University of São Paulo
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Phenotypic and transcriptomic profiling of the interaction involving a virulent *Meloidogyne javanica* and a tomato genotype carrying the *Mi-1.2* resistance gene

Pedro Marcus de Souza Confort

Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

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To Friends and Family

Close and Far…

…As odd as it may be

I dedicate my devotion to those worms

To thee.

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CONTENTS

RESUMO

Análise fenotípica e molecular da interação entre um isolado virulento de *Meloidogyne javanica* e um genótipo de tomate portador do gene de resistência *Mi-1.2*

A cultura do tomate encontra uma ameaça substancial nos nematoides das galhas radiculares (NGR), especialmente *Meloidogyne incognita* e *M. javanica*. Esses patógenos parasitam a raiz de seus hospedeiros, criando células nutridoras especializadas, acarretando crescimentos anormais da raiz chamados de galhas. O impacto dos NGR na produtividade do tomateiro varia significativamente com base em condições ambientais, em geral, infestações moderadas estão associadas a uma redução de rendimento da ordem de 20 a 30%. A utilização do gene de resistência *Mi-1.2* é a principal estratégia de controle usada no manejo desses fitonematoides. No entanto, o surgimento de populações de *M. javanica* e *M. incognita* que quebram a resistência conferida por este gene representam um obstáculo para a cultura. Este trabalho abrangeu duas linhas de investigação, a primeira foi voltada para o estudo da interação entre o genótipo suscetível de tomate "Santa Clara" (S) e o resistente "Santy" (R) em resposta a isolados de *M. incognita* e *M. javanica*, visando confirmar a capacidade de reprodução destes em relação ao gene *Mi-1.2* (R). Ainda nesta etapa, foi conduzido um estudo para observar os efeitos de isolados avirulentos e virulentos de *M. javanica* na produtividade de tomateiros S e R. Nestes experimentos realizados sob condição de estufa foram em um primeiro momento avaliadas as interações entre 4 isolados de *M. incognita* e 2 de *M. javanica* quando inoculadas em hospedeiros R e S. Tratamentos foram estabelecidos com base nos contrastes entre isolados e hospedeiros (6x2). Ao fim desta etapa observamos que um isolado de *M. javanica* (Vir) foi capaz de se reproduzir na presença do gene *Mi-1.2*. Uma segunda etapa avaliou o impacto da infecção por *M. javanica* na produtividade de tomateiros. Tratamentos consistiram dos contrastes entre hospedeiros R/S, isolados Vir/Avir (incapazes de reproduzir na presença do gene *Mi-1.2*) e duas densidades populacionais iniciais (6 e 9 nematoides/cm^3 de solo), adicionadalmente 2 tratamentos controle não infestados foram adicinados totalizando 10 tratamentos. O estudo demonstrou que uma população inicial de 6 ovos/juvenis de *M. javanica* por cm³ pode causar reduções na produtividade de tomates portadores do *Mi-1.2*. Na segunda etapa, o objetivo foi obter respostas sobre essa interação a nível molecular, um estudo subsequente explorou as respostas transcricionais do genótipo de tomate 'Santy' durante interações compatíveis e incompatíveis com isolados de *M. javanica*. A análiserevelou genes diferencialmente expressos (DEGs) associados a respostas biológicas cruciais a estresses bióticos. As descobertas incluíram o aumento da expressão de análogos de genes de resistência e calmodulinas na interação incompatível, sugerindo uma interação complexa entre os mecanismos de defesa da planta e fatores de virulência do nematoide, incluindo um efetor candidato por parte do isolado virulento de *M. javanica*. Em conclusão, este estudo produziu observações sobre as dinâmicas da resistência mediada pelo gene *Mi-1.2* em tomates contra NGRs. Até o momento, esta é a única análise transcritômica da interação entre *M. javanica* x *Solanum lycopersicum*, abrindo a perspectivade aprimorar a compreensão desta interação planta-patógeno, elucidando os mecanismos moleculares que facilitam o desenvolvimento da doença e a erosão da resistência *Mi-1.2*.

Palavras-chave: Fitopatologia, Nematoide das galhas, Genes de resistência, RNAseq

ABSTRACT

Phenotypic and transcriptomic profiling of the interaction involving a virulent *Meloidogyne javanica* and a tomato genotype carrying the *Mi-1.2* resistance gene

Global tomato crops face a substantial threat from root-knot nematodes (RKN), specially *Meloidogyne incognita* and *M. javanica*. These pathogens parasitize root cells, creating specialized feeding cells within abnormal root growths known as galls. This process results in diminished plant growth and, consequently, leads to yield losses. The impact of RKN on tomato yields fluctuates significantly based on specific environmental conditions. In general, medium infestations are associated with an overall yield reduction of 20-30%. The employment of the *Mi-1.2* resistance gene is a crucial control strategy used to overcome this challenge. Nevertheless, emerging populations of virulent *M. javanica* and *M. incognita* pose a hindrance to the effectiveness of *Mi-1.2*. This study encompassed two lines of investigation. The first focused on examining the interaction between the susceptible tomato genotype "Santa Clara" (S) and the resistant "Santy" (R) in response to isolates of *M. incognita* and *M. javanica*, aiming to confirm the reproductive capacity of some of them in relation to the *Mi-1.2* gene (R), present in the resistant tomato. In this stage, a productivity study was also conducted to observe the effects of avirulent and virulent isolates of *M. javanica* on the productivity of S and R tomato plants. In experiments conducted in a greenhouse, interactions were initially assessed between 4 isolates of *M. incognita* and 2 of *M. javanica* when inoculated in R and S hosts. Treatments were established based on the contrasts between isolates and hosts (6 x 2), with 8 biological replicas each. At the end of this stage, it was observed that an isolate of *M. javanica* (Vir) was able to reproduce in the presence of the *Mi-1.2* gene. A second stage evaluated the impact of *M. javanica* infection on the development and productivity of tomato plants. Treatments consisted of contrasts between R and S hosts, Vir and Avir isolates (unable to reproduce in the presence of the *Mi-1.2* gene), and two initial population densities (6 and 9 nematodes per cubic centimeter of soil). Additionally, 2 non-infested control treatments were added, totaling 10 treatments. The study demonstrated that an initial population of 6 specimens of *M. javanica* per cm^3 can cause reductions in the productivity of tomatoes carrying the *Mi-1.2* gene. To gain molecular insights, a following study explored the transcriptional responses of the 'Santy' tomato genotype during both compatible and incompatible interactions with *M. javanica* isolates. RNA sequencing revealed differentially expressed genes (DEGs) associated with crucial biological responses to biotic stresses. Findings included the upregulation of resistance gene analogs and calmodulins in the incompatible interaction, suggesting a complex interplay between the plant's defense mechanisms and nematode virulence factors, including a candidate effector. In conclusion, this comprehensive study provides insights into the intricate dynamics of *Mi-1.2* resistance in tomatoes against RNK. To this date, this is the only transcriptome analysis of the interaction between *M. javanica* x *Solanum lycopersicum*. And through it, we hope to enhances our comprehensive understanding of this plant-pathogen interactions, elucidating the molecular mechanisms that drive disease development and the erosion of *Mi-1.2* resistance.

Keywords: Phytopatology, Root gall nematodes, Resistance genes, RNAseq

1. GENERAL INTRODUCTION

The tomato plant (*Solanum lycopersicum* L.) is native to the Andean Region in western South America, and occurs naturally in Bolivia, Colombia, Ecuador, and Peru. The domestication of *S. lycopersicum*, however, took place in Mesoamerica, in a region that now encompasses the territory of Mexico, through the selection of phenotypes of interest by pre-Columbian cultures, similarly to other native crops of this region such as maize, potato, and peppers(Bergougnoux, 2014; Paran & Van Der Knaap, 2007). During the 16th century, with the Spanish colonization of the Americas, the crop spread across Europe and other regions of the world through maritime trade routes (Mccue, 1952; Rick, 1978). *S. lycopersicum* then began to be cultivated worldwide, being consumed fresh or used in sauces, proving to be a highly versatile food item (Nicola et al., 2009). The popularity of tomato goes beyond its gastronomic use, as there is a substantial body of research linking its consumption to several health benefits. The mature fruit is rich in antioxidants such as lycopene, carotenoids, and flavonoids, and the incorporation of this solanacea into the diet can reduce the risk of certain types of cancer, prevent arteriosclerosis and delay the formation of cataracts (Frusciante et al., 2007; Kim et al., 2003; Weisburger, 1998).

The primary tomato producers are situated in Asia, notably China, which produced 67 million tons of fruits in 2021, accounting for one-third of the global tomato production. Concerning its original region, only two South American nations, Mexico and Brazil, stand among the leading producers, both yielding approximately 4 million tons (FAOSTAT, 2021). In Brazil, this production spans 51 thousand hectares, primarily concentrated in São Paulo, Goiás, and Minas Gerais (Dossa & Fuchs, 2017).

Positive aspects related to tomato consumption are counterbalanced by the intensive use of pesticides in phytosanitary management due to the wide range of pathogens affecting the crop, presenting a risk both to rural workers due to exposure to toxic products and to consumers due to the presence of chemical residues in the food (Martins et al., 2008; Reis-Filho et al., 2009). Among these are fungi, bacteria, and nematodes, not to mention diseases caused by viruses and phytoplasmas, which occasionally require the application of insecticides to control vectors and insect pests (Kurozawa & Pavan, 2005). In this context, the genus Meloidogyne, comprising the group of root-knot nematodes, stands out as one of the most harmful pathogens to this crop, especially in Brazil, where their presence is ubiquitous. The most commonly occurring species, *Meloidogyne incognita* and *M. javanica*, are polyphagous, capable of completing their cycle in a wide range of hosts, including various vegetables. This poses a problem for the practice of crop rotation with non-host species if the pathogen gets established in a cultivation area (Lopes, C. A.;

Ávila, 2005). Nematode-infected roots develop swellings, which turns into galls where specialized nursing cells act as a source of nourishment for the pathogen, impacting the plant's ability to uptake water and nutrients, consequently limiting its growth and potential yield(Abad et al., 2009).

Chemical nematicides have been used in various crops, and the most used ones are biocides of high toxicity to the environment and humans (Ragoucy-Sengler et al., 2000; Sánchez-Moreno et al., 2009). Given the high effectiveness of these products, the improvement of other management techniques for nematode control, such as crop rotation, biological control, and fallowing, have not received much attention as control practises until recently (NOLING; BECKER, 1994; OKA et al., 2000), with the notable exception being the use of genetic control. Specifically, in the Meloidogyne x *S. lycopersicum* pathosystem, control is achieved by employing the major resistance (R) *Mi-1.2* gene, which confers multiple resistance to three species of the genus Meloidogyne: *M. incognita*, *M. javanica*, and M. arenaria (MILLIGAN et al., 1998).

The *Mi-1.2* gene, initially identified in Solanum peruvianum (PI 128657), was transferred to domesticated tomatoes through embryonic rescue, resulting in interspecific hybrids that gave rise to modern tomato varieties (Smith, 1944; Messegur et al., 1991). The *Mi-1.2* gene encodes a 1257-amino acid protein featuring NBS and LRR domains characteristic of the resistance gene class R. Notably, this protein lacks a signal peptide, suggesting its cytoplasmic localization (Williamson, 1999). Resistance is manifested as hypersensitivity in cells that would otherwise become feeding sites. Several days post-infection, the tissue surrounding the nematode undergoes necrosis, rendering it unsuitable as a nutrient source for the parasite. The sedentary phase of the nematode subsequently shares the fate of the surrounding cells (Chen et al., 2006).

Interestingly, despite the asexual mode of reproduction, phenotypic variability of *M. incognita* and *M. javanica* is common and has been reported since the mid-20th century (Martin, 1954). At that time, to elucidate how different populations of *M. incognita* would behave in distinct host crops, a Host Differential Test (HDT) was developed, which classifies populations of this nematode into four distinct races, each with different patterns of virulence according to the host (Sasser et al., 1983; Taylor & Sasser, 1978). This differential test, created at the University of North Carolina, aimed to guide breeding programs and provide more accurate management guidance to cotton, peanut, pepper, and tobacco producers, which are crops of importance in the region where the test was developed. The differential test was adopted in other countries as well as the nomenclature of races for *M. incognita*. Recent studies on the genome of this pathogen, however, have been shedding light on the plastiticy of its interaction with several hosts, despite the lack of changes in the *M. incognita* genome. The fact that these phenotypical

changes in relation to pre-stablished hosts (HDT) are not monophyletic and share no common ancestry, has led Koutsovoulos et al. (2018) to suggest the substitution of the termilogy race in favor of "pathotype" or "biotype".

The selection of virulent populations of *M. incognita* for certain resistance genes has been reported for decades (Jarquin-Barberena et al., 1991). Castagnone-Sereno et al. (1993))observed *M. incognita* isolates virulent to *Mi-1.2* andthe isolate maintained its virulence even after being multiplied for 18 generations in a tomato genotype not carrying the *Mi-1.2* gene, that is, without directional selection pressure, thus indicating that virulence towards this R gene is stable suggesting that this trait is not under Mendelian control, (R. Castagnone-Sereno et al., 1994). This hypothesis is supported by recent comparative genomics studies, in which the source of phenotypic variation could not be traced to changes in the sequence of genes of the nematode (Blanc-Mathieu et al., 2017; Castagnone-Sereno et al., 2019; Koutsovoulos et al., 2018; Szitenberg et al., n.d.). Virulent populations of *M. incognita* and *M. javanica* virulent to *Mi-1.2* are reported also at the field level after successive cultivation of resistant genotypes (Ornat & Verdejo-Lucas, 2001; Tzortzakakis et al., 2005). The identification of these virulent populations is sometimes hindered by the temperature-sensitive nature of the resistance coferred by *Mi-1.2*, which is inactivated above 28°C. Thus, a breakdown in resistance by *M. incognita* or *M. javanica* can be mistaken by a resistance breakdown due to high temperatures (Pinheiro et al., 2014).

In the absence of sexually derived genetic mechanisms that could account for the emergence of virulent strains, other mechanisms have been proposed to contribute to the adaptive capacity of Meloidogyne spp.(Goldberg et al., 2007), including changes in the number of copies of genes related to virulence, which can result in the loss or acquisition of virulence in regards to a specific host (Bekal et al., 2015; Castagnone-Sereno et al., 2019). In their 2019 study, Castagnone-Sereno et al. inoculated separately two isolates of *M. incognita*, one from Mexico and another from Russia on a tomato cultivar with the *Mi-1.2* gene and observed a significant decrease in the number of copies of multiple genes in both isolates across 120 generations. Despite originating from different places, this concomitant reduction was linked to the emergence of virulent phenotypes against the R gene. However, our understanding of how these changes contribute to Meloidogyne spp. adaptation to resistant hosts is still in its early stages, with research so far limited to *M. incognita.*

The sequencing of the tomato genome in 2012 by The Tomato Genome Consortium (2012) allowed deep insights into the intricate relationship between *S. lycopersicum* and a diverse array of plant pathogens, enabling the identification of key genes involved in the plant's defense mechanisms against diseases. Moreover, it also helped to unravel the diverse molecular

mechanisms governing the plant's tolerance to several biotic stresses, significantly advancing our understanding of these complex interactions (Adhikari et al., 2020; Chitwood-Brown et al., 2021; Seong et al., 2020; The Tomato Genome Consortium, 2012). These advances include, at this moment, two RNAseq studies, a transcriptional analysis of a *M. incognita* isolate able to overcome the *Mi-1.2* resistance (Guan et al., 2018), and full transcriptome analysis of the interaction of *S. lycopersicum* with *M. incognita* over the nematode life cycle (Shukla et al., 2018). As of january/2024 no RNAseq transcriptome of the interaction between *S. lycopersicum* x *M. javanica* has been published.

While studies in molecular genetics have aided to understand gene expression patterns in response to Meloidogyne spp. attack, , basic information is still needed to translate this information to applied research. Therefore, studies associating crop damage and productivity losses with information derived from molecular genetics studies should be conducted. Thus, this study aimed to 1) phenotypically characterize the interaction between virulent and avirulent isolates of *M. incognita* and *M. javanica* and tomato lines with of without the *Mi-1.2* gene; 2) estimate the negative effects of virulent and avirulent *M. javanica* colonization on a resistant genotype, in regard to growth and yield. 3) compare the patterns of gene expression during the initial phases of the interaction between virulent and avirulent *M. javanica* isolates (Vir and Avir) and an *Mi-1.2* carrying tomato variety by RNA sequencing (RNAseq). Following steps 1 and 2 we were able to identify a new *M. javanica* isolate and assesses that its negative impact on yield of resistant and susceptible tomatoes are of a similar magnitute, despite lower reproduction rates of the virulent isolate. While step 3 enabled us to shed light on key genes underlying the evasion of the HR response elicited by *Mi-1.2* gene when invaded by the virulent isolate, including a highly differentially up-expressed uncharacterized effector secreted in this compatible interaction.

