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Precrop Effect of Red Clover (*Trifolium pratense* L.) and Alfalfa (*Medicago sativa* L.) on the Population Development of the Northern Root-Knot Nematode *Meloidogyne hapla* Chitwood, 1949 and on Succeeding Crops—A Pot Study

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Abstract: The northern root-knot nematode, *Meloidogyne hapla*, is a major pest of many crop species. The objective of the study was to determine how *M. hapla* population dynamics is affected by two precrops, i.e., *Trifolium pratense* and *Medicago sativa*, in three crop durations: one, two and three years of continuous cultivation. Moreover, we set ourselves the task of evaluating the effect of the legume precrop soil on the growth of the succeeding tomato plant (*Solanum lycopersicum*) and on the nematode population. The experiment was performed outdoors in pots with naturally infected soil. Both precrop species investigated were found to modify the J2 nematode population density in the soil. The galls and nematode females with egg masses were observed on the roots of both studied plant species at the end of each growing season. They appeared to be more abundant on the red clover roots than on those of the alfalfa. The obtained data indicate that the spring soil sampling is more appropriate for the estimation of the *M. hapla* population density in the red clover precrop soil. The legume precrop soil had a limiting effect on tomato growth and fruit yield. The nematode population negatively influenced tomato growth. The experiment revealed that tomato plants could be planted in alfalfa precrop soil following at least three years of continuous alfalfa cultivation. The same cannot be said of the cultivation of red clover as a precrop for tomatoes.

Keywords: plant parasitic nematodes; population dynamics; crop rotation; legume precrops; tomato

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1. Introduction

Although red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) are Central Asian in origin, nowadays these plant species have a broad distribution worldwide, primary throughout temperate and subtropical regions [1–4]. They belong to the legume family and, like birdsfoot trefoil (*Lotus corniculatus* L.) for example, they represent the small-seeded legumes that produce herbage for harvesting as forage. They are grown for hay, dehydrated forage, pellets, silage, seeds and occasionally grazing. Their cultivation is known to significantly increase total soil nitrogen and organic carbon content, and improve soil structure, its water permeability and water retention capacity [5–14]. Both *T. pratense* and *M. sativa* can be grown in pure stand, but red clover is often mixed with tall-growing grasses such as Italian ryegrass (*Lolium multiflorum* Lam.), hybrid ryegrass (*Lolium boucheanum* Kunth.), timothy (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* Huds.) or tall fescue (*Festuca arundinacea* Schreb) [1,14,15].

Due to the high forage quality and the multiple effects of both alfalfa and red clover cultivation on soil condition, there is a substantial body of research into their cultivar development as well as the improvement in their cultivar yield [16–20].

The alfalfa and red clover cultivation have an impact on the diversity of the nematode community composition in the soil as well as on its seasonal fluctuations [21]. A wide range of the plant-parasitic nematodes were associated with alfalfa and red clover in various countries [21–26]. *Ditylenchus dipsci* (Kühn, 1857) and *Aphelenchoides ritzemabosi* (Swartz, 1911) are serious pests to the underground parts of plants, whereas *Heterodera trifolii* (Goffard, 1932), *Pratylenchus penetrans* (Cobb, 1917) and the root-knot nematodes *Meloidogyne* spp. infect roots and cause significant yield losses [27–31]. The genus *Meloidogyne* represents sedentary endoparasites with a group of around 10 damaging species, including the northern root-knot nematode *Meloidogyne hapla* (Chitwood, 1949). The annual economic losses due to *Meloidogyne* parasitism are estimated at several billion U.S. dollars [32]. *M. hapla* is considered a major pest to crops in regions with a temperate climate, especially roses (*Rosa* spp.), celery (*Apium graveolens* L.), carrot (*Daucus carota* L.), lettuce (*Lactuca sativa* L.) and tomato (*Solanum lycopersicum* L.) [33–37].

Tomatoes are one of the most important horticultural crops, with an estimated annual production of over 150 million tons globally [38] and 253 thousand tons in Poland [39], respectively. It is recommended for tomatoes to be precropped for one year with small-seeded legumes. However, if continued for two or three growing seasons, this treatment was proven to be unfavourable for the following tomato crop due to intensive reproduction of multivorous insects in the soil, i.e., larvae of Elateridae, Noctuinae and Bibionidae [40,41].

