Journal of Nematology 38(1):41–45. 2006.

Coiling is Not Essential to Anhydrobiosis by *Aphelenchus avenae* on Agar Amended with Sucrose

R. Otsubo, T. Yoshiga, E. Kondo, and N. Ishibashi

Abstract: The roles of preconditioning and coiling upon entrance into anhydrobiosis by *Aphelenchus avenae* were tested via video-assisted analysis at 25ºC. Fourth-stage juveniles or young adults of *A. avenae* were individually placed on 5% agar containing 0.8 M sucrose. Nematodes became quiescent within 3 hr, then gradually resumed a low level of activity and assumed a coiled posture. High desiccation survival rate was recorded when nematodes were incubated on agar for more than 6 hr; the survival rates were 0%, 3%, 73%, and 92% for 0, 2, 6, and 12 hr on agar, respectively. All nematodes placed on agar for 24 hr or more revived after rehydration following desiccation. Once nematodes were on agar for a sufficient time, no difference in desiccation survival was observed between nematodes taking a coiled posture and those uncoiled artificially. Based on these results, exposure to osmotic stress for 6 hr can prepare *A. aveae* physiologically for anhydrobiosis, but coiling does not appear to be a physiological requirement for desiccation in survival.

Key words: anhydrobiosis, *Aphelenchus avenae*, coiling, desiccation survival, osmobiosis.

Dryness is one of the major obstacles that retard the life of soil organisms. Some soil nematodes have developed desiccation survival strategies (Womersley et al., 1998; Wharton, 2002). For example, eggs and unhatched juveniles of cyst nematodes have dual mechanisms to slow drying that involve the eggshell and cyst or gelatinous matrix in which the eggs are laid. Root-knot nematodes produce gelatinous matrices that protect eggs from dryness and other environmental stresses. Entomopathogenic and animal-parasitic nematodes can enter an ensheathed, infective dauer stage that resists desiccation. Some free-living and stylet-bearing nematodes become inactive, taking a quiescent coiled posture, and may await the return of favorable conditions for a long period.

The fungivorous nematode, *Aphelenchus avenae*, is typical of species that assume a coiled posture under drying conditions. This nematode has been adopted as a model organism for studying anhydrobiosis (Crowe and Madin, 1975a; Crowe et al., 1978; Browne et al., 2002). Coiling is a competent trait for the preservation and/or delivery of this nematode as a bio-control agent for soil-borne fungal pathogens of plants (Ishibashi, 1998; Ishibashi et al., 2000). The role of coiling in desiccation survival, however, is not entirely clear. The study of anhydrobiotic survival has been limited mostly to masses or clumps of nematodes (Crowe and Madin, 1975a, 1975b; Womersley et al., 1982; Womersley, 1987). Coiling has generally been assumed to be a phenomenon that evolved from selection pressure to either further reduce the loss of water from the body surface (Wharton, 2002) or to retard water loss when clumping does not occur.

In the present study, change in motility, posture, and desiccation survival of *A. avenae* were monitored after transfer to and from a sucrose-amended substrate to test the hypothesis that coiling is essential to desiccation survival.

Materials and Methods

Nematodes: Aphelenchus avenae was collected from the Saga University farm in northern Kyushu, Japan, and cultured at 25°C on *Rhizoctonia solani* AG-4 on a vegetable medium composed of industrial waste (3:1:1 by weight potato waste: used green tea: pulp flock) in 5-liter glass jars (Ishibashi et al., 2000). Males did not develop at this temperature. Three to 4 wk after initiating the culture, nematodes were rinsed from the side of the jar and collected on a 44-µm (325-mesh) sieve. Fourth-stage juveniles (J4) or young adults (650 to 750-µm long) were used in experiments.

Sucrose-amended substrate: Prior to desiccation treatment, nematodes were placed on 5% water agar amended with different concentrations of sucrose. The range of sucrose concentration used for this study was determined according to Glazer and Salame (2000). To make the agar plates, 1, 2, 3, 4, or 5 g of agar was added to 100 ml of 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 M sucrose solution. The agar solution containing sucrose was autoclaved, and 5 ml of the agar solution was poured in a 6-cm-diam. petri dish and solidified. Agar plates were air-dried for 15 min in a laminar flow chamber to remove excess water on the surface.

