ORIGINAL RESEARCH

Effect of Slovenian climatic conditions on the development and survival of the root-knot nematode *Meloidogyne ethiopica*

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Abstract *Meloidogyne ethiopica* is a tropical root-knot nematode species which has recently been found in Europe. We examined its ability to survive in open fields located in regions with sub-Mediterranean and continental European climates. The outdoor microplot experiment consisted of two locations and lasted three growing and two winter seasons. It was demonstrated that *M. ethiopica* was able to survive at both locations and also that it retained its infection ability although temperatures below zero were recorded. The correct species was confirmed after each winter season by isozyme electrophoresis. Furthermore, the influence of temperature on the reproduction cycle of *M. ethiopica* was investigated. *Meloidogyne ethiopica* required 67, 48 and 36 days to complete the reproduction cycle at mean daily temperatures of 18.3, 22.7 and 26.3°C, respectively. At 13.9°C, *M. ethiopica* was not able to reproduce. The data obtained from these experiments were used to develop a correlation between temperature and the time needed for *M. ethiopica* to complete a reproduction cycle using a mathematical equation. Furthermore, eight vegetable crops that are important for agricultural production in Slovenia were tested for their suitability as hosts for *M. ethiopica*.

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Introduction

Root-knot nematodes (RKN, *Meloidogyne* spp.) are economically important plant parasites affecting a broad range of host plants including monocotyledons, dicotyledons, herbaceous and woody plants (Eisenback and Hirschmann 1991). They are obligate endoparasites causing substantial crop losses worldwide and attack plant roots by inducing characteristic galls (root-knots) where reproduction occurs. To date, more than 90 nominal species of the *Meloidogyne* genus have been described, of which 12 are considered to be emerging global plant pests: *M. acronea, M. arenaria, M. chitwoodi, M. enterolobii, M. ethiopica, M. exigua, M. fallax, M. graminicola, M. hapla, M. incognita, M. javanica* and *M. paranaensis*. These 12 species have received more attention than any other RKN because they are important agricultural pests that are distributed worldwide or have the potential to emerge as plant pests in the near future (Hunt and Handoo 2009).

Meloidogyne ethiopica was described by Whitehead in 1968 from the Mlalo region of Tanzania from a sample isolated from a single egg mass culture found on the type host tomato. It has been recorded in Africa in Tanzania, Kenya, Ethiopia, Mozambique, Zimbabwe and South Africa (Whitehead 1968, 1969); in Latin America in Brazil (Carneiro et al. 2003) and Chile

(Carneiro et al. 2004); and in Europe in Slovenia (Širca et al. 2004).

In Slovenia, *M. ethiopica* was recorded for the first time in 2003 on tomato plants in a greenhouse situated in the village of Dornberk with no linkage to imported plant material (Širca et al. 2004). In Chile, *M. ethiopica* was found to be the major root knot nematode parasite of grapevines (*Vitis vinifera*) and other crops (Carneiro et al. 2007). This species is currently widely distributed throughout various locations in Chile and is associated with difficulties in eradication due to the lack of a rootstock that has resistance to this species (Aballay et al. 2009). In Brazil, *M. ethiopica* affects the root growth, yield and quality of grapevines and kiwi (*Actinidia deliciosa*), causing serious crop damage and economic losses (Carneiro et al. 2003).

Meloidogyne ethiopica is considered a tropical, polyphagous pest that is able to parasitise at least 80 different host plants, including many economically important dicotyledons and monocotyledons in the families *Alliaceae, Amaranthaceae, Apiaceae, Asteraceae, Brassicaceae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Poaceae, Polygonaceae, Rosaceae* and *Solanaceae* (Whitehead 1968; O'Bannon 1975; Carneiro et al. 2003, 2004; Strajnar et al. 2009; Lima et al. 2009). However, few investigations have focused on host suitability to *M. ethiopica*. Host status is defined by the ability of the nematode species to multiply on the plant host (Liebanas and Castillo 2004), and it is measured as the final nematode population density compared to the initial population density (Pf/Pi), displaying the extent of nematode species multiplication that can occur on the certain host. The host status provides valuable information that can be used to build an efficient integrated pest management strategy. The cultivation of resistant non-host or poor hosts substantially reduces RKN populations, thus limiting the use of chemical nematicides. Therefore, our studies determined the host status of some crop species that are relevant to Slovenian and overall European agriculture.