The objectives of the study were (1) to estimate changes in the density of *M. hapla* population in the soil under the cultivation of *M. sativa* and *T. pratense* for one, two and three successive years, and to estimate *M. hapla* population development on the roots during *M. sativa* and *T. pratense* vegetation; (2) to evaluate the effect of *M. sativa* and *T. pratense* precrop soil for the tomato plant and the *M. hapla*.

2. Materials and Methods

2.1. Red Clover and Alfalfa Cultivation and Estimation of the Root-Knot Nematode Population Development

The population dynamics of *M. hapla* in the cultivation of *T. pratense* and *M. sativa* were assessed in a pot study. The experiment was conducted outdoors from 2016 to 2018, under natural conditions, at the Institute of Plant Protection-National Research Institute (IPP-NRI) in Poland (GPS 52°23′48″ N, 16°51′20″ E). It was designed according to the experiment planning scheme presented in Table 1.

Table 1. Scheme of experiments performed from 2016 to 2019.

Precrop	Precrop Treatment Duration			Year of *Solanum lycopersicum* Cultivation
Trifolium pratense			2016	2017
		2016	2017	2018
	2016	2017	2018	2019
Medicago sativa			2016	2017
		2016	2017	2018
	2016	2017	2018	2019
Bare fallow			2016	2017
		2016	2017	2018
	2016	2017	2018	2019

Seventy-two plastic pots, 30 cm in height and 25 cm in diameter, were filled with 10 dm^3 of sandy loam soil naturally infected with *M. hapla* at the density of 50 specimens of second stage juveniles (J2) per 200 cm^3 of soil (Pi—initial population density). Each pot was individually planted with three germinated seeds of *T. pratense* "Krynia" or *M. sativa* "Blue Moon" in April of 2016. All pots were fertilized before sowing and every October thereafter

from 2016 to 2018, with P_2O_5—0.4 g and K_2O—0.4 g per pot. Plants were watered during the summer to obtain an optimal water-holding capacity, as required. Each year, red clover and alfalfa crops were mowed twice using secateurs. Weeds were manually removed. Following the plant harvest, the contents of the pots were emptied into a litter box and the plant roots were gently cleaned to rinse away the adhering soil particles. The soil was mixed and placed back into the pots. Soil samples of 200 cm^3, consisting of four randomly taken subsamples of 50 cm^3, were taken to assess the density of the J2 stage. Nematodes were extracted from the soil using the sieve-centrifuge method [42] and counted using a stereoscopic microscope (Pf—final population density). In the study, bare fallow served as a control. The study was conducted in eight replicates. The initial population density of *M. hapla* in soil (Pi) was determined in mid-April and the final density (Pf) was assessed in the last week of September, for each year of the experiment.

In the last week of September, plants were removed from pots and root systems were examined for nematode gall and nematode female numbers. The roots of *T. pratense* and *M. sativa* were carefully washed of adhering soil particles and were stained with acid fuchsin in lactoglycerol [43]. The root galls and nematode females with egg masses attached were screened and counted with a stereoscopic microscope.

The assessment of *M. hapla* population in the soil and nematode development in roots were performed in the second and third year of cultivation, according to the methods described above. The pots containing soil infected with *M. hapla* were left for the following season and were used in order to evaluate tomato cultivation in red clover and alfalfa precrop soil, respectively, as well as in bare fallow soil.

2.2. Succeeding Plant Cultivation and Determination of the Nematode Population Development

The seedlings of tomato *S. lycopersicum* "Krakus" were planted in pots of 5 L capacity (21 cm in height and 17 cm in diameter) filled with precrop soil, bare fallow soil and control soil, according to standard crop recommendations (May 2017, 2018 and 2019). In the spring of 2017, samples of 200 cm^3 were collected from overwintered pots filled with soil infected with *M. hapla*. The J2 stage nematodes were isolated using the sieve-centrifuge method [42] and counted. Plastic pots were filled with soil infected with *M. hapla* at the density of 20 J2 specimens per 200 cm^3. The soil with the nematode population of the density corresponding to that in the alfalfa and clover precrop pots was used (bare fallow soil). Soil without nematodes was used as a control variant (control soil).

