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Original article (Orijinal araştırma)

Development of an *in vivo* bioassay to identify Turkish chickpea genotypes resistance to *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae)[1]

Ditylenchus dipsaci (Kühn, 1857) (Tylenchida: Anguinidae)'ye karşı dayanıklı Türk nohut genotiplerini belirlemek için *in vivo* biyolojik testlerin geliştirilmesi

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Abstract

Stem and bulb nematode, *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae) is one of the most damaging plant-parasitic nematodes and damage most legume crops such as chickpea. The aim of the study was to develop a protocol for *in-vivo* bioassay to investigate *D. dipsaci* interaction with chickpea. Nine accessions of wild and domesticated *Cicer* spp. including three *Cicer reticulatum* Ladiz., three *Cicer echinospermum* P.H.Davis and three *Cicer arietinum* L. (Fabales: Fabaceae) were used to evaluate chickpea genotype for resistance to *D. dipsaci* between 2019 and 2020 at Çukurova University, Nematology laboratory. The incubation time and inoculum density were determined to correlate with the size of the produced nematode population. It was concluded that the highest reproduction of *D. dipsaci* on carrot discs occurred between 20 and 25ºC at 45-60 days and an initial inoculum density were determined 100 nematodes per carrot disc. Also, the initial inoculum density of 300 and the growing time of 16 weeks was the best practice to identify reaction of chickpea genotypes to *D. dipsaci*. These methods provided that quick screening for resistance studies in chickpea against *D. dipsaci* to observe information of highest reproduction period of *D. dipsaci* as time, temperature, inoculum density.

Keywords: Carrot culture, chickpea, *Ditylenchus dipsaci*, inoculum density**,** screening methods

Öz

Soğan sak nematodu, *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae) en önemli zarar yapan bitki paraziti nematodlarından biridir ve nohut gibi birçok baklagillere zarar vermektedir. Çalışmanın amacı, *D. dipsaci*' nin nohut ile etkileşimini araştırmak için bir *in-vivo* biyolojik test protokolü geliştirmektir. *Ditylenchus dipsaci*'ye dayanıklılık açısından nohut genotipini değerlendirmek için üçü *Cicer reticulatum* Ladiz. (Fabales: Fabaceae), üçü *Cicer echinospermum* P. H. Davis (Fabales: Fabaceae) ve üçü *Cicer arietinum* L. (Fabales: Fabaceae) dahil olmak üzere yabani ve yerli *Cicer* türlerinin dokuz genotipi 2019-2020 yılları arasında Çukurova Üniversitesi Nematoloji laboratuvarında test edilmiştir. İnkübasyon zamanı ve inokulum yoğunluğunun, üretilen nematod popülasyonu miktarıyla bağlantılı olduğu belirlenmiştir. *Ditylenchus dipsaci*'nin havuç disklerinde en yüksek üremesinin, 20ºC ile 25ºC arasında 45-60 günde olduğu ve başlangıç inokulum yoğunluğunun havuç disk başına 100 nematod olarak saptanmıştır. Ayrıca, 300 adet bireyden oluşan inokulum yoğunluğu ve 16 hafta inkübasyon süresi nohut genotiplerinde *D. dipsaci* 'ye dayanıklılık için en iyi uygulama olmuştur. Bu yöntemler *D. dipsaci*'ye karşı dayanıklılık çalışmalarında hızlı testleme yapılması için; *D. dipsaci* en yüksek üreme periyodunun zaman, sıcaklık ve inokulum yoğunluğu olarak gözlenmesini sağlamıştır.

