

Impact of a conservation agriculture system on soil characteristics, rice yield, and root-parasitic nematodes in a Cambodian lowland rice field

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Abstract

Rice production in Southeast Asia is significantly affected by root-parasitic nematodes (RPN). The Green Revolution has encouraged new agricultural practices (e.g. intensive monoculture, high yielding rice variety) to respond to the high rice demand; however, these methods have promoted the spread of these pests. The recent banning of chemical nematicides resulted in a need for alternative sustainable control strategies. In the present study, we assessed the effects of a direct-seeding mulch-based cropping system (DMC) vs conventional plough-based tillages (CT) on soil properties, rice yield and RPN communities during a two-year trial in Cambodia. Our results show that on average the population densities of RPN were significantly higher in DMC than in CT. Molecular identification revealed only two RPN species associated with roots: *Meloidogyne graminicola*, not previously reported from Cambodia, was predominant and was present throughout the plant's development, whereas *Hirschmanniella mucronata* was only found at the tillering and milky stages. We conclude that DMC had a significant positive impact on rice yield, despite higher RPN short-term pressure. In order to increase the efficiency of such cropping systems, further studies and an evaluation of the long-term relationships between DMC, the nature of cover crops used, the soil biota including RPN, and rice yield should be conducted.

Keywords

Direct-seeding mulch-based cropping system, *Hirschmanniella mucronata*, Management, *Meloidogyne graminicola*, No-tillage, Population dynamics, Sustainable intensification.

Southeast (SE) Asian countries are the world's largest rice producers and consumers. For decades, socio-economic and environmental changes have been altering agricultural practices, including rice cultivation (Felkner et al., 2009; Satterthwaite et al., 2010; Reynolds et al., 2018; Flor et al., 2018). These alterations require closer scrutiny to understand and ultimately maximize agricultural output both locally and globally, and to more effectively utilize land and water resources, as population growth and urbanization increase competition for their use in agriculture. In order to meet global rice demands for a rapid growing world population, rice yields need to increase by approximately 42% by 2050 (Ray et al., 2013). Rice farming is facing a dual challenge of delivering sufficient

and nutritious food to meet the projected demands of population growth and markets, and overcoming issues such as climate change, soil fertility depletion and water scarcity through sustainable agricultural intensification. Thus, environmental-friendly strategies are needed to reduce the environmental burden associated with intensive rice cultivation without jeopardizing rice production, commoditization and global food security. In this context, rice diseases and pests are major biotic constraints reducing rice yields in Asia by about 37% (Gianessi, 2014). Among these pathogens, *Meloidogyne* spp. (Göeldi, 1892) and *Hirschmanniella* spp. (Luc and Goodey, 1963) are the two main groups of root-parasitic nematodes (RPN) that substantially affect rice production in SE Asia (De Waele and Elsen, 2007). The rice root-knot nematode, *Meloidogyne graminicola* (Golden and Birchfield, 1965), is the principal *Meloidogyne* species found on rice in Asia causing rice yield losses ranging between 16 and 80% of the total crop production (Plowright and Bridge, 1990; Prot and Matias, 1995; Soriano et al., 2000; Padgham et al., 2004; Mantelin et al., 2017), depending on the rice agroecosystems and agricultural practices. *Hirschmanniella* spp. can reduce rice yields by 20% on average (Prot, 1993). In lowland areas, *Hirschmanniella oryzae* (Breda De Haan, 1902) reduced the rice yield between 8.3 and 9.4% (Cho-Hen et al., 1994).

Rice agricultural practices have been significantly modified in SE Asia during the last decades, notably due to the Green Revolution (Pingali, 2012; Reynolds et al., 2018) and the increasing scarcity of water and labor (Thrall et al., 2010). For hundreds of years, farmers traditionally transplanted rice seedlings. Nowadays, with the support of agricultural mechanization and to offset the scarcity of labor, seed broadcasting on non-flooded rice fields is the most common practice. Seed broadcasting means that rice seeds are being directly sown in the field rather than by transplanting seedlings from a nursery. Soil is tilled before seeding, and the rice seeds are sown in lines or broadcasted.

Conservation agriculture (CA) is seen as a promising alternative to soil and crop management practices to enhance soil functions and to improve farm sustainability (Hobbs et al., 2008; Verhulst et al., 2010; Palm et al., 2014). CA is an alternative to traditional farming methods in which soil disturbance is minimized and cropping systems diversified, by crop rotations and cover crops mixtures for instance, to improve soil fertility and conserve water while reducing operational costs including labor inputs. CA has gained popularity throughout the world because it alleviates the negative impacts and high

production costs of the commonly used continuous plowing and crop monoculture. Although CA covered an estimated area of 180 million ha worldwide in 2016 (Kassam et al., 2019), few irrigated rice agroecosystems combine permanent no-till (NT) with cover crop management, a typical CA system. In the context of our study, we consider the terms CA and direct-seeding mulch-based cropping (DMC) systems as equivalent, the latter term having the advantage to be more explicit and refers to diversified cropping system managed under NT with a high biomass input. Biological soil processes are enhanced and maintained through the use of multifunctional cover crops and a higher degree of crop diversification (Séguy et al., 2006; Palm et al., 2014; Rabenarivo et al., 2014). The cover crops grown in between crop cycles provide a continuous flow of fresh organic matter increasing soil organic C and N concentrations (Hok et al., 2015; Sá et al., 2015; Patra et al., 2019) while they also enhance soil biodiversity (Lienhard et al., 2013a,b; Choudhary et al., 2018; Jiang et al., 2018). The effects of DMC on the abundance and diversity of soil microbial communities' benefit, in turn the plant's development, and thus yield (Hungria et al., 2009; Palm et al., 2014; Lienhard et al., 2014). Besides the micro-organisms, the relationships between DMC and the soil nematofauna have also been studied (Villenave et al., 2009; Ito, 2015; Ito et al., 2015). For instance, the examination of the long-term effects of contrasted DMC systems in Madagascar has shown that the soil food webs become more complex under DMC soils when compared with conventional plough-based tillage (CT), with an increasing abundance and diversity of soil microbial and nematode assemblages (Villenave et al., 2009).

