**CRISTIAN DAVID PLAZA PÉREZ**

# NANOPARTICLES OF ESSENTIAL AND NONESSENTIAL ELEMENTS IN THE MANAGEMENT OF PLANT DISEASES

**LAVRAS - MG**
**2019**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutor.

Prof. Dr. Edson Ampélio Pozza
Orientador

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| | |
|---|---|
| Dr. Wade Elmer | CAES/USA |
| Dr. Mário Lúcio Vilela de Resende | DFP/UFLA |
| Dr. Flávio Henrique Vasconcelos de Medeiros | DFP/UFLA |
| Dr. Teotonio Soares de Carvalho | DCS/UFLA |

Prof. Dr. Edson Ampélio Pozza
Orientador

**LAVRAS – MG**
**2019**

*À Deus, por todas as bençõe*s

*Ofereço*

*Aos meus pais Aleida Pérez e Aldemar Plaza*
*À minha irmã Claudia*
*Dedico*

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“A alegria que se tem em pensar e aprender faz-nos pensar e aprender ainda mais. ”

(Aristóteles)

“Empenhar-se ativamente para alcançar determinado objetivo dá à vida significado e substância. Quem quiser vencer deve aprender a lutar e perseverar. ”

(Bruce Lee)

## RESUMO

A nutrição correta e equilibrada das plantas deve constituir sempre a primeira linha de defesa contra os patógenos. Os micronutrientes são capazes de mediar a ativação de produtos de defesa da planta quando disponíveis em quantidades adequadas no tecido vegetal. Diferentes fontes desses micronutrientes e de elementos não essenciais, porém benéficos estão sendo avaliadas no mundo para verificar o seu efeito em reduzir a intensidade de doenças. Este trabalho teve como objetivo avaliar a efetividade da aplicação de micronutrientes e elementos não essenciais na forma iônica, sulfatada e/ou nanoparticulada, fornecidos às plantas via solução nutritiva ou pulverizados diretamente na parte aérea em dois patossistemas, ferrugem do cafeeiro e síndrome da morte súbita da soja. Os resultados desta pesquisa foram divididos em três artigos. O primeiro objetivou estudar o efeito do boro, manganês e zinco na redução da ferrugem do cafeeiro em mudas cultivadas em solução nutritiva. Foi verificada redução da severidade da doença ao usar os três micronutrientes, entretanto, a maior redução de 75% foi observada ao aplicar zinco. A maior produção de lignina e fenóis totais solúveis FTS foi associada à aplicação de manganês. No segundo artigo o objetivo foi comparar o potencial de nanopartículas de micronutrientes e elementos não essenciais diretamente no fungo *Fusarium virguiliforme* e na síndrome da morte súbita, doença causada por esse patógeno. I*n vitro*, foi verificada redução na biomassa seca de *F. virguiliforme* quando exposto à presença de boro, zinco e manganês. *In planta*, foi observado redução da podridão radicular associada à síndrome quando aplicado 500 mg $L^{-1}$ de nanopartículas de cobre em 4 de 4 experimentos, com zinco em 3 de 4, com boro em 2 de 3 e com prata em 2 de 2. No terceiro artigo, foi realizada a seleção das nanoparticulas promissoras na redução da ferrugem do cafeeiro e na germinação de esporos de *Hemileia vastatrix* a partir da avaliação inicial de 10 nanomateriais. Também foi comparado o efeito de diferentes fontes boro, zinco, manganês e cobre. Em mudas de cafeeiro expostas a nanopartículas de prata e zinco na concentração de 500 mg $L^{-1}$ foi observada redução da área abaixo da curva de progresso da ferrugem em 93 e 75%, respectivamente. Finalmente, as formas nanoparticuladas de boro, zinco, manganês e cobre reduziram significativamente a ferrugem comparado com suas respectivas formas iônicas. Dessa forma foi constatado o potencial da adequada nutrição no manejo de doenças de plantas.



**Palavras-chave**: Micronutrientes, patossistema, nanomateriais.

## ABSTRACT

Correct and balanced plant nutrition must always be the first line of defense against pathogens. Micronutrients are able to mediate the activation of plant defense products when available in suitable amounts in plant tissue. Different sources of these micronutrients and nonessential elements, however, are being evaluated in the world for their effect on reducing the intensity of disease. The objective of this work was to evaluate the effectiveness of micronutrients and nonessential elements in the ionic, sulfated and / or nanoparticulate form provided to the plants via nutrient solution or directly sprayed on the aerial part in two patosystems, coffee rust and sudden death syndrome of soybean SDS. The results of this research were divided into three manuscripts. The first one had the objective to study the effect of boron, manganese and zinc on the reduction of coffee rust on seedlings grown in nutrient solution. The severity of the disease was reduced by using the three micronutrients, however, the largest reduction of 75% was observed when applying zinc. The higher production of lignin and total soluble phenols was associated with the application of manganese. In the second manuscript, the objective was to compare the potential of nanoparticles of micronutrients and nonessential elements directly in the *Fusarium virguiliforme* fungus and in the SDS, a disease caused by this pathogen. *In vitro,* it was verified a reduction in the dry biomass of *F. virguiliforme* when exposed to the presence of boron, zinc and manganese. *In planta*, reduction of root rot associated with the SDS was observed when 500 mg $L^{-1}$ of copper nanoparticles when applied in 4 out of 4 experiments, with zinc in 3 out of 4, boron in 2 out of 3 and silver in 2 out of 2. In the third manuscript, the selection of promissory nanoparticles in the reduction of coffee rust and spore germination of *Hemileia vastatrix* was carried out from the initial evaluation of 10 nanomaterials. The effect of different boron, zinc, manganese and copper sources was also compared. In coffee seedlings exposed to silver and zinc nanoparticles at a concentration of 500 mg $L^{-1}$ a reduction of the area below the rust progress curve was observed in 93 and 75%, respectively. Finally, the nanoparticulate forms of boron, zinc, manganese and copper significantly reduced rust compared to their respective ionic forms. In this way, the potential of adequate nutrition in the management of plant diseases was verified.



**Key words:** Micronutrients, patosystem, nanomaterials.

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# PRIMEIRA PARTE – INTRODUÇÃO GERAL

## 1 GENERAL INTRODUCTION

Diseases are a major source of crop and plant damage that can be caused by a number of plant pathogenic (disease-causing) organisms. Fungi are the number one causal agent responsible by crops loss worldwide (STRANGE and SCOTT, 2005). Fungicide are being used as one of the main methods to plant disease management. However, its continuous use, often of the same chemical group, may develop pesticide resistance in pathogens, reducing the environmental sustainability of this control method. The aim of recent research in this area has been the development of alternative control strategies to reduce dependency on synthetic fungicides.

It´s a well-established fact that micronutrients like manganese, copper, boron, iron, molybdenum, zinc and others, are important for the growth and development of plants, but they also determine their resistance or susceptibility to disease, their histological or morphological structure or properties, and the ability of pathogens to survive on the host (BROADLEY et al., 2012). Passive and active mechanisms of disease control are active through nutrient management. Mineral nutrients are the components of plants, they regulate metabolic activity associated with resistance of plants, and virulence of pathogens. For example, the plants have ability to synthesize aromatic secondary metabolites, like phenols, phenolic acids, quinones, flavones, flavonoids, tannins and coumarins mediated through the Shikimate pathway (HUBER and THOMPSON, 2007). These groups of compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms. Nonetheless, adequate nutrition is generally required to maintain a high level of disease resistance.

As part of the environment, nutrients influence plant, pathogen, and microbial growth to remain an important factor in disease control. The interaction of nutrition in these components is dynamic and all essential nutrients are reported to influence the incidence or severity of some diseases (HUBER et al., 2012). A particular element may decrease the severity of some diseases, but increase others, and some have an opposite effect in different environments (HUBER, 1989; HUBER and GRAHAM 1999). Another potential alternative for disease management is the application of elements such as Ni, Ti, Al, Ce, Mo and Ag. The role that nonessential inorganic compounds may play in the activation of host defense mechanisms remains largely unexplored.

Biotechnological advancements in protection and nutrition strategies for plants have provided new tools, which are economic, eco-friendly, and sustainable approach for farmers (BARUAH and DUTTA, 2009). Nanotecnhology has been used in many fields of science like material development, medicine and agricultural and it is considered as the process to generate, manipulate and deploy nanomaterials, represents an area holding significant promise for the agricultural scenario. Nanotechnology includes nanoparticles (NPs) having one or more dimensions in the order of 100 nm or less (KUSMA, 2007). NPs hold great promise regarding their application in plant protection and nutrition due to their size-dependent qualities, high surface-to-volume ratio and unique optical properties in comparison to micron or millimeter sized particles present in their respective bulks (AUFFAN et al., 2009).

There are reports in the literature on the use of micronutrient nanoparticles and non-essential elements in increasing the plant disease resistance, reducing their severity in different patosystems around the world. For instance, the role of NP of Cu (GIANNOUSE et al., 2013; KAHNED et al., 2014), Zn (HE et al., 2010; DIMPKA et al., 2017), Mn (ELMER AND WHITE, 2016), Ag (LAMSAL et al., 2011), Si (SURIYAPRABHA et al., 2014) and Ti (PARET et al., 2013). Meanwhile, for crops of commercial importance in Brazil, research is still scarce.

According to the Brazilian Institute of Geography (IBGE, 2018), coffee and soybean crops will reach a record of production in 2018. Estimates of arabica coffee production totaled 43.4 million bags of 60 kg, while for soybean production rose to 115.8 million of tonnes, placing these commodities as the most important for Brazilian agribusiness. However, one of the main sources of losses in the production of these grains is attributed to the diseases that affect these crops. The main method of control for the different pathogens is the continuous use of systemic or contact fungicides. However, the world demand for less polluting methods has directed the control of diseases to areas still little explored like the use of nanoparticles as a parameter to reinforce the horizontal defense of the plant and thus reduce the intensity of the diseases.

In view of the above, the objective of the present study was to evaluate the effect of micronutrients provided via nutrient solution and nanoparticles of metal oxide and metalloids micronutrient and of nonessential elements supplied via foliar in the suppression of diseases in coffee and soybean as possible alternative method of management.

# 2 REFERENCIAL TEÓRICO

## 2.1 Nutrients for disease management.

In order to control the diseases of cultivable plants, in recent years the use of resistant varieties as a long-term measure has been implemented. However, the introduction of these materials into crops is still slow and many of the areas planted in Brazil still present susceptible cultivars, usually in monoculture and in full sun. Therefore, the use of short- and medium-term fungicides remains the main method of controlling diseases in different crops. Protective fungicides based on copper and systemic fungicides are applied in the field (POZZA et al., 2010). Both phytosanitary and crop management, mechanization, irrigation, soil management and fertilization give national agriculture high productivity.

However, Brazilian agriculture is one of the most demanding in relation to social and environmental sustainability. Consequently, the requirement for a reduction in the use of pesticides must be followed in accordance with environmental and labor legislation, aiming at the preservation of forests and native fauna, the control of soil erosion, as well as the protection of water resources, workers and consumers.

Until susceptible cultivars are replaced by resistant cultivars, an alternative to reduce the use of fungicides is cultural control, such as pruning to modify the canopy microclimate, rational use of irrigation water, and nutrient supply in equilibrium. The correct and balanced fertilization, besides being essential for the growth and development of plants, is considered as the primary component for the complete expression of the genetic resistance of plants and thus constitute their resistance barriers (HUBER and HANEKLAUS, 2007). Host nutrition associated with water supply is a primary component in the control of plant diseases and is the basis of horizontal resistance. The resistance phenotype is nothing more than the interaction of the genotype plus the environment. The expression of a gene, including those associated with barriers of physical or chemical resistance, depends on the presence of nutrients in equilibrium and water. Otherwise there is a great chance of even the DNA unrolling of the histones and opening its double helix for the synthesis of the messenger RNA (Pozza et al., 2004). Thus rendering the "Gene" inoperative and the plant susceptible to infection by pathogens. According to the aforementioned authors, in ideal concentrations, nutrients reinforce the physical and chemical barriers that constitute horizontal resistance, these factors being important in plant-disease interaction. This nutritional status may increase or reduce plant resistance to pathogens. The major changes, provided by mineral nutrition, responsible

for reducing disease intensity are thicker cell walls and cuticles, maintenance of soluble compounds such as simple sugars and amino acids within the cell, greater suberization, silification and lignification of tissues, greater synthesis and accumulation of phenolic compounds and frequency and duration of the stomata. In the case of diseases of fungal etiology, especially leaf blotches, the protection promoted by balanced mineral nutrition would be the result of: a) efficient physical barrier, inhibiting the penetration of the hyphae; b) better control of the permeability of the cytoplasmic membrane, thus avoiding the exit of sugars and amino acids into the intercellular space and c) chemical barrier, with production or formation of phenolic compounds with fungistatic properties, among others.

The effect of nutrients on plant physiology should be studied individually, that is, no standardization should occur. The action of each element in the plant-disease complex will depend on its concentration and the form available in plant tissues. A particular element may reduce infection by a particular pathogen, but increase the infection by others, depending on the species studied. In this way the response to diseases is specific, being subject to the etiological agent (fungus, bacteria, nematoid or virus). However, such increase or decrease may have the opposite effect if environmental conditions are modified and to avoid this, appropriate cultural practices are needed to improve the availability, uptake, distribution and utilization of nutrients in plants. According to Huber and Haneklaus (2007) and Pozza and Pozza (2012), both physical and chemical characteristics of soil and plant influence the interaction of nutrients, including organic matter, water availability and water management, irrigation, soil texture and structure, pH, number of plants per hectare, its way of driving, and climatic effects.

Thus, the function of each element should be understood as part of a balanced system, interdependent with the plant genetics and the environment, aiming to establish the nutrient balance to obtain the maximum productivity in each harvest. In view of the above, this review aims to highlight how adequate nutrition can influence the physiological processes for the greater development of plants, and to know better how the nutrients affect the intensity of the diseases besides the use of nanoparticles

**2.2 Nutrients in the constitution of resistance barriers against pathogens.**

Morphophysiological processes influenced by specific nutrients, which condition reactions to increase plant resistance to diseases are generally not known (MARSCHNER, 2012). Several studies have documented the speed and extent of plant defense reactions, often

decisive for the success or failure of the infection, as not only genetic control but influenced by mineral nutrients or climatic variables (DIETRICH et al., 2004). Thus, resistance can be increased by three mechanisms: 1) anatomical changes, for example, increased lignification and / or silification; 2) biochemical and physiological changes leading to nutrient concentration at specific sites; (3) restriction of the transfer of nutrients to the pathogens hindering their growth and development (AGRIOS, 2005).

### 2.2.1 Anatomical changes (physical barriers of resistance).

Biosynthesis and deposition of lignin in secondary cell walls are scheduled processes during plant development and are generally accepted as a mechanism to increase physical barriers against the initial colonization of pathogens (BONELLO et al., 2003; MIEDES et al., 2014). In the literature N deficiency is reported as inducing the expression of genes related to lignin biosynthesis (BHUIYAN et al., 2009). The lignification of infected cells prevents the propagation of toxins and hydrolytic enzymes from the pathogen into the host and at the same time prevents the transfer of water and nutrients from plant cells to the pathogen (SMITH et al., 2007).

On the other hand, silicon deposits (Si) also constitute physical resistance barriers of plants. Si can be accumulated in the cuticle forming the double Cuticle-Si layer and thus prevent the penetration of the pathogen through the mechanical barrier (CAI et al., 2009). Si is deposited as amorphous silica on cell walls. This element contributes to the mechanical properties, including stiffness and elasticity of the cell wall (TAIZ and ZEIGER, 2013). The positive effect of Si on plants has been attributed to the reduction of water loss due to cuticular transpiration due to the formation of Si deposits under the cuticle, a decrease in apoplastic flow and a reduced absorption of toxic minerals due to the formation of Si deposits on the root, and because of the increased rigidity and resistance of the plant cell wall (ROMERO et al., 2011).

### 2.2.2 Biochemical changes (Resistance biochemical barriers).

Biochemical compounds are synthesized when plant cell receptors recognize the pathogen (NEWMAN et al., 2010; NEWTON et al., 2013). These secondary metabolites protect plants against infections by pathogenic microorganisms and have been divided into three chemically distinct groups, terpenes, phenolic compounds and nitrogen compounds (TAIZ and ZIGER, 2013).

Phosphorus (P) is essential in the biosynthesis of terpenes. Manganese, copper, iron, cobalt and boron are involved in the metabolic pathway for the biosynthesis of phenolic compounds, including quinones, tannins and flavonoids (POZZA et al., 2004). The effect of manganese on the development of resistance in diseases of the root system and the area is widely described (HECKMAN et al., 2003). The increase in plant resistance mediated by Mn applications is explained in terms of activation of the peroxidase and polyphenoloxidase enzymes which increase phenolic concentrations in the roots (KALIM et al., 2003). In addition, Mn is responsible for controlling the biosynthesis of lignin and suberin by activating enzymes involved in the shikimic acid and phenylpropanoid route (MARSCHNER, 2012). Mn concentrations higher than the physiological demand of the plant may cause changes in the proteomics and metabolomics of the leaf apoplast and consequently affect the infection by pathogens. The increase of Mn increased the expression of proteins related to the pathogenesis (PR-proteins), among them glucanases and chitinases (FECHT-CHRISTOFFERS, 2003), besides improving the relation of the consumption and production of H2O2 - peroxidases within the apoplast of string bean leaves (FECHT-CHRISTOFFERS et al., 2006; FÜHRS et al., 2009). In relation to the pathogen, Mn inhibits aminopeptidase induction. This enzyme provides essential amino acids for the growth of fungi and pectin methylesterase, whose function is related to the degradation of host cell walls (DORDAS et al., 2008). Fertilization with Mn has been reported in the management of mildew, powdery mildew and covers diseases caused by *Gaeumannomyces graminis*. Reduction of common scabies in potatoes and reduction of *Fusarium spp* and *Sclerotinia sclerotiorum* infection in cotton and pumpkin, respectively, were also observed.

On the other hand, B has a direct function in the maintenance and stability of the cell wall and has a beneficial effect in reducing the severity of diseases (COOKSON and PHAM 1995; KARTAL et al., 2004; DORDAS et al., 2008). However, the physiological mechanisms involved in inhibiting the growth of pathogens are poorly understood. Bowen and Gauch (1966) observed inhibition in the growth of *Saccharomyces cerevisiae* and *Penicillium chrysogenum* when high concentrations of B were supplied. The aldolase enzyme was suggested as the target of high B levels, making fungi unable to utilize carbohydrates efficiently to maintain metabolic processes involved in its growth and reproduction. The effect of B on the fungus *Eutypa lata* was determined by mainly non-cellulose glucose consumption of the hemicellulosic fraction of the cell walls (ROLSHAUSEN et al., 2003). In the literature there are reports of the application of B to reduce diseases caused by *Plasmodiophora brassicae* in crucifers (WEBSTER and DIXON, 1991), Eutypa in grape

plant (ROLSHAUSEN and GUBLER, 2005), *Fusarium solani* in beans, *Verticillium albo* - tomato mosaic virus in tomato, *Gaeumannomyces graminis* and *Blumeria graminis* in wheat (MARSCHNER, 1996).

In the third chemical group are the phenols. These compounds have dual function, repel and attract different organisms in the plant environment. Phenols act as protective, inhibitory and toxic agents against nematodes, bacteria and phytopathogenic fungi (LATTANZIO et al., 2006). According to Karou et al. (2005), simple and complex phenolic compounds accumulate in tissues of plants acting mainly as phytoalexins of the type hydroxycuroins and hydroxycinnamate. Thus, the synthesis, release and accumulation of phenolic compounds, in particular salicylic acid, are fundamental for many plant defense strategies against pathogens (BHATTACHARYA et al., 2010).

### 2.2.3 Restriction of nutrient transfer

The nutrient transfer restriction is the main characteristic of vascular diseases as a consequence of the penetration of the pathogens through the root system in the radicels, with subsequent colonization of the xylem vessels, reducing their functionality, with reflexes in the transpiratory flow (WHEELER and RUSH, 2001 ). After the establishment in the xylem, fungi or bacteria produce cellulolytic and pectolytic enzymes to degrade the cell wall of the parenchyma, as a result the cytoplasm of the parenchymal cells becomes a hindrance to the flow of water and nutrients (MICHEREFF, 2005). In addition, some pathogens of the vascular system also can oxidize and polymerize phenolic compounds.

