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DIRECT AND INDIRECT EFFECTS OF HUMIC SUBSTANCES ON SOYBEAN, BEAN AND MAIZE GROWTH

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Fertilidade do Solo e Nutrição de Plantas, para obtenção do título de Doutor.

Prof. Dr. Carlos Alberto Silva
Orientador

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EFEITOS DIRETOS E INDIRETOS DE SUBSTÂNCIAS HUMICAS SOBRE CRESCIMENTO DA SOJA, FEIJÃO E MILHO

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À Francisca Dantas Rosa, minha mãe, pela mãe maravilhosa que sempre foi...

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Dedico

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"Ninguém é tão sábio que não tenha algo para aprender e nem tão tolo que não tenha algo para ensinar." (Blaise Pascal)

RESUMO

As substâncias húmicas (SH) desempenham importante papel na qualidade química, física e biológica do solo. Atuam na disponibilização de nutrientes, além de desempenharem papel de bioativadoras, regulando o crescimento vegetal e processos fisiológicos e bioquímicos das plantas. Fatores como material de origem e concentração de C-SH, espécie de planta e tipo de solo, são determinantes em modular a resposta das culturas à aplicação das SH. Este estudo teve por objetivo geral avaliar os efeitos direto e indireto de diferentes fontes e concentrações de SH em plantas de soja, milho e feijão. Foram avaliadas a disponibilidade de P no solo e o crescimento de soja em amostras de Latossolo Vermelho (LV) e de Latossolo Vermelho-Amarelo (LVA) tratados com cinco concentrações de C-ácido húmico (C-AH): 0; 5; 10; 50 ou 100 mg kg^{-1}, combinadas com duas fontes de P: fosfato natural de Araxá (FA) e superfosfato simples (SS). Os tratamentos foram incubados por 15 dias e, posteriormente, determinaram-se os teores de P, C, pH e condutividade elétrica (CE) da solução do solo, e o P disponível do solo. Em seguida, a soja foi semeada e, após 41 dias de cultivo, determinaram-se massa seca de: raiz (MSR), parte aérea (MSPA), total (MST), e P disponível ao final do experimento (P-residual). O segundo e terceiro experimento foram conduzidos com milho e feijão em solução nutritiva modificada de Hoagland & Arnon (1950), com a adição de AH p.a. Acros Organics® (AHA), AH Leonardita (AHL), e SHEA-substâncias húmicas extraídas com água, nas concentrações de 2, 5, 15, 40 e 75 mg L^{-1} de C-SH, além do controle. Foram avaliados em ambos os experimentos os teores de íons, pH e CE na solução nutritiva inicial, o índice SPAD, MSPA, MSR e MST e o acúmulo de nutrientes na parte aérea. Determinaram-se os ácidos orgânicos nos exsudados de raiz de milho com adição de SHEA e no feijão, com a adição de AHL. No LV adubado com SS, a adição de C-AH aumentou o P na solução do solo em até 17%, P-resina residual em 42%, MSPA, e o acúmulo de P e N. No LVA adubado com FA, houve um aumento de 18% do P residual. Em solução nutritiva a adição de SH aumentou a disponibilidade de P, K, Zn e Fe no meio de cultivo e o índice SPAD nas folhas de milho. Dentre as fontes estudadas, a SHEA aumentou em 29% a MSPA e a exsudação de ácidos orgânicos pelas raízes de milho. Na solução nutritiva cultivada com feijão, a adição de SH aumentou as concentrações de P, K, Ca, Mg, S, Fe e Mn. O AHL aumentou o índice SPAD nas folhas de feijão e a MST em até 29%. O aumento da disponibilidade P e crescimento de soja com a aplicação de AH depende do tipo de solo; a bioatividade de SH depende da fonte, concentração de SH e da espécie cultivada, sendo o milho mais responsivo do que as leguminosas.

Palavras-chave: Ácidos orgânicos de baixa massa molar. Ácido húmico. Fósforo. Solução nutritiva.

ABSTRACT

Humic substances (HS) play an important role in the chemical, physical and biological soil quality. They increase nutrient availability, act as bioactivators, regulating crop growth and several physiological and biochemical processes of plants. Factors such as HS source and concentration, plant species, and soil type are determining factors that modulate the plant response to HS application. The aim of this study was to evaluate the direct and indirect effects of different HS sources and concentrations on growth and nutrition of soybean, maize and bean. The soil P availability and soybean growth were evaluated in samples of Red Latosol (LV) and Red-Yellow Latosol (LVA) treated with five concentrations of C-HA: 0; 5; 10; 50 or 100 mg kg^{-1}, combined with two sources of P: Araxá phosphate rock (APR) and single superphosphate (SS). The treatments were incubated during 15 days and, then, soil solution P, C, pH and electrical conductivity (EC), and soil P availability were determined. After that, the soybean was sown, and 41 days later the root dry matter (RDM), shoot dry matter (SDM), total (TDM), residual P were determined. Using a modified Hoagland & Arnon (1950) nutrient solution, one experiment with maize and another with bean were carried out. It was tested the effects of the following HS sources: HA Acros Organics® (AHA), HA Leonardite (HAL), and WEHS-water extractable humic substances at the concentrations of 2, 5, 15, 40 and 75 mg L^{-1} C-HS, besides the control. It was evaluated in both experiments nutrient contents, EC and pH in the initial nutrient solution, SPAD index, SDM, RDM, TDM, and nutrient accumulation in shoot. The organic acids were determined in the maize root exudates of plants treated with WEHS and in the bean plants under the effect of HAL. In the LV fertilized with SS, the addition of C-HA concentrations increased the soil solution P up to 17%, residual P resin by 42%, SDM, and P and N accumulation in shoot. In the LVA samples fertilized with APR, residual P increased 18% over control. The addition of HS increased the P, K, Zn and Fe in nutrient solution and the maize SPAD index. Among the sources studied, WEHS increased maize SDM by 29% and root organic acid exudation. In nutrient solution grown with bean, the addition of HS increased the concentrations of P, K, Ca, Mg, S, Fe, and Mn. Only HAL increased the bean leaf SPAD index and TDM up to 29%. The increase in P availability and soybean growth with the application of HA depends on the type of soil, and the bioactivity of HS depend on the plant species, the HS source and concentration used. In general, maize is more responsive than soybean and bean to HS addition

Keywords: Low molecular weight organic acids. Humic acid. Phosphorus. Nutrient solution.

SUMÁRIO

1   PRIMEIRA SEÇÃO

1.1   INTRODUÇÃO

As substâncias húmicas (SH) são associações supramoleculares, estabilizadas por forças dispersas fracas, de moléculas heterogêneas e relativamente pequenas derivadas da degradação e decomposição de material biológico morto (PICCOLO, 2002). Num esquema de separação meramente analítico, são classificadas pelo grau de solubilidade de acordo com o pH do meio em humina, ácidos húmicos (AH) e ácidos fúlvicos (AF). Como frações da matéria orgânica, as SH desempenham importantes funções no solo, proporcionando melhoras na parte química, física e biológica. Atuam na agregação do solo, na retenção de água, na disponibilização de nutrientes, aumentam a CTC, atuam na complexação de íons presentes na solução do solo, como reserva energética e metabólica, e atuam como moléculas orgânicas promotoras do crescimento vegetal, dentre outras funções (OLAETXEA et al., 2018; SILVA; MENDONÇA, 2007; ZANDONADI et al., 2014). Diante disso, as SH vem sendo aplicadas na agricultura com o objetivo de regular a atividade fisiológica das plantas e aumentar o rendimento e qualidade das culturas (SHAH et al., 2018).

Os efeitos do uso de SH nas plantas são classificados como diretos e indiretos. Os efeitos diretos são localizados e resultantes da interação das SH com as membranas das células radiculares ou do tecido foliar (OLAETXEA et al., 2018), com ação sobre processos bioquímicos e moleculares no tecido das plantas (SHAH et al., 2018). Por outro lado, os efeitos indiretos são dependentes da estrutura e dos grupos funcionais presentes nas SH, pois são resultantes da interação dos íons e moléculas presentes no meio de cultivo que vão influenciar a disponibilidade de nutrientes (OLAETXEA et al., 2018; SHAH et al., 2018). A complexação de micronutrientes, como Zn, Mn, Cu e Fe e de macronutrientes,

como o P, são exemplos de efeitos indiretos das SH que aumentam ou reduzem a disponibilidade e aquisição de nutrientes pelas plantas (OLAETXEA et al., 2018; ROSA; SILVA; MALUF, 2018).

Os efeitos indiretos do uso de SH podem desempenhar importante papel nos solos tropicais. Em condições naturais, os solos tropicais apresentam baixa disponibilidade de P devido ao elevado grau de intemperismo e à predominância na argila de caulinita, gibbsita, hematita e goethita, minerais com elevada capacidade de retenção de ânions, notadamente de fosfato (NOVAIS; SMYTH, 1999). Uma vez liberado na solução do solo, o P tende a precipitar com Al, Fe ou Ca ou, ainda, ser adsorvido especificamente à superfície das partículas de argila e dos óxidos de Fe e Al (NOVAIS et al., 2007). Como consequência da fixação, o P passa a fazer parte de compostos de baixa solubilidade, dificultando sua absorção pelos vegetais. Diante disso, o uso de SH nos solos tropicais pode reduzir a adsorção de P e aumentar a disponibilidade do nutriente para as plantas (MALUF et al., 2018; ROSA; SILVA; MALUF, 2018).

Dessa forma, a aplicação SH, segundo Guppy et al. (2005), pode controlar a disponibilidade de P no solo por meio da sorção competitiva entre AH e P pelos mesmos sítios de adsorção no solo, através da complexação metálica e de reações de dissolução de óxidos de Fe e Al do solo, além da sorção de compostos da MO em minerais do solo, que aumentam as cargas negativas ou reduzem o PCZ médio do solo. Esses mecanismos podem aumentar a disponibilidade de P nos solos. No entanto, as SH podem formar pontes metálicas que ligam o P a cátions de número de oxidação elevado, adsorvidos na fase sólida de solos, por isso, dependendo da estabilidade do complexo formado, reduzem a disponibilidade de P (GUPPY et al., 2005; GERKE, 2010).

Por outro lado, os efeitos diretos da SH podem resultar no crescimento vegetal (CANELLAS et al., 2018; NARDI et al., 2016; ROSA; SILVA; MALUF, 2018). Estima-se que as SH aumentam em cerca de 20% tanto o

crescimento da parte aérea quanto da raiz (ROSE et al., 2014). O maior crescimento de plantas ocorre devido ao efeito bioestimulante das SH que é resultante da interação dessas com as plantas e, ou, microrganismos que pode resultar em estímulo ou inibição de atividades fisiológicas das plantas, refletindo em inibição ou estímulo de crescimento e/ou desenvolvimento vegetal (NARDI et al., 2009; ZANDONADI et al., 2014). A bioatividade de SH tem sido atribuída ao seu comportamento semelhante ao das auxinas (NARDI et al., 2016; OLAETXEA et al., 2018; SCAGLIA et al., 2016), ou a presença de ácido indol acético (AIA) e outros hormônios em sua estrutura, e em função também do aumento da atividade da H^+-ATPase na membrana plasmática de células radiculares (CANELLAS et al., 2002; ZANDONADI et al., 2007).

Com o aumento da atividade da H^+-ATPase os transportadores de íons secundários são energizados pelo gradiente eletroquímico gerado pelas bombas de prótons, favorecendo a absorção de nutrientes pela planta (RIMA et al., 2011). Além disso, o abaixamento do pH no apoplasto gerado pela hidrólise do ATP e transporte de H^+ por unidade de ATP hidrolisada favorece a ação de enzimas que realizam a hidrólise de polissacarídeos da parede celular, permitindo a expansão celular provocada pelo aumento da pressão de turgor (RAYLE; CLELAND, 1992). Essa é a base da teoria do crescimento ácido estabelecida por Rayle e Cleland (1992), que é acionada pela presença de auxinas.

As SH também atuam na regulação das espécies reativas de oxigênio (ROS), García et al. (2016) associaram a aplicação de ácido húmico (AH) oriundos de vermicomposto à regulação do metabolismo das ROS nas raízes de arroz, indicando que a aplicação de AH protege as plantas do estresse osmótico. Os autores relataram também que a aplicação de AH aumentou a produção de enzimas envolvidas na regulação da ROS, como SOD e POX; esse aumento não está relacionado com o efeito negativo do estresse no crescimento da planta e,

sim, com o maior crescimento de raízes, ocasionado pela aplicação de AH. Segundo Berbara e García (2014), contrário ao que se pensava anteriormente, as ROS podem regular os processos de crescimento das raízes por meio de rotas independentes dos fitohormônios, como as auxinas, dessa forma, as ROS produzidas pelo NADPH oxidase estimulam o influxo de Ca^{2+} na membrana plasmática apical que está associada ao crescimento de raiz (BERBARA; GARCÍA, 2014). A aplicação de elevadas concentrações de SH, todavia, pode aumentar a taxa de produção de ROS e desencadear a peroxidação lipídica, o que afeta negativamente o crescimento e o desenvolvimento da raiz (BERBARA; GARCÍA, 2014).

Os efeitos atribuídos à bioatividade não podem ser explicados apenas pelo que foi anteriormente relatado. Os efeitos descritos com a utilização de SH são superiores aos teores de AIA presentes nos AH (NARDI et al., 2009), além disso, de acordo com Shah et al. (2018), ácido abscísico (ABA), nitrato, NO, ROS, AIA e citocininas, regulam dinamicamente os eventos fisiológicos na raiz e parte aérea das plantas, desencadeando várias vias de resposta ao uso de SH. Existem ainda outras condicionantes que regulam a bioatividade de SH. Características como estádio fisiológico, espécie e órgão da planta, fonte e concentração de SH (ZANDONADI et al., 2014, ROSE et al., 2014). Quando aplicadas além da concentração ótima, as SH podem inibir o crescimento de plantas, em função de efeitos negativos na atividade fisiológica, que podem estar relacionados à presença de auxinas que, em altas concentrações, têm efeito inibitório no crescimento de plantas, supostamente atribuído à síntese do etileno (TAIZ; ZEIGER, 2010). Em relação às espécies, em monocotiledôneas, a bioatividade das SH é maior quando comparadas aos efeitos observados em dicotiledôneas. Rose et al. (2014), em estudos de meta análise, relataram que as monocotiledôneas apresentam maior resposta em termos de crescimento da parte aérea do que as dicotiledôneas. No entanto, não existe ainda uma explicação

clara com base molecular e fisiológica para essa diferença (CANELLAS et al., 2015), sendo necessários estudos mais específicos que expliquem a magnitude da bioatividade de SH entre as diferentes espécies de planta.

Há indícios também que o uso de SH altera o perfil de metabólitos exsudados pelas raízes das plantas. Mudanças no perfil de exsudação de ácido oxálico, cítrico, tartárico, maleico e fumárico foram observadas em plântulas de milho tratadas com ácido húmico (CANELLAS et al., 2008). Puglisi et al. (2013), estudando compostos oriundos da rizodeposição e a diversidade microbiológica em rizosfera de milho tratadas com SH, identificaram, por meio espectrômetro de massa acoplado a cromatografia gasosa (GC-MS), os ácidos oxálico, succínico, málico e cítrico. Além disso, os ácidos orgânicos exsudados pelas raízes também podem alterar as estrutura de SH, resultando em subunidades de menor massa molar e mais bioativas, algumas das quais podem ter atividade hormonal (PICOLLO et al., 2002; CANELLAS et al., 2008).

Diante dos efeitos benéficos observados com a utilização de SH na produção agrícola, tem-se a necessidade de entender melhor sua biotividade, mecanismos de ação na planta e em seu meio de cultivo, e aprofundar o entendimento dos fatores em solo e planta que magnificam a resposta em solo e das culturas às SH. Com isso, será possível antever as situações de cultivo onde será maior a eficiência agronômica das SH. Os objetivos do presente trabalho foram: i) avaliar o P disponível no solo e na solução do solo, bem como o crescimento de soja cultivada em Latossolos de texturas contrastantes adubados com superfosfato simples e fosfato natural de Araxá combinados com concentrações de C-ácido húmico; ii) avaliar o crescimento de milho e feijão em solução nutritiva com a adição de diferentes fontes e concentrações de SH, definindo-se a melhor fonte e concentração para cada espécie; e iii) identificar o perfil de exsudação de ácidos orgânicos da raiz do milho e feijão sob a melhor fonte de SH.

A tese está dividida em três capítulos apresentados na forma de artigos para a publicação em revistas científicas. O primeiro capítulo intitulado *"Phosphorus availability and soybean growth in contrasting Oxisols in response to humic acid concentrations combined with phosphate sources"*, trata do uso de concentrações de C-AH combinadas com o superfosfato simples e fosfato natural de Araxá, aplicados em solos de textura média e argilosa, constituindo dois experimentos distintos. O segundo e terceiro capítulos foram intitulados, respectivamente, como: *"Bioactivity of water extractable and Leonardite-derived humic substances on maize growth"* e "*Humic substances source effects on bean growth and on its root organic acid exudation profile"*. O segundo e terceiro capítulo avaliam a bioatividade de diferentes fontes e concentrações de SH no crescimento do milho e feijão, bem como o perfil de exsudação de ácidos orgânicos da melhor fonte de SH para ambas as espécies.

1.2   CONSIDERAÇÕES GERAIS

Poucos são os estudos que avaliam os efeitos dos ácidos húmicos na disponibilidade de P nos solos, principalmente em solos de texturas contrastantes. Em geral, os estudos avaliam a adsorção de P-AH em ensaios de laboratórios conduzidos em solução de equilíbrio, o que não representa de fato o comportamento P-AH nos solos tropicais, apenas dão indícios de seu comportamento. Diante disso, o presente estudo avaliou o P na solução do solo e o P disponível do solo após a incubação de AH e fontes de P antes do cultivo da soja, e ao final foi avaliado o P residual. Os resultados do presente estudo demonstram a potencialidade do AH em aumentar a disponibilidade e aquisição de P por plantas de soja em solos argilosos quando aplicado superfosfato simples. Além disso, o uso de AH aumentou o P residual de solo de textura média tratado com fosfato de Araxá.

Além de aumentar a disponibilidade de P em solos altamente intemperizados, o presente estudo demonstrou a potencialidade de substâncias húmicas (SH) em aumentar a disponibilidade de nutrientes em solução nutritiva e o crescimento de plantas de milho cultivadas sob diferentes fontes e concentrações de SH. Ressalta-se que o uso de "substâncias húmicas extraídas com água (SHEA)" foi a fonte que propiciou o máximo crescimento de plantas de milho. Trata-se de resultado de pesquisa relevante, por ser tratar de uma fonte altamente sustentável, de baixo custo e de fácil obtenção pelos produtores agrícolas. Além disso, o estudo do perfil de exsudação de ácidos orgânicos por plantas de milho tratadas com SHEA abre novos caminhos para a compreensão dos efeitos das SH no crescimento e desenvolvimento de plantas. O perfil de exsudação de ácidos orgânicos por plantas sob efeito de SH depende da espécie cultivada. Dessa forma, a condução de experimentos semelhantes com espécies distintas expõe cenário no qual o uso de SH é mais vantajoso. Entre as culturas

investigadas, o milho foi mais responsivo ao tratamento com SH do que o feijoeiro. Apesar disso, plantas de feijão cultivadas com ácido húmico extraído de Leonardita destacaram-se pelo expressivo crescimento do sistema radicular.

Diante disso, é importante ressaltar que os estudos com SH devem ser continuamente desenvolvidos, pois, devido à ampla gama de fatores que determinam os efeitos diretos e indiretos das SH nas plantas e nos solos, e preciso delinear cenários em que o uso de SH maximizará respostas positivas no crescimento e nutrição das culturas, com retorno econômico da adição de SH para o agricultor.

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2 SEGUNDA SEÇÃO – ARTIGOS

2.1 ARTIGO 1–Phosphorus availability and soybean growth in contrasting Oxisols in response to humic acid concentrations combined with phosphate sources

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Abstract

Humic acid (HA) use can improve phosphorus (P) availability in soils with high P fixation capacity. The aim of this study was to evaluate soil P availability and soybean growth in both medium-texture (MT) and clayey (CL) Oxisols under humic acid carbon (C-HA) concentrations: 0, 5, 10, 50, and 100 mg C. soil kg^{-1}, combined with Araxá phosphate rock (APR) and single superphosphate (SS). Mixtures of C-HA with P sources were incubated for 15 days. In sequence, the soil solution was extracted and analyzed for C (SSC), P (SSP), and electrical conductivity (EC). Soil initial resin-P and pH were determined before soybean sowing. After harvest, shoot (SDM), root (RDM), total dry matter (TDM), soil residual resin-P, and soil pH were determined. In CL fertilized with SS, the addition of C-HA increased SSP by 17%, residual resin-P by 42%, SDM, P and N accumulated in shoot. Addition of C-HA reduced SSP and initial resin-P in MT treated with SS and increased in 18% residual resin-P in MT fertilized with APR. Effects of HA on soil attributes, soybean growth and nutritional status rely on concentration, soil texture and P source used.

