BIOCHEMICAL, MORPHO-PHYSIOLOGICAL AND RESISTANCE RESPONSES OF DIFFERENT CHICKPEA VARIETIES AGAINST ROOT-KNOT NEMATODE *MELOIDOGYNE INCOGNITA*

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(Received 16th Jul 2022; accepted 20th Oct 2022)

Abstract. Twenty-seven genotypes of chickpea were assessed against the *Meloidogyne incognita* population in greenhouse. Three genotypes were found immune. Seven genotypes, i.e., Bital 98, CM-88, Hassan 2K, Noor-91, Wanhar-2000, Parbat-98 and Thal 2006 were found resistant (R) with Galling Index 1; five genotypes were moderately resistant (MR) with Galling Index 2 and five genotypes were moderately susceptible (MS) with Galling Index 3. Five genotypes, namely Dusht-98, Kark-98, Kark-2, C44 and KK-2 were susceptible (S) and two genotypes DG-89 and DG-92 were found to be highly susceptible (HS). Then, after screening chickpea genotypes, it was planned to determine the change in the profile of total phenolic, chlorophyll and protein contents of five resistant and five susceptible chickpea genotypes against the development of root knot nematode. There was decrease in total phenolic, chlorophyll and protein contents in susceptible genotypes. However, increase in these parameters was observed in resistant genotypes under nematode stress compared to un-inoculated ones. Moreover, it was also noted that this increase or decrease was in gradual manner. Total phenolic contents were measured highest in resistant genotype Hassan-2K and lowest in susceptible genotype Dusht-98 after the infection of *M. incognita* in comparison to un-inoculated. Observations indicated increase and decrease in chlorophyll contents over un-inoculated in resistant genotype Hassan-2K and in susceptible genotype C-44, respectively. Similarly, *M. incognita* reduced the protein contents in susceptible genotype C-44 and increased in resistant genotype Noor-91 after 7th day of inoculation. These parameters may be a good biomarker for *M. incognita* infection.
Keywords: *chickpea germplasm, M. incognita, resistant/susceptible, total phenolic contents, chlorophyll contents, protein contents*

Introduction

Chickpea (*Cicer arietinum* L.) is the second largest cultivated legume crop after dry beans globally (FAO, 2012). It is a very important source of protein and is grown on the marginal lands without continuous supply of irrigation water. It is grown in 54 countries as a rainfed, post-rainy season and winter crop in subtropical South Asia, parts of Africa and Australia and as a spring season crop in the temperate and Mediterranean regions (FAO, 2012). During 2012, chickpea covered a total of 13.11 million ha (M ha) area worldwide with a global production of 11.67 million tons (M t) and average productivity of 969.5 kg ha^{-1} (FAO, 2019), whereas in Pakistan it covered 0.873 M ha with the production of 0.261 M t in 2020-21 (Anonymous, 2021). Chickpea is the largest and one of the most important Rabi pulse crops, accounting for 76% of the total production of pulses in Pakistan during 2016-17 (Ali et al., 2009; Anonymous, 2017).

Various biotic and abiotic stresses affect stable and high yields of chickpea crop worldwide. Among the biotic stresses, but diseases are the major factor to reduce its productivity. Among enormous disease-causing agents, plant parasitic nematodes are the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2102_16091628

principle threat for the production of chickpea and other crop plants (Anwar et al., 2007; Ali et al., 2017, 2019). Chickpea is a host for over 100 species of plant-parasitic nematodes (Sikora and Greco, 1990; Nene et al., 1996). Plant-parasitic nematodes limit chickpea production, with annual yield losses predictable to be 14% of total worldwide production (Zwart et al., 2019). Root-knot nematodes belonging to genus *Meloidogyne* are serious menace to crop production around the globe (Ali et al., 2015). Among *Meloidogyne* species, *M. incognita* has been cited as leading limiting factor in crop production around the Globe (Maqbool, 1992; Ibrahim et al., 2011). The yearly yield losses produced by nematodes worldwide are projected to be about 14% (Zwart et al., 2019).

Nematicides application is avoided due to economic and environmental reasons. The most sustainable and effective and long-term approach to overcome limitations to chickpea production produced by plant-parasitic nematodes is the use of resilient cultivars (Zwart et al., 2019). Resistance is the capability of a plant to lessen nematode reproduction such that, no nematode reproduction happens in an extremely resilient plant, a low level of reproduction happens in a moderately resistant plant and unconstrained nematode reproduction happens in a vulnerable plant (Roberts, 2002). Growing resistant cultivars has the advantage of inhibiting nematode reproduction and reducing yield losses in the present produce. Furthermore, after developing resistant cultivars, nematode populations residual in the soil to harm succeeding crops are fewer than after susceptible cultivars, therefore profiting the whole agricultural system (Zwart et al., 2019).

Antagonistic to harmonious nematode–plant connections in susceptible hosts, the single dominant resistance genes from plants interact precisely with resultant avirulence (Avr) genes in the nematode, leading to a mismatched interface. This mismatched interface begins a force of plant reactions against the nematode defense approaches (Abd-Elgawad et al., 2022). Plants experience numerous modes of action for immunity and protection. A general innate/basal immune system can identify nematode-associated molecular patterns by pattern recognition receptors (PRRs) such as the primary defense line ‘layer’ against plant parasites. The extracellular receptor proteins (receptor-like kinases and receptor-like proteins) may be beginning dynamics to produce basal resistance, e.g., against Root Knot Nematodes (El-Sappah et al., 2019).

Sharma et al. (1993) evaluated 200 genotypes of *Cicer*s pp. including 173 chickpea advanced breeding lines and five cultivars for resistance to *Meloidogyne* spp. in a greenhouse. None of the tested lines was free of nematode damage. Variation in gall size was greater than that for gall number and galled area. Most of the tested lines showed symptoms of stress in terms of premature drying of leaves, cholorosis and stunting of plant. Plant growth of only two breeding lines was not affected by nematode parasitism, foliage of these genotypes remaining dark green and without premature leaf drop. Sharma and Mathur (1985) screened 35 new chickpea cultivars for resistance to *M. incognita* in pot experiments and categorized the genotypes into resistant, moderately resistant, susceptible and susceptible. Mani and Sethi (1984) studied pathogenicity of *M. incognita* on chickpea cultivar with five inoculum levels along with check and associated check. There was a progressive decrease in plant growth as the inoculum level of the nematode increased. An inoculum of 2 larvae per g soil was found to be the damaging threshold level. Ahmad and Kumar (1990) studied the impact of inoculum level on chickpea genotype with 500, 1000, 2000, or 4000 eggs of *M. incognita* on tomato fruits. Growth parameters including height, yield and vitamin C contents were observed. Based on toxicological and ecotoxicological profile, chemical nematicides are prone to a big threat

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2102_16091628

to human, animals and plants on Earth. Therefore, there is a dire need of the current era to find & environmentally safe control methods, resistant genotypes that could be the most effective and economical for our growers (Kamel et al., 2011; Ali et al., 2017, 2019).

