VIVIANE YUMI BABA

ANÁLISE DE TRANSCRIPTOMA E DE METABÓLITOS SECUNDÁRIOS EM FRUTOS DE *Capsicum annuum* NA INTERAÇÃO COM *Colletotrichum gloeosporioides*

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Agronomia, do Departamento de Agronomia, da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Doutora.

Orientador: Prof. Dr. Leandro Simões Azeredo Gonçalves.
Coorientador: Dr. Lukas Mueller - Cornell University.

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Londrina, 28 de fevereiro de 2018.

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BABA, V.Y. **Análise de transcriptoma e de metabólitos secundários em frutos de *Capsicum annuum* na interação com *Colletotrichum gloeosporioides*.** 2018. 91f. Tese (Doutorado em Agronomia) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

A antracnose, causada pelo fungo *Colletotrichum* spp., está entre as principais doenças que ocasionam perdas na produção de pré e pós-colheita em frutos de pimentas e pimentões. *Capsicum* spp. possuem mecanismos de defesa contra o ataque de patógenos com a produção de compostos especializados constitutivos ou induzidos que se acumulam no local de infecção. No entanto, o conhecimento sobre esses compostos permanece mal compreendido principalmente durante os diferentes estádios de maturação dos frutos de pimenta, os quais apresentam reações diferenciadas. Nesse sentido, o esclarecimento dos fatores bioquímicos e moleculares envolvidos na resposta de resistência durante a infecção do fungo pode contribuir para o desenvolvimento de genótipos de *Capsicum* com composições e características desejadas para a resistência, e estabelecer estratégias de controle da doença. Os objetivos do trabalho foram: *i)* avaliar a resposta de resistência à antracnose, em frutos verdes e maduros de *Capsicum* spp., e identificar fontes de resistência para o uso em programas de melhoramento; *ii)* caracterizar a quantidade de metabólitos secundários produzidos por frutos de *C. annuum* em resposta à antracnose e à maturação dos frutos; *iii)* analisar o transcriptoma de frutos de *C. annuum* em dois estádios de maturação (verde e maduro) durante a interação com *C. gloeosporioides*; e *iv)* avaliar o perfil transcricional de genes reprimidos e induzidos em frutos de pimenta sob a colonização do fungo. Para tanto, frutos verdes e maduros de 59 acessos de *Capsicum* spp. foram avaliados quanto a severidade dos sintomas da antracnose aos 35 e 50 dias após a antese. Dentre esses, dois acessos contrastantes de *C. annuum* (resistente e suscetível) foram selecionados para a caracterização dos seus respectivos metabólitos secundários quantificados por cromatografia líquida de ultra-alta eficiência (UHPLC-PDA). O acesso resitente foi utilizado no sequenciamento de mRNA (RNA-seq) para a identificação de genes diferencialmente expressos em resposta à infecção por *C. gloeosporioides* (24, 48, 72 e 96 horas após a inoculação). Genes envolvidos na biossíntese do capsidiol foram selecionados e seus perfis de transcritos foram validados por RT-qPCR em frutos de pimenta durante a interação com o fungo. Os resultados do trabalho apresentaram uma ampla variabilidade na resposta dos acessos de *Capsicum* spp. à infecção e um comportamento diferenciado destes frente aos estádios de maturação dos frutos. Frutos maduros apresentaram maior resistência à antracnose em relação aos frutos verdes. Seis acessos foram considerados resistentes à doença em ambos os estádios de maturação dos frutos. A concentração do perfil dos metabólitos secundários apresentou alteração em relação ao estádio de maturação e em resposta à resistência dos frutos de *C. annuum* ao fungo. Da mesma forma, os dados gerados por RNA-seq revelaram um aumento de transcritos relacionados à resposta de defesa em frutos maduros. Além disso, foi gerado um painel de genes candidatos relacionados às diversas vias metabólicas da pimenta, principalmente aquelas envolvidas na resistência contra *C. gloeosporioides*. Os resultados desse estudo permitiram um maior conhecimento sobre os compostos metabólitos e os genes possivelmente relacionados com a resistência de pimenta à antracnose e fornecem uma base para o desenvolvimento de melhores estratégias para controlar a doença em futuros programas de melhoramento.

Palavras-chave: Antracnose. Capsidiol. Compostos fenólicos. Fitoalexinas. Maturação de frutos. Pimentas. Pimentão. 3’ RNA-seq. RT-qPCR. UHPLC.

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ABSTRACT

Anthracnose, caused by *Colletotrichum* spp., is one of the major disease causing losses in chili and sweet peppers fruit production during pre- and post-harvest. *Capsicum* spp. have defense mechanisms responses against pathogens attack by the production of specialized constitutive or induced compounds that are accumulate in the pathogen infection sites. However, the spectrum of action of these compounds remains poorly understood mainly during pepper fruit development stages. In this way, the elucidation of biochemical and molecular mecanisms involved in the resistance response during fungus infection might contribute to the development of *Capsicum* genotypes with characteristics desired to improve fruits resistance and to establish disease control strategies. The objectives of this study were: *i)* to evaluate fruit resistance response to anthracnose disease in unripe and ripe fruits of *Capsicum* spp. and identify sources of resistance for breeding purposes; *ii)* to investigate the concentration of secondary metabolites produced by *C. annuum* fruits in response to anthracnose disease; *iii)* to analyze the transcriptome of *C. annuum* fruits in two development stages (unripe and ripe) during the interaction with *C. gloeosporioides*; and *iv)* to analyse the transcriptional profile of genes down- and up-regulated in pepper fruits in response to fungus colonization. We evaluated the severity symptons of 59 accessions of *Capsicum* spp. with fruits at 35 and 50 days after anthesis. Among them, two contrastants accessions of *C. annuum* (resistant and susceptible) were selected for secondary metabolites characterization quantified by ultra-high performance liquid chromatography (UHPLC-PDA). The resistant accession was used for mRNA sequencing (RNA-seq) in order to identify differentially expressed genes in response to *C. gloeosporioides* infection (24, 48, 72 and 96 hours post inoculation). Genes involved in capsidiol biosynthesis were selected and their transcriptional profiles were validated by RT-qPCR in pepper fruits in response to fungus colonization. Our results showed a wide variability in the *Capsicum* accessions responses and differentiated behavior against *C. gloeosporioides* infection were observed in both fruit development stages. Ripe fruit showed greater resistance to anthracnose in relation to unripe fruit. Six accessions were classified as disease resistant in both fruit development stages. The concentration of the secondary metabolites profile was different in relation to fruit development stage and intensity of resistance response of *C. annuum* fruits during fungus interaction. Further, the data generated by RNA-seq revealed an increasing of transcripts related to defense mechanisms in ripe fruits. In addition, a panel of candidate genes related to metabolic pathways of pepper was generated, especially those relevant for the improvement of resistance against this fungus. The results of this study improved our knowlegde about secondary metabolities and genes candidates related to pepper resistance against anthracnose and provide a basis to develop better strategies to control this disease in future breeding programs.

Keywords: Anthracnose. Capsidiol. Fruit ripening. Pepper. Phenolic compounds. Phytoalexins. 3' RNA-seq. RT-qPCR. UHPLC.

SUMÁRIO

1 INTRODUÇÃO

Capsicum é um gênero altamente diversificado pertencente à família Solanaceae, no qual estão inseridos os pimentões e as pimentas. Esse gênero possui elevada importância no cenário econômico das olerícolas, em razão principalmente da versatilidade de suas aplicações, além da grande variedade de seus produtos e subprodutos, usos e formas de consumo. Esse gênero compreende 38 espécies (USA-ARS, 2011), das quais apenas cinco são cultivadas para fins comerciais: *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. pubescens* Ruiz et Pavon e *C. baccatum* L.

Apesar dos avanços tecnológicos nos sistemas de produção de espécies domesticadas de *Capsicum*, os patógenos vêm sendo considerados um dos principais entraves para aumento da produtividade e qualidade dos frutos. Entre as várias doenças relatadas, a antracnose (*Colletotrichum* spp.) causa extensivas perdas pré e pós-colheita em plantas de pimentas e pimentões. Esta doença é considerada de etiologia complexa, pois diferentes espécies de *Colletotrichum* ocasionam doenças em distintos órgãos do mesmo hospedeiro. Dentre as espécies, *C. acutatum* e *C. gloeosporioides* infectam os frutos das pimenteiras em todos os estádios de maturação. Os sintomas típicos nos frutos são lesões deprimidas de formato circular com anéis concêntricos de acérvulos e com produção de massas de conídios rosadas ou alaranjadas.

Várias estratégias têm sido propostas para o controle da antracnose em *Capsicum*, tais como a utilização de sementes livres do patógeno, a rotação de culturas com espécies não hospedeiras, a eliminação de hospedeiros alternativos e de restos culturais, e a aplicação de fungicidas químicos e/ou biológicos. Contudo, o uso de cultivares resistentes é considerado o método mais efetivo de controle, pois além de diminuir as perdas, há um menor gasto com o uso de químicos e mão-de-obra no controle da doença.

Programas de melhoramento vêm sendo desenvolvidos visando à identificação e incorporação de genes relacionados com a resistência a doenças em pimentas e pimentões. Fontes de resistência a *Colletotrichum* spp. em acessos de *C. chinense* e *C. baccatum* já foram identificados. No entanto, estudos de patogenicidade evidenciaram reações diferenciais de resposta nos estádios de maturação dos frutos (verdes e maduros) quando um isolado de *Colletotrichum* foi inoculado em um mesmo genótipo de pimenta. Alguns isolados do fungo foram patogênicos em frutos verdes dos genótipos resistentes ‘PBC932’ (*C. chinense*), ‘PBC80’ e ‘PBC81’ (*C. baccatum*), mas não foram patogênicos nos frutos

maduros, sugerindo que a expressão dos genes de resistência pode ser diferencialmente modulada de acordo com os estádios de maturação dos frutos (TAYLOR et al., 2007).

Nesse contexto, a quantificação dos metabólitos secundários envolvidos nos mecanismos de defesa da planta contra a antracnose e a elucidação da regulação da expressão de genes sob o ataque do fungo, podem favorecer uma maior compreensão sobre os mecanismos de resposta de resistência dos frutos de *Capsicum*, nos diferentes estádios de maturação. Informações sobre os compostos químicos relacionados com a resistência à antracnose em *Capsicum* spp. ainda são escassos. Há relatos de que os níveis de compostos fenólicos na interação *C. annuum* x *C. gloeosporioides* sofreram distintas alterações, atuando como fitoalexinas e fitoanticipinas no mecanismo de defesa contra a antracnose (PARK et al., 2012). Observou-se também que a concentração de polifenóis torna-se intensa próxima ao tecido infectado por *C. coccodes*, o que dificulta a colonização do fungo para o tecido saudável de *C. annuum* (MIKULIC-PETKOVSEK et al., 2013).

Nos últimos anos, as novas tecnologias de sequenciamento do transcriptoma têm sido usadas como ferramentas poderosas para a identificação e caracterização de genes expressos em espécies de *Capsicum* (LIU et al., 2013; AHN et al., 2014; MARTÍNEZ-LÓPEZ et al., 2014). A combinação de informações fenotípicas, bioquímicas e genéticas pode auxiliar os melhoristas no desenvolvimento de cultivares resistentes e na elaboração de estratégias de controle do patógeno.

Os objetivos gerais do trabalho foram identificar fontes de resistência a *C. gloeosporioides*, bem como identificar compostos bioquímicos e mecanismos moleculares envolvidos na resistência em frutos de *Capsicum* em dois estádios de maturação. Os objetivos específicos foram: *i)* avaliar a resposta de resistência à antracnose (*C. gloeosporioides*), em frutos verdes e maduros de *Capsicum* spp., e identificar fontes de resistência para o uso em programas de melhoramento; *ii)* caracterizar os metabólitos secundários e correlaciona-los com os diferentes perfis de resistência à antracnose em frutos de *C. annuum*; *iii)* analisar o transcriptoma no patossistema *C. gloeosporioides* e frutos resistente de *C. annuum*, por RNA-seq, em dois estádios de maturação; e *iv)* validar o perfil transcricional de genes de frutos de pimenta, envolvidos na biossíntese de capsidiol (fitoalexina), por RT-qPCR.

2 REVISÃO DE LITERATURA

2.1 *CAPSICUM*

2.1.1 Aspectos Morfológicos

Capsicum é um gênero altamente diversificado, no qual estão inseridos os pimentões e as pimentas, sendo amplamente cultivados tanto em regiões tropicais e subtropicais. Sua provável origem é a América Central e do Sul (DJIAN-CAPORALINO et al., 2007), e acredita-se que esse gênero possui duas áreas de origem, uma chamada de centro primário e, posteriormente, introduzida a outras regiões chamadas de centros secundários. O Brasil é considerado centro secundário de diversidade deste gênero (DIAS et al., 2013). Após a colonização das Américas, sementes de *Capsicum* foram introduzidas em toda a Europa e, em seguida, espalhadas pela África e Ásia (SAXENA et al., 2016).

As espécies desse gênero pertencem à Divisão *Spermatophyta*, Filo *Angiospermae*, Classe *Dicotiledoneae*, Ordem *Solanales* e Família *Solanaceae* (ANDREWS, 1995). As plantas de pimentas são perenes e podem ser cultivadas durante todo o ano. As flores são hermafroditas, favorecendo a autopolinização, embora a taxa de polinização cruzada possa ser elevada, dependendo da ação de insetos polinizadores (FILGUEIRA, 2000). O genoma diploide de espécies domesticadas de *Capsicum* é constituído por n=12 cromossomos com um tamanho de genoma haplóide estimado em 3,0-3,6 Gb (MOSCONE et al., 2003; KIM et al., 2014; QIN et al., 2014).

Trinta e oito espécies de *Capsicum* foram descritas (USA-ARS, 2011), das quais apenas cinco são consideradas domesticadas (CARRIZO et al., 2013). Dentre as espécies domesticadas encontram-se: *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. pubescens* Ruiz et Pavon e *C. baccatum* L. (DeWITT & BOSLAND, 2009).

As espécies de *Capsicum* também são organizadas em três complexos baseados nas análises citogenéticas e de fertilidade cruzada. O complexo *C. annuum* contém *C. annuum*, *C. chinense*, *C. frutescens* e *C. galapagoense* Hunziker. As três primeiras espécies estão integradas por características morfológicas, derivadas de progenitores silvestres de espécies distintas. Essas espécies são potencialmente cruzadas com facilidade (ONUS & PICKERSGILL, 2004) e possuem a capacidade de produção de híbridos interespecíficos (HILL et al., 2013). O complexo *C. baccatum* é composto por *C. baccatum*, *C. praetermissum* Heiser et Smith e *C. tovarii* Eshbaugh, Smith et Nickrent. O terceiro complexo denominado

C. pubescens é constituído por *C. pubescens*, *C. cardenasii* Heiser et Smith e *C. eximium* Hunziker (WALSH & HOOT, 2001).

2.1.2 Importância Econômica

Pimentas e pimentões são considerados a primeira especiaria utilizada pelos seres humanos (PERRY et al., 2007), sendo amplamente consumidos no mundo devido aos atributos sensoriais (cor, pungência e aroma) e nutracêuticos (HOWARD et al., 2000; MENICHINI et al., 2009). A perspectiva do mercado de espécies de *Capsicum* é praticamente ilimitada, devido à versatilidade de suas aplicações culinárias, industriais, farmacêuticas e ornamentais. Além de ser segmentada e diversa, possuir uma grande variedade de produtos e subprodutos, usos e formas de consumo (BENTO et al., 2007; SUDRÉ et al., 2010).

Apesar da sua importância, os dados estatísticos de produção e comercialização de pimentas no Brasil e no mundo são escassos e, geralmente, apresentam-se em conjunto com pimentão, dificultando o entendimento das perspectivas para esse mercado específico (RUFINO & PENTEADO, 2006). Além disso, grande parte da produção é comercializada em mercados regionais e locais, e estes não fazem parte das estatísticas (DOMENICO et al., 2010). Dentre as espécies domesticadas de *Capsicum*, a espécie *C. annuum* é considerada a mais importante, sendo a quarta maior olerícola cultivada globalmente, com cerca de 400 variedades (SAXENA et al., 2016).

Segundo dados da FAOSTAT (2014), pimentas e pimentões *in natura* e desidratados foram estimados em 34 milhões de toneladas, em uma área total cultivada de 3,9 milhões de ha, com base nos dados de 2012. A Ásia é o maior produtor mundial *in natura*, com 66%, seguidos pela América e Europa com 13 e 9 %, respectivamente (FAOSTAT, 2014). Por outro lado, a India tem sido o principal produtor e exportador de pimenta desidratada no mundo com aproximadamente 21% de produção (FAOSTAT, 2014). Outros importantes produtores de pimentas são a China, México, Peru, Turquia, Tailândia, Indonésia, seguidos por países em regiões tropicais da África, principalmente Gana e Etiópia (SAXENA et al., 2106).

O agronegócio brasileiro de *Capsicum* ocupa uma área em torno de 12 mil ha, com produção de mais de 348 mil toneladas de frutos por ano (RIBEIRO et al., 2008). A crescente demanda tem impulsionado o aumento de área cultivada e o estabelecimento de agroindústrias (PANORAMA RURAL, 2006; RÊGO et al., 2011). O cultivo de pimenta ocorre praticamente em todas as regiões do Brasil e se ajusta ao modelo de agricultura

familiar e de integração do pequeno agricultor-agroindústria (REIFSCHNEIDER & RIBEIRO, 2008). Os principais estados produtores são Minas Gerais, Goiás, São Paulo, Ceará e Rio Grande do Sul (RUFINO & PENTEADO, 2006).

2.1.3 Importância Nutricional

Em relação aos aspectos nutricionais, um largo espectro de compostos antioxidantes está presente nos frutos de *Capsicum*, como por exemplo, a vitamina C, os carotenóides e a vitamina E. Também estão presentes as vitaminas do complexo B (tiamina, riboflavina, niacina, B-6 e ácido fólico), vitamina A, tocoferóis, flavonóides e capsaicinóides (WAHYUNI et al., 2013). É interessante ressaltar que frutos frescos de pimenta contém mais vitamina C do que nas frutas cítricas, enquanto frutos de pimenta vermelha apresentam um maior conteúdo de vitamina A do que o encontrado em cenouras (SAXENA, et al., 2016). Os frutos desse gênero possuem também alto teor de ferro, potássio e magnésio. Além disso, o consumo de pimentas pode estimular o sistema imunológico e diminuir os níveis de colesterol (MATERSKA & PERUCKA, 2005; SUN et al., 2007).

Os frutos são ainda fontes importantes de fibras, elementos essenciais no processo de digestão e prevenção de problemas intestinais, como úlcera gástrica (REIFSCHNEIDER, 2000). A aplicação farmacêutica da capsaicina tem atraído considerável atenção nas últimas décadas devido às suas propriedades quimioprotetoras contra certas doenças. Esse composto possui propriedades antioxidantes, e atuam na prevenção de doenças cardiovasculares, câncer, Alzheimer, diabetes e doenças degenerativas, quando ingeridos diariamente em quantidades adequadas (BAE et al., 2012; SIDDIQUI et al., 2015). Eles também podem ser usados como agentes anti-artrítico, anti-inflamatório e até mesmo como antídoto no caso de envenenamento. Outra forma de aplicação da capsaicina está na preparação de *sprays* de pimenta e na preparação de produtos naturais e pesticidas orgânicos (WELBAUM, 2015).

A característica pungência é exclusiva do gênero *Capsicum*, e é atribuída à presença de capsaicinóides, sendo a capsaicina e a diidrocapsaicina as moléculas predominantes, representando cerca de 90% do total de pungência (GIUFFRIDA et al., 2013). Esses alcalóides acumulam-se na superfície da placenta e são liberados quando o fruto sofre qualquer dano físico (CARVALHO et al., 2003). O teor de capsaicinóides pode ser avaliado pela escala de *Scoville Heat Units* (SHU), e sua concentração nos frutos é altamente variável, podendo ser categorizados em não pungente (pimentas doces), baixo, leve e altamente

pungente (HOWARD & WILDMAN, 2007). A variedade "Carolina Reaper" é uma das mais pungentes com cerca de 2,2 milhões de SHU (PUCKERBUTT PEPPER COMPANY, 2013).

Essa quantidade de capsaicinóides acumulada nos frutos é influenciada pelas condições ambientais, manejo da cultura, genótipo e do estádio de maturação do fruto (REYES-ESCOGIDO et al., 2011). A maturação do fruto influência na qualidade composicional, uma vez que, durante a maturação várias modificações (bioquímicas, fisiológicas e estruturais) podem determinar a concentração desses atributos (SIDDIQUI et al., 2013). A variação da coloração dos frutos é determinada pelo acúmulo de carotenóides específicos, como a capsantina encontrada em maior quantidade em frutos maduros e violaxantina em frutos amarelos (WAHYUNI et al., 2013).

2.2 DOENÇAS EM *CAPSICUM*

As doenças têm causado redução significativa no rendimento e na produção de plantas do gênero *Capsicum* em todo o mundo. As pimentas e pimentões podem ser atacados por várias doenças e pragas, as quais assumem diferentes graus de acomentimento, pois dependem principalmente do local e da época de plantio. Podem ocorrer desde a semeadura até a colheita e, se não controladas adequadamente, provocam perdas graves na produção.

As principais doenças em *Capsicum* podem ser causadas por bactérias, vírus, nematóides e fungos (AZEVEDO et al., 2005), e são influenciadas por falta ou excesso de fatores essenciais para o crescimento das plantas, tais como nutrientes, água e luz. No caso das pragas (insetos e ácaros) os danos podem ser diretos (danos nas raízes, caules, flores e frutos) ou indiretos (insetos vetores de doenças, em especial as viroses).

Dentre as principais doenças causadas por bactérias, pode-se citar a murcha-bacteriana (*Ralstonia solanacearum*) e a mancha-bacteriana (*Xanthomonas campestres* pv. *vesicatoria*). Os vírus de maior importância são *Potato virus* Y, agente causal do mosaico do pimentão e *Pepper yellow mosaic virus*. Os nematóides destacam-se os *Meloidogyne incognita* e *M. javanica*, e mais recentemente *M. enterolobii*, que tem causado sérios prejuízos no cultivo de pimentas (PINHEIRO et al., 2014). As doenças causadas por fungos têm destaque a murcha-de-fitóftora (*Phytophthora capsici* Leonian), o oídio (*Leveillula taurica*), e *Colletotrichum* spp., agente causal da antracnose.

2.2.1 Antracnose

A antracnose causa limitação na produção de pimenta e pimentão com grandes perdas de rendimento que podem chegar a 100% (PRUSKY, 1996). A perda pode ser alta devido à infecção do fungo nos frutos, causando severas perdas em pré-colheita de frutos verdes e maduros, e na etapa de conservação pós-colheita (GUIDARELLI et al., 2011; PEREIRA et al., 2011; PARK et al., 2012). A doença pode afetar diferentes partes aéreas das pimenteiras, no qual os frutos são os mais importantes economicamente, pois uma pequena lesão é suficiente para reduzir o seu valor de mercado, afetando diretamente o rendimento da cultura (MANANDHAR et al., 1995).

A antracnose é causada por espécies do gênero de *Colletotrichum* de fase assexuada que pertencem ao Reino *Fungi*, Filo *Ascomycota*, Classe *Coeleomycetes* de fungos imperfeitos (DEAN et al., 2012). As espécies deste gênero causam doenças em mais de 121 gêneros e 45 diferentes famílias de plantas (FARR et al., 2016). As condições ótimas para o desencadeamento bem sucedido da doença são temperaturas em torno de 27 °C e umidade relativa de 80% (ROBERTS et al., 2001).

A transmissão da doença pode ocorrer por sementes infectadas pelo patógeno, pelo vento e por respingos de água que podem espalhar facilmente os conídios do fungo de plantas infectadas para partes não infectadas (SAXENA, et al., 2016). Além disso, o solo e restos de vegetais podem conter esclerócitos do fungo como forma de sobrevivência em condições desfavoráveis, podendo também atuar na propagação da doença.

No patossistema *Colletotrichum*, diferentes espécies podem estar associadas no mesmo hospedeiro, ou uma única espécie pode infectar diferentes hospedeiros (SIMMONDS, 1965; FREEMAN et al., 1998; CANNON et al., 2000). Em *Capsicum* spp., a antracnose pode ser causada por mais de uma espécie de *Colletotrichum*, incluindo *C. acutatum* J.H. Simmonds, *C. capsici* (Syd). E.J. Butler & Bisby, *C. coccodes* (Wallr). S. Hughes e *C. gloeosporioides* Penz. (THAN et al., 2008a; MAHASUK et al., 2009; MONGKOLPORN, 2010; PARK et al., 2012).

Kim et al. (2004) relataram que diferentes espécies de *Colletotrichum* causam doenças em diferentes órgãos da planta de *Capsicum*. Por exemplo, *C. acutatum* e *C. gloeosporioides* podem infectar frutos de pimenta em todos os estádios de maturação, mas geralmente não infectam folhas ou caules, que são na maior parte infectadas por *C. coccodes*.

