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EFFECT OF DIFFERENT FACTORS ON THE NEMATODE
Heterorhabditis megidis (POINAR, JACKSON AND KLEIN, 1987)
– MUTUALISTIC BACTERIA *Photorhabdus luminescens*
(THOMAS AND POINAR, 1979) *IN VITRO* CULTURES

WPŁYW RÓŻNYCH CZYNNIKÓW NA UKŁAD
NICIEŃ *Heterorhabditis megidis* (POINAR, JACKSON I KLEIN, 1987)
– BAKTERIA MUTUALISTYCZNA *Photorhabdus luminescens*
(THOMAS I POINAR, 1979) W HODOWLI *IN VITRO*

Abstract: Entomopathogenic nematodes (EPN) of the families *Heterorhabditidae* and *Steinernematidae* mutualistically connected with bacteria of the genera *Photorhabdus* and *Xenorhabdus* have long been used in biological plant protection.

The aim of this study was to optimise the conditions of liquid culture of the nematode *Heterorhabditis megidis*. The performed studies were aimed at estimating the effect of biotic and abiotic parameters (temperature, aeration, the amount of the initial nematode dose) on the number of larvae undergoing further growth (larval recovery) and on the final yield of invasive larvae. It was found that the biotic parameter that directly and significantly affects initiation of further larval growth is the dose of nematodes introduced to the culture. Final productivity was significantly affected by aeration. The optimum set of parameters is: temperature of 25 $^\circ$C, aeration of 121 rpm and the initial dose of 1370 L3/cm^3.

Keywords: *Heterorhabditis megidis*, *Photorhabdus luminescens*, liquid *in vitro* cultures, optimisation of parameters

Dynamic development of methods of mass culture in artificial media that started in the 1980s was a key factor in commercial use of entomopathogenic nematodes. Now, biological means containing nematodes of the genera *Steinernema* and *Heterorhabditis* are produced by many firms worldwide [1–3]. From among many species only 6 (*S. carpocapsae* (Weiser, 1955)*, S. feltiae* (Filipjev, 1934)*, S. riobravis* (Cabanillas et al, 1994)*, S. scapterisci* (Nguyen i Smart, 1990)*, H. bacteriophora* (Poinar, 1976)*,

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H. megidis (Poinar, Jackson, Klein, 1979) found commercial application [4]. Essential precondition for succeeding in the production biopreparations of nematodes is the ability of their mass multiplication. Nematode cultures have been carried out for more than 70 years [5] and now nematodes are produced on a large scale using 3 methods: *in vivo*, *in vitro* on solid substratum and *in vitro* in liquid media [6, 7]. Each of these methods has its pros and contras resulting from: production costs, total financial input, required know-how, economy, product quality and, moreover, each of these methods might potentially be developed [8]. Optimisation of culture conditions in the case of nematodes of the family *Heterorhabditidae* should concentrate on the productivity of hermaphroditic larvae of the first generation. Though the next, second generation is amphimictic, males and females are not able to copulate in liquid media [9]. Maximising hermaphroditic individuals in liquid medium is inextricably linked with the larval recovery [10]. The percentage of recovered larvae *in vivo* is nearly 100 % [11] while in liquid media the phenomenon of departure from the invasive stage is unstable and varies from 0 to 86 % [10–13]. Differences in the number of recovered larvae are often found in experiments even if culture conditions remain the same. Hence, gaps should be filled in our knowledge of various aspects of nematode and bacterial physiology, of their mutual relationships and of a possibility of their reproduction under stress conditions prevailing in fermentors [14]. The aim of this study was to determine the effect of abiotic (temperature, aeration) and biotic (initial dose) factors on the number of recovered larvae and on the final yield of invasive larvae of the entomopathogenic nematode *H. megidis*.

Material and methods

Study material: a strain of entomopathogenic nematodes – monoxenic cultures of the nematode *Heterorhabditis megidis* (strain KV – 136) [15] were obtained from the firm Koppert Biological Systems B.V. (Netherlands); bacteria *Photorhabdus luminescens* were isolated from NBTA substratum and identified as pure phase I; liquid medium for nematode culture – 3 g of specific culture substratum (composition reserved by Koppert Biological Systems B.V., Netherlands), 3 cm^3 of maize oil, 75 cm^3 distilled water/300 cm^3 in Erlenmayer flasks.