In plants, phenolic substances are much stable products which are composed of a benzene ring with one hydroxyl group. Phenolic substances are secondary metabolites, these are one of the major groups which are synthesized in plants and are assembled finally within the plant cell chloroplasts (Kefeli et al., 2003). Phenolic compounds are famous for the major role in the modification of quality and nutritional value of the food like, aroma, taste, flavor and color (Sengul et al., 2009). They also retain health beneficial effects, effective antiviral, antibacterial, anti-inflammatory, antioxidant and anticancer activities (Sengul et al., 2009; Tlili et al., 2010, 2011; Chang et al., 2011). The above properties make them distinctive to activate the defensive system of the plants against microorganisms, insects, and other herbivores (Yang et al., 2002; Tlili et al., 2010). Chickpea also has phenolic contents for antioxidant activity (Malencic et al., 2007). High concentration of phenolic contents is recovered from resistant plants against diseases and pests attack (Wuyts et al., 2006; Mishra and Mohanty, 2007). Similarly, for plant development, photosynthetic pigments are vital to acquire the energy form light for photosynthesis. Quantification of chlorophyll is associated with the vegetative physiological status, estimation of productivity and species discrimination (Ferri et al., 2004). Due to abiotic and biotic stress, loss in chlorophyll content in infested plant leaves is reported, known as chlorosis (Schmitz et al., 2006; Hillnhutter et al., 2011). Moreover, the life of living organisms mainly depends upon availability of proteins after water, is necessary for the cell growth and repair of tissues (Hellmann and Estelle, 2002; Alex et al., 2004). In plants, initially proteins are stored in leaves & roots, and used in to biomass production and reproductive parts. Among plant protein sources, chickpea is the good one for high quality protein (Akande et al., 2007). This high-quality protein is affected by the root knot nematodes (Singh et al., 2010).

This study was planned to find out source of resistance against root knot nematode in chickpea genotypes and to estimate the total phenolic, chlorophyll and protein contents in inoculated and un-inoculated genotypes of chickpea upon infection of root knot nematode.

Materials and Methods

Evaluation of chickpea germplasm against Meloidogyne incognita

Seeds of twenty-seven chickpea cultivars/advanced lines were collected from Pulses Research Institute, AARI, Faisalabad. Experiment was conducted in green house of Department of Plant Pathology, University of Agriculture, Faisalabad (longitude 31°25' 0" N/latitude 73° 5' 0" E) at 25±5 °C. Three seeds of each chickpea cultivar were sown per pot in formalin sterilized sandy loam soil (85 percent sand, 10 percent silt and 5 percent clay) in clay pots (13-cm diameter) and were allowed to grow. Thinning was done up to one plant/pot after germination. At 4-5 leaf stage plants of uniform height were selected for experiment. Each treatment was replicated five times and was completely randomized on greenhouse bench.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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Nematode inoculum preparation

Mass culturing of *Meloidogyne* spp. was done on the roots of susceptible variety of eggplant cv. i.e., Dilnasheen. Eggs were collected from roots of eggplant using 0.5% NaOCl solution (Hussey and Barker, 1973). The desired inoculum density was prepared by stirring eggs suspension in distilled water. The inoculum density was prepared as 100 eggs per ml of water. After transplanting and establishing of plants in pots, they were inoculated with 5000 eggs per plant by making five holes equidistance around each plant and then holes were filled with sterilized sand (Kamran et al., 2011). Pots were immediately irrigated with water and allowed to grow for sixty days.

Data recorded

After 60 days, plants were taken off from the pots and washed in water carefully. Washed roots were blotted onto paper, damp-dried, and weighed. Number of galls, gall index (GI), egg masses, egg mass index (EMI), J2 (second-stage juvenile) per root system, J2 per 100 cc soil and Reproduction factor (Rf) were recorded.

After weighing, the root system was rated for gall and egg mass 0 to 5 scale where 0 = no galls or egg masses, 1 = 1-5, 2 = 6-15, 3 = 16-35, 4 = 36-65, and 5 = 65-100+ galls or egg masses per root system (Anwar et al., 2007). Root system of plants was stained with 0.15 g Phloxine B per liter of water (Holbrook et al., 1983) solution for 30 minutes to facilitate counting of egg masses. The entire root system was diced; chopped and composite root sample was incubated in a mist chamber for 5 days to hatch the eggs (Haq et al., 2011) by Modified Baermann Funnel extraction. The number of J2 per root system was determined by using a stereomicroscope. Each sample was thoroughly mixed and a 100 cc composite sample was processed through a 325-mesh sieve (pore size = 17 micrometer) followed by Modified Baermann Funnel extraction to collect J2 after 3 days and counted under 40X magnification. Reproduction factor was determined by the following formula according to Anwar and McKenry (2010):

$$\text{Reproductive factor} = \frac{\text{Final population of nematodes (Pf)}}{\text{Initial population of nematodes (Pi)}} \quad \text{(Eq.1)}$$

Biochemical characterization of chickpea varieties in response to nematode infection

For determination of chemical components, five resistant genotypes of chickpea viz. Bittal-98, CM-88, Hassan 2K, Noor-91 & Wanhaar-2000 and five chickpea susceptible genotypes, i.e., Dusht-98, Kark-98, C-44, KK-2 and Kark-2 were selected from the previous experiment to investigate the impact of *M. incognita* on biochemical changes. Formalin (40%) sterilized sandy loam soil was filled in 13-cm diameter clay pots and sowing was done with three seeds of each chickpea genotype per pot. After germination, thinning was performed up to one plant with 4-5 leaf. With completely randomized design, pots were replicated five times. Eggs suspension of desired inoculum density was prepared. A standard, 100 eggs per milliliter of water was prepared and by making five holes equidistance, plants were inoculated with five thousand eggs around each plant, sterilized sand was used to fill these holes. Un-inoculated plants served as control. Pots were immediately irrigated with ground water. Sampling of leaves was conducted on 1, 2, 3, 4, 5, 6 and 7 days after inoculation.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2102_16091628

Determination of total phenolic contents (TPC)

Leaf samples of both inoculated and un-inoculated plants (susceptible and resistant groups), total soluble phenols were determined according to the method of Ainsworth and Gillespie (2007). Fresh chickpea leaf samples were taken after 1, 2, 3, 4, 5, 6, & 7 days of inoculation, weighed individually (0.1 g of each sample) and extraction was conducted with 80% methanol (2 mL) using mortar and pestle. Centrifuged at 12,000 X*g* for 10 min and supernatant were taken in microfuge tube and stored at –20°C until used. Hundred microlitre (100 µL) of sample supernatant was taken, and mixed with 200 µL of 10% Folin-Ciocalteau's phenol reagent (Sigma, USA) and 800 µL of 700 m**M** Na_2CO_3. The mixture was incubated for two hours at room temperature. After that, each 200 µL sample transferred to a clear 96-well microplate and read the absorbance of each sample at 765 nm using µ-Quant microplate reader (BioTek, USA). The phenol concentration was determined from a gallic acid standard curve. The samples were replicated three times. Then phenolic contents were expressed as gallic acid equivalent (GAE).

Chlorophyll contents

Chlorophyll contents were determined from mature inoculated and un-inoculated plant leaves that were harvested at 1, 2, 3, 4, 5, 6 and 7 days after nematode inoculation for two selected groups (resistant/susceptible). Before extracting chlorophyll, one gram of sample was taken from selected inoculated and un-inculcated plants. Extraction was carried out in 2 mL of 80% acetone; extracted sample was taken in 2-mL micro-tube and then centrifuged the sample at 12,000X*g* for 10 min to remove debris. Supernatant was diluted to 10 mL with 80 % acetone. The absorbance of each sample was taken at 663 and 645 nm for chlorophyll a and b respectively using the µ-Quant microplate reader (BioTek, USA). Acetone (80%) was used as a blank for all of these measurements. The total chlorophyll per gram fresh weight was calculated, using following formulas as used by Molazem et al. (2010).

Total chlorophyll:

$$\text{mg/mL} = 0.0202A_{645} + 0.00802A_{663} \quad \text{(Eq.2)}$$

To calculate mg chlorophyll per gram fresh weight:

$$[\text{mg. chlor. /mL x volume of extract (mL)}] \div \text{Fresh weight (g)} \quad \text{(Eq.3)}$$

Protein contents

For protein extracts from chickpea leaves, one g of fresh tissue was grinded in a mortar after the addition of 5 ml of 0.05 **M**Tris - HCI buffer (pH: 7.5). Then, this suspension was centrifuged in Eppendorf tube at 4 °C for 25 min on 1000 X*g*. The obtained extract was subjected to measure the protein concentration.