Video-assisted analysis: For each analysis, nematodes were placed individually onto the agar surface using a dental file, and behavior and posture of the nematodes were recorded using a video recorder (VL-S100, Hitachi Ltd., Tokyo, Japan) with a video timer (VTG-10: FOR-A, Tokyo, Japan) attached to an Olympus SZX9 stereo microscope (Tokyo, Japan). Video recording of eight nematodes was done continuously for the first 10 min, then for 30-sec periods at 1-hr intervals for 24 hr after transfer. Nematode images were processed to obtain: (i) sequential computer images every hour, (ii) measured areas of overlap between images (25 images) by using Scion Image Beta 4.02 Win (Scion Corp., Fred-

Received for publication 11 December 2004.
Laboratory of Nematology, Saga University, Honjo-1, Saga 840-8502, Japan.
Presented at the 4th International Congress of Nematology at Tenerife, Spain, 2002.
E-mail: tyoshiga@cc.saga-u.ac.jp
This paper was edited by A. Skantar.

erick, MD), and (iii) calculated area overlap ([overlapped area between n and n −1 hr/area at n hr] ×100). If nematodes move, the difference in nematode images recorded is large and the percentage of overlapped area is small. A high percentage of area overlapped indicates low nematode activity.

Acquisition of desiccation tolerance on agar: Ten nematodes were placed on 5% agar containing 0.8 M sucrose for 0, 2, 6, 12, 18, 24, and 48 hr, and their posture was recorded at the end of each period. Nematodes were immobilized by carefully transferring them from agar to adhesive tape so as not to disturb the nematode posture on the substrate. Then nematodes were dried in a desiccating vessel containing silica gel (< 20% relative humidity, RH). After desiccation for 24 hr, nematodes were placed in deionized water to rehydrate. The effect of time on agar prior to desiccation on percentage survival was determined.

Scanning electron microscopy: Nematodes were placed individually onto the agar surface using a dental file and incubated for 0, 6, and 24 hr. After incubation, nematodes were individually transferred onto an agar film (dried 5% water agar pad); the nematode on the agar film was then dried for 24 hr in a desiccating vessel containing silica gel (< 20% RH). Dried nematodes were fixed in vapor of 2% OsO_4 in a solution of 2 M sucrose for 3 hr and post-fixed for 12 hr at 4°C in 5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2). After rinsing with the same buffer three times, specimens were dehydrated through a graded series of ethanol solutions, critical-point dried with carbon dioxide, sputter-coated with gold-palladium, and examined using SEM (Topcon ABT-32: JEOL Ltd., Tokyo, Japan) at 15 kV. As a control, nematodes without the drying treatment were fixed with 5% glutaraldehyde (0.1 M phosphate buffer pH 7.2) for 24 hr and post-fixed with 2% OsO_4 for 3 hr.

Survival of artificially uncoiled nematodes: Thirty coiled nematodes on the agar were uncoiled by using a dental file, stuck to adhesive tape to maintain posture, and desiccated at < 20% RH for 24 hr. Thirty coiled nematodes were subjected to the same desiccation treatment for comparison. Nematodes then were placed in deionized water for 24 hr and nematode survival was recorded. All experiments were conducted at 25°C.

Data analysis: Data on coiling and survival after desiccation treatments were analyzed with StatView J-4.5 (Abacus Concepts, Inc., Berkeley, CA). Scheffe's test was used to compare treatments when the analysis of variance showed significant differences among means ($p = 0.05$).

Results

Determination of the conditions for the pre-treatment: In our study, a high level of survival was recorded for the nematodes treated on agar containing 0.8 M sucrose (Fig. 1). Because the amount of solution around nematodes was smallest on 5% agar and nematode behavior was clearly observed, 5% agar containing 0.8 M sucrose was employed for behavioral analysis and pre-treatment of nematodes for desiccation survival.

Behavior analysis using a video recorder: Video-assisted analysis showed that all nematodes became quiescent for a few hours (Figs. 2,3). The quiescent nematodes took a loosely coiled or fishhook-like posture (Fig. 2). After this quiescence, the nematodes gradually resumed activity and then became coiled (Fig. 2), but continued to move within the coiled configuration (Figs. 2,3).

