**UNIVERSIDADE FEDERAL DO RIO DE JANEIRO**

**JOSÉ LEONARDO SANTOS JIMÉNEZ**

# Bioestimulação e indução de defesa em diferentes genótipos de maracujá sob condições de casa de vegetação e de campo

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**JOSÉ LEONARDO SANTOS JIMÉNEZ**

# Bioestimulação e indução de defesa em diferentes genótipos de maracujá sob condições de casa de vegetação e de campo

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***Orientador:***

Maite Vaslin de Fritas Silva

***Coorientador:***

Raul Castro Carriello Rosa

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**ATA DO EXAME DE DEFESA DE TESE DE DOUTORADO DE JOSÉ LEONARDO SANTOS JIMÉNEZ, COMO PARTE DOS REQUISITOS NECESSÁRIOS À OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS (BIOTECNOLOGIA VEGETAL E BIOPROCESSOS).**

Aos dezenove dias do mês de novembro do ano de dois mil e vinte e um, às 09 horas, reuniu-se via videoconferência, a Banca Examinadora abaixo discriminada, para avaliação da Tese de Doutorado do aluno **José Leonardo Santos Jiménez**, intitulada: "Bioestimulação e indução de defesa em diferentes genótipos de maracujá sob condições de casa de vegetação e de campo" desenvolvida sob a orientação da **Profª. Maite Vaslin de Freitas Silva** e co-orientação de **Dr. Raul Castro Carriello Rosa**. A apresentação feita pelo candidato foi acompanhada da arguição pelos componentes da Banca. Em seguida, esta se reuniu para sua avaliação e a tese foi ( **A** ) (inserir letra apropriada).

A) Aprovado;
B) Aprovado com pequenas modificações* a serem combinadas com o Presidente da Banca dentro de um mês;
C) Não aprovado ainda, é necessária a apresentação das modificações/correções* em uma nova versão do documento para o Presidente da Banca e uma carta do Presidente com prazo de máximo 3 meses para ser aprovada; se não reprovada
D) Não aprovado ainda, é necessária uma nova apresentação*, oral e escrita, para a mesma ou nova banca examinadora dentro de um prazo combinado com a comissão do Programa.
E) Reprovado, razões da reprovação* escritas no espaço destinado.

*As modificações/correções/razões para reprovação precisam ser discriminadas e o Presidente da Banca e o(a) aluno(a) precisam estar cientes.

E, para constar, foi lavrada a presente ata que vai devidamente assinada pelo coordenador, pelos membros da Comissão Examinadora e pela orientadora do aluno. O aluno deve ficar com uma cópia da ata e a outra deve ser entregue para a secretaria do PBV pelo Presidente da Banca.

Rio de Janeiro, 07 de dezembro de 2021.

**Universidade Federal do Rio de Janeiro**
**Centro de Ciências da Saúde**

**Coordenação de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos**

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**DEDICO**

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## RESUMO

SANTOS-JIMÉNEZ, José Leonardo. **Bioestimulação e indução de defesa em diferentes genótipos de maracujá sob condições de casa de vegetação e de campo.** 2021. Dissertação (Doutorado em Biotecnologia Vegetal e Bioprocessos). Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ. 2021.

A bioestimulação é uma ferramenta útil para apoiar o manejo ecológico na agricultura, aonde fatores bióticos e abióticos mantêm as plantas em condições de estresse permanente prejudicando a produtividade. O uso de bioinsumos que minimizem perdas reduzindo o uso de agrotóxicos, dispendiosos e muitas vezes ineficientes, é demanda urgente. Com a finalidade de encontrar novas alternativas, foi avaliado o efeito de um novo bioestimulante, uma peptidogalactomana, pGM, com ação elicitora de defesa e promotora de crescimento e do uso do ácido húmico com consórcio bacteriano, associado ou não à pGM, em maracujá (*Passiflora edulis*), espécie nativa do Brasil e que movimenta em torno de 1 bilhão de reais na economia do país. Plantas jovens foram submetidas aos tratamentos e suas respostas a nível molecular avaliadas por RT-qPCR para expressão dos genes relacionados à defesa *PR-3*, *POD12*, *SOD*, *PAL* e *LOX2* e de dois fitohormônios (*AUX* e *GA*). As plantas tratadas foram submetidas a infecções com o vírus cowpea aphid-borne mosaic virus (CABMV), causador da principal doença do maracujazeiro no Brasil, o endurecimento do fruto, ou com o fungo *Cladosporium herbarum* e a incidência e severidade das respectivas doenças avaliadas. Parâmetros morfológicos, de desenvolvimento e de produtividade das plantas tratadas sob condições de casa de vegetação e de campo foram avaliados. Primeiramente foi estabelecida a concentração de uso (100 $\mu g.ml^{-1}$) e o efeito da pGM extraída de *Cladosporium herbarum* sobre a indução de defesa em mudas de maracujá 'Redondo Amarelo'. Após 72 h do tratamento, as plantas foram inoculadas com CABMV. A análise da expressão dos genes relacionados a defesa mostrou que estes foram induzidos pelo tratamento antes e depois do inoculo viral. Plantas tratadas com a pGM e infectadas com o CABMV apresentaram sintomas leves ou ausentes da infecção viral e, supreendentemente, apresentaram os mesmos padrões de crescimento, desenvolvimento e biomassa que as plantas saudáveis não inoculadas, mostrando que a pGM leva as plantas a uma resposta robusta à infecção por CABMV, atenuando os sintomas da doença e mantendo o crescimento normal da planta. Testes de RT-PCR e ELISA para detecção do CABMV mostraram forte redução do vírus nas plantas tratadas (Capítulo I). O efeito da pGM como bioestimulador usado sozinho ou em combinação com ácido húmico (AH) e bactérias promotoras de crescimento (PGPB) foi também testado em maracujá em casa de vegetação e em campo em Campos dos Goytacazes, RJ (Capítulo II). A aplicação destes bioinsumos isoladamente ou em combinação, induziu a expressão dos mRNA de *AUX* e *GA* e dos genes relacionados a defesa. Os genes associados a fitohormônios estavam induzidos 16 semanas após os tratamentos em plantas crescidas em condições de campo, o que estava associado a um aumento na biomassa da raiz (aumento de 29% do peso seco) e do número de folhas (37%) e frutos (180%) das plantas. Esses resultados levaram à compreensão dos efeitos de AH mais consorcio bacteriano no crescimento de plantas, relatado na literatura, e mostram pela primeira vez seus efeitos sobre os mecanismos de defesa da planta. Ou seja, os tratamentos podem induzir as plantas a terem respostas mais robustas contra estresses bióticos e abióticos, isto acoplado ao aumento de suas biomassas. A terceira etapa desta tese (Capítulo III), avaliou

o efeito da pGM na mitigação dos danos e perdas na produtividade causados pelo CABMV nos genótipos de maracujá ‘FB300’ e ‘H09-110/111’ sob condições de casa de vegetação e de campo em sistema de produção de agricultura orgânica, em Seropédica, RJ. A pGM induziu a expressão de genes de defesa e fitohormônios após infecção por CABMV em campo, e as plantas tratadas responderam de forma eficiente superando os danos ao desenvolvimento associados ao vírus. A acumulação relativa do CABMV e a severidade da doença foram reduzidas. E, nas plantas tratadas, a infecção pelo vírus não levou a quedas nos padrões de altura, número de folhas, flores e frutos, o que gerou um aumento na produtividade de 65,7 e 114% em ‘FB300’, e 44 e 80% em ‘H09-110/11’, com uma e duas pulverizações, respectivamente, quando comparadas com os controles não tratados. Forma, tamanho e grau BRIX dos frutos não foram afetados pelo vírus nas plantas tratadas. Por fim, foi avaliado o efeito da pGM no controle da verrugose, doença causada pelo fungo *C. herbarum*, sob condições de casa de vegetação. A pGM mais uma vez foi capaz de mitigar a redução da altura, do número de folhas, diâmetro do caule, área foliar e biomassa decorrentes da presença do fungo. A incidência e severidade da verrugose foram também reduzidas pelo tratamento com pGM pós infecção (Capítulo IV). Portanto, a pGM mostrou ser capaz de estimular o crescimento e a defesa das plantas de forma robusta, tanto em casa de vegetação com em campo em duas distintas localidades. Nossos resultados sugerem que os tratamentos por meio dos bioinsumos avaliados, sozinhos ou em associação, oferecem estímulo à promoção de crescimento e proteção contra estresses bióticos em maracujá, evitando assim perdas na produção, diminuindo custos e a aplicação de agrotóxicos. O uso e disseminação dessas tecnologias pode elevar de forma significativa a produtividade do maracujá no Brasil, fortemente afetada por estas doenças, de forma ecologicamente sustentável.

**Palavras-chave:** *Passiflora edulis,* cowpea aphid-borne mosaic vírus, *Cladosporium herbarum,* defesa, fitohormônios, RT-qPCR, genes.

## ABSTRACT

SANTOS-JIMÉNEZ, José Leonardo. **Biostimulation and defense induction in different passion fruit genotypes under greenhouse and field conditions.** 2021. Dissertation (Doctor in Plant Biotechnology and Bioprocesses). Health Sciences Center, Federal University of Rio de Janeiro, Rio de Janeiro, RJ. 2021.

Biostimulation is a useful tool to support ecological management in agriculture, where biotic and abiotic factors keep plants under permanent stress conditions, impairing productivity. Bio-inputs that minimize losses by reducing the use of expensive and often inefficient pesticides is an urgent demand. In order to find new alternatives, the effect of a new biostimulant, a fungal peptidogalactoman, pGM, with defense-eliciting and growth-promoting action and the use of humic acid with bacterial consortium, associated or not with pGM, was evaluate in passion fruit (*Passiflora edulis*) plants, native from Brazil and with an economy of around in the country. Young plants submitted to treatments had their molecular responses evaluated by RT-qPCR for expression of the defense-related genes *PR-3*, *POD12*, *SOD*, *PAL* and *LOX2* and of two phytohormones (*AUX* and *GA*). Treated plants were submitted to infections with cowpea aphid-borne mosaic virus (CABMV), which causes the main passion fruit disease in Brazil, passion fruit woodiness, or with *Cladosporium herbarum* fungus, and the incidence and severity of the respective diseases were evaluated. Morphological, developmental and productivity parameters of plants treated under greenhouse and field conditions were evaluated. Firstly, the use concentration (100 $\mu$ g.ml-1) and the effect of pGM extracted from Cladosporium herbarum on the induction of defense in 'Redondo Amarelo' passion fruit were established. After 72 h of treatment, plants were inoculated with CABMV. Analysis of the expression of defense-related genes showed that they were induced by treatment before and after the viral inoculum. Plants treated with pGM and infected with CABMV showed mild or absent symptoms of viral infection and, surprisingly, showed the same patterns of growth, development and biomass as healthy uninoculated plants, showing that pGM drives plants to a robust response to CABMV infection, attenuating disease symptoms and maintaining normal plant growth. RT-PCR and ELISA tests for detection of CABMV showed a strong reduction of the virus in the treated plants (Chapter I). The effect of pGM as a biostimulator used alone or in combination with humic acid (HA) and growth-promoting bacteria (PGPB) was also tested in passion fruit in a greenhouse and in field, in Campos dos Goytacazes, RJ (Chapter II). The application of these bioinputs alone or in combination induced the expression of *AUX* and *GA* mRNA and defense-related genes. The genes associated with phytohormones were induced 16 weeks after treatments in plants grown under field conditions, which was associated with an increase in root biomass (29% increase in dry weight) and number of leaves (37%) and fruits (180%) of plants. These results lead to an understanding of the effects of HA plus bacterial consortium on plant growth, reported in the literature, and show for the first time its effects on plant defense mechanisms. In other words, treatments can induce plants to have more robust responses against biotic and abiotic stresses, coupled with an increase in their biomass. The third stage of this thesis (Chapter III), evaluated the effect of pGM in mitigating damage and losses in productivity caused by CABMV on passion fruit genotypes 'FB300' and 'H09-110/111' under greenhouse and field conditions in organic agriculture production system, in Seropédica, RJ. pGM induced the expression of defense genes and phytohormones after CABMV infection in the field, and the treated plants responded efficiently, overcoming the developmental damage associated with the virus. The relative accumulation of CABMV and disease severity were reduced. And, in treated plants, the virus infection did not lead to falls in height, number of leaves, flowers and fruits, which generated an increase in productivity of 65.7 and 114% in 'FB300', and 44 and 80 % in ‘H09-110/11’, with one and two sprays, respectively, when compared to untreated

controls. Fruit shape, size and BRIX degree were not affected by the virus in the treated plants. Finally, the effect of pGM in the control of scab, a disease caused by the fungus *C. herbarum*, under greenhouse conditions was evaluated. The pGM was once again able to mitigate the reduction in height, number of leaves, stem diameter, leaf area and biomass resulting from the presence of the fungus. The incidence and severity of scab were also reduced by treatment with pGM post infection (Chapter IV). Therefore, pGM was shown to be able to robustly stimulate the growth and defense of plants, both in the greenhouse and in the field in two different locations. Our results suggest that treatments using the evaluated bioinputs, alone or in association, offer growth promotion and protection against biotic stresses in passion fruit, thus avoiding production losses, reducing costs and the application of pesticides. The use and dissemination of these technologies can significantly increase the productivity of passion fruit in Brazil, which is heavily affected by these diseases, in an ecologically sustainable way.

**Key words:** *Passiflora edulis*, cowpea aphid-borne mosaic vírus, *Cladosporium herbarum*, defense, phytohormones, RT-qPCR, genes.

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# LISTA DE ABREVIATURAS

| | |
|---|---|
| ABA | Ácido abscísico |
| AFv | Ácido fúlvico |
| AH | Ácido húmico |
| AUX | Auxina |
| BR | Brassinosteroides |
| CABMV | Cowpea aphid-borne mosaic vírus |
| CTK | Citocininas |
| ET | Etileno |
| ETI | Imunidade desencadeada por um efetor do patógeno |
| GA | Giberelinas |
| $H_2O_2$ | Peróxido de hidrogênio |
| HR | Resposta de hipersensibilidade |
| IR | Resistência induzida |
| ISR | Resistência sistêmica induzida |
| JA | Ácido jasmônico |
| LOX | Lipoxigenase |
| MAMPs | Padrões moleculares conservados em microorganismos |
| NBLRR | Nucleotide-binding leucine rich repeat |
| $O_2^-$ | Radicais superóxido |
| PAL | Fenilalanina amônia-liase |
| PAMPs | Padrões moleculares conservados em patógenos |
| pGM | Peptidogalactomanana |
| PGPR | Rizobactérias promotoras de crescimento de plantas |
| PGPB | Bactérias promotoras de crescimento de plantas |
| POD | Peroxidase |
| PRs | Proteínas relacionadas à patogenicidade |
| PRRs | Receptores relacionados a patógenos |
| PTI | Padrões moleculares do patógeno |
| RNAi | RNA interferente |
| SA | Ácido salicílico |

| | |
|---|---|
| SAR | Resistência sistêmica adquirida |
| SOD | Superóxido dismutase |
| TMV | Tobacco mosaic virus |

**SUMARIO**

# 1. INTRODUÇÃO

## 1.1. Importância econômica da cultura de maracujá

O gênero *Passiflora* L. pertence à família Passifloraceae, sendo o gênero com maior número de espécies dentro da família, com cerca de 500 espécies. Dentre as espécies do gênero, a *Passiflora edulis* se destaca por sua importância econômica e medicinal (HE et al., 2020). Membros deste gênero conhecidos como maracujá são amplamente plantados em regiões tropicais e subtropicais em várias partes do mundo, especialmente na América do Sul, Caribe, sul da Flórida, África do Sul e Ásia (YUAN et al., 2017; HU et al., 2018). O centro de origem das Passifloraceae é o Brasil, com aproximadamente 200 espécies (COLARICCIO et al., 2020), sendo também o maior produtor mundial de maracujá como uma produtividade de 690.364 mil toneladas por ano, em uma área de 46.436 mil hectares e com uma produção nacional média de 14.8 toneladas por hectare (IBGE, 2020). Dentre as variedades de maracujá, o maracujá amarelo é o mais cultivado no Brasil e pertence a espécie *Passiflora edulis* Sims. Por ter frutos de casca amarela, recebe também a denominação de *Passiflora edulis* Sims forma "flavicarpa". Outra variedade cultivada é o maracujá roxo, *P. edulis* Sims. Estas são as duas variedades principais e comuns com considerável importância econômica (ZUCOLOTTO et al., 2009; CAZARIN et al., 2016). O maracujá amarelo tem 6–12 cm de comprimento e 4–7 cm de diâmetro; a casca é amarela brilhante, dura e espessa; as sementes são marrons; a polpa é ácida e possui forte sabor aromático. O maracujá roxo é relativamente pequeno (4–9 cm de comprimento e 3,5–7 cm de diâmetro); sua casca é roxa e a semente é preta (NARAIN et al., 2010).

O maracujá é produzido na maioria dos casos para o mercado *in natura*, onde, em geral, conseguem melhor preço e vendem os excedentes para a agroindústria (FERRAZ et al., 2002). O maracujazeiro é uma cultura de alto risco, devido à grande suscetibilidade a doenças e de ser necessário atender à exigência de qualidade dos mercados a que se destina. Ele é cultivado por pequenos produtores, com pomares de aproximadamente 5 hectares, no entanto, tem sido uma atividade bastante atrativa pelo alto valor agregado da produção (FONSECA, 2017). A mão de obra desta cultura é considerável, já que cada hectare de maracujá gera 4 empregos diretos aproximadamente e ocupa 7 a 8 pessoas nos diversos elos da cadeia produtiva (MELETTI, 2011), o que confere à cultura uma grande importância social.

## 1.2. Principais doenças do maracujazeiro

Várias doenças podem comprometer a produtividade e a longevidade da cultura do maracujazeiro. Dentre as doenças fúngicas encontramos as causadas por *Fusarium oxysporum* f. sp. *passiflorae, Fusarium solani* f. sp. *passiflorae, Phytophthora cinnamomi* Rands, *Rhizoctonia* sp., *Pythium* sp., *Phytophthora* sp e *Cladosporium herbarum*; dentre as principais doenças causadas por bactérias, destaca-se *Xantomonas axonopodis* pv. *passiflorae, Xanthomonas campestris* pv. *passiflorae*. As doenças causadas por vírus também causam impacto nesta cultura devido à dificuldade em controlar doenças virais em plantas, sendo o mais importante o cowpea aphid-borne mosaic virus (CABMV).

### 1.2.1. Viroses

Alguns vírus já foram descritos nesta espécie, sendo estes: cowpea aphid-borne mosaic virus (CABMV, *Potyvirus*) da família *Potyviridae;* passionfruit yellow mosaic virus (PFYMV, *Tymovirus*), da família *Tymoviridae;* passion fruit green spot virus (PFGSV, *Rhabdovirus*) da família *Kitaviridae;* passion flower little leaf mosaic virus (PLLMV, *Begomovirus*) e passion fruit severe leaf distortion virus (PFSLDV, *Begomovirus*), da família *Geminiviridae*, passion fruit vein clearing virus (PVCV, gênero não atribuído) da família *Rhabdoviridae*; cucumber mosaic virus (CMV, *Cucumovirus*) da família *Bromoviridae* (FISCHER; RESENDE, 2008; COLARICCIO et al., 2018) e lettuce chlorosis virus (LCV, *Crinivirus*) da família *Closteroviridae* (VIDAL et al., 2021). No entanto, a espécie mais prejudicial é o CABMV, uma espécie de vírus que ocorre em todas as regiões produtoras do mundo.

#### 1.2.1.1. Os Potyvirus

Os vírus pertencentes ao gênero *Potyvirus*, família *Potyviridae* são filamentosos, não envelopados, com aproximadamente 680-900 nm de comprimento e 12-15 nm de largura. Os virions contêm uma única molécula de RNA linear de sentido positivo, com aproximadamente 10.000 nucleotídeos, que codificam para uma única poliproteína (FISCHER; RESENDE, 2008). A poliproteína derivada do genoma é clivada em várias proteínas, algumas das quais formam corpos de inclusão amorfos e tipo cata-vento nas células (Figura 1) (FAUQUET et al. 2005; WYLIE et al., 2017).

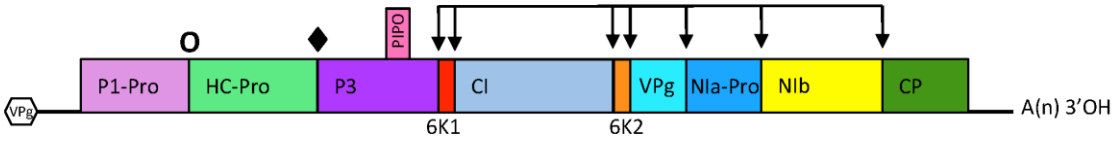


**Figura 1.** Mapa genômico de um membro típico do gênero *Potyvirus*, família *Potyviridae*. O genoma ssRNA é representado por uma linha e a poliproteína ORF por uma caixa aberta com os produtos proteolíticos maduros nomeados. VPg (proteína viral ligada ao genoma), a proteína viral ligada ao genoma covalentemente ligada ao nucleotídeo 5 'terminal é representada por um hexágono; P1-Pro (protease de proteína 1), uma proteína com atividade proteolítica de serina responsável pela clivagem em tipicamente Tyr / Phe-Ser (O); HC-Pro (protease de componente auxiliar), uma proteína com atividade de componente auxiliar de transmissão de pulgões e atividade proteolítica de cisteína responsável pela clivagem em Gly-Gly (◆); P3 (proteína 3); PIPO (ORF Potyviridae muito interessante); 6K (péptido de seis quilodaltons); CI (inclusão citoplasmática); NIa-Pro (protease de inclusão A nuclear), atividade proteolítica semelhante à cisteína responsável pela clivagem em Gln / Glu- (Ser / Gly / Ala) (↓); Nib (inclusão nuclear B), RNA polimerase dependente de RNA; CP (proteína de revestimento). Os locais de clivagem de P1-Pro, (O), HC-Pro (◆) e NIa-Pro (↓) são indicados (WYLIE et al., 2017).

### 1.2.1.2. Cowpea aphid-borne mosaic virus (CABMV)

Os isolados de CABMV causam perdas importantes na cultura do maracujá, devido aos graves sintomas de endurecimento dos frutos, em todas as regiões produtoras (CAVICHIOLI et al., 2011; COLARICCIO et al., 2018). Os sintomas foliares caracterizam-se pela presença de mosaico, e, em casos mais severos, este é acompanhado de bolhosidade e deformação da folha. Já os frutos podem apresentar bolhosidade, deformação, endurecimento e diminuição do tamanho (Figura 2). Plantas infectadas com CABMV têm redução da área foliar e do peso de fruto, com consequente redução do número, da qualidade e do valor comercial dos frutos (NASCIMENTO et al., 2004). O vírus é transmitido de forma não persistente por várias espécies de pulgões, e mecanicamente, durante a poda. A doença causa perdas severas de rendimento e reduz a vida útil da planta em aproximadamente 50% (FISCHER; REZENDE, 2008). O controle desta doença é problemático. Todas as cultivares de maracujá amarelo e doce são suscetíveis à infecção, e o controle químico de vetores é geralmente ineficiente devido à forma não persistente de transmissão. A proteção cruzada com cepas menos virulentas do vírus também não conseguiu controlar a doença (NOVAES et al., 2003). Entretanto, podem-se controlar os vetores, através de pulverização com inseticidas, porém, esse método não oferece

resultados satisfatórios e tem um alto custo, além da poluição ambiental e o risco para a saúde do operador (PINTO et al., 2008). SILVA et al. (2016) observaram que aplicações exógenas de ácido salicílico levou a indução de resposta de defesa em plantas de maracujá sob condições de casa de vegetação, diminuindo a severidade significativamente dos sintomas causados pelo CABMV. Várias medidas para controle da doença do endurecimento dos frutos foram adotadas, porém, até o momento, nenhuma teve resultado satisfatório no controle desta doença em campo. A melhor estratégia seria o desenvolvimento de cultivares resistentes ou tolerantes mediante programas de melhoramento genético, com alguns grupos apresentando sucesso (PREISIGKE et al., 2021; dos SANTOS et al., 2021). No entanto, alguns estudos têm mostrado que existe pouca variabilidade genética entre as cultivares de maracujazeiro-azedo para resistência a doenças (LEÃO et al., 2006; CERQUEIRA SILVA et al., 2008; CERQUEIRA SILVA et al., 2012). As principais estratégias de defesa em plantas frequentemente variam com as diferenças nas interações entre vírus específicos e seus respectivos hospedeiros. Até o momento, informações relativamente limitadas estão disponíveis sobre as estratégias de manejo e controle do CABMV na cultura do maracujazeiro.

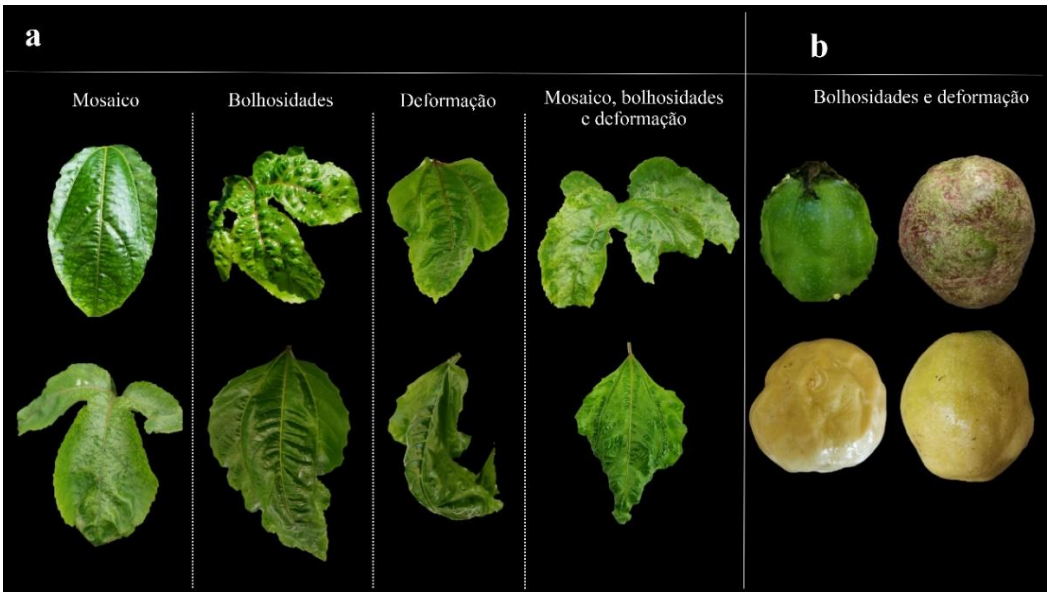


**Figura 2.** Sintomatologia induzida pelo cowpea aphid-borne mosaic virus (CABMV) em folhas (a) e frutos (b) de plantas de maracujá. Fonte: Acervo pessoal.

#### 1.2.1.3. Melhoramento genético do maracujazeiro para a resistência ao CABMV

Características agronômicas de interesse visando a identificar genótipos resistentes/tolerantes ao CABMV, devem ser estudadas enquanto a diversidade genética existente para futuros cruzamentos promissores (PAIVA et al., 2014a). Devido a estreita base genética de *Passiflora edulis* para resistência a doenças, os programas de melhoramento genético visam a usar espécies silvestres como fontes de genes de resistência, para utilizá-los em cruzamentos interespecíficos (SANTOS et al., 2014).

Alguns avanços significativos na resistência/tolerância desta doença em maracujá têm sido citados enquanto a produtividade e qualidade de frutos (SANTOS et al., 2015; FREITAS et al., 2015). Características de qualidade e de produtividade de frutos em *Passiflora edulis*, junto com as caraterísticas de resistência de outros genótipos como *Passiflora setácea*, podem ser usadas como estratégias para obter híbridos resistentes e que possam ter potencial e valor comercial (SANTOS et al., 2015). Estes mesmos autores tem mostrado avanços relacionados à resistência e tolerância às doenças e pragas importantes em maracujá.

Os programas de melhoramento genético do maracujá direcionam-se a obter fontes de resistência ao CABMV e a incorporação de genes de resistência em novas cultivares (VIDAL, 2021). Estudos recentes envolvendo um híbrido interespecífico (*P. edulis* x *P. cincinnata*) seguido de retrocruzamentos com *P. edulis* tem mostrado potencial para utilização em programas de melhoramento, por apresentarem vigor vegetativo e reprodutivo mais precoce, produção acumulada superior a 70 frutos por planta e resistência a CABMV (dos SANTOS et al., 2021). GONÇALVES et al., (2021) avaliaram diferentes genótipos do Banco Ativo de Germoplasma de Maracujá da Embrapa Mandioca e Fruticultura visando resistência ao CABMV, observando que após quatro inoculações mecânicas do CABMV, o vírus não foi detectado por RT-qPCR nas folhas superiores de plantas das espécies *P. pohlii* e *P. bahiensis*, indicando que essas espécies são provavelmente imunes ao CABMV.

### 1.2.2. Verrugose causada pelo *Cladosporium herbarum*

A Verrugose é uma doença causada pelo fungo *Cladosporium herbarum*, que ataca as folhas, os ramos e os frutos do maracujazeiro. A doença apresenta maior incidência quando a temperatura está amena, em torno de 15 a 25 ºC e umidade relativa alta. Segundo RHEINLÄNDER (2010), temperaturas de 20 °C são propícias para a infecção da planta pelo

fungo. SIMMONDS (1932) definiu entre 20 a 22 °C, a temperatura ótima para o patógeno. As folhas ficam com manchas circulares de 5 mm (Figura 3a), principalmente as folhas mais novas, as quais, mais tarde, sofrem necrose e caem. Os ramos, as gavinhas e os frutos apresentam lesões circulares e translúcidas que, depois, cobrem-se de tecido corticoso, áspero e saliente, de cor parda, com aspecto de "verrugas" (Figura 3b). A verrugose pode causar grandes perdas, pois atrasa o desenvolvimento da planta devido à diminuição de folhas novas, inclusive podendo provocar a morte das mudas. Nos frutos, apesar de não provocar podridão, a doença causa a desvalorização em razão da presença de verrugas e da deformação dos frutos (TEIXEIRA et al., 1994; PERUCH et al., 1997). Essa doença ocorre em todas as zonas produtoras do Brasil e, quando não controlada, exerce uma ação destrutiva nas folhas, ramos e frutos. Os frutos que conseguem desenvolver-se tornam-se imprestáveis para o comércio de frutas frescas (FERREIRA, 2016). A verrugose manifesta-se, principalmente, em tecidos em fase de crescimento, prejudicando o desenvolvimento da planta e reduzindo a produção. Diferentes regiões produtoras do Brasil e, diversos países são afetados por esta doença. Nas folhas, os sintomas se apresentam por lesões circulares medindo de 3 a 5 mm de diâmetro, inicialmente translúcidas, tornando-se necróticas posteriormente (FERREIRA, 2016). Outro sintoma da verrugose é a deformação (encarquilhamento) das folhas quando as lesões estão próximas ou sobre as nervuras. O rompimento no centro da lesão causa perfuração da folha (PIO-RIBEIRO; MARIANO, 1997). O controle, quando detectada a presença da doença, pode ser feito por meio do uso de fungicidas de efeito curativo (FERREIRA, 2016). Não se recomenda a aplicação de defensivos quando o destino de frutos é a indústria de sucos, pois as lesões limitam-se apenas à casca, não atingindo a polpa (FERREIRA, 2016). Deve-se dar atenção especial aos períodos em que os tecidos da planta são mais suscetíveis à doença, como quando as plantas são jovens, época de floração e período inicial da formação dos frutos.

Dentre os tratamentos para o manejo e controle desta doença, a maioria são por meio da aplicação de fungicidas. Algumas alternativas estudadas com sucesso para sistema de agricultura orgânica, é a aplicação de calda sulfocalcica em *Passiflora alata*, diminuindo a severidade da doença (MING et al., 2012). Entretanto, resultados promissórios no desenvolvimento de variedades de maracujá resistentes a esta doença têm sido estudados (BATISTTI et al., 2017). Porém, mais estudos são necessários para o uso destas variedades em um programa de melhoramento, visando à resistência à "verrugose".

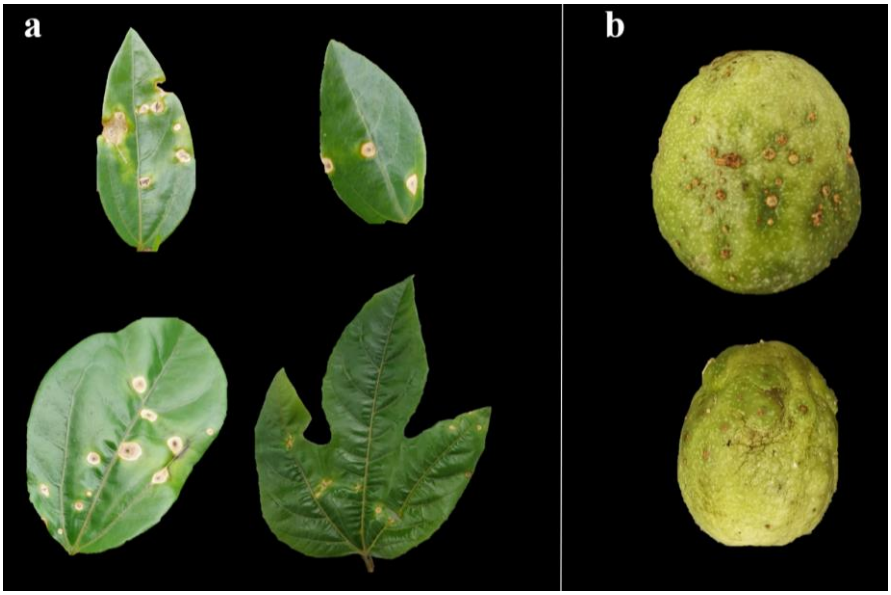


**Figura 3.** Sintomatologia da "verrugose" causada por *Cladosporium herbarum* em folhas (a) e frutos (b) de plantas de maracujá. Fonte: Acervo pessoal.

### 1.2.3. Bacteriose

A bacteriose é uma doença causada pela bactéria *Xanthomonas axonopodis* pv. *passiflorae*. Os sintomas iniciam nas folhas, com lesões pequenas, encharcadas, oleosas, translúcidas, frequentemente localizadas próximas às nervuras, com halos visíveis, podendo ocorrer o enegrecimento vascular a partir das bordas foliares (PIO-RIBEIRO; MARIANO, 1997). Essas lesões necrosam, assumindo tonalidade marrom avermelhada, principalmente na face dorsal da folha, podendo também formar um halo clorótico ao redor da mancha, de formato variado, raramente circulares, com tamanho médio de 3 a 4 mm (Figura 4) (VIANA et al., 2003). O desenvolvimento desta doença, leva à desfolha da planta, diminuindo consideravelmente a produtividade.

O tratamento preventivo desta doença pode ser realizado para evitar a contaminação por bacteriose, entre elas, se destacam o uso de sementes e mudas desenvolvidas por instituições certificadas (SÃO JOSE et al., 2011). Já, em casos mais severos o controle químico deve ser utilizado, aplicando quinquenalmente oxicloreto de cobre ou oxitetraciclina + estreptomicina (FISHER; KIMATI; REZENDE, 2005), mas o controle por meio destes antibióticos tem se mostrado altamente ineficiente (ARRIFANO, 2019).

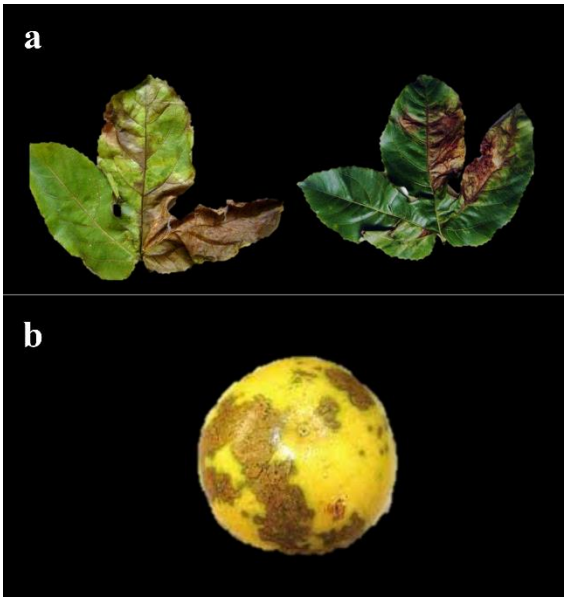


**Figura 4.** Sintomatologia de bacteriose causada pela bactéria *Xanthomonas axonopodis* pv. *passiflorae* em folhas (a) e frutos (b) de plantas de maracujá. Fonte: (a) https://www.agrolink.com.br/problemas/mancha-bacteriana_2047.html; (b) ALMEIDA at el., 1994.

### 1.3. Mecanismos de defesa das plantas

As plantas desenvolveram imunidades inatas e induzidas contra a infecção por patógenos. Tais sistemas de defesa incluem o RNA interferente (RNAi) que atua especialmente contra infecções virais; a imunidade associada a padrões moleculares do patógeno (PTI) e a imunidade desencadeada por um efetor do patógeno (ETI) (LI, et al., 2013). Tanto as plantas quanto os organismos associados contribuem com atores moleculares que ditam o resultado da interação planta-patógeno (WIN et al., 2012). Por exemplo, as plantas contribuem com a superfície celular e com os receptores imunes intracelulares, e os organismos colonizadores produzem um repertório de efetores que modulam os processos da planta, incluindo a indução e supressão das defesas da planta (Figura 5). A PTI é desencadeada através do reconhecimento por receptores das células vegetal, denominados PRRs (receptores relacionados a patógenos), de padrões moleculares conservados em patógenos e/ou microorganismos (PAMPs e/ou MAMPs), tais como a quitina presente na parede celular de fungos ou flagelina bacteriana (YANG et al., 2013; KAMATHAM et al., 2016). O reconhecimento destes padrões pode conter a colonização do patógeno e assim a doença, conferindo uma resistência basal. Já a segunda, ETI, imunidade desencadeada por um efetor, confere resistência duradoura, muitas vezes resultando em resposta de hipersensibilidade (HR) que a sua vez resulta na geração de estresse

oxidativo, disparando assim a morte celular. HR é resposta da planta realizada por meio de morte celular programada, como mecanismo de resistência onde está mata suas próprias células durante os estágios iniciais do ataque do patógeno, de forma que isola o patógeno dos nutrientes e seu crescimento cessa (SARKAR et al., 2015). As HR constituem na primeira etapa de respostas de defesa da planta que dispara uma série de outras alterações tais como: produção de proteínas relacionadas à patogenicidade (PRs), principalmente peroxidases, quitinases, β-1,3-glucanases; aumento na expressão de fenilalanina amônia liase; deposição de calose e lignina; produção de fitoalexinas e aumento dos níveis de ácido salicílico (VERBENE et al., 2000).

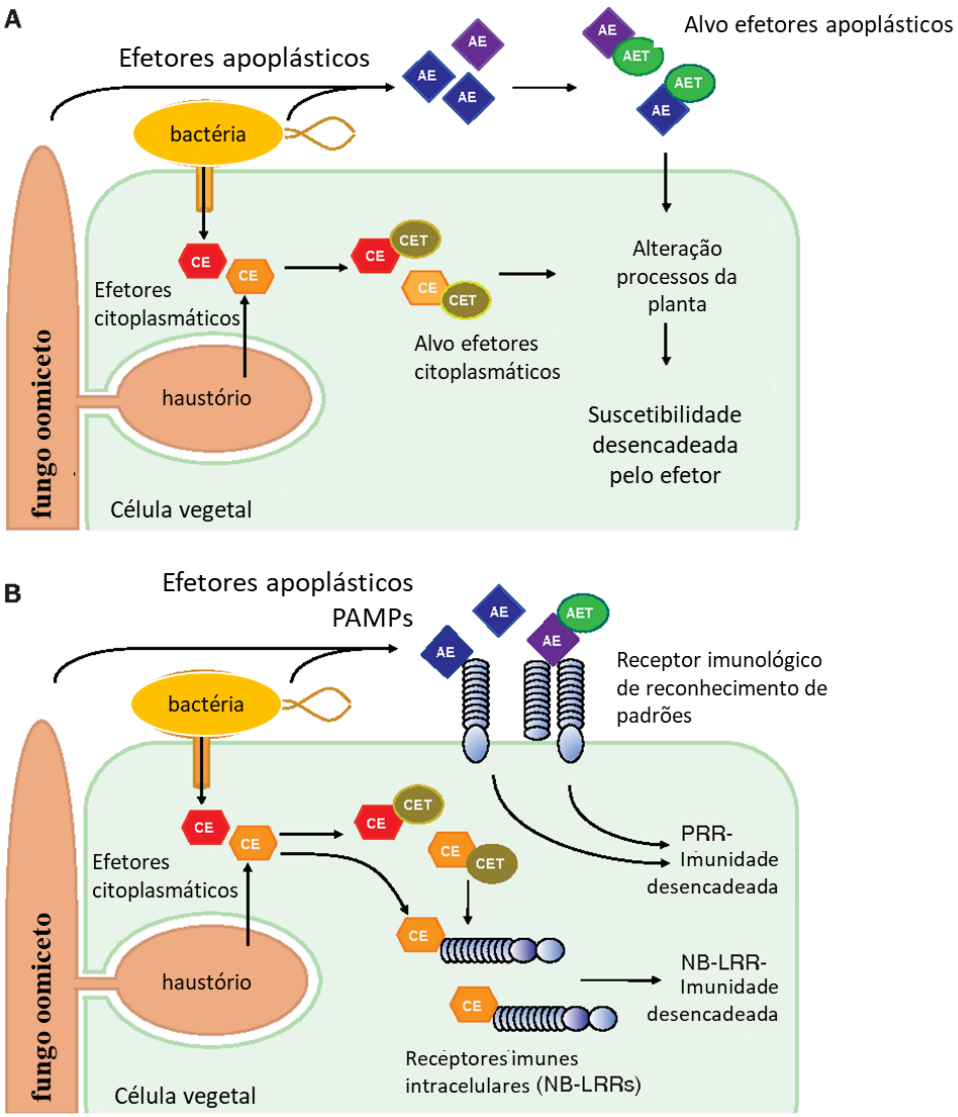


**Figura 5.** O conceito de efetores na imunidade das plantas. Patógenos infecciosos como bactérias, fungos, oomicetos e nematóides liberam moléculas efetoras na interface da planta

hospedeira (efetores apoplásticos, AE) ou dentro da célula (efetores citoplasmáticos, CE). Efetores translocados do hospedeiro (citoplasmáticos) são inseridos no citoplasma do hospedeiro por meio de um pilus de secreção do tipo III ou estruturas infecciosas especializadas chamadas haustórios que se formam dentro da célula. Os efetores do patógeno trafegam para vários compartimentos da planta, e ligam e manipulam diferentes proteínas do hospedeiro chamadas de alvos. Dependendo de sua localização nas células, esses alvos translocados são designados como alvo efetor apoplástico (AET) e alvo efetor citoplasmático (CET). As interações efetor-alvo impactam o resultado da interação entre o patógeno e seu hospedeiro. Em genótipos suscetíveis **(A)**, essas interações moleculares podem alterar os processos das células vegetais e suprimir as respostas imunológicas, levando à suscetibilidade desencadeada por efetores (ETS) e à colonização do hospedeiro. Em genótipos resistentes **(B)**, essas interações são percebidas pelos principais receptores de detecção do sistema imunológico que, por sua vez, interrompem o crescimento do patógeno. Receptores de reconhecimento de padrão de superfície celular (PRRs) detectam padrões moleculares associados a patógenos (PAMPs), efetores apoplásticos e/ou interações efetor-alvo apoplástico para iniciar a imunidade desencadeada por PRR (PTI). Receptores de ligação de nucleotídeos intracelulares (NB-LRR) induzem imunidade desencadeada por NB-LRR (ETI) no reconhecimento de efetores citoplasmáticos e/ou interações efetoras citoplasmáticos-alvo (Adaptado de WIN et al., 2012).

A imunidade associada diretamente aos efetores de patógenos é desencadeada através da identificação dos efeitos destes efetores nos alvos celulares do hospedeiro. Um tipo comum de ETI é o mediado pelo reconhecimento de efetores patogênicos por proteínas "*nucleotide-binding leucine rich repeat*" (NB-LRR), codificadas por genes de resistência a doenças. Este reconhecimento acaba acelerando e amplificando a resposta por PTI e levando, muitas vezes, a indução de HR e à resistência induzida (IR) pelo hospedeiro.

As plantas têm a capacidade de induzir resistência local e sistêmica ao ataque subsequente pelo mesmo ou por diferentes patógenos (WALTERS et al., 2007; HAMMERSCHMIDT, 2007). Essa IR pode controlar os patógenos diretamente ou gerar fatores que tornem o ambiente prejudicial ao patógeno. Este controle pode ser completo ou parcial (KUC, 1982; CHEN et al., 2014). Estudos revelaram que os genes expressos durante as respostas IR produzem proteínas como quitinase, glucanase e outras atividades enzimáticas que estão envolvidas nas reações de defesa a uma ampla gama de patógenos (IWUALA, et al., 2020; SAMARAH, et al., 2020; HANIN, et al., 2020; KHAN; UMAR, 2021). Existem diferentes maneiras de ativar o mecanismo de defesa das plantas. As duas formas comuns são chamadas de resistência sistêmica induzida (ISR) e resistência sistêmica adquirida (SAR) (PIETERSE et al., 2012). A ISR e a SAR são fenômenos diferentes, mas são respostas ativas de defesa da planta ao ataque do fitopatógeno.

A SAR fornece defesa a longo prazo contra um amplo espectro de agentes patogênicos (MUTHAMILARASAN; PRASAD, 2013; KAMATHAM et al., 2016). PTI e ETI induzem SAR através de complexas redes que envolvem uma variedade de sinais móveis e de interação cruzada, que culminam por ativar vias de sinalização imunológica como as mediadas por fitohormônios e restringir a invasão e proliferação microbiana (LI et al., 2013, CUI et al., 2014; BERENS et al., 2017; BOUTROT; ZIPFEL, 2017). Uma vez ocorrido o reconhecimento do patógeno ou microorganismo, é desencadeada pela planta uma resposta rápida e forte nos locais de infecção (ELVIRA et al., 2008). Embora ETI confira imunidade potente e robusta contra patógenos biotróficos, que se alimentam de tecidos hospedeiros vivos, a ETI é explorada por alguns patógenos necrotróficos que ativamente matam os tecidos do hospedeiro e se alimentam dos seus restos (NOBORI et al., 2018). Além disso, as respostas de defesa e morte celular associadas à ETI são estritamente reguladas de maneira espaço-temporal (BETSUYAKU et al., 2017; TSUDA, 2018). Portanto, pode ser possível que a regulação espaço-temporal de respostas ETI, incluindo morte celular, forneçam certa tolerância contra a infecção por patógenos necrotróficos. A SAR não é importante somente para a indução de resistência a fitopatógenos, já que é útil para as plantas conseguirem se recuperar destes (KAMLE et al., 2020). Entretanto, ISR é mediada por microrganismos benéficos presentes no solo, os quais interagem com as raízes das plantas, levando assim a uma indução de resposta imune nestas (ALOO et al., 2019; NEWITT et al., 2019).

