TÜBİTAK

Short Communication

Turk J Zool
2012; 36(4): 562-565
© TÜBİTAK
doi:10.3906/zoo-1103-10

An alternative storage method for entomopathogenic nematodes

Barış GÜLCÜ[1], Selçuk HAZIR[2,*]

[1]Department of Biology, Faculty of Arts and Sciences, Düzce University, 81620 Düzce - TURKEY

[2]Department of Biology, Faculty of Arts and Sciences, Adnan Menderes University, 09010 Aydın - TURKEY

Received: 21.03.2011

Abstract: Tetra Pak containers were evaluated as an alternative to tissue culture flasks for nematode storage. Our data showed that Tetra Pak containers were an excellent alternative to tissue culture flasks for storage of *H. bacteriophora* and will more than likely be useful for other entomopathogenic nematode species.

Key words: *Heterorhabditis bacteriophora*, nematode storage, Tetra Pak containers, tissue culture flasks

Entomopatojenik nematodların saklanmasında alternatif bir yöntem

Özet: Nematodların saklanmasında doku kültür kaplarına alternatif olarak tetra pak kutularının kullanılabilirliği araştırılmıştır. Elde ettiğimiz veriler *H. bacteriophora*'nın saklanmasında tetra pak kutularının doku kültür kaplarına çok iyi bir alternatif olduğunu göstermiştir. Bu kaplar diğer entomopatojenik nematod türlerinin saklanmasında da faydalı olacaktır.

Anahtar sözcükler: *Heterorhabditis bacteriophora*, nematode saklama, tetra pak kutular, doku kültür kapları.

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae are lethal insect parasites of soil insects and have a mutualistic relationship with a given genus of bacterium (*Xenorhabdus* for steinernematids and *Photorhabdus* for heterorhabditids) (Kaya and Gaugler, 1993). The infective juvenile (IJ) of the nematodes is the only free-living stage and has the capability to search for and infect an insect. Because these nematodes are effective biological control agents against a number of soil insect pests, several species are commercially available in many countries (Georgis et al., 2006; Kaya et al., 2006). For commercial production, the nematodes are produced in vivo or in vitro (Friedman, 1990; Gaugler and Han, 2002), and the IJs are usually formulated in a substrate that immobilizes them, enhances longevity, and makes for ease of shipping (Georgis and Kaya, 1998).

In research laboratories, the IJs can be held in liquid nitrogen for long-term storage (Wang and Grewal, 2002), or they can be stored in an aqueous suspension if they are being used for experiments on a regular basis (Kaya and Stock, 1997; Koppenhöfer, 2007). In the latter case, the IJs are usually stored in water at a concentration of 300-5000 IJs/mL in tissue culture flasks laid flat with the caps loosely in place and held at 4-15 °C. Under these conditions, most steinernematids can be stored for 6-12 months before a new batch of nematodes needs to be produced, whereas heterorhabditids can be stored for 3-4 months

* E-mail: shazir@adu.edu.tr

without much loss of activity (Kaya and Stock, 1997). However, it is recommended that IJs less than 4 weeks old be used for experimental purposes.

Tissue culture flasks, routinely used in most research laboratories for keeping aqueous nematode suspensions, are made from polystyrene (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Polystyrene is an aromatic polymer used to make many products including tissue culture flasks, petri dishes, insulation material, and automotive parts. Although polystyrene is a low-cost material, the price of tissue culture flasks is relatively expensive. On the other hand, Tetra Pak containers, which bear the company's name, are made of paper (75%) and polyethylene + aluminum (25%) (http://www.tetrapak.com.tr). Tetra Pak containers are used for the packaging and sale of liquid foods such as fruit juices and milk. These containers are not available through normal research channels, but they can be reused after the product within is consumed.

The aim of our study was to test Tetra Pak containers as an alternative to tissue culture flasks for short-term and long-term IJ storage. We hypothesized that the Tetra Pak containers provide storage conditions for *Heterorhabditis bacteriophora* IJs that are equal to or better than tissue culture flasks.

H. bacteriophora (Turkey isolate 09-48) was cultured in last instar wax moth (*Galleria mellonella*) larvae according to the method of Kaya and Stock (1997). A total of 5 wax worm larvae were placed in a plastic petri dish (100 mm × 15 mm), covered with 2 filter papers, inoculated with 500 IJs at room temperature (23 ± 1 °C), and replicated 9 times. After 3 days, the nematode-killed larvae were transferred to White traps to collect the emerging IJ progeny. IJs emerging from the cadavers were harvested from the White traps, pooled, washed 3 times by sedimentation, diluted to 2000 IJs/mL, and stored at 15 °C for 2-7 days before initiation of the experiments.

