EFFECT OF AMMONIUM NITRATE AND TIME OF HARVEST ON MASS PRODUCTION OF PASTEURIA PENETRANS[†]

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ABSTRACT

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Pasteuria penetrans, a biological control agent for root-knot nematodes, has great potential for development as a bionematicide. Previous studies have shown that high nitrogen levels affect the development of root-knot nematodes. The objectives of this study were to determine the effects of various levels of ammonium nitrate and of harvest time on development of *P. penetrans*. Five levels of ammonium nitrate (0.0, 0.2, 0.4, 0.8, 1.6 g/pot) and five different harvest times (37, 44, 51, 58, 65 days after inoculation) were tested separately with six replicates each for both experiments. Ammonium nitrate adversely affected the development of both *M. arenaria* race 1 and *P. penetrans*. A quadratic relationship was established between the number of females per root system and the nitrogen levels. Number of endospores per root system and number of endospores per female were negatively correlated with the nitrogen levels and decreased 1.1 million and 0.013 million, respectively, per 0.1 g ammonia nitrate increment. Numbers of endospores per female were correlated with the time of harvest. Endospores per female increased 0.031 million per 100 degree days increment based on a developmental threshold of 17°C. These results will be useful for the cultivation of *P. penetrans*.

Key words: ammonium nitrate, bacterium, biological control, cultivation, degree day, endospore, fertilizer, harvest time, *Lycopersicon esculentum*, mass production, *Meloidogyne arenaria*, nitrogen, *Pasteuria penetrans*, root-knot nematode, tomato.

RESUMEN

Chen, Z. X. y D. W. Dickson. 1977. Efecto del nitrato de amonio y el tiempo de cosecha en la producción masiva de *Pausteria penetrans*. Nematrópica 27:53-60.

Pausteria penetrans, un agente de control biológico para nematodos agalladores, posee un gran potencial para ser utilizado como bionematicida. Estudios previos han demostrado que altos niveles de nitrógeno, afectan el desarrollo de los nematodos agalladores. El objetivo de este estudio, fue determinar el efecto del uso de varios niveles de nitrato de amonio y del tiempo de cosecha en el desarrollo de *P. penetrans*. Cinco niveles de nitrato de amonio (0, 0.2, 0.4, 0.8 and 1.6g/maceta) así como cinco tiempos de cosecha (37, 44, 51, 58 y 65 días después de la inoculación) fueron ensayados separadamente, seis replicaciones fueron usadas cada vez en ambos experimentos. El nitrato de amonio tuvo un efecto adverso para el desarrollo de *M. arenaria* raza 1 y de *P. penetrans*. Se estableció una relación quadrática entre el número de hembras por sistema de raíces y los niveles de nitrógeno. El número de endosporas por sistema de raíces y por hembra, estuvo negativamente correlacionado con los niveles de nitrógeno, mostrando una disminución de 1.1 y 0.013 millones respectivamente, por cada gramo de nitrato de amonio incrementado. El número de endosporas por hembra, estuvo correlacio-

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nado con el tiempo de cosecha, estas aumentaron 0.031 millón por 100 días grado de incremento, basado en un umbral experimental de 17°C. Estos resultados serán de utilidad, para el cultivo de *P. penetrans.*

Palabras claves: bacteria, control biológico, cultivo, día grado, endosporas, fertilizante, tiempo de cosecha, *Lycopersicon esculentum, Meloidogyne arenaria,* nematodo agallador, nitrógeno, nitrato de amonio, *Pasteuria penetrans,* producción masiva, tomate.

INTRODUCTION

Plant-parasitic nematodes cause a $6 billion monetary loss to United States agriculture each year (Sasser and Freckman, 1987). The management of plant-parasitic nematodes currently relies largely on a combination of crop rotation and nematicide usage. Economic considerations and potential environmental hazards of nematicides, however, have led to great controversy about their continued use. Biological control of nematodes offers an alternative or supplemental management tactic to chemical or cultural control of nematode pathogens. One nematode antagonist in particular, *Pasteuria penetrans,* has shown great potential for biological control of root-knot nematodes (Dickson *et al.*, 1994; Mankau and Prasad, 1977; Sayre and Starr, 1985; Stirling, 1984, 1991). *Pasteuria penetrans* is an obligate, mycelial, endospore-forming bacterial parasite of *Meloidogyne* spp. Endospores of *P. penetrans* attach to the cuticle of the second-stage juvenile (J2) of *Meloidogyne* spp. in soil. Infection occurs after the J2 enters a plant root, where parasitized nematodes mostly develop into females but are incapable of reproduction (Bird, 1986). The females, and sometimes males (Hatz and Dickson, 1992), become filled with endospores, which are eventually released into the soil upon host disintegration.

