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IDENTIFICATION OF NOVELROOT-KNOT NEMATODE (*MELOIDOGYNE INCOGNITA*) RESISTANT TOMATO GENOTYPES

A. H. El- Sappah[1,2*], M. M. Islam[3], S. A. Rather[4], J. Li[1†], K. Yan[1], Z. Xianming[1], Yan Liang[3*] and M. Abbas[1*]

[1] College of Agriculture, Forestry and Food Engineering, Yibin University, Yibin, Sichuan 644000, China; [2]Genetics Department, Faculty of Agriculture, Zagazig University, 44511 Zagazig, Egypt; [3]College of Horticulture, Northwest A &F University, Yangling 712100, Shaanxi P. R., China; [4]College of Life Sciences, Northwest A&F University, Yangling 712100, Shaanxi P. R., China.
[*]Corresponding author's email: ahmed_elsappah2006@yahoo.com, , liangyan@nwsuaf.edu.cn, abbas2472@hotmail.com
[†]*Ahmed H. El-Sappah and Jia Li have contributed equally.*

ABSTRACT

Root-knot nematode is one of the most serious causes of biotic stress that negatively affect tomato production in China. The robust methodology to overcome this problem is growing resistant cultivars. This study's core purpose is to identify new root-knot nematode (RKN) resistant tomato genotypes out of all 13 available under controlled environmental conditions. After nematode inoculation; morphological, biochemical, and molecular analysis were performed. We observed obvious phenotypic changes in plant height, root length, and root dry weight among all13 genotypes. In comparison with control, nematode infection caused significant halt in plant height in all susceptible genotypes. Three genotypes M3, M7, and M11 were recorded with the lowest values of root gall index and reproduction index. These three genotypes displayed the significantly highest level of resistance compounds; total phenol, ortho-dihydroxy phenol, IAA oxidase, chlorogenic acid, and ascorbic acid contents as compared to the susceptible M82 genotype. Following six molecular markers *Mint-1*, *C&B*, *TG180*, *REX-1*, *JB-1*, and *Mi23*were employed to amplify *Mi*-genes. Only *Mint-1*marker successfully amplified a 622bp fragment in M3, M7, and M11genotypes. These findings proved that M3, M7, and M11 harbour root-knot nematode resistance gene *Mi 1.1*. So, we recommend only M3, M7, and M11 genotypes of tomato for future cultivation to avoid losses caused by RKN infection.

Keywords: Biochemical assay, *Meloidogyne incognita, Mi*-resistance genes, Molecular marker, *Solanum lycopersicum*.

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INTRODUCTION

Tomato is the second most essential vegetable in the world after potato with 177 million tons of production in the whole world (FAO STAT 2016). China is a leading producer of tomatoes with 32% of the total production (Cheng *et al*., 2020a; Liu *et al.*, 2020). Tomato is being cultivated on a large scale in different soil types and under different biotic and abiotic stress conditions (Cheng *et al*., 2020b). The root-knot nematode is the most devastating biotic stress factor that causes severe loss in tomato yield from 25 to 100% (Seid *et al*., 2015). The root-knot nematode belongs to the genus *Meloidogyne*, which is comprised upon 90 species of different races, while *M. incognita* is economically most devastating worldwide (El-Sappah *et al*., 2019). The root-knot nematode is a biotrophic parasite with more than 2000 host plant species. In many crops, nematode exerts significant deleterious effects on both plant health and growth. In order to obtain food, RKN penetrates the root, gives rise to giant cells, which lead to a severe halt in the physiological processes of the infected root (El-Sappah *et al*., 2019).

Root-knot nematode undergoes three morphogenic stages: the egg, four juvenile stages, and adult. Female nematode lay eggs inside a gelatinous mass nearby or over the root surface. Eggs hatch after a specific period of incubation and a number of juvenile stages move inside the soil or infect plant roots. Juvenile stage nematode penetrates the root with the help of its stylet in order to obtain food. After a few days, the juvenile's embedded head starts feeding on root sap (Seebold, 2014).

Consequently, enzymes secreted by juvenile leads to a significant increase in both cell size and number, known as giant cells. At maturity, the male nematode looks like a worm, while the female starts laying eggs. Adult nematode freely moves in the soil and is sometimes transported to different places with the help of different factors, like shoes or equipment (Seebold, 2014). Different varieties of tomatoes displayed different rates of damage under nematodes infection. Comparatively, tropical tomato species displayed the most severe damage rate under the nematode attack (Trudgill and Blok, 2001). Although there are several methods to control the nematodes, but screening of

resistant genotypes is a time-saving, cheapest and most promising method. *Solanum peruvianum* derived *Mi* genes are naturally available resources that confer resistance to tomato plants against a number of disease-causing nematodes such as *M. hapla*, *M. enterococci,* and *M. incognita* (Onkendi *et al*., 2014).

Till date, *Mi-1* is the only source of resistance against nematode and being widely employed in tomato improvement. Other members of the *mi*-gene family identified in tomato are *Mi-2*, *Mi-3*, *Mi4*, *Mi-5*, *Mi-6*, *Mi-7*, *Mi-8*, *Mi-9*, and *Mi-Ht*. However, seven *Mi*-genes, *Mi-2*, *Mi-3*, *Mi-4*, *Mi-5*, *Mi-6*, *Mi-9*, and *Mi-Ht,* are also involved in heat resistance (El-Sappah *et al*., 2019; Bozbuga *et al*., 2020). *Mi-3* was mapped on the short arm of chromosome 12 (Yaghoobi *et al*., 2005), while *Mi-9* was mapped on the short arm of chromosome 6 (Ammiraju *et al*., 2003), and both are homologs of *Mi-1* (Jablonska *et al.,* 2007). Finally, *Mi-HT* and *Mi-9* both were mapped on the short arm of chromosome 6 (Wang *et al*., 2013), but the rest heat-stable genes have not been mapped till today.

The nematode repellant biochemical compounds such as phenols, ortho-dihydroxy phenol, Indol acetic acid (IAA) oxidase, chlorogenic acid, and ascorbic acid are integral to tomato roots (Rani *et al*., 2008). Marker-assisted resistance gene identification is a powerful tool in plant breeding. DNA markers facilitate the identification of these resistance genes in several breeding programs (Szczechura *et al*., 2011). The *Rex-1* and *Mi23* are DNA markers employed in the detection of the *Mi-1* gene in several modern tomato cultivars (Williamson *et al.,* 1994; Seah *et al.,* 2007). *Mint-1* marker is being used to detect *Mi-1.1, 1.2, 1.4,* and *1.6* (Jablonska *et al.,* 2007 El-Sappah *et al*., 2019), while *C&B* marker is being used in the detection of the *Mi-9* gene (Kaloshian *et al.,* 1998). DNA marker *TG180* is being employed to detect the *Mi-3* gene in tomato (Yaghoobi *et al.,* 2005). A successful plant breeding program for nematode resistance solely depends on identifying those as mentioned earlier significant resistant genes (Niu *et al.,* 2007; El-Deeb *et al.,* 2018). In this study, wescreened13 tomato genotypes to identify resistance genes against root-knot nematodes.

