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Species characterization and population dynamics of *Hirschmanniella mucronata* in lowland rice fields managed under conservation agriculture in Cambodia

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ABSTRACT

The rice root nematodes, *Hirschmanniella* spp., are considered the predominant plant-parasitic nematode in the clay soils of Battambang's lowland rice fields in Cambodia. In this study, we compared the nematode population dynamics, rice yield parameters, and soil organic matter content in lowland rice fields under conservation agriculture (CA) system with conventional tillage systems with green manure management (GMCT) or with plough-based tillage (CT) systems. Results demonstrated that GMCT for one year (GMCT1) and the long-term CA for seven years (CA7) reduced nematode densities in both soil and in rice roots, almost throughout the study period, compared to the CT. In the GMCT with tillage for two years (GMCT2), however, the *Hirschmanniella* spp. densities were high at the beginning, but reduced at later stages of the cycle. For rice yield components and soil fertility, CA7 proved to be effective in increasing plant height, the number of tillers/plant and soil organic matter. Based on molecular, morphometric and morphological features, the nematodes were identified as *H. mucronata.* The phylogenetic trees of three nuclear markers displayed similarity among 18S, 18S-ITS1-5.8S and D2D3 regions, identifying nematodes as *H. mucronata* clade I (bootstrap values of 79–100) and related to *H. kwazuna* and *H. loofi* sisters. Morphologically the long body (1,512–2,665 μm), long stylet (25–29 μm) and obvious mucron at the end of terminus matched with *H. mucronata*. Therefore, long term CA, with reduced tillage and using leguminous cover crops provides a promising system to control *H. mucronata* and promote rice yields and soil organic matter in Cambodian lowland rice fields.

1. Introduction

Rice (*Oryza sativa* L.) is an economically important crop in many countries. Currently, rice cultivation faces a number of challenges, including soil fertility depletion and pest and disease issues due to continuous cultivation in the same fields over long periods (Shelley et al., 2016). Plant-parasitic nematodes (PPN) are among the most serious pests of rice reported from numerous countries (Jones et al., 2013; Bernard et al., 2017). *Hirschmanniella oryzae* and *H. mucronata,* or rice root nematodes, are commonly associated with damage to rice roots, with yield losses up to 70% reported (Mahapatra and Rao, 1980). These migratory nematodes occur frequently on rice, being found in approximately 80% of paddy rice fields and with a wide range of alternative hosts of over 30 plant species (Youssef and Eissa, 2013). Youssef and Eissa (2013) reported that yield losses caused by *H. oryzae* in Egypt amounted to approximately 25%, or 53,734 MT. Management of these nematodes, however, has proved difficult to effectively practice. Although chemical control is considered to be among the most popular due to convenience and ease of application, this method has environmental side-effects, contaminates waterways and is harmful to farmers and consumers (Aktar et al., 2009). The use of

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Biological Control Agents (BCA) is becoming increasingly accepted and widely used to control nematodes, such as *Arthrobotrys oligospora*, *Arthrobotrys dactyloides*, *Dactylaria brochopaga*, *Monacrosporium gephyropagum*, and *Lecanicillium muscarium*, which could control *Meloidogyne arenaria*, *M. graminicola*, *M. hapla* and *Heterodera schachtii* (Singh et al., 2007; Vouyoukalou, 1993; Hussain et al., 2017a; Hussain et al., 2017b). However, this method has been of limited interest in combining with agricultural practices for integrated control (Lewis and Papavizas, 1991). Additional methods to manage PPN include the use of leguminous crops, for instance, as cover crops in rice fields. Some species of cover crops (e.g. *Crotalaria juncea*) have been shown to suppress PPN by modifying the soil conditions, attracting predators or directly secreting allelopathic molecules or nematicides into the soil (Wang et al., 2002). In addition, cover crops lend themselves well to improving soil fertility and are environmentally compatible (Claudius-Cole and Fawole, 2016; Stagnari et al., 2017).

Conservation agriculture (CA) is a crop production system, which mainly focuses on proper soil management to increase the carbon levels by reducing tillage and increasing biomass return to the soil, by using a permanent soil cover (mulch) and suitable rotations (Hobbs et al., 2008; Friedrich et al., 2012). Leguminous cover crops are frequently used because they raise nitrogen levels through atmospheric nitrogen fixation via their mutualistic relationship with rhizobial bacteria (Liu et al., 2011; Stagnari et al., 2017). They also enhance organic matter contents in the soil when carbon input is higher than carbon output (Six et al., 2002). Other studies emphasized that green manure practice associated with incorporating sunhemp (*C. juncea*) leaf and stem residuals into the soil significantly reduced *Meloidogyne* spp. densities by 94% and reproduction by between 56 and 98% (Marla et al., 2008; Kankam et al., 2015; Patel and Dhillon, 2017). Claudius-Cole and Fawole (2016) also found that the use of *Stylosanthes guianensis* as a cover crop would reduce *Scutellonema bradys* densities in Nigerian yam production systems. Leguminous crops can, however, be susceptible to various pests and diseases, some of which may additionally attack the major crop, promoting a parasite bloom. *Crotalaria* spp., for instance, are known as hosts of *Spodoptera frugiperda* (Montezano et al., 2018) and certain PPN species, including *Pratylenchus* spp., *Helicotylenchus* spp., *Scutellonema* spp., and *Criconemella* spp. (Wang et al., 2002). With the advantage of CA and cover crops, our main research objective was to examine the effect of the CA strategy on *Hirschmanniella* population dynamics, soil organic carbon and rice yield components in lowland paddy rice in Cambodia. A secondary objective was to accurately characterize the *Hirschmanniella* species present in these rice fields.

