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SHORT COMMUNICATION

A novel virulent Brazilian pathotype of *Meloidogyne javanica* towards the tomato *Mi*-1.2 gene and pathogenicity to resistant rootstock

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Abstract

Very effective genetic control of some root-knot nematode (RKN) species (*Meloidogyne* spp.) is obtained by the employment of the tomato (*Solanum lycopersicum* L.) dominant *Mi*-1.2 gene. However, the phenotypic expression conferred by the *Mi*-1.2 gene may be impaired by high soil temperatures (above 28 °C) and by previously reported virulent RKN species and/or populations. Here, a putative *Mi*-1.2 gene-virulent RKN population was found inducing severe gall symptoms in roots of the tomato rootstock 'Guardião' (a homozygous *Mi*-1.2 hybrid of *S. lycopersicum* × *S. peruvianum*) under protected crop conditions in Southern Brazil. Females were extracted from severely infected roots displaying large galls and used for biochemical characterization of α-esterase (EST) and confirmed by SCAR markers. Additional pathogenicity assays were carried out in order to confirm the virulence (= resistance-breaking) feature of this RKN population by employing other or the same *Mi*-1.2 gene-carrying rootstocks. This virulent RKN population was identified as *M. javanica* (EST J3 and J2). The pathogenicity tests confirmed that this *M. javanica* population can overcome the *Mi*-1.2 resistance gene, and it is able to induce severe root-gall symptoms and to reproduce in two dominant resistant rootstocks ('Muralha' and 'Guardião') under greenhouse conditions. In addition, an initial inoculum of 2000–7000 eggs was considered ideal for future studies with the virulent *M. javanica* population. It is the first report of a *Mi*-1.2 gene-virulent *M. javanica* population in Brazil, which may represent a potential threat to the tomato agribusiness sector.

Keywords *Solanum lycopersicum* · Resistance · Root-knot nematodes · Virulence · Inoculum level

Introduction

The damage caused by root-knot nematode (RKN) species (*Meloidogyne* spp.) in the tomato (*Solanum lycopersicum* L.) crop has been efficiently managed in Brazil by genetic resistance (Trudgill 1986). The single dominant *Mi*-1.2 gene, introgressed from one accession of the wild tomato species *S. peruvianum* L. (Smith 1944), confers resistance against 13 *Meloidogyne* species (viz. *M. arenaria*, *M. ethiopica*, *M. exigua*, *M. hispanica*, *M. incognita*, *M. inornata*, *M. izalcoensis*, *M. javanica*, *M. konaensis*, *M. luci*, *M. morocciensis*, *M. paranaensis*, and *M. petuniae*), but not against *M. hapla* and *M. enterolobii* (Gabriel et al. 2020; Santos et al. 2020). This gene has been the only reliable source of RKN resistance for use in tomato breeding programs (Williamson 1998). Although the gene *Mi*-1.2 displays unusual broad-spectrum resistance to many RKN species, its phenotypic expression may be impaired by high soil temperatures (> 28 °C) and by the occurrence of virulent populations capable of suppressing and/or surpassing the action of this gene (Devran and Söğüt 2010; Cortada et al. 2011; Tzortzakakis et al. 2014). Furthermore, *Mi*-1.2-mediated resistance may be influenced by gene dosage, in which homozygous genotypes (with two

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copies of the dominant allele) show higher levels of resistance when compared to genotypes with only one copy of the resistant allele (Jacquet et al. 2005; Maleita et al. 2011; Iberkleid et al. 2014).

Virulence is defined as the ability of the nematode to reproduce in a host plant with one or more resistance genes. Virulent populations can occur naturally, even without previous exposure to a resistant cultivar (Ornat et al. 2001; Iberkleid et al. 2014). However, virulent populations can more often be selected after repeated exposure of the nematodes to plants carrying the resistance gene under field conditions (Karajeh et al. 2005; Verdejo-Lucas et al. 2009; Devran and Söğüt 2010; Tzortzakakis et al. 2014) or under laboratory-induced selection (Jarquin-Barberena et al. 1991; Williamson 1998).

