




# 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  $\mu\text{g ml}^{-1}$  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). *Cladosporium 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  $\beta$ 1-3 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  $27 \pm 2$  °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  $\mu\text{g ml}^{-1}$  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  $\mu\text{m}$  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  $\mu\text{l}$  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  $\mu\text{g ml}^{-1}$  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:  $DI = \frac{\sum(DS \times P)}{(TNP \times HGS)} \times 100$ , 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  $\text{leaf area} = 0.25 + 0.64(L \times W)$ . 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  $2^{-\Delta\Delta C_t}$  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 ( $0.5 \mu\text{g ml}^{-1}$ ) (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  $100 \mu\text{g ml}^{-1}$  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  $100 \mu\text{g ml}^{-1}$ . 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  $100 \mu\text{g ml}^{-1}$  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  $100 \mu\text{g ml}^{-1}$  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  $100 \mu\text{g.ml}^{-1}$  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<sup>-1</sup> of pGM on PWD disease severity (a) incidence (b) along the time

Treatment	Disease severity (%)			
	2 wai*	3 wai	4 wai	5 wai
<b>a</b>				
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 <sup>-1</sup> pGM + CABMV (1:10)	23.1 ± 41.4b	45.2 ± 41.1c	48.2 ± 42.9b	59.2 ± 37.9b
100 µg ml <sup>-1</sup> pGM + CABMV (1:50)	37.6 ± 41.0b	37.2 ± 39.7c	55.7 ± 41.9b	50.2 ± 36.0b
Treatment	Disease incidence (%)			
<b>b</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 <sup>-1</sup> pGM + CABMV (1:10)	20.0 ± 41.0b	60.0 ± 50.2b	65.0 ± 48.0b	75.0 ± 44.0b
100 µg ml <sup>-1</sup> 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  $\chi^2_5 = 59.03$ ,  $p < 0.0001$

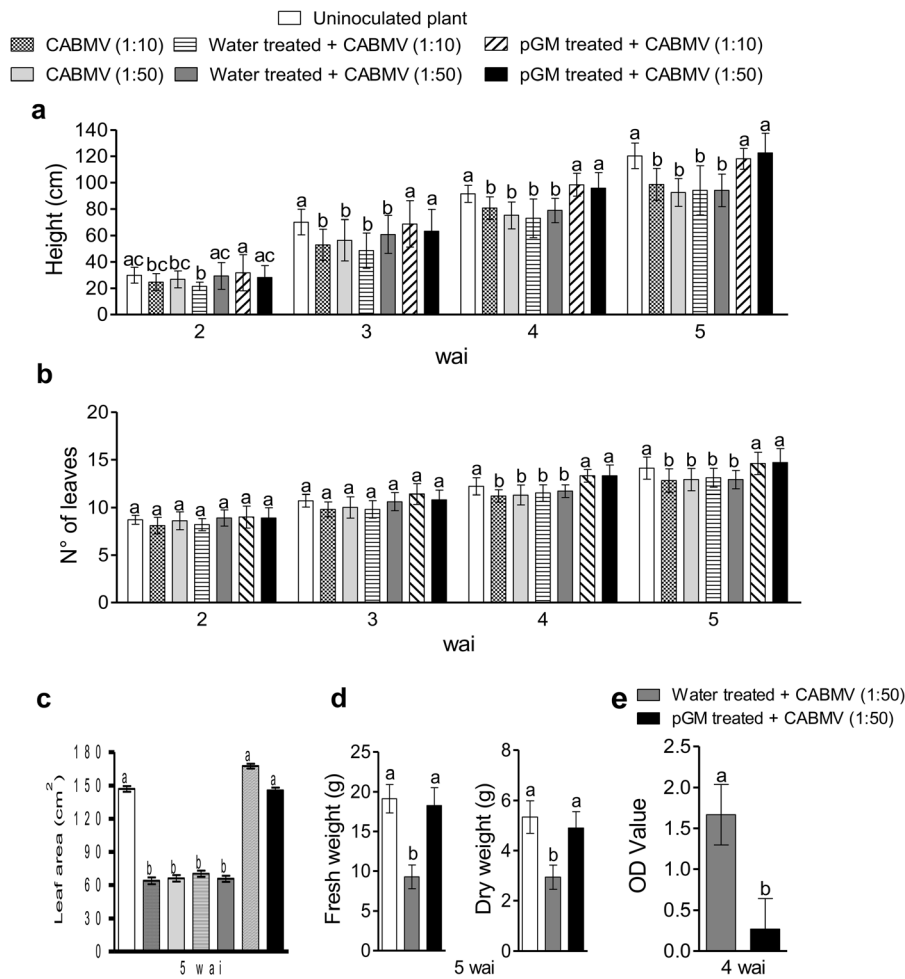
Corresponding results of One-way ANOVA. (b) Disease incidence at 2 wai:  $F_{5,114} = 28.77$ ,  $p < 0.0001$ ; at 3 wai:  $F_{5,114} = 12.80$ ,  $p < 0.0001$ ; at 4 wai:  $F_{5,114} = 8.52$ ,  $p < 0.0001$ ; at 5 wai  $F_{5,114} = 5.85$ ,  $p < 0.0001$

