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Mitigação da toxicidade de Pb por Mn e Zn em plantas jovens do genótipo clonal de cacau CCN 51 cultivadas no solo: fotossíntese, metabolismo antioxidativo, nutrição mineral e expressão gênica.

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ILHÉUS – BAHIA- BRASIL
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Área de Concentração: Genética e Melhoramento Vegetal

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DEDICATÓRIA

"Dedico cada una de estas líneas escritas en este trabajo de investigación, a mis abuelos, Alfredo, Alberto e Ilia, los cuales no estarán de cuerpo presente al final de este trabajo, pero su energía siempre habitará en mí, a ellos les dedico este título, por el cual tanto luché, espero que donde sea que se encuentren, estén orgullosos de mí, así como yo lo estoy de ellos, los amo."

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O chumbo (Pb) é altamente tóxico para seres humanos, animais e vegetais, além de ser um elemento não essencial para as plantas. Tem sido observado a presença de Pb em amêndoas de cacau e produtos à base de cacau, que acarreta riscos potenciais à saúde humana pela ingestão de produtos contaminados. O acúmulo de Pb em amêndoas de cacau depende não apenas do genótipo, mas também do local geográfico onde se cultiva as plantas. Pois, a presença de Pb no solo está associado tanto à rocha que deu origem ao solo e quanto às ações antrópicas. Por outro lado, Mn e Zn são elementos essenciais para as plantas e participam como cofatores enzimáticos em diversas rotas metabólicas. Pb é absorvido pelo sistema radicular através de transportadores de cátions divalentes e compete com minerais essenciais divalentes como Mn, Zn, Fe, Cu, Ca e Mg. Logo, o aumento da concentração de Mn ou Zn no solo pode reduzir a absorção de Pb pelo sistema radicular e mitigar a sua toxicidade nas plantas. O presente trabalho teve como objetivo principal avaliar a influência de Mn e Zn na mitigação da toxicidade de Pb em plantas jovens do genótipo clonal do cacau CCN 51, cultivadas em solo com diferentes doses de Pb, Mn, Zn, Pb+Mn e Pb+Zn, por meio de respostas fisiológicas, bioquímicas, nutricionais e moleculares. Verificou-se, no presente trabalho, que as plantas jovens do genótipo clonal de cacau CCN 51 acumularam Pb, Mn e Zn nas raízes e folhas. Além disso, a absorção de Pb, Mn e Zn pelas raízes e seus transportes para a parte aérea promoveu alterações nas trocas gasosas foliares, na emissão de fluorescência da clorofila, no teor de prolina, no balanço nutricional, no metabolismo antioxidante e na expressão gênica das plantas. Por outro lado, as toxicidades de Pb, Mn e Zn ativaram os mecanismos de defesa das plantas, alterando a expressão gênica de *psbA*, *psbO*, *met* e *Tpr1* e as atividades das enzimas SOD, GPX, APX, CAT e PPO envolvidas na desintoxicação celular, por meio da eliminação do excesso de espécies reativas de oxigênio. Além disso, constatou-se que doses adequadas de Mn+Pb e Zn+Pb aplicadas no solo mitigaram a toxicidade de Pb nas plantas. A mitigação da toxicidade de Pb por Mn e Zn se deveu à redução da absorção de Pb pelo sistema radicular, evitando que Pb se acumulasse em níveis tóxicos nas raízes e folhas das plantas. Por outro lado, altas doses de Pb e Zn aplicadas isoladamente ou em conjunto no solo foram altamente tóxicas para as plantas, levando, em alguns casos, à morte. Entretanto, não se observou toxicidade de Mn em plantas jovens do genótipo clonal do cacau CCN 51, mesmo em doses elevadas no solo. Logo, a aplicação de doses adequadas de Mn ou Zn no solo podem ser utilizadas para mitigar a toxicidade de Pb em solos contaminados.

Palavras-chave: *Theobroma cacao*, *metais pesados*, *trocas gasosas foliares*, estresse *oxidativo*, qPCR.

ABSTRACT

Apraez Muñoz Jose Julian, Mitigation of Pb toxicity by Mn and Zn in young plants of the cacao clonal CCN 51 genotype grown in soil: photosynthesis, antioxidative metabolism, mineral nutrition and gene expression. Advisor: Alex-Alan Furtado de Almeida. Co-Advisor: Dário Ahnert.

Lead (Pb) is highly toxic to humans, animals and plants, as well as being a non-essential element for plants. Presence of Pb in cocoa beans and cocoa-based products has been observed, which poses potential risks to human health through ingestion of contaminated products. Accumulation of Pb in cocoa beans depends not only on the genotype, but also on the geographical location where the plants are grown, once the presence of Pb in the soil is associated both with the rock that gave rise to the soil and with the anthropic actions. On the other hand, Mn and Zn are essential for plants and participate as enzymatic cofactors in several metabolic pathways. Pb is uptake by the root system through divalent cation transporters and competes with essential divalent minerals such as Mn, Zn, Fe, Cu, Ca and Mg. Therefore, increasing the concentration of Mn or Zn in the soil can reduce the Pb uptake by root system and mitigate its toxicity in plants. The present work had as main objective to evaluate the influence of Mn and Zn in the mitigation of Pb toxicity in young plants of the CCN 51 cacao clonal genotype, grown in soil with different doses of Pb, Mn, Zn, Pb+Mn and Pb+Zn, through physiological, biochemical, nutritional and molecular responses. It was found, in the present work, that young plants of the clonal cacao genotype CCN 51 accumulated Pb, Mn and Zn in the roots and leaves. In addition, the uptake of Pb, Mn and Zn by the roots and its transports to the aerial part promoted changes in leaf gas exchange, chlorophyll fluorescence emission, proline content, nutritional balance, antioxidant metabolism and gene expression of the plants. On the other hand, the toxicities of Pb, Mn and Zn activated the defense mechanisms of plants, altering the gene expression of *psbA*, *psbO*, *met* and *Tpr1* and the activities of the enzymes SOD, GPX, APX, CAT and PPO involved in the cell detoxification, through elimination of excess of reactive oxygen species. In addition, it was found that adequate doses of Mn+Pb and Zn+Pb applied to the soil mitigated the toxicity of Pb in plants. The mitigation of Pb toxicity by Mn and Zn was due to the reduction of Pb uptake by the root system, preventing Pb from accumulating in toxic levels in the roots and leaves of plants. On the other hand, high doses of Pb and Zn applied alone or together in the soil were highly toxic to plants, leading, in some cases, to death. However, no Mn toxicity was observed in young plants of the clonal cacao CCN 51 genotype, even at high doses in the soil. Therefore, the application of adequate doses of Mn or Zn in the soil can be used to mitigate the toxicity of Pb in contaminated soils.

Keywords: *Theobroma cacao*, heavy metals, leaf gas exchange, oxidative stress, *qPCR*.

1 – INTRODUÇÃO GERAL

O cacau (*Theobroma cacao* L.) é uma espécie lenhosa típica de clima tropical, diplóide (2n= 20), preferencialmente alógama, perene. Dentre as 22 espécies que compõem o gênero, apenas o cacau e o cupuaçu (*Theobroma grandiflorum* L.) são explorados comercialmente no Brasil (ALMEIDA; VALLE, 2007). Em condições de cultivo, geralmente chega a 5 m de altura, podendo atingir até 20 m quando em condições silvestres (BARTLEY, 2005). As importações mundiais de grãos de cacau totalizaram US $ 9.533 milhões em 2017, registrando um crescimento anual composto de 2,5% entre 2012 e 2017 (ICCO, 2018). Esta espécie é explorada principalmente para a produção de chocolate; entretanto, as amêndoas (sementes fermentadas e secas) podem ser utilizadas, também, para a produção de liquor, cosméticos, bebidas, geléia, cremes e sucos (ALMEIDA; VALLE, 2007). Amêndoas de cacau são fontes de carboidratos, gorduras, proteínas, minerais, flavonoides e vitaminas, que são as matérias-primas para uma economia industrial competitiva e multibilionária. O cacau é cultivado em plantações comerciais por toda parte do mundo tropical (DANTAS; GUERRA, 2010). As Américas produzem quase 13% da produção global de cacau, e o Brasil está incluso entre os principais países produtores no ranque mundial (ICCO, 2018).

A poluição do solo e da água por metais pesados é um grave problema ambiental. Os metais pesados são constituintes naturais da litosfera e ocorrem naturalmente no solo como elementos raros, cujos balanços dos ciclos geoquímicos e bioquímicos foram drasticamente alterados pela atividade humana. Neste cenário, as práticas agrícolas, deposição de lixo e a metalurgia contribuíram para a sua disseminação no meio ambiente (DALCORSO et al., 2013; ALMEIDA et al., 2013). Estas e outras atividades promovem um aumento na concentração desses elementos metálicos na biosfera, que, diferentemente da maioria dos poluentes, não são biodegradáveis e persistem no meio ambiente (PILON-SMITS, 2005). Além disso, são considerados como o principal grupo de poluentes inorgânicos e causam sérios problemas para os seres vivos, quando presentes na atmosfera, solo e água, incluindo a diminuição da atividade microbiana, fertilidade do solo, e produtividade das colheitas (YANG et al., 2005).

Os metais pesados constituem um grupo de elementos metálicos de densidade superior a 5 g cm^{-3}. Alguns deles são essenciais para o crescimento e o desenvolvimento normal das plantas, porque são integrantes de muitas enzimas e proteínas (KRAMER et

al., 2007). No entanto, concentrações elevadas de ambos os metais pesados essenciais e não essenciais conduzem a sintomas de toxicidade, afetando os processos de crescimento e desenvolvimento das plantas. Fitotoxicidade de metais pesados pode resultar de alterações de inúmeros processos fisiológicos causados em nível celular/molecular, incluindo a inativação de enzimas, o bloqueio de grupos funcionais de moléculas metabolicamente importantes, deslocando ou substituindo elementos essenciais e rompendo a integridade da membrana (DALCORSO et al., 2013; ALMEIDA et al., 2015).

Alguns metais como cobalto (Co), cobre (Cu), ferro (Fe), manganês (Mn), molibdênio (Mo), níquel (Ni) e Zinco (Zn) são necessários à vida em pequenas quantidades, entretanto, concentrações excessivas podem ser prejudiciais ao organismo. Já outros metais como chumbo (Pb), cádmio (Cd), mercúrio (Hg) e arsênico (As) não apresentam funções conhecidas nos seres vivos. Deste modo, mesmo em baixas concentrações podem causar danos em plantas e animais (CHIBUIKE; OBIORA, 2014). A absorção de Pb reduz a assimilação de outros elementos essenciais às plantas, como cálcio (Ca), Zn, Cu e potássio (K), que estão associados às proteínas estruturais e, ou a ativação enzimática e abertura e fechamento estomático, pela competição por canais de transportadores (KUMAR; KUMARI, 2015). A inibição da fotossíntese promovida pela toxicidade de Pb é bem conhecida. O dano ocorre de forma indireta, pois resultam da distorção da ultraestrutura do cloroplasto pela afinidade que Pb tem pelo ligante nitrogênio (N) e enxofre (S) de proteínas das membranas tilacoides, da obstrução do sistema de transporte de elétrons, do fechamento estomático inadequado, do aumento da atividade da clorofilase, do distúrbio no *status* hídrico etc. (HAN et al., 2013). Entretanto, há pouca relação com o teor de pigmentos fotossintéticos, sendo a clorofila **b** mais sensível do que a clorofila **a** (POURRUT et al., 2011).

Chumbo é um dos metais tóxicos mais presentes no ambiente (NORDBERG, 2009). Este elemento metálico ocorre naturalmente no solo, associado a outros elementos. Entretanto, as emissões antropogênicas têm aumentando as concentrações de Pb no solo, por meio de atividades de mineração, combustíveis fósseis, indústrias metalúrgicas e o uso intensivo de fertilizantes fosfatados (DIAS et al., 2013). Como Pb não pode ser degradado e a sua lixiviação é lenta, as concentrações nos solos aumentam de forma constante (MOULIS; THEVENOD, 2010). Além do teor total de Pb do solo, o pH, o teor de matéria orgânica e a textura do solo influenciam a disponibilidade de Pb

para as plantas (MOULIS; THEVENOD, 2010). Além disso, Pb é conhecido pela alta mobilidade no solo e por não se ligar fortemente à matéria orgânica, logo, é considerado como um dos elementos mais tóxico para os organismos vivos (GALLEGO et al., 2012). As plantas, na presença de Pb, apresentam efeitos genotóxicos e citotóxicos, entretanto, ao longo do processo evolutivo, foi desenvolvido mecanismos de tolerância ao excesso de metais (HASAN et al., 2009).

Chumbo existe em várias formas, com diferentes níveis de solubilidade e biodisponibilidade – dissolvido, com capacidade de trocas catiônicas com componentes orgânicos e inorgânicos, em componentes estruturais do solo, e como precipitados insolúveis (SHAHID et al., 2012). A assimilação de elementos metais traços pelas plantas varia muito em função das condições do solo. Altas concentrações de metais no solo nem sempre indicam níveis correspondentemente elevados destes metais nas plantas. Isto depende, por sua vez, de vários fatores, como pH, capacidade de troca catiônica, teores de matéria orgânica e argila no solo, entre outros (ALBASEL; COTTENIE, 1985).

As plantas absorvem Cd e Pb através de canais de cátions, competindo com os nutrientes essenciais como Fe, Mn, Ca, K, Zn, Cu e magnésio (Mg) (DalCORSO et al., 2013). A maior parte do Pb absorvido pelas plantas é acumulado nas raízes e apenas uma pequena fração é translocada para a parte aérea (PATRA et al., 2004). As toxicidades de Cd e Pb causam inibição e anormalidades no crescimento geral de muitas espécies vegetais, interferindo em processos fisiológicos e bioquímicos, inibindo a fotossíntese e a respiração e causando danos irreversíveis nas estruturas celulares (DIAS et al., 2013). Além disso, podem causar estresse oxidativo em plantas, embora estes elementos não participem ativamente das reações de Fenton – que produzem as espécies reativas de oxigênio (ERO), interferindo no equilíbrio entre a produção de ERO e o metabolismo antioxidativo (ZHOU et al., 2016).

Fatores bióticos como espécies vegetais, genótipos, atividade de raízes, padrões de enraizamento e microorganismos da rizosfera também podem afetar a disponibilidade de Cd para as plantas (HE et al., 2015). Vários estudos têm mostrado diferenças na absorção e distribuição de metais pesados na planta, tanto entre espécies quanto entre genótipos da mesma espécie (ZHOU et al., 2016). Particularmente para Cd, o efeito de cultivares na sua absorção tem sido demonstrado para muitas espécies, mas a maioria dos estudos foi realizada com culturas anuais (ARAO et al., 2008).

Os elementos minerais, como Zn e Mn, também são absorvidos através de canais de cátions (DALCORSO et al., 2013), de preferência como cations divalentes. Cádmio e Pb não são homogeneamente distribuídos entre diferentes tecidos em plantas. Na maioria das plantas, as concentrações mais elevadas são encontradas nas raízes, quantidades menores nas folhas e menores concentrações nas sementes (CLEMENS et al., 2013). A menor concentração de Cd nas sementes pode ser explicada pela translocação restrita de Cd das raízes, onde se acumula, às partes aéreas da planta (GRANT et al., 1997). As sementes em desenvolvimento só podem ser atingidas através do floema, que transloca nutrientes e outros compostos solúveis a partir de fontes (folhas) aos drenos metabólicos (frutos em crescimento) (BERSINSKY et al., 2008). Portanto, para alcançar a semente em desenvolvimento, o Pb é transferido do xilema para o floema ou remobilizado das folhas (BERSINSKY et al., 2008). Assim, além da retenção radicular e da atividade de carregamento do xilema, a transferência do xilema para o floema – que é sugerida ocorrer nos nós – é um processo chave para determinar a transferência de Pb para as sementes (CLEMENS, 2016).

Pesquisas recentes têm demonstrado contaminação de amêndoas de cacau por Cd e Pb em função, principalmente, do solo de origem e de sua contaminação pelo uso de fertilizantes fosfatados e fungicidas. Entretanto, a maioria das informações sobre a toxicidade e tolerância a estes elementos tóxicos é oriunda de plantas cultivadas em soluções nutritivas, cujo elemento está 100% disponível (CLEMENS, 2016).

Chumbo é absorvido pelo cacau do solo. Sua presença no solo é resultado de uma combinação de processos naturais e antrópicos. Os processos naturais incluem o intemperismo de rochas, atividade vulcânica, incêndios florestais, erosão e deposição em sedimentos de rios, enquanto os processos antrópicos incluem mineração e atividades industriais, bem como práticas agrícolas, como irrigação e fertilização. Em solos de cultivo de cacau, é provável que fontes naturais e antropogênicas participem do aumento de seu teor de Pb, com a importância relativa de diferentes fontes dependendo da área (CLEMENS, 2016).

Nem todo o Pb presente no solo está biodisponível para as plantas de cacau; ou seja, prontamente disponível para absorção pelas raízes. Enquanto níveis mais altos de teor total de Pb implicam em maior potencial de absorção deste elemento pelas plantas de cacau, altos níveis de Pb foram relatados em grãos de cacau de plantas crescendo em solo com teor de Pb total relativamente baixo. A biodisponibilidade do chumbo nas

plantas é influenciada por múltiplas propriedades do solo: pH, conteúdo de matéria orgânica, textura e mineralogia do solo, capacidade de troca catiônica, condutividade elétrica, conteúdo de macro e micronutrientes e presença de microrganismos. Por outro lado, a alteração de algumas dessas propriedades pode levar à redução da biodisponibilidade de Cd para as plantas de cacau (CLEMENS, 2016).

A seleção de cacau clonal foi identificada como sendo a forma mais rápida e eficaz para obter variedades melhoradas, por meio da fixação de genes desejáveis, especialmente aqueles relacionados com resistência. A partir de 2002, clones de cacau autocompatíveis e resistentes à vassoura de bruxa foram liberados aos produtores de cacau da região sul da Bahia, Brasil. Estes clones representam algumas seleções em fazenda, bem como seleções das populações-base melhoradas no Centro de Pesquisas do Cacau (CEPEC) e os clones equatorianos CCN 10 e CCN 51 (MONTEIRO; AHNERT, 2012). O clone CCN 51 foi selecionado no Equador, resultante do cruzamento do híbrido IMC 67 x ICS 95 com um genótipo equatoriano conhecido como “Canelos”. Trata-se de um clone que tem sido amplamente cultivado no Equador e está sendo usado como progenitor em muitos programas de melhoramento e seleção de cacau em outros países. Além disso, é altamente valorizado devido a sua alta produtividade, resistência a doenças, além de alta concentração de gordura em suas amêndoas (BOZA et al., 2014).

A presença destes elementos metálicos nestes produtos, oriundos de fontes naturais e, ou antropogênicas, pode ser proveniente da absorção radicular das plantas de cacau cultivadas em solos contaminados (DEVI et al., 2016) ou da contaminação durante processos de fabricação (YANUS et al., 2014). Em geral, Pb se encontra nos sólidos de cacau e manteiga de cacau não desnatados (YANUS et al., 2014). Embora os resultados dos estudos não permitam conclusões claras, parece que as práticas pós-colheita, como fermentação, secagem, torra e joeiramento podem afetar o teor de Pb das amêndoas de cacau (CLEMENS, 2016).

A exposição às altas concentrações de Pb está associada a uma ampla gama de impactos, incluindo vários efeitos comportamentais, mortalidade (principalmente de efeitos cardiovasculares), função renal prejudicada e hipertensão (EFSA, 2012). Para as crianças, concentrações elevadas de Pb no sangue estão associadas ao comprometimento do neurodesenvolvimento, especificamente a redução do quociente de inteligência ([ATSDR] *Agency for Toxic Substances Disease Registry* 2007). A *Food and Drugs Administration* (FDA) – USA emitiu um documento de orientação, em relação à

presença de Pb em doces que podem ser consumidos por crianças, o qual recomenda uma concentração máxima de 0,1 mg Pb kg^{-1}, incluindo doces com chocolate (2006a). Dados da indústria de chocolate dos USA afirmam que o chocolate amargo contém concentrações mais elevadas de Pb do que o chocolate ao leite (FDA, 2006b), provavelmente, devido às concentrações mais altas de sólidos de cacau. Embora não haja uma legislação que limite a concentração máxima permitida de Pb em cacau e seus subprodutos, FDA publicou um *Codex* que recomenda práticas agrícolas e de fabricação para prevenir e reduzir a contaminação de Pb em alimentos, incluindo recomendações aplicáveis ao cacau em pó e aos produtos de chocolate (CAC, 2004).

A exposição a concentrações elevadas de Cd pode estar associada a efeitos adversos à saúde humana, incluindo rins e ossos, e pode resultar em câncer (ATSDR, 2012). O Comitê Conjunto de Especialistas em Aditivos Alimentícios da Organização Mundial da Saúde/Organização das Nações Unidas para Agricultura e Alimentação (JECFA) avaliou as concentrações de Cd em cacau e produtos de cacau, além da concentração de Cd em outros alimentos (FAO/WHO, 2013). A dieta potencial para consumo de produtos de cacau e seus derivados contendo Cd foi estimada em 30-69% para ingestão mensal tolerável (25 μg/kg de peso corporal) para adultos e 96% para crianças de 0,5 a 12 anos. O comitê observou que a exposição ao Cd, pelo consumo de cacau e produtos derivados, foi, provavelmente, superestimada e não considerava preocupante (FAO/WHO, 2013).

O presente trabalho teve como objetivo principal avaliar a influência de Mn e Zn na mitigação da toxicidade de Pb em plantas jovens do genótipo clonal de cacau CCN 51, cultivadas em solo com diferentes doses de Pb, Mn, Zn, Pb+Mn e Pb+Zn, por meio de respostas fisiológicas, bioquímicas, nutricionais e moleculares. Além disso, teve como objetivos específicos (i) avaliar as trocas gasosas e a emissão de fluorescência da clorofila em nível foliar; (ii) determinar a concentração de macro e micronutrientes minerais e de Pb em raízes e folhas das plantas; (iii) determinar a atividade de enzimas envolvidas no metabolismo antioxidativo (SOD, APX, GPX, CAT e PPO) e o teor de prolina em folhas e raízes; e (iv) avaliar a expressão de genes associados à biossíntese de proteínas intrínseca (*psbA*) e extrínseca (*psbO*) ao fotossistema 2 (PS2) da fase fotoquímica da fotossíntese e de proteínas envolvidas na quelação de metais pesados nas folhas das plantas de cacau. A espécie *T. cacao* tem demonstrado ser exigente em Mn^{+2} e Zn^{+2}, que são absorvidos pelo sistema radicular, principalmente na forma divalente.

Como Pb^{+2} compete também por transportadores de íons divalentes, durante o processo de absorção efetuado pelo sistema radicular, a maior disponibilidade de Zn ou de Mn no solo contaminado por Pb pode mitigar a toxicidade deste elemento metálico pela maior absorção de Mn^{+2} ou Zn^{+2} em detrimento de Pb^{+2}.

2 - REVISÃO DE LITERATURA

2.1 - Toxicidade de metais em plantas.

As plantas, sendo organismos sésseis, estão expostas a diversos tipos de estresse biótico e abiótico. O estresse por metais tóxicos, sendo um estresse abiótico, tem recebido atenção diferenciada, porque afeta o crescimento, o desenvolvimento e a produção da planta. Além disso, estes metais tóxicos podem ser incorporados na cadeia alimentar e afetar os animais, inclusive o homem (DALCORSO et al., 2013). Os metais tóxicos estão naturalmente presentes no solo, entretanto as atividades geológicas e antropogênicas aumentam a concentração desses elementos, atingindo valores prejudiciais. A concentração total dos metais tóxicos no solo não está totalmente disponível para a absorção pelas plantas. Entre os íons metálicos tóxicos, alguns são mais móveis no solo e estão mais disponíveis para as plantas, como o Cd, enquanto outros apresentam menor mobilidade, a exemplo de Pb (THAKUR et al., 2016). Entretanto, a absorção de elementos metálicos tóxicos pelas plantas varia muito em função das condições físicas e químicas do solo.

O pH baixo do solo aumenta a disponibilidade do metal, já que o íon hidrogênio tem maior afinidade pelas cargas negativas dos coloides e compete com íons metálicos, deixando-os livres (PRASAD, 2013). Já o alto teor de matéria orgânica imobiliza os metais através de ligações com os ácidos húmicos e fúlvicos. Além disso, a maior capacidade de troca catiônica aumenta as possibilidades de ligações com cargas catiônicas (PRASAD, 2013). Todos estes fatores contribuem para a maior retenção do metal ao solo, reduzindo a absorção pela planta e, consequentemente, os efeitos tóxicos sobre o vegetal (CHIBUIKE; OBIORA, 2014).

Metais iônicos tóxicos penetram nas células utilizando os mesmos processos de absorção de micronutrientes essenciais iônicos. A quantidade absorvida pela planta depende da concentração e da especiação do metal na solução do solo, juntamente com o

seu movimento sucessivo do solo para o tecido radicular e da raiz para a parte aérea (PATRA et al., 2004). A translocação destes metais iônicos para a parte aérea depende da espécie vegetal, do metal envolvido e das condições ambientais (GILL, 2014). Os efeitos genotóxicos dependem do estado de oxidação do metal, da concentração e da duração de exposição, e estes são mais pronunciados em concentrações elevadas e após um maior tempo de exposição (COSIO et al., 2005).

Em solos de cultivo, é comum o uso de fertilizantes orgânicos e inorgânicos, para aumentar o crescimento e a produção das plantas. Esta atividade antrôpica é uma das principais fontes de metais pesados na agricultura, bem como o uso de pesticidas. Embora as concentrações de metais pesados sejam baixas nos fertilizantes e nos pesticidas, o uso contínuo pode aumentar as concentrações de Cd e Pb no solo, causando danos aos cultivos agrícolas (GILL, 2014).

Estresses abióticos podem ser considerados como as mais graves condições adversas que as plantas podem enfrentar. A gravidade de qualquer estresse abiótico pode ser intensificada em plantas, pois são organismos sésseis e, portanto, não podem se deslocar e evitar a perturbação do fator estressor, que interfere no seu metabolismo (SUZUKI et al., 2014). Como consequência, a exposição ao estresse abiótico, há um desencadeadamento do acúmulo excessivo de espécies reativas de oxigênio (ERO), levando a uma condição de estresse oxidativo (AZEVEDO et al., 2011).

O sistema de defesa antioxidante em plantas ajuda a acumular e tolerar os efeitos colaterais de altas concentrações internas de metal. O sistema antioxidante é ativado para combater os efeitos deletérios causados por ERO em função do estresse (AZEVEDO et al., 2011). Por outro lado, os metais podem interferir com nutrição mineral e alterar a concentração e a composição dos nutrientes das plantas. Além disso, os metais podem alterar também a conformação de proteínas, incluindo transportadores ou outras proteínas reguladoras (CLEMENS, 2013).

2.2 - Toxicidade de Pb em plantas.

O chumbo é encontrado naturalmente na crosta terrestre, entretanto, a sua concentração no solo vem aumentando por meio de ações antropogênicas, com a sua utilização em indústrias, tais como fabricação de baterias, mineração e fundição (CAUSSY et al., 2003); como resíduos urbanos e industriais; como fertilizantes e como

pesticidas e aditivos de combustíveis (WANG et al., 2015). O Pb no solo existe em várias formas com diferentes níveis de solubilidade e biodisponibilidade – dissolvido; com capacidade de troca catiônica com componentes orgânicos e inorgânicos; em componentes estruturais do solo; e como precipitados insolúveis (SHAHID et al., 2012).

Metais tóxicos estão entre as principais classes de agentes de estresse para organismos vivos, especialmente devido aos seus crescentes usos em atividades antrópicas (SOUZA et al., 2014). Consequentemente, a sua liberação no ambiente tem um potencial real para causar sérios danos aos animais e aos seres humanos, caso entrem na cadeia alimentar (GRATÃO et al., 2015). Tal potencial acúmulo de um metal tóxico é, portanto, um aspecto que deve ser levado sempre em consideração. A sua distribuição em tecidos/órgão vegetal, durante diferentes estágios de desenvolvimento, nunca deve ser negligenciada, especialmente quando se trata de cultivo alimentar. Seus efeitos podem afetar drasticamente a sinalização celular e causar danos irreversíveis aos sistemas (GILL et al., 2012).

Numerosos estudos foram realizados sobre a diversidade de espécies e a formação de vegetação metálica. Estes estudos mostraram que a riqueza e a composição de espécies vegetais mudaram drasticamente sob a influência da contaminação do solo com metais pesados. As pesquisas se concentraram principalmente em sítios abertos e metálicos, onde a sucessão de plantas estava nos estágios iniciais e onde as pastagens eram realizadas. Havia, há décadas atrás, lixões poluídos de escórias e objetos de pedras residuais (MAPAURE et al., 2011; VOLLAND et al., 2014; POLLE et al. 2013; GILL et al. 2012; QURESHI et al. 2010), ou lixões mais antigos (mais de cem anos), onde difíceis condições edafoclimáticas inibiram o processo de sucessão nos estágios iniciais (WOCH et al., 2016). Há, ainda, escassez de pesquisas ecológicas sobre os fatores que afetam as comunidades complexas, em sucessão avançada, como florestas decíduas desenvolvidas em locais com metal (WOCH et al., 2017).