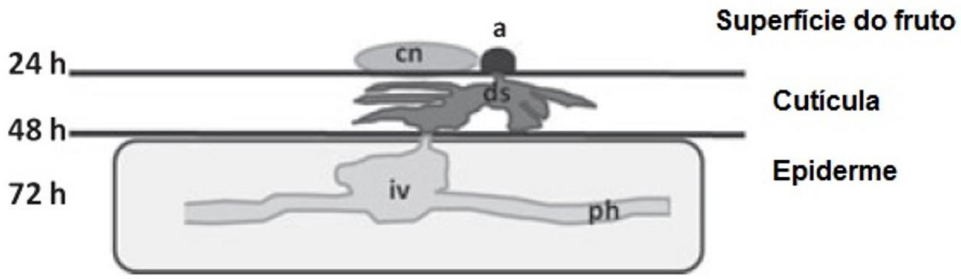
Colletotrichum gloeosporioides apresenta aproximadamente 470 espécies hospedeiras e é comercialmente importante, sendo o principal agente causal da doença em

abacate, banana, manga, café e morangos (HYDE et al., 2009). Em pimenta, essa espécie de fungo infecta os frutos em ambos os estádios de desenvolvimento (verde e maduro), causando severas perdas em pré e pós-colheita, por isso é considerada como uma das espécies mais destrutivas e amplamente distribuídas (VOORRIPS et al., 2004; BABU et al., 2011),

2.2.2 Estratégias de infecção de espécies de *Colletotrichum*

A estratégia de infecção e colonização empregada por espécies de *Colletotrichum* é descrito como hemibiotrófico, englobando tanto a fase biotrófica (adquiri nutrição diretamente das células vivas do hospedeiro) como a necrotrófica (mata as células do hospedeiro e se alimenta das células mortas) (PERFECT et al., 1999). Espécies deste gênero exibem diferentes mecanismos de infecção dependendo do hospedeiro infectado e do estádio de desenvolvimento do fruto. Geralmente, em frutos verdes ocorre a formação de apressório, hifas de penetração e quiescência, enquanto durante a maturação dos frutos o fungo desencadeia hifas secundárias e colonização no hospedeiro. Por exemplo, em frutos imaturos de morango, *C. acutatum* forma apressório na fase de quiescência, mas envolve a colonização necrotrófica em frutos maduros (GUIDARELLI et al., 2011). Do mesmo modo, foram observados processos distintos de infecção do fungo e respostas transcricionais diferenciais em frutos de tomateiro (*Solanum lycopersicum*) (ALKAN et al., 2015). A Figura 1 ilustra as estruturas formadas no processo de infecção inicial de espécies de *Colletotrichum* em frutos de *Capsicum*.

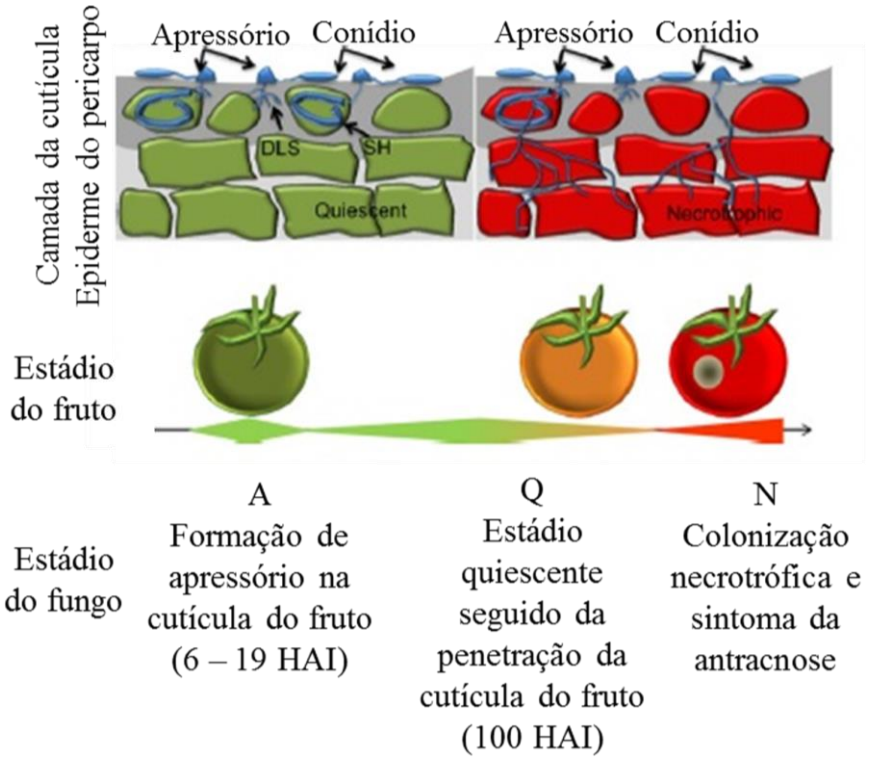
Figura 1. Processo de infecção em frutos de *Capsicum* inoculado com *Colletotrichum acutatum.* cn= conídio, a= apressório, ds= estrutura dentrítica, iv= vesícula de infecção, ph= hifa primária. Fonte: Liao et al. (2012).



Em frutos verdes, a germinação de conídios do fungo de espécies de *Colletotrichum* inicia-se em torno de 19 horas após a inoculação (HAI) com diferenciação de apressórios que penetram as células epidérmicas diretamente através da cutícula. Entre 40 e 48 HAI podem ser observados grampos de penetração do fungo e após três a cinco dias, hifas com estruturas dentríticas se formam na epiderme do fruto e estas se desenvolvem dentro das células hospedeiras vivas (fase biotrófica) (ALKAN et al., 2015). Estas estruturas especializadas do fungo permanecem em repouso (fase quiescente) por semanas até a maturação dos frutos, no qual se diferenciam em hifas longas, finas e ramificadas, com destruição do tecido hospedeiro (fase necrotrófica) e eventualmente culmina no desenvolvimento dos sintomas da doença (O'CONNELL et al., 2012; GAN e al., 2013; ALKAN et al., 2015). Quando *Colletotrichum* spp. germina na cutícula de frutos maduros de tomate, a infecção ocorre de forma mais rápida e as estruturas da fase quiescente mudam imediatamente para o crescimento necrotrófico (ALKAN et al., 2015). Todo o processo de colonização de *C. gloeosporioides* em frutos verdes e maduros de tomate descrito anteriormente pode ser observado na Figura 2.

Espécies de *Colletotrichum* desempenham reações de patogenicidade diferentes quando inoculados em frutos verdes e maduros de *Capsicum*. Alguns isolados foram patogênicos em frutos verdes, mas não patogênicos em maduros do mesmo genótipo, sugerindo que genes de resistência são diferencialmente expressos de acordo com os estádios de maturação dos frutos (KIM et al., 1999; TAYLOR et al., 2007; THAN et al., 2008b; SILVA et al., 2014; SUN et al., 2015).

Figura 2. Colonização de frutos de tomate (*Solanum lycopersicum*) verde e maduro por *Colletotrichum gloeosporioides*. Fonte: Alkan et al. (2015).



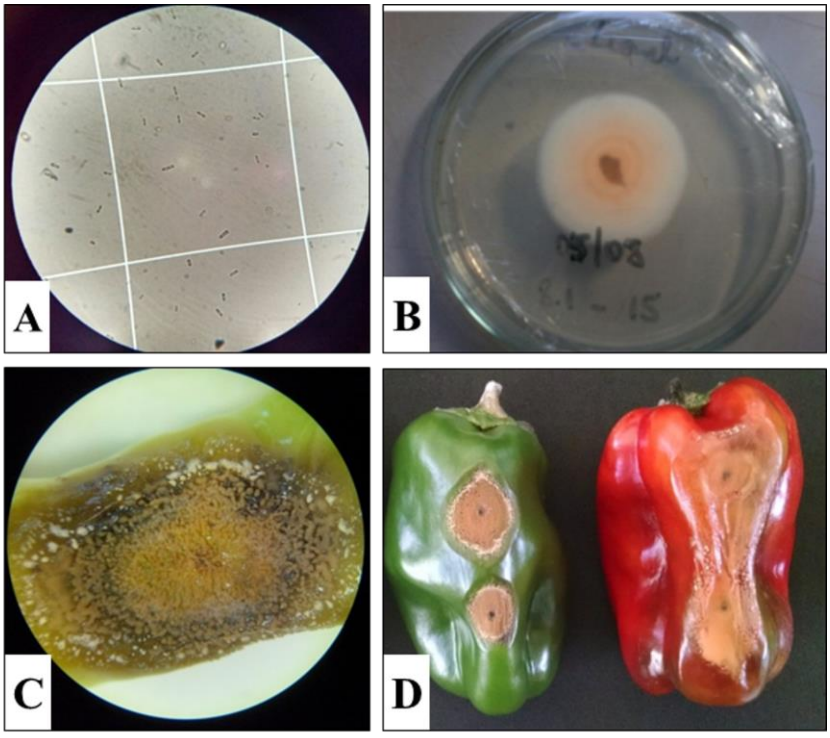
Genótipos resistentes de *C. chinense* (PBC932) e *C. baccatum* (PBC80 e PBC81) a *Colletotrichum* spp. apresentaram reações diferenciais de patogenicidade em frutos verdes e maduros (TAYLOR et al., 2007). Um resultado semelhante foi observado por Mahasuk et al. (2009), ao estudarem a herança da resistência de *C. capsici*, em diferentes estádios de maturação dos frutos, resultante do cruzamento entre *C. annuum* (suscetível) x *C. chinense* (resistente). Esses autores verificaram o envolvimento de dois genes responsáveis pela resistência nos frutos verdes e maduros, sugerindo que a mudança da maturação dos frutos pode ter desencadeado a expressão de diferentes genes.

2.2.3 Sintomas

Os sintomas de antracnose podem se manifestar nos frutos após a colheita, durante o armazenamento ou nas prateleiras, quando o produto atinge seu valor mais alto (GUIDARELLI et al., 2011). Esta manifestação dos sintomas da doença está relacionada com a capacidade de espécies de *Colletotrichum* em manter infecções latentes em frutos verdes, permanecendo em repouso até o amadurecimento dos frutos (PRUSKY, 1996).

Os sintomas característicos da antracnose em frutos de *Capsicum* spp. se iniciam com pequenas áreas circulares, deprimidas e necrosadas, que crescem rapidamente e podem atingir todo fruto. Em condições ideais de alta umidade, formam-se anéis concêntricos de acérvulos, contendo setas, coberto por uma massa de conídios alaranjados do fungo no centro das lesões (THAN et al., 2008b). Na Figura 3 estão representados os conidiósporos de *C. gloeosporioides*, colônia em meio de batata dextrose agar (BDA) e sintomas característicos da doença em frutos de *Capsicum*.

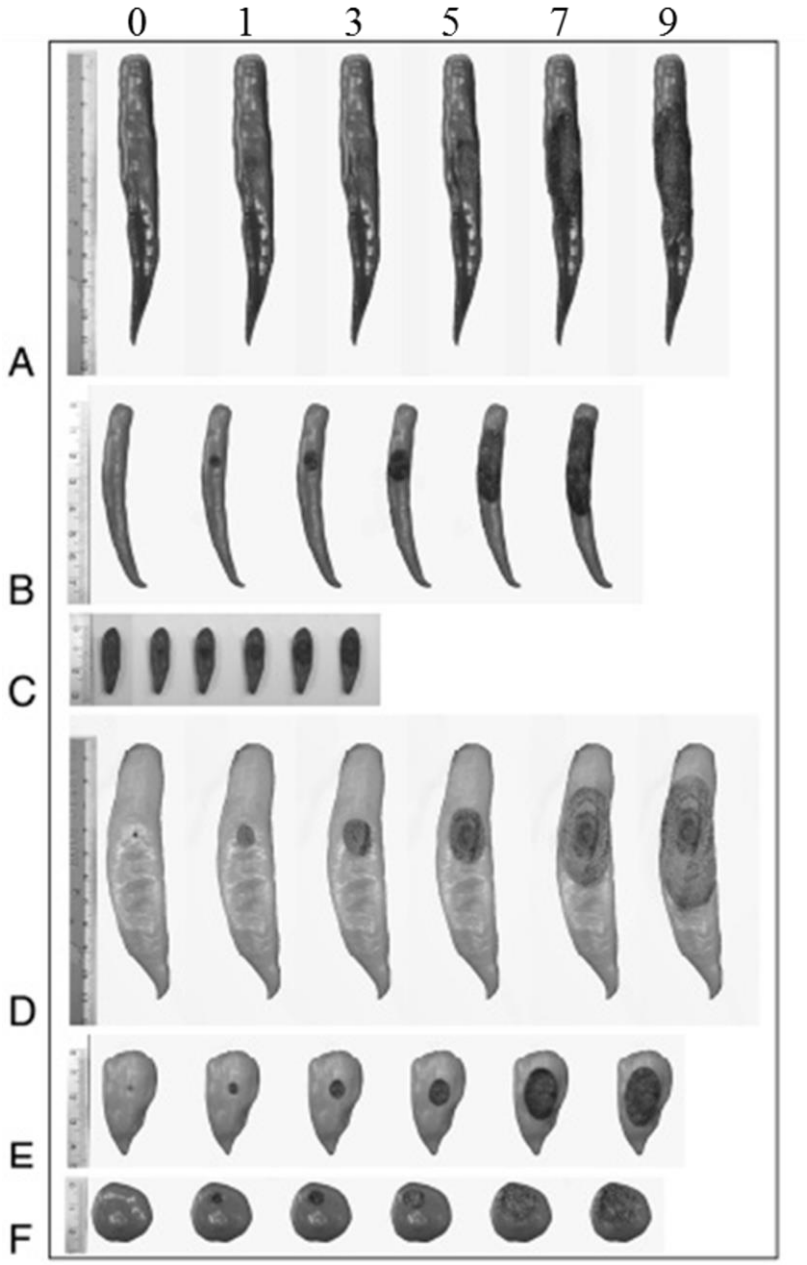
Figura 3. Morfologia e sintomas da antracnose (*Colletotrichum gloesoporioides*) em frutos de *Capsicum*. (A) Conidiósporos observados em microscopia ótica de luz (objetiva de 40x). (B) Colônia do fungo em meio batata dextrose ágar após sete dias de cultivo. (C) Fruto de *C. chinense* com sintomas da antracnose observados na lupa. (D) Sintomas característicos da doença em frutos de pimentão (*C. annuum*) verde e maduro. Fonte: própria autora.



Para a avaliação da severidade da antracnose em frutos de *Capsicum*, foi desenvolvido uma escala de notas da doença proposta por Montri et al. (2009), ilustrada na Figura 4. As notas atribuídas foram: 0– ausência de sintomas; 1– 1-2% da área do fruto com lesão necrótica ou com lesão encharcada de água em torno do local da infecção; 3– 2-5% da área do fruto com lesão necrótica, acérvulos podem estar presentes, ou lesão embebido em água em até 5% da superfície do fruto; 5– 5-15% da área do fruto com lesão necrótica,

presença de acérvulos ou lesão encharcada de água em até 25% da superfície do fruto; 7– 15-25% da área do fruto com lesão necrótica e acérvulos; 9– 25% da área do fruto com necrose, lesão muitas vezes circundando o fruto com acérvulos em abundância. Nesta escala, as notas 0 a 3 correspondem à reação de resistência de *Capsicum* a *C. capsici*, e as notas 5 a 9 reações de suscetibilidade.

Figura 4. Representação da escala de notas proposta por Montri et al (2009), na escala de 0 a 9, para caracterização da severidade dos sintomas da antracnose em diferentes espécies de frutos de *Capsicum*. (A) *C. annuum* Bang-chang; (B) *C. annuum* Jinda; (C) *C. frutescens* Kheenoo Suan; (D) *C. baccatum*; (E) *C. chinense* C04714; e (F) *C.chinense* PBC932.



2.2.4 Controle

Dentre as estratégias de controle da antracnose estão a utilização de sementes livres do patógeno, a rotação de culturas com espécies não hospedeiras, a eliminação de hospedeiros alternativos e de restos culturais de qualquer parte da planta infectada, e a aplicação de fungicidas químicos e biológicos. No entanto, o uso de cultivares resistentes e duradouras é o meio mais eficaz no controle da doença (THAN et al., 2008b; PARK et al., 2012), não apenas para reduzir os danos causados pela doença, como também para minimizar o controle químico e reduzir despesas mecânicas no controle da doença (AGRIOS, 2005).

2.3 MELHORAMENTO VISANDO A RESISTÊNCIA EM *CAPSICUM* X ANTRACNOSE

Diversos programas de melhoramento vêm sendo desenvolvidos visando à identificação e incorporação de genes de resistência as doenças em cultivares de pimenta e pimentão (PAKDEEVARAPORN et al., 2005; YOON & PARK, 2005; KIM et al., 2008; PEREIRA et al., 2011). Fontes de resistência contra a antracnose já foram identificadas em acessos de pimenta, como por exemplo, PBC932 (*C. chinense*), considerado resistente a *C. capsici*, e PBC80 e PBC81 (*C. baccatum*) resistentes a C. *gloeosporioides* (BABU et al., 2011). No entanto, até o momento, não houve sucesso no desenvolvimento de cultivares de *C. annuum* resistentes à antracnose, sendo a única espécie de *Capsicum* amplamente cultivada no mundo (PARK, 2007).

No Brasil, estudos voltados para a identificação de fontes de resistência para o uso em programas de melhoramento de plantas vêm sendo desenvolvido. Silva et al. (2014) investigaram a reação de patogenicidade de frutos verdes e maduros em uma coleção de acessos de *Capsicum* spp. a *C. gloeosporioides* durante os períodos pré e pós-colheita. Bento et al. (2017) identificaram acessos de *Capsicum* spp. com resistência à mancha bacteriana, antracnose e *Pepper yellow mosaic virus* (PepYMV), sendo o acesso de *C. annuum* (UENF 1381) resistente aos três agentes patogênicos.

Para o sucesso dos programas de melhoramento no desenvolvimento de cultivares resistentes de *Capsicum*, alguns requisitos são importantes, como o conhecimento de acessos resistentes em uma determinada região e os diferentes patótipos do patógeno encontrado nessa região. No entanto, no patossistema *Colletotrichum* spp. e frutos de *Capsicum* spp., a produção de cultivares resistentes é desafiadora, pois pode ocorrer a

associação de mais de uma espécie e também a variabilidade dentro da espécie do fungo ocasionando a doença (SAXENA, et al., 2014), juntamente com a habilidade de virulência diferencial do patógeno (MONTRI et al., 2009). Além disso, poucas fontes de resistências combinam todas as características necessárias para uma resistência completa, e a dificuldade aumenta com o aparecimento de novas raças de patógenos ou pela instabilidade dos genes de resistência em ambientes variáveis (STALL et al., 2009; TOMITA et al., 2011).

Outra informação importante é o conhecimento sobre os mecanismos de herança da resistência à antracnose, pois permite que os melhoristas decidam sobre os métodos a serem aplicados para a obtenção de cultivares resistentes. Contudo, a herança da resistência pode variar, dependendo da espécie do patógeno. Existem relatos que indicam que a resistência a *C. capsici* é controlada por um gene dominante (LIN et al., 2002) ou parcialmente dominante (PARK et al., 1990a). Para resistência a *C. gloeosporioides*, a herança foi descrita como sobredominante ou parcialmente dominante (PARK et al., 1990b). A presença de um gene recessivo controlando a resistência à *C. capsici* também já foi descrita em *C. chinense* (PAKDEEVARAPORN et al., 2005).

2.4 Compostos Bioquímicos de Defesa da Planta

As plantas são capazes de se defender dos ataques de patógenos por meio de um conjunto de mecanismos estruturais e bioquímicos pré (constitutivo) e/ou pós-formados (induzidos) (ANAND et al., 2009). Entre esses, a defesa bioquímica envolve a produção de substâncias que são potencialmente tóxicas para o agente patogênico (AHUJA et al., 2012). Esses compostos químicos, também chamados de bioativos, são provenientes do metabolismo secundário das plantas, e podem ser produzidos antes do ataque de patógenos (fitoanticipinas), ou produzidos e acumulados rapidamente em resposta ao ataque de patógenos (fitoalexinas) (MORRISSEY & OSBOURN, 1999; WITTS-TOCK & GERSCHENZON, 2002). As fitoanticipinas são sintetizadas pelas plantas durante seu crescimento e desenvolvimento, e acumuladas em tecidos específicos ou em estruturas especializadas (MORRISSEY & OSBOURN, 1999).

Os três principais grupos de compostos químicos produzidos pelas plantas para a defesa são os: compostos fenólicos, terpenóides e compostos nitrogênados (WALTERS, 2011). Os compostos fenólicos estão fortemente envolvidos na interação entre planta e patógeno. Eles são tóxicos para os organismos patogênicos e a sua produção pós-infecção e acumulação são mais intensos em cultivares de plantas resistentes do que em

suscetíveis (MIKULIC-PETKOVSEK et al., 2013). O ácido clorogênico, ácido caféico e ácido p-cumárico são exemplos de compostos fenólicos que podem inibir a germinação de esporos do fungo, o crescimento micelial, e também inativar enzimas específicas de ataque produzidas pelos patógenos (BÁIDEZ et al., 2006).

Em alguns estudos foram observados o aumento da concentração de fitoalexinas em tecidos da planta próximos à área infectada pelo patógeno, dificultando a colonização do fungo a partir das células infectadas para o tecido saudável (PARK et al., 2012; MIKULIC-PETKOVSEK et al., 2013). No entanto, o tempo da produção das fitoalexinas apresenta uma maior importância para o sistema de defesa da planta, do que a concentração final que se acumula no tecido vegetal.

Níveis de compostos fenólicos sofreram alterações em frutos de *C. annuum* infectados com o fungo *C. gloeosporioides* (PARK et al., 2012). A concentração dos compostos diminuiu rapidamente nas fases iniciais da infecção, indicando a utilização imediata desses compostos, antes mesmo do início da sua biossíntese em resposta ao ataque do patógeno. A quantidade de polifenóis aumentou gradualmente, com pico entre dois a quatro dias após a infecção, e diminuiu depois de cinco dias. Posteriormente, a diminuição progressiva dos polifenóis sugere que o equilíbrio da competição pode ter sido quebrado e que o mecanismo de defesa do fruto pode ser gradualmente vencido (PARK et al., 2012).

A produção de compostos fenólicos em frutos resistentes em momentos iniciais de infecção ajuda no processo de inibição do desenvolvimento do patógeno (KIM et al., 2004). Essa reação impede a sobrevivência e o crescimento do fungo biotrófico, mas não afeta os necrotróficos que utilizam células mortas como fonte de alimento. O'Connell et al. (2012) sugeriram que genes relacionados à patogenicidade, como enzimas de degradação da pectina e enzimas do metabolismo secundário, são induzidas antes da penetração e durante a fase biotrófica do fungo causador da antracnose, enquanto que a maioria das hidrolases e transportadores são regulados durante a fase necrotrófica.

No grupo dos terpenos, o capsidiol é considerado como a principal fitoalexina acumulada em frutos de pimentas maduras infectadas com *C. gloeosporioides*. A produção de capsidiol ocorre em torno do local de infecção e desempenha um papel de defesa contra agentes patogênicos (PARK et al., 2014; LEE et al., 2017).

Entre os compostos nitrogenados, a capsaicina presente em frutos de pimentas induz a expressão de genes relacionados com a defesa da planta aumentando as propriedades antimicrobianas e antifúngicas (TEWKSBURY et al., 2008; VELOSO et al., 2014). Um estudo relatou que o aumento de enzimas relacionadas com síntese de capsaicina

pode estar envolvido com as respostas de defesa contra um vírus em *C. annuum* (WIDANA GAMAGE et al., 2016). No entanto, não há estudos que relatam a relação de frutos de pimentas pungentes com a resistência contra patógenos.

Ko et al. (2016) sugeriram que a interação antagonista entre o ácido salicílico (SA) e o ácido jasmônico (JA)/ etileno (ET) é considerada um gatilho para desencadear respostas eficientes de defesa em células de plantas contra o fungo causador da antracnose. A defesa efetiva contra patógenos durante a interação biotrófica envolve a morte celular programada no hospedeiro e a ativação de respostas de defesa reguladas por vias dependentes do SA (GLAZEBROOK, 2005). Em contrapartida, após o desenvolvimento da fase necrotrófica, o patógeno secreta ativamente toxinas para matar o tecido hospedeiro, no qual um conjunto de diferentes respostas de defesa é ativado, incluindo o JA e vias de sinalização do ET. Ambos os tipos de respostas envolvem a ativação transcricional de um conjunto específico de genes relacionados à defesa, incluindo proteínas relacionadas à patogênese (PR).

As proteínas PR têm sido induzidas após a infecção por vários tipos de patógenos (oomicetos, fungos, bactérias e vírus), bem como pelo ataque de insetos, em muitas famílias de plantas (VAN LOON et al., 2006). Essas proteínas são comuns e foram classificadas em 17 famílias (VAN LOON et al., 2006). Em frutos de pimentas, os genes PR-3 *chitinase* (HONG et al., 2000) e PR-5 *thaumatin-like protein* (KIM et al., 2002) foram identificados como marcadores de defesa em resposta à infecção pela antracnose.

Outro gene identificado é o *pepper esterase* (*PepEST*), que está envolvido na hidrólise da camada externa das paredes celulares de *C. gloeosporioides*. Sua acumulação foi mais intensa na área de infecção de frutos maduros de *C. annuum* (SEO et al., 2017). Os potenciais efeitos antifúngicos de *PepEST* foram avaliados *in vitro* e evidenciaram a inibição do crescimento de patógenos fúngicos (KIM et al., 2001) e, mais recentemente, usando plantas transgênicas que superexpressaram *PepEST* na presença do fungo causador da antracnose (CHO et al., 2011; KO et al., 2016).