2. Materials and methods

2.1. Population dynamics of Hirschmanniella spp. in conservation agriculture fields

2.1.1. Description of the study sites

This research was conducted in a CA experimental field, established seven years previously by Department of Agricultural Land Resources Management (GDA/DALRM), team of the Conservation Agriculture Service Center (CASC) and the AIDA research unit of the French Agricultural Research for Development (CIRAD), Battambang province, Cambodia (Latitude 13°00′19.0″N, Longitude 103°04′20.0″E). The study was conducted between March 2018 – January 2019 in rice-based cropping systems consisting of: (1) conventional plough-based tillage (CT), (2) green manure cover crops plus tillage for one year (GMCT1), (3) green manure cover crops plus tillage for two years (GMCT2), and (4) conservation agriculture for seven years (CA7). The size of each rice plot was one hectare. In the CA system, three leguminous crops *Centrosema pascuorum*, *S. guianensis*, and *C. juncea* (6 kg $ha^{-1}$for *C. pascuorum* and *S. guianensis* and 15 kg ha^{-1} for *C. juncea*) were concomitantly grown, without tillage. In CT, leguminous crops were not planted, but tillage was performed three times/year (two ploughing and one harrowing). In GMCT, two cover crops were simultaneously planted and tillage conducted three times/year; in GMCT1 *S. guianensis* and *Sorghum bicolor* were used (December 2017) at the rate of 6 and 25 kg ha^{-1} respectively, while in GMCT2 *S. guianensis* and *Pennisetum typhoides* were used at the rate of 6 and 15 kg ha^{-1} respectively. The field soils had a clay soil texture, with 46–67% clay, 27–36% silt, and 6–23% sand (soil texture analysis was conducted by the Faculty of Agronomy, Royal University of Agriculture, Cambodia). Inorganic fertilizers, diammonium phosphate (18% N, 46% P_2O_5 and 0% K_2O) and potassium chloride (0% N, 0% P_2O_5 and 60% K_2O), were applied at the rate of 50 kg ha^{-1} each during rice transplantation. For controlling weeds, the herbicides 2,4 D (2,4-dichlorophenoxyacetic acid) and bispyribac sodium, were applied at the rate of 1 L ha^{-1} and 0.25 L ha^{-1}, respectively. The locally preferred jasmine rice cultivar "Phkar Rumdourl" was used in all treatments.

2.1.2. Soil sampling, soil analysis and yield component measurements

Eighty-four composite soil samples were collected at the depth of 10–30 cm from the soil surface from 14 plots (six composite soil samples/plot) of conservation agriculture, green manure cover crops plus tillage for one or two years, and conventional plough-based tillage (Fig. 1). Soil sampling was conducted four times: viz. in March 2018, at flowering stage of cover crops; in June 2018, during land preparation; in October 2018, at 120th day of rice cultivation (both soil and rice roots); and in January 2019, at 30th day of cover crop planting. Soil and rice root samples were placed in plastic bags and transported to the laboratory for nematode extraction.

Total organic carbon (TOC) from each plot was analyzed from soil samples (collected separately from those for nematode extraction and at the depth of between 0 and 30 cm from soil surface) using the Walkley and Black method (Walkley, 1947). In addition, rice yield components of plant height, number of tillers and one-thousand-seed weights were randomly measured according to Shahwani et al. (2014):

1. Plant height (cm): Recorded at crop maturity from a random selection of six locations per plot (six plants/location), using a measuring tape from bottom to tip of the spike.
2. Number of tillers per plant: Determined at crop maturity from six locations per plot (six plants/location).
3. One thousand rice seed weight (g): Six × 1000 seeds from each plot were collected at random and weighed.

2.1.3. Nematode processing

Nematodes were extracted from 150 g soil using the Cobb's Sieving and Decantation and the Modified Baermann's Funnel techniques (Christie and Perry, 1951). In brief, nematodes were extracted from soil by pouring the supernatants of soil suspensions through nested 250, 105 and 37 μm aperture sieves. Nematodes suspended on the 105 and 37 μm sieves were collected and placed on tissue papers lined on a wire screen, suspended on the funnels. Nematodes were collected from the bottom of the funnel after 48 h, observed, and counted under a compound microscope (Olympus BX50).

Rice roots were cut into about ~ 25 mm-long pieces, thoroughly mixed and six samples randomly selected from each plot in the field. Roots from each sample were rinsed free of soil under tap water, a 10 g sub-sample selected, which were further cut into 0.5–1 cm length, and then placed on the tissue papers lined on a plastic sieve in a tray. Water was then gently added into the tray