O acúmulo em excesso de Pb nas plantas pode interferir profundamente em uma série de processos fisiológicos, como a fotossíntese (FENG et al., 2010) e a respiração (VOLLAND et al., 2014), a absorção, o transporte e a assimilação de nutrientes minerais (NEDJIMI; DAOUD, 2009) e a absorção de água (POLLE et al., 2013). Além disso, o estresse de Pb altera genes (IRFAN et al., 2013) e a expressão proteica, induz ou inibe enzimas, aumenta o acúmulo de ERO, promove peroxidação lipídica e perturba o metabolismo (SEMANE et al., 2010).

Pb no solo pode induzir o estresse hídrico nas plantas, diminuindo a condutância estomática, a taxa de transpiração e o teor relativo de água na folha (DOMÍNGUEZ et al., 2011). Este é o resultado de danos fisiológicos promovidos pela diminuição dos espaços intracelulares e da quantidade de cloroplastos, bem como pelo aumento da célula (SANDALIO et al., 2001). Além disso, Pb também afeta a permeabilidade da membrana plasmática, causando uma redução no teor de água das plantas (FERNANDEZ et al., 2013). Polle et al. (2013) relataram que folhas de *Populus euphratica* apresentaram perda de água e murchamento sob estresse por Pb. A redução da embebição de água também foi relatada para as sementes de *Sorghum bicolor* (KURIAKOSE; PRASAD, 2008) e *Pisum sativum* (SIDDIQUI et al., 2009) expostas a Pb. Sun et al. (2013) mostraram que as células de *P. euphratica* tratadas com Pb exibiram uma clara retração do citoplasma, indicando perturbação do balanço hídrico. Além disso, Pb pode reduzir a área de superfície para absorção de água, inibindo o crescimento de pelos radiculares (GOUIA et al., 2003).

Pb pode se ligar competitivamente aos sítios essenciais de ligação de Ca no PSII, durante a fotoativação do sistema de oxidação da molécula de água (FALLER et al., 2005), e inibir diretamente a evolução fotossintética do oxigênio (PAGLIANO et al., 2006). Um número reduzido de grãos de amido e grana intacta, bem como tamanho reduzido de cloroplastos foram observados em plantas de *Sedum alfredii* (JIN et al., 2008), *P. sativum* (SANDALIO et al., 2001) e *Picris divaricata* (YANG et al., 2010), tratadas com 400, 50 e 75 mmol Pb L^{-1}, respectivamente, tendo $PbCl_2$ como fonte de Pb. Além disso, foi relatado que os grãos de amido granular acumulados e os grãos de amido modificados se ligam aos metais pesados, incluindo Cd (HAO et al., 2009). Além disso, verificou-se também que Pb pode afetar adversamente o teor de clorofila em *Pentas lanceolada* (CHANG et al., 2013). De acordo com Baryla et al. (2001) e Chen et al. (2008), Pb interfere na replicação dos cloroplastos, evidenciada pela diminuição das leituras do medidor de clorofila. A degradação de clorofilas ou a inibição de sua biossíntese tem sido proposta como responsável pela redução do crescimento das plantas submetidas ao estresse por Pb (WAN et al., 2012).

A diminuição do teor de clorofila induzida por Pb tem sido atribuída à redução da síntese de clorofila (SMIRI et al., 2009) ou ao aumento da degradação enzimática (QURESHI et al., 2010). Efeitos indiretos de Pb sobre o teor de clorofila, por meio da indução de deficiências de micronutrientes, também foram relatados. Além disso,

numerosos efeitos de Pb na fotossíntese se assemelham aos da deficiência de Fe e são caracterizados pela inibição e desorganização dos complexos clorofila-proteína, por meio da formação de um complexo de clorofila-Pb (KUPPER et al., 2007). Qureshi et al. (2010) relataram que Pb, em condições limitantes de Fe, poderia ser mais devastador para os complexos pigmento-multiproteínas existentes nas membranas tilacóides, uma vez que poderia danificar a maioria de seus componentes, resultando na perda de coordenação entre eles e afetando adversamente a homeostase de cloroplasto em *Brassica juncea*. No entanto, a presença de Fe auxiliou as plantas na supressão do estresse oxidativo e na retenção de cloroplastos e clorofilas em condições de estresse de Pb (QURESHI et al., 2010). Além disso, Volland et al. (2014) relataram também que a diminuição no teor de clorofila nas plantas tratadas com Pb foi atribuída ao fato de que o estresse de Pb reduziu a expressão do transportador de citrato FRD3 no parênquima do xilema radicular, levando à diminuição da translocação de Fe para a parte aérea.

O chumbo tem demonstrado causar graves distúrbios no crescimento, na morfogênese e nas atividades fotossintética e respiratória em *Micrasterias denticulata* (VOLLAND et al., 2014). A perturbação no metabolismo respiratório é uma causa contributiva dos efeitos deletérios do Pb na germinação de sementes (SMIRI et al., 2009). Pb interfere na atividade das enzimáticas do ciclo de Krebs (BANSAL et al., 2000) e nas cadeias de transporte de elétrons (BANSAL; SHARMA, 2000) em cotilédones, durante a germinação de sementes de *P. sativum*. Smiri et al. (2009) relataram que o estresse por Pb alterou as atividades de enzimas respiratórias, como as desidrogenases do malato e do succinato, as redutases da nicotinamida adenina dinucleotídeo (NADH), do succinato e do citocromo **c**, a oxidase do citocromo **c**, as desidrogenases da nicotinamida adenina dinucleotídeo fosfato (NADPH), da glicose-6-fosfato, do 6-fosfogluconato e a desidrogenase alcoólica no eixo embrionário de sementes de *P. sativum*.

O chumbo pode alterar também as características anatômicas e estruturais das células, o que é considerado o pior efeito tóxico de Pb (KUPPER et al., 2000). Shah e Dubey (1995) observaram baixo índice mitótico, divisão celular, proliferação celular e aberrações cromossômicas em várias espécies vegetais submetidas ao estresse por Pb. Além disso, números aumentados de nucléolos e vacúolos, citoplasma condensado, número reduzido de mitocôndrios, plasmólise, vacúolos aumentados, cloroplastos

desorganizados e envelope nuclear rompido foram encontrados nas células das raízes e folhas de diferentes cultivos em condições de estresse por Pb (LIU; KOTTKE, 2004).

A capacidade de detectar alterações genéticas significativas em plantas expostas a diferentes concentrações de Pb, antes do início dos efeitos fisiológicos, pode servir como um biomarcador molecular útil para a detecção precoce da exposição ao Pb e como indicador de efeitos biológicos relacionados (LIU et al., 2012). Numerosos estudos mostraram que a genotoxicidade de Pb está diretamente relacionada ao seu efeito na estrutura e função do DNA, tais como mutações pontuais, pequenas inserções e deleções, rearranjos, mudanças de ploidia, quebras de cadeia simples e dupla, substituições de bases, oxidação de bases, e até mesmo adutos volumosos em loci de DNA específico em organismos (LIU et al., 2012).

Pb é um elemento móvel e tem uma alta solubilidade em água, portanto, pode ser prontamente absorvido pelo sistema radicular das plantas e, eventualmente, suprimir o crescimento das plantas (GROPPA et al., 2012). Por outro lado, Pb é bastante tóxico para as plantas, uma vez que afeta muitos parâmetros fisiológicos e bioquímicos, como crescimento, fotossíntese, nutrição e *status* hídrico (MICHALACK, 2006). Pb tem alta afinidade para os grupos tiol e, portanto, afeta diretamente os grupos sulfidrila nas proteínas, o que leva à inibição da atividade ou à ruptura da estrutura (SHAH et al., 2006).

2.3 - Toxicidade de Pb em *T. cacao.*

A espécie *T. cacao* tem um alto valor econômico agregado às amêndoas, provenientes da fermentação das sementes de seus frutos, por causa de sua utilização na produção de manteiga de cacau e chocolate. Embora não sejam os únicos produtos comerciais extraído de suas amêndoas, sendo utilizadas também em indústrias de cosméticos e farmacêuticos e na fabricação de gêneros alimentícios, como geleias, bebidas e sorvetes (ALMEIDA; VALLE, 2007). Trata-se de uma espécie lenhosa típica de clima tropical, diploide (2n= 20), preferencialmente alógama, perene, nativa da região de floresta úmida da América do Sul e a única, dentre as 22 espécies do gênero, explorada comercialmente em larga escala. Pertence a família Malvaceae (ALVERSON et al., 1999), que contém cerca de 75 gêneros e, aproximadamente, 1.500 espécies, possui distribuição cosmopolita, predominando nos trópicos (SOUNIGO et al., 2003).

O gênero *Theobroma* é considerado como de origem exclusivamente neotropical, com dispersão natural em florestas úmidas, estendendo-se da bacia amazônica até o sul do México, entre as latitudes 18°N e 15°S (CUATRECASAS, 1964). Do seu provável centro de origem, na região do alto Amazonas (CHEESMAN, 1944), espalhou-se em duas principais direções, o que resultou nos dois principais grupos raciais: o 'Crioulo', cultivado na Venezuela, na Colômbia, no Equador, no norte da América Central e no México; e o 'Forasteiro', no norte do Brasil e nas Guianas (SOUNIGO et al., 2003). Um terceiro grupo, denominado 'Trinitário', também é apresentado por alguns autores como originário do cruzamento natural entre 'Crioulo' e 'Forasteiro' (MOTAMAYOR, 2001). A maior parte (85%) da produção mundial de cacau provém do grupo 'Forasteiro', sendo este predominante também nas plantações brasileiras. A espécie *T. cacao* foi introduzida no sul da Bahia, Brasil, em 1746, procedente do estado do Pará, Brasil, passando a ser cultivado inicialmente no município de Canavieiras e, posteriormente, em vários outros municípios do sul do estado, onde esses materiais genéticos receberam a denominação de 'Cacau Comum' da Bahia (LEAL, 2004).

A produção de cacau possui grande relevância na economia mundial, envolvendo muitos países no cultivo, comercialização e consumo. Os países produtores de cacau estão concentrados principalmente nas regiões tropicais dos continentes da África, América Central e do Sul, Ásia e Oceania (ICCO, 2017). Em 2017/2018, a África foi responsável por 74% da produção mundial, enquanto as Américas por 16% e Ásia e a Oceania por 10% (ICCO, 2016). Atualmente, no *ranking* dos países que cultivam *T. cacao*, encontra-se a Costa do Marfim como o principal produtor, que contribuiu com 39,3% da produção mundial, em seguida tem-se Gana (20,6%), Indonésia (8,3%), Camarões (6,3%), Equador (5,7%) e Nigéria (4,8%) (ICCO, 2016). O Brasil se posicionou no sétimo lugar, produzindo 135 mil toneladas, o que representa 3,4% do total (ICCO, 2017).

No Brasil, as regiões Norte e Nordeste são as principais regiões de produção de cacau. Em 2018, responderam por mais de 90% da produção total. Esta concentração da produção deve-se principalmente aos estados da Bahia e Pará (Anuário Brasileiro de Cacau, 2018), produzindo 122,8 mil toneladas na Bahia no ano 2018. Desde a introdução do cacaueiro, a região sul da Bahia ofereceu condições favoráveis ao seu desenvolvimento e permitiu que este estado viesse a se tornar o maior produtor nacional (SENA, 2011). Na década de 1980, o Brasil ocupava a segunda posição, como produtor

de cacau do mundo, atingindo valores próximos a 450 mil toneladas de amêndoas (SENA, 2011). No entanto, com o aparecimento de doenças no final da década de 1980, como a podridão parda e a vassoura de bruxa, tendo como agentes causais o oomiceto *Phytophthora* sp. e o fungo *Moniliophthora perniciosa*, respectivamente, um desastre econômico, social e ambiental atingiu esta região, e, como consequência, houve uma drástica redução na produção de amêndoas de cacau (PEREIRA, 1989). Desde então, várias medidas vêm sendo tomadas para restabelecer a produtividade de outrora, como seleção de materiais resistentes ou mesmo tolerantes à infecção, manejo e controle fitossanitário.

O escurecimento das raízes foi observado em muitas plantas submetidas à exposição ao Pb (CHANG et al., 2013). A toxicidade de Pb é frequentemente descrita como menor comprimento de raiz e massa seca, bem como diâmetro radicular aumentado (GRATÃO et al., 2009). A inibição do alongamento da raiz mostrou ser um dos primeiros e distintos sintomas da toxicidade do Pb (LUX et al., 2011). A inibição do alongamento radicular induzida pelo Pb pode ser atribuída à despolimerização dos microtúbulos do citoesqueleto celular e à formação de aberrações cromossômicas, que resultam em menores atividades mitóticas das células meristemáticas (SETH et al., 2008). O estresse por Pb resulta em maior diâmetro de raiz, devido ao aumento do tamanho das células parenquimatosas e dos tecidos corticais aumentados, que têm um papel funcional no aumento da resistência das plantas aos fluxos radiais de água. Sarwar et al (2010) fornecem uma discussão detalhada da natureza complexa do papel da nutrição mineral na redução da absorção de metais pesados. Alguns íons podem influenciar a absorção de chumbo diretamente por meio da competição por locais de troca de solo e quelação ou complexação com compostos de Pb. No entanto, prever o efeito nem sempre é simples, pois também depende do composto aplicado e do modo de aplicação que pode resultar em uma mudança no pH ou CTC e, portanto, afetar a biodisponibilidade do chumbo.

Como a capacidade de absorção das raízes está relacionada com o comprimento total e específico das raízes (GUERRERO-CAMPO et al., 2006), decréscimos no comprimento da raiz, área superficial, comprimento específico da raiz e número de pontas das raízes e aumento no diâmetro das raízes sugerem diminuição na aquisição de recursos hídricos, nutricionais, etc., em plantas submetidas ao estresse por Pb. Por outro lado, Cd também pode influenciar a arquitetura do sistema radicular em várias espécies

vegetais (WEI et al., 2012). Portanto, parâmetros sorológicos de raízes têm sido propostos como um indicador para avaliar a toxicidade de Pb (LU et al., 2013).

Poucos estudos sobre os efeitos de Pb no desenvolvimento de sementes sugerem que diminuições no rendimento de sementes e na taxa de germinação ocorrem quando as plantas são expostas ao Pb nos estágios de crescimento. Malan e Farrant (1998) relataram que concentrações mais baixas de Pb reduzem o tamanho da semente de *Glycine max*, mas não afetam o número médio de sementes por vagem. O rendimento de sementes também diminuiu em *Cicer arietinum* (WANI et al., 2007b) e em *Vigna radiata* (WANI et al., 2007a) cultivados em solo contaminado com Pb. Por outro lado, segundo estes autores, Pb inibiu a embebição das sementes e reduziu o teor de água nas plântulas, impactando assim a germinação e o plantio. Para uma semente germinar, o potencial hídrico do embrião precisa atingir um limiar crítico. Um modelo de tempo hidrotérmico mostrou que o potencial hídrico da semente estava intimamente relacionado com as mudanças na velocidade e na taxa de germinação e afetou a velocidade de alívio da dormência (ALVARADO; BRADFORD, 2005).

Há uma preocupação internacional recente com relação à presença de elementos traços (metais pesados) nos tecidos de cacau. Estudos recentes mostraram que o As, bismuto (Bi), cromo (Cr), Cd e Pb podem ser acumulados em amêndoas de cacau, casca do fruto e produtos à base de cacau (BERTOLDI et al., 2016). Entre estes elementos metálicos, o Pb, um metal traço não essencial, parece se acumular principalmente nas partes comestíveis do cacau, o que acarreta riscos potenciais à saúde humana pela ingestão de produtos contaminados. Também foi relatado que as amêndoas de cacau contêm diferentes concentrações de Pb, dependendo não apenas do genótipo, mas também do local geográfico, cujas concentrações médias alcançaram 1,4 mg kg^{-1} na América do Sul, 0,5 mg kg^{-1} na África Oriental e na América Central, 0,3 mg kg^{-1} na Ásia e 0,09 mg kg^{-1} na África Ocidental (BERTOLDI et al., 2016).

De fato, Pb é considerado um dos metais mais tóxicos que exibem efeitos adversos em todos os processos biológicos. Revela-se que há um impacto muito prejudicial de Pb ao meio ambiente e à qualidade dos alimentos (KABATA-PENDIAS; SZTEKE, 2015). Além de seus efeitos carcinogênicos, promove efeitos adversos observados no cérebro, rins e ossos. A União Européia classificou Pb e seus derivados clorados, oxigenados, sulfurados e sulfatados na categoria 1B. Da mesma forma, a Agência Internacional de Pesquisa sobre o Câncer e a Agência de Proteção Ambiental

dos Estados Unidos classificou o Cd no Grupo 1 e na Classe B, respectivamente. No entanto, a determinação da fração da concentração total de Pb que pode potencialmente afetar a saúde humana após a ingestão, ainda não foi estudada em produtos à base de cacau. Além da determinação das concentrações totais de metais traço dentro de um dado produto alimentício, as avaliações de risco à saúde também devem considerar sua bioacessibilidade gástrica (CABOCHE, 2009). Este parâmetro indica a quantidade máxima de um composto que, após ser liberado de sua matriz durante a digestão, pode ser absorvido pelo epitélio intestinal humano e então entrar na corrente sanguínea (PEIXOTO et al., 2016).

Oliveira et al. (2021) ao avaliar a ocorrência de Pb em amêndoas de cacau das principais regiões produtoras do Brasil, da África e do Equador demonstraram que a faixa de teor de Pb encontrada foi de 0,022 a 2,528 mg kg^{-1} MS e que em 66% das amostras os teores detectados foram superiores aos limites máximos permitidos. Além disso, estes autores verificaram também que os produtos derivados destas amêndoas apresentaram teor de Pb variando entre 0,022 e 0,136 mg kg^{-1} MS e que a ingestão de chocolate produzido a partir destas amêndoas contaminadas pode contribuir para a exposição do consumidor ao contaminante inorgânico. Segundo Peixoto et al. (2016), a bioacessibilidade intestinal de Pb varia de 3% a 11% em cinco tipos de achocolatados brasileiros, que contêm cacau misturado a outros ingredientes, como adoçantes artificiais que poderiam afetar a química de todo o processo pela formação de complexos solúveis ou insolúveis no trato gastrointestinal. Além disso, as diferenças entre os resultados de bioacessibilidade gástrica e intestinal são explicadas pelos diferentes protocolos analíticos utilizados para estimar esses percentuais. Xiong et al. (2016) optaram por medir apenas a bioacessibilidade gástrica de Pb, a fim de maximizar os riscos à saúde. Mesmo que a parte principal da absorção de Pb ocorra no jejuno e no íleo, o duodeno é um local de absorção não desprezível (DENYS et al., 2009). Como descrito por Caboche (2009), na saída do estômago, uma pequena quantidade de Pb contido na fase gástrica pode ser absorvida no duodeno por difusão paracelular passiva ou difusão transcelular passiva. Para elementos catiônicos como Pb, um aumento de pH na fase gastrointestinal induz a precipitação e, ou reabsorção de uma parte de Pb solubilizado na fase gástrica. Este precipitado de Pb não será absorvido e é então eliminado pelas fezes (DENYS et al., 2009).

2.4 - Alterações fisiológicas e ultraestruturais.

Uma vez absorvido pelo sistema radicular, Pb provoca vários efeitos diretos e indiretos sobre a germinação de sementes, o crescimento e o metabolismo das plantas (SHARMA; DUBEY, 2005). Os efeitos de Pb são descritos nas diferentes partes da planta, desde a raiz à parte aérea, embora haja uma maior concentração deste metal na raiz. A absorção de Pb reduz a assimilação de outros elementos essenciais às plantas, como Ca, Zn, Cu e K, que estão associados às proteínas estruturais e, ou à ativação enzimática e a abertura e fechamento estomático no caso do K, em detrimento da competição por canais de transporte (KUMAR; KUMARI, 2015). A inibição da fotossíntese é bem conhecida como consequência da toxidez de Pb, porém estudos recentes demonstram que este dano é indireto, pois resulta da distorção da ultraestrutura do cloroplasto, pela afinidade que Pb tem pelos ligantes N e S de proteínas das membranas tilacóides; da obstrução do sistema de transporte de elétrons, do fechamento estomático inadequado, do aumento da atividade da clorofilase, do distúrbio no status hídrico etc. (HAN et al., 2013). Por outro lado, há pouca relação com o teor de pigmentos cloroplastídicos, sendo que a clorofila **b** é mais sensível que a clorofila **a** (POURRUT et al., 2011).

As observações ultraestruturais demonstram que o excesso de Pb provoca muitas alterações em nível de cloroplasto, como deformações e, principalmente, aumento de plastoglóbulos, que tem como função o acúmulo de lipídios, síntese e reciclagem de proteínas sob estresse (REIS et al., 2015). Quanto aos mitocôndrios, há algumas controvérsias, pois, alguns estudos relatam que estes orgânulos celulares são mais tolerantes ao Pb do que os cloroplastos, mantendo a integridade das membranas mesmo sob altas concentrações deste elemento metálico (ERNST, 1998), enquanto outros demonstram a desintegração dos mitocôndrios na presença de Pb (KHAN et al., 2014). De acordo com Kaur et al. (2013), as alterações significativas em membranas mitocondriais e núcleos de células de raiz de *Triticum aestivum*, na presença de Pb, são justificadas pelo aumento do estresse oxidativo.

Muitos trabalhos demonstram que Pb causa estresse oxidativo, entretanto, o Pb não é um metal redox-ativo e, desta forma, não pode gerar ERO pela participação direta nas reações de Fenton, mas induz a formação ERO ao interferir nas atividades de transporte de elétrons (SHAHID et al., 2014). As espécies reativas de oxigênio causam

uma variedade de efeitos prejudiciais na planta, como peroxidação lipídica, redução de crescimento, clorose e escurecimento do sistema radicular (KAUR et al., 2013), inibição e aumento de atividades enzimáticas (POURRUT et al., 2011), inibição de produção de ATP e danos no DNA, podendo resultar em morte celular programada (KUMAR; KUMARI, 2015). Além destes efeitos na célula, Pb pode alterar a eficiência quântica de PS2, durante a fase fotoquímica da fotossíntese, obtida à partir da razão entre as fluorescências variável e máxima (Fv/Fm) da clorofila **a** de PS2, onde valores inferiores a 0,78 indicam situação de estresse em plantas (BAKER, 2008). Han et al. (2013) demonstraram que o aumento na concentração de Pb nas folhas reduz a eficiência quântica de PS2. Além disso, Pb provoca também redução no teor total de proteínas e inativação de enzimas e proteínas ao se ligar aos grupos tiois intracelulares (ASSCHE; CLIJSTERS, 1990). Além do mais, ao penetrar no núcleo, Pb se liga diretamente ao DNA ou indiretamente através de proteínas, causando distúrbios na replicação e transcrição, bem como a quebra da fita-dupla de DNA (POURRUT et al., 2011).

O impacto de Pb na fisiologia das plantas, manifestado principalmente por alterações nos antioxidantes (tióis, ácido ascórbico e metabolitos fenólicos) e concomitantemente no estado oxidativo, foi detectado por reagentes de fluorescência (BABULA et al., 2015). Devido ao amplo uso de Pb em estudos fisiológicos e sua ocorrência na natureza, este elemento metálico é usado como modelo estressor em estudos de detecção por reagentes de fluorescência. Pois, poucos estudos mostraram que metais podem afetar a resposta de alguns reagentes de fluorescência em tratamentos combinados (KOVÁČIK et al., 2014b). Vários reagentes de fluorescência foram amplamente utilizados para a detecção do estado oxidativo em raízes de plantas de *Zea mays* cultivadas com duas doses distintas de Pb, usando a espectrofotometria como padrão (KOVÁČIK et al., 2014b).

Doses de Pb elevadas no solo superficial e na água, mesmo em locais remotos, podem algumas vezes, resultar no transporte de longa distância de aerossóis contaminados (ATSDR, 2012) ou por insumos antropogênicos diretos. Como Pb está prontamente disponível a partir de fontes aéreas, a contribuição de Pb aéreo para Pb nas lavouras não pode ser negligenciada (KABATA-PENDIAS, 2011). A fim de considerar a deposição atmosférica como uma possível fonte de oligoelementos, a concentração natural no substrato do solo deve ser baixa e a sua absorção e acumulação pelas plantas não pode ser relacionada com os elementos do solo (DE TEMMERMAN et al., 2015).

Tem sido demonstrado que as partículas de poeira acumuladas na superfície foliar podem entrar na planta como íons solúveis através de estômatos, rachaduras cuticulares, lenticelas, ectodesmas e poros aquosos (SHAHID et al., 2017). Uma vez que os metais traço são acumulados no interior da planta, os mesmos podem ser transportados para o resto dos tecidos, contaminando partes comestíveis e se bioacumulando (XIONG et al., 2014).

2.5 - Alterações bioquímicas e moleculares.

As plantas diminuem ou neutralizam os efeitos tóxicos de Pb por meio de mecanismos específicos, de origem proteica e não-proteica. A parede celular é a primeira barreira para evitar os danos às células, por conter pectina com grupos carboxílicos, polissacarídeos e proteínas que adsorvem os íons Pb, diminuindo o movimento entre a membrana citoplasmática e o protoplasto, evitando os danos no protoplasto (WANG, 2015). A quelação de Pb, por meio de grupos carboxílicos pectínicos, é relatada como a principal via de tolerância da planta à toxidade do metal, cujos ácidos galacturônicos da pectina podem estar em nível variado de esterificação (WANG, 2015). Foi demonstrado que Pb^{+2} apresenta maior afinidade às cadeias não-metilesterificadas do que Ca^{+2}, logo Pb^{+2} pode substituir o Ca^{+2} da parede celular (RABEDA et al., 2015).

Estudos ultraestruturais mostram que a deposição de Pb, além de ocorrer nas paredes celulares, ocorre também nos vacúolos, protegendo os organelos mais sensíveis da célula. O acúmulo no vacúolo resulta predominantemente da ligação de íons metálicos no citosol em compostos de baixo peso molecular, como glutationa e fitoquelatinas, que permitem o transporte através do tonoplasto (ZHAO et al., 2015). As glutationas (GSH) são um grupo de tiois celular, que podem formar complexos com íons metálicos no citoplasma, reduzindo, assim, a toxicidade do metal em compartimentos celulares com metabolismo ativo (WOJCIK; TUKIENDORF, 2014). Em seguida, tais complexos são transferidos para o vacúolo ou são os principais doadores de íons metálicos para as fitoquelatinas (WOJCIK; TUKIENDORF, 2014). As fitoquelatinas (PC) são uma família de peptídeos ricos em cisteínas, sintetizados enzimaticamente, que utilizam GSH como precursor, apresentando importante papel na prevenção do estresse oxidativo em células vegetais (GUPTA et al., 2013). Tem sido proposto, *in vivo*, que as

PCs estão envolvidas na destoxificação e no acúmulo de vários metais, incluindo Pb, por causa da sua capacidade de formar complexos PC-metal. As PC sequestram Pb solúvel no citoplasma e transportam para o vacúolo e cloroplasto, diminuindo os efeitos deletérios do Pb^{+2} nas células (WANG et al., 2015). Entretanto, a capacidade das PCs de se ligarem aos diferentes metais se altera, pois foi demonstrado que os complexos de PC-Pb são mais fracos do que PC-Cd (SHARMA; DUBEY, 2005). Para algumas espécies vegetais, as PCs não conferem tolerância ao Pb (GUPTA et al., 2013).

Como mecanismos proteicos, têm-se as metalotioneínas e as enzimas do estresse oxidativo. As metalotioneínas (MT) se assemelham às fitoquelatinas, possuem alta afinidade com muitos metais, inclusive com o Pb. Entretanto, são proteínas de baixo peso molecular, com alto conteúdo de cisteína, e são superexpressas quando os organismos são submetidos a altas concentrações de metal. Embora seu papel na tolerância a metais pesados em plantas seja pouco conhecido, sabe-se que estas proteinas localizam-se no citosol e podem quelar os metais pesados, mas não estão envolvidas com o sequestro no vacúolo (AUGUY et al., 2013). De acordo com Auguy et al. (2013), mutantes para genes de metalotioneínas em *Arabidopsis thaliana* apresentavam maiores danos causados pelo Pb, sugerindo que estas proteínas estão relacionadas com a tolerância das plantas ao Pb. Para as espécies *Bruguiera gymnorrhiza*, *Avicennia marina* e *Kandelia candel* também ocorreu expressão destas proteínas na presença de Pb (AUGUY et al., 2013).

As enzimas do estresse oxidativo, como dismutase do superóxido (SOD), catalase (CAT), peroxidase do ascorbato (APX) e peroxidase do guaiacol (GPX), dentre outras, atenuam os efeitos de ERO, induzidos indiretamente por Pb (GILL; TUTEJA, 2010). A toxicidade induzida pelo Pb pode inibir ou ativar a atividade destas enzimas, assim como influenciar nas suas expressões e sínteses. Entretanto, estas ações dependerão da especiação do metal, da espécie vegetal e da duração/intensidade do tratamento (POURRUT et al., 2011). SOD é uma das enzimas chave das células vegetais, pois é responsável pela dismutação do superóxido ($O_2^{\bullet -}$**)** em H_2O_2 e O_2, enquanto que as peroxidases CAT, APX e POD são importantes para a eliminação de H_2O_2, por meio de mecanismos distintos (GILL; TUTEJA, 2010). A atividade destas enzimas mantém a integridade de membranas celulares e de moléculas importantes, tais como DNA e proteínas, evitando a peroxidação lipídica e a morte celular, reduzindo, assim, os danos provocados pelo Pb.

