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ROOT KNOT NEMATODE INFECTIONS PROMOTED BY AGRICULTURAL PRACTICE MODIFICATIONS IN VIETNAM AND THE IMPACTS ON RICE PRODUCTION

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ABSTRACT

A survey conducted on newly cultivated lowland rice fields by direct seeding method in Hai Duong Province, Viet Nam, in March 2017 revealed high devastation of the field. In these fields, farmers used an annual crop rotation cycle of rice-scallion-rice. Investigations on the devastated fields revealed that the chemical and physical soil properties were appropriate for rice cultivation. On the other hand, observations done on the root systems showed that the dead plants have symptomatic root galls suggesting the presence of plant parasitic nematodes. Sequencing of the internal transcribed spacer (ITS) region of the rDNA genes of the nematodes showed that the root nematodes extracted from the infested fields belonged to *Meloidogyne graminicola*. The reproductive factor of the isolated *M. graminicola* population on the IR64 rice variety (*Oryza sativa indica*) was normal, suggesting that the impact of this plant pest was not due to the emergence of an unusual virulent population. The combination of the three factors (wrong cropping choice for rotation, using rice variety susceptible to *M. graminicola* and direct seeding) were obviously promoting the nematode infection and its high proliferation in the surveyed fields. *Meloidogyne graminicola* could parasitize and propagate in scallions of Vietnam. Since this plant is annually cultivated on a paddy field for crop rotation, preventive measures or alternative plant for crop rottion is necessary.

Keywords: *Meloidogyne graminicola,* cropping sequence, rice, scallions, virulent.

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INTRODUCTION

Rice is the most cultivated cereal and the most important staple food in Vietnam. According to FAO statistics, Vietnam is ranked at the 5th among rice producing countries in terms of weights, after China, India, Indonesia and Bangladesh, with 42.8 million tonnes of paddy rice produced in 2017 (FAOSTAT, 2017). However, Vietnam ranks 26th among rice producing countries in terms of yields, at 55,476 hg/ha in 2017. Annually, pests such as plant parasitic nematodes (PPNs) are known to be responsible for agricultural losses of more than $US 80 billion (Nicol et al., 2011). they are particularly detrimental to rice (Mantelin et al., 2017). The most damaging PPN for rice is *Meloidogyne graminicola* (*M. graminicola*). This root-knot nematode (RKN) has a large host range and geographical distribution (Mantelin et al., 2017). RKNs are telluric obligate plant parasites that induce gall formation in the infected roots to facilitate female development (Bridge and Page, 1982). The sedentary female feeds on the plant cells in the root galls where they hijack the plant's metabolism making it weaker with a small root system and consequently severely compromise rice yields (Bridge Page, 1982).

In Vietnam, rice is cultivated in almost all provinces with two intensive production regions being the Red River Delta in the North and the Mekong Delta in the South. In the Red River Delta, farmers routinely have two rice crop productions a year with occasionally one crop rotation during the offseason (e.g. scallion, potato, sweet potato, pumpkin, corn, sesame (Nguyen, 2009; Pham et al., 2013). Due to the fast socio-economic changes in Vietnam, including urban migration and reduction of agricultural workforce (World Bank, 2019), in some provinces, farmers have recently stopped doing the traditional time-consuming transplanting and shifted to direct seedling practices. This practice saves time but is unfortunately accompanied by unwanted side effects like increased impacts by parasites such as PPN (De Waele & Elsen, 2007). Previous studies in Vietnam only noted the presence of *M. graminicola* species in paddy rice (Nguyen & Nguyen, 2000; Bellafiore et al., 2015), but damage assessment of *M. graminicola* in fields have not been conducted. In March 2017, our survey in Hai Duong Province revealed that several rice fields were highly devastated. Farmers were presented with the hypothesis that a nematode attack was compromising their rice production.

In this study, we analyzed the reasons that could explain the unusual proliferation of PPN and its observed impacts on rice in Hai Duong Province, Vietnam. Three main hypotheses were tested: (i) A pest (nematode) highly aggressive to rice was emerging, (ii) Farmers used rice genotypes highly susceptible to nematode infections, and (iii) Modification of the farmer practices lead to the proliferation of the pest.

