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Epigenetic and Metabolic Mechanisms in Plant Resistance to Root-Knot Nematodes

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Abstract: Two wild-type field populations of root-knot nematodes (*Mi-Vfield, Mj-TunC2field*), and two isolates selected for virulence in laboratory on resistant tomato cultivars (*SM2V, SM11C2*), were used to induce a resistance reaction in tomato to the soil-borne parasites. Epigenetic and metabolic mechanisms of resistance were detected and compared with those occurring in partially or fully successful infections. The activated epigenetic mechanisms in plant resistance, as opposed to those activated in infected plants, were detected by analysing the methylated status of total DNA, by ELISA methods, and the expression level of key genes involved in the methylation pathway, by qRT-PCR. DNA hypo-methylation and down-regulation of two methyl-transferase genes (*CMT2*, *DRM5*), characterized the only true resistant reaction obtained by inoculating the *Mi-1.2*-carrying resistant tomato cv Rossol with the avirulent field population *Mi-Vfield.* On the contrary, in the roots into which nematodes were allowed to develop and reproduce, total DNA was generally found to be hyper-methylated and methyl-transferase genes up-loaded. DNA hypo-methylation was considered to be the upstream mechanism that triggers the general gene over-expression observed in plant resistance. Gene silencing induced by nematodes may be obtained through DNA hyper-methylation and methyl-transferase gene activation. Plant resistance is also characterized by an inhibition of the anti-oxidant enzyme system and activation of the defence enzyme chitinase, as opposed to the activation of such a system and inhibition of the defence enzyme glucanase in roots infested by nematodes.

Keywords: antioxidant enzymes; DNA methylation; epigenetics; plant resistance; root-knot nematodes; ROS; tomato

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1. Introduction

Genetic natural resistance to root-knot nematodes (RKNs) is conferred in many plant species by a single dominant resistance gene (*R* gene) that specifically recognizes a corresponding avirulence (*Avr*) gene in the nematode. This 'gene-for-gene'interaction results in the initiation of a cascade of defenseresponses, which ultimately lead to the halt of nematode development. Most of our information on the mechanisms underlying defense response of resistant plants upon a nematodeattack is based on RKN-tomato interactions. A high number of genes (*Mi*series) has been identified in some clones of domesticated edible (*Lycopersicon esculentum* L.) and wild type (*L. peruvianum*) tomato[1]. However, the most diffused resistance gene which has been introduced in most commercial resistant tomato cultivars is *Mi-1.2*, conferring resistance against the three most diffused RKN species: *Meloidogyne incognita, M. javanica,* and *M. arenaria*.*Mi-1.2* also confers resistance to specific isolates of the potato aphid, *Macrosiphum euphorbiae* [2] and to two biotypes of the white fly, *Bemisia tabaci* [3]; *Mi-1.2* is the only known *R*-gene that confers resistance against such different groups of pests.Tomato resistance is expressed by a hypersensitive reaction (HR), leading to a rapid and localized cell death and tissue necrosis caused by a specific oxidative burst with enhanced generation and cellular concentration of reactive oxygen species (ROS). Salicylic acid (SA)-dependent defense pathway seems to play an important role in *Mi-1.2*-mediated resistance[4-6], as it has generally beenfound in most *R*-gene-mediated defenses [7]. SA over-production and spreading in root cells contribute to lesion formation and may cause the uncoupling and inhibition ofelectron transport detected in mitochondriaextracted from roots of resistant tomato plants inoculated with RKNs [8]. Host resistance mechanisms implicate a thorough rearrangement of gene expression which leads to the generation of an array of defense proteins involved in phytoalexin, lignin, proteinase inhibitors, and polyphenol biosynthesis [9]. Above all, immune reactionsare always characterized by a high production of pathogenesis-related (PR-)proteins, which are the executioners of plant immunity [10].

The first step in plant innate immunity against pest attacks isa relatively unspecific response, a basal defense, triggered by certain diffused pathogen-associated molecular patterns (PAMPs), known as PAMPs triggered immunity (PTI). A nematode-associated molecular pattern (NAMP, NemaWater) was recently reported to be an activatorof an early PTI response in plants correlated with hydrogen peroxide [11]. Such MPs are recognized in the apoplastic spaces by cell-surface receptorsknown as NLR proteins (nucleotide binding domain, NDB, leucine-rich repeats, LRR) [12]. However, adapted pathogens can circumvent PTI by delivering effector molecules directly into the cells. RKNs are able to suppress plant immune systemthrough an array of effectors directly injectedinto the cells by their stylet and/or secreted from the cuticle in the root apoplasm [13-15]. This suppression leads to silencing or down-regulation of many defense genes in the attacked susceptible plants [16-17]. In *R*-gene carrying plants, however, specific effectors can be recognized by intracellular NLRs in the so-called effector-triggered immunity (ETI). Although nematode penetration is allowed in immunized plants, a deleterious reaction against nematodes is triggeredwhen invading motile juveniles (J2) try to build up their feeding site. Specifically, in tomatoresistance conferred by *Mi-1.2* gene, this deleterious reaction is expressed by a HR, leading to a rapid and localized cell death, whose earliestvisible indications can be seen about 12 h after inoculation of roots with J2 [18].

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Although signaling and transcription factors leading to genome rearrangement and gene up-regulation in plant disease resistance have widely been described [7, 12, 19], the link between disease resistance and DNA methylation has only recently been focused [20]. Biotic interactions can impact plant epigenetic configuration, which, in turn,regulates biotic interactions by modulating plant response [21]. Epigenetics studies the heritable changes in gene function that do not depend on DNA sequence, such as DNA methylation and de-methylation, chromatin rearrangements, and histone modification. DNA methylation consists in the addition of a methyl group to the cytosine bases of DNA to form 5-methyl-deossi-cytosine. The amount of methylated DNA in plants is determined by *de novo* DNA methylation, methylation maintenance, and DNA de-methylation [22]. *De novo* methylation is catalyzed by DOMAINS REARRANGED METHYL-TRANSFERASES (DRMs), whilst maintenance is performed by three classes of enzymes: the most predominant CG methylation by methyl-transferase 1 (Met1), CHG methylation by chromo-methyl-transferases (CMT2and CMT3) and CHH methylation by DRM2 or CMT2.The RNA directed DNA methylation (RdDM) pathway promotes the sequence targeting by *DRMs*, through the synthesis of small-RNAs (smRNAs) [20].

The activation of different types of epigenetic mechanisms upon nematode infection has extensively been reported [23]. Gene silencing, produced in successful development of nematodes on susceptible plants through the manipulation of phyto-hormone pathways, has been ascribed to the activation of smRNAs and miRNAs pathways [16, 24]. In this study, we confronted the epigenetic and metabolic mechanisms exerted by susceptible and resistant tomato plants challenged by RKNs. To do so, we used either field-collected nematode populations or isolates selected for virulence that broke the resistance of the *Mi-1.2*-carrying tomato cultivars. Epigenetic changes seemed mainly to occur in plants attacked by wild-type field populations.

