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Investigação de genes de microRNAs envolvidos na interação entre feijão (*Phaseolus vulgaris* L.) com o patógeno *Colletotrichum lindemuthianum*

Florianópolis, Santa Catarina
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O presente trabalho em nível de Mestrado foi avaliado e aprovado, em 9 de julho de 2024, pela banca examinadora composta pelos seguintes membros:

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Ao meu pai, por me incentivar a seguir os meus sonhos

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RESUMO

A antracnose, causada pelo fungo hemibiotrófico *Colletotrichum lindemuthianum*, é uma doença altamente destrutiva para a cultura do feijão comum (*Phaseolus vulgaris* L.). A compreensão dos mecanismos moleculares envolvidos nas respostas de defesa é crucial para o desenvolvimento de tecnologias aplicadas ao melhoramento genético do feijão. Entre os mecanismos moleculares relacionados na interação planta-patógeno, os microRNAs (miRNAs) têm se destacado por atuarem na regulação pós-transcricional de genes envolvidos com respostas imunes de plantas. Este estudo visa investigar genes de miRNAs de *P. vulgaris* e sua atuação na regulação dos mecanismos de defesa do feijão durante a colonização e o desenvolvimento de *C. lindemuthianum*. Utilizando a técnica de RT-qPCR, foram analisados os perfis de expressão de genes de miRNAs em diferentes tempos após a inoculação do fungo. Estas análises revelaram uma modulação diferenciada para alguns dos miRNAs investigados. Além disso, a análise de predição *in silico* de potenciais alvos dos miRNAs selecionados proporcionou um melhor entendimento dos papéis que os miRNAs desempenham durante as respostas moleculares à colonização e ao desenvolvimento de *C. lindemuthianum*. O resultado da predição de alvos dos miRNAs de *P. vulgaris* foram corroborados por estudos que confirmam que os miRNAs miR160 e o miR393 modulam a sinalização de auxina, crucial para o equilíbrio entre crescimento e defesa da planta. Os alvos preditos para o miR482 incluem proteínas envolvidas em respostas ao estresse oxidativo e tolerância à seca e salinidade. Os miRNAs miR2118 e miR5374 estão envolvidos na regulação de genes de resistência *NBS-LRR*, componentes das vias de desenvolvimento e do sistema imunológico das plantas. O miR5374, específico de leguminosas, mostrou-se significativamente induzido durante a infecção fúngica, sugerindo sua importância na resposta imune do feijão. A compreensão detalhada dessas interações e suas implicações na defesa contra patógenos oferece novas perspectivas para o desenvolvimento de culturas resistentes, promovendo maior produtividade e sustentabilidade agrícola. Estes achados destacam o potencial dos miRNAs como alvos para intervenções genéticas e biotecnológicas no aprimoramento da resistência das plantas a doenças.

Palavras-chave: miRNA; antracnose; interação planta-patógeno; genes de resistência.

ABSTRACT

Anthracnose, caused by the hemibiotrophic fungus *Colletotrichum lindemuthianum*, is a highly destructive disease affecting common bean (*Phaseolus vulgaris* L.) cultivation. Understanding the molecular mechanisms involved in defense responses is crucial for developing technologies for the genetic improvement of beans. Among the molecular mechanisms related to plant-pathogen interactions, microRNAs (miRNAs) have emerged as key regulators of gene expression at the post-transcriptional level, particularly in modulating of plant immune responses. This study aims to investigate miRNA genes in *P. vulgaris* and their roles in regulating bean defense mechanisms during the colonization and development of *C. lindemuthianum*. Using RT-qPCR, the expression profiles of miRNA genes at different time points post-fungal inoculation were analyzed. These analyses revealed differential modulation for several miRNAs under investigation. Additionally, in silico prediction analysis of potential miRNA targets provided a better understanding of the roles these miRNAs play during the molecular responses to *C. lindemuthianum* colonization and development. The predicted miRNA targets in P. vulgaris were corroborated by studies confirming that miR160 and miR393 modulate auxin signaling, which is crucial for balancing plant growth and defense. The predicted targets for miR482 include proteins involved in responses to oxidative stress and tolerance to drought and salinity. The miRNAs miR2118 and miR5374 are involved in regulating NBS-LRR resistance genes, components of plant development pathways and immune systems. MiR5374, specific to legumes, was significantly induced during fungal infection, suggesting its importance in the bean immune response. A detailed understanding of these interactions and their implications for pathogen defense offers new perspectives for developing resistant crops, promoting higher productivity and agricultural sustainability. These findings highlight the potential of miRNAs as targets for genetic and biotechnological interventions in enhancing plant disease resistance.

Keywords: miRNA; anthracnose; plant-pathogen interaction; resistance genes.

SUMÁRIO

1 INTRODUÇÃO

1.1 CULTURA DO FEIJÃO

O feijão é uma cultura de destaque no cenário agrícola, sendo o Brasil o segundo maior produtor do mundo, atrás apenas da Índia (FAOSTAT 2022). Contudo, grande parte da produção nacional é direcionada para o consumo interno, uma vez que o feijão é um dos principais alimentos na dieta diária da população brasileira (CONAB 2024; Mantovani et al. 2024). Uma das variedades de feijão mais consumida no país é o feijão preto. Com relação à sua produção nacional, os maiores produtores estão concentrados nas regiões Sul, Sudeste e Centro-Oeste do Brasil. O estado do Paraná se destaca como o maior produtor da variedade no país, seguido por Santa Catarina (CONAB 2024). A variedade mais consumida em Santa Catarina também é o feijão-preto, e consequentemente, é a mais produzida no estado.

O tipo de solo, a temperatura e o balanço hídrico determinam as épocas de semeadura para as diferentes regiões, uma vez que o feijão apresenta pouca tolerância a condições ambientais extremas. Na região sul, recomenda-se evitar que o ciclo da cultura coincida com períodos de geadas, e que o período de floração não ocorra em temperaturas superiores a 32ºC ou em condições de déficit hídrico, pois tais fatores podem resultar no abortamento das vagens (CTSBF 2012; CONAB 2024). Além disso, a cultura do feijoeiro pode ser afetada por diferentes patógenos, que causam doenças geralmente associados às condições de temperatura e umidade.

1.2 ANTRACNOSE DO FEIJÃO

O principal patógeno do feijoeiro é o fungo hemibiotrófico *Colletotrichum lindemuthianum* (Sacc. & Magnus) Scrib., causador da antracnose. Essa é uma doença típica de regiões tropicais e subtropicais, com potencial devastador que tem como consequência a depreciação do produto, além da perda da produção poder chegar até 100% (Antunes Cruz et al. 2014; Lima et al. 2023; Kaur et al. 2023). Em virtude de ser hemibiotrófico, no estágio inicial da infecção (fase biotrófica) os conídios, estruturas reprodutivas assexuais do fungo, iniciam a germinação e formam apressório. O apressório é melanizado rapidamente e então forma uma hifa de penetração, a qual penetra a cutícula e invade as células da epiderme do hospedeiro (Fig. 1) (O'Connell et al. 1985; Cabral et al. 2024). Posteriormente ocorre a formação de hifas primárias, sem causar danos críticos à célula hospedeira (Spoel et al. 2007; O'Connell 2012; Pandey et al. 2023). A partir de 72 horas após a infecção, o fungo muda para

fase necrotrófica, produzindo hifas secundárias a partir das hifas primárias e secretando enzimas e toxinas responsáveis por degradar e matar células hospedeiras para obtenção de nutrientes (O'Connell 2012; Nabi et al. 2022; Guilengue et al. 2022; Pandey et al. 2023). O patógeno pode infectar todas as partes da planta em todos os estágios de desenvolvimento, sendo as plantas jovens as mais propensas ao estabelecimento do patógeno, em virtude dos tecidos serem menos lignificados que em plantas adultas (Antunes Cruz et al. 2014; Nabi et al. 2022).

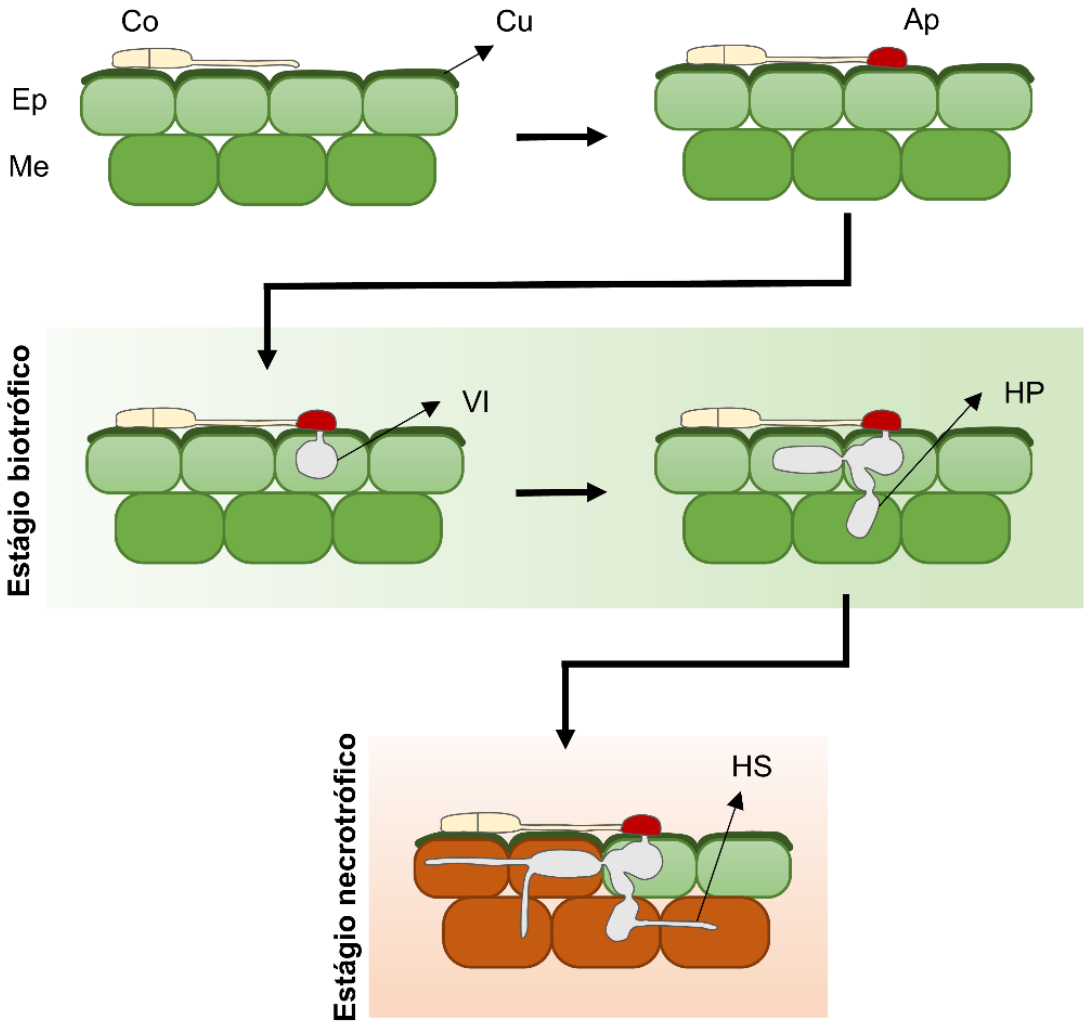

Figura 1. Ciclo infeccioso hemibiotrófico de *Colletotrichum lindemuthianum*. Após a germinação do conídio (Co) ocorre a formação de um apressório (Ap), o qual é melanizado e se adere à cutícula (Cu) para atravessar a parede celular de células da epiderme (Ep) foliar do hospedeiro. O estágio biotrófico se inicia a partir da penetração em células epidérmicas, seguida da formação de uma vesícula de infecção (VI) a qual dá origem às hifas primárias (HP). Posteriormente, hifas mais delgadas são produzidas a partir de hifas primárias e iniciam o processo de degradação de componentes celulares das células epidérmicas (Ep) e também do mesofilo (Me). Essas são chamadas hifas secundárias (HS), responsáveis por iniciar o estágio necrotrófico da doença. Fonte: Adaptado de Cabral et al. (2024).

Os sintomas característicos da antracnose são lesões com manchas marrom-escuro ou pardas em pecíolos e nervuras (Fig. 2A-D). Nas vagens ocorrem lesões circulares e deprimidas, circundadas por um anel escuro de borda marrom-avermelhada. A esporulação ocorre sob condições de umidade e temperatura favoráveis, formando uma massa de conídios rosados no centro das lesões (Fig. 2E). Além disso, o patógeno se desenvolve melhor em temperaturas entre 13ºC e 26ºC e alta umidade relativa do ar, fatores que acentuam sua ocorrência nas regiões do sudeste e sul brasileiro (CTSBF 2012; Thomazella et al. 2014; Shi et al. 2022).

