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RESEARCH PAPERS

Effect of Root-Knot Nematode *Meloidogyne incognita* on Barley: Morphological Alterations and the Role of Enzymes in Interactions

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Abstract– Plant-parasitic nematodes are devastating pests of most crops, and their management still encounters challenges. Unraveling the plant-nematode interactions can shed light on the pathogenesis and resistance mechanisms, particularly in the case of the southern root-knot nematode *Meloidogyne incognita* interactions with monocots such as barley (*Hordeum vulgare*), to manage them properly. In this research, plant growth characteristics (length and fresh/dry weight of shoot and root), nematode gall index, resistance index, and reproduction factor were studied in barley genotypes namely 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' at 60 days post-inoculation (dpi). Afterwards, the activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and phenylalanine ammonia-lyase (PAL) enzymes were measured at 0, 1, 2, 3, 4, and 10 dpi. The results showed that all plant growth characteristics except for the root weight decreased upon inoculation. While genotypes 'Reyhan', 'Nik' and 'Yousef' were proved to be resistant, moderately susceptible and susceptible, respectively, the remaining genotypes exhibited a moderate resistance response. In the 'Reyhan' genotype, the SOD, CAT, APX and GPX enzymes peaked at 1 dpi, whereas in other genotypes they increased over time. The genotype 'Reyhan' was completely different from the others in terms of peak time (except for PAL). The early enzymatic activity was likely related to the timely response of the resistant genotype 'Reyhan' to the nematode, while the late activity probably affected the tolerance to the nematode in the other genotypes. PAL exhibited an upward slope in the more resistant genotypes and probably had a positive correlation with resistance.

Keywords: *Hordeum vulgare*, antioxidant enzymes, gall index, phenylalanine ammonia-lyase, plant-nematode interactions

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INTRODUCTION

Barley (*Hordeum vulgare*; $2n = 2x = 14$) is considered to be the most economically important cereal after corn (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*), and hosts a wide range of pests including plant-parasitic nematodes [1]. Root-knot nematodes (RKN, *Meloidogyne* spp.) are a major group causing $173 billion in annual nematode damage to crops. By establishing a typical feeding structure in the plant roots, known as giant cells, they are capable to parasitise almost all cultivated plants [2]. The polyphagous *M. incognita* exhibits obligatory mitotic parthenogenesis mode of reproduction, and achieves the required function in parasitism through various mechanisms such as horizontal gene transfer, transposable elements, multiple hybridizations, and nucleotide divergence, allowing it to adapt to the different environmental conditions without sexual reproduction [3]. Upon exposure to biotic and abiotic stressors, plants activate signaling molecules such as reactive oxygen species (ROS), a group of free radicals, reactive molecules and ions derived mainly from molecular oxygen (O_2) to maintain normal growth and development [4]. ROS include free radicals, such as superoxide anion (O_2^-) and hydroxyl radical (OH), and non-radical molecules such as hydrogen peroxide (H_2O_2) and oxygen (O_2). However, pathogen invasion disturbs the balance between ROS production and inhibition, causing a rapid increase in internal ROS levels and oxidation of lipids, proteins, and nucleic acids, ultimately leading to irreversible damage. To cope with these inflicted damages, plants have developed an enzymatic scavenging system to regulate ROS levels, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; dpi, days post-inoculation; DW, dry weight; FW, fresh weight; GI, gall index; GPX, guaiacol peroxidase; L, length; RF, reproduction factor; RI, resistance index; RKN, root-knot nematodes; SOD, superoxide dismutase; PAL, phenylalanine ammonia-lyase.

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(GPX) and phenylalanine ammonia-lyase (PAL) [5]. Studying plant-nematode interactions leads to an understanding of the precise pathogenesis/resistance mechanism(s), paving the way for optimal management strategies [6]. The issue became outstanding when under the Montreal Protocol application of the effective fumigants was withdrawn due to the depletion of the Ozone layer and adverse effects on human health and the environment [7]. The initial step towards investigating the plant-nematode interactions is to study the morphological alterations such as plant growth parameters and host symptoms (resistance screening). In the case of the RKN, for instance, the typical symptom is the formation of galls in the roots. Thereafter, the subsequent step towards investigating plant-nematode interactions is studying biochemical changes like measuring enzyme activity.

Monocots and dicots may respond differently to pathogens; hence, it would be of interest to clarify the pathogenesis/resistance mechanism of *M. incognita*, with its unique reproduction and cosmopolitan identity. To the best of our knowledge, the information on the interaction of *M. incognita* with *H. vulgare* is rather scarce and the underlying mechanism(s) remains to be elucidated. The aims of this study were (i) to assess the effect of *M. incognita* on the growth characteristics of *H. vulgare* including shoot/root length, fresh and dry weight in inoculated and uninoculated samples, (ii) to screen the resistance of inoculated plants based on nematode gall index (GI), resistance index (RI) and reproduction factor (RF) based on several screening methods, and (iii) to keep track of activity of SOD, CAT, APX, GPX and PAL enzymes to investigate the responses of *H. vulgare* genotypes after inoculation with *M. incognita*.

MATERIALS AND METHODS

Barley genotypes. The barley genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow', and also the *M. incognita* isolate used in this research were kindly provided by the Seed and Plant Improvement Institute (SPII), Karaj, Iran, and the Nematology Lab at Ferdowsi University of Mashhad, Iran, respectively. The nematode isolate originated from pomegranate orchards in eastern Iran, e.g., Razavi, Northern and Southern Khorasan provinces.