Nas últimas duas décadas, SAR e ISR foram continuamente investigados em muitas culturas (GOZZO; FAORO, 2013; SARMA et al., 2016). Esses estudos mostram que os mecanismos moleculares envolvidos na SAR das diferentes plantas podem variar dependendo do agente indutor da resistência. Plantas de tabaco tratadas com biolicitores, leva a expressão proteínas relacionadas à patogênese (PR-proteína), induzindo SAR contra o TMV (WANG et al., 2021). Entretanto, ISR estabelece um estado preparado induzindo respostas de defesa mais rápidas após o ataque de patógenos (MAUCH-MANI et al., 2017). A SAR pode ser induzida pela infecção local de patógenos e envolve a sinalização do ácido salicílico (SA) e a expressão PRs (GAO et al., 2015). No entanto, outras proteínas PR, como quitinase ou PR5, também são expressas durante a SAR na infecção por patógenos. Por exemplo, *Puccinia triticina*, o patógeno da ferrugem da folha induz expressão do gene *PR5* após a infecção de tecidos de folhas de trigo (LI et al., 2015).

Portanto a IR se refere a um estado de "capacidade defensiva aprimorada" desenvolvido em uma planta devido a estímulos ambientais, por meio do qual as defesas inatas da planta são ativadas contra desafios bióticos subsequentes. Esta resistência é eficaz contra um grande número de patógenos, incluindo fungos, bactérias e vírus, (KUMARI et al., 2018a; KUMARI et al., 2018b; MEENA; SWAPNIL, 2019; SWAPNIL et al., 2021). A SAR e a ISR são os dois tipos de resistência induzida (Figura 6). A diferenciação é feita com base nas vias regulatórias envolvidas e na natureza do elicitor (KAMLE et al., 2020).

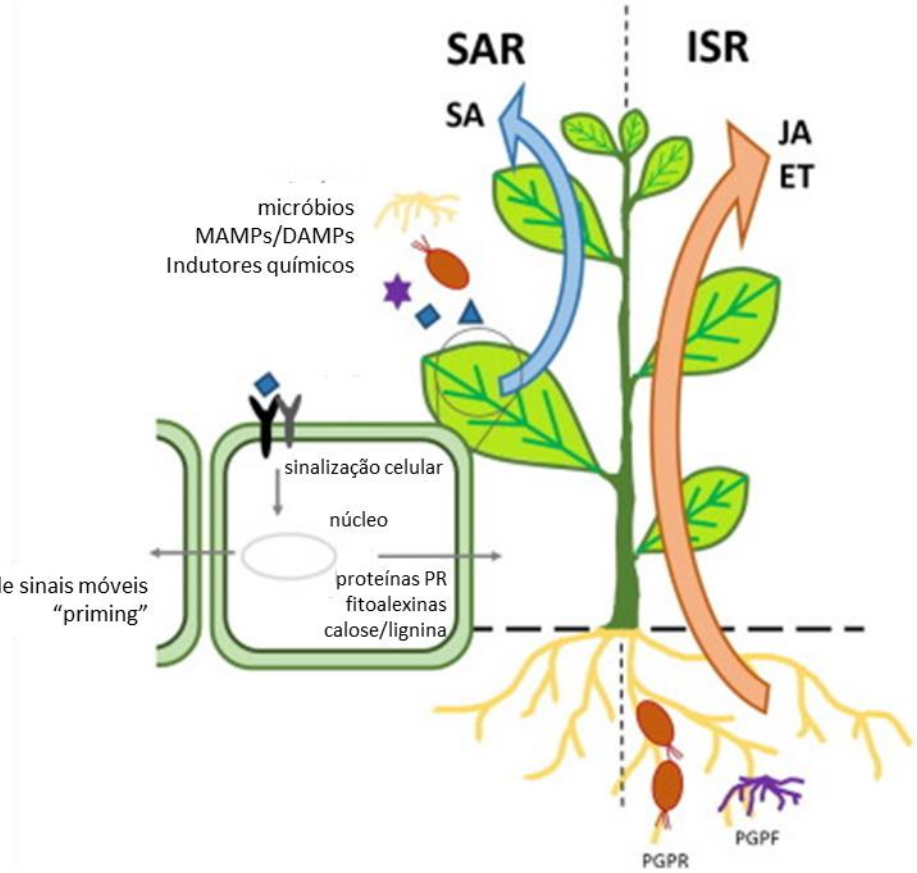


**Figura 6.** Esquema dos diferentes tipos de IR. A resistência sistêmica adquirida (SAR, a parte esquerda do esquema) é desencadeada por ataque de patógenos, tratamentos foliares de plantas com MAMPs, DAMPs, fitohormônios vegetais, ativadores químicos, entre outros. Após o reconhecimento por um sensor, a célula vegetal desencadeia uma sinalização complexa e respostas de defesa, incluindo a produção de proteínas relacionadas à patogênese (PRs), fitoalexinas antimicrobianas ou fortificação da parede celular com depósito de calose ou lignina. As defesas locais são seguidas pela produção de sinais móveis que são transportados via xilema a partes distais principais da planta para o acúmulo de compostos de defesa nestas regiões que não entraram em contato com o elicitor. A resistência sistêmica induzida (ISR, a parte direita do esquema) pode ser desencadeada pela colonização da raiz com rizobactérias ou fungos promotores de crescimento de plantas, PGPR ou fungos PGPF, respectivamente. ISR é

SA-independente, governado principalmente por ácido jasmônico (JA) e etileno (ET). Tanto o SAR quanto o ISR são defesas aprimoradas contra estresses bióticos e abióticos subsequentes. Redesenhado por BURKETOVA et al., 2015 e modificado de PIETERSE et al., 2009.

### 1.3.1. Proteínas relacionadas a defesa das plantas

As PRs são definidas como proteínas induzidas no hospedeiro em resposta à infecção por um patógeno ou por estímulos abióticos, estas atuam como marcadores nas vias de sinalização relacionadas à defesa e estão envolvidos na resistência de plantas a patógenos (DAI et al., 2016). Plantas resistentes respondem rapidamente às infecções, aumentando o acúmulo de transcritos de genes PR com a invasão do patógeno (LAVROVA et al., 2017; CUI et al., 2020; JIANG et al., 2019; HOU, et al., 2020). Essas proteínas PRs foram detectadas pela primeira vez no início da década de 70 em folhas de fumo (*Nicotina tabacum*) infectadas pelo tobacco mosaic virus (TMV) (VAN LOON; VAN KAMMEN, 1970). Atualmente são conhecidas 17 famílias de PRs em plantas, com diferentes funções (ZRIBI; GHORBEL; BRINI, 2020).

O papel das PRs na resistência de plantas contra microrganismos patogênicos pode ser tanto direto como indireto. Uma ação direta, como por exemplo, a inibição do crescimento do patógeno ou da germinação de esporos, representa uma concepção simplificada da defesa de plantas contra a entrada de agentes patogênicos. Neste sentido, em muitos casos as PRs apresentam atividade antimicrobiana *in vitro*. As PRs normalmente exercem uma maior atividade contra fungos, mas também tem relatos destas na defesa das plantas contra outros fitopatógenos, como bactérias, nematóides ou vírus (ZRIBI; GHORBEL; BRINI, 2020). Em sua maioria, PRs possuem uma maior importância na ação indireta, ou seja, no processo de indução de resistência, como por exemplo, na ação preventiva contra penetração de patógenos, por ação oxidativa de componentes da parede celular vegetal por meio de peroxidases (PR9); ou envolvimento na transdução de sinais durante a interação patógeno-hospedeiro, como na ação de oxalato oxidases (PR-15, PR-16) e de proteínas não específicas relacionadas com o transporte de lipídios (PR-14) (JAIN; KHURANA, 2018). Um número pequeno de famílias das PRs possui atividade enzimática como é o observado nas glucanases (PR-2), quitinases (PR-3, PR-8, PR-11), peroxidases (PR-9), ribonucleases (PR-10) e (PR-4), ou ação inibitória (PR-6). A atividade inibitória de proteases e α-amilases é atribuída às famílias PR-5, PR-12 e PR-14 (VAN LOON et al., 2006; GORJANOVIC, 2009; STANGARLIN et al., 2011).

As quitinases constituem o segundo maior grupo de proteínas antifúngicas. Elas catalisam a clivagem hidrolítica do β-1,4-glicosídeo, ligação presente em biopolímeros de N-acetil-D-glicosamina, principalmente em quitina (FERREIRA et al., 2007). As quitinases são um dos grupos importantes da proteína PR induzida em resposta ao estresse nas plantas. A infecção viral / fúngica induz a atividade da quitinase nas plantas devido à qual são consideradas como proteínas PR em geral (BORDOLOI et al., 2021). Assim, as quitinases de plantas inibem o crescimento de fungo invasores e geram oligossacarídeos de quitina que atuam como elicitores de defesa (FERREIRA et al., 2007).

O ataque de patógenos e diferentes condições de estresse podem ativar a produção de espécies reativas de oxigênio, o que pode danificar o DNA e as proteínas, e severamente comprometer a função da membrana. Em resposta ao aumento produção de radicais de oxigênio, as plantas ativam um sistema antioxidante complexo composto das moléculas capazes de limpar essas espécies, entre elas uma variedade de enzimas. As principais enzimas vegetais envolvidas na desintoxicação de tais radicais são as peroxidases (POD) e superóxido dismutase (SOD) (HIRAGA et al., 2001; ALMAGRO et al., 2009). A SOD parece ser a primeira a agir na linha de defesa em relação à eliminação de ROS, realizando a dismutação de radicais superóxido ($O_2^-$) em peróxido de hidrogênio ($H_2O_2$). Ela desempenha um papel importante na defesa contra espécies de oxigênio reduzindo seus efeitos tóxicos, e por isto, a SOD tem sido proposta como uma enzima importante para a tolerância ao estresse da planta (STEPHENIE et al., 2020). A regulação positiva de SOD está implicada no combate às espécies reativas de oxigênio (ROS) produzidas em excesso devido a estresses bióticos ou abióticos e tem um papel crucial na sobrevivência da planta em ambiente estressante (BELA et al., 2017). Um aumento significativo nas atividades totais de SOD foliar tem sido relatado em muitas espécies de plantas sob vários tipos de estresses abióticos em várias plantas como algodão (YI et al., 2016), cebola (RADY et al., 2018), *Camellia sinensis* (LI et al., 2018), entre outros. A superexpressão de SOD em plantas aumenta a tolerância ao estresse oxidativo e a outros estresses ambientais (WANG, et al., 2018). Houve muitos relatos no desenvolvimento de plantas tolerantes ao estresse com aumento da expressão de diferentes SODs em plantas (ZHOU et al., 2019; HUANG et al., 2020; HOU et al., 2020; MBAMBALALA, et al., 2021).

As PODs catalisam a oxidação de vários substratos usando peróxido de hidrogênio e estão envolvidas em uma variedade de processos biológicos (KALSOOM et al. 2015). As PODs

são específicas para plantas e são proteínas-chave que controlam a diferenciação e o desenvolvimento das plantas (OLIVEIRA et al., 2019; YAN et al., 2019; RENARD et al., 2020). Diferentes PODs têm atividades antagônicas que contribuem para o afrouxamento e o enrijecimento da parede celular que ocorre durante o crescimento da planta e a expansão celular (HERNÁNDEZ-ESQUIVEL et al., 2021).

Outra enzima relacionada aos mecanismos de defesa da planta é a fenilalanina amônia-liase (PAL), esta é uma enzima chave que controla o fluxo do metabolismo primário para o segundo metabolismo, que gera fitoalexinas antimicrobianas e desempenha papéis importantes na resposta ao ataque de patógenos (WU et al., 2017). Estudos tem indicado que esta enzima está envolvida nas respostas da planta ao ataque de patógenos (CASS et al., 2015; WANG et al., 2020; HE et al., 2020).

A lipoxigenase (LOX) é uma enzima da via dos octodecanoides que resulta na produção de JA. As LOXs foram identificadas em várias partes celulares que produzem moléculas de sinalização e estão envolvidas em diversas funções, que incluem crescimento e desenvolvimento, germinação de sementes, resposta ao estresse e ferimento biótico e abiótico, amadurecimento de frutos, senescência, morte celular e síntese de JA (VISWANATH et al., 2020). LOX induz modificações na parede celular para limitar a invasão de patógenos (VELLOSILLO et al., 2013). Em *Zea mays*, LOX desempenha um papel significativo na síntese de um nível mais alto de JA contra infecção por *Cochliobolus heterostrophus* (CHRISTENSEN et al., 2015). A via da LOX é crucial para o processo de peroxidação de lipídeos durante a resposta de defesa de plantas contra patógenos (HWANG; HWANG, 2010). Estudos genéticos revelaram o papel da via da LOX na defesa de plantas (WEI et al., 2020; JAN et al., 2020; ARANTES et al., 2020; SHABAN et al., 2021; CARAVACA-FUENTES et al., 2021).

Além das diferentes enzimas citadas acima, os fitohormônios também desempenham papéis essenciais para que o sistema de defesa seja bem desenvolvido nas plantas contra o ataque de fitopatógenos. O ácido salicílico (SA), o ácido jasmônico (JA) e o etileno (ET) são considerados atores-chave no sistema de defesa das plantas. Além disso, muitos outros fitormônios, incluindo auxina (AUX), ácido abscísico (ABA), citocininas (CTK), giberelinas (GA) e brassinosteroides (BR), também participam da defesa da planta contra diferentes tipos de patógenos (DEPUYDT S; HARDTKE CS, 2011; BERENS et al., 2017). Como componentes essenciais para o equilíbrio do estresse e aptidão das plantas, os fitohormônios influenciam

diferentes processos de crescimento e desenvolvimento, bem como estratégias de defesa nas plantas. Normalmente, SA, JA e ET estão principalmente envolvidos em mecanismos de defesa de plantas. Por outro lado, AUX, CTK, GA, ABA e BR também contribuem para as respostas de defesa da planta, mas tem papéis importantes no desenvolvimento e processos fisiológicos da planta (DURBAK et al., 2012; BERENS et al., 2016).

### 1.4. Importância dos fitohormônios em plantas

As plantas são vulneráveis a vários tipos de condições de estresse abiótico e biótico, estes têm impacto negativo na fisiologia e morfologia das plantas por meio de defeitos na regulação genética das vias celulares. Sem dúvida, as plantas empregam vários mecanismos e vias de tolerância para evitar os efeitos dos estresses que são desencadeados pelas alterações no metabolismo. Estes mecanismos conseguem detectar mudanças ambientais precisas e responder com a resposta ideal, minimizando assim os danos e conservando recursos para crescimento e desenvolvimento. A resposta das plantas a esses estresses é dinâmica e complexa. Uma resposta de defesa é iniciada por meio da modulação de eventos moleculares, que envolve a interação de moléculas de sinalização, incluindo fitormônios. Os fitohormônios são pequenas moléculas endógenas de baixo peso molecular, que desencadeiam uma resposta de defesa eficaz contra estresses bióticos e abióticos. Além da sinalização de defesa, esses fitormônios também são reguladores de crescimento, desenvolvimento e processos fisiológicos. Os fitohormônios, como AUX, CTK, GA, SA, JA, ET, ABA e BRs respondem ao estresse por via sinérgica e ações antagônicas (ARIF et al., 2020; SADIQ et al., 2020). Esses fitohormônios coordenam-se uns com os outros de maneira harmoniosa e respondem a estímulos ambientais e de desenvolvimento. Todas as respostas de defesa em plantas são o resultado da interação de muitos genes e famílias de genes bem orquestrados em uma rede. Os fitohormônios são importantes reguladores de crescimento sintetizados em órgãos definidos da planta que têm um impacto proeminente no metabolismo (KAZAN; MANNERS, 2013) e desempenham um papel importante na mitigação de estresses bióticos e abióticos (HU et al., 2013; SADIQ et al., 2020; JOGAWAT et al., 2021). No entanto, estresses causados na planta alteram os níveis endógenos de fitohormônios, como AUX, GA, ABA, JA e SA, o que causa perturbações no crescimento das plantas (KHAN et al., 2014; JOGAWAT et al., 2021). Em condições de estresse, a produção, distribuição ou transdução de sinal dos fitohormônios é afetada, levando a mudanças morfológicas, moleculares e fisiológicas que preparam as plantas para resistir às condições de

estresse (EYIDOGAN et al., 2012). Os fitohormônios, sendo transdutores de nível mais baixo, levam à ativação do sinal de estresse da cascata de sinalização que inicia o mecanismo de resposta (HARRISON 2012). Vários fitohormônios são conhecidos por desempenhar um papel na modulação dos genes. Além disso, por meio da mistura ideal de fitohormônios, as plantas mantêm a homeostase e se adaptam às mudanças ambientais. Isso só é possível por meio de uma conversa cruzada eficiente e sistêmica entre vários fitohormônios que ajudam as plantas a manter um equilíbrio crítico entre o crescimento e a resposta ambiental.

### 1.4.1. Auxinas (AUXs)

As auxinas são compostos orgânicos de baixo peso molecular e constituem um dos mais importantes e diversos grupos de fitohormônios geralmente encontrados em todas as plantas. Estão envolvidas em muitos processos de desenvolvimento, como expansão celular (MAJDA; ROBERT, 2018), controle da arquitetura do caule, desenvolvimento vascular e formação da raiz lateral por meio do controle da divisão celular (WOODWARD; BARTEL, 2005). As auxinas também controlam a senescência, a resposta a vários patógenos e ao estresse abiótico em plantas (WANG et al., 2010; FU e WANG, 2011). Além disso, também regula a formação de frutos (DE JONG et al., 2009). AUX atua de forma mutuamente antagônica com SA e compartilha muitos pontos em comum com JA durante a defesa da planta. Evidências sugerem que, após a infecção com alguns patógenos, as plantas produzem AUX ou aumentam a biossíntese desta como parte da defesa e seu desenvolvimento (VALLS et al., 2006; KAZAN; MANNERS, 2009; BIELACH et al., 2017; ISRAELI et al., 2020).

### 1.4.2. Giberelinas (GAs)

As giberelinas compõem uma das grandes famílias de compostos diterpenóides. Não é produzido apenas por plantas superiores, mas também por fungos e bactérias (MACMILLAN, 2001). É amplamente conhecida por promover alguns dos processos essenciais relacionados ao desenvolvimento floral, tempo de floração, alongamento do caule, desenvolvimento de tricomas e germinação de sementes (DAVIES, 2010). Vários estímulos ambientais levam à mudança nas concentrações de GA nas plantas, o que afeta ainda vários processos (DAVIES, 1995). As GAs são sintetizadas nos plastídios pela via do fosfato de metileritritol a partir do difosfato trans-geranilgeranil. Diversas análises sugerem que GA, como componente de sinalização, desempenha um papel importante na suscetibilidade e resistência às doenças em

plantas. A primeira descrição do papel da GA veio do trabalho de ZHU et al. (2005). Eles demonstraram que uma das proteínas do capsídeo do rice dwarf virus (RDV) interage com a ent-caureno oxidase da planta, resultando na diminuição dos níveis de GA nas plantas infectadas. GAs desempenham um papel no estresse oxidativo, e a sinalização de GA desempenha um papel importante no desenvolvimento da parede celular e reprime o relaxamento da parede celular, alterando a expressão de endotransglucosilase/endohidrolases (XTHs) e expansinas de xiloglucano. O afrouxamento da parede celular é, sem dúvida, importante para o crescimento celular. Através de muitos estudos biológicos utilizando linhagens mutantes COLEBRROK et al. (2014) observaram que as GAs são reguladoras importantes do crescimento das plantas.

### 1.5. Bioinsumos na agricultura

### 1.6. Bioelicitores ou Bioindutores de defesa em plantas

Bioinsumos derivados de compostos bioativos são substâncias de origem microbiana ou vegetal. Eles modulam o crescimento da planta e estão envolvidos nas respostas de defesa, incluindo a limitação do desenvolvimento do patógeno. Produtos com ação bioelicitora apoiam a agricultura fornecendo pelo menos uma das seguintes funções: melhorar a nutrição, conferir tolerância ao estresse abiótico ou abiótico e/ou melhorar as características de qualidade da cultura. Estes também regulam a defesa das plantas contra diferentes patógenos, porém, do ponto de vista regulatório, os reguladores e as partes interessadas têm mantido a bioestimulação e o biocontrole separados (KAUFFMAN et al., 2007).

Os bioelicitores seguem vários modos de ação, onde induzem um sinal para ativar os mecanismos de defesa da planta contra uma variedade de patógenos. Estes controlam fitopatógenos e ajudam na supressão de doenças através da produção de enzimas, compostos antimicrobianos, atividade antagonista envolvendo hiperparasitismo, resistência induzida, inibição competitiva, etc (ZEHRA et al., 2021)

A ação desses compostos naturais não é específica e seu efeito sobre os patógenos é versátil. Compostos bioativos naturais usados na proteção de plantas matam os patógenos (efeito fungicida) ou limitam seu desenvolvimento (efeito fungistático), bem como induzem reações de defesa das plantas como eliciadores (MALIK et al., 2020). Cada molécula ou composto que desencadeia ou estimula certos mecanismos de defesa em uma planta é chamado

de elicitor Como resultado da interação de um eliciador com um receptor da célula sobre a qual atua, é criado um estímulo metabólico, denominado “sinal”, devido à possibilidade de seu movimento intracelular, bem como intercelular e sistêmico. As plantas pulverizadas com esses compostos reagem rapidamente: os receptores de membrana das células vegetais ligam-se às moléculas elicitoras, induzem a resistência local e, subsequentemente, geram a resposta molecular da planta (BHASKAR et al., 2021).

Muitos compostos naturais atuam como elicitores de respostas de defesa em plantas (HEIMPEL; MILLS, 2017; BARUPAL et al., 2019). Os elicitores atuam induzindo ISR ou SAR (KUMARI et al., 2018a; KUMARI et al., 2018b; SWAPNIL et al., 2021). A ação indireta de compostos bioativos nas células vegetais estimula a liberação de elicitores proteicos e lipídicos. A síntese de fitoalexinas e proteínas relacionadas à patogênese (PR), o acúmulo de calosidades e lignificação da parede celular, bem como o aumento da atividade de várias enzimas de defesa são iniciadas nas células vegetais, o que protege as plantas contra patógenos (Figura 7) (WALTERS et al., 2007).

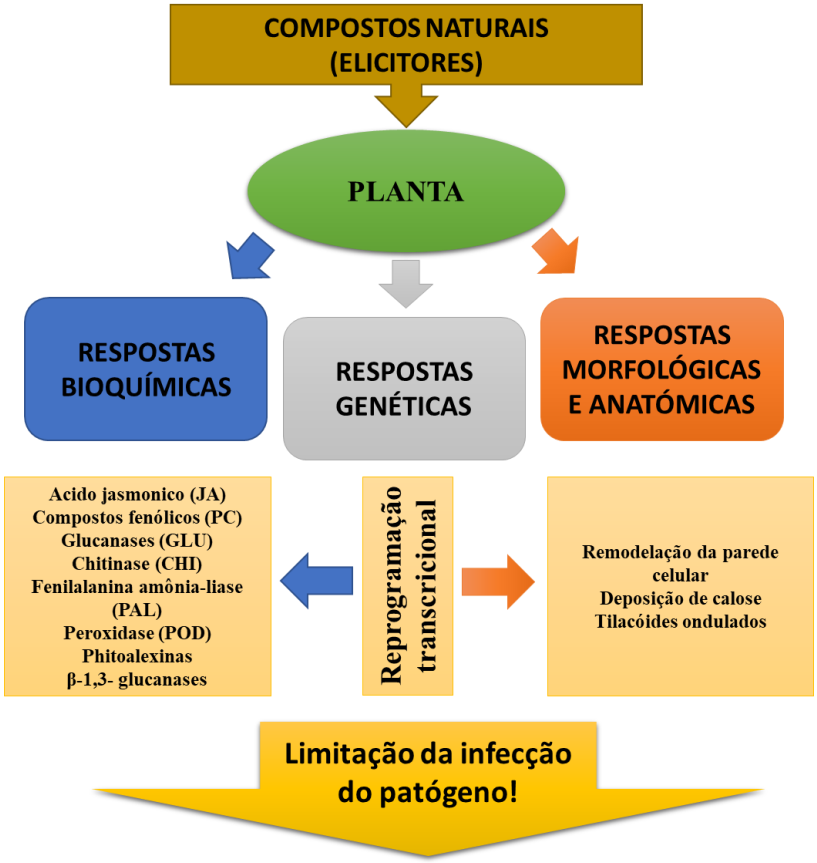


**Figura 7.** Resposta imune da planta sob a influência de eliciadores naturais. (Adaptado de WALTERS et al., 2007).

Elicitores diferem uns dos outros com base em sua fonte, natureza e estrutura molecular (THAKUR; SOHAL, 2013; PRŠIĆ; ONGENA, 2020). Eles são amplamente classificados como elicitores exógenos e elicitores endógenos. Os elicitores exógenos são compostos produzidos por patógenos, enquanto os elicitores endógenos são moléculas liberadas de plantas em resposta ao ataque patogênico (RAMIREZ-ESTRADA et al., 2016). Esses elicitores podem ser de natureza física ou química, biótica ou abiótica. Todos os elicitores de origem microbiana são considerados bióticos (bioelicitores). Muitos elicitores microbianos são componentes estruturais integrais ou determinantes primários de patogenicidade. Alguns destes ajudam na dispersão de agentes microbianos ou induzem sinais para evocar a imunidade das plantas. Bioelicitores têm o potencial de induzir mecanismos de defesa das plantas por meio da ação de seus compostos; eles servem como eliciadores microbianos na regulação de defesa de plantas (ZEHRA et al., 2017a; ZEHRA et al., 2017b; ABDUL MALIK et al., 2020). Os bioelicitores podem levar ao aumento da resistência a doenças em plantas sem um antagonismo direto ao patógeno, podendo aumentar os atributos funcionais destas e provocar resistência induzida contra fitopatógenos (MEENA; SWAPNIL, 2019).

Estresses bióticos causados por doenças em plantas podem ser muito destrutivos e causar grandes danos à produção e, às vezes, podem levar a perdas do 100%. As doenças nas plantas são geralmente controladas pela aplicação de produtos químicos antimicrobianos, que eventualmente entram na cadeia alimentar humana levando à toxicidade. O uso dos principais grupos de pesticidas (inseticidas, herbicidas, fungicidas, etc.) e de produtos químicos na agricultura aumentou em nível global de 2,2 milhões de toneladas em 1990 para 41 milhões de toneladas em 2017 (FAO, 2021). Portanto, estratégias de manejo bem definidas devem ser seguidas para combater aas diferentes doenças das culturas usando abordagens ambientalmente amigáveis (GILL HK; GARG H, 2014). A indução de sistemas de defesa natural pelas aplicações de “bioelicitores” pode servir como alternativa, sendo ecologicamente correta, biodegradável e não tóxica para a flora e fauna (MONDAL et al., 2015). Bioindutores servem como um valioso recurso renovável de inúmeros eliciadores para induzir respostas de defesa variadas.

A indução dos mecanismos de defesa das plantas pode ser ativada pelos elicitores dos patógenos ou do próprio hospedeiro para a aquisição do SAR (MODAFAR et al., 2012). Elicitores foram isolados de bactérias, fungos, oomicetos (NÜRNBERGER, 1999), algas

(ARMAN; QADER 2012) e podem ser amplamente categorizados como proteínas, peptídeos, ácidos graxos, glicoproteínas, lipídeos, oligossacarídeos e polissacarídeos (NÜRNBERGER, 1999; RADMAN et al., 2003; ARMAN; QADER 2012).

O uso de bioinsumos para controle de patógenos é muito atraente, e a disponibilidade de novas aplicações e técnicas moleculares abrem novos caminhos para abordagens na proteção das plantas. Muitos compostos orgânicos já foram comercializados e estão presentes no mercado como biofertilizantes ou bioestimulantes de crescimento de plantas. Estes, entre outros, incluem Biosept 33SL (extrato de toranja), Bio-Algeen S90 Plus, Labimar 10S, Kelpak SL, Lysodin Alga-Fert (extrato de algas marinhas), Bioczos BR (extrato de alho), Timorex Gold 24 EC (extrato de árvore do chá) e Vaxiplant SL (laminarina) (KOZIARA et al., 2006; TERZI et al., 2007; JAMIOŁKOWSKA, 2011). Diversos produtos sintéticos contendo moléculas indutoras de resistência ou análogas já foram desenvolvidos (Bion®, proAct®, Regalia®, Milsana®, Ecolife®40, Agro-mos®, fosfitos e silicatos, dentre outros) e estão sendo aplicados na agricultura. Alguns agentes de controle biológico que podem induzir resistência em plantas também estão registrados no Brasil, como o Serenade® (*Bacillus subtilis* QST 713, Basf SA), com resultados animadores no controle do mofo-branco (*Sclerotinia sclerotiorum*) em plantas de soja quando aplicado como controle preventivo, diminuindo a severidade desta doença (OTÁVIO et al., 2018). Essa bactéria produz uma proteína (surfactina) capaz de ativar respostas de defesa, em plantas, contra patógenos. Além do Serenade, estão registrados, no Brasil, produtos à base de Trichoderma, como o Quality (*T. asperellum*), o Tricovab (*T. stromatichum*) e o Trichodermil SC 1306 (*T. harzianum*). Esse gênero de fungo é conhecido na literatura por ser capaz de induzir resistência em plantas.

Indutores de resistência em plantas, geralmente são biodegradáveis, não tóxicos, não poluentes e não perigosas para vários organismos. Além disso, muitos deles não apenas mitigam as limitações induzidas pelo estresse e regulam/modificam os processos fisiológicos nas plantas para estimular o crescimento e aumentar a produtividade, mas também limitam diretamente o desenvolvimento de fitopatógenos (KOZIARA et al., 2006; YAKHIN et al., 2017).

O desenvolvimento de novos bioinsumos por meio de modificações químicas de biocompostos naturais contribuirá para a formação de compostos puros e estáveis e a criação de novos produtos para uso generalizado na agricultura (VILLAVERDE, et al., 2016). Dentre as vantagens desses bioprodutos é a segurança durante o uso e a ausência de resíduos tóxicos

para o meio ambiente e saúde. Alguns deles podem ser usados em misturas com agrotóxicos, o que não afeta sua eficácia, ao mesmo tempo que melhora a sua eficiência e reduzindo assim o uso indiscriminado dos pesticidas (SULTANA et al., 2011). Devido ao fenômeno de resistência às pragas e doenças e à retirada de muitas substâncias pesticidas ativas da produção vegetal, o uso preventivo de produtos à base de componentes naturais ou biológicos pode e deve se tornar uma alternativa ecologicamente amigável. Estudos prévios do nosso grupo mostraram que a peptidogalactomanana (pGM) de *C. herbarum* quando em contato com células de tabaco BY2 ou quando pulverizadas em plantas jovens de tabaco levaram à expressão de genes relacionados à defesa de plantas (MONTEBIANCO, 2017; MATTOS et al., 2018; MONTEBIANCO, 2021). As plantas pulverizadas com a pGM mostraram uma forte proteção à infecção pelo tobacco mosaic virus (TMV), com redução ou eliminação total dos sintomas. Em vista destes resultados positivos que mostraram que a pGM foi capaz de ativar a defesa da planta, foi necessário estudar a abrangência do espectro de proteção mediado pelo uso desta molécula frente a patógenos relevantes na agricultura. Desta forma o potencial da pGM na indução de genes relacionados a defesa em plantas de diferentes genótipos de maracujá foi avaliado, a fim de observar se após elicitação com a pGM, estas plantas estariam protegidas contra o ataque de fitopatógenos. A pGM, é composta de 76% de carboidratos, sendo manose, galactose e glicose seus principais monossacarídeos (relação molar, 52:36:12). A metilação e a análise de espectroscopia por ressonância magnética nuclear de 13C (RMN-13C) mostraram a presença de uma cadeia principal contendo resíduos de α-D-Manp ligados por (1 → 6), e resíduos β-D-Galf presentes como (1 → 5) correntes laterais interligadas (MATTOS et al., 2018).

Por tanto, o sucesso do uso desta estratégia poderia levar a uma baixa dos custos de produção e ao aumento de produtividade com a vantagem adicional de ser sustentável. Há no mercado internacional a indicação de um bioelicitor comercializado pela Syngenta Co. que teria atividade antiviral em maracujá, o Bion (PARKINSON et al., 2015). Entretanto, este produto na bula de sua recomendação não vem indicado ou testado para maracujá no Brasil, além disso, ele é um agente químico e classificado no MAPA no Brasil como “CLASSIFICAÇÃO TOXICOLÓGICA III – MEDIANAMENTE TÓXICO” e “CLASSIFICAÇÃO DO POTENCIAL DE PERICULOSIDADE AMBIENTAL III – PRODUTO PERIGOSO AO MEIO AMBIENTE”.

## 1.7. Substâncias húmicas

As substâncias húmicas são obtidas a partir do processo de humificação tanto de resíduos animais e/ou vegetais, formando estruturas moleculares (PICCOLO, 2016). O termo substâncias húmicas refere-se ao produtos transformados ou biomassa de matéria orgânica, que têm uma fase amorfa e não têm semelhanças morfológicas com as estruturas de onde eles foram derivados (EL-RAMADY et al., 2015). As substâncias húmicas possuem uma grande cadeia carbonácea com alta resistência à decomposição microbiana (AHMED MOSA et al., 2020). As substâncias húmicas contêm carbono, hidrogênio, oxigênio e nitrogênio com pequenas quantidades de enxofre e fósforo. Eles são uma mistura de ácidos que podem ser fracionados com base nas diferenças em sua solubilidade em ácido húmico (AH), ácido fúlvico (AFv) e frações de humina, além do ácido úlmico e alguns microelementos. O AH inclui agregados de cadeia longa, compostos de alto peso molecular, solúveis em álcali, enquanto o AFv é uma mistura de compostos de cadeia curta e baixo peso molecular, solúveis em soluções ácidas e alcalinas (GOEL; DHINGRA, 2021).

Devido aos seus efeitos vantajosos nas diferentes propriedades do solo, seu papel no crescimento das plantas tem sido reconhecido. Por várias décadas, produtos à base de AH comerciais estão disponíveis e são aplicados para aumentar o crescimento da safra e o rendimento econômico (OLK et al., 2018). Estes podem ser aplicados diretamente no solo, para que seja assimilado pelo sistema radicular da planta ou também em pulverizações foliares. A eficácia da aplicação direta no solo tem sido bastante pesquisada, entretanto, a pulverização foliar precisa ser explorada (OLK et al., 2018). Na aplicação foliar, AH não entram em contato com a rizosfera, onde ocorre efeito importante destas na disponibilidade de nutrientes (DE HITA et al., 2020). Duas hipóteses foram propostas para explicar o efeito dos AH no crescimento das plantas, a primeira, propõe que estes afetam as plantas devido ao seu efeito indireto nas propriedades do solo e nos nutrientes minerais disponíveis no solo (hipótese nutricional) e a segunda, propõe seu impacto direto no metabolismo das plantas através das raízes (hipótese do tipo hormonal) (GARCIA-MINA et al., 2012).

Ácidos húmicos (AHs) atuam principalmente sobre as proteínas envolvidas no transporte de nutrientes, assimilação de nitrogênio, divisão e desenvolvimento celular; H + -ATPases da membrana plasmática e vias hormonais (NARDI et al., 2021).

O efeito do AH na mitigação a estresses abióticos em plantas tem sido atribuído ao aumento de enzimas antioxidantes e alterações no equilíbrio iônico. Em vários estresses abióticos, as ROS são responsáveis por danos celulares. O dano oxidativo devido às ROS é prevenido pela indução de enzimas de limpeza ou antioxidantes, como SOD, POD, CAT e ascorbato peroxidase (APX), além de outras enzimas como, tocoferol, compostos fenólicos e produção de osmo-protetores como prolina, açúcares (GOEL; DHINGRA, 2021). A resposta ao tratamento com AH foi avaliada em variedades de melão tolerantes e sensíveis à seca e relatou aumento no peso do caule, área foliar, conteúdo de clorofila e atividades de enzimas antioxidantes (KIRAN et al., 2019). AHs aumentam a tolerância ao estresse, reduzindo a evaporação da água com efeito redutor na abertura estomática; ajudando a planta e o solo a reter mais umidade (LI, 2020). O estresse oxidativo é prevenido por substâncias húmicas por meio de seu efeito positivo na atividade da peroxidase e nos níveis de prolina; reduzindo assim os níveis de ROS e mantendo a homeostase celular (GARCÍA et al., 2012).

A exploração do impacto do *priming* químico com AH em mudas de milho em nível de transcrição, revelou que as vias de sinalização de vários hormônios como ABA, GA e AUX, além de genes responsivos ao estresse foram expressos, levando as plantas a tolerância a estresse por seca, metais pesados e salinidade (CANELLAS et al., 2020). A aplicação de ácido húmico conferiu tolerância eficaz às plantas de milho contra o estresse salino em termos de aumento da biomassa vegetal, conteúdo de clorofila, elementos minerais e atividades de enzimas antioxidantes (KAYA et al., 2018).

As propriedades específicas dos HAs qualificam seu uso na agricultura. Por tanto a exploração destes compostos como bioinsumos na indústria agrícola é de extrema importância.

### 1.8. Bactérias promotoras de crescimento (PGPB)

A rizosfera contém bactérias promotoras de crescimento de plantas (PGPG), que são um grupo especializado de comunidade bacteriana que estão presentes no solo (AHMAD et al., 2009). Essas bactérias formam colônias ao redor do sistema radicular das plantas (rizosfera), levando ao aumento e crescimento da planta (KOUR et al., 2020; YADAV et al., 2020).

PGPG participam na tolerância das plantas a diferentes tipos de estresse, como bióticos e abióticos (YADAV et al., 2017; KOUR et al., 2020; RANA et al., 2020). Além disso, também podem influenciar a bioquímica, a fisiologia e a morfologia das plantas em baixas concentrações, regulando o crescimento e o desenvolvimento das plantas, por síntese direta ou

indireta de fitohormônios (como AUX, GA, ET e CTK) (RAI et al., 2020). Por tanto, o papel fundamental das PGPR é a promoção do crescimento como um bioestimulador (ou promotor e regulador do crescimento da planta) (VERMA et al., 2017; YADAV; YADAV, 2018). As interações entre plantas e PGPG podem resultar na melhoria do desempenho da planta e maior resistência a estresses bióticos e abióticos, que são características importantes para as culturas cultivadas (GERMIDA et al., 2001). Diferentes cepas de PGPG são capazes de aumentar a produtividade das culturas, exibir biocontrole, aumentar a resistência a patógenos foliares, promover a nodulação em leguminosas e aumentar a emergência de mudas (VEJAN et al., 2016; KALAM et al., 2020; SWARNALAKSHMI et al., 2020).

As PGPB usam mecanismos diretos ou indiretos para ajudar no desenvolvimento de plantas por meio da produção de sideróforos, em que previnem as plantas da infecção por fitopatógenos, promovem a solubilização do fósforo, aumentam a regulação do crescimento das plantas por fitohormônios e aumentam a biomassa (ASHRAF et al., 2017). Os mecanismos diretos (promoção do crescimento vegetal) incluem a facilitação da aquisição de nutrientes e a síntese de hormônios (GLICK et al., 2012). Um dos principais problemas que as plantas enfrentam na aquisição de nutrientes é a fraca solubilidade dos elementos no solo. Por exemplo, o fósforo (P) é escasso em muitos solos do mundo, além de estar em formas insolúveis, limitando seu uso pelas plantas (OROZCO-MOSQUEDA et al., 2021). Portanto, o uso de PGPB, desempenha um papel fundamental na solubilização de formas insolúveis de P, principalmente por meio de mecanismos como a produção de fosfatases ácidas, que auxiliam na mineralização do fósforo orgânico no solo (KHATOON et al., 2020). A produção de fitohormônios e outros compostos voláteis que modulam o crescimento das plantas é um fator relevante para potenciais PGPB a serem usados como produtos bioestimulantes na agricultura (SANTOYO et al., 2019). Os principais hormônios que estimulam o desenvolvimento das plantas são as AUX, GA, CTK e alguns compostos orgânicos voláteis (COV). Cada um deles tem funções especiais para estimular o crescimento da planta, além de ser sintetizado pela planta e cumprir diversos processos fisiológicos. Entretanto, os mecanismos indiretos incluem o antagonismo das PGPB a potenciais fitopatógenos, seja restringindo o crescimento ou eliminando o fitopatógeno, para promover o crescimento e a saúde da planta. PGPB contém todo um arsenal de compostos e enzimas que têm a capacidade de restringir ou eliminar patógenos. Por exemplo, um mecanismo amplamente utilizado por PGPB com atividade

antifúngica é a produção de enzimas, como quitinases, celulases e β-1,3-glucanases, que degradam a parede celular fúngica. A quitinase degrada a quitina, um polímero linear insolúvel de β-1,4-N-acetil-glucosamina, conhecido por ser o principal componente das paredes celulares dos fungos. Várias bactérias que fazem parte do endobioma protetor da planta incluem espécies de *Bacillus*, como *B. licheniformis*, *B. cereus*, *B. subtilis* e *B. thuringiensis* (OROZCO-MOSQUEDA et al., 2021). A produção de outros compostos voláteis, como ET, SA e JA, pode induzir e controlar as respostas de defesa da planta (BITAS et al., 2013). Respostas de defesa da planta estimuladas por endófitos bacterianos são amplamente relatadas na literatura, e sua principal função é aumentar uma série de ações que permitem à planta se defender do ataque de patógenos (KLOEPPER et al., 2006).

Existem formulações sólidas (pó) ou líquidas, isso vai depender da situação em alguns casos, por exemplo, se a intenção é atacar fungos ou oomicetos do solo, é adequado aplicar em forma solida o bioinoculante com atividade antifúngica no solo, próximo à raiz. Por outro lado, se o potencial de infecção por patógenos for na parte aérea das plantas, o ideal é fazer pulverização foliar na forma líquida (OWEN et al., 2015). Em determinadas situações, a formulação depende do objetivo da aplicação, seja em casa de vegetação ou campo, seja em grandes ou pequenas áreas de terreno. Da mesma forma, o tempo de inoculação também é relevante, seja profilaticamente ou quando já existe uma determinada infecção causada por patógenos. No caso desta última situação, o efeito protetor não tem sido tão eficaz. O ideal em qualquer caso é que o uso de bioinoculantes seja antes de observar sintomas de doenças nas plantas (pre-tratamento), pois as evidências sugerem que sua ação é melhor quando o antagonista não está previamente presente, conseguindo proteger até mesmo os produtos na pós-colheita (SREEDHAR et al., 2014). Entretanto, se o que se deseja na hora de aplicar PGPB com ação estimuladora do crescimento e desenvolvimento da planta, a melhor opção é aplicar nas sementes ou pulverizar nas primeiras fases do crescimento da planta (mudas), e assim exercer um maior efeito de promoção (KAYMAK et al., 2010). Da mesma forma, alguns bioinoculantes podem ter dupla ação, ou seja, certas espécies de bactérias como *Pseudomonas* ou *Bacillus* podem exercer ação direta estimulando o crescimento da planta e ao mesmo tempo antagonizando patógenos e/ou estimulando as defesas das plantas (HERNÁNDEZ-LEÓN et al., 2015).

## 2. JUSTIFICATIVA

Em função dos altos prejuízos que podem ocasionar as doenças na cultura do maracujá, reduzindo fortemente a produtividade e inviabilizando a manutenção dos plantios, faz-se necessário o desenvolvimento de estratégias que possam mitigar os efeitos ocasionados por diferentes estreses bióticos no maracujá.

Em vista disso, a implementação de estratégias de indução de defesa e de estimulação de crescimento por meio de bioinsumos que possam ser utilizados na agricultura é muito promissora. A utilização de compostos bioativos com capacidade de proteger as plantas contra doenças possibilitando a expressão de mecanismos de defesa latentes é uma estratégia que vem sendo amplamente pesquisada no controle de pragas e doenças em culturas agrícolas (JAMIOŁKOWSKA, 2020; ZEHRAA, et al., 2021). Desta forma, é importante avaliar o potencial de diferentes produtos de origem biológica na indução de genes relacionados a defesa e de fitohormônios em diferentes genótipos de maracujá; a fim de observar se após bioestimulação ou bioindução, as plantas poderiam apresentar aumento de biomassa e/ou produtividade na ausência de patógenos, ou controlar/mitigar os danos causados por fitopatógenos restaurando os padrões de produtividade sob estresse biótico.

O sucesso do uso desta estratégia poderia levar a uma baixa dos custos de produção e ao aumento de produtividade com a vantagem adicional de ser sustentável. Por tanto, este trabalho estuda uma solução por meio do desenvolvimento de “bioinsumos” como alternativa na indução de defesa de plantas de maracujá, mitigando os danos no desenvolvimento e produtividade que podem ocasionar diferentes estreses bióticos nesta cultura. Sendo uma opção ecologicamente amigável com o meio ambiente, podendo ser usada tanto em agricultura orgânica como na convencional, ajudando na diminuição tanto dos custos de produção, como o uso indiscriminado de pesticidas.

Estudos prévios de nosso grupo utilizando a pGM de *C. herbarum* (MATTOS et al., 2028) e de colaboradores com o uso de ácido húmico associado ou não a um consórcio bacteriano (OLIVARES et al., 2015; da SILVA et al., 2021) mostraram que o uso destes bioinsumos apresenta benefícios para culturas de fumo e tomate, respectivamente. Em função destes resultados positivos, hipotetizamos que estes bioinsumos poderiam ser benéficos também para a cultura do maracujá.

# 3. OBJETIVOS

## 3.1. Objetivo geral

Avaliar a eficiência de bioestimulantes na indução de defesa e desenvolvimento em diferentes genótipos de maracujá sobre condições de casa de vegetação e campo.

## 3.2. Objetivos específicos

**3.2.1.** Avaliar o efeito da pGM na indução de genes relacionados a defesa e fitohormônios em plantas maracujá desafiadas com CABMV sob condições de casa de vegetação e campo.

**3.2.2.** Avaliar o efeito bioestimulador da pGM, ácido húmico e bactérias promotoras de crescimento, nos níveis de expressão de genes relacionados a defesa e fitohormônios, e na biomassa e produtividade de maracujá sob condições de casa de vegetação e campo.