The impact of DMC and rice RPN has not been studied so far. Some key practices in DMC could negatively affect the occurrence of RPN on rice. For instance, direct-seeded rice enhances the invasion and establishment of weeds (Farooq et al., 2011) such as *Echinochloa colona*, a host for *M. graminicola* (Negretti et al., 2014). In our study, we focus on a specific DMC system under consideration to be applied in Cambodia, where the rice yield is one of the lowest of SE Asian countries with only 2.5 Mg ha⁻¹ vs 3 Mg ha⁻¹ in Laos and Thailand, and 6 Mg ha⁻¹ in Vietnam (Anonymous, 2017). One of the limitations of rice production in Cambodia is the predominance of low-fertile soils. Most of the country's lowland rice growing areas are characterized by a poor sandy soil (Blair and Blair, 2010) that is favorable to RPN infestation (Pokharel, 2009). Our study aimed to evaluate the effects of the DMC system on soil nutrients and organic matter, and rice yield in relation

with the occurrence of RPN during two successive years as compared to a CT system on a poor sandy soil in Cambodia.

Materials and methods

Location and characteristics of the experimental field

The experimental field was located in Stung Chinith, Santuk district, Kampong Thom province, Cambodia (12°32'55"N and 105°08'47"E). Since April 2011, a study comparing DMC vs CT cropping systems has been implemented. The experiment was managed by agronomists of the General Directorate of Agriculture of Cambodia, the Department of Agricultural Land Resources Management (DALRM), the Conservation Agriculture Service Center (DALRM/CASC), and the AIDA research unit of the French Agricultural Research for Development (CIRAD). The field soil is a sandy podzolic soil ($\geq 70\%$ sand at 0- to 40-cm deep) belonging to the "Prey Khmer group" in the Cambodian agronomic soil classification system (White et al., 1997), equivalent to red-yellow podzols (Crocker, 1962), or fluvisols/arenosols according to the FAO soil taxonomy (Anonymous, 2006).

Annual rainfall in the Kampong Thom province was 1,489 mm in 2014 and 1,154 mm in 2015 (Ministry of Water Resources and Meteorology, Stung Chinit Station). The experiment was conducted during the rainy seasons (from June to November) of 2014 and 2015. During the dry season (from late November to the end of May), the experimental field remained dry before being progressively flooded during the rainy season with most water from the end of August until October due to heavy rains.

Experimental design

The experimental field was about 2.6 ha large, where nine plots (250 m² each) were randomly distributed and managed equitably either in the DMC (3 plots) or in the CT systems (2 × 3 plots) (Supplemental Material 1). In the DMC system, seeds of two leguminous cover crops (*Stylosanthes guianensis* cv. Nina and *Centrosema pascuorum* cv. Cavalcade) were broadcasted (4 to 6 kg ha⁻¹ and 6 to 8 kg ha⁻¹, respectively) at the end of the rainy season and before rice harvest (early November). The cover crops were grown during the dry season on the residual soil moisture (until early May). In total, 30 days prior to the sowing of the new rice crop, the cover crops were rolled down using a roller crimper and glyphosate (N-(phosphonomethyl) glycine)

at 0.96 kg ha⁻¹ and 2.4 D [2,4-dichlorophenoxyacetic acid] at 0.72 kg ha⁻¹ were applied. Approximately 15 to 25 Mg ha⁻¹ of fresh biomass was left to decompose on the soil's surface (F. Tivet, CIRAD, pers. comm.). In early June, rice seeds were directly seeded into the mulch with a NT planter.

The CT system consisted of a practice commonly used by the local rice farmers. After the harvest of the rice crop, rice stubbles were left in the field. One month before sowing rice (in May), the soil was ploughed to control weeds and incorporate the remaining rice stubbles into the soil. Then, soil plowing and harrowing were practiced again on the day of sowing (June). No cover crops were grown between the two rice crop cycles under CT management. Two rice planting/sowing methods were used: transplanting (three plots) and seed broadcasting (three plots). Before transplanting, seedlings were grown in a nursery on an adjacent field during two to three weeks. For seed-broadcasting plots, seeds were manually broadcasted directly onto prepared soil (plowing and harrowing). Then, a harrow was used to incorporate the seeds into the soil. Mineral fertilizer was applied (100 kg ha⁻¹ of diammonium phosphate; 18% N, 46% P₂O₅ and 0% K₂O) at sowing time under the DMC system, and 15 days after rice transplanting and at seed broadcasting under the CT system.

During the rice cycle, weed control was done three weeks after sowing under both treatments and Cyhalofop-butyl (2-4-4-cyano-2-fluorophenoxy phenoxy propanoic acid, butyl ester (R)) at 0.285 kg ha⁻¹ and 2.4 D (2,4-dichlorophenoxyacetic acid) at 0.504 kg ha⁻¹ were applied under unflooded conditions.

Root sampling

Roots were sampled four times per year in both 2014 and 2015, three to four years after establishment of the experiment. The 1st sampling was done when the rice plants were at the tillering stage (at the end of June in 2014 and mid-July in 2015), the 2nd sampling at the maximum tillering stage (late August), the 3rd sampling at the late reproductive phase or early milky stage (in October) and the 4th sampling at the ripening phase shortly before harvesting (November).