***M. incognita* inoculums.** In order to obtain *M. incognita* inoculum on the suitable host, 2000 second-stage juveniles (J2) were inoculated on the roots of tomato (*Solanum lycopersicum* cv. Rutgers) seedlings with four true-leaves and kept in the greenhouse. To hatch the juveniles and eggs required for the assays, the inoculated roots were cut into small pieces, blended and exposed to 1% NaOCl solution for 5 min. Thereafter, the mixture was carefully washed and maintained in a double-layered tissue in a sieve submerged in a container of half-filled distilled water. Eggs were hatched over water at 28°C for at least 3 days, thereupon, freshly hatched J2 nematodes were collected as inoculum [8].

***M. incognita* J2 preparation and inoculation.** Seeds of the *H. vulgare* genotypes were planted in pots containing sterile soil, sand and perlite (with 1 : 1 : 1; v : v : v). Afterwards, the roots at Zadoks growth stage 12 [9] were inoculated with 2000 freshly hatched J2 nematodes, the pots were kept in greenhouse conditions at 28 ± 4°C with 16-h photoperiod and 50% humidity, and checked daily for optimal growth conditions and pest/disease management until the end of assay. Non-inoculated plants were considered as controls.

Resistance screening based on the morphological characteristics of the plant. Sixty days after inoculation, the length and fresh weight (FW) of shoots and roots (i.e., all inoculated and non-inoculated samples) were measured. The samples were placed in paper bags and kept in an oven at 70°C. After 48 h, the dry weight (DW) of the shoot and root samples was recorded. The assay was conducted with at least five replicates and two biological repetitions were performed.

Resistance screening based on nematode gall index (GI), resistance index (RI) and reproduction factor (RF). Screening plant resistance to RKN was mainly carried out based on GI and nematode RF (ratio of the final population density to the initial population density of nematodes in inoculated plants). In the current research, the nematode-related traits were evaluated using three methods: (1) Mukhtar et al. method [10]; (2) the complementary methods described by Quesenberry et al. [11] and Taylor and Sasser [12]; (3) the method of Canto-Saenz [13].

According to the method 1, GI 0 = immune (no gall/root system), 1 = highly resistant (1–2 galls), 2 = resistant (3–10 galls), 3 = moderately resistant (11–30 galls), 4 = moderately susceptible (31–70 galls), 5 = susceptible (71–100 galls), 6 = highly susceptible genotype (>100 galls).

In method 2, the GI/egg mass index (EMI) was calculated as follows: 0 = no gall/egg mass, 1 = 1–2 galls/egg mass(es), 2 = 3–10 galls/egg masses, 3 = 11–30 galls/egg masses, 4 = 31–100 galls/egg masses, and 5 = more than 100 galls/egg masses/root system [12]. The resistance index (RI) was then determined using the following equation:

$$RI = \sqrt{\left(gall \middle| \middle| index^2 + eggmassindex^2 \right)}.$$

A RI of 0.09 was considered immune, RI in the range 1–1.9 demonstrates highly resistant, RI 2–2.9 is resistant, RI 3–3.9 is moderately resistant, RI 4–4.9 is intermediate resistance, RI 5–5.9 is moderately susceptible, RI 6–6.9 is susceptible, and RI > 7 was a highly susceptible genotype [11].

In the method 3, based on GI and RF, plants were regarded as resistant (for RF ≤ 1, GI ≤ 2), moderately resistant (for RF ≤ 1, GI > 2), tolerant (for RF > 1,

GI ≤ 2) and susceptible genotype (for RF > 1, GI > 2) [13].

Total protein extraction and enzyme activity assays. To evaluate the activity of SOD, CAT, APX, GPX and PAL enzymes, roots of the inoculated/non-inoculated plants were sampled at 5 time intervals, including 0, 1, 2, 3, 4, and 10 dpi. Total protein was extracted using the method described by Kar and Mishra [14]. Root samples were finely ground, using a sterile pestle and mortar in liquid nitrogen. The samples were then mixed with 100 mM potassium phosphate buffer (pH 6.8), vortexed and centrifuged at 15000 *g* (4°C) for 15 min. The supernatants (enzyme extracts) were transferred to new microtubes to measure the activity of SOD, CAT, APX and GPX enzymes. For the extraction of the PAL protein, 50 mM Tris-HCl buffer (pH 8.8) containing 15 mM β-mercaptoethanol was used [15].

Bovine serum albumin (BSA, Sigma) was used as a standard protein to measure the total protein concentration in the solution. Sample absorbance was recorded at 595 nm using a spectrophotometer Biowave II (VRW, United States) [16]. This assay was conducted with at least three replicates and two biological repetitions.

Superoxide dismutase (SOD). The reaction mixture, containing 0.013 M methionine, 6.3 μM nitroblue tetrazolium (NBT), 6.5 μM riboflavin, 0.1 mM EDTA and 0.05 M potassium phosphate buffer (pH 7.8). The mixture was incubated for 10 min at 30 under 6000 lux and read at A_{560} [17]. One unit of SOD activity was regarded as U SOD/(mg protein), the amount of the enzyme causing 50% inhibition of NBT reduction.

Catalase (CAT). 100 mM potassium phosphate buffer (pH 7) and 70 mM H_2O_2 were mixed in sterile distilled water. The absorbance rate of samples with/without enzyme extract was recorded at spectrophotometer for 3 min at 30 s intervals at 240 nm [18].