Climatic conditions, particularly temperature, are important for the development and distribution of RKN, especially in relation to their ability to survive cooler environmental conditions. *Meloidogyne ethiopica* is a tropical species that requires certain temperatures for successful reproduction.

As an additional objective of our studies, we investigated the ability of *M. ethiopica* to survive the natural environmental conditions at two distinct locations in Slovenia over two consecutive winter seasons. In addition, the length of the *M. ethiopica* reproduction cycle at optimal, low and high temperatures were investigated in growth chamber experiments.

Materials and methods

A population of *M. ethiopica* isolated from infected tomato roots from the Dornberk site (Širca et al. 2004) was grown from egg masses and maintained on beans (*Phaseolus vulgaris* cv. Top crop) planted in sterile sand kept at 20–25°C in a glasshouse for 50 days. The inoculum was prepared from an extraction of eggs taken from the infected bean roots using 1% sodium hypochlorite that was applied for 4 min to dissolve the gelatinous matrix surrounding the root-knot nematode eggs (Hussey and Barker 1973). The suspension of eggs was washed through 850, 250 and 32 μm banked sieves, and the eggs that were extracted on the lower sieve were washed with tap water to remove the NaOCl (Ehwaeti et al. 1998). The extracted eggs were rinsed in 40 ml of water, placed into 50 ml polycarbonate centrifuge tubes and centrifuged at 1500 rpm for 5 min. The pellets were re-suspended in 40 ml of sucrose solution (454 g sucrose per 1 l of tap water) and centrifuged at 1000 rpm for 1 min (McClure et al. 1973). The supernatant containing the eggs was poured through a 32 μm banked sieve and washed with tap water. Finally, the eggs were removed from the sieve, rinsed and counted.

Host plant test

Dicotyledonous and monocotyledonous plants were tested for their suitability as hosts for *M. ethiopica*. Plant species included beet, *Beta vulgaris var. conditiva* cv. Bikor; cauliflower, *Brassica oleracea var. botrytis* cv. Snežna kepa; chard, *Beta vulgaris var. cicla* cv. Srebrnolistna; kale, *Brassica oleracea var. subauda* cv. Železna glava; kohlrabi, *Brassica oleracea var. gongylodes* cv. Dunajska bela; maize, *Zea mays* cv. Benicia; spinach, *Spinacia oleracea* cv. Norvak; and tomato, *Solanum lycopersicum* cv. San Marzano F1. Seeds were planted in 16 cm diameter pots filled with a mixture of fine sterilised sand (0.25–1.0 mm) and hydroculture granules (size 1–4 mm) in a 3:1 volume ratio. The experiment was set up outdoors

under a roof to protect the plants against the rain and was conducted from May to July, 2009. During this period, the mean air temperature was 19.6°C, ranging from 12.9°C to 25.8°C in May, from 13.4°C to 24.3°C in June and from 15.4°C to 26.2°C in July. Temperature data were obtained from the Environmental Agency of Slovenia. The plants were watered and fertilized as needed. The nutrient concentration depended on the stage of plant development. Forty-five days old plants were inoculated with an aqueous suspension containing 5000 *M. ethiopica* eggs. The experiment was terminated 69 days after inoculation and the final population of *M. ethiopica* determined. Three to four test plants of each crop were evaluated for the nematode reproduction rate. Second stage juveniles were isolated from the substrate using a decanting method (Hržič 1973) followed by Bearman's funnel extraction. The nematode eggs were isolated as previously described for the inoculum preparation. The reproductive factor R=Pf/Pi was used as a measure of host suitability and was calculated as the final nematode population (eggs+J2) divided by the initial nematode population (eggs only). Four host categories were defined based on the R values (Ferris et al. 1993): $R>10$=excellent host; $10>R>1$=good host; R close to 1=maintenance host and $R<1$=poor host to non-host. Data were statistically analysed using an analysis of variance (ANOVA), and significant differences in the means of the reproduction factor values were ranked using Duncan's multiple range test at P=0.05 in the Statgraphics XV software package.

