UNIVERSIDADE FEDERAL DO PARANÁ

MARCELLE MICHELOTTI BETTONI

SUBSTÂNCIAS HÚMICAS E MICORRIZAÇÃO COMO BIOFERTILIZANTE PARA SISTEMA DE PRODUÇÃO DE CEBOLA CULTIVADA EM DIFERENTES NÍVEIS DE CO_2

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Agronomia, Área de Concentração em Produção Vegetal, Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para a obtenção do titulo de Doutora em Ciências.

Orientador: Prof. Dr. Átila Francisco Mógor.
Co-orientador: Prof. Dr. Volnei Pauletti.
Co-orientadora: Profª. Drª. Maria Nieves Preboste Goicoechea

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PARECER

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Agronomia - Produção Vegetal, reuniram-se para realizar a argüição da Tese de DOUTORADO, apresentada pela candidata **MARCELLE MICHELOTTI BETTONI**, sob o título **"SUBSTÂNCIAS HÚMICAS E MICORRIZAÇÃO COMO BIOFERTILIZANTES PARA SISTEMA DE PRODUÇÃO DE CEBOLA CULTIVADA EM DIFERENTES NÍVEIS DE CO_2"**, para obtenção do grau de Doutor em Ciências do Programa de Pós-Graduação em Agronomia - Produção Vegetal do Setor de Ciências Agrárias da Universidade Federal do Paraná.

Após haver analisado o referido trabalho e argüido a candidata são de parecer pela **"APROVAÇÃO"** da Tese.

Curitiba, 23 de Fevereiro de 2015.

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RESUMO

O aumento populacional e a crescente demanda por alimentos, fez com que o uso de fertilizantes sintéticos fosse disseminado, sendo frequentemente utilizados de maneira indiscriminada, acarretando problemas ambientais devido a lixiviação de nutrientes e eutrofização de corpos d'água. Neste sentido, o uso de alternativas biofertilizantes, como a fertilização com substâncias húmicas e fungos micorrízicos arbusculares são importantes para a redução do uso de fertilizantes minerais e sintéticos, bem como, diante dos desafios das mudanças climáticas globais, alterar possíveis efeitos negativos do aumento de CO_2 na atmosfera, sobre a produtividade e qualidade de plantas.Deste modo, o presente trabalho teve como objetivo avaliar o efeito da biofertilização com fungos micorrízicos arbusculares e substâncias húmicas em diferentes níveis de CO_2, como alternativa nutricional na qualidade e produtividade de mudas e bulbos de cebola. Avaliou-se a aplicação de substâncias húmicas e fungos micorrrízicos arbusculares, em dois níveis de CO_2 (ambiente e elevado) no tratamento de mudas e a campo. Para a avaliação das mudas foram determinadas as seguintes características: altura da parte área, comprimento de raiz, diâmetro do pseudocaule, massas seca e fresca da parte aérea e da raiz, áreas foliar e radicular, volumes foliar e radicular; conteúdo de água, amido, açúcares solúveis totais, proteínas solúveis totais, prolina, compostos fenólicos solúveis, atividade da fosfatase ácida, teor de clorofila a, clorofila b, clorofila total e carotenoides, atividade antioxidante (DPPH) e índice de eficiência micorrízica. Nos bulbos, foram avaliados: número de catáfilos, diâmetro do bulbo, conteúdo de água, massas fresca e seca, amido, açúcares solúveis totais, proteínas solúveis totais, prolina, compostos fenólicos solúveis, atividade da fosfatase ácida, pH, sólidos solúveis, acidez titulável, relação sólidos solúveis e acidez titulável e composição nutricional. Concluiu-se neste trabalho, que as substâncias húmicas e a inoculação micorrízica arbuscular podem ser utilizadas como alternativa biofertilizante para otimizar o sistema de produção de cebola, mesmo quando exposta a elevados níveis de CO_2, pois melhoram a qualidade tanto de mudas quanto de bulbos, bem como a produtividade final da cultura.

Palavras-chave: *Allium cepa* L., inoculação; ácido fúlvico; ácido húmico; metabólitos primários; metabólitos secundários; qualidade de mudas; qualidade de bulbos.

ABSTRACT

The increase in population and the growing demand for food, made with the use of synthetic fertilizers were disseminated, being frequently used so indiscriminately, causing environmental problems as nutrients leaching and eutrophication. In this sense, the use of biofertilizer alternatives, as fertilization with humic substances and arbuscular mycorrhizal fungi are important for reducing the use of mineral and synthetic fertilizers, as well facing the challenges of global climate change, modify possible negative effects of increased CO_2 in the atmosphere, on the productivity and quality of plants. In this way, the present study aimed to evaluate the effect of biofertilization with arbuscular mycorrhizal fungi and humic substancesat different levels of CO_2, as alternative nutritional in the quality and productivity of seedlings and bulbs of onion. Assessed the application of humic substances and arbuscular mycorrhizal fungi, on two levels of CO_2 (ambiental and elevate) in the treatment of seedlings and in the production of bulbs. For the evaluation of the seedlings were evaluated the following characteristics: shhot height, root length, diameter of the pseudostem, dry and fresh mass of shoot and root, foliar and root area, foliar and root volume; water contente of root and shoot, starch, total soluble sugars, total soluble proteins, proline, solubles phenolic compounds, acid phosphatase activity, chlorophyll a content, chlorophyll b, chlorophyll total and carotenoids, DPPH and mycorrhizal efficiency index. The bulbs were assessed: cataphyll number, bulb diameter, water contente of bulb, fresh and dry mass of bulb, starch, total soluble sugars, total soluble proteins, proline, solubles phenolic compounds, acid phosphatase activity, pH, solubles solids, titratable acidity, ratiosoluble solids andtitratable acidityand nutritional composition. In this study, it was concluded that humic substances andand the mycorrhizal arbuscular inoculation can be used as an biofertilizer alternative to optimize the production system of onion, even when exposed to high levels of CO_2, do improve the quality both seedlings and bulbs, as well as final productivity of culture.

Palavras-chave: *Allium cepa* L.; inoculation; fulvic acid; humic acid; primary metabolites; secondary metabolites; seedling quality; quality of bulbs.

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INTRODUÇÃO

Estudos afirmam que a população mundial chegará a 9,6 bilhões de pessoas em 2050 (GREENE, JOSHI e ROBLES, 2012), sendo um dos maiores desafios da agricultura a produção de alimentos que satisfaçam esta demanda.

Dentre as hortaliças, a cebola é a terceira mais produzida a nível mundial e altamente consumida pela população. Em 2012, foram cultivados cerca de 4 milhões de hectares e aproximadamente 80 milhões de toneladas produzidas, sendo que a produção brasileira foi de aproximadamente 1,5 milhão de toneladas (FAOSTAT, 2014).

Para atender a demanda crescente de alimentos provocada por este aumento exponencial da população mundial, tem-se utilizado intensamente insumos e fertilizantes minerais para incrementar os rendimentos dos cultivos agrícolas. Estas ações constituem em um grande problema na atualidade, provocado principalmente pelo uso indiscriminado destes produtos, resultando em contaminação ambiental, perdas de áreas produtivas, desequilíbrio ecológico, entre outros.

Ao mesmo tempo, a mudança climática global é um desafio adicional, uma vez que altera significativamente as condições ambientais que afetam culturas (COHEN, 2003). As previsões meteorológicas prevêem um aumento da concentração de CO_2 na atmosfera, em conjunto com um aumento da temperatura da superfície da Terra, secas mais frequentes e graves e precipitação mais intensa (AINSWORTH, ROGERS e LEAKEY, 2008). Assim, a produção agrícola mundial é profundamente influenciada (COHEN, 2003), na medida em que estas mudanças climáticas drásticas prognosticam uma incerteza crítica sobre a absorção de carbono pelos ecossistemas terrestres e sobre a produção de alimentos, exigindo sistemas agrícolas mais eficientes e adequados ao novo cenário climático (ELLIS, 2000).

Neste contexto, torna-se necessário buscar alternativas que supram as necessidades atuais, sem comprometer o meio ambiente, e ainda, que atendam um novo nicho de mercado de consumidores, que vem crescendo em função de estratégias de conscientização, os quais buscam alimentos frescos, livres de agroquímicos e com alto valor nutricional.

Dentre as alternativas viáveis, encontram-se os sistemas de produção agrícola que preconizam o uso de alternativas mais sustentáveis, como os biofertilizantes que são produtos, livres de compostos proibidos pela legislação específica, compostos por componentes ativos ou agentes biológicos capazes de atuar no metabolismo de plantas cultivadas, melhorando o sistema de produção (MAPA, 2012).O sistema agrícola de produçãoconsiste no conjunto das práticas realizadas para um cultivo, desde a escolha da espécie, cultivar, qualidade da semente e da muda, manejo nutricional, práticas culturais, colheita e pós-colheita.

A utilização de alternativas biofertilizantes em todas estas etapas, dentro de um sistema de produção, necessita de mais estudos para que se tenha a comprovação de sua eficiência.

Segundo Marcos Filho (1999), sementes de hortaliças, além de alto valor comercial, apresentam menores quantidades de reservas armazenadas, sendo importante seu máximo aproveitamento através da

produção de mudas, as quais determinam o desempenho final das plantas em canteiros de produção (CARMELLO, 1995).

Uma das principais estratégias de utilização dos FMA para o crescimento de plantas é a inoculação destes na fase de muda, onde é possível a produção de inóculo em quantidade suficiente.

Estima-se que, globalmente, FMAs possam ser responsáveis pelo dreno anual de cinco bilhões de toneladas de carbono (C) (BAGO *et al*., 2002), com conseqüências diretas nas propriedades do solo, nas plantas e nas relações referentes às mudanças globais, atuando no sequestro de C da atmosfera.

O crescente interesse pelo uso da micorrização como alternativa biotecnológica se deve, principalmente, pela capacidade das hifas externas das raízes colonizadas absorverem nutrientes do solo, translocando-os com maior eficiência para a parte aérea das plantas, promovendo maior desenvolvimento destas e permitindo a redução do uso de fertilizantes sintéticos e minerais.

O efeito nutricional dos FMA mais estudado, é o aumento da absorção de fósforo (P), elemento pouco móvel no solo, que limita o crescimento vegetal, em especial, de plantas com sistema radicular menos profundo. A falta de nitrogênio (N), juntamente com o P, representa uma das principais limitações na produção agrícola. Assim como o P, tanto as hifas, como as raízes micorrizadas são capazes de absorver N em várias formas e transferí-lo até a planta.

As substâncias húmicas, constituem entre 70 e 80% da matéria orgânica da maioria dos solos, sendo compostas pelas frações: ácidos fúlvicos, ácidos húmicos e humina. Tais substâncias exercem efeitos fisiológicos na permeabilidade das membranas das células, atividade enzimática e absorção de nutrientes, reduzindo a necessidade do uso de fertilizantes.

A cebola apresenta um sistema radicular fasciculado, o que é um fator limitante para a absorção de fósforo, além de ser altamente responsiva a este elemento, juntamente com o nitrogênio e o potássio, sendo necessário o uso de intensiva adubação para obtenção de altas produtividades.

Estudos anteriores realizados com cebola indicaram que a cultura é altamente responsiva a micorrização no crescimento das plantas e melhor rendimento (BOLANDNAZAR *et al.*, 2007; JAIME, HSIANG e MCDONALD, 2008; GOUSSOUS e MOHAMMAD, 2009; GALVÁN *et al.*, 2011).

O uso de substâncias húmicas em cebola também foi pesquisado (FEIBERT, SHOCK e SAUNDERS, 2003; SAJID *et al.*, 2012; KANDIL, SHARIEF e FATHALLA, 2013) e constatou-se que as mesmas promovem incrementos na produtividade e qualidade de bulbos.

O carbono é o mais importante substrato para plantas e o aumento em sua concentração pode aumentar a eficiência do aparelho fotossintético de plantas e consequente produção, porém, em alguns casos, este efeito não é verificado em função do processo de aclimatação.

Deste modo, buscando alternativas para otimizar o sistema de produção de cebola, o presente trabalho teve como objetivo aumentar a qualidade e produtividade de mudas e bulbos de cebola em função da biofertilização com fungos micorrízicos arbusculares e substâncias húmicas em diferentes níveis de CO_2, em associação ou isoladamente.

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REVISÃO DE LITERATURA

1.1 A CULTURA DA CEBOLA

A cebola, *Allium cepa* L., pertence à família Alliaceae, originária da Ásia Central, é uma espécie de grande interesse econômico, com cerca de 4 milhões de hectares de área colhida e aproximadamente 80 milhões de toneladas produzidas em 2012 (FAOSTAT, 2014).

É uma espécie diplóide (2n=2x=16), com poucos genes identificados, quando comparada a outras culturas (LEITE e ANTHONISEN, 2009), sendo seu germoplasma constituído de populações locais e cultivares desenvolvidas ao longo dos séculos adaptadas a diferentes latitudes, sistemas de cultivo e preferência dos consumidores.

O ciclo de crescimento e desenvolvimento desta cultura é bianual, sendo que, no primeiro ano são produzidos bulbos a partir de sementes, chamando-se fase vegetativa e, no segundo ano, ocorre o florescimento ou fase reprodutiva. A fase de formação de bulbos está relacionada com a interação entre a temperatura e o fotoperíodo, sendo este último o fator principal para a indução, formação e maturação do bulbo (GALMARINI, 1997).

Dentro de um sistema produtivo, diferentes etapas devem ser consideradas para a obtenção de uma produção com êxito e um produto de qualidade a ser ofertado, tais como a escolha da espécie, a escolha da cultivar, o sistema de produção de mudas, o manejo nutricional e cultural ao longo do ciclo, a colheita e a pós-colheita.

A escolha de cultivares, se não for adequada, ou seja, adaptada as condições locais, resultam em baixas produtividades e qualidade dos bulbos.

Neste sentido, a Embrapa Semi-Árido desenvolveu a cultivar BRS Alfa São Francisco, de dias curtos (DC) quanto a bulbificação (sua bulbificação é iniciada em dias com 11-12 horas de luz), lançada em 2004, para ser cultivada no segundo semestre do ano, no nordeste brasileiro, onde predominam altas temperaturas. Para a obtenção da cultivar realizou-se o método de seleção recorrente fenotípica para várias características dentro da cultivar Alfa Tropical, lançada em 1999 pela Embrapa Hortaliças e Epamig. Como características, a cultivar apresenta bulbos de cor amarelo/baia predominante, sendo arredondados, firmes e de bom aspecto comercial (COSTA *et al.*, 2005).

Verificou-se que esta cultivar tem potencial para ser cultivada na região Sul do país, inclusive em períodos de entressafra local, com semeaduras em janeiro e setembro, enquanto a época tradicional de semeadura ocorre nos meses de abril e junho (BETTONI *et al.*, 2013).

Após definido a espécie e a cultivar a ser implantada, a produção de mudas é a próxima etapa a ser realizada. Na horticultura, esta etapa é bastante difundida e de grande importância no sistema de produção, uma vez que o desempenho da cultura no campo depende da qualidade agronômica da muda (SOUZA *et al.*, 2006).

1.1.1 Produção de mudas

No caso da cebola, o processo de produção de mudas tem especial atenção principalmente porque suas sementes apresentam alto custo, além de tamanho reduzido, e são muito sensíveis aos constantes ciclos de hidratação-desidratação no solo (TRIGO, NEDEL e TRIGO, 1999). A produção destas mudas pode ser realizada através de sementeiras ou bandejas. No caso de sementeiras, a semeadura ocorre no solo, próximo à área de transplante, em campo aberto, enquanto a produção em bandejas ocorre em casa de vegetação, com um ambiente mais controlado.

Esta etapa preconiza a obtenção de mudas com máximo vigor e sanidade (NUNES e SANTOS, 2007), com adequado desenvolvimento e boa formação de sistema radicular, além de melhor capacidade de adaptação ao novo local após o transplantio.

Para a obtenção dos resultados requeridos, além da obtenção de sementes de qualidade, devem-se buscar características físicas, químicas e biológica adequadas no substrato a ser utilizado (LENZA e VALENTE, 2009).

Segundo Costa *et al.* (2009), há a necessidade de se verificar experimentalmente, para cada espécie vegetal, o tipo de substrato ou a melhor composição, que permita a obtenção de plantas vigorosas.

Bezerra *et al*. (2009) afirmam que, na maioria das vezes, são utilizados substratos artesanais produzidos pelos produtores com restos culturais, sem a utilização de nenhum tratamento para eliminar os fitopatógenos, e dependendo dos materiais usados na formulação, os teores de nutrientes nem sempre são suficientes para promover o desenvolvimento satisfatório das mudas.

Outro motivo para o produtor não investir em substrato de qualidade está no alto custo dos substratos comerciais, o que justifica a necessidade de produtos que proporcionem qualidade na obtenção de mudas, com menor custo (BERNARDES, REIS e RODRIGUES, 2011).

Uma alternativa econômica é adição de matéria orgânica ao substrato, a qual proporciona benefícios como o aumento da capacidade de retenção de umidade e da capacidade de troca catiônica dentre outros (PEREIRA *et al.*, 2010). Pode-se ainda utilizar substâncias húmicas (SH) ou fungos micorrízicos arbusculares (FMA), os quais permitem uma suplementação de nutrientes, além de benefícios na morfologia e fisiologia das plantas, gerando uma muda de melhor qualidade.

1.1.2 Nutrição e qualidade de cebola

Essas mesmas técnicas podem ser utilizadas durante o manejo nutricional da cultura, o qual consiste em uma etapa tão importante quanto às demais. Conhecer a extração e a exportação de nutrientes da cultivar é determinante para um adequado suprimento nutricional.

Segundo Vidigal, Moreira e Pereira (2010), a cultivar Alfa Tropical exportou pelos bulbos de cebola (em kg ha^{-1}): 70,42 de N; 57,39 de K; 25,09 de Ca; 14,69 de P; 12,29 de S; 4,50 de Mg; 0,63 de Fe; 0,21 de Zn; 0,19 de Mn e 0,03 de Cu. Estes valores podem servir de referência para um manejo nutricional adequado, fazendo com que sejam atingidas altas produtividades com menor uso de insumos, embora sejam variáveis com a época e o local de plantio, além das propriedades do solo.

Segundo Grangeiro *et al*. (2008) a qualidade da cebola está relacionada à aparência externa, ao tamanho do bulbo, cor, aroma, sabor, firmeza e sua composição química. Estes atributos, de acordo com Finger e Casali (2002), são determinados por fatores como o genótipo, manejo na pré-colheita, pela época adequada de colheita e por tratamentos na pós-colheita. Os mesmos atributos também são responsáveis pela escolha da cultivar e aceitação pelo mercado consumidor.

No caso da cebola e outras espécies do gênero *Allium*, a qualidade do produto final é importante, em função de suas propriedades profiláticas e medicinais, com alta visibilidade no mercado (KENDLER, 1987).

Atualmente existe uma demanda crescente por parte dos consumidores por alimentos seguros e nutritivos que melhorem o desempenho físico, reduzam os riscos de doenças e aumentem o tempo de vida, os quais são denominados alimentos funcionais ou nutracêuticos, ou seja, aqueles consumidos em uma dieta padrão e que fornecem benefícios além da nutrição básica (CARVALHO e MACHADO, 2004).

Tais alimentos são ricos em compostos que apresentam propriedades antioxidantes por eliminar os radicais livres, protegendo contra a oxidação celular, prevenindo o envelhecimento e atuando no tratamento e prevenção de doenças como câncer, doenças cardiovasculares, obesidade, colesterol, hipertensão, catarata e distúrbios do sistema digestivo (PAGANGA, MILLER e RICE-EVANS, 1999). Eles também apresentam propriedades antimicrobiana, antiviral, antifúngica e antioxidante, além de sequestradores de radicais livres e íons metálicos (HANDIQUE e BARUAH, 2002).

Tais compostos, parcialmente responsáveis pelas qualidades sensoriais e nutricionais dos alimentos vegetais, são denominados organosulfurados e flavonóides, também nomeados de fitoquímicos, e suas concentrações nestes alimentos são influenciadas por fatores genéticos e condições ambientais. Outros fatores, como germinação, nível de maturidade, variedade, também influenciam no conteúdo destes compostos (MAZZA e FRANCIS, 1995).

Segundo Griffiths *et al*. (2002), dois subgrupos de flavonóides foram encontrados em cebola, as antocianinas, que confere uma coloração do vermelho ao roxo em algumas cultivares, e as quercetinas e seus derivados, responsável pela coloração amarela e marrom das cascas de diferentes variedades e também, segundo Duthie e Dobson (1999), atrasam o dano oxidativo em células.

Muitos são os componentes naturalmente presentes nos alimentos que tem atividade antioxidante, como: carotenóides (α-caroteno, β-caroteno, luteína e licopeno), compostos fenólicos (ácido clorogênico e fenólico, cumarina, flavonoides, tirosol, antocianinas, taninos e catequina), compostos organosulfurados e tocoferol, sendo que nos bulbos de cebola, os componentes mais abundantes são compostos fenólicos e organosulfurados (SHAHIDI e WANASUNDARA, 1992).

Os antioxidantes mais conhecidos dos alimentos são os compostos fenólicos, que são comuns em plantas e atuam como mecanismos de defesa contra patógenos e condições de estresse ambientais (DUVAL, SHETTY e THOMAS, 1999), sendo variável conforme o estado fisiológico e do desenvolvimento da planta (EDREVA *et al.*, 2008), conforme a espécie, a cultivar, o local de plantio e a nutrição mineral (LEONG e SHUI, 2002).

No entanto, pesquisas comprovaram que alimentos cultivados em sistemas convencionais

apresentam menor teor destes compostos (BRANDT e MOLGAARD, 2001) em função do uso de insumos químicos, uma vez que as plantas não necessitam acionar seu mecanismo de defesa com tanta intensidade.

Albishi *et al.* (2013) testando diferentes cultivares observaram os seguintes valores de compostos fenólicos livres na casca de cebola pérola, vermelha, amarela e branca: 62,6, 23,7, 22,7 e 0,54 mg de ácido gálico equivalente g massa seca de bulbo^{-1}, respectivamente. Os mesmos autores encontraram valores de antocianinas na casca de cebola vermelha de 10,04 mg 100 g^{-1} de casca e, em cebola branca, de 0,06 mg 100 g^{-1}.

Outro composto acumulado em situações de estresse na planta é a prolina (TAYLOR, 1996). Sua função principal é a osmorregulação, porém, também atua na poteçãodo sistema endomembrana e de proteínas contra os efeitos adversos, o que aumenta a tolerância da planta a este tipo de situação, porém reduz sua capacidade produtiva, em função da realocação de fotoassimilados (EWERS, OREN e SPERRY, 2000), além da qualidade do bulbo.

Díaz *et al.* (2012) relataram um aumento da concentração de prolina em cebolas não aclimatadas, com consequente redução na concentração de carboidratos e açúcares, o que explica seu crescimento lento e maior capacidade de sobrevivência ao destinar os carboidratos para a formação de prolina, a qual protege as proteínas da membrana (CLAUSSEN, 2005).

Cerca de 65-80% da massa seca dos bulbos consiste em carboidratos não estruturais (BENKEBLIA, 2005). Os carboidratos não estruturais predominantes na cebola são: glucose, frutose, sucrose e frutanos de baixo peso molecular, enquanto que o amido e rafinose são praticamente ausentes. Os frutanos acumulam-se durante a bulbificação, seguido por catabolismo durante o crescimento e desenvolvimento dos bulbos (BENKEBLIA, 2005).

A qualidade do bulbo é algo relativo e a preferência por um determinado tipo de cebola varia em função da localização do mercado e seus consumidores. No Brasil, bulbos globulares, pungentes, de suave a doce, com tamanho médio e casca de amarelo a marrom escura, firmes, são preferidos para consumidores do mercado *in natura* (OLIVEIRA e BOITEUX, 2004).

Cebolas definidas como suave a doce também podem ser chamadas de fracas, quando classificadas segundo a pungência, conforme sugerido por Schwirmmer e Weston (1961). Tal classificação é baseada na quantidade de ácido pirúvico, dividindo a cebola em três grandes grupos: cebola fraca (2 a 4 µmolg^{-1}), intermediária (8 a 10 µmolg^{-1}) e forte (15 a 20 µmolg^{-1}).

O ácido pirúvico apresenta alta correlação com a percepção de sabor. O balanço entre os níveis de pungência e açúcares determinam a doçura de cebola e alta pungência pode ser diminuída por altos teores de açúcares e a cebola não é percebida como doce. Cebolas com baixa pungência e baixos teores de açúcares podem ser vistas como suaves. O ideal é que a cebola tenha altos níveis de açúcares e baixos níveis de pungência (VAGEN e SLIMESTAD, 2008).

Para Carvalho *et al.* (1987) a determinação dos teores de sólidos solúveis (SS) é um fator importante, pois é nessa fração que se encontram os açúcares responsáveis, em parte, pelo sabor, sendo a glicose, frutose e sacarose, juntamente com uma série de oligossacarídeos, os principais açúcares presentes

na cebola. A boa qualidade no armazenamento dos bulbos também é atribuída a altos teores de sólidos solúveis (CHAGAS, RESENDE e PEREIRA, 2004).

Schunemann *et al*. (2006), ao avaliarem 18 genótipos de cebola no Vale do Itajaí-SC, encontram valores de pH entre 5,44 até 5,61; 6,06 a 11,00 ºBrix de sólidos solúveis, 0,17 a 0,27% de acidez titulável (AT), 6,88 a 11,88 % de sólidos totais e 4,84 a 7,61 µmol g^{-1} de ácido pirúvico.

Grangeiro *et al.* (2008) avaliaram 18 genótipos de cebola na região Nordeste do Brasil e encontraram TSS variando entre 6,67 a 11,63 (º Brix), vitamina C de 22,7 a 46,81 (mg ácido ascórbico 100 mL^{-1} de suco) e a acidez total titulável de 0,19 a 0,45 (% de ácido pirúvico). Os mesmos autores verificaram valores de 9,7 ºBrix, 0,34% ácido pirúvico e 7,93 µmol g^{-1} de ácido pirúvico em cebolas Alfa São Francisco. Já Ribeiro *et al.* (2009) verificaram SS de 9,6 ºBrix e 0,12 % de ácido cítrico na mesma cultivar, também cultivada no Nordeste brasileiro

Caruso *et al.* (2014) encontraram valores entre 7,7 e 8,1 ºBrix; 1,97 e 2,23 Eq g^{-1} de ácido cítrico por 100 g de massa fresca de bulbo e 11,4 e 13,2 g de proteínas, em cebolas plantadas no primeiro trimestre do ano.

Segundo Costa *et al.* (2005), a cultivar Alfa São Francisco, produzida no Nordeste brasileiro caracteriza-se por bulbos firmes, com boa conservação pós-colheita e sólidos solúveis de 12,5 ºBrix, com teores de nutrientes de 24,3 g kg^{-1} de N, 5,7 g kg^{-1} de P, 11,0 g kg^{-1} de K, 11,6 g kg^{-1} de Na, 1,5 g kg^{-1} de Mg e 7,9 g kg^{-1} de S (SANTOS *et al.*, 2007).

Todas os aspectos relatados são importantes para à geração de bulbos de qualidade e com produtividade desejável.

1.2 ALTERNATIVAS BIOFERTILIZANTES

Segundo a legislação brasileira os biofertilizantes são definidos como produtos que contém componentes ativos ou agentes biológicos, capazes de atuar, direta ou indiretamente, sobre o todo ou parte das plantas cultivadas, melhorando o desempenho do sistema de produção e, que sejam isentos de substâncias proibidas pela regulamentação de orgânicos (MAPA, 2012).

Neste sentido, biofertilizantes são ferramentas biológicas que podemos utilizar para a melhoria do desenvolvimento agrícola, como os fungos micorrízicos arbusculares (FMA) e as substâncias húmicas (SH).

Por muitos anos, as estratégias utilizadas para aumentar a produtividade das culturas foi a de aumentar a área plantada e/ou o uso de fertilizantes químicos e sintéticos (AYALA e RAO, 2002). Porém, com a utilização de biofertilizantes, é possível melhorar a eficiência de um sistema produtivo e maximizar a absorção de nutrientes, sem a necessidade de incorporar maiores quantidades de fertilizantes.

Recentemente, este tipo de sistema intensivo tem sido questionada e novas estratégias tem sido adotadas para aumentar a produtividade na mesma área plantada, com a redução dos custos de produção e

aumento da eficiência dos insumos, sem comprometer a qualidade ambiental (SUTHAR, 2009) como o uso de biofertilizantes, SH e FMA.

1.2.1 Substâncias húmicas

As SH constituem 85 a 90 % da reserva total do C orgânico e são originadas da degradação química e biológica de resíduos orgânicos e da atividade da biota do solo, sendo que os produtos formados constituem estruturas complexas mais estáveis, de coloração escura, elevado peso molecular, sendo classificadas, segundo a solubilidade, em (SCHNITZER e POAPST, 1967):

• Humina: fração insolúvel em meio alcalino ou ácido diluído, com baixa capacidade de reação.

