LÍVIO DA SILVA AMARAL

ASPECTOS BIOQUÍMICOS DA INTERAÇÃO REPOLHO-XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de Doctor Scientiae.

VIÇOSA
MINAS GERAIS – BRASIL
2016

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T
A485a
2016

Amaral, Lívio da Silva, 1986-
Aspectos bioquímicos da interação repolho-*Xanthomonas campestris* pv. *campestris* / Lívio da Silva Amaral. – Viçosa, MG, 2016.
viii, 88f. : il. ; 29 cm.

Orientador: José Rogério de Oliveira.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Repolho - Doenças e pragas. 2. Repolho - Resistência a doença e pragas. 3. *Brassica*. 4. *Xanthomonas campestris*. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-graduação em Fitopatologia. II. Título.

CDD 22. ed. 635.34

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APROVADA: 25 de fevereiro de 2016.

Hélvio Gledson Maciel Ferraz

Maria Fernanda Antunes da Cruz

Jorge Luís Badel Pacheco

Fabrício de Ávila Rodrigues
(Coorientador)

José Rogério de Oliveira
(Orientador)

Não sabendo que era impossível,
foi lá e fez.
(Jean Cocteau).

À minha amada família,
minha mãe Aparecida,
meu pai Fernando,
minha tia Creusa,
meus irmãos Fernanda e Lucas,
e minha namorada Cristiane.

Dedico.

AGRADECIMENTO

A Deus, por ter me guiado por toda a minha vida e colocado pessoas tão especiais em meu caminho para chegar até aqui.

Aos meus pais Fernando e Aparecida, meus irmãos Fernanda e Lucas, e minha tia Creusa por todo amor, carinho e apoio incondicionais a mim dispensados.

À minha namorada Cristiane pelo amor, compreensão, dedicação, ajuda nas análises de resultados e todos os momentos compartilhados.

À Universidade Federal de Viçosa pela oportunidade a mim dada para me formar como Agrônomo e concluir o curso de Doutorado em Fitopatologia.

Ao professor José Rogério de Oliveira pela orientação nos trabalhos, mas principalmente por ter me inspirado a trabalhar com Bacteriologia de Plantas e me dar a oportunidade de trabalhar nesta área.

Ao professor Fabrício de Ávila Rodrigues por todo o apoio.

Aos membros da banca de defesa Prof. Jorge Luis Badel Pacheco, Dr^{a}. Maria Fernanda Antunes da Cruz e Dr. Hélvio Gledson Maciel Ferraz pela disponibilidade e conselhos.

Ao amigo Daniel Debona pela ajuda nas análises de resultados e, principalmente, pela parceria e amizade.

A todos os professores e funcionários do Departamento Fitopatologia pelos ensinamentos transmitidos e pela amizade.

Aos amigos Suely, Ana Luiza, Gabriel, Hélvio, Belarmina, Iane, Lilian, Letícia e Carol pelos ótimos momentos compartilhados no laboratório, e aos amigos da Pós-Graduação, pela amizade, pelas dificuldades e alegrias que compartilhamos.

A todos os meus grandes amigos Raul, Suzane, Wagner, Karine, Teu, Elisângela, Paulo Henrique, Sara, Hudson, Ana, Ramon, Daiana, Carlos, Patrícia, Élder e Carol pelos ensinamentos e pelos ótimos momentos vividos e que ainda vamos viver.

Aos amigos Victor, Alberto, Marcos, Tiago, Bruna e Adriana, além dos outros amigos conquistados na UFV por tornarem minha caminhada mais prazerosa.

Aos companheiros de futebol pela amizade e momentos de descontração.

A todos que contribuíram direta ou indiretamente para a minha formação.

Muito obrigado!

BIOGRAFIA

LÍVIO DA SILVA AMARAL, filho de Maria Aparecida da Silva Amaral e Fernando Antônio Rodrigues do Amaral, nasceu em Viçosa, Minas Gerais - Brasil em 17 de julho de 1986.

Em março de 2005, iniciou o curso de Agronomia na Universidade Federal de Viçosa (UFV) em Viçosa - Minas Gerais, graduando-se em janeiro de 2010.

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Em março de 2012, iniciou o Programa de Pós-Graduação em Fitopatologia, em nível de Doutorado, na Universidade Federal de Viçosa, com concentração na área de Bacteriologia de Plantas, sob orientação do Prof. José Rogério de Oliveira, submetendo-se à defesa da tese em 25 de fevereiro de 2016.

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RESUMO

AMARAL, Lívio da Silva, D.Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Aspectos bioquímicos da interação repolho-Xanthomonas campestris pv. campestris.** Orientador: José Rogério de Oliveira. Coorientador: Fabrício de Ávila Rodrigues.

O repolho (Brassica oleracea var. capitata) está entre as hortaliças mais produzidas e consumidas em todo o mundo. Xanthomonas campestris pv. campestris (Xcc) é o agente causal da podridão negra das brássicas, a doença mais destrutiva da cultura do repolho. O plantio de variedades resistentes é uma medida simples e barata para o controle de doenças de plantas. Fontes de resistência à podridão negra são escassas no genoma de B. oleracea. No entanto, o plantio de cultivares com maiores níveis de resistência ou a aplicação de indutores de resistência pode auxiliar no controle da doença. Embora essas medidas de controle sejam viáveis, estudos sobre os mecanismos de defesa basal de natureza bioquímicas do repolho e da indução de resistência à podridão negra são raros na literatura. O presente estudo visou encontrar variedades com maiores níveis de resistência à doença, observar a variabilidade patogênica de isolados de Xcc em relação a diferentes cultivares de repolho, verificar diferenças nas respostas bioquímicas de defesa do repolho a diferentes isolados de Xcc, verificar diferenças nas respostas bioquímicas de defesa de cultivares repolho com diferentes níveis de resistência à doença incitada por um isolado de Xcc, e observar a eficiência do Acibenzolar-S-Metil (ASM) no controle da podridão negra em repolho. Os cultivares ‘Fuyutokyo Kobayashi’, ‘Midori’ e ‘Coração de Boi Gigante’ apresentaram maiores níveis de resistência à podridão negra e os cultivares ‘Esmeralda’ e ’60 dias’ se mostraram altamente suscetíveis. Xcc induziu respostas de defesa em repolho, embora isolados mais agressivos foram capazes de modular essas respostas. Cultivares de repolho mais resistentes à podridão negra sofreram alterações significativas nas atividades das enzimas de defesa. As alterações que ocorreram em cultivares mais suscetíveis não foram suficientes para conter o processo infeccioso de Xcc. Plantas de repolho tratadas com ASM exibiram atividades enzimáticas significativamente maiores do que as não tratadas e apresentaram menor severidade da doença.

ABSTRACT

AMARAL, Lívio da Silva, D.Sc., Universidade Federal de Viçosa, February, 2016. **Biochemical aspects of the interaction cabbage-Xanthomonas campestris pv. campestris.** Adviser: José Rogério de Oliveira. Co-adviser: Fabrício de Ávila Rodrigues.

Cabbage (Brassica oleracea var. capitata) is among the most produced and consumed vegetables in the world. Xanthomonas campestris pv. campestris (Xcc) is the causal agente of the black rot of brassicas, the most destructive disease in cabbage crop. Planting resistant varieties is a simple and cheap way to control plant diseases. Sources of resistance to the black rot are scarce in the B. oleracea genome. However, planting varieties with higher resistance levels or applying resistance inducers may help to control the disease. Although these control measures are viable, studies on the basal defense mechanisms of biochemical nature in cabbage and resistance induction against black rot are rare in the literature. The present study aimed to find cultivars with higher resistance levels to the disease, determine the pathogenic variability of Xcc isolates in relation to different cabbage cultivars, verify differences in the cabbage biochemical defense responses with different resistance levels to the disease incited by a Xcc isolate, and observe the efficiency of Acibenzolar-S-Methyl (ASM) to control of the black rot in cabbage. Cultivars 'Fuyutokyo Kobayashi', 'Midori' and 'Coração de Boi Gigante' were more resistant to black rot and the cultivars 'Esmeralda' e '60 dias' were highly susceptible and may be used in pathogenicity tests for Xcc detection. Xcc induced defense responses in cabbage, however more aggressive isolates were able to modulate these defense responses. Cabbage cultivars more resistant to the black rot had significant alterations in the defense enzymes activities. The alterations that occurred in the more susceptible cultivars were not sufficient to contain the infectious process of Xcc. Cabbage plants treated with ASM exhibited enzymatic activities meaningfully higher than those non-treated and showed lower disease severity.

INTRODUÇÃO GERAL

A família Brassicaceae compreende várias espécies de plantas cultivadas e de importância econômica, bem como espécies de plantas daninhas e Arabidopsis thaliana, planta modelo para diversos estudos (Reis e Boiteux 2008). Dentre as espécies de importância econômica, encontram-se agrião (Nasturtium officinale), brócolis (Brassica oleracea var. italica), couve (Brassica oleracea), couve-flor (Brassica oleracea var. botrytis), nabo (Brassica napus), rabanete (Raphanus sativus) e repolho (Brassica oleracea var. capitata; Ribeiro 2010).

Dentre as principais hortaliças propagadas por sementes no Brasil, o repolho é a hortaliça com maior produção, totalizando 1,313 milhões de toneladas, sendo superado apenas pela melancia, pelo tomate e pela cebola. O repolho é a décima hortaliça com a qual mais se gasta com agroquímicos no país, alcançando R$ 18,4 milhões (ABCSEM 2011).

Dentre as doenças que afetam as brássicas, a podridão negra, causada por Xanthomonas campestris pv. campestris (Xcc), é a mais destrutiva nesse grupo de plantas, causando perdas na produção e na qualidade do produto (Villeth et al. 2009). O sintoma característico da doença é a mancha em forma de "V-invertido" a partir dos bordos da folha. A princípio, uma mancha clorótica evolui tornando-se necrótica com bordos amarelados e as nervuras tornam-se enegrecidas. O patógeno pode atingir os vasos condutores e infectar a planta sistemicamente causando manchas foliares e escurecimento dos vasos em qualquer parte da planta (Agrios 2005; Vicente e Holub 2013; Lange et al. 2015; Roohie e Umesha 2015).

Xanthomonas campestris é uma bactéria Gram negativa, de cor amarela, nitrato redutase negativa, catalase positiva e oxidase negativa (Garrity et al. 2004). Essa espécie compreende um grupo de organismos que causam doenças em diversas culturas

de importância econômica, dentre elas, plantas pertencentes à família Brassicaceae como repolho, couve, rabanete e couve-flor (Vauterin et al. 1995).

Xanthomonas campestris pv. campestris pode ser dispersada a curtas distâncias pela água da chuva ou irrigação, e a longas distâncias por meio de sementes contaminadas. Além disso, a bactéria pode sobreviver no solo, em restos culturais e como epífitas em plantas daninhas, consistindo em fontes de inóculo do patógeno (Gitaitis e Walcott 2007; Vicente e Holub 2013).

Para o manejo integrado da podridão negra em repolho são recomendados o manejo da irrigação, o plantio de mudas e sementes sadias, a rotação de culturas, a eliminação de restos culturais, a eliminação de plantas doentes e de plantas daninhas, dentre outros (Agrios 2005; Gitaitis e Walcott 2007; Vicente e Holub 2013). O uso de material com resistência qualitativa é limitado pela escassez de fontes de resistência em B. oleracea. Assim, uma alternativa interessante seria investigar os componentes bioquímicos da planta que são mais importantes na defesa contra Xcc e obter informações que podem ser utilizadas em programas de melhoramento que visem obter cultivares de repolho resistentes à podridão negra, ou no desenvolvimento de indutores de resistência (Vicente e Holub 2013).

Plantas possuem a capacidade de aumentar seus níveis de defesa basal para se protegerem contra o ataque de patógenos. Este processo, conhecido como indução de resistência (IR), pode ser dividido em duas classes denominadas resistência sistêmica adquirida (SAR) e resistência sistêmica induzida (ISR). A SAR é dependente da sinalização pelo ácido salicílico (SA) que promove a produção de proteínas relacionadas à patogênese (proteínas PR), enquanto a ISR é ativada por jasmonatos (JA) e etileno (ET), e não promove o aumento das proteínas PR (Pieterse et al. 2014). Essas respostas têm como objetivo evitar o progresso da doença e incluem a produção de compostos tóxicos, enzimas degradadoras do patógeno e até morte celular vegetal programada

(Freeman e Beattie 2008). Vários indutores de resistência têm sido desenvolvidos e sua aplicação tem resultado no controle de doenças em várias culturas, dentre as quais a podridão de Fusarium em trigo, doenças em tomate, mancha bacteriana em pimentão, vassoura-de-bruxa e murcha vascular em cacaueiro e mancha foliar em canola (Buonaurio et al. 2002; Resende et al. 2002; Huang et al. 2012; Oxley e Walters 2012; Moya-Elizondo e Jacobsen 2016). Dentre os indutores de resistência, o Acibenzolar-S-Metil (ASM, também conhecido como ácido benzo(1,2,3)tiadiazole-7-carboxílico, BTH) é uma substância sintética análoga do SA que tem exibido eficiência no controle de doenças em diversos patossistemas (Véronési et al. 2009).

O ataque de patógenos leva à indução de produção de espécies reativas de oxigênio (EROS) tais como peróxido de hidrogênio (H_2O_2), superóxido (O_2^-) e hidroxila (OH^-), as quais são responsáveis por causar danos na membrana celular (peroxidação de lipídeos), pigmentos, proteínas e ácido nucléico. Com o objetivo de remover o excesso de EROS decorrente da interação entre planta e patógeno, enzimas como superóxido dismutase (SOD), catalase (CAT), peroxidases (POX), glutationa-S-transferase (GST), ascorbato peroxidase (APX), glutationa redutase (GR), glutationa peroxidase (GPX) e lipoxigenases (LOX) são produzidas pela planta e a quantificação da atividade dessas enzimas pode nos dar informações sobre qual mecanismo de defesa é mais importante na resistência contra patógenos (García-Cristobal et al. 2015; Roohie e Umesha 2015). Outros componentes importantes da defesa da planta são as proteínas relacionadas à patogênese (proteínas-PR), tais como β-1,3-glucanases (GLU) e quitinases (CHI), as quais estão associadas à degradação de parede celular fúngica, e fenilalanina amônia-liases (PAL), a qual, por sua vez, está associada à produção de lignina, fitoalexinas e de compostos fenólicos (Hong e Hwang 2005; Kim et al. 2015).

Há estudos sobre a interação planta-bactérias fitopatogênicas (López-Gresa et al. 2011; Andrade et al. 2013; Raghavendra et al. 2013) porém poucos são os trabalhos que

tratam da interação Xcc-repolho (Conrads-Strauch et al. 1990; Newman et al. 1995). Conrads-Strauch et al. (1990) verificaram o aumento da atividade das enzimas β-1,3-glucanase e quitinase 24 horas após a inoculação de Xcc em folhas de nabo. Enquanto quitinases possuem atividades de lisozima e atuem diretamente sobre a bactéria, β-1,3-glucanase atuam na liberação de elicitores que amplificam as respostas de defesa na planta (Hong e Hwang 2005; Kim et al. 2015). Gay e Tuzun (2000) avaliaram a atividade de enzimas relacionadas ao estresse oxidativo (CAT, SOD e POX) e quantificaram H_2O_2 e lignina ao longo do tempo na interação Xcc-repolho. Os autores verificaram um aumento maior na atividade de SOD, POX e na deposição de lignina em cultivares mais resistentes do que cultivares mais suscetíveis após a infecção, sugerindo que estes componentes são importantes na defesa de plantas de repolho contra Xcc.

A falta de informação a respeito de como brássicas respondem ao ataque de bactérias desperta o interesse de pesquisadores sobre o assunto, pois as informações oriundas dessas pesquisas poderão direcionar o desenvolvimento de indutores de resistência e de cultivares resistentes.

Neste trabalho, procuramos entender quais os componentes da defesa de plantas de repolho são mais importantes contra a podridão negra, se variações na agressividade do patógeno estão relacionados a variações nos componentes de defesa ativados no repolho, se há cultivares resistentes dentre aqueles testados, se diferenças nos níveis de resistência de cultivares de repolho são reflexo de variações de alterações bioquímicas, e se a aplicação de ASM tem efeito na ativação de SAR contra a podridão negra em repolho.

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CAPÍTULO 1 - RESPOSTA DIFERENCIAL DE CULTIVARES DE REPOLHO À PODRIDÃO NEGRA E VARIABILIDADE PATOGÊNICA DE ISOLADOS DE XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS

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Resumo

O repolho é uma das hortaliças pertencente à família Brassicaceae mais produzidas e consumidas no mundo. Dentre os fatores que limitam sua produção, a podridão negra das brássicas (Xanthomonas campestris pv. campestris; Xcc) se destaca por ser a doença mais destrutiva na cultura. No Brasil, não há no mercado nenhum cultivar de repolho com resistência vertical comprovada. O objetivo deste estudo foi verificar a variabilidade patogênica de isolados de Xcc, a resistência de diferentes cultivares de repolho à podridão negra e propor cultivares que possam ser utilizados como padrão de suscetibilidade para testes de detecção de Xcc. Vinte e um isolados de Xcc foram inoculados em 12 cultivares de repolho e avaliou-se a incidência da doença, o período de incubação (PI) e a frequência de infecção (FI). Apenas seis isolados de Xcc não foram capazes de causar doença em todos os cultivares de repolho. Houve variação na gama de cultivares de repolho infectados e na agressividade entre isolados de Xcc. Os isolados Xcc 105 e S3 induziram sintomas na menor quantidade de cultivares e também foram os menos agressivos. Os cultivares '60 dias', 'Esmeralda' e 'Coração de Boi' apresentaram maior suscetibilidade à podridão negra. Para uso em testes de detecção, os cultivares '60 dias' e 'Esmeralda' se mostraram mais adequados por apresentarem suscetibilidade a todos os isolados de Xcc, menores valores de PI e maiores valores de FI. A padronização de testes para definir a resistência de cultivares de repolho à podridão negra é necessária e deve considerar a variabilidade patogênica dentro das populações de Xcc e as variações climáticas. Dentre os cultivares avaliados, 'Coração de Boi Gigante', 'Fuyutokyo Kobayashi' e 'Midori' foram infectados pela menor quantidade de isolados de Xcc e apresentaram os maiores valores de PI e os menores valores de FI, sendo, portanto, os mais resistentes à podridão negra.

