

The Effects of Various Organic and Inorganic Fertilizers on the Density of Entomopathogenic Nematodes Symbiont

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ABSTRACT

Entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* and their bacterial symbionts *Xenorhabdus nematophila* and *Photorhabdus luminescens* (*P. luminescens*), represent a specific agricultural niche. The successful integration of entomopathogenic nematodes (EPN) as regular use biological control agents require specific knowledge and understanding of the adaptation and establishment of applied biological control agents in agricultural ecosystems. For many years, the pest management capabilities of these pathogenic partnerships have been implemented in biological control settings. In this study, ten fertilizers were examined to explore the effects of nitrogen, phosphorus, and potassium (NPK) on the production of entomopathogenic bacteria *Xenorhabdus nematophila* and *Photorhabdus luminescens*. Laboratory exposure to fertilizer concentrations (0.5-2.5%) was used to determine the production of each bacterial species. Results conclude that *P. luminescens* are generally more sensitive to fertilizer than *X. nematophila*. Moreover, fertilizers containing high nitrogen suppressed bacterial densities more readily than those with lesser amounts. This paper summarizes the effects of the three important nutrients found in various concentrations of organic and inorganic fertilizers on entomopathogenic bacteria production.

Keywords: Entomopathogenic Nematodes, Inorganic Fertilizers, Organic Fertilizers, *Photorhabdus luminescens*, *Xenorhabdus nematophila*.

Submitted : February 15, 2022

Published : March 17, 2022

ISSN: 2684-1827

DOI: 10.24018/ejfood.2022.4.2.463

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I. INTRODUCTION

Steinernema and *Heterorhabditis* are insect pathogens with a host spectrum that includes over two hundred insect orders [1]. EPN's offer microbial advantages such as wide host ranges, host-seeking ability, easy storage, and non-toxicity to mammals [2]. These two families of nematodes are unique in that they carry a symbiotic pathogenic bacterium that excretes toxins and antimicrobial compounds that kill the insect host within 48 hr. of ingestion [3]. The majority of applied research has focused on their potential as inundatively applied augmentative biological control agents [4]. Understanding how fertilizers affect nematode communities is necessary if growers seek to convert from using market chemical insecticides to organic biopesticides like entomopathogenic nematodes. Research on the effects of fertilizers on entomopathogenic nematodes has been extremely limited, and little research has been conducted to compare the effects of different fertilizers on the bacterial symbionts of entomopathogenic nematodes [5].

Because entomopathogenic nematodes are adversely affected by nitrogen [2], the impact of various fertilizers on them must be determined before they can be considered as an alternative to commercial pesticides. It has been estimated that without added nitrogen, average yields for corn declined

by a staggering 41%, rice by 37%, barley by 19%, and wheat by 16% [6]. To date, research on the effect of various nitrogen sources on nematodes has been restricted to plant parasitic nematodes [7]. Nematicidal activity seems to be inversely related to C:N ratios of the amendment, but effects may vary with nitrogen source, rate of application, and nematode species [8]. Nitrogen is often the most limiting factor in crop production. [9]. Studies show that nitrogen compounds increase biotic activity of target areas which may alter the physical environment of the soil to the point of toxicity or by increasing the predation and parasitism on nematodes by microbes' parasites, and viruses [2]. The objective of this research was to determine the effect of commonly applied organic and inorganic fertilizers on *Xenorhabdus nematophila* and *P. luminescens*, bacterial symbionts of two of the most well-known and thoroughly studied entomopathogenic nematodes available for agricultural purposes [3].

A. Mutualistic Symbiosis

Photorhabdus luminescens and *Xenorhabdus nematophila* are biphasic, gram-negative bacterium that maintain a symbiotic relationship with the nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* [10]. Both *X. nematophilus* and *P. luminescens* bacterial genera undergo