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2. THE IMPACT ON TOMATO YIELD: INSIGHTS INTO THE INTERACTIONS BETWEEN RESISTANT, *MI-1.2* BEARING AND SUSCEPTIBLE GENOTYPES

Abstract

The *Mi-1.2* resistance gene is used to control the root-gall nematodes *Meloidogyne incognita* and *M. javanica*. However, the efficacy is limited as virulent populations of both species are often selected, leading to an increase in their population over several cycles in infested areas. We investigated the interactions of the susceptible tomato "Santa Clara" (S) and the resistant "Santy" (R) with a candidate *M. javanica* population for virulence against *Mi-1.2* gene (MjVi) comparing it to a *M. javanica* population unable to parasitize resistant plants (MjAv). Additionally, four *M. incognita* isolates were included, two known as virulent (MiVi1/MiVi2) and two avirulent (MiAv1/MiVi2). This stage encompasses the virulence phenotyping experiments (VPE1/VPE2). Cups of 500 cm³ were filled with steam-tread soil. For the inoculum, infected root tissue was processed in a blender and the aqueous suspension was sifted through 60 and 500 mesh sieves. Population densities were estimated by counting of the nematodes. The inoculum was introduced in the form of 1,500 juveniles+eggs, and the plants were transferred to a greenhouse. Nematode populations were determined 60 days after inoculation to obtain the reproduction factor (RF). The experiment consisted of 12 treatments (6 isolates x 2 hosts) in a completely randomized design. All isolates with an expected compatible interaction were able to reproduce on the R genotype. The highest RFs were observed in the MjAv-S interaction, with a RF of 303.78 and 98.46 for VPE1/2. In both experiments, the MjVi-R interaction resulted in FR's higher (FR: 29.87/26.91) than its susceptible contrast MjVi-S (FR: 5.78/16.01). The isolates (MjVi/MjAv) were included in the next study which had as an objective the assessment of the infection impact on tomato productivity, named Yield experiments (YE1/YE2). The installments resulted in 8 treatments (2 isolates x 2 hosts x 2 populational densities-d1/d2) and 2 additional non-inoculated controls were added for each host (Ctrl.R/Ctrl.S) totaling 10 treatments. Plants were grown in 7L pots with soil pre-infested using infected roots to reach the respective densities. The evaluations for YE included several parameters, mean fruit mass (MFM) for each plant and the number of nematodes per gram of root (NPGR). The lowest NPGR counts were observed in the Vi.R.d1 treatment, with means of 5,489 and 9,048 for YE1 and YE2, being consistently the treatment with the lowest populational densities. The Vi.S.d2 and Av.S.d2 treatments were significantly different from the Vi.R.d1, Vi.R.d2 and Av.S.d1 treatments for both experiments. These later three treatments presented the lowest final populations (FP) means. Ctrl.R was also consistently the treatment with higher MFM means, 83.5 and 90g for YE1/2 respectively. On the opposite spectrum, the Vi.S.d2 treatment showed the lowest means in both experiments, 30.03 and 28.65g per fruit on average. The Vi.S.d1 and Vi.S.d2 treatments exhibited no significant difference regarding both NPGR and FP, despite the latter showing an overall higher population. The current work presents a scenario in which an initial population of 6 eggs+juveniles *of M. javanica* per cm³ is sufficient to cause yield reductions in the *Mi-1.2* bearing host in the case of the virulent isolate.

Keywords: *Meloidogyne incognita*, *Meloidogyne javanica*, Plant resistance, tomato, yield reduction.

2.1. Introduction

As one of the most economically important species of the Solanaceae family, Tomato [*Solanum lycopersicum* (L.)], is the second most consumed vegetable crop in the world, second only to potato, with a world production of over 18.6 $x10^7$ t harvested in an area of $5x10^6$ ha as of 2020. The bulk of this production has its origin in Asia, being led by China, which is responsible for nearly one third of the total tomato world production (FAO, 2022; Padmanabhan et al., 2015). Despite being originated in the South and Central America, only two countries of these regions figure among the major world producers, Mexico and Brazil, both featuring a production of approximately 4x106t (Derli Dossa & Fuchs, n.d.; FAO, 2022).

Like other vegetable crops, tomato is host to a wide range of nematodes, including root-knot-nematodes (RKNs) of the Meloidogyne genus, a group of sedentary endoparasites able to colonize and reshape the root system (Hallmann & Meressa, 2018; Trudgill, 1997). Over 90 Meloidogyne species have been described so far, among those *M. incognita*, *M. javanica*, and *M. arenaria* pose a major threat to agricultural systems, accounting for 90% of all productivity losses caused by RKNs worldwide (Castagnone-Sereno et al., 1994). Accurate prediction of yield reductions, however, is difficult as tomato cultivars have different degrees of susceptibility and distinct Meloidogyne populations of the same species might show varying degrees of pathogenicity. (Seid et al., 2015)

To this date, successful management of RKN infestations in tomato growing areas has been achieved by using resistant genotypes carrying the major R gene *Mi-1.2*, which confers resistance to the three mentioned Meloidogyne species (Vos et al., 1998). This gene was transferred from the wild species *L. peruvianum* to domesticated tomato varieties via embryo rescue, resulting in a hybrid used as the source of this resistance gene for modern commercial varieties. (Messeguer et al., 1991; Milligan et al., 1998; Smith, 1944).

Despite of reproducing exclusively by means of mitotic parthenogenesis, *M. incognita* and *M. javanica* populations able to overcome the *Mi-1.2* resistance at the field level appear with frequency (P. Castagnone-Sereno, 2002; Hajihassani et al., 2022; Ornat & Verdejo-Lucas, 2001). In Brazil, until recently, only populations of *M. incognita* have been reported to be virulent on tomatoes with *Mi-1.2* (Silva et al., 2019), virulence here being defined as the capability of nematode juveniles to parasitize and reproduce on resistance host. However, seed producers have

recently reported the occurrence of virulent *M. javanica* populations as well and as of 2022, a *M. javanica* population able to reproduce on the resistant rootstock "Guardian" was reported (Gabriel et al., 2022).

Given the importance of RKNs as tomato pathogens and the absence of a detailed study on how virulent *M. javanica* isolates might impact this crop, the goal of this work was to characterize a virulent *M. javanica* population collected in the municipality of Bragança Paulista-SP, in contrast to avirulent population from Londrina-PR. And thus, to evaluate the effects of these distinct *M. javanica* isolates and their initial populations on susceptible and resistant tomato varieties.

2.2. Materials and Methods

2.2.1. Plant and nematode material

The commercial tomato cultivars "Santa Clara" (susceptible, without Mi 1.2) and "Santy" (resistant, with Mi 1.2) were used in the experiments.

Six Meloidogyne spp. isolates were used (Table 1). All isolates were originally obtained from distinct host crops and locations. Including 4 *M. incognita* isolates and 2 *M. javanica* isolates, their expected *Mi-1.2* interaction was based on field observation. All isolates were used in the virulence phenotyping experiments (VPE), while only the *M. javanica* isolates were used in the yield experiments (YE). Henceforth, all isolates of Meloidogyne will be classified as either virulent (Vi) or avirulent (Av) based on their abilityto reproduce on "Santy".

Table 1: List of isolates tested for compatibility with *Mi-1.2* bearing hosts.

Isolate	Species	Expected *Mi-1.2* interaction	Rearing culture	Origin
MjAv	*M. javanica*	Incompatible	Tomato	Londrina – PR
MjVi	*M. javanica*	Compatible	*Mi-1.2* bearing tomato	Bragança Paulista -SP
MiAv1	*M. incognita*	Incompatible	Tomato	Campo Verde - MT
MiAv2	*M. incognita*	Incompatible	Sugarcane	Campo Verde - MT
MiVi1	*M. incognita*	Compatible	*Mi-1.2* bearing tomato	Joanópolis – SP
MiVi2	*M. incognita*	Compatible	*Mi-1.2* bearing tomato	Caxias do Sul - RS

2.2.2. Virulence Phenotyping Experiments (VPE)

Greenhouse experiments were conducted during 2020 - 2022 to evaluate the reproduction of each isolate of *M. incognita* and *M. javanica* on Santy and Santa Clara. Inocula were obtained from roots of their respective rearing cultures routinely maintained in the greenhouse (Table 1) where plants are grown on a sandy-loam soil and are renewed every two months to maintain a high nematode population. Root tissue was collected and processed in a blender for 60s in 0.2% hypochlorite solution. The aqueous suspension was sifted through 60 and 500 mesh sieves in order to separate the females, juveniles and eggs from the larger pieces of root tissue. The filtered solution was centrifuged at 550 g for 5 minutes (Coolen and D'Herde, 1972). Population densities were estimated by counting the number of nematodes with the aid of a Peter's slide under a light microscope (Olympus CH2) 100x magnification.

Tomato seeds were sow in planting trays and transferred to plastic cups 10 days after emergence (DAE). Plastic cups had a volume of 500 cm3 (13.5 cm deep × 9 cm diam.) and were filled with steam-treated sandy-loam soil (121°C for 2 h). An initial population (Pi) comprised of 1,500 specimens of the respective isolates were evenly inoculated into two oblique holes of 2 and 4 cm flanking the plant in a 180º angle from each other, these holes were made approximately 1.5 cm away from the stem. Inoculation took place five days after transplant. The aqueous suspension containing the infective individuals for each plot was calibrated to a maximum of 2 mL. Nematode densities were determined 60 days after the inoculation (P60) by extracting nematodes from the roots using the method described above. And counting the Nematodes under a light microscope at 100× magnification with the aid of a Peters slide .

The experiment comprised 12 treatments representing the interaction of each isolate and the two tomato genotypes (6 isolates x 2 hosts) arranged in a completely randomized design with eight biological replicates, each replicate consisting of a single plant cultivated in a 500ml plastic cup as previously described. For naming purposes, each treatment was labeled as an abbreviation of the isolate identification (Table 1) followed by R or S, respectively for the "Santy" and "Santa Clara" tomato plants. This experiment was replicated once (VPE2) under the same conditions.

2.2.3. Yield experiments (YE)

Yield experiments were conducted to evaluate the effects of different densities of two *M. javanica* isolates on the yield of two tomato varieties the susceptible Santa Clara and the *Mi-1.2* bearing Santy.

Experimental design: The YE treatments comprised the contrasts between: 1) the isolates of *M. javanica* regarding virulence (Av/Vi) for avirulence and virulence, 2) host resistance (S/R) for susceptibility and resistance, and 3) populational density. In addition to the contrasting hosts and isolates variable, this third parameter was added to address the implications of distinct *M. javanica* population densities on yield components. Two populational densities were established: one (D1) with 3 specimens (eggs or juveniles) and a second (D2) with 6 specimens per cm^3 of soil, respectively.

The combination of these factors resulted in 8 treatments (2 isolates x 2 hosts x 2 densities) and 2 additional non-inoculated controls were added for each host (R/S) totaling 10 treatments. Biological replicates consisted of a single tomato plant transplanted to 7L pots as explained further in this section, a total of 6 biological replicates was established for both installments of the YE experiments (YE1/YE2). The experiments were conducted in a randomized block design.

Prior to transplanting plants were cultivated for 4 weeks in a growth chamber to ensure a more controlled environment. Tomato seeds were sown in $300cc^3$ plastic cups filled with steam treated soil, and the plants were inoculated 2 weeks after emergence following the same variables as the VPE with either 900 or 1800 juveniles and eggs to match the D1 and D2 densities. Plants were kept for 2 weeks in a growth chamber set to a 12/12h (day/night) photoperiod at a temperature of 24ºC and then were transferred to their final 7 liters plastic pots in the greenhouse filled with 6.5L of steam-treated sandy-loam soil (121°C for 2 h) in which a single "Santa Clara" plant was previously cultivated for 93 days to biologically condition the soil after steaming. Before transplanting these conditioning plants were removed, the pots were then emptied and the soil was homogenized prior to refilling.

Inoculum preparation and inoculation: For YE1 inoculum was prepared utilizing infected roots of each isolate. These roots were cut into 1cm long segments and homogenized. Four 5 g samples of each set of roots (AV and VI root systems) were processed to determine the average number of specimens per gram of root, following the extraction methodology described in the VPE section. These roots were manually incorporated into the soil of their respective treatments. Densities per gram of root were of 6,398 and 4,673 (J2 and eggs) for Av and Vi, respectively. To achieve the desired densities, d1/d2 treatments belonging to the Av received 3.05/6.10g of

infected roots, while its Vi counterparts received 4.17/8.34g . By the end of the YE1 experiment, the whole root system was removed for nematological evaluations, as detailed further in this section. After the removal of the roots, plots were separated by isolate (Av/Vi), then emptied, and homogenized separately. Once homogenized, four 200cc soil samples were taken from each contaminated soil (Av/Vi), quantification of individuals and eggs in these soil samples were conducted to assess the population densities in the homogenized soils with the objective of calibrating the initial densities of YE2 partially using the same substrate. This was performed 4 weeks after the end of the YE1. Mean densities of nematodes/cm3 were 9 for the virulent isolate and 7 for the avirulent one. An extra volume of steam-treated soil was added to each treatment as a way of reestablishing the D1 and D2 used in YE1.

Plant cultivation and nematode assessment: Plants were supported by bamboo stakes and had their side-shoots systematically pruned to maintain a single stem until they reached approximately 1.80m in height and bore 3 flowering branches, when the terminal bud was cut-off. Fertilization consisted of a pre-sowing addition of 10 g of a 4–14-8 NPK fertilizer (0.4 g N, 1.4 g P2O5, and 0.8 g K2O) per pot, additionally calcium nitrate was applied every 2 weeks after the first flowering, at a rate of 8 g per pot (30 mg Ca^{2+} and 19 mg $2NO^{-}{}_{3}$). Plants were watered daily and whiteflies were controlled by weekly applications of insecticides of the neonicotinoid and pyrethroid groups (Provado® 200sc/Karate Zeon® 50cs) in rotation.

Growth measurements were taken weekly until plants reached the maximum height, approximately by the tenth week, and the emission of floral branches annotated daily. Harvest was done continuously starting at 15-16th week and going for nearly 6 weeks when all tomatoes were removed. Tomatoes were harvested at fruit maturity and immediately weighed. Plants were cut off for weighting of their aerial parts(-fruits) and the roots were removed for nematological analysis and processing at 161 and 148 after emergence for YE1 and YE2, respectively. Fresh roots were weighted (FRW), while the aerial parts were dried for 72 hours at 60 ºC in a dry oven to reach a constant mass for the measurement of the aerial parts dry mass (APDM). The parameters used for fruit measurement were the total fruit mass (TFM), the total fruit count (TFC) and the mean fruit mass (MFM) for each plant. Final nematode densities (FP) as and the number of nematodes per gram of root (NPGR) were determined after FRW annotation through the extraction of nematodes from roots using the method described above.

2.2.4. Statistical analyses

The variables of the VPE and YE were subjected to an analysis of variance (ANOVA), and the treatment means were compared using the Tukey Honestly Significant Difference test (HSD), performed on the R software package (R: The R Project for Statistical Comp., 2017).

2.3. Results

Soil temperatures during the experiments were recorded by a digital thermometer and maximum and minimum temperature ranges were annotated daily (Table 2).

Table 2: Maximum, mean and minimum soil temperature throughout the experiments carried out in a greenhouse.

	Temperature	Experimental period
VPE1	Max: 36.7 ºC; Mean:26,7 ºC ;Min:21.3 ºC	Dez~Feb (2019-2020)
VPE2	Max:32.1 ºC; Mean:21,4 ºC; Min:12.8 ºC	Apr~Jun (2020)
YE1	Max: 31 ºC; Mean: 23.4 ºC; Min:16.1 ºC	Mar~Sep (2021)
YE2	Max: 35.2 ºC; Mean: 28.3; Min: 21.6ºC	Sep~Feb (2021-2022)

2.3.1. Virulence Phenotyping Experiments (VPE)

A heterogeneity of means test was conducted in a multifactorial manner to evaluate the interaction of all treatments between VPE1 and 2 in regard to its respective variables (RF/GI). Significant interactions were observed between the same treatments/variables across the two experiments; therefore data for VPE1 and 2 are presented separately . Mean reproduction factors and gall indexes differed between experiments, being lower in VPE2 possibly influenced by the lower temperatures (Tables 2 and 3).

All nematode isolates virulent on *Mi-1.2*, regardless of their rearing species, parasitized the resistant cultivar "Santy". The gall index (GI) values exhibited insufficient reliability as predictive indicators of the RF across various interactions, encompassing host dynamics, nematode species, and the Av and Vi isolates. In both experiments the avirulent *M. incognita* isolates MiAv1 and MiAv2 were unable to reproduce on the resistant Santy, but the *M. javanica* MjAv did so with RF's of 0.24 and 3.31 respectively (Table 3).

Across both experiments, the MiAv2-S interaction consistently displayed the highest gall indexes, registering values of 4.37 and 4.28 for VPE 1 and 2, respectively. However, it only

differed statistically from its avirulent *M. incognita* counterparts in the VPE2. These values placed MiAv2-S within the same statistical group as MiAv1-S for the RF, where MiAv1-S demonstrated gall index values of 2.75 and 2.7 for VPE 1 and 2, respectively. These varying ratios between GI and RF are even more exacerbated when comparing *M. incognita* and *M. javanica* , while on VPE 1 and 2 the MjAv-S interaction presented a RF of 303.78 and 98.46, and MiAv1-S a RF of 150.13 and 34.38, their respective gall indexes were of 3.25 and 3.14 for the MjAv-S and 4.37 and 4.28 for the MiAv2-S, as previously mentioned.

Isolates MiAv 1 and 2 reproduced on the susceptible hosts, as expected, but no statistical significance was found between their respective RF means in both experiments.

The highest reproduction rates were observed in the MjAv-S interaction, this treatment was consistently ranked in the group with the fastest populational increase in the 60-day span of the VPE1 and 2 experiments, with a RF of 303.78 and 98.46 respectively. Regarding the MjVi isolate, in both experiments the MjVi-R interaction resulted in FR's higher than its susceptible contrast, MjVi-S. MjVi-R presented a FR of 29.87 and 26.91, while the same parameter for MjVi-S was 5.78 and 16.01 for VPE1 and 2 respectively.

The MiVi 1 and 2 isolates showed discrepancy between the results in VPE 1 and 2, in both host interactions. Overall, the two isolates thrived in VPE1 being placed in the same THS group as the treatment MjAv-S, which showed the highest FR for VPE1. While in VPE2 the four treatments, MiVi1-S, MiVi1-R, MiVi2-S and MiVi2-R were set apart only from the incompatible interactions observed in MiAv1R and MiAv2-R treatments, being placed in the lowest reproduction group in the THS test (Table 3).