**3.2.3.** Avaliar a eficácia da pGM no controle e mitigação dos danos causados pelo CABMV no desenvolvimento, produtividade e rendimentos de plantas de maracujá sob condições de casa de vegetação e campo.

**3.2.4.** Avaliar o efeito bioprotetor da pGM no controle de *C. herbarum* em mudas de maracujá sob condições de casa de vegetação.

# 4. MATERIAL E MÉTODOS

## 4.1. Área de estudo e localização

A área de estudo localiza-se no Estado de Rio de Janeiro, os experimentos iniciais foram realizados em casa de vegetação com luz natural e temperatura controlada de 24 +/- 2 ºC na Universidade Federal do Rio de Janeiro, todos os estudos moleculares foram feitos no Laboratório de Virologia Molecular Vegetal (LVMV) da UFRJ, no município de Rio de Janeiro.

Outros experimentos (casa de vegetação e campo), foram realizados no SIPA – Sistema integrado de produção agroecológica, denominado "Fazendinha Agroecológica", que é uma área experimental da Empresa Brasileira de Pesquisa Agropecuária (Embrapa Agrobiologia), localizada no município de Seropédica. As coordenadas geográficas do local são 220 48'00'' de latitude Sul e 430 41'00'' de longitude Oeste. Apresenta altitude de aproximadamente 33 m e o solo da área classificado como argissolo vermelho-amarelo. Segundo a classificação de KÖPPEN (1948) o clima é Aw (chuvas concentradas no período novembro a março; precipitação média anual de 1.213 mm; temperatura média anual de 24,5°C).

Outro experimento sob condições campo foi realizado no Município de Campos dos Goytacazes, no norte do Estado do Rio de Janeiro, no distrito de Lagoa de Cima (21° 46'19'' de latitude sul e 41° 30' 5'' de latitude oeste; altitude 14 m). Segundo a classificação climática de KÖPPEN (1948), o clima da região é classificado como tropical úmido (Aw), com verão chuvoso e inverno seco.

## 4.2. Extração de glicoproteína de parede celular do fungo *Cladosporium herbarum* (pGM)

A cepa CBS 121621 do *C. herbarum*, foi fornecida pelo Dr. J. Guerra, Instituto de Estudos Avançados, Réus, Espanha. A qual foi crescido em frascos Erlenmeyer contendo 1 $L^{-1}$ de meio batata dextrose (PDB) por 07 dias e filtrado para obtenção da massa fúngica. Os lipídeos totais foram extraídos de aproximadamente 200 g da massa fúngica com clorofórmio/metanol 2:1 e 1:2 (v/v), por 2 h a temperatura ambiente, com agitação. A massa delipidada foi colocada em um balão com tampão fosfato de sódio 0.05M e pH 7.2, sob refluxo à 100ºC por 2 horas, para extração das glicoproteínas, de acordo com MATTOS (2011). Em seguida, o conteúdo foi filtrado e a massa fúngica guardada para posterior extração dos polissacarídeos. O filtrado foi concentrado a vácuo em rota-evaporador até um volume de 100

ml e a este filtrado concentrado foi adicionado 300 ml de etanol e mantido "overnight" a 4 ºC para precipitação da peptidogalactomanana. O precipitado foi separado por centrifugação, solubilizado em água e dialisado contra água por dois dias para retirada dos sais. O material foi liofilizado e este constitui a peptidogalactomanana para uso posterior (pGM) (HAIDO et al., 1998).

### 4.3. Extração e caraterização de ácido húmico (AH)

O AH foi fornecido pelo Professor Luciano P. Canellas do Núcleo de Desenvolvimento de Insumos Biológicos para a Agricultura da Universidade Estadual do Norte Fluminense (UENF). A extração e caracterização do AH foi feita seguindo a metodologia descrita por BAÍA et al. (2020). As substâncias húmicas foram extraídas do vermicomposto produzido com esterco bovino com NaOH 0,1 mol $L^{-1}$ 1:10 (v:v) sob atmosfera de $N_2$ por 4 h seguido de centrifugação (3000×g). O procedimento de extração foi repetido até que os extratos apresentassem absorbância zero a 280 e 465 nm. A separação dos ácidos húmicos do extrato alcalino foi obtida por acidificação em pH 1 com HCl 6 mol $L^{-1}$. A dissolução e a precipitação foram repetidas três vezes. Após centrifugação, a fração de HA foi lavada com água até o teste negativo com $AgNO_3$, dialisada (corte de massa molecular 1 kDa; Spectrapor, EUA) e liofilizada. A composição elementar do AH foi caracterizada em analisador CHN (Perkin-Elmer 1483; Perkin-Elmer, Norwalk, CT, EUA) e mineralizada em mufla a 750 °C por 8 h para medição do teor de cinzas. O AH continha 470 g $kg^{-1}$ C, 55 g $kg^{-1}$ de N, 451 g $kg^{-1}$ O e 5 g $kg^{-1}$ de cinzas. A caracterização molecular dos ácidos húmicos foi realizada por espectroscopia de RMN de estado sólido (13C-RMN) e rotação de ângulo mágico de polarização cruzada (CPMAS). O espectro 13C CPMAS NMR foi adquirido com um espectrômetro Bruker AVANCE 300 NMR equipado com uma sonda MAS de 4 mm de largura operando em uma frequência de ressonância 13C de 75,475 MHz. As amostras (100–200 mg) foram embaladas em rotores de zircônia de 4 mm com Kel-Fcaps e giradas a 13 kHz. Uma sequência de rampa de 1H foi usada durante um tempo de contato de 1 ms para explicar a possível falta de homogeneidade da condição de Hartmann-Hahn. Duas mil varreduras com 3.782 pontos de dados foram coletadas em um tempo de aquisição de 25 ms e um atraso de reciclagem de 2,0 s. O software Bruker Topspin 1.3 foi usado para coletar e elaborar os espectros. Todos os decaimentos de indução livre (FIDs) foram transformados pela aplicação de um preenchimento de zero de 4K e um alargamento de linha de 75 Hz. Os espectros foram integrados nos intervalos de ressonância de mudança

química (ppm): 187-162 (carbonilas de cetonas, quininas, aldeídos e carboxilas) = 8,7%, 162-112 (carbonos aromáticos e olefínicos) = 27%, 112-93 (anomérico carbonos) = 5%, 93–46 (sistemas C – O, como álcoois e éteres, grupos C – N e carbonos alifáticos complexos) = 36,3% e 46–0 ppm (carbono sp3, principalmente metileno e metil) = 23%.

### 4.4. Bactérias promotoras de crescimento (PGPB)

O consórcio bacteriano foi fornecido pelo Professor Fábio L. Olivares do Núcleo de Desenvolvimento de Insumos Biológicos para a Agricultura da Universidade Estadual do Norte Fluminense (UENF). A suspensão do consórcio de bactérias sintéticas foi obtida por uma combinação da cepa endofítica diazotrófica de *Herbaspirillum seropedicae* HRC 54 (Olivares et al. 2015), P-solubilizante quitinolítico *Serratia marcescens* cepa UENF 22GI (Mateolli et al., 2018) e *Bacillus* sp. cepa 77 obtida da coleção de cultura bacteriana do Laboratório de Biologia Celular e Tecidual (LBCT-UENF). A suspensão bacteriana foi preparada pelo crescimento das cepas bacterianas em frascos de vidro contendo meio líquido DIGYS sob agitação rotatória a 30 °C por 36 h a 150 rpm. As células bacterianas foram centrifugadas a 4.000 g por 15 min, e o pellet foi ressuspenso em solução salina esterilizada (NaCl, 0,85%) e ajustada espectrofotometricamente para uma densidade óptica (O.D) a 590 nm de 1.2, equivalente a 5 x 108 células $mL^{-1}$.

### 4.5. Material vegetal

Sementes de maracujá (*Passiflora edulis*) de diferentes genótipos (‘Redondo Amarelo’, ‘FB300’ e ‘H09-110/111’) foram germinadas em vasos plásticos. Para experimentos na UFRJ foi utilizado o substrato (Garden Plus, Turfa Fertil Co., Brazil/MECPLANT) e vermiculita na proporção (3:1), para os experimentos na Embrapa Agrobiologia (Município de Seropédica) e no Município de Campos dos Goytacazes foi usado substrato contendo 1/3 de vermiculita, 1/3 de húmus de minhoca e 1/3 de areia lavada. Em todos os experimentos as plantas foram mantidas em casa de vegetação, até atingirem aproximadamente 4 folhas verdadeiras totalmente abertas e expandidas para o início dos tratamentos.

### 4.6. Inoculação mecânica do CABMV

Em alguns experimentos, três dias após a pulverização de pGM, duas folhas por planta foram inoculadas mecanicamente com 100 μL de extrato obtido de amostras foliares congeladas em freezer -80 °C (amostras foliares de maracujá sintomáticas com o CABMV, trazidas do

estoque viral da EMBRAPA CENARGEN) na diluição 1:10 (folhas inoculadas com CABMV homogeneizadas em tampão de fosfato de sódio 10 mM, pH 7,0).

### 4.7. Avaliação de incidência e severidade do CABMV

Os dados de incidência e severidade da doença do endurecimento do fruto causada pelo CABMV foram coletados a partir da ocorrência dos primeiros sintomas. A incidência da doença foi determinada com base na presença de sintomas nas folhas das plantas inoculadas com o vírus. A proporção de plantas doentes foi estimada por: DI = (n / N) x 100 (DI = incidência; n = número de plantas doentes; N = número total de plantas avaliadas) (KONE et al., 2017).

A severidade da doença para CABMV foi determinada tomando-se uma escala de notas 1, 2, 3 e 4, onde 1 representava a ausência de sintomas, 2 representava a presença de sintomas de mosaico leves sem deformação foliar, 3 representava a presença de mosaico severo sem deformação foliar, e 4 representou a presença de mosaico severo, bolhas e deformação foliar, conforme proposto por GONÇALVES et al. (2018) (Figura 8). O índice de severidade foi determinado para cada tratamento usando a fórmula de acordo com MCKINNEY (1923): DS = $\sum$ (DS × P) / (TNP × HGS) × 100, onde DS = grau da escala 1 – 4 determinado para cada planta; P = número de plantas apresentando cada grau de infecção (nota); TNP = número total de folhas avaliadas; e HGS = grau mais alto da escala (nota máxima de infecção).

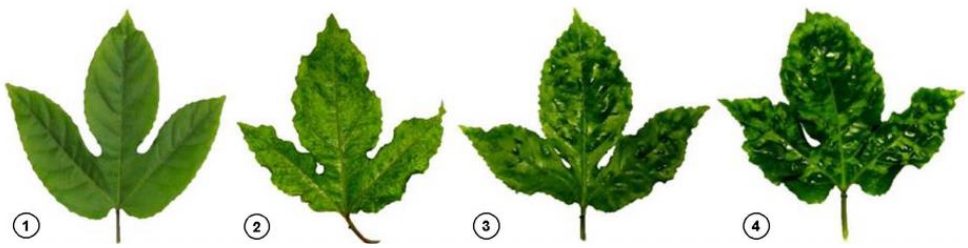


**Figura 8.** Folhas de *P. edulis* classificadas de acordo com a escala de notas: 1 = sem sintoma; 2 = mosaico leve sem deformações foliares; 3 = mosaico severo sem deformação foliar; 4 = mosaico severo, bolhas e deformações foliares. Escala retirada do método de GONÇALVES et al., 2018.

### 4.8. Visualização histoquímica de peróxido de hidrogênio ($H_2O_2$) e radical superóxido ($O_2^-$).

No primeiro experimento, para a avaliação histoquímica após o tratamento com pGM e antes da inoculação mecânico do CABMV, plantas de maracujá, tiveram suas duas folhas mais

jovens e plenamente expandidas coletadas após 24 e 72 horas após a pulverização. Para cada diferente tempo e para cada tratamento, foram utilizadas 5 plantas.

Para avaliação de $H_2O_2$, em seguida, foi preparado uma solução contendo diaminobenzidina (DAB) dissolvido em tampão fosfato de potássio 10 mM pH 3,8 na concentração de 1 $mg.ml^{-1}$.

Para a avaliação de $O_2^-$, foi preparado uma solução contendo o reagente nitro azul de tetrazólio (NBT) dissolvido em tampão fosfato de potássio 10 mM pH 7,8 na concentração de 0,5 $mg.ml^{-1}$.

As folhas coletadas foram submersas em placas de Petri contendo a solução (DAB ou NBT) e deixadas em uma câmara de vácuo por 1 hora no escuro. As folhas foram então submetidas a um tratamento com etanol absoluto fervente para a retirada da clorofila, proporcionando uma melhor visualização dos precipitados formados pelo DAB/$H_2O_2$ e pelo NBT/$O_2^-$. As folhas foram fotografadas após a retirada da clorofila (WANG et al., 2016).

### 4.9. Avaliação de expressão genica por meio de RT-qPCR

Foram coletadas amostras foliares em diferentes tempos em cada uns dos tratamentos (3 pools biológicos independentes de cada tratamento, cada pool composto por 3, 4 ou 5 plantas), mantidas em nitrogênio líquido e levadas ao laboratório para avaliação por meio de RT-qPCR. A expressão dos genes relacionados à defesa: *proteína relacionada à patogênese 3* (*PR-3*), *superóxido dismutase* (*SOD*), *peroxidase 12* (*POD12*), *lipoxigenase 2* (*LOX2*) e *fenilalanina amônia-liase* (*PAL*) foi avaliada. Para os genes *PR-3*, *SOD*, *POD12* e *LOX2*, foram utilizados os primers descritos por MUNHOZ (2013). Para amplificação do gene *PAL* foram desenhados primers com base na sequência de nucleotídeos, publicada por SANTOS et al. (2014), número de acesso ao GenBank KO457900.1. Os primers foram selecionados utilizando-se o Website GenScript® (https://www.genscript.com/). Dois genes envolvidos na via de sinalização de fitohormônios, *auxina* (*AUX*) e *giberelina* (*GA*) também foram analisados e os primers utilizados foram descritos por CHEN et al., (2021).

Todos os primers foram primeiramente validados por PCR convencional, onde se buscava à amplificação em amostras de cDNA oriundas de RNA total extraído de tecido foliar de plantas de maracujá (*P. edulis*). Testou-se diferentes concentrações de primers, temperaturas de anelamento e concentração de $MgCL_2$. Portanto, a mistura das reações consistia em 17,25

μL de $H_2O$ Milli-Q autoclavada, 2,5 μL de tampão KCl 10x, 1,5 μL de MgCL2 25mM, 0,5μL de dNTP 10mM, 1,0 μL de cada primer, 0,25 μL de Taq DNA polymerase 5U/μL (Invitrogen) e 1,0 μL de cDNA totalizando um volume final de 25 μl. A ciclagem consistia em: 95°C por 1 minuto, 95°C por 1 minuto de desnaturação, temperatura de anelamento dependendo do primer que variou 52°C a 68°C, 72 °C de extensão por 1 minuto, 72°C por 10 minutos. A visualização do produto da reação foi realizada em gel de agarose 0,8%, formulado com TAE 0,5X e brometo de etídio em fotodocumentador (Loccus).

Após validação por PCR convencional, os primers foram validados por PCR em tempo real quantitativo. Foi testada a melhor concentração de primers para gerar menor ciclo threshold, maior ΔRn e impedir a formação de dímeros de primers de acordo com as especificações do fabricante. As amplificações foram feitas utilizando 1,0 μL de cDNA diluído 1:25, 3.5 μL de água Milli-Q autoclavada, 0,25 μL de cada par de iniciadores a 10 mM e 6 μL de PowerUp SYBR® Green Master Mix (Applied Biosystems®) de acordo com as orientações do fabricante, totalizando um volume de 10 μL em equipamento 7500 Fast Real-Time PCR System (Life Technologies). A ciclagem foi de: 50°C a 2 minutos e 95°C por 2 minutos na fase de holding stage; 95°C por 15 segundos na fase de desnaturação; temperatura de anelamento de acordo com a determinação prévia de melhor temperatura, que variou de 52°C a 68°C; 95°C por 15 segundos, depois 60°C por 1 minutos, depois 95°C por 15 segundos e finalizando com 60°C por 15 segundos.

Para cada reação foi realizada uma curva de dissociação a fim de verificar contaminações e inespecificidades da reação (Figura suplementar 1 – Anexo 3). A curva de dissociação submete os produtos de PCR a um aumento gradual de temperatura até que 50% dos amplicons estejam na sua forma dissociada. Esta etapa realizada ao final do qPCR certifica que somente o alvo dos primers foi realmente amplificado, não sendo produtos inespecíficos ou dímeros de primers. As reações para cada par de primer foram realizadas em triplicata técnica e biológica.

### 4.9.1. Extração de RNA total

Para extração do RNA o protocolo utilizado seguiu as instruções do fabricante do TRIzol (Invitrogen®) como descrito a seguir: foi adicionado 1 mL do reagente TRIzol® (mantido a 4°C) ao tecido macerado (100 mg) e misturado com auxílio de um agitador de tubos do tipo vortex. Depois, as amostras foram mantidas por 5 min a temperatura ambiente (25°C)

para permitir a completa dissociação dos complexos de nucleoproteínas. Em seguida foram adicionados 200 µL de clorofórmio gelado (mantido a 4°C) às amostras, que foram homogeneizadas em equipamento tipo vortex por 1 min e, após, incubadas por 3 minutos a temperatura ambiente. Depois, as amostras foram centrifugadas a 12.000 g por 15 min a 4°C e o sobrenadante (fase aquosa) foi transferido para um novo tubo de 2 mL (o RNA total encontra-se exclusivamente nessa fase). Para precipitação do RNA foram adicionados 500 µL de isopropanol (mantido a -20ºC). Os tubos foram agitados cuidadosamente, por inversão, durante 15 segundos e mantidos a temperatura ambiente por 10 minutos. Depois, as amostras foram centrifugadas a 12.000 g por 10 min a 4°C e o RNA precipitado no fundo do tubo ("pellet"). O sobrenadante foi descartado e esse precipitado foi lavado com 1 mL de etanol 75% (mantido a 4°C). As amostras foram centrifugadas a 7.500 g por 5 min a 4°C e o sobrenadante foi imediatamente descartado. O precipitado foi seco por cerca de 10 min a temperatura ambiente e depois eluído em 50 µL de água Milli-Q autoclavada. Para auxiliar a eluição do RNA os tubos foram colocados a 55°C por 15min.

### 4.9.2. RT-qPCR precedida de transcrição reversa

SuperScriptTM VILO™ MasterMix (Invitrogen) e PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) foram usados para a síntese de cDNA e reações qPCR, respectivamente. As reações foram realizadas em um volume final de 10 µL, contendo 1 µl de cDNA (gerado de 100 ng.$\mu L^{-1}$ de RNA total), 0,25 µL (10 µM) de cada par de primers, 5 µL de PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) e 3,5 µL de $H_2O$ livre de DNAse e RNAse. Todas as reações para cada uma das amostras foram conduzidas em triplicata técnica no equipamento '7500 Fast Real-Time PCR System' (Applied Biosystems™) em uma 'MicroAmp™ Fast Optical 96-Well Reaction Plate' (0.1 mL; Applied Biosystems™). Como constitutivos o *ERS* (*Ethylene Response Sensor*), *NDID* (*NADP-dependent isocitrate dehydrogenase*) e *EF1a1* (*Translation elongation factor 1a-1)* foram usados para a normalização de qPCR (MUNHOZ et al., 2015). Todos os oligos utilizados nas reações de RT-qPCR estão listados na tabela 1. Foram utilizados 3 pools biológicos independentes (triplicata biológica). As reações de RT-qPCR foram realizadas em triplicata técnica no aparelho Applied Biosystems 7500 Fast Real-Time PCR (Thermo Fisher Scientific) com as seguintes condições de ciclagem: desnaturação inicial a 95 °C por 2 min, 40 ciclos de 95 °C por 15 seg, 53 – 68 °C por 30 seg e alongamento a 72 °C por 1 minuto. Os valores de Ct foram avaliados usando o

método $2^{-\Delta\Delta Ct}$ e representados como expressão relativa e fold change, conforme proposto por LIVAK; SCHMITGEN, (2001).

**Tabela 1.** Pares de oligonucleotidios usados para o RT-qPCR.

| Proteína | Sequência F (1a línea) e R (2a línea) (5'→ 3') | T° anelamento | Amplicom |
|---|---|---|---|
| ***Peroxidase 12* (*POD 12*)** | GTCTTGGTGGGGTCTTGG<br>CCTGCTTGAACTCTTTCTTG | 58 °C | 142 bp |
| ***Chitinase Class I (PR-3)*** | CGTCTCTAGTATTCTCGCTG<br>AGCACACGCACGACAGTTG | 55 °C | 173 bp |
| ***Lipoxygenase 2* (*LOX 2*)** | TCTACGCCTGGGGTTACTG<br>TGCCTTTTGGTTTTGATGGAC | 60 °C | 161 bp |
| ***Superoxide dismutase* (*SOD*)** | CGTTCTTAATAGCAGTGAGG<br>CAGCAGGATTGAAGTGTGG | 55 °C | 187 bp |
| ***Phenylalanine ammonia lyase* (*PAL*)** | CCTGAACTCCGCCACCATGCG<br>GGCCACGACCCTCTCAACTGG | 68 °C | 93 bp |
| ***AUX-responsive protein SAUR* (*AUX*)** | ACTGGTGAATAATCCTGAATGTT<br>CTTGGTGCTCTGCGATTG | 56 °C | 133 bp |
| ***GA 2-Beta dioxygenase (GA)*** | GGCAACAACCTGAAGTAG<br>CGTAGTTCCTGTCCATTG | 53 °C | 145 bp |
| ***qCABMV06*** | ATAGAATACAAGCCAGCACAAATCG<br>CCGTCCATCATAGTCCACACC | 55 °C | 200 bp |
| ***Ethylene Response Sensor* (*ERS*)** | GTATCTTGTGCTGTTGTGTC<br>CCATCTCCCTGTCAAGTTC | 58 °C | 95 bp |
| ***NADP-dependent isocitrate dehydrogenase* (*NDID*)** | GTCGTCACTCTCTCTTTACG<br>TCATTTCATCACCGTCCATC | 55 °C | 98 bp |
| ***Translation elongation factor* 1a-1 (*EF1a1*)** | GTTAAGGATTTGAAGCGTGG<br>ATGTGTGATGTGTGGCAGT | 55 °C | 97 bp |

### 4.10. Detecção qualitativa do CABMV por meio de RT-PCR

Amostras de folhas com infecção sistêmica (folhas novas), foram coletadas no tratamento com água + CABMV 1:50 e pGM + CABMV 1:50 as 1.5 e 4 semanas após inoculação viral, com a finalidade de determinar a prevalência do vírus nas folhas, mas novas da planta. A detecção do CABMV foi realizada por meio da técnica de RT-PCR. O procedimento foi conduzido com emprego do kit comercial Superscript® (Invitrogen by Life

Technologies, EUA), seguindo as recomendações do fabricante. As reações de RT-PCR foram realizadas em um volume final de 12,5 µL contendo 2,5 µL de RNA viral, 3,0 µL de $H_2O$ livre de DNAse e RNAse, 6,25 µL de PCR Master Mix (2x), 0,25 µL de Forward Primer 10 µM, 0,25 µL de Reverse Primer 10 µM, e 0,25 µL de Super Script IIITM One-Step RT-PCR System with Platinum Taq. Primers utilizados foram específicos para o capsídeo do CABMV: CABMV_M1MX3726F 5' GAGACACAAGCCAAAACACAAAATC 3' e CABMV_M1MX5029R 5' CGTTGCTACAAATTCTGGTATCTCC 3', que geram um amplicon esperado de 1311 bp (FONTENELE et al., 2018). Na amplificação a mistura foi submetida a 48 °C por 30 min, 95 °C por 10 min, seguida por 35 ciclos de PCR cada um composto de 95 °C por 15 s e 60 °C por 1 min. Cada conjunto de amostras submetido à amplificação foi acompanhado de um controle negativo, o qual continha todos os reagentes da mistura e água livre de DNAse e RNAse, e um controle positivo para CABMV. Os produtos amplificados foram submetidos à eletroforese em gel de agarose 1,3% (p/v) com brometo de etídio e visualizados sob luz ultravioleta (UV). O DNA Ladder Plus de 1 kb (LabAidTM) foi usado para verificar o tamanho dos amplicons.

### 4.11. Detecção semiquantitativa por meio de ELISA

A presença e acumulação relativa do CABMV foi determinada por meio do teste sorológico ELISA ("Enzyme Linked Immunosorbent Assay"). Foi utilizado o kit ELISA PathoScreen® (Agdia) para detecção específica de vírus do Grupo Potyvirus incluindo CABMV, conforme protocolo desenvolvido pelo fabricante. Amostras individuais de tecido vegetal coletadas as 4 semanas após inoculo viral e estocadas em freezer -80°C, do tratamento com água + CABMV 1:50 e pGM + CABMV 1:50, foram maceradas em almofariz e diluídas tampão de extração de amostra indireta (IEB) (pH 9,6), na proporção 1:100 (0,03 g de tecido vegetal, extraído com 3 ml de IEB). Foram colocados 100 µL em duplicata técnica na placa de microtitulação de alta resistência fornecida pelo fabricante, posteriormente foi incubada em câmara úmida por 1 hora em temperatura ambiente. Foram utilizadas amostras de plantas de maracujá infectadas com o CABMV como controle positivo e amostras de plantas sadias como controle negativo. Depois foi preparado o anticorpo de detecção e diluídos em tampão ECI (pH 7,5), utilizando 100 µL de anticorpo de detecção diluído por poço). Posteriormente foram lavadas as amostras da placa 8 vezes usando tampão de lavagem PBST (pH 7,5). Logo foi secada a placa, para posteriormente pipetar novamente 100 µL do anticorpo de detecção diluído

em cada poço da placa e incubada em câmara úmida por 2 horas em temperatura ambiente. Depois foi preparado o conjugado enzimático, diluído em tampão ECI (pH 7,5, utilizando 100 μL de conjugado enzimático diluído por poço. Logo a placa foi lavada 8 vezes usando tampão de lavagem PBST (pH 7,5). Posteriormente a placa foi secada e pipetaram-se novamente 100 μL do conjugado enzimático diluído em cada poço, a placa foi incubada em câmara úmida por 1 hora em temperatura ambiente. Depois foi preparado o substrato PNP, adicionando 1 comprimido de substrato PNP por 5 ml de tampão de substrato PNP (pH 9,8) e mantido no escuro até o uso. Foram utilizados 100 μL de solução PNP diluída por cada poço, depois a placa foi lavada 8 vezes usando tampão de lavagem PBST (pH 7,5) e posterior secagem, para novamente pipetar 100 μL de solução PNP dissolvida em cada poço e incubada (no escuro), por 1 hora em temperatura ambiente, onde ocorreu a reação enzimática. A absorbância de cada um dos poços (amostras) foi medida no espectrofotômetro (BIO RAD iMARK$^{TM}$ Microplate Reader), utilizando-se filtro de 415 nm. A reação foi considerada positiva, quando o valor médio da absorbância excedeu em 2 vezes o valor médio da absorbância do controle negativo (amostra de planta sem vírus).

### 4.12. Detecção quantitativa (RT-qPCR) do CABMV

Amostras foliares foram coletadas (3 pools biológicos independentes de cada tratamento, cada pool composto por 4 plantas), mantidas em gelo seco e levadas ao LVMV (UFRJ) para detecção quantitativa por meio de RT-qPCR. As reações de RT-qPCR a transcrição reversa foi realizada utilizando SuperScript$^{TM}$ VILO$^{TM}$ MasterMix (Invitrogen), seguindo as recomendações do fabricante. Foram utilizados o par de oligos qCABMV06_For 5' ATAGAATACAAGCCAGCACAAATCG 3' e qCABMV06_Rev 5' CCGTCCATCATAGTCCACACC 3', projetado para amplificar parte do gene da proteína de revestimento do CABMV de 200 pb (FREITAS, 2013). As reações foram realizadas em um volume final de 10 μL, contendo 1 μL de cDNA (gerado de 100 ng.μL$^{-1}$ de RNA total), 0,25 μL (10 μM) de cada par de primers, 5 μl de PowerUp$^{TM}$ SYBR$^{TM}$ Green Master Mix (Thermo Fisher Scientific) e 3,5 μL de $H_2O$ livre de DNAse e RNAse. Todas as reações para cada uma das amostras foram conduzidas em triplicata técnica no equipamento '7500 Fast Real-Time PCR System' (Applied Biosystems™) em uma 'MicroAmp™ Fast Optical 96-Well Reaction Plate' (0.1 mL; Applied Biosystems™). O programa utilizado para amplificação do gene da proteína capsidial do CABMV foi o padrão do equipamento no modo Standard, seguindo as

etapas: incubação a 50 °C por 2 min, ativação da taq DNA polimerase a 95 ºC por 10 min, seguida de 40 ciclos de desnaturação a 95 ºC por 15 seg, anelamento dos primers a 55 °C e extensão a 60 ºC por 1 min. Como constitutivos o *ERS* (*Ethylene Response Sensor*), *NDID* (*NADP-dependent isocitrate dehydrogenase*) e *EF1a1* (*Translation elongation factor 1a-1)* foram usados para a normalização de qPCR (MUNHOZ et al., 2015). Os valores de Ct foram avaliados usando o método $2^{-\Delta Ct}$ conforme proposto por LIVAK e SCHMITGEN (2001).

### 4.13. Avaliação de parâmetros morfológicos, desenvolvimento e produtividade

Alguns parâmetros morfológicos foram avaliados ao longo do tempo em todos os tratamentos. A altura, número de folhas, flores e frutos foi determinada ao longo do tempo, medindo com fita métrica e contagem individual de cada uma das plantas por cada tratamento, respectivamente. A área foliar foi avaliada em 5 folhas por cada tratamento seguindo o método não destrutivo descrito por SOUTO et al., (2017), onde foram medidos o comprimento da nervura central (L) e a maior largura da folha (W) das folhas lanceoladas e a área foliar foi determinada pela equação: área foliar = 0,25 + 0,64 (L x W). A biomassa das plantas foi determinada utilizando uma balança eletrônica de precisão (Bioprecisa - JA3003N), a parte aérea e o sistema radicular das plantas foram cortadas, as raízes foram lavadas e secadas a temperatura ambiente, em seguida foram pesadas para determinar peso fresco, posteriormente as amostras foram colocadas em sacos de papel individuais e secados a 65 °C por 48-72 horas para determinar peso seco destas. O diâmetro do tronco do caule foi medido com auxílio de paquímetro digital (Imports). Em outros experimentos a área foliar da quinta folha mais jovem formada de cada planta por cada tratamento (Contando de acima para abaixo) foi coletada e medida por meio de scanner de área foliar (LI- Cor Biosciences, modelo LI-3100c). Para os parâmetros de produtividade ou rendimentos em campo, 30 frutos de cada tratamento foram usados para avaliar algumas características como: peso (g), diâmetro (mm); comprimento (mm); peso (g) e rendimento (%) da polpa do fruto (estimado pela diferencia entre peso do fruto e o peso da polpa); sólidos solúveis (°Brix) e produção total (toneladas $ha^{-1}$). Diâmetro e comprimento foram medidos usando um paquímetro digital eletrônico de 0-150 mm, os pesos do fruto e polpa usando uma escala digital (Bioprecisa-JA3003N), os °Brix foram medidos usando um refratômetro portátil de sacarose com escala de 0-53 °Brix (PAL-1, ATAGO®). Além disso, o número de frutos produzidos por planta foi anotado em três momentos diferentes

antes da colheita. Foram contados apenas os frutos presentes cerca de 4 semanas após a floração. A produção de frutos por planta (kg) foi calculada multiplicando o número médio de frutos por planta pelo peso total médio para o respectivo genótipo. Finalmente, a produção de plantas individuais foi extrapolada em uma base por hectare como uma função do número de plantas por hectare para estimar o rendimento em t $ha^{-1}$.

### 4.14. Análise estatística

Two-way ANOVA foi realizada para avaliar a comparação entre as médias da expressão genica. Os dados obtidos para incidência, severidade, parâmetros morfológicos e produtividade em cada tratamento foram analisados pelo one-way ANOVA. Ambos seguidos pelo teste post-hoc de Bonferroni ($p$ <0.05). Para análise de severidade da doença causada pelo CABMV, baseada numa escala semiquantitativa, foi utilizado um teste não paramétrico, Kruskal-Wallis seguido pelo teste post-hoc de Dunn ($p$ <0.05). Experimentos onde só havia dois tratamentos (Controle e pGM) os dados obtidos para os parâmetros de desenvolvimento foram analisados por meio do teste t de Student ($p$ <0.05), este mesmo analise também foi utilizado para comparação da acumulação do vírus por RT-qPCR. Para a severidade da doença causada por *C. herbarum*, foi usado o teste não paramétrico de Wilcoxon ($p$ <0,05). O software GraphPad Prism versão 5.00 para Windows foi usado para análise estatística e elaboração dos gráficos.

# 5. RESULTADOS

## 5.1. Capítulo I. A fungal glycoprotein mitigates passion fruit woodiness disease caused by cowpea aphid-borne mosaic virus (CABMV) in *Passiflora edulis*

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# A fungal glycoprotein mitigates passion fruit woodiness disease caused by *Cowpea aphid-borne mosaic virus* (CABMV) in *Passiflora edulis*

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**Abstract** Passion fruit woodiness disease is responsible for severe losses in passion fruit production around the world. The disease is caused by *Cowpea aphid-borne mosaic virus* (CABMV), an aphid-transmitted potyvirus. Traditional sanitary measures against the disease, such as vector elimination and cross protection, have not been successful, resulting in elimination and replanting of passion fruit plants each season. To find new alternatives for disease control, we tested the use of a peptidogalactomannan (pGM) extracted from the fungus *Cladosporium herbarum* to activate passion fruit defense mechanisms, enabling plants to tolerate passion fruit woodiness disease (PWD). Passion fruit seedlings were spray-treated with pGM in a greenhouse three days before mechanical inoculation with CABMV. Experiments were set up in a completely randomized design, and disease incidence and severity were compared between water- and 100 µg ml$^{-1}$ pGM-treated plants. Woodiness symptoms and certain developmental parameters of water- and pGM-treated plants were evaluated over five weeks. pGM treatment did not affect plant normal development. Plants that were both treated with pGM and inoculated with the virus showed very mild or no foliar CABMV disease symptoms and had the same growth and developmental patterns as the healthy uninoculated control plants. pGM led to the accumulation of antioxidant enzymes such as peroxidase and superoxide dismutase in the leaf tissues as well as their respective mRNAs. In addition, a ten- and twofold transcription induction of the mRNAs of the defense-related genes such as *chitinase I* (*PR-3*) and *phenylalanine ammonia-lyase* (*PAL*), respectively, were observed in pGM-treated seedlings. These results

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suggested that pGM enables plants to respond more intensely to CABMV infection, mitigating woodiness symptoms and maintaining normal plant growth.

**Keywords** Peptidogalactomannan · pGM · Passion fruit · *Cladosporium herbarum* · Defence-related genes · PWD protection · Systemic acquired resistance

## Introduction

Passion fruit (*Passiflora edulis*), of the Passifloraceae family is a species of passion flower native to southern Brazil. Due to its economic importance associated with its special flavor, it is largely grown for fresh fruit consumption and juice production in almost all tropical places, including South America to Australia, Asia and Africa. Global production of passion fruit is estimated at 852 thousand tons, and the main passion fruit producing countries are Brazil, Mexico, Ecuador, Australia, Zimbabwe, Kenya and Colombia (Ramyashree et al. 2019). Brazil is the world's largest passion fruit producer, with a yield of 602,651 tons per year, an area of 43,248 hectares and an average national production of 14.1 tons per hectare. One of the most important phytosanitary problems facing this crop is passion fruit woodiness disease (PWD) caused by *Cowpea aphid-borne mosaic virus* (CABMV), which has an ~ 10 kb genome size and a polyA tail on the 3′-terminal end. PWD is a devastating disease and one of the main factors limiting passion fruit yields in South America and Africa. Infected plants have a reduced leaf area and fruit weight, with a consequent reduction in the number, quality, and commercial value of the fruits (Nascimento et al. 2006). PWD causes severe yield losses and reduces plant life by approximately 50% (Fischer and Rezende 2008). The virus is transmitted non-persistently by at least three aphid species: *Aphis gossypii* Glover, *A. fabae* Scopoli, and *A. craccivora* Bock (Hemiptera: Aphididae) and mechanically during pruning. All yellow and sweet passion fruit cultivars are susceptible to CABMV infection and chemical control of vectors is generally inefficient due to the distinct aphid species that can transmit the virus, making disease control difficult. Cross-protection with less virulent strains of the virus also failed to control the disease (Novaes and Rezende 2005).

Plants have developed innate and induced immunities against pathogen infection. Such defense systems include responses induced by pathogen-associated molecular patterns and by pathogen effectors that are designated pathogen- and effector-triggered immunity, PTI and ETI, respectively. PTI and ETI induce systemic acquired resistance (SAR) through complex networks that involve a variety of mobile and cross-interacting signals. which culminate in activating defensive signaling pathways to restrict microbial invasion and proliferation (Cui et al. 2015; Berens et al. 2017; Boutrot and Zipfel 2017). Typically, pathogenesis-related proteins (PR proteins) accumulate when plants exhibit SAR (Sticher et al. 1997). PR proteins are defined as host-induced proteins made in response to biotic or abiotic stimuli and may be correlated with non-specific host resistance to pathogens (Stangarlin et al. 2011).

The use of elicitors can trigger defense in non-host and host plants through the perceived presence of possible pathogens (Onaga and Wydra 2016; Dalio et al. 2017). Most elicitor molecules act through signaling pathways, involving a complex interaction among salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Reglinski et al. 2013). In species such as *Oryza sativa* and *Tsuga canadensis*, SA and JA have been used with some degree of success to control various plant pathogens (Stella de Freitas et al. 2019; Rigsby et al. 2019). In an effort to identify biogenic or biomolecules that can potentially protect plants against biotic stress, our group recently demonstrated that a *Cladosporium herbarum* (Capnodiales: Davidiellaceae) produced peptidogalactomannan (pGM), which induced defense-related genes in tobacco (*Nicotiana tabacum*, Solanales: Solanaceae) (Mattos et al. 2018). *Clasdosporium herbarum* pGM is present in the fungal cell wall and is composed of 76% carbohydrates, with mannose, galactose, and glucose as the main monosaccharides (molar ratio, 52:36:12) (Mattos et al. 2018). The treatment of BY2 tobacco cells and the spraying of whole tobacco plants with pGM was shown to lead to a strong induction of four PR genes: *PR-1a* (with unknown function), *PR-2* (a β1-3 endoglucanase), *PR-3* (an endochitinase), and *PR-5* (a thaumatin-like protein). In addition, the levels of *lipoxygenase* (*LOX*), *peroxidase* (*POD*) and *phenylalanine ammonia lyase* (*PAL*) transcripts were also highly increased. Here we analyzed whether *C. herbarum* pGM treatment could mitigate PWD

damage in passion fruit. We observed that spraying pGM onto passion fruit plants induced an important protective status, making them tolerant to PWD. Expression of defense-related genes and reactive oxygen species accumulation were observed in passion fruit pGM-sprayed plants, indicating that CABMV tolerance could be related to SAR induction.

## Materials and methods

### Cell wall glycoprotein (pGM) extraction

*Cladosporium herbarum* was grown in potato dextrose broth medium (PDB) for seven days to obtain the fungal mass. Glycoprotein extraction was performed according to Haido et al. (1998). Briefly, fungus mycelium was extracted with 0.05 M phosphate buffer, pH 7.2, at 100 °C for 2 h. After filtration, filtrate was vacuum evaporated and precipitated in 92.8% (v/v) ethanol at 4 °C. The precipitate was resuspended in Milli-Q water, dialyzed and freeze-dried to obtain the crude pGM. Freeze-dried pGM was store at − 20 °C until use.

### Plant materials

Experiments were carried out in a greenhouse at the Universidade Federal do Rio de Janeiro (UFRJ). *Passiflora edulis* plants were grown in plant substrate (Garden Plus, Turfa Fertil Co., Brazil) under tropical area natural light conditions with controlled air temperatures of 27 ± 2 °C. Treatments were performed in plants with 3–4 true leaves.

### pGM pulverization and virus inoculation

A pilot experiment was performed in which passion fruit young plants were sprayed with 25, 50, 100, 200 or 400 µg $ml^{-1}$ of pGM resuspended in Milli-Q water. Water sprayed plants and untreated plants were used as control. Suspensions were sterilized by filtration through a Millipore 0.20 µm filter before use. Pulverization was performed with a high-pressure device (W550, Wagner). As a control, Milli-Q water was sprayed using the same procedure (control treatment). The experimental design was randomized with ten plants for each treatment. Three days after pGM spray, two leaves per plant were each mechanically inoculated with 100 µl of a CABMV 1:10 dilution (CABMV-inoculated leaves homogenized in 10 mM sodium phosphate buffer, pH 7.0).

The following experiments were performed with a concentration of 100 µg $ml^{-1}$ of *C. herbarum* pGM following the same pulverization and CABMV infection procedures described above. The experimental design was randomized with ten plants for each treatment, and two independent experiments were performed. CABMV was inoculated at a 1:10 or 1:50 dilution on leaves at positions 3 and 4 (counting from the first fully expanded leaf from the top). The plants were monitored over time for PWD symptom appearance and/or plant morphological parameter evaluation.

### PWD incidence and severity assessments

Disease incidence and severity assessments were performed weekly after virus inoculation. Virus incidence was evaluated by considering the percentage of plants with at least one leaf showing mild to severe mosaic and/or deformation symptoms. The disease severity index (%) used a 1–4 scale as follows: 1 represented the absence of symptoms, 2 represented the presence of mild leaf mosaic symptoms without leaf deformation, 3 represented the presence of severe mosaic symptoms without leaf deformation, and 4 represented the presence of severe mosaic symptoms, blisters and leaf deformation, as proposed by Gonçalves et al. (2018). The disease severity index was calculated as proposed by Mckinney (1923), applying the following formula: $DI = \sum(DS \times P)/(TNP \times HGS) \times 100$, where DS = degree of the 1–4 scale determined for each plant, P = number of leaves showing each degree of infection (score), TNP = total number of plants, and HGS = highest grade of the scale (maximum infection score).

### Plant developmental parameter evaluation

Plant height, leaf number and leaf developmental status were evaluated for ten individual plants per treatment with two replications over time after virus inoculation. Leaf area evaluation was performed five weeks after CABMV inoculation (wai) on five leaves per plant for each treatment following the non-destructive method described by Souto et al. (2017), where the length of the midrib (L) and the greatest

width of the leaf (W) of lanceolate leaves were measured and the leaf area was determined by the equation leaf area = 0.25 + 0.64(L × W). The fresh and dry (after 48 h at 65 °C) weights were determined using an electronic scale.

### Histochemical detection of reactive species oxygen (ROS)

For histochemical tests, uninoculated pGM-treated leaf samples from five plants collected 24 and 72 h after treatment (hat) were vacuum-infiltrated with DAB (3,3-diaminobenzidine) or NBT (nitro blue tetrazolium) according to Wang et al. (2016). Leaves from untreated plants and water treated plants were used as controls.

### Evaluation of gene expression by real-time RT-qPCR

The expression of five defense-related genes was evaluated in leaves of CABMV uninoculated and inoculated plants after water and pGM treatment. Uninoculated plants were evaluated at 24 and 72 h after treatment (hat) and CABMV-inoculated plants were evaluated at 12 and 168 h after virus inoculation (hai). Total RNA was extracted with TRIzol® reagent (Thermo Fisher Scientific). SuperScript™ VILO™ MasterMix (Invitrogen) and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) were used for cDNA synthesis and qPCR, respectively. Three housekeeping genes: *ERS* (*ethylene response sensor*), *NDID* (NADP-*dependent isocitrate dehydrogenase*) and *EF1a1* (*translation elongation factor* 1a-1) were used for qPCR normalization. Oligonucleotides described previously by Munhoz et al. (2015) were used (Supplementary Table S1). qPCR of three independent biological pools, composed of five water- and/or pGM-treated plants each was performed for each point in technical triplicates on an Applied Biosystems 7500 Fast Real-Time PCR apparatus with the following cycling conditions: initial denaturation at 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 55–68 °C for 30 s and elongation at 72 °C for 1 min (Supplementary Table S1). Ct values were evaluated using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001) and represented as relative expression.

### Detection of CABMV prevalence

Ten systemic leaves from pGM and ten from water treated CABMV inoculated plants were collected 1.5 and four weeks after CABMV inoculation (wai), and virus presence was assayed by a semi-quantitative RT-PCR. Total RNA was extracted using TRIzol (Thermo Fisher Scientific). Multiplex RT-PCR was performed using a SuperScript III One-Step RT-PCR with Platinum DNA Polymerase Kit (Invitrogen), following the manufacturer's recommendations, CABMV capsid-specific oligos CABMV_M1 MX3726F 5′ GAGACACAAGCCAAAACACAAAATC 3′ and CABMV_M1 MX5029R 5′ CGTTGCTACA AATTCTGGTATCTCC 3′, which generate an expected amplicon of 1311 bp (Fontenele et al. 2018), and the plant constitutive *NDID* (NADP-*dependent isocitrate dehydrogenase*) oligos (Supplementary Table S1). The amplified products were subjected to 1.3% (w/v) agarose gel electrophoresis with ethidium bromide (0.5 μg $ml^{-1}$) (Promega) and visualized under UV light. The 1 kb DNA Ladder Plus (LabAid™) was used to check the amplicon size.

A semi-quantitative ELISA (PathoScreen® Agdia ELISA kit for specific detection of viruses of the potyvirus Group including CABMV, Elkhart, IN, USA) was performed to identify the presence and relative accumulation of CABMV in passion fruit plants following the Agdia protocol. Ten systemic leaf samples from pGM-treated/CABMV and ten from control (water/CABMV-inoculated) plants were collected at 4 wai. The ELISA was performed using three technical replicates per sample.

### Statistical analysis

Data obtained for DI, developmental parameters and gene expression were analyzed by the one-way or two-way ANOVA, respectively, followed by Bonferroni post-hoc correction to check if the different averages observed between water and pGM treatments were statistically supported. For DS and ELISA data comparisons, a non-parametric Kruskal–Wallis test was used with Dunn's post-hoc tests. GraphPad Prism software version 5.00 for Windows was used for statistical analysis.

## Results

The ability of five distinct concentrations of *C. herbarum* pGM in eliciting defense against PWD caused by CABMV was tested in passion fruit plants. Water treated and untreated plants were used as control. Plants sprayed with 100 μg $ml^{-1}$ pGM showed statistically supported milder leaf symptoms over time, presenting a lower DS index at 5 wai ($\chi^2_6 = 25.13, p < 0.001$) (Supplementary Table S2a).