Throughout the growing season, tomato plants were watered and fertilized at fortnightly intervals, using a complete nutrient solution (N 3%, K2%, Cu 70 mg/L, Fe 400 mg/L, Mn 170 mg/L, Mo 20 mg/L, Zn 150 mg/L) in a dilution of 1:400, at a dose of 50 mL per pot. At the flowering growth stage, flower shoots and flowers were counted at two-day intervals and the fruit mass was weighed weekly. After flowering and fruit production, the tomato plants were harvested, and their roots were examined for root galls and nematode females with egg masses using the method described by Hooper [43]. Plant development was characterized by the parameters of plant height, shoot weight and root weight. An evaluation of the nematode population dynamics, tomato plant development and fruit yield were performed after the second and the third year of the alfalfa and red clover precrop cultivation in 2018 and 2019, respectively.

2.3. Statistical Analysis

Data from pot experiments were subjected to an ANOVA variance analysis, and the significance of differences between means were assessed by Fisher's test at the level of $p \leq 0.05$.

3. Results

3.1. Red Clover and Alfalfa Cultivation and Estimation of the Root-Knot Nematode Population Development

The experiment showed that the cultivation of *T. pratense* and *M. sativa* affected the dynamics of the *M. hapla* J2 density in soil, the number of galls in the roots and the number of female nematodes. A marked decrease in the average J2 density was observed in the autumn of 2016, following the first growing season of red clover and alfalfa, as was the case for the bare fallow soil (Figure 1). Two consecutive growing seasons demonstrated that the J2 population dynamics found in the red clover precrop soil differed from changes in nematode population observed in the alfalfa precrop soil and in bare fallow soil. The mean number of J2 specimens per unit of red clover precrop soil was distinctly higher in spring than in autumn. In the alfalfa precrop soil, the J2 densities were low, and a significantly higher average number of J2 individuals per soil unit was isolated in the spring of the third growing season. Three consecutive fallow years resulted in the decrease in the nematode population density in the soil below the level of detection. The results are shown in Figure 1.

