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Multiplex RT-PCR identification of five viruses associated with the watermelon crops in the Brazilian Cerrado

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Watermelon plants can be naturally infected by several viruses in single or mixed infections, of which the diagnosis is difficult and require specific techniques. This study aims to detect and to verify the presence of *Cucumber mosaic virus* (CMV), *Papaya ringspot virus type W* (PRSV-W), *Watermelon mosaic virus* (WMV), *Zucchini lethal chlorosis virus* (ZLCV), *Zucchini yellow mosaic virus* (ZYMV), and the main cucurbit viruses in Brazil using multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay. Oligonucleotides were designed towards the conserved regions of the virus genomes. In the duplex-PCR, it was possible to detect all the virus combinations, except ZLCV with ZYMV. The amplified product sizes were 644 bp (CMV), 535 bp (WMV-2), 398 bp (PRSV-W), 244 bp (ZLCV), and 214 bp (ZYMV). To test the efficacy of the methodology, we analyzed plants with virus symptoms from four municipalities in the state of Tocantins, located at Brazilian Cerrado. Mixed infections were detected in 80% of samples in all the municipalities. The multiplex RT-PCR assay can be used to detect and differentiate watermelon viruses that affect crops in the state of Tocantins.

Key words: Cucumovirus, multiplex RT-PCR, Orthotospovirus, potyvirus, watermelon.

INTRODUCTION

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai) is a crop of significant importance to Brazil due to its economic and social aspects, mainly in the northern and northeastern regions. However, the crop in these regions is affected by viruses (Aguiar et al., 2013, 2015). The most important viruses that affect the watermelon plants are: (i) cucumovirus as *Cucumber mosaic virus* (CMV) (Roossinck, 2001); (ii) potyviruses as *Papaya ringspot virus type W* (PRSV-W) (Barnett, 1991; Bateson et al., 2002), *Watermelon mosaic virus* (WMV) (Desbiez

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and Lecoq, 2004; Purcifull and Hiebert, 1979), and *Zucchini yellow mosaic virus* (ZYMV) (Desbiez and Lecoq, 1997; Harth et al., 2017); and (iii) orthotospovirus as *Zucchini lethal chlorosis viruses* (ZLCV) (Camelo-García et al., 2015; Lima et al., 2016). These viruses are widely distributed in the cucurbit cultivation areas in Brazil, limiting the yield and fruit quality (Aguiar et al., 2013, 2015; Soares et al., 2016). Moreover, they have been reported to cause not only single but mixed infections in the cucurbits (Aguiar et al., 2015; Ali et al., 2012; Soares et al., 2016), that contribute not only to the maintenance of the population diversity but also associated with the predominance of these viruses in commercial crops (Gómez et al., 2009; Kumar et al., 2010). Therefore, the development of methods that allow the simultaneous detection and identification of different viruses are desirable for the routine diagnosis of cucurbit virus infections, particularly, one that would require less time, work, and costs.

Several methods are used to detect viruses such as enzyme-linked immunosorbent assay (ELISA), double-antibody sandwich ELISA (DAS-ELISA), reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time PCR (qRT-PCR), nucleic acid spot hybridization (NASH), macroarrays and microarray technologies (Ge et al., 2013). However, all these methods are slow and expensive to carry out in a standard research laboratory. To overcome these shortcomings and increase the diagnostic capacity of PCR, a variant method termed multiplex PCR (mPCR) has been developed wherein one target sequence can be amplified by including more than one single pair of primers in the reaction (Elnifro et al., 2000). Reverse Transcription reaction followed by mPCR (mRT-PCR) can be used for the simultaneous detection of multiple viruses, and it is more efficient and economical than the other conventional methods (Kwon et al., 2014; Zhao et al., 2016).

