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Effects of several ingredients on *in vitro* mass production of *Heterorhabditis bacteriophora* HBH strain

Bazı maddelerin *Heterorhabditis bacteriophora* HBH ırkının *in vitro* kitle üretimi üzerine etkileri

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ABSTRACT

Biological control is a great alternative to chemical pesticides in agriculture. Entomopathogenic nematodes (EPNs) are environmentally safe biocontrol agents that are used mostly against soil-dwelling insect pests. EPNs have been commercially mass produced for several decades. Mass production is generally done *in vitro* using solid and liquid cultures. After mass production, they are formulated with filler compounds and sold as commercial products. Most common substances for *in vitro* production are nutrient broth, yeast extract, soyflour and different plant oils for protein and lipid source. To increase the production yield of the mass culture, several compounds can be added to the media. In this study; lecithin, egg yolk and the both together were used in solid medium and effects of these additional compounds were examined on mass production of hybrid *Heterorhabditis bacteriophora* HBH strain, which was obtained from hybridization of Turkish isolates. Infective juveniles (IJs) of the strain were targeted as mass production yield criteria. According to the results, all treatments showed statistically better yields compared to control, and lecithin was statistically the best treatment among others. Thus, lecithin could be used as an additional compound in *in vitro* solid mass production media.

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ÖZ

Biyolojik mücadele, tarımda kimyasal pestisitlere karşı önemli bir alternatif yöntemdir. Entomopatojen nematodlar (EPN) çoğunlukla toprak kaynaklı zararlı böceklere karşı kullanılan doğa dostu biyolojik mücadele ajanlarıdır. Uzun yıllardan beri EPN'ler kitle halinde ticari olarak üretilmektedirler. Kitle üretimi genellik *in vitro* katı ve sıvı kültürler içerisinde yapılmaktadır. Kitle üretiminden sonra EPN'ler çeşitli dolgu maddeleri ile formüle edilmekte ve ticari ürün olarak satılmaktadır. Kitle üretiminde kullanılan en yaygın maddeler arasında protein ve yağ kaynağı olarak Nutrient Broth, Yeast ekstraktı, soya unu ve çeşitli bitkisel yağlar bulunmaktadır. Kitle üretiminin verimini artırmak için bu üretim ortamı içerisinde farklı maddeler eklenebilmektedir. Bu çalışmada, lesitin ve yumurta sarısı ve ikisinin birleşiminin *Heterorhabditis bacteriophora* HBH ırkının kitle üretiminin verimi üzerindeki etkileri incelenmiştir. Çalışmada *H. bacteriophora* türünün Türkiye'de farklı iklime sahip bölgelere ait izolatlarının hibritlenmesi ile elde edilmiş HBH ırkı kullanılmıştır. Kitle üretiminin veriminin hesaplanmasında infektif jüvenil (IJ) sayısı ölçüt olarak belirlenmiştir. Sonuçlar incelendiğinde tüm uygulamaların kontrole göre istatistiksel olarak daha etkili olduğu, en iyi uygulamanın ise lesitin kullanılan ortam ile elde edildiği belirlenmiştir. Bu sonuç ile lesitinin *in vitro* katı kitle üretiminde ek madde olarak kullanılabileceği tespit edilmiştir.

1. Introduction

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae have been used as safe biological control agents against many economically important insect pests (Ehlers 1996; Peters 1996). EPNs are obligate endoparasitic organisms and need an insect host to continue their lifecycle. They are safe to non-target organisms and they can actively seek for their host, features that are considered to be important for a biocontrol agent. They penetrate hosts from

natural openings, release their symbiotic bacteria and kill them in 24-36h (Kaya and Gaugler 1993). EPNs can be used to control especially soil-dwelling insect pests and they have substantial advantages over chemical insecticides, as they are environmentally safe. Considering the negative effects of chemicals on the environment and human health, EPNs have become popular organisms over the years. EPNs are considered as valuable organisms (Wright et al. 2005). Thus, there are many studies focused on improving genetic characteristics, efficacy, resistance, longevity, and reducing production costs and extending the shelf life of EPNs (Grewal 2000; Grewal et al. 2002; Susurluk and Ehlers 2008).

EPNs can be mass produced in large-scale liquid culture (Ehlers et al. 1998; Ferreira et al. 2014). EPNs can be cultured by using *in vivo* or *in vitro* techniques (Ehlers et al. 1998; Ehlers 2001; Gaugler et al. 2002). *In vivo* techniques are commonly used for small-scale application and laboratory studies. *In vitro* solid and liquid mass production techniques are used for large-scale applications and commercial production. Some compounds such as nutrient broth, agar, yeast extract, soy-flour and plant oil are used *in vitro* mass production of EPNs. However, there is a continuous search for newer compounds to improve production yield.

This study aimed to determine the effects of lecithin, egg yolk and both together on the yield of *in vitro* production of hybrid *H. bacteriophora* HBH strain that has already been patented by us. EPNs need protein and lipid for a long lifespan. It is also hoped that finding positive effects of newer compounds on *in vitro* mass production yield will have a useful contribution to commercial production.

