Cowpea aphid-borne mosaic virus (CABMV) in Passiflora by qPCR analysis reveals asymptomatic plants with viruses and new species with immunity

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Abstract

The passion fruit woodiness disease (*Cowpea aphid-borne mosaic virus* – CABMV) causes socioeconomic problems for Brazilian passion fruit crop. Understanding the temporal progress of the disease and identifying resistance sources to CABMV are essential steps to develop resistant varieties. The objective of the study was to evaluate temporal progress of passion fruit woodiness disease, identify *Passiflora* genotypes with CABMV resistance and to detect virus infection in asymptomatic plants by qPCR. The experiment was conducted in a greenhouse using 128 genotypes belonging to 12 species and three hybrids (inter and intraspecific) of *Passiflora* evaluated in five periods after inoculation. The symptoms severity was quantified from the disease index (DI%). The CABMV infection in symptomatic plants was confirmed by RT-PCR and in asymptomatic plants by qPCR. Progress rates and disease severity were lower in the species *P. cincinnata*, *P. gibertii*, *P. miersii* e *P. mucronata* compared to *P. edulis*, *P. alata*, *Passiflora* sp. and hybrids. Of the evaluated genotypes, 20.31% were resistant, with emphasis on the accessions of *P. suberosa*, *P. malacophylla*, *P. setacea*, *P. pohlii* e *P. bahiensis* that did not show symptoms of virus. The absence of symptoms does not imply immunity of plants to the virus, as the qPCR analysis confirmed infection by the virus in asymptomatic plants of *P. cincinnata*, *P. gibertii*, *P. miersii*, *P. mucronata*, *P. setacea*, *P. malacophylla* e *P. suberosa*. Even after four inoculations, the virus was not detected by qPCR in the upper leaves in plants of *P. pohlii* and *P. bahiensis* indicating that these species are immune to CABMV.

Introduction

Brazil stands out as the largest global producer of yellow passion fruit (*Passiflora edulis* Sims) [1, 2]. In 2018 the production was 602.651 t in an area of 42.731 ha. Despite being the largest worldwide producer, the average productivity of 14.1 t ha⁻¹ is low [3]. This low yield, in part, is associated with high severity of the passion fruit woodiness disease, attributed to *Cowpea aphid-borne mosaic virus* - CABMV [4–7].

CABMV (*Potyviridae* family and *Potyvirus* genus) has genome consisting of single stranded RNA (ssRNA), responsible for the expression of viral replication protein, accumulation, defense, viral movement and symptoms in infected plants [8, 9]. The virus acquisition and transmission are performed by aphides vectors (*Hemiptera* order and *Aphididae* family) in a non-circulatory and non-persistent way during the proof stunt [10–12]. Plants infected by CABMV present leaves with mosaic, blister or deformations, reduction in the plant size, deformed fruits, hardened and reduced size [10, 13].

Viruses is considered the disease with the highest socioeconomic impact for passion fruit cultivation in Brazil, because it reduces the longevity, productivity and fruit quality and there are no totally effective measures in the disease control [14, 15]. Indeed, there are important results in the adoption of disease management in field conditions, with the elimination of sick plants (roguing) and replacement with healthy plants, modifications in the plant conduction system as a measure of control and dissemination of the disease [16]. However, the implementation of these management strategies requires additional costs in the production, making it unfeasible for many producers that have low technology.

Despite advances in disease management, the use of resistant cultivars is considered the best strategy, because it does not demand increased production costs with manpower or chemicals [17, 18]. However, so far there are no yellow passion fruit cultivars (*P. edulis* Sims) with this attribute [18–20]. On the other hand, several studies indicate that wild passion tree species carry CABMV resistance genes [21–24], and it is an alternative to develop resistant cultivars [18, 25].

The evaluation of the Passion Fruit Germplasm Active Bank aiming to identify resistant wild genotypes is considered an indispensable step for the development of CABMV resistant cultivars [24]. However, the evaluation and accuracy in the quantification of viruses symptoms are highly dependent on the methodology used, which has direct relation to the quality of the data generated for subsequent manipulation and analysis [26]. In this pathosystem, the disease phytopathometric indices have been used to quantify the CABMV symptoms in *Passiflora* species [24, 26–28]. However, inferring the species reaction only with leaf symptoms evaluations has not been sufficient to determine the resistance level, because wild species with greater tolerance may not manifest leaf symptoms and are classified as immune, but the virus may be present in the tissues. In this context, the real time quantitative PCR (qPCR) opens the perspective for quantification of the viral load in plants due to the high sensitivity of the technique [29, 30]. Despite the relevance, there are few studies that identify the resistance and/or immunity of wild passion fruit species by means of qPCR.

Thus, this study had the following objectives: *i)* evaluate the progress of passion fruit woodiness disease symptoms (*Cowpea aphid-borne mosaic virus* – CABMV) in different *Passiflora* species, *ii)* perform screening in *Passiflora* spp. genotypes for identification of CABMV resistance sources, aiming to select genotypes with high resistance degree for using in interspecific hybridizations, and *iii)* detect the infection in plants with symptoms through RT-PCR and in asymptomatic plants from the quantitative real-time PCR (qPCR).

Material And Methods

Location and Plant material

The research was developed in Embrapa Mandioca e Fruticultura, located in Cruz das Almas, Bahia, Brazil (12°40'39" S, 39°06'23" O, 226 m altitude), with the climate of the transition region Am to Aw type (tropical sub-humid to dry) and annual average air temperature of 23.8 °C. One hundred and twenty eight *Passiflora* spp. genotypes were evaluated derived from the Passion fruit Germplasm Active Bank of Embrapa Mandioca e Fruticultura (GAB-Passion fruit) belonging to 12 species and three intra and interspecific hybrids, 54 genotypes of *Passiflora edulis* Sims., 27 interspecific hybrids (six simple hybrids (F_1) and 21 of the third generation of backcrossing [BC_3 - (*P.edulis* x *P.cincinnata*) x *P.edulis*]), six intraspecific hybrids, 21 of *P.cincinnata* Mast., three of *P. mucronata* Lam., *P. gibertii* N.E Brown., *P. alata* Curtis., and *P. setacea* DC., and one of *P. pohlii* Mast., *P. miersii* Mast., *P. bahiensis* Klotzsch., *P. malacophylla* Mast., *P. suberosa* L., *Passiflora* sp. Other two genotypes were used as control for the susceptibility reaction (*P. edulis*, cv. BRS Gigante Amarelo) and resistance (*P. cincinnata*, BGP200) [24]

during the entire evaluation of the symptoms caused by the passion fruit woodiness disease (*Cowpea aphid-borne mosaic virus* – CABMV) (Table 1).

Table 1. Relation of genotypes of *Passiflora* spp. used for the characterization of the reaction to passion fruit woodiness disease (*Cowpea aphid-borne mosaic virus* – CABMV).

Nº	Code*	Species	State	Nº	Code*	Species	State
1	BGP152	<i>P. sub</i>	SP	65	BC3.133	Inter H. ²	BA
2	BGP170	<i>P. mal</i>	SP	66	BGP449	<i>P. ed</i>	RJ
3	BGP434	<i>P. set</i>	BA	67	BC3.491	Inter H. ²	BA
4	BGP454	<i>P. poh</i>	BA	68	BGP418-S3	<i>P. ed</i>	BA
5	BGP477	<i>P. bah</i>	BA	69	BGP344	<i>P. ed</i>	BA
6	BGP244	<i>P. set</i>	BA	70	BGP161	<i>P. ed</i>	BA
7	BRS Pérola do Cerrado	<i>P. set</i>	DF	71	BGP399	<i>P. ed</i>	BA
8	BGP421	<i>P. cin</i>	BA	72	BC3Top-DX	Inter H. ²	BA
9	BGP422	<i>P. cin</i>	BA	73	BGP224	<i>P. ed</i>	BA
10	BGP279	<i>P. cin</i>	BA	74	BGP475	<i>P. ed</i>	BA
11	BGP276	<i>P. cin</i>	BA	75	BC3.584	Inter H. ²	BA
12	BGP085	<i>P. gib</i>	MG	76	BC3Top-51	Inter H. ²	BA
13	BGP290	<i>P. cin</i>	BA	77	H09-157	<i>P. ed</i>	RJ
14	BGP300	<i>P. cin</i>	BA	78	BGP418-S7	<i>P. ed</i>	BA
15	BGP243	<i>P. cin</i>	BA	79	BC3Top-5	Inter H. ²	BA
16	BGP478	<i>P. muc</i>	MG	80	BGP175	<i>P. ed</i>	BA

