER & ROSENQVIST, 2004).

Chlorophyll fluorescence is a marker of the ability of plants to convert light energy into biochemical energy via photosynthetic processes. Plants respond quickly to any change in culture conditions, resulting in alterations of the

photosynthetic processes and the photochemistry of the plant. These changes can be identified by alterations in the light/fluorescence emission of the plant indicating that the photosynthetic apparatus of the plants was altered (BAKER & ROSENQVIST, 2004; HAZEM & GUO 2008).

Several variables can be obtained along with the chlorophyll fluorescence analysis. Several studies have shown that the ratio between the emission peaks in the red (F685) and far-red (F735) regions is sensitive to stress caused by heavy metals in plants (CHERIF et al., 2010; CHERIF et al., 2012). The chlorophyll fluorescence technique, in addition to being non-destructive, is highly sensitive and simple to use, allowing for qualitative (emission spectra) and quantitative information (ratio Fr/FFr) to be obtained (FALQUETO et al., 2007). The variables obtained from the parameters between the peaks of the fluorescence bands can be used to predict the health status and stress levels of the plant (MISHRA & GOPAL, 2008). Because stress generally increases or decreases the levels of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids), this technique can use ratios of the levels of these pigments to detect stress (MAURYA et al., 2008).

The exposure of *Hydrilla verticillata* to As resulted in a reduction in the levels of photosynthetic pigments and changes in the parameters of the chlorophyll fluorescence analysis (SRIVASTAVA et al., 2012). The presence of As caused a decrease in the Fv/Fo and Fv/Fm ratios in maize plants, indicating a reduction in the activity function of photosystem II (STOEVA et al., 2003).

The chlorophyll fluorescence analysis is a promising tool for environmental monitoring, as the costs of its use are low, the instruments are simple to use and it does not generate waste; thus, it is a noninvasive and nondestructive technique that produces rapid results (TALIO et al., 2009). The aim of this study was to investigate the effects of As toxicity in maize plants grown in contaminated nutrient solutions and to evaluate the potential of the emission spectra and the ratio of Fr/FFr parameters obtained in the chlorophyll fluorescence analysis to determine the As stress level of maize plants.

MATERIAL E METHODS

Maize seeds (*Zea mays* L., cv. São José) were germinated between sheets of paper towel, with the bottom base immersed in a 0.67 mmol L^{-1} $Ca(NO_3)_2$ $4H_2O$ solution(VILELA & ANGHINONI, 1984). Seven days after sowing, two seedlings were transferred to a plastic container with 6 L of the modified Hoagland & Arnon (1950) nutrient solution, containing 105.05 mg L^{-1} N, 15.5 mg L^{-1} P, 117.3 mg L^{-1} K, 100.2 mg L^{-1} Ca, 24.3 mg L^{-1} Mg, 32.1 mg L^{-1} S, 0.325 mg L^{-1} Cl, 0.25 mg L^{-1} Mn, 0.025 mg L^{-1} Zn, 0.01 mg L^{-1} Cu, 0.25 mg L^{-1} B, 0.005 mg L^{-1} Mo and 7.53 mg L^{-1} Fe. The nutrient solution was replaced weekly and/or every time the electric conductivity reached 0.4 dS m^{-1}. Deionized water was added to the pot to replace the water lost by evapotranspiration. The pH was maintained close to 5.50 (+/- 0.2); adjustments were made with 1 mmol L^{-1} H_2SO_4 or NaOH.

After an 8-day adaptation period, doses of 0, 10, 20, 40, 80 and 120 µmol L^{-1} As were added to the pots, and the seeds were cultivated for an additional 21-day period.

Five chlorophyll fluorescence measurements were carried out during the experiment. The first was taken before As addition and the last was carried out one day before collection. Measurements were taken at night in order to ensure the deactivation of electron transport in the photosynthetic apparatus. In vivo chlorophyll fluorescence analyses were conducted with an ultraviolet LED device, with peaks at 685 ηm and 735 ηm wavelengths (Ocean Optics, USB 2000). The spectra were obtained by the Ocean Optics Spectra-Suite software and adjusted to two Gaussian curves corresponding to red (685 ηm) and far red (735 ηm). The ratio between the fluorescence intensity F685/F735 and peak height was calculated from the fitted curve for each As dose and used to infer to the effect of the element in the photosystem II, using the Origin software version 6.0.

Leaves were sampled to determine the levels of chlorophyll *a* and *b* and, through the sum, the total chlorophyll content (ARNON, 1949), at the middle third of the same leaf used for the chlorophyll fluorescence analysis.

Leaves, stems and roots were rinsed with tap water, followed by three washes with distilled water, before they were placed in paper bags. Subsequently, the samples were kept in an oven with forced air circulation at 65

°C until reaching constant weight for obtaining the dry matter of leaves, stems and roots, as well as the total dry matter.

Digestion of plant material was performed in nitric and hydrochloric acids in a microwave oven (Mars Xpress), according to Method 3051A (USEPA, 1998). The As content was determined in the digestion extract in an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800) coupled to a hydrides generator. The data were submitted to ANOVA and regression analyses.

RESULTS AND DISCUSSION

Dry matter production

The increasing doses of As added to the culture solution caused a linear reduction in plant dry matter (Figure 9). The toxic dose was defined as the dose resulting in a 50% reduction of total dry matter (KING, 1996); in this case, the dose was 68 µmol L^{-1} of As. The highest dose caused a 76% reduction in total dry matter. The standard deviations show that the dry matter was not significantly affected by the application of the first two doses of As (Figure 9). The toxic effect on the dry matter was apparent from a dose as low as 40 µmol L^{-1}, with no differences in the standard deviations of the 80 and 120 µmol L^{-1} doses.