Anahtar sözcükler: Havuç kültürü, nohut, *Ditylenchus dipsaci*, inokulum yoğunluğu, testleme yöntemleri

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Introduction

Legume crops are an important the nutrition of millions of people globally. Chickpea, *Cicer arietinum* L. (Fabales: Fabaceae), is one of the most important legume crops. Australia, Canada, Ethiopia, India, Iran, Mexico, Myanmar, Pakistan, Russia, Turkey and the USA are among the largest chickpea producing countries in the world (FAO, 2019). *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae) is an important migratory endoparasitic nematodes with the fourth-stage juveniles (J4) that have an important economic impact on agriculture crops, causing damage in below and above parts of the plant (Sikora et al., 2005). The number of wild and cultivated plant hosts for *D. dipsaci* is more than 1 200, and it is listed as an A2 quarantine nematode in the world (EPPO, 2015). In addition, it is widely observed in chickpea growing areas in Turkey. Recently, study from chickpea growing areas in Turkey indicated that 45% of the soil samples collected were infected with *D. dipsaci* (Behmand et al., 2019). Symptoms of *D. dipsaci* nematode infection in plants cause swelling and distortion of part plants such stem and leaves (Sturhan & Brzeski, 1991). However, the symptom of nematode damage depends on the plant species. For example, legume crops infected with *D. dipsaci* show symptoms such as swelling and deformation of stems and distortion on the surface plant (Sikora et al., 2005; Bridge & Starr, 2007; CABI, 2015). Thompson et al. (2000) showed that an integrated pest management strategy including rotation with non-host crops or fallow, and use of resistant cultivars is the best method to keep the nematode population below the economic threshold level in legume crops and cereals. A similar study by Trudgill, (1992) demonstrated that the use of tolerant cultivars is one of the best-integrated pest management strategies that can grow and yield well in the extremely infested region with plant-parasitic nematodes. Some sources of resistance to *D. dipsaci* are known for bean, clover, lucerne, oat and rye but none has been described for chickpea (Plowright et al., 2002). Providing useful information such as the improvement of an *in vivo* bioassay technique and clear source initial inoculum density of nematode can help the breeding program to make new resistance cultivars to the plant-parasitic nematode (Kühnhold et al., 2006). However, a chickpea improvement program for resistance cultivar to plant-parasitic nematodes is not easy because of the high genetic diversity of chickpeas. Generally, wild *Cicer* spp., *Cicer reticulatum* Ladiz. and *Cicer echinospermum* P. H. Davis (Fabales: Fabaceae), are more resistant genotypes to disease and are used in breeding programs (Rebecca et al., 2019). The levels of resistance to plant-parasitic nematodes have been diagnostic in wild *Cicer* spp. that are present in cultivated *C. arietinum* (Basandrai et al., 2011).

The effect of increasing population density of *D. dipsaci* and resistant chickpea cultivars was investigated. Investigation of factors such as the initial inoculum density of nematodes and harvest time to keep the nematode population below damage threshold levels is important in the development of a screening study in nematology. Estimating threshold levels and calculating economic thresholds for most nematodes/crops depend on the reproduction factor (RF). RF, the ratio of final and initial population density (Pf/Pi), is commonly used in nematology (Oostenbrink, 1966) as an indicator of the suitability of a host plant for a particular nematode. Thus, susceptible plants have RF > 1 and resistant or non-hosts, RF < 1 (Seinhorst, 1967). In addition, evaluation the relationship of Pi and Pf is basic to being able to predict yield reductions. Therefore, providing information about the relationship between these population densities of nematodes and crop performance is essential.

In addition, most plant-parasitic nematodes can develop in fresh root tissue. Nematodes can feed as migratory endoparasites and ectoparasites from plant tissue. Laboratory production of endoparasitic nematodes like *D. dipsaci* can be done in three ways: (1) on plants include alfalfa callus (Acosta & Malek, 1979; Draper & Smith, 1981), (2) root explants (Chitambar & Raski, 1985), and (3) carrot discs (Coolen, 1979). The latter method can give better production of endoparasitic nematodes such as *D. dipsaci* on carrot discs in Petri dishes in an incubator greater than root plants under laboratory conditions (Kühnhold et al., 2006). These nematodes reproduce easily on plant tissue like carrot discs and is the best and most practical way to reproduce migratory endoparasitic nematodes such as *D. dipsaci* in vitro on carrot discs. (O'Bannon & Taylor, 1968; Kühnhold et al., 2006). Likewise, it is one of the best ways the production of