2. Materials and Methods

2.1. Heterorhabditis bacteriophora HBH strain

Heterorhabditis bacteriophora is one of the most common EPN species all around the world. A hybrid strain of *H. bacteriophora* was used in the present study. The hybrid strain was obtained after hybridization of two Turkish native *H. bacteriophora* isolates from different climatic regions of Turkey. Moreover, HBH strain was patented due to its superior biological characters (TPMK Patent No: TR 2013 06141 B). Under laboratory conditions, it was reproduced using *in vivo* methods according to Kaya and Stock (1997). The last instar of great wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), larvae were used for reproduction of EPNs. Each larva was inoculated with approximately 50 IJs and incubated for four days at 24 °C. After incubation, dead larvae were transferred on White Trap and freshly emerged IJs were collected in a culture flask.

2.2. Egg isolation

Egg isolation is the most important process before *in vitro* mass production of EPNs. Before egg isolation, ten *G. mellonella* larvae were inoculated with 100 IJs per larva and incubated at 24 °C. The larvae were inoculated in a 24-well plate, filled with silver sand. Three to four days after incubation, all dead larvae were taken out from sand and rinsed with Ringer's solution. Under a stereomicroscope, all cadavers dissected and fertilized hermaphrodites were collected in a watch glass. On a magnetic stirrer, collected hermaphrodites were cut into small pieces with razor blades. The pieces of the hermaphrodites were filtered with a 70 µ sieve and all eggs were collected in a fresh Ringer's solution. The eggs were washed with sterile distilled water for several times, and their surfaces were sterilized with a sterilization solution. After surface sterilization, the eggs were transferred in a sterile well plate filled with a yeast extract solution (YS).

2.3. Preparation of solid media

There are several solid media for *in vitro* mass production of EPNs. Wouts agar is one of the most common solid media for general laboratory studies (Wouts 1981). Ingredients of 100ml Wouts agar can be seen in Table 1. In the present study, three different customized versions of Wouts agar were used for EPN production (Table 1). In addition to standard Wouts agar, lecithin (L), egg yolk (EY) and lecithin + egg yolk (L+EY) were added into media. Standard Wouts agar was used for control treatment. The ingredients of the solid media were mixed in a glass bottle and autoclaved for 15 minutes at 121 °C. After sterilization, all media were distributed evenly to 6 cm petri dishes in a laminar flow cabinet.

Table 1. Ingredients of solid media (100 ml).

	Solid Media			
	Wouts	**Wouts +Lecithin**	**Wouts +Egg Yolk**	**Wouts L+EY**
Nutrient Broth	1.6 g	1.6 g	1.6 g	1.6 g
Agar	1.2 g	1.2 g	1.2 g	1.2 g
Sunflower-oil	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Egg Yolk	-	-	0.5 g	0.5 g
Lecithin	-	0.5 g	-	0.5 g

2.4. Monoxenic cultures in solid media

The monoxenic culture of EPNs is a standard method for *in vitro* production (Lunau et al. 1993; Strauch and Ehlers 2000). Isolated and surface sterilized eggs were incubated in a sterile medium for three days to check contamination and hatching. Approximately 500 hatched and uncontaminated juveniles were transferred on each agar. Simultaneously, six droplets (~100 µl) of symbiotic bacteria were added on the surface of the agars (Lunau et al. 1993). As newly hatched live individuals were used, mortality rates of the juveniles were almost zero. Petri dishes were sealed with parafilm and incubated for a month at 24 °C. Nearly all phases of egg isolation and monoxenic culture preparation were performed under sterile conditions in a laminar flow cabinet. Each treatment, including control, was replicated three times and seven petri dishes were used for each replicate.

2.5. Yield calculation

Petri dish counts were continued until the end of IJ development to track the reproducing generations. The counting process was started one week after IJ inoculation on agar. Each petri dish was counted three times, and the time between each count was one week. After the end of IJ development, all agars were washed with Ringer solution and last IJs in collected suspensions were counted. Leica S8 Apo stereomicroscope was used for counts.

2.6. Statistical analysis

All data were analyzed using one way ANOVA, and means of the treatments were compared using LSD (Least Significant Differences) test (α= 0.05). JMP 7.0 software was used for all statistical analysis.

3. Results and Discussion

The infective juvenile number is one of the most important criteria for *in vitro* production yield. Among all treatments, lecithin significantly had the most positive impact on IJ yield after three counts of petri dishes. Even though egg yolk and L+EY were significantly lower than lecithin alone, all treatments showed better results than control treatment ($p<0.0001$) (Figure 1).

According to the results of the final agar count, lecithin was the best treatment by far and other treatments showed slightly better yield results compared to control ($p<0.0001$) (Figure 2). EPNs have symbiotic bacteria inside their intestine and the bacteria have a crucial role in EPN biology. The bacteria secrete many different metabolites and enzymes, including lecithinase (Boemare et al. 1996), which breaks down lecithin into fatty acids. Considering the positive effect of lipids on longevity and persistence of EPNs (Abu Hatab and Gaugler 2001), high yield of EPN production on lecithin can be explained with the lipid ratio of the media.

