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SEROLOGICAL DETECTION AND MOLECULAR CHARACTERIZATION OF A BEGOMOVIRUS ISOLATE OBTAINED FROM *Macroptilium lathyroides*

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ABSTRACT - The viruses from the genus *Begomovirus***,** family *Geminiviridae* are considered emergent pathogens, mainly because of the population explosion of their insect vectors. For this reason, more attention needs to be directed to the correct virus species identification inside the genus. The present paper had the objectives of serologically detecting a begomovirus in *Macroptilium lathyroides* plants in the State of Ceará, and developing biological, serological and molecular studies with a virus isolate obtained from *M. lathyroides.* Indirect ELISA with antiserum for *Macroptilium golden mosaic virus* (MaGMV) demonstrated that the samples collected from *M. lathyroides* showing golden mosaic in the field were infected with a begomovirus. The virus isolate obtained was transmitted by grafting to eight species of the family Leguminosae, four species of Solonaceae, and one species in the family Amaranthaceae. The virus also was transmitted from *M. lathyroides* to *M. lathyroides* by the whitefly *Bemisia tabaci* biotype B. A DNA fragment of 1.2 kb was obtained by PCR with the primers PAL1v 1978 and PAR1c 496 for component A, and a DNA fragment of 0.5 kb was obtained with the primers PBL1v 2040 and PCR cl for component B, confirming the presence of a begomovirus infecting *M. lathyroides.* Molecular studies indicated that the begomovirus isolate showed 77% genomic similarity with *Bean golden mosaic virus* and 75% with *Cowpea golden mosaic virus* for their *cp* and *rep* genes, indicating the possibility that the isolate is a distinct virus species of the *Begomovirus* genus.

Keywords: Weed plant. *Geminiviridae*. Source of virus. *Macroptilium golden mosaic virus*. MaGMV.

DETECÇÃO SOROLÓGICA E CARACTERIZAÇÃO MOLECULAR DE UM ISOLADO DE BEGOMOVIRUS OBTIDO DE *Macroptilium lathyroides*

RESUMO - Os vírus pertencentes ao gênero *Begomovirus,* família *Geminiviridae,* são considerados patógenos emergentes, sobretudo em razão da explosão populacional de seus vetores, sendo que maior atenção deve ser direcionada à identificação correta das espécies dentro do gênero *Begomovirus*. O presente trabalho teve por objetivo detectar por sorologia a presença de vírus do gênero *Begomovirus* em plantas de *Macroptilium lathyroides* no Estado do Ceará e efetuar estudos biológicos, sorológicos e moleculares com um isolado do vírus. Testes de ELISA indireto empregando anti-soro contra *Macroptilium golden mosaic virus* (MaGMV) foram positivos para as amostras de *M. lathyroides* exibindo mosaico dourado coletadas no campo, indicando que estavam infetadas por um vírus do gênero *Begomovirus*. Um isolado do vírus foi transmitido por enxertia para oito espécies da família Leguminoseae, quatro da família Solanaceae e uma da família Amaranthaceae. O vírus foi também transmitido por mosca branca (*Bemisia tabaci*) biótipo B de *M. lathyroides* para *M. lathyroides*. Um fragmento de aproximadamente 1,2 kb foi obtido por PCR com os oligonucleotideos PAL1v 1978 e PAR1c 496 para o DNA-A, enquanto que um fragmento de 0,5 kb foi amplificado com os oligonucleotideos PBL1v 2040 e PCR cl para o DNA-B, confirmando tratar-se de vírus do gênero *Begomovirus.* Estudos moleculares indicaram que o vírus isolado de *M. lathyroides* apresentou 77% de similaridade com o genoma do *Bean golden mosaic virus* e de 75% com *Cowpea golden mosaic virus* para as regiões dos genes *cp* e *rep*, revelando a possibilidade de ser uma espécie distinta de vírus do gênero *Begomovirus*.

Palavras-chave: Feijão de rola. *Geminiviridae*. Fonte de vírus. *Macroptilium golden mosaic virus.* MaGMV.