Keywords: low-grade phosphate rock, phosphate-metal-humic complexes, phosphorus-fixing soils, residual phosphorus, soil solution.

Introduction

   Phosphorus (P) is the one of most limiting nutrient for crop production nearly everywhere on the world due to low available P contents in soil (Smit et al. 2009). In highly weathered soils, a large portion of the added P-fertilizer is adsorbed as a result of the strong interaction between P and minerals of clay

fraction, such as kaolinite, gibbsite, goethite, and hematite, which strongly affect the supply of available P to crops over time (Batjes 2011). In such environment of low P use efficiency, soil management practices and strategies should be adopted to reduce P adsorption and increase the P-fertilizer recovery by crops in tropical soils (Roy et al. 2016; Withers et al. 2018). Thus, humic material use in soils may be a suitable strategy to minimize the P sorption (Wang et al. 2016; Maluf et al. 2018a) and, consequently, to increase P availability for plants (Hua et al. 2008).

Addition of humic acid (HA) to soils controls P availability through the competitive adsorption between HA and phosphate for binding sites onto soil colloids, complexation of Fe, Al, and Ca, preventing P precipitation, HA adsorption on soil minerals decreases point of zero charge of soil colloids, use of HA promotes steric hindrance on the mineral surfaces, and reduces the specific surface area of soil iron oxides (Guppy et al. 2005; Fu et al. 2013; Wang et al. 2016; Maluf et al. 2018a). These mechanisms increase soil P availability due to the reduction of soil phosphate sorption capacity and decreased colloid surface affinity for phosphate. On the other hand, HA may decrease P availability in soil due the formation of metal bridges that increase the phosphate adsorption sites (Guppy et al. 2005; Gerke, 2010). Formation of metal bridges can also reduce P availability, depending on the chemical stability of P-cation-HA complexes formed in soil (Guppy et al. 2005; Gerke 2010; Maluf et al. 2018a).

The magnitude of these processes rely mainly on P source, soil properties, HA concentration, and plant species (Rosa et al. 2018a; Maluf et al. 2018a). Phosphate fertilizers with high P soluble in water, as single superphosphate (SS) that fast release P in soil, are more prone to be adsorbed on colloid surfaces, reducing use efficiency of P-fertilizer by crops cultivated in Oxisols (Prochnow et al. 2006). According to Erro et al. (2012), the synthesized HA-SS complexes

enhanced the phosphate fertilizer use efficiency by decreasing P sorption in the soil and improving P acquisition by wheat.

On the other hand, the combined use of HA and low-grade phosphate rock (PR) in soil may optimize P use efficiency of low-agronomic value and less soluble phosphates. Use of PR *in natura* is not suitable to nourish short cycle crops since low-grade rocks cannot meet P demand from plants in a timely manner (Batjes 2011; Shen et al. 2011). However, the combined use of HA and PR in the soil can increase P-apatite solubilization through Ca-PR chelation by HA with the formation of compounds more water-soluble, increasing P release (Singh and Amberger 1997; Pramanik et al. 2009). HA also increases soil microbial activity (Giovannini et al. 2013), root growth and organic acids exudation (Canellas et al. 2008), which changes the root-soil-rock interface through acidification of the growth medium, favoring P-PR solubilization (Rafael et al. 2018).

The role played by HA in enhancing soil P availability and decreasing P adsorption is influenced by soil properties, such as particle size distribution and mineralogy of the clay fraction (Maluf et al. 2018a), which regulate the phosphate dynamics between soil liquid and solid phases. HA acts stimulating root and shoot growth, as well as nutrient acquisition by plants (Tavares et al. 2017; Olaetxea et al. 2018). This stimulus is regulated by crop type and HA rate, with the optimal concentration to be added to soil ranging from 25 to 750 mg kg^{-1} (HA soil^{-1}) (Rose et al. 2014). According to Prado et al. (2016), addition to the soil of 228 mg dm^{-3} P-fertilizer enriched with humic substances increased nutrient uptake, growth, and grain yield of soybean over control.

HA use in soils, thus, may be important to improve efficiency and sustainability of P management in tropical agriculture, crop growth, and P uptake by plants, especially taking into account that phosphate reservoirs are not renewable. However, little is known about the efficiency of HA application in

increasing the P availability in tropical soils, mainly in those with contrasting texture, neither the effect of HA on the growth and nutrient uptake by soybean. Therefore, the hypotheses of this study are: i) there is an optimum humic acid carbon (C-HA) concentration that increases the soil solution P content and its availability in soil fertilized with SS; ii) the combined effect of optimal C-HA concentration with the correct P source increases soybean growth and nutrients accumulated in shoot; iii) there is a C-HA concentration that favors P-PR solubilization and increases soil P availability for soybean. The aims of this study were to evaluate: i) chemical composition of soil solution and available P contents in the soil, and ii) growth and P acquisition by soybean plants fertilized with SS or PR combined with C-HA concentrations in Oxisols with different clay contents.

Material and methods

Soil characterization

Two experiments were carried out simultaneously with different soils under greenhouse conditions. Clayey Oxisol (CL) samples from the surface layer (0.0-0.1 m) and medium-texture Oxisol (MT) samples from the subsurface layer (0.2-0.4 m) were collected under native vegetation in Lavras, state of Minas Gerais, Brazil. The main attributes of the soils studied are shown in Table 1.

Humic acid and phosphate source characterization

The humic acid (HA) used in the experiment was a commercial product of Sigma-Aldrich®, which was characterized in laboratory and had the following characteristics: pH in water of 9.8 (HA:water); 376 g kg^{-1} C; 7.6 g kg^{-1} N; 0.3 g kg^{-1} P; 6.3 g kg^{-1} K; 5.9 g kg^{-1} Ca; 0.3 g kg^{-1} Mg; 4.4 g kg^{-1} S; 1.1 g kg^{-1} Fe; 40 mg kg^{-1} Cu; 15 mg kg^{-1} Mn; and 22 mg kg^{-1} Zn. Spectroscopic features of the HA sample were obtained by the attenuated total reflection Fourier transform infrared (ATR-FTIR) technique, in the region from 4000 to 400 cm^{-1}. Details

about the ATR-FTIR spectrum and main chemical groups recorded in the HA sample are described in Rosa et al. (2018a).

The P sources used were single superphosphate (SS), a high water-soluble P fertilizer, and the Araxá phosphate rock (APR), a fluorapatite Brazilian low-grade phosphate rock. SS fertilizer showed pH ($CaCl_2$) equal to 2.9, 8.3% of total P, water-soluble P content of 5.2%, neutral ammonium citrate plus water (NAC+H_2O)-soluble P of 7.4%, and P soluble in 2% citric acid solution (CA) of 6.1%. The APR had pH ($CaCl_2$) of 6.9, 11.9% of total P, water-soluble P content of 0.1%, NAC+H_2O-soluble P of 0.9 % and CA-soluble P of 1.3%. P contents of both sources were determined by the *vanadomolybdophosphoric* acid colorimetric method, following the analytical protocols described in Silva (2009).

Table 1 – Physical and chemical attributes of the clayey (CL) and medium-texture (MT) Oxisols samples under natural conditions

Soil	Soil attribute											
	pH	OM	P	K	Rem-P	Ca^{2+}	Mg^{2+}	Al^{3+}	CEC	Clay	Silt	Sand
		%	\multicolumn{2}{c}{mg dm^{-3}}	mg L^{-1}	---------- cmol$_c$ dm^{-3}----------				------ g kg^{-1}------		
CL	4.8	4.6	1.3	69	15.5	0.8	0.4	1.0	11.2	565	130	305
MT	5.1	0.7	0.3	42.7	13.6	0.1	0.1	0.1	2.0	270	50	680

pH in water 1:2.5 (v/v); OM: Organic matter by the modified Walkley-Black method; Soil available K and P levels extracted by the Mehlich-1 solution; Rem-P: Remaining P; Exchangeable Ca, Mg, and Al extracted by a 1 mol L^{-1} KCl solution; Cation exchange capacity at pH 7

Experimental design and treatments

In the two experiments, the treatments consisted of a 5 x 2 factorial arrangement through the combination of five HA carbon concentrations (0, 5, 10, 50, and 100 mg kg^{-1} C-HA.soil^{-1}) with two P sources: SS and APR. A randomized block experimental design was used, with four replicates. The amount of the P added to soil, 400 mg kg^{-1}, was calculated according to soluble P content in CA for APR, and in NAC+H_2O, for SS. Phosphorus rates added to

Oxisols were based on the remaining P soil test (Rem-P) (Table1), following guidelines of P fertilization for plants grown in pots used by Alvarez et al. (2010) to reach maximum plant growth in Brazilian highly weathered soils.

In the first incubation, soil samples were incubated with $CaCO_3$ and $MgCO_3$ (at 3:1 ratio) for 20 days to neutralize acidity and increase soil base saturation to 65%; soil moisture was maintained near 70% soil water-holding capacity (WHC). In sequence, soil samples were air-dried, passed through a 4 mm mesh sieve, and was performed the second incubation for 15 days with P sources and C-HA concentrations, keeping soil moisture near 70% WHC. After the second incubation, soluble sources of macro and micronutrients were added to Oxisols samples to attend soybean nutrition requirements grown in pots, as follows: 100 mg kg^{-1} N (NH_4NO_3) and K (KCl), 30 mg kg^{-1} S (($NH_4)_2SO_4$), 1 mg kg^{-1} B (H_3BO_3), 2 mg kg^{-1} Zn ($ZnSO_4.7H_2O$), 6 mg kg^{-1} Mn ($MnCl_2.4H_2O$) and 1.5 mg kg^{-1} Cu ($CuSO_4H_2O$). Then, soil samples were air-dried, passed through a 2 mm mesh sieve and a sample was taken from each experimental pot for further analysis.

The pots were filled with 1.2 kg of CL or 1.5 kg of MT and soil solution samplers (*Suolo Acqua*®) were installed in the center of the pots, leaving about 3 cm of soil between the sampler and the pot bottom. After keeping soil moisture near 100% WHC for 8 hours, aiming at the equilibrium between soil liquid and solid phases, soil solution samples were collected with 20 mL tubes previously conditioned to ~70 kPa in a vacuum pump. A needle placed at the outer end of each *Suolo Acqua*® samplers was inserted in the rubber-sealing cap of the soil solution sampling tube. In sequence, five soybean seeds (*Glycine max*L. cv. CD 250) were sown. Ten days after sowing (DAS), thinning was performed and two soybean plants per pot were cultivated for 38 days. Topdressing fertilization was performed by the addition of 100 mg kg^{-1} N (NH_4NO_3) and 100 mg kg^{-1} K (KCl), at 19 and 32 DAS.

Soil and plant analysis

Soil solution was characterized for electrical conductivity (EC) and soil soluble carbon (SSC), which were determined in an elemental automated analyzer (Elementar, Vario TOC Cube model, Germany), using the liquid module. Soil solution P contents (SSP) was measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). After 38 DAS, soybean growth was assessed by measuring shoot dry matter (SDM) and root dry matter (RDM). Roots were manually separated from soil and throughout washed with deionized water until soil particles were completely removed. Soybean root and shoot tissues were stored in paper bags and dried in an oven for 72 hours at 70 °C. After drying, biomasses were weighed to determine SDM and RDM, and total dry matter (TDM), summing SDM and RDM.

The SDM was ground in a Willey mill, and 500 mg of plant material was digested with 8 mL of a mixture of nitric and perchloric acid at a 4:1 ratio. In sequence, the total contents of P and S in soybean shoot were determined, following the analytical protocols described in Silva (2009). Briefly, contents of P in the shoot was determined by the molybdenum blue reaction method and S through reaction with a barium chloride solution, using an UV-visible spectrophotometry at 660 nm for P, and 420 nm for S quantification. The total content of N was determined by digestion of plant tissues in acid medium and distillation through the *Kjeldahl* method, according to the protocol described in Silva (2009).

At the end of the second incubation, a soil sample was collected from each experimental pot, dried, and passed in a 2 mm mesh sieve to determine initial soil pH and soil available P contents (initial resin-P). Contents of residual resin-P and final soil pH were measured after soybean cultivation. Initial and residual resin-P were extracted through the use of a mixed ionic exchange resin, following the analytical protocol described in Raij and Quaggio (2001), and

determined in an UV-visible spectrophotometer, according to Murphy and Riley (1962).

Statistical analysis

The dataset was subjected to analysis of variance and then to regression analysis, considering the possible relationships of soil or soybean attributes with C-HA concentrations combined with P sources added to the Oxisols. The SISVAR 5.6 (Ferreira 2014) computer program was used in all statistical steps. The regression model that best fit to the dataset was chosen based on the significance of the mathematical equation parameters ($p<0.05$), on the lowest value of the sum of squared errors, and on the equation with the highest adjusted coefficient of determination (R^2). Principal component analysis (PCA) was carried out using the Stats package version 3.4.0 of the R program (R Core Team 2014) to check the existence of multiple and linear relationships among SSC, SSP, EC, SDM, accumulation of P, initial and residual resin-P, and initial and final soil pH in Oxisols fertilized with different P sources.

Results

Soil solution

In the clayey Oxisol (CL) fertilized with single superphosphate (SS), the quadratic model was the best fitted to soil solution P contents (SSP) over humic acid carbon (C-HA) concentrations. Application of 51 mg kg^{-1} C-HA combined with SS increased SSP by 17% in relation to soil not treated with C-HA (Figure 1). However, the use of HA in the medium-texture Oxisol (MT) with SS reduced SSP up to 63 mg kg^{-1} C-HA. Application of Araxá phosphate rock (APR), in both CL and MT, did not change SSP over C-HA concentrations, which showed lower contents than Oxisols fertilized with SS.

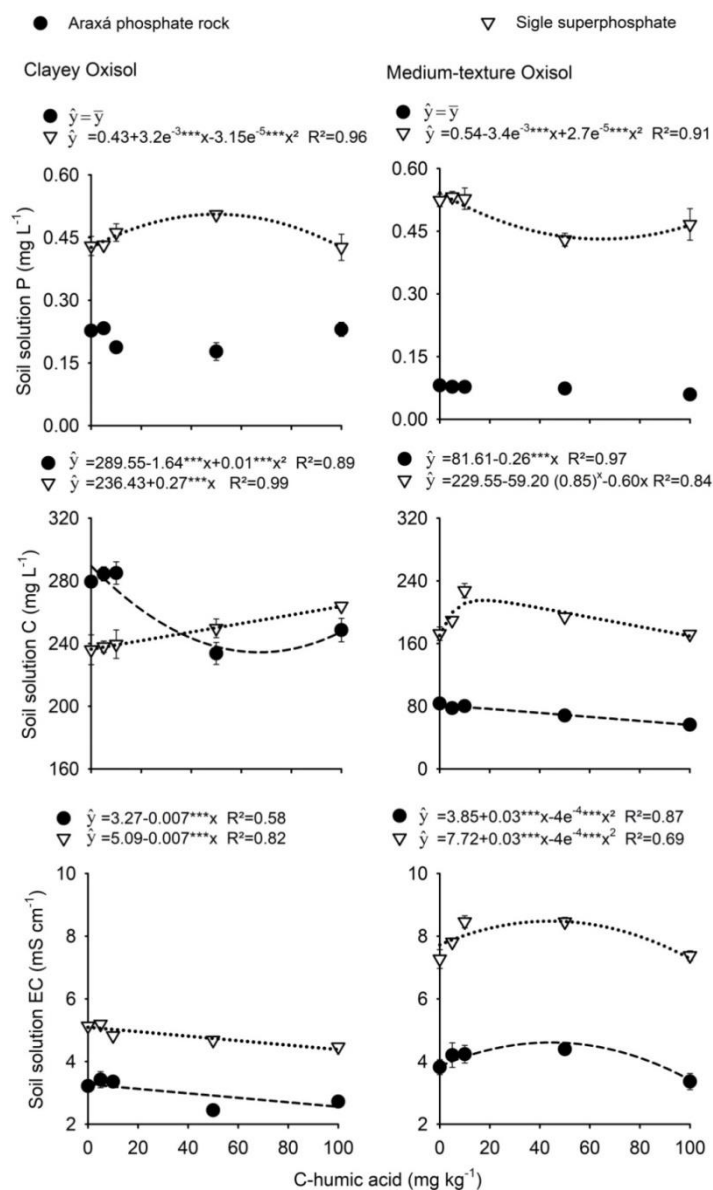**Figure 1**- Soil solution phosphorus, carbon, and electrical conductivity (EC) after treatments incubation in a clayey and medium-texture Oxisols fertilized with humic acid concentration (C-HA) and single superphosphate (SS) or Araxá phosphate rock (APR). *, ** and *** significance of the mathematical equation parameters to p<0.05, p<0.01 and p<0.001, respectively. Bars represent the standard error of the means.

Similarly to SSP, soil solution C contents (SSC) increased linearly over C-HA concentrations in CL fertilized with SS (Figure 1). The concentration of 100 mg kg^{-1} C-HA combined with SS increased by 12% SSC as compared to control. In the MT, use of SS increased SSC up to the concentration of 10 mg kg^{-1} C-HA, followed by a reduction, which can be related with SSP dynamics. When APR was applied to CL and MT samples, the quadratic and linear models were those that best fitted, respectively to each Oxisol, with a reduction of SSC over C-HA concentrations. Soil solution electrical conductivity (EC) in CL and MT was influenced by the isolated factors: C-HA concentrations and P sources ($p<0.05$; Figure 1). Thus, the use of C-HA concentrations in CL linearly reduced EC, regardless of the P source applied, while in MT, EC increased up to 42 mg kg^{-1} C-HA, followed by a decrease. In both, CL and MT fertilized with SS, EC was higher than when used APR.

Phosphorus availability and soil pH

Soil available P contents extracted by resin before soybean cultivation (initial resin-P) was not influenced by the C-HA concentrations-P source interaction in CL ($p>0.05$). Thus, when both factors were isolated, it was verified that initial resin-P contents in both Oxisols treated with SS were greater than with APR (Figure 2). Application of 100 mg kg^{-1} C-HA increased initial resin P by 19% compared to control, regardless of the P source applied. Soil P availability over treatments tested in the MT was different from those verified for CL samples (Figure 2). In the MT fertilized with SS reduced linearly initial resin-P contents over C-HA concentrations in comparison to soil without C-HA, while addition of C-HA did not change initial resin-P contents in soil fertilized with APR. The pH values were influenced only by P sources in CL ($p>0.05$) and were higher in soils treated with APR than with SS (Figure 2). In the MT, pH increased in a quadratic way over C-HA concentrations with the use of APR, reaching values near 7.0, whereas SS use did not alter the MT pH (pH 6.0) (Figure 2).
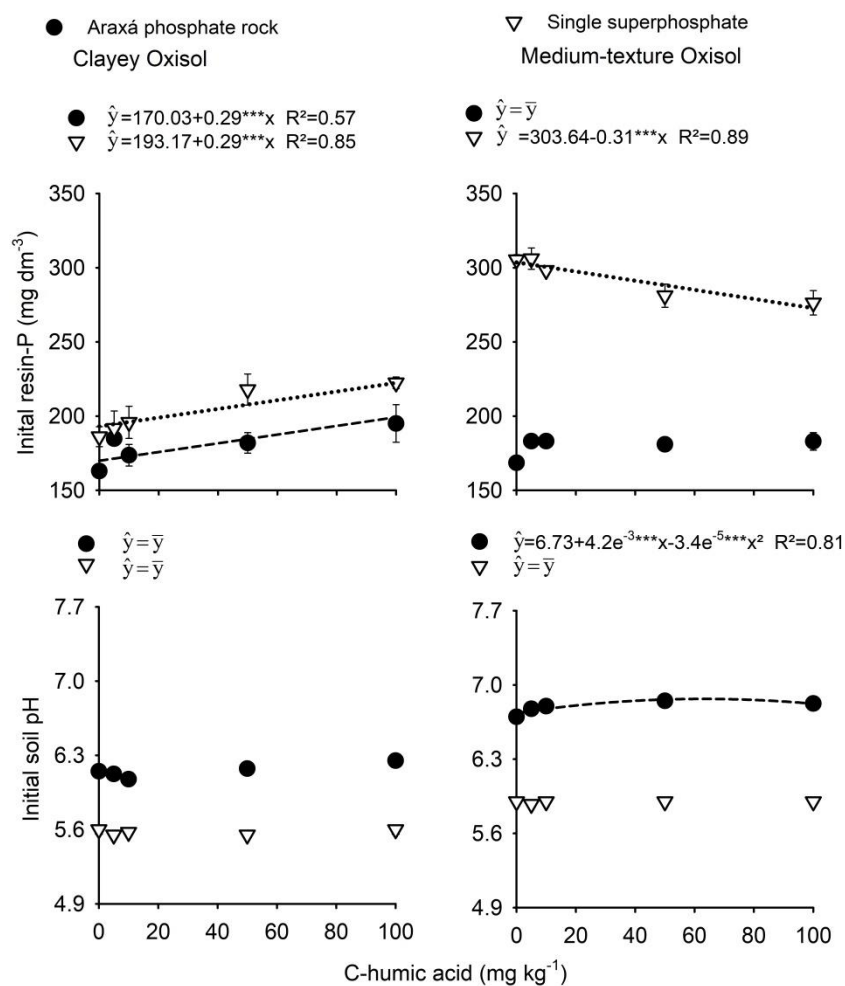
Figure 2- Initial resin-P and soil pH after treatments incubation in a clayey and medium-texture Oxisols fertilized with humic acid concentration (C-HA) and single superphosphate (SS) or Araxá phosphate rock (APR). *, ** and *** significance of the mathematical equation parameters to $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bars represent the standard error of the means.