Table 1. Continuation

Nº	Código*	Espécie	Estado	Nº	Código*	Espécie	Estado
17	BGP480	<i>P. cin</i>	BA	81	BC3Top-18	Inter H. ²	BA
18	BGP481	<i>P. cin</i>	BA	82	OTH-122	Inter H. ¹	BA
19	BGP414	<i>P. gib</i>	SP	83	BGP326	<i>P. ed</i>	SP
20	BGP114	<i>P. muc</i>	SP	84	BC3438	Inter H. ²	BA
21	BGP246	<i>P. cin</i>	BA	85	BGP203	<i>P. ed</i>	BA
22	BGP453	<i>P. mie</i>	RJ	86	BGP418	<i>P. ed</i>	BA
23	BGP008	<i>P. gib</i>	SP	87	BGP418-S4	<i>P. ed</i>	BA
24	BGP349	<i>P. cin</i>	BA	88	BC3Top-97	Inter H. ²	BA
25	BGP200 ³	<i>P. cin</i>	SP	89	BGP310	<i>P. ed</i>	BA
26	BGP268	<i>P. cin</i>	BA	90	BC3.52	Inter H. ²	BA
27	OTH-137	Inter H. ¹	BA	91	H09-125-S3	<i>P. ed</i>	BA
28	BGP389	<i>P. cin</i>	BA	92	BC3.507	Inter H. ²	BA
29	BGP294	<i>P. cin</i>	BA	93	H09-125-S1	<i>P. ed</i>	BA
30	BGP297	<i>P. cin</i>	BA	94	BGP347	<i>P. ed</i>	BA
31	BGP286	<i>P. cin</i>	BA	95	H09-156	<i>P. ed</i>	RJ
32	BGP398	<i>P. cin</i>	BA	96	H09-122-S2	<i>P. ed</i>	BA
33	BGP483	<i>P. cin</i>	MS	97	BGP190	<i>P. ed</i>	SP
34	BGP479	<i>P. muc</i>	BA	98	BGP338	<i>P. ed</i>	BA
35	BGP274	<i>P. cin</i>	BA	99	BGP337	<i>P. ed</i>	BA
36	BGP275	<i>P. cin</i>	BA	100	BC3Top-58	Inter H. ²	BA
37	BGP308	<i>P. cin</i>	BA	101	BGP450	<i>P. ed</i>	BA
38	BGP124	<i>P. ed</i>	BA	102	BGP436	<i>P. ed</i>	BA
39	BGP225	<i>P. ed</i>	BA	103	BC3.554	Inter H. ²	BA
40	BGP076	<i>P. ed</i>	BA	104	H09-123	<i>P. ed</i>	BA
41	BGP338	<i>P. ed</i>	BA	105	BGP235	<i>P. ala</i>	DF
42	BGP418-S1	<i>P. ed</i>	BA	106	H09-111	Intra H.	BA

43	BGP445	<i>P. ed</i>	BA	107	H09-122-S1	<i>P. ed</i>	BA
44	BGP402	<i>P. ed</i>	BA	108	BGP476	<i>P. ed</i>	BA
45	BGP482	<i>Passiflora sp.</i>	SP	109	BGP024	<i>P. ala</i>	DF
46	BGP423	<i>P. ed</i>	BA	110	OTH154	<i>P. ed</i>	BA
47	BGP418-S6	<i>P. ed</i>	BA	111	BGP429	<i>P. ed</i>	BA
48	BC3Top-46	Inter H. ²	BA	112	BGP214	<i>P. ed</i>	SP
49	BGP418-S2	<i>P. ed</i>	BA	113	BGP427	<i>P. ed</i>	BA
50	BC3Top-94	Inter H. ²	BA	114	BGP330	<i>P. ed</i>	BA
51	BGP188	<i>P. ed</i>	SP	115	H09-126	<i>P. ed</i>	RJ
52	BC3.112	Inter H. ²	BA	116	BRS Gigante Amarelo ⁴	Intra H.	BA
53	BGP093	<i>P. ed</i>	BA	117	OTH-101	Inter H. ¹	BA
54	BC3.183	Inter H. ²	BA	118	BGP393	<i>P. ala</i>	RJ
55	BC3Top-32	Inter H. ²	BA	119	BC3Top-34	Inter H. ²	BA
56	BGP424	<i>P. ed</i>	BA	120	BC3Top-44	Inter H. ²	BA
57	BGP418-S5	<i>P. ed</i>	BA	121	H09-158	Intra H.	BA
58	OTH-138	Inter H. ¹	BA	122	OTH-93	Inter H. ¹	BA
59	BGP047	<i>P. ed</i>	SP	123	H09-125-S2	<i>P. ed</i>	BA
60	BC3.507	Inter H. ²	BA	124	H09-02	Intra H.	BA
61	BGP302	<i>P. ed</i>	BA	125	H09-155	<i>P. ed</i>	BA
62	OTH-88	Inter H. ¹	BA	126	H09-110	Intra H.	BA
63	H09-154	<i>P. ed</i>	BA	127	H09-09	Intra H.	BA
64	BGP325	<i>P. ed</i>	DF	128	H09-112	Intra H.	BA

*Registration of the Passion fruit Germplasm Active Bank of Embrapa Mandioca and Fruticultura. ***P. sub* = *Passiflora suberosa*; *P. mal* = *P. malacophylla*; *P. set* = *P. setacea*; *P. poh* = *P. pohlii*; *P. bah* = *P. bahiensis*; *P. cin* = *P. cincinnata*; *P. gib* = *P. gibertii*; *P. muc* = *P. mucronata*; *P. mie* = *P. miersii*; ¹Inter H. = Simple interspecific Hybrid (F1); ²Inter H. = Interspecific hybrid of the third generation of backcrossing [BC₃ - (*P. edulis* x *P. cincinnata*) x *P. edulis*]; *P. ed* = *P. edulis* Sims; *P. ala* = *P. alata*; Intra H. = Intraspetic hybrid. BA = Bahia; RJ = Rio de Janeiro; MG = Minas Gerais; SP = São Paulo; MS = Mato Grosso do Sul; DF = Distrito Federal. ^{3,4}*P. cincinnata* genotype (BGP200) and yellow passion fruit cultivar (*P. edulis*, cv. BRS Gigante

Amarelo) used as resistance and susceptibility controls during the evaluations of leaf symptoms caused by CABMV.

Biological essay and sampling

Approximately 80 seeds of each genotype were soaked in 2 mL of the growth regulator GA₄₊₇ + N-(phenylmethyl) -aminopurine (Promalin[®]) at concentration of 400 mg.L⁻¹ for 24 hours [31]. After this period, the seeds were sown in 162 cells rigid polypropylene trays (50 mL of vol.) filled with coconut fiber mixture (Gold Mix[®]) and commercial substrate (Vivato[®]) in the ratio of 3:1 (v:v) with the addition of 50 g of slow release fertilizer (Osmocote[®]) for each 10 L of substrate. After emergence (40 days after sowing) the 30 most uniform plants were selected to compose the essay. Subsequently, the plants were transferred to polypropylene tubes (100 cm³) and conditioned in a vegetation house with temperature of 28 ± 2 °C and relative humidity (RH) of 75 ± 5% during the entire essay.

Inoculations and evaluations of the symptoms induced by CABMV

The inoculations with CABMV were performed artificially when the plants had at least four expanded leaves, approximately 60 days after the emergence of the seedlings. The CABMV isolate was derived from yellow passion fruit matrices plants (*P. edulis* Sims) with severe symptoms of the disease maintained in a greenhouse. The mechanical inoculation was performed as described by Gonçalves et al. [24], two inoculations per plant were performed with four days interval between the first and the second (Fig. 1).

The symptoms characterization was performed based on observations on the variation in the intensity of leaf symptoms in each evaluated leaf [24]. For that purpose, the diagram scale was used that varies from 1 (without symptoms) to 4 (severe symptoms) proposed by Novaes and Rezende [32], according to leaf symptoms demonstrated in Fig. 2. The evaluations started 12 days after the first inoculation (DAI) in all plants from the first leaf of the fully developed apex, totaling five leaves per plants. Subsequent evaluations were performed weekly and ended with 40 DAI.