There are many studies on enhancing *in vitro* solid and liquid mass production of EPNs(Wouts 1981; Ehlers et al. 2000; Ehlers 2001; El-Sadawy 2011; Sharma et al. 2011; Ramakuwela et al. 2016). Enhancing *in vitro* mass production generally based on optimization of environmental conditions and changing ingredients of the production media. Over the years, researchers have been reported different compounds including soy flour, milk powder, vegetable oil, egg yolk, liver and cholesterol (Sharma et al. 2011). Our study aimed to improve *in vitro* mass production of *H. bacteriophora* HBH strain, which has superior bio-ecological traits and native to Turkey (Susurluk et al. 2013a, 2013b; Susurluk and Ulu 2015). Compared to standard Wouts agar, lecithin and egg yolk were added to production media as protein and lipid source. These compounds were used as an additional ingredient for mass production for different EPN strains before (Han and Ehlers 2001; Johnigk et al. 2004; Chavarría-Hernández et al. 2006; Sharma et al. 2011). Most common reason for using these compound is because they are rich in nutrients, and protein is a must-have ingredient inmonoxenic culture (Chavarría-Hernández and De La Torre 2001; Shapiro-Ilan and Gaugler 2002). Another claim about egg yolk was the positive effect of nitrogen on the reproduction rate of EPNs, (Chavarría-Hernández and De La Torre 2001), but there is an ongoing debate about this claim. Even though there are many studies on mass production of EPNs using egg yolk and lecithin, these studies did not focus on individual effects of these ingredients. Each strain has its environmental adaptation and biological properties. Thus, responses of the new strains to different compounds are mostly unknown.

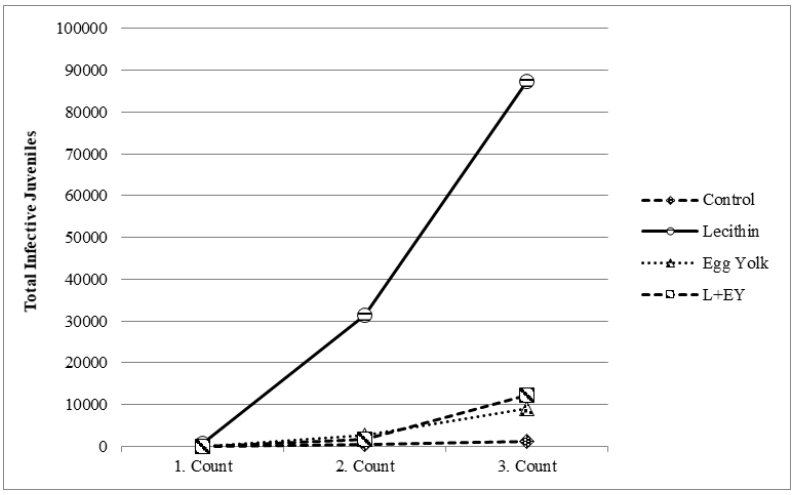

Figure 1. Infective juvenile numbers based on weekly petri dish counts.

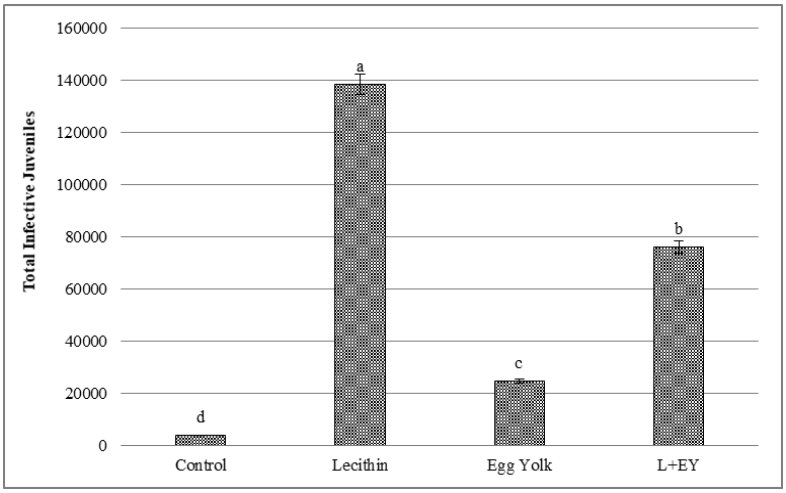

Figure 2. Infective juvenile numbers based on final agar count ($p<0.05$).

4. Conclusions

For sustainable agriculture, alternative management methods should be used efficiently to reduce chemical consumption. Biological control is a commonly used method for pest management. EPNs are important biocontrol agents against soil-dwelling insect pests. Their popularity has been rising with other biocontrol agents due to the negative effects of chemical pesticides. Even though EPNs have many important features, their mass production, formulation and storage expenses are still high. Thus, farmers mostly use EPNs with government subsidy, especially in Europe and the U.S.A. Our results showed the positive effects of lecithin and egg yolk on the mass production yield of EPNs. Considering accessibility and price of these compounds, we conclude that they can be used as additional substances for *in vitro* mass production. Nevertheless, we still need many studies to optimize the mass production of EPNs in the future.

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