Disease incidence was also evaluated and corresponded to the percentage of plants presenting mosaic and/or deformation symptoms on at least one leaf of pGM-treated/CABMV-inoculated plants over time (Supplementary Table S2b). The best results were also obtained in plants sprayed with a pGM concentration of 100 μg $ml^{-1}$. In the first 2 wai, the number of CABMV-inoculated plants showing at least one symptomatic leaf decreased by 60% at this pGM concentration when compared with the controls ($F_{6,63} = 4.47$, $p < 0.01$). However, at 5 wai only 10% of the 1:10 CABMV-inoculated plants treated with 100 μg $ml^{-1}$ pGM remained completely asymptomatic and 90% presented very mild mosaic symptoms in one or a few leaves. Disease incidence showed no significant differences between the CABMV control and all the treatments ($F_{6,63} = 0.83$, $p > 0.5$) at 5 wai. Based on these results, further experiments using 100 μg $ml^{-1}$ pGM were performed to better understand the effects on CABMV infection in pGM-treated passion fruit plants.

### Inoculum concentration does not interfere with CABMV protection

Passion fruit plants sprayed with water or 100 μg.$ml^{-1}$ pGM were inoculated with two CABMV dilutions: 1:10 and 1:50. Disease severity showed significant differences (Dunn's test $p < 0.0001$) between treated and control plants at all evaluated time points for both viral inoculum concentrations (Table 1a). A reduction in disease severity of 36.3 and 46.4% compared to the CABMV controls was observed at 5 wai for the 1:10 and 1:50 inocula, respectively ($\chi^2_5 = 59.03$, $p < 0.0001$). Thus, pGM treatment reduced the severity of the disease caused by CABMV with both the 1:10 and 1:50 inocula.

A decrease of 80 and 75% in the number of passion fruit plants showing leaf CABMV symptoms was observed in the pGM-treated plants when compared with the control in the first 2 wai using viral inocula at 1:10 and 1:50 dilutions, respectively ($F_{5,114} = 28.77$, $p < 0.0001$) (Table 1b). However, the incidence of treated plants presenting at least a mildly symptomatic leaf increased over time, with 75 and 70% disease incidence at 5 wai for the 1:10 and 1:50 dilutions, respectively ($F_{5,114} = 5.85$, $p < 0.0001$). Nevertheless, in all pGM-treated plants, leaf symptoms characteristic of PWD were less severe than those observed in untreated and water-treated control plants for both viral inoculum concentrations at 5 wai.

### Morphological and developmental parameters of the plants

As mentioned above, the PWD severity is usually evaluated by taking into account the presence of mosaic and leaf deformation. To our knowledge, there are no reports referring to how development and/or other morphological parameters are affected by the disease. Comparing uninfected healthy passion fruit plants to untreated plants inoculated with CABMV over five weeks, we observed that the height, number of leaves, leaf area and fresh and dry weights of CABMV-infected plants were mildly to drastically affected (Fig. 1). These data agree with the high productivity losses associated with PWD. Then, we explored the effect of pGM treatment on the mitigation of these parameters. Comparing the height of pGM-treated CABMV-inoculated plants with the height of water-treated or virus-inoculated control plants, we observed that they were significantly different since 2 wai for 1:10 CABMV inocula (Fig. 1a) (height, $F_{6,133} = 2.60$, $p < 0.05$). One week after, the untreated/CABMV- and water-treated/CABMV-inoculated plants show a reduction in height compared with that of the healthy and the pGM-treated/CABMV-inoculated plants from both inocula. At 5 wai, untreated/CABMV- and water-treated/CABMV-inoculated plants were 20–28 cm shorter than healthy and pGM-treated/CABMV-inoculated plants ($F_{6,133} = 24.80$, $p < 0.0001$). No differences in plant height could be detected between the pGM-treated/CABMV-inoculated and healthy uninoculated plants. Therefore, it seems that pGM treatment protects the plant by preventing the developmental delay imposed by virus infection.

**Table 1** Effect of 100 μg $ml^{-1}$ of pGM on PWD disease severity (a) incidence (b) along the time

| Treatment | Disease severity (%) | | | |
|---|---|---|---|---|
| | 2 wai* | 3 wai | 4 wai | 5 wai |
| a | | | | |
| CABMV (1:10) | 92.0 ± 8.0a | 93.7 ± 7.1a | 93.2 ± 5.9a | 93.0 ± 4.7a |
| CABMV (1:50) | 91.2 ± 8.5a | 90.3 ± 8.2a | 92.7 ± 8.4a | 93.6 ± 7.6a |
| Water + CABMV (1:10) | 75.2 ± 27.0a | 90.7 ± 19.2a | 93.5 ± 7.5a | 98.3 ± 2.6a |
| Water + CABMV (1:50) | 60.2 ± 24.1b | 72.8 ± 21.8b | 77.0 ± 18.4a | 88.7 ± 9.4a |
| 100 μg $ml^{-1}$ pGM + CABMV (1:10) | 23.1 ± 41.4b | 45.2 ± 41.1c | 48.2 ± 42.9b | 59.2 ± 37.9b |
| 100 μg $ml^{-1}$ pGM + CABMV (1:50) | 37.6 ± 41.0b | 37.2 ± 39.7c | 55.7 ± 41.9b | 50.2 ± 36.0b |
| Treatment | Disease incidence (%) | | | |
| b | | | | |
| CABMV (1:10) | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| CABMV (1:50) | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| Water + CABMV (1:10) | 85.0 ± 36.0a | 95.0 ± 22.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| Water + CABMV (1:50) | 85.0 ± 36.0a | 95.0 ± 22.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| 100 μg $ml^{-1}$ pGM + CABMV (1:10) | 20.0 ± 41.0b | 60.0 ± 50.2b | 65.0 ± 48.0b | 75.0 ± 44.0b |
| 100 μg $ml^{-1}$ pGM + CABMV (1:50) | 25.0 ± 45.0b | 50.0 ± 51.3b | 55.0 ± 51.0b | 70.0 ± 47.0b |

Passion fruit plants were mechanically infected with 1:10 and 1:50 CABMV inoculum 72 h after treatment

Each value is the means (± SD) of two replications (20 plants per treatment)

Different letters in columns indicate significant differences between treatments ($p < 0.05$)

Corresponding results of Kruskal–Wallis. (a) Disease severity at 2 wai: $\chi^2_5 = 45.16$, $p < 0.0001$; at 3 wai: $\chi^2_5 = 53.51$, $p < 0.0001$; at 4 wai: $\chi^2_5 = 27.78$, $p < 0.0001$; at 5 wai $\chi^2_5 = 59.03$, $p < 0.0001$

Corresponding results of One-way ANOVA. (b) Disease incidence at 2 wai: $F_{5,114} = 28.77$, $p < 0.0001$; at 3 wai: $F_{5,114} = 12.80$, $p < 0.0001$; at 4 wai: $F_{5,114} = 8.52$, $p < 0.0001$; at 5 wai $F_{5,114} = 5.85$, $p < 0.0001$

*Weeks after CABMV inoculation

Regarding the number of leaves per plant, we observed statistically significant differences at 4 and 5 wai (4 wai $F_{6,133} = 22.37$, $p < 0.0001$; 5 wai $F_{6,133} = 10.70$, $p < 0.0001$) over time between pGM-treated plants and inoculated controls (CABMV and water-treated plants) (Fig. 1b), while, once again, no differences were observed between pGM-treated virus-inoculated and uninoculated plants after 5 wai with CABMV. Leaf area, an important parameter for photosynthetic efficiency, was also evaluated at 5 wai (Fig. 1c). Our results showed that the leaf area of pGM-treated/CABMV plants was almost the double of the leaf area of untreated/CABMV and water/CABMV control plants ($F_{6,63} = 3180$, $p < 0.0001$). No differences were observed between pGM-treated/CABMV and healthy uninoculated plants regardless of the viral inoculum concentration. Fresh and dry weights of water/CABMV and pGM/CABMV plants showed that pGM treatment strongly reduced the weight loss associated with CABMV infection (Fig. 1d) (fresh weight $F_{2,27} = 85.05$, $p < 0.0001$; dry weight $F_{2,27} = 45.44$, $p < 0.0001$). pGM/CABMV and uninoculated plants showed similar weights. Additionally, pGM-treated/CABMV plants showed similar or even better development patterns than uninoculated plants in aerial parts and similar root formation (Supplementary Figure S1).

Therefore, these results suggest that pGM-treated CABMV-inoculated passion fruit plants have growth rates similar to those of healthy uninoculated plants with no reduction in plant height, leaf number, leaf area or fresh and dry weights. In addition to these normal parameters presented by pGM-treated/CABMV-inoculated plants, a normal leaf

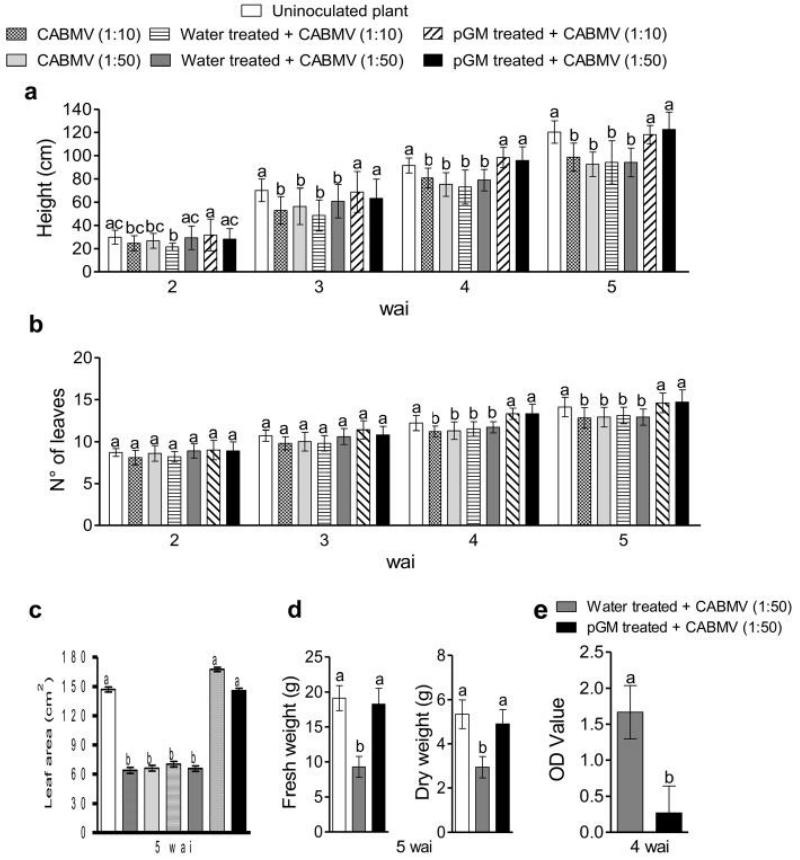


**Fig. 1** Analysis of the morphological parameters of pGM treated *P. edulis* inoculated -CABMV. Height (**a**) and number of leaves (**b**) of healthy (uninoculated plants), CABMV-inoculated plants (CABMV), water-treated and 100 μg $ml^{-1}$ pGM-treated CABMV-inoculated plants were evaluated at 2–5 weeks after virus inoculation (wai). In (**c**), values represent the mean area of five leaves from the middle part of ten independent plants at 5 wai. Two CABMV inoculum dilutions (1:10 and 1:50) were used. In (**d**), values represent the mean fresh and dry weights of ten plants per treatment and a 1:50 inoculum dilution at 5 wai. **e** Prevalence of CABMV in ten pGM-treated and 10 water-treated CABMV-inoculated passion fruit plants assessed by ELISA at 4 wai after CABMV inoculation. OD values were obtained at 415 nm. The bars represent the averages ± SD of two independent experiments using ten plants per treatment. Different letters indicate significant differences between treatments at each wai using one-Way ANOVA followed by Bonferroni post hoc correction ($p < 0.01$). For ELISA data comparisons, a non-parametric Kruskal–Wallis test was used with Dunn's post-hoc tests

development pattern was also observed on almost all the leaves of the pGM-treated plants. As shown in Supplementary Figure S2a and b, fully developed and young leaves of pGM-treated CABMV-inoculated plants did not present the abnormal developmental patterns observed in untreated and water-treated CABMV-inoculated plants.

## Histochemical detection of reactive oxygen species (ROS) after pGM treatment

Recent results from our research group showed that pGM is able to induce SAR in tobacco plants (Montebianco et al. in preparation). To better understand how pGM treatment induces virus protection in passion fruit plants and verify whether SAR is activated in these plants, we investigated the presence of superoxide anions ($O_2^-$) and hydrogen peroxide

($H_2O_2$) in the treated plants. The presence of $O_2^-$ radicals was observed 24 and 72 h after treatment, with leaf samples showing violet NBT substrate degradation areas (Supplementary Figure S2c). No violet spots were observed in untreated and water-treated control plants, indicating that the accumulation of superoxide radicals was induced by pGM treatment. We also observed the presence of small brown spots in DAB-incubated foliar samples, which shows that $H_2O_2$ accumulates in the leaves of pGM-treated plants 24 h after pGM spraying (Supplementary Figure S2d). Brown spots were not observed in untreated and water-treated control samples at any time or at 72 h after pGM treatment. Therefore, peroxidase seems to be induced only in the first 24 h after pGM treatment.

### pGM treatment effect on viral prevalence

As the symptoms observed in pGM-treated CABMV-inoculated plants were mild and development of these plants was almost unaffected, we then tested for CABMV prevalence. Using a multiplex RT-PCR assay it was possible to observe the presence of fragments corresponding to the CABMV coat protein gene in almost all the pGM- and water-treated CABMV-inoculated plants at 1.5 wai. At 4 wai, however, there was a reduction in the number of CABMV-positive in pGM-treated/CABMV plants (Supplementary Figure S3). Using a semi-quantitative ELISA, it was possible to observe a reduction of 83.7% in the relative CABMV accumulation in pGM-treated/CABMV plants compared to water-treated/CABMV plants ($\chi^2_1 = 14.42$, $p < 0.001$) (Fig. 1e).

### Expression of passion fruit defense-related genes

pGM treatment alone interferes with defense-related gene expression. Expression of two SAR marker genes, *PR-3* and *PAL*, was observed in the first hours after pGM treatment (hat) (Fig. 2). *PR-3* expression was strongly (tenfold) induced 24 h after treatment compared to the control (water-treated). However, 72 h after treatment, no significant differences were observed between pGM and water treatment. For *PAL*, the expression levels increased almost 2- and 4.2-fold at 24 and 72 h after treatment, respectively, compared with the water control. When examining gene expression in treated plants with subsequent CABMV infection (Fig. 2), 4- and ninefold induction of *PR-3* and *PAL* was observed, respectively, at 12 h after virus inoculation (hai) compared to water/CABMV plants. Gene expression differences were statistically supported by two-way ANOVA: $F_{1,64} = 868.1$, $p < 0.0001$ for PR-3 and $F_{1,64} = 679.2$, $p < 0.0001$ for *PAL*.

Genes associated with oxidative stress were also evaluated. Over-expression of *POD12* was observed at 24 and 72 h after pGM treatment alone (hat) and at 12 and 168 h after CABMV infection (hai) compared to water and water/CABMV treatments (*POD12*, $F_{1,64} = 519.6$, $p < 0.0001$). *SOD* was induced only at 72 hat. However, in the presence of the virus, an earlier induction was observed at 12 hai, which was sustained until 168 hai (*SOD,* $F_{1,64} = 63.11$, $p < 0.0001$). *LOX2,* a gene associated with biotic stress, showed no significant differential expression 24 h after pGM treatment in comparison with the water control but was repressed 72 h after treatment. In contrast, after virus infection, this gene was highly induced at 168 hai compared to water-treated/CABMV plants (*LOX2,* $F_{1,64} = 679.2$, $p < 0.0001$) (Fig. 2e).

## Discussion

Systemic acquired resistance activation may prepare the plant to respond successfully to pathogen attack, working as a biotechnological tool for plant protection. Here, we tested the role of *C. herbarum* pGM in protecting susceptible *P. edulis* against infection by CABMV, one of the most important passion fruit pathogens. Our results showed that plants treated with pGM present a strong mitigation of CABMV-related disease symptoms. Even when inoculated with a highly concentrated viral inoculum, passion fruit plants showed almost no symptoms and did not present the developmental delay characteristic of inoculated plants.

Studying the proteome of CABMV-inoculated susceptible and resistant passion fruit species, Carvalho et al. (2019) demonstrated that in susceptible *P. edulis* plants, basic defense response proteins are inhibited after virus infection, while others, such as glutathione peroxidase (GPX), are induced. Here, using a defense response elicitor, PWD developmental damage was turned off and defense-related genes were induced in CABMV-inoculated *P. edulis* plants,

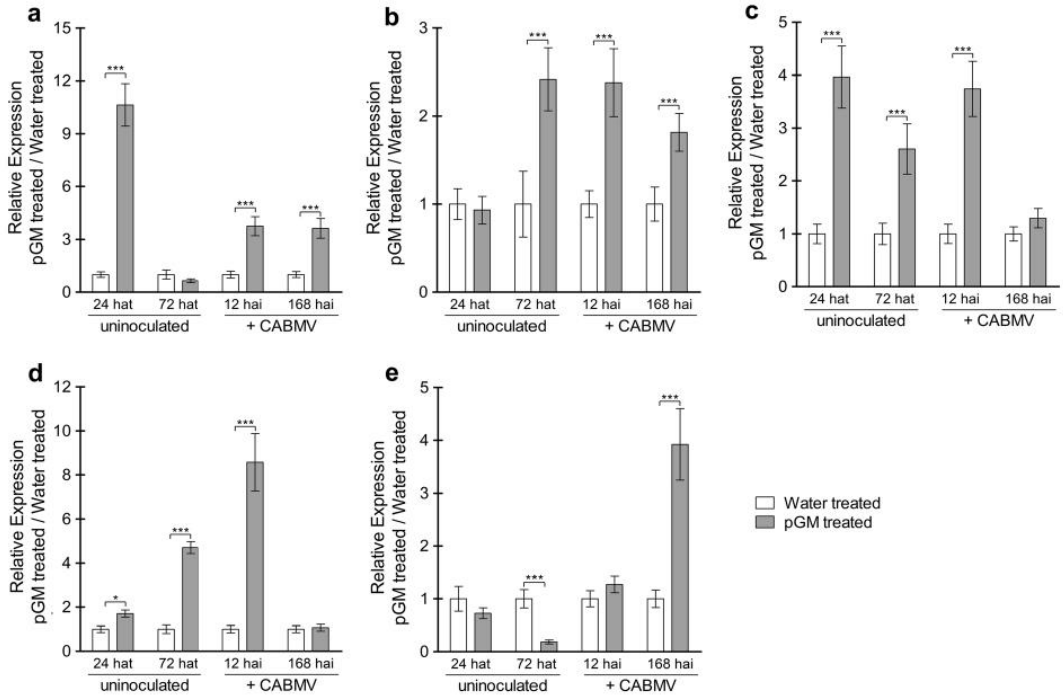


**Fig. 2** pGM treatment induced a defense response in passion fruit. Relative expression of **a** *PR3*, **b** *SOD*, **c** *POD12*, **d** *PAL* and **e** *LOX2* transcripts 24 and 72 h after 100 µg $ml^{-1}$ pGM treatment (hat—hours after treatment) and 12 and 168 h after viral CABMV inoculation (hai) relative to water. Ct values were normalized with *ERS*, *NDID* and *EF1a1*. Values represent the average (± SD) relative expression of mRNA transcripts between water and pGM treatment of three technical replicates from three biologically independent replicates of each treatment at each time point. * and *** represent significant differences of $p < 0.05$ and $p < 0.001$, respectively, comparing water and pGM treatment of each gene using two-way ANOVA followed by Bonferroni post-hoc correction (n = 9)

prompting the plants to show tolerance to the infection. pGM treatment led to a drastic mitigation in developmental parameters affected by CABMV infection and a 42% reduction in the disease severity index, which evaluates only leaf mosaic and deformations symptoms, compared to the controls at 5 wai (Table 1a). Moreover, regarding plant fitness parameters such as plant height, leaf number, foliar area and fresh and dry weights, pGM-treated CABMV-inoculated plants showed the same results as the uninoculated plants. The mitigation of virus-imposed developmental damage was accompanied by an important reduction in virus prevalence at 5 wai.

The expression profiles of five defense-related genes were analyzed to try to understand how pGM-treated *P. edulis* plants became tolerant to CABMV. We found that the *peroxidase* gene *POD12* was fourfold and 2.5-fold more highly expressed in pGM-treated plants at 24 h and 72 h after treatment, respectively. When the pGM-treated plants were inoculated with CABMV, the expression levels of these genes increased fourfold compared to water-treated inoculated plants (Fig. 2). Peroxidases are involved in regulating and maintaining the structural integrity of proteins, which assists the survival of cells under stress. POD induction is associated with various types of biotic and abiotic stresses, such as drought, salinity, cold, and pathogen attack (Park and Seo 2015). These enzymes play an important role in plant defense by mediating disease resistance signal transduction pathways, and the induction of *POD12* expression may have increased the ability of *P. edulis* to respond to CABMV infection. Moreover, one important biological function of peroxidases is

cellular $H_2O_2$ elimination. Plants use these enzymes to breakdown $H_2O_2$ in cells, reducing the toxicity of these compounds and modulating the cell oxidation state (Passaia and Margis-Pinheiro 2015). Histochemical assays of *P. edulis* leaves after pGM treatment showed very few small brown spots, which are indicative of $H_2O_2$ accumulation at 24 h and no spots at 72 h (Supplementary Figure S2c). The higher levels of *POD12* transcripts may suggest that POD12 enzyme over-expression catalyzes the reduction of $H_2O_2$ in water or alcohols and suppresses $H_2O_2$ accumulation. pGM treatment also induced the accumulation of $O_2^-$. In parallel, *SOD* was 2.5-fold more highly expressed in pGM than in the control uninoculated plants at 72 h after pGM treatment, but was induced after 12 hai and maintained until 168 hai (Fig. 2b). The increase in *SOD* levels seems to contribute to the $O_2^-$ decrease in passion fruit leaves after pGM treatment. The suppression of some detoxifying enzymes (such as POD and SOD) was already observed in some compatible plant-pathogen interactions as such as in the maize—*sugarcane mosaic virus* (SCMV) interaction (Wu et al. 2013). The suppression of these detoxifying enzymes may be crucial for the onset of programmed cell death, consequently leading to an inhibition of virus propagation in the plant (Apel and Hirt 2004). In the present study, *P. edulis* plants showed an increase in both the accumulation and gene expression of these detoxifying enzymes after elicitation with pGM. Similar data were obtained by Mofidnakhaei et al. (2016), who demonstrated that a defense inducer (potassium phosphite) sprayed on cucumber plants led to an increase in the activity of antioxidant enzymes such as SOD, POD, and CAT. Chitinase proteins belong to the *PR-3* group and are strongly induced in the host plant cells after pathogen attack, making them an important weapon against pathogens. In our analyses, *PR-3* expression levels were tenfold upregulated 24 h after treatment but could not be maintained at 72 h. Su et al. (2015) observed that chitinase transcripts accumulated to the maximal levels between 24 and 48 hai in the compatible interaction between *Saccharum* spp and *Sporisorium scitamineum,* returning to basal levels after that period. Surprisingly, we observed that in pGM-treated/CABMV-inoculated plants, the induced levels of the *PR-3* gene were maintained at 168 h after virus inoculation, suggesting that pGM treated plants can somehow continue to over-express this gene after contact with pGM and/or virus.

Expression of genes involved in the phenylpropanoid pathway may lead to the production of SA and phenolic compounds, such as phytoalexins and flavonoids, or may participate in the formation of polymers such as lignin, which makes cell walls more resistant to water loss and pathogen attack (Dixon et al. 2002). In this study, *PAL* expression was highly upregulated in pGM-treated uninoculated and CABMV-inoculated plants (Fig. 2), which is consistent with other studies showing significant increases in *PAL* after pathogen infection or treatment with elicitor substances (Gómez-Vásquez et al. 2004). Lima et al. (2018) found the maximum expression levels for *PAL* genes in *Phytopythium* sp.-elicited cassava roots at 48 h after elicitation. Our results in pGM-elicited *P. edulis* plants are consistent with those of other investigations with *PAL* accumulation being observed in the first hours after elicitation and/or virus contact. The increased levels of *PAL* observed after pGM treatment suggest a possible induction of the *P. edulis* plant defense response by isoflavonoid lignin and phytoalexin syntheses and/or SA production. Lipoxygenase (LOX) is an essential enzyme for JA biosynthesis. This enzyme catalyzes the oxygenation of fatty acids into hydroperoxy derivatives in JA biosynthesis pathway (Turner et al. 2002). *LOX2* gene expression was similar between water- and pGM-treated plants at 24 h and repressed at 72 h after the treatment. However, after virus inoculation, this gene was induced in pGM-treated plants. Thus, as observed for *PR-3* and *PAL*, viral contact triggered *LOX2* expression in pGM-treated plants. The presence of the virus may induce LOX accumulation and JA synthesis as already described in mono- and dicotyledon plants in response to the entry of fungal, bacterial and viral pathogens (Wallis and Browse 2002). In *Arabidopsis*, it is well known that *LOX* expression is triggered by exogenous application of JA due to the presence of a positive feedback loop that amplifies JA responses (Hickman et al. 2017). Therefore, we can speculate that CABMV may induce JA levels in passion fruit, consequently inducing LOX expression. However, the pre-treatment with pGM seems to prompt passion fruit plants to over-express *LOX* when challenged by the virus.

The results presented in this study show that pGM treatment strongly suppresses the negative effects of

CABMV on the development of passion fruit plants. Similar effects were previously reported using other inducers in different plants (Truong et al. 2012; Mofidnakhaei et al. 2016), which showed that disease control and recovery directly correlate with increased activity of the antioxidant system of the plant. Acibenzolar-S-methyl (Bion®), which has been able to reduce the severity of the disease caused by passion fruit woodiness virus (PWV) in *P. edulis* plants by 30% at 50 days after inoculation, also activated pathogenesis-related protein gene expression (Parkinson et al. 2015). Our results showed a low viral prevalence and an important relative virus titer reduction in the systematic leaves of the pGM-treated plants at 4 wai. Similar findings were reported by Bernardino et al. (2020) in tobacco plants (*N. tabacum* cv Xanthi) treated with a fungal elicitor and inoculated with tobacco mosaic virus, leading to a decrease in the virus titer in the plants. The activation of plant defense systems by bioagents such as pGM prior to attack by pathogens may prepare the host plant for pathogen invasion, allowing infected plants to develop in a similar manner as healthy plants. This is the first conclusive report demonstrating inducer-mediated resistance against CABMV infection, which results in severe economic losses in passion fruit crops. Our analysis also suggests that in addition to mosaic and leaf deformation parameters, a disease severity index for CABMV in passion fruit plants should consider also developmental parameters affected by virus infection, such as the size and number and area of leaves of the infected plants. With the identification of new resistance-inducing bioagents, ecologically friendly solutions for plant protection may become a reality within a few years.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Informed consent** All authors informed consent.

**Research involving human and animal participants** The research does not involved animal or human.

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**José Leonardo Santos-Jiménez** This research is part of the PhD projects of Jose Leonardo Santos Jiménez devoted to study the peptidogalactomannan (pGM) isolated from *Cladosporium herbarum* as a biostimulator of passion fruit plants during CABMV infection.

**Caroline de Barros Montebianco** This research is part of the PhD project of Caroline de Barros Montebianco devoted to study the ability of pGM in the inducing of plant protection against biotic stress.

**Andreza Henrique Vidal** was a master student at Embrapa Recursos Genéticos e Biotecnologia, EMBRAPA working with passion fruit virus.

**Simone G. Ribeiro** is the head of Plant Virus research group of Embrapa Recursos Genéticos e Biotecnologia, EMBRAPA.

**Eliana Barreto-Bergter** works on fungal cell wall components and is a full professor at Microbiology Institute of Universidade Federal do Rio de Janeiro.

**Maite Freitas Silva Vaslin** This work was carried out in the plant virology working group on biostimulators that mitigate virus disease damages of Dr. Maite Freitas Silva Vaslin at the Virology Department, Microbiology Institute from the Universidade Federal do Rio de Janeiro.

## Supplementary information

**Supplementary Table S1.** Oligonucleotide primer pairs used for RT-qPCR analysis.

| Gene name | Sequences F (1st line) and R (2nd line) (5 '→ 3') | Annealing temperature | Amplicon size |
|---|---|---|---|
| *Peroxidase* 12 (*POD 12*) | GTCTTGGTGGGGTCTTGG<br>CCTGCTTGAACTCTTTCTTG | 58 ºC | 142 bp |
| *Chitinase Class I* (*PR-3*) | CGTCTCTAGTATTCTCGCTG<br>AGCACACGCACGACAGTTG | 55 ºC | 173 bp |
| *Lipoxygenase* 2 (*LOX 2*) | TCTACGCCTGGGGTTACTG<br>TGCCTTTTGGTTTTGATGGAC | 60 ºC | 161 bp |
| *Superoxide dismutase* (*SOD*) | CGTTCTTAATAGCAGTGAGG<br>CAGCAGGATTGAAGTGTGG | 55 ºC | 187 bp |
| *Phenylalanine ammonia lyase* (*PAL*) | CCTGAACTCCGCCACCATGCG<br>GGCCACGACCCTCTCAACTGG | 68 ºC | 93 bp |
| *Ethylene response sensor* (*ERS*) | GTATCTTGTGCTGTTGTGTC<br>CCATCTCCCTGTCAAGTTC | 58 ºC | 95 bp |
| *NADP-dependent isocitrate dehydrogenase* (*NDID*) | GTCGTCACTCTCTCTTTACG<br>TCATTTCATCACCGTCCATC | 55 ºC | 98 bp |
| *Translation elongation factor* 1a-1 (*EF1a1*) | GTTAAGGATTTGAAGCGTGG<br>ATGTGTGATGTGTGGCAGT | 55 ºC | 97 bp |

**Supplementary Table S2.** Effect of distinct concentrations of pGM PWD severity (a) and incidence (b) passion fruit plants along the time.

| **a** | Disease severity % | | | |
|---|---|---|---|---|
| Treatments | 2 wai* | 3 wai | 4 wai | 5 wai |
| CABMV | 93.0 ± 10.7a | 89.2 ± 9.9a | 93.2 ± 12.5a | 94.2 ± 6.6a |
| Water + CABMV | 71.2 ± 20.4b | 78.2 ± 19.6a | 91.5 ± 7.5a | 93.0 ± 6.2a |
| 25 µg.ml$^{-1}$ pGM + CABMV | 86.2 ± 12.9b | 90.2 ± 15.3a | 90.2 ± 16.0a | 92.2 ± 16.0a |
| 50 µg.ml$^{-1}$ pGM + CABMV | 78.7 ± 19.5b | 87.5 ± 10.7a | 92.2 ± 9.9a | 92.2 ± 11.7a |
| 100 µg.ml$^{-1}$ pGM + CABMV | 33.7 ± 44.1b | 50.5 ± 43.8a | 54.7 ± 38.4b | 67.5 ± 24.4b |
| 200 µg.ml$^{-1}$ pGM + CABMV | 67.0 ± 38.0b | 78.5 ± 28.6a | 78.7 ± 28.6ab | 76.5 ± 28.3ab |
| 400 µg.ml$^{-1}$ pGM + CABMV | 65.2 ± 36.1b | 72.0 ± 28.1a | 74.5 ± 28.6ab | 80.2 ± 15.3ab |

| **b** | Disease incidence % | | | |
|---|---|---|---|---|
| Treatments | 2 wai* | 3 wai | 4 wai | 5 wai |
| CABMV | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| Water + CABMV | 90.0 ± 31.6a | 90.0 ± 31.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| 25 µg.ml$^{-1}$ pGM + CABMV | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| 50 µg.ml$^{-1}$ pGM + CABMV | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| 100 µg.ml$^{-1}$ pGM + CABMV | 40.0 ± 51.6b | 60.0 ± 51.0b | 70.0 ± 48.0a | 90.0 ± 31.0a |
| 200 µg.ml$^{-1}$ pGM + CABMV | 80.0 ± 42.1ab | 90.0 ± 31.0a | 90.0 ± 31.0a | 90.0 ± 31.0a |
| 400 µg.ml$^{-1}$ pGM + CABMV | 80.0 ± 42.1ab | 90.0 ± 31.0a | 90.0 ± 31.0a | 100.0 ± 0.0a |

* - weeks after CABMV inoculation

Passion fruit plants were mechanically infected with 1:10 CABMV inoculum 72 hours after treatments.

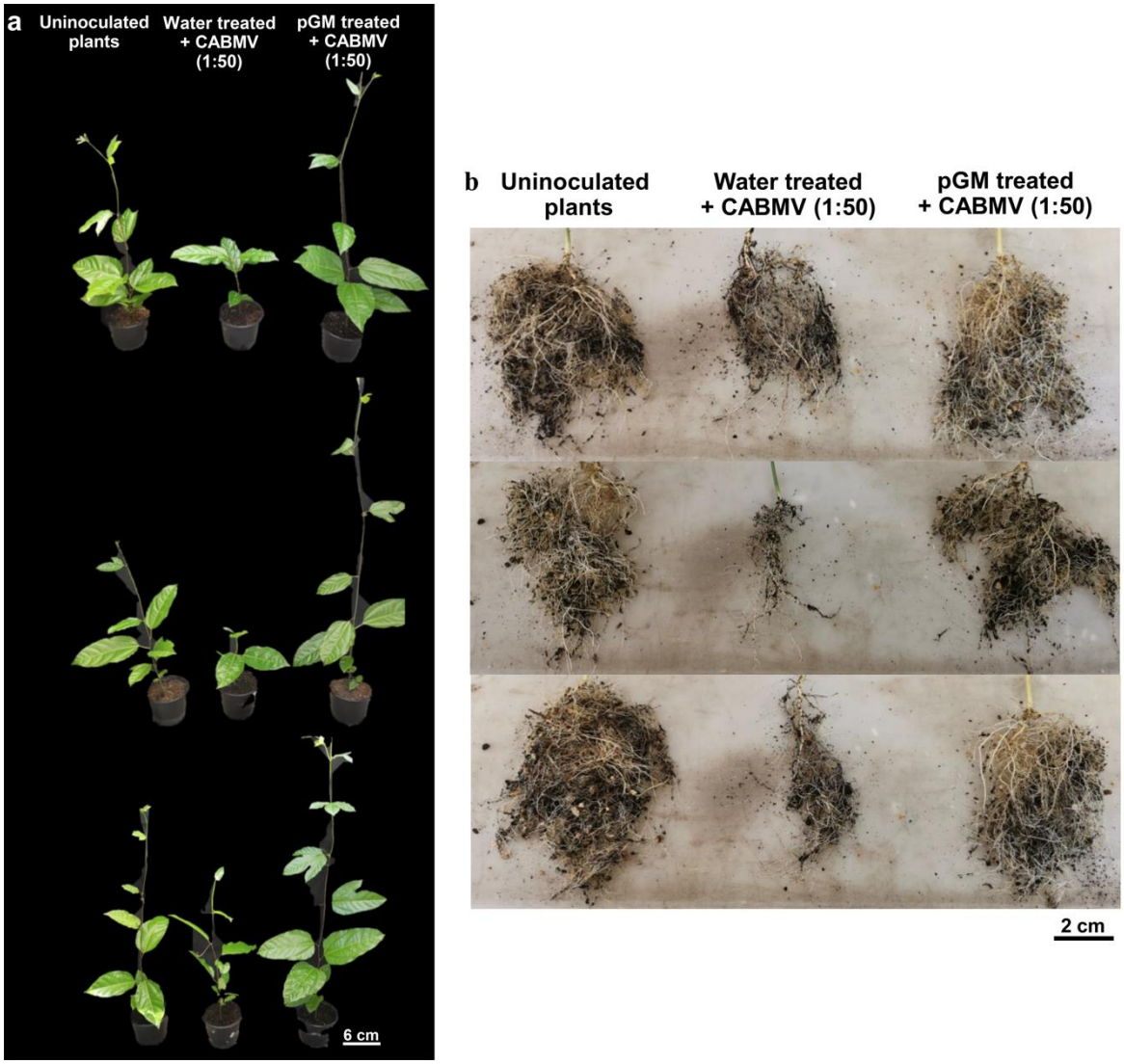
Each value is the means (± standard deviation) of an experiment using 10 plants per treatment.

Different letters in columns indicate significant differences between treatments.

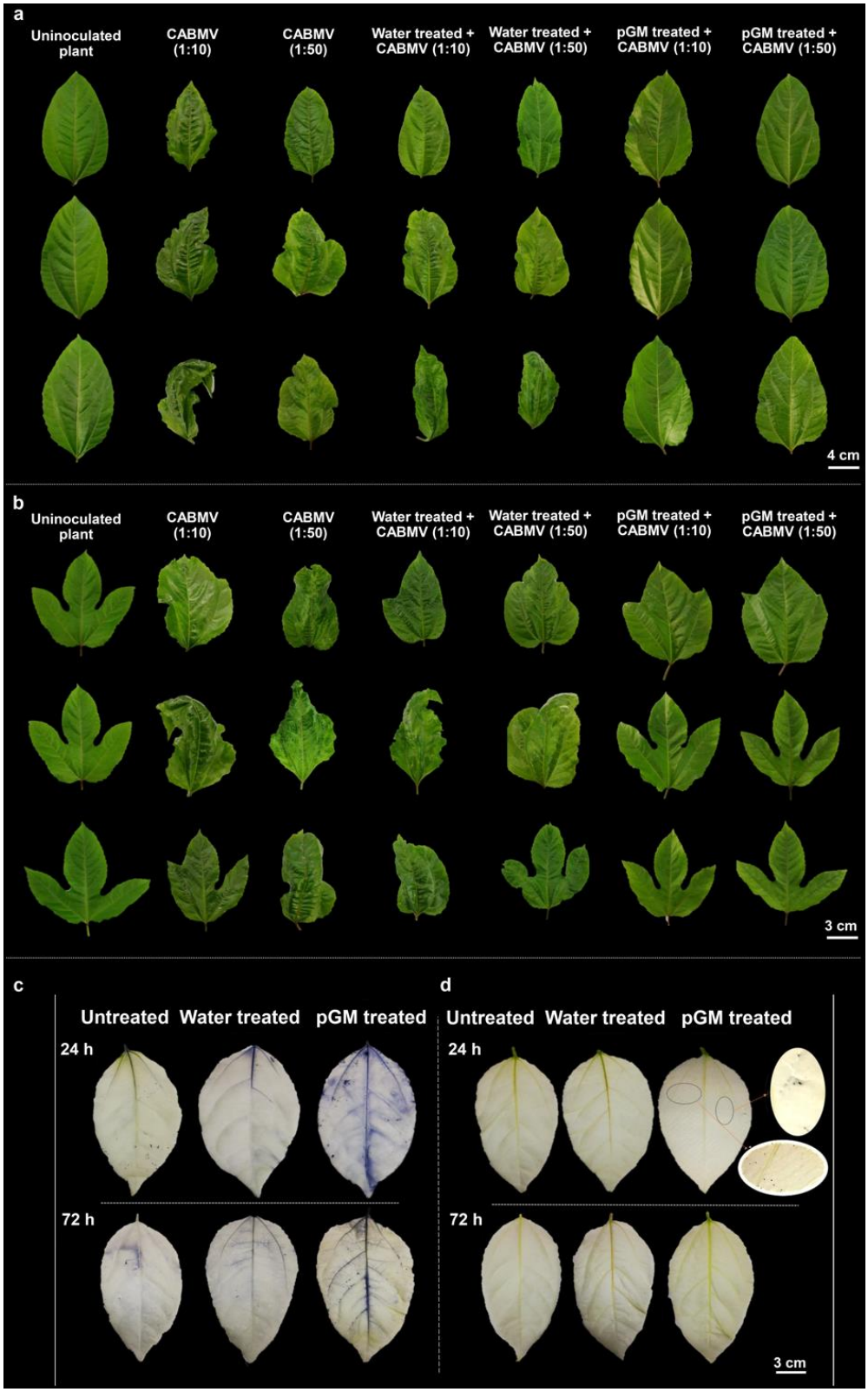
Corresponding results of Kruskal-Wallis. (a) Disease severity at 2 wai: $\chi^2_6 = 16.35$, $p < 0.05$; at 3 wai: $\chi^2_6 = 14.35$, $p < 0.05$; at 4 wai: $\chi^2_6 = 21.16$, $p < 0.01$; at 5 wai $\chi^2_6 = 25.13$, $p < 0.001$.

Corresponding results of One-way ANOVA. (b) Disease incidence at 2 wai: $F_{6,63} = 4.47$, $p < 0.001$; at 3 dai: $F_{6,63} = 3.21$, $p < 0.01$; at 4 wai: $F_{6,63} = 2.00$, $p < 0.05$; at 5 wai $F_{6,63} = 0.83$, $p < 0.05$.

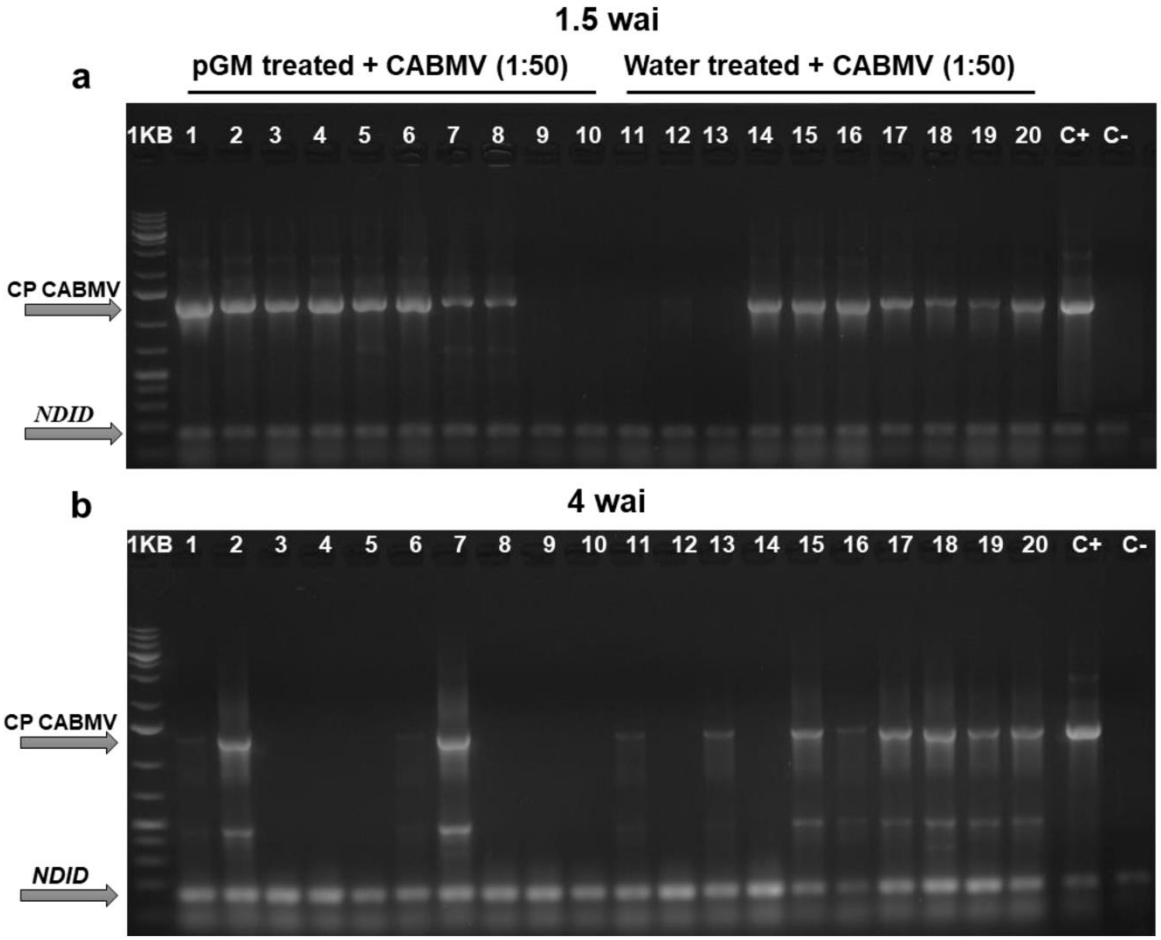
## Supplementary Figures




**Supplementary Figure S1. Development of plants at 5 weeks after viral inoculation**. (**a**) aerial part of the plants and (**b**) plant roots for each treatment. A single CABMV inoculum dilution (1:50) was evaluated. CABMV infection led to a reduction in the development of plants in terms of their size and roots in water-treated CABMV-inoculated plants, while it did not affect development in the 100 µg.ml$^{-1}$ pGM-treated CABMV-inoculated plants.


a
Uninoculated plant
CABMV (1:10)
CABMV (1:50)
Water treated + CABMV (1:10)
Water treated + CABMV (1:50)
pGM treated + CABMV (1:10)
pGM treated + CABMV (1:50)
4 cm
b
Uninoculated plant
CABMV (1:10)
CABMV (1:50)
Water treated + CABMV (1:10)
Water treated + CABMV (1:50)
pGM treated + CABMV (1:10)
pGM treated + CABMV (1:50)
3 cm
c
Untreated
Water treated
pGM treated
24 h
72 h
d
Untreated
Water treated
pGM treated
24 h
72 h
3 cm

**Supplementary Figure S2. The leaf morphology of pGM-treated CABMV-inoculated plants resembled that of healthy uninoculated plants.** Representative leaves of the middle part (**a**) and leaves of the upper part (**b**) of untreated uninoculated healthy plants and of untreated, water- or 100 µg.ml$^{-1}$ pGM-treated CABMV-inoculated passion fruit plants at 5 wai. Two CABMV inoculum dilutions (1:10 and 1:50) were evaluated. Typical symptoms of CABMV infection were observed in leaves from untreated and water-treated CABMV-inoculated plants that were drastically affected by viral infection, showing an abnormal developmental pattern. Leaves of pGM-treated/CABMV-inoculated plants, however, showed almost normal development patterns. Histochemical assay for $O^{2-}$ by NBT (**c**) and of $H_2O_2$ by DAB (**d**) in *P. edulis* leaves treated with water or 100 µg.ml$^{-1}$ pGM at 24 and 72 h after treatment.