Multiplex systems have been developed for the detection of multiple viral infections. Several genera have already been detected, for instance, astrovirus, rotavirus and reovirus (Jindal et al., 2012), carlavirus (Nam et al., 2015), crinivirus (Wintermantel and Hladky, 2010), nodavirus (Senapin et al., 2010), potyvirus, and allexivirus (Kumar et al., 2010), in animals (Jindal et al., 2012; Senapin et al., 2010) and plants (Kumar et al., 2010; Nam et al., 2015; Wintermantel and Hladky, 2010). According to the literature, the identification of viruses using mRT-PCR with specific primers has been widely used to detect viruses associated with different important crops around the world such as grapevine (*Vitis vinifera*) (Hajizadeh et al., 2012), pepino (*Solanum muricatum*) (Ge et al., 2013), potato (*Solanum tuberosum* L.) (Agindotan et al., 2007; Rigotti and Gugerli, 2007), *Prunus* spp (Jarosova and Kundu, 2010), sugarcane (*Saccharum* spp) (Xie et al., 2009) and tomato (*Solanum lycopersicum* L.) (Chen et al., 2011).

In the present study, we developed the mRT-PCR method for the simultaneous detection of five watermelon viruses (PRSV-W, WMV, ZYMV, CMV, and ZLCV), in the leaf tissue samples from infected watermelon from four different municipalities of Tocantins state, located at the Brazilian Cerrado.

MATERIALS AND METHODS

Plant and virus samples

Leaf samples from the infected watermelon plants in the reproductive stage, at the main watermelon producing regions of the municipalities of Gurupi, Porto Nacional, Lagoa da Confusão, and Formoso do Araguaia of Tocantins state (Figure 1) were collected as described by Aguiar et al. (2015).

CMV, PRSV-W, WMV, ZYMV, and ZLCV were obtained from Embrapa CNPH and Universidade de Brasília (UnB); they were provided by Dr. Mirtes Lima and Dr. Tatsuya Nagata. All the viruses were kept in a greenhouse at Universidade Federal do Tocantins-Gurupi in *Cucurbita pepo*, *Nicotine benthamiana*, and *Datura stramonium* plants by mechanical inoculation (Aguiar et al., 2015; Lima et al., 2009).

Primer design

The primers used in this study (Table 1) were designed from the specific nucleotide sequences data obtained from National Center for Biotechnology Information (NCBI) GenBank and checked by BLAST to confirm virus specificity. 11 accessions to CMV, 18 accessions to PMV-W, 18 accessions to WMV, 13 accessions to ZYMV, and 2 accessions to ZLCV (Supplemental Table 1) were used. The sequences were aligned by CLUSTAL W multiple sequence alignment program (Higgins and Sharp, 1989, 1988; Thompson et al., 1994) to find the genome regions specific for each virus (Table 1). Self-annealing and primer-dimer formation were tested in silico by using OligoCalc software (Kibbe, 2007). The primers were used here in several duplex combinations (Figures 4 and 5).

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA extraction was performed from the leaves of watermelon plants with symptoms of infection with CMV, PRSV-W, WMV, ZYMV, and ZLCV viruses (Figure 2) using the Plant RNA Purification Reagent Kit (Invitrogen Life Technologies, USA), according to the manufacturer's protocol. cDNA was synthesized from the total RNA extracted from the leaves with symptoms of infection of the viruses as previously described. First-strand cDNAs were synthesized using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen Life Technologies, USA) according to the manufacturer's instructions, with DEPC-treated water and random primers (NNNNNNN) (Invitrogen Life Technologies, USA) according to the supplied protocol.

Monoplex and multiplex PCR

PCR reactions were performed using the cDNA product with the addition of 0.4 µM of each virus-specific oligonucleotide, 10 µM of each dNTP, 2.5 µL of Taq DNA Polymerase Reaction Buffer, 2.0 mM of $MgCl_2$, and 1U of Taq DNA Polymerase (Invitrogen Life Technologies, USA), to a total volume of 25 µL.

Figure 1. Tocantins state's map indicating the four main producing municipalities from where the watermelon samples with viral infection symptoms, were harvested. Numbers on the map represent: 1, Porto Nacional; 2, Lagoa da Confusão; 3, Formoso do Araguaia; and 4, Gurupi.

Table 1. Oligonucleotides used in monoplex and multiplex RT-PCR for the detection of watermelon viruses.