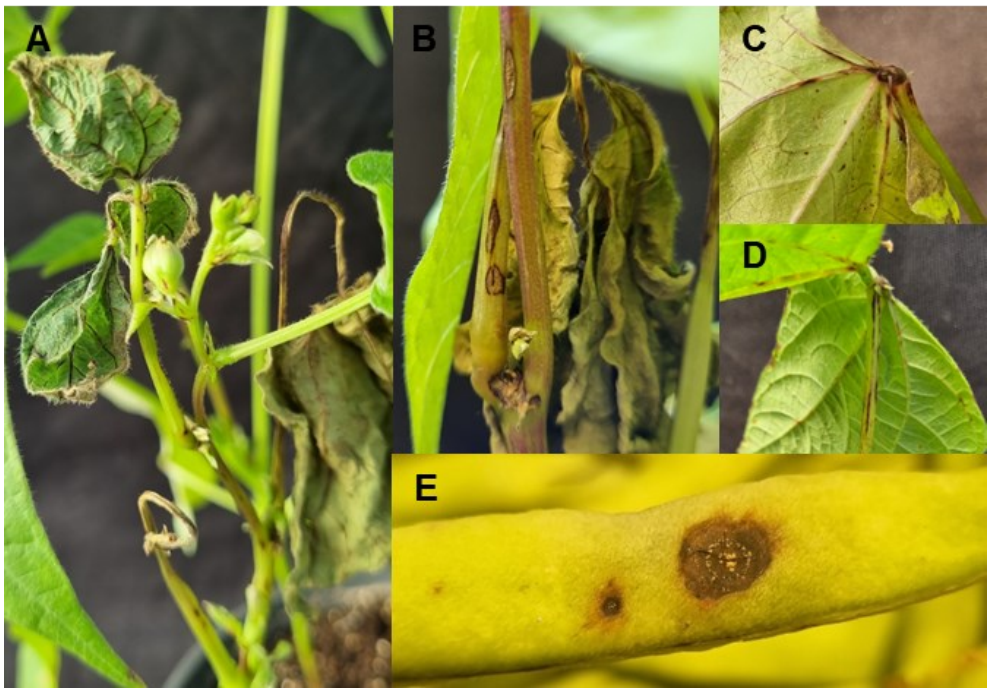

Figura 2. Sintomas da antracnose em plantas de *Phaseolus vulgaris* suscetíveis. (A) O patógeno pode afetar todas as partes aéreas da planta. Ao causar lesões necróticas em regiões do pecíolo o patógeno ocasiona intensa desfolha da planta hospedeira e compromete o transporte de nutrientes para demais regiões da planta. (B) Talos e pecíolos são afetados por *Colletotrichum lindemuthianum*. (C e D). Em folhas, o patógeno causa lesões necróticas características nas nervuras, de cor marrom-avermelhado. (E) Nas vagens, *C. lindemuthianum* produz lesões escuras arredondadas e deprimidas. Em condições favoráveis, ocorre a esporulação com produção de esporos rosados, como pode ser observado no centro da lesão. Fonte: da autora.

A disseminação de *C. lindemuthianum* pode ocorrer através de sementes, por respingos de chuva, vento e insetos (De Souza Filho 2013; Sohaliya et al. 2023). Além disso, o patógeno pode resistir de uma safra para outra em restos de culturas. As metodologias de controle da doença incluem o uso de sementes sadias, rotação de cultura, tratamento de plantas e sementes, aplicação de fungicidas, além da utilização de variedades resistentes (CTSBF 2012). Estudos indicam que a utilização de cultivares com resistência genética tem sido considerada a estratégia mais recomendada para o controle da antracnose (Rodríguez-

Suárez et al. 2007; Thomazella et al. 2014; Antunes Cruz et al. 2014; Nabi et al. 2022).O uso de variedades resistentes apresenta maiores vantagens por ser de fácil aplicação para o agricultor e não agredir o meio ambiente. Entretanto, a variedade de raças fisiológicas de *C. lindemuthianum* dificulta a obtenção de cultivares com resistência durável à antracnose (Antunes Cruz et al. 2014; Mungalu et al. 2020). Neste cenário, é necessária a compreensão dos mecanismos moleculares que atuam na defesa contra o patógeno para contribuir com o desenvolvimento de técnicas que visem o melhoramento genético do feijão.

1.3 MicroRNAs DE PLANTAS

Os microRNAs (miRNAs) constituem uma classe de RNAs endógenos não-codificantes que apresentam aproximadamente 19 a 24 nucleotídeos. Os genes de miRNAs ocorrem em todos os eucariotos e seus produtos atuam principalmente na regulação negativa da expressão gênica (Jones-Rhoades et al. 2006; Song et al. 2019; Gao et al. 2021; Bajczyk et al. 2023). Esses pequenos RNAs estão associados à regulação do crescimento e desenvolvimento das plantas, além de estarem envolvidos nas respostas a estresses bióticos e abióticos via regulação pós-transcricional de genes (Brodersen et al. 2008; Song et al. 2019; Gao et al. 2021; Bajczyk et al. 2023).

Os genes de miRNAs, *MIR,* são transcritos por uma RNA Polimerase II (Pol II), gerando transcritos primários, os pri-miRNAs (do inglês, *primary miRNAs*) (Lee et al. 2003; Jones-Rhoades et al. 2006). Os pri-miRNAs apresentam um eixo com regiões complementares (*stem*), formando uma dupla fita conectada por uma região de RNA fita simples sem hibridização (alça ou *loop*), formando a estrutura conhecida como *stem-loop* (grampo ou *hairpin*) (Figura 3) (Xie et al. 2005). São acrescentadas 7-metilguanosina (CAP) na região 5' terminal e cauda poli-A na extremidade 3', o que evita a degradação por endonucleases (Yang et al. 2006). O pri-miRNA interage com a proteína DAWDLE (DDL), responsável pela estabilização da estrutura do *stem-loop*. Diversos fatores de transcrição foram identificados como atuantes na transcrição de *MIR*, tais como SMALL 1 (SMA1), o complexo proteico PROTEIN PHOSPHATASE 4 (PP4), o complexo EMBRYONIC FLOWER 2 (EMF2), as proteínas NEGATIVE ON TATA LESS 2 (NOT2), e o complexo Transcription/Export Complex 2 (TREX-2), formado por um homólogo a Yeast Sac 3 A (SAC3A) e Tho2/Hpr2 Phenotype 1 (THP1) (Wang et al. 2013, 2019b; Xu et al. 2016a; Li et al. 2018; Zhang et al. 2020).

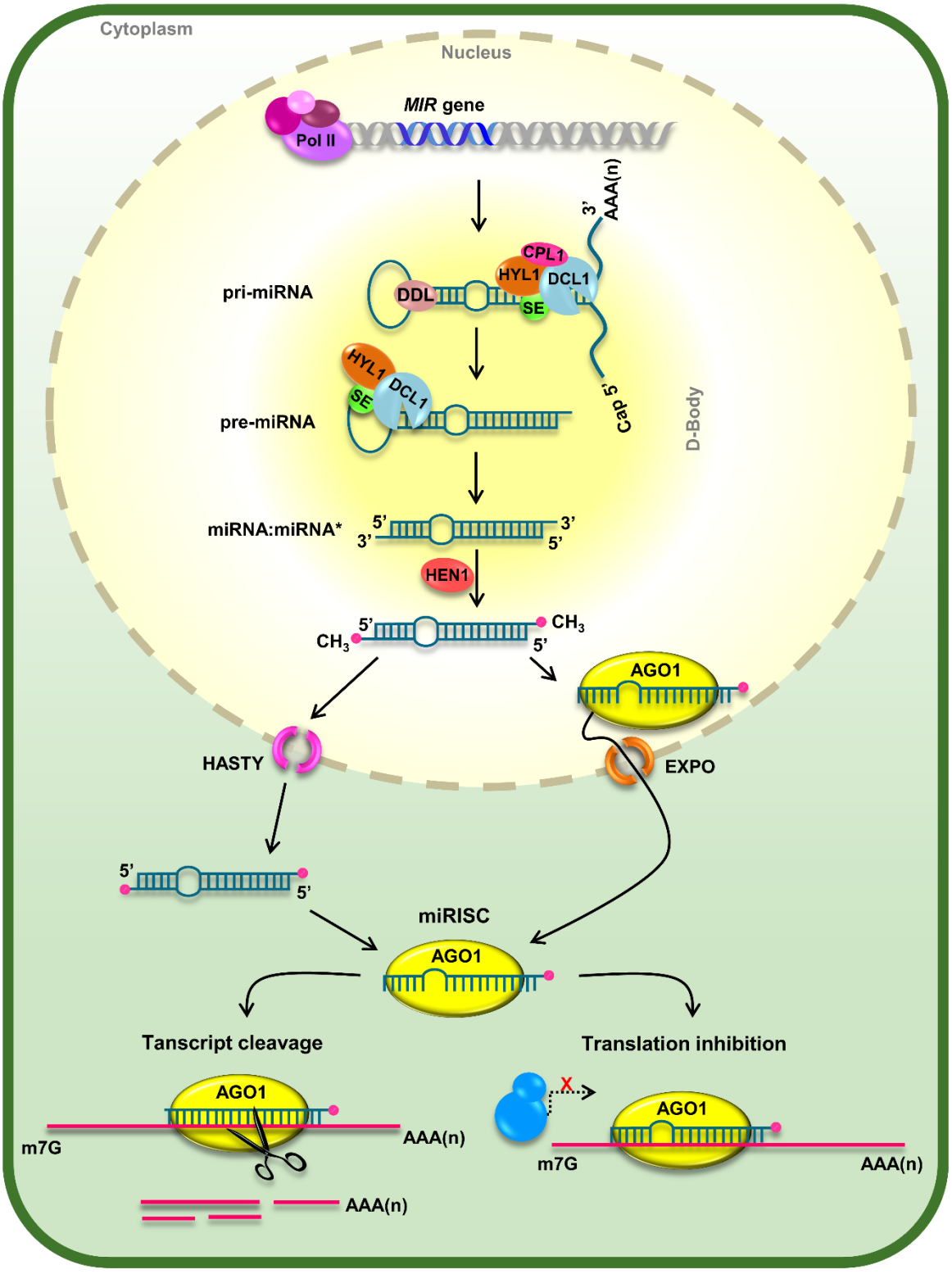

Figura 3. Biogênese de miRNAs e vias de atuação na regulação pós-transcricional. A biogênese de miRNA de plantas começa com a transcrição dos genes *MIR* pela RNA polimerase II (Pol II), produzindo um longo transcrito primário de miRNA de fita simples (pri-miRNA). Devido à sua auto-complementaridade, o pri-miRNA dobra-se em uma estrutura em forma de grampo, que é 5' capeada (Cap 5') e 3' poliadenilada (AAAn). O pri-miRNA interage com a proteína DAWDLE (DDL), que desempenha um papel na estabilização da estrutura em grampo. No núcleo, dentro dos *dicing bodies* nucleares (D-bodies), a enzima RNase III DICER-LIKE 1 (DCL1) processa a maioria dos pri-miRNAs por clivagem sequencial. DCL1, juntamente com a proteína ligante de dsRNA HYPONASTIC LEAVES1 (HYL1) e a proteína SERRATE (SE), clivam a estrutura em grampo, gerando o precursor do miRNA maduro (pre-miRNA). A clivagem adicional do pre-miRNA por DCL1

e seus cofatores respectivos produz um duplex miRNA*, com dois nucleotídeos desemparelhados na extremidade 3' de cada fita, tornando-os susceptíveis à degradação por exonucleases. Para estabilizar o duplex miRNA*, a metiltransferase de pequenos RNAs HUA ENHANCER 1 (HEN1) metila os nucleotídeos excedentes em cada fita dupla, prevenindo a uridilação e consequente degradação do miRNA. A fita de miRNA maduro é carregada em uma proteína ARGONAUTE (AGO) para formar um complexo de silenciamento induzido por miRNA (miRISC). O duplex miRNA* pode ser exportado para o citoplasma pela proteína de membrana HASTY, ou a fita guia de miRNA pode ser incorporada em AGO1 para formar o miRISC ainda no núcleo. O miRISC é então exportado para o citoplasma através da via CRM 1/EXPORTIN 1 (EXPO1). A regulação postranscricional de alvos ocorre através de dois mecanismos: clivagem do transcrito e repressão ou inibição da tradução. Fonte: Cabral, Hasse e Kulcheski (2023, capítulo de livro submetido para publicação).

Ainda no núcleo, a regulação do processamento dos miRNAs em plantas ocorre através da ação da endoribonuclease RNase III-like DICER-LIKE 1 (DCL1), que forma um complexo com a proteína HYPONASTIC LEAVES 1 (HYL1), também conhecida como DRB1, e SERRATE (SE), liberando assim um precursor de miRNA maduro, chamado pre-miRNA (Vazquez et al. 2004; Kurihara and Watanabe 2004; Yang et al. 2006; Voinnet 2009). O complexo DCL1-HYL1-SE ao finalmente interagir com TREX-2, promove a formação de *dicing bodies* (D-bodies) no núcleo, os quais desempenham um papel importante na biogênese de miRNAs (Fang and Spector 2007; Zhang et al. 2020; Xie et al. 2021). Posteriormente, ocorre a clivagem do pre-miRNA por DCL1, dando origem a um duplex de miRNA:miRNA* (Kurihara et al. 2005). A região 3' de cada sequência do duplex é estabilizada pela metiltransferase específica de pequenos RNAs HUA-ENHANCER 1 (HEN1), a qual é responsável por metilar o duplex miRNA:miRNA* e prevenir sua uridilação e degradação (Yu et al. 2005; Li et al. 2005; Baranauskė et al. 2015).

Geralmente a fita miRNA* é degradada enquanto a fita miRNA é associada à proteína ARGONAUTE (AGO), formando o Complexo de Silenciamento Induzido por RNA (RISC, do inglês *RNA-induced silencing complex*) (Baumberger and Baulcombe 2005; Vaucheret 2008). O fator que determina qual fita será associada à AGO e qual será degradada é a estabilidade termodinâmica na região terminal 5’ (Eamens et al. 2009). HYL1/DRB1 e a Phosphatase-like 1 (CPL1) estão envolvidas na seleção da fita com menor estabilidade na região 5’ (Eamens et al. 2009; Manavella et al. 2012). Há dois possíveis caminhos para a formação do complexo RISC. A exportação do duplex miRNA:miRNA* para o citoplasma pode se dar através da proteína de membrana HASTY, e posteriormente uma das fitas é acoplada à proteína AGO1 no complexo RISC (Bollman et al. 2003). A outra possibilidade inclui a translocação de AGO1 para o núcleo, incorporação do miRNA, e então a exportação de AGO1:miRNA para o citosol via CRM 1/EXPORTIN 1 (EXPO1) (Bologna et al. 2018).