## 2.3 Penetration and infection of pathogens prevented by mechanical barriers.

Physical barriers to avoid pathogen penetration include lignification, rapid wound healing, and calyx formation in vascular tissues. Several nutrients act as cofactors of the reactions, enzymatic activators or regulators of the biosynthetic route involved in the formation of barriers (POZZA and POZZA, 2012). These include calcium, silicon and some micronutrients.

## 2.4 Inhibition of disease progression by accumulation of inhibitory compounds around the site of infection or by direct contact with nutrients.

Nutrients are essential in several metabolic routes involved with the production of structural and / or biochemical defense mechanisms. Many compounds produced by secondary metabolic pathways are formed after the onset of infection and promote disease

resistance. These are the phytoalexins, flavonoids and auxins, capable of accumulating around the site of infection depending on the availability of various nutrients. Manganese, copper, iron, boron and cobalt may be involved in the synthesis of various phenolic, quinone and lignin synthesis enzymes whose function is linked to plant defense.

Copper, for example, is a component of ascorbic acid oxidase, tyrosinase, monoamine oxidase, uricase, cytochrome oxidase, phenolase, laccase and plastocyanin. In addition to nourishing the plant, Cu has been widely used as a fungicide. The amount required, however, is much higher compared to the nutritional requirement for the plant. The action of Cu as fungicide is based on direct application to the surface of the plant and the fungus in question. In the nutrient-deficient plants, a reduction in the synthesis of defense compounds, accumulation of soluble carbohydrates and reduction of lignification are observed in plants deficient in this nutrient, all of which contribute to the reduction of plant resistance to diseases (TAIZ and ZEIGER, 2013).

Another nutrient, zinc, may contribute to plant tolerance to abiotic stress factors by triggering defense mechanisms through gene activation and regulation (CAKMAK, 2000). But applying Zn also reduces the severity of diseases caused by biotic agents due to their effect being toxic to direct contact with pathogens rather than being mediated in the metabolic reactions of plants (GRAHAM and WEBB, 1991).

Plants absorb zinc in the divalent form ($Zn^{2+}$) (BROADLEY et al., 2012). Zn is involved in carbohydrate metabolism, maintenance of cell wall integrity, protein synthesis, regulation of auxin synthesis, pollen formation, as well as contributing to respiration, photosynthesis, chlorophyll formation, etc. (SAMANT, 2009) . The use of zinc sulfate in the management of peach gum caused by *Lasiodiplodia theobromae* inhibited the mycelial growth of this fungus and caused the formation of abnormal hyphae and tip swelling (LI et al., 2016). Chitin is the major polysaccharide in the cell wall. Abnormal morphology of hyphae in the presence of Zn can be caused by deposition of chitin as verified by Lanfranco et al. (2002). The mode of action of zinc sulfate against the fungus *L theobromae* was studied by Lew (2011). Zn ions at 50 mM concentration disrupt the internal hydrostatic pressure of the cell and inhibited the elongation of the fungal hyphae tips.

The application of Zn in the form ZnSO4.7H2O in the management of root rot (*Rhizoctonia spp*) in guar seedlings (*Cyamopsis tetragonoloba* L.) resulted in an increase in the resistance of the plants against the disease (WADHWA et al., 2014). Increasing the concentration of Zn from 10 to 20 mg kg -1 increased the activity of antioxidant enzymes (polyphenol oxidase, peroxidase, phenylalanine ammonia lyase (PAL) and tyrosine ammonia

lyase (TAL)). Antioxidant enzymes play an effective role against pathogen invasion. Peroxidase, for example, is involved in the synthesis of lignin at the site of penetration of the pathogen. PAL and TAL have a key role in the metabolism of phenylpropanoide, whose synthesis is related to plant defense (RAGHAVENDRA et al., 2007; KUMAR et al., 2010).

**2.5 Nanoparticles and their use in disease management**

Today nanotechnology is widely used in modern agriculture to make the concept of precision agriculture a reality. Nanotechnology includes nano particles (NPs) which have dimensions on the order of 100 nm or less (DUHAN et al., 2017). NPs can be divided into inorganic and organic. Inorganic NPs include metals (Al, Bi, Co, Cu, Au, Fe, In, Mo, Ni, Ag, Sn, Ti, W, Zn), metal oxides ($Al_2O_3$, $CeO_2$, CuO, $Cu_2O$, $In_2O_3$, $La_2O_3$, MgO, NiO, $TiO_2$, $SnO_2$, ZnO, $ZrO_2$) and quantum dots while fullerenes and carbon nanotubes are organic NPs (RAJPUT et al., 2018).

Many chemical methods are available for the synthesis of nanoparticles, which use toxic chemicals, requiring the implementation of environmentally correct routes. Researchers have developed biological techniques from bacteria, fungi, higher plants, actinomycetes and virus for the synthesis of NPs by reducing salts to the corresponding nanoparticle (BANSAL et al., 2014). The use of microbial cells for the synthesis of nanodimensioned material appeared as a new approach for the synthesis of metallic NPs. Several fungal strains have been used as promising sources for the manufacture of NPs, for example, *Fusarium*, *Aspergillus*, *Verticillium* and *Penicillium* (MOUSA et al., 2015). Different species of fungi are also candidates for the production of metallic NPs both intra- and extracellularly.

The NPs find application in plant protection, nutrition, handling and cultural practices due to the small size, high surface-volume relation and exclusive optical properties. "Phyton-nanotechnology" can help in the development of "intelligent" cultures (LI et al., 2016). Nanoscale materials can provide controlled times, specific targets, self-programmed regulation and multifunction capability. For example, some nanomaterials may provide agrochemicals (fertilizers, pesticides or herbicides) in an "under demand" manner, either through nutritional demand or protection against pests and pathogens (NAIR et al., 2010). Furthermore, the controlled release of nucleotides, proteins and other molecules have the potential for genetic modification and regulation of metabolism.

Different types of organic and inorganic salts have been used to control diseases for many years, however the use of nano particles is an innovative and effective approach with the advancement of nanotechnology application. NPs have suppressed some species of fungi

(DUHAN et al., 2017). On the other hand, Singh et al. (2013) reported a greater reduction in pea rust when $CuSO_4$ and $Na_2B_4O_7$ were applied among 15 nano forms of micronutrients. Manganese and zinc NPs reduced root tipping and root rot in sunflower plants (ABD EL-HAI et al., 2009). Ag NPs were evaluated in the control of *Candida albicans, C. krusei, C. tropicalis, C. glabrata* and *Aspergillus brasiliensis*. The fungicidal effect of zinc oxide (NPs) on two post-harvest pathogens, *Botrytis cinerea* and *Penicillium expansum*, was reported. ZnO (70 ± 15 nm) at concentrations higher than 3 mmol L-1 significantly reduced the growth of *B. cinerea* and *P. expansum*, in the latter fungus greater sensitivity to treatments was observed. The NPs caused deformation in the hyphae of *B. cinerea* and prevented the development of conidiophores and conidia in *P. expansum*, which consequently led to death of the hyphae (KHAN and RIVZI, 2014).

Krishnaraj et al. (2012) studied the effect of Ag NPs on *Alternaria alternata, Sclerotinia sclerotiorum, Macrophomina phaseolina, Rhizoctonia solani, B. cinerea* and *Curvularia luneta*. The authors observed greater inhibition in growth for all pathogens when doses of 15 mg $L^{-1}$ were applied. On the other hand, corn treated with nanosilica (20-40 nm) has been effective in reducing diseases caused by *Fusarium oxysporum* and *Aspergillus niger* when compared to silica salt. Nanosilver treated plants had higher expression of phenolic compounds (2056 and 743 mg $mL^{-1}$, respectively) and better expression of total phenols, phenylalanine ammonia lyase, peroxidase and polyphenol oxidase, at 10 and 50 kg $ha^{-1}$

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**2.6 Final considerations**

Mineral nutrients interfere with the anatomical, biochemical and physiological processes of plants, contributing to increased resistance to disease when available in adequate concentrations in plant tissues. The study of nutrient management in the control of the main coffee diseases of Brazil has allowed to improve the understanding of the dynamics of mineral nutrition in the plant-pathogen complex.

Moreover, to explore the genetic potential of cultivars, adequate nutrition should be explored for the complete genetic expression of plant resistance to disease. Correct and balanced fertilization accompanied by efficient agricultural practices to provide nutrients from the soil solution to the plants should continue to be studied. The use of nanoparticles has a promising future for modern agricultural practices, such as the precise supply of nutrients and the diagnosis of diseases at an early stage.

In sustainable agriculture, the nutritional balance is an essential component in some integrated crop protection programs because it represents a better cost / benefit ratio, and is an ecologically viable measure for the control of plant diseases by reducing the number of chemical pesticide applications.

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## SEGUNDA PARTE – ARTIGOS

### ARTIGO 1 Boro, zinc and manganese suppressing coffee rust in nutritive solution

**Cristian David Plaza Pérez[1], Edson Ampélio Pozza[1], Adélia Aziz Alexandre Pozza[2], Wade Elmer[3], Andreane Bastos Pereira[1], Daniel Guimarães[1], Ana Cristina Andrade Monteiro[1], Mário Lúcio Vilela de Rezende[1].**

✉**Edson Ampélio Pozza**
edsonpozza@gmail.com

[1] Department of Fitopatologia, Federal University of Lavras, Lavras, Minas Gerais 37200-000, Brazil

[2] Department of Ciência do Solo, Federal University of Lavras, Lavras, Minas Gerais 37200-000, Brazil

[3] Department of Plant Pathology and Ecology, The Connecticut Agricultural Experiment Station (CAES) — New Haven 06504, United States.

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[1]Departamento de Fitopatologia, Universidade Federal de Lavras, 37200-000, Lavras, MG, Brasil.

**Abstract**

The adequate nutritional status of coffee tree is a fundamental strategy to maintain plant health. The effect of five doses (0.05, 0.25, 0.50, 1.0, 2.0 and 4.0 mg $L^{-1}$) of boron, zinc and manganese on the severity of coffee rust was evaluated. Micronutrients were supplied via nutrient solution in seedlings with two pairs of fully developed leaves. *Hemileia vastatrix* was inoculated at $10^6$ spores/ml in this seedlings. Coffee rust assessments were performed from the 43rd day after inoculation, totaling five evaluations. Then, the area under the disease progress curve for severity (AUDPCS) was calculated. There was a statistical difference for AUDPCS ($p<0,05$) for all micronutrients. For boron and manganese, 12.1% and 52.3% reduction in severity was observed at 4.00 and 0,25 mg $L^{-1}$ dose respectively. At the same dose, however for zinc, was increased in 69.0% the AUDPCS. The three micronutrients significantly influenced the concentration of total soluble phenols, nevertheless, only manganese influenced the concentration of lignin.

**Keywords:** Alternative management, mineral nutrition, coffee rust.

## 1 Introduction

Coffee is one of the most traded agricultural commodities around the world. About 20 million rural poor farmers in the world depend on grain production, simply for their livelihoods, especially in tropical developing countries (FAO, 2015). However, for the 2012/2013 harvest, the rust caused by *Hemileia vastatrix* (Berkeley and Broome) affected 55% of the 593,037 hectares planted in Central America. Its high incidence was responsible for reducing coffee production and consequently the loss of income of more than two million small farmers in six nations of the region (USDA, 2016). The humanitarian crisis after the economic collapse caused the phenomenon of massive migration to the United States in search of better living conditions (ICO, 2014).

In Brazil, the world's largest producer and exporter of the *Coffea arabica* species, due to the continuous implantation of susceptible cultivars to coffee rust, producers have mainly adopted chemical control as a disease management measure in crops (Pozza and Pozza, 2012). However, the growing demand for environmentally friendly practices has led to the search for alternative methods to achieve crop sustainability, encouraging reduced spraying with fungicides. Among these alternatives, the use of balanced mineral nutrients for the management of diseases in plantations stands out. The adequate nutritional status of the plants is a fundamental strategy to maintain plant health. Thus, nutrition can define the borderline between the susceptibility and resistance of plants to disease (Datnoff, Elmer and Huber, 2007). Complete and balanced nutrition should always be the first line of defense against plant diseases (Elmer and Datnoff, 2014).

Micronutrients can contribute to reduce damage caused by pathogens. Directly by antagonism or indirectly by strengthening the defense mechanisms by acquired systemic resistance and / or by stimulating the antagonist population in the rhizosphere (Singh, 2009). One of the main functions of micronutrients is to catalyze or serve as a cofactor of enzymes involved in different metabolic reactions. Phenol metabolism and lignin biosynthesis, in the metabolic pathway of shikimic acid, are examples of defense products synthesized from boron, zinc, manganese and other enzyme systems.

The role of boron in reducing disease is attributed to different factors. The first consists in strengthening the structure of the cell wall by forming the carbohydrate-borate complex which regulates carbohydrate transport and protein metabolism in the cell wall. The second mechanism regulates the permeability, stability and function of the cell membrane and the third mechanism is due to the mediation of boron in the metabolism of phenols and lignin (Brown et al., 2008). In conditions of deficiency of this nutrient the cell wall is compromised.

According to Sanjeev and Eswaran (2008) the cells swell and divide, resulting in weakened intercellular space. Consequently the finer physical barrier will make it easier to infect and colonize the pathogen in plant tissues. The same authors verified the in vitro reduction of the mycelial growth of *Fusarium oxysporum* f.sp. *cubense* with the application of 500 and 700 mg $L^{-1}$ of boron.

Zinc is also associated with plant defense against plant pathogens. In fungi, it has a direct effect on growth and secondary metabolism. This micronutrient modulates the accumulation of nitric acid in fungi, stimulates the absorption of glucose and the production of antibiotics (Duffy, 2007). At concentrations below 0.5 mg $L^{-1}$ zinc induces accumulation of amino acids and reduces sugars in plant tissues benefiting fungal growth and sporulation (Marschner, 2012). On the other hand, Silva et al. (2016) observed a reduction of 55.9% of eucalyptus powdery mildew (*Oidium eucalypti*) by applying 2 ml $L^{-1}$ of zinc phosphite and reducing 45.3% when supplied with 2 g $L^{-1}$ of zinc sulphate in the soil.

The potential mechanisms of manganese in reducing plant diseases have not been fully elucidated. Manganese inhibits the formation of aminopeptidase, which provides essential amino acids for fungal growth and prevents the pathogen from forming the pectin methyl esterase, enzyme used to degrade the host cell walls (Vidhyasekaran, 2004). Manganese, as well as boron, also acts as an activator of different enzymes of the shikimic acid route for the synthesis of phenols, cyanogenic glycosides and leads to the lignin increase, constituting effective physical barriers to avoid pathogens penetration (Huber et al. al., 2007). The soil application of 10 mg $Kg^{-1}$ of manganese reduced the severity of root rot of common bean (*Vigna unguiculata*) caused by *Rhizoctonia solani* and *Rhizoctonia bataticola*, respectively in 42,7 and 42.0% (Kalim et al., 2003).

However, to isolate the effect of nutrients, and to make sure of its influence on the metabolic processes involved in the production routes of resistance compounds, it is necessary to keep all other essential elements in balance and without varying their content. Thus, in this type of study, it is essential to grow coffee in a nutrient solution (Alexander et al., 1939).

Understanding the influence of micronutrients on the host pathogen relationship can help to develop management strategies aimed at reducing fungicide sprays and consequently the environmental impact. Therefore, the objective of the present work was to evaluate the effect of boron, manganese and zinc micronutrients on the severity of coffee rust on seedlings grown in nutrient solution.

## 2 Material and methods

### 2.1 Experimental design and treatments.

Three experiments were conducted in a greenhouse. The treatments of each experiment consisted of the same doses of boron, zinc and manganese, at 0.05, 0.25, 0.50, 1.0, 2.0 and 4.0 mg $L^{-1}$. The experimental design, for the three experiments, was in randomized blocks, with four replicates, consisting of a 2.6-liter pot containing two seedlings. The experiments were repeated over time.

The nutrient solutions were prepared from the basic solution of Hoaugland and Arnon (1950) and the doses of boron, zinc and manganese balanced using respectively boric acid ($H_3BO_3$), zinc sulfate ($ZnSO_4.7H_2O$) and manganese sulphate ($MnSO_4$) as sources of these elements. The sources of macronutrients used were $NH_4NO_3$, $Ca(NO_3)_2.4H_2O$, $KNO_3$, KCl, $KH_2PO_4$, $MgSO_4.7H_2O$ e $CaCl_2.6H_2O$. The micronutrient salts used to compose the stock solution were $H_3BO_3$ (2,8 mg $L^{-1}$) $ZnSO_4.7H_2O$ (0,22 ml $L^{-1}$), $MnSO_4.4H_2O$ (3,0 ml $L^{-1}$), $CuSO_4.5H_2O$ (0,08 ml $L^{-1}$), $H_2MoO_4.H_2O$ (0,02 ml $L^{-1}$) and 1,0 ml. $L^{-1}$ Fe-EDTA.

An air compressor connected to the containers through hoses supplied the aeration of the solution in the pots continuously. The pH of the solution was maintained between 5.5 and 6.0 with the addition of 0.1 mol $L^{-1}$ HCl or 0.1 mol $L^{-1}$ NaOH, monitoring it weekly with a digital pH meter. When necessary, pot volume was supplemented with deionized water. The maximum, average and minimum temperature and relative air humidity were monitored by the HT-500 Instrutherm® datalogger located on the stands where the experiment was installed.

### 2.2 Obtaining seedlings and acclimatization.

Coffee seeds of Mundo Novo 376/4 cultivar were washed in tap water, disinfested with 50% alcohol for 50 seconds and 1% sodium hypochlorite for one minute and rinsed with sterile distilled water. Subsequently, they were sown in plastic trays (0.50 x 0.35 x 0.15m) containing washed sand. These trays were conditioned in a plant growth chamber with controlled temperature of 28 ° ± 2 ° C and photo period of 12 hours. After issuing the pair of cotyledon leaves, the seedlings were watered with basic solution of Hoagland and Arnon (1950) at 20% of the ionic strength. The application of this solution was done every ten days until the seedlings emitted the first pair of definitive leaves.

After 25 days, the seedlings were transferred to pots of 2.6 liters for acclimatization in the greenhouse in Hoagland basic solution at 50% ionic strength, in the respective treatments.

The seedlings remained for another 20 days in this solution. After this period 136 seedlings were selected according to the uniformity of size and the solution was replaced by 100% of the ionic strength.

Ion depletion of the nutrient solution was monitored weekly with Compaction Meter for K + (Horiba-CARDY®). When the depletion reached 30% of the initial value of K+ ions, the nutrient solution was changed in the different treatments.

**2.3 Obtaining inoculum and inoculation**

The pathogen was obtained from infected leaves in the field in the experimental areas of the Lavras municipality. The abaxial surface of the leaves was scraped with a brush to collect the uredospores and transferred to sterile distilled water. The suspension was calibrated in hemocytometer at a concentration of 1.0 x $10^5$ urospores $mL^{-1}$ and inoculated with a spray on the abaxial face of the first pairs of coffee leaves, fully developed to the point of drainage (Cruz Filho and Chaves, 1973). After inoculation, the pots were covered with black plastic bags to constitute the humid chamber. For this purpose, these bags were sprayed with distilled water on the inner face and wet cotton balls were placed over the Styrofoam caps in each 2.6 liter pot and held for 72 hours. After this period were withdrawn.

**Coffee rust assessments**

When the first rust symptoms were observed, severity assessments were performed at intervals of seven days for five weeks in the first and second pairs of fully developed new leaves, totaling four leaves per seedling and eight leaves per pot. The severity was evaluated using the scale proposed by Cunha et al (2001), being: 1 - from 0 to 3% of severity; 2 - from 3 to 6% severity; 3 - from 6 to 12% severity; 4 - from 12 to 25% severity; 5 - from 25 to 50% severity; 6 - more than 50% severity.

