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Sources of resistance in accessions of *Cucurbita* spp. to virus species from the genus *Potyvirus*[1]

Fontes de resistência em acessos de *Cucurbita* spp. a espécies de vírus do gênero *Potyvirus*

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ABSTRACT - The identification of source of resistance in cultivated cucurbits species is very important for the development of resistant cultivars to control diseases caused by virus from the genus *Potyvirus*. The present research had the objective to evaluate the phenotypic reactions and the behavior of pumpkin (*Cucurbita* spp.) accessions to the virus species from the genus *Potyvirus*: *Papaya ringspot virus* type Watermelon (PRSV-W), *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV). Twenty-eight accessions of pumpkin from the Cucurbit Germplasm Bank from Embrapa Semiárido, Petrolina, PE, Brazil were evaluated. Twelve young plants from each pumpkin accession were inoculated with each one of the virus species and were maintained at greenhouse for their symptom reaction evaluations. All possible virus infections or absence of infection were confirmed by plate-trapped antigen enzyme linked immunosorbent assay (PTA-ELISA) against antisera specific to PRSV-W, ZYMV and WMV at the Plant Virus Laboratory, Universidade Federal do Ceará. Three pumpkin accessions showed extreme resistant to WMV and eight accessions presented mild mosaic when inoculated with PRSV-W. The most severe symptoms were observed on those accessions inoculated with ZYMV and 50% of the accessions showed to be highly susceptible. On the other hand, the pumpkin accessions inoculated with WMV presented the mildest symptoms, indicating that 39% of them were resistant, and 39% were tolerant. The *Cucurbita* spp. accessions BGC 518, BGC 530, BGC 567, and BGC 683 that showed resistance to one or more than one virus species constitute promising sources of resistance for developing virus resistant pumpkin cultivars or hybrids.

Key words: PRSV-W. WMV. ZYMV. Cucurbits. Plant resistance.

RESUMO - A identificação de fontes de resistência em espécies cultivadas de cucurbitáceas é muito importante no desenvolvimento de cultivares resistentes para controlar doenças causadas por vírus do gênero *Potyvirus*. A presente pesquisa teve como objetivo a avaliação das reações fenotípicas e o comportamento de acessos de abóbora (*Cucurbita* spp.) quanto a inoculações mecânicas de vírus do gênero *Potyvirus*. Os ensaios foram conduzidos em casa de vegetação, do Laboratório de Virologia Vegetal, da Universidade Federal do Ceará, envolvendo 28 acessos de *Cucurbita* spp. provenientes do Banco Ativo de Germoplasma (BAG) de Cucurbitáceas, da Embrapa Semiárido, Petrolina, PE. Doze plantas de cada acesso foram inoculadas com cada um dos vírus: *Papaya ringspot virus* type Watermelon (PRSV-W), *Zucchini yellow mosaic virus* (ZYMV) e *Watermelon mosaic virus* (WMV) e avaliadas quanto às infecções virais por “plate-traped antigen enzyme linked immunosorbent assay” (PTA-ELISA) contra antissoros específicos para os três vírus. Três acessos de abóbora apresentaram extrema resistência a WMV e oito acessos apresentaram mosaico leve quando inoculados com PRSV-W. Os sintomas mais severos foram observados com os acessos inoculados com ZYMV, onde 50% deles mostraram-se altamente suscetíveis. De outra parte, os acessos inoculados com WMV exibiram sintomas menos severos, observando-se resistência e tolerância em 39% e 28% dos acessos, respectivamente. Os acessos de *Cucurbita* spp. BGC 518, BGC 530, BGC 567 e BGC 683 avaliados apresentaram resistência a uma ou mais de uma das espécies virais, constituindo-se em fontes promissoras de resistência para o desenvolvimento de cultivares de abóbora resistentes aos vírus do gênero *Potyvirus*.

Palavras-chave: PRSV-W. WMV. ZYMV. Cucurbitáceas. Resistência de plantas a doenças.

