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Artigo

Viruses occurring in watermelon in the tropical lowland of the Tocantins state, Brazil

Aline Torquato Tavares [1,*], Edgard Henrique Costa Silva [2,*], Tiago Alves Ferreira [1], Kleycianne Ribeiro Marques [1] e Ildon Rodrigues Nascimento [1]

[1] Universidade Federal do Tocantins, Palmas, Gurupi, TO
[2] Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, SP
***** Autor Correspondente: alinet4t@hotmail.com

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Abstract: The cucurbits are subject to various diseases caused by viruses and the identification of species that predominate in one region has great importance to direct the control strategies. The study aimed to diagnose by DAS-ELISA and RT-PCR tests virus isolates collected in commercial plantations of lowland tropical watermelon in the State of Tocantins. Were collected 20 leaf in the municipality of Formoso do Araguaia and 46 leaf samples from Lagoa da Confusão. The samples were packed in plastic bags properly identified and transferred to the Department of Plant Pathology DFP / UFLA and / or stored in a refrigerator at -80 °C. The samples were grown in a greenhouse for plants pumpkin *Curcubita pepo* cv. Caserta and were performed the DAS-ELISA test serology and molecular evaluation. No sample reacted with the antiserum for the cucumber mosaic virus (CMV), on the other hand reacted to sixteen mosaic virus-of-pumpkin (SqMV). The predominant virus in producing regions of watermelon grown in lowland tropical rainforest in the state of Tocantins were ZYMV and SqMV and the occurrence of mixed infection (ZYMV+SqMV) was achieved in 35% of samples in the municipality of Lagoa da Confusão. In the municipality of Formoso do Araguaia prevailed ZYMV.
Keywords: *Citrullus lanatus*. Identification of the viruses. SqMV. ZYMV.

Ocorrência de viroses em melancia nas várzeas do estado do Tocantins, Brasil

Resumo: As cucurbitáceas estão sujeitas a várias doenças causadas por vírus e a identificação das espécies que predominam em uma região é de grande relevância para direcionar as estratégias de controle. O trabalho teve por objetivo diagnosticar por meio de teste DAS-ELISA e RT-PCR isolados de vírus coletados em plantios comerciais de melancia em várzea tropical no Estado do Tocantins. Foram coletadas 20 amostras foliares do município de Formoso do Araguaia e 46 amostras foliares da Lagoa da Confusão. As amostras foram acondicionadas em sacos plásticos devidamente identificados e armazenadas em refrigerador a -80°C. As amostras foram multiplicadas em casa de vegetação em plantas de abóbora *Curcubita pepo* cv. Caserta e em seguida foram realizados o teste sorológico DAS-ELISA e a avaliação molecular. Nenhuma amostra reagiu com o antissoro para o vírus do mosaico do pepino (CMV), por outro lado dezesseis reagiram para o vírus do mosaico-da-abóbora (SqMV). Os vírus predominantes nas regiões produtoras de melancia cultivada em várzea tropical do Estado do Tocantins foram ZYMV e SqMV e a ocorrência de infecção mista (ZyMV+SqMV) foi verificada em 35% das amostras do município da Lagoa da Confusão. No município de Formoso do Araguaia predominou ZYMV.

Palavras-chave: *Citrullus lanatus* L. Identificação de viroses. SqMV. ZYMV.

1. INTRODUCTION

The cropping of watermelon [*Citrullus lanatus* L. (Thunb) Nakai & Matsum] in Brazil is carried out in all states. In the north of the country, the state of Tocantins is the main producer with over nine thousand hectares, in tropical lowland conditions with the use of subsurface irrigation, planting from April to July (IBGE, 2015). The two main producing municipalities are Lagoa da Confusão and Formoso do Araguaia, accounting together over 90% of the state production.

Despite the fact that the local temperatures extrapolate the limit set as ideal for the development of watermelon, higher yields are achieved due to the use of subirrigation, providing constant presence of moisture in the plant root system, causing this effect to be attenuated. With the use of cultivars, high fertility soils, favorable climate and sufficient water for the whole crop cycle, the harvesting is performed about 70 days after planting (Santos et al., 2010).