Microbial substrata: liquid medium YSE – 0.5 g of yeasts extract, 0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot (7H_2O)$, 5.0 g NaCl, 1.0 g lecitine, 5.0 g of maize oil, 1000 cm^3 distilled water; agar substratum NBTA – 37 g Nutrient agar, 25 mg *bromothymol blue* (BTB), 1000 cm^3 distilled water, 4.0 cm^3 1 % 2,3,5-triphenyltetra-zoliumchloride; Wouts agar – 19 g Bacto Nutrient Broth, 12 g Bacto Agar, 5 g of maize oil, 1000 cm^3 distilled water.

Culture parameters: temperature – 20^oC, 25 ^{o}C; aeration (expressed as rotations of the rotary shaker) – 121 rpm, 160 rpm and 200 rpm; initial dose of larvae L3/cm^3 – 1370 L3/cm^3; 2340 L3/cm^3; 4440 L3/cm^3.

In total 18 combinations of parameters were used. Each experiment lasted 26 days.

Population dynamics of *H. megidis* in the studied cultures were analysed by estimating their density and growth stages every third and second day, consecutively

(on days 3, 5, 8, 10, 13, 15, 18, 21, 23 and 26). Based on the obtained data, the following indices of population dynamics were calculated:

1) recovery percentage $R = (H/a) \cdot 100 \%$ [11],

where: R – larval recovery,
   H – the number of hermaphroditic individuals,
   a – initial dose of nematodes introduced to the culture on day 0.

2) final yield $W_k = l. \, l._{L3} \cdot V$,

where: W_k – final yield,
   l. $l._{L3}$ – the number of invasive (L3) larvae,
   V – volume of Erlenmeyer flask.

Result and discussion

The effect of physical parameters on population dynamics of nematodes in liquid media

The effect of physical parameters on recovery (H/cm^3)

Initial doses (1370 L3/cm^3, 2340 L3/cm^3, 4400 L3/cm^3) exerted a significant effect ($p < 0.05$) on recovery (Fig. 1). Statistically significant differences were found between
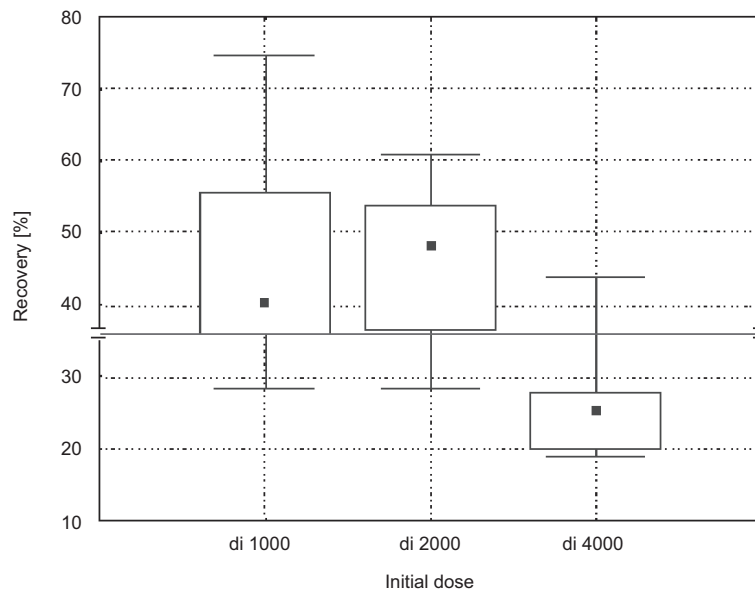

Fig. 1. The effect of initial dose (L3/cm^3) on recovery: di 1000 – initial dose 1370 L3/cm^3, di 2000 – initial dose 2340 L3/cm^3; di 4000 – initial dose 4400 L3/cm^3

doses 1370 L3/cm³ and 4400 L3/cm³ and between 2340 L3/cm³ and 4400 L3/cm³. The number of recovered larvae at the doses of 1370 L3/cm³ and 2340 L3/cm³ remained at the same level. The highest percentage of recovered larvae R = 74.8 % and R = 61.1 % were obtained at initial doses of 1370 L3/cm³ and 2340 L3/cm³, respectively. At the initial dose of 4400 L3/cm³ the percentage of recovered larvae did not exceed 50 %, being equal to R = 43.9 %.