Effect of temperature on *Meloidogyne ethiopica* development

The time needed for one reproduction cycle was determined in a growing chamber experiment at four separate experiments at mean daily temperatures: 13.9, 18.3, 22.7 and 26.3°C. Bean plants, *Phaseolus vulgaris* cv. Meraviglia di venezia nano; chicory, *Cichorium intybus var. foliosum* cv. Castelfranco; cucumber, *Cucumis sativus* cv. Dolge zelene and tomato, *Solanum lycopersicum* cv. Volovsko srce were used to determine the time. The plants were planted in 9 x 9 cm pots filled with 560 g fine sterilised sand (particle site 0.25–1.0 mm). The appropriate amount of water and nutrients for hydroponic growth were added according to the manufacturer's instructions (Flora

series, General hydroponics Europe) to achieve 15% moisture based on the dry sand weight. The growth conditions included 13 hours of illumination and 60% relative humidity at average daily temperatures of 13.9°C (15°C day/12.7°C night), 18.3°C (21°C day/15°C night), 22.7°C (25°C day/20°C night) and 26.3°C (30°C day/22°C night). Chicory was used as host plant for the experiment at 13.9°C, whereas bean, cucumber and tomato were used at 18.3, 22.7 and 26.3°C average temperature. The tomato, cucumber, chicory, bean plants were at 43, 43, 37 and 15 days after seeding, respectively inoculated with an aqueous suspension of 3000 *M. ethiopica* eggs per plant. The aqueous solution of eggs was prepared according to previously described methods. To evaluate the duration of the *M. ethiopica* reproduction cycle, the nematode egg development was examined in two pots of each host plant every fourth day starting at 20 days post inoculation (DPI) in the experiment at 26.3°C, 24 DPI at 22.7°C, 51 DPI at 18.3°C and 60 DPI at 13.9°C.

In addition, the temperature-dependent development equation was calculated by regressing the development times against temperature using TableCurve 2D (Systat Software, USA). The equation was based on best fit, algebraic simplicity and a logical description of the nature of the data.

Microplot experiments

The experiments to determine survival of *M. ethiopica* were conducted over three growing and two winter seasons in two locations in Slovenia. The first site, representing a continental climate was located in Ljubljana, the second site was located in a sub-Mediterranean climate in Bilje. For both locations two 50 l vessels were buried and filled with soil. In May 2007, two tomato plants (cv. Volovsko srce) were planted in the vessel and inoculated with eggs of *M. ethiopica*. In 2008 and 2009 new tomato seedling were re-planted in the vessels. Every year in the autumn, the tomato stems were cut, leaving the infected roots in the soil. The survival of the nematodes was examined after the winter seasons 2007/2008 and 2008/2009. A re-identification of the nematode species was done by isozyme phenotyping of young egg-laying females (Esbenshade and Triantaphyllou 1985) using PhastSystem (GE Healthcare Life Sciences) gel electrophoresis.

Results

Host plant test

Both, dicotyledonous and monocotyledonous plants were suitable hosts for *M. ethiopica*. Reproductive factors showed the highest reproduction rates on tomato (*R*=91.20), intermediate rates on spinach, beet and chard, and the lowest on cauliflower, kale, kohlrabi and maize (Table 1). In terms of host status tomato, spinach, chard and beet were considered as excellent hosts. Among the excellent hosts, tomato had reproductive factors 1.8, 5.0 and 7.6 times higher than spinach, chard and beet, respectively (Table 1). Maize and kohlrabi were identified as good hosts, but they had significantly lower reproductive factors of 2.87 and 4.47, respectively. Kale turned out to be a maintenance host with an R value above 1.0, while cauliflower was shown to be a poor host with an R value below 1.0 (Table 1).

Effect of temperature on *Meloidogyne ethiopica* development

No reproduction of *M. ethiopica* was observed at 13.9°C. For up to 120 DPI, chicory plants showed no root galling or signs of reproduction. At the average daily temperatures of 18.3, 22.7 and 26.3°C *M. ethiopica* was able to complete reproduction cycle. Eggs were observed after 51, 24 and 20 DPI at the average daily temperatures of 18.3, 22.7 and 26.3°C, respectively (Fig. 1).

In 18.3°C temperature experiment nematode eggs were observed at 51 DPI on tomato and bean plants. Eggs were detected on cucumber plants eight days later. Maximum of *M. ethiopica* eggs were obtained at 67 DPI on bean, cucumber and tomato plants (Fig. 1).

In the experiment at 22.7°C, the eggs were collected on bean plants at 24 DPI and four days later on cucumber and tomato plants. The maximum of nematode eggs were detected at 48 DPI (Fig. 1). Additionally, the highest nematode multiplication rate was determined in this experiment where the final number of eggs increased by 113 times on bean plants.