Tissue culture flasks (600 mL of Nunclon®) were obtained from NUNC® Company; new flasks were used. Tetra Prisma Aseptic model fruit juice containers (1000 mL) filled with orange or apple (100%) juice were obtained from a supermarket. Each type of container was washed with soap and rinsed 4 times with tap water (70-80 °C) before the experiment. Tissue culture flasks were filled with 100 mL of IJ suspension, whereas Tetra Pak containers were filled with 200 mL to take into consideration the difference between storage container volumes. They were held flat with caps loosely covered and kept at 2 different temperatures (10 and 15 °C) for 11 months. There were 3 replicates for each storage container and the experiment was conducted twice.

The following data sets were obtained monthly for each storage container: 1) IJ survival, 2) infectivity, and 3) penetration efficiency of IJs. For IJ survival, 2 subsamples of 0.5 mL of nematode suspension per storage container were transferred to a 100 mm × 15 mm petri dish, and the survival percentage of the IJs was determined by counting the dead and live nematodes at 40× magnification. Immobile IJs were touched with a fine-wire probe and recorded as dead if they did not respond. All IJs within a subsample were counted and recorded as dead or alive, and the 2 subsamples were averaged for each replicate. The volume of water removed was not replaced to prevent dilution of the nematode suspension.

For IJ infectivity, the wells of 24-well tissue culture plates (Corning Inc., Corning, NY, USA) were filled with 0.5 g of autoclaved and air-dried sand (Gungor et al., 2006). Fifty living IJs, collected with a 10-µL microdispenser (Drummond, Broomall, PA, USA) in 60 µL of distilled water, were pipetted into each well; 1 wax moth larva was added; and the plates were kept at 23 ± 1 °C. Ten larvae were used for each storage container with each larva representing a replicate. The mortality of the wax moth larvae was recorded after 72 h. For penetration efficiency, the cadavers from the infectivity tests were dissected and digested individually in a pepsin solution (Mauleon et al., 1993) to determine the number of nematodes that established in each nematode-killed larva. The nematodes were counted using a dissecting microscope at 40× magnification.

Data were analyzed using the general linear model (GLM). Means were compared at the $P = 0.05$ level and Tukey's test was used to separate means (SPSS, 2004). Arcsine transformation was carried out on survival and larval mortality before statistical analysis.

The results showed that the average survival for both storage containers was higher than 90% during the 11-month duration at 10 and 15 °C, respectively.

At 10 °C, IJ survival was 97.4% (95.5%-98.8%) and 95.4% (91.0%-8.3%) in Tetra Pak containers and tissue culture flasks, respectively. Statistically more IJs survived in Tetra Pak containers than in tissue culture flasks ($F = 25.96$; $df = 1, 110$; $P < 0.000$) (Figure 1A). There were significant differences in survival percentage between the 2 containers in months 1, 5, 6, and 11 ($P < 0.05$) (Figure 1A). At 15 °C, the mean survival for the IJs was 96.5% (93.0%-99.8%) in Tetra Pak containers and 92.8% (74.7%-99.0%) in tissue culture flasks at the end of 11 months. The difference between storage boxes was significant; the percentage of IJ survival was higher in Tetra Pak containers than in tissue culture flasks ($F = 23.91$; $df = 1, 110$; $P < 0.000$) (Figure 1B). When the survival percentage of IJs between the 2 containers was compared for each month, significant differences were observed between 2 the containers only in months 9 and 11 ($P < 0.05$) (Figure 1B). When all survival data obtained at 10 and 15 °C were combined and analyzed together, the survival percentage of the IJs in Tetra Pak containers was statistically higher than in tissue culture flasks ($F = 48.60$; $df = 1, 220$; $P < 0.000$).

At 10 °C, the mortality of wax worm larvae caused by IJs stored in different containers was 98.2% (93.3%-100%) for Tetra Pak containers and 98.9% (95.0%-100%) for tissue culture flasks. No significant difference was observed between the 2 types of containers ($F = 1.32$; $df = 1, 110$; $P < 0.253$). When larval mortality was compared for each month, a significant difference was observed only in month 8 ($P < 0.05$) (data not shown). The IJs stored in Tetra Pak containers and tissue culture flasks at 15 °C caused 96.8% (90%-100%) and 96.5% (76.7%-100%) larval mortality, respectively. No significant difference was observed between the 2 containers ($F = 0$; $df = 1, 110$; $P = 0.987$). When larval mortality was compared for each month, a statistical difference between containers was observed only in month 11 ($P < 0.05$) (data not shown). The combined data obtained from 10 and 15 °C showed no significant difference between Tetra Pak containers and tissue culture flasks ($F = 0.52$; $df = 1, 220$; $P = 0.471$).