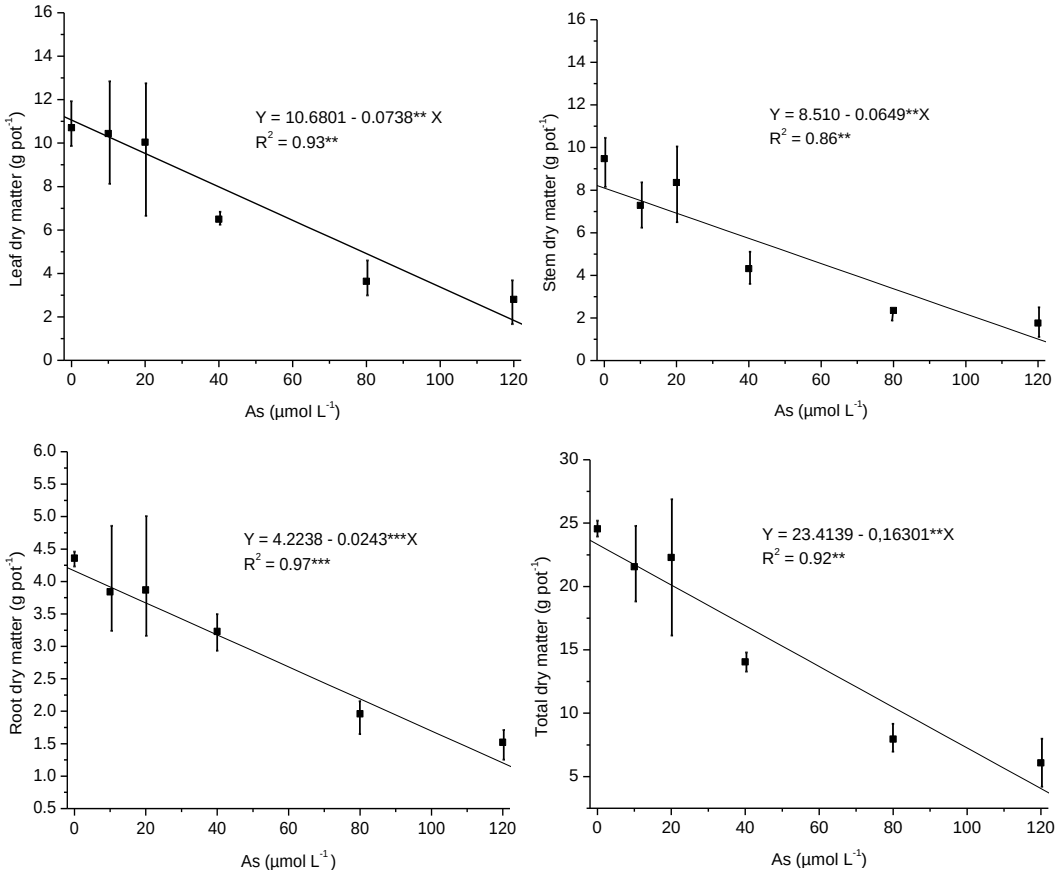

Figure 9. Leaf, stem and root dry matter production and total dry matter production of maize plants grown in a nutrient solution enriched with doses of As. **, *** - significant at 1 and 0.1% probability levels, respectively.

The absorption of As in rice plants grown in the presence of 0.5 mg L^{-1} of As (6.7 µmol L^{-1}) did not reduce the dry matter production of the plants (BOGDAN & SCHENK, 2012). As was the case in the present study, a dose of 67 µmol L^{-1} of As was toxic to castor bean plants, producing a 31% reduction in dry matter production (MELO et al., 2009).

Arsenic Levels

Arsenic levels in plants increased with the addition of As to the nutrient solution (Figure 10). The As levels producing a 50% reduction in dry matter were 1.34 mg kg^{-1}, 1.18 mg kg^{-1} or 86 mg kg^{-1} for the leaves, stems and roots, respectively. The standard deviations show that at doses higher than 20 µmol L^{-1}, the reduction in dry matter of the plant levels off.

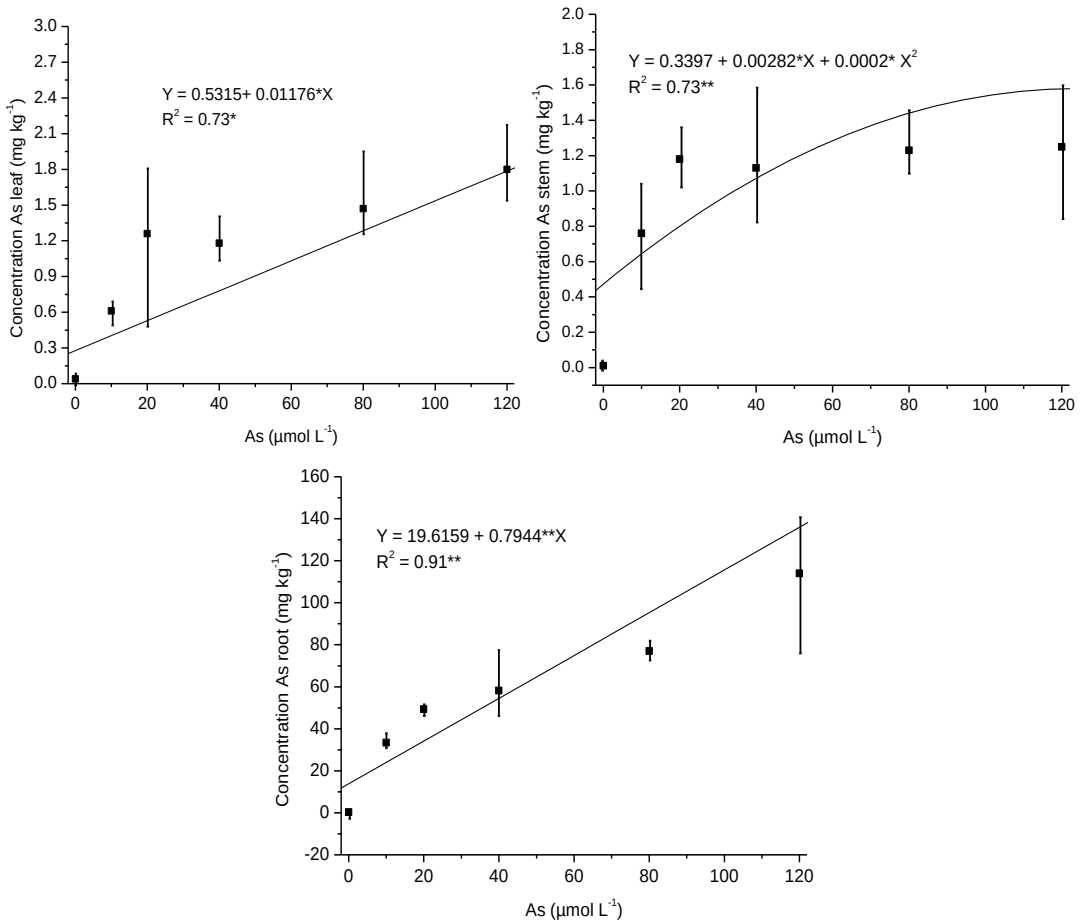

Figure 10. Arsenic levels in leaves, stems and roots of maize plants exposed to increasing doses of As in a nutrient solution over a period of 21 days. *, ** - Significance at 5 and 1% probability levels, respectively.