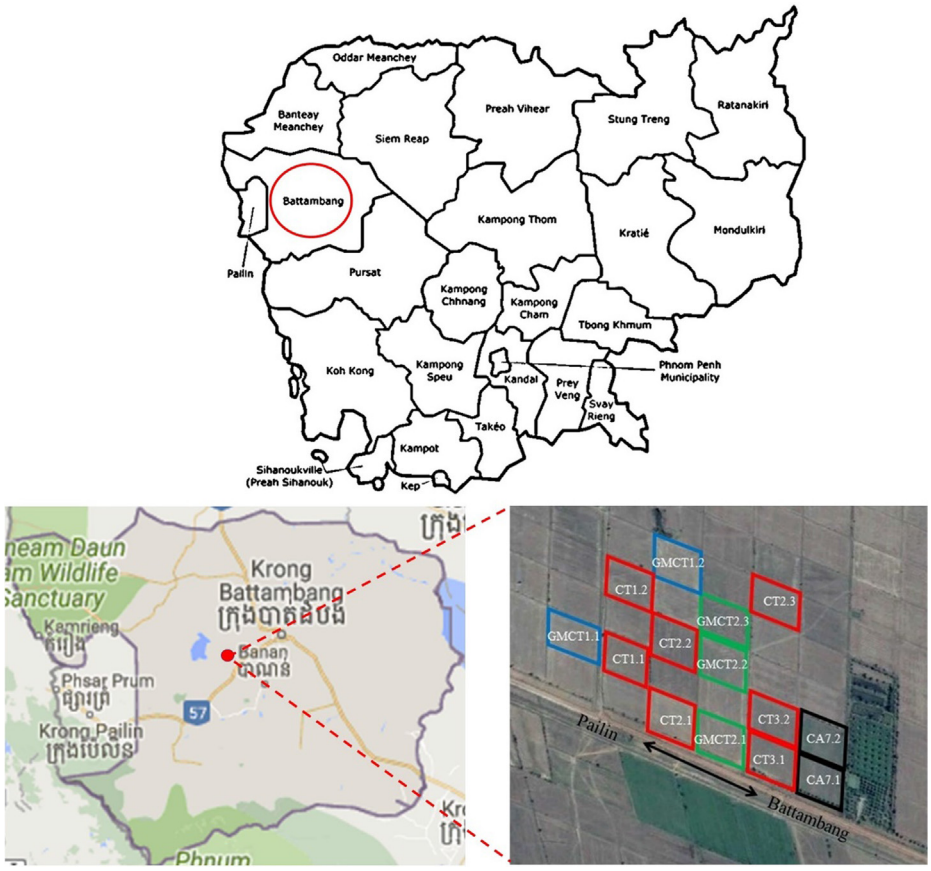

Fig. 1. Plot layout in Battambang province, Cambodia; CT = conventional plough-based tillage; GMCT1 = green manure cover crops plus tillage for one year; GMCT2 = green manure cover crops plus tillage for two years; CA7 = conservation agriculture for seven years (Veng, 2019).

until the roots were completely submerged (Coyne et al., 2007). After 48 h, nematodes suspended in tray were collected by 37 μm sieves, observed, and counted under a compound microscope (Olympus BX50).

2.1.4. Statistical analysis

Data were statistically analyzed using the SPSS software (version 16.0; SPSS Inc.; Chicago, IL, USA). Differences among CT, GMCT1 or GMCT2 and CA7 were determined by the Student's paired-plot design test at the 0.05 level as follows: GMCT1 with CT1, GMCT2 with CT2, and CA7 with CT3 (Fig. 1).

2.2. Identification of nematodes

2.2.1. Molecular characterization

A total of fifteen *Hirschmanniella* adults, randomly selected from a pool of the nematodes collected from the entire field of CA at Banan district, Battambang province, Cambodia, were used for DNA and phylogenetic analysis. In brief, one adult *Hirschmanniella* was placed in a 0.5 mL PCR tube filled with 25 μl of distilled water. Then, 25 μl of lysis buffer [200 mM NaCl (A&D Technology, Japan), 200 mM Tris-HCl pH 8.0 (A&D Technology, Japan), 1% (v/v) β-mercaptoethanol (Sigma, Japan), and 800 μg/ml proteinase K (Worthington Biochemical, USA)] was added. The reaction was incubated for 90 min at 65C, followed by 5 min at 99C (to deactivate the proteinase K) in a PCR Thermocycler (Biometra Tgradient Thermoblock). The extracted DNA was stored at −20 °C until used as DNA template (Holterman et al., 2006).

The polymerase chain reaction (PCR) was conducted by using extracted nematode DNA as the template (Five *Hirschmanniella* DNA samples/primer set). A 30 μl PCR reaction included 3 μl of DNA template, 9 μl of sterilized distilled water, 1.5 μl each of 10 μM forward and reverse primers [three primer sets including SSU18A/SSU26R for the 18S region (Mwangi et al., 2016), rDNA2/rDNA1.58 s for the 18S-ITS1-5.8S regions (Suong et al., 2019), and D2A/D3B for the D2D3 region (Subbotin et al., 2008)], and 15 μl of 2x PCR master mix with dye solution i-taq (Intron Biotechnology, Korea). The PCR condition was performed as follows: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s (all primers) and 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were purified and sequenced by Solgen Inc., Korea. Then, the DNA sequences were compared with those in the Genbank of the National Center for Biotechnology Information (NCBI), which was available online at https://www.ncbi.nlm.nih.gov/.

Multiple gene sequence alignments and phylogenetic reconstruction were performed by Molecular Evolutionary Genetics Analysis version 7.0. In brief, DNA sequences of four species of *Hirschmanniella* and plant-parasitic nematodes damaging rice roots based on 18S, 18S-ITS1-5.8S and D2D3 regions were selected from the Genbank. Then, the alignments were conducted to compare between these Genbank nucleotide sequences with those generated from each primer set using ClustalW. Phylogenetic trees were performed via the Maximum Likelihood (ML) methods based on Gamma distribution (GTR + G) model and the test of phylogeny conducted using the rapid bootstrap algorithm (1000 iterations) (Besnard et al., 2019).