MATERIALS AND METHODS

Experimental procedure: Seeds of thirteen test group M1, M2, M3, M4, M5, M6 M7, M8, M9, M10, M11, M12, M13, and one susceptible control M82 of tomato genotypes were obtained from tomatoes laboratory, Horticulture College, Northwest A&F University, China. A pot culture experiment was performed in greenhouse under controlled environmental conditions in the south campus of Northwest A&F University during 2018/2019. The experiment was conducted according to a completely randomized design (CRD). The analysis of variances (ANOVA) among different parameters was performed using the *SPSS* software suit. For germination, each genotype's seeds were placed on wet filter paper in Petri plates and incubated in the dark at 28°C. Twenty plants of each genotype and three replicates were maintained for further experiment. Seedlings were shifted to a growth chamber with a 16/8 h light and dark photoperiod and a 25/16°C temperature cycle. Two months old plants were inoculated with the root-knot nematode. The experiment was repeated to demonstrate reproducibility.

Nematode inoculation: Infected roots were cut into small pieces (2 cm long), and placed in NaOCl solution (0.5% v/v) (Sasser and Taylor, 1978). In order to dissolve the gelatinous matrix, infected roots were placed on a shaker for ~3 minutes. The eggs of RKNwere collected from egg mass, and incubated at 20-35°C temperature for 48 hours. Eggs were placed in petri dishes and aerated regularly using aerators to facilitate hatching process. The number of eggs hatched to J2 was adjusted by serial dilutions with the addition of water. In order of root inoculation, hatched J2 stage nematode were dumped in 2 cm depth near root rhizosphere, and covered with sterile sand.

Assessment of root-gall index and reproduction index: After forty-five days of inoculation, plants were removed carefully with minimum root damage and washed with tap water to remove soil particles. The growth parameters, plant height, root length, and dry weight were measured. Dry weight was determined after drying the plants in a hot air oven at 60°C for 72 hours. From the fresh root sample, a number of eggs per gram of root and a number of eggs per egg mass were counted under a stereoscopic microscope after staining with acid fuchsin lactophenol (Quesenberry *et al.,* 1989). Root gall index was determined and assigned a six-point rating scale (0–5) [0 = no gall or no infection (Immune; I); 1 = 1–2 galls (Highly Resistant; HR); 2 = 3–10 galls (Resistant; R); 3 = 11–30 galls (Moderately Resistant; MR); 4 = 31–100 galls (Susceptible; S), and 5 = 100 and above galls (Highly Susceptible; HS)] (Sasser and Taylor, 1978). The reproduction index (R.I) was calculated as the following

$$R.I. = \frac{m \quad o\ e \quad p \quad g \quad o\ to \quad r}{m \quad o\ e \quad p \quad g \quad o\ s \quad c \quad r} \times 100$$

Where the disease reaction is classified as RI = 0 (immune), RI < 1 (highly resistant), 1 < RI < 10 (very resistant), 10 < RI < 25 (moderately resistant), 25 < RI < 50 (slightly resistant) and RI > 50 (susceptible) (Taylor, 1967).

Biochemical characterization: Total phenol contents (µg/g) was determined with the help of the Folin-Ciocalteu method described by Magalhães *et al.,*(2010), with some modifications. Infected roots were dissolved in absolute methanol to extract phenol contents. Subsequently, 15 µl of the extractwas mixed in 750 µl of 1 N Folin-Ciocalteu reagent (1:10) and placed for 5 min

at 28°C (room temperature).Then 60 µl of Na_2CO_3 (7.5% v/v) was added to the extract and again incubated at 28°C for a half-hour. Multimode microplate reader Fluostar Omega (BMG Labtech, Chicago, IL, USA) was deployed to measure absorption spectra at765 nm. The concentration of total phenolic compounds was calculated using a standard curve of gallic acid equivalents (GAE) as milligrams per gram of extract (mg GAE/ge).

Ortho-dihydroxy phenol(µg/g) contents(O.D.) were measured according to Gutfinger,s method (1981). First of all, 1 ml of 0.1 M phosphate buffer (pH 6.5) and 2 ml of 5% $Na_2MO_4.2H_2O$ solution were mixed with extract and incubated for 15 min. The spectrophotometer was used to measure absorption spectra at 350 nm.IAA oxidase (µg/100 mg) contents were measured by the following methodology of Sadasivam and Manickam (1997). Following reaction mixture;0.1ml of 0.32 nm IAA, U.16 mm 2.4 dichlorophenol, 0.16 mM $MnCl_2$, 0.01 M H_2O_2, 0.1 ml phosphate buffer, and 0.5 ml enzyme sample was incubated at 30°C for 15 min, and absorption spectra were measured at 530 nm. Finally, a calibration curve was drawn for IAA contents data.

Chlorogenic acid(µg/g)extract and infusions were analyzed by an HPLC-UV system, as described by Farah *et al.*,(2005) at absorption spectra 325 nm. Similarly, ascorbic acid (µg/100 mg) contents were also measured in infected roots, according to Aono *et al*. 1995 method.Infected root tissue was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.0) containing 5 mM ascorbate, 5 mM DTT, 5 mM EDTA, 100 mM NaCl and 2% (w/v) polyvinyl pyrrolidone (PVP), followed by centrifugation at 15,000 × g for 15 min at 4ºC. Subsequently, a homogenized mixture was added 44 µM H_2O_2 for initiation of the reaction. Finally, absorption spectra were measured at 290 nm. The data obtained from each reaction was statistically analyzed on SPSS software.

DNA extraction and molecular detection: DNA was extracted from infected root tissues using the Wizard genomic DNA purification ki t. DNA concentration and purity were measured using Nanodrop (Abbas *et al*., 2019). The DNA quality was further confirmed by running a 2 µl sample on agarose gel (El-Sappah *et al*., 2012; Healey *et al.,* 2014). Six PCR primers were used for the amplification of *Mi*-genes (Table 1). Following markers *Mint-1*, *C&B*, *TG180*, *REX-1*, *JB-1*, and *Mi23* were analyzed by PCR (Devran *et al.,* 2013; El-Sappah *et al.,* 2019).

Total PCR mixture was 20 µl, containing 2 µl premix (Taq DNA polymerase, $MgCl_2$, dNTPs, KCl, stabilizer tracking dye and Tris-HCl),1 µl of forward and reverse primers (final concentration was 10 picomol/µl), 2 µl DNA template, and 14µl ddH_2O. PCR tubes with reaction mixture were mixed, vortexed, centrifuged, and finally placed in a PCR machine. PCR temperature conditions were optimized by providing three gradient temperatures for each primer set, and different times to achieve optimal amplification condition. Restriction enzymes Taq-1was used to digest *REX-1* and *JB-1* amplicons in order to analyze the condition of different alleles. Total digestion reaction was 20 µl containing 2µl 10X restriction buffer, 0.2µl acetylated BSA, 16.3µl ddH_2O, 1µl REX amplicons, and 0.5µl Taq1 RS. The reaction mixture was mixed gently, centrifuged and incubated at 6°C for 15 minutes to start activate enzyme activity, followed by incubation at room temperature for 30 minutes for digestion, and finally incubated at 95°C for 10 minutes to inactivate restriction enzymes. The digested product was electrophoresed on 3% agarose and visualized under a U.V. light documentation system (Huei-Mei *et al.,* 2015; El-Sappah *et al*., 2017).