root-lesion nematodes (Huettel, 1985). There are various studies on the reproduction of root-lesion nematodes on carrot discs (Chitambar & Raski, 1985; Kaplan & Davis, 1990; Verdejo-Lucas & Pinochet, 1992). Reproduction of *D. dipsaci* on carrot culture discs can be influenced by factors such as incubation time, temperature, and inoculum density. Abiotic factors like temperature are important for the development of *D dipsaci* on alfalfa and white clover plants (Griffith et al., 1997; Williams-Woodward & Gray, 1999). However, Blake (1962b) showed that the development population of this nematode also can affect by the growth of the host plant. Tenente & Evans (1998) indicated that the best optimum temperature for the development of this nematode is between 15 and 20ºC. It completes its life cycle (J1 to J4) in 21 to 28 days at 20ºC and between 28 to 34 days at 15ºC.

The first part of this study focused on establishing a reliable methodology for identification the reaction of wild and domesticated chickpea genotypes to *D. dipsaci* using different initial inoculation densities and times of assessment, and the second part aimed to determine (1) the effect of incubation time on the population density of *D. dipsaci*, (2) the effect of temperature on nematode reproduction, and (3) the effect of initial inoculum density on nematode reproduction on carrot discs. The second part of the study was designed to develop a protocol for carrot culture of *D. dipsaci* screening studies under sterile conditions to help attach a clean, identical and pure source of inoculum.

Materials and Methods

General Methods

Collection of nematodes and species determination

A population of *D. dipsaci* from a chickpea field in Şanlıurfa, Turkey was used in this study with nematodes extracted from 100 g of soil and 5 g roots by using a modified Baermann funnel (Hooper et al., 2005). The source population density of 2-70 nematodes/100 g of soil and 10-50 nematodes/5 g of the root. Nematodes were collected with a 5-µl micropipette using a stereomicroscope at 40× magnification and placed in a concave glass block. Standard morphological methods were to identify the plant-parasitic nematodes (Hooper, 1972; Fortuner, 1982; Brzeski, 1991). The extracted nematodes were placed in sterile falcon centrifuge tubes containing 0.1% streptomycin sulfate (w/v) and 0.1% amphotericin B (w/v) and allowed to stand at room temperature for 10 min and then rinsed three times with sterile water. The nematode suspensions were then kept at 4ºC until used.

***In vivo* inoculation system**

Nine accessions of Turkish domesticated and wild *Cicer* spp. including three *C. arietinum* (Azkan, Çağatay and Gökçe), three *C. echinospermum* (Karabahçe, Ortance and Destek), and three *C. reticulatum* (Fabales: Fabaceae) (Şırnak, Kallen and Eğil) were assessed for resistance to *D. dipsaci* under laboratory conditions. Genotypes used in this study were collected from three sites in Diyarbakır, Şanlıurfa and Şırnak provinces of Turkey. The seeds were scarified by making a small cut in the seed coat before germinating to improve water absorption and germination in the wild *Cicer* spp. The individual chickpea seeds were disinfected with hypochlorite (4%) and ethanol (30%). Also, to enhance seed germination, about 30 seeds of each accession were placed on the surface of wet filter paper at 4ºC for 3 days in sterile Petri dishes (Garcia et al., 2006). We incubated seeds at room temperature for 16 h before planting. The study was conducted as a completely randomized block design with four replicates. A germinated seed was then planted in the open-ended standard small tube (16 cm high and 2.5 cm in diameter) that contained 60 g of autoclaved (15 min at 121ºC) field soil, (73% clay, 17% silt and 10% sand) and supported by a frame. One week after planting, the nematodes were transferred at 25ºC, and plants were inoculated with either 150, 300 and 400 nematodes/tube in 1 ml water. The experiment was conducted concurrently with all accessions and grown in a growth room at 25ºC and 50% RH under a 16:8 h L:D photoperiod provided by high-pressure sodium lamps. After a further 16 and 20 weeks, the plants were harvested and nematodes from both soil and roots extracted using a Baermann funnel and counted (Hooper et al., 2005). RF was calculated as described above.

