Tolerance of proto-anhydrobiotic citrus nematodes to adverse conditions [1]

Bie Yun TSAI and Seymour D. VAN GUNDY

Department of Nematology, University of California, Riverside, CA 92521, USA.

SUMMARY

Coiled citrus nematodes *(Tylenchulus semipenetrans)* sampled on the day before irrigation from a citrus orchard were tested for their tolerance to extreme drought (0 % RH) and unfavorable temperatures (45°, 40°, 0°, and — 18°). The coiled nematodes appeared to be in a different physiological state from the non-coiled ones because they were more tolerant to freezing temperature (0° and — 18°). However, they did not survive a 24 h exposure to 0 % RH, therefore, they were not in the extreme anhydrobiosis (cryptobiosis). To avoid confusion with the cryptobiotic anhydrobiosis, the term " proto-anhydrobiosis " was selected to describe this early stage of anhydrobiosis of the citrus nematode naturally occurring in the citrus orchards. To test if the proto-anhydrobiotic citrus nematodes have the ability to proceed to cryptobiosis, field soil containing the coiled population was stored in polyethylene bags for sixteen months. The percentage of coiled nematodes increased after the storage (up to 92.7 %), however, none of the nematodes survived the soil moisture level of 2.6 % or less. Apparently the citrus nematodes were not able to proceed to cryptobiosis even with the very slow dehydration rate through the polyethylene bags. Artificial induction of anhydrobiosis by air drying the soil with different rates of moisture loss was not able to produce cryptobiotic citrus nematodes either.

RÉSUMÉ

La tolérance des nématodes protoanhydrobiotiques des citrus aux conditions adverses

Des nématodes des citrus *(Tylenchulus semipenetrans)* enroulés, prélevés dans un verger de citrus la veille de l'irrigation ont été testés pour leur tolérance à une sécheresse poussée (RH = 0 %) et à des températures non favorables (45°, 40°, O° et — 18°). Les nématodes enroulés paraissent être dans un état physiologique différent de ceux qui ne sont pas enroulés car ils sont plus tolérants envers les températures basses (0° et — 18°). Cependant, ils ne survivent pas à une exposition à un RH de 0 % pendant 24 h, et donc ne sont pas en état d'anhydrobiose extrême (cryptobiose). Pour éviter toute confusion avec l'anhydrobiose cryptobiotique, le terme de « proto-anhydrobiose » a été choisi pour décrire le stade primitif d'anhydrobiose de ces nématodes, lequel est trouvé, en nature, dans les vergers de citrus. Pour tester si ces nématodes proto-anhydrobiotiques sont capables de crypbiose, du sol naturel contenant une population de nématodes enroulés a été stocké dans des sacs en polyéthylène pendant seize mois. Le pourcentage de nématodes enroulés est plus élevé à la fin du stockage (jusqu'à 92,7 %), mais aucun nématodes ne survit à une humidité du sol inférieure ou égale à 2, 6 %. Apparemment donc, les nématodes des citrus ne sont pas capables d'entrer en cryptobiose, même lors d'une déshydratation très lente dans les sacs de polyéthylène. Des essais d'induction artificielle de l'anhydrobiose en faisant traverser le sol par un air à différents degrés d'humidité ont été négatifs, aucun nématode n'entrant en cryptobiose.

Anhydrobiotic nematodes have been reported to possess unusual abilities to withstand unfavorable conditions. *Anguina tritici* second-stage larvae in anhydrobiotic state maintained viability at 105° for 2 min and — 190° for 15 min (Bird & Buttrose, 1974). Similarly, anhydrobiotic *Aphelenchus avenae* survived exposure to — 196° for 15 min (O'Dell & Crowe, 1979). They also survived 0 % RH for 18 months (Crowe & Madin, 1975). Anhydrobiotic *Ditylenchus dipsaci* survived freezing for five years at — 80° (Bosher & McKeen, 1954). Anhydrobiotic *Scutellonema cavenessi* survived 0 % RH for one month (Demeure, 1980). The anhydrobiotic nematodes were all reported to possess a coiled shape.

Tsai and Van Gundy (1986) reported the discovery of coiled *Tylenchulus semipenetrans* Cobb, 1913 in citrus orchards between irrigation cycles. The coiled populations were negatively correlated to soil moisture in the orchards. The citrus nematodes were induced into cycles of anhydrobiosis between irrigations. This paper reports research to determine if the anhydrobiotic citrus nematodes in the cropped-field soil possessed the same tolerance to adverse conditions as the

(1) Portion of a Ph. D. dissertation by the senior author.

other anhydrobiotic nematodes previously described from dried plant materials or dry-fallowed soil.