2.2.2. Morphological identification

Live *Hirschmanniella* were killed by hot water at 50 °C and mounted on a drop of distilled water on a glass slide. Then, nematodes were observed and photographed using a digital camera

(Canon Power Shot A640) equipped with EOS Utility program and mounted on the compound microscope (Olympus BX50). Nematodes were measured via Axio Vision SE64 Rel. 4.9.1 program, and finally compared with the polytomous key (Khun et al., 2015). The morphometrics were then calculated as: L = Mean body length, a = Body length/Body width, b = Body length/Anterior end to pharyngo-intestinal junction (PIJ), b′ = Body length/Pharynx length, c = Body length/Tail length, c′ = Tail length/Maximum tail width, V% = Head to vulva length/Body length × 100, Stylet length, Maximum body width, Pharynx length, Anterior end to PIJ, Head to vulva length, Maximum tail width, and Tail length (Khun et al., 2015).

3. Results and discussion

3.1. Population dynamics of Hirschmanniella spp. in conservation agriculture fields

The GMCT1 and CA7 systems suppressed *Hirschmanniella* in the soil for almost the entire study period (Fig. 2A and C). Conversely, the GMCT2 system showed no reduction of *Hirschmanniella* densities in soil during the early period, compared to the CT system (Fig. 2B). A similar trend was observed in *Hirschmanniella* root densities, which showed the greatest reduction in densities, as com-

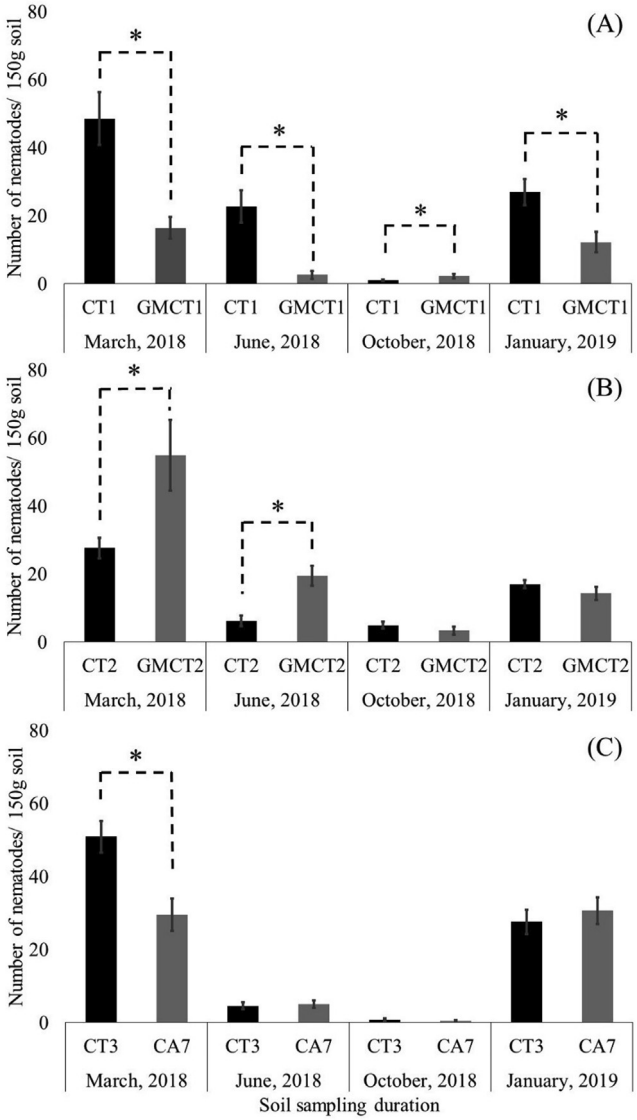

Fig. 2. Effect of (A) green manure cover crops plus tillage for one-year system (GMCT1), (B) green manure cover crops plus tillage for two-year system (GMCT2), and (C) conservation agriculture for seven years (CA7) on population dynamics of *Hirschmanniella mucronata* in 150 g soil, as compared with conventional plough-based tillage (CT). Bars refer to standard error of the mean of each treatment (n = 12 for GMCT1, CT1, CT3 and CA7; n = 18 for GMCT2 and CT2). *, significant at the 0.05 level.

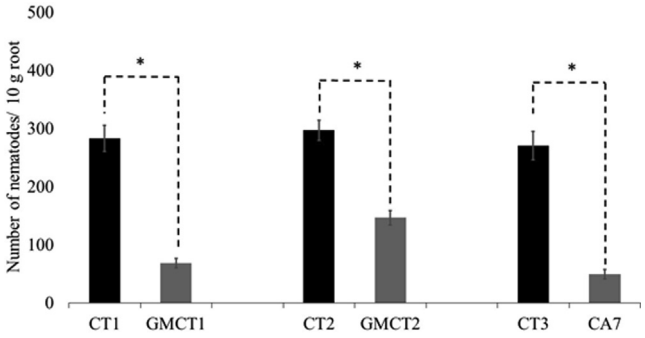

Fig. 3. Densities of *Hirschmanniella mucronata* per 10 g rice roots collected in October 2018. Bars refer to standard error of the mean of each treatment (n = 6). * significant at the 0.05 level; CT = conventional plough-based tillage; GMCT1 = green manure cover crops plus tillage for one-year system; GMCT2 = green manure cover crops plus tillage for two-year system; CA7 = conservation agriculture for seven years.

