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CARACTERIZAÇÃO BIOLÓGICA, SEROLÓGICA E MOLECULAR DE *Turnip mosaic virus* (TuMV) INFECTANDO RÚCULA, ALFACE E ACELGA

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Dissertação apresentada à Faculdade de Ciências Agronômicas da Unesp Câmpus de Botucatu, para obtenção do título de Mestre em Agronomia – Proteção de plantas.

Orientadora: Renate Krause Sakate

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TÍTULO: CARACTERIZAÇÃO BIOLÓGICA, SEROLÓGICA E MOLECULAR DE *Turnip mosaic vírus* (TuMV) INFECTANDO RÚCULA, ALFACE E ACELGA

AUTOR: MARCOS ROBERTO RIBEIRO JUNIOR
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“Tell me and I'll forget, teach me and I'll remember, involve me and I'll learn” -

Benjamin Franklin

Resumo

A partir do ano de 2016, anormalidades foram observadas em campos de produção de rúcula (*Eruca sativa*) no interior do estado de São Paulo, região de Botucatu. As plantas acometidas apresentavam mosaico, redução drástica no crescimento e deformação foliar, sintomas típicos da infeção por vírus. Associadas as essas plantas, também foram observadas altas populações de pulgões/afídeos e de plantas daninhas, como nabiça (*Raphanus raphanistrum*) e nabo forrageiro (*Brassica rapa*) apresentando sintomas de mosaico. Inicialmente, as plantas foram submetidas ao teste de ELISA indireto usando um antissoro para potyvirus, em seguida foi realizada a extração de RNA total e RT-PCR, utilizando as amostras ELISA-positivas. A sequência correspondente à região codificadora para a proteína capsidial foi obtida e o vírus foi identificado como *Turnip mosaic virus* (TuMV). Posteriormente outros campos de produção de folhosas foram visitados no Estado de São Paulo (região de Bragança Paulista e Mogi-Mirim) e o TuMV foi novamente identificado em rúcula, porém também em alface (*Lactuca sativa*) e acelga (*Beta vulgaris* subsp. *vulgaris*). Analises biológicas e moleculares agruparam ambos os isolados de TuMV no grupo *Brassica*-*Raphanus* (BR), que inclui isolados infectando espécies de *Brassica* e *Raphanus*. É apresentado aqui o primeiro relato de TuMV naturalmente infectando rúcula, alface e acelga no Brasil. De acordo com a análise filogenética, pelo menos duas introduções diferentes de isolados de TuMV ocorreram no Brasil, correspondendo aos tipos basal-BR e world-B, infectando *Brassica*/*Raphanus* e *Brassica*, respectivamente.

Palavras chave: *Raphanus*, *Eruca sativa*, *Lactuca sativa*, *Beta vulgaris* subsp. *vulgaris*, *Potyvirus*.

Abstract

Abnormalities have been observed in rocket (*Eruca sativa*) fields in Botucatu, Sao Paulo state, Brazil since 2016. Symptoms of mosaic, reduction in foliar growth and deformation, typical symptoms of virus infection were observed in rocket plants. Associated to these plants we also found high populations of aphids, as well as raphanus weeds (*Raphanus raphanistrum*) and forage turnip (*Brassica rapa*) with mosaic symptoms. Initially, the plants were submitted to indirect ELISA using a potyvirus antiserum, and then total RNA extraction and RT-PCR were performed using the ELISA-positive samples. The complete coat protein sequence was obtained and the virus was identified as *Turnip mosaic virus* (TuMV). Later, other fields were visited in Sao Paulo state (cities of Bragança Paulista and Mogi-Mirim) and TuMV was again identified not only in rocket, but also in lettuce (*Lactuca sativa*) and chard (*Beta vulgaris* subsp. *vulgaris*). Biological and molecular analysis grouped both TuMV isolates in the *Brassica*-*Raphanus* (BR) clade, which includes isolates infecting *Brassica* and *Raphanus* species. Here is the first report of rocket, lettuce and chard naturally infected with TuMV in Brazil. According to the phylogenetic analysis, at least two different introductions of TuMV isolates occurred in Brazil, corresponding to the basal-BR and world-B types, infecting *Brassica*/*Raphanus* and *Brassica*, respectively.

Keywords: *Raphanus*, *Eruca sativa*, *Lactuca sativa*, *Beta vulgaris* subsp. *vulgaris*, *Potyvirus*.

SUMÁRIO

INTRODUÇÃO GERAL

Os vírus pertencentes ao gênero *Potyvirus*, família Potyviridae*,* são capazes de infectar uma grande variedade de espécies de plantas monocotiledôneas e dicotiledôneas (GIBBS; OHSHIMA, 2010), são transmitidos por afídeos de forma não persistente e, em alguns casos, podem ser disseminados através de sementes e materiais vegetais (KING et al., 2011; SHUKLA; WARD; BRUNT, 1994).

