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Life Cycle and Mating Behavior of *Zygotylenchus guevarai* (Nematoda: Pratylenchidae) on Excised *Pisum sativum* Roots

Mehmet KARAKAŞ

Ankara Üniversitesi Fen Fakültesi Biyoloji Bölümü
mkarakas@science.ankara.edu.tr

Abstract

The life cycle and mating behavior of *Zygotylenchus guevarai* were observed in vitro on excised roots of *Pisum sativum* in gnotobiotic culture. Eggs hatched into larvae. Each larva stage was terminated by a molt. *Z. guevarai* had four larva stages. First molt occurred outside the egg shortly after hatching. After the final molt the larvae differentiated into adult males and females. Mating was required for reproduction. After mating, fertilized females began to lay eggs. The life cycle from second stage larva to second stage larva was completed in 39 days.

Key Words: Zygotylenchus guevarai, root lesion nematode, gnotobiotic culture, life cycle, mating behavior

Kesilmiş *Pisum sativum* Köklerinde *Zygotylenchus guevarai* (Nematoda: Pratylenchidae) nin Hayat Devri ve Çiftleşme Davranışı

Özet

Zygotylenchus guevarai'nin hayat devri ve çiftleşme davranışı gnotobiotik kültürde *Pisum sativum*'un kesilmiş köklerinde in vitro olarak gözlenmiştir. Yumurta açılımı ile larvalar ortaya çıkmıştır. Her bir larva safhası deri değişimi ile sonlanmıştır. *Z. guevarai* dört larva safhası göstermiştir. Birinci deri değişimi açılımdan kısa bir süre sonra yumurta dışında meydana gelmiştir. Son deri değişiminden sonra larvalar, ergin erkek ve dişilere farklılaşmışlardır. Çiftleşmenin üreme için gerekli olduğu gözlenmiştir. Çiftleşmeden sonra dişiler döllenmiş yumurtalarını bırakmıştır. Hayat devri ikinci devre larvadan, ikinci devre larvaya 39 günde tamamlanmıştır.

Anahtar Kelimeler: Zygotylenchus guevarai, kök lezyon nematodu, gnotobiotik kültür, hayat devri, çiftleşme davranışı

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1. Introduction

One of the most damaging plant parasitic nematodes of the world is the root lesion nematode *Zygotylenchus guevarai* (Tobar Jiménez, 1963) Braun and Loof, 1966. *Zygotylenchus guevarai* was recorded from most of the countries of the world around the roots of various hosts. It has a high damage potential at relatively low population densities and parasitizes a wide range of hosts among agricultural and horticultural crops such as carrot (*Daucus carota*), celery (*Apium graveolens*), corn (*Zea mays*), cotton (*Gossypium hirsutum*), kidney bean (*Phaseolus vulgaris*), melon (*Cucumis melo*), oats (*Avena sativa*), parsley (*Petroselinum crispum*), pea (*Pisum sativum*), pepper (*Capsicum annuum*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) and wheat (*Triticum durum*) [1,2]. Its development and reproduction occurs inside the root tissues of plant host. The migratory endoparasitic nematode *Z. guevarai* invades the root cortex causing root lesions and the formation of large cavities, destroying the cortical tissues [2].

Carrot disc cultures have been used to propagate a number of root lesion nematodes providing large numbers of highly infective nematodes [3,4,5,6]. Since contamination due to bacteria associated to the carrot tissue sometimes occurs in carrot disc cultures, *Agrobacterium rhizogenes* transformed potato root cultures were considered as an alternative to propagate *Z. guevarai*. Transformed root cultures have been successfully used to propagate sedentary endoparasitic nematodes [7,8,9,10,11]. The monoxenic culture of *Z. guevarai* in both carrot and transformed potato root is reported by Verdejo and Pinochet [12].

In this study, life cycle and mating behavior of *Z. guevarai* in gnotobiotic culture (with known associated organisms) were investigated.

