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TAMIRES LETÍCIA CUNHA LOPES

INIBIÇÃO DO CRESCIMENTO INDUZIDA PELA LIGNIFICAÇÃO EM PLANTAS DE SOJA (*Glycine max* L.) EXPOSTAS À NANOPARTÍCULAS DE ÓXIDO DE FERRO (γ-Fe_2O_3)

MARINGÁ
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Orientador: Prof. Dr. Rogério Marchiosi

Coorientador: Prof. Dr. Osvaldo Ferrarese-Filho

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RESUMO

As plantas estão constantemente expostas a perturbações ambientais que limitam seus crescimentos, e uma dessas condições é a exposição e interação com diferentes nanopartículas (NPs) que são abundantes e continuamente descartadas no meio ambiente. Até o momento, nenhum estudo foi realizado avaliando os efeitos das NPs de óxido de ferro (III) (γ-Fe_2O_3) no crescimento e na produção de lignina em plantas de soja, como propusemos aqui. Além disso, e para um propósito comparativo, submetemos plantas de soja ao cloreto de ferro (III) ($FeCl_3$), a contrapartida iônica do elemento ferro. A exposição das plantas às γ-Fe_2O_3 NPs estimulou a atividade da peroxidase ligada à parede celular (POD) das raízes, mas inibiu a atividade da fenilalanina amônia liase (PAL), que pode ser devido ao feedback negativo decorrente do acúmulo de compostos fenólicos. Contrariamente, a POD ligada à parede celular foi inibida pelo $FeCl_3$. Ambos os tratamentos (γ-Fe_2O_3 NPs e $FeCl_3$) aumentaram o teor de lignina nas raízes e nos caules. Uma inibição significativa do crescimento dos caules foi observada após a exposição à γ-Fe_2O_3 NP, o que provavelmente se deve a alterações na composição de monomérica da lignina. Nesse caso, as γ-Fe_2O_3 NPs diminuíram o teor de monômero guaiacil (G) nas raízes, mas aumentaram nos caules. Por sua vez, $FeCl_3$ aumentou o conteúdo de *p*-hidroxifenil (H) e de siringil (S) nas raízes. O alto teor de monômero G nos caules causado pelas γ-Fe_2O_3 NPs diminuiu a razão S:G, gerando uma lignina com maior número de ligações cruzadas, seguida pelo endurecimento da parede celular e inibição do crescimento. Opostamente, o aumento da razão S:G nas raízes das plantas submetidas ao $FeCl_3$ está de acordo com a ausência de efeitos sobre o crescimento devido à produção de uma lignina menos condensada. Em resumo, nossos achados mostraram que ambos γ-Fe_2O_3 NPs e $FeCl_3$ atuam sobre a soja por diferentes mecanismos.

Palavras-chave: fenilalanina amônia liase, peroxidases, composição de monômeros de lignina, razão S:G, vias fenilpropanoides, conteúdo fenólico total

ABSTRACT

Plants are constantly exposed to environmental perturbations that limit their growth, and one of these conditions is the exposure and interaction with different nanoparticles that are plenty and continuously discarded into the environment. Hitherto, no study has been carried out evaluating the effects of iron (III) oxide (γ-Fe_2O_3) NPs on soybean growth and lignin production, as we have proposed herein. Furthermore, and for a comparative purpose, we have submitted soybean plants to iron (III) chloride ($FeCl_3$), the ionic counterpart of element iron. Exposure of plants to γ-Fe_2O_3 NPs stimulated the activity of cell wall-bound peroxidase (POD) of roots, but inhibited the phenylalanine ammonia lyase (PAL) activity, which can be due to the negative feedback of accumulated phenolic compounds. By contrary, the cell-wall bound POD were inhibited by $FeCl_3$. Both γ-Fe_2O_3 NPs and $FeCl_3$ increased the lignin content in roots and stems. A significant growth inhibition of stems was noted after γ-Fe_2O_3 NPs exposure, which was due probably to changes in the lignin monomer composition. In this case, γ-Fe_2O_3 NPs decreased the content of guaiacyl (G) monomer in roots, but increased it in stems. In turn, $FeCl_3$ increased the contents of *p*-hydroxyphenyl (H) and syringyl (S) in roots. High content of monomer G in stems caused by γ-Fe_2O_3 NPs decreased the S:G ratios generating a more highly cross-linked lignin followed by the stiffening of the cell wall and growth inhibition. Contrarily, increase of S:G ratio in the roots of plants submitted to $FeCl_3$ are in agreement with the absence of effects on growth, due to production of a less condensed lignin. In brief, our findings showed that both γ-Fe_2O_3 NPs and $FeCl_3$ act differently in soybean.

Keywords: phenylalanine ammonia lyase, peroxidases, lignin monomer composition, S:G ratio, phenylpropanoid pathways, total phenolics content

BIOGRAFIA

Tamires Letícia Cunha Lopes nasceu em Maringá/PR em 17 de novembro de 1992. Possui graduação em Bacharelado em Bioquímica pela Universidade Estadual de Maringá (UEM) (2014). Tem experiência na área de botânica, atuando principalmente nos temas relacionados à Bioquímica Vegetal com ênfase no Metabolismo Secundário em Plantas. Iniciou o curso de Mestrado no Programa de Pós-graduação em Ciências Biológicas – área de concentração Biologia Celular e Molecular – em Março de 2015, desenvolvendo o trabalho "**Inibição do crescimento induzida pela lignificação em plantas de soja (*Glycine max* L.) expostas a nanopartículas de óxido de ferro (γ-Fe_2O_3)",** com defesa da dissertação realizada em agosto de 2017.

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4CL	4-Coumarate:CoA ligase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CAT	Catalase
CCR	Cinnamyl-CoA reductase
CoA	Coenzyme A
CuO	Cupper Oxide
$FeCl_3$	Iron (III) chloride
G	Guaiacyl monomer
H	*p*-Hydroxyphenyl monomer
HCl	Hydrogen chloride
HPLC	High-performance liquid chromatograph
INPs	Iron nanoparticles
IONPs	Iron oxide nanoparticles
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NPs	Nanoparticles
PAL	Phenylalanine ammonia lyase
POD	Peroxidase
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
S	Syringyl monomer
SOD	Superoxide dismutase
SPAD	Soil-plant analyses development unit
TEM	Transmission electron microscopy
UV	Ultraviolet
ZVI	Zero-valent iron
ε	Molar extinction coefficient

APRESENTAÇÃO

Esta dissertação é composta por um artigo científico que trata dos efeitos de nanopartículas (NPs) de óxido de ferro (γ-Fe_2O_3) sobre o crescimento e metabolismo de lignina em plantas de soja (*Glycine max* L.). O artigo será submetido à revista **Frontiers in Plant Science** (IF = 4,298), Qualis A2.

Tamires Letícia Cunha Lopes, Guilherme Henrique Gonçalves de Almeida, Gabriele Sauthier Romano de Melo, Rita de Cássia Siqueira-Soares, Osvaldo Ferrarese-Filho, and Rogério Marchiosi. Lignin Induced Growth-Inhibition in Soybean (*Glycine max* L.) Exposed to Iron Oxide Nanoparticles (γ-Fe_2O_3).

SUMÁRIO

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – Nanopartículas (NPs) são substâncias naturais ou construídas pelo homem que possuem pelo menos uma de suas dimensões com tamanho menor do que 100 nm. Devido ao seu formato e tamanho reduzidos elas possuem propriedades químicas distintas dos elementos químicos que as constituem. Características como alta área de superfície em relação ao volume e reatividade química têm permitido a sua utilização nas áreas das ciências físicas, químicas, biológicas e da saúde, além de outros campos interdisciplinares da ciência e engenharia. O potencial desta tecnologia é destacado pelos crescentes investimentos no setor, que em 2009 somaram aproximadamente 220 bilhões de dólares (US$ 220 bi). Ao mesmo tempo, pesquisadores e sociedade têm demonstrado preocupações sobre o impacto ambiental e toxicidade de produtos à base de nanomateriais. Atualmente os estudos na área de nanotoxicologia estão aumentando e muitos pesquisadores acreditam que a toxicidade e o destino dos nanomateriais devem ser estudados antes de dar atenção às suas aplicações. Há uma grave falta de informações sobre os impactos das NPs sobre a saúde humana e o ambiente. O potencial de nanomateriais para ser usado em uma ampla gama de setores significa que o número de possíveis rotas para as NPs entrarem em contato com os organismos e o ambiente é elevada. Eles podem ser descarregados diretamente para os rios ou na atmosfera pela indústria, ou inadvertidamente escapar de tintas, cosméticos, protetores solares e produtos farmacêuticos que são utilizados ou eliminados no meio ambiente. Como as NPs possuem um tamanho reduzido, a sua superfície em relação ao volume aumenta exponencialmente, o que torna estas partículas muito reativas e tóxicas. Além disso, à medida que o tamanho diminui, a capacidade das NPs de penetrarem nos tecidos vegetais e animais aumenta. De fato, pesquisas têm mostrado que as NPs podem ser bioacumuladas através da cadeia alimentar, e esse acúmulo poderia chegar aos organismos no topo da cadeia. Por serem organismos produtores, as plantas constituem uma importante rota de entrada e transporte de NPs na cadeia alimentar. Além disso, é atualmente questionado se a presença de NPs no ambiente pode acarretar perdas na produtividade das culturas, já que inúmeros trabalhos têm mostrado efeitos negativos das NPs sobre a germinação e o crescimento de uma diversidade de plantas. Neste contexto, futuros trabalhos que visem compreender os efeitos tóxicos das NPs sobre o crescimento e o desenvolvimento de

plantas cultivadas tais como a soja e o milho são essenciais. Assim, neste trabalho avaliamos os efeitos de NPs de óxido de ferro (γ-Fe_2O_3) sobre o crescimento e metabolismo de lignina em plantas de soja (*Glycine max* L.). As atividades da fenilalanina amônia liase (PAL), peroxidases (POD) solúvel e ligada à parede celular, conteúdo total de lignina e sua composição monomérica foram avaliadas para determinar o mecanismo de toxicidade das γ-Fe_2O_3 NPs. Experimentos adicionais com cloreto de ferro III ($FeCl_3$) foram realizados para distinguir os efeitos tóxicos das γ-Fe_2O_3 NPs do ferro iônico.