Os controles hormonais do desenvolvimento sob condições de estresses abióticos envolvem uma complexa cascata de sinalização e de percepção de estímulos à expressão gênica (AZEVEDO et al., 2012). Embora se tenha conhecimento de que vários genes responsivos ao estresse respondem aos hormônios, alguns dos quais já foram documentados mostrando que auxinas (AUX), giberelinas (GA), citocininas (CK), ácido abscísico (ABA), etileno (ET), brassinosteroides (BRs) e ácido salicílico (SA) fazem parte do processo de sinalização (FRAIRE-VELÁZQUEZ et al., 2011). Estresse por metais pesados em plantas modula severamente o padrão de expressão gênica (RAI et al., 2010). Nas plantas, muitos genes são regulados negativamente devido ao estresse por metais pesados (HALL, 2002). Estes genes estão relacionados com o metabolismo energético, metabolismo de carboidratos, biossíntese de lignina, metabolismo da fenilalanina, crescimento celular e morte, metabolismo lipídico, biodegradação de xenobióticos, metabolismo de aminoácidos etc. (HALL, 2002). Estresse abiótico induzido por metais pesados e elementos minerais produz uma grande quantidade de genes afetados, associados à biossíntese de metabolitos, especialmente a biossíntese de flavonóides, metabolismo lipídico, metabolismo de aminoácidos, metabolismo de carboidratos, biodegradação de xenobióticos, ascorbato e metabolismo de aldarato, transporte de membrana especialmente proteína de resistência a múltiples drogas, superfamília de facilitadores principais, transportadores ABC, metabolismo da GSH, via de sinalização MAPK (proteina-quinases ativadas por mitógeno), um grande número de GST etc. (RAI et al., 2010).

As plantas desenvolvem mecanismos celulares para tolerar e regular a absorção de metais pesados (HALL, 2002). No entanto, mecanismos e redes moleculares envolvidos na absorção e na desintoxicação de metais pesados permanecem pouco compreendidos. As fitoquelatinas (PC), uma classe de peptídeos de ligação a metais pesados ricos em cisteína, se liga a metais pesados e desintoxicam por sequestro vacuolar (HALL, 2002). A homeostase do enxofre (S) nas plantas resulta no aumento da produção de peptídeos ricos em S que se liga a metais, como GSH e PC, e fornecem tolerância a metais (RAI et al., 2010). A partir dos dados de expressão, foi demonstrado que a regulação positiva de um único citocromo O P450s, em diferentes estresses por metais pesados, é um dos principais mecanismos de desintoxicação (RAI et al., 2010). Nas plantas, o citocromo O P450 desempenha um papel importante no metabolismo de

várias vias biossintéticas, como flavonóides, cumarinas, antocianinas, isoflavonóides, fitoalexinas, ácido salicílico, ácido jasmónico e muitos outros (LI et al., 2007).

Um grande número de genes transportadores é diferencialmente regulado negativamente sob diferentes estresses por metais pesados, que incluem importantes genes facilitadores, transportadores de sulfato, transportadores de peptídeos, transportadores de nitrato, transportadores ABC, proteínas de resistência, transportadores de Zn, Nramp6 e extrusão de compostos tóxicos e proteínas multidrogas (MATE) da família de efluxo (NOCITO et al., 2004). Um dos nutrientes minerais essenciais necessários para o crescimento das plantas é o S, que entra na célula via transportadores de sulfato como sulfato inorgânico, e pode induzir a produção de peptídeos de ligação a metais ricos em S, como GSH e PC e, assim, fornecer defesa contra estresse por metais pesados (NOCITO et al., 2004). Segundo estes autores, ficou evidente, neste estudo, que cada metal pesado induziu transportadores de sulfato. Os transportadores de peptideos demonstraram transportar nitrato e tripeptideos como a GSH, que é um componente importante no metabolismo de S e de defesa durante o estresse (NOCTOR et al., 1998; CHIANG et al., 2004). Sugere-se que o transportador de nitrato desempenhe um papel na raiz para a translocação do nitrato, desempenhando assim um papel na toxicidade de Pb (LI et al., 2010).

Proteínas transportadoras ABC desempenham um papel importante no transporte de várias substâncias como lipídios, fitormônios, carboxilatos, metais pesados, catabólitos de clorofila etc., através de membranas biológicas (KRETZSCHMAR et al., 2009). Foi demonstrado que as proteínas Nramp são transportadores de metais bivalentes conservados (CAILLIATTE et al., 2009). NRAMP3 e NRAMP4 são relatadas como responsáveis pelo efluxo de Cd^{2+} do vacúolo (KRETZSCHMAR et al., 2009). Em *Oryza sativa* foram desenvolvidos transportadores de efluxo, para remover compostos tóxicos da célula (BROWN et al., 1999). Várias metiltransferases são diferencialmente moduladas quando as plantas são submetidas a diferentes tipos de estresses. Nas plantas, as metiltransferases constituem uma grande família de enzimas envolvidas na tolerância ao estresse, como relatado por Cailliatte et al. (2009). Metiltransferasas específicas catalisam a transferência de grupos metil, que estão envolvidos em várias vias metabólicas, que levam ao acúmulo de inositóis metilados, aminas quaternárias e espécies de sulfônio terciário, que desempenham um papel significativo na tolerância ao estresse (KRETZSCHMAR et al., 2009). Assim, a

modulação destes transcritos deve desempenhar um papel secundário na toxicidade de metais pesados. Durante diferentes estresses por metais pesados, esses metais induzem danos às membranas tilacoidais, levando ao aumento da peroxidação lipídica e, assim, causando uma regulação negativa das peroxidases (KRETZSCHMAR et al., 2009). Estes genes específicos da família das peroxidases podem desempenhar um papel fundamental na defesa enzimática da planta, para a eliminação de ERO durante as condições de estresse (KRETZSCHMAR et al., 2009). Proteínas de choque térmico (HSPs) em particular desempenham um papel importante na proteção das plantas contra o estresse, restabelecendo a conformação normal das proteínas e, portanto, a homeostase celular (KRETZSCHMAR et al., 2009).

Sabe-se que os metais pesados causam graves danos morfológicos, ultraestruturais, fisiológicos e bioquímicos nas plantas, cujos danos podem ser avaliados observando-se as mudanças na ocorrência da proteína do choque térmico 70s (Hsp70s), uma chaperona fundamental envolvida no dobramento de proteínas (BASILE et al., 2013). Isto, por sua vez, promove a homeostase celular em resposta ao estresse, como demonstrado em Bryophyta (ESPOSITO et al., 2012; BASILE et al., 2013). É bem conhecido que as Hsp70s são as proteínas mais abundantes das células que ganham sinapses, pois diferentes organismos produzem isoformas distintas e variáveis de Hsp70s (ESPOSITO et al., 2012). Além disso, uma classe destas proteínas, a Hsc70 cognata por choque térmico, tem sido descrita como um grupo de proteínas constitutivamente expressas, desempenhando um papel no correto dobramento dos polipeptídeos recém-sintetizados (HARTL, 1996). As Hsp70s são geralmente indicadas como as formas indutíveis pelo estresse e consideradas como tendo uma variedade de funções, incluindo o aumento da ocorrência de Hsc70, recuperação de proteínas parcialmente desdobradas e facilitação da remoção de proteínas desnaturadas (HARTL, 1996).

Em um estudo realizado com *Lemna minor* de flutuação livre, uma angiosperma monocotiledôneas-Arales, avaliou-se o acúmulo tissular, a localização intracelular e os efeitos tóxicos de Cd, Pb, Zn e Cu (BASILE et al., 2012a). Após as avaliações realizadas nessa espécie, danos ultraestruturais menores foram observados depois de exposição de *L. minor* ao Cd e Pb em baixas concentrações, mas quando as concentrações aumentaram para níveis maiores de acumulo dos metais, danos estruturais foram marcados e o dano foi detectado principalmente na forma dos cloroplastos, induzindo a transição de cloroplastos para cloroamiloplastos (APPENROTH et al., 2010).

Embora Pb não participe diretamente de reações redox celulares, o deslocamento de um elemento essencial da proteína leva indiretamente à formação de radicais livres e de ERO e causa dano oxidativo, como a peroxidação lipídica (SHAH et al., 2007). As plantas desenvolveram um mecanismo antioxidante enzimático e não enzimático para proteger seu componente celular e subcelular dos efeitos de ERO. O sistema de eliminação de ERO compreende enzimas como SOD, GPX, CAT e APX, bem como de compostos não enzimáticos como ácido ascórbico, carotenoides, α-tocoferóis e glutationa. As enzimas e os compostos antioxidantes são modulados por estresse oxidativo induzido por metais pesados e reduzem o dano por estresse (DIXIT et al., 2001).

Plantas induzem a síntese de compostos fenólicos quando expostos a metais pesados. Pb se acumula nos tecidos vegetais e também aumenta a quantidade de compostos fenólicos devido à sua concentração no solo (KOVÁČIK et al., 2009). Compostos fenólicos têm várias funções, incluindo atividade antioxidante, antimicrobiana e antimutagênica (KOVÁČIK et al., 2009). As propriedades antioxidantes dos compostos fenólicos são devidas ao seu alto potencial quelante e à capacidade de capturar radicais alcoxi-lipídicos que iniciam a peroxidação lipídica (KISA et al., 2016). Compostos fenólicos atuam como uma barreira à entrada de metais pesados na célula a partir da superfície celular e também desempenham um papel importante na captura de Pb (KISA et al., 2016). Devido à sua longa vida biológica e à retenção no solo, Pb se acumula facilmente em alimentos originários de plantas, que eventualmente afetam a saúde humana por meio da nutrição (SHAH et al., 2007).

2.6 - Mitigação da toxicidade de Pb por Mn e Zn.

A absorção de metal pelas plantas é modulada pela produção, exsudação e acúmulo de ácidos orgânicos de baixo peso molecular pela exposição aos elementos traços (KUTROWSKA; SZELAG, 2014). Os elementos metálicos Cu e Zn são importantes micronutrientes que participam do desenvolvimento normal da planta. Cu é um componente da plastocianina (PCY), citocromo oxidase (COX), óxidos de ascorbato (AAO) e tirosinase, enquanto Zn está presente na dismutase do superóxido (SOD) e na redutase do nitrito (PUIG; THIELE, 2002).

Metais são fortemente adsorvidos pela matéria orgânica, as argilas ou aos óxidos e, portanto, nestes casos, não estão disponíveis para a planta (CHEN et al., 2003). A exsudação de ácidos orgânicos de baixo peso molecular (LMWOAs) tem se mostrado um dos mais importantes fatores de mobilização e aumento da biodisponibilidade de nutrientes pouco solúveis, como fósforo (P), Fe e Zn. Também é relatado que os LMWOAs têm a capacidade para desintoxicar alguns metais nocivos, como Cd, Hg, As e Pb (KUTROWSKA; SZELAG, 2014). A exsudação de moléculas orgânicas pelas raízes é considerada como uma das estratégias mais importantes das plantas para tolerar a presença de oligoelementos (KUTROWSKA; SZELAG, 2014). Ácidos orgânicos são exsudados pelas raízes como ânions, cujo processo é equilibrado pela liberação de cátions (JANICKA-RUSSAK et al., 2008). Os ânions, em última análise, consomem prótons, particularmente quando o pH do substrato é baixo. Neste contexto, é provável que a liberação de ânions orgânicos, como LMWOAs, a partir das raízes das plantas, seja tratada como mecanismo de defesa da exposição a metais (UDDIN et al., 2015).

As estratégias para mitigar a absorção de Pb incluem a aplicação de emendas no solo e outras práticas culturais (XU et al., 2016). Chavez et al. (2016) demonstraram que em solos equatorianos a disponibilidade de Cd para as plantas de cacau foi afetada pelo pH do solo e pelo teor de matéria orgânica, enquanto Gramlich et al. (2018) relataram que o pH do solo e a geologia afetou a concentração de Pb em *Phaseolus vulgaris*. Isso sugere que a aplicação de emendas pode ser eficaz na mitigação da contaminação por Pb em cacau.

Mudanças nas condições ambientais afetam as respostas biológicas e fisiológicas das plantas. A toxicidade de metal em plantas de *O. sativa* inclui a absorção e os mecanismos de sequestro e as alterações bioquímicas, que ocorrem na planta (MARSCHNER, 2012). Para o seu crescimento e desenvolvimento global, as plantas requerem 14 elementos minerais diferentes (MARSCHNER, 2012). Esses elementos estão presentes no solo e são absorvidos pelas raízes, translocados para a parte aérea e distribuídos em diferentes órgãos e tecidos da planta, dependendo de suas necessidades (MARSCHNER, 2012). Minerais compreendem os metais e metalóides que são tóxicos para plantas e animais, mesmo em uma concentração muito baixa. Alguns desses metais pesados, como As, Cd, Hg, Pb e selênio (Se), não desempenham qualquer função fisiológica conhecida nas plantas e são chamados de metais não essenciais (MARSCHNER, 2012). Outros metais, como Co, Cu, Fe, Mn, Mo, Ni e Zn, são

elementos minerais essenciais, pois são necessários para o crescimento normal e metabolismo das plantas (MARSCHNER, 2012). Entretanto, os elementos minerais essenciais podem se tornarem tóxicos para as plantas, em altas concentrações. Por outro lado, Pb compete com outros elementos, especialmente com íons metálicos divalentes, mas a absorção de Pb não é inibida por Zn na espécie hiperacumuladora de Zn *Thlaspi caerulescens* (LOMBI et al., 2001) ou é ainda aumentada pela adição de Zn na espécie *Sedum alfredii* (LI et al., 2009).

Sabe-se que a toxicidade de Cd resulta de sua interação com nutrientes minerais em plantas, como Zn, Fe, Ca, K, Mn, Cu, silício (Si) e Mg (NEDJIMI; DAOUD, 2009). Cátions como Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} e Si^{2+} competem com Pb por locais de troca no solo (DEGRYSE et al., 2004). Em geral, a adsorção de Pb nos solos pode ser reduzida pela presença de íons competitivos. Feng et al. (2013) relataram que a adição de fertilizantes de cálcio, silício, fosfato de cálcio e magnésio poderia reduzir significativamente a concentração de Pb disponível nos solos. Óxidos de ferro, alumínio e manganês podem adsorver Pb na superfície, diminuindo assim a disponibilidade de Pb (HETTIARACHCHI; PIERZYNSKI, 2002). Por outro lado, a presença de Pb na solução nutritiva influencia o estado nutricional das folhas e raízes, provavelmente por inibir os transportadores de carregar outros metais na parte aérea das plantas e influenciar a produção de fitoquelatinas (SANDALIO et al., 2001).

A toxicidade de Pb nas plantas é altamente modificada pelo aumento da razão Zn/Pb (PAPOYAN et al., 2007). A absorção de Pb pelas raízes diminuiu com o aumento da concentração de Zn nos hiperacumuladores de Pb/Zn, incluindo *Arabidopsis halleri* e a maioria dos ecótipos de *Thlaspi caerulescens* (ZHAO et al., 2002), *Lonicera japonica* (LIU et al., 2011) e *Gynura pseudochina* (PANITLERTUMPAI et al., 2013), bem como nos cultivos não acumuladoras, como *T. aestivum* e *T. turgidum* (ZHAO et al., 2006), que indicou claramente que o influxo de Pb é largamente atribuído aos transportadores Zn, com uma forte preferência por Zn sobre Pb. Em geral, as plantas absorvem mais Pb se o teor de Zn é baixo, enquanto Zn em concentrações mais altas inibe a absorção de Pb (NAN et al., 2002). Ueno et al. (2008) observaram uma diminuição na concentração de Pb na seiva do xilema de *A. halleri* com o aumento da exposição ao Zn. Huguet et al. (2012) relataram a possibilidade de competição de Zn/Pb em baixa concentração de Pb (5 mol Pb L^{-1}) para *A. halleri*. No entanto, esse fenômeno não foi confirmado em 20 mol Pb L^{-1} (HUGUET et al., 2012). Homma e Hirata (1984) relataram que as taxas de

absorção de Pb pelas mudas de *O. sativa* foram tão altas quanto as de Zn, se as concentrações de ambas as espécies de íons fossem menores que 1 mol L^{-1}. Segundo estes autores, em concentrações mais altas, as taxas de absorção de Zn foram mais do que duas vezes as de Pb na mesma concentração em solução nutritiva. Chaney e Oliver (1996) analisaram culturas de *T. aestivum* e relataram que o nível de Pb não afetou a absorção e a translocação de Zn em raízes e parte aérea. No entanto, quando a atividade do Zn variou de 1 10 7: 6 a 1 10 5: 2 mol L^{-1}, a razão entre a concentração de Pb/Zn da raiz em *T. aestivum* diminuiu de 0,20 para 0,03. Além disso, Chaney e Oliver (1996) sugeriram que uma razão Pb/Zn <1,5% em alimentos, forneceu proteção contra impactos na saúde induzidos por Pb.

Assim, o antagonismo entre Zn e Pb oferece uma maneira de reduzir a absorção de Pb pelas plantas. No entanto, em plantas do ecótipo Ganges de *T. caerulescens*, que exibe uma capacidade excepcionalmente elevada para hiperacumular Pb na sua parte aérea, a absorção de Pb não foi inibida por Zn (LOMBI et al., 2001). Além disso, Xue e Harrison (1991) relataram um efeito sinérgico de Zn em relação à absorção de Pb. Segundo estes autores, o aumento da concentração de Zn (> 600 mg kg^{-1}) em solos contendo altas concentrações de Pb (10 mg kg^{-1}) resultou em uma maior concentração de Pb nas folhas de *Lactuca sativa*. Da mesma forma, Smilde et al. (1992) e Kachenko e Singh (2006) relataram altas concentrações de Pb em vegetais folhosos, com o aumento da concentração de Zn no solo. Além disso, Li et al. (2009) mostraram, em cultivos hidropônicos, que as interações de Zn e Pb em *S. alfredii* foram diferentes daquelas plantas cultivadas em condições normais e variaram notavelmente entre dois ecótipos contrastantes (HE e NHE) de *S. alfredii*. Interações positivas fortes entre Zn e Pb foram observadas em plantas de *S. alfredii* submetidas aos tratamentos com 100 mol Pb L^{-1} + 500 mol Zn L^{-1} no HE, e cuja absorção e translocação de Pb foram aumentadas pela adição de 500 mol Zn L^{-1} (LI et al. 2009). Por outro lado, o efeito das interações de Pb e Zn no crescimento radicular de NHE foi sinérgico, e 500 mol Zn L^{-1} inibiu a absorção de Pb, provavelmente devido à competição entre Cd e Zn (LI et al. 2009). Esses resultados sugeriram a presença de um sistema de transporte de Pb específico e eficiente (UENO et al., 2008).

Tem sido relatado que Pb influencia a absorção e o acúmulo de Cu, Zn e Fe em *B. juncea* (JIANG et al., 2004) e *Hordeum vulgare* (ASTOLFI et al., 2012). Concentrações de Cu nas raízes permaneceram inalteradas com a adição de Cd ao meio

de crescimento, enquanto um efeito antagônico de 200 mmol Pb L^{-1} ocorreu em nível de Fe nas raízes de *B. juncea* (MOHAMED et al., 2012). Savvas et al. (2012) relataram que Pb facilitou a deposição de Cu e Fe nas raízes de *Cucumis sativus* e restringiu sua translocação para a parte aérea da planta, tendo pouco efeito na absorção de Ca, Mg e K. Além disso, uma interação negativa de Pb-Mn tem sido amplamente relatada em plantas sob estresse de Pb (CHENG et al., 2009). Baszynski et al. (1980) afirmaram que o aumento da concentração de Mn poderia diminuir o acúmulo de Pb nas folhas da planta, possivelmente devido à competição pelos mesmos transportadores de membrana. Em contraste, Ramos et al. (2002) mostraram que um aumento na absorção de Mn foi observado sob estresse de Pb em *L. sativa*. Por outro lado, Li et al. (2011) observaram uma interação antagônica entre Pb e Mn nas raízes de *Lonicera japonica*; no entanto, a absorção e o acúmulo de Mn nas folhas tiveram um ligeiro aumento com o incremento das concentrações de Pb, indicando que algumas interações ocorreram entre Pb e Mn durante a absorção e a translocação.

3 - Chapter 1

Mitigation of Pb toxicity by Mn in young plants of CCN 51 clonal cacao genotype grown in soil: physiological, biochemical, nutritional and molecular responses

Abstract

Lead (Pb) is a highly toxic metal for humans and animals, but also for plants even at low concentrations in the soil. Evaluation of the occurrence of Pb contents in cocoa beans from main producing regions of Brazil, Africa and Ecuador, showed Pb contents higher than the maximum allowed limits. The ingestion of chocolate produced from contaminated beans can contribute to consumer exposure to Pb. On the other hand, Mn is an element essential for plants and participates as enzymatic cofactors in several metabolic pathways. Pb is uptake by the root system through divalent cation transporters and competes with essential divalent minerals such as Mn. Therefore, increasing the concentration of Mn in the soil can reduce the Pb uptake by root system and mitigate its toxicity in plants. The objective of this study was to evaluate the influence of Mn on mitigation of Pb toxicity in young plants of the cacao clonal CCN 51 genotype grown in soils with different doses of Pb, Mn and Mn+Pb, through physiological, biochemical, molecular and nutritional responses. It was found that the young plants of the cacao clonal CCN 51 genotype grown in soils with high Pb, Mn and Mn+Pb contents accumulated these heavy metals in the roots and leaves. Mn doses reduced the Pb uptake by root system and prevented that the Pb accumulated at toxic levels in the roots and leaves of the plants. On the other hand, high doses of Pb applied in soil were highly toxic to the plants, leading, in some cases, them to death. However, no Mn toxicity was observed in cocoa plants, even at high doses in the soil. Uptake of Pb and Mn by the roots and its transport into the aerial part of the plant promoted changes in photosynthesis, leaf gas exchange, respiration, carboxylation and in the instantaneous efficiency of carboxylation, reducing in the treatments with the highest concentrations of Pb, and the emission of chlorophyll fluorescence, affecting the efficiency of photosystem 2 and the production of photoassimilates. Besides that, Pb, Mn and Mn+Pb toxicities activated defense mechanisms in plants that alter the gene expression of *met*, *psbA* and *psbO*, increasing in plants subjected to high concentrations of Pb and the activity of the enzymes involved in the cellular detoxification of excess ROS at the leaf level, which were higher in treatments subjected to higher concentrations of heavy metals. In addition, high uptake of Mn by root system was found to reduced Pb uptake in plants grown with Mn+Pb in the soil, in the doses corresponding to 0.3 g Mn + 0.5 g Pb kg^{-1} soil and 0.15 g Mn + 0.75 g Pb kg^{-1} soil, and mitigated the damage caused by Pb. Therefore, application of Mn in the soil can be used to mitigate the Pb toxicity in young plants of the cacao clonal CCN 51 genotype grown in contaminated soils.

Keywords: *Heavy metal, photosynthesis, gene expression, antagonism, mineral nutrients, and physiological parameters.*

Introduction

Cacao (*Theobroma cacao* L.) is a woody species typical of tropical climate, diploid (2n = 20), preferably allogamous, perennial and among the 22 species that make up the genus, is the most commercially exploited in Brazil (Motamayor et al., 2003). Cacao is grown in commercial plantations around the world (Steinberg, 2002). The Americas produce around 16% of global cocoa production, with Brazil among the main producing countries (ICCO, 2018). It is considered one of the most important perennial crops on the planet, with an estimated cocoa beans production of 4.7 million tons in the 2017- 2018 harvest (ICCO, 2018). The southern region of Bahia is the main cocoa producing region in Brazil, with an estimated production of 143,000 tons in January 2018, 70% more than the previous harvest, in a total area of 530 thousand hectares (IBGE, 2018).

CCN 51 clonal genotype of cocoa has desirable agronomic characteristics such as high productivity, self-compatibility, witches' broom disease tolerance, and high butter content in its beans, besides being a widely used genotype by cocoa producers in Latin America (Boza et al., 2014). Clone CCN 51 was selected in Ecuador and is suggested as the result of crossing the IMC 67 x ICS 95 hybrid with an Ecuadorian cultivar known as "Canelos ". It is a cultivar that has been widely used in Ecuador and is being used as a parent in many cocoa breeding and selection programs in other countries.

Cocoa beans are the raw material for chocolate, as well as sources of carbohydrates, fats, proteins, natural minerals, flavonoids and vitamins used in the production of cosmetics, beverages, jellies, creams and juices (Almeida and Valle, 2007). However, recent research has revealed that soils in the cocoa growing area, beans and by-products such as sweets and chocolates are contaminated by heavy metals, especially lead (Pb), in the major cocoa producing and consuming regions of the world (Arévalo-Gardini et al., 2017), well above that recommended by the European Union (Caobisco, 2015). The main contamination routes of soils by heavy metals are represented by the rock of soil origin and by the anthropic action, in the deposition of industrial residues, application of sludge and phosphate fertilizers, which although essential to meet the need of the cultures may contain heavy metals, including Pb, which are incorporated into the soil indiscriminately (Kratz et al., 2016).

The possibility of detection of some heavy metals, such as Pb, in residues in cocoa cannot be avoided, due to the fact that most agricultural soils have these elements. Pb is easily absorbed by the roots and translocated to the aerial part of plants and can be stored in the components of the cacao pod. However, Mn may act on plant metabolism by reducing and/or eliminating the toxic effect of Pb, since Mn, even being a heavy metal, is absorbed in large quantities by the cacao tree without generally causing toxicity (Kratz et al., 2016). Therefore, a study is needed to elucidate the process of mitigation of Pb toxicity by Mn in clonal and seminal cacao genotypes, in order to reduce human health risks, due to the accumulation of Pb in beans and consequent contamination of the chocolate. Mn and Pb are metallic elements present in nature and have distinct functions in plants. Mn, usually present as Mn_2^+, is an essential micronutrient for all crops (Millaleo et al., 2010), being a toxic substance when in excess (Pendias and Pendias, 1992). Mn is naturally found in soils, the bivalent form (Mn_2^+) being the most soluble in the soil (Guest et al., 2002). Mn absorption by plant roots occurs from the soil solution, being transported as a divalent cation to the aerial part (Millaleo et al., 2010), where it usually occurs the greatest accumulation of this element. However, experiments with several species showed that Mn can be accumulated in the roots, but in a lower concentration (Page et al., 2006).

The excess of Mn and/or the presence of Pb in the plant systems triggers a series of reactions common to both metals, such as inhibition or decrease in growth rate (Gill et al., 2012), and to reduce the rate of photosynthesis and transpiration (Sanitá et al., 2010). The toxicity of both Mn and Pb can also trigger oxidative stress in plant cells, causing metabolic alterations and macromolecular damages, interfering in the activity of specific enzymes, generating excess reactive oxygen species (ROS), mainly OH^-, which disturb the cellular homeostasis (St Clair, 2004). ROS are naturally produced by plants as byproducts of various metabolic pathways located in different cell compartments such as mitochondria, chloroplasts and peroxisomes (Navrot et al., 2007). In stress condition, caused by metals (for example), there is an increase in ROS production, which causes imbalance to cellular structures, resulting in the generation of oxidative stress (Gill and Tuteja, 2010).

The plants have a range of potential mechanisms, such as induction and activation of plant defense antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX), which allow the detoxification of excess

ROS produced in response to absorption of soil metals by the roots (Schützendübel and Polle, 2002). In addition, plants accumulate a number of metabolites to deal with stressful conditions, especially proline (Yanga et al., 2009). Prolina is a multifunctional amino acid that acts as an osmolyte, stabilizer of proteins and cell membranes, eliminates free radicals, balances cellular homeostasis and acts as a signal in stressful conditions (Szabados and Savouré, 2009). In addition, the phytotoxicity of Pb can be minimized by the action of chelating agents such as phytochelatins (PCs) and metallothioneins (MTs).

The objective of this study was to evaluate the influence of Mn on mitigation of Pb toxicity in young plants of the CCN 51 clonal cocoa genotype grown in soil with different concentrations of Pb, Mn and Pb+Mn, through physiological, biochemical, molecular and nutritional responses.

Material and methods

Plant material and growing conditions

The experiment was carried out in a greenhouse at the State University of Santa Cruz (UESC), Ilhéus, Bahia, Brazil (14° 47' S, 39° 10' W).

The young plants of clonal cocoa were obtained from the rooting of stem cuttings from the ends of plagiotropic branches at the beginning of secondary growth, containing the apical bud, three auxiliary buds and three leaves, taken from five to 10 years old parent plants. The bottoms of the cuttings (~ 3 cm) were dipped into chemically inert talcum powder containing indol-3-butyric acid (IBA) at 4 g kg^{-1}. Afterwards, each cutting was transferred to a 288-cm^3 tubelike, black plastic pot containing organic substrate (turf+ grinded *Pinus* barks and grinded coconut fiber at 1:1 ratio), enriched with macro and micronutrients, according to the recommendations for cacao.