Survival and internal structural integrity after drying: High desiccation survival was acquired for the nematodes that were incubated on agar amended with sucrose for more than 6 hr before exposure to desiccation. Survival rates were 0%, 3%, 73%, and > 92% for the nematodes incubated on agar for 0, 2, 6, and 12 hr, respectively (Fig. 4). The rate of coiling increased with increase of incubation time on agar. While only 20% or 40% were coiled after 6 hr on agar, the corresponding survival rate after desiccation treatment was ≥ 70%

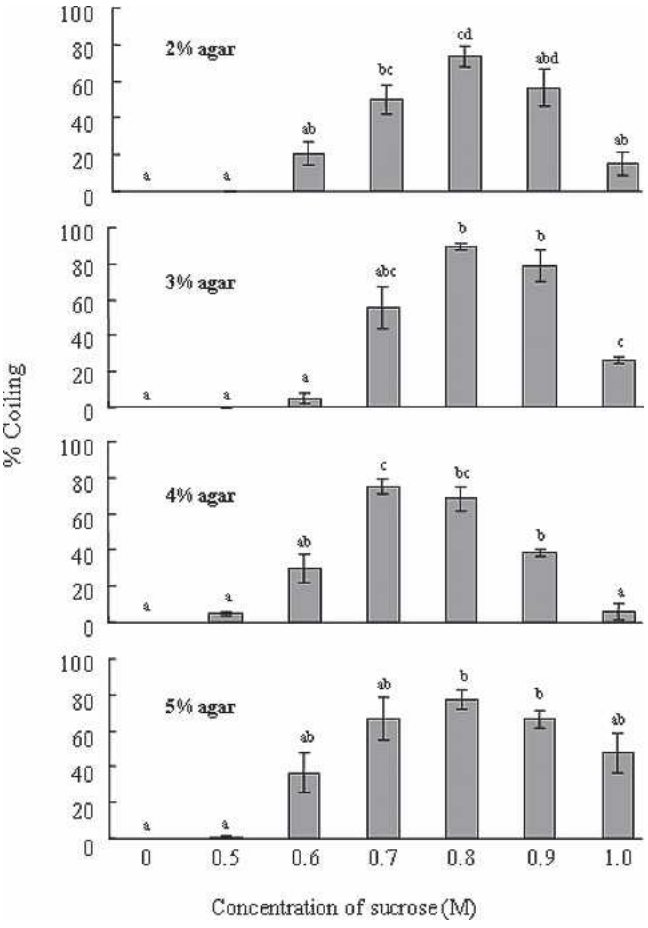

Fig. 1. Effect of sucrose concentration in the agar substrate on the coiling of *Aphelenchus avenae*. Bars (mean ± SE) in the same agar concentration with the same letter are not significantly different (Scheffe's test, $P < 0.05$).