In vitro culturing

The second part study examined the effect of incubation time, temperature and inoculum density on *D. dipsaci*) rearing on carrot cultures at Çukurova University, Nematology laboratory between 2019 and 2020. All equipment and materials were sterilized by autoclaving at 121ºC for 15 min. Also, all the working surfaces were cleaned with 70% ethanol. Moderately thick carrots were selected without cracks and washed in distilled water, then tap water, and surface sterilized in a 6% (v/v) NaOCl solution for 2 min, peeled, and soaked in 95% ethanol for 15 min. Then, 5 mm thick sections 3-4 cm in diameter were cut and transferred to sterilize Petri dishes (5-6 cm in diameter) with sterilize forceps. These were incubated in the dark for 3-4 weeks at 19-23ºC for 12-18 days until callus formed on the disc surface. The cultures were examined every week. Nematodes surface sterilized in glass measuring cylinders with 10 ml of water containing 6 mg of streptomycin with gentle mixing. After 1 h, this solution was changed to sterilized distilled water and reduced in volume, replenished with sterilized distilled water to 10 ml and left for 1 h. Fifty surface-sterilized females and males (1:1) were added to a drop of distilled water on carrot disc using a sterile needle. The discs were then incubated at 19 ± 1ºC. When the first nematodes egressed from the discs, the number of days after inoculation was recorded. The effect of time of incubation period on the development population density of *D. dipsaci* nematode was assessed at 20, 45, 60 and 90 days. Also, the effect of temperature on nematode reproduction was assessed at 15, 20, 25 and 30 ± 1ºC for 35 days. To determine the optimum number of *D. dipsaci* as initial inoculum carrot calluses were inoculated with 50, 100, 200, 300 and 500 nematodes per disc. Nematodes were inoculated in 100 µl sterile water with three drops of suspension were added to carrot disc. The average composition of life stage in the inoculum was 16% male, 57% juveniles, and 27% female. All the inoculated carrot discs were then incubated at 20 ± 1ºC in the dark for 35 days. Finally, carrot discs were cut into small pieces, and nematodes were extracted to determine the effect of time, temperature, and inoculum density on the reproduction of *D. dipsaci* nematodes. A completely randomized design with 10 replicates was conducted for the determination of these experiments.

Experimental design and analysis

The data (numbers of nematodes per tube and carrot discs) were analyzed using ANOVA in Genstat (V13). Significant differences between treatment and replication of data were calculated at $P < 0.05$. Outliers and variance distribution was assessed using residual plots. The data transformed to log10(x+1) values to provide normality.

Results

In vivo inoculation

The screening study for resistance to *D. dipsaci* indicated that there was no significant difference between growing time (16 and 20 weeks; $P = 0.539$) and *Cicer* spp. ($P = 0.601$; Table 1 and Figure 1). However, the population density of *D. dipsaci* in *C. arietinum* was higher than the any *Cicer* spp. (Figure 1). Also, the lowest population density was in *C. echinospermum* except for 16 weeks with 400 nematodes as the initial population after 16 and 20 weeks (Table 1 and Figure 1). Analysis of variance showed that initial nematode density has a large effect on final numbers (P linear = 0.009) and that there is some indication of an interaction between species and the linear effect of nematode density $P = 0.086$ (Table 1 and Figure 1). Figure 1 shows the RF increasing with 150-300, but not 400 nematodes applied, and that *C. echinospermum* is more responsive to the initial nematode density because it responds more steeply to the 150-300 change $P = 0.086$ (Table 1 and Figure 1). Thus at 150 nematode/plant *C. echinospermum* had lower final nematode counts than the other two species, while at 300 and 400 nematodes there is no significant difference. The data indicate that the initial inoculum of 300 nematodes/plant was optimal. The development of *D. dipsaci* was higher than at 150 nematodes in all species (Figure 1). The highest

population density of *D. dipsaci* in the inoculation density of 150 nematodes was observed in *C. arietinum* (Gökçe RF = 2.7, Çağatay RF = 2.6 and Menemen RF = 2.5) and the lowest population density observed in *C. echinospermum* (Karabahçe RF = 2.2, Ortance RF = 2.3 and Destek RF = 2.4).