After rooting (4 to 5 months of age), the young plants were transplanted to drilled plastic vessels with a capacity of 20 kg. The soil was fertilized with N, P and K (Supplementary material) and a mixture of $CaCO_3$ and $MgCO_3$, important to reach the Ca^{+2}: Mg^{+2} 4:1 ratio, and raising the soil base saturation value to 30%, which results in increased pH (4.7) results obtained after performing the soil analysis (Table 1). The level of fertilization for cacao was based on the needs of the crop during the 120 days of the experiment (Souza Junior, 2008) (Table 1). For the application of water to the plants,

first the field capacity of the soil was determined, to know the quantity of water application in each pot, when the plants were four months old, the treatments of Pb, Mn and Mn+Pb in the soil were applied in a volume of 500 mL/pot, with the following concentrations: treatment 1 (T1) (0.3 g Mn kg^{-1} soil + 0.5 g Pb kg^{-1} soil), treatment 2 (T2) (0.15 g Mn kg^{-1} soil + 0.75 g Pb kg^{-1} soil), treatment 3 (T3) (0.45 g Mn kg^{-1} soil + 0.25 g Pb kg^{-1} soil), treatment 4 (T4) (1.0 g Pb kg^{-1} soil) and treatment 5 (T5) (0.6 g Mn kg^{-1} soil), together with the control (T0) (without addition of Pb and Mn in the soil), totaling six treatments and having $PbCl_2$ and $MnCl_2$ as sources of Pb e Mn, respectively.

During the whole experimental period, young plants of cocoa were watered with rainwater previously stored. The photosynthetically active radiation (PAR), temperature and relative humidity inside the greenhouse were continuously monitored and recorded by with micrometeorological sensors (Hobo H8 Pro Series, Onset, USA). PAR, temperature and relative humidity means recorded during this period were 6±0.5 mol m^{-2} day^{-1}, 27±0.4°C and 78±0.7%, respectively.

Leaf gas exchange

During the experimental period, the net photosynthetic rate per unit leaf area (*A*), stomatal conductance to water vapor (estimated measurement) (*gs*) and leaf transpiration (*E*) were measured at 0, 15 and 30 days after application of treatments (AAT), between 08:00 and 12:00 h, in a fully expanded and mature leaf. Five plants per treatment were evaluates using a portable LI-6400 photosynthesis measurement system (Li-Cor, Nebraska, USA), equipped with a 6400-02B RedBlue artificial light source. The photosynthetic photon flux density and leaf temperature was set at 800 μmol photons m^{-2} s^{-1} and 26°C, respectively, using equipment accessories. The readings were record in the range of 2-3 min (coefficient of variation from 0.1% to 0.2%). The values of *A*, *gs* and *E* were used to calculate the intrinsic (*A/gs*) and instantaneous (*A/E*) efficiencies of water use and instantaneous carboxylation efficiency (*A/Ci*).

Chlorophyll fluorescence

Measurements of chlorophyll fluorescence emission was made on the same leaves used for gas exchange measurements, using a portable fluorometer (Pocket PEA Chlorophyll Fluorimeter - v 1.10 - Hansatech Instruments, Norfolk, UK), between 8 and 12 h. The selected leaves were adapted to the dark for a period of at least 20 min, for

reflection of incident solar radiation, decrease of leaf temperature and oxidation of the entire photosynthetic electron transport system, using appropriate clips. After dark adaptation, the leaf tissue was illuminated with a weak-modulated measuring beam (0.25 kHz, < 0.1 μmol m^{-2} s^{-1}, 650 nm, 1 s) to obtain the minimal fluorescence (F_0). A saturating white-light pulse (20 kHz; 3500 μmol m^{-2} s^{-1}, 650 nm, 1 s) was applied to ensure the variable (F_V) and maximum (F_m) fluorescence emissions. The maximum quantum yield of photosystem 2 (Fv/Fm) was calculated as [$Fv/F_m = (F_m - F_0)/F_m$)] (Baker, 2008). The fluorescence emission signals were recorded in the acquisition system of Pocket PEA data, using specific software.

Minerals nutrients and Pb

At the end of the experiment, 30 days after the application of the treatments, except treatments T3 and T4, which were collected at 18 days, since in this period of time the plants died, samples composed of roots and leaves were collected from different treatments. The dried and ground plant material were weigh out in triplicate (200 mg per sample) and placed in a 50 mL digestion tubes containing 3 mL of concentrated HNO_3 (Merck) (Yang et al., 2014). The tubes were capped with a cold finger, containing distilled water. During the digestion of the samples, the block temperature was gradually increased: (i) 50°C for 30 min, (ii) 80°C for 60 min, and (iii) 130°C for 45 min, plus 1 mL of 30% hydrogen peroxide (Merck). Soon after, the 30% hydrogen peroxide was added for a further 2x (1 mL) at 20 min intervals. Subsequently, after 15 min of the last addition of hydrogen peroxide, the digester block was turn off. When samples reached room temperature, they were transfer into Falcon tubes and volumated to 15 mL with ultrapure water. Subsequently, the macro and micronutrient content and Pb were analyzed in an Inductively-Coupled Plasma Optical Emission Spectrometer (ICP-OES) model Varian 710-ES.

Enzymes of antioxidative metabolism

In order to perform the enzymatic tests at leaf level, the plant tissue was collected at 0, 3, 6, 12, 24, 48 and 96 h AAT, immersed in liquid nitrogen and stored in ultrafreezer -80°C and subsequently lyophilized and stored in a freezer -20°C. The leaf samples were later macerated, in liquid nitrogen. The macerate was weighed, conditioned and then polyvinylpyrrolidone (PVP) was added to prevent macerate

oxidation. Immediately thereafter, the macerate was resuspended in extraction buffer (50 mM sodium phosphate buffer, pH 7.0 or 50 mM potassium phosphate buffer, pH 6.0), which varied according to the type of enzyme involved in the antioxidative metabolism, followed by shaking. Subsequently, the material was subjected to sonication, followed by centrifugation. Finally, the supernatant was collected, considering it as the crude extract, which was transferred to a 2 mL microtube, kept in Styrofoam with ice, and used immediately.

The activity of the enzymes guaiacol peroxidase (GPX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), polyphenol oxidase (PPO, EC 1.10.3.1) was determined according to the methodological procedures described by Amako et al. (1994), Nakano and Asada (1981), Siddiq et al. (1992), and Yao et al. (2012) respectively. The sample and standard readings were done with a UV–vis spectrophotometer (SpectraMax Paradigm Multi-Mode Microplate Reader, Molecular Device, USA).

Proline

Plant tissue was collected at 0, 3, 6, 12, 24, 48 and 96 h AAT, from the second or third mature leaf from the stem apex (approximately 100 mg), lyophilized and later macerated in liquid N. Immediately after, proline was extracted by adding 3% (w/v) sulfosalicylic acid to the samples of plant material. Then, samples were centrifuged and the resulting supernatant was used to determine proline concentration, according to the procedures described by Bates et al. (1973) with minor modifications (Khedr et al., 2003). Trials were performed in triplicates for each biological replicate.

Gene expression

After carrying out the evaluation of enzymatic activity and after analyzing the results, it was decided to compare the most contrasting treatments. Therefore, for the determination of genetic expression, mature leaves were collected at intervals of 0, and 96 h AAT. Samples were stored at - 80°C after immersion in liquid nitrogen and then lyophilized. For RNA extraction, RNAqueous® kit (Ambion) was use, following the manufacturer's recommendations. RNA samples were used for the synthesis of the cDNA with RevertAid ™ H minus M-MuLV Reverse Transcriptase (Fermentas), according to the manufacturer's instructions, using oligo d (T) 18 primers. Analyzes of

qPCR were performed on an Applied Biosystems 7500 Real-Time PCR System thermocycler using non-specific detection sequence (fluorophore) SYBR Green I. The reaction mix were composed of cDNA as a template, 0.5 µM of each primer and 12.5 µL of Maxima® SYBR Green/ROX qPCR Master Mix (2x). The temperature of PCR products was raised from 55°C to 99°C at a rate of 1°C/5s, and the resulting data were analyzed using the LightCycler software.

We only observed a single band with a characteristic melting point for each sample, indicating that the qPCR had produced a specific product for each used primer-pair. In order to confirm that the qPCR had only expressed the genes of interest, the PCR products were separated and visualized in agarose gel at 1%. The relative expression numbers of the genes were calculated as the number of times in relation to the control plant using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2013). Two endogenous genes were used as control in order to detect changes, *actin* and *β-Tubulin.* The abundance of transcripts was analyzed using specific primers (Table 2). In order to test the quality of these primers, the specificity and identity of the reverse transcription products, we have monitored the qPCR products after each PCR, using a melt-curve analysis distinguishing gene-specific from non-specific products.

Statistical analysis

The experimental design used was the randomized blocks with six treatments, 10 replicates and one plant per experimental unit, making a total of 60 clonal plants. Five replicates were used for the measurements of photosynthetic parameters, concentrations determinations of minerals nutrients and Pb and biochemical and molecular analysis. The data were submitted to ANOVA and the means values were compared by Tukey test ($p<0.01$ and $p<0.05$).

Results

Pb and mineral nutrients

The cacao plants in treatment T0 (i.e., without application of Pb to the soil) showed no accumulation of Pb in the leaves. Conversely, the highest leaf Pb accumulations were observed in T1 and T4, followed by T2 and T3. These Pb content values were directly associated with the dose of Pb applied to the soil. Treatment T5

showed the lowest accumulation of Pb in the leaves compared with the other treatments (Table 4).

Pb accumulation in the different organs of young cacao plants varied according to the doses of Pb applied to the soil. In roots, the highest accumulations of Pb were observed in treatments T1 (1827 mg dm^{-3} DM) and T4 (806 mg Pb dm^{-3} DM). For root Pb content in T2 and T3, significant statistical lower values were observed compared with the other treatments. No root Pb accumulation was observed for treatments T0 and T5 (0 mg kg^{-1} DM) (Table 3). There were significant statistical differences for the accumulation of micro and macronutrients in the roots and leaves of the cacao plants in response to increasing levels of soil Pb content (Table 3 and 4). However, no significant statistical differences in leaf N content were found across treatments. On the other hand, T2 and T3 showed the highest root K content compared to control, T1, T2, T4 and T5 treatments. High Ca accumulations in cacao leaves were evident in all treatments except T4 and in roots in T0 compared with the other treatments. Moreover, root Mg content in T0, T3 and T4 showed statistically significant differences in relation to the other treatments. In contrast, there were statistical differences for S accumulation in the roots between T0, T2, T3, T4, and T5 in comparison to T1 (Table 3). Treatments T0 and T1 and T2 and T3 and T5 displayed high root Cu accumulations, increased more than 1 mg dm^{-3}, compared to the other treatments, which were statistically not different from those of the other treatments. T4 Regarding Fe content in the roots, a higher accumulation of this element was found in T1 (3135 mg dm^{-3} DM) compared to T4, which received the highest dose of Mn applied to the soil and showed 50% less Fe content (Table 3). For root B content, the highest value of this microelement was found in T3 (57.2 mg B dm^{-3} DM), while the lowest value was found in T1 and T4 (26.1 and 27.8 mg B dm^{-3} DM).

There were significant differences in leaf P content for treatments T0, T2, T3, and T5 compared with T4, which contained the highest Pb dose in the soil and showed a P content of 0.1 dag kg^{-1} DM (Table 4). For leaf Ca content, T4 displayed low accumulation in contrast to the other treatments. The same tendency was observed for Mg, S, Cu, Fe, B, and Zn contents since T4 also showed the lowest values of these elements in the leaves (Table 4). Furthermore, in T1 and T4, CCN 51 clonal cacao plants displayed lower leaf Mn contents compared with T5, which showed the highest content of this metal in the leaves.

Leaf gas exchange

The application of different concentrations of Mn, Pb, and Mn + Pb to the soil promoted changes in leaf gas exchange in young clonal cacao plants. The evaluations were conducted on different days after application of treatment (AAT) and revealed differential responses of the plants across treatments, mainly in comparison with the control treatment. The different doses of Pb in the soil negatively influenced the net photosynthetic rate (*A*). Particularly, the plants grown under Pb treatments showed lower values of *A*. At 0, 15, and 30 days AAT, the application of Mn was associated with lower Pb doses and favored the increase in about 50% in *A* compared to the other treatments. However, at 21 days AAT in (0.15g Mn + 0.5 g Pb kg^{-1} soil and 0.45g Mn + 0.25 g Pb kg^{-1} soil, the young CCN 51 cacao plants died, due to toxicity of Pb and Mn in the soil (Figure 1A).

In general, T3 and T4 soil showed the lowest values of *A* compared with the other treatments; particularly, treatment 4 had a higher Pb dose per kg of soil at the time of application (day 0) (Figure A). This trend was maintained during the first 15 days of the experiment for the T3 treatment. After this period, there was a considerable reduction in *A*, mainly in 0.45g Mn + 0.25 g Pb kg^{-1} soil (T3) and 1.0 g Pb kg^{-1} soil (T4), at 30 days AAT. These two treatments had the highest Pb doses in soil, which induced toxicity in the young cacao plants, mainly in T3, where the plants eventually died.

At 0 h, there were statistical differences in *A* between T0 and the other treatments particularly T4, which had the highest Pb content per kg of soil; this behavior continued during the experiment until 30 days AAT and caused plant death at 30 days AAT due to Pb toxicity. The same behavior was observed for stomatal conductance (*gs*). On the other hand, statistical differences in instantaneous water-use efficiency (*A/E*) were observed at 15 days AAT when greater water-use efficiency was evident in T0 and T1 and T4 and T5 compared with 0.3 g Mn + 0.5 g kg^{-1} (T2) soil and 0.6 g Mn kg^{-1} (T3) soil (Figure 1F). Furthermore, differences in *A/E* were observed at 0 h, indicated by significantly higher values of *A/E* and *A/ci*, for 0.3 g Mn + 0.5 g kg^{-1} soil and 0.45 g Mn + 0.25 g kg^{-1} soil compared with 1.0 g kg^{-1} soil (Figure 1E and G). This behavior was more pronounced at 15 days AAT, leading to plant death at 30 days AAT caused by Pb toxicity, behavior that is repeated when observing Figure 1D for the variable *Ci/Ca*, in Figure 1D, in general, a recovery observed at 15 for all treatments, except the treatment of 1.0 g kg^{-1} soil.

Chlorophyll fluorescence emission

Regarding the initial fluorescence (F_0), statistically significant differences were observed at 15 days AAT, as shown by the lower values of T5 compared to the other treatments (Figure 2). On the other hand, there were no statistically significant differences for maximum efficiency of photosystem 2 (F_V/Fm) (Figure 2).

Antioxidant enzymes and proline content

The enzymatic activities of the SOD, APX and GPX enzymes were evaluated in the leaves of CCN 51 plants exposed to soil toxicity with Pb. The activity of the APX enzyme showed higher values in T0 and T1 at 0 AAT, compared to T3 (Figure 3A). However, at 3 h AAT, the activity of this enzyme in T3 increased by almost two-fold. Later, at 6 h AAT, the enzymatic activity returned to the level observed at the time of Pb and Mn applications (0H AAT). At 24 h AAT, the activity increased at T3 and decreased at T0, which at 48 h increased its activity considerably.

Guaiacol peroxidase activity was present from the beginning of the treatment applications. The highest activity of the GPX enzyme was observed in T1 compared to T3 and T5 (Figure 3B). However, T3 demonstrated a reduction in GPX activity at 3H AAT; At 6 h AAT, the activity of this enzyme was higher in T2, but decreased at 12 h AAT in the same treatment. Meanwhile, the control treatment (T0) showed the highest values of GPX activity and displayed the same tendency until 24 h AAT. However, at 48 h AAT, there was an increase in GPX enzyme activity in T4, followed by a reduction at 96 h AAT.

There were significant differences in CAT enzyme activity for T1 and T2 in contrast to T0 from 0 h to 3 h AAT (Figure 3C). However, at 6 h AAT, the enzyme activity increased considerably in T0, T1 and T3, and this trend was maintained until 24 h AAT for T0 and T1. At 48 hrs AAT, all treatments showed the same activities of the CAT enzyme. By the end of 96 h AAT, T1 showed an increase in CAT activity, differing from T0, T2, T4 and T5, which showed lower values.

PPO enzyme activity in T1 was very high at the time of treatment application, followed by T5. At 3 h AAT, there was an increase in PPO activity for T3, displaying values nearly twice higher compared with the enzyme activity at 0 h. At 6h AAT, PPO activity increased in T5, followed by a reduction at 12 h AAT. At 24 h AAT, T1 showed the highest enzyme activity, but at 48 h AAT, there were increases in PPO activity in T3

and T5. Finally, at 96 h AAT, the highest enzymatic activities occurred in T1 compared to the other treatments evaluated except T3 (Figure 3D).

At the beginning of the experiment, the highest SOD enzyme activities were observed in T4 and T5. At 3 h AAT, the tendency was maintained for T5, while SOD activity decreased by almost half in T4. At 6 h AAT, the lowest value of SOD activity was found in T2. However, at 24 h AAT, the highest SOD activities were observed in T0 and T3, but, at 48 hours AAT, the activity of this enzyme increased considerably in T4. At 96 hr AAT, T3 showed an increase in SOD activity compared to T0 and T2 (Figure 3E).

Finally, proline accumulation in leaves from plants grown under different Pb treatments was constant until 48 h AAT. At 96 h AAT, there was a large increase in the concentration of proline in T4 and T5 compared with the other treatments (Figure 3E).

Gene expression

The relative gene expressions of *psbA, psbO, Tpr1*, and *met* in leaves were evaluated at 0 and 96 h AAT. The results showed significant alterations ($p<0.05$) in the relative expressions of the four genes in leaves of young cacao plants of CCN 51 grown under different treatments of Mn, Pb, and Mn+Pb in soil.

At 0 h AAT, higher levels of *psbA* gene expression were observed in T2 but the expressions decreased dramatically at 96 h AAT. Meanwhile, T4 demonstrated the highest values of *psbA* gene expression in the leaves (Figure 4A). On the other hand, for *psbO*, the highest gene expressions at 0 h AAT were observed in T0, T2, and T5. However, at 96 h AAT, there was an increase the expressions of this gene in T1 and T5, which contained the highest doses of Mn (Figure 4B). The same results were also observed for *Tpr1* gene expression, which showed the highest values in T1, T2, T3, and T4. However, the expression of this gene was considerably reduced at 96 h AAT, showing no statistically significant differences across treatments (Figure 4C). For *met*, the highest gene expression values at 0 h AAT were found in T0, T2, T4, and T5. At 96 h AAT, only T4, which contained the highest Pb dose, had high expression of this gene (Figure 4D).

Discussion

Pb and accumulation of mineral nutrients

Excess Pb in the soil compromises seed germination and plant growth and development, causing serious problems for agriculture. In addition, many plant species will retain most of the absorbed Pb (approximately 95% or more) in the roots, while a small fraction is translocated to aerial parts of the plant (Pourrut et al., 2011). However, in this study, high doses of Pb and Mn applied to the soil resulted in higher contents of these metals in the leaves and roots. Pb has high affinity for the carboxylic groups of carbohydrates in the cell membrane and is accumulated in higher amounts near the endoderm; however, there are also reports of Pb transport via symplast and transmembrane, which explain its translocation to aerial parts of the plant (Chen et al., 2014). Moreover, the content of Pb in the plant tissues not decreased with Mn additions to the soil, demonstrated by a lower concentration of Pb in the leaves. This suggests competition between Pb and Mn during root uptake of these heavy metals from the soil, due to their similar electrochemical properties (load and valence). Therefore, a progressive increase in Mn uptake promotes a simultaneous decrease in Pb uptake and translocation to aerial parts of the plant. Consequently, this study demonstrated that Mn applied to the soil can be an alternative to mitigate Pb toxicity in cacao plants grown in contaminated soils (Chavez et al., 2016). Therefore, a progressive increase in the absorption of Mn does not promote a decrease in the absorption and translocation of Pb to the aerial parts of the plant. Consequently, this study showed that the Mn applied to the soil can be an alternative to mitigate the toxicity of Pb in cocoa plants grown in contaminated soils, for which the T1 treatment, in doses of 0.3 g Mn + 0.5 g Pb kg^{-1}, where these Mn doses present values that mitigate the Pb concentration applied to the soil.

Plants generally accumulate significantly more Pb in leaves than seeds (Clemens et al., 2013). This fact was also found in most previous studies on Pb accumulation in cacao beans, roots, stems, and leaves (Gramlich et al., 2017; Gramlich et al., 2018; Ramtahal et al., 2016). Nevertheless, Chavez et al. (2016) report even higher Pb concentrations in beans than in leaves of cacao trees in Ecuador. Cacao roots also show higher accumulated levels of Pb since roots are the first organ in contact with soil elements, constituting the first filter of nutrients to the plant. However, lower Pb

contents in treatments with Mn and Pb applications at different concentrations provide insight into how these elements compete within the diffuse double layer of the soil that contains the elements that will be absorbed by the roots.

The absorption of non-essential metals by plants interferes with the absorption of macro and micronutrients, due to antagonistic, additive and / or synergistic effects (Murakami et al., 2009). However, the results of this research show that the accumulation of Pb does not influence the concentration of macronutrients. This macronutrient is present in the plant cells since it is a main component of proteins, nucleic acids, and hormones. Thus, cacao plants may have increased protein synthesis in roots as a tolerance mechanism to Pb toxicity (Ovečka and Takáč, 2014). Similar results were obtained by Reis et al. (2015) for young cacao plants when seeds were soaked for 24 h in solutions with different concentrations of Pb, enriched with macro and micronutrients, Pb^{+2} competes for cation channels with elements such as K^{+}, Ca^{+2}, and Mg^{+2}, explaining the lower concentrations of these mineral macroelements (Pourrut et al., 2011).

The uptake of mineral elements, such as Mn, also occurs through cation channels (Dalcorso et al., 2013), preferably divalent cations. A higher Mn content was observed in the leaves of the plants in T3 (0.45 g Mn + 0.25 g Pb kg^{-1} soil), which was 14 times higher than in the control. Mn may have contributed to mitigate the effects of Pb toxicity on cacao plants since increased Mn uptake may have reduced soil Pb uptake through the root system (Ashraf et al., 2015). In addition, Mn acts on the oxygen-evolving complex in PS2 from the photochemical phase of photosynthesis, which can be displaced by Pb, dissociating it from the photosynthetic machinery (Ashraf et al., 2015).

A reduced Ca content in roots of cacao plants grown in T1 and T4 at 0.3 g Mn + 0.5 g Pb kg^{-1} soil and 1g Pb kg^{-1} soil, respectively, was also reported in previous studies on other plant species (Inoue et al., 2013). Besides competing for cation channels (e.g., with Ca^{+2}), Pb^{+2} also competes with Ca^{+2} for bonds to the non-methyl-esterified chains of galacturonic acids (Chen et al., 2014). The low-ester pectins containing negatively-charged carboxyl groups that would normally bind to Ca^{+2} will instead bind to Pb^{+2} because of their higher affinity to the latter metal (Krzesłowska et al., 2016). A study on *Populus* and other species of different plant genera exposed to Pb toxicity found that the cellular membrane of the root tissues of all species had remodeled and become thicker as a defense strategy (Krzesłowska et al., 2016). This strategy can also be accounted for in

this study, which reports high Pb and Ca contents in the roots; these tissues are the first filter for nutrients before being absorbed and structural modifications in roots are defense mechanisms against toxicity.

This research found no significant changes in N content in the roots and stems of young cacao plants. However, for P and K, T4 (1 g Pb kg^{-1} soil) showed the lowest contents of these macroelements. The same trend was observed for S, Cu, Fe, Mg, Zn, and Mn. According to Zhou et al. (2016), low Pb accumulation in aerial tissues of grafted apple trees depends on the contents of Pb and other micronutrients in the leaves and roots and the translocation of these elements relies on the combination between genotype and cultivar and a strong expression of genes involved in physiology of the plant.

Mineral micronutrients, such as Cu and Fe, usually act as enzyme cofactors; therefore, disturbances in the concentrations of these elements can inactivate enzymes and disrupt metabolic processes of plants. In addition, Cu and Fe participate in Fenton's reactions that produce reactive oxygen species; therefore, in excess, these metals may increase oxidative stress (Nagajyoti and Sreekanth, 2010). This study demonstrates an increase in the concentration of Cu in the roots, while Fe concentration decreased in the roots and increased in the leaves. The tendency of cacao trees to accumulate comparatively higher Pb concentrations in the beans and leaves was also observed in previous studies conducted in Ecuador, Malaysia, and Honduras (Arguello et al., 2019).

Pb and leaf gas exchange

In this work we show that the toxicity of Pb and the addition of Mn to the soil reduce the photosynthetic activity in young cacao plants, as can be observed at 0d AAT when the control is compared with the other treatments, taking AAT as critical time, since after this period of time two treatments died from toxicity (T2 and T3) where T3 decreases its photosynthetic activity considerably (figure 1). Net photosynthesis per unit area (*A*) and stomatal conductance (*gs*) were higher at T0 throughout the experiment, especially at 0 and 30d AAT.

Furthermore, critical periods were found at the beginning and the end of the evaluation period; specifically, at the end of the experiment, the plants in T2 and T3 died due to the toxicity generated by the addition of Pb and Mn to the soil. A considerable

reduction in transpiration (*E*) was observed across the different treatments, mainly in treatments in which the plants that died at the end of the experiment.

Reduced CO_2 assimilation is directly related to lower *gs* and *E*. In addition to the damage caused by Pb to the ultrastructure of mesophyll cells from cacao leaves, mainly at the chloroplast level, excess Pb in the soil and roots of cacao plants can alter the osmotic potential of the roots in relation to the soil matrix potential. This, in turn, can reduce water uptake by the root system and its translocation to the aerial parts, inducing partial and total closure of stomata and, consequently, reducing *gs* and *E* and the entry of CO_2 (Suzuki et al., 2014). Studies on *Populus* (Han et al., 2013; Emami et al., 2016) and *Mimosa caesalpiniae* plants (De Souza et al., 2012) exposed to soil Pb toxicity also report reduced *A*, *gs*, and *E* values.

Overall, Pb toxicity and reduced *A* lead to obstruction of the electron transport chain, inadequate stomatal closure, increased chlorophyllase activity, disturbance in water status, among others (Han et al., 2013), because the absorption of heavy metals uses the same transport channels of the essential elements for the plant, and Pb is not essential in any process and, on the contrary, generates toxicity, as seen in figure 3. Thus, this heavy metal plays an important role in the photosynthetic process since it is involved in the oxidation of the water molecule in PS2, providing sufficient amount electrons to the photosynthetic electron transport chain, in addition to participating in the activation of various enzymes involved in cell metabolism (Millaleo et al., 2010). However, it has been reported that excess Mn may result in reduced CO_2 uptake, which is one of the major phytotoxic effects of Mn (Millaleo et al., 2013). This fact was not proven here.

In this study, the proportions of CO_2 concentration in the foliar mesophyll and atmospheric CO_2 concentration (*Ci/Ca*), intrinsic efficiency of water use (*A/gs*), instantaneous efficiency of water use (*A/E*) and efficiency instantaneous carboxylation. (*A/Ci*) (Figure DG) decreased dramatically at the end of the experiment at 30 days AAT, in the control treatment and T5. This occurred mainly in T2 and T4 (i.e., with the highest dose of Pb applied to the soil). Furthermore, T0 and T5 (i.e., with the highest Mn content applied to the soil) exceeded by two-fold the values of T2 and T4 for these variables. Therefore, these results allow us to infer that high concentrations of Mn applied to the soil can generate toxicity to cocoa plants, because the treatments T2 and

T3 died at 15 d AAT, however T0 and T5 do not present statistical differences for *Ci/Ca, A/gs, A/E,* and *A/Ci* at 30 days AAT.

The results shown in this work allowed inferring that the exposure time and the dose of Pb applied to the soil are directly associated with the harmful effects of Pb toxicity on the photosynthetic parameters of cacao plants. This fact can be explained by the chelation of Pb, involving phytochelatins and metallothioneins, when there is low Pb content in soil, followed by storage of Pb in the vacuole and the irreversible adsorption of this heavy metal into the cell membrane of leaf tissues (Le Gall et al., 2015). According to Millaleo et al. (2013), excess Pb decreases photosynthesis, mainly leaf gas exchange parameters. Therefore, the results found here indicate that Pb is one of the major inorganic contaminants in the environment and its presence in the soil or atmosphere is considered a serious threat to agriculture.

Fluorescence emission

Chlorophyll fluorescence emission assessments provide valuable information on photosynthesis in stressed plants based on parameters such as minimum fluorescence (*Fo*), maximum fluorescence (*Fm*), and *Fv/Fm* ratio. These parameters evaluate the quantum efficiency of PS2; furthermore, ratio values below 0.78 indicate a stress condition for the plants (Baker, 2008). In this research, Pb toxicity in cacao leaves altered the initial chlorophyll fluorescence emission parameters of plants at 15 days AAT (figure 2A), indicated by a reduced *Fo* ratio in T4. The reduction reflects signs of photoinhibition and can be caused by oxidative stress that induces damage to important proteins and lipids of the photosynthetic machinery (Sytar et al., 2013). However, at 15 days AAT, reduced *Fo*, values were more pronounced for both doses of Pb applied to the soil. The decreased photosynthetic efficiency of plants exposed to Pb is caused by damage to the chloroplast ultrastructure and lipid composition of the thylakoid membranes, as well as a reduction in chlorophyll and carotenoid syntheses (Sharma and Dubey, 2005; Kalaji et al., 2012).