MATERIALS AND METHODS

Field description, plant and soil sampling

The survey was conducted on the 11th March, 2017 in Nam Sach district, Hai Duong Province (21°00'51.1"N and 106°19'33.0"E) (Figs. 1a, 1b). The Red River Delta of Northern Vietnam has a tropical monsoon climate. The three rice fields, where the survey was carried out, are inside a ten-ha area of land with three crops rotation per year: two rice and one scallion crop production cycle. For a decade, farmers have been growing scallion in the winter before cultivating two cycles of rice in spring and summer. Chemical fertilizers have been mainly used (from 8 to 8.5 × 100 kg NPK/ha/rice crop, and from 1 to 1.5 P_2O_5 × 1,000 kg + 300 kg Urea + 200 KCl/ha/scallion crop). Chemical pesticides were routinely applied to control plant pathogens whenever the epiphytotic of plants appeared in the field. For the first rice cropping cycle in 2017, 15 days after ploughing, the farmer planted the rice variety Bac Thom N°7 (*Oryza sativa indica)* by direct seeding. In the spring of 2017, due to unusual water scarcity, the fields were exposed to a drought stress for up to 20 days. Nearly four weeks after direct seeding, almost all seedlings died, presenting leaf chlorosis and small root systems with swelling galls Fig. 1c).

Plants and soil sampling

Three fields, 3,000 m^2 each, were surveyed from the rice cultivated area (Fig. 1b). Each field was subdivided in four plots of 100 m^2 each. 50 plants were randomly collected from each plot, i.e. a total of 600 plants were analyzed. In addition, a composite soil sample was taken per plot for physical and chemical properties analysis Each plant or soil sample was kept in a separate and labeled plastic bag at 4 ºC until laboratory analysis. Plant samples were analyzed at LMI RICE2 (Ha Noi, Vietnam), and soil properties were analyzed at the Soil Science Department, Faculty of Land Management in the Vietnam National University of Agriculture (VNUA, Ha Noi, Vietnam).

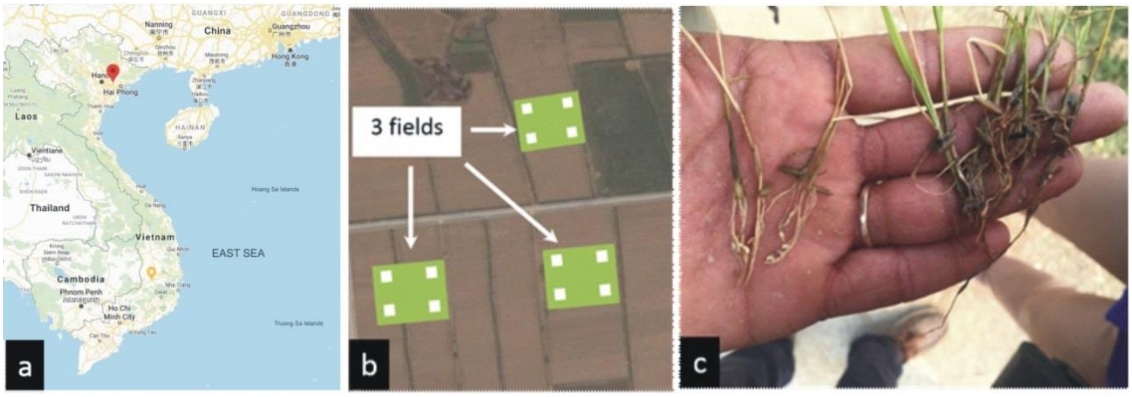

Figure 1. Rice fields of Hai Duong Province in Vietnam (a), location of the surveyed fields in Vietnam; (b) the three fields (in green) and the four plots (white) for each field where soil and plant samples were collected; (c) infested plants (left) with small terminal root galls and chlorosis leaves

Nematode extraction

Plants were picked up and scanned carefully for the presence of galls characteristic of RKN infection. The nematode extraction was carried out using the hypochlorite extraction method and a blender (McClure et al., 1973) with minor modifications (Bellafiore et al., 2015). Briefly, root galls collected in the field were carefully washed with tap water to remove soil then put in a 150 ml beaker containing 0.5% hypochlorite solution for two minutes before manually breaking the galls to extract nematode eggs and juveniles (J2). The mixture was then filtered through an 80 µm sieve to remove plant root tissues. Eggs and J2 were recovered on a second 25 µm sieve, rinsed several times with tap water in order to remove the hypochlorite solution. Eggs and J2 were placed on a strainer covered by two damp Kimwipe tissues on a 50 ml beaker filled with sterile ddH_2O. After being kept for two days in the dark at room temperature, nematodes were collected for further experiments.