Materials and methods

Coiled and non-coiled nematodes

Soil containing coiled second-stage larvae and adult males of the citrus nematodes, *T. semipenetrans*, was obtained directly from a citrus orchard (Field 10 B, Citrus Research Center, Riverside, California) eighteen days after furrow irrigation, i.e., one day before irrigation. The property of the soil and the moisture characteristic curve have been described in a previous paper (Tsai & Van Gundy, 1989). The soil was mixed thoroughly and divided into two equal parts. One part was referred to as the dry soil and was used directly for the tests of the natural coiled population. The other part was spread into a thin layer on wax paper and atomized with fine mist of water until the soil was thoroughly and uniformly wet to ensure complete uncoiling of the nematodes. The atomized soil was allowed to air-dry for approximately three hours to let excess moisture evaporate so that the soil was moist but not muddy. The moist soil was used as a control. Soil moisture of each kind of soils was determined by oven-drying at 105° for 24 h. Four 50 g samples of soil of each kind were processed in Baermann funnels to determine initial nematode populations. All the Baermann funnel extraction procedures in this research were carried out for seven days to allow sufficient time for the rehydration of the anhydrobiotic nematodes. Percentage of coiled nematodes in each kind of soil was determined with the hot formalin technique (Tsai & Van Gundy, 1989).

Tolerance to extreme drought

Twenty-five grams each of the dry and wet soil was placed in a small Petri dish in a desiccator to test the effects of extreme drought. Five dishes were placed in each desiccator, two of them for determining soil moisture, three for Baermann funnel extraction of nematodes. The soil samples were held in the desiccators for two to four days. Samples in one desiccator were processed each day until the soil was completely dry. Another set of soil samples were placed on the outside of the desiccator and sealed as the control. The experiment was repeated three times.

Tolerance to unfavorable temperatures

Fifty grams of the dry and wet soil each was sealed in small polyethylene bags for temperature tests. The bags were flattened to produce the same thickness of soil layer in each bag to enhance homogenity of the treatments. Four bags of each soil were exposed to temperature-duration treatments as follows : 45°, 24 h; 40°, 24 and 48 h; 0°, 5 h; and — 18°, 2 h. The live nematodes were extracted in the Baermann funnel. The percentage survival was based on the initial population. The t-test was performed after the log transformation of percentage survival.

Stored soils

To determine if the anhydrobiotic nematodes in the field have the ability to proceed to cryptobiosis, soils sampled 2, 7, 13, and 18 days after irrigation (DAI) in Field 10B were stored in double polyethylene bags at 23 ± 1° for sixteen months. Soil moisture, nematode population, and percentage of coiled nematode were determined on the day of sampling and sixteen months later. Percentage of survival was determined by Baermann funnel extraction.

Air drying

Different thicknesses of soil layers were prepared to give different rates of moisture loss. Three experiments were conducted with soil from Field 10 B. The soil was passed through a 1/4 inch screen, then mixed thoroughly. The initial population, percentage of coiled nematodes, and soil moisture were determined as described above. In the first experiment, the soil was spread evenly as a 1.2 cm thick layer on wax paper and air dried for 32 h. Soil moisture, percentage of coiled nematodes, and survival were determined at termination of the experiment. In the second experiment, the soil layer was increased to 2.5 cm thick and the air drying duration was reduced to 25.5 h. In the third experiment, three thickness of soil layer : 1.5, 3, and 9 cm, were layered in plastic shoe boxes. Samples were taken after a drying duration of 24 h and processed the same way as in the other two experiments. Three replications were made in all the above treatments.

Results

Tolerance to extreme drought

The 0 % RH treatment killed all citrus nematodes in both dry and wet soils. The coiled citrus nematode population did not demonstrate more tolerance to extreme drought than the non-coiled population.

Tolerance to adverse temperatures

The coiled population was significantly more tolerant to low temperatures, 0 and — 18°, than the non-coiled population (Tab. 1). There was 45.5 % survival in the coiled population but only 1.8 % survival in the non-coiled population after the treatment at — 18° for 2 h. Both the dry and wet soils were frozen in this treatment.

Although the numbers of surviving nematodes were greater in the dry soil than in the wet soil at high temperatures, 40° and 45°, there were no significant differences between the coiled and non-coiled populations in their tolerance to each temperature. There was a significant difference between 40° and 45°. Only 2 % of the citrus nematodes survived at 45° while 33 to 60 % survived at 40°.

Table 1

Survival of coiled and non-coiled second-stage larvae and adult males of *Tylenchulus semipenetrans* at unfavorable temperatures

Temp. (C)	*Duration (h)*	*% Survival* Coiled (1)	Non-coiled (2)	t-test (3)
45	24	2.0	1.6	NS
40	48	33.0	22.1	NS
40	24	60.2	51.1	NS
0	5	100.0	75.4	*
— 18	2	45.5	1.8	***

(1) Naturally coiled population in the soil at Field 10 B, a furrow-irrigated citrus orchard, obtained on the day before irrigation. The soil had a moisture of 4.8 % and contained 69.9 % coiled nematodes in the population.
(2) Non-coiled population obtained by atomizing half of the soil in 1) with water. The atomized soil had a moisture of 11.5 % and contained 4.3 % coiled nematodes in the population.
(3) Performed after log transformation.
NS = not significant
* = significant at 5 % level
*** = significant at 0.1 % level.

