Research Article

# Enzymatic Changes in Pomegranate Cultivars Infected with Phyto-Nematode Species with Emphasis on Role of Humic Acid Products in Controlling Root-Knot Nematode

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**Abstract** | Two pot experiments were conducted under greenhouse conditions. The first, three pomegranate cultivars (Assuity, Manfalouty, and Wonderful) were screened for *Meloidogyne incognita, Rotylenchulus reniformis,* and *Helicotylenchus indicus* reproductivity according to their nature of parasitism (endo, semi-endo, and ectoparasites). All cultivars reacted differently to nematode species infection, however, they were all susceptible. Manfalouty was the least to support the three nematodes' reproduction. Oxidative stress is represented in MDA and $H_2O_2$ contents raised by the infection of the three nematode species. This increase was the highest in the most susceptible cultivar (Assuity) infected with the spiral nematode in particular. In the second experiment, 4 commercial products of Humic acid (Actosol®, Decka™, Humic acid™, and Humo plus 40™) were applied as recommended (dose and application) against *M. incognita*. Soil drench application (Actosol® and Decka™) appeared to be more efficient on *M. incognita* reproduction than foliar spray (Humic acid™ and Humo plus 40™). On Assuity and Manfalouty, all treatments reduced significantly the nematode reproduction compared to Wonderful and nematode-treated checks regardless of the method of application. The MDA and $H_2O_2$ contents were significantly reduced after treatments while the antioxidant compounds GSH and AsA contents were significantly increased when compared with the check. Antioxidant defense enzymes, APX, SOD, and CAT showed a significant increase in their activities. Total phenol content and PPO were improved significantly in treated plants compared to nematode-treated checks. Herein, we recommend the implementation of the humic acid application as a natural product in the integrated nematode management programs.

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## Introduction

The root-knot nematode, *Meloidogyne incognita* is a sedentary endoparasite species, causing major economic damage to different crops worldwide (Rodríguez *et al*., 2009). The reniform nematode, *Rotylenchulus reniformis* is a semi-endoparasitic species, becoming a serious pest of multiple crops (Robinson, 2007). Also, the spiral nematode, *Helicotylenchus indicus* is an important and widely distributed ectoparasitic nematode, causing root damage and consequently limiting crop yield (Siddiqi, 2000; Diab *et al*., 2019). These nematode species were found the most dominant nematodes associated with the rhizosphere of different pomegranate cultivars in different regions in global survey studies (Ibrahim and Mokbel, 2009; Khan and Shaukat, 2010; Nayba *et al*., 2012; Korayem *et al*., 2014). Many research workers reported the damage of such nematode species to pomegranates (Shelke and Darekar, 2001; Khan and Shaukat, 2010; Nour El-Deen *et al*., 2016; El-Qurashi *et al*., 2017).

The incompatible resistant interactions to nematode infection may include many physiological defense actions, production of $H_2O_2$ (Peltzer *et al*., 2002), the formation of reactive oxygen species, ROS (Neill *et al*., 2002; Montes *et al*., 2004; Murgia *et al*., 2004), different enzymatic and non-enzymatic ascorbate (Yoshimura *et al*., 2004), increase in peroxidase and polyphenol oxidase level (Sarowar *et al*., 2005), antioxidant properties (Decker, 1997), polyphenols (Metodiewa *et al*., 1999).

Oxidative stress is an abnormal status associated with different organism infections; human diseases (Favier, 2006), phytoparasitic nematodes (Zhang *et al*., 2019), or even plant abiotic stress (Gechev and Petrov, 2020). In fact, inside the organism, there is an accurate balance between the ROS (free radicals) produced as byproducts of different metabolic pathways and the antioxidant scavenging system under homeostasis conditions (Reddy *et al*., 2005). However, the oxidative stress phenomenon is initiated when an excess of free radicals starts serial reactions damaging the essential molecules in the organism, for example, DNA and proteins. In this case, the antioxidant scavenging system is unable to suppress or scavenge all the serious free radicals produced (Halliwell and Gutteridge, 1999; Favier 2006). ROS reduced forms of atmospheric oxygen ($O_2$). The information includes the excitation of $O_2$ to form singlet oxygen ($O_2$) or form gaining one, two, or three electrons for $O_2$ to form, superoxide radical ($O_2$–), $H_2O_2$, or a hydroxyl radical (HO–), respectively. ROS plays two very different roles; serial damage or signaling the activation of a defensive reaction. The key factor controlling these different roles is the cellular levels of ROS (Dat *et al*., 2000). Antioxidants are a major milestone that enhances the ability of plants to overcome oxidative stress. The regulation of ROS in plants is governed by an antioxidant defense system composed of antioxidant enzymes and non-enzymatic antioxidants (Hasanuzzaman *et al*., 2017).

Results of Afify et al. (2014) revealed that different hosts reacted similarly to different nematode species; in which oxidants such as MDA, $H_2O_2$, and antioxidants such as GSH, AA, SOD, CAT, and APX were all increased. The enhancement of oxidative stress inside nematode bodies dramatically could increase the pathogenicity of *Bursaphelenchus xylophilus* on pine. The EU-Bx3 nematode isolates with $H_2O_2$ enhancement were the most pathogenic, causing 70% death of pine saplings. Furthermore, $H_2O_2$ (20 mM) significantly improved the fecundity of *Bu. xylophilus* (Zhang *et al*., 2019). On the contrary, Karajeh (2008) reported that hydrogen peroxide soil drench application affects *M. javanica* reproduction in tomatoes. Noureldeen *et al*. (2021) suggested that the bioprotective nature of *Pseudomonas fluorescens* for tomato plants against *M. incognita* is due to the increased expression of phenol content and defensive enzymes such as peroxidase and superoxide dismutase, and the significant reduction in MDA and $H_2O_2$ contents.