2. Materials and Methods

Zygotylenchus guevarai was obtained from infested pea roots (Karacabey-Turkey, 40° 13,2′ N; 28° 19.8 E′) and cultured on pea seedlings in sand-loam soil in the greenhouse (20 ± 2 °C and 70 % relative humidity). Then a population of *Z. guevarai* was established in petri dishes on excised pea roots (*Pisum sativum* L. cv. Asgrow Dorian) supported by Gamborg's B5 medium in 1.5 % agar adjusted to pH 6.5 and maintained in darkness at 20 ± 2 °C [13].

To study development, nematode eggs from the in vitro culture of *Z. guevarai* were aseptically transferred onto 1 % water agar plates and incubated at 20 ± 2 °C overnight. Hatched second stage larvae (L_2) were inoculated onto 10 day old pea root cultures. Five excised pea roots were cultured on each of four replicate culture dishes and were inoculated with 100 J2 of *Z. guevarai*. These plates were incubated under the above conditions. Nematode development and behavior in all replicates were observed daily with dissecting microscope and TZ 240 model Euromex binocular under cold light. The first occurrence of each molt and development stage among the replicates was the criterion used to determine the time periods of the life cycle which reflects a typical time course based on many repeated observations of each event.

Pea root culture dishes were prepared and divided into three groups with 10 replicates each: (1) one molting female fourth stage larva (L_4) in each dish, (2) one molting female L_4 and 10 males in each dish, (3) one molting female L_4 and 10 males in each dish with males removed after the first egg appeared in the medium in order to prevent further mating. Molting L_4 could be sexually differentiated on the basis of body morphology. After the female L_4 finished molting and developed into adults, they were considered virgin females and could only be fertilized by the males added to the same dish. These dishes were incubated as described above and nematode behavior was observed daily for 120 days. Once egg deposition in a dish occurred, the eggs were transferred onto corresponding 1.5 % agar dishes and observed for hatching. Hatched larvae were removed

immediately from this agar dish to avoid being mixed with larvae hatching later. The number of eggs produced by each female was also recorded.

3. Results and Discussion

The L_2 to L_2 life cycle of *Z. guevarai* was completed under gnotobiotic conditions at 20 ± 2 °C in 39 days (Table 1). The L_2 moved to the root tips and began feeding within 2.0 hour after inoculation. Feeding lasted for 12 to 24 hours, then the L_2 became immobile and remained positioned like a "C" or a closed circle. The second molt (M_2) started 4 days after inoculation. The most significant change during molting occurred in the oesophageal region. During the first 12 to 24 hours of molting, the stylet shaft, oesophageal lumen and median bulb became invisible. Only the stylet cone remained discernible.

Twelve hours later, the new cuticle became visible inside the old one, followed by the appearance of the new stylet shaft. Then the oesophageal lumen and the median bulb emerged and gradually became more distinctive. The larva body progressively elongated until it was confined by the old cuticle. At this time the new stylet began to probe the old cuticle at the rate of once every 5 to 10 seconds, associated with contraction of the median bulb once every 4 to 7 probings. The nematode finally broke through the old cuticle and migrated out. This molting period (M_2) lasted for 3 days. The third stage larva (L_3) began feeding again. At 12 days after inoculation the L_3 entered the third molting (M_3) period, which lasted for 4 days and resulted in the emergence of the fourth stage larvae (L_4). The L_4 started feeding on the roots again, followed by the fourth molting (M_4) period. Larvae that developed into males started the M_4 24 days after inoculation. By the end of the 4 days molting period, the male gonad, the spicules and the caudal alae had formed and the male migrated out of the old cuticle 28 days after inoculation. Larvae that developed into females started M_4 at 25 days after inoculation, which lasted for 5 days. By the end of the molting period, the female gonads and the vulva had formed. The female migrated out of the old cuticle 30 days after inoculation. Faster development of males than females has been observed with other nematodes, e.g., *Heterodera schachtii* [14] and *Belonolaimus longicaudatus* [15] in gnotobiotic cultures.