Bradford method was used to determine the soluble proteins of the samples. A 10 µL of each sample was taken in the sterilized test tube in triplicate form and Bradford reagent (1.0 mL) was added to each test tube. Along with the blank, all the sample solutions were incubated for 10-20 minutes at 37 °C. Standards were prepared from bovine serum albumin (BSA) for standard curve and sample solution. The absorbance was taken at 595 nm µ-Quant microplate reader (BioTek, USA). Above steps repeated for each of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2102_16091628

protein standards and for sample to be assayed. Absorbance was noted for each of standards and samples. All those samples were diluted by a known amount which showed absorbance at 595 nm higher than 2 and repeated the assay to determine the concentration of samples by using standard curve (Bradford, 1976).

Statistical analysis

Statistically analysis was performed on results for analysis of variance and comparison of means by using Fisher's Least Significant Difference (LSD) test at 5 percent probability by Statistix version 8.1 (Anonymous, 2005).

Results

Evaluation of chickpea germplasm against Meloidogyne incognita

Galls and egg masses on roots were present on chickpea lines/ cultivars tested against *M. incognita* infection. The production of root galls, egg masses, J2 population per root system, J2 population per 100 cc soil and reproduction rate were highly significant among the chickpea germplasm (*Table 1*). Fresh shoot & root weights and shoot & root lengths also showed significant differences from cultivar to cultivar (*Table 2*).

Table 1. *Mean squares of production parameters of 27 genotypes of chickpea*

Source of variation	DF	Mean squares						
		Per root system		Index		J2		Reproduction factor
		Galls	Egg mass	Galls	Egg mass	Root system	100 cc of soil	
Cultivars	26	1484.250**	1587.880**	6.269**	6.436**	57830000**	256141**	2.5395**
Error	54	27.910	48.800	0.123	0.160	568962	1950	0.0360
Total	80							

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)

Table 2. *Mean squares of different plant growth parameters of 27 genotypes of chickpea*

Source of variation	DF	Mean squares			
		Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)
Cultivars	26	37.085**	11.692**	35.313**	8.411**
Error	54	3.309	1.163	1.598	0.719
Total	80				

NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)

Three line/ cultivar (CM-2000, Paidar 91, Punjab-2000) were found to be highly resistant and two lines/ cultivars (DG-89, DG-92) were found highly susceptible (*Table 3*). Seven lines/ cultivars were resistant (Bital 98, CM-88, Hassan 2K, Noor-91, Wanhar-2000, Parbat-98, Thal2006) and five were susceptible (Dusht-98, Kark-98, Kark-2, C44, KK-2). Five lines/ cultivars (CM-98, Kark-3, KC-1, NIFA 88, Punjab 91) exhibited moderately resistant and five lines/ cultivars (Balkasar 2000, CM-72, NIFA 95, Lawaghar-2000, Sheengarh-2000) were exhibited moderately susceptible responses against *Meloidogyne incognita* infection (*Table 3*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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Table 3. *Reaction of chickpea cultivars*

Sr. #	Host Status	Gall index	No. of galls	No. of cultivars	Cultivars
1	Highly Resistant (HR)	0	0	3	CM-2000, Paidar 91, Punjab-2000
2	Resistant (R)	1	1-5	7	Bital 98, CM-88, Hassan 2K, Noor-91, Wanhar-2000, Parbat-98, Thal 2006
3	Moderately Resistant (MR)	2	6-15	5	CM-98, Kark-3, KC-1, NIFA 88, Punjab91,
4	Moderately Susceptible (MS)	3	16-35	5	Balkasar 2000, CM-72, NIFA 95, Lawaghar-2000, Sheengarh-2000
5	Susceptible (S)	4	36-65	5	Dusht-98, Kark-98, Kark-2, C44, KK-2
6	Highly Susceptible (HS)	5	65-100+	2	DG-89, DG-92

Root galls and gall index

Meloidogyne incognita was able to induce galls in all the twenty-seven chickpea cultivars tested. Two chickpea cultivars (DG-89, DG-92) had the highest number of galls per root system than all other cultivars (*Table 4*). However, three line/ cultivar (CM-2000, Paidar 91, Punjab-2000) had significantly (P = 0.05) lowest galls per root system.

Seven lines/ cultivars (Bital 98, CM-88, Hassan 2K, Noor-91, Wanhar-2000, Parbat-98, Thal 2006) produced ≤ 5 galls per root system which had the same (1) gall index and five cultivars (Dusht-98, Kark-98, Kark-2, C44, KK-2) produced galls per root system ranged between 35-65 which had the same (4) gall index. Five lines/ cultivars (CM-98, Kark-3, KC-1, NIFA 88, Punjab91) produced galls per root system ranged between 6-15 which had the same (2) galling index. Five lines/ cultivars (Balkasar 2000, CM-72, NIFA 95, Lawaghar-2000, Sheengarh-2000) had gall index (3).

Egg masses and egg mass index

The adult females of *M. incognita* produced significantly (P = 0.05) more egg masses on galled roots of chickpea cultivars DG-89 and DG-92 compared to other cultivars (*Table 4*). DG-89 and DG-92 do not differ statistically. Seven line/ cultivar (Bital 98, CM-88, Hassan 2K, Noor-91, Wanhar-2000, Parbat-98, Thal 2006) expressed significantly lowest egg masses with egg mass index (1). Five cultivars (Dusht-98, Kark-98, Kark-2, C44, KK-2) produced significantly different egg masses but classified within the egg mass index (4).

The cultivars i.e., CM-98, Kark-3, KC-1, NIFA 88 and Punjab91 produced egg masses within egg mass index (2). Egg mass index (3) had been showed by Balkasar 2000, CM-72, NIFA 95, Lawaghar-2000 and Sheengarh-2000 (*Table 4*).

J2 per root system

From the highly susceptible cultivar DG-92, the J2 population (13,337 J2) per plant was recovered which was significantly (P = 0.05) higher than the other cultivars. KK-2 cultivar the susceptible cultivar had also J2 more than 10,000 like the cultivars DG-89 and DG-92 that are highly susceptible cultivars. Cultivars i.e. Bital 98, CM-88, Hassan 2K, Noor-91, Wanhar-2000, Parbat-98 and Thal 2006 had less than 500 J2 per root system and were statistically alike. While highly resistance cultivars didn't showed infection thus there were 0 J2 per root system (*Table 4*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2102_16091628

Table 4. *Response of twenty-seven chickpea genotypes against Meloidogyne incognita infection*