MATERIAIS E MÉTODOS – Sementes de soja (*Glycine max* L.), *cv*. BRS-232, foram sanitizadas com hipoclorito de sódio ($HClO_3$) a 2% por 2 min e postas a germinar entre duas folhas de papel Germitest® previamente umedecidas com água destilada. Após três dias, plântulas viáveis foram transferidas para bandejas de plástico contendo células de 50 mL preenchidas com vermiculita e, em seguida, regadas com 20 mL de água destilada. As bandejas contendo as plântulas foram acondicionadas em uma sala de crescimento com temperatura de 25ºC, fotoperíodo de 12/12 h (claro/escuro) e irradiância de 300 μmol fótons m^{-2} s^{-1}, onde permaneceram por 16 dias. Os tratamentos das plantas de soja foram realizados a cada dois dias pela adição de 20 mL de solução nutritiva de Hoagland (pH 6,0) contendo diferentes concentrações (250, 500, 1000 e 1500 mg/L) de γ-Fe_2O_3 NPs. As plantas controle receberam apenas 20 mL de solução nutritiva. Para propósitos comparativos, plantas de soja também foram tratadas com 20 mL de uma solução de $FeCl_3$ à 1000 mg/L a cada dois dias. No 16º dia de cultivo as plantas foram retiradas do sistema experimental e separadas em raízes, caules e folhas para determinação dos comprimentos, das biomassas frescas e da área foliar. As biomassas secas de raízes, caules e folhas foram determinadas após desidratação dos tecidos em estufa a 70ºC por 72 h. A viabilidade das células do ápice radicular foi avaliada através do método de captação do corante azul de Evans. As atividades das PODs solúvel e ligada à parede celular de raízes e os teores de lignina de raízes e caules foram determinados espectrofotometricamente. Os compostos fenólicos totais foram quantificados pelo método de Folin Ciocalteau. A significância das diferenças observadas foi avaliada por ANOVA. As diferenças entre os parâmetros foram avaliadas pelo teste de comparação múltipla de Dunnet e os valores de $p \leq 0.05$ foram considerados estatisticamente significativos.

RESULTADOS E DISCUSSÃO – De modo geral, nossos resultados mostraram que as γ-Fe_2O_3 NPs e o $FeCl_3$ atuam sobre a soja por diferentes mecanismos. Em relação a via de fenilpropanoides, a exposição das plantas de soja às γ-Fe_2O_3 NPs estimulou a atividade da POD ligada à parede celular de raízes, mas inibiu a atividade da PAL. Contrariamente, a atividade da POD ligada à parede celular foi inibida por $FeCl_3$. O efeito inibitório das γ-Fe_2O_3 NPs e do $FeCl_3$ sobre a PAL deve-se, provavelmente, a um feedback negativo causado pelo acúmulo de compostos fenólicos nos tecidos. O conteúdo de lignina aumentou em raízes e caules em resposta a ambos os tratamentos, γ-Fe_2O_3 NPs e $FeCl_3$. Entretanto, significativa inibição do crescimento induzida pela lignificação foi observada apenas nos caules das plantas tratadas com γ-Fe_2O_3 NPs, provavelmente devido a alterações na composição monomérica da lignina. Neste caso, γ-Fe_2O_3 NPs reduziram o conteúdo do monômero guaiacil (G) nas raízes, enquanto aumentou seu conteúdo nos caules. Diferentemente, $FeCl_3$ aumentou o conteúdo dos monômeros *p*-hidroxifenil (H) e siringil (S) nas raízes. Em resumo, o maior conteúdo de monômero G nos caules das plantas tratadas com γ-Fe_2O_3 NPs levou à redução da razão S:G e à produção de uma lignina com maior número de ligações cruzadas, com consequente enrijecimento da parede celular e inibição do crescimento. Em contraste, o aumento da razão S:G nas raízes das plantas tratadas com $FeCl_3$ pode explicar a ausência de inibição do crescimento, pois sugere a síntese de uma lignina menos condensada e com menor capacidade de restringir a expansão celular.

CONCLUSÕES – Em resumo, a inibição do crescimento do caule verificada em plantas de soja expostas às γ-Fe_2O_3 NPs não pode ser explicada meramente pelo estímulo na deposição de lignina, mas também pelas alterações em sua composição monomérica. O maior conteúdo de monômero G nos caules levou a uma redução na razão S:G e a uma lignina com maior número de ligações cruzadas, com consequente enrijecimento da parede celular e inibição do crescimento. Em adição, os efeitos causados pelas γ-Fe_2O_3 NPs divergiram daqueles causados pelo metal de mesma valência ($FeCl_3$), mostrando que ambos atuam sobre a soja por diferentes mecanismos.

GENERAL ABSTRACT

INTRODUCTION AND OBJECTIVES – Nanoparticles (NPs) are natural or man-made substances that have at least one dimension in the size smaller than 100 nm. Due to their reduced size and shape they have different chemical properties than the chemical elements that make them up. Characteristics such as high surface area in relation to volume and chemical reactivity have allowed its use in the areas of physical, chemical, biological and health sciences, as well as other interdisciplinary fields of science and engineering. The potential of this technology is highlighted by the growing investments in the sector, which in 2009 amounted to approximately 220 billion dollars (US$ 220 bi). At the same time, researchers and society have shown concerns about the environmental impact and toxicity of nanomaterials-based products. Currently, studies in the field of nanotoxicology are increasing and many researchers believe the toxicity and fate of nanomaterials should be studied before giving attention to their applications. There is a serious lack of information on the impacts of NPs on human health and the environment. The potential of nanomaterials to be used in a wide range of sectors means that the number of possible routes for NPs to contact organisms and the environment is high. They can be discharged directly into the rivers or atmosphere by the industry, or inadvertently escape from paints, cosmetics, sunscreens and pharmaceuticals products that are used or disposed of in the environment. Because the nanoparticles have a small size, their surface area in relation to volume increases exponentially, which makes these particles very reactive and toxic. In addition, as the size decreases, the ability of NPs to penetrate plant and animal tissues increases. In fact, research has shown that NPs can be bioaccumulated through the food chain, and that accumulation could reach organisms at the top of the chain. Because they are producing organisms, plants constitute an important route of entry and transport of NPs in the food chain. In addition, it is currently questioned whether the presence of NPs in the environment can lead to losses in crop productivity, since many studies have shown negative effects of NPs on the germination and growth of a diversity of plants. In this context, future works aimed at understanding the toxic effects of NPs on the growth and development of cultivated plants such as soybean and corn are essential. Thus, in this work the toxic effects of γ-Fe_2O_3 NPs on the soybean growth and its relationship with the metabolism of lignin were evaluated. The activities of PAL, soluble and cell-wall bound PODs, total lignin

content, as well as the lignin monomer composition were determined to understand the mechanism of toxicity of the γ-Fe_2O_3 NPs. Further experiments with iron III chloride ($FeCl_3$) were carried out to distinguish the toxic effects of γ-Fe_2O_3 NPs from ionic iron.

MATERIALS AND METHODS - Soybean seeds (*Glycine max* L.), *cv*. BRS-232, were sanitized with 2% sodium hypochlorite ($HClO_3$) for 2 min and germinated between two sheets of Germitest® paper previously moistened with distilled water. After three days, viable seedlings were transferred to plastic trays containing 50 mL cells filled with vermiculite and, then, watered with 20 mL of distilled water. The trays containing the seedlings were conditioned in a growth room at 25°C, photoperiod of 12/12 h (light/dark) and irradiance of 300 μmol photons m^{-2} s^{-1}, where remained for 16 days. The treatments of soybean plants were carried out every other day by the addition of 20 mL of Hoagland nutrient solution (pH 6.0) containing different concentrations (250, 500, 1000 and 1500 mg/L) of γ-Fe_2O_3 NPs. The control plants received only 20 mL of nutrient solution. For comparative purposes, soybean plants were also treated with 20 mL of a 1000 mg/L $FeCl_3$ solution every other day. On the 16th day of cultivation the plants were removed from the experimental system and separated into roots, stems and leaves to determination of the lengths, fresh weights and leaf area. The dry weights of roots, stems and leaves were determined after dehydration of the plant tissues in an oven at 70ºC for 72 h. The viability of the root apex cells was evaluated by the Evans blue dye uptake method. The activities of soluble and cell-wall bound PODs of roots and the lignin content of roots and stems were determined spectrophotometrically. Total phenolic compounds were quantified by the Folin Ciocalteau method. The significance of the observed differences was evaluated by ANOVA. Differences between parameters were assessed by Dunnet's multiple comparison test and values of $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION - In general, our results showed that γ-Fe_2O_3 NPs and $FeCl_3$ act on soybean by different mechanisms. In relation to the phenylpropanoid pathway, the exposure of soybean plants to γ-Fe_2O_3 NPs stimulated the cell-wall bound POD activity, but inhibited the activity of PAL. In contrast, the activity of cell-wall bound POD was inhibited by $FeCl_3$. The inhibitory effect of γ-Fe_2O_3 NPs and $FeCl_3$ on PAL is probably due to negative feedback caused by the accumulation of phenolic compounds in plant tissues. The lignin content increased in roots and stems in response

to both treatments, γ-Fe_2O_3 NPs and $FeCl_3$. However, significant lignin-induced growth inhibition was observed only in the stems of plants treated with γ-Fe_2O_3 NPs, probably due to changes in the monomeric composition of lignin. In this case, γ-Fe_2O_3 NPs reduced the content of the guaiacyl monomer (G) in the roots, while increasing its content in the stems. In contrast, $FeCl_3$ increased the content of the *p*-hydroxyphenyl (H) and syringyl (S) monomers in the roots. In summary, the higher G monomer content in the stems of plants treated with γ-Fe_2O_3 NPs led to the reduction of the S:G ratio and the production of a more highly cross-linked lignin, with consequent cell wall stiffening and inhibition of growth. In contrast, the increase of the S:G ratio in the roots of the plants treated with $FeCl_3$ may explain the absence of inhibition of growth, since it suggests the synthesis of a less condensed lignin, with less capacity to restrict cell expansion.