Growing plants supplemented with fertilizers containing humic acid improved their resistance to nematode infection (Kesba and Al-Shalaby, 2008). Growing resistant pomegranate varieties supplemented with humic acid were supposed to result in better nematode management. Kesba and El-Beltagi (2012) reported that humic acid application reduced MDA and $H_2O_2$ in grapes while increasing GSH, ASA, TPH, APX, SOD, CAT, and PPO, which, in turn, reduced *M. incognita* and *R. reniformis* reproduction. Some studies pointed to the effect of humic substance's application on different plant hosts infected with the root-knot nematode. However, few studies investigated the antioxidant status associated with such application. Further, there

are contradictory results regarding the point, making it still unclear. This study was carried out to investigate the effect of type parasitism of three nematode species on nematode reproductivity, and oxidative and antioxidative enzymes in three pomegranate cultivars. Also, evaluate four commercial products of humic acid against the root-knot nematode, *M. incognita*, and pomegranate enzyme content under greenhouse conditions.

## Materials and Methods

*Nematode species sources*

Pure cultures of the endoparasitic root-knot nematode (RKN), *M. incognita* (Chitwood, 1949), the semi-endo parasitic reniform nematode (RN), *R. reniformis* (Adam *et al*., 2018), and the ectoparasitic spiral nematode (SN), *Helicotylenchus indicus* (Siddiqi, 1963) were obtained from isolates belonging to the Nematology Research Center, Faculty of Agriculture, Cairo University. Nematode species have been propagated separately, *M. incognita* on eggplant cv. Classic, *R. reniformis* on pigeon peas, and *H. indicus* on Italian cypress, plants were grown in 20 cm diameter clay pots filled with sterilized loamy soil. To avoid contamination, cultures of each nematode species were arranged separately, checked, and replenished periodically to ensure a continuous supply of inocula for the experimental work.

*Glasshouse experiments*

Three-month-old seedlings of 3 pomegranate cultivars (Assuity, Manfalouty, and Wonderful) with uniform size were obtained from Horticulture Research Institute, Agriculture Research Center and cultivated singly in 20 cm diameter clay pots filled with steam-sterilized sandy loam soil (1:1, v/v). After a week, 5 seedlings of each cultivar were inoculated separately with 5000 infective stages of *M. incognita*, *R. reniformis,* and *H. indicus* by pipetting the nematode water suspension into 4 holes around the root system which was immediately covered with soil. Pots were labeled and arranged randomly on a glasshouse clean bench, receiving similar horticulture treatments. Seedlings were left out after 3 months from inoculation. Soil population was extracted using (Hooper *et al*., 2005) and counted. The nematode-embedded stages of each species were also counted.

For testing the effect of commercial products of humic acid on the root-knot nematode development and reproduction, another 5 seedlings of each cultivar were inoculated with 5000 newly hatched $J_2$ of *M. incognita*/plant/pot. Two weeks after inoculation, four commercial products of humic acid were applied (recommended dose and method of application) as mentioned in (Table 1). All treatments were arranged in a fully randomized design on a clean bench in the glasshouse at 32±5°C receiving similar horticultural treatments. After 3 months, nematode soil populations were extracted and counted using a Hawksley counting slide, under a binocular microscope. A subsample (5 g) of roots from each plant was stained and gall numbers, embedded stages (developmental stages + eggmasses) per root were calculated, final population (embedded stages + nematodes in soil), nematode reproduction factor (Pf/Pi), an average of eggs/egg-mass were estimated.

*Plant chemical analysis*

Six subsamples of the fresh root of each treatment were chemically analyzed at the Central Chemistry Lab, Faculty of Agriculture Research Park (FARP), Faculty of Agriculture, Cairo University as follows:

Preparation of enzyme extracts: A sample of 1 g was homogenized in 3 ml of 50 mM phosphate buffer pH 7.0 containing 1.0 N NaCl, 1% PVP (Sigma), and 1 mM ascorbate (Sigma) at 4°C. After centrifugation at 15,000×g for 15 min, the supernatant was collected.

Assay of protein content: The protein was estimated by (Bradford, 1976) with standard curves prepared using bovine serum albumin.

**Table 1:** *Structure, dose, and application method of Humic commercial products.*

| Trade name | Structure | Recommended | |
|---|---|---|---|
| | | Dose/plant | Application method |
| Actosol® | (Humic acid and Fulvic acid) 20% + K 6% | 5 ml | Soil drench |
| Decka™ | Humic acid 10% + Fulvic acid 8% + Amino acids 8% + N 2% + P 4.2% + K 5% | 5 ml | Soil drench |
| Humic acid™ | Potassium humate 85% + Dissolved potassium 8% + Fulvic acid 3% | 2.5 g | Foliar spray |
| Humo plus 40™ | Humic acid 40% + N 1% + P 3% + K 5% + Fe 5000 ppm + Zn 1000 ppm + Mn1000 ppm + Cu 200 ppm + Mo 50 ppm | 2.5 ml | Foliar spray |

*Determination of oxidative burst*

Lipid Peroxide, MDA contents: The thiobarbituric acid (TBA) reaction is described by Heath and Packer (1968). Fresh mass (200 mg) from the culture was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12,000×g for 20 min. The obtained supernatant (1 ml) was mixed with an equal volume of TCA (10%) containing 0.5% (w/v) TBA or without TBA as the blank, and heated at 95°C for 30 min and then cooled in ice. The reaction product was centrifuged at 12,000×g for 15 min and the absorbance of the supernatant was measured at 400, 532, and 600 nm. The MDA equivalent was derived from the absorbance according to (Hodges *et al.*, 1999).

