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Original article (Orijinal araştırma)

Optimization of *in vitro* solid culture of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain[1]

Heterorhabditis bacteriophora Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hibrit ırkının *in vitro* katı kültürde üretiminin optimizasyonu

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Abstract

Entomopathogenic nematodes are soil-dwelling biocontrol agents. EPNs need an insect host to complete their life cycle, and they kill their host during its development. The major disadvantage of EPNs is the high cost of commercial products. Thus, there are many studies focused on reducing production costs by optimization of mass production. In a previous project, *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain was developed from local isolates. This hybrid strain was patented due to superior bioecological characteristics. This study aimed to optimize *in vitro* solid mass production of hybrid strain. All laboratory trials were performed between 2017 and 2018, in Nematology Laboratory of Bursa Uludağ University, Faculty of Agriculture, Department of Plant Protection. For optimization, additional supplementary ingredients (soy lecithin and egg yolk), temperature (24, 28 and 32°C) and medium pH (5, 7 and 9) were selected as production parameters. Optimization was evaluated based on hermaphrodite egg numbers, total infective juveniles (IJs), IJ body length and IJ virulence. Based on results, best production combination was found as agar containing soy lecithin, 28°C and pH 7. Also, agar media with pH 9 markedly reduced production yield. Consequently, optimum values for some important *in vitro* solid production parameters of HBH hybrid strain were determined.

Keywords: *Heterorhabditis bacteriophora*, mass production, monoxenic culture, optimization

Öz

Entomopatojen nematodlar (EPN), toprakta yaşayan biyolojik mücadele ajanlarıdır. EPN'ler yaşam döngüleri boyunca bir konukçu böceğe ihtiyaç duyarlar ve gelişimleri sırasında konukçusunu öldürürler. EPN'lerin en büyük dezavantajı, ticari ürünlerin yüksek maliyetidir. Bu nedenle kitle üretimin optimizasyonu ile üretim maliyetlerinin düşürülmesine odaklanan birçok çalışma bulunmaktadır. Daha önceki bir proje kapsamında iki yerel izolattan *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hibrit ırkı geliştirilmiştir. Bu hibrit ırk, üstün biyo-ekolojik özelliklerinden dolayı patentlidir. Bu çalışmada, hibrit HBH ırkının *in vitro* katı kültürde kitle üretiminin optimize edilmesi amaçlanmıştır. Tüm laboratuvar denemeleri 2017-2018 yılları arasında Bursa Uludağ Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü, Nematoloji Laboratuvarı'nda gerçekleştirilmiştir. Optimizasyonda standart ortama ek katkı maddeleri (soya lesitini ve yumurta sarısı), sıcaklık (24, 28 ve 32°C) ve ortam pH'ı (5, 7 ve 9) üretim parametreleri olarak seçilmiştir. Optimizasyon, hermafrodit yumurta sayıları, toplam infektif jüvenil (IJ) sayısı, IJ vücut uzunluğu ve IJ etkinliği ile değerlendirilmiştir. Sonuçlara göre, en iyi üretim kombinasyonu, soya lesitini içeren agar, 28°C ve pH 7 olarak bulunmuştur. Ayrıca pH 9'lu agarlar, üretim verimini önemli ölçüde azaltmıştır. Sonuç olarak, HBH hibrit ırkının bazı önemli *in vitro* katı üretim parametreleri için optimum değerler belirlenmiştir.

Anahtar sözcükler: *Heterorhabditis bacteriophora*, kitle üretimi, monoksenik kültür, optimizasyon

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Introduction

Today, the most widely used method against pathogens, pests and weeds in agriculture is chemical control (Sarwar, 2015). Until now, the most successful and applicable alternative method to chemical control has been biological control. Although the market share for biocontrol agents seems quite low, it is thought that the biological products will exceed chemical products in the 2050s (Glare et al., 2012; Olson, 2015). Chemical pesticides are preferred due to their high efficacy, quick mode of action and low price. For this reason, alternative methods to chemicals should be able to compete with pesticides. There are some major problems restricting the adoption of biological control agents such as high production costs, short shelf life, and slow effectiveness. For such reasons, many studies are conducted to overcome these disadvantages. A large part of this work is directed towards increasing the effectiveness of biological control agents, reducing production and storage costs, increasing production efficiency, and developing new formulations that improve ease of use (Grewal, 2002; Schumann et al., 2014).