\*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  $\mu\text{g ml}^{-1}$  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  $\pm$  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 ( $\text{O}_2^-$ ) and hydrogen peroxide

(H<sub>2</sub>O<sub>2</sub>) in the treated plants. The presence of O<sub>2</sub><sup>-</sup> 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<sub>2</sub>O<sub>2</sub> 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*.

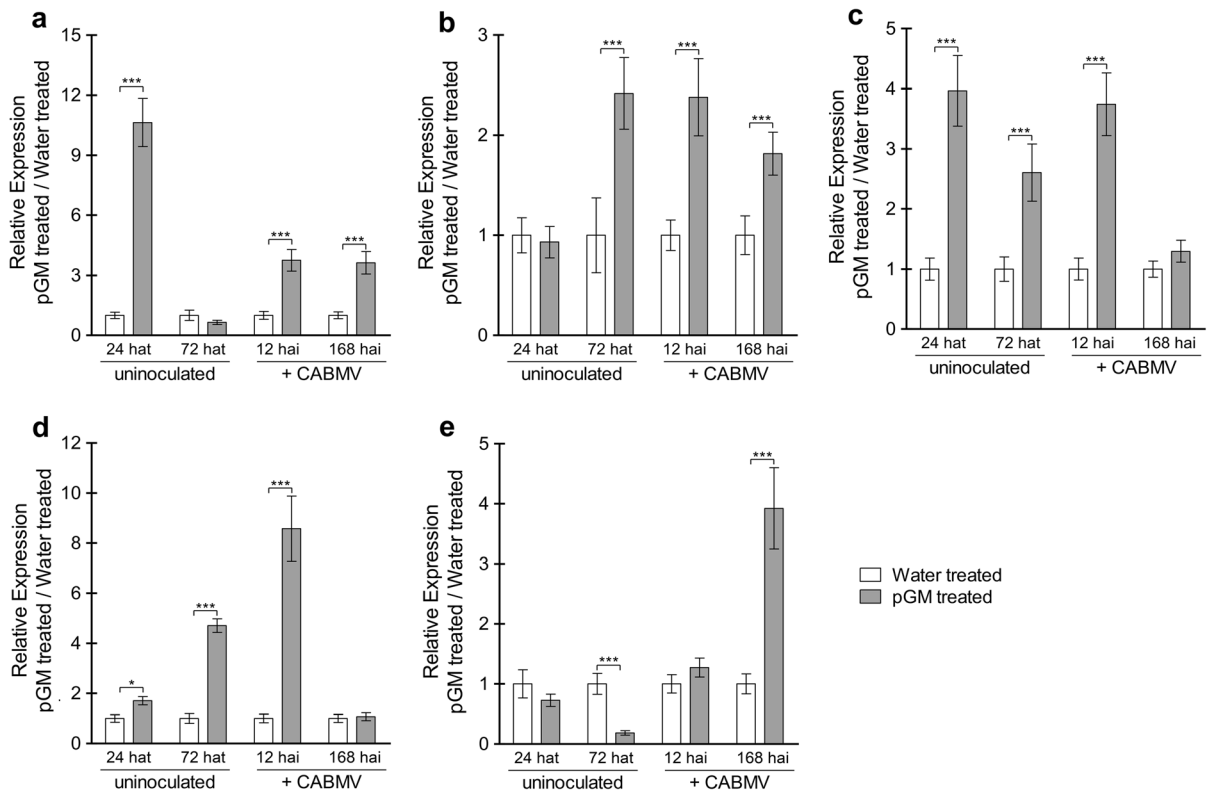
Genes associated with oxidative stress were also evaluated. Over-expression of *POD12* was observed at 24 and 72 h after pGM treatment alone (hat) and at 12 and 168 h after CABMV infection (hai) compared to water and water/CABMV treatments (*POD12*,  $F_{1,64} = 519.6$ ,  $p < 0.0001$ ). *SOD* was induced 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  $\mu\text{g ml}^{-1}$  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 ( $\pm$  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 Mofidnakhai 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 *Phytophthium* 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; Mofidnakhai 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 involve animal or human.

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