Table 3. Reproduction factor (RF) is here expressed by the ratio of final population over the initial inoculum. Followed by columns containing the mean gall index (GI) for each treatment.

	VPE 1		VPE 2	
Treatments	**RF**	**GI**	**RF**	**GI**
MjAv-S	303.7 ± 48.4 a[y]	3.2	98.4 ± 16.24 a	3.14
MiVi1-S	266.1 ± 38.3 a	3.5	4.3 ± 0.7 cd	3.4
MiVi2-R	176.2 ± 26.7 ab	3.7	2.0 ± 0.3 d	1.5
MiVi1-R	165.8 ± 30.3 ab	3.7	8.12± 0.9 cd	3.7
MiVi2-S	159.1 ± 21.5 ab	3.8	0.9 ± 0.2 d	1.4
MiAv2-S	150.1 ± 19.1 ab	4.4	34.4 ± 5.7 b	4.2
MiAv1-S	109.1 ± 14.4 bc	2.7	36.7 ± 5.1 b	2.7
MjVi-R	29.8 ± 5.2 c	1.3	26.9 ± 4.9 b	4.7
MjVi-S	5.7 ± 0.1 d	1.9	16.0 ± 3.1 c	3.7
MjAv-R	0.2 ± 0 .1 d	0.1	3.3 ± 0.8 d	3.3
MiAv1-R	0 [x]	0	0	0
MiAv2-R	0	0	0	0

y Means followed by the standard error of six replicates for each treatment. Means within a column followed by the same letter are not significantly different at P= 0.05.
x Means for the treatments exhibiting null reproduction factors were excluded from the statistical comparisons.

2.3.2. Yield experiments (YE)

Soil temperatures were on average 4.9º C higher during YE2 which was conducted between fall and summer (Table 2). These distinct temperatures likely contributed to a slightly longer experimental cycle during YE1 which spanned 161 days, 13 days longer than YE2.

2.3.2.1. Fresh Root Weight

No differences were detected among treatments for FRW in YE1 while in YE2 treatments Ctrl.R and Ctrl.S presented the higher values (Table 3). In contrast to the first experiment, the FRW for YE2 exhibited significant variations across all treatments. Noticeably, a gap between the non-inoculated resistant control (Ctrl.R) with the higher means of FRW and the Av.S.d2 interaction can be observed in Table 4, with these treatments exhibiting a per plant FRW of 115.6 and 35g respectively. Plants from the Av.S.d2 interactions presented severely damaged roots when compared to its respective control, Ctrl.S, as can be seen in Figure 1. The statistical grouping with the lowest root mass means had Av.S.d2 as its single member pertaining only to this group, with a mean mass of 35.01g. This group, however, had treatments with mean FRW

that went as high as 68.4g for Vi.S.d2, which was also place in the forementioned upper intermediate group, together with Av.R.d1 and Av.R.d2, with mass means of 67.42g and 60.65g, respectively as shown in table 4. This illustrates the high variance of this parameter, highly influenced by gall formation and senescence of older root systems.

Figure 1. Root systems of the susceptible control in constrast with its counterpart innoculated with the highest density of the avirulent isolate.

2.3.2.1. Nematodes per gram of root

For YE1 the NPGR means followed a similar pattern to FP means previously addressed, this is an expected result given that FRW did not differ statistically between the treatments in the first experiment. For YE2, however, the broad range of FRW means resulted in some differences between FP and NPGR for some of the treatments, namely Av.S.d2, which was placed in the same statistical group as Vi.S.d1 and Vi.S.d2, and was accounted for the highest NPGR numbers, reaching means of 43873 and 46033 eggs and juveniles for YE1 and YE2 respectively (Table 4).

The lowest NPGR counts were observed in the Vi.R.d1 treatment, with means of 5489 and 9048 for YE1 and YE2, being consistently the treatment with the lowest populational densities for both FP and NPGR.

2.3.2.2. Final nematode population

Repeating the pattern observed during the VPE experiments, the Av.S interaction was again placed in the Tukey HSD group with higher populational density means, except for FP during YE2. A notorious difference from the VPE is the behavior of the Vi.S interaction, which was not statistically differently from the Av.S regarding NPGR in both experiments and presented a higher FP during YE2.

As expected, no nematodes were detected in the non-inoculated controls and in the Av.R treatments inoculated with either D1 and D2 nematode population densities. At the end of YE1 the Av.S.d2 treatment showed the highest FP numbers, with a mean of $7.7x10^5$ specimens showing significant difference ($P \leq 0.05$) in comparison with the intermediate group treatments Vi.S.d1 and Vi.S.d2 ($3.2x10^5$ and $4.9x10^5$, respectively). For YE2, however, a trend inversion could be observed, with Av.S.d2 being, again, set apart from Vi.S.d1 and Vi.S.d2, but showing lower FP means ($1.5x10^5$) than the Vi.S treatments, in both densities (2.4x10 and 2.8x10, respectively for d1 and d2) as seen in table 4.

Vi.S.d2 and Av.S.d2 however were consistently significantly different ($P \leq 0.05$) from the Vi.R.d1, Vi.R.d2 and Av.S.d1 treatments for both experiments. These later three treatments presented the lowest FP means and were not significantly different ($P \leq 0.05$) during the YE1 and YE2 evaluations.

Treatments Av.S.d1, Vi.R.d1 and Vi.R.d2 were placed consistently in the statistical group which comprised the lowest FP, and even showed similar means in both experiments (Table 4).

Table 4: Quantification of the nematode infestation. Fresh root weight in grams (FRW), number of juveniles and eggs of *M. javanica* expressed in nematodes per gram of root (NPGR) and total final population (NPGRxFRW=FP).

Treatments	YE1			YE2		
	FRW (g)	**NPGR**	**FP**	**FRW (g)**	**NPGR**	**FP**
Ctrl.R	88.7 ± 3.9	0	0	115.68 ± 14.8 a	0	0
Ctrl.S	82.9 ± 3.8	0	0	102.32 ± 9.6 ab	0	0
Av.R.d1	62.8 ± 7.6	0	0	67.42 ± 3.2 bcde	0	0
Av.R.d2	80.1 ± 4.8	0	0	60.65 ± 7.2 bcde	0	0
Av.S.d1	76.8 ± 9	17841 ± 2501 cd	$2.6x10^6$ ± 127k cd	44.38 ± 6.5 de	27327 ± 1728 b	$1.2x10^6$ ± 140k c
Vi.R.d1	85.5 ± 4.6	5489 ± 1093 e	$8.7x10^5$ ± 67k d	75.51 ± 5.6 bcd	9048 ± 477 d	$6.8x10^5$ ± 70k c
Vi.R.d2	80.1 ± 2.6	9933 ± 1542 de	$1.6x10^6$ ± 32k cd	84.7 ± 4.3 abc	16150 ± 1281 c	$1.3x10^6$ ± 96k c
Av.S.d2	88.1 ± 7.3	43873 ± 4132 a	$7.7x10^6$ ± 87k a	35.01 ± 4.1 e	46033 ± 1916 a	$1.5x10^6$ ± 174k bc
Vi.S.d1	64.4 ± 9.8	25627 ± 2145 bc	$3.2x10^6$ ± 33k bc	60.20 ± 2.5 cde	41611 ± 6440 a	$2.4x10^6$ ± 377k ab
Vi.S.d2	73.6 ± 6.3	33666 ± 2916 ab	$4.9x10^6$ ± 48k b	68.4 ± 9.6 bcde	40633 ± 2183 a	$2.8x10^6$ ± 532k a

y Means followed by the standard error of six replicates for each treatment. Means within a column followed by the same letter do not differ (P= 0.05).

2.3.2.3. Aerial parts dry weight

The resistant and susceptible control were consistently placed in the statistical group that showed the highest APDW means for YE1 and YE2. Respectively with Ctrl.R presenting means of 78.1g and 123.3g and Ctrl.S 82.8g and 108.6g. The same can be observed for the Av.R.d1 interaction, with means of 71.8g and 112.6g corresponding to YE1 and 2 Av.R.d2 behaved similarly to its lower density counterpart for both YE1 and YE2, but was not included in the higher weight group in YE2 (Table 5).

Five treatments consistently presented the lowest APDW means for both YE1 and YE2, Av.S and Vi. S for both densities, and Vi.R.d2. Vi.R.d1 observations were contrasting from YE1 to 2, being placed apart from these groups in the YE1 analysis and set as an intermediate group. While in YE2 it was statistically grouped together with the other five previously mentioned treatments.

Table 5: Agronomical measurements in grams. Aerial Parts Dry Weight (APDW), Total Fruit Count (TFC), Mean Fruit Mass (MFC), Total Fruit Mass(TFM).

Treatments	YE1				YE2			
	APDW	**TFC**	**MFM**	**TFM**	**APDW**	**TFC**	**MFM**	**TFM**
Ctrl.R	78.1 ± 8.4 a	12.6 ± 0.6 abc	83,5 ± 8.3 a	1057 ± 27.2 a	122.3 ± 4.6 a	13.3 ± 0.3 ab	90 ± 4.7 a	1194 ± 45.1 a
Ctrl.S	82.8 ± 3.2 a	14.4 ± 0.3 ab	70.30 ± 3.2 b	1008 ± 39.2 a	108.6 ± 2.6 ab	15.6 ± 0.4 a	60.61 ± 2.1 b	946 ± 23.6 b
Av.R.d1	71.8 ± 5.9 abc	12.2 ± 0.4 abc	79.46 ± 5.9 ab	964 ± 44.1 a	112.6 ± 4.6 ab	13.16 ± 0.5 abc	66.86 ± 2.2 b	879 ± 40.4 b
Av.R.d2	73.6 ± 4.5 abc	12.5 ± 0.8 abc	74.72 ± 4.5 ab	929 ± 59.5 a	96.5 ± 5.7 bc	13.16 ± 0.7 abc	61.94 ± 2.6 b	811 ± 37.9 b
Av.S.d1	51.2 ± 3.7 d	14.7 ± 0.5 a	42.62 ± 3.7 cd	627 ± 37.3 b	56.1 ± 3.1 d	10.3 ± 0.6 cd	35.81 ± 2.2 c	367 ± 42.3 c
Vi.R.d1	60.2 ± 4.4 bcd	10.6 ± 0.7 c	49.27 ± 4.4 c	522 ± 31.8 bc	69.3 ± 3.4 d	13 ± 0.7 abc	38.11 ± 1.4 c	503 ± 20.8 cd
Vi.R.d2	44.1 ± 3.2 d	11.5 ± 0.7 c	42.52 ± 3.2 cd	458 ± 42.7 c	67.5 ± 5.9 d	11 ± 0.6 bcd	33.51 ± 2.1 c	352 ± 28.7 cd
Av.S.d2	49.8 ± 2.1 d	11.8 ± 0.8 abc	38.47 ± 2.0 cd	454 ± 50.4 c	61 ± 5.7 d	9.8 ± 1.1 d	29.14 ± 2.0 c	287 ± 31.4 d
Vi.S.d1	57.1 ± 6.7 cd	11.5 ± 0.7 bc	38.94 ± 6.7cd	446 ± 28.1 c	76. 3 ± 3.1 cd	9.5 ± 0.6 d	33.83 ± 2.2 c	317 ± 42.3 d
Vi.S.d2	49 ± 3.4 d	13 ± 0.5 abc	32.03 ± 3.4 d	415 ± 35.9 c	75 ± 4.9 cd	9 ± 0.3 d	28.65 ± 1.7 c	256 ± 13.8 d

y Means followed by the standard error of six replicates for each treatment. Means within a column followed by the same letter are not significantly different at P= 0.05.

2.3.2.4. Total fruit count

TFC means ranged from 10.6 to 14.7 fruits in YE1 (Vi.R.d1 and Av.S.d1) and 9 to 15.6 fruits in YE2 (Vi.S.d2 and Ctrl.R). Total fruit count varied greatly in YE1 placing the control groups among inoculated plants often. For YE2 however, the incompatible interactions had an average fruit count mean higher than most nematode infested treatments, with both densities of Vi.S and Av.S.d2 presenting the lowest numbers of fruit overall (Table 5).

2.3.2.5. Mean fruit mass

In both installments of the YE experiments, the Ctrl.R, Ctrl.S, and Av.R interactions displayed significantly higher MFM means compared to all other treatments where compatible interactions were anticipated (Tables 5 and 2). These four treatments were placed in the same statistical group for YE1, while in YE2 Ctrl.R distanced itself from the other three. Ctrl.R was also consistently the treatment with higher MFM means, 83.5 and 90g for YE1 and 2 respectively. On the opposite spectrum, the Vi.S.d2 treatment showed the lowest means in both experiments, 30.03 and 28.65g per fruit on average. The response of the compatible interactions in regards to the MFM criteria was very consistent between experiments, with the exception of Vi.R.d1 which distanced itself from the Vi.S.d2 treatment in YE1 (Table 5).

2.3.2.6. Total fruit mass

Similarly to the MFM criteria, the TFM means were found to be higher in the incompatible interactions and control treatments by the end of both experiments, respectively Av.R and Ctrl. (R and S). Ctrl.R exhibited the highest masses in YE1 (1057g) and YE2 (1194g) as shown in Table 5. Populational densities in the Vi.S interaction did not affect TFM, with both treatments exhibiting the same trend across YE1 and 2, being placed along with the Av.S.d2 and Vi.R.d2 on the statistical group that displayed the lowest TFM means. The treatment with the lowest yield per plant was Vi.S.d2, with TFM means of 415 and 256g for YE1 and 2 respectively. Av.S.d1 was consistently placed in between the groups with the highest and lowest TFM means, with a TFM mean of 627 and 367g for YE1 and 2.

2.4. Discussion

2.4.1. Virulence Phenotyping Experiments (VPE)

The VPEs confirmed most of the expected interactions between isolates and hosts described in Table 1. As shown in Table 3, MiAv1 and MiAv2 reproduction factors after 60 days were null except for the avirulent *M. javanica* isolate which was able to reproduce in the presence of Mi 1.2 as observed in both experiments, whitlow reproduction factor (RF=0.24) in VPE1 characteristic of a resistant reaction, but much higher in VPE2 (RF= 3.31). This fact is noteworthy, considering the lower temperature during VPE2, which resulted in lower RF means for all treatments in comparison to VPE1. Some possible explanations for this unexpected higher RF mean of this treatment under lower temperatures are: 1) temperature peaks over 35ºC recorded during VPE1 impacted egg hatching and compromised the mobility of juveniles. 2) From the host's perspective, plant metabolic pathways can be adversely affected by colder environments (Heidarvand & Maali Amiri, 2010; Ouellet, 2007). Such metabolic changes under specific conditions might negatively impact the resistance conferred by the *Mi-1.2* gene, which relies on a rapid cell signaling cascade to trigger a hypersensitive response (Williamson & Kumar, 2006).

Across experiments the MjAv-S interaction presented the highest reproduction factor (303.78 and 98.46, respectively). While in VPE1, it didn't statistically differ from other interactions, in VPE2, it differed from all other treatments ($P<0.05$). The greater fecundity of *M. javanica* in comparison to *M. incognita* on tomatoes has been extensively documented in previous studies (Cortada et al., 2009; Wajid Khan & Haider, 1991). *M. javanicaM. incognita* However, an interesting discussion arises from the overall lower RF means for all *M. incognita* isolates on VPE2 when compared to the VPE1. While the basal temperature for minimal *M. javanica* development is higher than for *M. incognita*, 13 and 10 ºC respectively, the total of heat sums (sum of degrees/day) required for *M. javanica* to complete its cycle is 60ºC heat sums lower than for *M. incognita*, which requires a total of approximately 410ºC days (Cortada et al., 2009; Ploeg & Maris, 1999b). Considering that even during VPE2 minimal daily temperature means were 12.8 º C, very close to the basal threshold for *M. javanica*, and the average temperature for the duration of the experiment was of 21.4ºC (Table 1), it is possible that for the second experiment the *M. javanica* isolates could have spawned an additional generation over their *M. incognita* counterparts.

The MiAv2 treatment was added to the experiment to evaluate possible effects on the fitness of this population over several generations on a rearing host culture other than tomato , but no differences were noticed between MiAV2 and MiAV1, indicating no loss of fitness.

Regarding the *Mi-1.2* virulent isolates, *M. javanica* presented lower fecundity than its respective avirulent contrast on a susceptible host. MjVi-S presented an RF mean approximately 60x lower than MjAv-S by the end of VPE1 (3.78 and 303.78, respectively). This pattern was repeated on VPE2 with a smaller RF difference between isolates, this time only 6x than the avirulent counterpart, but were still significantly different (P>0.0.5) . This points to a possible lower fitness which was previously reported for *M. incognita* parasitizing *Mi-1.2* bearing hosts (Djian-Caporalino et al., 2011.).

2.4.2. Yield experiments (YE)

A thorough analysis of agronomical characteristics highlighted some aspects of the two *M. javanica* isolates under study. All variables were significant compared to the controls except for the fresh root weight in YE1.