Ascorbate peroxidase (APX). The APX buffer contained 0.5 mM ascorbic acid, 50 mM potassium phosphate (pH 7) and 0.1 mM H_2O_2. By adding enzyme extract, the absorbance was readfor 180 s in 30 s intervals at absorbance 290 nm [19].

Guaiacol peroxidase (GPX). 100 mM potassium phosphate (pH 6.8), 100 mM guaiacol, 70 mM H_2O_2 and sterile distilled water were mixed to prepare the reaction buffer. Thereafter, GPX activity in samples with/without enzyme extract was recorded spectrophotometrically for 3 min with time intervals of 30 s at 470 nm [20].

The CAT, APX and GPX were expressed as μmol/(min mg protein). Each unit of CAT expressed the oxidation of 1 μM of H_2O_2/min. The required enzyme to oxidase 1 μmol ascorbic acid/min was regarded as an APX unit. One unit of GPX was expressed as the amount of enzyme that formed 1 μM of tetra guaiacol/min.

Phenylalanine ammonia-lyase (PAL). The reaction mixture included extraction buffer (50 mM Tris-HCl, 15 mM of β-mercaptoethanol, pH 8.8, 10 mM phenylalanine, 6 M HCl. The mixture (without HCl) was incubated at 37°C for 1 h. The reaction then was stopped by adding HCl to the samples and the absorbance was measured at 290 nm [15].

The reaction rate was determined by following the conversion of phenylalanine into *trans*-cinnamic acid. One unit of PAL expressed 1 μM of *trans*-cinnamic acid/min.

Statistical analysis. All experiments were laid out in factorial arrangements based on the completely randomized design (CRD). The resistance assay was conducted with at least five replicates and two biological repetitions. The biochemical assays were carried out with at least three replicates and two biological repetitions. Data were checked for normality and the homogeneity of variances using the Kolmogorov-Smirnov and Levene's tests respectively prior to performing the analysis of variance (ANOVA). Means were compared using the Tukey test at $P \leq 0.05$ in IBM SPSS Statistics 26 software for Windows. Values were expressed as means ± standard errors (SE).

RESULTS

Effect of M. incognita on Plant Growth Characteristics

The shoot length in the inoculated genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' decreased by 65, 88.9, 80.7, 66, 83, 95.5, 93.7, and 71.6% compared to their non-inoculated samples. Shoot lengths in inoculated genotypes except for 'Nik', 'Nimrouz' and 'Jolgeh' had no difference from the corresponding non-inoculated samples, but significant differences were detected in the other genotypes. 'Zarjow' inoculated samples were drastically different from other genotypes.

In non-inoculated 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow', the mean FW of shoots decreased by 30, 30.6, 19.3, 22.9, 51.6, 54.8, and 17.3% in the presence of *M. incognita*. In all inoculated genotypes, the shoot FW was drastically different compared to non-inoculated. The mean FW of the shoot in 'Bahman' and 'Jolgeh' was statistically different compared to other genotypes.

The mean DW in shoots of inoculated genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' decreased by 40.2, 76.1, 51.6, 31.2, 28.2, 29.6, 73.6, and 48.4% compared to the corresponding non-inoculated. In all inoculated genotypes, the shoot DW was statistically different compared to non-inoculated. A statistical difference was found in the mean of shoot DW in 'Nik' in comparison with genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Zarjow' and 'Nimrouz'.

In inoculated 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' the

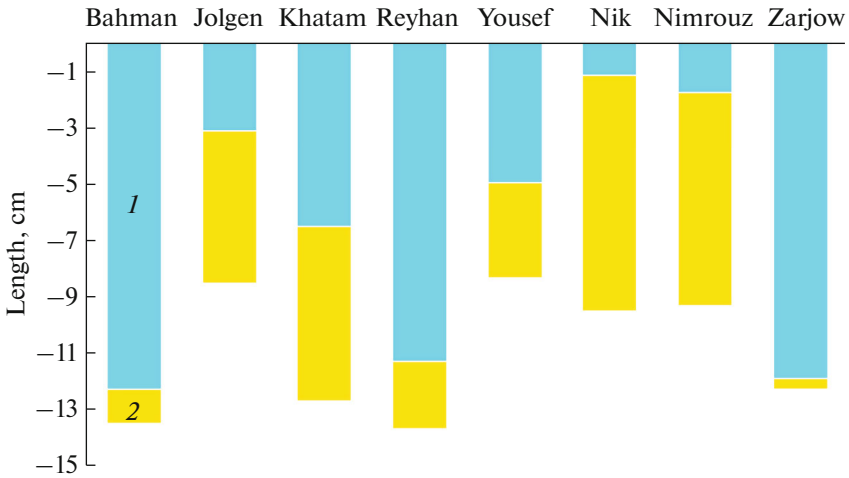

Fig. 1. Mean difference of (*1*) shoot length (blue) and (*2*) root length (yellow) in 8 barley genotypes in the absence and presence of root-knot nematode *Meloidogyne incognita*. The values are expressed according to cm.