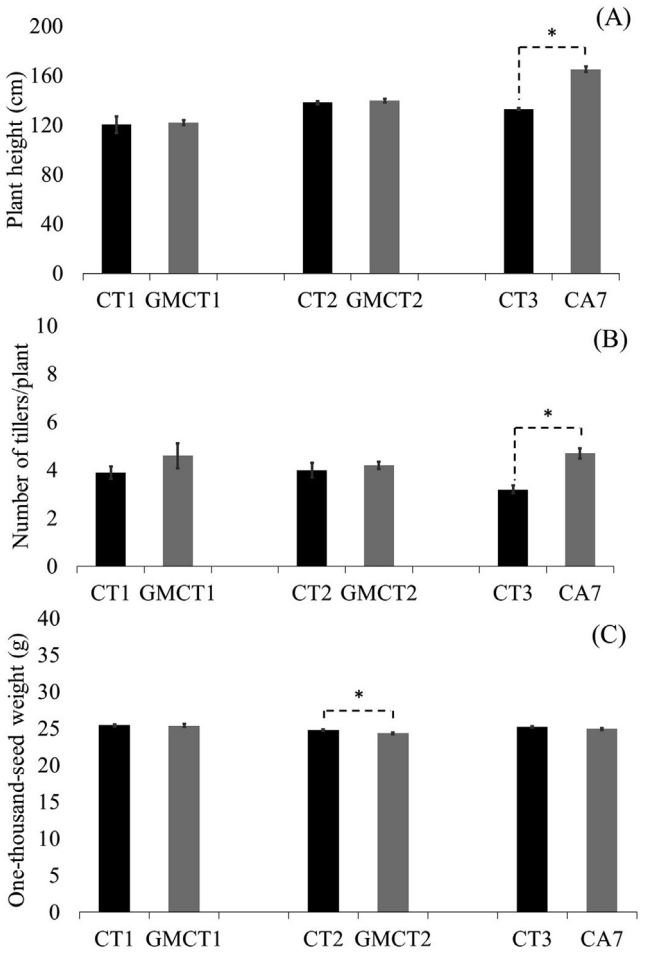

Fig. 4. Effects of conservation agriculture for seven years (CA7), green manure cover crops plus tillage for one year (GMCT1), green manure cover crops plus tillage for two years (GMCT2), and conventional plough-based tillage (CT) on plant height (A), number of tillers (B), and one-thousand-seed weights (C). Bars refer to standard error of the mean of each treatment (n = 12 for GMCT1, CT1, CT3 and CA7; n = 18 for GMCT2 and CT2). * significant at the 0.05 level.

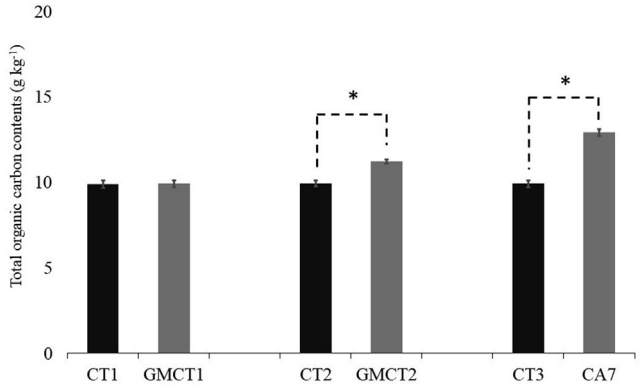

Fig. 5. Total organic carbon (TOC) (g kg^{-1}) in plots managed by the concept of conservation agriculture for seven years (CA7), green manure cover crops plus tillage for one year (GMCT1), and green manure cover crops plus tillage for two years (GMCT2), as compared to conventional plough-based tillage (CT). Bars refer to standard error of the mean of each treatment (n = 6 for GMCT1, CT1, CT3 and CA7; n = 9 for GMCT2 and CT2). * significant at the 0.05 level.

pared to CT, in CA7, followed by GMCT1 and GMCT2, respectively (Fig. 3). This result reflects that of Mcsorley and Gallaher (1993) and Govaerts et al. (2006), which found that crop residue retention under zero tillage reduced *Pratylenchus thornei* densities in maize fields, compared with conventional tillage, while conventional tillage with continuous maize and residue removal, the common farmer practice, had lower yields and dramatically higher *P. thornei* densities.

Results from our study demonstrated that CA strategies can influence the population densities of *Hirschmanniella* nematodes. At least three different scenarios could explain these impacts on *Hirschmanniella* densities. Firstly, secondary metabolites produced and released by cover crops roots (*C. pascuorum*, *S. guianensis* and *C. juncea*) affect the survival of plant-parasitic nematodes. This assumption was supported by reports from Wang et al. (2002), Marla et al. (2008) and Danahap and Wonang (2016), which demonstrated that *C. juncea* is suppressive to plant-parasitic nematodes viz. *Meloidogyne* spp., *Rotylenchulus reniformis* and *Radopholus similis* by acting as a non-host and by generating allelochemicals, including pyrrolizidine alkaloids and monocrotaline, that are toxic to PPN. Secondly, some leguminous crops recruited microbiomes that suppress soil-borne diseases, e.g. *Lotus corniculatus*, *Trifolium repens* and *Ononis repens*, additionally resulting in increased soil microbial diversity and persistence of entomopathogenic fungi (Vukicevich et al., 2016). Thirdly, the continuous practice of tillage could enhance the population build-up of *Hirschmanniella* spp., which was observed in GMCT2 and CT plots. This result coincided with the report by Zhong et al. (2017), which illustrated that reduction of tillage together with residue addition could control PPN (*Hirschmanniella* spp., *Rotylenchulus* spp. and *Tylenchorhynchus* spp.) in part through the promotion of bacterivorous, fungivorous and omnivorous nematodes in soil. Wang et al. (2012) reported that tillage had significant effects on soil properties and soil microbial communities, where