Os potyvirus são formados por partículas alongadas e flexuosas de aproximadamente 700 – 750 nm de comprimento, cada uma das quais contém uma única cópia do genoma viral, formada por uma molécula de RNA fita simples, sentido positivo, com cerca de 10.000 nucleotídeos (nt) de comprimento, envolto por um capsídeo formado por 2.200 cópias de um polipeptídeo de massa molecular em torno de 34 kDa (FAUQUET et al., 2005). Os genomas dos potyvirus possuem regiões terminais não traduzidas e uma única ORF (*Open Reading Frame*) que codifica uma poliproteína de aproximadamente 345 kDa (RIECHMANN; LAIN; GARCÍA, 1992), que é hidrolisada, após tradução, por proteinases codificadas pelo vírus, gerando 10 proteínas maduras e uma proteína incorporada na região codificadora para a proteína P3, intitulada PIPO, sendo traduzida na fase +2, onde posteriormente ocorre a sua expressão através da fusão da proteína P3 na região N-terminal (P3N-PIPO) (KING et al., 2011).

Turnip mosaic virus (TuMV), uma espécie pertencente ao gênero *Potyvirus,* foi relatado pela primeira fez infectando cultivos de Brassica em 1921 nos Estados Unidos (GARDNER; KENDRICK, 1921). É um importante patógeno que infecta diversas espécies de diferentes famílias de plantas, tanto cultivadas quanto ornamentais (OHSHIMA et al., 2002; TOMIMURA et al., 2003). Ele possui uma ampla gama de hospedeiros, maior que qualquer outro potyvirus, infectando cerca de 418 espécies de plantas (GIBBS; NGUYEN; OHSHIMA, 2015); apresenta grande diversidade genética, sendo classificado em diferentes estirpes e patótipos e é transmitido por mais de 80 espécies de afídeos de forma não-presistente (SHATTUCK, 2010).

Turnip mosaic virus causa sintomas variados em seus hospedeiros, sendo verificados em repolho anéis necróticos, geralmente não precedidos por clareamento

de nervuras. Em nabo, couve-de-bruxelas e mostarda, causa sintomas de mosaico, distorção foliar e subdesenvolvimento. Em couve, as folhas mais velhas não exibem sintomas e as folhas mais novas apresentam clareamento, mosaico, mosqueado e distorções (RIMMER; SHATTUCK; BUCHWALDT, 2007). Em diversas partes do mundo, assim como no Brasil, os cultivos de Brassicas e outras hortaliças folhosas são realizados em proximidade e muitas vezes durante o ano todo, o que resulta em reservatórios de plantas infectadas com TuMV, fornecendo fontes de inoculo constante aos afídeos vetores (WALSH, 1986).

Há diversas formas de classificação dos isolados de TuMV. Quatro "grupos de hospedeiros" ou do inglês "*host types*" são classificados de acordo com Nguyen et al. (2013); Ohshima et al. (2002). Os isolados do tipo (B) ocasionalmente infectam *Brassica*, muitas vezes de forma latente, mas não infectam *Raphanus*; os isolados do tipo B infectam a maioria das espécies de *Brassica*, mas não infectam *Raphanus*; os isolados do tipo B(R) infectam a maioria das espécies de *Brassica* e, ocasionalmente, também plantas de *Raphanus* de forma latente; e os isolados do tipo BR infectam ambas as espécies *Brassica* e *Raphanus.* Walsh (1989) distingue 4 grupos de TuMV de acordo com a interação em três genótipos diferenciais de *B. napus*. Utilizando estes três genótipos e um outro quarto de mais uma linhagem de *B. napus*, Jenner; Walsh (1996) caracterizaram 124 isolados coletados no mundo revelando 12 patótipos distintos, dos quais os patótipos 1,3 e 4 foram predominantes na Europa.

No Brasil, o TuMV já foi descrito causando danos em raiz-forte (*Armoracia rusticana*) (EIRAS et al., 2007); nabo (*Brassica rapa*) (LIMA; COSTA; KOEHLER, 1980); agrião (*Nasturtium officinale*) (COSTA et al., 2010); colza (*Brassica napus*) (LIMA, 1981) e na planta ornamental *Tropaeolum majus* (DUARTE et al., 2014). Com relação à sequencias genômicas de TuMV, só há dados parciais do TuMV isolado de agrião (chave de acesso: HM008961.1) e de *T. majus* (chave de acesso: KJ635891.1).

Em 2016, campos de produção de hortaliças da região de Botucatu foram visitadas, a pedido do produtor. Sintomas de mosaico, redução drástica no crescimento e deformação foliar foram observados em rúcula Astro (*Eruca sativa*) e por meio de testes sorológicos, moleculares e biológicos foi identificado, pela primeira vez no Brasil a presença de TuMV infectando naturalmente plantas de rúcula. Posteriormente outros campos de produção de folhosas foram visitados na região de

Bragança Paulista e Mogi-Mirim e o TuMV foi novamente identificado em rúcula, porém também em alface (*Lactuca sativa*) do tipo americana e acelga (*Beta vulgaris* subsp. *vulgaris*).

Rúcula (*Eruca sativa*), alface (*Lactuca sativa*), e acelga (*Beta vulgaris* subsp. v*ulgaris*) são vegetais folhosos, amplamente cultivados no Brasil, de forma convencional ou em sistema hidropônico. A rúcula é pertencente à família Brassicaceae, e ocupa a terceira posição entre as hortaliças folhosas cultivadas no Brasil, com área plantada de aproximadamente 40.000 hectares, segundo dados da Associação Brasileira do Comércio de Sementes e Mudas (ABCSEM, 2015). A alface, pertencente à família Asteraceae , é a folhosa mais cultivada no Brasil, com cerca de 90.000 hectares (ABCSEM, 2015). A acelga (*Beta vulgaris* subsp. *vulgaris*) pertence à família Amaranthaceae, no entanto, há poucos dados técnicos disponíveis sobre sua cultura.

