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Control of *Meloidogyne graminicola* a Root-Knot Nematode Using Rice Plants as Trap Crops: Preliminary Results

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Abstract: *Meloidogyne graminicola* is one of the most harmful organisms in rice cultivation throughout the world. This pest was detected for the first time in mainland Europe (Northern Italy) in 2016 and was subsequently added to the EPPO Alert List. To date, few methods are available for the control of *M. graminicola* and new solutions are required. In 2019, field trials using rice plants as trap crops were performed in a Lombardy region rice field where five plots for three different management approaches were staked out: (i) Uncultivated; (ii) Treated: three separate cycles of rice production where plants were sown and destroyed each time at the second leaf stage; (iii) Control: rice was sown and left to grow until the end of the three cycles in treated plots. The results showed that in the treated plots, the nematode density and the root gall index were lower than for the other two management approaches. Moreover, the plant population density and rice plant growth were higher than the uncultivated and control plots. In conclusion, the use of the trap crop technique for the control of *M. graminicola* gave good results and thus it could be a new phytosanitary measure to control this pest in rice crop areas.

Keywords: alien pest; Italy; *Oryza sativa*; phytosanitary measures; rice root-knot nematode; trap crop technique; upland rice cultivation

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1. Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are obligate plant-parasitic nematodes that cause serious damage and yield losses in a wide range of crops [1]. This group of nematodes presents a wide range of herbaceous and woody host plants, including monocotyledons and dicotyledons [2]. Due to the importance of their economic impact, different management strategies have been developing to control these plant-parasitic nematodes, such as application of live microbes (e.g., bacteria, fungi) and/or their secondary metabolites, essential oils, plant extracts, ozonated water, silicon, steaming, and solarization. These environmentally benign strategies can be considered for replacing the chemicals commonly used in agriculture [3].

Meloidogyne graminicola (which was first discovered by Golden and Birchfield in 1965 (Nematoda: Meloidogynidae)), commonly named as the rice RKN, is considered as one of the most important damaging parasites for upland, lowland, and deep-water rice cultivation throughout the world, particularly in South and Southeast Asia [4]. The second juvenile stage (J2) is the infective stage that hatches from the egg under favorable environmental conditions, finds the root, enters the meristematic zone, and induces the formation of giant galls by continuous feeding.

Rice is the most important host for rice RKN, but this nematode has a wide range of alternative hosts, including many weeds commonly found in rice fields that may offer refuge to these nematodes [5,6].

Italy is the main rice-growing country in Europe, with 217,195 ha of rice in 2018 [7]. The most important rice-growing area is the section of the Po River Valley straddling the regions of Lombardy and Piedmont (more than 202,000 hectares, 93% of the Italian rice surface [7]). *Meloidogyne graminicola* was detected for the first time in mainland Europe (in the Piedmont region, Northern Italy) in 2016 and was subsequently added to the EPPO Alert List [8,9]. To preserve the national rice production, the Italian National Plant Protection Organization (NPPO) quickly issued phytosanitary measures to limit *M. graminicola* damage and avoid its spread to new areas. The options to control *M. graminicola* are still limited and for many years, the use of nematicides has been the most efficient way to manage this pest. Due to their negative impact on the environment and the implementation of new directives and regulations to reduce chemical applications [10], alternative strategies are now needed to reduce RKN populations. Among the phytosanitary measures adopted by the Italian NPPO (reported in the Ministerial Decree of 6 July 2017), rice field flooding seems to be one of the most efficient techniques to control the size of the *M. graminicola* population, but in some areas of the Lombardy region, this practice is not applicable due to the soil structure characterized by a low water retention capacity [11]. For this reason, some field trials using rice plants as trap crops were conducted to identify new control strategies against this pest.

Trap cropping is a practice for pest nematode control that has been used since the late 1800s [12]. A susceptible host species is planted and nematode juveniles of a sedentary parasitic nematode such as root-knot nematodes are stimulated to hatch and invade the roots and establish a feeding site on the plant. Once this colonization has occurred, and the females begin to mature, they are unable to leave the plant root. Before the nematodes complete their life cycle, the crop is destroyed, avoiding a new soil infestation and thus reducing the nematode population.

In this study, among the various trap cropping techniques available, sequential trap cropping was chosen for the management of the rice RKN, since this technique involves plants that are highly attractive to the pest and that are sown earlier than the main crop [13].