*Hydrogen peroxide concentration check*

Hydrogen peroxide was measured by the method described by Capaldi and Taylor (1983), with a slight modification. The callus was ground in 5% TCA (2.5 ml per 0.5 g callus) with 50 mg active charcoal at 0°C, and centrifuged for 10 min at 15,000 × g. The supernatant was collected, neutralized with 4 N KOH to pH 3.6, and used for the $H_2O_2$ assay. The reaction mixture contained 200 µl of leaf extract and 100 µl of 3.4 mM 3-methyl benzothiazoline hydrazone (MBTH). The reaction was started by adding 500 µl of horseradish peroxidase solution (90 units per 100 ml) in 0.2 µl sodium acetate (pH 3.6). After two minutes 1400 µl of 1 N HCl was added. The absorbance was read at 630 nm after 15 min.

*Determination of total glutathione*

The level of total acid-soluble SH (glutathione GSH) was determined using Ellman's reagent (De Vos *et al.*, 1992). The buffer solution was mixed with 630 µl of 0.5 M $K_2HPO_4$ and 25 µl of 5 mM, 5'–dithiobis (2-nitrobenzoic acid) (final pH 7). The absorbance was read at 412 nm after 2 minutes. GSH was used as a standard.

*Determination of ascorbic acid*

Ascorbate content was measured using 2,4-dinitrophenol indophenol. The absorbance was measured at 520 nm according to Omaye *et al.* (1979).

*Phenol check*

The phenolic assay was performed following the method of Zieslin and Ben-Zaken (1993). The samples were homogenized at the rate of 0.1 g per 1 ml of 80% methanol and the methanolic extract was kept in a water bath at 70°C for 15 min with frequent stirring. One ml of methanolic extract was added to 5 ml of distilled water and 250 ml of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25°C for 30 min. Finally, 1 ml of a saturated solution of $Na_2CO_3$ and 1 ml of distilled water was added and the reaction mixture was incubated for 1 h at 25°C. After the blue color was developed, the absorbance was recorded at 725 nm. The contents of the total dissolved phenols were calculated according to a standard curve obtained from the reaction of Folin-Ciocalteau with a catechol solution. The phenol content was expressed as phenol equivalents in mg/g fresh weight of callus tissues.

*Determination of the activity of antioxidant defense enzymes*

SOD activity check: The activity of SOD was evaluated by measuring its ability to inhibit the photochemical reduction of NBT using a method (Beauchamp and Fridovich, 1971). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 1.0 mM EDTA, and 20 µl enzyme extract. Riboflavin was finally added and the reaction started by placing 30 cm tubes under 15 W fluorescent lamps. The reaction was started by turning on the light and allowed to work for 10 min. Turning off the light stopped the reaction and the tubes were covered with a black cloth. Unilluminated tubes were used as a control. The absorbance was read at 560 nm. The volume of enzyme extract corresponding to 50% reaction inhibition was taken as one enzyme unit.

*Ascorbate peroxidase (APX) activity assay*

The activity of ascorbate peroxidase was determined spectrophotometrically by decreasing the absorption at 265 nm (e = 13.7$mM^{-1}$ $cm^{-1}$) using the (Nakano *et al.*, 1981) method. The reaction mixture contains 50 mM potassium phosphate solution pH 7.0, 5 mM ascorbate, 0.5 mM $H_2O_2$, and enzyme extract. Started adding the reaction. The non-enzymatic oxidation rates of ascorbate were corrected by including a reaction mixture without the enzymatic extract.

*Catalase activity assay*

Catalase activity was determined by $H_2O_2$ consumption using the method of Dhindsa *et al.* (1981). The reaction mixture contains 50 mM potassium phosphate pH 7.0, 15 mM $H_2O_2$, and an enzyme extract. The consumption of $H_2O_2$ was monitored spectrophotometrically at 240 nm (e= 45.2$mM^{-1}$ $cm^{-1}$). Enzyme activity was expressed in µM $H_2O_2$ $min^{-1}$.

*Polyphenol oxidase activity assay*

Polyphenol oxidase was tested using the photochemical method as described by Coseteng and Lee (1987). The reaction mixture contains 50 mM potassium phosphate solution pH 6.2, 250 mM catechol, and enzyme extract. The increase in absorbance was measured at 420 nm. One unit of enzyme activity is defined as the amount of enzyme that causes an increase of 0.001 absorption units per minute at 25°C.

*Statistical analysis*

Data were compared by Duncan's Multiple Range Test (DMRT) at a 5% level of probability using MSTAT version 4 (1987).

## Results

The primary pathogenicity test for the three pomegranate cultivars against *M. incognita*, *R. reniformis*, and *H. indicus* (Table 2) revealed that all three cultivars were susceptible to the infection of the three nematode species. Nematodes were successfully able to penetrate, develop, reproduce, and consequently fold many times (at least 5) on the three cultivars during the experimental period. Assuity cultivar was the most susceptible host, recording RF of 6.7, 11.8, and 12.1 for *M. incognita*, *R. reniformis*, and *H. indicus*, respectively. The three cultivars almost reacted the same to the endoparasitic nematode, *M. incognita*. Yet Manfalouty achieved a low significance to the semi-endoparasitic nematode, *R. reniformis* parameters when compared with the other two cultivars (Assuity and Wonderful) which were similar in susceptibility. The ectoparasitic nematode, *H. indicus* severely infected the three cultivars, and the highest nematode numbers (embedded, in soil or final population) were recorded on Assutiy in particular.

