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Metabolismo de defesa em *Solanum tuberosum* em resposta à fitobactéria *Pectobacterium atrosepticum* e a indutores de resistência vegetal

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METABOLISMO DE DEFESA EM *Solanum tuberosum* EM RESPOSTA À FITOBACTÉRIA *Pectobacterium atrosepticum* E A INDUTORES DE RESISTÊNCIA VEGETAL

Dissertação apresentada como requisito para obtenção do título de Mestre em Biologia Celular e Molecular pela Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Prof. Dr. Leandro Vieira Astarita

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RESUMO

A batata destaca-se como o terceiro alimento mais consumido no mundo. Apesar de sua alta produtividade, inúmeros problemas fitossanitários vêm causando perdas na quantidade e na qualidade do produto. Sua alta suscetibilidade a patógenos, como a bactéria necrotrófica *Pectobacterium atrosepticum*, tem levado ao uso indiscriminado de agrotóxicos, causando danos ao meio ambiente e à população humana. Estudos com indutores de resistência vegetal têm representado uma alternativa para o controle de doenças em algumas culturas através da promoção do sistema inato de defesa contra patógenos. Compreender as modificações no metabolismo vegetal relacionado à defesa possibilita a avaliação e o desenvolvimento de novos recursos e produtos para o manejo fitossanitário. Este trabalho teve por objetivo avaliar alguns dos mecanismos envolvidos no metabolismo de defesa de *Solanum tuberosum* em resposta à fitopabactéria *Pectobacterium atrosepticum* e aos indutores XTH e ASM. Para tal, foram realizadas análises de atividade das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APx), das enzimas relacionadas ao metabolismo de defesa, polifenoloxidase (PPO), peroxidase (POX), fenilalanina amônia liase (PAL), e das proteínas relacionadas à patôgenese, β-1,3-glucanase e quitinase. Foi determinado também o acúmulo de compostos fenólicos e de ácido salicílico (AS), além da avaliação da manifestação da doença provocada pela fitobactéria em folhas pré-tratadas com XTH. Os resultados demonstraram que o indutor XTH foi capaz de retardar a progressão e os sintomas da doença provocados pela bactéria *P. atrosepticum* em folhas de batata. Os indutores XTH e ASM promoveram a ativação das enzimas antioxidantes, assim como a indução de enzimas relacionadas à defesa, anteriormente e posteriormente à inoculação de *P. atrosepticum*, diferindo do padrão de resposta observado nas folhas tratadas apenas com a bactéria patogênica. Além disso, foi observado um aumento dos níveis de AS livre nas folhas tratadas apenas com a fitobactéria, diferindo dos outros tratamentos. A partir destes resultados conclui-se que o indutor XTH promove resistência contra a bactéria necrotrófica *P. atrospeticum* através da ativação e modulação do metabolismo de defesa em *S. tuberosum*.

Palavras-chave: indutor biótico, ASM, enzimas antioxidantes, enzimas relacionadas à defesa, batata.

ABSTRACT

The potato is the third most important consumed food in the world. Despite its high productivity, many pest problems cause losses in the quantity and quality of the product. Its high susceptibility to pathogens such as the necrotrophic bacteria *Pectobacterium atrosepticum*, has led to the indiscriminate use of pesticides, causing damage to the environment and human population. Studies of plant resistance inducers represent an alternative for disease control in some cultures by promoting innate defense system against pathogens. Understanding the changes in plant metabolism related to defense enables the evaluation and development of new strategies and products to disease control. This work aimed was to evaluate some of the mechanisms involved in defense metabolism of *Solanum tuberosum* in response to the phytobacteria *Pectobacterium atrosepticum* as well as to the XTH and ASM elicitors. To this end, we analyzed the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APx), enzymes related to defense metabolism, polyphenol oxidase (PPO), peroxidase (POX), phenylalanine ammonia lyase (PAL), and pathogenesis-related proteins, β-1,3-glucanase and chitinase. It was also determined the synthesis of phenolic compounds and salicylic acid (SA), and the assessment of disease progression caused by the phytobacteria in leaves pretreated with XTH. Our results showed that the elicitor XTH was able to delay the disease progression and symptoms caused by the bacteria *P. atrosepticum*. The elicitors ASM and XTH promoted the activation of the antioxidant enzymes, as well as the induction of certain enzymes related to defense before and after the bacterial inoculation, differing from the response pattern observed in the leaves treated only with *P. atrosepticum*. In addition, we observed an increase in free SA levels in leaves treated only with the phytobacteria, differing from the other treatments. In conclusion, the elicitor XTH promotes resistance to the necrotrophic bacteria *P. atrospeticum* by the defense metabolism activation and modulation of *S. tuberosum*.

Key words: biotic elicitor, ASM, antioxidant enzymes, defense-related enzymes, potato.

LISTA DE ABREVIATURAS

APx - Ascorbato Peroxidase

AS - Ácido Salicílico

ASM - Acibenzolar-S-metil

CAT - Catalase

EROs - Espécies Reativas de Oxigênio

ET - Etileno

ETI - Imunidade Desencadeada por Efetor

FAO - Organização das Nações Unidas para a Agricultura e Alimentação

HR - Resposta de Hipersensibilidade

AJ – Ácido Jasmônico

LRR - Regiões Ricas em Repetições de Leucina

LysM - Regiões Ricas em Repetições de Lisina

MAMPs - Padrões Moleculares Associados a Microrganismos

PAL - Fenilalanina amônia liase

PAMPs – Padrões Moleculares Associados a Patógenos

POX - Peroxidase

PPO - Polifenoloxidase

PR - Proteínas Relacionadas à Patogênese

PRRs - Receptores de Padrões presentes na Membrana Plasmática

Proteínas R - Proteínas de Resistência

PTI - Imunidade Desencadeada por Padrões

SAR - Resistência Sistêmica Adquirida

SOD - Superóxido Dismutase

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Capítulo I

INTRODUÇÃO
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1. INTRODUÇÃO

1.1 *Solanum tuberosum*

A batata é uma planta dicotiledônia perene, embora seja cultivada bianualmente em algumas regiões. Sua parte aérea é herbácea, composta pelo caule e por folhas compostas, podendo atingir mais de 100 cm de altura na fase adulta. A parte subterrânea, que compreende o caule, dá origem aos tubérculos e raízes ramificadas. Pertencente à família Solanaceae, compartilha o gênero *Solanum* com várias outras espécies de importância econômica, como por exemplo, o tomate e a beringela. Taxonomicamente é classificada de acordo com o grau de ploidia, podendo ser diplóide, triplóide, tetraplóide ou pentaplóide. No grupo das tetraplóides encontram-se a subespécie *Solanum tuberosum* subsp. *andigena*, adaptada a dias curtos e cultivada apenas nos Andes, e a subespécie *Solanum tuberosum* subsp. *tuberosum*, amplamente cultivada no mundo e com grande importância econômica (Pereira e Daniels, 2003).

De acordo com relatos históricos, a batata foi levada para a Europa por colonizadores espanhóis durante o século XVI depois da invasão ao Império Inca, o qual já vinha desenvolvendo a bataticultura há oito milênios. Por ser originária da região do Lago Titicaca, entre a Bolívia e o Peru, acredita-se que a subespécie *Solanum tuberosum* subsp. *tuberosum* seja o resultado da introdução e adaptação da espécie *S. tuberosum* subsp. *andigena* às novas condições de clima e fotoperíodo. Após a adaptação às condições do continente europeu, cerca de 200 anos depois de sua introdução, a batata tornou-se um alimento básico na Europa, sendo, a partir de então, introduzida nos demais continentes. Contudo, paralelamente à sua adaptação, houve acentuada erosão genética, perdendo-se importantes genes responsáveis pela resistência a doenças e pragas – razão da elevada suscetibilidade das cultivares européias (FAO, 2008).

A batata destaca-se como o terceiro alimento mais consumido do mundo, ficando atrás apenas do trigo e do arroz (FAO, 2008*et al*). Segundo cálculos da Organização das Nações Unidas para a Agricultura e Alimentação (FAO), em 2003, a produção mundial da batata movimentou cerca US$ 63 bilhões (Nakano e Deleo, 2006). Em 2009, a área mundial plantada foi de 18.651.838 ha, e a quantidade produzida foi de 325.302.445 toneladas. Destacam-se na produção (avaliada por milhão de tonelada produzida; dados de 2009), a China (73.281.890 t), Índia (34.391.000 t), Federação Russa (31.134.000 t), Estados Unidos (19.569.100 t), e Alemanha (11.617.500 t) (Spooner, 2013).

Em 2008, devido à importância da batata em relação às questões relativas à fome, à miséria e às ameaças ao meio ambiente, a Organização das Nações Unidas (ONU) declarou o Ano Internacional da Batata. De acordo com Albuquerque (2008), a ONU acredita

que a batata deva ser um componente importante no combate à pobreza e à miséria, pois sua produção é ideal em regiões que apresentem escassez de terra e abundância de mão-de-obra, condições que caracterizam boa parte dos países em desenvolvimento. Além disso, a batata possui alto valor nutritivo, pouca gordura e é rica em micronutrientes, especialmente vitamina C, destacando-se também pela alta quantidade de proteína, semelhante à encontrada em outros cereais e mais alta quando comparada com outras raízes e tubérculos (Ezekiel *et al*., 2011; FAO, 2008).

No Brasil, cerca de 3,4 milhões de toneladas de tubérculos são produzidos em aproximadamente 123 mil ha ao ano, o que representa uma produtividade baixa em relação à média mundial. As regiões que mais se destacam em termos de produção são o Sudeste, Minas Gerais e Sul (IBGE, 2013). Atualmente, tem se observado a diminuição da área plantada e do número de agricultores familiares no cultivo da batata. Este abandono se deve ao alto custo de produção, alta suscetibilidade a patógenos e variação no preço de comercialização (Fioreze, 2005). Em 2006, o Projeto Hortifruti Brasil, do Centro de Estudos Avançados em Economia Aplicada (Cepea) estimou, junto aos principais produtores do Sudoeste Paulista, custos em torno de US$ 5,5 mil/ha para atingir uma produtividade média de 30 t/ha. Os maiores gastos estão relacionados à compra e à aplicação de defensivos químicos e fertilizantes (Nakano e Deleo, 2006). Contudo, apesar das práticas de manejo, inúmeros problemas fitossanitários vêm causando perdas na quantidade e na qualidade do produto.

1.2 *Pectobacterium atrosepticum*

A batata é afetada por muitas doenças que têm provocado perdas econômicas mundiais. Patógenos bacterianos, como do gênero *Pectobacterium*, são frequentes na maioria das áreas de produção e requerem múltiplas medidas de controle para mitigar sua incidência (Rivero *et al*., 2012). As bactérias do gênero *Pectobacterium* (anteriormente classificado como *Erwinia*) são bactérias pertencentes à família das Enterobacteriaceae, encontradas na superfície de plantas e em praticamente todos os solos cultiváveis (Ban *et al*., 2009; Bell *et al*., 2004). São bactérias necrotróficas, Gram-negativas, anaeróbias facultativas, que não formam esporos, e caracterizam-se pela produção de grandes quantidades de enzimas pectolíticas extracelulares. Elas baseiam-se, principalmente, na produção destas enzimas, em conjunto com uma ampla gama de outras enzimas de degradação da parede celular de plantas, para causar doença (Collmer e Keen, 1986). Além disso, podem atuar de maneira furtiva, manipulando a resistência da planta durante o processo de infecção (Liu *et al*., 2008).

As pectobactérias podem viver de forma epífita ou endofítica nas plantas, ou como saprófitas no solo ou em águas subterrâneas (Pérombelon, 2002). Segundo Pérombelon e Kelman (1980), bactérias do gênero *Pectobacterium* podem se espalhar superficialmente via águas pluviais através de partículas sólidas dispersas no vapor d'água, além da transmissão por insetos. A grande especificidade ao hospedeiro (batatas) e a fatores ecológicos favorece a sobrevivência da *Pectobacterium atrosepticum* de uma época para a outra, numa cultura vegetativa com produção em regiões temperadas frias. Sua patogenicidade é dependente da temperatura, ocasionando sintomas de doença abaixo de 25 °C (Pérombelon, 2002).

A *P. atrosepticum* é causadora de várias doenças de grande importância na cultura da batata, como a canela-preta e a podridão-mole (Oliveira, 2011). A canela-preta é caracterizada pelo enegrecimento da haste da planta na região do colo e descoloração dos vasos (Oliveira, 2011). Já a podridão-mole é uma doença no tubérculo caracterizada pelo apodrecimento aquoso dos tecidos (Oliveira, 2011). As pectobactérias infectam tubérculos e hastes da planta através dos estolões, de aberturas naturais, como as lenticelas, e de ferimentos causados pela queda de folhas a partir do ataque de insetos, por nematóides ou por práticas culturais (Chung *et al.*, 2013; Pereira e Daniels, 2003). As folhas da planta infectada tornam-se amarelas e murcham, causando um déficit na fotossíntese, comprometendo a formação de novos tubérculos. Atualmente, não há cultivares de batata resistentes à podridão mole causada por pectobactérias (Benelli *et al.*, 2004).

1.3 Indutores

A elevada suscetibilidade a patógenos dos principais cultivares agrícolas tem promovido o uso intenso de defensivos químicos, ocasionando aumento no custo de produção, surgimento de novas raças de patógenos mais resistentes e agressivas, além de aumentar os riscos de danos ao meio ambiente e à saúde da população (Aitbayeva *et alet al.*, 2013; Solla *et alet al.*, 2013; Thakur e Sohal, 2013). Neste cenário, o uso de indutores de respostas de defesa vegetal vem sendo largamente estudado, visando à promoção de resistência local ou sistêmica da planta, fornecendo ferramentas para o manejo de lavouras, promovendo a diminuição no uso de agroquímicos e preservando o uso de cultivares com alto potencial agrícola que apresentam alta suscetibilidade a patógenos (Cavalcanti *et alet al.*, 2005; Thakur e Sohal, 2013).

Indutores são moléculas que promovem respostas locais e/ou sistêmicas de defesa não-específicas em plantas, podendo proteger o vegetal contra ataques subsequentes de patógenos. Os indutores podem ser classificados em indutores abióticos e indutores bióticos. Os indutores abióticos incluem fatores de estresse ambiental, tais como radiação UV, temperatura, seca e metais, bem como compostos químicos que atuam como

hormônios ou moléculas sinalizadoras nos vegetais. Dentre os indutores abióticos, destaca-se o acibenzolar-S-metil (ASM), um análogo sintético do ácido salicílico, o qual permitiu a formulação do indutor comercial Bion® (Syngenta Proteção de Cultivos Ltda). Este indutor representa uma nova geração de protetores de plantas contra patógenos biotróficos e, principalmente, necrotróficos via resistência sistêmica adquirida (SAR) (Lyon e Newton, 1997). O ASM é um dos principais indutores abióticos testados extensivamente em diferentes condições de campo e interações planta-patógenos (Leskovar e Kolenda, 2002; Vallad e Goodman, 2004; Walters *et alet al.*, 2005). Rohilla *et alet al.* (2001) demonstraram que o efeito protetivo do ASM em arroz contra o fungo necrotrófico *Rhizoctonia solani* ocorreu devido à combinação entre a indução da resposta de defesa da planta e seu efeito adverso no crescimento e vigor do patógeno (Rohilla *et alet al.*, 2001). Luzzato *et alet al.* (2007) também observaram uma diminuição nos sintomas de doença e na proliferação da bactéria *Pectobacterium carotovorum* nas flores de copo-de-leite (*Zantedeschia aethiopica*) tratadas com Bion®.

Os indutores bióticos são representados por organismos vivos ou partes destes, patogênicos ou não, ativos ou inativados, ou por seus metabólitos, capazes de ativar a resposta de defesa local ou sistêmica da planta (Doke *et alet al.*, 1987). A indução da resistência através de indutores bióticos tem sido relatada em diversas espécies vegetais, como pepino, fumo, tomate, batata, melão, melancia, trigo, uva e cevada (Cavalcanti *et alet al.*, 2005). Sanchez *et alet al.* (2012) demonstraram que ramnolipídeos bacterianos promovem a ativação das respostas de defesa em *Arabidopsis thaliana* contra a bactéria hemibiotrófica *Pseudomonas syringae* pv. *tomato*, o oomiceto biotrófico *Hyaloperonospora arabidopsidis* e o fungo necrotrófico *Botrytis cinerea* (Sanchez *et alet al.*, 2012). Doke *et alet al.* (1987) induziram a resistência sistêmica em batatas contra *Phytophthora infestans* a partir de uma mistura contendo fragmentos de parede celular do fungo (Doke *et alet al.*, 1987). Outras pesquisas têm utilizado diferentes microrganismos para a promoção da defesa em diversas culturas. O uso de eliciadores da parede celular de *Saccharomyces cerevisiae* apresenta resultados promissores no controle de doenças em videira, batata e tomate, quando utilizados em conjunto com fungicidas tradicionais, possibilitando a redução do número de aplicações destes (Cavalcanti *et alet al.*, 2005).