This study aimed to conduct a first-time evaluation of rice plant use in trap crop techniques for the management of this nematode pest, in areas where the rice field flooding is not applicable.

2. Materials and Methods

2.1. Study Area

The study was carried out in 2019 in a rice-cultivated area at the Cascina Scalina farm, located in Garlasco (Pavia, Lombardy region, Italy) (45°19′ N, 08°89′ E, altitude ca. 43 m a.s.l.) within the rice crop district of Lomellina. The farm property consists of 227 hectares, distributed in 127 ha for maize and 100 ha for rice cultivation. This area is characterized by a high level of field fragmentation (55 rice field of variable surfaces) and an extensive network of canals for irrigation.

The local climate is humid subtropical (Cfa) according to the Köppen climate classification [14], with an average temperature of 21 °C and a cumulative rainfall depth of approximately 298 mm during the agricultural season (Data for April-September of 2014–2019, ARPA Lombardy—http://www.arpalombardia.it). The study area was classified as the Luvisol-Cambisol Region with Gleysols, developed on Alpine sediments that have been deposited north of the Po river [15]. The soils are coarsely textured (sand > 80%), with a pH from sub-acidic to neutral and a low retention capacity [11].

2.2. Experimental Design

In an upland rice field of 6 ha severely infected with *M. graminicola* in 2018 (root gall index 8 in a scale range of 0–10 [16]), the experimental layout was a randomized complete block design with 5 blocks. Each block was divided into three plots (5 × 5 m) where 3 management treatments were assigned randomly: Uncultivated (U); Treated (T), where three separate cycles of trap crop were carried out; Control (C), where the rice was sown and left to grow until the end of the three cycles in T plots.

The experimental area was located 15 m from the north edge of the selected field and plots were separated by 2-m-wide untreated buffers to avoid effects from the migration of nematodes, and to facilitate operations within the different plots.

At the end of April 2019 (T0), the experimental area was ploughed, the plots were delimited, and soil sampling was carried out as described below. In plots C and T, 0.60 kg/plot of long-grain rice cv. S. Andrea was sown, and only in T at the second leaf stage (BBCH-scale 12 [17]), after 15 days, rice plants were destroyed with a registered herbicide. This cycle was repeated three times, as illustrated in Figure 1. In particular, between the destruction of the rice plants of the previous cycle and the sowing of the next ones, a week was always allowed to pass.