Studies conducted by Sytar et al. (2013) showed that high doses of Pb in the soil can affect the functioning of the photosynthetic machinery. The results reported by the authors agree with those found in this study since the lowest values of *Fo* were also observed in the treatment with the highest dose of Mn in soil (T5), while treatments

without Pb in soil (e.g., T0) and a high concentration of Mn (e.g., T3, T2 and T1) showed the highest values of *F0*.

Pb and antioxidant enzymes

We evaluated the activity of the antioxidant enzymes SOD, APX, GPX, CAT, and PPO, as well as the proline content in leaves of cacao plants exposed to different Pb and Mn contents in soil (Table 2). There were significant statistical differences for enzyme activities between T0 (control) and the treatments with the highest doses of Pb and Mn (T3, T4, and T5). However, APX activity was maintained until 12 h AAT and between 48 and 96 h AAT. This tendency was also observed for the enzyme activities of GPX, SOD, and CAT. However, for T0 and T5 (i.e., with the highest dose of Mn applied to the soil), APX activity was low compared with the activity of the other enzymes (Figure 3A, B, C, and F).

Pourrut et al. (2011) demonstrated that plants exposed to Pb may increase or reduce the activity of antioxidant enzymes. In this work, the highest dose of Pb (treatment T4) was found to promote the enzyme activities of SOD and GPX in the leaves of young cacao plants. SOD acts as the first line of detoxification since it converts $O_2^{\bullet-}$ into H_2O_2, whereas GPX has H_2O_2 as substrate (Gill and Tuteja, 2010). On the other hand, APX, which also acts on H_2O_2, did not show changes in the leaves of cacao plants exposed to Pb toxicity in the soil. Although APX acts in the cytosol and mitochondria, several studies have shown that this enzyme plays a major role in chloroplasts (Caverzan et al., 2012). The high enzyme activities of SOD, GPX, and APX found here in the presence of Pb confirms previous findings for young cacao plants exposed to the toxic effects of Pb (Reis et al., 2015).

For PPO enzyme activity, the treatment with the highest dose of Pb applied to the soil (T4) showed lower values at 3 h AAT compared with the other treatments. This suggests that the activities of antioxidant enzymes are directly related to Pb uptake by the plant in the absence and/or presence of low Mn content in soil (e.g., treatment T0 without application of Mn and Pb to the soil). Pb is not a redox-active metal and cannot generate ROS by direct participation in Fenton's reactions; however, it induces ROS formation by interfering with the activity of electron transport chains (Shahid et al., 2014). ROS causes a variety of deleterious effects on plants, such as membrane lipid peroxidation, growth retardation, leaf chlorosis, darkening of the root system, and

inhibition of ATP production and DNA damage that results in programmed cell death (PCD) (Kumar and Kumari, 2015).

A high content of proline in plant tissue indicated stress induced by heavy metals that affected plant growth and development (Figure 3F). Therefore, proline, which is an essential osmolyte for plants under stress, acts as an important non-enzymatic antioxidant agent capable of eliminating excess ROS produced during the stress condition and inhibiting PCD (Gill and Tuteja, 2010). Treatments T4 and T5, with the highest doses of Pb and Mn, respectively, showed marked increases in proline content by the end of the experiment (96 h AAT) compared with the other treatments. Proline accumulation in response to heavy metal stress has been reported by several authors (Sharma and Dietz, 2008; Hossain et al., 2010). Proline reduces Pb stress not by sequestering the metal, but rather reducing damage through the formation of ROS and maintaining a rigorous reducing environment within the cell (Hossain et al., 2010). This fact was corroborated in this study.

Pb and gene expression

Gene expression analyses were performed after evaluating the enzyme activities involved in antioxidant metabolism. The most contrasting enzyme activities were observed at the time of treatment applications (0 h) and at the end of the experiment (96 h AAT) (Figure 4). The *psbA* gene displayed the highest expression in T2 (0.75 g Pb + 0.15 g Mn kg^{-1} soil). However, at 96 h AAT, the expression of this gene in T4 (i.e., with the highest dose of Pb applied to the soil) was twice higher compared with the other treatments. The *psbA* gene is in the chloroplast genome and encodes the D1 protein that plays a fundamental role in PS2 from the photochemical phase of photosynthesis, acting in the electron transport chain (Nelson and Yocum, 2006). The increased gene expression of *psbA* in leaves of cacao plants in T2 and T4 confirms the damage done to the quantum efficiency of PSII. Since this gene is of cytoplasmic (chloroplast) inheritance, the damage caused in this cellular organelle by Pb toxicity can reduce the transcript pool in its genome (Page et al., 2006).

The gene expression analyses of *psbO* revealed that it is linked to the dose of Mn applied to the soil. Treatment T5 showed higher expression of this gene from 0 h to 96 h AAT. However, T1, with 0.3 g Mn and 0.5 g Pb kg^{-1} soil, also triggered the activity of *psbO* at 96 h AAT. On the other hand, this gene was also expressed when cacao plants

were grown in Pb contaminated soil, but in fewer levels. The *psbO* gene is found in the nuclear genome and encodes the *psbO* protein, which is extrinsic to PS2 and is involved in the oxygen-evolving complex. Furthermore, *psbO* is also known as the Mn-stabilizing protein, which increases the efficiency of PS2 during the oxidation of the H_2O molecule (Popelkova and Yocum, 2011). Therefore, the increase in Mn concentration in the leaves of cacao plants exposed to Pb toxicity in the soil may have affected the signaling pathway for *psbO* gene expression (Li et al., 2015).

The gene expression of *Tpr1* only showed significant statistical differences at 0 h AAT, which allows inferring that the expression of this gene is done in short periods of time. According to Almeida et al. (2014), the regulation of *Tpr1* expression in plants submitted to stress conditions depends on the influx of Ca^{2+} into the cytosol, the activation of protein kinases, and protein phosphorylation, which involve regulatory mechanisms that can be activated within seconds and minutes (Almeida et al., 2014).

The gene expression of *met* was evaluated in the leaves of cacao plants in the presence and absence of Mn, Pb and Mn+Pb. This gene is involved in defense mechanisms against heavy metal toxicity. The high expressions of *met* in T4 and T5 at 0 h AAT were likely due to the high metal contents since these treatments had the highest concentrations of Pb and Mn kg^{-1} soil, respectively. Moreover, *met* is involved in the chelation of metals and showed overexpression in roots of cacao plants grown in Pb contaminated soil. Phytochelatins (PCs) have binding affinity for heavy metals when they are enzymatically synthesized using glutathione (GSH) as a precursor and form PC-metal complexes that are transported to the vacuole (Gupta et al., 2013). However, it has been shown that PC-Pb complexes are weaker than PC-Cd complexes (Sharma and Dubey, 2005); furthermore, in certain species, PCs do not confer tolerance to Pb toxicity (Gupta et al., 2013). On the other hand, metallothioneins (MTs) are proteins that also exhibit metal affinity but are not involved in metal sequestration into vacuoles (Lee et al., 2007; Auguy et al., 2013). Finally, the expression of *met* in cacao leaves in the presence of Pb was related to an increase in SOD enzyme activity. Accordingly, cacao plants exposed to Pb have high ROS production and maintain high gene expression of *met* (Bhaduri and Fulekar, 2012).

Conclusions

Young plants of the cacao clonal CCN 51 genotype grown in soils with high Pb, and Mn+Pb contents were found to accumulate these heavy metals in roots and leaves.

Mn has great potential for the mitigation of Pb in the soil, due to its divalent characteristics, but it must be managed in adequate doses since in high doses it can generate toxicity and cause the death of the young plants of the cacao clonal CCN 51 genotype.

Uptake of Pb and Mn by the roots and its transport into the aerial part of the plant promoted changes in leaf gas exchange and chlorophyll fluorescence emission, affecting the efficiency of photosystem 2 and the production of photoassimilates.

Pb, Mn and Mn+Pb toxicities activated defense mechanisms, in young plants of the cacao clonal CCN 51 genotype by altering the gene expression of *met*, *psbA*, and *psbO* and increasing the activity of enzymes involved in cellular detoxification of excess ROS in leaf level.

High uptake of Mn by root system was found to reduced Pb uptake in young plants of the cacao clonal CCN 51 genotype grown with Mn+Pb in the soil, in the doses corresponding to 0.3 g Mn + 0.5 g Pb kg^{-1} soil and 0.15 g Mn + 0.75 g Pb kg^{-1} soil, and mitigated the damage caused by Pb.

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Tables and Figures

Table 1 - Physical and chemical characteristics of the substrate for growth of cacao plants. P, Na, K, Fe, Zn, Mn, Cu (extracted by Mehlich 1), Ca, Mg, Al (extracted by KCl, 1 M) H + Al (extracted by Ca-acetate 0.5 M, pH 7.0), B (extracted by hot water). SB, sum of bases; t, effective cation exchange capacity; T, cation exchange capacity (pH 7.0); V, base saturation; m, Al saturation; ISNa, Na saturation index; OM, organic matter= Org C.×1.724; P-rem, remaining phosphorus;

pH	H_2O	4.7
P	mg dm^{-3}	5.9
K	mg dm^{-3}	22
Na	mg dm^{-3}	9
Ca^{+2}	$cmol_c$ dm^{-3}	1.1
Mg^{+2}	$cmol_c$ dm^{-3}	0.6
Al^{+3}	$cmol_c$ dm^{-3}	0.5
H+Al	$cmol_c$ dm^{-3}	4.13
CEC (t)	$cmol_c$ dm^{-3}	2.3
CEC (T)	$cmol_c$ dm^{-3}	5.9
V	%	30
M	%	22
ISNa	%	1.7
OM	dag kg^{-1}	2.29
P-rem	mg L^{-1}	38.4
Zn	mg dm^{-3}	2.3
Fe	mg dm^{-3}	145.1
Mn	mg dm^{-3}	15.3
Cu	mg dm^{-3}	1.5
B	mg dm^{-3}	0.2
S	mg dm^{-3}	15.9

Table 2 - Pairs gene-specific primers that were used in qRT-PCR analysis.

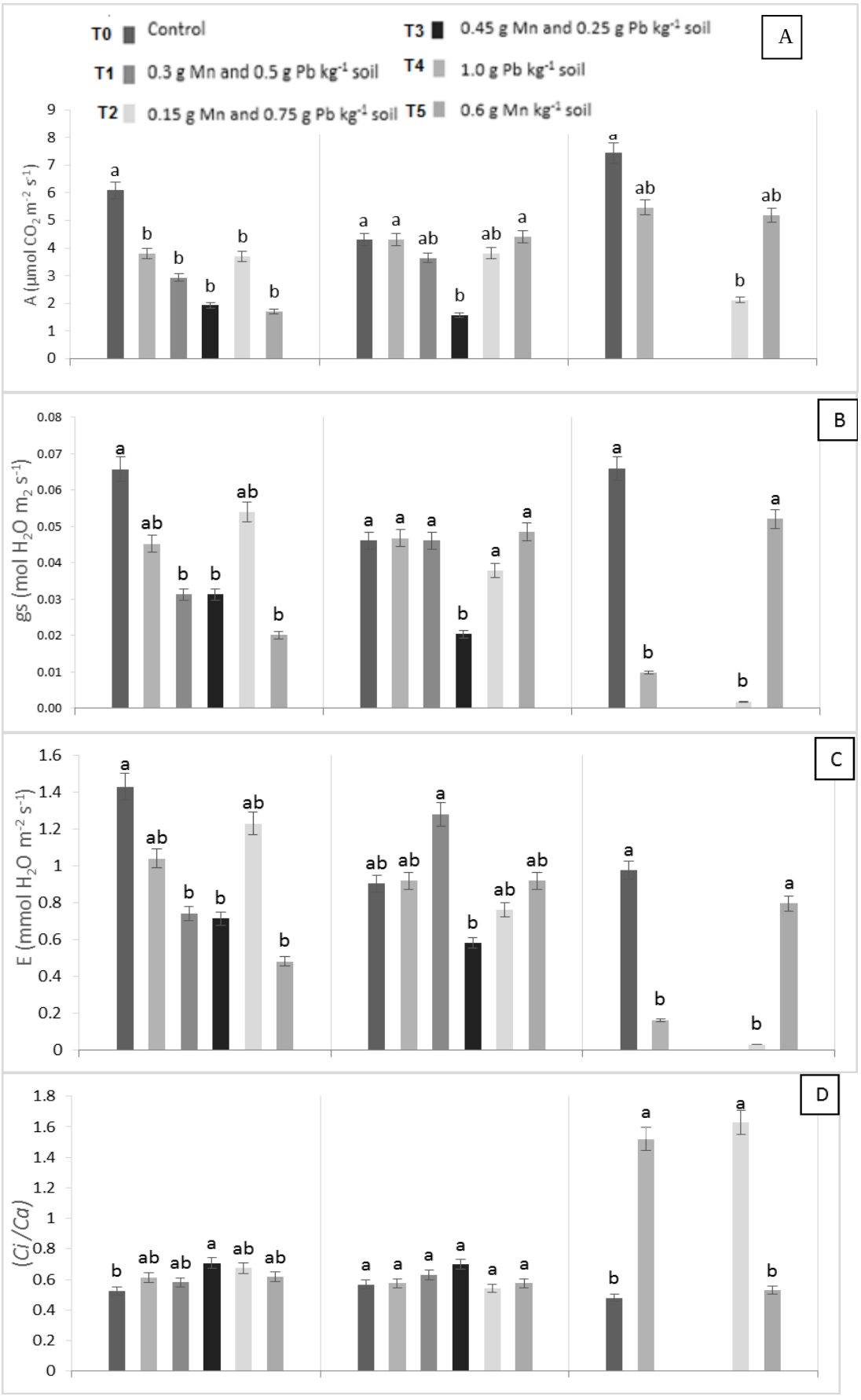
Gene	Access	Function	Primer
psbA	NC_014676.2[C]	Biosynthesis of the psbA protein or D1 protein.	F-5'GGTTTGCACTTTTACCCGA-3' R-5´CTCATAAGGACCGCCATT-3´
psbO	CL 326conting1[a]	Biosynthesis of the psbO protein.	F-5'GCAAACGCTGAAGGAGTT-3´ R-5'GGCTTGAAGGCAAATGAGTC-3´
Tpr	Q5NBT9	Associates both with chromatin in the HSP70 promoter and with mRNAs transcribed from this promoter under stress-induced conditions	F-5'ATGAGCAGCTAAAGCAGGGAA-3´ R-5'TTCCCTCCTTTACCTGCTCAT-3
Met		Ability to bind to both heavy physiological metals and xenobiotics.	F-5' AARAGYTGCTGYTCCTGCTG-3´ R-5' CAGCAGGARCAGCARCTYTT-3
B-tubulin	GU570572.1[C]	Endogen	F-5´TGCAACCATGAGTGGTGTCA-3´ R-5´CAGACGAGGGAAAGGAATGA-3´
Actina	Xm_ 018128615[c]	Endogen	F-5´TCCTCTTCCAGCCATCTCTC-3´ R-5´TCTCCTTGCTCATTCGGTCT-3´

Table 3 - Accumulation of Pb and changes in the contents of macronutrient and micronutrients in roots of CCN 51plants, submitted to doses of Pb, Mn and Pb+Mn in soil to the 30 days after application of treatments. Mean values of five replicates (± SE). Letters indicate comparisons among treatments by Tukey test ($p<0.05$).

	N	P	K	Ca	Mg	S	Zn	Mn	Pb	Cu	Fe	B
TRAT	----------cmolc dm^{-3}----------						----------mg dm^{-3}----------					
0	2.8ab	0.27b	1.7b	1.4a	0.68ab	0.12a	63.9a	663e	0e	3.7a	1393ab	29.3c
1	2.3b	0.19c	1.7b	0.9d	0.58c	0.08b	42.0c	1786d	1827a	4.01a	3135a	26.1c
2	2.7ab	0.32a	2.3a	1.1cd	0.66bc	0.13a	49.4b	2517c	773bc	3.3ab	1010ab	42.7b
3	3.2a	0.32a	2.3a	1.2abc	0.76a	0.13a	49.8b	3780b	302d	3.6ab	1056ab	57.2a
4	3 ab	0.25b	1.8b	1.1bc	0.69ab	0.12a	42.1c	582e	806b	2.7b	781c	27.8c
5	2.8ab	0.26b	1.95b	1.3ab	0.67b	0.12a	40.5c	6435a	0e	3.5ab	1676b	39.98b

Table 4 - Accumulation of Pb and changes in the contents of macronutrient and micronutrients in leaves of CCN 51 plants, submitted to doses of Pb, Mn and Pb+Mn in soil to the 30 days after application of treatments. Mean values of five replicates (± SE). Letters indicate comparisons among treatments by Tukey test ($p<0.05$).

	N	P	K	Ca	Mg	S	Zn	Mn	Pb	Cu	Fe	B
TRAT	----------dag kg^{-1}----------						----------mg kg^{-1}----------					
0	2.0a	0.21a	1.10a	0.50a	0.42a	0.12a	43.1a	128c	0e	19.8a	2694a	34.9ab
1	2.0a	0.22ab	0.85ab	0.50a	0.40a	0.11ab	36.9a	743c	3931a	20.2a	2460a	36.8a
2	2.1a	0.30a	1.20a	0.50a	0.40a	0.13a	43.7a	1031d	874c	20.1a	1473ab	47.8a
3	2.2a	0.30a	1.04a	0.47a	0.40a	0.13a	43.7a	1905b	755c	18.4a	1506ab	47.6a
4	2.0a	0.10b	0.50b	0.28b	0.15b	0.07b	14.7b	43.1e	1771b	2.20b	536b	17.7b
5	2.1a	0.25a	0.79ab	0.45ab	0.35a	0.12a	31.2ab	2366a	7.5d	18.9a	1819ab	37.9a


T0 Control
T1 0.3 g Mn and 0.5 g Pb kg-1 soil
T2 0.15 g Mn and 0.75 g Pb kg-1 soil
T3 0.45 g Mn and 0.25 g Pb kg-1 soil
T4 1.0 g Pb kg-1 soil
T5 0.6 g Mn kg-1 soil
A
A (µmol CO2 m-2 s-1)
B
gs (mol H2O m2 s-1)
C
E (mmol H2O m-2 s-1)
D
(Ci/Ca)

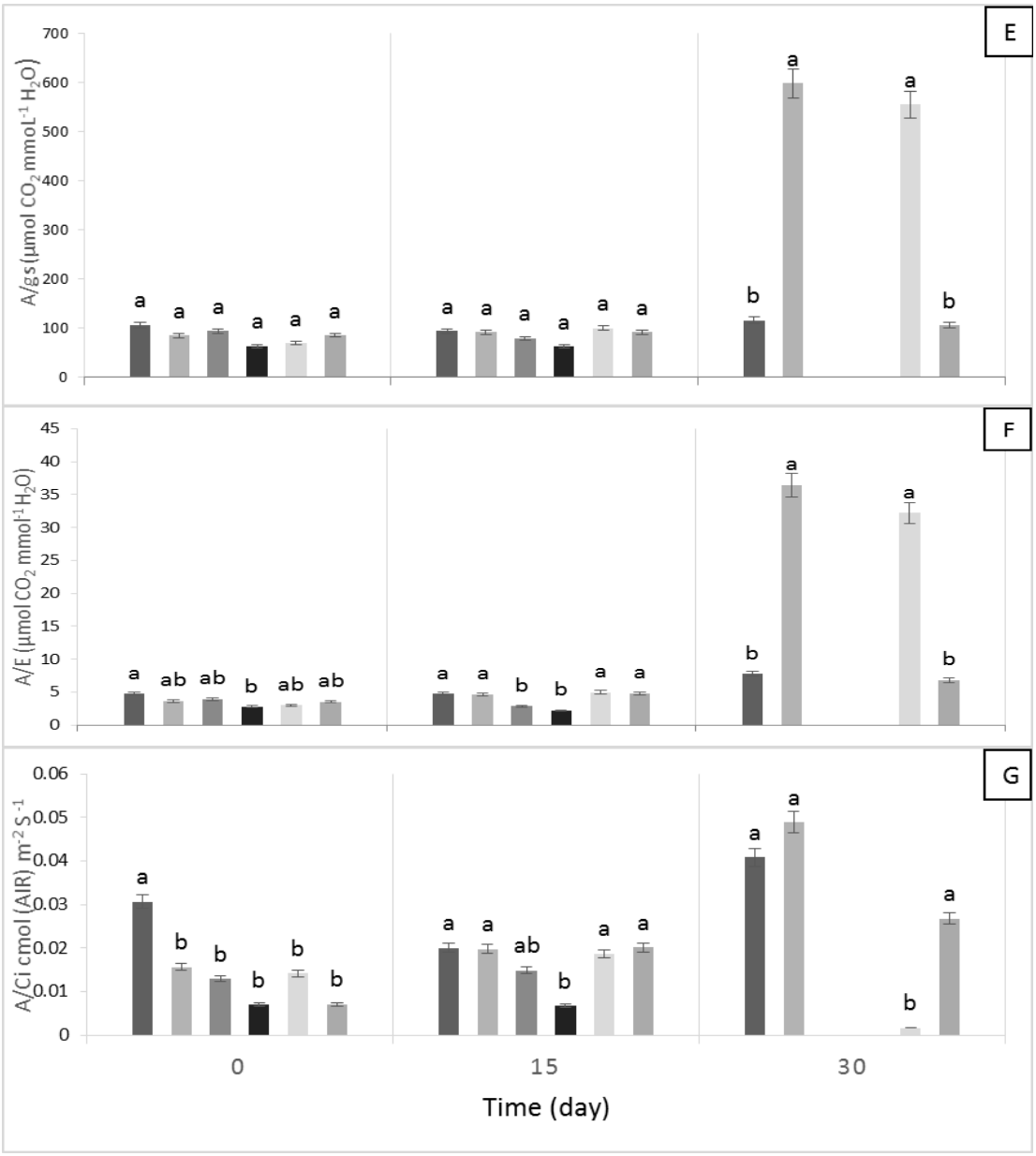


Figure 1 - (A) Net photosynthesis per unit leaf area (*A*), (B) stomatal conductance to water vapor (*gs*), (C) transpiration rate, (D) ratio of internal and atmospheric CO_2 concentration (*Ci/Ca*), (E) intrinsic efficiency of water use (*A/gs*), (F) instantaneous efficiency of water use (*A/E*), (G) instantaneous efficiency of carboxylation (A/Ci) in leaves of cacao young plants, submitted to doses of Pb, Mn and Pb+Mn in soil to the 0, 15 and 30 days after application of treatments. Mean values of four replicates (± SE). Lower case letters indicate comparisons between treatments and capital letters between time in days by Tukey test ($p<0.05$).

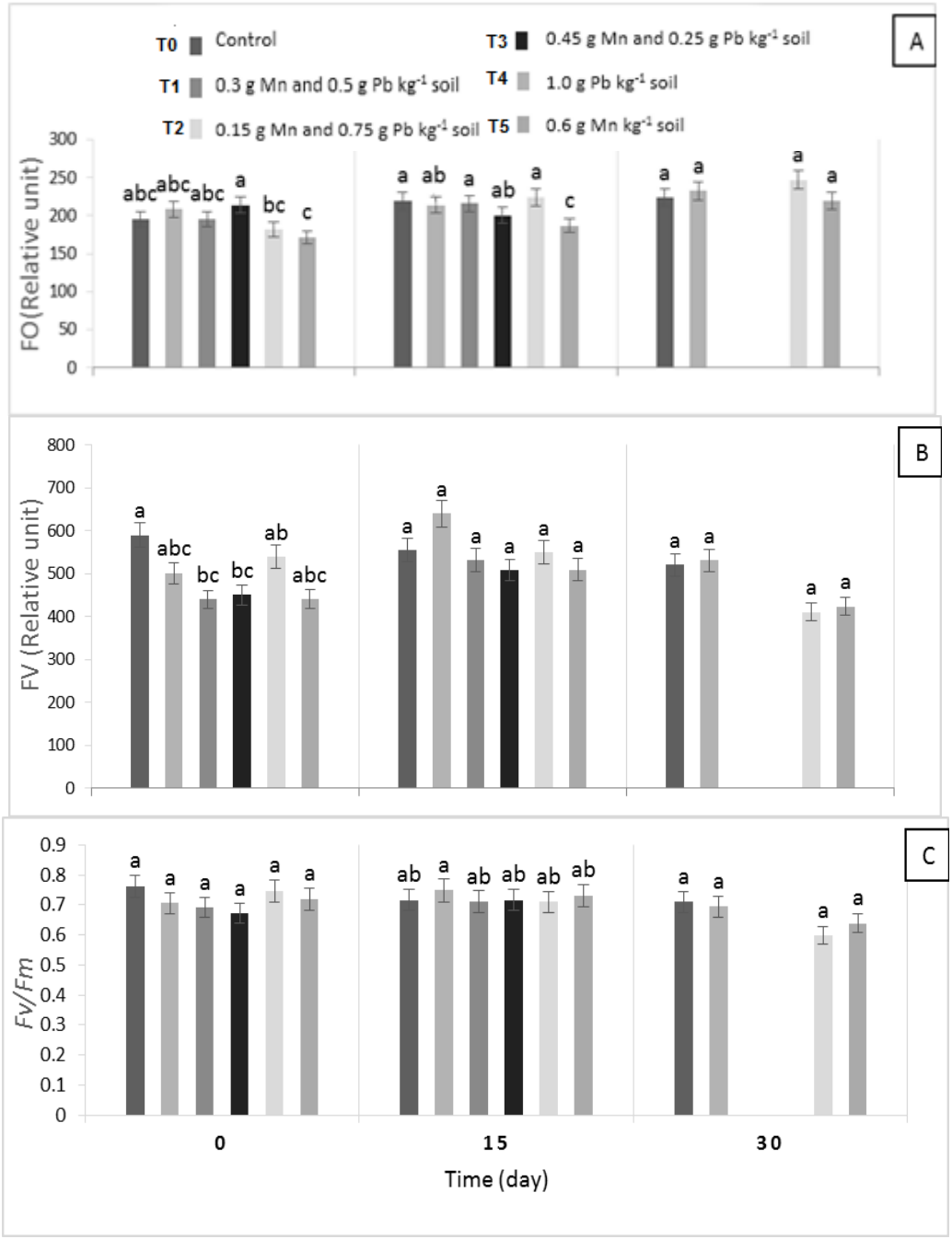
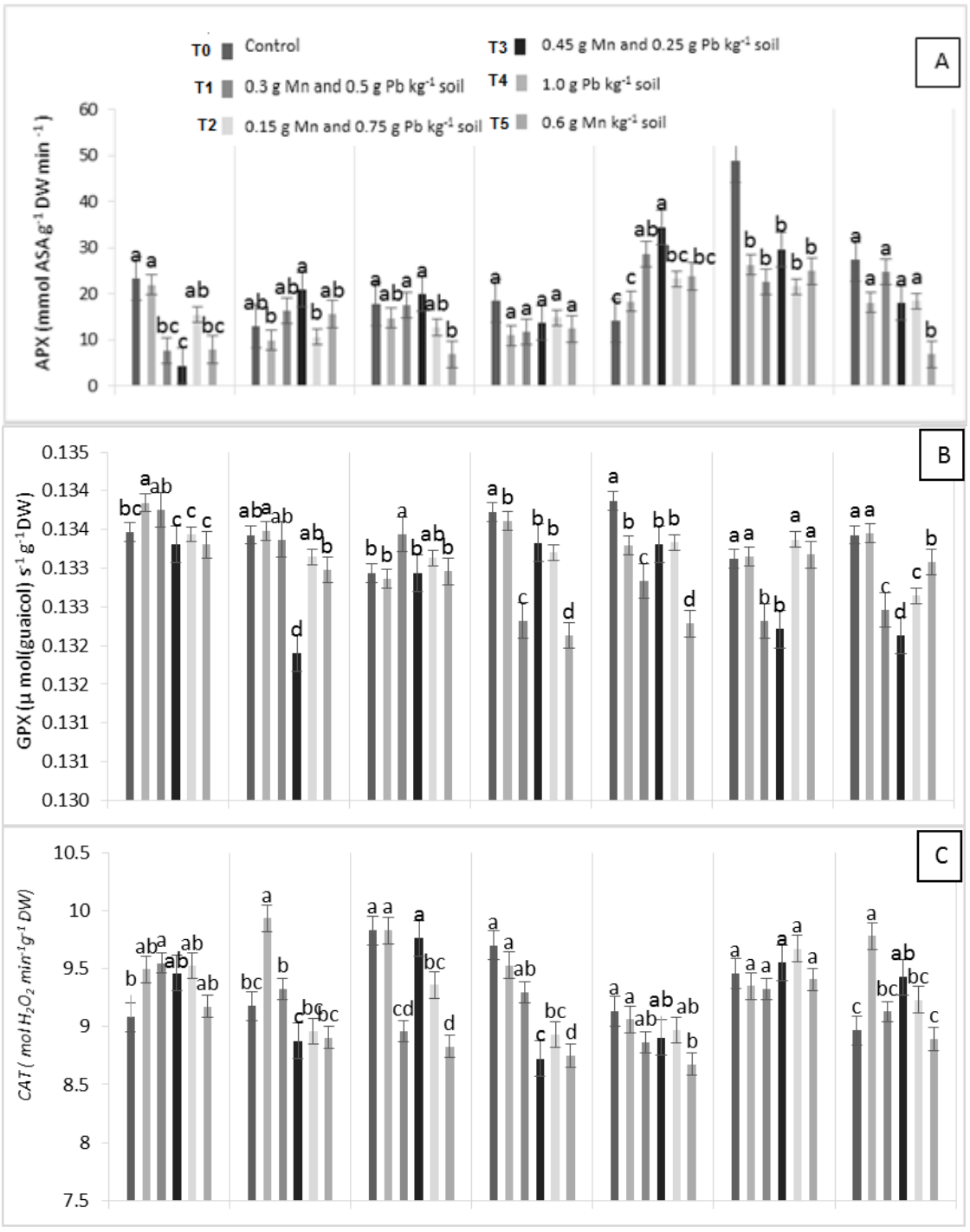


Figure 2 - (A) Initial fluorescence (F0), (B) variable fluorescence (Fv) and (C) maximum quantum yield of photosystem 2 (Fv/Fm) in leaves of cacao young plants, submitted to doses of Pb, Mn and Pb+Mn in soil to the 0, 15 and 30 days after application of treatments. Mean values of four replicates (± SE). Lower case letters indicate comparisons between treatments and capital letters between time in days by Tukey test ($p<0.05$).