1.4 Sistema imune vegetal

Os microrganismos patogênicos vêm evoluindo com as plantas há mais de 400 milhões de anos, a fim de retirar nutrientes de seu hospedeiro. Por sua vez, as plantas desenvolveram diferentes mecanismos para inibir a infecção por microrganismos patogênicos, sendo a cutícula e a parede celular as primeiras barreiras físicas. Quando os

patógenos superam estas barreiras, a planta recorre ao seu sistema imune inato. A imunidade inata é a primeira linha e a mais crítica na defesa da planta contra patógenos. Diferente dos mamíferos, as plantas não possuem células móveis e um sistema circulatório. Devido a isto, elas contam com a imunidade inata de cada célula e sinais sistêmicos que se deslocam a partir da região de infecção (Dangl *et al.*, 2013; Jones e Dangl, 2006; Spoel e Dong, 2012; Yu *et al.*, 2013).

Muitos componentes da superfície de bactérias como flagelina e lipopolissacarídeos (LPS) de bactérias Gram-negativas são reconhecidos e promovem a ativação do sistema imune inato de defesa das plantas. Estes componentes representam os Padrões Moleculares Associados a Patógenos (PAMPs). Estudos têm demonstrado que LPS e proteínas *harpin-like* presentes em *Xanthomonas axonopodis* pv. *citri* e *Xanthomonas campestris* pv. *campestris* atuam como PAMPs em diversas espécies vegetais (Kim *et al.*, 2003; Casabuono *et al.*, 2011). Proteínas *harpin-like* têm sido reportadas em todos os gêneros de *Xanthomonas.* Estes polipeptídios pertencem ao sistema de secreção de proteínas de virulência do tipo III (Alfano e Collmer, 2004).

Semelhante aos PAMPs, a imunidade inata da planta pode ser desencadeada a partir do reconhecimento extracelular de Padrões Moleculares Associados a Microrganismos (MAMPs) não patogênicos, como por exemplo, a quitina encontrada na parede celular de fungos e a flagelina encontrada no flagelo de bactérias (Faulkner e Robatzek, 2012). Este reconhecimento ocorre através de receptores de padrões presentes na membrana plasmática (PRRs), os quais apresentam um domínio extracelular contendo regiões ricas em repetições de leucina (LRR) ou lisina (LysM), responsável pelo reconhecimento de MAMPs e PAMPs e um domínio proteína quinase citosólico, requerido para a transdução do sinal. O reconhecimento de PAMPs e MAMPs inicia uma cascata de sinalização envolvendo, algumas vezes, MAP-quinases. Esta sinalização promove a indução de respostas de defesa, que incluem a produção de proteínas Relacionadas à Patogênese (proteínas PR) e de espécies reativas de oxigênio (EROs). As EROs podem atuar diretamente no patógeno e agir como sinalizadores para a indução da resposta de defesa na planta. Frequentemente, o reconhecimento inicial e as respostas de defesa ao patógeno, denominada de Imunidade Desencadeada por Padrões (PTI), são suficientes para eliminar ou deter a invasão do patógeno (Herman e Williams, 2012).

Para burlar ou interromper a PTI, alguns patógenos liberam dentro da célula ou no apoplasto do hospedeiro, proteínas efetoras que facilitam a aquisição de nutrientes e contribuem para dispersão patogênica no tecido vegetal (Dangl *et al.*, 2013). Muitos patógenos produzem diferentes efetores com diferentes funções, como por exemplo, os

efetores Avr2 e Ecp6 de *Cladosporium fulvum.* O efetor Avr2 age inibindo as proteases de defesa da planta no apoplasto e o Ecp6 atua se ligando à quitina da parede celular do fungo, impedindo o reconhecimento deste pelas PRRs (Herman e Williams, 2012). A bactéria necrotrófica *Pectobacterium carotovorum* e o fungo necrotrófico *Cochliobolus victoriae* utilizam seus efetores DspE e victorina, respectivamente, para causar morte celular no tecido, facilitando a aquisição de nutrientes (Hogan *et al.*, 2013; Lorang *et al.*, 2012). Os patógenos que utilizam efetores têm mais sucesso em seu estabelecimento, promovendo uma forte pressão seletiva nos seus hospedeiros.

Através da coevolução, as plantas desenvolveram mecanismos de reconhecimento dos efetores. As proteínas de Resistência (proteínas R) reconhecem diretamente os efetores ou reconhecem anormalidades em proteínas-alvo dos efetores, como por exemplo, a proteína R LOV1 de Arabidopsis, que é ativada após a ligação do efetor victorina na proteína TRX-5, uma tiorredoxina relacionada à defesa. Essa segunda linha de resposta denominada de Imunidade Desencadeada por Efetor (ETI) é semelhante à PTI, porém mais forte, mais rápida e prolongada (Herman e Williams, 2012; Lorang *et al.*, 2012; Spoel e Dong, 2012).

As proteínas R podem promover a síntese de moléculas sinalizadoras, como por exemplo, o ácido salicílico e EROs, que podem contribuir para a indução de genes de defesa e também iniciar uma Resposta de Hipersensibilidade (HR) (Herman e Williams, 2012). A HR é caracterizada pela síntese de moléculas antimicrobianas, como as enzimas hidrolíticas β-glucanase e quitinase, e pela morte das células no local de infecção. A HR protege o vegetal através da promoção de uma resistência local contra o patógeno (Spoel e Dong, 2012). Cabe ressaltar que a resposta de hipersensibilidade nem sempre é a melhor alternativa para impedir o crescimento do patógeno. No caso de patógenos necrotróficos, como a *P. atrosepticum,* a morte celular promoveria o desenvolvimento do patógeno no hospedeiro. Desta forma, alguns patógenos necrotróficos como *Cochliobolus victoriae* e *P. atrosepticum* utilizam do sistema de defesa da planta para promover a morte celular, mediada pelo reconhecimento de seus efetores através de proteínas R.

1.4.1 Resistência sistêmica adquirida

A Resistência Sistêmica Adquirida (SAR) é um sistema de defesa que prepara a planta contra ataques subsequentes em resposta ao primeiro contato com o patógeno, tornando-a mais resistente contra o mesmo ou contra outros patógenos (Frías *et al.*, 2013). Esta resposta foi descrita na década de 1960, quando folhas não infectadas de uma planta infectada por vírus apresentaram resistência a subsequentes infecções virais (Herman e Williams, 2012).

A SAR pode ser induzida por patógenos de diferentes estilos de vida (através de PTI ou ETI), promovendo uma resistência de amplo espectro contra fungos, oomicetos, vírus e bactérias (Herman e Williams, 2012; Kuc, 1987). Através de moléculas sinalizadoras como o ácido salicílico (AS), a SAR induz, em locais distantes daqueles que entraram em contato com o patógeno, alterações metabólicas e estruturais, tais como a formação de EROs, a biossíntese de fitoalexinas, o espessamento da parede celular, a deposição de calose, a síntese de enzimas de defesa e o acúmulo de proteínas PR (Thakur e Sohal, 2013).

A SAR pode durar semanas ou meses e, possivelmente, todo o período de crescimento da planta (Kuc, 1987). Yu *et al.* (1997) demonstraram que o AS é essencial para a ativação de SAR contra o oomiceto *Phytophthora infestans* em batata. Estes autores sugerem que a SAR está relacionada ao aumento na sensibilidade celular ao AS, pois os níveis basais elevados de AS nestes tecidos não garantem a resistência constitutiva nas plantas de batata saudáveis (Yu *et al.*, 1997). Além disso, estudos têm demonstrado que as defesas mediadas pelo AS também podem ser herdadas através de genes hipometilados, resultando na melhoria da resistência contra patógenos em gerações subsequentes (Fu and Dong, 2013; Luna et al., 2012). Alguns estudos têm demonstrado que a via de defesa através do AS não é a única utilizada pela planta como sinalizadora de resistência sistêmica. Sanchez *et al.* (2012) demonstraram que ramnolipídeos bacterianos induzem resistência em Arabidopsis através de diferentes sinais, dependendo do tipo de patógeno. Eles observaram que a resistência para o fungo necrotrófico *Botrytis cinerea* envolve os hormônios sinalizadores AS e ácido jasmônico (AJ), enquanto que os hormônios AS e etileno (ET) estavam envolvidos na via de sinalização de defesa para a bactéria hemibiotrófica *Pseudomonas syringae* pv. *tomato* e o oomiceto *Hyaloperonospora arabidopsis* (Sanchez *et al.*, 2012).

1.4.2 Metabolismo bioquímico da defesa vegetal

1.4.2.1 Superóxido dismutase, catalase e ascorbato peroxidase

As espécies reativas de oxigênio, como o peróxido de hidrogênio (H_2O_2) e o superóxido (O_2^-), são importantes moléculas sinalizadoras associadas à defesa vegetal, morte celular e abertura estomática (Foyer e Noctor, 2005; Karpinski *et al.*, 1999). O acúmulo de EROs nas células pode levar a uma resposta de hipersensibilidade, assim como a expressão de genes relacionados à defesa (Torres e Dangl, 2005). Estudos demonstraram que há uma rápida formação de O_2^- e H_2O_2 em respostas de HR em plantas inoculadas com fungos avirulentos, bactérias ou vírus (Lamb e Dixon, 1997). Em soja inoculada com raças avirulentas ou virulentas de *Pseudomonas syringae* pv. *glycinea* houve um fraco e transitório acúmulo de oxidantes. Porém, uma segunda inoculação com uma raça aviruleta levou a uma massiva e prolongada explosão oxidativa entre 3 e 6 horas após a inoculação (Baker e

Orlandi, 1995; Orlandi *et al*., 1992). Respostas semelhantes a esta explosão oxidativa também foram observadas em células de tabaco inoculadas com *Pseudomonas syringae pv. syringae* avirulenta (Atkinson *et al*., 1990; Keppler *et al*., 1989). Larroque *et al*. (2013) demonstraram que a resistência em Arabidopsis a *Phytophthora parasitica* é dependente da explosão oxidativa, porém a formação de EROs não está envolvida na morte celular. Contudo, as EROs também podem ser prejudiciais ao desenvolvimento da planta, pois níveis descontrolados podem causar danos às estruturas celulares do vegetal. Para o controle dos níveis de EROs, as células vegetais contam com enzimas antioxidantes como a superóxido dismutase (SOD), ascorbato peroxidase (APx) e catalase (CAT) (Lee *et al*., 2007).

A SOD cataliza a conversão de superóxido (O_2^-) à H_2O_2. Além do H_2O_2 ser mais estável e menos reativo que o O_2^-, ele desempenha um importante papel na patogênese da planta (Baker e Orlandi, 1995). O H_2O_2 está ligado a várias linhas de defesa das células vegetais. Ele pode atuar inibindo diretamente o crescimento do patógeno, atuar na via de produção de fitoalexinas, na expressão de genes de defesa, na morte celular em respostas de hipersensibilidade, no reforço da parede celular, na via de biossíntese do AS e, consequentemente, na SAR (Lamb e Dixon, 1997).

A APx pode ser encontrada no cloroplasto ou no citosol das células vegetais (Jespersen *et al*., 1997). Ela é responsável por reduzir o H_2O_2 a água e oxigênio através do ascorbato que atua como doador de elétrons (Tang *et al*., 2006). Além disso, apresenta um importante papel na defesa vegetal, protegendo as plantas contra agentes patogênicos, via SAR (Kvaratskhelia *et al*., 1997a). Sarowar *et al*. (2005) demonstraram que a superexpressão de APx em tabaco promove a tolerância ao estresse oxidativo e resistência ao oomiceto *Phytophthora nicotianae*. Além disso, demonstraram que, em plantas trangênicas com superexpressão de APx, houve um aumento na expressão de PR-1, comumente utilizada como marcador de SAR (Sarowar *et al*., 2005).

A CAT, assim como a APx, reduz o H_2O_2 à água e oxigênio. Também apresenta um importante papel na SAR mediada por AS (Witlekens *et al*., 1995). Chen *et al*. (1993) propuseram que o H_2O_2 pode funcionar como uma molécula sinalizadora para SAR através das seguintes observações: (1) AS e análogos se ligam e inativam a CAT *in vivo*; (2) AS leva ao aumento na produção de H_2O_2 *in vivo*; (3) H_2O_2 leva à indução da transcrição de proteínas PR. Niebel *et al*. (1995) demonstraram que os níveis de mRNA de *Cat2St* em batata são induzidos pela infecção com vários patógenos, indicando que a catalase pode interferir em certas funções do H_2O_2 durante as interações planta-patógeno. Além disso, o aumento da “responsividade” das células ao AS e a indução de SAR em plantas de batata

induzidas com o ácido aracdônico, contra *Phythophtora infestans,* está associada ao aumento da expressão da catalase, indicando que esta enzima atua de forma importante na sinalização das respostas de defesa (Yu *et al.*, 1999).

1.4.2.2 Peroxidase, polifenoloxidase e fenilalanina amônia liase

Uma das primeiras linhas de defesa da planta é a síntese de compostos fenólicos. A polimerização dos fenólicos, que leva ao aumento da rigidez da parede celular, é mediada pelas enzimas fenilalanina amônia liase (PAL), peroxidase (POX) e polifenoloxidase (PPO) (Matern e Kneusel, 1988). O aumento nas atividades da POX, PPO e PAL em tecidos vegetais tem sido demonstrado como um importante fator na indução de resistência contra patógenos (Song *et al.*, 2011).

A PAL é responsável por catalisar a conversão de fenilalanina em ácido cinâmico. Além de ser uma enzima chave na biossíntese de compostos fenólicos com atividade antimicrobiana, sua atividade fornece precursores para a biossíntese de lignina e do AS (Mauch-Mani e Slusarenko, 1996; Montesinos, 2000).

A partir do aumento dos níveis de H_2O_2 nas células, observa-se um aumento na atividade das POXs. Estas enzimas estão relacionadas com o aumento na síntese de lignina que fortalece a parede celular contra a ação de enzimas líticas produzidas pelos patógenos, levando ao aumento da resistência mecânica contra diversos patógenos (Kvaratskhelia *et al.*, 1997b; Passardi *et al.*, 2004). Além disso, trabalhos demonstram que peroxidases estão associadas à formação de intermediários reativos de oxigênio (Skelly e Loake, 2013). Bindschedler *et al.* (2006) demonstraram que houve redução na explosão oxidativa e diminuição na resistência contra fungo e bactéria quando os genes de duas peroxidases (PRX33 e PRX34) foram silenciados em Arabdopsis.

As PPOs são enzimas que catalizam duas reações diferentes: hidroxilação de monofenóis para difenóis e oxidação de difenóis a quinonas (Webb *et al.*, 2013). A oxidação de compostos fenólicos a quinonas gera compostos mais tóxicos aos patógenos, podendo levar à resistência vegetal (Mayer e Staples, 2002). Li e Steffens (2002) demonstraram que a superexpressão de PPO em tomate resultou em aumento significantivo da resistência contra *Pseudomonas syringae* pv. *tomato* em interações compatíveis.

1.4.2.3 Compostos fenólicos

Dentre os metabólitos de defesa, destacam-se os compostos fenólicos. Estes apresentam um importante papel na defesa vegetal, através do efeito tóxico direto sobre o patógeno, síntese de moléculas tóxicas catalisadas pela enzima PPO e como componente

na formação da lignina, responsável pelo fortalecimento da parede celular (Hückelhoven, 2007). Esta relação entre fenóis solúveis e a atividade das enzimas PPO, POX, PAL na defesa de plantas de batata contra *Pectobacterium carotovorum* subsp. *brasiliensis* foi demonstrada por Ngadze *et al.* (2012). Da mesma forma, a síntese de compostos fenólicos e de fitoalexinas está envolvida na resistência de batata contra *Phytophtora infestans* (Cheynier *et al.*, 2012).

1.4.2.4 Quitinase e β-1,3-glucanase

Quitinases e β-1,3-glucanases são proteínas PR com atividade antifúngica. Elas fragmentam a parede celular de fungos gerando oligossacarídeos, que atuam como elicitores com potencial de induzir respostas de defesa da planta (Ferreira *et al.*, 2007; Mohammadi *et al.*, 2002). Estas enzimas podem ser encontradas constitutivamente em alguns estágios de desenvolvimento e locais específicos na planta, assim como podem ser induzidas por patógenos ou eliciadores. Em plantas de tomate, o ácido β-aminobutírico induz o acúmulo de β-1,3-glucanases e quitinases, promovendo a resistência contra o oomiceto *Phytophthora infestans* (Cohen *et al.* 1994). Geralmente, quitinases e β-1,3-glucanases são observadas atuando em sinergismo, já que a quitina e o β-1,3-glucano, substratos da quitinase e da β-1,3-glucanase, respectivamente, são sintetizados simultaneamente no ápice do crescimento da hifa fúngica (Ferreira *et al.*, 2007). Em plantas de tomate transgênico foi demonstrado que apenas a expressão de genes de quitinase ou β-1,3-glucanase levava a suscetibilidade ao fungo *Fusarium oxysporum*, enquanto que a expressão dos dois genes levou ao aumento significativo na resistência (Jongedijk *et al.*, 1955).