Chickpea cultivars	Per root system		Index		J2		Reproduction factor
	Gall	Egg mass	Gall	Egg mass	Root system	100 cc of soil	
Bital 98	4.67±1.20ijk	5.67±1.20fg	1.33±0.33fg	1.33±0.33fg	277.7±31.06f	66.00±5.13g	0.12±0.05gh
Balkasar 2000	22.67±2.60e-h	19.67±0.88efg	3.00±0.00cde	3.00±0.00cde	7,086.0±1,148.54bc	470.67±34.36e	1.35±0.14f
CM-72	25.00±3.79d-g	31.67±4.70cde	3.00±0.00cde	3.33±0.33bcd	6,060.3±911.74cd	624.00±51.73cd	1.56±0.03def
CM-88	3.00±0.58jk	2.67±0.33fg	1.00±0.00gh	1.00±0.00gh	437.7±48.34f	43.33±4.33g	0.16±0.13gh
CM-98	8.00±1.53h-k	13.00±4.51efg	2.00±0.00efg	2.33±0.33def	3,371.0±153.73e	299.67±34.48f	0.62±0.07g
CM-2000	0.00±0.00k	0.00±0.00g	0.00±0.00h	0.00±0.00h	0.0±0.00f	22.67±3.48g	0.00±0.00h
Dusht-98	39.00±2.65cde	57.00±7.09ab	3.67±0.33bcd	4.33±0.33ab	9,201.3±331.82b	719.00±19.73cd	2.25±0.33abc
DG-89	68.33±5.24ab	67.33±12.20a	4.67±0.33ab	4.67±0.33a	13,277.7±577.50a	872.67±22.52ab	2.82±0.07a
DG-92	72.33±1.86a	68.67±4.63a	5.00±0.00a	4.67±0.33a	13,334.7±1,033.60a	896.67±31.31a	2.56±0.21ab
Kark-98	51.67±8.41bc	55.67±2.03ab	4.33±0.33ab	4.00±0.00abc	9,115.3±303.00b	738.67±34.91bc	2.05±0.07bcd
Hassan 2K	2.67±0.33jk	3.00±0.58fg	1.00±0.00gh	1.00±0.00gh	383.3±28.26f	51.67±6.36g	0.12±0.02gh
Kark-2	50.33±1.86c	44.00±3.79bcd	4.00±0.00abc	4.00±0.00abc	8,833.0±301.00b	659.33±31.74cd	1.72±0.06c-f
Kark-3	7.67±1.20h-k	15.00±5.51efg	2.33±0.33ef	2.00±0.00efg	3,904.3±447.52de	288.33±35.53f	0.62±0.04g
KC-1	9.00±1.53g-k	8.67±1.45fg	2.00±0.00efg	2.00±0.00efg	2,977.3±73.96e	262.67±28.18f	0.46±0.04gh
C44	55.33±3.48bc	62.67±3.28ab	4.00±0.00abc	4.33±0.33ab	8,829.3±468.85b	705.00±39.95cd	1.96±0.07b-e
Noor-91	3.33±0.33jk	4.67±2.19fg	1.00±0.00gh	1.33±0.33fg	479.3±16.76f	64.67±10.71g	0.14±0.02gh
NIFA 88	9.33±0.88g-k	15.00±1.73efg	2.00±0.00efg	2.33±0.33def	3,828.7±81.45de	252.00±40.50f	0.68±0.02g
Punjab91	10.00±1.53g-k	13.00±4.04efg	2.33±0.33ef	2.00±0.00efg	3,145.3±145.30e	240.67±30.94f	0.41±0.22gh
Paidar 91	0.00±0.00k	0.00±0.00g	0.00±0.00h	0.00±0.00h	0.0±0.00f	22.67±2.73g	0.00±0.00h
NIFA 95	21.33±2.40f-i	16.67±5.04efg	3.00±0.00cde	2.67±0.33de	6,825.0±170.91bc	459.67±26.03e	1.45±0.15def
Punjab-2000	0.00±0.00k	0.00±0.00g	0.00±0.00h	0.00±0.00h	0.0±0.00f	25.00±3.00g	0.00±0.00h
Wanhar-2000	4.33±1.45jk	2.67±0.33fg	1.33±0.33fg	1.00±0.00gh	432.3±27.45f	65.67±4.91g	0.12±0.05gh
Lawaghar-2000	18.67±5.04f-j	16.33±4.41efg	2.67±0.33de	2.67±0.33de	6,821.7±228.68bc	583.67±17.98de	1.40±0.16ef
Parbat-98	4.67±1.20ijk	5.00±0.58fg	1.33±0.33fg	1.00±0.00gh	373.3±28.98f	51.00±6.24g	0.15±0.01gh
Sheengarh-2000	30.67±1.76def	24.00±5.13def	3.00±0.00cde	2.67±0.33de	7,987.0±581.80bc	482.00±27.78e	1.48±0.05def
KK-2	41.00±7.51cd	49.67±3.84abc	3.67±0.33bcd	4.00±0.00abc	11,616.7±609.41a	241.67±6.36f	2.36±0.04ab
Thal 2006	3.33±0.67jk	6.00±1.15fg	1.00±0.00gh	1.33±0.33fg	435.7±39.75f	60.33±7.06g	0.12±0.04gh

Means sharing similar letters in a column are statistically non-significant (P>0.05)

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S 39-40, A3860, WARE and TN 81.142 produced statistically similar fresh root weight. HM-29 had 2.78 g fresh root weight which was significantly higher than other twenty-four varieties (*Table 5*). Root weight of chickpea cultivars were directly correlated to the number of J2 per root system, hatched from eggs produced by adult females (*Figure 1*).

Table 5. *Response of plant growth parameters of twenty-seven chickpea genotypes against Meloidogyne incognita*

Chickpea cultivars	Plant growth response			
	Length (cm)		Fresh weight (g)	
	Shoot	Root	Shoot	Root
Bital 98	41.33±0.88ab	19.67±0.33a-d	39.83±0.38ab	9.77±0.32ab
Balkasar 2000	34.93±0.93c-e	15.33±0.33ef	38.87±0.47a-d	7.53±0.32b-f
CM-72	32.37±0.46d-f	15.67±0.88ef	36.90±0.10b-e	7.70±0.35b-f
CM-88	41.00±0.58ab	20.00±1.00a-c	41.40±0.59a	10.77±0.30a
CM-98	32.40±1.01d-f	15.67±0.33ef	38.67±0.33a-d	5.80±0.85fg
CM-2000	34.27±0.55c-e	15.90±0.45ef	38.53±0.29a-d	6.30±0.64d-g
Dusht-98	37.33±1.76a-d	18.50±0.79a-e	39.07±0.64a-c	9.30±0.57a-c
DG-89	33.70±0.87c-f	15.40±0.06ef	39.17±1.64a-c	7.40±0.58b-g
DG-92	33.70±0.85c-f	16.27±0.54d-f	35.80±0.42c-e	7.20±1.11b-g
Kark-98	37.57±0.94a-d	17.07±1.03c-f	28.50±1.72f	5.77±0.22fg
Hassan 2K	43.00±0.87a	20.80±0.55a	41.57±0.87a	10.87±0.62a
Kark-2	30.63±0.38ef	17.33±0.33b-f	33.97±1.13e	6.77±0.47c-g
Kark-3	33.90±0.59c-f	17.43±1.33a-f	36.10±0.10b-e	6.27±0.38e-g
KC-1	36.30±0.58b-e	15.50±0.42ef	37.00±0.00b-e	6.37±0.09d-g
C44	28.17±0.77f	14.47±0.78f	25.73±0.67f	4.80±0.31g
Noor-91	37.17±3.23b-d	19.33±0.33a-d	39.63±0.54a-c	10.50±0.47a
NIFA 88	34.40±0.61c-e	15.90±0.50ef	37.67±0.33a-e	7.23±0.09b-g
Punjab91	35.07±0.23c-e	14.80±0.55f	37.27±0.47b-e	7.47±0.61b-g
Paidar 91	37.60±1.11a-d	14.63±0.22f	36.30±0.61b-e	5.93±0.20fg
NIFA 95	32.60±0.52c-f	16.27±0.61d-f	37.00±1.25b-e	7.27±0.19b-g
Punjab-2000	36.60±0.55b-d	15.27±0.55ef	35.00±0.58de	8.20±0.89a-f
Wanhar-2000	41.10±0.20ab	20.50±0.06ab	39.90±0.36ab	10.43±0.12a
Lawaghar-2000	36.97±0.38b-d	14.67±0.33f	38.20±0.49a-d	8.73±0.38a-e
Parbat-98	38.23±1.35a-c	16.33±0.33d-f	36.00±0.58b-e	7.30±0.12b-g
Sheengarh-2000	32.20±0.72def	14.33±0.67f	35.00±0.58de	8.93±0.22a-e
KK-2	32.77±1.37c-f	18.67±0.88a-e	36.90±0.85b-e	8.73±0.38a-e
Thal 2006	32.80±1.23c-f	15.40±0.60ef	38.67±0.33a-d	8.97±0.57a-d