phase variation in which their metabolic activities shift from an unstable, pathogenic state (phase I) to a stable, less pathogenic form (phase II). Phase I bacterial symbionts secrete various antimicrobials that help provide and maintain an environment for nematode reproduction by bioconverting the insect host into nutrition as well as preventing other organisms from invading the insect cadaver [11]. The process is as follows: after an insect is sensed the nematode ambushes it, sheds its outer cuticle, and enters the insect's hemocoel through its natural openings such as the anus or mouth [12]. Upon invasion, the infective stage of EPNs release the virulent bacteria symbiont within the host which is then metabolized in the insect hemolymph. There, the bacteria contribute to killing the insect, which provides nematodes with nutrients, protection, and favorable growth conditions [13]. Once the resources are consumed, and the EPN progeny that has developed into the specialized infective stage emerge from the insect carcass in search of another host and repeats the process. Although the bacteria are toxic to over 200 agricultural pests, they do not harm the environment, humans, livestock, or other beneficial insects such as bees or earthworms [14]-[16].

B. Fertilizer composition

The addition of soil amendments to improve soil fertility and plant growth is among the oldest of agricultural practices. The average percentage of yield attributed to fertilizer ranges from 40 to 60% [6]. Soil, as a result of over cultivation, may not be particularly nutrient rich, and therefore requires added nutrients. The three most important macronutrients necessary for plant growth are nitrogen, phosphorus, and potassium [17].

Of the three major nutrients, plants require nitrogen in the largest amounts because it aids in growth, increases leaf size and quality, hastens crop maturity, and promotes fruit and seed development. Soil contains low levels of nitrogen and requires annual applications to sustain crop growth. Little of the applied nitrogen is carried over to subsequent growing seasons due to crop removal, leaching and denitrification. Of all the elements required for crop production, nitrogen poses the greatest environmental threat through contamination of surface and ground water [18].

Phosphorus is also considered a primary nutrient for healthy plant growth, structural strength, crop quality, and seed production. Moreover, phosphorus encourages the growth of roots, promotes blooming, and is essential for nucleic acids synthesis. Plants deficient in phosphorus are stunted in growth and often have an abnormal dark-green color. Adding phosphorus to soil low in available phosphorus promotes root growth and winter hardiness, and often hastens maturity [19].

Potassium is necessary for photosynthesis because it regulates a wide range of growth related functions. Potassium deficiencies in plants result in defoliation, slow or stunted growth, chlorosis, weak roots, and the uneven ripening of plants. The primary source of inorganic phosphorus is phosphate rock and may be applied to soils directly. The primary inorganic source of potassium for use in NPK fertilizers is potash but can also be obtained by potassium sulfate and granite dust [20].

II. MATERIALS AND METHODS

A. Organic and Inorganic Fertilizers

TABLE I: THE FOLLOWING 10 FERTILIZERS WERE CHOSEN

Organic fertilizers	N.P.K	Inorganic	N.P.K
sea 90	0:0.01:1	Calcium Nitrate	15.5:0:0
Jobe's	5:2:3	Plant Starter	5:15:4
Mega Green	2:3:1	Ammonium Sulfate	21:0:0
Fish	5:1:1	Ammonium Nitrate	34:0:0
Fox Farm	0.01:0.03:0.07	Urea	46:0:0

B. Insects and Nematode

The last instars of the Lepidopteron insect *Galleria mellonella* were obtained from Carolina Biological Supply Company (Burlington, NC USA) and the nematodes (*Heterorhabditis bacteriophora* and *Steinernema carpocapse*) were obtained from Gardening Zone (Camarillo, CA USA).

C. Bacterial Isolation and Culture

In this study, in vivo culturing techniques were conducted to retrieve bacterial symbionts *P. luminescence* and *X. nematophila*. First, sanitized *H. bacteriophora* and *S. carpocapse* IJs (400-500IJs/ ml) were dropped with sterile pipettes on petri dishes containing 5 to 10 *Galleria mellonella* and incubated at 28 °C for 48-72 hours. A change of color (from yellow to tan for *X. nematophila* and reddish for *P. luminescence*) is characteristic of successful infection for EPNs [1], [3]. Once infection was verified, each batch of *G. mellonella* was surface sanitized by submerging them in multiple 70% ethanol mixtures for a few seconds each. Following sanitation, each carcass was aseptically dissected. For *X. nematophila*, a loop full of haemolymph was streaked onto nutrient agar (NA) (g/L: 2X Nutrient Broth and 1% olive oil). Each day samples were collected, Gram-stained, and observed until identical isolated colonies were obtained and transferred into 2X NB broth and incubated at 28 °C at 150 rpm on an orbital shaker. For *P. luminescence*, the haemolymph was streaked on both nutrient agar and a diagnostic agar medium known as NBTA (g/L: 8 nutrient agar; 0.025 bromothymol blue; 0.040 2, 3, 5 triphenyl-tetrazoliumchloride) and incubated at 28 °C that produces dark blue colonies when phase I cells are present. Once isolated, multiple colonies were transferred to 2x NB broth (in g/l: 5 peptone, 3 beef extract or yeast extract, 5 NaCl) and incubated at 28 °C at 150 rpm on orbital shaker (Certomat IS, Sartorius, Germany) for 24 hours. Master plates were used to prepare transfer tubes throughout the experiment [1,3].