Evaluation of CABMV severity and incidence

The leaf symptoms evaluation data of each plant (grades 1 to 4) in the five evaluation times (12, 19, 26, 33 and 40 DAI) were used to obtain the disease index (DI). The symptoms severity was measured from McKinney [33] disease severity index, considering the following formula: $DI(\%) = (DS \times L) / (TNL \times HGS)$; where: DS = degree of the determined scale for each leaf; L = number of leaves with each degree of symptoms (grades); TNL = total number of evaluated leaves, HGS = maximum infection degree (maximum grade). The incidence of the disease in genotypes was evaluated considering the percentage (%) of plants that presented typical CABMV symptoms.

Reinoculation and evaluation of asymptomatic plants

After finishing the leaf symptoms evaluations at 40 DAI, plants that did not manifest viruses symptoms (asymptomatic) were separated for new inoculations, in order to confirm plant resistance to CABMV (Fig. 3). These asymptomatic plants (n = 7 to 25) of the 34 genotypes (Table 3) were pruned at 15 cm (Fig. 3c). After 40 days of pruning (DAP), when plants had at least four leaves, inoculations and leaf symptoms evaluations were performed as described in item 2.3 and 2.4.

Detection of CABMV by qualitative RT-PCR

Extracting and treatment of total plant RNA

At 40 DAI, apical leaf tissue samples were collected from symptomatic inoculated plants (S-IP) and asymptomatic (A-IP), symptomatic reinoculated plants (S-RIP) and asymptomatic (A-RIP), and uninoculated plants (NIP – negative controls) of the 12 *Passiflora* spp. and hybrids (inter and intraspecific). RNA extractions were performed from the pool formation containing five apical leaves (about 0.5 g) representative of the five plants of each of the five sets (S-IP, A-IP, S-RIP, A-RIP, and NIP) (Fig. 3a-d), following the protocol of Ferreira et al. [34]. Pellets containing the RNAs were eluted between 40-80 μL with nuclease-free water (Promega[™]). The treatment was performed with 10 μL of total RNA (10 $\mu\text{g} \cdot \mu\text{L}^{-1}$) and 1.5 μL of the DNase enzyme (TURBO DNA-free[™] Kit – 2 $\text{U} \cdot \mu\text{L}^{-1}$) according to the manufacturer recommendations (Ambion[®]). To evaluate purity via ratio 260/280 and 260/230 (2.0 ± 1) as well as to estimate the total RNA concentration were performed three readings of each sample (1.0 μL) in NanoVue[™] Plus spectrophotometer (GE Healthcare), with the RNA concentration adjusted to approximately 100 $\text{ng} \cdot \mu\text{L}^{-1}$.

Obtaining complementary DNA (cDNA) and Polymerase Chain Reaction (PCR)

The cDNA was synthesized with 3.0 μL of total RNA (100 $\text{ng} \cdot \mu\text{L}^{-1}$), 1.0 μL of Oligo d(T)₁₈ and random primer, 1.0 μL of dNTP (2.5 mM) and 7 μL of water, heating at 65 °C for 5 min. Then we added 4.0 μL of buffer, 2.0 μL of DTT (0.1 M), 1.0 μL of RNase out[™] and 1.0 μL of M-MLV (Invitrogen[™], 200 $\text{U} \cdot \mu\text{L}^{-1}$), under thermocycling of 37 °C for 50 min. and inactivation of the enzyme at 70 °C for 15 min. PCR reactions were performed with specific primers [CABMV/M1MX3726_F (5' GAGACACAAGCCAAAACACAAAATC 3') and CABMV/M1MX5039_R (5' CGTTGCTACAAATTCTGGTATCTCC 3')] that amplifies part of the CABMV cylindrical inclusion gene of 1311 pb [35] (Fig. 4).

PCR reactions were performed with 3.0 μL of cDNA (diluted in 1:10), 0.5 μL of primer (10 μM) and Taq polymerase (5 $\text{U} \cdot \mu\text{L}^{-1}$ – Invitrogen[™]) according to Gonçalves et al. [24]. The amplification program was 95 °C for 3 min., followed by 38 cycles at 94 °C for 45 sec., 54 °C for 45 sec., 72 °C for 1:30 sec., followed by a final extension of 72 °C for 4 min. As positive control (PC) was used leaf tissue pool of matrix plants (n = 5, *P. edulis*) with symptoms of the disease collected at 40 DAI, with the cDNA concentration adjusted to 200 $\text{ng} \cdot \mu\text{L}^{-1}$. The PCR products were analyzed by electrophoresis (80 volts, 2h:30 min.) and agarose gel 1% (Tris-borate and EDTA, pH 8.0), and the amplicons were visualized by Kodak GeoLogic-220 pro photo

documenter (Carestream). The amplicons size was determined with 5.0 μL of Low Mass DNA Ladder of 1 Kb (Invitrogen[™]).

Quantitative real-time PCR (Polymerase Chain Reaction - qPCR)

Amplification efficiency of the real-time PCR essay (qPCR)

For the amplification efficiency essay a standard sample was prepared for using in decimal serial dilutions (10^n). The total RNA sample was used ($100 \text{ ng} \cdot \mu\text{L}^{-1}$) of *P. edulis* 40 DAI. Then four cDNA syntheses of the same sample were proceeded (2.6.2). In the sequence, PCR reactions of each of the four repetitions (2.6.2) were performed, and analyzed in agarose gel 1% to verify the formation of unique amplicons. Finally, the four cDNA repetitions were gathered forming a composite sample (pool), quantified (3x) (NanoVue[™] Plus Spectrophotometer) and the concentration fixed to $200 \text{ ng} \cdot \mu\text{L}^{-1}$.

The essay was performed obtaining a curve with five points (10^{10} to 10^6) of dilution in series of ten times; 1:20, 1:200, 1:2000 and 1:20,000, starting from 200 to $0.02 \text{ ng} \cdot \mu\text{L}^{-1}$. The primer pair used was qCABMV07_For (5' CTGGTAGAGTGCTTCTCAATTTGG 3') and qCABMV07_Rev (5' CTCTCCCTTGATGGCCTCAA 3') designed to amplify part of the CABMV coat protein gene of 121 pb [37] (Fig. 4). The tests were performed by RT-qPCR Sybr[®] Green reagent in technical triplicates (7500 Fast Real-Time PCR System – Applied Biosystems[™]). The amplifications efficiency calculation was performed automatically through Software 7500 Fast v2.0.6 (Applied Biosystems[™]) using the Slope obtained in the linear regression from the following formula: $[\text{Efficiency (E)} = 10^{(-1/\text{slope}) - 1}] \times 100\%$ [38, 39].

Standard curve of the real-time PCR essay (qPCR)

In the assembly of the standard curve essay a standard sample (defined concentration) was prepared for using in the quantification of CABMV in plants. Initially, a total RNA sample was used ($100 \text{ ng} \cdot \mu\text{L}^{-1}$) of *P. edulis* 40 DAI. The cDNA synthesis of the sample was proceeded (4 repetitions) and then four PCR reactions were performed for final volume of $50 \mu\text{L}$, next $5.0 \mu\text{L}$ of each reaction in agarose gel 2% was applied (verify presence of unique amplicons). The samples were gathered originating two samples with $90 \mu\text{L}$ each, and the final products of the PCR were purified with KitPureLink[™] PCR Purification, following the manufacturer specifications (Invitrogen[™]). After purification, the samples were again gathered ($80 \mu\text{L}$). The sample was quantified in agarose gel 1% ($5.0 \mu\text{L}$) and spectrophotometer (3x) (NanoVue[™] Plus; GE Healthcare), obtaining an approximate concentration of $30 \text{ ng} \cdot \mu\text{L}^{-1}$.

The standard curve was assembled with five points (10^9 to 10^5 copies) of serial dilutions in ten times; 1:10, 1:100, 1:1000 and 1:10,000 (1 to $0.0001 \text{ ng} \cdot \mu\text{L}^{-1}$). Subsequently linear regression calculation was applied regarding the Cycle threshold variables (Ct) vs. Log of cDNA concentrations ($\text{ng} \cdot \mu\text{L}^{-1}$) to determine the correlation coefficient (R^2) among the points of each dilution and the curve inclination (Slope) [39]. The concentration of the number of copies of CABMV ($\text{copies} \cdot \mu\text{L}^{-1}$) in each point of the serial

dilution was determined with application of the following formula: Copy Numbers (CN. μL^{-1}) = (Sample concentration [ng] x 6.022×10^{23}) / (fragment size [pb] x 1×10^9 x 660 g/mol) [40 41].