Pasteuria penetrans has been observed in nematode-suppressive soils and successfully tested against root-knot nematodes in greenhouse and field microplot experiments (Brown *et al.*, 1985; Chen *et al.*, 1996b; Chen *et al.*, 1997; Dickson *et al.*, 1994; Stirling, 1984). Experiments have demonstrated that the suppressiveness of *P. penetrans* infested soil to nematodes can be increased following continuous planting of susceptible hosts of root-knot nematodes (Oostendorp *et al.*, 1991). The number of endospores of *P. penetrans* attached per J2 increased with a corresponding increase in soil suppressiveness. Furthermore, using various techniques to discriminatively eliminate nematodes, fungi, and other bacterial antagonists, *P. penetrans* has been successfully documented as the leading suppressive agent in a nematode-suppressive soil (Weibelzahl-Fulton *et al.*, 1996). *Pasteuria penetrans* also has suppressed root-knot nematodes following application of endospores in field microplots. Reduction of nematodes, root galls, and pod galls with corresponding increases in peanut yield have been correlated with increasing levels of *P. penetrans* (Chen *et al.*, 1996b). The capability of *P. penetrans* to suppress root-knot nematodes underscores its potential as a successful bionematicide. As an example, Chen *et al.* (1996b) demonstrated that applications of 10 000 endospores per gram of soil effectively suppressed root-knot nematodes in microplots cropped to peanut.

The major obstacle for the practical application of *P. penetrans* to soil infested with root-knot nematodes is the inability to produce sufficient numbers of endospores. Currently, the parasite must be produced *in vivo* in *Meloidogyne* spp., which, in turn, must be reared on susceptible plants in pot culture (Stirling and Wachtel, 1980), in

hydroponic culture (Serracin *et al.*, 1997), or in nematode-excised root cultures (Verdejo and Jaffee, 1988). The mass production system developed by Stirling and Wachtel (1980) is widely used to produce inoculum for experimental purposes, but several factors affect the mass production of *P. penetrans*. Sharma and Stirling (1991) suggested that nematode inoculum density, plant species, and time of harvest are the main factors influencing endospore production. The optimum conditions suggested are 5 000 J2/pot of inoculated tomato plants harvested at 900 degree-days (Sharma and Stirling, 1991). Davies *et al.* (1991) observed that a watering regime using less water favors the development of *P. penetrans*. The development of endospores increased with increasing temperature between 20°C and 35°C, with optimum temperature between 28 and 35°C (Hatz and Dickson, 1992; Serracin *et al.*, 1997). Nitrogen fertilizer is an important factor in greenhouse culture practice. The effect of nitrogen on development of *P. penetrans* remains unknown. In this study, we investigated the effects of various levels of ammonium nitrate and time of harvest on the production of *P. penetrans* using a greenhouse pot culture technique.

MATERIALS AND METHODS

Nematode and Pasteuria penetrans *isolates:* Isolates of *P. penetrans* (designated P-20) and *Meloidogyne arenaria* (Neal) Chitwood race 1, each derived from a peanut field in Levy County, Florida, were used. *Meloidogyne arenaria* was cultured on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a steam-pasteurized potting soil and *P. penetrans* was propagated on *M. arenaria* race 1 growing on tomato plants in a greenhouse. Maintenance of *M. arenaria* and *P. penetrans* was the same as previously reported (Chen *et al.*, 1996b).

Fertilizer test: The experiment was conducted in a quonset shaped fiberglass covered structure with screened ends (quonset house) at the University of Florida, Gainesville. Fifty-day-old tomato seedlings were transplanted into 15-cm-diameter pots. After 2 days, the potted tomato plants were fertilized with 0.4 g of a 20-20-20 (N-P-K) mixture/pot. The plants were acclimated for 18 days before treatments were applied. Endospore-encumbered J2 (up to 5 days old) were obtained by a centrifugal technique (Hewlett and Dickson, 1993). On 1 August 1994, tomato plants were inoculated with endospore-encumbered J2 at 1 500 J2/pot. The incidence of endospore attachment was 3.4 ± 2.1 endospores/J2 and each J2 had at least one endospore attached. After 1 week, tomato plants were treated with various levels of ammonium nitrate.