As β-1,3-glucanases (PR-2) podem ser encontradas no vacúolo da célula vegetal (proteínas básicas, pertencentes à Classe I) e extracelularmente (proteínas ácidas, pertencentes às Classes II e III). As proteínas da Classe I apresentam atividade antifúngica *in vitro* e *in vivo*, enquanto que as da Classe II exibem atividade antifúngica apenas quando na presença de quitinases ou β-1,3-glucanases da Classe I (Ferreira *et al.*, 2007).

As quitinases são o segundo maior grupo de proteínas antifúngicas. Além de serem agrupadas em famílias de PR (PR-3, PR-8, PR-11 e PR-4), são também classificadas em classes (Ferreira *et al.*, 2007). As quitinases ácidas, pertencentes às Classes Ib, II, III, IV e VI, são secretadas no apoplasto, enquanto que as básicas, pertencentes as Classes Ia, III e VI estão localizadas no vacúolo (Arie *et al.*, 2000). Aparentemente, as quitinases do apoplasto estão envolvidas nos estágios iniciais do processo de resposta à patogênese. Por outro lado, as quitinases vacuolares são liberadas devido à penetração das hifas na célula,

reprimindo diretamente o crescimento do fungo através da degradação das cadeias de quitina recém-sintetizadas (Collinge *et al.*, 1993).

2. JUSTIFICATIVA

A batata possui grande importância econômica, sendo o terceiro alimento mais consumido no mundo. Apesar disso, ela apresenta grande suscetibilidade a patógenos devido à perda de genes de resistência em virtude da seleção de cultivares mais produtivos e adaptados ao ambiente no qual foi introduzida. No Brasil, sua produção é baixa em relação à média mundial, devido às condições de cultivo e à alta suscetibilidade das cultivares a patógenos, ocasionando perdas na quantidade e na qualidade do produto, provocando o aumento do custo de produção. Além disso, o uso indiscriminado de agrotóxicos pode levar à contaminação do solo, danos ao meio ambiente e à intoxicação da população humana. Neste contexto, o uso de moléculas promotoras do metabolismo de defesa vegetal pode representar uma ferramenta auxiliar para o manejo de lavouras de batata. Contudo, esta abordagem depende do conhecimento dos mecanismos celulares vegetais envolvidos nas respostas de defesa promovidos por indutores de resistência.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar o metabolismo vegetal relacionado à defesa e à indução de resistência em folhas destacadas de *Solanum tuberosum* em resposta ao eliciador bacteriano *Xanthomonas axonopodis* (XTH), ao indutor comercial Acibenzolar-S-metil (ASM) e à fitobactéria *Pectobacterium atrosepticum.*

3.2 Objetivos específicos

1. Determinar a atividade das enzimas β-1,3-glucanase, polifenoloxidase, fenilalanina amônia liase, peroxidase, superóxido dismutase, catalase, quitinase e ascorbato peroxidase em folhas destacadas de *S. tuberosum* tratadas com XTH, ASM e *P. atrosepticum*;

2. Determinar a síntese de compostos fenólicos e ácido salicílico em folhas destacadas de *S. tuberosum* tratadas com XTH, ASM e *P. atrosepticum*;

3. Avaliar a manifestação da doença provocada por *P. atrosepticum* em folhas destacadas de *S. tuberosum* pré-tratadas com XTH;

4. Determinar a atividade das enzimas β-1,3-glucanase, polifenoloxidase, fenilalanina amônia liase, peroxidase, superóxido dismutase, catalase, quitinase e ascorbato peroxidase em folhas destacadas de *S. tuberosum* pré-tratadas com XTH e ASM, e posteriormente desafiadas com *P. atrosepticum*;

5. Determinar a síntese de compostos fenólicos e ácido salicílico em folhas destacadas de *S. tuberosum* pré-tratadas com XTH e ASM, e posteriormente desafiadas com *P. atrosepticum*.

Capítulo II

ARTIGO CIENTÍFICO 1
ARTIGO CIENTÍFICO 2

ARTIGO CIENTÍFICO 1

Modulation of defense metabolism in leaves of *Solanum tuberosum* promoted by Acibenzolar-S-methyl and *Xanthomonas axonopodis* extract

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Modulation of defense metabolism in leaves of *Solanum tuberosum* promoted by Acibenzolar-S-methyl and and *Xanthomonas axonopodis* extract

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Abstract

The induction of the plant natural immune system has been related to an alternative approach for disease control, holding a promise due to its broad action spectrum. In this study, we investigated the modulation of defense metabolism in leaves of *Solanum tuberosum* induced by the *Xanthomonas axonopodis* pv. *citri* extract (XTH). Acibenzolar-S-methyl (ASM) was used as reference to compare the effectiveness and mode of action of the biotic elicitor. The potato pathogenic bacteria *P. atrosepticum* was used to evaluate changes in the plant defense metabolism. According to the results, the elicitors XTH and ASM were able to activate antioxidant enzymes (SOD, CAT and APx) and promote enzymes related to plant defense. These responses differed from the observed in leaves inoculated with the phytobacteria. Moreover, the increment of free SA level was only observed in leaves treated with *P. atrosepticum*. These results demonstrated that the XTH biotic elicitor was able to modulate the defense metabolism of *S. tuberosum* similarly to the observed in the commercial formulation of ASM and differently from the necrotrophic bacteria *P. atrosepticum*.

Key words: biotic elicitor, ASM, *Pectobacterium atrosepticum*, antioxidant enzymes, defense-related enzymes

1. Introduction

Potato (*Solanum tuberosum*) is affected by many diseases, producing substantial economic losses worldwide. Bacterial pathogens, like the genus *Pectobacterium* are frequent in most production areas and require multiple control measures to mitigate their incidence. *Pectobacterium carotovorum* is the causal agent of blackleg and soft-rot diseases, involving severe symptoms in foliar and tuber tissues, respectively (Rivero *et al*., 2012).

The induction of the natural immune system, as an alternative strategy to disease control in plants, holds promise because of its broad spectrum. The effectiveness of some elicitors for promoting plant resistance has been reported, suggesting the feasibility of adopting this approach for disease control in certain crops. Pretreatment of susceptible plants with avirulent pathogens (biotic elicitors) or certain chemicals (abiotic elicitors), such as acibenzolar-S-methyl (ASM), can enhance resistance to subsequent attack not only at the site of treatment but also in tissues distant from the initial treated sites (Malolepsza, 2009). Depending on the efficacy of the elicitors, these compounds can be used in fields either alone or in combination with fungicides. Biotic elicitors, such as the commercial Elexa® (chitosan), Messenger® (harpin from *Erwinia amylovora*), Ecolife® (citric biomass) and Milsana® (extract from the plant *Reynoutria sachalinensis*) have been effectively used to protect a range of crops against important pathogens. Additionaly, elicitors derived from the yeast *Saccharomyces cerevisiae* have been shown to control plant diseases (Walters *et al*., 2005). The better understanding of plant signalling pathways has led to the discovery of natural and synthetic elicitors that induce similar defense responses in plants as those triggered by the pathogen infection (Gómez-Vásquez *et al*., 2004). Previous studies have demonstrated the

ability of *Xanthomas axonopodis* pv. *citri* in promoting the increased polyphenol oxidase activity and total phenolic compounds in *S. tuberosum* plants (Poiatti *et al*., 2009). Potato plants sprayed with this bacterial formulation also showed a reduction (above 60%) in the disease symptoms caused by *Pectobacterium atrosepticum*, without apparent plant toxicity (CN102256495A, US8932844B2).

Promotion of disease resistance in plants is associated with a wide array of defense responses that slow down or halt infection at certain stages of the host–pathogen interaction. These responses include changes in the accumulation of reactive oxygen species (ROS), signal hormone pathways, such as salicylic acid (SA), synthesis of pathogenesis-related (PR) proteins and enhanced activity of various defense-related enzymes (Baysal *et al*., 2003).

In response to biotic stresses and elicitors, a plant triggers a number of defense reactions which may be related either to direct immune responses or to a specific signal transduction pathway. The oxidative burst is one of the first plant defense responses and result in increased activity of enzymes involved in ROS scavenging, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APx). SOD is the first enzyme in the ROS detoxifying process that converts superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2), whereas APx and CAT enzymes act reducing H_2O_2 to water. Intensive generation of ROS may increase resistance to some biotrophic pathogens, although cell death might promote growth of necrotrophic pathogens (Lamb and Dixon, 1997; Nowogórska and Patykowski, 2015). SA is an important hormone involved in the induction of resistance responses in plants. SA levels increase during many avirulent and some virulent infections, leading to the establishment of systemic acquired resistance (SAR) and activation of various defense-related genes. SAR is characterized by the induction of PR proteins, such as glucanases and chitinases, and many other enzymes involved in the defense metabolism, such

as phenylalanine ammonia lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO) (Sticher *et al*., 1997).

In order to characterize the crude autoclaved extracts of *X. axonopodis* pv. *citri* (XTH) as elicitor, activities of the enzymes SOD, CAT, APx, PAL, PPO, POX, β-1,3-glucanase, and chitinase, as well as the SA levels were evaluated. ASM, from a commercial formulation, was used as reference to compare the effectiveness and mode of action of the biotic elicitor XTH. The pathogenic bacteria *Pectobacterium atrosepticum* was inoculated in leaves of *S. tuberosum* in order to compare and evaluate the changes in the defense metabolism.

2. Materials and Methods

2.1 Plant material

Seed tubers of *Solanum tuberosum* cv. Agata were provided by potato growers (Sao Francisco de Paula, RS, Brazil, 29°26'49"S 50°34'44"W). Tubers were cultivated in pots (8 L) with commercial organic soil and maintained in greenhouse for three or four weeks (stolon and tuber formation phases). Expanded leaves were detached and placed in sterile Petri dishes (15 x 2.5 cm) with two layers of moistened filter paper (15 ml of sterile distilled water). The petioles were covered with wet cotton in order to reduce the leaf dehydration within the plate. Two leaves were placed in each plate. All plates were maintained under photoperiod (16h light) and controlled temperature (25 °C).

2.2 Microorganism

Pectobacterium atrosepticum (provided by Dr. Valmir Duarte, Laboratory of Phytopathology, UFRGS/Brazil), was grown in liquid LB medium (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ NaCl, 10 $g.L^{-1}$ sucrose) at 25 °C for 16 h under 100 rpm. Cultures were centrifuged at 4,000 *xg* for 10 min. Supernatants were discarded, pellets were re-suspended in sterile distilled water and washed three times. In the final step, the pellet was re-suspended in sterile distilled water and the bacterial concentration was adjusted to 10^{10} $cfu.mL^{-1}$ (OD_{600nm} =

0.5) (Poiatti *et al*., 2009). The suspensions of *P. atrosepticum* were immediately used for inoculation of potato detached leaves.

2.3 Preparation of elicitors

Biotic and synthetic elicitors were tested on the detached leaves. *Xanthomonas axonopodis* pv. *citri* was cultivated and cell suspension prepared as previously described for *P. atrosepticum* (except for 48 h of cultivation). Bacterial concentration was adjusted to 10^{20} cfu.mL^{-1} (OD_{600nm} = 1.0) and inactivated by autoclaving for one hour at 121°C. The inactivated bacterial suspension (XTH) was used as the biotic elicitor. The synthetic elicitor was prepared by dissolving Acibenzolar-S-methyl (Bion® 500 WG, Syngenta S.A.) in sterile distilled water (50 mg. L^{-1}).

2.4 Treatment of detached leaves

Elicitor treatments consisted of Acibenzolar-S-methyl (ASM) or XTH sprayed on detached leaves (1 ml/leaf). The suspension of phytobacterium *P. atrosepticum* was inoculated (100 µL) using a needleless syringe on the abaxial surface of the lateral leaflet. Leaves from the control treatment were inoculated with sterile water.

Leaf samples were collected at 0, 12, 24, 72 and 96 hpt (hours post-treatment) and analyzed for the enzyme activities of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX), β-1,3-glucanase and chitinase. Salicylic acid (SA) was also evaluated. Activities of the enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxide (APx) were analyzed in leaf samples collected at 0, 0.5, 1, 3, and 6 hpt.

2.5 Enzyme assays

Phenylalanine ammonia lyase (PAL) - Leaf samples (0.3 g) were homogenized in 3 ml of 50 mM Tris-HCL (pH 8.8) containing 1 mM EDTA, 1% polyvinylpyrrolidone (PVP), and 0.2% Triton X-100. Extracts were centrifuged at 3,200 *xg* for 20 min at 4 °C, and the supernatants were used for enzyme assays. PAL activity was evaluated following the method

of Navarre *et al*. (2013) with modifications. The reaction containing 200 µl of extract, 0.2 M phenylalanine and 50 mM sodium borate buffer (pH 8.8) was incubated for 1 h at 37 °C. The enzyme activity unit was defined as the amount of enzyme needed to produce 1 nmol of cinnamic acid per mL of reaction per minute per mg of protein at 290 nm. Protein concentration was estimated using Bradford reagent with bovine serum albumin (BSA) as standard.

Polyphenol oxidase (PPO) and Peroxidase (POX) - Leaf samples (0.5 g) were homogenized in 2.5 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 1% PVP. Extracts were filtered and centrifuged at 3,200 *xg* for 20 min at 4 °C, and the supernatants were used for enzyme assays. Polyphenol oxidase activity was determined according to Salla *et al*. (2014) spectrophotometrically at 400 nm using chlorogenic acid as substrate. The samples were incubated for 30 min at 30 °C. The enzyme activity unit was defined as the change in absorbance.min^{-1}.mg^{-1} protein. Peroxidase activity was determined spectrophotometrically by the oxidation of guaiacol at 420 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 6.0), 0.1M guaiacol as substrate and 10 mM hydrogen peroxide. The enzyme activity unit was expressed in µkatal.mg^{-1} protein. One katal (kat) corresponds to the quantity of enzyme activity that transforms 1 mol.s^{1} of substrate (Salla *et al*., 2014). Protein concentration was estimated using Bradford reagent with bovine serum albumin (BSA) as standard.

Superoxide dismutase (SOD) - Leaf samples (0.5 g) were homogenized in 2.5 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 1% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 3,200 *xg* for 20 min at 4 °C and the supernatants were used for enzyme assays. SOD activity was determined according to Beyer and Fridovich (1987) in a reaction containing 50 mM potassium phosphate buffer (pH 7.8), 57 µM nitro blue tetrazolium (NBT), 9.9 mM methionine, 1% Triton X-100, 0.9 µM riboflavin and 200 µl of

extracts. Absorbance was recorded at 560 nm after white illumination for 15 min. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT photoreduction. Protein concentration was estimated using Bradford reagent with bovine serum albumin (BSA) as standard.

Catalase (CAT) and Ascorbate peroxidase (APx) - Leaf samples (0.6 g) were homogenized in 1.8 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 3,200 *xg* for 20 min at 4 °C and the supernatants were used for enzyme assays. The activity of CAT was estimated by measuring the rate of decomposition of H_2O_2 at 240 nm (Havir and McHale, 1987). The reaction medium contained 50 mM potassium phosphate buffer (pH 7.0) 12 mM H_2O_2 and 50 µl extract. Total APx activity was estimated by monitoring the decline in absorbance at 290 nm (Nakano and Asada, 1981). The reaction contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM H_2O_2, 20 µl ascorbate (2 mM) and 50 µl extract. One unit of APx was defined as the amount of enzyme required to oxidize 1 µmol (ascorbate).min^{-1}. Protein concentration was estimated using Bradford reagent with bovine serum albumin (BSA) as standard.

β-1,3-Glucanase and Chitinase - Leaf samples (0.5 g) were homogenized in 2.5 mL of 50 mM sodium acetate buffer (pH 5.0) containing 1% PVP. Extracts were filtered and centrifuged at 3,200 *xg* for 20 min at 4 °C, and the supernatants were used for enzyme assays. The β-1,3-glucanase activity was determined according to the methodology described by Fink *et al*. (1988) with modifications. The reaction mixture consisted of 50 µL extract, 170 µl laminarin (2 mg.mL^{-1} in buffer sodium acetate 50 mM pH 5.0) and buffer of sodium acetate 50 mM pH 5.0. The reaction was incubated at 37 °C for 1 h. Then copper reagent was added to the solution and boiled in a water bath for 10 min. The mixture was kept to return to room temperature and arsen-molybdate solution was added. Samples were diluted in 1.5 mL of distilled water and read at 500 nm. For the determination of chitinase activity, the reaction

solution consisted of 1.8 mg of chitin azure (Sigma), 140 µl of 50 mM sodium acetate pH 5.0 and 70 µl of extract. The reaction solution was incubated at 37 °C for 24 h. Then 100 µl of 2 M HCl and 300 µl of 50 mM sodium acetate pH 5.0 were added in order to stop the reaction. Samples were centrifugated at 14,000 *xg* for 10 min and the absorbance of the supernatant was measured at 550 nm (Li *et al*., 2014).