STORED SOILS

The results are shown in Table 2. Soils from three trees sampled on each sampling date were not combined because each sample had a different soil moisture. Overall, the percentage of coiled nematodes increased as soil moisture decreased. Many of the nematodes from 13 and 18 DAI appeared to have lost their body contents and only the empty cuticles remained in coiled shape after 16-months storage. In spite of the high percentage of coils in these samples (as high as 92.7 % coil), there was no survival when soil moisture was 2.6 % or lower.

AIR DRYING

Nematodes exposed to air drying in Experiment 1 in a 1.2 cm layer of soil for 32 h were coiled (73.5 %) but none survived the treatment. Similar result was obtained in Experiment 3 with a soil layer of 1.5 cm exposed for 24 h (Tab. 3). Some citrus nematodes survived in soil layers of 2.5 and 3.0 cm. The greatest survival occurred in soil layer of 9.0 cm, however, coiling was greatly reduced. The nematodes responded to desiccation by coiling but they were not able to survive soil moisture lower than 2.8 %. No nematodes survived for 24 h when the air drying rate was 0.22 % or higher per hour. When a moisture loss was 0.07 % per hour, 78 % of the nematodes survived.

Discussion

Nematodes are known to be able to survive unfavorable conditions in a quiescent state in which the metabolism is inhibited and the nematodes are suspended in life. Conditions which may induce quiescence are : desiccation, low temperature, lack of oxygen, and high salt concentration, or the combination of any of the four factors (Van Gundy, 1965; Cooper & Van Gundy, 1971). Giard (1894) proposed the term « anhydrobiosis » to describe the quiescence induced by the loss of water. Keilin (1959) proposed the terms « cryobiosis », « anoxybiosis », and « osmobiosis » to describe respectively the other three kinds of quiescence induced by the last three factors mentioned in the above. Cryptobiosis is the extreme form of any type of quiescence (Cooper & Van Gundy, 1971; Demeure, Freckman & Van Gundy, 1979). Nematodes in cryptobiosis are known to be resistant to extreme conditions, e.g., cold, heat, drought, radiation (Demeure, Freckman & Van Gundy, 1979; Demeure & Freckman, 1981). Anhydrobiotic *A. avenae* have survived for eighteen months at extreme 0 % RH (Crowe & Madin, 1975) and 15 min at — 196° (O'Dell & Crowe, 1979). Those nematodes were in a fully cryptobiotic state via anhydrobiosis. Coiled citrus nematodes found in citrus orchards between irrigation cycles apparently were not in a cryptobiotic state since they could not survive a 24 h exposure at 0 % RH. Temperature tests showed that they were more resistant to freezing temperatures (0° & — 18°) than the non-coiled population (Tab. 1) but not to higher temperatures (40-45°). This agrees with Townshend's report (1984) on the anhydrobiotic *Pratylenchus penetrans* which had increased resistance to — 4° but not to 40°. This experiment indicated that coiled citrus nematodes in the orchards were in the earlier stages of anhydrobiosis but not in cryptobiosis. Historically, all anhydrobiotic nematodes have been capable of going into cryptobiosis, such as *Anguina, Ditylenchus,* and *Aphelenchus.* Therefore, it has been assumed that anhydrobiosis always leads to cryptobiosis. In reality, nematodes in early stages of anhydrobiosis are not well prepared for extreme conditions. To avoid confusion, the term « proto-anhydrobiosis » is proposed here to describe the early stage of anhydrobiosis found in irrigated field soil. « Proto- » means « early » or « beginning form ». Using a prefix for anhydrobiosis has the advantage of dis-

Table 2

Effect of 16-months storage in double polyethylene bags on the percentage of coil and survival of the second-stage citrus nematode larvae and adult males.

Sampling date (1)	Replicate no.	% Coil		% Soil moisture		% Survival
		0 day	16 months	0 day	16 months	
2 DAI	1	20.4	52.3	12.0	8.7	66.3
	2	15.1	39.2	12.9	7.0	100
	3	22.6	59.7	11.5	5.7	23.4
7 DAI	1	17.1	50.1	9.3	7.7	100
	2	22.6	46.4	10.7	8.6	88.4
	3	31.9	39.2	9.5	7.5	83.1
13 DAI	1	21.4	76.1	5.7	2.6	0
	2	30.3	92.7	5.8	2.5	0
	3	22.7	80.0	7.4	3.1	0.7
18 DAI	1	31.2	90.8	5.1	2.5	0
	2	61.6	73.0	9.2	6.6	17.8
	3	15.6	85.1	5.5	2.6	0

(1) Soils sampled from 10 B, a citrus orchard naturally infested with *Tylenchulus semipenetrans*.
DAI - Days after irrigation.