**Supplementary Figure S3. CABMV detection by RT-PCR.** Ten samples of pGM-treated/CABMV inoculated (lanes 1-10) and 10 of control (water-treated)/CABMV inoculated plants (lanes 11-20) at 1.5 (**a**) and 4 wai (**b**) were assayed by RT-PCR for amplification of CABMV CP and the constitutive gene NDID. Fragments amplified by this multiplex RT-PCR assay were subjected to 0.8% agarose gel electrophoresis.

## 5.2. Capítulo II. Passion fruit treatment with biostimulants induces defense-related and phytohormone-associated genes.

(Trabalho submetido no *Plant Gene*)

Plant Gene

# Passion fruit treatment with biostimulants induces defense-related and phytohormone-associated genes

--Manuscript Draft--

| | |
|---|---|
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| Article Type: | Research paper |
| Keywords: | humic acid; bacterial consortia; peptidogalactomannan; biostimulation; RT-qPCR |
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| Abstract: | Biostimulation is a relevant technology to support the ecological intensification of agriculture, especially when biotic or abiotic factors keep plants under conditions of permanent stress that impair crop productivity. Biostimulants promote molecular, biochemical, physiological, and morphological changes in the plant, leading to better adaptation to adverse conditions and increasing growth and yields. This work evaluated how the combined application of humic acids and a synthetic bacterial consortium (sym) or humic acids (HA) plus sym suspension and peptidogalactomannan (pGM) of a cell wall fungus affected some defense-related and phytohormone gene expression profiles in passion fruit plants under greenhouse and field conditions. Both treatments induced the expression of all defense-related genes evaluated 24 h after treatment, as shown by RT–qPCR assays. Interestingly, both auxin and gibberellin precursor mRNAs were observed to be upregulated 16 weeks after the treatments, which was associated with an increase in shoot and root biomass and the number of leaves. These results may explain how these treatments can improve the plant growth reported in the literature and elucidate the putative effect on plant defense mechanisms, which can induce plants to have stronger responses against biotic stress coupled with growth traits. Our results suggest that disseminating these technologies may improve passion fruit productivity in an ecologically friendly manner. |

# Passion fruit treatment with biostimulants induces defense-related and phytohormone-associated genes

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## ABSTRACT

Biostimulation is a relevant technology to support the ecological intensification of agriculture, especially when biotic or abiotic factors keep plants under conditions of permanent stress that impair crop productivity. Biostimulants promote molecular, biochemical, physiological, and morphological changes in the plant, leading to better adaptation to adverse conditions and increasing growth and yields. This work evaluated how the combined application of humic acids and a synthetic bacterial consortium (sym) or humic acids (HA) plus sym suspension and peptidogalactomannan (pGM) of a cell wall fungus affected some defense-related and phytohormone gene expression profiles in passion fruit plants under greenhouse and field conditions. Both treatments induced the expression of all defense-related genes evaluated 24 h after treatment, as shown by RT–qPCR assays. Interestingly, both auxin and gibberellin precursor mRNAs were observed to be upregulated 16 weeks after the treatments, which was associated with an increase in shoot and root biomass and the number of leaves. These results may explain how these treatments can improve the plant growth reported in the literature and elucidate the putative effect on plant defense mechanisms, which can induce plants to have stronger responses against biotic stress coupled with growth traits. Our results suggest that disseminating these technologies may improve passion fruit productivity in an ecologically friendly manner.

**Keywords:** humic acid; bacterial consortia; peptidogalactomannan; biostimulation; RT–qPCR

## 1. Introduction

Passion fruits belong to the genus *Passiflora* L. and are the most representative and abundant genus of the family Passifloraceae, which includes approximately 500 species, with *Passiflora edulis* predominating for its medicinal and nutritional significance (He et al., 2020). The most planted species are yellow passion fruit (*Passiflora edulis* f. flavicarpa), purple passion fruit (*Passiflora edulis*), and fragrant gradanilla (*Passiflora alata*) (Ribeiro et al., 2019). The center of origin of passion fruit is South America, but it is widely cultivated in subtropical and tropical areas worldwide (Ortiz et al., 2012; Silva et al., 2014).

Despite the severe phytosanitary problems in passion fruit due to viral diseases, such as Cowpea aphid-borne mosaic virus (CABMV) reducing yield and lifespan, no efficient chemical controls are available. Infected plants produce fewer fruits, and fruits are small, woody and deformed, becoming undesirable for the market (Nascimento et al., 2006; Santos-Jiménez et al., 2021). Other important pests and diseases include caterpillars (*Dione juno juno* and *Agraulis vanillae vanillae*), bugs (*Diactor bilineatus*, *Holhymenia clavigera* and *H. histrio*), anthracnose, scab, sptoria, powdery mildew, fusariosis, and bacterial disease: *Xanthomonas campestris pv. passiflorae* and *Ralstonia solanacearum* (bacterial wilt*)*. The use of natural organic matter and beneficial microorganisms that trigger physiological responses that improve nutritional efficiency and stress tolerance stimulate metabolism and favor plant performance without impacting planted areas and/or animal/human alimentation as a biological solution (Van Oosten et al., 2017). Biostimulating agents with positive results in mitigation of all these issues include microorganisms, fractions of humified organic matter, protein hydrolysates, amino acids, and other nitrogenous compounds, oligosaccharides, inorganic compounds, green algae extracts and botanical products, and fungal cell wall molecules (Calvo et al., 2014; Du Jardin, 2015; Nardi et al., 2016; Mattos et al., 2018; Bernardino et al., 2020; Santos-Jiménez et al., 2021).

Beneficial bacteria can boost plant growth and development in several ways. Direct promotion involves biofertilization effects based on increased nutrient availability through nitrogen fixation, phosphorus solubilization, and iron absorption. Biostimulation also promotes the modulation of phytohormones such as auxins, ethylene, and gibberellins. Concomitant induction of plant resistance mechanisms is frequently observed and may offer extra indirect positive effects (Pathania et al., 2020). Two recent examples of these positive effects are a significant decrease in the incidence of tomato spotted wilt virus (TSWV) in *Solanum*

*lycopersicum*-infected plants after the application of *Bacillus amyloliquefaciens* strain MBI600 (Beris et al., 2018) and an increase in dry biomass of the aerial parts and roots in *Phaseolus vulgaris* under greenhouse conditions by intercropping *Pantoea* sp. and *Erwinia* sp. plant growth-promoting bacteria (Emmer et al., 2021).

In other approaches, the use of humic acids has stimulated plant growth and systemic defense against stress. For example, in maize and lettuce plants, humic acids induced the metabolism of phenylpropanoids, as confirmed by the induction of *phenylalanine ammonia-lyase* expression (Schiavon et al., 2010; Hernandez et al., 2015). Furthermore, the supramolecular structure of humic acids is thought to determine their biostimulant action, leading to proliferation, elongation and changes in root architecture, resulting in increased absorption and transport of ions and stimulation of primary metabolism, regulation of molecular processes and induction of synthesis of secondary metabolites, which results in the promotion of systemic responses to stress due to the "chemical priming" effect (Piedade et al., 2017; Shah et al., 2018; Aguiar et al., 2018; Canellas et al., 2020). These beneficial effects were also observed in passion fruit plants, where spraying humic acids on the leaves induced an increase in the biomass of *P. edulis* seedlings (Cavalcante et al., 2013). In another study, the use of concomitant humic substances and nitrogen led to higher concentrations of nitrogen in the leaves, with an increase in stem diameter and yields of the treated plants (Silva et al., 2016).

Our group observed that simultaneous treatment of tomato plants with humic acids (HA) and the plant growth-promoting bacteria (PGPB) *Herbaspirillum seropedicae* acted as an elicitor, activating plant defense mechanisms against *Phytophthora infestans* and *Xanthomonas euvesicatoria* infection and reducing leaf symptoms and the incidence of bacterial spots (Olivares et al., 2015; da Silva et al., 2021). However, the genes modulated by HA plus bacteria have not been determined.

Previous studies by the group also showed that foliar application of peptidogalactomannan (pGM) from *Cladosporium herbarum* induces the expression of *pathogen-related 1, 2, 3* and *5* and *peroxidase* genes in BY-2 tobacco cells and passion fruit plants (Mattos et al., 2018; Santos-Jiménez et al., 2021). Furthermore, the strong induction of some of these defense-related genes was associated with mitigation of tobacco mosaic virus (TMV) and cowpea aphid born mosaic virus (CABMV) symptoms in tobacco and passion fruit, respectively.

This work aimed to evaluate whether treatment with HA in association with a synthetic bacterial consortium (sym) with or without *C. herbarum* peptidogalactomannan (pGM) can induce defense-related and phytohormone pathway genes in passion fruit plants, highlighting the pathways associated with defense triggered and growing stimulation responses shown by treated plants.

## 2. Materials and methods

### 2.1. Plant material

Experiments were carried out in a greenhouse at the University Federal of Rio de Janeiro under field conditions in the Campos dos Goytacazes, Rio de Janeiro state. Hybrid H09-110/111 purple passion fruit plants (*P. edulis*) developed by EMBRAPA were grown in plant substrate (1/3 vermiculite, 1/3 earthworm humus and 1/3 washed sand) under tropical area natural light and temperature conditions.

### 2.2. Microorganisms

The synthetic bacteria consortium (sym) suspension was obtained by a combination of the endophytic diazotrophic *Herbaspirillum seropedicae* strain HRC 54 (Olivares et al. 2015), P-solubilizing chitinolytic *Serratia marcescens* strain UENF 22GI (Mateolli et al., 2018) and *Bacillus* sp. strain 77 obtained from the bacterial culture collection of the Laboratório de Biologia Celular e Tecidual (LBCT-UENF). The bacterial suspension was prepared by growing the bacterial strains in glass flasks containing DIGYS liquid medium under a rotatory shaker at 30 °C for 36 h at 150 rpm. Bacterial cells were centrifuged at 4,000 *g* for 15 min, and the pellet was resuspended in sterilized saline solution (NaCl, 0.85%) and adjusted spectrophotometrically to an optical density (O. D) at 590 nm of 1.2, equivalent to $5 \times 10^8$ cells. $mL^{-1}$.

The CBS 121621 strain of the fungus *Cladosporium herbarum* was made available by Dr. Joseph Guarro from the Microbiology Unit, Faculty of Medicine, Institute of Advanced Studies, Spain, and was grown in Erlenmeyer flasks containing 1 $L^{-1}$ potato dextrose medium (PDB) for 7 days under a rotatory shaker at room temperature.

### 2.3. Peptidogalactomannan (pGM) extraction

*Cladosporium herbarum* growth and peptidogalactomannan (pGM) extraction were performed according to Haido et al. (1998) and Mattos (2011).

### 2.4. Extraction and characterization of humic acids (HA)

Extraction and characterization of humic substances were carried out following the methodology described by Baía et al. (2020).

### 2.5. Inoculant preparation

pGM was prepared by diluting 100 $\mu g^{-1}$ in tap water per $mL^{-1}$. The HA plus sym was prepared by diluting 200 $mL^{-1}$ bacteria in 800 $mL^{-1}$ humic acids (4 mM C $L^{-1}$) at pH 6.8-7.0 for a final bacterial concentration of 1 $\times 10^8$ cells. $mL^{-1}$.

### 2.6. Treatments

Two-month-old H09-110/11 passion fruit plants were subjected to one of the following three foliar spray treatments: 100 $\mu g.mL^{-1}$ pGM resuspended in tap water, humic acid + bacteria complex (HA + sym), or 100 $\mu g.mL^{-1}$ pGM resuspended in HA + sym at the 4–5 true leaf stage. Treatments were performed with a costal manual sprayer (Jacto - XP). Control plants were treated with tap water foliar spray.

### 2.7. Field experiment

Two months after sowing, the seedlings were transplanted to the field and placed in an espalier on 12 wires fixed at 2.0 m from the ground. In each line, wooden posts were fixed every 6.0 m. Part of the plants was transferred to a greenhouse. A field experiment was carried out on an Ultisol located in the Lagoa de Cima district of Campos dos Goytacazes, Rio de Janeiro, Brazil (21°46'19"S  41°30'56"W; altitude 14 m). The experiment was set up in a completely randomized block design with four replications, one line per block, containing five plants per plot. Holes (0.40 x 0.40 x 0.40 m) were drilled in rows spaced 3.5 m apart with six plants per plot. The holes were fertilized with 20 L of cattle manure and 100 g of dolomitic limestone one month before transplanting the seedlings. Treatments were imposed in the field one day after transplanting using one application of isolated HA from vermicompost at 4 mmol C $L^{-1}$ plus sym (*H. seropedicae* spp., *Serratia* spp. *Bulkholderia* spp.) at a final concentration of $10^9$ cells $mL^{-1}$ of each strain described below. Cover fertilization was carried out twice a month with superficial application of 2 liters of cattle manure around the plant.

### 2.8. Greenhouse experiment

Part of the two months after sowing, seedlings were maintained in plastic bags, grouped in boxes, and sprayed with each treatment. Then, these plants were taken to the Laboratory of Plant Molecular Virology from UFRJ (Rio de Janeiro, RJ) and kept in a greenhouse. Twenty-four hours after treatment (hat), leaf samples were collected for gene expression analysis, and the plants were transferred to 9-$L^{-1}$ plastic pots containing a substrate for further biomass analysis. The experiment in a greenhouse was carried out in a completely randomized design with five plants per treatment.

### 2.9. Real-time RT–PCR

Expression of the defense-related genes *pathogenesis-related protein 3* (*PR-3), superoxide dismutase (SOD), peroxidase 12 (POD12), lipoxygenase 2* (*LOX2)* (Munhoz, 2013) and *phenylalanine ammonia-lyase (PAL)* (Santos-Jiménez et al., 2021) was evaluated in leaves 24 hat (hours after treatment) (under greenhouse conditions) and 16 wat (weeks after treatment) (under field conditions). Additionally, the expression of two genes involved in the phytohormone signaling pathway, *auxin* (*AUX)* and *gibberellin* (*GA*) (Chen et al., 2021), was evaluated at 16 wat (under field conditions). Total RNA was extracted with TRIzol® reagent (Thermo Fisher Scientific). cDNA synthesis and qPCR conditions followed Santos-Jiménez et al. (2021). *NDID* (NADP-*dependent isocitrate dehydrogenase*), *EF1a1* (*translation elongation Factor* 1a-1) and *ERS* (*ethylene response sensor*) (Munhoz, 2013) were used as housekeeping genes for qPCR normalization. Three independent biological pools composed of 5 plants from each treatment were performed for each point evaluated in technical triplicates for the qPCRs using the Applied Biosystems 7500 Fast Real-Time PCR thermocycler (Thermo Fisher Scientific). The $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmitgen (2001) was used to evaluate the Ct values, and the results were expressed as relative expression levels.

### 2.10. Evaluation of morphological characters

To evaluate the effects of the treatments on plant development under greenhouse conditions, the fresh and dry weights of the aerial parts and roots of five plants per treatment grown in a greenhouse were evaluated at 7 weeks after treatment (wat). Plant shoots and root systems were cut and placed in individual paper bags, the fresh mass weight was obtained, and plant parts were oven-dried at 60 °C for 72 h for dry weight determination. Weights of the aerial

part and root system were determined using an accurate electronic scale (Bioprecisa – JA3003N).

Under field conditions, the number of leaves and number of fruits were evaluated at 16 and 36 wat through individual counting of plants per treatment, respectively.

### 2.11. Statistical analysis

To verify whether the different means observed between the treatments were statistically significant, one-way ANOVA was used to analyze the data obtained in the morphological characters such as fresh and dry weights and the numbers of leaves and fruits, while for the analysis of gene expression, two-way ANOVA was used. All ANOVAs were followed by Bonferroni post hoc tests ($p$ <0.05).

## 3. Results

### 3.1. Passion fruit defense-related gene expression in hybrid H09-110/111 under different treatments

We first analyzed whether treatment with pGM, HA + sym, and their combination interfered with defense-related gene expression in the first hours after elicitation (under greenhouse contitions). *PR-3* and *PAL* transcripts, which are considered systemic acquired resistance (SAR) markers, were induced 24 hours after all treatments (Figure 1). Strong *PR-3* induction was observed (17.8 times) after pGM + (HA + sym) combination treatment compared to water treatment. In pGM-treated plants, this gene was strongly induced, but minor induction was observed with the pGM + (HA + sym) combination. In HA + sym-treated plants, 2-fold induction was observed (Figure 1a). Therefore, all the treatments induced *PR3* expression, generating precociously activated SAR responses in passion fruit plants.

The expression levels of *PAL*, which encodes the key enzyme for phenylpropanoid pathway activation, increased almost 7.5, 4.3 and 3.1 times with the pGM, HA + sym and pGM + (HA + sym) treatments, respectively, compared with the water control (Figure 1b). Thus, induction of *PAL* mRNA was already associated with pGM and HA + sym treatments. However, the combination of pGM + (HA + sym) here showed minor stimulation of *PAL* expression compared with the isolated treatments, showing the absence of synergic effects and even a decrease by the association of the three biostimulants.

Genes involved in oxidative stress were evaluated. Overexpression of *POD12* by 8.3- and 3.7-fold was observed with pGM and pGM + (HA + sym), respectively, while with HA +

sym treatment, no induction was observed (Figure 1c). HA + sym treatment alone or in combination with pGM did not induce *SOD* expression; its expression was induced only in pGM-treated (6.9 times) plants (Figure 1d).

*LOX2,* a gene involved in various aspects of plant physiology, such as growth and development as well as pest resistance, showed significant differential expression with pGM + (HA + sym) treatment compared with water treatment, with two-times higher expression with the former. In contrast, in pGM-treated, an induction of 4.7 times was observed. However, *LOX2* expression was repressed (1.8-fold) in HA + sym-treated(Figure 1e) ($F_{3,160}$ = 413.1 and $p < 0.001$).

When examining all these genes (under field conditions) at several weeks after treatments (16 wat), no significant differences between treatments and the control were observed, suggesting that all the treatments induced an efficient defense response a few hours after elicitor contact, which may activate an important defense pathway. However, the genes involved in activation of these pathway were not overexpressed later ($p > 0.05$).

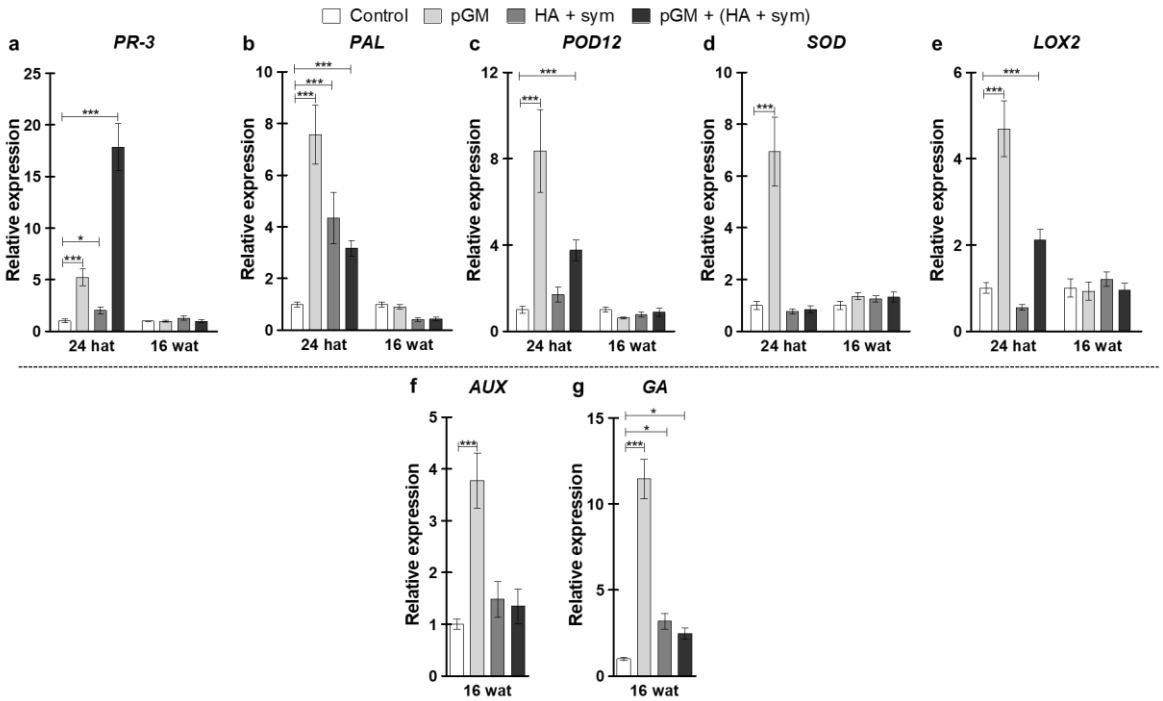


**Figure 1. RT–qPCR detection of the relative expression levels of defense-related (a) *PR-3*, (b) *PAL*, (c) *POD12*, (d) *SOD*, (e) *LOX2* and phytohormone pathways (f) *AUX*, and (g) *GA* gene transcripts at 24 h (hat) and 16 weeks (wat) after pGM, HA+ sym, and pGM + (HA+ sym) treatments relative to those with water treatment (Control).** Ct values were normalized using the combination of reference genes with *EF1a1*, *NDID* and *ERS*. Values represent the average of mRNA transcripts between the control and treatments with three

technical replicates from three biologically independent replicates of each treatment. Error bars represent the standard deviation. * and *** represent significant differences at $p < 0.05$ and $p < 0.001$, respectively, when comparing Control vs. treatments for each gene using two-way ANOVA followed by Bonferroni post hoc test.

### 3.2. Levels of mRNA from gibberellic acid precursors are upregulated by treatments

Under field conditions at 16 wat, genes related to the phytohormone pathway were expressed compared to gene expression in the control (Figure 1f, g) ($p < 0.001$). The gene encoding *AUX* was expressed in pGM-treated plants (3.7 times), while with other treatments, no significant difference with respect to the water control was found (Figure 1f). With *GA*, we observed its expression with all treatments, which was higher with the pGM treatment (11.4 times) (Figure 1g).

### 3.3. All the treatments induced an increase in shoot and root biomass and in the numbers of leaves and fruits

As the HA and sym used here are well-known plant-growth promoters, the biomass of aerial parts and roots was evaluated. For this experiment, passion fruit plants were maintained under greenhouse conditions after treatment. Significant differences were observed in the fresh and/or dry weights of the aerial part and root system of pGM, HA + sym, and pGM + (HA+ sym) treated plants compared to the water control at 7 wat. Regarding the fresh weight of the aerial part, pGM alone, HA + sym and pGM + (HA + sym) induced almost equal biomass (Figure 2a). Stronger biomass induction was observed for root fresh weight, where once again, all the treatments induced similar biomass increases (fresh weight aerial part, $F_{3,16} = 12.37$, $p < 0.001$; fresh weight root, $F_{3,16} = 7.96$, $p < 0.005$). Any synergic effect was observed upon combining the three elicitors in pGM + (HA + sym) treatment.

Although no significant differences were observed between all treatments and the control treatment in the dry weight of the aerial parts (Figure 2b) (dry weight aerial part, $F_{3,16} = 2.696$, $p > 0.05$), significant increases of approximately 32% were observed in root dry biomass after all the treatments compared to the control treatment (Figure 2d) (dry weight root, $F_{3,16} = 5.478$, $p < 0.01$). Figure 2e shows the appearance of the roots after the treatments.

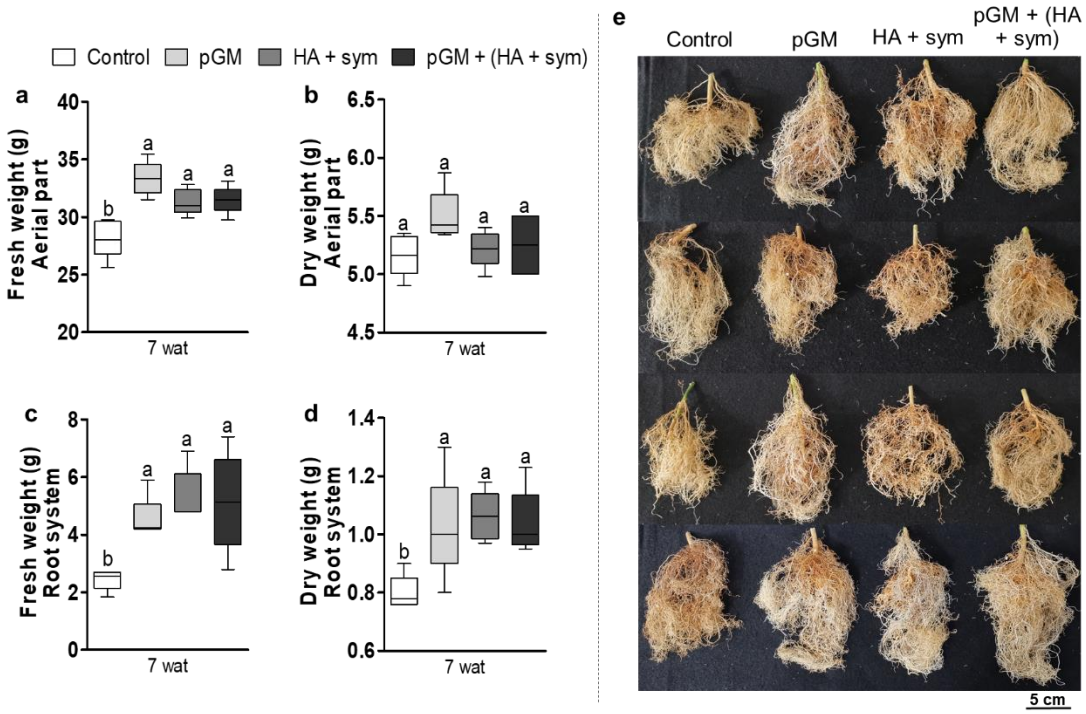


**Figure 2. Analysis of biomass in passion fruit under greenhouse conditions.** Fresh and dry masses of the aerial part (a, b), the root system (c, d) and the appearance of roots (e) in passion fruit in the independent experiment using 5 plants per treatment under greenhouse conditions at 7 weeks after treatment (wat). Treatments consisted of water (control), pGM, HA + sym, and pGM + (HA + sym). Bars represent the averages of the plants per treatment, and different letters represent a significant difference at $p$ <0.05. The borders in the boxes (lower and upper) represent the first and third quartiles, and the average is represented by the horizontal line within the box. Whiskers extend to the largest and smallest data points in the interquartile range.

The number of leaves per plant was evaluated under field conditions at 16 wat. An increase in the number of leaves of the plants submitted to all treatments was observed, with an increase of approximately 38.7% (Figure 3) (number of leaves, $F_{3,58} = 5.225$, $p$ <0.05). A similar result was observed for the number of fruits, with a significant increase greater than 100% among all treatments with respect to the control treatment (Figure 4) (number of fruits, $F_{3,79} = 8.6$, $p$ <0.05).

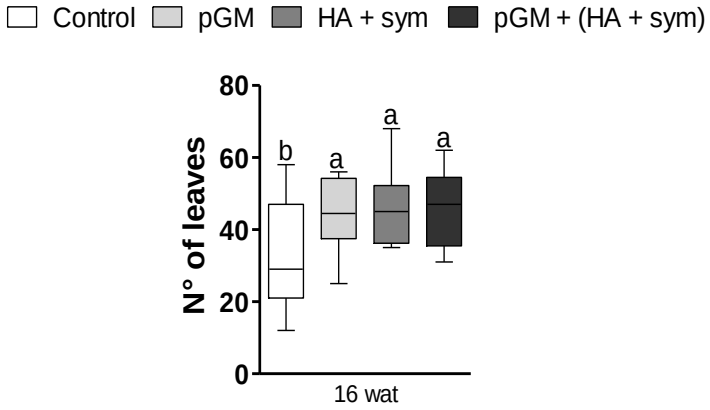


**Figure 3. Analysis of the number of leaves in passion fruit under field conditions.** Number of leaves of an independent experiment using 20 plants per treatment in field conditions at 16 wat. Treatments consisted of water (control), pGM, HA + sym, and pGM + (HA + sym). Bars represent the averages of the plants per treatment each, and different letters represent a significant difference at $p$ <0.05. The borders in the boxes (lower and upper) represent the first and third quartiles, the average being represented by the horizontal line within the box. Whiskers extend to the largest and smallest data points in the interquartile range.

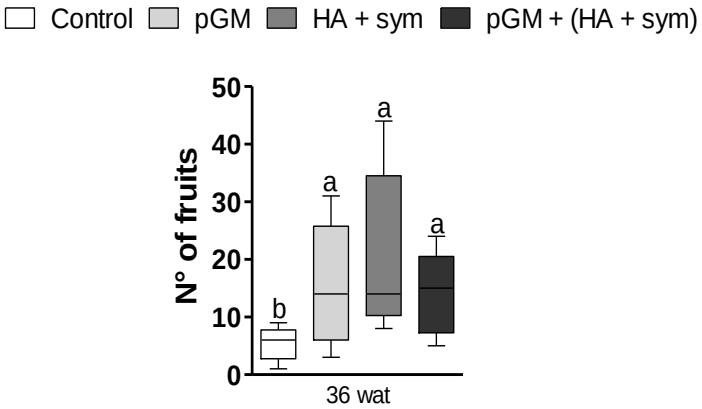


**Figure 4. Analysis of the number of fruits in passion fruit under field conditions.** The number of fruits in an independent experiment using 20 plants per treatment under field conditions at 36 wat. Treatments consisted of water (control), pGM, (HA + sym, and pGM + (HA + sym). Bars represent the averages of the plants per treatment, and different letters represent a significant difference at $p$ <0.05. The borders in the boxes (lower and upper) represent the first and third quartiles, and the average is represented by the horizontal line within the box. Whiskers extend to the largest and smallest data points in the interquartile range.

No differences were observed between the biostimulant treatments, indicating that the combination of pGM + (HA + sym) seems to improve the biomass, number of leaves, and number of fruits similarly to pGM or HA + sym alone.

## 4. Discussion

As sessile organisms, plants are constantly exposed to unfavorable environmental conditions and subject to biotic and abiotic stress. Ecologically friendly technologies, such as the exogenous application of biostimulants, can be studied and evaluated to help plants control or mitigate these problems. However, the understanding of the molecular pathway activated by distinct biostimulants is still very incipient. Therefore, the present study investigated how genes from distinct pathways were associated with gene defense and stress responses and plant growth responses to treatment in association of HA and sym alone or with the addition of a pGM extracted from cell wall fungi.

In association with the sym, HA induced the expression of all the genes studied, with less *SOD*. When pGM was added to HA plus sym, a synergistic effect was observed, with transcriptional upregulation of *PR-3, POD12* and *LOX2* compared to the effects of treatment with HA plus sym at 24 hat (Figure 1). However, compared to the pGM effect alone, some genes were less expressed in the HA + sym or pGM + (HA + sym) group. *PR-3* was a unique gene and was more highly expressed when plants were treated with the three biostimulating agents simultaneously. *PR-3* is an important marker of SAR induction, and the strength of this gene indicates that in addition to a growth-promoting effect, treatment with HA plus sym may induce biotic stress resistance. Another important indication of activated biotic stress resistance in the treated plants is upregulation of the *PAL* and *POD* genes. Both PAL and POD enzymes are associated with defense mechanisms in plants against biotic and abiotic stresses (Almagro et al., 2009). As the key enzyme of the phenylpropanoid pathway, PAL activation can modulate salicylic acid (SA) and several secondary metabolites acting in pathogen defense. A macroalgae (*Ascophyllum nodosum*) used as a biostimulant alone and in conjunction with chitosan led to an increase in the expression of the *TaGlu2, TaPR1.1, TaPR2* and *TaPR3* genes and of the PAL enzyme in *Triticum sativum* seedlings (Gunupuru et al., 2019). *Ascophyllum nodosum* seems to trigger defense mechanisms in plants, possibly due to its complex polysaccharides, such as alginates and fucans (Mercier et al., 2001; Shukla et al., 2016). Application of chitosan to the leaves of potato and tomato plants infected by *Meloidogyne incognita* or *Phytophthora infestans* led to the induction of some defense enzymes, such as lipoxygenase, β-1,3-glucanase and chitinase (Vasyukova et al., 2001). In another approach, *Daucus carota* plants treated with *Ascophyllum nodosum* extracts under greenhouse conditions showed an increase in defense-

associated genes such as *PAL, NPR-1, PR-1, PR-5, chalcone synthase, lipid transfer protein* and *chitinase* and a significant decrease in the incidence of diseases caused by *Botrytis cinerea* and *Alternaria radicina* fungi (Jayaraj et al., 2008). A biostimulant extracted from collagen-derived protein hydrolysate (APR), used as a preventive treatment, prepares plants for reactive oxygen species (ROS), positively regulating some genes involved in oxidative stress, such as *superoxide dismutase 1* (Trevisan et al., 2019).

The treatments used in this work induced upregulation of defense-related genes including *PR-3, PAL, POD12, SOD* and *LOX2* in the first hours after treatment (24 hat). However, this upregulation was not sustained at the later time of evaluation (16 wat) (Figure 1). At this time point, plants were starting the flowering stage. At this stage, the literature shows that plants reduce their defense mechanisms to favor flowering. Wang et al. (2017) observed that SOD was suppressed at the flowering stage in rice plants under drought stress. A transcriptional analysis of potential regulatory networks and genes associated with *Lemna gibba* flowering showed that several cell wall genes, including *PAL*, which is involved in lignin biosynthesis, are downregulated during this stage (Fu et al., 2020). Our results suggested that the transcription of defense genes declined in the initial flowering stage, while genes involved in the phytohormone pathway were upregulated, which can facilitate floral development.

On the other hand, the auxin and gibberellin pathways were activated by all the treatments at 16 wat (Figure 1f, g). *AUX* was upregulated in pGM-treated plants, while *GA* was upregulated with all treatments compared to the control treatment. Phytohormones such as AUX and GA can influence different growth processes and responses to biotic or abiotic stresses, but among their main roles are the regulation of physiological processes and plant development (Durbak et al., 2012; Berens, 2016). GA has a fundamental role in regulating the development, flowering, and senescence of plants (Shu et al., 2018). In response to the bioinoculant treatments in this work, gibberellin precursor mRNA increased, showing a putative positive effect on passion fruit plant development.

Beneficial plant growth-promoting bacteria (PGPB) and fungi applied to plants can lead to changes in auxins, gibberellins and cytokinins, demonstrating that their use as biostimulants can lead to increased defense against abiotic stresses, facilitating the availability and absorption of nutrients and promoting increased plant productivity (Rouphael et al., 2015; Saia et al., 2020). The use of biostimulants in plants has already been reported to facilitate the development

of root systems, increasing their biomass due to the regulation of auxins, gibberellins and/or cytokinins (Rouphael et al., 2015; López-Bucio et al., 2015). Here, we observed that all treatments significantly stimulated the biomass of the root system in passion fruit plants under greenhouse conditions compared to the control (Figure 2). Trevisan et al. (2017) observed that treatment with the collagen-derived protein hydrolyzed biostimulant in *Zea mays* seedlings under abiotic stress in a climatic chamber led to significant increases in the dry biomass of the radicular system and the root/shoot ratio. In a previous study, pGM spraying was shown to improve the root and aerial part biomass of passion fruit plants under virus biotic stress in greenhouse conditions (Santos-Jiménez et al. 2021). Previous studies have shown that treatment with humic substances improves root development and increases tolerance to environmental stresses such as drought (Colombo et al., 2015). In another assay, HA treatment of onion plants significantly increased the root weight (Bettoni et al., 2016). In contrast, studies carried out on *Lycopersicon esculentum* treated with humic acids under field conditions did not showsignificant increases in plant dry biomass, fruit production or nutrient uptake by the plants (Hartz and Bottoms, 2010). Here, under field conditions, we observed that the leaf and fruit numbers of passion fruit-treated plants significantly improved with all treatments (Figures 3 and 4).

Biostimulants that promote growth cannot be considered fertilizers, as they do not directly provide nutrients to plants. However, they significantly improve root nutrient uptake and water absorption, prompting the plant to better use or uptake nutrients that are already in the soil. This improvement in nutrient utilization by plants induces morphological changes in root architecture (Du Jardin, 2015). Biostimulants promote biological effects on plants, improving antioxidant activities and improving the development of the root system, leaves, flowering, and fruiting induction (Yakhin et al., 2017).

## 5. Conclusions

Our results indicate that HA plus sym or HA plus sym plus pGM together induce biotic stress pathways and auxin and gibberellin phytohormones, which can explain the productivity increase observed with these treatments. Thus, these biostimulant products may represent a promising tool for both the improvement of plant defense and development in a sustainable manner.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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## 5.3. Capítulo III. Induction of tolerance against cowpea aphid-borne mosaic virus (CABMV) in different genotypes of passion fruit in organic production system

(Trabalho a ser submetido no *Plant Science*)

# Induction of tolerance against cowpea aphid-borne mosaic virus in different genotypes of passion fruit in organic production system

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## Highlights

- CABMV suppresses defense-related and phytohormones-associated genes in passion fruit plants.
- Peptidogalactomannan treatment induces defense genes and phytohormones in different passion fruit genotypes, keeping them increased over time in CABMV-infected passion fruit plants.
- Peptidogalactomannan treatment mitigates damage caused by CABMV-infection on passion fruit development and productivity under greenhouse and field conditions.
- Peptidogalactomannan treatment reduces the CABMV relative accumulation in young leaves of passion fruit plants.

## ABSTRACT

The productivity of passion fruit (*Passiflora edulis*) is drastically affected by infection with cowpea aphid-borne mosaic virus (CABMV), an aphid transmitted potyvirus. This disease causes decreased productivity and deformation of the fruits and is associated with the occurrence of leaf mosaic and deformation symptoms, accompanied by blisters, a marked reduction in plant development and productivity. This study aimed to evaluate the eliciting effect of peptidogalactomannan (pGM) on the mitigation of damage and losses in yield caused by CABMV in different genotypes of passion fruit under greenhouse and field conditions. In an organic farming production system, the plants were mechanically inoculated with the CABMV 72 h after receiving pGM treatment. The experiment was carried out in a randomized block design in a split plot scheme. pGM-treatment induced the expression of 5 defense-related genes before and after CABMV-inoculum, and also led to the expression of genes asociated to phytohormones. pGM-treated plants were able to respond efficiently to overcome CABMV-associated damage in development, such as height, number of leaves, flowers, and in

productivity parameters, such as numbers of fruit. Furthermore, CABMV relative accumulation and disease severity was reduced with pGM treatment. pGM-treated/CABMV infected plants were able to increase their yields by approximately 70% when compared to controls. These results suggested that pGM allows plants to respond more efficiently against CABMV infection, mitigating woody symptoms and maintaining normal plant growth without affecting yields caused by viruses.

**Key words:** Peptidogalactomannan; *Passiflora edulis*; CABMV; defense-related genes; phytohormones.

## 1. Introduction

The genus *Passiflora* L. belongs to the Passifloraceae family, comprising about 500 species, among which, *Passiflora edulis* stands out for its economic and medicinal importance [1]. It is widely planted in tropical and subtropical regions around the world, especially in South America, the Caribbean, southern Florida, South Africa, and Asia [2,3]. With approximately 200 species, Brazil is the country where the passion fruit originates [4]. Among the different varieties of passion fruit, the yellow-fruited *P. edulis* f. flavicarpa and the purple-fruited type, *P. edulis* Sims, are the cultivars that have greater economic and commercial importance [5,6]. Yellow-fruited, have a bright yellow peel, with a hard and thick consistency, measuring approximately 6 to 12 cm in length and 4 to 7 cm in diameter, their seeds are brown, the pulp is acidic and possess a strong aromatic flavor. However, the purple-fruited have a purple peel, are smaller in size, measuring approximately 4 to 9 cm in length and 3.5 to 7 cm in diameter, and their seeds are black [7].

Brazil stands out as the world's largest producer of passion fruit (690,364 t $year^{-1}$), accounting for 70% of total world production and with an average national production of 14.8 t $ha^{-1}$ [8]. Under organic production systems, there is a big fluctuation in productivity, ranging from 10.2 t $ha^{-1}$ [9] to 21.6 t $ha^{-1}$ [10]. *Passiflora edulis* is the main species cultivated commercially, and some viruses have already been described infecting this species, specifically: passion fruit yellow mosaic virus (PFYMV); passion fruit vein clearing virus (PVCV); passion fruit green spot virus (PFGSV); passion fruit severe leaf distortion virus (PFSLDV); purple granadilla mosaic virus (PGMV); passion fruit leaf mosaic virus (PLLMV); cucumber mosaic

virus (CMV); passion fruit chlorotic mottle virus (PCMoV); cucurbit aphid-borne yellows virus (CABYV); lettuce chlorosis virus (LCV) and cowpea aphid-borne mosaic virus (CABMV), [11,12,13,14,15]. The most harmful is CABMV, a virus belonging to the genus *Potyvirus* in the family Potyviridae. Its genome consists of a positive-sense single-stranded positive-sense RNA molecule of approximately 10,000 nucleotides [16]. Passion fruit woodiness disease (PWD) caused by CABMV causes significant losses in the passion fruit crops, due to severe symptoms of fruit hardening in all producing regions where the disease is detected [12,17].

CABMV is transmitted and disseminated mechanically during pruning and by some species of aphids (*Aphididae* family, *Hemiptera* order, *Animalia*), in a non-persistent manner. Although there are not aphid species colonizing *Passiflora* spp., a few species have been describe as CABMV-vectors, principally: *Aphis gossypii* Glover, *A. craccivora* Bock, *A. fabae* Scopoli, *Myzus persicae* Shulzer, *Uroleucon ambrosiae* Thomas, *U. sonchi* L., *Toxoptera citricidus* Kilkaldy and *Myzus nicotianae* Blackman [18,19].

CABMV causes serious damage to passion fruit, it decreases fruit size and causes deformations associated with the occurrence of mosaic symptoms, accompanied by leaf malformation, blisters, a marked reduction in plant development, and woodiness in some fruits [20]. The damage caused by the virus in plant development is probably due to the suppression of several key genes in the phytohormone pathway. Chen et al. [21] observed in passion fruit plants after CMV infection that some genes involved in auxin and gibberellin signaling were notably suppressed after virus infection, causing serious damage to passion fruit development.

Plants have developed systems to fight infections, activating specific defensive pathways aimed at various types of threats. The handling of plant cell by biotic elicitors is a utility approach to enhance the production or accumulation of important secondary metabolites [22]. Elicitors can play various roles after application to plants and can be used to improve the synthesis of important secondary metabolites. The elicitor treatment alters the physiological conditions in plants to protect them against various biotic and abiotic stresses, such as attacks by pathogens, wounds, heavy metals, air pollutants, ultraviolet rays, and adverse environmental conditions [23].

After infection by a phytopathogen or by the elicitation of inducing molecules, plants can activate various proteins, phytohormones and signaling molecules that are strictly related to the plant's immunity or defense. Flags such as salicylic acid (SA) and jasmonic acid (JA),

lead to the accumulation of defense proteins, known as pathogen-related proteins (PRs). Based on biochemical, regulatory and sequence similarity, an abundance of PR proteins was quickly reported in many plants, including gymnosperms, monocots, dicots and mosses [24]. These proteins minimize the multiplication of pathogens in uninfected plant organs. Therefore, the production, accumulation or expression of PRs is a fundamental step in plant defense induction [25]. Among the 17 families of PRs known today, many are associated or classified to different biochemical functions such as glucanase, chitinase, peroxidase, also to different biological functions such as antibacterial or antifungal activities. In addition to participating in the plant's defense system, the phytohormones that play an important role in almost every aspect of plant development and growth processes [26, 27].

To identify biogenic or biomolecules that can potentially protect plants against biotic stress, our group recently demonstrated that a *C. herbarum* (Davidiellaceae family, Capnodiales Order, *Fungi*) derived peptidogalactomannan (pGM) induced defense-related genes in passion fruit (*Passiflora edulis* Sims *f. flavicarpa* Deg) in greenhouse conditions that enable the plant to mitigate symptoms of CABMV-infection [28]. However, it is not yet known if these positive effects of pGM elicitation would be maintained in the field, where plants are submitted to multiple adverse conditions as temperature variation, water stress and pathogen attack. Here we determine whether peptidogalactomannan (pGM) leads to defense gene induction in CABMV-inoculated passion fruit plants from different genotypes under field conditions. In addition, we analyzed whether pGM-conferred protection was sufficient to attenuate the damages caused by CABMV in terms of yield and productivity in an organic production system.

## 2. Materials and methods

### 2.1. Location of experimental areas

A part of the experiments was carried out under greenhouse conditions, in the city of Rio de Janeiro, at Universidade Federal do Rio de Janeiro (UFRJ).

Other experiments in greenhouse and field conditions was carried out at SIPA - Integrated system of agroecological production “Fazendinha Agroecológica”, which is an experimental area of Empresa Brasileira de Pesquisa Agropecuaria (Embrapa Agrobiologia), located in the municipality of Seropédica, Rio de Janeiro State, Brazil. The geographical coordinates are 220 48’00’’ South latitude and 430 41’00’’ West longitude. It has an altitude of

approximately 33 m and the soil is classified as Red-yellow Argisol. According to the Köppen classification, the climate is Aw (rainfall concentrated from November to March; average annual rainfall of 1.213 mm; average annual temperature of 24.5 °C).

### 2.2. Peptidogalactomannan (pGM) extraction

*Cladosporium herbarum* fungus strain CBS 121621, was grown in potato dextrose broth medium (PDB) for 7 days and 3MM paper filtered to obtain the fungal mass. The pGM extraction was performed according to Haido et al. [29]. Briefly, fungus mycelium was extracted with 0.05 M phosphate buffer, pH 7.2 at 100 °C for 2 h. After filtration, filtrate was vacuum evaporated and precipitated in 92.8 % (v/v) ethanol at 4 °C. The precipitate was resuspended in distillated water, dialyzed, and freeze-dried to obtain the crude pGM.

### 2.3. Plant materials

Experiments were carried out in greenhouse and field conditions. Different *Passiflora edulis* genotypes were used in this study, two commercial varieties of yellow passion fruit such as 'Redondo Amarelo' and 'FB300', plus 'H09-100/111' an intraspecific hybrid from the Passion fruit Germplasm Active Bank of Embrapa. All the experiments were performed in an organic production system according to the recommendations for the yellow passion fruit cultivation in Brazil and to the federal laws of organic agriculture [30]. In field conditions, control of spontaneous vegetation was carried out by means of mechanized weeding between rows and hoeing in rows when needed and pollination was naturally performed by bumblebees (*Xylocopa* spp.). No insecticides, fungicides or chemical fertilizers were used during all the experiments periods.

### 2.4. pGM-treatment

In all experiments, passion fruit plants were foliar sprayed with 100 $\mu g.mL^{-1}$ of pGM resuspended in tap water when plants had 3 or 4 true leaves. This dose was selected based on Santos-Jiménez et al. [28]. The treatments were performed with a costal manual sprayer (Jacto - XP). As a control, tap water was foliar sprayed using the same procedure (water treatment).