2.6 Salicylic acid analysis

The method for the analysis of salicylic acid (SA) was carried out as described by Verbene *et al*. (2002). Briefly, the quantification of SA was performed using a Phenomenex (Torrance, CA, USA) column, type LUNA 3µC_{18}(2) (150 x 4.60 mm i.d.; 3 µm) with a Phenomenex SecurityGuard pre-column. The eluent was 0.2 M sodium acetate buffer pH 5.5 (90%) with methanol (10%) at a flow-rate of 0.8 mL.min^{-1}. A spectrofluorometric detector was used with an excitation wavelength of 305 nm and emission wavelength of 407 nm.

2.7 Statistical Analysis

Means were determined from four to six replicates of a *pool* of eight leaves. The homogeneity of variances was determined by the Levene's test and when necessary, data were transformed to adjust to the normal distribution. The occurrence of extreme values was determined by BoxPlot test. Means were compared using analysis of variance (ANOVA) complemented by Duncan`s test, with $p \leq 0.05$. The correlation between activities of antioxidative enzymes was analyzed using Pearson product-moment correlation coefficient. Data were analyzed using the SPSS software version 18.

3. Results

3.1 Antioxidant enzymes

In general, leaves treated with *P. atrosepticum* showed a delay in the increment of SOD and APx activity (3 hpt), compared to elicitors treatment (1 hpt). However, *P. atrosepticum* treatment did not promote any CAT activity increment.

The SOD activity was analyzed within 6 hpt (Figure 1A). The inoculation of leaves with *P. atrosepticum* led to a reduction in SOD activity at 0.5, 1 and 6 hpt compared to the control. However, SOD activity increased and decreased over the time in the leaves treated with the elicitors XTH and ASM (Figure 1A). The reduction in SOD activity could indicate the accumulation of hydrogen peroxide in the tissues.

CAT activity was higher in the leaves sprayed with XTH in the first 3 hpt (Figure 1B) when compared to the control. However, CAT activity was not promoted when leaves were inoculated with *P. atrosepticum.* The spraying with ASM resulted to an increment in CAT activity at 0.5, 1 and 6 hpt (Figure 1B).

Leaves treated with *P. atrosepticum* showed a decrease and an increase in APx activity at 0.5 and 3 hpt, respectively (Figure 1C), compared to control treatment. Opposite response was observed when leaves were sprayed with XTH, showing an increase and decrease in APx activity at 1 and 6 hpt, respectively. However, leaves treated with ASM showed only an increment at 1 hpt.

There was a negative correlation between the activities of CAT and SOD in leaves treated with *P. atrosepticum* ($r = -0.62$), XTH ($r = -0.503$) and ASM ($r = -0.613$) ($p < 0.05$). Moreover, a strong positive correlation was observed between the activities of APx and CAT in XTH treatment ($r = 0.742$), and ASM ($r = 0.671$) ($p < 0.01$). The control treatment did not show any correlation in all the parameters analyzed.

3.2 Salycilic acid

The levels of salicylic acid (SA) were measured at 12, 24, 72 and 96 hpt (Figure 2). Free SA levels in leaves treated with *P. atrosepticum* increased at 12 hpt when compared to control treatment (Figure 2A). On the other hand, all treatments presented low levels of conjugated SA at 12 hpt (Figure 2B). An increment in free SA with a concomitant decrease of conjugate SA levels was observed at 72 hpt in leaves treated with *P. atrosepticum*.

Free SA levels in leaves sprayed with XTH were similar to that observed in the leaves treated with *P. atrosepticum* at all times analyzed (Figure 2A). However, levels of conjugated SA were higher in leaves sprayed with XTH than in leaves treated with *P. atrosepticum* at 72 hpt (Figure 2B). At 96 hpt, the leaves sprayed with XTH increased the levels of conjugated SA, compared to the control and leaves sprayed with ASM.

Free SA levels in leaves sprayed with ASM resembled the control at all times analyzed, except at 24 hpt (Figure 2A). Conjugated SA levels in ASM treated leaves were similar to the control at 72 and 96 hpt (Figure 2B).

3.3 Enzymes related to defense responses

In general, the elicitor XTH induced PAL, PPO and chitinase, while *P. atrosepticum* treatment increased β-1,3-glucanase activity and promoted a later POX and PAL increment.

The elicitors XTH and ASM promoted a significant increase in PAL activity at 24 ($p=0.001$), 72 ($p=0.005$) and 96 ($p<0.001$) hours post-treatment (Figure 3A), compared to control treatment. However, the PAL activity in leaves treated with *P. atrosepticum* showed differences to the control at 96 hpt ($p<0.001$). The activity of PAL in leaves sprayed with XTH and ASM did not differ within the time analyzed (Figure 3A).

PPO activity was promoted by the elicitors XTH and ASM at 24 hpt ($p=0.001$), compared to the control (Figure 3B). However, at 72 hpt only leaves sprayed with ASM showed a significant increase, compared to the other treatments ($p=0.001$). Furthermore, leaves treated with *P. atrosepticum* showed no alteration in the PPO activity along the timecourse. Nevertheless, when compared to the other treatments at 96 hpt, leaves treated with the phytobacterium showed the lowest PPO activity ($p<0.05$) (Figure 3B).

POX activity was promoted in leaves of *S. tuberosum* treated with ASM ($p<0.001$) at 72 and 96 hpt when compared to the other treatments (Figure 3C). At 96 hpt, however, POX activity was also induced in treatments with *P. atrosepticum* ($p<0.001$). Leaves treated with

XTH presented a similar behavior to the control treatment, with no significant differences along the analyzed time (Figure 3C).

Chitinase activity showed similar behavior on leaves sprayed with ASM and XTH along the time (Figure 3D). However, only leaves treated with XTH presented changes in chitinase activity at 12 and 72 hpt compared to control ($p=0.008$ and $p=0.041$, respectively).

Activity of β-1,3-glucanase was significantly lower in all treatments ($p<0.001$) at 72 and 96 hpt compared to control group (Figure 3E). The activity of this enzyme was promoted in 12 hpt in ASM treatment ($p=0.027$). However, a significant decrease of this enzyme activity occurred in the leaves treated with ASM at 72 hpt ($p<0.001$), compared to the other treatments. Interestingly, only leaves treated with *P. atrosepticum* showed an increase of β-1,3-glucanase activity at 24 hpt ($p <0.001$) (Figure 3E).

4. Discussion

This study assessed the effects of XTH, Acibenzolar-S-methyl (ASM) and *P. atrosepticum* in the defense metabolism of *S. tuberosum* leaves. ASM is a known plant defense activator and it has been successfully used to induce resistance against a wide spectrum of pathogens in many monocotyledonous and dicotyledonous plants (Iriti and Faoro, 2003; Ishi *et al.*, 1999; Lawton *et al.*, 1996; Lyon and Newton, 1997). *Xanthomonas axonopodis pv. citri* (XTH) has already been proposed as an inducer of defense responses in potato (Poiatti *et al.* 2009; CN102256495A, US8932844B2).

One of the earliest plant defense responses involves ROS formation and induction of oxidative stress-related genes. In this study, the activity of antioxidant enzymes of potato leaves in response to the pathogen *P. atrosepticum* and the elicitors XTH and ASM were evaluated. In general, SOD activity decreased in response to *P. atrosepticum* treatment. On the other hand, variation on SOD activity was observed along the timecourse when leaves were treated with ASM and XTH. Interestingly, XTH induced the highest SOD activity at 1

hpt. Ge *et al*. (2015) reported an increment of SOD activity in muskmelon fruit treated with ASM. Malolepsza (2005) observed no significant changes in SOD activity in tomato plants treated with the pathogen *Botritys cinerea* or the elicitor *o*-hydroxyethylorutin. In contrast, an increase in SOD activity was observed in postharvest apple treated with algal saccharides (Abouraïcha *et al*., 2015). Although contradictory results in SOD activity were report by these authors, all plants showed an increment in the natural resistance against necrotrophic pathogens. Variation in SOD activity could be explained by the well-known phenomenon of Cu/ZnSOD inactivation by H_2O_2 accumulation (Yim *et al*., 1990). Furthermore, a negative correlation between SOD and CAT activity was observed in all treatments, except in the control. Moreover, a positive correlation between CAT and APx activity was recorded in leaves treated with the elicitors. These correlations suggest a H_2O_2 accumulation when SOD is activated. It has been reported that controlled levels of H_2O_2 may act as a second messenger, mediating the systemic expression of various defense-related genes, inducing programmed cell death, stomatal closure and up-regulation of calmodulin gene. In addition, H_2O_2 is involved in redox signaling, interacting with cysteine residues within proteins affecting its activity and activation of specific transcription factors (Apel and Hirt, 2004; Hung *et al*., 2005). The accumulation of H_2O_2 is under control of CAT and APx in order to protect host cells from oxidative damage. Intensive generation of ROS may increase plant resistance to some biotrophic pathogens (Lamb and Dixon 1997). However, cell death can promote growth of necrotrophic pathogens (Nowogórska and Patykowski, 2015), such as *P. atrosepticum.* Bacterial, like *P. atrosepticum*, secretes T3SS protein into the host cytoplasm leading to the death of tissues and necrosis (Lindeberg *et al*., 2008; Whisson *et al*., 2007). In fact, works showed that a functional *P. syringae* T3SS can elicit a strong ROS production in *Arabidopsis thaliana* and *Nicotiana benthamiana* plants (Degrave *et al*., 2008; Oh *et al*., 2010).

In the present study, we report a general increment in CAT activity in the leaves treated with elicitors. However, results did not demonstrate an increase in CAT activity in potato leaves treated with *P. atrosepticum*. Li *et al*. (2014) have associated the CAT increase activity and plant resistance against *Pectobacterium carotovorum* subsp. *carotovorum* in Chinese cabbage treated with the elicitor Trichokonins. It has been reported that *Phytophthora sojae* secretes two effectors to regulate plant programmed cell death and H_2O_2 homeostasis through direct interaction with *Nicotiana benthamiana* catalases (Zhang *et al*., 2015). These authors also observed the enhancement of susceptibility to *Phytophthora capsici* by silencing of catalase genes, indicating that catalases are essential for plant resistance. Additionally, transgenic antisense tobacco plants with reduced CAT or APx expression displayed higher sensitivity to cell death during bacteria-induced HR (Mittler *et al.,* 1999). According to Wen (2012) ROS was useful for *Sclerotinia sclerotiorum* to infect *Brassica napus* tissues, and the spread of the Sclerotinia disease was reduced with the increase of the efficiency of the plant antioxidative system. It has also been reported that pathogenicity of the two necrotrophs, *S. sclerotiorum* and *B. cinerea*, was directly dependent on the level of superoxide and H_2O_2 in the cells (Wen, 2012).

After the recognition and activation of the antioxidant metabolism a specific signaling pathway has to be activated to trigger resistance against a specific pathogen in a particular host. Specific hormone signaling system determines the outcome of plant-pathogen interactions, culminating in either disease development or disease resistance (Vidhyasekaran, 2015). Generally, it is accepted that the reactions of plants to necrotrophic pathogens are associated to jasmonic acid (JA) and/or ethylene signal transduction pathways, while biotrophic pathogens induce salicylic acid (SA) pathway (Glazebrook, 2005). Our results demonstrated that levels of free SA in potato leaves were increased only in the treatment with *P. atrosepticum*. Free SA can promote and potentiate the programmed cell death (Brodersen

et al., 2005). Host cell death results in the formation or expanding of necrotic lesions in the infected tissue by necrotrophic pathogens, thereby some necrotrophs promote virulence by using the plant HR machinery as a strategy (Mengiste, 2012). Treatments with culture filtrates of *Erwinia carotovora* induces defense-related gene expression and cell death in various plants, including *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Physcomitrella patens* (Hirakawa *et al*., 2015). Interestingly, the elicitors XTH and ASM were not effective in promoting SA increment. According to Halim *et al* (2009), potato plants treated with the biotic elicitor Pep-13, a PAMP from *P. infestans*, requires both SA and JA pathways for plant defense. On the other hand, PAMPs from bacteria, such as flg22, are well-known promoters of SA accumulation in Arabidopsis (Tsuda *et al*, 2008). There are evidences that ASM mimics the SA step, acting at the site or downstream of SA accumulation. Salicylic acid and ASM induce the PR-1a gene promoter and use similar *cis*-acting elements (Cole, 1999). Du and Klessig (1997) have found that ASM competes very successfully with SA for binding to an SA-binding protein (SABP2) and this ability was correlated with the activation of defense gene expression. Therefore, SA-mediated defense responses require not only a sufficiently high level of SA but also an effective SA signal perception and transduction mechanism. Studies demonstrated that potato plants have a high constitutive level of SA that does not lead to constitutive resistance in healthy plants. Besides, potato plants may have a poor SA signal perception and/or transduction mechanism (Yu *et al*., 1997).

One outcome of defense signaling pathways is the production an important group of defense-related enzymes such as phenylalanine ammonia-lyase (PAL), polyphenol oxidases (PPO), peroxidases (POX), β-1,3-glucanases and chitinases (Raju *et al*., 2008). The present study showed an early increment in PAL activity in leaves treated with elicitors (XTH and ASM) compared to *P. atrosepticum* treatment. The slow buildup of PAL activity in the *P. atrosepticum* treatment allows the bacteria to multiply within the host tissues and reach the

critical population density required for the production of pectic enzymes (Ngadze, 2012). In genus *Solanaceae*, the nature of plant defenses to pathogens are not restricted to the interaction type (compatible or incompatible). Defenses are generally induced earlier and to a greater extent in incompatible than in compatible interactions. This is well described for the activation of PAL during the interaction of potato tubers with compatible or incompatible races of *Phytophthora infestans* (Kröner *et al*., 2011). PAL is a key enzyme of the phenylpropanoid pathway, which leads to the synthesis of antimicrobial compounds, particularly chlorogenic acid that is related to the reduction of *P. atrosepticum* growth (Kröner *et al*., 2012). The increase in PAL activity in potato tubers induced by filtrates of *Phytophthora infestans* resulted in resistance against *P. atrosepticum* (Kröner *et al*., 2011; Saubeau *et al*., 2014). The late and slight increase in PAL activity in leaves inoculated with *P. atrosepticum* is similar to the results observed in tomato plants inoculated with *Clavibacter michiganensis* ssp. *michiganensis*. In this study, the pathogen inoculation resulted in a slight increase of PAL activity 3 days after treatment and then subsequently decreased (Baysal *et al*, 2005). The authors also demonstrated an early and prolonged induction of PAL activity in plants treated with DL-β-aminobutyric acid elicitor.

The elicitors XTH and ASM promoted an increment in PPO activity. However, leaves treated with *P. atrosepticum* did not show any increment. PPO enzymes are involved in the oxidation of polyphenols into quinones (antimicrobial compounds) (Mohammadi and Kazemi, 2002). The increase in PPO activity in tobacco plants, induced by the elicitor linoleic acid, presented an increment in resistance against *P. carotovorum* subsp. *carotovorum* (Sumayo *et al*., 2014). Induction of PPO following chemical and biological treatments has also been observed in postharvest tomato, pear and mango fruit which was correlated to increased disease resistance (Zeng *et al*., 2006, Lin *et al*., 2011 and Liu *et al*., 2007).

ASM was efficient in promoting POX activity in the treated leaves. Contrarily, XTH did not show any effect. Increases in POX activity are often associated with plant cell wall reinforcement processes, increasing the mechanical barrier to physical penetration into the cell (Malolepsza, 2009). The enzyme is believed to catalyse the last step in lignin formation and cross-linking of plant cell wall proteins (Nicholson and Hammerschmidt, 1992; Wojtaszek 1997). Tomato plants treated with ASM showed a close correlation between the increases in POX activity and plant resistance against *Clavibacter michiganensis* subsp. *michiganensis* (Soylu *et al*., 2003). The same correlation has also been observed in other pathosystems (Brisset *et al*., 2000; Resende *et al*., 2002). In turn, Poiatti *et al*. (2009) observed an increase and no significant difference in POX activity of potato plants treated with *Erwinia carotovora* and *Xanthomonas axonopodis* pv. *citri*, respectively.