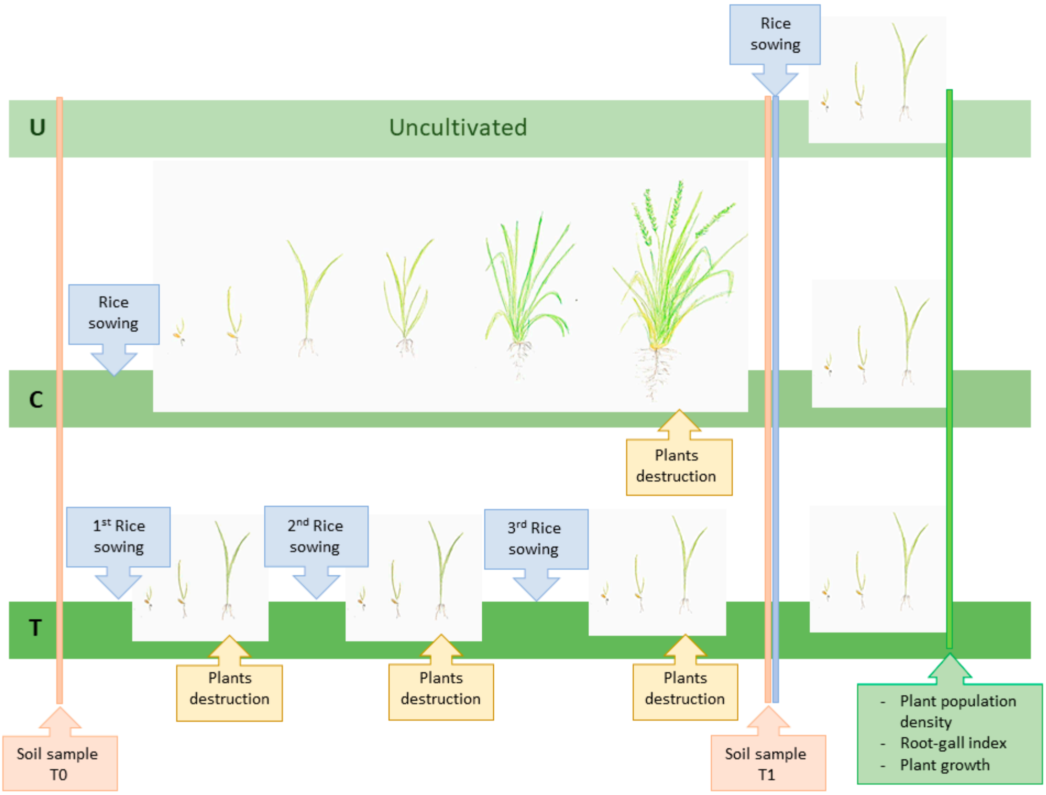

Figure 1. Illustration of the experimental design used to evaluate the trap crop technique in the management of *Meloidogyne graminicola*. Uncultivated (U), Control (C), and Treated (T). (Drawings by Giuseppe Mazza).

2.3. Evaluation of Nematode Density in the Soil

To evaluate the number of eggs and juveniles of *M. graminicola* and compare the population density before (T0) and after (T1) the trap crop technique experiment, in each plot, three soil samples (approximately 0.5 kilo/sample) were randomly collected using a hand shovel. All samples were individually placed in a plastic bag, labeled, and then brought to the laboratory of the Minoprio Foundation (Como, Italy). These materials were stored in a climatic chamber at about +4 °C until they were processed for analysis. For each sample, 200 cc of soil were placed in a plastic bucket, and 6 L of water were added.

The resulting slurry was vigorously swirled for about 30 s, and after 45 s of sedimentation time, the supernatant suspension was decanted through a 40 µm sieve. Water was again added to the soil in the bucket and the process was repeated twice.

To dissolve the gelatinous matrices of the egg masses and obtain the suspension with nematodes, the sodium hypochlorite (5% NaOCI) technique described in Byrd et al. [18] and the centrifugal flotation method [19] were carried out. Nematodes were collected in a glass dish for examination and counted under an optical microscope LEICA MZ12 (Leica Microsystems, Heerbrugg, Switzerland).

2.4. Evaluation of the Plant Population Density

After the third cycle of trap crop, all experimental areas were mechanically worked to destroy rice plants and weeds, taking care not to transport soil from one plot to another with the machines. Subsequently, each plot was sown with the same amount of rice (see above) at the same time. At the second leaf stage of the plants, in order to record the number of rice plants per unit area, a circle frame (0.3 m^2) quadrant was used. It was randomly launched five times in each plot and all plants rooted inside the circle were counted.

2.5. Evaluation of Root-Gall Index and Plant Growth

To evaluate the damages on plants, the gall index was assessed on the rooting system and the plant growth was measured on the aerial part of the same plants.

At the same time of the evaluation of the plant population density, a representative sample (20–23 rice plants/plot) was collected with the whole root system. Plants of each plot were placed in a labeled plastic bag and analyzed in the lab within 24 h of collection.

The roots were rinsed with tap water, placed on paper towels to eliminate excess water, and observed to assess the severity of root damage caused by the amount of galling. The evaluation of root gall indices was studied visually using the root evaluation chart developed by Bridge and Page [16].

The same plants were individually photographed (Canon PowerShot G3—Ōta, Tokyo, Japan) with a bare scale, and the plant length (distance from the coleoptilar node to the tallest leaf) was recorded using ImageJ program (Image Processing and Analysis in Java) Version 1.53a (Wayne Rasband, National Institute of Health, Washington, DC, USA).

2.6. Statistical Analysis

To assess the influence of the managements (U, C, and T, see above) and the time (T0 and T1) on the total number of *M. graminicola* (eggs and juveniles) a generalized mixed model (statistic: F), with a negative binomial probability distribution and a log link function was run. The total number of nematodes was the dependent variable, while the management and the time was the fixed effect. Moreover, we considered the interaction management x time.

To assess the influence of management on plant population density, a generalized mixed model (statistic: F), with a negative binomial distribution and a log link function was run. The density of each case (each group of plants) was the dependent variable, while management (U, C, and T) was the fixed effect. The block id (5 different plots, each with 5 groups of plants measured for each management) was included as a random effect.

The root-gall index in the soil among managements (U, C, and T) was compared with the Kruskal-Wallis test (statistic: H) and post hoc Mann-Whitney pairwise (statistic: U; raw *p* values, sequential Bonferroni significance).