Soybean nutrition and growth

Root dry matter (RDM) was higher when soybean was grown in the CL samples fertilized with SS as compared to APR (Figure 3). With the SS use,

shoot dry matter (SDM) and total dry matter (TDM) increased over C-HA concentrations. Thus, the addition of 100 mg kg^{-1} C-HA in the CL, SDM and TDM production increased by 10 and 9%, respectively, compared to the soybean grown in the control (Figure 3). Use of APR in CL did not change SDM and TDM over C-HA concentrations. In MT samples, C-HA concentrations and P sources interaction did not influence RDM, SDM, and TDM (Figure 3, $p>0.05$). On the contrary, RDM, SDM, TDM in MT decreased up to 75, 52 and 57 mg kg^{-1} C-HA, respectively. For soybean fertilized with SS the parameters RDM, SDM, and TDM were higher than the soybean biomass produced with the use of APR in the MT. The use of APR in CL and MT increased RDM/SDM ratio over C-HA concentrations. Regardless of the soil fertilized with SS, RDM/SDM ratio is not regulated by C-HA concentrations.

Besides different responses to soybean growth, the use of C-HA concentrations combined with P sources, in both Oxisols, also affected soybean nutrition. Application of 51 and 65 mg kg^{-1} C-HA with SS in CL increased the accumulation of P and N in a shoot by 11 and 13%, respectively, over control (Figure 4) and no effect was observed when using APR in the CL. In the MT fertilized with APR, the amounts of P in shoot did not change over C-HA concentrations (Figure 4). On the other hand, in MT fertilized with SS, the quadratic model was adjusted to amounts of N in shoot over C-HA concentrations. The increase of N uptake by soybean plants was 27% greater with 49 mg kg^{-1} C-HA than N acquired by soybean in MT not treated with C-HA. Nevertheless, in the MT fertilized with APR, N contents in soybean shoot reduced in a quadratic manner over C-HA concentrations. In addition to N and P uptake, S acquisition by soybean cultivated in the MT reduced over C-HA concentrations, regardless of the P source used. In both CL and MT, accumulation of S was greater in plants fertilized with SS than in soybean fertilized with APR.
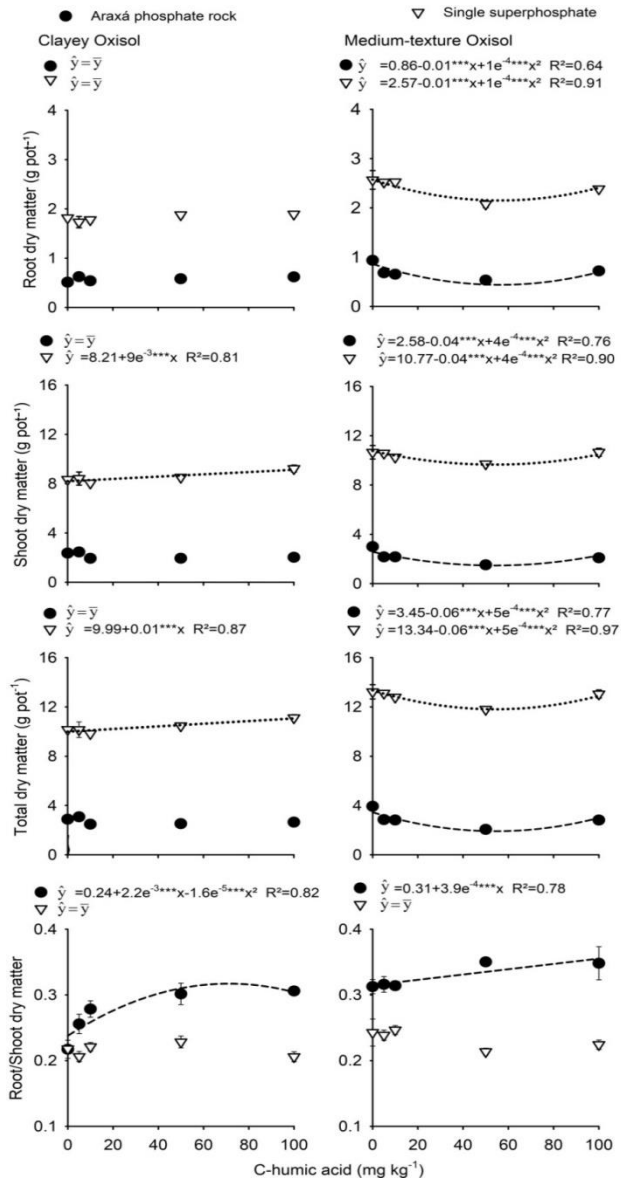
Figure 3- Root dry matter (RDM), shoot dry matter (SDM), total dry matter (TDM) and root/shoot dry matter ratio (RDM/SDM) of soybean plants cultivated in a clayey and medium-texture Oxisols fertilized with humic acid concentration (C-HA) and single superphosphate (SS) or Araxá phosphate rock (APR). *, ** and *** significance of the mathematical equation parameters to p<0.05, p<0.01 and p<0.001, respectively. Bars represent the standard error of the means.
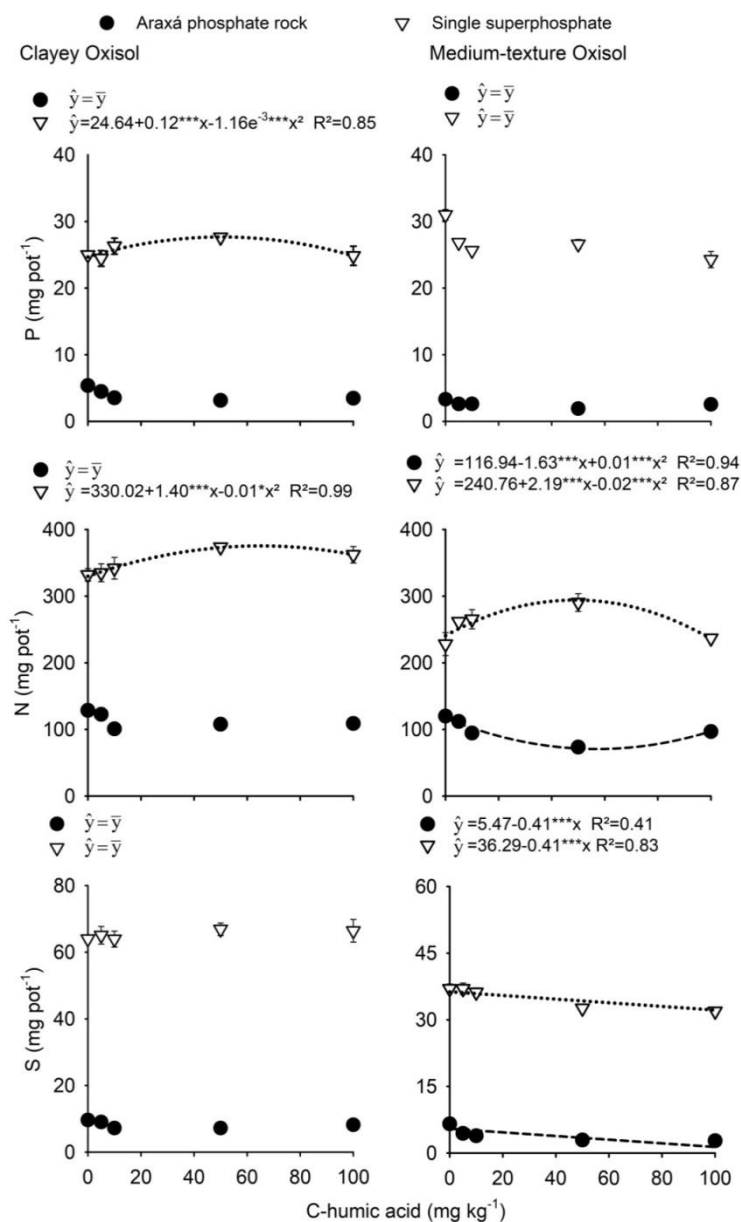
Figure 4- Accumulation of P, N and S in soybean shoot grown in a clayey and medium-texture Oxisols fertilized with humic acid concentration (C-HA) and single superphosphate (SS) or Araxá phosphate rock (APR). *, ** and *** significance of the mathematical equation parameters to $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bars represent the standard error of the means.

Residual P and soil pH after soybean cultivation

In both soils APR presented higher pH values than soil fertilized with SS (Figure 5). With APR use, pH reached values near to 5 in the CL, and close to 6 in the MT, whereas SS fertilization reduced pH values to 4.3 in CL and 5.2 in MT. Available P contents extracted by resin after soybean cultivation (residual resin-P) were direct and positively related to C-HA concentrations in CL fertilized with SS (Figure 5). Residual resin-P contents were 42% greater with 100 mg kg^{-1} C-HA over control in CL fertilized with SS. At the same soil treated with APR, residual resin-P contents did not change as C-HA concentrations increased, and it showed lower contents in soil than SS use. Inverse results were verified in MT samples, residual resin-P levels remained unchanged in soil under HA use combined with SS, whereas 43 mg kg^{-1} C-HA increased P availability with APR use (Figure 5). With this result, optimum C-HA concentration added to MT samples treated with APR increased residual resin-P content in 18% as compared to the same soil not treated with HA.

Principal component analysis

Principal component analyses (PCA) showed a clustering of P sources, SS and APR, used in CL and MT, and explained 78.3% and 97.2% of the total variance of data set for the CL and MT, respectively (Figure 6). In the CL, SSP, initial resin-P, EC, SDM, accumulation of P, and residual resin-P were positively related to the use of SS. Increase in SSC, initial and final soil pH were more related to APR. In addition, PCA indicated in CL an inverse relation between SSP and initial and final soil pH. In MT, similar to CL, SDM, SSP, and accumulation of P were the variables mostly linked to samples fertilized with SS as well as SSC. The initial and final soil pH were the variables which most associated to APR addition to MT.
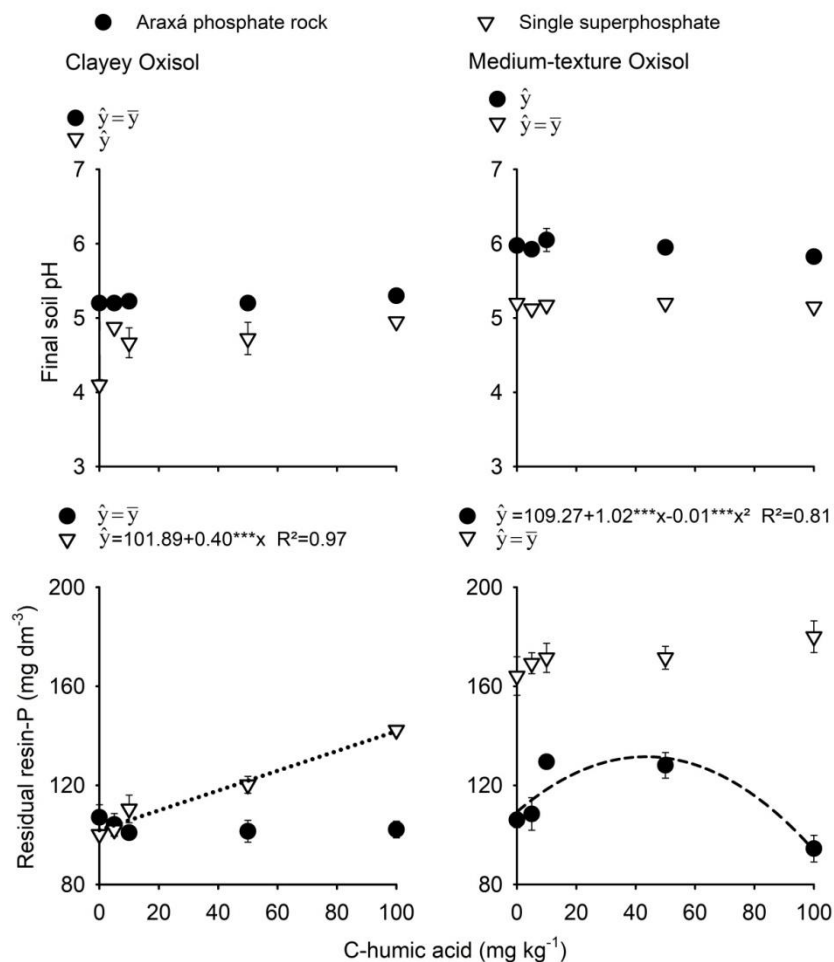
Figure 5- Residual resin-P and final soil pH of a clayey and medium-texture Oxisols fertilized with humic acid concentration (C-HA) and single superphosphate (SS) or Araxá phosphate rock (APR) after soybean cultivation. *, ** and *** significance of the mathematical equation parameters to $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bars represent the standard error of the means.
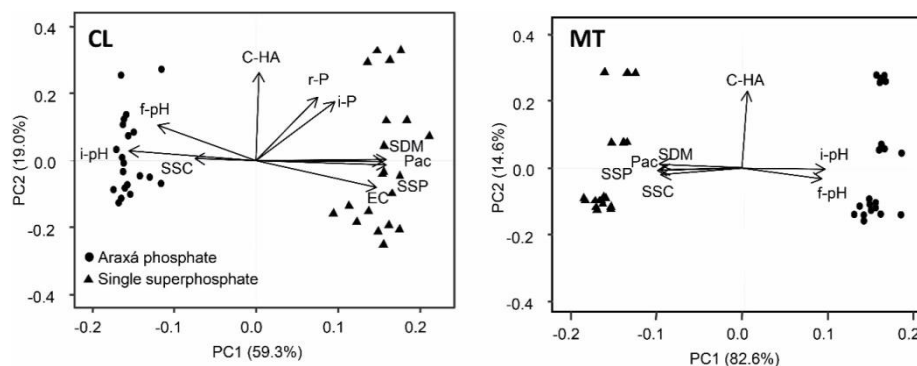
Figure 6- Principal component analyses between initial resin-P (Pi), residual resin-P (Pr), soil solution P (SSP), soil solution C (SSC), eletrical condutivity (EC), initial soil pH (i-pH), final soil pH (f-pH), shoot dry matter (SDM), P acumulated (Pac) in the shoot of soybean cultivated in the clayey (CL) and medium-texture (MT) Oxisols fertilized with single superphosphate (SS) or Araxá phosphate rock (APR).

Discussion

Humic acid (HA) addition in CL fertilized with single superphosphate (SS) increases soil solution P (SSP) possibly due to competition between HA and phosphate for adsorption sites in the soil solid phase (Figure 1). Polycarboxylic groups found in HA can inhibit P sorption in tropical soils, blocking phosphate binding sites, decreasing colloid surfaces affinity for P and reducing precipitation of P with Ca, Al and Fe (Hua et al. 2008; Erro et al. 2012; Wang et al. 2016; Maluf et al. 2018a). Furthermore, HA develops a repulsive negative electrostatic field around the plane of adsorption of the mineral as was suggested by Fu et al. (2013), maintaining more P-fertilizer in soil solution. Hua et al. (2008) reported that the addition of humic substances (HS)-based products with monocalcium phosphate increased the P availability in acidic soils up to 90%.

The increase of 17% in SSP in CL fertilized with SS and C-HA highlights the possibility to assure SSP concentrations greater than the critical level of 0.2 mg L^{-1}, established for most crops grown in soils (Beckwith 1965). After the

optimum concentration of 51 mg kg^{-1} C-HA, the reduction of SSP (Figure 1) may also be associated with the formation of low solubility HA-metal-P complexes (Guppy et al. 2005), as a result of the increased amount of organic ligands available in high HA concentrations added to soils. The formation of these HA-metal-P complexes in Oxisol was suggested by Maluf et al. (2018a) since resin-P contents decreased or tended to stabilize with the addition of 110 mg kg^{-1} C-HA from Leonardite.

In CL fertilized with Araxá phosphate rock (APR), the SSP was not affected by HA application (Figure 1) due to slow P-APR release. Solubilization of phosphate-rock (PR) is regulated by PR-soil particles contact and soil acid conditions (Prochnow et al. 2006; Chien et al. 2010). Moreover, the CL high clay and organic matter (OM) contents (Table 1) and the use of an alkaline fraction like HA contributes to the maintenance of soil pH values higher than 6 (Figure 2). Values of pH near neutral tend to maintain the integrity of the apatite, preventing P-APR solubilization (Prochnow et al. 2006; Chien et al. 2010; Maluf et al. 2018b).

Oxisol properties regulated the SSP content, which was verified with SSP reduction over C-HA concentrations in the MT (Figure 1). The MT had half the clay content of CL, that increases the competition between HA and P-SS for P-fixing sites, which contributed to the reduction in SSP over C-HA concentrations. This indicates that soil colloidal surface has a greater affinity for P than HA, which phosphate from SS replaces organic anions at the sorption sites, releasing C to the soil solution. Displacement of C to soil solution due to SS application occurred in both soils, CL and MT, increasing SSC. A similar effect was observed by Affif et al. (1995) and Maluf et al (2018a), where the role played by HA in blocking P binding sites can be transient since attraction between HA and soil colloid adsorption sites is not effective in competing with phosphate (Lidergren and Persson 2009). Guan et al. (2006) reported that the

presence of phosphate reduced the adsorption of HA by 21 to 66% on the surface of synthetic aluminum hydroxide. In contrast, slow-release of P-APR and low SSP due to conditions related in CL, increase the C adsorption in the soil solid phase, reducing SSC and did not change SSP in CL and MT (Figure 1). Higher clay content it explains the intense reduction of SSC in CL fertilized with APR as compared to MT.

Moreover, SSP and SSC dynamics influenced soil electrical conductivity (EC) (Figure 1). Soil EC indirectly expresses the ions and salts concentrations in the soil liquid phase (Carmo et al. 2016) and can be influenced by C-HA concentration. Addition of HA on soil can form complexes of variable stability with cations and anions (Olaetxea et al. 2018), and, depending on the stability, organic complexes (OCs) formation can reduce ion concentration in soil solution as C-HA concentration is increased, consequently reducing soil solution EC. The greater OM content in CL than in MT increase the amount of complexes between ions and organic ligands from the native OM (Figure 1). In the MT, due to low soil OM and clay contents (Table 1), the addition of HA enhance cations and anions in the soil solution, increasing the EC up to 42 mg kg^{-1} C-HA (Figure 1). Application of low rates of HS increases cation availability, and after the optimum C-HA concentration, the high content of organic ligands lead to a formation of less soluble OCs, reducing micronutrient availability (Rose et al. 2014). In both Oxisols, the EC was higher in soil fertilized with SS than APR use, due to the difference in water solubility of P and ions found in the P sources investigated.

The increase of 19% in initial resin-P contents are related to SSP increase in CL fertilized with SS (Figure 2), and it can be explained by the HA adsorption on soil minerals, complexation with Fe and Al, the same mechanisms reported in SSP (Guppy et al. 2005). These effects are in accordance to the results found by Maluf et al. (2018a), which verified that resin-P contents increased by 17% with

the application of ~110 mg kg^{-1} C-HA in a clayey Oxisol. Other fractions of HSs also improve soil P availability, such as Fulvic acid (FA), a polycarboxylic fraction with lower molecular weight than HA. This effect was shown by Yang et al. (2013) since the combined use of FA with KH_2PO_4 in an acid soil increased soil pH and reduced exchangeable Al concentration along with a concomitant increase in available P contents in soil. Initial resin-P in CL fertilized with APR increased by 19%, however, SSP did not change over C-HA concentrations (Figure 1).

In addition to the increase of initial resin-P contents in soil with HA addition, residual resin-P increased by 42% in the CL fertilized with SS and 100 mg kg^{-1} C-HA, which was twice as high as the control P levels (Figure 5). Enhance in residual P shows the potential of HA combined with SS in increasing P availability to subsequent crops in the clayey Oxisol, which contributes to the legacy of phosphate fertilization. In soil with properties contrasting to Oxisols used in this study, Kumar and Singh (2017) observed an increase until 25% in P content at the end of rice cultivation in an alkaline (pH 7.9) and sandy loam texture soil treated with 5 mg kg^{-1} of C-potassium humate. In CL fertilized with APR, residual resin-P contents were not affected by HA addition (Figure 2 and 5). Thus, the increase of initial resin-P in CL fertilized with APR did not contribute to improve residual P. During the cultivation, the soil pH decreased from 6.1 to 5.2 in CL (Figure 5), but this reduction did not favor the P-APR solubilization, probably due to the high capacity of the CL soil in fixing phosphate.

