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Influence of soil temperature on initial energy reserves of *Globodera rostochiensis* larvae

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Summary — Computer-aided image analysis was used to estimate the influence of soil temperature on initial energy reserves of the larvae of *Globodera rostochiensis*. The larvae were reared at four soil temperatures and during three different developmental times. The initial lipid reserves of the larvae at 20 °C and 24 °C were higher than that at 12 °C or 16 °C. The reproduction of the nematode was optimal at 20 °C. Developmental time of the female did not have a significant effect on the initial energy reserves of the larvae she produced, but a longer developmental time period increased the multiplication of the nematode. The results suggested that for *G. rostochiensis* in Finland the optimum soil temperature is 20 °C, and that the normal field temperature (16 °C) may result in low initial energy reserves and may lead to a reduced pest potential of *G. rostochiensis*. The results emphasize clearly the usefulness of lipid estimation when assessing the economic thresholds for the control of *G. rostochiensis* in cool climatic conditions.

Résumé — ***Influence de la température du sol sur les réserves énergétiques initiales des larves de* Globodera rostochiensis** — L'analyse d'image assistée par ordinateur a été utilisée pour estimer l'influence de la température sur les réserves énergétiques initiales de *Globodera rostochiensis*. Les larves sont élevées à quatre températures de sol et pendant trois périodes de développement de durées différentes. Les réserves énergétiques initiales des larves sont plus élevées à 20 °C et 24 °C qu'à 12 °C et 16 °C. La température optimale pour la reproduction est de 20 °C. La durée de développement de la femelle n'a pas d'effet significatif sur les réserves énergétiques initiales des larves qu'elle produit, mais une période de développement longue augmente la multiplication du nématode. Ces résultats suggèrent que la température optimale pour *G. rostochiensis* est, en Finlande, de 20 °C, et que la température normale des sols (16 °C) est responsable des faibles réserves énergétiques initiales, ce qui réduit le pouvoir parasitaire du nématode. Ces résultats soulignent clairement l'utilité d'une estimation du contenu lipidique pour l'établissement de seuils économiques en vue du contrôle de *G. rostochiensis* dans les sols froids.

Key-words : Temperature, reserves, *Globodera*.

Potato cyst nematode (PCN) was first found in Finland at Hyvinkää (60° 40′ N, 24° 50′ E) in 1946 (Vappula, 1954) and by the early 1980's it could be found throughout the southern parts of the country to some areas in Central Finland (Tiilikkala & Aapro, 1980). The northernmost discovery of *Globodera rostochiensis* has been made near the town Rovaniemi, located a few kilometers north of the Polar Circle (Sarakoski, 1976). *G. rostochiensis* is the only PCN species which has been found and the pathotype Ro 1 is dominant, although a few *andigena*-resistance breaking pathotypes have been established, too (Heikkilä & Tiilikkala, 1991). In Finland, the most noxious animal pest in commercial potato production is PCN. It has been controlled by various cultural methods : crop rotation, resistant cultivars and early varieties. No nematicide is registered for PCN control. In a field study where potatoes were grown every third year alternating susceptible and resistant varieties, nematode multiplication was inhibited and a high and stable yield was assured (Tiilikkala, 1991).

Tiilikkala (1987) has shown that in Finland *G. rostochiensis* tolerates low soil temperatures, but the shortness of the growing season may reduce the multiplication of the nematode. No studies have examined how the climatic conditions of Finland affect the infectivity of larvae or the duration of their infective life. It is well known that the infectivity of parasitic nematodes depends on their energy reserves (Van Gundy *et al.*, 1967; Croll & Matthews, 1973; Storey, 1984; Storey & Atkinson, 1984) which mainly consist of neutral lipid reserves (Barrett, 1976; Lee & Atkinson, 1976; Reversat *et al.*, 1980). Robinson *et al.* (1987*a*, *b*) have described how lipid reserves influence the activity of PCN larvae; the 65 % level of the initial lipid content was critical to infectivity. Energy reserves may also affect the susceptibility of larvae to control methods as Alphey (1983) described. It has been found that larval lipid reserves are an important factor which must be taken into account when assessing the economic thresholds during crop rotations (Storey, 1984).