At each sampling date, 20 individual rice plants were randomly uprooted per plot. At the 1st and 2nd sampling dates, whole root systems were collected, whereas at the 3rd and 4th sampling dates, a composite sample of 100 g was collected for each plant due to the large size of the root systems. Composite samples were made after cutting the roots into small pieces and mixing the pieces thoroughly

before taking a representative sub-sample of 100g. The roots were carefully washed in running tap water and placed in labeled plastic bags before being transported in cool iceboxes to the laboratory.

Assessment of the population densities of root-parasitic nematodes

Eggs were recovered from the root systems of 20, individually sampled, plants using a hypochlorite extraction method and a blender (McClure et al., 1973) with minor modifications (Bellafiore et al., 2015). After being filtered through 500µm and 250µm sieves, eggs and juveniles were recovered on a third 25µm sieve. The nematode suspension obtained after extraction was homogenized in 40ml tap water and 1 ml of this suspension placed in a counting cell chamber to count the number of eggs, second-stage juveniles (J2), and males using a stereomicroscope. Based on the total number of eggs, J2 and males, RPN population density per gram of fresh roots (RPN/g roots) was calculated.

Molecular identification of the root-parasitic nematodes

From each plot, two infected rice plants (the infection determined by the presence of RPN after counting) were randomly selected. Using a stereomicroscope, 20 J2 were randomly and individually collected from each plant and put into 1 ml tubes with 0.001% of Tween-20 solution and stored at -80°C until DNA extraction (Bellafiore et al., 2015). Ten microliters of pre-mix 2X lysis buffer (20mM Tris-HCl at pH 8.0, 100mM KCl, 3mM MgCl₂, 2mM DTT, 0.9% Tween 20) were added to each 1 ml tube containing a single J2, 10µl of dH₂O and 0.001% Tween 20. Zero-point five microliter of Proteinase K (100µg/µl in stock solution, Thermo Scientific (Waltham, MA) and 0.025µl of DTT (1 M) were added, and the tubes were incubated at 55°C for 3hr before being transferred to 98°C for 15min. One microliter of RNase type A (10mg/ml; Thermo Fisher Scientific, Waltham, MA) was then added to each tube and incubated at 37°C for 1 hr. The extracted DNA was immediately used for polymerase chain reaction (PCR) and then conserved at -20°C for future study.

PCR was performed on each J2 DNA extract using *M. graminicola*'s specific SCAR-MgFw/Rev marker (Sequence Characterized Amplified Regions) (Bellafiore et al., 2015). In the case of no amplification, suggesting the presence of other plant parasitic nematodes, another PCR with the Internal Transcribed Spacer (ITS) primers rDNA2 (5'-TTGATTACGTCCCTGCCCTTT-3') and rDNA1.58s

(5'-ACGAGCCCGAGTGATCCACCG-3') (Vrain et al., 1992; Powers, 2004; Pokharel et al., 2007) was then performed to sequence the amplification product and compare it to the NCBI databases. PCR products were purified after gel electrophoresis using Thermo Scientific GeneJET Gel extraction kit (Thermo Fisher Scientific, Waltham, MA) and sent for sequencing using standard procedures and rDNA2 primers (Macrogen Inc., Seoul, South Korea) (chromatograms and sequences are available on the "Harvard Dataverse": <https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/ZHLQVM>).

Using standard nucleotide BLAST, the sequences were compared to a collection of ITS sequences from *Hirschmanniella* spp. and imported from GenBank database (for *H. oryzae* accession number DQ309588, EU722286 and for *H. mucronata* (Das, 1960) accession number KP179331, DQ309589, EU722287). ITS sequences alignment, phylogenetic, and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013).

Assessment of the population dynamics of the root-parasitic nematodes

In conjunction with the molecular identification of the RPN species, the population dynamics of the RPN were studied using the RPN population densities at four rice development stages: seedling, tillering, milky and harvest. Eggs extracted from infected roots were hatched under controlled conditions (Bellafiore et al., 2015). For the tillering stage in 2014 and the harvest stage in both 2014 and 2015, the hatching was compromised and we were not able to collect J2. The average number of J2 per 100g of fresh roots was calculated for each plant stage. Based on the percentage of each RPN species from the molecular analyses and taking into account the RPN population density per gram of fresh roots previously formulated, the population density of each RPN species was calculated.

Soil sampling and soil analyses

Each plot was subdivided into four sub-plots. Five soil samples were collected from each sub-plot at 0 to 5cm depth in early December 2014 and these were mixed to create a composite sample. The samples were air-dried at room-temperature, passed through a 2-mm sieve and homogenized. Soil organic C and total N concentrations were determined with the dry combustion method (Nelson and Sommers, 1996) using a Sumigraph NC-80 Analyzer (Sumitomo Chemical Co, LTD, Osaka, Japan). Total organic carbon (TOC) and total

nitrogen (TN) were estimated and computed on an equivalent soil mass-depth basis according to Ellert and Bettany (1995). The labile C (LC) fraction was determined using the potassium permanganate oxidizable C (POXC) procedure modified by Culman et al. (2012) at 0.2M KMnO_4 . Briefly, 2.5g of air-dried soil was transferred into a 50ml centrifuge tube, to which 18ml of deionized water and 2ml of 0.2M KMnO_4 stock solution were added. The tube was shaken at 120rpm with a horizontal shaker for 2min, then left to settle for 10min. After settling, 0.5ml of the supernatant was transferred into another 50ml centrifuge tube containing 49.5ml of deionized water. The absorbance of the samples was read by a Spectrophotometer UV-1200 (Shimadzu Inc., Kyoto, Japan) at 550nm.