2. Results and Discussion

2.1 Resistance and susceptibilityof tomato to RKNs

Plants are defined as resistant when the attacking nematodes show reduced levels of reproduction [25]. Only when reproduction is as low as 50-25% of that on a fully susceptible cultivar, tomato plants are generally considered as slightly resistant to RKNs; full resistant plants usually support a reproduction lowerthan 10%.In nature, resistance-breaking virulent populations are selected from avirulent wild-type populations by repeated exposures to *R*-genes. In a study involving tens of *M. incognita* fieldpopulations, this “natural” selection has been mimicked in controlled greenhouse conditionsby repeated inoculations of the progeny of those individuals which were able to develop andreproduce on *Mi-1*-carrying tomato [26]. It was possible to produce, from most of the tested avirulent field population, virulent isolates that reached their full reproductivepotential within the second/third generation developed on resistant tomato. Some *M. incognita* populations exist in tropical or subtropical countries, commonly referred to as “natural virulent”, that may or may not have had a previous and not documented exposure to *Mi-1* gene or analogues.

We used two couples of field populations/virulent isolates to test resistant (Rossol cv) and susceptible (Roma VF cv) tomato plants. We produced partial and full resistant and susceptible responses and compared the associated epigenetic and metabolic mechanisms. The virulent isolate *SM2V* was selected from an avirulent field population of *M. incognita* (*Mi-Vfield*), collected from

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Venezuela. Conversely, the virulent isolate *SM11C2* was selected from a partial virulent wild-type population of *M. javanica* (*Mj-TunC2field*), collected from Tunisia. We tested these RKN populations/isolates in their reproduction potential (RP), their ability to produce egg masses (EMs/RS), and in their female fecundity (FF), after infestation on resistant and susceptible tomato plants (Figures 1-2).

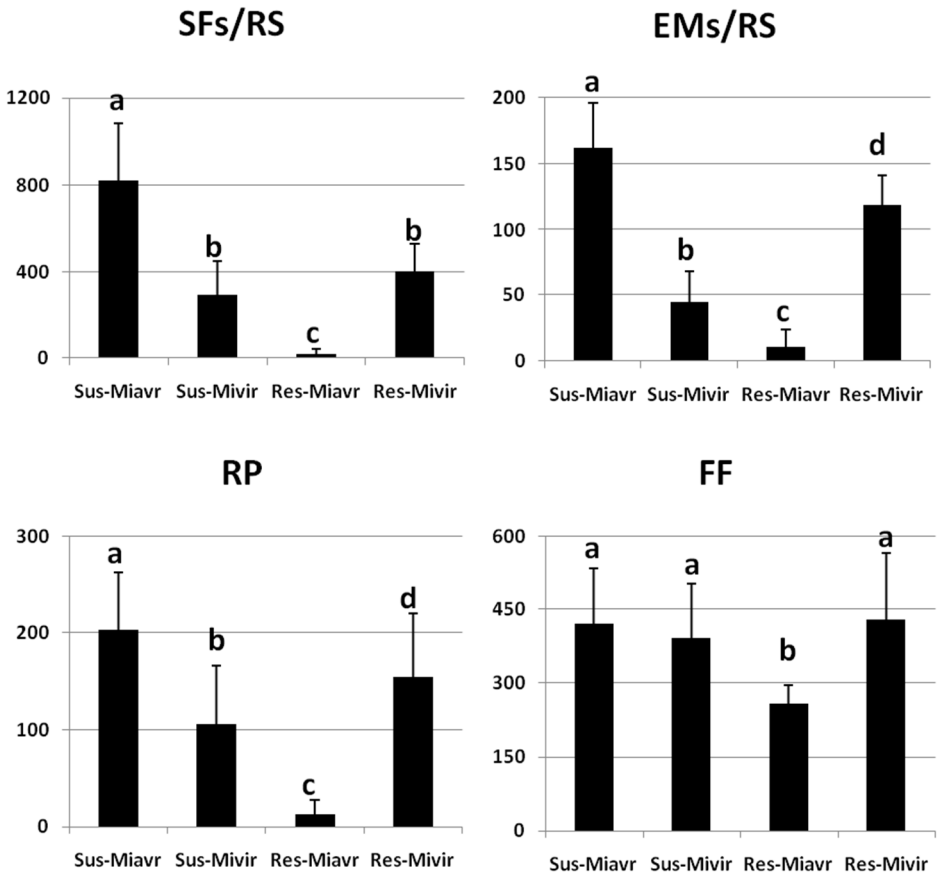

Figure 1. Infection factors of susceptible and resistant tomato plants inoculated with the avirulent *Meloidogyne incognita* field population *Mi-Vfield* and the selected virulent isolate *SM2V*. Infection factors of four interactions are shown: Roma VF/*Mi-Vfield* (Sus-Miavr); Roma VF/*SM2V* (Sus-Mivir); Rossol/*Mi-Vfield* (Res-Miavr); Rossol/*SM2V* (Res-Mivir). Infection level was characterized by the numbers of Sedentary Forms per Root System (SFs/RS), Egg Masses per Root System (EMs/RS), Reproduction Potential (RP), and Female Fecundity (FF). Values are expressed as means (n=9) ± standard deviations. Means were separated by a Duncan's Test (Significance Level: 0.05).

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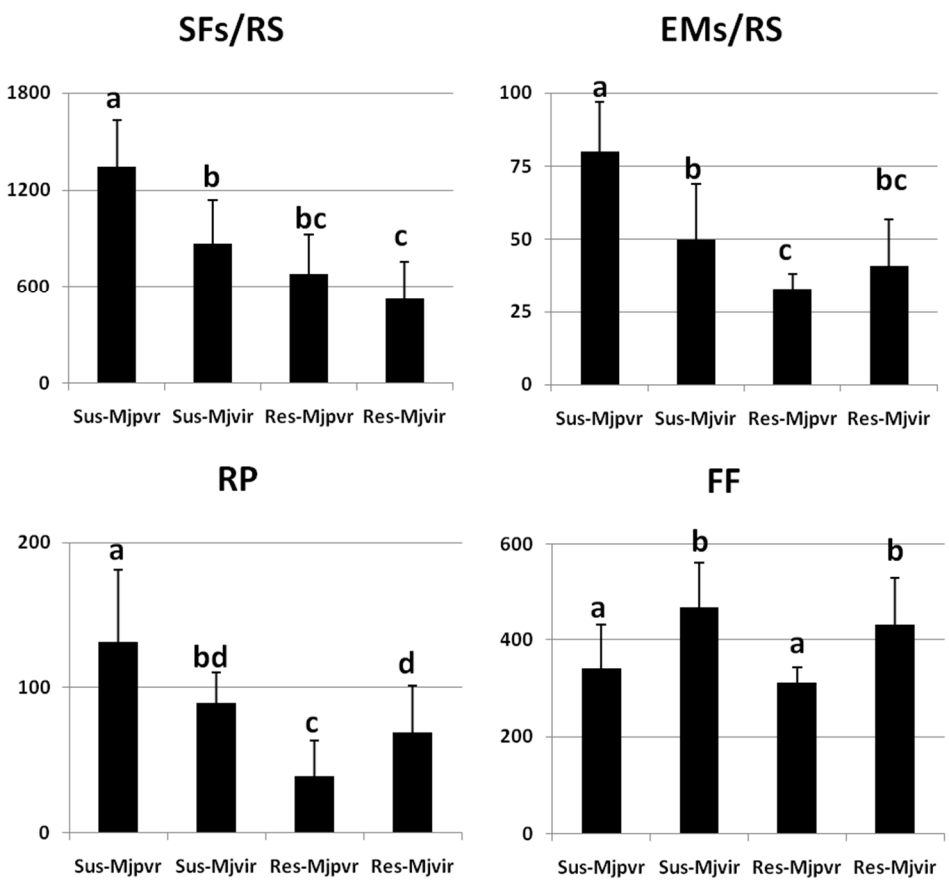