• Ácidos húmicos (AH): fração escura solúvel em meio alcalino, precipitando-se como produto escuro e amorfo em meio ácido. São formados por compostos aromáticos e alifáticos com elevado peso molecular, e grande capacidade de troca catiônica, podendo associar-se a elementos metálicos, formando humatos, que podem precipitar ou permanecer em dispersão coloidal. Apresentam maior conteúdo de C e menor de O, e consequentemente, uma massa maior que os ácidos fúlvicos.

• Ácidos fúlvicos (AF): fração marrom solúvel em meio alcalino ou ácido diluído. Constituídos por polissacarídeos, aminoácidos, compostos fenólicos, apresentando alto conteúdo de grupos carboxílicos, com peso molecular relativamente baixo. Têm menos C e N e maior conteúdo de grupos funcionais contendo oxigênio (CO_2H, OH, C=O) por unidade de peso que as outras frações húmicas. Possuem maior acidez total e capacidade de troca catiônica (CTC) que os ácidos húmicos.

De maneira geral, as SH apresentam grande quantidade de grupos de ácidos carboxílicos e fenólicos, responsáveis pelas suas propriedades de complexação e de troca iónica, além de apresentarem características tanto hidrofóbicas, como hidrofílicas. Pesquisas também revelaram a presença de espaços nas estruturas destas substâncias, as quais poderiam alojar outros compostos como carboidratos, proteínas, lipídeos, agrotóxicos e outros poluentes, além de elementos inorgânicos como argilas e óxidos-hidróxido (SCHULTEN e SCHNITZER, 1997).

As SH apresentam distintas ações na planta e no solo. No solo, elas reduzem a evaporação de água, aumentando sua capacidade de retenção de umidade (KHALED e FAWY, 2011); reduzem a erosão do solo, aumentando as forças de coesão das partículas do solo, melhorando sua estrutura e suas propriedades físicas; auxiliam no transporte e absorção de nutrientes (EYHERAGUIBEL, SILVESTRE e MORARD, 2008) como ferro e zinco (BALDOTTO *et al.*, 2009), entre outros micronutrientes (LIMA *et al.*, 2011); e aumentam a disponibilidade de nutrientes pela sua ação complexadora e quelatizante, reduzindo a necessidade de aplicação de fertilizantes sintéticos e minerais (ZHANG *et al.*, 2013).

Além disso, as SH têm efeito sobre a biota do solo. A adição de SH, como o AH, influencia a esporulação, produção de micélio externo e colonização micorrízica (GRYNDLER *et al.*, 2005, 2009). Rodriguez e Ortuño (2007) também verificaram melhoria na micorrização de plantas de cebola com a adição de SH, e os dois fatores associados levaram a incrementos de produção da cultura, melhorando a eficiência

do uso de adubos. Para Nobre *et al.* (2013), a produção de glomerosporos aumenta com doses entre 20 e 80 mg C L^{-1}. Abdel-Razzak e El-Sharkawy (2012) observaram que a interação entre bactérias fixadoras de N e AH via foliar, aumentaram a produtividade, qualidade e durabilidade de alho.

Nas plantas, as SH têm importante ação sobre o metabolismo celular do N, aumentando o teor de NO_3^- (PICCOLO *et al.*, 1993) devido à redução do pH na superfície da raiz, facilitando, assim, a H^+ / NO_3^- simporte (QUAGGIOTTI *et al.*, 2004). Ertani *et al.* (2011) observaram, em milho tratado com SH, aumento de 65% na atividade da glutamina sintetase (GS) da raiz e aumentos de 176 e 204% na atividade da glutamato sintase (GOGAT) da raiz e das folhas, respectivamente.

Esta ação das SH sobre o metabolismo do N está diretamente ligada á redução de impactos ambientais, uma vez que tal mecanismo aumenta a eficiência da utilização do N. Normalmente, menos de 50% do N aplicado sob a forma de fertilizante é utilizado pelas culturas, sendo este elemento perdido pela lixiviação de nitrato, volatilização de amônia e emissão de N_2, N_2O e outros óxidos de nitrogênio (ANGHINONI, 1986).

Na planta, elas diminuem a deterioração causada pelo stress, com indução da atividade da esterase (MUSCOLO *et al.*, 1993) e a proteção contra o stress oxidativo (GARCÍA *et al.*, 2012); aumentam a respiração e a velocidade das reações enzimáticas do ciclo de Krebs (NARDI, MUSCOLO e VACCARO, 2007), resultando em maior produção de ATP nas células radiculares; aumentam o conteúdo de clorofila (BALDOTTO *et al.*, 2009); atuam na síntese protéica (FAÇANHA *et al.*, 2002); aumentam a velocidade e taxa de germinação de sementes (YOUNG e CHEN, 1997); atuam no metabolismo secundário de plantas, agindo na biossíntese de compostos fenólicos, com aumentos na expressão da fenilalanina (tirosina) amoniliase (PAL/TAL) (SCHIAVON *et al.*, 2010); ativam hormônios como citocininas, giberilinas (ARANCON *et al.*, 2012), poliaminas e ácido abscísico em plantas (MORA *et al.*, 2010, 2013), além de etileno (MORA *et al.*, 2012, 2013).

As SH também promovem o crescimento de plantas, através do maior desenvolvimento da parte aérea e radicular (DAUR e BAKHASHWAIN, 2013), com aumento na emissão de raízes secundárias (SILVA *et al.*, 2011).

A promoção do crescimento e desenvolvimento de plantas tem sido atribuída, por diversos pesquisadores, ao efeito similar às auxinas, interagindo com receptores na superfície celular, aumentando a transcrição de mRNA da H^+-ATPase na membrana plasmática, promovendo a acidificação do apoplasto e o conseqüente aumento da plasticidade da parede celular (SCHIAVON *et al.*, 2010; SILVA *et al.*, 2011). O crescimento de plantas pode, também, ser atribuído a presença de poliaminas, tais como putrescinas, espermidina e espermina encontradas em SH (YOUNG e CHEN, 1997), as quais, segundo Martens e Frankenberger (1994) atuam como reguladores de plantas (KUMAR, IMTIYAZ e KUMAR, 2007). Dobbss *et al.* (2007) atribuem o crescimento às alquilamidas, uma nova classe de compostos com ação hormonal, que apresentam estímulo do crescimento da raiz principal e de forma independente da sinalização auxínica (RAMÍREZ-CHÁVEZ *et al.*, 2004).

O uso de SH também tem efeito sobre a pós-colheita de hortaliças, conforme comprovado por Pinto

et al. (2008), que observaram menor perda de massa e maior firmeza da polpa de melões tratados com 50 L ha^{-1} de SH, e Lima *et al.* (2011) que observaram maior relação sólidos solúveis (SS) e acidez titulável (AT) em tomates tratados com AH, atribuindo tal efeito ao estímulo da fotossíntese, resultando em uma maior taxa de assimilados em folhas e exportação para o tomate, que aumentou o teor de SS. Aminifard *et al.* (2012) comprovaram o efeito de AH na qualidade de pimentão, afetando a AT, os SS, carboidratos, licopeno e beta-caroteno, quando aplicado via solo. Canellas *et al.* (2002) comprovaram que plantas tratadas com SH tiveram a frutose, glicose e amido reduzidos. Nardi, Muscolo e Vaccaro (2007) e Ertani *et al.* (2011) também constataram a redução do amido, enquanto a atividade da amilase e os teores de açúcares solúveis aumentaram.

Parandian e Samavat (2012), utilizando doses de AH e AF nas doses de 0, 0,5 e 1% na mergulhia e 10x menos na pulverização foliar em lírios, observaram incrementos de μ-amilase, açúcares solúveis e Zn com a imersão de plantas em SH, quando comparadas as plantas pulverizadas, que apresentaram maiores teores de antocianinas. O AH apresentou maior teor de açúcares solúveis na planta quando comparado ao AF.

Yang *et al.* (2004) estudando espécies arbóreas observaram que as SH estimulam a clorofilase *a* e *b*, sendo que o AF tem maior estímulo sobre a clorofilase *a*, enquanto o AH, sobre a clorofilase *b*, na dose de 0,5 mg mL^{-1}.

Osman, EL-Masry e Khatab (2013), utilizando diferentes doses de AH e AF, observaram que os mesmos podem ser aplicados diretamente nas folhas ou via solo, ajudando na melhoria da fertilização, além de reduzir custos, com efeito mais pronunciado quando utilizam a aplicação foliar, em plantas de arroz.

Young e Chen (1997) e Rodda *et al.* (2006) comprovaram que a aplicação de 100 mg L^{-1} de C melhorou o crescimento de alface. Sajid *et al.* (2012) verificaram que a aplicação de AH aumenta o rendimento de cebolas e a disponibilidade de nutrientes para as plantas, com 2 kg ha^{-1} de AH. Estudos prévios mostraram que a aplicação ótima de AH varia de 11 a 100 mg L^{-1} (RODDA *et al.*, 2006; EYHERAGUIBEL, SILVESTRE e MORARD, 2008; ROSA *et al.*, 2009; NOBRE *et al.*, 2013), sendo a faixa de concentração de substâncias húmicas em solução que, normalmente, inibe o crescimento de plantas encontra-se acima de 500 mg L^{-1} de C (CHEN e AVIAD, 1990).

Feibert, Shock e Saunders (2003) comprovaram efeitos benéficos no crescimento e produtividade de cebolas cultivadas com adição de SH. Kandil, Sharief e Fathalla (2013) revelaram que a pulverização foliar de 18,5% de AH, aplicados via foliar aos 60 e 80 DAT, em cebola, aumentou seu crescimento vegetativo, produção de bulbos, qualidade cebola e composição química, verificando-se maior rendimento total e comercial de bulbos, bem como, maiores massas médias de bulbos, maior teor de SS, massa seca e menor perda de peso. No entanto, Osvalde *et al.* (2012) não verificaram efeito das SH aplicadas via solo no momento do preparo das mudas e via foliar (duas ou três vezes), na concentração de 0,2%.

Diante do contexto, as SH podem proporcionar distintas vantagens para as plantas, com respostas dependentes da espécie, da cultivar, do modo de aplicação, do tipo de SH utilizada e dose, sendo necessária a realização de experimentos que comprovem seus efeitos em relação a condição experimental desenvolvida.

1.2.2 Fungos micorrízicos arbusculares

A relação micorrízica é um evento mutuamente benéfico, onde as plantas fornecem compostos de C para o fungo, enquanto os fungos suprem às plantas de nutrientes. Esta simbiose planta-fungo deve-se ao fato do fungo produzir hifas intra e extra-radiculares (HARLEY e SMITH, 1983) que absorvem elementos minerais do solo e os transferem para o ambiente radicular, onde são absorvidos.

No espaço intra-radicular, a troca bidirecional ocorre no arbúsculo, que é uma estrutura formada pela interação de hifas de fungos micorrízicos arbusculares (FMA) e a plasmalema de algumas células do córtex, sendo que sua formação depende da interação genética e funcional entre combinações fungo-planta (HARRISON, 1999). Após penetrar a parede celular, a hifa se torna extremamente fina, com diâmetro menor que 1 μm, que se ramifica e forma uma matriz de troca com a plasmalema da célula vegetal.

O micélio externo também é responsável pela exsudação de glicoproteínas hidrofóbicas chamadas de *glomalinas* (LEAKE *et al.*, 2004), que representam uma forma estável de armazenar C no solo (RILLIG, 2004).

Os FMA geram muitos benefícios para as plantas, tais como: melhor crescimento e desenvolvimento (WANG *et al.*, 2008); maior tolerância de plantas aos diferentes tipos stress (hídrico, salino ou térmico) (GIANINAZZI-PEARSON, 1996); incrementos à resistência de plantas diante do ataque patogênico (JAIME, HSIANG e MCDONALD, 2008); imobilização de metais pesados; melhoria na eficiência do uso da água e dos níveis de fotossíntese (JEZDINSKÝ *et al.*, 2012); incrementos na ação das H^+-ATPases (BLEE e ANDERSON, 2002); aumentos de compostos antioxidantes (BASLAM *et al.*, 2011), além de melhorar a absorção e translocação de nutrientes como: N, P, K, Ca, Mg, S, Zn, Cu, Mo, Fe, Mn, entre outros (GAMPER *et al.*, 2004; JÚNIOR *et al.*, 2011).

No caso do N, os FMAs absorvem e deslocam às plantas significativas quantidades deste nutriente, seja na forma de NH_4^+, seja na de NO_3^-. As enzimas de assimilação de N estão presentes tanto em raízes como em estruturas do FMA, sendo que o elemento pode ser acumulado pelos fungos, garantindo gradientes de concentração entre o espaço extra e intracelular, bem como entre células do córtex (GÜNTER e OVODOV, 2005).

Em relação ao P, nutriente estrutural que constituí ácidos nucléicos, fosfolipídeos, assim como de diversas enzimas (LEHNINGER, NELSON e COX, 1995) (e está envolvido nos processos de fosforilação e, portanto, no metabolismo energético, na transdução de sinais e na regulação da atividade celular), sua falta ocasiona significante declínio no conteúdo de ATP (-74 %) e ADP (-91 %), bem como dos conteúdos de enzimas (DUFF *et al.*, 1989), sendo importante a melhoria da absorção deste elemento.

Altas aplicações de P aumentam seu enriquecimento nas águas, levando a eutrofização dos rios. Neste sentido, é necessário o desenvolvimento sustentável de tecnologias para o uso de P (TAWARAYA, HIROSE e WAGATSUMA, 2012). Essas características fazem com que a simbiose micorrízica arbuscular tenha potencial biotecnológico e ecológico a ser explorado.

Os FMA aumentam a taxa de absorção de P em plantas (NIELSEN, JONER e DECLERCK, 2002)

em função: do aumento do volume de solo explorado pela quantidade hifas do fungo (BAGO, PFEFFER e SHACHAR-HILL, 2000), e por sua característica de menor diâmetro em relação às raízes; da formação de polifosfatos ou moléculas orgânicas sintetizadas pelos FMA ricas em P, que reduzem a concentração de Pi no interior das hifas, remobilizando este em condições de estresse, permitindo, um fluxo contínuo deste nutriente para a planta; e da produção de enzimas como fosfatases, que catalisam a liberação de P dos complexos orgânicos, permitindo sua absorção na forma iônica pelas plantas nas unidades arbusculares (MARSCHNER e DELL, 1994). Karandashov e Bucher (2005) também atribuem a maior taxa de absorção de P a regulação genética dos mecanismos de transporte de Pi controlada diretamente pelo FMA.

Estudos realizados por Jolicoeur *et al.* (2002) demonstram que os teores de Pi (e possivelmente outros nutrientes), além de açúcares intracelulares, regulam a orientação do fungo em produzir hifas ou cessar seu crescimento.

Outros estudos revelam que a absorção de Pi pelo FMA e sua transferência à planta são estimuladas pela transferência de C da planta para o fungo (BÜCKING e SHACHAR-HILL, 2005). Diante da maior oferta de C, o fungo diminui a síntese de polifosfatases, levando a aumentos nos teores de Pi citoplasmáticos, bem como à sua incorporação em fosfolipídeos e polifosfatos (poli P) (VIERECK, HANSEN e JAKOBSEN, 2004). Já a planta, que pode perder até 20 % do C fixado pelo simbionte, responde a este dreno com aumentos na sua taxa fotossintética, e redução no teor de SS (explicado pela possível translocação do açúcar das raízes para a parte aérea, além da estrutura de suporte das micorrizas (BASLAM *et al.*, 2011), ocasionando aumentos da produtividade primária e no dreno de C da atmosfera (JAKOBSEN, SMITH e SMITH, 2003), variável importante e pouco estudada diante dos processos de mudanças climáticas (LEAKE *et al.*, 2004).

O efeito benéfico da micorriza depende de diversos fatores, tais como: tipo de planta (espécie e cultivar), espécie de FMA, prática cultural, regime de água, época de cultivo e fertilização (BASLAM, GARMENDIA e GOICOECHEA, 2013).

Em relação ao tipo de planta, as pertencentes a família Aliaceae, como cebola e alho, apresentam um sistema radicular menos desenvolvido que outras (GREENWOOD *et al.*, 1982), e a inoculação com FMA reduz a necessidade de fertilização com N e P, uma vez que apresenta efeitos benefícios equivalentes (GOUSSOUS e MOHAMMAD, 2009), além dos custos de produção (TAWARAYA, HIROSE e WAGATSUMA, 2012).

Estudos mostram que a inoculação com FMA incrementaram, além do P, outros nutrientes e aumentaram o crescimento de plantas de *Allium cepa* (GOUSSOUS e MOHAMMAD, 2009; SHUAB *et al.*, 2014), *Allium fistulosum* (BORDE, DUDHANE e JITE, 2010) e *Allium porrum* (JEZDINSKÝ *et al.*, 2012).

Outro fator que afeta o efeito benéfico da micorriza é a espécie de FMA. Em plantas de *A. cepa* inoculadas com *Glomus versiforme*, foi observado maior teor de N, P e Zn, além de maior firmeza dos bulbos quando comparadas as plantas inoculadas com *Glomus intraradices*, enquanto esta última apresentou maior teor de Mn (CHARRON *et al.*, 2001).

Arandia *et al.* (2007) observaram maior diâmetro do pseudocaule e altura de plantas de cebola inoculadas com FMA, já Suhail e Mahdi (2013), além de maior altura de plantas, também verificaram maiores massas fresca e seca de cebolas micorrizadas, cultivadas em diferentes níveis de salinidade. Shuab *et al.* (2014) também observaram maior crescimento, desenvolvimento e teores de clorofila em plantas de cebolas inoculadas com FMA, além de maior produtividade e diâmetro de bulbos. Becerra e Casierra-Posada (2004) observaram maior firmeza e maior tolerância à podridão em bulbos de plantas micorrizadas.

Tawaraya e Saito (1994) observaram menor acúmulo de prolina em raízes de cebola colonizadas com FMA, atribuindo esta resposta a redução do efeito negativo do estresse em plantas. Borde, Dudhane e Jite (2010) observaram maiores teores de prolina, bem como incrementos nas enzimas antioxidante, na biomassa, na atividade fotossintética e na atividade das fosfatases ácida e alcalina, em alho micorrizado.

O aumento da atividade das fosfatases é uma resposta importante para as plantas, pois as fosfatases são enzimas que hidrolisam o fosfato terminal dos fosfomonoésteres, liberando assim, o P inorgânico. No FMA, a fosfatase ácida está presente nos terminais de ramos arbusculares, e esta atividade é reduzida em função do envelhecimento dos ramos arbusculares. Já a fosfatase alcalina, ausente em hifas imaturas, ocorre dentro do vacúolo dos fungos, aumentando a atividade conforme aumenta a maturidade (GIANINAZZI, GIANINAZZI-PEARSON e DEXHEIMER, 1979).

Outro fator que afeta a simbiose FMA-planta, é a fertilização. A adição de P e maior teor de matéria orgânica no solo afetam a colonização micorrízica de *Allium cepa* (CHARRON *et al.*, 2001). A falta de P em plantas de *Allium cepa*, leva a planta a produzir exsudatos que contém compostos hidrófobos, os quais auxiliam tanto a formação de apressórios, como estimulam o crescimento das hifas, facilitando o desenvolvimento da micorriza (TAWARAYA, HASHIMOTO e WAGATSUMA, 1998).

Diante deste contexto, a utilização de FMA pode ser uma importante ferramenta biotecnológica no cultivo de cebola, melhorando aspectos produtivos e qualitativos da cultura e reduzindo a aplicação de fertilizantes.

1.3 CO_2 ELEVADO

O intenso uso dos recursos naturais, a emissão de combustíveis fósseis e outras emissões químicas geradas pela industrialização vêm enriquecendo a atmosfera com os gases do efeito estufa (KÖRNER *et al.*, 2005), ocasionando alterações climáticas, e o CO_2 é o maior contribuinte para o agravamento deste efeito.

Atualmente o nível de CO_2 na atmosfera é de 396,33 ppm (NOAA, 2014), e deverá atingir 700 ppm até ao final do século (IPCC, 2007).

Tais aumentos apresentam efeito direto sobre as plantas, uma vez que as mesmas necessitam do CO_2 da atmosfera para realizar a fotossíntese e obter carbono (C) orgânico. Desta forma, o aumento da concentração de CO_2 pode estimular a taxa de fotossíntese e aumentar a produtividade dos sistemas agrícolas e naturais (GRIFFIN e SEEMANN, 1996), ocorrendo maior incorporação de C.

O processo de fixação de C na fotossíntese, em plantas C3, como a cebola, ocorre pela carboxilação

da ribulose-1,5-bifosfato (RuBP) através da enzima ribulose-1,5-bifosfato carboxilase/oxigenase (Rubisco). Os produtos desta reação são ácidos orgânicos com três C, que formarão a sacarose, a qual poderá seguir três caminhos principais: entrar na respiração, ser armazenada na forma de carboidratos intracelulares (sacarose, compostos da série rafinósica e frutanos nos vacúolos e amido em amiloplastos do citoplasma) e extracelulares (polissacarídeos de parede celular) ou formar parte da planta, sendo incorporada nos polissacarídeos da parede celular (TAIZ e ZEIGER, 2010).

Existem basicamente duas vias de fixação do CO_2 pelas plantas durante a fotossíntese. Durante o processo, o C sofre uma discriminação isotópica, gerando uma redução na concentração de 13C e em consequência um aumento da concentração de 12C. Esta discriminação isotópica do $13CO_2$ pode ser utilizada para diferenciá-las. No ciclo C3 (ou Ciclo de Calvin), que utiliza a RuBP como molécula receptora de CO_2 atmosférico, o gás carbônico é absorvido nas plantas por difusão, nos estômatos e passa ao interior das células para a síntese de carboidratos, os quais fornecem massa e energia às plantas. Nesta rota bioquímica, o primeiro composto orgânico sintetizado contém três átomos de carbono. Um caminho alternativo a este ciclo é o ciclo C4 (ou Ciclo de Hatch-Slack), que utiliza o oxalacetato como molécula receptora de CO_2, sendo que os primeiros açúcares formados apresentam quatro carbonos. Plantas C3 apresentam valores de d13C entre –32 e –25 ‰, enquanto as do ciclo fotossintético C4 mostram valores de d13C próximos a –12‰ (BRAND, 1996).

O indicador do processo de assimilação de C como um todo é a razão isotópica entre 13C e 12C na massa seca das plantas, registrada em partes por mil, tendo por referência um padrão. A ocorrência do 13C na massa vegetal é baixa, porque durante a carboxilação, o 12C é favorecido pelas enzimas. A Rubisco favorece mais fortemente o 12C, discriminando mais o 13C, quando comparada com a PEP-carboxilase (-28‰ e -9‰, respectivamente), comparando enzimas de plantas C3 e C4 (LANIGAN *et al.*, 2008).

Em função da dinâmica do ciclo, as plantas com metabolismo C3 são mais beneficiadas pelo aumento de CO_2 atmosférico do que plantas com metabolismo C4 (STRECK, 2005). No entanto, se o aumento da concentração de CO_2 for acompanhado de aumento da temperatura do ar, poderá não haver aumento no crescimento e no rendimento das culturas, principalmente em razão do encurtamento do seu ciclo de desenvolvimento (BUTTERFIELD e MORISON, 1992) e aumento da respiração do tecido vegetal (STRECK, 2005; TAIZ e ZEIGER, 2010). Em alguns casos, o aumento da taxa de crescimento não ocorre em função da aclimatação fotossintética, porém, mesmo nestes casos de aclimatação, a taxa de fotossíntese é mais elevada em altas concentrações de CO_2 quando comparada CO_2 ambiente.

Segundo Farquhar, Caemmerer e Berry (1980), a taxa de assimilação de C pela Rubisco é regulada por 3 fatores: a quantidade e/ou o estado de ativação da Rubisco; a regeneração da RuBP (aceptor primário do CO_2) ou a capacidade do cloroplasto em trocar trioses fosfato/ Pi (fosfato inorgânico) com o citoplasma.

A Rubisco também catalisa a oxigenação da RuBP, sendo a etapa inicial da fotorrespiração. Embora a Rubisco tenha mais afinidade pelo CO_2 do que pelo O_2, o O_2 ocorre em concentração muito maior, fazendo com que a competição entre eles pela enzima seja um dos fatores determinantes da eficiência da fotossíntese nas atuais concentrações de CO_2 atmosférico (GRIFFIN e SEEMANN, 1996). Uma vez que a reação com o

oxigênio é inibida competitivamente pelo CO_2 (STITT, 1991; DRAKE, GONZALEZ-MELER e LONG, 1997), espera-se que, se a fotossíntese for limitada pela Rubisco, um aumento da concentração atmosférica de CO_2 aumente a taxa de assimilação de C. Já se a fotossíntese for limitada pela troca de trioses fosfato/ Pi com o citoplasma, esta não deve aumentar com o incremento na concentração de CO_2 atmosférica (STITT, 1991).

Outro fator que interfere na resposta da planta ao incremento de CO_2 é a regulação negativa. Quando as plantas são cultivadas em alto CO_2 existe um estímulo inicial da fotossíntese, que pode ser perdido ou diminuído em função da regulação, limitando o potencial das plantas de suavizar os efeitos danosos do excesso de CO_2 na atmosfera (DRAKE, GONZALEZ-MELER e LONG, 1997; NORBY *et al.*, 1999).

Esta regulação negativa também é denominada aclimatação da fotossíntese e está associada com mudanças na concentração de carboidrato das folhas (MOORE *et al.*, 1999). Pesquisas revelaram maior acúmulo de carboidratos, devido ao aumento da taxa fotossintética, em folhas de plantas cultivadas em CO_2 elevado, (JACOB, GREITNER e DRAKE, 1995), sendo este acúmulo considerado um mecanismo de *feedback* (STITT, 1991). A alta concentração de carboidratos aumenta a capacidade dos drenos já existentes e estimula a formação de novos drenos até um determinado ponto, porém a incapacidade do organismo de criar novos drenos (ou aumentar os existentes) leva ao acúmulo de carboidratos nas folhas o que resulta na inibição da taxa fotossintética (STITT, 1991; PAUL e FOYER, 2001).

Aumentos nos níveis de CO_2 apresentam incrementos na atividade fotossintética e no crescimento de plantas, em função das maiores taxas de carboxilação e redução na fotorrespiração pela menor taxa de oxigenação catalisada pela Rubisco (AINSWORTH e LONG, 2005), além de incrementos na produtividade (THOMAS e STRAIN, 1991; SILVA *et al.*, 2011; MAMATHA *et al.*, 2014), biomassa (THOMAS E STRAIN, 1991; ZISKA *et al.*, 1991), área foliar (SILVA *et al.*, 2011), conteúdos de clorofila a, b e total (MAMATHA *et al.*, 2014), nos teores de sacarose, amido e celulose (THOMAS e STRAIN, 1991; AIDAR *et al.*, 2002), alterações no metabolismo secundário de plantas, redução da condutância estomática, o aumento da eficiência de uso da água, redução nas taxas de transpiração (ENOCH e HURD, 1979), aumento de P e da proporção C/N (LI *et al.*, 2013).

O cultivo com elevado CO_2, após algum tempo, também pode apresentar respostas distintas, em função do processo de aclimatação. A redução no índice estomático é uma das respostas da aclimatação, uma vez que reduz a capacidade total de entrada de CO_2 nas folhas ao longo de um período de alta concentração de CO_2 (AIDAR *et al.*, 2002). Outra resposta a este processo é a redução dos níveis de N, e consequentes reduções na concentração de proteína foliar, devido ao incremento de C nos tecidos vegetais e as consequentes mudanças na relação C/N nos tecidos vegetais (LINCOLN, COUVET e SIONIT, 1986).

Para a produção de espécies hortícolas, a economia de carboidratos é pouco estudada e é de grande importância, devido ao potencial de modificação na alocação de carbono na planta, com reflexos no aumento ou diminuição da produção comercial (SILVA *et al.*, 2011).

Em cebola foram observados aumentos em compostos voláteis como derivados sulfúricos que são fontes de pungência e sabor na cultura (IMAI *et al.*, 2002). Jasoni *et al.* (2004) também, observaram

incrementos na biomassa total, taxa fotossintética, teor de C assimilado e flavonóides em cebola (cv. Purplette) cultivadas em alto CO_2 (1000 µmol mol^{-1}). Wheeler *et al.* (2004) constataram aumentos na área foliar, taxa fotossintética e carboidratos não estruturais em bulbos de cebola (cv. Hysam) cultivadas em elevado CO_2 (532 µmol mol^{-1}), porém o teor de N foliar foi menor (0.064% e 1,41%, em CO_2 elevado e ambiente, respectivamente). Plantas do gênero *Allium* (*A. cepa, A. fistulosum* e *A. schoenoprasum*) cultivadas em alto CO_2 (1200 ppm) tiveram maior biomassa total e teor de flavonoides totais (THOMPSON *et al.*, 2004).

Identificar cultivares que respondam bem ao CO_2 elevado é importante para a adaptação às alterações climáticas (ZISKA *et al.*, 2012; TAUSZ *et al.*, 2013) e pode levar a maior eficiência do uso de recursos (DRAKE, GONZALEZ-MELER e LONG, 1997), tornando-se importante o desenvolvimento de pesquisas neste sentido.