Soil pH was determined using a 1:2.5 ratio of soil/distilled water and measured with a pH meter D-51 (Horiba Ltd., Kyoto, Japan). Phosphorus content in each sample was determined using the Olsen method to extract the available soil phosphorus (Olsen et al., 1954). The extracted soil phosphorus was measured by the Murphy and Riley (1962) method. Exchangeable base cations Ca^{2+} and Mg^{2+} were extracted with 1 mol/liter KCl and K^+ with Mehlich-1 solution. Exchangeable Ca^{2+} and Mg^{2+} were determined by titration using 0.025 mol/liter EDTA. K^+ was determined by flame photometry.

In addition, for each sub-plot the central point was sampled using a core sampler topsoil (0–5cm depth) to measure soil bulk density (ρ_b) as assessed by the core method (Blake and Hartge, 1986). These soil samples were oven-dried at 105°C for 24hr, and dry weights were assessed to calculate the bulk density. In the field, soil conductivity (Cond), redox (Redox) and quantity of salt in parts per million (ppm) were recorded for each plot topsoil (0-5cm depth) at each sampling date with Hanna probes (Hanna Inc., Woonsocket, Rhode Island).

Statistical analysis

All statistical analyses were done using the software R (R Core Team, 2015; version 3.3.3). The RPN population density per g of fresh roots did not follow a normal distribution due to the high number of non-infected plants (zero value), and the data was therefore transformed into a binary distribution (0 for non-infected and 1 for infected plants) before performing a generalized linear model (GLM) following a binomial distribution. We tested the effect of the sampling by year (2014 vs 2015), cropping system (DMC vs CT), plot and rice developmental stage on RPN population densities by considering

both non-infected and infected plants. The same tests were also performed with only infected plants. Then, the relationship between yield and number of RPN per gram of fresh roots was evaluated using Kendall's test.

Plot parameters were further investigated using a principal component analysis (PCA) to summarize the information conveyed by all the variables to a reduced number of dimensions. The analysis was carried out with the library *ade4* (Dray and Dufour, 2007). A multivariate analysis was conducted to further analyze the contribution of the different plot parameters to the *a priori* classification of plots according to the agricultural system (DMC vs CT), since our main objective was to compare the effects of these contrasted practices on RPN. We compared the three DMC plots to the six CT plots. In order to have all the parameters, we conducted the PCA using plot means of both years. A between-class analysis was performed to investigate whether families cluster according to the agricultural system (DMC vs CT). The between-till structure was tested using permutations tests (Monte–Carlo tests; *ade4*, Dray and Dufour, 2007). Pairwise comparison of the number of RPN and the rice yield in a DMC system vs two CT systems was performed using the Kruskal–Wallis non-parametric test due to non-normal data distribution.

Results

Impact on soil nutrients and soil organic matter

The PCA showed an impact of the DMC and CT cropping systems on soil nutrient concentrations, TOC, TN, pH, labile soil organic C and bulk density (Fig. 1). The two axes explained 63.6% of the variance (first: 42.7%; second: 20.9%). The significance of this grouping (DMC vs CT) was significant (Monte–Carlo test, $p=0.016$).

The DMC cropping system resulted in higher TOC concentration, LC, TN, Mg and K concentrations (Table 1). In contrast, the pH was lower in the DMC cropping system compared with the CT cropping system.

Impact on rice yield

For all cropping systems, the yield was significantly higher in 2014 (2,572 kg ha^{-1}) compared to 2015 (2,299 kg ha^{-1} , $p<0.001$) (Fig. 2). When both evaluated CT systems are combined, the PCA emphasized higher yields under the DMC system compared

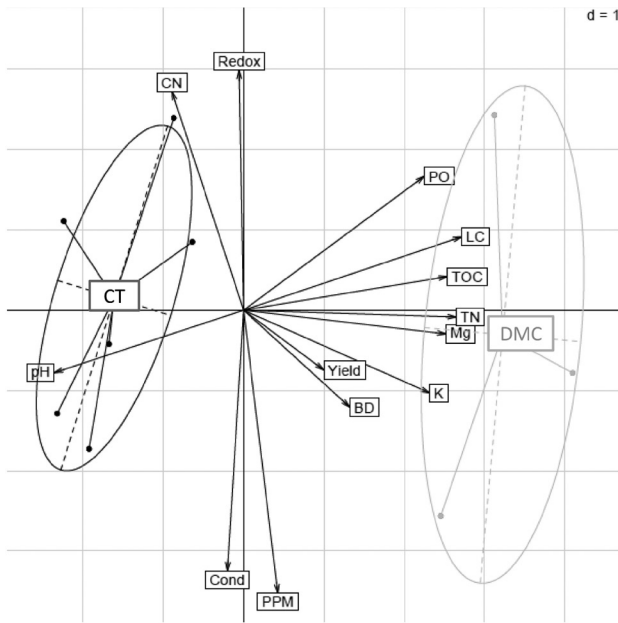


Figure 1: Principal component analysis (PCA) between plots belonging to conventional plough-based tillage (CT) and direct-seeding mulch-based cropping (DMC) systems and other variables in Stung Chinith field experiment. The first two dimension captures 43.3 and 21.9% of the total variance, respectively. Each dot is a plot; plots are regrouped by an ellipse considering the cultivation mode (DMC vs CT, see text for details). The variables were measured from samples at 10 cm soil layer/depth during each nematode survey: Cond is soil conductivity, Redox is the redaction-oxidation reaction, and ppm is the amount of salt in parts per million. Other variables were calculated from 0 to 5 cm soil layer in the laboratory: pH, BD (soil bulk density (ρ_b)), K (available potassium (K^+)), Mg (available magnesium (Mg^{2+})), TN (total nitrogen), TOC (total organic carbon), LC (labile carbon), PO (diphosphorus dioxide (P_2O_2)), and CN (ratio between carbon and nitrogen). Yield is rice yield calculated in kilogram per hectare ($kg\ ha^{-1}$). Groups constituted by the cultivation mode (DMC vs CT) are significantly different (Monte-Carlo test $p=0.016$).

to the CT system (Fig. 1). Still merging both CT systems, when one considers the rice yield it is not significantly different ($p=0.671$) in 2014 between DMC and CT but in 2015, it becomes significantly higher under DMC ($p<0.001$) (Fig. 2). Using the plot means of all plots combined (DMC and CT), a low negative correlation was found between the rice yield and the number of RPN, but it was not significant (correlation -0.25 ; $p=0.364$). Using only the plot means of the DMC system, a significant negative correlation was found between the rice yield and the number of RPN (correlation -0.89 ; $P=0.039$). When observed individually, the conventional tillage-seed broadcasting method (CT-B) gave the lowest rice yield for both studied years (Fig. 2).

