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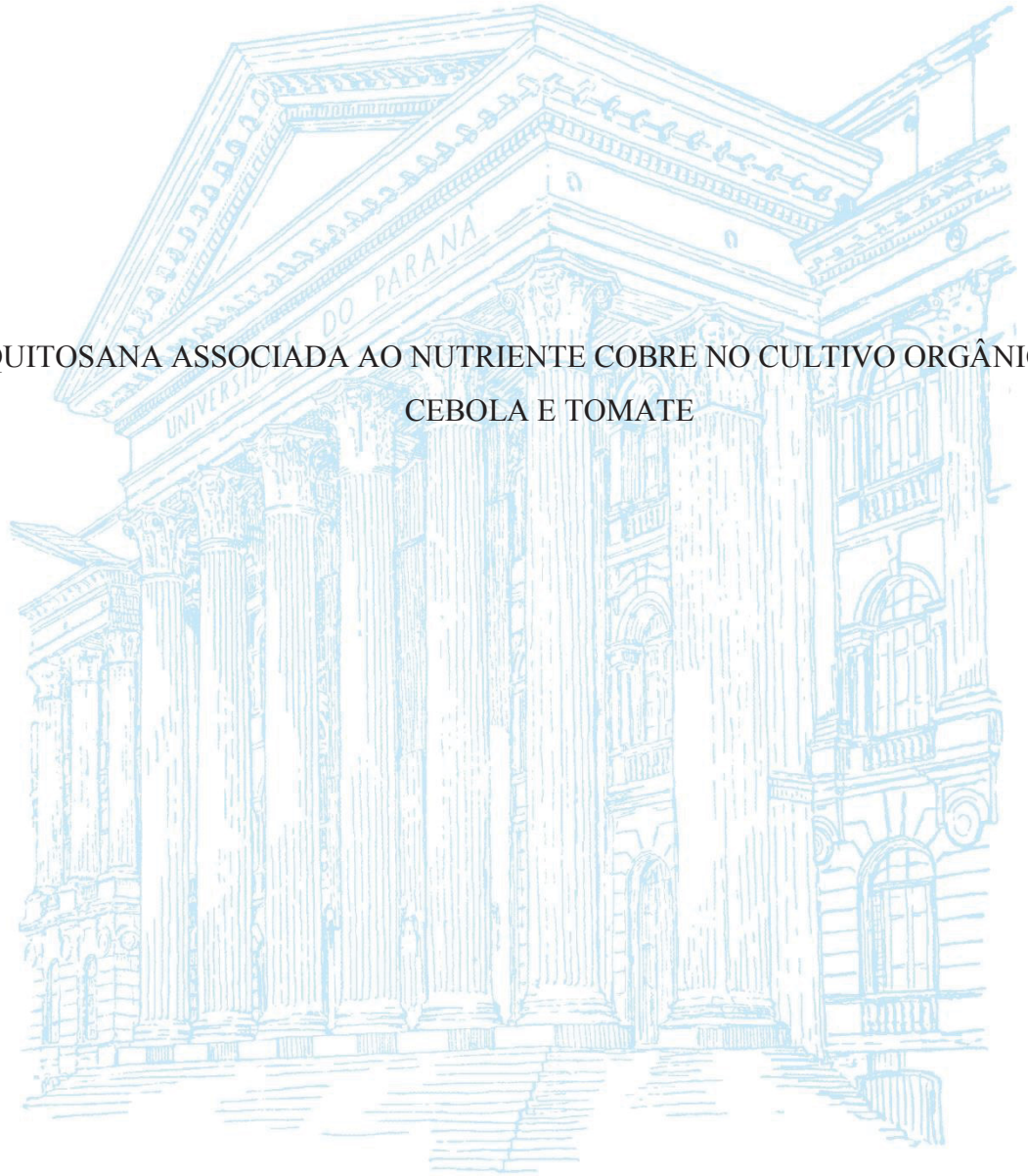
LAÍS GOMES ADAMUCHIO



QUITOSANA ASSOCIADA AO NUTRIENTE COBRE NO CULTIVO ORGÂNICO DE CEBOLA E TOMATE

CURITIBA

2020

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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 obtenção do título de Doutor em Ciências.

Orientador: Prof. Dr. Átila Francisco Mógor
Co-orientadores: Prof. Dr. Bruno Francisco Sant'Anna-Santos
Prof. Dr. Sérgio Miguel Mazaro

CURITIBA

2020

Oliveira, Laís Gomes Adamuchio de
Quitosana associada ao nutriente cobre no cultivo orgânico de cebola e tomate. / Laís Gomes Adamuchio de Oliveira. - Curitiba, 2020.

Tese (Doutorado) - Universidade Federal do Paraná. Setor de Ciências Agrárias, Programa de Pós-Graduação em Agronomia.

Orientador: Átila Francisco Mógor.

Coorientadores: Bruno Francisco Sant'Anna-Santos; Sérgio Miguel Mazaro.

1. Tomate. 2. Cebola. 3. Plantas - Resistência a doenças e pragas. 4. Enzimas. 5. Nutrientes. I. Mógor, Átila Francisco. II. Sant'Anna-Santos , Bruno Francisco. III. Mazaro, Sérgio Miguel. IV. Título. V. Universidade Federal do Paraná.

Sistema de Bibliotecas/UFPR
Guilherme Luiz Cintra Neves - CRB9/1572

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIENCIAS AGRARIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO AGRONOMIA
(PRODUÇÃO VEGETAL) - 40001016031P6

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LAIS GOMES ADAMUCHIO** intitulada: **QUITOSANA ASSOCIADA AO NUTRIENTE COBRE NO CULTIVO ORGÂNICO DE CEBOLA E TOMATE**, sob orientação do Prof. Dr. ÁTILA FRANCISCO MÓGOR, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Fevereiro de 2020.

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A Deus, na pessoa do Senhor Jesus Cristo, meu Salvador, refúgio e fortaleza.
Ofereço.

Aos meus pais Laércio e Maria Aparecida, pelo amor e apoio incondicionais.
Ao meu esposo Edson, amor da minha vida.
Dedico.

AGRADECIMENTOS

Em primeiro lugar agradeço a Deus por ter me dado a salvação por meio de seu filho Jesus, que em sua infinita misericórdia tem me sustentado a cada dia guiando meus passos para trilhar os caminhos que Ele sonhou para mim. Sem a sua graça, força e orientação nada seria possível. Agradeço a Ele por ter permitido que as pessoas abaixo citadas fizessem parte de minha vida.

Agradeço aos meus pais Laércio e Maria Aparecida por todo amor, carinho, dedicação, orações, apoio e incentivo em toda minha caminhada. Seus ensinamentos são a base de quem sou.

A meu amado esposo Edson, em especial, por seu companheirismo em todos os momentos. Seu incentivo, paciência e amor tornaram os meus dias mais leves.

Aos meus queridos irmãos Jessé e Juliano pelo carinho e apoio.

A Universidade Federal do Paraná e ao Curso de Pós-Graduação em Agronomia-Produção Vegetal, pela oportunidade de realizar o doutorado.

Ao Professor Dr. Átila Francisco Mógor, pela oportunidade, orientação, confiança, amizade, incentivo, paciência e ensinamentos que foram fundamentais para meu crescimento profissional e para o desenvolvimento do projeto.

Ao Professor Dr. Sérgio Miguel Mazaro, Dr. Bruno Francisco Sant'Anna-Santos, Dra. Lucimeris Ruaro, Dr Henrique da Silva Silveira Duarte, Dr Cícero Deschamps por todo apoio e orientações durante o desenvolvimento deste trabalho.

A Dra. Gilda Mógor pela amizade, apoio, conselhos, auxílio e contribuições no desenvolvimento do projeto.

A Dra Francine Lorena Cuquel e Dra Katia Christina Zuffellato-Ribas pela amizade, conselhos e incentivo durante essa trajetória.

Aos demais professores do curso de Pós-Graduação em Produção Vegetal da Universidade Federal do Paraná, pelos ensinamentos que contribuíram no aperfeiçoamento de minha formação profissional.

Aos técnicos de laboratório, Roger Raupp Cipriano, Carlos Eduardo Maduro, Maria Emília Kudla pela amizade e apoio que foram fundamentais para o desenvolvimento do projeto.

Aos meus queridos colegas Juliana de Oliveira Amatussi, Luiz Gabriel Gemin, Ely Cristina Negrelli Cordeiro, Gabriel Lara, Aline Novaski, Cristiane Maria Ronkoski, Luiza Prado, Tamires Santos pela amizade, companheirismo, alegria, descontração e apoio nos trabalhos de laboratório, campo e em todas as atividades que realizamos juntos.

A querida Lucimara pela amizade, atenção e por realizar um trabalho de excelência à todos da Pós-Graduação em Agronomia – Produção Vegetal.

A Emater Paraná por disponibilizar o tempo necessário para conclusão da tese. Aos colegas extensionistas Mariana Elisa Muller, Julian Mattos, Rodrigo Rossi, Amauri Ferreira Pinto, Milton Satoshi Matsushita, Luiz Fernando Brondani e Richard Golba pelo incentivo e apoio.

A todos que de alguma maneira contribuíram para que esse trabalho fosse possível, minha gratidão.

RESUMO

Técnicas que promovam melhorias na produtividade com uso eficiente de insumos e que não comprometam a sustentabilidade ambiental, devem ser priorizadas na produção de hortaliças. A quitosana é um biopolímero natural que tem sido utilizado em plantas com efeito benéfico e é reconhecido como importante indutor de resistência a doenças. O cobre é um importante micronutriente essencial relacionado à produção de compostos do metabolismo secundário vegetal e um nutriente chave nas respostas de defesa vegetal. O objetivo desse trabalho foi avaliar o efeito da aplicação foliar de quitosana associada ao cobre-EDTA na cultura do tomateiro e da cebola. Foram avaliados parâmetros biométricos, bioquímicos, anatômicos e fitossanitários para o tomateiro. Para a cultura da cebola foram avaliados parâmetros biométricos, produtivos, enzimáticos nas folhas, bioquímicos em folhas e bulbos, bem como aspectos nutricionais dos bulbos. Quitosana associada ao cobre quelatizado apresentou maior eficiência na ativação de enzimas relacionadas à patogenicidade no tomateiro do que a quitosana ou o cobre isoladamente. A quitosana associada ao cobre quelatizado promoveu o acúmulo de compostos fenólicos, o aumento da espessura da parede periclinal externa e da cutícula nas folhas do tomateiro, bem como alterou a proporção do tecido de revestimento em relação ao mesofilo. Esses fatores combinados permitiram reduzir concomitantemente a severidade do oídio, sem afetar os parâmetros biométricos e bioquímicos relacionados ao metabolismo primário. Verificou-se que as aplicações de quitosana isoladamente promoveram maior acúmulo de compostos relacionados ao metabolismo de defesa das plantas de cebola, com maior atividade de enzimas relacionadas à patogenicidade nas folhas. Houve redução do crescimento das plantas em função do aumento da dose. Ocorreram diferenças de comportamento entre as cultivares quanto ao acúmulo de peroxidase nas folhas e compostos fenólicos nos bulbos. Também ocorreram alterações nos teores de prolina nas folhas e bulbos. A aplicação de quitosana associada ao cobre via foliar aumentou os teores de P, K, Mn e Zn nos bulbos e alterações metabólicas decorrentes não promoveram modificações no metabolismo primário a ponto de afetar a produtividade, bem como o teor médio de água. Houve aumento de compostos fenólicos, que estão diretamente ligados a qualidade dos bulbos. Concluiu-se que a aplicação foliar de quitosana associada ao quelato de cobre pode ser uma técnica eficiente para a indução de resistência no cultivo de tomate. A aplicação de quitosana via foliar afetou o metabolismo primário e secundário das plantas de cebola e pode induzir respostas de defesa nas folhas pelo aumento de proteínas relacionadas à patogenicidade e aumento da resistência a estresses abióticos (como seca, frio, salinidade e outros) em bulbos pelo maior acúmulo de prolina. O uso de quitosana associada ao cobre é uma técnica eficiente para melhorar a qualidade nutricional de bulbos de cebola.

Palavras-chave: *Solanum lycopersicum* L., *Allium cepa*, indução de resistência, enzimas, nutriente.

ABSTRACT

Efficient cultivation techniques that promote productivity improvements with efficient use of inputs without compromising environmental sustainability have been sought in vegetable production. Chitosan is a natural biopolymer that has been used in plants with beneficial effect besides being recognized as an important inducer of disease resistance. Copper is an important essential micronutrient related to the production of plant secondary metabolism compounds and a key nutrient in plant defense responses. The objective of this work was to evaluate the effect of foliar application of chitosan associated with copper-EDTA on tomato and onion crops. Biometric, biochemical, enzymatic, anatomical and phytosanitary parameters were evaluated for tomato. For onion culture, biometric, productive, leaf enzymatic, leaf biochemical and bulb parameters as well as nutritional aspects of the bulbs were evaluated. Chlelated copper-associated chitosan showed higher efficiency in the activation of pathogenicity-related enzymes in tomato than chitosan or copper alone. Chelated copper-associated chitosan increased the accumulation of phenolic compounds, the thickness of the external periclinal wall and the cuticle in tomato leaves, as well as the proportion of epidermal tissues in relation to leaf limb. These combined factors allowed the reduction of mildew severity simultaneously, without affecting the biometric and biochemical parameters related to primary metabolism. Chitosan applications alone promoted greater accumulation of compounds related to the defense metabolism of onion plants, with higher activity of enzymes related to pathogenicity in leaves. A reduction was found in plant growth as a function of increasing doses. Differences were found in behavior between cultivars regarding leaf peroxidase accumulation and bulb phenolic compounds. There were also changes in proline levels in leaves and bulbs. The application of copper-associated chitosan through leaves increased the contents of P, K, Mn and Zn in the bulbs and consequent metabolic alterations did not promote changes in the primary metabolism to the point of affecting the productivity as well as the average water content. There was an increase in phenolic compounds, which are directly linked to bulb quality. It was concluded that leaf application of chelated copper-associated chitosan can be an efficient technique for resistance induction in healthy tomato cultivation. Leaf-applied chitosan affects onion primary and secondary metabolism and can induce leaf defense responses by increasing pathogenicity-related proteins and increasing resistance to abiotic stress in bulbs by means of an increase in proline accumulation. Copper- associated chitosan appears as a valid horticultural technique to improve the nutritional quality of onion bulbs.

Keywords: *Solanum lycopersicum* L., *Allium cepa*, induced resistance, enzymes, nutrient.

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1. INTRODUÇÃO GERAL

O total de habitantes do planeta deve passar dos atuais 7,7 bilhões para 9,7 bilhões em 2050, aumentando em 2 bilhões de pessoas nos próximos 30 anos, afirma um relatório das Nações Unidas, publicado pelo Departamento de Assuntos Econômicos e Sociais da ONU, DESA (2019), sendo um dos maiores desafios da agricultura a produção de alimentos que satisfaçam esta demanda.

Para atender a demanda crescente de alimentos provocada por este aumento exponencial da população mundial, tem-se utilizado intensamente vários insumos, como 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.

A área de produção agrícola no Brasil representa 34% da área total do país (289.374.000 hectares) (FAOSTAT 2019a). Os últimos dados da FAO (Food and Agriculture Organization of the United Nations) de 2017 descrevem que o Brasil é o décimo maior produtor de tomates do mundo (4.230.150 toneladas) e o décimo segundo maior produtor de cebola (1.622.106 toneladas) (FAOSTAT, 2019a).

Na Lei da Agricultura Orgânica Lei 10.831 de 2003, a definição encontrada é:

> Considera-se sistema orgânico de produção agropecuária todo aquele em que se adotam técnicas específicas, mediante a otimização do uso dos recursos naturais e socioeconômicos disponíveis e o respeito à integridade cultural das comunidades rurais, tendo por objetivo a sustentabilidade econômica e ecológica, a maximização dos benefícios sociais, a minimização da dependência de energia não-renovável, empregando, sempre que possível, métodos culturais, biológicos e mecânicos, em contraposição ao uso de materiais sintéticos, a eliminação do uso de organismos geneticamente modificados e radiações ionizantes, em qualquer fase do processo de produção, processamento, armazenamento, distribuição e comercialização, e a proteção do meio ambiente.

Segundo a Federação Internacional de Movimentos da Agricultura Orgânica (IFOAM, 2018) estão identificados cerca de 3 milhões de produtores orgânicos em um universo de 181 países. A agricultura orgânica cresceu em todos os continentes atingindo área recorde de 70 milhões de hectares, aproximadamente (IFOAM, 2018).

Dados do Censo Agropecuário do IBGE mostram que, de 2006 a 2017, o número de estabelecimentos agropecuários com a certificação de produção orgânica cresceu mais de 1.000% no Brasil, saltando de 5.106 para 68.716. O Brasil é o líder do mercado de orgânicos da América Latina e possui em extensão de terra destinada à agricultura orgânica 1.136.857 hectares, ocupando

a 12º no mundo em área (IFOAM, 2018). Esse sistema de produção tem crescido continuamente, em função de uma demanda cada vez maior por produtos ecologicamente corretos.

Para tornar o sistema de produção orgânico viável, os produtores encontram algumas limitações como a carência de fertilizantes orgânicos que forneçam os nutrientes necessários ao crescimento das plantas, o controle de espécies invasoras, o controle fitossanitário e a carência de cultivares adaptadas às condições edafoclimáticas das distintas regiões (WORDELL FILHO et al., 2006; GONÇALVES et al., 2008; VIDIGAL et al., 2010).

O controle de doenças é um fator essencial para obter alta produtividade. O uso de fungicidas sintéticos tem sido a principal alternativa de controle acarretando alto custo de produção, danos à saúde humana e ao meio ambiente e além de que o uso indiscriminado desses produtos pode acarretar em cepas resistentes (ZANIN, 2017).

O uso de produtos naturais para o controle de doenças tem sido amplamente estudado e a quitosana tem sido relatada como uma alternativa eficiente possui baixo custo e importantes propriedades biológicas como a biodegradabilidade, biocompatibilidade e não-alergicidade (XING, 2015). A ação antimicrobiana desse importante biopolímero depende de muitos fatores como as condições ambientais, como pH, tipo de microrganismo e componentes vizinhos, suas condições estruturais, como peso molecular, grau de desacetilação, forma derivada, concentração e fonte originaria (HOSSEINNEJAD & JAFARI, 2016).

O cobre é um nutriente essencial para o desenvolvimento das plantas como cofator de numerosas proteínas e metabolismo de carboidratos (PRINTZ et al., 2016). Os micronutrientes são usados em pequenas quantidades, as plantas em condições normais, tem 100ppm de cobre e 20ppm quando apresentam deficiência (VIJAY, et al, 2017).

As vias de ação do cobre em plantas também estão relacionadas aos mecanismos de defesa e qualidade, pois é um nutriente chave, fornecendo alterações na estrutura e forma das paredes celulares e participando de enzimas como a fenilalanina amônia-liase (PRINTZ et al 2016). Outro efeito do cobre na resistência à doenças é devido à sua participação na síntese de lignina, uma barreira parcial à penetração nos tecidos das plantas (GRAHAM & WEBB, 1991).

O objetivo desse trabalho foi avaliar o efeito da aplicação foliar de quitosana associada ao cobre-EDTA na cultura do tomateiro e da cebola. Foram avaliados parâmetros biométricos, bioquímicos, anatômicos e fitossanitários para o tomateiro. Para a cultura da cebola foram avaliados parâmetros biométricos, produtivos, enzimáticos nas folhas, bioquímicos em folhas e bulbos, bem como aspectos nutricionais dos bulbos.

2. REVISÃO DE LITERATURA

2.1. A cultura da cebola

A cebola (A*llium cepa* L.*)* é uma espécie herbácea monocotiledônea pertencente à família Alliaceae, originária da Ásia Central e considerada como uma das mais antigas plantas cultivadas, com registros datados de cerca de 5.000 a. C. no Antigo Egito (SHIGYO & KIK, 2008). A palavra cebola é originária do latim e significa "grande pérola" (SHIGYO & KIK, 2008). Devido às suas propriedades terapêuticas e características específicas quanto ao sabor, aroma e pungência, o bulbo é consumido em todos os continentes, compondo os mais diversos pratos da culinária mundial. A cebola é consumida *in natura* na forma de saladas, desidratada, processada e industrializada, dá origem a uma gama de produtos usados como condimentos na alimentação humana (RESENDE et al., 2007).

A cebola apresenta em sua composição as lectinas (proteína de maior concentração), prostaglandinas, frutanos, pectina, adenosina, vitaminas B1, B2, B6, C, E, biotina, ácido nicotínico, ácidos graxos, glicolípidios, fosfolipídios, aminoácidos essenciais, compostos sulfurosos e principalmente os compostos fenólicos (CORZO-MARTINEZ et al., 2007; PATRA et al., 2013). Nas cebolas estão presentes também várias fitomoléculas ativas como os ácidos fenólicos, flavonoides, tiosulfinato, compostos organosulfurosos e antocianinas (PEREZ-GREGÓRIO,2014). Os principais flavonoides encontrados na cebola são quercetina, miricetina e kaempferol (SANTAS et al., 2010).

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).