Lee et al. (2017) sugeriram que a energia do metabolismo primário é desviada e pode ser usada na ativação de respostas de defesa, como a acumulação de alguns carboidratos, aminoácidos e ácidos graxos. Essa acumulação reage positivamente na expressão de genes relacionados na resistência contra patógenos, para cascatas de sinalização de defesa envolvendo SA, JA, genes PRs, elicitação de respostas de hipersensibilidade (HR) e precursores de defesa mediados por espécies reativas de oxigênio (ROS).

2.5 TRANSCRIPTOMA

Com os avanços recentes das tecnologias de sequenciamento de alto rendimento, a custos acessíveis, diversos estudos com montagem de genomas completos e análises globais de transcriptomas têm sido disponibilizados. Com isso, diferentes genomas de espécies de plantas e de fungos fitopatogênicos já foram sequenciados nos últimos anos. Em *Capsicum* spp. dois genomas em *C. annuum* estão disponíveis em banco de dados públicos (KIM e al., 2014; QIN et al., 2014). Ambos os materiais sequenciados são portadores de múltiplas resistências a patógenos, possuem altos teores de pungência e apresentaram tamanho próximo de 3 Gb.

Nos últimos anos, as tecnologias de sequenciamento de mRNA (RNA-seq) têm sido utilizadas como ferramentas poderosas para a identificação e caracterização de genes relacionados à expressão tecido-específica (VORHÖLTER, 2013). Dentre as técnicas para análises de expressão gênica, RNA-Seq gera sequências a partir de amostras de RNAs obtidas em um estágio específico do desenvolvimento da planta ou condição fisiológica específica, compreendendo o conjunto completo de transcritos daquele momento biológico. Esta ferramenta possibilita a expressão dos níveis de RNA, com o benefício adicional de que todo o transcriptoma é analisado sem qualquer conhecimento *a priori* das regiões transcritas (WILHELM & LANDRY, 2009). O transcriptoma pode variar dependendo do tipo de célula, bem como em resposta a estímulos externos e internos durante o desenvolvimento do tecido biológico analisado (MARTÍNEZ-LÓPEZ et al., 2014).

Recentes métodos de RNA-seq têm sido desenvolvidos, incluindo 3’ RNA-seq, no qual gera sequências próximas da extremidade 3’ de RNAs poliadenilados. Essa metodologia pode determinar a expressão gênica de espécies com precisão e ao menor custo quando comparado com outros métodos. Além disso, a normalização do tamanho das sequências não é necessária, uma vez que o método 3’ RNA-seq gera apenas um fragmento por transcrição, no qual a quantificação da expressão gênica é baseada (MOLL et al., 2014). Este método ignora a limitação do método RNA-seq tradicional que resulta em uma estimativa tendenciosa dos níveis de expressão (TANDONNET et al., 2017). Estes mesmos autores confirmaram que o método 3’ RNA-seq representa uma ferramenta válida para investigar genes diferencialmente expressos (DEGs) e também pode se tornar um método de escolha para estudos de RNA-seq usando sequências de transcrição completa. Além disso, os autores sugerem que, para uma espécie modelo com um genoma disponível e bem anotado, o método 3’ RNA-seq pode apresentar um maior poder de detecção de DEGs e produzir níveis

de expressão mais precisos. Para uma espécie não modelo com pouca informação genômica disponível, o método tradicional de RNA-seq apresenta um melhor custo-benefício, pois garante informações mais completas sobre as transcrições. Apesar de 3’ RNA-seq ser um método recente, alguns estudos de expressão gênica foram realizados tanto em plantas, como voltados para a saúde humana (FORMENT et al., 2017; ILIAS et al., 2017).

Alguns trabalhos de transcriptoma em espécies de *Capsicum* já foram realizados. Góngora-Castillo et al. (2012) observaram diferenças nas expressão de genes em folhas de pimentas infectadas por *Pepper golden mosaic virus* (PepGMV); Liu et al. (2013) analisaram transcritos da placenta e do pericarpo de *C. frutescens* relacionados à identificação de genes envolvidos na biossíntese de capsaicinas; Ahn et al. (2014) analisaram o transcriptoma com o intuito de identificar novos genes, os quais podem ser úteis para o mapeamento genético e para o desenvolvimento de novas variedades.

Martínez-López et al. (2014) estudaram o transcriptoma de frutos ao longo da maturação dos frutos. Genes relacionados com a biossíntese de capsaicinóides e ácido ascórbico foram superexpressos em 20 dias após a antese (DAA), enquanto os relacionados com a biossíntese de carotenóides foram altamente expressos nos frutos com 40-60 DAA (maduro). Esse período foi caracterizado por uma diminuição global na expressão dos genes, o que indica o fim da maturação e o início da senescência dos frutos de pimenta.

Transcriptoma de espécies de *Colletotrichum* já foram estudados com foco no estilo de vida do patógeno e a sua patogenicidade (O’CONNELL et al., 2012; ALKAN et al., 2013; GAN et al., 2013). Alkan et al. (2015) analisaram simultaneamente o transcriptoma de *C. gloeosporioides* em frutos de tomate durante diferentes fases de infecção do fungo. Esse trabalho apresentou estratégias de patogenicidade e virulência (ataque) do fungo, assim como mecanismos de defesa do fruto. Embora um estudo de transcriptoma durante o amadurecimento de frutos de *Capsicum* já tenha sido realizado (MARTÍNEZ-LÓPEZ et al., 2014), os mecanismos de resposta de defesa dos frutos contra a infecção por espécies de *Colletotrichum* ainda permanecem mal compreendidos.

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3. ARTIGO A: *Capsicum-Colletotrichum* INTERACTION: IDENTIFICATION OF RESISTANCE SOURCES AND QUANTIFICATION OF SECONDARY METABOLITES IN UNRIPE AND RIPE FRUITS IN RESPONSE TO ANTHRACNOSE INFECTION (submitted to PloS One)

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Abstract

Anthracnose, caused by *Colletotrichum* species complex, is one of the main fungal diseases in pepper (*Capsicum* spp.) crops, resulting in extensive fruit losses during pre- and post-harvesting. Plants have structural and biochemical defense mechanisms produced before and/or after the pathogen attack. Biochemical defense involve the production of compounds that accumulate at the site of infection and are toxic to the pathogen. However, the accumulation and biosynthesis of these compounds during *Capsicum-Colletotrichum* interaction, especially during fruit development stages remain poorly understood. In order to identify potential resistant genotypes and to improve our knowledge about the metabolites produced by pepper fruits against fungus infection, we inoculated unripe and ripe fruits of 59 accessions of *Capsicum* spp. with *C. gloeosporioides* and analyzed the disease severity during 8 days after inoculation. In this study, we observed a wide variability of fungus resistance

response in unripe and ripe fruits of *Capsicum* spp. accessions, and ripe fruits presented greater resistance to anthracnose than unripe. Six accessions (GBUEL06, GBUEL28, GBUEL73, GBUEL87, GBUEL104 and GBUEL106) were considered as resistant for both fruits development stages and have potential to be used in future breeding programs. In addition, we selected two *C. annuum* accessions GBUEL103 (susceptible) and GBUEL104 (resistant), to describe the histological aspects of *C. gloeosporioides* infection in pepper fruits, followed by the quantification of secondary metabolites produced during this plant-pathogen interaction, using light microscopy and ultra-high performance liquid chromatography (UHPLC), respectively. The quantification of secondary metabolites produced in pepper fruits during fungus infection showed different values according genotype characteristic (susceptible or resistant), fruit development stages (unripe and ripe) and time (1^{st} and 8^{th} day post-inoculation). Interestingly, high concentrations of caffeic and chlorogenic acid were quantified in unripe and ripe fruits characterized as resistant genotype, showing that these biochemical compounds are putatively involved in fruit defense mechanism in response to anthracnose disease.

Introduction

Capsicum spp. (sweet and chili pepper) is one of the most popular and widely horticultural crops cultivated worldwide, due to the great variety of products, uses and consumption forms [1]. This genus was originated from the tropical zones of South and Central America, and has 38 species described with a wide range of morphological variability, mainly observed in the fruits and related to color, size, shape and pungency levels [2] (http://www.theplantlist.org). Among these species, only five are considered as domesticated plants: *C. annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. pubescens* Ruiz et Pav. and *C. baccatum* L. (var. *pendulum*) [3].

Biotic stress represent one of the main challenges for *Capsicum* spp. production [4, 5], and the anthracnose disease, caused by *Colletotrichum* spp., is an important fungal disease that cause significant yield losses during pre- and post-harvest [6, 7, 8]. This disease has a complex etiology, since different *Colletotrichum* species can cause anthracnose in several *Capsicum* species [7, 9, 10, 11, 12]. However, *C. capsici* (Syd.) Butler & Bisby, *C. acutatum* J.H. Simmonds and *C. gloeosporioides* Penz species are the most destructive and widely distributed [4, 8, 13].

Colletotrichum spp. are considered hemibiotrophic fungi, showing an initial biotrophic phase of infection in the plant host tissues and a second phase denominated necrotrophic, that culminates with the destruction of tissues and the emergence of symptoms [14]. *Colletotrichum* species infect plants through spore germination, appressoria formation, followed by hyphal penetration in the epidermis host [14, 15]. The disease symptoms vary according to the plant part affected, causing seedlings tumbling, dark circular spots in the leaves, stem necrosis, and fruit lesions [13]. However, the most common and economically important damages caused by anthracnose occur in the fruit, in the form of dark, depressed and necrotic lesions with concentric acervuli rings, leading to pre and post-harvest rottenness [13, 16].

Different management strategies have been used to control anthracnose in *Capsicum* spp., such as adoption of different crop management (adequate soil drainage, crop rotation, and removal parts of infected plants in the field), biological and/or chemical control agents, and the use of resistant genotypes [8, 13]. However, the most economically and environmentally sustainable strategy is the development of resistant cultivars, since this strategy not only minimizes the losses caused by the disease, but also reduces the chemical and mechanical expenses used to control the disease [16].

The main source of anthracnose resistant have been identified in *C. chinense* and *C. baccatum* [17, 18, 19], and these species have been used in inheritance studies [20, 21, 22, 23]. However, the genetic control of resistance to *Colletotrichum* spp. is highly dependent on a series of factors such as the pathogen/pathotypes species, the source of resistance, the inoculation method and the fruit development stages [22, 24]. Pathogenicity studies showed different reactions in unripe and ripe fruits when the same isolate of *Colletotrichum* was inoculated in the same pepper genotype [9, 22].

Plant genetic resistance against pathogens has been developed through a pre-existent (constitutive) and induced defense system. Constitutive defenses are characterized by physical structures and chemical compounds denominated phytoanticipins [16]. The induced defense responses are triggered after the recognition of a wide range of compounds denominated elicitors, which can lead to hypersensitive responses, phytoalexins production, lignification and cell wall strengthening, reactive oxygen species (ROS) production, and biosynthesis of pathogenesis-related proteins (PRs) [25].

Information about the chemical compounds that confers resistance characteristic to the *Capsicum* spp. against anthracnose disease remain poorly understood [4, 7]. In the *C. annuum*

x *C. gloeosporioides* interaction, it was already reported that the phenolic compounds act as phytoalexins and phytoanticipins in the plant defense mechanism against anthracnose [7]. Moreover, the concentration of polyphenols becomes intense in the bordering of *C. annuum* tissue infected by *C. coccodes*, making fungus colonization restricted to spread from the infected cells into the healthy tissue [26].

In this study, we classified 59 accessions of *Capsicum* spp. (unripe and ripe fruits) as being susceptible or resistant genotypes against anthracnose disease based on their symptoms. The histological analysis of the *C. gloeosporioides* infection process showed distinct fungal structures in unripe and ripe fruits of susceptible (GBUEL103) and resistant (GBUEL104) *C. annuum* accessions. In addition, we characterized the quantity of 19 secondary metabolites produced by susceptible and resistant pepper fruits during fungus infection. Interestingly, that was a pattern for the resistant genotypes, all of them showed high amounts of caffeic and chlorogenic acids.

Material and Methods

Plant material

We used in this study unripe (35 days after anthesis - DAA) and ripe (50 DAA) fruits of 59 *Capsicum* spp. accessions (53 of *C. chinense*, four *C. baccatum* var. *pendulum* and two *C. annuum*), from the Universidade Estadual de Londrina (UEL) gene bank were used in this study in response to *C. gloeosporioides* infection (S1 Table).

S1 Table. Identification and origin of 59 *Capsicum* spp. accessions from Universidade Estadual de Londrina (UEL) gene bank.

Accessions	Origin	Species	Accessions	Origin	Species
GBUEL02	Mato Grosso	*C. chinense*	GBUEL68	Maranhão	*C. chinense*
GBUEL05	Mato Grosso	*C. chinense*	GBUEL69	Maranhão	*C. chinense*
GBUEL06	Mato Grosso	*C. chinense*	GBUEL85	Maranhão	*C. chinense*
GBUEL11	Mato Grosso	*C. chinense*	GBUEL86	Maranhão	*C. chinense*
GBUEL14	Mato Grosso	*C. chinense*	GBUEL87	Maranhão	*C. chinense*
GBUEL15	Mato Grosso	*C. chinense*	GBUEL95	Maranhão	*C. chinense*
GBUEL25	Mato Grosso	*C. chinense*	GBUEL42	Bahia	*C. chinense*
GBUEL26	Mato Grosso	*C. chinense*	GBUEL46	Bahia	*C. chinense*
GBUEL27	Mato Grosso	*C. chinense*	GBUEL46b	Bahia	*C. chinense*
GBUEL28	Mato Grosso	*C. chinense*	GBUEL53	Bahia	*C. chinense*
GBUEL30	Mato Grosso	*C. chinense*	GBUEL88	Bahia	*C. chinense*
GBUEL34	Mato Grosso	*C. chinense*	GBUEL89	Bahia	*C. chinense*

GBUEL72	Mato Grosso	*C. chinense*	GBUEL49	Pará	*C. chinense*
GBUEL73	Mato Grosso	*C. chinense*	GBUEL50	Pará	*C. chinense*
GBUEL74	Mato Grosso	*C. chinense*	GBUEL58	Pará	*C. chinense*
GBUEL75	Mato Grosso	*C. chinense*	GBUEL90	Pará	*C. chinense*

S1 Table continuation

Accessions	**Origin**	**Species**	**Accessions**	**Origin**	**Species**
GBUEL76	Mato Grosso	*C. chinense*	GBUEL91	Pará	*C. chinense*
GBUEL77	Mato Grosso	*C. chinense*	GBUEL92	Pará	*C. chinense*
GBUEL78	Mato Grosso	*C. chinense*	GBUEL93	Pará	*C. chinense*
GBUEL79	Mato Grosso	*C. chinense*	GBUEL94	Pará	*C. chinense*
GBUEL80	Mato Grosso	*C. chinense*	GBUEL48	Rio de Janeiro	*C. chinense*
GBUEL81	Mato Grosso	*C. chinense*	GBUEL96	Rio de Janeiro	*C. chinense*
GBUEL35	Goiás	*C. chinense*	GBUEL97	Rio de Janeiro	*C. chinense*
GBUEL47	Espírito Santo	*C. chinense*	GBUEL103	Comercial	*C. annuum*
GBUEL47b	Espírito Santo	*C. chinense*	GBUEL104	México	*C. annuum*
GBUEL82	Espírito Santo	*C. chinense*	GBUEL105		*C. baccatum* var. *pendulum*
GBUEL83	Espírito Santo	*C. chinense*	GBUEL106	Rio de Janeiro	*C. baccatum* var. *pendulum*
GBUEL84	Minas Gerais	*C. chinense*	GBUEL107	Minas Gerais	*C. baccatum* var. *pendulum*
GBUEL61	Maranhão	*C. chinense*	GBUEL108	Paraná	*C. baccatum* var. *pendulum*
GBUEL64	Maranhão	*C. chinense*			

Capsicum accessions seeds were sown in polystyrene trays, with organic plants substrates, and after the emergence of two pairs of definitive leaves, seedlings were transferred individually to plastic pots containing a mixture of soil and substrate (2:1 ratio). Plants were grown in a greenhouse following practices recommended for pepper cultivation.

Identification of resistance source in *Capsicum* species in response to *C. gloeosporioides* infection

Unripe and ripe fruits from 59 *Capsicum* spp. accessions were detached from the plant, and disinfected superficially in 1% (w/v) sodium hypochlorite solution for five min, followed by three washes with distilled water for one min [19]. Five fruits from each accession and each fruit development stages were evaluated, and one fruit was used as control (mock inoculation with sterilized distilled water).

The virulent isolate "8.1" of *C. gloeosporioides*, provided by Genetics and Plant Breeding Laboratory of Universidade Estadual do Norte Fluminense *Darcy Ribeiro* (UENF),

was grown in a potato dextrose agar (PDA) culture medium, pH 7.0, and incubated in the dark at 25 ºC, until colonies formation, to use for inoculum suspension.

Fruit inoculation was performed under laboratory conditions by the injection method [27] in the central part of the fruit using a Micro Syringe model 1705 TLL (Hamilton, Bonaduz, Switzerland). The needle depth was fixed at 1 mm to ensure inoculum volume and lesion size uniformity. Conidia suspension was prepared minutes before inoculation at a concentration of 1 x 10^6 conidia.mL^{-1}, adjusted by counting in a *Neubauer* chamber. After inoculation, *Capsicum* spp. fruits were incubated in the dark for 24 h at 25 ºC. The subsequent cycle of photoperiod was settled in 12 h light/dark, and fruits were kept in a humid chamber.

Anthracnose severity in *Capsicum* spp. fruits was evaluated every 24 h, among the 1st and 8th day after inoculation (DAI), and values were based on score scale proposed by Montri et al. [17], where 1= highly resistant; 3= resistant; 4= moderately resistant; 6= moderately susceptible; 8= susceptible; and 10= highly susceptible.

Histological analysis and secondary metabolites quantification in *C. annuum* fruits in response to *C. gloeosporioides* infection

Unripe and ripe fruits from GBUEL103 (susceptible) and GBUEL104 (resistant) accessions of *C. annuum* were selected for histological and secondary metabolites quantification analyses and both were inoculated with *C. gloeosporioides*, as previously described. Five fruits from each accession and each fruit development stages were used for both analyses. Control fruits were similarly treated using sterilized distilled water for mock inoculation. After fungus inoculation, 1 cm^2 of each *C. annuum* fruit tissues were collected at 1st and 8th DAI for histological and secondary metabolites quantification analyses.

Histological sections of fruits were fixed in 70% FAA (37% formaldehyde, 100% glacial acetic acid, 70% alcohol - 1:1:18 v/v/v) for 48 h, at room temperature, and transferred to 70% alcohol, maintained at 4 ºC. The samples were dehydrated through a graded ethanol series (80, 90 e 100%) for 30 min each, and then infiltrated in paraffin. Longitudinal sections (15 µm) of *C. annuum* fruits were obtained with a steel blade (Kasvi, São José do Pinhais, PR, Brazil) in a manual microtome (Ancap, São Paulo, SP, Brazil). The fruits sections were deparaffinized with xylol, stained with 50% silver nitrate and lactophenol cotton blue, and mount in Canada balsam. Sections observation was performed by light microscopy DM 4500 B (Leica Microsystems, Wetzlar, Germany). The images generated were captured with DFC

300FX (Leica Microsystems, Wetzlar, Germany) camera, using the LAS v4.3 (Leica Microsystems, Wetzlar, Germany) software.

Secondary metabolites of fruits were extracted (0.1 g) with 2 mL 80% (v/v) ethanol, with agitation TE-145 (Tecnal, Piracicaba, SP, Brazil) at 120 rpm for 2 h, at room temperature. The extract was centrifuged at 2500 rpm for 5 min, filtered in nylon membrane 0.22 µm (Micropore Technologies, High Bridge, NJ, USA), and stored at a -12 ^{o}C [28]. The compounds quantification was carried out by external standardization method using the standards: gallic acid (GALL), protocatechuic acid (PROT), epigallocatechin (EPI), catechin (CAT), epicatechin (EPICAT), quercetin (QUER), caffeic acid (ACAF), rutin (RUT), kaempferol (KAEMP), chlorogenic acid (CHLO), p-coumaric acid (PCO), ferrulic acid (FERR), synaptic acid (SYN), myricetin (MYR), trigonelline (TRIGO), theobromine (THEO), paraxanthine (PAR), caffeine (CAF), and ascorbic acid (ASC). All standards used showed degree of purity >99% (Sigma-Aldrich, St. Louis, MO, USA).

The separation of compounds was performed on ultra-high performance liquid chromatography (UHPLC), using Acquity UPLC I Class Waters© Alliance e2695 (Milford, MA, EUA) equipment, and HSS C18 1.8 µm 2.1×100 mm (Waters©, Milford, MA, EUA) column. Injection volume was 1 µL with flow of 0.4 mL.min^{-1}, using two solvents as mobile phase: ultrapure water (A) and methanol (B) (JT Baker HPLC grade, Philipsburg, NJ, USA), acidified with 0.05% and 0.1% formic acid (JT Baker HPLC grade, Philipsburg, NJ, USA), respectively. Mobile phase gradient conditions were 0 to 10 min, with 95% of phase A and 5% of phase B; 10 to 10.10 min, with 5% of phase A and 95% of phase B; 10.10 to 13 min, with 95% of phase A and 5% of phase B. The total sample run time was 13 min. A photodiode array detector (UHPLC-DAD) was used to scan the wavelengths between 190 and 700 nm and to read the wavelengths at 270 and 320 nm.

Data analysis

Anthracnose severity scores were used to calculate the area under the disease progress curve (AUDPC), according to Campbell e Madden [29]. An anova-type statistics (ATS) analysis was performed for the non-parametric data, and then the averages were compared by the Dunnett test (p<0.05), with the resistant accession control (GBUEL73). The Spearman correlation was used on the anthracnose variables (disease severity scores and AUDPC), in two fruit development stages (unripe and ripe fruits). Secondary metabolites quantification data were submitted to variance analysis, principal component analysis (PCA) and, clustering

heat map analysis. All analyses were performed using R software (www.r-project.org) and *agricolae*, *nparLD*, *nparcomp*, *gplots*, *corrplot*, and *FactoMineR* packages.

Results

Identification of resistance source in *Capsicum* species in response to *C. gloeosporioides* infection

Non-parametric variance analysis showed a significant effect of the variation sources: accessions (A), fruit development stages (FDS) and interaction of A x FDS for anthracnose average severity scores and AUDPC (Table 1). Unripe fruits showed greater susceptibility to *C. gloeosporioides* in relation to ripe fruits, with disease average severity scores of 6.16 and 3.59, respectively, and AUDPC of 23.30 and 12.24, respectively (Table 1).

Table 1. Non-parametric analysis of the averages values for the variation sources of the disease severity scores and area under the disease progress curve (AUDPC) related to anthracnose resistance response of 59 accessions of unripe and ripe fruits of *Capsicum* spp.

Sources of variation	**Chi-square**	**Degree of freedom**	***P*-value**
Scores			
Accessions (A)	6.97	28.17	<0.0001
Fruit development stages (FDS)	200.55	1.00	<0.0001
A x FDS	5.41	33.00	<0.0001
AUDPC			
Accessions (A)	7.49	26.47	<0.0001
Fruit development stages (FDS)	313.56	1.00	<0.0001
A x FDS	5.90	35.19	<0.0001
Averages	**Scores**	**AUDPC**	
Unripe fruits	6.16	23.30	
Ripe fruits	3.59	12.24	

The correlation analysis showed high association between the AUDPC variables and disease severity scores for the anthracnose symptoms in unripe and ripe fruits (0.93 and 0.94, respectively) (Fig 1). Low correlation was observed comparing unripe and ripe fruits, supporting the differential fungus response in pepper fruits development stages.

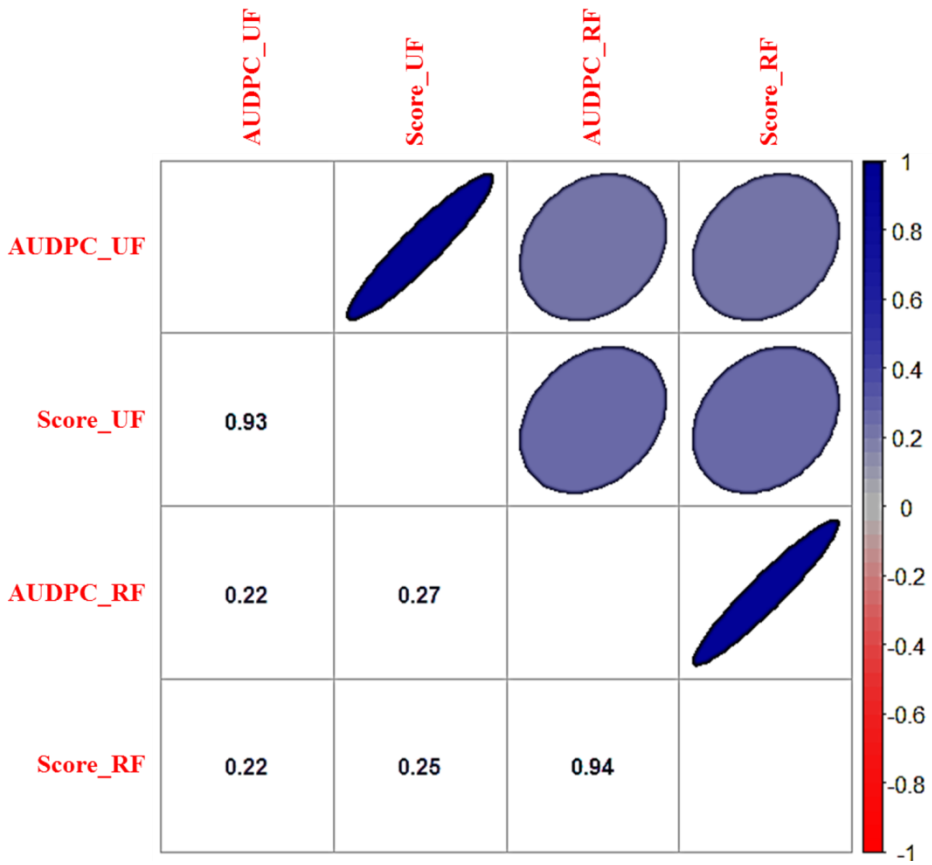


Fig 1. Spearman correlation between the variables: anthracnose severity scores (Scores) and area under the disease progress curve (AUDPC), in unripe fruits (UF) and ripe fruits (RF) of 59 accessions of *Capsicum* species.