The experiment had five treatments, six replicates per treatment, arranged in a randomized complete block design. Ammonium nitrate (35% N) was used as the nitrogen source and it was applied weekly in 150 ml of water at 0.0, 0.2, 0.4, 0.8, or 1.6 g/pot. The recommended rate of nitrogen for commercial production of tomato is 90.8 kg/ha, which is equivalent to 1.13 g of NH_4NO_3 per pot. Other plant nutrient elements were not used. Fertilizer application was stopped 1 week before harvest; hence, five treatments with a total amount of 0, 1.0, 2.0, 4.0, and 8.0 g of ammonium nitrate per pot were applied during the experimental period. Tomato root systems were harvested 51 days after inoculation and stored at 4°C until examined. The root material was incubated in 50 ml of a 12.5% cytolase PCL5 solution (Genencor International, Rochester, NY) for 2 days. The material was then decanted onto a sieve with 600-µm-pore openings nested in a sieve with 75-µm-pore openings and subjected to a high-pressure spray of

water to dislodge the females (Hussey, 1973). All the recovered females were hand-picked with a pipette and counted with the aid of a microscope at ×20. They were ground in deionized water using a glass-tissue grinder. Concentrations of endospores in the suspensions were determined by counting four 0.1-µl aliquots using a hemocytometer (Fisher Scientific, Atlanta, GA) at ×450.

Time of harvest test: The experiment was conducted in the same quonset house and at the same time as the fertilizer test and the same experimental protocol as stated above. Tomato plants were fertilized weekly with 1.4 g of a 20-20-20 (N-P-K) mixture/pot beginning 1 week after inoculation of endospore-encumbered J2. The root systems were harvested at 37, 44, 51, 58, and 65 days after inoculation and stored at 4°C.

Aerial temperature in the quonset house was recorded daily using a maximum-minimum thermometer. Phytotoxicity was observed in the treatment with 1.6 g ammonium nitrate/pot in the fertilizer test, and some second-generation females appeared at the harvest time of 65 days; consequently, these two treatments were excluded from data analyses. Before analysis of each data set, numbers of females per root system, numbers of endospores per root system, and numbers of endospores per female were transformed to their natural logarithms. Data presented in the text were back-transformed. Regression analysis was used, and an equation was provided if the correlation was significant at $P \leq 0.05$. Duncan's multiple-range test was used to compare differences among treatment means.

RESULTS AND DISCUSSION

Increasing levels of ammonium nitrate adversely affected the number of females per root system, number of endospores per root system, and number of endospores per female ($P \leq 0.05$) (Fig. 1). A quadratic relationship existed between the number of females per root system and the ammonium nitrate levels (Fig. 1A). The number of endospores per root system and the number of endospores per female were negatively correlated with the ammonium nitrate levels (Fig. 1B,C). Based on the linear regressions, the number of endospores per root system and the number of endospores per female decreased 1.1 million and 0.013 million, respectively, per 0.1 g ammonium nitrate increment.

Supplemental nitrogen is known to suppress the development of plant-parasitic nematodes (Khan *et al.*, 1986; Riispere, 1990; Rodríguez-Kábana, 1986; Talavera *et al.*, 1984; Verma and Gupta, 1987). The suppression mechanisms have not been elucidated. The suppression probably results from either the direct toxic effects of nitrogen on nematodes (Talavera *et al.*, 1984; Verma and Gupta, 1987), the indirect mediation of the physiological state of the feeding site, or from impacting the entire host plant (Riispere, 1990). Usually, the best conditions for development of nematodes occur when the host is under moderate stress (Riispere, 1990). Our results indicate that such suboptimal conditions may also favor the development of *P. penetrans*. Davies *et al.* (1991) observed that the development of *P. penetrans* in infected females was slower when soil moisture was maintained at field capacity. Since *P. penetrans* is an obligate endoparasite of root-knot nematodes, coevolution of the parasite and the host may have resulted in similar environmental requirements for development.

The highest level of ammonium nitrate was remarkably detrimental to the development of root-knot nematode females (Fig. 1A). The number of females per root

system at 0.8 g of ammonium nitrate/pot was less than those at other ammonium nitrate levels ($P \leq 0.05$). This may have resulted from direct injury to the nematode (Talavera *et al.*, 1984). Numbers of females per root system at 0.2 to 0.4 g ammonium nitrate/pot were similar to that in the control (Fig. 1A). Verma and Gupta (1987) reported that nitrogen was capable of inhibiting the reproduction of root-knot nematodes.

Endospore production also decreased with increasing ammonium nitrate levels (Fig. 1B,C). Endospore production per root system is related to the number of female nematodes in the root system, however, the fact that the number of endospores per female was dramatically decreased suggests that development of *P. penetrans* is hampered by ammonium nitrate. Thus, the reduction in the number of endospores per root system resulted from two factors: a reduction in the number of females per root system at the highest level of ammonium nitrate and a reduction of endospore produced per female. The mode of action of ammonium nitrate on development of *P. penetrans* is unknown, but it probably results from the modification of the physiological state of the nematode hosts.