The fresh weight of roots (FRW) parasitized by root gall nematodes can be a misleading variable if certain aspects of this relationship are neglected; in this type of association, for instance, a high root mass does not necessarily reflect a healthy plant. Structural changes promoted by the feeding sites of this pathogen leads to a growth in coarse root tissue and a decline in fine roots formation (Abad et al., 2009; Strajnar et al., 2012). Therefore, it is not unusual that non inoculated treatments weight nearly the same as infected roots by the end of an evaluation period (Jose´- et al., 2006), this phenomenon was observed in this study, as the Ctrl.S and Av.S.d2 presented similar values in YE1 (Table 4, Figure 1), which results from heavy galling in the presence *M. javanica*. It is worth stressing that despite its unreliability, fresh root weight is still a key interaction factor when analyzed taking into account the total nematode population in the root system, as this combination results in the variable nematode per gram of root , discussed further in this study. The lack of differences among treatments for FRW in YE1 but its significance in YE2 (Table 4) can be interpreted from an environmental scope. Similarly to the VPE, the YEs were subjected to different mean temperatures. YE1 took place under maximum mean temperature of 31 °C and an average of 23.4 ºC while in YE2, the maximum was 35.2 ºC and the average 28.3 ºC. Thus, for FRW the biotic stress caused by the formation of galls and the nematode feeding sites was exacerbated by the extended periods of high temperatures observed in YE2. As result of Meloidogyne parasitism, tissues adjacent to the feeding cells have their metabolism altered, undergoing hyperplasia and hypertrophy, which results in a disruption of the vascular cylinder, affecting the ability to absorb nutrients and water (Caillaud et al., 2008). This

assumption is further backed by casual visual observations of mild wilting during peak temperature hours affecting most inoculated treatments with exception of Av.R.d1 and Av.R.d2.

The density of nematodes did not affect FRW. However, in the incompatible interactions (Av.R) both densities presented a significant effect when compared to non-inoculated control (Table 4), presenting root systems substantially lighter, despite the absence of nematodes and galls by the end of the experiment. To better clarify the implications of this interaction, in which root growth was affected despite a null final population, two aspects should be discussed. First, during the previously discussed VPE experiments, we observed that the MjAvR interaction was not entirely incompatible, with the MjAv-R presenting FRs of 0.2 and 3.3 for VPE 1 and 2 respectively (Cortada et al., 2009). And secondly, the nature of the resistance conferred by the *Mi-1.2* gene, which does not prevent penetration of the nematode and is expressed only after the initial formation of the feeding cells (El-Sappah et al., 2019; Paulson & Webster, 1972). Perhaps this brief interaction is enough to impact on the vegetative development of the root (Balint-Kurti, 2019).

Unlike the VPE experiments, in the incompatible interactions of *M. javanica* (MjAv) did not reproduce during the course of both experiments (161 and 148 days for YE 1 and 2, respectively). It is reported that, once an avirulent population of *M. incognita* overcomes the resistance conferred by the *Mi-1.2* gene, this trait becomes stable, and the following generations will remain virulent during this specific interaction (Castagnone-Sereno et al., 1993). However, it is unlikely that the low, but noticeable, reproduction factors presented by the Mj.Av isolates in VPE 1 and 2 is the starting point of a selection for virulence in this population, especially considering that naturally occurring populations of *M. javanica* seem to be able to reproduce at similar degree to what was observed in this study when facing a resistant host (Devran et al., n.d.; Iberkleid et al., 2014). It is possible that contrary to the VPE experiments, in the YEs the populations declined and by the time of evaluation no new juveniles were able to develop additional feeding sites in the tomato roots, which would signal possible secondary cycles of the disease.

Regarding the initial nematode densities (d1/d2), the Av.S interactions presented an interesting interplay between nematodes per gram of root (NPGR) and final populations (FP). Whereas NPGR was not affected by the different densities in both YE1 and 2, FP was reduced in YE2, especially when plants were inoculated with the highest density. suggesting that under a moderate abiotic stress the higher initial density of this isolate has a lethal potential to the host over its cropping cycle (Seinhorst, 1970). Different from the compatible Av.S interactions, in the incompatible interactions nematode densities did not impact both NPGR and FP.

The YE confirmed the virulence to the *Mi-1.2* gene by the MjVi isolate observed during the VPE experiments. Overall the Mj.R compatible interactions presented the lowest reproductive numbers, which is in accordance with similar works (Iberkleid et al., 2014). Taking into consideration the FP and NPGR of this same isolate when parasitizing a host without the *Mi-1.2* gene (Vi.S.d 1 and 2), this could signal to a partial, rather than complete, resistance break event, or perhaps a loss of reproductivity fitness by the virulent isolate. This is expected, as the acquisition of the virulence trait to the *Mi-1.2* gene could lead to other phenotypical changes in the host x pathogen relations, despite the absence of the R gene (Djian-Caporalino et al., 2011; Hussey & Janssen, 2002).

Reflex symptoms are commonly the first observable sign of a nematode infestation and are often an indicative of potential yield losses. Here, the analysis of the dry weight of the aerial parts showed a positive correlation between nematode parasitism and reduction in vegetative growth as in both experiments the APDW of the controls and of the incompatible interactions were higher than in the compatible interactions (Table 4), with a few exceptions such as Vi.S.d1 and Vi..R.d1 being placed in an intermediate group together with the Av.R interactions in YE1.

Total fruit count (TFC) varied greatly among treatments, but was higher under the compatible interactions. Notably the Av.S.d1 treatment presented a mean number of fruits similar to its Ctrl.S counterpart during YE1. This is not entirely unexpected, as flower abortion on tomato correlates to water stress (Ganeva et al., 2019), which did not occur in YE1. Overall, for the YE2 experiment, TFC was lower on most of the compatible interactions. It is possible that the simultaneous biotic and abiotic stresses caused to the plant by the presence of the nematode and higher daily temperature could be responsible for the lower number of fruits in the heavily infested treatments. This resulted in treatments Vi.S.d1, Vi.S.d2 and Av.S.d2 presenting roughly 3-4 less fruits on average when compared to the incompatible interactions.

The absence of correlation between nematode densities and TFC during YE1 was not observed for mean fruit mass (MFM). Across both experiments nematode populations densities directly affected individual fruit mass. For some interactions such as Vi.S and Av.S.d2 the nematode infestation led to a 2 to 3-fold reduction when compared to their corresponding controls in YE1 and 2 respectively, this degree of fruit weight reduction has been observed in similar works (Gharabadiyan et al., 2013).

Total yield expressed as total fruit mass (TFM) was calculated by combining TFC and MFC. The TFM exhibited consistent patterns in both experiments, with a notable reduction in yield observed in all compatible interactions across both host varieties. It is noteworthy, however, that the Vi.R (d1/d2) interactions had similar yield reductions despite presenting a much lower

degree of nematode infestation. For instance, Vi.R.d2 presented a NPGR mean of 9993 and 16150 for YE1 and 2 respectively, being consistently placed in the same Tukey HSD test as Vi.S during both experiments, while these interactions presented a NPGR 2-3 times higher than Vi.R.d2. This could point to a lower populational threshold in regard to tomato yield reduction, in the case of this virulent isolate. Meaning that despite its lower reproduction it was able negatively affect tomato yield in the same manner as higher populations of avirulent isolate in the compatible interaction (Ferris et al., n.d.; Seinhorst, 1965). Overall, these findings provide valuable insights into the interaction between Meloidogyne spp. isolates and tomato hosts, highlighting the complex nature of plant-parasitic nematode interactions and the influence of environmental factors on the outcomes. It should be noted that while the characterization of this resistance-breaking phenomenon has been studied over the past decades, specially at the molecular level (Castagnone-Sereno et al., 2019; Noling, 2000; Williamson, 1998), most of these efforts focused on *M. incognita*. Despite their close phylogenetic relation, the virulence of *M. javanica* towards *Mi-1.2* showed differences when compared to *M. incognita* in the study here presented. In this sense, there is a lack of studies aiming to characterize the *Mi-1.2* x *M. javanica* pathosystem. This lack of data is specially important for growing regions in which *M. javanica* is the prevalent Meloidogyne species. In Brazil, for instance, this species is reported to occur in 50% of the tomato production fields, while *M. incognita* is present in 28% (Pinheiro et al. 2014).

The current work presents a scenario in which an initial population of 6 eggs/juveniles per cubic centimeter of soil is sufficient to cause yield reductions in the *Mi-1.2* bearing cultivar "Santy". The study of this agricultural threat is a good opportunity to highlight that overreliance on a single R gene might be catastrophic in the safeguard of its longevity. With the effectiveness of the *Mi-1.2* gene in mind, key aspects of an integrated disease management for this pathogen should be considered, such as crop rotation or fallow, the incorporation of organic soil amendments and the use of biological or chemical nematicides.

In conclusion, this study should be able to provide growers with valuable insight into this pathosystem and its economic implications by presenting predictions on potential yield losses under *M. javanica* parasitism on a resistant and a susceptible host, while also showing several production parameters and how they are affected by distinct isolates of this pathogen. Additionally, we hope that the data discussed here is able to provide potential guidelines for researchers interested in studying new ways to understand the erosion of the *Mi-1.2* mediated resistance by *M. javanica* and related species.

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3. TRANSCRIPTOME PROFILING OF TOMATO-*Meloidogyne javanica INTERACTIONS*: INSIGHTS OF THE RESISTANCE CONFERRED BY THE *MI-1.2* GENE AND ITS BREAKDOWN

Abstract

Root-knot nematodes, particularly *Meloidogyne incognita* and *M. javanica* present a great threat to tomato crops. Control is achieved by employing the major resistance *Mi-1.2* gene. In this study, we investigated the transcriptional responses of a resistant variety carrying *Mi-1.2* to an avirulent (avir) and a resistance-breaking (vir) isolates of *M. javanica* at 3 and 7 days after inoculation (DAI) with 1,000 second stage juveniles (J2). Histological analyses of roots were also performed. Penetration of roots was observed in both isolates at 3 DAI, and very few individuals of the avir isolate could be observed with no signal of gall induction at 7 DAI. In total, 6,402 and 2,394 genes were differentially expressed (DEGs) between the vir and avir interactions at 3 and 7 DAI, respectively. At 3 DAI up-regulated DEGs were predominantly observed in the incompatible interaction, with this trend reversing at 7 DAI, at which point more up-regulated DEGs were observed in the compatible interaction. Functional annotation showed an enrichment of resistance gene analogs at 3 DAI, including one gene homologous to *Mi-1.2* and another to the tomato spotted wilt resistance-5 (*Sw-5*) gene. At 3 DAI in the compatible interaction our analysis revealed the suppression of genes associated with pathogen perception and signaling suggesting interference with the plant immune system. Also, during the 3 DAI evaluation gene groups of the expansin and xyloglucan endotransglucosylase/hydrolase (XTH) families were diversely affected by nematode parasitism, with expansins up-regulation more pronounced in the compatible vir response, while XTH up-expression was more intense in the incompatible avir interaction. A total of 4 DEGs resulting in transcripts encoding cytokinin oxidases were upregulated in the compatible interaction at 3 DAI, increasing to 5 DEGs at 7 DAI. Calmodulins were upregulated in the incompatible interaction at both time points. Gene ontology (GO) analysis highlighted terms associated with key biological responses to biotic stresses, including "cell wall modification," "carbohydrate metabolic process," and "cytokinin dehydrogenase activity" all presenting DEGs up-expressed at 7 DAI in the compatible interaction. A candidate effector homolog of the *M. incognita* gene *Minc0482* involved in apoptosis suppression was detected as up-expressed in the virulent interaction both at 3 and 7 DAI. This first transcriptomic analysis of tomato responses to *M. javanica* provides novel insights into the mechanisms underlying disease development and *Mi-1.2* resistance breakdown.

Keywords: root-knot nematode; *Solanum lycopersicum*; defense response, RNAseq

3.1. Introduction

The genus *Meloidogyne* harbors plant parasitic nematodes commonly known as root-knot-nematodes (RKN) because its members share a similar mode of parasitism which relies on the formation of a specialized tissue within the host root called giant feeding cell. The successful establishment of this feeding site is essential for the development of these nematodes, as they provide nourishment through its life cycle for months depending on the species and climate conditions (P. Castagnone-Sereno et al., 2013; Hussey & Janssen, 2009). Unlike other biotrophic pathogens, certain species of *Meloidogyne*, such as *M. incognita* and *M. javanica* parasitize multiple crops of different plant families, which could be attributed to the fact that these species

manipulate basal conserved cell cycle mechanisms of the host cell and carry a diverse arsenal of effectors (Abad et al., 2009; Caillaud et al., 2008).

M. incognita and *M. javanica* pose a significant threat to various plant species, with tomatoes being particularly noteworthy due to its economic significance, even though one of the most well-studied resistance (R) gene has been used to control those species (Williamson, 1998). This gene, named *Mi-1.2*, was introduced into commercial tomato lines from the wild relative *Solanum peruvianum* (Gheysen & Jones, 2013; Seid et al., 2015). These species reproduce through mitotic parthenogenesis, but exhibit significant phenotypic variability, a phenomenon recognized since the mid-20th century (Martin, 1954). A host differentiation test (HDT) was developed to classify *M. incognita* populations into four distinct races based on the reaction of different hosts (Taylor & Sasser, 1978). This phenotypic variability enables *M. incognita* and *M. javanica* to overcome the resistance conferred by the *Mi-1.2* (R. Castagnone-Sereno et al., 1994; Jarquin-Barberena et al., 1991). In Brazil, the first report of the breakdown of *Mi-1.2* in tomato was reported in 1993 for *M. incognita* (Carneiro & Moraes, 1993), while for *M. javanica* it occurred nearly 3 decades later (Gabriel et al., 2022). This time lapse most likely reflects the fact that the interaction of tomato with *M. incognita* is more studied than with *M. javanica*. A subject search on the Web of Science for this pathosystem returned 1,979 publications for *M. incognita* and 827 publications for *M. javanica* (as of Dec.2023). This predominance of scientific literature studying *M. incognita* x Tomato is possibly linked to the fact that the *Mi-1.2*, hence its name, was first described in this pathosystem. Taking in account that both species share a similar host range, distribution, and temperature preferences, there is not a clear reason for the lack of studies focusing on *M. javanica* (Karssen et al., 2013). This disparity extends to studies in the molecular field as well, as transcriptome studies have been conducted only for the interaction between *S. lycopersicum* and *M. incognita* (Shukla et al., 2018b; Guan et al., 2017),

RNA sequencing (RNAseq) is a valuable tool to gain insights of the intricate molecular interplay between plants and pathogens, allowing to identify key genetic factors controlling their interactions. This technique enables us to obtain knowledge not only on how the genetic manipulation of the host by the pathogen occurs, but also how the host responds to avoid a compatible interaction (Santini et al., 2015; Shukla et al., 2018a; Zambounis et al., 2020). This can be achieved by observing gene expression in resistance responses, such as those elicited by pattern recognition receptors (PRRs), which respond to pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) caused by the invading organism's disturbance, both of which can trigger a pattern-triggered immunity (PTI), conferring resistance to the host. On the other hand, pathogens employ effectors that overcome PTI and enable a

successful interaction, a phenomenon named effector-triggered susceptibility (ETS). At a deeper defense layer the host will answer with detection mechanisms to these effectors, usually through the employment of major resistance R genes, which interact with effectors or avirulence factors, triggering an effector-triggered immunity (ETI). ETI's are often characterized by a quick hypersensitivity reaction (HR) that leads to cell death and culminates in the pathogen's death (Jones & Dangl, 2006; Kaloshian, 2004; Siddique et al., 2022). In the Meloidogyne x tomato interaction, it is proposed that the *Mi-1.2* gene triggers ETI to impede the formation of the giant feeding cells through HR (Williamson, 1999). The exact molecular pattern to which the *Mi-1.2* gene responds is still unknown, however it has been proposed that it either follows the Guard R gene model, which monitors other proteins altered by the invading pathogen, or is part of a surveillance cascade that is activated after the recognition of a nematode associated pattern. These assumptions are largely based on the fact that transgenically expressed *Mi-1.2* resistance to root knot nematodes has only been achieved by introduction on related Solanaceae species, such as *Solanum melongena*, meaning that other conserved proteins such as guards, decoys or signaling cascade components exclusive to this family are required for this resistance to occur (Tomczak et al., 2008).

The present study, through a RNA-seq analysis, compared the transcriptome profiles of a *Mi-1.2* bearing host in response to the infection of two distinct isolates of *M. javanica,* one avirulent and another virulent. We were able to identify several differentially expressed tomato genes (DEGs) up-expressed in the incompatible interaction, among those, over 70 resistance gene analogs (RGAs), a group of calmodulin and calmodulin like proteins, and an abundance of genes encoding proteins containing binding motifs involved in signaling and regulatory pathways. From the nematode's perspective, an up-expressed candidate effector gene was observed only in the virulent isolate during the compatible interaction.

3.2. Materials and Methods

3.2.1. Plant material, nematode populations and experimental design

This study utilized the resistant commercial hybrid "Santy" carrying the *Mi-1.2* gene. Seeds were sown in trays filled with a steam-sterilized substrate (120°C, 2 hours) consisting of a sandy loam soil mixture (70% sand, 20% loam, 10% silt) and kept in a growth chamber. Approximately 7 days after emergence, the seedlings were transferred to 300cc plastic cups containing the same substrate. Plants were watered daily and kept under a 12h photoperiod with

temperatures averaging 24°C for 4 weeks. The two *M. javanica* populations used in this study were characterized in detail in a previous study, one virulent and another avirulent on "Santy". The experimental design was completely randomized with treatments consisting of the contrast between the two isolates and two evaluation times, 3 and 7 days after the inoculation (DAI). Therefore, a total of four treatments were established, with 3 biological replicates each comprised by two plants cultivated on independent plastic cups. Additionally, each treatment had two extra replicates used in the histological analysis.

3.2.2. Recovery of second stage juveniles and inoculation

Second stage juveniles (J2) were used as inoculum for the RNAseq experiment instead of eggs to avoid the continuous hatching which could introduce new feeding individuals over a 3 to 7-day period. To acquire an adequate quantity of J2s, hatching chambers were set up using watch glasses filled with distilled water (Figure 1). A plastic sieve covered with paper, each containing 10 egg masses, was positioned over the glasses in contact with the water film beneath to allow the newly hatched J2 to migrate to the water. The chambers were kept at 28°C for 48 hours and the J2 suspension was calibrated using a Peter's slide under a light microscope. The 4-week-old plants were inoculated with a total of a 1,000 J2s. The aqueous suspension of nematodes was divided into two oblique holes of 2 and 4 cm flanking the plant in a 180° angle from each other, at an approximately distance of 1.5 cm away from the stem.