The mean number of IJs established in the cadavers at 10 °C was 17.8 (9.7-29.7 °C) for Tetra Pak containers and 16.8 °C (4.2-23.8 °C) for tissue culture flasks. There was no significant difference between the 2 containers ($F = 3.08$; $df = 1, 416$; $P = 0.08$). When established percentages between the 2 containers were compared for each month, significant differences were observed in months 1, 4, 9, 10, and 11 ($P < 0.05$) (Figure 2A). At 15 °C, the average number of IJs established in the cadavers was 17.6 (7.2-23.4) and 17.3 (9.8-25.1) for Tetra Pak containers and tissue culture flasks, respectively. No significant difference was observed between the 2 containers ($F = 0.20$; $df = 1, 408$; $P = 0.65$). When the number of IJs that had penetrated into the larvae was compared for each month, statistical differences were observed in months 1, 3, and 7 ($P < 0.05$) (Figure 2B). When the establishment data obtained from 10 and 15 °C were combined, no significant difference was observed between the 2 storage containers ($F = 2.27$; $df = 1, 824$; $P = 0.132$).

Our data showed that Tetra Pak containers were excellent alternatives to tissue culture flasks for storage of *H. bacteriophora* and will more than likely be useful for other entomopathogenic nematode

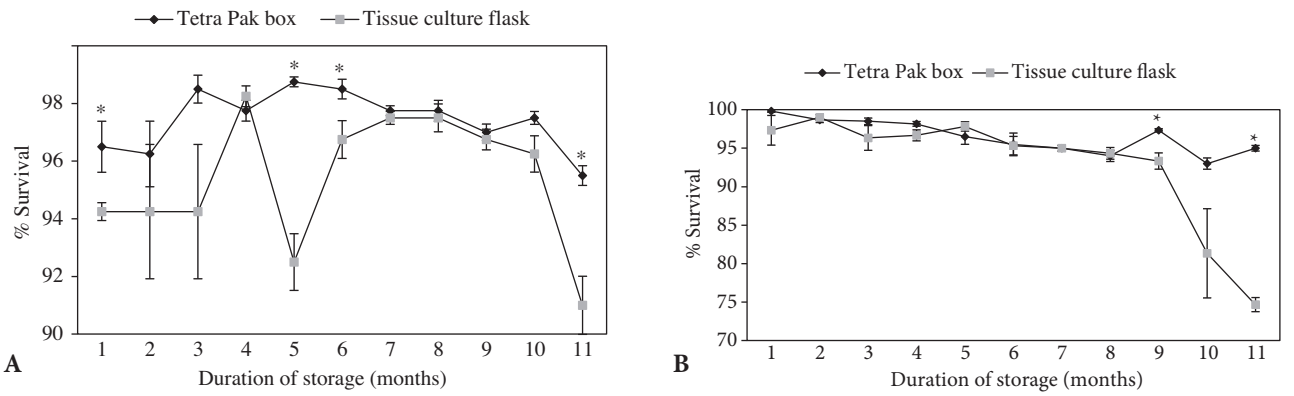

Figure 1. Survival (%) of IJs in Tetra Pak containers and tissue culture flasks at 10 °C (A) and 15 °C (B); * indicates significant difference.