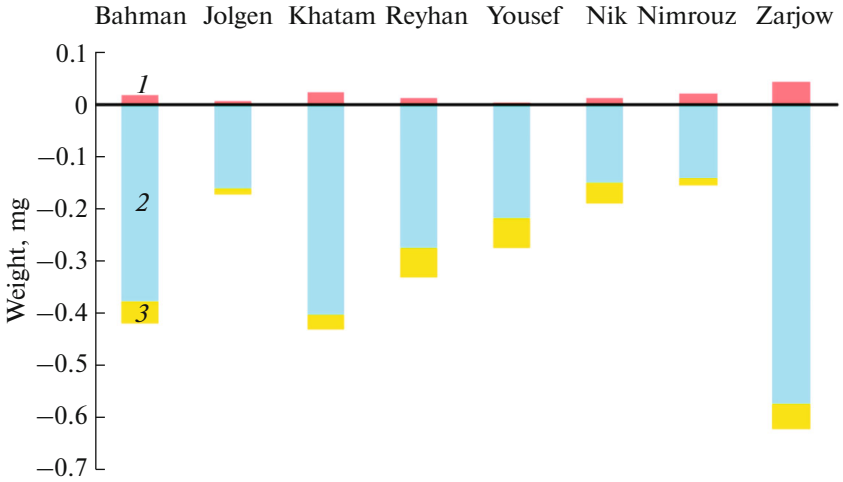

Fig. 2. Mean difference of (*1*) root fresh weight, (*2*) shoot fresh weight, and (*3*) shoot dry weight in 8 barley genotypes in the absence and presence of root-knot nematode *Meloidogyne incognita*. The values are expressed according to mg.

mean root length decreased by 89.2, 64.9, 80.1, 84.2, 77.3, 53.3, 56.3, and 97.1% compared to the non-inoculated. Except for inoculated 'Bahman', 'Reyhan' and 'Yousef', which did not have a statistically significant difference in the mean root length, differences were observed in the other genotypes. The mean root length of 'Khatam' had differences compared to all genotypes.

The mean root FW of non-inoculated 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' decreased by 45.1, 74, 42.1, 50, 88.8, 59.2, 40 and 32.2% compared to the corresponding inoculated, respectively. On the other hand, the nematode increased the rootFW. Except for 'Nik' and 'Yousef', significant statistical differences were observed among the inoculated and non-inoculated genotypes. A statistical difference was found in the root FW of 'Zarjow' in comparison with all genotypes ($P \leq 0.05$).

The differences between each inoculated genotype and the corresponding non-inoculated genotype and the other inoculated genotypes were calculated for all the plant growth characteristics (Table 1, Figs. 1 and 2). Root FW was calculated, but root DW was excluded for all genotypes as less than 0.001 g.

Nematode-Related Factors

The method 1 of Mukhtar et al. [10], the complementary methods 2 of Taylor and Sasser [12] and Quesenberry et al. [11], and method 3 of Canto-Saenz [13] were employed to screen *H. vulgare* genotypes for resistance to *M. incognita*. Considering the RI in the method 2, 'Reyhan' (IR 2.8), 'Yousef' (IR 6.4) and 'Nik' (IR 5) were considered resistant, susceptible, and moderately susceptible, respectively. While varieties 'Bahman', 'Jolgeh', 'Khatam', 'Nimrouz' and

Table 1. Analysis of variance of barley growth factors in genotypes inoculated/uninoculated with the root-knot nematode *Meloidogyne incognita* L.

Genotype	Sample type	Shoot L, cm	Shoot FW, mg	Shoot DW, mg	Root L, cm	Root FW, mg
Bahman	Control	35.6 ± 0.81b	535 ± 0.022b	72 ± 0.003bg	11.2 ± 0.01def	14 ± 0.0007ef
Bahman	Inoculated	23.2 ± 0.663gh**	159 ± 0.012e**	29 ± 0.001h**	10 ± 0.948ef$^{n.s}$	31 ± 0.002bcd**
Jolgeh	Control	29 ± 0.707def	232 ± 0.006d	42 ± 0.001ef	15.4 ± 1.02cd	20 ± 0.001def
Jolgeh	Inoculated	25.8 ± 0.96efgh$^{n.s}$	71 ± 0.003g**	32 ± 0.001fgh**	10 ± 0.707ef**	27 ± 0.0009bcde*
Khatam	Control	34.2 ± 0.86bc	497 ± 0.018b	60 ± 0.001c	31.2 ± 0.8a	16 ± 0.001ef
Khatam	Inoculated	27.6 ± 1.02defg**	96 ± 0.002fg**	31 ± 0.001gh**	25 ± 0.707b**	38 ± 0.001b**
Reyhan	Control	33.6 ± 1.12bc	357 ± 0.012c	80 ± 0.001b	15.2 ± 0.8cd	12 ± 0.008f
Reyhan	Inoculated	22.2± 0.86h**	82 ± 0.0001fg**	25 ± 0.002hi**	12.8 ± 1.01def$^{n.s}$	24 ± 0.003cdef**
Yousef	Control	30 ± 0.707cde	358 ± 0.016c	78 ± 0.001b	15 ± 1.04cd	24 ± 0.0009cdef
Yousef	Inoculated	25 ± 0.707fgh**	140 ± 0.005ef**	22 ± 0.001hi**	11.6 ± 0.6def$^{n.s}$	27 ± 0.0009bcde$^{n.s}$
Nik	Control	27.2 ± 0.86defg	310 ± 0.01c	54 ± 0.002cd	18 ± 0.83c	16 ± 0.0007ef
Nik	Inoculated	26 ± 0.07 defg$^{n.s}$	160 ± 0.02e**	16 ± 0.007i**	9.6 ± 0.87f**	27 ± 0.0007bcde$^{n.s}$
Nimrouz	Control	29 ± 1.3def	310 ± 0.1c	57 ± 0.002cd	17.4 ± 092c	14 ± 0.014ef
Nimrouz	Inoculated	27.2 ± 1.0 defg$^{n.s}$	170 ± 0.004de**	42 ± 0.003efg**	9.8 ±0.58f**	35 ± 0.002bc*
Zarjow	Control	42.4 ± 0.92a	690 ± 0.2a	97 ± 0.004a	14.2 ± 0.58cde	20 ± 0.023cdef
Zarjow	Inoculated	30.4 ± 0.92cd**	120 ± 0.002efg**	47 ± 0.001de**	13.8 ± 0.66cdef**	62 ± 0.009a**

The results are expressed as mean ± standard error based on a completely randomized design. ns, non-significant, * significant at levels $P < 0.05$ and ** at $P < 0.01$ between inoculated and non-inoculated samples according to Tukey's test. Means followed by different letters in column are significantly different.