Means sharing similar letters in a column are statistically non-significant (P>0.05)

J2 per 100cc of soil and rate of reproduction

The J2 population per 100cc of soil and rate of reproduction of nematode was significantly (P = 0.05) greater on roots of chickpea cultivars DG-89 and DG-92 compared to that of roots of all other twenty-five cultivars (*Table 4*). Highly Susceptible cultivars i.e., CM-2000, Paidar 91 and Punjab-2000 had almost statistically same count of J2 per 100cc of soil and rate of reproduction, however these were tremendously lower than the remaining cultivars. The J2 per 100cc of soil and rate of reproduction of nematode was directly correlated. The soil nematode population was increased due to nematode reproduction (*Figure 2*).

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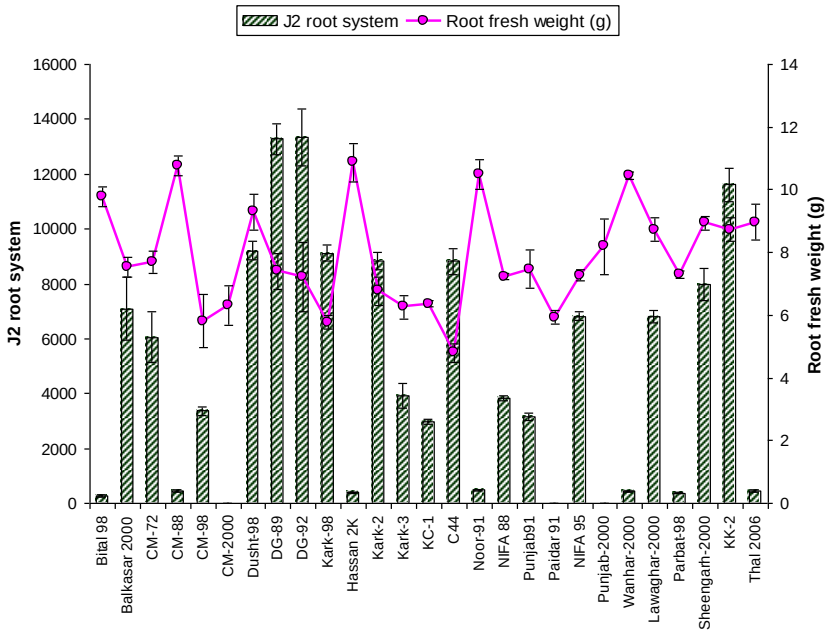

Figure 1. *Effect of M. Incognita on J2 per root system and root fresh weight (g) on 27 chickpea genotypes*

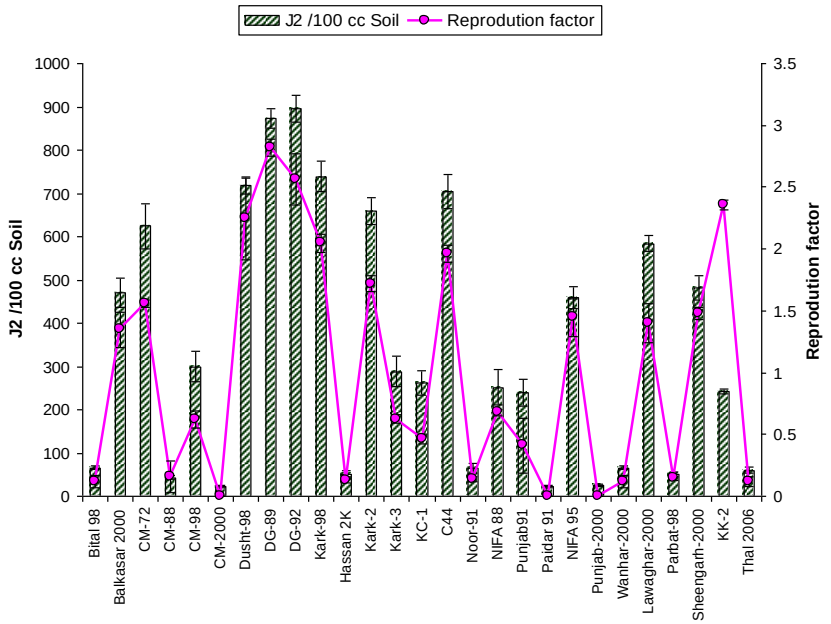

Figure 2. *Effect of M. Incognita on J2/100 cc soil and reproduction factor on 27 chickpea genotypes*

Shoot and root length (cm)

Shoot length was from 28.17 cm to 43.00 cm and root length ranged from 14.47 cm to 20.80 cm on all cultivars. Shoot and root length was significantly (P = 0.05) more on Hassan 2K and less on C44 (*Table 5*). *Fig. 3* showed the reduction in root and shoot length as the number of galls increased on different cultivars.

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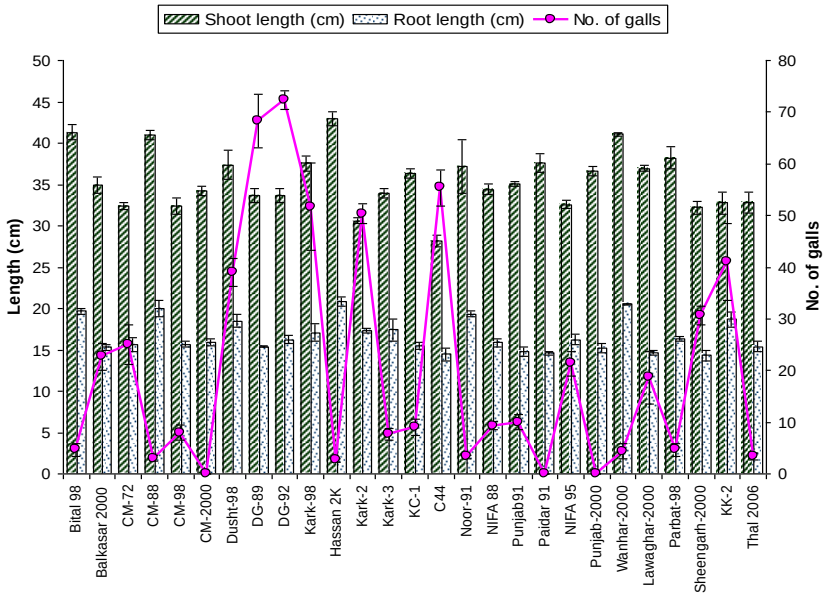

Figure 3. *Effect of M. Incognita on No. of galls, shoot length and root length on 27 chickpea genotypes*

Fresh root and shoot weight (g)

Root weight of chickpea cultivars were directly correlated to the number of J2 per root system, hatched from eggs produced by adult females (*Figure 1*). Fresh shoot and root weights fluctuated from 28.50 g to 41.57 g and 5.77 g to 10.87 g on all cultivars, respectively. Significantly (P = 0.05) high fresh shoot weight produced by Hassan 2K and lowest by Kark-98 and same cultivars showed the highest and lowest fresh root weights (*Table 5*). Inversely proportional relationship was depicted by the number of galls to fresh shoot weight but directly proportional relationship to fresh root weight (*Figure 4*).

