DIFFERENT LEVELS OF ANTI-OXIDANT ENZYME ACTIVITIES IN TOMATO GENOTYPES SUSCEPTIBLE AND RESISTANT TO ROOT-KNOT NEMATODES

M. M. M. Abd-Elgawad[1]*, S. S. A. Kabeil[2], E. Fanelli[3], and S. Molinari[3]

1Plant Pathology Department, National Research Centre, Dokki 12622, Giza, Egypt. 2City for Scientific Research and Technology, Borg-Elarab, Alexandria, Egypt. 3CNR, Istituto per la Protezione delle Piante, U.O.S. Bari, Via G. Amendola 122/D, 70126 Bari, Italy. *Corresponding author: mahfouzian2000@yahoo.com

ABSTRACT

Abd-Elgawad, M. M. M., S. S. A. Kabeil, E. Fanelli, and S. Molinari. 2012. Different levels of anti-oxidant enzyme activities in tomato genotypes susceptible and resistant to root-knot nematodes. Nematropica 42:330-336.

Seven tomato cultivars and 9 hybrids coming from a breeding programme, susceptible or resistant to root-knot nematode by carrying the resistance gene *Mi-1*, were compared by determining free phenol content and catalase (CAT) activity in their leaves. A host suitability test based on the determination of the number of egg masses per root system (EM), after inoculation with an avirulent field population of *Meloidogyne incognita*, confirmed the response to root-knot nematodes of the positive and negative controls, and revealed that all the hybrids except one were highly resistant to the infection. Catalase activity and free phenols extracted from leaves were detected on 3 different groups of tomato plants: 1) known susceptible cultivars; 2) known resistant cultivars; 3) accessions from the breeding programme. The level of catalase activity of the groups 2 and 3 was similar and approximately 2-fold higher than that of the group 1. Conversely, no consistent difference was found in free phenol content among the 3 groups. Moreover, one pair of susceptible and resistant tomato cultivars was used to check changes of anti-oxidant activities due to nematode infection. Catalase and peroxidase activities were measured in roots 5 days after inoculation with 300 and 600 juveniles (J2) of a *M. incognita* population, and in uninoculated roots used as controls. Catalase was found to be inhibited and enhanced, respectively, in infected resistant and susceptible roots, with respect to controls. The degree of inhibition was higher in the resistant roots inoculated with 600 J2. Guaiacol peroxidase activity did not change after inoculation of susceptible plants, whereas it was enhanced in infected resistant roots compared with controls. The use of catalase activity as a biochemical marker of *Mi-1*-mediated resistance in tomato is proposed.

Key words: enzymes, genotypes, host suitability, *Meloidogyne, Solanum lycopersicum.*

RESUMEN

Abd-Elgawad, M. M. M., S. S. A. Kabeil, E. Fanelli, and S. Molinari. 2012. Diferentes niveles de actividad enzimática anti-oxidante en genotipos de tomate susceptibles y resistentes a nematodos agalladores. Nematropica 42:330-336.

Se comparó el contenido de fenoles y la actividad de catalasa (CAT) en las hojas de siete cultivares y nueve híbridos de tomate con el gen de resistencia *Mi-1*. Una evaluación de la susceptibilidad, basada en el número de masas de huevos por raíz, confirmó la respuesta de los controles y reveló que todos los híbridos, excepto uno, eran altamente resistentes. Se midió la actividad de catalasa y los fenoles libres en tres grupos de plantas de tomate: 1) cultivares susceptibles conocidos; 2) cultivares resistentes conocidos; 3) accesiones del programa de mejoramiento. El nivel de actividad de catalasa de los grupos 2 y 3 fue similar y aproximadamente el doble del observado en el grupo 1. Si embargo, no se encontraron diferencias consistentes entre los tres grupos para los fenoles libres. Se usaron cultivares susceptibles y resistentes para verificar los cambios en la actividad anti-oxidante en respuesta a la infección de los nematodos. Se midió la actividad de catalasa y peroxidasa en raíces 5 días después de la inoculación con 300 y 600 juveniles (J2) de *M. incognita*, y en raíces sin inocular. Se encontró que la catalasa se inhibe en plantas resistentes y se estimula en plantas susceptibles, con respect a los controles. El grado de inhibición fue mayor en raíces resistentes inoculadas con 600 J2. La actividad de peroxidasa guaiacol no cambió en plantas susceptibles luego de la inoculación, pero aumentó en plantas resistentes. Proponemos el uso de la actividad de catalasa como marcador biquímico de la resistencia mediada por el gen *Mi-1* en tomate.