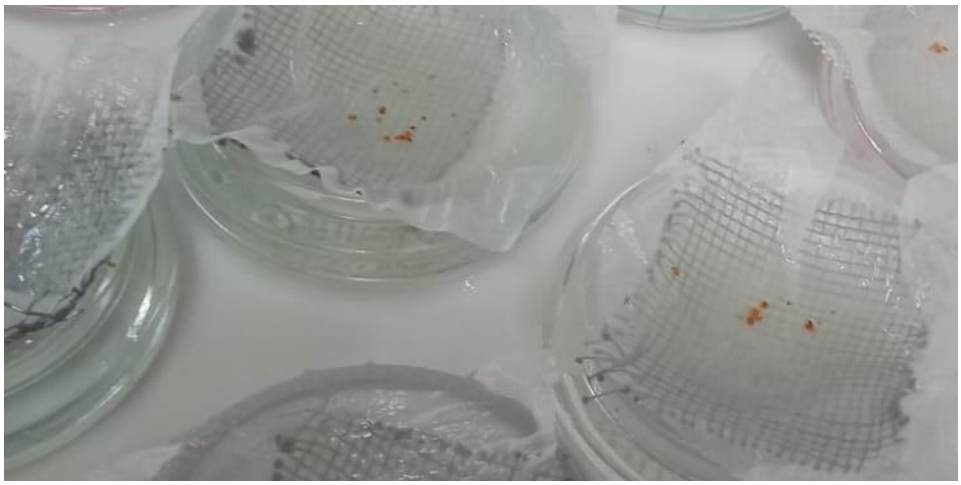

Figure 1: Hatching chambers designed to separate egg masses and viable second stage juveniles in abundance.

3.2.3. Histological comparison of roots infected by nematodes

Two replicates from each treatment were used for the histological analyses of the plant x pathogen interaction. Nematode in roots were stained with acid fuchsin following Byrd et al., (1983) with modifications. Roots were washed with running water, submerged in a 1 % sodium hypochlorite solution for 6 minutes under stirring and rinsed over a 60 mesh sieve. Roots were transferred to a beaker filled with enough water to submerge them and kept for 15 minutes to ensure the removal of hypochlorite. Roots were transferred to a beaker filled with the staining solution (acid fuchsin) previously heated until boiling and kept at room temperature during 10 minutes for cooling. A final washing was carried out to remove the excess of staining and rehydrate the tissue by adding acidified glycerin (40 ml glycerin + 2 drops of 10N of HCl). Roots were sectioned into multiple 1cm segments and mounted onto microscope slides for imaging using a light microscope outfitted with a digital camera to document observations (100x) on the stage of the parasitic interaction.

3.2.4. RNA extraction and sample preparation

Prior to RNA extraction roots were washed as described, blot dry, flash frozen in liquid nitrogen, and stored at -80ºC until RNA extraction. The tissue was macerated using a pestle in a mortar and total RNA extraction and purification was performed using the MiniKit PureLink (Invitrogen) + Trizol (Ambion), following the manufacturer's protocol. The purified RNA was treated with RNAse-free DNAse (DNAse I Kit, Sigma-Aldrich). RNA integrity was assessed on agarose gel and quantified in a NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA integrity number (RIN) was determined using the Agilent Bioanalyzer with the aid of the TapeStation Analysis Software 4.1.1.

3.2.5. RNA sequencing and construction of transcriptome libraries

Transcript libraries were constructed by poli A enrichment using the TruSeq Stranded mRNA kit (Illumina) followed by clustering and sequencing of paired end reads (2x100bp) in a NovaSeq 6000 equipment, yielding a total of 12 libraries.

3.2.6. Transcriptome data analysis

Data analysis was performed on the nf-core/rnaseq pipeline (Ewels et al., 2020). Quality control of the raw data was run on the FastQC (v0.12.1) (2 × 100 bp PE reads), and low-quality sequences were discarded by Trim-Galore! The reference genome of choice for the alignment of tomato reads was the SL4.0 assembly (Hosmani et al., 2019) and the ITAG4.1 annotation (SGN-Sol Genomics Network). For the nematode alignments the reference genome was the ASM369362v1 assembly (Szitenberg et al., 2017) and the 2019-01-WormBase available online at the Wormbase platform. Reads were aligned to the reference genomes using the STAR software (Dobin et al., 2013) and transcript abundance was quantified with the aid of Salmon (Patro et al., 2017). Aligned reads were organized into a matrix for each respective GFF annotation file (ITAG4.1/2019-01-WormBase). Data processing was carried out remotely, through the Ohio Supercomputer (OSC) and its OnDemand interface.

3.2.7. Analysis of differentially expressed genes (DEGs) and functional enrichment analysis

Differential gene expression across treatments was assessed by the DESeq2 (Love et al., 2014) package (version 1.42.0) via R/Bioconductor, which defined genes differentially expressed assuming an adjusted p-value <0.05. Normalized gene counts values were adjusted to compensate for library sizes in order to compare gene expression data across different samples, allowing a more accurate identification of DEGs. Principal component analysis (PCA) was performed using the PCAtools. Transcripts of interest were categorized based on annotations of the tomato reference genome and confirmed through manual inspection using UniProt, PFAM terms and the InterProScan databases for functional classification. For the tomato reads, a Gene Ontology (GO) enrichment analysis was conducted using the Goseq (1.54.0) R/Bioconductor package (Young et al., 2010).

3.3. Results

3.3.1. Histological analysis of infected roots

Microscopical visualization of stained roots allowed to determine the stages of infection at 3 and 7 days after inoculation (DAI). Overall, penetration of roots was observed for both

interactions at 3 DAI, with a few individuals of the virulent isolate already beginning to develop their respective feeding sites (<20%), but most still at the motile J2 stage (>70%). For the avirulent interaction only motile J2 were observed. At 7 DAI, few individuals sparsely located of the avirulent isolate were observed, some of which were tentatively stablishing feeding sites, with no signal of gall induction. On the other hand, several individuals of the virulent isolate had already developed into J3-J4 (<15%), most, however, were sessile J2 already feeding (>80%) and gall formation was visible to the naked eye (Figure 2).

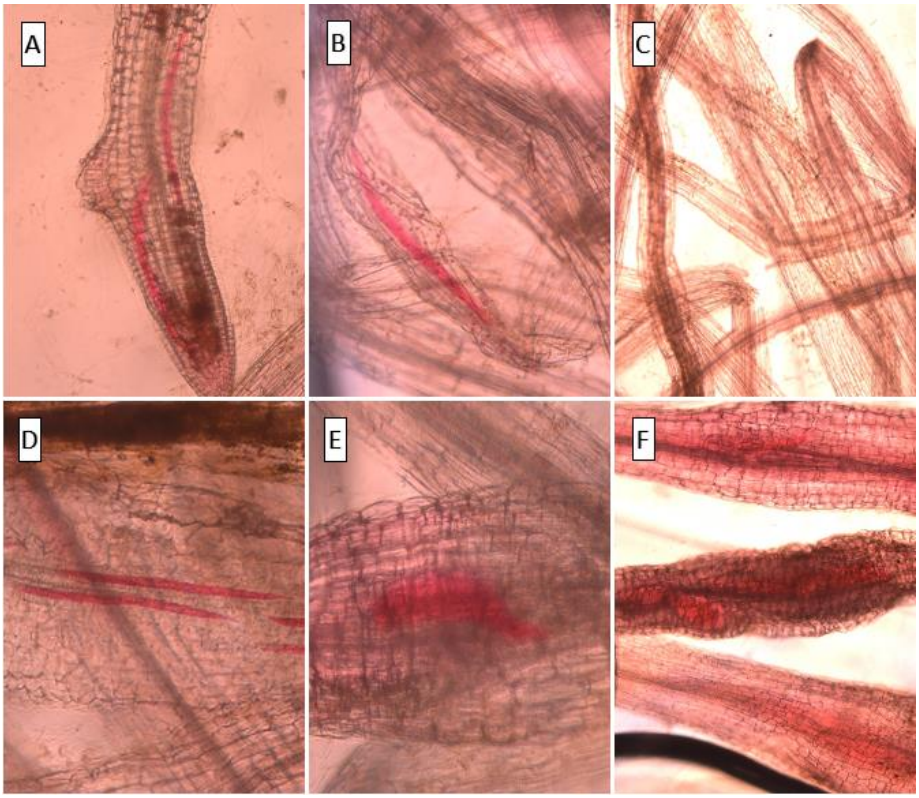

Figure 2. Stages of the plant x nematode interaction A) J2 individuals of the avirulent isolate in a root tip at 3 DAI. B) A malnourished avirulent J2 lodged in a root tip at 7 DAI with no visible gall or feeding site formation. C) most of the roots parasitized by the avirulent isolated were unpopulated at 7 DAI. D) several J2's of the virulent isolate migrating through the root cortex. E) A virulent J3 or J4 in the middle of a root gall in a feeding site. F) Several individuals of the virulent isolate successfully feeding on the roots. Images are representative of observations of 6 slides from each treatment.

3.3.2. Data sequencing and DEG profiles

Sequencing of the RNA-seq libraries produced a total of 394 million paired-end reads aligned to the tomato genome sequence SL4.0. These reads were divided between the four treatments as presented in table 1, Avir3, Vir3, Avir7 and Vir7. Reads aligned to the nematode

genome varied greatly between isolates, with the incompatible interaction (avir) contributing far less to the total of mapped reads than the virulent one. For instance, at 3 and 7 DAI, reads of the avir isolate ranged from 38 to 50 thousand alignments, while reads of the vir isolate varied from 400 thousand to 1 million for the same periods, respectively. Therefore, conclusions regarding the nematode's DEG profiles are far more limited, as expected, and were interpreted carefully.

Table 1: Total and average counts of reads according to the treatments inoculated with avirulent and virulent isolates of *M. javanica* at 3 and 7 days after inoculation.

Treatment	DAI	Replicate	Read count	Total read count	Read average
Avir3	3	1	35,744,748	94,934,333	31,644,777
	3	2	28,633,187		
	3	3	30,556,398		
Vir3	3	1	33,479,867	100,519,914	33,506,638
	3	2	32,640,549		
	3	3	34,399,498		
Avir7	7	1	32,050,766	105,165,358	35,055,119
	7	2	36,554,194		
	7	3	36,560,398		
Vir7	7	1	29,188,489	93,291,607	31,097,202
	7	2	28,996,720		
	7	3	35,106,398		

DEGs transcription levels were expressed as Log_2-fold change (LFC) based on the incompatible interaction (Avir). Therefore, positive LFC's are attributed to genes up-expressed in this interaction and negative values indicate genes up-expressed in the compatible interactions (Vir) (Figure 3). A total of 6,402 and 2,394 DEGs were detected in Santy inoculated with the Avir and Vir isolates at 3 and 7 DAI (p-value $\geq$ 0.05). At 3 DAI, 3,477 had positive and 2,925 had negative LFC's, whereas at 7 DAI there were 1,082 positive and 1,312 negative LFCs. Therefore, between evaluations the number of DEGs not only decreased but also shifted in regard to which interaction presented the highest number. While at 3 DAI the incompatible interaction presented 552 DEGs over the compatible interaction, at 7 DAI the compatible interaction presented 230 additional DEGs when compared its incompatible counterpart. Tables of the tomato and nematode DEGs are available as supplementary material.

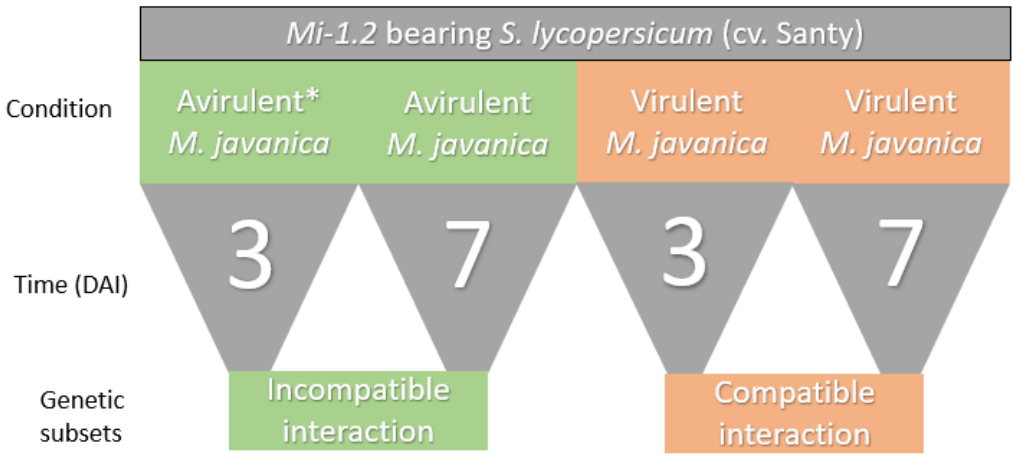

Figure 3: Visual representation of the approach used to identify sample subsets for the analysis of differentially expressed genes in the tomato interaction between the two contrasting *M. javanica* isolates. * Virulence and avirulence based on their abilityto successfully reproduce on tomato "Santy".

Principal Component Analysis (PCA) results shed light on the underlying patterns in gene expression across our samples. PC1 shows the influence of the isolates on the observable data, explaining a substantial portion of the overall variance in our dataset at 51.13%. This suggests that the major differences or patterns in gene expression are comprised by this dominant principal component. This is not unexpected, as most of this variation has its origin on the compatible interaction at 7 DAI, at which point expression patterns could be distinctly affected by temporal tipping points in the host's metabolisms hijacked by the nematode. In PC 2 it is possible to observe the diversely affected time component (DAI 3 and 7). While the incompatible interactions (red) remain closely clustered together, the compatible interaction (blue) presents its 3 and 7 DAI clusters largely apart, highlighting the difference between those treatments. Additionally, volcano plots were employed to detect genes demonstrating substantial alterations in expression levels (Figure 4).

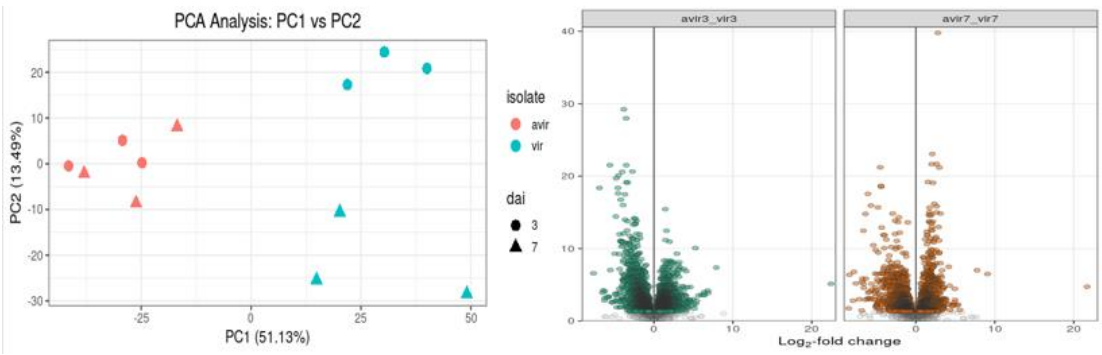

Figure 4. A) clustering of the independent libraries based on the PCA analysis of the DEGs across all interactions. B) volcano plot of the DEGs expression patterns between isolates at 3 and 7 DAI.

3.3.3. Gene ontology analysis reveals overrepresentation of sets involved in key biological responses to biotic stresses

A total of 27 enriched GO terms were detected at 3 DAI, 10 belonging to biological process (BP), 6 to cellular component (CC) and 11 to molecular functions (MF). All processes were upregulated in the compatible interaction, but only the MF terms "ADP binding" (GO: 0043531) and "binding" (GO: 0005488) were upregulated in the incompatible interaction. The ADP binding term covers several resistance gene analogs (RGAs) and will be further discussed in the next section, while the binding GO term comprises several genes involved in cell signaling and regulatory processes.

The 3 DAI enrichment analysis made it clear that the compatible interaction affects the hosts metabolisms in a very complex way, with 25 GO terms being unique to this contrast. Among these, the BP's "cytoplasmic microtubule organization" (GO:0031122), "carbohydrate metabolic process" (GO:0005975), "plant-type cell wall organization" (GO: 0009664), "microtubule-based process" (GO: 0007017) and "transport" with 65 DEGs (GO: 0006810). GO terms of CC category such as "dynein complex" (GO: 0030286), "microtubule associated complex" (GO: 0005875), "gamma-tubulin complex" (GO: 0000930) and "nucleosome" (GO: 0000786). And for the MF category "protein heterodimerization activity" (GO:0046982), "transmembrane transporter activity" (GO: 0022857), "transporter activity" (GO: 0005215) and "structural constituent of cytoskeleton" (GO: 0005200). During the 3 DAI evaluation, the amount of enriched terms in the compatible interaction goes in contrast with the two terms afore mentioned for the compatible interaction, this could point to a very coordinated response in the incompatible interaction, in which most up-regulated genes encoding RGAs and cell signaling/regulatory proteins. While in the compatible interaction many diverse cellular activities involving hosts metabolism changes can be observed within the enriched terms.

At 7 DAI 18 GO terms were identified, being 7, 1 and 11 for the BP, CC and MF categories, respectively. At least 9 of these terms were associated to both interactions, whereas of the remaining, 7 were in the compatible and 2 on the incompatible interaction. Among the shared terms were "defense response" (GO: 0006952), "response to oxidative stress" (GO: 0006979), and "peroxidase activity" (GO: 0004601). The term "Cytokinin dehydrogenase activity" (GO: 0019139) was enriched only in the compatible interaction and can be seen containing the same 5 DEGs shown in the previous section 3.3.7, as well as the BP "plant-type cell wall organization" (GO: 0009664), also present in the 3 DAI and presented in section 3.3.6.