Abstract

Cabbage is one of the vegetables belonging to the family Brassicaceae most produced and consumed in the world. Among the factors that limit the yield, the black rot of brassicas (Xanthomonas campestris pv. campestris; Xcc) stands out for being the most destructive disease in this crop. In Brazil, there is no cabbage cultivar with proven vertical resistance in the market. The objective of this study was to verify the pathogenic variability among isolates of Xcc, as well as the resistance of different cabbage cultivars to black rot and to propose cultivars that may be used as susceptibility patterns in detection tests for Xcc. Twenty one isolates of Xcc were inoculated onto 12 cabbage cultivars and the disease incidence, the incubation period (IP) and the infection frequency (IF) were assessed. Only six Xcc isolates were not able to cause disease in all cabbage cultivars. There was variation in the cultivars range and in the aggressiveness among isolates of Xcc. The isolates Xcc 105 and S3 induced symptoms in the least amount of cultivars and were the less aggressive. The cultivars '60 dias', 'Esmeralda' and 'Coração de Boi' exhibited the highest susceptibility to black rot. For the use in detection tests, the cultivars '60 dias' and 'Esmeralda' were the most adequate by exhibiting susceptibility to all isolates of Xcc, the lowest IP values and the highest FI values. The standardization of tests to define the resistance of cabbage cultivars to black rot is necessary and must consider the pathogenic variability inside the populations of Xcc and the climatic variations. Among the cultivars evaluated, 'Coração de Boi Gigante', 'Fuyutokyo Kobayashi' and 'Midori' were infected by the least amount of isolates of Xcc and exhibited the highest IP values and the lowest IF values, being, therefore, the most resistant to black rot.

Introdução

O repolho (Brassica oleracea var. capitata) é uma das hortaliças mais plantadas e consumidas em todo mundo. Segundo a FAO (2013) a produção mundial em 2013 foi de 70 milhões de toneladas, sendo a China o maior produtor dessa hortaliça e a Lituânia o maior produtor de sementes de repolho. No Brasil, a produção de repolho superou os 1,3 milhões de toneladas no ano de 2011. Só no Estado de São Paulo no ano de 2012, a produção foi de 278.855 toneladas em uma área plantada de 7.143 ha, correspondendo a uma produtividade de 39,0 kg/ha (Carvalho et al. 2013).

Dentre as doenças que afetam e limitam a produção do repolho, a podridão negra, causada por Xanthomonas campestris pv. campestris (Xcc) (Pammel) Dowson, é considerada a doença mais destrutiva para a cultura em todas as regiões do mundo onde esta hortaliça é cultivada. (Lange et al. 2015; Roohie and Umesha 2015; Saha et al. 2016). Normalmente, a bactéria penetra pelos hidatódios, coloniza o tecido e pode se translocar dentro da planta causando sintoma tanto próximo quanto distante do sítio de infecção. Uma mancha clorótica pode ser visualizada ao redor do sítio de infecção, progredindo para uma lesão em forma de "V" com o vértice voltado para a nervura central. O centro da lesão torna-se necrótico e pode ser visto o enegrecimento de nervuras dentro e em torno da lesão. (Agrios 2005; Vicente e Holub, 2013; Lange et al. 2015; Roohie e Umesha 2015).

X. campestris pv. campestris pode ser dispersa por água de chuva e irrigação a curtas distâncias. Para evitar a disseminação da doença a curtas distâncias é importante o manejo da irrigação e do espaçamento entre plantas (Gitaitis e Walcott 2007; Lee et al. 2009; Vicente e Holub 2013). A longas distâncias Xcc é dispersa pelas sementes. Como a presença de apenas três sementes contaminadas pode resultar na incidência da doença em até 10.000 plantas no campo, além de introduzir a doença em um local onde ela

ainda não ocorre, o desenvolvimento de uma metodologia eficiente para detecção do patógeno bem como a desinfecção das sementes são fatores preponderantes para evitar a disseminação da podridão negra (Chitarra et al. 2002; Massomo et al. 2004; Agrios 2005; Gitaitis e Walcott 2007). O uso de materiais resistentes é uma alternativa interessante e barata para controle de doenças em qualquer cultura. Fontes de resistência vertical em repolho são escassas, mas o desenvolvimento de cultivares de repolho com maiores níveis de resistência contra a podridão negra são estratégias de controle possível e o plantio dessas cultivares pode atrasar o progresso da doença no campo (Williams et al. 1972; Massomo et al. 2004; Shimelis 2005; Vicente e Holub 2013).

Além de Xcc, outros cinco patovares já foram descritos como sendo patogênicos a brássicas, sendo eles X. campestris pv. aberrans, X. campestris pv. armoraciae, X. campestris pv. barbareae, X. campestris pv. incanae e X. campestris pv. raphani (Vauterin et al. 1995). Também tem sido relatada alta variabilidade nas populações de Xcc, o que dificulta ainda mais o emprego de variedades resistentes para o controle satisfatório da doença (Singh et al. 2011). Fargier e Manceau (2007) propuseram a existência de nove raças de Xcc causando diferentes sintomas em uma gama de hospedeiros variada que inclui Brassica spp., Raphanus sativus (rabanete), Armoracia rusticana (raiz-forte), Matthiola incana (goivo), Capsicum annuum (pimentão), Solanum lycopersicum (tomate), dentre outros.

Doze cultivares de repolho foram inoculadas com 21 isolados de Xcc, com o intuito de verificar a resistência desses cultivares à podridão negra, a variabilidade patogênica dos isolados de Xcc, bem como propor um cultivar como padrão de suscetibilidade para ser utilizado em testes de detecção da bactéria.

Material e Métodos

Coleção, isolamento e identificação dos isolados de Xcc

Isolados de Xcc foram obtidos de folhas de brássicas exibindo sintomas de podridão negra recebidas de diversas regiões do Brasil. Isolados pertencentes à coleção de isolados bacterianos do Laboratório de Bacteriologia de Plantas da Universidade Federal de Viçosa também foram utilizados.

O isolamento de Xcc foi realizado conforme o método descrito por Romeiro (2001). Colônias apresentando características típicas de Xcc (amarelas, brilhantes, elevadas, circulares e com bordos regulares) foram repicadas para tubos de ensaio contendo meio 523 (Kado e Heskett 1970) inclinado e incubadas por 48 h a 28°C. Os tubos contendo os isolados foram armazenados em geladeira (5-10°C) e repicados para novos tubos a cada 30 dias.

Os isolados foram preservados em glicerol 30% e em óleo mineral. Isolados cultivados em meio 523 a 28°C por 48 h foram repicados com auxílio de uma alça de repicagem previamente flambada para tubos criogênicos contendo 1 ml de glicerol 30% estéril, agitados em vórtex e imediatamente armazenados em ultrafreezer a -80°C. Os isolados também foram repicados para tubos de vidro (tipo penicilina) estéreis contendo meio 523 e cultivados por 48 h a 28°C. Após o cultivo, foi adicionado 1 ml de óleo mineral estéril sobre o crescimento bacteriano, o tubo foi fechado com tampa de borracha estéril, selado com lacre de alumínio e armazenado em geladeira.

Os isolados foram submetidos a testes morfológicos, bioquímicos, moleculares e de patogenicidade para identificação. Após 48 h de cultivo a 28°C em meio 523, as características típicas de colônias de Xcc foram avaliadas. Os isolados foram repicados para tubos com 5 ml de meio de cultura líquido contendo asparagina como única fonte de carbono e nitrogênio (Rachid e Ahmed 2005). Após sete dias de incubação a 28°C, 50 µl da suspensão de cada isolado foram transferidos para novos tubos contendo o mesmo meio e estes foram novamente incubados a 28°C por sete dias. A avaliação

visual foi feita comparando a turbidez dos tubos contendo os isolados com os tubos controle (apenas o meio de cultura).

A extração do DNA bacteriano foi feita utilizando o Wizard® Genomic DNA Purification Kit (Promega), de acordo com as instruções do fabricante. Após a extração, o DNA foi armazenado a -20°C. As reações de PCR foram montadas utilizando 2 µl de DNA (20 ng/ µl), 1 µl de cada primer e o GoTaq® Green Master Mix (Promega), seguindo as instruções do fabricante para reações com volume de 25 µl. Os primers específicos utilizados foram XCF (5'-CGATTCGGCCATGAATGACT-3') e XCR (5'-CTGTTGATGGTGGTCTGCAA-3') e as reações foram realizadas em termociclador com a desnaturação inicial ocorrendo a 94°C por 5 minutos precedente aos 35 ciclos, cada um com 15 segundos a 94°C, 15 segundos a 58°C e 30 segundos a 72°C, e a extensão final a 72°C por 5 minutos (Park et al. 2004). Alíquotas de cada amplificação foram analisadas através da eletroforese em gel de agarose 1%, voltagem a 100 V constante e corrente a 400 mA. Adicionou-se 2 µl do corante GelRed (1 µl/mL) a 4 µl da amplificação e utilizou-se marcador de 1 kb (Invitrogen™). O tamanho esperado da banda foi de 535 pb. O gel foi fotografado em fotodocumentador L Pix (Loccus Biotecnologia) para posterior análise.

Para confirmação da patogenicidade, foi feita uma inoculação cruzada dos isolados de Xcc com os hospedeiros dos quais eles foram isolados. Sementes de acelga 'Loura', couve 'Manteiga da Geórgia', couve-flor 'Bola de Neve', couve-brócoli 'Piracicaba Precoce' e dos cultivares '60 dias' e 'Esmeralda' de repolho foram semeadas em vasos plásticos contendo substrato Tropstrato HT Hortaliças (VidaVerde®) e mantidos em casa de vegetação por 45 dias até a inoculação. As plantas foram mantidas em saco plástico por 24 h antes e após a inoculação para formação e manutenção da câmara úmida. Isolados cultivados por 48 h a 28°C em meio 523 foram inoculados com auxílio

de palito de madeira estéril. Foram inoculados os bordos de duas folhas de cada planta em cinco pontos equidistantes. Cada isolado foi inoculado em duas plantas de cada espécie/cultivar, totalizando quatro folhas inoculadas e 20 pontos de inoculação. O controle consistiu de plantas feridas com palito estéril sem inóculo. As plantas foram mantidas em casa de vegetação por 21 dias quando foi avaliada a incidência da doença.

Inoculação dos cultivares de repolho

Sementes de 12 cultivares de repolho com diferentes níveis de resistência comercial (resistência informada pela empresa produtora da semente) à podridão negra, a saber 'Esmeralda', 'Louco de Verão', 'Veloce F1', 'Musachi', 'Midori F1', 'Fuyutokio Kobayashi', 'Sekai F1', 'Coração-de-Boi', 'Chato de Quintal', '60 dias', 'Crespo de Milão', 'Coração de Boi Gigante' e 'Blue Canyon' foram semeadas e as plantas cultivadas como descrito anteriormente. As plantas foram transferidas para câmara de nevoeiro e lá permaneceram por 24 h antes e 24 h após a inoculação para formação e manutenção da câmara úmida. Após este período, as plantas foram transportadas de volta à casa de vegetação onde foram mantidas até o final das avaliações. Os isolados identificados como Xcc foram cultivados e inoculados como descrito anteriormente. A inoculação foi feita em cinco pontos equidistantes nos bordos das folhas de repolho, sendo inoculadas duas folhas por planta (duas pseudo-repetições) e duas plantas por isolado, totalizando duas repetições por tratamento. Foi avaliada a incidência (aparecimento ou não de sintomas em pelo menos um dos pontos inoculados), o período de incubação (PI; tempo decorrente da inoculação até o aparecimento do sintoma em cada ponto inoculado) e a frequência de infecção (FI; porcentagem de pontos inoculados que resultaram em lesões típicas da doença) até os 13 dias após a inoculação (dai).

Análises estatísticas

Para comparação entre isolados, foi feita análise de variância (ANOVA), as médias de PI foram comparadas por meio do teste de Tukey ($P \leq 0,05$) e as médias de FI foram comparadas por meio do teste de Kruskal-Wallis ($P \leq 0,05$). A comparação dos valores de PI entre cada isolado de Xcc e cada cultivar de repolho foi feita por meio de ANOVA e as médias foram comparadas por meio do teste de Tukey ($P \leq 0,05$). Foi feita uma análise de agrupamento (distância Euclidiana quadrática) pelo método Ward (método da distância mínima) para diferenciar/agrupar os isolados de Xcc quanto à gama de hospedeiros e quantidade de cultivares infectados.

Resultados

Identificação dos isolados

Nenhum dos isolados de Xcc foi capaz de utilizar asparagina como única fonte de carbono e nitrogênio, e todos eles isolados apresentaram colônias arredondadas, elevadas, com bordos regulares, coloração amarelo-palha e brilhantes em meio 523 (Kado e Heskett, 1970). Todos os isolados apresentaram a banda de 535 pb como resultado da amplificação utilizando os primers XCF/XCR. Todos os isolados foram patogênicos a brássicas e nenhum foi capaz de infectar acelga, inclusive o isolado Xcc 14. Estes resultados permitiram confirmar a identidade dos isolados de Xcc. A Tabela 1 exibe informações sobre os isolados utilizados neste estudo.

Inoculação cruzada

Todos os isolados de Xcc foram capazes de causar doença em pelo menos cinco cultivares de repolho. Apenas os isolados Xcc 10, Xcc 105, S3, Xcc 103, UFPR 4 e UFU B7 não foram capazes de causar doença em todos os cultivares. O isolado Xcc 105 causou doença em apenas 5 cultivares, apresentando a menor incidência, seguido do isolado S3, o qual foi capaz de infectar apenas 8 cultivares (Tabela 2). Além da

quantidade, a gama de cultivares infectados também variou entre isolados e essa variação permitiu a diferenciação de seis grupos de similaridade por meio da análise de agrupamento. Os três primeiros grupos correspondem aos isolados Xcc 105, S3 e UFU B7, os quais foram patogênicos a 5, 8 e 10 cultivares, respectivamente. Os isolados UFPR 4, Xcc 103 e Xcc 10 causaram doença em 11 cultivares porém foram separados em dois grupos distintos. Isso ocorreu porque UFPR 4 não foi capaz de infectar o cultivar 'Coração de Boi Gigante', enquanto que Xcc 103 e Xcc 10 não infectaram o cultivar 'Fuyutokyo Kobayashi'. Os demais isolados infectaram todos os cultivares e foram agrupados juntos (Figura 1).

A gama de isolados capazes de causar doença variou entre os cultivares de repolho inoculados (Tabela 3). Os cultivares '60 dias', 'Esmeralda' e 'Chato de Quintal' foram suscetíveis a todos os isolados inoculados. O cultivar 'Fuyutokyo Kobayashi' foi suscetível à menor quantidade de isolados, apresentando sintomas como resultado da inoculação de 18 dos 21 isolados (Tabela 4).

Período de incubação (PI)

O isolado que apresentou o maior valor de PI em relação a todos os cultivares de repolho inoculados foi o isolado Xcc 105 (11,67 dai). Doze isolados de Xcc apresentaram valores médios de PI estatisticamente iguais, porém inferiores aos outros nove isolados inoculados. Os valores de PI para esses 12 isolados variaram entre 6,48 e 7,16 dai (Tabela 2). A comparação entre os valores de PI entre isolados de Xcc por cada cultivar de repolho inoculado pode ser observada na Tabela 5. O cultivar que apresentou o menor valor médio de PI em relação a todos os isolados inoculados foi o cultivar 'Esmeralda' (4,84 dai). O aparecimento dos sintomas foi mais tardio para os cultivares 'Midori', 'Fuyutokyo Kobayashi' e 'Coração de Boi Gigante', com 8,65, 8,5 e 8,29 dai, respectivamente (Tabela 4).

Frequência de infecção (FI)

Os isolados Xcc 105 e S3 apresentaram os menores valores de FI quando feita a comparação entre isolados em relação a todos os cultivares de repolho (4,17 e 8,75%, respectivamente). O isolado Xcc 10 apresentou valor de FI igual a 28,33%, sendo significativamente superior a Xcc 105 e S3 e inferior a todos os outros isolados (Tabela 2). Os cultivares que apresentaram os maiores valores de FI foram 'Esmeralda', 'Chato de Quintal', 'Coração de Boi' e '60 dias', com valores variando entre 70,71 e 61,67%. Os cultivares 'Coração de Boi Gigante', 'Musachi', 'Midori' e 'Fuyutokyo Kobayashi' apresentaram os menores valores de FI (38,57 a 30%; Tabela 4).

Resistência de cultivares de repolho à podridão negra

Os resultados de incidência, PI e FI foram utilizados para separar os cultivares de repolho em quatro grupos de resistência, a saber: altamente suscetível (Incidência > 95%; PI > 6; FI > 60%), medianamente suscetível (Incidência > 95%; PI > 8; FI > 60%), medianamente resistente (Incidência > 90%; PI > 6; FI < 60%) e altamente resistente (Incidência < 91%; PI > 8; FI < 40%). Os cultivares 'Esmeralda', 'Coração de Boi' e '60 dias' foram classificados como altamente suscetíveis enquanto os cultivares 'Coração de Boi', 'Fuyutokyo Kobayashi' e 'Midori' foram classificados como altamente resistentes. Apenas o cultivar 'Chato de Quintal' foi classificado como medianamente suscetível e os demais cultivares foram agrupados na classe medianamente resistente.