Desta forma, o presente trabalho foi dividido em dois capítulos intitulados: 1) "**Biological and molecular characterization of a basal-Brassica/Raphanus *Turnip mosaic virus* isolate from *Eruca sativa*.**" redigido em inglês e publicado na revista Tropical Plant Pathology, disponível através do DOI: http://dx.doi.org/10.1007/s40858-017-0207-8 e 2) "**First report of a basal-Brassica/Raphanus *Turnip mosaic virus* naturally infecting lettuce and chard plants in Brazil.**" redigido em Inglês na forma de Disease note.

CAPÍTULO 1 - Biological and molecular characterization of a basal-Brassica/Raphanus *Turnip mosaic virus* isolate from *Eruca sativa*

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Abstract

Eruca sativa (rocket salad or arugula) and *Raphanus raphanistrum* (raphanus) plants with mosaic symptoms were found in the field during 2016 in São Paulo State, Brazil. Initially, the plants were submitted to indirect ELISA using a potyvirus antiserum, and

then total RNA extraction and RT-PCR were performed using the ELISA-positive samples. The complete coat protein sequence was obtained and the virus was identified as *Turnip mosaic virus* (TuMV). Biological and Bayesian analysis grouped the TuMV rocket isolate in the *Brassica-Raphanus* (BR) clade that includes isolates infecting *Brassica* and *Raphanus* species. This clade has two sub-clusters, the basal-*Brassica*/*Raphanus* (basal-BR) and the Asian-*Brassica*/*Raphanus* (Asian-BR), and the rocket isolate was placed in the basal-BR cluster. TuMV from rocket was aphid-transmitted to raphanus and rocket, and sap-transmitted to *Chenopodium quinoa*, *C. amaranticolor*, *Nicotiana benthamiana*, *N. tabacum*, *N. glutinosa*, *Datura stramonium*, *Beta vulgaris subsp. vulgaris*, *Brassica rapa* and to seven rocket cultivars, which were heavily affected by the virus. Cabbage and cauliflower were not infected by the virus. According to the phylogenetic analysis, at least two different introductions of TuMV isolates occurred in Brazil, corresponding to the basal-BR and world-B types, infecting *Brassica*/*Raphanus* and *Brassica*, respectively.

Keywords

Raphanus, Rocket, TuMV, Variability

Rocket or arugula (*Eruca sativa*) is a leafy vegetable classified into the Brassicaceae family. Cultivation can be by hydroponic or conventional systems. In Brazil (where it is known as rúcula) it is the third most important leafy vegetable, occupying around 40.000 ha (ABCSEM 2015). Different viruses have been reported infecting rocket in the world, such as the ophiovirus *Mirafiori lettuce big-vein virus* (MiLBVV) (Umar et al. 2017), the potyvirus *Turnip mosaic virus* (TuMV) (Gardner and Kendrick 1921), the crinivirus *Tomato chlorosis virus* (ToCV) (Boiteux et al. 2016) and

the comovirus *Turnip ringspot virus* (TuRSV) (Picoli et al. 2012). The last two have been detected naturally infecting rocket in Brazil.

In commercial production fields in Botucatu, São Paulo state, rocket salad and raphanus (*Raphanus raphanistrum*) were found showing symptoms of mosaic, leaf deformation and growth reduction, typically caused by viruses (Fig. 1). To investigate the etiology of the disease, the extract from symptomatic rocket leaves was analyzed by transmission electron microscopy using the leaf dip methodology (Brandes 1957). Elongated, flexuous particles with 700–750 nm in length were observed, indicating infection by a potyvirus. Fifteen plants collected from the field were tested by ELISA using an antiserum which detects most aphid-transmitted potyvirids classified in the Potyvirus genus (Agdia). All symptomatic plants were ELISA-positive.