Palabras clave: enzimas, genotipos, *Meloidogyne, Solanum lycopersicum*, susceptibilidad

INTRODUCTION

Root-knot nematodes (RKNs), *Meloidogyne* spp., are considered the most damaging nematode group in the world as they cause severe yield losses to many economically important plant species in subtropical and tropical regions (Luc *et al*., 2005). Their infection on tomato (*Solanum lycopersicum* L.) is common in Egypt, Italy and worldwide and cause high crop damage especially in light soils (e.g., Netscher and Sikora, 1990; Abd-Elgawad and Aboul-Eid, 2001). Root-knot nematodes can be managed effectively by chemical treatments but many of the nematicides are expensive or pose human and environmental risk (Greco *et al*., 1992; Abd-Elgawad, 2008). Conversely, host-plant resistance shows major advantages with respect to chemical, biological, cultural, and regulatory control components of nematode management programmes because of the "self-protection" of the resistant crop, which can provide an effective and economical method for managing nematodes in both high and low value cropping systems (Molinari, 2011a).

All currently available fresh-market and processing tomato resistant to RKNs originate from the progeny of a single F1 plant in which the resistance gene *Mi-1* was introgressed from the wild relative of tomato using embryo rescue (Smith, 1944). Moreover, *Mi-1* was the first root-knot nematode resistance gene to be cloned (Milligan *et al*., 1998; Vos *et al*., 1998), as well as the first plant R-gene proved to confer resistance to pests very different from nematodes, such as the potato aphid *Macrosiphum euphorbiae* (Rossi *et al*., 1998) and the white fly *Bemisia tabaci* (Nombela *et al*., 2003). Detection of germplasm carrying *Mi-1*-mediated resistance still relies on comparison with known susceptible cultivars on which RKN infection produces specific and clearly visible root symptoms, such as the typical root knots or galls. Reproduction is measured as the amount of eggs that each gravid female lays outside the roots in special gelatinous matrices named egg masses. Therefore, evaluation of resistant genotypes is based on the comparative determination of the degree of galling, egg mass number, or total eggs collected from the root system (Molinari, 2009). Although an attempt to provide a complete array of damage and reproductive indices has recently been reported (Molinari, 2011b), the rating of host suitability based on bioassays always shows a conspicuous number of drawbacks (Abd-Elgawad and Molinari, 2008), the most evident of which is the long time required to allow nematode reproduction. A practical alternative is the finding of biochemical markers based on enzyme activities strictly associated with the resistance status or response. Catalase activity has already been proposed as such a marker for *Mi-1*-mediated resistance to RKNs in tomato (Molinari and Abd-Elgawad, 2007), although, in this previous report, the evaluation of a differential catalase activity between resistant and susceptible tomato was limited to root extracts. In this study, we focus on catalase extracted from leaves of a wide number of tomato cultivars and accessions coming from a breeding programme, because such a procedure is much more suitable at maintaining the plants alive for further studies and applications. Moreover, we investigated on the changes of anti-oxidant activities, such as catalase and peroxidase, that are specifically involved in the resistant response of tomato to RKNs.

MATERIALS AND METHODS

Plant material

Tomato plants used in this study were either susceptible or resistant to root-knot nematodes in that they carried the gene *Mi-1.2* conferring resistance to *M. incognita, M. javanica* and *M. arenaria* and potato aphids (Vos *et al*., 1998). Cultivars with a known response to root-knot nematodes, used as positive and negative controls, were divided into susceptible cultivars, such as Roma VF, Super Strain B, and the parent susceptible accession 2789 gently provided by S.A.I.S. S.p.A. (Società Agricola Italiana Sementi, Cesena, Italy), and resistant cultivars such as Motelle, VFN8, Nematode-1400, and Small Fry (Petoseed Co., Inc., California, USA). Super Strain B and Nematode-1400 have previously been reported as susceptible and resistant to RKNs, respectively (Mohamed *et al*., 1999; Abd-Elgawad and Kabeil, 2010). Nine accessions (2729, 2730, 2731, 2733, 2737, 2738, 2739, 2748, 2745) were hybrids coming from a breeding programme for root-knot nematode (RKN) resistance carried out by S.A.I.S. These accessions were tested for RKN resistance together with the positive and negative controls. Control resistant and susceptible cultivars together with all the hybrids were used for biochemical tests, such as content of phenols and catalase activity in leaves. Moreover, Super Strain B and Nematode-1400 were inoculated with 300 and 600 J2 per plant of an avirulent *M. incognita* population to monitor root catalase and peroxidase activity changes 5 days after inoculation.