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INTRODUCTION

Pumpkin (*Cucurbita* spp.) has significant economic importance for the Northeast of Brazil, especially as familiar agriculture because of its rusticity, nutritional values, post-harvesting conservation and the use of the crop refuges and excesses to feed animals (DIAS *et al*., 2006). This region has a detached position in the production of high variability of pumpkin including *Cucurbita moschata* Duch., and *C. maxima* Duch. (RAMOS *et al*., 1999).

However, the climate conditions and the production systems adopted in the region for cucurbit crops with subsequent plantings in closed agriculture fields favor the distribution and maintenance of source of virus all year around and from one crop field to another with consequent disseminations by the aphids which are the natural and efficient vector for virus species from the genus *Potyvirus* (LIMA, 2015).

Among several disease that occur in cucurbits, those caused by virus affect seriously the quality and quantity of fruit production, representing one of the most important limiting factor for the crop production. More than 20 virus species can naturally infect cucurbit crops and those that belong to the genus *Potyvirus* have been demonstrated to be the most important (FINETTI-SIALER *et al*., 2012; MOURA *et al*., 2005). Therefore, the following virus species from the genus *Potyvirus* are the most important virus species detected in cucurbit field crops in the Northeastern Brazil (LIMA *et al*., 2012a; OLIVEIRA *et al*., 2000): *Papaya ringspot virus* type Watermelon (PRSV-W), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV). Those virus species cause several symptoms in infected plants including mosaic, mottle, blistering, leaf and fruit deformations (KING *et al*., 2012; LIMA, 2015).

The damages and crop lost caused by virus infections can reach high levels and the virus incidences in cucurbit field were already detected in several regions of Brazil, including the Northeast (RAMOS; LIMA; GONÇALVES, 2003; SILVEIRA *et al*., 2009). The severity of symptoms and the damages in the crop production are serious, when the plants are infected in the first stages of development (OLIVEIRA; QUEIROZ; LIMA, 2002) and when they are infected for more than one virus, in mixed infection. When mixed virus infection does lead to severe disease symptoms, however, it sometimes is referred to as being synergistic (MURPHY; BOWEN, 2006).

The development of virus resistant cucurbit cultivars is usually a complex and long process. As an initial phase it is necessary to select appropriated source of resistance to particular virus specie or resistant to more than one species, which should be controlled (NOGUEIRA *et al*., 2011; PACHNER; LELLEY, 2004; SILVEIRA *et al*., 2009).

The incorporation of virus resistant genes in commercial hybrids will greatly reduce the lost caused by virus infection and eliminate the insecticide application by the growers. The identification of possible genes of resistance can be done by indexing cucurbit accessions from Germplasm Collections (MOURA *et al*., 2005). The Embrapa Semiárido has great cucurbit variability in its Cucurbit Germoplasm Bank, with several pumpkin accessions and other cultivated cucurbit species, which were collected in different States from Northeastern Brazil. For these reason, the present research had the objective to evaluate the phenotypic reactions and the behavior of several *Cucurbita* spp. accessions to mechanical inoculation of PRSV-W, WMV and ZYMV.

MATERIAL AND METHODS

Source of Viruses

The virus isolates used in this study belong to the active plant viruses collection from the Plant Virus Laboratory, from the Universidade Federal do Ceará (UFC), Fortaleza, Ceará, Brazil, and included isolates of PRSV-W, WMV and ZYMV, all species from the genus *Potyvirus* obtained from natural infected cultivated cucurbits in the Northeast of Brazil (OLIVEIRA *et al*., 2000; OLIVEIRA; QUEIROZ; LIMA, 2002; RAMOS; LIMA; GONÇALVES, 2003). All virus isolates were maintained at greenhouse conditions by periodical transferences from infected to healthy *Cucurbita pepo* L. 'Caserta', by mechanical inoculations. The degrees of purity of all sources of viruses were also periodically confirmed by serology.