According the AUDPC values, six accessions showed no significant difference by the Dunnett test (p<0.05), presenting the lowest values for unripe fruits, considered resistant by the anthracnose severity scores (Table 2). Twenty four accessions showed the lowest values for AUDPC and showed no significant difference by the Dunnett test (p<0.05) for ripe fruits (Table 2). Among these accessions, only GBUEL25, GBUEL48, GBUEL64 and GBUEL79 were considered resistant by the disease severity scores while the others accessions were classified as highly resistant (Table 2). GBUEL06, GBUEL28, GBUEL73, GBUEL87, GBUEL104 and, GBUEL106 accessions were considered highly resistant for both fruit development stages (Table 2).

Table 2. Area under the disease progress curve (AUDPC) and anthracnose severity scores in unripe and ripe fruits of 59 *Capsicum* spp. accessions in response to *C. gloeosporioides* infection.

Accessions	Unripe fruits			Ripe fruits		
	AUDPC	Disease severity scores[1]		AUDPC	Disease severity scores[1]	
GBUEL73[2]	7.00*	1.00	HR	7.00*	1.00	HR
GBUEL02	32.70	8.40	S	13.50	4.00	MR
GBUEL05	27.10	9.20	S	8.70*	2.40	HR
GBUEL06	7.00*	1.00	HR	10.70*	2.80	HR
GBUEL11	26.30	8.40	S	8.70*	2.40	HR
GBUEL14	17.30	4.00	MR	7.60*	1.40	HR
GBUEL15	27.90	8.80	S	23.30	7.20	MS
GBUEL25	28.50	8.00	S	10.50*	3.60	R
GBUEL26	25.50	8.00	S	8.20*	1.80	HR
GBUEL27	18.00	3.00	R	7.80*	2.60	HR
GBUEL28	7.00*	1.00	HR	10.70*	2.40	HR
GBUEL30	41.10	10.00	HS	16.00	5.80	MR
GBUEL34	12.70*	2.80	HR	11.10	4.00	MR
GBUEL35	37.30	10.0	HS	14.90	5.60	MR
GBUEL42	34.50	8.00	S	7.20*	1.40	HR
GBUEL46	21.00	5.00	MR	9.60*	2.60	HR
GBUEL46b	18.10	5.20	MR	11.00	3.40	R
GBUEL47	31.10	9.20	S	11.20	3.00	R
GBUEL47b	33.30	9.60	S	10.00*	2.60	HR
GBUEL48	14.60*	4.60	MR	10.50*	3.20	R
GBUEL49	32.10	8.40	S	8.00*	2.20	HR
GBUEL50	19.60	4.60	MR	12.90	4.00	MR
GBUEL53	16.10	4.80	MR	7.00*	1.00	HR
GBUEL58	23.50	8.00	S	9.10*	2.00	HR
GBUEL61	22.30	5.60	MR	11.90	3.60	R
GBUEL64	30.90	8.00	S	9.70*	3.20	R
GBUEL68	17.40	5.80	MR	8.30*	2.00	HR
GBUEL69	7.00*	1.00	HR	11.40	3.00	R
GBUEL72	7.00*	1.00	HR	16.30	5.60	MR
GBUEL74	30.90	7.60	MS	11.70	3.60	R
GBUEL75	7.00*	1.00	HR	23.40	7.80	MS
GBUEL76	11.50*	2.80	HR	12.10	4.80	MR
GBUEL77	17.10	3.60	R	7.60*	2.20	HR
GBUEL78	32.10	10.00	HS	20.50	6.80	MS
GBUEL79	21.10	6.80	MS	9.80*	3.00	R
GBUEL80	44.90	10.00	HS	8.90*	2.00	HR
GBUEL81	30.60	8.20	S	7.00*	1.00	HR
GBUEL82	26.30	6.80	MS	10.20*	2.60	HR
GBUEL83	18.20	5.00	MR	16.00	4.60	MR
GBUEL84	27.50	7.20	MS	12.80	5.00	MR
GBUEL85	19.30	6.00	MS	14.30	4.00	MR
GBUEL86	19.00	4.20	MR	9.30*	1.60	HR
GBUEL87	12.40*	3.00	R	9.10*	2.00	HR
GBUEL88	25.90	8.00	S	18.30	6.00	MS

Table 2 continuation

Accessions	Unripe fruits			Ripe fruits		
	AUDPC	Disease severity scores[1]		AUDPC	Disease severity scores[1]	
GBUEL89	26.30	8.40	S	10.60*	2.60	HR
GBUEL90	33.70	8.80	S	17.10	4.80	MR
GBUEL91	32.50	8.00	S	12.20	4.20	MR
GBUEL92	28.00	8.20	S	13.20	3.80	R
GBUEL93	23.40	6.20	MS	11.10	4.40	MR
GBUEL94	25.30	7.60	MS	7.00*	1.00	HR
GBUEL95	29.00	6.20	MS	14.30	4.40	MR
GBUEL96	42.50	10.00	HS	21.90	8.00	S
GBUEL97	36.50	9.20	S	18.30	7.20	MS
GBUEL103	31.10	8.40	S	29.10	8.00	S
GBUEL104	8.40*	1.40	HR	8.60*	1.80	HR
GBUEL105	25.90	8.40	S	10.60*	1.80	HR
GBUEL106	7.00*	1.00	HR	7.50*	2.00	HR
GBUEL107	9.90*	2.00	HR	17.10	4.40	MR
GBUEL108	27.30	7.20	MS	20.10	6.40	MS

*Averages with asterisks showed no difference from the control by the Dunnett test ($p<0.05$).
[1]Scores scale proposed by Montri et al. [18]. HR= highly resistant; R= resistant; MR= moderately resistant; MS= moderately susceptible; S= susceptible; HS= highly susceptible.
[2]Accession used as control (resistant) for unripe and ripe fruits.

Unripe and ripe fruits of bell pepper GBUEL103 showed typical symptoms of anthracnose at 8th DAI, considered susceptible to this fungus disease with average scores of 8.40 and 8.00, respectively (Figs 2A and 2B). In contrast, chili pepper GBUEL104 fruits showed a small brown spot, during both fruit development stages, with average disease severity scores of 1.40 and 1.80 for unripe and ripe fruits, respectively. GBUEL104 was considered as highly resistant to anthracnose (Figs 2C and 2D). In this way, GBUEL103 and GBUEL104 accessions were selected for further analyses during *C. gloeosporioides* interaction.

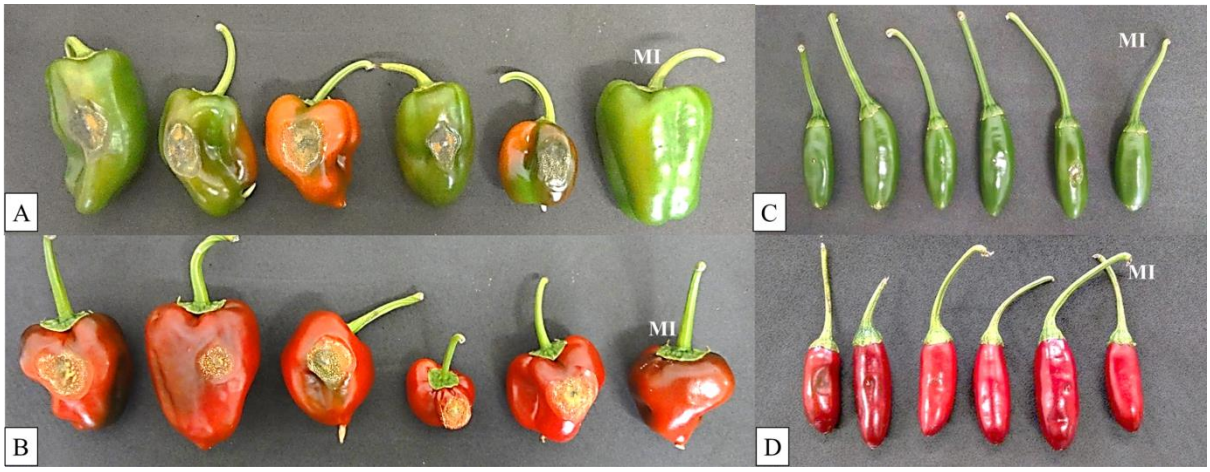


Fig 2. Fruit of *C. annuum* bell pepper GBUEL103 (susceptible) and chili pepper GBUEL104 (resistant) at eight days after inoculation with *C. gloeosporioides* in unripe (A and C) and ripe fruits (B and D). Characteristic symptoms of anthracnose can be observed in susceptible fruits (A and B). In resistant fruits (C and D) showed brown spots. MI = Mock inoculation (autoclaved distilled water).

Histologic analysis of *C. annuum* fruits in response to *C. gloeosporioides* infection

No fungal structures in unripe and ripe fruits tissues of *C. annuum* susceptible (GBUEL103) and resistant (GBUEL104) to anthracnose were observed at 1st DAI (Figs 3A, 3C, 3E e 3G; 4A, 4C, 4E and 4G). However, both accessions showed structural morphological differences in infected fruits at 8th DAI (Figs 3D, 3H, 4D and 4H). Alterations in pepper cell morphology were greater in susceptible compared to resistant accession, and between unripe and ripe fruits (Fig 3 and 4).

Inter and intra-cellular fungus colonization was observed, in which they differed rapidly into long and branched hyphae (necrotrophic phase) (Figs 3D, 4D and S1), and acervuli formation developed in the tissues cuticle of susceptible fruits, in both fruit development stages, on the 8th DAI (S1 Fig). Fungal structures were not observed in ripe resistant fruits under pathogen infection (Fig 4H). In resistant unripe fruits, hyphae penetration occurred only in the parenchyma cells (Fig 3H) at 8th DAI, corroborating with the disease severity scores and AUDPC in *Capsicum* spp. fruits, being the unripe fruits more susceptible to anthracnose.

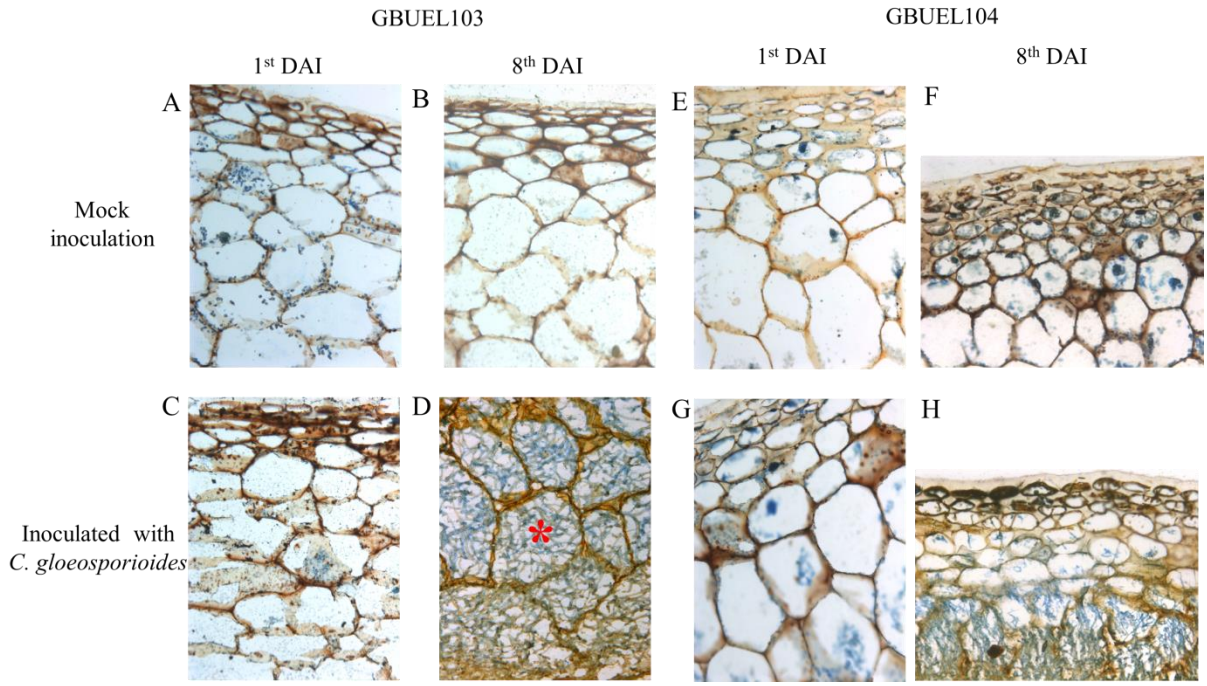


Fig 3. Light micrographs with longitudinal sections (15 µm) in unripe fruits of *C. annuum* GBUEL103 (A, B, C and D) and GBUEL104 (E, F, G and H) at 1st and 8th day after inoculation (DAI) with *C. gloeosporioides* (C, D, G and H) and mock inoculation (A, B, E and F). Asterisks (*) indicate the formation of secondary branched hyphae.

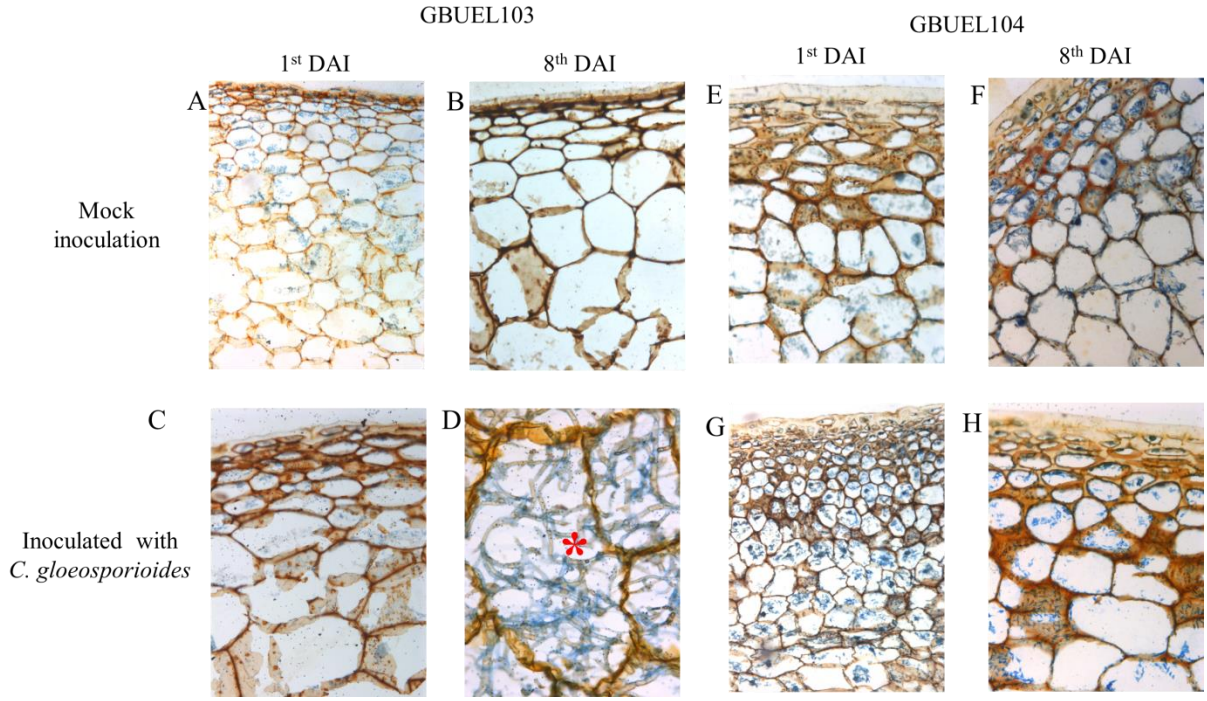
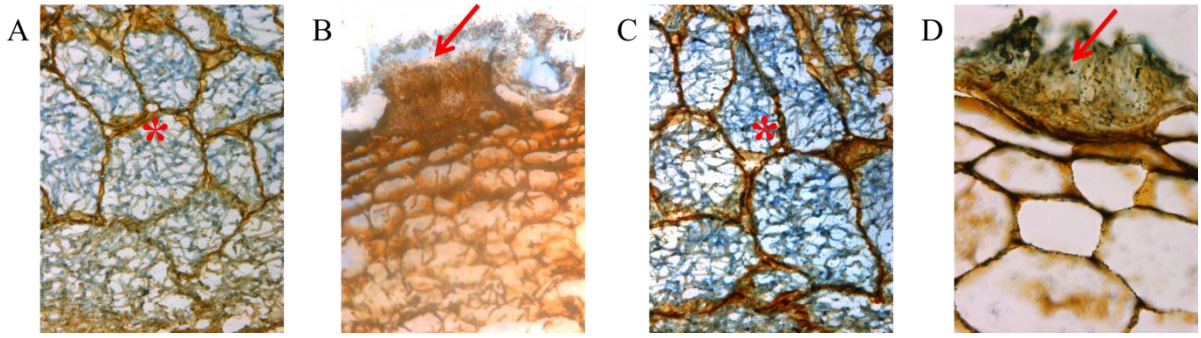


Fig 4. Light micrographs with longitudinal sections (15 µm) in ripe fruits of *C. annuum* GBUEL103 (A, B, C and D) and GBUEL104 (E, F, G and H) at the 1st and 8th day after inoculation (DAI) with *C. gloeosporioides* (C, D, G and H) and mock inoculation (A, B, E and F). Asterisks (*) indicate the formation of secondary branched hyphae.



S1 Fig. Light micrographs with longitudinal sections (15 µm) in unripe (A and B) ripe (C and D) fruits of *C. annuum* GBUEL103 on the 8th day after inoculation with *C. gloeosporioides*. Asterisks indicate the formation of secondary branched hyphae. The arrows indicate the formation of acervuli in the fruit cuticle.

Quantification of secondary metabolites in *C. annuum* fruits in response to *C. gloeosporioides* infection

The principal component analysis (PCA – components 1 and 2) explained 63.14 and 59.61% of secondary metabolites concentrations observed in unripe (Figs 5A and 5B) and ripe (Figs 5C and 5D) fruits, respectively. Metabolites evaluated in unripe fruits showed clusters for treatments in the resistant accession (GBUEL104), showing high concentrations of ferrulic acid (FERR), gallic acid (GALL), caffeic acid (ACAF), chlorogenic acid (CHLO), trigonelline (TRIGO) and paraxanthine (PAR) for the 1st and 8th DAI, compared to the susceptible accession (GBUEL103) (Figs 5A and 5B).

Among these metabolites, FERR, CHLO, GALL and PAR showed no difference (p<0.05) between *C. gloeosporioides* and mock inoculation, while ACAF showed a concentration reduction under fungus inoculation (S2 Table). However, for this metabolite, the resistant inoculated accession at the 1st (RI1) and 8th (RI8) DAI, obtained 203.50 and 220.70 mg.Kg^{-1}, respectively, while the susceptible inoculated (SI1 and SI8) showed low values for the same parameters (87.67 and 2.13 mg.Kg^{-1}) (S2 Table). And also, TRIGO levels increased in the resistant accession under fungus inoculation at 8th DAI (RI8) (S2 Table).

ACAF, CAF, GALL and TRIGO metabolites showed the highest concentration values in the resistant compared to susceptible accession at the 8th DAI (Fig 5D). Among these metabolites, TRIGO concentration did not show difference (p<0.05) in response to fungus and mock inoculation in the resistant accession (S2 Table). In the other hand, ACAF, CAF and GALL metabolites showed a concentration increase in RI8 treatment when compared to RM8. The metabolites for susceptible accession, under fungus inoculation fruits, at 8th DAI showed a difference, with high concentrations of epicatechin (EPICAT), theobromine

(THEO), ascorbic acid (ASC), rutin (RUT), synaptic acid (SYN), myricetin (MYR) and epigallocatechin (EPI) (Fig 5D).

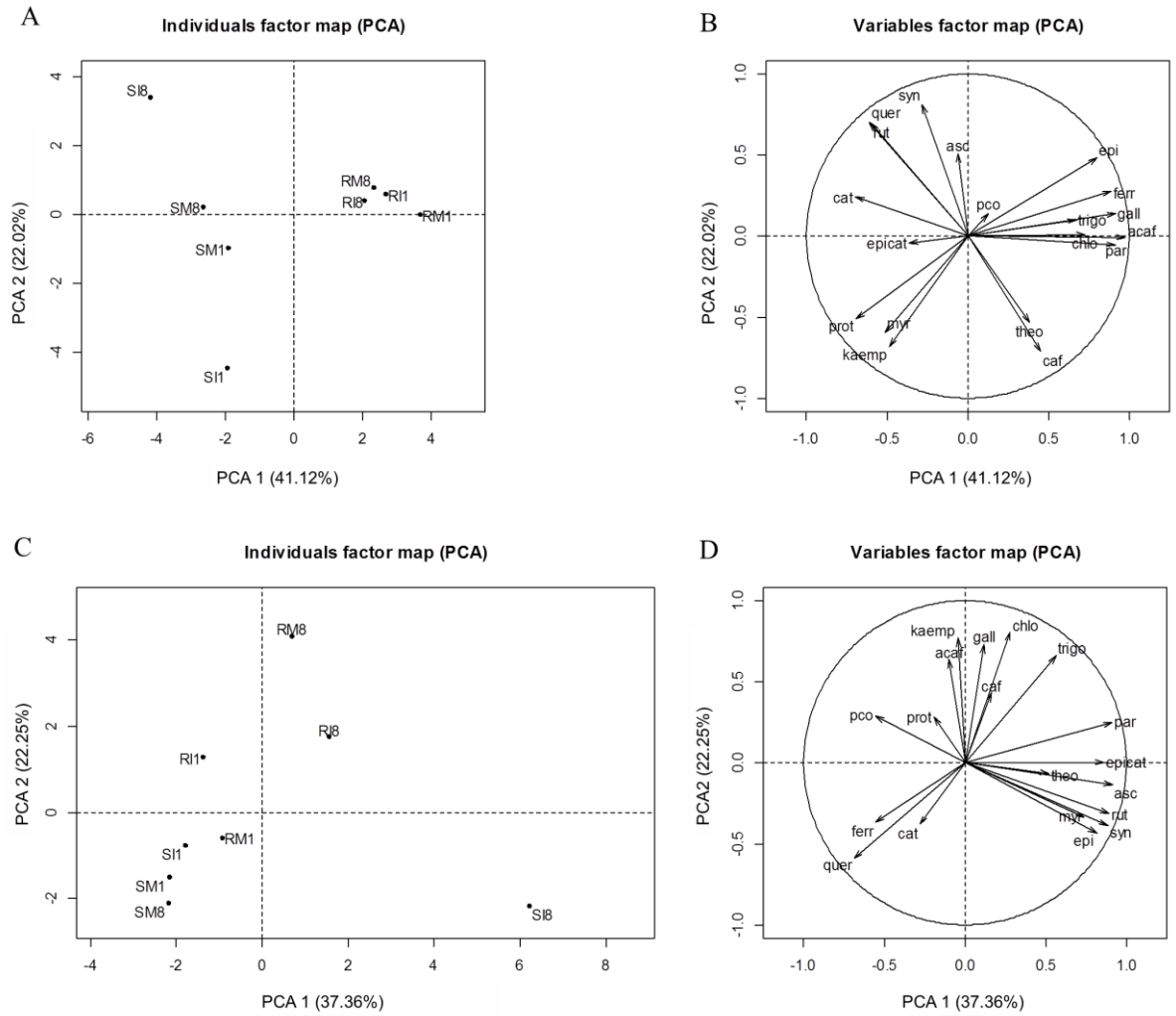


Fig 5. Principal components analysis of 19 secondary metabolites produced by *C. annuum* susceptible (GBUEL103) and resistant (GBUEL104) accession during anthracnose infection. Biochemical compounds were quantified at the 1st and 8th DAI with *C. gloeosporioides* in unripe (A and B) and ripe (C and D) fruits. Treatments diagrams (A and C) and correlation between the secondary metabolites (B and D).