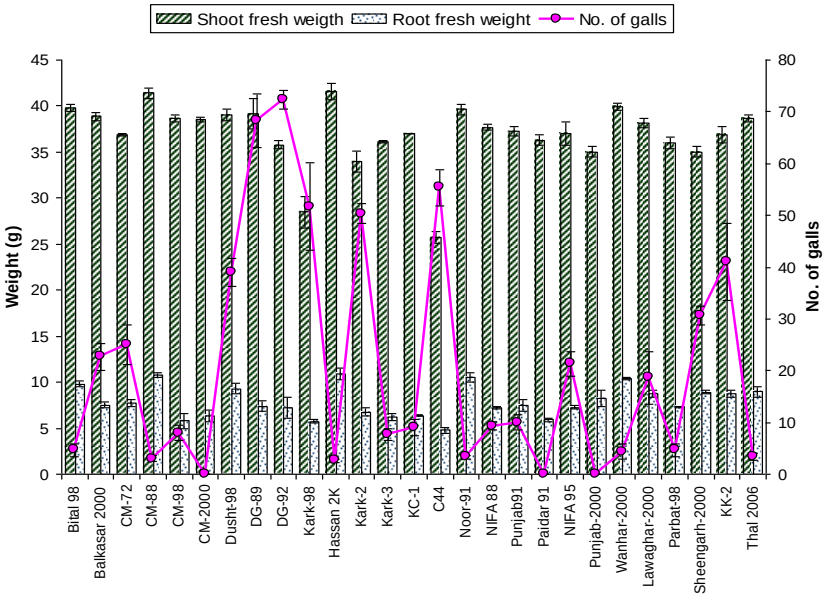

Figure 4. *Effect of M. Incognita on shoot fresh weight and root fresh weight (g) on 27 chickpea genotypes*

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Determination of chemical components

Total phenolic contents

Leaves extraction by methanol (80%) was standardized for their phenolic compounds with gallic acid. The standard curve has linear relationship with gallic acid for the range from 0 to 100 µg/mL, showed correlation coefficient (R^2) of 0.997. Infection of *M. incognita* on all the resistant and susceptible chickpea genotypes showed the phenolic compounds production in varying amount. The highest phenolic contents recovered from the chickpea genotype Hassan-2K in resistant group and lowest from chickpea genotype Dusht-98 in susceptible group than other genotypes (*Fig. 5*).

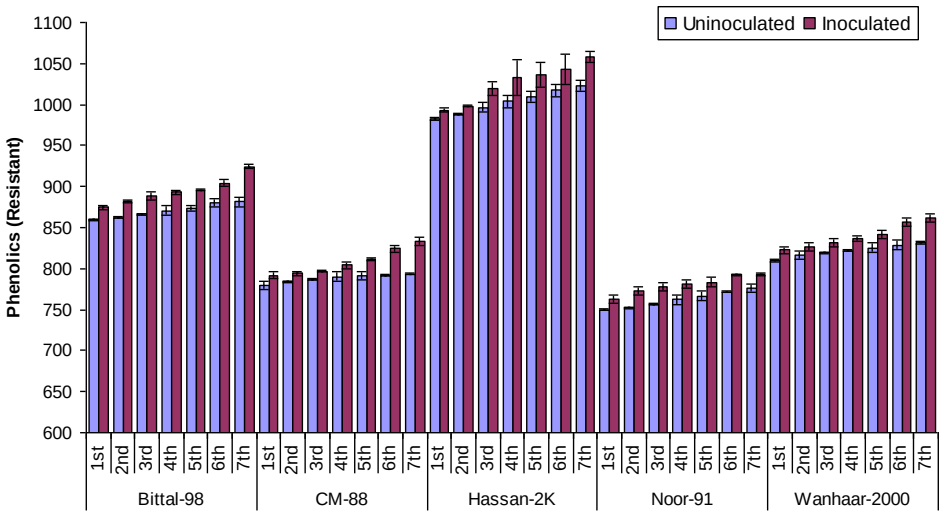

Figure 5. *Effect of M. incognita on total phenolic contents in five resistant chickpea genotypes i.e., Bittal-98, CM-88, Hassan-2K, Noor-91 and Wanhhar-2000*

On the 1st day after application of *M. incognita* on resistant genotypes, all inoculated genotypes showed the increase in the phenolic contents than un-inoculated. Similarly, in the next consecutive days after inoculation in resistant group, all inoculated genotypes also exhibited the increase in production as compared to un-inoculated group. The highest difference in the phenolic contents recovered on 7th day after inoculation and the highest increase value over un-inoculated presented by the genotypes in this order Hassan-2K > Bittal-98 > Wanhaar-2000 > CM-88 > Noor-91.

In susceptible group, all genotypes obtained low amount of phenolic contents upon infection than un-inoculated. *Figure 6* indicated the day-by-day loss in the phenolics on all inoculated genotypes. Less than 1% decrease in phenolic contents on 1st day after inoculation was observed on all five genotypes. The results of 7th day showed the decreases of 469.67, 543.33, 618.33, 709.17 and 724.50 in total phenolic contents were measured from genotypes; Dusht-98, C-44, Kark-2, Kark-98 and KK-2, respectively, compared with the corresponding controls (*Fig. 6*). Compare to their corresponding un-inoculated controls, increase in total phenolic contents in resistant group and decrease in susceptible group was detected after the infection of *M. incognita*.

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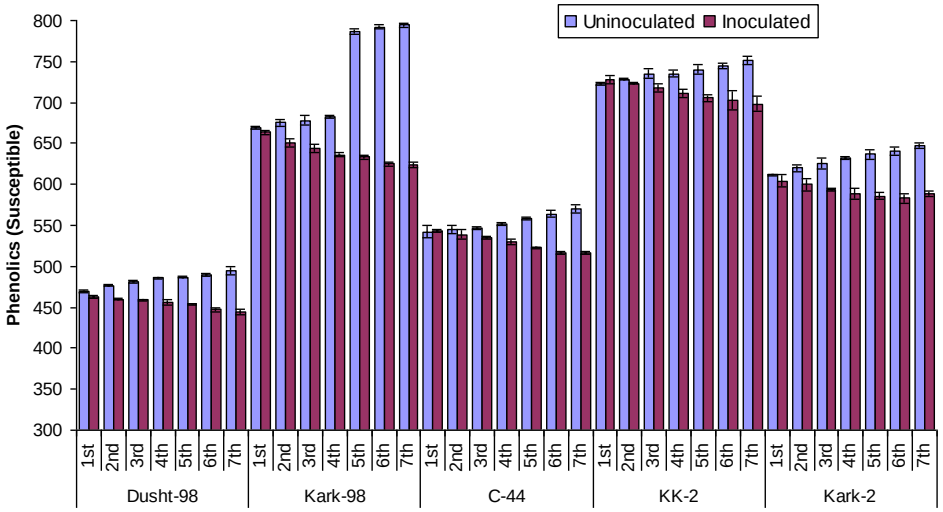

Figure 6. *Effect of M. incognita on total phenolic contents in five susceptible chickpea genotypes i.e., Dusht-98, Kark-98, C-44, KK-2 and Kark-2*

Chlorophyll contents

The estimation of chlorophyll contents in all genotypes of both groups showed the variation in response. The inoculated genotypes of resistant group had higher chlorophyll content compared to corresponding un-inoculated plants. All genotypes disclosed the increase in amount of chlorophyll contents after inoculation with *M. incognita* and gradual increase. After 7th day of inoculation, highest increase in chlorophyll over un-inoculated was observed in genotype Hassan-2K than from genotypes Bittal-98, CM-88, Wanhaar-2000 and Noor-91 over control (*Fig. 7*).