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2 ONION (*ALLIUM CEPA* L.) SEEDLING GROWTH USING HUMIC SUBSTANCES

Crecimiento de plántulas de cebolla (*Allium cepa* L.) *con uso de sustancias húmicas*

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2.1 ABSTRACT

Humic substances can increment plant development, promoting the growth of shoots and roots, but their use in the production of seedlings is little studied. The aim of this study was to evaluate the effect of different doses of humic substances in promoting the growth of onion seedlings of the Alfa São Francisco Ciclo VIII variety. The experimental design was completely randomized, with five replications and six treatments: control with application of water and 5 doses (5, 10, 15, 20 and 25 mL L^{-1}) of humic substances containing 10% fulvic acid. The application was performed 28 days after sowing (DAS), by immersion of trays with the seedlings in the solution according to each treatment. The following characteristics were evaluated at 48 DAS: shoot height, root length, pseudostem diameter, shoot fresh mass, shoot dry mass, root fresh mass, root dry mass, foliar area, foliar volume, root volume and root area. The use of humic substances influenced the evaluated characteristics. The greatedy growth promotion of onion seedlings occurred between the concentrations of 13.8 and 24.5 mL L-¹ of humic substances in the immersion solution.
Key words: Horticulture**.** Biofertilizer. Immersion solution.

RESUMEN

Las sustancias húmicas pueden favorecer el desarrollo vegetativo, promoviendo el crecimiento de la parte aérea y raíces, pero su uso en la producción de plántulas es poco estudiado. Por lo tanto, el objetivo de este trabajo fue evaluar el efecto de la aplicación de diferentes dosis de las sustancias húmicas en el desarrollo de las plántulas de cebolla. El diseño experimental fue completamente al azar, con cinco repeticiones y seis tratamientos, siendo ellos: testigo, con aplicación de agua y 5 dosis de las sustancias húmicas que contenga 10% de ácido fúlvico (5, 10, 15, 20 y 25 mL L^{-1}) en 'Alfa São Francisco Ciclo VIII'. La aplicación se realizó a 28 días después de la siembra (DDS) a través de la inmersión de las bandejas con las plántulas en la solución según cada tratamiento. A los 48 DDS fueron evaluados: altura de la parte aérea, la longitud de las raíces, diámetro de pseudocaule, masa fresca y seca de la parte aérea, masa fresca y seca de la raíz, área y volumen de la hoja y área y volumen de la raíz. El uso de las sustancias húmicas influencian las características evaluadas. La mayoría de los efectos en lo crecimiento de plántulas de cebollas fue promovido por las concentraciones entre 13,8 y 24,5 mL L^{-1} de las sustancias húmicas en la

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solución de inmersión de plántulas de cebolla.

Palabras clave: Horticultura. Biofertilizante. Solución de inmersión.

2.2 INTRODUCTION

In horticulture, seedling production is one of the stages of the production and is a widespread system of great importance, since the performance of the crop in the field depends on the agronomic quality of the seedlings. In the case of the onion, the seedling production process has received particular attention, largely because its seeds are of high cost and reduced size, and are very sensitive to the constant cycles of hydration-dehydration in soil (Trigo *et al*., 1999). This step requires obtaining seedlings with maximum force and sanity, with appropriate development, good root system formation and better ability to adapt to the new location after transplanting (Pereira *et al.*, 2010).

According to Costa *et al.* (2009) there is a need to verify in a practical way, for each species, the type of substrate or the best composition that allows obtaining vigorous plants. Bezerra *et al*. (2009) claim that the use of handmade substrates produced with cultural remains, depending on the materials used in the formulation, does not always provide sufficient nutrient levels to promote the satisfactory development of the seedlings. Another reason why producers do not invest in commercial substrates is their high cost, which justifies the need to provide quality products to obtain seedlings with the lowest cost (Baldotto *et al*., 2009; Bernardes *et al*., 2011).

A cost-effective alternative is the addition of organic matter or biofertilizer as humic substances (HS) to the substrate, which provides benefits such as increasing the capacity of moisture retention and cationic exchange capacity, among others (Pereira *et al.*, 2010). Humic substances are composed of humic acids, fulvic acids and humin from biochemical transformations of compounds of soil organic matter such as lignin, cellulose, hemicellulose, sugars and amino acids (Primo et al., 2011). Humic substances increase the capacity of moisture retention in the soil or substrate (Khaled and Fawy, 2011), assist in the transport and absorption of nutrients Chen *et al*., 2004; Baldotto *et al*., 2009) due to the formation of complexes and chelates, reducing the need for chemical fertilizer application (Zhang *et al.*, 2013). In addition, HS have important action in the cellular metabolism of N, increasing the level of NO_3^- (Piccolo *et al*., 1993), increasing respiration and the speed of enzymatic reactions of the Krebs cycle (Nardi *et al*., 2007), increasing the content of chlorophyll (Baldotto *et al.*, 2009), acting on protein synthesis (Canellas *et al.*, 2002; Façanha *et al.*, 2002) and active hormones such as auxin, cytokinins, gibberillins (Arancon *et al.*, 2012), polyamines and abscisic acid (Mora et al. 2010, 2013).

HS also promote the growth of plants, through the greater development of shoot and root (Baldotto *et al*., 2009; Gulser *et al*., 2010; Ortiz *et al.*, 2010; Bernardes *et al*., 2011; Daur and Bakhashwain, 2013), with an increase in the production of secondary roots (Canellas *et al.*, 2002; Zandonadi *et al*., 2007; Oliveira Aguiar *et al*., 2009; Rosa e*t al*., 2009). Venter *et al.* (1991) observed greater root length of seedlings of onion cv. Texas Grano that were obtained in seed treatment with humic substances, but there are no reports about

the effects of these substances applied to onion seedlings. The objective of this study was to evaluate the effect of different doses of humic substances to promote the growth of seedlings of onion cv. Alfa São Francisco Ciclo VIII.

2.3 MATERIALS AND METHODS

Onion cv. Alfa São Francisco Ciclo VIII' (Embrapa) seeds were sown on May 15, 2012 in polystyrene trays containing 288 cells, cut into plots of 5 x 11 cells, each plot consisted of 55 cells. The cells were filled with commercial substrate Plantmax® (Agroads, Brazil) and the trays were kept in a greenhouse with micro sprinkler timed irrigation with intervals of two hours. The experimental design was completely randomized with 6 treatments and 5 replicates, composed of the control with application of water, and five doses of product containing humic substances: 0, 5, 10, 15, 20 and 25 mL L^{-1}. The original commercial solution had 10% fulvic acid, 90% humic substances and pH 4.0, originating from Leonardite (Nutriplant®), with 34.4% C; 3.8% H and 2.3% N.

The application was performed 28 days after sowing (DAS) through the immersion of trays in 2 L of syrup for 60 seconds. Ten plants per replication were collected 48 DAS and the following characteristics were evaluated: shoot height (SH), root length (RL), diameter of pseudostem (DP), shoot fresh mass (SFM), shoot dry mass (SDM), root fresh mass (RFM), root dry mass (RDM), leaf area (LA), foliar volume (FV), root volume (RV) and root area (RA).

For RL evaluation we used a graduated ruler, taking the average measurement of the 3 largest roots. To obtain the LA, FV, RV and RA the samples were analyzed in the image analysis computer program WinRhizo®, coupled to a Scanner LA1600. The leaves were taken separately and pressed for reading; the value obtained was multiplied by 2, because the leaves are cylindrical. The roots were immersed in water for easy reading. The data obtained were evaluated for homogeneity of variances by the Bartlett test and subsequently subjected to regression analysis.

2.4 RESULTS AND DISCUSSION

The maximum pseudostem diameter (DP) was obtained with the humic substance concentration of 17.1 mL L^{-1}, with an increase of more than 10 mm compared to the control (Figure 1A).

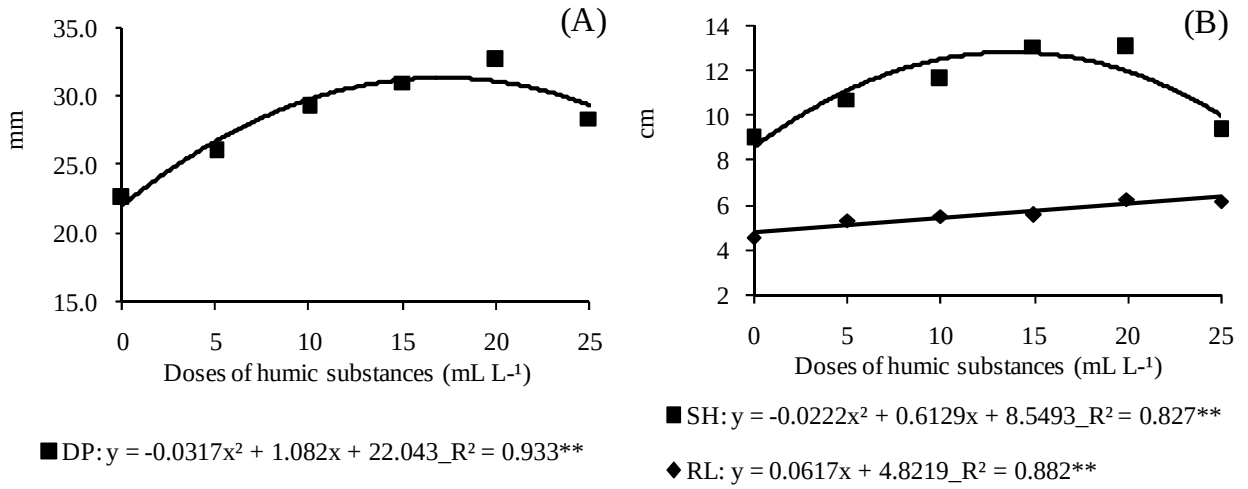

Figure 1: Diameter of pseudostem (DP) (A), shoot height (SH) and root length (RL) (B) of onion seedlings 'Alfa São Francisco Ciclo VIII' in function of the immersion in humic substances, 48 days after sowing. Curitiba, 2013.

The maximum shoot height was obtained with the concentration of 13.8 mL L-¹ (Figure 1B), while root length increased linearly with increasing concentration of humic substances in the substrate. These data reflect the effect of humic substances, probably promoted by the presence of auxin compounds, as detected by Quaggiotti *et al*. (2004) in tests with corn, that activate the H^+-ATPase the plasmatic membrane, acidifying the apoplast and activating enzymes that act directly on the cell wall, allowing greater plasticity and leading to cell elongation.

Plant growth may also be attributed to the presence of polyamines such as spermidine, spermine and putrescine found in HS (Young and Chen, 1997), which act as regulators of plants. For Dobbss et al. (2007) the growth is attributed to alkylamides, a new class of compounds with hormonal action, producing the main root growth stimulation independently of auxin signaling (Ramírez-Chávez et al. 2004).

Both leaf area and root area increased with the increase in concentration of HS, up to 17.7 and 19.2 mL L-¹, respectively (Figure 2A). This increase in area was nearly 60% for both shoots and roots, corroborating the results of Atiyeh *et al.* (2002), who obtained greater leaf area with the application of 500 mg kg^{-1} of humate. In an experiment with humic acids in corn, Zandonadi *et al.* (2007) also observed similar results, with increased proliferation of secondary roots resulting in greater root area. This increase in surface area of the root system of onion seedlings is explained by the emergence of new roots, probably as a function of stimulus of H^+- ATPase due to the presence of HS of low molecular weight.

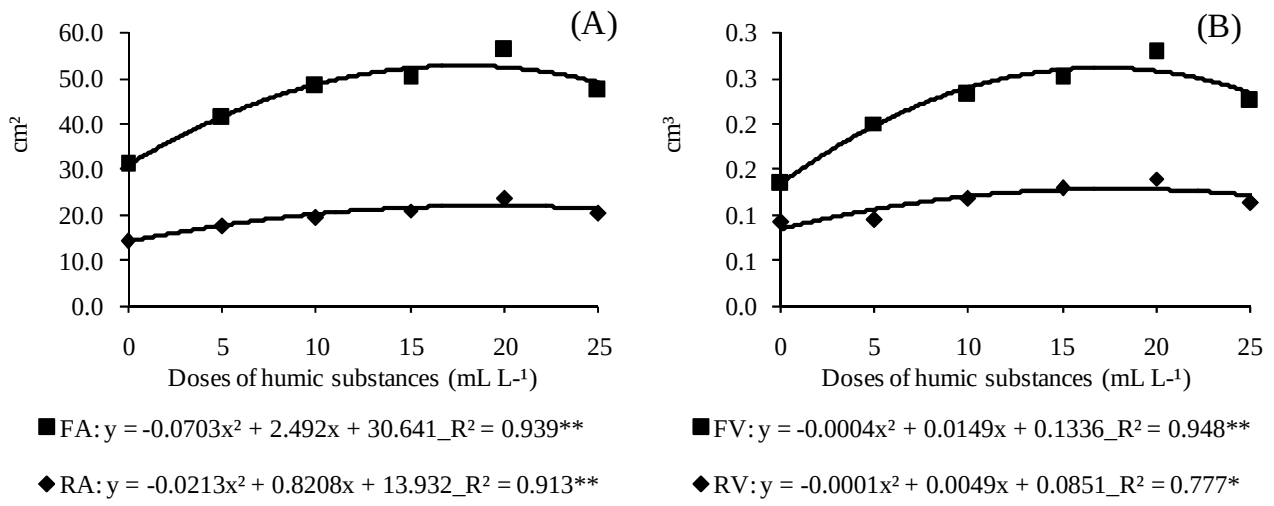

Figure 2: Foliar area (FA), root area (RA) (A)**,** foliar volume (FV) and root volume (RV) (B) of onion seedlings 'Alfa São Francisco Ciclo VIII' in function of the immersion in humic substances, 48 days after sowing. Curitiba, 2013.

The foliar and root volume increased as a function of the dose of humic substances (Figure 2B), with highest values at doses of 18.6 and 24.5 mL L-¹, respectively, probably as a consequence of an increase in the area of these two fractions of plants (Figure 2A).

The volume and foliar area, as well as shoot height, could be influenced by the auxin effect on enzyme expansin, responsible for cell elongation. Acidification of the cell wall caused by humic substances probably stimulated this enzyme, which justifies the increases observed by the application of these substances (Quaggiotti et al. 2004).

Root and shoot fresh mass of onions increased until the concentration of humic substances of 18.38 and 21.38 mL L-¹, respectively (Figure 3A). Similarly, shoot and root dry mass increased by more than three times with the application of humic substances, but with the maximum obtained at somewhat larger doses of 18.73 and 20.06 mL L-¹, respectively (Figure 3B), agreeing with results found by Rosa *et al.* (2009) in bean plants treated with humic substances.

Similar results were obtained Eyheraguibel *et al.* (2008), who observed positive effects of humic treatment on the shoot and root length of corn, in addition to greater fresh and dry mass which, according to these authors can be explained by possible interaction with plant growth regulators, as auxin present in the humic molecules.

All variables except root length, showed a quadratic behavior, with reductions values in dose 25 mL L^{-1}, which could be explained by a possible cause toxic effects.

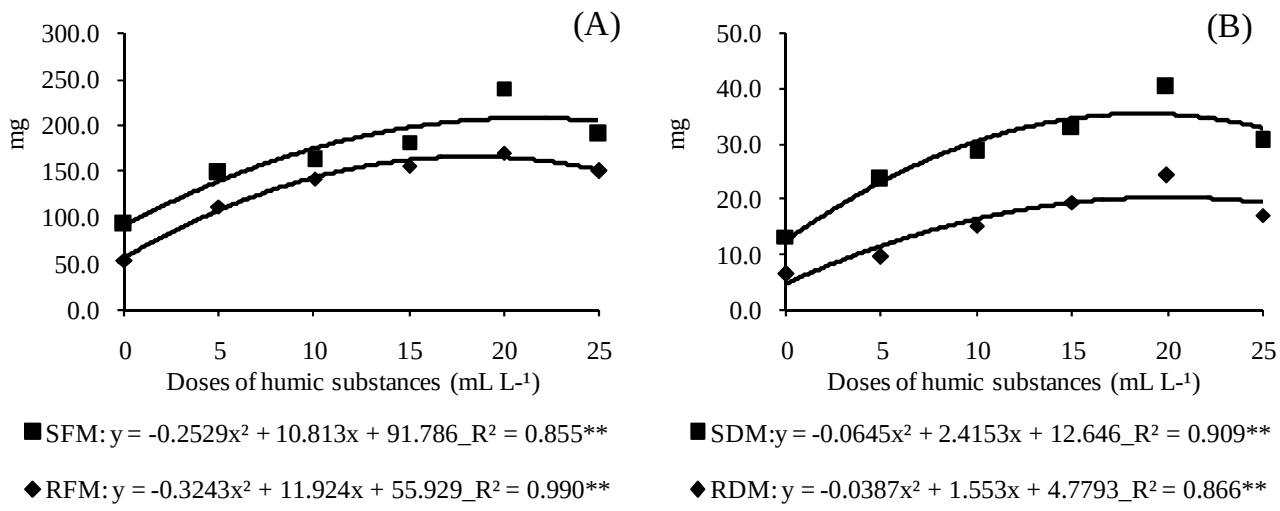

Figure 3: Shoot fresh mass (SFM), root fresh mass (RFM) (A), shoot dry mass (SDM) and root dry mass (RDM) (B) of onion seedlings 'Alfa São Francisco Ciclo VIII' in function of the immersion in humic substances, 48 days after sowing. Curitiba, 2013.

The greatest effects of application of HS occurred between the doses of 13.8 and 24.5 mL L-¹, indicating this range of dose to be the most suitable for the production of onion seedlings. The application of humic substances during the production of onion seedlings, cv. Alfa São Francisco Ciclo VIII positively influenced the growth of plants.

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3 PRODUCTIVITY AND NUTRITIONAL AND CHEMICAL QUALITY OF ONION AFFECTED BY DIFFERENT METHODS AND DOSES OF HUMIC SUBSTANCES

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3.1 ABSTRACT

Application of doses of humic substances and their methods of application are two factors that can influence the productivity and mineral and chemical quality of onion. Therefore, our main objective was to assess the effect of each of the abovementioned factors, separately or interacting, on the productivity and mineral and metabolism of onion cultivated in organic systems. The experimental design was completely randomized in a factorial, 2 x 3, with two methods of application of humic substances containing 10% fulvic acid and two doses design and control, with 5 replicates. The methods consisted of immersion of seedlings associated with the foliar pulverization the field (IM +FP) or only to the foliar pulverization the field (FP). Doses of humic substances immersion were: 0, 10, and 20 mL L^{-1}. For foliar pulverization doses was ten times less concentrated. The product was composed of 10% fulvic acid. At 95 days after transplantation plants were harvested. The following characteristics were evaluated: bulb fresh weight, bulb dry matter, water content, productivity, starch, total soluble proteins, total soluble sugars, proline and mineral nutrients. The use and method application of humic substances influenced the evaluated characteristics. The different

application and doses of humic substances have interaction with each other and affect on the productivity and mineral and chemical quality of onion.

Keywords: *Allium cepa*, fulvic acid, organic solutes, vegetative growth.

Abbreviations: DM = dry matter; FA = fulvic acid; FW = fresh weight; FP = foliar pulverization; 0FP= without application of foliar pulverization of humic substances; 1FP= with 1 mL L-¹ of foliar pulverization of humic substances; 2FP= with 2 mL L-¹ of foliar pulverization of humic substances; IM+FP = immersion and foliar pulverization; HA = humic acids; HS = humic substances; 0IM+0FP= without application of foliar pulverization or immersion of humic substances ; 10IM+1FP= with 10 mL L-¹ by immersion and 1 mL L^{-1} of foliar pulverization of humic substances; 20IM+2FP= with 20 mL L-¹ by immersion and 2 mL L^{-1} of foliar pulverization of humic substances; 0HS = without application of humic substances; TSP= total soluble proteins; TSS = total soluble sugars; WC = water content.

3.2 INTRODUCTION

Onion (*Allium cepa* L) is an extremely important vegetable crop at the worldwide level. It occupies a mundial area of 239594 ha, with one production of 4342135 t $yaer^{-1}$ and one mean productivity 18.12 t ha^{-1} in 2012, which generated $2139 million (FAOSTAT, 2014).

For many years, the strategies used to increase the productivity of crops was to increase the area planted and / or using chemical fertilizers (Ayala and Rao, 2002). Recently, the use of humic substances (HS) may be a viable alternative to increase productivity, with a reduction in production costs and increased efficiency of inputs, without compromising environmental quality.

These substances are composed of humic acid, fulvic acid and humic, derived from biochemical transformations of compounds of soil organic matter, such as lignin, cellulose, hemicelluloses, sugars and amino acids (Schulten and Schnitzer, 1997), addition to hormones Façanha et al., 2002;; Elena et al., 2009; Schiavon et al., 2010; Silva et al., 2011), polyamines (Young and Chen, 1997) and alkylamides (Dobbss *et al.*, 2007).

The HS promoted the growth and developed of plants (Mora et al., 2012; Daur and Bakhashwain, 2013), with effect in the primary (Canellas et al., 2002; Façanha et al., 2002) and secondary metabolism (Schiavon et al., 2010); with action in enzymatic reactions (Nardi, Muscolo and Vaccaro, 2007), more chlorophyll and ATP production (Baldotto *et al.*, 2009; Ertani *et al.*, 2011) and activation of hormones auxin (Schiavon et al., 2010; Silva et al., 2011), cytokinin, gibberellin (Arancon et al., 2012), polyamines, abscisic acid (Mora et al., 2010, 2013) and ethylene (Mora et al., 2012, 2013).

Canellas and Santos (2005) also reported that these substances can promote better absorption of nutrients by plants by cell wall acidification process, increasing the permeability to nutrient, and increase its availability by complexed and chelated action, as well as help transport and absorption of nutrients (Eyheraguibel, Silvestre and Morard, 2008), with effect in N metabolism (Ertani *et al.*, 2011), as well as iron and zinc (Chen et al., 2004; Baldotto et al., 2009), among other micronutrients (Lima et al., 2011).

The use of HS also has effect on the quality of plants, affecting solids and soluble sugars (Lima et al., 2011), carbohydrates (Aminifard et al., 2012) starch (Canellas et al., 2002; ; Ertani et al., 2011).

Different results were verified in function of method of application of HS. Parandian and Samavat (2012), studying immersion and pulverization methods, separated and pulverization with doses ten times less concentrated with HS in Lilium found that immersion method is more effective on nutrient uptake, soluble sugar and ∝-amylase than pulverization method. However, Osman, EL-Masry and Khatab (2013), observed that the more pronounced effect was using foliar application in rice.

In onion, the application in seedlings, by soil, promoted more productivity (Feibert, Shock and Saunders, 2003). Sajid et al. (2012) observed more productivity and nutrient concentration in onion when used 2 kg ha^{-1} de AH in the sowing. Osvalde et al. (2012) not found effect of HS applied to soil while preparing the seedlings and the leaves (two or three times) at a concentration of 0.2%. Kandil, Sharief and Fathalla (2013) revealed that foliar application of 18.5% HA, applied at 60 and 80 days after transplant (DAT) in onion, increased vegetative growth, bulb yield, quality and chemical composition of onion, verifying highest total and marketable yield of bulbs as well as higher average masses of bulbs, higher soluble sugars (SS) content, dry matter and less weight loss.For Grangeiro et al. (2008) the onion quality is related to the external appearance as the bulb size, color, flavor, firmness and chemical composition. These attributes, according to Finger and Casali, (2002), are determined by factors such as genotype, pre-harvest management, the proper harvesting time and post-harvest treatments. Than, the humic substances can affect both the productivity and the quality of onions.

The main objective of our study was to assess the effect of each of the abovementioned factors, different application and doses of humic substances (HS), separately or interacting, on the productivity and mineral and chemical quality of onion. Special attention was paid to the levels of starch, sugars, proteins and proline in the bulb as well as to nutritional composition of bulbs.

3.3 MATERIALS AND METHODS

3.3.1 Plant material and growth conditions

The experiment was performed from August 2012 and February 2013, in the Organic Vegetables production area at the Canguiri Experimental Stations of the Federal University of Paraná, in Pinhais, State of Paraná, Brazil. It is located in the physiographic region called first 'Paranaense Plateau', located at 25º25' S, y 49°08' W, elevation 930m. According to the classification of Köeppen the climate is temperate Cfb with marked seasons. Mean monthly temperatures for period between were August 2012 and February 2013: 15.5º; 16.1º; 18.3º; 18.3º; 21.6º; 19.1º and 20.4 ºC, respectively (Simepar, 2013). The photoperiod between May 2012 and February 2013 was: 10:02h/August; 11:46h/September; 13:36h/October; 15:15h/November; 16:08h/December, 15:04h/January and 14:11h/February (Observatorio Nacional, 2013). Soil was prepared two weeks before seedling transplant, and consisted of 200 kg ha^{-1} magnesium thermo phosphate (YOORIN

MASTER 1, with 17% P_2O_5) and 8 t ha^{-1} of organic compounds, whose analysis yielded: N = 14.4 g kg^{-1}; P = 10.6 g kg^{-1}; K = 11.3 g kg^{-1}; Ca = 31.7 g kg^{-1}; Mg = 6.8 g kg^{-1}; C = 384 g kg^{-1}; pH = 7.1; C/N = 27.6. This fertilization followed the recommendation proposed by Raij et al. (1996). Four rows of plants per plot, with 30 cm spacing between rows and 15 cm between plants, comprising a population of 222.222 plants per hectare. Seedlings were transplanted when they reached a height of 18-20 cm (Ferreira and Minami, 2000) in 18th October 2012.

The soil is classified as 'Latosol red-yellow alico' with clay texture and gentle rolling hills (EMBRAPA, 2006), whose chemical analysis in a 0-15 cm soil profile in the first cycle indicated: pH ($CaCl_2$) = 5.9; pH SMP = 6.0; Al^{+3} = 0; H+Al = 4.0 $cmol_c$ dm^{-3}; Ca^{+2} = 10.7 $cmol_c$ dm^{-3}; Mg^{+2}= 4.5 $cmol_c$ dm^{-3}; K^+ = 1.32 $cmol_c$ dm^{-3}; P = 32.6 mg dm^{-3}; C = 23.2 g dm^{-3}; Boron = 0.98 mg dm^{-3}; V% = 81.0 and CTC= 20.52 $cmol_c$ dm^{-3}.

The sowing of onion cultivar 'Alpha San Francisco Cycle VIII' (Embrapa), was held on August 17th 2012 in polystyrene trays containing 288 cells. The cells were filled with commercial substrate Plantmax® (Agroads, Brazil) and the trays were kept in a greenhouse with irrigation micro sprinkler timer with intervals of two hours.

The experimental design was completely randomized in a factorial design 2 x 3, with 5 replicates, comparing two methods of application of humic substances and two doses design and control. The methods consisted of immersion of seedlings associated with the foliar pulverization in plants the field (IM +FP) or only to the foliar pulverization in plants the field (FP). The original commercial solution had 10% FA, 90% HS and pH 4.0, originating from Leonardite (Nutriplant®), 34.4% C; 3.8% H and 2.3% N. Doses of humic substances immersion were: 0, 10, and 20 mL L^{-1}. For foliar pulverization doses was ten times less concentrated.

For IM method, the dives were performed at the time of sowing and at 30 and 60 days after. For IM+FP method, the immersion was the same the IM method and more FP that followed up to 63 days after transplanting (DAT), with intervals of 15 days, with the first application at 7 DAT.

Therefore, there were six different treatments in total: (1) 0FP; (2) 1FP; (3) 2FP; (4) 0 IM+0FP; (5) 10IM+1FP and (6) 20IM+2FP.

Final harvest of all onion was carried out on day 95th after transplant, when about 85% of the plants in the experimental field reached the stage of "snap", softening of the pseudostem, indicating the end of the cycle.

3.3.2 Growth parameters and water status

At harvest, ten plants of each treatment were randomly selected for determination of bulb fresh weight (FW), bulb dry matter (DM) and mean productivity (MP). This parameter was estimated by measuring the fresh weight of ten bulbs and multiplying by number of plants per hectare (222.222 plants). DM was determined after drying plant material in oven at 80°C until weight was constant. Water status of

seedlings was determined by calculating water content (WC) of bulb: FW of bulb – DM of bulb/ DM of bulb. Bulb WC was expressed as g of water g^{-1} DM.

3.3.3 Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline in bulb

Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline were quantified in potassium phosphate buffer (KPB; 50 mM, pH 7.5) extracts of dry bulb (0.5 g per bulb, five bulb per treatment, with two readings each). These extracts were filtered through four cheesecloth layers and centrifuged at 38720 *g* for 10 min at 4ºC. The pellet was used for starch determination (Jarvis and Walker, 1993). The supernatant was collected and stored at 4ºC for TSS, TSP and proline determinations. TSS were analyzed with the anthrone reagent in a Spectronic 2000 (Bausch and Lomb, Rochester, NY) according to Yemm and Willis (1954). TSP was measured by the protein dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The free proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrine reaction (Irigoyen, Emerich and Sánchez-Díaz, 1992). The results were expressed as mg of starch, TSS or TSP per g of leaf DM and as μmol of proline per g of bulb DM.