**2.4 Area under the disease progress curve.**

After the five rust severity assessments performed weekly, the area under the disease progress curve for severity (AUDPCS) was calculated according to equation 1 (Shanner and Finey, 1977).

$$AUDPCS = \Sigma(i + Yi)/2 \times (t_{i+1} - t_i),$$

where:

AUDPC = area below the disease progress curve;

Yi = proportion of disease in the i-th observation;

Ti = time, in days, in the i-th observation;
n = total number of observations

**2.5 Lignin and total soluble phenols biochemical analyzes.**

A pair of leaves of the middle third, fully developed, was collected after the last assessment of disease severity. During the collection, the leaves were protected with aluminum foil, placed in a polystyrene box containing liquid nitrogen and taken to the laboratory, where they were stored at -80 ° C until the biochemical analyzes were performed. The material frozen at -80 ° C was macerated in liquid nitrogen with mortar and pestle until fine powder was obtained. Immediately, the samples were lyophilized for 16 hours. An aliquot of 30 mg of lyophilized material was transferred to 2 ml micro tubes, 1.5 ml of methanol was added and shaken at 150 rpm for 16 hours protected from light at room temperature. The suspension was centrifuged at 12,000 rpm for five minutes.The supernatant was transferred to a new microtube for the determination of total soluble phenols (Spanos and Wrolstad, 1990), while the solid residue was used for lignin determination (Doster and Bostock, 1988).

**2.6 Nutrient leaf analysis**

After finishing the evaluations, the plants were cut down and the leaves, stems and roots washed with distilled water, placed in paper bags and oven dried at 60ºC until reaching constant weight. The leaves of each treatment were ground and routed to determine their macro and micronutrient contents, according to methodology proposed by Malavolta et al (1997).

**2.7 Statistical analysis.**

The variables AUDPCS, dry plant weight, foliar nutrient content, lignin, and total soluble phenols were submitted to the Shapiro-Wilk test to verify the assumptions of analysis of variance. After verifying the homogeneity and normality, the data of these variables and the repeated experiments over time were submitted to a joint analysis to determine if there was statistical difference between them. The means of the treatments were compared by F test ($p < 0.05$) and the significant quantitative variables were submitted to regression analysis to fit linear models. The data were analyzed using the software R (R DEVELOPMENT CORE TEAM).

## 3 Results

No differences were noted in the joint analysis of the data between the experiments repeated over time ($P > 0.05$); therefore, the variables given below refer to their means.

### 3.1 Progression curve of disease severity.

The first symptoms of coffee rust were observed at 43 days after inoculation, with increased disease severity over time for the three micronutrients evaluated (Fig 1).

The severity of the disease increased over time, ranging from 0.8 to 26% depending on the dose of the element (Figs. 1A, B and C), reaching a maximum of 25.5% at 64 days after inoculation in the experiment manganese (Fig. 1C). For B, the lowest progression of disease was observed at a dose of 0.05 mg $L^{-1}$, while for Zn and Mn it was at the intermediate doses of 0.50 to 2.00 and 0.25 to 0.50 mg $L^{-1}$, respectively.

### 3.2 Area under the severity progress curve.

The behavior of B was different from Zn to AUDPCS. For both, quadratic regression models were fitted with doses of boron ($p < 0.009$) (Fig 2 •) and zinc ($p < 0.05$) (Figure 2).

An increase in the coffee rust AUDPCS was observed with increasing boron doses, from 0.05 mg $L^{-1}$ to 2.00 mg $L^{-1}$. For zinc, however, there was a 78% reduction in coffee rust severity of 0.05 mg $L^{-1}$ of zinc to the intermediate dose of 2.00 mg $L^{-1}$. The higher dose (4.00 mg $L^{-1}$ of zinc) resulted in an increase in the AUDPCS value from 61.68 to 170.4, ie 63.8%. In relation to the experiment evaluating manganese doses, there was a statistical difference between the treatments ($p <0.05$), but there was no adjustment for a simple linear model ($R^2$ = 0,04) or quadratic ($R^2$ = 0,40). However, two main reductions (52.3 and 47.0%) were observed at concentrations of 0.25 and 0.50 mg $L^{-1}$ of manganese, respectably. Followed by increase.

### 3.3 Dried plant weight.

There was no statistically significant difference in the dry weight of the plants compared to the increase of micronutrients in nutrient solution, boron ($p = 0.22$), zinc ($p = 0.96$) and manganese ($p = 0.76$).

**3.4 Nutritional aspects of coffee seedlings.**

There was a quadratic increase ($p < 0.05$) in the boron (Fig 3A), zinc (Fig 3B) and manganese contents (Fig 3C) with increasing doses of the respective element in the nutrient solution. The leaf contents of N, P, K, Mg, S as well as Cu and Fe were not significantly influenced ($p > 0.05$) when applying boron, zinc and manganese.

**3.5 Content of total soluble phenols (TSP) and lignin.**

There was a statistical difference ($p < 0.05$) in total soluble phenol content (TSP) with increased supply of boron, zinc and manganese in nutrient solution (Fig 4). For the three micronutrients, a quadratic linear model was fitted. The highest values of FST were obtained with manganese. In this assay the TSP increased 26.56% with the increase of the manganese dose from 0.05 to 2.00 mg $L^{-1}$, followed by a small reduction of this compound at an excess dose of 4.00 mg $L^{-1}$. As for boron, the TSP content remained practically stable between doses of 0.05 to 2.00 mg $L^{-1}$, reducing the dose of 4.00 mg $L^{-1}$ by more than 10%. However, it was observed a directly proportional increase of the TSP with the increment of the supply of zinc, in nutrient solution, until the maximum dose.

In addition, for the lignin content in the three experiments there was statistical difference ($p < 0.05$). However, only the manganese model fit (Fig. 5). It was observed a quadratic increase in lignin concentration with increasing doses of manganese, except at the dose 1.00 mg $L^{-1}$ in which the concentration of this compound decreased from 3.39 to 2.90 μg mg $MS^{-1}$. Of general formaldehyde was found 41.2% lignin increase from the lowest dose (0.05 mg $L^{-1}$) to the highest dose (4.00 mg $L^{-1}$).

**4 Discussion**

The supply of nutrients in equilibrium, being an environmental factor, is essential for the expression of the plant genotype in resistance phenotypes. The biosynthesis of defense-related compounds, constituents of physical and chemical barriers is mediated by micronutrients. In this study, was observed the effect of boron, zinc and manganese on reducing the severity of coffee rust on seedlings cultivated in nutrient solution and on the content of phenols and lignins, two important compounds associated with resistance, produced in the shikimic acid route (Graham, 1983; Graham and Weber, 1991) especially for biotrophic pathogens such as *H. vastatrix*.

Of the three micronutrients, zinc had a greater effect and reduced rust severity by up to 78%. The lowest AUDPCS of the disease were observed in the three intermediate doses, between 0.25 and 2 mg $L^{-1}$ of zinc, whereas in those considered deficient (0.05 mg $L^{-1}$) and excessive (4.0 mg $L^{-1}$) there were increased disease. The effect of balanced doses of zinc on the reduction of coffee rust was also observed by Carvalho et al (2008), but in the field in foliar applications. According to these authors, the severity of the disease in coffee trees, at 12 years of age, was reduced in a quadratic form with the foliar application of zinc sulfate (ZnSO4). This element was provided in four sprays at 30 day intervals at doses 0, 1200, 1500 and 3000 mg $L^{-1}$, also observing increased severity at the zero dose and at the greater dose. Reaffirming the importance of balanced nutrition with zinc in reducing the severity of coffee rust in order to promote plant health.

The supply of zinc in the nutrient solution also directly increased the concentration of FST in the leaves of the coffee tree, constituting an effective response to avoid the colonization of *H. vastatrix*. According to Michalak (2006) phenolic compounds of low molecular weight (salicylic acid, caffeic acid, gallic acid, vanillic acid, coumaric acid, cinnamic acid, among others) are precursors of lignin polymers and free radicals produced during polymerization, the which can inactivate the membranes of the fungus, preventing its growth, its enzymes, toxins and / or elicitors. Lignin polymers synthesized from these phenolic compounds may have antifungal activity by lignifying the tips of the hyphae and then leading to the loss of the plasticity required for their growth (Gottlieb, 1951). Simultaneously, the mycelium can absorb lignin, and some compounds present in the cell wall of the fungus, such as chitin, cellulose and hydroxyproline, rich in proteins, can also serve as matrices to polymerize it. In the host, lignified cell walls may restrict the flow of enzymes and toxins from the fungus into plant cells, and water and nutrients from the host to the fungus. This restriction will consequently limit fungal growth and establishment of the host pathogen relationship.

Besides lignin acting as a physical barrier, its biochemical activity in the dissociation of fungal enzymes is also known. According to Friend (1976) the esterification of cell wall polysaccharides involving cinnamic acid or derivatives such as ferulic acid and coniferyl alcohol, lignin precursor phenolic compounds, may positively alter the ability of the polysaccharides to serve as a substrate for covalently binding to the glycoprotein of the cell wall. This process will physically protect the saccharides from the action of the enzymes saccharidases of the fungus. However, specific studies evaluating resistance mechanisms involving lignified coffee cell walls to prevent penetration by *H. vastatrix* are still scarce.

Among the micronutrients, manganese had the greatest influence on lignin and FST biosynthesis in coffee leaves compared to boron and zinc. According to Leina et al. (1996), manganese is a cofactor of phenylalanine ammonilase among other phenolic compounds and also of the enzyme peroxidase, involved in the polymerization of cinnamon to lignin biosynthesis, which can be activated in response to fungal invasion. Kalim et al. (2003) published a study evaluating the effect of manganese on reducing diseases and its association with the production of plant defense compounds. According to the authors, the severity of root rot caused by *Rhizoctonia solani* and *R. bataticola* in string bean (*Vigna unguiculata*) was reduced by 42.7 and 42% respectively, when compared to the control, when 10 μg g-1 of manganese using $MnSO_4 \cdot H_2O$ as the micronutrient source. The reduction in disease severity was associated with increased levels of the polyphenyl oxidase, peroxidase, and total phenols. For another pathosystem, wheat caused by *Gaeumannomyces graminis* was also found to reduce disease severity by 20% in untreated plants to 5% in wheat fertilized with 2,4 g $L^{-1}$ of MnSO4 applied in the alone (Hill et al. 1999). The results described above, although with necrotrophic pathogens, resemble those observed in the present study in relation to the effect of manganese applications on reducing diseases and their relation with the increase of defense compounds, mainly phenols and lignin.

Boron has also been little cited in the control of coffee diseases and its influence on plant defense mechanisms against pathogens. Nonetheless, Vasco et al. (2018) studied the combined effects of boron and potassium doses on rust severity in coffee seedlings grown in nutrient solution. These authors found the highest value for AUDPCS in the intermediate dose of 2.00 mg $L^{-1}$ of boron, while the lowest values were observed at doses considered deficient and in excess of 0.05 to 4.00 mg $L^{-1}$, respectively. However, in this last dose evaluated by these authors, when associated to high doses of K, also occurred a reduction of the leaf area and the weight of the dry plant, that is, it was toxic. These results were similar to those obtained in the present work applying the same doses of boron, however without the combination with other cations, especially at the lowest dose, of 0.05 mg $L^{-1}$. In other words, doses higher than 0.05 mg L-1 when applied to the root system may be associated with phytotoxicity.

Finally, the translocation of boron, zinc and manganese from the roots to the aerial part was verified, after quantifying their leaf contents. In addition, there was no influence on the contents of other macro and micronutrients with the application of the different doses of boron, zinc and manganese used in the present test. The leaf contents of each micronutrient ranged from 28.00 to 140.00 mg $Kg^{-1}$ for boron, from 9.00 to 11.00 mg $Kg^{-1}$ for zinc and

60.00 and 340.00 mg $kg^{-1}$ for manganese with the increase of 0.05 to 4.00 mg $L^{-1}$ of the corresponding micronutrient in the nutrient solution. These values were compared with those found in other studies for coffee plants grown in nutrient solution using the formula proposed by Hoagland and Arnon (1950). In the research conducted by Gontijo et al (2008), the boron and zinc leaf contents were 64.90 and 5.31 mg $Kg^{-1}$, respectively, after the complete solution was supplied. In another study carried out by Cunha et al. (2012) the leaf contents found were 11.52 mg $Kg^{-1}$ for zinc and 68.56 mg $Kg^{-1}$ for manganese. The variations of foliar contents in these studies in relation to those observed in the present investigation may be related to the specific conditions of cultivation, for example, solution pH, aeration, solution temperature, among others. The values of boron, zinc and manganese obtained in this work were also compared with the values found in the research conducted by Martinez et al (2003) in the field after one year of high and one year of low production in four crops in the state of Minas Gerais. The mean values of foliar values of micronutrients after two years were in the range of 40.00 to 68.00 mg $Kg^{-1}$ for boron, 9.00 to 21.00 mg $Kg^{-1}$ for zinc and 109 to 200 mg $Kg^{-1}$ for manganese. In the present study, the highest leaf manganese value was 340.00 mg $Kg^{-1}$ at the highest applied dose of 4.00 mg $L^{-1}$, considered in excess, representing an increase of 170% compared to the aforementioned study. According to Martinez et al (2003) the manganese contents were generally deficient in the areas evaluated by them. This is probably due to high pH (> 6) conditions, redox potential and soil aeration. However, culture conditions in nutrient solution allowed monitoring the pH weekly to be maintained between 5.5 to 6.0 and the aeration of the solutions constant with the objective of providing suitable conditions for the absorption of the applied ions, leading to the translocation of the nutrients after the application of different doses. However, in all cases, the foliar contents found did not compromise the growth of the plants, nor were foliar visual symptoms of deficiency or excess of the respective micronutrients observed.

The results obtained in this research demonstrated the importance of balanced mineral nutrition as a possible rust management strategy in order to reduce dependence on the use of pesticides and to contribute to the sustainability of the coffee crop. From these results, with the isolated effects in nutrient solution and the results obtained with the reduction of the disease, field research should be carried out to verify the combined effect of these micronutrients under equilibrium conditions to reduce the severity of the rust and therefore increase the range of fungicide sprays, and may even eliminate applications and thus increase the environmental sustainability of the crop.

## 5 Conclusions

In nutrient solution.

- Boron, zinc and manganese influenced the severity of the rust. However, the highest reduction of 78% in the AUDPCS of the disease was observed with the application of zinc up to the dose of 2.00 mg $L^{-1}$. This dose, but from boron, a reduction of 15% of AUDPCS was verified. For manganese the lowest AUDPCS value of rust was found at the intermediate dose of 0.25 mg $L^{-1}$.
- Manganese had greater effect on the synthesis of total soluble phenols (TSP) and lignin in coffee seedlings inoculated with *H. vastatrix*.

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## 7 Compliance with ethical standards

**7.1 Conflict of interest.** The authors declare that they have no conflict of interest.

**7.2 Human and animal studies.** The conducted research does not involve human participants or animal

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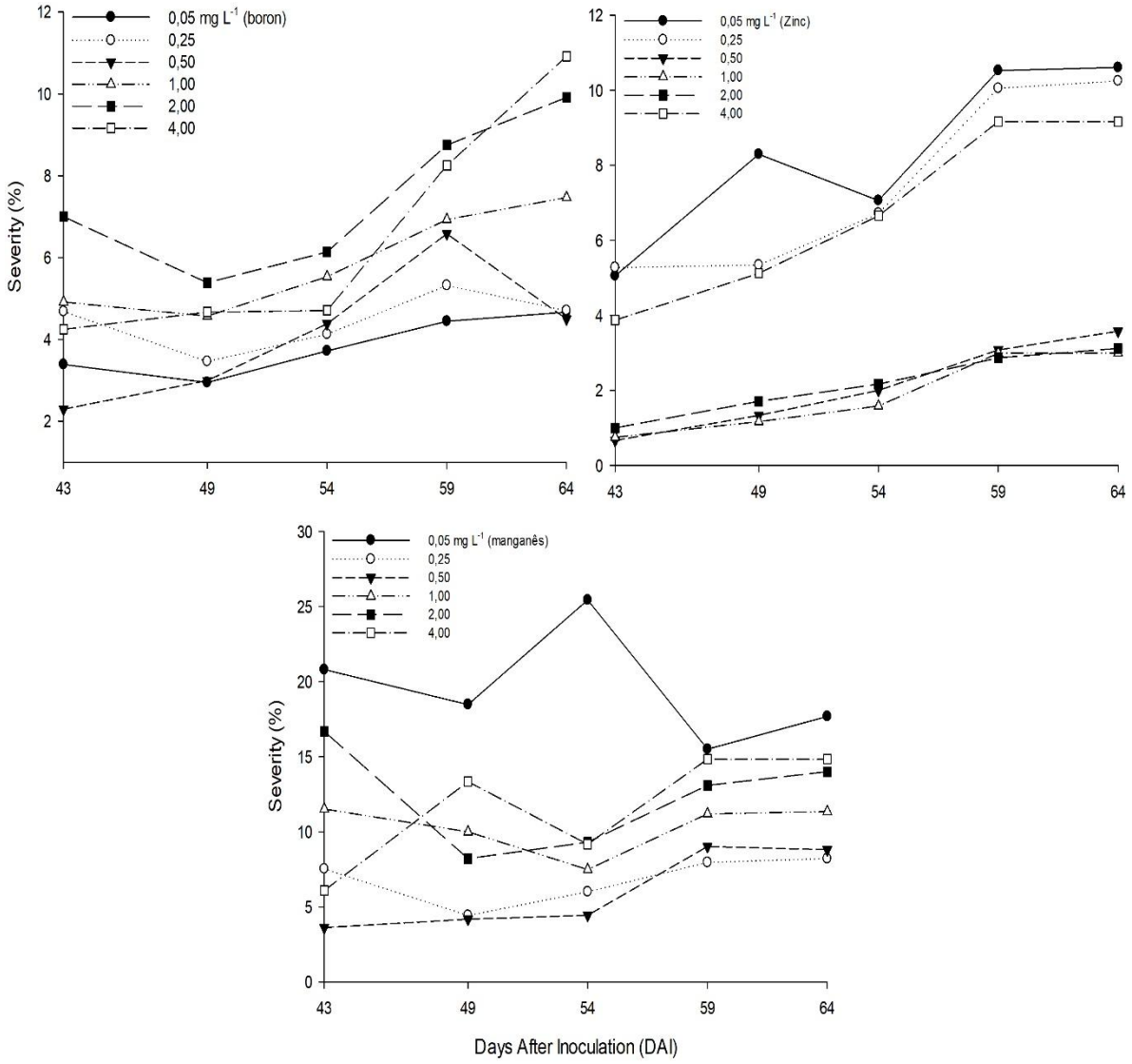


**Fig. 1.** Curve of the severity (%) of coffee rust (*Hemileia vastatrix*) over time in different doses of boron (A), zinc (B) and manganese (C) in coffee plants grown in nutrient solution.

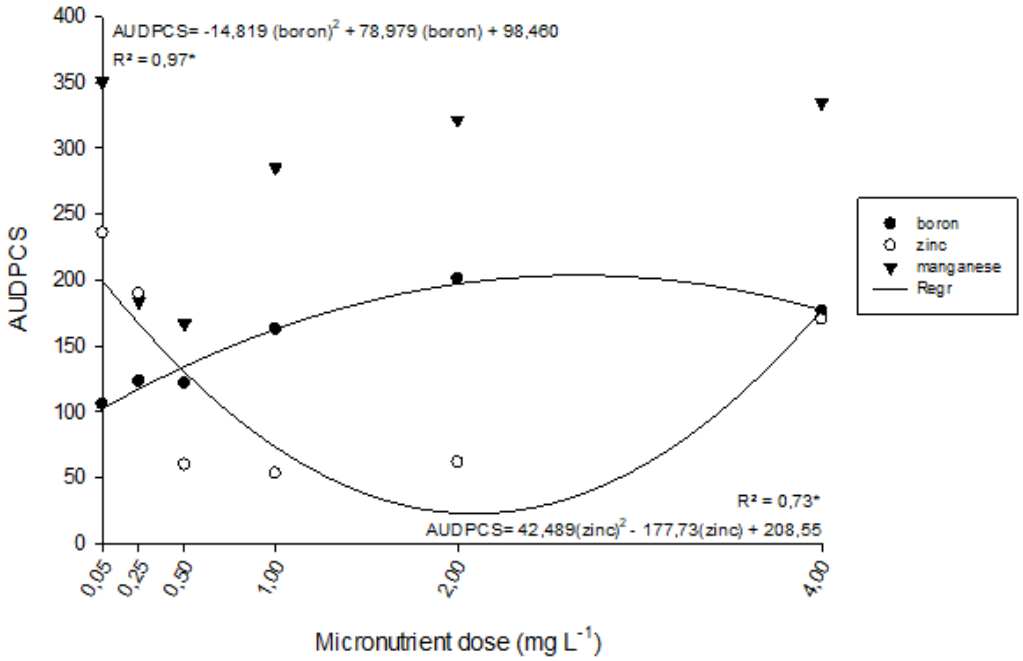


**Fig. 2.** Area under the disease progression curve for severity (AUDPCS) of coffee rust (*Hemileia vastatrix*) as a function of boron (•) and zinc (◦) doses in seedlings grown in nutrient solution.