Table 1. Analysis of Variance for chickpea resistant to *Ditylenchus dipsaci*

Source of variation	Degree of Freedom (df)	Sum Square (SS)	Mean Square (MS)	F Ratio	P Value
Period	1	0.088	0.088	0.07	0.539
Density	2	12.154	5.077	4.55	0.016
Linear	1	10.959	10.959	9.81	0.009
Species	1	0.690	0.690	0.30	0.601
Period.Density	2	0.331	0.165	0.14	0.316
Period.Linear	1	0.101	0.101	0.09	0.587
Period.Species	2	5.791	2.895	2.59	0.137
Linear.Species	2	5.447	2.723	2.44	0.086
Residual	18	20.074	1.115		
Total	30	55.634			

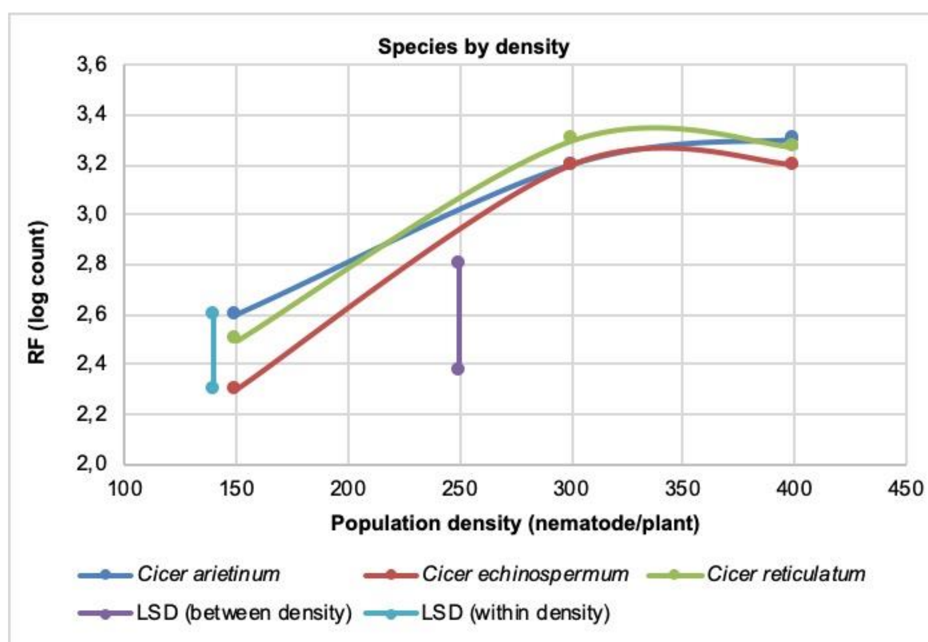

Figure 1. Effect of inoculation density on the reproduction factor (RF) of *Ditylenchus dipsaci* on chickpea species.

In vitro culturing

The population density and indicates of *D. dipsaci* increased with time 20 days after incubation at 19 ± 1ºC (Table 2 and Figure 2). The highest and lowest RF (RF = 644 and 7.4) occurred after 90 and 20 days after incubation respectively. The RF of *D. dipsaci* was 600 after 45-60 days, compared to 7.4 after 20 and 59.2 after 40 days. A higher number of nematodes and RF occurred after 45 and 60 days than after 20 and 40 days, and there were no differences observed between 60 and 90 or 45 and 60 days (Table 2 and Figure 2).

Table 2. Effect of incubation period on reproduction of *Ditylenchus dipsaci* on carrot discs after incubation with 50 nematodes

	Number of nematodes per carrot disk				
Incubation period (day)	Female	Juvenile	Male	Pf	RF
20	67	220	30	370	7.4
45	860	1 200	100	2 960	59.2
60	4 800	1 300	12 200	30 000	600.0
90	5 200	15 500	11 500	32 200	644.0
$LSD_{0.05}$	1.353	5.645	4.127	11.126	222.5

Pf, final population; Pi, initial population; and RF, reproduction factor as Pf/Pi.