Table 2. Mean nematode-related factors in barley genotypes inoculated with 2000 second-stage juveniles of the root-knot nematode *Meloidogyne incognita*

Genotype	Gall mean/root	GI	RI	Method 1	Method 2	Method 3
Bahman	30 ± 0.435c	3	3.6	MR	MR	MR
Jolgeh	12 ± 0.316e	3	3.1	MR	MR	MR
Khatam	20.6 ± 0.509d	3	3.1	MR	MR	MR
Reyhan	5.5 ± 0.223f	2	2.8	R	R	R
Yousef	78.6 ± 0.509a	4	6.4	MS	S	MR
Nik	38.4 ± 1.36b	4	5	MS	MS	MR
Nimrouz	12.8 ± 0.96e	3	3.1	MR	MR	MR
Zarjow	14 ± 1.14e	3	3.1	MR	MR	MR

The results are expressed as mean ± standard error based on a completely randomized design. Means followed by dissimilar letters in column are significantly different according to Tukey's test; GI, gall index; RI, resistance index; MR, moderately resistant; MS, moderately susceptible; R, resistant; S, susceptible.

'Zarjow' (IR 3–3.9) showed a moderately resistant response. These results were also confirmed by other methods with minor differences. The difference in the methods was more apparent in the genotypes 'Yousef' and 'Nik'. Genotype 'Yousef' was determined as moderately susceptible in method 1, susceptible in method 2, and moderately resistant in method 3. According to methods 1 and 2, the genotype 'Nik' was regarded as moderately susceptible and moderately resistant based on method 3. Due to more accuracy, we considered method 2 as the basis of resistance screening. In all genotypes, RF was less than one, which means that the nematode had not successfully reproduced and the final population density was less than the initial population density. Except for the 'Zarjow', 'Jolgeh' and 'Nimrouz', gall mean/root was significantly different in the other genotypes (Table 2).

Effect of M. incognita on Enzymes Activity

The SOD activity in genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' had the maximum amount of 2.68, 2.09, 2.43, 3, 2.89, 2.73, 1.91 and 2.15 U/(mg protein) at 4, 3, 4, 1, 4, 4, 4, and 3 dpi, respectively. In 'Reyhan', the enzyme activity reached its maximum at 1 dpi for 3 U/(mg protein), then decreased to stabilize at 4 and 10 dpi for 2 and 2.1 U/(mg protein), respectively. The SOD activity in the 'Yousef', 'Nik' and 'Khatam' genotypes increased gradually and reached the peak at 4 dpi for 2.89, 2.7 and 2.4 U/(mg protein), respectively. Genotypes 'Nik', 'Zarjow' and 'Nimrouz' exhibited nearly linear patterns. The highest SOD activity was recorded at 1 dpi for genotype 'Reyhan' and in genotypes 'Reyhan', 'Jolgeh', 'Yousef' and 'Zarjow' was statistically different at all time points considered ($P \leq 0.05$) (Fig. 3a).

In 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow', the CAT exhibited the highest activity for 0.19, 0.11, 0.18, 0.24, 0.21, 0.20, 0.09 and 0.11 μmol/(min mg protein) at 4, 4, 4, 1, 4, 4, 4 and 4 dpi, respectively. The enzyme activity in 'Reyhan' first increased at 1 dpi for 0.24 μmol/(min mg protein), and then decreased to reach relative stability; however, the reactions in all time points showed profound differences. The CAT activity in 'Bahman', 'Jolgeh', 'Khatam', 'Yousef', 'Nik' and partially 'Zarjow' grew with a high gradient to be maximized at 4 dpi, the reactions at 3–4 dpi had the most significant difference from others. CAT activity was nearly linear in the 'Nimrouz', therefore, the reactions showed no statistical difference on any day ($P \leq 0.05$) (Fig. 3b).

APX with values of 0.27, 0.27, 0.27, 0.23, 0.29, 0.26, 0.27 and 0.27 μmol/(min mg protein) exhibited the highest activity in genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow' at the 4, 4, 3, 1, 4, 4 and 4 dpi, respectively. The enzyme increased in genotypes with a steep and almost similar slope except for 'Reyhan', which peaked earlier at 1 dpi. The reactions had statistical differences between most of the time points ($P \leq 0.05$) (Fig. 3c).

GPX exhibited the highest activity 0.31, 0.34, 0.36, 0.35, 0.33, 0.31, 0.27 and 0.27 μmol/(min mg protein) on days 3, 4, 3, 1, 4, 4, 4, and 3–4 in the genotypes 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow', respectively. GPX had the maximum activity at 1 dpi in 'Reyhan' and then decreased. However, its activity was still at high level for 0.33, 0.32, 0.3, 0.3 μmol/(min mg protein) at 2, 3, 4 and 10 dpi, respectively. In other genotypes, the enzyme peaked later at 3 and/or 4 dpi, and among them, the highest enzyme activity 0.36 μmol/(min mg protein) was recorded for the 'Khatam' genotype. The reactions at the 3–4 dpi were drastically different in all genotypes ($P \leq 0.05$) (Fig. 3d).