Statistical analysis : Data analysis was performed by using SPSS software suti.Analysis of variance (ANOVA)displayed significant differences among treatments, and Least Significant Difference (LSD) at 5% was compared with means.

Table 1: Set of 6 markers of detecting *Mi*-genes.

No.	Primer	Gene	Applications size (bp)	Oligonucleotide	reference
1	*Mint-1* (Intron)	*Mi-1.1, 1.2, 1.4* and *1.6*	1353, 981, 1137 1186, 1372, 622 , 1410	F.TTCTCTAGCTAAACTTCAGCC R.TTTTCGTTTTTCCATGATTCTAC	Jablonska *et al.,*(2007); El-Sappah *et al.,*(2019)
2	*C&B*	*Mi-9*	400, 360	F.TACCCACGCCCCATCAATG R.TGCAAGAGGGTGATATTAGTGC	Ammiraju *et al.,*(2003); El-Sappah *et al*., (2019)
3	*REX*	*Mi-1.2*	750, 570, 160	F.TCGGAGCCTTGGTCTGAATT R.GCCAGAGATGATTCGTGAGA	Ammiraju *et al.,*(2003); El-Sappah *et al.,*(2019)
4	*TG180*	*Mi-3*	1200	F.ATACTTCTTTRCAGGAACAGCTCA R.ACTTAGTGATCATAAAGTACCA	Yaghoobi *et al.,*(2005); El-Sappah *et al.,*(2019)
5	*JB-1*	*Mi-1*	420, 900	F.AACCATTATCCGGTTCACTC R.TTTCCATTCCTTGTTTCTCTG	Devran, Z. and Sö˘güt,(2014)
6	*Mi23*	*Mi-1.2*	380, 430	F.TGGAAAAATGTTGAATTTCTTTTG R.GCATACTATATGGCTTTTTACCC	Seah *et al.,*(2007)

RESULTS

RNK resistance phenotypic characters: Some morphological changes in tomato plant's height, root length, and weight were noticed after the infection by RKN (Fig. 1). Significant phenotypic changes were observed at the 45th day of post-infection among 13test genotypes compared to susceptible M82 genotype. We observed very obvious phenotypic differences between resistant and susceptible genotypes. Except for M3, M7 and M11, all other tested genotypes displayed significant loss in growth parameters. We observed a significant loss in height in all tested genotypes except M3, M7, and M11(Table 2). The root showed the highest variation in its length and weight among all tested genotypes pre and post-infection.

Noticeably, M3, M7, and M11displayed non-significant variations in their root physical parameters such as root length and weight compared to other tested genotypes(Table 2). Infection to resistance rate was recorded for each genotype; all parameters significantly decreased in resistant genotypes compared to susceptible. The infection's extent reflects plant resistance to the nematode, which was estimated with identification of root-knot nematode gall index, number of eggmasses, and number of eggs per eggmass. Gall index, eggmasses, and a number of eggs per eggmass, all these factors were significantly decreased in the following three genotypes M3, M7, and M11as compared to all other genotypes. Root gall index values in M3, M7, and M11 genotypes were 0.4, 0.7, and 1.0 %, respectively (Table 3). The differences within the numbers of eggmasses per gram of root and eggs per egg mass were observed among all tested genotypes (Table 3). The number of eggmasses per gram root displayed variable values between 0.01 and 580.98, with an average of 250.46. But lowest eggmasses 580.98 were recorded among these three genotypes M3, M7, and M11 as compared to M82 susceptible control. A number of eggs per eggmass were also more and less, with a mean of 1062.21(Table 3). Noticeably, M3, M7, and M11 did not possess any eggmasses, that's why these three genotypes also do not possess eggs.

Biochemical characters for resistance: Biochemical parameters, such as total phenol contents, Ortho-dihydroxy phenol, IAA oxidase, chlorogenic acid, and ascorbic acid were determined which are responsible for resistance against nematode (Table 4,5, and 6). Total phenol contents were determined at the 3rd, 8th, and 15th days of post-infection (3, 8, and 15 days post-infection). The highest phenolic contents were recorded in M3, M7, and M11, which were 110.39, 119.25, and 125.93 µg/g, respectively, at the 8th day of post-infection. Noticeably, at the 3rd and 15th day of post-infection phenolic contents were almost similar, such as 98.20, 102.30, and 104.61 µg/g on 3rd post-infection day, and 88.32, 90.33 and 93.98 µg/g on the 15th post-infection day. The second key biochemical compound responsible for resistance against nematode is ortho-dihydroxy. M3, M7, and M11 have also been recorded with the highest ortho-dihydroxy content at all three intervals. The highest ortho-dihydroxy contents in M3, M7, and M11 were 39.41, 53.34, and 44.87 µg/g, recorded on the 8th day of post-infection.

Figure1:Root morphology45 days post-infection;(A)Resistance plant and (B) Susceptible plant showed the formation of galls due to nematode feeding.

Table 2: Mean performance growth parameters (± STDEV) of 14 tomato genotypes after 45 days of infection.

Genotype	Plant height (cm)		Root length (cm)		Root weight (g)	
	Non	Infected	Non	Infected	Non	Infected
M1	77.09±2.9	72.13±1.39	11.32±1.43	9.23±.93	12.63±1.22	15.98±.32
M2	68.87±1.03	63.98±1.54	18,93±.83	13.33±.04	9.68±.03	13.88±1.38
M3	55.49±0.9	55.84±.92	10.32±1.34	10.21±1.23	14.75±1.44	14.81±1.55
M4	76.27±2.21	69.98±.29	14.96±1.03	12,23±1.29	12.32±1.98	15.91±1.36
M5	80.43±1.24	77.6±.53	19.44±1.39	14.85±1.83	12.87±2.01	16.90±1.74
M6	67.08±.84	65.5±1.75	16.34±2.05	11.39±1.45	8.05±1.45	10.54±1.54
M7	72.9±.34	72±2.21	12.34±1.44	12.49±.23	8.03±1.32	7,86±.88
M8	59.9±1.29	47.1±1.85	19.54±.04	18.82±1.84	13.01±.03	17.91±.05
M9	67.1±.96	41.3±.93	22.37±1.36	16.43±1.22	15.75±.12	18.06±1.28
M10	79.2±.02	54±.55	11.35±.47	8.95±1.76	12.08±1.21	15.92±1.35
M11	84.07±1.53	83.9±1.06	25.32±.45	24.98±2.02	16.09±.92	15.94±1.56
M12	65.4±.33	58.1±1.28	19.44±1.32	15.14±1.33	12.92±1.29	16.05±.43
M13	87.1±.85	78.4±.09	16.38±1.39	10.87±1.34	9.08±.83	12.84±.32
M82 (Control)	83.8±.44	78±1.43	24.65±1.21	20.75±.99	15.93±1.42	18.08±1.54
Mean	71.67	63.94	17.34	14.26	12.37	15.05
SE	4.85	2.15	1.31	1.24	0.74	0.78

Table 3: Evaluation of tested tomato genotypes for RKN infection under greenhouse conditions at 45 days post-infection (dpi).