Virulent *Meloidogyne* populations capable of reproducing in plants carrying the gene *Mi*-1.2 have been reported in several European countries (Castagnone-Sereno et al. 1994; Ornat et al. 2001; Robertson et al. 2006; Bleve-Zacheo et al. 2007; Devran and Söğüt 2010; Tzortzakakis et al. 2014). Studies carried out by Pinheiro et al. (2014) in areas of tomato production in Brazil indicated the predominance of *M. javanica* (50%), followed by *M. incognita* (28.5%), *M. ethiopica* (14.2%), *M. enterolobii* (7.1%), and *M. morocciensis* (3.6%). So far, the reports of *Meloidogyne* populations virulent to the gene *Mi*-1.2 in Brazil are restricted to one *M. incognita* population (Silva et al. 2019) with no reports of virulent populations from other *Meloidogyne* species under field conditions. Considering that the use of resistant varieties has been the most economical and sustainable strategy for controlling RKN in tomato production (Devran and Söğüt 2010), monitoring virulent populations in cultivated areas is a fundamental research action; this action can improve management practices, using crop rotations with non-host plants. In this context, the main objectives of the present study were: (i) to identify the RKN species associated with severe root symptoms observed in the resistant tomato rootstock 'Guardião' carrying the *Mi*-1.2 gene and susceptible tomato 'Santa Cruz' group, in the northwestern region of Rio Grande do Sul state, Brazil; (ii) to confirm the virulent (resistance-breaking) nature of this RKN population, conducting pathogenicity bioassays in tomato rootstock 'Muralha', carrying the homozygous *Mi*-1.2 gene under greenhouse conditions; and (iii) to study the effect of different inoculum levels on the reproduction of the virulent *Meloidogyne* population in the resistant tomato rootstock 'Guardião'.

Material and methods

Meloidogyne spp. populations

Samples from five localities with tomato roots displaying severe RKN symptoms of infection were collected from tomato monocultures in open fields and greenhouses in the northwestern region of Rio Grande do Sul state, in the municipalities of Frederico Westphalen, Rodeio Bonito, and Palmeira das Missões (Table 1). Ten subsamples (roots and soil) were taken at the depth of 0–20 cm, and placed in plastic bags and transported to the laboratory. The roots were separated and washed, and 10 g of the roots was used to extract the nematodes, according to the modified methodology of Hussey and Barker (1973), in which the roots were ground in a blender with a solution of 0.5% sodium hypochlorite (NaOCl) for 30 s. A fraction of 200 cm^3 of soil replicated three times was processed by sieving and flotation in a centrifuge with a saccharose solution (Jenkins 1964). To evaluate the nematode population from the field, the number of second-stage juveniles (J2) in the soil and eggs + J2 in the roots was counted using Astel Peters slides, under a light microscope. The different populations were maintained on tomato 'Santa Clara', under greenhouse conditions for 60–90 days and the virulent population of *M. javanica* (a mixture of EST J3 + EST J2) also.

Biochemical identification

Twenty females were handpicked individually from infected tomato plants that are obtained first from field and after from greenhouse and characterized biochemically by electrophoretic analysis of non-specific esterase (EST) phenotype,

Table 1 Geographical localization of samples collected in tomato cultivars in Rio Grande do Sul

Sample[a]	Geographic origin	Coordinates	Cultivar[c]
1	Frederico Westphalen[b]	27° 21′ 32" S/53° 23′ 38" O	Guardião (R)
2	Rodeio Bonito	27° 28′ 15" S/53° 10′ 08" O	Santa Cruz Kada (S)
3	Palmeira das Missões	27° 53′ 56" S/53° 18′ 50" O	Santa Cruz Santa Clara (S)
4	Frederico Westphalen	27° 21′ 32" S/53° 23′ 38" O	Santa Cruz Santa Clara I5300 (S)
5	Rodeio Bonito	27° 28′15" S/53° 10′ 08" O	Santa Cruz Kada (S)

[a]*Meloidogyne* species are included in Table 2, according to the samples

[b]1 from greenhouse in field, the other (2–5) from field

[c]R: resistant (homozygous *Mi*-1.2/*Mi*-1.2); S: Susceptible (homozygous *mi*-1.2/*mi*-1.2)

in polyacrylamide gels, according to the methodology described by Carneiro and Almeida (2001). The isozyme profiles were compared to the ones described in the literature (Carneiro et al. 2016).