The effect of physical parameters on final productivity (L3/cm³)

It was found that aeration had a significant impact ($p < 0.05$) on the final productivity of invasive larvae in culture (Fig. 2). Statistically significant differences were found between aeration of 121 rpm and 200 rpm and between 160 rpm and 200 rpm. The highest final yield of invasive larvae (190560.0 L3/cm³) was obtained at aeration of 160 rpm. Similarly high yields (174760.0 L3/cm³, 175260.0 L3/cm³, and 189700.0 L3/cm³) were obtained at 121 rpm. When applied aeration was 200 rpm the productivity of invasive larvae was very low and equalled 72800 L3/cm³.
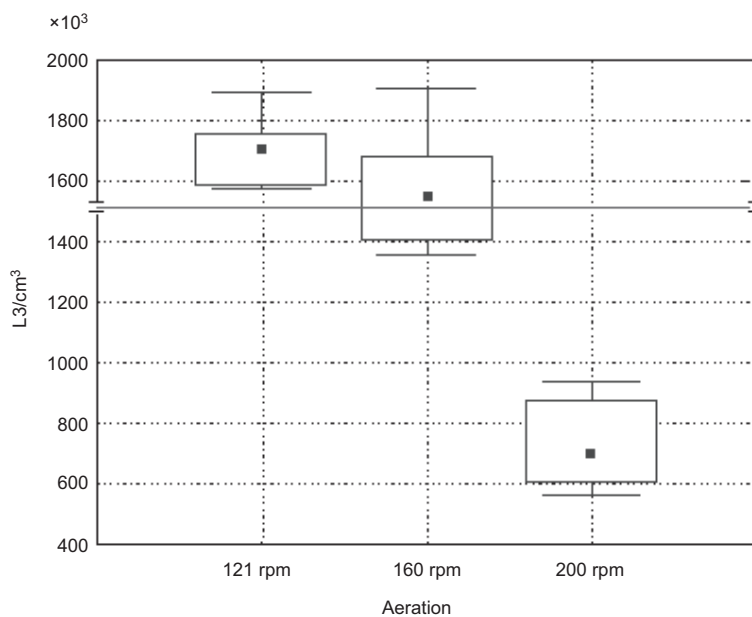

Fig. 2. The effect of aeration on final productivity of invasive larvae, L3/cm³

Correlation between larval recovery (H/cm³) and final productivity (L3/cm³) of invasive larvae

Correlation between the recovered larvae (H/cm³) and final yield (L3/cm³) showed that the number of hermaphroditic individuals significantly ($p < 0.05$) affected final productivity (Fig. 3). In almost all cases high numbers of hermaphroditic individuals
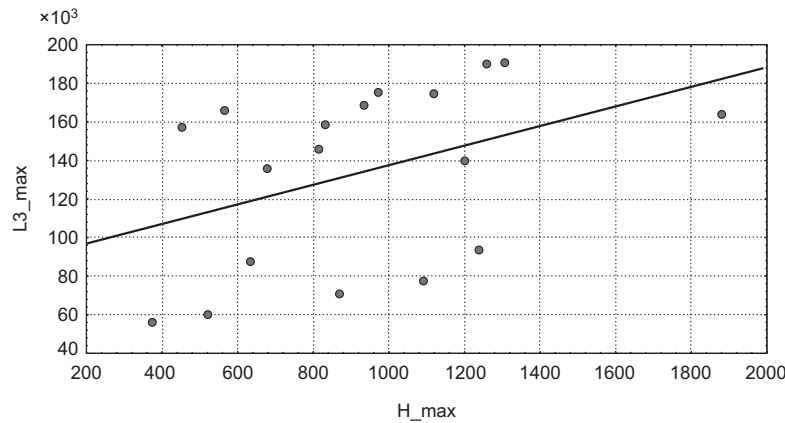
Fig. 3. Correlation between recovery (H/cm³), and final productivity (L3/cm³) of invasive larvae