Detection and quantification of CABMV by real-time PCR (qPCR)

The qPCR reactions to quantify CABMV in asymptomatic plants and asymptomatic reinoculated plants were performed in the equipment '7500 Fast Real-Time PCR System' (Applied Biosystems™, Foster City, USA) on a 'MicroAmp™ Fast Optical 96-Well Reaction Plate' (0.1 mL; Applied Biosystems™), programmed for analysis of the type "Quantitation - Standard Curve".

The plate was mounted with 1.0 μL of cDNA (generated from 100 ng. μL^{-1} of total RNA), 0.5 μL of each primer at 2.0 μM (final concentration of 0.1 μM), 5.0 μL of GoTaq® qPCR Master Mix (Promega®), 0.2 μL of CXR Reference Dye and 2.8 μL of water (q.s. 10 μL) (Nuclease-Free Water – Promega®). To ensure that there was no unspecific amplification or contamination in the preparation of the reactions, it was used as negative control a cDNA sample derived from plant not inoculated with CABMV (*P. edulis*) and control without mold was also included (Non Template Control – NTC). All reactions for each of the samples 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 °C for 10 min, followed by 40 denaturation cycles at 95 °C for 15 sec, annealing of the primers and extension at 60 °C for 1 min. To check the occurrence of primers dimers, specificity in generating unique amplicons and determine the amplicons average fusion point, the samples were submitted to analysis of the dissociation curve at 95 °C for 15 seconds, 60 °C for 20 sec and 95 °C for 15 sec.

After end of the qPCR reaction and dissociation curve, the technical triplicates of each sample were gathered in a single sample. Then 4.0 μL of dye was applied (5X Green GoTaq® – Promega™) to the reaction products and it was applied in agarose gel 2%, submitted to electric field (80 V) for 1h 30 minutes. Therefore, the gel was photo documented to view the presence or absence of amplicons. The fragments size was determined by means of the molecular weight marker (5.0 μL) of 100 pb (Ludwig™).

Design and Data Analysis

The experimental design used was entirely randomized considering each of the 25 plants inoculated as a repetition. Other five plants were maintained as control (not inoculated with CABMV). The control plants (of all species) and the asymptomatic plants of *P. edulis* were not considered in the severity analyses.

The means of the disease index (DI%) in each evaluation period (12, 19, 26, 33 and 40 DAI) were plotted in logarithmic curves to determine the disease evolution in the twelve *Passiflora* species and in the three hybrids (inter and intraspecific). To calculate the rate of viruses progress in the leaves, the severity values (DI %) were used due to the five evaluation time for species with symptoms (*P. mucronata*, *P. miersii*, *P. cincinnata*, *P. gibertii*, *Passiflora* sp., *P. edulis*, *P. alata*, intraspecific hybrid and interspecific hybrid [F₁ and

BC₃]). The original severity or linearized data ($Y = \text{severity}/100$), were tested by the Gompertz empirical models ($Y = -\ln[-\ln(y)]$), Monomolecular ($Y = \ln[1/(1 - y)]$) and Logistic ($Y = \ln[y / (1 - y)]$) and adjusted for simple linear regression models [42]. Determination of R^2 and exponent, logit, monit and gompit coefficients were performed through a regression among real values and evaluation period in days (DAI). They were adjusted according to the original or linearized data, obtaining the determination coefficients (R^2) of the regression analysis [42]. Using the best adjustment, the disease progress rate was estimated (r), and determined by the ' b ' (angular coefficient) parameter of the regression equation (R^2) and by biological interpretations of the data.

The DI (%) estimates at 40 DAI were used for the comparison of averages by the Scott-Knott test ($p \leq 0.05$). From the DI (%) at 40 DAI, the intervals were generated for the classification of genotypes such as: resistant - R (DI varying from 0.0 – 15.9%), moderately resistant - MR (DI varying from 16.0 – 31.9%), susceptible - S (DI varying from 32.0 – 50.9%) and highly susceptible - HS (DI $\geq$ 51.0%). The analyses were performed in R environment using the 'ExpDes.pt' package [43]. The grouping of genotypes was performed based on the Gower index [44] and hierarchical grouping method Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dissimilarity matrix was obtained with the Genes program [45] and from the matrix the MEGA7.0 program was used to obtain the dendrogram [46].

After the qPCR reactions for asymptomatic inoculated species, the raw data were exported in Excel worksheet (Microsoft®), treated, and the estimated viral load in the species were determined from the average value of the viral cDNA quantity ($\text{ng} \cdot \mu\text{L}^{-1}$) having as parameter the values relative to each point of the standard curve and after the calculations the number of viral copies was converted to Log 10 scale.

Results

Temporal progress of CABMV in *Passiflora* species

Considering the evolution of the disease, it was null for the species belonging to group 1 (*P. suberosa*, *P. setacea*, *P. pohlii*, *P. malacophylla*, *P. bahiensis*, *P. gibertii* and *P. miersii*) and stable in group 2 (*P. mucronata* and *P. cincinnata*). The evolution of the disease was more progressive in the species of group 3 (*P. alata*, *P. edulis*, Interspecific hybrid - BC₃, Interspecific hybrid - F₁ and *Passiflora* sp.) and of group 4 (Intraspecific hybrid) (Fig. 5).

Regarding the disease progress rate based on the values of or among pairs of species (Table 2), it was possible to verify significant differences among the disease progress rates being lower in *P. cincinnata* than in *P. alata* and *Passiflora* sp. The species *P. gibertii* presented lower progress rate compared to the intraspecific hybrid, *Passiflora* sp. and *P. alata*, but with higher rate when compared to *P. miersii* and *P. mucronata* species. On the other hand, *P. miersii* and *P. mucronata* presented lower rate compared to intraspecific hybrids; *P. alata*; *P. edulis* and *Passiflora* sp., while the interspecific hybrid (F₁) presented

higher rate than *P. mucronata*. *P. miersii* and *P. mucronata* species obtained higher rates compared to *P. cincinnata* (Table 2). Other comparisons did not present significant differences (Table 2).

Table 2 Comparisons of the viruses progress rates (and)¹ among the species evaluated.

Combinations (species vs. species)	Limit		θ_1	θ_2	Sig. ($p \leq 0.05$)
	Lowest	Highest			
<i>P. cincinnata</i> vs. <i>P. alata</i>	0.00712	0.01408	0.00489	<u>0.01549</u>	*
<i>P. cincinnata</i> vs. <i>P. miersii</i>	0.00454	0.00690	<u>0.00489</u>	-0.00083	*
<i>P. cincinnata</i> vs. <i>P. mucronata</i>	0.00362	0.00755	<u>0.00489</u>	-0.00069	*
<i>P. cincinnata</i> vs. <i>Passiflora</i> sp.	0.00719	0.02030	0.00489	<u>0.01864</u>	*
<i>P. gibertii</i> vs. Intra H.	0.00202	0.01364	0.00188	<u>0.00971</u>	*
<i>P. gibertii</i> vs. <i>P. alata</i>	0.01012	0.01710	0.00188	<u>0.01549</u>	*
<i>P. gibertii</i> vs. <i>P. miersii</i>	0.00150	0.00392	<u>0.00188</u>	-0.00083	*
<i>P. gibertii</i> vs. <i>P. mucronata</i>	0.00059	0.00455	<u>0.00188</u>	-0.00069	*
<i>P. gibertii</i> vs. <i>Passiflora</i> sp.	0.02331	0.01020	0.00188	<u>0.01864</u>	*
<i>P. miersii</i> vs. Intra H.	0.00482	0.01626	-0.00083	<u>0.00971</u>	*
<i>P. miersii</i> vs. <i>P. alata</i>	0.01298	0.01966	-0.00083	<u>0.01549</u>	*
<i>P. miersii</i> vs. <i>P. edulis</i>	0.00021	0.01914	-0.00083	<u>0.00885</u>	*
<i>P. miersii</i> vs. <i>Passiflora</i> sp.	0.01299	0.02595	-0.00083	<u>0.01864</u>	*
<i>P. mucronata</i> vs. Intra H.	0.00447	0.01634	-0.00069	<u>0.00971</u>	*
<i>P. mucronata</i> vs. <i>P. alata</i>	0.01249	0.01987	-0.00069	<u>0.01549</u>	*
<i>P. mucronata</i> vs. <i>P. edulis</i>	-0.00006	0.01913	-0.00069	<u>0.00885</u>	*
<i>P. mucronata</i> vs. <i>Passiflora</i> sp.	0.01266	0.02600	-0.00069	<u>0.01864</u>	*
<i>P. mucronata</i> vs. Inter H. (F_1).	-0.00421	0.02331	-0.00069	<u>0.00886</u>	*
Other 31 combinations	-	-	-	-	ns

¹ Values greater of the disease progress rate (or are underlined in the table.