Discussão

Neste trabalho, nós verificamos a existência de cultivares de repolho com maiores níveis de resistência à podridão negra. A variabilidade dos cultivares de repolho em relação à resistência à podridão negra das brássicas causada por tem sido observada em outros estudos (Henz e Melo 1994; Massomo et al. 2004; Griffiths and Roe 2005;

Shimelis 2005) e os resultados têm indicado uma escassez de fontes de resistência dentro do genoma de Brassica oleracea. A maioria dos estudos visando encontrar genes de resistência tem focado no genoma C de brássicas (genoma de B. oleracea). Os genomas A e B são encontrados em espécies selvagens ou de menor importância econômica comparadas ao repolho e têm sido menos estudados, embora sejam fontes de resistência potenciais e sejam utilizadas na diferenciação de raças de Xcc (Fargier e Manceau 2007). Devido a essas limitações, pesquisas sobre resistência do repolho à podridão negra têm visado o aumento da resistência basal pela aplicação de indutores de resistência ou buscar por variedades mais resistentes (Vicente e Holub 2013).

Alguns isolados de Xcc não foram capazes de causar doença em todos os cultivares e ainda apresentaram variações na agressividade quando comparados os valores de PI e de FI. Essa variabilidade tem sido verificada em outros estudos (Massomo et al. 2004; Griffiths e Roe 2005). Embora Xcc seja o agente causal da podridão negra de ocorrência mais comum no Brasil (Miguel-Wruck et al. 2010), há relatos de seis patovares de Xanthomonas campestris causando doença em brássicas, a saber: pv. campestris, pv. aberrans, pv. armoraciae, pv. barbareae, pv. incanae e pv. raphani. Além disso, nove raças de Xcc foram descritas até agora (Vicente e Holub 2013). Estas raças são diferenciadas com base na capacidade de infectar seis cultivares, a saber 'Wirosa F1' (B. oleracea), 'Just Right Hybrid Turnip' (B. rapa), 'Seven Top Turnip' (B. rapa), 'PI 199947' (B. carinata), 'Florida Broad Leaf Mustard' (B. juncea) e 'Miracle F1' (B. oleracea), e também com base no tipo de sintoma induzido (podridão negra, crestamento bacteriano ou pinta bacteriana; Fargier e Manceau 2007). Dentre todas as características utilizadas para definir esses patovares e raças, a gama de hospedeiros tem papel importante uma vez que há variabilidade na capacidade de isolados de Xcc em causar doença em espécies e cultivares de brássicas (Jensen et al. 2010). Essa variabilidade genética, tanto dos patógenos quantos dos hospedeiros, deve ser explorada

em programas de melhoramento com o intuito de encontrar genes de resistência à podridão negra das brássicas.

Os cultivares ‘60 dias’, ‘Chato de Quintal’ e ‘Esmeralda’ foram suscetíveis a todos os isolados inoculados enquanto que o cultivar ‘Fuyutokyo Kobayashi’ foi infectado pelo menor número de isolados de Xcc. Massomo et al. (2004), utilizando a incidência da doença nas folhas de repolho como um dos parâmetros para avaliação da resistência, observaram que cinco entre os 31 cultivares de repolho estudados quanto à resistência à podridão negra foram parcialmente resistentes. Em outro estudo, Griffiths e Roe (2005) verificaram que nenhum dos cultivares de repolho inoculados com Xcc apresentou resistência vertical à podridão negra, porém a severidade da doença variou significativamente indicando a existência de cultivares com maiores níveis de resistência. As interações incompatíveis entre isolados de Xcc e cultivares de repolho observadas no presente estudo sugerem a ocorrência de interação gene-a-gene (Avr x R). Recentemente, variedades de B. oleracea (brócolis, couve de Bruxelas, couve-flor e repolho) foram investigadas quanto à resistência à podridão negra. Apenas um cultivar de couve-flor apresentou resistência vertical à raça 1 de Xcc, evidenciando a ocorrência de genes R no genoma C de B. oleracea (Saha et al. 2016). Embora, no presente estudo, as avaliações tenham ocorrido apenas até os 13 dai, os cultivares que não exibiram sintomas da doença para pelo menos um dos isolados inoculados devem ser investigados quanto à ocorrência de genes de resistência ou aos mecanismos de defesa eficientes contra a podridão negra.

O aparecimento de sintomas foi mais tardio nos cultivares ‘Midori’, ‘Fuyutokyo Kobayashi’ e ‘Coração de Boi Gigante’. O cultivar ‘Esmeralda’ apresentou o menor valor de PI. Santos et al. (2008) verificaram que os cultivares ‘Chato de Quintal’ e ‘60 dias’ apresentaram PI igual a 3,67 e 4,33, respectivamente. No presente trabalho, nós

verificamos que o cultivar ‘Chato de Quintal’ apresentou PI igual a 8,04 e o cultivar ‘60 dias’ apresentou PI igual a 5,61. Essa variação pode ser explicada baseando-se nos componentes do ‘Triângulo de Doença’, ou seja, nas características do patógeno, do hospedeiro e do ambiente, e na interação entre eles. Neste trabalho, nós inoculamos 21 isolados com diferentes níveis de agressividade enquanto que Santos et al. (2008) utilizaram apenas um isolado para avaliar a resistência desses cultivares à podridão negra. Além disso, as plantas foram inoculadas por pulverização da suspensão bacteriana de Xcc enquanto neste trabalho a inoculação foi feita por ferimento utilizando palitos de madeira. Santos et al. (2008) conduziram seus experimentos no Estado de Pernambuco, onde as características climáticas são diferentes das encontradas na região da Zona da Mata no Estado de Minas Gerais. A idade da planta na época da inoculação também pode ser um fator relevante, uma vez que Santos et al. (2008) inocularam plantas aos 30 dias após a semeadura (das) e nós inoculamos aos 45 das. De acordo com essas observações, a avaliação da resistência de cultivares de repolho à podridão negra deveria respeitar a variabilidade genética dentro das populações de Xcc, as características ambientais das diferentes regiões onde o repolho é plantado e o estádio fenológico da planta. Além disso, uma metodologia padrão de inoculação de Xcc deve ser adotada quando o objetivo avaliar a resistência de cultivares de repolho. Nós recomendamos a inoculação por palito como metodologia padrão de inoculação por dispensar o tempo gasto com o preparo de suspensão bacteriana, garantir a inoculação do patógeno dentro da planta, permitir a visualização do sintoma típico da podridão negra e evitar a contaminação de plantas quando diversos isolados de Xcc forem inoculados em vários cultivares de repolho ao mesmo tempo e no mesmo local onde a resistência será avaliada.

Os valores de FI indicaram os cultivares ‘60 dias’, ‘Chato de Quintal’, ‘Coração de Boi’ e ‘Esmeralda’ como os mais suscetíveis à podridão negra. Os cultivares ‘Coração de Boi

Gigante', 'Fuyutokyo Kobayashi', 'Midori' e 'Musachi' apresentaram os menores valores de FI. Essas observações reforçam a informação de que há variabilidade entre cultivares de repolho quanto à resistência à podridão negra. O Ministério de Agricultura, Pecuária e Abastecimento (MAPA 2009) recomenda a realização do teste de patogenicidade inoculando o isolado suspeito em plântulas de brássica suscetível por meio de ferimento nos bordos da folha como parte do método de detecção de Xcc em Brassica spp., porém não indica uma espécie ou um cultivar de brássica como padrão de suscetibilidade. De acordo com os resultados obtidos neste estudo, nós recomendamos a utilização dos cultivares de repolho '60 dias' e 'Esmeralda' para a realização de testes de patogenicidade por terem apresentarem os maiores valores de incidência e FI, e os menores valores de PI.

A informação fornecida por algumas empresas produtoras de sementes sobre a resistência do cultivar à podridão negra diferiu, em alguns casos, dos resultados obtidos neste trabalho. O cultivar 'Esmeralda', que deveria apresentar resistência à podridão negra, apresentou alta suscetibilidade. Os cultivares 'Coração de Boi Gigante' e 'Fuyutokyo Kobayashi', no entanto, apresentaram alta resistência à doença, porém essa informação não é apresentada pelas empresas que as produzem. Incoerências como estas observadas no presente estudo foram observadas anteriormente quando se avaliou a reação de cultivares de repolho, brócolis e couve-chinesa à podridão negra (Henz e Melo 1994; Jr. Seabra et al. 2008; Santos et al. 2008). Essas observações reforçam a necessidade de padronização da metodologia e das condições para avaliação da resistência de cultivares de repolho à podridão negra.

No presente estudo nós verificamos a ocorrência de variabilidade patogênica de isolados de Xcc e a existência de cultivares de repolho com maiores níveis de resistência à podridão negra. Pesquisas futuras devem explorar a variabilidade genética do patógeno

e do hospedeiro com o objetivo de possibilitar o desenvolvimento de cultivares de repolho resistentes ou com maiores níveis de resistência à podridão negra das brássicas.

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Figuras e Tabelas

Tabela 1. Origem dos isolados de Xanthomonas campestris pv. campestris utilizados.

Código	Origem	Hospedeiro	Instituição	Colaborador
C13	Camocim-PE	Repolho	UFRPE	Rosa de Lima R. Mariano
S3	Camocim-PE	Repolho	UFRPE	Mariano, R. L. R.
S4	Camocim-PE	Repolho	UFRPE	Mariano, R. L. R.
UFPR 3	Pinhais - PR	Brócolis	UFPR	Tiago Miguel Jarek
UFPR 4	S. J. Pinhais-PR	Couve	UFPR	Jarek, T. M.
UFPR 5	S. J. Pinhais-PR	Repolho	UFPR	Jarek, T. M.
UFSC 1	Florianópolis-SC	B. oleracea	UFSC	Marciel João Stadnik
UFSC 2	Florianópolis-SC	B. oleracea	UFSC	Stadnik, M. J.
UFU B7	Uberlândia-MG	Repolho	UFU	Nilvanira Tebaldi
UFU D12	Uberlândia-MG	Couve	UFU	Tebaldi, N.
UNESP 2909	Botucatu - SP	Couve	UNESP	Antônio Carlos Maringoni
UNESP 3159	Botucatu - SP	Brócolis	UNESP	Maringoni, A. C.
X 01	Curitiba-PR	Repolho	UFPR	Lucimeris Ruaro
X 03	Florianópolis-SC	Couve	UFSC	Robson Marcelo Di Piero
Xcc 10	V. do Sto. Antônio-PE	Couve-flor	Embrapa	Alice Kazuko Inoue Nagata
Xcc 103	Rancho Queimado-SC	Brássica	Embrapa	Nagata, A. K. I.
Xcc 105	Rancho Queimado-SC	Repolho	Embrapa	Nagata, A. K. I.
Xcc 14	Brasília-DF	Acelga	Embrapa	Nagata, A. K. I.
Xcc 2	Manaus-AM	Repolho	Embrapa	Nagata, A. K. I.
Xcc 320	Sergipe-PE	Couve	Embrapa	Nagata, A. K. I.
Xcc 8	São Luís-MA	Repolho	Embrapa	Nagata, A. K. I.

UFRPE, Universidade Federal Rural de Pernambuco; UFPR, Universidade Federal do Paraná; UFSC, Universidade Federal de Santa Catarina; UFU, Universidade Federal de Uberlândia; UNESP, Universidade Estadual Paulista "Júlio Mesquita Filho" – Botucatu-SP; Embrapa, Embrapa Hortaliças.

Tabela 2. Comparação da incidência, período de incubação (PI) e frequência de infecção (FI) entre isolados de Xanthomonas campestris pv. campestris inoculados em repolho.

Isolado	Incidência [a]	Incidência (%)	PI (dai) [b,c]	FI (%) [d]
Xcc 14	12	100	6,48 d	57,50 a
S 4	12	100	6,50 d	62,08 a
UFPR 5	12	100	6,54 d	68,75 a
Xcc 2	12	100	6,59 d	64,58 a
X 03	12	100	6,69 d	65,83 a
UFU D12	12	100	6,75 d	60,83 a
UNESP 2909	12	100	6,79 d	60,47 a
UFPR3	12	100	6,93 d	64,17 a
UFU B7	10	83,3	6,97 d	49,58 b
C 13	12	100	7,06 d	61,25 a
UNESP 3159	12	100	7,12 d	48,33 b
Xcc 103	11	91,7	7,16 d	54,17 a
Xcc 320	12	100	7,36 c	59,58 a
UFSC 2	12	100	7,54 c	55,83 a
UFPR 4	11	91,7	7,68 c	41,25 b
X 01	12	100	7,77 c	54,17 a
UFSC 1	12	100	7,83 c	45,00 b
Xcc 8	12	100	8,14 c	43,33 b
Xcc 10	11	91,7	8,31 c	28,33 c
S 3	8	66,7	10,39 b	8,75 d
Xcc 105	5	41,7	11,67 a	4,17 d

[a] = Número de cultivares que apresentaram sintomas da doença.
[b] = Dias após a inoculação.
[c] = Médias seguidas de letras diferentes são significativamente diferentes (Tukey, $P < 0{\cdot}05$).
[d] = Médias seguidas de letras diferentes são significativamente diferentes (Kruskal-Wallis, $P<0.05$).

Tabela 3. Inoculação cruzada de 12 cultivares de repolho com 21 isolados de Xanthomonas campestris pv. campestris.

Isolados	Cultivares											
	60D	CB	ESM	CRM	LV	CQ	SK	CBG	FTK	VEL	MID	MUS
S 4	+	+	+	+	+	+	+	+	+	+	+	+
UNESP 2909	+	+	+	+	+	+	+	+	+	+	+	+
C 13	+	+	+	+	+	+	+	+	+	+	+	+
Xcc 2	+	+	+	+	+	+	+	+	+	+	+	+
UFSC 1	+	+	+	+	+	+	+	+	+	+	+	+
UFPR3	+	+	+	+	+	+	+	+	+	+	+	+
Xcc 10	+	+	+	+	+	+	+	+	-	+	+	+
Xcc 105	+	-	+	+	+	+	-	-	-	-	-	-
Xcc 8	+	+	+	+	+	+	+	+	+	+	+	+
X 01	+	+	+	+	+	+	+	+	+	+	+	+
S 3	+	+	+	-	-	+	-	+	+	+	-	+
Xcc 14	+	+	+	+	+	+	+	+	+	+	+	+
X 03	+	+	+	+	+	+	+	+	+	+	+	+
Xcc 320	+	+	+	+	+	+	+	+	+	+	+	+
UFPR 5	+	+	+	+	+	+	+	+	+	+	+	+
Xcc 103	+	+	+	+	+	+	+	+	-	+	+	+
UFPR 4	+	+	+	+	+	+	+	-	+	+	+	+
UFU B7	+	+	+	+	-	+	+	+	+	-	+	+
UFU D12	+	+	+	+	+	+	+	+	+	+	+	+
UFSC 2	+	+	+	+	+	+	+	+	+	+	+	+
UNESP 3159	+	+	+	+	+	+	+	+	+	+	+	+

(+) = plantas apresentando sintomas típicos de podridão negra aos 15 dai (-) = plantas assintomáticas. 60D = cv. '60 dias', CB = cv. 'Coração de Boi', ESM = cv. 'Esmeralda', CRM = cv. 'Crespo de Milão', LV = cv. 'Louco de Verão', CQ = cv. 'Chato de Quintal', SK = cv. 'Sekai F1', CBG = cv. 'Coração de Boi Gigante', FTK = cv. 'Fuyutokyo Kobayashi', VEL = cv. 'Veloce F1', MID = cv. 'Midori' e MUS = cv. 'Musachi'.

Tabela 4. Comparação da incidência, período de incubação (PI) e frequência de infecção (FI) entre cultivares de repolho inoculados com Xanthomonas campestris pv. campestris.

Cultivar	Incidência [a]	Incidência (%)	PI (dai[b])	FI (%)
MID	19	90,5	8,65	33,57
FTK	18	85,7	8,50	30,00
CBG	19	90,5	8,29	38,57
CQ	21	100	8,04	69,29
LV	19	90,5	8,01	49,29
SK	19	90,5	7,86	51,90
CRM	20	95,2	7,82	46,43
MUS	20	95,2	7,51	37,62
VEL	19	90,5	6,97	49,76
CB	20	95,2	5,65	65,71
60D	21	100	5,61	61,67
ESM	21	100	4,84	70,71

[a] = Número e porcentagem de isolados que causaram doença nos cultivares de repolho testados.
[b] = Dias após a inoculação.
60D = cv. '60 dias', CB = cv. 'Coração de Boi', ESM = cv. 'Esmeralda', CRM = cv. 'Crespo de Milão', LV = cv. 'Louco de Verão', CQ = cv. 'Chato de Quintal', SK = cv. 'Sekai F1', CBG = cv. 'Coração de Boi Gigante', FTK = cv. 'Fuyutokyo Kobayashi', VEL = cv. 'Veloce F1', MID = cv. 'Midori' e MUS = cv. 'Musachi'.

Tabela 5. Período de incubação (PI) da podridão negra causada por 21 isolados de Xanthomonas campestris pv. campestris em 12 cultivares de repolho.