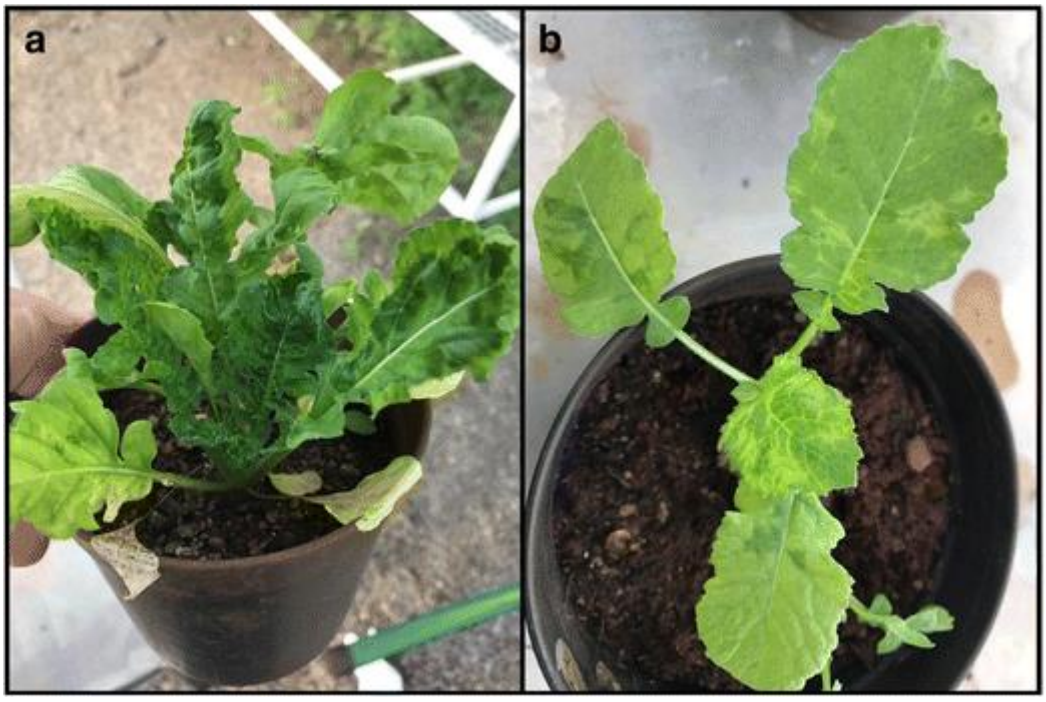

Fig. 1 Symptoms observed in (a) *Eruca sativa* and (b) *Raphanus raphanistrum* plants collected in Botucatu, São Paulo state, Brazil

Total RNA extraction (Bertheau and Frechon 1998) was performed from symptomatic rocket and raphanus leaves followed by a single step RT-PCR reaction using the potyvirus universal primers W-CIEN (5′-ATG GTT TGG TGY ATY GAR AAT-3′) and PV-1 (5′-GAT TTA GGT GAC ACT ATA $GT_{(17)}$-3′) (Gibbs and Mackenzie 1997; Mota et al. 2004), which amplify a fragment with approximately 850 bp corresponding to part of the capsid protein coding region. The reaction conditions were identical to those recommended by the authors. The fragment obtained shared 99% sequence identity with *Turnip mosaic virus* (TuMV, GenBank accession number EU734433).

Turnip mosaic virus is classified in the genus *Potyvirus* of the *Potyviridae* family, having flexuous filamentous particles 700–750 nm long and a genome comprised of a single-stranded positive-sense RNA with approximately 10.000 nt (Wylie et al. 2017). The virus was first reported in the United States infecting *Brassica* spp. (Gardner and Kendrick 1921) and is aphid-transmitted in a non-persistent manner (Yoshii 1966). The TuMV isolate collected in rocket was transmitted by *Aphis gossypii* from raphanus to rocket, and from rocket to rocket plants, using twenty aphids that were fasted for one hour before they fed for one more hour on TuMV-infected plants, and then transferred to twenty individual plants. Twenty days after the transmission, all plants tested were potyvirus-positive by RT-PCR.

TuMV presents a large variety of natural and experimental hosts, mostly, but not exclusively, plants species from the Brassicaceae family (Nguyen et al. 2013). We used symptomatic leaves of rocket plants ground in 0.05 M potassium phosphate buffer pH 8.0 as inoculum source for sap transmission to five plants each of *Beta vulgaris* subsp. *vulgaris*, *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Datura stramonium*, *Nicotiana benthamiana*, *Nicotiana glutinosa*, *Nicotiana tabacum*, *Brassica oleracea*, *Brassica oleracea* var. *botrytis*, *Brassica oleracea* var. *capitata*, *Brassica*

oleracea var. *capitata* f. *rubra*, *Brassica rapa*, *Raphanus raphanistrum* and seven cultivars of *Eruca sativa* (Table 1). The plants were pre-dusted with carborundum (600 mesh) and kept in the greenhouse. For the biological purification, a single local lesion collected from *C. amaranticolor* was used for back-inoculation in *Eruca sativa*. The virus induced leaf deformation, mosaic and stunting on the seven rocket cultivars tested. *Brassica oleracea* var. *capitata* (green and red cabbages) and cauliflower were not infected by the TuMV-rocket isolate. Cabbage was previously demonstrated to be a non-host of TuMV (Nguyen et al. 2013), but in Turkey, TuMV was reported infecting cabbage plants (Sevik and Deligoz 2016). Chlorotic spots and necrotic local lesions were observed on *C. quinoa* and *C. amaranticolor* leaves. Usually, plants of the Solanaceae family display a wide range of symptoms, and in our case *N. benthamiana* presented mosaic and stunting; *N. tabacum*, necrotic local lesions; *N. glutinosa*, chlorotic spots; and *D. stramonium* presented chlorotic and necrotic local lesions. In *B. vulgaris* subsp. *vulgaris*, the symptoms observed included leaf deformation, mosaic, necrotic local lesions and stunting. *B. rapa* developed mosaic symptoms. Raphanus plants displayed mosaic symptoms and leaf deformation, as observed on naturally infected plants. The field of rocket salad in Botucatu was highly infested with this weed. The incidence of weeds of the species *R. raphanistrum* has increased significantly in commercial rocket crops in Brazil due to its vigorous growth, and the recent discovery of resistance to herbicides, such as 2,4-D, which hinders its control (Busi and Powles 2017). Since TuMV is not transmitted by seed, these weeds are the most likely primary source of inoculum, and aphids are responsible for disseminating the virus in the field. The incidence of TuMV in rocket in Turkey was also associated with the presence of weeds, including raphanus (Sevik 2016).