In view of the host response to nematode infection, the root content of MDA, $H_2O_2$, GSH, TAA, and TPH was very much higher than in the healthy (nematode-free) plants. Obviously, under all three nematode species infections, the oxidative stress on the most susceptible cultivar 'Assuity' represented in MDA and $H_2O_2$ contents had the highest and the most significant values, while it was not for the antioxidants GSH, TAA, and TPH. The response of the cultivars (as measured by the antioxidant content) for the three nematode species was significantly increased and different from each other in the three pomegranate cultivars. Also, significant differences were noticed among the cultivars as a response to the same nematode species (Table 3). Likewise, in Table 4, the enzymatic antioxidants response due to the nematode infection followed the same awarding trend. The enzymatic antioxidant content due to *H. indicus* infection stress was the highest in all cultivars. It is also clear from Tables 3 and 4 that the biochemical response in the three cultivars due to the same nematode species infection was significantly different.

**Table 2:** *Reproductivity of M. incognita, R. reniformis, and H. indicus on pomegranate cultivars.*

| Cultivar | Galls/ Root | Embedded stages/ Root | Soil population | Final population (Pf) | RF (Pf/ Pi) |
|---|---|---|---|---|---|
| ***M. incognita*** | | | | | |
| Assuity | 1588 b | 2108 a | 31410 a | 33518 a | 6.7 a |
| Std.D. | 96.1 | 114.0 | 1561.0 | 1569.5 | 0.3 |
| Manfalouty | 1086 c | 1499 b | 27500 b | 28999 b | 5.8 b |
| Std.D. | 102.2 | 91.3 | 1731.6 | 1735.9 | 0.3 |
| Wonderful | 1802 a | 2262 a | 31650 a | 33912 a | 6.8 a |
| Std.D. | 93.8 | 169.1 | 1401.9 | 1454.5 | 0.2 |
| ***R. reniformis*** | | | | | |
| Assuity | - | 2419 a | 56475 a | 58894 a | 11.8 a |
| Std.D. | | 146.0 | 3180.2 | 3258.7 | 0.6 |
| Manfalouty | - | 1474 c | 33500 c | 34974 c | 7.0 c |
| Std.D. | | 165.9 | 4038.7 | 4088.8 | 0.8 |
| Wonderful | - | 1854 b | 51650 b | 53504 b | 10.7 b |
| Std.D. | | 163.4 | 385.0 | 389.7 | 0.1 |
| ***H. indicus*** | | | | | |
| Assuity | - | 2263 a | 58475 a | 60738 a | 12.1 a |
| Std.D. | | 178.2 | 1788.8 | 1681.8 | 0.3 |
| Manfalouty | - | 1484 b | 32000 c | 33484 c | 6.7 c |
| Std.D. | | 201.1 | 685.9 | 877.8 | 0.1 |
| Wonderful | - | 2058 a | 41650 b | 43708 b | 8.7 b |
| Std.D. | | 138.5 | 921.6 | 951.3 | 0.1 |

*Means followed by the same letter(s) within a column of each nematode species are not significantly different (P ≤ 0.05) according to Duncans' multiple range test. Final population (PF) = Embedded stages + Soil population, Rf (Reproduction factor) = Pf (Final population)/Pi (Initial population), Std.D.= Standard deviation.*

Data in Table 5 showed the effects of humic acid application on the reproduction of *M. incognita* on pomegranate. It is clear that application of all humic acid commercial products significantly reduced nematode criteria in terms of galls, final population, and reproduction factor, whether it was applied as a soil drench (Actosol® and Decka™) or foliar spray (Humic acid™ and Humo plus™), with

many exceptions in numbers of eggs per egg mass which showed non-significant differences in several treatments when compared to control. Drench application showed more superiority in reducing nematode parameters (Actosol® and Decka™) than that of foliar spray products (Humic acid™ and Humo plus™), regardless that the products Humic acid™ and Humo plus™ were richer in such ratio than the other two drench products (Table 1). This, in turn, reflects the higher efficacy of humic acid against nematode when applied as a drench application. However, the reductions in nematode numbers were significant compared to control, the nematode final population was still high in all treatments.

**Table 3:** *Effect of M. incognita, R. reniformis, and H. indicus on pomegranate cultivars root contents of non-enzymatic antioxidants.*

| Nematode species | MDA (µ mol/g FW) | $H_2O_2$ (µ mol/g FW) | GSH (µ mol/g FW) | TAA (mg/g FW) | TPH (mg/g FW) |
|---|---|---|---|---|---|
| **Assuity** | | | | | |
| *M. incognita* | 8.61 a | 235.12 a | 6.29 i | 10.89 f | 5.36 g |
| Std.D. | 0.07 | 0.85 | 0.10 | 0.07 | 0.08 |
| *R. reniformis* | 8.19 b | 229.49 b | 8.78 f | 14.42 c | 7.76 d |
| Std.D. | 0.03 | 1.43 | 0.05 | 0.67 | 0.13 |
| *H. indicus* | 8.08 c | 223.06 c | 11.45 c | 17.59 a | 9.98 b |
| Std.D. | 0.06 | 1.06 | 0.07 | 0.30 | 0.07 |
| Healthy | 2.95 h | 90.46 k | 2.98 k | 3.97 gh | 1.30 i |
| Std.D. | 0.06 | 0.52 | 0.08 | 0.13 | 0.03 |
| **Manfalouty** | | | | | |
| *M. incognita* | 7.54 d | 208.10 d | 7.58 g | 12.85 d | 6.09 f |
| Std.D. | 0.07 | 0.84 | 0.08 | 0.52 | 0.24 |
| *R. reniformis* | 7.46 d | 196.46 e | 10.81 d | 14.92 c | 7.35 e |
| Std.D. | 0.06 | 0.78 | 0.06 | 0.21 | 0.14 |
| *H. indicus* | 7.56 d | 181.07 g | 12.78 a | 15.72 b | 8.97 c |
| Std.D. | 0.04 | 1.03 | 0.03 | 0.11 | 0.32 |
| Healthy | 2.40 i | 96.24 j | 2.84 l | 4.37 g | 1.69 h |
| Std.D. | 0.03 | 0.88 | 0.15 | 0.06 | 0.07 |
| **Wonderful** | | | | | |
| *M. incognita* | 6.93 e | 188.04 f | 7.33 h | 12.24 e | 5.97 f |
| Std.D. | 0.06 | 1.26 | 0.07 | 0.21 | 0.06 |
| *R. reniformis* | 6.77 f | 163.42 h | 9.08 e | 14.96 c | 8.94 c |
| Std.D. | 0.10 | 1.25 | 0.05 | 0.21 | 0.07 |
| *H. indicus* | 6.08 g | 153.02 i | 12.28 b | 17.15 a | 11.01 a |
| Std.D. | 0.08 | 1.07 | 0.05 | 0.25 | 0.03 |
| Healthy | 2.14 j | 96.73 j | 3.28 j | 3.76 h | 1.61 h |
| Std.D. | 0.03 | 1.12 | 0.04 | 0.04 | 0.12 |