Classification of *Passiflora* species and genotypes regarding the severity of CABMV

Based on the severity results associated with the disease index (DI%) at 40 DAI, it was observed genetic variability among the *Passiflora* spp. and intra and interspecific hybrids, with variation in the DI from 0.0

to 70.80%, and 26 genotypes (20.3%) were classified as resistant (R; DI ranging from 0.0 to 15.75%). Considering the reaction within this same group, it was highlighted the BGP152 (*P. suberosa*), BGP170 (*P. malacophylla*), BGP434, BGP244 and BRS Pérola do Cerrado (*P. setacea*), BGP454 (*P. pohlii*) and BGP477 (*P. bahiensis*) genotypes, which did not manifest typical CABMV symptoms (DI: 0.0%). Other 12 genotypes (9.4%) were moderately resistant (MR; DI ranging from 16.3 to 31.1%), 42 (32.8%) susceptible (S; DI ranging from 33.3 to 50.9%) and 48 (37.5%) highly susceptible (HS; DI ranging from 51.2 to 70.8%) (Figs. 6a, b and Sup. 1).

Assuming the *P. edulis* genotypes, only the BGP124 was considered as moderately resistant, with DI of 31.11%. Other 53 genotypes (41.4%) presented some degree of susceptibility, of which 27 (21.1%) were susceptible and 26 (20.3%) were highly susceptible. All genotypes belonging to the intra-specific hybrids, interspecific hybrids (F1) and interspecific hybrids of the third generation of backcrossing [BC₃ (*P. edulis* x *P. cincinnata*) x *P. edulis*] were classified as susceptible and highly susceptible (Fig. 6 and Sup. 1). The genotypes used as controls for resistance reactions (BGP200) and susceptibility (cv. BRS Gigante Amarelo) presented severity within the expected level, with mean DI of 15.30% and 62.20%, respectively.

Among the species evaluated, seven (46.67%) were classified as resistant (*P. bahiensis*, *P. malacophylla*, *P. pohlii*, *P. setacea*, *P. suberosa*, *P. gibertii* and *P. miersii*) with DI of 0.0 to 14.80%; two species (13.33%) were classified as moderately resistant (*P. cincinnata* and *P. mucronata*) with DI of 16.10 to 18.70%; three (20%) (*Passiflora* sp., *P. edulis* and interspecific hybrids of the third generation of backcrossing [BC₃]) were classified as susceptible to CABMV, with DI that ranged from 37.60 to 50.40% and three (20%) were considered highly susceptible to CABMV with mean DI of 51.90 to 64.90% (hybrids [F₁], *P. alata* and intraspecific hybrids) (Fig. 6c).

Severity and classification of genotypes reinoculated with CABMV

The reinoculated genotypes demonstrated typical viruses symptoms, specifically those that are belonging to the species *P. gibertii*, *P. cincinnata*, *P. mucronata*, *P. miersii* and the interspecific hybrid (OTH-137), the latter with 100% incidence in reinoculated plants (Table 3). The average severity of 34 reinoculated genotypes ranged from 0.0 to 60.74% (Table 3). Among the genotypes evaluated 55.88% (n = 19) were considered as resistant, with DI ranging between 0.0 and 15.83%. Within this same group, the BGP152 (*P. suberosa*), BGP170 (*P. malacophylla*), BRS Pérola do Cerrado, BGP434 and BGP244 (*P. setacea*), BGP454 (*P. pohlii*) and BGP477 (*P. bahiensis*) genotypes even after reinoculation did not manifest symptoms of the disease, maintaining the classification as resistant (Table 3). Other 13 genotypes (38.24%) were classified as moderately resistant, with DI of 17.50 to 30.29% and two genotypes (BGP275 and OTH-137) were classified as susceptible (DI: 36.74%) and highly susceptible (DI: 60.74%) (Table 3).

Table 3 Minimum, maximum and average severity value (DI) and resistance phenotypic classification of 34 *Passiflora* spp., reinoculated with the passion fruit woodiness disease (*Cowpea aphid-borne mosaic virus* – CABMV).

Nº	Code*	Species	DI (%) ^a			Class ^b
			Min.	Max.	Mean	
1	BGP152	<i>P. suberosa</i>	0.00	0.00	0.00a	R
2	BGP170	<i>P. malacophylla</i>	0.00	0.00	0.00a	R
3	BRS Pérola do Cerrado	<i>P. setacea</i>	0.00	0.00	0.00a	R
4	BGP434	<i>P. setacea</i>	0.00	0.00	0.00a	R
5	BGP454	<i>P. pohlii</i>	0.00	0.00	0.00a	R
6	BGP477	<i>P. bahiensis</i>	0.00	0.00	0.00a	R
7	BGP244	<i>P. setacea</i>	0.00	0.00	0.00a	R
8	BGP085	<i>P. gibertii</i>	0.00	33.33	5.18a	R
9	BGP421	<i>P. cincinnata</i>	0.00	26.67	7.33b	R
10	BGP300	<i>P. cincinnata</i>	0.00	33.33	8.23b	R
11	BGP290	<i>P. cincinnata</i>	0.00	33.33	11.42b	R
12	BGP243	<i>P. cincinnata</i>	0.00	33.33	12.82b	R
13	BGP422	<i>P. cincinnata</i>	0.00	33.33	12.85b	R
14	BGP008	<i>P. gibertii</i>	0.00	60.00	13.33b	R
15	BGP414	<i>P. gibertii</i>	0.00	33.33	13.33b	R
16	BGP389	<i>P. cincinnata</i>	0.00	40.00	13.33b	R
17	BGP268	<i>P. cincinnata</i>	0.00	66.67	14.22b	R
18	BGP478	<i>P. mucronata</i>	0.00	33.33	14.44b	R
19	BGP114	<i>P. mucronata</i>	0.00	33.33	15.83b	R
20	BGP279	<i>P. cincinnata</i>	0.00	26.67	17.50c	MR
21	BGP453	<i>P. miersii</i>	0.00	33.33	18.33c	MR
22	BGP480	<i>P. cincinnata</i>	0.00	53.33	19.04c	MR
23	BGP483	<i>P. cincinnata</i>	0.00	40.00	19.33c	MR
24	BGP246	<i>P. cincinnata</i>	0.00	33.33	19.99c	MR
25	BGP276	<i>P. cincinnata</i>	0.00	40.00	20.55c	MR

26	BGP308	<i>P. cincinnata</i>	0.00	53.33	20.95c	MR
27	BGP398	<i>P. cincinnata</i>	0.00	60.00	21.25c	MR
28	BGP297	<i>P. cincinnata</i>	0.00	33.33	23.80c	MR
29	BGP286	<i>P. cincinnata</i>	0.00	86.67	24.16c	MR
30	BGP274	<i>P. cincinnata</i>	0.00	66.67	25.55c	MR
31	BGP349	<i>P. cincinnata</i>	0.00	33.33	28.33d	MR
32	BGP294	<i>P. cincinnata</i>	0.00	33.33	30.29d	MR
33	BGP275	<i>P. cincinnata</i>	33.33	40.00	36.74d	S
34	OTH-137	Interspecific H.	46.67	80.00	60.74e	HS

*Code of Passion fruit Germplasm Active Bank of Embrapa Mandioca e Fruticultura. ^aDI (%) = Disease index at 40 days after reinoculation (DARI) of asymptomatic plants; ^bR = resistant; MR = moderately resistant; S = susceptible; HS = highly susceptible. Mean followed by the same letter in the column belong to the same group by the Scott-Knott test ($p \leq 0.05$).

Detection of CABMV by qualitative RT-PCR

The molecular analysis by qualitative RT-PCR and primers CABMV/M1MX3726_F/CABMV/M1MX5039_R confirmed the viral infection in symptomatic inoculated plants, with amplification of the genomic fragments related to the cylindrical inclusion region (CI) of CABMV with expected size of 1311 pb (Fig. 7a). In asymptomatic inoculated plants and negative control, the systemic replication of CABMV was not confirmed (Figs. 7b and c). Asymptomatic plants were submitted to real time quantitative PCR analysis (qPCR) to confirm the infection (Fig. 7b).