Entomopathogenic nematodes (EPN) are obligate endoparasites that spend more than 90% of their life cycle in the soil and need an insect host to continue their development. EPNs can be found in many regions and climates around the world (Griffin et al., 1990; Hominick, 2002), they have a wide host range (Peters, 1996), no negative effects on other non-targeted organisms (Lacey et al., 2015), they have active host search capabilities (Lewis et al., 1992), they can be easily applicable with many agricultural equipment (Georgis, 1990; Wright et al., 2005) and they are compatible with pesticides (García del Pino & Jové, 2005; Atwa et al., 2013).

EPNs are suitable organisms for mass production, and improvements in production techniques have been going on for many years (Ehlers, 2001; Shapiro-Ilan et al., 2002). The most widely used *in vitro* production method of EPNs, which have been produced by a range of methods, was developed by Lunau et al. (1993). EPNs are mass produced *in vivo* or *in vitro*, depending on their intended use (Ehlers, 2001). While *in vivo* methods are preferred for laboratory studies, greenhouse and small-scale field trials, *in vitro* methods are preferred for commercial production and large-scale applications (Gaugler et al., 2002). *In vitro* methods are basically divided into two groups using solid and liquid media. Solid media production is generally conducted in Petri dishes of certain sizes. In some conditions, solid media are preferred to liquid media due to being more convenient and risk-free.

Although mass production of a biological control agent is an important feature, expensive commercial products are less likely to become popular in the market. There have been many developments in the production of EPNs in artificial environment over the years (Chavarría-Hernández et al., 2011; Testa & Shields, 2017). Most of these developments have been realized on increasing production efficiency, reducing production costs, or increasing the quality of the product. Some of these studies examined the effects of mass production in artificial environment and environmental factors during the production process (Hirao & Ehlers, 2009; Leite et al., 2017).

There are many factors that affect the mass production of EPNs using solid and liquid media. Some of these factors can be summarized as temperature, humidity, pH, viscosity, salinity, electrical conductivity, agitation speed, pressure, media content, type, or race of EPN used (Shapiro-Ilan et al., 2012). All these factors directly affect the yield during production and the quality of the product after production. With the optimization of mass production of EPNs, commercial biological products have gained ground to compete with pesticides and increase their market share.

This study aimed to optimize the *in vitro* solid mass production of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain. The HBH hybrid strain was patented in 2018 due to its superior bioecological features (Patent No: TR 2013 06141 B). Egg yolk and soy lecithin were added as protein and fat sources into the standard solid medium used for optimization. In addition, the effects of temperature and pH on production efficiency were also investigated. The effects of these

variables were evaluated using parameters such as the number of eggs of hermaphrodites, the body length of infective juveniles (IJs), the total number of IJs produced and the effectiveness of IJs. By this study, it was also aimed to reveal some optimum production parameters of our local EPN strain and speed up liquid production processes in future.

Materials and Methods

Heterorhabditis bacteriophora HBH hybrid strain

Heterorhabditis bacteriophora is one the most abundant EPN species in Turkey. Two local Turkish isolates of *H. bacteriophora* were hybridized and HBH strain obtained with superior bioecological traits. HBH hybrid strain has high virulence, reproduction capacity and longevity compared to commercial EPN strains. *H. bacteriophora* HBH strain was *in vivo* reared on last instar of *Galleria mellonella (*L., 1758) (Lepidoptera: Pyralidae) larvae (Kaya & Stock, 1997).

Egg isolation and bacteria cultures

Egg and bacteria isolation are the first steps of monoxenic cultures. Eggs are usually extracted from the first-generation hermaphrodites (*Heterorhabditis*) or gravid females (*Steinernema*). For egg isolation, each *G. mellonella* larva was inoculated with 100 IJs of the hybrid strain. Four days after inoculation, dead cadavers were dissected in a Petri dish and fertilized hermaphrodites were collected in a tube. Hermaphrodites were rinsed with Ringer's solution several times and they were cut into little pieces with razor blades using a vortex. Dispersed eggs were filtered through a 50-µm sieve and washed with distilled water to eliminate hermaphrodite particles. Finally, surface of the eggs was sterilized with sterilization solution, and they were incubated in sterile YS medium until they hatch contamination-free (Lunau et al., 1993).

Freshly hatched eggs need to feed immediately. So, frozen bacterial cultures were thawed, and YS medium inoculated with symbiotic bacteria. To establish food source for nematodes, modified agar media inoculated with precultured bacteria and incubated for 2 days before nematode transfer (Hirao & Ehlers, 2009).