Caracteriza-se por ser uma espécie polimórfica que exibe diferenças quanto à cor e nível de cerosidade das folhas, ao formato, tamanho e cor dos bulbos, e à reação ao comprimento do dia (MELO, 2007). É uma espécie herbácea com cerca de 60 cm de altura que apresenta folhas grandes dispostas alternadamente em duas fileiras, podendo ser cerosas ou não. O caule verdadeiro está localizado abaixo da superfície do solo, sendo este um disco compacto com formato cônico, situado na base inferior do bulbo de onde partem as raízes. As bainhas foliares formam um pseudocaule cuja parte inferior é o próprio bulbo (FILGUEIRA, 2008).

A espécie foi introduzida no Brasil pelos portugueses no século XVI, é uma das hortaliças mais importantes, sendo cultivada na maioria das regiões brasileiras (BOITEUX & MELO, 2004). Ocupa o terceiro lugar entre as hortaliças de maior expressão econômica do Brasil e constitui

atividade socioeconômica de grande relevância para os estados da região sul. O cultivo da cebola tem importância socioeconômica, uma vez que cultivada por pequenos agricultores a necessidade de mão-de-obra é grande, gerando emprego e renda. Já na agricultura empresarial, a cebola tem importância significativa na geração de empregos de forma direta e indireta (EL BALLA, 2013).

Segundo os dados do último censo agropecuário realizado em 2017 a área plantada no Brasil foi de 58.001 hectares alcançando uma produção total de 1.719.440 toneladas (IBGE, 2019). Segundo os dados do DERAL (Departamento de Economia Rural da Secretaria de Agricultura do Estado do Paraná), em 2018 a área plantada no Paraná foi de 4819 hectares e produção total de 125.737,5 toneladas.

No cultivo convencional para tratamento fitossanitário se recomenda a pulverização semanal alternada até o final do ciclo dos fungicidas metalaxil-m mancozebe e metalaxil-m clorotalonil; e a partir do final de setembro ao fim do ciclo de forma alternada dos inseticidas lambdacialotrina e imidacloprido (MENEZES-JUNIOR et al., 2018). Contudo, a racionalização de produtos fitossanitários possibilita a obtenção de altas produtividades na cultura da cebola com redução do uso de insumos (MENEZES-JUNIOR et al., 2018).

2.2. A cultura do tomate

O tomateiro pertence à família Solanaceae (TAYLOR, 1986). O tomateiro é uma planta perene, de porte arbustivo, sendo cultivada como planta anual. Pode ser conduzida na forma rasteira, semi-rasteira e ereta. Apresenta dois hábitos de crescimento, sendo limitado nas variedades de crescimento determinado e ilimitado nas variedades de crescimento indeterminado (ALVARENGA, 2004).

Está entre as hortaliças mais consumidas no mundo, possui excelentes compostos para a saúde humana, como vitamina C e licopeno (YA-DAN et al., 2017). O consumo de tomate nas últimas décadas seja *in natura* seja processado aumentou de forma progressiva, com tendência a continuar crescendo e criar ainda mais espaço para o desenvolvimento do mercado.

Considerando a demanda de consumo, geração de emprego, renda e participação expressiva no agronegócio, o tomate ocupa posição de grande importância socioeconômica (SILVA & GIORDANO, 2006). O tomateiro é uma das principais hortaliças consumidas no Brasil, quer seja na forma fresca quanto na forma processada (SOARES et al., 2012), tendo destaque especial, tanto do ponto de vista econômico quanto social, pelo volume de produção e geração de empregos (BARROS et al., 2014). Em 1990, a produção nacional era de 34,6 $t.ha^{-1}$ (EMBRAPA, 2019), alcançando em 2014 produção de 66,7 $t.ha^{-1}$ (IBGE, 2019), equivalendo ao aumento de 92,77%, crescimento que está relacionando, dentre outros fatores, às técnicas de cultivo criadas e disponibilizadas para a tomaticultura, proporcionando maiores rendimentos.

Segundo os dados do último censo agropecuário realizado em 2017 a área plantada no Brasil foi de 22.026 hectares alcançando uma produção total de 1.143.922 toneladas (IBGE, 2019). Segundo os dados do DERAL (Departamento de Economia Rural da Secretaria de Agricultura do Estado do Paraná), em 2018 a área plantada no Paraná foi de 3.990 hectares e produção total de 235.626,4 toneladas.

A cultura do tomate demanda cuidados constantes, pois está sujeita ao ataque de grande número de doenças, pragas e desordens fisiológicas. É importante destacar que os defensivos agrícolas respondem hoje por mais de 35% do custo total de uma lavoura de um hectare de tomate estaqueado, utiliza-se três a quatro aplicações de mistura de fungicidas e inseticidas por semana (NASCIMENTO et al., 2013).

2.3. Produção orgânica de cebola

O cultivo de cebola orgânica vem aumentando devido à expansão da demanda dos consumidores, possivelmente devido ao aumento da população e aumento da renda dos consumidores. Segundo Gonçalves & Wamser (2007), a produção de cebola orgânica, quando comparada com o cultivo convencional, atinge níveis de produtividade semelhantes, com menor uso de insumos e menor custo de produção, porém, com maior necessidade de mão-de-obra devido à realização de capinas manuais.

Dentre as características levadas em consideração para classificar as cebolas, estão as exigências fotoperiódicas, o padrão genético, a preferência e a forma de consumo (OLIVEIRA et al., 2004). A cultivar a ser plantada deve ser escolhida em função das condições climáticas da região e da exigência do mercado com relação ao tipo de bulbo (COSTA et al., 2002).

Costa et al. (2008) avaliaram o desempenho de diferentes cultivares de cebola em sistema orgânico nas condições edafoclimáticas do Vale do São Francisco, e obtiveram produtividades comerciais superiores a 26 e 17 $t.ha^{-1}$,respectivamente em um Argissolo e um Vertissolo, sendo as cultivares Brisa IPA-12, São Paulo, Botucatu-150, Pira Ouro (Argissolo), Texas Grano PRR e IPA-10 (Vertissolo) as mais produtivas.

Ao avaliar seis cultivares de cebola sob manejo orgânico, na região metropolitana do estado do Rio de Janeiro, Paula et al. (2005, 2009) observaram que todas produziram alta proporção de bulbos com boa aceitação comercial, indicando que a região apresenta grande potencial para o cultivo de cebola orgânica.

Vidigal et al. (2010), pesquisaram desempenho da cultivar CNPH 6400 em sistema orgânico, submetida a doses de composto orgânico à base de dejetos sólidos de suínos em Oratórios, Zona da Mata de Minas Gerais, de maio a outubro de 2005; concluíram que é possível cultivar cebola com boas características produtivas e nutricionais em sistema orgânico. Em

contrapartida, Rodrigues et al. (2006a), avaliando 16 genótipos de cebola no município de Viçosa, Zona da Mata, Minas Gerais, nos sistemas orgânico e convencional, concluíram que as cebolas obtidas no sistema convencional apresentaram maior peso de bulbos mais adequados à indústria, quando comparados aos bulbos obtidos no sistema orgânico.

Em termos de sanidade da cultura, uma das principais doenças da cebola em regiões de clima tropical e subtropical é a mancha-púrpura ou crestamento, causada pelo fungo *Alternaria porri*, capaz de gerar perdas de 50-100% em plantações com cultivares suscetíveis e condições ambientais favoráveis. A umidade e temperatura são as condições ambientais mais importantes para o surgimento da doença, pois o fungo é dependente de água para a germinação do esporo e para a esporulação na superfície da planta, sendo a temperatura ideal para seu desenvolvimento situada entre 21 e 30 ºC, sendo que o fungo pode crescer entre 6 e 34ºC (REIS & HENZ, 2009).

2.4. Produção orgânica de tomate

Caracterizada como uma cultura bastante responsiva à adubação mineral e suscetível a pragas e doenças; a tomaticultura convencional tem figurado dentre as culturas de maior demanda por fertilizantes e agrotóxicos (ANVISA, 2002). O uso indiscriminado de insumos na tomaticultura convencional contribui para elevação dos custos de produção e traz em pauta o questionamento a cerca da qualidade e dos riscos associados ao consumo *in natura* do tomate.

Estudos realizados por Vargas et al. (2004), no Rio de Janeiro, a cerca da caracterização agronômica de genótipos de tomateiro "Heirloom", cultivados sob manejo orgânico, constataram boa sanidade das plantas, principalmente em relação a doenças de folhagens. A produtividade variou de 0,7 a 2,7 kg.planta^{-1}, nos genótipos de frutos grandes e de 0,5 a 1,9 kg.planta^{-1} nos genótipos do tipo cereja. Os frutos obtidos apresentaram bons resultados tanto em termos quantitativos como qualitativos, pois além da produtividade, 80% dos frutos possuíam padrão comercial. Segundo estes autores, tais resultados indicaram boa adaptabilidade dos genótipos para produção orgânica, bem como, boa aceitação pelos consumidores do Rio de Janeiro. Avaliando as características nutricionais da cultivar Carmem, produzida sob manejo orgânico e convencional, Borguini (2002) concluiu que os frutos produzidos organicamente apresentaram um teor mais elevado de vitamina C e licopeno, comparado àqueles frutos obtidos no sistema convencional.

O tomate ocupa lugar de destaque na mesa do consumidor, que a cada dia tem despertado para o desejo de obter alimentos produzidos de forma a valorizar a diversidade biológica, livre de agressões ao meio ambiente e sobretudo isento de resíduos nocivos à saúde. Neste sentido, tem-se a eminência de um nicho de mercado e uma tendência que favorece a criação de novas oportunidades, como emprego e renda aos produtores, em especial da agricultura familiar.

Assim, diante dos pressupostos já citados, no que tange ao potencial econômico, às limitações técnicas de cultivo, bem como às exigências mercadológicas envolvidas na produção do tomate, tem-se no sistema de cultivo orgânico uma eficiente alternativa para obtenção de alimentos saudáveis, dentro de um sistema sustentável e economicamente viável (LUZ et. al., 2007). Para minimizar o entrave fitossanitário, o emprego de coberturas, se aplicaria como uma medida física de controle preventivo ao ataque de pragas e doenças, o que possivelmente representaria um incremento no investimento inicial da lavoura, mas que poderia ser diluído ao longo do tempo de cultivo e, recompensado com a minimização do uso de agrotóxicos e um maior preço do produto orgânico.

O tomateiro é classificado como uma cultura cosmopolita, pela tolerância às variações climáticas, conduzido em regiões de clima tropical, subtropical e temperado (FILGUEIRA, 2008). Estudos a cerca da viabilidade técnica e financeira de diferentes condições de cultivo e manejo, se mostram necessários de modo a elucidar a melhor alternativa produtiva, econômica, social e ambiental, dentro dos pilares gerais de um sistema sustentável. Nesse sentido, a identificação de substâncias que levem a resistência a patógenos vai ao encontro das demandas da agricultura orgânica para a cultura.

2.5. Quitosana como fator de indução a resistência

A quitosana é a forma desacetilada da quitina, um biopolímero que ocorre naturalmente como um componente das paredes celulares dos fungos, insetos-esqueletos e conchas de crustáceos. A caracterização e aplicação da quitosana estão em andamento há décadas, levando ao uso mundial da quitosana em muitos setores, incluindo agricultura, indústria e medicina (PICHYANGKURA & CHADCHAWAN, 2015).

A quitosana tem sido extensivamente estudada como um meio de inibir o crescimento microbiano e diminuir a integridade da membrana microbiana (XU et al., 2007; PALMA-GUERRERO et al., 2008), reduzindo a incidência e severidade da doença em muitas culturas (ABD-ALLA & HAGGAG, 2010; ALI et al., 2010, 2012, 2013, 2014, 2015; BAUTISTA-BANÕS et al., 2003; BELL et al., 1998; BENHAMOU & THÉRIAULT, 1992; BHASKARA-REDDY et al., 1999; LI et al., 2009; MAQBOOL et al., 2010; PRAPAGDEE et al., 2007).

A quitosana, obtida a partir da desacetilação alcalina da quitina, é um polissacarídeo funcional com grande potencial para aplicações em alimentos e requisitos de embalagem. É o polissacarídeo mais investigado para o desenvolvimento de filmes e revestimentos antimicrobianos comestíveis, devido às suas propriedades antimicrobianas e antifúngicas e capacidade de formação de filme (FERNÁNDEZ-PAN et al., 2015).

A quitosana é uma base fraca e insolúvel em água e em solventes orgânicos. No entanto, é solúvel em soluções ácidas diluídas e aquosas (pH <6,5), que podem converter as unidades de glucosamina na forma solúvel R-NH^{3+} (KUMAR et al., 2004). No entanto, a quitosana mostra atividade antibacteriana apenas em um meio ácido, o que geralmente é atribuído à baixa solubilidade de quitosana em pH alto (QIN et al., 2006).

Os mecanismos de ação antimicrobiana da quitosana não estão bem elucidados, mas várias hipóteses têm sido apresentadas. Esses mecanismos dependem muito da espécie mas de maneira geral a hipótese que apresenta maior viabilidade está relacionada à indução de resistência. Nesse processo a quitosana age ligando-se a receptores presentes na membrana celular das plantas, mimetizando o fenômeno de reconhecimento que ocorre em uma interação incompatível entre a planta e o patógeno (LABANCA, 2002). A quitosana pode inibir a proteinases, alterar o metabolismo das fitoalexinas, promover a lignificação (TERRY & JOYCE, 2004), induzir a formação de compostos fenólicos (BAUTISTA-BAÑOS et al., 2006), ativar as enzimas quitinases e β-1,3-glucanases (EL GHAOUTH et al.,1992; ZHANG & QUANTICK, 1998), fenilalanina amônia-liase (ROMANAZZI et al., 2002) e peroxidase (ZHANG & QUANTICK, 1997). Pode atuar ainda na inibição de enzimas de desestruturação da parede celular sintetizada por fungos, como a poligalacturonase, pectinaliase e celulase, e nos compostos tais como ácidos orgânicos (oxálico e fumárico) e toxinas específicas, como alternariol (BHASKARA-REDDY et al., 1998).

Outra hipótese é que as moléculas de quitosana de anti-carga positiva interferem com os resíduos carregados negativamente na superfície bacteriana fazendo com que a quitosana interaja com a membrana das bactérias para alterar a permeabilidade das células (SEVERINO et al., 2015). Outro mecanismo proposto é a interação de produtos de hidrólise difusa com DNA microbiano, o que leva à inibição do mRNA e da síntese de proteínas (YUAN, et al., 2016). Quitosana também inibe o crescimento microbiano pela quelação de nutrientes e metais essenciais (YUAN, et al., 2016, CHIEN et al., 2015), além disso quitosana na superfície da célula pode formar uma membrana polimérica que impede nutrientes de entrar na célula (EL-TAHLAWY, et al., 2005)ou atua como uma barreira ao oxigênio que pode inibir o crescimento de bactérias aeróbicas (DEVLIEGHERE, et al., 2004).

Na olericultura, a quitosana vem sendo largamente estudada, como em alface minimamente processada, aonde prolongou a vida pós-colheita, estendendo-se por quatro dias o período de armazenamento (DEVLIEGHERE et al., 2004). Em pepinos houve controle da podridão de raízes causada por *Pythium aphanidermatum*, indução de respostas de defesas, incluindo a indução de barreiras estruturais nos tecidos das raízes e estímulo das hidrolases (quitinases e β-1-3-glucanases) tanto nas raízes como nas folhas, além de não ter causado fitotoxidade na planta (EL GHAOUTH et al., 1997). Em pimenta, o uso de quitosana sobre *Botrytis cinerea* causou severos

danos citológicos a hifa, inibindo a habilidade do patógeno de colonizar os tecidos (EL GHAOUTH et al., 1997).

2.6. O cobre

A utilização do cobre é vastamente difundida na agricultura, como nutriente essencial, mas também pelo efeito fungicida e bactericida que possui alta eficiência no controle de muitas doenças. Os micronutrientes são usados em pequenas quantidades, as plantas em condições normais tem 100ppm de cobre e 20ppm quando apresentam deficiência (VIJAY et al., 2017). O efeito desse micronutriente sobre o patógeno pode ser direto (GRAHAM & WEBB, 1991), haja vista que a quantidade de cobre requerida pelos microrganismos é inferior à da planta (10 a 20 ppm), além de serem pouco tolerantes ao excesso desse elemento.

Os fungicidas à base de cobre são ferramentas essenciais na prevenção de doenças e tratamento em uma grande variedade de plantas (BORKOW & GABBAY, 2005). Contudo, o uso indiscriminado de fungicidas cúpricos pode ter efeitos nocivos à alimentação humana e animal, além de outros danos ambientais.

A mistura de sulfato de cobre e hidróxido de cálcio denominada "calda bordalesa" tem sido utilizada com sucesso em vinhedos desde seu primeiro uso em 1885 contra o míldio, uma doença oomiceta afetando uma grande variedade de plantas, incluindo uvas (MILLARDET, 1885). Embora a aplicação da mistura de sulfato de cobre e hidróxido de cálcio seja permitida em sistemas de cultivos orgânicos, os compostos a base de cobre podem ser acumulados no solo e não devem exceder aos limites estabelecidos para metais pesados (BRUNEL et al., 2013).

O excesso de metais pesados como cobre nas células vegetais pode causar uma variedade de alterações tóxicas, por exemplo, a geração de oxigênio reativo (ROS), resultando em estresse oxidativo (RUCIN´SKA-SOBKOWIAK & PUKACKI, 2006; PRZYMUSIN´SKI et al., 2007), o que leva à destruição das membranas celulares: a membrana plasmática, sistema endomembranar e organelas membranosas, como cloroplastos e mitocôndria (HALL, 2002; PATRA et al., 2004) e núcleo (PRASAD, 2004). Além disso, eles podem destruir a conformação de ácido nucleico (PAWLAK & DECKERT, 2007), alterar o balanço hídrico e perturbar muitas outras funções das células vegetais (HALL, 2002; PATRA et al., 2004). Todas essas alterações podem levar à inibição do crescimento das plantas, necrose e, finalmente, à morte de toda a planta (PRASAD, 2004). Vale ressaltar, no entanto, que em uma dose baixa o estresse pode causar um efeito hormonal, ou seja, melhor funcionamento dos organismos vegetais (SCHWARZEROVA´ et al., 2002).

Uma aplicação de doses mínimas que causem efeitos no metabolismo secundário vegetal e responda positivamente ao controle de doenças é desejável. Portanto, muitos compostos e complexos de cobre foram desenvolvidos para melhorar a eficácia dos produtos à base de cobre,

reduzindo concomitantemente a quantidade de cobre necessária para controle da doença (BENNS et al., 1960; IVANOV et al., 1989).

Alguns estudos mostram o efeito antimicrobiano de quitosana associada ao cobre no controle de *Alternaria alternata*, *Macrophomina phaseolina*, *Rhizoctonia solani* (SAHARAN et al 2013) e *Fusarium graminearum* (BRUNEL et al, 2013). Esses trabalhos revelaram efeitos diretos somente sobre os patógenos, porém não aprofundaram os efeitos dos produtos como indutores de resistência à doenças em plantas.

O cobre possui efeitos sobre o metabolismo secundário por atuar no controle do estado redox, protegendo contra o estresse oxidativo, além de proporcionar mudanças na estrutura e no formato da parede celular, fazer parte e atuar como co-fator de importantes enzimas relacionadas com atuação de defesa vegetal como a fenilalanina amônia-liase que por sua vez pode estimular o efeito na atividade de peroxidases, super óxido-desmutase, entre outras (PRINTZ et al., 2016).

As vias de ação do cobre em plantas também estão relacionadas aos mecanismos de defesa e qualidade, pois é um nutriente chave, fornecendo alterações na estrutura e no formato da parede celular, participação na síntese de lignina que é uma barreira parcial à penetração de patógenos nos tecidos das plantas (GRAHAM & WEBB, 1991).

O cobre influencia a atividade da peroxidase e da catalase, a qual é reduzida sob alto teor de cobre, fato que resulta no acúmulo de peróxidos, substância altamente bactericida, que se forma a partir do estímulo provocado pelo aumento da respiração de tecidos infectados (ZAMBOLIM & VENTURA, 1996).

A toxicidade do cobre tem sido amplamente atribuída às suas propriedades redox. Pode catalisar a produção de radicais hidroxila altamente reativos que podem subsequentemente danificar lipídios, proteínas, DNA e outros biomoléculas (BORKOW & GABBAY, 2005). Portanto, é possível pequenas quantidades de quitosana associada ao cobre podem causar interrupção na integridade da membrana, permitindo que mais cobre obtenha através da membrana fúngica causando danos extensos dentro a célula (BRUNEL et al., 2013). Portanto a associação de cobre e quitosana pode também ser considerada uma estratégia muito promissora para o controle do crescimento de microorganismos.