Numbers of endospores produced per root system and per female at low levels of nitrogen were comparable to those observed in previous studies (Chen *et al.*, 1994; Chen *et al.*, 1996a; Hatz and Dickson, 1992; Verdejo and Jaffee, 1988), but lower than numbers produced in others (Davies *et al.*, 1988; Mankau and Prasad, 1977; Stirling, 1981; Stirling and Wachtel, 1980). The numbers of reported endospores per female vary from 0.9 million (Davies *et al.*, 1988) to 2.4 million (Stirling, 1981). Reports of ratios of numbers of females to numbers of J2 inoculated ranged from 0.004 (Davies *et al.*, 1991) to 0.34 (Stirling,

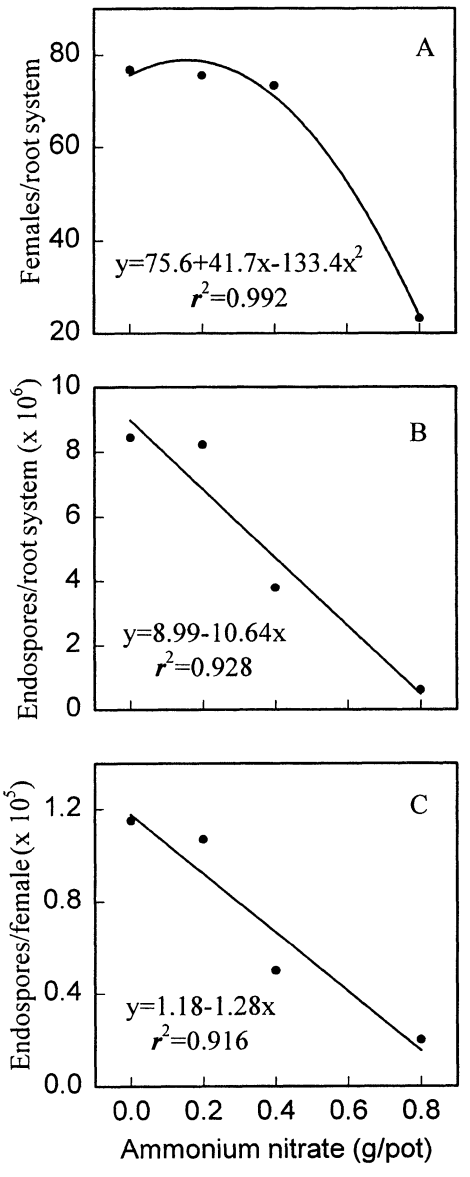

Fig. 1. Effect of ammonium nitrate on *Pasteuria penetrans* grown in *Meloidogyne arenaria* race 1, which in turn was reared on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a quonset house. All of the regressions were significant at $P \leq 0.05$. A) A quadratic relationship between the number of females per root system and the ammonium nitrate levels. B) Linear regression of number of endospores per root system on ammonium nitrate levels. C) Linear regression of number of endospores per female on ammonium nitrate levels.

1984) for J2 infested with *P. penetrans* endospores and 0.15 (Davies *et al.*, 1991) to 0.66 (Davies *et al.*, 1988) for noninfested J2. Apparently, fewer females developed when J2 were encumbered with *P. penetrans* endospores. The ratio of numbers of females to numbers of inoculated J2 in this study was 0.016 to 0.088, which is consistent with the reported ranges. The variations among the different reports on number of females per root system and number of endospores per female could result from differences in *P. penetrans* isolates, nematode species, plant hosts, and cultural conditions.

During the experiment, the daily high temperature, low temperature, and mean temperature recorded in the quonset house were 34.5 ± 3.5°C, 23.1 ± 1.4°C, and 28.8 ± 2.2°C, respectively. The base threshold temperature of 10°C, which was originally reported for *M. arenaria* (Ferris *et al.*, 1978), has been used widely to calculate the accumulative degree days for the development of *P. penetrans* (Bird, 1986; Serracin *et al.*, 1997; Sharma and Stirling, 1991; Stirling, 1981). Recently, we estimated the base threshold temperature for the development of *P. penetrans* to be 17°C (Chen and Dickson, 1997), which was used in this study. The accumulative degree days above 17°C were 469, 551, 627, and 684 for harvest times of 37, 44, 51, and 58 days, respectively.