Optimization parameters

Production media has significant impact on yield (Ramakuwela et al., 2016; Alsaidi et al., 2018). There are many different solid media for EPN production. Wouts agar is mostly used for small-scale production (Wouts, 1981; El-Sadawy, 2011). Thus, we modified Wouts agar to improve yield and quality of IJs (Yoo et al., 2000). In addition to the ingredients of Wouts agar, we used egg yolk for additional protein and soy lecithin for fat source. List of the modified agar media is given in Table 1. Standard Wouts agar was used as control.

Table 1. Modified Wouts agar media for *in vitro* solid culture (100 ml)

	W	WL	WE	WLE
Nutrient broth	1.6	1.6	1.6	1.6
Agar (g)	1.2	1.2	1.2	1.2
Sunflower oil	0.5	0.5	0.5	0.5
Egg yolk (g)	-	-	0.5	0.5
Soy lecithin (g)	-	0.5	-	0.5

W, Wouts (control); WL, Wouts + lecithin; WE, Wouts + egg yolk; and WLE, Wouts + lecithin + egg yolk.

Production temperature is another important parameter (Serwe-Rodriguez et al., 2004; Anbesse et al., 2013). Since different EPN species have different temperature adaptations, *in vitro* productions were conducted at 24, 28 and 32°C to find the optimum temperature. Incubators were preheated, and agar media were incubated at each temperature during production.

Lastly, even slight changes of pH may have detrimental effect on bacteria and nematodes (Yoo et al., 2000; Hirao et al., 2010). Though pH is more important for liquid cultures, it is also an important parameter for solid production optimization. It is known that bacteria secrete different metabolites under different pH values, which will eventually change production yield. We prepared agar media with 3 pH values (5, 7 and 9). The pH of the agar media was adjusted with ascorbic acid and sodium bicarbonate during preparation.

Evaluation of the optimization

First-generation hermaphrodites of *Heterorhabditis* genus highly correlate with the yield of reproduction (Zioni et al., 1992; Strauch & Ehlers, 2000; Ferreira et al., 2014). Thus, one criterion of the evaluation was the eggs of the hermaphrodites. Five first-generation hermaphrodites were collected from surface of each agar and dissected separately in Petri dishes. Dispersed eggs were counted under microscope and average egg number was calculated.

Each EPN production aims to achieve maximum IJ yield. Although the number of hermaphrodite eggs is closely related with final yield, a linear relationship cannot be established between the total number of IJs and the number of hermaphrodite eggs due to many reasons during production. After 14 days of incubation, the Petri dishes were washed with distilled water and all produced IJs were transferred to a tube. Six 10 µl samples were taken from the tubes and counted under the microscope. The average number of IJ obtained from the samples was scaled to the total suspension in the tube and the total number of IJ obtained from the Petri dish was calculated.

Another part of the evaluation is to measure the length of IJs (Hirao et al., 2010; Ferreira et al., 2014). IJs are the only free-living, non-feeding and infective stage of EPNs. IJs consume their fat reserves to stay alive and higher body fat ratio provides longer life. Body length of ten in vitro produced IJs from each Petri were measured under an invert microscope using Leica Application Suite v3.2.

Virulence of the IJs is an important indicator for the quality of production. We tested the virulence of *in vitro* produced IJs on the larvae of *G. mellonella*. Each larva inoculated with 50 IJs and incubated at room temperature. After incubated, dead cadavers were dissected, and mortality rates was calculated. All experiments were repeated three times.

Statistical analyses

Results were subjected to one-way and full factorial ANOVA. Means of the treatments were compared using least significant difference at $\alpha = 0.05$ level. All analyses were done using JMP v11.0 software.

Results

Hermaphrodite eggs

WL agar containing lecithin had a statistically significant effect compared to the W (control) agar as well as the other agar media. However, WE agar was not statistically different from the control group W medium in which you were evaluated in terms of the number of hermaphrodite eggs ($F = 144$; $df = 3, 104$; $p < 0.0001$). When the effect of temperature on the number of hermaphrodite eggs was examined, 28°C had positive effect compared to other temperatures and was statistically different. The most detrimental temperature was 32°C ($F = 39.2$; $df = 2, 105$; $p < 0.0001$). There were significant differences between the effect of pH values on the number of hermaphrodite eggs ($F = 5780$; $df = 2, 105$; $p < 0.0001$). In the pH 9 agar media, a statistically significant decrease occurred, and the number of hermaphrodite eggs was approximately three times lower than the control, pH 7 (Figure 1).