Isolados	Cultivares de Repolho											
	60D	CB	ESM	CRM	LV	CQ	SK	CBG	FTK	VEL	MID	MUS
S 4	4 d	4 d	4 de	7 bc	6.67 cd	6.67 efg	6.67 ab	7 bcd	9 bcd	7 bcd	8.67 a	7 bc
UNESP 2909	4.3 d	4.3 d	4 de	6.67 bc	7.33 bcd	7 defg	8.67 ab	8 bcd	9.33 bc	6.67 cd	7.67 a	6.67 bc
C 13	4 d	4 d	4 de	8.67 b	7.67 bcd	7.33 defg	8 ab	7 bcd	8 cde	6.33 cd	7.67 a	8.33 bc
Xcc 2	4 d	4.67 cd	4 de	6 c	7.67 bcd	6.67 efg	7.33 ab	8.33 bc	8 cde	6 cd	8.33 a	6 c
UFSC 1	4.67 d	8 abc	5 bcd	7.67 bc	7.67 bcd	9.33 bc	7.67 ab	7 bcd	9.33 bc	6.33 cd	9.33 a	7.33 bc
UFPR3	4 d	4 d	4.33 de	7.67 bc	8 bcd	7.67 cdefg	6.67 ab	9 b	6.67 e	6 cd	8.67 a	8.67 ab
Xcc 10	6.67 bcd	8.33 abc	5 bcd	8.33 bc	9.33 ab	9.33 bc	7.67 ab	12.67 a	nd	5.33 d	8.33 a	6.67 bc
Xcc 105	11 a	nd	11.33 a	12 a	10.67 a	12.5 a	nd	nd	nd	nd	nd	nd
Xcc 8	6.33 cd	6.33 bcd	5 bcd	8.67 b	8 bcd	7.67 cdefg	10 a	7.67 bcd	7 de	8.67 b	9.33 a	7 bc
X 01	5.33 d	7.00 abcd	6.33 b	8.33 bc	8.67 abc	8.33 bcde	6.67 ab	7.67 bcd	6.67 e	7.00 bcd	10.33 a	7.67 bc
S 3	9.5 ab	10 a	6 bcd	nd	nd	10 b	nd	12 a	13 a	13 a	nd	11 a
Xcc 14	4.3 d	4.67 cd	3 e	6.33 bc	6 d	7.33 defg	6.33 ab	8 bcd	8 cde	6.33 cd	6.67 a	7 bc
X 03	4.67 d	4.67 cd	4 de	8 bc	8 bcd	7.33 defg	5.33 b	8 bcd	6 e	6 cd	6.67 a	6.33 bc
Xcc 320	4.3 d	4.5 d	4.33 de	7 bc	7.67 bcd	7 defg	9 ab	7.67 bcd	7.67 cde	7.33 bc	7.33 a	8.33 bc
UFPR 5	4.67 d	5 bcd	4.33 de	6 c	6 d	6.33 fg	6.67 ab	5.33 d	12.5 a	6 cd	7.67 a	6 c
Xcc 103	4.3 d	4.33 d	4.33 de	7 bc	7.67 bcd	8 cdef	9 ab	8 bcd	nd	7 bcd	8.33 a	7 bc
UFPR 4	6.67 bcd	4.67 cd	5 bcd	8 bc	9.67 ab	8.67 bcd	10 a	nd	11 ab	6.67 cd	7.67 a	7 bc
UFU B7	9.33 abc	4 d	4 de	7 bc	nd	7.33 defg	6.33 ab	6.67 bcd	11 ab	nd	7.67 a	6.67 bc
UFU D12	5.33 d	4.3 d	4.67 cd	6 c	6 d	6 g	7 ab	5.67 cd	6.33 e	7 bcd	8.33 a	7 bc
UFSC 2	5 d	6.33 bcd	4.33 de	8 bc	8 bcd	8 cdef	6.33 ab	8.33 bc	7.67 cde	7 bcd	8 a	6.33 bc
UNESP 3159	4.67 d	5.33 bcd	5 bcd	6.67 bc	7.67 bcd	7.67 cdefg	7 ab	8 bcd	7 de	6.67 cd	9 a	6.67 bc

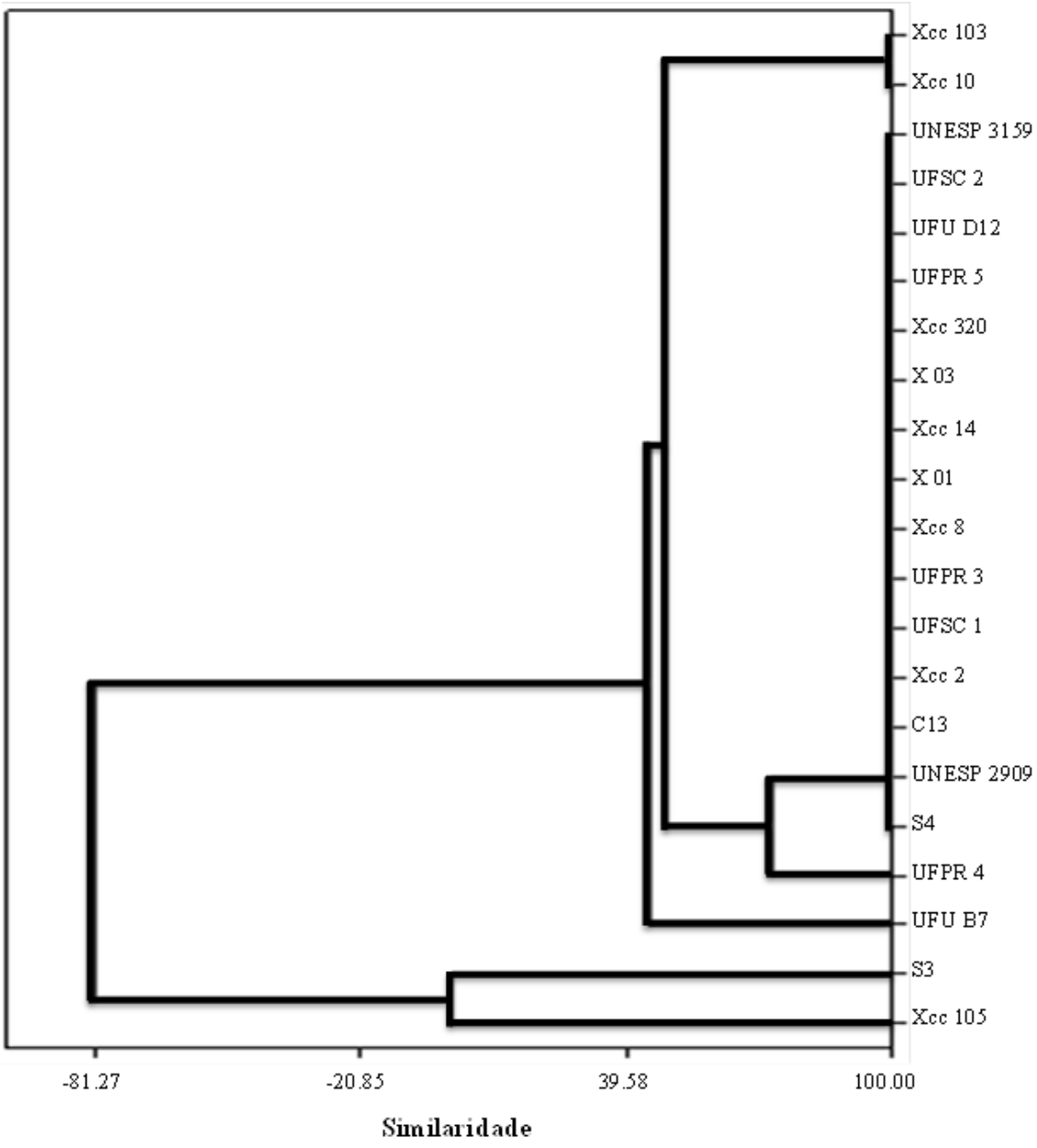
Letras minúsculas representam entre isolados por cada cultivar (coluna); médias seguidas da mesma letra não são significativamente diferentes (Tukey, $P < 0{\cdot}05$). 60D = cv. '60 dias', CB = cv. 'Coração de Boi', ESM = cv. 'Esmeralda', CRM = cv. 'Crespo de Milão', LV = cv. 'Louco de Verão', CQ = cv. 'Chato de Quintal', SK = cv. 'Sekai F1', CBG = cv. 'Coração de Boi Gigante', FTK = cv. 'Fuyutokyo Kobayashi', VEL = cv. 'Veloce F1', MID = cv. 'Midori' e MUS = cv. 'Musachi'. nd = não definido (não exibiu sintomas da doença até o fim das avaliações).

Tabela 6. Comparação entre a resistência comercial e a resistência observada de 12 cultivares de repolho.

Cultivar	Resistência Comercial	Resistência Observada
60D	Suscetível	AS
CB	Suscetível	AS
ESM	Resistente	AS
CRM	Suscetível	MR
LV	Tolerante	MR
CQ	Suscetível	MS
SK	Resistente	MR
CBG	Suscetível	AR
FTK	Suscetível	AR
VEL	Resistente	MR
MID	Resistente	AR
MUS	Alta Resistência	MR

60D = cv. '60 dias', CB = cv. 'Coração de Boi', ESM = cv. 'Esmeralda', CRM = cv. 'Crespo de Milão', LV = cv. 'Louco de Verão', CQ = cv. 'Chato de Quintal', SK = cv. 'Sekai F1', CBG = cv. 'Coração de Boi Gigante', FTK = cv. 'Fuyutokyo Kobayashi', VEL = cv. 'Veloce F1', MID = cv. 'Midori' e MUS = cv. 'Musachi'. AS = altamente suscetível (Incidência > 95%; PI > 6; FI > 60%), MS = medianamente suscetível (Incidência > 95%; PI > 8; FI > 60%), MR = medianamente resistente (Incidência > 90%; PI > 6; FI < 60%) e AR = altamente resistente (Incidência < 91%; PI > 8; FI < 40%).

Figura 1. Dendrograma originado da análise Euclidiana quadrática de 21 isolados de Xanthomonas campestris pv. campestris inoculados em 12 cultivares de repolho.



CHAPTER 2 - BIOCHEMICAL CHANGES IN THE CABBAGE-XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS INTERACTION AND THE EFFECT OF ACIBENZOLAR-S-METHYL UPON THE BLACK ROT PROGRESS

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Abstract

Xanthomonas campestris pv. campestris (Xcc) is the causal agent of black rot in crucifers, the most devastating disease that afflicts brassicas. As information about cabbage defense mechanisms against Xcc infection is still limited, we aimed to investigate the activity of antioxidant enzymes, pathogenesis-related proteins (PR-proteins) and concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) during the infectious process of Xcc in cabbage leaves. This study was subdivided in three experiments. In the 'Experiment 1', we assessed the biochemical changes during the interaction of cabbage plants from cv. 'Esmeralda' inoculated with two isolates of Xcc: UFPR 5 (more aggressive), and Xcc 10 (less aggressive). In the 'Experiment 2', we observed the biochemical responses of the cabbage cultivars 'Louco de Verão' (cv. "LV"; more resistant) and 'Chato de Quintal' (cv. "CQ"; more susceptible) to the inoculation with Xcc. Finally, in the 'Experiment 3', we verified the effect of Acibenzolar-S-Methyl (ASM) upon the antioxidant system and PR-proteins activities during the cabbage-Xcc interaction. As the results of the 'Experiment 1', plants inoculated with Xcc 10 exhibited lower values of severity and area under the disease progress curve (AUDPC) compared to those inoculated with UFPR 5, evidencing the difference in the aggressiveness of both isolates. This difference may be explained by the biochemical changes during the pathogenic interaction. Plants inoculated with Xcc 10 exhibited higher values of superoxide dismutases (SOD), peroxidases (POX) and ascorbate peroxidases activities (APX) activities, and lower concentrations of H_2O_2 and MDA compared to the plants inoculated with UFPR 5. In contrast, the activities of chitinases (CHI), β-1,3-glucanases (GLU) and polyphenoloxidases (PPO) were higher in plants inoculated with UFPR 5 compared to the control and to those inoculated with Xcc 10. Taken together, these results suggest that the enzymes studied here are important for the defense of cabbage to limit the black rot progress, and that Xcc can

modulate the defense mechanisms in cabbage to cause disease. In the 'Experiment 2', inoculated plants from cv. "LV" exhibited lower values of severity and AUDPC compared to those from cv. "CQ". This result may be explained by the higher increment of CHI, GLU and PPO activities in plants from cv. "LV". Inoculated plants from cv. "CQ" displayed higher values of SOD, POX and APX activities compared to those from cv. "LV". However, the concentrations of H_2O_2 and MDA were higher in plants from cv. "CQ", suggesting that the antioxidant system of this cultivar was not efficient to limit the cellular damage and, consequently, the disease progress. Inoculated plants from cv. "LV" displayed a slight increment in the POX activity compared to the non-inoculated ones whereas SOD and APX activities remained constant, suggesting that other antioxidant enzymes may be involved in the removal of reactive oxygen species and, thus, in limiting the Xcc infection. The first report of the effectiveness of ASM application on the control of the black rot in cabbage could be observed in the 'Experiment 3'. With the exception of SOD, ASM treated plants displayed an increment in the enzymes activities, which resulted in concentrations of H_2O_2 and MDA at constant levels and, thus, lower values of severity and AUDPC compared to non-treated plants. In conclusion, the PR-proteins studied here are important components of the defense mechanisms in cabbage to prevent Xcc infection. These PR-proteins may be used as biomarkers in breeding programs that aim to develop resistant cabbage cultivars and in researches that aim to develop and test resistance inducers. Further research will provide information about recommended dose, applications intervals, and about the impact of the ASM application upon cabbage attributes such as plant growth, yield and nutritional composition.

Introduction

Cabbage (Brassica oleracea var. capitata) is the most planted and consumed crop among the Brassicaceae (Lee et al. 2015). Global cabbage production was more than 70 million tons in 2013, when China produced 31.7 million tons (biggest cabbage plant producer) and Lithuania 2,300 tons of seeds (biggest seed producer; FAO 2013). The most limiting factor for cabbage production is black rot of crucifers caused by Xanthomonas campestris pv. campestris (Xcc) (Pammel) Dowson. Black rot occurs wherever cabbage is cultivated and causes heavy yield losses especially during warm and humid seasons (Lange et al. 2015; Roohie and Umesha 2015; Saha et al. 2016). Xcc penetrates through hydathodes to colonize the plant tissue. The typical black rot symptom is V-shaped with its vertex turned to the center of the symptomatic leaf. The lesion has chlorotic edges with a necrotic center and black veins (Vicente and Holub 2013; Lange et al. 2015; Roohie and Umesha 2015). Strategies for black rot management include the control of irrigation, crop rotation and sowing pathogen-free seeds (Gitaitis and Walcott 2007; Lee et al. 2009; Vicente and Holub 2013). Unfortunately, vertical resistance is very rare in cabbage (Vicente and Holub 2013), but the use of cultivars with higher resistance levels is possible (Massomo et al. 2004). An important factor that contributes to difficult the search for resistance in cabbage is the variability among isolates of Xanthomonas campestris pathogenic to cabbage. Besides Xcc, X. campestris pv. aberrans, X. campestris pv. armoraciae, X. campestris pv. barbareae, X. campestris pv. incanae e X. campestris pv. raphani are also causal agents of black rot in brassicas (Vauterin et al. 1995). Furthermore, Xcc has high variability and at least six races were described, which makes the search for cultivars more resistant to black rot difficult (Singh et al. 2011).

Plants are able to enhance their basal defense levels to protect themselves against pathogen attacks (van Loon and van Strien 1999). This phenomenon is known as induced resistance and can be divided into two classes known as systemic acquired resistance (SAR), which is salicylic acid (SA)-dependent for signaling and promotes the production of pathogenesis-related proteins (PR-proteins), and induced systemic resistance (ISR), which is activated by jasmonates (JA) and ethylene (ET), but does not enhance PR protein levels (Pieterse et al. 2014). These defense responses are aimed at constraining progress of the disease and include the production of toxic compounds, pathogen-degrading enzymes and programed cell death (Freeman and Beattie 2008). Pathogen attack causes increase in reactive oxygen species (ROS) production as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (OH^-), which are responsible for damaging cell membrane (lipid peroxidation), pigments, proteins and nucleic acids. In order to counteract the deleterious effects of ROS produced during the plant-pathogen interaction, enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) are produced by the infected plant and the determination of their activities can give us information about what defense mechanisms are operating in plant disease resistance (García-Cristobal et al. 2015; Roohie and Umesha 2015). Other important components in plant defense include PR-proteins like chitinase (CHI) and β-1,3-glucanase (GLU), which are involved in degradation of the fungal cell wall, and contribute to the production of elicitors which amplify defense responses in plants (Hong and Hwang 2005; Kim et al. 2015).

Resistance inducers have been developed and their application has resulted in control of plant diseases in a number of crops. Examples include citrus canker in orange, bacterial speck in lettuce, bacterial wilt and bacterial spot in tomato, and fire blight in apple trees (Francis et al. 2009; Yigit 2011; Mandal et al. 2013; Chandrashekar and Umesha 2014; Acimovic et al. 2015). Acibenzolar-S-Methyl (ASM, also known as benzothiadiazole,

BTH) is a synthetic substance analogous to SA which has shown efficacy in the control of fungal, both viral and bacterial, in various crops (Véronési et al. 2009).

Biochemical components involved in the resistance of cabbage to Xcc are not well documented and neither is the effect of the application of ASM to black rot control. This study is divided in three experiments that aimed to: (1) observe differences in the activities of antioxidant enzymes and PR-proteins, and the concentrations of H_2O_2 and MDA induced by two isolates of Xcc contrasting in their levels of aggressiveness in a cabbage cultivar; (2) observe differences in the activities of antioxidant enzymes and PR-proteins, and the concentrations of H_2O_2 and MDA among two cabbage cultivars with different levels of resistance to black rot; (3) determine the effect of ASM on the progress of black rot, the activities of antioxidant enzymes and PR-proteins, and the concentration of H_2O_2 and MDA in cabbage plants.

Material and Methods

Cabbage growth

Cabbage seeds were sown in plastic pots containing the substrate Tropstrato HT Hortaliças (VidaVerde®; Brazil) and the plants were cultivated in a greenhouse for 45 days. Plants were irrigated daily and fertilized once a week (Niphokan 10-08-08; Fênix®; Brazil; following the manufacturer instructions). For the inoculation, plants were transported to a mist chamber and were kept there for 24 hours before and after the inoculation, after which they were transported to a growth chamber under a controlled temperature (28°C) and photoperiod of 12 hours. The plants were maintained in this growth chamber until the evaluations were completed.