Numbers of females recovered from root systems were 41, 133, 30, and 28 for the harvest times of 37, 44, 51, and 58 days after inoculation, respectively (Fig. 2A). Although the number of females per root system at 44 days was greater than that at any other harvest time ($P \leq 0.05$), it may be an aberrant estimate caused by experimental variation. Generally speaking, for the time periods considered, the number of females per root system should decrease with increasing harvest time because of natural mortality of females. Numbers of endospores per root system were 0.08, 2.74, 0.78, and 2.16 million for the harvest times of 37, 44, 51, and 58 days, respectively (Fig. 2B). The number of endospores per root system at 37 days was lower than that at other harvest times ($P \leq 0.05$), but there were no differences in endospore production among harvest times of 44, 51, and 58 days ($P > 0.05$). Although the number of females per root system and the number of endospores per root system were not correlated with the accumulated degree days ($P > 0.05$), the number of endospores per female correlated with the accumulative degree days ($P \leq 0.05$) (Fig. 2C). The number of endospores per female increased 0.031 million per 100-degree-day increment.

The recommended harvest time for the mass production of *P. penetrans* was 7 to 8 weeks after inoculation (Stirling and Wachtel, 1980) or 900 degree days above 10°C (Sharma and Stirling, 1991). Neither of these studies provided information on the relationship of number of endospores per root system to various harvest times. Thus, the suggested harvest time was rather empirical. The results in the present study suggest that the optimum harvest time might be even longer. Higher numbers of endospores per female might be obtained with a longer plant production time (Fig. 2C). Hatz and Dickson (1992) observed that isolate P-20 of *P. penetrans*, which was used in this study, required a long period of time (36-54 days) until mature endospores were first observed. This was confirmed by Serracin *et al.* (1997), working with a different isolate of *P. penetrans* and a different species of *Meloidogyne*. Differences in endospore production over time may be induced by various *P. penetrans* isolates, nematode species, plant hosts, and cultural conditions. Certainly, the growth and development of *P.*

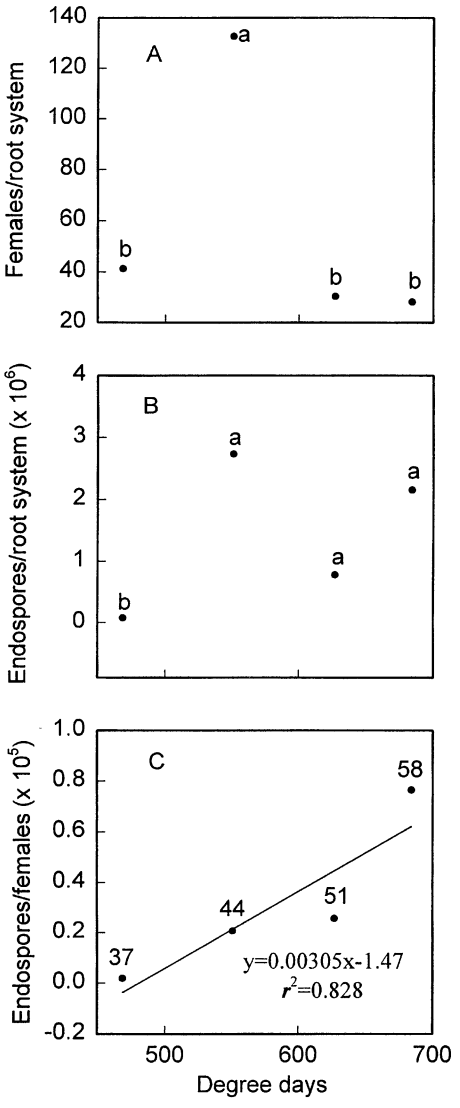

Fig. 2. Effect of harvest time on *Pasteuria penetrans* grown on *Meloidogyne arenaria* race 1, which in turn was reared on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a quonset house. The developmental threshold used for calculating degree days was 17°C. The same letter in each graph indicates no difference according to Duncan's multiple-range tests ($P > 0.05$). A) Correlation between number of females per root system and degree days was not significant ($P > 0.05$). B) Correlation between number of endospores per root system and degree days was not significant ($P > 0.05$). C) Linear regression of number of endospores per female on degree days ($P \leq 0.05$, days after inoculation shown above data points).

penetrans and *M. arenaria* under fluctuating temperature conditions such as those that existed in the quonset house may differ from those that occur under constant temperature conditions. Nevertheless, these results provide useful information on the cultivation of *P. penetrans* under conditions that cannot be controlled carefully but will likely exist in large scale production houses for mass rearing of the organism.

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