Genotype	Number of eggmasses /plant	Number of eggs/ gram of root	Root gall index (G.I.)	Reproduction Index (R.I.)	Disease reaction GI/RI
M1	351.41± 3.04	1329.6±2.33	3.2±.87	62.05±1.98	HS/S
M2	421.63±2.94	1587.9±2.89	2.9±.12	74.58±2.54	HS/S
M3	0.01±0.002**	10±.28**	0.4±.02**	0.47±.01**	HR/HR
M4	276.23±2.93	1167.9±2.54	3.4±.42	54.85±2.43	HS/S
M5	323.83±3.09	1181.4±2.73	3.8±.86	55.49±2.19	HS/S
M6	181.32±1.93	1248.9±1.92	4.4±.09	58.66±2.27	HS/S
M7	0.02±.01**	15.3±.04**	0.7±.04**	0.72±.03**	HR/HR
M8	462.09±2.55	1497.3±1.35	3.3±.37	70.33±3.01	HS/S
M9	230.13±2.65	1138.5±1.53	4.7±.64	53.47±1.39	HS/S
M10	391.71±1.86	1271.4±2.58	3.1±.98	59.72±2.04	HS/S
M11	3.04±0.6**	27.42±.56**	1.0±.11*	1.92±.03**	HR/VR
M12	401.65±2.98	1109.4±2.88	3.2±.43	52.11±1.44	HS/S
M13	215.89±2.78	1187.4±2.99	4.3±.54	55.77±2.43	HS/S
M82 (Control)	580.98±3.94	2129.1±3.01	5.0±.84	100±3.87	HS/S
Mean	250.46	1062.21	3.1	49.86	
SE	45.83	168.07	0.39	7.89	

*P<0.05; **P<0.01

Root gall index scale (0-5); [0 = no gall or no infection (Immune; I); 1 = 1–2 galls (Highly Resistant; HR); 2 = 3–10 galls (Resistant; R); 3 = 11–30 galls (Moderately Resistant; MR); 4 = 31–100 galls (Susceptible; S), and 5 = 100 and above galls (Highly Susceptible; HS)The reproduction index scale, RI = 0 (immune), RI < 1 (highly resistant), 1 < RI < 10 (very resistant), 10 < RI < 25 (moderately resistant), 25 < RI < 50 (slightly resistant) and RI > 50 (susceptible).

IAA is another biochemical agent relevant to nematode infection. In M3, M7, and M11, the highest contents of IAA were recorded after each interval of post-inoculation as a response to nematode infection. Highest IAA contents 74.59, and 80.85 µg/100g were recorded on the 8th day of post-infection in M3, M7, and M11 genotypes. Similarly, the highest chlorogenic acid contents were also observed on the 8th day of post-infection in M3, M7, and M11 genotypes, which were 66.79, 61.48, and 81.55 µg/g, respectively 3rd day of post-

infection IAA contents were 60.34, 58.20 and 65.84 µg/g, and at 15thday of post-infection were 75.09, 49.21 and 54.21 µg/g in M3, M7 and M11, respectively. Ascorbic acid is an indicator for the detection of resistance mechanisms in tomato after nematode infection. The highest ascorbic acid contents were also recorded in M3, M7, and M11 at each post-infection test interval (Tables 4,5 and 6). The highest IAA contents in M3, M7, and M11 were 46.65, 59.99, and 48.21 mg/100g, recorded on the 8th day of post-infection.

Table 4: Mean performance (± STDVE) of 14 genotypes for biochemical root-knot nematode resistance characters at three days post-infection

Genotype	Total Phenol (µg/g)	Ortho- dihydroxy phenol (µg/g)	IAA oxidase (µg/100 g)	Chlorogenic acid (µg/g)	Ascorbic acid in roots (mg/100g)
M1	28.35±1.23	20.29±.07	20.29±1.03	13.09±1.98	20.13±.32
M2	40.24±2.03	13.21±.03	24.41±.23	20.92±2.01	33.56±1.66
M3	98.20±.03**	35.28±.18*	65.20±.09**	60.34±1.03**	40.62±1.52*
M4	51.61±.87	28.91±.24	35.20±.27	18.81±.96	22.90±1.68
M5	35.91±1.38	25.62±.49	40.30±1.38	30.73±1.30	28.81±.67
M6	35.60±1.34	23.91±1.04	18.49±1.94	25.84±.36*	32.23±.88
M7	102.30±1.94**	50.17±.83**	60.34±.92**	58.20±.29	46.09±1.77**
M8	60.21±1.05	30.60±.64	25.69±.39	40.58±.30	38.87±.73
M9	27.02±.83	14.56±.49	30.48±.59	35.45±.45	29.82±1.91
M10	32.26±1.92	14.27±.86	41.30±.18	16.64±.55	37.39±1.04
M11	104.61±.05**	40.43±1.55**	70.36±1.91**	70.38±1.23**	41.66±1.08*
M12	29.02±1.96	10.20±1.33	22.29±1.82	28.33±.44	31.24±1.19
M13	38.01±1.04	23.45±.05	28.34±1.4	25.15±1.54	27.80±1.23
M82	42.25±1.23	26.24±.55	36.19±1.29	21.02±1.53	31.22±1.44
Mean	51.83	25.51	37.06	33.25	33.02
SE	8.10	3.61	4.53	5.26	2.38

*P<0.05; **P<0.01

Molecular evaluation of tomato genotypes: Six molecular markers (*Mint-1*, *C&B*, *TG180*, *REX-1*, *JB-1*, and *Mi23*) were employed to amplify *Mi*-genes. Mint-1 marker amplified 622 bp only in M3, M7, and M11 genotypes (Fig. 2). M14 genotype displayed three bands of 1300,1100 and 981 bp size. C&B marker amplified 360 bp band in all genotypes that means all genotypes are same (Fig. 3).*TG180* marker amplified 1100 bp fragment in all genotypes which means all genotypes are same (Fig. 4).*REX-1* marker amplified 720 bp fragment in all tomato genotypes, which means all tested genotypes are same (Fig.5A). After digestion of the PCR product with the Taq1 enzyme (Figure 6B), all plants produced two bands of 300 and 720bp.