Treatments: RI1 = resistant accession in the 1st DAI inoculated with *C. gloeosporioides*; RI8 = resistant accession in the 8th DAI inoculated with *C. gloeosporioides*; RMI = resistant accession in the 1st DAI inoculated with water (mock inoculation); RM8 = resistant accession in the 8th DAI inoculated with water (mock inoculation); SI1 = susceptible accession in the 1st DAI inoculated with *C. gloeosporioides*; SI8 = susceptible accession in the 8th DAI inoculated with *C. gloeosporioides*; SM1 = susceptible accession in the 1st DAI inoculated with water (mock inoculation); SM8 = susceptible accession in the 8th DAI inoculated with water (mock inoculation).

Secondary metabolites: trigo = Trigonelline; asc = Ascorbic acid; gall = Gallic acid; prot = Protocatechuic acid; theo = Theobromine; par = Paraxanthine; epi = Epigallocatechin; cat = Catechin; chlo = Chlorogenic acid; acaf = Caffeic acid; caf = Caffeine; epicat = Epicatechin; pco = p-coumaric acid; ferr = Ferrulic acid; syn = Synaptic acid; rut = Rutin; myr =Myricetin; quer = Quercetin; kaemp = Kaempferol.

Concentration variation of some secondary metabolites, for susceptible accession, in unripe fruits was observed under fungus inoculation in relation to mock inoculated fruits (Fig 5A). Protocatechuic acid (PROT), kaempferol (KAEMP) and MYR compounds were in higher concentrations in the inoculated susceptible (SI1) than in mock susceptible (SM1) on the 1st DAI in unripe fruits. While on the 8th DAI, higher levels of RUT, quercetin (QUER), SYN, catechin (CAT) and ASC were observed in SI1 compared to SM1 (Fig 5B).

S2 Table. Secondary metabolites concentrations quantified in the susceptible (GBUEL103) and resistant (GBUEL104) accessions in response to *C. gloeosporioides* infection.

Fruit stage	Secondary metabolites	mg.Kg^{-1}							
		RI1	**RI8**	**RM1**	**RM8**	**SI1**	**SI8**	**SM1**	**SM8**
Unripe	acaf	203.50	220.70	232.00	230.70	87.67	2.13	29.66	39.20
	asc	134.70	550.70	76.80	176.10	102.40	432.70	69.67	106.53
	caf	1.91	2.93	2.83	2.48	4.28	0.69	1.32	0.67
	cat	15.72	10.63	18.33	11.16	34.93	61.53	17.16	18.09
	chlo	19.16	6.94	16.51	7.93	7.45	4.67	4.00	7.03
	epi	3.66	2.43	3.99	3.42	nd	2.03	nd	nd
	epicat	10.98	10.88	3.05	3.44	13.42	13.65	4.66	4.96
	ferr	3.79	4.24	3.49	2.77	0.87	1.33	1.67	1.35
	gall	19.26	10.82	20.62	17.41	6.19	4.77	2.07	1.53
	kaemp	1.06	1.21	0.82	1.19	6.67	2.71	3.30	0.49
	myr	3.36	3.05	2.09	2.78	7.85	3.76	2.30	5.51
	par	3.35	4.10	3.37	4.03	1.87	nd	nd	nd
	pco	1.23	0.73	1.21	1.67	0.78	0.87	1.47	1.56
	prot	nd	5.33	nd	nd	8.12	3.21	5.96	7.70
	quer	2.63	1.15	0.53	3.06	0.94	5.81	2.47	3.57
	rut	0.46	0.50	0.13	0.42	nd	5.93	nd	1.51
	syn	10.22	10.76	12.19	6.16	1.64	28.13	1.19	13.52
	theo	6.95	nd	9.68	nd	9.23	nd	nd	nd
	trigo	28.40	66.07	34.93	35.13	19.61	16.41	18.61	17.16
Ripe	acaf	164.80	252.30	246.20	161.70	41.47	2.52	39.07	47.73
	asc	89.20	477.70	121.40	111.00	63.93	623.30	101.10	109.20
	caf	0.88	2.77	1.51	1.57	1.33	1.09	1.42	0.97
	cat	4.73	14.29	11.51	10.21	8.90	9.76	23.60	17.43
	chlo	6.64	8.77	6.16	25.07	5.15	8.25	5.05	4.05
	epi	nd	nd	1.01	nd	nd	2.61	nd	nd
	epicat	3.13	7.15	2.23	8.00	3.11	12.13	6.45	4.42
	ferr	0.70	1.86	3.51	1.72	0.88	0.33	4.31	5.95
	gall	6.85	4.91	3.84	5.16	2.67	3.55	2.75	2.73
	kaemp	5.07	1.69	0.47	22.47	5.62	0.75	3.01	2.43
	myr	0.46	3.38	3.05	0.66	4.56	7.14	0.86	nd
	par	nd	1.59	nd	1.15	0.31	1.93	nd	nd
	pco	0.91	0.71	0.72	0.93	1.09	0.62	0.95	0.68
	prot	6.03	5.74	nd	6.07	3.84	3.21	5.04	7.23
	quer	1.30	0.33	1.37	nd	4.69	nd	6.37	6.83
	rut	1.09	0.94	nd	1.23	nd	8.57	nd	1.49
	syn	2.69	3.26	8.27	3.84	4.58	45.40	2.30	1.36
	theo	5.96	9.51	nd	10.41	nd	14.61	9.35	12.76
	trigo	17.65	30.73	20.59	35.93	16.97	26.67	21.93	17.51

Treatments: RI1 = resistant accession in the 1st DAI inoculated with *C. gloeosporioides*; RI8 = resistant accession in the 8th DAI inoculated with *C. gloeosporioides*; RMI = resistant accession in the 1st DAI inoculated with water (mock inoculation); RM8 = resistant accession in the 8th DAI inoculated with water (mock inoculation); SI1 = susceptible accession in the 1st DAI inoculated with *C. gloeosporioides*; SI8 = susceptible

accession in the 8th DAI inoculated with *C. gloeosporioides*; SM1 = susceptible accession in the 1st DAI inoculated with water (mock inoculation); SM8 = susceptible accession in the 8th DAI inoculated with water (mock inoculation); nd = not detected.
Secondary metabolites: trigo = Trigonelline; asc = Ascorbic acid; gall = Gallic acid; prot = Protocatechuic acid; theo = Theobromine; par = Paraxanthine; epi = Epigallocatechin; cat = Catechin; chlo = Chlorogenic acid; acaf = Caffeic acid; caf = Caffeine; epicat = Epicatechin; pco = p-coumaric acid; ferr = Ferrulic acid; syn = Synaptic acid; rut = Rutin; myr =Myricetin; quer = Quercetin; kaemp = Kaempferol.

Heat map results corroborates with PCA analysis for unripe and ripe fruits (Fig 6). Unripe fruits showed four clusters for treatments and two clusters of secondary metabolites (Fig 6A). Ripe fruits showed six clusters for treatments and three clusters of secondary metabolites (Fig 6B).

Unripe fruits showed better clustering of metabolites in relation to resistant and susceptible accessions compared to ripe fruits (Fig 6A). Resistant accession treatments were clustered and showed higher metabolites concentrations of GALL, EPI, FERR, PAR, ACAF and CAF. In addition, RI8 treatment also showed high concentrations of TRIGO and ASC, RM1 and RI1 treatments showed high concentrations of CHLO and THEO, and the opposite was observed for treatments in the 8th DAI (RM8 and RI8) (Fig 6A). For the susceptible accession, SI1 treatment showed an increase of KAEMP, MYR and CAF metabolites concentration.

CAF, ACAF, ASC and PAR metabolites showed the highest concentrations for RI8 treatment in ripe fruits (Fig 6B), while KAEMP, CHLO and TRIGO metabolites showed high concentrations for RM8 treatment. RI1 treatment showed an increase concentration for GALL and RM1 for ACAF compound. Treatments SM1 and SM8 showed high values for THEO, PROT, FERR, CAT and QUER metabolites. In the other hand, SI1 treatment showed increase levels of p-coumaric acid (PCO), MYR and QUER (Fig 6B).

Susceptible fruits inoculated with fungus and analyzed at 8th DAI (SI8) were the most distant treatment cluster observed for both fruit development stages. SI8 showed high concentrations of EPICAT, ASC, RUT, and SYN in unripe and ripe fruits (Figs 6A and 6B). In addition, SI8 unripe fruits showed high values of CAT and QUER (Fig 6A), and SI8 ripe fruits for EPI, MYR, PAR and THEO (Fig 6B).

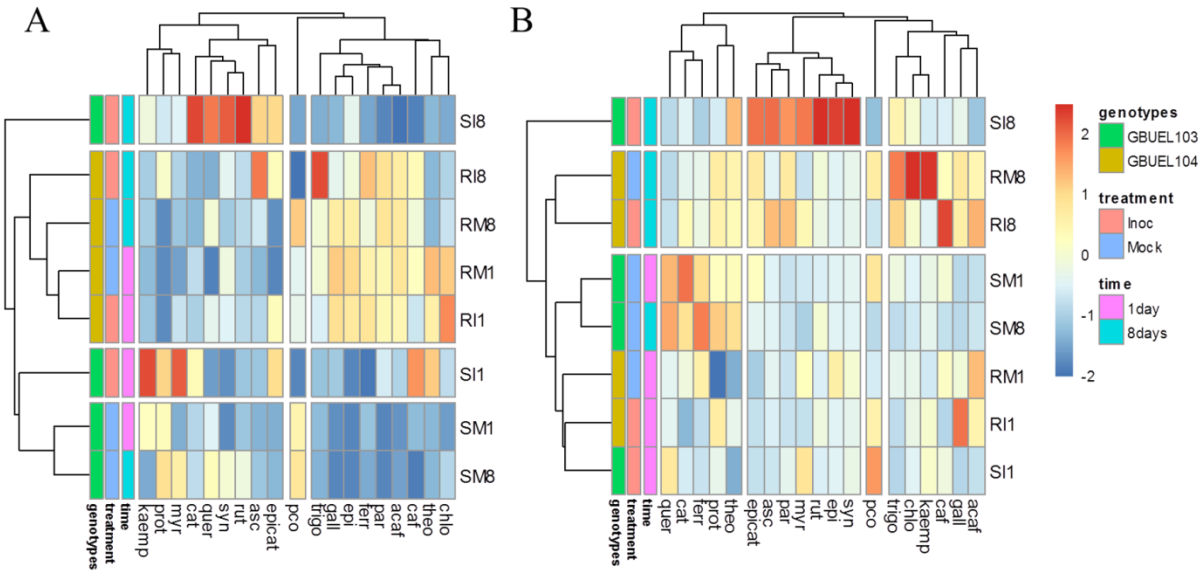


Fig 6. Heat map analysis of unripe (A) and ripe (B) fruits of *C. annuum* susceptible (GBUEL103) and resistant (GBUEL104) accessions under anthracnose infection.
Treatments: RI1 = resistant accession in the 1st DAI inoculated with *C. gloeosporioides*; RI8 = resistant accession in the 8th DAI inoculated with *C. gloeosporioides*; RMI = resistant accession in the 1st DAI inoculated with water (mock inoculation); RM8 = resistant accession in the 8th DAI inoculated with water (mock inoculation); SI1 = susceptible accession in the 1st DAI inoculated with *C. gloeosporioides*; SI8 = susceptible accession in the 8th DAI inoculated with *C. gloeosporioides*; SM1 = susceptible accession in the 1st DAI inoculated with water (mock inoculation); SM8 = susceptible accession in the 8th DAI inoculated with water (mock inoculation).
Secondary metabolites: trigo = Trigonelline; asc = Ascorbic acid; gall = Gallic acid; prot = Protocatechuic acid; theo = Theobromine; par = Paraxanthine; epi = Epigallocatechin; cat = Catechin; chlo = Chlorogenic acid; acaf = Caffeic acid; caf = Caffeine; epicat = Epicatechin; pco = p-coumaric acid; ferr = Ferrulic acid; syn = Synaptic acid; rut = Rutin; myr =Myricetin; quer = Quercetin; kaemp = Kaempferol.

Discussion

A wide variability of response against anthracnose fungus infection was observed for unripe and ripe fruits of 59 *Capsicum* spp. accessions (Table 1). Some studies have reported that distinct genes control the genetic resistance mechanism for different fruit development stages during plant-pathogen interaction [9, 11, 19, 22]. We observed that ripe fruits showed to be more resistant to anthracnose compared to unripe fruits (Table 1), as previously observed in other studies with *Capsicum* spp. [19, 22, 30].

Ripe fruits present higher resistance against anthracnose and it is probably related to the accumulation of resistance compound, considering that in resistant genotypes the defense response is rapidly activated. Antioxidants concentration increases during fruit maturation process of *Capsicum* spp. [31], as an important biochemical compound to prevent oxidative degradation of fruit caused by biotic stress.

We observed that *C. chinense* (GBUEL06, GBUEL28, GBUEL73 and GBUEL87), *C. annuum* (GBUEL104) and *C. baccatum* var. *pendulum* (GBUEL106) accessions were considered highly resistant to anthracnose in both fruit development stages (Table 2). These accessions are considered promising for genetic inheritance studies and future use in pepper genetic breeding programs, due to the high incidence of this pathogen in Brazil and worldwide.

Subsequently, we selected GBUEL103 (susceptible) and GBUEL104 (resistant) accessions for histological and secondary metabolites quantification analysis, since both are *C. annuum*, highly acceptable by the consumer market, and globally cultivated.

In contrast, fruits of resistant accession showed smaller lesions that can be related to hypersensitivity reaction (Fig 2). Programmed cell death occurs from the 1st DAI in fruits under fungus inoculation, and this reaction prevents biotrophic fungus survival and growth [32], but does not affect the necrotrophic that use dead cells as nutrition source.

The absence of fungal structure in susceptible and resistant accessions at the 1st DAI can be related to the germination time of the *C. gloeosporioides* conidia (Figs 3C, 3G, 4C and 4G). In general, fungus germination of *Colletotrichum* species starts around 19 h after inoculation (HAI) with appressoria differentiation [14, 15]. A fungal penetration peg (biotrophic phase) can be observed between 40 and 48 HAI [14, 15]. Within three to five days, hyphae with dendritic-like structures are formed in the fruit epidermis, where they remain at quiescent (quiescent phase) until fruit ripening, and then differentiate into long and thin branched hyphae, with the destruction of the host tissue (necrotrophic phase) [14, 15]. Unripe and ripe fruits tissues from the susceptible accession (GBUEL103), in the 8th DAI, were colonized by the fungus, conducting the alteration in pepper cell morphology (Figs 3D, 4D and S1). These alterations can be related to the necrotrophic form of fungus colonization and to the reduction of phenolic compounds concentration during late phases of anthracnose infection [7].

We observed an alteration of secondary metabolites concentration in *C. annuum* fruits infected with *C. gloeosporioides* according to fruit development stages, types of plant-pathogen interaction (compatible and incompatible), and hemobiotrophic fungus infection process (Figs 5 and 6). In addition, compounds levels variations due to the competition between the plant host and the phytopathogenic agent, associated to the production of phytoanticipins and phytoalexins [7, 26].

Concentrations of compounds activated in the *C. annuum* and *C. gloeosporioides* interaction decreased in the early stage of fungus infection, indicating the degradation/recycling of compounds before the initiation of their biosynthesis in response to the pathogen attack [7]. The levels of polyphenols increased gradually and then decreased, suggesting that the competition balance between pathogen metabolic reaction and the biosynthesis of plant defense may have been broken, and consequently, fruit defense mechanism was gradually defeated [7].

Phenolic acids produced by plants act as signaling molecules during the symbiosis process, and as plant defense agents against biotic and abiotic stresses [33]. In unripe fruits, the phenolic acids (FERR, GALL, ACAF and CHLO), trigonelline (TRIGO) and paraxanthyne (PAR) were associated to resistant accession (GBUEL104), and these compounds were probably acting as phytoanticipins (Figs 5B and 6A). Similarly, ACAF, GALL, TRIGO and PAR also showed high concentration in the resistant ripe fruits (Figs 5D and 6B). Phenolic compounds production in resistant fruits at initial stages of infection (biotrophic phase) helps the inhibition process of *Colletotrichum* species growth [14, 32]. Phytoalexins induced after fungus inoculation is more intense in resistant than in susceptible plants [26]. In addition, the higher content of phenolic compounds in the pathogen infection zone [7, 26, 34], makes it difficult to spread from infected cells to healthy tissue [26].

CHLO and ACAF are important phytoanticipins against *Alternaria alternata* [35], and their presence reduced fungus colonization in resistant tomato fruits. CHLO is considered as one of the main secondary metabolite components related to plant defense [36]. A previous study observed high amounts of CHLO in the bordering zone of *C. coccodes* infection in chili peppers tissues, and their presence inhibited fungus spread [26]. The derived compound of ACAF on the defense mechanism of *C. annuum* infected with the *C. gloeosporioides* fungus is considered as phytoalexins [7], showing antimicrobial activity, and participating in lignin biosynthesis [37]. Lignification can contribute to fruit resistance against pathogens in several ways, such as: *i)* degradation of enzymes secreted by the pathogen; *ii)* barrier preventing the free nutrient movement and therefore helping to starve the pathogen; and *iii)* slowing down the penetration process [38]. In addition, a previous transcriptome analysis of *C. gloeosporioides* and tomato fruits pathosystem showed that plant defense pathways were intensified during the quiescent phase, including the phenylpropanoid pathway as precursors of phytolexin and lignin [15].

ACAF, CHLO and p-coumaric acids (PCO) inhibit fungal spore germination, mycelia growth, enzymes production and also on the inactivation of specific enzymes produced by the pathogens [39]. Under *in vitro* conditions, ACAF also inhibited the zoospore germination of *Phytophtora capsici*, *P. megakarya* and *P. palmivora* [40]. The quickness of phytoalexins production plays a crucial role in the early than in the final stage of plant defense.

Conclusion

Our results showed a wide variability of *Capsicum* spp. accessions in relation to the resistance response to anthracnose disease and we observed stage-genotype-specific responses in *C. annuum* fruits infected with *C. gloesoporioides* fungus. The resistance responses are influenced by genotype, fruit development stages, and its biochemical composition.

Ripe *Capsicum* spp. fruits showed to be more resistant to anthracnose in relation to unripe fruits. Resistant accession presented high concentration of ACAF and CHLO, two well-known secondary metabolites related to improve plant defense response against pathogens. More studies of these secondary metabolites produced by pepper fruits and related to defense compounds are needed to improve the knowledge about the mechanisms involved in the resistance process of *Capsicum* spp. to anthracnose interaction.

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4. ARTIGO B: CAPSIDIOL-RELATED GENES ARE HIGHLY EXPRESSED IN RESPONSE TO C*olletotrichum gloeosporioides* DURING *Capsicum annuum* FRUIT DEVELOPMENT STAGES (Scientific Reports – in preparation)

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Abstract

Capsicum annuum (sweet and chili pepper) is one of the most important horticultural crops worldwide. Anthracnose disease (*Colletotrichum gloeosporioides*) is a major constraint for chili production, causing substantial losses. Capsidiol is a sesquiterpene phytoalexin present in pepper fruits that can enhance resistance against anthracnose disease. However, the genetic mechanisms involved in the biosynthesis of this compound are still poorly understood, especially during pepper fruit development stages. Our hypothesis is that peppers produce more capsidiol in order to reduce fungus infection by up-regulating capsidiol-related genes. In this study, we employed novel 3' RNA-sequencing and developed an analysis pipeline to generate *C. annuum* transcriptomes to uncover the transcriptional profile of genes in response to *C. gloeosporioides* in unripe and ripe fruits. We observed 4,845 up-regulated and 4,720 down-regulated genes in unripe fruit, and 2,560 up-regulated and 1,762 down-regulated genes in ripe fruits under anthracnose inoculation. Seven genes related to capsidiol biosynthesis were observed in the top 10 up-regulated genes under fungal interaction (three *CaEAS* and four *CaEAH*). Of these genes, we selected *CA12g05030* and *CA02g09520* related to 5-epi-aristolochene synthase (*CaEAS*) genes and *CA12g05070* and *CA01g05990* related to 5-epi-aristolochene 1,3-dihydroxylase (*CaEAH*) genes to validate their profiles using RT-qPCR. *CA12g05030* and *CA01g05990* showed rapid induction and higher transcriptional activity in the beginning of pathogen inoculation (24 hours post-inoculation - HPI) in ripe fruits, and their expression was induced more than 68-fold and 53-fold at 96 HPI, respectively. In unripe fruits, all genes expression showed a late response to anthracnose (96 HPI), but their activity accumulated more than 700-fold for *CA12g05030* gene at this time point. This is the first high-throughput effort to enhance the knowledge about genes transcriptionally regulated in response to fungal interaction in unripe and ripe pepper fruits. Moreover, our transcriptome dataset proved to be a powerful tool to discover resistance-related genes that might contribute to future disease control strategies and to improve pepper breeding programs.

Keywords: Pepper, anthracnose, 3' RNA-seq, 5-epi-aristolochene synthase, 5-epi-aristolochene 1,3-dihydroxylase, RT-qPCR, gene cluster.

Introduction

Capsicum annuum L. (sweet and chili pepper) is an important crop for the human diet, and it is also used for other purposes like medicines, beverages and ornamental plants. Pepper fruits have several nutraceutical benefits for human health, due to a variety of antioxidant, anti-inflammatory, antimicrobial, anti-carcinogenic and cardio-protective properties (Khan et al., 2014).

Anthracnose, caused by *Colletotrichum* spp., is a major disease of chili fruit worldwide and it causes significant yield loss and reduces the marketability of peppers (Saxena et al., 2016). *Colletotrichum* species are able to infect several organisms in the genus *Capsicum* and the disease has a complex etiology (Kim et al., 2004; Mongkolporn et al., 2010). *Colletotrichum* species tend to engage distinct strategies during fruit development stages: in unripe fruit, there is an appressoria formation, hyphal penetration and quiescence phase, while fruit ripening triggers fungus active infection and colonization. For example, in white strawberry fruits, *C. acutatum* is quiescent and forms appressoria, but engages in necrotrophic colonization in red fruits (Guidarelli et al., 2011). Similarly, distinct processes of infection and related transcriptional responses have been observed in tomato fruit (Alkan et al., 2015).

Plants can defend themselves with a vast array of chemical compounds, which range from direct defenses, such as toxins, to indirect defenses, such as phenolic compounds, alkaloids and terpenoids (Mithöfer and Maffei, 2017). The levels of these biochemical compounds can vary according to pepper fruit development stages (Park et al., 2014). One of these compounds is capsidiol (sesquiterpenoid), which has already described as being related to antifungal activity against *C. gloeosporioides* in pepper fruits (Park et al., 2014). Two key enzymes are responsible for capsidiol biosynthesis: 5-epi-aristolochene synthase (*EAS*) and 5-epi-aristolochene dihydroxylase (*EAH*) (Takahashi et al., 2005; Park et al., 2014). *EAS* was significantly induced in ripe fruits infected with *C. gloeosporioides*, and there was a negative relation between the capsidiol level and lesion size in fruits (Park et al., 2014).

However, though overall transcriptional changes during the development of pepper fruits are starting to be characterized (Martínez-López et al., 2014), the specific effects of anthracnose interaction on transcriptome-level responses still remain poorly understood. While studies of individual expression defense-related genes have provided insights into pepper responses to anthracnose (Ko et al., 2005; Mishra et al., 2017), large scale transcriptome studies provide more information to elucidate of the contrasting responses

involved in ripe and unripe fruits. This is an interesting subject for pepper breeding purposes, since anthracnose is caused mainly by *C. gloeosporioides* and can affect unripe and ripe fruits stages; red pepper fruits seem to be more resistant than green fruits (Taylor et al., 2007; Silva et al., 2014).

To address this question, our main goal was to elucidate the distinct pepper transcriptional responses to anthracnose in ripe and unripe fruits by studying metabolic pathways through the use of a recently developed 3' RNA-seq method and developing a panel of candidate genes for future pepper breeding programs. In addition, we analyzed the transcriptional activity of capsidiol-related genes (*CaEAS* and *CaEAH*) at both fruit development stages under *C. gloeosporioides* interaction. *CaEAS* (*CA12g05030* and *CA02g09520*) and *CaEAH* (*CA12g05070* and *CA01g05990*) had their digital gene expression pattern verified by real time quantitative polymerase chain reaction (RT-qPCR). This *C. annuum* transcriptome data constitutes an important high-throughput dataset of distinct transcriptional responses to anthracnose and provides important clues to identify candidate genes related to several pepper metabolic pathways that could be relevant to improve pepper resistance mechanism against *C. gloeosporioides* in the future. Furthermore, the results will provide a basis to develop better strategies for pepper breeding focusing on anthracnose disease control.