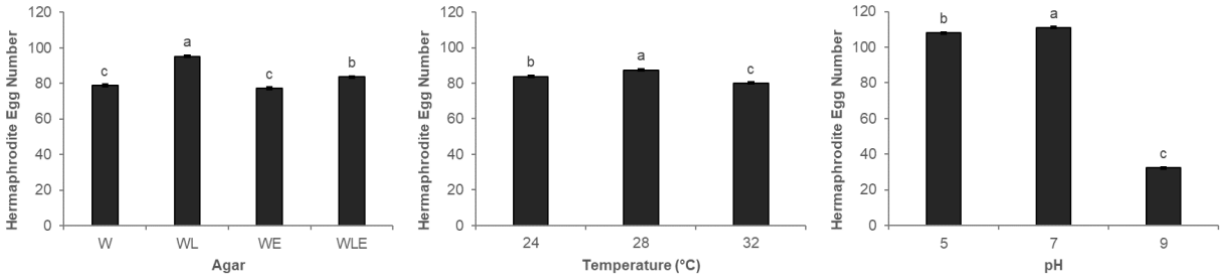

Figure 1. Effect of optimization parameters on hermaphrodite egg numbers.

Total IJs

Considering the total number of IJs, the agar ingredients had significant effect on IJ yield. Like the number of hermaphrodite eggs, it was determined that the WL agar containing lecithin gave better results than the other media in total IJ number and provided greater production efficiency ($F = 146$; $df = 3, 104$; $p < 0.0001$). When the effect of temperature on the total IJ number was examined, it was determined that the yield was statistically higher at 28°C than the other two temperatures. No statistical difference was found between the other two temperatures, 24 and 32°C ($F = 7.97$; $df = 2, 105$; $p = 0.0007$). Similar to the results of hermaphrodite egg count, the result with the lowest total IJs was at pH 9 ($F = 2840$; $df = 2, 105$; $p < 0.0001$). While no statistical difference was found between the other two pH values, the pH 9 value resulted in lower production yields (Figure 2).

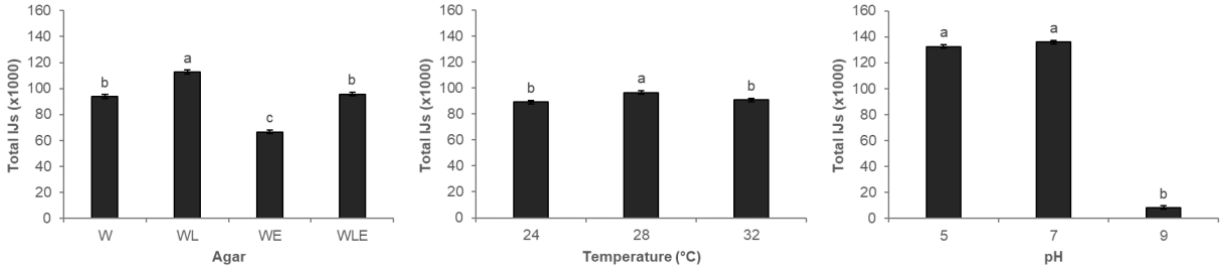

Figure 2. Effect of optimization parameters on total infective juveniles.

Body length of IJs

When the measurements were evaluated, it was determined that the media content ($F = 0.090$; $df = 3, 104$; $p = 0.97$) and temperature ($F = 0.460$; $df = 2, 105$; $p = 0.63$) did not have a statistically significant effect on the length of the produced IJs. Considering that IJs feed on bacteria growing on solid media, this result shows that media content and temperature do not have a positive or negative effect on the chemical compounds secreted by bacteria (Cabral et al., 2004). When the effect of pH value on IJ length was examined, it was determined that a negative effect occurred especially at pH 9 ($F = 7.73$; $df = 2, 105$; $p = 0.0009$). Although the optimum pH demand of symbiotic bacteria varies according to the species, it varies between 6 and 7 (Yoo et al., 2001). The chemical compounds secreted by the bacteria during development cause the pH value to change and have a negative effect in the long term. For this reason, pH is constantly monitored in liquid culture production and the production process is directed accordingly. Due to the high pH value of the agar in this study, it is thought that the length of the IJs was decreased (588.17 µ for pH 9 and a mean of 592.42 µ for the other treatments), especially due to the negative effects during the development of symbiotic bacteria.