Xanthomonas campestris pv. campestris inoculation

The isolates of Xcc used in this study had been previously tested for aggressiveness against 12 cabbage cultivars. The isolates were grown in Petri dishes containing medium 523 (Kado and Heskett 1970) for 48 hours at 28°C in a chamber under controlled temperature. Bacterial colonies were used as source of inoculum and sterile toothpicks were used to inoculate the plants. Each isolate of Xcc was inoculated in the cabbage plants in five points per leaf and four replicates (cabbage plants) were used for each treatment. Plants used for the evaluation of severity and AUDPC were inoculated in the second and the third leaves (from the top to the base) those plants used for the determination of the biochemical parameters were inoculated from the second to the sixth leaf (from the top to the base).

Application of the resistance inducer

Only for the 'Experiment 3', cabbage plants were sprayed with distilled water (controls) or 0.05 g a.i. l^{-1} ASM (Bion®; Syngenta; Brazil; as 50% active ingredients in WP formulation). Tween 20 (0.1%, v/v) was added as a surfactant. A manual atomizer (Pressure Sprayer; DSC®; Brazil) was used to spray the plants 24 h before they were inoculated with Xcc (Soylu et al. 2003). The plants from each treatment were kept separate from the application of the inducer and water until 1 day after the inoculation (dai).

Quantification of epidemiological variables

The black rot severity was assessed in the second and third leaves (from the top to the base) of each plant every day after inoculation up to 15 dai. Severity was assessed with the aid of a diagrammatic scale previously developed for assessing black rot. The area under the disease progress curve (AUDPC) was calculated using the equation of Campbell and Madden (1990).

Determination of the activities of peroxidases (POX, EC1.11.1.7), superoxide dismutases (SOD, EC 1.15.1.1), ascorbate peroxidases (APX, EC 1.11.1.11), polyphenoloxidases (PPO, EC 1.10.3.1), chitinases (CHI, EC 3.2.1.14) and β-1,3-glucanases (GLU, EC 3.2.1.39)

To perform the determination of the enzymatic activities and the concentrations of the compounds, cabbage leaf fragments were collected at 1, 5, 10 and 15 dai, placed in liquid nitrogen an stored in ultrafreezer (-80°C) until the biochemical analysis.

To obtain the extracts used to determine the activities of POX, SOD, APX and PPO, 200-300 mg of leaf tissue was macerated in liquid N_2 with the aid of a mortar and pestle to obtain a fine powder. The powder was homogenized in 2 ml of 100 mM potassium phosphate (pH 6.8) containing 1 mM phenylmethylsulfonicfluoride (PMSF), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and polyvinylpolypyrrolidone (PVP) 1% (w/v). The homogenized material was centrifuged at 12,000 g for 15 min at 4ºC and the supernatant was used for enzyme determination.

POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Miashra (1976), with a number of modifications. The reaction started after the addition of 5 µl of the enzymatic extract to the reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2 in a volume of 245 µl, and POX activity was determined through the absorbance of colored purpurogallin detected at 420 nm for 1 min at 25°C. A molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate the POX activity, which was expressed as µmol purpurogallin min^{-1} mg^{-1} of protein.

Formazan blue is the product of the photochemical reduction of p-nitrotetrazole blue (NTB) by SOD activity (Del Longo et al. 1993). The reaction was started after the addition of 2 µl of the enzymatic extract to 248 µl of a mixture containing 50 mM

potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NTB, 0.1 mM EDTA and 2 µM riboflavin. The reaction occurred at 25°C under a 15-W lamp light. After 5 min of light exposure, the light was interrupted, and the production of formazan blue was monitored by the increase in absorbance at 560 nm. The reaction mixture for the control samples was kept in the same conditions for 5 min, and the absorbance measured at 560 nm. The amount of enzyme necessary to inhibit NBT photoreduction by 50% is considered one unit of SOD (Beauchamp and Fridovich 1971).

APX activity was determined based on the method described by Nakano and Asada (1981). The reaction started after the addition of 8 µl of the enzyme extract to a reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.8), 1 mM H_2O_2, and 0.8 mM ascorbate in a volume of 242 µl. APX activity was obtained by measuring the rate of ascorbate oxidation at 290 nm for 1 min at 25°C and was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µmol min^{-1} mg of protein.

For PPO activity, 8 µl of the enzyme extract was added to a mixture containing distilled water, 100 mM potassium phosphate buffer (pH 6.8) and 100 mM pyrogallol in a volume of 242 µl. The absorbance was at 420 nm every 10 seconds for 1 min after adding the extract to the mixture in a total of seven readings. A molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate PPO activity, which was expressed in mmol purpurogallin min^{-1} mg^{-1} of protein (Kar and Miashra 1976).

To obtain the extracts used to determine the activities of CHI and GLU, 200 to 300 mg of leaf tissue was also macerated in liquid N_2 to obtain a fine powder. The powder was homogenized in 2 ml of 50 mM sodium phosphate (pH 6.5) containing 1 mM phenylmethylsulfonicfluoride (PMSF), 0.1 mM ethylenediaminetetraacetic acid, (EDTA) and polyvinylpolypyrrolidone (PVP) 1% (w/v). The homogenized material was

centrifuged at 20,000 g for 25 min at 4ºC and the supernatant used for enzyme determination.

The CHI activity was determined following the method described by Harman et al. (1993) with a few modifications. The reaction was started by the addition of 5 µl of enzyme extract to a mixture containing 50 mM sodium acetate buffer (pH 5.0) and p-nitrofenil-β-D-N-N'-diacetilquitobiose 2 mg ml^{-1} in a volume of 120 µl. The reaction mixture was incubated in a water bath at 37ºC for 2 h and then stopped by the addition of 125 µl of 0.2 M sodium carbonate. The activity of CHI was determined by measuring the absorbance of the end products released during the reaction at 410 nm. An extinction coefficient of 7×10^4 mM^{-1} cm^{-1} was used to calculate CHI activity, which was expressed as mmol of p-nitrophenyl min^{-1} mg^{-1} of protein.

The reaction to determine GLU activity was initiated by the addition of 5 µl aliquots of the supernatant to a mixture of 100 mM sodium acetate (pH 5.0) and of the substrate laminarin in a concentration of 4 mg ml^{-1} in a volume of 120 µl and was incubated in a water bath for 30 min at 45ºC. After the incubation period, the amount of reducing sugars originated from the reaction was determined by adding 125 µl of dinitrosalicylic acid (DNS) to the mixture which was incubated in a water bath for 15 min at 100ºC. In the control samples, the reaction mixture was the same but the extract was added only after heating the mixture at 100ºC. An ice bath was used to cool and stop the reactions. The product released by GLU was measured at 540 nm and its activity was expressed in absorbance min^{-1} mg^{-1} of protein (Lever 1972).

Protein was quantified according to the method developed by Bradford (1976). Briefly, 2 µl of the crude extract was mixed with 198 µl water and 1000 µl Bradford´s reagent. A wavelength of 595 nm was used to determine the quantity of protein that was

expressed in milligrams. Bovine serum albumin (BSA) was used as the standard protein.

Measurement of H_2O_2

Leaf samples of cabbage plants (100-150 mg) were ground into a fine powder in liquid nitrogen using a mortar and pestle. The powder was homogenized in a volume of 2 ml of 50 mM potassium phosphate buffer (pH 6.5) amended with 1 mM hydroxylamine and the resulting suspension was centrifuged at 10,000 g for 15 min at 4ºC. The reaction consisted of 12.5 µl of the supernatant was added to a mixture containing 100 µM ferric ammonium sulfate ($FeNH_4(SO_4)$), 25 mM sulfuric acid, 250 µM xylenol orange, and 100 mM sorbitol in a volume of 250 µl. After 30 min kept in the dark, the absorbances of the samples were measured at 560 nm. The controls were prepared under the same conditions and subtracted from the samples. The hydrogen peroxide concentration was estimated based on a standard curve of H_2O_2 expressed as µmol H_2O_2 kg^{-1} of fresh weight (Gay and Gerbicki 2000).

Determination of the concentration of malondialdehyde (MDA)

Leaf samples were collected at each time of collection and placed in liquid nitrogen (N_2) from sampling time to storing in an ultra-low freezer (-80°C) where the samples were kept until they were processed. Oxidative damage to the lipids in the leaf cells was estimated as the total content of 2-thiobarbituric acid (TBA) reactive substances and expressed as malondialdehyde (MDA) equivalents, according to Cakmak & Horst (1991) with a number of modifications. Fragments of cabbage leaves (200-300 mg) were ground into a fine powder in liquid nitrogen using a mortar and pestle, and the powder was homogenized in 2 ml 0.1% (w/v) trichloroacetic acid (TCA) solution at 4ºC. The suspension was centrifuged at 10,000 g for 15 min and 250 µl of the supernatant was mixed with 750 µl of TBA (0.5% in 20% TCA) for 2.5 h in a

Thermomix (Eppendorff) at 99°C. The reaction was stopped in an ice bath. The samples were centrifuged at 13,000 g for 4 min, and the specific absorbance of the supernatant was measured at 532 nm. Non-specific absorbance was measured at 600 nm and subtracted from the specific absorbance. The concentration of MDA formed in each sample was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}, and was expressed as µmol MDA kg^{-1} of fresh matter.

Experimental design and data analysis

Experiment 1

The experiment consisted of three treatments: cabbage plants from cultivar 'Esmeralda' inoculated with the isolate UFPR 5 (more aggressive), cabbage plants inoculated with Xcc 10 (less aggressive) and non-inoculated plants. The experiment was arranged in a completely randomized design with four replicates per treatment. The data from all variables were analyzed by analysis of variance (ANOVA; Table 1), and the means from the treatments compared by the Tukey's test ($P \leq 0.05$), except for severity and AUDPC, for which the means were compared by the Student's t-test. For ANOVA of the severity, the design was considered to be a 2 × 6 factorial experiment, consisting of two isolates of Xcc and six evaluation times (10, 11, 12, 13, 14 and 15 dai). For AUDPC, a one-way experimental design was adopted, in which only the two isolates were analyzed. For POX, SOD, APX, PPO, CHI, and GLU activities as well as for the H_2O_2, and MDA concentrations, the design was considered to be a 3 × 4 factorial experiment for the ANOVA, consisting of three treatments and four sampling times (1, 5, 10 and 15 dai).

Experiment 2

A 2 × 2 factorial experiment, consisting of the cabbage cultivars ‘Louco de Verão’ (cv. “LV”; more resistant) and ‘Chato de Quintal’ (cv. “CQ”; more susceptible) and plants that were non-inoculated or inoculated with the Xcc isolate S3, was arranged in a completely randomized design with four replications. Data from all variables were analyzed by ANOVA (Table 2), and the means were compared by Tukey’s test ($P \leq 0.05$). For ANOVA of the variables severity and AUDPC the means from the treatments were compared by the Student’s t-test. For POX, SOD, APX, PPO, CHI, and GLU activities as well as for the H_2O_2 and MDA concentrations, the experimental design was considered to be a 2 × 2 × 4 factorial for ANOVA, consisting of two cabbage cultivars, non-inoculated or inoculated plants, and four sampling times (1, 5, 10 and 15 dai).

Experiment 3

Cabbage plants from cultivar ‘Esmeralda’ and the isolate UFPR 5 of Xcc were used in this experiment. The treatments defined for the experiment were: non-inoculated plants + distilled water; non-inoculated plants + ASM; inoculated plants + distilled water; and inoculated plants + ASM. The experiment was arranged in a completely randomized design with four treatments and four replications. Data from all variables were analyzed by ANOVA (Table 3), and the means from the treatments were compared by Tukey’s test ($P \leq 0.05$). For ANOVA of severity and AUDPC the means were compared by the Student’s t-test. For POX, SOD, APX, PPO, CHI, and GLU activity, as well as for the H_2O_2, and MDA concentrations, the experimental design was considered to be a 4 × 4 factorial experiment for the ANOVA, consisting of four treatments and four sampling times (1, 5, 10 and 15 dai).

Results

Experiment 1

Epidemiological variables

Black rot severity was significantly higher in leaves inoculated with the isolate UFPR 5 than in those inoculated with Xcc 10 at 13, 14 and 15 dai (Fig. 1A). Leaves inoculated with the isolate UFPR 5 displayed AUDPC values that were significantly higher compared with those of Xcc 10 (35 vs. 8, respectively; Fig. 1B).

Enzyme activities

Activities of the antioxidant enzymes significantly increased in response to the infection by Xcc 10, recording increases from 1 to 15 dai for SOD (34-162%; Fig. 2A), for POX (67-376%; Fig. 2C) and for APX (33-55%; Fig. 2E) compared with the control plants. The activities of SOD, POX and APX were not altered in plants inoculated with UFPR 5 regardless of sampling time. A different trend was observed for the defense enzymes. In this case, plants that were inoculated with the isolate UFPR 5, displayed activities that were significantly higher for CHI at 10 and 15 dai (167-65%; Fig. 2B), for GLU at 1, 5, 10 and 15 dai (70, 76, 461 and 283%, respectively; Fig. 2D) and for PPO at 10 and 15 dai (136-33%; Fig. 2F), relative to the control plants. Plants inoculated with Xcc 10 displayed significant increases in the activities of CHI at 10 dai (47%; Fig. 2B), of GLU at 10 and 15 dai (135 and 178%, respectively; Fig. 2D) and of PPO at 15 dai (46%; Fig. 2F).

Oxidative stress-related compounds

Significant increases in the concentrations of oxidative stress-related compounds were observed only in plants inoculated with the isolate UFPR 5 (Fig. 3). Concentrations of H_2O_2 and MDA were increased at 10 and 15 dai by 29 and 52% (Fig. 3A), and by 35 and 48% (Fig. 3B) in response to the inoculation with the isolate UFPR 5, respectively.

Experiment 2

Epidemiological variables

Black rot severity was significantly lower by 65, 73 and 76% in leaves from cv. “LV” than in those from cv. “CQ” at 13, 14, and 15 dai, respectively (Fig. 4A). Cabbage plants from cv. “LV” showed an AUDPC that was significantly lower by 67% compared with leaves from cv. “CQ” (Fig. 4B).

Enzyme activities

Among antioxidant enzymes analyzed in the cv. “LV”, only the POX activity was significantly higher in inoculated leaves by 18 and 20% at 5 and 15 dai, respectively, compared with the non-inoculated ones (Fig. 5C). Significant increases in the cv. “LV” in response to Xcc inoculation were also recorded for CHI (119-359%; Fig. 5G) and for GLU (85-183%; Fig. 5I) from 1 to 15 dai, and for PPO (25-147%; Fig. 5K) at 5, 10 and 15 dai. For cv. “CQ”, the SOD activity was higher for the inoculated than for the non-inoculated plants at 10 and 15 dai (Fig. 5B). The inoculated plants from cv. “CQ” displayed higher POX activity from 1 to 15 (Fig. 5D), and higher APX activity than the non-inoculated ones at 5, 10 and 15 dai (Fig. 5F). CHI and PPO activities were significantly increased in response to Xcc inoculation in the cv. “CQ” only at 15 dai, when there were increases of 24 and 62% compared to the non-inoculated leaves (Figs. 5H and 5L). SOD and APX activities were significantly higher in inoculated leaves from cv. “CQ” from 5 to 15 dai by 24-124% and 42-142%, respectively, compared with those from cv. “LV” (Figs. 5A, 5B, 5E and 5F). POX activity in inoculated leaves from cv. “CQ” was significantly higher by 25-266% from 1 to 15 dai relative to their counterparts from cv. “LV” (Figs. 5C and 5D). Inoculated leaves from cv. “LV” displayed higher activities of CHI (at 5, 10 and 15 dai; Figs. 5G and 5H), GLU (from 1 to 15 dai; Figs. 5I and 5J) and PPO (at 10 and 15 dai; Figs. 5 K and 5L) compared with the inoculated ones from cv. “CQ”.

Oxidative-stress related metabolites

Inoculated leaves from cv. "LV" exhibited concentrations of H_2O_2 and MDA that were significantly higher by 8 and 32% at 15 dai than their non-inoculated counterparts (Figs. 6A and 6C). The inoculation of cv. "CQ" leaves with Xcc resulted in significantly higher concentrations of H_2O_2 and MDA at 10 and 15 dai compared to the non-inoculated ones (Figs. 6B and 6D). Concentrations of H_2O_2 (Figs. 6A and 6B) and MDA (Figs. 6C and 6D) in inoculated leaves from cv. "LV" were significantly lower by 17 and 40% at 10 and 15 dai, respectively, compared with inoculated leaves from cv. "CQ".

Experiment 3

Epidemiological variables

Black rot severity was significantly lower in leaves from ASM-treated plants compared with those non-sprayed from 10 to 15 dai (Fig. 7A). Leaves from ASM-treated plants showed an AUDPC that was significantly lower by 66% compared with leaves in the control plants (Fig. 7B).

Enzyme activities

Among antioxidant enzymes, there were no differences in SOD activities in leaves from non-inoculated plants sprayed with ASM compared to those non-inoculated and non-sprayed at all sampling times (Fig. 8A). Leaves from inoculated plants sprayed with ASM showed SOD activities which were significantly lower by 13 and 31%, compared with those non-inoculated and sprayed, at 10 and 15 dai, respectively (Fig. 8A and B). Inoculated plants sprayed with ASM had lower SOD activity compared to those non-inoculated at 15 dai (Fig. 8A and B). Inoculated plants sprayed with ASM increased POX activity (27-60%) from 1 to 15 dai and APX activity (25-36%) from 5 to 15 dai

compared with the non-inoculated and non-sprayed plants (Fig. 8C, D, E and F). However, APX activity was found to be depressed by 8% in inoculated and sprayed plants at 1 dai compared with those non-inoculated and non-sprayed (Fig 8E and F). Enzyme activity in leaves from inoculated and sprayed plants were higher for CHI (from 1 to 15 dai; Fig. 8G and H), GLU (at 1 and 5 dai; Fig. 8I and J) and PPO (at 5 and 10 dai; Fig. 8K and L) relative to leaves from non-inoculated and non-sprayed plants.

Oxidative-stress related metabolites

Concentrations of H_2O_2 and MDA in leaves from inoculated but non-sprayed plants were significantly increased at 10 and 15 dai compared with leaves from non-inoculated and non-sprayed plants (Fig. 9). Leaves from inoculated and sprayed plants did not show any increase in concentrations of H_2O_2 and MDA compared with non-inoculated and non-sprayed plants.