3.3.4 Mineral analyses in bulbs

For mineral analyses, samples (0.5 g DM) of three bulbs per treatment were dry-ashed and dissolved in HCl according to Duque (1971). Phosphorus, potassium, magnesium, calcium, manganese, iron, zinc and copper were determined using a Perkin Elmer Optima 4300 inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin Elmer, USA). The operating parameters of the ICP-OES were: radio frequency power, 1300 W; nebulizer flow, 0.85 L min^{-1}; nebulizer pressure, 30 psi; auxiliary gas flow, 0.2 L min^{-1}; sample introduction, 1 mL min^{-1} and three replicates per sample.

3.3.5 Carbon, nitrogen, ratio carbon and nitrogen, carbon 13 and nitrogen 15 in bulbs

Bulb samples were dried at 60ºC over 48h and were weighed in small tin capsules. Samples were analysed to determine the carbon and nitrogen isotope composition using an Flash 1112 Elemental Analyser (Carbo Erba, Milan) coupled to an IRMS Delta C isotope ratio mass spectrometer through a Conflo III Interface (Thermo-Finnigan, Germany).

Results of carbon isotope ratio analyses are reported in per mile (‰) on the relative δ-scale, as $\delta^{13}C$ and refer to the international standard V-PDB (Vienna Pee Dee Belemnite) according to the following equation:

$$Š^{13}C = \left(\frac{R_{sample}}{R_{standard}} \right) - 1 \quad \text{(Eq.1)}$$

where R is the $^{13}C/^{12}C$ ratio.

Carbon isotope discrimination ($\Delta^{13}C$) of shoot TOM was calculated from δ_a and δ_p (Farquhar et al. 1980) as:

$$\Delta^{13}C = \frac{\delta_a - \delta_p}{\delta_p + 1} \quad \text{(Eq.2)}$$

where *a* and *p* refer to air and plant, respectively.

Nitrogen results were also expressed in δ notation ($\delta^{15}N$) using international secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) referred to N_2 in air:

$$Š^{15}N = \left(\frac{R_{sample}}{R_{standard}} \right) - 1 \quad \text{(Eq.3)}$$

where R is the $^{15}N/^{14}N$ ratio.

3.3.6 Statistical analysis

Data were subjected to a two-factor ANOVA (factorial 2 x 3, Assistat Beta 7.7). The variance was related to the main treatments (different application methods of humic substances, IM+FP or just FP, and different application doses of humic substances 0, 10 and 20 mL L^{-1} when use immersion and 0, 1 and 2 mL L^{-1} for FP) and to the interaction between them (Methods × HS). Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Tukey´s test was applied.

3.4 RESULTS

3.4.1 Growth parameters and water status

Data shown in Table 1 indicate that the two factors applied in the study (Methods and Doses HS) increased bulb productivity of onion when applied separately or together, being this enhancement mainly due to improved biomass of bulbs (Methods, $P \leq 0.01$; Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$ for bulb FW and bulb DM). The same factors were significantly influenced in the water accumulated in bulbs and mean productivity of bulbs. The highest values of bulb FW were achieved by onion grown with 10IM+1FP (77.22 g $bulb^{-1}$). Similar results were observed in mean productivity of bulbs, with the highest

value on the same treatments (17.16 t ha^{-1}). The bulb DM obtained the highest value when used 10IM+1FP and 20IM+2FP (5.26 g $bulb^{-1}$ DM and 5.10 g $bulb^{-1}$ DM, respectively). The application of 1FP or 10IM+1FP increased the value of the water accumulated in bulbs (1306.01 g H_2O g-¹ FM and 1367.69 g H_2O g-¹ FM, respectively).

3.4.2 Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline in bulb

Concentrations of starch in bulbs were always clearly lower than those of soluble sugars in all onion bulbs (Table 1). When cultivated with IM+FP method and HS addition the accumulation of starch, being additive effects of both factors when compared with the control. Therefore, with IM+FP method, the highest levels of starch were found in bulbs that received 10IM+1FP and 20IM+2FP (2.64 mg $bulb^{-1}$ DM and 2.41 mg $bulb^{-1}$ DM, respectively).

Levels of TSS were very sensitive to each factor applied (Methods or Doses HS) and also were to the interactions between different factors (Methods, $P \leq 0.01$; Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$ for TSS). The highest concentrations of TSS were found in bulbs of plants that received 10IM+1FP (148.92 mg g^{-1} bulb DM).

Similarly to findings with TSS, there were positive effects of different factors, separately or interacting among them, on the protein levels of onion bulbs (Methods, $P \leq 0.01$; Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$ for TSP) (Table 1). The lowest content of proteins in bulbs corresponded to plants grown without HS application (3.07 mg g^{-1} bulb DM for FP and 2.30 mg g^{-1} bulb DM for IM+FP). The highest value for TSP was of 11.23 mg g^{-1} bulb DM, when used 10IM+1FP.

The effect of doses HS and the interaction Methods x Doses HS was verified for proline (Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$ for proline) (Table 2). Bulbs growth without HS showed the highest values (6.60 mg g^{-1} bulb DM for FP and 7.23 mg g^{-1} bulb DM for IM+FP).

3.4.3 Mineral analyses in bulbs

In the Table 2 is possible see the effect of doses HS and the interaction with the Methods (Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$) in nutrients P, K, Fe and Na. The isolated factors or the duo interaction between them (Methods, $P \leq 0.01$; Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$ for TSP) (Table 2) enhanced the amount the concentration of Ca, Mg, Mn and Zn in bulbs (Methods, $P \leq 0.01$; Doses HS, $P \leq 0.01$ and Methods x Doses HS, $P \leq 0.01$ for Ca, Mg and Mn and $P \leq 0.05$ for Zn). For Cu and Ni was not observed the effect of interaction of factors, just the isolated effect of methods and doses HS (Methods, $P \leq 0.05$; Doses HS, $P \leq 0.01$ for Cu and Doses HS, $P \leq 0.05$ for Ni). Boron was not affected by any of the factors did not differ significantly among treatments, with values between 20.13 and 21.89 mg g^{-1}

DM (Table 2).

The lowest values of P and Mg were found in cultivated bulbs without HS (3.24 g kg-¹ DM for FP and 2.97 g kg-¹ for IM+FP when used for P and 1.82 g kg-¹ DM for FP and 2.00 g kg-¹ for IM+FP when used for Mg). In opposite, the 20IM+2FP application showed the highest values for both minerals when compared with control (4.00 g kg-¹ for P and 3.18 g kg-¹ for Mg) (Table 2). The 20IM+2FP also showed the increment in concentrations for K (11.65 g kg-¹), Ca (6.13 g kg-¹) and Fe (4.78 g kg-¹) (Table 2).

The HS dose 1FP or 10IM+1FP, independent of method, increased the concentrations of Cu (17.94 mg g-¹ for FP and 18.62 mg g-¹ for IM+FP) and Na (405.78 mg g-¹ for FP and 401.63 mg g-¹ for IM+FP) and, for Ni the same dose affected the concentration, but just in the IM+FP method, when compared of control for the three nutrients.

The IM+FP method with HS application showed the highest values of Mn (38.31 mg g-¹ for 10IM+1FP and 37.34 mg g-¹ for 20IM+2FP) and Zn (43.85 mg g-¹ for 10IM+1FP and 44.68 mg g-¹ for 20IM+2FP).

3.4.4 Carbon, nitrogen, ratio carbon and nitrogen, carbon 13 and nitrogen 15 in bulbs

The Table 3 show that the Doses HS factor was significantly influenced in the nitrogen and ratio carbon and nitrogen accumulated in bulbs (Doses HS, $P \leq 0.05$). The Data shown in Table 3 indicate that the two factors applied in the study (Methods and Doses HS) increased the carbon 13 and nitrogen 15 in onion bulbs when applied separately (Methods, $P \leq 0.01$ and Doses HS, $P \leq 0.01$). Carbon contents were not affected by any of factors and no significant differences were observed between treatments, which had averages ranging between 39.94% and 43.05% (Table 3). The nitrogen values also were similar between treatments, with means between 1.66% and 2.22%. The highest value of ratio carbon and nitrogen was 26.43 when the bulbs were cultivate in IM+FP without HS, but haven´t differences significantly compared to the other treatments, except the treatment 20IM+2FP, with the lowest value (19.43).

The FP method with 1FA reached the highest value for the carbon 13 (-28.88), not being observed difference for treatment with 2FP (-28.99) and 10IM+1FP (-29.30) (Table 3). The highest values for nitrogen 15 were verified with FP method (7.30 for 0FP, 7.79 for 1FP and 8.04 for 2FP, respectively)

3.5 DISCUSSION

In the present study, the application of humic substances promoted the growth and productivity of onion. The values found in these experiment at bulb FW and mean productivity when used the 10IM+1FP (Table 1) are greater than the value found for Bettoni et al (2013) studying the same cultivar (Alfa São

Francisco- Ciclo VIII) (57.25 g $bulb^{-1}$ for bulb FW and 15.46 t ha^{-1} for mean productivity) and the productivity is close to mundial average that is 18.12 t ha^{-1} in 2012 (FAOSTAT., 2014). These positive effect on growth and productivity is due to humic substances and its frequency of application, probably promoted by the presence of auxins, which activates the H^+-ATPase of the plasma membrane, acidifying the apoplast and activating enzymes that act directly on the cell wall, allowing greater plasticity of this, leading to cell elongation (Façanha et al., 2002; Elena et al., 2009; Schiavon et al., 2010; Silva et al., 2011).

The plant growth may also be due to the presence of polyamines, such as putrescine, spermidine and spermine found in HS (Young and Chen, 1997), which, according to Martens and Frankenberger (1994) act as regulators of plants (Kumar, Imtiyaz and Kumar, 2007). Dobbss et al. (2007) attributed the growth alkylamides, a new class of compounds with hormonal action, which provide stimulating root growth and independently of auxin signal (Ramírez-Chávez *et al.*, 2004). Thus, a hypothesis can be formulated that it is related to the HS containing substances alkylamides.

The IM+FP of HS showed the higher concentrations of non-structural sugars (starch and soluble sugars) in bulbs (Table 1). The higher values of soluble sugars in plants which receiving the application HS was also observed by other researchers (Ertani et al., 2011; Parandian and Samavat, 2012) and they attribute such increments of the promotion of photosynthesis with increased activity of chlorophyll and Rubisco (Ertani *et al.*, 2011), with increased production and accumulation of assimilates.

Protein concentrations in bulbs of onion also were higher with 10IM+1FP (Table 1), which indicates that the HS have action about cell metabolism N, with the highest NO_3^- concentration in plants (Piccolo et al., 1993), due to the reduction of the pH on the root surface, thus facilitating H^+/NO_3^- symport (Quaggiotti et al., 2004), addition to the increase in the activity of the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT), which act in the availability of NH_4^+, resulting in increases in N organic compounds (as chlorophyll and proteins) (Ertani *et al.*, 2011). Researches also proved more synthesis of protein when use HS (Façanha et al., 2002)

When not used HS, the values of proline were higher (Table 1). This results can be explicated is one answer to a metabolic imbalance. In cabbage, the same result was observed, with the accumulation of proline in one situation unfavorable, when cultivated in conventional system, since when cultivated with humic substances don't have highest values of proline, because had one situation of equilibrium (Vilanova and Da Silva Junior, 2010).

Plants in situations of stress or metabolic imbalance has a slower growth because they mobilize carbohydrates and sugars for the synthesis of proline, as can be observed in this work, in the treatments without HS (Díaz *et al.*, 2012).

Accumulation of mineral nutrients was variable in function of different treatments, but, in general, the more concentration was when use 20IM+2FP (Table 2), that is the major dose with the major frequency. Sajid et al. (2012) also observed more nutrient concentration in onion when used HS.

According Canellas and Santos (2005) this result occur because the HS stimulates the H^+-ATPase and promotes the acidification of the cell wall, which in turn increases its permeability, thereby allowing the

entry of nutrients. Another factor associated with greater absorption of nutrients is the action of the chelate and complexed of HS, making the nutrients enter the cells more easily (Eyheraguibel, Silvestre and Morard, 2008).

The HS are also especially important because of their ability to chelate micronutrients, thus increasing their bio-availability. Plants receiving HS can absorb more mineral elements, due to the better development of its roots, and its effect on membrane permeability due to the presence of hydrophilic and hydrophobic sites in these substances (Chen and Schnitzer, 1978), causing them to interact with the structures of phospholipids of cell membranes and reacted as carriers of nutrients through them.

Bulb concentration of N and C not had different with HS application (Table 3). This fact can be explained by the mobilization of these nutrients for the synthesis of other compounds such as sugars and proteins. The same result occur with the C/N.

The largest accumulation of 13C and 15N was observed in treatments with HS when treated with FP, which means that the bulbs were considered preferred drains the photoassimilate accumulation when compared to other treatments (Silva *et al.*, 2011).

3.6 CONCLUSIONS

Doses and application method of humic substances fertilization appear as valid horticultural techniques for improving productivity and chemical and nutritional quality of onion bulbs. The immersion onion plant in a dose 10 mL L^{-1} associated with the same dose results in increases in 1 mL L^{-1} foliar pulverization bulb fresh and dry mass as well as the average water content and productivity. The same treatment also had a positive effect on the chemical composition of onion bulbs, with higher starch, total soluble sugars and soluble proteins. As the nutritional composition, the use of humic substances in the immersion method associated with the foliar pulverization was the most effective for most nutrients.

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3.8 TABLES

Table 1. Productivity parameters, water status, concentrations of starch (mg g^{-1} DM), total soluble sugars (TSS) (mg g^{-1} DM), total soluble proteins (TSP) (mg g^{-1} DM) and proline (μg g^{-1} DM) in bulbs of onion humic substances foliar pulverization (FP) or immersion more foliar pulverization (IM+FP) with different doses (0, 10 and 20 mL-¹ HS for IM and 0, 1 and 2 mL-¹ HS for FP). Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; *and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. FW = fresh weight; DM = dry matter; WC = water content; MP = Mean productivity.

Treatments		**Bulb FW** (g)	**Bulb DM** (g)	**Bulb WC** (g H_2O g-¹ FW)	**MP** (t ha-¹)	**STARCH** (mg g^{-1} Bulb DM)	**TSS** (mg g-¹ DM)	**TSP** (mg g-¹ DM)	**PROLINE** (mg g-¹ DM)
FP	**0**	34.56 ± 0.37 e	3.22 ± 0.02 d	972.77 ± 6.75 c	7.68 ± 0.08 e	1.67 ± 0.03 de	109.09 ± 3.08 b	3.07 ± 0.39 de	6.60 ± 0.66 ab
	1	56.60 ± 1.48 b	4.03 ± 0.06 b	1306.01 ± 39.31 ab	12.58 ± 0.33 b	1.57 ± 0.04 e	112.08 ± 1.82 b	3.77 ± 0.05 cd	4.14 ± 0.17 c
	2	41.98 ± 1.76 d	3.18 ± 0.05 d	1217.56 ± 39.96 b	9.33 ± 0.39 d	2.22 ± 0.06 bc	85.50 ± 3.23 c	6.72 ± 0.36 b	4.38 ± 0.49 c
IM+FP	**0+0**	33.94 ± 0.19 e	3.58 ± 0.01 c	849.21 ± 2.25 c	7.54 ± 0.04 e	1.94 ± 0.06 cd	113.28 ± 2.59 b	2.30 ± 0.11 e	7.23 ± 0.43 a
	10+1	77.22 ± 1.67 a	5.26 ± 0.05 a	1367.69 ± 38.83 a	17.16 ± 0.37 a	2.64 ± 0.12 a	148.92 ± 2.18 a	11.23 ± 0.24 a	4.78 ± 0.29 bc
	20+2	49.32 ± 1.03 c	5.10 ± 0.03 a	868.11 ± 22.55 c	10.96 ± 0.23 c	2.41 ± 0.03 ab	96.72 ± 3.72 bc	4.56 ± 0.27 c	5.02 ± 0.38 bc
Methods		**	**	**	**	**	**	**	ns
Doses HS		**	**	**	**	**	**	**	**
Methods x Doses HS		**	**	**	**	**	**	**	**

Table 2. Concentrations of mineral nutrients in bulbs of onion humic substances foliar pulverization (FP) or immersion more foliar pulverization (IM+FP) with different doses (0, 10 and 20 mL-¹ HS for IM and 0, 1 and 2 mL-¹ HS for FP). Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$, and $P \leq 0.01$, respectively. DM = dry matter.

Treatments		**P** (g kg-¹ DM)	**K** (g kg-¹ DM)	**Ca** (g kg-¹ DM)	**Mg** (g kg-¹ DM)	**Fe** (g kg-¹ DM)	**Cu** (mg kg^{-1} DM)	**Mn** (mg kg^{-1} DM)	**Zn** (mg kg^{-1} DM)	**B** (mg kg^{-1} DM)	**Ni** (mg kg^{-1} DM)	**Na** (mg kg^{-1} DM)
FP	**0**	3.24 ± 0.018 c	10.30 ± 0.020 c	5.10 ± 0.013 cd	1.82 ± 0.048 e	3.93 ± 0.052 c	16.23 ± 0.11 b	25.84 ± 0.29 b	37.84 ± 0.08 b	20.13 ± 0.35 a	7.83 ± 0.19 b	354.06 ± 9.75 c
	1	3.78 ± 0.028 b	10.80 ± 0.023 bc	5.33 ± 0.025 c	2.84 ± 0.014 bc	4.36 ± 0.047 b	17.94 ± 0.16 a	24.27 ± 0.26 bc	39.03 ± 0.42 b	21.89 ± 0.85 a	8.46 ± 0.27 ab	405.78 ± 7.90 a
	2	3.85 ± 0.008 ab	11.24 ± 0.245 ab	4.88 ± 0.088 d	2.71 ± 0.034 c	4.46 ± 0.038 b	15.38 ± 0.20 b	20.98 ± 1.16 c	38.99 ± 0.48 b	21.61 ± 0.71 a	7.83 ± 0.37 b	385.85 ± 5.84 abc
IM+FP	**0+0**	2.97 ± 0.045 d	9.66 ± 0.070 d	5.21 ± 0.037 c	2.00 ± 0.036 d	3.83 ± 0.112 c	16.26 ± 0.26 b	26.57 ± 0.75 b	40.04 ± 0.83 b	20.41 ± 0.35 a	8.50 ± 0.38 ab	396.92 ± 9.36 ab
	10+1	3.84 ± 0.063 ab	11.23 ± 0.056 ab	5.84 ± 0.093 b	2.89 ± 0.040 b	4.05 ± 0.053 c	18.62 ± 0.40 a	38.31 ± 1.14 a	43.85 ± 0.63 a	20.16 ± 0.42 a	9.41 ± 0.28 a	401.63 ± 6.71 a
	20+2	4.00 ± 0.006 a	11.65 ± 0.161 a	6.13 ± 0.039 a	3.18 ± 0.016 a	4.78 ± 0.041 a	16.25 ± 0.26 b	37.34 ± 0.73 a	44.68 ± 0.80 a	22.30 ± 0.96 a	8.16 ± 0.14 ab	364.52 ± 4.60 bc
Methods		ns	ns	**	**	ns	*	**	**	ns	*	ns
Doses HS		**	**	**	**	**	**	**	**	ns	*	**
Methods x Doses HS		**	**	**	**	**	ns	**	*	ns	ns	**

Table 3. Concentrations of nitrogen, carbon, ratio carbon and nitrogen, carbon 13 and nitrogen 15 of bulbs of onion humic substances foliar pulverization (FP) or immersion more foliar pulverization (IM+FP) with different doses (0, 10 and 20 mL-¹ HS for IM and 0, 1 and 2 mL-¹ HS for FP). Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$, and $P \leq 0.01$, respectively.

Treatments		**N (%)**	**C (%)**	**C/N**	**d13C***	**d15N**
FP	**0**	1.83 ± 0.10 a	41.89 ± 0.46 a	23.04 ± 1.48 ab	-29.39 ± 0.04 bc	7.30 ± 0.08 ab
	1	2.09 ± 0.14 a	42.18 ± 0.68 a	20.30 ± 1.09 ab	-28.88 ± 0.06 a	7.79 ± 0.22 a
	2	2.22 ± 0.09 a	42.96 ± 0.25 a	19.43 ± 0.87 b	-28.99 ± 0.08 ab	8.04 ± 0.08 a
IM+FP	**0+0**	1.66 ± 0.12 a	43.45 ± 0.21 a	26.43 ± 1.66 a	-29.98 ± 0.08 d	6.44 ± 0.09 c
	10+1	1.87 ± 0.14 a	39.94 ± 3.78 a	21.44 ± 1.62 ab	-29.30 ± 0.14 abc	6.99 ± 0.28 bc
	20+2	2.09 ± 0.15 a	43.03 ± 0.49 a	20.82 ± 1.68 ab	-29.69 ± 0.15 cd	6.97 ± 0.13 bc
Methods		ns	ns	ns	**	**
Doses HS		*	ns	*	**	**
Methods x Doses HS		ns	ns	ns	ns	ns

*transformated datos x+100

4 GROWTH AND METABOLISM OF ONION SEEDLINGS AS AFFECTED BY THE APPLICATION OF HUMIC SUBSTANCES, MYCORRHIZAL INOCULATION AND ELEVATED CO_2[3]

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4.1 ABSTRACT

Onion (*Allium cepa* L.) is a crop with great economic importance over the world. The vigor of seedlings plays a crucial role in the posterior growth and quality of bulbs. Application of humic substance (HS), inoculation of arbuscular mycorrhizal fungi (AMF) in the soil and enhancement of atmospheric CO_2 are three factors that can influence plant growth and development. Therefore, our main objective was to assess the effect of each of the abovementioned factors, separately or interacting, on the metabolism and growth of onion seedlings before bulb formation and under greenhouse conditions. Results showed that these three factors appear as valid horticultural techniques for improving growth and quality of onion seedlings cultivated in greenhouse even when mycorrhizal colonization of roots does not achieve high rates. Beneficial effects of HS were additive to those of mycorrhizal inoculation or elevated CO_2 (ECO_2) on shoot and root biomass production. The triple interaction between exogenous HS application, mycorrhizal inoculation and ECO_2 induced the highest accumulation of soluble sugars, proteins and proline in leaves, suggesting that such interaction was the most effective for increasing the quality of onion shoots as source organs for posterior growth and quality of bulbs and also for enhancing the tolerance of onion seedlings to environmental stresses.

Keywords: *Allium cepa*, arbuscular mycorrhizae, carbon dioxide, humic substances, organic solutes,

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vegetative growth.

Abbreviations: ACO_2 = ambient CO_2; AMF = arbuscular mycorrhizal fungi; BSA = bovine serum albumin; Chl = chlorophyll; DM = dry matter; DPPH = α, α-Diphenil-β-picrylhydrazyl radical scavenging activity; E = extension of mycorrhizal colonization; EC = electrical conductivity; ECO2 = elevated CO_2; FA = fulvic acid; FW = fresh weight; HA = humic acids; HS = humic substances; 0HS = without application of humic substances; 20HS = with application of humic substances; I = incidence of mycorrhizal colonization; Int = intensity of mycorrhizal colonization; KPB = potassium phosphate buffer; L = mycorrhizal colonization in length; MEI = mycorrhizal efficiency index; +M = inoculated with mycorrhizal fungi; -M = non-inoculated with mycorrhizal fungi; TSP= total soluble proteins; TSS = total soluble sugars; W = mycorrhizal colonization in width; WC = water content.

4.2 INTRODUCTION

Onion (*Allium cepa* L.) is a crop with great economic importance. It is the second most important vegetable crop in the world, with approximately 4 million Ha of harvested area and about 80 million tons produced in 2012. Only in Brazil the production exceeded 1.5 million tons and in Spain production almost reached 1.2 million tons, being this last country one of the main producing countries of onions in the European Union (FAOSTAT 2014).

The size of the onion bulb is dependent upon the number and size of the green leaves at the time of maturity. There is a ring of onion for every leaf and the larger the size of the leaf, the bigger will be the ring (Addai et al., 2014). Therefore, the vigor of seedlings plays a crucial role in the posterior growth and quality of bulbs. Humic substances (HS), arbuscular mycorrhizal fungi (AMF) and enhanced atmospheric CO_2 (ECO_2) are three factors that may strongly influence growth and metabolism of onion seedlings.

Humic substances (HS) have been included among the natural molecules that exert physiological influences on plant growth (Nardi et al., 2002). They are derived from the degradation and decomposition of dead biological material in soils and are considered as supramolecular associations of self-assembling heterogenous and relatively small molecules (Piccolo, 2001). It has been reported that the HS can affect plant physiology because they may exert hormone-like effects (Nardi et al., 2002; Zandonadi et al., 2007), influence photosynthesis (Ferretti et al., 1991), activate some enzymes (Vaughan et al., 1985), increase mineral availability (Varanini and Pinton, 2001; Murillo et al., 2005; Eyheraguibel et al., 2008) and/or stimulate beneficial soil microorganisms (Linderman and Davis, 2001). Among HS, the fulvic acids (FA) appear to confer a specific bioactivity to HS because of their loose conformation and hydrophilic nature. In addition, due to their substantial structural flexibility and heteregenous composition, FA are likely to release mobile hormone-like molecules, which may enter the biological pathways related to auxin activities (Zancani et al., 2011).

Mycorrhizal fungi colonize the roots of over 80% of plant species mostly to the mutual benefit of both plant host and fungus (Smith and Read, 2008). The most common are the arbuscular mycorrhizas (AM), which are formed by the majority of crop and horticultural plants, including onion. The association of onion with AMF can enhance plant height, leaf area index, chlorophyll content, total plant biomass as well as bulb

dry mass and diameter (Bolandnazar et al., 2007). In addition, mycorrhizal symbiosis can improve the defence responses of onion plants against bacterial and fungal pathogens due to increased chitinase activity (Dumas-Gaudot et al., 1992), higher antimicrobial activity and enhanced levels of phenolic compounds (Lokhandwala et al., 2014) in roots. Mycorrhizal association can also favour the nitrate uptake by onions cultivated on dry soils (Azcón and Tobar, 1998). However, the effectiveness of AM for improving nutrient uptake and yield of onions can vary according to the type of fungus inoculated to plants. Charron et al. (2001a, b) found that bulbs of onions inoculated with *Glomus versiforme* were firmer than those inoculated with *Rhizophagus intraradices* (formerly *G. intraradices*) and the P, N and Zn concentrations were higher in onion plants colonized by *G. versiforme* than in those colonized by *R. intraradices*. The markedly mycotrophic character of onions has led to use this culture to enrich soil with mycorrhizal propagules prior planting some fruit trees (Panja and Chaudhuri, 2004).

The increase of atmospheric CO_2 as a consequence of global change and/or horticultural practices affects plant growth and development. The enhanced CO_2 concentration (ECO_2) increases the potential net photosynthesis in C3 plants, such as onion (Drake et al., 1997) and therefore can improve yield over short-term exposures (Oliveira et al., 2010). In an assay performed under greenhouse conditions, Savé et al. (2007) concluded that CO_2 fertilization shows interesting perspectives to improve horticultural techniques in order to enhance plant medicinal productivity. However, when the synthesis of carbohydrates in plants exposed to ECO_2 exceeds the capacity to produce new sinks, the plants decrease their photosynthetic rate so as to balance source activity and sink capacity (Thomas and Strain, 1991). In onion, sink effect of bulbs may counteract at least in part the expected acclimation of photosynthesis over medium or long-term exposures to ECO_2 and the presence of AMF colonizing onion roots would presumably increase the sink effect.

The main objective of our study was to assess the effect of each of the abovementioned factors, application of humic substances (20HS), mycorrhizal inoculation (+M) and elevated CO_2 (ECO_2), separately or interacting, on the metabolism and growth of onion seedlings before bulb formation. Special attention was paid to the levels of photosynthetic pigments, sugars, proteins and proline in leaves as well as to acid phosphatase in roots. Levels of soluble phenolics and the total antioxidant capacity of leaves were also tested.

4.3 MATERIALS AND METHODS

4.3.1 Plant material and growth conditions

Seeds from *Allium cepa* L. cv. Alfa São Francisco- Cycle VIII (Embrapa Semi-Árido) were germinated on a mixture of light peat and sand (1:1, v:v) (on 26th March 2013). Peat (Floragard, Vilassar de Mar, Barcelona, Spain) had a pH of 5.2-6.0, 70-150 mg L^{-1} of nitrogen, 80-180 mg L^{-1} P_2O_5 and 140-220 mg L^{-1} K_2O and it was previously sterilized at 100ºC for 1 h on three consecutive days. Twenty two days after sowing (on 17th April 2013), seedlings were transferred to trays filled with a mixture of vermiculite- siliceous

sand- sterilized light peat (2.5:2.5:1, v:v:v). Each tray had 60 cells with a capacity of 60 mL each one. The experiment was conducted in a completely randomized design in a factorial 2 × 2 × 2, with four replications for each treatment. Factors were 'mycorrhizal inoculation, M', 'humic substances, HS' and 'CO_2 concentration in the atmosphere, CO_2'.