Virulence of IJs

When the results were examined, it was determined that the agar content did not have any effect on the virulence of IJs on the *G. mellonella* larvae ($F = 1.00$; $df = 3, 104$; $p = 0.398$). Similar results were obtained in the temperature experiment ($F = 1.00$; $df = 2, 105$; $p = 0.398$). When the pH test results were examined, no statistically significant effect was observed on the efficacy, similar to the results in the other two criteria ($F = 1.00$; $df = 2, 105$; $p = 0.373$). All larvae were dead and virulence of all treatments was 100%.

Discussion

One of the obstacles to the widespread use of biological control is that biological products are expensive and unattractive to the producers. EPNs cannot compete with chemical products, especially due to the high production, formulation, and storage costs. By improving virulence, storage life or reproduction capacity, these costs can be also reduced indirectly (Shapiro-Ilan et al., 2012; Blackburn et al., 2016). In this study, it was aimed to optimize the *in vitro* solid mass production of the *H. bacteriophora* HBH hybrid strain. Effect of modified agar media, temperature and pH was tested on hermaphrodite egg number, total IJ number, IJ body length and virulence of IJs.

Optimization parameters have statistically significant effect on the number of hermaphrodite eggs. Hermaphrodite egg number is a key indicator in *in vitro* solid and liquid production (Zioni et al., 1992; Ciche et al., 2008; Clarke, 2008). The source of IJs is the eggs in hermaphrodites (Ehlers, 2001). Even though there are many other factors, a good hermaphrodite development has the potential to increase production efficiency.

The total number of IJs is generally accepted as one of the most important production criteria of *in vitro* production (Hirao & Ehlers, 2010; Addis et al., 2016; Leite et al., 2017). Although the number of hermaphrodite eggs and the total number of IJs can be seen as two directly related criteria, sometimes a parallel relationship cannot be established between these two criteria due to various reasons related to symbiotic bacteria (Johnigk & Ehlers, 1999; Ehlers et al., 2000). During the *in vitro* solid and liquid production process, the metabolites secreted by the bacteria change due to the changes in the content of the medium, and this result directly affect the production efficiency. Actually, when the criteria for the number of hermaphrodite eggs and the total number of IJs are examined, the results vary. It is thought that adverse conditions for the development of IJs occur in the later stages of production because of differences in media content and the effects of pH on symbiotic bacteria. However, when the yield results in the WL medium containing lecithin, which is the most efficient production medium, are examined, approximately 10,000 IJs are produced per 1 g medium. These results are comparable with the yields obtained in a previous study (El-Sadawy, 2011). In some studies, more than 100,000 IJ per 1 g medium was produced in solid culture (Tabassum & Shahina, 2004). The main reason is that the solid medium used in their work is sponge instead of agar. Sponge media is both a lower density than agar and an environment that allows three-dimensional production. In the agar, growth mostly occurs on the surface.

IJs are the only stage that can survive under the soil for a long time without feeding and are essential for EPNs to colonize insects. It is known that IJs use the lipid reserves in their bodies during survival without nutrition (Smart, 1995; Qiu & Bedding, 2002). In addition, EPNs accumulate lipids from the environment where they are fed (Blackburn et al., 2016). For this reason, providing more nutrients can increase the production quality (Yoo et al., 2000; Singh & Upadhyay, 2018). In the current study, lecithin was used as a fat source. It has been shown that symbiotic bacteria break down lecithin and reveal fatty acids due to secretion of lecithinase enzymes (Boemare et al., 1996). In the data obtained in the present study, the positive effect of the agar media containing lecithin was consistent with previous studies.

When the production yield was examined, there was very little production in some experiments. Despite low production, high virulence ratios can cause confusion. The main reason for this discrepancy is the use of 50 IJs per larva for all media, regardless of production yield. This result can be evaluated from different perspectives. One of the important features of the final product of *in vivo* or *in vitro* production is virulence. The data obtained from the virulence trials show that the efficacy of IJs produced under control combination (W-24-7) is not statistically different when compared with all other interactions. High virulence can be considered as a positive trait. Although the agar content was not suitable for the growth of IJs, ingredients of the agar did not adversely affect the symbiotic bacteria. However, since the production yield is very low, the importance of efficiency falls into the background. This has also been emphasized in some studies (Susurluk et al., 2013; Ulu & Susurluk, 2014). Based on the results of the current study, the best combination was WL-28-7 for the hybrid strain.

This study is one of the first studies in Turkey on the optimization of mass production of EPNs. Although studies have been conducted for many years around the world, it is thought that the study is important due to the use of a patented strain. There are many parameters used in *in vitro* production, however, the most important parameters for solid and liquid culture have been used to find optimized conditions for hybrid strain. More work is needed to improve the production efficiency and the quality of the IJs for efficient field application.

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