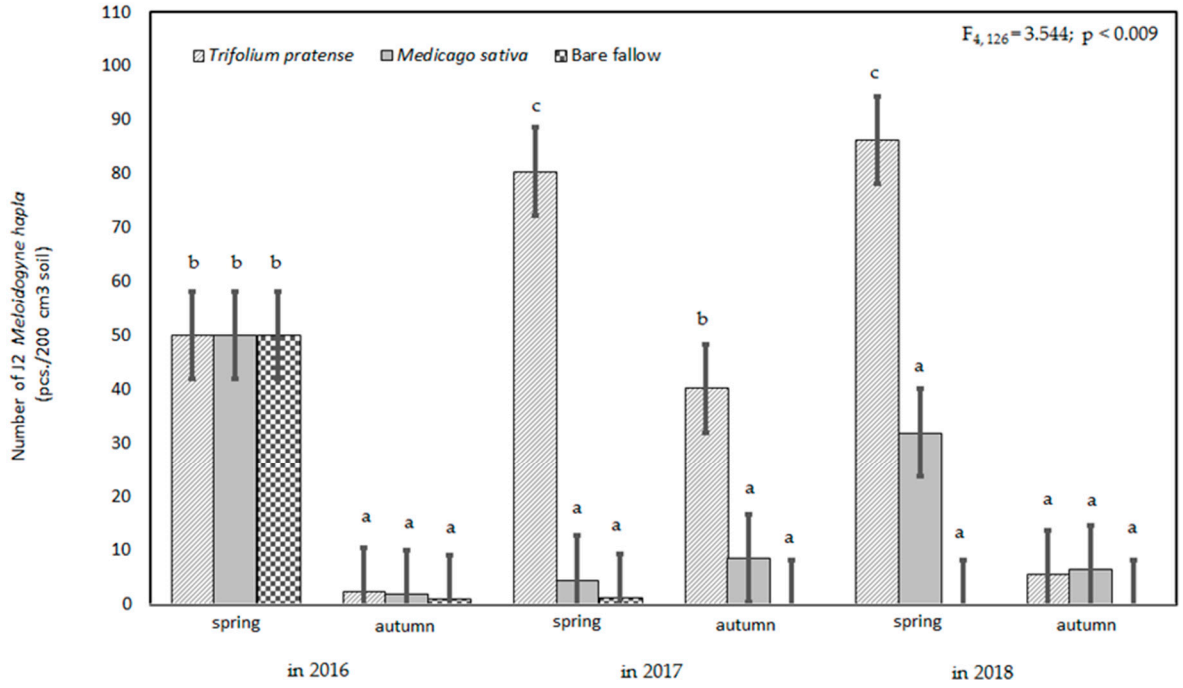

Figure 1. Population dynamic of J2 juveniles of *Meloidogyne hapla* in soil with cultivation of *Trifolium pratense*, *Medicago sativa* and in bare fallow (mean ± SE). Bares with different letters above are statistically different from each other (Fisher's test, $p \leq 0.05$).

The galls and the female nematodes with egg masses were observed on the roots of all investigated plant species. The galls were counted throughout the duration of the experiment, but the differences were not statistically significant (Figure 2). The females with egg masses were statistically more numerous on roots of *T. pratense* (Figure 3).

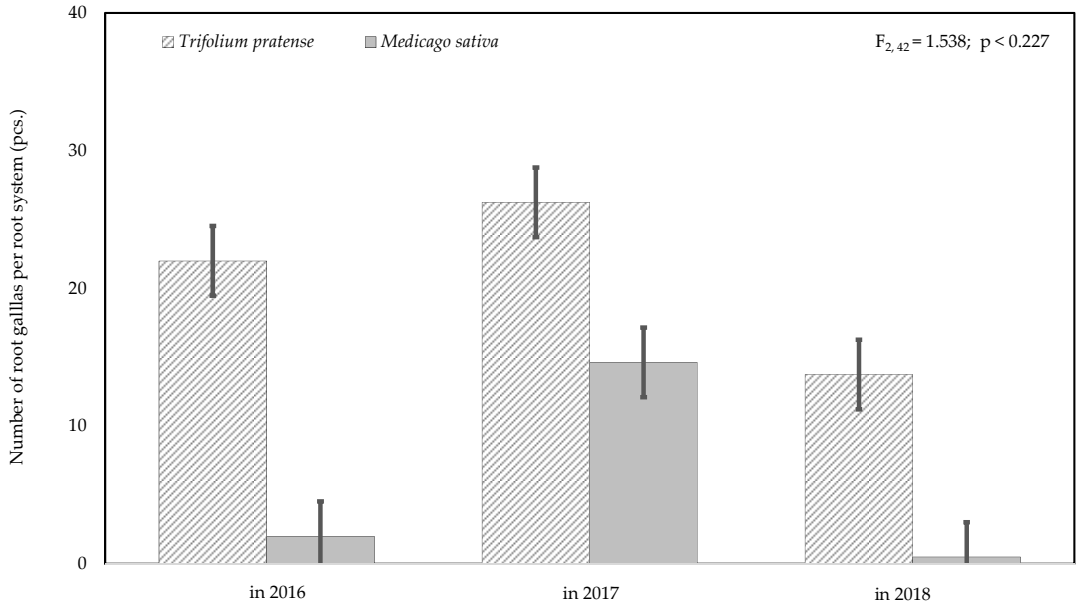

Figure 2. Root galling by *Meloidogyne hapla* after first, second and third year of plant vegetation (mean ± SE).