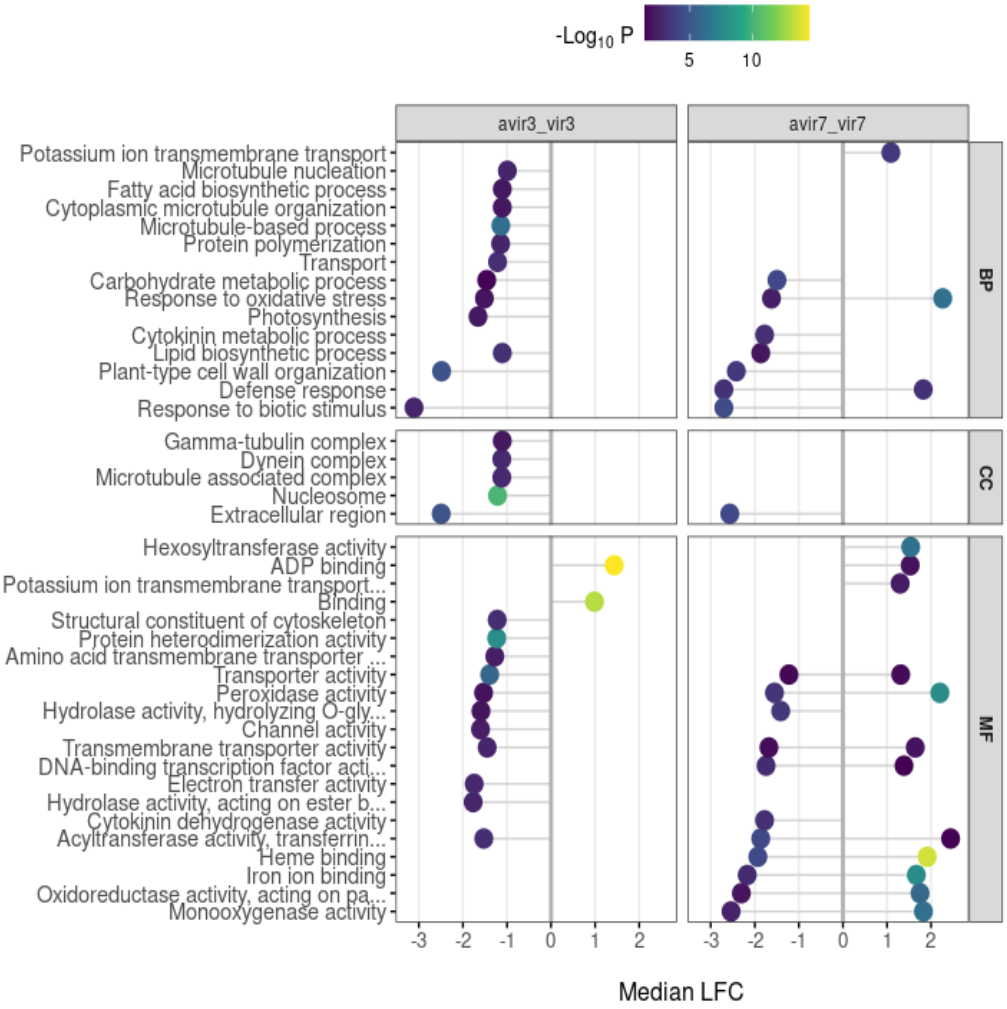

Figure 5. Cleveland dot plots containing enriched GO terms at 3 and 7 DAI (P>0.01). Median LFC signals the direction and expression rates of genes contained in each GO term regarding the interactions. Positive LFCs indicate up-expression in the incompatible and negative in the compatible interaction.

3.3.4. Differential expression of resistance gene analogs (RGAs) can be observed as early as 3 dai

DEGs with typical defense related motifs such as nucleotide binding and leucine rich repeats could be detected at both times, totaling 71 DEGs of which 70 were upregulated in the incompatible interaction at 3 DAI. Among those 71 DEGs at 3 DAI, 19 remained differentially expressed at 7 DAI, most of them (18 DEGs) still up-expressed in the incompatible interaction (Figure 5). The only gene of this class up-expressed in the compatible interaction was the Solyc12g097000, a TIR-NBS-LRR RGA.

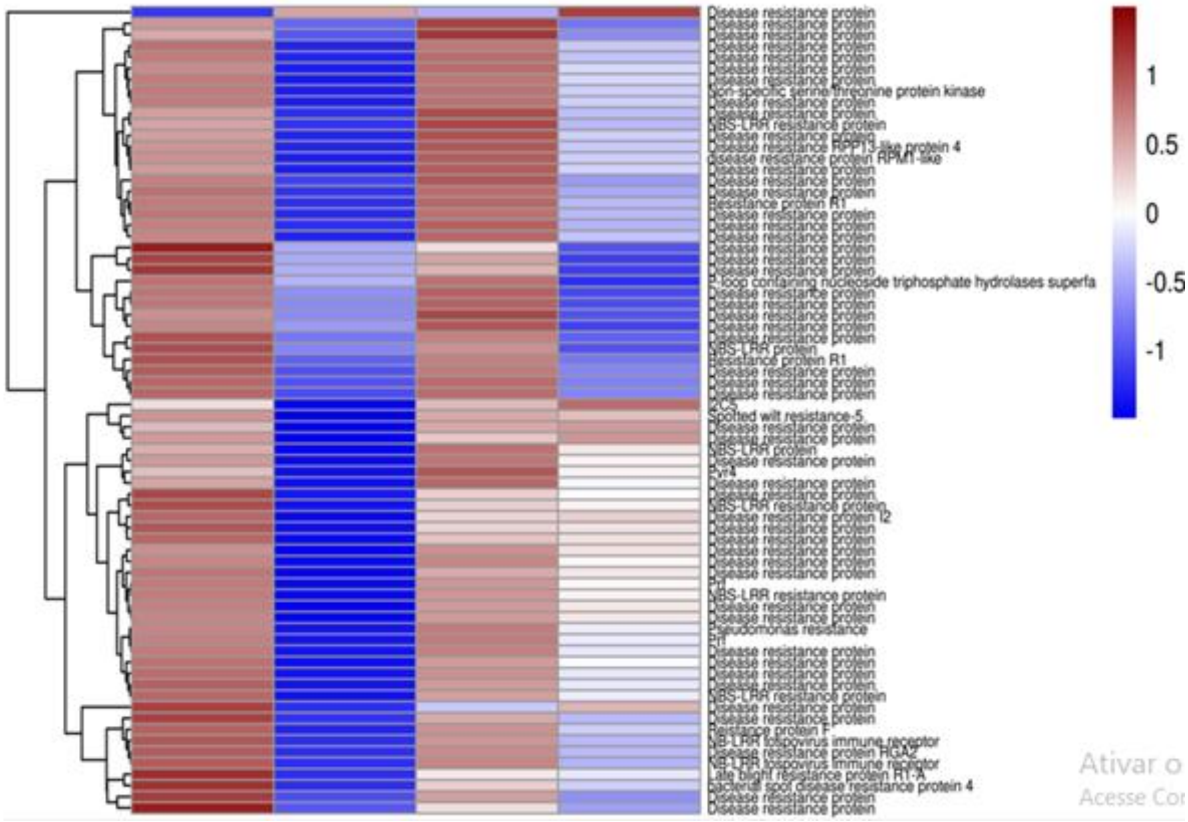

Figure 5: Heatmap of Differentially Expressed Genes with ADP Binding Function at 3 and 7 Days After Inoculation (DAI). The intensity of the color indicates the upregulation (red) or downregulation (blue) of each gene under each condition. The heatmap employs gene-wise z-scores for standardized comparison across samples

Among the DEGs at the earlier time that were not detected at 7 DAI are two noteworthy genes: Solyc06g008390.4 which aligned to the *Mi*-1B gene (UniProt: A1Y9Q8_SOLLC), a nonfunctional copy of *Mi-1.2*, (Seah et al., 2007) and Solyc09g098130.3, identified as the tomato spotted wilt resistance-5 (*Sw-5*) gene, that confers broad resistance to tospoviruses. This transcript presented one of the highest expression rates at 3 DAI with an LFC of 5.4 (Figure 6), this gene is also a homolog of *Mi-1.2* (Brommonschenkel et al., 2000).

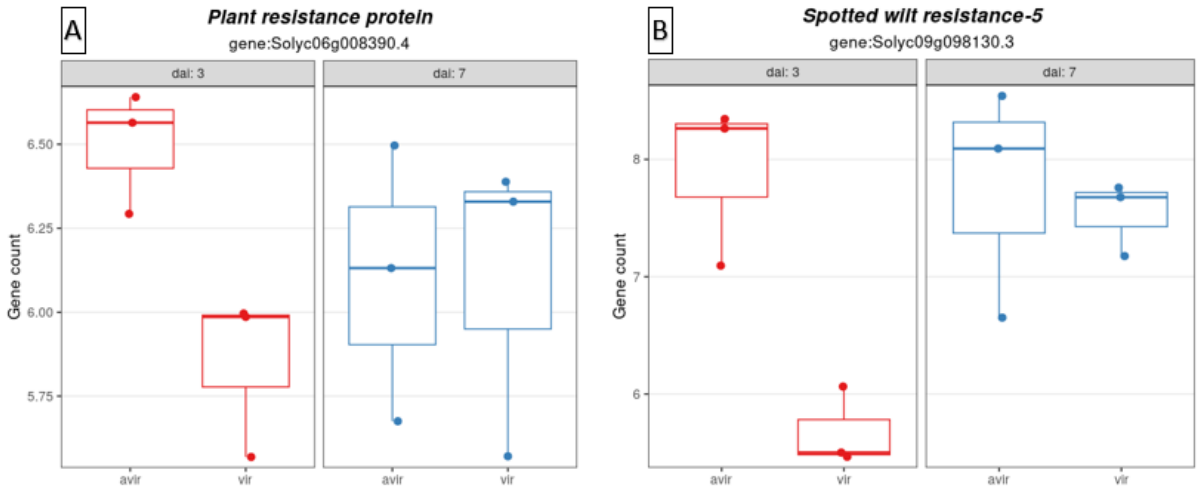

Figure 6: Boxplot of normalized gene counts. Up-expression of *Mi-1.2* (A) and of SW5 (B) at 3 DAI in the incompatible interaction.

3.3.5. Intense signaling cascades and regulatory changes occur in an incompatible interaction

In the incompatible interaction, 134 DEGs related to regulatory and signaling gene pathways were detected at 3 DAI in sharp contrast with the compatible, with only 24. (Figure 7). The number of DEGs in this category was much lower at 7 DAI , only 13, with 9 and 4 being up-expressed in the compatible and incompatible interactions, respectively. Another group of genes strongly related to cell signaling are the calcium-binding proteins (calmodulins). Ten and 4 calmodulins were up expressed in the incompatible interaction at 3 and 7 DAI, respectively. Of the ones detected at 7 DAI, 1 was up-expressed in the compatible interaction as well (Figure 8). At both times, Solyc02g088580, annotated as a calmodulin-binding domain-containing protein (UniProt: A0A3Q7F947_SOLLC), showed the highest LFC values among all DEGs, 22.45 and 21.72 at 3 and 7 DAI respectively in the incompatible interaction. Among the DEGs found under the binding GO term, a few standed out as up-expressed in the incompatible interaction, 11 coding for a RING-type E3 ubiquitin transferase, 18 ARM-repeat/Tetratricopeptide(Armadillo) repeat and 2 U-box domain-containing proteins. A similar signaling and defense related pattern of expression was observed in a transcriptome analysis conducted by Shukla et al. (2018), it is important to note however, that in this work, the stages of interaction with *M. incognita* are slightly delayed in comparison to our study. A delay to be likely attributed to interspecific differences between the nematodes and/or temperature, as his study was conducted at 22ºC, a slightly colder temperature compared to the present work.

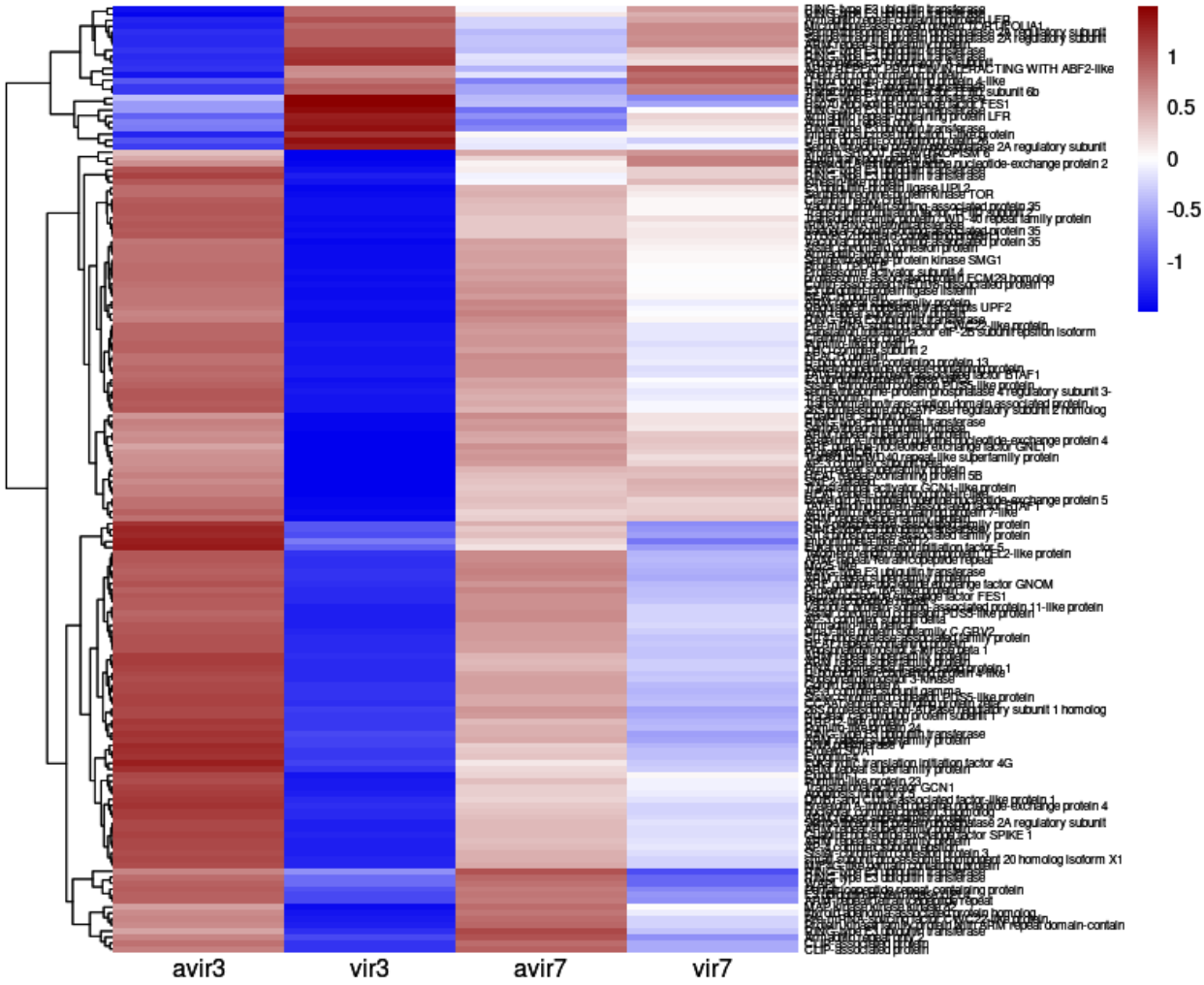

Figure 7: Heatmap of Differentially Expressed Genes with binding function at 3 and 7 days after onoculation (DAI). The intensity of the color indicates the upregulation (red) or downregulation (blue) of each gene under each condition.

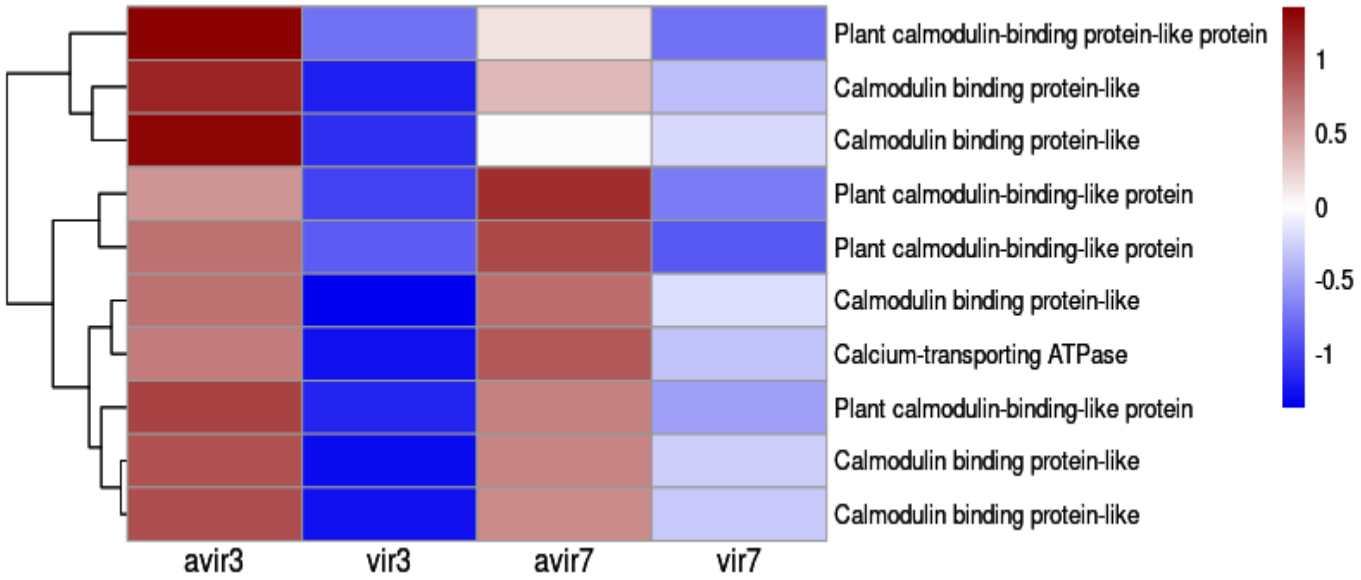

Figure 8: Heatmap of differentially expressed genes of the calmodulin complex at 3 and 7 DAI. The intensity of the color indicates the upregulation (red) or downregulation (blue) of each gene under each condition.