Cucurbita spp. Accessions Evaluation

Accessions of *Cucurbita* spp. were evaluated against PRSV-W, WMV and ZYMV through greenhouse experiments in the Plant Virus Laboratory, with temperature varying from 30 ºC during the day to 26 ºC during the night, during the period of February 2012 to January 2014.

The following 28 accessions of *Cucurbita* spp. from the Active Cucurbit Germplasm Bank (Banco Ativo de Germoplasma de Cucurbitáceas para o Nordeste brasileiro - BGC - Embrapa Semiárido") were evaluated: BGC 229, BGC 299, BGC 460, BGC 506, BGC 512, BGC 518, BGC 527, BGC 529, BGC 530, BGC 531, BGC 537, BGC 552, BGC 553, BGC 562, BGC 565, BGC 566, BGC 567, BGC

569, BGC 571, BGC 578, BGC 586, BGC 620, BGC 623, BGC 624, BGC 629, BGC 683, BGC 749 and BGC 1418. These accessions were collected in 11 Northeastern counties from States of Bahia, Maranhão, Pernambuco, and Rio Grande do Norte.

Seeds from the *Cucurbita* spp. accessions were seeded in plastic trails with 128 cells containing a sterile substrate, using a seed per cell. After eight days, germinated seedlings with the first true leaves were transplanted to plastic pots containing a mixture of one part of sterile manure with two parts of sterile soil. The experiments were developed in a complete casual design with four plants per pot (parcel) and three replicates for each *Cucurbita* spp. accession. Two healthy plants of each accession were maintained as negative control.

A total of 12 plants from each *Cucurbita* spp. accession were mechanically inoculated with extracts from *C. pepo* 'Caserta' plants infected with each virus species (PRSV-W, WMV, and ZYMV). All virus inoculations were performed by rubbing the expanded cotyledon leaves at 10 days after sowing (DAS) with extracts from corresponded virus infected *C. pepo* 'Caserta' plants. The extracts containing the viruses were obtained by grinding leaf tissues from infected plants with a mortar and pistil in the presence of potassium phosphate buffer (KPO_4) 0.5 M, pH 7.5, in the proportion of 1,0 g of tissues per 2.0 mL of buffer. The obtained extracts were strained through a double tissue cloth and a small amount of carborundum was added into the extracts to function as an abrasive. All prepared virus inocula were maintained in cold temperature and the inoculations were performed by rubbing the adaxial part of the leaves with a piece of tissue cloth embedded with the virus inocula.

Extracts from each virus inoculated plants were prepared in the proportion of 1:10 (p/v) with the extraction carbonate buffer, pH 9.6 (0.15 M of Na_2CO_3, 0.035 M of $NaHCO_3$ and 0.007 M of sodium diethyldithiocarbamate), and 100 µL of the obtained extracts from the inoculated plants were placed in the bottom of the ELISA polystyrene plate wells. The plates were incubated at 37 ^{o}C for 1 h, after which they were washed three times with PBS-Tween buffer, and 100 µL of virus polyclonal antisera, previously absorbed by extracts from healthy plants, diluted to 1,000 were added into the wells. The plates were incubated again at 37 ^{o}C for 1 h, after which they were washed three times with PBS-Tween. After drying, 100 µL of anti-rabbit IgG produced in goat conjugated to alkaline phosphatase, diluted in the proportion of 1:7,500 in a buffer contain 2% of polyvinylpyrrolidone, 0.2% of albumin, and 0.02% of sodium azide were added into the wells. The plates were incubated once more at 37 ^{o}C for 1 h, and washed again three times with PBS-Tween. Finally, it was added into the wells, 100 µL of a mixture containing p-nitrophenyl phosphate substrate in the concentration of 0.5 mg/mL dissolved in a buffer containing 12% of diethanolamine, and 0.25% of sodium azide, pH 9.8. After 40 and 60 min the plates were analyzed in the ELISA plate reader apparatus (Labsystems Multiskan MS), using a filter for 405 nm wave length. The absorption readings were considered positive when the readings for the samples were 2.5 times the average of the absorption values obtained for the extracts from healthy plants used as negative control. The antisera used for PRSV-W, WMV and ZYMV were previously absorbed with extracts from healthy *C. pepo* 'Caserta' plants and used in the dilution of 1:4,000.