Nematode identification

Firstly, rice root galls extracted from fields were stained with acid fuchsin (Byrd et al. 1983) to confirm the presence of PPN. Secondly, under a stereomicroscope, freshly extracted J2 were observed and individually collected. Nematodes were fixed in TAF (91 ml H_2O; 7 ml of 40% formalin; 2 ml of triethanolamine) and transferred to anhydrous glycerine to make permanent slides following Seinhorst (1959). Perineal patterns of the swollen females were cut, cleaned, and mounted in glycerine following Hartman & Sasser (1985).

Twenty single J2 were picked up in 10 µl of ddH_2O and transferred individually in twenty PCR tubes. 10 µl of 2X DNA lysis buffer was then added to each PCR tube to proceed DNA extraction by a proteinase K method as described by Bellafiore et al. (2015). Primers rDNA2 (5'-TTGATTACGTCCCTGCCCTTT- 3') and rDNA 1.58s (5'-ACGAGCCCGAGTGATCCACCG-3') were used to amplify the internal transcribed spacer (ITS) region of the rDNA gene (Vrain et al., 1992). PCR was performed following Bellafiore et al. (2015) with 35 PCR cycles of 95 ºC for 30 seconds, 54 ºC for 30 sec and 72 ºC for 1 min followed by one step at 72 ºC for 10 min. Amplicons were gel-purified and seven samples having good result after purification were directly sent for sequencing (Macrogen, South Korea) using primer rDNA2. ITS sequences were blasted against NCBI's nucleotide-collection (nr/nt) database, aligned with reference accession numbers *M. graminicola* MgVN18 KF250488, *M. graminicola* MgVN13 KF250481; *M. graminicola* HM623442.1; *M. naasi*, JN157863; *M. arenaria*, AF387092; *M. incognita*, KC464469; *M. javanica* AY438555; *M. hapla* LC030362.1 and LC030359; *Hirschmanniella oryzae*, EU722286 and *Globodera rostochiensis* GQ294519.1 using MUSCLE v3.8.31 (Edgar, 2004) and cleaned with GBLOCKS (Castresana, 2000). The phylogenetic tree using the ITS sequence of *Meloidogyne* isolated at Hai Duong and other nematode species was constructed using Maximum Likelihood (ML) analysis in MEGA 6 software with 1000 bootstrap replications.

Reproduction factor and virulence test

The scallion cultivar and IR64 cultivar (*Oryza sativa*) were grown to assess their susceptibility to *M. graminicola* under controlled conditions (28 ºC, 16 hours light-8 hours dark). Before transplanting, the scallion bulbs were treated for 10 min in 1% aqueous sodium hypochlorite solution before being rinsed several times with tap water. Scallion were grown in 20 × 20 cm pots previously filled with autoclaved sandy soil made of 50% sand and 50% potting soil and watered every three days in order to conserve a non-saturated soil. Two weeks after planting, each plant was inoculated with 200 J2 (initial population "Pi"). Concurrently, 10 days old IR64 seedlings cultivated in small columns containing autoclaved sand were inoculated with 200 freshly hatched J2s. At 27 days, post-inoculation (dpi) roots were collected, one gram of root was stained with acid fuchsin (Byrd et al., 1983).

Nematode in rice and scallion roots were extracted according to the method described above. Under stereomicroscope, for each root system, eggs and nematodes were counted and the sum of eggs and J2 gave the final population density "Pf". The reproductive factor (Rf) was calculated according to the ratio: Rf = Pf/Pi. This experiment was repeated twice. Five plants for scallion and 10 plants for rice genotype were used for each repeat. Plants with Rf < 1 were considered resistant, and Rf > 1 as susceptible (Soriano et al., 1999). The Rf of first repeat was present in the result.

Statistical analyses for the reproductive factor and soil properties

All statistical analyses were performed using R software (R core Team, 2015). Two sample Student's t-tests were used to compare the different means in Rf of rice and scallion with 95 percent confidence interval. Variance analysis was used to compare the three fields for the different parameters of soil properties using the Kruskal Wallis test.

RESULTS

Soil characteristics

Measured pH (pH H_2O and pH KCl) as well as chemical contents, including organic carbon (OC), organic matter (OM), nitrogen (N), sulfur (S) and cation exchange capacity (CEC), were in a range suitable for rice growing (McCall 1980; Dwevedi et al., 2017; Mccauley et al., 2017). No significant differences were observed for each parameter in the four repeats of each field and among the three fields ($p > 0.05$). The average of the four repeats in each field for each property is summarized in table 1. Only phosphorous (P), measured by P_2O_5

(%), were present in a relatively high level in the three prospected fields. With a pH below 6.5, phosphorus uptake by the plant is optimum and therefore the field did not need any more chemical P input (Pagliari et al., 2017).