SCAR marker identification

For the DNA studies, the eggs were extracted from infested roots of tomato obtained from greenhouse and stored at −80 °C, until use. For each nematode population, genomic DNA was extracted from 200 to 300 µl of eggs (Randig et al. 2002). The *M. javanica* and *M incognita* populations analyzed in this study (Table 1) were tested with species-specific SCAR (Sequence Characterized Amplified Region) primers: OPA-01_{700} (Zijlstra et al. 2000), incK14F/R (Randig et al. 2002). The PCR reactions were performed in 25-µl volume, containing 5 ng of total genomic DNA, 0.5U of Taq polymerase (Invitrogen), 1 × Taq polymerase reaction buffer, 200 µM of each deoxynucleotide triphosphate (Pharmacia Biotec), 0.05 µM of $MgCl_2$ and 8 µM of the primer (Life Technologies). The amplifications were made using the PTC-100 programmable thermal controller (MJ Research), and the PCR conditions were the same as those used by Zijlstra et al. (2000) and Randig et al. (2002).

Meloidogyne javanica virulence and influence of inoculum level on pathogenicity

In order to confirm the resistance-breaking nature of the *M. javanica* population from Frederico Westphalen, two bioassays were carried out using tomato seedlings at three-leaf stage. These were transplanted to plastic pots (2 L) with sterilized soil and the commercial Bioplant substrate (ingredients: *Sphagnum* peat, coconut fiber, rice husk, pine bark, vermiculite, agricultural gypsum, calcium carbonate, magnesium and fertilizers) (1:1), under greenhouse conditions (average temperature 25-30ºC). In the first assay, the response of the tomato rootstock 'Muralha', homozygous at the *Mi*-1.2 locus (*Mi*-1.2/*Mi*-1.2; Gabriel 2020; Bhavana et al. 2019) and the control 'Santa Clara' (mi-1.2/mi-1.2, recessive form, susceptible) were evaluated against the virulent *M. javanica.* In the second assay, the effect of different initial population densities (Pi) of the virulent population of *M. javanica* was also evaluated on nematode reproduction on the homozygous resistant (*Mi*-1.2/*Mi*-1.2) tomato rootstock 'Guardião' (Gabriel 2020; Bhavana et al. 2019). The susceptible cultivar Santa Clara (*mi*-1.2/*mi*-1.2) was used as control. The Pi was 5000 eggs + J2 per plant, in the first assay, and 0, 250, 2,000, 5000, 7000, and 10,000 eggs + J2 per plant in the second. The bioassays were installed in a completely randomized design, with six replicates/treatment. Sixty-five days after inoculation, the roots were washed and evaluated for fresh weight. Gall and egg mass index per root system were evaluated (Taylor and Sasser 1978). Subsequently, they were processed according to the modified methodology of Hussey and Barker (1973) and the total number of eggs + J2 (final population, Pf) was evaluated. The reproduction factor (RF = Pf/Pi) was calculated (Oostenbrink 1966). The data were subjected to analysis of variance. Normality was determined by the Shapiro–Wilk test. Subsequently, the treatment averages were grouped using the Scott-Knott test (at 5% probability). The statistical analyses were performed using the SISVAR software (Ferreira 2011).