At 26.3°C, the eggs were first observed on tomato plants at 20 DPI followed by cucumber plants four days later. The number of eggs reached maximum at 36 and 40 DPI on tomato and cucumber plants, respectively (Fig. 1). Beans were excluded from the experiment at 26.3°C since growth conditions were unsuitable for bean plants (plants wilted and declined). The results demonstrated that approximately 67, 48 and 36 days are required for the reproductive cycle to be completed at 18.3, 22.7 and 26.3°C, respectively (Fig. 1). These results were used for the estimation of a correlation between temperature and the duration of the *M. ethiopica* reproduction cycle. The reproduction curve was defined with the following equation:

$$\ln(y) = 5.2115 - 0.01892 * x * \ln(x).$$

The adjusted regression coefficient and the standard error for the calculated equation were 0.9999 and 0.05,

Table 1 Reproductive factors and host status of *Meloidogyne ethiopica* for eight plant species inoculated with 5000 eggs per plant

Plant species	Cultivar or breeding line	Num. of replicates	*R*[1]	Host status[2]
Beet	Bikor	4	12.06 b[3]	E
Cauliflower	Snežna kepa	4	0.33 a	P
Chard	Srebrnolistna	4	18.26 b	E
Kale	Železna glava	3	1.48 a	M
Kohlrabi	Dunajska bela	3	4.47 a	G
Maize	PR38F70 SI	4	2.87 a	G
Spinach	Norvak	3	49.32 c	E
Tomato	San Marzano F1	3	91.20 d	E

[1] *R* = Pf/Pi (final population/initial population).

[2] Host status categories: E = Excellent (*R*>10); G = good (10>*R*>1); M = maintenance (*R*+/− 1); P = poor to nonhost (1>*R*>0).

[3] In each column, means followed by the same letter are not significantly different according to Duncan's multiple range test (*P*=0.05).
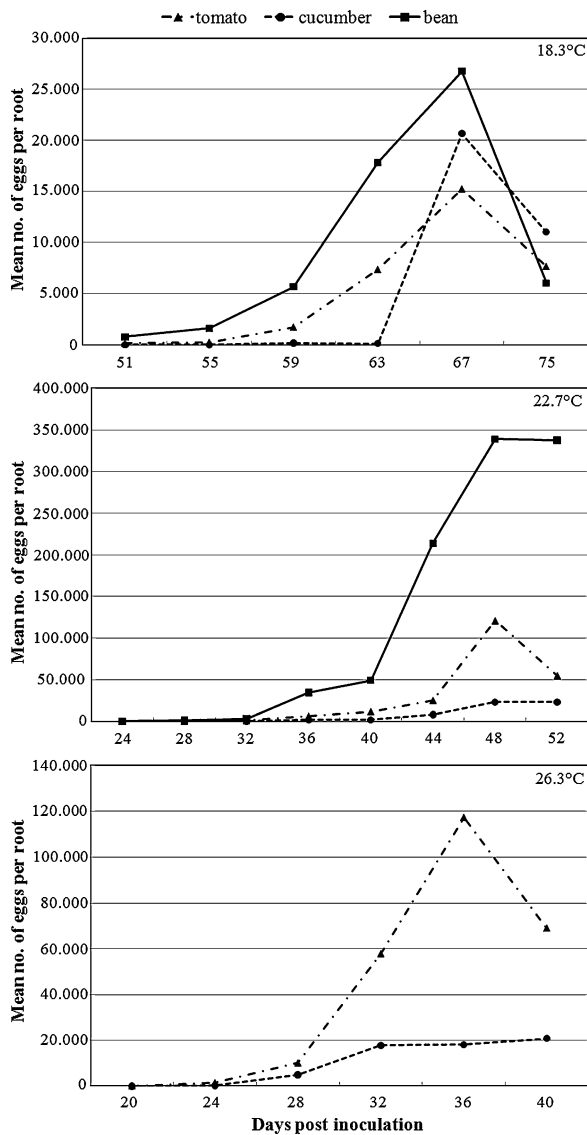
Fig. 1 Number of *Meloidogyne ethiopica* eggs after inoculation with 3000 eggs from tomato, cucumber and bean plants in three experiments with mean daily temperatures of 18.3, 22.7 and 26.3°C. The graphs show the number of days needed for development of maximum number of eggs indicating completed reproduction cycle at certain temperature

respectively. The reproduction curve covers temperatures ranging between 18.3°C and 26.3°C (Fig. 2).