Material and Methods

Plant material

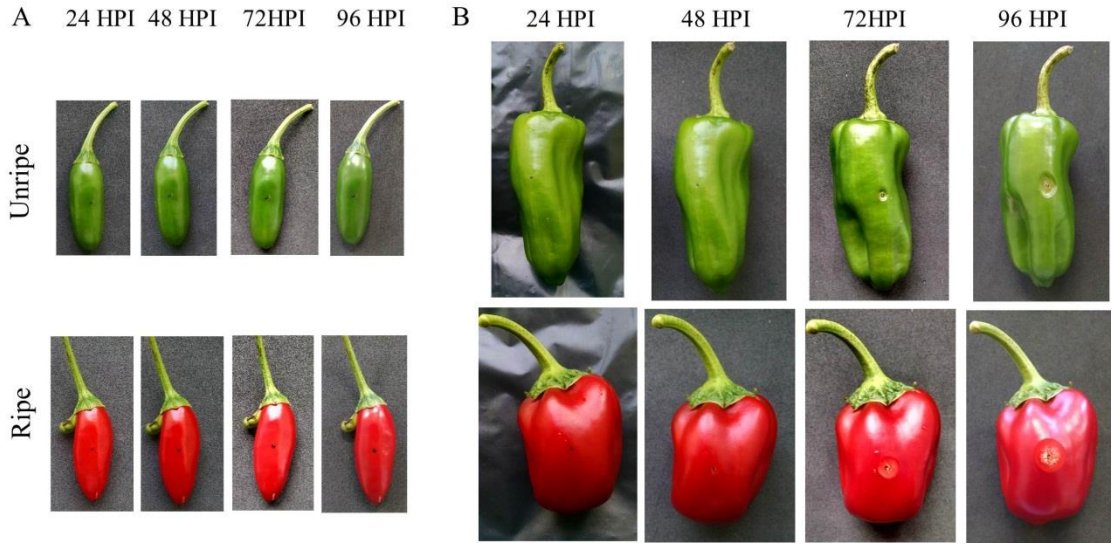
Capsicum annuum accession GBUEL104 from the Universidade Estadual de Londrina (UEL) gene bank, considered resistant to multiple pathogens, including anthracnose, in two fruit development stages (Bento et al., 2017), was used for RNA-seq and RT-qPCR analyses. We used unripe (35 days after anthesis - DAA) and ripe (50 DAA) fruits for both analyses. GBUEL104 seeds were sown on a tray with organic plant substrates and, after the emergence of two pairs of definitive leaves, seedlings were transferred individually to plastic pots containing a mixture of soil and substrate (2:1 ratio). Plants were grown in a greenhouse following practices recommended for pepper cultivation.

Anthracnose inoculation

Unripe and ripe pepper fruits were detached from the plant and were sterilized in 1% (w/v) sodium hypochlorite solution for five min, followed by three washes with distilled

water for one min. *C. gloeosporioides* spore suspension was prepared with a virulent isolate "8.1", provided by the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), at a concentration of 1 x 10^6 conidia.mL^{-1}. Inoculation was performed under laboratory conditions by the injection method in the central part of the fruit, using a Micro Syringe Model 1705 TLL (Hamilton, Switzerland). The needle depth was fixed at 1 mm to ensure inoculum volume and uniformity of lesion size. Control fruits were similarly treated and processed with distilled water for mock inoculation.

Pepper fruits were incubated in the dark for 24 h at 25 °C and, for subsequent 12 h light/dark cycles, were kept in a humid chamber. Fruits of the two development stages were sampled at 24, 48, 72 and 96 hours post-inoculation (HPI). All samples were frozen immediately in liquid nitrogen and stored at -80 °C until RNA extraction. A susceptible cultivar (GBUEL103) was treated using the same inoculation conditions to validate successful pathogen inoculation in the resistant accession (GBUEL104) (see Supplementary Fig. 1).



Supplementary Figure 1. *C. gloeosporioides* inoculation in unripe (35 DAA) and ripe pepper fruits (50 DAA) of the resistant accession GBUEL104 (A) and susceptible accession GBUEL103 (B). Photographs were taken at 24, 48, 72 and 96 hours post-inoculation (HPI).

RNA extraction, library construction and sequencing procedures

Total RNA of resistant pepper fruits (GBUEL104) was extracted using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and purified using the PureLink RNA Mini kit (Thermo Fisher Scientific, Waltham, MA, USA). All samples were treated with DNase I (RNase-free, Invitrogen, Carlsbad, California, USA). RNA quantity, purity and integrity were verified by spectrophotometry using NanoDrop® ND-1000 (Thermo Fisher

Scientific, Waltham, MA, USA), Qubit fluorometric quantitation (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer Chip DNA 1000 series II (Agilent Technologies, Santa Clara, California, USA). All reagents were used according to the manufacturer's instructions.

48 libraries from the *C. annuum* accession GBUEL104 under mock (water) and *C. gloeosporioides* inoculation were sequenced. Two stages of fruit development (unripe and ripe) at four time points post-inoculation (24, 48, 72 and 96 h) with three biological replicates for each inoculation-by-stage-by-time condition were used.

mRNA sequencing was performed at the Biotechnology Resource Center, Institute of Biotechnology, Cornell University (http://www.biotech.cornell.edu/). For each sample, 2 µg of total RNA was used to prepare mRNA libraries, using the QuantSeq 3' RNA-seq kit by Lexogen (Moll et al., 2014) to generate sequences close to the 3' end of polyadenylated RNAs. High-throughput sequencing was performed using the Illumina NextSeq 500 platform, yielding single-end 75 base pair (bp) reads.

Transcriptome data analysis

The 3' RNA-seq data were processed according to the data analysis workflow recommended by Moll et al. (2014). Raw reads were trimmed and filtered for quality and adaptor contamination using BBDuk v37.36 (http://sourceforge.net/projects/bbmap/). The first 12 bp were trimmed from each sequence read. Subsequently, quality trimming of reads was performed using the Phred algorithm, set to Q20. Trimmed reads with a length of less than 35 bp were discarded. FastQC v0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to evaluate the quality of reads before and after trimming.

High-quality reads were mapped to the pepper reference genome *C. annuum* cv. CM334 v1.55 (Kim et al., 2014) available at the Sol Genomics Network website (https://solgenomics.net/organism/Capsicum_annuum/genome) using STAR v2.4.2a (Dobin et al., 2013). Mapped reads were quantified by HTSeq-count (Anders et al., 2015) to obtain digital gene expression read counts from uniquely aligned reads. In order to adequately capture reads mapping to 3' ends, the GTF file was modified to include 300 bp extensions after the CDS (coding sequences) using the BEDTools *slop* function (Aaron and Quinlan, 2010; Quinlan, 2014) to increase the size of each feature in the file.

Differentially expressed genes

Analysis of differentially expressed genes (DEGs) was performed for both fruit development stages in response to anthracnose interaction. The DEGs (FDR<0.05) were determined for pairwise comparisons between mock and inoculated samples, and they were analyzed in two different ways: 1) using DESeq2 (Anders & Huber, 2010) comparing unripe and ripe fruits; 2) using edgeR (Robinson et al., 2010) at each time point (24, 48, 72 and 96 HPI) in unripe and ripe fruits. Prior to DEG analysis using DESeq2 and edgeR, library size normalization was conducted using the calcNormFactors function in edgR and accounted for using sample-specific size factors in the DESeq function of DESeq2. Sequence length normalization, as is for example used when calculating expression in fragments per kilobase of transcript per million mapped reads (FPKM), is not appropriate here, particularly since the 3' sequencing method generates only one fragment per transcript (Moll et al., 2014). In addition, we annotated the top 10 up-regulated genes at each time point analyzed.

All samples were included in a principal component analysis (PCA) and hierarchical clustering heatmap analyses using DESeq2. Venn diagrams were developed using Calculate and Draw custom Venn Diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/). Gene ontology (GO) overrepresentation analyses for up-regulated genes in response to fungal interaction of unripe and ripe fruits were performed using topGO R package ($p<0.05$) (Alexa et al., 2006). Additional hierarchical clustering of the 100 most variable genes across the samples was conducted using the pheatmap function. All analyzes were performed in R.

Identification and annotation of capsidiol biosynthesis-related genes

Protein coding sequences of 5-epi-aristolochene synthase (NCBI accession number: O65323.1) and 5-epi-aristolochene 1,3-dihydroxylase (NCBI accession number: Q94FM7.2) genes previously described in plants (Back et al., 1998; Takahashi et al., 2005) were used as query sequences to search for their respective orthologs in our pepper transcriptome dataset. A manual annotation was performed for capsidiol biosynthesis-related genes in the *C. annuum* transcriptome using tBlastN at NCBI (UniProtKB/Swissprot) and BlastN in the Sol Genomics Network for pepper databases (https://solgenomics.net/tools/blast/?db_id=217), since we observed that several genes related to capsidiol biosynthesis were incorrectly annotated in the existing *C. annuum* genome database. We have used a cutoff of minimum score of 500 as parameters, as well as requiring the presence of the conserved domain

(pfam03936 and pfam00067) in the protein sequence to manually annotate capsidiol-related genes.

RT-qPCR transcriptional validation

Primers from capsidiol-related genes (*CaEAS* and *CaEAH*) were designed using CLC Genomics Workbench 9.5.3 (https://www.qiagenbioinformatics.com/) to amplify nucleotide sequences ranging from 100 to 207 bp with annealing Tm of 55 ^{o}C ± 2 ^{o}C (see Supplementary Table 1).

Supplementary Table 1. Primer sequences of candidate genes for capsidiol biosynthesis used for real time quantitative PCR analyses in pepper fruits.

Gene name	Gene annotation	Gene ID	Acession number	Primer Forward and Reverse	Average efficiency (%)	Amplicon size (bp)
CaEAS	*5-epi-aristolochene synthase*	*CA02g09520*	O65323.1	F - ATGGCCTCAGTTGTAGTTGG R - AAACGATCACCCCATAGACT	110	101
CaEAS	*5-epi-aristolochene synthase*	*CA12g05030*	O65323.1	F - ATGGCCTCAGTTGCAGT R - AACGATCACCCCATAGACT	110	100
CaEAH	*5-epi-aristolochene 1,3-dihydroxylase*	*CA01g05990*	Q94FM7.2	F - AGAATCACCAAACACTCCCA R - CATGGACCTGGAGGCAA	110	132
CaEAH	*5-epi-aristolochene 1,3-dihydroxylase*	*CA12g05070*	Q94FM7.2	F - CTCTCCCAAAATGCAATTCTTC R - GGAGGCAATTTTTTGGTTTGG	110	111
CaUEP	*Ubiquitin extension protein*	*CA12g21050*	DQ975458	F - CCGACTACAACATCCAGAAG R - CACACTCAGCATTAGGACAC	100	207
CaEF1 α	*Elongation factor 1-alpha*	*CA06g07620*	AY496125	F - TGAAGAATGGTGATGCTGGC R - GACAACACCAACAGCAACAG	100	132

Complementary DNAs (cDNAs) of all samples were synthesized using GoScript™ Reverse Transcription System Kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions, in a final volume of 20 µl and using 2.5 µg of total RNA.

Transcriptional profiles of genes were analyzed using ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) equipment. The reactions consisted of a total volume of 15 µL with 7.5 µL of GoTaq® qPCR Master Mix (Promega, Madison, Wisconsin, USA), 0.5 µL of forward and reverse primer (10 µM), 1 µL of cDNA (25 ng/µL) and 5.5 µL of nuclease-free water. The amplification conditions were 94 ºC for 5 min, followed by 40 cycles of 94 ºC for 30 s, 55 ºC for 45 s and 72 ºC for 30 s, followed by melting curve analysis to verify the presence of a single RT-qPCR product. All reactions were performed with three biological replicates, and followed MIQE guidelines for RT-qPCR experiments (Bustin et al., 2009).

Relative expression levels of capsidiol-related genes were analyzed by GenEx 6.1 software (MultiD Analyses AB, Göteborg, Sweden) according to the default parameters. Gene

normalization analysis was performed using *CaEF1α* and *CaUEP* gene expression profiles as reference genes (Bin et al., 2012). The value 1 was established to the library 24 HPI mock inoculation from unripe and ripe fruits, as calibrator samples. The amplification efficiency was calculated using LinReg (Ramakers et al., 2003) (see Supplementary Table 1).

Results

Transcriptome sequencing and data mining

A total of 386,333,505 raw reads were obtained from 3' RNA-sequencing in unripe and ripe fruits of *C. annuum* in response to *C. gloeosporioides* inoculation (Table 1). In summary, 205,118,058 and 181,215,447 reads from unripe and ripe fruits, respectively, were trimmed.

A high proportion of quality reads (362,449,581 - 94%) were mapped to the reference genome of *C. annuum* (Kim et al., 2014) to estimate the transcriptional activity of genes under anthracnose (Table 1). Also, a large number of uniquely mapped reads (283,656,019 - 77%) was obtained (Table 1) and 30,242 active genes (87%) in unripe and ripe fruits were identified in the transcriptome from the public genome (Kim et al., 2014) available at the Sol Genomics Network website.

Transcriptome samples clustering analysis

Clustering distance was evaluated for all 3' RNA-seq pepper samples using PCA and heatmap analysis. In the principal component analysis, components 1 and 2 explained 90% of data variance for treatment (mock vs. inoculated), stage (unripe vs. ripe), and time post-inoculation (24, 48, 72 and 96 h) (see Supplementary Fig. 2A and 2B).

We observed two distinct clusters for unripe and ripe fruits showing potential differences in the response to anthracnose (see Supplementary Fig. 2A and 2B). Ripe fruits showed distinct clusters for mock and inoculated treatment while unripe fruits showed clusters for early (24 and 48 HPI) and later (72 and 96 HPI) times post-inoculation. Mock and inoculated samples at 24 and 48 HPI showed greater initial transcriptional responses in ripe fruits. Unripe fruits showed greater responses at 72 and 96 HPI for inoculated samples, as well as for ripe fruits at 96 HPI (see Supplementary Fig. 2B), in which the degree of response to fungal inoculation was clearly greater than other post-inoculation times. Also, we identified a cluster with all mock and inoculated samples at 24 and 48 HPI for unripe fruits. Biological replicates showed no sample outliers.

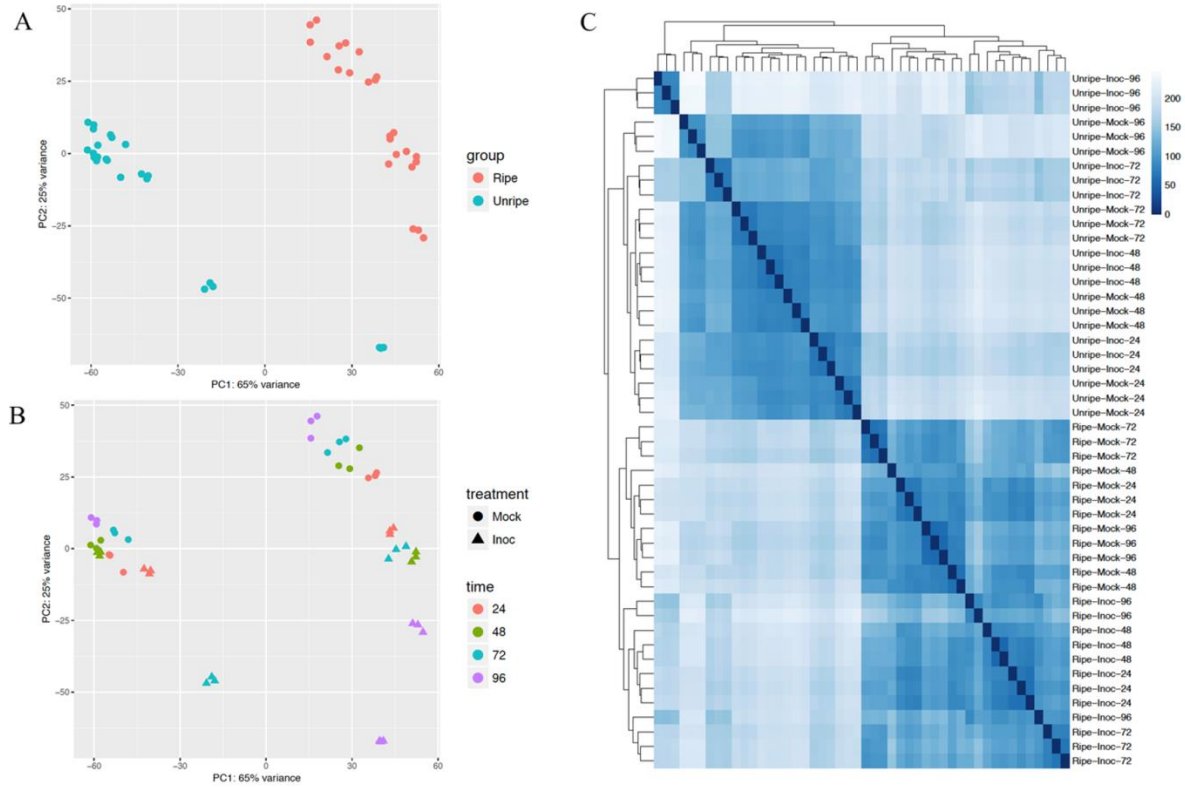
Table 1. *C. annuum* transcriptome data analysis.

Treatment	Inoculation	Time post-inoculation (h)	Repeat	Raw reads	High-quality reads	% of high-quality reads	Uniquely mapped reads	% mapped reads	% feature reads
Unripe	Mock	24	1	12,990,889	12,184,527	94	9,584,189	79	63
			2	3,966,942	3,699,549	93	2,889,847	78	63
			3	14,606,216	13,862,879	95	11,085,840	80	64
		48	1	12,995,915	12,130,332	93	9,418,354	78	63
			2	6,933,438	6,483,410	94	5,120,107	79	65
			3	12,525,732	11,858,690	95	9,241,647	78	64
		72	1	7,424,660	7,009,182	94	5,419,311	77	65
			2	7,479,304	7,054,619	94	5,468,677	78	65
			3	9,634,301	9,028,962	94	7,127,772	79	66
		96	1	13,181,365	12,285,148	93	9,650,275	79	66
			2	10,527,138	9,682,324	92	7,691,708	79	67
			3	6,523,917	6,059,695	93	4,745,300	78	66
	Inoc	24	1	9,850,667	9,198,742	93	7,080,806	77	62
			2	6,827,939	6,508,457	95	5,112,657	79	64
			3	12,611,459	11,800,483	94	9,387,955	80	65
		48	1	7,627,569	7,215,392	95	5,744,296	80	65
			2	9,035,442	8,589,423	95	6,844,742	80	65
			3	10,053,727	9,431,511	94	7,505,322	80	65
		72	1	6,154,448	5,786,816	94	4,399,277	76	63
			2	3,734,928	3,534,320	95	2,710,166	77	64
			3	4,443,351	4,183,856	94	3,276,723	78	65
		96	1	5,386,086	5,030,122	93	2,317,923	46	65
			2	3,554,552	3,271,355	92	1,573,903	48	66
			3	7,048,073	6,563,789	93	2,771,888	42	65
Average per unripe samples				8,546,586	8,018,899	94	6,090,362	74	65
Ripe	Mock	24	1	8,830,408	8,342,243	94	6,827,228	82	67
			2	12,485,224	11,782,806	94	9,628,905	82	66
			3	9,733,605	9,063,522	93	7,485,642	83	68
		48	1	5,781,929	5,432,891	94	4,256,856	78	68
			2	7,052,114	6,672,680	95	5,591,035	84	66
			3	12,456,326	11,779,954	95	9,928,591	84	67
		72	1	11,630,104	10,681,045	92	8,518,160	80	62
			2	5,431,606	4,961,725	91	3,924,629	79	62
			3	6,594,686	6,104,440	93	4,859,535	80	61
		96	1	9,650,320	9,167,362	95	7,558,657	82	66
			2	8,751,650	8,288,542	95	6,808,386	82	67
			3	9,525,509	9,034,162	95	7,528,533	83	66
	Inoc	24	1	4,264,122	4,026,402	94	3,349,044	83	66
			2	4,531,714	4,282,299	94	3,527,047	82	67
			3	7,987,388	7,608,792	95	6,381,044	84	67
		48	1	4,688,392	4,365,719	93	3,484,262	80	65
			2	6,353,887	5,982,699	94	4,962,403	83	66
			3	7,389,005	6,990,993	95	5,799,937	83	66
		72	1	5,355,723	4,910,216	92	3,733,959	76	58
			2	6,281,458	5,610,643	89	4,337,099	77	61
			3	6,021,957	5,645,650	94	4,470,158	79	61
		96	1	4,803,738	4,575,877	95	3,385,361	74	65
			2	7,213,368	6,829,997	95	5,324,138	78	67
			3	8,401,214	7,855,339	94	5,816,725	74	68
Average per ripe samples				7,550,644	7,083,167	94	5,728,639	81	65
Total				386,333,505	362,449,581		283,656,019		

Clustering distance was evaluated for all 3’ RNA-seq pepper samples using PCA and heatmap analysis. In the principal component analysis, components 1 and 2 explained 90% of data variance for treatment (mock vs. inoculated), stage (unripe vs. ripe), and time post-inoculation (24, 48, 72 and 96 h) (see Supplementary Fig. 2A and 2B).

We observed two distinct clusters for unripe and ripe fruits showing potential differences in the response to anthracnose (see Supplementary Fig. 2A and 2B). Ripe fruits showed distinct clusters for mock and inoculated treatment while unripe fruits showed clusters for early (24 and 48 HPI) and later (72 and 96 HPI) times post-inoculation. Mock and inoculated samples at 24 and 48 HPI showed greater initial transcriptional responses in ripe fruits. Unripe fruits showed greater responses at 72 and 96 HPI for inoculated samples, as well as for ripe fruits at 96 HPI (see Supplementary Fig. 2B), in which the degree of response to fungal inoculation was clearly greater than other post-inoculation times. Also, we identified a cluster with all mock and inoculated samples at 24 and 48 HPI for unripe fruits. Biological replicates showed no sample outliers.

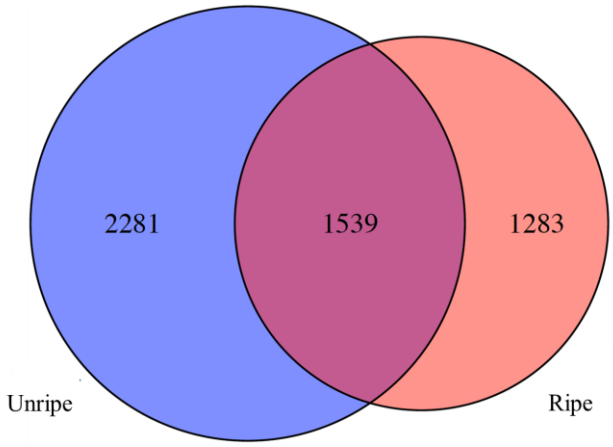
Heatmap analysis showed a concordance with PCA analysis for unripe and ripe fruits (see Supplementary Fig. 2C). Clusters were observed for fruit development stages based on the inoculation treatment for ripe fruits and based on time post-inoculation for unripe fruits. Three biological unripe replicates at 96 HPI were the most distinct from the other samples.



Supplementary Figure 2. Pepper transcriptome clustering analysis among 48 libraries. PCA analysis for fruit development stages: unripe (blue color) and ripe (red color) (A); and for inoculated with *C. gloeosporioides* (inoc - triangle) and with water (mock - circles) and time post-inoculation (24, 48, 72 and 96 h) comparisons (B). Heatmap analysis of cluster distance for all pepper samples (C). The intensity of blue color is proportional to the similarities between samples.

Differential gene expression profiles among fruit development stages in response to fungal interaction

A panel of statistically significant DEGs (FDR<0.05) were obtained using DESeq2 analysis in response to anthracnose. The Venn diagram of *C. annuum* transcripts for each fruit development stage showed an overlap between unripe and ripe fruits (1,539), but also revealed distinct stage-specific expression, in which 2,281 DEGs were unique to unripe pepper fruits, while 1,283 transcripts were unique to ripe fruits (see Supplementary Fig 3). In this way, there was a higher number of regulated genes specific to the unripe and ripe pepper fruits under fungal interaction.



Supplementary Figure 3. Venn diagram of differentially expressed genes for inoculation (mock x inoc) from unripe (blue) and ripe (red) pepper fruit transcriptomes in response to *C. gloeosporioides*.

In addition, we performed another transcript abundance analysis using edgeR to identify genes that were significantly (FDR<0.05) up- (Fig. 1A) and down-regulated (Fig. 1B) from both fruit stages and at each time post-inoculation (24 to 96 HPI). A different gene expression profile was observed in response to fungal interaction at each time-point analyzed. The highest total number of up-regulated genes was found in unripe (4,845) compared with ripe fruits (2,560) (Fig. 1A and 1C). Similar result was observed for down-regulated, in which 4,720 genes were observed for unripe and 1,762 for ripe fruits (Fig. 1B and 1D).

The total number of genes at 72 and 96 HPI in unripe fruits increased by more than 2-fold in relation to ripe fruits in the same time post-inoculation. The highest number of unique DEGs were observed at 96 HPI for both fruit development stages (Fig. 1). However, unripe fruits showed more down- (2,105) than up-regulated (1,788) genes at 96 HPI (Fig. 1A and

1B). The opposite occurred for ripe fruits in which the number of unique genes was higher for up- (803) than down-regulated (535) at 96 HPI (Fig. 1C and 1D).

Also, the number of up- and down-regulated unique genes at 24, 48 and 72 HPI showed the opposite pattern. Unripe fruits showed more up- (461, 228 and 362, respectively) than down-regulated unique genes (405, 142 and 332, respectively) (Fig 1A and 1B). On the other hand, ripe fruits revealed more down- (499, 120 and 272, respectively) than up-regulated unique genes (390, 93 and173, respectively) (Fig. 1C and 1D). In general, the number of DEGs showed little overlap at each time post-inoculation, indicating high distinct transcripts for each fruit development stage, except for 72 and 96 HPI up- and down-regulated unripe genes.