CONCLUSION – In summary, the stem growth-inhibition verified in soybean plants exposed to γ-Fe_2O_3 NPs cannot be explained merely by the stimulus in lignin deposition, but also by changes in its monomer composition. The higher G monomer content in the stems led to a reduction in the S:G ratio and a more highly cross-linked lignin, with consequent stiffening of the cell wall and inhibition of growth. In addition, the effects caused by the γ-Fe_2O_3 NPs diverged from those caused by the metal of the same valence ($FeCl_3$), showing that both act on the soybean by different mechanisms.

Lignin Induced Growth-Inhibition in Soybean (*Glycine max* L.) Exposed to Iron Oxide Nanoparticles (γ-Fe_2O_3)

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ABSTRACT

Plants are constantly exposed to environmental perturbations that limit their growth, and one of these conditions is the exposure and interaction with different nanoparticles that are plenty and continuously discarded into the environment. Hitherto, no study has been carried out evaluating the effects of iron (III) oxide (γ-Fe_2O_3) NPs on soybean growth and lignin production, as we have proposed herein. Furthermore, and for a comparative purpose, we have submitted soybean plants to iron (III) chloride ($FeCl_3$), the ionic counterpart of element iron. Exposure of plants to γ-Fe_2O_3 NPs stimulated the activity of cell wall-bound peroxidase (POD) of roots, but inhibited the phenylalanine ammonia lyase (PAL) activity, which can be due to the negative feedback of accumulated phenolic compounds. By contrary, the cell-wall bound POD were inhibited by $FeCl_3$. Both γ-Fe_2O_3 NPs and $FeCl_3$ increased the lignin content in roots and stems. A significant growth inhibition of stems was noted after γ-Fe_2O_3 NPs exposure, which was due probably to changes in the lignin monomer composition. In this case, γ-Fe_2O_3 NPs decreased the content of guaiacyl (G) monomer in roots, but increased it in stems. In turn, $FeCl_3$ increased the contents of *p*-hydroxyphenyl (H) and syringyl (S) in roots. High content of monomer G in stems caused by γ-Fe_2O_3 NPs decreased the S:G ratios generating a more highly cross-alinked lignin followed by the stiffening of the cell wall and growth inhibition. Contrarily, increase of S:G ratio in the roots of plants submitted to $FeCl_3$ are in agreement with the absence of effects on growth, due to production of a less condensed lignin. In brief, our findings showed that both γ-Fe_2O_3 NPs and $FeCl_3$ act differently in soybean.

INTRODUCTION

Nanoparticles (NPs) are natural or man-made substances that have at least one dimension in the size smaller than 100 nm (Ju-Nam and Lead, 2008; Arruda et al., 2015; Khan et al., 2017). According to their chemical composition, NPs can be divided into four groups: 1) inorganic NPs, 2) carbon-based NPs, 3) quantum dots and 4) nanopolymers. They exist in fused, aggregated or agglomerated forms, and can have a spherical, tubular or irregular shape (Nowack and Bucheli, 2007; Hatami et al., 2016). The shape and the reduced size of the NPs give to them different chemical properties from their constituent elements (Brunner et al., 2006). Among the main characteristics are high chemical reactivity and surface energy, high surface area in relation to volume, as well as catalytic and magnetic properties, among others (Bombin et al., 2015). These properties have allowed the wide use of NPs in industry, medicine and agriculture (Arruda et al., 2015; Anwaar et al., 2016; Fraceto et al., 2016; Peters et al., 2016). In fact, investments in nanotechnology exceeded $224 billion in 2009, with NPs production estimated to reach 58,000 tons in 2011-2020 (Bombin et al., 2015).

Industrially, NPs can be used in cosmetics as sunscreens. Titanium dioxide (TiO_2) and zinc oxide (ZnO) do not absorb visible light and block UV light, improving the efficiency of sunscreens without presenting the whitish appearance of other blockers (Ko et al., 2012). One of the most exciting applications of NPs are drug loading and delivery systems, with relevant use in medical field. In addition to protecting the drug against premature degradation in the body, encapsulation in nanosystems helps to direct it to the target tissue or cell, increasing its absorption (Shaffer, 2005; Wilczewska et al., 2012). In agriculture, NPs increase productivity, improve soil quality, stimulate plant growth and providing an intelligent monitoring system for humidity, pH, temperature and light exposure (Fraceto et al., 2016).

Iron NPs (INPs) exist as zero valent iron (ZVI) and iron oxides, such as maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4). They are among the most widely used NPs, being useful in photocatalysis (Nie et al., 2010), gas sensor (Zhou et al., 2014), drug delivery and as contrast agent for magnetic resonance imaging (Bombin et al., 2015). They are also important in environmental remediation technologies due to the low cost and effectiveness. For example, they can destruct or stabilize substances as organochlorine pesticides, polychlorinated biphenyls, chlorinated organic solvents, halogenated hydrocarbons, carbon tetrachloride, arsenite, organic dyes, some inorganic compounds

and metal ions (Yuvakkumar et al., 2011; Cheng et al., 2015; Ruttkay-Nedecky et al., 2017). In addition, the use γ-Fe_2O_3 NPs has been proposed as an alternative for the substitution of traditional iron fertilizers (Rui et al., 2016; Ruttkay-Nedecky et al., 2017). Due to its wide use, it is evident the increasing concentration of INPs in soil and water, being necessary the evaluation of their toxicity on plants, especially crops. In fact, several studies reveal that INPs affect germination and growth of different plant species (Trujillo-Reyes et al., 2014; Li et al., 2016; Rui et al., 2016; Hu et al., 2017).

Lignification, the metabolic process of sealing a plant cell wall by lignin deposition, is a crucial step during plant growth (Lima et al., 2013). Lignin, a complex phenolic polymer essential for the mechanical and chemical resistance to the plant cell wall, is a product of the phenylpropanoid pathway (Boerjan et al., 2003). The first step in this pathway is the deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL) to produce *t*-cinnamate. In turn, cinnamate 4-hydroxylase (C4H) converts *t*-cinnamate to *p*-coumarate, which is esterified with coenzyme A (CoA) by the action of 4-coumarate:CoA ligase (4CL). Subsequent steps consist of hydroxylation and methoxylation of the pathway intermediates to successively produce caffeate, ferulate, 5-hydroxyferulate and sinapate. Further, cinnamyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) convert these intermediates into the corresponding monolignols (*p*-coumaryl, coniferyl and sinapyl alcohols). By action of cell wall-bound peroxidase (POD) these three monolignols are polymerized to form, respectively, the *p*-hydroxyphenyl (H), guaiacyl (G) and (S) syringyl monomers of the lignin (Salvador et al., 2013).

Although excessive lignification is often associated with the reduction of plant growth under abiotic stresses (Gall et al., 2015), few studies have attempted to evaluate the role of this metabolic process on growth of plants exposed to NPs. For example, a first and recent study carried out for this purpose demonstrated that the root growth inhibition of soybean by copper oxide (CuO) NPs was correlated to increased lignification and expression of *PAL*, *C4H*, *CAD* and *POD* genes (Nair and Chung, 2014). To gain a deeper insight about the mechanism of toxicity of the γ-Fe_2O_3 NPs, we evaluated herein its effects on soybean growth and lignin metabolism. For this, we determined the activities of PAL, soluble and cell-wall bound PODs, total lignin content and its monomeric composition. We also compared the effects of iron (III) chloride ($FeCl_3$) with those observed with γ-Fe_2O_3 NPs.

MATERIALS AND METHODS

Characterization of γ-Fe_2O_3 NPs

The γ-Fe_2O_3 NPs (particle size <50 nm (BET), surface area of 50-245 m^2/g, product number 544884) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). The shape and size of the NPs were determined by transmission electron microscopy (TEM). For this, 15 mg/L of γ-Fe_2O_3 NPs were suspended in distilled water and sonicated for 15 min. Then, a drop of NPs suspension was placed in a Formvar-coated copper grid, drained with a filter paper and examined on a high-resolution transmission electron microscope (JE 1400) adjusted for an accelerating voltage of 120 kV.

General Procedures

Soybean (*Glycine max* L. Merril) seeds, *cv*. BRS-232, were sanitized with 2% sodium hypochlorite for 2 min, rinsed extensively with deionized water, and evenly distributed on two Germitest® paper sheets previously moistened with deionized water. The seeds were covered with an additional sheet of paper, rolled and packed in plastic tubes containing a small water film to maintain moisture. The seeds were transferred to germination chambers where remained in darkness at 25°C for 72 h. After germination, uniform seedlings were selected, transferred into 50 mL plastic trays filled with substrate vermiculite, and watered with 20 mL of distilled water. The trays were kept in a plant growth room at 25°C with a light/dark photoperiod of 12/12 h and a photon flux density of 300 μmol m^{-2} s^{-1} for 16 days. The plants were treated every other day with different concentrations of γ-Fe_2O_3 NPs (250, 500, 1000 and 1500 mg/L), dissolved in Hoagland nutrient solution (pH 6.0) and sonicated for 15 min. The control plants were watered every other day with 50 mL of Hoagland nutrient solution. For comparative purposes, soybean plants were treated with 1000 mg/L ferric chloride ($FeCl_3$). All reagents were of the purest grade available or of chromatographic grade, and used as received.

Plant Growth Parameters

After 16 days of cultivation with of γ-Fe_2O_3 NPs or $FeCl_3$, the soybean plants were divided into roots, stems and leaves. The lengths and fresh weights of roots and stems were determined immediately. The dry weights were recorded after oven-dehydration of the plant tissues at 70ºC for 72 h. The leaf area of the first trifolium was determined with the aid of a leaf area integrator (LI 3100, LI-COR).

Cell Viability

The loss of viability of 16-d old soybean root cells was determined by the Evans blue (a nonpermeating dye) staining spectrophotometric assay (Neves et al., 2012). First, the excised roots were incubated in 15 mL of 0.25% Evans blue solution for 15 min. After, the roots were dipped in deionized water for 30 min to remove the excess of unbound dye to the dead cells. Then, root tips (0.5 cm) were excised and transferred to eppendorf tubes containing 1 mL of *N*,*N*-dimethylformamide for 50 min, at room temperature, to extract the unbound dye. The absorbance was measured at 600 nm, using deionized water as a blank. The loss of cell viability was expressed as absorbance of treated roots in relation to control.