The following day after transferring seedlings to trays (on 18th April 2013) half of cells (80 seedlings) received 5 mL of a solution containing 20% HS (20HS). This solution was applied by using a syringe. The original commercial solution had 10% FA, 90% HS and pH 4.0, originating from Leonardita (Nutriplant®), 34.4% C; 3.8% H and 2.3% N. Other 80 seedlings did not receive this solution (0HS). Two days after transferring seedlings to trays (on 19th April 2013), half of 0HS cells (40 seedlings) and half of 20HS cells (40 seedlings) were inoculated with the liquid mycorrhizal inoculum 'Glomygel Intensivo' (Mycovitro S.L., Pinos Puente, Granada, Spain) (+M seedlings). The concentrated commercial inoculum derived from an *in vitro* culture of arbuscular mycorrhizal fungi (AMF): *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüβler comb. nov. (Krüger et al., 2012). It contained around 2,000 mycorrhizal propagules (inert pieces of roots colonized by AMF, spores and vegetative mycelium) per mL of inoculum. In order to facilitate its application, the concentrated commercial inoculum was diluted with distillate water until obtaining a resultant mycorrhizal inoculum with around 250 propagules per mL. Each +M seedling received 5 mL of the diluted mycorrhizal inoculum close to the roots thus making a total of 1,250 propagules. A filtrate was added to seedlings that did not receive the mycorrhizal inoculum (-M seedlings) in an attempt to restore other soil free-living microorganisms accompanying AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a layer of 15-20 µm filter papers (Whatman, GE Healthcare, UK) and each –M seedling received 5 mL of filtrate close to the roots. The selection of in vitro-produced inoculum of *R. intraradices* was based on two expected benefits: (1) easy application of the product and (2) low colonization of onion roots by contaminant fungi (Vimard et al., 1999).

Subsequently all trays were transferred to four [CO_2] controlled greenhouses located at the University of Navarra campus (42.80 N, 1.66 W; Pamplona, Spain). The design of the greenhouses was similar to that described by Sanz-Sáez et al. (2012) and based on Aranjuelo et al. (2005). Half of seedlings (20 0HS +M seedlings, 20 0HS -M seedlings, 20 20HS +M seedlings and 20 20HS -M seedlings) were divided into two greenhouses where no CO_2 was added and [CO_2] was maintained at ambient conditions (~360 µmol mol^{-1}) (ACO_2). The other half (20 0HS +M seedlings, 20 0HS -M seedlings, 20 20HS +M seedlings and 20 20HS -M seedlings) were divided into two greenhouses where [CO_2] was increased to ~700 µmol mol^{-1} by injecting pure CO_2 (purity up to 99.99%) from cylinder-gases (34 L of CO_2 per cylinder) at the two inlet fans during the light hours (ECO_2). Injection of CO_2 to greenhouses began when light intensity was equal or superior to 5 watts m^{-2} as measured by a Silicon Pyranometer PYR-S (APOGEE Instruments, Inc., Logan, UT, USA). The CO_2 was provided by Air Liquide (Bilbao, Spain). The [CO_2] was continuously monitored using a Guardian Plus gas monitor (Edinburgh Instruments Ltd, Livingston, UK). The monitor's signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans.

Therefore, there were eight different treatments in total: (1) 0HS+M seedlings grown at ACO_2; (2) 0HS-M seedlings grown at ACO_2; (3) 0HS+M seedlings grown under ECO_2; (4) 0HS-M seedlings grown under ECO_2; (5) 20HS+M seedlings grown at ACO_2; (6) 20HS-M seedlings grown at ACO_2; (7) 20HS+M seedlings grown under ECO_2; and (8) 20HS-M seedlings grown under ECO_2.

All seedlings received 30 mL of modified Hewitt's solution (Baslam et al., 2011) twice a week and other 30 mL of distilled water once a week. The nutrient solution contained 6 mM $Ca(NO_3)_2$, 6 mM $CaCl_2$, 3 mM KNO_3, 2.3 mM K_2SO_4,1.5 mM $MgSO_4$, 1.3 mM NaH_2PO_4, 68 µM EDTA-Fe, 13 µM $MnSO_4$, 9 µM H_3BO_4, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM Na_2MoO_4. The original pH of the nutrient solution (5.2 ± 0.1) was adjusted to 7.0. The EC of the nutrient solution adjusted to 7.0 was 140 ± 15 µS cm^{-1} as determined with a conductivity meter 524 Crison (Crison Instruments S.A., Alella, Spain). Seedlings were alternatively irrigated with distilled water and nutrient solution to avoid salt accumulation. Final harvest of all seedlings was carried out on day 72nd after sowing.

4.3.2 Growth Parameters, Water Status, Mycorrhizal Colonization and Mycorrhizal Efficiency Index (MEI)

At harvest, five plants of each treatment were randomly selected for determination of shoot height, number of leaves per plant, shoot and root fresh weight (FW), shoot and root dry matter (DM), and the ratio root to shoot DM. DM was determined after drying plant material in oven at 80°C until weight was constant. Water status of seedlings was determined by calculating water content (WC) of shoots or roots: FW of shoot or root – DM of shoot or root/ DM of shoot or root. Shoot or root WC was expressed as g of water g^{-1} DM.

Root samples were cleared and stained (Phillips and Hayman, 1970) and mycorrhizal colonization was determined by examining 1 cm root segments (n = 45 per pot) under the microscope. Extension (E), incidence (I) and intensity (Int) of mycorrhizal colonization were calculated for each pot as described by Baslam et al. (2014). The E of mycorrhizal colonization was firstly determined for every root segment and it was calculated as the product between value of mycorrhizal colonization in width (W) and value of mycorrhizal colonization in length (L). Values of mycorrhizal colonization in width (W) and length (L) were ascribed according a scale in which 0 meant complete absence of fungal structures and 10 meant that fungal structures occupied the full length or width of the root segment. Afterwards, total E per pot was calculated as $E = \sum (W \times L)/n$, where 'n' was the total number of root segments observed under the microscope (n = 45 per pot) and it was expressed as a percentage. Incidence (I) of mycorrhizal colonization per pot was calculated as the ratio between number of root segments with fungal structures (arbuscules, vesicles and/or hyphae) and total number of root segments observed under the microscope (n = 45 per pot). Finally, the intensity (Int) of mycorrhizal colonization per pot was calculated as the product between E and I ($Int = E \times I$) and results were expressed as percentage of infection (Hayman et al., 1976). The Mycorrhizal Efficiency Index (MEI) was estimated according to Bagyaraj (1994): MEI = DM of +M seedlings − DM of –M seedlings × 100/DM of

+M seedlings. Determination of MEI allows assessment of the growth improvement brought about by inoculation of plants with a mycorrhizal fungus.

4.3.3 Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline in leaves

Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline were quantified in potassium phosphate buffer (KPB; 50 mM, pH 7.5) extracts of fresh leaves (0.5 g per seedling, five seedlings per treatment). These extracts were filtered through four cheesecloth layers and centrifuged at 38720 *g* for 10 min at 4ºC. The pellet was used for starch determination (Jarvis and Walker, 1993). The supernatant was collected and stored at 4ºC for TSS, TSP and proline determinations. TSS were analyzed with the anthrone reagent in a Spectronic 2000 (Bausch and Lomb, Rochester, NY) according to Yemm and Willis (1954). TSP were measured by the protein dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The free proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrine reaction (Irigoyen et al., 1992). The results were expressed as mg of starch, TSS or TSP per g of leaf DM and as μmol of proline per g of leaf DM.

4.3.4 Chlorophylls and carotenoids in leaves

Samples of 100 mg of fresh leaves were immersed in 5 mL of 96% ethanol at 80ºC during 10 min to extract chlorophylls (chl a, chl b) and carotenoids (Séstak et al., 1971). For every extract absorbance was measured at 470, 649, 665 and 750 nm using a Spectronic 2000 (Bausch and Lomb, Rochester, NY, USA). Estimation of chl a, chl b, chl a+b and total carotenoids in the same extract was performed by using the extinction coefficients and equations described by Lichtenthaler (1987). Results were expressed as mg of chlorophylls or carotenoids per g of leaf DM.

4.3.5 Total soluble phenolic compounds and total antioxidant capacity of leaves

Total phenolic compounds were extracted according to Chapuis-Lardy et al. (2002) with some modifications. One sample (0.5 g of FW) of shoot per seedling (four seedlings per treatment) were pulverized in liquid nitrogen, mixed with 20 mL of 80% methanol, and homogenized at room temperature for 1 min. After filtration, 0.5 mL of each sample was mixed with 10 mL of distilled water. The total phenolic content was determined from aqueous solutions by spectrophotometric analysis at 760 nm with Folin Ciocalteu reagent (Waterman and Mole, 1994). The results are expressed as mg of gallic acid per g of DM.

The total antioxidant capacity was evaluated by applying the free α, α-Diphenil-β-picrylhydrazyl radical scavenging activity ($DPPH^{\bullet}$ assay) in the same leaf extracts used for determining the content of total soluble phenolic compounds. The free radical scavenging activity using the free radical $DPPH^{\bullet}$ (Brand-Williams et al., 1995) was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction in parafilm-sealed glass cuvettes (to avoid methanol evaporation) at 25ºC (Espín et al., 2000). The reaction was started by adding 20 μL of the corresponding sample to the cuvette containing 80 μM (methanol solution) (980 μL) of the free radical ($DPPH^{\bullet}$) (Llorach et al., 2004). The final volume of the assay was 1 mL. Reaction was followed with a spectrophotometer (Jasco V-630, Analytical Instruments, Easton, MD, USA). Calibration curve was made using gallic acid as standard. Results were expressed as μg of gallic acid per g of DM.

4.3.6 Acid phosphatase activity in roots

The acid phosphatase (EC.3.11.3.2) activity in roots was measured as described by Dodd et al. (1987) and results were expressed as the amount of *p*-nitrophenol released during incubation.

4.3.7 Statistical Analysis

Data on mycorrhizal colonization and MEI were subjected to a two-factor ANOVA, being atmospheric CO_2 concentration, CO_2, and application of humic substances, HS, the main factors; the interaction between them (CO_2 × HS) was also studied. Data on growth, water status, starch, total soluble sugars (TSS), total soluble proteins (TSP), proline, phenolics and antioxidant capacity in leaves as well as acid phosphatase in roots were subjected to a three-factor ANOVA (factorial 2 x 2 x 2, Assistat Beta 7.7). The variance was related to the main treatments (atmospheric CO_2 concentration, CO_2, inoculation of mycorrhizal fungi, M, and application of humic substances, HS, and to the interaction between them (CO_2 × M, CO_2 × HS, M × HS, CO_2 × M × HS). Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Tukey´s test was applied. Tests were considered significant at $P \leq 0.05$. Significance levels for ANOVAs were: ns = not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

4.4 RESULTS

4.4.1 Growth parameters, water status, mycorrhizal colonization and mycorrhizal efficiency index (MEI)

Data shown in Table 4 indicate that the three factors applied in the study (ECO_2, HS or AMF) increased shoot growth of onion seedlings when applied separately, being this enhancement mainly due to improved biomass (CO_2, $P \leq 0.01$; M, $P \leq 0.01$ and HS, $P \leq 0.01$ for shoot DM). In contrast, none of these factors significantly influenced the production of leaves per plant and only mycorrhizal inoculation increased the amount of water accumulated in shoot tissues (M, $P \leq 0.01$ for shoot WC). There were also some additive effects between different factors on shoot biomass (CO_2 × HS, $P \leq 0.01$; M × HS, $P \leq 0.01$ for shoot DM). The highest values of shoot DM were achieved by onion seedlings grown under ECO_2 and inoculated with AMF (78 mg $plant^{-1}$ without HS addition and almost 80 mg $plant^{-1}$ when HS were added). Similar results were observed in roots. Root biomass increased after applying HS, inoculating AMF or exposing plants to ECO_2 (HS, $P \leq 0.01$; M, $P \leq 0.01$ and CO_2, $P \leq 0.01$ for root DM). The application of HS increased the positive effect of ECO_2 or AMF inoculation on root development (CO_2 × HS, $P \leq 0.01$; M × HS, $P \leq 0.01$ for root DM). Root WC was similar in all onion seedlings. The highest root to shoot biomass ratio corresponded to seedlings inoculated with AMF, amended with HS and grown under ECO_2.

Microscopic observations of cleared and stained roots revealed that there were few fungal structures (hyphae, arbuscules and vesicles) in root tissues. Percentages of mycorrhizal colonization ranged from 15.8% in onion not amended with HS and grown under ECO_2 to 19.5% in onion not supplied with HS and cultivated at ACO_2. There were not significant effects of main factors on mycorrhizal colonization (HS, ns; CO_2, ns). Mycorrhizal structures were never detected in roots of onion plants that did not receive mycorrhizal inoculum (Table 5). Results of Mycorrhizal Efficiency Index (MEI) are represented in Fig. 4 and they have been divided into leaves, roots and the whole plant. Inoculation of onions with AMF always enhanced plant growth so that +M seedlings produced around 35-40% more total biomass than –M plants, regardless they were grown at ACO_2 (white histograms) or under ECO_2 (black histograms) and independently they were (20HS) or not (0HS) amended with HS (Fig. 4, 'Plant'). However, this beneficial effect differed when plants were cultivated at ACO_2 or under ECO_2. At ACO_2 (white histograms) leaves were the organs that most beneficed from mycorrhizal inoculation (Fig. 4, 'Leaf), so that +M onions produced up to 25% of shoot biomass than –M seedlings, irrespectively of HS addition. Under ECO_2 (black histograms) shoot biomass in +M onions was 20% higher than in –M seedlings in absence of HS (0HS) and this value lowered to 15% when seedlings received HS (20HS) (Fig. 4, 'Leaf'). In contrast, the highest efficiency of AMF in improving root biomass (Fig. 1, 'Root') was observed when onions were cultivated under ECO_2 (black histograms) and HS were applied to the substrate (20HS) (root DM was 30% greater in +M than in –M onions).

4.4.2 Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline in leaves

Concentrations of starch in leaves were always clearly lower than those of soluble sugars in all onion seedlings (Table 6). When cultivated at ACO_2, HS addition and AMF inoculation enhanced the accumulation of starch, being additive effects of both factors. Therefore, at ACO_2 the highest levels of starch were found in +M seedlings that received HS (20HS) (11.45 mg g^{-1} leaf DM). Under ECO_2 the supply of HS increased starch concentrations but +M plants accumulated similar starch contents than their respective –M plants. Levels of TSS were very sensitive to each factor applied (CO_2, AMF or HS) and also were to the interactions between different factors. The highest concentrations of TSS (87.47 mg g^{-1} leaf DM) were found in leaves of seedlings inoculated with AMF (+M), grown under ECO_2 and that received HS (20HS) ($CO_2 \times M \times HS$, $P \leq 0.01$ for TSS). In contrast, the lowest levels of TSS were observed in leaves of seedlings non-inoculated with AMF (-M) and cultivated at ACO_2 without receiving HS (0HS) (32 mg g^{-1} leaf DM).

Similarly to findings with TSS, there were positive effects of different factors, separately or interacting among them, on the protein levels of onion seedlings (Table 6). The lowest content of proteins in leaves corresponded to seedlings grown at ACO_2 without neither HS application (0HS) nor mycorrhizal inoculation (-M) (10.64 mg g^{-1} leaf DM). In the opposite, interaction between ECO_2, HS and AMF induced the accumulation of proteins in leaves (18.72 mg g^{-1} leaf DM in +M 20HS seedlings under ECO_2). This triple interaction also enhanced the amount of proline in leaves (10.68 μg g^{-1} leaf DM).

4.4.3 Chlorophylls (Chl) and carotenoids in leaves

The highest contents of Chl a, Chl b and total Chl (a+b) were found in leaves of onion seedlings inoculated with AMF (+M), fertilized with HS (20HS) and grown under ECO_2 (5.09 mg Chl a g^{-1} leaf DM, 2.39 mg Chl b g^{-1} leaf DM, 7.49 mg Chl a+b g^{-1} leaf DM) (Table 7). In contrast, the lowest levels of these pigments were found in seedlings non-inoculated with AMF (-M), without HS addition (0HS) and cultivated at ACO_2 (2.54 mg Chl a g^{-1} leaf DM, 1.01 mg Chl b g^{-1} leaf DM, 3.55 mg Chl a+b g^{-1} leaf DM). ANOVA results corroborated the significant influence of CO_2, mycorrhization (M) and HS on Chl levels (CO_2, $P \leq 0.01$, M, $P \leq 0.01$ and HS, $P \leq 0.01$ for the concentrations of Chl a, Chl b and Chl a+b). The total concentrations of Chl (a+b) were more sensitive to the interactions between different factors than levels of Chl a or Chl b separately. The concentrations of total carotenoids increased in leaves of onions after inoculation with AMF (+M), regardless they received (20HS) or not (0HS) HS; however, these enhancements were significant only at ACO_2 (Table 7). The application of HS (20HS) induced the accumulation of carotenoids only in leaves of +M seedlings grown at ACO_2. ECO_2 increased the levels of carotenoids in –M onions fertilized (20HS) or not (0HS) with HS.

4.4.4 Total soluble phenolic compounds and total antioxidant capacity of leaves

ECO_2 (black histograms), mycorrhizal inoculation (+M) and HS addition (20HS) induced the accumulation of total soluble phenolics in leaves of onion seedlings (Fig. 5A) and these increases were observed when factors were applied separately or when they were interacting (Table 8). Therefore, the lowest levels of these secondary metabolites were measured in seedlings non-inoculated with AMF (-M), without HS addition (0HS) and cultivated at ACO_2 (white histograms) (550 mg g^{-1} leaf DM, Fig. 2A) and the highest concentrations were found in +M seedlings that received HS (20HS) and were exposed to ECO_2 (black histograms) (970 mg g^{-1} leaf DM, Fig. 2A).

The total antioxidant capacity measured in leaves of onion seedlings (Fig. 5B) was not correlated with the amount of phenolics (Fig. 5A). ECO_2, mycorrhizal inoculation (M) and application of HS exerted a significant influence on the antioxidant capacity in onion leaves either separately or when they interacted among them (Table 8). However, the effects of different factors were sometimes opposite. Mycorrhizal inoculation (+M) increased the antioxidant capacity in absence of HS (0HS) and when plants were grown at ACO_2 (white histograms) (Fig. 5B). The addition of HS (20HS) enhanced the antioxidant capacity at both ACO_2 (white histograms) and ECO_2 (black histograms) but only in –M plants (Fig. 5B). In contrast, ECO_2 (black histograms) diminished the antioxidant capacity in both –M and +M seedlings when HS were not supplied (0HS) (Fig. 5B).

4.4.5 Acid phosphatase activity in roots

The application of HS (20HS) always increased the acid phosphatase activity in roots, regardless mycorrhizal inoculation or CO_2 concentration in the atmosphere (Fig. 6). Similarly, mycorrhizal inoculation (+M) enhanced this enzymatic activity in roots of seedlings fertilized (20HS) or not (0HS) with HS at both ACO_2 (white histograms) or ECO_2 (black histograms). However, increases induced by AMF were smaller than those induced by HS addition. ECO_2 (black histograms) increased acid phosphate activity in roots in both –M and + M plants but only in absence of HS fertilization (0HS).

4.5 DISCUSSION

In the present study, the percentage of mycorrhizal colonization in onion roots never exceeded 20% (Table 5). These values are clearly lower than those measured by Charron et al. (2001a) in onion inoculated with root segments that came from leek plants colonized by *R. intraradices* (from 39 to 53%). In our study, the main component of the mycorrhizal inoculum was spores of *R. intraradices*. Therefore differences in root

colonization may be at least partially due to the different type of inocula applied to onion plants in both studies. On the other hand, Vimard et al. (1999) working with leek affirmed that monoxenically-produced spores of *R. intraradices* as mycorrhizal inoculum can yield percentages of mycorrhizal colonization (43.5%) similar to a root-segment inoculum (58.3%) in a soil mix growth medium (peat-perlite-turface-soil, 1:1:1:1) after 16 weeks; in this case, the highest increase in the percentage of mycorrhizal colonization occurred between weeks 12 and 16. In our experiment the substrate used did not contain soil and onion seedlings were harvested 10 weeks after sowing. Both the beginning of root colonization and the percentage of roots colonized by AMF in onion are also highly dependent on the species of mycorrhizal fungus (Afek et al., 1990). The application of nutrient solution can also influence the percentage of mycorrhizal colonization. In a recent assay performed by Navarro et al. (2012) with carnation plants inoculated with a very similar in vitro-produced commercial inoculum (Glomygel Garden, *R. intraradices*, from MYCOVITRO) than that used in our study (Glomygel Intensivo, *R. intraradices*, from MYCOVITRO), the highest mycorrhizal colonization (26%) was reached in plants irrigated with fresh water, whereas increases of salinity in the irrigation water caused decreased levels of mycorrhizal colonization. In our study, however, such effect is expected to be inappreciable due to the low EC of the applied nutrient solution and the alternation of nutrient solution and water for irrigating onion seedlings. Although we did not observe significant differences between the percentages of mycorrhizal colonization in nonamended onions (0HS) and onions that received HS (20HS), previous data revealed that the application of organic substances with high HA content to onions can improve root colonization by *R. intraradices* (Linderman and Davis, 2001). However, as explained by the later authors, fungal response depends on the organic substances applied and soil (or substrate) characteristics.

In agreement with Linderman and Davis (2001), mycorrhizal inoculation (+M) and application of HS interacted synergistically and enhanced plant growth more in combination than separately (M × HS, $P \leq 0.01$ for shoot height, shoot and root DM) when onions were cultivated at ACO_2 (Table 5).

Anjum et al. (2011) observed that foliar FA applications enhanced the chlorophyll contents in maize under both deficient and optimal water regimes. Similarly, our results showed higher amount of chl a, chl b and total chl in onion seedlings supplied with 20HS than in those non-amended with HS (0HS) (Table 7). Enhanced contents of chl may have improved photosynthesis rates in onion seedlings that received exogenous application of HS with 10% FA (Anjum et al., 2011), which would explain the higher concentrations of non-structural sugars (starch and soluble sugars) found in leaves of seedlings fertilized with HS (20HS) (Table 6). In +M seedlings, however, the application of HS increased the levels of total chl only when onions were exposed to ECO_2. Onions inoculated with AMF (+M) accumulated higher amount of soluble sugars in leaves than their respective –M controls, regardless seedlings were (20HS) or not (0HS) fertilized with HS or cultivated at ACO_2 or under ECO_2 (Table 6). Increased levels of soluble sugars in leaves of +M plants compared with –M plants have been previously found (Baslam et al., 2011, 2012) and can be a consequence of enhanced photosynthesis in +M plants (Sánchez-Díaz et al., 1990). Positive effects of HS and mycorrhizal inoculation in increasing the levels of non-structural sugars were additive may be due

to the auxin-like effect of FA present in the commercial HS applied. Gay et al. (1994) observed that auxin overproducer mutants of an ectomycorrhizal fungus increased mycorrhizal activity. In our study, the enhanced acid phosphatase activity in +M plants compared with that of the respective –M plants (Fig. 6) could have provided more phosphorus (P) to the leaves thus beneficing photosynthesis. Higher phosphatase activity in +M plants has been reported for different plant species such as corn, soybeans (Khalil et al., 1994), alfalfa (Goicoechea et al., 1996) or barley (Goicoechea et al., 2004) and may be due to the greater phosphatase activity of the internal hyphae produced by mycorrhizal fungi in host roots (Saito, 1995). Enhanced acid phosphatase activity in +M onions may be crucial under ECO_2. According to Cavagnaro et al. (2011) decreased phosphorus (P) concentrations in tissues of plants grown under ECO_2 can be alleviated by the formation of mycorrhizal symbiosis, thus reducing or delaying photosynthetic acclimation in these plants. Beneficial effects of HS application, mycorrhizal inoculation and ECO_2 (separately or interacting among them) on photosynthetic rates would improve plant growth and biomass production, as observed in our study (Table 4).

Phosphorus is not the only elemental nutrient whose levels in plant tissues can increase after HS application or mycorrhizal symbiosis. Xu et al. (2012) reported that the addition of HS stimulated the accumulation of nitrogen (N) in cucumber and several studies have demonstrated significant N enrichment in +M crops such as tomato, cucumber, lettuce, wheat or onion in comparison with –M plants (see Azcón et al., 2008 for more details). Improved uptake and translocation of N from roots to shoots in onion seedlings that received HS and/or were inoculated with AMF would explain the enhanced amount of proteins found in leaves of +M and/or 20 HS seedlings compared with those found in leaves of 0HS and/or –M seedlings (Table 6). Moreover, protein concentrations in leaves of onion seedlings were higher under ECO_2 than at ACO_2 (Table 4), which indicates that there was not any N limitation in leaves, one of the possible causes of photosynthetic down-regulation under ECO_2 (Sanz-Sáez et al., 2010). In addition to soluble proteins, the levels of proline also increased in leaves of onions fertilized with HS (20HS), inoculated with AMF (+M) or both and such enhancements were more evident under ECO_2 than at ACO_2 (Table 6). Likewise, Anjum et al. (2011) found that FA application improved the proline accumulation in leaves of maize under both well-watered and drought conditions. Mycorrhizal association can also induce the accumulation of proline in roots and leaves of host plants, being these increases especially evident in plants undergoing water deficit (Goicoechea et al., 1998). Several possible physiological functions have been ascribed to this amino acid accumulation, such as osmoregulation, a sink for energy and nitrogen, a signal of senescence and an indicator of drought resistance and/or stress sensor (Aspinall and Paleg, 1981).

Baslam et al. (2012) observed that the accumulation of mineral nutrients (P, Cu, Fe), carotenoids and phenolics induced by mycorrhizal symbiosis in leaves of lettuces cultivated at ACO_2 diminished or disappeared under ECO_2 suggesting that a relevant quantity of photoassimilates were used for improving plant growth and spreading mycorrhizal colonization in detriment to the secondary metabolism under ECO_2. In contrast, onion seedlings exposed to ECO_2 showed improved growth and biomass production together with enhanced accumulation of phenolics. In a recent review, Cheynier et al. (2013) postulate a link between

primary and secondary metabolism that couples the accumulation of proline with the energy transfer toward phenylpropanoid biosynthesis via the oxidative pentose phosphate pathway: proline synthesis is accompanied by the oxidation of NADPH and an increased $NADP^+$/NADPH ratio may enhance activity of the oxidative pentose phosphate pathway providing precursors for phenolic biosynthesis via the shikimic acid pathway. In our study, there was a positive relationship ($r = 0.867$, $P \leq 0.01$) between the levels of proline (Table 6) and soluble phenolics in leaves (Fig. 2A). However, concentrations of phenolic compounds were not correlated to the total antioxidant capacity (Fig. 3B), suggesting that other compounds would be implied in the total antioxidant capacity in onion leaves. According to our results, the accumulation of such compounds seems to be negatively affected by the interaction between exogenous HS application and mycorrhizal inoculation.

4.6 CONCLUSIONS

Application of HS, addition of in vitro-produced mycorrhizal inoculum to substrate and atmospheric CO_2 fertilization appear as valid horticultural techniques for improving growth and quality of onion seedlings under greenhouse conditions even when mycorrhizal colonization of roots does not achieve high rates. Beneficial effects of HS were additive to those of mycorrhizal inoculation or ECO_2 on shoot and root biomass production. The triple interaction between HS application, mycorrhizal inoculation and ECO_2 induced the highest accumulation of soluble sugars, proteins and proline in leaves, suggesting that such interaction was the most effective for increasing the quality of onion shoots as source organs for posterior growth and quality of bulbs and also for enhancing the tolerance of onion seedlings to environmental stresses.

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4.8 TABLES

Table 4. Growth parameters and water status in onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO2) or under elevated (ECO2) CO_2 in the atmosphere. Values are means (n = 4) ± SE. Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$, and $P \leq 0.01$, respectively. FW = fresh weight; DM = dry matter; WC = water content; R/S = root DM/shoot DM.