A regulação postranscricional via miRNAs pode ocorrer a partir de duas possíveis vias: clivagem e degradação do transcrito alvo ou inibição da tradução (Yu et al. 2017). Os

miRNAs de plantas apresentam alta complementaridade aos seus transcritos alvos, essencial para o controle da expressão através da clivagem por AGO1 (Mallory et al. 2004; Fahlgren and Carrington 2010). O pareamento entre miRNAs e seus alvos é caracterizado pela hibridização quase perfeita na região 5’ (com máximo de um erro de pareamento de bases) e um pareamento mais livre na região 3’, podendo ocorrer a formação de pequenas bolhas (máximo de 4 erros de pareamento) (Schwab et al. 2005; Axtell and Meyers 2018; Wang et al. 2019a). Após a clivagem guiada por miRNA, o fragmento 5’ não capeado é uridilado por HEN SUPPRESSOR 1 (HEN1) e degradado por RISC-INTERACTING CLEARING 3'-5' EXORIBONUCLEASE 1 e 2 (RICE1 e RICE2) (Zhang et al. 2017). A degradação dos fragmentos 3’ não capeados, por sua vez, é mediada por uma exoribonuclease 4 (XRN4) (Souret et al. 2004). Contudo, fragmentos 3’ não capeados podem ser convertidos em RNAs de fita-dupla (dsRNA, do inglês *double-stranded*) através da atuação da RNA polimerase dependente de RNA 6 (RDR6), a qual possibilita a biossíntese de outros pequenos RNAs de interferência chamados *phased secondary* siRNAs (phasiRNAs) (Liu et al. 2020; Zhan and Meyers 2023).

A outra via de silenciamento via miRNAs muito comum em plantas é através da inibição da tradução de mRNAs alvos (Brodersen et al. 2008). Os miRNAs de plantas podem apresentar pareamentos perfeitos nas regiões 5’ e 3’ e conservar erros de pareamento entre os nucleotídeos centrais, possibilitando a hibridização de AGO:miRNA ao mRNA porém impedindo sua clivagem (Axtell et al. 2006; Franco-Zorrilla et al. 2007). Portanto, a inibição da tradução pode ocorrer devido ao miRNA impedir a leitura do mRNA pelo ribossomo (Yu et al. 2017).

1.3.1 MicroRNAs e infecções fúngicas em plantas

As plantas apresentam diversos mecanismos de defesa contra diferentes patógenos, como vírus, bactérias e fungos. Dentre estes mecanismos há a imunidade iniciada pelo reconhecimento de padrões moleculares associados a patógenos (PAMPs) (Schwessinger and Zipfel 2008; Li et al. 2023). O processo de detecção é mediado por receptores de reconhecimento de padrões (PRRs) codificados pelo hospedeiro, que resulta em imunidade desencadeada por PAMP (PTI) (Bari and Jones 2009; Bigeard et al. 2015; Jones et al. 2016; Li et al. 2023). Há a produção de proteínas relacionadas à patogênese (PR), cuja ativação se dá a partir do ataque de patógenos, ferimentos ou outras situações de estresse. Além disso, o silenciamento via RNAi também é um dos mecanismos de defesa de plantas contra patógenos

(Ding and Voinnet 2007; Mann et al. 2023). Como parte da imunidade inata das plantas, o silenciamento de RNA requer a ação de proteínas como Argonautas, RNA polimerases RNA-dependentes (RDRs, do inglês *RNA-dependent RNA polymerases*) e DCLs (Musidlak et al. 2017). A complexa maquinaria de silenciamento que controla a expressão gênica pode ocorrer após a transcrição, mediada por miRNAs ou siRNAs (Raja et al. 2014; Mann et al. 2023).

O papel dos miRNAs na resistência a doenças de plantas foi descrito pela primeira vez por Navarro *et al*. (2006) em *Arabidopsis thaliana* na interação com a bactéria *Pseudomonas syringae*. Nas últimas décadas diversos trabalhos associaram miRNAs aos mecanismos de defesa contra patógenos fúngicos em plantas. Foram identificados miRNAs durante a interação planta-patógeno em soja (Kulcheski et al. 2011; Yin et al. 2013), algodão (Yin et al. 2012), arroz (Campo et al. 2013; Sun et al. 2016), entre outras culturas de importância econômica (Zhang et al. 2023a; Jiang et al. 2023).

1.3.2 MicroRNAs de *Phaseolus vulgaris*

Nas últimas décadas, os miRNAs de *P. vulgaris* têm sido alvo de estudos com o objetivo de identificação de seus genes, bem como sua importância e o papel que desempenham. Diversos estudos identificaram famílias de miRNAs no genoma do feijão, incluindo miRNAs conservados em outras plantas e miRNAs aparentemente exclusivos de leguminosas. Foram identificados miRNAs relacionados a diferentes estresses abióticos, como deficiência de nutrientes e toxidez por manganês (Valdés-López et al. 2010), toxidez por alumínio (Mendoza-Soto et al. 2015), seca (Arenas-Huertero et al. 2009; Wu et al. 2017; De la Rosa et al. 2019) e alagamento (De la Rosa et al. 2019). A respeito dos miRNAs de *P. vulgaris* relacionados a defesa contra infecções fúngicas, os miRNAs miR5374 e miR5658 foram relacionados com um possível papel na resistência a doenças a partir da regulação de proteínas com repetições ricas em leucina (Han et al. 2014). Além disso, um estudo identificou 14 miRNAs responsivos à infecção pelo vírus *mungbean yellow mosaic India virus* (MYIMV): miR156a, miR156e, miR159a, miR160j, miR164c, miR166c, miR167a, miR172ad, miR319p, miR390d, miR395f, miR398a, miR5368 e miR5368b (Patwa et al. 2019). Entre os miRNAs identificados *in silico*, destacam-se os miRNAs miR159, miR165/166, miR170/171, miR319, miR1514a, miR482, miR2118, miR2119, miR5374 e miR5658 pelo seu provável envolvimento com estresse biótico. Os miRNAs identificados em *P. vulgaris* estão anotados nos bancos de dados online miRBase (https://mirbase.org) e PmiREN (https://pmiren.com).

Embora diversas pesquisas tenham sido realizadas sobre miRNAs de plantas em interações planta-patógeno, pouco se sabe a respeito dos miRNAs de *P. vulgaris* e seus respectivos alvos durante os processos de infecção fúngica, principalmente considerando seus papeis durante a interação com *C. lindemuthianum.* Dada a importância da cultura do feijão no Brasil e no mundo, torna-se necessário desenvolver técnicas e ferramentas a partir do entendimento dos mecanismos envolvidos nos processos celulares e moleculares de defesa do feijão. As análises moleculares são ferramentas essenciais para compreender como os miRNAs atuam na regulação dos processos biológicos. O modelo *P. vulgaris – C. lindemuthianum* permite investigar a modulação da expressão de genes de miRNAs por meio da comparação entre plantas infectadas e não infectadas. Essas análises poderão auxiliar na identificação de miRNAs que fazem parte dos mecanismos de defesa do feijão durante o estresse biótico. Compreender a regulação desses miRNAs em resposta à infecção fúngica e identificar seus possíveis alvos é essencial para elucidar os mecanismos moleculares envolvidos nas respostas do feijão ao *C. lindemuthianum*. Este conhecimento é fundamental para o desenvolvimento de estratégias de melhoramento genético voltadas para o aumento da resistência do feijão-preto a doenças, promovendo assim maior produtividade e sustentabilidade agrícola.

2 HIPÓTESE

A hipótese científica deste trabalho é que ensaios com *P. vulgaris* infectado com o fungo *C. lindemuthianum* revelarão miRNAs diferencialmente regulados, que podem ser importantes fatores na interação fitopatogênica. Além disso, a avaliação da germinação e do desenvolvimento do fungo ao longo do tempo proporcionará uma melhor compreensão dos papéis dos miRNAs durante diferentes estágios da infecção.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar a modulação da expressão de genes de miRNAs e suas vias regulatórias em *P. vulgaris* durante o processo infeccioso de *C. lindemuthianum*.

3.2 OBJETIVOS ESPECÍFICOS

- Analisar o processo infeccioso de *C. lindemuthianum* em feijão preto;
- Analisar o padrão de expressão dos genes selecionados em um cultivar de feijão preto suscetível à antracnose (cv. Uirapuru);
- Relacionar o desenvolvimento de *C. lindemuthianum* com a expressão de genes de miRNAs de *P. vulgaris*;
- Realizar predição de alvos de miRNAs diferencialmente expressos em situação de inoculação e controle.

4 CONTEXTUALIZAÇÃO DO ARTIGO CIENTÍFICO

O feijão (*Phaseolus vulgaris*) é uma leguminosa de grande importância econômica e nutricional para a população brasileira. Contudo, a produção do feijão tem como principal obstáculo a ocorrência da antracnose, a principal doença do feijoeiro. A antracnose é causada pelo fungo hemibiotrófico *Colletotrichum lindemuthianum*, o qual pode causar danos consideráveis às plantas e impactar a produção. No presente estudo, visamos investigar as vias moleculares envolvidas na regulação da interação feijão x *C. lindemuthianum.* O silenciamento via RNAi tem sido descrito como um importante mecanismo de regulação das interações moleculares entre planta-patógeno. Entre os pequenos RNAs envolvidos na regulação da expressão gênica, os miRNA se destacam como importantes reguladores de processos de crescimento, desenvolvimento e respostas a estresses biótico e abiótico. Apesar de diversos miRNAs terem sido descritos como reguladores de vias cruciais durante o estabelecimento e desenvolvimento de patógenos, até o presente momento não há miRNAs de feijão descritos como atuantes na regulação das respostas durante a interação com *C. lindemuthianum*. A partir disso, foram realizados ensaios de infecção com plantas de feijão inoculadas com *C. lindemuthianum* para identificar miRNAs expressos diferencialmente em comparação com plantas controle. A partir da identificação de miRNAs modulados, foi realizada a predição de alvos dos miRNA destacados para a melhor compreensão do papel desses genes na regulação das respostas do feijão durante a antracnose. Os resultados obtidos neste trabalho contribuem com a identificação de vias envolvidas na regulação de mecanismos celulares e moleculares cruciais para as respostas de resistência e susceptibilidade do feijão contra *C. lindemuthianum.*

5 ARTIGO CIENTÍFICO

Artigo elaborado para submissão em revista científica da área.

MicroRNAs regulate common bean (*Phaseolus vulgaris* L.) immunity during anthracnose

ABSTRACT
Anthracnose, caused by the hemibiotrophic fungus *Colletotrichum lindemuthianum*, poses a severe threat to common bean (*Phaseolus vulgaris* L.) cultivation. A thorough understanding of the molecular mechanisms underlying plant defense responses is critical for advancing genetic resistance improvement technologies in beans. Among these mechanisms, microRNAs (miRNAs) have gained prominence due to their role in post-transcriptional regulation of genes during plant immune responses. This study investigates the expression and regulatory roles of miRNAs in *P. vulgaris* during the development of *C. lindemuthianum*. Through the application of RT-qPCR, the expression profiles of various miRNAs were analyzed at multiple time points post-inoculation, revealing dynamic regulatory patterns. The *in silico* prediction analysis of potential miRNA targets provided insights into the specific roles these miRNAs play in the plant's molecular response to fungal colonization and development. Our findings indicate that upregulation of miR160 and miR393 might be involved in the modulation of auxin signaling, a pathway essential for balancing plant growth and defense mechanisms. Additionally, miR482, miR2118 and miR5374 are potential regulators of NBS-LRR resistance genes, which are key components of both plant development and immune responses. This study also identified that miR5374, a legume-specific miRNA, was significantly induced during fungal infection, underscoring its potential importance in the immune response of beans during the initial necrotrophic phase of *C. lindemuthianum*. This research highlights the promising potential of miRNAs as targets for improving plant disease resistance through advanced genetic modification strategies.

Keywords: miRNA; anthracnose; plant-pathogen interaction; resistance genes.

5.1 INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) holds the importance of the most consumed grain legume in human diet (Bitocchi et al. 2017; Lovatto et al. 2024). Bean grains serve as a crucial source of protein, dietary fiber, and complex carbohydrates, which are essential for human nutrition (Vaz Patto et al. 2015; Vidigal Filho et al. 2020; Lovatto et al. 2024). This is particularly significant for low-income populations in regions such as South America and Africa, where beans play a vital role in providing affordable and nutrient-dense food options.

This leguminous crop is vulnerable to numerous pathogens, particularly under favorable temperature and humidity conditions. Among the most significant diseases affecting this crop is anthracnose, caused by the hemibiotrophic *Colletotrichum lindemuthianum*.

Predominantly found in tropical and subtropical regions, anthracnose poses a severe threat to bean production, leading to reduced plant growth and yield (Antunes Cruz et al. 2014; Lima et al. 2023; Kaur et al. 2023). The infection process of *C. lindemuthianum* begins with the germination of conidia, followed by the formation of pre-infective structures: the germinative tube and appressoria. The appressoria rapidly melanize and generate penetration pegs that breach the host leaf cuticle and invade epidermal cells (O'Connell et al. 1985; Cabral et al. 2024). During the initial biotrophic phase, the fungus forms primary hyphae, which proliferate without causing significant damage to host cells (Spoel et al. 2007; O'Connell 2012; Pandey et al. 2023). However, at 72 hours after the infection, as the pathogen transitions to the necrotrophic phase, it develops secondary hyphae that secrete enzymes and toxins, leading to the degradation of host cells (O'Connell 2012; Nabi et al. 2022; Guilengue et al. 2022; Pandey et al. 2023). The use of cultivars with genetic resistance is considered the most recommended strategy for anthracnose management (Rodríguez-Suárez et al. 2007; Thomazella et al. 2014; Antunes Cruz et al. 2014; Nabi et al. 2022). However, the diverse physiological races of *C. lindemuthianum* challenges the development of cultivars with durable resistance to anthracnose (Mungalu et al. 2020). In this context, a comprehensive understanding of the molecular mechanisms underlying plant defense is crucial.