Enzymatic Assays

The activity of phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5) was determined as described by Ferrarese et al. (2000). Fresh roots (2.0 g) were homogenized in 5,0 mL of 0.1 M sodium borate buffer (pH 8.8) at 4°C. The homogenate was centrifuged at 2200×*g* for 15 min and the supernatant used as an enzyme preparation. The reaction mixture contained 1,0 mL of 0.1 M borate buffer (pH 8.7), 0,25 mL enzyme preparation and 0,25 mL of 50 mM L-phenylalanine as substrate, for a final volume of 1,5 mL. Prior to addition of the substrate, the reaction mixture was incubated at 40°C for 5 min. To start the reaction, substrate was added; and it was stopped after 1 h by adding 50 µL of 5 M HCl.

Samples (20 µL) were filtered through of polyvinylidene fluoride (PVDF) membranes with 0.45 µm porosity and then analyzed in a high-performance liquid chromatograph (Prominence HPLC system, Shimadzu®, Tokyo, Japan) equipped with a quaternary gradient pump (LC-20AT), auto-sampler (SIL-20A), photodiode array detector (SPD-M20A), column oven (CTO-20A), degasser (DGU-20A), communications bus module

(CBM-20A) and an LcSolution workstation system. A reversed-phase CLC-ODS (M) column (150 × 4.6 mm, with 5 μm particles), equipped with an equivalent pre-column (10 x 4.6 mm), was used at 30°C. The mobile phase was methanol:water (70:30, *v*:*v*) with a flow of 0.5 mL min^{-1}. Absorption was monitored at 275 nm. *t*-Cinnamate, the product of the enzymatic reaction, was identified by comparing its retention time with that of a Sigma-Aldrich® standard (St. Louis, MO, USA, Product number C80857). The results were expressed as μmol *t*-cinnamate h^{-1} g^{-1} fresh weight.

Soluble and cell wall-bound peroxidases (POD, EC. 1.11.1.7) activities were determined as described by (Santos et al., 2008). Fresh roots (0.5 g) were ground in 5 mL of 67 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 2200×*g* for 5 min at 4°C, and the supernatant was used to determine the soluble POD activity. The pellet was resuspended with 2 mL of 1 M NaCl and incubated at 4°C for 1 h. After centrifugation (2200×*g* for 5 min at 4°C) the supernatant was used to determine the cell wall-bound POD activity. The reaction mixture, with a final volume of 3.0 mL, contained 25 mM phosphate buffer (pH 6.8), 2.58 mM guaiacol, 10 mM H_2O_2 and 400 μL of enzyme preparation appropriately diluted. The reaction was started by the addition of the enzyme preparation, and the oxidation of guaiacol was followed spectrophotometrically, at 470 nm, for 5 min. The enzyme activity was calculated from a molar extinction coefficient (ε) of 25.5 mM cm^{-1}. The POD activities were expressed as μmol tetraguaiacol min^{-1} g^{-1} fresh weight.

Total Phenolics Quantification

To quantify total phenolics, dry roots or stems (0.25 g) were ground in 5.0 mL of 2 M HCl and transferred to screw-cap glass tubes. Samples were boiled for 30 min followed by cooling in water for 5 min. After filtration through a Whatman® paper, 2.5 mL of samples appropriately diluted were mixed with 350 μL of 1.9 M sodium carbonate plus 125 μL of Folin-Ciocalteau phenol reagent. The reaction was kept in darkness for 1 h. The absorbance was read at 750 nm, against deionized water as blank. The total phenolics content was calculated from a molar extinction coefficient (ε) of 13.665 mM cm^{-1}, obtained from a standard curve of ferulic acid (Sigma-Aldrich®, Product number 128708). The results were expressed as mg total phenolics g^{-1} dry weight.

Quantification of Lignin and Monomer Composition

An adequate obtaining of the protein-free cell wall is crucial to quantify lignin and its monomer composition (Ferrarese et al., 2002). For this, dry roots (0.3 g) were homogenized in 7 mL of 50 mM phosphate buffer (pH 7.0) and transferred to 15 mL centrifuge tubes. The homogenate was centrifuged at 1400×*g* for 5 min and subsequently washed by successive stirring and centrifugation as follows: 2 × with 7 mL of 50 mM phosphate buffer (pH 7,0); 3 × with 1% Triton® (*v*:*v*) prepared in 50 mM phosphate buffer (pH 7,0); 2 × with 7,0 mL of 1,0 M NaCl also in 50 mM phosphate buffer (pH 7,0); 2 × with 7,0 mL of distilled water and 2 × with 5,0 mL of acetone. The pellet was oven dried at 60°C for 24 h and cooled in a vacuum desiccator. The dry matter obtained was defined as the protein-free cell wall fraction.

To determine the lignin content, protein-free cell wall tissues (20 mg) were placed in screw-cap centrifuge tubes containing 0.5 mL of 25% acetyl bromide reagent prepared in glacial acetic acid. The reaction occurred in a water bath at 70°C for 30 min, and it was stopped on ice by adding 0.9 mL of 2.0 M NaOH. Next, 0.1 mL of 7.5 M hydroxylamine-HCl and 4 mL of glacial acetic acid were added to the mixture. The samples were centrifuged at 1000×*g* for 5 min, and the absorbance of the supernatant was measured at 280 nm, after appropriate dilution. The lignin content was determined using a calibration curve with lignin (alkali, 2-hydroxy-propyl ether, Sigma-Aldrich®, Product number 37096-7). The results were expressed as mg/g of protein-free cell wall (Moreira-Vilar et al., 2014).

The method of alkaline nitrobenzene oxidation (Dean, 1997) was used to quantify the lignin monomer composition. Protein-free cell wall samples (50 mg) were transferred to Pyrex® ampoules containing 1 mL of 2 M NaOH plus 100 μL of nitrobenzene. The ampoules were sealed by a gas torch and heated at 170°C for 150 min. A stirring was performed after halftime. Then, the samples were cooled at room temperature and washed twice with chloroform. After acidification to pH 3–4 with 5 M HCl, the samples were extracted twice with chloroform; the organic extracts were combined and dried by using a rotary evaporator. Next, the samples were resuspended in 1.0 mL of methanol, filtered through a 0.45-μm PVDF membranes analyzed by HPLC, as described earlier. The mobile phase consisted of a mixture of methanol:4% acetic acid (20:80, *v*:*v*) with a flow rate of 1.2 mL min^{-1}. Absorption of the monomer aldehyde products (*p*-hydroxybenzaldehyde, vanillin and syringaldehyde) was recorded at 290 nm using the

corresponding standards (Sigma-Aldrich®, St. Louis, MO, USA, Product numbers 54590, V1104 and S7602, respectively). The results were expressed as mg monomer g^{-1} protein-free cell wall.

Statistical Analysis

The experimental design was completely randomized, and each plot was represented by a 50mL plastic tray with one plant. Data were expressed as the mean of 4 to 7 independent experiments ± standard error of the mean. One-way analysis of variance (ANOVA) was done to test the significance of the observed differences using the *GraphPad Prism*® software package (version 5.01 GraphPad Software Inc., USA). Dunnett´s post-doc test was done to determine the differences between parameters, and a level of probability of $p \leq 0.05$ was considered as statistically significant.

RESULTS

Characterization of γ-Fe_2O_3 NPs

The TEM micrographs shown that the γ-Fe_2O_3 NPs have a spherical morphology with an average diameter of 50 nm (**Figure 1A–D**). This agrees with the manufacturer (Sigma-Aldrich®) specifications. However, the images also revealed a low homogeneity of NPs, which varies from 10 to about 100 nm.

The γ-Fe_2O_3 NPs Affected the Soybean Growth and Cell Viability

By and large, the γ-Fe_2O_3 NPs stimulated the root growth while stems were reduced (**Figure 2**). When compared to the control, the root length increased by 22% after 1000 mg/L γ-Fe_2O_3 NPs exposure (**Figure 2A**). After exposure to 1000 and 1500 mg/L γ-Fe_2O_3 NPs, the fresh and dry weights of roots increased by 20% and 26% (**Figure 2C**) and by 34% and 40% (**Figure 2E**), respectively, in comparison to the corresponding controls. The effects from 250 to 1500 mg/L γ-Fe_2O_3 NPs were also evident on stem lengths, which significantly decreased from 16% to 30%, respect to control (**Figure 2B**). While the stem fresh weight was reduced by 14% at 250 mg of γ-Fe_2O_3

NPs (**Figure 2D**), the dry weights were reduced about 16% regardless of the concentration, when compared to the corresponding controls. The $FeCl_3$ did not affect all growth parameters, except for an increase of 32% in the root dry weight (**Figure 2E**).

The γ-Fe_2O_3 NPs did not affect the leaf area (**Figure 3A**). However, the fresh and dry weights of leaves were increased by 60% and 69% (**Figure 3B**) and by 42% and 40% (**Figure 3C**) by action of 1000 and 1500 mg/L γ-Fe_2O_3 NPs, respectively, when compared to the corresponding controls. Exposure of plants to $FeCl_3$ did not affect these parameters.

The results revealed that the viability of root cells was affected by both γ-Fe_2O_3 NPs and $FeCl_3$ (**Figure 4**). Uptake of the Evans blue dye in roots exposed to 500–1500 mg/L γ-Fe_2O_3 NPs was, in mean, 120% higher than that observed in control plants. Already, the dye uptake was 225% higher than control after $FeCl_3$ exposure, indicating significant loss of the cell viability.