In a survey conducted by Lima and Alves (2011) in 21 municipalities covering seven states, plus the Federal District, in the period 2008-2010, with a total of 564 samples, using dot-ELISA with polyclonal antibodies, was checked virus occurrence in 323 samples, representing 57.3%. Among the species of virus, PRSV-W was found in 182 samples (32.3%), WMV in 156 (27.7%) and ZYMV in 156 (27.7%) of the samples. The presence of CMV was identified in 121 plants (21.5%) while ZLCV occurred in 74 (19.8%). Mixed infection with the involvement of more than one species of virus in the plant was detected in 31.4% of samples. The three potyvirus and the CMV were detected in crops of all sampled states and the Federal District. These results confirm the wide dissemination of these viruses in cucurbit crops in Brazil.

Alencar et al. (2012) performed a biological and molecular identification of cucurbits leaves containing virus, from the State of Tocantins. In 56% of the samples was identified Squash mosaic virus (SqMV). The remaining isolates were identified as Zucchini yellow mosaic virus (ZYMV). Tavares et al. (2014) evaluating phenotypic symptoms of watermelon samples collected in these two Tocantins state producing regions also noted the severity with which these viruses attack the watermelon crop, highlighting the importance of further studies on these pathogens.

The presence of favorable climatic conditions and the presence of alternative hosts have hindered the management and control of viruses in lowlands commercial crops. Thus, the molecular characterization of virus isolates collected in commercial watermelon plantings in tropical lowland in the state of Tocantins, represents an important tool for breeding programs developed at the Federal University of Tocantins (UFT) and also for the management and control of these viruses by local farmers.

The viruses impair substantially the yield of watermelon, so the local producers, which is the main producing region of watermelon in the State of Tocantins, need to take preventive measures, such as the use of certified seeds to mitigate the damage caused by occurrence of viruses.

The objective was to diagnose by DAS-ELISA and RT-PCR tests, the virus species that occur in commercial watermelon crops in tropical lowland conditions, in the state of Tocantins.

2. MATERIAL AND METHODS

It was collected sixty-six well-developed true leaves leaf samples of watermelon plants with symptoms of virus, obtained from commercial crops in lowland conditions, being 20 from Formoso do Araguaia and 46 from Lagoa da Confusão (Table 1). It was subsequently packed in plastic bags and labeled with date and place of collection, and then stored in a refrigerator at -80° C.

Table 1. Identification and local of collections of virus isolates collected from plants of watermelon commercial crops on tropical lowland conditions in the municipalities of Formoso do Araguaia and Lagoa da Confusão.