*Means followed by the same letter(s) within a column are not significantly different (P ≤ 0.05) according to Duncans' multiple range test. MDA=Lipid peroxidation, $H_2O_2$=Hydrogen peroxide, GSH = Glutathione, TAA = Total ascorbic acid, TPH = Total phenols, Std.D.= Standard division.*

**Table 4:** *Effect of M. incognita, R. reniformis, or H. indicus on pomegranate cultivars root contents of antioxidant enzymes.*

| Nematode species | APX (unit/mg protein) | CAT (unit/mg protein) | PPO (unit/mg protein) | SOD (unit/mg protein) |
|---|---|---|---|---|
| **Assuity** | | | | |
| *M. incognita* | 16.49 h | 52.93 e | 14.26 h | 217.99 f |
| Std.D. | 0.17 | 0.59 | 0.24 | 1.48 |
| *R. reniformis* | 26.41 e | 68.78 c | 28.86 e | 247.35 d |
| Std.D. | 0.11 | 2.09 | 0.13 | 8.73 |
| *H. indicus* | 35.79 b | 84.82 a | 43.11 b | 277.08 b |
| Std.D. | 0.25 | 1.02 | 3.32 | 8.35 |
| Healthy | 9.88 j | 24.86 i | 3.11 i | 127.46 j |
| Std.D. | 0.11 | 0.91 | 0.16 | 0.42 |
| **Manfalouty** | | | | |
| *M. incognita* | 15.83 i | 42.64 g | 13.11 h | 199.82 g |
| Std.D. | 0.22 | 2.53 | 0.19 | 0.24 |
| *R. reniformis* | 24.73 f | 61.06 d | 26.84 f | 228.32 e |
| Std.D. | 0.29 | 1.21 | 0.17 | 2.86 |
| *H. indicus* | 32.90 c | 80.57 b | 39.85 c | 256.63 c |
| Std.D. | 0.22 | 0.37 | 0.88 | 3.24 |
| Healthy | 9.66 j | 24.72 i | 3.75 i | 144.46 i |
| Std.D. | 0.06 | 0.42 | 0.15 | 0.13 |
| **Wonderful** | | | | |
| *M. incognita* | 17.54 g | 46.94 f | 16.22 g | 225.89 e |
| Std.D. | 0.04 | 1.31 | 0.70 | 1.50 |
| *R. reniformis* | 30.47 d | 66.80 c | 31.05 d | 281.30 b |
| Std.D. | 0.09 | 0.50 | 1.32 | 1.37 |
| *H. indicus* | 43.94 a | 85.23 a | 46.78 a | 336.18 a |
| Std.D. | 0.15 | 1.56 | 0.25 | 1.86 |
| Healthy | 8.92 k | 30.64 h | 2.80 i | 154.15 h |
| Std.D. | 0.20 | 0.67 | 0.12 | 0.49 |

*Means followed by the same letter(s) within a column are not significantly different (P ≤ 0.05) according to Duncans multiple range test.APX = Ascorbat peroxidase, CAT = Catalase, PPO = Polyphenol oxidase, SOD = Superoxide dismutase, Std.D.= Standard division.*

Data in Tables 5, 6, and 7 indicated that nematode reproduction had a prominent positive relationship with MDA and $H_2O_2$ contents and a negative relationship with GSH, TAA, TPH, APX, CAT, PPO, and SOD contents of root tissues when compared with the untreated inoculated plants. Drench application treatments of Actosol® and Decka™ products caused the highest significant increases in all antioxidants activities (GSH, TAA, TPH, APX, CAT, PPO, and SOD). These increments were the most in the Manfaloutycultivar. Simultaneously, oxidative stress

(MDA, $H_2O_2$) was lower due to humic acid product application. The lowest values of MDA and $H_2O_2$ were achieved in the Manfalouty cultivar. Significant differences were achieved in root contents of oxidant and antioxidant enzymes either between cultivars or nematode species, treated or not treated.

## Discussion

In this study, the three pomegranate cultivars (Assuity, Manfalouty, and Wonderful) were suitable hosts for parasitism of *M. incognita*, *R. reniformis,* and *H. indicus*, which was evidenced by the measured high reproduction factor for all nematode species.