Fig. 7 Products of amplification by RT-PCR in agarose gel 1% using primers for the genomic fragment of the cylindrical inclusion of CABMV (*Cowpea aphid-borne mosaic virus*) of 1311pb. A) pool of leaf samples of symptomatic inoculated plants (S-IP) at 40 days after inoculated (DAI); B) pool of leaf samples of asymptomatic inoculated plants (A-IP); C) pool of leaf samples of not inoculated plants with CABMV (NIP, negative controls). PC: Positive control (Matrices plants of *P. edulis* with CABMV symptoms – cDNA 200 ng.µL⁻¹). M = Molecular DNA Marker Ladder of 1Kb (Invitrogen™). *P. ed* = *Passiflora edulis*; Intra H. = Intraspecific Hybrid; Inter H. = Interspecific Hybrid; *P. ala* = *P. alata*; *P. mie* = *P. miersii*; *P. muc* = *P. mucronata*; *P. cin* = *P. cincinnata*; *P. gib* = *P. gibertii*; *P. set* = *P. setacea*; *P. poh* = *P. pohlii*; *P. mala* = *P. malacophylla*; *P. sub* = *P. suberosa*; *P. bah* = *P. bahiensis*. *Unregistered leaf tissue (*Passiflora* sp - BGP482).

Standardization of the qPCR technique for CABMV quantification

The amplifications with the primer qCABMV07 were quite uniform (Fig. 8a) and the amplification efficiency obtained Slope = -3.53, determination coefficient (R^2) = 0.997, Efficiency (E) = 91.96% and the standard deviation (SD) of the Cycle threshold (Ct) of the technical triplicates in each dilution (10^{10} to 10^6) was 0.08 to 0.32 (Fig. 8b). The dissociation curve of amplifications was uniform, without formation of primers dimers or nonspecific peaks (Fig. 8c).

The essay of the purified PCR standard curve was prepared with seven dilution points (10^{10} , 10^9 , 10^8 , 10^7 , 10^6 , 10^5 and 10^4 copies). However, points 10^{10} (10 ng.µL⁻¹) and 10^4 (0.001 ng.µL⁻¹) were disregarded. Point 10^{10} despite presenting amplifications of replicates considerable uniform, did not present linearity in the spacing of -3.32 between the dilutions 10^{10} and 10^9 . Point 10^4 did not present uniformity in the amplifications of the technical repetitions (Figs. 9a and b). Linear regression ratio between the mean values of the Ct and the concentration of the CABMV genic product was observed with determination coefficient (R^2 = 0.998) and an Slope of -3.33, demonstrated efficiency (E) of 99.37% of the qPCR reaction (Fig. 9a). The dissociation curve of amplifications also demonstrated that there was high specificity of qPCR for the standard curve, observed through the uniformity of amplifications, formation of unique peaks and without presence of primers dimers (Fig. 9b). The quantification of the number of copies of part of the CABMV coat protein gene in the serial dilutions of the standard curve was equivalent to 3.28×10^{33} to 3.28×10^{29} copies.µL⁻¹ with mean values of the Ct of the technical triplicates obtained in a very reproducible way and standard deviation (SD) of the Ct varied from 0.03 to 0.34 (Table 4). These quantifications were used for absolute quantification of the viral load in the evaluated species.

Table 4 Mean values and standard deviation (SD) of the Cycle threshold (Ct) of the three technical replicates and number of copies of the CABMV virus (Copies.µL⁻¹) expressed in Log¹⁰ scale of each serial dilution of the standard curve of the CABMV coat protein gene.

Dilutions	10^9	10^8	10^7	10^6	10^5
Ct±SD	20.15±0.07	23.55±0.03	26.86±0.09	30.44±0.34	33.39±0.28
Nº Copies.µL ⁻¹	3.28×10^{33}	3.28×10^{32}	3.28×10^{31}	3.28×10^{30}	3.28×10^{29}

Detection and quantification of CABMV by real-time PCR (qPCR)

The qPCR reactions in asymptomatic inoculated plants (Fig. 3b), demonstrate quality and precision in the amplification obtaining curve inclination (Slope) of -3.28, determination coefficient (R^2) of 0.998, efficiency (E) of 101.74% and the dissociation curve demonstrated the lack of nonspecific products and absence of primer dimers (Fig. 10a). The standard deviation of the Ct of the three technical replicates of each of the samples varied from 0.07 to 0.65 (Fig. 10b).

Regarding the quantity of CAMBV in asymptomatic species, it was observed variation of 1×10^0 to 1.59×10^{31} copies of viruses per microliter (Fig. 10b). There was little variation in the number of copies of the

virus for the interspecific hybrid (OTH-137) and the species *P. cincinnata*, *P. gibertii* and *P. miersii*, with 1.59×10^{31} , 1.41×10^{31} , 1.33×10^{31} and 1.11×10^{31} copies of viruses. μL^{-1} , respectively (Fig. 10b). Regarding the *P. mucronata*, *P. setacea* and *P. malacophylla* species, it was observed lower quantity of virus than the previously mentioned species, with 1.18×10^{30} , 1.34×10^{29} and 4.35×10^{28} copies. μL^{-1} , respectively (Fig. 10b). In the *P. pohlii*, *P. suberosa* and *P. bahiensis* species, it was not detected fragment corresponding to CABMV, showing that there was no infection after two inoculation attempts (Figs. 10a and b). The result in agarose gel confirms the specificity in the amplifications, with unique amplicons of expected size of 121 pb in the interspecific hybrid (OTH-137) and the *P. cincinnata*, *P. gibertii*, *P. miersii*, *P. mucronata*, *P. setacea* and *P. malacophylla* species (Fig. 10c).

From the molecular analysis of RT-PCR there was confirmation of the viral infection in plants that presented symptoms after the third and fourth reinoculation (Fig. 11a). However, numerous plants of *P. cincinnata*, *P. gibertii*, *P. miersii*, *P. mucronata*, *P. setacea*, *P. malacophylla*, *P. suberosa*, *P. pohlii* and *P. bahiensis* did not manifest viruses symptoms, and, therefore, were submitted to the analysis of qPCR, except for interspecific hybrid (OTH-137) that had all plants with symptoms.

The parameters of quality definition and accuracy of the qPCR technique for reinoculated asymptomatic plants (Fig. 3c) demonstrated reproductive and accurate results, obtained Slope = -3.35, determination coefficient (R^2) = 0.998 and Efficiency (E) = 98.84%, and the dissociation curves also demonstrated high specificity of amplification of the CABMV coat protein gene (Fig. 11b). The standard deviation of the Ct of the three technical replicates of each of the samples varied from 0.02 to 0.92 (Fig. 11c). The analysis of qPCR in reinoculated asymptomatic plants (Fig. 3d), detected CABMV in *P. cincinnata*, *P. gibertii*, *P. miersii*, *P. mucronata*, *P. setacea*, *P. malacophylla* and *P. suberosa*, but was not detected in *P. pohlii* and *P. bahiensis* (Figs. 10c and d). The viral load varied from 1×10^0 to 1.83×10^{31} copies. μL^{-1} (Fig. 10c).

The variation in the number of copies of the virus among the evaluated species was low, with 1.83×10^{31} for *P. cincinnata*, 1.81×10^{31} in *P. gibertii*, 1.78×10^{31} compared to *P. miersii* and 1.59×10^{31} for *P. mucronata*. *P. setacea* and *P. malacophylla* species presented mild decrease in the viral load compared to the species mentioned above, with 3.49×10^{30} and 2.11×10^{29} copies of viruses. μL^{-1} . In *P. suberosa*, there was the viral detection only after plant reinoculation, with viral load of 7.78×10^{28} copies of viruses. μL^{-1} (Fig. 10c). The result demonstrated in Fig. 10d, evidences the formation of unique amplicons of CABMV coat protein of 121 pb in species *P. cincinnata*, *P. gibertii*, *P. miersii*, *P. mucronata*, *P. setacea*, *P. malacophylla* and *P. suberosa*.

Discussion

Temporal progress and identification of resistance source in *Passiflora* spp. species and genotypes

The differences in evolution curves and viruses progress rates (Fig. 5 and Table 2) demonstrate high variability of *Passiflora* spp. for resistance/susceptibility to CABMV. The results of Spadotti et al. [16] demonstrated that the incubation time of CABMV in *P. edulis* plants (FB-200) is, on average, 8 DAi.