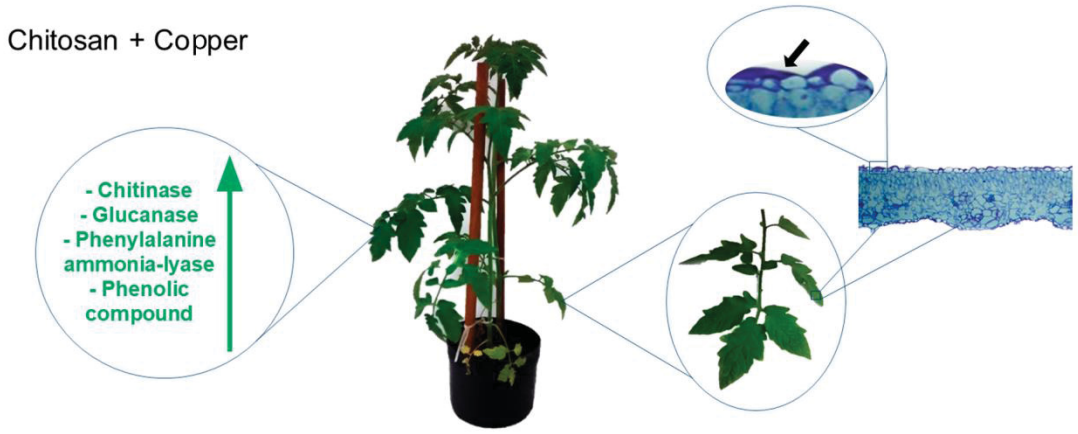
3. CAPÍTULO I - CHITOSAN ASSOCIATED WITH QUELATED COPPER APPLIED ON TOMATOES: ENZYMATIC AND ANATOMICAL CHANGES RELATED TO PLANT DEFENSE RESPONSES

Abstract

The aim of this work was study the use of an experimental product containig chitosan and quelated cooper (Cu) as a sustainable tool for tomato plants protection, understanding which are the mechanisms involved in resistance promotion and what could be the anatomical changes on tomato leaves related to those defense responses. The experiment was conducted in greenhouse with tomato plants cultivated in pots and subjected to weekly foliar sprays of suspension containig chitosan associated with Cu-EDTA (ChiCu). Increased phenolic compound concentrations and phenylalanine ammonia-lyase (PAL) enzyme activities were detected already 24 hours after. In addition, a increase was found in the activity of chitinase and β-1,3-glucanase, enzymes related to defenses responses. There was an increase in the thickness of the external periclinal wall on of the adaxial epidermal surface in leaves submitted to 4 ml L^{-1} ChiCu. The proportion of the epiderm to the leaf blade was also higher. Although, pathogenicity-related enzymes were activated as well as phenolic compounds had increased, these changes did not affected the biometric and biochemical parameters related to the primary metabolism and essential for the maintenance of productive potential of tomato plants. The increase in the external periclinal wall as a result of ChiCu sprays could contribute to the resistance against diseases since it is the first barrier to the infection of a wide of pathogens.

Keywords : *Solanum lycopersicum* L., resistance induction, enzymes, nutrient, powdery mildew.

GRAPHICAL ABSTRACT



Graphical abstract - Tomato plant submitted to chitosan applications with copper. The arrow indicates cuticle thickness.

1. Introduction

The demand for the use of natural inducers of disease resistance in plants has increased in order to produce healthier foods. Chitosan is a product of natural origin found in the exoskeleton of crustaceans and has become a sustainable alternative for preventing fungal diseases inducing plant defense, being biodegradable, biocompatible and not presenting toxicity to humans (XING et al., 2015). More than 20 species of vegetables have already been studied under the effect of chitosan (PICHYANGKURA & CHADCHAWAN, 2015).

The mechanisms of defence triggered by chitosan have not been fully elucidated yet and depend on characteristics such as its degree of deacetylation and applied concentration. Its also depends on the crop and their stage of development (REGE et al., 2003). Some mechanisms of action have already been reported such as activation of pathogenicity-related enzymes, formation of phytoalexins and lignification (TERRY & JOYCE, 2004).

Some studies show the effect of chitosan associated with Cu based fungicides in some plant pathogens such as *Alternaria alternata, Macrophomina phaseolina, Rhizoctonia solanie* (SAHARAN et al., 2013) *and Fusarium graminearum* (BRUNEL et al., 2013). These studies

focussed on direct effects on pathogens, but did not characterise the effect of Cu as a nutrient related to plant immune responses and chitosan on plant resistance mechanisms.

Cu is used in farming as a nutrient, but widely also as fungicide and bactericide in controlling of diseases (KRISTL et al., 2019). The indiscriminate use of Cu fungicides may have harmful effects on human and animal diets, as well as other environmental damages (MAHER, 2018). Although the application of a mixture of Cu-sulfate and calcium (Ca) hydroxide, better known as "Bordeaux mixture" is allowed in organic cropping systems, the Cu-based compounds can be accumulated in the soil (BRUNEL et al., 2013). In this sense, an application of minimum doses of Cu as a nutrient, that cause effects on plants defense responses is desirable.

One factor that can affect leaf absorption of nutrients is the characteristic of the solution to be sprayed. Chelatization has as its basic function to protect cationic nutrients (such as Cu^{2+}) so that they are less subject to precipitation or insolubilization reactions and thus maintain their availability to plants and can be absorbed efficiently. Among the most commonly used chelates is EDTA (Ethylene Diamino Tetracetic Acid) (CAO et al., 2019).

As a nutrient, Cu is a key for plant defense, by providing changes in structure and shape of the cell walls and participating in enzymes such as PAL (PRINTZ et al., 2016). Other effect of Cu on resistance to infection is due to its participation in lignin synthesis, a partial barrier to penetration into plant tissues (GRAHAM & WEBB, 1991).

Molecules described as elicitors such as chitosan, promote the induction of disease resistance soon after pant cell recognition, usually by activating signal transduction pathways that generate the formation of reactive oxygen species and biosynthesis of phytoalexins, reinforcement in the plant cell wall associated with the compounds of the route of the phenylpropanoids, callose deposition, synthesis of enzymes and accumulation of proteins related to the pathogenicity (THAKUR & SOHAL, 2013).

Chitosan and the nutrient Cu have several paths of action in common in the plant defense metabolism, thus the objective of this work was to verify the effect of foliar sprays of chitosan

associated with EDTA-quelated Cu in tomato plants in order to answer the following questions: Does the application of chitosan associated with the nutrient Cu induce tomato resistance to phytopathogens? What are the pathways that promote this resistance? Would anatomical changes occur?

2. Materials and methods

2.1 Experimental conditions

Experiments were done in 2017 and 2018 at the Canguiri Experimental Station (25º 23' S and 49º 07' W, Köeppen temperate type climate - Cfb) of Federal University of Parana, Pinhais, Paraná State, Brazil.

Trials were conducted in greenhouse using 5-liter pots, filled with commercial substrate (Provaso®) and soil at ratio 1:1. The chemical analysis of mixture showed values adequate to growing tomato: pH H_2O= 6,5; Al^{+3}= 0; $H^{+}Al^{+3}$= 2,1 $cmol_c\ dm^{-3}$; Ca^{2+}= 6,70 $cmol_c\ dm^{-3}$; Mg^{2+}= 3,8 $cmol_c\ dm^{-3}$; K^{+} = 0,40 $cmol_c\ dm^{-3}$; P (Mehlich)= 8,0 mg dm^{-3} ; S= 5,8 mg dm^{-3} ; C= 32 $g.dm^{-3}$; organic matter = 5,5%; base saturation= 83,85, CEC= 13 cmolc dm^{-3}. The irrigation was performed using drip tape by keeping the moisture of the pots near to the field capacity using tensiometer. The tomato cultivar used was the Compack hybrid (Seminis®), which presents indeterminate growth. Sowing was done in trays and keept in nursery by 30 days. After that, one seedling per pot was transplanted.

The chitosan solution on acetic acid with 1,5 % (w/v) presented deacetylation degree of 93.2% calculated by potentiometric titration method, carried out in the Biopolymers Laboratory of Chemistry Department at the Federal University of Paraná, Brazil. The Cu source was obtained by quelation reaction of copper sulfate ($CuSO_4$) with EDTA ($C_{10}H_{16}N_2O_8$) (Cu-EDTA 5% w/v), supplied by Biocross of Brazil Fertilizers®.

2.2. Experiments

For all experiments, eight foliar applications were carried out weekly with a pressurized sprayer at constant pressure (40 psi), with application volume varying from 20 to 66 mL $plant^{-1}$, increasing

according plant growth. At all experiments controls were sprayed with distilled water.

2.2.1. Experiment 1

The first experiment consisted of doses: chitosan 1; 2; 3 and 4 ml L^{-1} (respectively 15, 30, 45 and 60 ppm), Cu-EDTA 1; 2; 3 and 4 ml L^{-1} (respectively 50, 100, 150 and 200 ppm) and chitosan associated with Cu-EDTA 1; 2; 3; 4 ml L^{-1} (respectively: chitosan 15 ppm associated with 50 ppm Cu+EDTA, chitosan 30 ppm associated with 100 ppm Cu-EDTA, chitosan 45 ppm associated with 150 ppm Cu-EDTA and chitosan 60 ppm associated with 200 ppm Cu-EDTA), totaling 13 treatments and 4 replicates with a completely randomized design. This experiment analyzed the content of phenolic compounds and activity of the enzymes PAL, chitinase, and β-1,3-glucanase.

2.2.2. Experiment 2

Based on the results obtained in the first experiment, the treatments chitosan 2ml L^{-1} and 4 ml L^{-1} (respectively chitosan 45 and 60 ppm); Cu-EDTA 2ml L^{-1} and 4 ml L^{-1} (respectively 150 and 200 ppm) and the doses of the combination of chitosan plus Cu- EDTA 2ml L^{-1} and 4 ml L^{-1} (respectively chitosan 45 ppm associated with 150 ppm Cu-EDTA and chitosan 60 ppm associated with Cu-EDTA 200 ppm) were selected, composing 7 treatments with 4 replicates. The experiment was repeated twice and the results were the mean of both experiments.

The content of phenolic compounds, total sugar, proteins, amino acids and the activity of the enzymes PAL, chitinase, β-1,3-glucanase were analyzed. The content of phenolic compounds and the activity of the enzyme PAL were evaluated 0; 24; 48; 96 and 192 hours after the last foliar application. The biometric parameters of height, leaf area, fresh and dry mass were determined. Leaf segments of chitosan and chitosan plus Cu treatments were collected for micromorphometric anatomical analysis.

2.2.3. Experiment 3

A third experiment was carried out using doses chitosan 2ml L^{-1} and 4ml L^{-1} (respectively chitosan 45 and 60 ppm) and the dose of chitosan plus Cu-EDTA 2ml L^{-1} and 4 ml L^{-1} (respectively chitosan 45 ppm associated with 150 ppm Cu-EDTA and chitosan 60 ppm associated

with Cu-EDTA 200 ppm), totaling 5 treatments and 4 replicates. In this experiment, natural infection of powdery mildew (*Leveillula taurica*) occurred, since tomato plants were kept in a greenhouse with high inoculum and none extra inoculum was necessary. Greenhouse conditions are ideal for spread of conidia; air circulation is good enough to disseminate spores but not enough to keep humid air from accumulating around plant surfaces (LINDE & SHISHKOFF, 2017). Several authors report evaluating the severity of powdery mildew from natural inoculation (BOYD et al., 2013; YANAR et al., 2011; LAGE et al., 2019; IBRAHIM et al., 2019). Disease severity was evaluated 8 days after the last application of the treatments. In order to prove that the infestation was homogeneous, 3 leaves from the middle third (between nodes 4 to 8 from the base to the apex) of three plants per repetition were evaluated, four replicates per treatment distributed at random. The content of phenolic compounds and the activity of PAL, chitinase, β-1, 3-glucanase enzymes were analyzed.

2.3. Biochemical and enzymatic analyzes

Biochemical analyses were performed for total proteins, phenolic compounds and PAL, chitinase and β-1,3-glucanase at the Biochemistry and Phytosanitary Laboratory at Federal Technological University of Parana (UTFPR), Dois Vizinhos City, Parana State, Brazil. The analyses of total free amino acids and total sugars were carried out in the Biofertilizers Laboratory at UFPR, Parana, Brazil. For all analyzes, samples of 0.3 g of plant material were used.

Total protein analysis was performed using the Bradford method (BRADFORD, 1976). The quantification of total phenols used the method adapted from Bieleski and Turner (1966). The quantification of the activity of the enzyme PAL was performed according to Rodrigues (2006b). For the enzymes chitinase and β-1,3-glucanase, the methodology described by Wirth and Wolf (1992) and with the procedure described by Guzzo and Martins (1996).

For the analysis of total free amino acids, the amino acids were extracted according to Winters et al., (2002) and the colorimetric reaction with the reading of the absorbance was made at

570 nm. The standard curve was performed with 2 mM glutamine with values ranging from 28 and 140 μg mL^{-1}.

The total sugars were quantified according to Maldonade et al., (2013) preceded by acidic hydrolysis of the sample using 3,5-dinitro salicylic acid (DNS).The standard curve for reducing and total sugars was made with glucose at 1 mg mL^{-1} (5.5 mM) with values between 50 and 800 μg mL^{-1}.

2.4. Leaf anatomy

For the micromorphometric analysis, the leaf samples were collected from two plants per repetition of the third experiment, eight days after the last application, 60 days after transplanting. Fragments of 0.5 cm^2 were sectioned from the medial region of the main leaflet of leaves at the middle third of the plant. The samples were fixed in Karnovsky solution (KARNOVSKY, 1965) and, after 24 hours, dehydrated in ethyl series and included in methacrylate (Historesina, Leica Instruments). Cross sections (8 micrometers thick) were stained with 0.1% toluidine blue in 5% borax and the blades were assembled in 500® stained glass varnish for analysis and photographic recording (PAIVA et al., 2006). The thickness of the external periclinal wall of the adaxial and abaxial surfaces of the epidermis, the palisade and spongy parenchyma were measured as well as the total thickness of the limbus. The thickness data (μm) were obtained with the aid of ANATI QUANTI software (AGUIAR et al., 2007), making a total of 100 observations for each structure evaluated per treatment. The images were digitalized in a photomicroscope (Zeiss Axiolab model) with a coupled digital camera (Sony Cybershot 7.2 ned mb model) at the Laboratory of Anatomy and Plant Biomechanics of the Botany Department of the Federal University of Paraná, Brazil.

2.5. Biometric analysis

Fresh mass, dry mass, height, and leaf area were measured 8 days after the last application, 60 days after transplanting. The height was measured in 3 plants per repetition and the aerial part of the plants was collected, which after weighing on a precision scale, they were dried in an oven at 65ºC using forced ventilation until constant weight and then the dry mass was determined. For the

determination of the leaf area, all leaves of two plants were collected per replicate. Leaf area was estimated using Winrhizo Pro® software version 2013, coupled to an Epson® dual-lens scanner (model V700 PHOTO), and performed at Biofertilizers Lab. of UFPR.

2.6. Powdery Mildew (*Leveillula taurica*) Severity

Disease severity was determined from a diagrammatic scale 8 days after the last application of the treatments (COSTA-LAGE et al., 2015). Three definitive leaves located in the middle third of three plants per repetition were evaluated to determine the severity of the disease.

2.7. Data analysis

The data were tested for their homogeneity of variances by Bartlett test and then submitted to ANOVA and to regression analysis. The concentrations related to the maximum efficiency of the assessed variables when of quadratic distributions were determined by the first derivative of the regression equations, equaled to zero. The means were compared by the Scott-Knott test at the 5% level. Statistical analyses were performed in Assistat® 7.7 Beta software (SILVA & AZEVEDO, 2016).

3. Results

3.1. Experiment 1

The plants showed no visible symptoms of diseases, indicating that the achievements were an effect induced by the applied products and not by pathogens.

The content of phenolic compounds at the chitosan plus Cu treatments (ChiCu) presented a linear growth to the 4 ml L^{-1}. On the other hand, the chitosan (Chi) treatment showed a polynomial distribution, while the effect of Cu alone (Cu) showed a polynomial response (Figure 1a).

To the activity of PAL enzyme at Chi and ChiCu treatments, it was possible to estimate the maximum efficiency doses in 2.55 ml L^{-1} and 2.41 ml L^{-1} respectively, whereas the increasing doses of Cu alone showed polynomial response again (Figure 1.b). The activity of chitinase showed polynomial distribution at both Chi and Cu alone, while ChiCu maximum efficiency dose was of

2.5 ml L^{-1} to induce the highest enzyme activity (Figure 1.c). The activity of the enzyme β-1,3-glucanase could not be explained by regressions.

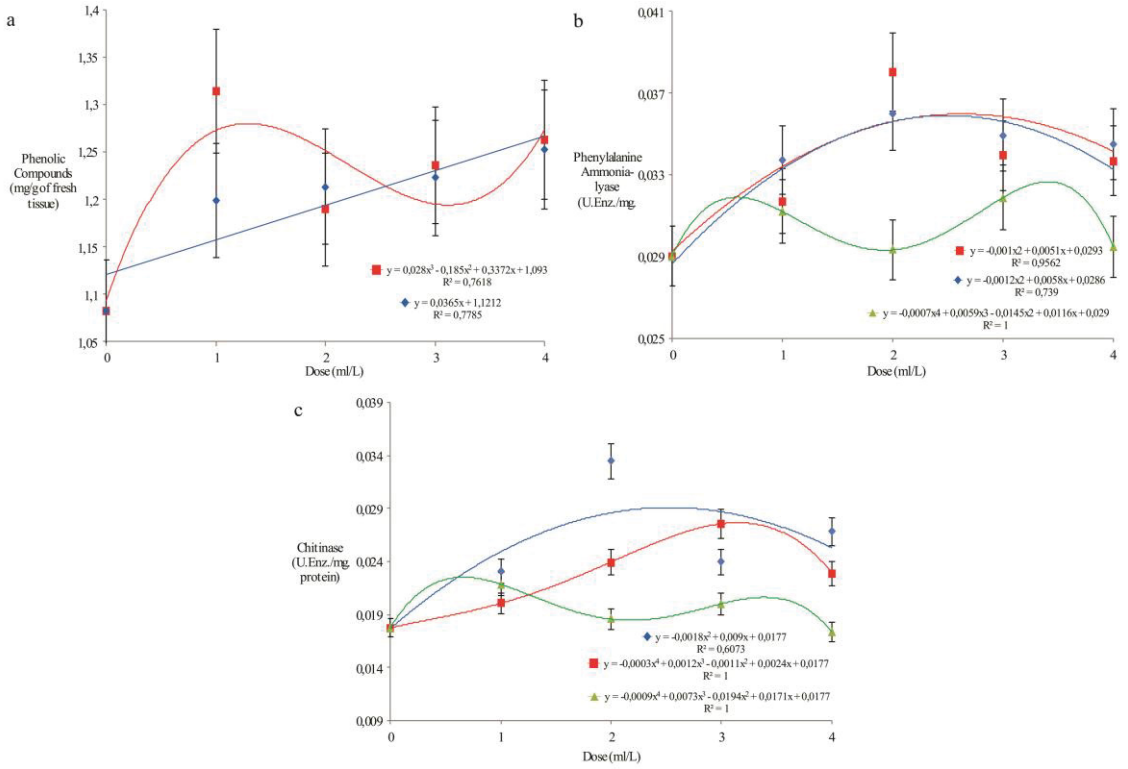


Figure 1 - Changes in the content of phenolic compounds (a), phenylalanine ammonia-lyase activity (b) and chitinase activity (c) in tomato leaves, 63 days after transplantation, submitted to leaf sprays of chitosan (red lines), copper (green lines) and chitosan associated with copper (blue lines).

3.2. Experiment 2

Based on these previous results, a better effect on phenolic compounds and on the activity of PAL and chitinase were obtained among 2 and 4 ml L^{-1} doses, highlighting ChiCu.

A significant increase was found in the contents of phenolic compounds at doses of 2 and 4 ml L^{-1} for all treatments if comparing to control (Figure 2.a).