Figure 2. Infection factors of susceptible and resistant tomato plants inoculated with the partially virulent *Meloidogyne javanica* field population *Mj-Tunc2field* and the selected virulent isolate *SM11C2*. Infection factors of four interactions are shown: Roma VF/*Mj-TunC2field* (Sus-Mjpvr); Roma VF/*SM11C2* (Sus-Mjvir); Rossol/*Mj-TunC2field* (Res-Mjpvr); Rossol/*SM11C2* (Res-Mjvir). Infection level was characterized by the numbers of Sedentary Forms per Root System (SFs/RS), Egg Masses per Root System (EMs/RS), Reproduction Potential (RP), and Female Fecundity (FF). Values are expressed as means (n=9) ± standard deviations. Means were separated by a Duncan's Test (Significance Level: 0.05).

Such tests have to give information also on factors that can be related to actual disease severity and plant damage. The extent of galling in RKN-plant interactions determines root damage that impairs nutrient and water transports along the plant, thus causing poor shoot growth and yield loss. The common gall index, detected by a direct visual analysis of the roots, may be more suitable to "yes or no" statements of nematode infestation, but seems a rather subjective indicator in distinguishing intermediate degrees of galling, as it occurs in partial resistance or susceptibility [27]. Therefore, we used the number of developed sedentary individuals extracted from roots (SF/RS) as a more quantitative factor of root damage and gall index, since galling is a reaction to motile individuals that enter the roots, establish a feeding site, and turn into sedentary developmental stages [26].

The avirulent field population *Mi-Vfield* showed a high RP and high numbers of SFs/RS on susceptible tomato (approx. 200 and 800, respectively) but very low ones (approx. 13 and 17) on resistant tomato, as expected (Figure 1). The ability to develop and reproduce on resistant tomato drastically increased in the virulent isolate *SM2V*. The partial virulent wild-type population *Mj-*

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TunC2field was very aggressive on susceptible tomato with more than 1200 SFs/RS (Figure 2). Increased competition for food lessened both FF and RP of *Mj-TunC2field*, with respect to those of *Mi-Vfield*. Of course, *Mj-TunC2field* was much less aggressive on resistant tomato, showing a relatively low RP value. The virulent isolate *SM11C2* developed on resistant tomato at a level similar to its starting field population *Mj-TunC2field*; conversely, because of its higher FF value, its overall reproductive rate resulted higher. The selective pressure operated on both isolates and the resulting genetic homogeneity may have been the cause of the observed diminished aggressiveness and reproduction rate on susceptible plants, with respect to the respective starting field populations (Figure 1-2). *Mi-1.2*-conferred resistance was broken by those virulent isolates, although they could not achieve the reproduction rates occurring in full compatibility. Actually, full compatibility was realized only in the interactions between field populations and susceptible tomato, whilst full incompatibility in the interaction between *Mi-Vfield* and Rossol.

2.2 DNA methylation in resistance and susceptibility of tomato to RKNs

Generally, resistance reaction, HR and plant priming against RKNs are associated with a diffused up-regulation of defense genes, and, in particular, of pathogenesis-related genes (*PR*-genes) [6,17, 28, 29]; in contrast, susceptibility is characterized by *PR*-gene down-loading and SA-signaling inhibition, although some genes, encoding for anti-oxidant enzymes and their activities, were found to be activated [17].

Epigenetic changes were shown to regulate the expression of genes involved in plant resistance response [20]. DNA methylation, besides many other biological processes, is involved in mechanisms underlying plant response to pathogen and nematode attacks [20, 23]. Methylation on promoter regions negatively correlates with gene expression levels [30], whereas DNA hypo-methylation has been associated with plant defense against nematodes [23]. Hypo-methylation of resistance genes enhances their expression to rapidly respond to environmental factors [20].

Relative 5-mdC immunofluorescence, indicating the methylation state of total DNA, was detected in roots collected from a series of compatible and one incompatible tomato-RKN interactions (Figure 3). A significant hypo-methylation was observed in the incompatible interaction Res/Miavr, with respect to uninfected roots (Figure 3A), whereas hyper-methylation characterized fully successful nematode infections (Sus/Miavr, Sus/Mjpvr), already at 7 DAI (Figure 3A, B). Interestingly, infections by virulent isolates to both resistant and susceptible plants (Res-Sus/Mivir, Res-Sus/Mjvir) did not produce significant changes in DNA methylation of roots, with respect to uninfected controls (Figure 3). These data show that DNA hypo-methylation characterizes enhanced defense gene expression in immune response, triggered by *R*-genes (ETI) against RKNs. Therefore, our results bring additional evidence to the reported finding that DNA hypo-methylation is part of a conserved PTI response in monocot and dicot plants [23], indicating that such a mechanism is involved also in plant genetic resistance to RKNs (ETI). In contrast, gene silencing, induced by nematodes in host plants, may result from the hyper-methylation of total DNA observed in the present study in successfully infected susceptible plants. Unfortunately, no data exist on changes in defense gene expression of roots attacked by selected virulent isolates. Surely, infections caused by such isolates are much less severe than standard ones (Figures 1-2), although nematode development and reproduction is somehow allowed in such interactions. Taken together, these data