Results

Biochemical and molecular identifications

Three EST phenotypes were observed in the isoenzymatic characterization of the *Meloidogyne* from field and greenhouse populations. The putative virulent *Mi*-1.2 population from the resistant tomato 'Guardião' rootstock displayed a mix of two *M. javanica* EST phenotypes: EST J3 (Rm: 1.00; 1.20, 1.40) and EST J2 (Rm: 1.00; 1.20), and the population from susceptible 'Kada' tomato revealed only *M. javanica* EST J3. EST I2 (Rm: 1.0; 1.06), corresponding to *M. incognita* (Fig. 1), was observed in another three field samples not associated with resistant cultivars (Tables 1 and 2).

Meloidogyne javanica and *M. incognita* populations were tested with the species-specific molecular marker, type SCAR, developed for these species (Zijlstra et al. 2000; Randig et al. 2002). Using the primers OPA-$01_{700,}$ the two populations of *M. javanica* were identified by the fragments of 700 bp; the primers incB06F/R amplified one fragment of 1200 bp for the three populations of *M. incognita* (Tables 1 and 2) and confirmed the identification using esterase phenotypes.

Nematode population densities in field samples

The highest nematode population levels were observed in sample 1 coming from Frederico Westphalen cultivated with 'Guardião' rootstock (carrying the gene *Mi*-1.2) with greater reproduction than the susceptible cultivars (Table 2).

Meloidogyne javanica virulence and influence of inoculum level on pathogenicity

The number of nematodes per gram of root and the reproduction factor (RF) were higher in the homozygous (*mi*-1.2/*mi*-1.2) susceptible cultivar Santa Clara than in the homozygous resistant (*Mi*-1.2/*Mi*-1.2) rootstock 'Muralha' (Table 3). This rootstock also allowed high reproduction of the virulent *M. javanica*, confirming its virulence towards the resistance gene. When the RF is very high, as in Table 3,