Impact on root-parasitic nematodes

GLM analysis showed the effects of cropping system, rice developmental stage, year of sampling, and plot on RPN. Interactions were revealed between all variables measured (Table 2). When only the nematode-infected plants were considered, GLM analysis showed no interaction between the variables measured. All variables had an effect on RPN, except the sampling year (Table 2). The average number of RPN per gram of roots was significantly higher in 2014 than in 2015 for all cropping systems ($p<0.001$; Fig. 3). When comparing the three methods (DMC system, hand-broadcasting and transplanting in the CT systems), higher numbers of RPN were observed following DMC ($p<0.001$). Within the CT systems, the number of RPN was not different between hand-broadcasting or transplanting. Using rDNA barcoding, 360 and 480 J2 were analyzed in 2014 and 2015, respectively. This molecular identification revealed that the main nematodes associated with the rice roots were either root-knot nematodes (*Meloidogyne* spp.) or *Hirschmanniella* spp, albeit with lower abundance. Sequence alignment using Blastn revealed that only two RPN species, *M. graminicola* and *H. mucronata*, were present and *M. graminicola* was dominating. The occurrence of *M. graminicola* was higher than *H. mucronata* in both years and in both cropping systems (Fig. 4). The frequency of occurrence of *M. graminicola* decreased from 2014 to 2015 in both cropping systems, while the frequency of occurrence of *H. mucronata*, instead increased (Fig. 4). *M. graminicola* was present during all plant developmental stages, whereas *H. mucronata* was only found at the tillering and milky stages. In both 2014 and 2015, the total number of RPN per gram of roots was highest at the seedling stage when the plants were 2 weeks old, and lowest at harvest

Table 1. Comparison of soil minerals in DMC versus CT in sandy field experiment in Cambodia.

Systems	Plots ID	TOC	TN	CN	LC	pH	PO	BD	Ca ²⁺	Mg ²⁺	K ⁺
DMC	42L	8.78	0.91	9.62	370.33	4.57	27.64	1.72	0.89	0.22	0.13
DMC	52L	8.38	0.91	9.16	254.94	4.50	15.21	1.78	1.20	0.22	0.14
DMC	62L	13.14	1.43	9.19	312.46	4.19	17.79	1.64	0.92	0.20	0.14
CT	41LB	4.62	0.46	9.94	139.35	4.91	11.53	1.68	0.53	0.14	0.10
CT	41LT	4.43	0.45	9.81	139.91	4.96	9.53	1.62	0.51	0.14	0.10
CT	51LB	5.53	0.59	9.32	110.56	4.97	12.86	1.62	0.90	0.15	0.10
CT	51LT	4.69	0.52	9.04	107.12	5.05	9.51	1.62	0.70	0.18	0.10
CT	61LB	8.30	0.75	10.99	174.19	4.45	13.39	1.49	0.56	0.15	0.10
CT	61LT	8.17	0.78	10.54	169.73	4.57	15.41	1.72	0.51	0.14	0.07
DMC	Plot mean	10.10	1.09	9.33	312.58	4.42	20.21	1.72	1.00	0.21	0.14
CT	Plot mean	5.71	0.57	9.92	139.57	4.84	11.86	1.63	0.60	0.15	0.09

DMC, direct-seeded mulch-based cropping system; CT, conventional tillage; TOC, total organic carbon; TN, total nitrogen; CN, ratio between carbon and nitrogen; LC, labile carbon; pH, potential of hydrogen; PO, diphosphorus dioxide (P₂O₂); BD, soil bulk density (*pb*); Ca²⁺, calcium ions; Mg²⁺, available magnesium (Mg²⁺); and K⁺, available potassium (K⁺). Soil properties at 0–5-cm soil depth.

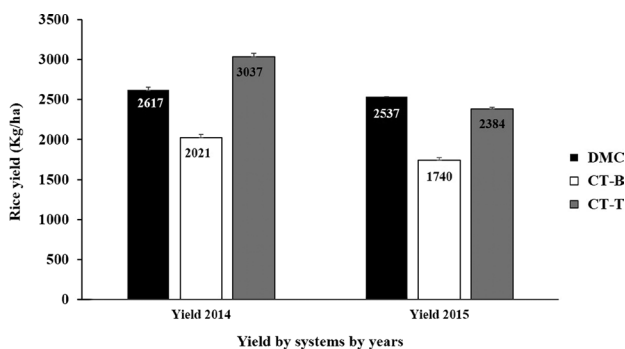


Figure 2: Comparison of yield in direct mulch-based cropping systems (DMC) vs conventional plough-based tillage (CT) plots. Pairwise comparison of rice production in 2014 and 2015 in the DMC system vs two CT systems using the Kruskal–Wallis non-parametric test. DMC: direct-seeding mulch-based cropping system; CT-B: conventional tillage-seed broadcasting method; CT-T: conventional tillage transplanting method. Values are in kg ha⁻¹ and in the form: mean ± standard error.