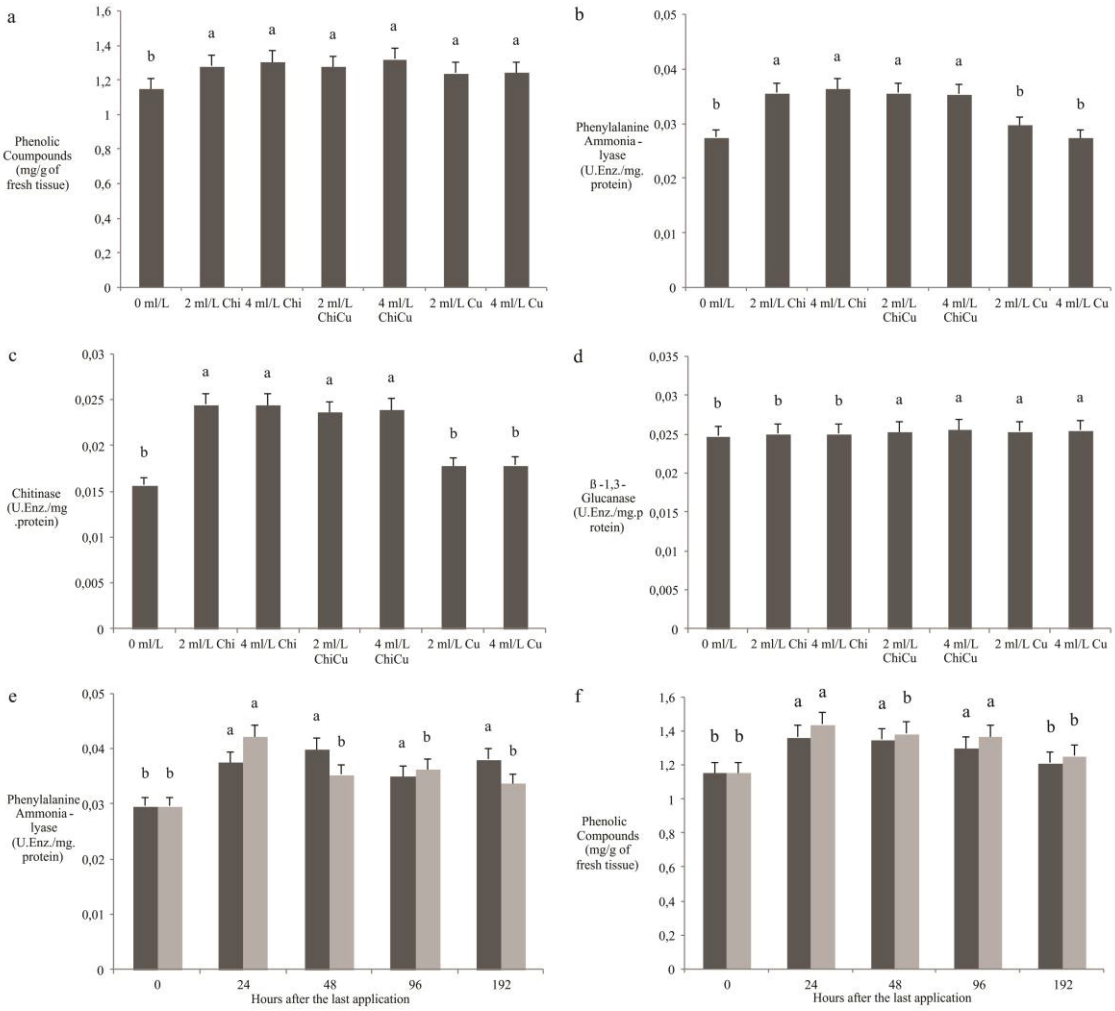


Figure 2 - Effect of foliar application of chitosan, chitosan associated with copper and copper on phenolic compounds (a) and phenylalanine ammonia-lyase (b) enzymes, chitinase (c) and β-1,3-glucanase (d) on tomato leaves 63 days after transplanting. Columns with the same letter do not differ statistically ($p < 0.01$) according to Scott-Knott test (n=3). Effect of foliar application of chitosan associated with copper on time after application on phenylalanine ammonia-lyase enzyme (e) and phenolic compounds (f) (dark gray column = 2ml.L^{-1} chitosan associated with copper and light gray column = 4ml.L^{-1} chitosan associated with copper. Chi = Chitosan, ChiCu= Chitosan associated with Copper and Cu= Copper. Bars represent standard error. The ANOVA analysis indicated in: (a) Time = **, Treatments = ** and Interactions = ns, (b) Time = **, Treatments = ** and Interactions = *, (c) Time = **, Treatments=** and Interactions=** and (d) Time=**, Treatments=** and Interactions = ns, (e) Time = **, Treatments = ** and Interactions = ns, (f) Time = **, Treatments = ** and Interactions = * where ns=not significant, * and ** = significant at p=0.05 and p=0.01, respectively.

The enzyme PAL and chitinase showed highest activity at Chi and ChiCu. The Cu alone has not affected the activity of these enzymes in relation to the control, thus, indicating that chitosan was an essential factor for the increase in the activity of these enzymes (Figure 2.b and c). The enzyme β-1,3-glucanase showed higher activity at ChiCu and Cu alone, indicating that Cu as a nutrient had a direct effect on the increase in the activity of this enzyme, but no synergistic effect was noted with the association to chitosan (Figure 2.d).

A significant increase was found in the content of phenolic compounds from 24 to 96 hours after the last application of ChiCu at 2 and 4 ml L^{-1} (Figure 2.e). The activity of PAL increased

from 24 to 192 hours after ChiCu 2 ml L^{-1} last application, while 4 ml L^{-1} increased the activity just at 24 hours.

The micromorphometric analysis of the tomato leaves submitted to the treatments showed a greater thickening of the outer periclinal wall of the adaxial side of the epidermis (which includes the cuticle) in the treatments 4 ml L^{-1} of Chi (Figure 3b and Figure 4a) and 4 ml L^{-1} of ChiCu (Figure 3 and Figure 4a).

Proportionally, the epiderm occupied a greater percentage of the limbus with ChiCu 4 ml L^{-1} (Figure 4 b) and, consequently, a lower percentage of mesophyll (Figure 4c).

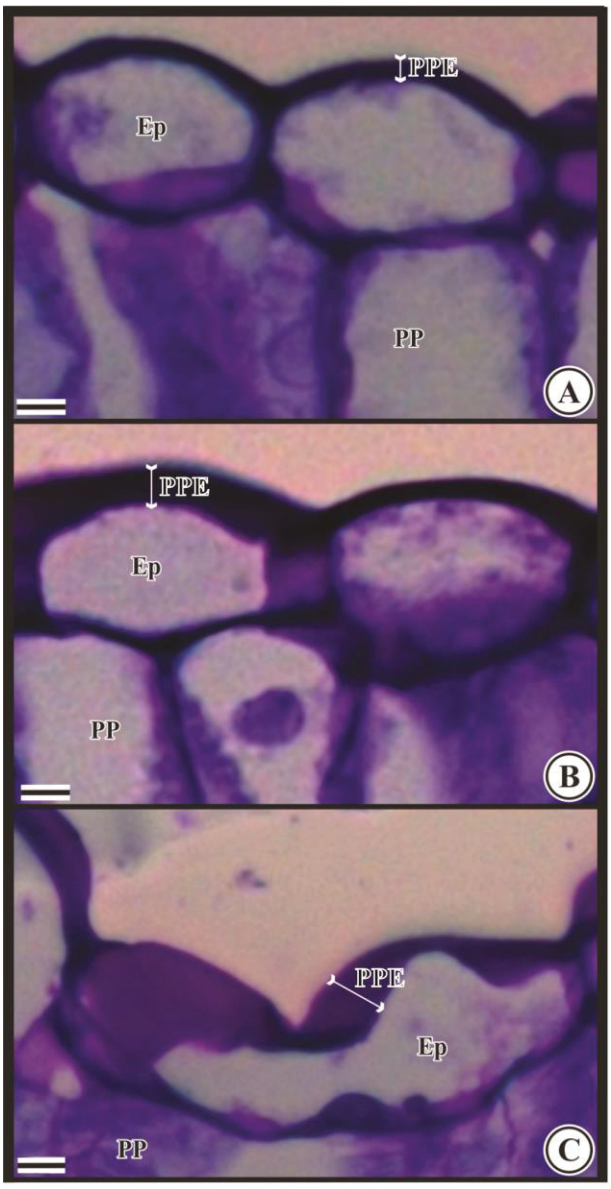


Figure 3- Palisade parenchyma (PP), adaxial face of epidermis (Ep) and external periclinal wall (PPE) of tomato leaves (light microscopy) 63 days after transplanting. Control (a), leaves under foliar application of 4 ml.-1 of chitosan (b) and 4 ml.-1 of chitosan associated with copper (c). Bar= 5 micrometers.

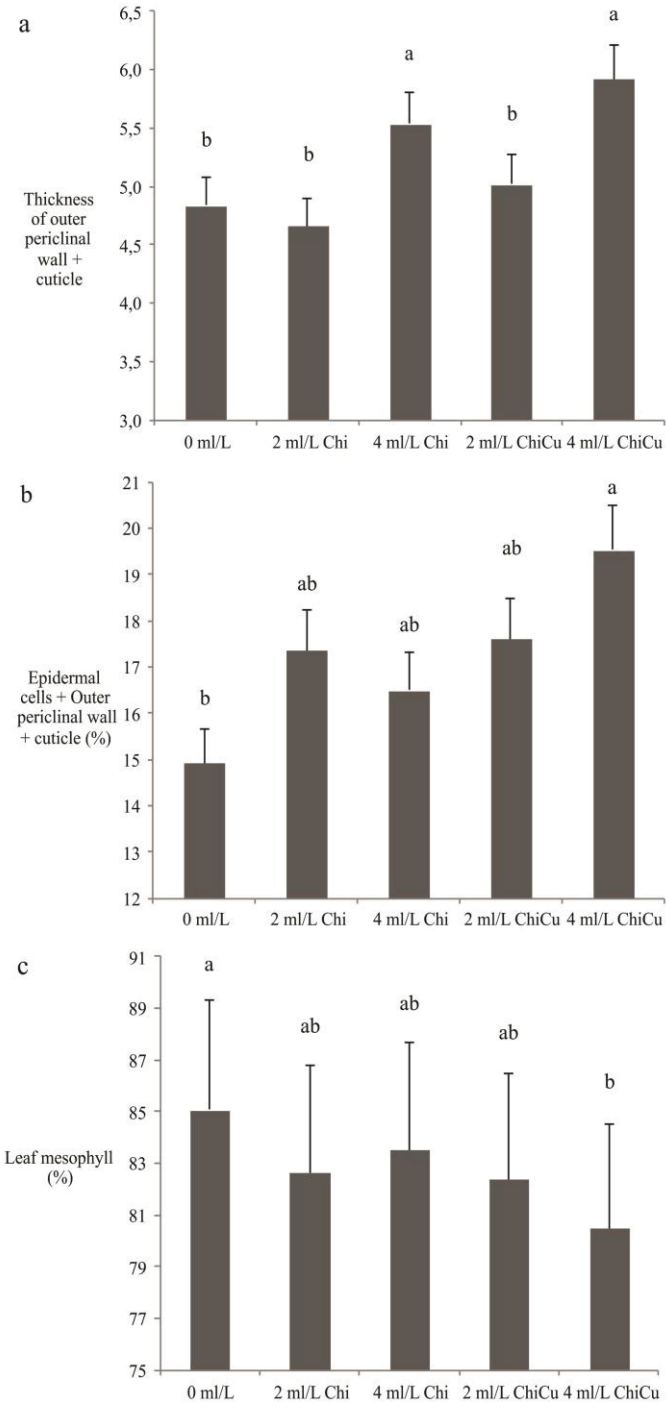


Figure 4 - Effect of foliar application of chitosan, chitosan associated with copper and copper alone on external periclinal wall thickness and adaxial cuticle (a), percentage of epidermal cells, external periclinal wall and cuticle in relation to leaf blade thickness (b) and percentage of leaf mesophyll (Palisade parenchyma and lacunar parenchyma) in relation to leaf blade thickness (c), in leaves of tomato 63 days after transplanting. Chi = Chitosan and ChiCu= Chitosan associated with Copper. The averages followed by the same letter do not differ from each other. The Scott-Knott test was used at $p < 0.05$.

The tomato plants biometric parameters of fresh mass, dry mass, height, and leaf area, as well as the contents of total sugars, proteins, and amino acids were not affected by the weekly sprays of Chi and ChiCu (Figure 5).

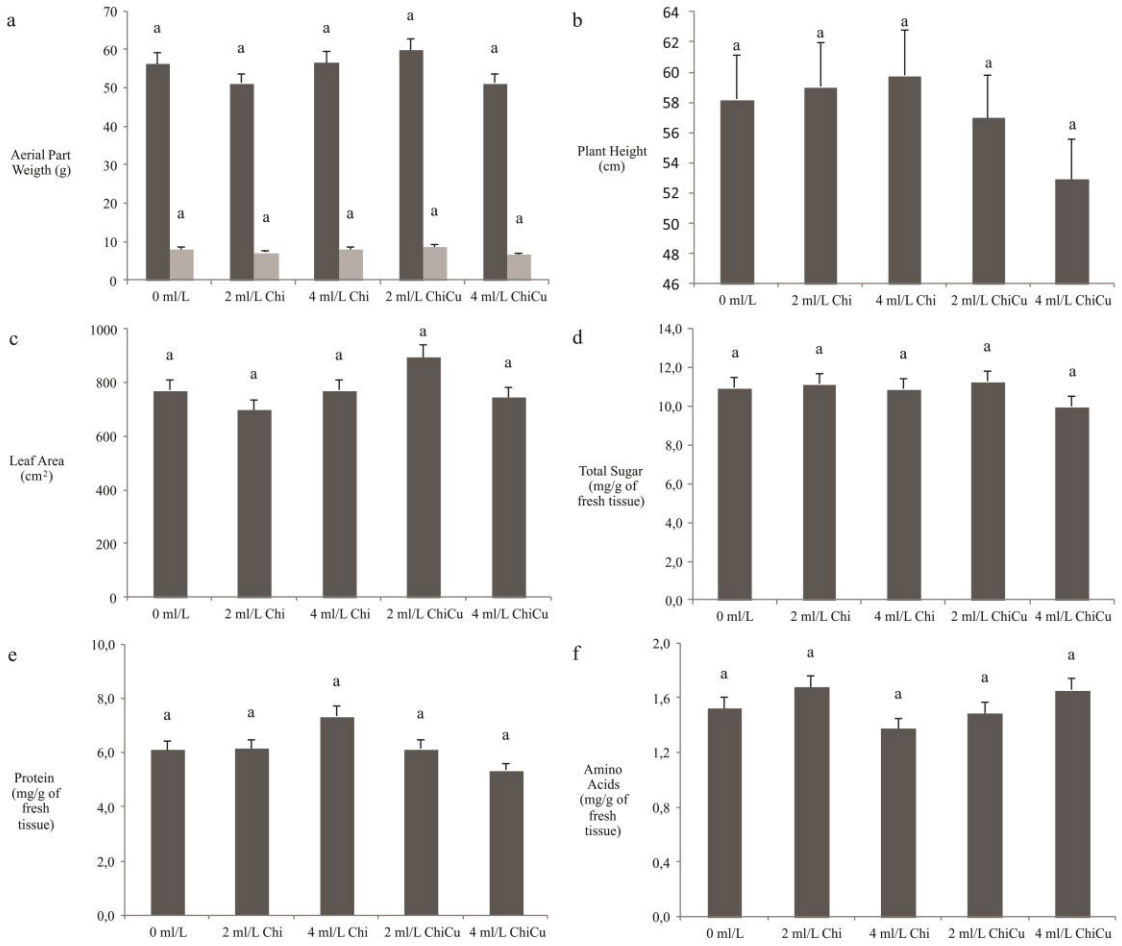


Figure 5- Effect of the weekly foliar application of chitosan and chitosan associated with copper on biometric and biochemical parameters related to the primary metabolism of tomato 48 days after transplanting. a- Dry and fresh mass of the aerial part (dark gray column = fresh matter and light gray column= dry matter); b- Height of plants; c- Leaf area; d- Total sugar; e- Total Proteins; f- Total Amino Acids. Chi = Chitosan and ChiCu= Chitosan associated with Copper. The averages followed by the same letter do not differ from each other. The Scott-Knott test was used at $p < 0.05$. Bars represent standard error.

3.3. Experiment 3

Favorable climatic conditions associated with the environmental characteristics of the protected crop allowed the natural inoculation of powdery mildew (*Leveillula taurica*) in plants that were submitted to treatments of foliar application of 0 (non tratament sprayed with distilled water), 2 and 4 ml L^{-1} of Chi and ChiCu. A reduction was observed in the severity of powdery mildew for all treatments comparing to control, and the doses of ChiCu showed values close to zero, statistically differing from the treatments with Chi alone (Figure 6 a).

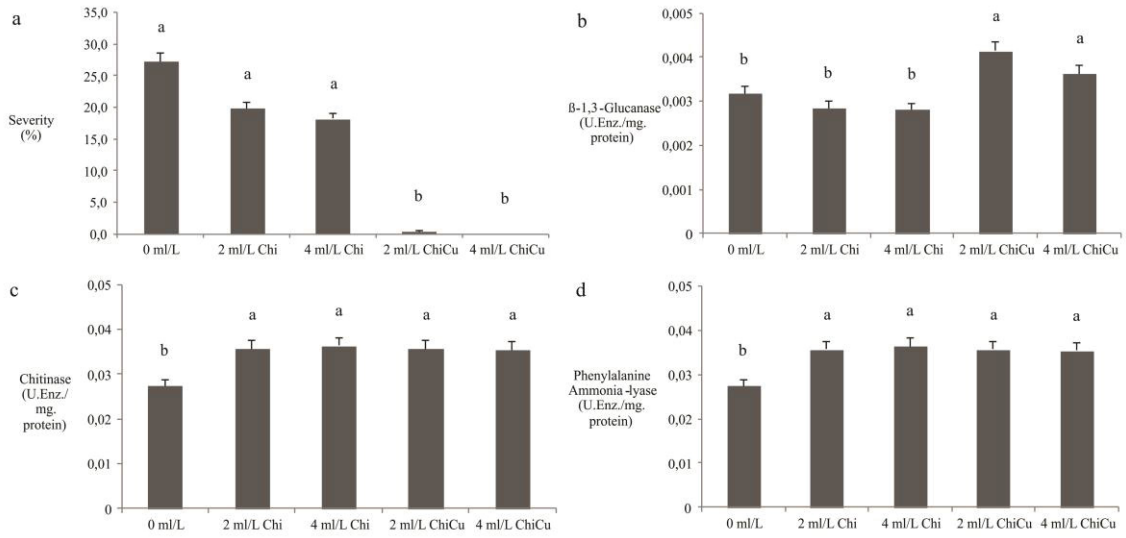


Figure 6- Severity of powdery mildew (*Leveillula taurica*) (a), β-1,3-glucanase enzyme activity (b), chitinase enzyme activity (c) and phenylalanine ammonia-lyase enzyme activity (d) in tomato leaves 48 days after transplantation and submitted to weekly leaf application of chitosan and chitosan with copper. Chi = Chitosan and ChiCu= Chitosan associated with Copper. The averages followed by the same letter do not differ from each other. The Scott-Knott test was applied at the 5% probability level.

The enzyme β-1,3-glucanase had its activity increased by ChiCu in relation to the control and Chi treatments (Figure 6 b). The Chi and ChiCu significantly increased the activity of chitinase (Figure 6 c) and PAL (Figure 6 d) comparing to control.

4. Discussion

The foliar application of chitosan associated with copper, synergistically enabled the activation of pathogenicity-related proteins, increased the content of phenolic compounds promoting changes in the cell wall and cuticle of leaves, as well reducing mildew severity.

When plants are exposed to the resistance-inducing agents, there is an increase in the activity of metabolic pathways involved in the perception of the presence of potential pathogens, which can trigger the synthesis of antimicrobial compounds, such as pathogenicity-related proteins (MACAGNAN et al., 2008) as occurred in this work due to the application of chitosan associated with copper.

Some enzymes have been considered as indicators of the state of induction, among which, the lytic enzymes chitinases and β-1,3-glucanases are mentioned, being generically called PR-proteins (MAZARO et al., 2012). These enzymes have received progressive attention as important components of the arsenal of plant defense proteins, catalyzing the hydrolysis of the main carbohydrates in the fungal cell wall: chitin and β-1,3-glucan (CAMPOS et al., 2009).

The production and accumulation of PR proteins in plants is one of the main plant defense mechanisms (OLIVEIRA et al., 2016). Compounds called resistance inducers generally lead to the production of reactive oxygen species, phytoalexin biosynthesis, reinforcement of plant cell wall associated with phenyl propanoid compounds, deposition of callose, synthesis of defense enzymes, and the accumulation of pathogenesis-related (PR) proteins (VAN-LOON & VAN-STRIEN, 1999; MADHUSUDHAN et al., 2008; ARYAL et al., 2011), as shown in Figures 1, 2 and 6 b.

Chitosan could trigger several defensive responses in plants, such as increasing in glucanase and chitinase enzimes (PICHYANGKURA & CHADCHAWAN, 2015). It also induces many enzymes in the reactive oxygen species scavenging system, such as superoxide dismutase, catalase and peroxidase (FERRARI et al., 2013).

The PR proteins accumulate not only locally in the infected (or on site where there was resistance inductor action) and surrounding tissues but also in remote uninfected tissues. Production of PR proteins in the uninfected parts of plants can prevent from further infection (EBRAHIM et al., 2011). The treatments with Chi and ChiCu allowed simultaneous increases in the enzymatic activity of PAL, chitinase and β-1,3- glucanase. In several studies, the increase in β-1,3-glucanase or chitinase levels was associated with the increase in resistance, therefore suggesting that chitosan-induced resistance to diseases are potentially based on the induction of these enzymes (PICHYANGKURA & CHADCHAWAN, 2015).

In experiment 3 when there was the presence of pathogen, the enzyme glucanase showed higher activity at ChiCu. The lower severity of diseases in the treatments and the greater activity of the enzyme β-1,3-glucanase indicates that this may have been an important factor to reduce powdery mildew severity. The β-1,3-glucanase acts on the glucan bounds of the fungi cell walls causing the death of pathogen (KARAKAYA, 2004).

The changes on contents of phenolic compounds and on activity of PAL over the time vary according to the metabolism of the plant species and the type of elicitor (SINGH & SINGH, 2018),

as shown with the increase in the activity of PAL on tomato plants 24 hours after sprays with ChiCu 4 ml L^{-1} (Figure 2).

Phenolic compounds have their origin in the shikimic acid pathway and are the precursors of the phenylpropanoids by PAL activity, in which the amino acid phenylalanine is deaminated to cinnamic acid, the precursor of lignin, flavonoids and several other compounds related to the plant defense (KARAKAYA, 2004). The application of chitosan as an inducer can elicit responses that mimic an attack on the plant, resulting in accumulation of phenolic compounds as well as the induction of PAL activity. This effect has been reported in several species of plants, including tomato (BADAWYA & RABEA, 2009), as also found in this study as shown in the Figures 1 and 2.

A common response of the several plants to different pathogens includes thickening of cell wall (BARROS et al., 2010). The epidermis wall shows an impregnation formed predominantly by cutin (a polymer made of fatty acid molecules) called cuticle on its external face (EVERT, 2006). The main functions of the cuticle are protection against excessive water loss and protection against damage caused by pathogens (KERSTIENS, 1996). From a chemical viewpoint, the cuticle is formed by an array of compounds with different physico-chemical properties (FERNÁDEZ et al., 2016). These compounds can be waxes, cutin and/or cutan, polysaccharides, phenolics and mineral elements (ESPAÑA et al., 2014; GUZMÁN et al., 2014, GUZMÁN-DELGADO et al., 2016).