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INTRODUCTION

Several viruses from the genus *Begomovirus,* family *Geminiviridae* are considered emergent pathogens, mainly because of the population explosion of their insect vectors. For constituting an emergent group of viruses, more attention needs to be directed to the correct virus species identification within the genus (MORALES; ANDERSON, 2001; MORALES; JONES, 2004). Genetic diversity among virus species and isolates of the *Begomovirus* has been observed in Brazil (RIBEIRO et al., 2003; AMBROZEVICIUS et al., 2002; MONCI et al., 2002; COLARICCIO et al., 2007; SILVA et al., 2010). The emergence of begomoviruses in Brazil started in 1990 in association with the appearance of a new whitefly species or biotype, *Bemisia tabaci* biotype B (BELLOWS JR. et al., 1994; FARIA et al., 2000; RIBEIRO et al., 2003). The presence of *B. tabaci* biotype B was first observed in 1990 and 1991, in the State of São Paulo, probably introduced through the importation of ornamental plants from Europe (MELO, 1992). The biotype B of *B. tabaci* is capable of colonizing more than 500 plant species distributed among 84 botanical families (HAJI et al., 2004). The diseases caused by begomoviruses are considered limiting factors for many plant crops in several Brazilian regions (FARIA et al., 2000; LIMA et al., 2000; ARNAUD et al., 2007; SILVA et al., 2010).

The whitefly is included among the most important plant virus vectors, and can reach high population levels, especially when the weather conditions are hot and dry, favoring virus dissemination. The adults and nymphs of the whitefly cause damage by feeding on plants and by serving as important virus vectors in the genus *Begomovirus* (STANSLY, 2004).

The diseases caused by begomoviruses can be identified by serological methods and the enzyme linked immunosorbent assay (ELISA) is the most used technique for large scale diagnosis because of its elevated sensitivity, simplicity and the capacity for testing a dreat number of plant samples in a relatively short period of time (CANCINO et al., 1995). Zerbini et al. (2001), however, stated that molecular analyses were more sensitive and appropriate for virus diagnosis, especially for members of the genus *Begomovirus*.

Several begomovirus species isolated from weeds in Florida were characterized, including two isolates from *Sida rhombifolia* L. and one from *M. lathyroides* (ABOUZID; HIEBERT, 1993; CANCINO et al., 1995). The isolate from *M. lathyroides* was designated *Macroptilium golden mosaic virus* (MaGMV). Several weed species have been identified as natural hosts for begomovirus in Brazilian regions, including the Northeast (SANTOS et al., 2003; ASSUNÇÃO et al., 2006; SILVA et al., 2010).

The objectives of the present study were to serologically detect a virus from the genus *Begomovirus* in *M. lathyroides* plants in the State of Ceará, and to develop biological, serological and molecular studies with a virus isolated from an infected plant.

MATERIAL AND METHODS

Different counties were visited in the State of Ceará with the objective of evaluating *M. lathyroides* plants which exibited golden mosaic symptoms. None cultivated fields and cowpea (*Vigna unguiculata* (L.) Walp. susp. *unguiculata*) cultivated areas were visited. Samples from plants showing golden mosaic were collected and taken to the Plant Virus Laboratory at the Federal University of Ceará. Leaf samples were harvested from 318 different *M. lathyroides* plants exhibiting golden mosaic from the following counties: Aracoiaba (14), Fortaleza (15), Iguatu (55), Itapiuna (25), Jaguaribe (24), Mauriti (10), Morada Nova (18), Pacajus (23), Quixadá (62) and Quixeramubim (72). All leaf samples were tested by indirect ELISA (Almeida, 2001) with polyclonal antiserum raised against MaGMV (CANCINO et al., 1995). The reactions were considered positive when the absorption values were higher than two and half times the average values obtained for the healthy *M. lathyroides* plant extracts used as control (ALMEIDA, 2001).

A leaf sample positive for MaGMV in indirect ELISA, collected in the Quixeramobim County, was graft-inoculated onto healthy *M. lathyroides* plants grown under greenhouse conditions. The grafting method used consisted of putting together internal stem tissues with removed epidermis of both healthy and infected plants which were joined by PARAFILM®. The inoculated plants were maintained under greenhouse conditions, evaluated daily for symptom development and 30 days after inoculation they were tested by indirect ELISA against antiserum for MaGMV and by PCR.

