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Orijinal araştırma (Original article)

Quality control of *in vitro* produced *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) strains isolated from Turkey[1]

Türkiye'den izole edilen ve *in vitro* olarak üretilen *Heterorhabditis bacteriophora* izolatlarının kalite kontrolü

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Summary

H.b. 6 (Antalya), H.b. 17 (Kırklareli), H.b. 10 (Adana), H.b. HIZ (İzmir), H.b. HSU (Şanlıurfa) and H.b. 876 (Çanakkale) strains of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae), were produced with *in vivo* and *in vitro* mass production techniques. Effectiveness and reproduction capacities of the nematodes obtained from *in vivo* and *in vitro* cultures were compared in order to determinethe quality of *in vitro* productionThe difference in infectivity of the strains produced with *in vivo* and *in vitro* techniques was not statistically significant, except H.b. 6, which was lower in *in vitro*. Some *in vitro* produced strains had significantly higher reproduction capacity than *in vivo* strains but some strains were not significantly different as *in vivo* produced strains. It was generally detected that the *in vitro* cultured Turkish strains maintained their prerequisites on effectiveness and reproduction capacities as their quality control standards.

Key words: *Heterorhabditis bacteriophora*, *in vivo*, *in vitro*, mass production, effectiveness, reproduction capacity.

Özet

Heterorhabditis bacteriophora Poinar, 1976 (Rhabditida: Heterorhabditidae) türünün H.b. 6 (Antalya), H.b. 17 (Kırklareli), H.b. 10 (Adana), H.b. HIZ (İzmir), H.b. HSU (Şanlıurfa) ve H.b. 876 (Çanakkale) ırkları *in vivo* ve *in vitro* kitle üretim teknikleri kullanılarak üretilmiş ve üretilen kültürler üzerinde yapılan etkinlik ve üreme kapasitesi denemelerinden elde edilen sonuçların karşılaştırılması ile *in vitro* olarak üretilen ırkların kalite kontrolleri yapılmıştır. *In vivo* ve *in vitro* olarak üretilen ırkların üzerinde yapılan etkinlik denemelerinin sonuçları H.b. 6 ırkı haricinde diğer tüm ırklarda etkinliğin istatistiksel olarak aynı olduğunu göstermiştir. Üreme kapasitesi denemelerinde ise *in vitro* olarak üretilen bazı ırkların *in vivo* olarak üretilen ırklardan istatistiksel olarak daha üstün olduğu, geri kalan ırkların ise *in vivo* olarak üretilen ırklar ile istatistiksel olarak aynı üreme kapasitesine sahip olduğu belirlenmiştir. Genel olarak, *in vitro* yöntemle üretilen Türk ırklarının, kalite kontrol unsurlarından en önemli ikisi olan mevcut etkinlik ve üreme kapasitesi özelliklerini korudukları tespit edilmiştir.

Anahtar sözcükler: *Heterorhabditis bacteriophora*, *in vivo*, *in vitro*, kitle üretim, etkinlik, üreme kapasitesi.

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Introduction

There are very few environmentally safe insecticides with low toxicity and short-term persistence, low mobility in the soil to prevent ground-water contamination and limited effects on non-target organisms. These characteristics have reduced the probability of obtaining new insecticidal compounds registered, especially for soil application. Therefore, entomopathogenic nematodes (EPNs) can be used as an alternative to control soil insect pests and even have substantial advantages over chemical compounds.

Entomopathogenic nematodes have a high control potential against various insect pests, which are economically important (Klein, 1990; Shapiro-Ilan et al., 2002). Nowadays, *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) Poinar, 1976 is used in biological control to overcome agricultural insect pest species belonging to a number of orders, especially Lepidoptera, Coleoptera, Diptera and Thysanoptera (Gaugler & Kaya, 1990; Ehlers & Peters, 1994; Peters, 1995; Ehlers, 1996).