Table 3

Effect of different soil thicknesses on the desiccation survival and percentage of coiled second-stage larvae and adult males of *Tylenchulus semipenetrans*

Soil thickness (cm)	Duration of air drying (h)	Soil moisture (%)	Rate of soil moisture loss (%/h)	% Coil	% Survival (1)
Experiment 1					
1.2	0	9.3	—	16.2	100
	32	1.6	0.22	73.5	0
Experiment 2					
2.5	0	7.7	—	2.6	100
	25.5	3.0	0.17	57.6	12.6
Experiment 3					
all three	0	7.3	—	2.6	100
1.5	24	1.5	0.22	75.7	0
3.0	24	2.8	0.17	68.0	1.8
9.0	24	5.5	0.07	29.5	78.0

1) Based on the living population at 0 h.

tinguishing from the extreme form of anhydrobiosis (cryptobiosis) without creating a completely new term.

Examples of nematodes in earlier stages of anhydrobiosis which are less tolerant to extreme conditions than those in deeper anhydrobiosis can be found in O'Dell and Crowe's report (1979) on *A. avenae*. They found that 91 % of the anhydrobiotic *A. avenae* which had less than 21 % water content survived the exposure of — 196° for 15 min while those with 28 % water content only had 25 % survival under the same treatment. Although not mentioned by O'Dell and Crowe, it was apparent from the data that the nematodes with less than 21 % water content were in deeper anhydrobiosis than those with 28 % water content. Demeure, Freckman and

Van Gundy (1979) viewed anhydrobiosis as a continuum rather than a specific point. This agrees with the concept of passing through proto-anhydrobiosis before reaching cryptobiotic anhydrobiosis.

It is now well accepted that coiled morphology and physiological adaptations are two features accompanying anhydrobiosis (Crowe & Madin, 1975; Demeure, Freckman & Van Gundy, 1979; Evans & Womersley, 1980; Demeure & Freckman, 1981; Crowe & Crowe, 1982). Crowe and Madin (1975) showed that if *A. avenae* were dehydrated too rapidly, they did not form coils and were killed by the dehydration; while those which were dehydrated slowly at 97 % RH for 72 h formed coils and survived exposure to dry air. Our study (Tab. 2 & 3) indicated that coil formation alone was not sufficient to protect the nematodes in dehydration. The percentage of coiled citrus nematodes was as high as 92.7 % in the stored soil (Tab. 2) but all nematodes died at low soil moistures. This was further confirmed by the air-drying tests (Tab. 3). In all cases, high percentage of coiling did not accompany high survival. Without accompanying physiological changes, the coiled nematodes with compacted cuticles can not survive severe adverse conditions. This was also evident in *A. avenae.* Crowe and Madin (1975) reported that when slowly dehydrated at 97 % RH, coil formation of *A. avenae* was completed in 36 h but only 20 % of them survived subsequent treatment at 0 % RH. The increase in the level of trehalose and glycerol was completed in 72 h at 97 % RH (Crowe & Madin, 1975) and more than 95 % of the nematodes survived subsequent treatment at 0 % RH. Although not pointed out by Crowe and Madin (1975), the level of trehalose and glycerol at 36 h was apparently not high enough to protect *A. avenae* from the extreme drought.

Slow dehydration at high relative humidities prior to desiccation at 0 % RH has been reported to enhance the survival of nematodes (Crowe & Madin, 1975; Robinson, Orr & Heintz, 1984). The very slow rate of moisture loss in the sealed double polyethylene bags during 16-months storage did not enable the citrus nematodes to survive soil moisture below 2.8 % (Tab. 2). This is a strong indication that the citrus nematodes may not have the ability to proceed to cryptobiosis after proto-anhydrobiosis. Artificial induction of anhydrobiosis by air drying the soil with different rates of moisture loss was not able to produce cryptobiotic citrus nematodes. Our other research indicated that the anhydrobiotic ability of the citrus nematodes was much weaker than many other plant parasitic nematodes. The citrus nematodes could not survive relative humidities lower than 97 % RH in a 24 h exposure. However, they were capable of surviving the permanent wilting point in the field (Tsai & Van Gundy, 1988).

The occurrence of proto-anhydrobiosis between irrigation cycles in cropped-fields soil extends the role of anhydrobiosis in agroecosystems which was known only in dry-fallowed field in the past. This phenomenon may be expected to exist in most cropped fields in arid and semi-arid regions in the world regardless of the species of soil plant parasitic nematodes.

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