(Fig. 5A). For both years, only *M. graminicola* was found in the roots of the young rice plants two weeks after sowing (Fig. 5B). No data for 2014 are available but in 2015, the RPN population at the tillering stage consisted of *M. graminicola* (73%) and *H. mucronata* (27%). At the milky stage, *H. mucronata* was present in 2014 (15% of the RPN population), but not in 2015.

Discussion

In our study, the abundance of RPN was higher under the DMC system compared to the CT system. Several causes could be emphasized to explain this observation. First, the two cover crops used in the DMC system, *S. guianensis* and *C. pascuorum*, could have acted as reservoirs for RPN between the two rice crops. Both cover crops were checked and recognized as non-hosts of *M. graminicola* (M. Suong, pers. comm.). In addition, other studies have shown *S. guianensis* to have a nematicidal effect on another root-knot nematode species, *M. exigua*, in Brazil (Amaral et al., 2009), and it cannot be excluded that this is also the case for *M. graminicola*. Therefore, additional studies are needed to evaluate the direct effect of the cover crops. These two cover crops

Table 2. Generalized Linear Model (GLM) results with the p -value associated for the four principal factors and the interaction between them, considering the plants are infected or not.

Variables	Log-likelihood ratio test (χ^2)	df	Pr ($>\chi^2$)
Cropping system	32.039	1	$p < 0.001$
Plot	85.259	13	$p < 0.001$
Year	12.900	1	$p < 0.001$
Rice developmental stage	72.845	2	$p < 0.001$
Year \times Plot	132.994	13	$p < 0.001$
Year \times Rice developmental stage	11.432	2	$p = 0.003$
Plot \times Rice developmental stage	227.402	26	$p < 0.001$
Plot \times Cropping system	68.299	26	$p < 0.001$
Year \times Cropping system	3.514	1	$p = 0.060$
Rice Developmental stage \times Cropping system	2.366	2	$p = 0.030$
Cropping system	52.274	1	$p < 0.001$
Plot	99.796	13	$p < 0.001$
Year	3.024	1	$p = 0.082$
Rice developmental stage	32.079	2	$p < 0.001$

GLM results using both non-infested and infested plants (coded 0 for non-infested and 1 for infested plants) were highlight in gray. The model tested the effects of the sampling by year (2014 and 2015), cropping system (DMC and CT), plot and rice developmental stage on RPN population densities and their interactions. The results obtained from the analysis of Deviance Table (Type II tests), the significance was considered at $p < 0.05$ (presented in bold in the Table). Other non-highlighted color was GLM results using only infested plants. The model tested the effect of the sampling by year (2014 and 2015), cropping system (DMC and CT), plot and rice developmental stage on RPN population densities. The significance was considered at $p < 0.05$ (presented in bold in the Table). No interactions were detected between variables.

could have benefitted RPN abundance indirectly through their effect on soil nutrient and organic matter content. The effects of soil nutrients and organic matter on nematode assemblages have been reported elsewhere (Cadet et al., 2004; Asif et al., 2015).

Second, prevalence of some weeds species was observed both under DMC and CT and those could have a positive impact on RPN abundance. Specifically, *Cynodon dactylon*, *Cyperus iria*, *Echinochloa colona*, *Fimbristylis miliacea* and *Panicum repens* were found in both systems and an examination of their root systems also revealed the presence of *M. graminicola* on these plants (Supplemental Material 2). During the offseason (dry season), a higher abundance of *P. repens* was observed in the DMC system. *Commelina* spp., *Cyperus rotundus*, *Digitaria* spp.,

Eleusine indica, *Leptochloa chinensis*, *Marsilea minuta*, *Mimosa diplotricha* and *Paspalum distichum* were also present, but no infection with *M. graminicola* was observed. However, *Commelina* spp., *C. rotundus*, *Digitaria* spp., *E. indica* and *P. distichum* have been previously described as susceptible to *M. graminicola* and may therefore also serve as reservoirs (Bridge et al., 2005). Similarly, *Hirschmaniella* spp. susceptible weeds (Bridge et al., 2005; Anwar et al., 2011) were identified (i.e. *C. iria*, *C. rotundus*, *Digitaria* spp., *E. colona*, *E. indica*, *F. miliacea*, *M. minuta*, and *P. distichum*) and may have played the role of refuge plants (Yik and Birchfield 1979; Anwar et al., 2011). Despite the use of cover crops, an improvement of the weed management under DMC should be targeted (Sims et al., 2018).

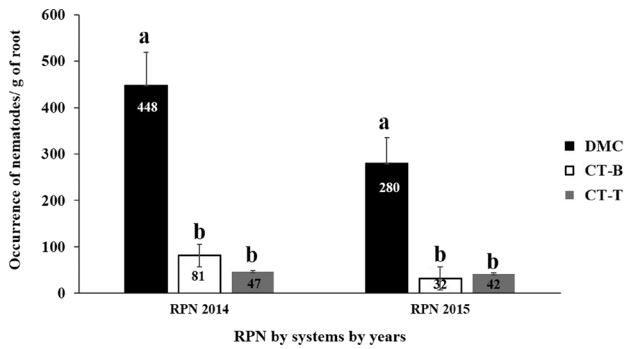


Figure 3: Comparison of the average number of root-parasitic nematodes between DMC vs conventional tillage plots in both year sequences. RPN stands for root-parasitic nematodes extracted from rice roots. The number of nematodes indicated is the average of RPN per gram of root. Different letter on histogram (a, b) indicates significant difference using Kruskal–Wallis non-parametric test. Values are the mean \pm standard error. DMC: direct-seeding mulch-based cropping system; CT-B: conventional tillage-seed broadcasting method; CT-T: conventional tillage transplanting method.