The level of initial energy reserves is influenced by host cultivar and nematode characteristics (Storey & Atkinson, 1984), however, it is also possible that the temperature during embryonated development affects the initial lipid reserves of the fully developed second stage larvae. At minimal temperatures a part of the energy reserves may be used for the completion of the development of the larvae as Richards (1964) found after studying the development of insect larvae. Thus

PCN larvae, which develop at low soil temperature and during a short growing season like those typical to Northern Europe, may have low initial energy reserves and thus a reduced pest potential.

This work investigates the influence of soil temperature on the initial energy reserves of *Globodera rostochiensis* larvae. The effect of soil temperature and developmental time on the size and initial energy reserves of the larvae were studied and the multiplication of a PCN population at different soil temperatures was determined. The use of lipid reserve estimation for the planning of nematode control is discussed.

Material and methods

The PCN population used in the study was obtained from a field where susceptible potato had been grown continuously for ten years. The infected soil was taken from the field on April 11th and the soil was dried at 22 °C for two weeks. Cysts were extracted from the soil with a Fenwick can and set on Baermann funnels which were filled with potato root diffusate (PRD). The PRD was obtained from potato plants cv. Sabina and diluted with distilled water (1 in 4). The larvae that hatched in 24 hours during the fifth day on the funnel were collected and used for inoculation.

On April 13th seed potatoes cv. Sabina were planted in pots (13 cm diameter and 11 cm deep) which were filled with a fine steam sterilized sand (particle size 100-400 μm). After planting, the pots were placed in controlled temperature water baths so that the level of the soil was below that of the water. A thermocouple was inserted into one pot in each of the four baths and records of soil temperatures were printed on paper four times per hour. The temperature was kept at 15 °C from the planting day to the inoculation of the nematode larvae and one week after inoculation. Four-week-old potato plants were infected by using a hypodermic syringe to inoculate a total of 1000 larvae per pot in 10 ml water distributed at several points around each plant. After the incubation period of one week at 15 °C, the temperatures of the baths were set to 12, 16, 20 and 24 °C; one bath per temperature. Measured temperatures deviated less than 0.5 °C from the adjusted temperatures. There were 15 randomly distributed pots in every bath; three for three different developmental time periods (59, 69 and 79 days) and five for the replications. The daytime (12 h) light intensity was 3400 lux and the air temperature 22 °C, night temperature 17 °C. All pots were irrigated with a drip irrigation system (ITU), and the irrigation water was manured with a fertilizer (N 17 %, P 4 %, K 28 %).

Five pots at a time were removed from the water baths 59, 69 and 79 days after the incubation period to a cold-storage room with an air temperature of 3 °C. The stems were cut off ten days before the removals. On the 79th day all of the pots were moved to a drying cabinet where the air temperature was 22 °C, and the soil was then dried for two weeks after which the cysts were collected with a Fenwick can. The cysts were picked under a binocular microscope and squashed by an electrically stirred perspex rod in glass tubes to release the larvae. The larvae were counted, stained with Oil Red O; and finally mounted on microscope slides for the analysis of lipid reserves as described in Alphey (1982). The computer-aided analysis of the stained fractions was carried out with IMAGIST of Princeton Gamma-Tech, a SUN workstation-based analyzer that runs under the UNIX operating system. The program had a display of four full resolution images with 256 independent gray levels on each. The image resolution was 512 × 512 pixels. The principles of image analysis are described, for example, in Moran *et al.* (1989).

The image of a single larva was transferred from a microscope (Nikon Optiphot) to the computer. Before every image transfer, the brightness value was optimized to give the best possible wave form for the image. Light intensity of the microscope and the contrast value of the system were kept constant. Cuticular outline of the larvae was digitized by moving a pointer on the screen, and the digitized area was copied out from its back-

Table 1. Accumulated soil temperatures (threshold 4.4 °C) in the potato pots grown in water baths with four different temperatures and for three different growing periods.