Isolates	Local of collection	Isolates	Local of collection
1-FA	Formoso do Araguaia	14-LC	Lagoa da Confusão
2-FA	Formoso do Araguaia	15-LC	Lagoa da Confusão
3-FA	Formoso do Araguaia	16-LC	Lagoa da Confusão
4-FA	Formoso do Araguaia	17-LC	Lagoa da Confusão
5-FA	Formoso do Araguaia	18-LC	Lagoa da Confusão
6-FA	Formoso do Araguaia	19-LC	Lagoa da Confusão
7-FA	Formoso do Araguaia	20-LC	Lagoa da Confusão
8-FA	Formoso do Araguaia	21-LC	Lagoa da Confusão
9-FA	Formoso do Araguaia	22-LC	Lagoa da Confusão
10-FA	Formoso do Araguaia	23-LC	Lagoa da Confusão
11-FA	Formoso do Araguaia	24-LC	Lagoa da Confusão
12-FA	Formoso do Araguaia	25-LC	Lagoa da Confusão
13-FA	Formoso do Araguaia	26-LC	Lagoa da Confusão
14-FA	Formoso do Araguaia	27-LC	Lagoa da Confusão
15-FA	Formoso do Araguaia	28-LC	Lagoa da Confusão
16-FA	Formoso do Araguaia	29-LC	Lagoa da Confusão
17-FA	Formoso do Araguaia	30-LC	Lagoa da Confusão
18-FA	Formoso do Araguaia	31-LC	Lagoa da Confusão
19-FA	Formoso do Araguaia	32-LC	Lagoa da Confusão
20-FA	Formoso do Araguaia	33-LC	Lagoa da Confusão
1-LC	Lagoa da Confusão	34-LC	Lagoa da Confusão
2-LC	Lagoa da Confusão	35-LC	Lagoa da Confusão
3-LC	Lagoa da Confusão	36-LC	Lagoa da Confusão
4-LC	Lagoa da Confusão	37-LC	Lagoa da Confusão
5-LC	Lagoa da Confusão	38-LC	Lagoa da Confusão
6-LC	Lagoa da Confusão	39-LC	Lagoa da Confusão
7-LC	Lagoa da Confusão	40-LC	Lagoa da Confusão
8-LC	Lagoa da Confusão	41-LC	Lagoa da Confusão
9-LC	Lagoa da Confusão	42-LC	Lagoa da Confusão
10-LC	Lagoa da Confusão	43-LC	Lagoa da Confusão
11-LC	Lagoa da Confusão	44-LC	Lagoa da Confusão
12-LC	Lagoa da Confusão	45-LC	Lagoa da Confusão
13-LC	Lagoa da Confusão	46-LC	Lagoa da Confusão

At inoculation, the leaves of each isolate were macerated with addition of potassium phosphate buffer (K_2HPO_4) 0.01 M, pH 7.0, 0.1% sodium sulphite (Na_2SO_3). The inoculation was done by rubbing the viral suspension on cotyledons leaves, previously dusted with the abrasive carborundum, 400-mesh abrasiveness. After inoculation, withdrew, via washing, the excess of abrasive. These plants were kept in a greenhouse with an aphid-proof net.

After the onset of symptoms in the inoculated plants, the leaves were collected and subjected to diagnostic DAS-ELISA ytest Clark and Adams (1977) with antisera specific for SqMV and CMV, produced by Agdia®, and the procedure was according to the manufacturer's recommendation. The sample was considered positive in DAS-ELISA test when the read value of the absorbance was at least twice superior to the average value of the absorbance recorded for healthy plant extract used as a negative control.

In the diagnostic with RT-PCR, it was used primers designed based on the squash mosaic virus genomes (SqMV: Foward: 5'-TTTGACGGCATGGTC-3' and Reverse: 5'-GGAAAGAAGCCACAAC-3'), on zucchini yellow mosaic virus (ZYMV : Foward: 5'-GATTTGAATGAGCAACAGATGG-3' and Reverse: 5'-CTCCGCTGCATCTGAGAAGT-3') and papaya ringspot virus strain W (PRSV-W: Foward: 5'-GATTTGAATGAGCAACAGATGG-3' and Reverse: 5'-CTCCGCTGCATCTGAGAAGT-3'). The complementary DNA (cDNA) was synthesized from total RNA extracted using the reverse primer 5'-CTCCGCTGCATCTGAGAAGT-3' and the enzyme M-MMLV reverse transcriptase (Promega, São Paulo / SP, Brazil), according to the manufacturer's recommendations.

For RT-PCR analysis, the total RNA extraction of leaf samples of the isolates was carried out by maceration of 0.2 g of young leaves with approximately ten days, with viruses characteristic symptoms of each isolated in a pestle in presence of liquid nitrogen. To the obtained product was added Trizol® solution (aqueous solution containing 38% saturated phenol, 0.8M of guanidiniun thiocyanate, 0.4M of ammonium thiocyanate and 0.1M sodium acetate, pH 5.5% of glycerol) in 1g10mL ratio. Subsequently, the microtubes (2 ml) were incubated at room temperature, for 5 minutes, and then centrifuged at 12,000 RPM for 10 minutes, at 4ºC. The obtained precipitate was discarded, adding 300 µl of chloroform to each microtube, which was later vortexed and left at room temperature for 3 minutes.