Initial resin-P contents in MT fertilized with SS decreased over C-HA concentrations, similar to SSP, and with APR initial resin-P did not change over C-HA concentrations (Figure 2). Using medium-texture soil, Rosa et al. (2018b) observed that incubation of soil samples with HA concentrations and APR, before wheat cultivation, did not influence the soil available P. These results

confirm that P availability due to HA application depends on soil properties, such as clay content, that rules the soil solid phase affinity for P. On this are possible to affirm there is no significant amount of P in the HA to contribute to the available P in Oxisols studied. The role played by HA in supplying P cannot be discharged, but the HA used in the present studied had lower P contents than the HA reported by He et al. (2015). Even at the highest concentration of HA used in this study (100 mg kg^{-1} C-AH) had and input of only 0.08 mg kg^{-1} P. Thus, the amount P added by HA is too small when compared to the P from fertilizers added to the Oxisols studied.

Residual resin-P was not affected by HA addition in MT fertilized with SS, on the other hand, the soybean cultivation contributed to the increase of residual resin-P up to 18% in the MT fertilized with APR over control. According to Shen et al. (2011), legumes, such as soybean, uptake more cations than anions, resulting in proton release and rhizosphere acidification. Moreover, plant under a P-deficiency, such as in the soil fertilized with APR, roots tend to exudate more organic anions and ligands, which increases P solubility and may enhance P acquisition by plants (Gerke 2015; Rafael et al. 2018). These facts may have contributed to release more P-APR, especially in MT. The MT is less buffered than CL, which favors the rhizosphere pH reduction, and, due to the lower MT clay content in relation to CL, P adsorption is reduced, consequently, increasing soil P availability. Based on the results discussed before, HA application with APR is a powerful strategy to increase P availability in the medium-texture Oxisol to crops grown in succession to soybean. According to Rosa et al. (2018b), in medium-texture Oxisol, the combination of 56 mg kg^{-1} C-HA with APR ensured wheat biomass production similar to that reached by SS fertilized plants.

The increase in SSP and initial resin-P contents in CL fertilized with SS contributed to increase in 10% shoot dry matter (SDM) and in 9% the total dry

matter (TDM) of soybean (Figure 3). The higher root dry matter (RDM) of soybean fertilized with SS in CL in relation to APR use is also related with greater P availability promoted by SS application. In contrast, the reduction of SSP in MT fertilized with SS and no changes in SSP and initial resin-P in this soil with APR reflected in the reduction of soybean RDM, SDM, and TDM (Figure 3). The effects of HA on soybean growth are more prone to be linked to changes in soil chemical of physicochemical properties, mainly those related to the forms and availability of P in soil and in solution, both controlled by HA concentrations and P source. In this study, HA action on plant physiology that drives plant growth was not verified as reported in other studies (Tavares et al. 2017; Olaetxea et al. 2018). Plant responses due to HA use depends on the physiological stage, plant species, HA physicochemical characteristics, HA concentration and growth medium (Rose et al. 2014). Prado et al. (2016) found an increased soybean SDM of 30 and 15% in the stages of flowering and maturation, respectively, cultivated in sandy soil under water stress with the use of P fertilizer enriched with HS.

After soybean growth, it was verified the increase in RDM/SDM ratio with the addition of C-HA concentrations in both soils fertilized with APR (Figure 3). This result is indicative of low P availability in the soil since soybean plants develop more root than shoot to increase P uptake efficiency (Gerke, 2015). Higher root than shoot growth could mean a higher soil volume explored by roots and a greater contact of soybean root with APR particles, which may favor P-apatite solubilization. On the other hand, increase in RDM/SDM ratio can damage the soybean growth, since photoassimilates and nutrients that could be used for shoot growth and grain production were spent to produce more roots (Nikbakht et al. 2008).

Increase in soil P availability improved soybean phosphate and nitrogen uptake in CL with SS use, besides increasing shoot N accumulation in plants

fertilized with SS in the MT (Figure 4). A similar result was found by Prado et al. (2016) since the use of a fertilizer enriched with HS increased the accumulation of N and P in soybean shoot. This increase of N accumulation by soybean shoot cultivated in both soils fertilized with SS can be related to sulphur (S) from SS, which synergistically enhances N uptake by plants (Fageria 2006) since SS is composed by $Ca(H_2PO_4)_2$ and $CaSO_4.2H_2O$. In addition, the HS presence increases plant enzyme activity involved in N assimilation, such as GS and GOGAT, which may enhance the incorporation of NH_4^+ in plant organic compounds (Conselvan et al. 2017; Olaetxea et al. 2018) and HA use also improves NO_3^- acquisition (Tavares et al. 2017).

Principal component analysis (PCA) clustered variables analyzed based on the different chemical properties and capacity to P sources to supply P to soybean plants (Figure 6). In CL, PCA also confirms that the non-response of soybean to C-HA combined with APR was related to soil pH, due to initial and final soil pH were more related to the use of APR. The soil pH controls apatite solubilization and, consequently, the effects of HA on P availability in soils treated with APR. Moreover, in CL there is an inverse relation among APR addition and SSP, SDM and accumulation of P in the shoot, showing that APR use hampers soybean growth and nutrition and decrease levels of SSP. On the other hand, the use of SS influenced initial and residual resin-P, and SSP, which correlated with C-HA concentrations, confirms that the HA addition to CL increases P availability and SDM production. In the MT, changes with APR use were more related to soil pH as it was verified in CL samples. The greatest variations observed for soil and plant attributes were more related to SS application to soils than to APR.

Contrary to the results of the positive interaction of HA-APR in improving wheat growth (Rosa et al. 2018b) even combined with high concentrations of HA, APR is not a suitable fertilizer to meet soybean plants P requirement in a

timely way. However, there is a synergy between HA and P-SS in the CL, and, according to our results, for crops in succession to soybean, cost of phosphate fertilization in soils treated with HA maybe reduced in almost 50%. In field cultivation to increase in 10% soybean total biomass, if consider the HA addition only in soil strip, at the 0-0.1 m soil depth where the SS is applied, the C-HA concentration required is about 13 kg ha^{-1}. Thus, HA combined with SS can be economically feasible, mainly if one consider the improvement in soil physical and chemical attributes with successive and long-term HA addition in crops. Further studies with the HA use in soil cultivated with annual crops are necessary to better understand the dynamics of this humic fraction in real crop field conditions in the agroecosystem.

Conclusion

The increase of soil P availability over HA addition depends on the soil particle size distribution and P source used. Application of HA combined with SS increase SSP and, initial and residual resin-P levels in the clayey Oxisol, but it has no beneficial effect in the medium-texture Oxisol. The increase of P availability in the clayey Oxisol with SS and 100 mg kg^{-1} C-HA increased roughly in 10% soybean biomass and N and P accumulation in soybean shoot. In the clayey Oxisol fertilized with APR, use of HA did not favor solubilization of phosphate rock, while in the medium-texture Oxisol the application of APR with 40 mg kg^{-1} C-HA increased available residual resin-P levels without improving soybean growth. Use of HA is an important strategy to keep threshold levels of available and solution P to nourish soybean plants, especially in the clayey Oxisol treated with SS.

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2.2 ARTIGO 2- Bioactivity of water extractable and Leonardite-derived humic substances on maize growth

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Abstract

The biostimulant effect of humic substances (HS) on maize plants (*Zea mays*) depends on the source and concentration of HS. The aim of this study was to evaluate maize growth and nutrient content in growth medium treated with Leonardite-derived HS sources and water extractable HS (WEHS). Maize plants were cultivated during 21 days in nutrient solution with the addition of Acros Organics® humic acid p.a. (AHA), HA extracted from Leonardite (HAL) and WEHS at concentrations of 2, 5, 15, 40 and 75 mg L^{-1} C-HS, plus control treatment (No HS addition). Electrical conductivity (EC), pH, C (SC) and nutrient availability were determined in nutrient solution after maize cultivation. The SPAD index, shoot dry matter (SDM), root dry matter (RDM) and nutrient accumulation in maize shoot were measured. Organic acid root exudate was determined for plants treated with WEHS. Application of WEHS increased P, K and Zn concentration in nutrient solution; Fe contents in the growth medium increase with the application of HAL and AHA. The SPAD index increased about 50% for all HS sources. Addition of 40 mg L^{-1} C-HAL and C-WEHS to the nutrient solution increased maize shoot growth roughly in 21 and 29 %, respectively. Use of WEHS increased maize root exudation of citric, isocitric, malic, maleic-fumaric, succinic, oxalic and tartaric acids in comparison to control. The most efficient HS source to cultivated maize in nutrient solution was WEHS due to its positive effects on the availability of nutrients in solution and on maize biomass and nutrition, besides being a renewable HS source extracted from compost.

Keywords: Compost tea, humic acid rate, root exudate, SPAD index.

1. Introduction

The increasing demand for food and crops with higher yield and better quality is pushing agricultural research towards sustainable and eco-friendly fertilizers (Xu and Geelen, 2018). Biostimulants are a class of compounds of various origins or microorganism applied to plants with the aim to improving plant growth, with higher water and nutrient use efficiency, enhance primary and secondary metabolism, increase abiotic stress tolerance and crop quality regardless of its nutrients content (du Jardin, 2015). In the last years, the use of biostimulants has been constantly increasing for sustainable agriculture. Among biostimulants, humic substances (HS) or humates demonstrated a positive effect on the uptake of macro and micronutrients that considerably improve the growth and yields of relevant agricultural crops (Nardi *et al*., 2009).

HS are supramolecular structures of heterogeneous molecules held together by weak hydrophobic interactions and hydrogen bonds (Piccolo, 2002). HS naturally occur in soil and water reservoirs and can be extracted from soil organic matter (SOM), composts, organic wastes, peat, agricultural by-products, fresh organic matter from plants, animals, and secondary coal like lignite (Shah *et al*., 2018). Humic fractions are responsible for many complex chemical reactions intrinsically associated with several chemical, physical and biological soil properties (Halpern *et al*., 2015). Besides improving soil quality, HS act directly on plant growth and physiology, playing the role of biostimulants (Nardi *et al*., 2016; Olaetxea *et al*., 2018). HS bioactivity has been demonstrated by several studies (Jindo *et al*., 2012; Aguiar *et al*., 2013; Zandonadi *et al*., 2014; Shah *et al*., 2018). An increase of about 23% in the growth rate of primary maize roots was found by Canellas and Olivares (2017) when earthworm compost-derived humic acid (HA; 12 mg L^{-1}) was added in the growth medium as

compared to plants not treated with HA. It is estimated that the use HS can augment shoot and root growth ~20% (Rose *et al*., 2014).

The mechanisms of action involved in HS and other biostimulant activity are not yet totally elucidated. The bioactivity of HS has been attributed to the capacity of these fractions to act as auxins in plant physiology (Aguiar *et al*., 2016; Canellas *et al*., 2018), or due to the presence of indol acetic acid (IAA) in HS structure (Jindo *et al*., 2012). However, the effects attributed to the bioactivity of HS cannot be explained only by the presence of IAA since the effects described with the use of HS are higher than the levels of the hormone present in HS, thus, there are other factors that regulate the bioactivity of HS (Nardi *et al*., 2009). Nitric oxide signaling (Zandonadi *et al*., 2010) has been proposed to be involved in HS-induced increase in plasma membrane (PM) H^+-ATPase activity in the root and Reactive Oxygen Species (ROS) have been suggested to have a signaling role as mediators of HS-induced responses (García *et al*., 2016a).

Biostimulant HS have been extracted for study purposes from several sources, among which earthworm faeces (Carletti *et al*., 2008; Roomi *et al*., 2018), leonardites from different areas (Conselvan *et al*., 2017) and compost of various origin (Jindo *et al*., 2012; Canellas *et al*., 2015). In addition to strong Na or K alkaline solution, HS can be extracted from compost also through water. The water extractable humic substances (WEHS), also known as compost tea, consists of a series of bioactive molecules, like IAA, cytokinins, and gibberellins, as well as microbial populations derived from the parent material (Arancon *et al*., 2012; Pant *et al*., 2012). WEHS increased germination percentage and seedling growth of tomato and lettuce (Arancon *et al*., 2012) and increased of shoot length and plant fresh weight of tomato and lettuce (Traversa *et al*., 2010). Moreover, many HS based products are currently available in the market (Olk *et al*., 2018). Properties related to HS sources such as the degree of

humification, the origin of organic waste, and chemical and physicochemical properties are factors determining HS bioactivity (Jindo *et al*., 2012; Conselvan *et al*., 2017). Concentration is another factor that regulates HS bioactivity because, when applied beyond the optimum concentration, HS may inhibit plant growth (Canellas and Olivares 2017).

Taking into account that bioactivity of HS is expressed by increments or decreases in plant physiological parameters, root exudation can help to understand these effects, since exudates are a marker of biotic and abiotic stresses in the soil (Adeleke *et al*., 2017). Canellas *et al*. (2008) observed changes in the profile of organic acids exudation by maize seedlings treated with HS. In addition, the root exudation of organic acids can alter HS structures, resulting in more bioactive subunits, some of which may have hormonal activity (Canellas *et al*., 2008; Piccolo, 2002).

Studying plant responses to the bioactivity of HS from different sources, based on the best concentration and source, might help the development of new generation of bioactive compounds aiming at greater agronomic efficiency and crop yield. Thus, the aims of this study were to i) evaluate the changes in nutrient solution, growth and SPAD index of maize treated with HS from different sources at increasing concentrations; ii) identify the optimum HS source and concentration to improve nutritional status and maize growth; and iii) characterize the maize root organic acids exudation profile by plants under the best HS source.

2. Material and methods

2.1. Humic substances extraction

Acros Organics® humic acid (AHA) is a commercial product pure to analyses with 45-70% sodium salt. Humic acid was extracted from Leonardite (HAL) through the use of a 0.5 mol L^{-1} KOH solution, and, in sequence,

purified, following the method recommended by the Internacional Humic Substances Society (Swift, 1996). WEHS was extracted from compost formulated with 60% (v/v) coffee husk, 30% chicken manure, and 10% biochar incubated for 150 days, dried, milled and passed through a sieve with a 2 mm mesh. To obtain WEHS, compost and water at 80°C were mixed in a ratio of 1:10 (w/v), stirred for 4 hours on a horizontal shaker at 90 rpm, then, centrifuged for 15 minutes at 2800 rpm, and filtered on GF1 glass fiber microfilter for further analysis.

2.2. Plant material and growth conditions

The experiment was carried out with maize (30F53) under greenhouse conditions at Department of Soil Science/Federal University of Lavras. Treatments were arranged in a 3 x 5 + 1 factorial scheme, through the combination of three humic substances (HS) sources: AHA; HAL and WEHS at five HS carbon concentrations as follows: 2; 5; 15; 40 and 75 mg L^{-1} C-HS, plus the control treatment without HS, with four replicates.

Maize was sown on vermiculite and seven-day-old seedlings were transferred to pots with 1.3 L of a Hoagland and Arnon (1950) modified nutrient solution with pH adjusted to 5.5 ± 0.3, under the influence of the treatments aforementioned. Nutrient solution has the following nutrient concentrations: 210 mg L^{-1} N; 31 mg L^{-1} P; 234 mg L^{-1} K; 160 mg L^{-1} Ca; 49 mg L^{-1} Mg; 65 mg L^{-1} S; 0.80 mg L^{-1} B; 1 mg L^{-1} Mn; 0.2 mg L^{-1} Zn; 0.05 mg L^{-1} Cu; 0.01 mg L^{-1} Mo and 5 mg L^{-1} Fe. One maize plant was grown per pot and plants were constantly and individualized aerated throughout the experimental period. The volume of each pot was completed on a daily basis using deionized water. After seven days of maize cultivation, nutrient solution was renewed and HS were reapplied. The maize plants were cultivated for 24 days.

2.3. HS chemical characterization

Electrical conductivity (EC) and pH of AHA and HAL were determined using deionized water and humic fraction: water ratio of 1: 2.5 (1g : 2.5 mL). The HA + water mixtures were shaken for 30 seconds, kept at rest for 30 min and stirred for 30 s. In sequence, the pH and EC of mixtures and in an aliquot of WEHS was measured. The C of AHA and AHL was determined in a dry combustion analyzer (Elementar, model Vario TOC Cube). Total C-WEHS were determined in the TOC analyzer liquid module. To determine macro and micronutrients in the HS, 500 mg of AHA and HAL was digested with 8 mL of a mixture of nitric and perchloric acids at a 4:1 ratio. Contents of P was determined by the molybdenum blue reaction method, S through reaction with a barium chloride solution, and B through reaction with azomethine-H using in an UV-visible spectrophotometer at 660 nm for P, 420 nm for S, and 460 nm for B. The total content of N was determined by digestion in acid medium and distillation using the *Kjeldahl* method. The contents of Ca, Mg, Cu, Fe, Mn and Zn were determined through atomic absorption spectrometry with acetylene and K and Na through flame photometry. To determine water-soluble nutrient contents of the AHA and HAL, 0.04 g was diluted in 50 mL, and then the nutrient determination, as well as the nutrient contents in the WEHS, was performed in Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

2.4. Nutrient solution and plant analysis

After one day of maize cultivation, an aliquot of nutrient solution was collected from each experimental pot for pH, EC, and ion concentration determinations. Carbon was determined in an elemental automated analyzer TOC using the liquid module, and P, K, Ca, Mg, S, Fe, Mn and Zn were measured by ICP-OES. At the end of the experiment, leaf greenness content was determined using a SPAD (Minolta, SPAD-502, Osaka, Japan) through analysis

of the last expanded leaf of maize plants. Six measurements were performed for each plant.

In sequence, plant roots were washed with ultrapure water and dipped into pots containing 230 mL of ultrapure water, protected from sunlight, kept for about 3 hours under oxygenation to obtain root exudates. Then, the plants were transferred to the respective treatments. Maize growth was assessed by measuring shoot dry matter (SDM) and root dry matter (RDM). The maize root and shoot tissues were stored in paper bags and dried in an oven at 60°C until constant weight. After drying, biomass was weighed to determine SDM and RDM, and total dry matter (TDM) through the sum of SDM and RDM. The SDM was ground in a Willey mill. The plant material digestion and macro and micronutrient determination were done following analytical procedures described in the manual of chemical analysis of soil, plants and fertilizers (Silva, 2009), as was performed before to determination of HS nutrient contents. The accumulation of each nutrient in shoot was determined by multiplying nutrient concentration in the plant tissue by its respective dry matter.

2.5. Root exudates chromatographic analysis

The chromatographic analyses of the exudates solutions were performed on a liquid chromatograph. The root exudates were filtered in a 0.45 μm filter and stored in 80 mL bottles in an ultra-freezer. The root exudates from plants treated with WEHS were lyophilized (freezer Enterprise 1- Terroni) until the total water withdrawal, to concentrate the organic acids. In the lyophilized materials, it was added 1 mL of ultrapure water for the solubilization, and, then, the samples were filtered through 0.2 μm filter membrane. Ten μL of each sample were injected in an UHPLC-MS. The UHPLC Acquity coupled with a TQD mass spectrometer Acquity (Micromass-Waters Manchester, England), and an ESI detector. It was used a C8 column BEH Waters Acquity (2.1 mm x 50 mm x 1.7 μm particle size) at 30 °C. During the elution, mobile phase A (Mili-Q purified water with

0.01% ac.) and mobile phase B (HPLC grade methanol) were used in the gradient starting from 100% A to 2.5 min, changing to 40% A up to 3.5 min, holding up to 4 min, returning to initial conditions and stabilizing up to 6 min. In mass spectrometry detection, electrospray ionization with capillary negative mode was used at 2.8 KV and 25 V, temperature of the source at 150°C and desolvation at 350°C. In root exudates, it was analyzed oxalic, lactic, tartaric, malonic, malic, maleic-fumaric, succinic, citric and isocitric acids. Analytical organic acid certified samples were used to build calibration curves. The calibration curves were generated with organic acids concentrations ranging from 0.01 to 50 µg mL^{-1}. The standard analytical curve was obtained by relating different concentrations of the certified organic acids with their respective peak area in the chromatogram.

2.6. Statistical analysis

The data set was subjected to analyses of variance and regression ($p<0.05$), to verify the interaction between HS sources and concentrations, as well as the difference between the control treatment and the factorial on the growth and photosynthetic parameters of maize. A contrast analysis was performed to identify if addition of HS are different from control treatment. The regression model that best fit to the data set was chosen based on the significance of the mathematical equation parameters ($p<0.05$), on the lowest value of the sum of squared errors, and on the equation with the highest adjusted coefficient of determination (R^2). The treatments were grouped according to the level of similarity, following the hierarchical clustering technique. The data were standardized in standard score (z-scale) and dendrograms was constructed by using Euclidian distance and Ward agglomeration method. Choice of method was based on the assumption that the cophenetic matrix generated by that method had a higher Pearson correlation with the original distances compared to the others (Landeiro 2011; Provete *et al*., 2011). Statistical analyses were

performed through the computer program R Studio 1.0.136, using the ExpDes package 1.1.2 (Ferreira *et al*., 2013) and Ape package 5.0 (Paradis *et al*., 2018), and SISVAR 5.6 (Ferreira, 2014).