Virus	Oligonucleotide sequence (5' - 3')*	Position	Amplicon size (bp)	Temperature of annealing (ºC)
CMV	F: ACTNTTAACCACCCAACCTT R: TTAGCCGTAAGCTGGATGGA	1379-1399 2003-2023	644	53.6 - 55.8
PRSV-W	F: TGGGTTATGATGGATGGGGA R: ATACCCAGGAGAGAGTGCAT	9703-9723 10081-10101	398	55.5 - 55.1
WMV	F: TTRTTGTTGAATGCTGTCCT R: GCTGCACAAATTGCCTCAG	8164-8184 8680-8699	535	52.6 - 55.1
ZYMV	F: CATACATGCCGAGGTATGGTTT R: GTGTGCCGTTCAGTGTCTT	9123-9145 9318-9337	214	55.0 - 55.6
ZLCV	F: GAGTTTCACTGTAATGTTCCATAGC R: AGYTTTGAGATGATCAGTGTTGT	454-479 675-698	244	53.9 - 52.5

*Nucleotides in degenerate positions are represented by a single letter of the code: R = A and G; Y = C and T; B = C, G, and T; D = A, G, and T; N = G, A, C, and T.

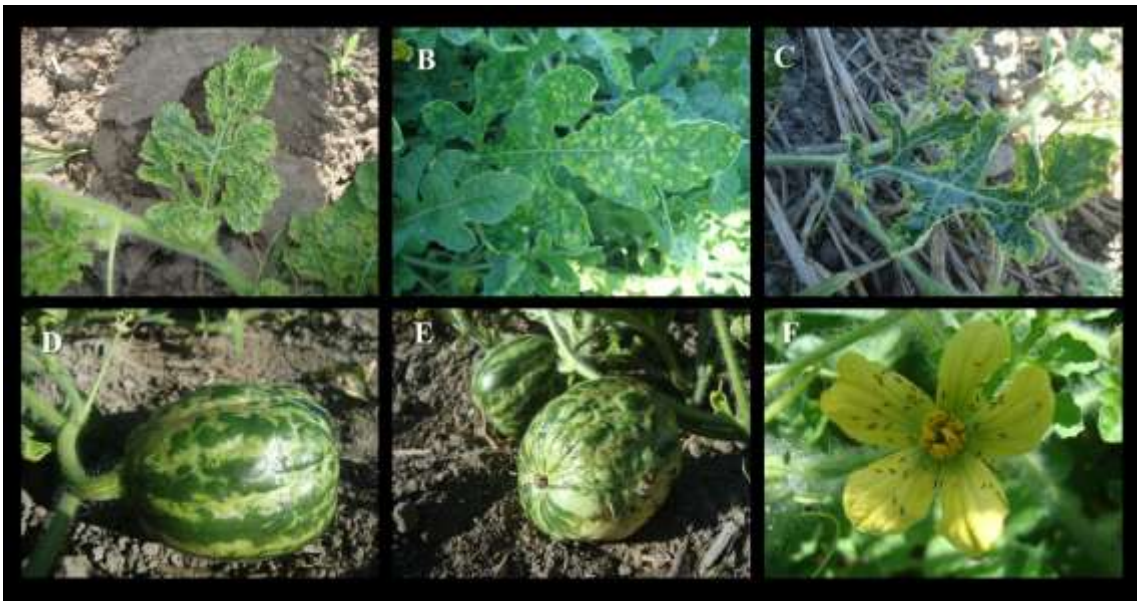

Figure 2. Symptoms of the viral infection of watermelon plants obtained from the main watermelon producing regions in the state of Tocantins. **(A)** leaves with symptoms of mosaic and chlorotic rings; **(B)** leaves with symptoms of bloom, foliar deformation, a slight mosaic in the lower parts, and underdevelopment; **(C)** leaves with symptoms of severe mosaic, bloom, leaf foliage, furrowed edges, and foliar deformation followed by necrosis; **(D)** watermelon fruit with symptoms of nutrient starvation; **(E)** watermelon fruit with symptoms of nutrient starvation and deformation of the bark; and **(F)** watermelon flower with trips infestation.