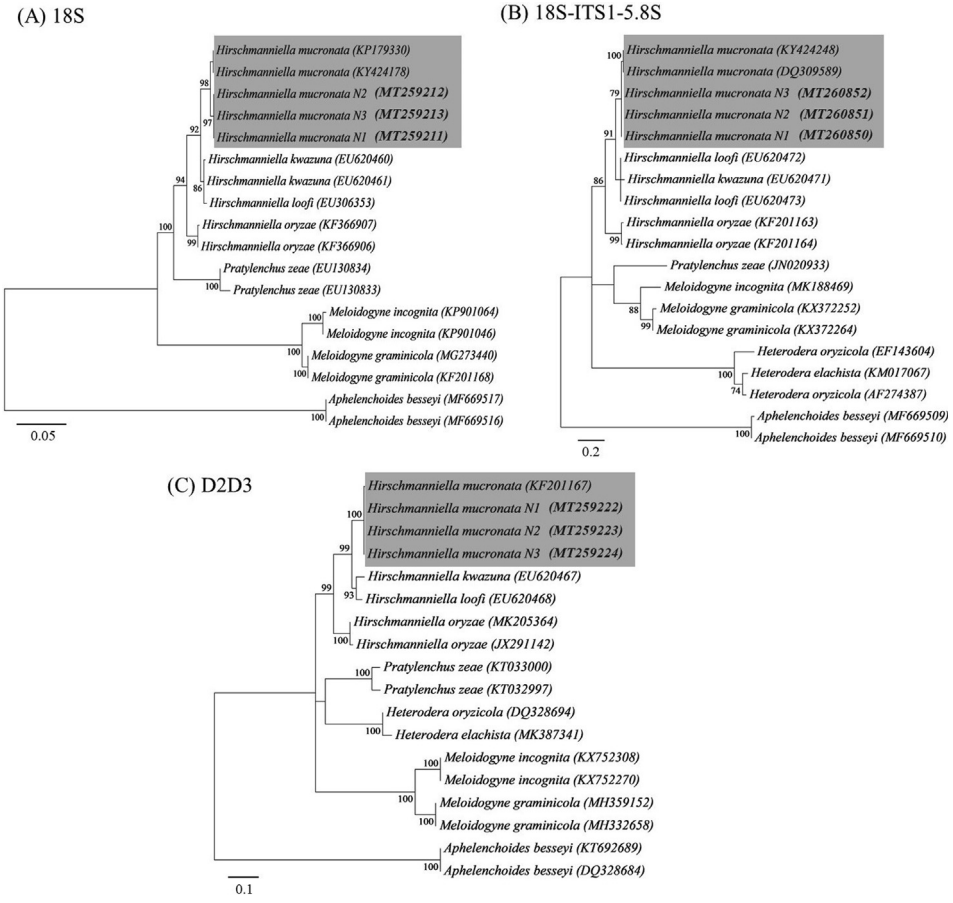

Fig. 6. Phylogenetic reconstruction based on: (A) 18S, (B) 18S-ITS1-5.8S, and (C) D2D3 regions of *Hirschmanniella* spp. and some other plant-parasitic nematodes in rice. Numbers beside branches represent ML bootstrap support values ≥ 70%. Scale bar represents substitutions per nucleotide position. NCBI accession numbers are listed behind the species names.

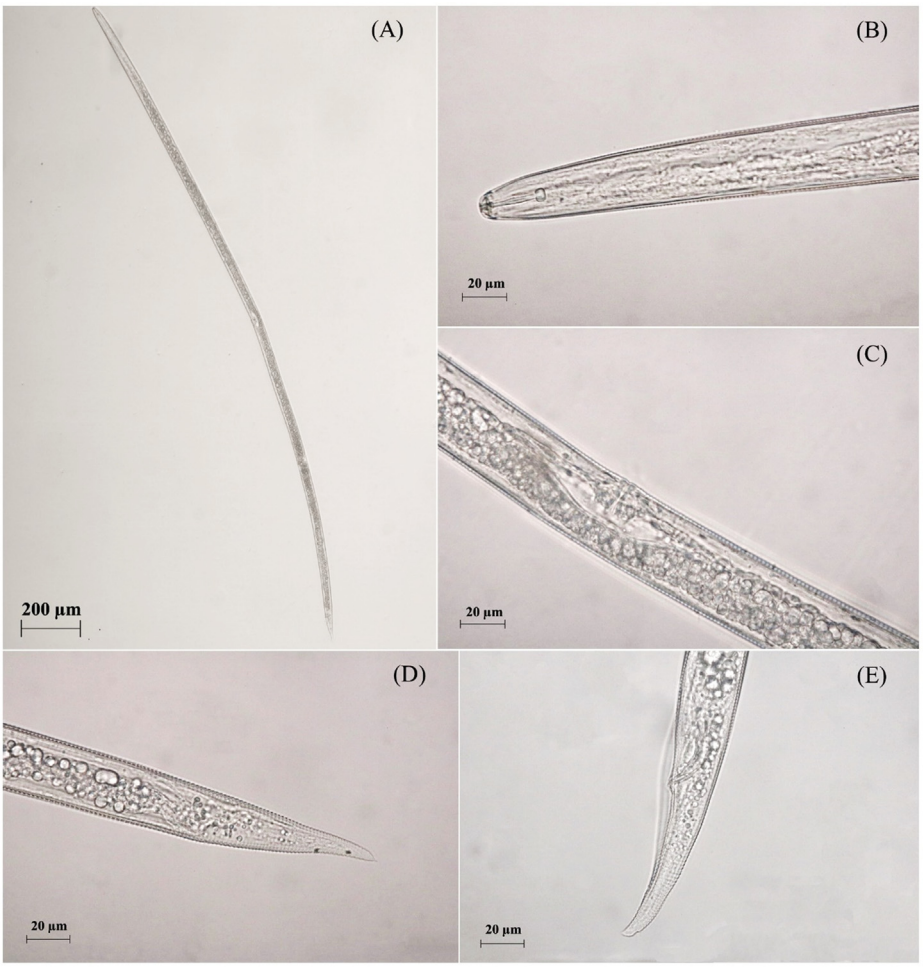

Fig. 7. Photomicrographs of *Hirschmanniella mucronata* found at Banan district, Battambang province, Cambodia. A: Whole body (100 ×); B: Anterior region (1000 ×); C: Middle region (1000 ×); D: Female tail (1000 ×); E: Male tail (1000 ×).