Twenty-two days after inoculation (DAI), leaf samples from each *Cucurbita* spp. accession plant were individually tested by plate-trapped antigen enzyme linked immunosorbent assay (PTA-ELISA) against antiserum to PRSV-W, WMV, and ZYMV according to Lima *et al*. (2012b).

All 28 accessions of *Cucurbita* spp. inoculated plants were evaluated daily and the observed symptoms were classified as: mM- mild mosaic; M- mosaic; sM- severe mosaic; B- bubbles on the leaves, and LfD- leaf deformations. According to the symptoms the inoculated plants were classified according the following scale: I- Immune or extreme resistance: absence of local and systemic symptoms, with the absence of the virus confirmed by PTA-ELISA; R- Resistance: local lesions in the inoculated leaves and no systemic virus infection or absence of symptoms, confirmed by PTA-ELISA; T- Tolerant: virus replication and systemic distribution with very mild symptoms or absence of symptoms, confirmed by PTA-ELISA; S- Susceptible: virus replication and systemic distribution in the plant with symptoms of mosaic and or severe mosaic, confirmed by PTA-ELISA; HS- Highly susceptible: virus replication and systemic distribution in the plant with severe mosaic with other systemic symptoms as leaf deformations, confirmed by PTA-ELISA.

RESULTS AND DISCUSSION

The lowest levels of susceptibility in *Cucurbita* ssp. accessions were observed for WMV, and PRSV-W isolates, with the absence of symptoms and the occurrence of mild mosaic. The presence and absence of the viruses were confirmed by PTA-ELISA (Table 1).

The average absorption reading values in PTA-ELISA showed significant differences at 5% level of probability in the Scott-Knott test to estimate the virus concentrations in the infected plants. According to the symptoms and the serological results, neither one of the pumpkins accessions showed to be immune or extremely resistant to PRSV-W and ZYMV (Table 1).

Absorbance reading values in PTA-ELISA of 16 pumpkins accessions classified as tolerant to PRSV-W, depending on the severity of symptoms were similar to absorbance reading values of accessions considered susceptible/highly susceptible to the virus. Similarly, the absorbance reading values obtained for eight accessions shown to be tolerant to WMV by symptom evaluation, where they were similar to the absorbance reading values obtained by susceptible/highly susceptible accessions to the same virus (Table 1). These results indicate that asymptomatic plants classified as resistant or tolerant may have the same concentration of virus in host cells, differing only by symptomatic reactions, revealing that even classified as resistant or tolerant to viruses, it may continue acting as sources of the virus in the field.

Vieira, Ávila and Silva (2010) observed that PRSV-W showed high replication levels, although the infected watermelon genotypes did not present severe symptoms, demonstrating the absence of relationship of symptoms and virus concentration evaluated by PTA-ELISA absorption readings. Therefore, the symptoms severity in systemically virus infected plants is not directly correlated with the virus concentration, neither with its replication rates.

Eight pumpkin accessions (28,5%) presented mild mosaic when inoculated with PRSV-W and 39% of the accessions were considered susceptible or highly susceptible, exhibiting severe mosaic and leaf deformations. Similar symptoms were observed by Nascimento *et al*. (2012) in susceptible *Cucurbita* sp. genotypes. Even considered less severe than ZYMV, the PRSV-W is considered a limiting factor for several cucurbit productions mainly when its infection occurs during the first life cycle stages (OLIVEIRA *et al*., 2000).