The transmission tests with whiteflies were performed inside small screen cages maintained in the greenhouse, with adults of *B. tabaci* biotype B and virus sources in *M. lathyroides* plants previously graft-inoculated with infected plant tissue. Non-viruliferous insects maintained in healthy melon (*Cucumis melo* L.) were submitted to an acquisition period of 48 h on infected plants, and transferred to four healthy *M. lathyroides* plants maintained in a separated screen cage, using five whiteflies per healthy plant for similar period of time. The inoculated plants were observed daily for symptom development and 30 days after inoculation they were tested by indirect ELISA and by PCR.

Host range studies were performed in green-

house conditions with the virus isolate obtained from *M. lathyroides* in Quixeramubim County. Stem tissues from infected plants were graft-inoculated onto plant species of the following botanical families: Amaranthaceae: *Gomphrena globosa* L.; Fabaceae: *Canavalia ensiformis* DC., *Cassia occidentalis* L., *C. tora* L., *Leucaena* spp., *Macroptilium atropurpureum* DC., *Phaseolus lunatus* L. and *Phaseolus vulgaris* L.; and Solanaceae: *Capsicum frutescens* L.; *Datura stramonium* L.; *Solanum lycopersicum* L.; *Nicotiana benthamiana* Domin; *N. debneyi* Domin; *N. glutinosa* L.; *N. tabacum* L. and *Solanum melongena* L. The selected plants are relevant virus indicator hosts, common weeds or important crops for the Northeast. The plants were maintained in plastic pots containing a mixture of soil and manure at a 2:1 (w/w) ratio, sterilized at 120° C, for 120 min. Four plants of each species were graft-inoculated with virus-infected plant tissue, and two plants were not inoculated to serve as negative controls. During the experiments plants were sprayed once a week with systemic insecticide and the inoculated plants were observed daily for symptom development and 30 days after the inoculations they were tested by indirect ELISA.

Leaf samples from *M. lathyroides* plants inoculated with the virus isolate were used for DNA extraction with TRIZOL® according to manufacturer's instructions, which was used for virus amplification by polymerase chain reactions (PCR), using specific primers for detection of components A and B of begomovirus genome (ROJAS et al., 1993). Approximately 40 mg tissue samples from young leaves were used for the total DNA extraction. The precipitated DNA was dried under vacuum and resuspended in 100 ml of Milli-Q sterile water. One microliter of extracted DNA was mixed with 45 ml of PCR SuperMix (Life Technologies), 2.0 ml of Milli-Q sterile water and 1.0 ml each of the primers PAL1v1978- 5'GCATCTGCAGGCCCA-CATYGTCTTLYCCNGT 3' and PAR 1 C496 - 5' AATACTGCAGGGCTTYCTRTACATRGG 3' (ROJAS et al., 1993) for the amplification of a fragment from the DNA-A. The primers PBL1 v2040 – 5'GCTCTGCAGCART-GRTCKATCTTCCATACA3' and PCRc1– 5'C-TAGCTGCAGCATATTTACRARWATGCCA3' were used for the amplification of a DNA-B fragment (ROJAS et al., 1993).

The amplified DNA fragments were analyzed in a 1.0% agarose gel electrophoresis at 96 volts in a GIBCO BRL horizontal system during 55 min. The gel was stained with ethidium bromide, analyzed and photographed in a BIO-RAD mini-transilluminator.

The amplified virus DNA fragments were cloned in the pGEM-T® Easy Vector System I (Promega, Madison, WI) according to the manufacturer's instructions and were transformed into *Escherichia coli* JM109 competent cells. Recombinant plasmids were identified by enzymatic cleavage using *Eco*R I, and the viral insert was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The indirect ELISA for MaGMV indicated that the 318 leaf samples collected from *M. lathyroides* plants showing golden mosaic from the field and four from graft-inoculated plants were infected with a begomovirus, serologically related to MaGMV. All leaf samples from *M. lathyroides* exhibiting golden mosaic collected in all the different counties reacted specifically with the antiserum, with absorption readings at 405 nm (A_{405nm}) varying from 0.850 to 1.2, while the readings for extracts from healthy plants presented an average of 0.180. These data indicated the presence of a begomovirus in all *M. lathyroides* plants analyzed. The symptoms observed in *M. lathyroides* (Figure 1) were similar to those described for MaGMV detected in Florida (CANCINO et al., 1995). According to Cancino et al. (1995) the MaGMV antiserum also reacted with other begomovirus isolates obtained from other plant species. Although indirect ELISA was shown to be a practical and efficient method for detection of plant viruses (LIMA et al., 1996; NASCIMENTO et al., 2010) including the begomoviruses, its limiting factor is the need to produce monoclonal or polyclonal specific antisera (POLSTON; ANDERSON, 1997). However, Zerbini et al. (2001) stated that the molecular analysis was more sensitive and appropriate for virus diagnosis, especially for begomoviruses. On the other hand, the broader serological relationship among the virus species from the genus *Begomovirus* makes it possible to utilize a polyclonal antiserum produced one virus species to be used to identify related virus species infecting different crops (FAUQUET et al., 2005). Naturally infected *M. lathyroides* plants exhibiting golden mosaic were detected in all the counties visited. Besides confirming the presence of a possible MaGMV isolate naturally infecting *M. lathyroides* in the State of Ceará, these results suggest the importance of this weed as potential reservoir of virus in the field. A virus of the genus *Begomovirus* was also detected in tomato (*Solanum lycopersicum*) in the Ibiapaba Hills in the State of Ceará, using the antiserum for MaGMV (LIMA et al., 2000).