At 10 dpi PAL had the highest activity with values of 15, 16, 15.2, 21, 14, 14.5, 15 and 18 unit/(g protein) in 'Bahman', 'Jolgeh', 'Khatam', 'Reyhan', 'Yousef', 'Nik', 'Nimrouz' and 'Zarjow'. PAL activity in 'Rey-

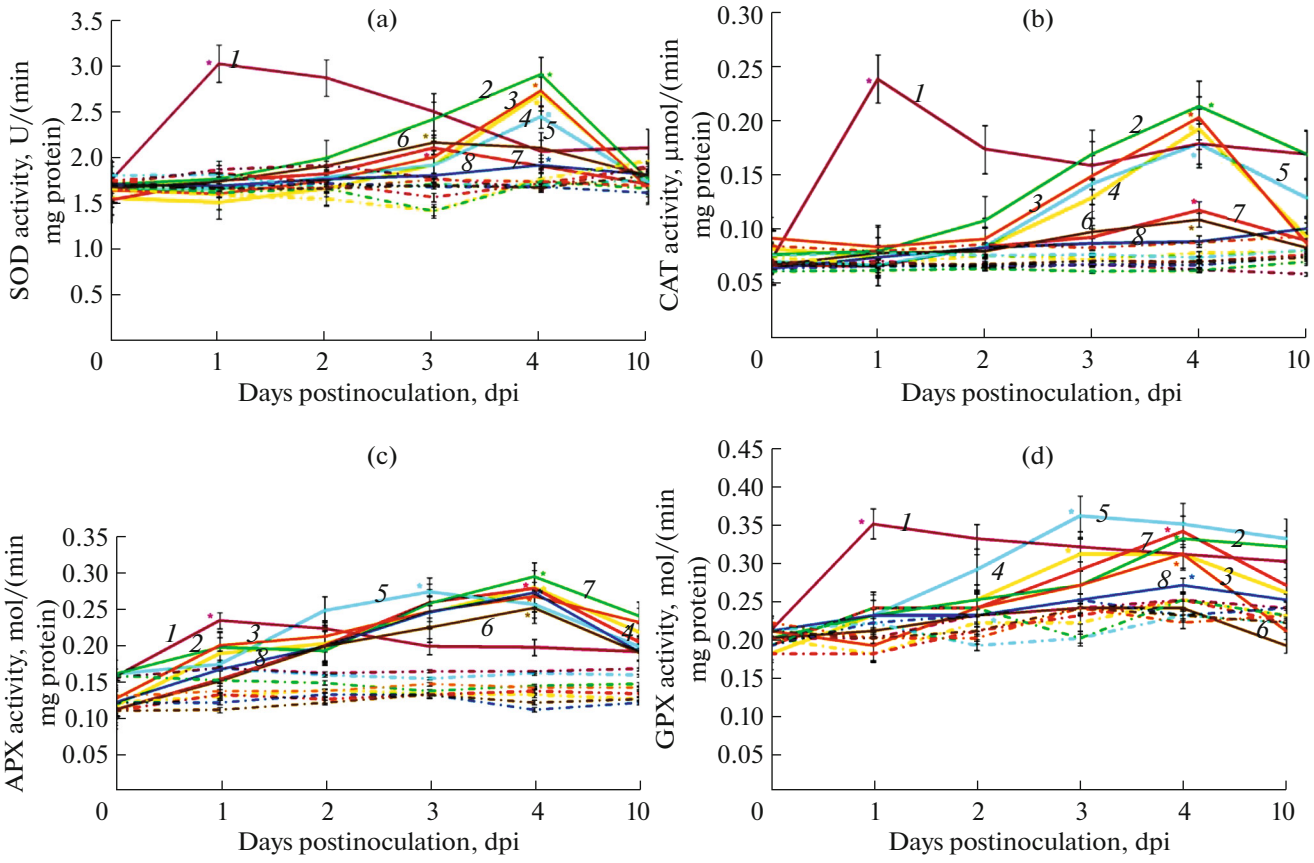

Fig. 3. Antioxidant enzymes activity in barley genotypes inoculated (solid line)/non-inoculated (dash-dot line) with the root-knot nematode *Meloidogyne incognita*. (a) Superoxide dismutase (SOD). (b) Catalase (CAT). (c) Ascorbate peroxidase (APX). (d) Guaiacol peroxidase (GPX) at 0, 1, 2, 3, 4, and 10 days post-inoculation (dpi). (*1*) 'Reyhan' (purple); (*2*) 'Yousef' (green); (*3*) 'Nik' (orange); (*4*) 'Bahman' (yellow); (*5*) 'Khatam' (cyan); (*6*) 'Zarjow' (braun); (*7*) 'Jolgeh' (red); (*8*) 'Nimrouz' (blue). The mean that are followed by asterisk (*) for each evaluation time are significantly different as determined by Tukey's test.

han' as the resistance genotype was higher than in others ('Reyhan' > 'Zarjow' > 'Jolgeh' > 'Nimrouz' > 'Bahman' > 'Nik' > 'Khatam' > 'Yousef'). There was a drastic difference between the reactions at some time points especially on the fourth day compared to the day before inoculation in 'Jolgeh', 'Zarjow', 'Nimrouz' and 'Reyhan' ($P \leq 0.05$). The enzyme activity exhibited an increasing trend in all genotypes across all time points tested (Fig. 4).