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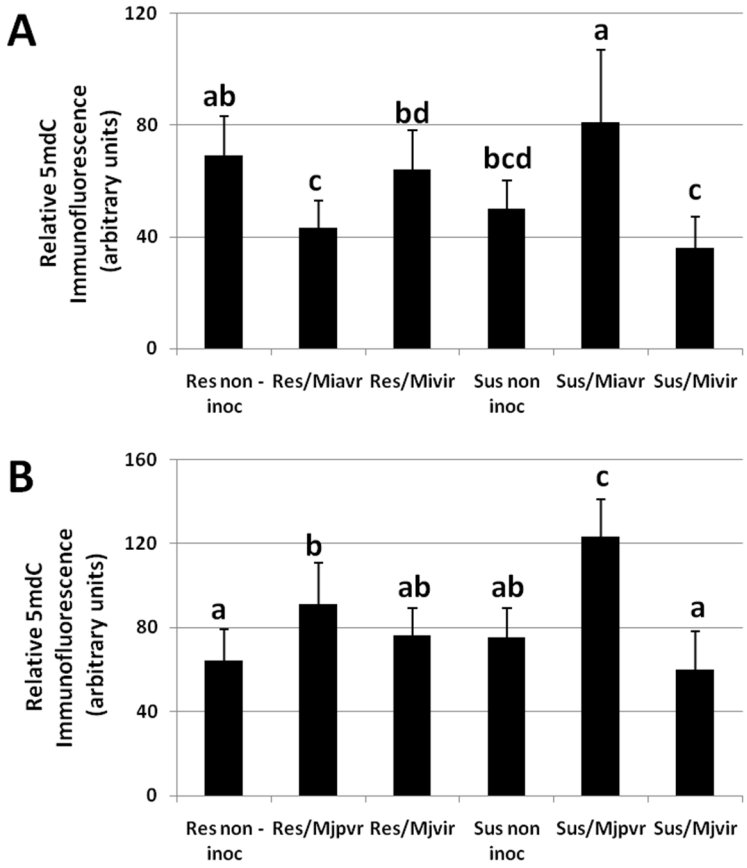

Figure 3. Percentages of DNA methylation on total DNA in roots of susceptible and resistant tomato plants un-inoculated or inoculated with 2 couples of field populations/virulent isolates of RKNs. Data were taken at 7 DAI. DNA was extracted from resistant (Res non-inoc, Rossol) and susceptible (Sus non-inoc, Roma VF) un-inoculated plants. In A, DNA was also extracted from roots of Rossol and Roma VF plants inoculated with the avirulent *Meloidogyne incognita* field population *Mi-Vfield* and the selected virulent isolate *SM2V*: Rossol/*Mi-Vfield* (Res-Miavr); Rossol/*SM2V* (Res-Mivir); Roma VF/*Mi-Vfield* (Sus-Miavr); Roma VF/*SM2V* (Sus-Mivir). In B, DNA was also extracted from roots of Rossol and Roma VF plants inoculated with the partially virulent *Meloidogyne javanica* field population *Mj-Tunc2field* and the selected virulent isolate*SM11C2*: Rossol/*Mj-TunC2field* (Res-Mjpvr); Rossol/*SM11C2* (Res-Mjvir); Roma VF/*Mj-TunC2field* (Sus-Mjpvr); Roma VF/*SM11C2* (Sus-Mjvir). Values are expressed as arbitrary units of relative 5mdC immunofluorescence and as means (n=6) ± SD. Means were separated by a Duncan's Test (Significance Level: 0.05).

suggest that DNA hypo-and hyper-methylation occur only in full resistance and full susceptibility, respectively.

2.3 Expression of genes involved in DNA methylation

We detected the expression of *Met1* (A), *CMT2* (B), and *DRM5* (C) genes in roots of resistant (Res) and susceptible (Sus) plants, infected by the avirulent field population *Mi-Vfield* (Miavr) and the selected virulent isolate *SM2V* (Mivir), and confronted these gene expressions with the ones from un-infected roots (Figure 4). The most interesting result is the drastic repression of *DRM5* and the halving of the *CMT2* transcript amounts in the incompatible interaction with respect to the un-infected resistant roots (Res-Miavr, Figures 4B-4C). The reported DNA hypo-methylation in this same interaction can undoubtedly be associated with the suppression of *de novo* methylation and the

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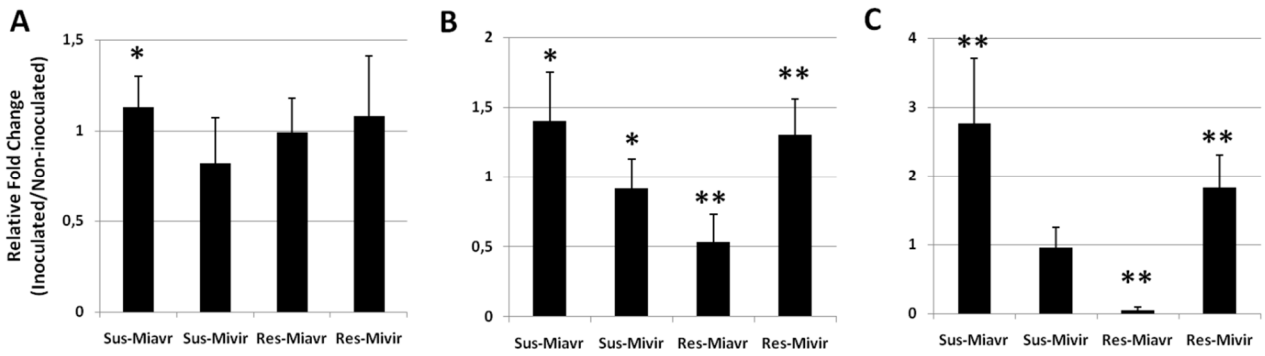

Figure 4. Expression of *Met1* (A), *CMT2* (B), and *DRM 5* (C) genes in susceptible (Sus) and resistant (Res) tomato roots inoculated with the RKN field population *Mi-Vfield* (Miavr) and the virulent isolate *SM2V* (Mivir). Gene expression was detected at 7 DAI by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Data are the mean fold changes (n=6) ± SD in gene transcript levels of tissues from inoculated plants compared with tissues from non-inoculated control plants (the value of 1 indicates no change). Asterisks indicate that the mean fold change is significantly different from 1 as determined by a t test (*P<0.05; **P<0.01).

inhibition of methylation maintenance. On the contrary, these genes had their expression enhanced in all the tested compatible interactions (Sus-Miavr, Sus-Mivir, Res-Mivir). Full compatible interaction (Sus-Miavr) showed the highest expression of all tested genes, thus supporting the finding of a high DNA methylation state in such an interaction with the possible consequent gene silencing.