no-till systems increased microbial biomass carbon, total nitrogen, abundance of microbial communities, and arbuscular mycorrhiza fungi. Thierfelder and Wall (2010) and Singh and Dhumal (2019), concluded that ploughing resulted in soil compaction, reducing water infiltration, soil aeration and SOM content. Moreover, ploughing disturbed the habitat of soil microbial communities and faunas, thereby causing loss of microbial abundance and diversity. On the other hand, Ligowe et al. (2017) explained that CA increased pH, SOM content, soil aggregation and the abundance of earthworms. Consequently, different circumstances and situations can behave differently.

Although the current study used a range of cover crops, *S. guianensis* was incorporated in all treatments. Suong et al. (2019) reported that *S. guianensis* and *C. pascuorum* acted as non-hosts of *Meloidogyne graminicola*. Amaral et al. (2009) illustrated that *S. guianensis* produced compounds that were active against J2 of *M. exigua,* while Claudius-Cole and Fawole (2016) found that *S. guianensis* controlled *Scutellonema bradys* on yam by interrupting its life cycle over a 45-d duration. Sritharan et al. (2007) reported that crop rotation with pearl millet cv. CFPM 101 effectively controlled root-lesion nematodes (*Pratylenchus penetrans*) in on-farm trials with potato in Canada. Using sorghum as a green manure was also effective at decreasing root-knot nematode infestation in the soil (Djian-Caporalino et al., 2019). However, additional studies are needed to evaluate the direct and indirect effects of these cover crops on *Hirschmanniella* spp.

Aside from reducing the *Hirschmanniella* densities, CA7 also improved some rice yield components; significantly increasing rice plant height and numbers of tillers, compared to CT. GMCT1 and GMCT2, however, had no effect on height and tiller number, relative to CT (Fig. 4). For one thousand seed weights, these were not significantly different among all treatments, except GMCT2 that was slightly lower than in CT. Total organic carbon (TOC) was significantly improved in CA7 and GMCT2 ($p < 0.05$) (Fig. 5). These results are similar to those of Samal et al. (2017), who reported that long term CA increased total soil organic carbon (SOC) content, passive pool of SOC and rice yields. Increased level of SOC also activates and diversifies important soil biological activities by promoting new energy sources, which supports soil microbial activity, leading to higher densities of free-living nematodes and lower PPN densities (Widmer et al., 2002).

3.2. Identification of nematodes

3.2.1. Molecular characterization

Three primer sets (SSU18A/SSU26R, rDNA2/rDNA1.58 s and D2A/D3B) were used to amplify three different DNA targets of the nuclear marker (18S, 18S-ITS1-5.8S, and D2D3 regions). Comparison of the sequences (GenBank accession no. MT259211-MT259213, MT259222-MT259224, and MT260850-MT260852) with those in databases (NCBI) showed high homology with *Hirschmanniella mucronata* (more than 98–99% similarity). These results were similar to previous reports in Asia viz. Cambodia (Takeo and Kampong Thom provinces), Philippines, and China, where *H. mucronata* was identified from lowland paddy rice fields (Khun et al., 2015; Suong et al., 2019; Pascual et al., 2014; Chen

Table 1
Morphometrics of *Hirschmanniella mucronata* isolated from Battambang, Cambodia, and their comparison with those from other reports from Takeo–Cambodia, Luzon–Philippines and Puyan–China. All measurements are in μm and in the form of mean ± S.D. (max.-min.)