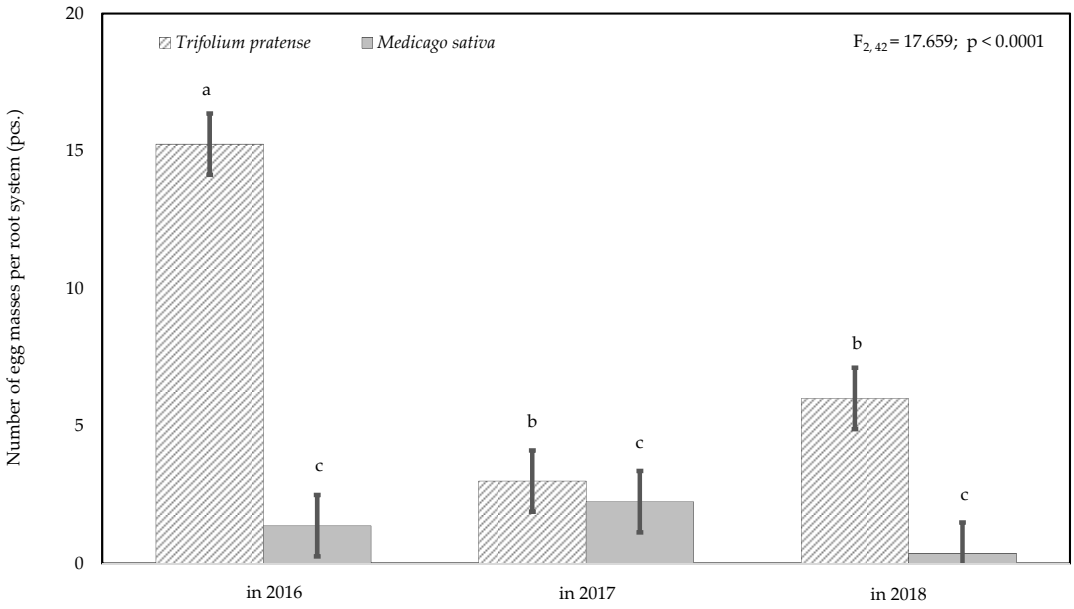

Figure 3. Egg masses of *Meloidogyne hapla* in roots of *Trifolium pratense* and *Medicago sativa* after first, second and third year of plant vegetation (mean ± SE). Bars with different letters above them are significantly different from each other (Fisher's test, $p \leq 0.05$).

3.2. Succeeding Crop Cultivation—Tomato and Evaluation of the Nematode Population Development

The results of the experiment revealed statistically important differences among the mean values of characters defined as plant biomass/development (plant height $F_{3,90} = 35.75$, $p < 0.0001$; fresh weight of shoots $F_{3,90} = 34.184$, $p < 0.0001$; fresh weight of roots $F_{3,90} = 3.638$, $p < 0.016$); flowering phase (number of flower shoots $F_{3,90} = 8.615$, $p < 0.0001$; number of flowers $F_{3,90} = 26.271$, $p < 0.0001$) and fruit weight ($F_{3,90} = 65.956$, $p < 0.0001$ of tomato plants cultivated in precrop soil (red clover, alfalfa), in bare fallow soil and in control soil (Table 2).

On average, the tomato plants were taller when precropped with red clover and alfalfa for three years. The shoot weight and total weight of tomato plants cultivated in

red clover and alfalfa precrop soils were lower than those in plants from bare fallow soil and control soil at the end of the first and the second growing seasons. The root weight was significantly lower when the tomato plants were precropped with alfalfa for one year. The tomato plants were characterised with a distinctly higher number of flower shoots in the control, but flowers appeared more often on plants both in a control and bare fallow treatment after the second year of experiment. The experiment revealed differences in fruit yield. During the first and at the second harvest, tomato plants growing in the legume precrop soil yielded significantly less than those cultivated in bare fallow and control soils. During the third harvest, there was no difference between fruit yield obtained from tomato plants kept in the alfalfa precrop soil and those in control soil. The results are shown in Figure 4.