2.4 Metabolic mechanisms in resistance and susceptibility of tomato to RKNs

Catalase activity (CAT) changes seems to be strictly associated with tomato metabolic response to RKNs. Inhibition of CAT was found to occur in resistance response, whilst CAT was enhanced in successful infections (Figure 5C, D). CAT inhibition was found also in leaves of inoculated resistant plants as early as 1 day after inoculation (DAI). SA is a known inhibitor of root and leaf CAT [31]. SA was found to be over-produced in roots and shoots of resistant tomato attacked by *M. incognita* [32]. Actually, most of the SA produced in roots may rapidly be transferred to leaves, in analogy with the SA adsorbed by roots from a solution [31]. SA is the main mediator for systemic acquired resistance (SAR), which provides long-term resistance to hemi-biotrophic pathogens and pests, and is correlated to the activation of *PR*-genes [17]. SAR has been reported to occur in upper leaves, in response to the attacks of pathogens at lower leaves, causing cell death and tissue necrosis [33]. Cell death and tissue necrosis result in roots from the HR triggered by immune response to RKNs and are most likely caused by an elevated concentration of SA, through the potentiation of ROS generation [34]. Over-expression of *PR*-genes in leaves of *Mi-1.2*-carrying tomato plants has been reported 5 DAI with *M. incognita*; thus, induction of SAR in leaves is likely to be the result of SA generation in roots and its movement upwards [6]. Successful defense reaction can be explicated only if ROS level is maintained high by ROS over-production and inhibition of anti-oxidant metabolic mechanisms. In addition to CAT, SOD was also inhibited at the earliest stage of an incompatible interaction, whilst APX restriction seems not to be involved in the resistance

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response (Figures 5A, 5F). Conversely, successful infection required high activities of the anti-oxidant enzymes in roots, CAT included (Figures 5B, C, E).

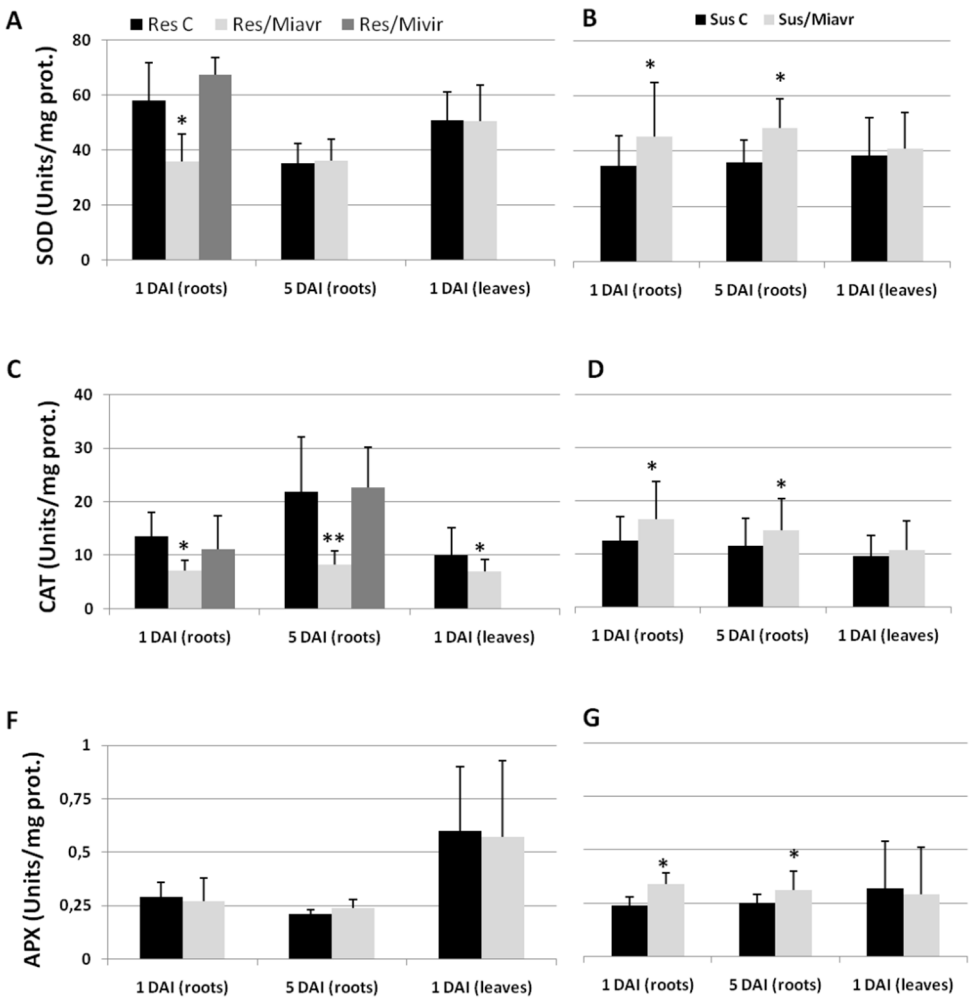

Figure 5. Anti-oxidant enzyme activities of resistant (Res C) and susceptible (SuS C) un-inoculated control plants compared with those of plants inoculated with the avirulent field population *Mi-Vfield* (Res/-Sus/Miavr) and with the virulent isolate *SM2V* (Res/Mivir). Superoxide dismutase (SOD - A, B), catalase (CAT – C, D), and ascorbate peroxidase (APX – F, G) activities are expressed as Units x mg^{-1} prot. Protein extraction was carried out using roots 1 and 5 DAI and using leaves 1 DAI. Values are shown as means (n=9) ± SD. Means coming from the inoculated roots were separated from those of un-inoculated control roots by a t-test. Asterisks indicate significant difference compared with controls (*P<0.05; **P<0.01).

High CAT activity may result from the reported up-loading of *CAT* gene in compatible tomato-RKN interactions, which was analogously observed only in infected roots [17]. Activation of anti-oxidant enzymes, induced by nematodes in the host plants, is functional for their protection from the ROS typically generated in the early response to biotic challenges. For instance, RKNs have been reported to secrete an effector (MjTTL5), which interacts directly with another anti-oxidant enzyme in *Arabidopsis*, the ferrodoxin : thioredoxin reductase catalytic subunit (FTRc), to induce an enhanced ROS-scavenging activity [36].

GLU and CHI are defense-induced enzymes in plants. GLU increased in leaves of infested plants at 1 DAI; root GLU was left unaffected (Figure 6A). CHI was found to be enhanced in roots of

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resistant plants inoculated with RKNs at 1 and 5 DAI, with respect to un-inoculated plants (Figure 6C). Activation of CHI and over-expression of its encoding gene *PR-3* have already been reported in genetic and induced plant resistance to RKNs [17, 36]. On the contrary, in susceptible tomato-*M. incognita* interaction, GLU resulted inhibited in the roots (5 DAI) and in the leaves (1 DAI) of infested plants (Figure 6B). No change in CHI was detected (Figure 6D).