Amplification of the fragments for virus detection was performed with an initial denaturation step at 94°C for 5 min followed by 35 cycles at 95 °C for 30 s, 52 °C for 1 min and 30 s, and 72 °C for 4 min. A final elongation step was performed at 72°C for 8 min and 4ºC at the end until the samples were analyzed.

For the mRT-PCR assay, some conditions were modified in order to optimize the Taq DNA Polymerase amplification of cDNA template on two or more simultaneous viruses. The amount of each oligonucleotide specific for each virus was changed to 0.5 mM and 2.5µM of each dNTP and 2.0 mM of $MgCl_2$, to a total volume of 25 µL.

Analysis of mRT-PCR

The products of PCR (5 µL) were analyzed on 1% agarose gel in TBE buffer (0.1 M boric acid, 0.02 mM EDTA, pH 8.3) by electrophoresis at 80V. The molecular size of the amplified fragments was determined by comparison with 1kb Ladder Marker (Invitrogen Life Technologies, USA). The gel was stained with ethidium bromide solution (0.1 µL/mL) and visualized in a UV light transilluminator (Molecular Image LPIX-Locus Biotechnology). Moreover, the PCR products were sequenced to confirm the virus specificity and the nucleotides sequences of each target virus were compared with NCBI GenBank Database by using BLAST tool.

RESULTS

A range of symptoms caused by watermelon viruses were observed in the collected plant samples from the different municipalities of Tocantins state (Figure 1). The most frequently here observed symptoms were ruffled edges, blistering, spur, leaf narrowing, shoestring, leaf distortion, leaf rolling, necrosis, mosaic, and stunting, corresponding to viral infections symptoms in watermelon (Ali et al., 2012).

The virus-specific oligonucleotides were designed towards the conserved regions of CMV, WMV, PRSV-W, ZYMV, and ZLCV obtained from the sequences deposited in NCBI GenBank. The most conserved genome regions used for the development of oligonucleotides of the potyviruses (PRSV-W, ZYMV, and WMV) correspond to the regions of the protein coat genes. The oligonucleotides were developed for the rapid differentiation and detection of the watermelon viruses with each analyzed virus having a specific amplicon size.

The oligonucleotides designed to identify the watermelon virus were confirmed by both monoplex (single PCR) and mRT-PCR, in agarose gel electrophoresis (Figure 3). Using duplex combinations of oligonucleotides, the mRT-PCR assay was used for virus identification in infected plants samples from four different locales of watermelon production in the state of Tocantins (Figures 4 and 5).

In Gurupi, 80% of the leaf samples were positive for mixed infections (occurrence of two or more virus species) (Figure 4A). In all plants, the fragments of the expected size were amplified, especially PRSV- W, which was detected by mixed or single PCR in 80% of samples, WMV virus in 30%, ZLCV in 20%, and CMV in 10% samples (Figure 4A and Table 2). It was observed that