Table 2. Influence of precrop soil on biometrical measurements of *Solanum lycopersicum.*

Precrop Soil	Trait					
	Plant Height [cm]	Fresh Weight of Shoots [g]	Fresh Weight of Roots [g]	Number of Flower Shoots [pcs.]	Number of Flowers [pcs.]	Fruit Weight [g]
T. pratense	47.69 ± 9.64 a	52.33 ± 22.24 a	22.55 ± 6.99 b	3.5 ± 0.98 a	16.5 ± 7.99 a	253.75 ± 109.84 a
M. sativa	48.54 ± 11.14 a	55.08 ± 25.64 a	18.60 ± 7.13 a	3.6 ± 1.13 a	18.6 ± 8.97 a	418.75 ± 275.14 b
Bare fallow	59.63 ± 9.31 b	81.63 ± 8.68 b	18.79 ± 2.83 a	4.2 ± 1.01 a	24.7 ± 4.82 b	595.42 ± 80.01 c
Control soil *	73.79 ± 10.4 c	108.63 ± 30.76 c	18.79 ± 2.46 a	5.0 ± 1.30 b	32.1 ± 9.23 c	793.50 ± 118.70 d

* Control (soil without nematode); mean value ± SD with different letters in the columns are significantly different represent ranking by the Fisher's LSD post hoc test at $p < 0.05$.

Significant differences occurred among mean values of the number of root galls according to precrop ($F_{3,84} = 18.391$, $p < 0.002$), and precrop × years interaction ($F_{6,84} = 9.960$, $p < 0.0001$) as well as mean values of the females with egg masses in precrop × years interaction ($F_{6,84} = 36.251$, $p < 0.0001$). On the tomato roots subjected to bare fallow treatment, the galls were abundant at the end of each growing season. When precropped with legumes, the highest number of root galls occurred in tomato plants succeeding the three-year cultivation of red clover precrop. Female nematodes with egg masses were found to be less numerous in the legume precrop treatment than in bare fallow soil during the first and second harvest. They reached approximately 27% and 22 % of bare fallow for red clover and alfalfa, respectively. At the third harvest, the number of female nematodes in the red clover and alfalfa precrop treatment was significantly higher and accounted for 84% and 37% of the bare fallow result, respectively. The results are presented in Table 3.

Table 3. Development of *Meloidogyne hapla* on roots of *Solanum lycopersicum* growing in soil after cultivation of *Trifolium pratense*, *Medicago sativa* and in bare fallow.

Precrop Soil	Mean Values on Tomato Roots	
	Number of Galls	Number of Egg Masses
T. pratense, one-year-old	6.88 ± 4.05 b	8.75 ± 5.52 b
M. sativa, one-year-old	8.75 ± 8.01 b	9.25 ± 7.85 b
Bare fallow, one-year-old	43.63 ± 7.61 e	24.00 ± 5.61 cd
Control soil	0 ± 0 a	0 ± 0 a
T. pratense, two-year-old	16.63 ± 6.65 c	3.50 ± 1.60 ab
M. sativa, two-year-old	5.13 ± 2.90 ab	2.13 ± 0.83 ab
Bare fallow, two-year-old	40.88 ± 8.41 de	25.62 ± 8.02 cd
Control soil	0 ± 0 a	0 ± 0a
T. pratense, three-year-old	36.81 ± 10.66 d	61.13 ± 19.84 d
M. sativa, three-year-old	19.38 ± 5.01 c	30.00 ± 6.50 c
Bare fallow, three-year-old	44.00 ± 10.92 e	22.50 ± 3.74 c
Control soil	0 ± 0 a	0 ± a

In columns (mean ± SD), values followed by the same latter are not statistically different ($p < 0.05$); letters represent ranking by the Fisher's LSD post hoc test.