Character	Battambang, Cambodia (This study)		Takeo, Cambodia (Khun et al., 2015)		Luzon, Philippines (Pascual et al., 2014)	Puyan, China (Chen et al., 2006)
	Female	Male	Female	Male	Female	Female
n	25	20	30	21	1	21
L	2,217 ± 206.8 (2,665–1,881)	1,988 ± 191.6 (2,270–1,512)	1,775 ± 188 (2,160–1,260)	1,734 ± 186 (2,109–1,421)	2,136	2,020 ± 20 (2,370–1,770)
V (%)	51.8 ± 2.5 (57.3–46.6)	–	52 ± 2.3 (59–49)	–	50.3	51 ± 2.4 (55.2–43.5)
a	66.2 ± 5.7 (80.9–51.9)	64.8 ± 5.9 (80.4–54)	58 ± 5.2 (67–47)	59 ± 7.6 (81–45)	69.6	60.6 ± 6.1 (76.8–49.5)
b	17.3 ± 1.9 (21.8–12.7)	16.2 ± 1.5 (19.2–13.9)	14 ± 1.1 (16–12)	13.9 ± 1.7 (16.7–11)	–	23.5 ± 1.4 (26.6–20.9)
b'	6.1 ± 0.6 (7.9–4.9)	6 ± 0.6 (7.3–4.8)	5.9 ± 0.7 (7.4–4.4)	5.8 ± 0.7 (7–4.5)	–	6.3 ± 0.4 (6.8–5.6)
c	25.8 ± 2.1 (33.5–22.7)	24.5 ± 2.4 (30.1–21.1)	22 ± 2.7 (28–16)	21 ± 2.2 (25–17)	28.3	22.0 ± 2.4 (26.4–18.4)
c'	4.1 ± 0.4 (4.9–3.2)	5 ± 0.5 (6–3.8)	3.7 ± 0.4 (5–2.8)	4.4 ± 0.4 (5.1–3.5)	3.5	3.8 ± 0.3 (4.4–3.3)
Stylet length	27.5 ± 1.1 (29.4–25)	27.3 ± 1 (29–25)	22.2 ± 0.6 (23–21)	23 ± 1.2 (26–21)	25.4	24.7 ± 0.8 (26.7–23.3)
Maximum body width	33.6 ± 2.9 (38.3–28)	30.7 ± 2 (34–28)	30.5 ± 2.3 (35–25)	30 ± 3.6 (35–22)	30.7	–
Pharynx length	362.8 ± 34.5 (427–286)	335.4 ± 34.1 (396–266)	300 ± 40 (399–229)	299 ± 38 (371–215)	–	–
Anterior end to PIJ	128.5 ± 9.9 (153.6–109)	123.1 ± 7.5 (136–109)	124 ± 12 (147–84)	125 ± 10.9 (144–108)	–	121 ± 13 (137–98)
Anterior to vulva length	1,146.1 ± 95.5 (1,334–998)	–	936 ± 104 (1,160–630)	–	–	–
Spicule length	–	32 ± 1.8 (35–28)	–	34 ± 1.6 (37–31)	–	–
Maximum tail width	21.2 ± 2.8 (26.1–15)	22.04 ± 1.7 (24–19.4)	22 ± 2.3 (27–18)	18.5 ± 1 (20–16)	–	24 ± 2 (28–22)
Tail length	86.2 ± 9 (104.5–71.1)	80.7 ± 6.2 (88.7–69.4)	81 ± 8.2 (99–60)	82.6 ± 9.9 (98–63)	75.5	92 ± 9 (108–77)

et al., 2006). However, only *H. oryzae* was reported from Myanmar, based on identifications using morphological characteristics (Maung et al., 2010).

The phylogenetic reconstruction for *Hirschmanniella* was similar among 18S, 18S-ITS1-5.8S and D2D3 regions (Fig. 6). Three samples collected from Battambang province, Cambodia, were identified as *H. mucronata* clade I and related to *H. kwazuna* and *H. loofi* sisters, with all nodes significantly supported (bootstrap values of 79–100) even though clade II showed only *H. oryzae* with a low support to clade I. Our results correspond with those of Khun et al. (2015), who also showed that *H. mucronata* shared a clade with *H. kwazuna* and *H. loofi*, the single nucleotide polymorphisms (SNPs) at D2-D3 region of *H. mucronata* differed from *H. loofi* and *H. kwazuna* by 7.7%–8.3% and 8.1%–8.7%, respectively, while at ITS1-5.8S-ITS2 were 22.8%–23.4% and 22.7%–23%, respectively. Moreover, Pascual et al. (2014) reported that the sister phylogenetic position of *H. mucronata* to the clade of *H. loofi* and *H. kwazuna* was observed, while *H. oryzae* is located in a different clade.

3.2.2. Morphological identification

Morphological characteristics of males and females had similar (Fig. 7 and Table 1) body length ranging from 1,512–2,665 μm, stylet length from 25 to 29.4 μm depending on body size of nematodes, basal knob round, distinctly offset, the mean values of anterior end to pharyngo-intestinal junction (PIJ) 109–153 μm, long overlapping of esophagus over intestine, pharyngeal glands elongated, pharynx lengths 266–427 μm, tail lengths 69.4–104.5 μm, maximum tail widths 15–26.1 μm and the end of tail terminal an obvious mucron. For female, the position of vulva was located at approximately 50% of body length with two ovaries, while *Hirschmanniella* male tail had a distinct bursa and spicules of 32 ± 1.8 μm long.

The morphometric characteristics of the *Hirschmanniella* specimens were: L = 1,512–2,665 μm, V% = 46.6–57.3, a = 51.9–80.9, b = 12.7–21.8, b' = 4.8–7.9, c = 21.1–33.5 and c' = 3.2–6. These values correspond with the descriptions by Khun et al. (2015) in Takeo province, Cambodia, Pascual et al. (2014) in Luzon province, Philippines and Chen et al. (2006) in Puyan province, China for *H. mucronata*. However, the size of nematodes in our study was larger than in these reports. So et al. (2012) reported that nematode size is dependent on food, sex and temperature. Consequently, *Hirschmanniella,* found in rice fields in Banan district, Battambang province, Cambodia, was identified by using nematode morphological characteristics as *H. mucronata*.

4. Conclusion and recommendation

This current study demonstrates that long-term CA for seven years, incorporating leguminous crops, such as *C. pascuorum*, *S. guianensis*, and *C. juncea* as cover crops and no tillage, significantly lowers *H. mucronata* densities and improves rice yields (plant heights and number of tillers) and soil organic carbon, when compared with a system using green manure cover crops plus tillage and conventional plough-based tillage. Regarding the identification of nematode species, based on molecular, morphometric, and morphological characteristics, *H. mucronata* is the main *Hirschmaniella* species in Battambang's lowland rice fields, Cambodia. Further studies are needed, especially to determine the effect of these leguminous crops on the growth and development of *H.*

mucronata and to elucidate the mechanisms of CA practice in suppressing plant-parasitic nematodes in rice fields.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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