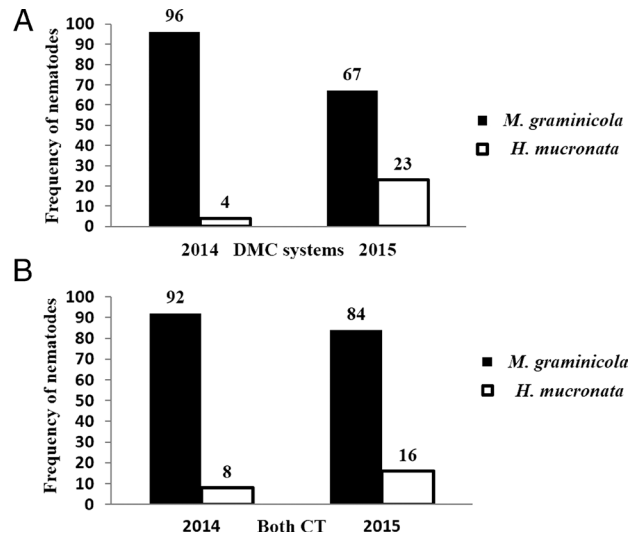


Figure 4: Comparison of the percentage of *Meloidogyne graminicola* vs *Hirschmanniella mucronata*. (A) represents the percentage of *M. graminicola* vs *H. mucronata* in directmulch-based cropping systems (DMC). (B) represents the percentage of *M. graminicola* vs *H. mucronata* in conventional plough-based tillage (CT).

Our third hypothesis is that the DMC system may have changed the microbial soil community in such a way that this benefitted the RPN. This cropping system may have resulted in more complex soil food webs compared to the CT system (Villenave et al., 2009; Ito et al., 2015). Higher root development in DMC systems (F. Tivet, pers. comm.) could also increase RPN population densities, as more roots offer more infection sites. Higher rice yields in the DMC system suggest that the increase in soil nutrients and organic matter observed may have allowed the rice plants to compensate for damage by the increased RPN numbers, also observed by Asif et al. (2015).

The molecular identification revealed that two RPN species were associated with rice roots. *Meloidogyne graminicola* was the predominant species although, to our knowledge, not reported from Cambodia prior to our study. This observation is in agreement with previous reports (De Waele and Elsen, 2007; Mantelin et al., 2017) stating that this root-knot nematode species is predominant in other regions of SE Asia. The migratory root endoparasitic nematode *H. mucronata*, previously reported in Cambodia (Khun et al., 2015), was the other RPN species found

in our study. This species was at least three times less abundant than *M. graminicola* in this lowland rice field characterized by a poor sandy soil.

Meloidogyne graminicola was observed at all rice developmental stages. Its population density was highest during the vegetative phase (from seeding to stem elongation), whereas *H. mucronata* was only observed at the end of the reproductive phase and beginning of seed ripening. Win et al. (2013) reported that the population densities of another *Hirschmanniella* species, *H. oryzae*, were highest during the maximum tillering and heading stages of rice cultivated in Myanmar. It has been shown that water regime plays an important role in the population dynamics of both *M. graminicola* and *Hirschmanniella* species (Prot and Matias, 1995; Win et al., 2013). *Meloidogyne graminicola* is well-adapted to intermediate and wet soil conditions (Soriano and Reversat, 2004; De Waele and Elsen, 2007). In fields with a poor sandy soil, water drainage is fast. Thus, in the early season from June to July, the experimental field was wet but not flooded, representing optimal conditions for *M. graminicola* infection (Soriano and Reversat, 2004; De Waele and Elsen, 2007). This

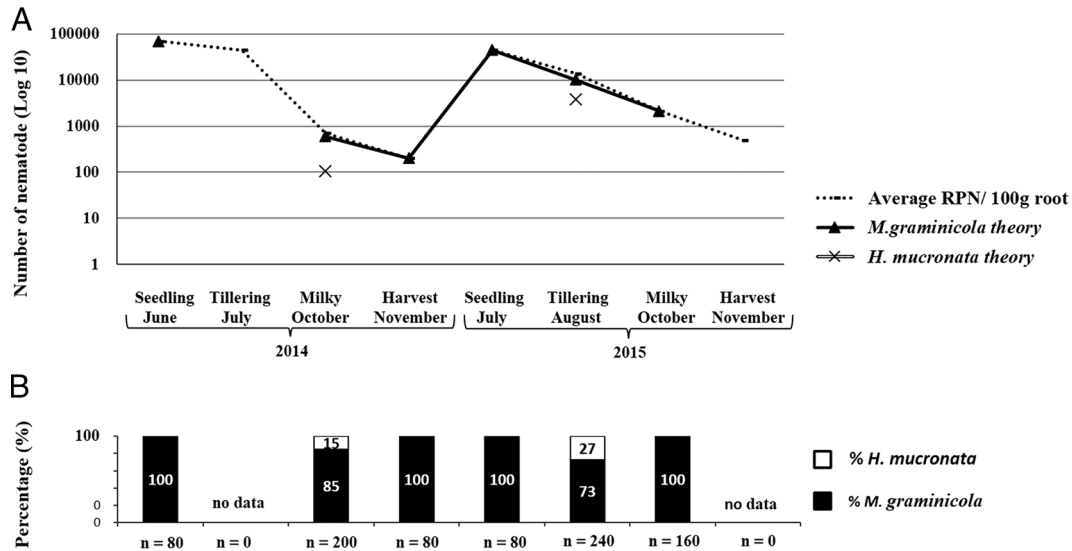


Figure 5: Comparison of the dynamic of *Meloidogyne graminicola* vs *Hirschmanniella mucronata* following rice development stages. (A) represents the dynamic of *M. graminicola* theory (continue line with the black triangle symbols), of *H. mucronata* theory (continue line with the cross-black symbol), and of the average of root-parasitic nematodes (RPN) per 100g of root (discontinued line with squared-white symbols). *M. graminicola* theory and *H. mucronata* theory were calculated by multiplying the ratio of *M. graminicola* and *H. mucronata* identified by PCR with the total number of RPN then divided by 100. The survey times and the rice development stages are indicated below the lines. The discontinued graphs for *M. graminicola* and *H. mucronata* are due to the fact that no J2 were collected during tillering stage 2014 and harvest stage 2015 because the hatching was compromised and we were not able to recover J2 for PCR/Blastn performance. (B) represents the percentage of *M. graminicola* (black histograms) vs *H. mucronata* (white histograms) during both eight surveys. *n* represents the total number of J2 successes identified by PCR and sequencing. No data means that no J2 were collected during tillering stage in 2014 and harvest stage in 2015, because the hatching was compromised and we were not able to collect J2.