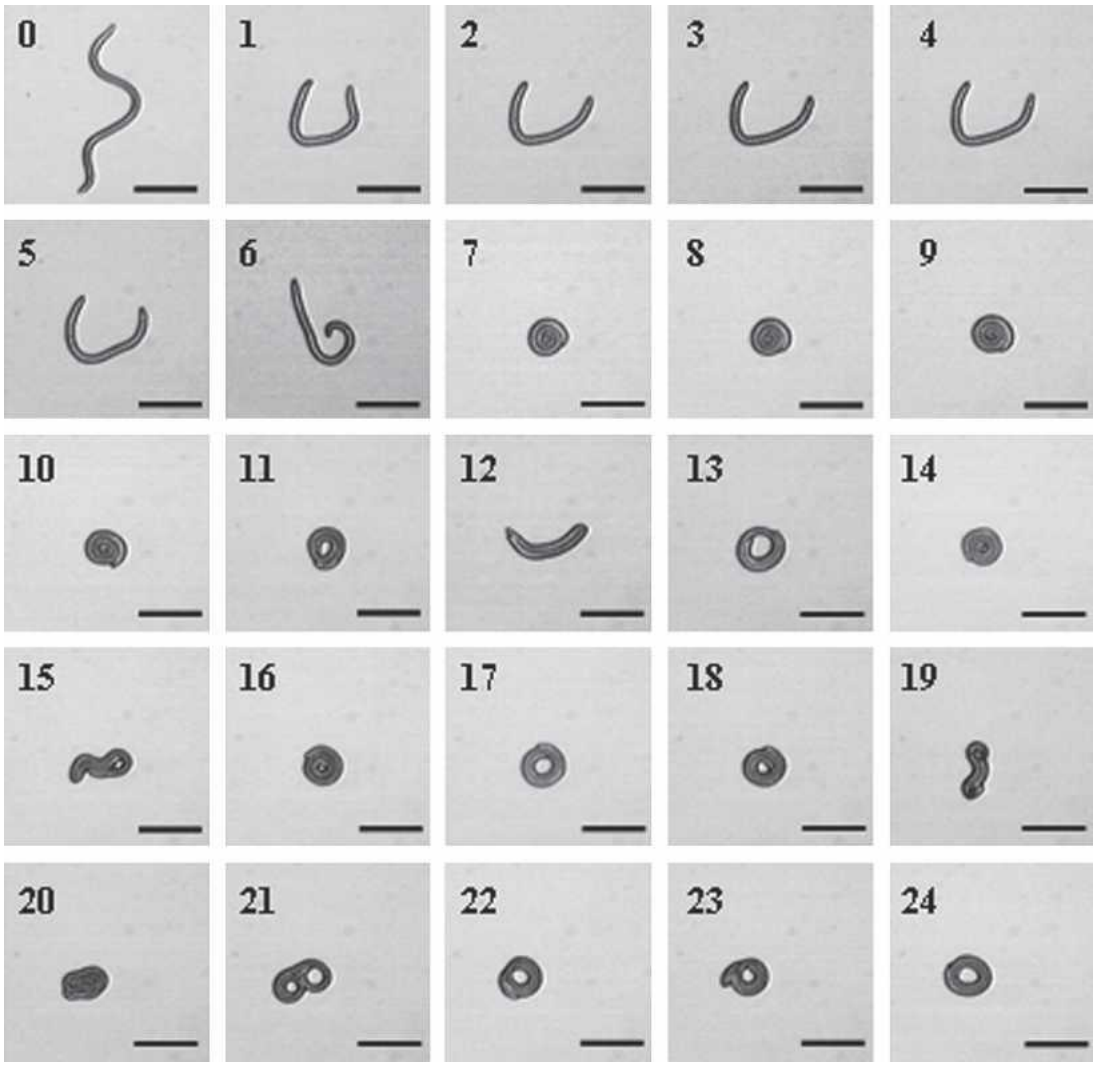

FIG. 2. Postures of *Aphelenchus avenae* young adults on the 5% agar substrate amended with 0.8M sucrose. Numbers indicate the time in hr after being placed on the substrate. The photos were taken of one nematode that was typical of the eight examined in the study. Scale bars indicate 200 μm.

(Fig. 4). Without pre-exposure on the agar, no nematodes survived desiccation and rehydration.

After rehydration, dead nematodes contained extensive vacuolar-like non-refractive regions in their bodies, and the esophagus was not visible (Fig. 5). Nematodes placed on the substrate for only 6 hr also exhibited the vacuole-like non-refractive spaces (Fig. 5), but the esophagus and intestine appeared normal. All nematodes incubated on agar for 24 hr looked normal (Fig. 5).

SEM observation of nematodes: The cuticular surface of nematodes desiccated directly or after incubation on agar for less than 4 hr collapsed and appeared irregular and misshapen (Fig. 5). The body surface of the nematodes desiccated following 6 hr or more of incubation on agar exhibited a regular shrinkage (Fig. 5).

Desiccation survival of artificially uncoiled nematodes: All nematodes pre-exposed for 24 hr or longer on the substrate regained motility following rehydration. There was no difference between the survival rates of coiled nematodes and those of coiled nematodes that had been uncoiled with a dental file. The survival rate of both groups was ≥ 99% (data not shown).

DISCUSSION

The nematodes incubated on the sucrose-amended agar prior to desiccation were subjected to osmobiosis. Both osmobiosis and anhydrobiosis are known to accompany the dehydration process, and osmobiotic nematodes also show desiccation survival. Therefore, this situation is predicted to be similar to events that occur during the course of anhydrobiosis because physiological changes enabling osmobiosis also would favor desiccation survival. Because nematodes use salts in the external medium for normal volume regulation (Wright and Newall, 1980), those incubated in nonelec-