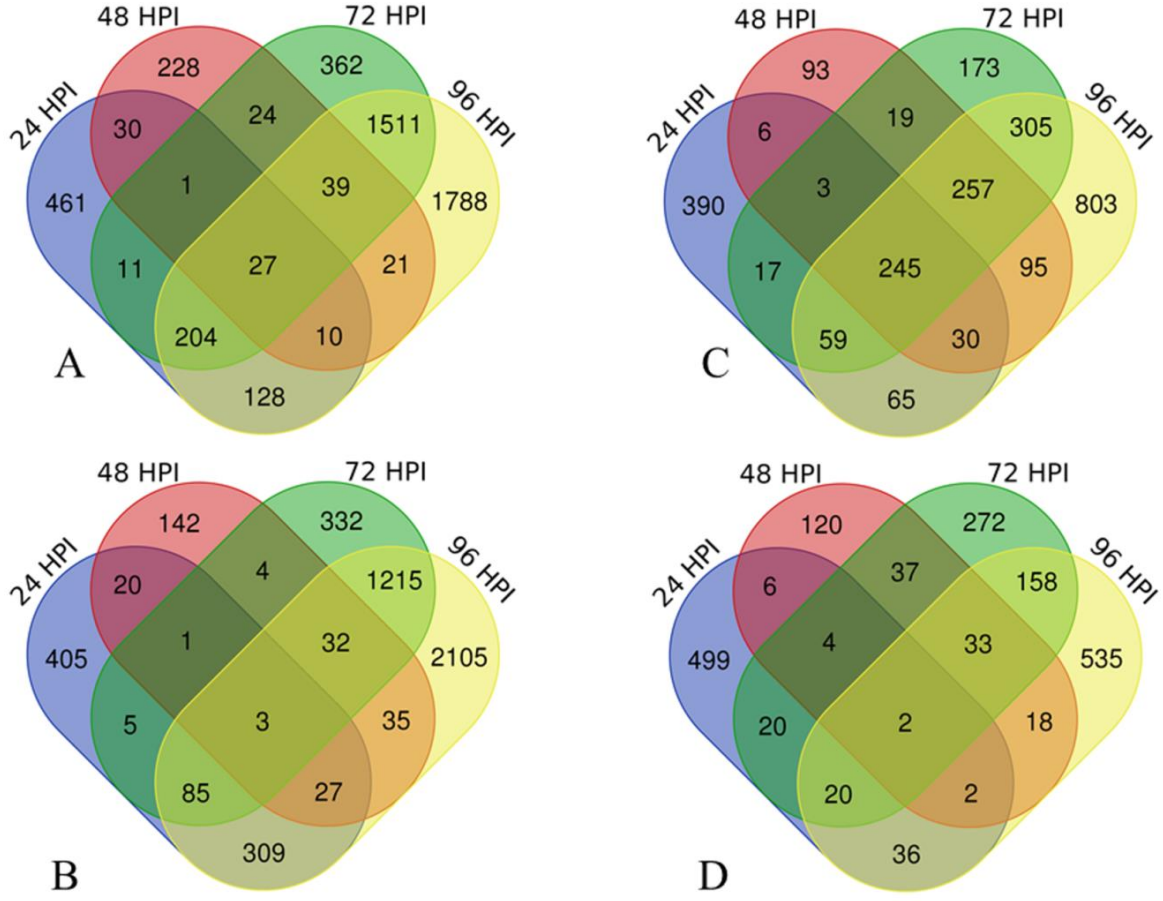


Figure 1. Venn diagrams of differentially expressed genes for inoculation (mock x inoc) in unripe (left) and ripe (right) pepper fruit tissues. Genes up- (A) and down-regulated (B) in unripe fruit inoculated with *C. gloeosporioides* (24, 48, 72 and 96 HPI). Genes up- (C) and down-regulated (D) in ripe fruit inoculated with *C. gloeosporioides* (24, 48, 72 and 96 HPI).

We also listed the top 10 differentially expressed genes for each time point post-inoculation in unripe and ripe fruits (Table 2). Ripe fruits showed defense response genes at

all post-inoculation times, including binding protein, resistance protein, pathogenesis-related protein, pepper esterase, ethylene response factor, cytochrome P450, fatty acid, 5-epi-aristolochene synthase (*EAS*) and 5-epi-aristolochene 1,3-dihydroxylase (*EAH*) genes. Seven candidate genes for capsidiol biosynthesis were also observed in this list (Table 2), three for *EAS* (*CA02g09520, CA12g05030* and *CA12g05260*) and four for *EAH* (*CA01g05990, CA02g09570, CA12g05070, CA12g05140*).

In unripe fruits, the defense response genes were highly expressed, especially at 96 HPI, including *CA02g09520* capsidiol-related genes (Table 2). However, in ripe fruits, we observed more up-regulated genes related to capsidiol biosynthesis than in unripe fruits (Table 2). Among the up-regulated genes in ripe fruits under pathogen inoculation, we found one *EAS* (*CA02g09529*) and five *EAH* (*CA01g05990, CA02g09570, CA12g05030, CA12g05070* and *CA12g05140*) genes at 24 HPI, one *EAS* (*CA12g05260*) and one *EAH* (*CA01g05990*) gene at 72 HPI (2 genes), and two *EAS* (*CA02g09520* and *CA12g05030*) genes at 96 HPI.

Transcriptome gene enrichment analysis

In order to verify pepper metabolic pathways that were enriched under anthracnose inoculation, we performed a gene enrichment analysis using topGO ($p<0.05$). The dataset for this analysis was only genes up-regulated in response to fungal interaction. We observed 32 and 27 significantly descriptive GO terms in the biological processes level that were overrepresented under *C. gloeosporioides* for unripe and ripe fruits, respectively. In the molecular function level, 48 and 41 significantly descriptive GO terms were enriched for unripe and ripe fruits, respectively (see Supplementary Table 2).

Defense metabolic pathways were enriched in the biological processes level for both fruits development stages, such as defense response to fungus, L-phenylalanine metabolic process, chitin catabolic process and isoprenoid biosynthetic process. Another defense enriched pathways observed for ripe fruits was ethylene-activated signaling pathway. For molecular function, some of the significant enriched pathways for unripe and ripe fruits were protein serine/threonine kinase activity, related to plant defense response to a pathogen, and chitinase activity, connected with fungus digestion cell walls, potent inhibitors of fungal growth.

Table 2. List of the top 10 up-regulated genes for each time point post-inoculation in unripe and ripe fruits.

Gene ID	Annotation	p-value
UNRIPE		
24 HPI		
CA09g18430	Unknown protein	8.01E-99
CA02g28000	Detected protein of unknown function	7.07E-89
CA04g04080	Phytoene synthase	5.24E-87
CA11g18070	Serine carboxypeptidase III	2.79E-73
CA04g21250	Detected protein of confused Function	1.57E-72
CA07g15720	CASP-like protein VIT_01s0010g01870-like	1.66E-63
CA03g06040	Cyanidin-3-O-glucoside 2-O-glucuronosyltransferase-like	5.57E-63
CA08g13840	Germin-like protein subfamily 1 member 20	2.42E-57
CA10g09450	Auxin efflux carrier component, auxin transport protein	9.76E-57
CA05g02660	PREDICTED: BURP domain-containing protein 17-like	3.66E-47
48 HPI		
CA08g17070	18.5 kDa class I heat shock protein-like	3.69E-69
CA03g08390	Translocator protein homolog	8.30E-66
CA03g30260	Heat shock protein, putative	3.60E-47
CA09g08990	Glycerol-3-phosphate acyltransferase 6	1.07E-45
CA11g18770	Ripening-related protein grip22	5.26E-42
CA02g16190	Detected protein of unknown function	3.22E-40
CA03g21390	Heat shock protein 26 (Type I)	4.17E-40
CA08g07920	BAG family molecular chaperone regulator 6-like	7.60E-40
CA03g27140	Detected protein of unknown function	6.04E-36
CA05g01800	Universal stress protein MJ0531-like isoform 1	3.09E-35
72 HPI		
CA05g04810	Zeatin O-glucosyltransferase-like	0
CA05g04830	Multiprotein-bridging factor 1c-like	0
CA07g11250	1-aminocyclopropane-1-carboxylic acid oxidase	0
CA09g04530	Ca^{2+}-binding protein 1	0
CA12g06260	UDP-glucose:flavonoid 3-O-glucosyltransferase	0
CA02g04610	Tau class glutathione transferase GSTU15	0
CA03g03950	UDP-sugar:glycosyltransferase	0
CA02g09520*	5-epi-aristolochene synthase	0
CA02g22240	Unknown protein	0
CA05g03050	Cytochrome P450 CYP736A54	0
96 HPI		
CA05g03050	Cytochrome P450 CYP736A54	0.00E+00
CA11g14520	Cytochrome P450	0.00E+00
CA04g13070	Pathogen-related protein-like	7.53E-306
CA03g35110	DNA binding protein homolog	3.77E-285
CA02g09520*	5-epi-aristolochene synthase	1.31E-274
CA02g04360	Ethylene response factor ERF2	6.86E-263
CA08g04180	Omega-6 fatty acid desaturase, endoplasmic reticulum isozyme 2-like	8.72E-257
CA07g11250	1-aminocyclopropane-1-carboxylic acid oxidase	6.02E-256
CA12g22670	Protein ECERIFERUM 1-like	1.20E-251
CA02g15780	Polyphenol oxidase	7.66E-245
RIPE		
24 HPI		
CA08g18080	Allene oxide synthase	5.71E-91
CA02g09570*	5-epi-aristolochene 1,3-dihydroxylase	4.54E-81
CA12g05070*	5-epi-aristolochene 1,3-dihydroxylase	1.99E-74
CA01g05990*	5-epi-aristolochene 1,3-dihydroxylase	1.80E-61
CA12g05030*	5-epi-aristolochene synthase	2.04E-57
CA05g20080	Isopentenyl diphosphate isomerase	4.18E-57
CA03g35110	DNA binding protein homolog	1.01E-54
CA12g05140*	5-epi-aristolochene 1,3-dihydroxylase	3.91E-53

CA02g09520*	5-epi-aristolochene synthase	2.96E-51

Table 2 continuation

Gene ID	Annotation	p-value
CA02g22240	Unknown protein	3.27E-51
48 HPI		
CA05g17820	UTP:alpha-D-glucose-1-phosphate uridylyltransferase	1.26E-75
CA03g01800	Pleiotropic drug resistance protein 1-like	4.19E-65
CA07g11250	1-aminocyclopropane-1-carboxylic acid oxidase	1.64E-60
CA05g18370	Unknown protein	1.86E-55
CA08g18080	Allene oxide synthase (Fragment)	8.15E-54
CA09g03220	Pathogenesis-related leaf protein 4-like	2.61E-51
CA01g04790	Invertase	5.31E-49
CA04g10620	Pepper esterase	5.37E-46
CA03g04260	Pathogenesis-related protein STH-2-like	6.63E-46
CA02g04360	Ethylene response factor ERF2	2.76E-45
72 HPI		
CA02g15780	Polyphenol oxidase	9.08E-173
CA02g00210	Carbonic anhydrase	3.94E-137
CA03g03950	UDP-sugar:glycosyltransferase	1.15E-121
CA08g10220	Wound-induced protein WIN2	6.72E-120
CA08g18080	Allene oxide synthase	1.95E-112
CA04g10620	Pepper esterase	1.98E-109
CA12g05260*	5-epi-aristolochene synthase	2.02E-104
CA03g29750	Em protein H5-like	4.45E-103
CA12g05270	UV-induced sesquiterpene cyclase	3.71E-99
CA01g05990*	5-epi-aristolochene 1,3-dihydroxylase	1.13E-98
96 HPI		
CA05g04830	Multiprotein-bridging factor 1c-like	2.19E-157
CA05g03050	Cytochrome P450 CYP736A54	9.58E-148
CA02g15780	Polyphenol oxidase	1.13E-147
CA08g18080	Allene oxide synthase	2.49E-147
CA08g04180	PREDICTED: omega-6 fatty acid desaturase, endoplasmic reticulum Isozyme 2-like	1.16E-146
CA11g14520	Cytochrome P450	9.97E-146
CA12g05030*	5-epi-aristolochene synthase	2.29E-140
CA07g11250	1-aminocyclopropane-1-carboxylic acid oxidase	3.38E-139
CA02g00210	Carbonic anhydrase	2.39E-138
CA02g09520*	5-epi-aristolochene synthase	6.56E-136

*candidate genes for capisidiol biosynthesis up-regulated under *C. gloeosporioides* interaction.

Supplementary Table 2. Enriched GO terms of up-regulated genes in unripe and ripe fruits in response to *C. gloeosporioides* inoculation.

GO ID	Term	p-value
UNRIPE		
Biological Process		
GO:0009070	serine family amino acid biosynthetic pr...	8.10E-06
GO:0006534	cysteine metabolic process	5.00E-05
GO:0016567	protein ubiquitination	7.30E-05
GO:0006563	L-serine metabolic process	0.00011
GO:0009073	aromatic amino acid family biosynthetic ...	0.00021
GO:0050832	defense response to fungus	0.00022
GO:0042742	defense response to bacterium	0.00022
GO:0006596	polyamine biosynthetic process	0.00033
GO:0048544	recognition of pollen	0.00047
GO:0042823	pyridoxal phosphate biosynthetic process	0.00057
GO:0006558	L-phenylalanine metabolic process	0.00068
GO:0006525	arginine metabolic process	0.00068
GO:0008652	cellular amino acid biosynthetic process	0.00107

GO:0051205	protein insertion into membrane	0.00212
GO:0006090	pyruvate metabolic process	0.0026

Supplementary Table 2 continuation

GO ID	Term	p-value
GO:0009408	response to heat	0.00302
GO:0006099	tricarboxylic acid cycle	0.00332
GO:0006720	isoprenoid metabolic process	0.00459
GO:0046417	chorismate metabolic process	0.00684
GO:0019419	sulfate reduction	0.00684
GO:0034613	cellular protein localization	0.00944
GO:0006413	translational initiation	0.014
GO:0016311	dephosphorylation	0.01514
GO:0006591	ornithine metabolic process	0.0194
GO:0042255	ribosome assembly	0.0194
GO:0006002	fructose 6-phosphate metabolic process	0.02307
GO:0005992	trehalose biosynthetic process	0.02559
GO:0006032	chitin catabolic process	0.02559
GO:0045454	cell redox homeostasis	0.03605
GO:0006222	UMP biosynthetic process	0.03668
GO:0051188	cofactor biosynthetic process	0.04192
GO:0006614	SRP-dependent cotranslational protein ta...	0.04365
Molecular Function		
GO:0030170	pyridoxal phosphate binding	5.50E-06
GO:0043565	sequence-specific DNA binding	1.10E-05
GO:0005509	calcium ion binding	7.50E-05
GO:0046912	transferase activity, transferring acyl ...	0.00012
GO:0003700	transcription factor activity, sequence-...	0.00023
GO:0004842	ubiquitin-protein transferase activity	0.00023
GO:0004385	guanylate kinase activity	0.00041
GO:0016705	oxidoreductase activity, acting on paire...	0.00049
GO:0004722	protein serine/threonine phosphatase act...	0.00068
GO:0005506	iron ion binding	0.00074
GO:0004298	threonine-type endopeptidase activity	0.00097
GO:0004775	succinate-CoA ligase (ADP-forming) activ...	0.00156
GO:0016410	N-acyltransferase activity	0.00288
GO:0003743	translation initiation factor activity	0.00385
GO:0003924	GTPase activity	0.00385
GO:0004222	metalloendopeptidase activity	0.00457
GO:0016597	amino acid binding	0.00529
GO:0004674	protein serine/threonine kinase activity	0.00545
GO:0004158	dihydroorotate oxidase activity	0.00555
GO:0004106	chorismate mutase activity	0.00555
GO:0008898	S-adenosylmethionine-homocysteine S-meth...	0.00555
GO:0051082	unfolded protein binding	0.00595
GO:0016841	ammonia-lyase activity	0.00662
GO:0020037	heme binding	0.00664
GO:0016407	acetyltransferase activity	0.00674
GO:0016831	carboxy-lyase activity	0.00774
GO:0051087	chaperone binding	0.00935
GO:0008483	transaminase activity	0.0155
GO:0004549	tRNA-specific ribonuclease activity	0.01582
GO:0004019	adenylosuccinate synthase activity	0.01582
GO:0004617	phosphoglycerate dehydrogenase activity	0.01582
GO:0004568	chitinase activity	0.01696
GO:0030976	thiamine pyrophosphate binding	0.01739
GO:0051536	iron-sulfur cluster binding	0.01894
GO:0000287	magnesium ion binding	0.01949
GO:0015662	ATPase activity, coupled to transmembran...	0.02064
GO:0016628	oxidoreductase activity, acting on the C...	0.02708
GO:0035639	purine ribonucleoside triphosphate bindi...	0.02927

GO:0004556	alpha-amylase activity	0.03007
GO:0004571	mannosyl-oligosaccharide 1,2-alpha-manno...	0.03007

Supplementary Table 2 continuation

GO ID	Term	p-value
GO:0008083	growth factor activity	0.03007
GO:0004664	prephenate dehydratase activity	0.03007
GO:0030151	molybdenum ion binding	0.03007
GO:0009001	serine O-acetyltransferase activity	0.03007
GO:0003872	6-phosphofructokinase activity	0.03331
GO:0016758	transferase activity, transferring hexos...	0.04245
GO:0008312	7S RNA binding	0.04767
GO:0050662	coenzyme binding	0.04789
RIPE		
Biological Process		
GO:0016567	protein ubiquitination	4.70E-06
GO:0009070	serine family amino acid biosynthetic pr...	4.70E-05
GO:0050832	defense response to fungus	0.00014
GO:0042742	defense response to bacterium	0.00014
GO:0048544	recognition of pollen	0.00014
GO:0006596	polyamine biosynthetic process	0.00018
GO:0006563	L-serine metabolic process	0.00041
GO:0006558	L-phenylalanine metabolic process	0.00042
GO:0006032	chitin catabolic process	0.00057
GO:0016998	cell wall macromolecule catabolic proces...	0.0024
GO:0009095	aromatic amino acid family biosynthetic ...	0.00285
GO:0006412	translation	0.00603
GO:0006662	glycerol ether metabolic process	0.01136
GO:0009435	NAD biosynthetic process	0.0115
GO:0006536	glutamate metabolic process	0.0115
GO:0055114	oxidation-reduction process	0.01194
GO:0042823	pyridoxal phosphate biosynthetic process	0.01582
GO:0007205	protein kinase C-activating G-protein co...	0.01678
GO:0008299	isoprenoid biosynthetic process	0.01913
GO:0006816	calcium ion transport	0.02479
GO:0006096	glycolytic process	0.02678
GO:0006820	anion transport	0.03397
GO:0008283	cell proliferation	0.0477
GO:0000103	sulfate assimilation	0.0477
GO:0009873	ethylene-activated signaling pathway	0.0477
GO:0006542	glutamine biosynthetic process	0.0477
GO:0019318	hexose metabolic process	0.04793
Molecular Function		
GO:0004674	protein serine/threonine kinase activity	2.90E-08
GO:0048037	cofactor binding	3.00E-07
GO:0003735	structural constituent of ribosome	1.50E-06
GO:0005506	iron ion binding	1.90E-06
GO:0016705	oxidoreductase activity, acting on paire...	3.90E-06
GO:0005509	calcium ion binding	6.80E-06
GO:0020037	heme binding	1.30E-05
GO:0004842	ubiquitin-protein transferase activity	3.10E-05
GO:0004568	chitinase activity	0.00022
GO:0015662	ATPase activity, coupled to transmembran...	0.00031
GO:0043565	sequence-specific DNA binding	0.00032
GO:0003700	transcription factor activity, sequence-...	0.00039
GO:0046912	transferase activity, transferring acyl ...	0.00044
GO:0016857	racemase and epimerase activity, acting ...	0.00045
GO:0016831	carboxy-lyase activity	0.00095
GO:0008061	chitin binding	0.00157
GO:0000287	magnesium ion binding	0.00313
GO:0016841	ammonia-lyase activity	0.00377

GO:0004722	protein serine/threonine phosphatase act...	0.00386
GO:0004781	sulfate adenylyltransferase (ATP) activi...	0.00406

Supplementary Table 2 continuation

GO ID	Term	p-value
GO:0046915	transition metal ion transmembrane trans...	0.00506
GO:0004143	diacylglycerol kinase activity	0.00982
GO:0030060	L-malate dehydrogenase activity	0.01135
GO:0004385	guanylate kinase activity	0.01166
GO:0004124	cysteine synthase activity	0.01166
GO:0008378	galactosyltransferase activity	0.01605
GO:0003951	NAD+ kinase activity	0.02004
GO:0008483	transaminase activity	0.02184
GO:0004402	histone acetyltransferase activity	0.0221
GO:0004556	alpha-amylase activity	0.02234
GO:0004775	succinate-CoA ligase (ADP-forming) activ...	0.02234
GO:0008083	growth factor activity	0.02234
GO:0016842	amidine-lyase activity	0.02234
GO:0004664	prephenate dehydratase activity	0.02234
GO:0008977	prephenate dehydrogenase activity	0.03567
GO:0004665	prephenate dehydrogenase (NADP+) activit...	0.03567
GO:0008312	7S RNA binding	0.03567
GO:0004866	endopeptidase inhibitor activity	0.0358
GO:0008308	voltage-gated anion channel activity	0.03684
GO:0004743	pyruvate kinase activity	0.03684
GO:0030955	potassium ion binding	0.03684

Top 100 most heterogeneously expressed genes

We selected the top 100 genes that had the most variable transcription across samples in the 3' RNA-seq dataset to verify if there was a pattern between up- and down-regulated genes (Fig. 2). Our results allowed us to observe the presence of at least three well-defined groups of genes in the gene expression profiles.

One group was composed by 49 genes that were induced only for unripe fruits, except for inoculated samples at 96 HPI. In general, most of the genes were related with the chlorophyll a/b binding protein that was already described as being related to appressoria formation in the pepper-fungal interaction (S3 Table). The second group showed an opposite pattern, where 14 were up-regulated genes in ripe fruits and down-regulated in unripe fruits. In the last group, we observed 37 genes that were down-regulated in all mock samples (unripe and ripe fruits) and at early time points (24 and 48 HPI) under fungal interaction in unripe fruits. These same genes were up-regulated at all time points under *C. gloeosporioides* inoculation in ripe fruits and particularly at 72 and 96 HPI in unripe fruits.

Interestingly, most of the genes in this group were involved in response to pathogen attack, such as cytochrome P450, pathogen-related protein-like, pepper esterase, and ethylene response factor (see Supplementary Table 3). More importantly, six candidate genes related to capsidiol biosynthesis were identified in this group: three *EAS* genes (*CA01g05990*,

CA02g09520 and *CA12g0503*) and three *EAH* genes (*CA12g05070*, *CA12g05140* and *CA02g09570*). These genes were up-regulated under fungal interaction for both fruit development stages (unripe and ripe), especially at 96 HPI. For this transcriptome study, these capsidiol-related genes were considered as good candidate genes for capsidiol biosynthesis.

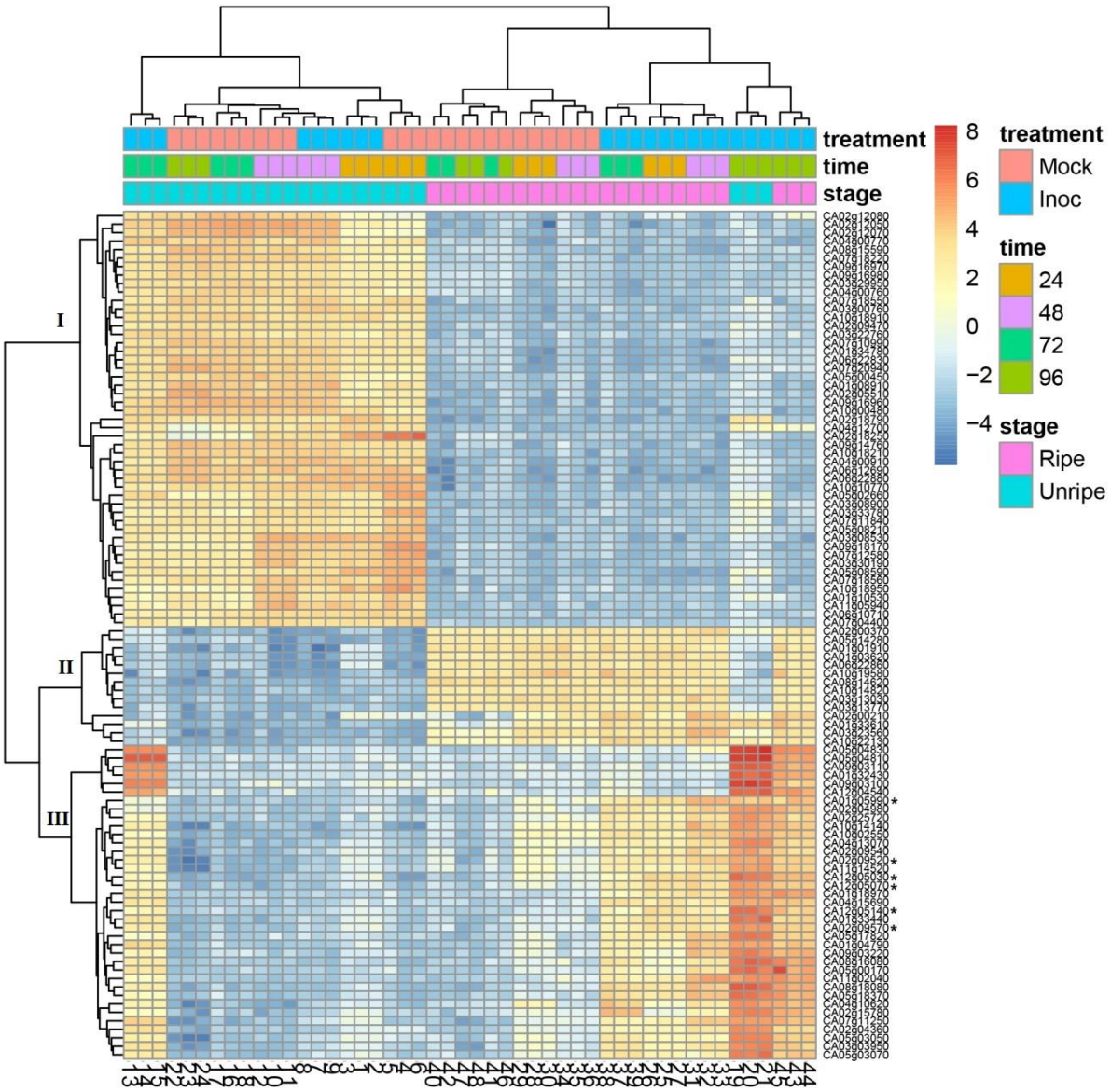


Figure 2. Heatmap analysis representing the transcriptional activity of the 100 most variable genes in unripe and ripe fruits of *C. annuum* after 24, 48, 72 and 96 hours post-inoculation with *C. gloeosporoides* and mock inoculation. Rows are genes and columns are samples. Red color indicates high row mean-centered expression levels and blue fields indicate lower row mean-centered expression. Asterisks denote capsidiol-related genes (*CA01g05990*, *CA02g09520*, *CA12g05030*, *CA12g05070*, *CA12g05140*, *CA02g09570*).