In carrot cultures development population density of *D. dipsaci* was greater at 19 and 25ºC than 15 and 30ºC. There were no other significant differences observed i between 20 and 25ºC or between 15 and 30ºC (Table 3). The highest and lowest numbers of *D. dipsaci* were observed with inoculum of 100 and 500 *D. dipsaci* per disc (Table 4 and Figure 2). The maximum numbers were obtained after 35 days at 20ºC with 100 nematodes per disc. Also, it was found that by increasing nematode inoculum density from 100 to 500 the reproduction rate of *D. dipsaci* declined.

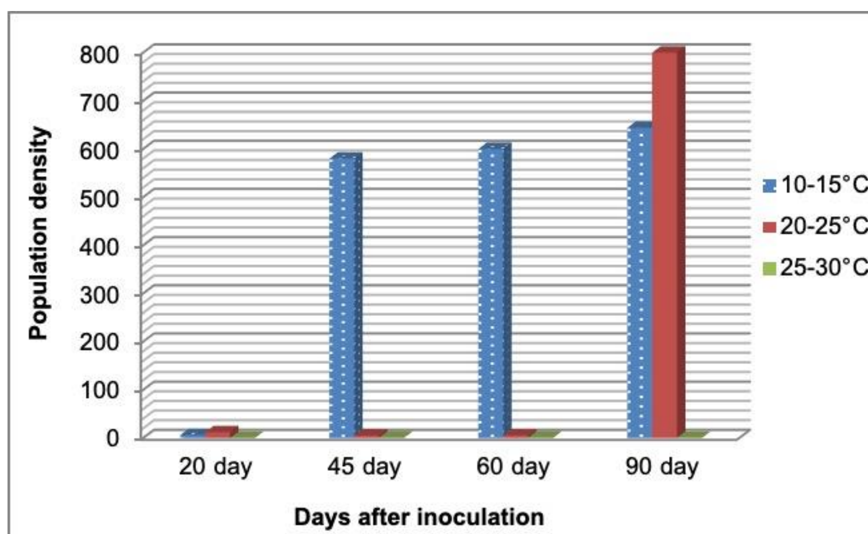

Figure 2. Effect of incubation time (20, 45, 60 and 90 days) and temperature (10, 15, 20, 25 and 30ºC) on the population density of *Ditylenchus dipsaci* nematode on carrot discs.

Table 3. The effect of temperature on reproduction of *Ditylenchus dipsaci* on carrot discs after incubation 50 nematodes

	Number of nematodes per carrot disk				
Temperature (ºC)	Female	Juvenile	Male	PF	RF
15	15	9	30	54	1.1
20	68	120	80	268	5.4
25	60	92	72	224	4.5
30	8	4	3	15	0.3
$LSD_{0.05}$	12.5	35.7	22.8	71.0	1.4

Pf, final population; Pi, initial population; and RF, reproduction factor as Pf/Pi.

Table 4. Effect of inoculum density on reproduction of *Ditylenchus dipsaci* on carrot discs at 20 ± 1ºC

	Number of nematodes per carrot disk				
Inoculum density	Female	Juvenile	Male	PF	RF
50	137	260	290	687	13.74
100	240	1 580	960	2 780	27.80
200	540	2 200	1 880	4 620	23.10
300	885	3 200	2 200	6 285	21.60
500	1 100	5 100	3 420	9 620	19.24
$LSD_{0.05}$	390.000	1.902	880	3.172	4.26

Pf, final population; Pi, initial population; and RF, reproduction factor as Pf/Pi.

Discussion

The present study was design to develop a standard protocol for culture *D. dipsaci* for use in a screening study. This method allows providing a clean, uniform, and pure source of high numbers of *D. dipsaci in vitro* rearing under sterile conditions. Kagoda et al. (2010) reported that the carrot discs provided as a food source for reproduction high number of population nematodes that used in screening study.