Treatments			Shoot heigth (cm)	Leaves per plant	Shoot FW (mg plant^{-1})	Shoot DM (mg plant^{-1})	Root FW (mg plant^{-1})	Root DM (mg plant^{-1})	Shoot WC (g H_2O g^{-1} DM)	Root WC (g H_2O g^{-1} DM)	R/S
ACO_2	**-M**	**0HS**	13.8 ± 0.23 cd	2.9 ± 0.16	405.2 ± 3.79 f	42.8 ± 0.97 f	249.6 ± 3.53 g	25.3 ± 0.35 f	8.5 ± 0.13 b	8.9 ± 0.06 a	0.62 ± 0.01 e
		20HS	15.0 ± 0.20 b	2.9 ± 0.16	472.8 ± 2.04 d	49.6 ± 0.75 de	355.0 ± 1.80 e	36.4 ± 0.17 d	8.5 ± 0.14 b	8.7 ± 0.07 a	0.75 ± 0.01 d
	+M	**0HS**	13.4 ± 0.24 d	2.9 ± 0.16	554.3 ± 2.15 c	57.3 ± 0.29 bc	311.9 ± 0.64 f	32.8 ± 0.34 e	8.7 ± 0.08 b	8.5 ± 0.12 a	0.56 ± 0.00 f
		20HS	15.4 ± 0.15 ab	3.3 ± 0.24	598.6 ± 4.96 b	60.2 ± 0.51 b	505.1 ± 1.75 b	52.3 ± 0.72 b	8.9 ± 0.01 ab	8.7 ± 0.16 a	0.84 ± 0.01 c
ECO_2	**-M**	**0HS**	14.1 ± 0.15 c	2.9 ± 0.16	448.7 ± 3.20 e	46.9 ± 0.74 ef	393.2 ± 1.37 d	41.1 ± 0.47 c	8.6 ± 0.16 b	8.6 ± 0.12 a	0.88 ± 0.01 b
		20HS	15.3 ± 0.22 ab	3.0 ± 0.12	558.0 ± 1.53 c	53.8 ± 0.55 cd	482.6 ± 0.61 c	51.3 ± 1.10 b	9.4 ± 0.09 a	8.4 ± 0.20 a	0.87 ± 0.00 bc
	+M	**0HS**	14.2 ± 0.19 c	3.0 ± 0.24	756.3 ± 1.13 a	78.3 ± 1.57 a	478.0 ± 3.91 c	51.4 ± 0.56 b	8.7 ± 0.20 b	8.3 ± 0.16 a	0.63 ± 0.01 e
		20HS	15.8 ± 0.22 a	2.9 ± 0.16	770.6 ± 4.72 a	79.9 ± 1.19 a	708.4 ± 1.76 a	73.2 ± 0.82 a	8.6 ± 0.10 b	8.7 ± 0.10 a	0.92 ± 0.01 a
CO_2			**	ns	**	**	**	**	ns	*	**
M			ns	ns	**	**	**	**	**	ns	**
HS			**	ns	**	**	**	**	ns	ns	**
CO_2 × M			ns	ns	ns	ns	**	ns	ns	ns	**
CO_2 × HS			ns	ns	**	**	**	**	**	ns	**
M × HS			**	ns	**	**	**	**	ns	*	**
CO_2 × M × HS			ns	ns	**	ns	**	ns	**	ns	**

Table 5. Mycorrhizal colonization (%) in roots of onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means (n = 45 root fragments) ± SE. ANOVA: ns = not significant ($P \leq 0.05$). ND = not detected.

Treatments			Mycorrhizal colonization (%)
ACO_2	**-M**	**0HS**	ND
		20HS	ND
	+M	**0HS**	19.5 ± 3.2
		20HS	18.6 ± 2.7
ECO_2	**-M**	**0HS**	ND
		20HS	ND
	+M	**0HS**	15.8 ± 3.6
		20HS	16.2 ± 4.3
CO_2			ns
HS			ns
CO_2 × HS			ns

Table 6. Concentrations of starch (mg g-1 DM), total soluble sugars (TSS) (mg g-1 DM), total soluble proteins (TSP) (mg g-1 DM) and proline (µg g-1 DM) in leaves of onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means (n = 4) ± SE. Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$, and $P \leq 0.01$, respectively. DM = dry matter.

Treatments			Starch (mg g^{-1} DM)	TSS (mg g^{-1} DM)	TSP (mg g^{-1} DM)	Proline (µmol g^{-1} DM)
ACO_2	**-M**	**0HS**	4.04 ± 0.07 e	32.07 ± 0.35 g	10.64 ± 0.12 g	3.51 ± 0.04 e
		20HS	8.40 ± 0.33 b	47.12 ± 0.93 e	13.50 ± 0.11 d	4.23 ± 0.04 e
	+M	**0HS**	6.73 ± 0.13 c	36.71 ± 0.61 f	11.58 ± 0.12 f	3.76 ± 0.04 e
		20HS	11.45 ± 0.24 a	58.86 ± 0.30 c	14.50 ± 0.10 c	5.83 ± 0.06 cd
ECO_2	**-M**	**0HS**	4.46 ± 0.18 de	50.65 ± 0.76 d	12.62 ± 0.12 e	5.15 ± 0.08 d
		20HS	6.60 ± 0.18 c	73.18 ± 0.68 b	15.68 ± 0.24 b	9.83 ± 0.25 b
	+M	**0HS**	5.05 ± 0.21 d	59.73 ± 1.08 c	14.54 ± 0.24 c	6.19 ± 0.13 c
		20HS	6.76 ± 0.02 c	87.47 ± 0.90 a	18.72 ± 0.09 a	10.68 ± 0.37 a
CO_2			**	**	**	**
M			**	**	**	**
HS			**	**	**	**
CO_2 × M			**	**	**	**
CO_2 × HS			**	**	**	ns
M × HS			ns	**	*	*
CO_2 × M × HS			ns	**	*	**

Table 7. Concentrations of chlorophyll a (Chl a) (mg g-1 DM), chlorophyll b (Chl b) (mg g-1 DM), total chlorophylls (Chl a+b) (mg g-1 DM) and total carotenoides (mg g-1 DM) in leaves of onion seedlings non-amended (0HS) or amended (20HS) with humic (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means (n = 4) ± SE. Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; *and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. DM = dry matter.

Treatments			Chl a (mg g^{-1} DM)	Chl b (mg g^{-1} DM)	Chl a+b (mg g^{-1} DM)	Carotenoids (mg g^{-1} DM)
ACO_2	**-M**	**0HS**	2.54 ± 0.05 f	1.01 ± 0.01 e	3.55 ± 0.05 f	1.40 ± 0.01 d
		20HS	3.15 ± 0.06 e	1.19 ± 0.03 d	4.34 ± 0.07 e	1.57 ± 0.03 d
	+M	**0HS**	4.08 ± 0.04 c	1.73 ± 0.06 c	5.81 ± 0.05 c	2.20 ± 0.03 c
		20HS	4.58 ± 0.01 b	1.30 ± 0.00 d	5.88 ± 0.02 c	3.13 ± 0.01 a
ECO_2	**-M**	**0HS**	3.57 ± 0.06 d	1.19 ± 0.02 d	4.75 ± 0.08 d	2.61 ± 0.14 b
		20HS	4.01 ± 0.07 c	2.11 ± 0.02 b	6.12 ± 0.09 bc	2.82 ± 0.04 ab
	+M	**0HS**	4.68 ± 0.09 b	1.68 ± 0.04 c	6.36 ± 0.13 b	2.82 ± 0.11 ab
		20HS	5.09 ± 0.05 a	2.39 ± 0.05 a	7.49 ± 0.10 a	3.13 ± 0.15 a
CO_2			**	**	**	**
M			**	**	**	**
HS			**	**	**	**
CO_2 × M			ns	ns	**	*
CO_2 × HS			**	**	**	**
M × HS			ns	**	**	**
CO_2 × M × HS			ns	**	*	*

Table 8. Significance of three-factor ANOVA showing effects of CO2, arbuscular mycorrhizal fungi (M), humic substances application (HS) and their interactions on the total soluble phenolics and antioxidant activity in leaves as well as on acid phosphatase activity in roots of onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. ANOVA: ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

	Soluble phenolics in leaves	**Antioxidant activity in leaves**	**Acid phosphatase in roots**
CO_2	**	**	**
M	**	**	**
HS	**	**	**
CO_2 × M	*	**	**
CO_2 × HS	*	**	ns
M × HS	*	**	ns
CO_2 ×M × HS	*	**	ns

4.9 FIGURE CAPTIONS

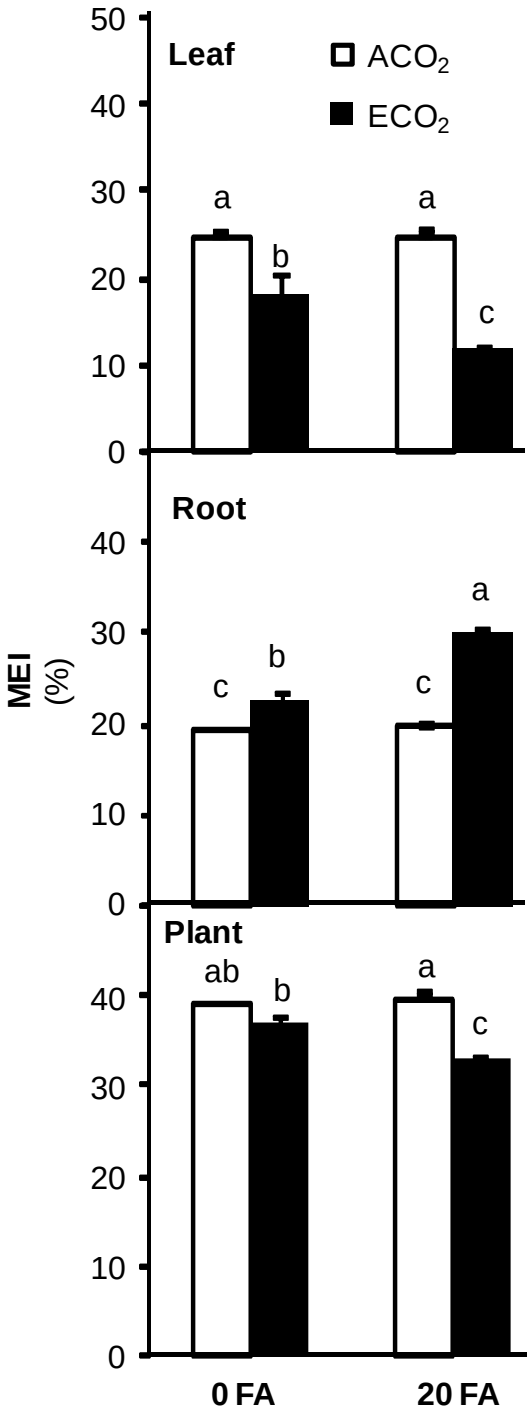

Fig. 4: Mycorrhizal Efficiency Index (MEI) (%) on leaves, roots and whole plant biomass production in onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), and grown either at ambient (ACO_2) (white histograms) or under elevated (ECO_2) (black histograms) CO_2 in the atmosphere. Values are means (n= 4) ± SE. Within each figure data followed by the same letter indicate that values are similar ($P \leq 0.05$).

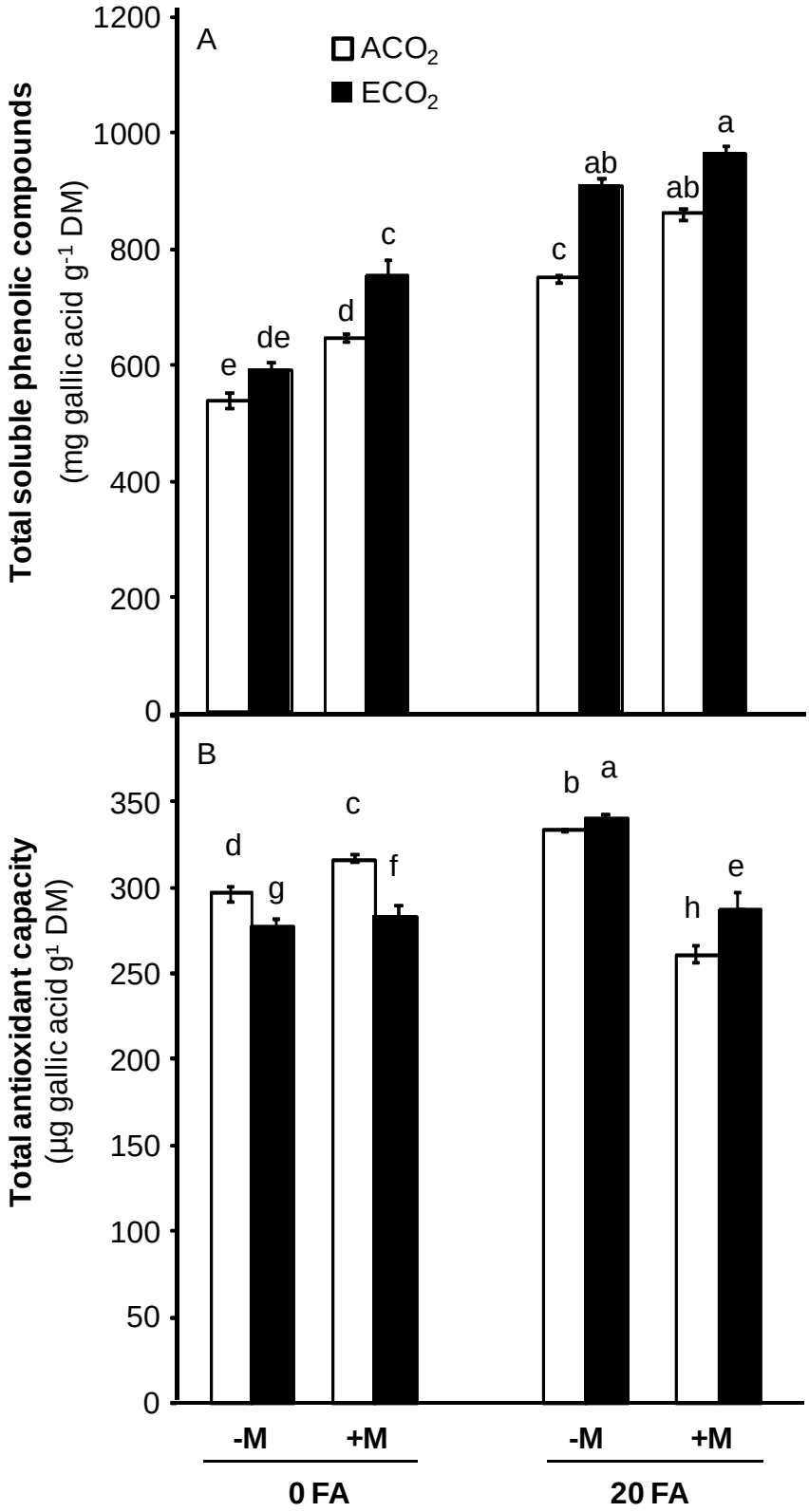

Fig. 5. Total soluble phenolic compounds (mg gallic acid g-1 DM) (Fig. A) and total antioxidant capacity (µg gallic acid g-1 DM) (Fig. B) in leaves of onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) (white histograms) or under elevated (ECO_2) (black histograms) CO2 in the atmosphere. Values are means (n = 4) ± SE. Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). DM = dry matter.

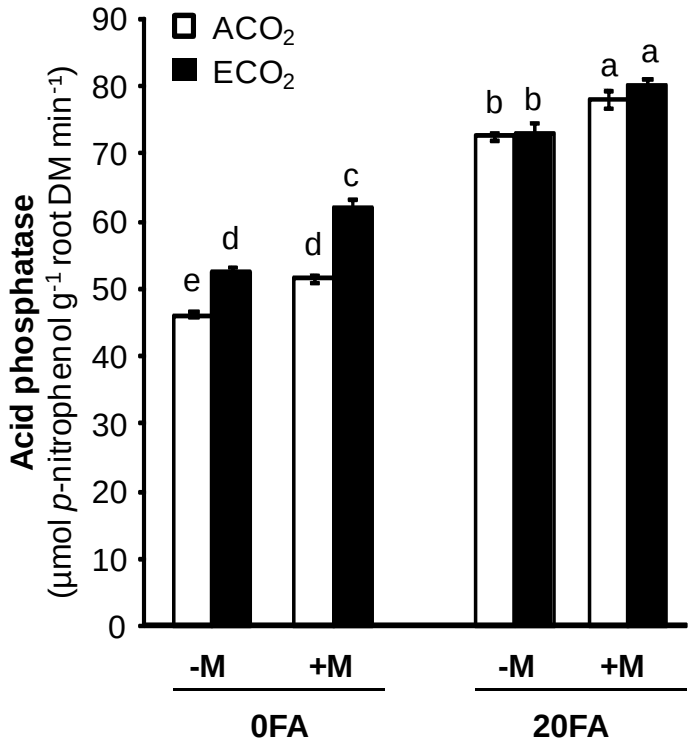

Fig. 6. Acid phosphatase activity (µmol p-nitrophenol g-1 DM min-1) in roots of onion seedlings non-amended (0HS) or amended (20HS) with humic substances (HS), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) (white histograms) or under elevated (ECO_2) (black histograms) CO_2 in the atmosphere. Values are means (n = 4) ± SE. Data followed by the same letter indicate that values are similar ($P \leq 0.05$). DM = dry matter.

5 ELEVATED CO_2, HUMIC ACIDS AND MYCORRHIZAL SYMBIOSIS INFLUENCE GROWTH, PRODUCTIVITY AND QUALITY OF ONION

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5.1 ABSTRACT

Onion (*Allium cepa* L.) is a crop with great economic importance over the world. The growth and development of seedlings is important for obtained greater productivity and quality of bulbs. Application of humic acids (HA), inoculation of arbuscular mycorrhizal fungi (AMF) in the soil and enhancement of atmospheric CO_2 are three factors that can influence plant growth, development and greater productivity and quality of bulbs. Therefore, our main objective was to assess the effect of each of the abovementioned factors, separately or interacting, on the metabolism and growth of onion seedlings and bulb under greenhouse conditions. Results showed that these three factors appear as valid horticultural techniques for improving growth and quality of onion seedlings and bulb cultivated in greenhouse even when mycorrhizal colonization of roots does not achieve high rates. Beneficial effects of humic acid were additive to those of mycorrhizal inoculation or elevated CO_2 on shoot biomass and bulb production. The triple interactionamong mycorrhizal inoculation, humic acid application and elevated CO_2 induced the highest accumulation of starch and soluble phenolics compounds in leaves of seedlings and bulbs, and of solubles sugars, proteins solubles solids in bulbs, suggesting that the three factor was effective for increasing the quality of onion seedlings and for growth and quality of bulbs.

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Keywords: *Allium cepa*, arbuscular mycorrhizae, carbon dioxide, humic substances

5.2 INTRODUCTION

The onion (*Allium cepa* L.) is second only to tomato in importance as a vegetable in the tropics, with approximately 80 million tons harvested in 2012, in world (FAOSTAT, 2014).

One of the most important stages of production system is the production of seedlings, which directly influences the final performance of plants. In this step, the use of containers as polystyrene tray has been shown effective under various aspects, such as economy substrate and space inside the house a greenhouse, lower spending on plant protection products, production seedlings with high quality and high level of fixation after transplantation. In addition to size the container, the type of substrate to be used is important because these factors directly affect development and system architecture root as well as the supply of nutrients (Maggioni et al., 2014).

The system tray provides utmost care during germination and emergence, because activity is developed under protected environment, eliminating the risk of frosts in early stage of culture, in addition to providing lower cost in the control of pests and diseases, high rate of cost provider after the transplant and greater uniformity in the field (Reghin et al., 2007).

Among the alternatives to achieve best qualities of both seedlings and bulbs are the incorporation of humic substances and inoculation with arbuscular mycorrhizal fungi (AMF) on onion biofertilization associated with elevated CO_2.

Many experiments have demonstrated the positive effect of humic acids (HA) on the physiology and growth of plants (Oliveira Aguiar et al., 2009; Hernandez et al., 2014). It was reported that the humic substances can have an effect on the plants, affecting various physiological processes, such as activation of enzymes (Ertani et al., 2011), the photosynthesis (Baldotto et al., 2009) and mineral nutrition greater absorption of nutrients (Baldotto et al., 2009; Lima et al., 2011; Zhang et al., 2013) and hormone biostimulation (Nardi *et al.*, 2002).

Research has shown that onions is highly responsive to mycorrhizal inoculation (Charron et al., 2001; Bolandnazar et al., 2007; Suhail and Mahdi, 2013), but the response its varied in function of plant genotype and fungal species used (Linderman and Davis, 2001). Some results showed that the symbiotic association between fungi and root provides a significant contribution to plant nutrition and growth (Wang et al., 2008; Júnior et al., 2011). It was found that the inoculation of FMA increases levels of chlorophylls and carotenoid (Baslam et al., 2011), in total soluble sugars, protein, proline, nutrients (Baslam, Garmendia and Goicoechea, 2011) and increased antioxidant activity (Baslam and Goicoechea, 2012).

How to CO_2, as photosynthesis in particularly C3 plants is not saturated at present-day atmospheric CO_2 concentrations (Long et al., 2004; DaMatta et al., 2010), continued increases in CO_2 have the potential to enhance the yield of many crops (Long *et al.*, 2004).

The main objective of our study was to assess the effect of each of the abovementioned factors, application of fractions humic acids (HA), mycorrhizal inoculation (+M) and elevated CO_2 (ECO_2), separately or interacting, on the metabolism and growth of onion seedlings and bulb.

5.3 MATERIALS AND METHODS

5.3.1 Plant material and growth conditions

Seeds from Allium cepa L. cv. Alfa São Francisco- Cycle VIII (Embrapa Semi-Árido) were germinated on a mixture of light peat (Floragard, Vilassar de Mar, Barcelona, Spain) and sand (on 12th April 2013). Peat was previously sterilized at 100ºC for 1 h on three consecutive days. Twenty two days after sowing (on 4th May 2013), seedlings were transferred to trays filled with a mixture of vermiculite- siliceous sand- light peat (2.5:2.5:1, v:v:v). Each tray had 60 cells with a capacity of 60 mL each one. The experiment was conducted in a completely randomized design in a factorial 2 × 2 × 2, with five replications. Factors were 'mycorrhizal inoculation, M', 'humic acid, HA' and 'CO_2 concentration in the atmosphere, CO_2'.

Two days after transferring seedlings to trays (on 6th may 2013) half of cells (160 seedlings) received 5 mL of a solution containing 10% HA (10HA). This solution was applied by using a syringe. The product with the humic acids, obtained from Leonardita was employed in the experiments. A specific amount of HA (100 mg) was extracted and purified using the IHSS methodology, as described in Mora et al. (2010), 32.5% C; 3.2% H and 0.8% N). Other 160 seedlings did not receive this solution (0HA). Two days after transferring seedlings to trays (on 8th May 2013), half of 0HA cells (80 seedlings) and half of 10HA cells (80 seedlings) were inoculated with the liquid mycorrhizal inoculum 'Glomygel Intensivo' (Mycovitro S.L., Pinos Puente, Granada, Spain) (+M seedlings). The concentrated commercial inoculum derived from an in vitro culture of arbuscular mycorrhizal fungi (AMF) and contained around 2,000 mycorrhizal propagules (inert pieces of roots colonized by AMF, spores and vegetative mycelium) per mL of inoculum. In order to facilitate its application, the concentrated commercial inoculum was diluted with distillate water until obtaining a resultant mycorrhizal inoculum with around 250 propagules per mL. Each +M seedling received 5 mL of the diluted mycorrhizal inoculum close to the roots thus making a total of 1,250 propagules. A filtrate was added to seedlings that did not receive the mycorrhizal inoculum (-M seedlings) in an attempt to restore other soil free-living microorganisms accompanying AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a layer of 15-20 µm filter papers (Whatman, GE Healthcare, UK) and each –M seedling received 5 mL of filtrate close to the roots.

Subsequently all trays were transferred to four [CO_2] controlled greenhouses located at the University of Navarra campus (42.80 N, 1.66 W; Pamplona, Spain). The design of the greenhouses was similar to that described by Sanz-Sáez et al. (2012) and based on Aranjuelo et al. (2005). Half of seedlings (20 0HA +M seedlings, 20 0HA -M seedlings, 20 10HA +M seedlings and 20 10HA -M seedlings) and more 20 seedlings of each treatment were transferred for 2.5 L pots for evaluation of bulbs were divided into two greenhouses where no CO_2 was added and [CO_2] was maintained at ambient conditions (~360 µmol mol^{-1}) (ACO_2). The other half (20 0HA +M seedlings, 20 0HA -M seedlings, 20 10HA +M seedlings and 20 10HA -M seedlings) and more 20 seedlings of each treatment were transferred for 2.5 L pots were divided into two greenhouses where [CO_2] was increased to ~700 µmol mol^{-1} by injecting pure CO_2 at the two inlet fans

during the light hours (ECO_2). The CO_2 was provided by Air Liquide (Bilbao, Spain). The [CO_2] was continuously monitored using a Guardian Plus gas monitor (Edinburgh Instruments Ltd, Livingston, UK). The monitor's signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans.

Therefore, there were eight different treatments in total: (1) 0HA-M seedlings grown at ACO_2; (2) 0HA+M seedlings grown at ACO_2; (3) 0HA_M seedlings grown under ECO_2; (4) 0HA+M seedlings grown under ECO_2; (5) 10HA-M seedlings grown at ACO_2; (6) 10HA+M seedlings grown at ACO_2; (7) 10HA-M seedlings grown under ECO_2; and (8) 10HA+M seedlings grown under ECO_2.

All seedlings received 30 mL of modified Hewitt's solution (Baslam, Garmendia and Goicoechea, 2011) twice a week and other 30 mL of distilled water twice a week. The nutrient solution contained 6 mM $Ca(NO_3)_2$, 6 mM $CaCl_2$, 3 mM KNO_3, 2.3 mM K_2SO_4,1.5 mM $MgSO_4$, 1.3 mM NaH_2PO_4, 68 µM EDTA-Fe, 13 µM $MnSO_4$, 9 µM H_3BO_4, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM Na_2MoO_4. The original pH of the nutrient solution (5.2 ± 0.1) was adjusted to 7.0. The EC of the nutrient solution adjusted to 7.0 was 140 ± 15 µS cm^{-1} as determined with a conductivity meter 524 Crison (Crison Instruments S.A., Alella, Spain). Seedlings were alternatively irrigated with distilled water and nutrient solution to avoid salt accumulation. Final harvest of all seedlings was carried out on day 60th after sowing.

The other potted plants continued in the greenhouse getting the nutrient solution with the same frequency, just adjusting the amount in relation to the volume of pots (2.5 L). The HA also continued being applied at regular intervals of 15 days, adapting the amount in relation to the volume of pots. The bulbs were harvest with 128 days after sowing (20t^{h} August 2014)

5.3.2 Growth parameters, water status and mycorrhizal efficiency index (MEI) of seedlings and bulb

At harvest of seedlings, five plants of each treatment were randomly selected for determination of shoot height, number of leaves per plant, shoot and root fresh weight (FW), shoot and root dry matter (DM), and the ratio root to shoot DM. DM was determined after drying plant material in oven at 80°C until weight was constant. Water status of seedlings was determined by calculating water content (WC) of shoots or roots: FW of shoot or root – DM of shoot or root/ DM of shoot or root. Shoot or root WC was expressed as g of water g^{-1} DM.

At harvest of bulb, five plants of each treatment were randomly selected for determination of, bulb and root fresh weight (FW), bulb and root dry matter (DM), transverse diameter (TD) and cataphyll number (CN). DM was determined after drying plant material in oven at 80°C until weight was constant. Water status of bulb was determined by calculating water content (WC) of bulb: FW of bulb – DM bulb/ DM of bulb. Bulb WC was expressed as g of water g^{-1} DM.

Root samples were cleared and stained according to Phillips and Hayman (1970) for visualizing mycorrhizal structures. The Mycorrhizal Efficiency Index (MEI) was estimated according to Bagyaraj (1994): MEI = DM of +M seedlings − DM of –M seedlings × 100/DM of +M seedlings. Determination of

MEI allows assessment of the growth improvement brought about by inoculation of plants with a mycorrhizal fungus.

5.3.3 Starch, total soluble sugars (TSS), total soluble proteins (TSP), proline and soluble phenolic compounds (SPC) in leaves seedlings and bulbs and pH, soluble solids (SS), titratable acidity (TA) and ratio soluble solids (SS) and titratable acidity (TA) in bulb

Starch, total soluble sugars (TSS), total soluble proteins (TSP) and proline were quantified in potassium phosphate buffer (KPB; 50 mM, pH 7.5) extracts of fresh leaves (0.5 g per seedling, five seedlings per treatment) and fresh bulbs (0.5 g per bulb, five bulbs per treatment). These extracts were filtered through four cheesecloth layers and centrifuged at 38720 g for 10 min at 4ºC. The pellet was used for starch determination (Jarvis and Walker, 1993). The supernatant was collected and stored at 4ºC for TSS, TSP and proline determinations. TSS were analyzed with the anthrone reagent in a Spectronic 2000 (Bausch and Lomb, Rochester, NY) according to Yemm and Willis (1954). TSP were measured by the protein dye-binding method of Bradford, (1976) using bovine serum albumin (BSA) as a standard. The free proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrine reaction (Irigoyen, Emerich and Sánchez-Díaz, 1992). The results were expressed as mg of starch, TSS or TSP per g of leaf DM and as µmol of proline per g of leaf DM.

Soluble phenolic compounds were extracted according to Chapuis-Lardy et al., (2002) with some modifications. One sample (0.5 g of FW) of shoot per seedling (five seedlings per treatment) and (0.5 g of FW) of bulb per plant (five bulbs per treatment) were pulverized in liquid nitrogen, mixed with 20 mL of 80% methanol, and homogenized at room temperature for 1 min. After filtration, 0.5 mL of each sample was mixed with 10 mL of distilled water. The total phenolic content was determined from aqueous solutions by spectrophotometric analysis at 760 nm with Folin Ciocalteu reagent (Waterman and Mole, 1994). The results are expressed as mg of gallic acid per g of DM.