Time as days	Temperatures			
	12 °C	16 °C	20 °C	24 °C
59	448	684	920	1156
69	524	800	1076	1352
79	600	916	1232	1548

Table 2. Monthly means of soil temperatures (depth 10 cm) and accumulated temperatures (threshold 4.4 °C) in Jokioinen calculated from the meteorological data for the years 1981-1989.

	Monthly means	Accumulated temperatures
May	8.3 (5.2-9.8)	122 (48-164)
June	13.5 (11.7-15.8)	395 (267-487)
July	16.4 (15.3-19.3)	767 (619-948)
August	15.0 (13.2-15.9)	1016 (892-1294)
September	10.6 (8.6-12.1)	1278 (1023-1525)

ground. The gray levels from 5 to 256 were grouped into 16 subsets and the subset-areas of gray-levels 5-96 (colors from dark red to orange) were used in the estimation of the stained areas. The absolute size of every subset and their proportion of the whole nematode were measured, the sum of the absolute sizes being the estimate for the size of a larva. The size value was an image unit representing a proportion of a standard image of the processor. Altogether 300 larvae, 25 per treatment, were analyzed and the computer-aided estimation was then compared to a manual estimation of 300 larvae. In the manual estimation the stained areas were marked on graph paper using a Wild 20 microscope with a drawing tube and the areas were determined from the drawings on the paper.

The differences in the numbers of larvae and in the estimates for size and lipid contents were analyzed with SAS statistics (ANOVA/Contrast) and the regression of accumulated soil temperature on lipid reserves was calculated with SPSSX statistics.

Accumulated soil temperatures of the pots were compared to field data from the years 1981-1989. The soil temperature data was obtained from the routine measurements made by the Finnish Meteorological Institute at Jokioinen. The mean soil temperatures and heat accumulation were calculated as described in Tiilikkala (1987). In the pot experiment the effective heat sum (over the base 4.4 °C) varied from 448 to 1548 day degrees and the soil temperature 16 °C represented well the typical outdoor temperature measured for the growing seasons 1981-1989 (Tables 1, 2).

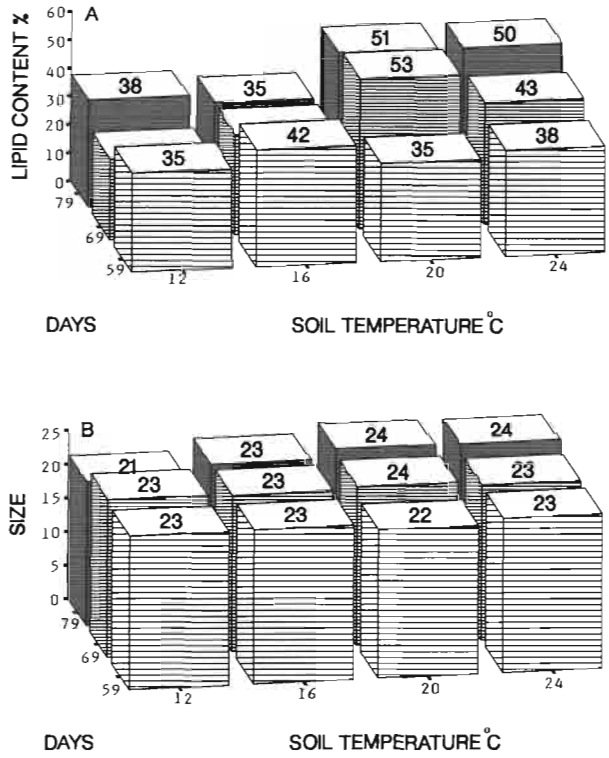

Fig. 1. Lipid content (A) and size (B) of *Globodera rostochiensis* larvae developed at four soil temperatures and for three developmental time periods. Lipid content was estimated as percent of the total area of a larva and size as percent of total image.