The linear relationship between As levels in plant tissues and water-soluble As levels in the soil solution indicate that the translocation of As occurs passively via translocation in water (KABATA-PENDIAS, 2010); indeed, As accumulates in a linear manner in both the roots and leaves of maize plants. Aquaporins (proteins responsible for water intake in plants) are the main routes of As entry into the plants (ALI et al., 2009).

Production of photosynthetic pigments

The production of photosynthetic pigments and chlorophyll a was altered in plants under stress. Chlorophyll b levels, however, did not change (Table 5). The reduction in levels of photosynthetic pigments could be observed at a dose as low as 20 µmol L^{-1}, demonstrating that damage to these pigments occurred at doses lower than 40 µmol L^{-1} (the dose that causes a reduction in dry matter

production). Interestingly, this dose is associated with a trend of increased production of all pigments, suggesting that the plant responds to the decreased synthesis caused by the toxicity. As toxicity increases, this response is no longer possible and the pigment contents decrease further.

Table 5. Photosynthetic pigments (chlorophyll a, b and total) levels in maize leaves exposed to As in the nutrient solution

As Doses	Chlorophyll content		
	A**	B^{ns}	Total*
µmol L^{-1}	----------mg g^{-1}----------		
0	1.34	0.59	1.92
10	1.23	0.98	2.21
20	0.80	0.58	1.38
40	0.96	0.87	1.83
80	0.79	0.46	1.25
120	0.87	0.64	1.51
C.V. (%)	17.77	20.71	17.23

*,**- significant at 5 and 1% probability levels, respectively

Despite the reduction in growth experienced by the plants (Figure 11), the plants did not show pronounced symptoms of chlorosis in the leaves at the end of the experiment. The only visual symptoms of As toxicity observed at the two highest doses were a wilting of older leaves, subsequent necrosis of the leaves from the tip to the base through the edges, brown roots and greatly reduced growth. Kabata-Pendias (2010) reported wilted leaves, violet coloration (high levels of anthocyanin), root discoloration and cell plasmolysis in plants exposed to As; the most commonly observed symptom was the restriction of plant growth.

Figure 11. Details of the drastic reduction in the growth of maize plants exposed to increasing doses of As in the nutrient solution after 21 days of treatment.

Monitoring by chlorophyll fluorescence

The analysis of the chlorophyll fluorescence spectra performed on day 20 of As exposure showed changes in the red (Fr) and far-red (FFr) emission peaks, indicating changes in the photosynthetic apparatus of plants (Figure 12). These results demonstrate the ability of the chlorophyll fluorescence analysis to detect stress caused by As in maize plants. Changes in chlorophyll fluorescence parameters were observed in *Hydrilla verticillata* exposed to As, followed by a reduction in photosynthetic pigment levels (SRIVASTAVA et al., 2012).

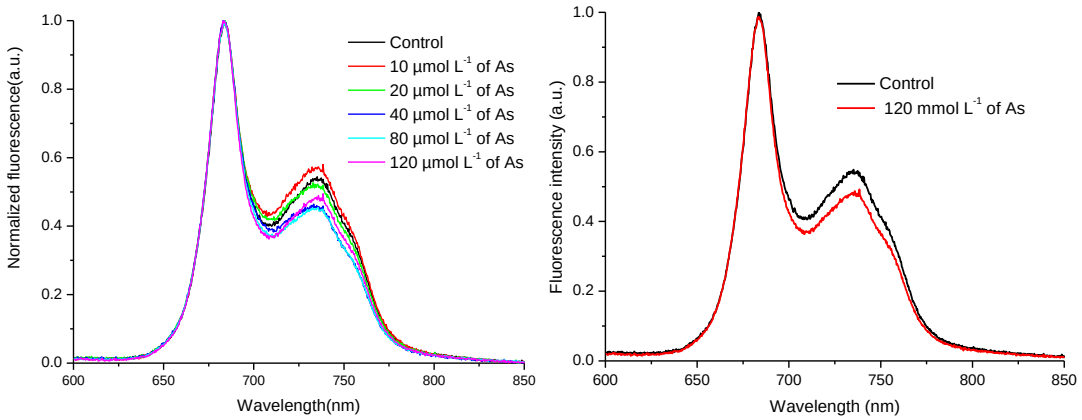

Figure 12. Average of chlorophyll fluorescence spectra in maize plants exposed to increasing doses of As in the nutrient solution and maximum intensity of chlorophyll fluorescence in maize plants grown with and without As in the nutrient solution.

The results of this analysis confirm that this technique is able to detect stress caused by other elements. Differences in the spectra and the Fr/FFr ratio were observed in a study of Cd toxicity in maize plants (SILVA et al., 2012). Changes in the spectra of tomato plants were observed when these plants were exposed to Cd and Zn (CHERIF et al., 2010; CHERIF et al., 2012).

The chlorophyll fluorescence analysis is a viable technique for identifying stress in plants, as it detects changes only five days after the addition of As to the nutrient solution (Figure 13). The wilting of the leaves and subsequent necrosis, in contrast, appeared eight days after the plants were exposed to two higher doses; the reduced plant growth was also evident only after eight days.

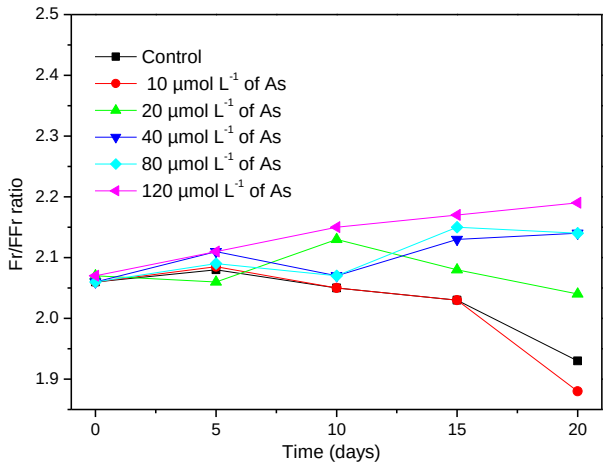

Figure 13. Ratio of chlorophyll fluorescence spectra as a function of the cultivation time in maize plants exposed to increasing doses of As in the nutrient solution; the ratio refers to the red and far-red readings (Fr/FFr) at the wavelengths of F685 and F735 ηm, respectively.