The identification of weeds as sources of virus is of fundamental importance to understanding disease epidemiology and how viruses survive in the field. Viruses of the genus *Begomovirus* have a wide host range. It has been demonstrated that one virus species could infect several plant species from differ-

ent botanical families, many of which are weeds. These weeds could serve as natural reservoirs of virus that could infect cultivated crops by whitefly transmission (ASSUNÇÃO et al., 2006).

Among 18 plant species graft-inoculated, the virus infected eight species from the family Leguminosae: *C. ensiformis, C. tora*, *C. ternatae*, *Leucaena sp., M. atropurpureum*, *M. lathyroides, P. lunatus* and *P. vulgaris;* one from the family Amaranthaceae: *G. globosa*; and four from the family Solanaceae: *S. lycopersicum*; *N. debneyi*, *N. glutinosa* and *N. tabacum* (Table 1). These results indicated that the virus could infect *C. ternatae*, *P. vulgaris* and *S. lycopersicum* plants in the field (Table 1).

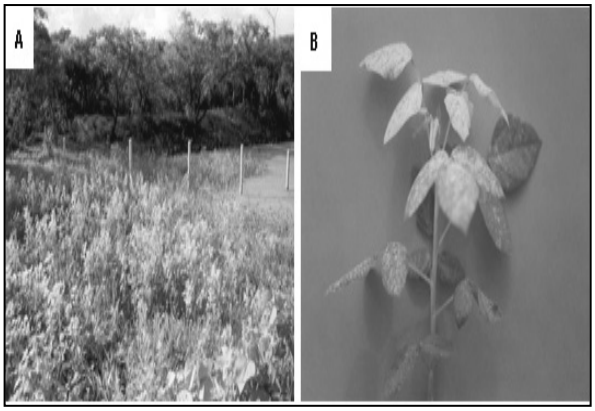

Figure 1. *Macroptilium lathyroides* plants showing golden mosaic symptoms. **A-** View of a field in the State of Ceará countryside with a large population of plants with golden mosaic. **B-** Plant of *M. lathyroides* exhibiting golden mosaic symptoms that was graft-inoculated with tissue from a virus-infected plant.

The most severe symptoms were observed in *G. globosa* graft-inoculated plants that showed yellow mosaic and leaf distortion (Table 1). Although *C. tora* only exhibited mild symptoms, the virus was serologically detected in the leaves, indicating that this species could function as a potential alternative host for the virus, considering its large distribution in the Northeast Region.

The grafting transmission studies facilitated the biological characterization of the virus. According to Faria and Zerbini (2000), the absence of mechanical transmission for most of the begomoviruses has limited studies of their host range and grafting has been considered an important method of inoculation.

Besides the symptoms, the serological and PCR results showed the efficiency of grafting and the whitefly transmission of the virus, confirming their importance for biological begomovirus studies, considering the absence of or its low conventional mechanical transmission (FARIA; ZERBINI, 2000).