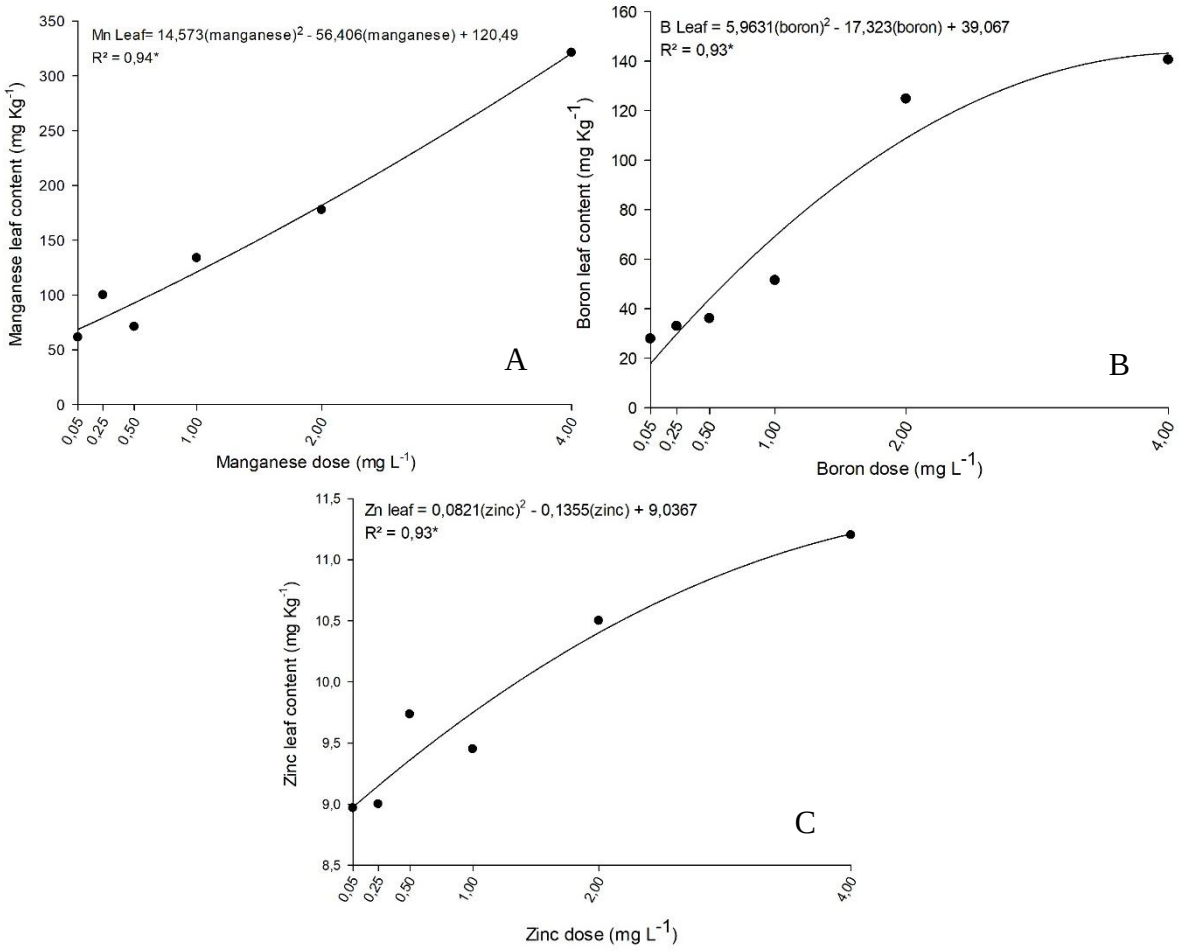


**Fig. 3.** Foliar contents of manganese (A), boron (B) and zinc (C) of coffee seedlings inoculated with *Hemileia vastatrix* as a function of the doses of the respective micronutrients supplied via nutrient solution.

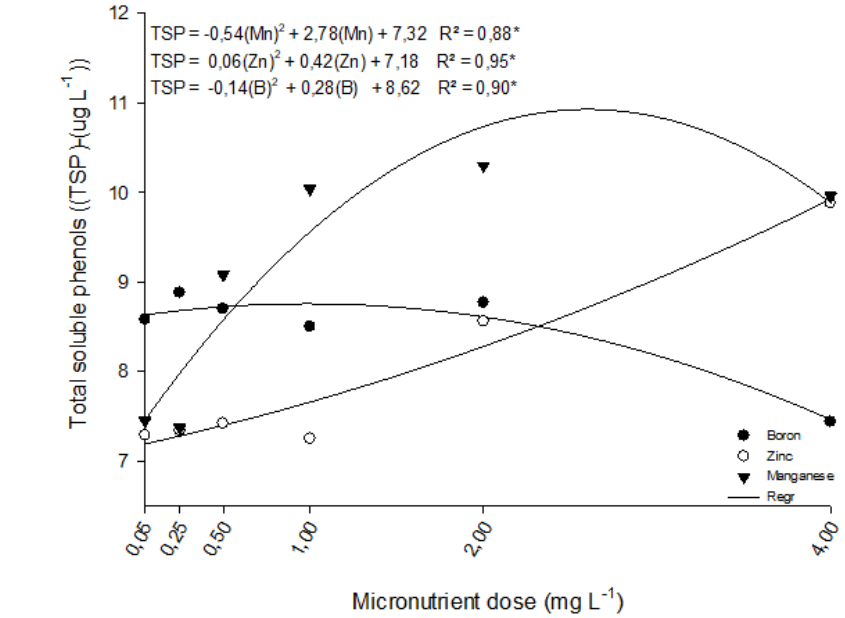


**Fig 4.** Total soluble phenols (TSP) contents in coffee leaves inoculated with *Hemileia vastatrix* as a function of doses of boron, zinc and manganese.

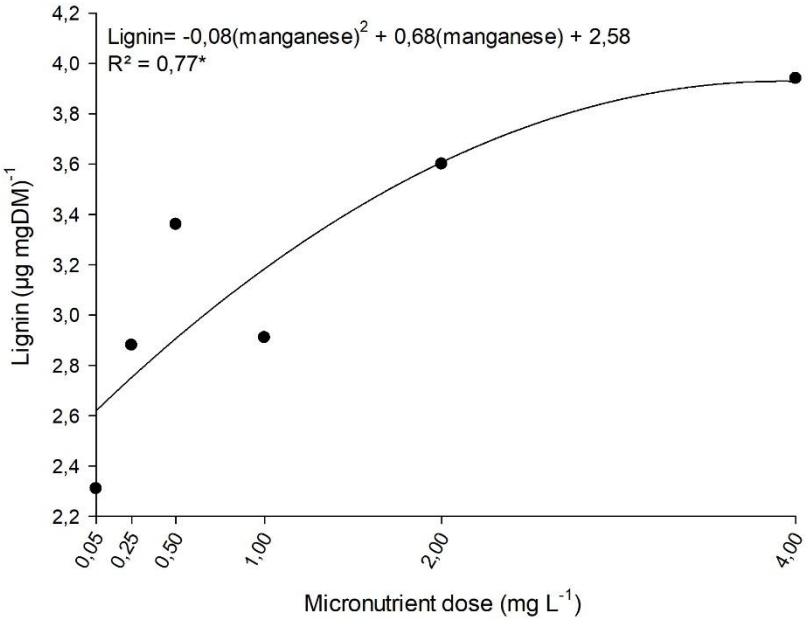


**Fig. 5.** Concentration of lignin (µg mg $MS^{-1}$ (dry mass)) in coffee leaves inoculated with *Hemileia vastatrix* as a function of manganese doses.

ARTIGO 2 **Suppression of Sudden Death Syndrome and root rot of soybean with metalloid and metal oxide nanoparticles.**

**C. D. P. Pérez[1,3], R. D. L. Torre-Roche[2], N. Zuverza-Mena[2], C. Ma[2], J. C. White[2], E. A. Pozza [1], A. A. A. Pozza[4], and W. H. Elmer[3]**

[1]Dept. of Phytopathology, UFLA, Lavras, Brazil, [2]Dept. of Analytical Chemistry, The Connecticut Agricultural Experiment Station (CAES), The Center for Sustainable Nanotechnology, New Haven, Connecticut USA; [3]Dept. of Plant Pathology and Ecology, CAES, Center for Sustainable Nanotechnology, New Haven, Connecticut USA; [4]Dept of Soil Science, UFLA, Lavras, Brazil

Contact person Wade.Elmer@ct.gov

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[1]Departamento de Fitopatologia, Universidade Federal de Lavras, 37200-000, Lavras, MG, Brasil.

**Abstract**

Metal based nanoparticles (NPs) have unique functions and when applied to plants, can suppress disease and promote growth. In the greenhouse, we explored foliar applications of metalloid and metal oxide NPs of the essential elements boron (B), copper (Cu), manganese (Mn), and zinc (Zn), and of the nonessential metals, silver (Ag) and cerium ($CeO_2$), to determine their effect on sudden death syndrome (SDS) in soybean caused by *Fusarium virguliforme.* Four different greenhouse studies were conducted where a single foliar application of NPs (500 µg/ml) was applied to a V3 stage soybean plant (1 to 2 ml per plant). Although not all eight NPs were evaluated in each of the four experiments, we observed suppression of root rot associated with SDS with NP CuO in 4 out of 4 experiments, with ZnO in 3 out of 4 experiments, with B in 2 out of 3 experiments, and with Ag in 2 out of 2 experiments. MnO and $CeO_2$ did not consistently affect soybean growth or SDS. The root rot rating was a more sensitive measurement to the effect of NPs than fresh weight determinations which were more variable. *In vitro* assays that examined NPs B, CuO, MnO, and ZnO on mycelial biomass found that only NPs of ZnO had toxicity at 1,000 µg/ml, suggesting that there was minimal fungicide activity of the NPs in disease suppression. Foliar-applied NPs to young plants may be a new tool in promoting root health in soybeans by reducing root rot caused by *F. virguliforme*.

**Key words**: Mineral nutrition, alternative management, nanomaterials.

## 1 Introduction

Nanoparticle (NP) forms of metalloid and metallic oxides of essential micronutrients have been shown to have important applications in plant protection and nutrition. More rapid dissolution and greater efficiency of NP forms leads to improvement in growth and metabolic function (Elmer and White 2018; Servin et al. 2015). Given that the role of micronutrients in plant metabolism and host defense directly affects the production of the phenolics, lignin, quinones, tannins, and flavonoids, as well as membrane and cell wall stability (Graham and Webb 1991; Roemheld and Marschner 1991), continued efforts to further tune and enhance their availability and function in nanoscale form are warranted. The rate at which these elements can activate the physiological and biochemical processes can often control the level of host resistance and eventual disease (Graham and Webb 1991).

Although the role of micronutrients in suppression of crop disease is well documented (Elmer and Datnoff 2014), obstacles exist in delivering and distributing these elements to the infected tissues. Most micronutrients are poorly translocated to the roots following foliar application (Bukovac and Wittwer 1957) and for soil applications to be effective, the rates must be very high due to micronutrient precipitation as oxides in slightly acid to neutral soils (Leeper 1952). Foliar feeding to enhance plant health is an established practice (Reuveni and Reuveni 1998), but incorporating NPs of micronutrients is a novel approach. Work from our group has demonstrated that applying NPs of copper oxide (CuO), copper phosphate nanosheets ($Cu_3(PO_4)_2$), and zinc oxides (ZnO) improved uptake, translocation, and function of the micronutrient when compared to the larger bulk equivalent or salt forms (Borgatta et al. 2018; Elmer and White 2016; Elmer et al. 2018).

Nonessential elements, such as silver (Ag) and cerium (Ce), also enhance plant growth when applied in nanoscale form (Adisa et al. 2018; Ali et al. 2015; Lamsal et al. 2011a & b). Of all the reports mentioned in a recent review on NPs and plant disease, Ag, CuO, and ZnO have shown more consistent effects suppressing crop diseases than other NPs (Elmer and White 2018). For example, NPs of Ag were first used on 2006 to suppress powdery mildew of pumpkin (Park et al. 2006). Different nanoscale forms of Cu applied to eggplant, tomatoes, and watermelon suppressed Fusarium and Verticillium wilts (Borgatta et al. 2018; Elmer and White 2016; Elmer et al. 2018). Graham et al. (2016) demonstrated that foliar application of NP ZnO on citrus reduced citrus canker after *Xanthomonas citri* subsp. *citri* was injected into the leaf intercellular space; notably, this work has led to a commercially available nanoscale Zn formulation (Zinkicide®).

Sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Meer) caused by *Fusarium virguliforme* has increased in distribution and economic importance in the Midwest of the United States. Since 2014, between 200 to 700 million dollars has been are estimated lost due to SDS in the United States alone (Wrather et al. 2001; 2003). *F. virguliforme* (*Fv*) is the causal agent of SDS in North America, but the species complex differs in the southern hemisphere (Aoki et al 2003). Early symptoms include poor root development and root rot that may progress into foliar symptoms, including interveinal chlorosis and necrosis, defoliation, and early death (Roy et al. 1997); importantly, foliar symptoms can be quite variable (Gongora-Canul et al., 2010).

Management of SDS has been difficult, although significant advances have been made (Hartman et al. 2015). Selecting for host-plant resistance has been successful in identifying some cultivars with modest tolerance, but screening for resistance is difficult because disease onset and expression are strongly dependent on environmental factors (Scherm and Yang 1999). Fungicides as seed treatments have value (Kandel et al. 2016; Weems et al. 2015), and crop rotation provide suppression in some fields (Xing and Westphal 2006; Hartman et al. 2015). Cultural management of field parameters, such as improving soil drainage and reducing soil compaction, can reduce the severity of SDS (Hartman et al. 2015; Leandro et al. 2012), but no management strategy has consistently suppressed disease in all areas. Consequently, growers are encouraged to combine management strategies. New and novel strategies are needed, especially in light of the predictions that global food demand will need to double in the next several decades (Tillman et al. 2011). Additionally, host nutrition is another component that can affect SDS severity (Rupe et al., 1993; Sanogo and Yang 2001). The role of micronutrients in nanoscale metal oxide form as a foliar treatment strategy for soybean diseases has never been evaluated. Their small size and large surface area have created new opportunities for nanotechnology in plant pathology yet considerable testing and evaluation are still needed (Auffan et al 2009; Elmer and White 2018)

Our objectives for this report were to first determine the optimal inoculum concentration of *F. virguliforme* on three soybean cultivars to promote consistent root rot so that foliar applications of NP B, CuO, MnO, and ZnO can be compared on soybeans and on SDS; and to compare NPs of other nonessential metals (Ag, and $CeO_2$) on SDS). Measured endpoints included plant growth and root rot severity, as well as the elemental composition of roots and stems to assess how NPs application affect mineral uptake. Lastly, we assessed the *in vitro* antifungal activity of B, CuO, MnO, and ZnO NPs on the growth *F. virguliforme*.

## 2 Material and methods

### 2.1 Nanoparticles, plants, and inoculum.

NPs of Ag (20 nm), B (100 nm), CuO (40 nm), $CeO_2$ (25 nm), MnO (30 nm), and ZnO (10-30 nm) were obtained from US Research Nanomaterials Inc. (Houston, TX). Suspensions of NPs were prepared at 500 µg/ml distilled water that was amended with a nonionic surfactant (1 ml/liter) (Regulaid®, Kalo Inc., Overland Park, KS). Suspensions were sonicated for 2 min in a probe sonicator (Fisher Scientific, FB505) at 50% amplitude immediately before application to achieve a stable dispersion.

Seeds of soybean cultivars 'Seedranch' (Seedranch, Odessa, FL), 'Sloan' (provided by Dr. Glen Hartman, University of IL) and 'Spencer' (provided by Dr. Martin Chilvers, Michigan State University) were germinated in 36 cell (5.66 x 4.93 x 5.66 cm) plastic liners (1 plant/cell) filled with soilless potting mix (ProMix BX, Premier Hort Tech, Quakertown, PA, USA). Potting mix analyses revealed the pH = 6.5, $NO_3$-N = 3 µg/g, $NH_4$-N = 12 µg/g, P = 100 µg/g, K = 180 µg/g, Ca = 1,66 µg/g, Mg = 125 µg/g, and soluble salts = 0.3 ms/cm as determined by Morgan Test (Lunt et al. 1950). Plants were fertilized once after three weeks with 40 ml of Peters' soluble 20-10-20 (N-P-K) fertilizer (R.J. Peters, Inc., Allentown, PA). Greenhouse temperatures averaged 17 to 22 °C night and 19 to 25 °C day. Plants in the V3 leaf stage (Fehr and Caviness 1977) were used in all studies.

The pathogen inoculum was prepared on Japanese millet that had been autoclaved with distilled water (1:1 wt/vol) for 1 hour on two consecutive days (Elmer and White 2016). The millet was seeded with three agar plugs colonized by *F. virguliforme* (Isolate Mont-1) (Gray and Achenbach 1996). The culture was allowed to grow for 2 weeks at 22-25 °C and the millet was air-dried, and ground in a coffee mill for 30 sec. The millet inoculum was thoroughly mixed by hand into potting mix (ProMix BX, without mycorrhizae, Premier Hort. Tech, Quakertown, PA, USA). Imidacloprid was applied (0.3 g/pot) once as a granular amendment to suppress fungus gnats.

### 2.2 Greenhouse experiments.

Five experiments were conducted. The first greenhouse experiment was designed to determine the optimal inoculation rate for future studies. Soybean cultivars 'Seedranch', 'Sloan' and 'Spencer' were transplanted at the V3 stage into 1-liter plastic pots filled 0.8 liters of potting mix and infested with 0, 0.5, 1.0, 2.0 or 3.0 g millet inoculum/liter. Inoculum was enumerated by serially diluting potting mix onto Peptone PCNB agar plates (Leslie and

Summerell 2006), incubating them for 5 days, and counting colonies of *F. virguliforme.* The colony forming unit (CFU) of *F. virguliforme* /g potting mix was calculated. There were two soil samples per inoculum concentration and three plates per dilution at $10^{-2}$ or $10^{-3}$ ml/g soil (oven dry weight equivalent) were prepared. Plants of each cultivar were transplanted into a pot filled with each inoculum density/concentration and were set on greenhouse bench in a 3 (soybean varieties) X 5 (inoculum concentrations) randomized complete block design with six replicates per treatment. Each pot received 50 ml of a complete fertilizer solution (20–20–20, N-P-K) once per month. The experiment was repeated eight months later with three replicates. After 5 weeks of growth, the experiments were terminated and the plants were removed from pots, washed in tap water to remove all potting mix, and weighed. The root systems were rated for the percentage root rot as the percent root area with reddish-brown discoloration. The root systems and above ground tissue were weighed bagged separately, dried to constant weights at 50 °C, and re-weighed.

With this information on the optimal inoculum concentration, we conducted four greenhouse experiments to determine the effect of foliar applications of NPs of CuO, MnO, and ZnO. Experiment 2 used Sloan and was designed to determine the effect of NPs B, CuO, MnO, and ZnO sprayed onto soybeans plant. The data collected included plant growth, SDS severity (percent root rot), and elemental composition of roots and above ground tissue. Polyvinylidene chloride film (Saran$^{TM}$ wrap) was securely fitted around the stem of each plant to cover the soil and prevent NP contamination of the growth media. Plants were sprayed using plastic spray atomizers until leaves were wet (1 to 2 ml per plant); the plants were allowed to dry and the film was removed. Control plants were sprayed with sonicated distilled water. Three plants of each treatment were immediately transplanted into non-infested potting mix or to potting mix infested with 2 g/liter of millet inoculum. Plants were individually irrigated to avoid wetting the leaves. Each pot received 50 ml of a complete fertilizer solution (20–20–20 N-P-K) every 2 wks.

The experiment was arranged on a greenhouse bench as a randomized block design with five NPs treatments (untreated control, B, CuO, MnO, ZnO) (x) two inoculum levels (infested with *F. virguliforme* or not infested). Imidacloprid was applied (0.3 g/pot) once as a granular amendment to suppress fungus gnats. After 5 weeks, plants were harvested and fresh and dry weights were measured as described above. The roots were washed free of potting mix, weighed, and the percent root rot was determined. Dried root and above ground tissue were analyzed for elemental composition described below.