Chitinases and β-1,3-glucanases are PR proteins strongly induced by treatment with activators of systemic acquired resistance, as well as in response to biotic and abiotic elicitors (Magnin-Robert *et al*., 2007). The present study showed an increase in chitinase and β-1,3-glucanase activity in leaves treated with XTH and ASM, respectively. The β-1,3-glucanase activity was also induced in leaves treated with *P. atrosepticum.* Chitinases and β-1,3-glucanase degrade the major cell-wall constituents (i.e. chitin and β-1,3-glucan) of most filamentous fungi, inhibiting their growth and generating oligosaccharides, which act as elicitors with potential to induce plant defense responses (Ferreira *et al*., 2007; Mohammadi *et al*., 2002). Some plant chitinases also have lysozyme activity and can, therefore, hydrolyse bacterial cell walls (Baysal *et al.*, 2003). In contrast, β-1,3-glucanases are probably not directly involved in resistance against bacteria, but their significant local and systemic accumulation is an indication of an induction of overall resistance to pathogen attack (Brisset *et al*., 2000). According to Ott *et al*. (2006), chitinases participate in early basal resistance induced by PAMPs against virulent bacteria. An early increase in chitinases and β-1,3-

glucanases activity in the first hours post treatment with ASM and biotic elicitors has also been reported in tomato leaves (Cavalcanti *et al.*, 2006).

In conclusion, the biotic elicitor XTH, as well as ASM, were able to modulate the defense metabolism of *S. tuberosum*. This modulation was different from that observed for the *P. atrosepticum* treatment. According to the antioxidant enzyme activities, we suggest that XTH elicitor promotes a relative low and transient H_2O_2 accumulation during the development of early basal defense response, preventing the cell death and HR. This low and transient H_2O_2 accumulation is presumably a SA-independent response, avoiding cell death and promoting an increment in enzymes activities related to the defense metabolism (PAL, PPO and chitinase). Taking all these findings, we suggest that XTH has the ability in promoting resistance against *P. atrosepticum*. However, the efficiency of the elicitor XTH in plant protection must still be elucidated.

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FIGURES

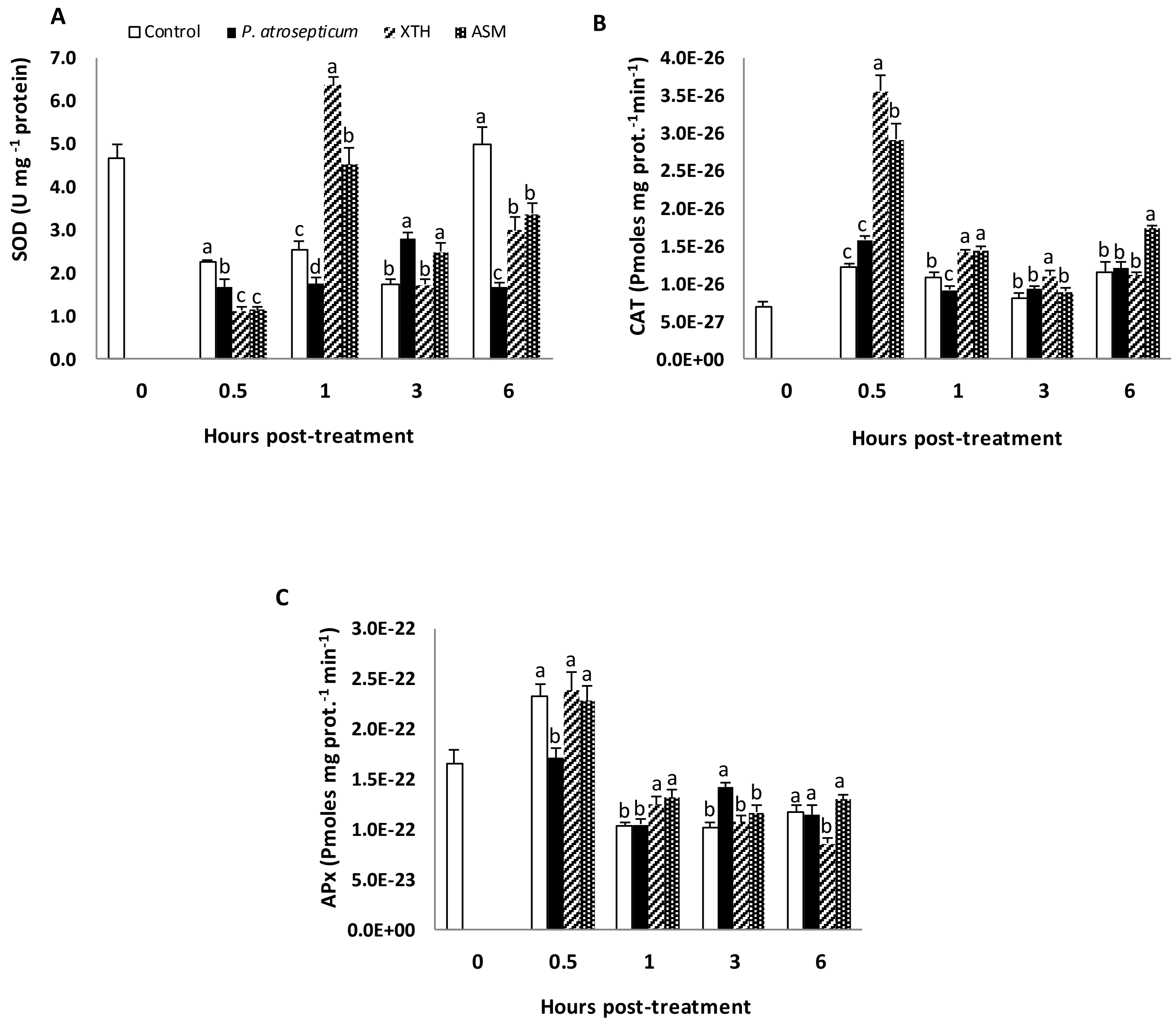

Figure 1 – Activity of antioxidative enzymes (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) ascobate peroxidase (APx) in detached leaves of *S. tuberosum* treated with sterile water (control), *P. atrosepticum*, XTH (bacterial extract) or Acibenzolar-S-methyl (ASM). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

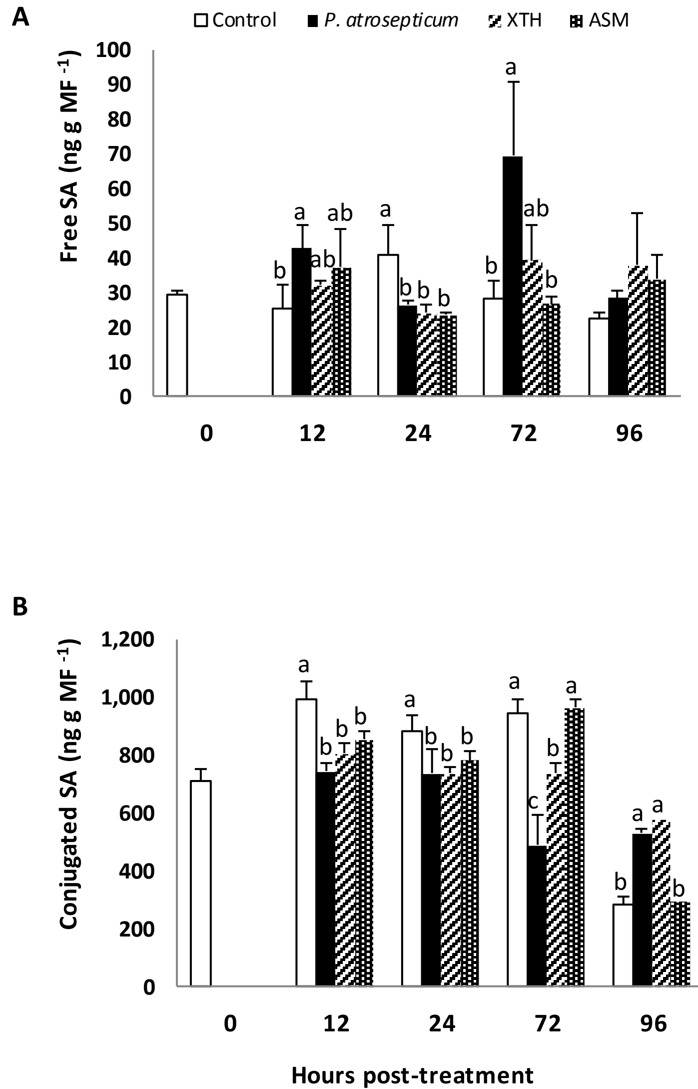

Figure 2 – Levels of (A) free and (B) conjugated salicylic acid (SA) in detached leaves of *S. tuberosum* treated with sterile water (control), *P. atrosepticum*, XTH (bacterial extract) or Acibenzolar-S-methyl (ASM). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

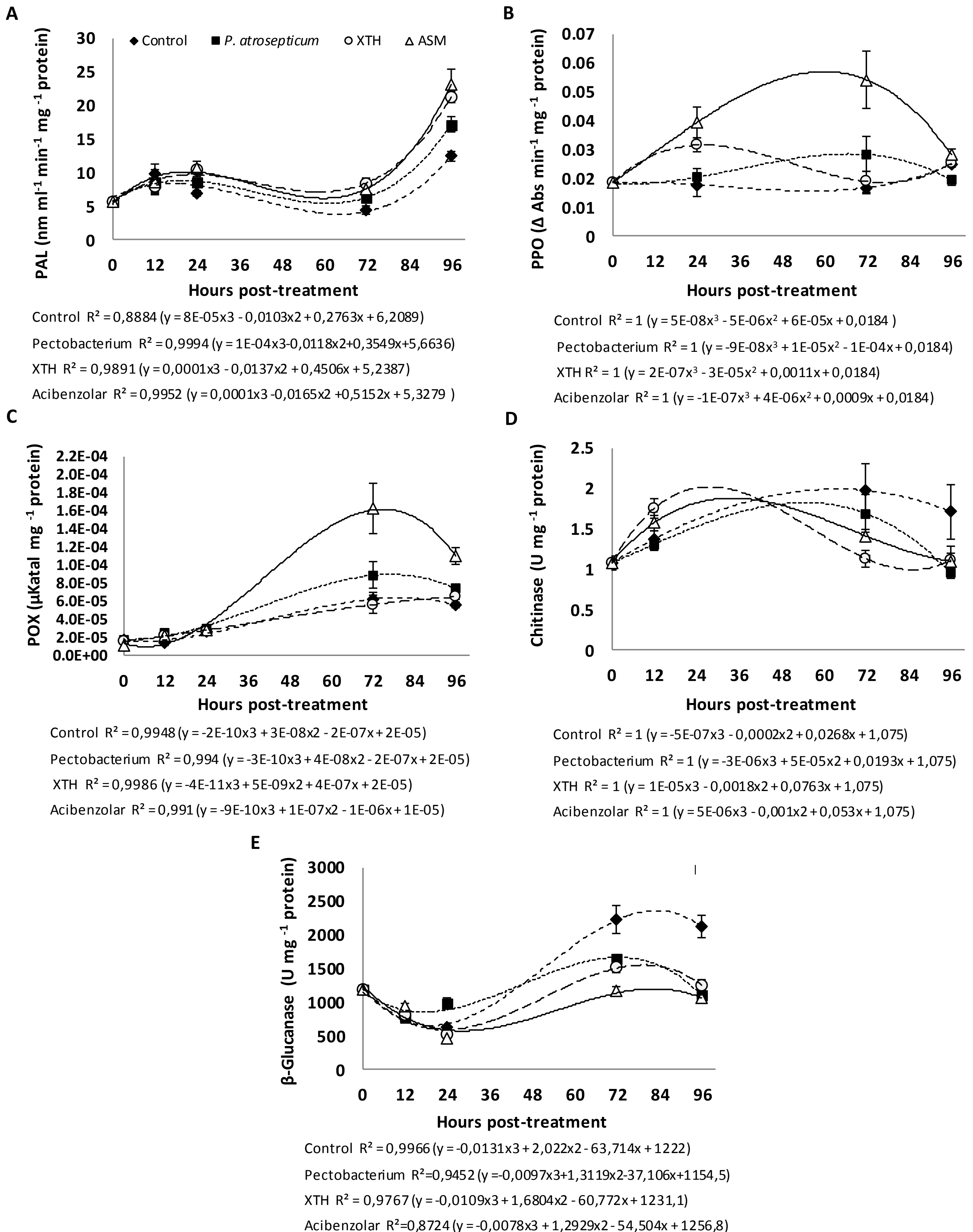

Figure 3 - Polynomial regression of the enzymes activity (A) phenylalanine ammonia lyase (PAL), (B) polyphenol oxidase (PPO), (C) peroxidase (POX), (D) chitinase and (E) β-1,3-glucanase in detached leaves of *S. tuberosum* treated with sterile water (control), *P. atrosepticum*, XTH (bacterial extract) or Acibenzolar-S-methyl (ASM). Equations of regression are shown for each treatment.

ARTIGO CIENTÍFICO 2

Extract of *Xanthomonas axonopodis* induces resistance in *Solanum tuberosum* against *Pectobacterium atrosepticum*

*O manuscrito será submetido para a revista Plant Pathology, cujo fator de impacto é 2,969.

Extract of *Xanthomonas axonopodis* induces resistance in *Solanum tuberosum* against *Pectobacterium atrosepticum*

Running head: *X. axonopodis* extract induces resistance

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Abstract

Potato (*Solanum tuberosum*) is affected by many pathogens, such as the necrotrophic bacteria *Pectobacterium atrosepticum*, producing substantial economic losses. Induction of natural disease resistance in harvested horticultural crops using biological and chemical elicitors has received increasing attention over recent years, due to the low environment toxicity in the disease management. In this work we evaluate the ability of the biotic inducer XTH in delay the disease caused by *P. atrosepticum* and the mechanism by which the elicitor promote resistance in *S. tuberosum* detached leaves. Acibenzolar-S-methyl (ASM), from a commercial formulation, was used as reference to compare the effectiveness and mode of action of the biotic elicitor. Our results demonstrated that the XTH reduced the progression and disease symptoms caused by *P. atrosepticum*. This effect can be related to the early activation of antioxidant enzymes and the induction of the activities of enzymes related to defense. We also observed that the increase in free SA level occurred only in *P. atrosepticum*

treatment, differing of the leaves pretreated with the elicitors and inoculated with the phytobacteria. According to our results the XTH mode action is similar to the observed in acknowledged elicitor ASM, with some peculiarities.

Key words: biotic elicitor, ASM, antioxidant enzymes, defense-related enzymes

1. Introduction

Pectobacterium atrosepticum, formerly was named *Erwinia carotovora* subsp. *atroseptica,* is a causal agent of bacterial soft rot and black leg, affecting both potato yield and tuber quality. *P. atrosepticum*, a necrotrophic pectinolytic Gram-negative bacterium, produces extracellular enzymes such as pectate-lyases, pectinases, cellulases and proteases, resulting in tissue maceration and rot symptoms affecting postharvest storage and causing significant economic losses (Saubeau *et al*., 2014).

Induction of natural disease resistance in horticultural crops using biological and/or chemical elicitors represents an auxiliary tool for plant disease management. Elicitors are compounds specifically recognized by plant that subsequently induce defense responses against pathogens or herbivores. Elicitors may lead to a systemic expression of a wide range of antimicrobial genes in distal tissues, protecting the whole plant from secondary infection. This phenomenon is called systemic acquired resistance (SAR). The importance of induced or SAR in plants has been long documented. However, only recently its potential has been widely recognized (Terry & Daryl, 2004).

Depending on the efficacy of elicitor, these compounds can be used in field crops either alone or in combination with fungicides. The novel synthetic elicitor benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), also known as acibenzolar-S-methyl (ASM), is an effective activator of SAR in a variety of plant–pathogen systems (Terry & Daryl, 2004). According to Hajhamed *et al*. (2007), ASM significantly decreased severity of

bacterial soft rot disease in potato. The better understanding of plant signaling pathways has led to the discovery of natural and synthetic elicitors that induce similar defense responses in plants as induced by the pathogen infection.

In addition to synthetic elicitors, different classes of biotic inducers are also recognized by the cell surface–localized pattern-recognition receptors (PRRs) through pathogen-associated molecular patterns (PAMPs). Common features of PAMPs are their highly conserved structures, such as bacterial lipopolysaccharide (LPS), lipoproteins and flagellin. They are of functional importance for microorganisms and are present in a broad range of microbial species (Casabuono *et al*., 2011). Commercial biotic inducers based on these natural defense responses, such as Elexa® (chitosan) and Messenger® (harpin from *Erwinia amylovora*), have been used to protect a range of crops against important pathogens. In our previous studies with *Xanthomonas axonopodis* pv. *citri* (XTH) we have reported the efficiency of XTH in promoting the defense metabolism of *Solanum tuberosum* plants and detached leaves (data not published). Potato plants sprayed with this bacterium formulation have shown a reduction (above 60%) in the disease symptoms caused by *Pectobacterium atrosepticum*, without apparent plant toxicity (CN102256495A, US8932844B2).