DISCUSSION

Investigating the enzymatic activity of SOD, CAT, GPX, APX, and PAL in interactions with *M. incognita* is of paramount importance to attain a better understanding of the role(s) of desired enzymes in plant defense. According to our findings, all plant growth characteristics decreased significantly except for the root weight upon inoculation with *M. incognita*. RKN induce the formation of galls in roots to parasitize their hosts leading to disruption of water and nutrient transportation and thus plant weakening [21]. Results of

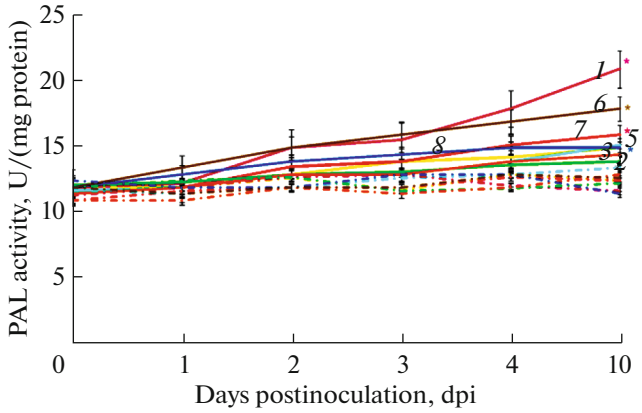

Fig. 4. Enzymatic activity phenylalanine ammonia-lyase (PAL) activity at 0, 1, 2, 3, 4, and 10 days post-inoculation (dpi). (*1*) 'Reyhan' (purple); (*2*) 'Yousef' (green); (*3*) 'Nik' (orange); (*4*) 'Bahman' (yellow); (*5*) 'Khatam' (cyan); (*6*) 'Zarjow' (braun); (*7*) 'Jolgeh' (red); (*8*) 'Nimrouz' (blue). The mean that are followed by asterisk (*) for each evaluation time are significantly different as determined by Tukey's test.

ANOVA, however, indicated that root weight was increased by *M. incognita*. It has also been well-documented that nematodes are able to manipulate plant growth regulators such as auxins and cytokinins, which are required to establish feeding structures in the roots for their benefit following inducing pathogenicity [22]. Accumulation of plant regulators and also the formation of galls more likely contribute to heavier roots in the *M. incognita*-inoculated plants [23]. In this study, based on method 2, the genotypes 'Reyhan', 'Yousef' and 'Nik' were considered resistant, susceptible, and moderately susceptible, respectively. Other genotypes (i.e., 'Bahman', 'Jolgeh', 'Khatam', 'Nimrouz' and 'Zarjow') were regarded as moderately resistant. The moderate resistance and resistance of most barley genotypes are presumably attributed to the presence of callose papillae and lignin in the cell wall [24]. Our data highlighted that the genotype 'Reyhan' differed in terms of SOD higher activity and peak time, the enzyme activity in the resistant genotype 'Reyhan' maximized at 1 dpi, while in other genotypes peaked at 3 ('Jolgeh' and 'Zarjow') or 4 dpi ('Bahman', 'Khatam', 'Yousef', 'Nik' and 'Nimrouz'). SOD by scavenging the primary form of ROS, O_2^-, affects pathogen survival and virulence. Prior studies demonstrated SOD activity was alleviated in resistant genotypes of cowpea and tomato when exposed to *M. incognita* [25, 26], possibly to accumulate ROS at the early inoculation stage to inhibit nematode further penetration by killing the plant cells around in oxidative explosions [27]. Labudda et al. [28] reported similar results as they compared *Heterodera filipjevi*-inoculated barley to non-inoculated. The reduction of SOD gene expression in *M. arenaria*-inoculated genotypes, both resistant and susceptible, has been recorded in the first 24 h post-inoculation [29]. Furthermore, Zacheo and Bleve-Zacheo [25] attributed the activity of SOD to preserving the susceptible genotypes from the destructive effects of ROS. Our findings did not support the data obtained by the aforementioned studies (except [25]), in which resistant genotypes decreased SOD activity, meanwhile, the early peak time in 'Reyhan' was likely related to the timely response to nematode invasion. Late SOD activity in barley non-resistant genotypes (i.e., moderately resistant, moderately susceptible and susceptible genotypes) was probably attributed to signaling and preserving the plant.

CAT enzyme in the genotype 'Reyhan' reached the peak at 1 dpi, subsequently after decreasing, yet showed relatively higher activity, whilst other genotypes declined sharply after the peak point at 4 dpi. The highest turnover rate among enzymes scavenging H_2O_2 has been assigned to CAT [5, 30]. The research conducted so far has shown conflicting reports on the role of CAT in plant-nematode interactions. Molinari [31] declared that at the first 24 h post RKN inoculation (i.e., *M. incognita* and *M. hapla*), the CAT activity in the tomato-resistant genotype decreased, while high activity was noted in the susceptible one. Similarly, cowpea's resistant genotype alleviated CAT activity against *M. incognita*, as compared to less resistant and non-inoculated genotypes [26]. Similar results were obtained when barley was inoculated with *H. filipjevi* [28]. These results are consistent with the view that in dicotyledonous resistant genotypes [32], high salicylic acid concentration inhibits CAT activity to accumulate H_2O_2, subsequently inducing hypersensitive reaction and producing antimicrobial compounds against the nematode [31]. *M. arenaria*-inoculated maize-susceptible and resistant genotypes also lowered CAT activity, particularly at the first 24 h post-inoculation [29]. Interestingly, Scandalios et al. [33] reported in comparison with dicots, the mechanism of salicylic acid associated with CAT is different in monocots, i.e., slight CAT inhibition was recorded in resistant genotypes. Taken together, it seems CAT's higher activity in resistant genotype 'Reyhan' was more likely related to the lack of inhibition in monocotyledonous barley. On the other hand, the lower and late activity of CAT in other genotypes was probably correlated with the inadequate response to the nematode.