**Table 5:** *Reproductivity of M. incognita on pomegranate cultivars as influenced by the addition of commercial products of humic acid.*

| Treatment | Dose/Plant | Galls/Root | Embedded stages/Root | Final population (Pf) | Rf (Pf/Pi) | Eggs/ Egg-mass |
|---|---|---|---|---|---|---|
| **Assuity** | | | | | | |
| Actosol® | 5ml (SD) | 714 h | 895 f | 17815 g | 3.6 g | 339 abc |
| Std.D. | | 25.35 | 34.82 | 725.06 | 0.15 | 30.70 |
| Decka™ | 5ml (SD) | 826 fg | 1156 ef | 19596 f | 3.9 f | 321 bc |
| Std.D. | | 39.27 | 108.82 | 614.20 | 0.12 | 40.52 |
| Humic acid™ | 2.5g (FS) | 847 f | 1103 ef | 20703 f | 4.1 f | 357 abc |
| Std.D. | | 45.66 | 118.17 | 615.19 | 0.13 | 31.38 |
| Humo plus 40™ | 2.5 ml (FS) | 867 ef | 1099 ef | 22499 e | 4.5 e | 351 abc |
| Std.D. | | 46.72 | 97.69 | 905.84 | 0.18 | 36.64 |
| Inoculated only | | 1588 b | 2108 ab | 33518 a | 6.7 a | 367 ab |
| Std.D. | | 67.69 | 1168.62 | 566.00 | 0.11 | 25.88 |
| **Manfalouty** | | | | | | |
| Actosol® | 5ml (SD) | 734 gh | 979 f | 17699 g | 3.5 g | 314 c |
| Std.D. | | 61.68 | 32.29 | 721.69 | 0.14 | 26.55 |
| Decka™ | 5ml (SD) | 923 ef | 1200 def | 18360 g | 3.7 g | 326 bc |
| Std.D. | | 31.74 | 91.79 | 878.59 | 0.18 | 41.59 |
| Humic acid™ | 2.5g (FS) | 930 ef | 1230 def | 19850 f | 4.0 f | 346 abc |
| Std.D. | | 31.61 | 264.19 | 953.10 | 0.19 | 16.73 |
| Humo plus 40™ | 2.5 ml (FS) | 1051 cd | 1325 cdef | 20425 f | 4.1 f | 349 abc |
| Std.D. | | 79.87 | 79.06 | 1069.46 | 0.21 | 7.42 |
| Inoculated only | | 1086 c | 1499 cde | 28999 b | 5.8 b | 336 abc |
| Std.D. | | 87.85 | 90.56 | 1278.46 | 0.25 | 33.81 |
| **Wonderful** | | | | | | |
| Actosol® | 5ml (SD) | 848 f | 1554 cde | 26934 d | 5.4 d | 324 cd |
| Std.D. | | 42.51 | 134.60 | 1041.07 | 0.21 | 48.40 |
| Decka™ | 5ml (SD) | 916 ef | 1572 cde | 27052 cd | 5.4 cd | 330 abc |
| Std.D. | | 42.49 | 122.86 | 1334.05 | 0.27 | 57.01 |
| Humic acid™ | 2.5g (FS) | 969 de | 1672 bcd | 28092 bcd | 5.6 bcd | 379 a |
| Std.D. | | 29.03 | 155.99 | 575.53 | 0.12 | 6.36 |
| Humo plus 40™ | 2.5 ml (FS) | 1059 cd | 1730 bc | 28310 bc | 5.7 bc | 344 abc |
| Std.D. | | 111.77 | 166.25 | 1184.33 | 0.24 | 31.10 |
| Inoculated only | | 1802 a | 2262 a | 33912 a | 6.8 a | 341 abc |
| Std.D. | | 215.13 | 218.65 | 1518.49 | 0.30 | 34.89 |

*Means followed by the same letter(s) within a column are not significantly different (P ≤ 0.05) according to Duncans' multiple range test. Final population (PF) = Embedded stages + Soil population, Rf (Reproduction factor) = Pf (Final population)/Pi (Initial population), Std.D.= Standard division, SD = Soil drench, FS = Foliar spray.*

**Table 6:** *Effect of commercial products of humic acid on infected pomegranate cultivars root contents of non-enzymatic antioxidants.*