After incubation, the microtubes were centrifuged again, at 12,000 RPM, for 10 minutes, at 4 ºC, and the supernatant obtained was transferred to another microtube, adding to half of its volume isopropanol and 0.8M of sodium citrate / 1.2 M of NaCl. The tubes were gently mixed by inversion and left at room temperature for 10 minutes, so that there was precipitation of the RNA. After this time, the microtubes were centrifuged at 12,000 RPM, at 4 °C, for 10 min, discarding, subsequently, the supernatant. The precipitate was washed with cold 75% ethanol, centrifuged at 12,000 rpm, at 4 °C, for 10 minutes. The supernatant was discarded and the tube was dried in vacuum. The pellet obtained was resuspended in 25µL of ultrapure water treated with diethylpyrocarbonate (DEPC). The total RNA extracted was visualized on 0.7% agarose gel.

The reverse transcription was done using 0.5 µL of the reverse primer, 1.0 µL of extracted RNA and 4.0 µL DEPC-treated ultrapure water in a microtube that was initially incubated for 5 minutes, at 75 °C, and after this time, it was immediately transferred to ice. Then it is added 2.0 µL of reverse transcriptase buffer (RT M-MLV USB buffer), 0.5 µL of 10mM dNTP, 0.2 µL of the RT enzyme (USB) and 1.8 µL of DEPC-treated ultrapure water. The tubes were incubated at 42 °C, for 30 minutes, then at 95 °C, for 5 min, and transferred to ice.

Thereafter, it was made the PCR amplification with a buffer, MgCl2 (25 mM,) dNTP10 mM, primer sense (forward primer) and antisense (reverse primer), the enzyme Go Taq Flexi DNA polymerase using an initial denaturing at 95 °C, for 2 minutes, followed by 35 cycles: 95 °C for 45 seconds, 50,7 ºC for a minute, 72 °C for 1 minute, and final extension at 72 °C for 5 minutes. The obtained product was analyzed by electrophoresis on 0.7% agarose gel stained with Gel Red (Biotium®).

3. RESULTS AND DISCUSSION

In DAS-ELISA test performed, none of the samples reacted with the antiserum for the cucumber mosaic virus (CMV), in the two municipalities. On the other hand, sixteen of the 46 samples from Lagoa da Confusion, that is, 35% were identified as squash mosaic virus (SqMV) (Table 2).

Table 2 - Serological identification by DAS-ELISA of watermelon leaf samples with virus symptoms collected in areas of commercial production of watermelon in the State of Tocantins

Local of collection	Number of tested samples	Number of negative samples	Number of samples with virus and Percentage of incidence	
			CMV	SqMV
Lagoa da Confusão	46	30	0 (-)	16 (35*)
Formoso do Araguaia	20	20	0 (-)	0 (-)
Total	66	50	0 (-)	16 (35*)

* percentage of samples with virus

This result is alarming since, a few years ago there was no report of this virus in the region. According to Lima and Amaral (1985), the virus dissemination is carried by some species of insects, beetles chrysomelids (*Diabrotica* spp. and *Acalymma* spp.), by a coccinellid beetle and by seeds. The origin of seeds become alerting, as long distance introduction is facilitated via seeds and the virus presence was diagnosed in other neighboring statesIn work carried out by Silveira et al., (2009), non occurrence of expressive values of squash mosaic virus were observed. When evaluating cucurbit leaf samples from the southern state of Tocantins, Alencar et al. (2012) reported the occurrence of SqMV in 56% of samples, corroborating the data of this work.

The SqMV has been considered less important in the Brazilian territory, so that in some regions, such as northeastern Brazil, it is considered that the improvement of breeding programs should consider only the PRSV, strains W and P, and ZYMV Halfeld-Vieira et al., (2004). However, the results obtained here showed that this is not the same reality that occurs in the state of Tocantins, where a significant portion of the samples was affected with this virus. This demonstrates that the SqMV may have found the ideal conditions in the Tocantins region to disseminate and infect the local hosts.