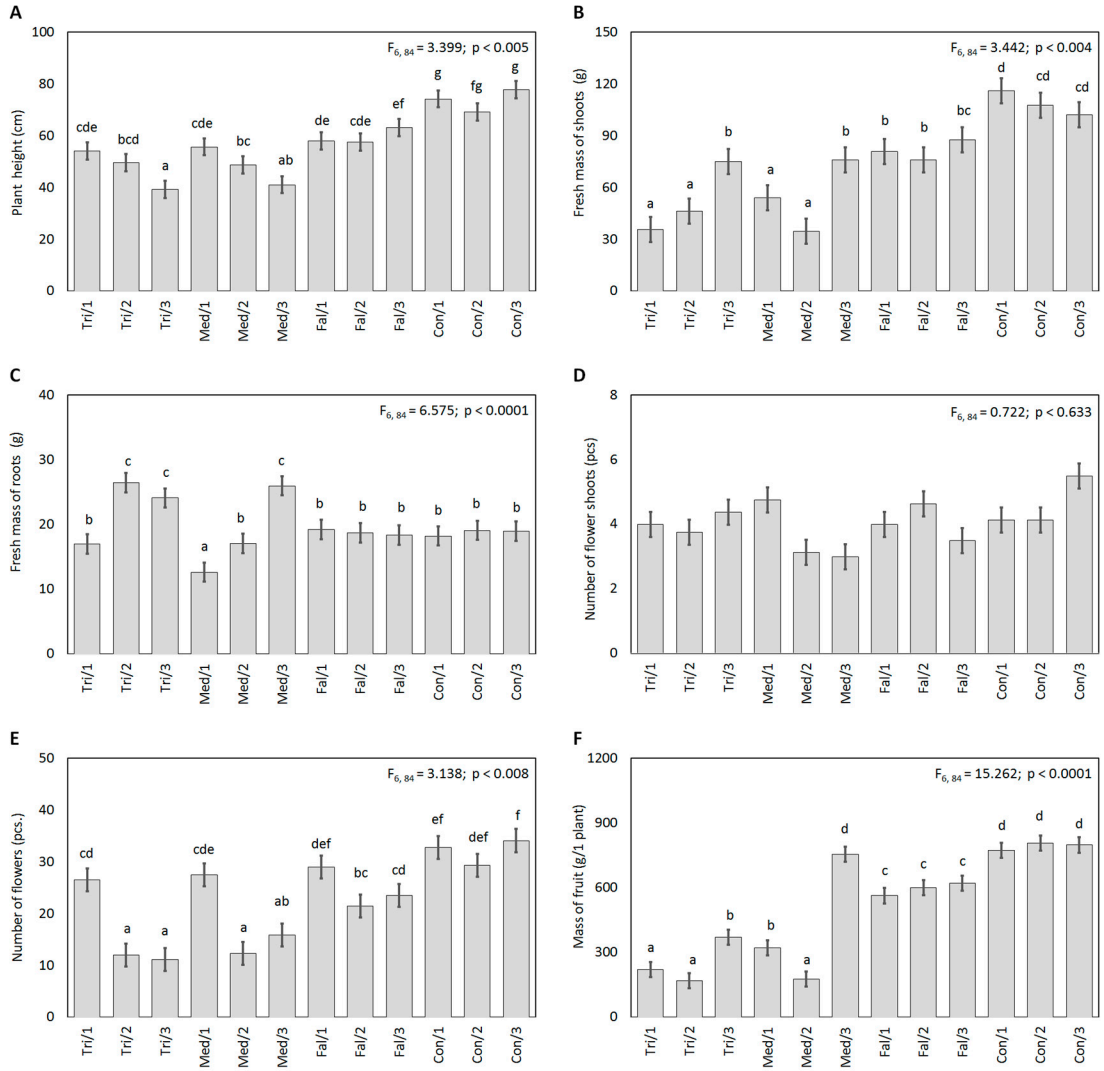

Figure 4. The influence of precrop soil (*Trifolium pratense*, *Medicago sativa* and bare fallow) infested with *Meloidoyne hapla* on biometric measurements of tomato *Solanum lycopersicum*. (**A**) Plant height, (**B**) Fresh weight of shoots, (**C**) Fresh weight of roots, (**D**) Number of flower shoots, (**E**) Number of flowers, (**F**) Fruit weight. Means bearing different letters differ significantly ($p < 0.05$) according to Fisher's test. Tri/1—*T. pratense*, one-year-old; Tri/2—*T. pratense*, two-year-old; Tri/3—*T. pratense*, three-year-old; Med/1—*M. sativa*, one-year-old; Med/2—*M. sativa*, two-year-old; Med/3—*M. sativa*, three-year-old; Fal/1—bare fallow, one-year-old; Fal/2—bare fallow two-year-old; Fal/3—bare fallow, three-year-old; Con/1—control soil, one-year-old; Con/2—control soil, two-year-old; Con/3—control soil, three-year-old.