Among the molecular mechanisms involved in plant-pathogen interactions, microRNAs (miRNAs) have gained prominence due to their role in the post-transcriptional regulation of genes associated with plant immune responses. MiRNAs are non-coding RNAs of 19 to 24 nucleotides long that act as regulators of diverse cell pathways involved in plant growth, development and responses to biotic and abiotic stress. Plant miRNAs exert control over target genes at the post-transcriptional level through two primary mechanisms: transcript cleavage and translation repression (Yu et al. 2017). Transcript cleavage reduces the levels of specific mRNAs, while translation repression leads to a decreased accumulation of proteins synthesized from the target mRNAs (Song et al. 2019).

Plant miRNAs exhibit extensive complementarity to their target sequences throughout their entire length (Fahlgren and Carrington 2010). This high degree of complementarity plays a vital role in effectively regulating mRNA expression, (Mallory et al. 2004). The mature miRNA strand is loaded on an ARGONAUTE (AGO) protein to form the RNA-induced silencing complex (RISC). Typically, the binding between miRNAs and their respective target mRNAs follows strict base-pairing rules in plants (Schwab et al. 2005; Axtell and Meyers 2018; Wang et al. 2019a). Plant miRNAs typically cleave their mRNA targets by binding to the open reading frame (ORF) region, usually targeting a single binding site per

mRNA (Millar and Waterhouse 2005; Ajila et al. 2023). Another way miRNAs regulate target gene expression is by inhibiting translation, a process shown to be prevalent in plants (Brodersen et al., 2008). This inhibition can occur either by preventing the initiation of translation or by obstructing ribosome movement along the mRNA (Yu et al. 2017). Several miRNAs are transcriptionally induced or repressed during PAMP-Triggered Immunity (PTI) or Effector-Triggered Immunity (ETI) responses, reflecting the diverse pathways that plants employ to combat a multitude of pathogenic microorganisms. (Jones and Dangl 2006; Camargo-Ramírez et al. 2018).

Over the past decades, the miRNAs of *P. vulgaris* have been explored to identify their genes and elucidate their significance and roles in regulating biological processes. Various studies have identified miRNA families in the common bean genome by the *in silico* approach, revealing both miRNAs conserved across other plant species and those seemingly exclusive to legumes (Sunkar and Jagadeeswaran 2008; Arenas-Huertero et al. 2009; Peláez et al. 2012; Han et al. 2014; de Sousa Cardoso et al. 2016). Regarding the miRNAs of *P. vulgaris* associated with defense against fungal infections, an *in silico* prediction described miR5374 and miR5658 as potential modulators of disease resistance by regulating leucine-rich repeat (LRR) proteins (Han et al. 2014). Despite significant research on plant miRNAs in plant-pathogen interactions, little is known about the specific miRNAs of *P. vulgaris* and their targets during fungal infection processes, especially in the context of interactions with *C. lindemuthianum*. The cultural and economic importance of bean culture highlights the importance of further investigations to elucidate the molecular mechanisms by which these miRNAs contribute to the plant's immune responses during the interaction with fungal pathogens.

5.2 MATERIALS AND METHODS

5.2.1 Plant material and fungal inoculation

Seeds of common bean (*Phaseolus vulgaris* L.) from the susceptible cultivar ‘Uirapuru’ were provided by the Plant Pathology Laboratory (Florianópolis, Santa Catarina, Brazil). The seeds surface were sterilized by washing with 70% ethanol and 1% sodium hypochlorite for 1 minute each, followed by three rinses with sterile distilled water (Migliorini et al. 2017). Three plants per pot were germinated in two-liter plastic pots containing a mixture of organic compost and vermiculite (4:1, v/v). The plants were maintained in a greenhouse at a temperature of 25±5ºC (de Freitas et al. 2011).

The fungus *C. lindemuthianum* Race 73 was obtained from the monospore isolate MANE 001-03. The fungus was transferred to Petri dishes containing potato dextrose agar (PDA) medium and incubated in a growth chamber at 20±1ºC with a 12-hour photoperiod. After 15 days, PDA discs with *C. lindemuthianum* mycelium were placed in contact with autoclaved bean pods and kept in closed test tubes containing agar medium for 20 days. After sporulation, 15 mL of sterilized purified water was added to each tube, followed by vortexing. The solution was filtered through gauze and homogenized using a magnetic stirrer. Subsequently, the conidial concentration was adjusted to 6×10^6 conidia.mL^{-1} using a Neubauer chamber (Migliorini et al. 2017).

Plants of the Uirapuru cultivar, known for its susceptibility to anthracnose, were used. The conidial solution, prepared at a concentration of 6×10^6 conidia.mL^{-1}, was sprayed onto the plants designated for the inoculated group. The control group was treated by spraying the plants with water. Each sample represented a pool of three plants. Following the treatments, the plants were maintained in separated humid chambers at 28°±2°C with humidity levels above 90%. Using a randomized block design, the first fully extended leaves from Uirapuru cultivar were collected at 12, 24, 48 and 96 hours after inoculation (hai) for relative expression analysis via RT-qPCR. Immediately after collection, all samples were frozen in liquid nitrogen and stored at -80°C.

5.2.2 *C. lindemuthianum* development and disease evaluation

To compare the development of *C. lindemuthianum* with the expression of candidate miRNA genes, leaf discs from each sample of the inoculated Uirapuru cultivar were excised at 24, 42, and 96 hai. The leaf discs were immediately placed in a solution of ethanol and acetic acid (3:1, v/v) for tissue clearing, with the solution being changed daily for three days. Following this, the clearing solution was replaced with a preservation solution comprising lactic acid, glycerol and water (1:1:1; v/v/v). The discs were the stained with Aman blue for 10 min and observed under bright field microscopy using an OLYMPUS® BX41 microscope at 400x magnification. Images were captured with a 3.3-megapixel digital camera using Q-capture Pro 5.1 Q-imaging® software. The conidial germination and formation of pre-infective structures were randomly assessed for 100 conidia per leaf disc. Five discs were examined per experimental unit, which consisted of leaves from three biological replicates. The assessment consisted in determining the percentage of germinated conidia and germling with or without appressoria. Conidia were considered germinated when formed a sessile

appressorium or a germ tube. Appressoria were categorized based on their melanization status.

5.2.3 RT-qPCR analysis

Total RNA was extracted from *P. vulgaris* leaves using the TRIzol reagent (Invitrogen), following the manufacturer's protocol. The integrity of the total RNA samples was assessed by electrophoresis on a 1% agarose gel. The samples were then treated with DNase I to remove any contaminating DNA, followed by quantification using spectrophotometry on the NanoDrop equipment (Nanodrop Technologies). The miRNAs previously identified as pathogen responsive in Fabaceae and other plants were selected for investigation in *P. vulgaris* genome in the Phytozome database (*Phaseolus vulgaris* v2.1, Phytozome genome ID: 442). The cDNA synthesis was carried out by multiplex technique (Chen 2005) from 1 µg of total RNA from each sample. Each reaction was primed with a pool of 0.5 µM eight miRNAs' gene-specific stem-loop primers. The Reverse Transcription (RT) reaction was set up using the SuperScript™ IV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Briefly, 1 µg of total RNA was mixed with 0.5 µM stem-loop RT primer and 5 mM dNTPs. The reaction mix was incubated at 65ºC for 5 minutes and immediately placed on ice. Then, it was added 5x Buffer, 100 mM DTT and 200 U SuperScript™ IV Reverse Transcriptase. The solution was incubated at 16°C for 30 minutes, followed by 42°C for 30 minutes, and then 80°C for 10 minutes to inactivate the enzyme. All cDNA samples were 100-fold diluted with RNase-free water before being used as a template in qPCR analysis.

Reactions were completed in a volume of 20 µL containing 10 µL of diluted cDNA (1:100), 1x SYBR Green I (Invitrogen), 0.025 mM dNTP, 1x PCR Buffer, 3 mM MgCl2, 0.25 U Platinum Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primer. The universal reverse primer (5' GTGCAGGGTCCGAGGT 3') was used in all RT-qPCR reactions. Samples were analyzed in biological triplicate in a 96-well plate, and a no-template control was included. The conditions were set as the following: an initial polymerase activation step for 5 minutes at 94°C, 40 cycles for 15 seconds at 94°C for denaturation, 10 seconds at 60°C for annealing and 15 seconds at 72°C for elongation. A melting curve analysis was programmed at the end of the PCR run over the range 50-99°C, increasing the temperature stepwise by 0.3°C. Threshold and baselines were manually determined using the StepOnePlus™ Real-Time PCR System Software v 2.3 (Applied Biosystems).To calculate the

relative expression of the miRNAs was employed the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and miR156abc-5p and miR169eafgm were used as reference genes (Kulcheski et al. 2010).

5.2.4 miRNA target prediction

To predict miRNA targets in *P. vulgaris* genome (DFCI Gene index, Unigene version 4), the psRNATarget (2017 version) database were consulted. For each miRNA, potential target sites within the *P. vulgaris* transcriptome were identified, considering factors such as sequence complementarity and the thermodynamic stability of miRNA-mRNA duplexes. The penalty for wobble pairing (G=U) was 0,5, and for other mismatches the penalty was 1. Penalty higher than 1,5 was considered for the seed region, with only two mismatches between nucleotides 2 and 13. Two points were allowed per bubbles. The translational inhibition region was considered between nucleotides 10 and 11, and the cut-off for potential targets was 3.

Target gene sequences were cross-referenced with the Phytozome database to obtain detailed annotations (accession *Phaseolus vulgaris* v2.1, Phytozome genome ID: 442). This included the target sequence itself, associated protein IDs, and classification into protein families and classes based on PANTHER (https://pantherdb.org) and KEGG (https://genome.jp/kegg/) databases. Gene Ontology (GO) annotations were retrieved using QuickGO (https://ebi.ac.uk/QuickGO/) to provide insights into the biological processes, molecular functions, and cellular components associated with each target gene. Additionally, protein domains were identified using InterPro (https://ebi.ac.uk/interpro) to further characterize the functional domains present within the target proteins.

5.2.5 Statistical analysis

To assess differences in gene expression, the Student's t-test was used to compare the means of inoculated *versus* mock groups at each time point. For comparisons over time, the Kruskal-Wallis test was employed to evaluate differences in distributions between groups, ranking data points without assuming normality. Analyses were conducted using GraphPad Prism (v9) and IBM® Statistical Package for the Social Sciences (SPSS, v. 25.0), with significance set at $p < 0.05$.

5.3 RESULTS

5.3.1 Fungal development and infection process

Conidial germination and the formation of pre-infective structures on bean leaves began at 48 hai and increased significantly by 96 hai (Fig. 1A). During this period, the pathogen initiated its infection process by developing structures essential for host invasion, such as germinative tube and appressoria. Despite a delay in the formation of both structures (Fig. 1B), the appressoria that did form underwent rapid melanization, a process critical for successful infection. Appressorial melanization, a key factor in the pathogen's ability to penetrate host tissues, was notably higher at 48 hai, with 67% of the appressoria showing melanization compared to 51% at 96 hai (Fig. 1C). This observation suggests a complex dynamic in the pathogen's development. By 96 hai, although there was an overall increase in the formation of pre-infective structures, the rate of melanization decreased.

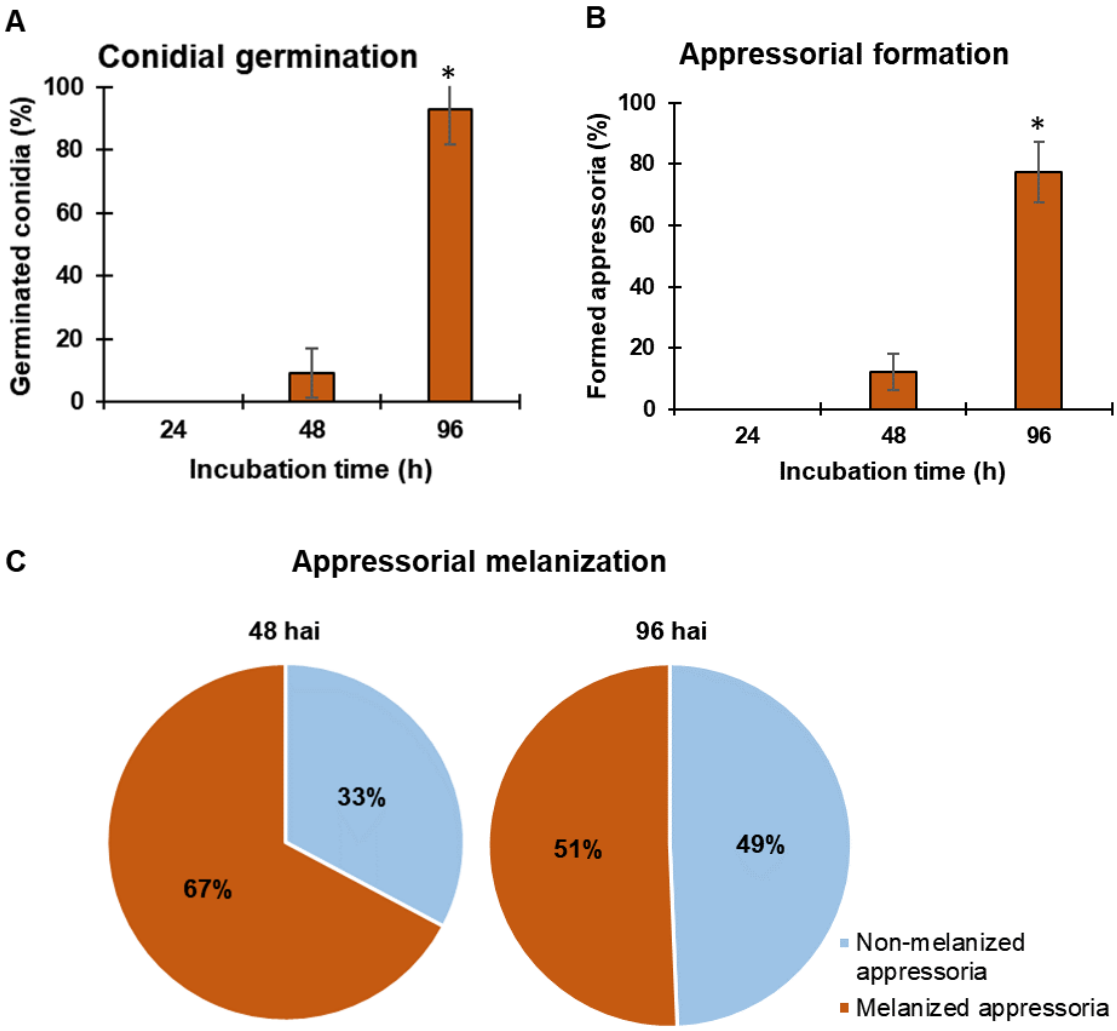

Figure 1. Germination index and appressorial melanization of *Colletotrichum lindemuthianum* in *Phaseolus vulgaris* leaves. Statistical differences between mock and treated groups at a determined time point ($P < 0.05$) were highlighted with an asterisk (*). Bars represent the standard error of five independent biological replicates.