The experiment was set up again with some changes. NP B were not included and there were six replicates per treatment instead of three. The experiment was arranged on a greenhouse bench as a randomized block design with three NPs (CuO, MnO, ZnO) x two inoculum levels (infested with *F. virguliforme* or not infested). Untreated plants were also grown in infested and non-infested potting mixes to yield a total of eight treatments. Plants were grown as described above and the experiment was terminated after 5 weeks. The roots were washed free of potting mix, weighed, and rated for SDS. Fresh biomass and the percent root rot was determined as described above and dried root and above ground tissue were analyzed for elemental composition described below.

Experiment 4 compared NPs of the essential elements B, CuO, MnO, or ZnO to the nonessential elements Ag and CeO2, on soybean growth, percent root rot, and elemental composition of stems and roots. There were six replicates and plants were arranged on greenhouse benches as a seven NP (Untreated control, Ag, B, $CeO_2$, CuO, MnO, and ZnO) X two inoculum levels (infested with *F. virguliforme* or not infested) factorial randomized complete block design. As revealed below, we used 3 g of millet/liter inoculum because Spencer was more tolerant of SDS. After 5 weeks, plants were harvested for fresh and dry weight determination; as above, roots were washed, weighed, and rated for the percent root rot. Dried root and above ground tissue root were assayed for elemental composition as described below.

Experiment 5 reexamined the same NP treatments used in experiment 4, but the cultivar Seedranch was used instead of Spencer. In addition, plants were exposed to the NP treatment by inverting the plant and the immersing the leaves into the NP suspensions for 3-5 seconds, and hung upside down until dry. Plants were then transplanted into infested or non-infested potting mix. The technique sought to provide more complete foliar coverage than spraying the shoots. There were nine replicates and plants were arranged on greenhouse benches as a six NP (Untreated control, Ag, B, $CeO_2$, CuO, and ZnO) X two inoculum levels (infested with *F. virguliforme* or not infested) factorial randomized complete block design.

Experiment 6 was conducted to determine the *in vitro* toxicity of B, CuO, MnO, or ZnO NPs to *F. virguliforme* in shake culture. Fifty ml of sterile potato dextrose broth (Difco Laboratories, Livonia, MI) was added to 125-ml Erlenmeyer flasks were then amended with 0, 100, and 1,000 µg/ml of each NP. Flasks were seeded with a colonized agar plug of *F. virguliforme* and were set on a platform shaker at 125 rpm for 5 days at 22 °C. Mycelial mats were harvested under vacuum onto pre-weighed Whatman® #1 filter paper that had been dried at 50 $^{o}$C for 18 hr. The mycelia-containing filter papers were re-dried at 50 $^{o}$C for at least 18

hours and weighed again. The dried mycelial weights were then calculated after subtracting the weight of NP treatment that was added to the flask. There were three replicate flasks per NP type and concentration. The experiment was repeated to confirm findings.

**2.3 Elemental analysis.**

Root and foliar tissues from greenhouse experiments 2, 3, 4, and 5 were analyzed for the elemental composition. Tissues were dried in an oven at 50 $^{o}$C, ground in a Wiley mill, and passed through a 1 mm sieve. Acid digestion of ground samples (0.5 g) were done in 50 ml polypropylene digestion tubes with 5 ml of concentrated nitric acid at 115 °C for 45 min using a hot block (DigiPREP System; SCP Science, Champlain, NY). The elements Ag, B, Ca, Ce, Cu, Fe, K, Mg, Mn, Mo, P, S, and Zn were quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES) on an iCAP 6500 (Thermo Fisher Scientific, Waltham, MA). Elemental content was expressed as μg/g (dry plant weight). In experiment 4, tissue from replicates 1 and 2, 3 and 4, and 5 and 6 were composited, yielding three replicates per treatment. In experiment 5 tissue from replicates 1, 2 and 3; 4, 5, and 6; and 7, 8, and 9 were composited, yielding three replicates per treatment. Tissue from the other studies were not composited over replicates.

**2.4 Statistical analyses.**

Data sets of biomass and elemental composition were subjected to Shapiro-Wilk's Test for equality of variance. Normally distributed data with equal variance were analyzed for treatment (NPs and *F. virguliforme* infestation) using ANOVA for a mixed model factorial blocked design. Treatment effects (NP and Fusarium inoculation) were tested as fixed variables with block and replication as random effects. Means separated using Tukey's Honestly Significant Difference Test at $P < 0.05$. Disease severity values (percent root rot) were analyzed non-parametrically using Wilcoxon Signed Fisher's Test at ($P = 0.05$) (Conover and Iman 1981). Regression analysis was used to analyze the inoculum concentration on SDS and recovery of *F. virguliforme* from potting mix. All statistical analyses were performed using SYSTAT V.10 (Cranes Software International Limited, Bangalore, Karnataka, INDIA)

# 3 RESULTADOS

## 3.1 Experiment 1.

The first study sought to determine the optimal millet inoculum added to potting mix at 0, 0.5, 1.0, 2.0 and 3.0 g of per liter. A curvilinear increase was observed in the recovered CFU of *F. virguliforme* per g of oven dried potting mix (Figure 1 lower panel). The data were best fit by the polynomial equation CFU = $184.8x^2 – 155.5x$ ($R^2 = 0.98$, $P = 0.001$) where Y = CFU and X equal millet inoculum/g mix (dry weight equivalent). Root rot ratings for soybean cultivars 'Seedranch' and 'Sloan' reached their peak of 50 to 75 % at 2.0 g inoculum/liter potting mix and were not significantly different at 3.0 g inoculum/liter potting mix, despite the increase in recovered colonies of *F. virguliforme* from 300 to 1,200 CFU/ g potting mix (Figure 1 upper panel). We observed that 'Seedranch' and 'Sloan' were very susceptible followed by 'Spencer'. Given these findings, we found there were no large differences in susceptibility between the 2 g and 3 g millet rated for 'Seedranch' and 'Sloan' so 2 g per liter potting mixes was used. We opted to use 3 g per liter potting mix for Spencer given it had more tolerance to SDS.

## 3.2 Experiment 2.

The fresh and dry weight data yielded the same statistical differences so only the fresh weight data are presented (Figure 2 upper panel). Significant interactions between the NP treatment and infestation with *F. virguliforme* were detected ($P < 0.001$) and were evident by the strong effect of the NP treatment on the weights of noninfested plants that was not observed with the pathogen was present. Main treatment effect were also significant (NP treatment = $P < 0.001$ and infestation with *F. virguliforme* = $P < 0.001$). Healthy plants exposed to NPs B, CuO, and ZnO were 32, 46, and 35 % larger in fresh weight, respectively, when compared to controls, but did not differ in the presence of the pathogen. The NP MnO treatment did not differ from untreated controls in infested or noninfested potting mixes. The presence of the pathogen *F. virguliforme* reduced the overall fresh weights from an average of 30.9 g to 10.0 g. The root rot severity ratings were analyzed using Wilcoxon rank test (P = 0.05). Only NP of CuO and ZnO resulted in a 26% and 27% reduction, respectively, in the percent root rot compared to untreated plants (Figure 2 lower panel). We observed no effect of NP B or MnO on the percent root rot in this experiment. Averaging over all treatments we observed infesting the potting mix resulted in an overall 134% increase in root rot compared to noninfested potting mix (T-test $P < 0.001$).

Significant interactions were observed for B ($P$ <0.001) and Cu ($P$ <0.001) concentrations for the NP treatment x the tissue which was evident by the lower level of B and higher level of Cu in the above ground tissue than in control roots (Table 1). An interaction between the tissue x infestation with *F. virguliforme* was also seen for Cu level since higher levels were observed in infested above ground tissue than in noninfested roots. The main effects of NP treatment, infestation, and tissue type were significant B and Cu. Tissue digests of the roots and above ground tissue were assayed for Ag, Al, As, B, Ca, Cd, Ce, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, Si, Ti, Zn to determine if the NPs affect the elemental concentration. No NP treatment affected the uptake of any element except for NP B and CuO. For plants growing in noninfested potting mixes, NP B and CuO increased the above ground concentration of B and CuO, respectively, but not in the roots (Table 1). The NP MnO and ZnO did not affect the tissue concentrations of any element assayed. Averaging over all treatments and plant parts, infesting the mix with *F. virguliforme* increased B, Ca, Cu, Fe, Mo, and P, but decreased Mg (data not shown). Roots had lower levels of B, Ca, Fe, Mn, and Mo and higher levels of Cu, K, Na, and S when averaged over treatment and infestation.

**3.3 Experiment 3.**

When NP CuO, MnO and ZnO treatments were re-examined, the fresh and dry weight data yielded the same statistical differences so only the fresh weight data are presented. No significant interactions were detected ($P$ < 0.001) and only the *F. virguliforme* treatment was significant ($P$ = 0.001) and reduced the overall averaged fresh weight by 16%. When the percent root rot of plants were analyzed using Wilcoxon Rank Test, plants treated with NP CuO or ZnO had 29 or 30 % reduction in the percent root rot, respectively, compared to untreated infested controls.

In reference to the Cu, Mn, and Zn concentrations in the tissue, there were strong interactions between the NP treatments x plant tissue, likely due to the elevated foliar levels verses the roots (Table 2). Significant main effects were also detected for NP treatment for Cu (P< 0.001), Mn (P < 0.001), and Zn ($P$ = 0.005). In potting mix that was either infested with *F. virguliforme* or not infested, there were higher above ground tissue concentrations of Cu and Mn when the respective NPs were applied (Table 2). In NP CuO-treated plants, Cu concentrations were 23 or 8 times higher than controls for both infested or nonninfested potting mix treatments, respectively. Similarly, above ground Mn levels were 1.6 or 2 times higher for plants grown in infested or nonninfested mix, respectively. Zn concentrations were twice as high in NP ZnO-treated plants, but only when plants were grown in noninfested

potting mix. Although the root tissue was more distal to the site of exposure, Cu and Mn was still 6.5 and 1.5 times greater, respectively, than control plants, but only for plant grown in infested potting mixs. Compared to controls, root concentrations of Zn show an increased trend of 20 % when NP ZnO-treated plants were grown in infested mix, but differences just missed significance ($P = 0.081$).

**3.4 Experiment 4.**

Soybeans treated with the NP treatments of essential and nonessential element had greater fresh and dry weights in the absence of *F. virguliforme* when compared to the untreated control; only fresh weights are presented (Table 3). The main effects of NP treatment ($P < 0.001$) and the infestation with *F. virguliforme* ($P < 0.001$) were significant, but there was no interaction ($P = 0.898$). Compared to the untreated controls grown in non-infested potting mix, the fresh weights were increased 24, 33, 37, 29, 31, 30, and 29% for plants treated with Ag, B, $CeO_2$, CuO, MnO, and ZnO, respectively. For plants grown in infested potting mix and treated with NP B, a 30 % increase in fresh weight resulted when compared to controls. Plants exposed to NP Ag, CuO, and MnO, had a 26, 10, and 9% reduction in the percent root rot, respectively, when compared untreated plants grown in infested potting soils. Plants grown in noninfested potting had less than 10 % root rot and did not differ by NP treatment so data were not shown. In experiment 4, in the absence of any treatment, the infestation with *F. virguliforme* resulted in a 10% ($P < 0.001$) reduction in fresh weights (Table 3).

**3.5 Experiment 5.**

When the NPs Ag, B, CeO, CuO, and ZnO were re-examined on ‘Seedranch’ in experiment 5, the fresh weights were not affected by NP treatment in infested or noninfested potting mix. However, the percent root rot ratings were again reduced 24, 41, 21, 21, and 33% for plants treated with NPs Ag, B, CeO, CuO, and ZnO, respectively. For experiment 5, in the absence of any treatment, the infestation with *F. virguliforme* resulted in a 30% ($P < 0.001$) reduction in fresh weights (Table 3). Plants grown in noninfested potting had less than 8% root rot and did not differ by NP treatment so data were not shown Only NP Ag and CuO reduced root rot severity in both experiment 4 and 5 (26 and 24% for NPs of Ag, and 10 and 21% for CuO, respectively) (Table 3). In experiment 4, both Ag and Cu were present at significantly greater levels in the roots of soybean plants and grown in infested mix when they were treated with the respective NP treatment; Cu levels were also higher in the non-infested

mix (Table 2). We also noted a significant increase in Cu in plant roots treated with NP Ag. As expected, we also observed an increase of Ag and Cu in the foliage of treated plants grown in infested and non-infested potting mixes. However, the difference in root levels were not observed in experiment 5.

### 3.6 Experiment 6

*In vitro* growth of *F. virguliforme* was assessed at three concentrations (0, 100 and 1000 µg/ml) of NP B, CuO, MnO, and ZnO in two separate experiments (Figure 5). No interaction was detected between the experiment and the treatments so data sets were combined. NPs ZnO were the most toxic to *F. virguliforme,* completely inhibiting growth at the 1000 µg/ml, followed by CuO and B. NPs of Mn were unexpectedly stimulatory to *F. virguliforme* in both repetitions of the study.

## 4 Discussion

Management of SDS have included the use of moderately resistant cultivars, fungicides, and cultural rotation strategies, but none of these approaches are consistently effective in all sites (Hartman et al. 2015; Kandel et al. 2016; Leandro et al. 2012; Weems et al. 2015; Xing and Westphal 2006). We explored the use of foliar sprays of metalloid and metal oxide NP to determine their efficacy in suppressing SDS. After determining the optimal inoculum load (2 g/millet) to produce consistent root rot symptoms, we conducted four greenhouse experiments that demonstrated the effects of nanoscale micronutrients and nonessential elements on SDS of soybean. Estimates of percent root rot was more sensitive endpoint to the NP treatments than fresh weights which was more variable from experiment to experiment. Although not all NPs were evaluated in each experiment, we observed disease suppression of root rot following treatment of NP CuO (4 out of 4 experiments) and ZnO (3 out of 4 experiments). NP of B and MnO were evaluated three times and reduction in the percent root rot were observed for each NP only once. NP of Ag were examined in two experiments and both times resulted in a reduction in the percent root rot.

This finding that NPs of CuO and ZnO suppress SDS follows many other reports where foliarly applied NPs of CuO or ZnO suppressed plant disease. NP of CuO suppressed Fusarium and Verticillium diseases in tomato, eggplant, and watermelon (Borgatta et al 2018; Elmer et al. 2018; Elmer and White 2016). In most cases, the disease suppression was associated with increased yield. In the current study, root tissue analysis revealed higher

levels of Cu in experiment 2, 3, and 4, which agrees with past studies (Elmer et al. 2018; Elmer and White 2016). In experiment 2, the NP CuO treatment resulted in a six-fold increase in root Cu as compared to the untreated controls (Table 2). Hong et al (2016) noted that CuO NPs applied at lower concentrations (50 – 200 μg/ml) to cucumber (*Cucumis sativus*) plants did not significantly change the amount of Cu in the roots suggesting a threshold may reached and that lower rates may be as useful. Wang et al. (2012) suggested that the shoot-to-root transport of CuO NPs takes place via phloem, although the mechanism is still largely unexplored. There is limited information on the interactions of plants with Cu-based NPs applied foliarly in comparison to root exposure studies (Keller et al. 2017). In roots, the uptake of Cu can occur as intact NPs or as ions released from Cu-based NPs (Zuverza-Mena et al. 2017). In Bt-transgenic cotton, NP CuO applied to leaves is taken up by endocytosis, while the NPs are retained in the cell wall of conventional cotton (Le Van et al. 2016). In the current study, it is not known whether the NPs are remaining on the leaf surface and slowly dissolving and releasing ions into the leaf through stomatal openings with subsequent ion transport to the roots, or if the NPs themselves are being transferred through the plant. In watermelon, the increased Cu root levels following treatment with NPs of CuO were associated with strong up-regulation of polyphenol oxidase and PR1 genes but only when NP CuO and *F. oxysporum* f. sp. *niveum* were both present in the roots (Elmer et al. 2018). A similar mechanism may be occurring with soybean. Cu availability was shown to be a strong driver of polyphenol oxidase activity in soybean (Marziah and Lam 1987). Copper serves as a cofactor for plastocyanins, peroxidases, and multi-Cu oxidases (Evans et al. 2007); all of which, in turn, serve as key components of host defense. Interestingly, it appears that nanoscale Cu-induced defense reactions are somewhat non-specific with regard to pathogen and may serve as a highly useful management option in a range of disease systems (Elmer and White 2018). Newer Cu composites may further enhance defense reaction by allowing release of Cu ions to be delivered (Strayer-Scheyer et al. 2018).

Zn nutrition has long been associated with suppression disease and functions as cofactors in superoxide dismutase (SOD) enzymes suppressing free radicals (Duffy 2007). Delivering Zn to plant in the nanoscale form has been shown to enhance host resistance in citrus, rose, and sugarbeets (Derbalah et al. 2013; Graham et al. 2016; Paret et al. 2013), but information on its uptake in nano-form versus ionic form is lacking. We also observed that NP ZnO had a significant positive effect on soybean resistance to SDS. In experiment 2, 3 and 5, we observed significant reductions in root rot following NP ZnO. Despite probe sonication of the NP suspension for 2 min before application, NPs of ZnO tend to coalesce into larger

non-nano size aggregates, which in turn, reduce the dissolution of the Zn ion. This raises the possibility that NP of ZnO may have more activity than observed here if better formulation could maintain dispersion. Researchers in Florida have begun to overcome this obstacle by formulating ZnO with coatings (Graham et al. 2016).

There is a history of Mn nutrition being associated with suppression disease (Thompson and Huber 2007). Mn is an activator of Phenylalanine ammonia lyase and phenol synthesis (Thompson and Huber 2007). The association between Mn and root health has been demonstrated in asparagus, beets, eggplant, strawberries, and wheat (Elmer and Datnoff 2013; Thompson and Huber 2007). However, the benefits of using NPs of Mn to suppress SDS are not evident based on these findings. In fact, the observation the *F. virguliforme* was stimulated *in vitro* by NP MnO suggest NP MnO may not have value in SDS management.

The role of B in plant disease was reviewed by Stangoulis and Graham (2007) and found that in 20 reports where B was studied, 18 (90%) were associated with disease suppression. In those reports, disease was incited by foliar and root infecting fungi, bacteria, and viruses, suggesting B nutrition may affect a wide array of defense mechanisms. Since that review, Bellaloui et al. (2012) reported that soybean plants with enhanced B nutrition were more tolerant to the charcoal rot disease caused by *Macrophomina phaseolina* and had higher levels of phenolics, seed coat lignin, isoflavones, and sugars. Therefore, our finding that the foliar treated NPs of B tended to suppressed SDS may not be surprising. In three experiments (experiments 2, 4, and 5), foliar-treated NPs of B reduced the root rot ratings in 2 experiments, but only increased the corresponding fresh weights in one trial (experiment 4). Elmer et al. (2018) examined NPs of B on watermelon in a single field trial in soil deficient in B to determine effects on growth, yield, and Fusarium wilt. When compared to untreated controls, a reduction in disease rankings was observed, but no effect on yield was detected. Given that soybeans are responsive to B application (Ross et al. 2006) even in the absence of disease, NPs of B show promise as nano-fertilizer to promote crop health.

NPs Ag were examined in only two trials, but we observed a reduction in root rot in both. The antimicrobial properties of NPs Ag are well known (Jo et al. 2013), and it was the first NP to be used on a plant disease (Park et al. 2006). Many have used NPs Ag in creative ways. Ocsoy et al. (2013) used NPs of Ag to functionalize graphene oxide NPs which then were sprayed to suppress *Xanthomonas perforans* on tomatoes. In the current report, the mechanism of disease suppression is not known. There could be an induced resistance stimulated by Ag amendment, although the physiological basis for this effect is not known. Future root physiological and transcriptomic analyses are needed to validate the hypothesis

that NPs Ag can induce resistance. The detection of Ag in the root (experiment 4) may suggest some host defense mechanism could be activated.

Ce, a nonessential element, has recently received attention as health promoting element in plants when applied in nano form (Adisa et al, 2018; Rico et al. 2013). Our findings report NPs $CeO_2$ decreased root rot in only one of two trials. However, Adisa et al. (2018) first showed that NPs $CeO_2$ was suppressive to Fusarium wilt of tomato, was increased chlorophyll, lycopene, catalase, peroxidase, polyphenol oxidase, fruit production, and total biomass when compared to untreated plants or to Ce acetate. It is unclear if what use NPs $CeO_2$ may have in disease management platforms, but additional research is certainly warranted.