To assess the influence of management on plant growth, a generalized mixed model (statistic: F), with a normal probability distribution and an identity link function was run. The growth of each plant was the dependent variable, while the management (U, C, and T) was the fixed effect. The block id (5 different plots, each with a range of 17 to 23 plants measured for each management) was included as a random effect.

To calculate the effect size, Cohen's d as: $d = (ma - mb)/s.d.$ was computed, where ma and mb are the estimated marginal means of each category within the pairwise comparison, and s.d. is the pooled standard deviation. According to Cohen [20], the interpretation of d is as follows: d = 0.2: small effect, d = 0.5: medium effect, d = 0.8: large effect. Pairwise post-hoc comparisons between each couple of categories were performed using Bonferroni's sequential correction. Statistical analyses were performed in SPSS 20.0 [21] and PAST 3.25 [22].

3. Results

3.1. Evaluation of Nematode Density in the Soil

The management and the time had a significant effect on the total number of nematodes (F = 13.641, df = 2, 81, $p < 0.0001$ and F = 38.563, df = 1, 81, $p < 0.0001$, respectively). Moreover, there was a significant interaction between management and time (F = 8.086, df = 2, 81, $p < 0.0001$). In T0 no differences were found among managements (U, C, and T), confirming the similar distribution of nematodes in the experiment area. In T1, the number of nematodes were again similar in C and U, while a significant reduction was assessed in T (Table 1, Figure 2).

Table 1. Pairwise comparisons among managements (Uncultivated: U, Control: C, and Treated: T) before (T0) and after (T1) the trap crop experiment. Significant differences are indicated in bold.

Time	Pairwise Comparisons	*p*
T0	C vs. U	0.698
T0	C vs. T	0.603
T0	U vs. T	0.350
T1	C vs. U	0.769
T1	**C vs. T**	**0.002**
T1	**U vs. T**	**0.001**

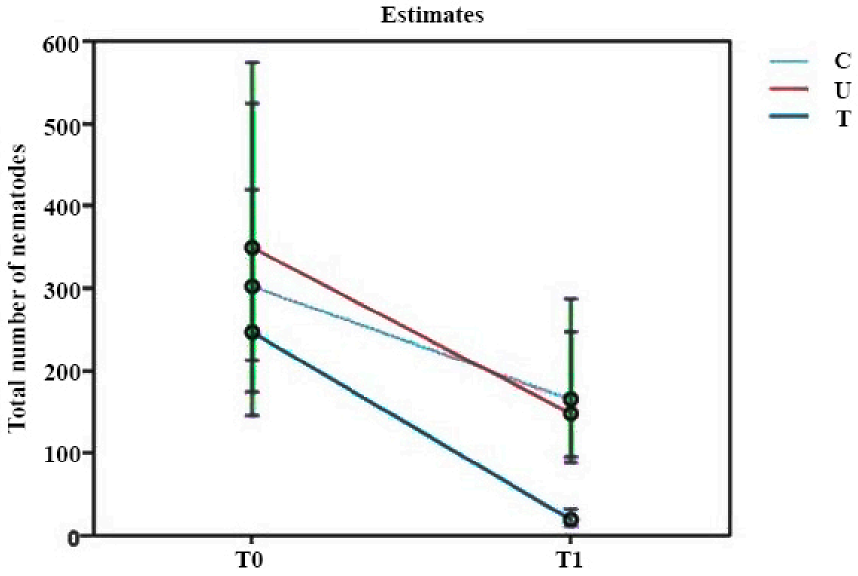

Figure 2. Interactive effect of time (before = T0 and after = T1 the three-trap crop cycles) and managements (Control: C, Uncultivated: U, and Treated: T) on the total number of *Meloidogyne graminicola* (eggs and juveniles) (average and 95% confidence interval are shown).

3.2. Evaluation of the Plant Population Density

The management had a significant effect on the plant population density per unit area (F = 21.509, df = 2, 72, $p < 0.0001$). Density was higher in treated plots (T) in comparison to both plant density in the U ($p < 0.0001$) and those in the C plots ($p < 0.001$). No significant difference was found when comparing density between C and U plants ($p = 0.114$) (Figure 3). Effect size: d = 0.20 (T vs. U) and d = 0.16 (T vs. C).