Results

Energy reserves

The estimated energy reserves of the larvae which developed at four different soil temperatures and for three different time periods are presented in Figure 1 *a*. Mean lipid contents were 33.6 at 12 °C, 37.6 at 16 °C, 46.0 at 20 °C and 44.2 at 24 °C when all developmental times were pooled at a temperature. Two factors, temperature ($F = 10.07^{***}$) and the interaction of time and temperature ($F = 4.93^{***}$) have a significant effect on the estimated lipid content of the larvae. The effect of developmental time alone was not significant ($F = 2.44$). The highest lipid content value (mean 53 %) was estimated in the treatment where larvae developed at 20 °C for 69 days. It was significantly higher than in all other treatments except in those where the larvae developed at 20 °C for 79 days and at 24 °C for 79 days. The relationship between accumulated soil temperature and estimated lipid content of the larvae is shown in the Figure 2 *a*. It indicated that the increase of accumulated temperature raised the lipid content of the larvae. The quadratic regression model gave a result consistent with the linear model and omitting the highest temperature, did not improve the coefficient of determination. In addition, a positive regression ($P < 0.001$) was found between the soil temperature and lipid content of the larvae with a developmental time of 69 and 79 days (Fig. 2 *b*). The regression coefficient was insignificant for the larvae allowed to develop for 59 days. No correlation between the tuber yield and lipid estimates was found ($r = 0.117$).

Size of the larvae

The size estimates of the larvae are presented in the Figure 1 B. The mean size of larvae by temperature were (image units) : 22.4 at 12 °C, 22.9 at 16 °C, 23.2 at 20 °C and 23.3 at 24 °C. The analysis of variance (contrast) showed that larvae which developed at 12 °C for 79 days were significantly smaller than all of the others. No other differences were found. Temperature and developmental time alone did not have a significant effect on the size of the larvae, but their interaction did ($F = 3.66^{**}$). The interaction can be expressed as accumulated soil temperature in this case. A positive and significant ($P = 0.010$) regression between the size of the larvae and accumulated soil temperature was found.

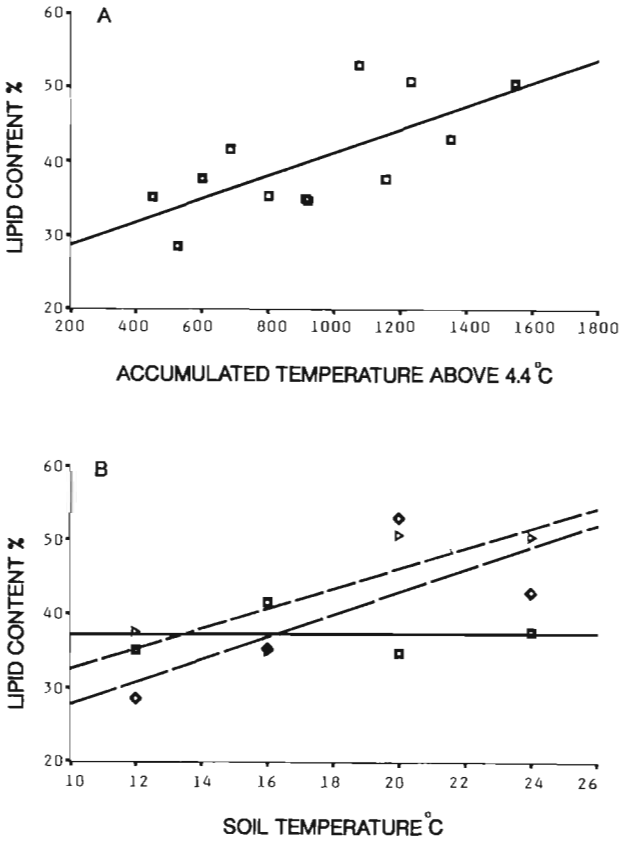

Fig. 2. Relationships between the initial lipid content of *Globodera rostochiensis* larvae and (A) accumulated soil temperature, and (B) temperature during the development of the larvae. In the regression *A* : RSQ = 0.495, P = 0.001 and *Y = 25.5 + 0.016 X*. In the regression *B* : 59 days displayed (----), *Y = 36.9 + 0.02 X;* 69 days displayed (-- --), *Y = 12.5 + 1.52 X;* 79 days displayed (- - -), *Y = 18.9 + 1.36 X*.