These nematodes have a relationship with specific bacterium species.When Glaser (1931) reported the *in vitro* culture of *Steinernema glaseri* (Rhabditida: Steinernematidae), scientists working on EPNs did not know of the existence of the symbiotic bacteria. A significant breakthrough in biotechnology of EPNs was the discovery of a symbiotic bacterium by Poinar and Thomas (1965). This symbiotic relationship is very important for the production of EPNs. Although *Steinernema* spp., can reproduce and kill insect hosts without the symbiotic bacteria, *Heterorhabditis* spp. have an obligate relationship with bacteria so they cannot complete their life cycles and cannot kill to their hosts without the related bacterium (Glaser, 1940; Poinar & Thomas, 1966). Therefore, the symbiotic bacteria play a key role in producing *Heterorhabditis* spp. *Heterorhabditis bacteriophora* is symbiotically associated with the bacterium *Photorhabdus luminescens* that is carried in the intestine of the third-stage infective dauer juvenile (Thomas & Poinar, 1979; Poinar, 1990; Boemare et al., 1993, Ciche et al., 2006). The dauer juvenile (DJ) is a free-living and non-feeding stage, which enters the host via natural openings such as the mouth, anus, spiracles, or occasionally directly through the cuticle (Poinar, 1990; Bedding & Molyneux, 1982; Peters & Ehlers, 1997). Immediately after dauer juveniles enter into the haemocoel of insects, they release their symbiotic bacterium that kills the host by means of septicemia within 24-48 h after penetration (Akhurst, 1983; Kaya & Gaugler, 1993; Glazer & Lewis, 2000; Dowds & Peters, 2002). The bacteria grow in the host and produce suitable conditions for nematode development and reproduction. Dauer juveniles feed on bacterium and host tissues and then develop to self-fertile hermaphrodites, which are the female phenotype (Poinar, 1975). In addition these individuals give rise to a second generation consisting of amphimictic males, females and juvenile stages (Poinar, 1990; Dix et al., 1992; Strauch et al., 1994).

Entomopathogenic nematodes can be cultured by using *in vivo* or *in vitro* techniques (Friedman, 1990; Ehlers, 2001; Gaugler & Han, 2002; Shapiro-Ilan & Gaugler, 2002). In general, *in vivo* production has been used in the White trap method (White, 1927). In this method, highly susceptible Galleria *mellonella* (Lepidoptera: Pyralidae) last instar larvae are inoculated with DJs in a moist environment. The other technique to produce EPN is *in vitro* on solid or liquid cultures. Today, a few companies still produce *in vitro* solid and liquid cultures of EPNs on a large scale.

Effective quality control is essential for the success of EPNs as biological control agents. The quality of *in vitro* produced EPNs has been an important issue for biotechnological producition and users. Quality indicators of EPNs include especially the infectivity of the DJs to host insects and reproductive capability in a host. The aim of this study was to compare the efficacy and reproductive capacities of *in vivo* and *in vitro* produced *H. bacteriophora* strains isolated from different regions of Turkey, in order to determine the quality of *in vitro* produced *H. bacteriophora* strains.

Material and Methods

Nematode strains and insects

Six *H. bacteriophora* strains from different regions of Turkey were used in the experiments. *H. bacteriophora* H.b. 6, H.b. 17, H.b. 10, H.b. HIZ, H.b. HSU and H.b. 876 were isolated from Antalya, Kırklareli, Adana, İzmir, Şanlıurfa and Çanakkale Provinces, respectively. All strains were identified by the molecular technique (PCR-RFLP) (unpublished data).

The greater wax moth larvae were reared as the host insect in 1000 ml glass containers at 30±2ºC on an artificial medium, according to Wiesner (1993) and weights of the *G. mellonella* larvae used in the experiments were 150±10 mg.

In vivo production

All of *H. bacteriophora* strains were reared on last instar larvae of *G. mellonella*. The larvae were inoculated with each nematode strain at the rate of 100 DJs per larva in each well of the 24 well plate, according to Susurluk et al. (2001, 2003) and incubated at 25 ºC for 4 days. Six larvae were used per strain. After incubation, each dead larva was transferred to a White trap (White 1927). When the DJs emerged, they were harvested and stored in Ringer's solution (NaCl, 9 g; KCl, 0.42 g; $CaCl_2$ x $2H_2O$, 0.37 g; $NaHCO_3$, 0.2 g and distilled water, 1000 ml) at 4 ºC (Kaya & Stock, 1997).