The micromorphometric analysis of the tomato leaves submitted to the treatments showed a greater thickening of the outer periclinal wall of the adaxial side of the epidermis (which includes the cuticle) in the treatments 4 ml L^{-1} of Chi (Figure 3b and Figure 4a) and 4 ml L^{-1} of ChiCu (Figure 3 and Figure 4a), and can therefore be an important factor for plant defense against pathogens in tomatoes.

The deposition of lignin is related to the nutrient Cu and seems to increase the resistance of the cell wall to the digestive enzymes of pathogens (BARROS et al., 2010). In studies using *Arabdopsis* it has been described that for the formation of lignin, the polymerization of monolignite

units and these molecules are oxidized in the apoplast by enzymes of the Cu-containing laccase and/or peroxidase family (ZHAO et al., 2013).

The foliar application of Cu can increase the yield of plants and under stress conditions can increase the activity of antioxidant enzymes (HERNÁNDEZ-HERNÁNDEZ et al., 2017). In this work, the foliar application of Cu-EDTA (without chitosan) increased the enzyme activity of β-1,3-glucanase as shown in Figure 2 d.

The results in this work point to a greater formation not only of epiderm but also cell wall (which includes the cuticle) which are associated with protection of the leaf against pathogens (KERSTIENS, 1996). The alteration of the biochemical, enzymatic and anatomical factors as a consequence of sprays with Chi and ChiCu contributed to the induction of resistance to diseases since the external periclinal wall (which includes the cuticle) (Figure 3) is the first barrier to the entry of fungi and bacteria.

The participation of nutrient Cu in several stages in the routes of secondary metabolism has been described. Besides directly affecting the pathways of lignin formation, which have as precursors the phenolic compounds, Cu also influences the activity of PAL (COSIO & DUNAND, 2009; HADŽI-TAŠKOVI´C ŠUKALOVI´C et al., 2010).

Among the different noble metals studied, copper and its complexes have been used widely since ancient time for their antimicrobial properties (FAÚNDEZ et al., 2004). In general, copper is applied in plants to control diseases in high concentrations and in large volumes, which has caused high levels of pollution to the environment. In this work it was possible to demonstrate that the application in low doses (150 to 200 ppm) of chelated Cu associated with chitosan, were sufficient to promote enzymatic activity and thus contribute to plant defense.

The set of chitosan effects in biometric aspects in the crops is very variable and depends on several factors like the vegetal species, mode of application and dose of chitosan (PICHYANGKURA & CHADCHAWAN, 2015). The results using Chi and ChiCu indicate that although there was an energy shift from primary to secondary metabolism due to the higher

accumulation of defense compounds, this did not affect productive parameters on tomato plants (Figure 5).

4. Conclusions

The foliar sprays of chitosan associated with Cu quelate on tomato plants had efficient in the activation of enzymes related to pathogenicity than that chitosan or Cu alone. Chitosan associated with Cu-EDTA increased the accumulation of phenolic compounds, external periclinal wall thickness, and cuticle in tomato leaves, as well as the proportion of coating tissues in relation to leaf limbus. These factors in combination allowed concomitantly reducing the severity of powdery mildew, without affecting biometric and biochemical parameters related to the primary metabolism. The foliar application of chitosan associated to Cu chelate can be an efficient technique for the induction of resistance in healthier tomato cultivation.

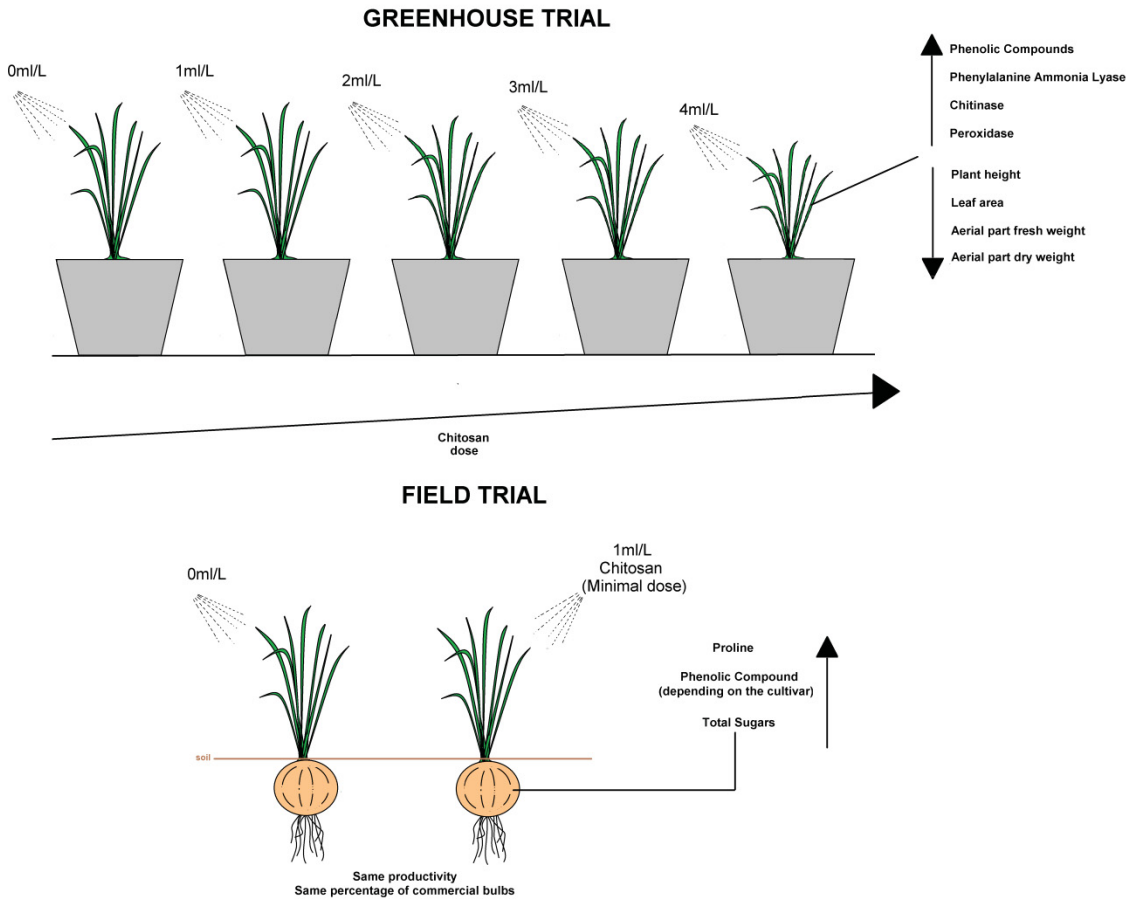
4. CAPÍTULO II - Chitosan through onion leaf: enzymatic, biochemical and biometric changes related to defense responses.

Abstract

The use of natural resistance inducers has been studied to reduce the use of synthetic products for plant deseases control, that may be harmful to the environment and consumers. Chitosan, a biopolymer derived from crustacean exoskeleton, has been reported as sustainable alternative in plant disease management. In this work was evaluated the effect of chitosan foliar sprays on organically grown onion (*Allium cepa* L.). Two experiments were conducted: i) in a pot-grown greenhouse, testing increasing doses of chitosan-based product (1.5%) (0; 1; 2; 3; and 4 $ml.L^{-1}$) with two onion cultivars, evaluating enzymatic activity, biochemical and biometric parameters, ii) in the field in organic system with four cultivars, to determine yield and biochemical changes in bulbs. Chitosan applications promoted greater accumulation of compounds related to the defense metabolism of onion plants, with higher activity of enzymes related to pathogenicity in leaves. There was a reduction in plant growth as a function of increased dose. Differences were found among cultivars regarding accumulation of peroxidase enzyme in leaves and phenolic compounds in bulbs, as well changes in proline levels in leaves and bulbs. It was concluded that leaf-applied chitosan affects primary and secondary metabolism of onion and may induce leaf defense responses by increasing pathogenicity-related proteins and could increase tolerance to abiotic stress in bulbs by means of an increased proline accumulation.

Keywords: *Allium cepa*, enzymes, organic production, induced resistance, proline.

GRAPHICAL ABSTRACT



1. Introduction

Onion (*Allium cepa* L.) is one of the main vegetable grown worlwide. It is consumed in its fresh or used as a condiment. The leading producer countries are China, India and USA. Brazil ranks as the 12th producer with a production of 1.622.106 tones and 51.957 hectares (FAOSTAT, 2019b). In this sense, the search for new natural tools to improve organic onion production, gains relevance facing the challenges to supply a growing demand for healthy food.

Disease control is key for the achievement of a high yield. In general, the use of synthetic fungicides has been the first control alternative, resulting in high production cost, damage to the human health and to the environment. Also, the uncontrolled use of those products may result in resistant strains (ZANIN et al., 2017).

The use of natural products for the control of diseases has been widely studied. Moreover, chitosan has been reported as an efficient alternative as it has low cost and important biological

properties such as biodegradability, biocompatibility and non- allergenicity (XING et al., 2014). It is obtained through the reaction of partial chitin- deacetylation, which is a substance found in shrimps, insects, mollusks and on the cell wall of fungi (KUMARASWAMY et al., 2018).

Chitosan can act on the plants systemic resistance mechanisms, when acting on the path of salicylic acid and on the activation of proteins related to pathogenicity (KUMARASWAMY et al., 2018). Several works have reported the involvement of molecules as oxygen reactive species, Ca^{2+}, nitric oxide, phytohormones in signaling path mediated by chitosan. Effects related to tolerance to abiotic stress have also been reported (MULEY et al., 2019; PEYKANI & SEPEHR, 2018; NGUYEN et al., 2011). Besides promoting alterations in the secondary metabolism, chitosan can also promote growth or generate toxicity in plant species, therefore, affecting yield (KATIYAR et al., 2015).

Although the potential of chitosan has already been describe in several crops, there is a need for more information about its use for onion cultivation, mainly under organic production system. Thus, the aim of this work was to evaluate through enzymatic, biochemical, biometric and yield measurements, the effect of foliar sprays of chitosan in organically grown onions.

2. Materials and methods

2.1. Experimental conditions

The experiments were conducted in 2016 and 2017 in the Organic Vegetables Research Area, under organic system since 2006, at Federal University of Paraná, Pinhais municipality, Paraná state, Brazil (25º 23’ S and 49º 07’ W). The climate in the region is classified as Cfb temperate with well-defined seasons according to Köeppen’s classification.

A chitosan solution on acetic acid (1.5%) (Biocross®) was used, where the chitosan originated from the exoskeleton of crustaceans and showing a deacetylation degree of 93.2%.

2.1.1 Pot experiment

The experiment was conducted in greenhouse in 3-L pots filled with a 1:1 organic substrate mixture (Provaso®) and soil (pH ($CaCl_2$) which chemical analysis presenting = 5.80; pH H_2O= 6.5;

Al^{+3}= 0; $H^{+}Al^{+3}$= 2.1 cmolc dm^{-3} ; Ca^{2+}= 6.70 cmolc dm^{-3} ; Mg^{2+}= 3.8 cmolc dm^{-3} ; K^{+} = 0.40 cmolc dm^{-3} ; P (Mehlich)= 8.0 mg dm^{-3} ; S= 5.8 mg dm^{-3} ; C= 32 g.dm^{-3} ; Organic matter = 5.5 %; V%= 83.85 and CEC= 13 cmolc dm^{-3}). Irrigation was carried out using dripping tape keeping the moisture of the pots at 80% of field capacity using tensiometer.

The onion cultivars used were BR29® and Perfecta® (Agristar Brasil®), common among organic growers in South of Brazil. Sowing was carried out in beds and after 40 days, two seedlings showing five leaves each were transplanted per pot.

The experiment were arranged in a completely randomized design and factorial scheme with five treatments (control sprayed with distilled water; 1; 2; 3; and 4 $ml.L^{-1}$ of chitosan 1.5%), two cultivars (BR29®, Perfecta®) and five replications, each being a 4 pots.

Twelve foliar sprays started 30 days after transplanting were performed at weekly frequency using a pressurized sprayer at constant pressure (40 psi), with volume varying from 10 to 30 mL pot^{-1}, increasing according to plant growth, which. After the 12th application (112 days after transplanting), biometric evaluations were carried out and the second and third leaves (from the youngest to the oldest) were collected, which were pressed in liquid nitrogen and frozen at -80º C for further biochemical and enzymatic analyses.

2.1.2 Field experiment

The soil analysis showed high fertility, as a result of twelve years under organic system: (pH (CaCl2) = 5.80; pH H_2O= 6.5; Al^{+3}= 0; $H^{+}Al^{+3}$= 2.1 cmolc dm^{-3} ; Ca^{2+}= 6.70 cmolc dm^{-3} ; Mg^{2+}= 3.8 cmolc dm^{-3} ; K^{+} = 0.40 cmolc dm^{-3} ; P (Mehlich)= 8.0 mg dm^{-3} ; S= 5.8 mg dm^{-3} ; C= 32 g.dm^{-3} ; Organic matter = 5.5 %; V%= 83.85 and CEC= 13 cmolc dm^{-3}). For soil fertility maintenance and support onion growth, Seven days prior to transplanting, the soil was prepared according to the Brazilian regulation for organic agriculture, with the incorporation of 8 t ha^{-1} organic compost with the following average values: C=30.3 g kg^{-1}; N=30.3 g kg^{-1}; P =8.5 g kg^{-1}; K =6.6 g kg^{-1}; Ca =8.1 g kg^{-1}; Mg=4.1 g kg^{-1}.

Seeds of BR29®, Perfecta®, Maragogi® (Bejo Brasil®) and Alvará® (Bejo Brasil ®) cultivars were sown in seedbeds in June 2016 and transplanted after 60 days.

The seedlings were transplanted in beds with a dimension of 1.20 x 24 m, spacing of 30 cm between rows and 10 cm between plants, distributed in four planting rows, equivalent to a plant population of 230.000 per hectare.

After 60 days from transplant, solutions containing 1ml. L^{-1} chitosan 1,5% were applied at fortnightly frequencies, totaling five application, using a pressurized sprayer at constant pressure (40 psi), with application volume varying from 250 to 400 L ha^{-1}, increasing according to plant growth.

The experiment was organized in a completely randomized design and factorial scheme: dose (control sprayed with distilled water and 1 ml L^{-1} of chitosan 1.5%) vs cultivar (BR29®, Perfecta®, Maragogi® and Alvará®), with 4 replications, each composed of 24 plants.

Harvest was done at 120 days after transplanting, when about 75% of the plants shown pseudostem collapse indicating the proper harvest time. Plants were classified, weighed and bulbs samples were collected for biochemical analysis. Bulbs were classified according to the diameter in class or garbage: smaller than 15 mm; class 1: 15 to 35 mm; class 2: 35 to 50 mm; class 3: 50 to 70 mm and class 4: larger than 70 mm. The results were expressed in percentage of the total number of bulbs produced. Class 3 and 4 were considered as commercial sized bulbs.

2.3. Biochemicals and enzymatic analysis

Biochemical analysis of total proteins, phenolic compounds and enzymes phenylalanine ammonia-liase, chitinase and β-1,3-glucanase were carried out in the Biochemical and Plant Health laboratory at UTFPR in Dois Vizinhos, Paraná, Brazil. Analysis of total free amino acids, proline and total sugars were carried out in biofertilier laboratory at UFPR, in Pinhais, Paraná, Brazil.

Total proteins analysis was carried out through the Bradford (BRADFORD, 1976) method. Total phenols were quantified through the method adapted from Bieleski & Turner (1966). The activity of phenylalanine ammonia-lyase enzyme was quantified according to Rodrigues et al.

(2006). For the enzymes chitinase and β-1,3-glucanase, the methodology described by Wirth & Wolf (1992) was used.

Total free amino acids were analyzed through extraction according to Winters et. al (2002) and the colorimetric reaction with absorbance reading taken at 570 nm. The standard curve was performed with 2 mM glutamine with values between 28 and 140 μg mL^{-1}. The free proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrine reaction (IRIGOYEN et al., 1992).

Total sugars were quantified according to Maldonade et al. (2013) preceded by acid hydrolysis of the sample using 3,5-dinitro salicylic acid (DNS). The standard curve for reducing and total sugars was made with glucose at 1 mg/mL (5.5 mM) with values between 50 and 800 μg /mL.

2.4 Statistics analysis

Data were subjected to analysis of variance and means compared by the Scott-knott test at 5% level. Data were processed using Assistat version 7.7 beta software.

3. Results and discussion

3.1 Pot experiment

The increasing doses of chitosan promoted greater accumulation of phenolic compounds and an increment in the activity of pathogenicity-related enzymes: phenylalanine ammonia-lyase, chitinase, peroxidase in onion leaves (Figure 1.a, b, c and d). There was a difference between cultivars for phenolic compounds content (lower for BR29®) and peroxidase (higher for BR29®).

The rise in the content of phenolic compounds indicates that chitosan foliar sprays provided activation in the phenylpropanoid pathway, triggering the activity of the enzyme phenylalanine ammonia-lyase. By the action of this enzyme, the phenylalanine molecule is deaminated to cinnamic acid, which is a precursor of lignin, flavonoids and various other key phenolic compounds, important for plant defense (KARAKAYA, 2004).

Phenolic compounds are precursors to the flavonoid route, but the flavonoid content were not affected in the leaves from the application of the tested doses suggesting that this was not the preferred defense compound for this species. It was reported in sage (*Salvia officinalis* L.) that the increase in flavonoids due to the chitosan application correlated with the increase in water stress (VOSOUGHI et al., 2018). As in this experiment the onion plants were not under water stress situation, this may have been the reason why these compounds were not pronounced.

As presented in Figure 1 c and d, the increase in chitinase and peroxidase activity has also been reported as an effect of chitosan application on species such as tomato (CHUN, 2019; SATHIYABAMA et al., 2014), potato (MULEY, 2019), grape (TROTEL-AZIZ et al., 2006) and pear (MENG et al., 2010).

A reduction was observed in protein content by chitosan sprays (Figure 1.e) which may have occurred due to the deviation to enzyme production, since the analysis performed only measures protein macromolecules. There was interaction only between the applied doses and the two cultivars tested for amino acid accumulation (Figure 1.e), which was greater for the cultivar BR29® at doses 3 and 4 ml. L^{-1}, smaller for Perfecta® cultivar at doses 0, 1 and 2 ml.L^{-1}.

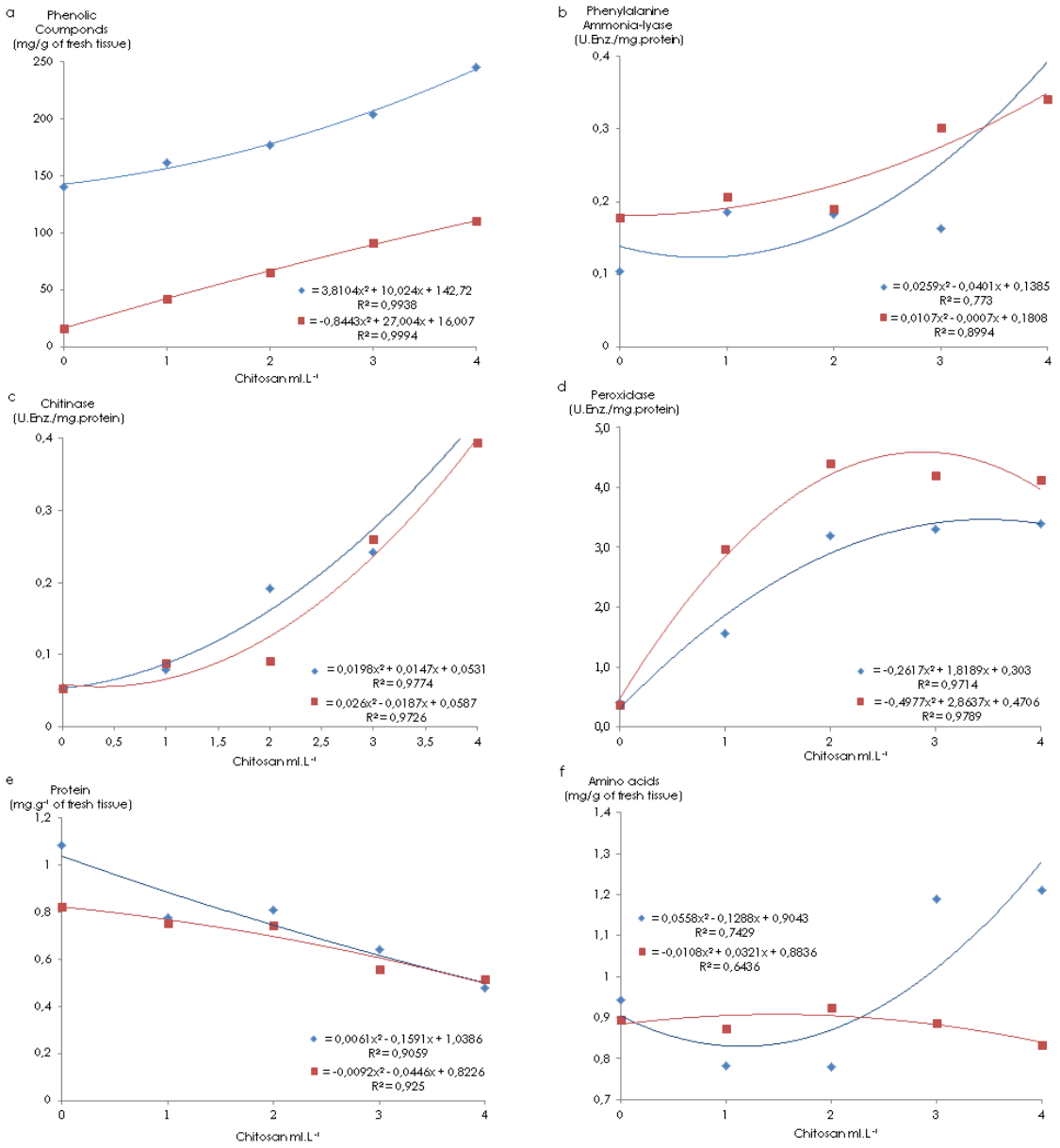


Figure 1- Effect of the application of increasing doses of chitosan on the content of a- phenolic compounds and enzimes activity of b- phenylalanine ammonia lyase, c- chitinase, d- peroxidase, content of e- proteins and f- amino acids in onion leaves grown in pots 84 days after transplanting. BR29 cultivar (red lines), Perfecta cultivar (blue lines).