Tests of resistance and nematode inoculation

All the tomato cultivars and accessions were tested for host suitability with an avirulent *M. incognita* field population. Tests were conducted with single tomato seedling transplanted in 100-cm^3 clay pots filled with steam-sterilized loamy sand. Each seedling was inoculated by 300 active J2 (3 J2/cm^3 soil). Plants were allowed to grow on temperature-controlled benches in a glasshouse (soil temperature 23-26°C), providing a regular regime of 12 h light/day. Pots were arranged in a randomized block design and in

each experiment the same tomato-RKN interaction had at least six replicates. Plants were harvested 7 weeks after inoculation. The roots were washed free of soil debris and separately chopped into pieces of about 1 cm length. Samples of 1 g per plant (replicate) were immersed in a solution (0.1 g/L) of the dye Eosin Yellow (Roberts *et al*., 1990) and stored for at least 1 h in a refrigerator. Red-coloured egg masses were then counted under a stereoscope (x 6 magnification) and referred to as egg masses per root system (EM). EM values are expressed as averages ± standard deviations (n = 6). The egg mass index (EI) was calculated according to Hadisoganda and Sasser (1982), as follows: 0=no egg masses, 1=1-2 egg masses, 2=3-10, 3=11-30, 4=31-100, 5=more than 100 egg masses. Based on the range of EI values the estimation of the degree of resistance (DR) was determined as follows: 0-1 EI=highly resistant (HR), 1-3 EI very resistant (VR), 3-3.5= moderately resistant (MR), 3.6-4=slightly resistant (SR), 4.1-5= susceptible (S).

To detect changes of anti-oxidant enzyme activities due to nematode infection, seeds of the tomato cvs Super Strain B and Nematode-1400 were surface-sterilized with successive washes for 4 min in 95% ethanol, for 10 min in 10% sodium hypochlorite and 7 times with sterile water. The seeds were separately germinated in 10-cm diameter earthen pots, each containing 0.7 Kg of sterilized sandy soil. Plants were maintained under greenhouse conditions and periodically watered with Hoagland's nutrient solution. Very young seedlings of each cultivar were inoculated separately with either 300 or 600 freshly hatched second stage juveniles (J2) of *Meloidogyne incognita* while their corresponding inoculated checks served as control. The nematodes were propagated originally from a single egg-mass progeny and maintained on tomato cv. Strain B. J2 used for inoculation were obtained by incubation of RKN egg masses in tap water at 27°C in the dark. They were collected every 2 days and concentrated in small volumes of sterilized water by filtering through 1 µm filters (Whatman type) and collecting them after repeated washes (Molinari, 2009).

Biochemical assays on leaf extracts

Seeds were germinated in sterilized quartz sand. Afterwards, single seedlings were transplanted into 100 cm^3-pots filled with steam-sterilized loamy sand and allowed to grow up to the six-true-leaf stage for approx. 1 month in a greenhouse (soil temperature 23-26°C), providing a regular regime of 12 h light/day. Then, 3 samples of leaves per each cultivar and one sample per each accession were collected, rinsed with tap water, weighed, placed in porcelain mortars and reduced to powder by grinding after immersion in liquid nitrogen. Leaf powder was suspended (1:5 w/v) in a grinding buffer of 0.1 M K-phosphate, pH 7.0, and ground by a Polytron® PT-10-35 (Kinematica GmbH, Switzerland). Coarse homogenates were filtered through four layers of gauze and centrifuged at 9000 g for 15 min in order to remove heavy particulate fractions. Aliquots (2 ml) of the supernatants (leaf extracts) were ultrafiltered through YM-ultrafiltration membranes (10,000 molecular weight cut-off, Amicon Co.) at 4°C and separated into a retained fraction, containing most of the proteins, and an ultrafiltrate fraction, containing mostly free phenols and low molecular weight components. Protein content was determined in both leaf extracts and retained fractions by the Folin reagent method, according to Lowry *et al.* (1951) with slight modifications (Molinari and Abd-Elgawad, 2007). Three aliquots were tested from each extract and corresponding retained fraction. Free phenol content of each sample was calculated by subtracting the values of unfiltered extracts from those of retained fractions. Values were expressed per each cultivar or accessions as mg free phenols per g of leaf fresh weight ± SD (n = 9). Catalase (CAT) activity was detected in leaf retained fractions as the initial rate of disappearance of hydrogen peroxide (Chance and Mahley, 1955). The reaction mixture consisted of 20 mM H_2O_2 and 25-50 µl enzyme suspension in 0.5 ml of 0.1 M Na-phosphate buffer, pH 7.0. A Perkin-Elmer 557 double-beam spectrophotometer was used to continuously detect the rate of H_2O_2 disappearance as decrease in absorbance at 240 nm. Negative controls were carried out with boiled samples. Oxidation of 1 µmole H_2O_2 x min^{-1} ($\varepsilon = 0.038\ mM^{-1}$) represented one unit of enzyme. Three enzyme tests was performed on each leaf sample, then, values of CAT activity per each cultivar or accession was expressed as units x g leaf FW ± SD (n = 9).