The knowledge of a virus transmission methods is strategic for understanding its epidemiology and important for biological studies under greenhouse conditions. In the present research, the use of grafting transmission confirmed to be an excellent method to partially evaluate the host range of the virus isolate obtained from *M. lathyroides*. The experiment with whiteflies also proved to be efficient for transmitting the virus from infected to healthy *M. lathyroides* plants, but requires the maintenance of a permanent non-viruliferous whitefly colony. The

Table 1. Symptoms and serological results with antiserum for *Macroptilium golden mosaic virus* of plant species graft-inoculated with a virus isolated from *Macroptilium lathyroides* with golden mosaic.

Plant Species	Symptom*	ELISA – Absorption**
Family Amaranthaceae		
Gomphrena globosa	MM	0.898 (+)
Family Fabaceae		
Canavalia ensiformis	Cl	1.556 (+)
Cassia occidentalis	NS	0.154 (-)
Cassia tora	MM, Ld	0.866 (+)
Clitoria ternatea	MM	0.986 (+)
Leucaena spp.	Cl	0.882 (+)
Macroptilium atropurpureum	Cl	0.731 (+)
M. lathyroides	GM	0.837 (+)
Phaseolus lunatus	Cl	1.772 (+)
P. vulgaris	MM	1.387 (+)
Family Solanaceae		
Capsicum frutescens	NS	0.189 (-)
Datura stramonium	NS	0.203 (-)
Solanum lycopersicum	MM	0.987 (+)
Nicotiana benthamiana	NS	0.187 (-)
N. debneyi	NS	0.895 (+)
N. glutinosa	NS	0.956 (+)
N. tabacum	MM	0.978 (+)
Solanum melongena	NS	0.198 (-)
Control: Healthy *M. lathyroides*	NS	0.111 (-)

* Cl- chlorotic lesions; GM- golden mosaic; Ld- leaf distortion; MM- mild mosaic; NS- no symptoms.
** Absorbance reading 60 min after the addition of the substrate. ELISA results were considered positive when absorption values were higher than 2.5 times the average values for the controls.

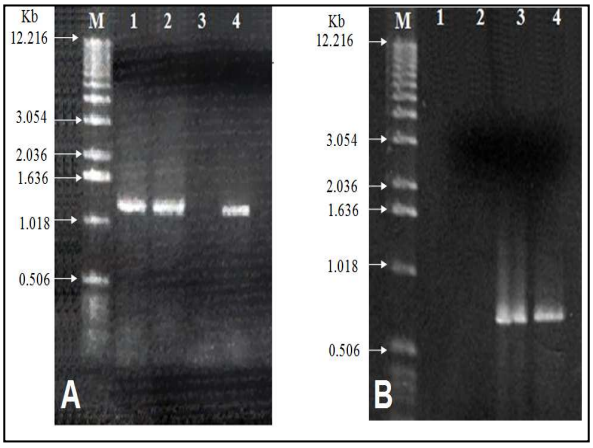

Figure 2. Virus DNA fragments amplified by polymerase chain reactions (PCR) of *Macroptilium lathyroides* plants with golden mosaic. **A-** PCR products obtained by using primers PAL1v1978 and PAR1c496 for DNA-A: Lane M- DNA ladder in kb; Lanes 1 and 2 - DNA fragment of 1.2 kb from *M. lathyroides* with golden mosaic in the field; Lane 3 - Healthy *M. lathyroides*; Lane 4 - DNA fragment of 1.2 Kb from *M. lathyroides* with golden mosaic, which had been graft inoculated in the greenhouse. **B-** PCR products using the primers PBL1v2040 and PCRc1 for DNA B: Lane M- DNA ladder with standards of indicated length in Kb; Lanes 1 and 2 - Healthy *M. lathyroides*; Lane 3- Virus DNA fragment of 0.5 Kb from *M. lathyroides* with golden mosaic, in the field; 4 - Virus DNA fragment of 0.5 Kb from *M. lathyroides* with golden mosaic, which had been graft inoculated in the greenhouse.

presence of virus in eight symptomatic *M. lathyroides* plants of ten whitefly inoculated plants was confirmed by indirect ELISA with antiserum for MaGMV and PCR with the primers PAL1v 1978 and PAR1c 496 for the virus DNA-A component, and with the primers PBL1v 2040 and PCR cl for the virus DNA-B component.

The PCR results with symptomatic graft-inoculated plants revealed the presence of a 1.2 kb DNA fragment amplified with the primers PAL1v 1978 and PAR1c 496 for the virus DNA-A component, and a 0.5 kb fragment amplified with the primers PBL1v 2040 and PCR cl for the virus DNA-B component (Figure 2), confirming the presence of a begomovirus infecting plants of *M. lathyroides* from Quixeramobim showing golden mosaic.