T0 Control
T1 0.3 g Mn and 0.5 g Pb kg-1 soil
T2 0.15 g Mn and 0.75 g Pb kg-1 soil
T3 0.45 g Mn and 0.25 g Pb kg-1 soil
T4 1.0 g Pb kg-1 soil
T5 0.6 g Mn kg-1 soil
A
APX (mmol ASA g-1 DW min-1)
B
GPX (µ mol(guaicol) s-1 g-1DW)
C
CAT (mol H2O2 min-1g-1 DW)

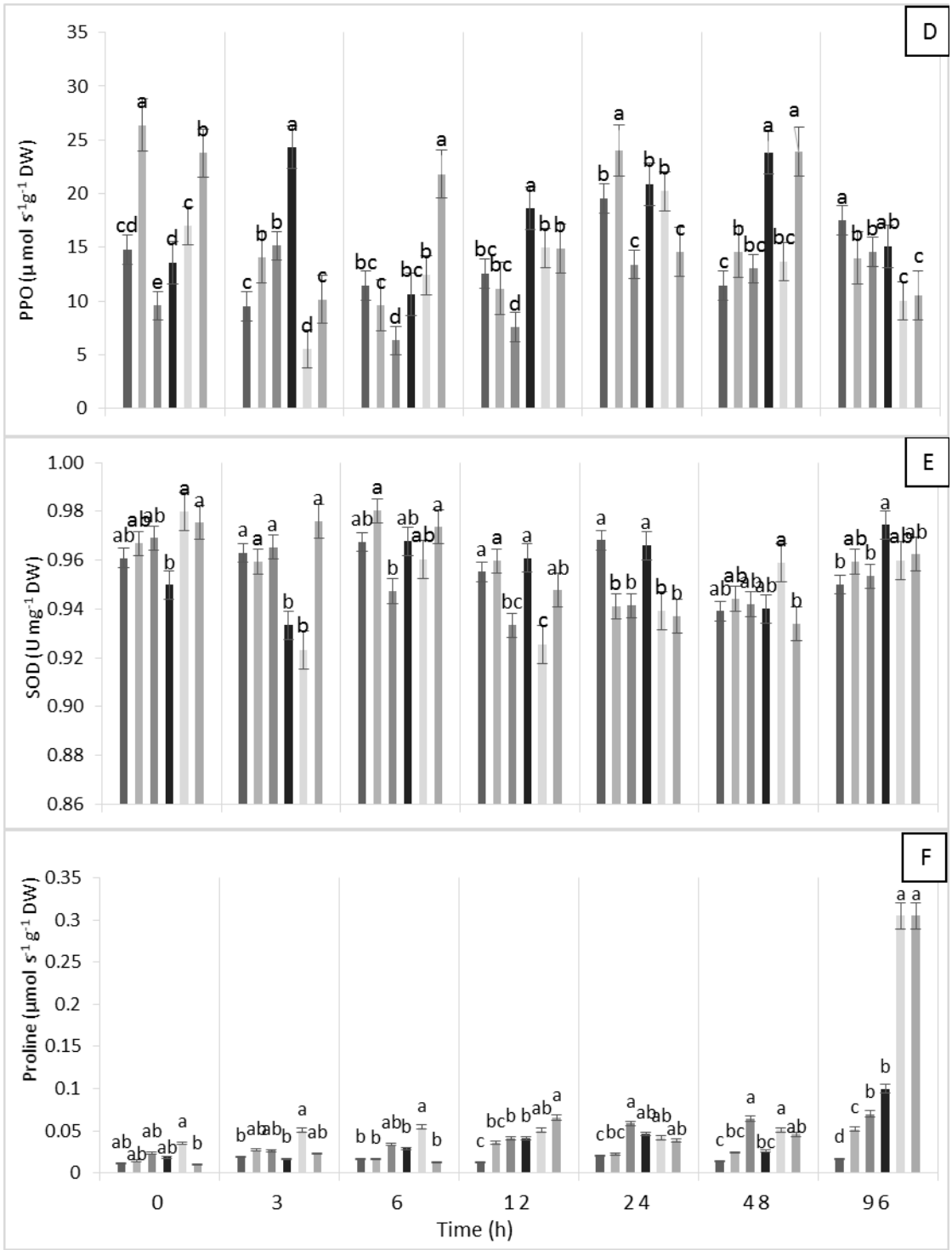


Figure 3 - Enzymatic activity of APX, ascorbate peroxidase (A), GPX, guaiacol peroxidase (B), CAT, catalase (C), PPO, polyphenol oxidase (D), SOD, superoxide dismutase and proline content (F) in leaves of cacao young plants submitted to different doses of Pb, Mn and Pb+Mn in soil to 0, 3, 6, 12, 24, 48 and 96 h after application of treatments. Mean values of five replicates (± SE). Letters indicate comparisons between treatments by Tukey test ($p<0.05$).

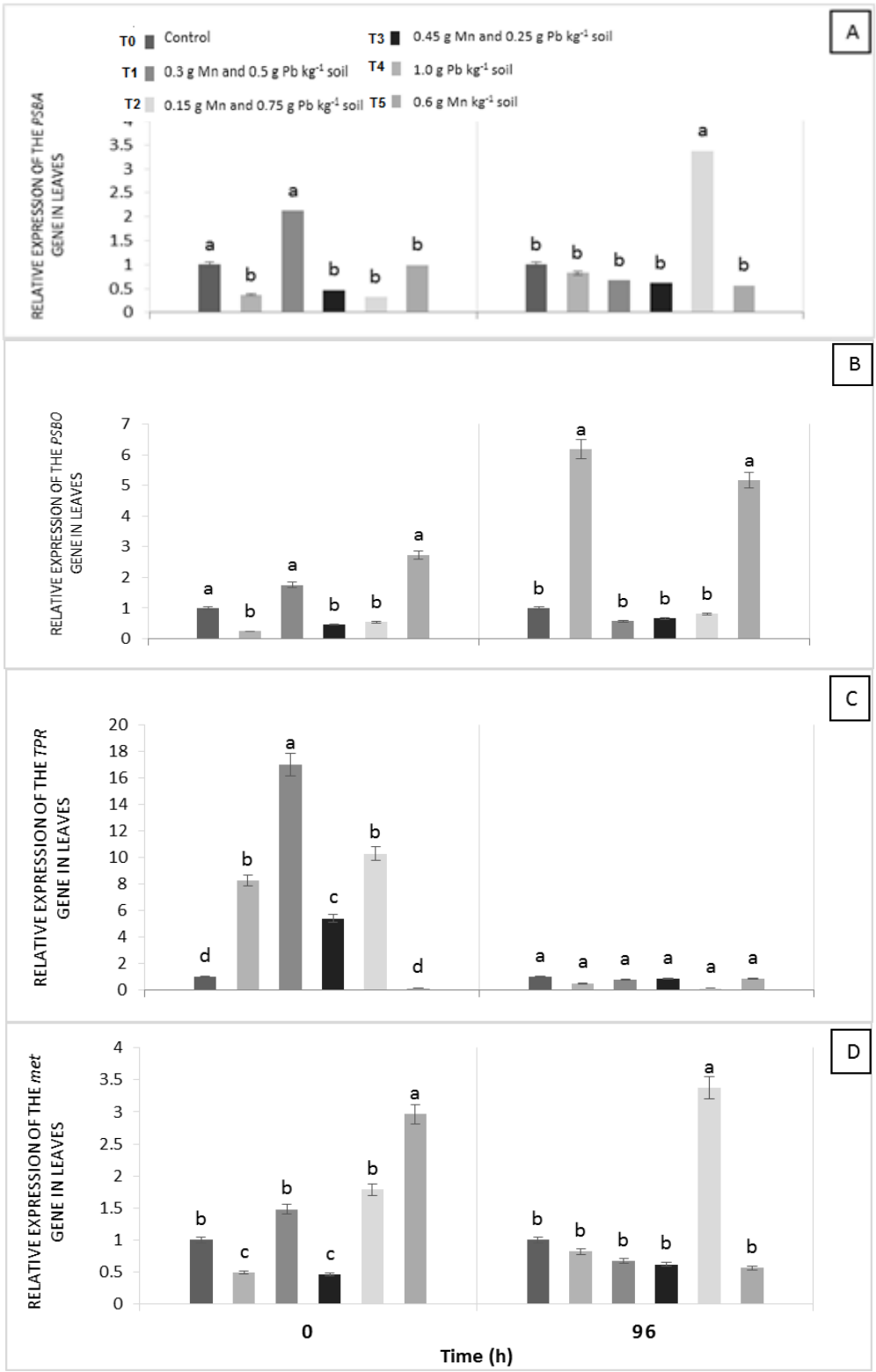


Figure 4 - Relative expression of genes *PsbA* (A), *PsbO* (b), *Tpr* (C) and *met* (D) in leaves of cacao young plants, submitted to doses of Pb, Mn and Pb+Mn in soil at 0 and 96 h after application of treatments. Mean values of four replicates (± SE).The statistical significance was determined by Tukey test ($p<0.05$).

4- Chapter 2

Influence of Zn in mitigating Pb toxicity in young plants of CCN 51 cacao clonal genotype grown in soil: physiological, biochemical, nutritional and molecular responses

Abstract

The soil is among the main contamination sources of Pb in cocoa beans, which carries potential risks to human health from ingesting contaminated cocoa products. Therefore, the Pb contents in cocoa beans depend not only on the genotype, but also on the geographic location. Pb toxicity in plants is highly modified by increasing the Zn/Pb ratio. Pb uptake by the roots decreases with the increase in the Zn content in hyperaccumulator plant species of Pb/Zn, as well as in non-accumulator species, clearly indicating that the inflow of Pb is largely attributed to Zn transporters, with a strong preference for Zn at the Pb detriment. The objective of this study was to evaluate the influence of Zn on mitigation of Pb toxicity in young plants of the cacao clonal CCN 51 genotype grown in soils with different doses of Pb, Zn and Zn+Pb, through physiological, biochemical, molecular and nutritional responses. It was observed in the present work that the young plants of the cacao clonal CCN 51 genotype grown in soils with high Pb, Zn and Zn+Pb contents were found to accumulate these heavy metals in the roots and leaves. Uptake of Pb and Zn by the roots and its transports into the aerial part promoted to significant physiological changes in the photosynthesis, nutritional balance, antioxidant metabolism, and gene expression of plants. Increased SOD enzyme activity and proline content in leaves contributed to mitigate Pb and Zn toxicities at the highest doses of these metallic elements applied in soil. In addition, Zn+Pb adequate doses applied in soil mitigated the toxicity of Pb in plants. On the other hand, Zn+Pb and Zn doses applied in soil, corresponding to 0.45 g Zn + 0.25 g Pb kg^{-1} soil and 0.6 g Zn kg^{-1} soil, respectively, induced the death of young plants of the cacao clonal CCN 51 genotype at 15 days after application of treatments. Soon, application of Zn in adequate doses in the soil can be used to mitigate the Pb toxicity in young plants of the cacao clonal CCN 51 genotype growns in contaminated soils.

Keywords: *Metal toxicity, photosynthesis, antioxidant metabolism, gene expression, mineral nutrients.*

Introduction

Theobroma cacao L. is a woody species typical of tropical climate and preferably allogamous. Previously the species was classified into the family Sterculiaceae (Cuatrecasas, 1964), but recently was reclassified into the Malvaceae family (Alverson et al., 1999). Under natural conditions, the cocoa tree can reach 20 to 25 m in height (Lachenaud et al., 1997), while under cultivation it normally varies from 3 to 5 m. The geographical origin of cocoa is South America, where several wild populations can be found in the regions of Amazonia and Guianas (Motamayor et al., 2002). '*CCN 51*' is a highly productive, disease-resistant, and precocious cultivar that produces large pods and beans after only two years of transplanting to the field. The beans have high butter content (54%), one of the highest yields for the cacao butter industry (Marita et al., 2001). With appropriate pre-drying before box or ''yute'' (sack) fermentation, the cultivar produces low acidity and acceptable flavor but does not meet the qualification of fine-flavored beans (Amores et al., 2011). Cacao OR cocoa (as you wish) is considered one of the most important perennial crop on the planet, with an estimated global production of more than 4 million tons (ICCO, 2018), and is cultivated predominantly in tropical areas of South America, Central America, Asia and Africa (Marita et al., 2001).

Lead (Pb) is found naturally in the earth's crust. However, the levels of Pb in the soil have been increasing through anthropogenic actions in industries such as battery manufacturing, metal mining and smelting (Caussy et al., 2003), urban and industrial waste, fertilizers, pesticides, and additives (Shahid et al., 2012). Pb has no known biological function, so it is not an essential element for plants. However, many studies have shown that certain plant species can absorb it and then show signs of toxicity (Wang, 2015). The absorption of Pb by the roots is regulated by pH, particle size and cation exchange capacity in the soil, as well as by exudation and other physicochemical parameters (Gupta et al., 2013). Once absorbed by the plants, Pb causes several direct and indirect effects on seed germination, growth and metabolism (Patra et al., 2004). As its concentration in the soil increases, Pb can begin to impair the ultrastructure of subcellular components such as chloroplast, mitochondria, nucleus and membranes in plants. This damage can induce loss of function in the organelles and eventually affect

normal physiological functions such as photosynthesis, respiration, protein synthesis, cell division, inducing programmed cell death (Salazar and Pignata, 2014).

The plants either diminish or neutralize the effects of Pb by means of specific mechanisms of protein and non-protein origin. The cell wall is the first barrier to prevent damage to the cells by containing pectin with certain carboxylic groups, polysaccharides and proteins that fix Pb ions, reducing movement between the cytoplasm and avoiding damage of the protoplast (Wang, 2015)**.** There are non-protein thiols such as phytochelatin, which is a family of peptides rich in cysteine that are synthesized enzymatically from glutathione (GSH), which play an important role in the prevention of oxidative stress in plant cells. Further they are involved in detoxification and accumulation of several metals in vacuoles, including Pb (Gupta et al., 2013). In addition to phytoquelatins, there is the role of metallothioneins (MT) and of the antioxidant metabolism involved in the elimination of reactive oxygen species (ROS). Functions of MT resemble phytochelatins; they have high affinity for many metals, including Pb. They are low-molecular-weight proteins with high cysteine content that are overexpressed when organisms are subjected to high concentrations of metal (Auguy et al., 2016). Enzymes of antioxidative metabolism, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidases (GPX) among others, can attenuate the ROS effects (Gill and Tuteja, 2010).

Pb-induced toxicity may either inhibit or activate the activity of enzymes involved in the antioxidative metabolism and influence the expression and synthesis of these enzymes. However, these actions will depend on metal speciation, plant species and duration and/or metal concentrations (Pourrut et al., 2011). The activity of these enzymes maintains the integrity of cell membranes and important molecules, such as DNA and proteins, avoiding lipid peroxidation and cell death, and therefore reducing the damage caused by Pb (Gill and Tuteja, 2010). Scientific research has recently demonstrated the presence of Pb in soils used for cocoa grown and in products derived of cocoa beans, the example of chocolate (Yanus et al., 2014). The presence of Pb in the soil can be justified by the original rock during soil genesis, by the use of phosphate fertilizers and Cu-based fungicides. These fungicides are applied by cocoa producers with the aim to reduce the attack of diseases such as black pod rot and witches' broom, which have *Phytophthora* spp., and *Moniliophthora perniciosa* as causal agents, respectively. The present study had as main objective to evaluate the influence of Zn on

mitigation of Pb toxicity in young plants of the CCN 51 clonal cocoa genotype, cultivated in the soil with different concentrations of Pb, Zn and Zn+Pb, through physiological, biochemical, molecular and nutritional responses.

Material and methods

Plant material and growing conditions

The experiment was carried out in a greenhouse at the State University of Santa Cruz (UESC), Ilhéus, Bahia, Brazil (14° 47' S, 39° 10' W).

The young plants of CCN 51 were obtained from the rooting of stem cuttings from the ends of plagiotropic branches at the beginning of secondary growth, containing the apical bud, three auxiliary buds and three leaves, taken from five- to 10-years old parent plants. The bottoms of the cuttings (~ 3 cm) were dipped into chemically inert talcum powder containing indol-3-butyric acid (IBA) at 4 g kg^{-1}. Afterwards, each cutting was transferred to a 288-cm^3 tubelike, black plastic pot containing organic substrate (turf + grinded *Pinus* spp., barks and grinded coconut fiber at 1:1 ratio), enriched with macro and micronutrients, according to the recommendations for cacao (Souza Junior, 2008)

After rooting (4 to 5 months of age), the young plants of CCN 51 were transplanted to drilled plastic vessels with a capacity of 20 kg. The soil was fertilized with N, P and K (Supplementary material) and a mixture of $CaCO_3$ and $MgCO_3$, important to reach the Ca^{+2}: Mg^{+2} 4:1 ratio, and raising the soil base saturation value to 30%, which results in increased pH (4.7) results that were obtained after the analysis of the soil used for this experiment. (Table 1). The level of fertilization for cacao was based on the needs of the crop during the 120 days of the experiment (Souza Junior, 2008) (Table 1). For the application of water to the plants, first the field capacity of the soil was measured, to know the quantity to be applied in each pot. When the plants were four-months old, the treatments of Pb, Mn and Mn+Pb in the soil were applied in a volume of 500 mL/pot, with the following concentrations: treatment 1 (T1) (0.3 g Zn kg^{-1} soil + 0.5 g Pb kg^{-1} soil), treatment 2 (T2) (0.15 g Zn kg^{-1} soil + 0.75 g Pb kg^{-1} soil), treatment 3 (T3) (0.45 g Zn kg^{-1} soil + 0.25 g Pb kg^{-1} soil), treatment 4 (T4) (1.0 g Pb kg^{-1} soil) and treatment 5 (T5) (0.6 g Zn kg^{-1} soil), together with the control (T0)

(without addition of Pb and Mn in the soil), totaling six treatments and having $PbCl_2$ and $ZnCl_2$ as sources of Pb e Zn, respectively.

During the whole experimental period, young plants of cocoa were watered with rainwater previously stored. The photosynthetically active radiation (PAR), temperature and relative humidity inside the greenhouse were continuously monitored and recorded by means of micrometeorological sensors (Hobo H8 Pro Series, Onset, USA). PAR, temperature and relative humidity means recorded during this period were 6±0.5 mol m^{-2} day^{-1}, 27±0.4°C and 78±0.7%, respectively.

Leaf gas exchange

During the experimental period, the net photosynthetic rate per unit leaf area (*A*), stomatal conductance to water vapor (estimated measurement) (*gs*) and leaf transpiration (*E*) were measured at 0, 15 and 30 days after application of treatments (AAT), between 08:00 and 12:00 h, in a fully expanded and mature leaf. Five plants per treatment were evaluates using a portable LI-6400 photosynthesis measurement system (Li-Cor, Nebraska, USA), equipped with a 6400-02B RedBlue artificial light source. The photosynthetic photon flux density and leaf temperature set at 800 μmol photons m^{-2} s^{-1} and 26°C, respectively, using equipment accessories. The readings were record in the range of 2-3 min (coefficient of variation from 0.1% to 0.2%). The values of *A*, *gs* and *E* were used to calculate the intrinsic (*A/gs*) and instantaneous (*A/E*) efficiencies of water use and instantaneous carboxylation efficiency (*A/Ci*).

Chlorophyll fluorescence

Measurements of chlorophyll fluorescence emission was done or performed on the same leaves used for gas exchange measurements, using a portable fluorometer (Pocket PEA Chlorophyll Fluorimeter - v 1.10 - Hansatech Instruments, Norfolk, UK), between 08:00 and 12:00 h. The selected leaves were adapted to the dark for a period of at least 20 min, for reflection of incident solar radiation, decrease of leaf temperature and oxidation of the entire photosynthetic electron transport system, using appropriate clips. After dark adaptation, the leaf tissue was illuminated with a weak-modulated measuring beam (0.25 kHz, < 0.1 μmol m^{-2} s^{-1}, 650 nm, 1 s) to obtain the minimal fluorescence (F_0). A saturating white-light pulse (20 kHz; 3500 μmol m^{-2} s^{-1}, 650 nm, 1 s) was applied to ensure the variable (F_V) and maximum (F_m) fluorescence emissions.

The maximum quantum yield of photosystem 2 (Fv/Fm) was calculated as [Fv/Fm = (Fm − F_0)/Fm)] (Baker, 2008). The fluorescence emission signals were recorded in the acquisition system of Pocket PEA data, using specific software.

Minerals nutrients and Pb

At the end of the experiment, 30 days after the application of the treatments, except treatments T3 and T4, which were collected at 18 days, since in this period of time the plants died, samples composed of roots and leaves were collected from different treatments. The dried and ground plant material were weigh out in triplicate (200 mg per sample) and placed in a 50 mL digestion tubes containing 3 mL of concentrated HNO_3 (Merck). The tubes were capped with a cold finger, containing distilled water. During the digestion of the samples, the block temperature was gradually increased: (i) 50°C for 30 min, (ii) 80°C for 60 min, and (iii) 130°C for 45 min, plus 1 mL of 30% hydrogen peroxide (Merck) for each temperature regime (Yang et al., 2014). Soon after, the 30% hydrogen peroxide was added for a further 2x (1 mL) at 20 min intervals. Subsequently, after 15 min of the last addition of hydrogen peroxide, the digester block was turn off. When samples reached room temperature, they were transfer into Falcon tubes and volumated to 15 mL with ultrapure water. Subsequently, the macro and micronutrient content and Pb were analyzed in an Inductively-Coupled Plasma Optical Emission Spectrometer (ICP-OES) model Varian 710-ES.

Enzymes of antioxidative metabolism

In order to perform the enzymatic tests at leaf level, the plant tissue was collected at 0, 3, 6, 12, 24, 48 and 96 h AAT, immersed in liquid nitrogen and stored in ultrafreezer - 80°C and subsequently lyophilized and stored in a freezer - 20°C. The samples were macerated in liquid nitrogen. The macerate was weigh, conditioned and then polyvinylpyrrolidone (PVP), 0.5 gr per 2 ml tube, was adding to prevent oxidation of the macerate. Immediately thereafter, the macerate was resuspended in extraction buffer (50 mM sodium phosphate buffer, pH 7.0 or 50 mM potassium phosphate buffer, pH 6.0), which varied according to the type of enzyme involved in the antioxidative metabolism, followed by shaking. Subsequently, the material was subjected to sonication, followed by centrifugation. Finally, the supernatant was collected,

considering it as the crude extract, which was transferred to a 2 mL microtube, kept in Styrofoam with ice, and used immediately.

The activity of the enzymes guaiacol peroxidase (GPX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), polyphenol oxidase (PPO, EC 1.10.3.1) was determined according to the methodological procedures described by Amako et al. (1994), Nakano and Asada (1981), Siddiq et al. (1992), and Yao et al. (2012), respectively. The sample and standard readings were done with a UV–vis spectrophotometer (SpectraMax Paradigm Multi-Mode Microplate Reader, Molecular Device, USA).

Proline

Plant tissue was collected at 0, 3, 6, 12, 24, 48 and 96 h AAT. Lyophilized samples of the second or third mature leaf from the stem apex (approximately 100 mg), were macerated in liquid N. immediately after, proline was extracted by adding 3% (w/v) sulfosalicylic acid to the samples. Then, the samples were centrifuged and the resulting supernatant was used to determine proline concentration, according to the procedures described by Bates et al. (1973) with minor modifications (Khedr et al., 2003). Trials were performed in triplicates for each biological replicate.

Gene expression

After carrying out the evaluation of enzymatic activity and after analyzing their results, it was decided to compare the most contrasting treatments (0h and 96h) for the determination of genetic expression, mature leaves were collected at intervals of 0, and 96 h AAT. Samples were stored at - 80°C after immersion in liquid nitrogen and then lyophilized. For RNA extraction, RNAqueous® kit (Ambion) was use, following the manufacturer's recommendations. RNA samples were used for the synthesis of the cDNA with RevertAid™ H minus M-MuLV Reverse Transcriptase (Fermentas), according to the manufacturer's instructions, using oligo d (T) 18 primers (Table 2). Analyzes of qPCR were performed on an Applied Biosystems 7500 Real-Time PCR System thermocycler using non-specific detection sequence (fluorophore) SYBR Green I. The reaction mix were composed of cDNA as a template, 0.5 µM of each primer and 12.5 µL of Maxima® SYBR Green/ROX qPCR Master Mix (2x). The temperature of

PCR products was raised from 55ºC to 99°C at a rate of 1°C/5s, and the resulting data were analyzed using the LightCycler software.

We have only observed a single band with a characteristic melting point for each sample, indicating that the qPCR had produced a specific product for each used primer-pair. In order to confirm that the qPCR had only produced the genes of interest, the PCR products were separated and visualized in agarose gel at 1%. The relative expression numbers of the genes were calculated as the number of times in relation to the control plant using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2013). Two endogenous genes were used as control in order to detect changes, *actin* and *β-Tubulin.* The abundance of transcripts was analyzed using specific primers (Table 2). In order to test the quality of these primers, the specificity and identity of the reverse transcription products, we have monitored the qPCR products after each PCR, using a melt-curve analysis distinguishing gene-specific from non-specific products.

Statistical analysis

The experimental design used was the randomized blocks with six treatments, 10 replicates and one plant per experimental unit, making a total of 60 CCN 51 clonal plants. Five replicates were used for the measurements of photosynthetics parameters, concentration determinations of minerals nutrients and Pb and biochemical and molecular analysis. The data were submitted to ANOVA and the mean values were compared by Tukey test ($p<0.01$ and $p<0.05$).

Results

Pb concentration

Pb accumulations in the roots and leaves of the young clonal cacao plants were found to vary according to the doses of Pb, Zn and Zn+Pb applied to the soil. Treatments T1 (0.3 g Zn + 0.5 g Pb kg^{-1} soil) and T4 (1 g Pb kg^{-1} soil) showed higher Pb accumulations in the roots (843 and 1112 mg dm^{-3} DW, respectively). On the other hand, T0 (control) and T5 (0.6 g Pb kg^{-1} soil) (i.e., with no application of Pb to the soil) did not show Pb accumulation in the roots and leaves (Tables 3 and 4). Leaf Pb accumulation exhibited the same tendency as in the roots; for instance, T1 and T4 showed very high values of Pb contents in the leaves which present accumulations three

times higher in relation to the other treatments. However, the accumulation of Pb in the leaves was twice higher than in the roots.

Mineral nutrients

Significant statistical differences in the accumulation of macro and micronutrients in the roots and leaves of young clonal cacao plants were found across treatments in response to increasing doses of Pb applied to the soil. However, there were no significant differences for N accumulation in the roots and leaves for all treatments (Tables 3 and 4). On the other hand, T3 (0.45 g of Zn + 0.25 g Pb kg^{-1} soil) and T4 (1 g Pb kg^{-1} soil) showed the highest P accumulations in the roots (Table 3). The same tendency was observed for the leaves; for example, T3 showed the highest accumulation of P in leaves than T2 and T5 (Table 4). Moreover, T2 (0.15 g Zn + 0.75 g Pb kg^{-1} soil) and T3 (0.45 g Zn + 0.25 g Pb kg^{-1} soil) had the highest K accumulations (1.96 and 2.4 mg dm^{-3} DW, respectively) in roots (Table 3), although these treatments also showed the lowest K contents in leaves. In contrast, T0 showed the highest accumulation of this element in the leaves (Table 4).

Only T0 and T1 differed from the others in relation to S content in roots (Table 3). The lowest S concentrations were found in the roots (Table 3). On the other hand, the highest S accumulation in the leaves was observed mainly in T4, which had the highest dose of Pb applied to the soil (1g Pb kg^{-1} soil). Moreover, higher values of Ca content in the roots were observed in T3 and T4 (Table 4), while higher Ca accumulations in the leaves occurred in T1 and T2 with moderate Zn and Pb doses applied to the soil (Table 4).