Even the producers of the Tocantins state being technified, they use second-generation hybrids seeds of other regions, which may explain the introduction of SqMV in the state. In the first survey carried out in Brazil for the incidence of viral diseases, it was found only the presence of potyvirus in Tocantins state, but lately, the work carried out included the SqMV.

The SqMV can be transmitted through commercial seeds in a percentage of up to 10% (Blancard et al., 1996). In the state of Rio Grande do Norte, the SqMV was probably introduced via imported commercial seeds (Florindo and Lima 1993). This may have occurred due to use of uncertified seeds from other regions. Although only a minority of host pathogen interactions result in the infection process, the transmission of viruses via seeds can be considered important with serious economic consequences for the farmer. The low transmission rate is not a good epidemiological indicator, since, jointly with the presence of vectors in the cultivated area, may result in the introduction of viruses into new areas, generating and causing epidemic dissemination to more distant locations. For example, in the case of lettuce mosaic virus (potyvirus), whose vector transmits the virus in a non-persistent mode, the incidence of 0.001% of affected seeds can lead to impairment of cropping. In this case, besides the yield loss, there is also the loss of product quality. Therefore, unlike other pathogens-caused diseases, the viruses can not be controlled with pesticides when they are already present in the field. Thus, the use of certified seeds, demonstrably free of viruses, is far more efficient and preventive control, in order to avoid or delay the maximum the entry of the virus into a production area (Machado, 2010).

In the diagnosis by molecular testing with RT-PCR, eleven samples from the municipality of Formoso do Araguaia amplified bands that characterize the presence of ZYMV (bands of 200 bp), representing 55% of total

samples of that municipality and 17% of the total analyzed. Regarding the five samples from the municipality of Lagoa da Confusão, only the sample 2-LC has not amplified band that characterize this virus (Figure 1).

All the 66 samples, although they were tested with primers for PRSV-W and SqMV, showed negative result, with no amplification bands for any of these viruses. Twenty-seven samples from municipality of Lagoa da Confusão have amplified bands with the pair of primers used to ZYMV, representing 59% of the samples of the municipality and 41% of the total (Figure 1, 2 and 3).