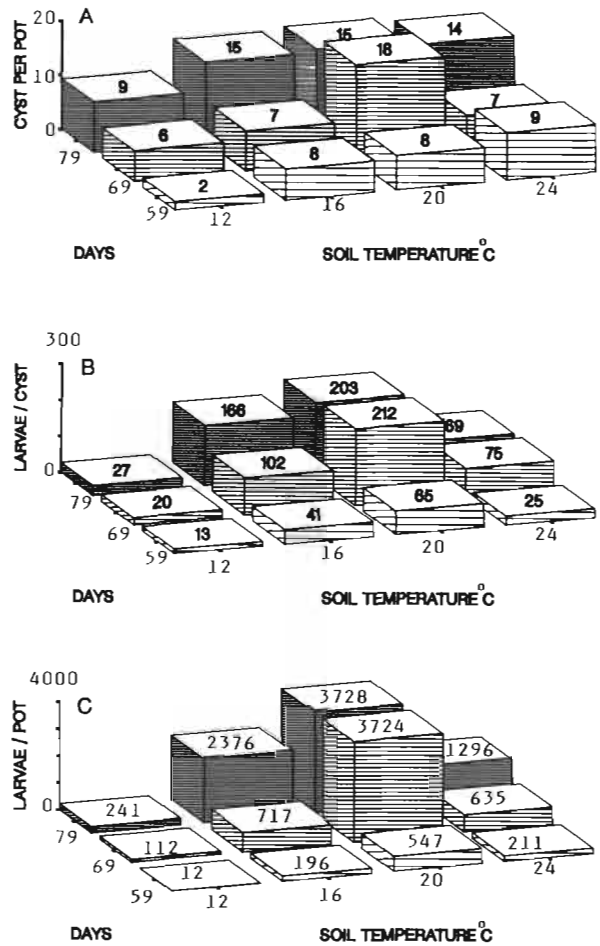

Fig. 3. Final population density of *Globodera rostochiensis* in potato pots grown at four soil temperatures and for tree growing time periods. A : Cysts per pot; B : Larvae per cyst; C : Larvae per pot.

Population density

The number of cysts per pot, larvae per cyst and larvae per pot are presented in the Figure 3. The highest cyst numbers (18/pot) were found in the pots kept at 20 °C for 69 days. The analysis of variances showed this maximum cyst amount to be significantly higher than any other except for that in the pots with the longest developmental time at 16, 20 and 24 °C (Fig. 3 A). All three factors, temperature (F = 4.30**) time (F = 8.60***) and their interaction (F = 3.20***) had a significant effect on the amount of cysts per pot.

The number of the larvae per cyst was highest on the potatoes grown for 69 and 79 days at 20 °C (Fig. 3 B). All of the analyzed factors : temperature (F = 15.79***), time (F = 9.40***) and their interaction (F = 7.12***), had a significant effect on cyst contents. Similarly, the effect of temperature (F = 12.01***), time (F = 9.61***) and their interaction (F = 5.60***) had a significant effect on the number of larvae/pot (Fig. 3 C). The maximum final population density (Pf) was found in the pots grown at 20 °C for 69 (3724 larvae) and 79 days (3727 larvae) and those two densities were significantly higher than the density in the other pots (Fig. 3 C). The number of the larvae per pot was higher the greater the accumulated soil temperature; a positive linear regression between Pf and the accumulated temperature was found (P = 0.006, RSQ = 0.123). The quadratic regression model was significant as well (P = 0.000, RSQ = 0.199). When the highest temperature (24 °C) was not included in the analysis the suitability of fit for both of the regression models was improved : for linear model P = 0.000 and RSQ = 0.474, for the quadratic model the value were P = 0.000 and RSQ =

0.507. The results indicated that 24 °C was detrimentally high for the multiplication of the nematode.

Discussion

The present results showed that the larvae which developed at low soil temperatures had lower energy reserves than those which developed at higher temperatures. Thus the hypothesis presented in Richards (1964), " relatively more energy is required for complete development of insect larvae at minimal temperatures " apparently concerns nematodes, too. It is known, according to general thermodynamic laws, that the lower the temperature the greater the energy requirement for chemical reactions; especially the energy of activation of enzyme-catalyzed reactions is high at minimal temperatures (Watson, 1977). The PCN larvae that developed at 24 °C had high initial lipid contents, although the females reproduced fewer eggs than at 20 °C. This proved that the temperature did not affect lipid reserves by changing the vitality of females, but instead changed the utilization of energy during the completion of larval development. The material which was used in this study to test the influence of temperature on the energy reserves was limited, yet large enough to show the statistical significance of the phenomenon.