### 2.5. CABMV mechanical inoculation

Three days after pGM treatment, 100 μL of a CABMV 1:50 dilution inoculum was mechanically inoculated into the first two leaves of each plant using Celite (Sigma) as an abrasive. The inoculum was prepared by macerating CABMV-infected leaves in 10 mM sodium phosphate buffer, pH 7.0. The same procedure was used for mock inoculations of control plants with sodium phosphate buffer.

### 2.6. Greenhouse experiment

In order to understand the molecular damage that CABMV can cause in passion fruit plants, leading to developmental delays and how treatment with pGM can lead to protection of plants against virus infection, experiments under greenhouse conditions at UFRJ and EMBRAPA was carried out with the *P. edulis* plants. In the UFRJ experiments the seedlings of 'Redondo Amarelo' and 'H09-110/111' were germinated in 9 L plastic pots, containing commercial substrate (MECPLANT), and kept in a greenhouse under conditions of natural light and controlled temperature 27 ± 2 °C. Three days after treatment, plants received mechanical CABMV inoculum for further analysis of defense-related and phytohormones-associated genes at 12 and 168 hours after inoculation (hai) and biomass evaluation at 7 weeks after inoculation (wai). The experimental design was randomized in biological replicates with 10 plants of 'Redondo Amarelo' and 4 plants of "H09-110/111" for each treatment.

However, another experiment under greenhouse conditions at Embrapa was conducted. Passion fruit seedlings were grown in 15 L plastic pots containing 1/3 vermiculite substrate, 1/3 earthworm humus and 1/3 washed sand, under natural temperature and light conditions of a tropical area. Three days after treatment plants were mechanically inoculated with CABMV. Plants were conducted in a vertical trellis system with a wire 2.0 m above the plastic pot of each plant. Cover fertilizations were carried out every 10 days with worm-bed leachate (earthworm leachate solution) (1 L seedling$^{-1}$) and castor bean cake (residue from the biodiesel production) (50 g seedling-1). Three passion fruit genotypes 'FB300', 'H09-110-111' and 'BGP473' were used, and thus to evaluate the effect of pGM in the induction of defense genes in the first hours after treatment (24 and 72 hat) and to observe whether the effect this in the protection against CABMV is maintained in all genotypes, analyzing morphological characters such as height and

number of leaves over time. The experimental design was randomized in biological replicates, with 6 plants of 'FB300' and 'BGP473', and 9 plants of 'H09-110/111' for each treatment.

In all experiments under greenhouse conditions, the treatments consisted of: a) Uninoculated plant; b) Water-treated + CABMV-inoculated; and c) pGM-treated + CABMV-inoculated.

### 2.7. Field experiment

In order to assess the protection of pGM against CABMV in passion fruit plants under field conditions, and whether the induction of defense genes and phytohormones can be maintained at later weeks after viral inoculation (8 and 12 wai), and whether the treatment can attenuate or mitigate the damage caused by the virus in the productivity of this crop, an experiment was set up in the experimental fields of EMBRAPA. Passion fruit plants 'FB300' and 'H09-110/111' were grown in bags and kept in a greenhouse until they had 3 to 4 true leaves, were foliar sprayed with pGM-treatment. Three days after treatment two leaves per plant were mechanically inoculated with CABMV. One week after CABMV-inoculation (wai), plants were transplanted to the field, at 2.5 m x 1.0 m tree spacing (4000 plants $ha^{-1}$). Cultivation in field was conducted in espalier on wire threads number 12 fixed at 2.0 m above the ground. Pits (0.4 m x 0.4 m x 0.4 m) were fertilized with 150 g of thermophosphate (Yoorin® Master 1), 300 g of phonolytic rock powder (Yoorin® eKoSil) and 1 kg of corral manure. After planting, cover fertilizations were carried out three times every 45 days with corral manure (1 kg $seedling^{-1}$) and castor bean cake (200 g $seedling^{-1}$). A drip irrigation system was installed, using flow drippers (4 L $hour^{-1}$ $plant^{-1}$), being activated 1 hour per day. The experimental design was in randomized blocks, with 4 treatments, three replicates and 4 plants per plot (n=12 plants per treatment). Treatments consisted of: a) Uninoculated plant; b) Water-treated + CABMV-inoculated; c) pGM-treated + CABMV-inoculated (once sprayed); and d) pGM-treated + CABMV (twice sprayed, with an interval of 12 weeks).

The plants were monitored over time for CABMV disease symptoms appearance and/or plant morphological parameters evaluation.

### 2.8. RT-qPCR gene expression

The expression of five defense-related genes such as *phenylalanine ammonia-lyase (PAL)* [28], *peroxidase 12 (POD12), lipoxygenase 2* (*LOX2), pathogenesis-related protein 3* (*PR3)* and, *superoxide dismutase (SOD)* [31] and two genes involved in phytohormone signaling pathways such as auxin (*AUX-responsive protein SAUR)* and *gibberellin* (*GA 2-beta dioxygenase*) [21] was evaluated in leaves of the plants per each treatment. Leaf samples were collected in biological triplicate (under greenhouse conditions) and quadruplicate (under field conditions) for gene expression analysis. Total RNA was extracted with TRIzol® reagent (ThermoFisher Scientific). SuperScript$^{TM}$ VILO$^{TM}$ MasterMix (Invitrogen) and PowerUp$^{TM}$ SYBR$^{TM}$ Green Master Mix (Thermo Fisher Scientific) were used for cDNA synthesis and qPCR reactions, respectively. Three internal controls: *NDID* (NADP-*dependent isocitrate dehydrogenase*), *ERS* (*Ethylene Response Sensor*) and, *EF1a1* (*Translation elongation factor* 1a-1) [31] were used for qPCR normalization. qPCR of 3 independent biological pools, were performed for each evaluated point in technical triplicates on Applied Biosystems 7500 Fast Real-Time PCR apparatus with the following cycling conditions: initial denaturation at 95 °C for 2 min, 40 cycles of 95 °C for 15 sec, 52 °C–68 °C for 1 minute and elongation at 72 °C for 1 minute. Ct values were evaluated using the $2^{-\Delta\Delta Ct}$ method and represented as fold change as proposed by Livak and Schmitgen, [32].

### 2.9. Qualitative, semi-quantitative and quantitative detection of CABMV

Young leaves from each treatment were collected at different time. Under greenhouse conditions, samples of 'Redondo Amarelo' plants, were collected at 12 and 168 hai. Under field conditions samples of 'FB300' and 'H09-110/111' plants were collected at 4, 8 and 12 wai.

For the qualitative RT-PCR diagnosis of CABMV, the total RNA from passion fruit samples was extracted using TRIzol (Thermo Fisher Scientific). RT-PCR was performed using a SuperScript III One-Step RT-PCR with Platinum DNA Polymerase Kit (Invitrogen) and CABMV capsid-specific oligos, CABMV_M1 MX3726F 5' GAGACACAAGCCAAAACACAAAATC 3' and CABMV_M1 MX5029R 5' CGTTGCTACAAATTCTGGTATCTCC 3', which generate an expected amplicon of 1311 bp [13]. The amplified products were run in 1% (w/v) agarose gel electrophoresis with ethidium

bromide (0.5 µg.mL$^{-1}$) (Promega) and visualized under ultraviolet light (UV). The molecular weight standard 1 kb DNA Ladder Plus (LabAid$^{TM}$) was used to check the amplicon size.

PathoScreen® (Agdia) ELISA kit for specific detection of viruses of the potyvirus group including CABMV was used to identify the presence and relative accumulation (Absorbance at $OD_{415}$) of CABMV in passion fruit plants following Agdia protocol.

The RT-qPCR reactions to quantify CABMV in treatments were performed using the pair primer qCABMV06_For 5' ATAGAATACAAGCCAGCACAAATCG 3' and qCABMV06_Rev 5' CCGTCCATCATAGTCCACACC 3', designed to amplify a 200 pb part of the CABMV coat protein gene [33]. All reactions were conducted in technical triplicates. The program used for amplification of the CABMV coat protein gene was the standard of the equipment in Standard mode, following the stages: incubation at 50 °C for 2 min, activation of taq DNA polymerase at 95 ºC for 10 min, followed by 40 denaturation cycles at 95 ºC for 15 sec, annealing of the primers 55 °C and extension at 60 ºC for 1 min. Ct values were evaluated using the $2^{-\Delta Ct}$ method as proposed by Livak and Schmitgen [32].

### 2.10. pGM-effect in development and productivity evaluation

In the experiment in a greenhouse, in passion fruit 'H09-110/111', the fresh and dry biomass of the aerial part and root system of the plants was evaluated for each treatment at 7 wai. To measure biomass weight, an electronic scale (Bioprecisa – JA3003N) was used. The samples were weighed after washing and wiped-dried for the determination of fresh weight. Then, the sample was oven-dried at 65 °C for 72 h, and the dry weight was measured. However, in passion fruit 'BGP473', 'FB300' and, 'H09-110/111', developmental characters such as height (estimate with a measuring tape) and counting the number of leaves over time were evaluated.

Under field conditions, development and productivity characters were evaluated as height, number of leaves, number of flower buds and number of fruits. The harvest of the fruits was done 43 wai. 30 fruits per each treatment were used to evaluate: weight fresh (WF, in g); weight pulp (WP, in g), yield of fruit pulp (YP, calculated through the difference between WP and WF); soluble solids (SS, in °Brix), and total yield (TY, in tons ha$^{-1}$). WF and WP were measured using a digital scale (Bioprecisa – JA3003N), and SS was measured using a portable sucrose refractometer with 0–53 °Brix scale (PAL-1, ATAGO®). In addition, the number of fruits produced per plant was checked at three different times prior to harvesting and counting

only those fruits present at about 4 weeks after blooming. Fruit production per plant (in kg) was calculated by multiplying the average number of fruits per plant (TNF) by the mean WF for the respective treatment. Finally, individual plant production was extrapolated on a per hectare basis as a function of the number of plants per hectare for estimating yield in t $ha^{-1}$.

### 2.11. CABMV disease rating

These parameters were estimated from typical leaf symptoms of the virus. Data were collected from 2 weeks after CABMV-inoculation (wai). Disease incidence (DI%) was determined based on the symptoms on diseased plants (at least one leaf showing mild to severe mosaic and/or deformation). The proportion of diseased plants was estimated by: $DI = (n/N) \times 100$ (DI = incidence; n = number of diseased plants; N = total number of plants assessed) [34]. Disease severity (DS%) was calculate using a symptom severity rating scale of 1, 2, 3 e 4 were: 1 represented the absence of symptoms, 2 represented the presence of mild mosaic symptoms without leaf deformation, 3 represented the presence of severe mosaic without leaf deformation, and 4 represented the presence of severe mosaic, blisters, and leaf deformation, as proposed by Gonçalves et al. [35]. DS index was then determined for each treatment using the formula according to Mckinney [36] as shown below: $DS = \sum(DS \times L)/(TNP \times HGS) \times 100$, where DS = degree of the 1-4 scale determined for each plant; L = number of leaves showing each degree of infection (score); TNP = total number of leaves evaluated; and HGS = highest grade of the scale (maximum infection score).

### 2.12. Data analysis

Data obtained for gene expression and DI, development, productivity and/or yield parameters were analyzed by the parametric Two-way and One-way ANOVA tests, respectively, followed by Bonferroni post-hoc test ($p < 0.05$) to check if the different averages observed between water and pGM treatments were statistically supported. The data obtained for the compare CABMV accumulation by RT-qPCR were analyzed using Student's t test with a significance level of $p < 0.05$. For DS and ELISA data comparisons, a non-parametric ANOVA Kruskal-Wallis test was used with Dunn's post-hoc tests ($p < 0.05$). For statistical data analysis GraphPad Prism software version 5.00 was used.

## 3. Results

### 3.1. pGM treatment mitigates the damage caused by CABMV on morphological and productivity parameters

Among the damages caused by the CABMV, an important delay in plant development occurs [28]. In order to observe the effect of pGM in mitigating the damage caused by CABMV in two genotypes of *P. edulis* both under greenhouse and field conditions, different characters of development and plant productivity were evaluated. Seven weeks after CABMV inoculation (wai) in passion fruit 'H09-110/111' under greenhouse conditions, pGM-treated + CABMV plants showed similar or even better development patterns than uninoculated plants in the root formation (Figure 1a). Additionally, in the biomass (fresh and dry weights) of the aerial part and the root system were evaluated, noting that in water-treated + CABMV plants showed strong weight reduction associated with CABMV-infection. In the aerial parts, the fresh and dry weight were 86 and 33% higher in pGM-treated + CABMV than in water-treated + CABMV plants, respectively (Figure 1b and 1c) ($p$ <0.001). Also, we observed that the root system (fresh and dry weight) showed an increase of 100 and 71%, respectively, in pGM + CABMV plants when compared with water + CABMV plants (Figure 1d and 1e) ($p$ <0.01).

Morphological parameters such as height and number of leaves were also evaluated and similar data were found both in greenhouse and field conditions, with a significant increase in pGM + CABMV compared to water + CABMV ($p$ <0.05), observing an increase of 17 and 26% in height at 5 wai in passion fruit 'H09-110/111' and 'FB300', respectively (Figure 1f and g), and more than 30% in both genotypes in the number of leaves at 9 wai, under greenhouse conditions (Figure 1h and i). Similar results were observed under field conditions between 4 and 10 wai, with a significant increase in pGM + CABMV plants 'FB300' and 'H09-110/111' of approximately 30% in height (Figure 2a and b) and 40% in the number of leaves (Figure 2c and d), when compared with water + CABMV.

These parameters of height and number of leaves under field conditions were evaluated in all treatments, and the times evaluated were before the second spraying (up to 10 wai) in the other group of plants destined for this treatment, as this was applied 12 wai, and at this time these parameters were impossible to measure due to plant development.

An important effect observed in pGM + CABMV (once and twice sprayed) under field conditions, was the induction of flowering in passion fruit plants, here we observed that treated

plants started to form flower buds earlier when compared to controls (uninoculated plants and water + CABMV), they started to appear from 10 wai onwards. Statistical differences starting at 12 wai and noting a significant increase of approximately 133% in both genotypes at 16 wai (Figure 2e and f) ($p$ <0.001).

In general, under field conditions, the appearance of the passion fruit plants ('H09-110/111' and 'FB300') over time was better in pGM + CABMV (once and twice sprayed), observing plants with larger foliar and better developed when compared to the controls (Supplementary Figure S1).

Looking at the number of fruits (TNF), a similar result to that obtained in the number of flower buds was observed, with an increase in pGM + CABMV (once and twice sprayed) when compared to the controls (uninoculated plants and water + CABMV) both in 'FB300' and 'H09-110/111', since the pGM + CABMV (twice sprayed) had a higher mean number of fruits compared to the other treatments (Table 2) ($p$ <0.001).

Despite the differences observed in development and productivity between the controls (uninoculated and water + CABMV) and pGM + CABMV plants, no strong differences were observed between the symptoms caused by CABMV in the fruit appearance, probably due to good nutritional management or the inoculated strain or the natural infection in the field. Fruits from all the treatments didn't show hardening and blisters, characteristic symptoms of CABMV in the physical appearance of the fruits. However, it should be noted that fruits from pGM + CABMV (once and two sprayed) looked better (Supplementary Figure S2) and had higher fresh weight of fruit (WF), higher weight of pulp (WP) and higher pulp yield (PY%) (Table 2) ($p$ <0.001). Thus, mitigating the damage caused by CABMV in fruit yield. However, in the evaluation of soluble solids (SS), no significant differences were observed in any of the treatments ($p$ >0.05), inferring that neither the pGM-treatment nor the CABMV infection alters this parameter in passion fruit pulp.

In the estimated productivity in a single season (total yields, TY $ha^{-1}$), we observed that the CABMV does not affect this parameter after pGM + CABMV (once and twice sprayed), being better in twice sprayed, leading to yields of approximately 15.5 and once sprayed it was approximately 12 tons $ha^{-1}$ in both genotypes. While the controls were approximately 7 at 8 tons $ha^{-1}$ (Table 2) ($p$ <0.0001).

### 3.2. pGM-treated plants showed less CABMV-accumulation

The CABMV-accumulation was analyzed in young leaves of the plants. Using a qualitative RT-PCR, semi-quantitative ELISA and quantitative RT-qPCR assay it was possible to observe the presence and accumulation of CABMV in each treatment. Under field conditions the presence of CABMV wasn't detected by ELISA assays at 4 wai in pGM + CABMV 'FB300' and 'H09-110/111' plants, while in water + CABMV it was detected only in 'FB300' (Figure 3a and b; Supplementary Figure S3a and b). However, at 8 wai in 'FB300', in all plants of the pGM and water treatment, the presence of the virus was detected (Supplementary Figure S3c). There was an increase in CABMV-accumulation in pGM + CABMV, there are no significant differences when compared to water + CABMV (Figure 3a). Nevertheless, this same genotype in 12 wai we observed a decrease in the CABMV-accumulation in pGM + CABMV (once and twice sprayed), with significant reduction of 28.9 and 22.7%, respectively, when compared to water + CABMV (Figure 3a) ($p$ <0.001). Meanwhile, in 'H09-110/111' at 8 wai, we observed CABMV detected in all plants in water + CABMV and six plants only in pGM + CABMV (Supplementary Figure S3d), but with a low CABMV-accumulation of 78% less in pGM + CABMV when compared to water + CABMV, maintained at 12 wai (once and twice sprayed) with 18 and 14% less, respectively (Figure 3b) ($p$ <0.001).

Likewise, in the quantitative RT-qPCR, showed a decrease in the CABMV-relative accumulation in pGM + CABMV plants both in greenhouse and field conditions compared to water + CABMV. Under greenhouse conditions in 'Redondo Amarelo' plants, at 12 hai it was not possible to detect the presence of CABMV in any of the treatments, while a significance decrease in pGM + CABMV was observed at 168 hai (Figure 3c) ($p$ <0.05). As well, under field conditions and later times after CABMV-inoculation, the CABMV relative accumulation were much greater in water + CABMV than in pGM + CABMV plants, with statistically differences starting at 12 wai in 'FB300' (Figure 3d) and 'H09-110/111' (Figure 3e) ($p$ <0.05).

Therefore, we infer that pGM-treated seems to prepare the plant to withstand viral infection and making it difficult to replication and translocation of the virus in the younger leaves of the plant.

### 3.3. pGM treatment is effective in the decrease of CABMV disease incidence and severity

In view of the effect of pGM in decreasing the prevalence and accumulation of CABMV in passion fruit plants, we wanted to observe whether this same response could be maintained in the incidence and severity of disease caused by CABMV in plant leaves, as one of the characteristic symptoms of this virus is the appearance of leaf symptoms (such as mosaic, deformation, blisters, etc.). Passion fruit woodiness disease incidence (DI) and severity (DS) were evaluated in greenhouse and field conditions. First, under greenhouse, significant differences (Dunn's test $p < 0.0001$) in both parameters were observed between pGM + CABMV and water + CABMV plants at all evaluated time points (Supplementary Table S1). The DI has always remained lower in pGM + CABMV over time. Same, in DS a reduction of 32 and 47% in pGM + CABMV compared to water + CABMV was observed at 9 wai in 'H09-110/111' and 'FB300', respectively (Supplementary Table S1).

In the same way, under field conditions we observed that DI and DS in pGM + CABMV was lower with respect to the water + CABMV, with significant differences at 4 wai in both 'FB300' and 'H09-110/111' (Table 1). However, no difference in DI and DS was observed between pGM + CABMV (once and twice sprayed).

### 3.4. CABMV-suppression of defense-related genes and phytohormones signaling pathways

In view of the damage caused by CABMV in plant development observed both in the greenhouse and in the field (Figure 4), we wanted to understand molecularly the flaws that the CABMV can take both in the immune system (defense-related genes) and in the pathways involved in the regulation of plant development (phytohormones-associated genes). Under greenhouse conditions, analyzing and comparing CABMV-inoculated and uninoculated plants, we observed that the CABMV-infection led to suppression of three defense-related, such as, *PR-3, PAL* and *LOX2*, and phytohormones-associated genes such, *AUX* and *GA* at 12 hai (Figure 4a). Although CABMV at this time was not detected by RT-qPCR (Figure 3c), we observed that the virus quickly began to cause molecular failures in the plant. Nonetheless, the suppression of *PR-3*, *PAL* and *GA* was maintained at 168 hai (Figure 4a). On the other hand,

*SOD* was induced at both times, this is because SOD is a crucial enzyme in plant survival in a stressful environment. In the same way, under field conditions, in 'FB300' CABMV-inoculated plants, a similar effect was observed with the suppression of the expression of all defense-related genes and *AUX* (Figure 4b). While in 'H09-110/111' CABMV-inoculated plants, were down-regulated of *SOD*, *AUX* and *GA*. Surprisingly we found that *PR-3,* and *POX12* was induced at this time, inferring that this genotype has a better immune-related gene expression profiles than 'FB300' in CABMV-infection (Figure 4b). Passion fruit 'Redondo Amarelo' and 'FB300' are CABMV susceptible varieties, and the gene modulation observed may reflect a virus-induced inhibition of important defense and phytohormone pathways that benefit the disease of CABMV-infection.

### 3.5. pGM induction of defense-related and phytohormone signaling pathways genes in different genotypes of passion fruit under greenhouse and field conditions

Previously, we showed that defense related genes are induced in *P. edulis* 'Redondo Amarelo' after pGM-treatment under greenhouse conditions [28]. In this study, we wanted to see if this pGM-mediated induction of defense genes could be induced in other passion fruit genotypes. The profile of five defense-related genes was evaluated 24 and 72 hat in pGM-treated plants 'FB300' and 'H09-110/111' under greenhouse conditions. Here, we can observe that the passion fruit 'H09-110/111', showed a faster response in the induction of defense, since all genes were expressed within 24 hat, returning to baseline levels at 72 hours with the exception of *PAL* (Figure 5). However, in passion fruit 'FB300' the gene expression profile was better at 72 hours, with all the evaluated defense genes being expressed.

After observing the induction of defense-related genes in the first hours after pGM-treatment (Figure 5), we looked to understand if pGM treatment was able to sustain this induction after CABMV-inoculation at later times and under field conditions. Previously we observed that CABMV-infection leads to suppression of both defense genes and phytohormone signaling pathway (Figure 4). Here, we wanted to understand if pGM-treatment could avoid the suppression of several genes involved in plant defense and phytohormones pathway, since it was observed that the treatment led to protection and mitigation of damage caused by CABMV-infection in plant development both in a greenhouse or in the field conditions (Figure1; Figure

2; Figure 3). Surprisingly, we found that pGM + CABMV the virus failed to suppress these defense genes (Figure 6). At 8 wai, in 'H09-110/111' two genes such as *PAL* and *LOX2* were overexpressed, whereas *PR-3*, *SOD* and *POD2* were at their basal levels but not being suppressed by CABMV-infection. However, in 'FB300' all genes were overexpressed except for *SOD* which was at its basal levels (Figure 6 a-e). Looking at the results 12 wai, where there are two groups of plants treated with pGM (once and twice sprayed), we observed that in pGM + CABMV (once sprayed) in 'H09-110/111' plants showed an increase of the five genes, *PR-3, SOD, POD12, PAL* and *LOX2*, of 10.9, 52, 21.3, 15.2 and 21.2 times, respectively (Figure 6f-j). However, in 'FB300', we observed that only *PAL* (5.5 times) and *LOX2* (1.8 times) was induced in pGM + CABMV (once sprayed). Nevertheless, in pGM + CABMV (twice sprayed) only the induction of *SOD* in 'H09-100/111' and *PAL* in 'FB300' was maintained, the other genes were at their basal levels (Figure 6j-j). Even so, the pGM + CABMV (once and twice sprayed) avoided the inhibition or suppression of these defense genes in the passion fruit plants, inferring that the pGM can control CABMV-infection, thus explaining why pGM-effect led to the protection of the plant, decreasing the disease severity caused by CABMV-infection observed in this study (Table 1; Supplementary Table S1).

### 3.6. pGM is able to modulate auxin and gibberellic acid mRNAs expression

As previously mentioned, CABMV-infection leads to the suppression of phytohormone signaling pathway genes, and here we observed that pGM + CABMV led to induction of *AUX* and *GA* mRNAs, with an overexpression in the first hours after CABMV-inoculation (12 and 168 hai) in greenhouse conditions (Figure 7a). However, under field conditions in 'H09-110/111' and 'FB300' with pGM + CABMV (once or twice sprayed), a significant increase in the expression of these genes was observed at 12 wai (Figure 7b). In the same way observed in the defense-related genes, pGM + CABMV (once and twice sprayed) avoided the suppression of genes-associated with the pathway of phytohormones such as *auxin* and *gibberellin*, in all at both initial times (under greenhouse conditions) and later times (under field conditions), these were overexpressed and, thus understanding the mitigation of damage caused by CABMV-infection in the development and productivity of passion fruit plants, since pGM-effect led to an increase in morphological and productivity parameters (Figure 1; Figure 2) where these

phytohormones have a fundamental role, such as cell expansion, floral modulation and fruit formation.

## 4. Discussion

Viral diseases are one of the most serious phytosanitary problems of passion fruit, however, there have been few previous studies on the genetic-molecular mechanisms of the passion fruit-virus interaction and fewer still on protective treatments. Here, we evaluated how a peptidogalactomannan (pGM) might protect passion fruit plants susceptible to CABMV infections. CABMV leads to suppression of both defense-related and phytohormone pathway genes (Figure 4), as well, to decreasing root system and plant growth (Figure 1 and Figure 2). Our results showed that pGM-treated plants strongly induced defense-related genes before and after CABMV-inoculation (Figure 5 and Figure 6), reducing disease severity and virus symptoms (Table 1 and Table 2). In addition, pGM-treatment also prevented CABMV-infection from suppressing critical phytohormone signaling pathways (Figure 7), thus minimizing effects on plant development and subsequent productivity, characteristic of CABMV-infected plants. These results are in agreement with other molecular studies on virus-plant interaction. Paiva et al. [37] who analyzed the interaction between *Vigna unguiculata* and Cowpea severe mosaic virus (CPSMV), show that, compared to healthy plants, infected plants undergo significant changes in protein synthesis involved in signaling, metabolism and defense plants. The inhibited expression of several genes has been previously observed in other pathosystems, such as cucumber plants infected with Cucumber mosaic virus (CMV) and tomato plants inoculated with Tomato yellow leaf curl virus (TYLCV), occurring simultaneously with the virus replication in the plant [38,39]. The disease caused by CABMV manifests itself due to a failure in the signaling system for susceptible species [40]. Here, using a pGM, we observed that there was no inhibition of 5 defense-related genes 8 and 12 wai in pGM + CABMV-inoculated plants (Figure 6), previous studies by our group in greenhouse conditions and at earlier times after CABMV-inoculated in passion fruit plants “Redondo Amarelo”, observed that 12 and 168 h after CABMV-inoculated, these defense genes were overexpressed in pGM-treatment [28].

Pathogenesis-related protein (PRs) are considered the main weapons in plant defense tactics against pathogens [24]. Here we see that *PR-3* (*Chitinase I)* and *PR-9* (*Peroxidase 12* - *POD12*) were upregulated in the first hours after pGM-treatment in different passion fruit

genotypes under greenhouse conditions (Figure 5), and field conditions these genes were still expressed (Figure 6).

Parkinson et al. [41], evaluated the efficacy of acibenzolar-S-methyl (Bion®) in passion fruit plants infected with the potyvirus Passionfruit woodiness virus (PWV), managed to activate pathogenesis-related proteins such as *PR-3* and reduce the disease severity by 30% at 50 dai and the relative concentration of viruses determined by DAS-ELISA in the untreated upper leaves (new growth) above the inoculation site was reduced by 22% in treated plants. In our results, we observed that in addition to the induction of *PR-3* and other defense genes before and after CABMV-inoculation in pGM-treated plants and a decrease in disease severity over time, there was also a reduction in the relative concentration of the virus in young leaves of the plants, at 168 hai in greenhouse conditions (Figure 3c). However, under field conditions pGM + CABMV at 4 wai the presence of the virus was not detected (Figure 3a-b and Supplementary Figure S3a-b), while with the water + CABMV the virus was detected (Figure 3a and Supplementary Figure S3a). In later times, at 8 and 12 wai there was a decrease of the relative CABMV-accumulation in pGM + CABMV passion fruit plants (Figure 3). Inferring that probably the pGM-effect hinders the translocation and replication of CABMV in young leaves of passion fruit plants. It appears that the manifestation of CABMV symptoms has been "turned off" and defense-related genes have been induced in pGM + CABMV passion fruit plants, leading the plants to show tolerance to CABMV-infection. pGM + CABMV led to a drastic reduction in disease symptoms, showing a normal development pattern and leading to 32% and 37% reduction in DS compared to water-treated/CABMV at 9 wai in 'H09-110/111' and 'FB300', respectively, under greenhouse conditions (Supplementary Table S1) and 18% and 21% at 16 wai under field conditions in 'H09-110/111' and 'FB300' plants, respectively (Table 1).

Many studies have confirmed that antioxidant metabolism is involved in the defense response of plants. For example, increased *POD* activity was involved in resistance to the Pepper yellow mosaic virus (PYMV) in *Capsicum baccatum* 'pendulum' [42]. Our results indicated that pGM-treatment before and after CABMV-inoculation significantly increased the mRNA transcripts of the ROS-eliminating enzymes (*POD12* and *SOD*) (Figure 5b-c and Figure 6c, g, h). These results suggest that antioxidant metabolism is involved in the passion fruit response to CABMV.

Various researchers have identified a complex positive role of SA (Salicylic acid) in plants against viral pathogens [43,44]. The increased levels of *PAL* mRNAs found after pGM-treatment (Figure 5d) suggest a possible induction of the defense response of *P. edulis* by the synthesis of isoflavonoid and phytoalexin lignin and/or SA production. Normally, SA is thought to mediate defense signaling in response to biotrophic and hemibiotrophic pathogens, while JA (Jasmonic acid) and ET (Ethylene) are always associated with necrotrophic defense responses [45]. However, for growth, proper development, and better control against the attack of phytopathogens, plant hormones do not act in isolation, but through synergistic or antagonistic interference, and acclimating to each other in biosynthesis or responses, leading to fine adjustment of the response of complex defense [46]. Based on previous studies on plant hormones in other plants, probably the signaling pathways induced by pGM and related to SA, JA and ET may play important roles in the passion fruit response to CABMV-infection.

Plant-virus interactions are known to slow plant growth through rapid changes in phytohormones and their signaling pathways [44]. A viewpoint that is growing is that the interruption and alteration of the virus-mediated plant development weakens the plant defense strategies through improved replication and systemic spread of viral particles. Phytohormones are vital regulators of plant-pathogen interactions. Chen et al. [21], analyzed the DEGs associated with the phytohormone signaling pathways in *P. edulis*, noting that some genes involved in *AUX* signaling, including *AUX SAUR* responsive protein, *AUX*-induced protein, and *AUX ABP* binding protein, were notably down-regulated after infection by CMV, these authors indicated that the CMV-infection caused serious cellular damage in passion fruit, these same authors, based on histochemical analysis, suggest that CMV-infection leads to delay or decrease in the growth and development of passion fruit seedlings. Therefore, we propose that the patterns of differential expression of phytohormone signaling pathways may have a close relationship with the depressed growth and development of passion fruit caused by CABMV-infection in *P. edulis*, we observed that there was a suppression of *AUX* and *GA* at 12 and 168 hai under greenhouse conditions (Figure 4a), maintaining this for 12 wai in field conditions (Figure 4b) agreeing with the results of these authors in the suppression of these genes in passion fruit by virus-infection. Here, we observe the pGM-effect on plant preparation, such that when plants are CABMV-inoculated, it is not successful in inhibiting or suppressing these genes associated with phytohormones, such as *AUX* and *GA* (Figure 7) so the plant managed to

develop well, without severe damage to the root system, aerial part, height, etc. (Figure 1 and Figure 2a-d), and due to the stimulation of these phytohormones, the plants manage to increase the number of flowers and fruits, helping to increase crop yields (Figure 2e-f and Table 2). Inferring that there was a response of the plant in pGM-treatments to the control of the affects of these parameters caused by CABMV-infection. As previously described, considering that phytohormones participate in the defense of the plant against pathogens by crosstalk with the SA-JA-ET cascades [21], understand how well they orchestrate the growth and defense of passion fruit plants after pGM-treatment and/or CABMV-inoculation need to be clarified in future studies.

In this study, we managed to maintain good fruit quality and increase yields in pGM + CABMV plants by 65.7% (once sprayed) and 114% (twice sprayed) on 'FB300', while in 'H09-110/111' there was an increase 44% (one sprayed) and 80% (two sprayed) when compared to water + CABMV. We also observed similar significant differences when compared to the uninoculated plants (Naturally infected) (Table 2). Uchôa, et al. [47], obtained average yields of 14.5 tons in yellow passion fruit plants under an organic production system (Planted at a 3 x 3 m spacing), with an irrigation system and a good nutrition plan, these authors did not report any problems caused by viruses. We obtained yields of 15.5 tons in two evaluated genotypes of *P. edulis*. These results are above the national average, 14.8 tons $ha^{-1}$ [8].

## 5. Conclusions

This is the first study to analyze the fungal glycoprotein-effect on the mitigation of CABMV-infection in distinct genotypes of *P. edulis* under field conditions. Protein signal transduction, detoxification, phenylpropanoid biosynthesis, as well as some other important defense-related and phytohormone signals seem to be involved in this plant's immune improvement responses. Although the underlying mechanisms of defense-related pathways that regulate the passion fruit's response to the viral pathogen still require further research, the knowledge gained from this study will serve as a useful tool to combat CABMV in passion fruit crops.

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## Tables and legends

**Table 1.** pGM effect on the CABMV disease incidence (DI) and disease severity (DS) in passion fruit plants in field conditions through the time.

| **a. 'FB300'** | | **DI** | | | **DS** | | | |
|---|---|---|---|---|---|---|---|---|
| **Treatments** | **4 wai** | **5 wai** | **7 wai** | **9 wai** | **4 wai** | **8 wai** | **12 wai** | **16 wai** |
| Uninoculated | 50.0 ± 52.2ab | 58.3 ± 51.4a | 83.3 ± 38.9a | 100 ± 0.0a | 31.2 ± 33.9ab | 78.1 ± 16.1a | 81.0 ± 19.1a | 73.5 ± 15.6ab |
| Water + CABMV | 91.6 ± 28.8a | 100 ± 0.0a | 100 ± 0.0a | 100 ± 0.0a | 66.6 ± 28.8a | 83.3 ± 14.2a | 87.7 ± 18.1a | 79.1 ± 13.7a |
| pGM + CABMV | 25.0 ± 45.2b | 58.3 ± 51.4a | 83.3 ± 38.9a | 100 ± 0.0a | 17.7 ± 33.0b | 56.2 ± 30.3b | 71.2 ± 20.3a | 60.0 ± 16.1b |
| pGMx2 + CABMV | 33.3 ± 49.2b | 58.3 ± 51.4a | 83.3 ± 38.9a | 100 ± 0.0a | 22.9 ± 34.4b | 51.0 ± 26.9b | 78.7 ± 19.2a | 60.6 ± 14.5b |
| **P value (n=12)** | *0.0035 | *0.0627 | *0.5377 | *0.5770 | ≠0.0068 | ≠0.0015 | ≠0.1160 | ≠0.0055 |
| **b. 'H09-110/111'** | | **DI** | | | **DS** | | | |
| Uninoculated | 33.3 ± 49.2b | 50.0 ± 52.2ab | 83.3 ± 38.4a | 100 ± 0.0a | 18.5 ± 29.7b | 51.9 ± 26.4b | 70.0 ± 17.7a | 60.7 ± 15.4a |
| Water + CABMV | 91.6 ± 28.8a | 100 ± 0.0a | 100 ± 0.0a | 100 ± 0.0a | 68.7 ± 34.3a | 86.4 ± 11.2a | 80.4 ± 18.1a | 67.0 ± 22.9a |
| pGM + CABMV | 33.3 ± 49.2b | 41.6 ± 51.4b | 75.5 ± 45.2a | 100 ± 0.0a | 21.8 ± 33.7b | 63.5 ± 25.2b | 61.6 ± 20.1a | 55.2 ± 14.4a |
| pGMx2 + CABMV | 41.6 ± 51.4b | 41.6 ± 51.4b | 76.5 ± 43.2a | 100 ± 0.0a | 29.1 ± 36.6b | 57.2 ± 33.0b | 63.9 ± 18.2a | 54.5 ± 10.9a |
| **P value (n=12)** | *0.0074 | *0.0062 | *0.3248 | *0.5770 | ≠0.0032 | ≠0.0021 | ≠0.0811 | ≠0.5676 |

Each value is the means (± SD) of an independent experiment. Different letters in columns indicate significant differences between treatments.

*Corresponding results of One-Way ANOVA. Significant differences according to using the Bonferroni post-hoc test ($p$ <0.05). FB300 at 4 wai: $F_{3,44} = 5.25$, $p = 0.0035$; at 5 wai: $F_{3,44} = 2.61$, $p = 0.0627$; at 7 wai: $F_{3,44} = 0.73$, $p = 0.5377$; at 9 wai: $F_{3,44} = 0.66$, $p = 0.5770$. H09-110/111 at 4 wai: $F_{3,44} = 4.53$, $p = 0.0074$; at 5 wai: $F_{3,44} = 4.70$, $p = 0.0062$; at 7 wai; $F_{3,44} = 1.189$, $p = 0.3248$; at 9 wai: $F_{3,44} = 0.66$, $p = 0.5770$.

**Table 2.** pGM effect in the estimated productivity of passion fruit (a) cv. FB300 and (b) hybrid H09-110/111 in field conditions. Total number of fruits (TNF), Total yields (TY) weight fruit (WF); weight pulp (WP), pulp yield (PY), soluble solids content - °Brix (SS), length (LF), diameter (DF).

| **a. FB300** | | | | | | |
|---|---|---|---|---|---|---|
| Treatments | **TNF** | **TY (Tons ha$^{-1}$)** | **WF (g)** | **WP (g)** | **PY (%)** | **SS (°Brix)** |
| Uninoculated | 10.83 ± 1.46 b | 7.54 ± 1.47c | 161.8 ± 53.92b | 70.85 ± 27.89b | 43.34 ± 8.64a | 13.73 ± 2.53a |
| Water-treated + CABMV | 10.50 ± 5.46 b | 7.27 ± 2.21c | 168.0 ± 62.33b | 58.87 ± 27.92b | 34.40 ± 12.54b | 14.88 ± 1.71a |
| pGM-treated + CABMV | 16.42 ± 4.68 a | 12.05 ± 2.32b | 215.6 ± 81.57a | 99.96 ± 36.43a | 46.66 ± 9.62a | 13.59 ± 1.74a |
| pGM-treated x2 + CABMV | 20.67 ± 3.79 a | 15.58 ± 2.90a | 217.7 ± 70.58a | 101.5 ± 37.71a | 46.49 ± 3.43a | 14.35 ± 1.18a |
| **P value*** | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0311 |

| **b. H09-110/111** | | | | | | |
|---|---|---|---|---|---|---|
| Treatments | **TNF** | **TY (Tons ha$^{-1}$)** | **WF (g)** | **WP (g)** | **PY (%)** | **SS (°Brix)** |
| Uninoculated | 11.83 ± 3.38 a | 7.43 ± 1.01c | 158.9 ± 24.52a | 60.77 ± 13.17b | 38.05 ± 4.74c | 14.15 ± 1.76a |
| Water-treated + CABMV | 13.67 ± 4.25 ab | 8.61 ± 1.70c | 145.5 ± 31.20ac | 55.94 ± 15.57b | 41.41 ± 9.61bc | 15.12 ± 1.87a |
| pGM-treated + CABMV | 17.50 ± 6.44 bc | 12.44 ± 1.51b | 188.7 ± 54.08ab | 78.07 ± 26.16a | 47.22 ± 4.30a | 14.45 ± 1.46a |
| pGM-treated x2 + CABMV | 19.42 ± 3.28 c | 15.50 ± 2.36a | 174.2 ± 45.60ab | 66.74 ± 24.15ab | 43.93 ± 6.09ab | 15.19 ± 1.13a |
| **P value*** | 0.0006 | 0.0001 | 0.0005 | 0.0004 | 0.0001 | 0.0293 |

Each value is the means (± standard deviation) of one experiment using 12 plants per treatment. The average of WF, WP, PY, SS, LF and DF are 30 fruits per treatment. Different letters in columns indicate significant differences between treatments according to using the Bonferroni post-hoc test ($p$ <0.05).

*Corresponding results of One-Way ANOVA.

## Figures and legends

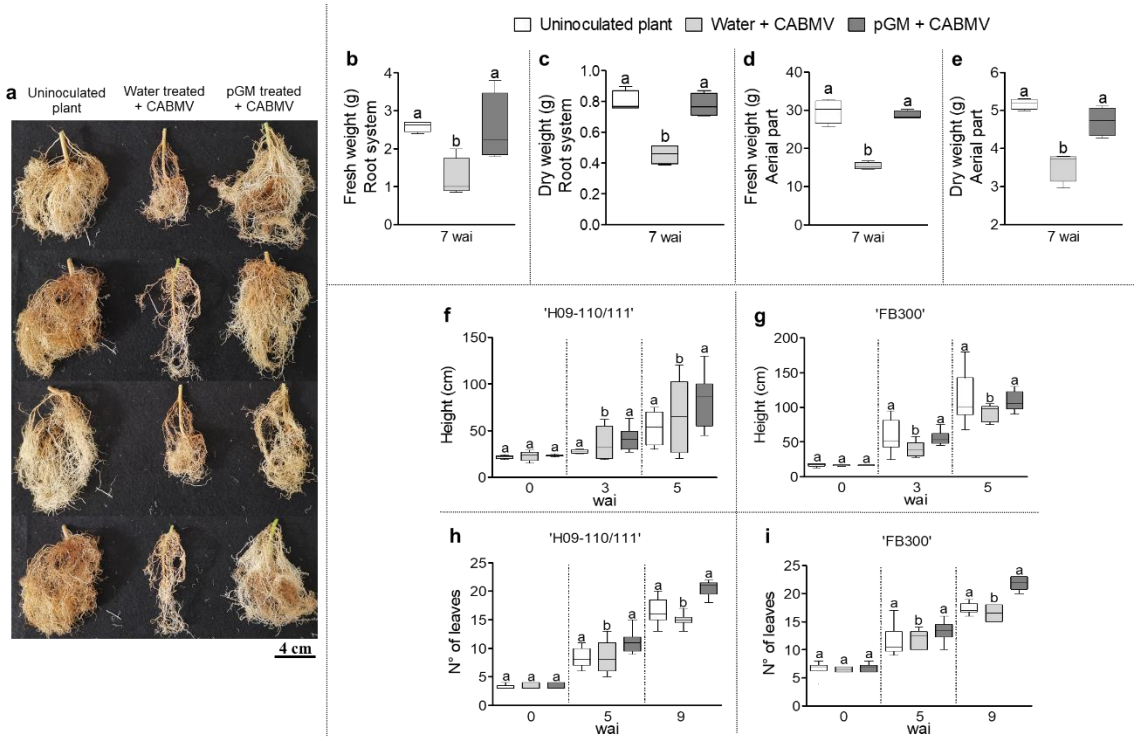



**Figure 1. pGM-effect in morphological parameters in passion fruit plants under greenhouse conditions.** Root system morphology of 'H09-110/111' plants were evaluated at 7 weeks after CABMV-inoculation (a). Analysis of the biomass in 'H09-110/111'; fresh and dry weight of root system (b-c) and aerial part (d-e). Development parameters as height at 0 - 5 wai in 'H09-110/111' (f) and 'FB300' (g); number of leaves at 0 - 9 wai in 'H09-110/111' (h) and 'FB300' (i). The bars represent the averages of the independent experiment using 4 plants per treatment (b), 9 plants per treatment (f and h) and 6 plants per treatment (g and i). Bars represent the averages of the plants per treatment, and different letters represent a significant difference at $p$ <0.05. The borders in the boxes (lower and upper) represent the first and third quartiles, and the average is represented by the horizontal line within the box. Whiskers extend to the largest and smallest data points in the interquartile range.

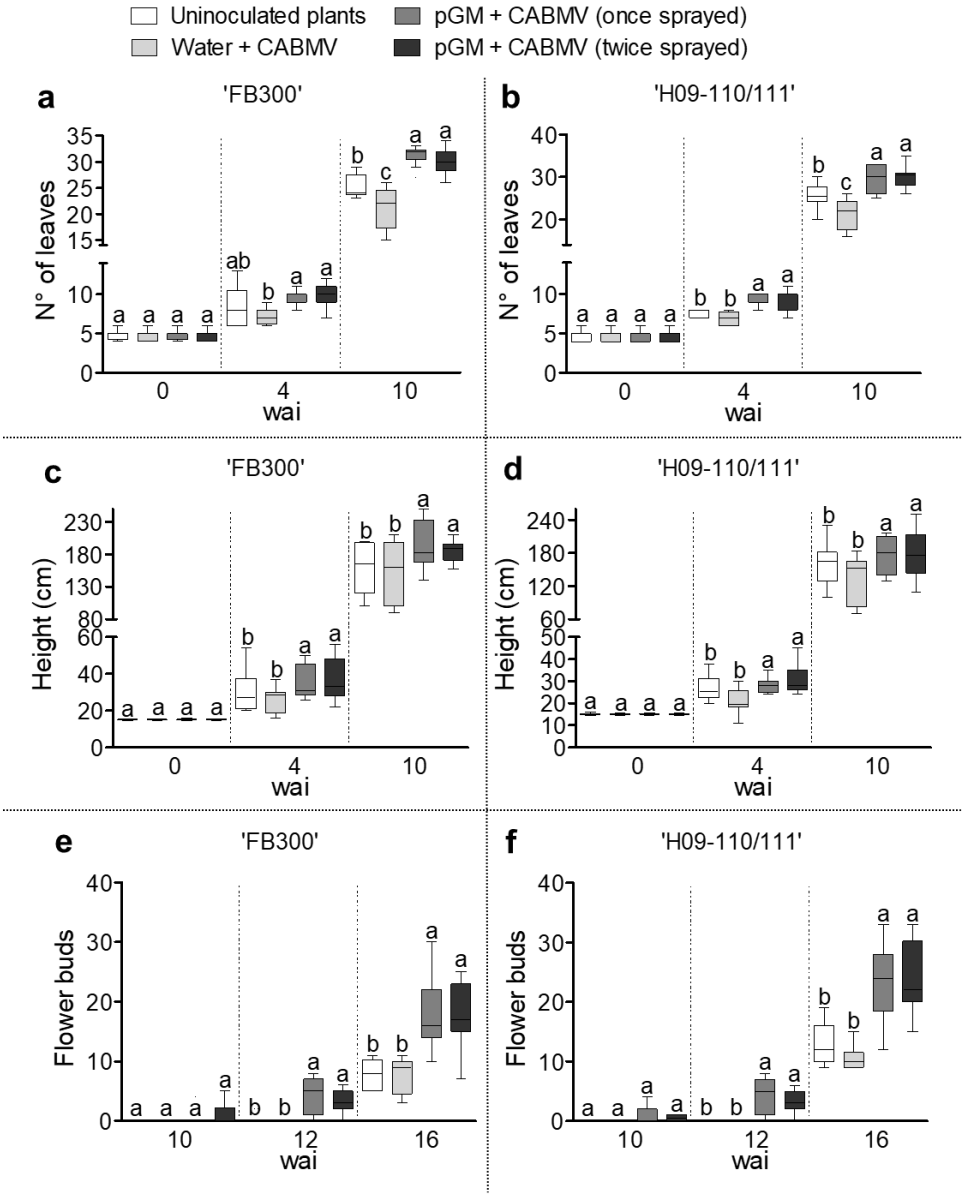



**Figure 2. pGM-effect in morphological parameters in passion fruit plants under field conditions.** Number of leaves in 'FB300' (a) and 'H09-110/111' (b) at 0 - 10 wai; height in 'FB300' (c) and 'H09-110/111' (d) at 0 - 10 wai; flower buds in 'FB300' (e) and 'H09-110/111' (f) at 10 - 16 wai. Bars represent the averages of the independent experiment using 12 plants per treatment, and different letters represent a significant difference at $p < 0.05$. The borders in the boxes (lower and upper) represent the first and third quartiles, and the average is represented by the horizontal line within the box. Whiskers extend to the largest and smallest data points in the interquartile range.