In both repetitions of the experiment, we found the dried mycelial biomass of *F. virguliforme* was relatively unaffected at the 100 and 1,000 µg/ml concentrations for NPs of B, slightly inhibited by NPs of CuO, but actually stimulated by NPs of MnO. The greatest inhibition was observed with NPs ZnO at the highest level where no fungal growth occurred. Others have also found that NPs of ZnO were inhibitory to *F. graminearum* (Dimkpa et al. 2013), and to *Botrytis cinerea* and *Penicillium expansum* (He et al. 2011). Elmer and White (2016) incorporated NPs of CuO, MnO and ZnO into 25% potato dextrose agar and also found that Zn was inhibitory to the radial expansion of *F. oxysporum* f. sp. *lycopersici*. In that study and the current one, NPs MnO were stimulatory to *Fusarium.* Given that NPs of CuO were not toxic up to 1,000 µg/ml, it is unlikely there was direct fungicidal effect on the pathogen. Indirect effects through increased host defense are more likely.

Fertilization and plant nutrition are often overlooked as components of disease suppression (Elmer and Datnoff 2014). This oversight may result from reports where the addition of micronutrients in the absence of disease do not increase yield (Sutradhar et al. 2017). As a result, the use of nutrition to influence plant disease is significantly underutilized as a disease management strategy. Considering all the studies herein, the positive effects of NPs of the essential micronutrients Cu and Zn hold promise in the suppression of soybean SDS more than $MnO_2$ or $CeO_2$. NPs of Ag and B need more study, but may have potential. Future studies are currently designed to explore more tunable forms and shapes of many different nano-elements. Field studies are planned to determine the role of these NPs on enhancing yield. Although formulation and delivery of NPs will require considerable attention by the chemical industry before wide-scale acceptance by soybean growers, it has become increasingly clear that the role of NPs in plant health has great potential as a new tool for US growers as foliarly applied nanofertilizers. Simple applications could increase yield without

increasing dependence for more chemical inputs, water use, or tillable land (Elmer and White 2018).

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**Table 1.** Foliar and root levels of Cu, Mn and Zn levels (µg/g tissue) for soybean plants treated with nanoparticle (NP) or bulk suspensions of CuO, MnO, or ZnO (Experiment 2).

| | Above ground | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Nanoparticle | Infested | | | | Not Infested | | | |
| | B | Cu | Mn | Zn | B | Cu | Mn | Zn |
| Control | 49.3 a | 4.4 a | 84.0 a | 122.7 a | 37.5 a | 4.1 a | 107.3 a | 117.1 a |
| B | 147.5 b | 3.9 a | 75.7 a | 107.2 a | 68.1 b | 4.1 a | 95.9 a | 133.1 a |
| CuO | 63.0 a | 43.2 b | 125.9 a | 75.1 a | 36.5 a | 26.4 b | 113.1 a | 128.3 a |
| MnO | 81.1 a | 4.1 a | 122.3 a | 114.9 a | 36.7 a | 4.0 a | 110.0 a | 124.7 a |
| ZnO | 49.1 a | 4.0 a | 72.7 a | 202.4 a | 38.0 a | 4.3 a | 116.0 a | 172.0 a |
| | | | | | | | | |
| | Roots | | | | | | | |
| Nanoparticle | Infested | | | | Not Infested | | | |
| | B | Cu | Mn | Zn | B | Cu | Mn | Zn |
| Control | 12.4 a | 18.6 a | 75.4 a | 271.5 a | 12.9 a | 13.2 a | 53.9 a | 123.0 a |
| B | 14.9 a | 18.7 a | 38.0 a | 122.1 a | 17.0 a | 15.6 ab | 42.3 a | 151.5 a |
| CuO | 14.4 a | 24.2 b | 46.8 a | 141.1 a | 14.9 a | 18.0 b | 63.2 a | 147.9 a |
| MnO | 15.5 a | 19.8 ab | 48.9 a | 140.1 | 16.0 a | 16.5 ab | 40.0 a | 148.2 a |
| ZnO | 13.5 a | 21.5 ab | 33.1 a | 123.7 | 14.0 a | 13.4 a | 66.6 a | 131.0 a |

| ANOVA | | | | | |
|---|---|---|---|---|---|
| Source (*P*) | Df | B | Cu | Mn | Zn |
| NP | 4 | 0.001 | <0.001 | 0.378 | 0.722 |
| Infestation (I) | 1 | 0.002 | 0.051 | 0.295 | 0.312 |
| Tissue (T) | 1 | <0.001 | 0.001 | <0.001 | 0.953 |
| NP x I | 4 | 0.246 | 0.573 | 0.383 | 0.570 |
| NP x T | 4 | 0.002 | <0.001 | 0.522 | 0.357 |
| T x I | 1 | 0.678 | 0.001 | 0.640 | 0.794 |
| NP x T x I | 4 | 0.215 | 0.698 | 0.700 | 0.449 |

Nanoparticles (500 µg/ml) were foliarly applied in distilled water with Regulaid surfactant (1ml/l)

[y] Soil was infested with a ground millet colonized by *Fusarium virguliforme* at 2 g/liter potting mix

[z] Values represent the means of four composited samples (two replicates); values followed by differing letters are significantly different according the Tukey;s Honest Significant Difference test.

**Table 2**. Foliar and root levels of Cu, Mn and Zn levels (µg/g tissue) for soybean plants treated with nanoparticle (NP) of CuO, MnO, or ZnO (Experiment 3)

| | Above ground tissue (µg/g) | | | | | |
|---|---|---|---|---|---|---|
| | Infested | | | Not infested | | |
| Nanoparticle | Cu | Mn | Zn | Cu | Mn | Zn |
| Untreated | 5.9 a[x] | 92.3 a | 144.7 a | 8.55 a | 114.1 a | 101.9 a |
| CuO | 133.5 b | 115.9 a | 94.5 a | 69.6 b | 182.0 b | 101.8 a |
| MnO | 9.2 a | 236.4 b | 85.9 a | 8.17 a | 228.6 c | 80.8 a |
| ZnO | 5.7 a | 81.0 a | 197.2 a | 8.42 a | 94.5 ab | 209.0 b |
| | Roots (µg/g) | | | | | |
| | Infested | | | Not infested | | |
| Untreated | 11.2 a | 108.4 a | 108.0 ab | 12.2 a | 121.0 a | 65.2 a |
| CuO | 73.6 b | 116.8 a | 75.9 a | 15.2 a | 145.7 a | 68.3 a |
| MnO | 11.7 a | 166.4 b | 80.2 a | 11.3 a | 131.8 a | 66.3 a |
| ZnO | 9.6 a | 92.1 a | 129.7 b[y] | 10.2 a | 136.1 a | 73.1 a |
| | | | | | | |

| ANOVA | | | | |
|---|---|---|---|---|
| Source (*P*) | Df | Cu | Mn | Zn |
| NP | 3 | <0.001 | <0.001 | 0.005 |
| Infestation (I) | 1 | 0.886 | 0.099 | 0.929 |
| Tissue (T) | 1 | <0.001 | 0.142 | <0.001 |
| NP x I | 3 | 0.997 | 0.804 | 0.622 |
| NP x T | 3 | <0.001 | <0.001 | 0.006 |
| T x I | 1 | 0.718 | 0.661 | 0.933 |
| NP x T x I | 3 | 0.987 | 0.666 | 0.683 |

[x]Values for foliar or roots represent the mean of three replicates composed of bulked foliage and root samples from replicates 1 and 2, 3 and 4, and 5 and 6; values boldfaced are significantly different according to Tukey Honest Significant difference test at $P = 0.05$.
[y] was statistically different from the untreated control at $P = 0.081$

**Table 3**. The effect of nanoparticles (NPs) of essential micronutrients and non-essential metallic oxides on the fresh weights and percent root rot ratings of soybean grown in soil with and without *Fusarium virguliforme* (experiments 4 and 5).

| | **Experiment 4** | | | **Experiment 5** | | |
|---|---|---|---|---|---|---|
| | Fresh weight (g) | | | Fresh weight (g) | | |
| Nanoparticle | Noninfested soil | Infested soil | % Root rot severity | Noninfested soil | Infested soil | % Root rot severity |
| Untreated | 25.3 a[w] | 24.8 a | 73 b | 23.4 a[x] | 16.5 a | 54 a |
| Ag | 31.5 ab | 28.0 ab | 54 d[y] | 24.6 a | 15.1 a | 41 b |
| B | 33.9 b | 32.4 b | 86 a | 22.9 a | 15.9 a | 32 b |
| Ce | 34.7 b | 29.6 ab | 78 b | 23.4 a | 17.9 a | 43 b |
| Cu | 32.7 b | 28.7 a | 66 c | 26.9 a | 13.7 a | 43 b |
| Mn | 33.3 b | 28.7 a | 67 c | --[z] | -- | -- |
| Zn | 32.8 b | 29.0 ab | 81 a | 24.2 a | 15.9 a | 35 b |

[w] values represent the mean of six replicates; values followed by different letters are significant different according to the Wilcoxon Rank test at P = 0.05;

[x] values represent the mean of nine replicates; values followed by different letters are significant different according to the Wilcoxon Rank test at *P* = 0.05.

[y] bold face values represent values that were significantly different from their respective controls in both repetitions of the experiment (experiment 4 and experiment 5)

[z] Mn treatment was not done

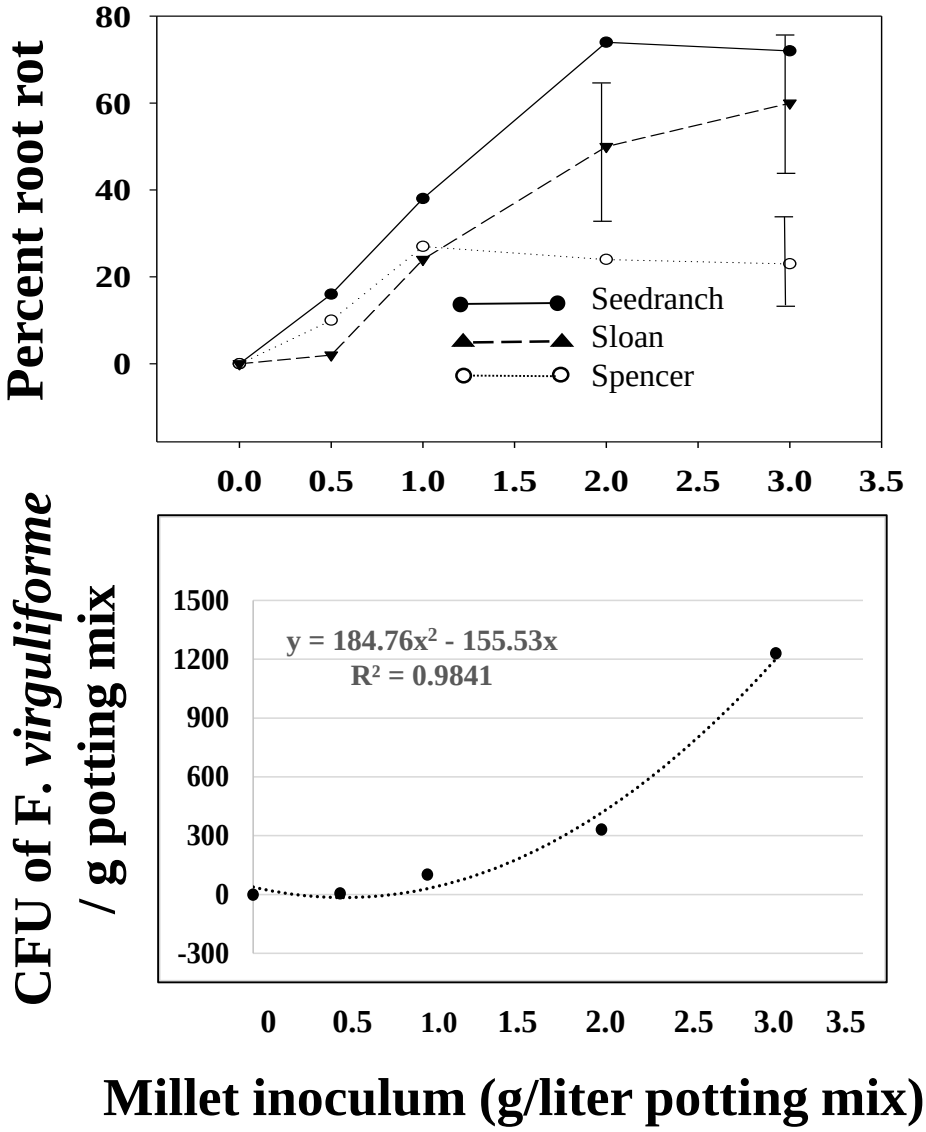


Figure 1. (Upper panel) The effect of increasing rates of millet inoculum of *Fusarium virguliforme* on the percent root of three soybean cultivars, Seedranch, Sloan, and Spencer; values represent the mean of six replicates error bars represent the standard error of the mean; (Lower panel) the effect of increasing rates of millet inoculum of the recovery *Fusarium virguliforme* following serial dilutions on agar; values represent the mean of four replicates.

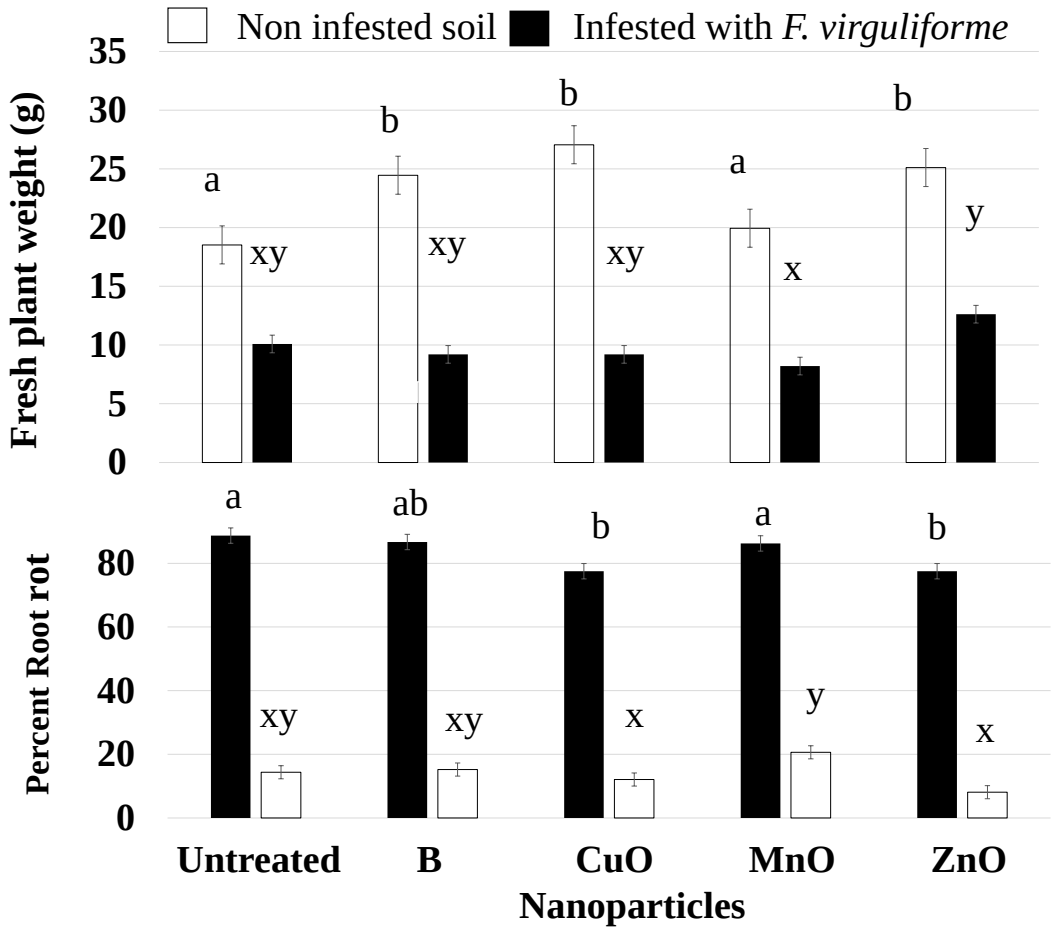


Figure 2. The effect of foliarly applied nanoparticle of B, CuO, MnO or ZnO (500 µg/ml) on the fresh weights (g) (upper pannel) and percent root rot (bottom panel) of soybean plants grown in potting mix infested with *Fusarium virguliforme* or not infested.

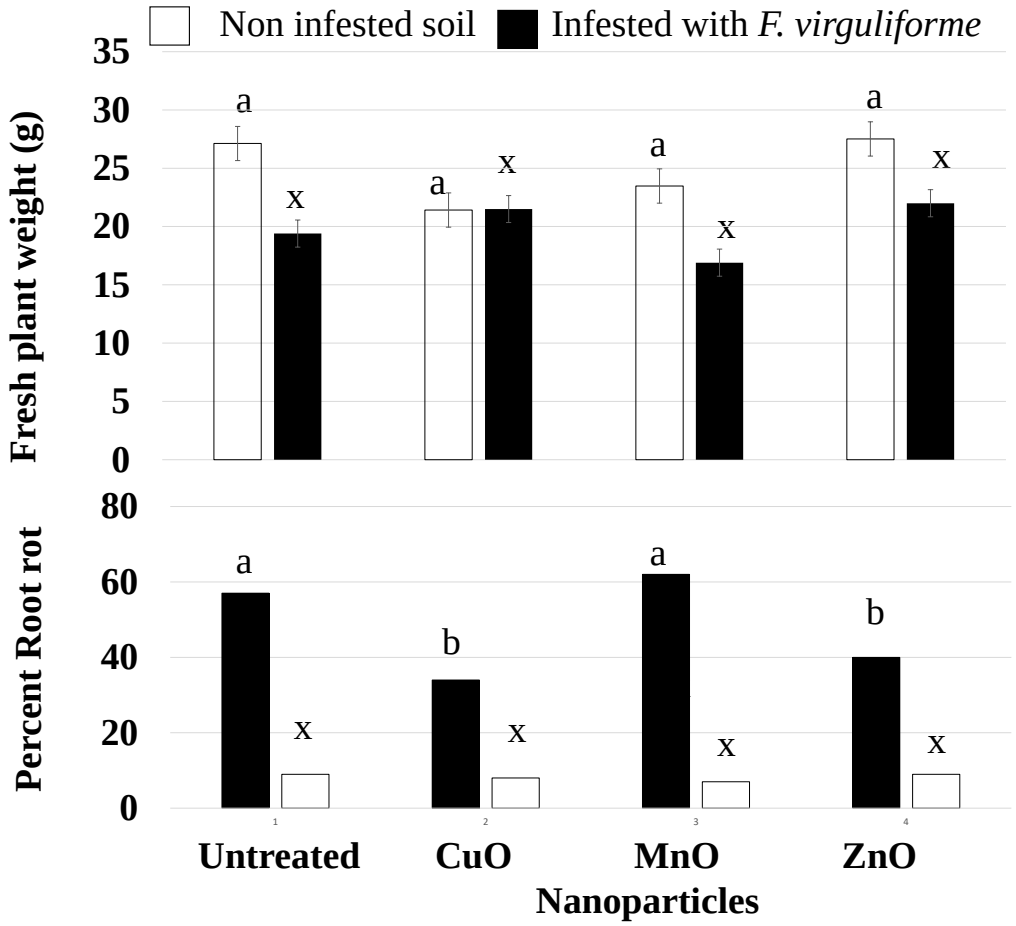


Figure 3 The effect of foliarly applied nanoparticle of CuO, MnO or ZnO (500 µg/ml) on the fresh weights (g) (upper pannel) and percent root rot (bottom panel) of soybean plants grown in potting mix infested with *Fusarium virguliforme* or not infested.