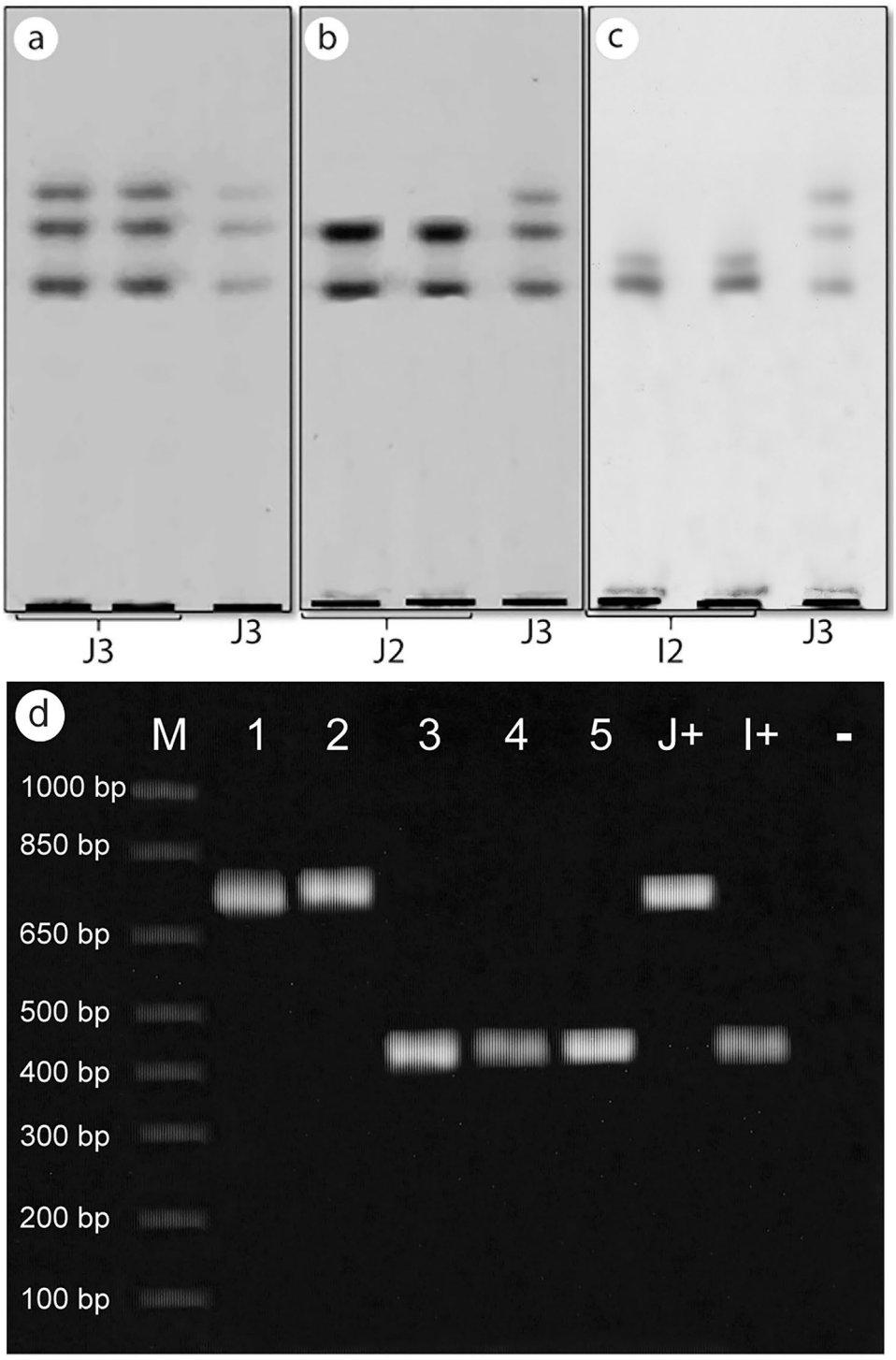

Fig. 1 **a**, **b** Esterase phenotypes (Est) of the virulent population of *Meloidogyne javanica* (Est J3 and J2) and **c** *M. incognita* (Est I2). *M. javanica* (Est J3) was used as reference in each gel. **d** SCAR-PCR amplification pattern for virulent *M. javanica* (lane 1, 2, Far/Rjav for *M. javanica*, Zijlstra et al. 2000); *M. incognita* (lanes 3,4,5, Inck14 F/R, Randig et al. 2002). Positive controls: J + = *M. javanica*, I + = *M. incognita*; − = water negative control, M = 1 kb DNA extension ladder (Invitrogen)

the GI and EMI index always reach their maximum values (5). The results revealed also that the virulent population of *M. javanica* reproduced in all tested densities and induced a high number of root galls (GI = 5) in the resistant tomato rootstock 'Guardião', confirming the reaction observed under field conditions (Table 4). The increase in the initial nematode populations (IP) resulted in significant increases in the number of eggs + J2 per gram of root (NNGR), except for the inoculum level of 10,000 eggs/J2, which caused a reduction in NNGR compared to the other inocula (5000–7000). Considering the RF, three levels of inoculum can be highlighted: very low (Pi = 250) corresponding to very high RF (121.50), intermediate (Pi = 2000–7000), corresponding to medium RF (73.78–79.43) and high inoculum

Table 2 *Meloidogyne incognita* and *M. javanica* populations found in soil and roots of tomato cultivars and rootstocks sampled in production areas in Rio Grande do Sul

Sample	Species (Esterase phenotype)	Cultivars (allelic composition)[a]	NNGR[b]	J2/100 cm^3 of soil
1	*M. javanica* (J3 and J2)	Guardião (*Mi*-1.2/*Mi*-1.2)	15,686	938
2	*M. javanica* (J3)	Kada (*mi*-1.2/*mi*-1.2)	8452	412
3	*M. incognita* (I2)	Santa Clara (*mi*-1.2/*mi*-1.2)	6540	186
4	*M. incognita* (I2)	Santa Clara *I5300* (*mi*-1.2/*mi*-1.2)	3859	274
5	*M. incognita* (I2)	Kada (*mi*-1.2/*mi*-1.2)	2345	213