The formation of pre-infective structures of *C. lindemuthianum* was evaluated over time, providing insights into the pathogen's developmental stages (Fig. 2). Observations at 48 hai revealed that the majority of non-melanized appressoria remained sessile, either with or without a germ tube. In contrast, melanized appressoria were predominantly found detached from the conidia (Fig. 2C). The progression of *C. lindemuthianum* within the host was further monitored by tracking the formation and spread of primary hyphae. The initiation of primary hyphae formation was detected at 48 hai. These structures marked the biotrophic stage of the fungal invasion into the host tissues. As the infection advanced, the primary hyphae continued to proliferate, with significant expansion observed by 96 hai (Fig. 2D). At 96 hai, a critical transition was noted with the presence of secondary hyphae (Fig. 2D). These secondary

structures were observed penetrating adjacent cells to those where the primary hyphae had initially formed.

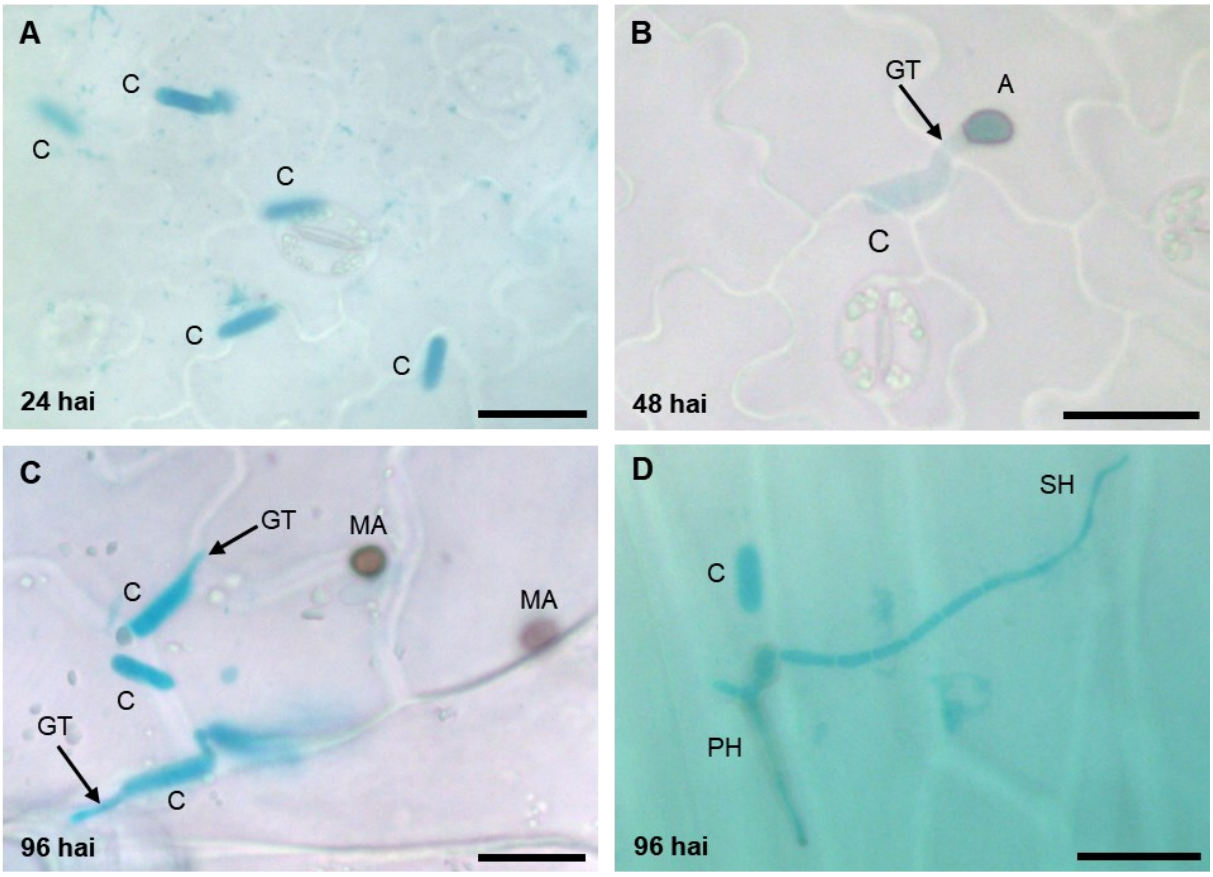

Figure 2. Germination and development of *Colletotrichum lindemuthianum* pre-infective and infective structures on *Phaseolus vulgaris* leaves. Eight-millimeter diameter leaf discs stained with Aman blue were observed at (**A**) 24, (**B**) 48, and (**C**) 96 hours after inoculation (hai) with a conidial suspension of *C. lindemuthianum* (1×10^6 conidia.mL^{-1}). Conidia were considered germinated if they exhibited a germ tube (GT), appressorium (A), or melanized appressorium (M). (**D**) Formation of primary (PH) and secondary (SH) hyphae on *P. vulgaris* leaf surface. Scale bars represent 20 µm.

To evaluate the severity of anthracnose in *P. vulgaris*, plants were observed over a period extending from 12 hai to 14 days after inoculation (dai). The progression of disease symptoms was meticulously documented throughout this period (Fig. 3). The first visible symptoms manifested at 5 dai, characterized by the appearance of minor necrotic spots on the stems, petioles, and leaves (Fig. 3A). As the infection progressed, more severe symptoms became evident. By 7 dai, significant changes were observed as the first trifoliate leaves began to exhibit signs of desiccation (Fig. 3B). The severity of the infection had escalated considerably at 10 dai. Several leaves were completely dry and began to abscise from the plant, suggesting extensive damage and cell death caused by the pathogen (Fig. 3B). Meanwhile, new necrotic lesions continued to appear on other leaves, demonstrating the

ongoing spread of the infection. By 14 dai, the overall health and vigor of the plants had noticeably declined (Fig. 3B). The cumulative effects of the infection were apparent, as the plants exhibited significant signs of stress and reduced vitality.

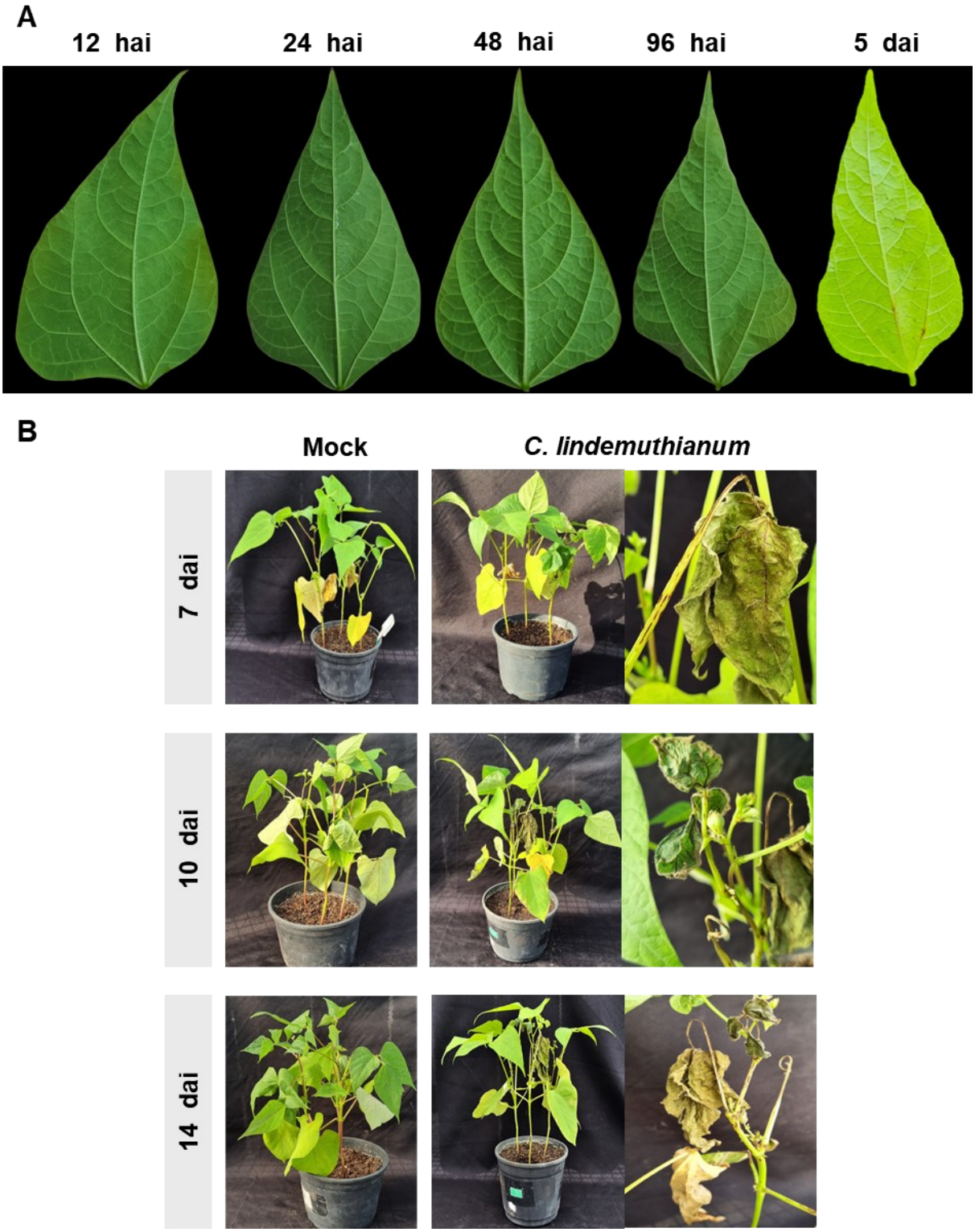

Figure 3. Antracnose progression evaluation. *Phaseolus vulgaris* plants inoculated with *Colletotrichum lindemuthianum* were monitored from 12 hours after inoculation (hai) until 14 days after inoculation (dai) to

assess disease progression. (**A**) Initial symptoms appeared at 5 dai as minor necrotic spots on stems, petioles, and leaves. (**B**) By 7 dai, the first trifoliates began to wither. At 10 dai, plants inoculated with *C. lindemuthianum* were visibly less vigorous than control plants. In inoculated plants, some leaves were completely dry and started to fall, while new symptoms continued to develop on other leaves. By 14 dai, the plants showed reduced vigor due to the cumulative effects of the infection.

5.3.2 Gene expression analysis

Among the eight miRNA genes analyzed, five showed differential gene expression between the infected and control groups at various time points following the inoculation of *C. lindemuthianum* (Fig. 4). Notably, miR160abc-5p exhibited suppression at 48 hai, followed by a significant induction at 96 hai under *C. lindemuthianum* infection (Fig. 4A). Additionally, miR393abcd-5p showed induction at both 12 hai and 96 hai in the inoculated group (Fig. 4B). The miR396abc-5p, miR398-3p and miR2119-3p did not show significant modulation in treated groups compared to mock (Fig. 4C-D). However, analysis within mock and inoculated groups and between mock and inoculated groups shows that these miRNAs expression patterns might be time-dependent during the plant interaction with the pathogen. Both miR482-5p and miR2118-3p displayed similar expression dynamics over time, with reduced expression observed at 48 hai (Fig. 4E-F). Conversely, miR5374 was specifically induced in the inoculated group at 96 hai (Fig. 4H).