Discussion

This is the first study to present evidence that Xcc modifies cabbage basal defense levels and that these defense responses vary among different isolates of Xcc and among cabbage cultivars. Although many studies have investigated the biochemical changes related to the basal defense of plants against pathogens (Moya-Elizondo and Jacobsen 2016) a few studies have focused on identifying these changes in the Brassica spp.-Xcc interaction (Conrads-Strauch et al. 1990; Newman et al. 1995; Gay and Tuzun 2000). Furthermore, here we present the first evidence that Acibenzolar-S-Methyl is efficient in inducing resistance in cabbage against black rot.

Experiment 1

The higher severity and AUDPC values for plants inoculated with UFPR 5 compared to Xcc 10 support the view that these isolates are more and less aggressive, respectively, in

cabbage. The AUDPC reflects the effect of the host defense mechanisms on disease intensity over a period of time (Campbell and Madden 1990). The difference between the AUDPC's for UFPR 5 and Xcc 10 suggests that there is variability in the aggressiveness among isolates of Xcc and that cabbage enhances its basal defense levels in response to Xcc infection to prevent black rot progress. This variability also occurs among isolates of Xanthomonas axonopodis pv. passiflorae, causal agent of bacterial spot in passion fruit (Nakatani et al. 2009).

The enhancement of PR-proteins in plants, such as CHI and GLU, is associated with the increment in the resistance of plants against diseases and these proteins are used as biochemical markers of induced resistance (Aleandri et al. 2010). Chitin and β-1,3-glucan are compounds present in the cell wall of many fungi and CHI and GLU are enzymes that hydrolyze these compounds, respectively, releasing elicitors of defense response in plants (Keen and Yoshikawa 1983). Although the increase of these enzyme levels was higher in plants inoculated with UFPR 5, plants inoculated with Xcc 10 also exhibited an increase in CHI and GLU compared to the control. A significant increment of CHI and GLU activities with a subsequent decrease were observed in plants inoculated with UFPR 5. Meanwhile, GLU and CHI activities started to increase at 10 dai but a decrease was not in plants inoculated with Xcc 10. These results suggest that these enzymes are important to cabbage defense against black rot but Xcc modifies these defense responses so as to cause disease. Similar results were obtained by Conrads-Strauch et al. (1990). The authors verified higher levels of CHI and GLU in turnip leaves (Brassica campestris) inoculated with Xcc compared with non-inoculated plants and that their activities were more intense in turnip leaves inoculated with X. campestris pv. vitians, non-pathogenic to turnip. An increment in the GLU levels was also observed in tomato plants as a response to Pseudomonas syringae pv. tomato (Pst) infection until 7 dai, with a decrease at 10 dai (Andrade et al. 2013).

PPO is involved in the production of quinones through the oxidation of phenolic compounds. These products are able to inactivate pectolytic enzymes and have antimicrobial activity (Cavalcante et al. 2014). Although the PPO activity was higher in the inoculated plants compared to the non-inoculated ones, there was no difference among plants inoculated with Xcc 10 or UFPR 5. These results indicate that PPO plays a role in the cabbage defense against Xcc infection. The increment of PPO activity is also important for the defense of snap bean and tomato plants against Pst and Xanthomonas axonopodis pv. phaseoli, respectively (Vigo et al. 2012; Andrade et al. 2013).

The increase in SOD, APX and POX activities suggest that these enzymes are important to the cabbage's defense against Xcc and that this bacterium is able to modify this defensive response and cause black rot. SOD constitutes the first line of defense against oxidative stress by converting O_2^- into H_2O_2, which is removed by enzymes like POX and APX (Alscher et al. 2002). APX is able to reduce H_2O_2 to H_2O and is important for H_2O_2 removal from chloroplasts, peroxisomes and mitochondria (Chandrashekar and Umesha 2014). Polymerization of phenolics is caused by POX and results in biosynthesis of lignin, of phytoalexins and removal of ROS (Higara et al. 2001). The activities of SOD, APX and POX increased along the time in plants inoculated with Xcc 10 and were higher than in the control and in plants inoculated with UFPR 5 at all collection times. These enzyme activities showed no differences between UFPR 5 and the control plants. An increment in POX and SOD activities were already observed in cabbage plants inoculated with Xcc (Gay and Tuzun 2000). Similar results were obtained by Silveira et al. (2015) while studying the activities of APX and POX in tomato plants from cultivar 'Santa Clara', which is susceptible to Xanthomonas gardneri. The APX and POX activities exhibited the same dynamics in cotton plants inoculated with Xanthomonas citri pv. malvacearum (Oliveira et al. 2012). The SOD

activity increased until 5 dai with a decrease after this in grapefruit plants inoculated with Xanthomonas axonopodis pv. citri, a similar trend observed in this study (Kumar et al. 2011).

MDA is a product of lipid peroxidation and is an indicator of membrane degradation and, consequently, cellular damage (Mandal et al. 2008). MDA concentration was higher in plants inoculated with UFPR 5 indicating that the cellular damage caused by ROS was higher for these plants than for those inoculated with Xcc 10 and the control. SOD, APX and POX activities are related to ROS degradation and the lower concentrations of H_2O_2 and MDA in plants inoculated with Xcc 10, compared to the plants inoculated with UFPR 5, indicate that these enzymes are important to prevent cellular damage, to limit the pathogen infection and the disease progress. The correlation between MDA concentration and disease intensity was also observed in grapefruit plants inoculated with Xanthomonas axonopodis pv. citri as well as between the MDA concentration and the activity of SOD, APX and POX (Kumar et al. 2011).

An increase in H_2O_2 concentration during the infectious process contributes to the increase of MDA concentration (Kumar et al. 2011). The concentrations of H_2O_2 and MDA were higher in plants inoculated with UFPR 5 than in the control at 10 dai and 15 dai. The concentration of these compounds in plants inoculated with Xcc 10 did not differ from the control plants at any collection time. The increase in the activity of enzymes related to oxidative stress (SOD, POX and APX) prevented H_2O_2 accumulation in plants inoculated with Xcc 10 demonstrating the importance of these enzymes in preventing oxidative stress and cell damage in cabbage leaves. Also, the increase of H_2O_2 and MDA concentrations and the maintenance of SOD, POX and APX activities at low levels in plants inoculated with UFPR 5 suggest that Xcc has the ability to modify the antioxidant system in cabbage so as to cause disease. Corroborating with

the results obtained here, Gay and Tuzun (2000) demonstrated that higher activities of SOD and POX in cabbage are associated with lower concentrations of H_2O_2 in plants inoculated with Xcc. Similar association was observed in grapefruit plants inoculated with Xanthomonas axonopodis pv. citri (Kumar et al. 2011).

The results of this study suggest that cabbage limits Xcc infection and prevents cell damage by increasing the activity of antioxidant enzymes (removal of ROS excess) and PR-enzymes. On the other hand, Xcc is able modify these defense responses so as to facilitate the infectious process and this ability varies between isolates of this bacterium. Further studies will reveal the signal exchanges between cabbage and Xcc that results in the increment and/or the decrease of the PR-proteins activity and, consequently, affects the black rot progress. These results may contribute to cabbage breeding research programs aimed at searching for a resistance inducer which uses these enzymes as biochemical markers to select cultivars with higher levels of resistance and to enhance the resistance of cabbage plants to black rot.

Experiment 2

The AUDPC and severity values were higher for cv. “CQ” compared with cv. “LV”, supporting the information that these cabbage cultivars are, respectively, more susceptible and more resistant to the isolate S3. The differences in AUDPC and severity of cv. “CQ” and cv. “LV” suggest that the defense mechanisms against disease severity during the period of assessment were more effective for limiting the progress of black rot in cv. “LV” plants. Corroborating our results, the black rot severity was higher for the cabbage cultivar ‘Perfect Ball’ (susceptible) than for the cultivar ‘Hancock’ (resistant) suggesting that the intensity of the plant defense responses differed among these cultivars (Gay and Tuzun 2000). The difference in disease severity among different plant genotypes were also observed in the Citrus spp.-Xanthomonas citri pv.

citri interaction (de Carvalho et al. 2015), in the common bean-Xanthomomas axonopodis pv. phaseoli interaction and in the common bean Pseudomonas savastanoi pv. phaseolicola interaction (Donmez et al. 2013).

The reduction in black rot intensity in cabbage is a result of the increment in CHI, GLU and PPO activities. Although CHI has activity against fungal cell wall and nematode eggs, such enzyme also has lysozyme activity and are able to hydrolyze the bacterial cell wall peptidoglycan (Düring 1993). CHI activity was higher for inoculated plants from cv. “LV” in comparison with those from cv. “CQ” and the control plants from both cultivars. The activity of this enzyme was significantly higher for inoculated plants from cv. “CQ” compared to the non-inoculated only at 15 dai. Similar results were obtained when assessing GLU activity. This enzyme has an indirect action upon bacteria (Hong and Hwang 2005). GLU is able to hydrolyze fungal an plant cell walls and the fragments released as a consequence of the enzyme action functions as signal molecules that will activate other defense mechanisms in the plant (Keen and Yoshikawa 1983; Hong and Hwang 2005). For cv. “LV”, GLU activity was higher in inoculated plants in comparison with the non-inoculated plants from the same cultivar, and with inoculated and non-inoculated plants of cv. “CQ” at all sampling times. GLU activities in cv. “CQ” did not differ between inoculated and non-inoculated plants. Other studies presented similar results. Turnip leaves (Brassica campestris) inoculated with Xcc exhibited higher CHI and GLU activities in comparison with the non-inoculated ones (Conrads-Strauch et al. 1990). Baysal et al. (2003) obtained similar results comparing CHI activity in tomato plants inoculated and non-inoculated with Clavibacter michiganensis subsp. michiganensis (Cmm) and concluded that the increment in CHI activity is related to higher resistance levels of tomato plants against the bacterial canker. O’Garro and Charlemange (1994) compared inoculated and non-inoculated tomato plants to three different cultivars with X. campestris pv. vesicatoria

(Xcv) and verified the increment in CHI and GLU activities at similar rates. However, the Xcv growth rate decreased in one cultivar only, demonstrating that other defense mechanisms may be involved in the resistance of tomato against bacterial blight.

A number of the products of PPO activity in plants, such as quinones, can deactivate pectolytic enzymes and have antimicrobial activity (Leatham et al. 1980; Vaughn and Duke 1988). In comparisons between the inoculated and non-inoculated plants from both cultivars, the increment in the PPO activity was faster and higher for the plants from cv. "LV" than for those from cv. "CQ", indicating that this enzyme is important to contributing to defense responses to Xcc and that the efficacy of these responses is host-genotype dependent. The increments in PPO activity were also associated with resistance of potato to Pectobacterium atrosepticum, P. carotovorum subsp. brasiliensis and Dickeya spp. infection (Ngadze et al. 2011).

Antioxidant enzyme activities were, in general, significantly higher for the inoculated plants from cv. "CQ" in comparison with the non-inoculated ones and with plants from cv. "LV". Although SOD activity did not differ between inoculated and non-inoculated plants from cv. "LV", plants from cv. "CQ" inoculated with Xcc had higher SOD activity compared with the non-inoculated plants and with inoculated and non-inoculated plants from cv. "LV". Accumulation of ROS is common in plant cells during the infectious process of bacteria (García-Cristobal et al 2015). APX, POX and SOD activities can remove the excess of ROS, inhibit damages in the cell membrane and restrict pathogen colonization (Kumar et al. 2011). The resistant cabbage cv. 'Hancock' showed higher SOD activity compared to the susceptible cv. 'Perfect Ball' and the same trend was observed in the assessment of H_2O_2 concentration. The SOD catalyzes the conversion of O_2^- into O_2 and H_2O_2 as a first step in the removal of excess ROS justifying the increment in SOD activity and H_2O_2 concentration in cv. 'Hancock' plants

(Gay and Tuzun 2000). The enhancement of SOD activity in inoculated plants from cv. "CQ" resulted in an increment in H_2O_2 concentration but the increment in POX and APX activities were not sufficient to remove the excess of H_2O_2 and to limit black rot progress.

The antioxidant system of cv. "CQ" is not efficient in inhibiting the infectious process. In contrast, the slightly enhanced POX activity in cv. "LV" may explain the reduced cellular damage but other antioxidant enzymes may be involved. For the cv. "LV", POX activity was higher for inoculated plants compared to the non-inoculated ones. The inoculated plants from cv. "CQ" exhibited higher POX activity during all sampling times in comparison with the non-inoculated plants from the same cultivar and the plants from cv. "LV". The increment in POX activity was also observed during the infectious process of Cmm in tomato plants (Baysal et al. 2003). In contrast to the results obtained here, higher POX activity was observed in the resistant cabbage cv. 'Hancock' in comparison with other cultivars with lower resistance levels (Gay and Tuzun 2000). The results obtained here suggest that the increment in POX activity in the susceptible cabbage cv. "CQ" was not sufficient to inhibit pathogen colonization and, thus, the progress of the disease.

APX activity was higher in plants from cv. "CQ" inoculated with Xcc compared with the non-inoculated and the inoculated and non-inoculated plants from cv. "LV". Differences in APX activity were also observed among different accessions of tobacco inoculated Pseudomonas syringae pv, phaseolicola (Psp; non-pathogenic to tobbaco) and these differences permitted the authors to conclude that the increment in the APX activity is important to prevent the infection. Furthermore, accessions with elevated levels of APX activity were less affected by Psp (Zhang et al. 2015). Kazemi et al. (2010) observed that the increment in APX activity in canola plants (B. napus) is

important for preventing oxidative stress induced by nickel. In contrast to the results obtained by these authors, in the present study the increment in APX activity was not sufficient to inhibit the infectious process during the cabbage-Xcc interaction up to 15 dai.

The increment in MDA and H_2O_2 concentrations in plants are indicators of lipid peroxidation and cellular damage (Shi et al. 2015; Silveira et al. 2015). The MDA and H_2O_2 concentrations were higher in inoculated plants from cv. “CQ” at 10 and 15 dai compared with the non-inoculated and inoculated and non-inoculated plants from cv. “LV”. The increment in the concentrations of these compounds is related to the higher AUDPC and severity in cabbage plants from cv. “CQ”, indicating that this cultivar is more susceptible to the black rot in comparison to cv. “LV”. The enhancement in H_2O_2 concentration contributes to an increment in MDA concentration and results in higher disease severity in other pathosystems like grapefruit-Xanthomonas axonopodis pv. citri and tomato-Xanthomonas gardneri. As a defense response to the oxidative stress, the activities of enzymes related to the plant antioxidant system, like SOD, POX and APX, enhances as an attempt to limit the cellular damage (Kumar et al. 2011; Silveira et al. 2015). Although the activities of the antioxidant enzymes had an enhancing effect during this experiment, the increment in APX, POX and SOD activities was not sufficient to prevent the accumulation of MDA and H_2O_2, and to inhibit black rot progress. These results indicate that the slight cellular damage provoked by Xcc in plants from cv. “LV” is not reduced by APX and SOD activities. POX activities and other antioxidant enzymes may be related to the inhibition of cellular damage and to the prevention of the black rot progress.

Further studies will investigate role of the cabbage antioxidant system in plant-Xcc interaction. CHI, GLU and PPO can be used as biochemical markers for testing

resistance inducers in cabbage to black rot and in breeding programs that aim to develop cabbage cultivars with higher resistance levels to this disease.

Experiment 3

Here, we present the first report of black rot control in cabbage after ASM application. The results obtained in this experiment indicate that spraying cabbage with ASM was effective in reducing black rot severity and was associated with the increment in APX, POX, CHI, GLU and PPO activities. ASM application significantly reduced black rot severity in comparison with non-treated plants and the AUDPC was greater for cabbage plants that were not sprayed with ASM. The effect of ASM application in plants to induce SAR and reduce disease progress has been studied in many pathosystems. In lettuce, the application of ASM was responsible for a significant reduction in the bacterial leaf spot, caused by Xanthomonas campestris pv. vitians (Yigit 2011). The severity of the tomato bacterial spot, caused by Xanthomonas campestris pv. vesicatoria, was significantly reduced due to the application of ASM (Cavalcanti et al. 2006). The reduction in the disease intensity through the application of ASM was observed in other pathosystems like orange-Xantomonas citri subsp. citri and kiwifruit-Pseudomonas syringae pv. actinidae (Graham and Myers 2013; Cellini et al. 2014).

The enzyme that acts in the first line of the antioxidant system in the plant is SOD. This enzyme generates H_2O_2 through the dismutation of superoxide (O_2^-; Hafez et al. 2012). In this experiment, the ASM was not effective in enhance the SOD activity. This result contrasts with those verified in other studies. Tomato plants inoculated with Cmm and treated with ASM exhibited an increment in the SOD activity and a reduction in the bacterial canker severity (Soylu et al. 2003). The enhancement of the SOD activity as a consequence of the application of a resistance inducer was observed in cotton plants treated with silicon and inoculated with Xanthomonas citri subsp. malvacearum

(Oliveira et al. 2012), and in rice plants treated with 2-(methyl sulfonyl)-5-(4-fluorophenyl)-1,3,4-oxadiazole or bismerthiazol and inoculated with Xanthomonas oryzae pv. oryzae (Shi et al. 2015).

After SOD convert O_2^- in H_2O_2, other enzymes related to the antioxidant system convert the peroxide in water (Roohie and Umesha 2015). Two of these enzymes related to oxidative stress (APX and POX) exhibited an enhancement in their activities in plants sprayed with ASM. APX activity was higher in inoculated plants sprayed with ASM in comparison with those inoculated but non-sprayed. This enzyme plays a role in the removal of H_2O_2 from chloroplasts, peroxisomes and mitochondria, and is an important component in the scavenging of ROS in tomato plants inoculated with Xanthomonas gardneri (Silveira et al. 2015). Our results are also in line with those of Chandrashekar and Umesha (2014) who verified that the application of 2,6-dichloroisonicotinic acid enhances the APX activity and that this increment is important for tomato plants to limit the Xanthomonas perforans infection. This result indicates that ASM induces the increment in APX activity in cabbage and that this enzyme is an important component of the antioxidant system of cabbage in the plant-Xcc interaction.