The Fr/FFr ratio obtained on the last day of evaluation illustrates the response of the plant to the addition of As (Figure 13). The ratio tended to decrease with the first two doses in a similar manner to the control (without As); this result is in agreement with the reduction in the dry matter produced by these conditions (Figure 9). In contrast, the ratio tended to increase with the other doses, confirming the toxic effect of As and the reduction in dry matter with As doses higher than 40 µmol L^{-1}.

When the Fr/FFr ratio increases, the plant is experiencing some type of stress. For example, the increased fluorescence emission may indicate a lower CO_2 assimilation caused by changes in the photosynthetic apparatus; indeed, a dysfunctional photosynthetic apparatus loses the ability to perform electron transport and consequently reduces the production of dry matter. The relationship between dry matter production and the Fr/FFr ratio illustrates this concept (Figure 14).

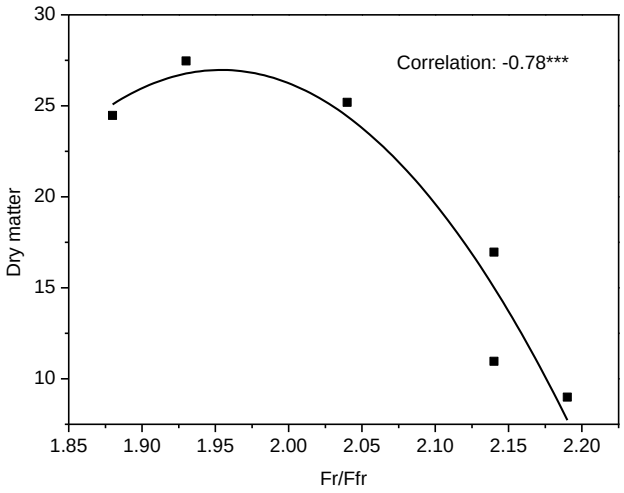

Figure 14. Relationship between total dry matter production and the Fr/FFr ratio of maize plants exposed to increasing doses of As in the nutrient solution. *** - Significant at the 0.1% probability level.

Cherif et al. (2012) studied the toxic effect of the interaction between Cd and Zn in tomato plants and found that the chlorophyll fluorescence analysis was an important method for detecting stress when the ratios in the region near the F690/F735 are measured. The results of this investigation show that the intensity of the fluorescence spectra depends on the chlorophyll levels in the leaves. An increase in the F690/F735 ratio indicates not only a reduction in chlorophyll levels but also a disturbance of the quantum conversion processes of photosynthesis.

Arsenic caused a decrease in the Fv/Fo and Fv/Fm ratios in maize plants, indicating a reduction in the activity of photosystem II (STOEVA et al., 2004). The advantages of this technique in measuring the physiological state of the plant include the rapidity of the analysis, the ability to measure changes, its non-invasiveness and its non-destructiveness. This technique allows an evaluation of the physiological conditions of the photosynthetic apparatus of the plants

over time, which makes it useful for the successful detection of stress in plants (BAKER & ROSENQVIST, 2004; GORBEA & CALATAYUD, 2012).

CONCLUSIONS

Arsenic was found to be highly toxic to maize plants grown in a contaminated nutrient solution, resulting in a high reduction in the dry matter production of plants and a direct effect on the photosynthetic apparatus. The toxic dose of 68 µmol L^{-1} (5.1 mg L^{-1}) resulted in a 50% reduction in dry matter. The chlorophyll fluorescence analysis was shown to be a sensitive tool for detecting stress caused by As, as the reduction in the production of photosynthetic pigments was caused by the presence of As in the solution. The Fr/FFr ratio is a very useful measure for identifying changes in plants; in addition, this method is sensitive and it monitors changes in the plant over time in a practical, efficient and low cost way.

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CAPÍTULO IV

Mitigation effects of silicon on Arsenic toxicity assessed by chlorophyll fluorescence spectra

Mitigation effects of silicon on Arsenic toxicity assessed by chlorophyll fluorescence spectra

RESUMO

Arsênio (As) é um metaloide de grande potencial de toxidez para plantas e animais, causando redução do crescimento vegetal e diversos problemas de saúde humana e animal. O silício (Si), por sua vez, tem se destacado na amenização de estresse causado por elementos tóxicos em plantas. O objetivo deste trabalho foi investigar os efeitos do Si na amenização do estresse causado por As em plantas de milho cultivadas em solução nutritiva e avaliar o potencial dos parâmetros de emissão espectral e da razão Fr/FFr obtidos na análise da fluorescência da clorofila na determinação dessa interação. Para isso foi montado um experimento em solução nutritiva com uma dose tóxica de As (68µmol L^{-1} de As) e seis doses crescentes de Si (0; 0,25; 0,5; 1; 1,5 e 2 mmol L^{-1} de Si), sendo avaliados a produção de matéria seca, teores de As, Si e pigmentos fotossintéticos. Medidas de fluorescência da clorofila foram tomadas ao longo do cultivo. O silício promove efeitos positivos na amenização do estresse causado por As em plantas de milho, comprovado pelo incremento de pigmentos fotossintéticos. A aplicação de Si proporcionou maiores teores de As no tecido vegetal e a sua utilização em estudos de técnicas de fitorremediação de solos contaminados pode ser promissora. A análise de fluorescência da clorofila demonstrou ser uma ferramenta sensível, podendo ser empregada com sucesso no estudo dos efeitos amenizantes do Si na proteção de plantas, sendo a razão Fr/FFr a variável recomendada na identificação de alterações temporais em plantas.

Palavras-chave: metais pesados, metaloides**,** pigmentos fotossintéticos, elementos traços.