Table 1. Physical and chemical properties of soil in Nam Sach, Hai Duong

Field	pH H_2O	pH KCl	Total content					Avail. $SO4^{2-}$ (mg/100 g)	CEC (meq/100 g)	< 0.002 mm	0.002–0.02 mm	0.02–2 mm
			OC (%)	OM (%)	N (%)	P_2O_5 (%)	S (%)			%		
1	6.33[a]	5.68[a]	1.63[a]	2.81[a]	0.15[a]	0.29[a]	0.02[a]	30.36[a]	13.15[a]	20.9[a]	43.2[a]	35.9[a]
2	6.23[a]	5.75[a]	1.47[a]	2.53[a]	0.15[a]	0.26[a]	0.02[a]	39.64[a]	12.65[a]	20.4[a]	42.6[a]	37.0[a]
3	6.18[a]	5.70[a]	1.54[a]	2.65[a]	0.15[a]	0.27[a]	0.02[a]	43.21[a]	13.28[a]	22.7[a]	42.4[a]	35.0[a]

Note: Column numbers followed by the same letter ([a]) are not significantly different at $P = 0.05$ as determined by Kruskal-Wallis test.

Comparison of the soil texture with the 12 major textural classes and particle size scale (Malla, 2017) revealed that the three fields in Hai Duong Province were characterized by a loamy soil which is appropriate for growing most plant varieties including rice and scallion (Brown, 2007).

Morphology characters and molecular identification

Morphological characters of *M. gramminicola* Golden & Birchfield fit descriptions by Hirschmann (1985), Nguyen & Nguyen (2000) and Perry at al. (2009). Females with pearly white body, small neck, body length (L = 570.09 ± 54.11 µm) (Fig. 2A). Lip region smooth, anteriorly flattened, not distinctly set off from neck (Fig. 2B). Rounded stylet knobs with posteriorly sloping anterior margins, 11.03 ± 1.1 µm long (Fig. 2B). Excretory-secretory pore very distinct, generally located about one and one-half-stylet lengths or more from base of unprotruded stylet (Fig. 2B). Perineal pattern prominent with distinct and characteristic striations (Fig. 2C). The J2 character by body cylindrical vermiform, tapering markedly toward posterior end (L = 464.57 ± 42 µm). Stylet slender; knobs small, oval-shaped and backwardly sloping, stylet length (11.07 ± 0.69 µm). Lip region flat anteriorly, continuous with body, and weakly sclerotized (Fig. 2D); 0Tail shape and tail terminus rounded, often slightly clavate with tail length (68.84 ± 5.77 µm), hyaline tail length (20.20 ± 2.87 µm) (Fig. 2E).

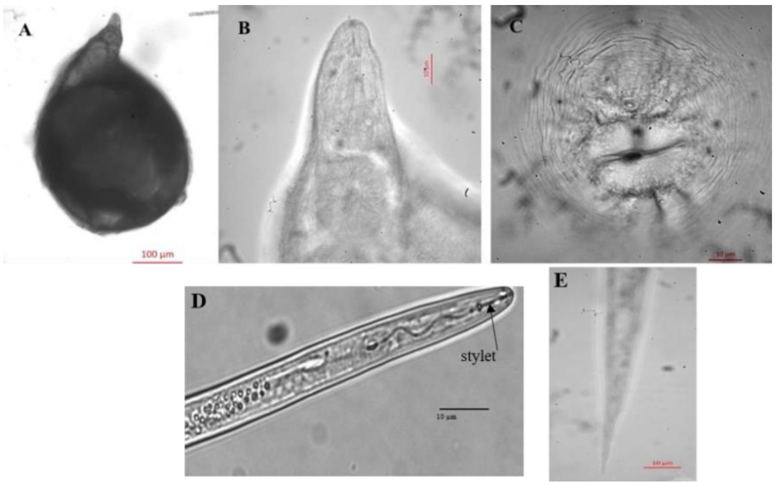

Figure 2. Morphological character of *M. graminicola* females from Hai Duong. A: Entire body, B: Head region, C: Perineal pattern, D: Anterior end of juvenile stage 2, E: Juvenile tail tip