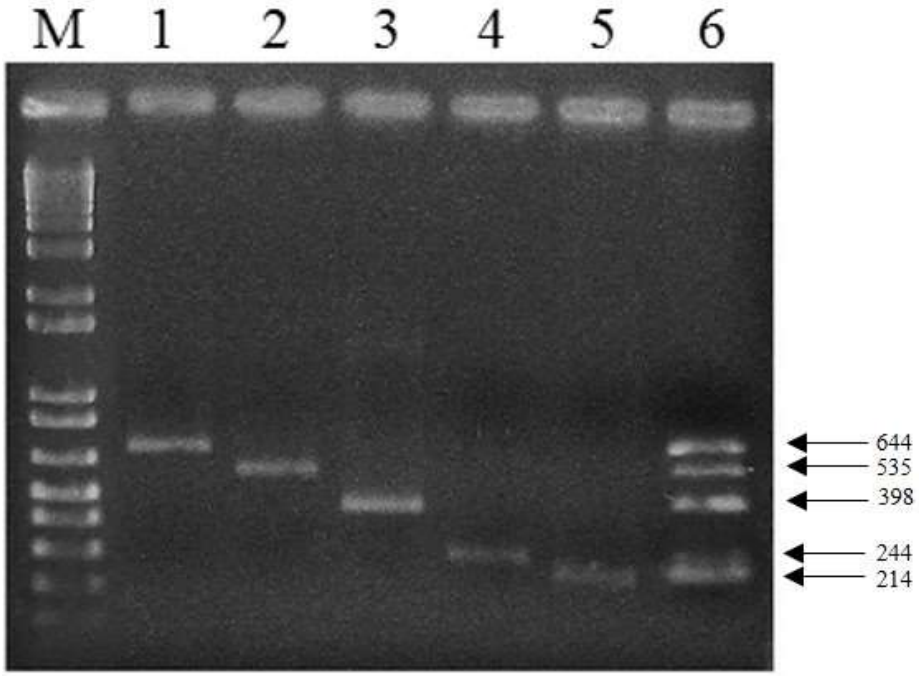

Figure 3. RT-PCR products obtained from specific primers. Viral RNA was used as a template for monoplex and multiplex RT-PCR. **M**, 1Kb marker; **1**, CMV (644 bp); **2**, WMV (535 bp); **3**, PRSV-W (398 bp); **4**, ZLCV (244 bp); **5**, ZYMV (214 bp); **6**, multiplex RT-PCR of all five viruses.

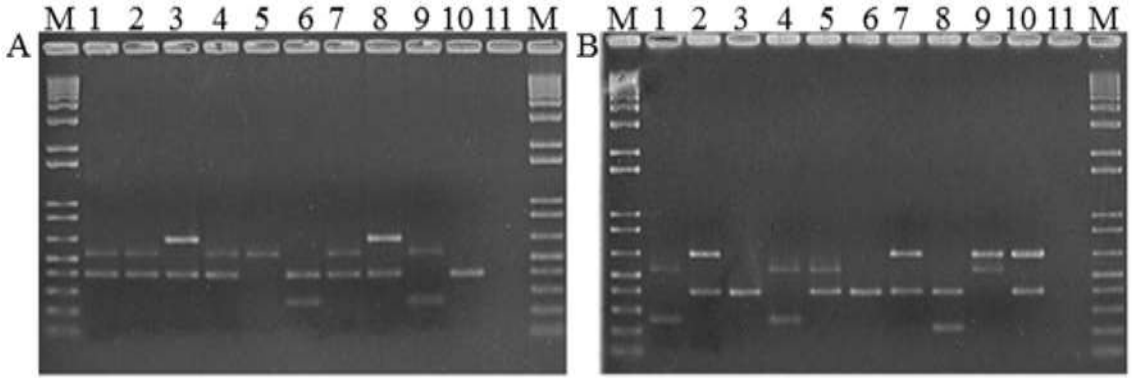

Figure 4. Multiplex RT-PCR viral detection on watermelon leaves of one sample from different cities. **(A)** Gurupi: **M**, 1Kb marker; **1**, WMV + PRSV-W; **2**, WMV + PRSV-W; **3**, CMV + PRSV-W; **4**, WMV + PRSV-W; **5**, WMV; **6**, PRSV-W + ZLCV; **7**, WMV + PRSV-W; **8**, CMV + PRSV-W; **9**, WMV + ZLCV; **10,** PRSV-W; and **11**, negative control. **(B)** Porto Nacional: **M**, 1Kb marker; **1**, WMV + ZLCV; **2**, CMV + PRSV-W; **3**, PRSV-W; **4**, WMV + ZLCV; **5**, WMV + PRSV-W; **6**, PRSV-W; **7**, CMV + PRSV-W; **8**, PRSV-W + ZYMV; **9**, CMV + WMV; **10**, CMV + PRSV-W; and **11**, negative control.

ZYMV was not present in the Gurupi samples.

In the city of Porto Nacional, 80% of the leaf samples had mixed infections as detected by mRT-PCR (duplex type) (Figure 4B). The PRSV-W was present in 70% of the samples with 30% of them in the mixed form (Figure 3B and Table 2). Interestingly, all the five viruses were found in the samples from the city of Porto Nacional.

In the city of Lagoa da Confusão, 100% of the leaf samples were found to have mixed infections by mRT-PCR (duplex type) (Figure 5A). WMV was present in 80% of the samples, followed by PRSV-W in 70% and ZLCV in 50%. Besides, the absence of CMV and ZYMV was observed at this locale (Figure 5A and Table 2).