Microplot experiments

The microplot experiments were conducted from May 2007 to June 2009. The monthly soil temperatures at a depth of 5 cm in Ljubljana were approximately 2°C lower compared to the Bilje site. In the first season

(May–October 2007), the average soil temperatures in Bilje ranged from 13°C to 27°C and from 11.6°C to 24.3°C in Ljubljana (Fig. 3). The temperatures during the winter were the key factors affecting the survival of *M. ethiopica*. In the winter of 2007/2008, the soil temperatures in Ljubljana were below zero for 20 days, while at the Bilje location, the soil temperatures were always above zero. After the first winter season, re-infection of tomato plants was found at both locations. Re-identification confirmed that plants were indeed infected by *M. ethiopica*. In the 2008 growing season, the average temperatures ranged from 12.6°C to 23.7°C in Ljubljana and from 14.8°C to 26.4°C in Bilje (Fig. 3). The temperatures in the second winter 2008/2009 were below zero for 20 days in Ljubljana and for 10 days in Bilje. In the 2009 growing season, a re-identification of the nematodes confirmed that the *M. ethiopica* population had survived the low winter temperatures and retained infection ability in the open field at both locations.

Discussion

In these studies, the effects of temperature and host plants on survival and reproduction of *M. ethiopica* were investigated. *Meloidogyne ethiopica* was first observed in Tanzania, Africa. Published reports from South America have described plant damage caused by *M. ethiopica,* and the species has recently been classified into the group of pest species that require special attention (Hunt and Handoo 2009). *Meloidogyne ethiopica* infestations in Chilean vineyards are a permanent problem (Carneiro et al. 2007;
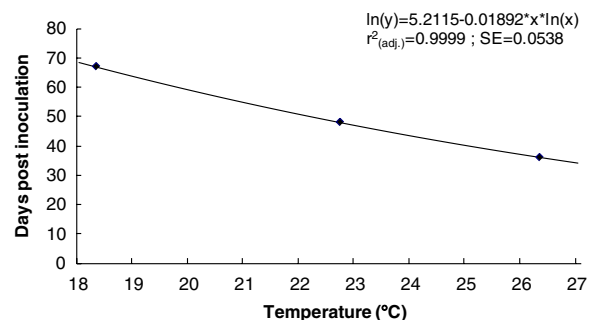
Fig. 2 The reproduction curve describing the correlation between temperatures ranging from 18.3°C to 26.3°C and the duration (days) for *M. ethiopica* to complete the reproduction cycle

Fig. 3 The monthly average, minimum and maximum soil temperature at a depth of 5 cm for Ljubljana and Bilje from May 2007 to June 2009. Data were obtained from the Environmental Agency of the Republic of Slovenia
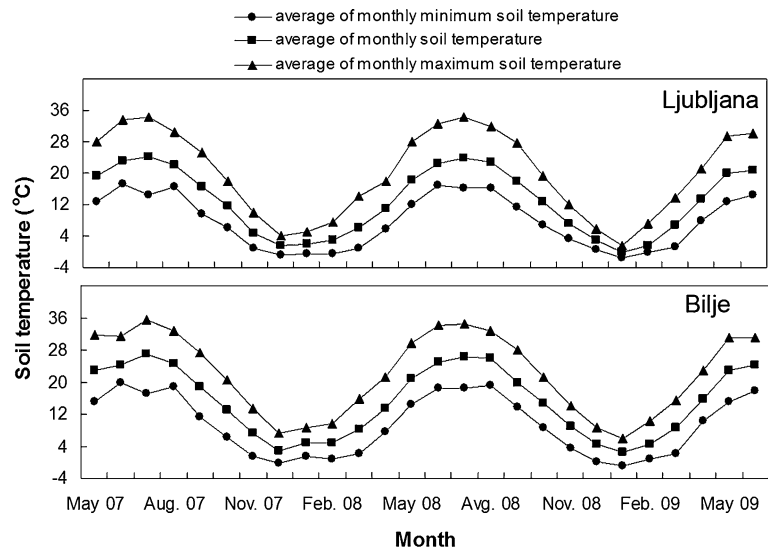
Aballay et al. 2009) and also represent a serious risk to grapevine production in Brazil, where there are 67,800 ha in cultivation (Carneiro et al. 2003; Lima et al. 2009). In Europe, *M. ethiopica* has only been recorded in Slovenia (Širca et al. 2004).