Peroxidases are involved in the removal of excess ROS and in lignin biosynthesis (Roohie and Umesha 2015). In the present study we observed that POX activity was significantly higher in inoculated plants sprayed with ASM in comparison with the other treatments at all sampling times, except when compared to the non-inoculated plants sprayed with ASM. The increment in POX activity as a result of ASM application and the importance of this increase in plant defense were also observed in tomato plants inoculated with Cmm (Baysal et al. 2003). A similar result was observed in tomato plants inoculated with Ralstonia solanacearum and sprayed with salicylic acid (Mandal et al. 2013). Our results suggest that ASM induces the increment in the

POX activity and that this enzyme is the major component of the antioxidant system of cabbage in the defense against Xcc.

Polyphenoloxidases are involved in the production of quinones that have antimicrobial activities and can inactivate pectolytic enzymes (Cavalcante et al. 2014). PPO activity was higher for inoculated plants sprayed with ASM than the other treatments. Similar results were obtained in tomato plants sprayed with ASM and inoculated with Pseudomonas syringae pv. tomato (Cavalcante et al. 2014) or Xanthomonas gardneri (Luiz et al. 2012).The effect of ASM upon the increment in PPO activity and disease reduction were observed in snap bean inoculated with Xanthomonas axonopodis pv. phaseoli (Vigo et al. 2012). In this study we observed that ASM also promotes an increment in PPO activity and that this increase is important for controlling black rot.

The enzymes CHI and GLU have antifungal activity and may release elicitors of defense responses in plants (Keen and Yoshikawa 1983). Here we observed that inoculated plants sprayed with ASM exhibited significantly higher CHI activity than the other treatments at all sampling times except in comparison with the inoculated but non-sprayed (1 and 15 dai) and with the sprayed but non-inoculated (10 dai). The application of ASM in tomato plants inoculated with Cmm also induced an increment in CHI activity leading to 76% of bacterial canker control (Baysal et al. 2003). Acimovic et al. (2015) observed an increment in the pr-8 (class III chitinase gene) expression and a reduction in the fire blight severity in apple trees trunk-injected with ASM. In this study we verified that ASM induces the increase in CHI activity in cabbage plants and this increment is an important component of the defense of plants against Xcc infection.

The increment in the GLU activity was significantly faster and higher for inoculated plants sprayed with ASM in comparison with all the other treatments. Conrads-Strauch et al. (1990) also observed that the increment in GLU activity in turnip plants is an

important defense mechanism against Xcc infection. Our results are in line with those obtained by Cavalcante et al. (2014) in which the application of ASM enhanced the GLU activity and reduced the bacterial leaf speck intensity in tomato plants. Francis et al. (2009) verified an increment in the pr-2 (β-1,3-glucanase gene) expression in orange plants inoculated with Xanthomonas citri subsp. citri and treated with ASM. Herein, we present evidence that ASM application induces the increment in GLU activity in cabbage plants infected with Xcc.

The increment in H_2O_2 and MDA concentrations are indicative of lipid peroxidation and cellular damage (Silveira et al. 2015). In the present study, the concentrations of H_2O_2 and MDA were significantly higher only for the inoculated plants without ASM application in comparison with the other treatments. Corroborating our results, the application of resistance inducers also reduced the concentrations of H_2O_2 and MDA in rice plants inoculated with Xanthomonas oryzae pv. oryzae and this reduction positively correlates with less disease severity (Shi et al. 2015). In another study, the oxidative stress induced by nickel in canola plants was evidenced by the increment in H_2O_2 and MDA concentrations and the application of salicylic acid induces the increment of APX activity and reduction in concentration of these compounds (Kazemi et al. 2010). Herein, we present evidence that the increment in POX and APX activity induced by ASM application is efficient in maintaining H_2O_2 and MDA concentrations at constant levels.

Further research will provide information about recommended dose, applications intervals, and about the impact of the ASM application upon other important characteristics such as plant growth, yield and nutritional composition.

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Figures and Tables

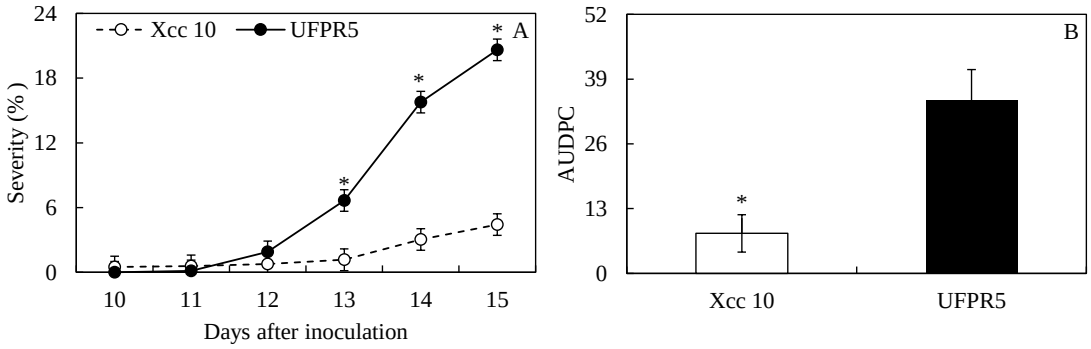

Figure 1. Progress of black rot (A) and area under diseases progress curve (AUDPC; B) on leaves of cabbage plants (cv. 'Esmeralda') that were inoculated with a less aggressive (Xcc 10) or a more aggressive (UFPR 5) isolate of Xanthomonas campestris pv. campestris. Means of isolates followed by an asterisk (*) are significantly different based on the Student's t-test. Bars represent the standard errors of the means.

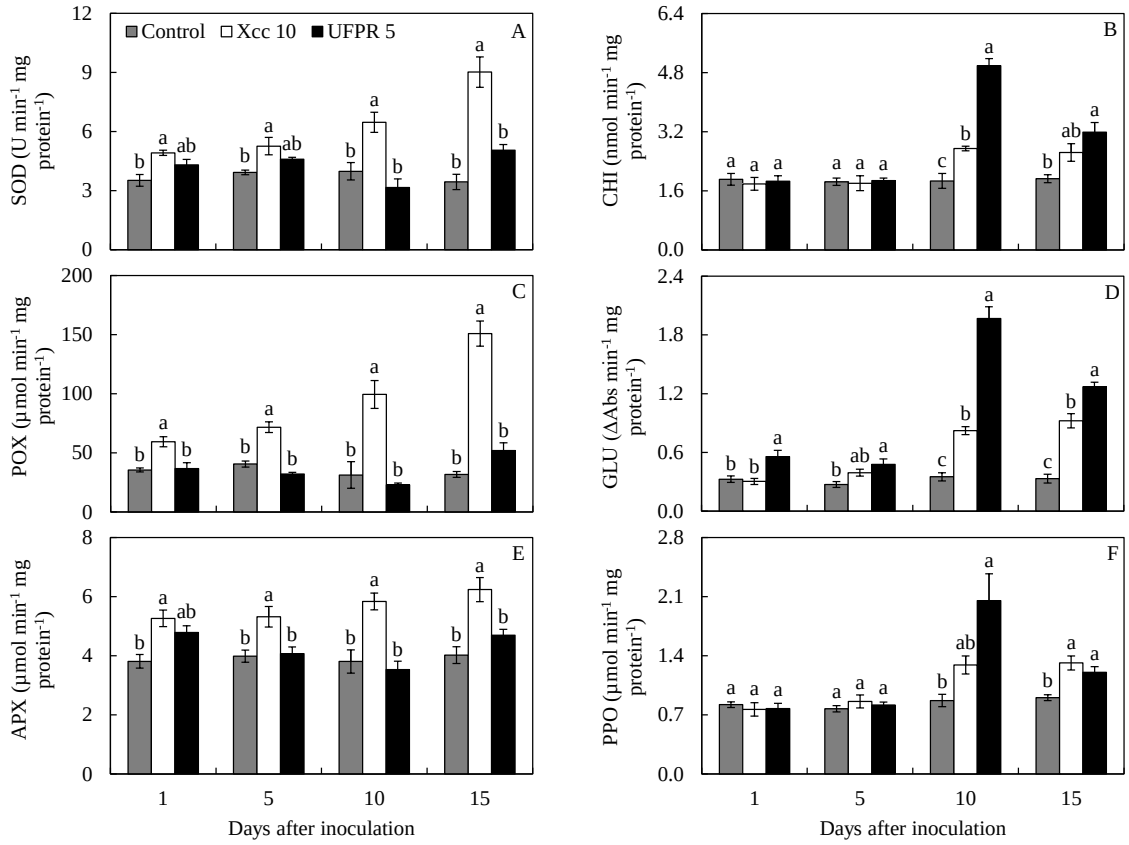

Figure 2. Activities of superoxide dismutases (SOD; A), peroxidases (POX; C), ascorbate peroxidases (APX; E), chitinases (CHI; B), *β*-1,3-glucanases (GLU; D) and polyphenoloxidases (PPO; F) in leaves of cabbage plants (cv. 'Esmeralda') that were either non-inoculated (control) or inoculated with a less aggressive (Xcc 10) or a more aggressive (UFPR 5) isolate of Xanthomonas campestris pv. campestris. Means of the treatments followed by the same letter within each sampling time are not significantly different based on the Tukey's test. Bars represent the standard errors of the means.

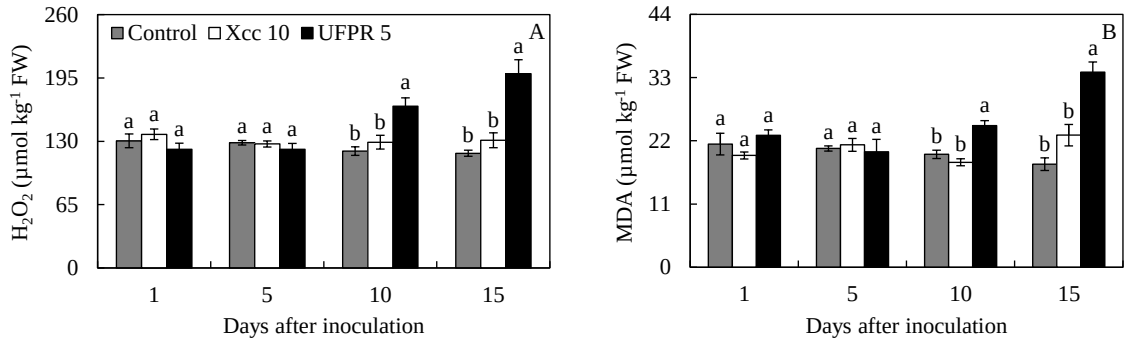

Figure 3. Concentrations of hydrogen peroxide (H_2O_2) (A) and malondialdehyde (MDA) (B) in leaves of cabbage plants (cv. 'Esmeralda') that were either non-inoculated (control) or inoculated with a less aggressive (Xcc 10) or a more aggressive (UFPR 5) isolate of Xanthomonas campestris pv. campestris. FW = fresh weight. Means of the treatments followed by the same letter within each sampling time are not significantly different based on the Tukey's test. Bars represent the standard errors of the means.

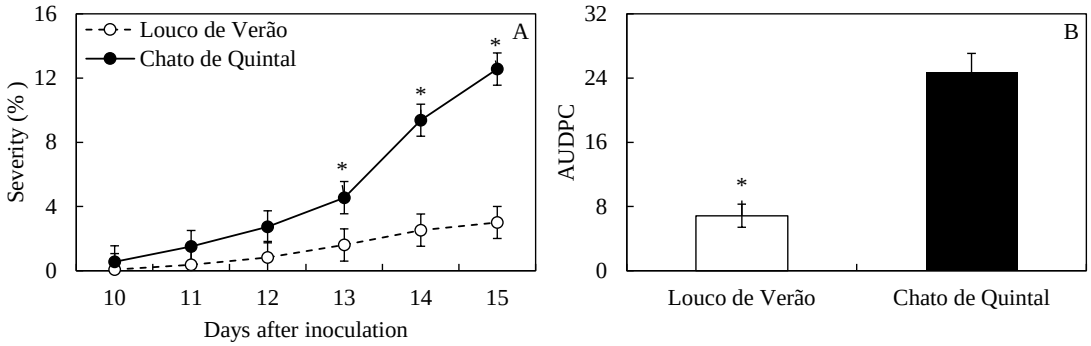

Figure 4. Progress of black rot (A) and area under disease progress curve (AUDPC; B) on leaves of cabbage plants from cultivars ‘Louco de Verão’ and ‘Chato de Quintal’ inoculated with the isolate S3 of Xanthomonas campestris pv. campestris. Means of cultivars followed by an asterisk (*) are significantly different based on the Student’s t-test. Bars represent the standard errors of the means.

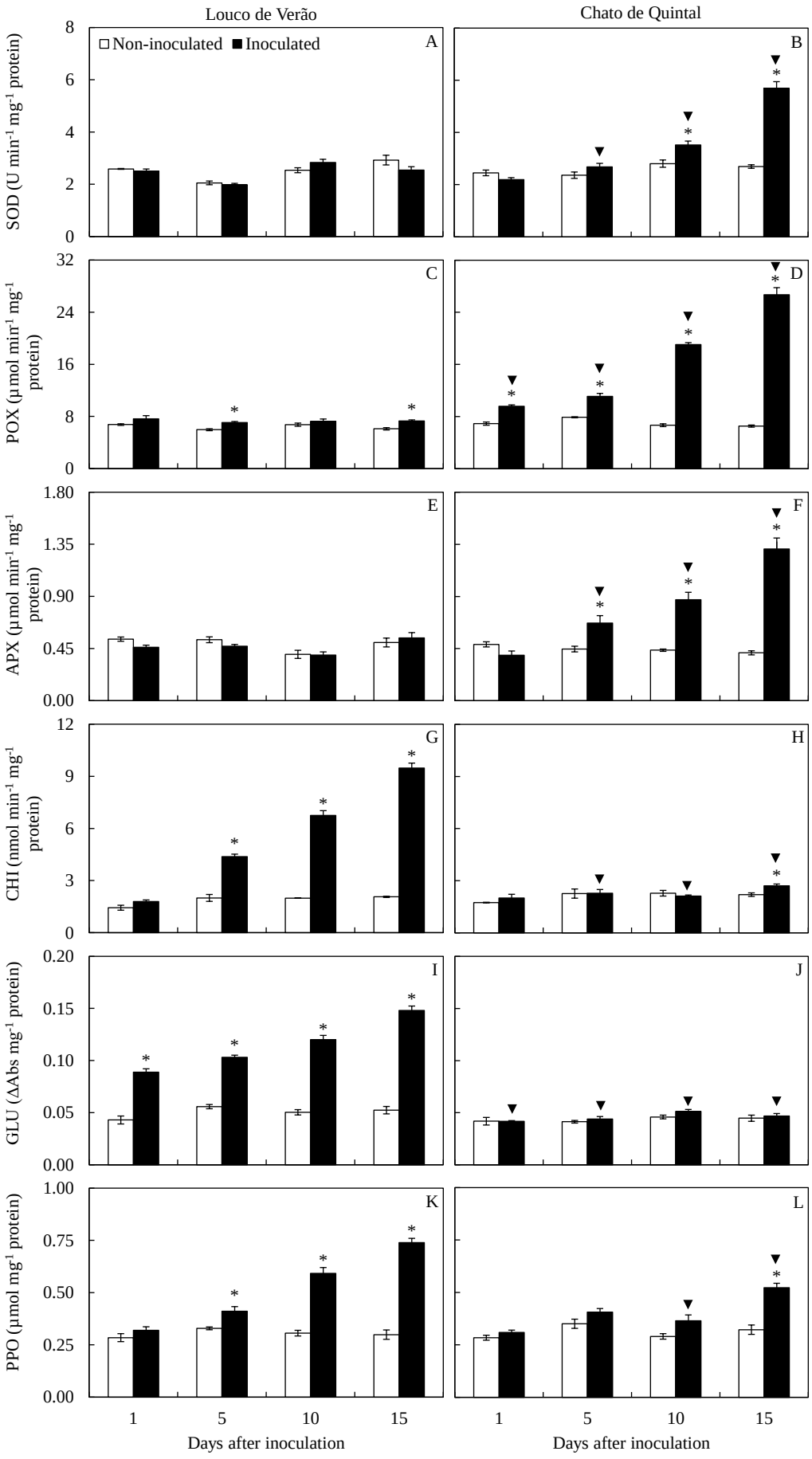
Louco de Verão
Chato de Quintal
Non-inoculated
Inoculated
A
B
C
D
E
F
G
H
I
J
K
L
SOD (U min-1 mg-1 protein)
POX (µmol min-1 mg-1 protein)
APX (µmol min-1 mg-1 protein)
CHI (nmol min-1 mg-1 protein)
GLU (ΔAbs mg-1 protein)
PPO (µmol mg-1 protein)
Days after inoculation
Days after inoculation

Figure 5. Activities of superoxide dismutases (SOD; A and B), peroxidases (POX; C and D), ascorbate peroxidases (APX; E and F), chitinases (CHI; G and H), *β*-1,3-glucanases (GLU; I and J) and polyphenoloxidases (PPO; K and L) in cabbage plant leaves from cv. 'Louco de Verão' (A, C, E, G, I and K) and cv. 'Chato de Quintal' (B, D, F, H, J and L) that were either non-inoculated or inoculated with Xanthomonas campestris pv. campestris. Means of the non-inoculated and inoculated treatments followed by an asterisk (*) in each sampling time and cultivar are significantly different based on the Student's t-test. Means of the cvs. 'Louco de Verão' and 'Chato de Quintal' followed by the symbol ▼ in each sampling time and cultivar are significantly different based on the Student's t-test. Bars represent the standard errors of the means.