In vitro production

Bacteria isolation: Five *G. mellonella* last instar larvae were buried in moist sand (particle size: 300-400 µm) (10% relative humidity) in 24 well plates (each well 1.6 cm diameter, 1.8 cm high). Approximately 100 DJs per larva were applied and incubated at 25ºC for 2 days. Then, the well plates were opened and all larvae were washed with ringer solutions. To isolate the bacteria, the treated larvae were sterilized with 70% ethanol for 5 minutes. After sterilization, each larva was injected with a sterile needle and one drop of haemolymph was taken in aid of inoculation loop and transferred on NBTA (Standard-I-Nutrient Agar, 37.0 g; Bromthymolblue, 25.0 mg; distilled water, 1 l; 2,3,5 Triphenyl-tetracoliumchloride solution, 4.0 ml) . The bacteria on the NBTA were incubated at 25ºC in the dark. After colonization by bacteria, one colony was transferred to YS-medium (yeast extract, 5.0g; NaCl, 5.0 g; $NH_4H_2PO_4$, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4x7H_2O$, 0.2 g and distilled water, 1 l) and incubated for 1 or 2 days at 25ºC in an orbital shaker at 200 rpm in the dark (Akhurst, 1980; Ehlers et al., 1998).

Egg isolation: Five *G. mellonella* last instar larvae were put with moist sand (10% w/v moisture) in each well of the plates described above. Approximately 100 DJs were added for each larva and incubated at 25ºC for 4 days. After the incubation, infected larvae were dissected and hermaphrodites were collected. The collected hermaphrodites were transferred to a glass tube contained pieces of razor blades and ringer solutions. The glass tube was treated with vortex for a few minutes to disrupt the hermaphrodites and release their eggst. After the eggs were released, the egg - ringer suspension was centrifuged for 2 min at 3000 rpm to sediment the eggs. They were removed and the sterilization solution was added (12% NaOCl, 0.5 ml; 4 M NaOH, 1.5 ml; distilled water, 10 ml) to the eggs until the supernatant remained clean. The sterilization solution was removed and replaced with YS-medium understerile conditions and repeated centrifugation at the same as bacterial isolation. Finally, the eggs were transferred to sterile well plates filled with YS-medium and incubated at 25 ºC for 2 days (Lunau et al., 1993).

Preparation of monoxenic cultures: Two days after egg isolation, the YS - medium was inoculated with the bacterium *Photorhabdus* sp. and incubated in a shaker at 200 rpm and 25 ºC in the dark for 24 h. After the bacteria grew in the YS, WOUTS (Bacto® Nutrient Broth, 16.0 g; Bacto® Agar, 12.0 g; Corn-oil or sunflower-oil, 5.0 g; distilled water, 1 l), agar was inoculated with 2 drops of the bacterial suspension and approximately 50-100 J1 (first instar juvenile) under sterile conditions. The plates were then closed with parafilm and incubated at 25 ºC (Lunau et al., 1993; Kaya & Stock, 1997; Ehlers et al., 1998).

Determination of efficacy of the *in vivo*- and *in vitro*- produced *Heterorhabditis bacteriophora* strains

Galleria mellonella last instars larvae were also used for the experiments as a sensitive test insect. The experiments were performed in the 24 well plates described above. One larva of *G. mellonella* was placed in the bottom of each well and covered with sterile sand, which had a water content of 3% (Susurluk et al., 2001). The efficacy of each nematode isolate was tested at the rate of 50 DJs per larva, according to protocol of single dose infectivity (Peters, 2005). DJs were applied in 300 µl ringer solution on the surface of sand in each well. The wells were closed and sealed with parafilm and then incubated at 25 ºC for 4 days. Each experiment was replicated three times, with twenty larvae each replication. The larvae were removed from the sand after incubation and all insects killed by the nematode were counted to determine effectiveness of each strain. The control wells were treated with distilled water only (Susurluk, 2008).