Among the 28 accessions inoculate with WMV, only three (BGC 460, BGC567, and BGC 620) showed to be immune or extremely resistant, according to the absence of symptoms and negative results in PTA-ELISA (Table 1). The plant reactions to infection by virus species from the genus *Potyvirus* could be variable, presenting a serial of symptoms, including absence of macroscopic symptoms in those tolerant genotypes (OLIVEIRA *et al*., 2000). Approximately 39% and 28% of the pumpkins accessions showed to be moderately resistant and tolerant, respectively, with symptoms less severe than those caused by PRSV-W and ZYMV, and only three accessions demonstrated to be highly susceptible (Table 1). Mosaic without leaf deformations was the predominant symptom induced by WMV, although this virus can cause different types of mosaic with leaf and fruit deformations which could be miss mixed with other virus species that infect cucurbits, including ZYMV (OLIVEIRA *et al.*, 2000). WMV presents strong serological relationship with ZYMV but it is serologically distinct from PRSV-W (LIMA *et al*., 2012a).

The severest symptoms in the present research were caused by ZYMV, which is known to cause a variable kind of symptoms and has been observed to be severest than those caused by PRSV-W and WMV (MOURA *et al*., 2005; OLIVEIRA *et al*., 2000; YAKOUBI *et al*., 2008). They were found in approximately 50% of the evaluated accessions which were considered highly susceptible, with symptoms similar to those reported by Ramos, Lima and Gonçalves (2003) in squash cv. Caserta, and melons cvs. Hy Mark, Gold Mine, and Orange Flesh. Only one pumpkin accession shown was resistant to ZYMV (Table 1). Similarly, Moura *et al*. (2005) evaluated 100 *Cucurbita* sp. accessions and found immunity to ZYMV only in three accessions (BGH-1934, BGH-1937 and BGH-1943) from the Active Germplasm Bank from the Universidade Federal de Viçosa, while 26 were found to be resistant and 48 tolerant to ZYMV. Similar symptoms were observed in pumpkin and melon plants naturally infected with PRSV-W, ZYMV, and WMV (TAVARES *et al.*, 2014) and in mechanically inoculated melon and watermelon genotypes (RABELO FILHO *et al*., 2010). Ramos, Lima and Gonçalves (2003) confirmed the ZYMV severity by comparing its symptoms in single and double infections, with PRSV-W and WMV and stated that ZYMV is responsible for the most important and destructive cucurbit virus disease in the tropics, as well as in temperate regions.

None of the genotypes showed immunities or resistances to all virus isolates, but the variable behaviors observed among the pumpkin accessions demonstrated the possible existence of sources of resistance to each virus alone or double resistance to the virus combinations: PRSV-W + WMV and WMV + ZYMV, since 15 accessions were considered resistant or tolerant to two virus species and 11 resistant to only one virus specie. The pumpkin accessions BGC 518, BGC 530, BGC 567, and BGC 683 deserve special attention as sources of immunity and resistance to WMV (Table 1). Besides to be immune to WMV, the accession BGC 518, BGC 530, and BGC 683 showed to be tolerant to PRSV-W, and ZYMV, while the accession BGC 567 was resistant to ZYMV and tolerant to PRSV-W (Table 1). Even considering the biological diversity of plant viruses, including those from the genus *Potyvirus,* resistant cultivars represent the best way to control virus from the genus *Potyvirus* (OLIVEIRA; QUEIROZ; LIMA, 2002). Over the past few years, many cultivars and hybrids of pumpkin and other cultivated cucurbits have been developed as resistant to one or more viruses from the genus *Potyvirus* (COUTTS; JONES, 2005; SVOBODA; LEISOVA-SVOBODOVA; AMANO, 2013; WU *et al*., 2010). Pachner and Lelley (2004) showed that *C. moschata* 'Soler,' which was developed by L. Wessel-Beaver of the University of Puerto Rico, carries a recessive gene that confers resistance to ZYMV in crosses with *C. moschata* 'Waltham Butternut'. Nogueira *et al*. (2011) developed summer-squash hybrids among lines resistant to PRSV-W, derived from the 'Caserta' x 'Whitaker' cross.