The spread of a plant disease is driven by the plant ability on inducing a complex array of defense responses after the detection of a microorganism via the recognition of elicitor molecules released during the plant–pathogen interaction. Following elicitor perception, the activation of signal transduction pathways leads to the production of reactive oxygen species (ROS). The strength and lifetime of the ROS signaling pool depends on balance between production and removal by the antioxidant system. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APx), are responsible for the maintenance of the ROS balance (Mittler, 2002). Moreover, the increment of antioxidant enzyme activities might be an important component in the defense strategy (Cavalcanti *et al*.,

2006). Hormone signals, such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) are activated in the cell after ROS imbalance. These signals lead to the increment of enzymes activities related to defense, such as phenylalanine ammonia lyase (PAL), polyphenol oxidases (PPO) and peroxidases (POX) and the accumulation of pathogenesis-related (PR) proteins (β-1,3-glucanase and chitinase). All these defense reactions, which sometimes are associated with a localized cell death known as the hypersensitive reaction (HR), are considered to be important responses for delimiting the pathogen's growth. However, if these defense reactions do not occur in a suitable time to impair the pathogen, the infection process will spread successfully (Soylu *et al*., 2003).

The present study aimed to evaluate the ability of the biotic inducer XTH in delay the disease caused by *P. atrosepticum* in *S. tuberosum* detached leaves. In order to elucidate the mechanisms involved in resistance of potato leaves against *P. atrospeticum* we analyzed the changes in the activity of antioxidant (SOD, CAT and APx) and defense related (PAL, PPO, POX, β-1,3-glucanase and chitinase) enzymes, as well the level of salicylic acid (SA) and total phenolic compounds. ASM, from a commercial formulation, was used as reference to compare the effectiveness and mode of action of the biotic elicitor XTH.

2. Materials and Methods

2.1 Plant material

Seed tubers of *Solanum tuberosum* cv. Agata were provided by potato growers (São Francisco de Paula, RS, Brazil, 29°26'49"S 50°34'44"W). Tubers were cultivated in pots (8 L) with commercial organic soil and maintained in greenhouse for three or four weeks (stolon and tuber formation phases). Plants were pretreated with elicitors and expanded leaves were detached, challenged with pathogen and placed in sterile Petri dishes (15 x 2.5 cm) with two layers of moistened filter paper (15 ml of sterile distilled water). The petioles were covered

with wet cotton in order to reduce leaf dehydration. Two leaves were placed in each plate. All plates were maintained under photoperiod (16 h light) and controlled temperature (25 °C).

2.2 Microorganism

Pectobacterium atrosepticum (Pba) was grown in liquid LB medium (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ NaCl, 10 $g.L^{-1}$ sucrose) at 25 °C for 16 h under 100 rpm. Bacterial culture was centrifuged at 4,000 *xg* for 10 min, supernatant was discarded, pellet was re-suspended in sterile distilled water and rinsed three times. In the final step, the pellet was re-suspended in sterile distilled water and the bacterial concentration was adjusted to 10^{10} $cfu.mL^{-1}$ (OD_{600nm} = 0.5) (Poiatti *et al.*, 2009). The suspensions of Pba were immediately used for inoculation of potato detached leaves.

2.3 Elicitors

Biotic and synthetic elicitors were sprayed on potato plants (pretreatment). *Xanthomonas axonopodis* pv. *citri* was cultivated and cell suspension prepared as previously described for *P. atrosepticum* (except for 48 h of cultivation). Bacterial concentration was adjusted to 10^{20} $cfu.mL^{-1}$ (OD_{600nm} = 1.0) and inactivated by autoclaving for one hour at 121°C. The inactivated bacterial suspension (XTH) was used as the biotic elicitor. The synthetic elicitor was prepared by dissolving Acibenzolar-S-methyl (Bion® 500 WG, Syngenta S.A.) in sterile distilled water (100 $mg.L^{-1}$).

2.4 Elicitors pretreatment and bacterial inoculation

Acibenzolar-S-methyl (ASM treatment) or *X. axonopodis* extract (XTH treatment) were used as elicitors. Potted potato plants (3 to 4-week-old) were pretreated by spraying 5 $mL.plant^{-1}$ of the elicitor solution and maintained in a greenhouse. Control-pretreatment (Cp) consisted in plants sprayed with distilled sterile water. Four days after spraying, leaves were detached, transferred to Petri dishes and inoculated with the pathogenic bacterium *P. atrosepticum* (Pba treatment). The suspension of phytobacterium *P. atrosepticum* was

inoculated (100 μL) using a needleless syringe in the abaxial surface of the lateral leaflet. Control treatment (Ct) consisted in detached leaves from the control-pretreated plants, inoculated with distilled sterile water, using a needleless syringe, as previously described.

Leaf samples were collected at 0, 12, 24, 72 and 96 hpt (hours post-treatment) and analyzed for the total phenolic compounds and enzyme activities of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX), β-1,3-glucanase and chitinase. Salicylic acid (SA) was also evaluated. Activities of the enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxide (APx) were analyzed in leaf samples collected at 0, 0.5, 1, 3, and 6 hpt. Time point 0 consisted in leaves detached four days after the pretreatment (with elicitors or water) and before the treatment with the challenger *P. atrosepticum*.

2.5 Disease assessment

Detached leaves maintained in Petri dishes were inoculated with *P. atrosepticum*, as previously described. The disease symptoms (chlorosis) were evaluated along 5 days after the pathogen infection. The disease incidence was determined by estimating the leaf area (%) showing the symptom. The area under the disease progress curve (AUDPC) was estimated.

2.6 Enzyme assays

Phenylalanine ammonia lyase (PAL) **-** Leaf samples (0.3 g) were homogenized in 3 ml of 50 mM Tris-HCL (pH 8.8) containing 1 mM EDTA, 1% polyvinylpyrrolidone (PVP), and 0.2% Triton X-100. Extracts were centrifuged at 3,200 *xg* for 20 min at 4 °C, and the supernatants were used for enzyme assays. PAL activity was evaluated following the method of Navarre *et al.* (2013) with modifications. The reaction containing 200 μl of extract, 0.2 M phenylalanine and 50 mM sodium borate buffer (pH 8.8) was incubated for 1 h at 37 °C. The enzyme activity unit was defined as the amount of enzyme needed to produce 1 nmol of cinnamic acid per mL of reaction per minute per mg of protein at 290 nm. Protein

concentration was estimated using Bradford reagent with *bovine serum albumin* (BSA) as standard.

Polyphenol oxidase (PPO) and Peroxidase (POX) - Leaf samples (0.5 g) were homogenized in 2.5 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 1% PVP. Extracts were filtered and centrifuged at 3,200 *xg* for 20 min at 4 °C, and the supernatants were used for enzyme assays. Polyphenol oxidase activity was determined according to Salla *et al*. (2014) spectrophotometrically at 400 nm using chlorogenic acid as substrate. The samples were incubated for 30 min at 30 °C. The enzyme activity unit was defined as the change in absorbance.min^{-1}.mg^{-1} protein. Peroxidase activity was determined spectrophotometrically by the oxidation of guaiacol at 420 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 6.0), 0.1M guaiacol as substrate and 10 mM hydrogen peroxide. The enzyme activity unit was expressed in µkatal.mg^{-1} protein. One katal (kat) corresponds to the quantity of enzyme activity that transforms 1 mol.s^{1} of substrate (Salla *et al*., 2014). Protein concentration was estimated using Bradford reagent with *bovine serum albumin* (BSA) as standard.

Superoxide dismutase (SOD) - Leaf samples (0.5 g) were homogenized in 2.5 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 1% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 3,200 *xg* for 20 min at 4 °C and the supernatants were used for enzyme assays. SOD activity was determined according to Beyer and Fridovich (1987) in a reaction containing 50 mM potassium phosphate buffer (pH 7.8), 57 µM nitro blue tetrazolium (NBT), 9.9 mM methionine, 1% Triton X-100, 0.9 µM riboflavin and 200 µl of extracts. Absorbance was recorded at 560 nm after white illumination for 15 min. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT photoreduction. Protein concentration was estimated using Bradford reagent with *bovine serum albumin* (BSA) as standard.

Catalase (CAT) and Ascorbate peroxidase (APx) - Leaf samples (0.6 g) were homogenized in 1.8 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 3,200 *xg* for 20 min at 4 °C and the supernatants were used for enzyme assays. The activity of CAT was estimated by measuring the rate of decomposition of H_2O_2 at 240 nm (Havir and McHale, 1987). The reaction medium contained 50 mM potassium phosphate buffer (pH 7.0) 12 mM H_2O_2 and 50 µl extract. Total APx activity was estimated by monitoring the decline in absorbance at 290 nm (Nakano & Asada,1981). The reaction contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM H_2O_2, 20 µl ascorbate (2 mM) and 50 µl extract. One unit of APx was defined as the amount of enzyme required to oxidize 1 µmol (ascorbate).min^{-1}. Protein concentration was estimated using Bradford reagent with *bovine serum albumin* (BSA) as standard.

β-1,3-Glucanase and Chitinase - Leaf samples (0.5 g) were homogenized in 2.5 mL of 50 mM sodium acetate buffer (pH 5.0) containing 1% PVP. Extracts were filtered and centrifuged at 3,200 *xg* for 20 min at 4 °C, and the supernatants were used for enzyme assays. The β-1,3-glucanase activity was determined according to the methodology described by Fink *et al*. (1988) with modifications. The reaction mixture consisted of 50 µL extract, 170 µl laminarin (2 $mg.mL^{-1}$ in buffer sodium acetate 50 mM pH 5.0) and buffer of sodium acetate 50 mM pH 5.0. The reaction was incubated at 37 ° C for 1 h. Then copper reagent was added to the solution and boiled in a water bath for 10 min. The mixture was kept to return to room temperature and arsen-molybdate solution was added. Samples were diluted in 1.5 mL of distilled water and read at 500 nm. For the determination of chitinase activity, the reaction solution consisted of 1.8 mg of chitin azure (Sigma), 140 µl of 50 mM sodium acetate pH 5.0 and 70 µl of extract. The reaction solution was incubated at 37 °C for 24 h. Then 100 µl of 2 M HCl and 300 µl of 50 mM sodium acetate pH 5.0 were added in order to stop the reaction.

Samples were centrifugated at 14,000 *xg* for 10 min and the absorbance of the supernatant was measured at 550 nm.

2.7 Phenolic compounds

Leaf samples (0.25 g) were homogeneized in 5 mL of 80% (v/v) aqueous methanol. Extracts were filtered and centrifuged at 4,000 *xg* for 15 min at 15 °C. Phenolic compounds were quantified by the colorimetric technique using Folin-Ciocalteu reagents and Na_2C0_3 (20% w/v). Absorbancy (765 nm) was determined after 30 min incubation at 25 °C in the dark (Sartor *et al.*, 2013). Gallic acid was used as standard.

2.8 Salicylic acid analysis

The method for the analysis of salicylic acid (SA) was carried out as described by Verberne *et al.* (2002). Briefly, the quantification of SA was performed using a Phenomenex (Torrance, CA, USA) column, type LUNA $3\mu C_{18}(2)$ (150 x 4.60 mm i.d.; 3 µm) with a Phenomenex SecurityGuard pre-column. The eluent was 0.2 M sodium acetate buffer pH 5.5 (90%) with methanol (10%) at a flow-rate of 0.8 $mL.min^{-1}$. A spectrofluorometric detector was used with an excitation wavelength of 305 nm and emission wavelength of 407 nm.

2.9 Statistical Analysis

Means were determined from four to six replicates of a *pool* of eight leaves per treatment. The homogeneity of variances was determined by the Levene's test and when necessary, data were transformed to adjust to the normal distribution. The occurrence of extreme values was determined by BoxPlot test. Means were compared using analysis of variance (ANOVA) complemented by Duncan`s test, with $p \leq 0.05$. The correlation between activities of antioxidative enzymes was analyzed using Pearson product-moment correlation coefficient. Data were analyzed using the SPSS software version 18.

3. Results

3.1 Promotion of plant resistance

Detached leaves from the XTH+Pba treatment showed a significant delay in disease progression, compared to the treatment Cp+Pba (Fig. 1; Table 1). Treatment Cp+Pba resulted in 55.5% of chlorosis in leaf area on the third day after pathogen challenging. However, leaves from the treatment XTH+Pba did not differ statistically from control (Cp+Ct) until the fourth day of disease assessment. Leaf senescence (chlorosis symptoms) of detached leaves was observed on the fifth day post-detachment in the Cp+Ct treatment (Fig. 1).

3.2 Antioxidant enzymes

SOD activity in leaves pretreated with ASM and XTH (Fig. 2a) decreased at 0 hpt, compared to control (Cp). After bacterial challenging, leaves pretreated with XTH (treatment XTH+Pba) continued not showing any increase in SOD activity during the analyzed time points. However, SOD activity increased in leaves of ASM+Pba treatment at 1 and 3 hpt. The SOD activity increment was also observed in leaves from the treatment Cp+Pba at 3 hpt. In general, CAT activity increased at 0.5 hpt when potato leaves were challenged with *P. atrosepticum* (treatments Cp+Pba, XTH+Pba and ASM+Pba). This activity promotion was also observed at 1 and 3 hpt in the treatments ASM+Pba and XTH+Pba, respectively (Fig. 2b). The APx activities in leaves pretreated with ASM and XTH (Fig. 2c) showed an increase and decrease, respectively, at 0 hpt, compared to Cp treatment. Leaves from ASM+Pba treatment had APx activity increased from 1 to 6 hpt, although in XTH+Pba and Cp+Pba treatments this increase was only observed at 3 and 6 hpt, respectively.

SOD and CAT activities presented a negative correlation in XTH+Pba treatment ($r = -0.491$, $p<0.05$), result that was not observed in Cp+Ct and ASM+Pba treatments. The Cp+Pba treatment also showed a strong negative correlation between SOD and CAT activities ($r = -0.620$, $p<0.01$), but the correlation was not detected in CAT and APx. The ASM+Pba and XTH+Pba treatments showed a positive correlation between CAT and APx activities, whereas

XTH+Pba treatment presented the strongest correlation ($r = 0.526$, $p<0.05$ and $r = 0.709$, $p<0.01$, respectively).

3.3 Salicylic Acid

The levels of free and conjugated salicylic acid (SA) were measured at 0, 12, 24, 72 and 96 hpt. The free SA increased only in leaves from Cp+Pba treatment at 12 and 72 hpt when compared to the Cp+Ct treatment (Fig. 3a). Whilst, in leaves treated with Cp+Pba and XTH+Pba the increment of conjugated SA levels was detected only at 96 hpt (Fig. 3b). On the other hand, pretreatment with ASM increased the conjugated SA at 0 hpt, although after the bacterial pathogenic challenged (treatment ASM+Pba) no increment was observed in conjugated SA. Levels of conjugated SA decreased in all treatments at 12, 24 and 72 hpt, compared to the control Cp+Ct.

3.4 Plant defense metabolism

Alterations on plant defense metabolism regarding changes in the activity of enzymes, such as PAL, PPO and POX, and in the production of antimicrobial molecules (phenolic compounds) were observed in detached leaves of *S. tuberosum*. Changes in the levels of phenolic compounds occurred only at 96 hpt, the time point where the highest level of total phenolic compounds in leaves treated with ASM+Pba was detected (Fig. 4a).

Contrary to the phenolic compounds, defense-related enzymes varied significantly in detached leaves. The treatment Cp+Pba promoted PAL activity at 72 and 96 hpt, while the leaves pretreated with elicitors (XTH+Pba and ASM+Pba) showed an earliest increment from 24 hpt on Fig. 4b). However, only leaves from the treatment XTH+Pba presented levels of PAL activity higher than the Cp+Pba treatment at 24 and 96 hpt and the highest enzyme activity at 96 hpt. The PAL activity observed in the ASM+Pba treatment did not differ from the Cp+Pba treatment during the analyzed time points. Variation on PPO activity was observed among treatments and time points (Fig 4c). Early response was detected in leaves

from ASM+Pba, where PPO activity increased from 24 hpt on. Moreover, in this same treatment was observed the highest PPO activity at 72 hpt. XTH+Pba treatment resulted in a delay on PPO activity (at 96 hpt), while Cp+Pba treatment was not effective on promoting PPO activity. However, on POX analyses, the treaments ASM+Pba and Cp+Pba significantly affected the enzyme activity (at 24 and 96 hpt), whilst no difference was noted in leaves from XTH+Pba when compared to the Cp+Ct treatment (Fig. 4d). The POX activities in both treatments (ASM+Pba and Cp+Pba) were similar at 24 and 72 hpt, although the highest POX activity was observed at 96 hpt in the ASM+Pba treatment.