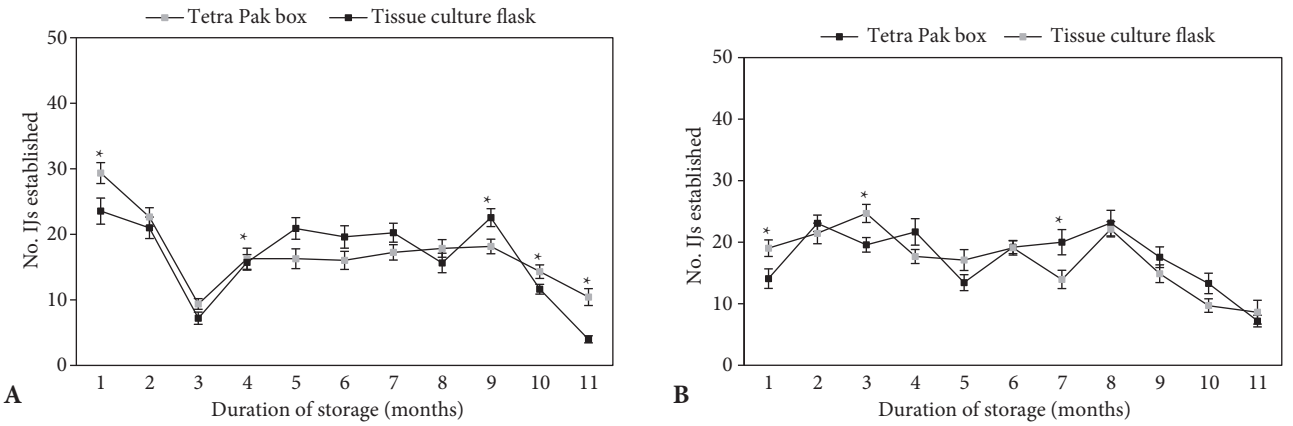

Figure 2. Number of IJs penetrated into larvae at 10 °C (A) and 15 °C (B); * indicates significant difference.

species. The advantages of Tetra Paks are: 1) the rectangular shape allows the container to be laid flat, providing more exposure of the IJ suspension to aeration, similar to tissue culture flasks; 2) they are inexpensive; 3) they are reusable; 4) soap or bleach can be used for sterilization; 5) for long-term storage of IJs, they are comparable to or better than tissue culture flasks. The disadvantages of Tetra Paks are: 1) it is not possible to check the IJ suspension directly; 2) they are available only through commercial food outlets. Tetra Pak containers will be useful for scientists who have a limited budget for purchasing tissue culture flasks. They should be readily available in most countries, can be used multiple times after the liquid food content has been consumed, and can be washed in hot water.

Acknowledgments

We would like to thank Dr Harry K. Kaya for editing the manuscript, Dr İbrahim Çakmak for his excellent assistance with the statistical analyses, and Ms Derya Aşıcı and Mr Karthik Raja Ramalingam for their assistance in the experiments.

References

Friedman, M.J. 1990. Commercial production and development. In: Entomopathogenic Nematodes in Biological Control (Eds. R. Gaugler and H.K. Kaya). CRC Press, Boca Raton, Florida, USA, pp. 153-172.

Gaugler, R. and Han, R. 2002. Production technology. In: Entomopathogenic Nematology (Ed. R. Gaugler). CABI Publishing, Wallingford, UK, pp. 289-310.

Georgis, R. and Kaya, H.K. 1998. Advances in entomopathogenic nematode formulation. In: Formulation of Microbial Biopesticides, Beneficial Microorganisms Nematodes and Seed Treatments (Ed. H.D. Burges). Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 289-308.

Georgis, R., Koppenhöfer, A.M., Lacey, L.A., Belair, G., Duncan, L.W., Grewal, P.S., Samish, M., Tan, L., Torr, P. and Van Tol, R.W.H.M. 2006. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control 38: 103-123.

Gungor, D.S., Keskin, N. and Hazir, S. 2006. Ecological characterization of *Steinernema anatoliense* (Rhabditidae: Steinernematidae). J. Invertebr. Pathol. 92: 39-44.

Kaya, H.K., Aguillera, M.M., Alumai, A., Choo, H.Y., De la Torre, M., Fodor, A., Ganguly, S., Hazır, S., Lakatos, T., Pye, A., Wilson, M., Yamanaka, S., Yang, H. and Ehlers, R.-U. 2006. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biol. Control 38: 134-155.

Kaya, H.K. and Gaugler, R. 1993. Entomopathogenic nematodes. An. Rev. Entomol. 38: 181-206.

Kaya, H.K. and Stock, S.P. 1997. Techniques in insect nematology. In: Manual of Techniques in Insect Pathology (Ed. L. Lacey). Academic Press, San Diego, California, USA, pp. 281-324.

Koppenhöfer, A.M. 2007. Nematodes. In: Field Manual of Techniques in Invertebrate Pathology (Eds. L.A. Lacey and H.K. Kaya). Springer, Germany, pp. 249-264.

Mauleon, H., Briand, S., Laumond, C. and Bonifassi, E. 1993. Utilisation d'enzyme digestives pour l'e ´tude du parasitisme des Steinernematidae et Heterorhabditidae envers les larves d'insectes. Fund. Appl. Nematol. 16: 185-186.

SPSS. 2004. SPSS v.13.0 for Windows. SPSS Inc., Chicago, IL, USA.

Wang, X. and Grewal, P.S. 2002. Rapid genetic deterioration of environmental tolerance and reproductive potential of an entomopathogenic nematode during laboratory maintenance. Biol. Control 23: 71-78.