The POD and PAL Activities Were Altered by γ-Fe_2O_3 NPs

In roots, the activities of soluble and cell-wall bound PODs and PAL were significantly altered by γ-Fe_2O_3 NPs and $FeCl_3$. The soluble POD (**Figure 5A**) and cell wall-bound POD (**Figure 5B**) activities increased by 34% and 42% and by 36% and 56%, respectively, after 1000 and 1500 mg/L γ-Fe_2O_3 NPs treatments, when compared to the corresponding controls. The PAL activities decreased in a dose-dependent manner, *i.e.*, from 22% to 60% with 250–1500 mg/L of γ-Fe_2O_3 NPs, in comparison to the control (**Figure 5A**). The same figures reveal that $FeCl_3$ decreased activities of soluble POD (25%), cell wall-bound POD (24%) and PAL (52%), in comparison to the controls.

The γ-Fe_2O_3 NPs Increased Lignin and Changed its Monomeric Composition

Because of γ-Fe_2O_3 NPs exposure, the lignin contents in roots and stems were significantly different from those of the controls (**Figure 6**). The exposure of soybean roots to 1000 and 1500 mg/L γ-Fe_2O_3 NPs increased lignin content by 18% and 26%, respectively (**Figure 6A**). At the same concentrations, γ-Fe_2O_3 NPs increased the lignin contents in stems by 61% and 53% (**Figure 6B**). Similarly, $FeCl_3$ increased lignin contents of roots and stems by 28% and 53%, respectively.

Data revealed that γ-Fe_2O_3 NPs also affected lignin monomer composition of roots and stems of soybean (**Figure 7**). The main change noted in roots was a reduction of the G contents, in mean 27%, regardless of the concentration, and compared to control (**Figure 7A**). The γ-Fe_2O_3 NPs did not affect the H and S contents, except for a reduction (28%) in the S units after 250 mg/L exposure. From 250 to 1500 mg/L γ-Fe_2O_3 NPs exposures, the G contents in stems were, in mean, 29% higher than that observed in control plants (**Figure 7B**). The H unit increased 38% after 1500 mg/L γ-Fe_2O_3 NPs treatment, while no change was noted in the S contents. Finally, $FeCl_3$ increased the monomers H (63%) and S (97%), and reduced G (32%) in roots, but without any effect on stems.

Table 1 summarizes the measurements of lignin (referred to as the sum of H+G+S) and the S:G ratios. In roots, the γ-Fe_2O_3 NPs reduced the H+G+S contents by 39%, 28%, 34% and 25% from 250 to 1500 mg/L treatments, respectively, compared with the control. The S:G ratios were not affected by the γ-Fe_2O_3 NPs. By contrary, in stems, the γ-Fe_2O_3 NPs increased, in mean, 22% the H+G+S contents after 250, 1000 and 1500 mg/L treatments, when compared to the control. In addition, the S:G ratios were reduced by 24% to 11% from 250 to 1500 mg/L treatments, respect to control. In turn, and only in roots, $FeCl_3$ increased 155% the S:G ratio, without affect other parameters.

Finally, the phenolics contents were significantly higher in plants exposed to γ-Fe_2O_3 NPs than in the control plants (**Figure 8**). In roots, γ-Fe_2O_3 NPs increased the phenolics contents by 22% (in mean) after 250 to 1500 mg/L treatments (**Figure 8A**). In stems, the increases of phenolics contents were more evident, *i.e.*, by 84% to 124% from 250 to 1500 mg/L exposures, in comparison to the control (**Figure 8B**). The $FeCl_3$ increased by 12% and 158% the phenolics contents of roots and stems, respectively, when compared to the corresponding controls.

DISCUSSION

At large, our findings revealed that γ-Fe_2O_3 NPs significantly affected the growth of soybean plants, *i.e.*, stimulated roots and inhibited stems (**Figure 2**), and increased the fresh and dry weights of leaves, without any changes on foliar areas (**Figure 3**). Stimulatory or inhibitory effects on growth have been reported after exposure of several plants to NPs, in most cases in a dose-dependent manner to the type, concentration and exposure of NPs, and the plant organ/tissue analyzed. For instance, γ-Fe_2O_3 NPs

increased root length, plant height, biomass and the soil plant analysis development (SPAD) index of *Arachis hypogaea* (Rui et al., 2016). In soybean, 2000 mg/L γ-Fe_2O_3 NPs stimulated by 40% the root length, after 5 days of treatment. In addition, from 500 to 1000 mg/L it increased root and stem dry weights, leaf area and SPAD index after 14 days (Alidoust and Isoda, 2013).

It has been proposed that increases of root length are related to high solubility of γ-Fe_2O_3 NPs, reduced size which allows their uptake by the roots, and a greater availability of iron (Dehner et al., 2011; Alidoust and Isoda, 2013). On the issue of the size, there is a broad consensus that above 20 nm, these NPs do not overpass the plant cell wall (Zuverza-Mena et al., 2017). Remarkably, the uptake of 20.2 nm γ-Fe_2O_3 NPs was demonstrated in *Citrus maxima* plants, but they were not translocated from the roots to the stems (Hu et al., 2017). Herein, the MET analysis showed γ-Fe_2O_3 NPs with 50 nm of average size, but a lot of them have less than 20 nm (**Figure 1**). Likewise, a good dispersion of the NPs was evident after sonication of the suspension for 15 min; a procedure performed before MET analyses and soybean plants exposures. Size, aggregation and sedimentation are important factors that should be monitored in studies with NPs because they can alter the effectiveness of the concentration tested (Alidoust and Isoda, 2013). Our findings suggest that γ-Fe_2O_3 can have been absorbed, at least in part, by the soybean roots, stimulated the growth of roots and leaves and increased the availability of iron. In addition, soybean plants exposed to high concentration of ionic iron (1000 mg/L $FeCl_3$) were not affected (**Figures 2** and **3**), except for an increase in the root dry weights (**Figure 2E**). Yet about our results on soybean growth, a hypothesis cannot have ruled out; γ-Fe_2O_3 NPs can influence the growth by changing the levels of phytohormones, and evidences has been revealed in *Arabidopsis thaliana* (Lei et al., 2014), *Oriza sativa* (Gui et al., 2015) and *Arachis hipogaea* (Rui et al., 2016). However, this clue should be properly investigated in soybean.

Despite the stimulatory effect on roots, we observed significant losses of cell viability after exposure of plants with 500 to 1500 mg/L of γ-Fe_2O_3 NPs, and a more pronounced effect with 1000 mg/L $FeCl_3$ (**Figure 4**). It is known that iron oxide NPs (IONPs) reduce the hydraulic conductivity of roots and the uptake of water and nutrients due to mechanical rupture of membranes and cell walls of roots (Martínez-Fernández and Komárek, 2016; Zuverza-Mena et al., 2017). Most likely, this effect is due to the ability of IONPs and their aggregates adhere to negatively charged surface of the roots by electrostatic attraction, and connect to cation exchange sites (Trakal et al.,

2015). As noted here, the reduction in the cell viability seems not be sufficient to impair water and nutrient absorption, even under $FeCl_3$ exposure. In fact, a 10-fold increase in losses of cell viability was required to inhibit soybean root growth by salt stress (Neves et al., 2012). Our data also revealed that 1000 mg/L FeCl3 was more toxic than γ-Fe2O3 NPs for soybean root cells (**Figure 4**). Although iron is an essential micronutrient for biosynthesis of heme and iron-sulfur centers, it can be toxic to plants at high concentration (Qin et al., 2015).

One of supposed mechanisms for toxicity of NPs, including INPs, is an excessive generation of reactive oxygen species (ROS), which induce oxidative stress in plants (Fu et al., 2014; Hatami et al., 2016). Increased activities of antioxidant enzymes, such as superoxide dismutase, peroxidase (POD) and catalase, among others, are an adaptive response of the plants to scavenger ROS, by reducing thus possible cellular damages (Soares et al., 2011). SOD is responsible for the dismutation of $O_2^{\bullet-}$ in H_2O_2, while POD and CAT detoxify the latter in H_2O (Apel and Hirt, 2004). As noted herein, increased soluble POD activity (**Figure 5B**) suggests a possible oxidative stress in soybean plants exposed to γ-Fe_2O_3 NPs. This is corroborated by an increase of total phenolics content (**Figure 8**), which can act as electron donors for the H_2O_2 detoxification through guaiacol-type PODs (Sakihama et al., 2002; Kováčik et al., 2010). In addition, the loss of the cell viability by action of γ-Fe_2O_3 NPs (**Figure 4**) suggests, at least in principle, a slight impairment of membrane integrity due to lipid peroxidation. In agreement with our findings, increases in POD, CAT and SOD activities were observed in *Zea mays* and *Oriza sativa* submitted to γ-Fe_2O_3 NPs (Gui et al., 2015; Li et al., 2016). The clear finding of the current study is that γ-Fe_2O_3 NPs and ionic iron ($FeCl_3$) act distinctly on antioxidant metabolism of soybean. In this sense, the inhibition of soluble POD activity by $FeCl_3$ (**Figure 5B**) indicates no oxidative stress.

A remarkable fact noted here is that the γ-Fe_2O_3 NPs influenced the soybean lignification, *i.e.*, significantly increased the lignin contents of roots and stems (**Figure 6**). In truth, this is the first study evaluating the effects of γ-Fe_2O_3 NPs on soybean lignin. Increased lignification in plants has been considered as a defense against abiotic stress. There are evidences that allelochemicals, such as cinnamic acid and derivatives, impair soybean plant growth associated with premature cell wall lignification (dos Santos et al., 2008; Zanardo et al., 2009; Bubna et al., 2011; Salvador et al., 2013). When compared to the corresponding controls, γ-Fe_2O_3 NPs greatly increased lignin content in stems in comparison to roots (**Figure 6**). Because stress-induced lignification

limits cell expansion, nutrient uptake and plant growth, this finding could explain the stem growth-inhibition caused by γ-Fe_2O_3 NPs (**Figure 2B**). In turn, the observed root lignification seems to be insufficient to limit its longitudinal growth, although it has contributed to an increase in their fresh and dry weights (**Figures 2A, C, E**).

A high lignin content is related in general to increased activity of PAL, that plays a key role in the phenylpropanoid pathway (Boerjan et al., 2003; Lima et al., 2013; Salvador et al., 2013). However, we have noted that PAL activity was reduced by the γ-Fe_2O_3 NPs (**Figure 5C**), despite increased lignin production (**Figure 6**). Similar behavior has been observed in soybean roots submitted to metabolites of the phenylpropanoid pathway, such as cinnamic and benzoic acids derivatives that inhibit PAL activity, both *in vitro* (Sato et al., 1982) and *in vivo* (Zanardo et al., 2009; Bubna et al., 2011). So, a plausible explanation for PAL inhibition by γ-Fe_2O_3 NPs can be a negative feedback caused by phenolic compounds accumulated in tissues (**Figure 8**).