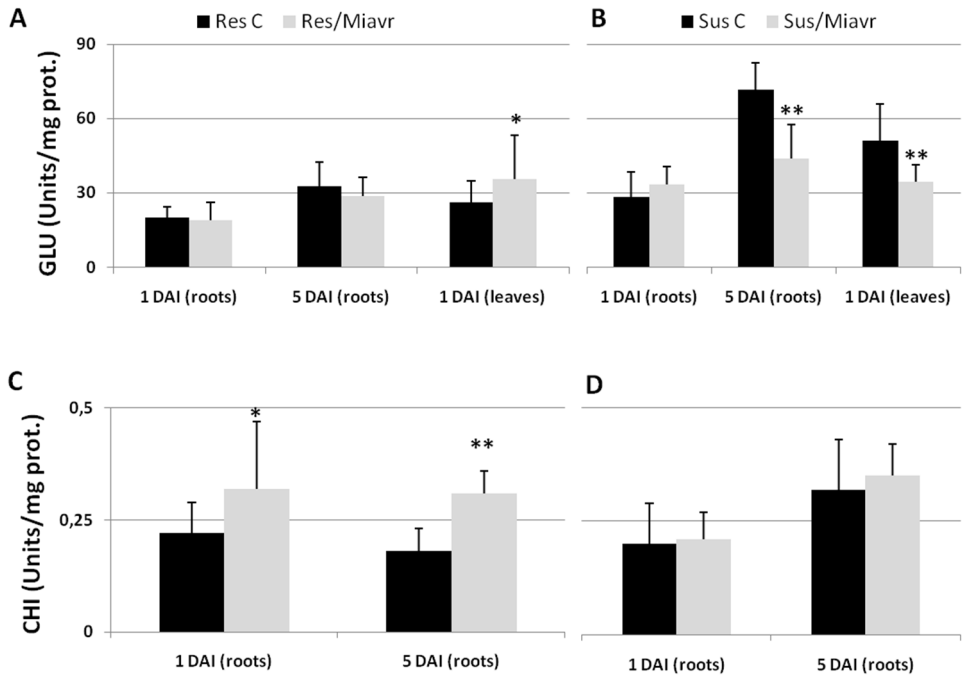

Figure 6. Defense enzyme activities of resistant (Res C) and susceptible (SuS C) un-inoculated control plants and of plants inoculated with the avirulent field population *Mi-Vfield* (Res/-Sus/Miavr) or with the virulent isolate *SM2V* (Res/Mivir). Glucanase (GLU – A, B), and chitinase (CHI – C, D) activities are expressed as Units x mg^{-1} prot. Protein extraction was carried out using roots 1 and 5 DAI and using leaves 1 DAI, as it concerned GLU; CHI was assayed only in roots 1 and 5 DAI. Values are shown as means (n=9) ± SD. Means coming from the inoculated roots were separated from those of un-inoculated control roots by a t-test. Asterisks indicate significant difference compared with controls (*P<0.05; **P<0.01).

Accordingly, *PR-2*, the gene encoding for endoglucanases, was found to be down-loaded in roots of susceptible tomato plants at 5 DAI with *M. incognita*, but over-expressed in leaves of inoculated resistant plants [6].

2.5 Concluding Remarks

Epigenetic changes in total DNA methylation are being recognized of paramount importance in determining the outcome of nematode-plant interactions. DNA hypo-methylation, probably caused by the inhibition of methyl-transferase gene expressions, seems to trigger the enhanced expression of defense genes in PTI and ETI of plants against RKNs. On the contrary, gene silencing in nematode heavily infected plants is likely to be preceded by DNA hyper-methylation and activation of methyl-transferase gene expressions. Interestingly, in infections caused by genetically-

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homogeneous selected isolates to both susceptible or resistant plants, which were characterized by a less consistent numbers of galls, DNA methylation general status of roots seems not to be enhanced, although the expression of some methyl-transferase genes was found to be increased.

It is now generally recognized that plant epigenome can influence plant phenotype and biotic interactions. However, soil-borne endo-parasites, such as RKNs, can in turn induce epigenetic changes to address plant metabolism in order to favoring the most suitable conditions for their own development. Genetic resistance do not allow RKNs to impair plant immune reaction by rapidly producing PR-proteins and ROS, toxic to the invading J2. Defense gene expression seems to be supported by a consistent de-methylation of total DNA. Moreover, the general process of plant priming against pests, diseases, and abiotic stresses may be based on similar epigenetic modifications that suppress or enhance the transcription of key regulators of the immune system. These environment-induced epigenetic changes may be transmitted to next generations for acclimation to a changing environment. Research into the relationship between epigenetics and biotic interactions should be supported to have information on plant adaptation and crop improvement to face the increasing emergence of local or alien pests, also in view of the climate changes we are experiencing. The knowledge of such mechanisms would lead to the arrangements of environmental-friendly strategies for sustainable protection against (a)biotic challenges based on the long-lasting immune memory in plants.

3. Materials and Methods

3.1 Nematode populations

Two virulent isolates (*SM2V, SM11C2*) were selected from 2 field populations (*Mi-Vfield* and *Mj-TunC2field*, respectively) by repeated mass inoculation on *Mi*-carrying resistant tomato cvs, as described in [37]. *Mi-Vfield* and *Mj-TunC2field* were collected from infested plants located in fields in Venezuela and Tunisia, respectively, maintained on susceptible tomato in a glasshouse. Nematodes were species identified as *M. incognita* (*Mi-Vfield*) and *M. javanica* (*Mj-TunC2field*) by means of isozyme electrophoretic patterns of esterase and malate dehydrogenase. *Mi-Vfield* had an initial negligible reproduction on resistant tomato, therefore it was classified as “avirulent” field population; in contrast, *Mj-TunC2field* had an initial consistent reproduction on resistant tomato and was classified as “natural partially virulent field population”. A higher number of repeated inoculations on resistant tomato occurred to select *SM2V* from *Mi-Vfield* than those needed to select *SM11C2* from *Mj-TunC2field*. Selection was considered to be completed when the selected isolate reached a reproduction rate, on resistant tomato, that could not significantly be exceeded by the next generation.

3.2 Preparation of plants and nematode inoculations

Seedlings of the cv Roma VF were used as the tomato (*Solanum lycopersicum* L.) line susceptible to root-knot nematodes (RKNs), whilst the *Mi1.2*-carrying resistant cvs used were Motelle, VFN8, and Rossol [38]. All resistant cvs were used to select virulent isolates from RKN field populations. Rossol was used as the tomato line resistant to RKNs in all experiments. Seeds were surface sterilized and sown in a sterilized mixture of peat and soil at 23–25 °C in a glasshouse.

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Plantlets were transplanted to 100 cm^3 clay pots filled with an autoclaved mixture of loamy soil and sand (1+1 by volume). Pots were randomly placed on temperature-controlled benches (soil temperature 24–25 °C), located in a glasshouse. A regular regime of 12 h light/day was set, and plants were regularly watered with Hoagland's solution. Plants, before being inoculated with nematodes, were allowed to grow to the 3–5 compound leaves stage and to 2-4 g fresh weight. Field nematode populations and their respective virulent isolates were used to inoculate both Roma VF (Sus) and Rossol (Res) plants. Eight different tomato-RKN interactions were then analyzed and named as follows:1) Roma VF/*Mi-Vfield* (Sus-Miavr); 2) Roma VF/*SM2V* (Sus-Mivir); 3) Rossol/*Mi-Vfield* (Res-Miavr); 4) Rossol/*SM2V* (Res-Mivir); 5) Roma VF/*Mj-TunC2field* (Sus-Mjpvr); 6) Roma VF/*SM11C2* (Sus-Mjvir); 7) Rossol/ *Mj-TunC2field* (Res-Mjpvr); 8) Rossol/*SM11C2* (Res-Mjvir). Inoculations were carried out by pouring, into 2 holes made at the base of each plant, few milliliters of a stirring water suspension containing 250 active J2. J2 used for inoculation were obtained by incubation of the respective egg masses in tap water at 27°C for 2–3 days.