Finally, 80% of the leaf samples from the City of

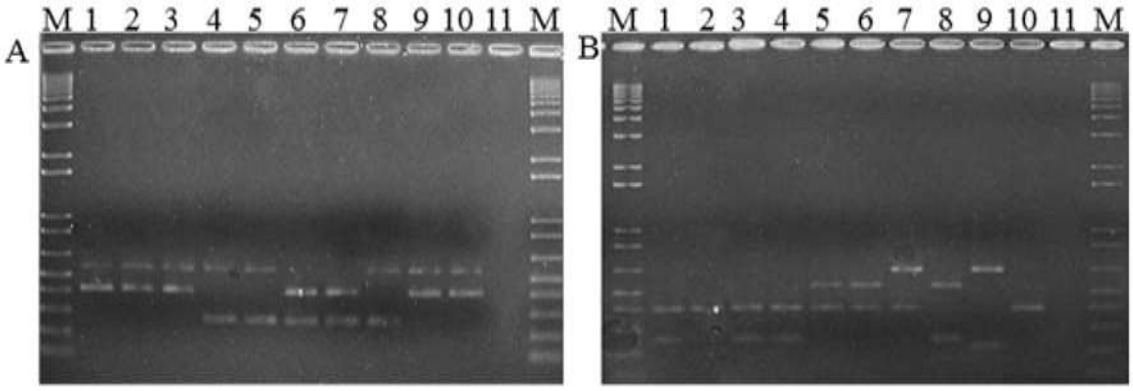

Figure 5. Multiplex RT-PCR viral detection on watermelon leaves of one sample from different cities tested with several possible combinations. **(A)** Lagoa da Confusão: **M**, 1Kb marker; **1**, WMV + PRSV-W; **2**, WMV + PRSV-W; **3**, WMV + PRSV-W; **4**, WMV + ZLCV; **5**, WMV + ZLCV; **6**, PRSV-W + ZLCV; **7**, PRSV-W + ZLCV; **8**, WMV + ZLCV; **9**, WMV + PRSV-W; **10**, WMV + PRSV-W; and **11**, negative control. **(B)** Formoso do Araguaia: **M**, 1Kb marker; **1**, PRSV-W + ZLCV; **2**, PRSV-W; **3**, PRSV-W + ZLCV; **4**, PRSV-W + ZLCV; **5**, WMV + PRSV-W; **6**, WMV + PRSV-W; **7**, CMV + PRSV-W; **8**, WMV + ZLCV; **9**, CMV + ZYMV; **10**, PRSV-W; and **11**, negative control.

Table 2. Multiplex RT-PCR viral detection incidence in watermelon plant samples (n = 40) with symptoms of infection harvested in four cities of the Tocantins state.

Local	Virus presence (%)					Mixed infection	Single infection
	CMV	PRSV-W	WMV	ZLCV	ZYMV	(%)	(%)
Gurupi	20.0	80.0	60.0	20.0	0.0	80.0	20.0
Porto Nacional	40.0	70.0	40.0	20.0	10.0	80.0	20.0
Lagoa da Confusão	0.0	70.0	80.0	50.0	0.0	100.0	0.0
Formoso do Araguaia	20.0	80.0	30.0	40.0	10.0	80.0	20.0
Total	20.0	75.0	52.5	32.5	5.0	85.0	15.0

Formoso do Araguaia were identified with mixed infections by mRT-PCR (duplex type) (Figure 5B). PRSV-W was present in 80% of the samples, followed by ZCLV in 40%, WMV in 30%, CMV in 20%, and ZYMV in 10% of the samples. So, all the viruses were detected in this locale.

At the state level, 85% of the total infections were of mixed type while the rest were of single type (Table 2). The CMV occurred in 20% and ZLCV in 32.5% of the total samples analyzed (Table 2).