There was a reduction in leaf proline at the dose of 3ml.L^{-1} of chitosan for the cultivar Perfecta® (Figure 2). Although this amino acid correlates with the biotic and abiotic defense mechanisms (ANWAR-HOSSAIN et al., 2014), these results indicate that this path was not activated and also that it was not the change in this amino acid that may have caused variation in the total amino acid content. One hypothesis for proline reduction in the leaf is the translocation of this osmolyte to the bulb. There was no variation in the levels of total sugars in the leaves.

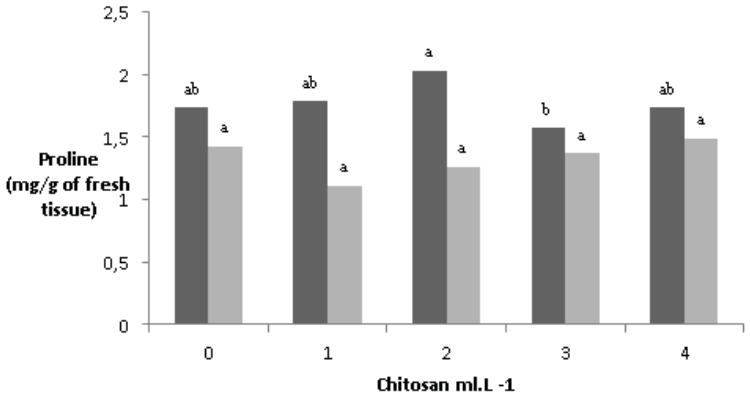


Figure 2 - Effect of the application of increasing doses of chitosan on the content of proline in onion leaves of cultivars Perfecta ® (dark gray) and BR29® (light gray), grown in pots 84 days after transplanting. Means followed by the same letter do not differ from each other. The Scott-Knott test was applied at the 5% probability level.

A reduction was observed in height, leaf area, fresh mass and dry mass of the aerial part as doses of chitosan increased (Figure 3). The reduction in these biometric parameters of the aerial part in the vegetative growth phase indicates that the activation of secondary metabolism affected onion primary metabolism. The resistance induction process generates costs for the plant and the allocation of plant resources for growth or defenses is determined by competition for common substrate and energy, and the plant must balance investments in these processes (GAYLER et al., 2004). Besides the energy cost, there is the metabolic cost, which is explained by the repression of some genes (KUHN & PASCHOLATI, 2007). This repression may occur to balance total metabolism and balance costs within the plant system as a compensatory effect (SHIH, 2018).

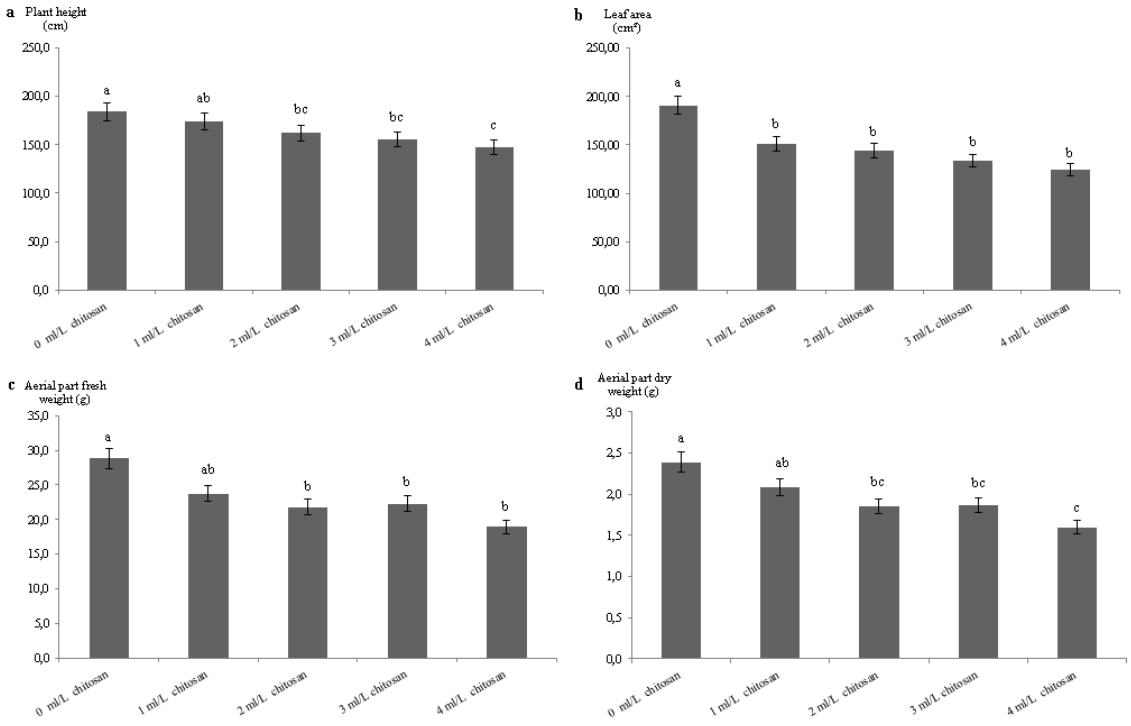


Figure 3- Effect of the application of increasing chitosan doses on the biometric parameters: a- height, b- leaf area, c-fresh weight of the aerial part, d-dry weight of the aerial part of onion plants in pots 84 days after transplanting. Means followed by the same letter do not differ from each other. The Scott-Knott test was applied at the 5% probability level.

3.2 Field experiment

It has been shown in the pot experiment that chitosan activates secondary metabolism, but, depending on the dose, it may cause a reduction in biometric parameters. In order to evaluate the productive and biochemical effects of leaf sprays of chitosan on bulbs, the field experiment was set by testing the chitosan dose (1ml L^{-1}) (which was the lowest dose tested in the pot experiment) in four onion cultivars (BR29®, Perfecta®, Maragogi®, Alvará®).

There was no difference between control and chitosan dose of 1ml.L^{-1} in terms of total yield and commercial yield, percentage of commercial bulbs (Table 1) and no interaction was found between dose factor and cultivars. Differences occurred between cultivars, so that cultivar BR29® obtained lower percentage of commercial bulbs (Table 1).

Table 1- Total and commercial productivity between cultivars and between control and treatment containing chitosan. Percentage of commercial bulbs between cultivars and chitosan-containing treatment.

		Total Productivity (Kg. ha^{-1})[1]	Comercial Productivity (Kg. ha^{-1}) [1]	Commercial Class (%) Bulbs[1]
Cultivars	BR29®	14147,96 a	11256,20 b	71,61 a
	Maragogi®	16630,33 a	15892,70 a	87,30 a
	Perfecta®	18863,32 a	14703,01 a	89,96 a
	Alvará®	19067,68 a	16532,78 a	81,42 a
Doses	Control	16626,52 a	14349,01 a	83,50 a
	Chitosan 1ml.L^{-1}	17728,14 a	14843,33 a	81,65 a
	CV%	21,98	14,37	13,10

[1]Means followed by the same letter do not significantly differ by the test of Scott-Knott at 5% probability.

Regarding the biochemical analysis of bulbs, the total sugar content was not affected by the application of 1 ml.L^{-1} of chitosan. However, there was a reduction in reducing sugars content and an increase in proline content (Figure 4), there was no interaction between the applied chitosan dose and the cultivars.

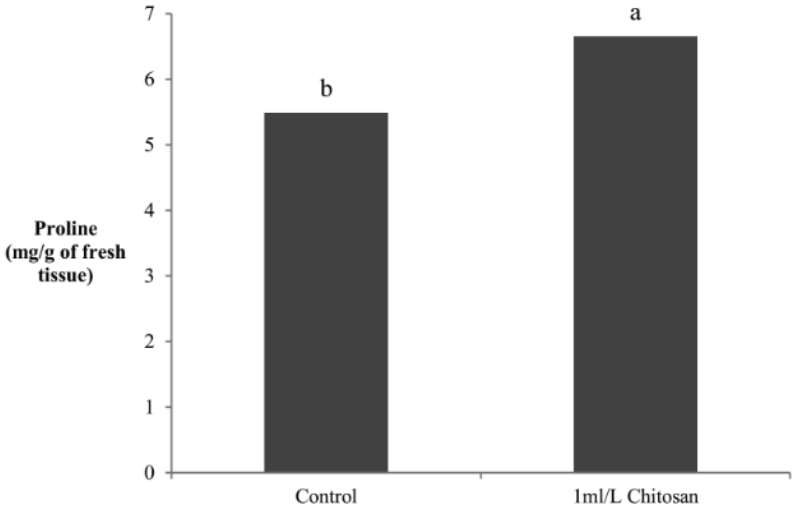


Figure 4 - Effect of leaf application of chitosan (1ml.L^{-1}) on the proline content in onion bulbs under organic cultivation 150 days after planting. Means followed by the same letter do not differ from each other. The Scott-Knott test was applied at the 5% probability level.

Among the functions of osmoprotectants, such as proline, it should be highlighted the role of preventing free radical production or sequestering reactive oxygen species, such as superoxide ion,

hydrogen peroxide, hydroxyl radicals, and peroxyl, which characterize a secondary stress oxidative stress (ANWAR-HOSSAIN et al., 2014). Given that oxidative stress with the increase in reactive oxygen species is one of the chitosan signaling pathways, the increase in proline levels in the bulbs can be attributed to foliar sprays, therefore, suggesting a primer effect of chitosan for increases in the levels of these amino acids in the bulb.

In pot experiment, a reduction in proline content in the leaf was observed with an increase in chitosan dose, which may have occurred due to the translocation of the osmolyte to the bulb. Besides the response correlation to oxidative stress, proline accumulation in the bulb may be related to the fact that proline acts under non-stressful conditions on reproductive development (MATTIOLI et al., 2009) mediated by the action of salicylic acid (MARTINEZ et al., 2014). Since chitosan promotes inducing action on salicylic acid mediated pathways this may have induced the increase in proline accumulation in the bulb.

An interaction was found between cultivars and doses for total sugar, amino acids and phenolic compounds in the bulbs (Figure 5). In relation to total sugar, there was no difference between doses, variation occurred only between cultivars (Figure 5a). A higher and lower content was found for total sugar and reducing sugar (Figure 5b), respectively for cultivar BR29, and this may have been due to the fact that this cultivar had a smaller percentage commercial sized bulbs, therefore indicating that the bulbs were still in the development phase and therefore had a higher reducing sugar content, which is a feature of mature bulbs.

Content of total amino acid was reduced (Figure 5c) in the bulbs due to the application of 1 ml.L of chitosan to the Perfecta® cultivar. This same cultivar showed higher leaf amino acid contents as dose of chitosan dose applied via leaf in the pot increased. This may suggest that as a result of chitosan application, this cultivar accumulates or maintains more amino acids in the aerial part than translocates to the bulb.

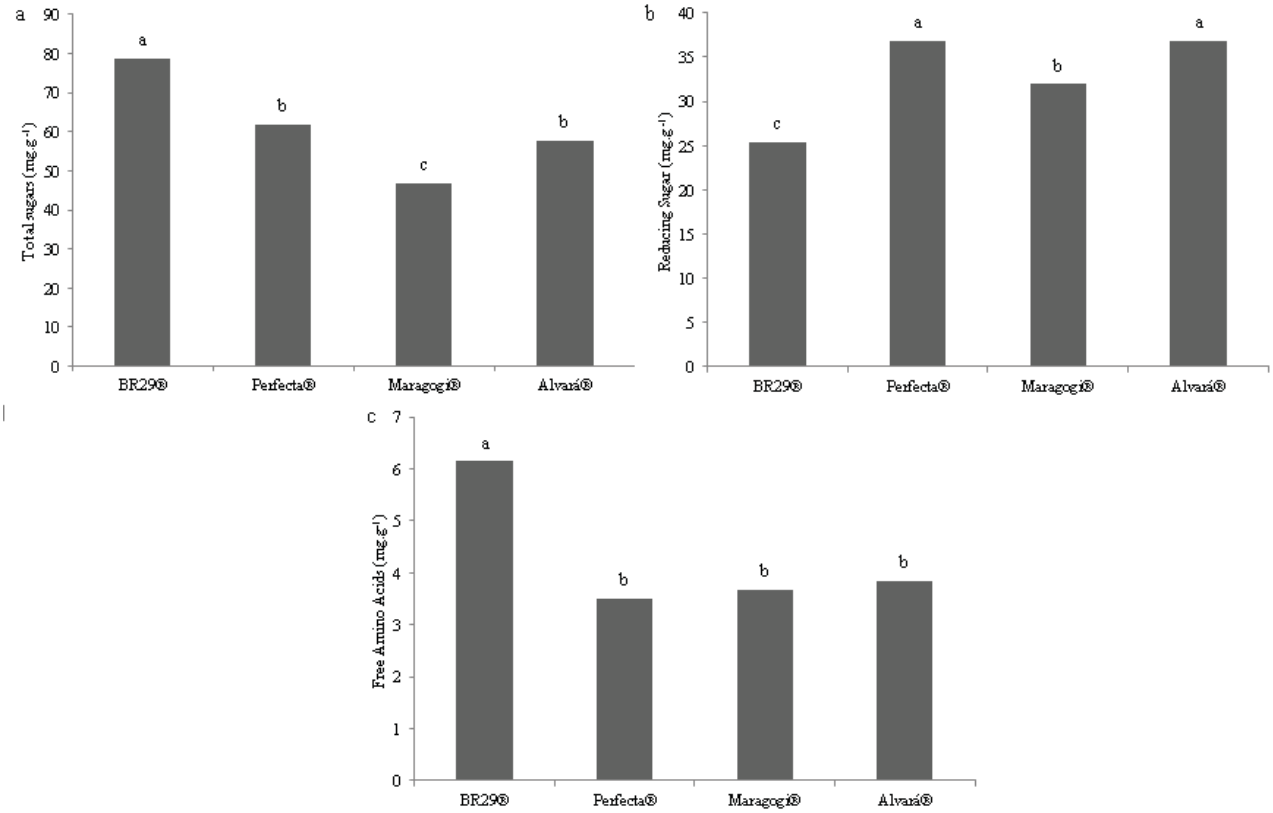


Figure 5- Contents of total sugars (a), reducing sugar (b), total free amino acids (c) on onion bulbs cultivars BR29®, Perfecta®, Maragogi ®and Alvará® under organic cultivation 150 days after planting. Means followed by the same letter do not differ from each other. The Scott-Knott test was applied at the 5% probability level.

An increment was found in the phenolic compounds content in the 1ml.l^{-1} chitosan treatment only for Perfecta® cultivar. On the other hand, the cultivar Maragogi® obtained a reduction in phenolic compounds (Table 2). The variation of accumulation of phenolic compounds in the bulb between cultivars, under the effect of chitosan application, demonstrates that the genetic factor directly affects chitosan action mechanism for this species and should be a factor to be considered in order to optimize the application of the product.

Table 2 – Effect of leaf application of chitosan (1ml.L^{-1}) on the phenolic compounds content in onion bulbs under organic cultivation 150 days after planting.

		Phenolic compounds[1]
Cultivars	**BR29®**	1.86 b
	Maragogi®	1.10 c
	Perfecta®	2.43 a
	Alvará®	0.71 c
Dose	**Control**	1.55 a
	Chitosan 1ml.L^{-1}	1.50 a

Interaction Cultivars x Dose			
		Dose [2]	
		Control	**Chitosan 1ml.L^{-1}**
Cultivars	**BR29®**	1.8801 aA	1.85 bA
	Maragogi®	1.8317 aA	0.37 cB
	Perfecta®	1.7923 aB	3.07 aA
	Alvará®	0.6867 bA	0.73 cA
CV%		29.18	

[1]Means followed by the same letter do not significantly differ by the test of Scott-Knott at 5% probability.
[2]Means followed by the same lower-case letter in the column and upper-case letter in the line do not significantly differ by the test of Scott-Knott at 5% probability.

4. Conclusion

The application of chitosan in onion promotes greater accumulation of compounds related to defense metabolism, with greater activity of pathogenicity-related enzymes in leaves. Doses above 1m.L-1 of chitosan promotes an energy deviation that results in the reduction of biometric parameters of the crop, however, at this dose, it does not cause losses related to total and commercial yield, and percentage of commercial bulbs. Chitosan promotes changes in proline levels in onion. There are differences in behavior among cultivars regarding the greater accumulation of peroxidase in leaves and phenolic compounds in the bulbs due to chitosan application. It was concluded that leaf-applied chitosan affects primary and secondary metabolism of the onion and can induce leaf defense responses through the increase in pathogenicity-related proteins and resistance to abiotic stress in bulbs by incrementing proline accumulation.

5. CAPÍTULO III - BIOFORTIFICATION, PRODUCTIVITY AND PHENOLIC COMPOUND CONTENTS IN ONIONS AFFECTED BY FOLIAR APPLICATION OF CHITOSAN AND COPPER-ASSOCIATED CHITOSAN

Abstract

Efficient cultivation techniques that promote productivity improvements, nutritional quality of bulbs with efficient use of inputs without compromising environmental sustainability have been sought for onion cultivation. Chitosan is a natural biopolymer that has been widely used in the production of metabolites in plants with beneficial effect on crops. Copper is an important essential micronutrient related to the production of compounds of plant secondary metabolism. The objective of this work was to evaluate the effect of foliar application of chitosan and copper-associated chitosan on the yield, nutritional quality of bulbs and phenolic compounds production in onion cultivated under organic production system. Experiments were carried out in greenhouse evaluating two cultivars in increasing doses of chitosan for analysis of phenolic compounds in the leaves and in parallel, field experiments were performed by testing four cultivars and evaluating the effects on yield and nutritional quality of bulbs. Application of copper-associated chitosan (1 $ml.L^{-1}$) appears as a valid horticultural technique for improving the nutritional quality of onion bulbs without altering primary metabolism without affecting productivity. The contents of phenolic compounds increased in leaves and bulbs.

Key-words: *Allium cepa* L.; Bulb yield; Mineral elements; Organic system; Nutrient content.

1. Introduction

The onion (*Allium cepa* L.) belongs to the *Alliaceae* family, which includes, garlic, spring onion and leeks. It is one of the most important vegetable crops in the world and the main areas of production are China, India and the USA. Brazil is ranked twelfth with a production of 1,622,106 tons and 51,957 hectares (FAOSTAT, 2019).

Onion quality is related to some factors such as external appearance, bulb size, color, taste, firmness and chemical composition and those characteristics may be influenced by genotype, pre-harvest management, appropriate harvesting and post-harvest treatments (GRANGEIRO et al., 2008, FINGER & CASALI, 2002). Irrigation system (ENCISO et al., 2009), postharvest treatments (NEGA et al., 2015), K application (DESHPANDE et al., 2013) or saline stress (COCA et al., 2012) may change the pungency and/or sweetness of the onion bulb. Over the years, the most common way to improve productivity and/or planted area has been the indiscriminate use of synthetic fertilizers (AYALA & RAO, 2002).

Productivity improvement at reduced cost, efficient use of inputs without compromising environmental sustainability are the goals to be achieved in current agriculture (XING et al, 2015). Extensive use of agrochemicals in agriculture results in a number of damages such as erosion of soil fertility, loss of biodiversity, pesticide runoff causing contamination that affects the environment and human resources (BOUSSEMART et al., 2013).

Chitosan is a natural chitin-derived biopolymer as a component of the cell walls of fungi, skeletal-insects and shells of crustaceans. Chitosan has been characterized and applied for decades. The use of use of chitosan worldwide occurs in many sectors, including farming, industry and medicine (PICHYANGKURA & CHADCHAWAN, 2015). Chitosan use in plants has been reported for various applications, acting as growth promoter and generating responses in primary and secondary metabolism in the presence or absence of stressful conditions (MUKHTAR AHMED et al, 2019).