Table 1 Host reaction of the *Turnip mosaic virus* isolate obtained from rocket (*Eruca sativa*).

Family	**Species**	**Symptom***	**PCR**
Chenopodiaceae	*Chenopodium amaranticolor*	CS/NLL	+
	C. quinoa	CS/NLL	+
Solanaceae	*Datura stramonium*	CS/NLL	+
	Nicotiana benthamiana	M/St	+
	N. glutinosa	CS	+
	N. tabacum	NLL	+
Amaranthaceae	*Beta vulgaris* subsp. *vulgaris*	LD/M/NLL/St	+
Brassicaceae	*Brassica oleracea*	-	-
	B. oleracea var. *botrytis*	-	-
	B. oleracea var. *capitata*	-	-
	B. oleracea var. *capitata* f. *rubra*	-	-
	Brassica rapa	M	+
	Raphanus raphanistrum	M	+
	Eruca sativa cv. Astro	LD/M/St	+
	E. sativa cv. Rococó	LD/M/St	+
	E. sativa cv. Selecta	LD/M/St	+
	E. sativa cv. Rokita	LD/M/St	+
	E. sativa cv. Donatella	LD/M/St	+
	E. sativa cv. Antonella	LD/M/St	+
	E. sativa cv. Roka	LD/M/St	+

* Symptoms in inoculated leaves. At least five plants of each host species were inoculated. CS, chlorotic spots; LD, leaf deformation; M, mosaic; NLL, necrotic local lesions; St, stunting; -, not infected.

TuMV isolates can be grouped into four “host types” (Ohshima et al. 2002; Nguyen et al. 2013). Isolates from host type (B) occasionally infect *Brassica* plants, often latently, but do not infect *Raphanus*; isolates from host type B infect most *Brassica* species, but do not infect *Raphanus*; isolates from host type B(R) infect most

Brassica species, and occasionally also *Raphanus* plants latently; and isolates from host type BR infect both *Brassica* and *Raphanus*. Since the TuMV isolate found in rocket salad infects both *Brassicae* and *Raphanus*, it should be classified as belonging to host type BR. The four host types correlate well with the four phylogenetic lineages determined based on the P1 and CP coding regions: the paraphyletic basal-*Brassica* (basal-B), which includes isolates from Brassicacea and also from species in other botanical families; world-*Brassica* (world-B), which is the more variable and widespread cluster; basal-*Brassica*/*Raphanus* (basal-BR); and Asian-*Brassica*/*Raphanus* (Asian-BR) (Nguyen et al. 2013). A monophyletic lineage named Orchis (O), comprised of isolates infecting wild orchids, is considered as an ancestor of TuMV (Nguyen et al. 2013).

Although the complete genome sequence of TuMV is the most suitable for phylogenetic inference (Nguyen et al. 2013), we analyzed the region corresponding to the complete capsid protein of the TuMV rocket isolate. For this, a primer pair was synthesized (TuMV8750: 5'-ACA GAT GAG CAG AAA CA GGC-3′ and TuMV9371: 5′-AAT CAA ATG CGT AC CG AGC-3′) that amplifies a 621 bp fragment corresponding to part of the 3'NIb-5'CP coding regions. The PCR reaction was performed with 12.5 µL of master mix buffer, 1 mM of each oligonucleotide, 1 unit of AMV reverse transcriptase (Promega) and DEPC-treated water (to make up the volume to 25 µL), and the following cycle parameters: 42 °C for 30 min (RT step), 94 °C for 1 min (hot start to PCR), then 35 cycles of amplification (1 min at 92 °C for denaturation, 30 s at 52 °C for annealing and 30 s at 72 °C for extension), followed by a final cycle at 72 °C for 10 min. The amplified PCR products were purified from the gel (Gel Extraction Kit, Qiagen) and directly sequenced in both forward and reverse directions using the same primers used for amplification. The complete nucleotide sequence of the CP coding

region (~960 bp; accession number MF773494) was obtained using the primer pairs WCIEN/PV1 and TuMV8750/TuMV9371. The sequence was compared to a dataset of 28 TuMV reference sequences (CP region) using MAFFT v7.222 within the Geneious v.9.1.3 software, and Bayesian phylogenetic analysis was performed using Mr. Bayes 3.2.2. The Bayesian analysis grouped the Brazilian TuMV rocket isolate into the basal-BR cluster (Fig. 2). The Brazilian TuMV isolate reported in watercress, *Nasturtium officinale* (HM008961; Costa et al. 2010), was placed in the world-B cluster (Fig. 2).