Determination of reproduction of the *in vivo*- and *in vitro*-produced *Heterorhabditis bacteriophora* strains

Reproduction capacity of all strains used in the present study was assessed on *G. mellonella* last instar larvae. These experiments were also performed in the 24 well plates. One larva of *G. mellonella* was put on the bottom of each well filled with sterile sand having a water content of 3%. The reproduction capacity of each nematode isolate was tested at the rate of 50 DJs per larva. The plates were incubated at 25 º C for 4 days. The insects were removed from the sand after incubation and each cadaver was rinsed and put on a 90 mm Petri dish containing 10 ml ringer solution, according to White (1927). All of the traps were incubated at 25 ºC in the dark for 10 days. All emerged DJs from a single host insect were recovered over a period of 10 days and stored in a 50 ml flask (Boff et al., 2000). The content of each flask (nematode suspension from individual cadavers) was mixed thoroughly using air bubbles. Eight samples of 10 µl from each suspension were examined under a stereomicroscope and the total number of DJs per cadaver was counted. This treatment was replicated five times. Five larvae were used in each replication (Susurluk, 2006).

Statistical analyses

The data for reproduction and percentage of infectivity were normalized by log transformation (Log_{10}) and then subjected to analysis of variance (breakdown one way ANOVA),followed by a Least Significant Difference (LSD) test as post-hoc comparisons using JMP® 7.0. The minimum level of significance was taken as $p<0.05$.

Results

Determination of the effectiveness of *Heterorhabditis bacteriophora* strains

Differences in effectiveness of the *in vivo* and *in vitro* produced strains were not statistically significant, except for H.b. 6. It was detected that *in vivo* produced H.b. 6 was significantly more effective than that produced *in vitro*. Insect mortality varied from nearly 76% to 100 % in this experiment. *In vivo* and *in vitro* produced H.b. 876 caused 100% mortality. There was no mortality in the control (Table 1).

Table 1. Mortality (%) of *Galleria mellonella* larvae caused by *in vivo* and *in vitro* produced *Heterorhabditis bacteriophora* strains*

H. bacteriophora strains	Insect mortality (%) caused by strains (Mean ± SE)		Statistical data
	In vivo produced	*In vitro* produced	
H.b. 6	100±0.0 a	86.67±1.8 b	F=13.57; df=1, 4; P=0.021
H.b. 17	96.67±1.8 a	86.67±4.8 a	F=1.14; df=1, 4; P=0.346
H.b. 10	96.67±1.8 a	83.33±3.6 a	F=2.87; df=1, 4; P=0.165
H.b. HIZ	86.67±1.8 a	93.33±1.8 a	F=1.99; df=1, 4; P=0.230
H.b. HSU	93.33±1.8 a	76.67±3.6 a	F=4.98; df=1, 4; P=0.090
H.b. 876	100±0.0 a	90±2.24 a	F=2.89; df=1, 4; P=0.165

* For each strain, means in the same row followed by the same letter are not statistically different.

Determination of reproduction of *H. bacteriophora* strains

The results of the reproductive potential experiment indicated that differences in the number of offspring of H.b.6, H.b. 17 and H.b. 876 strains between *in vivo* and *in vitro* production were statistically significant, so *in vivo* produced strains had statistically lower production capability than *in vitro* produced of those did. However, differences in reproduction capability of the other strains (H.b 10, H.b. HIZ and H.b. HSU) between *in vivo* and *in vitro* production were not significantly different (Table 2).

Table 2. Reproduction capacities of *Heterorhabditis bacteriophroa* strains produced in *vivo* and *in vitro* conditions*

H. bacteriophora strains	Nematode reproduction (Mean ± SE)		Statistical data
	In vitro production	*In vivo* production	
H.b. 6	116861±6109 a	6401±428 b	F=1607.75; df=1, 8; P=0.0001
H.b. 17	69093±8556 a	47411±3597 b	F=6.50; df=1, 8; P=0.0342
H.b. 10	82558±10203 a	75306±13895 a	F=0.31; df=1, 8; P=0.5916
H.b. HIZ	88656±8556 a	68122±3597 a	F=1.62; df=1, 8; P=0.2379
H.b. HSU	46669±9339 a	66290±7559 a	F=2.69; df=1, 8; P=0.1394
H.b. 876	36477±8838 a	11736±2087 b	F=14.82; df=1, 8; P=0.0049

* For each strain, means in the same row followed by the same letter are not statistically different.

Discussion

Quality control is one of the key issues in the recent industrial development. With living biological products, which are intrinsically variable in performance, quality control will never provide the same product stability as with technical products. Quality of EPNs can be defined as their performance against target pests, persistence and reproductive potentials in practice (Jung, 1999).