Application of chitosan and its oligosaccharides may improve plant growth (IBRAHEIM & MOHSEN, 2015; MONDAL et al., 2012; MONIRUL et al., 2018; Sathiyabama, et al., 2014), seed germination (GUAN et al., 2009), chlorophyll content (MOHAMED, 2018), nitrogen fixation (OHTA et al, 1999) and nutrient uptake (ABU-MURIEFAH, 2013). In addition, chitosan may affect the content of secondary metabolites such as essential oil (terpenoids) (BISTGANI et al, 2017), phenolic (ALI et al., 2012; ZHANG et al, 2018). In addition, chitosan has been reported as a biopestiside for various species acting as antibacterial, antifungal and antiviral agents to decrease the intensity of plant diseases and restrict pathogen proliferation, thus preserving crop yield and quality (MUKHTAR-AHMED et al., 2019).

Copper is an essential nutrient for plant development as a cofactor of numerous proteins and carbohydrate metabolism (PRINTZ et al., 2016). Copper pathways in plants are also related to defense mechanisms and quality, as it is a key nutrient, providing changes in the structure and shape of cell walls and participating in enzymes such as phenylalanine ammonia-lyase (PRINTZ et al 2016). Another effect of copper on disease resistance is due to its participation in lignin synthesis, a partial barrier to penetration into plant tissue (GRAHAM & WEBB, 1991).

The objective of this work was to evaluate the effect of chitosan foliar application and chitosan associated with copper on the yield, nutritional quality of bulbs and phenolic compounds production in onion cultivated under organic production system.

2. Materials and methods

2.1 Experimental conditions

The chitosan solution on acetic acid with 1.5 % (w/v) showed a deacetylation degree of 93.2% calculated through potentiometric titration method, carried out in the Biopolymers Laboratory in the Chemistry Department at the Federal University of Paraná, Brazil. The cooper source was obtained through quelation reaction of copper sulfate with EDTA (Cu-EDTA 5% w/v), supplied by Biocross®.

For all experiments, eight foliar applications were carried out weekly with a pressurized sprayer at constant pressure (40 psi), with application volume varying from 20 to 40 mL $plant^{-1}$, increasing according to plant growth.

Experiments were conducted in 2016 and 2017, in the organic vegetable production research area, where an organic system was implemented 13 years ago at the Federal University of Paraná, at the Canguiri Experimental Station (25º 23' S and 49º 07' W, Köeppen temperate type climate – Cfb, at an altitude of 920 m).

In August 2016 and 2017, onion 'BR-29' (open-pollinated cultivar), 'Perfecta F1' (hybrid) (Topseed®), 'Maragogi' (Bejo Brasil®) and 'Alvará' (Bejo Brasil ®) both commonly used by growers in Southern Brazil were sown in a nursery-type seedbed under a polyethylene tunnel. Thirty days after sowing (DAS) the seedlings were collected and used for pot and after 60 DAS to field experiments, which were conducted simultaneously.

2.2 Greenhouse experiment

The cultivars 'BR29' e 'Perfecta' were conducted in greenhouse using 5-liter pots, filled with commercial substrate (Provaso®) and soil at 1:1 ratio. The chemical analysis of mixture showed values adequate to growing onion: pH H_2O= 6.5; Al^{+3}= 0; $H^{+}Al^{+3}$= 2.1 $cmol_c$ dm^{-3} ; Ca^{2+}= 6.70 $cmol_c$ dm^{-3} ; Mg^{2+}= 3.8 $cmol_c$ dm^{-3} ; K^{+} = 0.40 $cmol_c$ dm^{-3} ; P (Mehlich)= 8.0 mg dm^{-3} ; S= 5.8 mg dm^{-3} ; C= 32 g.dm^{-3} ; organic matter = 5.5%; base saturation= 83.85; CEC= 13 cmolc dm^{-3}. The

irrigation was performed using drip-tape by keeping the moisture of the pots near to the field capacity using tensiometer.

Twelve applications were performed weekly which started 30 days after transplantation. They consisted of the following doses: chitosan 1; 2; 3 and 4 $ml.L^{-1}$ (respectively 15, 30, 45 and 60ppm), totaling 5 treatments and 4 replicates in a completely randomized design. This experiment analyzed the content of phenolic compounds. The experiment was repeated twice and the results were the mean of both experiments.

2.3 Field experiment

Cultivars 'BR29', 'Perfecta', 'Maragogi' and 'Alvara' were used in the field experiment. Chemical analysis of the 0–20 cm soil layer in the field indicated the average values: 6.30 pH (H2O); 33.30 $g.dm^{-3}$ organic matter; 133.10 $mg.dm^{-3}$ P; 1.44 $cmol_c.\ dm^{-3}$ K; 9.30 $cmol_c.\ dm^{-3}$ Ca; 4.30$cmol_c.\ dm^{-3}$ Mg; 0 $cmol_c.\ dm^{-3}$ Al; 3.7 $cmol_c.\ dm^{-3}$ $Al^{+}H$; 18.34 $cmol_c.\ dm^{-3}$ CEC and 80% base saturation. Seven days prior to transplanting, the soil was tillaged by incorporating 8 t ha^{-1} organic compost with the following average values: C=30.3 g kg^{-1}; N=30.3 g kg^{-1}; P =8.5 g kg^{-1}; K =6.6 g kg^{-1}; Ca =8.1 g kg^{-1}; Mg=4.1 g kg^{-1}. Soil fertilization was done according to the Brazilian regulation for organic agriculture. The onion seedlings were transplanted in beds with a dimension of 1.20 x 24 m, spacing of 30 cm between rows and 10 cm between plants, distributed in 4 planting rows, equivalent to a plant population of 230.000 per hectare.

The treatments were distributed in 1.20×1.0-m plots in a completely randomized design (n=3) and factorial scheme. They consisted of the following doses: 1$ml.L^{-1}$ chitosan and 1$ml.L^{-1}$ chitosan plus Cu-EDTA (respectively 15 ppm chitosan and chitosan 15 ppm associated with 50 ppm Cu-EDTA).

2.3.1. Biometric and yield analysis

At 150 days after transplanting, about 80% of the plants showed pseudostem collapse indicating proper harvest time. The plants were harvested and kept on plots over the soil for a week for complete leaf senescence. Eight bulbs were collected per plot for fresh and dry mass determination using a digital scale, and the ratio between fresh and dry mass was calculated. Another twelve bulbs per plot were collected for bulb classification according their diameters (caliber) as follows: caliber 2 (35–50 mm), caliber 3 (50–70 mm) and caliber 4 (70–90 mm) according to Brazilian market bulbs classification, and the total and commercial (caliber 3–5) yield was determined. Four commercial bulbs from each treatment were stored in plastic boxes and then in a room with an average temperature of 23.5°C and relative humidity of 74%±5. The bulbs were stored and evaluated after 8 weeks.

2.3.2. Chemical analysis

Four onion plants per plot were randomly collected to quantify macro and micronutrient contents (K, P, Ca, Mg, Cu, Mn, Fe, Zn and B) of bulbs and leaves. After drying in a forced air circulation oven (65℃±5℃), samples with 0.3 g of dry mass were diluted in HNO_3 and dissolved in H_2O_2, subsequently read by means of Perkin Elmer Optima 4300 Induction Plasma Optical Emission Spectroscopy (Perkin Elmer®, USA) in triplicate. The quantification of nitrogen (N-total) was carried out by combustion in the CHONS analyzer (model Vario EL III).

2.3.3. Biochemical analysis

For the determination of the total and reducing sugars in bulbs, the methodology described by Maldonade et al. (2013) was used. The free amino acids in bulbs were extracted following Winters et al. (2002) and the colorimetric reaction was performed according to Magné & Larher (1992). Soluble proteins were determined using the methodology described by Bradford (1976). For all analyzes, samples of 0.3 g of plant material were used.

Data on biometric, chemical and biochemical analysis were tested using the Bartllet test and ANOVA. The Scott-knott mean test ($p < 0.01$) was applied. All were processed by Assistat 7.7 beta software.

3. Results

3.1 Productivity and water status

The data shown in Figure 1 demonstrate that there was no difference between commercial bulb yield (Figure 1.a), commercial bulb percentage (Figure 1.b) and bulb dry matter (DM) percentage (Figure 1.c) among the treatments containing chitosan and copper-associated chitosan; however, productivity varied among cultivars, in which ‘BR29’ achieved the lowest one.

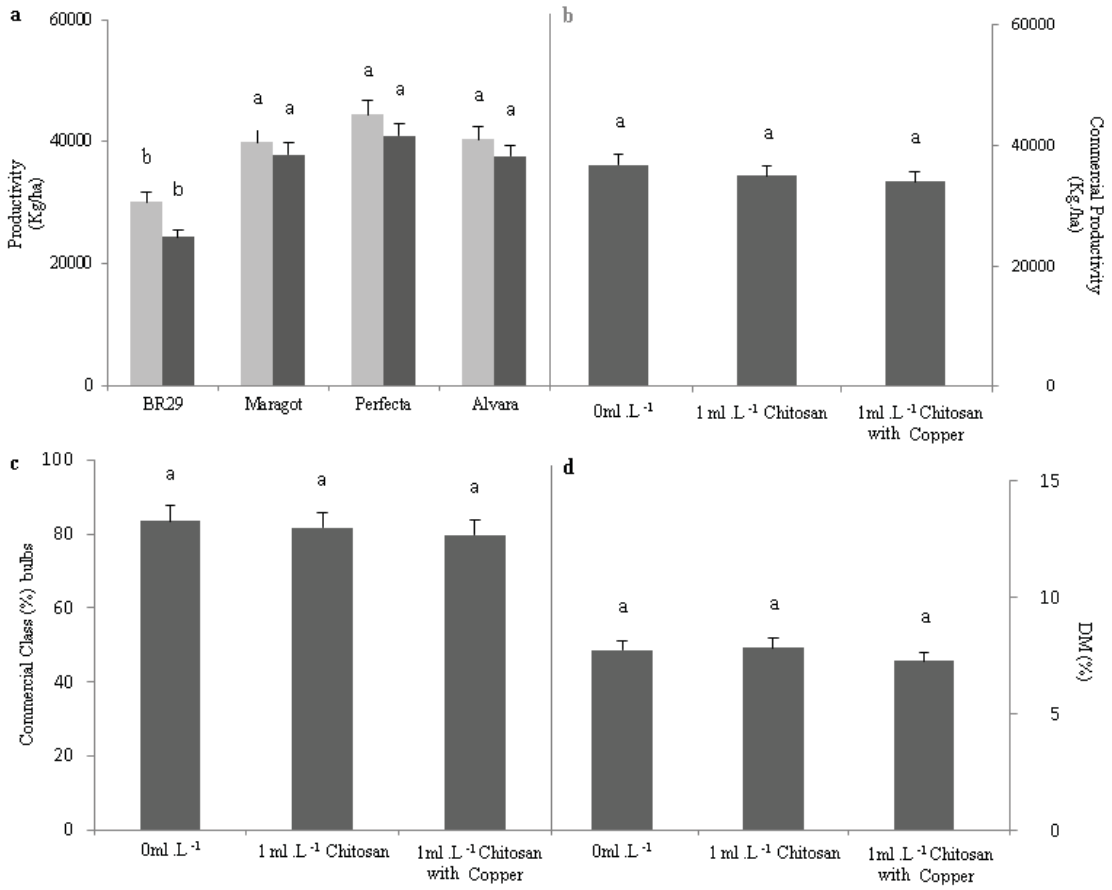


Figure 1- Total productivity (light gray) and commercial productivity (dark gray) on cultivars (a), effect of foliar application of chitosan and copper-associated chitosan on commercial productivity (b). Percentage of commercial size bulbs (c) and percentage of dry matter content (d) of onion organic system-grown 150 days after planting. Columns with the same letter do not differ statistically ($p < 0.01$) according to Scott-Knott test. Bars represent standard error. The figures a and b: Caliber: 1(< 35 mm), 2 (35–50 mm), 3 (50–70 mm) and 4 (70–90 mm). Calibers 3 and 4 are considered marketable.

3.2. Total sugars, reducing sugar, total free amino acids and soluble proteins in bulbs

No variation was found in the contents of total sugars (Figure 2.a), reducing sugar (Figure 2.b), total free amino acids (Figure 2.c) and protein (Figure 2.d) for the tested chitosan and copper-chitosan. They only occurred between cultivars. Cultivar 'BR29' had higher levels of total sugars and lower levels of reducing sugars, because it was the cultivar that presented lower productivity, indicating that the bulbs were possibly late in physiological maturity in relation to the other cultivars.

Free amino acid levels were higher for cultivar 'BR29' when treated with chitosan and copper-associated chitosan. Cultivar 'BR29' obtained significantly higher amino acid content than the others by evaluating the copper-associated chitosan treatment alone.

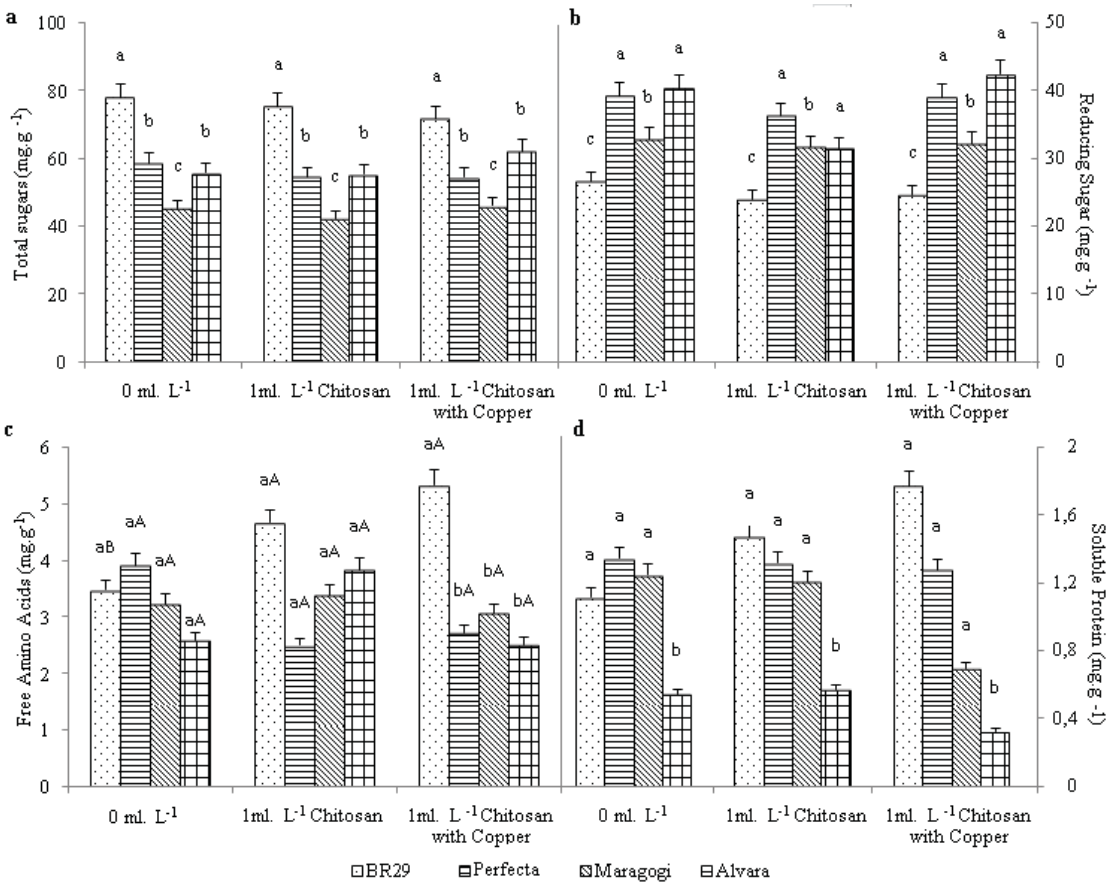


Figure 2- Contents of total sugars (a), reducing sugar (b), total free amino acids (c) and soluble proteins (d) on bulbs of organically grown onion cultivars submitted to foliar application of chitosan 1ml.L^{-1} and copper-associated chitosan 1ml.L^{1}. Columns with the same letter do not differ statistically ($p < 0.01$) according to Scott-Knott test (n=3). Capital letters = Treatments. Lowercase letters = cultivars. Bars represent standard error. The ANOVA analysis indicated in: (a) Cultivars = **, Treatments = ns and Interactions = ns, (b) Cultivars = **, Treatments = ns and Interactions = ns, (c) Cultivars = **, Treatments = ns and Interactions = * and (d) Cultivars=**, Treatments=ns and Interactions = ns, where ns=not significant, * and ** = significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

3.3 Mineral analyses

The foliar application of 1 ml.L^{-1} of copper-associated chitosan altered the nutrient accumulation in onion bulbs in the tested cultivars, therefore, increasing the phosphorus, potassium, copper, manganese and zinc contents (Table 1).

A variation was found in nutrient content as the cultivar was tested for phosphorus, potassium, copper, manganese and zinc (Table 1). Cultivar 'Perfecta' showed lower phosphorus, manganese and zinc contents. Cultivars 'Maragogi' and 'Alvara' had higher potassium contents. Copper contents were higher for the cultivar 'Alvara'.

The interaction between cultivars and doses can be observed for phosphorus and manganese (Table 2). The cultivar 'Maragogi' obtained the highest content of both nutrients for the treatment of copper-associated chitosan.

Table 1- Nutrient contents in bulbs of organically grown onion cultivars after weekly foliar spraying of 0.1 ml.L^{-1} chitosan and 1 ml.L^{-1} copper-associated chitosan. The same lower case letters (treatments) are not different according to Scott-Knott test at 5% probability ($p < 0.05$) (n=4). Means with * are different by the Scott-Knott test at 5% probability ($p < 0.05$). ANOVA: ns = not significant; * and ** = significant at $p < 0.05$ and $p < 0.01$, respectively. DM = dry matter.

Treatment		N (g.Kg^{-1} DM)	P (g.Kg^{-1}DM)	K (g.Kg^{-1}DM)	Ca (g.Kg^{-1}DM)	Mg (g.Kg^{-1}DM)	Fe (mg.Kg^{-1}DM)	Cu (mg.Kg^{-1}DM)	Mn (mg.Kg^{-1}DM)	Zn (mg.Kg^{-1}DM)
Cultivars	'BR29'	15.33 a	3.82 a	9.26 b	2.32 a	1.34 b	22.02 a	3.40 c	5.16 a	20.95 a
	'Perfecta'	16.70 a	3.21 b	10.50 b	2.05 a	1.33 b	13.29 b	2.38 c	3.72 b	16.76 b
	'Maragogi'	17.87 a	4.54 a	11.58 a	2.35 a	1.61 a	6.96 b	4.27 b	4.74 a	24.64 a
	'Alvara'	16.22 a	3.97 a	11.78 a	2.41 a	1.62 a	13.80 b	5.60 a	4.66 a	21.39 a
Dose	0	15.42 b	3.48 b	10.16 b	2.17 a	1.36 a	12.98 a	2.60 b	4.05 b	17.88 b
	1 ml. L^{-1} Chitosan	15.98 b	3.78 b	10.58 b	2.27 a	1.45 a	10.37 a	3.71 b	4.51 b	20.23 b
	1 ml. L^{-1} Chitosan with Copper	18.19 a	4.40 a	11.60 a	2.40 a	1.61 a	18.70 a	5.42 a	5.15 a	24.69 a
Cultivars		ns	**	**	ns	*	*	**	*	*
Doses		ns	**	*	ns	ns	ns	**	*	**
Cultivars x Dose		ns	*	ns	ns	ns	ns	ns	*	ns

Table 2- Phosphorus and manganese contents in onion bulbs, cultivars, doses of weekly foliar spraying (0, 1ml.L^{-1} chitosan and 1ml.L^{-1} of copper-associated chitosan). Means followed by the same capital letter in the line and lower case in the column are not different according to Scott-Knott test at 5% probability ($p < 0.05$). DM = dry matter.

		P (g.Kg^{-1}DM)		
		Doses		
		0	1 ml. L^{-1} Chitosan	1 ml. L^{-1} Copper-associated chitosan
Cultivars	'BR29'	3.3648 aA	3.9263 aA	4.1541 bA
	'Perfecta'	3.2501 aA	3.24 aA	3.154 bA
	'Maragogi'	3.5185 aB	4.0023 aB	6.0845 aA
	'Alvara'	3.7669 aA	3.9339 aA	4.2066 bA
CV%			17.53	
		Mn (mg.Kg^{-1}DM)		
		Doses		
		0	1 ml. L^{-1} Chitosan	1 ml. L^{-1} Copper-associated chitosan
Cultivars	'BR29'	5.0862 aA	4.7511 aA	5.6303 aA
	'Perfecta'	3.8845 aA	4.1001 aA	3.1847 bA
	'Maragogi'	3.0292 aB	4.457 aB	6.7257 aA
	'Alvara'	4.1932 aA	4.7253 aA	5.0609 aA
CV%			21.84	

3.4. Phenolic compounds in leaves and bulbs

The application of increasing doses of chitosan in potted onion leaves promoted an increase in leaf phenolic compounds for 'BR29' and 'Perfecta' cultivars, ranging from 42.36 mg.g^{-1} (1ml.L^{-1}) up to 110.05 mg.g^{-1} (4ml.l^{-1}) and from 140.59 mg.g^{-1} (1ml.L^{-1}) to 245.64 mg.g^{-1} (4ml.l^{-1}) respectively.

Considering that the application of 1ml.L^{-1} of chitosan generated an increase in the phenolic compounds content for both cultivars, this same dose was applied to the plants in the field and the phenolic compounds contents in the bulbs were evaluated as well as the copper-associated chitosan treatment.