3.3.6. Transcription of genes involved in cell wall modification and biogenesis are diversely affected in response to the avirulent and virulent isolates

Several genes involved in cell wall modification were differently expressed in the incompatible and compatible interaction. At 3 DAI the group of xyloglucans endotransglucosylases/hydrolases (XTH) involved in wall biogenesis presented 14 DEGs, most of which 11 were up-expressed in the incompatible interaction and 3 in the compatible. The number of DEGs in this group decreased to 10 at 7 DAI, with 7 being up-expressed in the incompatible interaction (Figure 9) and 3 in the compatible. In contrast to these genes, of the ones involved in plant cell wall organization, mainly expansin-coding genes, presented 16 DEGs at 3 DAI, only 3 of which were up-expressed in the incompatible interaction, while 13 were up-expressed in the compatible interaction. At 7 DAI this number was reduced to 9 of which 1 was up-expressed in the incompatible interaction, and 8 in the compatible (Figure 9).

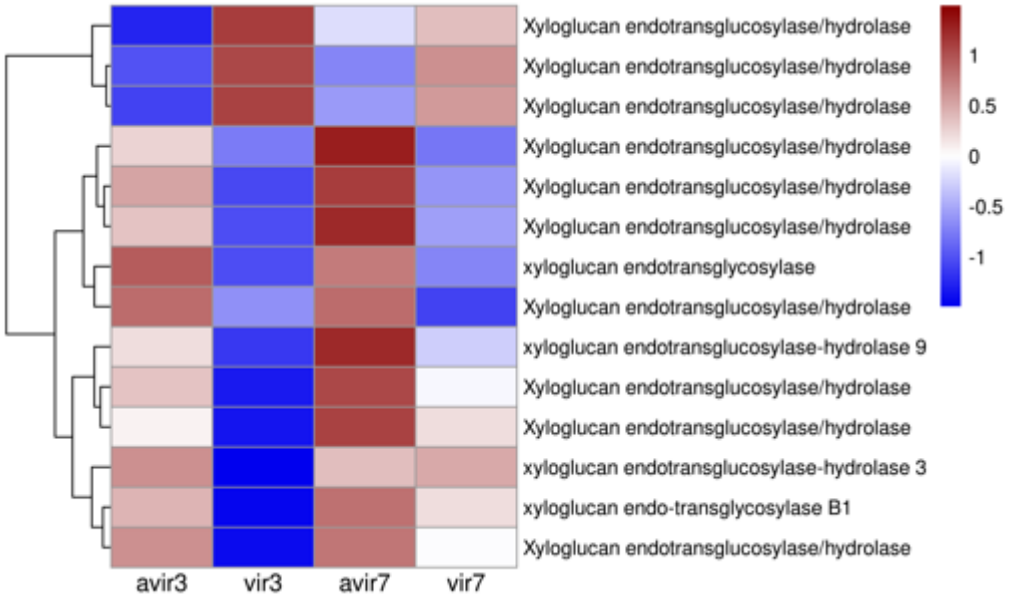

Figure 9: Heatmap of differentially expressed genes with cell wall biogenesis function at 3 and 7 DAI. The intensity of the color indicates the upregulation (red) or downregulation (blue) of each gene under each condition. The heatmap employs gene-wise z-scores for standardized comparison across samples.

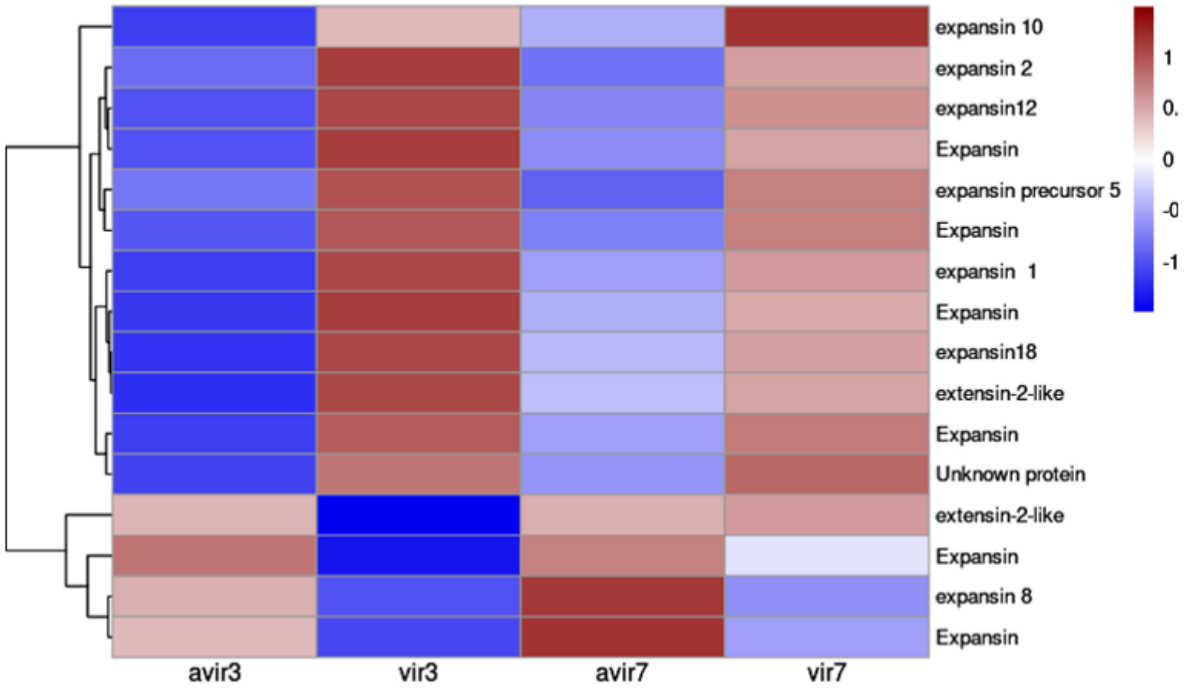

Figure 10: Heatmap of differentially expressed genes related to plant cell wall organization functions at 3 and 7 DAI. The intensity of the color indicates the upregulation (red) or downregulation (blue) of each gene under each condition. The heatmap employs gene-wise z-scores for standardized comparison across samples.

3.3.7. Increased expression of cytokinin oxidase genes occurs only in the compatible interaction

Four DEGs corresponding to transcripts involved in regulation of cytokinins were found upregulated in the compatible interaction at 3 DAI and increased to 5 at 7 DAI. The Solyc12g008900.2 DEG, annotated as cytokinin oxidase 6 presented an LFC higher than 4. The additional DEG detected at 7 DAI was Solyc01g088160.5, a cytokinin oxidase/dehydrogenase (Figure 10). One of the many functions of cytokinin is the regulation of root growth, overall architecture and regulation of the cellular cycle. Upregulation of this phytohormone signals for the emission of lateral roots, which in the context of *Meloidogyne* spp. parasitism equals new potential feeding sites. Additionally, as a regulator of cell cycle, the presence of cytokinins is a key component in the formation of the nematode's feed cells (Gheysen & Mitchum, 2019). Interestingly, in the transcriptome analysis conducted by Shukla et al. (2018), it was observed that cytokinin oxidases at similar stages of evaluation were downregulated in the compatible interaction with *M. incognita*.

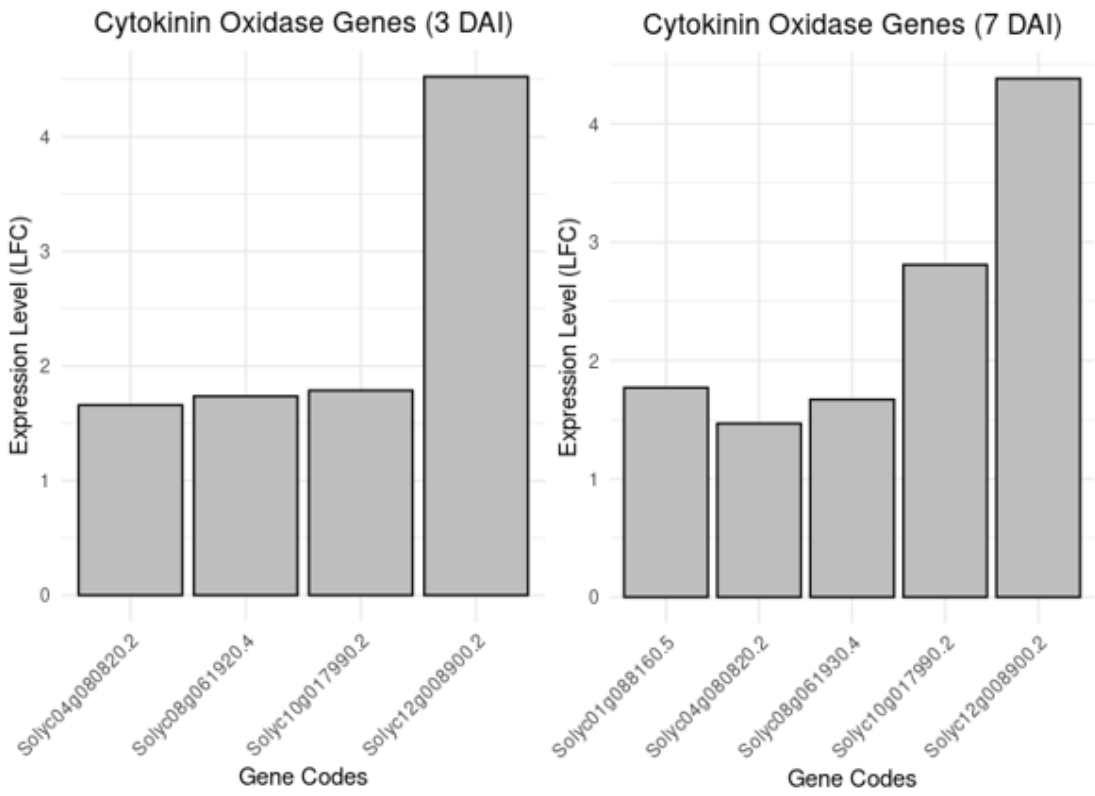

Figure 11: Upregulation levels of cytokinin oxidase over the course of the two evaluations in the compatible interaction.

3.3.8. A candidate effector gene of *M. javanica* is up-regulated in the compatible interaction

From the nematode perspective, two interesting DEGs were observed as upregulated in the compatible interaction (virulent isolate). First, the gene positioned at the scaffold6430_ cov211.g10831 a candidate secreted effector of *M. javanica* (Uniprot: A0A915MHQ4_MELJA), the predicted function of the gene in question is derived from its similarity to the previously characterized *M. incognita* gene, *Minc04822*, with which it exhibits 100% identity. The second, scaffold10369_cov160.g14738 is a protein of the caveolin family (Uniprot: A0A915LEI4_MELJA) associated with membrane organization and plasma membrane functions (Figure 14).

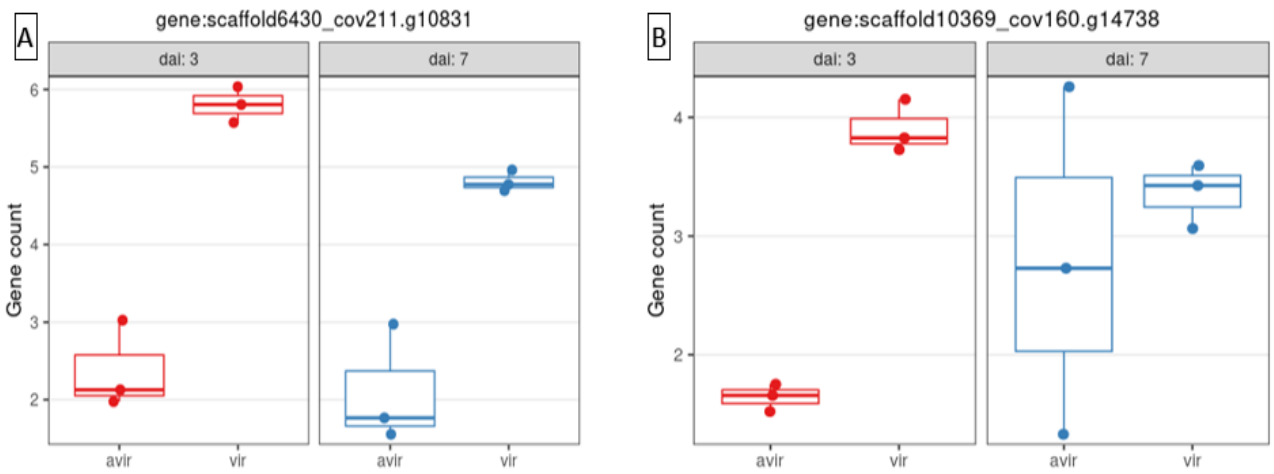

Figure 12: Boxplot of normalized gene counts. A) Differential expression of the candidate *M. javanica* effector upregulated at 3 and 7 DAI in the compatible interaction. B) High expression rates of the caveolin gene at DAI in the compatible interaction.

3.4 Discussion

The gene *Mi-1.2* has served as a durable resistance resource for the control of nematodes of the *Meloidogyne* genus, traditionally against *M. javanica*, *M. incognita* and M. *arenaria* species (Milligan et al., 1998; Vos et al., 1998). Recent studies expanded the resistance spectrum of *Mi-1.2* including *M. ethiopica* and *M. luci* (Santos et al., 2020). However, this resistance is being challenged by a combination of factors. Intense cultivation cycles exert selective pressure on virulent isolates while average global temperatures rises from year to year. This situation poses not only a general problem for plant disease management but also a specific problem in the case of the thermosensitive action of *Mi-1.2*, which is inactivated when temperatures surpass 28°C. These factors likely favored the appearance of populations of *M. javanica* and *M. incognita* virulent on *Mi-1.2* (Iberkleid et al., 2014; Juroszek et al., 2020; Ornat et al., 2001). Despite the potential yield losses caused by *M. javanica* in tomatoes, there is a notable absence of transcriptome analyses examining differentially expressed genes of tomato genotypes carrying *Mi-1.2* when challenged with virulent *M. javanica* isolates.

Histological analysis of infected roots through nematode staining allowed to compare a virulent (vir, compatible reaction) and an avirulent (avir, incompatible) isolate of *M. javanica* and select meaningful stages of the interaction to establish a parallel between expression patterns and the observed outcome of the interaction (Aparecida Beneventi et al., 2013; Petitot et al., 2016; Shukla et al., 2018). The staining of nematodes allowed us to observe the compatible interaction of our resistance breaking isolate of *M. javanica* and the contrast with its avirulent counterpart, specially at the later evaluation, at which point not a single specimen of the avirulent isolate was

observed successfully developing into more mature stages, albeit some few juveniles still in the second stage could be observed on root tips (Figure 2). This is in line with observations made by Iberkleid et al. (2014) in which avirulent isolates of *M. javanica* could occasionally survive and thrive on a *Mi-1.2* host, although at a very low reproduction rate. On the other hand, from the 3 DAI specimens of the virulent isolate could be observed initiating feeding sites and becoming swollen second-stage juveniles, and by 7 DAI third stage juveniles were most found, all accompanied by an initial root galling. The observation of later stages of development of the nematode and gall formation confirms the overcoming of the resistance conferred by the *Mi-1.2* gene (Castagnone-Sereno et al., 1993). This difference in development between the Avir and Vir isolates likely contributed to the low rate of transcripts aligning to *M. javanica* in the incompatible interaction when compared to the compatible one.

Transcript alignment yielded 6,402 and 2,394 DEGs at 3 and 7 DAI respectively, indicating that These numbers reveal the first underlying pattern observed between the interactions, at 3 DAI, 54.39% of the differentially expressed genes were upregulated in the compatible interaction, whereas at 7 DAI 54.84% were upregulated in the incompatible interaction. Despite a reduction of 62.59% in the total of DEGs from 3 to 7 DAI, the shift to upregulation from the incompatible interaction to the compatible one followed a similar pattern. The higher number of DEGs at 3 DAI reflects the challenging of both isolates against the host immune system. In the presence of *Mi-1.2* , the anticipated outcome involves the recognition of a pathogen effector which activates an effector-triggered immunity (ETI) ultimately ending the infectious process (Jones & Dangl, 2006). Under our experimental circumstances, this is in line with the incompatible interaction, in which the host is challenged by the avirulent isolate. While in the compatible interaction the virulent isolate is able to bypass this ETI and stablish a compatible interaction. A higher number of up-expressed genes during an ETI mediated incompatibility is not unexpected, as this type of responses carried by the presence of an R gene is very robust and leads to intense transcriptional changes (Pombo et al., 2014). The shift in up-expressed DEGs towards the compatible interaction at 7 DAI coincides with physiological changes induced by the nematode in stablishing stable plant x pathogen interactions (I. H. Lee et al., 2019; Li et al., 2009).