D. Experimental Set-up

For each fertilizer, the below concentrations were used in this study based on manufacturer recommendations provided on the package to apply in the field.

TABLE II: DEPICTS THE OPTIMAL CONCENTRATIONS OF EACH FERTILIZER AS RECOMMENDED BY THE MANUFACTURER

Organic fertilizers	N.P.K	Inorganic	N.P.K
Jobe's	0.30%	Calcium Nitrate	0.60%
Sea 90	1.50%	Urea	1.98%
Mega Green	0.50%	Plant Starter	2.24%
Fish	0.75%	Ammonium Sulfate	0.75%
Fox Farm	0.37%	Ammonium Nitrate	0.80%

E. Media Preparation, Inoculation, and Collection

The effect of NPK (nitrogen, potassium, phosphate) levels within organic and inorganic fertilizers on bacterial survivability was demonstrated using *X. nematophila* and *P. luminescens* bacterial symbionts. After determining the manufacturers' recommended fertilizer concentration for 1sqft of foliage (Table II), the following concentrations of each fertilizer (0%, 0.50%, 1.0% 1.5%, 2.0% and 2.5%) were prepared in 100 mL flasks containing 25 mL of 2X NB and the required amount of fertilizer to obtain the proper concentration (Table II), set to a pH of 7.5, covered, and then autoclaved. Fresh bacterial stock cultures were prepared and held in 3 mL of 2X NB and incubated at 28 °C for 24 hours and agitated at 150 rpm prior to each experiment. Bacterial health was determined using simple gram staining techniques and then used to inoculate tubes containing 3 mL of each fertilizer concentration. For each standard there was one control tube, one tube for *X. nematophila* bacteria, and one for *P. luminescens* bacteria. Each tube contained 3 mL of media and was inoculated with 50 µL of either bacterium for a total of 18 tubes. After inoculation, the tubes were placed back in the incubator at 28 °C for 24 hours and agitated at 150 rpm. Following 24 h of agitation, a control was blanked, and the contents of each flask were subjected to spectrophotometry at 600 nm to determine the density of bacteria in a 1 mL sample.

III. RESULTS

A. Inorganic Fertilizer Results

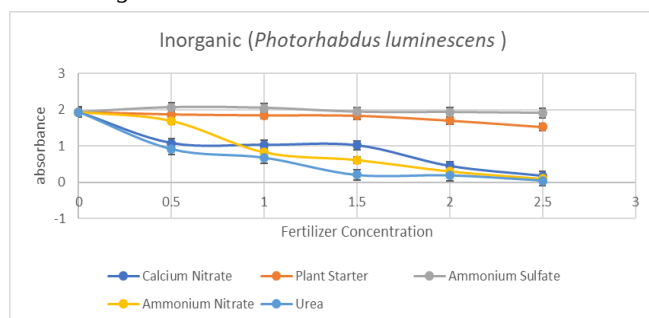


Fig. 1. Effect of inorganic fertilizers on *Photorhabdus luminescens*.