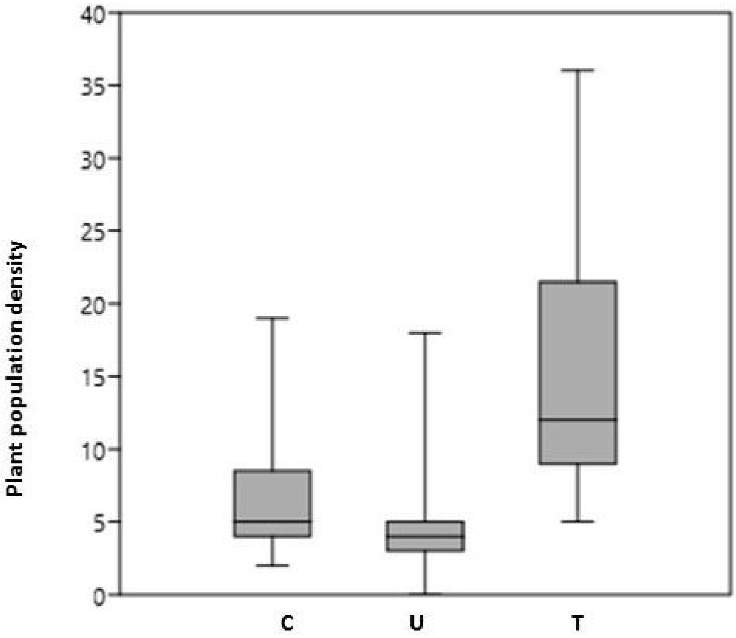

Figure 3. Influence of the managements (Uncultivated: U, Control: C, and Treated: T) on plant population density.

3.3. Evaluation of Root-Gall Index and Plant Growth

There was a significant difference in the root-gall index among management (H = 63.81, df = 301, $p < 0.0001$). In particular, C vs. U (U = 4284; $p = 0.03$), C vs. T (U = 2766; $p < 0.0001$), and U vs. T (U = 1919; $p < 0.0001$) (Figure 4).

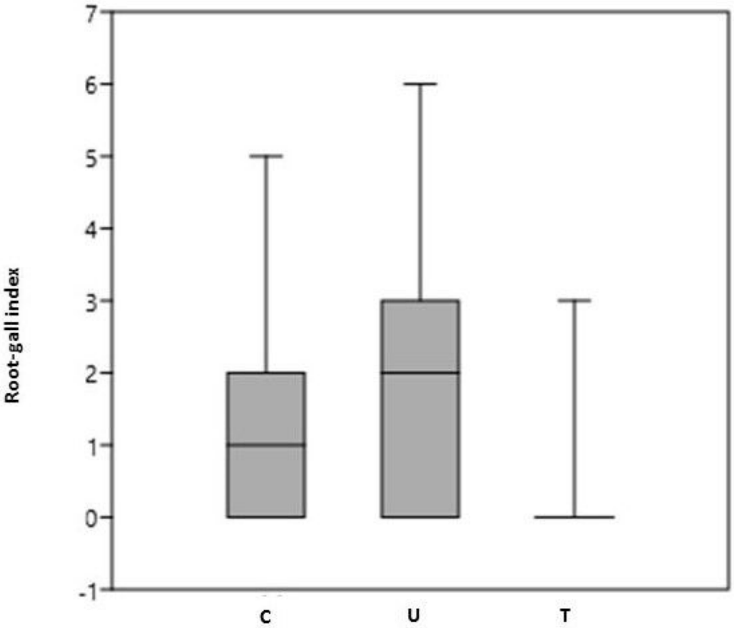

Figure 4. Influence of the managements (Uncultivated: U, Control: C, and Treated: T) on root-gall index.

The management had a significant effect on plant growth (F = 37.107, df = 2, 300, $p < 0.0001$). Plants were higher in T than both plants in U ($p < 0.0001$) and C ($p < 0.0001$). No significant difference was found when comparing plant growth between C and U ($p = 0.105$) (Figure 5). Effect size: d = 0.12 (T vs. U) and d = 0.10 (T vs. C).