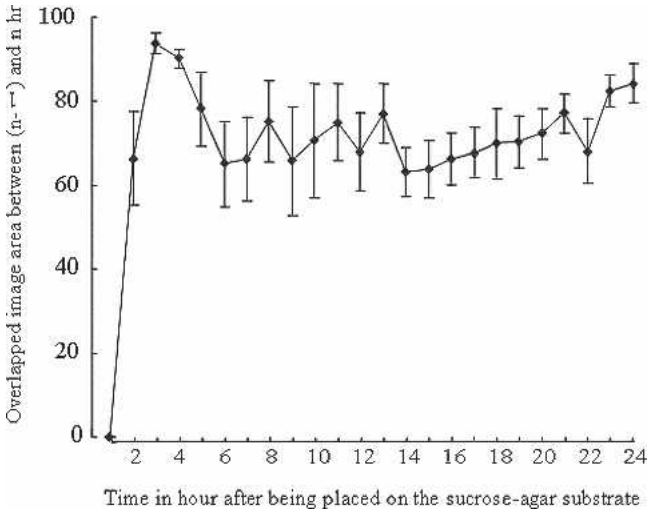

FIG. 3. Overlapped area (%) of *Aphelenchus avenae* with time on the 5% agar substrate amended with 0.8M sucrose. (overlapped image area at [*n* - 1] hr/image area at *n* hr) x 100. If a nematode did not move during 1 hr, then the overlapped area becomes 100%.

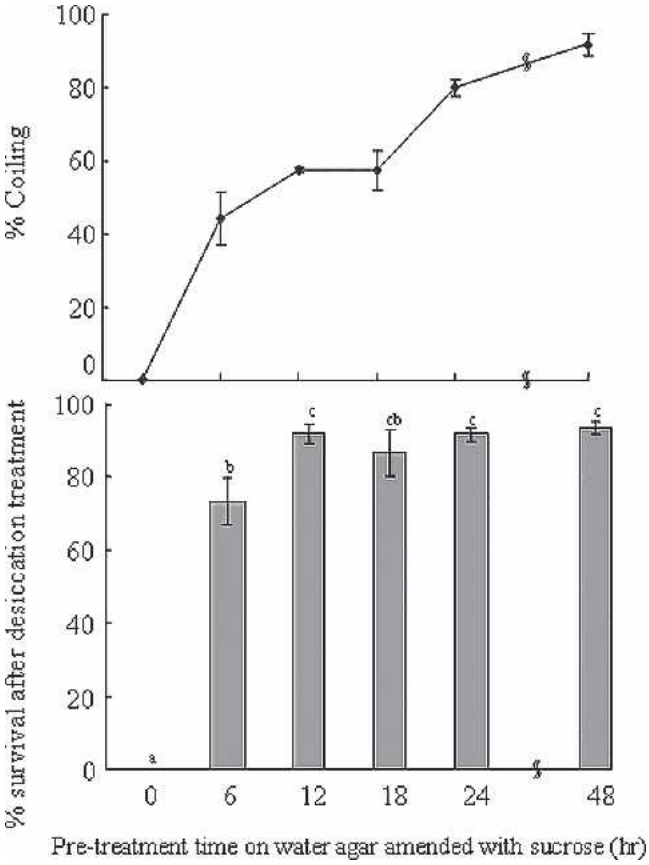

FIG. 4. Effect of pre-treatment time on coiling (A) and survival rate after desiccation treatment (B) of *Aphelenchus avenae*. Bars (mean ± SE) with the same letter are not significantly different (Scheffe's test, $P < 0.05$).

trolytes such as sucrose, without balanced salts, sometimes cannot regulate their volume. In the present study, however, nematodes incubated on the agar amended only with sucrose kept high viability and there was no apparent effect of solution without salts. In *Steinernema carpocapsae*, IJ incubated in 1.2 M sucrose solution for 3 d maintain high viability (Glazer and Salame, 2000). However, agar made with balanced salts may still be necessary for longer incubation of nematodes.

Womersley (1987) divided anhydrobiotic nematodes into two groups, slow-dehydration strategists and fast-dehydration strategists. *Aphelenchus avenae* was classified into the former group, which requires gradual drying to enter anhydrobiosis. A slow rate of water loss may allow the synthesis of trehalose (Womersley, 1978; Crowe et al. 1984; Nicholas and Stewart, 1989; Higa and Womersley, 1993; Womersley and Higa, 1998; Womersley et al., 1998) and the commencement of other protective mechanisms that limit damage during water loss (Wharton, 2002). To identify anhydrobiotic *A. avenae*, a nematode clump was observed after several days of slow and progressive drying (Crowe and Madin, 1975a, 1975b; Crowe et al., 1984). Our study demonstrated that even a single nematode could enter the anhydrobiotic state. With incubation on sucrose-amended agar for $\leq$ 4 hr, all nematodes were killed by desiccation. After rehydration, these dead nematodes appeared physically damaged, with an unclear esophagus and the formation of many vacuole-like non-refractive spaces in the body. Conversely, nematodes that were exposed to osmotic stress on the sucrose-amended agar prior to desiccation appeared to retain normal structural integrity. In the present study, uniform shrinkage was observed for the nematodes placed for 6 hr on the agar, suggesting that the maintenance of structural integrity and tolerance to desiccation was acquired during the 6-hr incubation period of osmotic stress. Therefore, uniform shrinkage associated with regular contraction of the cuticle during osmotic stress appears to be necessary for reanimation.