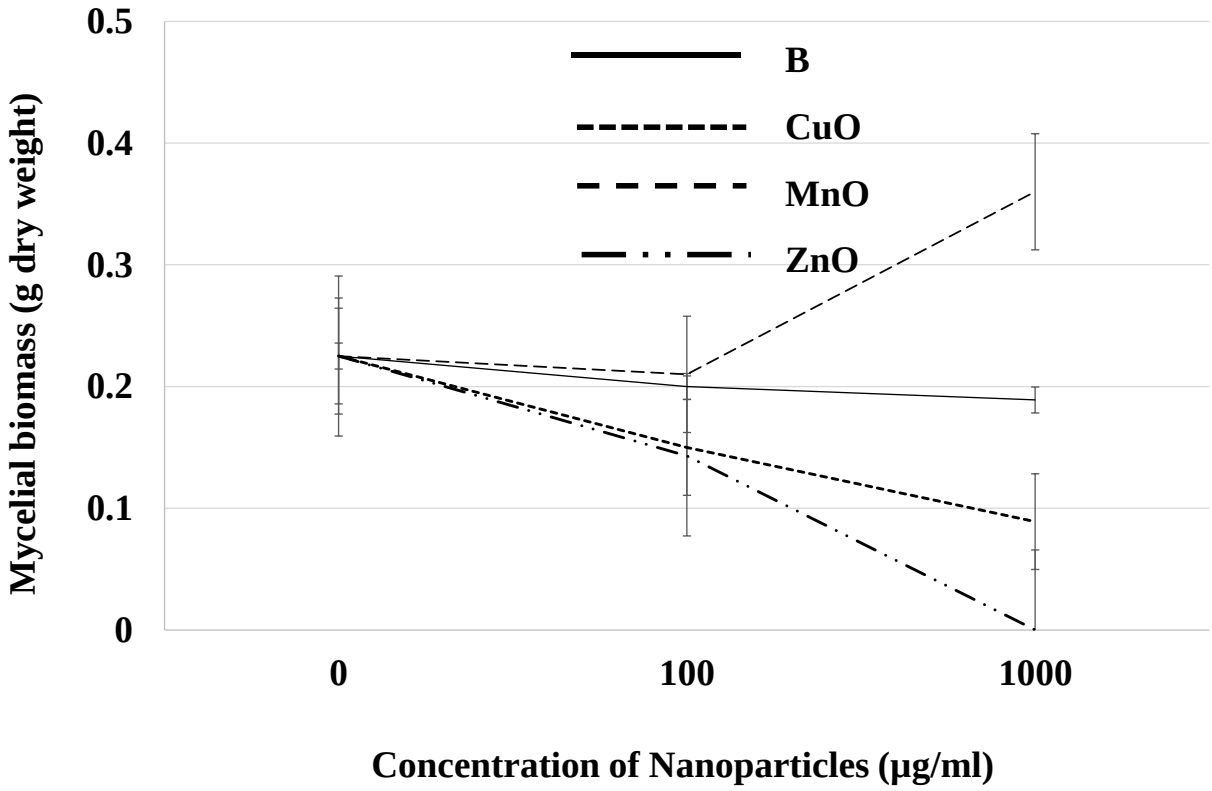


Figure 4. The effect of rate of B, CuO, MnO, and ZnO nanoparticles on the mycelial biomass of *Fusarium virguliforme;* each point represents the mean of six replicates. Error bars represent the standard error of the mean.

ARTIGO 3 **Suppression of coffee rust with metalloid and metal oxide nanoparticles**

**C. D. P. Pérez[1], E. A. Pozza[1], A. A. A. Pozza[2], W. Elmer[3], A. B. Pereira[1], D. Guimarães[1], A. C. A. Monteiro[1], M. L. V. Rezende[1].**

✉**Edson Ampélio Pozza**
edsonpozza@gmail.com

[1] Department of Fitopatologia, Federal University of Lavras, Lavras, Minas Gerais 37200-000, Brazil

[2] Department of Ciência do Solo, Federal University of Lavras, Lavras, Minas Gerais 37200-000, Brazil

[3] Department of Plant Pathology and Ecology, The Connecticut Agricultural Experiment Station (CAES) — New Haven 06504, United States.

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[1]Departamento de Fitopatologia, Universidade Federal de Lavras, 37200-000, Lavras, MG, Brasil.

**Abstract**

The use of balanced nutritions should be considered as a priority plan in the management of diseases, but achieving balance of micronutrients can be difficult. Nanomaterials are an efficient way to deliver micronutrients compared to conventional forms. In the present research, one *In vitro* experiment and three *In planta* -experiments were carried out with the objective of verifying the effect of nanoparticles of metal oxides and metalloids in reducing the spore germination of *Hemileia vastatrix* and the severity of coffee rust. The first *In planta* trial consisted of the selection of promising nanoparticles. Experiment two had the objective of evaluating the effect of nanoparticles compared to their respective bulk and sulphides. For the third experiment we compared different sources of copper in the management of coffee rust. In the *In vitro* assay, spore germination was reduced by all nanoparticles from 100 to 1000 mg $L^{-1}$. Nonetheless, silver, copper, zinc, manganese and molybdenum were able to reduce germination by less than 50% with the lowest dose. In the first *In planta* trial, reductions in disease severity were observed with the application of silver nanoparticles (93%) and, zinc (75%) when, compared to the control (water) treatment. In the second experiment, a reduction of AUDPC was observed in 56 and 41% when applied nanoparticles of manganese and zinc, respectively. In the third *In planta* trial, copper (500mg $L^{-1}$) was applied as NP of copper oxide, copper oxychloride, EDTA copper chelate, and as copper hydroxide (Kocide®) along with a untreated control and a conventional fungicide. The nanoparticle form of CuO was able to reduce AUDPC by 79,1%. Nanomaterials of Ag, Cu, Mn and Zn may have great potencial in disease management of coffee rust.

## 1 Introduction

Resistance to plant diseases is governed by host genetics. However, resistant cultivars could be less productive compared to the susceptible ones, requiring the fungicides spraying to diseases control. One strategy to reduce the use of fungicides and strengthen the defense barriers of these cultivars is through of managing plant nutrition (Roemheld and Marschner 1991). Micronutrients, such as B, Zn, Cu and Mn are responsible for activating plant defense responses, highlighting the production of phenols, lignins, quinones and tannins, flavonoids, among other secondary metabolites (Graham and Webb 1991). Another potential alternative for disease management is the application elements such as Ni, Ti, Al, Ce, Mo, and Ag. The role that nonessential inorganic compounds may play in the activation of host defense mechanisms remains largely unexplored.

Although the bulks of these elements have already been successfully tested for their ability to reduce diseases in different crops, several well-characterized bulk materials have been found showing outstanding results in the suppression of plant diseases when studied at the nanoscale. The use of nanoscale forms is a technology adopted by modern agriculture to make the concept of precision agriculture a reality. In comparison to bulk materials, nanoparticles hold great promise regarding their application in plant protection and nutrition due to their size-dependent qualities high surface-to-volume ratio and unique optical properties. (Pomastowski et al. 2017). Nanotechnology employs nanoparticles (NPs) having one or more dimensions in the order of 100 nm or less (Auffan et al. 2009). NPs offers an approach for plant disease management due to their strong antimicrobial properties (Navrotsky 2000).

The advantage of using nanoparticles is that they can inhibit the action of pathogenic fungi when used in small concentrations through fungicidal and nutritional mechanisms. Moreover, these are durable and show great selectivity and heat resistance. For instance, Elmer and White (2016) observed that CuO, MnO, or ZnO NPs (1000 mg $L^{-1}$) reduced the area under the disease progress curve (AUDPC) of tomato plants grown in soil with *Fusarium oxysporum* f. sp. *lycopersici* by 31, 28, or 28%, respectively, when compared to untreated controls.

Several reports have revealed enhanced effectiveness toward plant pathogens as a function of non-essential element nanoparticles. Jo et al. (2011) showed that Ag NPs (≥50 and 100 mg $L^{-1}$, respectively) significantly affected in vitro colony formation and disease progress of plant-pathogenic fungi *Magnaporthe grisea* and *Bipolaris sorokiniana*.

$CeO_2$ NPs mycosynthesized using fungal plant pathogen *Curvularia lunata* also showed antibacterial activity against three gram positive (*Staphylococcus aureus, Streptococcus pneumoniae* and *Bacillus subtilis*) and gram negative (*Pseudomonas aeruginosa, Proteus vulgaris* and *Klebsiella pneumoniae*) bacterial pathogens using three different concentrations 10, 50 and 100 mg $L^{-1}$ of $CeO_2$ NPs (Munusamy et al 2014). On the other side, $TiO_2$ NPs (32.58 nm) from the aqueous leaf extract of *Psidium guajava* were tested against bacteria *Aeromonas hydrophila*, *Proteus mirabilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The maximum zone of inhibition was observed against *S. aureus* and *E. coli* when $TiO_2$ NPs were used at 20 mg $mL^{-1}$ concentration (Santhoshkumar et al. 2014). However, there are few reports in the literature showing effects in the suppression of pathogens when applied nanoparticles of Ni, Al and Mo.

Coffee rust caused by *Hemileia vastatrix* is present in every coffee-growing region in the world. Between 2011 to 2014, coffee production in Central America fell 17%, from 20,2 million bags in 2011 to 16,8 million bags in 2014. Consequently, coffee prices collapsed by half. The International Coffee Organization estimates that the rust disease has cost Central America more than $616 million (International Coffee Organization 2014). These crises contributed to food stress and food insecurity in parts of Guatemala and Honduras. A deeper humanitarian crisis was forestalled by action from the World Food Program, international agencies, and smaller nongovernmental organizations (Avelino et al. 2015).

Among the measures available for coffee leaf rust control, the most widely used is the application of protectant and systemic fungicides (Pozza et al. 2010; Capucho et al. 2013). Nonetheless, the challenge for contemporary coffee researchers will be to develop strategies to control coffee rust that reflect the volatile economic and ecological conditions of the early $21^{st}$ century. Nanotechnology, by virtue of nanomaterial related properties, has potential agro-biotechnological applications for alleviation of these problems.

Then, the objectives for the present study were to determine *in vitro* the inhibition of germination from various NPs of Ag, B, CuO, $CeO_2$, MnO, $TiO_2$, ZnO, AlO, and $MoO_3$ against *H. vastatrix* and *in planta* on coffee seedlings affected by the coffee rust. More specifically, we determined effects on the host, disease severity, growth, lignin and total soluble phenol. We also determined element tissue levels.

## 2 Materials and methods

### 2.1 Nanoparticles and plant materials.

NPs of Ag (20 nm), B (100 nm), CuO (40 nm), $CeO_2$ (25 nm), MnO (30 nm), $TiO_2$ (amorphorous) (10-25 nm), ZnO (10-30 nm), AlO (20 nm) and $MoO_3$ (13-80 nm) were obtained from US Research Nanomaterials Inc. (Houston, TX). An initial stock solution at 1000 mg $L^{-1}$ of all the nanoparticles was prepared. All solutions were stored at 4°C until use. The working concentration of NPs (500 mg $L^{-1}$) were prepared by diluting the original stock solutions. Bulk oxide equivalents were obtained from Merck® (São Paulo, Brazil). Bulked suspension were applied at the same concentrations as the respective NP. Sulfate salts were adjusted so the respective metal concentration was constant. The fungicide a.i. Epoxiconazole + Pyraclostrobin (3,75 ml $L^{-1}$) was used as positive control and distilled water was used as negative control.

### 2.2 Inoculum and viability.

To obtain the inoculum, urediniospores of *H. vastatrix* from naturally infected leaves were collected from coffee plants in the field, and their viability was assessed before the plants were inoculated. For this purpose, a total of 125µl of a suspension of urediniospores was transferred to five Petri dishes containing a potato dextrose agar media. The suspension was homogeneously distributed on each dish using a Drigalski glass stick. Petri dishes were incubated in complete darkness at 23°C. After 16h, lactophenol was added to the plates to stop urediniospores germination. Two hundred urediniospores were randomly examined from each Petri dish under a microscope (Carl Zeiss Axio Imager A1, Berlin, Germany) at 400x magnification. A urediniospore was considered to be germinated when the germ tube was longer than its diameter (Capucho et al. 2009).

### 2.3 *In vitro* assessment of NP on Hemileia vastatrix.

To evaluate the toxicity on *H. vastatrix* germination, NP of Ag, B, $CeO_2$, CuO, MnO, $MoO_3$, NiO, AlO, $TiO_2$, and ZnO were amended into 1.0% (w/v) agar-water medium (AW) at 100, 300, 500, 800 e 1000 mg $L^{-1}$ poured into petri dishes (6 cm diam.), and allowed to cool. After the solidification of the medium, 200 µL of the conidial suspension of the pathogen was deposited on its surface and spread with a Drigalski spatula. Next, the dishes were incubated at 23º C, in the dark for 16 hours. The experiment was conducted in a completely randomized design, with two dishes for each treatment, and each one divided into four quadrants, where

50 urediniospores per quadrant were appraised, to a total of eight replications. After incubation, germination was stopped by the addition of four drops of lactoglycerol solution and the urediniospore germination percentage appraised under a light microscope.

**2.4 *In planta* assessment of NP on coffee rust intensity.**

Three separate experiments were conducted and each was repeated. The first greenhouse trial was designed to examined NPs of the essential elements B, ZnO, MnO, CuO or $MoO_3$, along with the nonessential elements Ag, $CeO_2$, $TiO_2$, AlO, NiO on coffee growth, coffee rust and elemental composition of tops. Suspensions of NP (500 mg $L^{-1}$) were applied to seedlings coffee. There were five replicates and plants were arranged on plastic bags as a 12 NP (Untreated control, fungicide, Ag, B, $CeO_2$, CuO, MnO, $MoO_3$, NiO, AlO, $TiO_2$, and ZnO) X 2 inoculum levels (inoculated with *H. vastatrix* or not inoculated) factorial randomized complete block design. After five weeks, plants were harvested for fresh and dry weight and elemental analysis.

The second experiment was to compare NPs CuO, MnO, ZnO, or B to their larger bulk equivalents on coffee rust. Seedlings were treated with 500 mg $L^{-1}$ of NPs, larger bulks and sulfate salts. For boron only two sources were compared, being a B NP versus $H_3BO_3$ in the same concentration of metal. The experiment was arranged on a greenhouse as a randomized block design with six NPs (Untreated control, fungicide, CuO, MnO, ZnO e B); three metal form (NP x bulk form x sulfate salt), except boron (just NP versus $H_3BO_3$) and two inoculum level (inoculated with *H. vastatrix* or not). After five weeks, plants were harvested as described above.

The third experiment was carried out to compare different sources of copper. We compared CuO NP (CuO, 99%, 40nm), copper oxychloride (Recop), EDTA copper chelate (Kellus Coper) and copper hydroxide (Kocide®). The sources were standardized at 500 mg $L^{-1}$ of copper. As a positive control, a fungicide based on Epoxiconazole + Pyraclostrobin was used. The experiment was arranged as a randomized block design with six treatments (Untreated control, fungicide, CuO-NP, $Cu_2Cl(OH)_3$, Cu-EDTA and $Cu(OH)_2$) and two inoculum level (inoculated with *H. vastatrix* or not). After the end of the disease evaluations, leaves were collected for the lignin and phenol analyzes and the plants were conditioned to take to the oven, to dry and quantify the nutrients present in the aerial part.

**2.5 Obtaining seedlings and acclimatization.**

Coffee seeds of the cultivar Mundo Novo 376/4 were germinated in a moist sand bed for 60 days. After this period, the coffee plants were transplanted to plastic bags containing 0,5 kg of a mixture of soil, manure and sand (2.5 : 1.0 : 0.5 proportions). Soil pH was corrected by adding 0.75 g of dolomitic limestone to each bag at 30 days before seedling transplant. The coffee plants were fertilized at 3 days after transplanting with 25 ml of nutritive solution (Novais et al. 1991) and then again every 7 days until the end of the experiment.

When plants reached three pairs of fully open leaves, medium size plants were selected. The treatments (NPs) were applied using a hand sprayer up to the dripping point on both sides of the leaves. *H. vastatrix* was inoculated 48 h after the spraying treatments. For inoculation, urediniospores were removed from the leaves using a soft bristle brush. A suspension of urediniospores of *H. vastatrix* was prepared at a concentration of $1x10^5$ urediniospores in distilled water containing Tween 80 (0.025%). This concentration was used in all the experiments. Then, inoculated plants were held in a moist chamber (relative humidity of 95 ± 5%, 24 ± 1°C) for five days, with the aid of dark plastic bags. The greenhouse used for these experiments was maintained at 25-32°C (day) and 17-24°C (night) with natural light.

**2.6 Area under the disease progress curve.**

As symptoms of disease developed, plants were rated for severity once per week for five weeks on a scale of 1 to 6, where 1 = 0 - 3%, 2 = 3 – 6%, 3 = 6 – 12%, 4 = 12 – 25%, 5 = 25 – 50% and 6 = higher than 50% (Cunha et al 2000). The progress of disease developed was plotted over time and the area-under-the-disease-progress-curve (AUDPC) was calculated using the trapezoid rule where: $AUDPC = \Sigma(Yi + Yi)/2 \times (t_{i+1} - t_i)$, where $Y_i$ = disease rating at time $t_i$. (Shanner and Finey, 1977).

**2.7 Biochemical and nutrient leaves content.**

When disease assessments were completed, leaves were collected for the enzymatic analyzes and the plants were conditioned to take to the oven, to dry and quantify the nutrients present in the leaves.

For this purpose, a pair of leaves of the middle third, fully developed, was collected after the last assessment of disease severity. During harvest, the leaves were protected with aluminum foil, placed in a polystyrene box containing liquid nitrogen and taken to the

laboratory where the samples were stored at -80°C until the biochemical analyzes were performed. The material frozen at -80°C was macerated in liquid nitrogen with mortar and pestle until fine powder was obtained. Immediately, the samples were lyophilized for 16 hours. An aliquot of 30 mg of lyophilized material was transferred to 2 ml micro tubes, 1.5 ml of methanol added and shaken at 150 rpm for 16 hours protected from light at room temperature.

The suspension was centrifuged at 12,000 rpm for five minutes. The supernatant was transferred to a new microtube for the determination of total soluble phenols (Spanos and Wrolstad 1990), while the solid residue was used for lignin determination (Doster and Bostock, 1988). After finishing the evaluations, the plants were cut down and the leaves, stems and roots washed with distilled water, placed in paper bags and oven dried at 60ºC until reaching constant weight. The leaves of each treatment were ground and routed to determine their macro and micronutrient contents, according to methodology proposed by Malavolta et al (1997).

### 2.8 Statistical analyses

The variables AUDPC, dry plant weight, foliar nutrient content, lignin, and total soluble phenols were submitted to the Shapiro-Wilk test to verify the assumptions of analysis of variance. After verifying the homogeneity and normality, the data of these variables and the repeated experiments over time were submitted to a joint analysis to determine if there was statistical difference between them. The means of the treatments were compared by F test ($p < 0.05$) and the significant quantitative variables were submitted to regression analysis to fit linear models. The data were analyzed using the software R.

## 3 Results

No difference ($p > 0.05$) between the repeated experiments were detected over time for all variables analyzed. Thus, the results presented refer to the mean of these trials.

### 3.1 *In vitro* assessment of NP on *Hemileia vastatrix*.

There were differences ($p < 0.05$) between the concentrations for the different treatments in the *in vitro* experiment evaluating the spore germination of *H. vastatrix* (Figure 1). Nanoparticles of Ag, Zn, Mo, Ce, and Cu reduced the germination below 50% from the lowest tested concentration of 100 mg $L^{-1}$. However, 200 mg $L^{-1}$ of Ag and Mo reduced

germination of *H. vastratix* spores below 2%. When applying Ni, spore germination remained stable (77%) up to the 300 mg $L^{-1}$ dose. The mean spore germination in the control was 73,2%.

**3.2 Area under the disease progress curve.**

The first symptoms of coffee rust were observed at 38 days post inoculation, with increased disease severity over time for all three experiments (Figure 2).

The severity of the disease increased over time, varying from 0.8 to 10% depending on the element and source applied (Figures 2A, B and C), reaching a maximum of 9,98% at 66 days after inoculation experiment after evaluating 10 nanoparticles 500 mg $L^{-1}$ (Figure 2A). In general, the values of the lowest severity in this study (<4.0%) were observed in the experiment comparing the nanoparticles with their respective bulks and sulfate salts (Figure 2B). No evidence of phytotoxicity was noted in these trials.

**3.3 *In planta* assessment of NP on coffee rust intensity.**

Eight nanoparticles were effective in reducing the AUDPC coffee rust (Figure 3A), but the suppression was the greatest for silver and copper (93.0 and 75.0 %, respectively) when compared to the control. NP of Al and Ni had no effect on coffee rust reduction ($p > 0,05$). The second experiment (Figure 3B) consisted in evaluating different sources of boron, zinc, and manganese at the same concentration in reducing the disease. There was a statistical difference between nutrients and sources ($p < 0.05$) . The coffee rust was reduced by 56% and 41% with the application of NPs of Mn and Zn respectively. B (NP) did not differ statistically compared to bulks and sulfide salts. When different copper sources were tested (Figure 3C), the greatest reductions compared to the control in coffee rust severity (AUDPC) were observed with Cu NP (79.1%) and $Cu(OH)_2$ (74.2%). No coffee rust symptoms were observed with fungicide application in all three experiments.