Our data also revealed a stimulatory effect of γ-Fe_2O_3 NPs on cell-wall bound POD activities (**Figure 5B**). To polymerize monolignols into cell wall, this enzyme uses H_2O_2 that, at reduced level during lignin biosynthesis, can indirectly down-regulate the PAL activity (Zanardo et al., 2009; Kováčik et al., 2011). In line with our results, increased lignin content in soybean submitted to copper oxide (CuO) NPs was related to decreased growth of roots and stems, and increased levels of transcripts for anionic and cationic PODs (Nair and Chung, 2014).

Another striking observation is the fact that the γ-Fe_2O_3 NPs altered the lignin monomer composition (**Figure 7**). In this sense, γ-Fe_2O_3 NPs and $FeCl_3$ appear to act oppositely because the monomeric profiles of roots and stems were clearly different. In stems, the most evident change caused by γ-Fe_2O_3 NP was an increased G monomer content (**Figure 7B**), resulting in low S:G ratio (**Table 1**) and, therefore, a lignin more cross-linked and rigid (Cesarino et al., 2012). These findings are evidence that a lignin polymer with high degree of cross-linking can contribute for a reduced cell wall expansion and of the longitudinal growth of stems, as noted herein. Otherwise, γ-Fe_2O_3 NPs decreased the G content in roots (**Figure 7A**) generating a lignin less cross-linked. So, this fact can explain, at least in part, the absence of effects on root growth. No change in the root growth under action of $FeCl_3$ can be related to the formation of less cross-linked lignin, since an relevant increase in the S:G ratio (0,87) was observed in the current work (**Table 1**).

CONCLUSION

In brief, most interesting findings of the current work indicate that the stem growth-inhibition observed in soybean plants exposed to γ-Fe_2O_3 NPs cannot be solely explained by the increased lignin production, but also by changes in its monomer composition. The high G monomer content in stems lowed the S:G ratio generating a more highly cross-linked lignin, with subsequent stiffening of the cell wall and inhibition of the growth. In addition, the effects caused by the γ-Fe_2O_3 NPs diverged from those caused by the metal of the same valence ($FeCl_3$), evidencing that both act differently in soybean.

AUTHOR CONTRIBUTION

TLCL carried the experiments, GHGA and GSRM performed microscopy analyzes, RCSS helped to carry out the experiments, OFF revised the manuscript, RM idealized the experiments and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TABLES

Table 1. Monomer (H+G+S) composition and S:G ratios in soybean plants submitted to γ-Fe_2O_3 NPs for 16 days. The results are expressed as mg monomer g^{-1} cell wall.

			γ-Fe_2O_3 NPs (mg/L)			**$FeCl_3$ (mg/L)**
Root	**0**	**250**	**500**	**1000**	**1500**	**1000**
H+G+S	2.56 ± 0.22	1.55 ± 0.061*	1.84 ± 0.055*	1.69 ± 0,14*	1.92 ± 0.024*	2.50 ± 0.055
S:G	0.34 ± 0.019	0.29 ± 0.022	0.29 ± 0.014	0.39 ± 0.014	0.39 ± 0.003	0.87 ± 0.045*
Stem						
H+G+S	9.03 ± 0.39	11.11 ± 0.29*	10.56 ± 0.38	11.14 ± 0.22*	10.88 ± 0.68*	10.1 ± 0.46
S:G	0.63 ± 0.043	0.48 ± 0.038*	0.51 ± 0.023*	0.51 ± 0.016*	0.53 ± 0.017*	0.56 ± 0.017

H, *p*-hydroxyphenyl; G, guaiacyl; S, syringyl. Mean (n = 3–4 ± SE) values significantly different from the control ($p \leq 0.05$, Dunnett's multiple comparison test) are marked with an asterisk (*).

FIGURE CAPTIONS

Figure 1. Transmission electron microscope image of γ-Fe_2O_3 NPs. The nanoparticles are shown in magnification of 100 **(A)**, 200 **(B)**, 300 **(C)** and 500 **(D)** thousand times.

Figure 2. Effects of γ-Fe_2O_3 NPs on soybean growth. Root length **(A),** stem length **(B),** fresh root weight **(C),** fresh stem weight **(D),** dry root weight **(E)** and dry stem weight **(F)** of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. *Mean (n = 4–7 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$).

Figure 3. Effects of γ-Fe_2O_3 NPs on soybean leaf. Leaf area **(A)**, fresh leaf weight **(B)** and dry leaf weight **(C)** of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. *Mean (n = 4–7 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$).

Figure 4. Effects of γ-Fe_2O_3 NPs on cell viability. Loss of cell viability of roots of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. Increases in absorbance values indicate loss of the cell viability, respect to control. *Mean (n = 4–7 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$).

Figure 5. Effect of γ-Fe_2O_3 NPs on enzyme activities of soybean roots. Activities of soluble peroxidase (POD) **(A)**, cell wall-bound POD **(B)** and phenylalanine ammonia-lyase (PAL) **(C)** of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. *Mean (n = 4–7 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$).

Figure 6. Effects of γ-Fe_2O_3 NPs on lignin content of soybean. Lignin content of roots **(A)** and stems **(B)** of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. *Mean (n = 4–7 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$).

Figure 7. Effects of γ-Fe_2O_3 NPs on soybean lignin monomer composition. Lignin monomer composition of roots **(A)** and stems **(B)** of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. *Mean (n = 3–4 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$). H, *p*-hydroxyphenyl; G, guaiacyl; S, syringyl.

Figure 8. Effects of γ-Fe_2O_3 NPs on the content of total phenolics of soybean. Content of total phenolics in the roots **(A)** and stems **(B)** of soybean plants exposed to different concentrations of γ-Fe_2O_3 NPs or 1000 mg/L $FeCl_3$ for 16 days. *Mean (n = 4–7 ± SE) values differ statistically (Dunnett´s multiple comparison test) from the control ($p \leq 0.05$).