To categorize gene-sets among the 9 thousand DEGs, taking together the differential expressions at 3 and 7, a GO analysis was conducted. Despite the higher number of up-expressed genes in the incompatible interaction at 3 DAI, only 2 GO terms were significant corresponding to the "binding" and "ADP binding" molecular functions. Genes within these groups will be addressed in detail further over this discussion section, however, their up-expression at 3 DAI in

the incompatible interaction is in line with an intense defense reaction. The broad "binding" GO term encompasses genes involved in the interaction between a binding protein and its substrate, which could be DNA, RNA, another protein, or an ion. While no conclusive inference can be made solely on the upregulation of this GO term, several recent work report it in resistance responses to plant pathogens of multiple etiologies (Cui et al., 2020; Du et al., 2015; I. H. Lee et al., 2019). The "ADP binding" GO term harbors several functions, including genes with nucleotide binding (NB) functions such as resistance gene analogs, which are marked by their NB and leucine rich repeat (LRR) domains. The GO term for "nucleotide biding" (GO:0000166), often appears with genes up-expressed in incompatible interactions, and are helpful fine tuning the search for RGAs (Tiwari et al., 2021), in our analysis however, this go term was not enriched at ($P>0.01$).

At this time, in the compatible interaction the up-expression of genes involved in the transcription of proteins related to cell wall organization, microtubular and cytoskeletal functions, nucleosome and transport indicated a successful establishment of feeding cell asthese categories are directly associated with giant cell (GC) (Nabor-Romero et al., 2022; Przybylska & Spychalski, 2021; Tiwari et al., 2021). During the formation of GC, undifferentiated meristematic cells are reprogrammed by the invading nematode. And the cells undergo several cycles of partial cell division, in which karyokinesis occurs without completing the cytokinesis, resulting in an enlarged multinucleated cell (Abad et al., 2009).

At 7 DAI, the hosts in the incompatible and compatible interactions are faced with distinct prospects. The incompatible interaction is no longer being intensively challenged by the nematode, while the compatible interaction is nourishing a developing nematode. Despite these differences, several GO terms were simultaneously enriched in both interactions, including the GO term "defense response", which, as will be presented later, could signal a late defense response in the compatible interaction. Interestingly, one of the few exclusively up-expressed GO terms at 7 DAI is the "carbohydrate metabolic process" in the compatible interaction, which features several enzymes responsible for the conversion and processing of several forms of sugar. This GO term has been often observed as enriched in compatible plant x pathogen interactions (Fang et al., 2021; Yang et al., 2015; Zhang et al., 2018). A DEG related to carbohydrate transport that was not attributed to any enriched GO term in our analysis was the Solyc08g082770 gene, which codes a Bidirectional sugar transporter SWEET protein (UniProt: A0A3Q7HWZ6_SOLLC). This gene had the highest up-expression in the incompatible interaction at 3 DAI with a LFC of 7.65, and ranked 4th at 7 DAI for the same interaction with a LFC of 7.82. Proteins of this family are responsible for the transmembrane transport of sugar

molecules and are known to be exploited by pathogens of various etymologies (Breia et al., 2021; L. Chen, 2014; L.-Q. Chen et al., 2010). More recently Zhou et al. (2023), investigated the role of the Arabdopsis thaliana SWEET family of genes (AtSWEETs) and observed that 13 genes of this family were upregulated in a compatible interaction with *M. incognita* at 3 DAI, with AtSWEET1 presenting the highest expression. Using a reporter gene (GUS) Zhou et al. (2023) was able to observe that AtSWEET1 was being expressed on the nematode feeding. In the same study a mutant for the AtSWEE1 gene with near zero expression (atsweet1) was able to reduce the number of galls and nematodes by approximately 20% and significantly delay the development of adult females at 18 DAI.

Among the several resistance gene analogs (RGAs) differentially expressed at 3 and 7 DAI, only one was up-expressed in the virulent interaction. Solyc12g097000 is a TIR-NB-LRR (UniProt: A0A3Q7JE37_SOLLC) and was associated to a resistant response in tomato against *Xanthomonas perforans* (Shi & Panthee., 2020). Interestingly, the response to this necrotrophic pathogen correlated to the production of peroxidases and genes involved in cell detoxification. The same could occur in the compatible interaction of *M. javanica* and *Mi-1.2* mediated HR, as it has been reported that virulent isolates stimulate an overexpression of a key peroxidase gene in tomato to avoid apoptosis of the host cell (Guan et al., 2017). In our study we found no pattern of peroxidase overexpression in the compatible interaction, with exception of three ascorbate peroxidase of the APX family (Solyc02g083630.3, Solyc02g083620.3, Solyc09g007270.3), all up-expressed at 3 and 7 DAI in the incompatible interaction. Those enzymes also play a role in H2O2 scavenging (Gomes et al., 2022). The occurrence of resistance R genes involved in susceptibility responses is not an uncommon phenomenon. Considering the intricate signaling cascades initiated by individual R genes, this behavior may signal the exploitation of a latent vulnerability in the plant's defense mechanisms by a pathogenic agent. It is important to consider that the activation of NB-LRR receptors results in a network of cross-talk regarding the deployment of possible response pathways, which is partially responsible by the differentiation between a biotrophic or necrotrophic pathogen attack (Glazebrook, 2005). The identification and examination of these occurrences, in which R genes are overexpressed in a compatible interaction, may provide valuable insights into potential blind spots in the plant's immune response, shedding light on novel avenues for understanding plant-pathogen interactions. (Bent & Mackey, 2007; Eckardt, 2002; Sweat et al., 2008).

All other DE RGAs were upregulated in the incompatible interaction at 3 DAI, with their numbers declining by 74% at 7 DAI. This reduction can be explained from an ETI response, in which the recognition of the invading pathogen triggers a cascade of cellular signals

that culminate in cell death usually in less than 24h (Ngou et al., 2022). Thus, it is expected that these RGA's would not be differentially expressed at this time of the interaction.

The SD-CC-NB-LLR are a class of R genes observed in the Solanaceae family. Described by Mucyn et al., (2006) these genes are named after the Solanaceae Domain (SD), a peculiar motif after their coiled-coil (CC) motif at its extended N-terminal portion. Four Solanaceae members are described as homologs. These include *Mi-1.2* and *Sw5*, along with *R1* known for conferring resistance to late blight in tomatoes, and *Prf* associated with Pseudomonas syringae resistance and all are known to elicit a rapid HR reaction in response to their respective pathogens (Ballvora et al., 2002; de Oliveira et al., 2018; Sheikh et al., 2023). Among the 71 RGA class DEGs observed in the 3 DAI interaction all four were upregulated in the incompatible interaction, the Solyc05g012890 which is translated into the R1 protein (LFC:2.62), and Solyc05g005130 for the Prf gene (LFC:1.74). The expression of multiple tomato R genes is directly related to a successful defense response. In their 2023 study, von Dahlen et al. (2023) conducted a meta-analysis of gene expression patterns, exploring various transcriptome libraries and reported that approximately 10 % of the 940 annotated R genes exhibited differential expression in response to biotic stress. This agrees with our findings, in which 8% of the R genes (71 out of 940) were differentially expressed at 3 DAI.

After an ETI response, the outcome of a pathogen interaction with its host will be determined by subsequent changes in the cells targeted by the pathogen (Jones & Dangl, 2006; Mazarei et al., 2008). In our study several DEGs previously reported as belonging to the GO "binding" category were found to be functionally annotated as pathway regulators and cell signaling proteins. In the incompatible interaction most of those genes were up-expressed at 3 DAI. Among those DEGs a group of 27 proteins of the Arm-repeat/Armadillo family were observed. Armadillo repeat proteins are involved in multiple regulatory functions, including biotic and abiotic stress responses, transduction of extracellular signals to the nucleus, and are also associated with ubiquitination processes in plants (Mandal et al., 2018; Sharma & Pandey, 2016a; Tewari et al., 2010). Eleven ubiquitin transferases of the E3 group were up-expressed at 3 DAI in the incompatible interaction. Among their functions, E3 ubiquitin transferases are fast-acting enzymes responsible for post-translational modifications in other proteins, marking them for cell signaling, degradation or trafficking functions. (Kelley, 2018; Mazzucotelli et al., 2006). Besides the more evident roles that Armadillo and E3 ubiquitin transferases have in the downstream signaling after a pathogen recognition, the interaction between both proteins and U-box domain proteins in plants has been strongly associated with the maintenance of cytokinin homeostasis (Kim et al., 2003; J. H. Lee et al., 2003; Sharma & Pandey, 2016b). In our study the two highest

up-regulated genes in the "binding" category at 3 DAI for the incompatible interaction were the U-box domain proteins Solyc03g043700 and Solyc06g051090 (LFC: 3.2 and 2.8). The maintenance of cytokinin levels seems like a logical response in the incompatible interaction, as the formation of the nematode feeding sites requires an abundance of this hormone in order to occur (Bird, 2004; Gheysen & Mitchum, 2019). The role of cytokinins in the establishment of compatible interactions with the host is so crucial that second stage juveniles of *M. incognita* produce and secrete cytokinins (Chinnapandi et al., 2017; De Meutter et al., 2003).

Regarding cytokinin catabolism, at 3 and 7 DAI genes involved in cytokinin degradation were up-expressed in the compatible interaction, with the enriched GO terms "BP: cytokinin metabolic process" and "MF: cytokinin dehydrogenase activity" appearing at 7 DAI). Overexpression of cytokinin oxidases are reported in compatible interactions with Meloidogyne (Guimarães et al., 2010; Kumar et al., 2019). It is unclear, however, if the expression of these enzymes is a response to the high concentration of cytokinins caused by the nematode's parasistism, or a specific defense response. In a work by Shukla et al., (2018), the presence of cytokinin oxidases was observed in an incompatible interaction of *M incognita* and tomato at similar stages, this could point to a partial defense response in the compatible interaction of our study. What is clear, however, is that upregulation of cytokinin oxidases are linked to a reduction in gall formations and impaired development of the nematode (Dowd et al., 2017; Lohar et al., 2004). Interestingly, in a previous work, our visual observations of the galls formed in the *Mi-1.2* carrying host "Santy", used in this study were generally much smaller than the galls formed in the susceptible "Santa Clara" when parasitized by the virulent isolate.

Another pathway that was strongly up-expressed in the incompatible interaction was that of the free-calcium (Ca2+) mediated signaling, which includes genes of the calmodulin (CaM) and CaM-like families of eukaryotes, with CaM-like genes being especially abundant in higher plants (Day et al., 2002; Zhao et al., 2013). Changes in the levels of intracellular free calcium (Ca2+) triggered by various stimuli function as secondary signals for a wide range of biological processes in eukaryotic cells. Through a variety of CaM binding proteins acting as intracellular Ca2+ sensors and transducers, these calcium-signaling cascades can regulate plant defense mechanisms and an elicit ETI response that results in programmed cell death (PCD), in a typical HR response (Zhivotovsky & Orrenius, 2011). Together with mitogen-activated protein kinases (MAPKs), calcium binding proteins coordinate transcriptional reprograming of defense genes by triggering the activation of various transcriptional factors (Yuan et al., 2022). The role of CaM and CaM-like genes positively involved in the defense response of plants has been a subject of interest for breeding programs interested in nematode resistance (Ali et al., 2017). In a study

by Matthews et al., (2013), several target soybean genes were overexpressed with the goal of increasing resistance to the soybean cyst nematode *Heterodera glycines*. Among these, Glyma19g19680, encoding the Calmodulin SCaM-3 protein, was able to reduce the number of mature females by 50%. In our study, the suppression of the CaM mediated pathways in the compatible interaction shows that overcoming the resistance triggered by *Mi-1.2* involves, at least to some degree, the down regulation of the calcium signaling network especially the CaM-like Solyc02g088580 gene, which was highly overexpressed in the incompatible interaction for both 3 and 7 DAI.

The onset of a compatible interaction relates to intense physiological changes in the host root system. Several cell wall modifying (CWM) enzymes are known to be exploited by plant nematodes, including *Meloidogyne* spp., they are pectinases, hemicellulases, endoglucanases and expansins. In our study we found several DEGs encoding expansins, most of them up-expressed in the compatible interaction, but not all. Expansins, classified as cell-wall loosening proteins, play a crucial role in facilitating cell wall elongation and expansion, being crucial in the formation of feeding cells (Gheysen & Mitchum, 2009). Additionally, much like cytokinins, these CWMs are also produced and secreted by several phytonematodes (Davis et al., 2011; Hewezi et al., 2008). In their study Gal et al., (2006) localized the expression of plant expansins to the cells surrounding the feeding cells from 4-10 days after the infection of tomato roots by *M. incognita*, with expression rates dropping along that period. Our data followed a similar pattern as expansins DEGs dropped from 3 to 7 DAI. Despite the upregulation of the of expansins, in the compatible interaction at 3 DAI, three were highly up-expressed in the incompatible interaction at the same time as well. Namely Solyc10g084780 (LFC: 3.92) , Solyc12g089380 (LFC:2.94) and Solyc08g080060 (LFC: 1.69). The upregulation of these genes in the incompatible interaction is especially interesting in light of a recent study that reported a high degree of resistance to *M. incognita* of a transgenic soybean overexpressing the expansin (GmEXPA1). In the same study, transgenic lines of *A. thaliana* expressing GmEXPA1 were able to reduce *M. incognita* reproduction factor by 60-90%, while *N. tabacum* lines reduced this same factor by 30-90%. by (Basso et al., 2023). Recently, the longstanding paradigm that plant expansins are solely a facilitator of nematode parasitism has been challenged, as some genes within this class have been described reducing susceptibility to this pathogen (Brasileiro et al., 2021; Guimaraes et al., 2017). In the context of this study, the identified expansins, which were found to be upregulated in the incompatible interaction between *M. javanica* and *S. lycopersicum*, may serve as potential candidate genes implicated in reducing susceptibility to nematodes in tomato genotypes.

Another CWM family that presented several DEGs during our evaluation times was that of the of xyloglucan endotransglucosylase/hydrolases (XTH). The modification of xyloglucans interlaced with cellulose microfibrils of the cell wall by XTH enzymes is a key mechanism of cell wall extension essential for cell elongation during plant growth, fruit development, and response to biotic and abiotic stimuli (Eklöf & Brumer, 2010). In a recent work, higher transcript levels of the soybean GmXTH43 increased resistance to the soybean cyst nematode (SCN) in a susceptible genotype, while lower levels induced through RNA interference increased the susceptibility of a resistant one (Niraula et al., 2021). Besides, in the same work they linked this increase in resistance to the SCN due to higher levels of GmXTH42 transcripts to a rigidity in cell wall that limit the growth of the syncytia cells, which are the equivalent of the feeding cells for *Heterodera* spp. From our data two hypotheses can be drawn regarding the expression of XTH: 1) the higher number of up-expressed transcripts of this gene family could result from the host resuming its natural growth and cellular expression after an incompatible interaction. 2) On the other hand, the decrease in transcripts from 14 to 10 between 3 and 7 DAI, may suggest a diminishing defense response over time following an incompatible interaction, meaning that the pathogen is no longer perceived as a threat by the host.

Despite the limited number of reads aligned to the *M. javanica* genome, a candidate effector gene was detected as up-expressed in the interaction between the virulent isolate and the host with the *Mi-1.2* gene. The gene, encodes a protein with 100% homology to the four identical copies of Minc genes of *M. incognita* of the Minc18876 family. Their predicted protein codes a small glycine and cysteine-rich effector (MiSGCR1) that accumulates in the dorsal gland of second stage juveniles, the expected location for nematode effector production (Nguyen et al., 2018; Hussey & Janssen, 2009). Silencing Minc genes of the Minc18876 family reduced both the population of adult females and the number of egg masses in tomato roots. Furthermore, when introducing this particular gene into transgenic *Nicotiana benthamiana*, its expression in the leaves effectively suppressed programmed cell death induced by Pseudomonas syringae. The up-regulation of this MiSGCR1 in our study at 3 and 7 DAI (LFCs: 5.4/4.8), is in line with the previously mentioned study, in which this effector was found up-expressed in the compatible interaction for juveniles of the same stages we observed in the histological analysis.

A second interesting nematode DEG was also up-expressed (LFC:4.37) by the virulent isolate in the compatible interaction at 3 DAI. This corresponds to a gene of the caveolin family of integral membrane proteins that play a crucial role in the formation and function of caveolae, which are small invaginations or flask-shaped lipid rafts on the cell membrane. Caveolae are involved in various cellular processes, including endocytosis, signal transduction, and lipid

regulation (Chidlow & Sessa, 2010). The role of caveolins in pathogenesis has been an ongoing debate for both animal and plant pathogens (Darwiche et al., 2017; Wilbers et al., 2018). The role of this caveolin in the compatible interaction could be further ascertained by cell localization studies, to verify if its expression occurs in one of the nematode sites for production of effectors, such as dorsal, subventral or pharyngeal glands. It might be interesting to address, however, that invaginations are a common feature in the cell walls of nematode-induced giant cells, which increases overall surface contact and facilitates cell-to-cell transferences (Vilela et al., 2019). Notwithstading, no studies on plant nematode caveolins and its role in parasitism have been conducted to this date.

This research explored the intricate molecular networks during a compatible and an incompatible interaction between a *Mi-1.2* carrying tomato and two *M. javanica* isolates, revealing contrasting interactions and novel genes when compared to previous works involving the *M. incognita* x *S. lycopersicum* pathosystem. Our findings establish a groundwork for subsequent functional investigations aiming to elucidate the specific roles of highlighted genes in facilitating susceptibility to *M. javanica* parasitism. Beyond enhancing our understanding of plant-nematode interactions, these insights pave the way for future studies towards developing targeted control strategies against *Meloidogyne* species, especially the management of the broadly used *Mi-1.2* gene.

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