Biochemical assays on root extracts

Six young seedlings of both uninoculated and inoculated Super Strain B and Nematode-1400 tomato cvs were gently uprooted and washed thoroughly with distilled water 5 days after nematode inoculation. Roots were separated from the shoots with a scalpel (3-5 cm of terminal root) and kept in an ice bath for protein extraction and enzyme tests. Roots were placed in an ice-cold 0.05 M phosphate buffer, pH 6.5 and cut with scissors to obtain a coarse homogenate. This homogenate was ground by hand in a porcelain mortar followed by centrifugation under cooling conditions for 10 min at 9,000 G. The supernatants were saved and the precipitates were re-extracted with the same buffer and re-centrifuged. The two supernatants were pooled and designated as crude extracts (Mohamed *et al.*, 1999). Peroxidase (PEX) activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm due to oxidation of guaiacol, according to Lee (1973). One unit of enzyme activity was defined as the amount of enzyme that caused 1.0

O.D. min^{-1} change under standard assay conditions. Specific activity was expressed in units mg-1 protein. Protein content and catalase activity were determined in the roots as previously mentioned.

Statistical analysis

The experiments were arranged according to a completely randomized block design. Values of leaf phenols and CAT were divided into 3 groups: 1) data from known resistant cultivars; 2) data from known susceptible cultivars; 3) data from unknown hybrid accessions. Therefore values are expressed as the averages ± standard deviation of a number of tests that is, respectively, 12, 9, and 9 for the 3 groups. Data of root CAT and PEX come from two experiments with 6 replicates each (n = 12). No significant difference occurred between the repeated experiments; therefore, data from duplicate tests were pooled for analysis. A two-way analysis of variance (ANOVA) was performed on the data followed by mean separation with Duncan's New Multiple Test to compare means among treatments. Differences are reported at $P \leq 0.05$.

RESULTS

Host suitability tests were carried out on all the tomato cultivars and accessions used in this study (Table 1). As expected, the known susceptible cultivars and the susceptible accession 2789 were heavily infected by the *M. incognita* avirulent field population used for inoculation, with average numbers of egg masses per plant (EM) ranging from 139 to 197. Conversely, resistant cultivars resulted from highly to very resistant according to the method of assessing degree of resistance (DR) reported in Hadisoganda and Sasser (1982). Hybrids coming from a breeding program for nematode resistance resulted accordingly highly resistant, except for one accession (2745) that was found susceptible to RKNs. Therefore, tomato plants were divided into three groups: known resistant plants, known susceptible plants and hybrids. Catalase and phenols were extracted from the leaves of these plants. Catalase activity extracted from resistant and hybrid leaves was found to be approx. 2-fold higher than that extracted from susceptible leaves (Fig. 1). High leaf catalase activity was then associated with the presence of the resistant gene *Mi-1.2*. Conversely, no apparent difference was found in phenol content among the three groups considered.

Catalase and peroxidase activities were detected in roots of the susceptible tomato cv. Super Strain B and the resistant tomato cv. Nematode-1400, uninoculated or inoculated with increasing amount of *M. incognita* J2 (Table 2). Catalase activity of susceptible roots significantly increased five days after inoculation with both 300 and 600 J2, with respect to

Table 1. Response in terms of numbers of egg masses/ root system (EM) of the tomato cultivars and accessions used in this study to inoculation with a *M. incognita* avirulent field population. Degree of Resistance (DR) is given according to Hadisoganda and Sasser (1982).