3.3 Tests of tomato resistance and susceptibility to RKNs

Plants were harvested approximately 7 weeks after inoculation to let nematodes complete their life-cycle and plants be infested by the second generation. Roots were cut from shoots and washed free of soil debris. Weights of roots and shoots were measured. Two root systems of plants from the same interaction were chopped into pieces of about 2 cm length and accurately mixed to be used for nematode life-stage extractions and counting. Three different samples of about 2 g were separated from the mix to be used for: i) egg masses (EMs) counting; ii) eggs extraction; iii) developed sedentary forms (SFs) extraction. For EMs counting, root samples were immersed in a solution (0.1 g L^{-1}) of the colorant Eosin Yellow for at least 1 h in a refrigerator. Red-colored EMs were then counted under a stereoscope (6 × magnification). Eggs were extracted by sodium hypochloride, according to the protocol described in [39]. Extraction of sedentary J3, J4 and swollen females from roots was carried out by incubation with pectinase and cellulase enzyme mixture at 37° C in an orbital shaker to soften the roots. After a brief homogenization in physiological solution, SFs were collected on a 90 µm sieve and counted under a stereoscope (12 × magnification). Eggs suspensions were counted under a stereoscope at 25 × magnification. This counting allowed to have values of EMs, eggs, and SFs per root system (RS) for the eight tested nematode-tomato interactions. These values were used to calculate 2 additional infection factors:

1. Reproduction Potential = n. eggs (root system)/n. inoculated J2 (RP); this factor indicates the number of times the initial population (Pi) multiplies at the end of the experimental time (Pf). RP is particularly important to predict the population density to which the next crop will be exposed
2. Female Fecundity = n. eggs (root system)/n. EMs (root system) (FF); it indicates the average number of the eggs laid by a single female.

In the experimental conditions adopted in this study, only the inoculated J2 can reach the reproductive stage (gravid females producing eggs embedded in EMs). The juveniles hatched in pots from these eggs can develop into sedentary forms, but cannot reach the reproductive stage.This is why SFs/RS can exceed the one thousand units as compared with the 250 J2 inoculated per plant in a fully compatible interaction (field populations versus susceptible tomato). Furthermore, approximately 50% of the inoculated J2 reach the reproductive stage in a fully compatible

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interaction. On the other hand, when a plant-nematode interaction produces a RP 50-25% lower than that from a fully compatible interaction, a partial resistance response can be predicted. Actually, values of EMs, RP, and FF are indicative of the infection level caused by the first generation produced by the artificial inoculation and the reproduction rate of the populations/isolates. Conversely, SFs gives an indication of the aggressiveness of the second generation of the invasive J2 hatched in the soil, as well as the level of root galling and plant damage caused by the populations/isolates.

3.4 5-mdC ELISA-based immunoassays

Roots from Rossol and RomaVF tomato cvs, un-inoculated and inoculated with the field population/virulent isolate couples (*Mi-Vfield/SM2V, Mj-TunC2field/SM11C2*), were used, at the 7th day after inoculation, to extract total DNA by a plant genomic DNA extraction kit (DNA-easy Plant Mini, Qiagen), according to the manufacturer's instructions.To compare relative levels of global DNA methylation between healthy and infested roots, the 5-mdC DNA ELISA kit D5325 (Zymo Research Corporation, Irvine,California, U.S.A.) was used, according to the manufacturer's instruction. DNAaliquots (100 ng) were denaturated and incubated with a mix consisting of anti-5-deoxy-methylcytosine (5-mC) and secondary (horseradish peroxidase conjugate) antibodies. After incubation, these mixtures were added to ELISA plates. Percentages of methylated DNA could be measured by reading the absorbance in an ELISA plate reader at 450 nm. A standard curve of absorbance at 450 nm, as a function of known percentages of 5-mC, had previously to be plotted. The % 5-mC of unknown samples could be calculated by a complex equation derived from the logarithmic second-order regression standard curve. Negative control readings were subtracted from the readings of the sample and the standard. The reported values are the means of the absorbance taken at 45 and 60 min since the start of the reactions. Technical duplicated or triplicate DNA samples were obtained from three independent biological assays.

3.5 RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction

RNA isolation was carried outfrom the roots of susceptible (Roma VF) and resistant (Rossol) tomato plants, un-inoculated and inoculated with the field population/virulent isolate couple (*Mi-Vfield/SM2V*), at the 7th DAI. An RNA-easy Plant Mini Kit (Qiagen, Germany), according to the instructions specified by the manufacturer, was used. RNA quality was verified by electrophoresis runs on 1.0% agarose gel and quantified using a Nano-drop spectrophotometer. cDNAs were obtained using the QuantiTect Reverse Transcripton Kit (Qiagen, Germany). qRT-PCR was carried out with the SYBR Select Master Mix (Applied Biosystems, Italy) according to supplier's indications, using an Applied Biosystems 1 StepOne™ instrument. Amplification conditions were as follows: PCR cycling consisted in an initial denaturation step at 95 ˚C (10 min); 40 cycles at 95 ˚C (30 s), at 58 ˚C (30 s), at 72 ˚C (30 s), with the final step at 60 ˚C (1 min), denaturation at 95°C for 10 min. Oligonucleotide primers are described in Table S1. The following genes were tested: cytosine-5 DNA methyl-transferase 1 (*Met1*), chromo methyl-transferase 2 (*CMT2*), and DOMAINS REARRANGED METHYL-TRANSFERASE 5(*DRM5*). Three biological replicates were done for each gene. For each oligonucleotide set, a no-template water control was used. Quantification was carried out using the Actin as reference gene for the experimental conditions

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(infected versus uninfected) used in this work and the threshold cycle number (C_t) for each transcript quantification were examined.The relative fold changes in gene expression was calculated by the $2^{-\Delta\Delta CT}$ method [40].

3.6 Protein extraction and enzyme activity assays

Proteins were extracted from roots and leaves of un-inoculated and inoculated plants 1 and 5 DAI, and 1 DAI, respectively. Roots and leaves were separated from shoots. Tissues from each RKN-tomato interaction were collected, dried, weighed and put on ice. Tissue samples were immediately used for protein extractions or stored at -80˚C. Samples were ground in porcelain mortars by immersion in liquid nitrogen. Powdered samples were suspended in a grinding buffer (1:5 w:v) of 0.1M K-phosphate buffer (pH 6.0), 4% poly-vinyl-pyrrolidone and the protease inhibitor phenyl-methane-sulfonyl fluoride (PMSF, 1 mM). Suspensions were further ground using a Polytron1 PT–10–35 (Kinematica GmbH, Switzerland), and filtered through four layers of gauze. Filtrates were centrifuged at 12000 x *g* for 15 min. Supernatants were filtered through 0.45 µm nitrocellulose filters applied to 10-ml syringes. These filtrates were ultra-filtered at 4˚C through 20-ml Vivaspin micro-concentrators (10,000 molecular weight cut off, Sartorius Stedim, Biotech GmbH, Germany). Retained protein suspensions were used for protein content and enzyme assays. Protein content was determined by the enhanced alkaline copper protein assay, with bovine serum albumin, as the standard [41].