The funigivorous nematode, *Ditylenchus myceliophagous*, becomes inactive in a straight posture for about 2 wk before swarming onto the glass walls of a culture vessel and then coiling (Womersley and Higa, 1998). In the present study on *A. avenae*, we also observed an inactive phase during the process of osmobiosis. After this inactive phase, nematodes regain motility and thus may be able to regulate osmosis and/or partially dehydrate. Motility suppression caused by osmotic stress may be a necessary prelude to the physiological changes that accompany osmotic stress and facilitate anhydrobiosis.

Survival after desiccation was correlated with coiling. However, survival was also recorded for noncoiled nematodes that were exposed initially to osmotic stress on sucrose-amended agar. Even when coiled nematodes were manually uncoiled, the survival rate was comparable to the rate observed for coiled nematodes. This result supports the interpretation that maintenance of coiling is not critically important for desiccation survival once the biochemical changes requisite for desiccation survival are complete.

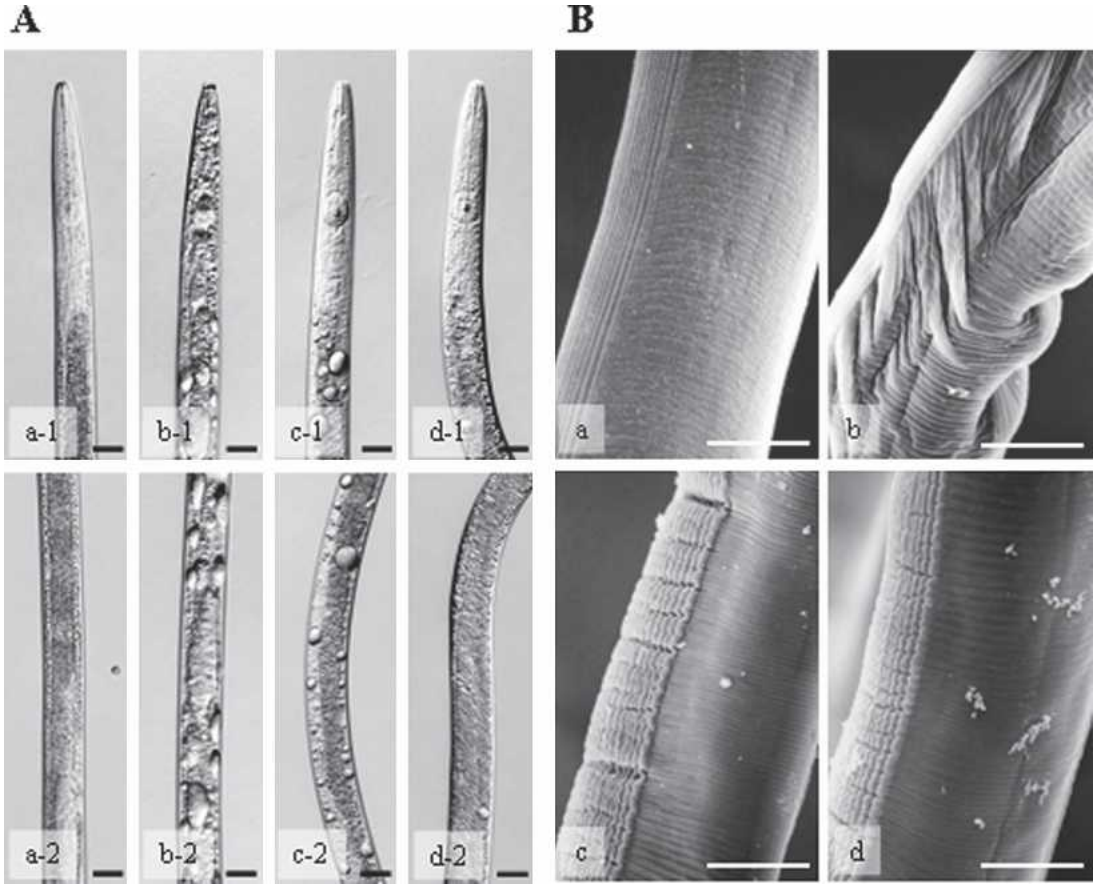

FIG. 5. Interference micrographs (A) and SEM pictures (B) of the lateral profiles of *Aphelenchus avenae* rehydrated after desiccation treatment for 24 hr. a) intact nematode without desiccation treatment, b) direct desiccation treatment without pre-treatment on the substrate, c) desiccation treatment after pre-treatment on the substrate for 6 hr, d) desiccation treatment after pre-treatment on the above sucrose-agar substrate for 24 hr. 1 and 2 indicate the anterior and mid part of the nematodes, respectively. Scale bars indicate 20 μm.

LITERATURE CITED

Browne, J., Tunnacliffe, A., and Burnell, A. 2002. Anhydrobiosis: Plant desiccation gene found in a nematode. Nature 416:38.

Crowe, J. H., Crowe, L. M., and Chapman, D. 1984. Preservation of membranes in anhydrobiotic organisms: The role of trehalose. Science 223:701–703.

Crowe, J. H., Lambert, D. T., and Crowe, L. M. 1978. Ultrastractual freeze fracture studies on anhydrobiotic nematodes. Pp. 23–51 *in* J. H. Crowe and J. S. Clegg, eds. Dry biological systems. New York: Academic Press.

Crowe, J. H., and Madin, K. A. C. 1975a. Anhydrobiosis in nematodes: Evaporative water loss and survival. Journal of Experimental Zoology 193:323–334.