The ability of *M. ethiopica* to survive under continental climate conditions that are characterised as mild, with hot summers and cold winters, as well as in a sub-Mediterranean climate characterised by hot summers and mild winters defines the potential risks of spread and establishment for this tropical root-knot nematode species. *Meloidogyne ethiopica* survived under field conditions despite the fact that the winter temperatures fell below zero degrees on several occasions. The survival of the nematode population was monitored for two winter and three growing seasons by root galling which is an indicator of nematode infection. In the second growing season increased root infestation was observed at both field locations. The resultant observations were contrary to our expectation that low temperatures in the winter season would reduce the nematode population. Instead, our results suggest that *M. ethiopica* can survive through the winter period in Europe and, therefore, represent a serious risk for agriculture production, especially in southern Europe.

Approximately eighty plant species have been reported as hosts for *M. ethiopica* (Whitehead 1968; O'Bannon 1975; Carneiro et al. 2003, 2004; Strajnar et al. 2009; Lima et al. 2009), including several crops

grown in moderate climatic zones. Strajnar et al. (2009) tested several vegetable crops that are relevant to Slovenian agricultural production, and all of the tested plants proved to be suitable hosts for *M. ethiopica*. This wide host range is similar to other major RKN species such as *M. incognita* and *M. javanica* which also reproduce by mitotic parthenogenesis. Mitotic parthenogenesis is believed to drive and maintain a wide host range because it slows coevolution and genetic drift (Trudgill 1997). In this study, we determined the host suitability of eight crops that are important to Slovenian agricultural production. A high reproduction rate of *M. ethiopica* was observed on plants from the family *Chenopodiaceae* and *Solanaceae*. Bean plants proved to be excellent hosts, enabling the nematode population to increase 113 times in a single reproduction cycle. Lima et al. (2009) also determined that plants from the *Fabaceae* family are susceptible to *M. ethiopica* infection. Cauliflower and kale were poor or maintenance hosts for *M. ethiopica*; they are members of the *Brassicaceae* family. One of the potential explanations for the reduction in the population of these plants could be the biocidal activity of *Brassicaceae* plant cells that possess a glucosinolate-myrosinase system and therefore are capable of producing a number of biologically active compounds including isothiocyanates, nitriles, epithionitriles and thiocyanates (Fahey et al. 2001). Mojtahedi et al. (1993) established a reduction in a *M. chitwoodi* population with glucosinolate degradation

products. Therefore, *Brassicaceae* plants could represent an efficient approach for *M. ethiopica* management.

Our study determined the generation times at low, optimal and high temperatures, and the reproduction curve between 18.3°C and 26.3°C was calculated. The data also indicated that plant species did not influence the duration of the nematode reproduction cycle. The highest nematode population was obtained at a daily temperature of 22.7°C, which is considered to be the optimal temperature for *M. ethiopica* reproduction. The correlation between temperature and RKN development time has been studied for *M. javanica* by Madulu and Trudgill (1994). In these experiments, the time from J2 inoculation to the development of the first J2 of the next generation was defined as a complete reproduction cycle. These experiments were carried out at temperatures ranging from 18°C to 30°C, and their results showed a linear relationship between developmental time and temperature. The first-hatched J2 were collected after 44, 32, 25 and 24 days at temperatures of 21.1, 23.8, 26.9 and 29.9°C, respectively. Similar results were observed in our study when the first eggs were collected after 51, 24 and 20 days at temperatures of 18.3, 22.7 and 26.3°C, respectively, suggesting that *M. ethiopica* has a developmental time similar to *M. javanica*.

The reproduction curve determined from our results represents a useful tool for further applied research on this pest species. The reproduction curve defined the duration of *M. ethiopica* reproduction cycles at certain temperatures ranging from 18.3 to 26.3°C. These results can be used in further tests of host status determinations, tests of host resistance to *M. ethiopica*, or any tests where the maximum number of the final pest population is important. The reproductive curve can also be used to predict the number of *M. ethiopica* generations that might occur on crops in a growing season.

These results confirm the importance of this pest species, not only for tropical areas but also for Europe. The facts that *M. ethiopica* can survive and maintain its infection ability under climatic conditions present in Slovenia together with its polyphagous nature underpin the importance of this RKN species. The data obtained in this study represent groundwork for more intensive studies needed for a thorough pest risk analyses (PRA) to determine the threat this pest poses to agricultural production in Europe.

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