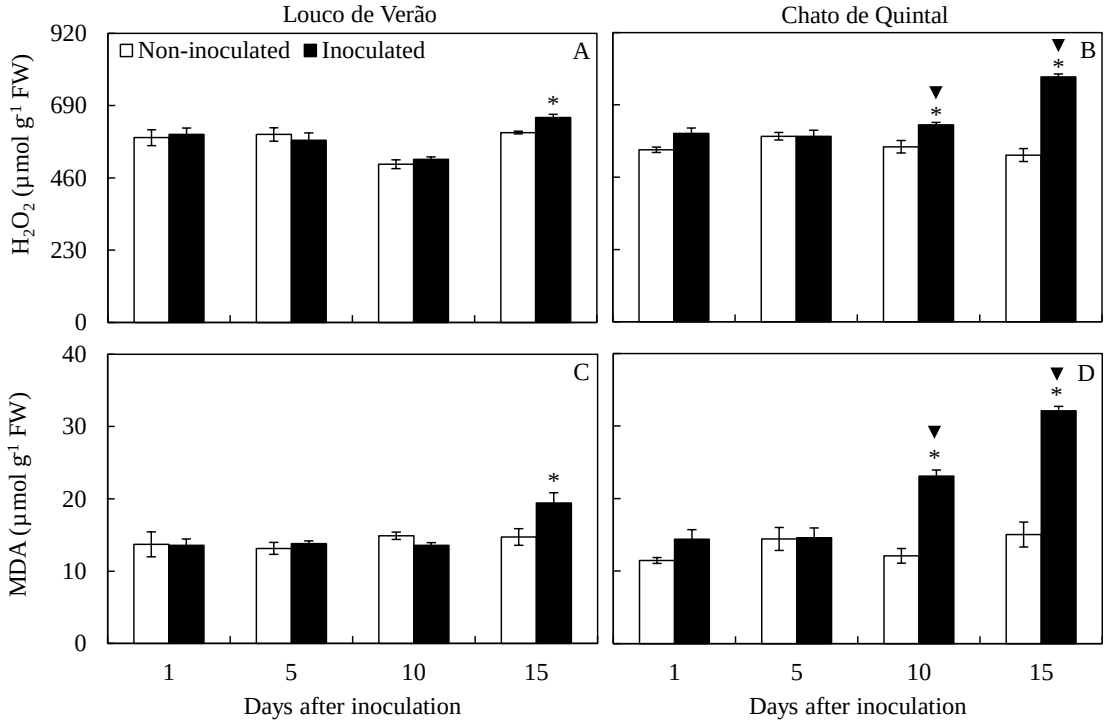

Figure 6. Concentrations of hydrogen peroxide (H_2O_2; A and B) and malondialdehyde (MDA; C and D) in leaves of cabbage plants from cv. 'Louco de Verão' (A and C) and cv. 'Chato de Quintal' (B and D) that were either non-inoculated or inoculated with Xanthomonas campestris pv. campestris. FW = fresh weight. Means of the non-inoculated and inoculated treatments followed by an asterisk (*) in each sampling time and cultivar are significantly different based on the Student's t-test. Means of the cvs. 'Louco de Verão' and 'Chato de Quintal' followed by the symbol ▼ in each sampling time and cultivar are significantly different based on the Student's t-test. Bars represent the standard errors of the means.

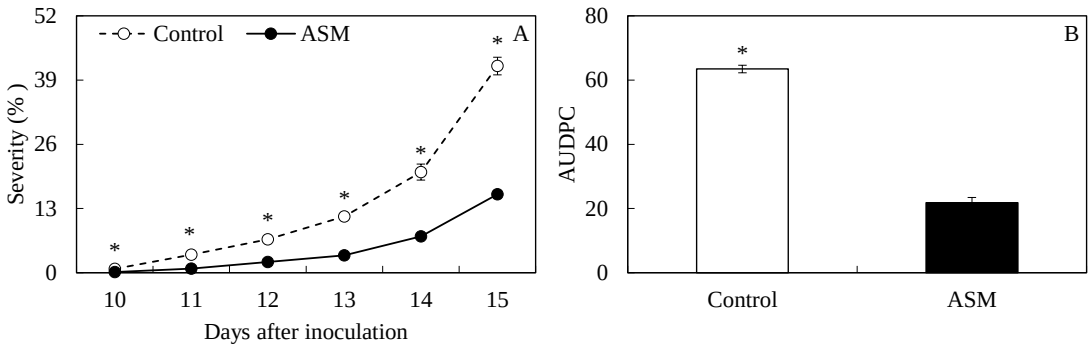

Figure 7. Progress of black rot (A) and area under disease progress curve (AUDPC; B) on leaves of cabbage plants (cv. ‘Esmeralda’) that were not treated (Control) or treated with Acibenzolar-S-Methyl (ASM) and inoculated with Xanthomonas campestris pv. campestris. Means of isolates followed by an asterisk (*) are significantly different based on the Student’s t-test. Bars represent the standard errors of the means.

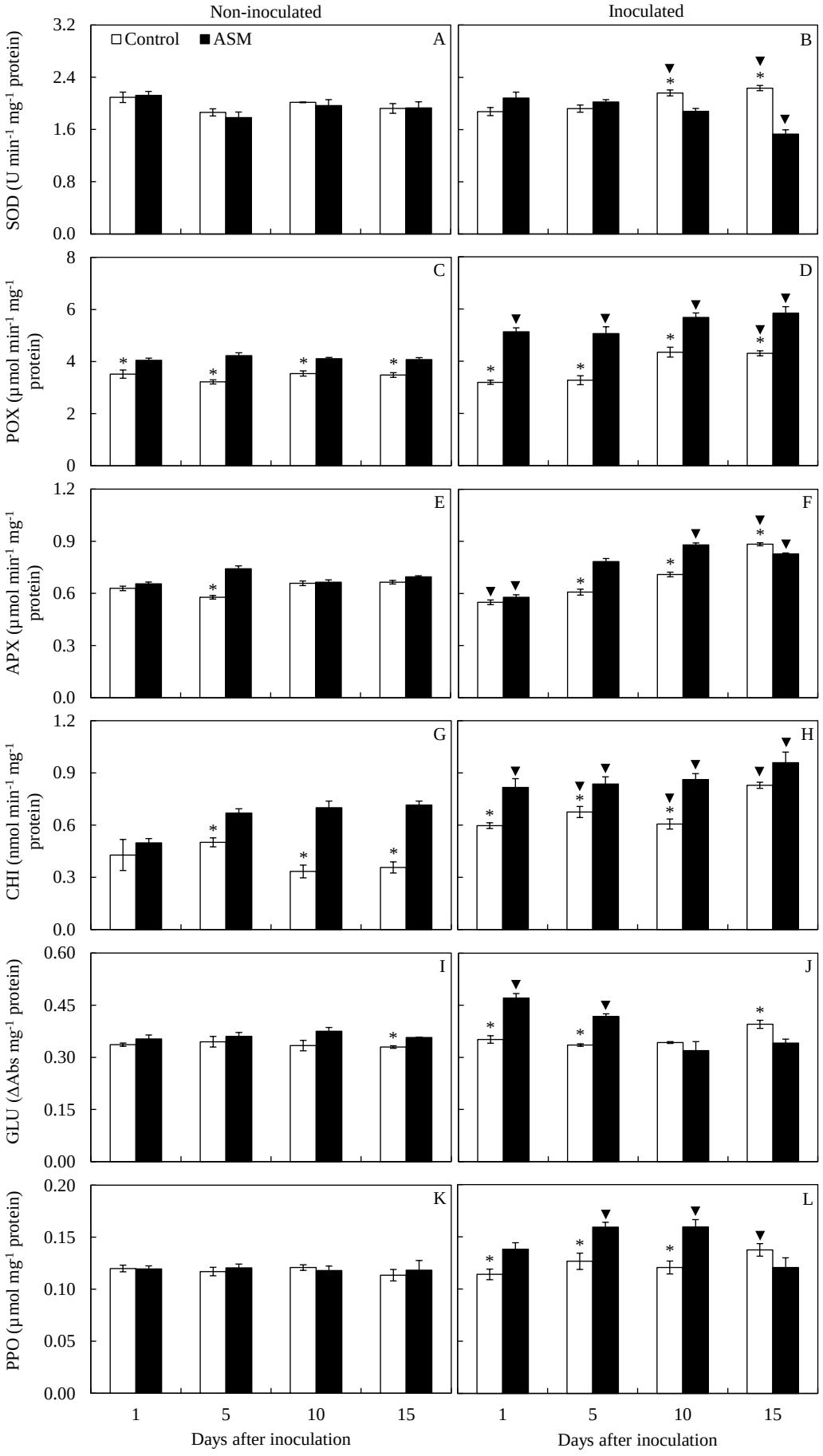
Non-inoculated
Inoculated
Control
ASM
A
B
C
D
E
F
G
H
I
J
K
L
SOD (U min-1 mg-1 protein)
POX (μmol min-1 mg-1 protein)
APX (μmol min-1 mg-1 protein)
CHI (nmol min-1 mg-1 protein)
GLU (ΔAbs mg-1 protein)
PPO (μmol mg-1 protein)
Days after inoculation
Days after inoculation

Figure 8. Activities of superoxide dismutases (SOD; A and B), peroxidases (POX; C and D), ascorbate peroxidases (APX; E and F), chitinases (CHI; G and H), *β*-1,3-glucanases (GLU; I and J) and polyphenoloxidases (PPO; K and L) in leaves of cabbage plants (cv. 'Esmeralda') that were either non-inoculated (A, C, E, G, I and K) or inoculated (B, D, F, H, J and L) with Xanthomonas campestris pv. campestris. Means of the control and Acibenzolar-S-Methyl (ASM) treatments followed by an asterisk (*) in each sampling time are significantly different based on the Student's t-test. Means of the inoculated and non-inoculated treatments followed by the symbol ▼ in each sampling time are significantly different based on the Student's t-test. Bars represent the standard errors of the means.

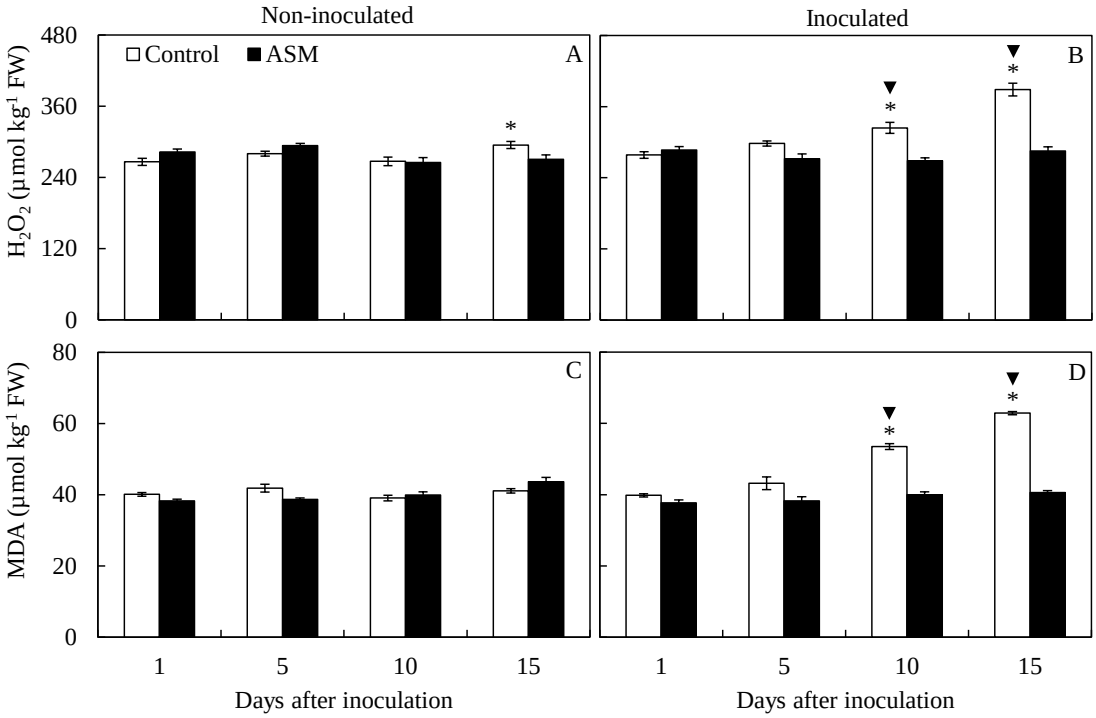

Figure 9. Concentrations of hydrogen peroxide (H_2O_2; A and B) and malondialdehyde (MDA; C and D) in leaves of cabbage plants (cv. 'Esmeralda') that were either non-inoculated (A and C) or inoculated (B and D) with Xanthomonas campestris pv. campestris. FW = fresh weight. Means of the control and Acibenzolar-S-Methyl (ASM) treatments followed by an asterisk (*) in each sampling time are significantly different based on the Student's t-test. Means of the inoculated and non-inoculated treatments followed by the symbol ▼ in each sampling time are significantly different based on the Student's t-test. Bars represent the standard errors of the means.

Table 1. Analysis of variance of the effects of isolates of Xanthomonas campestris pv. campestris, evaluation time and its interaction in the severity (SEV), area under disease progress curve (AUDPC) activities of superoxide dismutases (SOD), peroxidases (POX), ascorbate peroxidases (APX), chitinases (CHI), *β*-1,3-glucanases (GLU) and polyphenoloxidases (PPO), and concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA).

	F values[z]		
Variables	Isolates	Evaluation time	Isolates × Evaluation time
SEV	**39.22**	**21.05**	**10.07**
AUDPC	**12.14**	-	-
SOD	**39.11**	**7.18**	**6.49**
POX	**85.20**	**12.83**	**9.71**
APX	**31.06**	0.15	0.17
CHI	**31.88**	**33.12**	**15.13**
GLU	**132.14**	**76.75**	**29.28**
PPO	**8.38**	**15.53**	**5.41**
H_2O_2	**13.23**	**5.03**	**8.91**
MDA	**16.12**	**5.43**	**6.68**

[z]Bold values are significant ($P < 0.01$).

Table 2. Analysis of variance of the effects of cabbage cultivar (C), plant inoculation (PI) and evaluation time (ET) and its interaction in the severity (SEV), area under disease progress curve (AUDPC) and activities of superoxide dismutases (SOD), peroxidases (POX), ascorbate peroxidases (APX), chitinases (CHI), β-1,3-glucanases (GLU) and polyphenoloxidases (PPO).

	F values[z]						
Variables	C	PI	ET	C × PI	C × ET	PI × ET	C × PI × ET
SEV	**144.07**	**-**	**58.07**	**-**	**21.38**	**-**	**-**
AUDPC	**36.39**	-	-	-	-	-	-
SOD	**59.29**	**38.17**	**56.46**	**48.99**	**23.27**	**20.11**	**31.41**
POX	**524.46**	**592.63**	**72.29**	**402.31**	**79.68**	**94.11**	**92.49**
APX	**39.70**	**51.35**	**16.36**	**66.27**	**14.37**	**23.64**	**14.76**
CHI	**244.02**	**386.96**	**101.31**	**326.73**	**61.16**	**63.43**	**8.80**
GLU	**533.79**	**416.61**	**24.83**	**357.73**	**14.41**	**13.46**	**12.08**
PPO	**23.15**	**179.64**	**39.44**	**29.61**	**8.40**	**33.95**	**7.21**

[z]Bold values are significant ($P < 0.01$).

Table 3. Analysis of variance of the effects of resistance inducer (RI), plant inoculation (PI) and evaluation time (ET) and their interactions in the severity (SEV), area under disease progress curve (AUDPC), activities of superoxide dismutases (SOD), peroxidases (POX), ascorbate peroxidases (APX), chitinases (CHI), β-1,3-glucanases (GLU) and polyphenoloxidases (PPO) and concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA).

	F values[z]						
Variables	RI	PI	ET	RI × PI	RI × ET	PI × ET	RI × PI × ET
SEV	**389.32**	**-**	**345.45**	**-**	**66.90**	**-**	**-**
AUDPC	**270.14**	-	-	-	-	-	-
SOD	**6.40**	0.01	**3.73**	**3.71**	**7.29**	2.39	**7.81**
POX	**189.29**	**95.52**	**10.12**	**33.07**	1.34	**8.12**	0.78
APX	**86.22**	**81.73**	**92.65**	2.52	**29.02**	**58.74**	**12.48**
CHI	**87.51**	**114.82**	**5.89**	1.13	2.73	**2.95**	**3.01**
GLU	**17.94**	**11.92**	**4.93**	0.20	**7.71**	**7.62**	**12.09**
PPO	**9.68**	**22.61**	1.71	**7.42**	**2.84**	1.39	**4.21**
H_2O_2	**30.92**	**34.54**	**13.58**	**33.99**	**18.15**	**10.57**	**879.30**
MDA	**119.06**	**66.91**	**48.27**	**425.22**	**46.30**	**95.13**	**133.22**

[z]Bold values are significant ($P < 0.01$).

CONCLUSÕES GERAIS

1) Isolados de Xcc apresentam variabilidade quanto à sua patogenicidade e agressividade a diferentes cultivares de repolho.

2) Os cultivares ‘Coração de Boi Gigante’, ‘Fuyutokyo Kobayashi’ e ‘Midori’ apresentaram maior resistência à podridão.

3) Os cultivares ‘60 dias’ e ‘Esmeralda’, por apresentarem alta suscetibilidade a todos os isolados de Xcc, apresentam potencial para utilização em testes de patogenicidade para detecção da bactéria.

4) O repolho limita a infecção por Xcc e evita o dano celular pelo aumento da atividade de enzimas antioxidantes (remoção do excesso de EROs) e de enzimas de defesa.

5) A redução da intensidade da podridão negra em repolho é resultado do incremento nas atividades de CHI, GLU e PPO.

6) A aplicação de ASM em repolho foi eficiente na redução dos sintomas da podridão negra, a qual foi associada à maior atividade de APX, POX, CHI, GLU e PPO.