For bulb measures, extracts were centrifuged, and the supernatants used for the following determinations: soluble solids (SS) (°Bx) measured by a refractometer (Zuzi model nº 315, Digital ABBE); pH (pH-meter); titratable acidity (TA) determined by titrating 10 mL of extract against NaOH 0.1 N, and converted to a weighed quantity of piruvic acid.

5.3.4 Chlorophylls and carotenoids in leaves of seedlings

Samples of 100 mg of fresh leaves were immersed in 5 mL of 96% ethanol at 80ºC during 10 min to extract chlorophylls (chl a, chl b) and carotenoids (Séstak, Càtsky and Jarvis, 1971). For every extract absorbance was measured at 470, 649, 665 and 750 nm using a Spectronic 2000 (Bausch and Lomb, Rochester, NY, USA). Estimation of chl a, chl b, chl a+b and total carotenoids in the same extract was performed by using the extinction coefficients and equations described by Lichtenthaler (1987). Results were

expressed as mg of chlorophylls or carotenoids per g of leaf DM.

5.3.5 Acid phosphatase activity in roots of seedlings and bulb

The acid phosphatase (EC.3.11.3.2) activity in roots was measured as described by Dodd et al. (1987) and results were expressed as the amount of *p*-nitrophenol released during incubation.

5.3.6 Statistical analysis

Data were subjected to a three-factor ANOVA (factorial 2 x 2 x 2, Assistat Beta 7.7). The variance was related to the main treatments (atmospheric CO_2 concentration, CO_2, inoculation of mycorrhizal fungi, M, and application of fulvic acids, FA, and to the interaction between them (CO_2 × M, CO_2 × HA, M × HA, CO_2 × M × HA). Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Tukey´s test was applied. Tests were considered significant at $P \leq 0.05$.

5.4 RESULTS

5.4.1 Growth parameters, water status and mycorrhizal efficiency index (MEI) of seedlings and bulb

Data shown in Table 9 indicate that the three factors applied in the study (ECO_2, HA or M) increased shoot growth of onion seedlings when applied together or separately. Being this increase mainly due to improved shoot height (M, $P \leq 0.01$, CO_2 × M, $P \leq 0.01$ and M × HA, $P \leq 0.05$). In contrast, just the shoot DW was affected to mycorrhizal colonization separately DW (M, $P \leq 0.01$), while shoot FW and leaves per plant was affected by mycorrhizal colonization and mycorrhizal colonization together elevated CO_2 (M, $P \leq 0.01$, CO_2 × M, $P \leq 0.05$). None of these factors significantly influenced the root DM and only humic acid affected of water accumulated in root tissues (M, $P \leq 0.05$ for shoot WC). Root WC and R/S was affected by the three factors applied in the study (ECO_2, HA or M) increased root growth when applied together (CO_2 x FA x M, $P \leq 0.01$). The highest values of shoot FM were achieved by onion seedlings grown under ACO_2 non-inoculated and inoculated with HA (1992.7 mg $plant^{-1}$ without and 2026.2 mg $plant^{-1}$, respectively) and ECO_2 inoculated with HA (1791.5 mg $plant^{-1}$) when compared with the control. Similar results were observed in shoot FW with the highest values for ACO_2 non-inoculated with HA (187.0 mg $plant^{-1}$) and ECO_2 inoculated with HA (192.6 mg $plant^{-1}$) when compared with the control. The leaves for plant, root FM and DM obtained similar results, independent of factor, with values between 2.8 and 3.5 for leaves for plant, 862.1 and 1227.8 mg $plant^{-1}$ for root FM and 61.8 and 95.9 mg $plant^{-1}$ for root DM

Shoot heigth increased after applying HA, inoculating or exposing plants to ECO_2 (M, $P \leq 0.01$; CO_2 × M, $P \leq 0.01$ and M × HA, $P \leq 0.05$), except for non-inoculated with HA that also increased. The applying HA also increased the shoot heigth in ACO_2. The highest value was 24.7 cm for ACO_2 non-inoculated with

HA, followed by 22.5 cm for ACO_2 iinoculated with HA and ECO_2 inoculated without HA with 21.5 cm. The root WC was better for all treatments when compared with control, being the most value in ACO_2 and inoculated without HA was 13.5 g H_2O g^{-1} DM. In R/S, the control had the most value (0.97).

In Table 10 it is possible to check the data on the growth parameters of bulbs, valued at 128 days after sowing. The three factors applied in the study (ECO_2, HA and M) increased bulb growth of onion when applied together. Being this increase mainly due to improved bulb FW and DW (CO_2 x HA x M, $P \leq 0.01$). The bulbs of higher weight were influenced by the ECO_2 (CO_2, $P \leq 0.01$) and by applying HA (HA, $P \leq 0.01$), being the treatment ECO_2 + M 10HA the largest value found (81.66 g $bulb^{-1}$). The same behavior can be observed in bulb DM, with higher DM in plants cultivated in ECO_2 (CO_2, $P \leq 0.01$) and with the application of HA (HA, $P \leq 0.01$), independent of mycorrhizal inoculation (5.75 g $bulb^{-1}$ non-inoculated and 5.52 g $bulb^{-1}$ inoculated, respectively).

The root FW was affected by the interaction of three factors (CO_2 x HA x M, $P \leq 0.05$) and by CO_2 and by inoculation of AMF, separately (CO_2, $P \leq 0.01$ and M, $P \leq 0.01$). Inoculated plants without the application of HA, independent of CO_2 in the atmosphere had higher value root FW (5.52 g $bulb^{-1}$, in both cases). The root DM was affected by three factors applied in the study (ECO_2, HA or M) separately, being the largest verified dry mass in treating ACO_2 + M 0HA (0.295 g $plant^{-1}$).

The water content of bulbs was influenced by the interaction between AMF and HA (M x HA, $P \leq 0.05$) and the cataphyll number was affected only by CO_2 (CO2, $P \leq 0.05$), however, in any of these parameters was found significant differences among the treatments. In contrast, the transverse diameter of the bulbs was affected by three factors separately (CO_2, $P \leq 0.01$; M, $P \leq 0.01$ and HA, $P \leq 0.01$). The highest values of bulb TD were achieved by onion grown under ECO_2 with HA (54.18 mm non-inoculated and 55.09 mm inoculated, respectively).

Microscopic observations of cleared and stained roots revealed that there were few fungal structures colonizing root tissues so that percentage of mycorrhizal colonization did not exceed 20% (data not shown), in both seedlings and bulbs. Results of Mycorrhizal Efficiency Index (MEI) are represented in Fig. 7 and they have been divided into leaves, seedlings and bulb roots, the whole plant and total (bulb+root).

Inoculation of onions with AMF always enhanced plant growth so that +M seedlings produced around 27-33% more total biomass than –M plants, regardless they were grown at ECO_2 (black histograms) or under ACO_2 (white histograms) and independently they were (10HA) or not (0HA) amended with HA (Fig. 7, 'Plant'). Inoculation of onions with AMF always enhanced plant growth so that +M bulbs produced around 33-48% more total biomass than –M plants, regardless they were grown at ECO_2 (black histograms) or under ACO_2 (white histograms) and independently they were (10HA) or not (0HA) amended with HA (Fig. 7, 'Total'). The same behavior can be observed in bulbs root (Fig. 7, 'Root'), so that +M onions produced up to 45% of root biomass than –M bulb.

In leaves were the organs that most benefited from mycorrhizal inoculation (Fig. 7, 'Leaf), so that +M onions produced up to 25% of shoot biomass than –M seedlings, being higher in leaves with ECO_2 without HA (black histograms) and ACO_2 without of HA addition (white histograms). The bulbs were the

organs that less benefited from mycorrhizal inoculation (Fig. 7, 'Bulb'), so that +M onions produced just 11% of bulb biomass than –M bulb, being higher in bulb with ACO_2 (white histograms), whith or not HA.

In contrast, the highest efficiency of AMF in improving root biomass (Fig. 7, 'Root') was observed when onions were cultivated under ACO_2 (white histograms) and without HA (root DM was 10% greater in +M than in –M onions). The highest value was on onion seedlings in ACO_2 without HA (white histograms), but this treatments had the lowest value for leaves.

5.4.2 Starch, total soluble sugars (TSS), total soluble proteins (TSP), proline and soluble phenolic compounds (SPC) in leaves seedlings and bulbs and pH, soluble solids (SS), titratable acidity (TA) and ratio soluble solids (SS) and titratable acidity (TA) in bulb

Data shown in Table 11 indicate that the three factors applied in the study (ECO_2, HA or M) increased shoot growth of onion seedlings when applied separately or together (CO_2, $P \leq 0.01$; M, $P \leq 0.01$ and HA, $P \leq 0.01$, CO_2 x M x AH, $P \leq 0.05$).Concentrations of starch in leaves were always clearly lower than those of soluble sugars in all onion seedlings and bulb (Table 11).

When cultivated at ECO_2 with HA addition and ACO_2, HA addition and AMF inoculation enhanced the accumulation of starch, being additive effects of both factors. Therefore, at ACO_2 the highest levels of starch were found in +M seedlings that received HA (10HA) (9.27 mg g^{-1} leaf DM). Under ECO_2 the supply of HA increased starch concentrations but +M plants accumulated similar starch contents than their respective –M plants (8.38 and 9.10 g^{-1} leaf DM, respectively). The highest concentrations of TSS (79.83 mg g^{-1} leaf DM) were found in leaves of seedlings inoculated with AMF (+M), grown under ACO_2 and that received HA (10HA) ($CO_2 \times M \times HA$, $P \leq 0.01$ for TSS). In contrast, the lowest levels of TSS were observed in leaves of seedlings non-inoculated (-M) and cultivated at ACO_2 without receiving HA (0HA) (50.16 mg g^{-1} leaf DM), similar at seedlings non-inoculated (-M) and cultivated at ECO_2 without receiving HA (0HA) (55.50 mg g^{-1} leaf DM).

Similarly to findings with TSS, there were positive effects of different factors, separately or interacting among them, on the protein levels of onion seedlings (Table 11). The lowest content of proteins in leaves corresponded to seedlings grown at ACO_2 without neither HA application (0HA) nor mycorrhizal inoculation (-M) (7.56 mg g^{-1} leaf DM), similar at seedlings with ECO_2, -M and 0HA (8.68 mg g^{-1} leaf DM).

In the opposite, interaction between HA with ACO_2 or ECO_2 and inoculated or non-inoculated induced the accumulation of proteins in leaves (14.50 mg g^{-1} leaf DM in ACO_2 +M 10HA, than 12.89 mg g^{-1} leaf DM in ECO_2 -M 10HA and 12.10 mg g^{-1} leaf DM in ACO_2 -M 10H or ECO_2 +M 10H).

Levels of proline were very sensitive to each factor applied (CO_2, AMF or HA) and also were to the interactions between different factors both in seedlings and in bulbs. This triple interaction also enhanced the amount of proline in leaves cultivated at ACO_2 (13.53 μg g^{-1} leaf DM with AMF and with HA, 12.95 μg g^{-1} leaf DM with AMF and without HA, 12.45 μg g^{-1} leaf DM at control and 9.63 μg g^{-1} leaf DM without AMF and with HA). In the bulbs, the highest value was observed when plants were grown in ACO_2 inoculation

without HA (Table 12) (5.21 μg g^{-1} bulb DM).

The starch in the bulbs (Table 12) was influenced by CO_2, the interaction between HA and mycorrhizal inoculation and the triple interaction of factors (CO_2, $P \leq 0.01$; M x HA, $P \leq 0.05$ and CO_2 x M x HA, $P \leq 0.05$), where as those grown in ECO_2 without HA and non-inoculated plants and ECO_2 and inoculation with HA addition showed the highest values (3.21 mg g^{-1} DM bulb and 2.99 mg g^{-1} DM bulb, respectively) when compared to plants grown in ACO_2 without HA, regardless of mycorrhizal inoculation.

Levels of TSS, TSP, SPC, SS and SS/TA in bulbs were influenced by each factor applied (CO_2, MFA or HA) were isolated and also to the interactions between them. The highest values of bulb these variables were achieved by onion grown under ECO_2 with HA and inoculation (588.85 mg g^{-1} DM bulb for TSS, 31.74 mg g^{-1} DM bulb for TSP, 3121.79 mg g^{-1} DM bulb for SPC, 8.50ºBx for SS and 94.90 for SS/TA).

The pH when grown in ECO_2 inoculated, independent of HA addiction, showed the highest values when compared to control (5.55 without HA and HA with 5.57). Similar results were observed in TA, the highest value recorded in the treatment ECO_2 -M 0HA (0.111 mg piruvic acid g^{-1} Bulb FW).

5.4.3 Chlorophylls and carotenoids in leaves

The highest contents of Chl a, Chl b and total Chl (a+b) and carotenoids were found in leaves of onion seedlings grown under ECO_2 when compared with plants grown in ACO_2. The plants grown under ECO_2 inoculated with HA showed 74.1% mg Chl a g^{-1} leaf DM more than control (Figure 8).

The largest increase in Chl b was 96% g^{-1} leaf DM more than control, in treatment ECO_2 –M 10HA. The ECO_2 with HA inoculated and no-inoculated and the ECO_2 without HA inoculated were the treatments with greater increases compared to control (62.8% mg Chl a+b g^{-1} leaf DM more than control for ECO_2 –M 10HA, 48.0% mg Chl a+b g^{-1} leaf DM more than control for ECO_2 +M 0HA and 63.4% mg Chl a+b g^{-1} leaf DM more than control for ECO_2 +M 10HA) (Figura 8).

The concentrations of total carotenoids increased in leaves of onions after they received HA non-inoculated grown under ECO_2 with 75.2 % g^{-1} leaf DM more than control (Figura 8).

ANOVA results corroborated the significant influence of CO_2, mycorrhization (M) and HA on Chl a, Chl b, total Chl (a+b) and carotenoids levels (CO_2, $P \leq 0.01$, for the concentrations of all parameters; M, $P \leq 0.01$, for the concentrations of all parameters except Chl b; HA, $P \leq 0.01$, for the concentrations of all parameters and CO_2, x M x HA, $P \leq 0.01$, for the concentrations of all parameters except Chl a).

5.4.4 Acid phosphatase activity in roots of seedlings and bulb

The acid phosphatase activity in roots of seedlings were affected by the M and HA, separately (M, $P \leq 0.01$ and HA, $P \leq 0.01$), while in roots of bulb were affected by the CO_2 and by inoculation of AMF, separately (CO_2, $P \leq 0.01$ and M, $P \leq 0.01$).

The inoculation of AMF with HA fertilization increased acid phosphate activity in roots of seedlings (11.07 µmol de paranitrofenol DM de plant-¹ min-¹ for ACO_2 and 11.38 µmol de paranitrofenol DM de plant-¹ min-¹ for ECO_2). However, in the bulbs, the highest acid phosphatase activity was with ECO_2 and HA, non-inoculated (3706.70 µmol de paranitrofenol DM de plant-¹ min-¹) and inoculated (3441.90 µmol de paranitrofenol DM de plant-¹ min-¹) (Figure 9).

5.5 DISCUSSION

In this study, the HA increased the growth of shoot seedling only when associated with mycorrhizal inoculation (+M) (Table 9).

These results can being explained by effect of HA, with similar effect the auxin, that has the function the activation of mRNA of H+-ATPase in the plasma membrane, with acidifying the apoplast and increased cell wall plasticity (Schiavon et al., 2010; Silva et al., 2011), promoting, thus, the growth and elongation of cell.

Another hypothesis that explains the growth of seedlings is the presence of polyamines in HA, such as putrescines, spermidine and spermine (Young and Chen, 1997), which, according to Martens and Frankenberger (1994) act as regulators of plants (Kumar, Imtiyaz and Kumar, 2007). Dobbss et al. (2007) attribute the growth to alkylamides, one new class of compounds with hormone action, with growth stimulation of plant, regardless of auxin signaling (Ramírez-Chávez et al., 2004).

The associated between mycorrhizal inoculation (+M) and HA, also has positive effect, proving that the addition of HA not undertakes mycorrhizal inoculation, and can be used in conjunction to promote the growth of plants, agreeing with the results observed in other polls (Gryndler et al., 2005; Nobre et al., 2013; Rodriguez and Ortuño, 2007). When cultivated under ECO_2, the positive effect of the other two factors is maintained about the shoot FW and DM of seedlings, indicating that increases in levels of atmospheric CO_2 will not compromising the growth of shoot of onion seedlings when treated with HA and mycorrhizal inoculation.

The leaves for plant in the seedlings no ranged with the treatments (Table 9), as well as the CN (Table 10), what can be attributed to genetic characteristic of the plant itself and agree with results observed for Bettoni et al. (2013) in the same cultivar.

The root FW and DM of seedlings were not affected by treatments (Table 9), what can be explained by low content of mycorrhizal colonization (less than 20% - no data presented). Already the effect of HA, reported by issuing secondary roots (Oliveira Aguiar et al., 2009; Silva et al., 2011; Zandonadi, Canellas and Façanha, 2007), may have occurred to the detriment of growth of principal roots, not being observed the difference in weight of the same.

Linderman and Davis (2001) observed values between 20 to 39% for root colonization by *R. intraradices*. In our study, In our study, the main component of the mycorrhizal inoculum was the same

spores, but the mycorrhizal colonization was lower. Charron et al. (2001) obtained levels of inoculation of 39 a 53% using root segments of leek colonized by *R. intraradices*, but the onion cultivar was other ('Improved Autumn Spice'). For Linderman and Davis (2004), the degree of responses varied in function of plant genotype and fungal species used, which may explain the lower rate of mycorrhizal infection of this study.

The plants that receveid HA under ECO_2 had the largest MEI in root in detriment of the leaves in seedlings, and the same ocurr with bulbs (Figure 7), being the mycorrhyzal inoculations of roots favoured by adding HA and elevated CO_2.

The positive effect of humic substances about mycorrhizal was observed for Gryndler et al. (2005) and Nobre et al. (2013). Rodriguez and Ortuño (2007) also there have been improvement in mycorrhizal of onion plants with the addition of humic substances. The ECO_2 also increases the positive impact of mycorrhizal on plant growth (Treseder, 2004) in fuction of increased availability of carbon promoted the increase in the levels of CO_2.

Although the rate of mycorrhizal infection has been small, the mycorrhizal inoculation (+M) increased the WC of seedlings root (Table 9), agreeing with Bolandnazar et al. (2007), confirming by improvement in the efficiency of water use and stomatal conductance (Nowak, 2004; Jezdinský et al., 2012). The same effect was observed in the seedlings shoot, but when use HA application, which, also, act as osmotic regulators (Santoyo et al., 1998), demonstrating effect additive of HA about mycorrhizae.

The ECO_2 don't have effect in growth parameters of seedlings, but for FM, DM and TD bulb had increments obtained when grown in ECO_2 (Table 10). The HA and mycorrhizae, together with ECO_2, also showed positive effects on these parameters.

These results in seedlings can be explained by regulator mechanisms. Plants grown in elevated CO_2 have a negative regulatory mechanism or acclimation (Norby et al., 1999; Goicoechea et al., 2014) and is associated with accumulation of carbohydrates in leaves (Moore et al., 1999) which results in the inhibition of photosynthetic rate (Stitt, 1991; Paul and Foyer, 2001).

This mechanism is reversible, so it is possible to observe the effect of increasing the CO_2 in bulbs, in function the physiological mechanism of the plant, since the same need CO_2 of the atmosphere for photosynthesis and obtain organic carbon (C). In this way, the increase in concentration of CO_2 can stimulate the rate of photosynthesis and accumulate more fresh and dry mass (Griffin and Seemann, 1996).

The increase promoted when use HA can be attributed the auxin or polyamines, acidify the apoplast and increased cell wall plasticity (Schiavon et al., 2010; Silva et al., 2011), promoting, thus, the elongation of cell, resulting in bulbs larger and consequently more weights. The mycorrhizal associated it has additive effect on function the increased of cytokinin activity observed in mycorrhizal plants (Shaul-Keinan et al., 2002), which increases cell division.

Largest shoot DM resulted in higher bulb DM ($r = 0.7438$, $P \leq 0.05$) (Table 9), proving that the seedling production is directly related to final production of culture, agreeing with Reghin et al. (2007).

The root FW was greater with mycorrhizal (+M) (Table 10), without HA, independent of CO_2 level.

The observed increments with mycorrhizal inoculation (+M) can be explained by greater cell

division promoted by activating cytokinin activity, promoted by mycorrhizal .

The mycorrhizal also increased the root DM, in ACO_2. Baslam et al. (2012) observed increments in root DM of alfafa mycorrhizal when cultivated under ECO_2. The same results were observed by Goicoechea et al. (2014), in lettuce. However, in our work, these increments were not checked, and may have been a greater accumulation of DM in the shoot of plant over the root, however this didn't compromise the final production of culture.

Already when the plants cultivated in ECO_2 received HA, the increased of root DM was verified.

The increased in root DM with HA application can be explained by issuing secondary roots promoted by the HA (Oliveira Aguiar et al., 2009; Silva et al., 2011, 1999). The additive effect promoted by ECO_2 is in function the higher rates of carboxylation and reduction in photorespiration the lower rate of oxygenation catalyzed by Rubisco (Jasoni et al., 2004; Ainsworth and Long, 2005), resulting in higher concentration of assimilates in plants.

Copetta et al. (2011) observed that mycorrhizal inoculum and green compost (rich in humic substances) increased the nitrate concentration, which is quickly incorporated into aminoacids and organic compounds. The same results were observed in this work.

Onion seedlings +M with addition of HA showed higher levels of starch, solubles sugars, proteins and phenolic compounds (Table 11).

The ECO_2, associated with the other two factors resulted in high levels of starch and phenolic compounds in seedlings and bulbs, in addition to soluble sugars, protein, soluble solids, pH and SS/AT in bulbs (Table 12). Goicoechea et al. (2014) also observed increased in soluble sugars and proteins in alfafa mycorrhizal cultivated under ECO_2.

The concentrations of metabolites in seedlings presented positive correlation with shoot FW and DM (Shoot FW x TSS: $r = 0.7988$, $P \leq 0.05$; Shoot FW x TSP: $r = 0.8794$, $P \leq 0.01$; Shoot FW x SPC: $r = 0.7662$, $P \leq 0.05$; Shoot DM x SPC: $r = 0.7848$, $P \leq 0.05$).

The same ocurr with bulb (Bulb FW x TSS: $r = 0.9274$, $P \leq 0.01$; Bulb DM x TSS: $r = 0.8154$, $P \leq 0.05$; Bulb FW x TSP: $r = 0.8843$, $P \leq 0.01$; Bulb DM x TSP: $r = 0.7933$, $P \leq 0.05$; Bulb FW x SPC: $r = 0.7631$, $P \leq 0.05$; Bulb DM x SPC: $r = 0.8257$, $P \leq 0.01$; Bulb FW x SS: $r = 0.7895$, $P \leq 0.05$), suggesting the probable osmotic adjustment of the plant, where the accumulation of intracellular metabolites decreases the osmotic potential (ψs), helping maintaining turgor pressure, leading to cell elongation and expansion (Premachandra et al., 1992).

Levels of proline in onion seedlings were higher under ACO_2 when compared with ECO_2 (Table 11), already in adults plants, the proline content was lower in all treatments, except in control. This content in seedlings in presented negative correlation with bulb FW ($r = -0.9271$, $P \leq 0.01$), solubles sugars in bulb ($r = -0.8696$, $P \leq 0.01$) and proteins solubles ($r = -0.7471$, $P \leq 0.05$)

Broetto et al. (1995) and Brito et al. (2008) observed higher concentrations of proline are correlates with increased of hydrolysis proteins and reduction in their synthesis, in stressful situations. In our work, the same was observed in seedlings and adults plants (Table 10).

The proline in adults plants had correlation negative with bulbs metabolites (Bulb proteins x Bulb Proline: r= -0.8368, P ≤ 0.01; Bulb starch x Bulb Proline: r= -0.8734, P ≤ 0.01; Bulb TSS x Bulb Proline: r= -0.7342, P ≤ 0.05; Bulb SPC x Bulb Proline: r= -0.8913, P ≤ 0.01 and SS x Bulb Proline: r= -0.7481, P ≤ 0.05).

According to Camara et al. (1998), the decrease in contents of proline result in the accumulation of other solutes, as observed in this work, indicating that the onion seedlings grow in ECO_2 redirect the energy saved in the synthesis of proline to the formation of other metabolites, the same occur in adults plants of onion when received HA and/or +M.

The higher concentrations of metabolites can be attributed the greatest photosynthetic activity, resulting in increased photoassimilated accumulation in plants, explained by the observed correlation between levels of chlorophyll (Figure 8) and the metabolites (Seedlings Starch x Chl a: r = 0.8529, P ≤ 0.05; Seedlings Starch x Chl total: r = 0.8603, P ≤ 0.05; Seedlings Starch x Carotenoids: r = 0.8061, P ≤ 0.05; Seedlings TSS x Carotenoids: r = 0.7479, P ≤ 0.05; Seedlings SPC x Chl a: r = 0.8380, P ≤ 0.01; Seedlings SPC x Chl total: r = 0.8404, P ≤ 0.01; Seedlings SPC x Carotenoids: r = 0.7531, P ≤ 0.01; Bulb Starch x Chl b: r = 0.7674, P ≤ 0.05; Bulb Starch x Chl total: r = 0.8226, P ≤ 0.05; Bulb TSS x Carotenoids: r = 0.7481, P ≤ 0.05; Bulb TSP x Chl a: r = 0.8055, P ≤ 0.05; Bulb TSP x Chl total: r = 0.8936, P ≤ 0.01; Bulb TSP x Carotenoids: r = 0.8130, P ≤ 0.05; Bulb SPC x Chl a: r = 0.8594, P ≤ 0.01; Bulb SPC x Chl total: r = 0.8509, P ≤ 0.01; Bulb SPC x Carotenoids: r = 0.7831, P ≤ 0.01;SS x Chl a: r = 0.7679, P ≤ 0.05; SS x Chl total: r = 0.7794, P ≤ 0.01).

Higher rates of photosynthesis have been observed in plants cultivated under ECO_2 (Nowak et al., 2004; Ainsworth and Long, 2005),, biofertilizer with HA (Baldotto et al., 2009) and inoculated with mycorrhizal (Nowak, 2004; Jezdinský et al., 2012). In our work, the three factors interacted with each other and resulted in greater levels of Chl total (Figure 8).

Plants +M with HA application cultivated under ECO2 showed higher SS and pH when compared with control, and the TA decreased (Table 10). The increment of SS and decreased acidity results in better flavor and the ratio between SS and TA is one important characteristic for evaluate the quality of in fruits and vegetables (Chitarra and Chitarra, 2005). Chagas et al. (2004) claim that greater SS means better quality of bulbs, in this way, treated their quality improved bulbs in relation to control.

The use of HA in tomatoes resulted in higher ratio SS/TA according to Lima et al. (2011), that they attributed this effect to the stimulation of photosynthesis, resulting in a higher rate of assimilated into leaves and export to the tomato, with increased content of SS in fruit. Aminifard et al. (2012) also proved the effect of humic substances on quality of capsicum, affecting the TA, SS, carbohydrates, lycopene and beta-carotene. Increased of SS in strawberry also was verified in +M plants (Guohui et al., 2001) and plants under ECO_2 (Sun et al., 2012).

Seedlings +M with HA showed the greater acid phosphatase activity (APA). Adult plants treated with HA mycorrhizal or not, when cultivated in ECO_2 showed higher values of APA (Figure 9).

The APA in seedlings showed positive correlation with chl a (r= 0.9278, P ≤ 0.01), chl total (r=

0.8536, $P \leq 0.01$) and carotenoids (r= 0.7384, $P \leq 0.05$), indicating that the largest APA in root can increment the photosynthetic activity, resulting in higher output of phtoassimilates, that also showed positive correlation with the APA (APA x Seedlings TSP: r= 0.8088, $P \leq 0.05$; APA x Seedlings TSS: r= 0.7839, $P \leq 0.05$; APA x Seedlings Starch: r= 0.814, $P \leq 0.05$; Seedlings SPC: r= 0.9589, $P \leq 0.01$). In bulbs, the APA in seedlings had positive correlation with SPC (r= 0.8047, $P \leq 0.05$), and when evaluated in adult plants had positive correlation with soluble proteins content (r= 0.7381, $P \leq 0.05$).

This effect can assigned to a possible increase in the content of phosphorus (P) in leaves, that beneficing photosynthesis.

Humic acids stimulate the APA (Malcom and Vaughan, 1979). The same ocurr in +M plants (Goicoechea et al., 2004). Kang et al. (2001) observed reduction in APA under ECO_2. In our work, the HA and +M increased the APA in plants under ECO_2.