| Treatment | Dose / Plant | MDA (µ mol/g FW) | $H_2O_2$ (µ mol/g FW) | GSH (µ mol/g FW) | TAA (mg/g FW) | TPH (mg/g FW) |
|---|---|---|---|---|---|---|
| **Assuity** | | | | | | |
| Actosol® | 5ml (SD) | 2.33 l | 133.44 f | 17.12 b | 21.68 b | 12.57 e |
| Std.D. | | 0.02 | 0.05 | 0.46 | 0.08 | 0.03 |
| Decka™ | 5ml (SD) | 3.52 f | 146.37 e | 17.04 b | 21.47 b | 12.91 d |
| Std.D. | | 0.04 | 0.06 | 0.65 | 0.02 | 0.06 |
| Humic acid™ | 2.5g (FS) | 3.66 e | 148.60 d | 11.18 e | 16.97 cd | 9.73 i |
| Std.D. | | 0.04 | 0.01 | 0.13 | 0.06 | 0.01 |
| Humo plus 40™ | 2.5 ml (FS) | 4.35 d | 152.39 c | 11.16 e | 17.33 c | 9.85 h |
| Std.D. | | 0.03 | 0.29 | 0.24 | 0.17 | 0.07 |
| Inoculated only | | 8.31 a | 226.80 a | 5.57 h | 9.65 j | 4.75 m |
| Std.D. | | 0.02 | 0.03 | 0.06 | 0.06 | 0.05 |
| **Manfalouty** | | | | | | |
| Actosol® | 5ml (SD) | 2.47 k | 104.24 k | 18.36 a | 23.55 a | 15.64 a |
| Std.D. | | 0.03 | 1.45 | 0.13 | 0.51 | 0.10 |
| Decka™ | 5ml (SD) | 2.54 j | 104.79 k | 18.24 a | 23.48 a | 15.71 a |
| Std.D. | | 0.03 | 0.14 | 0.92 | 0.26 | 0.09 |
| Humic acid™ | 2.5g (FS) | 3.30 g | 116.55 i | 12.36 cd | 14.60 f | 8.80 j |
| Std.D. | | 0.03 | 0.03 | 0.04 | 0.12 | 0.05 |
| Humo plus 40™ | 2.5 ml (FS) | 3.35 g | 119.44 h | 12.54 c | 15.41 e | 8.53 k |
| Std.D. | | 0.02 | 0.05 | 0.12 | 0.02 | 0.03 |
| Inoculated only | | 7.90 b | 221.37 b | 6.71 fg | 11.39 h | 5.40 l |
| Std.D. | | 0.04 | 0.88 | 0.05 | 0.03 | 0.03 |
| **Wonderful** | | | | | | |
| Actosol® | 5ml (SD) | 2.54 j | 114.16 j | 11.86 d | 16.64 d | 13.60 b |
| Std.D. | | 0.02 | 0.06 | 0.23 | 0.49 | 0.10 |
| Decka™ | 5ml (SD) | 2.74 i | 117.27 i | 12.08 cd | 16.68 d | 13.33 c |
| Std.D. | | 0.02 | 0.10 | 0.08 | 0.04 | 0.10 |
| Humic acid™ | 2.5g (FS) | 2.99 h | 119.01 h | 7.06 fg | 11.76 gh | 10.78 f |
| Std.D. | | 0.01 | 0.08 | 0.31 | 0.09 | 0.06 |
| Humo plus 40™ | 2.5 ml (FS) | 3.31 g | 125.71 g | 7.28 f | 12.13 g | 10.58 g |
| Std.D. | | 0.03 | 0.05 | 0.08 | 0.06 | 0.03 |
| Inoculated only | | 7.19 c | 215.17 c | 6.50 g | 10.85 i | 5.29 l |
| Std.D. | | 0.02 | 0.04 | 0.07 | 0.05 | 0.05 |

*Means followed by the same letter(s) within a column are not significantly different (P ≤ 0.05) according to Duncans multiple range test. MDA=Lipid peroxidation, $H_2O_2$=Hydrogen peroxide, GSH = Glutathione, TAA = Total ascorbic acid, TPH = Total phenols, Std.D.= Standard division, SD = Soil drench, FS = Foliar spray.*

As a result of the three nematode species parasitism, contents of lipid peroxidation and hydrogen peroxide were raised in all tested cultivars. Also, non-enzymatic antioxidants; Glutathione (GSH), Total ascorbic acid (TAA), and Total phenols (TPH) were sharply increased. Regarding MDA and $H_2O_2$, the over-production of reactive oxygen species due to nematode penetration wounds and/or secretions could interpret the higher amounts of MDA and $H_2O_2$ (Davis *et al.*, 2000; Huang *et al.*, 2004). It maybe considered a defensive tactic leading to programmed cell death (Borden and Higgins, 2002; Mellersh *et al.*, 2002) in a trial of the plants to overcome nematode infection. From another point of view, the overproduction of

**Table 7:** *Effect of commercial products of humic acid on infected pomegranate cultivars root contents of antioxidants enzymes.*

| Treatment | Dose / Plant | APX (unit/mg protein) | CAT (unit/mg protein) | PPO (unit/mg protein) | SOD (unit/mg protein) |
|---|---|---|---|---|---|
| **Assuity** | | | | | |
| Actosol® | 5ml (SD) | 34.91 e | 84.16 cd | 37.28 e | 300.26 d |
| Std.D. | | 0.11 | 1.65 | 1.48 | 2.45 |
| Decka™ | 5ml (SD) | 33.62 f | 85.23 c | 45.73 c | 308.31 c |
| Std.D. | | 0.07 | 3.12 | 2.50 | 1.55 |
| Humic acid™ | 2.5g (FS) | 16.16 j | 41.89 hi | 15.73 gh | 231.63 gh |
| Std.D. | | 0.14 | 0.77 | 1.08 | 9.56 |
| Humo plus 40™ | 2.5 ml (FS) | 15.73 k | 49.30 f | 12.60 ij | 228.32 h |
| Std.D. | | 0.19 | 2.52 | 0.73 | 1.70 |
| Inoculated only | | 14.62 l | 46.90 fg | 12.63 ij | 210.27 j |
| Std.D. | | 0.11 | 4.01 | 0.13 | 5.24 |
| **Manfalouty** | | | | | |
| Actosol® | 5ml (SD) | 45.70 a | 97.38 a | 48.49 b | 381.40 a |
| Std.D. | | 0.20 | 1.71 | 0.62 | 4.96 |
| Decka™ | 5ml (SD) | 40.90 d | 92.57 b | 50.60 a | 375.93 a |
| Std.D. | | 0.12 | 1.65 | 2.39 | 4.74 |
| Humic acid™ | 2.5g (FS) | 16.40 i | 54.43 e | 17.70 fg | 244.97 e |
| Std.D. | | 0.13 | 5.01 | 0.54 | 2.48 |
| Humo plus 40™ | 2.5 ml (FS) | 17.63 g | 50.69 ef | 18.44 f | 236.81 fg |
| Std.D. | | 0.32 | 1.66 | 1.32 | 1.68 |
| Inoculated only | | 14.03 m | 37.79 i | 11.62 j | 192.74 k |
| Std.D. | | 0.06 | 0.64 | 1.03 | 2.22 |
| **Wonderful** | | | | | |
| Actosol® | 5ml (SD) | 44.50 b | 79.90 d | 43.31 d | 359.72 b |
| Std.D. | | 0.09 | 4.19 | 0.63 | 6.24 |
| Decka™ | 5ml (SD) | 42.11 c | 82.94 cd | 46.97 bc | 358.66 b |
| Std.D. | | 0.09 | 0.83 | 0.87 | 5.89 |
| Humic acid™ | 2.5g (FS) | 17.00 h | 47.97 f | 16.83 fg | 239.99 ef |
| Std.D. | | 0.11 | 1.83 | 1.28 | 3.06 |
| Humo plus 40™ | 2.5 ml (FS) | 17.13 h | 43.53 gh | 15.75 gh | 236.36 fg |
| Std.D. | | 0.05 | 1.26 | 0.33 | 1.30 |
| Inoculated only | | 15.54 k | 41.59 hi | 14.38 hi | 217.89 i |
| Std.D. | | 0.13 | 1.65 | 0.65 | 2.18 |