In the mass production of EPNs for use in biological control or integrated pest management, during their producing systems as *in vitro* conditions or their applications in the field, the objective is to obtain EPNs of good quality. The questions are: What is good? How do we define quality? How do we maintain a high level of quality? Today, producers and users of EPNs are concerned with having good quality nematode material for use in biological control. It has been expected that *in vitro* produced EPNs would be better than *in vivo* produced or be same as from *in vivo* culture. If it is not, the nematode species must be changed or the *in vitro* production should be stopped since the produced culture is not good quality for users and producers in biocontrol. Although some EPNs can maintain some their characteristics for a long time, many species can quickly lose their abilities (Susurluk and Ehlers, 2008; Jung, 1999). Variability among the EPNs depends on their geographic regions of origin and their lipid reserves (Mukuka et al., 2010; Patel et al., 1997). In order to understand *in vitro* produced EPNs, some characters of *in vivo* and *in vitro* produced species should be compared with each other. The most important features of EPNs are their effectiveness and reproduction capabilities because they play key roles in the success of biological control.

In this study, one dose (50 DJs per larva) was used, because probable differences of their effectiveness cannot be detected at lower and higher numbers than 50 DJs/larva. Moreover, due to the fact that this is a comparative study, one dose is enough for the infectivity experiment, according to Peters (2005) who used single dose infectivity test with larvae of the lesser mealworm, *Tenebrio molitor* (Col.: Tenebrionidae). The current study indicated that *H. bacteriophora* strains produced both *in vivo* and *in vitro* techniques caused high mortality (from 76 to 100%) on *G. mellonella* larvae. Results also showed that there is no difference in the effectiveness of *in vivo* and *in vitro* produced strains (except H.b.6 strain). In other words, *in vitro* produced strains kept their effectiveness in the present study. This result is not accordance with Susurluk and Ehlers, 2008. Their results indicated that the infectivity and reproduction ability of *in vivo* produced *H. bacteriophora* isolated in Germany were significantly higher than for *in vitro* produced culture. This may possibly be explained by the difference of origin of the strains.

The reproduction abilities of *in vitro* produced *H. bacteriophora* strains were statistically higher than those of *in vivo* produced strains or not different to *in vitro* produced strains in the present study. Considering their reproduction capabilities, *in vitro* culture of the strains was not negatively affected by *in vitro* mass production procedures. Similar results were found by Anbesse et al. (2013); they stated that the desiccation tolerance of *H. bacteriophora* improved only with selection pressure in both *in vivo* and *in vitro.* Moreover, heat tolerance was successfully increased both *in vivo* and *in vitro* propagated *H. bacteriophora*. According to their results, besides infectivity and reproduction features of *H. bacteriophora* strains, heat and desiccation tolerances were not negatively affected by *in vitro* mass production. Moreover, recent studies on the *in vitro* production of *H. bacteriophora* have been carried out in order to improve the media for *in vitro* production so that *in vivo* propagated *H. bacteriophora* can also maintain their biological features in *in vitro* production (Inman et al., 2012). These results are very encouraging for mass production techniques. By this means, it is expected to get improved success of sustainable biological control with EPNs, because of their high reproduction also in treated fields.

Susurluk and Ehlers (2008) stated that significant differences were recorded between *in-vitro* produced and recovered populations one year after the application of one-week-old fermented *H. bacteriophora* regardinginfectivity and persistence. However, in reproduction capacities of the species, there was no significant difference between them at the dose of 50 DJs per *G. mellonella* larva. This result is similar to that of the present study. There are very few studies about comparisons of reproduction

capacities between *in vivo* and *in vitro* mass cultured EPNs. However, there have been many studies on the relationship between application doses and reproduction capacities (Selvan et al., 1993; Boff et al., 2000; Susurluk, 2006).

The present study showed that Turkish strains of *H. bacteriophora* cultured *in vitro* can sustain their effectiveness and reproductive capabilities in the framework of quality control. Therefore, it appears that this advantage supports the production of the Turkish *H. bacteriophora* strains on a large scale with *in vitro* production systems with a low risk of quality problems.

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