The application of Pb and Zn metals in isolation favored significant increases in leaves and roots (Tables 3 and 4). On the other hand, the highest Mn contents in the roots were observed in T3 and T4, while the content of foliar Mn was the same for all treatments (Table 4).

Treatments T3, T4, and T5 showed higher Cu contents in the roots compared with T0 and T1 (Table 3). A significantly higher Cu content in the leaves was observed only in T1, which had moderate Zn and Pb doses applied to the soil (0.3 g Zn + 0.5 g Pb kg^{-1} soil). In addition, T5, T4 and T3 displayed a significantly higher Fe content in the roots of young clonal cacao plants compared with the other treatments, showing there is some connection between the balances of metals in relation to Fe (Table 3). Likewise, a higher Fe content in the leaves was found in T5 (0.6 g Zn kg^{-1} soil) compared with the

other treatments (Table 4). Finally, B accumulation in the roots and leaves was highest in plants grown in T3 (0.3 g Zn + 0.5 g Pb kg^{-1} soil) (Tables 3 and 4).

Leaf gas exchange

There were significant statistical differences for net photosynthesis per unit leaf area (*A*) at 15 days AAT (Figure 1A). Treatment T5 (0.6 g Zn kg^{-1} soil) showed a marked reduction of *A*, which continuously decreased leading to plant death as a result of high Zn toxicity (Figure 1A). At 0 days AAT, statistical differences in the transpiration rate (*E*) were found between the control and T5; this trend was maintained during the experiment (Figure 1C). However, at 30 days AAT, the plants in T3 and T5 died due to Pb and Zn toxicity. Stomatal conductance to water vapor (*gs*) showed the same tendency as *E*; specifically, the lowest *gs* values were found in T5 from day 0 until plant death (Figure 1B). On the other hand, at 15 days AAT, there were statistical differences for the relationship between intracellular CO_2 concentration and atmospheric CO_2 concentration (*Ci / Ca*), where T5 presented the highest values (Figure 1D). While for the variables of intrinsic efficiencies (*A/gs*) and instantaneous (*A/E*) in the use of water this same treatment (T5) at 15d AAT, presented the lowest values (Figures 1E and 1F). However, the instantaneous efficiency of carboxylation (*A/Ci*) showed statistical differences between T0 and T5 at 0 and 15 days AAT (Figure 1G). Furthermore, there were statistical differences in *A/Ci* between T0, T3 and T4 (1 g Pb kg^{-1} soil), showing the highest value, and T1, T3 and T5, with the lowest values. In contrast, by the end of the experiment at 30 days AAT, the control and T4 had the highest *A/Ci* values.

Fluorescence emission

Treatment T4 (1 g Pb kg^{-1} soil) showed significant statistical differences ($p<0.05$) at 15 days AAT for initial fluorescence (*F0*) (Figure 2A) with the highest values. The plants in T4 (1.0 g Pb kg^{-1} soil) and T5 (0.6 g Zn kg^{-1} soil) died at 18 days AAT due to toxicity caused by high content of Pb and Zn in the soil. Therefore, there are no values for chlorophyll fluorescence emission at 30 days AAT for these treatments, however, when observing the behavior of *Fv* and *Fv / Fm*, no significant statistical differences are found

Antioxidant enzymes and proline content

Significant differences were found for APX enzyme activity in leaves of CCN 51 plants grown under different doses of Pb, Zn and Zn+Pb in the soil. The highest APX activities were observed at 0 T4, 3 T0 and T4, and 6 T0, T3, T4 and T5 and at 12 h AAT for T3. In contrast, at 24 and 96 h AAT, the highest APX activity was seen in the control treatment. Furthermore, the leaves of plants in the control treatment displayed the highest APX activity in most of the time periods evaluated. The other treatments showed APX activities lower than half of those observed in the control after 24 hours AAT (Figure 3A). GPX enzyme activity in leaves did not show significant changes in response to the treatments applied. In general, however, T2 showed low enzyme activity during most the experiment compared with T0, T4 and T5 (Figure 3B). The plants grown in the control treatment (T0) showed a low activity of the CAT enzyme at 0 and 3 h AAT. Moreover, CAT activity was different between the treatments evaluated. For instance, T1 showed an increase at 96 h AAT, while T2 and T3 both showed high and low enzyme activities during the evaluation period. Conversely, T4 demonstrated higher enzyme activity compared with the other treatments as the hours progressed and this activity only decreased at the end of the experiment. Finally, in T5, CAT activity decreased after 3 h AAT until the end of the evaluation (Figure 3C). In plants subjected to T0 and T3, lower PPO enzyme activity was found compared with the other treatments for the initial period, increasing only at 12 h AAT. On the other hand, plants in T4 showed lower values at most evaluation hours compared with T2. Higher PPO activities were found in plants grown in T5 at 3, 6, 12 and 48 h AAT. However, at 0 h, 24 and 96 h AAT, the activity of this enzyme decreased significantly in this treatment (Figure 3D). The highest SOD enzyme activities were observed in plants subjected to T4 (at 3 h AAT), T3 (at 6 h AAT), and T5 (at 96 h AAT), which showed significantly higher values than in the other treatments (Figure 3E).

The highest proline content was observed in leaves of CCN 51 plants in T5 at 48 and 96 h AAT, while lower proline accumulation was observed in leaves of the control treatment (T0) during the evaluation period (Figure 3F).

Gene expression

There were significant differences ($p<0.05$) in the relative gene expression levels of *psbA*, *psbO*, *Tpr1*, and *met* in leaves in response to applications of Pb, Zn and Zn+Pb

to the soil (Figure 4). The expressions of *psbA* at 0 h AAT were almost twice higher in T5 and T1 compared with T0 and T2. However, these expression levels were reduced by more than half at 96 h AAT in T1 and T5 (Figure 4A). The gene expressions of *psbO* were higher at 0 h AAT in T3, T4 and T5. Conversely, T2 did not show expression for this gene. The highest relative gene expression of PsbO was observed in T2 at 96 h AAT compared with the other treatments (Figure 4B). On the other hand, *Tpr* gene expressions were found in T2, T3, and T4, which showed the highest values at 0 h AAT. However, a peak in the expression of this gene was observed in T4 at 96 h AAT, with a value that exceeded the other treatments (Figure 4C). Finally, the highest expressions of *met* occurred at 0 h AAT for T0 and T1 and at 96 h AAT for T2, T4, and T5, with values almost twice higher compared with the control (Figure 4D).

Discussion

Many plant species will retain most of the absorbed Pb (approximately 95% or more) in the roots, while a small fraction is translocated to the aerial parts (Pourrut et al., 2011). Pb toxicity depends on the concentration and exposure time to this heavy metal, as well as soil physical and chemical characteristics, such as pH, cation exchange capacity, and organic matter content, which affect soil-plant absorption (He et al., 2015). The addition of 0.45 g Zn + 0.25 g Pb kg^{-1} soil and 0.6 g Zn kg^{-1} soil were toxic to CCN 51 cacao clonal plants. The results showed that Pb and Zn were absorbed by the root system and accumulated not equally in the roots and substantially more in leaves of cacao plants, demonstrating a high translocation rate for this species. A higher accumulation of metals in the roots is typical of intolerant plants, whereas the ability to translocate metals to the aerial parts is considered a tolerance factor (Verbruggen et al., 2009).

The soils containing cultivated plants with high Zn reserves had more availability of nutrients (N, K and P) under normal and stressed conditions, as observed in Tables 3 and 4, where the plants have lower Zn reserves and this it is due to better root growth, as mentioned by White and Veneklaas (2012) and Rehman et al. (2018a), which determined that a larger root surface increases access to nutrients. Furthermore, the availability of N was higher in soils where plants with high Zn were planted, possibly due to a positive interaction between Zn and N (Guo et al. 2015). Furthermore, higher N and Zn contents in the leaves of plants cultivated under high concentrations of Zn are

possibly due to a greater exudation of organic acids from the roots, which could accelerate microbial activity and promote greater Zn solubilization (Rehman et al. 2018b) and N mineralization. On the other hand, the positive interaction between Zn and K improves the state of exchangeable K in soil since Zn reduces leakage of amides and K, as well as maintains membrane integrity (Cakmak and Marschner 1998).

Root uptake of Pb occurs through bivalent cation transporters found in the plasma membrane, such as ZIP, Zn, and Fe transporters (Parmar et al., 2013). Besides, Pb can also compete for Ca^{+2}, Mg^{+2}, Mn^{+2}, and Cu^{+2} transporters (Clemens, 2016). In the specific case of the clonal genotype of cocoa CCN 51, this competition affected the absorption of Zn and Mg, where it accumulates in a greater way in the treatments with more Zn applied and similar behavior observed in the concentration of Fe in leaves. Zn is an essential cofactor in the oxidation pathway of the water molecule at the PSII level (Dučić and Polle, 2005). The higher absorption of Mn may be associated with mechanisms that reduce the toxic effects of Cd (Sarwar et al., 2010). On the other hand, Pb can substitute Ca^{+2} in calmodulin-dependent signaling pathways, such as enzymatic activation and gene expression control (Dalcorso et al., 2013). Thus, changes in Ca content may interfere in the response of cacao plants to Pb toxicity since the translocation factor for Ca was higher in the presence of Pb. A reduced K content in cacao roots was also reported in *Genipa americana* plants subjected to increasing doses of Pb in nutrient solution (Souza et al., 2011).

Moreover, the data in this study demonstrated that Pb and Zn did not interfere with root contents of P and N. However, the treatments that present doses of Pb and Zn applied to the soil have a higher content of P in the roots than the control to which these elements were not applied. The translocation of these metals was affected since P contents increased in roots and leaves in the presence of Pb. The plants subjected to T3 (0.45 g Zn + 0.25 g Pb kg^{-1} soil) and T5 (0.6 g Zn kg^{-1} soil) died at 23 days AAT due to the toxicity of these metals at high concentrations. On the other hand, the addition of zinc to the soil increases photosynthesis in young cocoa plants from 0 to 30 days AAT. However, there are reports that excess Zn may result in reduced CO_2 uptake, one of the major phytotoxic effects of Zn (Li et al., 2010). This fact was not confirmed in this study. In contrast, Pb accumulation in cacao leaves caused severe damage to the photosynthetic machinery, as previously described in the literature (Clemens, 2016). This effect may be due to a reduced expression of important genes (for example, *psbA*),

as observed in Figure 4, due to the inactivation of enzymes involved in carboxylation, results that are related to those reported by Gallego et al. (2012), who evaluated lipid peroxidation and antioxidant defense disorder and found similar behaviors between the expression of these genes when subjected to oxidative stress by heavy metals. In addition, in this research, the damage was influenced by the concentration of Pb and the exposure time had a significant influence on the enzymatic activity and the genetic expression.

The young plants of the CCN 51 showed variations in net photosynthesis (*A*) after applying the treatments with Pb, Zn and Zn + Pb. In treatments containing high Zn and Pb doses in the soil, uptake of Zn and Pb by the roots and the consequent accumulation of these metals in cacao leaf dry biomass promoted reduced photosynthesis in clonal cacao plants (Figure 1). According to Moradi and Ehsanzadeh (2015), as can be seen in Figure 1, the high applications of Pb and Zn promote alterations in PSII and partial closure of the stomata, which consequently limit the assimilation of CO_2 as evidenced in this investigation**,** when observing that the variables *A*, *E* and *gs* decrease their values in the treatments to which Pb was applied, as can be observed when comparing it with the control. In addition, Pb toxicity can inhibit chlorophyll synthesis and disrupt the photosynthetic process (Moradi and Ehsanzade, 2015).

In a recent study that evaluated Pb toxicity in *Populus* plants grown in nutrient solution with increasing concentrations of the heavy metal, the authors reported low variation in *Fv/Fm* ratios (Jiao et al., 2015). Nevertheless, the study was conducted under hydroponic conditions, the toxic metal was 100% available in solution, the pH of the solution was under control, and there was a short time of exposure to the metal.

Pb is not a redox-active metal and cannot generate ROS by direct participation in Fenton's reactions. However, it induces ROS formation by interfering with the electron transport chain in the reaction centers of photosynthesis. (Shahid et al., 2014). ROS cause a variety of deleterious effects on plants, such as lipid peroxidation, growth retardation, chlorosis, darkening of the root system, inhibition of ATP production, and DNA damage, resulting in programmed cell death (Kumar and Kumari, 2015). To prevent ROS from irreversibly damaging the photosynthetic machinery, there are enzymatic and non-enzymatic metabolic pathways that eliminate ROS (Gill and Tuteja, 2011). In this regard, proline, which is an essential osmolyte for plants under stress, acts

as an important non-enzymatic antioxidant capable of eliminating ROS produced during the stress condition and inhibiting PCD (Gill and Tuteja, 2011)

Accumulation of Pb in plant tissues negatively interferes with several essential metabolic processes, promoting oxidative damage caused by ROS (Gill and Tuteja, 2011). In this sense, the increased activity of antioxidant enzymes is considered a generalized response to excess trace metals in the soil, resulting in protection against cell disturbance and damage (Mittler, 2002). Therefore, excess Zn, as a toxic metal, can cause metabolic disturbances and macromolecule damage derived from overproduction of ROS (Gill et al., 2011).

Pourrut et al. (2011) demonstrated that plants exposed to Pb may increase or reduce the activity of antioxidant enzymes. In this work, the enzyme activities were lower in treatments with Pb addition to the soil compared with treatments containing Pb + Zn or in the absence of Pb (Figure 3). The high concentrations of these reactive species that exceed the capacity of antioxidant defense enzymes disturb redox homeostasis, which could trigger damage to macromolecules, such as membrane lipids, proteins and nucleic acids, and ultimately result in nitro-oxidative stress and plant cell death (EL-Esawi et al., 2020). These findings suggest that Zn is a potent mineral that can counteract the absorption of Pb in soils with high concentrations of the latter. Thus, Zn addition to the soil can become an effective tool to reduce toxicity and prevent the negative effects of Pb on the metabolic system of the plant. Superoxide dismutase (SOD), is usually considered to be the first line of defense against oxidative stress, converting O^{2-} species into H_2O_2 that can then be converted to water by peroxidases and catalase, the increase in SOD activity in different *Brassica* species and different metals, although there are some exceptions, but the subsequent decomposition of hydrogen peroxide to water seems to be more general and metal dependent (EL-Esawi et al., 2020).

The enzymatic activities of APX, GPX, CAT, and PPO were lower when high amounts of Pb were added to the soil (i.e., inducing stress). However, in treatments with high doses of Zn applied to the soil (T3 and T5), this heavy metal became toxic to the clonal cacao plants. Therefore, to mitigate Pb toxicity, Zn should be applied to the soil in small amounts due to its counterproductive effect (Caverzan et al., 2012). On the other hand, when plants are under heavy metal stress, antioxidant enzyme activities and proline content are increased (Caverzan et al., 2012). In this study, important changes

were found in the activities of the enzymes involved in antioxidant metabolism in the leaves of clonal cocoa plants exposed to Pb toxicity in the soil; except SOD, which showed high activity at the highest doses of Zn and Pb applied to the soil. Probably, the low activity of the antioxidant enzymes may have been compensated by the increased activity of the non-enzymatic system involved in the elimination of excess ROS that were produced during Pb toxicity in plants of the clonal CCN 51 cacao genotype.

This study demonstrated that plants with high concentrations of Zn can ameliorate the adverse effects of Pb stress by reducing ROS damage through the accumulation of proline and total soluble phenolics to reduce oxidative stress. Furthermore, the superior performance of plants with high concentrations of Zn was linked to increased CEC and nutrient uptake, which improve plant growth and grain yield (Faran et al. 2019). The presence of Zn limits oxidative damage through higher accumulation of osmolytes (proline and phenolic) and reduction of total antioxidant activities and lipid peroxidation (Faran et al., 2019). However, Zn can also generate adverse effects, as observed in treatment T5 containing higher concentrations of Zn; therefore, the dosage of this micronutrient must be well regulated.

The gene expression analyses were based in the most contrasting collection time in relation to the control (0 and 96 h AAT), which was determined after evaluating enzyme activities. At 0 h AAT, *psbA* showed the highest gene expression in T5 (i.e., with the highest dose of Zn applied to the soil), followed by T1. However, the activity of this gene decreased at 96 h AAT. The control treatment (T0), maintained stable values of gene expression during the experiment. These results demonstrate that plants subjected to variable concentrations of Zn and Pb show changes in expression of *psbA* gene, since these heavy metals cause oxidative stress.

Gene expression regulation in plants under stress conditions depends on the influx of Ca^{2+} into the cytosol, the activation of protein kinases, and protein phosphorylation. These processes have regulatory mechanisms that can be activated within seconds or minutes (Almeida et al., 2014). The *psbA* gene is located in the chloroplast genome and encodes the D1 protein that plays a fundamental role in PS2 from the photochemical phase of photosynthesis, acting in the electron transport chain (Nelson and Yocum, 2006). However, when cacao plants were exposed to 1 g Pb kg^{-1} soil, there was no increase in the gene expression of *psbA* in the leaves. Since this gene

is of cytoplasmic (chloroplast) inheritance, the damage caused to this cellular organelle by Pb toxicity can reduce the transcript pool in its genome (Page et al., 2016).

Higher gene expressions of *psbO* were observed at 0 h AAT in T3, T4, and T5. Treatment T2 did not show significant expression, but, by the end of the experiment (96 h AAT), this treatment stood out from the other treatments. The PsbO nuclear gene encodes the PsbO protein, which is extrinsic to PSII. This protein is involved in the oxygen-evolving complex and is also known as the Mn-stabilizing protein, which increases the efficiency of PSII during the oxidation of the H_2O molecule (Popelkova and Yocum, 2011). Therefore, the increase in leaf Zn content in clonal cacao plants exposed to Pb toxicity in the soil may have affected the signaling pathways for PsbO expression in this organ (Li et al., 2015). Nascimento et al. (2018) found changes in the gene expression levels of *sod*, *psbA psbO* in response to the toxicity generated by heavy metals in the soil. According to this authors, the changes in gene expression altered CO_2 assimilation, through decreased in *gs* values and enzyme activities. Furthermore, found that the highest expression levels of *psbA* and *met* occurred at higher doses of heavy metals applied to the soil at 24 and 96 h AAT. These results are similar to those found in this research since the highest expressions of the genes *met,* were observed at 96 h AAT in treatments containing the highest concentrations of Pb and Zn applied to the soil.

Since *psbA* is responsible for electron transfer during photosynthesis and *psbO* is in charge of regulating the oxygen-evolving complex (Cheng et al., 2016) during oxidation of the water molecule, then variations in the concentration of *psbO* directly affect photosynthesis and plant growth (Rehem et al., 2011a, 2011b). Several studies with cocoa plants suggest that the toxicity generated by heavy metals (Cd or Pb) promotes underexpression of PsbO as a mechanism to protect D1 protein, which participate in oxidative stress by regulating ROS to prevent damage to PSII (Araujo et al., 2017). Similar results were found in this investigation since plants subjected to higher Pb contents in soil altered gene expression of PsbO (Figure 4b), which may be a strategy to prevent metabolic alterations caused by Pb absorption in plants.

The relative expressions of *Tpr* gene were high at 96 h AAT in T1 and T4 with high doses of Pb applied to the soil. Similarly, the highest expression levels of *met* were observed at 96 h AAT for all treatments containing Zn and Pb applied to the soil. These findings suggest that these heavy metals influenced response mechanisms of the plants to the stress. In addition, metallothioneins are proteins that also exhibit metal binding

affinity, but are not involved in metal sequestration to the vacuole (Auguy et al., 2016). The expression of *met* in cacao leaves exposed to Pb showed a similar pattern in the roots, whereas the expression of *sodcyt* in cacao leaves corresponded with high SOD activity in the same organ. Therefore, it is likely that the increased concentration of Fe in cacao leaves in the presence of Pb promoted the production of ROS, maintaining high expression levels of *sod* (Bhaduri and Fulekar, 2012). The relative expression of *met* and *sod* genes in cocoa leaves coincided with the responses of these enzymes in the leaves of the plants evaluated. This study determined that the underexpression of these genes is related to the exposure of plants to Pb in the soil. This was also reported by Castro et al. (2015), when seeds from cocoa progenies derived from the self-pollination of 'Catongo'x 'Catongo' and of the crossing between CCN-10 x SCA-6 were immersed for 24 h in different Cd doses and by Souza et al. (2014), when seedlings of CCN 51 cocoa genotype were grown under greenhouse conditions and exposed to increasing Cu doses in nutrient solution.

The overexpression of genes such as *met* under high concentrations of heavy metals in the soil is a tolerance mechanism of Pb toxicity (Pereira et al., 2017). Phytoquelatins are non-protein metal chelators that are enzymatically synthesized using glutathione as a substrate. Phytoquelatins genes are constitutively expressed, but enzymatic activation depends on the presence of the metal cofactor (Sunthia et al., 2012). On the other hand, metallothioneins are polypeptides that are generally synthesized under stress conditions (Dalcorso et al., 2013). Phytoquelatins and metallothioneins act by sequestering Pb, thus, inactivating this heavy metal in the cytoplasm or transporting it to vacuoles (Sunitha et al., 2012). In addition, the role of phytoquelatins in the transport of heavy metal to the aerial parts of the plant has been well studied and demonstrated (Lopez-Climent et al., 2014).

Overall, this study demonstrated that the toxicity generated by the presence of Pb in the soil can be mitigated by the addition of Zn. The competition of these heavy metals for root absorption in cocoa plants results in lower uptake of Pb and higher availability of Zn in the soil. However, the addition of Zn can also generate toxicity in plants and cause oxidative stress, which leads to overexpression of genes such as *met* and *Tpr* (Figure 4C and 4D). On the other hand, moderate additions of Zn (0.15, 0.30 g Zn kg^{-1} of soil) can promote the mitigation of Pb toxicity by increasing the expression of these genes and activating all the mechanisms to stop the damages caused by oxidative stress.

The presence of heavy metals in the soil considerably alters the cellular metabolism of cocoa plants, leading to death in some cases, as mentioned by Pereira et al. (2017).

Conclusions

Young plants of the CCN 51 cacao clonal genotype grown in soils with high Pb, and Zn+Pb contents were found to accumulate these heavy metals in the roots and leaves.

Uptake of Pb and Zn by the roots and its transports into the aerial part promoted significant physiological changes in the photosynthesis, nutritional balance, antioxidant metabolism, and gene expression of the young plants of CCN 5.

Increased SOD enzyme activity and proline content in leaves of the young plants of CCN 51 cacao clonal genotype contributed to mitigate Pb and Zn toxicities at the highest doses of these metallic elements applied in soil.

Zn adequate doses applied in soil mitigated the toxicity of Pb in young plants of CCN 51.

The application of Zn in adequate dosages can be a good strategy to mitigate toxicity caused by Pb, however, the age of the crop, the genotype and the concentrations must be taken into account, since elevated Zn (T4, 0.6 g kg^{-1} soil) and Zn + Pb, (T3, 0.45 g Zn + 0.25 g Pb kg^{-1} soil) can cause the death of plants in a short period of time, due to high toxicity.

5 - References

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Tables and Figures

Table 1 - Physical and chemical characteristics of the substrate for growth of cacao plants. P, Na, K, Fe, Zn, Mn, Cu (extracted by Mehlich 1), Ca, Mg, Al (extracted by KCl, 1 M) H + Al (extracted by Ca-acetate 0.5 M, pH 7.0), B (extracted by hot water). SB, sum of bases; t, effective cation exchange capacity; T, cation exchange capacity (pH 7.0); V, base saturation; m, Al saturation; NaSI, Na saturation index; OM, organic matter= Org C.×1.724; P-rem, remaining phosphorus.

pH	H_2O	4.7
P	mg dm^{-3}	5.9
K	mg dm^{-3}	22
Na	mg dm^{-3}	9
Ca^{+2}	$cmol_c$ dm^{-3}	1.1
Mg^{+2}	$cmol_c$ dm^{-3}	0.6
Al^{+3}	$cmol_c$ dm^{-3}	0.5
H+Al	$cmol_c$ dm^{-3}	4.13
CEC (t)	$cmol_c$ dm^{-3}	2.3
CEC (T)	$cmol_c$ dm^{-3}	5.9
V	%	30
M	%	22
ISNa	%	1.7
OM	dag kg^{-1}	2.29
P-rem	mg L^{-1}	38.4
Zn	mg dm^{-3}	2.3
Fe	mg dm^{-3}	145.1
Mn	mg dm^{-3}	15.3
Cu	mg dm^{-3}	1.5
B	mg dm^{-3}	0.2
S	mg dm^{-3}	15.9

Table 2 - Pairs gene-specific primers that were used in qRT-PCR analysis.

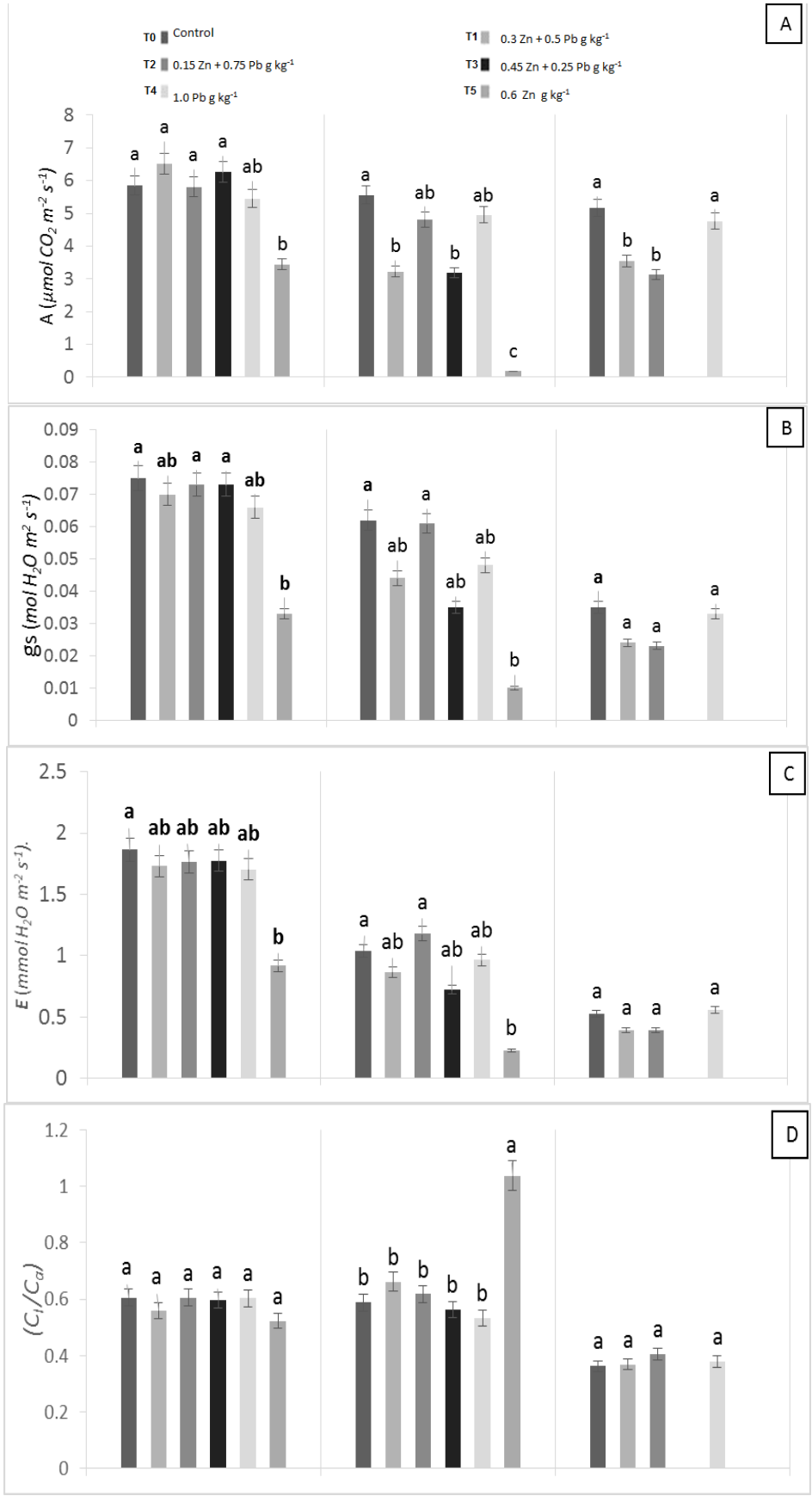
Gene	Access	Function	Primer
psbA	NC_014676.2[C]	Biosynthesis of the psbA protein or D1 protein.	F-5'GGTTTGCACTTTTACCCGA-3' R-5´CTCATAAGGACCGCCATT-3´
psbO	CL 326conting1[a]	Biosynthesis of the psbO protein.	F-5'GCAAACGCTGAAGGAGTT-3´ R-5'GGCTTGAAGGCAAATGAGTC-3´
Tpr	Q5NBT9	Associates both with chromatin in the HSP70 promoter and with mRNAs transcribed from this promoter under stress-induced conditions	F-5'ATGAGCAGCTAAAGCAGGGAA-3´ R-5'TTCCCTCCTTTACCTGCTCAT-3
Met		Ability to bind to both heavy physiological metals and xenobiotics.	F-5' AARAGYTGCTGYTCCTGCTG-3´ R-5' CAGCAGGARCAGCARCTYTT-3
B tubulina	GU570572.1[C]	Endogen	F-5´TGCAACCATGAGTGGTGTCA-3´ R-5´CAGACGAGGGAAAGGAATGA-3´
Actina	Xm_ 018128615[c]	Endogen	F-5´TCCTCTTCCAGCCATCTCTC-3´ R-5´TCTCCTTGCTCATTCGGTCT-3´

Table 3 - Accumulation of Pb and changes in the contents of macronutrient and micronutrients in roots of young cacao plants, submitted to doses of Pb, Zn and Pb+Zn in the soil at 30 days after application of treatments. Mean values of five replicates (± SE). Letters indicate comparisons between treatments by Tukey test ($p<0.05$).