*Means followed by the same letter(s) within a column are not significantly different (P ≤ 0.05) according to Duncans multiple range test. APX = Ascorbat peroxidase, CAT = Catalase, PPO = Polyphenol oxidase, SOD = Superoxide dismutase, Std.D.= Standard division, SD = Soil drench, FS = Foliar spray.*

ROS may be a signal for defensive reaction activation. Such a dual role, according to (Dat *et al*., 2000), is controlled by the cellular level of the ROS. Suggesting the hypothesis of ROS signaling, we can conclude that the significant increase of non-enzymatic antioxidants (GSH, TAA, and TPH) may have resulted from the enhancement of MDA and $H_2O_2$ production after nematode infection.

Enzymatic antioxidants (APX, CAT, PPO, and SOD) activities showed an upwards trend in plants infected with the three nematode species. The excess

superoxide anion ($O_2$-) produced through plant-nematode interaction is scavenged by SOD in the susceptible cultivars. Similarly, ascorbate peroxidase and catalase scavenge hydrogen peroxide (Zacheo and Bleve-Zacheo, 1988). Chemically, the enzymatic detoxification process of ROS includes sequential steps; for example, superoxide dismutase transforms the superoxide anion into hydrogen peroxide, which is finally transformed into atmospheric oxygen and water by catalase (Zacheo *et al*., 1987). The fluctuations in antioxidant activities among cultivars due to the same commercial products may reflect the effect of the genetic variations among different cultivars. Herein, one could conclude that the biochemical response is nematode species-dependent.

The antioxidant response due to ectoparasitic nematode, *H. indicus* in all cultivars, recorded the highest significant values when compared to the other two nematode species regardless of the host type. This may be due to its feeding habits, where *Helicotylenchus* spp. used to feed externally on the root cortical layer producing small necrotic lesions (Bridge and Gowen 1993). In a single feed time, their stylet is repetitively inserted until having enough meal, then after being hungry, they return to the same actions, causing large numbers of wounds. These wounds may force the plant to produce much higher responsive molecules. Such a case could be supported by Sango *et al*. (2004) findings which found the high damage potential of *H. multicinctus* was achieved in relatively low populations.

When comparing the pomegranate cultivar's reaction against the endoparasitic nematode, *M. incognita* infection, the highest oxidative stress (MDA, and $H_2O_2$ contents) was noticed in the most susceptible cultivar 'Assuity'. This reflects the supporting role of oxidative stress in nematode pathogenicity.

The application of commercial humic acid products either through foliar spray or soil drench greatly enhanced the antioxidant activities (enzymatic and non-enzymatic) on the RKN-infected cultivars while reducing the MDA and hydrogen peroxide contents. These alterations were significant and sharply altered when compared to the untreated plants. In fact, ROS/antioxidant activities seem to be extremely dynamic, complex, and rather very correlative. Stimulation of the antioxidant system by humic acid products application increased the scavenging capacity inside plants which reduced the oxidative stress represented in MDA and $H_2O_2$. Decreasing ROS could hinder nematode pathogenicity and reproduction (Zhang *et al*., 2019), which is why RKN was partially suppressed due to humic acid treatments. In conclusion, we can implement humic acid products in the IPM programs as a secondary approach useful in managing the root-knot nematode.

## Conclusion

The findings of this study have several important implications for the management of plant-parasitic nematodes in pomegranate orchards. The identification of susceptible cultivars can guide growers in selecting resistant or tolerant varieties. Monitoring the levels of antioxidant enzymes and oxidative stress markers can provide valuable insights into the plant's defense response against nematode infection.

Furthermore, the study highlights the potential of humic acid as a natural nematicide for pomegranate. Future research should focus on optimizing the application rates and timing of humic acid for maximum efficacy against different phyto-nematode species. Additionally, investigations into the mechanisms by which humic acid enhances plant resistance to nematodes are warranted.

By integrating these findings into an integrated pest management (IPM) program, pomegranate growers can develop sustainable strategies to control plant-parasitic nematodes and improve crop health and productivity.

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## Novelty Statement

This study provides novel insights by comprehensively linking the type of nematode parasitism (endo, semi-endo, and ecto) to the specific oxidative and antioxidative responses in different pomegranate cultivars. Unlike previous research that often focuses on a single nematode species, we demonstrate that the ectoparasite *Helicotylenchus indicus* induces the most severe biochemical defense response, likely due to its feeding behavior. Furthermore, we present a comparative efficacy analysis of four distinct

commercial humic acid products, revealing that soil drench applications are significantly more effective than foliar sprays in modulating the plant's antioxidant defense system to suppress root-knot nematode reproduction. These findings offer a new, physiology-based strategy for integrating specific humic acid products into integrated pest management programs for pomegranate.

## Author's Contribution

All authors carried out the experiments, Kesba, H.H. and El-Ganainy, S.M. designed and performed the statistical analysis, Diab, S.F. prepared results tables, Abdel-rahman, A.A. prepared the first look of the manuiscript. All authors revised and approved the manuscript.

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