[a]In Gabriel 2020, [b]NNGR = number of nematodes (eggs + J2) per gram of root

Table 3 Fresh tomato root weight, number of nematodes (eggs/J2) per gram of root (NNGR), gall index (GI), egg mass index (EMI), reproduction factor (RF) of the rootstock 'Muralha' (resistant) and cultivar Santa Clara (susceptible), 65 days after inoculation with 5000 eggs/J2 of the virulent *Meloidogyne javanica* population from Fredericо Westphalen

Cultivars (allelic composition)	Fresh root weight (g)	NNGR	GI	EMI	RF
Muralha (*Mi*-1.2/*Mi*-1.2)	29.80 b	28,861 b	5	5	171.79 b
Santa Clara (*mi*-1.2/*mi*-1.2)	38.28 a	57,561 a	5	5	436.57 a
Coefficient of variation. (%)	7.80	24.33			21.84

Mean values (six replications) followed by the same lowercase letter in the column do not differ statistically by the Scott–Knott test at 5% probability

GI and EMI (0–5): 0 = no gall/egg mass, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, 5 > 100 galls/egg masses per root system (Taylor and Sasser 1978)

Table 4 Fresh tomato root weight, number of nematodes (eggs/J2) per gram of root (NNGR), gall index (GI) and reproduction factor (RF) of the rootstock 'Guardião' (resistant), 65 days after inoculation with different initial populations (Pi) of the virulent population of *Meloidogyne javanica*,

Pi	Fresh root weight (g)	NNGR*	GI	RF*
0	21.73 a	–	–	–
250	14.04 b	2233 d	5	121.50 a
2000	14.80 b	11,760 c	5	73.78 b
5000	15.20 b	26,474 b	5	78.67 b
7000	15.62 b	41,770 a	5	79.43 b
10,000	16.43 b	17.678 c	5	28.71 c
CV %	11.73	20.22		18.41

CV% coefficient of variation

*Mean values followed by the same lowercase letter in the column do not differ statistically by the Scott–Knott test at 5% probability. GI and EMI (0–5): 0 = no gall/egg mass, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, 5 > 100 galls/egg masses per root system (Taylor and Sasser 1978)

level (Pi = 10,000) presenting low RF (28.71) (Table 4). The inoculum level of 10,000 eggs/J2 per plant was considered excessive, since it significantly reduced NNGR and RF. The GI was the maximum (5) for all inoculum densities tested, not reflecting the NNGR.

Discussion

Using biochemical analysis, the presence of *M. javanica* and *M. incognita* species was confirmed in the three tomato fields sampled in Rio Grande do Sul state. These findings are in agreement with surveys that indicated *M. javanica* and *M. incognita* species as the predominant RKN species in major tomato-producing areas in Brazil (Pinheiro et al. 2014).

The presence of EST profile polymorphisms has already been reported for *M. javanica* species as well as for *M. incognita* (Santos et al. 2012). According to Carneiro et al. (1996), the EST J3 phenotype is the most frequently observed profile in *M. javanica* populations associated with tomato crops in Brazil, while the EST J2 phenotype is less frequent. This J2 phenotype has been reported in Brazil in other crops, such as rice, sugarcane, corn, blackberry, cassava, pepper, tobacco (Carneiro et al. 1996, 1998), soybean (Castro et al. 2003), banana (Cofcewicz et al. 2004), okra (Oliveira et al. 2007), fig tree (Gomes et al. 2009), grapevine (Somavilla 2011), and potato (Medina et al. 2017). The occurrence of a *M. javanica* population inducing severe symptoms in 'Guardião' rootstock (carrying the gene *Mi*-1.2) is, to our knowledge, the first record of a virulent *M. javanica* (mixture of EST J3 and J2) population in Brazil.