ABSTRACT

Arsenic (As) is a metalloid highly toxic to plants and animals, causing reduced plant growth and various problems to human and animal health. Yet, silicon (Si) has excelled in alleviating stress caused by toxic elements in plants. The aim of this study was to investigate the effects of Si in alleviating As stress in maize plants grown in a nutrient solution, and evaluate the potential of spectral emission parameters and the ratio Fr/FFr obtained from the chlorophyll fluorescence analysis in determining this interaction. An experiment was carried out in a nutrient solution containing a toxic dose of As (68 µmol L^{-1}) and six increasing doses of Si (0, 0.25, 0.5, 1, 1.5 and 2 mmol L^{-1}). Dry matter production, concentrations of As, Si and photosynthetic pigments were then evaluated. Chlorophyll fluorescence measurements were taken along plant growth. Si promotes positive effects in alleviating As stress in maize plants, evidenced by the increase of photosynthetic pigments. Si application resulted in higher As levels in plant tissue; therefore, using Si on soil phytoremediation may be a promising choice. Chlorophyll fluorescence analysis proved to be a sensitive tool, and it can be successfully used in the study of ameliorating effects of Si on plant protection, with the ratio Fr/FFr as the recommended variable to identify temporal changes in plants.

Keywords: heavy metals, metalloids, photosynthetic pigments, trace element.

INTRODUCTION

Millions of people around the world are exposed to As. A classic case is the contamination of groundwater in Bangladesh, which is an important public health problem affecting 35 to 75 million people (CHEN et al., 2009). As is one of the most carcinogenic elements to humans, being linked to several adverse health effects, including skin, lung, bladder, liver and kidney cancers (AHSAN & STEINMAUS, 2013; MALKONIAN et al., 2010). Diabetes, cardiovascular diseases, pre-natal complications and decreased intellectual function in children are also related to As exposure (CHEN et al., 2009). The risk is greater for people living near mining areas, where As exposure of plants and animals is high. The concentration of As in these people's hair and urine (2.92 mg kg^{-1} and 164 µg L^{-1}, respectively) suggests health damage to these populations (LIU et al., 2010).

Si has been recognized as a toxicity alleviating agent in plants, for retaining heavy metals in roots, inhibiting their translocation to the aerial parts (SHI et al., 2005a), depositing SiO_2 in the root apoplast and leaf surface, forming a barrier to the apoplastic flux of metallic ions and to the transpiration flux (LUX et al., 2002; SHI et al., 2005a). It also contributes for the co-precipitation of Si-metal complexes in the cell wall, compartmentalization of metals bound to organic acids in the vacuole (NEUMAM & NEIDEN, 2001), more homogeneous distribution of metals, formation of Si-polyphenol complexes in tissues (MAKSIMOVIC et al., 2007) and reduced lipid peroxidation in the membrane, via stimulation of enzymatic and non-enzymatic antioxidants (SHI et al., 2005b). Si is linked to a lower As translocation to rice straw and grains (ALI et al., 2009), proving its beneficial effect on food safety. Increased production of antioxidant enzymes, reduced lipid peroxidation and reduced As level in rice plants were observed when Si was added to the culture solution (TRIPATHI et al., 2013).

The chlorophyll fluorescence emission by the leaves is altered when biotic and abiotic stress cause disruption to the photosynthetic apparatus, either directly or indirectly (BAKER & ROSENQVIST, 2004). The chlorophyll fluorescence technique, in addition to being nondestructive, is highly sensitive and simple to use, generating qualitative and quantitative information about the physiological status of the photosynthetic apparatus of plants (FALQUETO et

al., 2007). Many parameters can be obtained from the chlorophyll fluorescence analysis. Several studies have shown the ratio between the emission peaks in the red (F685) and far red (F735) regions as a sensitive variable in the identification of stress caused by heavy metals in plants (CHERIF et al., 2010; CHERIF et al., 2012; SILVA et al., 2012).

Chlorophyll fluorescence analysis has proven to be a promising tool for detecting Cd toxicity in maize plants (SILVA et al., 2012). Variations in the emission peaks of chlorophyll fluorescence and their respective ratios were efficient when detecting Cd-related stress in maize (MAURYA et al., 2008). The technique was also efficient in the analysis of As toxicity in maize (STOEVAL et al., 2003).

The chlorophyll fluorescence analysis has also shown significant results when identifying Si benefits to plant nutrition, especially when under stress. For example, Si has caused beneficial effects, such increased quantum yield and effective maximum quantum, on the photosynthesis of cucumber plants grown in the presence of Cd (FENG et al., 2010). Si was also effective in alleviating Cr stress, by increasing the concentrations of photosynthetic pigments and the efficiency of chlorophyll fluorescence parameters (ALI et al., 2013). Si application increased Fv/Fm and qP parameters in rice plants under Cd stress (NWUGO & HUERTA, 2008a).

The aim of this study was to investigate the effects of Si in alleviating As stress in maize plants grown in a nutrient solution. Also, to evaluate the potential of spectral emission parameters and the Fr/FFr ratio obtained in the analysis of chlorophyll fluorescence in determining the Si-As interaction in maize plants under an As toxic dose.

MATERIAL AND METHODS

Maize seeds (*Zea mays* L., cv. São José) were germinated between sheets of paper towel, with the bottom base immersed in a 0.67 mmol L^{-1} $Ca(NO_3)_2$ $4H_2O$ solution(VILELA & ANGHINONI, 1984). Seven days after sowing, two seedlings were transferred to a plastic container with 6 L of the modified Hoagland & Arnon (1950) nutrient solution, containing 105.05 mg L^{-1} N, 15.5 mg L^{-1} P, 117.3 mg L^{-1} K, 100.2 mg L^{-1} Ca, 24.3 mg L^{-1} Mg, 32.1 mg L^{-1} S, 0.65 mg L^{-1} Cl, 0.5 mg L^{-1} Mn, 0.05 mg L^{-1} Zn, 0.02 mg L^{-1} Cu, 0.5 mg L^{-1} B,

0.01 mg L^{-1} Mo and 7.53 mg L^{-1} Fe. The nutrient solution was replaced weekly and/or every time the electric conductivity reached 0.4 dS m^{-1}. Deionized water was added to the pot to replace the water lost by evapotranspiration. The pH was maintained close to 5.50 (+/- 0.2); adjustments were made with 1 mmol L^{-1} H_2SO_4 or NaOH.

After 12 days for plant adaptation to the nutrient solution, the toxic As dose obtained in the previous experiment (68 µmol L^{-1}) and the Si (K_2SiO_3) doses of 0, 0.25, 0.5, 1, 1.5 and 2 mmol L^{-1} were added to the solution. Maize plants were then grown for 21 days.