FIGURES

Figure 1

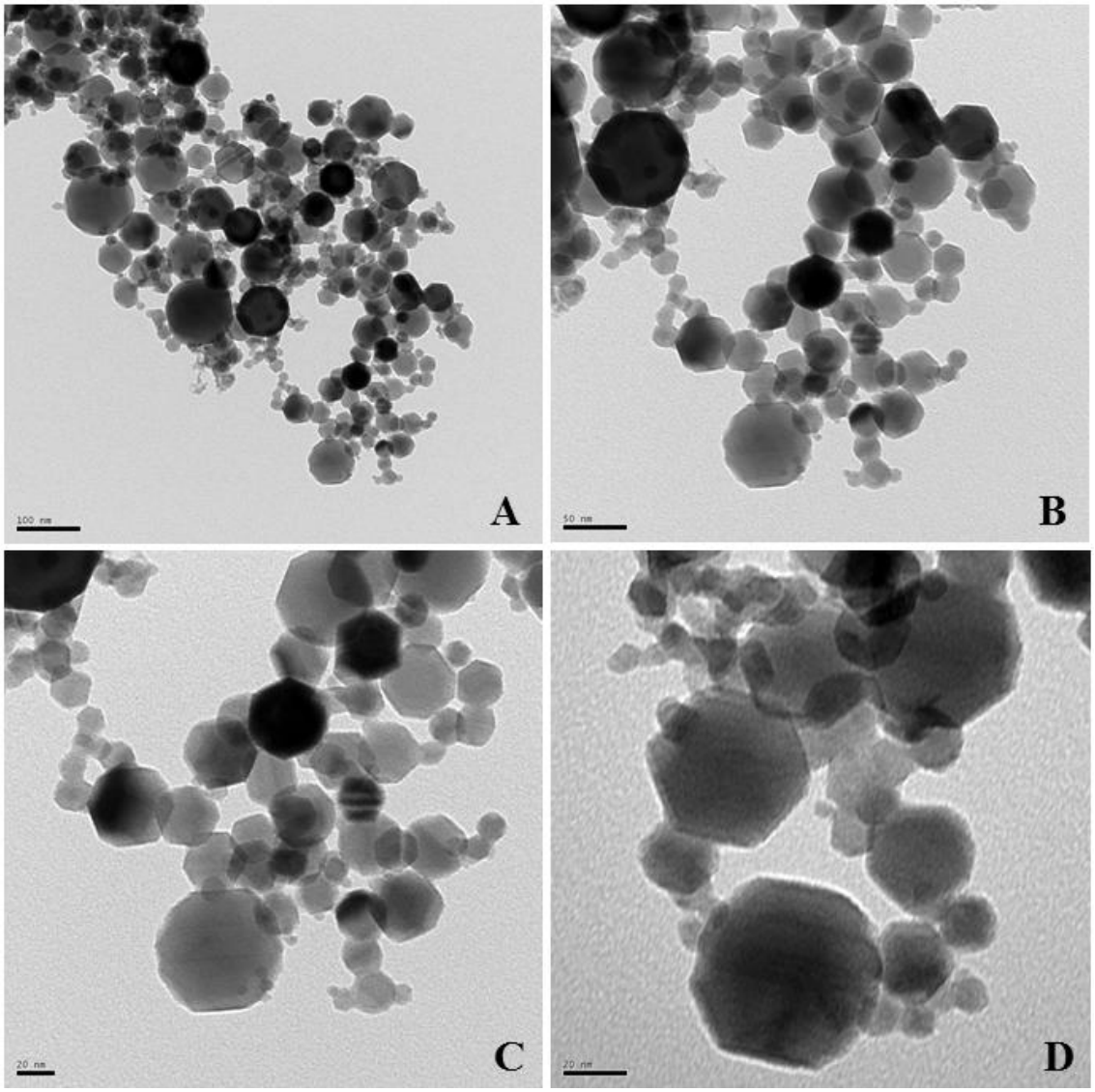


Figure 2

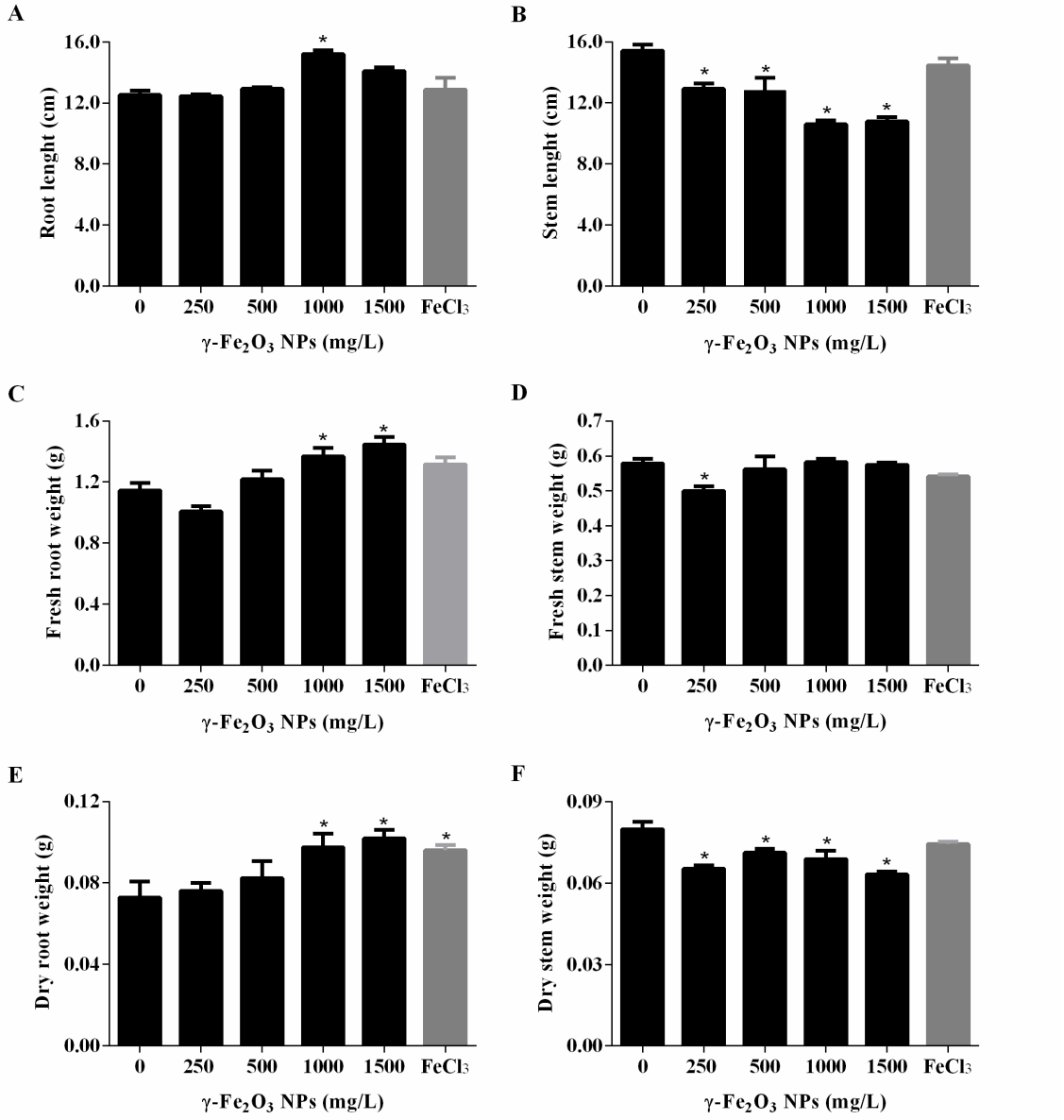

A
Root lenght (cm)
16.0
12.0
8.0
4.0
0.0
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
B
Stem lenght (cm)
C
Fresh root weight (g)
1.6
1.2
0.8
0.4
D
Fresh stem weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
E
Dry root weight (g)
0.12
0.08
0.04
0.00
F
Dry stem weight (g)
0.09
0.06
0.03

Figure 3

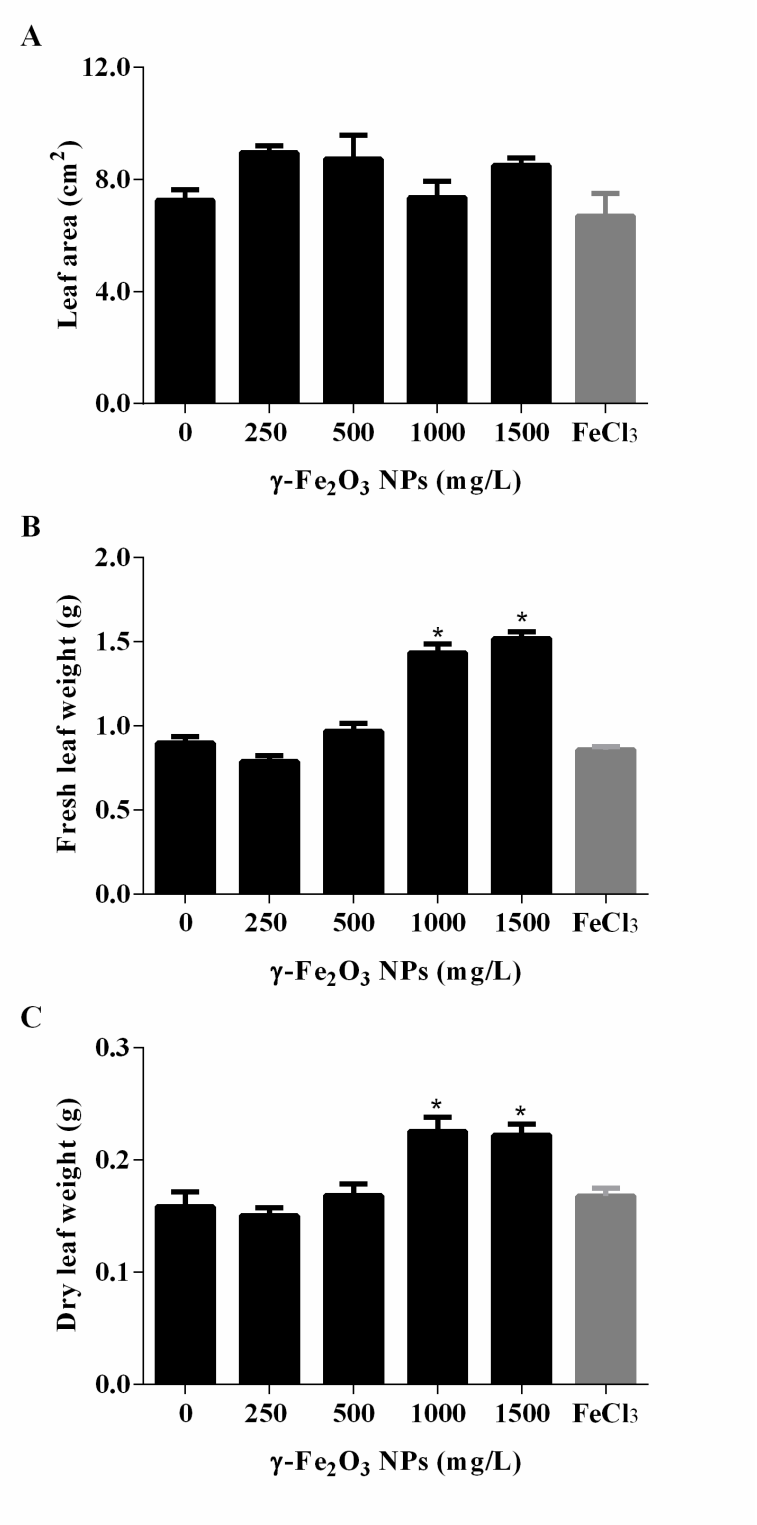

A
12.0
8.0
4.0
0.0
Leaf area (cm2)
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
B
2.0
1.5
1.0
0.5
0.0
Fresh leaf weight (g)
*
*
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
C
0.3
0.2
0.1
0.0
Dry leaf weight (g)
*
*
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)

Figure 4

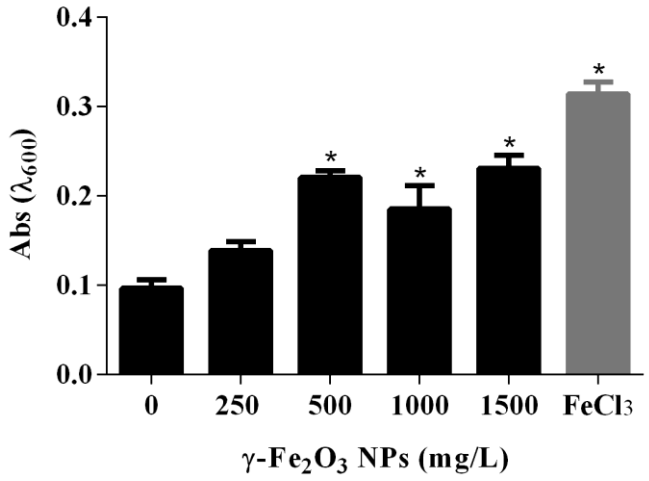

0.4
0.3
0.2
0.1
0.0
Abs (λ_{600})
*
*
*
*
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)