With the aim of confirming the pathogenicity of virulent *M. javanica* to the rootstock 'Guardião' and better understanding the reproduction behavior of this population, a study with different initial population densities (Pi) was carried out. Our results indicated that the breakdown of the *Mi*-1.2-mediated resistance was not related to the Pi of *M. javanica*. The highest Pi (except 10,000 eggs + J2 per plant) showed an increase in the NNGR and RF, corroborating previous studies (Charegani et al. 2012; Kamran

et al. 2013). Values of Pi between 2000 and 7000 eggs/J2 showed to be the best inoculum densities for future screening studies of resistant varieties. The Pi of 10,000 eggs/J2 per plant showed the most suppressive effect on RF, which could be explained by a competition for feeding sites (Seinhorst 1970), considering the small size of the roots when the tomato plants were inoculated. The ability to overcome resistance in tomato cultivars and rootstocks with the *Mi*-1.2 gene may be related to several factors. Among them may be the intraspecific variability of the local RKN populations (Castagnone-Sereno 2002, 2006), high soil temperature (> 28ºC) (Araujo et al. 1982; Verdejo-Lucas et al. 2013), or selection pressure favoring virulent biotypes, due to the continuous use of resistant cultivars (Xu et al. 2001).

Although Ornat et al. (2001) and Iberkleid et al. (2014) reported populations of *Meloidogyne* species that were naturally virulent to gene *Mi*-1.2, other studies showed that populations can also break down the resistance conferred by gene *Mi*-1.2 after repeated exposure to resistant plants (Karajeh et al. 2005; Verdejo-Lucas et al. 2009; Devran and Söğüt 2010; Tzortzakakis et al. 2014). This seems to be the most plausible hypothesis to explain this virulent profile of our *M. javanica* population, since the cultivar 'Guardião' (carrying the *Mi*-1.2 gene) was employed by the farmers in the sampled region for three consecutive years, which corresponds to ≈27 reproductive cycles of the nematode.

In fact, studies have indicated that the ability of *Meloidogyne* populations to reproduce in plants carrying the gene *Mi*-1.2 can be developed either gradually or rapidly (Williamson 1998). Eddaoudi et al. (1997) obtained some virulent populations of *M. javanica* naturally selected in fields previously cultivated with resistant tomato cultivars for at least 1 year. In central Florida, a virulent population of *M. incognita* was selected after five reproductive cycles on the resistant tomato 'Sanibel' (Noling 2000). In the laboratory, artificial selection experiments showed that changes from avirulence to virulence were progressively established over 5–10 generations of recurrent inoculations of *M. incognita* in tomato plants carrying the *Mi*-1.2 gene (Jarquin-Barberena et al. 1991; Castagnone-Sereno et al. 1994). Although most *Meloidogyne* species (including *M. javanica*) display obligatory mitotic parthenogenetic reproduction, they have a high capacity to adapt to environmental constraints, including their ability to surpass host resistance genes (Castagnone-Sereno 2006). The adaptation of RKN populations to gene *Mi*-1.2 may be related to a loss of putative avirulence genes. Castagnone-Sereno et al. (2009) studied two *Mi*-1.2-avirulent *M. incognita* populations from different regions (Russia and Mexico) and subjected them to selection pressure, making them virulent after 40 generations. These populations showed the deletion of genomic regions containing some allelic variants of the gene *map*-1 (*map*-1.2 and *map*-1.3). According to these authors, the fact that the same deletion was observed in two *M. incognita* lines, independent of the geographical origin and subjected to the same selection pressure, suggested that the loss of genes after the selection stage is not a random event, but the result of an adaptive mechanism that allows the nematode to develop in resistant plants.

More recently, the comparative genomic analysis of two *M. incognita* populations virulent to the gene *Mi*-1.2, as well as two almost isogenic avirulent populations, allowed the identification of 33 genes that decreased their copy numbers in the virulent populations but not in the avirulent ones (Castagnone-Sereno et al. 2019). In conclusion, the present study is the first confirmation of a *Mi*-1.2 gene-virulent *M. javanica* pathotype in Brazil, which may represent a potential threat for the tomato agribusiness. In this scenario, the adoption of management practices, such as rotation with non-host crops and a preemptive search for new sources of resistance to these *Meloidogyne* populations are mandatory research actions that may increase the longevity of the crucial gene for tomato cultivation in the tropics.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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