	N	**P**	**K**	**Ca**	**Mg**	**S**	**Zn**	**Mn**	**Pb**	**Cu**	**Fe**	**B**
TRAT	----------cmolc dm^{-3}----------						----------mg dm^{-3}----------					
0	2.96a	0.08c	0.94b	0.76d	0.0c	0.13b	20d	38.2d	0.0e	0.53b	101d	19.7c
1	2.90a	0.08c	1.09b	0.78d	0.0c	0.15b	26d	63.7c	843a	0.58b	142c	21.8c
2	3.10a	0.15b	1.96a	0.98cd	0.25bc	0.17a	217b	244b	206bc	1.40ab	193c	25.2b
3	2.84a	0.33a	2.4a	1.34ab	0.79a	0.23a	1520a	681a	156cd	4.05a	921b	43.4a
4	2.79ab	0.25a	1.81b	1.19ab	0.66b	0.18a	70c	605a	1112a	2.90a	1106b	26.6b
5	2.47ab	0.21b	2.03b	0.89bc	0.54b	0.18a	2041a	427e	0.0e	3.70a	3269a	28.5c

Table 4 - Accumulation of Pb and changes in the contents of macronutrient and micronutrients in leaves of young cacao plants, submitted to doses of Pb, Zn and Pb+Zn at 30 days after application of treatments. Mean values of five replicates (± SE). Letters indicate comparisons between treatments by Tukey test ($p<0.05$).

	N	P	K	Ca	Mg	S	Zn	Mn	Pb	Cu	Fe	B
TRAT	----------dag kg^{-1}----------						----------mg kg^{-1}----------					
0	2.1a	0.24ab	1.27a	0.49b	0.52b	0.15b	63.7e	101ab	0.0c	18.2b	2747b	24.1bc
1	1.9ab	0.23ab	0.82bc	0.60a	0.44b	0.15b	669d	97.6ab	2090a	27.3a	2548b	28.4ab
2	1.9ab	0.21bc	0.63c	0.56a	0.52b	0.15b	1146c	89.5ab	715b	25.4ab	2973b	27.3abc
3	1.8ab	0.25a	0.57c	0.43c	0.44b	0.15b	1980b	74.9b	688b	22.4ab	2355b	30.5a
4	1.9ab	0.22ab	0.93b	0.46bc	0.48b	0.17a	76.5e	89.9ab	3270a	18.9b	2338b	27.5abc
5	1.6ab	0.18c	0.33d	0.43c	1.48a	0.12c	2386a	104ab	0.0c	22.4ab	6402a	22.4c


A
T0 Control
T1 0.3 Zn + 0.5 Pb g kg-1
T2 0.15 Zn + 0.75 Pb g kg-1
T3 0.45 Zn + 0.25 Pb g kg-1
T4 1.0 Pb g kg-1
T5 0.6 Zn g kg-1
A (µmol CO2 m-2 s-1)
B
gs (mol H2O m2 s-1)
C
E (mmol H2O m-2 s-1),
D
(Ci/Ca)

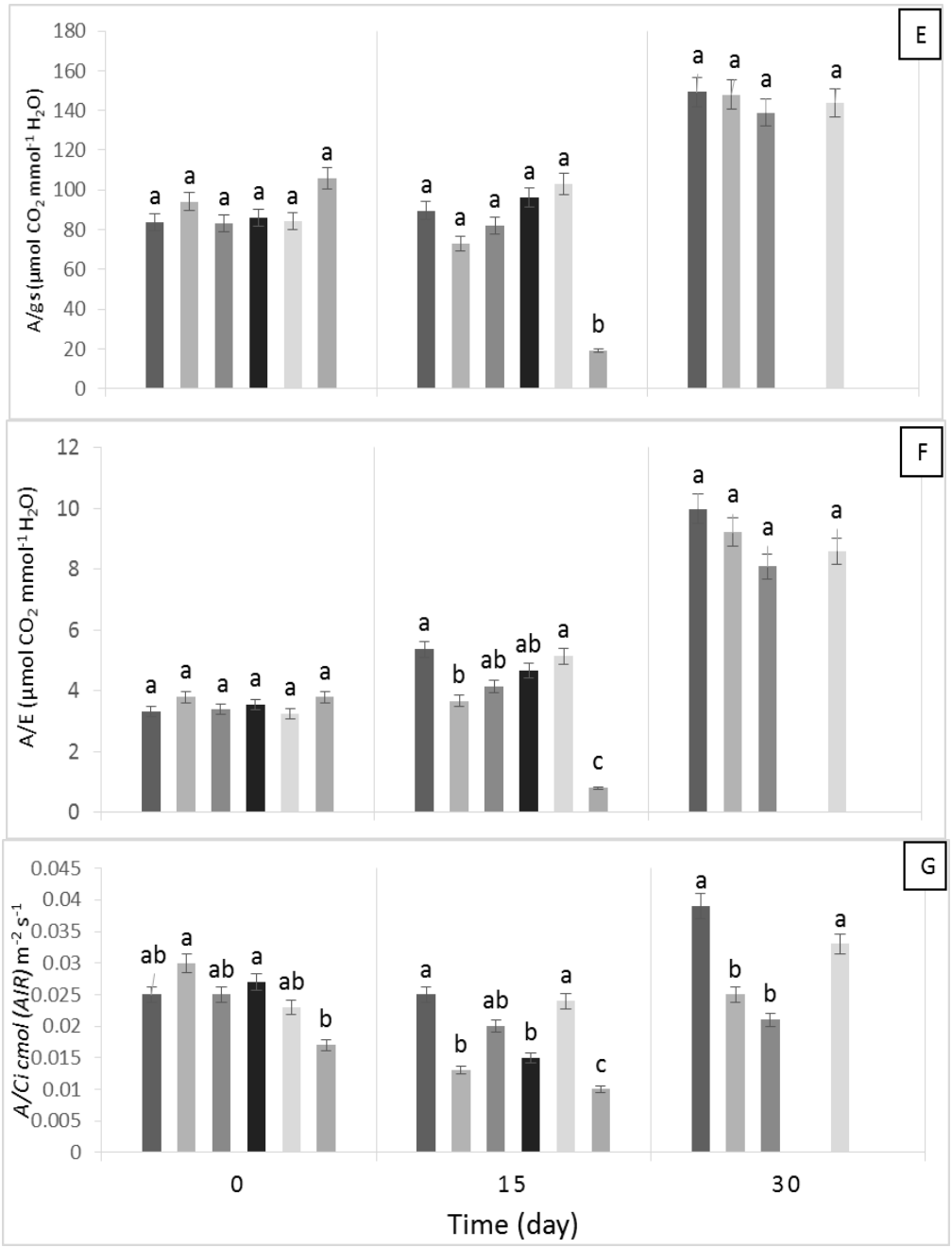


Figure 1 - (A) Net photosynthesis per unit leaf area (*A*), (B) stomatal conductance to water vapor (*gs*), (C) transpiration rate (*E*), (D) ratio of internal and atmospheric CO_2 concentration (*Ci/Ca*), (E) intrinsic efficiency of water use (*A/gs*), (F) instantaneous efficiency of water use (*A/E*), (G) instantaneous efficiency of carboxylation (*A/Ci*) in leaves of young cacao plants, submitted to doses of Pb, Zn and Pb+Zn in soil at 0, 15 and 30 days after application of treatments. Mean values of four replicates (± SE). Lower case letters indicate comparisons between treatments by Tukey test ($p<0.05$).

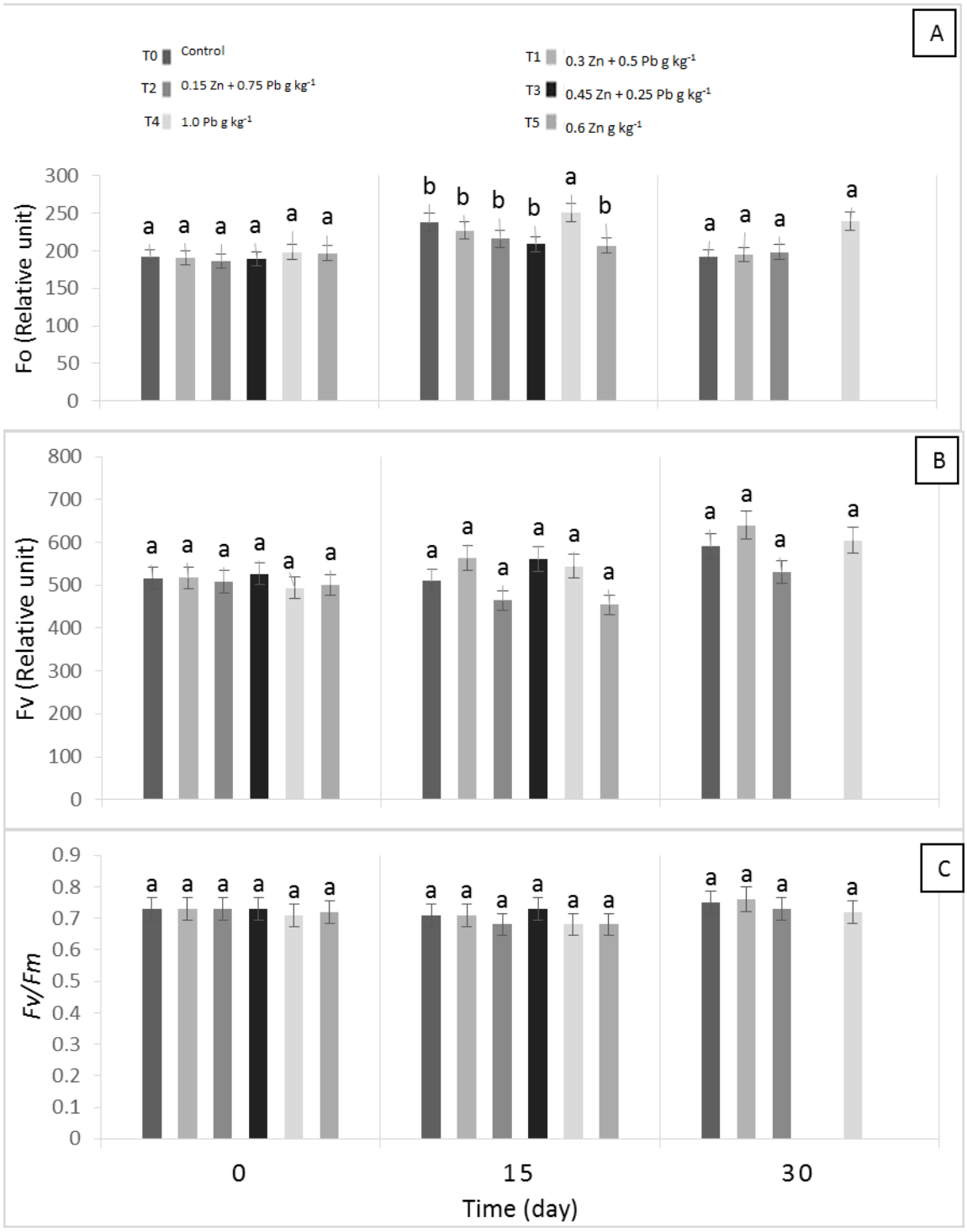
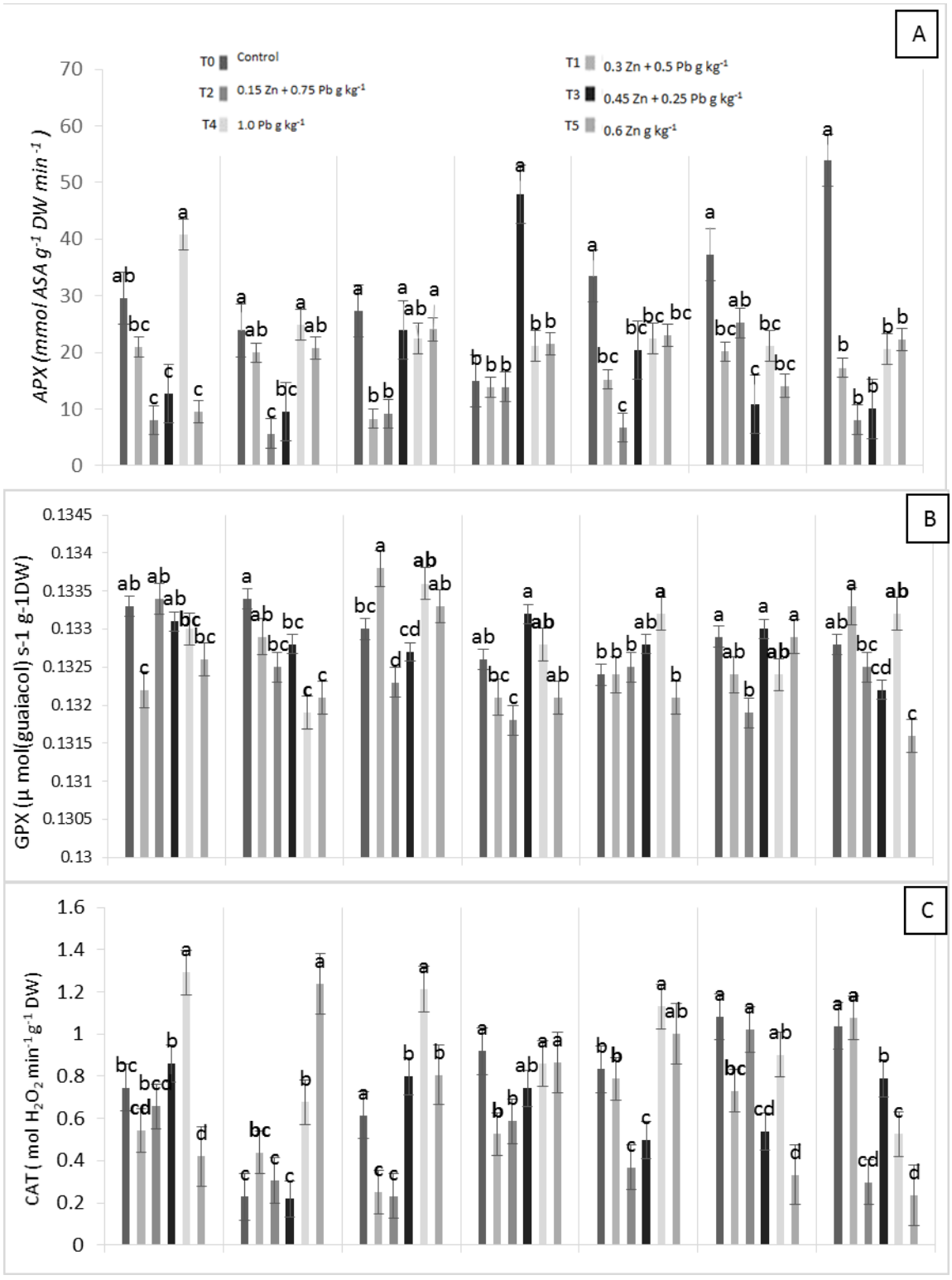


Figure 2 - (A) Initial fluorescence (F0), (B) variable fluorescence (Fv) and (C) maximum quantum yield of photosystem 2 (Fv/Fm) in leaves of young cacao plants, submitted to doses of Pb, Zn and Pb+Zn in soil at 0, 15 and 30 days after application of treatments. Mean values of four replicates (± SE). Lower case letters indicate comparisons between treatments by Tukey test ($p<0.05$).


A
T0 Control
T1 0.3 Zn + 0.5 Pb g kg-1
T2 0.15 Zn + 0.75 Pb g kg-1
T3 0.45 Zn + 0.25 Pb g kg-1
T4 1.0 Pb g kg-1
T5 0.6 Zn g kg-1
APX (mmol ASA g-1 DW min-1)
B
GPX (µ mol(guaiacol) s-1 g-1DW)
C
CAT (mol H2O2 min-1 g-1 DW)

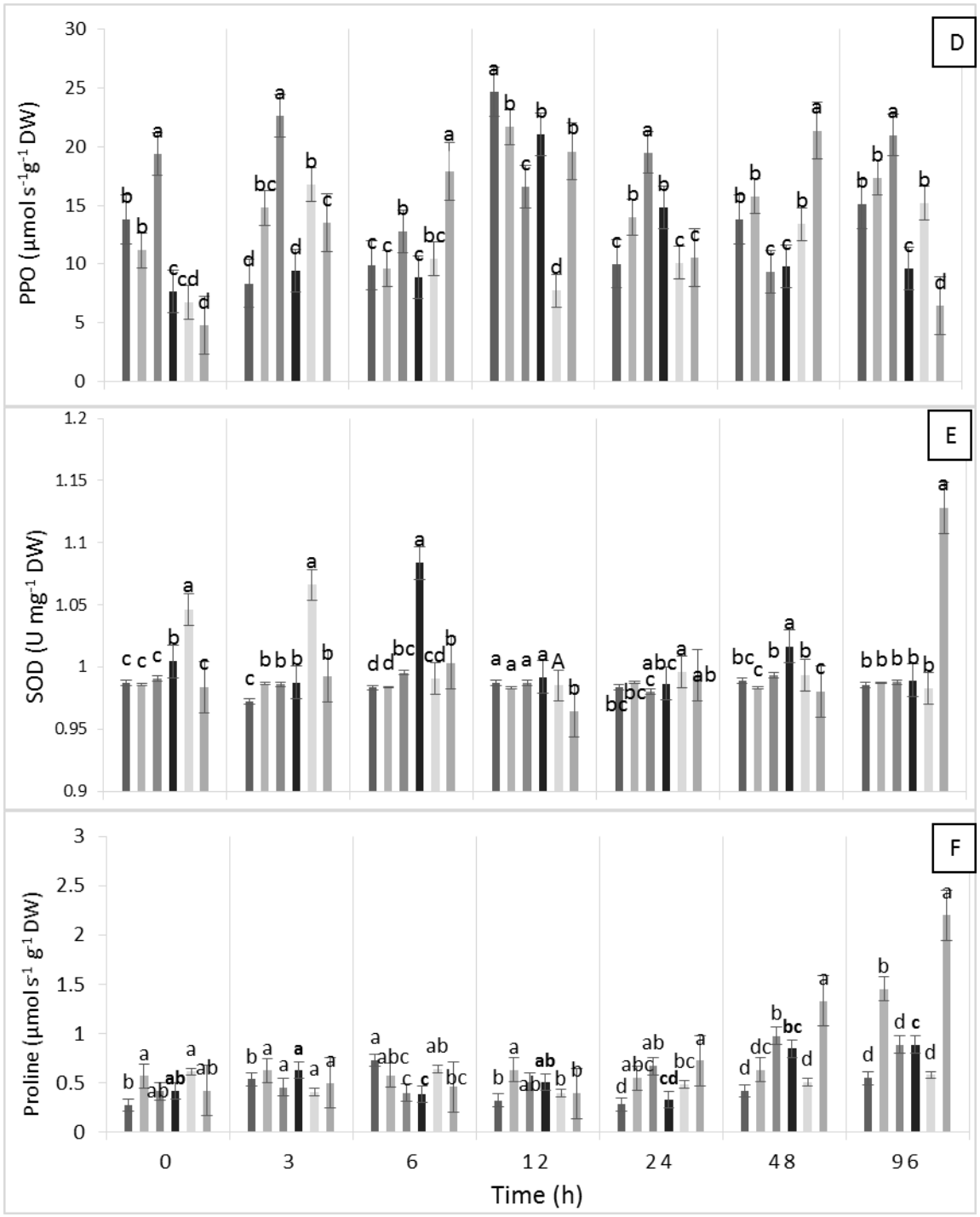


Figure 3 - Enzymatic activity of APX, ascorbate peroxidase (A), GPX, guaiacol peroxidase (B), CAT, catalase (C), PPO, polyphenol oxidase (D), SOD, superoxide dismutase and proline content (F) in leaves of cacao young plants submitted to different doses of Pb, Zn and Pb+Zn in soil to 0, 3, 6, 12, 24, 48 and 96 h after application of treatments. Mean values of five replicates (± SE). Letters indicate comparisons among treatments by Tukey test ($p<0.05$).

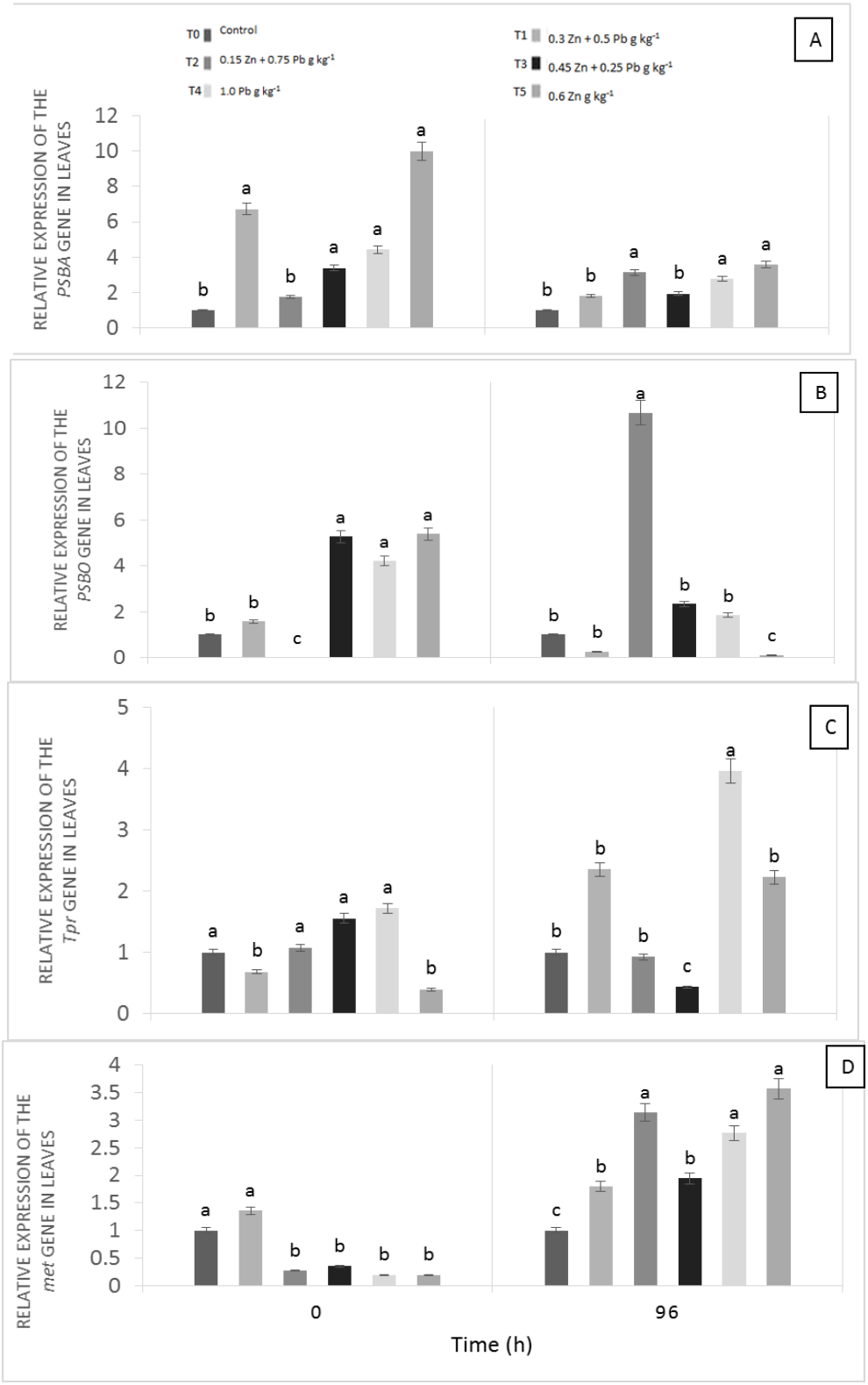


Figure 4 - Relative expression of genes *PsbA* (A), *PsbO* (b), *Tpr* (C) and *met* (D) in leaves of cacao young plants, submitted to doses of Pb, Zn and Pb+Zn in soil at 0 and 96 h after application of treatments. Mean values of four replicates (± SE). The statistical significance was determined by Tukey test ($p<0.05$).

6 - General conclusion

Young plants of the cacao clonal CCN 51 genotype grown in soils with high Pb, Mn, Zn, Mn+Pb and Zn+Pb contents were found to accumulate these heavy metals in roots and leaves.

Uptake of Pb, Mn and Zn by the roots and its transports into the aerial part promoted changes in leaf gas exchange, chlorophyll fluorescence emission, proline content, nutritional balance, antioxidant metabolism and gene expression of the young plants of the cacao clonal CCN 51 genotype.

Pb, Mn and Zn toxicities activated the defense mechanisms in young plants of the cacao clonal CCN 51 genotype by altering the gene expression of *psbA*, *psbO*, *met* and *Tpr1* and the enzyme activities SOD, GPX, APX, CAT and PPO involved in cellular detoxification of ROS excess in leaf level.

Adequate doses of Mn+Pb and Zn+Pb applied in soil mitigated the toxicity of Pb in young plants of the cacao clonal CCN 51 genotype.

Mitigation of Pb toxicity by Mn and Zn was due to the reduction of Pb uptake by the root system, preventing Pb from accumulating in toxic levels in the roots and leaves of young plants of the cacao clonal CCN 51 genotype.

High doses of Pb, Mn and Zn alone or together applied to the soil were highly toxic to the young plants of the cacao clonal CCN 51 genotype, leading, in some cases, them to death. However, no Mn toxicity was observed in cocoa plants, even at high doses in the soil.

Application of adequate doses of Mn or Zn in the soil can be used to mitigate the Pb toxicity in young plants of the cacao clonal CCN 51 genotype grown in contaminated soils.

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Anexos

Fator de Bioconcentração (FBC) da raiz e da parte aérea:

- $FBC\ raiz$ (%) = $[Metal]\ raiz$/ $[Metal]\ solo\ x$ 100
- $FBC\ parte\ aérea$ = $[Metal]\ parte\ aérea$ / $[Metal]\ solo\ x$ 100

Onde: [Metal] raiz - concentração do elemento tóxico na raiz; [Metal] parte aérea - concentração do elemento tóxico na parte aérea e [Metal] solo - concentração do elemento tóxico no solo (Yoon et al., 2006).

Fator de translocação:

- FT (%) = $[Metal]\ parte\ aérea$ / $[Metal]\ raiz$

Onde: [Metal] parte aérea - concentração de chumbo na massa seca da parte aérea e [Metal] raiz - concentração de chumbo na massa seca de raiz (Abichequer e Bohnen, 1998).

Tabela anexa 1 - Fator de Bioconcentração do capitulo 1 "Mitigation of Pb toxicity by Mn in young plants of the cacao clonal CCN 51 genotype grown in soil: physiological, biochemical, nutritional and molecular responses".

	Solo	**[Pb]**		**[Mn]**	
TRAT	[Pb] mg*kg^{-1}	[Folha]	[Raiz]	[Folha]	[Raiz]
0	0	0	0	0	0
1	500	786,2	365,3	148,6	357,2
2	750	116,55	103,0	137,47	335,6
3	250	301,84	120,8	762	1511,9
4	1000	177,1	80,6	4,31	58,2
5	0	0	0	0	0.0

Tabela anexa 2 - Fator de translocação do capitulo 1 "Mitigation of Pb toxicity by Mn in young plants of the cacao clonal CCN 51 genotype grown in soil: physiological, biochemical, nutritional and molecular responses".

	FT (%)	
TRAT	[Pb]	[Mn]
0	0	0,19
1	2,15	0,42
2	1,13	0,41
3	2,50	0,50
4	2,20	0,07
5	0	0,37

Tabela anexa 3 - Fator de Bioconcentração do capitulo 2 “Mitigation of Pb toxicity by Zn in young plants of the cacao clonal CCN 51 genotype grown in soil: physiological, biochemical, nutritional and molecular responses”.

	Solo	**[Pb] %**		**[Zn] %**	
TRAT	[Pb]	[Folha]	[Raiz]	[Folha]	[Raiz]
0	0	0	0	0	0
1	500	417,92	168,6	133,9	5,2
2	750	95,35	27,5	152,84	29,0
3	250	275,16	62,4	791,96	608,1
4	1000	326,97	111,2	7,65	7,0
5	0	0	0	0	0

Tabela anexa 4 - Fator de translocação do capitulo 2 “Mitigation of Pb toxicity by Zn in young plants of the cacao clonal CCN 51 genotype grown in soil: physiological, biochemical, nutritional and molecular responses”.

	FT (%)	
TRAT	[Pb]	[Zn]
0	0	3,16
1	2,48	25,88
2	3,47	5,27
3	4,41	1,30
4	2,94	1,09
5	0	1,17