DISCUSSION

Watermelon culture is affected by several viruses that directly influence its yield and fruit quality. In the present study, CMV, PRSV-W, WMV, ZYMV, and ZLCV were detected that affect watermelon yield in several places, such as Asia (Kwon et al., 2014; Zhao et al., 2016), Africa (Levi et al., 2016), and North America (Ali et al., 2012). Here we focused on the occurrence of these viruses in the Brazilian Cerrado in the state of Tocantins, a large producer of this crop. It is important to consider that the identification of a specific virus affecting watermelon crops is very difficult, or impossible, because of the possibility of asymptomatic or mixed virus infections, different virus strains, and confusion with abiotic disorders, such as nutrient deficiencies (Ali et al., 2012).

The design of oligonucleotides for effective detection of CMV, WMV, PRSV-W, ZYMV, and ZLCV becomes necessary because of the natural occurrence of these viruses in mixed infections, an interesting phenomenon of occurrence of synergism between two or more virus species (Ali et al., 2012). Thus, specific target oligonucleotides must be designed for the conserved regions of each virus genome. The oligonucleotides used here could detect and differentiate mixed viral infections by amplifying the products during mRT-PCR (duplex type) without interacting between them, the ideal for this technique (Wei et al., 2008). Even for ZLCV and ZYMV whose fragment sizes (244 and 214 bp, respectively) are very close, it is possible to differentiate the amplicons on agarose gel. It is important to note that alterations in the concentrations of oligonucleotides, cDNA, and/or Mg^{2+}

source, for instance, may improve the efficiency of PCR (Arezi et al., 2003), as the results here show.

Several researchers around the world developed methods with mRT-PCR to simultaneously identify and differentiate plant viruses for various crops, for instance the identification of pospiviroids in grapevine, with detection rate superior to 60% in four of five viroid samples analyzed (Hajizadeh et al., 2012), potexvirus, carlavirus, and tobamovirus in pepino, with 5% to 40% virus detection (Ge et al., 2013), potyvirus and polerovirus in sugarcane (Xie et al., 2009), cucumivirus and tobamovirus in tomato, with detection superior to 75% (Chen et al., 2011); potyvirus and allexivirus in onion (Kumar et al., 2010) and garlic (Nam et al., 2015), and crinivirus in tomato, lettuce (*Lactuca sativa* L), and melon (*Cucumis melo* L.) (Wintermantel and Hladky, 2010). Here, we detected all the five viruses tested (CMV, PRSV-W, WMV, ZLCV, and ZYMV) in almost all locales, except CMV in Lagoa da Confusão and ZYMV in Lagoa da Confusão and Gurupi.

ZYMV virus was found only in two samples, in the mixed infection form, at Porto Nacional and Formoso do Araguaia, in combination with PRSV-W and CMV. It is important to mention that the distance between the locations of these two cities is large enough, and infection is not possible by the same host insects from one place to another. Moreover, PRSV-W had the highest number of infected leaves samples, was present in all cities studied, with infection of 75% of the samples tested here (Table 2), and these results indicate the predominance of this virus in the commercial plantations of cucurbits in Tocantins state. Besides, the quality of our multiplex results is similar to that demonstrated by other studies in cucurbits (Kwon et al., 2014; Wang et al., 2010). Moreover, the capacity of PRSV-W infection and association with other viruses makes it difficult to identify the infection by symptoms. Another key factor is the mixed infections caused by this virus (Figures 3 and 4), which was obtained in all combinations (PRSV-W + WMV; PRSV-W + CMV; PRSV-W + ZLCV; and PRSV-W + ZYMV).