5.6 CONCLUSIONS

Mycorrhizal inoculation associated the humic acid applied in substrate and atmospheric CO_2 appear as valid horticultural techniques for improving growth and quality of onion seedlings and bulbs under greenhouse conditions. Beneficial effects of humic acid were additive to those of mycorrhizal inoculation or elevated CO_2 on shoot biomass and bulb production. The triple interaction among mycorrhizal inoculation, humic acid application, and elevated CO_2 induced the highest accumulation of starch and soluble phenolics compounds in leaves of seedlings and bulbs, and of solubles sugars, proteins solubles and solubles solids in bulbs, suggesting that the three factor was effective for increasing the quality of onion seedlings and for growth and quality of bulbs.

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5.8 TABLES

Table 9: Growth parameters and water status in onion seedlings non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$, and $P \leq 0.01$, respectively. FW = fresh weight; DM = dry matter; WC = water content; R/S = root DM/shoot DM.

Treatments			Shoot heigth (cm)	Leaves per plant	Shoot FW (mg plant^{-1})	Shoot DM (mg plant^{-1})	Root FW (mg plant^{-1})	Root DM (mg plant^{-1})	Shoot WC (g H_2O g^{-1} DM)	Root WC (g H_2O g^{-1} DM)	R/S
ACO_2	**-M**	**0HA**	17.2 ± 0.39 c	2.8 ± 0.25 a	930.9 ± 0.10 b	90.5 ± 0.01 b	862.1 ± 0.09 a	83.3 ± 0.01 a	9.3 ± 0.18 bc	9.3 ± 0.35 c	0.97 ± 0.02 a
		10HA	24.7 ± 0.34 a	3.3 ± 0.25 a	1992.7 ± 0.27 a	187.0 ± 0.03 a	901.5 ± 0.12 a	74.1 ± 0.01 a	9.7 ± 0.19 b	11.2 ± 0.19 bc	0.41 ± 0.01 e
	+M	**0HA**	19.5 ± 0.92 bc	3.0 ± 0.00 a	1323.1 ± 0.16 ab	122.0 ± 0.01 ab	889.9 ± 0.09 a	61.8 ± 0.01 a	9.8 ± 0.26 b	13.5 ± 0.19 a	0.51 ± 0.01 cd
		10HA	22.5 ± 1.11 ab	3.3 ± 0.25 a	2026.2 ± 0.22 a	164.8 ± 0.02 ab	1141.7 ± 0.11 a	95.9 ± 0.01 a	11.3 ± 0.34 a	11.0 ± 0.44 bc	0.60 ± 0.01 bc
ECO_2	**-M**	**0HA**	20.0 ± 1.21 bc	3.0 ± 0.00 a	1510.9 ± 0.16 ab	149.8 ± 0.01 ab	870.5 ± 0.06 a	68.8 ± 0.00 a	9.1 ± 0.28 bc	11.7 ± 0.51 ab	0.47 ± 0.03 de
		10HA	20.8 ± 0.83 abc	2.8 ± 0.25 a	1732.4 ± 0.08 ab	158.7 ± 0.01 ab	1008.8 ± 0.13 a	73.7 ± 0.02 a	9.9 ± 0.41 b	11.1 ± 0.78 bc	0.54 ± 0.02 bcd
	+M	**0HA**	21.5 ± 1.12 ab	3.5 ± 0.29 a	1626.2 ± 0.24 ab	157.1 ± 0.03 ab	1128.8 ± 0.14 a	72.0 ± 0.01 a	9.4 ± 0.16 bc	10.8 ± 0.21 bc	0.63 ± 0.03 b
		10HA	21.0 ± 0.21 abc	3.0 ± 0.00 a	1791.5 ± 0.13 a	192.6 ± 0.02 a	1227.8 ± 0.14 a	87.7 ± 0.01 a	8.3 ± 0.23 c	10.9 ± 0.47 bc	0.54 ± 0.02 bcd
CO_2			ns	**	ns	ns	ns	ns	**	ns	**
M			**	**	**	**	ns	ns	*	ns	**
HA			ns	ns	ns	ns	*	ns	ns	*	ns
CO_2xM			**	*	*	ns	ns	ns	**	ns	**
CO_2xHA			ns	ns	ns	ns	ns	ns	**	**	**
MxHA			*	ns	ns	ns	ns	ns	ns	**	**
CO_2xMxHA			ns	**	ns	ns	ns	ns	**	**	**

Table 10: Growth parameters and water status in onion bulb non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$and $P \leq 0.01$, respectively. FW = fresh weight; DM = dry matter; WC = water content; TD = transverse diameter; CN = cataphyll number.

Treatments			Bulb FW (g plant^{-1})	Bulb DM (g plant^{-1})	Root FW (g plant^{-1})	Root DM (g plant^{-1})	Bulb WC (g H_2O g^{-1} DM)	Bulb TD (mm)	CN (un)
ACO_2	**-M**	**0HA**	43.39 ± 1.81 e	3.62 ± 0.12 c	4.35 ± 0.22 bcd	0.211 ± 0.004 cd	944.16 ± 21.93 a	50.85 ± 0.45 c	5.90 ± 0.19 a
		10HA	65.30 ± 1.33 cd	4.80 ± 0.13 b	3.34 ± 0.11 d	0.084 ± 0.005 f	979.67 ± 128.57 a	52.49 ± 0.28 b	6.20 ± 0.20 a
	+M	**0HA**	54.44 ± 2.50 de	4.22 ± 0.18 b	5.52 ± 0.46 a	0.295 ± 0.010 a	998.69 ± 92.72 a	43.97 ± 0.33 e	6.30 ± 0.20 a
		10HA	48.88 ± 2.25 e	4.52 ± 0.12 b	4.62 ± 0.12 abc	0.161 ± 0.005 e	905.71 ± 115.19 a	48.00 ± 0.35 d	6.00 ± 0.22 a
ECO_2	**-M**	**0HA**	70.25 ± 2.98 bc	4.77 ± 0.11 b	3.86 ± 0.31 cd	0.264 ± 0.005 ab	1271.23 ± 187.81 a	52.56 ± 0.43 b	6.30 ± 0.12 a
		10HA	78.79 ± 2.52 ab	5.75 ± 0.04 a	5.27 ± 0.30 ab	0.255 ± 0.011 b	1017.68 ± 149.51 a	54.18 ± 0.20 a	6.70 ± 0.20 a
	+M	**0HA**	73.10 ± 3.07 abc	4.33 ± 0.15 b	5.52 ± 0.12 a	0.237 ± 0.010 bc	1188.73 ± 159.19 a	51.26 ± 0.39 bc	6.20 ± 0.20 a
		10HA	81.66 ± 2.90 a	5.52 ± 0.14 a	5.20 ± 0.17 ab	0.183 ± 0.003 de	1055.67 ± 140.94 a	55.09 ± 0.29 a	6.80 ± 0.34 a
CO_2			**	**	**	**	ns	**	*
M			ns	ns	**	**	ns	**	ns
HA			**	**	ns	**	ns	**	ns
CO_2xM			ns	*	ns	**	ns	**	ns
CO_2xHA			ns	ns	**	**	ns	ns	ns
MxHA			**	ns	*	*	*	**	ns
CO_2xMxHA			**	**	*	ns	ns	ns	ns

Table 11: Concentrations of starch (mg g^{-1} DM), total soluble sugars (TSS) (mg g^{-1} DM), total soluble proteins (TSP) (mg g^{-1} DM), proline (µg g^{-1} DM) and soluble phenolics compounds (SPC) (mg g^{-1} DM) in leaves of onion seedlings non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. DM = dry matter.

Treatments			STARCH (mg g^{-1} Shoot DM)	TSS (mg g^{-1} Shoot DM)	TSP (mg g^{-1} Shoot DM)	PROLINE (µmol g^{-1} Shoot DM min^{-1})	SPC (mg g^{-1} Shoot DM)
ACO_2	**-M**	**0HA**	5.06 ± 0.16 d	50.16 ± 1.26 e	7.56 ± 0.18 d	12.45 ± 0.09 a	548.76 ± 33.29 d
		10HA	6.49 ± 0.05 c	64.72 ± 0.97 c	12.10 ± 0.13 b	9.63 ± 0.32 b	712.42 ± 19.15 c
	+M	**0HA**	7.31 ± 0.24 c	63.17 ± 1.42 cd	9.44 ± 0.21 c	12.95 ± 0.34 a	505.69 ± 23.77 d
		10HA	9.27 ± 0.15 a	79.83 ± 2.24 a	14.50 ± 0.45 a	13.53 ± 0.33 a	915.75 ± 32.95 ab
ECO_2	**-M**	**0HA**	6.98 ± 0.18 c	55.50 ± 1.55 de	8.68 ± 0.35 cd	6.53 ± 0.20 c	633.97 ± 57.38 cd
		10HA	9.10 ± 0.35 a	73.14 ± 2.80 ab	12.89 ± 0.49 b	6.90 ± 0.09 c	790.05 ± 30.97 bc
	+M	**0HA**	7.38 ± 0.20 bc	65.62 ± 0.91 bc	9.65 ± 0.30 c	7.70 ± 0.33 c	753.23 ± 37.55 c
		10HA	8.38 ± 0.28 ab	65.89 ± 1.75 bc	12.10 ± 0.40 b	6.98 ± 0.18 c	966.94 ± 16.98 a
CO_2			**	ns	ns	**	**
M			**	**	**	**	**
HA			**	**	**	**	**
CO_2xM			ns	*	**	*	ns
CO_2xHA			**	**	**	**	*
MxHA			ns	**	ns	**	**
CO_2xMxHA			*	**	*	**	ns

Table 12: Concentrations of starch (mg g^{-1} DM), total soluble sugars (TSS) (mg g^{-1} DM), total soluble proteins (TSP) (mg g^{-1} DM) , proline (µg g^{-1} DM), soluble phenolics compounds (SPC) (mg g^{-1} DM), pH, soluble solids (SS) titratable acidity (TA) (mg piruvic acid g^{-1} FM) and SS/TA in bulbs of onion non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. Values are means ± SE (n= 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). ANOVA: ns = not significant; * and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. DM = dry matter, FW = fresh weight.

Treatments			STARCH (mg g^{-1} Bulb DM)	TSS (mg g^{-1} Bulb DM)	TSP (mg g^{-1} Bulb DM)	PROLINE (µmol g^{-1} Bulb DM min^{-1})	SPC (mg g^{-1} Bulb DM)	pH	SS (ºBx)	TA (mg piruvic acid g^{-1} Bulb FM)	SS/TA
ACO_2	**-M**	**0HA**	1.26 ± 0.06 b	297.28 ± 0.50 f	4.49 ± 0.10 g	5.208 ± 0.07 a	1895.55 ± 25.49 e	5.32 ± 0.04 b	5.38 ± 0.09 f	0.101 ± 0.001 b	53.50 ± 0.34 f
		10HA	1.99 ± 0.29 ab	349.66 ± 5.33 d	9.38 ± 0.22 f	4.260 ± 0.05 b	2539.59 ± 9.13 cd	5.31 ± 0.04 b	6.36 ± 0.06 de	0.106 ± 0.002 ab	60.05 ± 1.31 de
	+M	**0HA**	1.57 ± 0.12 b	324.74 ± 0.97 e	12.43 ± 0.10 e	3.405 ± 0.07 c	2441.45 ± 13.28 d	5.45 ± 0.03 ab	5.86 ± 0.10 ef	0.104 ± 0.004 ab	56.42 ± 1.49 ef
		10HA	2.38 ± 0.39 ab	353.35 ± 7.28 d	13.44 ± 0.08 e	3.255 ± 0.11 cd	2639.55 ± 15.93 bc	5.46 ± 0.09 ab	6.74 ± 0.17 cd	0.090 ± 0.002 c	75.26 ± 0.96 c
ECO_2	**-M**	**0HA**	3.21 ± 0.33 a	469.01 ± 5.24 c	17.71 ± 0.33 d	3.425 ± 0.13 c	2463.56 ± 13.76 d	5.55 ± 0.04 a	6.94 ± 0.08 bc	0.111 ± 0.002 a	62.82 ± 1.45 d
		10HA	2.24 ± 0.30 ab	535.17 ± 6.53 b	26.28 ± 0.29 b	3.012 ± 0.20 cd	2722.75 ± 58.73 b	5.58 ± 0.01 a	6.74 ± 0.15 cd	0.082 ± 0.001 c	81.92 ± 1.24 b
	+M	**0HA**	2.03 ± 0.31 ab	478.76 ± 1.34 c	23.13 ± 0.14 c	3.546 ± 0.21 c	2605.45 ± 33.82 bc	5.55 ± 0.03 a	7.46 ± 0.17 b	0.088 ± 0.002 c	84.72 ± 1.25 b
		10HA	2.99 ± 0.42 a	588.85 ± 2.52 a	31.74 ± 0.38 a	2.658 ± 0.12 d	3121.79 ± 23.00 a	5.57 ± 0.06 a	8.50 ± 0.10 a	0.090 ± 0.001 c	94.90 ± 1.02 a
CO_2			**	**	**	**	**	**	**	**	**
M			ns	**	**	**	**	*	**	**	**
HA			ns	**	**	**	**	ns	**	**	**
CO_2xM			ns	*	ns	**	ns	*	**	ns	**
CO_2xHA			ns	**	**	ns	ns	ns	**	**	ns
MxHA			*	ns	**	ns	*	ns	**	ns	ns
CO_2xMxHA			*	**	**	**	**	ns	**	**	**

Table 13: Significance of two-factor ANOVA showing effects of CO_2, humic acids application (HA) and their interactions on Mycorrhizal Efficiency Index (MEI) (%) on leaves, roots and whole plant biomass production in onion seedlings and on bulb, roots and total biomass production in onion bulbs non-amended (0HA) or amended (10HA) with humic acids (HA) and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. ANOVA: ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

Treatments	MEI LEAVES	MEI ROOT SEEDLINGS	MEI PLANT SEEDLINGS	MEI BULB	MEI ROOT BULB	MEI TOTAL
CO_2	*	**	**	**	**	**
HA	**	**	ns	**	**	**
CO_2xHA	**	**	ns	**	**	**

Table 14: Significance of three-factor ANOVA showing effects of CO_2, arbuscular mycorrhizal fungi (M), humic acids application (HA) and their interactions on the chlorophyll a (Chl a) (mg g^{-1} DM), chlorophyll b (Chl b) (mg g^{-1} DM), total chlorophylls (Chl a+b) (mg g^{-1} DM), total carotenoides (mg g^{-1} DM) in leaves of onion seedlings and acid phosphatase activity (APA) in roots seedlings and bulb non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2 in the atmosphere. ANOVA: ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

Treatments	Chl a	Chl b	Chl a+b	Carotenoids	APA SEEDLINGS	APA BULB
CO_2	**	**	**	**	ns	**
M	**	*	**	**	**	**
HA	**	**	**	**	**	ns
CO_2xM	ns	ns	ns	ns	ns	**
CO_2xHA	*	ns	ns	ns	ns	**
MxHA	ns	*	ns	**	ns	**
CO_2xMxHA	ns	**	**	**	ns	ns

5.9 FIGURES CAPTIONS

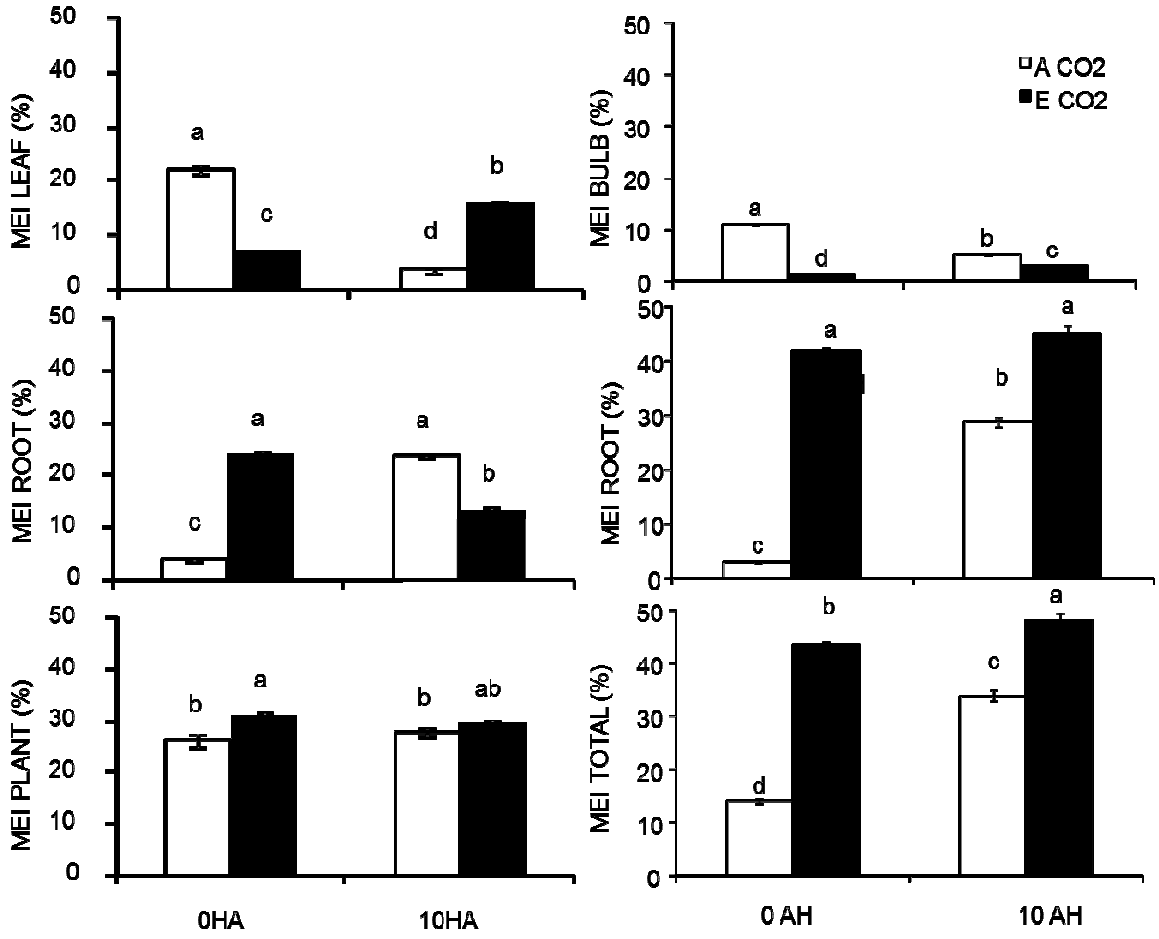

Fig. 7: Mycorrhizal Efficiency Index (MEI) (%)on leaves, roots and whole plant biomass production in onion seedlings and on bulb, roots and total biomass production in onion bulbs non-amended (0HA) or amended (10HA) with humic acids (HA), and grown either at ambient (ACO_2) (white histograms) or under elevated (ECO_2) (black histograms) CO_2 in the atmosphere. Values are means ± SE (n= 5). Within each figure data followed by the same letter indicate that values are similar ($P \leq 0.05$).

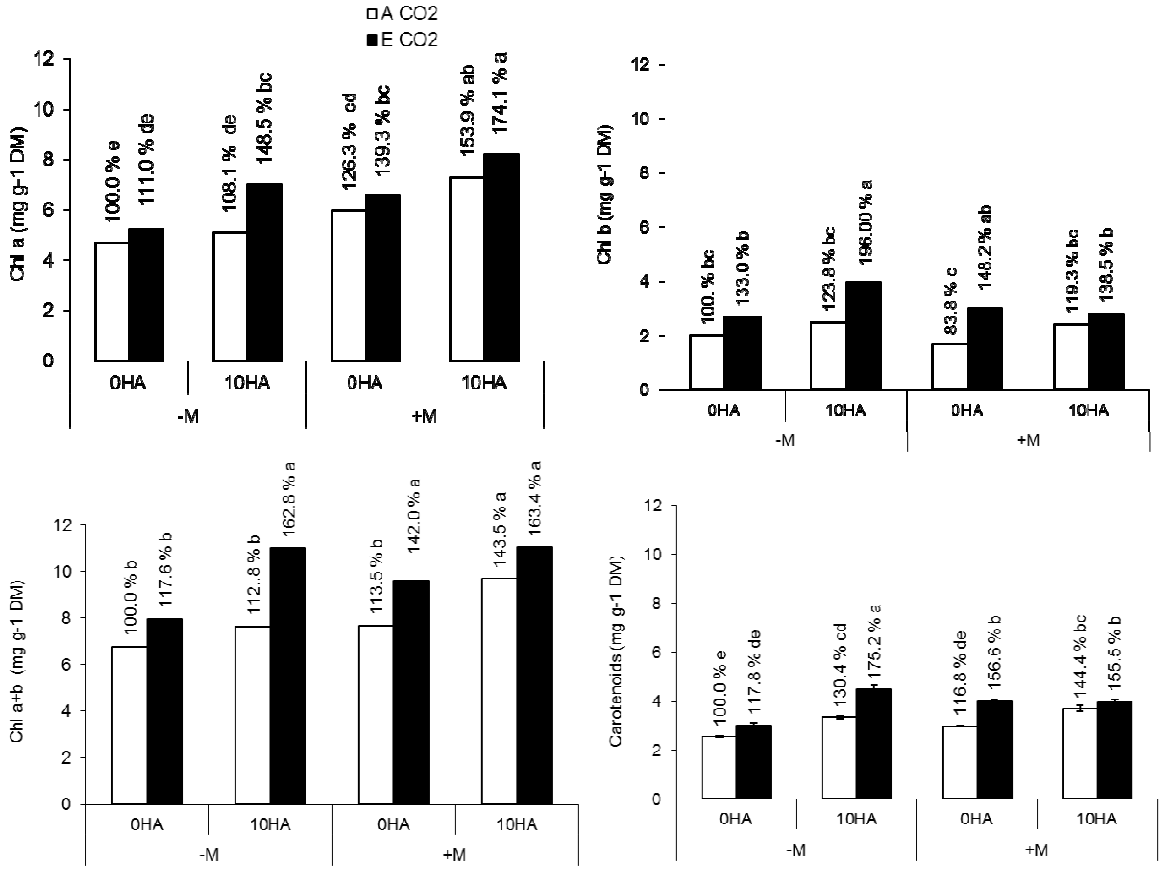

Fig. 8: Chlorophyll a (Chl a) (mg g^{-1} DM), chlorophyll b (Chl b) (mg g^{-1} DM), total chlorophylls (Chl a+b) (mg g^{-1} DM) and total carotenoides (mg g^{-1} DM) in leaves of onion seedlings non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) (white histograms) or under elevated (ECO_2) (black histograms) CO_2 in the atmosphere. Values are means ± SE (n = 5). Within each parameter data followed by the same letter indicate that values are similar ($P \leq 0.05$). DM = dry matter.

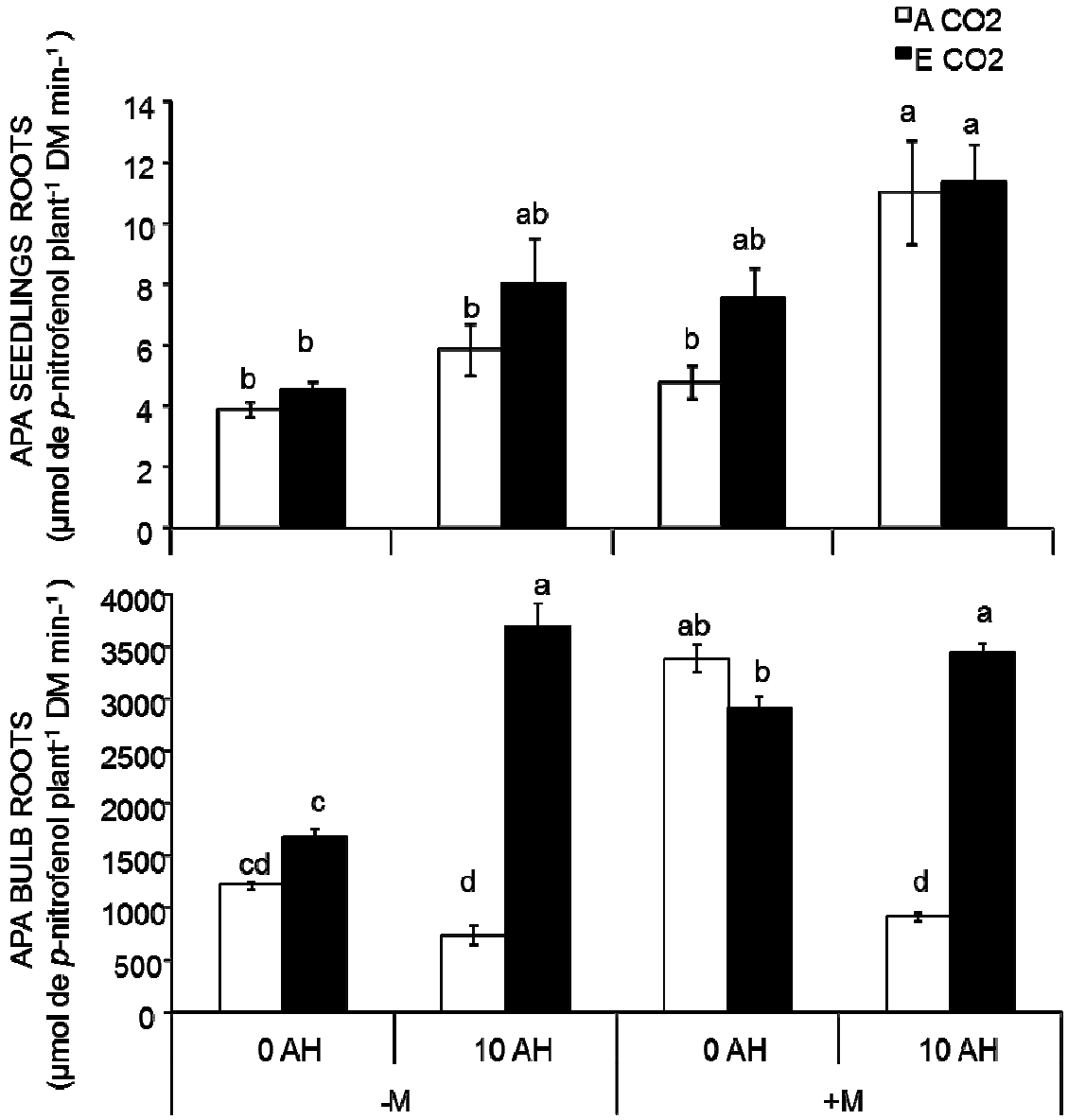

Fig. 9: Acid phosphatase activity (µmol p-nitrophenol g-1 DM min-1) in roots of onion seedlings and onion bulbs non-amended (0HA) or amended (10HA) with humic acids (HA), non-inoculated (-M) or inoculated (+M) with mycorrhizal fungi and grown either at ambient (ACO_2) (white histograms) or under elevated (ECO_2) (black histograms) CO_2 in the atmosphere. Values are means ± SE (n = 5). Data followed by the same letter indicate that values are similar ($P \leq 0.05$). DM = dry matter.

CONCLUSÃO

A aplicação de substâncias húmicas, via imersão de bandejas, promove a melhoria das características de crescimento de mudas de cebola cv. Alfa São Francisco Ciclo VIII quando aplicada entre as concentrações 13,8 e 24,5 mL L^{-1}.

O método de aplicação das substâncias húmicas influencia a qualidade e a produtividade de bulbos, sendo o ideal, associar a aplicação das substâncias via imersão da bandeja com posteriores pulverizações foliares à campo.

Substâncias húmicas e inoculação micorrízica arbuscular podem ser utilizados como alternativas biofertilizantes para a produção de mudas e bulbos de cebola, com efeitos aditivos quando cultivadas em elevados níveis de CO_2.

As substâncias húmicas juntamente com a inoculação micorrízica arbuscular melhoram características de crescimento de mudas e qualidade de mudas e bulbos de cebola cv. Alfa São Francisco Ciclo VIII, mesmo quando cultivados em CO_2 elevado.

CONSIDERAÇÕES FINAIS

Em função dos resultados observados neste estudo, recomenda-se a continuidade de pesquisas que preconizem a utilização de substâncias húmicas e fungos micorrízicos arbusculares com o objetivo de melhorar a eficiência da absorção de nutrientes pelas plantas, bemcomo seu metabolismo.

A associação destas ferramentas biofertilizantes pode responder de diferentes maneiras em função da espécie cultivada, da cultivar, da espécie de fungo, entre outros fatores, sendo necessária a realização de estudos específicos neste sentido.

É importante também saber o custo benefício da utilização destes métodos e sua aplicabilidade à campo, em diferentes regiões.

A utilização do CO_2 elevado, por sua vez, gera benefícios quando associada as substâncias húmicas e a micorrização, porém seu alto custo inviabiliza a adoção desta prática à campo.

Em contrapartida, estruturas utiizadas no armazenamento pós-colheita de frutas e hortaliças poderiam ser aproveitadas, enquanto ociosas, para acelerar a produção de mudas, por exemplo.

Outra constatação importante a ser feita para outras cultivares ou até mesmo para outras espécies é a resposta destas a elevação do CO_2, o que poderia nos ajudar a selecionar materiais mais responsivos a estes aumentose que sejam adaptados a futura realidade.