The variation of PR proteins β-1,3-glucanase and chitinase activities differed of the other enzymes related to defense described above. β-1,3-glucanase and chitinase activity was increased and decreased in leaves only pretreated with ASM and XTH, respectively, at 0 hpt (Fig. 4e and 4f). After the phytopathogen inoculation, earliest promotion of β-1,3-glucanase activity was observed in leaves treated with ASM+Pba. The treatments XTH+Pba and ASM+Pba also induced the chitinase and β-1,3-glucanase activities at 12 and 24 hpt, respectively. However, at 24 hpt, the activity of β-1,3-glucanase in ASM+Pba was similar to the Cp+Pba and higher to the XTH+Pba treatment. The treatment Cp+Pba did not increase chitinase activity during the analyzed time points. In general, the activity of β-1,3-glucanase and chitinase decreased in all treatments at 72 and 96 hpt compared to control Cp+Ct.

4. Discussion

The use of avirulent or attenuated strains of either pathogenic or saprophytic microorganisms to induce SAR in vegetative host tissue has been relatively well documented (Terry & Daryl, 2004). According to our previously study, inactivated extract of *Xanthomonas axonopodis* pv. *citri* (XTH) acted as an inducer, modulating the defense metabolism in leaves of *S. tuberosum* (data not published).

Pre-treatment of susceptible plants with avirulent pathogens (a biotic inducer) can enhance resistance to subsequent attack not only at the site of treatment, but also in tissues distant from the initial infection sites. Typically, this inducible resistance system, known as systemic acquired resistance (SAR), is effective against diverse pathogens including viruses, bacteria, and fungi (Soylu *et al*., 2003). At this present work, we showed that leaves pretreated with XTH four days before the inoculation with *P. atrosepticum* significantly delayed the symptoms and disease progression caused by the phytopathogen. It has been reported that lipopolysaccharides (LPS), indispensable components of the cell surface of Gram-negative bacteria, trigger some plant defense-related responses, such as SAR and PR-proteins accumulation. Furthermore, studies with LPS from *Xanthomonas axonopodis* pv. *citri* and *X. campestris* pv. *campestris* demonstrated that they are involved in activation of immune response (Casabuono *et al*., 2011). However, the mechanism involved in the defense metabolism during the infection by *P. atrosepticum* in leaves pretreated with XTH is complex and remains unclear.

Plants respond to pathogen attack by the induction of a range of responses that include the production of reactive oxygen species (ROS). Generation of ROS is thought to be an early event that can fundamentally influence the balance of the interaction between the plant and the pathogen (Cavalcanti *et al*., 2006). In order to evaluate this first line of plant defense, we analyzed the activity of antioxidant enzymes SOD, CAT and APx in potato. ASM was used as a commercial reference to compare the effectiveness and mode of action of the biotic elicitor XTH. Prior inoculation with *P. atrosepticum* (time point 0), we observed that leaves pretreated with the elicitors XTH and ASM showed lower SOD activity than leaves from the control treatment. Moreover, no increment in SOD activity was observed in leaves from XTH+Pba treatment. However, Cp+Pba and ASM+Pba treatments showed an increase in SOD activity. In tomato plants, leaves pretreated with the elicitor *o*-hydroxyethylorutin and

inoculated with *Botrytis cinerea*, showed a delayed in disease symptoms caused by this fungus, although no significant changes were observed in SOD activity (Malolepsza, 2005). Contrarily, Soylu *et al*. (2003) observed an increase in SOD activity in tomato leaves infected with *Clavibacter michiganensis* subsp. *michiganens* (Cmm) and Cmm-infected leaves that were previously treated with ASM. SOD plays an important role in plant pathogenesis, since it converts O_2^- into H_2O_2 and this product acts on inducing programmed cell death (PCD) and hypersensitive response (HR) in plants (Mittler, 2002). According to Wen (2012) PCD and HR promote the growth of necrotrophic pathogen, like *P. atrosepticum*, resulting in the formation or expanding of necrotic lesions in the infected plant tissue. Likewise, it has been reported that LPS from Gram-negative bacteria, like *Xanthomonas axonopodis* utilized in this work, acts preventing the HR and PCD responses (Newman *et al*., 2007). LPS promotes a significant acidification of potato, tomato and tobacco extracellular media, without induction of ROS formation (Saubeau *et al*., 2013).

The activity of antioxidant enzymes is promoted as scavengers of ROS generated by the increase of bacterial spot lesions caused by the advance of bacterial colonization on susceptible tomato leaves (Cavalcanti *et al*., 2006). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and H_2O_2 (Mittler, 2002). The present results showed a negative correlation between SOD and CAT activities in Cp+Pba and XTH+Pba treatments. However in Cp+Pba treatment the negative correlation was stronger than the negative correlation observed in XTH+Pba treatment. A positive correlation was also observed between CAT and APx in XTH+Pba and ASM+Pba. These results suggest a relative low and transient H_2O_2 accumulation caused by XTH+Pba treatment and a strong H_2O_2 accumulation as consequence of Cp+Pba treatment. Low and transient levels of H_2O_2 may induce stress acclimation, while high levels may trigger PCD (Dat *et al*., 2000). The prolonged production of ROS for several hours induces a necrotic

process. CAT and APx acts protecting the cell from the detrimental effects of H_2O_2 accumulation by scavenging this molecule (Dat *et al*., 2000). The present work showed that leaves pretreated with elicitors and challenged with the phytobacteria increased CAT activity in almost all analyzed time points. The treatments XTH+Pba and ASM+Pba promoted the highest CAT and APx activity, respectively, when compare to the other treatments. However, CAT and APx activities in leaves of Cp+Pba treatment were similar to the control leaves (Cp+Ct). In *Arabidopsis thaliana* CAT inhibited cell death and conferred evident disease resistance against the necrotrophic bacterial *Pectobacterium carotovorum* (Ahn, 2007). Moreover, the increase in CAT and APx activities was described in resistant lines of *Brassica napus* to the necrotrophic pathogen *Sclerotinia sclerotiorum* (Wen, 2012). The pathogenicities of the two necrotrophs *S. sclerotiorum* and *B. cinerea* were directly dependent on the level of H_2O_2 in cells (Wen, 2012). It has been suggested that the inhibition of APx and CAT activities results in the accumulation of H_2O_2 and the activation and acceleration of cell death/HR (Ahn, 2007). Bacterial necrothophic, like the genus *Pectobacterium,* could induces cell death and ROS accumulation in order to infect tissues (Davidsson *et al*., 2013). HR and ROS such as H_2O_2 have been reported to benefit the infection by necrotrophic pathogens, which may even be able to produce ROS themselves or stimulate the host to do so. Additionally, ROS act synergistically in a signal amplification loop with SA to drive the HR in many plant species (Shetty *et al*., 2008).

Levels of resistance in whole plants are also influenced by systemic signals mediated by plant hormones. Classically, salicylic acid (SA) signaling triggers resistance against biotrophic and hemibiotrophic pathogens, whereas a combination of jasmonic acid (JA) and ethylene (ET) signaling activates resistance against necrotrophic pathogens. These two pathways are mostly antagonistic: elevated biotroph resistance is often correlated with increased necrotroph susceptibility, and elevated necrotroph resistance is often correlated with

enhanced susceptibility to biotrophs (Robert-Seilaniantz *et al*., 2011). The present results showed an increment in free SA (active form) in Cp+Pba treatment. Free SA can promote and potentiate the programmed cell death, benefiting *P. carotovorum* infection (Davidsson *et al*., 2013). Several pathogens have acquired the ability to modify plant hormone signaling and commander host hormonal crosstalk mechanisms as a virulence strategy (Garavaglia *et al*., 2010). Interestingly, leaves pretreated with elicitors ASM and XTH and inoculated with *P. atrosepticum* did not show any increase in free SA. In turn, there is some evidence that ASM mimic the SA step, acting at the site or downstream of SA accumulation (Terry & Daryl, 2004). Norman-Setterblad *et al*. (2000) demonstrated that ethylene and JA have a central role in regulating plant defense gene expression as well as resistance against *Erwinia carotovora* subsp. *carotovora* in Arabidopsis. They also suggest that lower levels of SA might have a crucial role in modulating other types of defense. However, activation of plant innate immunity systems including both SA and JA/ET-mediated defenses appears to play a central role in attenuation of *Pectobacterium* virulence (Davidsson *et al*., 2013). Pavlo *et al*. (2011) also demonstrated that both SA and JA/ET-dependent pathways were required in resistant endophyte-infected *Arabidopsis* after challenging with *P. atrosepticum*. It has been demonstrated that potato plants have a high constitutive level of SA that does not lead to constitutive resistance in healthy plants, suggesting potato plants may have a poor SA signal perception and/or transduction mechanism (Yu *et al*., 1997). Therefore, SA-mediated defense responses require not only a sufficiently high level of SA but also an effective SA signal perception and transduction mechanism.

Plant resistance is usually correlated with activation of some defense-related enzymes, like PAL, PPO, POX, chitinase and β-1,3-glucanase. Our results demonstrated that PAL activity was earliest and highest increased in XTH+Pba treatment. ASM+Pba treatment also resulted in an increase in PAL activity when compared to the control, but this activity was

always similar to the Cp+Pba treatment. Similarly, Vayda *et al*. (1992) observed an increment in PAL activity during the interaction with living *P. atrosepticum* cells and potato tubers. Resistance of plants to attempted microbial attack is usually associated with more rapid and more intense induction of defense responses (Newman *et al*., 2007). In *Solanaceae*, plant defenses are generally induced earlier and to a greater extent in incompatible than in compatible interactions (Desender *et al*., 2007). Studies demonstrated that increase in PAL activity was associated with decrease disease severity caused by *P. atrosepticum* (Kroner *et al*., 2012; Saubeau *et al*., 2014). PAL is the key enzyme of the phenylpropanoid pathway catalyzing the transformation of phenylalanine into cinnamic acid, which is the core molecule for the synthesis of phenolics. It has been shown to be involved in defense reactions, either as physical and chemical barriers or by acting as signal molecules (Kroner *et al*., 2012). The present results showed an increment in total phenolic compounds in Cp+Pba, XTH+Pba and ASM+Pba treatments, with the highest level in ASM+Pba. Phenolic compounds are often considered to play an important role in resistance to many plant pathogens, including potato resistance against *Pectobacterium*. Phenolics can directly inhibit bacterial growth, by inhibiting wall-degrading enzymes, or as precursors in the formation of lignin and suberin, which are both involved in the formation of physical barriers that can completely block the spread of pectobacteria (Meziani *et al*., 2015; Wegener & Jansen, 2007). According to Meziani *et al* (2015), potato tuber infected by *P. atrosepticum* accumulates phenolics. Andreu *et al*. (2001) also suggested a role for phenolics in resistance of potato to *P. infestans*, since the amount of phenolics induced after infection was lower in a susceptible potato cultivar, compared to a resistant cultivar. Indeed, total phenolic content was negatively correlated with tuber rot severity due to *P. atrosepticum* (Kroner *et al*., 2012).

Phenolic compounds are potential substrates of POX and PPO enzymes. The present results showed an increase in PPO activity in ASM+Pba and XTH+Pba, while leaves in the

Cp+Pba treatment did not exhibit any increase. PPOs catalyze the oxidation of phenols to quinones, molecules more toxic to pathogens (Li & Steffens 2002). PPO is important in the initial stage of plant defense, where membrane damage causes release of phenols (Wegener & Jansen, 2007). PPO appears to play a role in resistance to soft rot caused by *P. carotovorum* in potato tuber (Wegener & Jansen, 2007). Li and Steffens (2002) reported that the over-expression of a potato PPO gene in a transgenic tomato plants, improved the plant resistance against *Pseudomonas syringae pv. tomato.* Potato susceptibility to soft rot and defense responses are modulated by their faculty to produce phytoalexins, and to induce key enzymes like PPO and PAL (Baz *et al*., 2012). POX is also related to defense against pathogen attack through physiological processes such as lignification, suberization and cross-linking of cell wall proteins. POX catalyzes the polymerization of phenolics to produce a variety of bioactive products, contributing to chemical as well as physical defenses (Jang *et al*., 2004). The present results demonstrated that POX activity increased in ASM+Pba and Cp+Pba treatments. Similar result was described by Malolepsza (2006), whom observed an increment in POX activity in tomato plants pretreated with ASM and challenged with *Botrytis cinerea*. According to Jang *et al*. (2004), the increase in POX activity in sweet potato after *Pectobacterium chrysanthemi* inoculation, suggested that POXs are involved in defense mechanism in relation to pathogenesis of *P. chrysanthemi*. However, POX activity was not correlated with potato resistance against *P. carotovorum* (Wegener & Jansen, 2007). Poiatti *et al*. (2009) also observed an increment in POX activity during *Erwinia carotovora* infection in potato plants, and no differences in POX activity in plants inoculated with *Xanthomonas axonopodis* pv. *citri*.

Many biochemical changes occur during systemic acquired resistance. PR-proteins including acidic β-1,3-glucanase and chitinase are secreted in the intercellular spaces, where they act against fungal and or bacterial pathogens at early stage of infection process

(Hajhamed *et al*., 2007). The present results showed an early increase in β-1,3-glucanase and chitinase activity in potato leaves pretreated with ASM or XTH and challenged with *P. atrosepticum*. While Cp+Pba treatment induced only β-1,3-glucanase. The enzymatic activities of β-1,3-glucanases and chitinases possess direct antimicrobial activity by degrading microbial cell wall components. Some plant chitinases also have lysozyme activity and can therefore hydrolyse bacterial cell walls (Baysal *et al*., 2003). Studies have been shown that ASM and biotic elicitors promote early accumulation and activity of chitinase and glucanase in other plant–pathogen interactions (Cavalcanti *et al*., 2006). In turnip (*Brassica campestris*) and pepper, LPS from *Xanthomonas campestris* pv. *campestris* induced expression of a gene encoding a defense-related β-1,3-glucanase when applied to leaves (Newman *et al*., 2007). Interestingly, many works suggest that β-1,3-glucanases are probably not directly involved in resistance against bacteria, while chitinases participate in early basal resistance induced by PAMPs against virulent bacteria (Baysal *et al*., 2003).

In conclusion, the elicitor XTH was able to delay disease symptoms and progression caused by *P. atrosepticum*. This resistance promoted by XTH could be explained by a previous cellular-response modulation, where the *P. atrosepticum* was enable to promote oxidative burst and plant cell death (HR). On the other hand, this previous XTH-modulation led to activation of enzymes related to defense, such as PAL, PPO and chitinase after plant infection with *P. atrosepticum*. Inducers are a new tool for plant disease control, leading to the development of new commercial products. Although the majority of mechanisms related to plant resistance mediated by elicitors still remains elusive, further studies in field conditions must be performed with XTH formulations in order to evaluate any non desired effect on plant growth.

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FIGURES AND TABLES

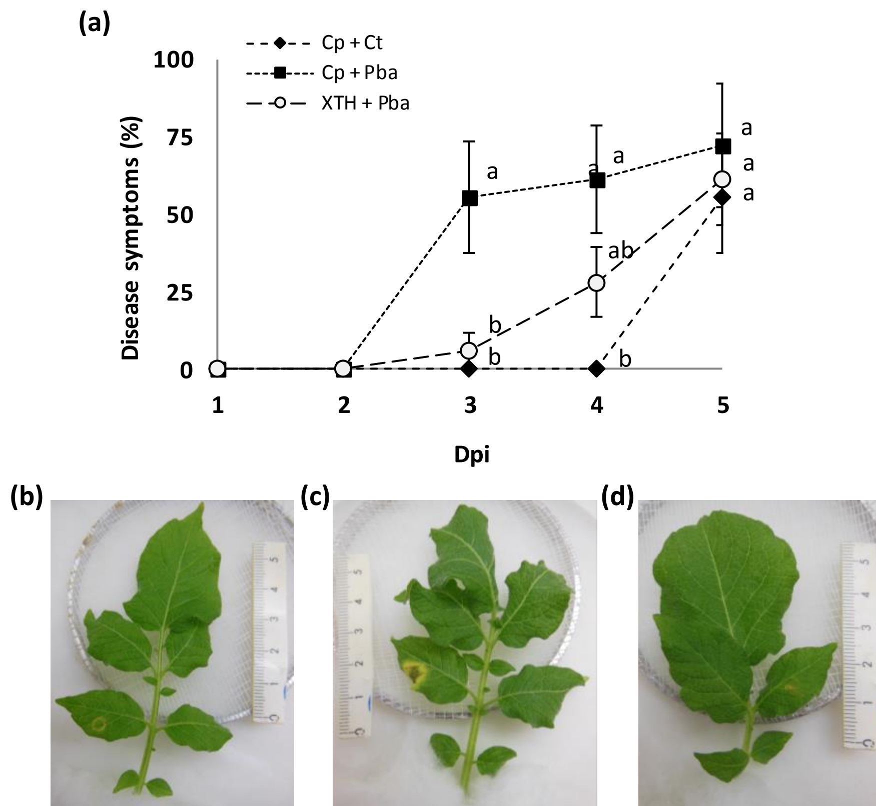

Figure 1 – (a) Disease symptoms in detached leaves of *S. tuberosum* pretreated with XTH (bacterial extract) or sterile water (Cp), and inoculated with *P. atrosepticum* (Pba) or sterile water (Ct). Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$). Representative images of leaves at 3 dpi: (b) Cp+Ct, (c) Cp+Pba, infected leaf with necrotic spot in the leaflet, (d) XTH+Pba.