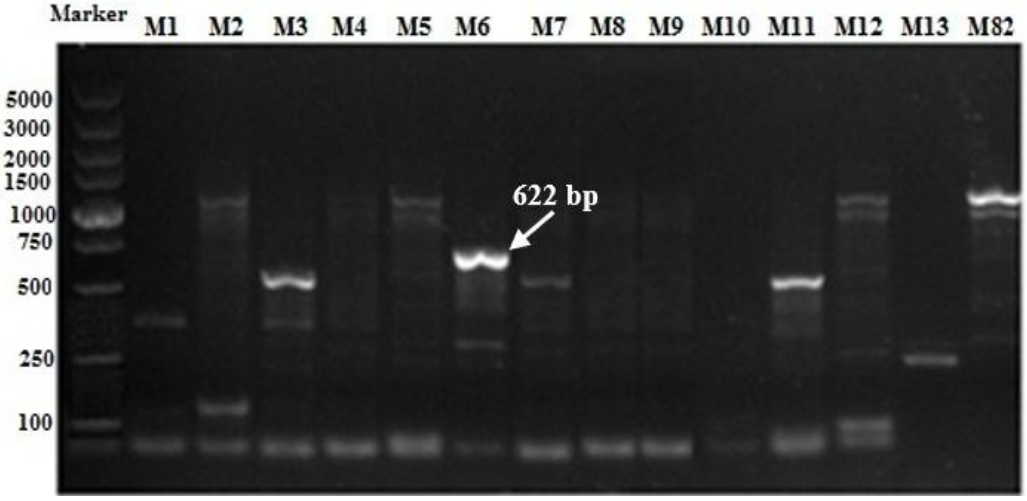

Figure 2: PCR product obtaining using *Mint-1* marker with 14 tomato genotypes; the appearance of bands 622 bp in the three genotypesM3, M6, and M11 indicating for *Mi-1.1.*

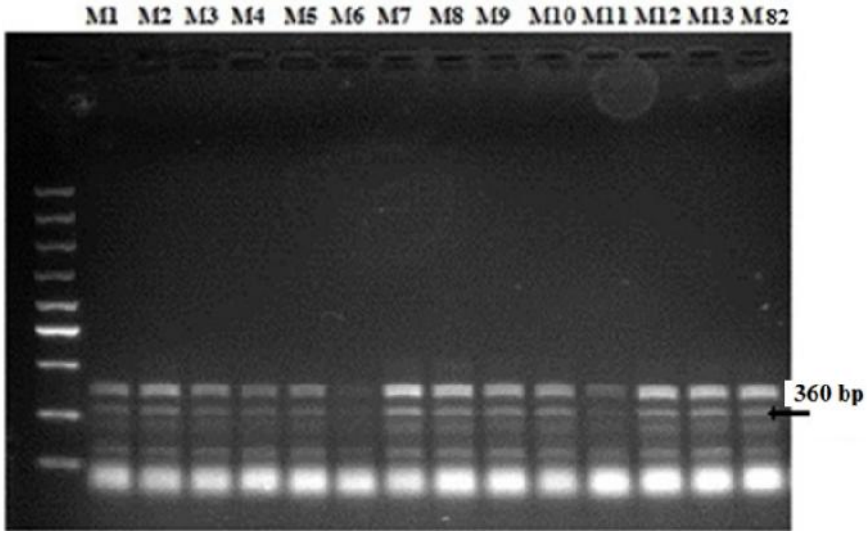

Figure 3: PCR product obtaining using *C&B* marker with 14 tomato genotypes; the appearance of the common band at 360 bp, indicated that these lines susceptible to *Mi-9*

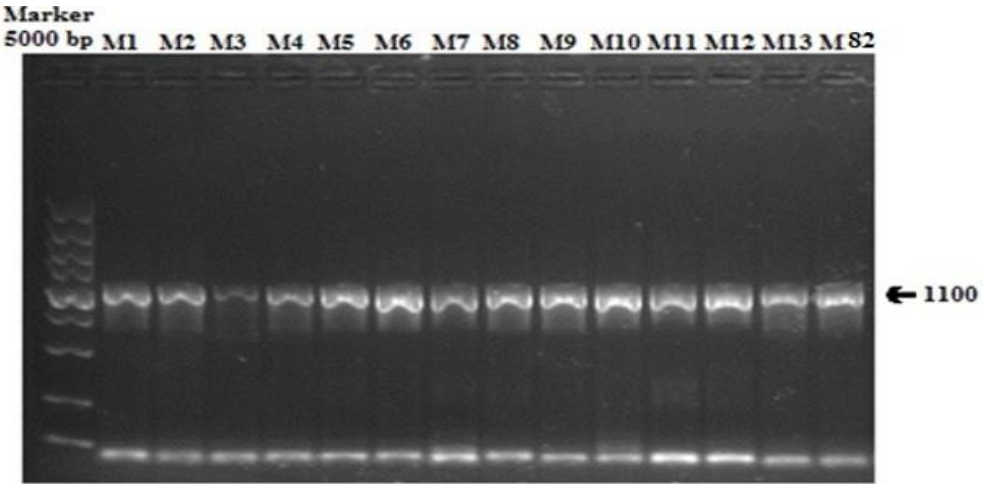

Figure 4: PCR product obtaining using *TG180* marker with 14 tomato genotypes; the appearance of 1100 bp and the disappearance of 1200bp indicated that all tested genotypes have not the *Mi-3*. The resistant plant that contains *Mi-3* should show one band at 1200 bp.

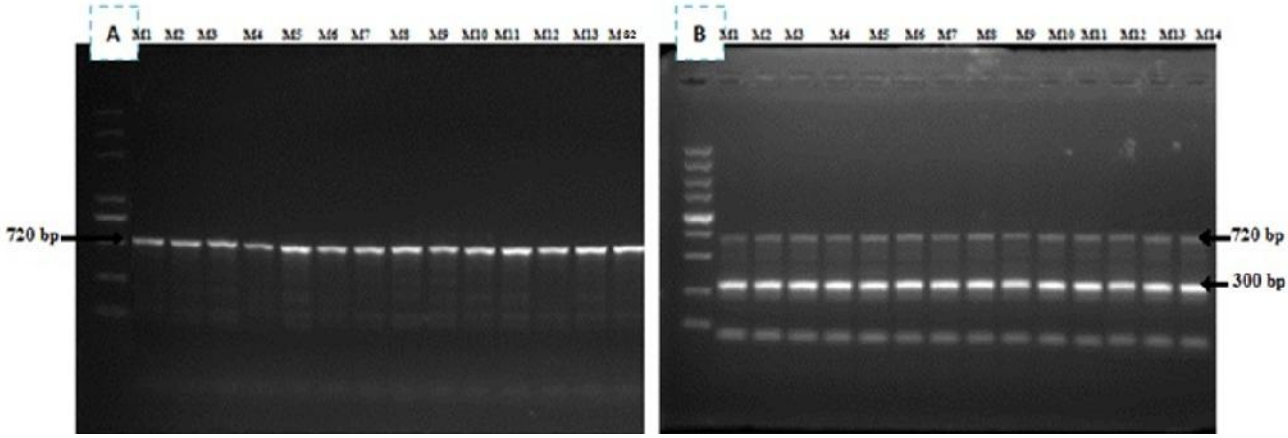

Figure 5: (A) PCR product obtaining using *REX-1* marker and (B) Digestion of *REX-1* PCR products with *Taq*1; the appearance of common bands at 720 bp after restriction with *Taq*1, indicated the susceptible of al tested genotypes to *Mi1.2*.