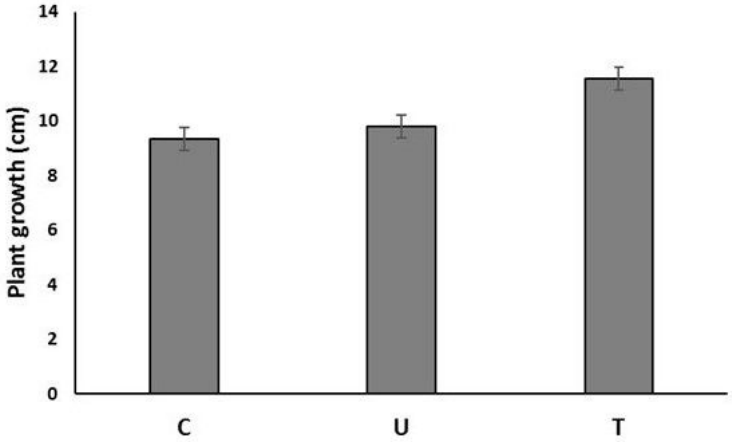

Figure 5. Influence of the managements (Uncultivated: U, Control: C, and Treated: T) on plant growth. Bars indicate mean ± standard error.

4. Discussion

Trap cropping is a technique used in both ecological and agronomic fields and it is based on the use of plant species, particularly attractants and species susceptible towards certain pests, insects, or nematodes [23]. According to the characteristics of the plant used and the time or space of deploying, different modalities of trap cropping (perimeter, sequential, multiple, and push-pull) are reported for the management of different pests [13]. Although trap cropping has usually been employed to control insect pests, few studies were previously performed on nematodes, such as cyst nematodes [24–26], and root-knot nematodes [27–29].

In the present research, the trap crop technique was evaluated to reduce the population density of *Meloidogyne graminicola* in upland rice field experiments where continuous flooding cannot be applied as a management practice to control this pest.

To select the most successful trap cropping method, the host range, biology, development, and multiplication, spread and survival strategies of the pest are pivotal information for the correct management [30].

Among the numerous host plants reported, *Oryza sativa* has been recorded to be the most attractive and susceptible one to *M. graminicola* [6,31]. For this reason, it was selected in this study as the trap crop plant. Concerning the time prior to the destruction, a sufficient period is required for the host plants to attract free-living second-stage juveniles (J2) and permit the root colonization to occur before nematode reproduction. In this work, the choice to destruct the rice plant at the second leaf stage (about 16–17 days from sowing to the trap crop destruction) was based on bibliographic information [32,33], *M. graminicola* cycle observations in the field, and analysis in the laboratory during the 2019 mandatory monitoring of this pest in the Lombardy region (Sacchi S, pers. obs.). In fact, Dabur et al. [32] observed that J2 of *M. graminicola* in the soil can enter the roots of host plants from the 5th day of sprouted rice seed sowing, increasing their number in the roots up to the 12th day of sowing. Moreover, from our observations, at the second/beginning third leaf stage, only J2 were found inside the roots, while at the end third/beginning fourth leaf stage, mostly J3, J4, and males were present. The female presence was observed from the fourth leaf unfolded stage.

At the end of the experiment, a reduction of the total number of *M. graminicola* was recorded only in the treated plots. Uncultivated and control managements gave similar results and are perhaps related to the several weeds present in the uncultivated plots. Some of them, such as *Echinocloa* spp. and *Cyperus* spp. are known as host plants of *M. graminicola* [6], and therefore the nematode can survive and reproduce in these alternative hosts. This result confirms and encourages efficient weed management as an important tool to maintain a low nematode population in infested fields [29].

Concerning the reduction of the rice RKN number in the soil, the results also highlighted positive consequences directly on the plant health *status*, and plant population density per unit area, due to the lower stress. Indeed, the rice plants grown after the three trap crop cycles showed a significantly lower infestation index in treated plots than both control and uncultivated ones, notwithstanding the low root-gall index in all the plots due to the second leaf stage of the plants. Also, the rice plants grown in the treated plots were taller by about 12% than both plants in the control and uncultivated plots at this stage of plant development. Moreover, in the treated plots the plant population density increased by 25% and 34% compared to the control and uncultivated ones, respectively.

5. Conclusions

In conclusion, these results show the efficacy of trap cropping for the management of the rice RKN phytosanitary problem in most rice-growing areas, especially those with water shortages. In climatic and pedological areas similar to the Lombardy region, the duration of the three trap crop cycles could be just over two months. This technique of decreasing the nematode population density in the soil, therefore, has a much shorter time of action than flooding method (as indicated among the phytosanitary measures reported in Ministerial Decree of 6 July 2017). However, future studies are necessary to establish the most effective number of trap crop cycles that are useful to reduce the presence of *M. graminicola* in the infested soils, maintaining its density below the level that allows the optimal growth of rice plants. Moreover, this technique could be also inserted in integrated pest management programs as a low environmental impact agronomic practice, compared to the flooding method, to control this damaging rice pest.

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