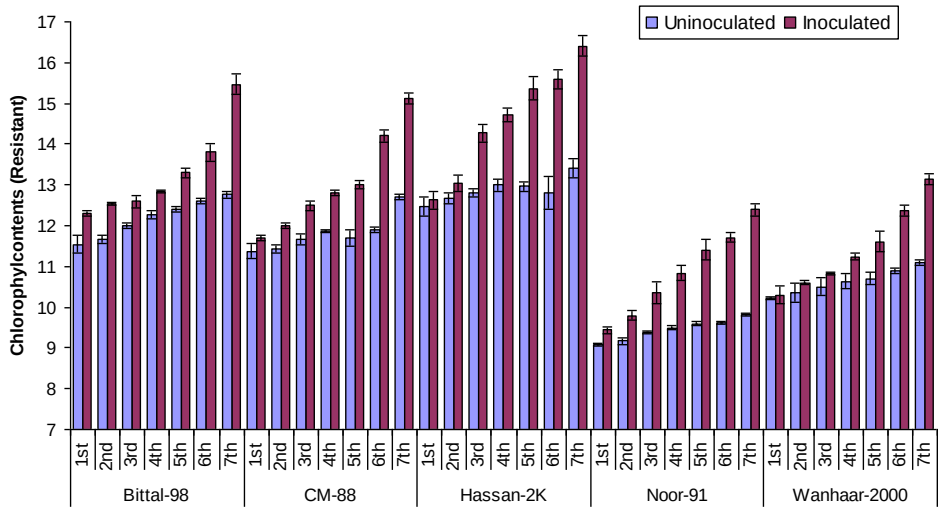

Figure 7. *Effect of M. incognita on chlorophyll contents in five resistant chickpea genotypes i.e., Bittal-98, CM-88, Hassan-2K, Noor-91 and Wanhhar-2000*

In the susceptible group, *M. incognita* infection reduced the chlorophyll contents over respective control. There was a tendency that chlorophyll content decreased with the

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development of *M. incognita* in infected genotypes. A reduction was calculated at 1st day for chlorophyll production. Reduction range of chlorophyll contents increases day by day and at 7th day chlorophyll contents decreased to a great extent. The highest chlorophyll content reduction was noted in C-44 genotype as compared to other genotypes (*Figure 8*).

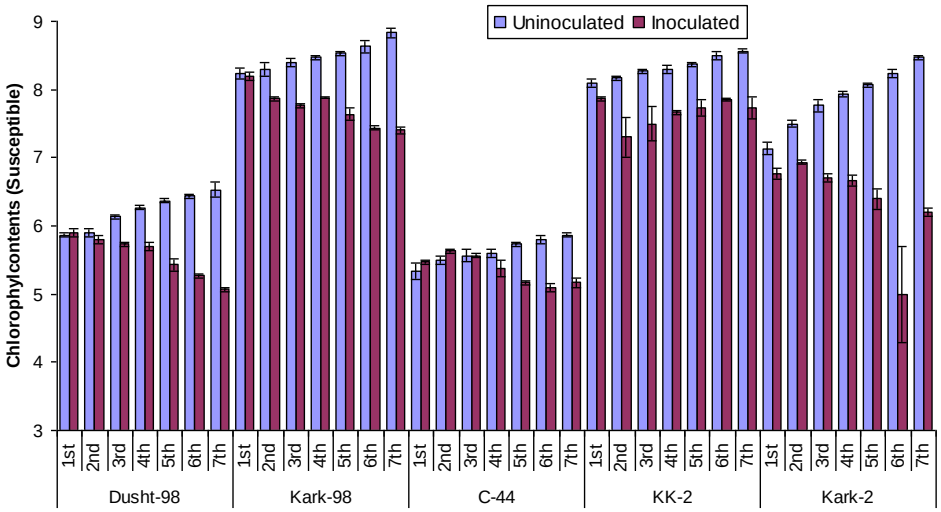

Figure 8. *Effect of M. incognita on chlorophyll contents in five susceptible chickpea genotypes i.e., Dusht-98, Kark-98, C-44, KK-2 and Kark-2*

Protein contents

Protein contents were high in inoculated plants as compared to un-inoculated plants in resistant group but in susceptible group protein contents reduced. In resistant genotype's group, the increased percentage in all genotypes was very low at 1st day but later on this increase was observed progressively. At 7th day, protein contents were found maximum which was higher than pervious (*Figure 9*). The highest increase of protein contents was recovered in genotype Noor-91 in encounter to healthy.

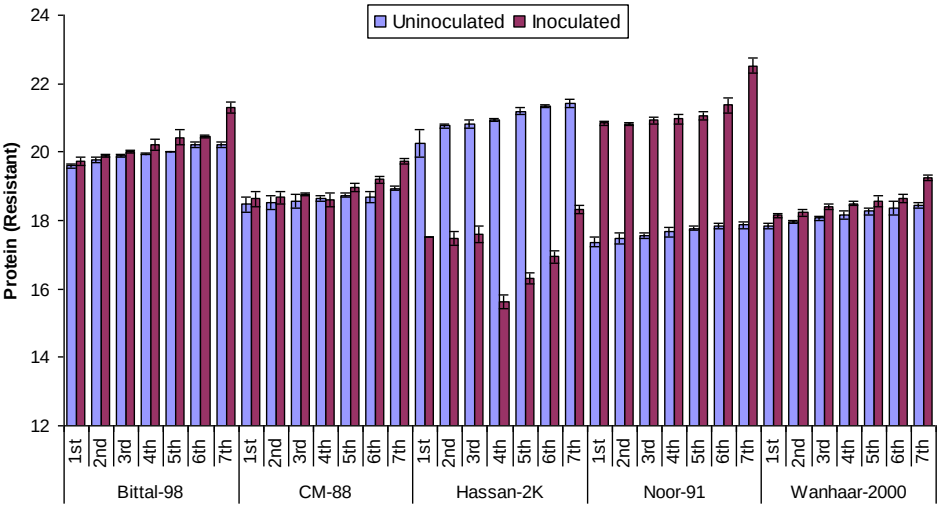

Figure 9. *Effect of M. incognita on protein contents in five resistant chickpea genotypes i.e., Bittal-98, CM-88, Hassan-2K, Noor-91 and Wanhhar-2000*

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Reduction was observed in susceptible group. Lowest reduction was observed on 1st day but gradually rise in reduction was noted as showed in *Figure 10*. The lowest amount of protein obtained from genotype C-44 as compared to un-inoculated. The un-inoculated plants in all genotypes showed increase in protein contents but this increase was very little in amount on day basis.

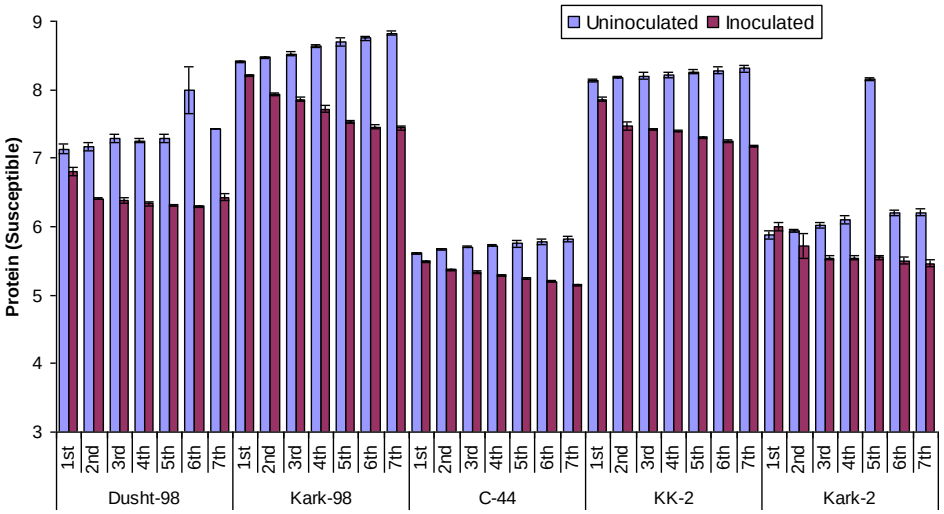

Figure 10. *Effect of M. incognita on protein contents in five susceptible chickpea genotypes i.e., Dusht-98, Kark-98, C-44, KK-2 and Kark-2*

Discussion

Resistance response to chickpea germplasm against Meloidogyne incognita

Production of genetically modified (GM) crop plants is negligible in Pakistan due to several reasons, like; GM cultivars are lower in yield than conventional cultivars (Carpenter, 2001). So, the conventional breeding methods have been used and are still in use for crop improvement to enhance yield and resistance against pests (Ali et al., 2009). Evaluation/ screening of new germplasm against root knot nematode have provided an effective method to pinpoint the new sources of resistance through greenhouse screening procedures (Mukhtar et al., 2017).