However, the incubation time of CABMV for most of the *Passiflora* spp. it is not known with accuracy, and the initial expression of symptoms is dependent on the plant age, genotype or isolated used and affected directly by environmental conditions and nutrition of plants [16, 28]. In the initial evaluation (12 DAI), most species had already manifested the typical symptoms of the disease, however, it is possible that some of the species had already expressed symptoms before this period. *P. suberosa*, *P. setacea*, *P. pohlii*, *P. malacophylla* and *P. bahiensis* species did not manifest symptoms over time and *Passiflora* sp. was at 19 DAI.

Species classified as resistant and moderately resistant are wild, and naturally carry resistance alleles [47] and in this study 38 genotypes (29.70%) are in these two classes. Highlighting the species such as *P. bahiensis* (BGP477), *P. malacophylla* (BGP170), *P. pohlii* (BGP454), *P. setacea* (BGP244, BGP434 and BRS Pérola do Cerrado) and *P. suberosa* (BGP152) that did not manifest viruses symptoms in the leaves (DI: 0.0%). On the other hand, *P. gibertii* (BGP085, BGP008 and BGP414), *P. miersii* (BGP453), *P. mucronata* (BGP478 and BGP114) and *P. cincinnata* species (BGP421, BGP422, BGP279, BGP276, BGP290, BGP300, BGP243, BGP480, BGP481, BGP246, BGP349 and BGP268) exhibited light mosaic throughout the evaluations, indicating tolerance to CABMV virus (Fig. 6c). According to Oliveira et al. [22] a resistant genotype can present mild viruses symptoms, but has mechanisms that prevents the multiplication of the pathogen. With this in mind, genotypes that presented mild symptoms (DI from 4.19 to 15.75%) should be considered for selection aiming resistance to CABMV from interspecific hybridization. Previous studies reported resistance to CABMV in genotypes belonging to *P. setacea* [22, 48], *P. cincinnata* [22, 24], *P. gibertii* [24] and immunity in *P. suberosa* [21]. This research is pioneer in reporting immunity to CABMV in *P. bahiensis* and *P. pohlii* and moderately resistant in *P. miersii*, amplifying the number of wild species of *Passiflora* spp. characterized for CABMV reaction.

Species with null or low severity can be used to intensify the genetic improvement programs of *Passiflora* via interspecific crosses. However, interspecific hybridizations with commercial species, in some cases, may not succeed due to differences in the number of chromosomes (*P. pohlii* [2n = 12 and 36] and *P. suberosa* [2n = 12, 24 and 36]) and belong to different subgenera [49, 50] and in the same subgenre can occur barriers of crossings or lack of synchronism in the flowering [51]. However, in some cases the interspecific barriers of incompatibility are relatively fragile, and there may be success in hybridization [52].

The understanding of cytogenetic aspects involved in the crossing of *P. edulis* x *P. cincinnata* demonstrate the possibility of obtaining hybrids and thus transferring resistance alleles or other characters of the wild species [53]. Studies using *P. setacea* and *P. cincinnata* (both 2n = 18), as donors of CABMV resistance alleles for *P. edulis* (2n = 18) were also successful [18–20, 25, 54]. *P. malacophylla* and *P. bahiensis* species (belonging to the genus *Passiflora*) present potential for using genetic improvement programs of passion fruit, because they are resistant to CABMV (DI: 0.0%) and have chromosomal analogy with *P. edulis* (2n = 18) [55], opening possibilities of obtaining resistant commercial hybrids. However, complementary studies should be performed in these two species to confirm the real chance of obtaining viable hybrids.

It was observed relation between incidence and severity of CABMV for most of the evaluated genotypes, already in *P. mucronata* (BGP479) and *P. edulis* (BGP124) even with incidence of 100% the plants presented only mild mosaic (grade 2), being classified as moderately resistant (Sup. 1). The occurrence of this case allows to indicate that the incidence cannot always be related to the severity.

Most of the genotypes of *P. edulis* presented susceptibility to CABMV (Fig. 6), except for the BGP124 genotype that was moderately resistant. Results in the literature demonstrated different resistance levels among *P. edulis* genotypes, being classified from resistant to highly susceptible [22, 24, 56, 57]. This indicates there is intraspecific genetic variability in the resistance to CABMV. For this reason, the evaluation of *P. edulis* genotypes in GABs should be performed to explore low severity levels of the disease, and thus reduce the time for obtaining resistant cultivars [18, 24, 25].

The susceptibility observed in the six intraspecific hybrids of *P. edulis* (H09-111, H09-02, H09-110, H09-09, H09-112 and H09-158) is directly related to the selection of parentals, taking into consideration only agronomic attributes of vigor and production [58, 59]. Interspecific hybrids of the third generation of backcrossing (BC₃) despite having contrasting genitors, BGP330 (*P. edulis*; susceptible) and BGP077 (*P. cincinnata*; resistant) [24] did not present resistance to CABMV, probably related to the low number of evaluated progenies (n = 21) or fact that there are few genes or resistance losses in BC₃, since 93.75% of the genome involved in the backcrossing belongs to the susceptible recurrent genitor (*P. edulis*). Fonseca et al. [60] also did not verify the resistance to CABMV in genotypes of the fourth and fifth generation of backcrossing (BC₄ and BC₅) involving *P. edulis* x *P. setacea*, due to the loss of resistance as new backcrossing are performed, probably due to the polygenic heritage of the character [18–20]. Therefore, that there are gains associated with resistance to CABMV it is necessary to evaluate a very large number of progenies as the backcrossing generations advance [19]. However, the genetic heritage for resistance to CABMV of most of the *Passiflora* species is still unknown, being an open field for researches in genetic improvement programs.

The variation in the resistance level to CABMV within and among the evaluated genotypes can be associated with the genetic variability of the passifloras, since they are self-incompatible plants [51, 61, 62]. Moreover, different studies attribute the passion fruit response to the use of different isolates [21, 56], latency period and virus incubation [16], individual resistance level of genotypes [24], genetic and environmental factors (such as temperature and relative humidity) [19, 25, 63], nutritional condition and age difference among plants [64]. Alone or together, these factors can influence the virulence of the pathogen and in the manifestation of the disease symptoms. In this study many plants were observed without viruses symptoms, especially in wild species. However, some of these factors may not be the cause, since the evaluated genotypes had the same age and the environmental and nutritional conditions were the same.

Conclusions

Progress rates and mean disease severity in symptomatic plants were lower in *P. cincinnata*, *P. gibertii*, *P. miersii* and *P. mucronata* in relation to the species *P. edulis*, *P. alata*, inter and intraspecific hybrids and *Passiflora* sp. The accessions belonging to the species *P. suberosa*, *P. malacophylla*, *P. setacea*, *P. pohlii* e *P. bahiensis* did not manifest visual symptoms of the disease after artificial inoculation. Some asymptomatic plants (*P. cincinnata*, *P. gibertii*, *P. miersii* and *P. mucronata*) after new inoculation cycles (third and fourth inoculation) had lower mean disease severity in relation to the severity of the symptoms of the two initial inoculations and this may indicate some control mechanism like pre-immunization. The absence of visual symptoms of the disease in some plants or accessions does not indicate immunity, because asymptomatic plants may exhibit systemic infection by CABMV as detected by qPCR analysis. The species *P. pohlii* and *P. bahiensis* even after four inoculations, showed no disease symptoms and no systemic infection of the virus by qPCR analysis, revealing that they are immune to CABMV.

Declarations

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Authors' contributions

All authors contributed to writing, as well as toward interpreting the results, revising, and improving the paper. ZSG carried out the installation of the experiment, assessment of the severity of the disease, molecular analysis of virus detection and quantification and writing of the paper. ZSG, ONJ, LKSL and participated in the statistical analysis, organization and elaboration of tables and figures, as well as data interpretation. ONJ, LKSL and RXC corrected the paper. ONJ and RXC were the creators of this research.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical standards

The authors declare that the present work complies with ethical standards of committee on publication ethics (COPE) and complies with the company's ethical standards Universidade Estadual de Santa Cruz and Embrapa Mandioca e Fruticultura.

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Figures

Figure 1

Artificial inoculations scheme with the passion fruit woodiness disease (Cowpea aphid-borne mosaic virus – CABMV) in the 128 *Passiflora* spp. genotypes. A) inoculation performed from the leaf extract preparation with viruses symptoms and cellite; B) inoculation with buffer and abrasive in plants used as control (negative control). DAFI: Days after the first inoculation.