4. Discussion

Particular species of legumes have a different impact on the dynamics of *M. hapla* development in the soil [44]. The population dynamics of the northern root-knot nematode in the soil under annual and two- or three-year red clover and alfalfa precrop cultivation, involves the host suitability of each of those plant species to northern root-knot nematode, the precrop effects for succeeding crops and the timing of the sampling to determine the abundance of this nematode in soil towards assessing its damage potential to crops.

Root galls and egg masses of *M. hapla* counted on the roots of red clover and alfalfa plants indicate differences in the susceptibility of the studied plant species to colonization by this nematode. According to the Willis' scale [45] used in nematological studies, the tested clover species can be described as moderately susceptible, while alfalfa, as the resistant species, is moderately susceptible. Despite the presence of root galls, no negative impact of the root-knot nematode on red clover growth and development was observed.

M. hapla, present in small numbers on alfalfa roots, also did not pose a threat to its growth. However, sensitive alfalfa varieties are known in cases of plant death caused by *M. hapla* feeding [46]. The low susceptibility of the tested alfalfa variety to infection by *M. hapla* may be determined by the chemical properties of the variety. It has been shown that the chemical composition differs between the genotypes of alfalfa [47]. The limiting effects of alfalfa have also been described for *Xiphinema* and *M. incognita* (Kofoid and White, 1919) [48,49].

The soil left over from the red clover or alfalfa precrop cultivation affects the succeeding crops. Although the number of invasive forms of J2 in the soil did not exceed the damage threshold of 20 individuals in 200 mL of soil [50], the values of the parameters characterizing the vegetation of tomato plants and the weight of the fruit were lower than the values obtained in the bare fallow variant and in the control soil without nematodes. Only the weight of fruit harvested from plants grown in alfalfa precrop soil (three-year precrop cultivation) did not differ from the fruit weight calculated for the control, although the number of root galls and female nematodes on the roots were more numerous. It can be assumed that the roots of legume plants present in the soil changed its properties by introducing root secretions, thus leading to a reduction in the quality of tomato vegetation. Changes in soil properties caused by the presence of alfalfa, leading to a decrease in potassium uptake, were previously observed in *Vicia faba* L. plants [51]. Other studies have found alfalfa a good precrop for tomato plants, enriching the soil with nitrogen and limiting the occurrence of weeds [52,53]. In our study, the weaker growth and development of tomato plants succeeding bare fallow was observed. Since the initial abundance of J2 forms in the soil was low, it is difficult to directly ascribe the observed result only to the negative effect of the nematode. It can be assumed that the obtained result was also influenced by the individual susceptibility of the tomato cultivar to nematode colonization and the individual infection capacity by the root-knot nematode population used in the study.

Determining the population density of nematodes in the soil, in the context of their damage potential to succeeding crops, is an important action which needs to be implemented to ensure the long-term success of the plantation (boosting crop protection and minimizing losses). Given that alfalfa cultivation keeps the northern root-knot nematode population density relatively low, soil nematode testing can be performed both after the end of vegetation in the autumn, and in the following spring prior to planting the succeeding crop. When comparing the J2 concentration in the soil with the available data, it can be stated that it is not detrimental to the cultivation of carrots and roses [54,55]. Based on the results obtained from the observations using the red clover precrop soil, it can be advisable to test the soil for the presence of the root-knot nematode in spring, before the introduction of the succeeding crop.

5. Conclusions

The three-year red clover and alfalfa precrop cultivation decreased the abundance of the northern root-knot nematode in the soil. Based on the results of the observation of the population dynamics of invasive forms of nematodes in the soil, it is recommended that attempts to estimate the number of nematodes in red clover precrop soil should be made in spring. The legume precrop soil has a limiting effect on the growth and fruiting of tomato plants and the population of the nematode negatively influenced the tomato vegetation. Satisfactory results of tomato fruit yield are obtained only following the three years of alfalfa precrop cultivation.

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