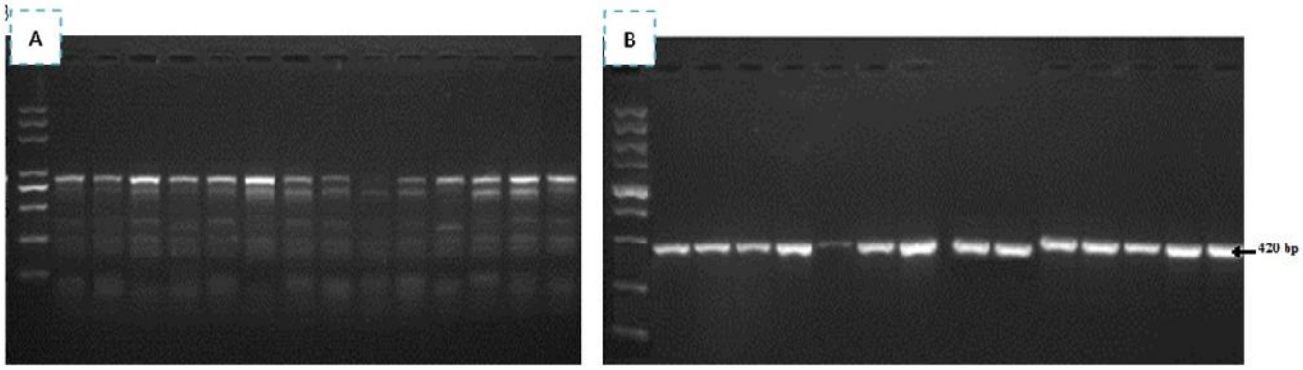

Figure 6: (A) PCR product obtaining using *JB-1* marker and (B) Digestion of *JB-1* PCR products with *Taq*1; after restriction with Taq1 enzyme, *JB-1*produced only 420 bp indicated that all tested genotypes susceptible to*Mi-1*.The resistant plants should show two bands at 420 and 900 bp.

Table5: Mean performance (± STDVE) of 14 genotypes for biochemical root-knot nematode resistance characters ateight days post-infection.

Genotype	Total Phenol (µg/g)	Ortho- dihydroxy phenol (µg/g)	IAA oxidase (µg/100 g)	Chlorogenic acid (µg/g)	Ascorbic acid in roots (mg/100g)
M1	26.32±1.39	23.43±1.32	24.34±1.76	10.23±1.02	17.29±1.54
M2	42.92±.98	15.83±2.42	22.36±.84	24.99±1.32	36.32±1.98
M3	110.39±1.54**	39.41±1.29*	70.53±1.34**	66.79±2.31**	48.21±2.18*
M4	49.01±.75	25.32±.67	38.95±2.05	14.74±.88	26.07±1.29
M5	30.26±1.64	22.98±.92	38.79±1.12	35.33±2.03	29.87±1.44
M6	34.34±.54	16.09±1.43	21.77±1.66	17.98±.03	28.62±.94
M7	119.25±2.96**	53.34±1.09**	74.59±2.01**	61.48±.83**	59.99±2.06**
M8	62.74±2.05	32.14±.54	46.39±1.11	48.95±1.76	40.43±1.36
M9	24.66±1.90	16.74±1.23	28.18±1.38	31.11±1.65	31.59±1.78
M10	35.98±1.46	13.22±1.69	45.03±1.64	19.38±1.84	38.66±1.91
M11	125.93±1.28**	44.87±1.99**	80.85±.55**	81.55±.65**	46.65±2.61*
M12	23.66±1.43	12.20±.09	18.74±1.73	31.84±.18	34.91±1.05
M13	41.30±1.94	24.90±.16	33.69±2.04	24.63±1.43	22.56±2.95
M82	38.54±2.53	21.03±1.29	39.43±1.22	25.03±1.65	35.98±1.45
Mean	54.66	25.82	41.69	35.29	35.51
SE	10.04	4.021	5.37	6.08	3.44

*P<0.05; **P<0.01

Table 6: Mean performance (± STDVE) of 14 genotypes for biochemical root-knot nematode resistance characters at 15 days post-infection.

Genotype	Total Phenol (µg/g)	Ortho- dihydroxy phenol (µg/g)	IAA oxidase (µg/100 g)	Chlorogenic acid (µg/g)	Ascorbic acid (mg/100g)
M1	23.25±1.11	17.36±1.45	18.83±1.29	12.32±.02	15.34±1.04
M2	37.65±2.04	10.30±1.49	24.02±2.01	16.22±.83	30.60±1.03
M3	88.32±1.95**	31.78±1.73*	61.34±1.77**	54.21±3.01**	37.49±1.54*
M4	42.09±1.35	30.19±1.39*	30.22±1.87	20.49±2.29	20.04±1.43
M5	29.94±1.07	22.91±.06	37.88±2.04	28.83±1.66	29.46±.04
M6	33.38±2.03	19.90±1.25	23.31±1.64	20.19±1.21	26.39±.53
M7	90.33±1.29**	46.82±1.75**	58.05±1.89**	49.21±2.34*	48.47±1.35*
M8	57.01±2.43	23.95±1.05	40.55±2.45	44.36±2.42	35.55±.95
M9	24.86±1.63	9.33±.06	25.43±1.40	30.49±1.63	21.95±.29
M10	30.06±1.74	14.99±1.02	35.78±1.24	13.27±.95	29.93±.58

M11	93.98±1.24**	33.21±2.03*	66.93±3.67**	75.09±3.95**	43.87±1.09*
M12	31.34±2.07	12.38±1.40	15.84±.37	24.02±1.34	22.64±.98
M13	40.54±1.77	17.88±.98	27.91±.88	18.63±1.64	30.78±2.05
M82	39.79±1.54	21.93±1.21	30.02±1.65	26.96±.54	27.03
Mean	47.32	22.35	35.44	31.02	29.97
SE	6.84	3.38	4.36	5.19	2.74

*P<0.05; **P<0.01

JB-1 marker amplified multiple bands(Fig.6A), and at digestion with Taq1 restriction enzymes produced bands of420 bp in all genotypes (Fig. 6B). *Mi23* is a co-dominant marker being widely employed in the amplification of *Mi-1.2* in tomato plants. Mi-23 amplified 430 bp in all genotypes, which means there is no difference in all tested genotypes (Fig. 7).