Supplementary Table 3. Annotation of the top 100 most heterogeneously expressed genes in unripe and ripe fruits in response to *C. gloeosporioides* inoculation.

Gene ID	Annotation	Cluster
CA02g12080	Light harvesting chlorophyll a/b-binding protein	I
CA02g12050	Light harvesting chlorophyll a/b-binding protein	I
CA02g12070	Chlorophyll a/b binding protein	I
CA04g00770	Light harvesting chlorophyll a/b-binding protein	I
CA08g15590	Chloroplast pigment-binding protein CP24	I
CA07g18220	Chlorophyll a/b-binding protein	I
CA09g16970	Unknown protein	I
CA09g16980	Unknown protein	I
CA03g29950	Chlorophyll a/b-binding protein (cab-11)	I
CA04g00760	Light harvesting chlorophyll a/b-binding protein	I
CA07g18550	Unknown protein	I
CA03g00760	Detected protein of unknown function	I
CA10g18910	Cytochrome P450	I
CA02g09470	Cucumisin, putative	I
CA03g22760	UPA16	I
CA07g10990	Chlorophyll a/b-binding protein	I
CA01g34780	Unknown protein	I
CA06g22830	Unknown protein	I
CA07g20940	Photosystem I reaction center subunit V, chloroplastic-like	I
CA05g00450	Unknown protein	I
CA01g08910	Carotenoid cleavage dioxygenase 4	I
CA02g05510	Ribulose bisphosphate carboxylase small chain 2C, chloroplastic-like	I
CA09g16960	Unknown protein	I
CA10g00480	NADPH:protochlorophyllide oxidoreductase	I
CA02g18790	1-aminocyclopropane-1-carboxylate oxidase	I
CA04g12700	Extensin	I
CA02g18250	Secretory peroxidase	I
CA09g14760	21 kDa protein, putative	I
CA10g18210	Tropinone reductase I	I
CA04g00910	MLP-like protein 34-like	I
CA06g12690	Non-specific lipid-transfer protein 2-like	I
CA06g22880	Detected protein of unknown function	I
CA10g10770	Non-specific lipid-transfer protein 2-like	I
CA05g02660	BURP domain-containing protein 17-like	I
CA03g08900	CASP-like protein VIT_07s0104g01350-like	I
CA03g33780	Aspartic proteinase nepenthesin-1, putative	I
CA07g11840	DNA binding protein, putative	I
CA05g08210	Kirola-like	I
CA03g08530	Putative aminotransferase	I
CA09g18170	Zeatin O-glucosyltransferase-like	I
CA07g12580	Xyloglucan endotransglycosylase	I
CA03g30190	42kDa chitin-binding protein	I
CA05g08590	Kirola-like	I
CA07g18560	Major facilitator superfamily	I
CA10g18950	Kunitz-type protease inhibitor KPI-D2.2	I
CA01g10530	Lipoxygenase	I
CA11g05940	Acyltransferase 1	I
CA06g10710	Acyl-[acyl-carrier-protein] desaturase	I
CA07g04400	Tetratricopeptide repeat-like superfamily protein]	I
CA02g00370	Zinc/iron transporter, putative	II
CA05g14280	Probable peroxygenase 5-like	II
CA01g01910	Endoglucanase-like	II
CA01g03620	Green flesh protein	II
CA06g22860	Capsanthin/capsorubin synthase	II
CA10g19580	Polygalacturonase	II
CA08g14620	13S globulin seed storage protein 2-like	II

CA10g14820	Cysteine protease	II

Supplementary Table 3 continuation

Gene ID	Annotation	Cluster
CA03g13030	Mads box protein, putative	II
CA03g13770	Protein phosphatase 2C	II
CA02g00210	Carbonic anhydrase	II
CA01g33610	Detected protein of unknown function	II
CA03g23560	Biotic cell death-associated protein	II
CA10g22130	CYP98A33v1	II
CA05g04830	Multiprotein-bridging factor 1c-like	III
CA05g04810	Zeatin O-glucosyltransferase-like	III
CA09g03110	Putative glutathione S-transferase T2	III
CA01g32430	Transcription factor, putative	III
CA09g03100	Glutathione S-transferase-like protein	III
CA12g04540	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16-like	III
CA01g05990*	5-epi-aristolochene 1,3-dihydroxylase	III
CA02g04980	Reticuline oxidase-like protein-like	III
CA02g25720	Patatin-like protein 3	III
CA10g14140	Unknown protein	III
CA10g02550	Cytochrome P450	III
CA04g13070	Pathogen-related protein-like	III
CA02g09540	Premnaspirodiene oxygenase-like	III
CA02g09520*	5-epi-aristolochene synthase	III
CA11g14520	Cytochrome P450	III
CA12g05030*	5-epi-aristolochene synthase	III
CA12g05070*	5-epi-aristolochene 1,3-dihydroxylase	III
CA01g18970	BON1-associated protein 2-like	III
CA04g15690	Unknown protein	III
CA12g05140*	5-epi-aristolochene 1,3-dihydroxylase	III
CA01g33440	3-hydroxy-3-methylglutaryl coenzyme A synthase	III
CA02g09570*	5-epi-aristolochene 1,3-dihydroxylase	III
CA05g17820	UTP:alpha-D-glucose-1-phosphate uridylyltransferase	III
CA01g04790	Invertase	III
CA09g03220	Pathogenesis-related leaf protein 4-like	III
CA08g16080	AT3	III
CA05g00170	Unknown protein	III
CA11g02040	RING-H2 finger protein ATL74-like	III
CA08g18080	Allene oxide synthase	III
CA05g18370	Unknown protein	III
CA04g10620	Pepper esterase	III
CA02g15780	Polyphenol oxidase	III
CA07g11250	1-aminocyclopropane-1-carboxylic acid oxidase	III
CA02g04360	Ethylene response factor ERF2	III
CA05g03050	Cytochrome P450 CYP736A54	III
CA03g03950	UDP-sugar:glycosyltransferase	III
CA05g03070	Cytochrome P450 CYP736A54	III

*candidate genes for capisidiol biosynthesis up-regulated under *C. gloeosporioides* interaction.

Identification and annotation of capsidiol biosynthesis-related gene candidates

We performed a manual identification and annotation of all capsidiol candidate genes, 5-epi-aristolochene synthase (*CaEAS*) and 5-epi-aristolochene 1,3-dihydroxylase (*CaEAH*) in this transcriptome dataset (Table 3), since we observed that some were incorrectly annotated in the *C. annuum* genome data. Eleven *EAS* and 14 *EAH* genes showed high e-values (0.0) and scores above 500. In addition, all candidate genes presented the specific conserved

domain in their protein sequences, pfam03936 (*EAS*) and pfam00067 (*EAH*), both already described in other plants.

Table 3. Description of annotated *C. annuum* candidate genes related to capsidiol biosynthesis.

Gene ID	Acession Number	Manual annotation	Genome annotation	E-value	Score	Protein Size	Conserved Domain
CA12g05020	O65323.1	5-epiaristolochene synthase	Vetispiradiene synthase	0.0	1154	559	pfam03936
CA12g05150	O65323.1	5-epiaristolochene synthase	Vetispiradiene synthase	0.0	1051	559	pfam03936
CA12g05060	O65323.1	5-epiaristolochene synthase	5-epi-aristolochene synthase	0.0	1050	563	pfam03936
*CA12g05030**	O65323.1	5-epiaristolochene synthase	UV-induced sesquiterpene cyclase	0.0	1050	559	pfam03936
*CA02g09520**	O65323.1	5-epiaristolochene synthase	UV-induced sesquiterpene cyclase	0.0	1011	563	pfam03936
CA08g05300	O65323.1	5-epiaristolochene synthase	UV-induced sesquiterpene cyclase	0.0	827	472	pfam03936
CA12g05310	O65323.1	5-epiaristolochene synthase	Vetispiradiene synthase	0.0	816	510	pfam03936
CA12g05260	O65323.1	5-epiaristolochene synthase	5-epi-aristolochene synthase	0.0	635	382	pfam03936
CA12g05170	O65323.1	5-epiaristolochene synthase	Viridiflorene synthase-like	0.0	609	379	pfam03936
CA12g09360	O65323.1	5-epiaristolochene synthase	Terpene synthase	0.0	565	552	pfam03936
CA12g09250	O65323.1	5-epiaristolochene synthase	Terpene synthase	0.0	524	481	pfam03936
*CA01g05990**	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D51v2	0.0	830	515	pfam00067
*CA12g05140**	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	Cytochrome P450 71D7-like	0.0	799	501	pfam00067
*CA12g05070**	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D51v2	0.0	769	514	pfam00067
CA12g05220	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D51v2	0.0	761	513	pfam00067
*CA02g09570**	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D51v2	0.0	760	515	pfam00067
CA01g12720	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	Cytochrome P450	0.0	717	493	pfam00067
CA01g12560	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	Premnaspirodiene oxygenase-like	0.0	709	495	pfam00067
CA06g13700	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D49v1	0.0	589	496	pfam00067
CA07g03270	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D48v2	0.0	577	493	pfam00067
CA07g11990	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D47v1	0.0	573	498	pfam00067
CA01g08100	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	CYP71D48v1	0.0	572	504	pfam00067
CA10g06850	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	Cytochrome P450, putative	0.0	536	502	pfam00067
CA02g19590	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	Cytochrome P450	0.0	531	509	pfam00067
CA02g19610	Q94FM7.2	5-epiaristolochene 1,3-dihydroxylase	Cytochrome P450	0.0	523	514	pfam00067

*candidate genes for capisidiol biosynthesis up-regulated in pepper fruits under *C. gloeosporioides* interaction.

Capsidiol biosynthesis-related genes transcriptional validation

To validate the digital expression profile of the 3' RNA-seq data, we selected four candidate genes from capsidiol biosynthesis: two *CaEAS* (*CA12g05030* and *CA02g09520*) and two *CaEAH* (*CA12g05070* and *CA01g05990*) for RT-qPCR analysis (Fig. 3A). RT-qPCR of capsidiol candidate genes showed stage-specific expression profile consistent to those predicted by 3' RNA-seq (Fig. 3B) in the DEG analysis.

We observed that *CA12g05030* gene showed a higher number of transcripts in both fruit development stages. Unripe fruits showed a late response to anthracnose, but showed the highest expression levels for both capsidiol-related genes (*CaEAS* and *CaEAH*) at 72 and mainly at 96 HPI under *C. gloeosporioides* inoculation (Fig. 3B). In relation to ripe fruits, *CaEAS* and *CaEAH* transcripts abundance in response to fungal interaction was rapidly induced starting at 24 HPI (Fig. 3D and 3E). Similarly to unripe fruits, the transcript peak was detected at 96 HPI.

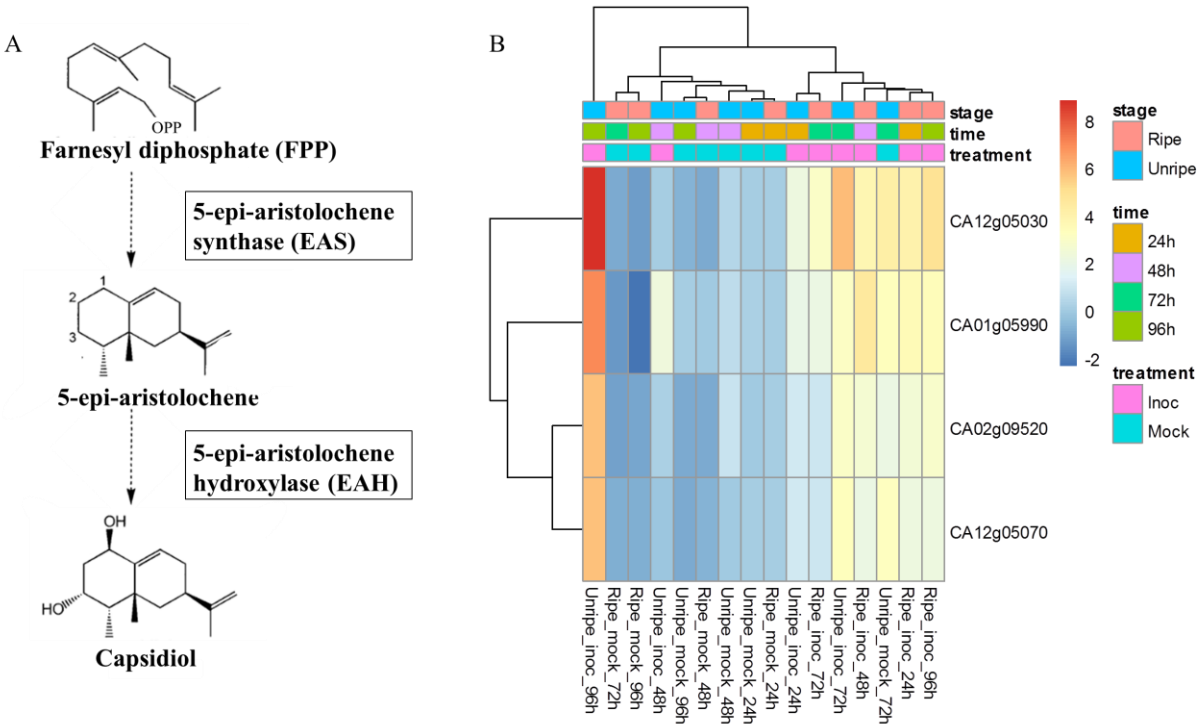


Figure 3. Diagram of capsidiol metabolic pathway (A) including capsidiol-related genes expression profile (B). *CaEAS* and *CaEAH* gene expression patterns obtained using RT-qPCR analysis for unripe and ripe fruits analyzed at 24, 48, 72 and 96 hours post-inoculation (HPI). The mean values for *CaEAS* and *CaEAH* relative expression were normalized using *CaEF1α* and *CaUEP*. 24 HPI mock inoculation was set to 1, used as calibrator.

Discussion

A global view of *C. annuum* pathways activated in response to *C. gloeosporioides* in unripe and ripe fruits was acquired by sequencing pepper transcriptomes, using the 3' RNA-seq method, and by developing a successfully analysis pipeline. This method can be used for further transcriptome analysis with an available and well annotated genome, since it is a powerful method to detect DEGs and for accurately determine gene expression at a low cost (Tandonnet et al., 2017).

Clustering distance analyses (PCA and heatmap) based on the pepper transcriptome expression profile showed different patterns for unripe and ripe fruits (Supplementary Fig. 2A and 2C), and also for mock and inoculated treatments (Supplementary Fig 2B). This result was already expected since unripe and ripe fruits are phenotypically and biochemically different (Park et al., 2014). Ripe fruits present higher amounts of some biochemical compounds (e.g. capsidiol) relative to unripe fruits, and those compounds can be positively related to fruit resistance against fungus disease (Howard et al., 2000; Park et al., 2014; Lee et al., 2017).

Unripe fruits showed a distinct transcriptomic response to anthracnose from that of ripe fruits (Supplementary Fig. 3). In a previous analysis of transcriptomes across fruit developmental stages by Martínez-López et al. (2014), distinctive transcriptomic profiles were also observed, where pepper fruits ripening from 40 to 60 DAA were characterized predominantly by a global decrease in gene expression, signaling the end of maturation and the beginning of senescence of chili pepper fruit. Red pepper fruit showed more specialized and less diverse genes (Martínez-López et al., 2014). In addition, in the initial fruit development stage, compounds are still being produced and several genes are being expressed, as has been observed during coffee (Ivamoto et al., 2017) and strawberry fruits development stages in response to *C. acutatum* (Guidarelli et al., 2011).

It is worth mentioning that the transcript accumulation in unripe and ripe fruits is dependent on the infection and colonization strategies employed by *Colletotrichum* species, described as hemibiotrophic, that involve both biotrophic and necrotrophic phases. In unripe fruit, there is formation of appressoria, hyphal penetration and a quiescence phase, while fruit ripening triggers fungus active infection and colonization (Guidarelli et al., 2011; Gan e al., 2013; Alkan et al., 2015). Simultaneous transcriptome analysis of *C. gloeosporioides* and tomato fruit also reveals defense genes induced in stage-specific fungal colonization (Alkan, et al., 2015).

Salicylic acid (SA) is associated with resistance to biotrophs and hemibiotrophs, while jasmonic acid (JA) and ethylene (ET) regulate defense during necrotrophic infection (Antico et al., 2012; Pandey et al., 2016). The transcript accumulation of JA and ET responsive genes such as plant defensin 1.2 (PDF1.2), Lypoxygenase 3 (Lox3), Allene oxide synthase (AOS), ACC synthase 2 (ACS2), phenylalanine ammonia-lyase 3 (PAL3), and pathogenesis related proteins (PR2 and PR5) were more rapid and higher induced in the resistant cultivar of chilli and *C. truncatum* pathosystem (Mishra et al., 2017). These genes, related to pepper defense response against fungal interaction, were also induced in ripe fruits starting at 24 HPI, while in unripe fruits the response was delayed and observed at 96 HPI (Table 2).

Another up-regulated gene in ripe fruits was pepper esterase (*PepEST*), which was already described as being high expressed in ripe pepper fruits under *C. gloeosporioides* interaction (Kim et al., 2001). *PepEST* is involved in the hydrolysis of the external layer of fungal cell walls, leading to inhibition of appressoria formation and activating the defense signaling pathways (Ko et al., 2016; Seo et al., 2017). Resistance in ripe fruits might also be

related to the accumulation of ethylene in non-climacteric pepper fruits, and can act as a defense hormone providing resistance to diseases (Alkan et al., 2015).

Cytochrome P450 genes were described as being induced in unripe fruit of *C. annuum* under incompatible interaction with *C. gloeosporioides* (Oh et al., 1999), and also esterase and MADS-box TFs, genes related to antifungal defenses, were induced in this same species-pathogen interaction (Oh et al., 2003). All these genes were also induced in the 3' RNA-sequencing for ripe fruits (Supplementary Table 3).

Capsidiol-related genes, 5-epi-aristolochene synthase (*EAS*) and the cytochrome P450 from subfamily CYP71D also known as 5-epi-aristolochene 1,3-dihydroxylase (*EAH*), were already described to improve anthracnose resistance in ripe pepper fruits (Park et al., 2014). In the transcriptome data, we observed that capsidiol biosynthesis-related genes were ranked in the top 10 list of genes more up-regulated under *C. gloeosporiodes* interaction (Table 2) and in the heatmap of the top 100 most heterogeneously expressed genes (Fig. 2). Capsidiol is a phytoalexin (sesquiterpene) produced by several Solanaceous plants, including pepper, during pathogen interaction (Park et al., 2014). This compound can exhibit fungistatic activity for many fungal species (Takahashi et al., 2005; Park et al., 2014) and capsidiol-related genes are considered an important gene involved in pepper tolerance against anthracnose disease. *EAS* is responsible for transforming farnesyl diphosphate (FPP) in 5-epi-aristolochene, and *EAH* is a cytochrome P450 responsible for transforming 5-epi-aristolochene into the sesquiterpene capsidiol (Takahashi et al., 2005; Fig. 3A).

We also observed the presence of genes in clusters mentioned by a previous study (Lee et al., 2017) and composed of multiple copies of highly induced *EAS/EAH* genes that includes *CA12g05030* (*CaEAS*) and *CA12g05070* (*CaEAH)*. These gene clusters are located in a 1.3Mb expanded region of *C. annuum* on chromosome 12 and is composed of four *CaEAS* (*CA12g05020, CA12g05030, CA12g05060, CA12g05150*) and two *CaEAH* (*CA12g05070, CA12g05140*) genes (Lee et al., 2017). In addition, the capsidiol biosynthetic pathway is stimulated during the nonhost interaction between pepper and pathogen infection (Lee et al., 2017). These authors showed that a subset of *EAS/EAH* gene family members was highly induced upon *Phytophthora infestans* attack in parallel with capsidiol accumulation. They also suggested that *EAS* and *EAH* genes formed a chemical barrier of nonhost resistance against *P. infestans* in which the fungus could not overcome the toxicity.

Considering the importance of capsidiol candidate genes to pepper resistance against anthracnose disease and of the need to improve knowledge about the genetic mechanisms

involved in this plant-pathogen interaction, we validated the *CaEAS* and *CaEAH* expression profile (Fig. 2) in the DEG analysis as being consistent with RT-qPCR results (Fig. 3B, 3C, 3D and 3E). As the GBUEL104 accession is resistant to anthracnose in both pepper fruit development stage, we observed a late and high up-regulation of *CaEAS* and *CaEAH* in response to *C. gloeosporioides* in unripe fruits (96 HPI), while in ripe fruits, the response was rapidly induced beginning with the initial time post-inoculation (24 HPI). This result could provide a reasonable explanation as to why ripe fruits are more resistant than unripe fruits against *C. gloeosporioides*. Pepper plants, by increasing expression of key capsidiol biosynthesis genes, likely increase the capacity to produce capsidiol during fruit development stages and to accumulate it in ripe fruits. *EAS* was already described as a key enzyme involved in capsidiol biosynthesis and seems to be associated with the enhanced synthesis of capsidiol in response to *C. gloeosporioides* in ripe fruits (Park et al., 2014; Lee et al., 2017).

The transcriptome dataset produced here can serve as a powerful tool for future analysis of several metabolic pathways mentioned in this study, as was demonstrated using capsidiol-related genes as an example. It opens new possibilities to focus on genes that could be used for pepper breeding programs in the future, to improve its resistance against *C. gloeosporioides*.

Conclusion

Our results provide a transcriptome-level overview of the changes in *C. annuum* gene expression profiles under fungal interaction using a pipeline for 3’ RNA-seq analysis. Overall, the analysis reveals distinct stage-specific gene expression in unripe and ripe pepper fruits in response to the pathogen using genetic mechanisms to produce defense proteins. In particular, we identified and selected capsidiol-related genes to validate their differentially expressed profile by using RT-qPCR analysis. In this way, we generated a reliable panel of up- and down-regulated candidate genes that can be used in future projects to improve the knowledge about *C. annuum* x *C. gloeosporioides* interactions.

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5. CONSIDERAÇÕES FINAIS

A combinação de dados de transcriptoma aliados aos dados de quantificações bioquímicas de metabólitos secundários presentes em frutos de *Capsicum* infectados com a antracnose revelou ferramentas importantes para o melhor entendimento dos mecanismos de defesa frente à maturação dos frutos de pimenta e pimentão.

Em ambas as análises foram observadas respostas distintas estádio-específica, sugerindo que o ataque do fungo é influenciado pelo estádio de desenvolvimento de frutos de *C. annuum*. Frutos maduros de *Capsicum* spp. apresentaram maior resistência à antracnose em relação aos frutos verdes. Adicionalmente, frutos maduros resistentes apresentaram maior concetração dos níveis de metabólitos secundários e indução dos transcritos relacionados aos genes de resposta de defesa a doenças.

O transcriptoma gerado por 3’ RNA-seq mostrou-se um método eficiente para a identificação e caracterização de genes relacionados a resposta de defesa à antracnose, como por exemplo, genes envolvidos na biossíntese do capsidiol, no qual o perfil de expressão foi validado por RT-qPCR. Além disso, esses genes pertencem à mesma família gênica e foram regulados principalmente em frutos maduros.

Os resultados obtidos são importantes informações para compreensão da base bioquímica e molecular do metabolismo secundário em relação ao patossistema *C. gloeosporioides* e frutos *C. annuum* e suas relações com o aumento da tolerância a estresses bióticos.

O painel de genes candidatos gerado é uma fonte confiável, no qual pode ser usada em futuras análises transcricionais envolvendo diferentes rotas metabólica para o melhor entendendimento da interação *C. annuum* x *C. gloeosporioides* visando o melhoramento de pimentas e pimentões para obetenção de frutos com maior qualidade e tolerantes a estresses bióticos.