Superoxide Dismutase activity (SOD) was assessed as the percentage of inhibition on the reduction of cytochrome *c* (80 µM) by the xanthine (1 mM)-xanthine oxidase (20 mU) system in 1 ml assay medium of 0.1 M Na-K-phosphate buffer (pH 7.8), 20 mM NaN_3 and 1 mM EDTA. Standard reactions were carried out with 25-50 µl of extraction buffer. Reactions were started by adding xanthine oxidase and monitored at 550/540 nm, in a 557 Perkin-Emer double-beam spectrophotometer. One unit of SOD activity represents the amount of enzyme able to produce a 50% inhibition on standard reaction [42]. Catalase activity (CAT) of tissue extracts was measured as the initial rate of disappearance of hydrogen peroxide [43], using 20 mM H_2O_2 and 25 ml sample in 0.1 M Na-phosphate buffer, pH 7.0; the rate of H_2O_2 disappearance was followed as decrease in the absorbance at 240 nm and oxidation of 1 mmole H_2O_2 min^{-1} (e = 0.038 mM^{-1} cm^{-1}) represented one unit of enzyme. Ascorbate peroxidase activity (APX) was determined as the rate of oxidation of ascorbate by H_2O_2 [44]. Reaction mixtures contained 0.1M TES, pH 7.0, 0.1 mM EDTA, 1 mM ascorbate, 0.1 mM H_2O_2, 10–20 µl tissue extracts, in 0.5 ml final volume. Ascorbate oxidation was monitored as a decrease in absorbance at 298 nm; 1unit of enzyme expresses the oxidation of 1 µmole ascorbate min^{-1} (ε = 0.8 mM^{-1} cm^{-1}).

β-1,3-Endoglucanase activity (GLU) was measured as the amount of glucose released from laminarin (Sigma, Italy) used as substrate. One hundred µl tissue extracts were added to 300 µl 0.1M Na-acetate (pH 5.2) and laminarin (0.4 mg) in plastic eppendorfs, which were incubated at 37˚C for 30 min. After incubation for glucose production, Nelson alkaline copper reagent (300 µl) was added and the mixtures kept at 100˚C for 10 min. After cooling at room temperature, Nelson chromogenic reagent (100 µl) was added for reducing sugars assays [45]. Negative and positive controls consisted of grinding buffer and laminarinase (2 U/ml), respectively. Enzyme activity was expressed as µmol glucose equivalents released min^{-1}, according to a standard curve created with known amounts (10–200 µg ml-1) of commercial glucose (Sigma, Italy).

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Chitinase activity (CHI) was measured by a colorimetric procedure that detects N-acetyl-D-glucosamine (NAG) [46]. The hydrolytic action of chitinase produces chitobiose which is converted into NAG by the β-glucuronidase introduced in the reaction mixture. Na-acetate buffer (150 µl, 0.05M, pH 5.2) containing 0.5 M NaCl was added with suspended chitin (250 µl, 10 mg/ml) from shrimp shells (Sigma-Aldrich, Italy). The mixtures were prepared in eppendorfs, which were incubated for 1 h at 37˚C in an orbital incubator to let the reaction occur at the most suitable temperature. The reaction was stopped by boiling at 100˚C for 5 min in a water bath. Eppendorfs were centrifuged at 10000 x *g* for 5 min at room temperature. Supernatants (300 µl) were collected and added with 5 µl β-glucuronidase (Sigma, type HP-2S, 9.8 units/ml). After incubation and boiling, as previously described, reaction mixtures were cooled at room temperature. After adding 60 µl of 0.8M K-tetraborate (pH 9.1), mixtures were again heated to 100˚C for 3 min and cooled to room temperature. Then, 1% 4-dimethylaminobenzaldehyde (1.2 ml, DMAB, Sigma) was added, and mixtures incubated at 37˚C for 20 min.Absorbance was read at 585 nm (DU-70, Bechman), and the amount of produced NAG was determined by means of a standard curve obtained with known concentrations (4.5–90 nmoles) of commercial NAG (Sigma). Blanks (negative controls) were mixtures in which tissue extracts were not added; positive controls were arranged by adding 10 µl chitinase from *Streptomyces griseus* (Sigma, 200 units/g). One unit of activity was defined as 1.0 nmol NAG produced per second at 37˚C. All the enzyme activities were expressed as Units mg^{-1} protein.

3.7 Experimental design and statistical analysis

Experiments of resistance bioassays were designed to use 6 plants for each of the 8 tested RKN-tomato interactions. Three subsequent experiments were carried out. Three replications per experiment were arranged; values of infection factors are expressed as means (n=9) ± standard deviation. Means for each tested infection factor, characterizing the 2 nematode field population/virulent isolate couples (*Mi-Vfield/SM2V, Mj-TunC2field/SM11C2*) infesting both Roma VF and Rossol, were separated by a Duncan's test (Significance Level: 0.05) carried out by the X-Stat software.

DNA extractions were carried out from bunches of roots from un-inoculated and inoculated plants (6 resistant and 6 susceptible) by two nematode field population/virulent isolate couples (*Mi-Vfield/SM2V, Mj-TunC2field/SM11C2*). Two DNA extractions were performed from 2 bioassays. Each DNA sample had 3 replicate readings. Values were expressed as means (n=6) ± standard deviations. Means were separated by a Duncan's test (Significance Level: 0.05) carried out by the X-Stat software.

RNA extractions were carried out from single susceptible and resistant roots un-inoculated or inoculated with the field population/virulent isolate *Mi-Vfield/SM2V* couple. Three extractions per bioassay from 2 bioassays were analyzed for gene expression. qRT-PCR data are expressed as means (n=6) ± standard deviations of $2^{-\Delta\Delta Ct}$ values of each group from inoculated plants, considering as 1 the values of each group from un-inoculated plants; significant difference with respect to the un-inoculated controls was determined by a t-test (*P<0.05; **P<0.01).

Protein extractions were carried out from mixed tissues coming from 2 un-inoculated or inoculated plants, in order to have 3 extractions per experiment. From each of the 3 protein extracts, one value of enzyme activity was determined by 3 technical replicates at the spectrophotometer.

Three bioassays were performed in order to have 9 values for each enzyme activity. Means ± standard deviations were calculated out of these values. Means of the un-inoculated controls were separated from those of inoculated plants by a *t*-test (*P<0.05; **P<0.01).

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