Figure 5

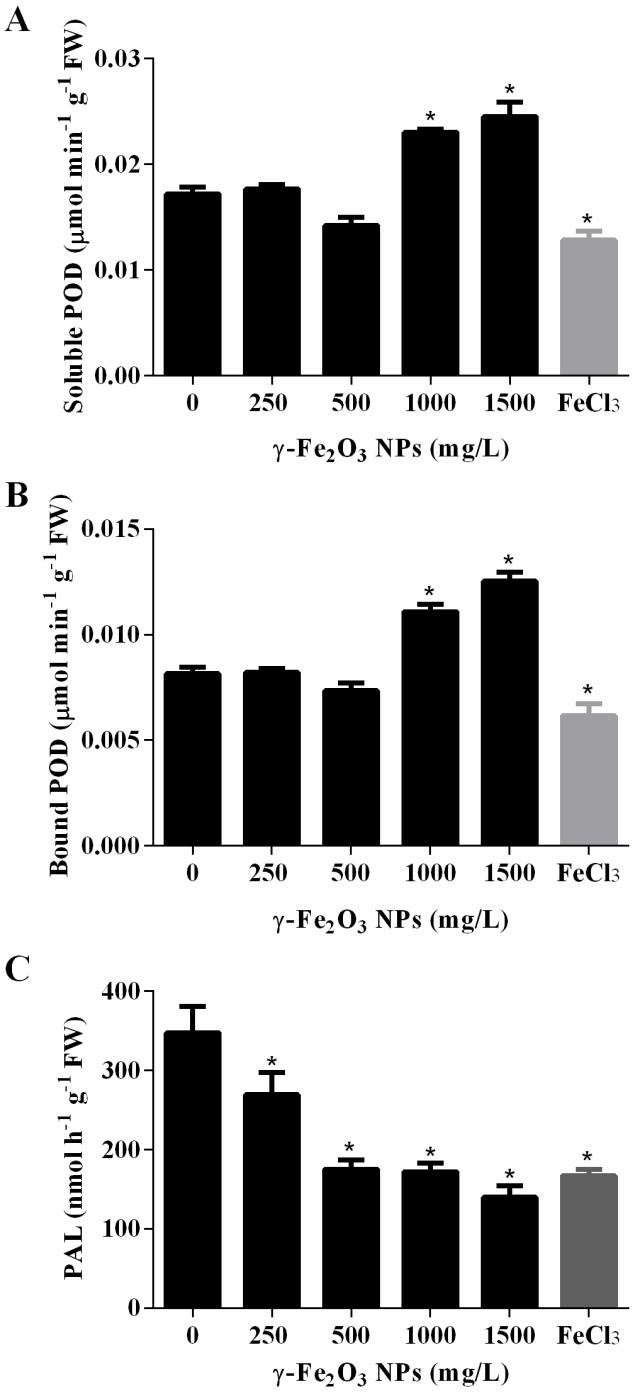

A
Soluble POD (μmol min-1 g-1 FW)
0.03
0.02
0.01
0.00
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
B
Bound POD (μmol min-1 g-1 FW)
0.015
0.010
0.005
0.000
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
C
PAL (nmol h-1 g-1 FW)
400
300
200
100
0
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)

Figure 6

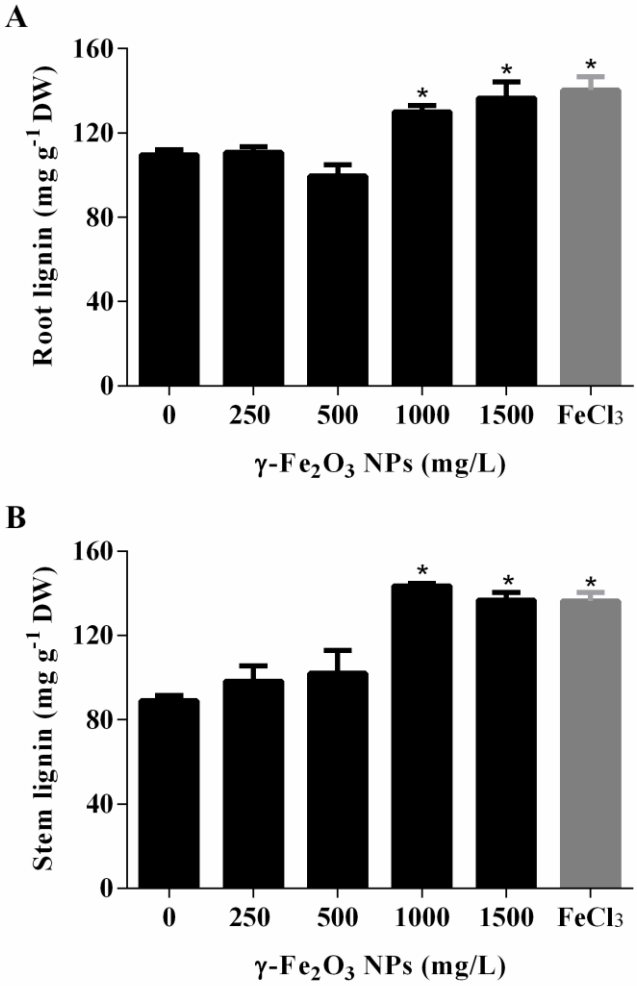

A
Root lignin (mg g-1 DW)
160
120
80
40
0
*
*
*
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
B
Stem lignin (mg g-1 DW)
160
120
80
40
0
*
*
*
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)

Figure 7

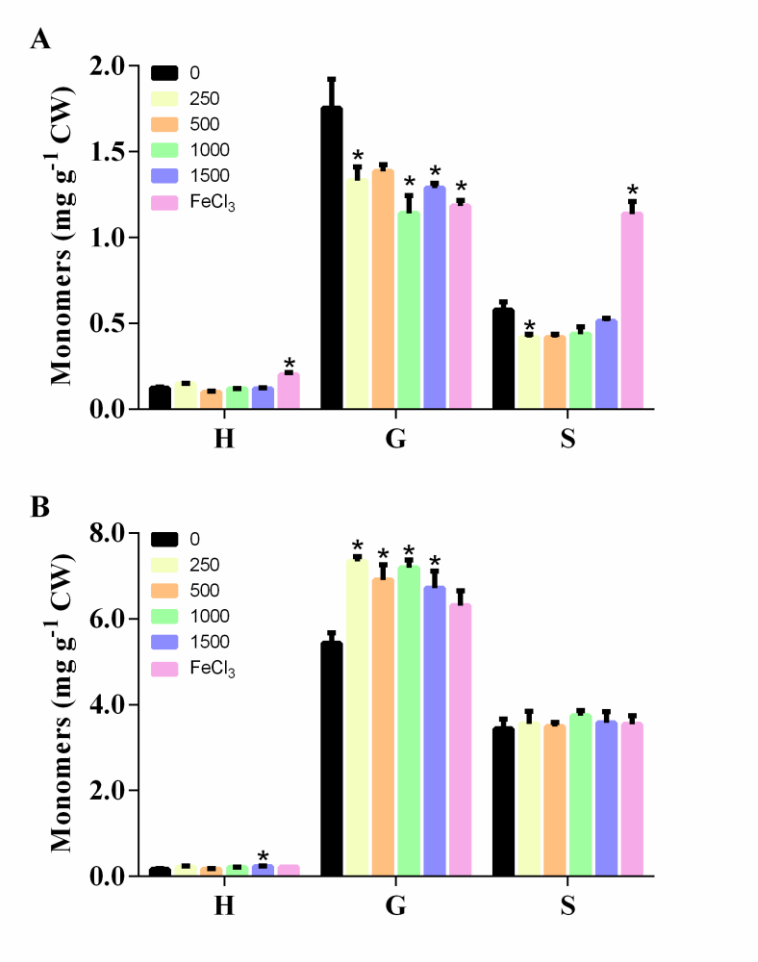
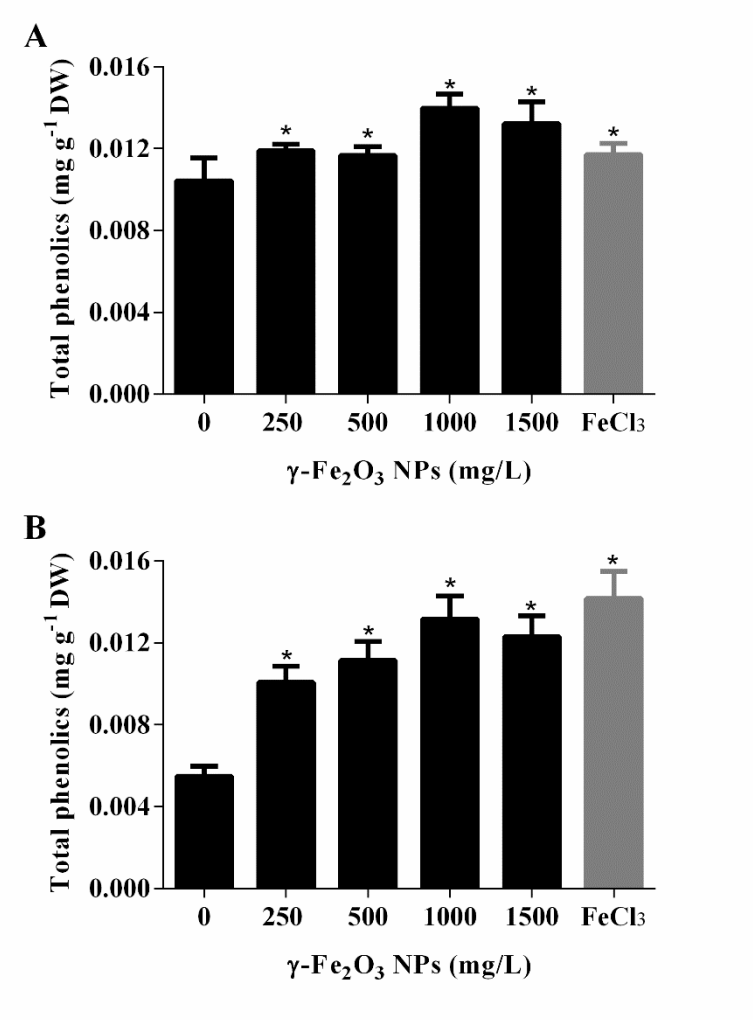

A
Monomers (mg g-1 CW)
2.0
1.5
1.0
0.5
0.0
0
250
500
1000
1500
FeCl3
H
G
S
B
Monomers (mg g-1 CW)
8.0
6.0
4.0
2.0
0.0
0
250
500
1000
1500
FeCl3
H
G
S

Figure 8


A
Total phenolics (mg g-1 DW)
0.016
0.012
0.008
0.004
0.000
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)
B
Total phenolics (mg g-1 DW)
0.016
0.012
0.008
0.004
0.000
0
250
500
1000
1500
FeCl3
γ-Fe2O3 NPs (mg/L)