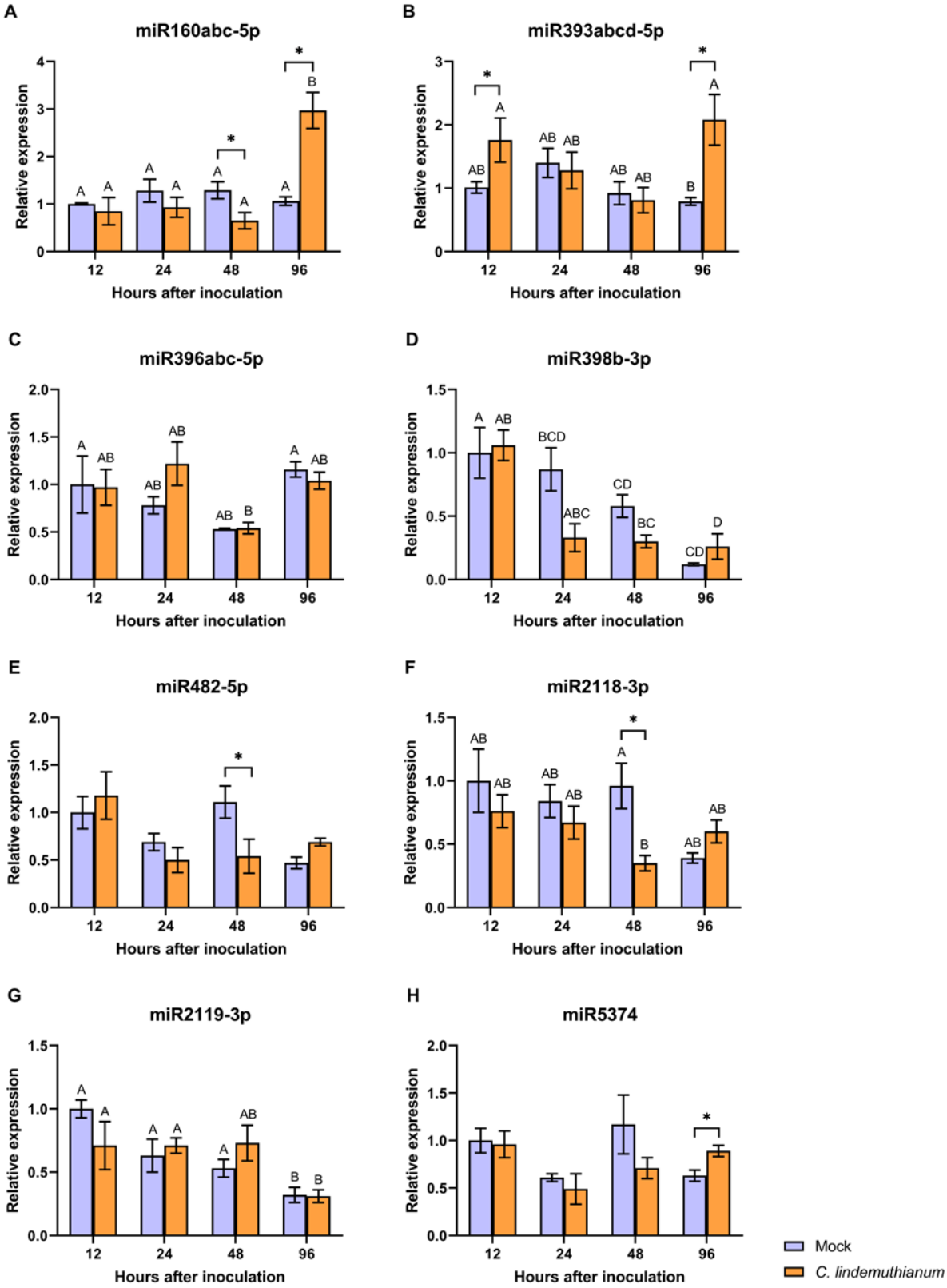

Figure 4. Expression analysis of miRNA genes in *Phaseolus vulgaris* during the infection process of *Colletotrichum lindemuthianum*. The expression of eight miRNA genes was analyzed and compared between infected and control groups at various time points post-inoculation. Statistical differences between mock and treated groups at specific time point were determined by Student's t test ($P < 0.05$) are marked with an asterisk (*). The same uppercase letters indicate no significant differences among the groups across the time course. Bars represent the standard error of three independent biological replicates.

5.3.3 miRNA target prediction

Target prediction was performed for the miRNAs that were modulated in infected groups when compared to control. The miR160abc-5p has six potential targets, including auxin response factors (ARFs), crucial transcription factors that regulate auxin signaling and transcription (Table 1). Specifically, miR160abc-5p targets Auxin response factor 17 (ARF17) encoded by Phvul.009G026200.1 and Phvul.008G168700.1. Additionally, it regulates transcripts related to Auxin response factor 10 (Phvul.005G134500.1, Phvul.011G080100.1, Phvul.007G095000.1, and Phvul.007G149000.1). miR393abcd-5p has three potential targets, the GRR1-like protein 1-related (Phvul.001G087000.1) and Transport inhibitor response 1 (Phvul.007G166200.1 and Phvul.001G202600.1), which are involved in hormone signal transduction (Table 1).

The miR482 regulates oxidoreductase activity and lipid metabolic processes by targeting flavodoxin-related (Phvul.008G004800.1), a multifunctional dehydrogenase peroxisomal fatty acid beta-oxidation protein (Phvul.004G164800.1), and lysosomal acid lipase-related (Phvul.002G315000.1) (Table 1). It also targets transcripts related to the phosphatidylinositol/phosphatidylcholine transfer protein (Phvul.009G006000.1), involved in intracellular transport. miR2118 and miR5374 predominantly target proteins with a leucine-rich repeat (LRR) domain, crucial for antimicrobial responses (Table 1). miR2118 specifically targets proteins with Toll/interleukin-1 and nucleotide-binding site (NBS) domains, including the TIR-NBS-LRR class of disease resistance proteins often with NB-ARC domain, which has ATPase activity typical of plant resistance genes. Interestingly, miR2118 may also regulate Suppressor of gene silencing 3 (SGS3), an endoribonuclease involved in gene silencing mediated by non-coding RNA.

Table 1. New *Phaseolus vulgaris* miRNA targets identified by *in silico* prediction

Pvu-miRNA	Target locus (psRNATarget)	Protein ID (Phytozome)	Protein family (PANTHER/KEGG)	Protein function (QuickG/InterPro)
miR160abc-5p	Phvul.005G134500.1	Auxin response factor 10-related	Auxin response factor-related (PTHR31384:SF27)	Auxin signaling; DNA binding; Regulation of transcription, DNA-templated; Response to hormone
	Phvul.011G080100.1			
	Phvul.007G095000.1			
	Phvul.009G026200.1	Auxin response factor 17	Auxin response factor (PTHR31384:SF19)	
	Phvul.008G168700.1			
	Phvul.007G149000.1	Auxin response factor 10-related	Auxin response factor-related (PTHR31384:SF27)	

miR393abcd-5p	Phvul.001G087000.1	Grr1-like protein 1-related	Ubiquitin carboxyl-terminal hydrolase (PTHR24006); GRR1-like protein 1-related (PTHR24006:SF456)	Cysteine protease; Protein binding
	Phvul.007G166200.1	Transport inhibitor response 1	Transport inhibitor response 1 (K14485)	Plant hormone signal transduction
	Phvul.001G202600.1			
miR482-5p	Phvul.009G006000.1	Phosphatidylinositol/phosphatidylcholine transfer protein sfh6-related	SEC14 related protein (PTHR23324)	Transfer/carrier protein
	Phvul.008G004800.1	Flavodoxin-related	Nitric oxide synthase-related (PTHR19384), NADPH-dependent diflavin oxidoreductase 1 (PTHR19384:SF10)	Oxidoreductase; FMN binding; Iron ion binding; Oxidation-reduction process; Oxidoreductase activity
	Phvul.004G164800.1	Peroxisomal fatty acid β-oxidation multifunctional protein AIM1	3-hydroxyacyl-CoA dehyrogenase (PTHR23309),	Dehydrogenase; Fatty acid metabolic process; 3-hydroxyacyl-CoA dehydrogenase activity; Oxidation-reduction process; Oxidoreductase activity
	Phvul.002G315000.1	AB-hydrolase associated lipase region containing protein	Lysosomal acid lipase-related (PTHR11005)	Lipase; Lipid metabolic process

miR2118-3p	Phvul.002G079200.1	TIR-NBS-LRR class disease resistance protein-related	Leucine-rich repeat-containing protein (PTHR11017)	Signal transduction; Protein binding
	Phvul.002G279500.1	Protein suppressor of gene silencing 3	Ribonuclease P subunit P38 (PTHR21596)	Endoribonuclease; Regulatory ncRNA-mediated gene silencing
	Phvul.011G201000.1	Leucine-rich repeat-containing protein	Disease resistance protein RP (PTHR23155)	Antimicrobial response protein; ADP binding
	Phvul.003G072500.1	TIR-NBS-LRR class disease resistance protein-related	Leucine-rich repeat-containing protein (PTHR11017)	ADP binding; Protein binding; Signal transduction
	Phvul.010G136700.1	Leucine-rich repeat-containing protein		
	Phvul.006G066800.1		Disease resistance protein RP (PTHR23155)	Antimicrobial response protein; ADP binding
	Phvul.011G181500.1			

	Phvul.010G025000.1		Leucine-rich repeat-containing protein (PTHR11017)	ADP binding; Protein binding; Signal transduction
	Phvul.002G131100.1		Disease resistance protein RP (PTHR23155)	Antimicrobial response protein; ADP binding
	Phvul.002G129200.1			
	Phvul.002G131200.2			
	Phvul.011G030000.1		Leucine-rich repeat-containing protein (PTHR11017)	Signal transduction; Protein binding
	Phvul.010G025700.1			ADP binding; Protein binding; Signal transduction
	Phvul.010G008700.2			

	Phvul.L002637.1			
	Phvul.002G130400.1		Disease resistance protein RP (PTHR23155)	Antimicrobial response protein; ADP binding
	Phvul.002G130300.1			
	Phvul.002G129500.1			
	Phvul.002G130100.1			
	Phvul.002G131000.1			
	Phvul.002G129700.1			

	Phvul.002G129900.1			
	Phvul.005G030500.1		Leucine-rich repeat-containing protein (PTHR11017)	ADP binding; Protein binding; Signal transduction
miR5374	Phvul.003G247200.2	Leucine-rich repeat-containing protein	Disease resistance protein RP (PTHR23155)	Antimicrobial response protein; ADP binding

Pvu – *Phaseolus vulgaris*; SFH6-related – SEC FOURTEEN-HOMOLOG 6-related; AMI1 – ABNORMAL INFLOURESCENCE 1.

5.4 DISCUSSION

The infection assay revealed that *C. lindemuthianum* exhibited late germination, primarily at 48 hai and 96 hai. Notably, appressorial melanization occurred promptly as the appressoria were formed. The higher percentage of melanized appressoria at 48 hai implies that once initiated, melanization occurs swiftly, equipping the pathogen with the necessary structures to breach the plant's defenses effectively. Nevertheless, rapid melanization is essential for protecting the fungus against environmental stresses such as hydrogen peroxide (H_2O_2) oxidative stress, osmotic stress, ultraviolet (UV) radiation and extreme temperatures (Gessler et al. 2014; Wang et al. 2021a). Appressorial melanization is crucial for pathogen adhesion to the surface and mechanical penetration of host epidermal cells (Wang et al. 2020). The melanized appressorium reduces the permeability of the host's epidermal cell wall, facilitating osmolyte accumulation and generating higher turgor pressure within the host cell (Wang et al. 2021b; Zhang et al. 2023b). The rapid melanization of appressoria at 48 hai highlights a critical window during which the pathogen prepares for aggressive invasion, underscoring the importance of timely defense responses in the host plant.

The withering of the trifoliates at 10 dai marked a critical point in the disease progression, indicating that the pathogen was advancing within the plant tissues and disrupting normal physiological processes. The extensive leaf drop and widespread necrosis at 14 dai highlighted the destructive impact of *C. lindemuthianum* on *P. vulgaris*, underscoring the pathogen's ability to severely impair plant growth and productivity.

At 48 hai, the pathogen initiated the penetration of host cells and began to establish biotrophic primary hyphae. At this time, was observed the downregulation of miR160, miR482, and miR2118, which might be involved in upregulation of their targets as a response to pathogen penetration during the biotrophic stage (Fig. 5). Meanwhile, the upregulation of miR160, miR393, and miR5374 at 96 hai was parallel to the pathogen's transition to the necrotrophic stage of infection (Fig. 5).

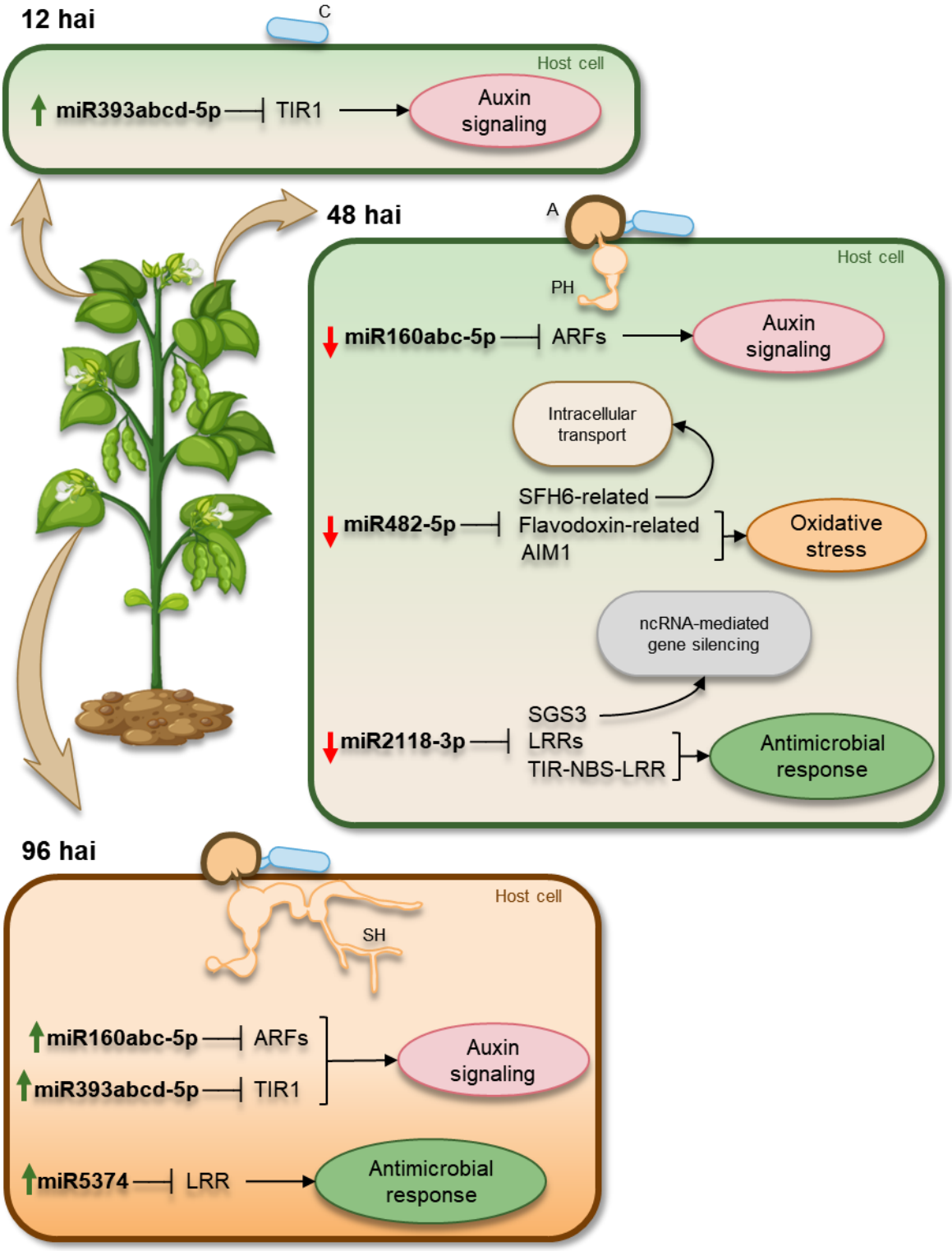

Figure 5. Modulation of *Phaseolus vulgaris* miRNAs during *Colletotrichum lindemuthianum* infection and regulatory pathways during plant-pathogen interaction. At 12 hours after inoculation (hai) miR393abcd-5p was induced and is involved in the regulation of its predicted target, the Transport inhibitor response 1 (TIR1) transcription factor. After germination of the conidium (C) and melanization of the appressorium (A) at 48 hai, the pathogen begins to spread biotrophic primary hyphae (PH). At this period, there was a suppression of miR160abc-5p, miR482-5p and miR2118-3p. The miR160abc-5p was predicted to target Auxin response factors (ARFs), which are involved in auxin signaling and activation of auxin responsive genes. The miR482-5p potential targets include the SEC FOURTEEN-HOMOLOG 6-related (SFH6-related) protein, flavodoxin-related and an acid beta-oxidation multifunctional protein ABNORMAL INFLOURESCENCE MERISTEM 1 (AMI1). The miR2118-3p targets are SUPRESSOR OF GENE SILENCING 3 (SGS3) and proteins with leucine rich