The foliar application of copper-associated chitosan affected the phenolic compounds content in onion bulbs. The cultivar 'BR29' was 4.4 times higher than the control and 3.0 times higher than the treatment containing chitosan. In relation to the

other cultivars, in the copper-associated chitosan treatment, cultivars 'BR29' and 'Perfecta' had the highest content of phenolic compounds.

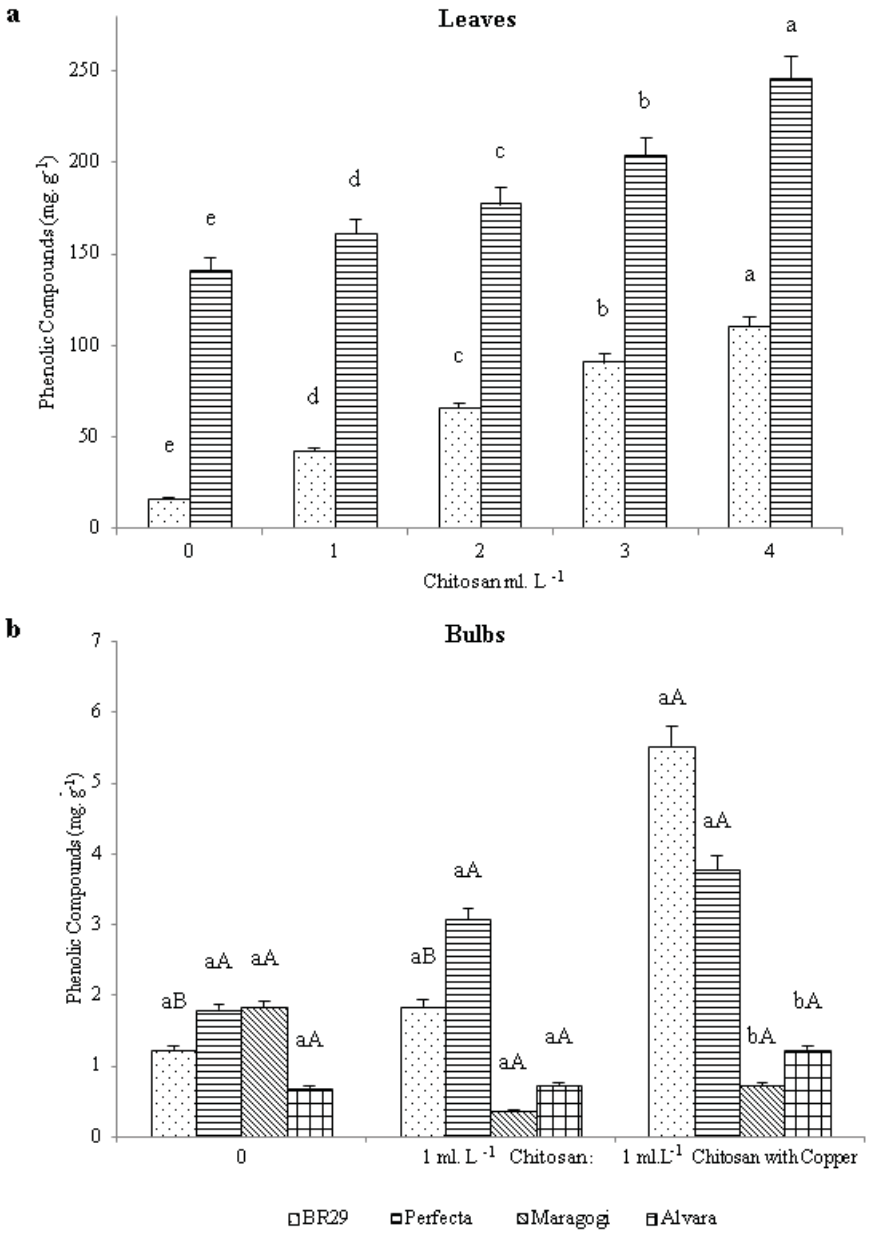


Figure 3- Phenolic compounds content in onion leaves (a), grown in pots 84 days after transplantation under chitosan application and phenolic compounds content in onion bulbs (b) under foliar application of chitosan and copper-associated chitosan under organic cultivation 150 days after planting. Columns with the same letter do not differ statistically ($p < 0.01$) according to Scott-Knott test (n=3). Capital letters = Treatments. Lowercase letters = cultivars. Bars represent standard error. The ANOVA analysis indicated in: (a) Cultivars = **, Treatments = ** and Interactions = ns, (b) Cultivars = **, Treatments = * and Interactions = *, where ns=not significant, * and ** = significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

The contents of total sugars, free amino acids, soluble proteins and flavonoids were evaluated at harvest and eight weeks after storage. The bulbs analyzed after 8 weeks of storage had higher levels of total sugars and amino acids, while the bulbs analyzed at harvest had higher levels of proteins and flavonoids (Figure 4). There was no interaction between the evaluated cultivars to chitosan and copper-associated chitosan doses and the content of these compounds at harvest and postharvest.

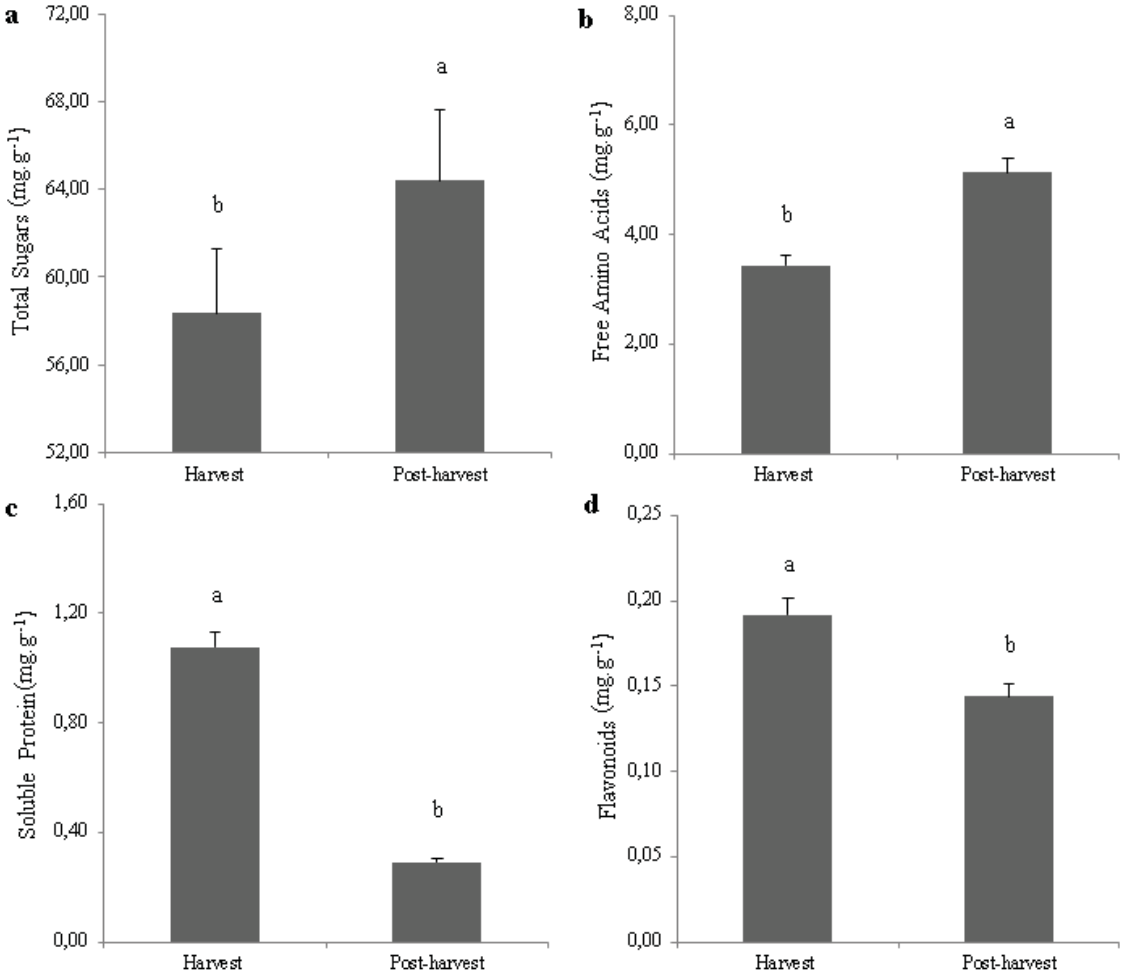


Figure 4- Contents of total sugars (a) total free amino acids (b), soluble proteins (c) and flavonoids (d) in organically cultivated onion bulbs, analyzed after harvest (harvest) and eight-week storage (post-harvest). Columns with the same letter do not differ statistically ($p < 0.01$) according to Scott-Knott test (n=3). Bars represent standard error.

4. Discussion

The application of 1ml.L^{-1} chitosan and 1ml.L^{-1} copper-associated chitosan did not affect onion growth and yield. Among the several functions of chitosan for plants, its use as a growth promoter is one of them (SALACHNA & ZAWADZIŃSKA, 2014),

but when the intention is to promote changes in secondary metabolism aimed at inducing disease resistance, it is important that primary metabolism (yield, percentage of commercial bulbs, dry mass, as well as total and reducing sugars, total free amino acids and soluble proteins) are not reduced, thus demonstrating that energy expenditure on defense did not hinder plant development, which occurred in this work.

The concentration of Cu found in plant tissues is between 1-5 mg g^{-1} dry matter (DM) and the average amount found in the leaves is 10 mg g-1 DM (5-20 mg $.g^{-1}$ DM), although these concentrations may vary between species and varieties of plants of the same species and the ideal copper content in onions is between 10 and 30 mg. kg^{-1}. (YRUELA, 2005). Although copper is an essential nutrient with photosynthesis-related functions (YRUELA, 2019), the applied concentration did not promote increase in bulb yield, percentage of commercial bulbs or increase in the contents of total and reducing sugars, total free amino acids and soluble proteins. The copper values in dry matter suggest that in this work the plants were not in deficiency or toxicity conditions.

About 80% of the onion bulb dry matter consists of non-structural carbohydrates, in which glucose and fructose reducing sugars are the main components as well as non-reducing sucrose and fructooligosaccharides (ZHANG et al., 2018). Sugars are the main products of photosynthesis, and their transport from source to drain is determinant for plant growth, transported in phloem and accumulating in the drain, therefore, stimulating water inflow into the drain (LEMOINE et al., 2013), as observed in Figure 2 a and b, respectively for total and reducing sugars. In the most productive cultivars ‘Perfecta’, ‘Maragogi’ and ‘Alvara’, there was less accumulation of total sugars and greater accumulation of reducing sugars, thus, reflecting gains in size and yield as these carbohydrates participate in the water balance of bulbs.

The increase in P, K, Mn and Zn contents due to the application of copper-associated chitosan indicates that even in association with chitosan, copper had biochemically affected the accumulation of these nutrients. Ballabh et al. (2013) studied the effects of foliar application of micronutrients (Cu, Zn, B, Fe and Mn) on onion growth, yield and quality and indicated that foliar application with micronutrients significantly improved the parameters of vegetative growth, total yield and content of quality in bulb tissues compared to control treatment. El-Hadidi, 2016 states that the 50ppm leaf application of copper allowed to maximize its yield, quality and absorption of nutrients nitrogen, phosphorus, potassium and copper for onion.

Eradication of “hidden hunger” (a term used to describe inherent malnutrition in human diets that is adequate in calories but lacks vitamins and/or mineral nutrients such as Ca, Mg, Fe, Zn, Cu, Se or I), represents a target aspect of food security programs (WHITE & BROADLEY, 2009). Many people in developed countries (for example, the UK or the USA) do not consume adequate amounts of copper (COPPER DEVELOPMENT ASSOCIATION, 2011). Iron deficiency is a major public health issue in over 130 nations, including developed countries, and nearly 50% of the world's population is at risk of inadequate Zn absorption (FAO/WHO, 2001). In this sense, the combination of chitosan with copper can be considered as a suitable method to increase the levels of various minerals in onion bulbs, including P, K, Mn and Z.

Besides favoring the accumulation of nutrients important for human nutrition, the application of copper-associated chitosan results in important physiological interactions for the vegetable. Copper plays an essential role in photosynthesis and respiratory chains of electron transport, ethylene sensor and cell wall metabolism (BERNAL, et al., 2007; BOUAZIZI, et al., 2010; GHORBANPOUR et al., 2016; KE et al., 2007). In addition, copper is part of signaling for protein transcription and transport, iron mobilization and cell-level oxidative phosphorylation in plants (YRUELA, 2005). MONNI et al (2000) found that copper plays an important role in the system as a structural component. Higher copper concentration may act as a stress factor that inhibits growth. The binding of metals to sulfhydryl groups in the protein causes structure disruption and inhibition of protein activity (YRUELA, 2009).

Some correlations with the nutrients that had higher accumulation in this work and application of copper-associated chitosan may be performed. The increase in phosphorus accumulation due to the application of copper-associated chitosan may be due to the role of phosphorus in building energy for plant growth metabolism through cellular productions such as ATP and ADP from early to late life stages of the plant (MARSCHNER, 1995).

Rodrigo-Moreno et al. (2013), demonstrated that copper interferes with the flow of cations such as Ca^{2+} and K^{+} in the root tips of *Arabidopsis thaliana* L. that were subjected to copper foliar applications. In addition to the direct correlations that may be related to K accumulation over application of chitosan with copper, there may have been indirect correlations with other nutrients in which there was an increase in content, such as phosphorus which is also highly linked to essential functions in the plants.

The interactions between Cu-Mn in plant nutrition are reported in the literature to be both synergistic and antagonistic (ADHIKARY & KARKI, 2006). Manganese acts as a cofactor for various enzymes such as superoxide-dismutase, Mn catalase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. The positive correlation between Cu and Mn for both accession organs suggests a possible role of Mn as an antioxidant agent capable of detoxifying reactive oxygen species (TANAKA et al., 1995; YU et al., 1998; YU; RENGEL, 1999).

Copper together with Zn is a constituent of the enzyme superoxide dismutase (Cu/ZnSOD). Ions of Cu and Zn are essential for the transport of photosynthetic electrons to oxygen in the Haber-Mehler reaction. In chloroplasts, superoxide radicals (O_2^-) are formed even under normal metabolism. The Cu/ZnSOD, located near photosystem I, considerably accelerates the decomposition of O_2^- and consequently forms hydrogen peroxide (H_2O_2), O_2 and reactive hydroxyl radical (OH^-) (HÄNSCH & MENDEL (2009).

Onions are rich in polyphenol compounds that encompass antioxidant potential (CHENG et al., 2013). For onion growing, phenolic compounds play a role in aroma and flavor, shelf life and product action as a functional food, notably as an antioxidant. Phenolic concentration is correlated with antioxidant capacity and can be used to monitor product quality in the postharvest phase (CHITARRA & CHITARRA, 2005).

Pichyangkura (2015) describes a likely chitosan chain reaction as a chemical signaler that generates the formation of compounds related to plant defense. Chitosan binds to the plant cell membrane, initiating a secondary messenger signal in the cell and generating hydrogen peroxide (H_2O_2) through the octadecanoid pathway and nitric oxide in the chloroplast. The H_2O_2 triggers the reactive oxygen species elimination system and the abscisic acid (ABA) synthesis pathway, while nitric oxide regulates phosphatidic acid synthesis via the phospholipase C and diacylglycerol kinase pathways. Phosphatidic acid enhances the action of ABA by inhibiting ABI1 (the ABA negative regulator). Thus, ABA induces stomatal closure and other responses to abiotic stress. H_2O_2 coordinates the activity with jasminic acid (JA), synthesized by the octadecanoid pathway, regulating the expression of biotic stress-responsive genes (for example, chitinase or glucanase).

The signal transduction pathway, hydrogen peroxide is considered a secondary messenger of defense signals by stimulating phenylpropanoid pathway genes

(APOSTOL et al.,1989), which also greatly increases PAL activity - a key enzyme in phenolic biosynthesis (CAMM & TORRES, 1973; HAO et al., 2015). Chitosan application also improved biosynthesis of phenols in various plants such as *Thymus daenensis*, sunflower, tomato, apricot and stevia (MUKHTAR AHMED et al., 2019), as observed in the greenhouse and field experiment.

An important relationship between copper nutrient and phenolic compounds is in the chemical and enzymatic mechanism of protection against oxidation. The enzymatic system is formed by several enzymes, especially superoxide dismutase (SOD) that acts by transforming two superoxide radical anions into a hydrogen peroxide, which is a normal reaction at physiological pH but very accelerated through this enzyme. Superoxide dismutase can occur in three ways, depending on the metal associated with it (copper and zinc in eukaryotic cytoplasm, manganese in mitochondrial matrix, iron in bacteria) (PRINTZ et al., 2016).

Storage is a factor that may alter the content of compounds in onion bulbs (RODRIGUES et al, 2010). The bulbs analyzed after 8 weeks of storage had higher levels of total sugars and amino acids, while the bulbs analyzed at harvest had higher levels of proteins and flavonoids (Figure 4). Onions contain high levels of flavonoids, an important class of non-nutrient antioxidants (RODRIGUES et al, 2010) but the susceptibility to induction of these phytochemicals in vegetables depends on several factors such as cultivar type, stage of maturity, initial phenolic levels in the tissues of the plants and stress intensity, among others (CISNEROS-ZEVALLOS, 2003). The interest in the role of antioxidants in human health has led to an effort to evaluate the antioxidant properties of fruits and vegetables and to determine whether these properties can be maintained or improved, for example, with or without postharvest storage periods (HAGEN et al., 2007; HIGASHIO et al., 2005). This work demonstrated that the highest flavonoid contents occur at harvest and decrease during storage.

5. Conclusions

The application of copper-associated chitosan ($1ml.L^{-1}$) appears as a valid horticultural technique to improve the nutritional quality of onion bulbs by increasing the contents of P, K, Mn and Zn. The resulting metabolic changes did not promote changes in primary metabolism to the point of affecting productivity as well as the average water content. There was an increase in phenolic compounds, which are directly

linked to bulb quality. Biochemical evaluations related to plant defense metabolic pathways, pathogenicity-related enzymes, as well as studies related to postharvest improvement of bulbs are recommended to elucidate the mechanisms of action of the product used in this work.

6. CONCLUSÕES GERAIS

A quitosana associada ao cobre apresentou maior eficiência na ativação de enzimas relacionadas à patogenicidade em tomateiro do que a quitosana ou cobre isoladamente. A quitosana associada ao cobre EDTA aumentou o acúmulo de compostos fenólicos, a espessura da parede periclinal externa e da cutícula nas folhas de tomateiro, bem como a proporção que a epiderme ocupa no limbo. Esses fatores combinados permitiram reduzir concomitantemente a gravidade do oídio, sem afetar os parâmetros biométricos e bioquímicos relacionados ao metabolismo primário.

A aplicação de quitosana na cebola promove maior acúmulo de compostos relacionados ao metabolismo da defesa, com maior atividade de enzimas relacionadas à patogenicidade nas folhas. A aplicação de quitosana em campo, em doses acima de $1m.L^{-1}$, promove um desvio de energia que resulta na redução dos parâmetros biométricos da cultura, porém, nessa dose, não causa perdas relacionadas ao rendimento total e comercial, e porcentagem de bulbos comerciais. A quitosana promove alterações nos níveis de prolina na cebola, que podem estar relacionadas ao estresse oxidativo e à ativação da rota do ácido salicílico nos órgãos reprodutivos. Existem diferenças de comportamento entre as cultivares quanto ao maior acúmulo de peroxidase nas folhas e compostos fenólicos nos bulbos devido à aplicação de quitosana.

A aplicação de quitosana associada ao cobre ($1ml.L^{-1}$) aparece como uma técnica hortícola válida para melhorar a qualidade nutricional de bulbos de cebola aumentando os teores de P, K, Mn e Zn. Alterações metabólicas decorrentes não promoveram modificações no metabolismo primário a ponto de afetar a produtividade, bem como o teor médio de água. Houve aumento de compostos fenólicos, que estão diretamente ligados a qualidade dos bulbos.

7. CONSIDERAÇÕES FINAIS

Este trabalho está em consonância com os trabalhos recentes de pesquisa que indicam que a quitosana pode ser benéfica aos cultivos vegetais e que sua atuação no metabolismo secundário pode resultar em melhor capacidade de defesa contra doenças bem como melhorias na qualidade. Foram obtidos dados inéditos para o cultivo de tomate e cebola no que se diz respeito às vantagens da associação de quitosana ao micronutriente cobre quelatizado.

A aplicação foliar de quitosana associada ao quelato de cobre pode ser uma técnica eficiente para a indução de resistência no cultivo orgânico de tomate.

A quitosana aplicada nas folhas das plantas de cebola afeta o metabolismo primário e secundário e pode induzir respostas de defesa foliar através do aumento de proteínas relacionadas à patogenicidade e resistência ao estresse abiótico nos bulbos, aumentando a acumulação de prolina. Quando associada ao cobre a quitosana pode favorecer o acúmulo de nutrientes no bulbo bem como o aumento no teor de compostos fenólicos.

Avaliações bioquímicas relacionadas a enzimas relacionadas à patogenicidade na interação de quitosana com outros micronutrientes, bem como estudos relacionados a controle de doenças que afetam as diversas regiões produtoras são recomendadas para elucidação dos mecanismos de ação do produto utilizado neste trabalho e para o desenvolvimento de inovações tecnológicas para o produtor rural.

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