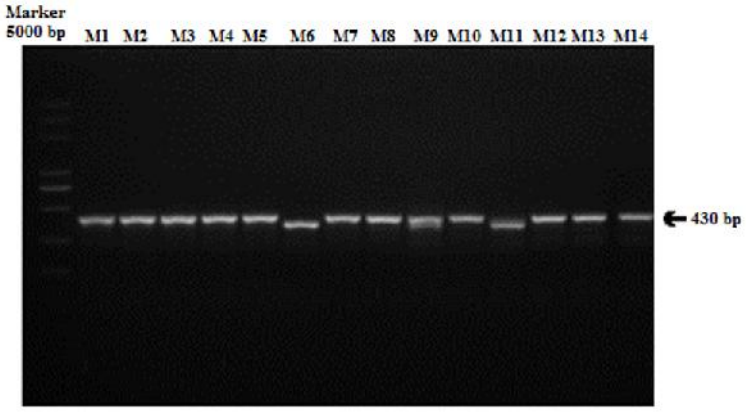

Figure7: PCR product obtaining using Mi23 marker with 14 tomato genotypes; *Mi23* produced only 430 bp indicated that all tested genotypes susceptible to*Mi1.2*. The resistant plant should show two bands at 430 and 380 bp.

DISCUSSION

Plant growth, such as shoot length, root length, and root weight was significantly affected by *M. incognita* infection of tested and susceptible tomato genotypes for nematode-resistance.Plant height was seriously affected under root-knot nematode infection in all tested genotypes except M3, M7, and M11. In general, shoot length is an indicator of plant growth and development, and dwarf stature is only due to significant disease or undernutrition. Similarly, root length was also tremendously affected due to RKN infection. In all tested genotypes, a severe reduction in root length was observed in all RKN infected plants as compared to control. Three genotypes, M3, M7, and M11 did not display any significant loss in root length, which is evidence of these genotype's resistance against RKN. Loss in root and shoot length in susceptible plants is obviously due to RKN infection, which also induced giant cells at nematode infiltration sites within the root vascular system, and galls were also formed inthe root system. These physiological destructions in the root system resulted in a severe halt in water and nutrients uptake and their transport from roots to aerial parts of plants (Abad *et al.,*2003; Rodiuc *et al.,* 2014). Insufficient water and nutrients supply to leaves resulted in perturbation in photosynthetic processes (Hussey and Williamson, 1998; Strajnar *et al.,* 2012), which was observed in all susceptible genotypes (Melakeberhan *et al.,* 1987). Root weight was significantly increased in all tested genotypes except M3, M7, and M11due to their resistance against RKN. Plant,s resistance to RKN is directly related to the following parameters; root gall index, number of eggmasses per/g ininfected roots, and number of eggs/eggmass. Out of all tested genotypes, only M3, M7, and M11 showed a relatively low percentage of root gal index, due to retaining resistance against RKN. These results are in accordance with all previous findingsof root gall index and nematode infection. The level ofroot gall index is always higher in susceptible plants and *vice vers* (Chen *et al.,* 2004).

The other parameters are the number of eggmasses per/g of root and the number of eggs /eggmass. Following three RKN resistant genotypes, M3, M7, and M11 did not show the presence of eggmasses. In the RNK susceptible plants, multiple juveniles infiltrate roots, which results in the formation of giant cells that appear like galls (knot) on roots, so resistance to RNK is dependent on its penetration (Indu Rani *et al.,* 2009). Usually, gall formation is a kind of host response to root-

knot nematode infestation. Laying eggs in root cells ensures the nematode's ability to accomplish its life cycle, also known as host-parasite suitability. Root gall count is the simplest method to evaluate a tomato genotype for its resistance against RKN, explained by Heald *et al*., 1989. In resistant genotypes, retaining functional *Mi* genes, localized tissue necrosis at the feeding site was occurred, which resulted in the nematode,s death or migration to the next host, and no giant cells were formed (Milligan *et al.,*1998; Lopez-Perez *et al.,* 2005).

Measurement of biochemical compounds is also a reliable indicator of infection rate. Total phenol, ortho-dihydroxy phenol, IAA oxidase, chlorogenic acid, and ascorbic acid contents indicate the host's resistance level against the parasite. Among all biochemical compounds, total phenol contents measurement of the host plant is the most reliable indicator of the degree of resistance against nematode (Masood and Husain, 1976; Ramesh *et al.,* 2008).A positive correlation between the degree of host resistance to the pathogen and total phenolic contents of infected tissues (Giebel, 1974). A distinct correlation may also exist due to;(a)secretion of hydrolytic enzymes during feeding, which excretes out phenol (Acedo and Rohde, 1971), and (b) sudden degradation of phenolic compounds or alteration in phenol biosynthesis pathway resulted in the formation of different compounds such as lignin, which plays a significant role in resistant (Nayak, 2015). Total phenol contents in M3, M7, and M11 genotypes were comparatively higher, which proved their resistance against nematode, and these results are in accordance with previous studies (Ganguly and Dasgupta, 1982). Moreover, InduRani *et al.,*2009, in their evaluation of some genotypes, reported that total phenol content increased in the resistance genotypes. Higher phenol content accumulation in infected roots was due to RKN infection, which plays a key role in the plant's defense mechanisms *via* affecting a pathogen's metabolism (Gopinatha *et al.,* 2002).

Higher contents of ortho-dihydroxy phenol in M3, M7, and M11also proved their resistance against these genotypes. Higherortho-dihydroxy phenol level was due to a defensive reaction towards nematode infection. Ortho-dihydroxy phenols play the antagonistic role of oxidation and produce quinones, which are comparatively more toxic to the pathogen (Chen *et al.,*2004). Our these findings are promising with Lakshmanan (1981), who analyzed a broad range of phytochemicals that play a key role in protecting the host against nematodes. These phenolic compounds also have antifungal, antibacterial, and antiviral activities. Accumulation of higher ortho-didoxyphenol contents in RKN resistant genotypes was also observed by Ranchana in 2015while evaluating resistant tuberose genotypes against nematode infection.

A high level of IAA was also observed in all tested genotypes, and a comparatively higher level was recorded in M3, M7, and M11. Nematode secretes hormones against IAA to induce their infection. But, cyst nematode secretions could not induce changes in tobacco protoplasts against IAA (Goverse *et al.,* 1999). Additionally, local IAA production is also induced during the infection of juveniles of plant-parasitic nematodes. Juveniles release auxin or auxin-like compounds inside the outermost host cells on infection initiation (Goverse *et al.,* 1999). Similarly, cyst and root-knot nematodes also secrete hormones inside the primary host cell to manipulate auxin and local IAA levels. Local incrementing IAA level transactivates polar auxin transport(Goverse *et al.,* 2000b).