repeats (LRRs) and proteins with domains Toll/interleukin-1, nucleotide-binding site and LRR (TIR-NBS-LRR). At 96 hai, the pathogen has switched to the necrotrophic stage of the infection and started to spread secondary hyphae (SH). At the same time, miR160abc-5p, miR393abcd-5p and miR5374 were induced. The miR5374 predicted target is a LRR, involved in antimicrobial response. Arrows indicate upregulation, blunt-ended arrows indicate downregulation.

The role of miR160 and miR393 in regulation of auxin pathway has been reported to be essential for various plant processes, including growth, development, and defense mechanisms (Jodder 2020; Jiang et al. 2022; Hao et al. 2022; Cabral et al. 2024). The pioneering study by Navarro et al. (2006) identified miR393 as a key regulator of auxin signaling and plant immune response by targeting TIR1. The regulation of ARFs by miR160 is crucial for modulating plant growth and developmental processes, ensuring a balance between these cellular processes with the need to mount effective defense responses during fungal infections (Hao et al. 2022). The present study revealed that miR160abc-5p and miR393abcd-5p exhibit dynamic expression patterns following inoculation with *C. lindemuthianum*, highlighting their involvement in *P. vulgaris* defense mechanisms (Fig. 5).

The auxin signaling regulatory network depends on the fine tuning of miR160 and miR393 altogether (Luo et al. 2022). When auxin is absence, the Auxin/indole-3-acetic acid (Aux/IAA) transcriptional repressors bind to ARFs to inhibit the transcription of ARF-mediated auxin responsive genes. When auxin is present, it is perceived by an F-box domain of TIR1/Auxin Signaling F-box Protein (TIR1/AFB) complex and promotes its interaction with Aux/IAA (Wang and Estelle 2014; Luo et al. 2022). The increase of TIR activity promotes ubiquitination and degradation of Aux/IAA repressors, leading to ARF release and activation of auxin-response genes (Calderón Villalobos et al. 2012; Luo et al. 2022).

The role of auxin signaling during biotic stress have been explored in different pathosystems. Studies have shown that miR160 is upregulated in response to pathogen attack, leading to the downregulation of ARFs and subsequently altering auxin signaling to prioritize defense over growth (Jodder 2020). This response has been observed in various pathosystems, including *Arabidopsis*, banana, and cassava, in response to infection by *Botrytis cinerea, Fusarium oxysporum* and *Colletotrichum gloeosporioides*, respectively (Zhang et al. 2011; Pinweha et al. 2015; Xue and Yi 2018; Cheng et al. 2019). In the present study, the upregulation of miR160/miR393 was observed in *P. vulgaris* susceptible cultivar ‘Uirapuru’, which implicates that auxin regulatory mechanism might be involved in bean immune response system during the necrotrophic stage of the pathogen. During *C. gloeosporioides* invasion in cassava, the accumulation of ARF10 and TIR1 causes a hormonal imbalance (Pinweha et al. 2015). The previous research from Pinweha et al. (2015) highlighted that the

downregulation of miR160 and miR393 at 48 and 96 hai was associated with higher susceptibility to *C. gloeosporioides*, while their induction at the same time points was related to resistance by reducing ARF and TIR1 (Pinweha et al. 2015). In this case, low ARF levels mediated by miR160/miR393 lead to the suppression of auxin-responsive genes to *C. gloeosporioides* infection and resulted in enhanced resistance (Pinweha et al. 2015). The induction of miR160 and miR393 at 48 hai in susceptible bean cultivar and resistant cassava cultivar during *Colletotrichum* spp. infection indicates that the upregulation of theses miRNAs might be a consequence of pathogen presence and the infection phase, and not necessarily related to resistant or susceptible genotypes.

Overall, the observed changes in miRNA expression and their predicted target interactions underscore the intricate regulatory network governing plant responses to pathogen infection. The dynamic modulation of gene expression by miRNAs such as miR160abc-5p and miR393abcd-5p highlights their crucial roles in orchestrating defense mechanisms through the regulation of key signaling pathways and transcription factors. These findings collectively emphasize the importance of miR160/miR393 in plant defense, particularly its role in targeting TIR1 and ARFs transcripts to modulate hormone signaling and enhance resistance against fungal pathogens. This regulatory mechanism not only maintains plant growth and development plants but also combat infections, ensuring survival and productivity.

Both miR482-5p and miR2118-3p displayed similar expression dynamics over time, with reduced expression observed at 48 hai (Fig. 5). The predicted targets of miR482-5p in bean are SEC FOURTEEN-HOMOLOG 6 (SFH6), flavodoxin-related protein and ABNORMAL INFLOURESCENCE MERISTEM 1 (AIM1). The miR482-5p putative target SFH6 is a phosphatidylinositol/phosphatidylcholine transfer protein from the SECRETION 14-related (SEC14-related) protein family of intracellular transporters. The SEC14-related proteins are involved in developmental robustness in plants (Liu et al. 2023), and were associated with plant responses to abiotic stress, such as salinity and phosphate deficiency (Yang et al. 2023; Qin et al. 2023). Other predicted targets for miR482-5p are involved in important cellular processes, including lipid metabolic processes and oxidoreductase activity. The iron ion binding flavodoxin-related protein belongs to a family of electron-carrier proteins involved in oxidative stress response and tolerance to drought and salinity (Lodeyro et al. 2012; Battu et al. 2021; Niazian et al. 2021; Eggers et al. 2021). The peroxisomal fatty acid β-oxidation multifunctional protein AIM1 plays an important role in phenylalanine ammonia-lyase (PAL) pathway during basal salicylic acid (SA) biosynthesis in Arabidopsis

(Bussell et al. 2014; Xu et al. 2023). Additionally, the PAL pathway is crucial for pathogen-induced SA biosynthesis during soybean interaction with the hemibiotrophic pathogens *Pseudomonas syringae* and *Phytophthora sojae* (Shine et al. 2016; Xu et al. 2023).

Although our findings suggest that bean miR482 regulates the targets discussed above, the miR482 and miR2118 are considered part of a superfamily due to their evolutionary conservation, functional similarities, and their coordinated role in regulating plant immune responses, particularly through the modulation of NBS-LRR disease resistance genes (Fei et al. 2013; Zhang et al. 2016; Rodrigues et al. 2024). It has been reported that both miR482 and miR2118 generate secondary small interfering RNAs (phasiRNAs) from their target transcripts, which further mediate gene silencing, amplifying their regulatory effects (Fei et al. 2013; Zhang et al. 2016; Rodrigues et al. 2024). The NBS-LRRs are known group of NC-ARC domain R proteins that are pivotal in recognizing PAMPs and PTI and ETI (van Ooijen et al. 2008; Suo et al. 2023). NBS-LRRs mediates ETI responses after the interaction with pathogen effector proteins by activating hypersensitive response (HR) and local cell death, thereby inhibiting pathogen growth (Dangl and Jones 2001; Zhang et al. 2016; Rodrigues et al. 2024). The miR2118 is involved in regulating NBS-LRR genes and other stress-related genes. This miRNA functions in a feedback loop where pathogen infection induces its expression, leading to the cleavage of target mRNAs and the generation phasiRNAs (Fei et al. 2013; Zhao et al. 2015). These phasiRNAs further reinforce the silencing of NBS-LRR genes, amplifying the plant's defense response. The role of miR482/2118 in triggering the production of phasiRNAs was recently reviewed by Zhang et al. (2022) and Liao et al. (2022), which underscored its importance in fine-tuning immune responses. It is crucial to conserve NBS-LRR homeostasis in order to maintain plant growth (Li et al. 2015). Therefore, this homeostasis is achieved by keeping low NBS-LRR expression level under normal conditions, and induction gene expression upon pathogen attack (López-Márquez et al. 2023). The fine tuning of such intricated pathway is important to prevent plant autoimmunity as a consequence of a constitutive or dysregulated activation of NBS-LRRs, which may cause severe growth suppression (Tian et al. 2003; Palma et al. 2010; López-Márquez et al. 2023).

Interestingly, target prediction analysis revealed that *P. vulgaris* miR2118 could target the RNA-binding protein SGS3. The SGS3 plays a critical role in the RNA silencing pathway, which is essential for plant defense mechanisms against biotic stresses (Tan et al. 2023). SGS3 is involved in the biogenesis of small interfering RNAs (siRNAs) that are crucial components of the RISC complex, regulating gene expression to maintain genome integrity and in response to stress conditions (Béclin et al. 2002; Peragine et al. 2004; Tan et al. 2023).

RNA dependent RNA Polymerases (RDRs) form siRNA bodies with SGS3 during the regulation of plant development and biogenesis of small interfering RNAs (siRNAs) and is a key component of RNA silencing pathways essential for plant defense against biotic stress (Peragine et al. 2004; Yoshikawa et al. 2021; Jin et al. 2022; Tan et al. 2023). The miR2118 modulation of SGS3 is involved in the production of secondary siRNAs, indicating its significant role in the plant's defense mechanisms (Vazquez and Hohn 2013; Tan et al. 2023).

In summary, SGS3 is integral to the RNA silencing pathway, crucial for generating ta-siRNAs and phasiRNAs, including those derived from miR2118. This positions SGS3 as a key player in regulating gene expression in response to pathogen attacks, ensuring an effective and balanced defense response in plants. The interplay between miR482 and miR2118 reflects a sophisticated regulatory mechanism that ensures plants can mount a robust defense against pathogens while minimizing the adverse effects on growth and development. By modulating the expression of key resistance genes and generating secondary small RNAs, these miRNAs help plants adapt to biotic stresses efficiently. Understanding these interactions in bean responses to anthracnose offers valuable insights into developing crops with enhanced disease resistance through biotechnological approaches.

The miR5374 was induced in the inoculated group at 96 hai, highlighting its potential involvement in the later stages of the plant's defense response (Fig. 5). The miR5374 is an emerging player in the regulation of plant responses to biotic and abiotic stress (Xu et al. 2016b; Chen et al. 2016; Das et al. 2021). Recent studies have demonstrated that miR5374 modulates plant defense mechanisms, particularly during fungal infections. The miR5374 is considered a legume-specific miRNA (Mishra et al. 2022), and degradome sequencing analysis revealed that soybean miR5374 targets a disease resistance protein from the coiled coil (CC)-NBS-LRR class during *Soybean mosaic virus* and *Phytophthora sojae* infection (Chen et al. 2016; Zhang et al. 2023c).

Recent research by Zhang et al. (2023) indicates that miR5374 may interact with other miRNAs and regulatory networks to amplify defense signals, thereby creating a coordinated and multi-layered defense strategy. This study revealed that soybean miR5374 acts together with miR5041, targeting the CDS region of a *NBS-LRR* gene (named Glyma05g17470) in roots (Zhang et al. 2023c). This interplay ensures that plants can mount a comprehensive defense while minimizing the impact on their growth and development.

In the present work with *P. vulgaris*, it was identified that miR5374 is upregulated at 96 hai during fungal infection, further corroborating its role in plant biotic stress responses. This upregulation suggests that miR5374 plays a significant part in the plant's late defense

mechanisms against pathogens. Overall, the involvement of miR5374 in modulating key components of the plant immune system highlights its potential as a target for improving disease resistance in crops. The continuous discovery of miR5374's interactions and regulatory functions provides valuable insights into the sophisticated defense strategies employed by plants.

5.5 CONCLUSION

The dynamic expression and regulatory roles of miR160, miR393, miR482, miR2118, and miR5374 illustrate their involvement in bean defense mechanisms during anthracnose. The dynamic expression patterns of miR160abc-5p and miR393abcd-5p highlight their involvement in bean defense mechanisms during *C. lindemuthianum* infection by regulating auxin signaling. The involvement of miR2118-3p and miR5374 in the regulation of LRRs is key for understanding their role in bean responses during pathogen invasion and colonization. These miRNAs might orchestrate complex networks that balance growth and immunity, providing valuable targets for genetic and biotechnological interventions aimed at enhancing crop resistance to pathogens. Advances in elucidating the regulatory functions and interactions of these miRNAs will provide a foundation for developing crops with enhanced resistance to biotic stresses, thereby promoting higher productivity and sustainability in agriculture.