Five chlorophyll fluorescence measurements were carried out during the experiment. The first was taken before As addition and the last was carried out one day before collection. Measurements were taken at night in order to ensure the deactivation of electron transport in the photosynthetic apparatus. In vivo chlorophyll fluorescence analyses were conducted with an ultraviolet LED device, with peaks at 685 ηm and 735 ηm wavelengths (Ocean Optics, USB 2000). The spectra were obtained by the Ocean Optics Spectra-Suite software and adjusted to two Gaussian curves corresponding to red (685 ηm) and far red (735 ηm). The ratio between the fluorescence intensity F685/F735 and peak height was calculated from the fitted curve for each Si dose and used to infer to the effect of the element in the photosystem II, using the Origin software version 6.0.

Leaves were sampled to determine the levels of chlorophyll *a* and *b* and, through the sum, the total chlorophyll content (ARNON, 1949), at the middle third of the same leaf used for the chlorophyll fluorescence analysis.

Leaves, stems and roots were rinsed with tap water, followed by three washes with distilled water, before they were placed in paper bags. Subsequently, the samples were kept in an oven with forced air circulation at 65 °C until reaching constant weight for obtaining the dry matter of leaves, stems and roots, as well as the total dry matter.

Digestion of plant material was performed in nitric and hydrochloric acids in a microwave oven (Mars Xpress), according to Method 3051A (USEPA, 1998). The As content was determined in the digestion extract in an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800) coupled to a hydrides generator. Si digestion in the plant tissue was carried out with hydrogen peroxide (H_2O_2) and sodium hydroxide (NaOH) in an autoclave.

Dosing was done in a colorimeter using ammonium molybdate as a complexing agent (KORNDÖRFER et al., 2004). The data were submitted to ANOVA and regression analyses.

RESULTS AND DISCUSSION

Dry matter production

The production of dry matter from leaves and roots was not affected by the increasing Si doses (Table 6); a positive effect was only observed for the stem and total dry matter. Si accumulation in plants does not always cause positive effects on dry matter production (MELO et al., 2009; ARAÚJO et al., 2011), but it can bring other beneficial effects, such as protection against physiological stress by improving the photosynthetic apparatus (MATTSON & LEATHERWOOD, 2010).

Table 6. Dry matter production of maize plants under As stress and Si doses

Si doses	Dry matter production (g pot^{-1})			
mmol L^{-1}	Leaf ^{ns}	Stem *	Root ^{ns}	Total *
0	34.97	30.72	22.99	88.69
0.25	29.64	24.19	17.97	71.79
0.50	30.87	28.04	20.73	79.64
1.00	30.08	25.48	19.22	74.78
1.50	33.01	29.96	21.11	84.08
2.00	30.53	28.90	21.42	80.85
C.V. (%)	9.67	8.39	9.52	7.36

* - significant at 5% probability. ^{ns} - non-significant.

Silicon and Arsenic accumulation in plants

Maize plants responded positively to Si application in the nutrient solution (Figure 15). Silicon content greater than 1% in the aerial parts characterizes the species as a accumulator of the element (MA et al., 2001). Furthermore, the greatest Si accumulation in the aerial parts (leaves and stems) indicates that

maize plants have a mechanism of active transport for this element, similar to rice (NWUGO & HUERTA et al., 2008b).

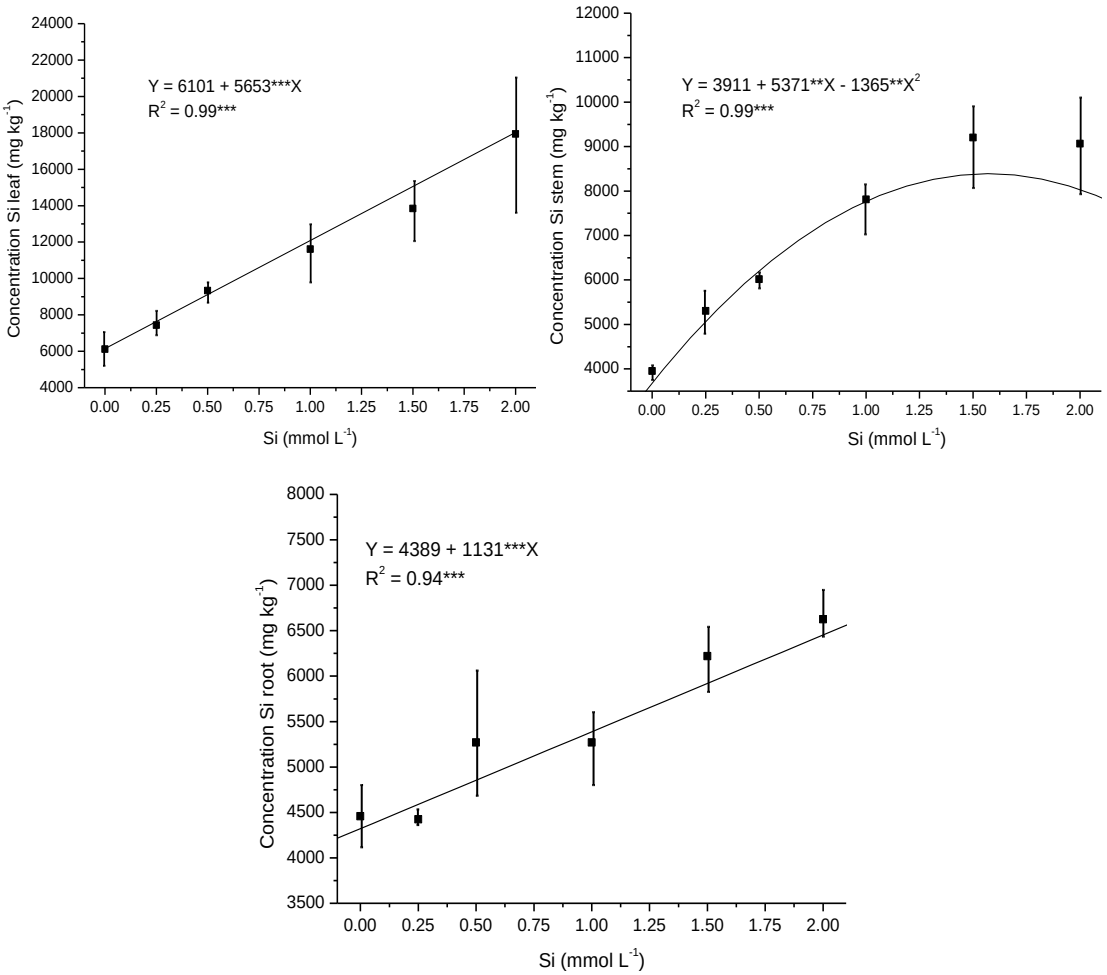

Figure 15. Silicon content in leaves, stems and roots of maize plants grown in a nutrient solution contaminated by As and under increasing doses of Si. (** - significant at 1% probability; *** - significant at 0.1 probability).