3. Results

3.1 Nutrients and soluble C in growth media

Humic substances (HS) principal properties as well as their nutrient contents soluble in water are shown in Table 1.

Table 1- Chemical properties of Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL), water extractable humic substances (WEHS) and water-soluble nutrient content found in 1 g L^{-1} AHA and HAL

Chemical properties	Humic substance source				
	AHA	HAL	WEHS	W-AHA	W-AHL
pH	9.3	9.7	8.7	-	-
EC(dS m^{-1})	23	36	0.43	-	-
	------ g kg^{-1}------		-----------mg L^{-1}-----------		
C	330	350	2400	-	-
N	7.5	5.2	-	-	-
P	0.21	0.05	8.9	0.07	<QL
K	3.9	41.6	54.2	2.9	144
Ca	5.6	1.4	3.5	4.6	0.30
Mg	0.8	2.6	39.6	1.2	2.9
S	4.5	2.8	5.3	3.7	2.9
Fe	1.2	2.7	-	9.6	1.9
Na	71.4	3.2	-	-	-
	-------mg kg^{-1}------				
Al	-	-	1.80	-	-
Cu	45.7	7.62	0	0.03	<QL
B	12.5	41.6	1.4	-	-
Mn	18.9	21	0.8	0.03	0.02
Zn	25.3	76.3	1.7	0.01	0.01

*1 g L^{-1}; QL quantification limit.

Nutrient solution carbon (SC) one day after addition of treatments were affected by HS sources and concentrations ($p<0.05$) (Figure 1). Regardless of the HS source used, the mathematical equation that best fitted to the SC was the quadratic model. SC contents in HAL and WEHS treated solutions were significantly higher than control, whereas SC in the nutrient solution treated with WEHS was higher than in the growth medium treated with HAL and AHA (Figure 1). One day after the addition of treatments to nutrient solution, electrical conductivity (EC) and pH were influenced by HS source-concentration interaction ($p<0.05$; Figure 1). The quadratic model was adjusted to the pH increase over C-WEHS and C-HAL concentrations. Application of 75 mg L^{-1} C-WEHS increased pH in one unit in relation to control. EC increased linearly as C-WEHS was increased and decreased with the use of AHA. In addition, nutrient solution EC treated with AHA and WEHS were higher than control.
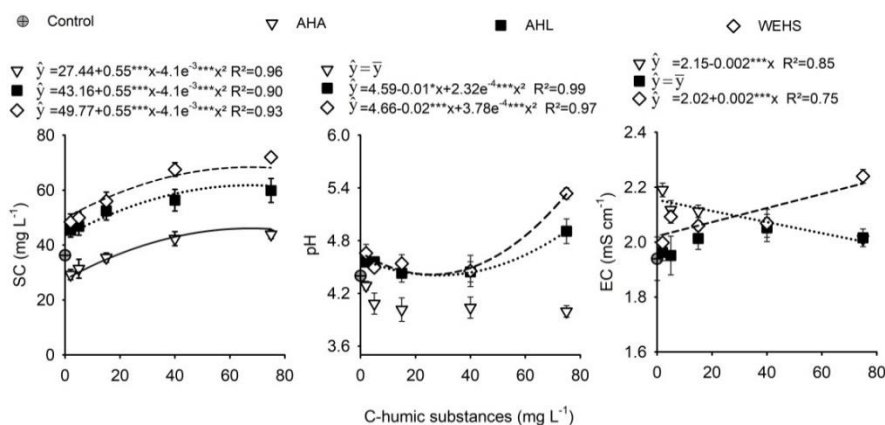
Figure 1–Nutrient solution carbon (SC), pH and electrical conductivity (EC) one day after the addition to nutrient solution of Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS) over increasing C-humic substances concentrations. *, ** and *** significance of the mathematical equation parameters with $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bars represents the standard error of the mean.

The interaction between HS source-concentration (p<0.05) influenced P, K, Ca, Mg, S, Fe, and Zn concentrations in nutrient solution one day after the treatments addition (Figure 2). The Mn concentrations in nutrient solution were affected only by HS sources. Concentrations of P, K, Ca, Mg, S and Zn increased over C-WEHS, and application of 75 mg L^{-1} C-WEHS lead to a 26, 20 and 141% increase in P, K and Zn concentration, respectively, in relation to control. K and Fe contents in nutrient solution increased over C-HAL. The optimal concentration of HAL in the nutrient solution, ~53 mg L^{-1} C, increases in 49% Fe concentration in comparison to its content in control. The other nutrient concentrations did not change over C-HAL. Sulfur and Fe concentrations in nutrient solution increased over C-AHA, Fe contents increased by 43% until 46 mg L^{-1} C-AHA over control, and S contents in nutrient solution were lower than control. Application of C-AHA concentrations did not affect the contents of P, K, Ca, Mg, S, and Zn, and all this nutrient concentration were lower than those measured in the medium not treated with HS. With the use of WEHS, the Mn contents in nutrient solution were higher than those determined for the other HS sources.

3.2. SPAD index and maize growth

Leaf greenness, measured in terms of SPAD values, was influenced by the HS source-concentration interaction (p<0.05; Figure 3). The quadratic model was the one that best fitted to SPAD index for all HS sources. With the AHA use, SPAD decreased as C-AHA concentration increased. Despite this, all HS sources present SPAD index greater than control. The concentrations of about 33 mg L^{-1} C-HAL and 32 mg L^{-1} C-WEHS were responsible for the maximum SPAD index. The leaf greenness increased by 53%, 43% and 46% over control for AHA, HAL and WEHS sources, respectively.

Control ⊕   HAH ▽   HAL ■   WEHS ◇

Legend: Control, HAH, HAL, WEHS

Mn panel with bars labeled B, B, A

P panel:
▽ $\hat{y} = \bar{y}$
■ $\hat{y} = \bar{y}$
◇ $\hat{y} = 29.83 + 0.12^{***}x$ $R^2 = 0.93$

K panel:
▽ $\hat{y} = \bar{y}$
■ $\hat{y} = 194.65 + 0.27^{***}x$ $R^2 = 0.92$
◇ $\hat{y} = 186.25 + 0.69^{***}x$ $R^2 = 0.98$

S panel:
▽ $\hat{y} = 65.53 + 0.12^{***}x$ $R^2 = 0.78$
■ $\hat{y} = \bar{y}$
◇ $\hat{y} = 67.93 + 0.15^{***}x$ $R^2 = 0.90$

Mg panel:
▽ $\hat{y} = \bar{y}$
■ $\hat{y} = \bar{y}$
◇ $\hat{y} = 44.80 + 0.12^{***}x$ $R^2 = 0.93$

Ca panel:
▽ $\hat{y} = \bar{y}$
■ $\hat{y} = \bar{y}$
◇ $\hat{y} = 170.44 + 0.31^{***}x$ $R^2 = 0.95$

Fe panel:
▽ $\hat{y} = 5.09 + 0.09^{***}x - 9.8e^{-4}$ $R^2 = 0.96$
■ $\hat{y} = 5.11 + 0.08^{***}x - 7.5e^{-4}x^2$ $R^2 = 0.89$
◇ $\hat{y} = \bar{y}$

Zn panel:
▽ $\hat{y} = \bar{y}$
■ $\hat{y} = \bar{y}$
◇ $\hat{y} = 0.59 - 0.007^{***}x + 1.62e^{-4***}x^2$ $R^2 = 0.98$

C-humic substances (mg L^{-1})

Figure 2– Nutrient solution manganese (Mn), phosphorus (P), potassium (K), sulphur (S), magnesium (Mg), calcium (Ca), iron (Fe), and zinc (Zn), contents one day after the addition to nutrient solution of Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS) over increasing C-humic substances concentrations. *, ** and *** significance of the mathematical equation parameters with $p<0.05$, $p<0.01$ and $p<0.001$, respectively. The value presented for each source, in Mn, corresponds for averages for all concentrations. Means followed by the same letter are not statistically different by the Scott-Knott test ($p<0.05$). Bars represents the standard error of the mean.
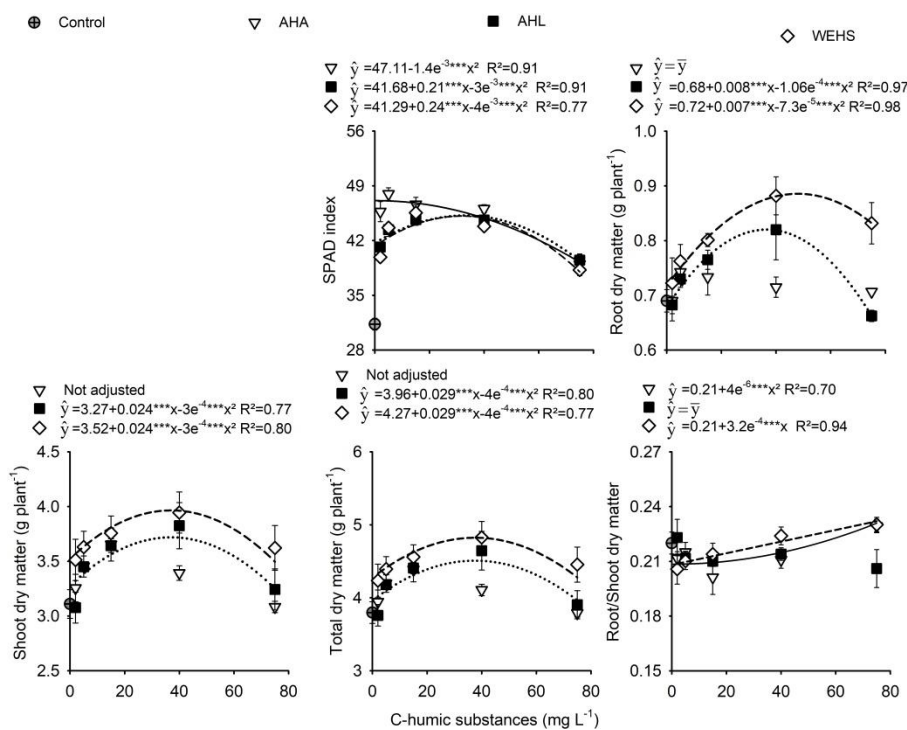
Figure 3–SPAD index, shoot dry matter, root dry matter, total dry matter, root/shoot dry matter ratio of maize plants cultivated in nutrient solution with Acros Organics® humic acid (AHA), humic acid extracted from leonardite (HAL) and water extractable humic substances (WEHS) over increasing C-humic substances concentrations.*, ** and *** significance of the mathematical equation parameters with $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bars represent the standard errors of the mean.

Root dry matter (RDM) and root/shoot dry matter ratio (RDM/SDM) were influenced by the interaction HS sources-concentrations ($p<0.05$). Shoot dry matter (SDM) and total dry matter (TDM) were not affected by the interaction but were influenced by HS source and concentration ($p<0.05$). The quadratic model was adjusted to RDM over C concentrations added as HAL and WEHS (Figure 3). The concentration of 36 mg L^{-1} C-HAL and 48 mg L^{-1} C-WEHS provided the highest root growth, with an increase of about 19% and 28% in

HAL and WEHS treated plants, respectively, in relation to control. RDM did not change over C-AHA concentrations. SDM and TDM increased in a quadratic manner over C-AHL and C-WEHS concentrations. The highest SDM and TDM among the studied HS sources were found for plants treated with WEHS. The concentration of 40 mg L^{-1} C-HAL and C-WEHS led to a 21 and 29% increase in SDM, respectively, in relation to maize plants cultivated in nutrient solution untreated with HS. In addition to, TDM increased 19 and 26% when 37 mg L^{-1} C-HAL and C-WEHS were added to nutrient solution, respectively, over control. In plants treated with HS, SDM/RDM ratio did not change in relation to control, however, with WEHS and AHA use, the ratio increased as C-HS concentration increased (Figure 3).

3.3. Maize nutritional status

Accumulation of N and K contents in maize shoot were affected by HS sources and concentrations ($p<0.05$; Figure 4). No mathematical model was fitted to the accumulation of N in maize shoot as a function of increasing concentrations of HS. Amounts of N in maize treated with HAL and WEHS were higher than plants cultivated in the control treatment. N in AHA was the lowest among HS sources. Increase in accumulation of K in maize shoot can came to 31% and 25% in maize plants treated with 51 mg L^{-1} C-AHA and C-HAL, respectively. Among the HS sources studied, the accumulation of K in maize plants treated with WEHS was the lowest. Amounts of P, S, Ca and Mg in maize shoots were influenced by the interaction HS sources-concentrations ($p<0.05$; Figure 4). Accumulation of S and Ca increased until 30 mg L^{-1} C-AHA followed by a decrease, but the values were not higher than amounts of S and C without HA treatment. Over C-WEHS, Ca accumulated decreased and S accumulated increased in a quadratic manner, with an increase of about 50% of S compared to control at 52 mg L^{-1} C-WEHS. The quadratic model was best fitted to P, Ca, and Mg amounts in maize shoots as C-HAL increased. The

concentrations of 33, 31, and 42 mg L^{-1} C-HAL provided the highest accumulation of P, Ca, and Mg, respectively, and represents an increase of 21% for P, 16% for Ca and 22% for Mg in the shoot over control.

Amounts of Mn in the shoot were influenced by the HS source-concentration interaction. Cu, Fe, and Zn contents were not affected by HS source and concentrations interaction ($p>0.05$; Figure 4), thus the factors studied were isolated to evaluate the influence of concentrations on Cu, Fe and Zn contents. The amounts of Mn in the shoot with all the HS were higher than control. Mn content reduced over C-AHA concentrations, however, in relation to control, the amounts of Mn in the shoot was 16% higher with 15 mg L^{-1} C-AHA. With WEHS, amounts of Mn led to an increase of 48% with the application of 75 mg L^{-1} C-WEHS, compared to plants cultivated in control treatment. The quadratic model was the best fitted to Cu contents with AHA use, amounts of Fe in maize shoot treated with WEHS, and Cu, Fe and Zn content with HAL application. Nevertheless, amounts of Cu, Fe, and Zn in maize shoot under the influence of different HS concentration did not differ from those amounts determined for the control plants.

3.4. Clustering of HS effects on maize and growth media

Euclidean distance of 13 separated the treatment in three well-defined groups that can be observed in dendrogram (Figure 5). The first, consisting of three treatments: 15 and 40 mg L^{-1} C-WEHS with high similarity, and 75 mg L^{-1} C-WEHS. The second group was composed of five treatments, sub-divided in two groups: 2, 5 mg L^{-1} C-AHA; and 15, 40 mg L^{-1} C-AHA, 40 mg L^{-1} C-AHL. The last one group was composed by eight treatments sub-divided in three groups: 75 mg L^{-1} C-AHL, 2, 5 mg L^{-1} C-WEHS; 2, 5 and 15 mg L^{-1} C-AHL, finally 75 mg L^{-1} C-AHA and control treatment.
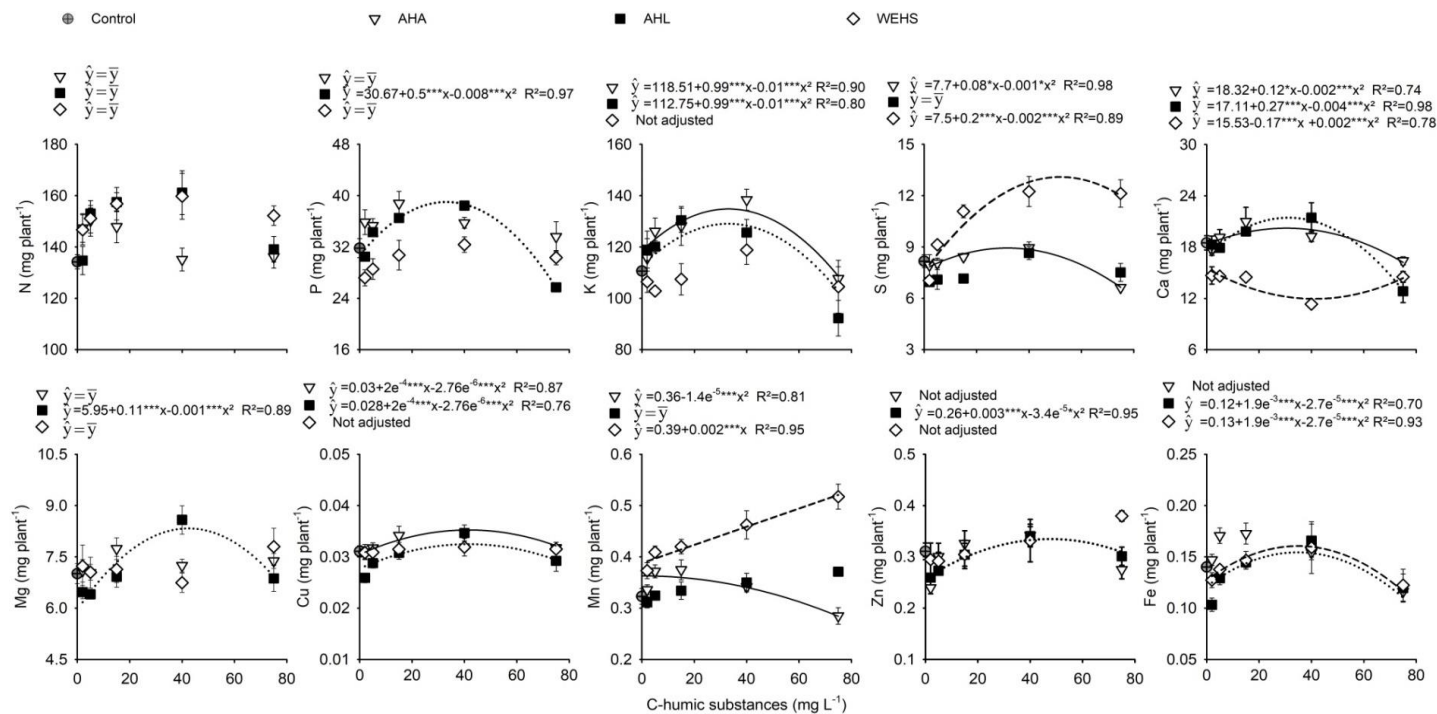
Figure 4–Accumulation of Nitrogen (N), phosphorus (P), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg),copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) in maize shoot cultivated in nutrient solution with Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS) over increasing C-humic substances concentrations. *, ** and *** significance of the mathematical equation parameters with *p*<0.05, *p*<0.01 and *p*<0.001, respectively. Bars represent the standard errors of the mean.
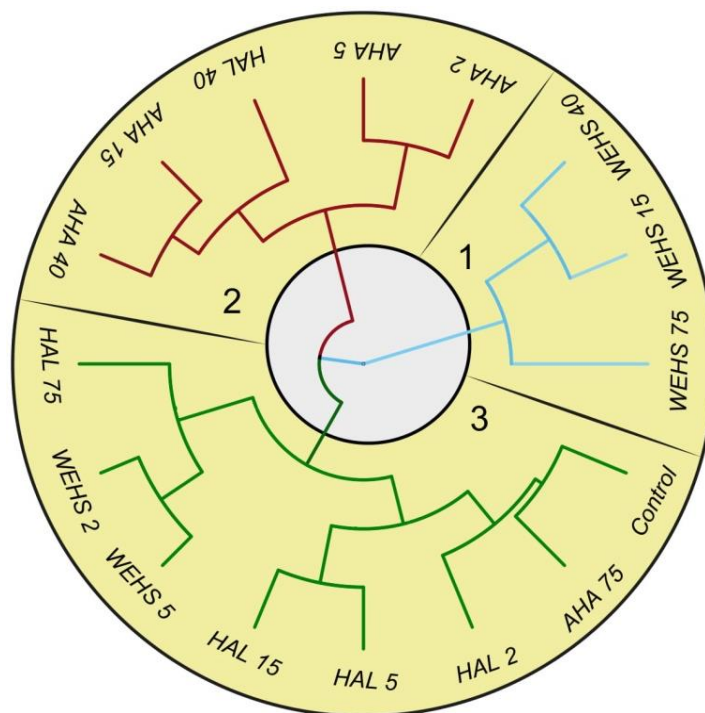
Figure 5- Dendrograms of hierarchical clustering analysis for Acros Organics®
humic acid (AHA), humic acid extracted from Leonardite (HAL) and water
extractable humic substances (WEHS) in concentrations of 2, 5, 15, 40 and 75
mg L^{-1} C and control treatment, according to Euclidean distance using 27
treatments that were self-scaled by the mean and the standard deviation.