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Supplementary material

Supplementary Table 1. Primers list.

Pvu-miRNA	miRNA sequence (5'-3')	Oligo sequence (5'-3')
miR156abc-5p	GGCCGGTGACAGAAGAGAGT	UGACAGAAGAGAGUGAGCAC
miR160abc-5p	TGCCTGGCTCCCTGTATGCCA	TGCCTGGCTCCCTGT
miR169eafgm	CAGCCAAGGAUGACUUGCCGG	gcgAGCCAAGGATGACT
miR393abcd-5p	TCCAAAGGGATCGCATTGATC	gTCCAAAGGGATCGCA
miR396abc-5p	TTCCACAGCTTTCTTGAACTG	ggcTTCCACAGCTTTCTT
miR398b-3p	CCGGGTGTGTTCTCAGGTCG	UGUGUUCUCAGGUCGCCCCCG
miR482-5p	GCCGGAATGGGCTGATTGG	GGAAUGGGCUGAUUGGGAAGCA
miR2118-3p	CGGTTGCCGATTCCACCCA	UUGCCGAUUCCACCCAUUCCUA
miR2119-3p	CGGCCGTCAAAGGGAGTTGTA	UCAAAGGGAGUUGUAGGGGAA
miR5374	TTATAGTCTGACATCTGGAAT	ggccgTTATAGTCTGACATC

Supplementary Table 2. Target prediction analysis.

miRNA and mature sequence	Tagert locus	E-value	Alignment (psRNATarget)	Control mode	Target protein family (PANTHER/KEGG)	Gene Ontology (QuickGO)
miR160abc-5p TGCCTGGCTCCCTGTATGCCA	Phvul.005G134500.1	0	5' ACCGUAUGUCCCUCGGUCCGU 3' -||||||||||||||||||| 3'...AGGCAUACAGGGAGCCAGGCA...5'	Cleavage	Auxin response factor-related (PTHR31384:SF27)	DNA binding (GO:0003677); Regulation of transcription, DNA-templated (GO:0006355); Response to hormone (GO:0009725)
	Phvul.011G080100.1	0	5' ACCGUAUGUCCCUCGGUCCGU 3' -||||||||||||||||||| 3'...AGGCAUACAGGGAGCCAGGCA...5'	Cleavage		
	Phvul.007G095000.1	0	5' ACCGUAUGUCCCUCGGUCCGU 3' ||||||||||||||||||| 3'...AGGCAUACAGGGAGCCAGGCA...5'	Cleavage		
	Phvul.009G026200.1	0.5	5' ACCGUAUGUCCCUCGGUCCGU 3' ||||||:||||||||||| 3'...UGGCAUGCAGGGAGCCAGGCA...5'	Cleavage	Auxin response factor (PTHR31384:SF19)	
	Phvul.008G168700.1	0.5	5' ACCGUAUGUCCCUCGGUCCGU 3' ||||||:||||||||||| 3'...UGGCAUGCAGGGAGCCAGGCA...5'	Cleavage		
	Phvul.007G149000.1	0.5	5' ACCGUAUGUCCCUCGGUCCGU 3' ||||||:||||||||||| 3'...UGGCAUGCAGGGAGCCAGGCA...5'	Cleavage	Auxin response factor-related (PTHR31384:SF27)	
miR393abcd-5p TCCAAAGGGATCGCATTGATC	Phvul.001G087000.1	1.0	5' CUAGUUACGCUAGGGAAACCU 3' -|-|||||||||||||||| 3'...AAACAAUGCGAUCCCUUUGGA...5'	Cleavage	Ubiquitin carboxyl-terminal hydrolase (PTHR24006); GRR1-like protein 1-related (PTHR24006:SF456)	Protein binding (GO:0005515)
	Phvul.007G166200.1	1.0	5' CUAGUUACGCUAGGGAAACCU 3' -:-||||||||||||||||| 3'...AGACAAUGCGAUCCCUUUGGA...5'	Cleavage	Transport inhibitor response 1 (K14485)	
	Phvul.001G202600.1	1.0	5' CUAGUUACGCUAGGGAAACCU 3' -:-||||||||||||||||| 3'...AGACAAUGCGAUCCCUUUGGA...5'	Cleavage		

			3'...AGACAAUGCGAUCCCUUUGGA...5'			
miR482-5p GGAAUGGGCUGAUUGGGAAGCA	Phvul.009G006000.1	2.5	5' ACGAAGGGUUAGUCGGGUAAGG 3' -||||:|||||::||:|||||: 3'...CGCUUUCCAAUUGGCUCAUUCU...5'	Cleavage	SEC14 related protein (PTHR23324)	Transport (GO:0006810); Intracellular (GO:0005622); Transporter activity (GO:0005215)
	Phvul.008G004800.1	2.5	5' ACGAAGGGUUAGUCGGGUAAGG 3' |-||||:||||||||:|||-|| 3'...UUCUUCUCAAUCAGCUCAUCCC...5'	Cleavage	Nitric oxide synthase-related (PTHR19384), NADPH-dependent diflavin oxidoreductase 1 (PTHR19384:SF10)	FMN binding (GO:0010181); Iron ion binding (GO:0005506); Oxidation-reduction process (GO:0055114); Oxidoreductase activity (GO:0016491)
	Phvul.004G164800.1	3.0	5' ACGAAGGGUUAGUCGGGUAAGG 3' -||||:||||||:|:||-|||| 3'...GGCUUUCCAAUCGGUCCUUUCC...5'	Cleavage	3-hydroxyacyl-CoA dehyrogenase (PTHR23309),	Fatty acid metabolic process (GO:0006631); 3-hydroxyacyl-CoA dehydrogenase activity (GO:0003857); Oxidation-reduction process (GO:0055114); Oxidoreductase activity (GO:0016491)
	Phvul.002G315000.1	3.0	5' ACGAAGGGUUAGUCGGGUAAGG 3' |-:||:|||||:||:::||||:	Cleavage	Lysosomal acid lipase-related	Lipid metabolic

			3'...UCUUUUCCAAUUAGUUUAUUCU...5'		(PTHR11005)	process (GO:0006629)
miR2118-3p UUGCCGAUUCCACCCAUUCCUA	Phvul.002G079200.1	1.5	5' AUCCUUACCCACCUUAGCCGUU 3' --||||||||||||:|:||:|| 3'...AUGGAAUGGGUGGAGUUGGUAA...5'	Cleavage	Leucine-rich repeat-containing protein (PTHR11017)	Signal transduction (GO:0007165); Protein binding (GO:0005515)
	Phvul.002G279500.1	1.5	5' AUCCUUACCCACCUUAGCCGUU 3' -:-|-||||||||||:||||| 3'...GGAGCAUGGGUGGAAUUGGCAA...5'	Cleavage	Ribonuclease P subunit P38 (PTHR21596)	Regulatory ncRNA-mediated gene silencing (GO:0031047)
	Phvul.011G201000.1	2.0	5' AUCCUUACCCACCUUAGCCGUU 3' |:|||||||||||:|-|||| 3'...UGGGAAUGGGUGGAGUGGGCAA...5'	Cleavage	Disease resistance protein RP (PTHR23155)	ADP binding (GO:0043531)
	Phvul.003G072500.1	2.0	5' AUCCUUACCCACCUUAGCCGUU 3' --||:||||||||::|:||||| 3'...AUGGGAUGGGUGGGGUUGGCAA...5'	Cleavage	Leucine-rich repeat-containing protein (PTHR11017)	ADP binding (GO:0043531); Protein binding (GO:0005515); Signal transduction (GO:0007165)
	Phvul.010G136700.1	2.0	5' AUCCUUACCCACCUUAGCCGUU 3' -:||-||||||||||:||:|| 3'...GGGGUAUGGGUGGAAUUGGUAA...5'	Cleavage		
	Phvul.006G066800.1	2.5	5' AUCCUUACCCACCUUAGCCGUU 3' |:|||||||||||:|-||:|| 3'...UGGGAAUGGGUGGAGUGGGUAA...5'	Cleavage	Disease resistance protein RP (PTHR23155)	ADP binding (GO:0043531)
	Phvul.011G181500.1	2.5	5' AUCCUUACCCACCUUAGCCGUU 3' |:|||||||||||:|-||:|| 3'...UGGGAAUGGGUGGAGUGGGUAA...5'	Cleavage		
	Phvul.010G025000.1	2.5	5' AUCCUUACCCACCUUAGCCGUU 3' -:|||||||-||:||:||||| 3'...GGGGAAUGGGAGGGAUUGGCAA...5'	Cleavage	Leucine-rich repeat-containing protein (PTHR11017)	ADP binding (GO:0043531); Protein binding (GO:0005515); Signal transduction (GO:0007165)
	Phvul.002G131100.1	2.5	5' AUCCUUACCCACCUUAGCCGUU 3'	Cleavage	Disease resistance	ADP binding

			|-||-|||||||||-||||||| 3'...UUGGUAUGGGUGGACUCGGCAA...5'		protein RP (PTHR23155)	(GO:0043531)
	Phvul.002G129200.1	2.5	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|||||| 3'...UUGGUAUGGGUGGACUCGGCAA...5'	Cleavage		
	Phvul.002G131200.2	2.5	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|||||| 3'...UUGGUAUGGGUGGACUCGGCAA...5'	Cleavage		
	Phvul.011G030000.1	2.5	5' AUCCUUACCCACCUUAGCCGUU 3' --||||--|||||||:|||| 3'...AUGGAACAGGUGGAAUUGGCAA...5'	Cleavage	Leucine-rich repeat-containing protein (PTHR11017)	Signal transduction (GO:0007165); Protein binding (GO:0005515)
	Phvul.010G025700.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' -:|||||||-||:||:||:|| 3'...GGGGAAUGGGAGGGAUUGGUAA...5'	Cleavage		ADP binding (GO:0043531); Protein binding (GO:0005515); Signal transduction (GO:0007165)
	Phvul.010G008700.2	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' -:||:|||||-|||||:||:|| 3'...GGGGGAUGGGAGGAAUUGGUAA...5'	Cleavage		
	Phvul.L002637.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' -:||-||||||||||-||:|| 3'...GGGGCAUGGGUGGAAUAGGUAA...5'	Cleavage		
	Phvul.002G130400.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:||||| 3'...UUGGUAUGGGUGGACUUGGCAA...5'	Cleavage	Disease resistance protein RP (PTHR23155)	ADP binding (GO:0043531)
	Phvul.002G130300.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:||||| 3'...UUGGUAUGGGUGGACUUGGCAA...5'	Cleavage		
	Phvul.002G129500.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:|||||	Cleavage		

			3'...UUGGUAUGGGUGGACUUGGCAA...5'			
	Phvul.002G130100.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:||||| 3'...UUGGUAUGGGUGGACUUGGCAA...5'	Cleavage		
	Phvul.002G131000.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:||||| 3'...UUGGUAUGGGUGGACUUGGCAA...5'	Cleavage		
	Phvul.002G129700.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:||||| 3'...UUGGUAUGGGUGGACUUGGCAA...5'	Cleavage		
	Phvul.002G129900.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' |-||-||||||||-|:||||| 3'...UUGGUAUGGGUGGACUUGGCAA...5'	Cleavage		
	Phvul.005G030500.1	3.0	5' AUCCUUACCCACCUUAGCCGUU 3' --|||:|-|||||||-||||| 3'...GUGGAGUUGGUGGAAUAGGCAA...5'	Cleavage	Leucine-rich repeat-containing protein (PTHR11017)	ADP binding (GO:0043531); Protein binding (GO:0005515); Signal transduction (GO:0007165)
miR5374 TTATAGTCTGACATCTGGAAT	Phvul.003G247100.1	0	5' UAAGGUCUACAGUCUGAUAUU 3' |||||||||||||||||||| 3'...AUUCCAGAUGUCAGACUAUAA...5'	Cleavage	No information available	No information available
	Phvul.003G247200.2	2.0	5' UAAGGUCUACAGUCUGAUAUU 3' |||||||||||-||:||||| 3'...AUUCCAGAUGUCUGAUUAUAA...5'	Cleavage	Disease resistance protein RP (PTHR23155)	ADP binding (GO:0043531)
	Phvul.007G177800.1	3.0	5' UAAGGUCUACAGUCUGAUAUU 3' --|:-|-:|||||||||||| 3'...CAUUGAAGUGUCAGACUAUAA...5'	Cleavage	No information available	Protein binding (GO:0005515)

6 CONCLUSÃO

A partir dos dados obtidos nos ensaios de infecção realizados neste trabalho, foi possível identificar cinco miRNAs de *P. vulgaris* envolvidos nas respostas moleculares durante a interação com *C. lindemuthianum*. A regulação de miR160, miR393, miR482, miR2118 e miR5374, demonstra um potencial significativo para aprimorar a resistência de *P. vulgaris* a antracnose. Esses miRNAs desempenham um papel crucial na modulação dos mecanismos de defesa das plantas por meio de várias vias, regulando vias de sinalização de hormonal e outros genes de defesa.

Os miR160 e miR393 atuam em conjunto na regulação de ARFs e TIR1, respectivamente, durante ataques de patógenos, priorizando a defesa sobre o crescimento, garantindo uma resposta eficaz às infecções fúngicas. Os miR482 e miR2118 ajustam finamente as respostas de defesa da planta, direcionando genes de resistência a doenças NBS-LRR e gerenciando a resistência a patógenos. Já o miR5374, emergindo como um regulador significativo em leguminosas, amplifica os sinais de defesa durante infecções fúngicas, destacando seu papel na imunidade das plantas.

Os miRNAs destacados neste estudo apresentam grande potencial de orquestrarem redes complexas que equilibram crescimento e imunidade, proporcionando alvos valiosos para intervenções genéticas e biotecnológicas. A partir da validação dos alvos preditos e avanços na compreensão de suas funções regulatórias é possível o desenvolvimento de culturas com resistência aprimorada a estresses bióticos, garantindo maior produtividade e sustentabilidade na produção de *P. vulgaris*.

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