Chlorogenic acid, a hydroxycinnamic acid family derivatives considered an essential phenolic compound that is comprised of caffeinated and quinic acid (Santanagálvez *et al.,* 2017). Chlorogenic acid contents are also robust tools of resistance against nematode infection. Chlorogenic acids oxidized due to oxidase enzyme secreted by nematode or host polyphenol oxidase, which produces brown-colored melanin at the site of infection. These resistance-related compounds inhibit nematode activity and refrain nematode larva from penetrating and give rise to giant cells (Acedo and Rohde, 1971). All tested plants were recorded witha high level of Chlorogenic acid, and the highest contents were recorded in M3, M7, and M11. Our these results are also in accordance with Indu Rani *et al.,*(2009), who recordedhigher chlorogenic acid contents in resistance tomato genotypes, and Pegard *et al.,*(2005), who recorded a higher level of chlorogenic acid, which prohibited penetration of root-knot nematode in pepper (*Capsicum annuum*). Ascorbic acid regulates biosynthesis of hydroxyproline-proteins, involved in plant defense (Arrigoni, 1979; Aono, 1995), growth (Smirnoff *et al.,*2001), and analysis of its contents in tomato on nematode infections a robust tool to estimate resistance against RKN (InduRani *et al.,* 2009). A significantly higher level of ascorbic acid among M3, M7, and M11 genotypes is a promising factor of resistance against RKN, and our these results are consistent with Ramesh *et al.,*(2008).

Marker-assisted selection (MAS) is a robust tool for confirming *Mi* genes in tomato plants (Chen *et al.,*2012; Michael *et al.,* 2017). Deployment of *Mint-1* marker came out with successful amplification of 622 bp long heterozygous RKN resistance *Mi1.1* geneonly in RKN resistant genotypes M3, M7, and M11. Different fragments of *Mi 1.1*, *1.6*, *1.4*, and *1.2* with variable-length 1300, 1100, and 981 bp were also amplified in the M14 genotype, which representsM14 is a susceptible homozygous genotype. In past studies, 1410 bp long genes were also amplified in *Mi 1.6*, 1186 bp in *Mi1.4*, 1372 bp in *Mi 1.2*, and 622 bp in *Mi 1.1*. If selectable markers amplify 1353, 981, and 1137 bp long fragments, its means-tested genotype is susceptible. Similarly, if the band's size is 1186 and 981 bp while amplifying the *Mi-*

1.4 gene, it shows the tested genotype is heterozygous resistant to RKN. If amplification of *Mi-1.2* gene produces 1372 bp long fragment, its means-tested genotype is resistant, 1137 bp long amplicon means susceptible genotype, and two bands of 1372 and 1137 bp means heterozygous RKN resistant genotype. Pure lines and hybrids which do not possess a 622 bp long fragment of the *Mi1.1* gene are susceptible to RKN (Ammiraju *et al.,* 2003; Yaghobi, 2005; Chen *et al.,* 2012). All RKN resistant genotypes tested for the *Mi-1.1* marker yielded 1372bp long fragments, and all susceptible genotypes yielded 1137bp long fragments; our these findings are in agreement with Inaddhahirabood (2018).

C&B marker was employed for amplification of the *Mi-9* gene. All tested genotypes yielded only one 360bp long fragment, which proved that these lines are susceptible to RKN. *C&B* marker is responsible for resistance against nematode and activated when soil temperature is>28ºC. Primer pair of *C&B* marker confirmed the presence of 400bp long *Mi-9* gene located on chromosome number 6. Normally, mi/mi homozygous susceptible plants yield 360bp long fragments, but we observed two 400 and 360bp long amplicons in all tested genotypes, which proved that these are heterozygous resistant genotypes. Our findings of the presence of the *Mi* gene in resistant genotypes are in accordance with Ammiraju *et al.,*(2003), genotypes retaining *Mi* marker gene are resistant to RKN attack at >28ºC and recommended for areas with high temperature such as Iraq. Similarly, the *TG180* marker amplifies *Mi-3*, which confers resistance against RKN in tomato at >32ºC. According to Yaghoobi *et al.,*(2005), allele one of *Mi-3* is 1200bp long, associated with resistance against RKN. All genotypes tested with*TG180* marker amplified a 1100bp fragment, so all tested genotypes are susceptible to RKN at >32ºC. *REX-1* marker amplified a 720bp long *Mi-1.2* gene in all tested tomato genotypes. Amplification of three bands of 160, 570, and 750 bp size means-tested genotype is heterozygous resistant. Amplification of only one 750bp fragment means-tested genotype is homozygous susceptible, and two amplification of two fragments of 570 and 160bp means-tested genotype is homozygous resistant (Williamson *et al.,* 1994). In a recent study, Bhavana *et al.*(2019) employed *REX-1*marker on ten different tomato genotypes, amplified three 160, 570 and 750bp in resistant genotypes, and only one 720bp long *Mi-1.2* in susceptible genotypes.

JB-1 marker can be used in the screening of *Ty-1* gene responsible for resistance against Tomato Yello Leaf Curl Virus (TYLCV) (Perez de Castro *et al.,* 2007), and *Mi-1*gene (Devran *et al.,*2013). But it can not differentiate between resistant and susceptible plants against RKN (Bhavana *et al.,* 2019), so it can not be used in this study. Similar to *REX1*, a co-dominant marker *Mi-23* can also amplify380bp fragment of *Mi-1.2*gene in homozygous resistance genotypes and 430bp fragment of *Mi-1.2* in susceptible genotypes (Seah *et al.,* 2007; Devran and Elekçioğlu, 2004), but it is non desired because its results are the same with *REX1*, and it is in accordance with Danso *et al.,*(2011). Danso *et al.,* employed specific primers (Mi23/F//Mi23/R) for screening of few tomato genotypes. The role of*Mi-23* marker is also controversial similar to *JB-1* used for amplification of *Ty-1* (Yu *et al.,* 2008). A contradictory statement about the role of *JB-1*was given in previous studies that this marker can be employed in study of RKN and TYLCV. However, markers associated to the *Mi-1* gene did not provide consistent results in the detection of *Ty-1*(Perez de Castro *et al.,* 2007; Yu *et al.,* 2008).

Conclusions: In this study, 13 novel tomato genotypes were evaluated for resistance against the root-knot nematode (RKN). Infected plants were analyzed at; morphological, biochemical, and molecular levels. The difference in plant height was more significant in susceptible genotypes as compared to resistant genotypes. Plant height and root length of all tested genotypes were significantly reduced except M3, M7, and M11. Similarly, the root weight of all infected genotypes was increased after infection due to galls and giant cell formation in roots except M3, M7, and M11. Biochemical analysis revealed accumulation of defense compounds against nematode such as phenol, ortho-dihydroxy phenol, IAA oxidase, chlorogenic acid, and ascorbic acid only in M3, M7, and M11 out of all 13 tested genotypes. Finally, six molecular markers *Mint-1*, *C&B*, *TG180*, *REX-1*, *JB-1*, and *Mi23* were employed to amplify genes. *Mint-1* marker amplified a622 bp fragment, which proved the presence of resistance gene*Mi-1.1* in resistant genotypes. Our study proved, M3, M7, and M11 are only novel tomato genotypes resistant to RKN infection.

Abbreviation: J2: juvenile stage two, STDEV: standard deviation, S.E.: standard error, TYLCV: Tomato yellow leaf curl virus

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