The maximum APX enzyme activity in the resistant genotype 'Reyhan' was recorded at 1 dpi and in other genotypes at 3 or 4 dpi. Moreover, the enzyme activity in 'Reyhan' exhibited lower activity than others. APX, an H_2O_2 scavenging peroxidase with a high affinity, plays an eminent role in tuning ROS in the plant [30]. *H. filipjevi*-inoculated barley genotype declined APX activity, as compared with the non-inoculated [28]. More than 70% of *H. glycines* female index decreased, as a soybean gene encoding for APX was overexpressed, showing the importance of the enzyme in plant defense [34]. Studying on effects of *H. avenae* on hexaploid wheat genotypes, Simonetti et al. [35] attributed the early growing activity of APX in the resistant genotype to inducing the hypersensitivity reaction. Our findings were consistent with Simonetti et al. [35], in which the resistant genotype induced APX activity at the early time, meanwhile the enzyme lower activity in the genotype 'Reyhan' did not agree with the rest of the aforementioned research.

In 'Reyhan', the GPX enzyme exhibited the highest activity at 1 dpi, whereas in other genotypes, it peaked later at 3 or 4 dpi. GPX scavenges H_2O_2 during lignin biosynthesis by H_2O_2-dependent polymerization and strengthens the cell wall [26]. Previous studies demonstrated that the enzyme activated highly in inoculated plants when compared to non-inoculated, as the effect of *M. incognita* on plants.

There are reports that plant growth-promoting bacteria-treated inoculated plants enhanced GPX activated. For example, Abbasi et al. [36] obtained similar results as they tested *Bacillus* spp. (i.e., *B. subtilis*, *B. firmus*, *B. coagulans*) against *M. javanica* on eggplants.

Our findings were in agreement with the previous research, and probably the early reaction of 'Reyhan' to strengthen the cell wall and prevent nematode penetration caused the early and higher activity of GPX.

In this study, PALs activity increased in all genotypes, especially in the resistant genotype 'Reyhan', whilst the lowest was detected in the susceptible genotype 'Yousef'. According to Starr et al. [37], although in *M. incognita*-resistant corn inbred lines the PAL-related genes namely ZmPAL1, ZmPAL2, and ZmPAL5 were continuously expressed, in the medium-susceptible lines; temporary expression of PAL-related genes was noted. Working on African rice (*Oryza glaberrima*), Petitot et al. [38] reported a drastic increment in phenylpropanoid biosynthesis, relatively simple secondary metabolites regulated by the PAL, in RKN-resistant genotypes as compared to the susceptible genotypes, suggesting a link between PAL expression and resistance to *M. graminicola*. Synthesizing lignin, as a part of the phenylpropanoid biosynthesis pathway creates a mechanical barrier against pathogens by depositing in the intercellular cavities between the cell wall polymers, thus, the higher resistance possibly influenced by elevated expression of PAL as the key enzyme in the pathway [39]. The activity of phenylpropanoid-related genes has been reported following inoculation by pathogens in a wide range of plant species [40], hereto, the injuries inflicted by *M. incognita* possibly affected PAL activity, particularly in the susceptible genotype 'Yousef'.

Briefly, none of the barley genotypes exhibited immunity to *M. incognita*, although the formation of galls on the roots was often few and small; so that among the genotypes tested, only genotypes 'Yousef' and 'Nik' was classified as susceptible and moderately susceptible, respectively. The nematode had a detrimental impact on various plant growth factors, i.e., shoot length, fresh/dry weight, and root length. However, it exhibited a slight positive effect on root weight. Necessarily, the alterations in plant growth factors were not consistently associated with the susceptibility of the genotypes. Upon nematode invasion, the activity of SOD, CAT, APX and GPX enzymes increased in all resistant, moderately resistant, susceptible, and moderately susceptible genotypes. However, the resistant genotype 'Reyhan' with the lowest gall count, exhibited a quicker response to the nematode compared to other genotypes. Additionally, the peak activity of the aforementioned enzymes occurred at a later stage in the remaining genotypes. The activity of the PAL enzyme increased over time in all genotypes, suggesting a potential correlation with the resistance level of the genotypes.

CONCLUSIONS

In this study, *M. incognita* decreased all plant growth characteristics except for the root weight. Furthermore, in most genotypes, the enzymes SOD, CAT, APX, and GPX were activated later, when compared to the resistant genotype 'Reyhan', likely due to the inability of the plant to react adequately. PAL elevated in all genotypes as time goes by, although, it was higher in the resistant genotype 'Reyhan' as compared to the other susceptible, moderately susceptible, and moderately resistant genotypes. Nematode-infected plants undergo profound morphological, biochemical, and transcriptomic changes. The abovementioned findings confirmed the effect of *M. incognita* on the morphological and biochemical characteristics of the barley, although the transcriptomic changes deserve further investigation.

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AUTHOR CONTRIBUTIONS

All authors contributed equally and approved the manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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