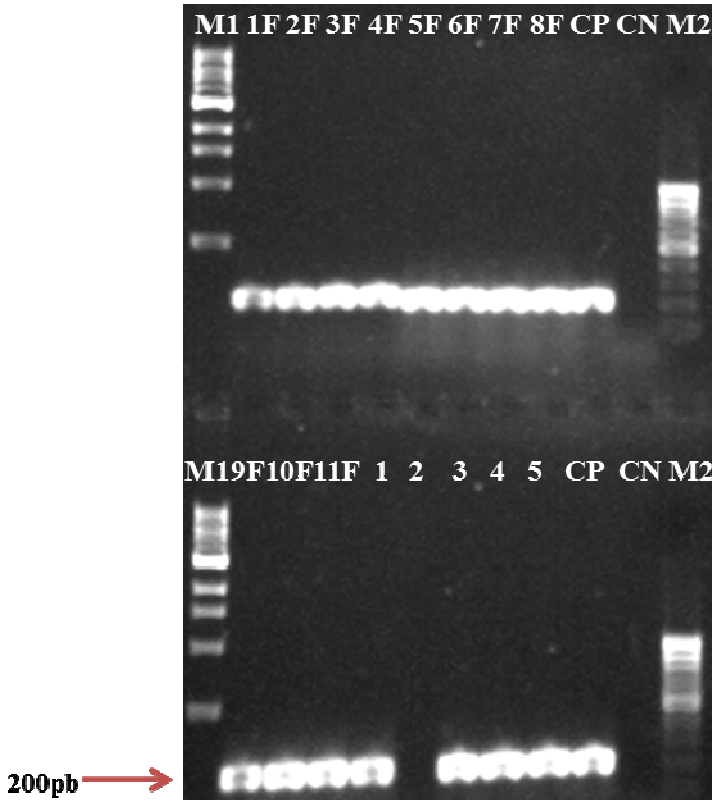

Figure 1 - Banding pattern of amplified electrophoretic analysis with primer to Zucchini yellow mosaic virus (ZYMV). M1: 1Kb DNA ladder marker; 1F to 11F 2-FA samples; 3-FA; 4-FA; 5-FA; 6-FA; 7-FA; 8-FA; 9-FA; 10-FA and 11-FA represent isolates from Formoso do Araguaia; 1-5 samples: 1-LC; 2-LC; LC-3; 4-5-LC and LC represent isolated from Lagoa da Confusão; CP (positive control); CN (negative control) and M2: 100 pb Marker.

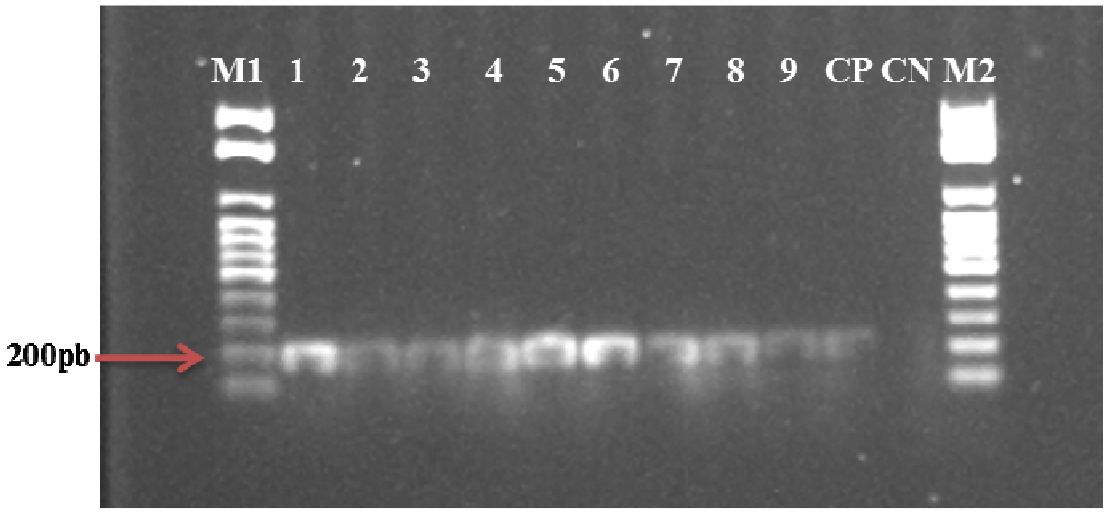

Figure 2 - Banding pattern of amplified electrophoretic analysis with primer to Zucchini yellow mosaic virus (ZYMV). M1 and M2: 100pb Marker; 1 to 9: samples 6-LC, 7-LC, 8-LC, 9-LC, 10-LC, 11-LC, 12-LC, 13-LC and 14-LC from Lagoa da Confusão. CP (positive control); CN (negative control)