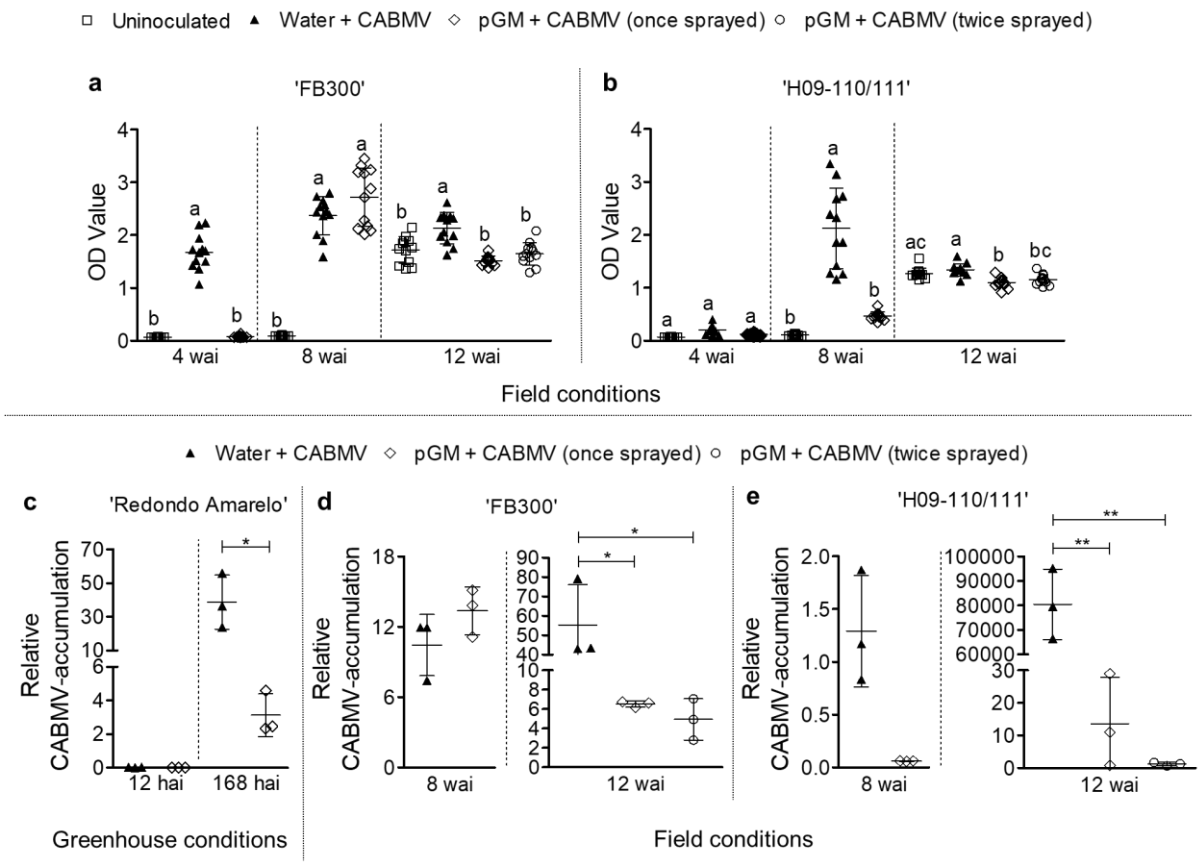



**Figure 3. pGM effect in the decrease of the relative CABMV-accumulation in passion fruit after CABMV-inoculated in greenhouse and field conditions.** Semi-quantitative and quantitative detection of CABMV. Standardized OD values of tissue samples from CABMV-inoculated (water and pGM-treatment) tested for Potyvirus CABMV-infection by ELISA at 4, 8 and 12 wai in *P. edulis* (a) 'FB300' and (b) 'H09-110/111'. The result of RT-qPCR is the relative CABMV-accumulation of water + CABMV relative to pGM + CABMV in *P. edulis* (c) 'Redondo Amarelo' at 12 and 168 hai under greenhouse conditions; (d) 'FB300' and (e) 'H09-110/111' at 8 and 12 wai under field conditions. Ct values were normalized with *ERS*, *NDID* and *EF1a1*. Bars with different letters and/or asterisks denote groups between which there is a significant difference. Dots represent individual data points, with the horizontal line inside representing the mean value. Whiskers extend to the highest and lowest data points the interquartile range. * and ** represent significant statistical differences with $p < 0.05$ and $p < 0.01$, respectively.

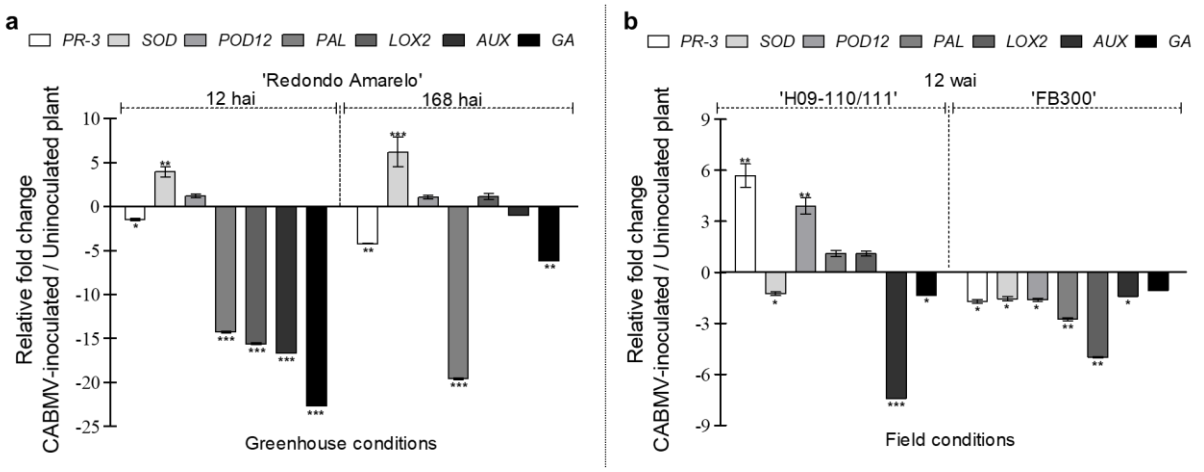



**Figure 4. CABMV-inoculated suppresses a defense-related and phytohormones signal pathways genes in passion fruit plants in greenhouse and field conditions.** RT-qPCR in CABMV-inoculated relative fold change to uninoculated plants of *PR-3*, *SOD*, *POD12*, *PAL*, *LOX2*, *AUX* and *GA* genes expressions transcripts. Passion fruit plants (a) 'Redondo Amarelo' at 12 and 168 hai under greenhouse conditions; (b) 'H09-110/111' and 'FB300' at 12 wai under field conditions. Ct values were normalized with *ERS*, *NDID* and *EF1a1*. Values represent the average relative fold change of mRNA transcripts of three technical replicates from four biologically independent replicates of each treatment at each time point. Error bars represent the standard deviation. *, ** and *** represent significant statistical differences with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

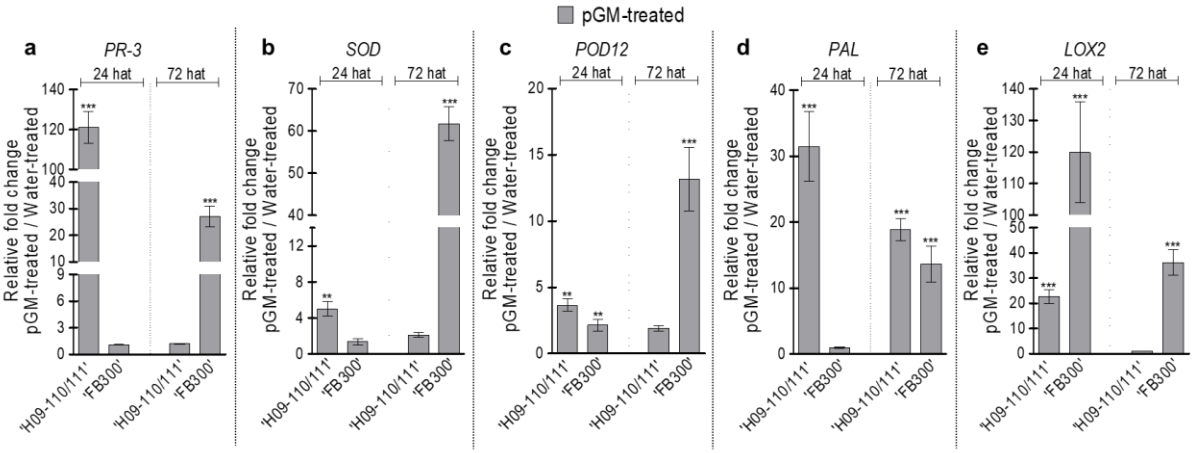



**Figure 5. pGM-induction of defense-related genes in passion fruit plants in greenhouse conditions.** RT-qPCR in pGM + CABMV relative fold change to water + CABMV of (a) *PR-3*, (b) *SOD*, (c) *POD12*, (d) *PAL* and (e) *LOX2* genes expressions transcripts at 24 and 72 hai. Ct values were normalized with *ERS*, *NDID* and *EF1a1*. Values represent the average relative fold change of mRNA transcripts of three technical replicates from three biologically independent replicates of each treatment at each time point. Error bars represent the standard deviation. ** and *** represent significant statistical differences with $p$ <0.01 and $p$ <0.001, respectively.

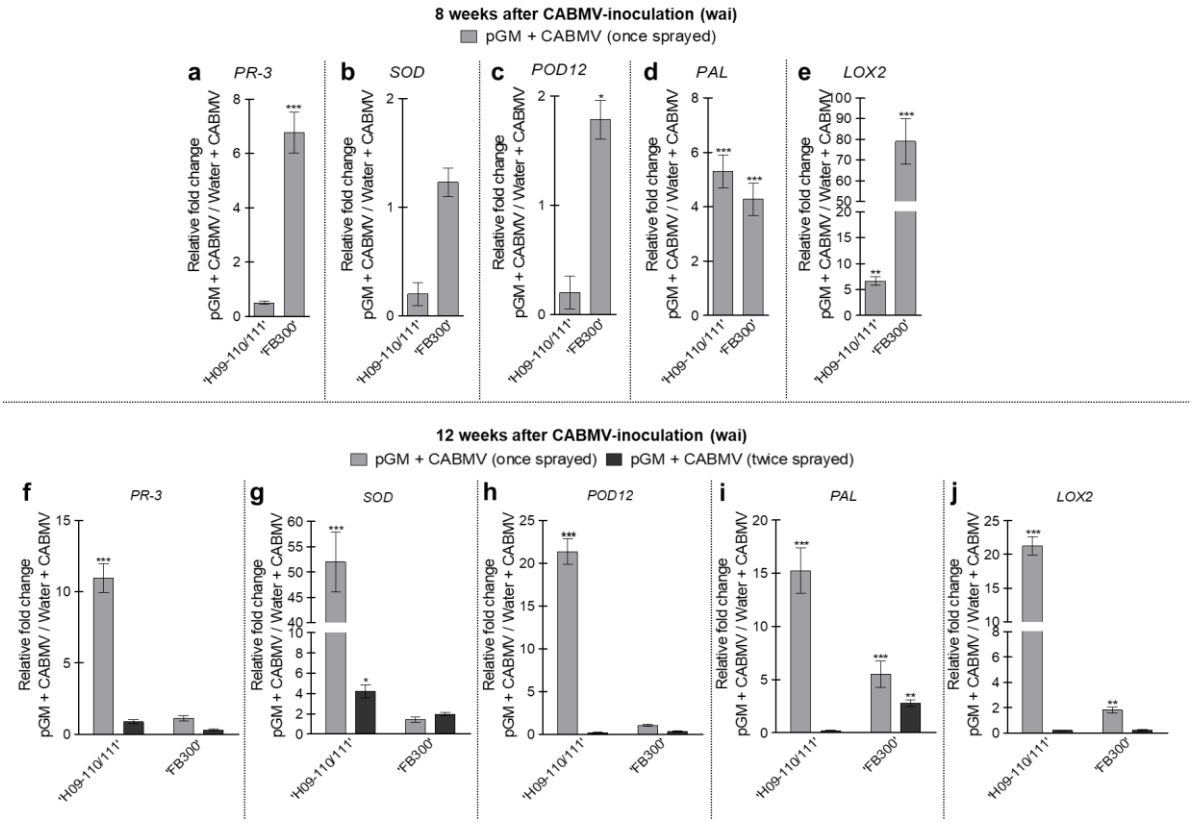



**Figure 6. pGM-induction of defense-related genes in passion fruit plants after CABMV-inoculated in field conditions.** RT-qPCR in pGM + CABMV (once sprayed) relative fold change to water + CABMV of (a) *PR-3*, (b) *SOD*, (c) *POD12*, (d) *PAL* and (e) *LOX2* genes expressions transcripts at 8 wai; RT-qPCR in pGM + CABMV (once and/or twice sprayed) relative fold change to water + CABMV of (f) *PR-3*, (g) *SOD*, (h) *POD12*, (i) *PAL* and (j) *LOX2* genes expressions transcripts at 12 wai. Ct values were normalized with *ERS*, *NDID* and *EF1a1*. Values represent the average relative fold change of mRNA transcripts of three technical replicates from three biologically independent replicates of each treatment at each time point. Error bars represent the standard deviation. *, ** and *** represent significant statistical differences with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

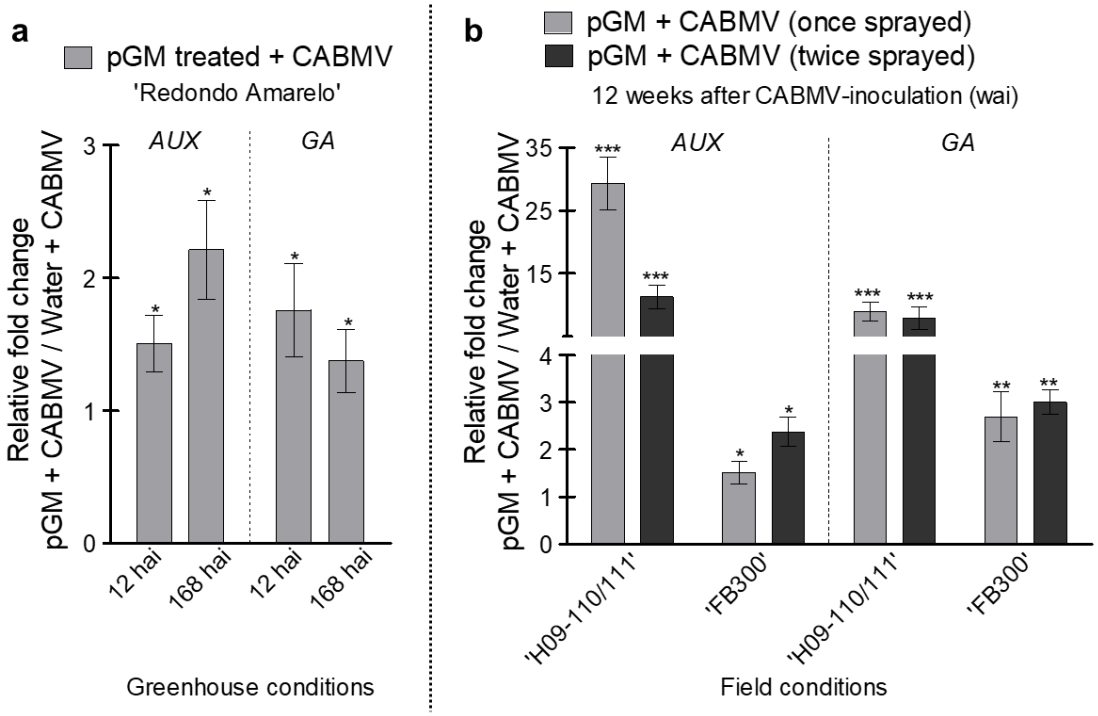



**Figure 7. pGM-induction of phytohormones signal pathways genes in passion fruit plants after CABMV-inoculated in field conditions.** (a) RT-qPCR in pGM + CABMV relative fold change to water + CABMV of *AUX* and *GA* genes expressions transcripts at 12 and 168 hai in passion fruit 'Redondo Amarelo' plants under greenhouse conditions. (b) RT-qPCR in pGM + CABMV (once and/or twice sprayed) relative fold change to water + CABMV of *AUX* and *GA* genes expressions transcripts at 12 wai in passion fruit 'H09-110/111' and 'FB300' plants under field conditions. Ct values were normalized with *ERS*, *NDID* and *EF1a1*. Values represent the average relative fold change of mRNA transcripts of three technical replicates from three biologically independent replicates of each treatment at each time point. Error bars represent the standard deviation. *, ** and *** represent significant statistical differences with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

## Supplementary information

**Supplementary Table S1.** pGM effect on the CABMV disease incidence (DI%) and disease severity (DS%) in three genotypes of passion fruit plants in greenhouse conditions. 2 at 9 weeks after CABMV inoculation (wai) are shown.

| a. 'FB300' | 2 wai | | 4 wai | | 6 wai | | 9 wai | |
|---|---|---|---|---|---|---|---|---|
| **Treatments** | **DI%** | **DS%** | **DI%** | **DS%** | **DI%** | **DS%** | **DI%** | **DS%** |
| Uninoculated plants | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0c | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0b |
| Water + CABMV | 66.6 ± 51.6a | 52.0 ± 40.6a | 100 ± 0.0a | 81.6 ± 15.5a | 100 ± 0.0a | 83.7 ± 18.9a | 100 ± 0.0a | 95.0 ± 5.4a |
| pGM + CABMV | 0.0 ± 0.0b | 0.0 ± 0.0b | 16.6 ± 40.8b | 13.7 ± 33.6b | 50.0 ± 54.7b | 31.2 ± 36.8ab | 100 ± 0.0a | 57.5 ± 20.7ab |
| **P value (n=6)** | *0.0017 | #0.0082 | *0.0001 | #0.0008 | *0.0003 | #0.0022 | *0.0001 | #0.0003 |

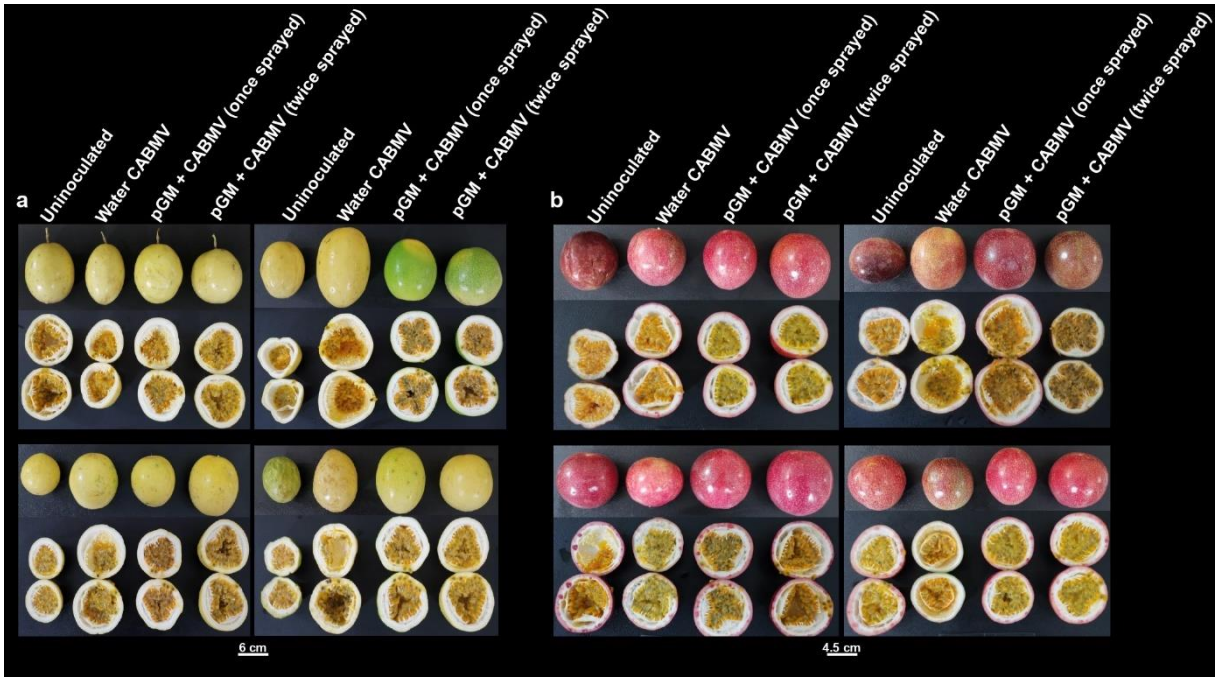
| a. 'H09-110/111' | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Treatments** | **DI%** | **DS%** | **DI%** | **DS%** | **DI%** | **DS%** | **DI%** | **DS%** |
| Uninoculated plants | 0.0 ± 0.0c | 0.0 ± 0.0b | 0.0 ± 0.0c | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0b | 0.0 ± 0.0b |
| Water + CABMV | 100 ± 0.0a | 86.1 ± 18.1a | 100 ± 0.0a | 98.6 ± 4.1a | 100 ± 0.0a | 98.6 ± 4.1a | 100 ± 0.0a | 98.6 ± 1.3a |
| pGM + CABMV | 55.5 ± 52.7b | 37.5 ± 35.9b | 55.5 ± 52.7b | 56.1 ± 42.3b | 77.7 ± 44.1a | 70.8 ± 40.9a | 88.9 ± 33.3a | 66.6 ± 38.0b |
| **P value (n=9)** | *0.0001 | #0.0001 | *0.0001 | #0.0001 | *0.0001 | #0.0001 | *0.0003 | #0.0001 |

Each value is the means (± standard deviation). Different letters in columns indicate significant differences between treatments.

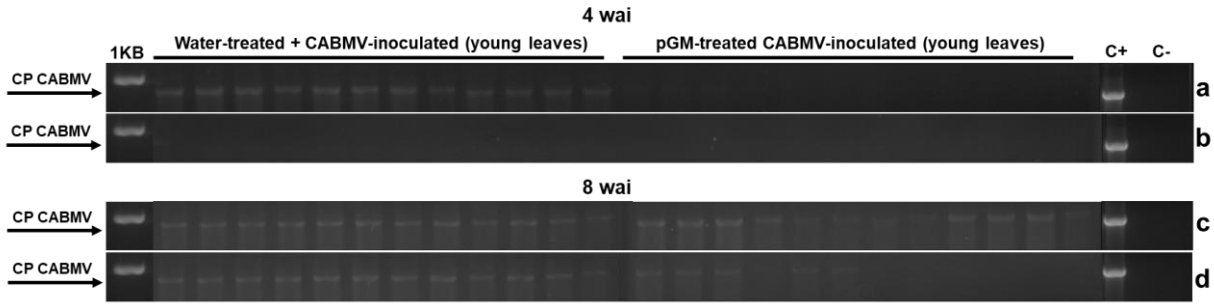
*Corresponding results of One-Way ANOVA. Significant differences according to using the Bonferroni post-hoc test ($p$ <0.05).

#The P value is based on the Kruskal–Wallis test. Significant Dunn's multiple comparison test ($p$ <0.05).


**Supplementary Figure 1. Development of whole passion fruit plants per treatment 18 and 25 wai under field conditions.** (a, b) 'FB300' and (c, d) 'H09-110/111'. CABMV-infection leads to a reduction in the development of plants in uninoculated (Natural infection), water + CABMV, while in pGM + CABMV (once and twice sprayed) it did not affect the development and plant vigour.




**Supplementary Figure 2. Qualitative description of fruits in *Passiflora edulis*** (a) 'FB300' (yellow peel) and (b) 'H09-110/111' (purple peel) in each treatment.




**Supplementary Figure 3. CABMV detection by RT-PCR in passion fruit plants in field conditions.** Young leaf samples from pGM + CABMV and water + CABMV at 4 wai in (a) 'FB300' and (b) 'H09-110/111'; and at 8 wai in (d) 'FB300' and (e) 'H09-110/111'. Amplicons of 1311 bp denote the presence of CABMV CP. The amplified products were submitted to 1% (w/v) agarose gel electrophoresis with ethidium bromide and visualized under ultraviolet light (UV). 1KB - 1 kb DNA Ladder Plus (LabAid$^{TM}$). C+ (CABMV positive control) and C – (Negative control).

## 5.4. Capítulo IV. Passion fruit treatment with biostimulants induces defense-related and phytohormone-associated genes.

(Trabalho submetido na *Pesquisa Agropecuária Brasileira – PAB)*

Pesquisa Agropecuária Brasileira


# Effect of a fungal glycoprotein on the control and mitigation of damage caused by Cladosporium herbarum in passion fruit plants under greenhouse conditions

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| Manuscript Type: | Original Article |
| Area of knowledge: | POMOLOGY, PHYTOPATHOLOGY, DISEASES CONTROL, CROP SCIENCE |
| Keyword: | Peptidogalactomannan, Passiflora edulis, scab, bioelicitor, Cladosporium herbarum |
| | |
| Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online. | |
| Supplementary Figure 1 (300dpi).tif<br>Supplementary Figure 2 (300dpi).tif | |

# Effect of a fungal glycoprotein on the control and mitigation of damage caused by *Cladosporium herbarum* in passion fruit plants under greenhouse conditions

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## Abstract

Phytosanitary problems drastically strengthen passion fruit cultivation around the world. Among them, scab, a fungal disease that attacks the aerial part of plants, especially the younger leaves, impairs development and reduces plant productivity. The objective of this work was to evaluate the effect of a fungal cell wall glycoprotein in the control of *Cladosporum hebarum* infection, which causes scab, in two *Passiflora edulis* genotypes, 'H09-110/111' and 'FB300', under greenhouse conditions. pGM treatment was able to mitigate the damage caused to plant development in parameters such as height, number of leaves, stem diameter, leaf area and biomass in the 'H09-110/111' genotype when compared to the control. However, in the genotype 'FB300', no significant differences were observed with respect to the control. Passion fruit scab disease incidence and severity were also reduced by pGM treatment. Therefore, this study suggests that the use of pGM can lead to control and attenuation of the damage caused by this fungus in the early stages of passion fruit plants 'H09-110/111', that are more susceptible to attack by phytopathogens, increasing their defense and mitigating future delays in plant development.

**Index terms:** Peptidogalactomannan; *Passiflora edulis*; scab, bioelicitor, *Cladosporium herbarum*.

## Resumo

Problemas fitossanitários atingem drasticamente a cultura do maracujazeiro em todo o mundo. Entre elas, a verrugose, doença fúngica que ataca a parte aérea das plantas, principalmente as folhas mais jovens, prejudica o desenvolvimento e reduz a produtividade das plantas. O objetivo deste trabalho foi avaliar o efeito de uma glicoproteína de parede celular fúngica no controle da

infecção por *Cladosporum hebarum*, causadora da verrugose, em dois genótipos de *Passiflora edulis*, 'H09-110/111' e 'FB300', sob condições de casa de vegetação. O tratamento com pGM foi capaz de mitigar os danos causados ao desenvolvimento das plantas em parâmetros como altura, número de folhas, diâmetro do caule, área foliar e biomassa no genótipo 'H09-110/111' quando comparado com o controle. Porém, no genótipo 'FB300', não foram observadas diferenças significativas em relação ao controle. A incidência e severidade da doença também foram reduzidas pelo tratamento com pGM. Portanto, este estudo sugere que o uso da pGM pode levar ao controle e atenuação dos danos causados por este fungo nas fases iniciais do maracujá 'H09-110/111', quando são mais suscetíveis ao ataque de fitopatógenos, aumentando sua defesa e mitigando atrasos no desenvolvimento da planta.

**Termos para indexação:** Peptideogalactomanana, Passiflora edulis, verrugose, bioelicitor, *Cladosporium herbarum*.

## Introduction

Passion fruit (*Passiflora edulis* Sims) is one of the most important fruits grown in Brazil. Although Brazilian production is quite significant in relation to other producing countries (IBGE, 2020), the volume produced is insufficient to meet the domestic demand for fresh fruits, as well as for concentrated juice. Productivity is impaired by several diseases that can compromise plant development and fruit quality. Diseases that affect this culture in the initial stages of plant growth are a major problem, as they affect the aerial and leaf parts of the plant, causing physiological distress in processes such as photosynthesis and resulting in plant development delay. Among the fungal diseases affecting passion fruit plants, scab is relevant in the fresh fruit market due to the visual appearance of the fruit, which presents rough lesions, with a cork appearance, which does not favor the choice of the product by consumers. The causal agent of scab is *Cladosporium herbarum*, this is a ubiquitous genus in the family *Cladosporiaceae* of the recently proposed order *Cladosporiales* in the *Dothideomycetes* (Abdollahzadeh et al., 2020). This fungus, in addition to causing lesions on the fruits, also affects the aerial part of the young passion fruit plant, causing lesions on the branches, leaves and flowers (Joy et al., 2016). Fungal diseases of the aerial part have been basically controlled by the application of pesticides, imposing the risks of overspraying, increased production costs and large yield losses. Therefore, it is important to address new alternatives for the control of

these diseases by strengthening measures based on good agricultural practices and eco-friendly products, which help to control, manage and mitigate damage caused by them. *C. herbarum* fungus prefers mild temperatures between 15 and 25 °C and high humidity. The spread of the pathogen occurs mainly through contaminated seeds and seedlings and by wind. In addition, when scabs occur in passion fruit seedlings, they can cause symptoms on the stems and leaves, which can cause the seedlings to die (Joy et al., 2016).

Products developed from natural or biological sources have a high potential to control plant diseases. Bioelicitors act by activating plant resistance mechanisms, thus generating a cascade of signals that culminate in the activation of the host plant's defense genes. This phenomenon is known as resistance induction. It is a natural phenomenon of plant response to pathogen attack, which is activated as soon as the interaction between host and pathogen occurs (Pascholati et al., 2011). The success in plant infection is due to the pathogen's ability to overcome the protective barriers imposed by the plant, whether structural or biochemical, pre- or postformed. In addition, much of this success is linked to the speed of the plant's response at the time of interaction with the virulent microorganism; that is, the faster the plant's response is, the lower the probability of occurrence of the disease (Melo et al., 2017). Due to the demands of organic farming and pesticide-free fruit in the consumer market, there is growing interest in developing safer and more effective alternative compounds to control plant diseases. The exploration of natural products, such as pGM, in the control of fungi is of great importance in the alternative control of passion fruit seedlings. The aim of this study was to evaluate the effect of a fungal cell wall glycoprotein, a peptidogalactomannan (pGM) in controlling the severity of the disease and mitigating the damage caused by the fungus *Cladosporium herbarum* in two genotypes of passion fruit seedlings under greenhouse conditions.

**Materials and methods**

Experiments were carried out in a greenhouse in Embrapa Agrobiologia (Empresa Brasileira de Pesquisa Agropecuária), located in the municipality of Seropédica, Rio de Janeiro State. The geographical coordinates are 220 48’00’’ south latitude and 430 41’00’’ west longitude. Two genotypes of *Passiflora edulis* hybrid ‘H09-110/111’ and cultivar ‘FB300’ were grown in tubes containing 1/3 vermiculite, 1/3 earthworm humus and 1/3 washed sand and maintained in greenhouse conditions under tropical area natural light and temperature conditions. Two months later, the seedlings were transplanted to plants grown in coconut fiber

substrate in plastic pots of 40 liters containing 100 g of phosphorus and 800 g of organic compound (Organosolo). One month after treatment, 800 g of earthworm humus was added to each pot. Two plants of different genotypes were placed in each pot and kept in a greenhouse under tropical area natural light and temperature conditions.

For the peptidogalactomannan (pGM) extraction, *Cladosporium herbarum* fungus was grown in potato dextrose broth medium (PDB) for 7 days, and the fungal mass was obtained using 3MM paper filtration. Glycoprotein extraction was performed according to Haido et al. (1998). Briefly, fungus mycelium was extracted with 0.05 M phosphate buffer, pH 7.2, at 100 °C for 2 h. After filtration, the filtrate was vacuum evaporated and precipitated in 92.8% (v/v) ethanol at 4 °C. The precipitate was resuspended in water, dialyzed, and freeze-dried to obtain crude pGM.

During the first stages of plant development and before starting the treatments, due to favorable weather conditions for fungal diseases, a natural *Cladosporium* infection occurred, appearing symptoms of brown spots on the leaves of all plants destined for the experiment (Supplementary Figure 1). To avoid interfering with the effects of the treatments, no solution was applied to control this fungus. All the plants in the treatment were infected, showing symptoms of the fungus.

Treatments were performed in plants with 2 to 3 true leaves, with a foliar spray of 100 $\mu g.mL^{-1}$ pGM, performed with a costal manual sprayer (Jacto - XP). Control plants were treated with foliar spray tap water. The experimental design was randomized using 12 plants for each genotype and for each treatment, and all the plants of the treatments (water treatment and pGM treatment) had the same level of severity (number of spots) and development (height and number of leaves) before starting foliar spray.

Disease incidence (DI%) was determined based on leaf symptoms (the three youngest leaves) on diseased plants (at least one leaf showing typical disease spots). The proportion of diseased plants was estimated by $DI = (n/N) \times 100$ (DI = incidence; n = number of diseased plants; N = total number of plants assessed) (Kone et al. 2017). The severity disease of "scabs" was evaluated before and after starting the treatment in the first three youngest leaves of the plant, according to a scale of score: 1= 0 - 3 spots; 2= 3 - 6 spots; 3= 6 - 12 spots; 4= 12 - 25 spots; 5= 25 - 50 spots, under the occurrence of a natural inoculum source. This scale corresponds to part of the grading scale cited by Negreiros et al. (2004).

Plant development was evaluated over time, height, number of leaves, stem diameter, and leaf area of the youngest fifth leaf (count from above to below) dry biomass of aerial parts and roots. Height was measured with the aid of a measuring tape, the leaves were manually counted, the diameter of the stem was measured with the aid of a digital caliper (MTX-316119), the leaf area was measured by means of a leaf area scanner (LI-Cor Biosciences, model LI-3100c) for biomass were determined using accuracy electronic scale (Bioprecisa – JA3003N), the plants shoots and root system were cut, placed in individual paper bags and dried at 60 °C for 72 h, before weight dry. Weights of the aerial part and root system.

Data obtained for development parameters and disease incidence were analyzed using a paired Student's t-test at a significance level of $p < 0.05$; for disease severity, a nonparametric Wilcoxon rank sum test was used ($p < 0.05$). GraphPad Prism software version 5.00 for Windows was used for statistical analysis.

**Results and Discussion**

Passion fruit seedlings growing in a greenhouse naturally infected by *C. herbarum* were evaluated for scab disease and showed a disease index of 100% for all plants from the two passion fruit genotypes. On day 0, half of the plants were treated with 100 µg.ml of pGM, and half were treated with water. In 'H09-110/111', the incidence of the disease decreased considerably with the treatment, with a decrease of 75% at 5 weeks after treatment (wat) compared to day 0 evaluation, with significant differences from the control (Table 1a) (Disease incidence, 5 wat: $F_{1,21} = 8.65$, $p < 0.05$) and continued to decrease in the following weeks, where the treated plants did not present symptoms in the systemic leaves. The symptoms also decreased in water-treated plants, probably because the relative humidity started to fall during these weeks (INMET, 2021), and the fungal conditions were more favorable with high moisture.

Regarding the disease severity caused by the fungus, it was observed that in 'H09-110/111', there was a decrease in DS at 5 wat in pGM-treated plants compared to the control. These differences, however, were not observed at 7 wat because the severity in the leaves of the control also decreased; moreover, at 9 wat in the control, the severity began to increase, and significant differences were observed at this evaluation time (Table 2a) (Wilcoxon rank sum test, at 5 wat: $W = 33$; $p < 0.05$; at 7 wat: $W = 3$; $p > 0.05$; at 9 wat: $W = 26$; $p < 0.05$). However, in 'FB300', differences between water- and pGM-treated plant responses were initially

observed at 2 wat. The severity of the control also decreased in the following weeks, without significant differences between pGM and water-treated plants. However, at 9 wat, the severity began to increase in the water-treated plants, leading to significant differences between treatments (Table 2b) (Wilcoxon rank sum test, at 2 weeks: $W = 57$; $p < 0.05$; at 9 weeks: $W = 21$; $p < 0.05$).

The concentration of conidia in the air showed a high correlation with relative humidity of approximately 80%, and temperatures between 23 - 29 °C were an indication of the favorable conditions of the *Cladosporium* spp. (Sadyś et al., 2015). Here, we observed that in 'H09-110/111', the disease incidence and disease severity significantly decreased at 5 wat compared to the control ($p$ <0.05) (Table 1a; Table 2a). However, in 'FB300', although the incidence started to decrease after 5 wat on pGM treatment, no significant differences were observed over time, since the incidence also decreased in the control ($p$ >0.05) (Table 1b). On the other hand, in the disease severity, we observed significant differences at 2 and 9 wat ($p$ <0.05) (Table 2b).

The results obtained in this study showed the inhibitory effect on *Cladosporium herbarum* severity in young leaves of passion fruit seedlings under greenhouse conditions. Plants treated with pGM presented a decrease in severity at various evaluation times after treatment. To date, few studies have reported the control of this disease. Willingham et al. 2002 observed that in fields with favorable climatic conditions for fungal diseases, the incidence of scab was significantly reduced in passion fruit plants with the use of chemical fungicides such as azoxystrobin, acibenzolar and trifloxystrobin. These same authors observed that chemical control with acibenzolar was able to induce protection of passion fruit seedlings in decreasing the severity caused by scab. Campo-Arana et al. (2019) observed that the application of Mancozeb alternating with potassium phosphite and Mancozeb alternating with azoxistrobin showed outstanding control of anthracnose (*Colletotrichum gloesporioides*) by significantly reducing the levels of severity in yellow passion fruit. In the same way, Mengal et al. (2020), observed that Iprovalicarb + Propineb, Neem extract and *Neurospora* sp. can be used for *Cladosporium* control in grapes. However, Pérez and Lannacone (2006) reported that the application of fungicides is not a fully efficient alternative to control fungal diseases caused by *Colletotrichum*, for example.

Development parameters were evaluated throughout the experiment. The plant height showed significant differences in 'H09-110/111' plants 5 weeks after pGM treatment,

remaining constant until 9 wat, compared to water-treated plants (Figure 1a) (height, at 5 wat: $t = 3.85$, $df = 11$, $p < 0.05$; at 7 wat: $t = 3.81$, $df = 11$, $p < 0.05$; at 9 wat: $t = 3.17$, $df = 11$, $p < 0.05$). Nonetheless, in 'FB300', although the mean pGM-treated plant height was greater at 9 wat, no significant differences were observed with respect to the control (Figure 1b) ($p > 0.05$). With respect to leaf emission, it was observed that there was a greater number of leaves in pGM-treated of 'H09-110/111' plants in 5 at 9 wat (Figure 1c) (Number of leaves at 5 wat: $t = 3.39$, $df = 11$, $p < 0.05$; at 7 wat: $t = 3.73$, $df = 11$, $p < 0.05$; at 9 wat: $t = 3.11$, $df = 11$, $p < 0.05$). These same differences were not observed in 'FB300', where the number of leaves per plant was similar in the treatments (Figure 1d) ($p > 0.05$). Another developmental parameter evaluated was the stem diameter, observing significant differences in pGM at 7 and 9 wat in 'H09-110/111' (Figure 1e) (stem diameter, at 7 wats: $t = 3.71$, $df = 11$, $p < 0.05$; at 9 wats: $t = 4.46$, $df = 11$, $p < 0.05$). Though, in 'FB300', no statistically significant differences were observed (Figure 1f) ($p > 0.05$). In the size of the leaf area of the youngest fifth leaf at 9 wat, we observed significant differences in 'H09-110/111' in pGM treatment with respect to the control, while in 'FB300' no significant differences were observed. (Figure 2) (Leaf area, 'H09-110/111' at 9 wat: $t = 2.80$, $df = 11$, $p < 0.05$; 'FB300' at 9 wat: $t = 1.45$, $df = 11$, $p > 0.05$). In the plant biomass parameter at 9 wat, differences were observed in the dry weight of the root system in plants treated with pGM in 'H09-110/111', while in 'FB300' the root dry weight was similar between treatments (Figure 3a) (Dry weight of root system, 'H09-110/111' at 9 wat: $t = 4.08$, $df = 11$, $p < 0.05$; 'FB300' at 9 wat: $t = 0.88$, $df = 11$, $p > 0.05$). Regarding the biomass of the aboveground part of the plant, significant differences were maintained in 'H09-110/111', while in 'FB300', no differences were observed between treatments (Figure 3b) (dry weight of aerial part, H09-110/111 at 9 wat: $t = 2.91$, $df = 11$, $p < 0.05$; FB300 at 9 wat: $t = 1.44$, $df = 11$, $p > 0.05$). The general appearance of the roots was better in the pGM treatment than in the control, especially in 'H09-110/111' (Figure 3c).

When the control agent is applied before germination and establishment of fungal diseases, it could be more effective than when applied after pathogen establishment (Agrios, 2005). Here, we demonstrate the effect of pGM in the control after natural infection with sacb disease, leading to reduced severity and mitigating the damage caused by the fungus in the development of passion fruit 'H09-110/111' seedlings in terms of height parameters, number of leaves, diameter of the stem, biomass and leaf area (Figures 1, 2, and 3). Passion fruit plants

at 9 wat looked better than the control, being more developed plants, especially in 'H09-110/111' (Supplementary Figure 2). The increase in plant development parameters associated with the use of pGM after scab infection in a greenhouse can be attributed to the ability of this bioelicitor to delay or harm the fungal infection process in passion fruit seedlings. Studies carried out in the pretreatment of passion fruit for the biocontrol of *Fusarium* wilt using *Trichoderma harzianum* led to a decrease in the severity of this disease and an increase in plant height and dry weight (Wasike, 2014). Possible explanations for the increase in plant development parameters using defense bioinducers may be that pathogen control leads to stronger growth, soil nutrient uptake and growth hormone production (Dewen et al., 2017).

This is the first report on scab in passion fruit plants treated with a bioelicitor after infection.

**Conclusions**

1. Fungal glycoprotein (pGM) may be used in passion fruit plants 'H09-110/111' infected with *Cladosporum herbarum* to eliminate scab disease progression.
2. pGM treatment led to a decrease in fungal severity and an increase in plant development parameters compared to the control.
3. Results of pGM treatment observed for the 'H09-110//111' genotype may be explained by a faster activation of the defense response induced by pGM in this genotype.

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**Tables and Figures**

**Table 1.** Effect of pGM treatment on the scab disease incidence (DI%) in passion fruit plants (a) 'H09-110/111' and (b) 'FB300' 0 at 9 weeks after treatment (wat).

| | | | | | |
|---|---|---|---|---|---|
| *Temperature (°C) | 21.3 | 19.2 | 19.8 | 18.9 | 23.2 |
| *Relative humidity (%) | 76 | 74 | 69.9 | 66.5 | 61.5 |
| a. 'H09110/111' | Disease incidence (DI%) | | | | |
| Treatments | 0 wat | 2 wat | 5 wat | 7 wat | 9 wat |
| Water treatment | 100 ± 0a | 100 ± 0a | 83.3 ± 38.9a | 25 ± 45.0a | 41.6 ± 51.4a |
| pGM treatment | 100 ± 0a | 100 ± 0a | 25.0 ± 45.2b | 0.0 ± 0.0a | 0.0 ± 0.0b |

| | | | | | |
|---|---|---|---|---|---|
| b. 'FB300' | Disease incidence (DI%) | | | | |
| Treatments | 0 wat | 2 wat | 5 wat | 7 wat | 9 wat |
| Water treatment | 100 ± 0a | 100 ± 0a | 83.3 ± 38.9a | 8.3 ± 28.8a | 41.6 ± 51.4a |
| pGM treatment | 100 ± 0a | 100 ± 0a | 58.3 ± 51.4a | 0.0 ± 0a | 16.6 ± 38.9a |

Each value is the means (± standard deviation) of an independent experiment using 12 plants per treatment. Different letters in columns indicate significant differences between treatments according to t-test ($p$ >0.05).

Ccorresponding to t-test, (a) H09-110/111, 0 wat: $t$=1.0, $df$=11, $p$ >0.05; 2 wat: $t$=1.0, $df$=11, $p$ >0.05; 5 wat: $t$=3.0, $df$=11, $p$ <0.05; 7 wat: $t$=1.9, $df$=11, $p$ >0.05; 9 wat: $t$=2.8, $df$=11, $p$ <0.05. (b) FB300, 0 wat: $t$=1.0, $df$=11, $p$ >0.05; 2 wat: $t$=1.0, $df$=11, $p$ >0.05; 5 wat: $t$=1.3, $df$=11, $p$ >0.05; 7 wat: $t$=1.0, $df$=11, $p$ >0.05; 9 wat: $t$=1.9, $df$=11, $p$ >0.05.

*Temperature (°C) and relative humidity (%) data obtained from the INMET (Instituto Nacional de Meteorologia) database, 2021.