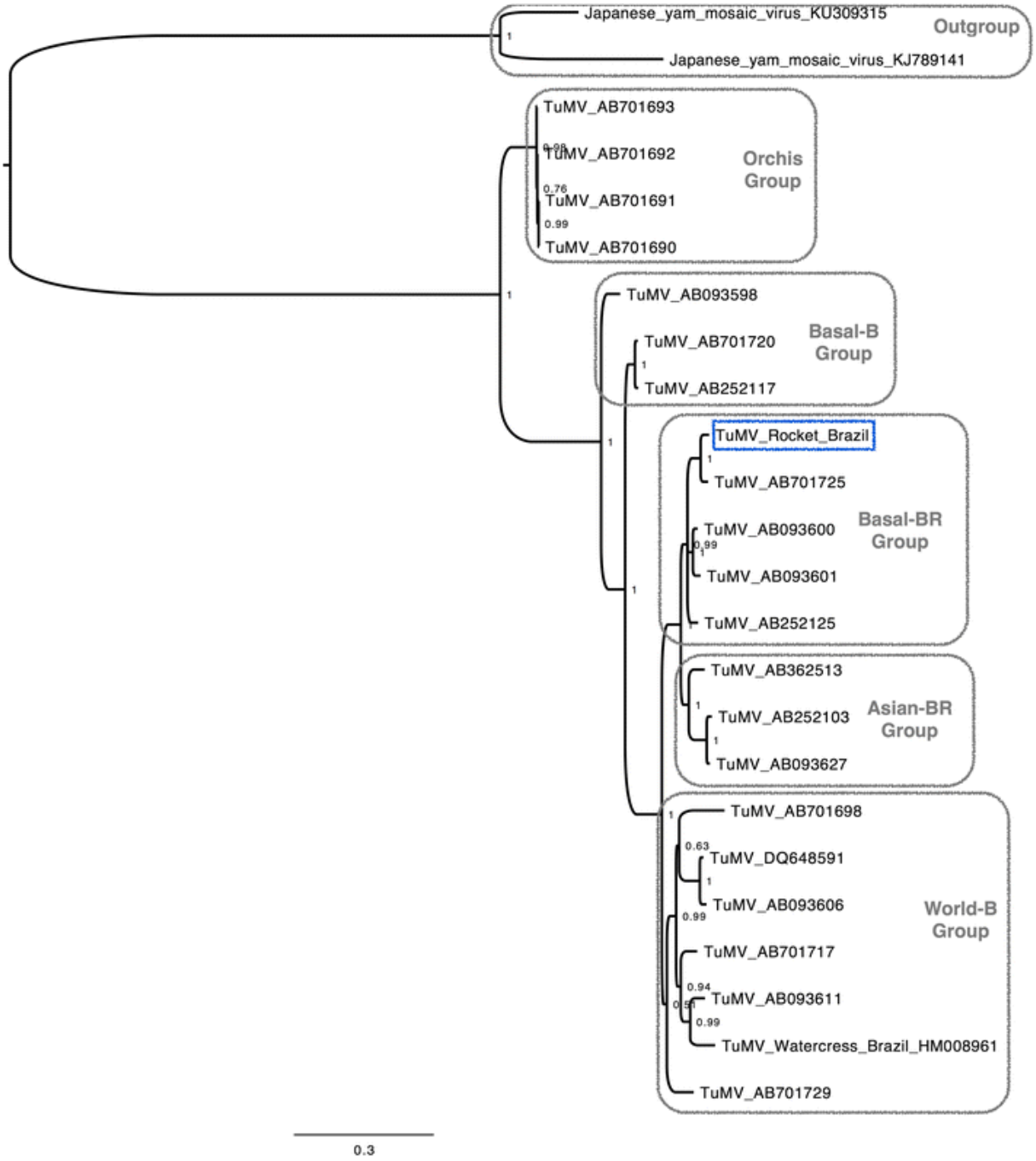

Fig. 2 Bayesian phylogenetic tree of a dataset consisting of *Turnip mosaic virus* reference sequences and the isolate of TuMV found in rocket in this work (highlighted in blue). The boxes indicate the different phylogenetic lineages of TuMV according to Nguyen et al. (2013).

TuMV is the world's most widespread and damaging virus of brassicas (Ohshima et al. 2002; Nguyen et al. 2013) but is still poorly studied in Brazil. Here we

present evidence that at least two different introductions of TuMV isolates occurred in the country, basal-BR and world-B isolates infecting *Brassica*/*Raphanus* and *Brassica*, respectively. A TuMV isolate was also recently found infecting *Tropaeolum majus* (Duarte et al. 2014), but no coat protein sequence is available to determine its phylogenetic lineage. The ability of TuMV to infect brassicas, which are dicotyledonous plants, is probably a recent adaptation, because in a genomic potyvirus phylogeny, TuMV's closest relatives infect monocots (Gibbs and Ohshima 2010; Nguyen et al. 2013). As far as we know, this is the first report of rocket naturally infected with TuMV in Brazil and, at least among the tested varieties, all were heavily affected by the virus. It is important to evaluate the occurrence of this virus in other producing areas of Brazil, and new genotypes need to be tested for resistance to the virus.

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CAPÍTULO 2 - First report of a basal-Brassica/Raphanus *Turnip mosaic virus* naturally infecting lettuce and chard plants in Brazil