(1306.4 H/cm³, 1259.6 H/cm³, and 1120.0 H/cm³) resulted in the highest final yields (190560.0 L3/cm³, 189700.0 L3/cm³, and 174750 L3/cm³, respectively). The only difference could be observed at aeration of 200 rpm at which high number of hermaphroditic individuals (1000 H/cm³) gave low yields of invasive larvae (irrespect-ively of the initial dose and temperature).

Literature data on the effect of environmental factors on the size and stability of larval recovery indicate that the *in vitro* recovery is highly variable ranging from 18 to 90 % [16]. The performed studies demonstrated that the initial dose of nematodes introduced to the culture is the factor directly and significantly affecting recovery percentage. It was shown that the lowest initial dose (1370 L3/cm³) gave best results and that in these cultures the highest recovery percentage was recorded. Johnigh et al [17] studying the effect of the initial dose on recovery also found that increasing the initial dose was accompanied by the decrease of recovered larvae. Statistical analysis of the effects of temperature and aeration on recovery percentage did not show a significant impact of these parameters. Aeration was found to be the only parameter significantly affecting the final yield of invasive larvae. Strauch and Ehlers [18] in their study also pointed to the effect of this parameter on the final yield. The maximum final yield (190560 L3/cm³) was obtained in performed tests at aeration of 160 rpm. Aeration at 121 rpm gave slightly lower yields. It, however, provided the highest stability of final yield that remained at a level of 157320–189700 L3/cm³. Aeration at 200 rpm gave the lowest yields.

Considering profitability ie taking into account the ratio of introduced to finally obtained larvae the use of the lowest dose of 1370 L3/cm³ allows for obtaining highest efficiency between 11220.7 % and 13560.5 % of hermaphroditic larvae of the I generation.

The maximum final yield obtained in the performed cultures (190560 L3/cm³) was relatively high as compared with the literature data (138000 L3/cm³ [11]).

Conclusion

1. The physical parameter directly and significantly affecting:

– larval recovery is the number of nematodes introduced to the culture. The best results were obtained at the lowest dose of 1370 L3/cm^3.

– final yield of invasive larvae is strongly dependent on aeration. Application of 160 rpm aeration allowed for obtaining the highest final yields of invasive larvae.

2. The final yield of invasive larvae significantly depends on the number of hermaphroditic individuals.

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W HODOWLI *IN VITRO***

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Abstract: Nicienie entomopatogenne (EPN) z rodziny *Heterorhabditidae* i *Steinernematidae* związane mutualistycznie z bakteriami należącymi do rodzaju *Photorhabdus* i *Xenorhabdus* od wielu lat są stosowane w biologicznej ochronie roślin. Celem badań była optymalizacja warunków hodowli nicieni *Heterorhabditis megidis* w ciekłym środowisku. Prowadzone badania zmierzały do określenia wpływu parametrów biotycznych i abiotycznych (temperatury, napowietrzania oraz dawki inicjalnej nicieni) na liczbę larw przechodzących dalszy rozwój i na wydajność końcową larw inwazyjnych. Określono również korelację między rozwojem nicieni *H. megidis* a ich bakteriami mutualistycznymi *P. luminescens* w warunkach *in vitro*.

W wyniku przeprowadzonych badań stwierdzono m.in., że parametrem biotycznym, który bezpośrednio i istotnie wpływa na inicjowanie przechodzenia larw inwazyjnych w dalszy rozwój jest dawka nicieni wprowadzonych do hodowli. Na wydajność końcową hodowli istotnie wpływa napowietrzanie. Optymalnym układem parametrów jest: temperatura 25 ^{o}C, napowietrzanie 121 rpm, dawka inicjalna 1370 L3/cm^3.

Słowa kluczowe: *Heterorhabditis megidis*, *Photorhabdus luminescens,* plynne hodowle *in vitro,* optymalizacja parametrów