Responses of various chickpea genotypes to *M. incognita* infection was assessed by the production of root galls, gall and egg mass indices, eggs per root system and per 100cc of soil and rate of reproduction was considerably variable among the genotypes. There was no immune genotype to nematode infection (Bendezu et al., 2004; Stirling et al., 2006; Kiewnick et al., 2009). Our results showed that different chickpea genotypes responded differently against infection of *M. incognita*, variation in genetic response was observed. This variation in nematode infection to cultivars/ lines might be associated to genetic make-up based on the availability of resistance genes (Abad et al., 2003; Ali et al., 2017) and the resistance level of a particular germplasm (Anwar and Mckenry, 2002). Resistant/ susceptible chickpea germplasm has resistance/ susceptible genes in their genome which confers resistance or susceptible responses to *M. incognita* (Das et al., 2011; Mazarei et al., 2011).

Five chickpea cultivars illustrated resistant response to *M. incognita* infection. This may be due to a series of physiological and biochemical reactions which are carried out

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in plants upon infection of nematodes (Wuyts et al., 2006; Mazid et al., 2011). There are several different types of secondary metabolites involved in the resistant reaction of plant against nematode. Some of these metabolites are involved in the resistance against nematodes in early infections (Ali et al., 2018). Phenolic contents, defense enzymes (hydrolase, oxidase, and dehydrogenase), phytoalexins synthesis, superoxide dismutase (SOD), peroxidase dismutase (POD) catalase, and polyphenol oxidase are investigated from the roots of chickpea against nematodes which are used as the precursor by a resistant plant (Wu and Duan, 2011; reviewed by Ali et al., 2018).

All chickpea cultivars had variation in galls quantity on roots and this variation in root galls per plant presented the status of host plant (Buenna et al., 2007) which was influenced by the nematode reproduction rate (Davis et al., 2003) or the final population of nematodes at harvest (Pathan et al., 2004; Sharma et al., 2004; Khan et al., 2010; Kamran et al., 2011). *M. incognita* egg masses production was observed with variation on all chickpea genotypes. This variation in eggs number laid by a female was governed by the nematode species, nematode genus, populations involved, host status and other environmental factors (Anwar et al., 2000). The susceptibility of line/cultivar is also depending upon egg mass index (Niyaz et al., 2011). Galling & egg mass indices inconsistency among the chickpea gemplasm and egg production consistency proposed that eggs quantity was better and useful indication of resistance against root knot nematode (Shazad et al., 2011).

After 60 days of inoculation, infective second-stage juveniles (J2) of *Meloidogyne incognita* penetration were evident for the establishment on all chickpea cultivars. J2 penetrated into roots of the host and migrated intercellularly to the vascular cylinder (Hemaprabha and Balasaraswathi, 2008; Jones and Goto, 2011). Resistant cultivars have fewer J2 of *M. incognita* than susceptible cultivar because J2 developed slowly in resistant cultivar (Abbas et al., 2008).

Among twenty-five chickpea lines/ cultivars evaluated against M. incognita in this study, no one was immune according to reproduction rate and host status. Those resistant genotypes which have low reproduction rate may be due to hypersensitive response as they exhibited root galls but inhibited reproduction of nematodes (Anwar et al., 2000; Hussey and Janssen, 2004).

Results indicate that chickpea plant growth also affected by the root knot nematode like other crops (Pandey and Kalra, 2003). This happens due to high populations of J2 which can actually cease root elongation and development by damaging meristematic cells at the root tip (Shazad et al., 2011). The root weight was significantly increased by the root knot nematode infection as a result of gall (Gutierrez et al., 2011). Root weight was directly proportional to number of galls whereas shoot & root length and fresh shoot weight were inversely proportional. This indicates that root weight is a good parameter (El-Sherif et al., 2007). Root weight is the better measurement for nematode reproduction due to presence of galls on roots (Olaniyi et al., 2005; Tobih et al., 2011).

M. incognita infection to all chickpea lines/ cultivars has showed that these lines/ cultivars are deficient in resistant genes. This finding indicates that breeders should work on the transfer of resistant character/ gene into new lines to combat the problem.

Determination of chemical components

Pathogen infection to plants stimulates the enzymes which synthesize a burst of defensive primary and secondary metabolites including phenolics to combat pathogens. Therefore, phenols production is promoted in infected plant's tissues (Ashry and

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Mohamed, 2011). This high concentration of phenolic contents in resistant genotypes gives them resistance against pathogen (Ramanathan et al., 2000; El-Modafar and El-Boustani, 2005; Singh et al., 2011). As a result of these phenolic compounds, nematode population decreases (Shaukat and Siddiqui, 2001). In contrast phenolic compounds decreased in susceptible genotypes (Singh et al., 2003; Khan et al., 2005; Lattanzio et al., 2006; Rathod and Vakharia, 2011).

On infection, mostly leaves cease synthesized of chlorophyll pigment and show chlorosis symptoms (Silva et al., 2010) due to shrink in nutrient concentrations in leaves by the infection of nematodes (Talwana et al., 2003). Chlorophyll contents were increased to pathogenic infection in resistant genotypes and declined in susceptible genotypes (Jalali et al., 2007; Alaei, 2011) and high chlorophyll contents provide plant resistance/tolerance to stress (Roy and Kirchner, 2000; Lattanzio et al., 2006). Higher chlorophyll contents have been reported in un-inoculated plants as compared to inoculated in susceptible genotypes (Selvaraj et al., 2009).

Root knot nematodes enter into roots of host plant and establish feeding site (Gheysen and Fenoll, 2002). These feeding sites, giant cells, work as food source for nematodes (Caillaud et al., 2008a,b). Nematodes block the nutrient uptake especially nitrogen, halt the nodulation formation and also affect the development of Rhizobia (Ibewiro et al., 2000). Nitrogen is necessary for protein production, for appropriate leaves growth and play critical functions in photosynthesis of plant (Acikgoz and Deveci, 2011). So, upon infection of nematodes degradation of the tissues occur, nitrogen reduces and ultimate reduction in protein contents (Singh et al., 2011) in infected susceptible genotypes.

Conclusion

In this study, increase of phenolics, chlorophyll contents and protein contents in resistant genotypes and decrease in susceptible genotypes suggest that these traits can be used as biomarkers for the identification for chickpea resistance sources against *M. incognita*. Moreover, detection studies are recommended for further exploration of the different enzymes as other biomarkers.

Acknowledgements. This study was funded by Higher Education Commission, Islamabad through Indigenous Scholarship Programme of HEC. The authors are thankful to Higher Education Commission, Islamabad for providing funds through the prestigious scholarship programme.

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