Figure 2

Grade scale (1 to 4), description of the leaf symptoms observed in *Passiflora edulis* (a), *P. alata* (b) and *P. cincinnata* (c) plants inoculated with CABMV (Cowpea aphid-borne mosaic virus) and phenotypic class associated with each gradient of leaf symptoms. R = resistant; MR = moderately resistant; S = susceptible and HS = highly susceptible.

Figure 3

Representative scheme of the steps for the detection of CABMV infection in symptomatic and asymptomatic inoculated plants. A) inoculations (1 and 2) and evaluations of viruses symptoms in passion fruit plants; B) apical leaf tissue collection of plants inoculated with and without symptoms after 40 DAI; C) separation of plants that did not present symptoms, pruning and reinoculation (inoculation 3 and 4) of plants after 40 DAP; D) apical leaf tissue collection of symptomatic and asymptomatic reinoculated plants after 40 DAI for CABMV amplification test through RT-PCR and qPCR. Inoc. = Inoculation; DAI = Days after inoculation; RT-PCR = Reverse Transcriptase - Polymerase Chain Reaction; qPCR = Real Time quantitative Polymerase Chain Reaction.

Figure 4

Schematic design of the annealing position of the two primer pairs used in the RT-PCR and qPCR reactions to amplify the gene portion of the cylindrical inclusion (CI) and coat protein (CP) of Cowpea aphid-borne mosaic virus (CABMV). For = Forward; Rev = Reverse. The scheme of the Potyvirus genome organization illustrated above was adapted from ViralZone© [36]. Available at: www.expasy.org/viralzone.

Figure 5

Logarithmic regression for the disease index (DI %) in 12 species of *Passiflora* spp. and three hybrids (inter and intraspecific) in different periods after inoculation (DAI) with the passion fruit woodiness disease (Cowpea aphid-borne mosaic virus – CABMV). Group 1 (resistant – R: *P. suberosa*, *P. setacea*, *P. pohlii*, *P. malacophylla*, *P. bahiensis*, *P. gibertii* and *P. miersii*); Group 2 (moderately resistant – MR: *P. mucronata* and *P. cincinnata*); Group 3 (susceptible – S: *P. alata*, Interspecific hybrids BC3 and F1, *P. edulis* and *Passiflora sp.*); Group 4 (highly susceptible – HS: Intraspecific hybrid). The values in brackets represent the amplitude of the disease index (DI %) of the species that compound each group.

Figure 6

Cluster and severity values (DI%) in 128 genotypes of *Passiflora* spp. (A); mean values and number of genotypes in the resistant classes (R), moderately resistant (MR), susceptible (S) and highly susceptible (HS) (B) and mean values and their distribution in the classes R, MR, S and HS of the 12 species of *Passiflora* spp., an intraspecific hybrid, a simple interspecific hybrid (F1) and an interspecific hybrid of the third generation of backcrossing [BC3 - (*P. edulis* x *P. cincinnata*) x *P. edulis*]] and incidence of viruses in the species (D).

Figure 7

Products of amplification by RT-PCR in agarose gel 1% using primers for the genomic fragment of the cylindrical inclusion of CABMV (Cowpea aphid-borne mosaic virus) of 1311pb. A) pool of leaf samples of symptomatic inoculated plants (S-IP) at 40 days after inoculated (DAI); B) pool of leaf samples of asymptomatic inoculated plants (A-IP); C) pool of leaf samples of not inoculated plants with CABMV (NIP, negative controls). PC: Positive control (Matrices plants of *P. edulis* with CABMV symptoms – cDNA 200 ng.μL⁻¹). M = Molecular DNA Marker Ladder of 1Kb (Invitrogen™). *P. ed* = *Passiflora edulis*; Intra H. = Intraspecific Hybrid; Inter H. = Interspecific Hybrid; *P. ala* = *P. alata*; *P. mie* = *P. miersii*; *P. muc* = *P. mucronata*; *P. cin* = *P. cincinnata*; *P. gib* = *P. gibertii*; *P. set* = *P. setacea*; *P. poh* = *P. pohlii*; *P. mala* = *P. malacophylla*; *P. sub* = *P. suberosa*; *P. bah* = *P. bahiensis*. *Unregistered leaf tissue (*Passiflora* sp - BGP482).

Figure 8

Quantitative real-time PCR (qPCR) of the serial dilutions for amplification efficiency of the primer qCABMV07. A) Graphic of amplifications obtained from the serial dilutions of cDNA (200 ng.μL⁻¹); B) Curve of the serial dilutions of cDNA and quality and precision parameters of amplification of the primer qCABMV07 in qPCR; C) Dissociation curves (Curve melting) of the serial dilutions. M = Molecular DNA Marker Ladder of 100 pb (Ludwig®); NTC (Non Template Control) = without mold cDNA. The numbers on the left side of the curve represent the mean values of the Ct of the technical replicates of each point of the serial dilution and the standard deviation of the Ct.

Figure 9

A) Standard curve of serial dilutions at 10 times (1:10) and quality and accuracy parameters of the qPCR reaction of part of the CABMV coat protein gene with primer qCABMV07; B) Dissociation curves (Melting curve) of the amplifications of the serial dilutions. The values on the left side of the standard curve represent the concentrations (ng.μL⁻¹) of each serial dilution.

Figure 10

Detection and quantification of CABMV in plants of *Passiflora* spp. Asymptomatic inoculated from quantitative real-time PCR technique (qPCR). A) Amplification graph of the standard curve and of the samples, standard curve with the quality and precision parameters of the qPCR reaction and dissociation curves (Melting curve) of the amplifications of the CABMV coat protein gene; B) Copies numbers (copies. μL^{-1}) of the CABMV virus expressed in Log10 scale in *Passiflora* spp. asymptomatic inoculated; C) Genic products from part of the genomic region of the CABMV coat protein of 121 pb. Agarose 2%. M = Molecular DNA Marker Ladder of 100 pb (LudwigTM). NTC (No Template Control) = without cDNA as mold; NC (Negative control) = sample of *P. edulis* not inoculated with CABMV. Inter H = Interspecific Hybrid (OTH-137); *P. cin* = *P. cincinnata*; *P. gib* = *P. gibertii*; *P. mie* = *P. miersii*; *P. muc* = *P. mucronata*; *P. set* = *P. setacea*; *P. mal* = *P. malacophylla*; *P. poh* = *P. pohlii*; *P. sub* = *P. suberosa*; *P. bah* = *P. bahiensis*. The numbers above the bars represent the mean values of the Cycle threshold (Ct) of the three technical replicates and the standard deviation (SD) of the Ct .

Figure 11

Detection and quantification of CABMV in symptomatic and asymptomatic reinoculated plants by means of qualitative RT-PCR techniques and quantitative real-time PCR (qPCR) at 40 days after reinoculation (DARI). A) Amplification products by RT-PCR of the cylindrical inclusion of CABMV of 1311 pb in symptomatic reinoculated plants – S-RIP. Agarose 1%. M = Molecular DNA Marker Ladder of 1 Kb (Invitrogen™). PC = positive control (Matrix plant with severe CABMV symptoms – cDNA 200 ng.μL⁻¹);

B) Amplification of the standard curve and of the samples, standard curve with the quality and precision parameters of the qPCR reaction and dissociation curves (Melting curve) of the amplifications of the CABMV coat protein gene; C) Copies numbers (copies.μL⁻¹) of the CABMV virus in the asymptomatic reinoculated *Passiflora* spp. species; D) Genic products of the genomic region of the CABMV coat protein of 121 pb. Agarose 2%. M = Molecular DNA Marker Ladder of 100 pb (LudwigTM). NTC (No Template Control) = without mold cDNA; NC (Negative Control) = sample of *P. edulis* not inoculated with CABMV. Inter H. = Interspecific Hybrid (OTH-137); *P. mie* = *P. miersii*; *P. muc* = *P. mucronata*; *P. cin* = *P. cincinnata*; *P. gib* = *P. gibertii*; *P. set* = *P. setacea*; *P. mal* = *P. malacophylla*; *P. sub* = *P. suberosa*; *P. poh* = *P. pohlii*; *P. bah* = *P. bahiensis*. The numbers above the bars represent the mean values of the Cycle threshold (Ct) of the three technical replicates and the standard deviation (SD) of the Ct of each sample.

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