The accessions BGC 518, BGC 530, BGC 567, and BGC 683 that showed resistance to one or more than one virus species constitute promising source of resistance for production of virus resistant pumpkin cultivars, with larger and more stable resistance to viruses. Since those genotypes did not show resistance or immunity to PRSV-W, there is the need to search for more systematically resistance to this virus in *Cucurbita* spp. accessions

Table 1 - Symptom reactions, absorption readings in plate-trapped antigen enzyme linked immunosorbent assay (PTA-ELISA) and behavior of *Cucurbita* spp. accessions to *Papaya ringspot virus* type Watermelon (PRSV-W), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV) in greenhouse experiments

Cucurbita spp. accessions	PRSV-W			WMV			ZYMV		
	Symptom reactions[1]	PTA-ELISA[2]	Behavior[3]	Symptom reactions[1]	PTA-ELISA[2]	Behavior[3]	Symptom reactions[1]	PTA-ELISA[2]	Behavior[3]
BGC 229	M	+++	T	mM	+	R	sM	+++	S
BGC 299	M	+++	T	M	+++	T	M	++	T
BGC 460	mM	+++	T	NS	-	I	sM/ LfD	+++	HS
BGC 506	M	+++	T	mM	+	R	sM/ LfD	+++	HS
BGC 512	mM	+++	T	M	+++	T	sM/B/ LfD	+++	HS
BGC 518	M	+++	T	mM	+	R	M	++	T
BGC 527	mM	+++	T	M	+++	T	sM/B/ LfD	+++	HS
BGC 529	mM	+++	T	mM	+	R	sM	+++	S
BGC 530	mM	+++	T	mM	+	R	M	+++	T
BGC 531	sM/B	+++	HS	sM/B	+++	HS	sM/B/ LfD	+++	HS
BGC 537	M/ LfD	+++	HS	mM	+++	T	sM/ LfD	+++	HS
BGC 552	sM/ LfD	+++	HS	mM	+++	T	sM/B/ LfD	+++	HS
BGC 553	M/B/ LfD	+++	HS	mM	+++	T	sM/ LfD	+++	HS
BGC 562	M	+++	T	mM	+	R	sM	+++	S
BGC 565	sM/ LfD	+++	HS	sM	+++	S	sM/B/ LfD	+++	HS
BGC 566	sM/B/ LfD	+++	HS	sM/B	+++	HS	sM/B/ LfD	+++	HS
BGC 567	M	+++	T	NS	-	I	mM	+	R
BGC 569	sM/B	+++	HS	mM	+	R	M	++	T
BGC 571	sM	+++	S	sM/B	+++	HS	sM	+++	S
BGC 578	sM	+++	S	sM	++	S	sM/B/ LfD	+++	HS
BGC 586	mM	+++	T	M	+++	T	sM	+++	S
BGC 620	mM	++	T	NS	-	I	sM/ LfD	+++	HS
BGC 623	M	+++	T	NS	+	R	M/ LfD	+++	HS
BGC 624	sM/B	+++	HS	mM	+++	T	sM/ LfD	+++	HS
BGC 629	sM/B	+++	HS	mM	+++	S	M	++	T
BGC 683	M	+++	T	mM	+	R	M	++	T
BGC 749	M	+++	T	NS	+	R	M	+++	S
BGC 1418	mM	+++	T	mM	+	R	sM	+++	S

[1]mM = mild mosaic; M = mosaic; sM = severe mosaic; B = blistering; LfD = leaf deformation; NS = no symptom. [2]Absorbance reading values below 0.25 (-) correspond to negative results while minimum values between 0.25 and 1.00 (+), medium between 1.00 and 2.00 (++), and maximum above 2.00 (+++) are equivalent to positive results by PTA-ELISA. [3]I = immune or extreme resistance; R= resistant; T = tolerant; S = susceptible; HS = highly susceptible

available in BAGs from the Brazilian Northeast. If immunity or resistance to this virus specie is not identified, other alternatives could be incorporated from another source of resistance or by genetic engineering.

CONCLUSION

The *Cucurbita* spp. accessions BGC 518, BGC 530, BGC 567, and BGC 683 are a good alternative for the production of new pumpkin cultivars or hybrids resistant to one or more of the virus species of the genus *Potyvirus*.

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