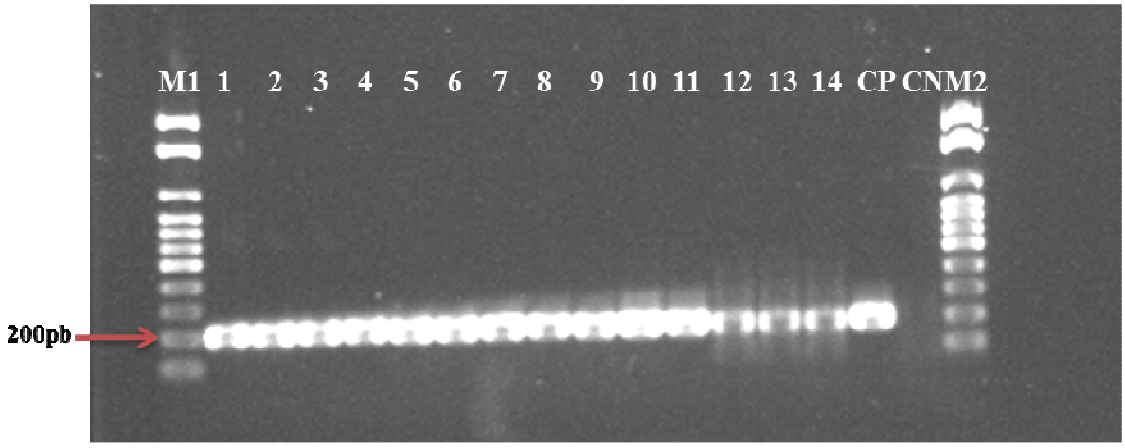

Figure 3 - Banding pattern of amplified electrophoretic analysis with primer to Zucchini yellow mosaic virus (ZYMV). M1 and M2: 100pb Marker; samples 1 to 14: 15-LC, 16-LC, 17-LC, 18-LC, 19-LC, 20-LC, 21-LC, 22-LC, 23-LC, 24-LC, 25-LC, 26-LC, 27-LC and 28-LC from Lagoa da Confusão. CP (positive control); CN (negative control)

The ZYMV has been identified in other regions of the country. Halfeld-Vieira et al. (2004) in Roraima and Amazonas Lima et al. (2010) in the Lower Basin of São Francisco Region, and Silveira et al. (2009) and Moura et al. (2001) in Maranhão. Recently Alencar et al. (2012) found 44% of cucurbits leaf samples in the state of Tocantins with the presence of ZYMV, corroborating the results of this work.

Sixteen samples from the municipality of Lagoa da Confusão that were positive for the SqMV antiserum in DAS-ELISA were also positive by RT-PCR molecular test for the virus ZYMV, demonstrating mixed infection in 24% of all samples and 35% of samples from the municipality of Lagoa da Confusão. Dikova and Hristova (2002) evaluated the presence of ZYMV, SqMV and CMV virus in cucurbit seeds, they found a very significant presence, reaching 91% of ZYMV and SqMV and 95% of CMV in a commercial cultivar in Bulgaria.

Ramos et al. (2003) argue that strategies for coping with these viruses originated from mixed infection should not consider viruses separately, since both are occurring simultaneously and may cause more damage when they affect the same plant at the same time. Halfeld-Vieira et al. (2004) analyzing samples collected at different times in the years 2003/2004 in the State of Roraima verified the presence of PRSV-W, WMV and ZYMV in single and mixed infections. The mixed infection that was found can be from the interaction of viruses belonging to different genres, which are transmitted by insects in different ways, being the ZYMV transmitted non-persistently by aphids. Regarding the SqMV that is transmitted semi-persistently by aphids and also by seeds, yet these two viruses found ways for its dissemination in the Tocantins area.

The transmission of SqMV occurs through insects of the order Coleoptera, the genera Diabrotica (*D. speciosa, D. bivitula*) and Epilachma (*Epilachma cacica*), in a persistent or circulative way (Zitter et al., 1996), by beetles Chrysomelids (*Diabrotica* spp and *Acalymma* spp) (Freitag, 1956; Lastra, 1968; Sitterly, 1960; Stoner, 1963), by a coccinellid beetle (Cohen and Nitzany, 1963) and leafhopper (Stoner, 1963). It has been shown that all viruses transmitted by seed so are by the pollen grain or the egg. The pollen as a vehicle for viruses implies the production of contaminated seeds and the infection of virus-free plants, and insects contribute to this dissemination.

4. CONCLUSIONS

The predominant viruses in watermelon crops in tropical lowlands of the state of Tocantins are the ZYMV and the SqMV.

Mixed infection (ZYMV + SqMV) represents 35% of the samples in the municipality of Lagoa da Confusão. In the municipality of Formoso do Araguaia predominates the ZYMV.

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