Si absorbed by the plants is translocated to the aerial parts by a water flow; in the leaves, the element concentration is determined by the loss of water through the stomata. This causes Si polymerization in the apoplast of leaves, forming an important barrier to protect plants against various types of stress (MITANI et al., 2005) or causing co-precipitation of toxic elements. This can be observed for As in all plant parts (Table 7), which showed increased concentrations of the element to up to 1 mmol L^{-1} Si in solution. Si addition was effective in alleviating As stress in rice plants, showing to be effective in reducing As levels in aerial parts, with accumulation of antioxidant enzymes. Furthermore, Si addition increased the concentrations of cysteine and reduced

the lipid peroxidation (TRIPATHI et al., 2013). Si hyperaccumulators, such as rice, accumulate As at higher levels than many other species, because As and silicic acid share the same carrier (CHEN et al., 2012).

Table 7. As content in leaves, stems and roots of maize plants grown in nutrient solutions with silicon

Si doses	As content		
	Leaf**	Stem ^{ns}	Root ^{ns}
mmol L^{-1}	----------mg kg^{-1}----------		
0.00	0.66	1.37	75.22
0.25	0.81	1.73	88.89
0.50	1.07	1.92	93.78
1.00	1.20	2.13	99.78
1.50	0.89	1.92	91.95
2.00	0.93	1.69	91.03
C.V. (%)	15.90	24.97	21.29

**- significant at 1% probability. ^{ns} - non-significant.

A higher level of As in plants demonstrates the potential use of Si in soil remediation techniques, such as phytostabilization and phytoextraction. Interestingly, a reduction in tissue levels of As for the two highest doses was observed, showing an effect of decreased As absorption when high Si doses are applied.

The results imply that Si changes the forms of As absorbed and accumulated in different plant parts. The vegetative growth is not affected and no toxicity symptoms are observed, even with a higher As content in the tissue. The various chemical forms of As may represent different toxicity levels (JEDYNAK et al., 2012). The biotransformation of As^{3+} into the less toxic As^{5+} through oxidation, mainly using Fe and sulfates, is one of the mechanisms activated in both prokaryotic and eukaryotic microorganisms (HALTER et al., 2012).

Production of photosynthetic pigments

The addition of Si to the nutrient solution increased the levels of photosynthetic pigments (Table 8), proving the beneficial effects of this element in alleviating As stress. The levels of chlorophyll *a* and total were altered, whereas chlorophyll *d* (accessory pigment) did not vary with Si addition. Photosynthetic pigments are greatly responsible for the photosynthetic metabolism in plants; chlorophyll *a* is the main responsible for allowing this interaction between the capture of solar energy and the process initiated within the chloroplasts, whereas chlorophyll *b* acts as an accessory pigment in the process of electron transfer to chlorophyll *a* (CAIRES et al., 2009; BOHR, 2011).

Table 8. Photosynthetic pigments content as a function of Si doses in maize plants grown in a nutrient solution with Arsenic

Si doses	Chlorophyll content		
	A*	B ^{ns}	Total *
mmol L^{-1}	----------mg g^{-1}----------		
0.00	1.12	1.24	2.36
0.25	1.80	1.51	3.31
0.50	1.79	1.21	3.00
1.00	1.95	1.44	3.39
1.50	1.81	1.46	3.27
2.00	1.63	1.49	3.11
C.V. (%)	15.07	16.69	9.95

*- significant at 5% probability. ^{ns} - non-significant.

The results show that Si addition to the nutrient solution promotes a higher content of photosynthetic pigments for all applied Si doses, mainly to chlorophyll *a* and total; the highest levels for all pigments were found with 1 mmol L^{-1} Si.

Si was important in alleviating the visual symptoms of Mn toxicity on cucumber plants, reflecting an increase in the levels of photosynthetic pigments when 1 mmol L^{-1} was applied (FENG et al., 2009). An increase in the content of photosynthetic pigments was also observed after Si application to cucumber

plants under Cd stress (FENG et al., 2010). These authors observed that Si provided improvements in gas exchange, favoring a lower Cd accumulation in the leaves, reducing the damage to chloroplasts and protecting their ultrastructure. Reduced symptoms of As toxicity, even with higher levels of the element in leaves, was observed in this study, indicating that a decrease in As levels occurred where photosynthesis is most active, thus resulting in stress alleviation in leaf structures.

Chlorophyll fluorescence monitoring

Alleviation of As stress to the photosynthetic apparatus by Si application varied according to the used doses. There was a reduction in the spectrum emission at 1 mmol L^{-1} Si (Figure 16), even though this dose caused a greater As accumulation in plant leaves (Table 7). It is observed that Si doses greater than 1 mmol L^{-1} cause more stress than the presence of As, indicating the existence of an optimal dose for stress alleviation. This result corresponds to the best response of chlorophyll *a* and total (Table 8), which corroborates the findings. Increase in photochemical efficiency and electron transport of photosystem II were caused by the addition of 1 mmol L^{-1} Si to cucumber plants grown under Mn stress (FENG et al., 2009).

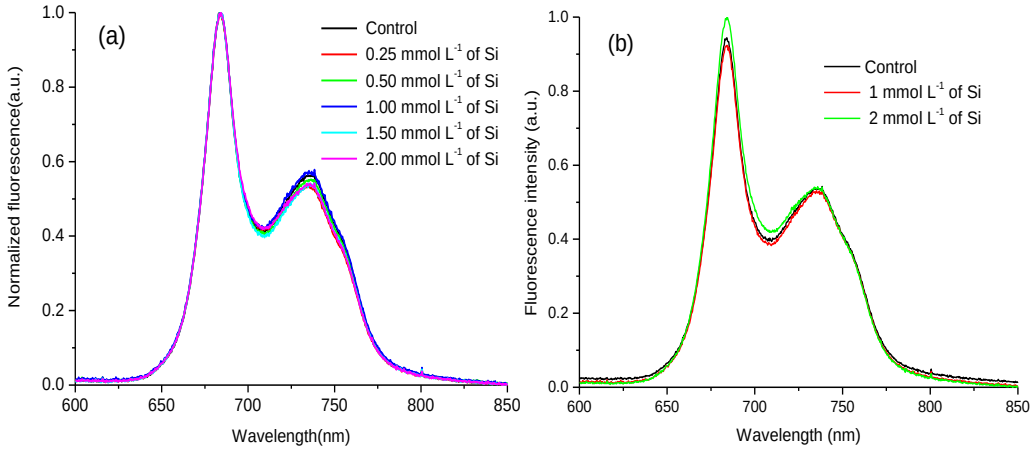

Figure 16. (a) Spectra of chlorophyll fluorescence in maize plants grown under different Si doses in a nutrient solution contaminated by As. (b) Maximum intensity of chlorophyll fluorescence for the highest Si doses in the nutrient solution.