Generally, the results suggested that the multiplication of a PCN population depends on the length of the growing season and the soil temperature up to 20 °C; whereas the initial energy reserves of the larvae depend mainly on the soil temperature during the season. A 100 °C increase in accumulated soil temperature raised the lipid estimate by 1.6 units, which was about 3 % of the maximum average lipid content found in this experiment. Other factors such as host cultivar and nematode characteristic may also have an influence on the initial energy reserves of the larvae as Storey (1983) has reported.

For the development of PCN in Finland the soil temperature of 20 °C might be close to the optimum, because all of the measured qualities : reproduction, size and energy reserves of the larvae, reached the maximum values at 20 °C. Inagaki (1984) has also recorded the highest reproduction of PCN at 20 °C and found that the reproduction was generally vigorous at this soil temperature. The regression analysis on the effect of accumulated soil temperature on Pf showed that 24 °C is detrimentally high for the reproduction of the PCN population studied. It is known that different ecotypes of PCN vary in many ways (Mugniéry, 1982) and that PCN populations from separate localities have temperature optimums of their own (Hominick, 1982). Nevertheless, Robinson *et al.* (1987*b*) found that 20 °C is the optimum for the lipid utilization of *G. rostochiensis* and Fenwick (1951) has reported that temperatures above 21 °C greatly restricted the development of PCN in plant tissue.

The energy reserves of the larvae which developed at 16 °C were lower than at 20 °C. Because 16 °C is the normal soil temperature in Finnish field conditions (Table 1) it seems probable that the pest potential of PCN is reduced because of the low initial energy reserves of the larvae. Robinson *et al.* (1987*b*) showed that when the lipid reserves fell below 65 % of the original level, the motility and invasion of the larvae were reduced. In the present experiment, the highest lipid content was 53 % (average). The 65 % level of 53 % lipid content is 34.4 %, and this " critical content " was reached by 46 % of the larvae at 12 °C, by 60 % at 16 °C, by 73 % at 20 °C and by 76 % of the larvae that developed at 24 °C. This suggests that in Northern Europe a part of the larvae of field populations may not be infective at all, or their infective life is short. This might be one of the reasons why PCN has been controlled successfully with short crop rotation (1:3) in Finland (Tiilikkala, 1991), and why a higher proportion of PCN larvae are parasitized by fungi in the northern potato growing areas (Andersson, 1987; Dackman, 1988).

In the future, the warming of the climate as described in Kettunen *et al.* (1989) may greatly increase the pest potential of PCN and other plant parasitic nematodes in Northern Europe. In contrast, the control efficiency of entomoparasitic nematodes in field conditions may be low now, but higher in the future.

The present results showed, as Storey (1984) has already suggested, that the measurement of lipid reserves is of value for modifying the economic thresholds. However, the measurements have not been widely used possibly because of the difficulties of the estimation methods. The computer-aided image analysis which was used in this experiment for the estimation of the lipid reserves of nematode larvae is a rather novel procedure. It turned out to be quick and it gave consistent results, at least with the manual estimation. In the often used scanning microdensitometry technique (Croll, 1972) only the density of stained areas in individual larvae is estimated and the previously used camera technique (Van Gundy *et al.*, 1967; Reversat, 1980) estimates only the area of staining. The image analyses offer both; the intensity of light and the area of staining within the estimate, and the estimation can be done on an individual larva or on many larvae at a time.

Further work is needed to investigate the influence of crop rotation on the lipid reserves of PCN larvae and the use of lipid estimation in the planning of PCN control. In field experiments, also other factors (Croll & Matthews, 1973) affecting the infective life of nematodes must be considered.

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