Table 1 – Mean AUDPC (Area Under the Disease Progress Curve) values and standard errors (in parenthesis) represent the disease progression in detached leaves of *S. tuberosum* pretreated with XTH (bacterial extract) or sterile water (Cp), and inoculated with *P. atrosepticum* (Pba) or sterile water (Ct). Different letters indicate significant differences between the treatments (Duncan's test, $p \leq 0.05$).

Treatments	AUDPC
Cp + Ct	111,00 (80,65) b
Cp + Pba	496,72 (314,22) a
XTH + Pba	233,10 (151,50) b

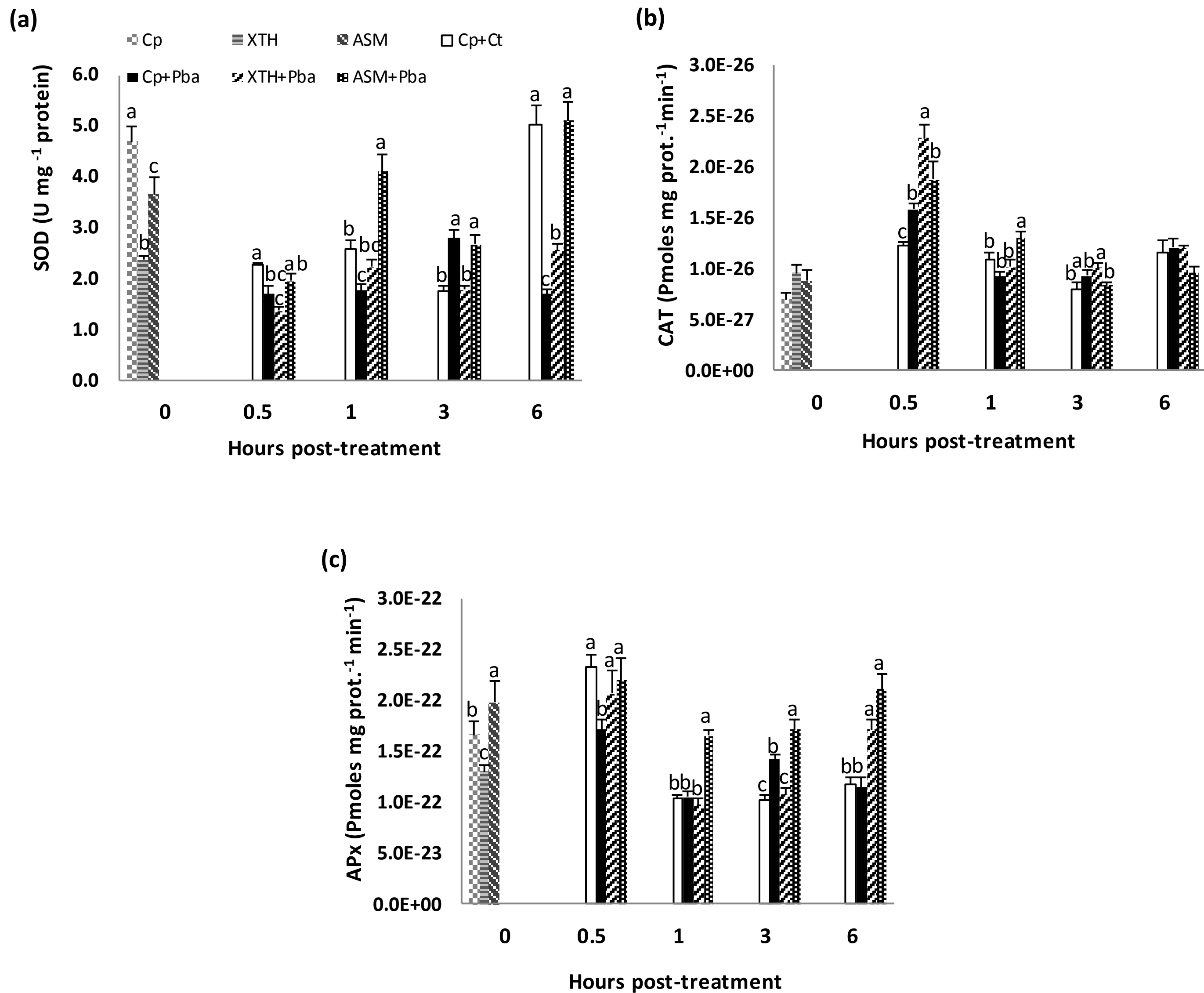

Figure 2 – Activity of antioxidant enzymes (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) ascorbate peroxidase (APx) in detached leaves. Plants were pretreated with extract of *Xanthomonas axonopodis* (XTH), Acibenzolar-S-methyl (ASM) or water (Control, Cp). After four days, leaves were detached and treated with *P. atrosepticum* (Pba) or water (Control, Ct). Time point 0 consisted in leaves detached four days after the pretreatment (with elicitors or water) and before the treatment with the challenger. Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

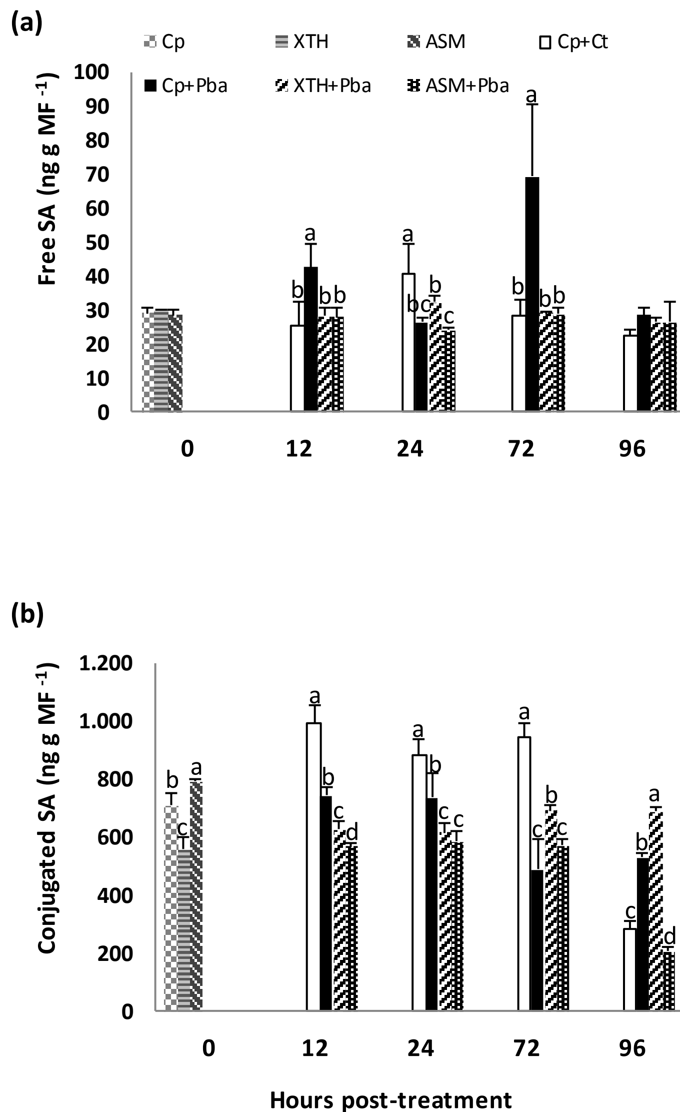

Figure 3 – Levels of (a) free and (b) conjugated salicylic acid (SA) in detached leaves. Plants were pretreated with extract of *Xanthomonas axonopodis* (XTH), Acibenzolar-S-methyl (ASM) or water (Control, Cp). After four days, leaves were detached and treated with *P. atrosepticum* (Pba) or water (Control, Ct). Time point 0 consisted in leaves detached four days after the pretreatment (with elicitors or water) and before the treatment with the challenger. Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

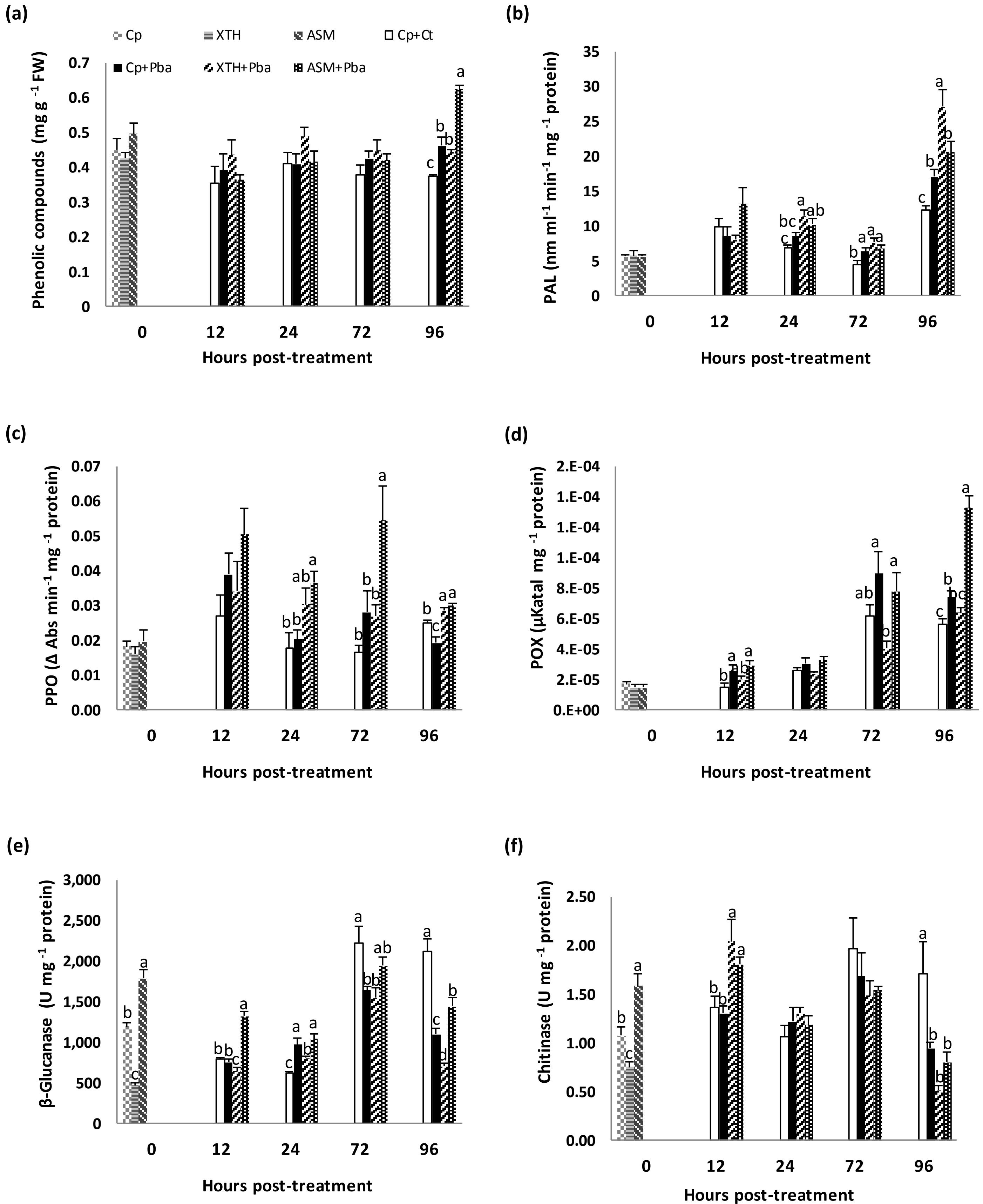

Figure 4 – Level of (a) phenolic compounds and activity of (b) phenylalanine ammonia lyase (PAL), (c) polyphenol oxidase (PPO), (d) peroxidase (POX), (e) β-1,3-glucanase and (f) chitinase in detached leaves. Plants were pretreated with extract of *Xanthomonas axonopodis* (XTH), Acibenzolar-S-methyl (ASM) or water (Control, Cp). After four days, leaves were detached and treated with *P. atrosepticum* (Pba) or water (Control, Ct). Time point 0 consisted in leaves detached four days after the pretreatment and before the treatment with the challenger. Different letters indicate significant differences within the same time point (Duncan's test, $p \leq 0.05$).

Capítulo III

CONSIDERAÇÕES FINAIS

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Há cerca de 9.000 anos o homem vem domesticando, cultivando e selecionando as plantas para fins de consumo alimentício. O melhoramento empreendido pelo homem, desde as mais remotas épocas da agricultura, permitiu que gradualmente as culturas se tornassem mais produtivas. A seleção de plantas mais adaptadas ao ambiente e com maior potencial de produção de sementes, tubérculos, folhas, raízes, etc, contribuiu para a rápida transição do homem à condição civilizada e ao aumento da população humana.

Devido à seleção, iniciou-se o acúmulo de linhagens vegetais que diferiam dos ancestrais selvagens. Com as guerras, o comércio e as viagens empreendidas a distâncias cada vez maiores, as linhagens melhoradas tornaram-se cada vez mais dispersas. A seleção de linhagens mais produtivas e adaptadas culminou na acentuada erosão genética, perdendo-se importantes genes responsáveis pela resistência a doenças e pragas.

O uso indiscriminado de agrotóxicos, que matam e controlam a disseminação de plantas invasoras, insetos e fungos nas plantações, tem preocupado as principais instituições de saúde pública e do meio ambiente, assim como a população em geral. O uso extensivo de agroquímicos nas lavouras aumenta o custo de produção, e pode levar à contaminação do solo, danos ao meio ambiente e intoxicação humana e animal.

Com este cenário, o uso de indutores de defesa vegetal vem sendo largamente estudado, visando à promoção de resistência local ou sistêmica da planta, fornecendo ferramentas para o manejo de lavouras, promovendo uma diminuição no uso de agroquímicos e preservando o uso de cultivares com alto potencial agrícola que apresentam alta suscetibilidade a patógenos.

O indutor biótico XTH, desenvolvido a partir de uma solução bacteriana de *Xanthomonas axonopodis* pv. *citri* autoclavada, vem sendo extensivamente estudado na modulação de respostas de defesa e no retardo de doenças provocadas por patógenos, como a bactéria necrotrófica *Pectobacterium atrosepticum,* em plantas de *Solanum tuberosum*.

Neste estudo, foi demonstrado que o indutor XTH promove a ativação de enzimas antioxidantes, levando a um rápido e transiente acúmulo de espécies reativas de oxigênio nas células das folhas de plantas de batata. Além disso, o indutor biótico pareceu não envolver o estabelecimento de respostas de hipersensibilidade e a via do ácido salicílico na ativação de respostas defesa. Contudo, estudos mais detalhados sobre a via hormonal ativada pelo XTH, ainda devem ser realizados. O XTH também promoveu a atividade de enzimas relacionadas à defesa, como fenilalanina amônia liase, polifenoloxidase e quitinase. O mesmo padrão de resposta relatado foi observado nas folhas pré-tratadas com XTH e desafiadas com a bactéria *P. atrosepticum*, indicando que o retardo da doença promovido

pelo indutor pode estar relacionado com os mecanismos discutidos anteriormente. Além disso, foi observado que o indutor XTH difere do indutor Acibenzolar-S-metil (ASM) na ativação de alguns padrões de resposta, como por exemplo, na atividade de peroxidases. Apesar disso, os dois indutores foram capazes de modular rapidamente o metabolismo de defesa antes e após o desafio com a bactéria em folhas destacadas de *S. tuberosum.*

Estudos prévios em casa de vegetação demonstraram o potencial do XTH na indução de resistência de plantas de *S. tuberosum* contra *P. atrosepticum*. Contudo, ainda não foram realizados experimentos em condição de campo (lavoura experimental). Além disso, se faz necessário verificar a eficácia do XTH em tubérculos de batata, nos quais doenças provocadas por patógenos, como a *P. atrosepticum,* são frequentes. Estudos sobre a promoção de resistência sistêmica e a durabilidade da proteção proporcionada pelo indutor biótico também são aspectos a serem examinados em futuros experimentos. Ainda, seria de crucial interesse verificar a indução de resistência pelo XTH em outras interações planta-patógeno antes de considerarmos o seu uso em formulações comerciais. Considerando estas perspectivas, é necessário otimizar o cultivo em escala comercial de *Xanthomonas axonopodis* pv. *citri*, avaliando a produção de moléculas extracelulares solúveis e com capacidade eliciadora.

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