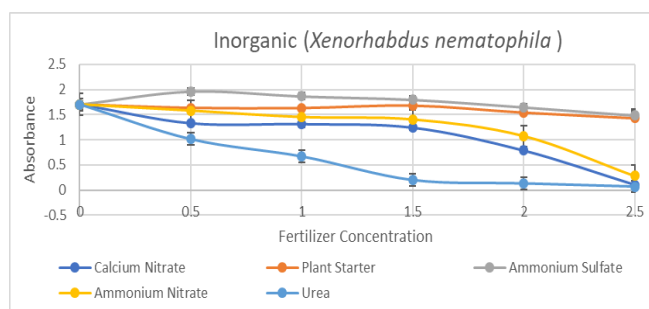


Fig. 2. Effects of inorganic fertilizer on *Xenorhabdus nematophila*.

Ammonium nitrate, urea, and calcium nitrate are common fertilizers used in regions with moderate to high rain fall. In this experiment, 10 fertilizers (five organic and five inorganic) were chosen based on the most commonly used soil amendments in North Carolina and used to determine the effect of NPK on two entomopathogenic nematode populations. After determining the recommended amount of fertilizer for 1ft2 of foliage, parameters were set (0 to 2.5%)

which encompassed the optimal concentrations for each fertilizer. Bacterial concentrations of each species began with an absorbance between 1.5 and 2.0, were subjected to identical concentrations of fertilizer (0 -2.5%) and analyzed daily until the absorbance value decreased approximately 0.

Although nitrogen is a very important micronutrient for the successful growth and sustainability of most crops, research suggests it is also significantly toxic to entomopathogenic nematode populations. Research on the relative effects of different nitrogen sources on nematodes has been restricted to studies on plant parasitic nematodes. Generally, nitrites are more toxic than ammonia compounds, and nitrates are less toxic than ammonia compounds [18]. In this study, with the exception of fish fertilizer, (see graph 3) bacterial symbionts exposed to inorganic (synthetic) fertilizers had greater population declines as well as greater mortality rates than those treated with organic amendments. Both *X. nematophila* and *P. luminescens* were negatively impacted by fertilizers that contained high concentrations of nitrogen. Urea, with the most available nitrate, presented the quickest decline and eradication of living bacteria colonies in both *X. nematophila* and *P. luminescens*. However, ammonium sulfate, with the third highest amount of nitrogen, and plant starter with the only source of potassium yield the highest bacteria production and maintain nearly the same number of bacteria throughout all concentrations of nitrogen.

B. Organic Fertilizer Results

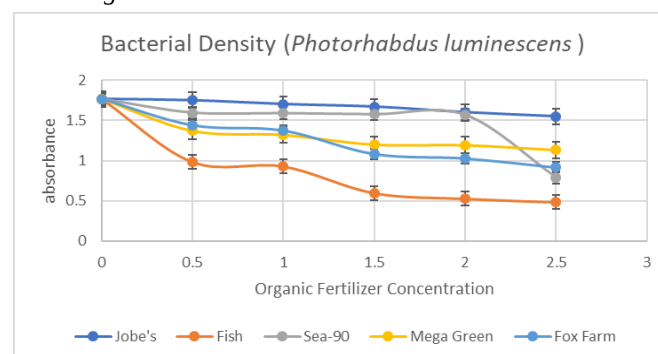


Fig. 3. Effect of organic fertilizers on *Photorhabdus luminescens*.

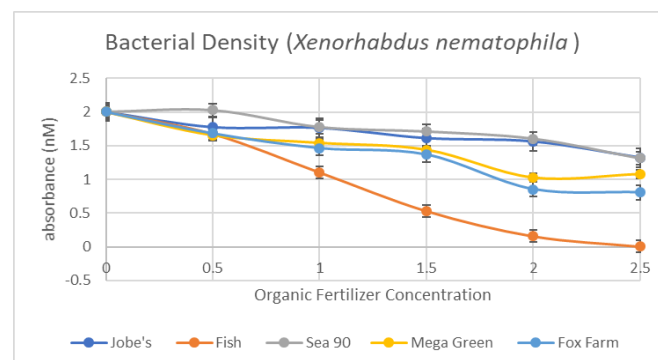


Fig. 4. Effect of organic fertilizers on *Xenorhabdus nematophila*.