Males of sting nematode *Belonolaimus longicaudatus* Rau. approached females soon after the females finished the last molt. Often two or more males surrounded a female, which caused more competition for mates [16,17,15]. These findings were similar with *Z. guevarai*. The males seemed to be directly attracted by the females and gathered around them quickly. The males moved around the female and began to intensely rub the side of the female body with the lateral side of their lip region. The rubbing movement of the female head was perpendicular to the axis of the female body. In the meantime one of the males moved toward the female head so that its bursa finally touched the female body. This male would move farther ahead, continuously rubbing the female body until its bursa reached the vulva region of the female. The male then moved back and forth and the female also twisted its body until finally the spicules penetrated through the vulva with the bursa covering the area around the vulva. Then, the body movement of both nematodes slowed down. Mating in this manner lasted for 10-12 minutes, during which fertilization presumably took place. The male then withdrew its spicules and both nematodes moved away. This mating behavior was observed at least 6 times and each time the mating occurred on the surface of the culturing medium. It was not determined whether a female mated more than once during its life.

After mating, both females and males fed on the surface of the culturing medium. Meanwhile, eggs began forming within female uteri and were clearly visible. Before females began to lay eggs, they stopped feeding and moved slowly within or on the surface of the medium. The eggs in the uterus were pushed toward the vagina. The egg-shall was very flexible and was squeezed to pass the shallow lumen of the vagina and delivered through the vulva. Egg deposition was completed in

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approximately 5 minutes, during which the female did not move. The egg resumed its shape outside the female body. The first eggs were laid 34 days after inoculation. All larva stages as well as the adult stages of both genders fed on the host.

Table 1. Life cycle period of *Zygotylenchus guevarai*

LIFE STAGES (Female)	LIFE PERIOD (Day/Days)	L[a] (mm)	LIFE STAGES (Male)	LIFE PERIOD (Day/Days)	L (mm)
First stage larva (L_1)	1	0.17	First stage larva (L_1)	1	0.14
First molt (M_1)	1		First molt (M_1)	1	
Second stage larva (L_2)	4	0.30	Second stage larva (L_2)	4	0.28
Second molt (M_2)	3		Second molt (M_2)	3	
Third stage larva (L_3)	5	0.41	Third stage larva (L_3)	5	0.38
Third molt (M_3)	4		Third molt (M_3)	4	
Forth stage larva (L_4)	9	0.50	Fourth stage larva (L_4)	8	0.47
Fourth molt (M_4)	5		Fourth molt (M_4)	4	
Mating Egg-laying } Feeding	4	0.58 [b]	Mating } Feeding	9	0.54 [c]
Embryo	3				

a: Total Body Length (Data are means of five replicates for each stage) **b:** Adult Female **c:** Adult Male

Zuckermann and Strich-Harari [18] found no evidence that females of banana spiral nematode could reproduce in the absence of males. The present study allowed examination of this hypothesis in more detail and confirmed that sexual reproduction was obligatory. In the treatment with only one female in each of 10 dishes the females produced in total only 0.5 ± 0.4 eggs during 90 days incubation, and none of these eggs hatched. When males were always present in the medium, each female produced an average of 125 ± 15 eggs in 90 days, and all the eggs hatched. When the males were removed after the first eggs appeared, each female still produced an average of 132 ± 18 eggs in 90 days, and all the eggs hatched. The number of eggs in treatments with fertilized females were not significantly different from each other (Tukey's test, P = 0.01). Therefore fertilized females produced eggs without the continued presence of males during the 90-day observation. Females collected from field locations almost always have spermathecae filled with sperm [19]. Under the present culture conditions, each fertilized female produced an average of 2.06 ± 0.26 eggs per day for 90 days. It was not determined how many eggs a female can produce throughout its life time.

In vitro culture of plant nematodes allows continuous observation of the nematodes and has been utilized in nematological studies in the 1950s [20]. It has proven to be helpful in studying nematode life cycle and host-parasite relationships. By means of this technique, the life cycle of *Heterodera zeae*, *H. glycines* and *H. multicinctus* have been described in detail [21,22,23] . However only a few strictly ectoparasitic phytonematodes such as *Criconemella xenoplax* [24] and *H. multicinctus* [23] have been successfully cultured on excised roots. This technique not only provided sterile nematode inocula for well controlled

host-nematode relationship studies but also allowed direct observation of the nematode behavior without the interference of soil flora and fauna [13]. Results must be interpreted with caution however, since the metabolic response of the host might be quite from that of an intact host. However, no obvious changes were observed during the course of this study in terms of behavior and parasitism.

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