**3.4 Biochemical and nutrient leaves content.**

In order to carry out the biochemical analysis, the promising treatments to reduce coffee rust were selected from the results of AUDPC (Table 1). Thus, for experiment 1 the four essential elements (B, Zn, Cu and Mn) and silver NP were selected. In experiment 2, NPs of Zn and Mn and for experiment 3, Cu (NP) and copper hydroxide were selected. There was a significant difference ($P < 0.05$) for the total soluble phenol and lignin variables among the treatments in the different tests conducted. In experiment 1, in plants inoculated with *H.*

*vastatrix*, a difference was observed for the concentrations of the two compounds with the zinc nanoparticle being the nutrient with the highest concentration of lignin.

For total soluble phenol, copper and boron increased the concentration of this compound by 22.61 and 27.3%, respectively, compared to the control. In experiment 2, there was a significant difference only for total soluble phenol. Both nanoparticles Mn and Zn increased the production of this compound by 3.14 and 4.26%. Finally, in experiment 3 there was difference only for lignin concentration. Treatment using CuO and fungicide were the most effective in the synthesis of this compound.

## 4 Discussion

The use of nanoparticles in the management of coffee rust was verified in the present work. Our *In vitro* test found that the application of AgNPs at the minimum tested dose of 100 mg $L^{-1}$ was sufficient to reduce the germination of *H. vastatrix* spores below 10%. The direct use of nanoparticles on the germination or sporulation of different pathogens has already been observed in *In vitro* and *In plant* experiments. Lamsal et al. (2011), evaluated the effect of different concentrations of AgNPs on reducing the severity against Powdery Mildews on cucumber (*Golovinomyces cichoracearum* and *Sphaerotheca fusca*) in-field and *In vitro* experiments. For both assays, the concentration of 100 mg $L^{-1}$ was the most effective in reducing the severity of the disease in the field and in reducing mycelial growth and conidial germination in petri dishes trials. Ali et al. (2015) also investigated the potential of AgNPs against several *Phytophthora* spp. In *In vitro* dose-response tests conducted in microtiter plates, 10 mg $L^{-1}$ of AgNPs inhibited mycelial growth of *P. parasitica, P. infestans, P. palmivora, P. cinnamoni, P. tropicalis, P. capsici and P. katsurae*. For *P. parasitica* and *P. capsici* after application of 2.1 to 8.3 mg $L^{-1}$, 100% efficiency in inhibition of mycelial growth, spore germination, germ tube efficacy, and zoospore production was observed. We considered it important to conduct future tests evaluating concentrations lower than those tested in this study in order to determine the concentration required for inhibition of 50% ($IC_{50}$) of spore germination.

Silver nanoparticles showed significant antifungal activity in both *In vitro* petri dish assays and *In planta* inoculation experiments. AgNPs may directly attach to and penetrate the cell membrane to kill spores, although penetration of AgNPs into microbial cell membranes is not completely understood. However, a previous study observed that silver nanoparticles disrupt transport systems, including ion efflux (Hwang et al. 2008). The dysfunction of ion efflux can cause rapid accumulation of silver ions, which interrupts cellular process such as

metabolism and respiration by reacting with molecules (Lamsal et al. 2011). Also, silver ions can produce reactive oxygen species via their reactions with oxygen, which are detrimental to cells, causing damage to proteins, lipids, and nucleic acids (Morones et al 2005).

Nanoparticles of zinc, manganese and copper also had a marked reduction compared to the rest tested. Several studies on petri dishes have confirmed the effect of these nanoparticles on various plant pathogens. According to He et al. (2011), concentrations higher than 250 mg $L^{-1}$ of ZnNPs can significantly inhibit the growth of *Botrytis cinerea* and *Penicillium expansum.* Kanhed et al (2014) found that CuNPs could inhibit the growth of *Phoma destructiva*, *Curvularia lunata, Alternaria alternata* and *Fusarium oxysporum*. The antimicrobial effect of manganese nanoparticles has been tested primarily on bacteria that cause disease in humans (Kunkalekar et al. 2013; Azhir et al. 2015). A greenhouse experiment carried out by Elmer and White (2016 found that, a reduction of 31, 28 and 28% in AUDPC of Verticillium wilt of eggplant resulted when seedling were exposed to 1000 mg $L^{-1}$ of Cu, Mn and Zn (NPs), respectively. In this same research was also compared the effect of the application of nanoparticles of Zn and Cu versus their respective bulks on the disease. Both nanoparticles were more effective in reducing the severity of the Verticillium wilt fungus. These results are similar to those observed in our research for coffee rust. In general, the nanoparticulate sources had better reduction compared to their respective bulks and ionic forms.

The detailed mechanism of these NPs mode of action is yet to be fully understood. Previous reports on zinc oxide nanoparticles, for example, suggests that there is a good correlation between antimicrobial efficacy and surface area of the material (Xie et al. 2011) Greater efficacy of NPs could be therefore linked to the increased bioavailability of metallic due to high surface area of nano-size oxide material (Sirelkhatim et al. 2015). On the other hand, several studies reported that integration of metal oxides nanoparticles into bacteria may induce continuous release of membrane lipids and proteins, which changes the membrane permeability of bacterial cells (Brayner et al. 2006). For fungi, it is suggested that nanoparticles may affect cellular functions and finally cause the increase of nucleic acid and carbohydrates contents. The increment of nucleic acid may due to stress response of fungal hyphae. The increase of carbohydrate may be due to the self-protecting mechanism against the NPs (Alvarez-Peral et al 2002).

Regardless of the source or size of the particle, nano, micro or milliscale micronutrients used to suppress crop disease, the relationship between nutritional status and plant disease must be explored to understand how the mineral nutrients act in defense against

the pathogens in order to activate physical or biochemical responses and thus to reinforce the horizontal resistance barriers inherent to the plant genetics. We quantified the contents of total soluble phenol and lignin to verify the relationship between the production of these compounds with the application of micronutrient nanoparticles and some non - essential elements like Ag. The differences found in the concentration of these compounds compared to inoculated plants and not with *H. vastatrix* and with their respective control allowed to verify the effect of the micronutrients (Cu, Zn and Mn - NPs) and the AgNPs as constituent of vegetal defense.

Micronutrients are critical in the defense vegetal against crop disease, with tissue infection inducing a cascade of reactions commonly resulting in the production of inhibitory secondary metabolites (Servin et al. 2015) such as those tested in the present study (total soluble phenol and lignin). Notably, these metabolites are often generated by enzymes that require activation by micronutrient cofactors. For example, Mn, Cu, and Zn enhance disease resistance by activating the host defense enzymes phenylalanine ammonia lyase and polyphenol oxidases (Huber and Thompson, 2007). Importantly, the role that nonessential inorganic compounds, such as Ag, may play in the activation of the host defense mechanism, as found in this research, however these mechanisms remain largely unexplored. Consequently, the use of nanoparticle-based micronutrient or nonessential inorganic compounds formulations may offer a highly effective novel platform for crop disease suppression and yield enhancement through more targeted and strategic nutrition-based promotion of host resistance (Servin et al. 2015).

This study demostrated a descrease in coffee rust severity and spore germination of *H. vastatrix* by using nanoparticles of Ag, Zn, Mn and Cu. Investigations aimed at discovering alternatives to chemical fungicides against plant pathogens are highly desirable. Based on this, we suggest spraying of the nanoparticles in field experiments in order to consider its use as an alternative method for the management of coffee rust in Brazil.

## 5 Conclusions

- An initial screen with 10 nanoparticles was tested. The effect of the AgNPs on the *H. vastatrix* reduced the severity of the coffee rust. There was a 92% reduction of AUDPC and inhibition of 90% in the lowest dose evaluated *In vitro* of 100mg $L^{-1}$.
- Copper, zinc and manganese nanoparticles were also effective in reducing the severity of the disease and inhibiting the germination of spores of *H. vastatrix* and the

influence of these micronutrients on the total soluble phenol and lignin biosynthesis was verified.

- Nanoparticle spraying could be considered as an eco-friendly alternative to coffee rust management.

## 6 Acknowledgements

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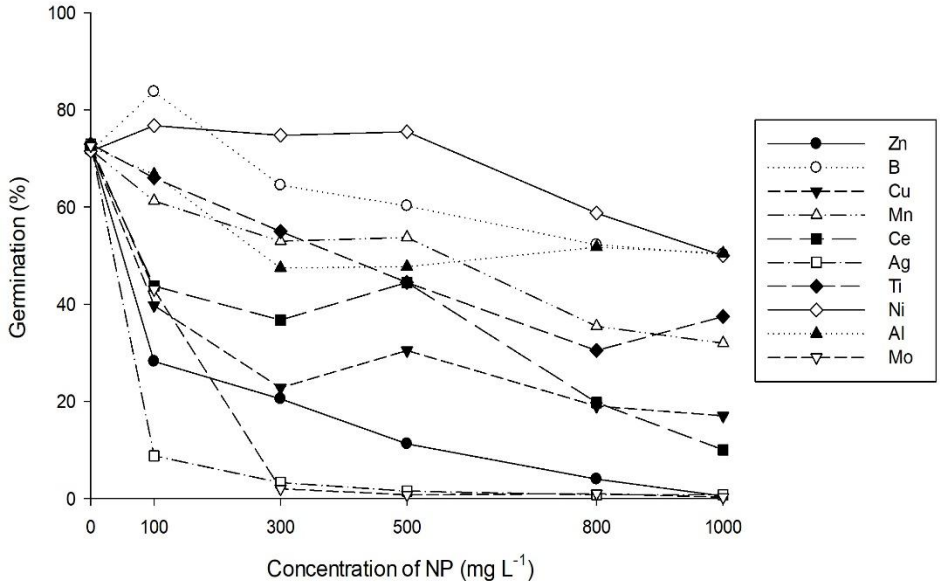


Figure 1. Germination of *Hemileia vastatrix* spores in different concentrations of metal oxide nanoparticles and metalloids of micronutrients and nonessential elements.

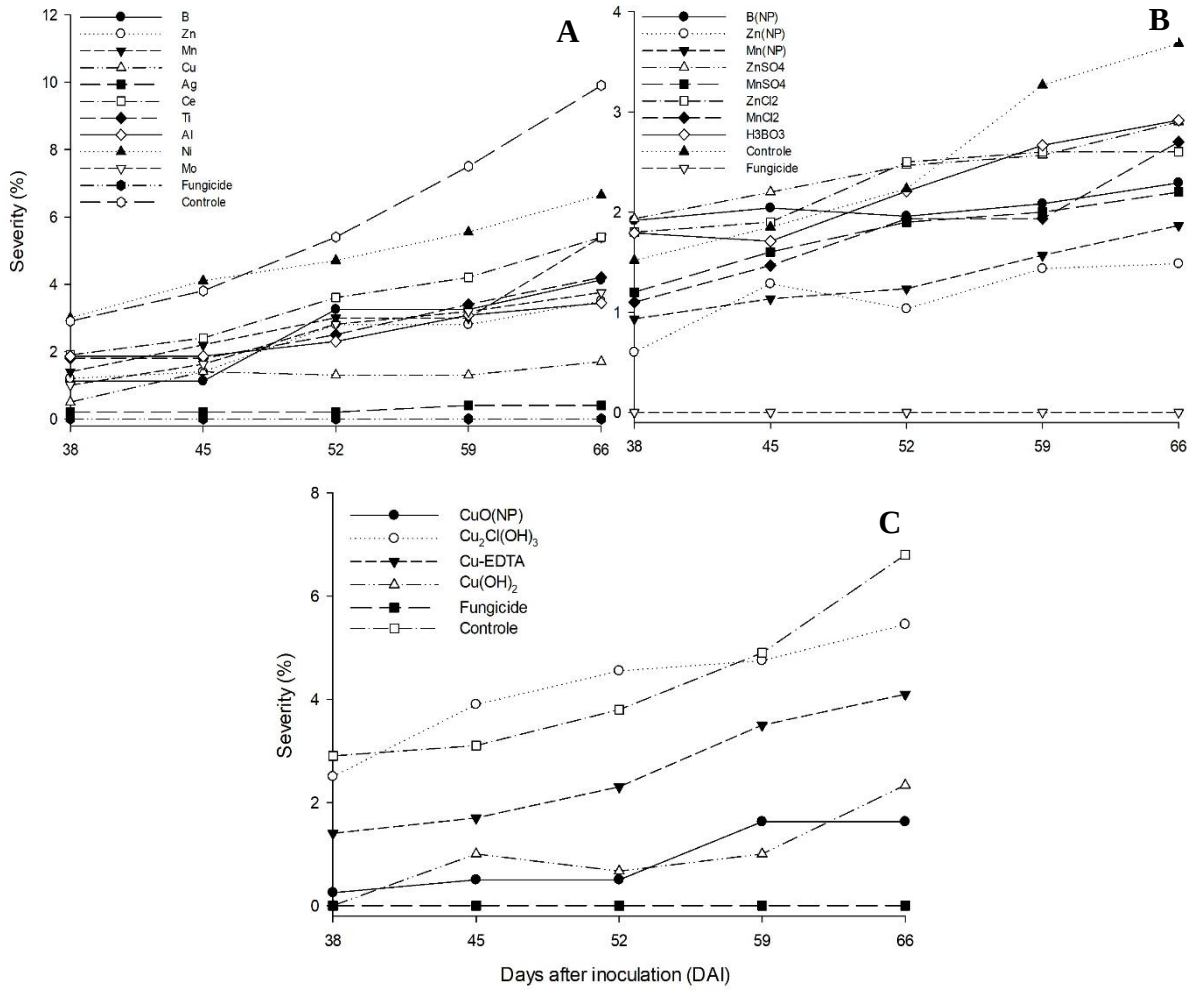


Figure 2. Curve of severity (%) of coffee rust (*Hemileia vastatrix*) over time in three experiments. 1A. Ten nanoparticles. 1B. Nanoparticles vs bulks and sulfate salts. 1C. Different sources of copper. In all experiments the concentration of nutrients was kept constant (500 mg $L^{-1}$).

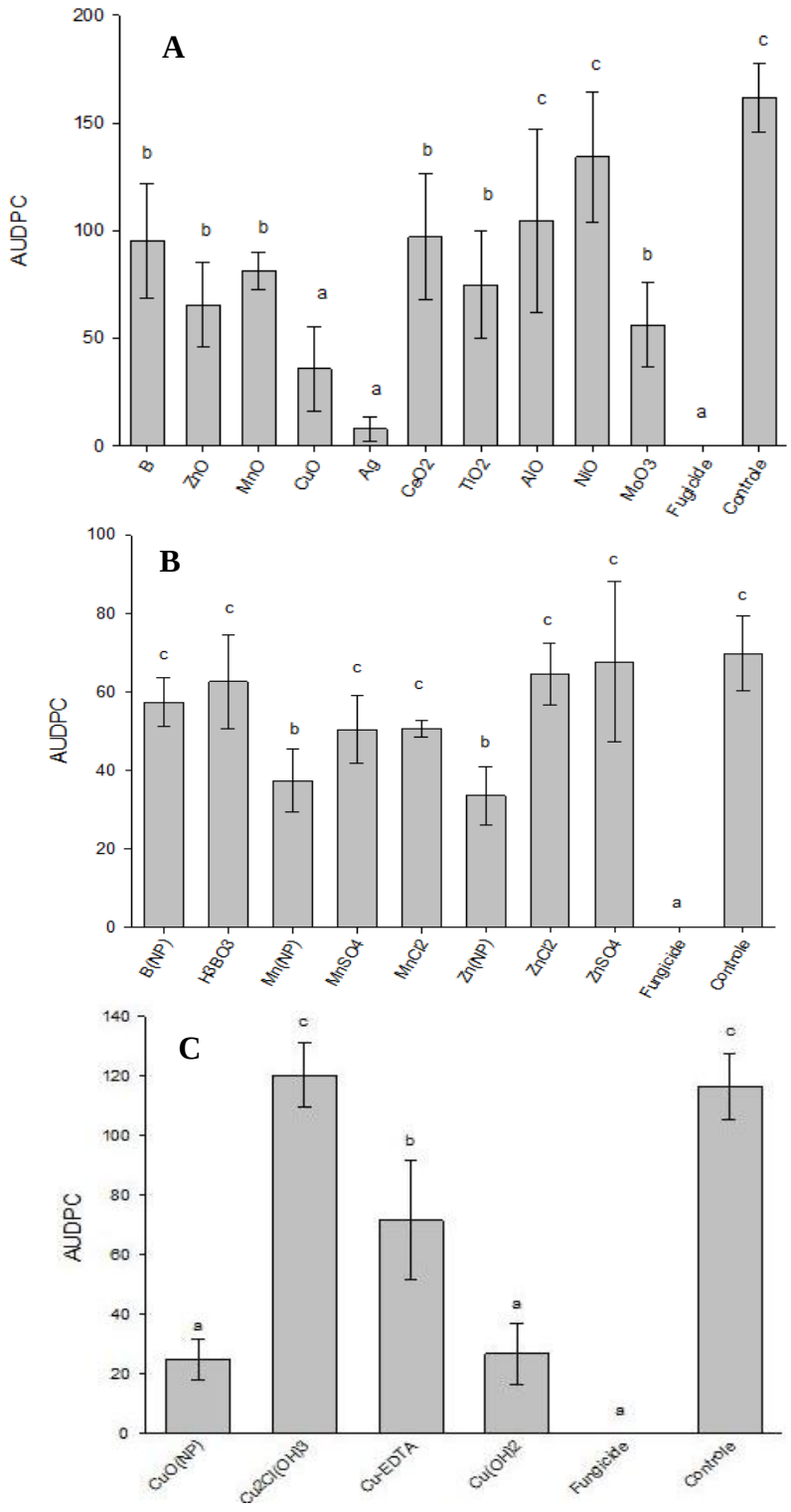


Figure 3. The area-under-the-disease-progress-curve (AUDPC) of coffee rust (*Hemileia vastatrix*) in three experiments. 1a. Ten nanoparticles. 1b. Nanoparticles vs bulks and sulfate salts. 1c. Different sources of copper. In all experiments the concentration of nutrients was kept constant (500 mg $L^{-1}$).

Table 1. Concentration of lignin and total soluble phenol (total soluble phenol) in coffee seedlings inoculated and not with *Hemileia vastatrix* and sprayed with different sources of micronutrients or nonessential elements.

| Experiment | Lignin (µg mgDM)$^{-1}$ | | Total soluble phenols (µg L$^{-1}$) | |
|---|---|---|---|---|
| | *H. vastatrix* | Not inoculated | *H. vastatrix* | Not inoculated |
| B* | 0,673 b | 0,874 c | 1,072 d | 0,864 a |
| ZnO | 1,173 d | 0,627 a | 0,892 a | 0,916 b |
| MnO | 0,728 b | 0,695 b | 0,959 b | 0,923 b |
| CuO | 0,713 b | 0,711 b | 1,037 d | 0,863 a |
| Ag | 0,939 c | 0,681 b | 0,932 b | 0,934 b |
| Fungicide | 0,935 c | 0,571 a | 0,973 c | 0,967 c |
| Control | 0,535 a | 0,559 a | 0,849 a | 0,833 a |
| ZnO** | 0,861 a | 0,561 a | 1,027 b | 0,912 a |
| MnO | 0,619 a | 0,767 a | 1,016 b | 0,964 b |
| Fungicide | 0,803 a | 0,846 a | 0,996 a | 0,908 a |
| Control | 0,665 a | 0,846 a | 0,985 a | 0,981 b |
| CuO*** | 0,761 b | 0,784 b | 0,643 a | 0,669 a |
| Kocide | 0,472 a | 0,426 a | 0,623 a | 0,579 a |
| Fungicide | 0,895 b | 0,413 a | 0,710 a | 0,699 a |
| Control | 0,591 a | 0,664 a | 0,684 a | 0,703 a |

Values followed by differing letters are significantly different by Scott-Knott´s test at P = 0.05.

* Experiment 1. NPs of essential and nonessential elements

** Experiment 2. NPs x bulk form x sulfate salt.

*** Experiment 3. Different sources of copper.