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Article Type: Disease note

Turnip mosaic virus (TuMV) belongs to the *Potyvirus* genus, and is a major pathogen that infects at least 418 host species of cultivated or wild plants (Gibbs, Nguyen and Ohshima 2015). Recently, symptoms of mosaic were observed in lettuce (*Lactuca sativa*) and in chard (*Beta vulgaris* subsp. *vulgaris*) fields, during the spring of 2017 in Sao Paulo State (Brazil). Subsequent analyzes indicated that the causal agent was an isolate of *Turnip mosaic virus* (TuMV). Initially, the collected symptomatic leaves were submitted to indirect ELISA test, using a polyclonal potyvirus antiserum (Agdia, Inc), and all the plants were found positive. Total RNA was extracted (Bertheau and Frechon 1998), followed by RT-PCR reaction using universal primers W-CIEN (5′-ATG GTT TGG TGYATY GAR AAT-3′) and PV-1 (5′-GAT TTA GGT GAC ACTATA $GT_{(17)}$-3′) (Mota et al. 2004; Gibbs and Mackenzie 1997) designed to amplify part of the potyvirus

capsid protein (CP) gene, and a fragment of the expected size (~850 bp) was obtained. This fragment was sequenced for lettuce and chard samples, and both showed nucleotide identity of 96% with TuMV (GenBank: AB701725.1). Further, the complete sequence of the CP gene (~1238 bp) from both isolates was amplified using a specific primers pair (Fwd: 5'-TAC CTA CAA GCA ATC TTTG-3' and Rev: 5'-GGC AAT CGA GAT ACT ATC TC-3'). These amplicons were purified (Gel Extraction Kit, Qiagen) and directly sequenced in forward and reverse directions using the PCR primers, confirming the presence of TuMV in the symptomatic lettuce and chard plants. The sequences obtained were compared to a dataset of 29 TuMV reference sequences (CP region) using MAFFT v7.222 within the Geneious v.9.1.3 software, and Bayesian phylogenetic analysis was performed using Mr. Bayes 3.2.2. The TuMV isolates from lettuce and chard were both grouped in the *Brassica-Raphanus*-[BR] clade. In our previous study (Ribeiro-Junior et al. 2018), we identified for the first time a basal-BR TuMV naturally infecting *Eruca sativa* (rocket salad) and *Raphanus raphanistrum* (raphanus) plants in Brazil and as far as we know, only two different introductions of TuMV are known in Brazil, the basal-BR and world-B types, infecting *Brassica*/*Raphanus* and *Brassica*, respectively. The TuMV isolate for lettuce and chard were successfully sap-transmitted to *Raphanus sativus* and *Eruca sativa* and the infection was confirmed by RT-PCR. To our knowledge, this is the first occurrence of TuMV naturally infecting lettuce and chard plants in Brazil.

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CONSIDERAÇÕES FINAIS

Trata-se da primeira evidencia de infecção natural de rúcula, alface e acelga por TuMV no Brasil.

Pelo menos duas introduções diferentes de isolados de TuMV ocorreram no Brasil, correspondendo aos tipos basal-BR e world-B, infectando *Brassica*/*Raphanus* e *Brassica*, respectivamente.

Os isolados encontrados em rúcula, nabiça, nabo, alface e acelga pertencem ao grupo filogenético basal-BR.