3.5. *Organic acid exudation*

Organic acid exudation was analyzed only in plants treated with WEHS.
Nine organic acids were analyzed, however, malonic and lactic acids were
below the equipment quantification limit. Malic, isocitric and succinic acid
concentrations increased and tartaric acid concentrations decreased over WEHS
($p<0.05$; Figure 6). Citric and oxalic acid did not change over WEHS
concentrations ($p>0.05$). Maleic-fumaric acid did not fit to the mathematical

models studied. In addition, all organic acids exudate by maize roots were higher than concentrations measured for plants grown in control treatment.
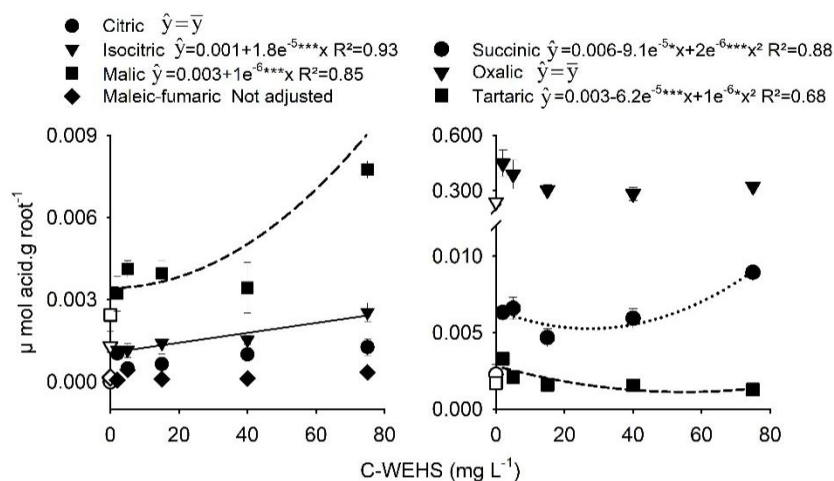
Figure 6– Organic acid maize root exudate cultivated in nutrient solution with increase concentration of water extractable humic substances (WEHS). White symbols represent control treatment.*, ** and *** significance of the mathematical equation parameters with *p*<0.05, *p*<0.01 and *p*<0.001, respectively. Bars represent the standard errors of the mean.

4   Discussion

As expected, higher concentrations of humic substances (HS) in treated pots lead to a higher nutrient solution carbon (SC) (Figure 1). Among the HS sources, water extractable humic substances (WEHS) keep more C in nutrient solution, due to high solubility in medium growth. These results indicated that WEHS was the HS source most adequate to be applied at high concentrations in nutrient solution. The increasing concentration of C-WEHS and C-HAL buffered the nutrient solution pH, which reduced one unit of pH value over the adjusted pH of control, 5.5 ± 0.3 to 4.4 (Figure 1). A similar result was found by Morais *et al*. (2018) using increasing concentrations of HA-Leonardite in nutrient solution

cultivated with eucalyptus. Furthermore, the pH increase was expected with the addition of alkaline materials such as HS. The high solubility of nutrients present in WEHS may explain the increase of electrical conductivity (EC) over C-WEHS concentrations (Figure 1) since ions and salts concentrations in the nutrient solution are indirectly expressed by EC (Carmo *et al*., 2016). On the other hand, the EC reductions over C-AHA can be associated with precipitation of HA with nutrient solution ions. HA can form complexes of variable stability with cations and anions (Olaetxea *et al*., 2018), which increases as C-HS concentration, reducing the nutrient solution EC.

The soluble nutrients in WEHS contribute to augmentation of P, K, Ca, Mg, S and Zn concentration in nutrient solution (Figure 2). However only P, K and Zn concentrations in nutrition solution with C-WEHS were higher than control, thus WEHS can increase these nutrients contents and, probably, nutrient uptake by maize. HAL due to high K contents in the solid phase (Table 1) increased K concentration in the growth medium. Furthermore, Fe concentration in growth media increases over C-AHA and C-HAL concentration in almost 50% in relation to control, indicating that HS sources can provide Fe to maize plants cultivated in nutrient solution. According to Ingham (2005), small fragments of low molecular weight HA fractions can release nutrients adsorbed in the HS structure. WEHS is known for its capacity to supply nutrients to roots (Ingham, 2005; Pant *et al*., 2009). In line with Pant *et al*. (2009), compost tea, extracted by different methods, supplied N, P, K, Ca, Mg, S, Mn, Zn, Cu and B to Pak choi (*Brassica rapa* cv. Bonsai, Chinensis group).

HS application increasing SPAD index in maize plants and the concentration of about 33 mg L^{-1} C-HS increased by 50% the SPAD index for the three HS tested (Figure 3). The results of this study are in line with data reported in the literature which showed an increase in photosynthesis in response to HS addition (Ertani *et al*., 2013; Aguiar *et al*., 2016). Aguiar *et al*. (2016) studying a

combination of growth promoting bacteria and HA observed an increase transpiration rate and stomatal conductance, a consequence of stomatal opening by plasma membrane H^+-ATPase activity in sugarcane treated with HA induced by the high auxin-like activity of HS, which increase net photosynthesis. An increase in leaf chlorophyll content and photosynthesis due to HS application lead to formation of ATP, amino acids, carbohydrates, and proteins (Nardi *et al*., 2009). The augment in photosynthesis is important to increase biomass production as reported by Berbara and García (2014) and Fan *et al*. (2014).

Application of 48 and 40 mg C-WEHS L^{-1} in nutrient solution leads to an increase of 28% of maize shoot dry matter (SDM) and 29 % and root dry matter (RDM), respectively, the highest growth observed, followed by HAL source, which increases maize shoot and root biomass by 19 and 21% (Figure 3). The increased production RDM with HS application is in line with results published by Jindo *et al*. (2012), which showed that addition of 20 mg C L^{-1} HA increased by 25 to 30% seven-day-old maize RDM with respect to control. Zanin *et al*. (2018) observed an increase of 12% in maize fresh weight treated two days with WEHS over control. In other studies with monocotyledon species, HS enhance the plant growth at different environmental conditions (Tavares *et al*., 2017; Aguiar *et al*., 2016). Rice plants cultivated in nutrient solution 80 mg L^{-1} HA increase the total length, area, volume and number of lateral roots compared with control treatment (Tavares *et al*., 2017). Aguiar *et al*. (2016) observed an increase of 50% of SDM and 173% of RDM in sugarcane treated with a combination of growth promoting bacteria and HA cultivated in Ultisol.

The H^+-ATPase in the plasma membrane of root cells is an indicator of HA bioactivity used in several works (Zandonadi *et al*., 2007; Busato *et al*., 2010; Ramos *et al*., 2015) and may explain the increase of RDM in maize treated with HAL and WEHS. According to Rayle and Cleland (1992) hydrolysis of ATP and ATP H^+ transport favors the action of enzymes that hydrolyze

polysaccharides from the cell wall, allowing cell expansion and plant root growth. Various signaling entities such as abscisic acid, nitrate root-shoot distribution, NO, reactive oxygen species, indole-3-acetic acid and cytokinins, dynamically regulate the root and shoot physiological events by triggering several pathways in response to HS (Olaetxea *et al*., 2018; Shah *et al*., 2018). As well as, HS can induce the production of endogenous hormones that influence plant growth and affect several plant physiological process (Zandonadi *et al*., 2014). Besides that, the drop observed in maize growth before optimum concentration are related to negative effects of HS on physiological processes like excessive auxin production, according to Taiz and Zeiger (2010) excessive auxin production reduces plant shoot and root growth, probably explained by the enhanced synthesis of ethylene. In addition, lipid peroxidation is triggering by application of high concentrations of HS, due to an increase of ROS production (Berbara and García, 2014), which imbalance delays the root cell differentiation process, reducing plant growth and development (Berbara and García 2014; García *et al*. 2016b).

In addition to maize growth, use of HAL in nutrient solution increased uptake of N, P, K, Mg and Ca which contributed to maize growth (Figure 4). AHA source increase accumulation of K due to their composition. Only accumulation of N and S increased with WEHS use. Excepting of accumulation of Mn, the other micronutrients studied did not show greater accumulation in maize shoot due to the addition of HS concentrations over control (Figure 4). According to Halpern *et al*. (2015)**,** the plant physiology mechanisms through which HS affect nutrient uptake include changes in root morphology and an increase in root activity of H^+ATPase and activity of NO_3^- assimilation enzymes. According to Ramos *et al*. (2015), the HA induce PM H^+-ATPase activity that controls the H^+ efflux and root surface pH, and as a consequence, controls the anions fluxes.

This hyperpolarization increases cytosolic Ca^{2+}, which acts as a second messenger response like CDPK activities and anion channel activation.

These results indicated that the high maize growth with HAL was related to increasing in accumulation of nutrients; on the other hand, the growth increase of plant treated with WEHS was probably related to the bioactivity of this humic fraction, since WEHS are supposed to have lower molecular weight, higher polarity and charged organic functional groups than humic fractions extracted with several different matrices with strong alkaline Na and K solutions.

The dendrogram summarises all results and indicate the better source and concentration to be used on maize cultivation (Figure 5). The first group is separated due to high concentrations of WEHS are responsible for the better results obtained with variables studied, represented by an increase in relation to control treatment. The concentration of 75 mL^{-1} C-WEHS was responsible to increase nutrients in growth medium, and the optimal concentration of variables such as dry matter, SPAD index, and accumulation of S, Fe, and Cu are in the range of 15 and 40 mg L^{-1} C-WEHS. Due to this, it is possible to infer that WEHS is the best source because it presented the high dissimilarity in relation to control treatment, based on Euclidean distance. Four of the five concentrations used of AHA are in the second group, thus, these concentrations were similar due this concentrations did not change the most of variables studied. In the third group, the treatments are more similar to control than the other groups, thus these results indicate that application of these treatments is not so efficient in improving maize growth and nutrition as was WEHS.

Plants exudate organic molecules due to active (Adeleke *et al*., 2017) and passive process (Neumann and Römheld, 1999; Bertin *et al*., 2003). The increase of malic, maleic-fumaric, and succinic acid over C-WEHS may be associated with HS interference in this process of exudation by roots. The increase in H^{+}ATPase activity and H^{+} extrusion by increasing concentrations of

WEHS may favor the outward diffusion of carboxylate anions (Bertin *et al*. 2003). The increase in oxalic, citric and tartaric acid in relation to control is in agreement to results found by Canellas *et al*. (2008), which found an increase in oxalic, citric and tartaric acid exudate in maize plants treated with 50 mg L^{-1} C-HA in relation to control. However, different from this study, the authors found succinic and malic acid only in control treatment. Erro *et al*. (2010) studying organic acid root exudation in P uptake from rhizosphere-controlled fertilizer with HS found malic and citric acid in maize root exudates. Organic acids are able to fragment HA in small bioactive units allowing plants to access bioactivity substances that affect plants morphology and physiology (Piccolo, 2002; Canellas *et al*., 2008). According to Canellas *et al*. (2008), there is a cross-talk between HA and plant cells in which HA effects on plant metabolism depend on changes in root organic acid exudation which in turn leads to changes in HA structure.

5   Conclusion

The best concentration to cultivate maize in nutrient solution was about 40 mg L^{-1} C-WEHS. Use of WEHS increased availability of P and K, and, mainly, of Zn in the nutrient solution, SPAD index and promoting a 26% higher maize biomass over control. Use of WEHS also increased maize root organic acids exudation, mainly malic, isocitric and succinic acids, whose concentrations in the nutrient solution are WEHS concentration-dependent. Enhancement in plant growth by Leonardite is explained by the changes in the availability of nutrients in the growth medium and nutrient accumulation in maize shoot. However, the best source to cultivated maize in nutrient solution was WEHS, due to effects in maize growth, besides being a highly sustainable source extracted from compost. In future studies, the effect of type and contents of organic acids exudate by

maize roots in fragmenting humic substances in smaller bioactive units should be investigated.

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2.3   ARTIGO 3- Humic substances source effects on bean growth and on its root organic acid exudation profile

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Abstract

Humic substances (HS) can improve plant growth and nutrient uptake, but its biostimulant effect depends on the concentration, source, among other HS properties. The aim of this study was to evaluate bean (*Phaseolus vulgaris*) growth, nutrient uptake and growth medium treated with different HS sources and root organic acid exudation profile. Bean plants were cultivated during 35 days in nutrient solution with the addition of Acros Organics® humic acid (AHA), HA extracted from Leonardite (HAL) and water extractable humic substances (WEHS) at concentrations of 2, 5, 15, 40 and 75 mg L^{-1} C-HS, plus control. In nutrient solution, before bean cultivation, it was determined the electrical conductivity (EC), pH and nutrient contents. It was also measured the SPAD index, shoot dry matter (SDM), root dry matter (RDM) and nutrient accumulation in shoot. Root organic acid exudation was determined in plants treated with HAL. Addition of C-HS increases the EC, nutrient solution P, K, Ca, Mg, S, Fe and Mn contents, whose magnitude of effects is HS source-dependent. HAL improved the SPAD index, RDM up to 29%, and slightly increased SDM. The addition of HS was not effective in improving nutrient accumulation in bean plants. The bean plant cultivated with HAL exudate acetic, D-malic, oxalic, quinic, and succinic acids whose amounts were dependent on HAL concentration added to the nutrient solution.

Keywords: compost tea; humic acid optimum concentration; Leonardite-derived humic substances; root exudates; SPAD.

Introduction

Humic substances (HS) are used in production systems to regulate the physiologic process and improving field crops yield and quality (Shah et al. 2018), due to capacity to improve plant mineral nutrition, acts to attenuate plant

abiotic stress, prevent heavy metal toxicity, improve root and shoot growth among other effects (Halpern et al. 2015; Shah et al. 2018; Olaetxea et al. 2018; Canellas et al. 2018). Some of these effects result from the interaction among HS, plants, and microorganisms those results in increase or decrease in physiological processes or plant growth, that corresponds to HS bioactivity (Nardi et al. 2009; Zandonadi et al. 2014). HS bioactivity is explained, among other factors, due to the presence of growth hormones in its structure (Jindo et al. 2012), production or modulation of several plant growth hormones, such as auxins, gibberellins, and cytokinins, which promote changes in the plant physiology (Shah et al. 2018; Olaetxea et al. 2018; Canellas et al. 2018).

Positive effects of HS application on bean growth were already verified (Rosa et al. 2009; Qian et al. 2015; Melo et al. 2017) and also for another plant species (García, Santos, et al. 2016; Morais et al. 2018; Rosa et al. 2018; Canellas et al. 2018). Qian et al. (2015) found augments in leaf and bean root growth regardless of the HA-Leonardite molecular weight applied. Rosa et al. (2009) reported a 30% increase in bean shoot and 41% in root biomass cultivated in nutrient solution with 11 and 20 mg L^{-1} C-HS charcoal-derived, respectively. On the contrary, Akinremi et al. (2000) observed no significant effect on bean dry matter in soil treated with HA-Leonardite.

Increase in plant growth and nutrient assimilation due to HS application arises from the direct and indirect effects of HS on soil and plants. The direct effect is localized on plant cell membranes, physiology, and biochemical processes and the indirect effects are linked to the role played by HS in interacting with ions and molecules or due to changes in the growth medium, which affect nutrient bioavailability and acquisition by plants (Shah et al. 2018; Olaetxea et al. 2018). Both effects, direct and indirect, depends on HS chemical properties, concentration and source, and plant physiological stage, species and organ (Rose et al. 2014; Zandonadi et al. 2014). Thus, it is important to know

the bioactivity of different HS sources and the optimum HS concentration, because high concentrations may inhibit plant growth (Canellas and Olivares, 2017). In addition, optimum concentration is crop-dependent since in nutrient solution some authors reported that the optimum HS are in the range of 5 to 50 mg L^{-1} C (Façanha et al. 2002; Canellas et al. 2008; Pinheiro et al. 2010; Canellas and Olivares, 2017).

The response of crops to HS application is another important factor to be investigated because the effects of HS on monocotyledons is greater than in dicotyledons, however, there is no clear molecular or physiological basis to explain this difference (Rose et al. 2014; Canellas et al. 2015). Based on this assumption, it is important to study the effects of HS in bean plants since only a few studies have focused on the action of humic materials on this crop (Qian et al. 2015; Melo et al. 2017). Besides, in Brazil, common bean is a crop of great economic impact, only in 2018, it was harvested 3 million hectares in Brazil (CONAB, 2018). Furthermore, bean root organic acid exudation can be a key route to understand more about HS biostimulant effect, since root exudation is influenced by biotic and abiotic stress (Adeleke et al. 2017), and some HS effects are associated to plant stress. HS addition can change the nature and amount of low molecular weight compounds, and the magnitude of effects depend on the HS source (Puglisi et al. 2013). Low molecular weight organic acids are able to fragment supramolecular HA structures in small bioactive units (Piccolo 2002; Canellas et al. 2008).

Thus, it is important to study the bioactivity of HS as a function of source and concentration in bean plants, to outline conditions where HS application in beans plants is more responsive. Thus, the aims of this study were to: i) evaluate the changes in the nutrient solution, growth, and SPAD index of bean cultivated in nutrient solution treated with different humic substances sources and concentrations; ii) identify the optimum HS source and concentration to improve

bean nutritional status and growth; and iii) analyze the profile of root organic acids exudation of the best HS source.

Material and methods

Humic substances sources and extraction

Acros Organics ® humic acid p.a (AHA) is a commercial product pure to analyses with 45-70% sodium salt, and humic acid extracted from Leonardite (AHL) through the use of a 0.5 mol L^{-1} KOH solution, and, in sequence, purified, following the method recommended by Internacional Humic Substances Society (Swift, 1996). Water extractable humic substances (WEHS) was extracted from a 150 days-humified dried and ground compost formulated with 60% (v/v) coffee husk, 30% chicken manure, and 10% biochar. To obtain WEHS, compost and water at 80°C were mixed at a ratio 1:10 (w/v), shaked for 4 hours on a horizontal shaker at 90 rpm, then, centrifuged for 15 minutes at 2800 rpm and filtered on GF1 glass fiber microfilter.

Plant material and growth conditions

The experiment was carried out with bean (BRS Notável) cultivated in nutrient solution under greenhouse conditions at the Department of Soil Science/Federal University of Lavras. Treatments were arranged in a 3 x 5 + 1 factorial scheme with four replicates, through the combination of three humic substances (HS) sources: AHA; HAL and WEHS, and five HS carbon concentrations: 2; 5; 15; 40 and 75 mg L^{-1} C-HS, plus the control treatment in which no HS was added to the nutrient solution.

Previously to cultivation, the bean was sown on vermiculite, and twelve-day-old seedlings were transferred to trays containing half strength of modified Hoagland and Arnon (1950) nutrient solution in which plants remained seven days for adaptation. Nutrient contents in the nutrient solution used to grow bean plants were: 210 mg L^{-1} N; 31 mg L^{-1} P; 234 mg L^{-1} K; 160 mg L^{-1} Ca; 49 mg L^{-1}

Mg; 65 mg L^{-1} S; 0.80 mg L^{-1} B; 1 mg L^{-1} Mn; 0.2 mg L^{-1} Zn; 0.05 mg L^{-1} Cu; 0.01 mg L^{-1} Mo and 5 mg L^{-1} Fe. After the adaptation phase in the nutrient solution, bean seedlings were transferred to pots with 1.3 L of the modified Hoagland and Arnon (1950) nutrient solution with pH adjusted to 5.5 ± 0.3 and the respective treatments. One bean plant was grown per pot and aeration was constant and individualized throughout the experimental period. The volume of each pot was completed daily using deionized water.

HS Chemical characterization

Electrical conductivity (EC) and pH of AHA and HAL were determined using deionized water and HA: water ratio of 1: 2.5 (1g: 2.5 mL), in an aliquot of WEHS was determined directly. The C of HS was determined in analyzer of the Elemental brand, model Vario TOC Cube, the total C of AHA and AHL was determined in solid mode and C-WEHS in liquid mode. To determination of HA macro and micronutrients contents 500 mg of AHA and HAL was digested with 8 mL of a mixture of nitric and perchloric acids at a 4:1 ratio. Contents of P was determined by the molybdenum blue reaction method, S through reaction with a barium chloride solution, and B through reaction with azomethine-H using an UV-visible spectrophotometry at 660 nm for P, 420 nm for S and 460 nm for B. The total content of N was determined by digestion in acid medium and distillation through the *Kjeldahl* method. The contents of Ca, Mg, Cu, Fe, Mn, and Zn were determined through atomic absorption spectrometry with air-acetylene and K and Na through flame photometry. To determine the water-soluble nutrient content of AHA and HAL 0.04 g was diluted in 50 mL, then the macro and micronutrient determination, as well as the nutrients of WEHS, was performed in Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

Nutrient solution and plant analyses

After one day of bean cultivation, an aliquot of the nutrient solution was collected from each experimental pot for pH and EC determination, and P, K, Ca, Mg, S, Fe, Mn, and Zn contents was quantification through the use of ICP-OES. The bean plants were cultivated for 35 days. In the end of the experiment leaf greenness content was determined using SPAD (Minolta, SPAD-502, Osaka, Japan), which was quantified at the last expanded leaf in six replicates for each plant. In sequence, the plant roots cultivated with HAL were washed with ultrapure water and dipped into pots containing 230 mL of ultrapure water, protected from sunlight, and kept for 3 hours under oxygenation to obtain root exudates. The root exudates were filtered in a 0.45 μm filter and stored in 45 mL bottles and ultrafreezed for posterior analysis.