Tomato cv./acc.	EM	DR
Roma VF	139±19	S
Super Strain B	197±39	S
parent susceptible 2789	175±82	S
VFN8	0	HR
Motelle	0	HR
Nematode-1400	0	HR
Small Fry	9±5	VR
2729	0	HR
2730	0	HR
2731	0	HR
2748	0	HR
2738	0	HR
2739	0	HR
2733	0	HR
2737	1	HR
2745	170±110	S

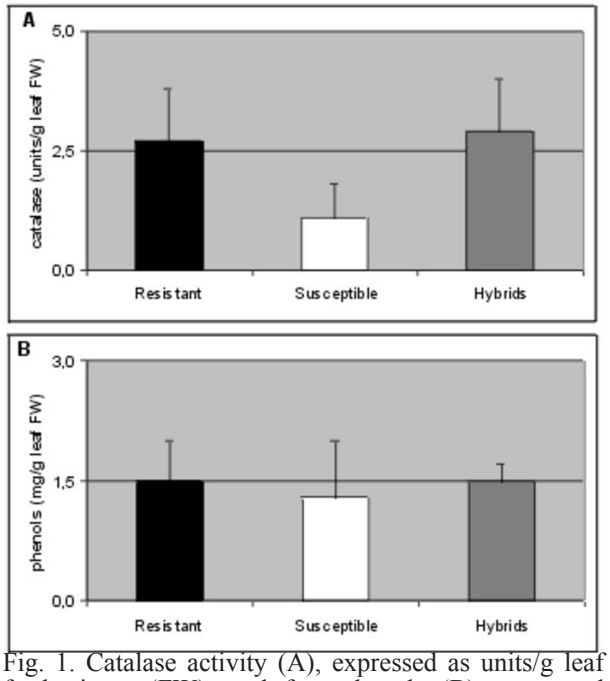

Fig. 1. Catalase activity (A), expressed as units/g leaf fresh tissue (FW), and free phenols (B), expressed as mg/g leaf FW, of leaf extracts from tomato plants resistant and susceptible to root-knot nematodes, and 9 accessions coming from a breeding programme for nematode resistance (Hybrids). Values are averages of the 3 groups ± standard deviations.

Table 2. Catalase and peroxidase activities of root extracts of tomato (cvs. Super Strain B and Nematode 1400) recorded 5 days after inoculation with *Meloidogyne incognita* and compared to those of untreated seedlings (controls)[z].

	Catalase			Peroxidase		
Tomato cultivar	Control	300 J2	600 J2	Control	300 J2	600 J2
Super Strain B	14±3.5°*	18.2±5.7b	18.5±5.8b	11.4±5.4a	11.9±6.1a	12.2±5.9a
Nematode-1400	15±4.8°	11.5±3.4b	8.2±2.9c	24.1±7.6a	38.5±13.9b	43.1±15.8b

[z] Values are given as averages ± SD (n = 12). Catalase and peroxidase activity are expressed as units × mg-1 protein.
*For each enzyme, in a row, means indicated by a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

uninoculated roots. Conversely, roots from resistant plants inoculated with 300 J2 showed a decreased catalase activity compared with uninoculated roots; the inhibition of root catalase was even higher in roots inoculated with 600 J2. No changes in root guaiacol peroxidase activity was detected due to nematode inoculation of susceptible plants, whilst this activity increased in infected resistant plants with no difference between the two ranges of inoculum tested.

DISCUSSION

The general evaluation of host suitability to *Meloidogyne* spp. is based on elaborate and time-consuming procedures that imply long-term applications in greenhouses and not easy arrangements of data. The search of fast detectable markers of resistance in plant-nematode interactions, in which the genetic of resistance is widely known, as it occurs in tomato-root knot nematode interaction, should be highly supported for its multi-tasking applications, first of all the detection of products from a breeding programme. It has long been recognized that tomato *Mi-1*-mediated resistance to nematodes is expressed by a hypersensitive reaction (HR), leading to a rapid and localized cell death, whose earliest visible indications can be seen about 12 h after inoculation of roots with J2, while they attempt to establish a feeding site (Paulson and Webster, 1972). As for many other R-gene-mediated resistance, an enhanced production of reactive oxygen species (ROS) with altered timing is associated with nematode infection of resistant compared with susceptible tomato roots (Molinari, 2001; Melillo *et al.*, 2006). The increase of ROS in root cells may be caused by a specific and very early inhibition of H2O2-degrading enzymes, such as catalase (Molinari and Loffredo, 2006; Molinari and Abd-Elgawad, 2007). Data reported here actually confirm that a catalase inhibition specifically occurs in incompatible tomato-RKN interaction; moreover, in this paper the level of catalase inhibition has been found to be proportional to the degree of challenge that resistant roots must face in terms of inoculum density.