Cucurbit crops are cultivated throughout the world with watermelon being mainly produced in South America; however, the yield and fruit quality are affected by viral diseases (Romay et al., 2014). CMV, PRSV-W, WMV, ZYMV, and ZLCV are the main viruses that infect watermelon through aphids (CMV, PRSV-W, WMV) and trips (ZLCV) as vectors (Romay et al., 2014). In Brazil, ELISA method is often used to detect watermelon virus. Previous studies report PRSV-W and WMV as the main viruses infecting watermelon and other cucurbits in different regions such as the northeast (Moura et al., 2001; Silveira et al., 2009; Soares et al., 2016), southeast (Lima and Alves, 2011), midwest (Lima and Alves, 2011) and north (Aguiar et al., 2013, 2015; Lima and Alves, 2011). On the other hand, it was reported in Paraiba that ZYMV was the main virus infecting watermelon crop (Soares et al., 2016) contrary to other reports from the same region (Moura et al., 2001; Silveira et al., 2009). Here, we mainly described PRSV-W and, especially in Tocantins, it has been reported that CMV causes more damage to watermelon plants (Aguiar et al., 2013), and the pattern of infection frequency is the same: PRSV-W with the highest rate followed by WMV, ZLCV, CMV, and ZYMV (Aguiar et al., 2015). For instance, PRSV-W was detected in 22% of symptomatic plants by ELISA method, while mRT-PCR found it in 75% (Table 2). It is worth mentioning that weeds can act as natural reservoirs of these watermelon viruses, particularly ZYMV and PRSV-W (Aguiar et al., 2018). The distribution of virus in the state can be attributed to the mode of transmission, non-persistent by several species of aphids, of these viruses (CMV, PRSV-W, WMV, and ZYMV). In this way, the potyviruses (PRSV-W, WMV, and ZYMV) were detected in all the samples analyzed, in single or mixed infections, and these results indicate the prevalence of potyvirus infecting cucurbits in Tocantins.

Virus detection by rapid and reliable techniques can be important tools in plant virology for disease control in production fields to phytosanitary barriers. Current panorama of watermelon production in Brazil and the world indicates that an effective simultaneous detection and differentiation of CMV, PRSV-W, WMV, ZLCV, and ZYMV is required due to the natural occurrence of these viruses in mixed infections. To our knowledge, this is the first report on the use of mRT-PCR for simultaneous detection of these five viruses that affect watermelon crop in Tocantins, an important agricultural region in Brazil. The mRT-PCR assays developed in this study will provide a simple method for the detection of multiple viruses in watermelon culture in Brazil. Moreover, the specificity of the oligonucleotides allows the development of epidemiological, spatial, and temporal distributions of the virus under study.

CONFLICT OF INTERESTS

The authors did not declare any conflict of interest.

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ABBREVIATIONS

CMV, *Cucumber mosaic virus*; **DAS-ELISA,** double-antibody sandwich ELISA; **ELISA,** enzyme-linked immunosorbent assay; **M-MLV,** Moloney Murine Leukemia Virus; **mPCR,** multiplex PCR; **mRT-PCR,** reverse transcription reaction followed by mPCR; **NASH,**

nucleic acid spot hybridization; **PRSV-W,** *Papaya ringspot virus type W*; **qRT-PCR**, quantitative real-time PCR; **RT-PCR,** reverse transcription polymerase chain reaction; **WMV,** *Watermelon mosaic virus*; **ZLCV,** *Zucchini lethal chlorosis virus*; **ZYMV,** *Zucchini yellow mosaic virus*.

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Supplemental Table 1. Accession numbers obtained from National Center for Biotechnology Information (NCBI) GenBank used for the oligonucleotides design.

Virus	CMV	PRSV-W	WMV	ZYMV	ZLCV
Accession	AB006813	AB369277	AB218280	AB188115	AF067069
	AF103991	AY010722	AB369278	AB188116	D00645
	AF127977	AY027810	AY437609	AB369279	
	AJ304399	AY162218	DQ399708	AF014811	
	AJ585522	AY231130	EU660578	AF127929	
	AJ831578	DQ340769	EU660579	AY188994	
	AM183116	DQ340770	EU660580	AY278998	
	D00385	DQ340771	EU660581	AY278999	
	D10538	DQ374152	EU660582	AY279000	
	D10539	DQ374153	EU660583	DQ124239	
	NC001440	EF017707	EU660584	EF062582	
		EF183499	EU660585	EF062583	
		EU126128	EU660586	NC003224	
		EU475877	EU660587		
		EU882728	EU660588		
		NC_001785	EU660589		
		X67673	EU660590		
		X97251	NC006262		

CMV, *Cucumber mosaic virus*; **PRSV-W,** *Papaya ringspot virus type W*; **WMV,** *Watermelon mosaic virus*; **ZYMV,** *Zucchini yellow mosaic virus*; **ZLCV,** *Zucchini lethal chlorosis virus*