Bean growth was assessed by measuring shoot dry matter (SDM) and root dry matter (RDM). The bean root and shoot tissues were stored in paper bags and dried in an oven at 60°C until constant weight. After drying, biomass was weighed to determine SDM and RDM, and total dry matter (TDM) through the sum of SDM and RDM. The SDM was ground in a Willey mill. The plant material, 500 mg, was digested with 8 mL of a mixture of nitric and perchloric acids at a 4:1 ratio to the determination of macro and micronutrients in the bean shoot. The N content was determined using the *Kjeldahl* method. The macro and micronutrients were determined according to the methods described at the Embrapa manual for chemical analysis of soil, plants, and fertilizers (Silva, 2009). The accumulation of each nutrient was determined in shoot through multiplication of nutrient concentration in the plant tissue by its respective dry matter.

Root exudates chromatographic analysis

The root exudates (45 mL) from bean plants treated with C-HAL were lyophilized (freezer Enterprise 1- Terroni) until the total water withdrawal to concentrate the organic acids. In the lyophilized material, it was added 1 mL of

ultrapure water to solubilize the material. The organic acids root exudate were determined by HPLC (Agilient 1220 Infinity), using the Synergi-Hydro RP 80A (250 x 4.6 nmid; 4 μm) column and the UV-visible at a wavelength of 220 nm. The mobile phase used was a KH_2PO_4, 20 mM L^{-1} solution, pH 2.9 at a flow rate of 0.7 mL min^{-1}. It was injected 100 μL of each sample and the run time was 16 min. As analytical standards, certified samples were used, being analyzed the acetic, citric, fumaric, maleic, D-malic, oxalic, quinic, shikimic, and succinic acids. The standard analytical curves were obtained by relating different concentrations certified organic acids with their respective area in the chromatogram. Mathematical equations obtained for each certified organic acid are show in Table 1. Detection limits (DL) and quantification limits (QL) for each organic acid were estimated according to Ribani et al. (2004): DL = 3.3 (s/S) and DQ = 10 (s/S), where "s" is a estimation of the standard deviation of response obtained by the linear coefficient of equation, and "S" is the angular coefficient of the analytical curve (Slope) (Table 1). The identification of organic acids in the samples was made based on the retention time of each organic acid in the chromatogram with the certified samples.

Statistical analysis

Organic acid exudation was calculated based on its release per unit of root dry weight. In some samples, the concentration of organic acids was below the quantification limit, so no statistical test was performed, and the results are represented only with bar errors. Total contents of organic acid exudate were correlated to shoot dry matter. The dataset was subjected to analysis of variance and regression ($p<0.05$), to verify the interaction between HS sources and concentrations, as well as the difference between the control treatment and the factorial on the bean growth attributes, nutrient solution and SPAD index. A contrast analysis was performed to identify if the effect HS addition is different from the control treatment. The regression model that best fitted to the dataset

was chosen based on the significance of the mathematical equation parameters ($p<0.05$), on the lowest value of the sum of squared errors, and on the equation with the highest adjusted coefficient of determination (R^2). Statistical analyses were performed using the computer program R Studio 1.0.136 using the ExpDes package (Ferreira et al. 2013) and Sisvar 5.6 (Ferreira 2014).

Table 1- Linearity, regression equation (chromatogram area *vs* organic acid concentration), detection limit (DL), quantification limit (QL) and organic acid retention time (Rt)

Organic acid	Linearity (μ mol L^{-1})	Regression equation	r	DL	QL	Rt (min)
Oxalic	58-2888	y=3.8023x + 137.43	0.99	38	114	3.6
Quinic	204-1223	y=0.6657x + 37.929	0.99	47	143	4.3
D-malic	216-1618	y = 0.6579x - 13.774	0.99	50	153	5.3
Shikimic	12-1191	y = 61.602x + 361.51	0.99	27	82	6.2
Acetic	291-8742	y = 0.231x - 1.695	1	32	98	7.3
Citric	208-2084	y = 1.5029x - 24.586	0.99	79	239	8.4
Maleic	12-1818	y = 82.437x + 2904.5	0.99	79	240	8.7
Fumaric	11-1706	y = 76.821x + 5030.6	0.98	246	746	9.6
Succinic	267-2007	y=0.3494x+0.303	1	12	38	11.3

Results

Nutrient solution

The humic substances (HS) pH was 9.3, 9.7, and 8.7 to Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL), water extractable humic substances (WEHS) respectively. Eletrical conductivity (EC) of HS materials was: 23 dS m^{-1} to AHA, 36 dS m^{-1} to AHL, and 0.43 dS m^{-1} to

WEHS. Principal properties as well as their nutrient contents soluble in water are shown in Table 2.

Table 2- Chemical and physicochemical properties of humic substance source (HS): Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL), water extractable humic substances (WEHS) and water-soluble nutrient content present in 1 g L^{-1} of AHA* and HAL*

HS	Property											
	C	N	P	K	Ca	Mg	S	Fe	Na	Cu	Mn	Zn
	---------------------------- g kg^{-1} ----------------------------									------ g kg^{-1} ------		
AHA	330	7.5	0.21	3.9	5.6	0.8	4.5	1.2	71	46	19	25
AHL	350	5.2	0.05	41.6	1.4	2.6	2.8	2.7	3.2	7.6	21	76
	-- mg L^{-1} --											
WEHS	2400		8.9	54.2	3.5	40	5.3	-	-	0	0.8	1.7
AHA*	-		0.07	2.9	4.6	1.2	3.7	9.6	-	0.03	0.03	0.01
HAL*	-		<QL	144	0.30	2.94	2.9	1.9	-	<QL	0.02	0.01

*1 g L^{-1}; QL: quantification limit.

The electrical conductivity (EC) and pH of the growth medium at one day after cultivation were influenced by the interaction between the humic substances (HS) sources and concentrations (Figure 1; $p<0.05$). The linear model was adjusted to the pH increase over C-AHA concentration and application of 75 mg L^{-1} increased pH in one unit. The pH of the nutrient solution decreased over C-HAL in a quadratic manner, and did not change over C-WEHS. Nutrient solution EC augmented over C-HS concentrations, and application of 75 mg L^{-1} C-WEHS increased ~5% EC in comparison to control.

The addition of HS sources in increasing C concentration in nutrient solution changed the concentrations of P, K, S, Ca, Mg, Cu, Fe, Mn, and Zn one day of the treatment addition ($p<0.05$; Figure 2). Concentrations of P, K, S, Ca, Mg, and Fe over HS concentrations were higher compared to control. The quadratic model fitted better to P, S, Ca, Mg, and Fe concentrations in the nutrient solution treated with AHA. The concentration of about 45, 50, 40, 42, and 35 mg L^{-1}
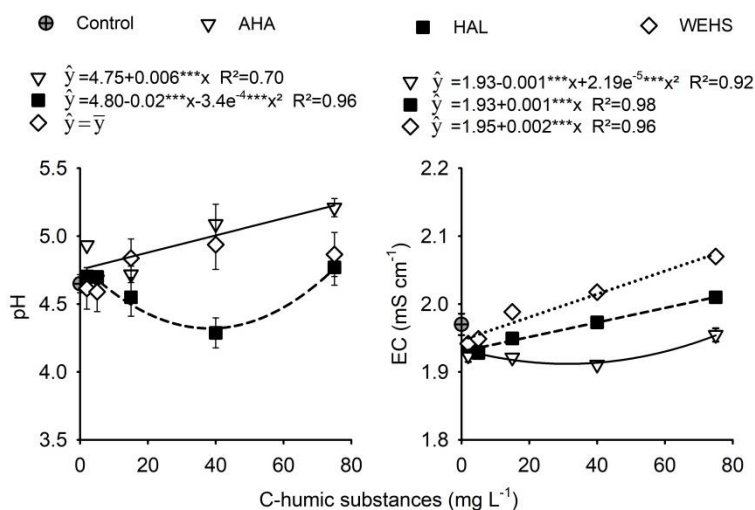
Figure 1– Nutrient solution pH and electrical conductivity (EC) one day after the addition in nutrient solution of Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS) at different concentrations, and in the control for bean cultivation.*, ** and *** significance of the mathematical equation parameters at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bar represents the mean standard error.

AHA can increased in 8, 9, 8, 9, and 19% P, S, Ca, Mg, and Fe concentrations, respectively, in relation to control, Cu and Zn contents in nutrient solution decreased over C-AHA, while Mn contents did not change over C-AHA concentrations. With the C-HAL addition to nutrient solution, P, Ca, Mg, Cu, Mn and Zn, contents decreased and S and Fe contents did not change. However, 2 mg L^{-1} HAL increased in 11% P and Ca, 10% S and Mn, 12% Mg, and 15% Fe contents in comparison to control. K content in nutrient solution increased 15% with 75 mg L^{-1} C-HAL. The linear model was the best fit for the increase of P, K, and S over C-WEHS concentration. The application of 75 mg L^{-1} C-WEHS leads to an increase of 26, 23 and 12 % of P, K and S contents in the nutrient solution, respectively. Cu content decreased in a quadratic manner over
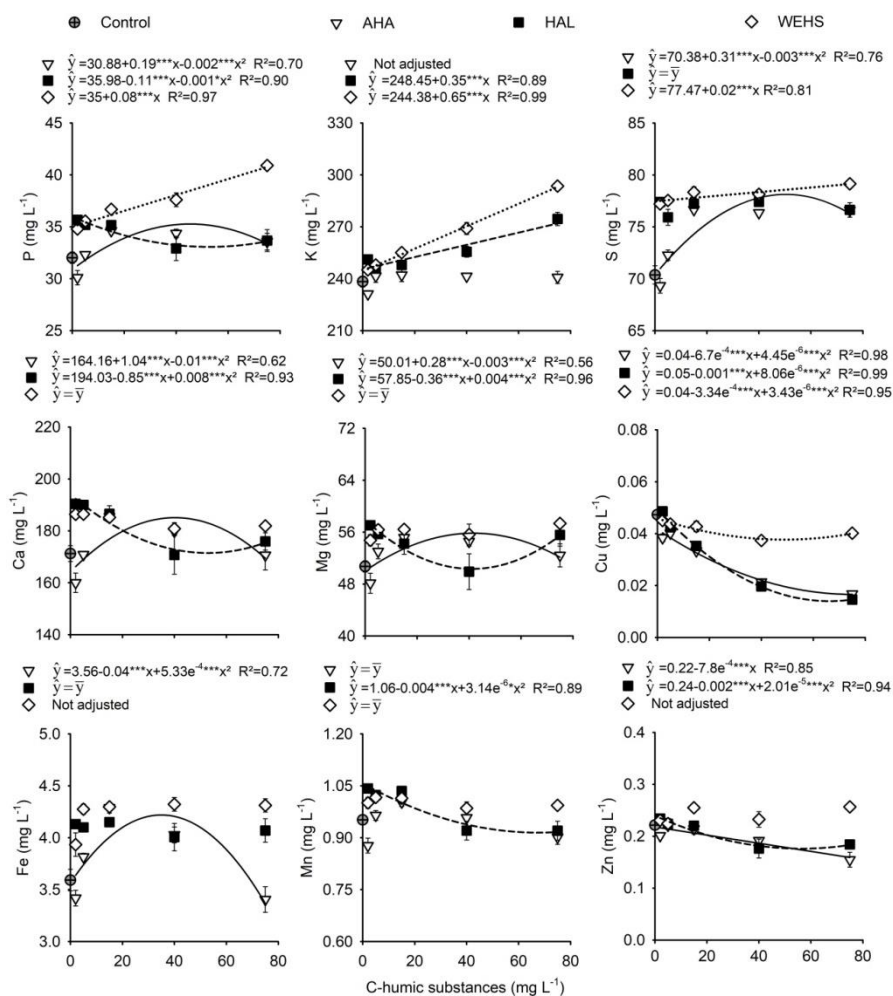
Figure 2– Nutrient solution phosphorus (P), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) contents one day after the addition in nutrient solution of Acros Organics® humic acid (AHA), humic acid extracted from leonardite (HAL) and water extractable humic substances (WEHS) at different concentrations, and in the control for bean cultivation.*, ** and *** significance of the mathematical equation parameters at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Bar represents the mean standard error.

C- WEHS concentrations, while Ca, Mg, and Mn contents did not change, but they were higher than the control. Five mg L^{-1} C-WEHS increased by 19% Fe

contents in the nutrient solution, though Fe and Zn contents did not adjust to any of mathematical model tested.
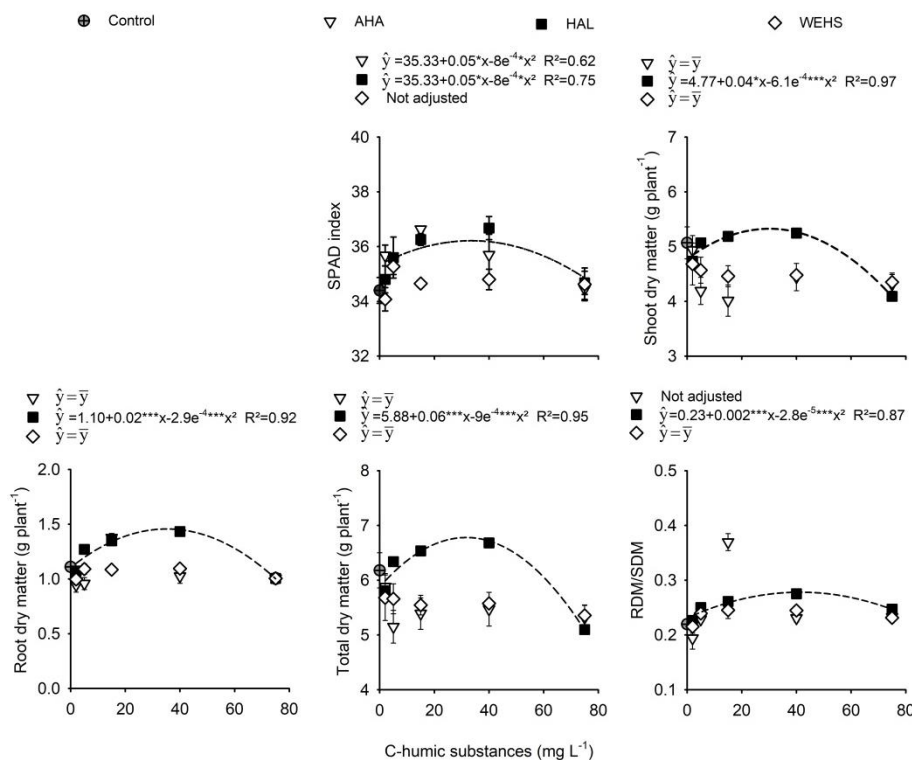
Figure 3 – SPAD index, shoot dry matter, root dry matter, total dry matter, root/shoot dry matter ratio of bean cultivated in nutrient solution treated with Acros Organics® humic acid (AHA), humic acid extracted from leonardite (HAL) and water extractable humic substances (WEHS) at different C-humic substances concentrations, and control.*, ** and *** significance of the mathematical equation parameters with $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Error bars represent the standard errors of the mean.

Leaf greenness content (SPAD index) and maize growth

The SPAD index was indirectly used to infer the content of chlorophyll in the bean leaf. Thus, bean leaf greenness content was influenced by HS source and concentrations ($p<0.05$; Figure 3). The SPAD index followed a quadratic model

over C-AHA and C-HAL concentrations and reaches a maximum at 33 mg L^{-1} C-HAL and C-AHA, both presented leaf greenness content higher than control. Shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM) and root/shoot dry matter ratio (RDM/SDM) were influenced by the interaction HS sources-concentrations ($p<0.05$; Figure 3). The quadratic model was the one that best fitted to increased biomass over C-HAL concentrations. The augment of RDM and RDM/SDM with the use of HAL were higher than control. The concentration of 35 mg L^{-1} C-HAL can increase in 29% RDM, however, SDM only 4% in comparison to control. Bean dry matter did not change over AHA and WEHS concentrations.

Bean nutrition

Accumulation of P, Cu, Fe, and Zn in bean shoot were influenced by the interaction HS sources-concentrations ($p<0.05$; Figure 4). Amounts of P and Cu in bean shoot decrease with the application of C-AHA and C-HAL and did not change with WEHS application. In addition, the accumulation Fe and Zn in the bean shoot linearly decreases over C-HAL, however the addition of 5 mg L^{-1} C-HAL increased in 46% the amount of Fe in relation to control. Use of AHA and WEHS did not influence the accumulation of Fe and Zn in bean shoot. The amounts of N, K, S, Ca, and Mg in bean shoot was affected by HS sources ($p<0.05$; Figure 5). Accumulations of N and K in bean shoot treated with HS were lower than the control, but with WEHS application, the accumulation of K was higher than those of other HS sources. In addition, the accumulation of N in plants treated with WEHS and HAL were higher than bean treated with AHA. The accumulations of Ca, Mg, and S in bean shoot treated with HS were similar to control. With the use of AHL, the bean plants accumulated more Ca, Mg and S than plants treated with HAL and WEHS.
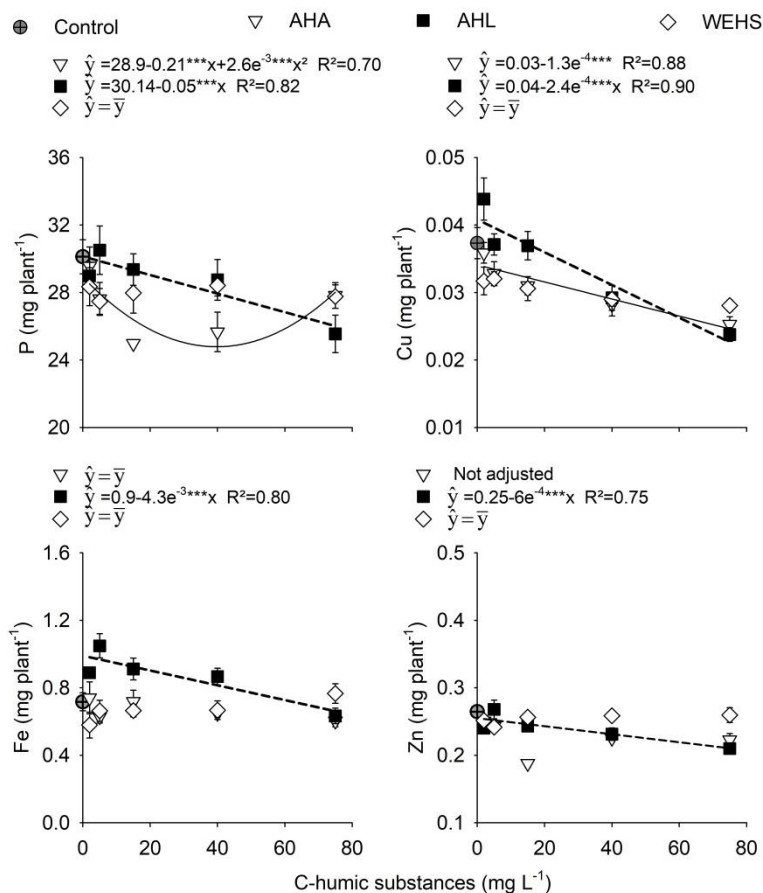
Figure 4 –Phosphorus (P), copper (Cu), iron (Fe), and zinc (Zn) content, in bean shoot cultivated in nutrient solution with Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS) at different C-humic substances concentrations, and control. *, ** and *** significance of the mathematical equation parameters with $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Error bars represent the standard errors of the mean.
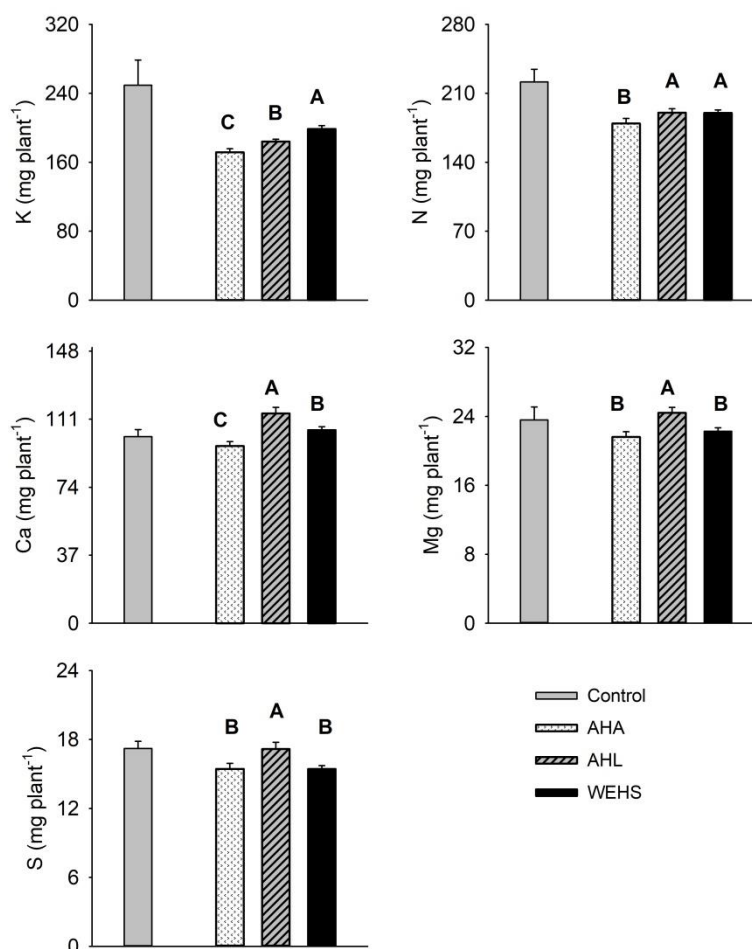
Figure 5 –Nitrogen (N), potassium (K), sulphur (S), calcium (Ca) and manganese (Mn) content in bean shoot cultivated in nutrient solution with Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS) at different C-humic substances concentrations, and control. The value presented for each source corresponds for averages for all concentrations. Means followed by the same letter are not statistically different by the Scott-Knott test ($p<0.05$). Error bars represent the standard errors of the mean.