could explain why *M. graminicola* population densities were highest at the seedling stage. In contrast, heavy rains started in August until the beginning of October, flooding the experimental field and promoting *H. mucronata* infection (Bridge et al., 2005). Thus, *M. graminicola* infection took place earlier, during the seedling stage, allowing the nematodes to remain in the roots and multiply until harvest. In contrast, *H. mucronata* infected the rice roots at a later stage, probably during tillering when the rice fields were flooded. *Meloidogyne graminicola* generally takes only 20 to 29 days to reproduce (Fernandez et al., 2014), whereas *Hirschmanniella* spp. take longer, from 28 to 48 days (Prot and Matias, 1995). During a 6-month rice growing period, *M. graminicola* can reach a maximum of six to nine life cycles, whereas *Hirschmanniella* spp. can only reach three to six life cycles. Based on the time of infection observed in our study, we speculate that *M. graminicola* could have reached 6 to 9 life cycles vs only one or two life cycles for *H. mucronata*.

We can conclude that the DMC system applied in our study improved, at least in the short term, the soil fertility and soil quality in such a way that also promoted the occurrence of RPN. In this short-term transition, rice yields were significantly higher ($p < 0.05$) under DMC when compared with CT. It will be of particular interest to investigate whether, during a long-term transition to DMC, recolonization by beneficial bacterial and fungal species, as well as mesofauna, can suppress nematode infection, as observed in other CA management (Verhulst et al., 2010; Palm et al., 2014). Indeed, the abundance and diversity of natural biocontrol agents may be greater in CA soils (Verhulst et al., 2010). Studies have shown an initial increase in pests and diseases under CA, followed by a gradual decrease (Palm et al., 2014).

In order to enhance the efficiency of such DMC system, attention should be paid to weed management during the dry season that can grow jointly with cover crops, since the DMC system has

been shown to be more favorable to the occurrence of some weed species (i.e. *Panicum repens*), and to the most efficient combination of cover crops to reach different objectives (weeds management, overall increase of soil biota, nutrient cycling, among others). The host status to RPN of weeds and cover crops should be examined, an aspect often neglected by agronomists. Increasing quantity and diversity of cover/relay crops are also of paramount importance under CA management to enhance for this depleted sandy soil the overall soil biota activity and move to an integrated management of pests (weeds, insects, parasitic nematodes) and diseases. Appropriate-scale mechanization is also a fundamental pillar of a sustainable intensification of rice farming including the use of roller crimper to manage high amount of biomass inputs reducing the use of herbicides. In fields infested with *M. graminicola*, water regime or the correct timing of planting may offer an efficient physical control method to limit the infection of rice seedlings by this pathogen. However, it may not always be possible to apply the correct water regime in all agroecosystems.

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Appendix

Supplemental Material 1



Figure S1: Distribution of plots in the experimental field (28,850 m²) in Stung Chinith, Cambodia.

Supplemental Material 2

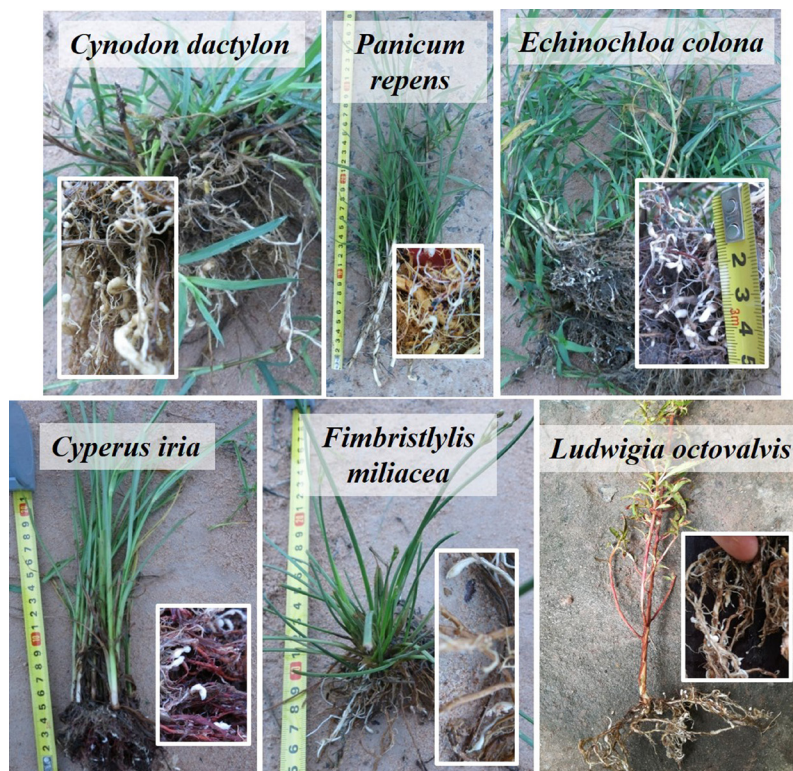


Figure S2: The typical formation of root galls caused by *M. graminicola* infection in six weed species commonly found in the lowland experimental paddy field.