Overall, our results indicate that the carrot disc culture can be used to produce *D. dipsaci* obtained from chickpea. The maximum population density of *D. dipsaci* in carrot disc cultures was after 45 and 60 days incubation with an initial inoculum density of 100 nematodes at 20ºC. These results indicated that the reproduction of *D. dipsaci* between 20 and 25ºC at 45-60 days after inoculation was nearly 4 times the rate after 20 days inoculation at 20ºC. The population density of plant-parasitic nematodes depends on their complete life cycle. In plant-parasitic nematodes, passing through ecdysis (molting) from the first stage juvenile (J1) to the second stage depends on the temperature and period time (Trudgill, 1995). The study found that the population of males was more than females in carrot cultures at 25ºC indicating that a temperature rise may impact male number. A similar study by Tenente & Evans (1998) showed that the population density of males was more than females in onion tissues at 26ºC 28 days after inoculation. Laughlin et al. (1969) showed that the temperature could affect the number of females to males during the mating process. Blake (1962a) showed that the reproduction of *D. dipsaci* was highly affected by some factors such as temperature and moisture. Griffin (1974) showed that the temperature range can affect the activity and reproduction rate of *D. dipsaci* on alfalfa. Similarly, studies indicated the optimum temperature for development was between 15 and 20ºC and there was minimal activity of this nematode between 3 and 5ºC (Yüksel, 1960; Griffith et al., 1997). The initial inoculum density of nematodes, different sizes of carrots could affect food sources and cause nutrients to become depleted and initiate nematode migration or not reproduce from the carrot (Coyne et al., 2014). Similarly, Castillo et al. (1995) showed that the population density of root-lesion nematodes, *Pratylenchus thornei* Sher & Allen, 1953 (Tylenchida: Pratylenchidae) decreased with an increasing initial inoculum density of 100 nematodes on chickpea under laboratory conditions.

Interaction between initial and final population density of nematodes is important on resistance or susceptibility of different cultivars. Toktay et al. (2012) showed that optimization of these factors is necessary for the development methodology for resistance screening and to keep the population density of nematode below the damage threshold level in the wheat breeding programs. It was reported the effect of temperature on egg production, hatching and the life cycle of *Ditylenchus destructor* Thorne, 1945 (Tylenchida: Anguinidae) isolated from groundnut *in vitro* (Waele & Wilken, 1990). Also, Schomaker & Been (2006) indicated that the information on the initial population density of nematodes and predication of damage under specific conditions for specific crops is essential for an integrated pest management strategy. The screening of the different chickpea genotypes did not show a significant difference in *D. dipsaci* resistance at 16 and 20 weeks and between the initial inoculum densities of 300 and 400 nematodes/tube. However, accessions of *C. echinospermum* had more resistance than *C. arietinum* and

C. reticulatum. Thompson et al. (2011) observed that the response of the root lesion nematodes for resistance was the difference between *Cicer* spp. A similar study by Behmand et al. (2019) indicated that there were differences between *Cicer* spp. to *P. thornei* sensitivity, and both of *C. arietinum* and *C. reticulatum* accessions were more susceptible than any of the *C. echinospermum* accessions.

Based on the presented data, the standard protocol now implemented in our laboratory for production of *D. dipsaci* uses an initial inoculum density of 300 nematodes per tube with harvesting after 16 weeks. The results are similar to those obtained study by Behmand et al. (2019), who reported that there was no significant difference observed between growing times 16 and 20 weeks and between the initial inoculum density of 225 and 300 nematodes/tube to identify chickpea resistance to *P. thornei*. Chickpea is cool-season crop and requires a growing time of 100 days to reach maturity (Singh & Ocampo, 1997). Reen & Thompson (2009) indicated that the maximum difference in population density of *P. thornei* among cultivars of chickpea was observed after 18 to 20 weeks under laboratory conditions. This study was the first to assess chickpea genotypes collected from Turkey for resistance to *D. dipsaci*, some of which offer new sources of *D. dipsaci* resistance and genetic diversity useful.

These days, genomic tools and standard plant breeding technologies make it possible to study the genotypes associated with desirable phenotypes. Standard breeding methods have been highly successful for developing new cultivars. The use of standard pre-genomic breeding methods has yielded improvements to modern cultivars that allow for a dramatic increase in staple crop yields. These standard methods can help future breeding efforts aimed at *D. dipsaci* prevention. The identification of resistant *D, dipsaci* germplasm to nematode diseases is a fundamental task for breeding nematode resistant cultivars.

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