As the use of organic and non-GMO initiatives spread across the nation, producers face decisions pertaining to how best to care for their crops while meeting yield expectations. In this study, five organic fertilizers were applied to entomopathogenic bacterial populations to determine the effects of each on their survivability. Results conclude that although the bacteria populations exposed to fertilizers with higher concentrations of nitrogen experienced a greater

decline in numbers than those with lesser amounts, it does not play as significant of a role in organic amendments as it does in inorganic. Out of the 5 fertilizers, Jobe's and Fish fertilizer had the highest nitrogen content. For both *Xenorhabdus nematophila* and *Photorhabdus luminescens*, Fish produced the least number of bacteria, had the most rapid bacterial reduction rate, and the lowest overall survivability of the group. Jobe's fertilizer with the same percentage of nitrogen, deviated from this trend showing instead the highest production of bacteria and lowest death rate.

An important difference to recognize between the fertilizers used is that the organic fertilizers used in this experiment all were supplemented with potassium. Jobe's, although having one of the highest nitrogen percentages, also has the highest Potassium and Phosphate values. This observation indicates that potassium is an important factor in bacterial growth. The inorganic fertilizers, aside from Plant starter, which also deviated from the expected results, was not supplement with potassium and all had much faster reduction rates and less overall survivability. Results conclude that in all but Fish fertilizer, the bacterial symbionts treated with organic amendments had the highest initial growth rate and had a mortality rate of approximately 45%. This is quite significant when compared to the bacteria fertilized with inorganic compounds which were observed to have 70% mortality rate.

IV. DISCUSSION

Various environmental factors in the soil such as fertilizer decomposition products, increase abiotic activity and parasitism on nematodes (due to fertilizer application), pH changes, and increased moisture within the soil [7], may reduce nematode survival and thereby reduce the ability of entomopathogenic nematodes to suppress insect pests [21]. Minimal impact may be expected if nematodes are applied as biological insecticides to achieve short-term results (i.e., inundative biological control) in combating pest outbreaks such as fleas on lawns however, the effect of chemical fertilizers on nematodes may be more significant when used for inoculative biological control, where establishment and recycling are the objectives [9]. Studies indicate that certain factors may have been more important than others in reducing nematode virulence. For example, it is unlikely that slight differences in pH is sufficient to reduced nematode virulence, however it is likely that nitrogen compounds caused some the differences observed in the [7]. In this study, the pH of each solution was set to 7.5 to eliminate that environmental stressor from the equation.

Inorganic fertilizers tend to contain higher percentages of nitrogen (Fig. 3) than their organic counterparts. Inorganic fertilizers can reduce the effectiveness of entomopathogenic nematodes as biological control agents in the soil, but this impact is exposure-dependent [9]. Research also suggests that urea, another readily used additive, is toxic to EPN's due to the decomposition chemistry that may reduce oxygen availability. Because of this, the efficacy of entomopathogenic nematodes may also be reduced if they are applied at the same time as fresh manure or chemical pesticides [7].

Organic amendments can have positive, neutral, or

negative effects on soil properties including nematode communities. All amendments tend to increase availability of nutrients, such as nitrogen, microbial biomass, and abundance of bacterivore and fungivore nematodes [22], [23]. Manure adds organic matter to the soil which improves soil quality by increasing granulation, water infiltration, nutrient content, soil bioactivity, and fertility and productivity [7]. Studies suggest that when applied at recommended rates, fertilizers have little impact on entomopathogenic nematode efficacy [24], and may even encourage nematode establishment and recycling, and might be a tool useful for conservation biological control [25].

V. CONCLUSIONS

Today, it is impossible to ensure sustainable growth of crop yields without the application of fertilizers. However, when using soil amendments, it is necessary to select the ones that will complement the existing environment to prevent negatively affecting the crops but also the structure and agrochemical conditions of the soil and the soil biota. Overall, it is apparent that nitrogen negatively affects the bacterial symbionts, but it is unknown whether or not potassium counteracts the toxicity, or if the bacterial growth was affected by something else. Results suggest that organic fertilizers pair better with *Xenorhabdus nematophila* and *Photorhabdus luminescens*, but they may lack too much nitrogen for the plants to be able to produce.

ACKNOWLEDGMENT

The authors would like to thank The Biotechnology Research and Training Center, Department of Chemistry and Physics at The University of North Carolina at Pembroke, NC, USA for providing laboratory space and equipment.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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