Peroxidases have been reported to oxidize phenols, and consequently have been assigned a role analogous to that proposed for polyphenol oxidases in the necrotic reaction (Goodman *et al.*, 1986). They play a major role in four physiological processes; auxin catabolism, defence mechanism against pathogens, some respiratory processes and synthesis of lignin as well as other terpenoids involved in phytoalexin production (Bashan *et al.*, 1987; Siddiqui and Mahmood, 1992). Various isoperoxidases, oxidizing guaiacol, syringaldazine, and *p*-phenylenediamine-pyrocathecol (PPD-PC), were detected in roots of young seedlings of the tomato resistant cv. Rossol and in the susceptible cv. Roma VF uninoculated and inoculated by *M. incognita* (Molinari, 1991). Guaicol peroxidase was found to increase both in cytoplasmic and cell-wall fraction of infected resistant roots with respect to uninfected roots. Such an increase in root extracts from infetced resistant roots has been confirmed in the data reported here; however, the level of enhancement of PEX seems not to be dependent on the inoculum density. Increase of PEX in incompatible tomato-RKN interactions may favor polymerization of phenols and their precipitation on particulate cellular fractions; free phenols were found to be drastically reduced in root extracts deprived of particulate fractions from resistant tomato roots infected by RKNs (Molinari and Abd-Elgawad, 2007).

Catalases from roots and leaves of tomato plants resistant and susceptible to RKNs were extracted, purified and characterized (Molinari and Loffredo, 2006). Catalases from the same tissue of resistant and susceptible plants showed marked difference in their kinetic parameters and sensitivity to salicylic acid. Moreover, the amount of root catalase activity was found to be more consistent in resistant tomato cvs, such as Motelle, Rossol, and Small Fry, than in susceptible tomato cvs, such as Moneymaker, Roma VF, and NemaSol (Molinari and Abd-Elgawad, 2007). Isoelectrofocusing (IEF) profiles of CAT isozymes extracted from roots belonging to plants of the near-isogenic lines Motelle/Moneymaker revealed the same number of bands, although the basic isoforms of Motelle were much more stained

than the corresponding bands of Moneymaker. As extractions from roots of this potential marker activity are impossible without killing the plants, it was decided to test leaf catalase of tomato cultivars known in their response to RKNs and a series of accessions coming from a breeding programme. Of course, seedlings of all samples were grown together in the same environmental conditions and to the same age to avoid biased comparison due to other factors influencing the level of CAT activity except the carrying in the genome of the resistance gene *Mi-1*. Actually, it had already been reported that CAT activity of green tissues from one resistant tomato cv. was approximately twice that of the susceptible counterpart (Molinari, 2001). The screening reported here confirmed the occurrence of a much higher CAT activity in leaves of well-known resistant cultivars with respect to corresponding susceptible cultivars. Unknown hybrids showed CAT activities very similar to those of resistant cultivars, almost 2-fold higher than those of susceptible cultivars. Accordingly, all the hybrids but one resulted to be highly resistant to nematode attack. Leaf catalase may be used to detect *Mi-1*-mediated resistance of unknown accessions coming from breeding programmes, if leaf catalase is contextually detected in parents or well-known resistant and susceptible cvs grown in the strictly same conditions; moreover, tests must be carried out on plants of the same age. Conversely, the other biochemical parameter tested, the amount of phenols in leaf extracts, did not result a suitable tool to differentiate tomato resistant and susceptible to RKNs. Screening of CAT activity on seeds will be the next step to support the use of such enzyme activity in rating plant genotypes for nematode resistance as biochemical markers especially to expedite breeding programmes (Mohamed *et al*., 1999; Abd-Elgawad and Molinari, 2008).

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