**Table 2.** Effect of pGM treatment on the sacb disease severity (DS) in passion fruit plants (a) 'H09-110/111' and (b) 'FB300' 0 at 9 weeks after treatment (wat).

| | | | | | |
|---|---|---|---|---|---|
| *Temperature (°C) | 21.3 | 19.2 | 19.8 | 18.9 | 23.2 |
| *Relative humidity (%) | 76 | 74 | 69.9 | 66.5 | 61.5 |
| **a.** 'H09-110/111' | Disease severity (DS%) | | | | |
| Treatments | 0 wat | 2 wat | 5 wat | 7 wat | 9 wat |
| Water treatment | 2.08 ± 0.76a | 1.56 ± 0.58a | 1.45 ± 0.40a | 1.02 ± 0.9a | 1.2 ± 0.26a |
| pGM treatment | 2.16 ± 0.78a | 1.66 ± 0.65a | 1.05 ± 0.12b | 1.0 ± 0.0a | 1.0 ± 0.0b |
| | | | | | |
| **b.** 'FB300' | Disease severity (DS%) | | | | |
| Treatments | 0 wat | 2 wat | 5 wat | 7 wat | 9 wat |
| Water treatment | 2.25 ± 0.58a | 1.80 ± 0.47a | 1.22 ± 0.32a | 1.08 ± 0.2a | 1.38 ± 0.50a |
| pGM treatment | 2.33 ± 0.61a | 1.29 ± 0.45b | 1.22 ± 0.32a | 1.0 ± 0.0a | 1.02 ± 0.09b |

Each value is the means (± standard deviation) of an independent experiment using 12 plants per treatment. Different letters in columns indicate significant differences between treatments according to non-parametric Wilcoxon rank sum test ($p$ <0.05).

*Temperature (°C) and relative humidity (%) data obtained from the INMET (Instituto Nacional de Meteorologia) database, 2021.

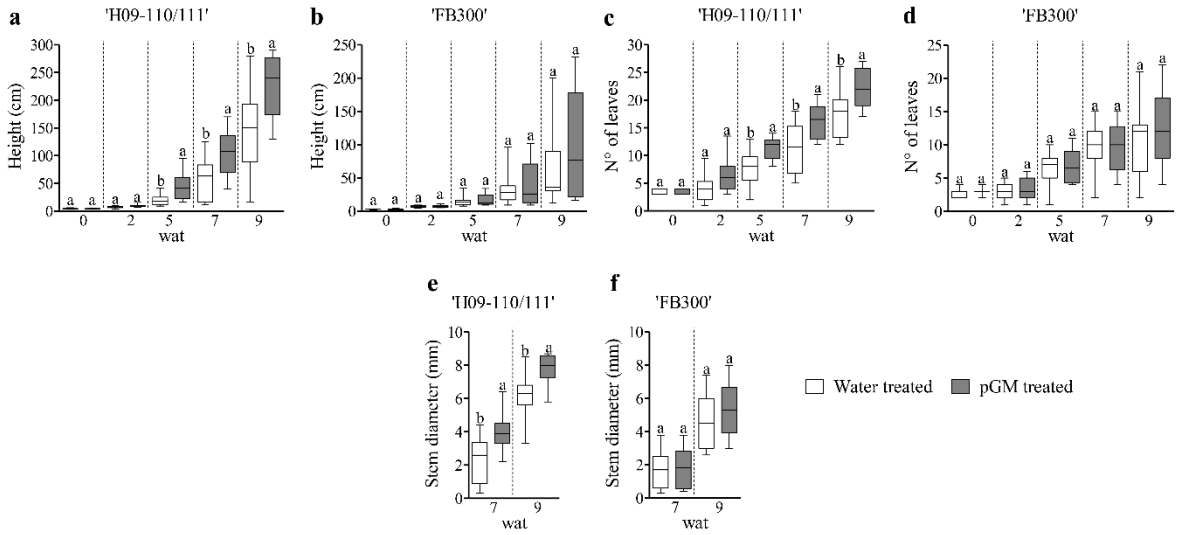



**Figure 1.** Effect of pGM on plant development parameters in passion fruit infected with scab disease. Height of 'H09-110/111' (a) and 'FB300' (b); number of leaves of 'H09-110/111' (c) and 'FB300' (d); stem diameter of 'H09-110/111' (e) and 'FB300' (f). Averages correspond to independent experiments using 12 plants per treatment in greenhouse conditions through the time after treatment. Bars represent the averages of the plants per treatment each, and different letters denote groups between which there is a significant difference at $p < 0.05$. The lower and upper edges of boxes represent the first and third quartiles, with the horizontal line inside representing the average value. Whiskers extend to the highest and lowest data points in the interquartile range.

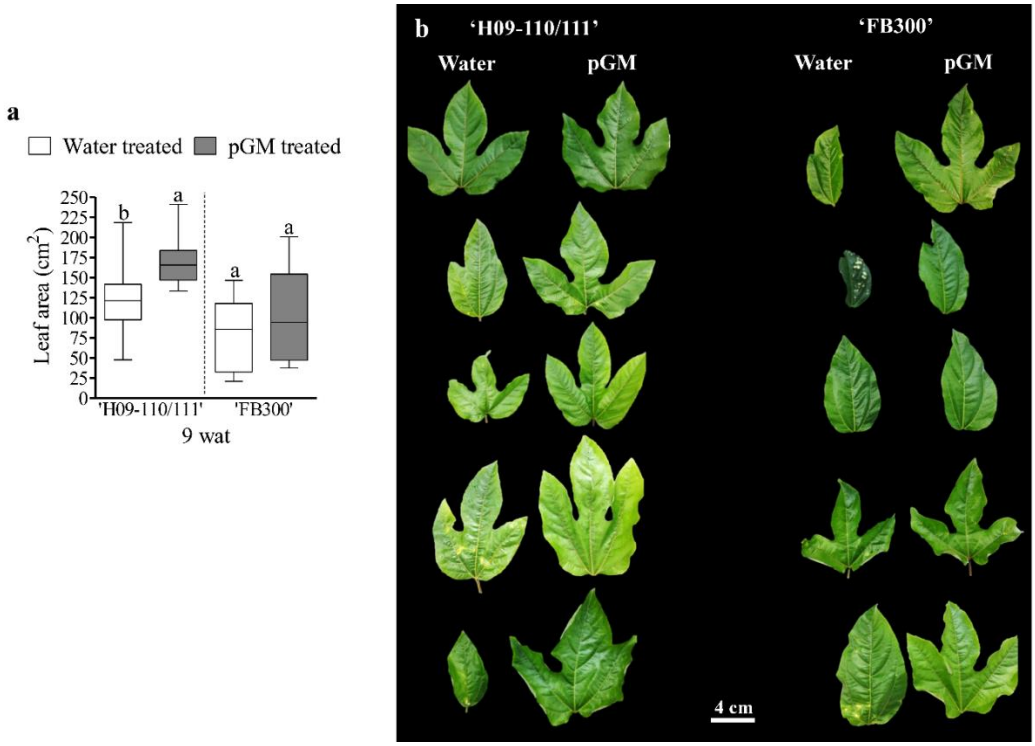



**Figure 2.** pGM effect in the leaf area on the fifth youngest leaf of passion fruit plants 'H09-110/111' and 'FB300' (a) of the independent experiment using 12 leaves per treatment in greenhouse conditions at 9 weeks after treatment (wat). Visual representation of some leaves collected for leaf area reading (b). Bars represent the averages of the plants per treatment each, and different letters denote groups between which there is a significant difference at $p < 0.05$. The lower and upper edges of boxes represent the first and third quartiles, with the horizontal line inside representing the average value. Whiskers extend to the highest and lowest data points in the interquartile range.

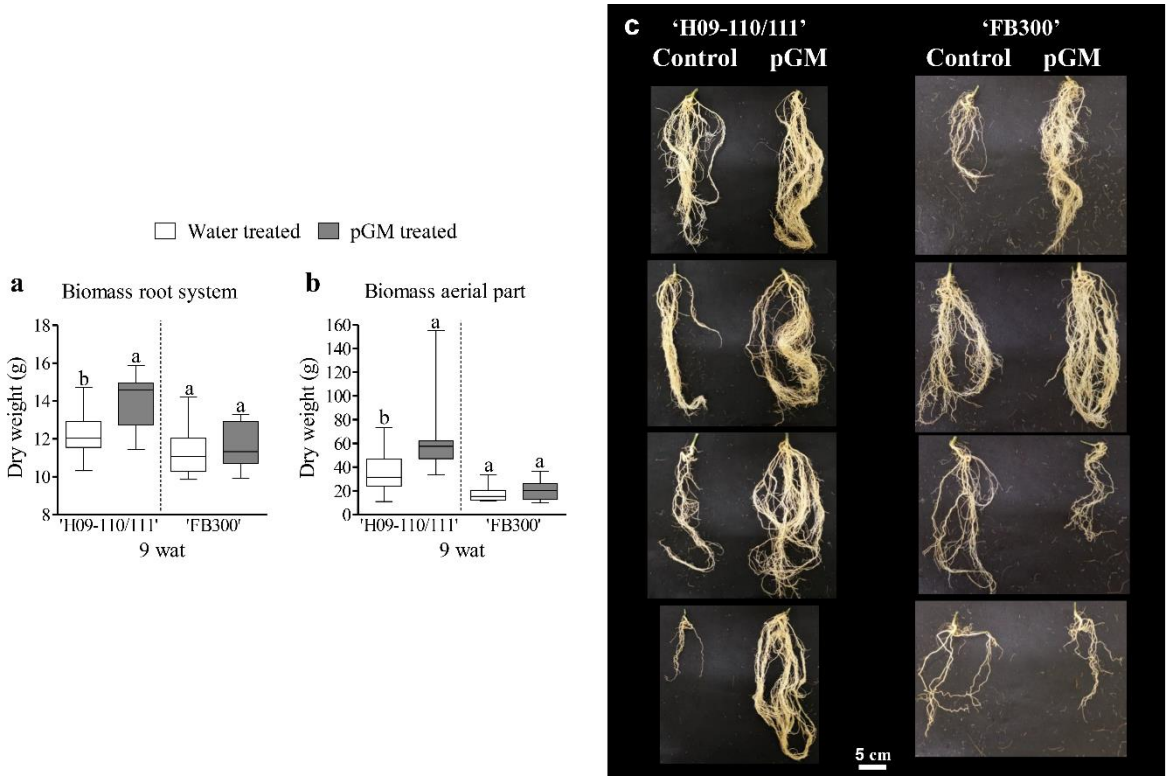
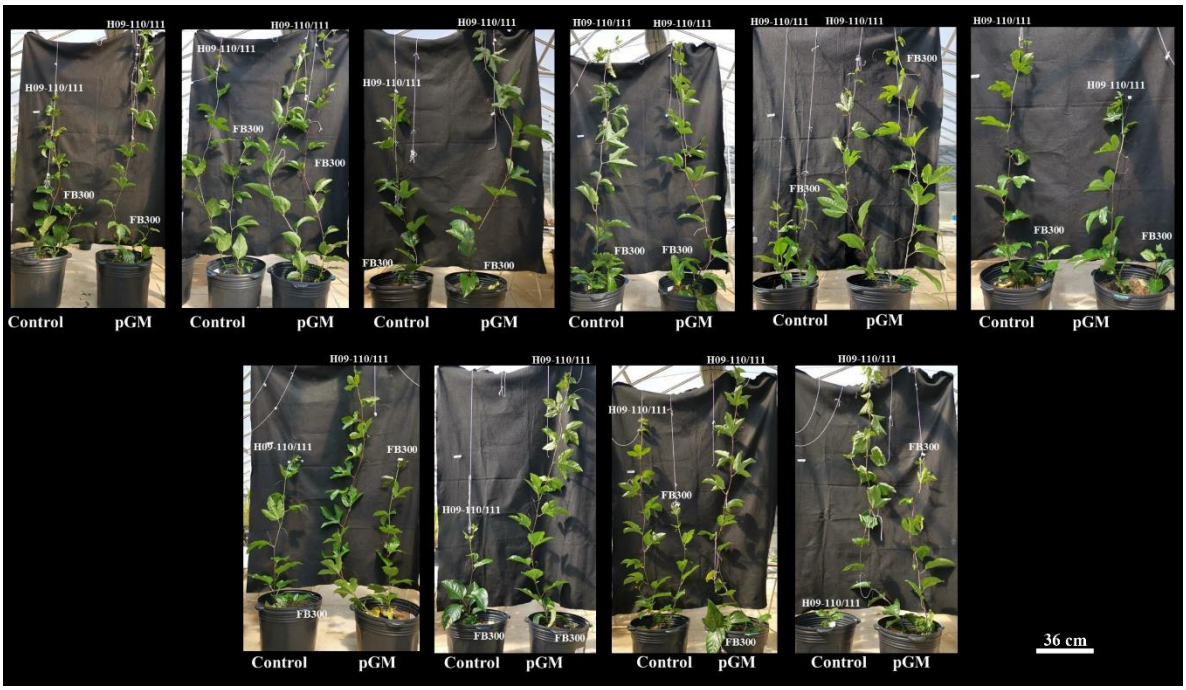



**Figure 3. Evaluation of biomass in passion fruit under greenhouse conditions.** Dry weight of the root system (a), aerial part (b), and appearance of root (e) in passion fruit 'H09-110/111' and 'FB300' of the independent experiment using 12 plants per treatment in greenhouse conditions at 9 weeks after treatment (wat). Bars represent the averages of the plants per treatment each, and different letters denote groups between which there is a significant difference at $p < 0.05$. The lower and upper edges of boxes represent the first and third quartiles, with the horizontal line inside representing the average value. Whiskers extend to the highest and lowest data points in the interquartile range.


**Supplementary Figure 1.** Leaf symptomatology and infection status or scab severity caused by *Cladosporium herbarum* fungus in passion fruit (a) 'H09-110/111' and (b) 'FB300' seedlings before starting treatments. Plant leaves showing circular, translucent, and necrotic spots, characteristic symptoms of scab in leaf tissue of the plant.




**Supplementary Figure 2.** General development status of passion fruit plants 'H09-110/111' and 'FB300' at 9 weeks after treatment under greenhouse conditions.

## 6. DISCUSSÃO

O gênero *Passiflora* é originário da América do Sul, e a maior parte deste gênero encontra-se no Brasil, sendo o maior centro de diversidade genética desse gênero (FALEIRO et al., 2015). O Brasil é o maior produtor e consumidor mundial de maracujá com uma produção de 690.364 mil toneladas por ano, numa área plantada de 46.530 hectares e com rendimentos médios de 14,8 toneladas por hectare (IBGE, 2020), no entanto, nos últimos dez anos, está cultura tem baixado sua produção e área plantada, já que para o ano 2010 a produção era de 922.334 mil toneladas por ano, e uma área destinada de 62.401 mil hectares, havendo uma diminuição do 25% em ambos parâmetros durante os últimos dez anos (IBGE, 2020). Esta diminuição, deve-se em grande parte aos problemas fitossanitários que atingem esta cultura. Atualmente, uma das principais doenças no maracujazeiro é causada por vírus, especificamente o cowpea aphid-borne mosaic virus (CABMV), causador da doença do endurecimento dos frutos, chegando a ser uns dos fatores mais limitantes em algumas regiões produtoras (DAMATTO JUNIOR et al., 2014). Uma vez instalada na cultura, o CABMV dissemina-se com muita rapidez e pode em apenas quatro meses infectar todas as plantas (NARITA et al., 2012). As perdas na produtividade podem atingir até 80% (NASCIMENTO, 2017). Quanto mais cedo as mudas forem infectadas, maiores serão os danos causados a elas (GIORIA et al., 2000). Além do declínio na produção, o CABMV também compromete a qualidade do fruto, a produtividade, a longevidade dos pomares e reduz o valor comercial do fruto (FALEIRO et al., 2011; SANTOS et al., 2015b; FREITAS et al., 2016).

Geralmente, agricultores de maracujá, produzem as mudas em campo, muitas vezes sem a devida proteção, sendo o plantio com mudas pequenas com 4 – 5 folhas verdadeiras e entre 20 e 30 cm de altura aproximadamente, facilitando a infecção destas plantas e a disseminação do CABMV, já que, até essas plantas atingirem a maturidade e desenvolvimento para que iniciem sua produção, passam muito tempo em campo, estando expostas a contaminação viral. A sequência e o escalonamento do plantio tornam as culturas mais velhas como grandes fontes de inoculo, já que o CABMV é praticamente exclusivo do maracujá (NASCIMENTO, 2017). A falta de uma época definida de plantio faz com que haja culturas de diversas idades numa mesma região, e isso faz com que permanentemente haja fontes do vírus.

Neste trabalho observamos o efeito bioestimulante tanto de defesa como no desenvolvimento das plantas tratadas com bioinoculantes como a pGM, ácido húmico (AH) e

bactérias promotoras de crescimento (PGPB) no maracujazeiro. Além, disso, observamos uma forte indução do sistema de defesa de plantas de diferentes genótipos de maracujá mediado pelo tratamento com pGM, levando as plantas a apresentarem tolerância ao CABMV, permitindo que plantas tratadas e infectadas se desenvolvessem de maneira semelhante às plantas saudáveis em condições de casa de vegetação e campo, aumentando a produtividade destas sob condições de campo e mitigando os danos causados pelo vírus.

Este é o primeiro relato conclusivo demonstrando tolerância mediada por um indutor de defesa contra a infecção pelo CABMV em diferentes genótipos de maracujá, que resulta em graves perdas econômicas nesta cultura. Com a identificação de novos bioestimulantes elicitores de defesa, soluções ecologicamente corretas para a proteção de plantas podem se tornar uma realidade em poucos anos. No geral, este estudo também é o primeiro a analisar o efeito de uma glicoproteína de parede celular fúngica na indução de tolerância em plantas de maracujá e como estas plantas tratadas respondem à infecção por CABMV em condições de campo. Transdução de sinal de proteína, desintoxicação, biossíntese de fenilpropanóide, bem como algumas outras vias importantes relacionadas à defesa e fitohormônios, foram encontradas diferencialmente expressas e podem desempenhar um papel na regulação dessas vias. Embora os mecanismos subjacentes de como esses genes e vias relacionadas afetam ou regulam a resposta do maracujá ao patógeno viral ainda exijam mais investigação em estudos futuros, o conhecimento obtido neste estudo servirá como um recurso útil para facilitar futuras pesquisas, e o tratamento com elicitores em resposta à infecção por vírus fornece muitas direções possíveis sobre a interação maracujá-vírus.

Devido à ineficiência no controle do CAMBV e do seu vetor, e que ainda não há disponível para os passicultores cultivares comercias que sejam resistentes ao CABMV, o lançamento de um produto que mitigue os danos causados pela doença causada por este vírus, visando reduzir as perdas na produção e na qualidade dos frutos é muito útil. Em função dos resultados positivos e promissores obtidos em trabalhos anteriores do grupo e neste trabalho, foi criada a startup **“TolVeg”** a qual atualmente está na etapa experimental no escalonamento e eficiência na produção da pGM, realizando vários testes preliminares para definir as melhores condições de crescimento e produção de massa fúngica para extração da molécula. Em dados preliminares da TolVeg, a produção de 1 grama de pGM custa ao redor de R$43,67. Segundo cálculos baseados nos experimentos feitos neste trabalho, para pulverização de 1 ha em mudas

de maracujá, são necessários aproximadamente 6 gramas de pGM (diluídos em 60 L de água), chegando a um custo de produção da molécula de aproximadamente R$262,02/ha. Mais testes de eficiência na produção, onde diversas condições juntas como temperatura, pH, agitação e quantidade de inoculo estão sendo realizados pela empresa, com a finalidade de diminuir os custos da extração e produção da pGM, para que prontamente esteja disponível aos produtores de maracujá a pGM. Este produto seria uma alternativa para o manejo do CABMV, com a vantagem de ser ecologicamente amigável com o meio ambiente, podendo ser utilizado em qualquer sistema de produção seja orgânico o convencional.

## 7. CONCLUSÕES

Os resultados obtidos permitem concluir que

a) A pGM de *C. herbarum* apresenta efeito bioestimulante e protetor quando pulverizada na concentração de 100 ug.ml-1 na parte aérea de plantas de maracujá em casa de vegetação e em campo. O efeito em campo se mostrou robusto em duas distintas localidades do estado do Rio de Janeiro com distância de aproximadamente 350 km e apresentando condições climáticas e de solo distintas;

b) Os efeitos bioestimulante e protetor foram observados em 3 distintos genótipos de maracujá (‘Redondo Amarelo’, ‘FB 300’ e ‘H09-110/111’);

c) Genes *PR*-3, *POD12*, *SOD*, *LOX2* e *PAL* ligados a defesa e os genes que codificam para os fitohormônios *auxina* (*AUX*) e *giberelina* (*GA*) foram induzidos pelos tratamentos nos distintos genótipos;

d) Quando confrontadas com o vírus CABMV (causador do endurecimento do fruto) por inoculação mecânica, as plantas tratadas com pGM mostraram forte tolerância, acumulando até 80% menos de vírus e mostrando forte atenuação na severidade e incidência da doença;

e) As plantas tratadas com pGM e infectadas com o CABMV apresentaram perfil de desenvolvimento em casa de vegetação e em campo semelhante ao das plantas saudáveis não infectadas;

f) O tratamento com pGM levou a um aumento de produtividade de 65,7 e 114% em ‘FB300’, e 44 e 80% em ‘H09-110/11’, com uma e duas pulverizações, respectivamente, quando comparadas com os controles não tratados.

g) Os bioinsumos AH + consórcio bacteriano mostraram ação bioestimulante e bioindutora de defesa em maracujá sozinhos e em associação com a pGM, mostrando que estes bioinsumos podem ser usados em conjunto;

h) pGM foi capaz de inibir a verrugose em plantas jovens de maracujá quando utilizada após a colonização do fungo.

Os resultados obtidos desta tese, portanto, evidenciaram um importante papel da aplicação de bioestimulantes na cultura do maracujazeiro; e que podem ser estendidos a outras culturas agrícolas.

Nossos resultados indicam que os bioinsumos pGM e ácido húmico mais consórcio bacteriano combinados ou não, induzem fitohormônios e vias de defesa contra estresses bióticos, o que pode explicar o aumento de produtividade observado com esses tratamentos. Dessa forma, esses produtos bioestimulantes podem representar uma ferramenta promissora tanto para o aprimoramento da defesa quanto para o desenvolvimento e ganho de biomassa de plantas de forma sustentável.

Este é o primeiro estudo a analisar o efeito da glicoproteína fúngica (pGM) na mitigação dos danos causados tanto a nível molecular, como no desenvolvimento e produtividade, pelo CABMV em diferentes genótipos de *P. edulis* sob condições de casa de vegetação e campo. Além disso, a pGM mostrou resultado positivo quando usada em plantas de maracujá infectadas com *Cladosporum herbarum*, eliminando a progressão da doença causada por este fungo, diminuindo severidade e mitigando os danos causados no desenvolvimento das plantas sob condições de casa de vegetação.

Transdução de sinal de proteína, desintoxicação, biossíntese de fenilpropanoides, bem como alguns outros sinais importantes relacionados à defesa e fitohormônios, parecem estar envolvidos nas respostas de melhoria imunológica das plantas tratadas. Embora os mecanismos subjacentes das vias relacionadas à defesa que regulam a resposta do maracujá a estresses bióticos ainda requeiram mais pesquisas, o conhecimento obtido com este estudo servirá como uma ferramenta útil na cultura do maracujá.

Com a identificação de novos bioestimulantes, soluções ecologicamente corretas para a proteção e estimulação de desenvolvimento das culturas, podem se tornar uma realidade em poucos anos.

## 8. REFERÊNCIAS BIBLIOGRÁFICAS

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# 9. ANEXOS

## 9.1. Anexo 1. Deposito de pedido de patente.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

22/12/2020 870200160006 12:02

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**Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT**

**Número do Processo:** BR 10 2020 026358 7

**Dados do Depositante (71)**

**Depositante 1 de 1**

**Nome ou Razão Social:** UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
**Tipo de Pessoa:** Pessoa Jurídica
**CPF/CNPJ:** 33663683000116
**Nacionalidade:** Brasileira
**Qualificação Jurídica:** Instituição de Ensino e Pesquisa
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PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 22/12/2020 às 12:02, Petição 870200160006

## Dados do Pedido

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| **Natureza Patente:** | 10 - Patente de Invenção (PI) |
| **Título da Invenção ou Modelo de Utilidade (54):** | COMPOSIÇÃO BIOINDUTORA, MÉTODO DE APLICAÇÃO DA COMPOSIÇÃO BIOINDUTORA E SEU USO |
| **Resumo:** | A presente invenção revela uma composição bioindutora que compreende galactomanana e peptídeo extraídos do fungo Cladosporium herbarum e pelo menos um ou todos os compostos selecionados do seguinte grupo: solvente polar, cálcio, carbonatos, sulfatos, sódio e boro. Adicionalmente, também é revelado um método de aplicação da referida composição e seu uso para proteção de plantas contra doenças e pragas |
| **Figura a publicar:** | 1 |

**PETICIONAMENTO ELETRÔNICO** Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 22/12/2020 às 12:02, Petição 870200160006

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## Documentos anexados

| Tipo Anexo | Nome |
|---|---|
| Comprovante de pagamento de GRU 200 | 29409161927703602 GRU-200 Nat10.pdf |
| Relatório Descritivo | Relatório Descritivo - Minuta Final - 690.26.pdf |
| Reivindicação | Reivindicações - Minuta Final - 690.26.pdf |
| Desenho | Desenhos - Minuta Final - 690.26.pdf |
| Resumo | Resumo - Minuta Final - 690.26.pdf |

**PETICIONAMENTO ELETRÔNICO** Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 22/12/2020 às 12:02, Petição 870200160006

## 9.2. Anexo 2. "Review paper" publicado durante o doutorado na revista "*Biotechnology Research & Innovation*".

Biotechnology Research and Innovation (2020) 3, 19–26

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

REVIEW ARTICLE

# Biotechnological solutions for major cotton (*Gossypium hirsutum*) pathogens and pests

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Received 25 October 2019; accepted 16 January 2020
Available online 1 February 2020

**Abstract** Cotton (*Gossypium* spp. L.) is the largest source of natural fibers in the world, with a planted area of more than 33 million hectares in 2019. Biotic stress caused by a variety of pathogens and pests has considerable negative impacts on cotton, and control measures increase global production costs. Among the most important diseases affecting cotton are bacteria and fungi that infect leaves, stems, roots and fruits. In addition, viruses, nematodes, insects and mites cause considerable losses. Here, we summarize the diversity of biotic stresses affecting the cotton crop and highlight present and future biotechnological solutions for disease control, including transgenes, RNAi, gene editing and bioagents. We demonstrate that ''Ag Biotech'' solutions help keep the cotton industry sustainable in cotton-producing countries.

## Major cotton pathogens and pests

Cotton (*Gossypium* spp. L., Malvaceae) is the largest source of natural fibers in the world. In addition, cotton is one of the most important speculative annual crops, generating substantial economic returns. The cotton industrial chain involves approximately 150 countries and provides income for approximately 100 million families. The worldwide cotton planting area in 2019 occupies more than 33 million hectares, and the most important cotton-growing regions are in Central and East Asia, the southern United States (USA), the Brazilian Savanna and West Africa. The USA, along with Brazil, India, Uzbekistan and Australia, are the most important cotton fiber exporters. India is the greatest cotton producer, followed by China, the USA and Pakistan (USDA, 2019a).

As is the case for many major crops, biotic stress in cotton caused by pests and diseases contributes to annual losses worldwide of between 10 and 30%. Among the most important diseases affecting cotton are those caused by bacteria, such as bacterial blight, caused by *Xanthomonas citri* pv. *malvacearum* (Jalloul, Sayegh, Champion, & Nicole, 2015). Fungal pathogens are also of considerable importance, causing major diseases such as Fusarium wilt (caused by *Fusarium oxysporum* f. sp. *vasinfectum*) (Cox, Babilonia, Wheeler, He, & Shan, 2019), Verticillium

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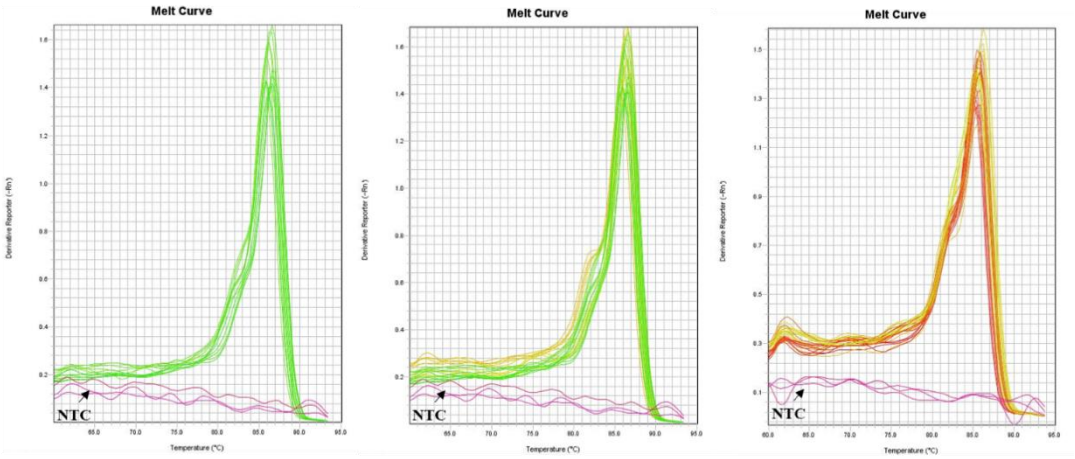
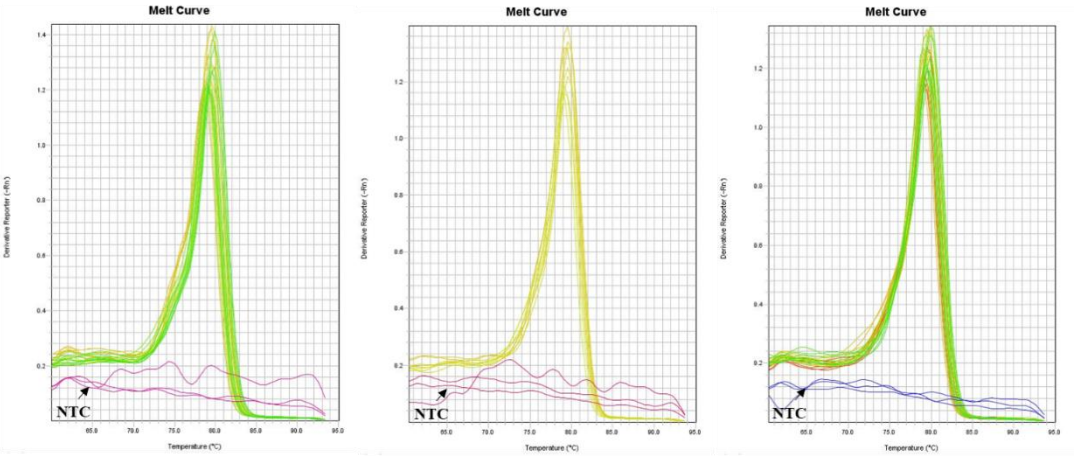
## 9.3. Anexo 3. Especificidade de amplificação da RT-qPCR com os cDNAs das amostras avaliadas em cada experimento.

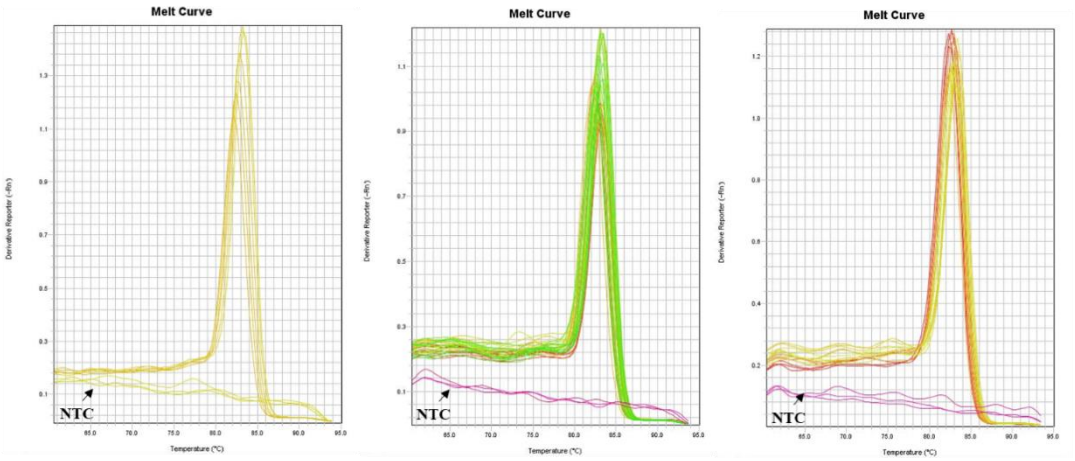
*PR-3*



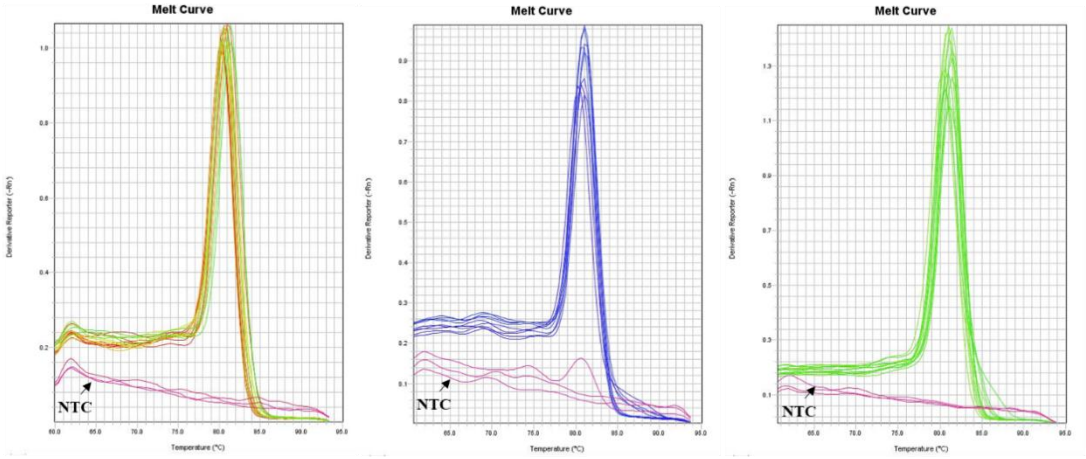

*POD12*




*SOD*



*PAL*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Melt Curve
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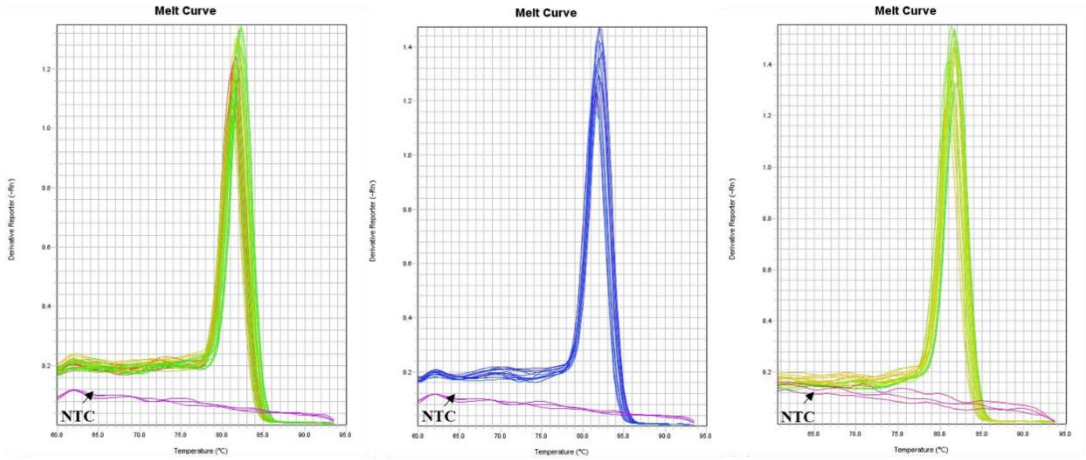
Temperature (°C)
Temperature (°C)

*LOX2*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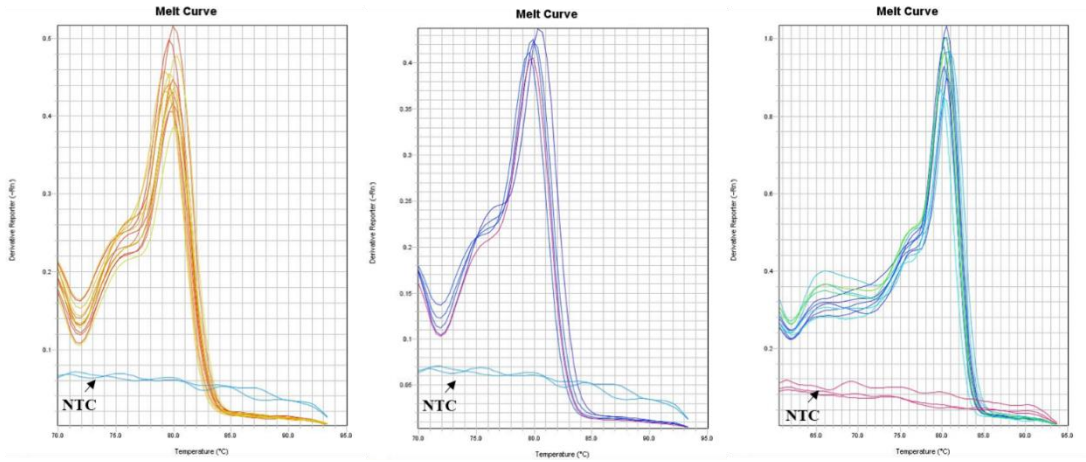
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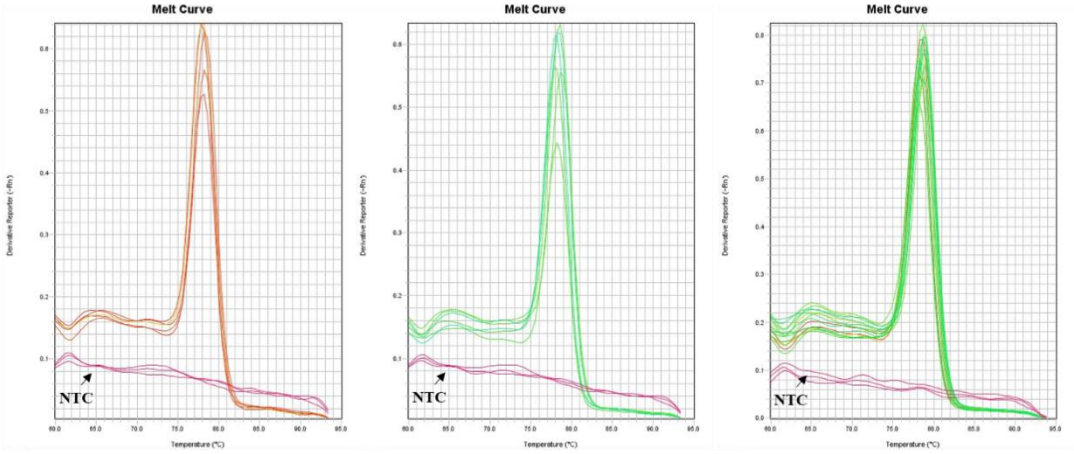
Temperature (°C)

*GA*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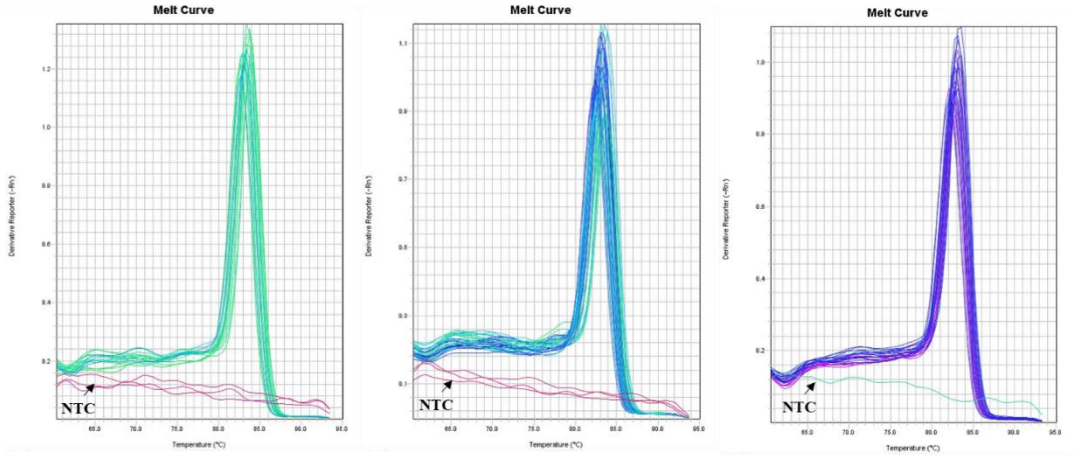
*NDID*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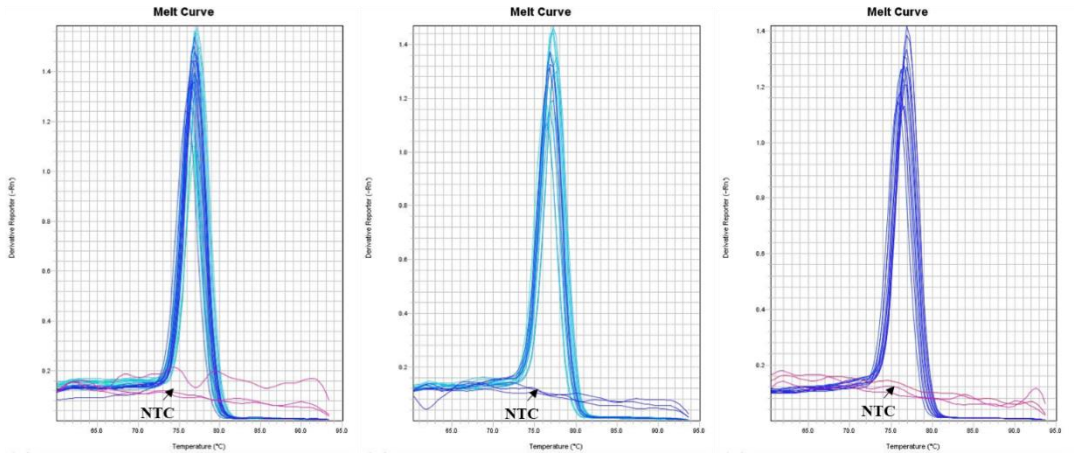
NTC

*ERS*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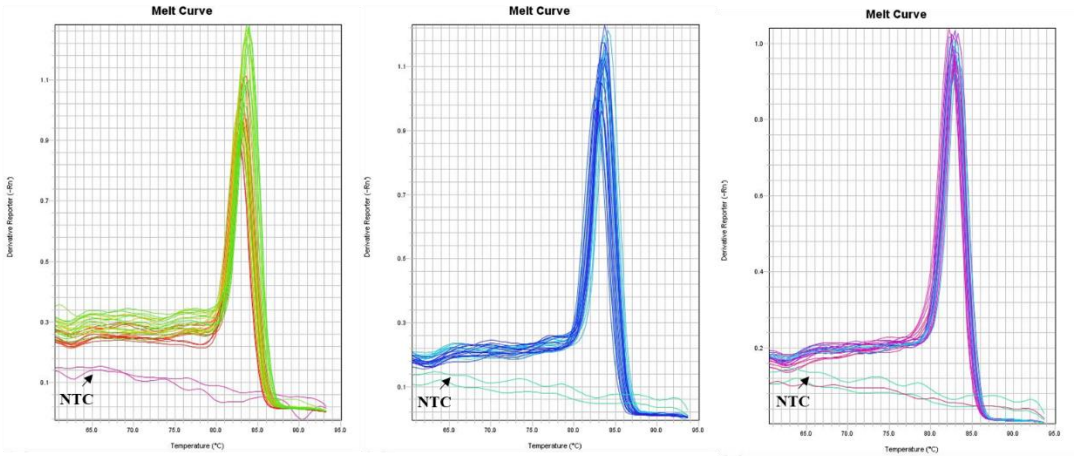
NTC

*EF1a1*




*qCABMV06*

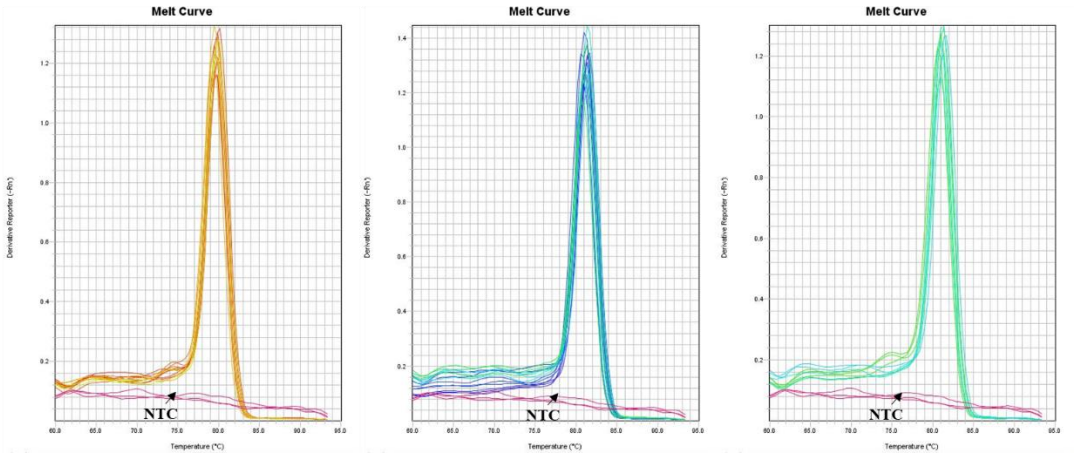



**Figura suplementar 1.** Especificidade dos primers desenhados para o estudo. Especificidade dos primers desenhados para o estudo. Curvas de dissociação (Meltings) dos primers dos genes estudados (*PR-3, POD12*, *SOD*, *PAL*, *LOX2*, *AUX* e *GA*), genes de referência utilizados no estudo (*NDID*, *EF1a1* e *ERS*) e genes utilizados para diagnostico do CABMV (qCABMV06), gerados pelo programa do aparelho 7500 Fast Real-Time PCR (Applied Biosystems). No eixo "y" está a derivada que indica a taxa de mudança na fluorescência do SYBR Green em função da temperatura. No eixo "x" está representada a temperatura. NTC representa o controle da reação (Branco).