Root organic acid exudation

It was analyzed in the bean root exudates: acetic, D-malic, oxalic, quinic, and succinic acids, but citric, fumaric, maleic and shikimic acids contents were above the quantification limit (Figure 6). The concentration of organic acids ranged from 0.88 µmol g root dry matter $(RDM)^{-1}$ (D-malic) to 34 µmol g RDM^{-1} (Oxalic). Plants treated with 2 mg L^{-1} C-HAL released up to 9 µmol g RDM^{-1} of succinic acid and with 5 mg L^{-1} C-HAL released the highest amount of D-malic acid (3.7 µmol g RDM^{-1}). In general, at 75 mg L^{-1} C-HAL caused the highest exudation of organic acid. Acetic, oxalic and quinic acids in plants cultivated with 75 mg L^{-1} C-HAL released 27, 34 and 5.4 µmol acid g RDM^{-1}, respectively. Significant and negative correlation was found between total organic acid exudation and SDM (r =-0.96 $p<0.05$).

Discussion

One day after treatment application, the pH of control reduced from 5.5 to 4.6, and thus the addition of Acros Organics® humic acid concentrations (AHA) buffered the solution near the initial pH of 5.5 (Figure 1). Depending on the rate applied, the addition of alkaline humic materials is expected to increase the pH of nutrient solution. Morais et al. (2018) reported the HA buffer capacity in the nutrient solution used to cultivate eucalyptus as the HA concentration was increased. Electrical conductivity also increases over C-HS addition, which is related to the increase of ions in nutrient solution added by the HA, mainly by water extractable humic substance (WEHS). EC expresses the ions and salts concentrations dissolved in the nutrient solution (Carmo et al. 2016), in this situation, possibly induced by amounts of nutrients added HS concentrations. These results are in line with those reported by Morais et al. (2018) and Pinheiro et al. (2010) in cultivation of eucalyptus in nutrient solution.
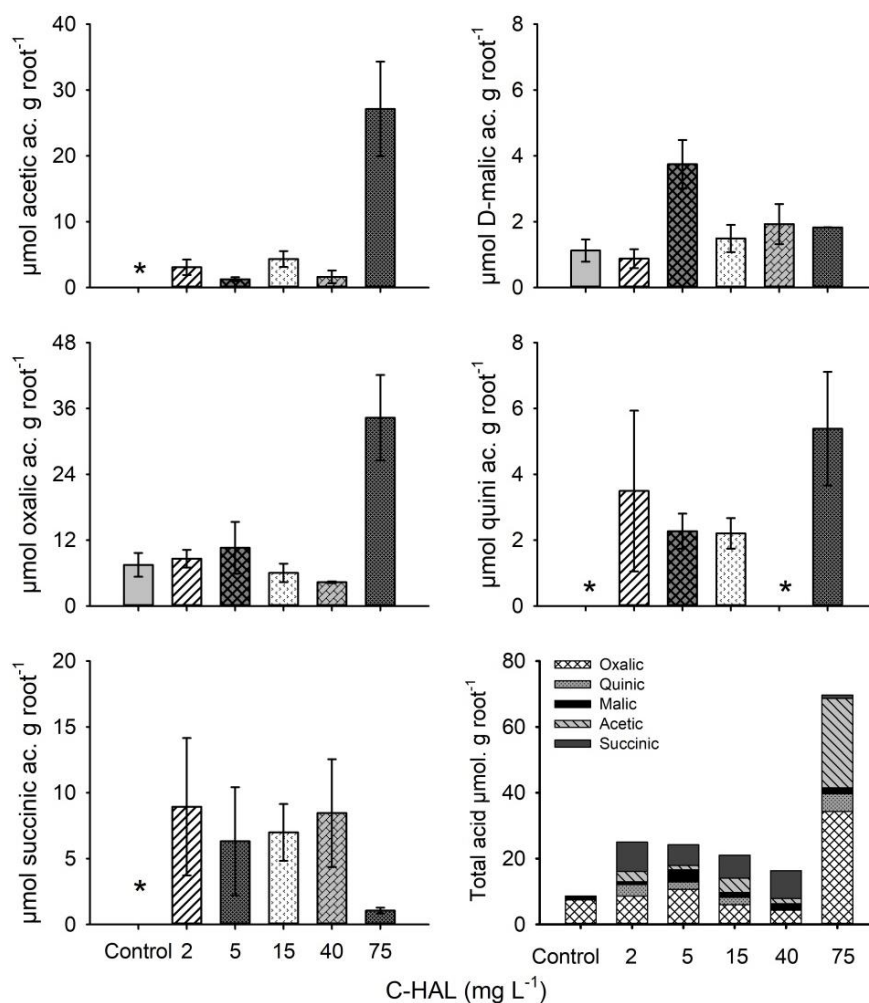
Figure 6- Organic acids exudation of oxalic, quinic, malic, acetic, succinic acids and total organic acid by bean roots cultivated in nutrient solution with increased concentrations of humic acid extracted from leonardite (HAL). Bars represent the standard errors of the mean. * more than two replicates below the detection limit.

Optimal concentrations of the HS sources added to the growth media contribute to increase macronutrients, Fe and Mn, which results in EC augment (Figure 2 and 3). AHA supplied more Fe and HAL supplied more K due to their

composition (Table 2). The results demonstrate that WEHS can be a suitable source of P, K, and S in a nutrient solution, and AHA can be a suitable Fe source to bean. In some studies, it was already reported the capacity of HS in supplying nutrients to plants in several growth medium (Pant et al. 2009; Rose et al. 2014), depending on the contents of nutrients found in the HS (Ingham 2005). According to Olaetxea et al. (2018) and Shah et al. (2018), the capacity of HS in supplying nutrients is classified as an indirect effect of HS on plant growth. In line with Pant et al. (2009), compost tea, here called WEHS, extracted by different methods, supplied efficiently N, P, K, Ca, Mg, S, Mn, Zn, Cu and B to Pak choi (*Brassica rapa* cv.).

On the other hand, the addition of HS in nutrient solution reduced the contents of Cu and Zn. These results can be explained by the capacity of organic matter fractions to form complexes of variable chemical stability with cations and anions (Olaetxea et al. 2018). The stability of the bonds in the organic metallic complexes (OMCs) depends on the type of cation and the reactivity and functional groups dissociation degree of humic ligands (Guppy et al. 2005; Urrutia et al. 2014). Studying the HS influence on the bioavailability of Cu and Zn during composting of sewage and maize straw, Kang et al. (2011) found that HA-Cu and HA-Zn increased as composting period increased. Such effect may be associated with the high affinity of humified organic matter in complexing these micronutrients.

HS application also enhanced leaf greenness (SPAD index) of bean plants (Figure 3), that is associated with chlorophyll content in leaf. In other studies, it was already reported the effect of HS in increasing the SPAD index (Azcona et al. 2011; Fan et al. 2014; Aguiar et al. 2016). The augment in photosynthesis rate is a key factor to enhance biomass production (Berbara and García 2014; Fan et al. 2014). Fan et al. (2014) observed that improving photosynthetic characteristics and chlorophyll content over HA use increased chrysanthemum

growth. Azcona et al. (2011) also associated a greater pepper growth due to the increase of photosynthetic activity in plants treated with HS over control.

Only HAL improved the bean growth with a maximum increase in roots of ~29% over control (Figure 3). The increased production of bean root in response to HA application is in line with results published by Qian et al. (2015) who verified that different fractions of HA-Leonardite stimulate leaf and bean root growth; HA with low-molecular weight was more effective in improving root length and surface area, while leaf growth was affected by HA with the highest molecular weight. Melo et al. (2017) found that the use of co-inoculation of rhizobia and *Herbaspirillum seropedicae* with HS-vermicompost promoted a significant increase of shoot and root bean dry biomasses in plants with full water supply, but the enhancement was more pronounced under water-stress conditions. HAL presented groups of carboxilic acid, and high content of carboxylic groups in the HA structure has been related to increasing auxin activity (Conselvan et al. 2017).

The shoot growth increase verified in this study due to HAL application was too small. According to Rose et al. (2014), the shoot growth is affected by HS source and concentration, because, multiple chemical functional groups of humic material behave completely differently under different environmental conditions. The increase in bean root growth treated with HAL may be associated to increase of H^+-ATPase activity reported to some authors that are linked to auxin (Busato et al. 2010; Ramos et al. 2015), which results in cellular expansion (Rayle and Cleland, 1992). The role played by HS in increasing plant growth is attributed to their capacity to produce or modulate several plant growth hormones or contains in its structure, or other signaling molecules (Shah et al. 2018; Olaetxea et al. 2018; Canellas et al. 2018), as well as can induce the production of endogenous hormones (Zandonadi et al., 2014). According to Berbara and García (2014), ROS can regulate root growth by an independent

route of auxins; additionally, ROS produced by NADPH oxidases stimulating Ca^{2+} influx in the apical plasma membrane, which are associated to root growth. The action of HA on plant growth involves a combined effect indole-3-acetic acid, cytokinins, ROS, NO abscisic acid signaling pathways in the root, and nitrate root-shoot distribution (Olaetxea et al. 2018; Shah et al. 2018).

The increase in nutrient contents in medium growth (Figure 2) did not reflect in augment in bean growth and nutrient accumulation in bean shoot. The reduction in Cu and Zn accumulation are in agreement to initial decrease of these nutrients in the growth medium (Figure 2), which possibly is associated with the high affinity of C to these micronutrients, forming in sequence OMCs (Kang et al. 2011). Only Fe content in bean shoot increase with HAL use. In general, the nutrient content of bean plants cultivated with HS is equal or lower then nutrient contents found in plants without HS treatment. These results is in line with those results published by Azcona et al. (2011) that verified no effects in pepper leaf nutrients accumulation treated with HS derived from composted sludge and Leonardite.

According to Bertin et al. (2003), the extrusion of protons through ATPase generates a positive charge gradient that releases carboxylate anions. Thus, exudation of acetic, D-malic, oxalic, quinic, and succinic acids may be associated with HS interference in this process of exudates released by roots. High amounts of acetic, oxalic and succinic acids were found in response to the highest concentration of C-HAL. It is likely that the exudation of these acids is related to plant stress due to high concentrations of HAL application, since, in this work, shoot growth decreased over the increase of total organic acid exudation ($r = -0.96$), in addition, high HAL concentrations decrease acquisition of some nutrients (Figure 2 and 3). According to Adeleke et al. (2017), the root organic acid exudation is influenced by biotic and abiotic stress. On the other hand, D-malic and succinic acid were in higher quantity in small C-HAL

concentrations, which may be associated with an improvement in nutrient concentration in the nutrient solution and bean growth.

Moreover, root exudate of organic acids can fragments HA in small bioactive units, allowing plants to access bioactivity substances, that affect plants morphology and physiology (Piccolo 2002; Canellas et al. 2008). Amphiphilic properties of the acids interact with the hydrophilic and the hydrophobic domains of humic aggregates that disrupting weak forces which stabilize the HA (Piccolo 2002). According to Canellas et al. (2008), there is a cross-talk between HA and plant cells in which HA effects on plant metabolism depend on changes in root organic acid exudation which, in turn, leads to changes in HA structure.

Conclusions

The availability of nutrients in the nutrient solution depends on the source of HS used, and did not affect the accumulation of nutrient in bean shoot. Acros Organics HA and water extractable HS were not effective in improving bean growth and nutrient uptake. Bean growth, especially root biomass treated with HAL, increased ~29% in relation to control and the amount of biomass produced is HA concentration-dependent. Humic acid extracted from Leonardite improving the exudation of acetic, D- malic, quinic, oxalic and succinic acids by bean roots. Addition of 32 mg L^{-1} C-HAL assured the bean biomass production.

Acknowledgments

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APÊNDICE A- Tabela de análise de variância do artigo 2 – Milho

Table 1 – *p* value and coefficient of variation of nutrient solution and maize growth variables treated with different sources and humic substances concentrations

SV	df							*p* value						
		SC	pH	EC	NsP	NsK	NsCa	NsMg	NsS	NsFe	NsZn	NsMn	SPAD	RDM
Block	3	0.291	0.084	0.621	0.581	0.793	0.830	0.850	0.197	0.008	0.193	0.051	0.496	0.031
HS sources	2	0	0	<0.001	0	0	0.001	0.001	0.002	0.001	0	0.005	0	0
[C-HS]	4	0	0	0.787	<0.001	0	0.258	0.370	<0.001	0.001	0.016	0.257	0	<0.001
HS	8	0.542	<0.001	0.001	<0.001	0	0.006	0.020	0.003	0.038	0	0.057	0.018	0.033
Control	1	0	0.598	0.005	0.880	0.696	0.046	0.353	0.044	<0.001	0.015	0.124	0	0.039
Residues	45													
CV (%)		4.1	4.3	3.9	6.9	3.9	5.3	7.8	4.7	12.3	17	13	3.8	7.3
		SDM	TDM	R/S	N.a	P.a	K.a	Ca.a	Mg.a	S.a	Cu.a	Mn.a	Zn.a	Fe.a
Block	3	0.023	0.018	0.528	0.624	0.868	0.371	0.015	0.432	0.676	0.412	0.060	0.075	0.075
HS sources	2	0.001	<0.001	0.584	0.029	0	0.001	0	0.539	0	0.005	0	0.118	0.016
[C-HS]	4	<0.001	<0.001	0.122	0.040	0	0	0	0.108	0	0.001	0.001	0.001	0
HS	8	0.382	0.307	0.047	0.089	0.004	0.170	0	0.008	0	0.255	0	0.105	0.134
Control	1	0.007	0.007	0.218	0.019	0.470	0.321	0.069	0.651	0.364	0.986	0.003	0.765	0.921
Residues	45													
CV (%)		7.7	7.3	5.9	7.7	9	9.6	9.8	9.9	10.7	10.1	8.03	13.7	14.9

*$p<0.005$ are significant. SV- source of variation; df – degrees of freedom; [C-HS] humic substances carbon concentration; CV – coefficient of variation; SC – solution C; Ns – nutrient solution; SPAD – SPAD index; RDM – root dry matter; SDM – shoot dry matter; TDM – total dry matter; R/S DM – root/shoot dry matter; .a- nutrient accumulation in maize shoot

APÊNDICE B- Tabela de análise de contrate do artigo 2 - Milho

Table 2 – p value of contrast analysis between control and humic substances treatments, Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS), of nutrient solution and maize growth variables

Treatment	p value												
	SC	pH	EC	NsP	NsK	NsCa	NsMg	NsS	NsFe	NsZn	SPAD	RDM	SDM
AHA	0.957	0.004	0.001	0.159	0.027	0.002	0.032	0.002	0.008	0.979	0	0.361	0.085
HAL	0	0.097	0.188	0.871	0.189	0.146	0.586	0.224	0.006	0.135	0	0.166	0.026
WEHS	0	0.007	0.001	0.048	0.045	0.284	0.913	0.164	0	0	0	0.001	0.000

	p value											
	TDM	R/S DM	N.a	P.a	K.a	Ca.a	Mg.a	S.a	Cu.a	Mn.a	Zn.a	Fe.a
AHA	0.097	0.198	0.150	0. 016	0.045	0.842	0.464	0.741	0.350	0.266	0.544	0.294
HAL	0.029	0.168	0.022	0.436	0.270	0.636	0.925	0.174	0.333	0.372	0.510	0.511
WEHS	0.000	0.403	0.004	0.227	0.739	0	0.650	0	0.323	0	0.675	0.911

*$p<0.005$ the treatment are different to control. SC – solution C; Ns – nutrient solution; SPAD – SPAD index; RDM – root dry matter; SDM – shoot dry matter; TDM – total dry matter; R/S DM – root/shoot dry matter; .a- nutrient accumulation in maize shoot.

APÊNDICE C- Tabela de análise de variância do artigo 3 – Feijão

Table 3 – *p* value and coefficient of variation of nutrient solution and bean growth variables treated with different sources and humic substances concentrations

SV	df	*p* value												
		pH	**EC**	**NsP**	**NsK**	**NsS**	**NsCa**	**NsMg**	**NsCu**	**NsFe**	**NsMn**	**NsZn**	**SPAD**	**RDM**
Block	3	0.022	0.014	0.121	0.204	0.156	0.027	0.348	0.025	0.988	0.311	0.212	0.271	0.942
HS sources	2	<0.001	0	0	0	0	0	<0.001	0	0	0	0	0.002	0
[C-HS]	4	0.021	0	0	0	0	0.001	0.150	0	0	0	<0.001	0.002	0
HS	8	0.004	0	0	0	0	0	0.001	0	0	0	0	0.125	<0.001
Control	1	0.304	0.514	0	0	0	0.004	0.008	0	0	0.114	0.459	0.052	0.902
Residues	45													
CV (%)		4.5	0.68	3.2	2.1	1.9	3.11	4.5	5.5	4	3	8.32	2.5	9.1
		SDM	**TDM**	**R/S**	**N.a**	**P.a**	**K.a**	**Ca.a**	**Mg.a**	**S.a**	**Cu.a**	**Mn.a**	**Zn.a**	**Fe.a**
Block	3	0.015	0.035	0.016	0.015	0.626	0.633	0.457	0.519	0.619	0.822	0.735	0.287	0.337
HS sources	2	0.003	<0.001	0.024	0.025	0.070	<0.001	0	0.002	0.011	0.002	0.077	0	0
[C-HS]	4	0.048	0.024	0	0.289	0.128	0.053	0.178	0.343	0.242	0	0.517	0.002	0.151
HS	8	0.031	0.026	0	0.270	0.025	0.064	0.109	0.141	0.114	0.006	0.323	0	0.001
Control	1	0.040	0.072	0.020	<0.001	0.039	0	0.517	0.509	0.243	0.006	0.637	0.002	0.756
Residues	45													
CV (%)		9.6	8.8	8.6	8.5	7.3	9.8	10.3	10.6	12.4	11.3	11	6.1	15.4

*$p<0.005$ are significant. SV- source of variation; df – degrees of freedom; [C-HS] humic substances carbon concentration; CV – coefficient of variation; SC – solution C; Ns – nutrient solution; SPAD – SPAD index; RDM – root dry matter; SDM – shoot dry matter; TDM – total dry matter; R/S DM – root/shoot dry matter; .a- nutrient accumulation in bean shoot.

APÊNDICE D- Tabela de análise de contrate do artigo 3 – Feijão

Table 4 – *p* value of contrast analysis between control and humic substances treatments, Acros Organics® humic acid (AHA), humic acid extracted from Leonardite (HAL) and water extractable humic substances (WEHS), of nutrient solution and bean growth variables

Treatment	*p* value												
	pH	**EC**	**NsP**	**NsK**	**NsS**	**NsCa**	**NsMg**	**NsCu**	**NsFe**	**NsM**	**NsZ**	**SPA**	**RD**
AHA	0.02	0	0.126	0.725	<0.00	0.500	0.175	<0.00	0.031	0.494	0.01	0.015	0.41
HAL	0.68	0.456	<0.00	<0.00	<0.00	<0.00	0.010	<0.00	<0.00	0.029	0.16	0.015	0.03
WEHS	0.31	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	0.003	0.07	0.552	0.34
	p value												
	SD	**TDM**	**R/S**	**N.a**	**P.a**	**K.a**	**Ca.a**	**Mg.a**	**S.a**	**Cu.a**	**Mn.**	**Zn.a**	**Fe.a**
AHA	0.00	0.011	0.010	0	0.009	0	0.387	0.143	0.108	0.002	0.82	0	0.42
HAL	0.40	0.758	0.007	0.001	0.182	0	0.039	0.546	0.955	0.131	0.26	0.002	0.01
WEHS	0.02	0.031	0.183	0.001	0.059	0	0.554	0.324	0.107	0.001	0.64	0.177	0.43

*$p < 0.005$ the treatment are different to control. SC – solution C; Ns – nutrient solution; SPAD – SPAD index; RDM – root dry matter; SDM – shoot dry matter; TDM – total dry matter; R/S DM – root/shoot dry matter; .a- nutrient accumulation in maize shoot.