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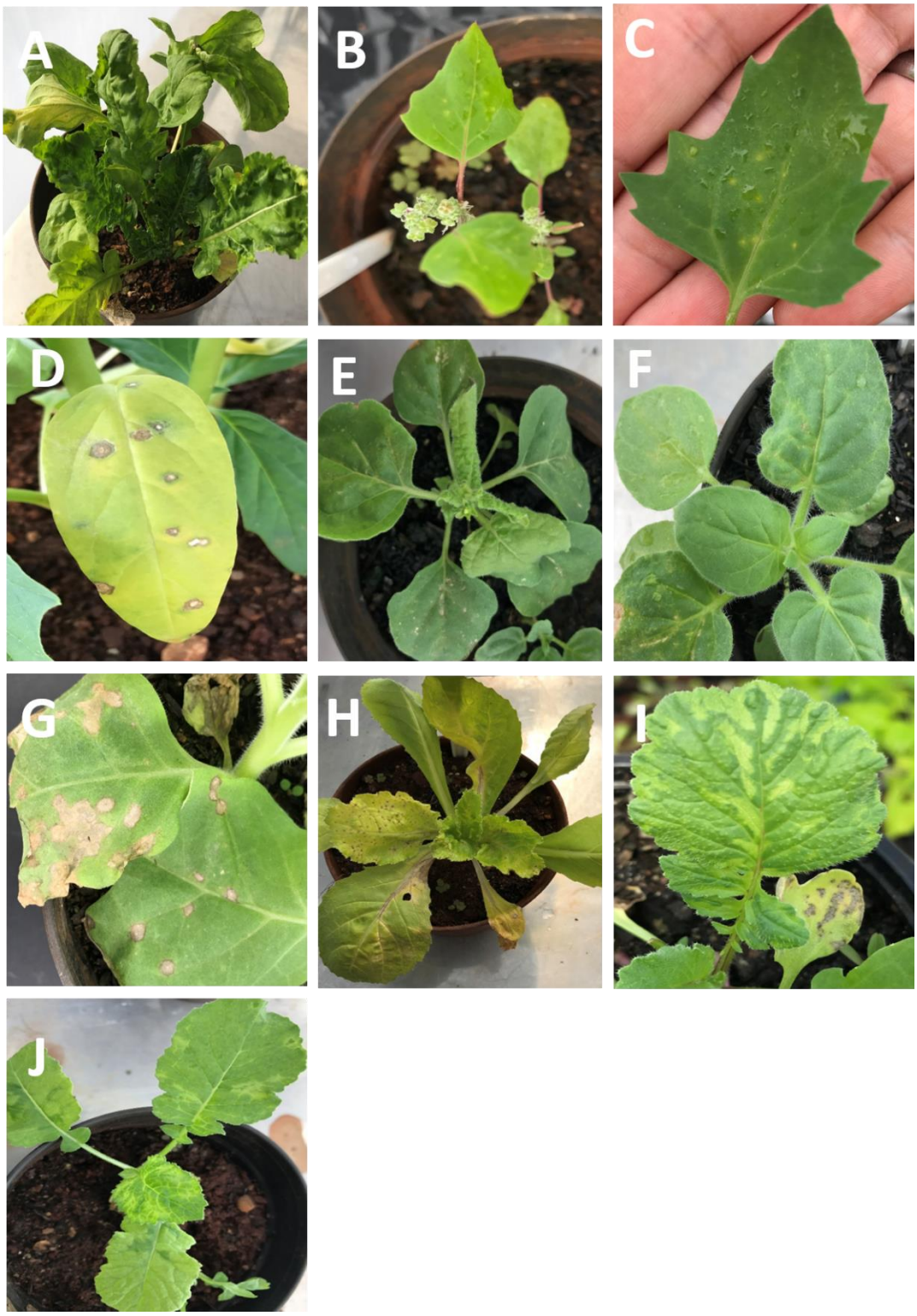
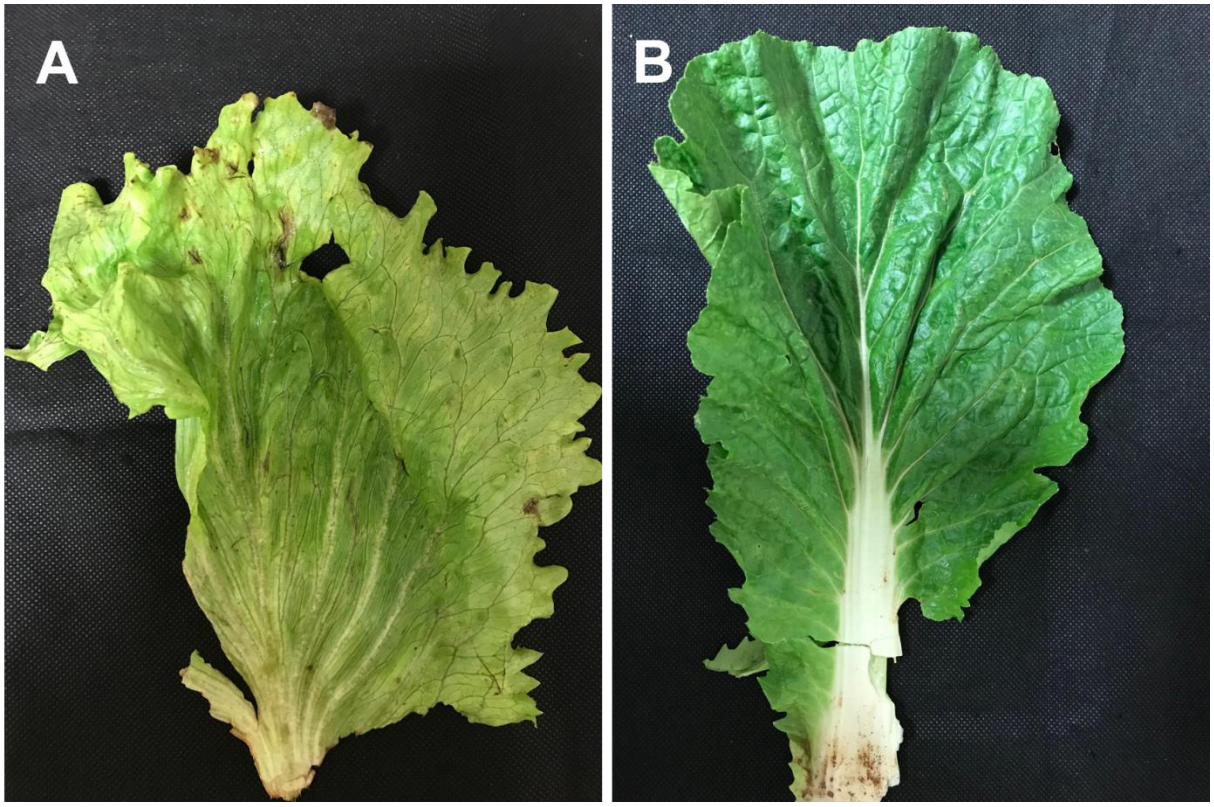
APÊNDICE A – **Sintomas observados em plantas inoculadas com extrato vegetal proveniente de plantas de rúcula (*Eruca sativa*) (A): *C. ammaranticolor* (B), *C. quinoa* (C), *D. stramonium* (D), *N. benthamiana* (E), *N. glutinosa* (F), *N. tabacum* (G), *B. vulgaris* (H), *B. rapa* (I), *R. raphanistrum* (J).**



APÊNDICE B – Amostras de alface (*Lactuca sativa*) (A) e acelga (*Beta vulgaris* subsp. *vulgaris*) (B) infectadas com *Turnip mosaic virus*.



APÊNDICE C – Árvore filogenética bayesiana descrita no Capítulo 2, constituída de sequências de referência do *Turnip mosaic virus* (TuMV) e os isolados de TuMV encontrados em rúcula, alface, acelga, nabo e nabiça (destacado em azul). As caixas indicam as diferentes linhagens filogenéticas de TuMV de acordo com Nguyen et al. (2013).