Crowe, J. H., and Madin, K. A. C. 1975b. Anhydobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration. Journal of Experimental Zoology 193:335–342.

Glazer, I., and Salame, L. 2000. Osmotic survival of the entomopathogenic nematode, *Steinernema carpocapsae*. Biological Control 18: 251–257.

Higa, L. M., and Womersley, C. Z. 1993. New insights into the anhydrobiotic phenomenon: The effect of trehalose content and differential rates of evaporative water loss on the survival of *Aphelenchus avenae*. Journal of Experimental Zoology 267:120–129.

Ishibashi, N. 1998. Possible integration of beneficial nematodes and microbial agents for biocontrol of soil pests. Japanese Journal of Nematology 28 (special issue):22–28.

Ishibashi, N., Ali, M. R., and Saramoto, M. 2000. Mass-production of fungivorous nematode, *Aphelenchus avenae* Bastian 1865, on industrial vegetable wastes. Japanese Journal of Nematology 30:8–17.

Nicholas, W. L., and Stewart, A. C. 1989. Experiments on anhydrobiosis in *Acrobeloides nanus* (de Man, 1880) Anderson, 1986. Nematologica 35:489–491.

Wharton, D. A. 2002. Nematode survival strategies. Pp. 389–411 *in* D. L. Lee, ed. The biology of nematodes. New York: Taylor & Francis.

Womersley, C. 1978. A comparison of the rate of drying of four nematode species using a liquid paraffin technique. Annals of Applied Biology 90:401–405.

Womersley, C. 1987. A re-evaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. Pp. 165–173 *in* J. Veech and D. W. Dickson, eds. Vistas on nematology. Hyattsville, MD: Society of Nematologists.

Womersley, C. Z., and Higa, L. M. 1998. Trehalose: Its role in the anhydrobiotic survival of *Ditylenchus myceliophagus*. Nematologica 44: 269–292.

Womersley, C., Thompson, S. N., and Smith L. 1982. Anhydrobiosis in nematodes II: Carbohydrate and lipid analysis in undesiccated nematodes. Journal of Nematology 14:145–153.

Womersley, C., Wharton, D. A., and Higa, L. M. 1998. Survival biology. Pp. 271–302 *in* R. N. Perry and D. J. Wright, eds. The physiology and biochemistry of free-living and plant-parasitic nematodes. Wallingford, UK: CAB International.

Wright, D. J., and Newall, D. R. 1980. Osmotic and ionic regulation in nematodes. Pp. 143–162 *in* B. M. Zuckerman, ed. Nematodes as biological models, vol. 2. New York: Academic Press.