The cloning of virus DNA fragments was confirmed by the plasmid DNA cleavage with the restriction enzyme Eco-R1. A sequence fragment of 761 nt from DNA-A showed 77% similarity with the corresponding part of the region from the coat protein gene (*cp*) and part of the replicase gene (*rep*) of a *Bean golden mosaic virus* isolate (BGMV, GenBank access n^o BGU92531) and 75% with a similar region of a Brazilian *Cowpea golden mosaic virus* isolate (CGMV, GenBank access n^o AF188708) (Table 2).

The DNA-B fragment sequence was 550 nt and presented 87% of similarity with a region from the gene responsible for the cell-to-cell movement of the BGMV DNA identified in Brazil (GenBank access MBGBRBL) and 77% with the same region of a *Macroptilium yellow mosaic virus* isolate (MYMV) obtained in Jamaica (GenBank access n^o EF585291) (Table 2).

The homologies and the differences in the nucleotide sequences of a virus genome are considered relevant factors for identification and molecular characterization of viruses from the genus *Begomovirus* and their strains (PADIDAM et al., 1995). According to the criteria for virus species differen-

Table 2. Percentage similarity between nucleotide sequences of part of the genome from the virus fragment obtained from *Macroptilium lathyroides* and the sequences of other viruses from the genus *Begomovirus* deposited in GenBank.

Virus genome	Virus name	Virus isolate	Genbank code	Origin	Similarity (%)
DNA-A	*Bean golden mosaic virus*	BGMV-BRII	BGU92531	Brazil	77
	Bean golden mosaic virus	SAdG	FJ665283	Brazil	75
	Cowpea golden mosaic virus	CGMV-BR	AF188708	Brazil	75
	Tomato mottle leaf curl virus	PE-Pt3	AY049215	Brazil	74
DNA-B	*Bean golden mosaic virus*	Brazil	MBGBRBL	Brazil	87
	Macroptilium yellow mosaic virus	St. Thomas	EF585291	Jamaica	77
	Passionfruit severe leaf distortion virus	BR:LNS2:Pas:01	FJ972768	Brazil	79
	Sida micrantha mosaic virus segment B	A2B2	AJ557453	Germany	78

tiation in the genus *Begomovirus,* nucleotide sequence homology below 90% is an indication of the existence of a new virus species (FAUQUET, 2000). Ramos et al. (2002), using degenerate primers PAL1v1978-PAR1c715 and PAL1c1960-PAR1v722, found that the DNA-A sequence of a virus isolated in Villa Clara (GenBank Accession No. AJ344452) had the highest percentage identity when compared to *Bean golden yellow mosaic virus* strains (BGYMV, GenBank Accession Nos. AF173555, M91604, and L01635), with 85 to 87% and 93 to 94% nucleotide and amino acid sequence identity, respectively. Therefore, Ramos et al. (2002) proposed that the virus isolate could be considered as a new species of the genus *Begomovirus* named Macroptilium yellow mosaic virus (MaYMV). Although the nucleotide sequence of the DNA-B fragment from the virus isolated from *M. lathyroides* had shown 87% of similarity with DNA-B from the BGMV isolate identified in Brazil, it could be considered as a distinct virus species (FAUQUET, et al., 2003). According to Fauquet et al. (2003) the description of a new species in the genus *Begomovirus* should be accepted when its nucleotide sequence shows less than 90% identity. Studies are ongoing at the Plant Virus Laboratory at Federal University of Ceará to obtain the complete genome sequence of this isolate.

CONCLUSIONS

The virus from the genus *Begomovirus* serologically detected and confirmed by PCR in *M. lathyroides* was related to MaGMV;

The results indicated that the virus could potentially cause problems in cultivated plants *C. ternatae*, *P. vulgaris* and *S. lycopersicum,* and several weeds could serve as natural sources of the virus in the field;

The transmission of the virus by grafting was of strategic importance for determining its biological properties in greenhouse conditions;

The molecular results confirmed the presence of a virus from the genus *Begomovirus* in *M. lathyroides* plants, showing golden mosaic, and the sequencing analysis data, and indicated that possibly it is a distinct species.

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