Over time, this difference is much more explicit, showing the importance of the Fr/FFr ratio as the most sensitive parameter in the identification of As stress and its alleviation by Si (Figure 17). The difference in the ratio can be observed in the reading taken at five days of culture under As and Si doses. The reduction of the Fr/FFr ratio confirms Si beneficial effects to the photosynthetic apparatus of maize plants. As the control showed the highest Fr/FFr ratio, all Si doses were effective in alleviating stress.

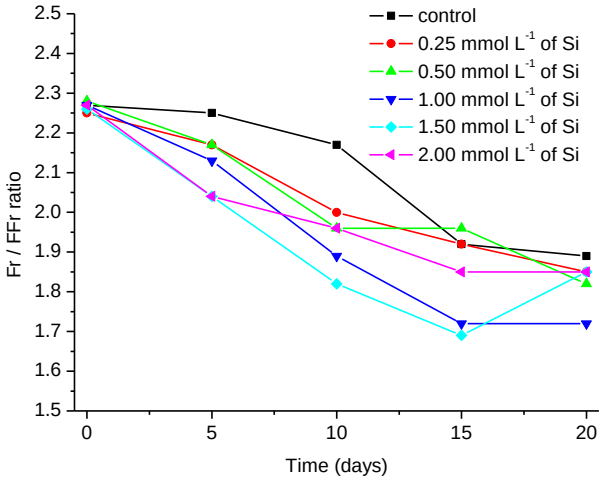

Figure 17. Spectra of chlorophyll fluorescence ratio as a function of growing time of maize plants under increasing Si doses, in a nutrient solution contaminated by As (Fr/FFr at F685 ηm and F735 ηm wavelengths, respectively).

The effects of Si on growth, photosynthesis and chlorophyll *a* fluorescence parameters in plants under Cd stress play an important role in the protection of the photosynthetic apparatus, since its application increases the quantum yield and the maximum effective quantum yield of the photosystem II (FENG et al., 2010). This result can be confirmed for As stress in maize plants.

Si was also effective in alleviating Cr toxicity and improving the photosynthetic parameters and the efficiency of chlorophyll fluorescence parameters. This is possible because Si has the ability to alleviate the damage caused to cell ultrastructure in leaves and roots (ALI et al., 2013). The chlorophyll fluorescence analysis suggests that Si alleviates Cd toxicity in rice plants under low stress levels, as Fo reduction, Fv/Fm and qP increase have

been observed, thus improving the efficiency on light use (NWUGO & HUERTA, 2008a).

Morphological differences between maize leaves with and without supplemental Si showed different results on Mn alleviation. The increased thickness of the epidermal layers suggests an important role for these cells in Mn tolerance, both in the genetically Mn tolerant and in the Si-induced tolerant (DONCHEVA et al. 2009). In the presence of Si, *Brassica* plants showed a more accelerated endoderm development, compared with plants grown only in the presence of Cd. This may have led to a lower Cd absorption and reduced toxicity, due to a suberin lamella formed in the endodermis of plants supplied with Si (VATEHOVÁ et al., 2012).

The results of this study confirm that Si is an important element in alleviating As stress in maize plants, since Si provided greater efficiency in the photosynthetic parameters and an increase in photosynthetic pigments levels in plants.

CONCLUSIONS

The use of Si to alleviate As stress in maize plants grown in a nutrient solution showed positive results when protecting the photosynthetic apparatus. Si alleviated the deleterious As effect, which resulted in a higher production of photosynthetic pigments in the leaves. The chlorophyll fluorescence analysis has proven to be a sensitive tool, thus the technique can be successfully employed in the study of Si effects on toxicity alleviation in plants. This is possible thanks to the positive effects of Si in protecting the photosynthetic apparatus, with the Fr/FFr ratio the recommended variable to identify temporal changes in plants. This technique has the advantage of being non-invasive and non-destructive, allowing its use over time in the evaluation of the same plant. Because Si application provided higher levels of As in plant tissue, using the element in studies on soil phytoremediation may be a promising choice.

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CONCLUSÕES GERAIS

O estudo da toxidez de Cd e As em plantas foi importante para comprovar o efeito fitotóxico destes dois elemento, além de possibilitar a comprovação da amenização da toxidez de Cd e As por Si em plantas de milho, sendo um estudo inédito para a utilização da razão Fr/FFr como parâmetro sensível na detecção e no monitoramento da toxidez de Cd e As, principalmente sobre o efeito positivo do Si na amenização do estresse causado por estes dois elementos fitotóxicos.

O Si é capaz de amenizar o estresse de Cd e As em plantas de milho, podendo ser utilizado como medida protetiva de plantas cultivadas sob estresse causado por estes elementos. Os resultados mostraram que o Si tem a capacidade de influenciar positivamente o metabolismo fotossintético das plantas, reduzir a absorção e translocação para a parte aérea e contribuir na proteção da cadeia trófica, além de possibilitar sua aplicação em programas de fitorremediação de solos contaminados por Cd e As.

A análise da fluorescência da clorofila é uma ferramenta promissora na avaliação do estresse causado por Cd e As em plantas de milho, sendo também sensível na detecção da amenização da toxidez de Cd e As favorecida por Si de plantas de milho cultivadas em solução nutritiva. A razão espectral obtido entre os picos de emissão espectral no vermelho e vermelho distante, obtidos na análise da fluorescência da clorofila é o parâmetro indicado no estudo de impactos ambientais causado por estes elementos, tendo como vantagem a possibilidade de análise ao longo do tempo e rápida obtenção dos resultados.