500 base pair (bp.) in the ITS region of Hai Duong PPN was amplified by PCR and sequenced. Comparison of the amplified sequences with other available sequences using Nucleotide Basic Local Alignment Search Tool (BLASTN) (http://blast.ncbi.nlm.nih.gov/) revealed that among the seven PPN sequenced, all were *M. graminicola* with a high level of similarity from 99.67% to 100%. Sequence alignment against reference *M. graminicola* populations (MgVN18 KF250488) did not present intraspecific variation. The sequenced rDNA region was identical to that of *M. graminicola* VN13 (accession number KF250481) a population previously isolated from the same region (Bellafiore et al., 2015). The phylogenetic trees showed that the seven Hai Duong *Meloidogyne* isolates were in the same clade as the three reference *M. graminicola* (KF250488, KF250481 and HM623442). In this tree, the closest but significantly distant RKN species is *M. naasi* (JN157863) and the RKN isolated from Hai Duong are more distant from *Meloidogyne hapla* LC030362.1 LC030359.1; *Meloidogyne javanica* (AY438555), *Meloidogyne incognita* (KC464469) and *Meloidogyne arenaria* (AF387092) (Fig. 3).

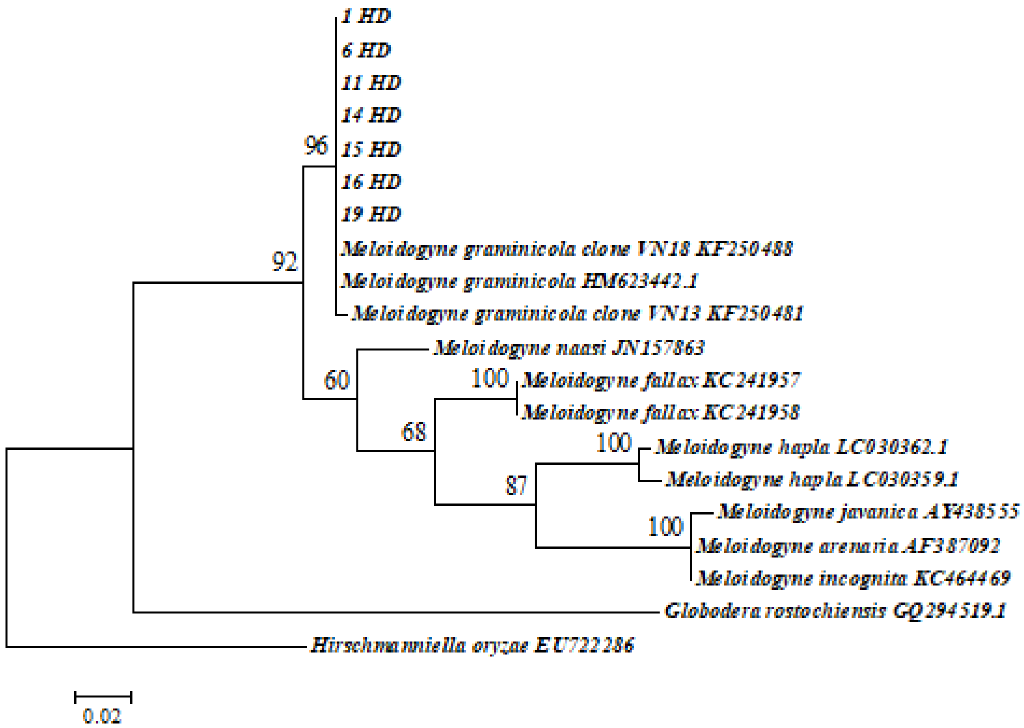

Figure 3. Evolutionary relationships of ITS sequences are estimated using maximum-likelihood. Branches with bootstrap support > 70% are indicated (1000 replications). The scale bar denotes 0.02 substitutions per nucleotide position. All positions containing gaps and missing data were eliminated. (1 HD, 6 HD, 11 HD, 14 HD, 15 HD, 16 HD, 19 HD: the sequence of *Meloidogyne* collected in Hai Duong rice field)

Reproduction and pathogenicity of *M. graminicola*

At the time of the survey, only rice was cultivated and some unplanted scallion bulbs remained on the edges of the fields. Therefore, the susceptibility to *M. graminicola* of the scallion used during the crop rotation in winter was tested under controlled conditions in a grow chamber.

After 27 dpi, small galls were easily identified in the root system (Fig. 4B). Acid fuchsin staining confirmed the susceptibility of scallion and rice varieties cultivated by farmers in this field (Figs. 4A, 4B). *M. graminicola* eggs and females were present in abundance in the roots of the scallion plants (Fig. 4C).

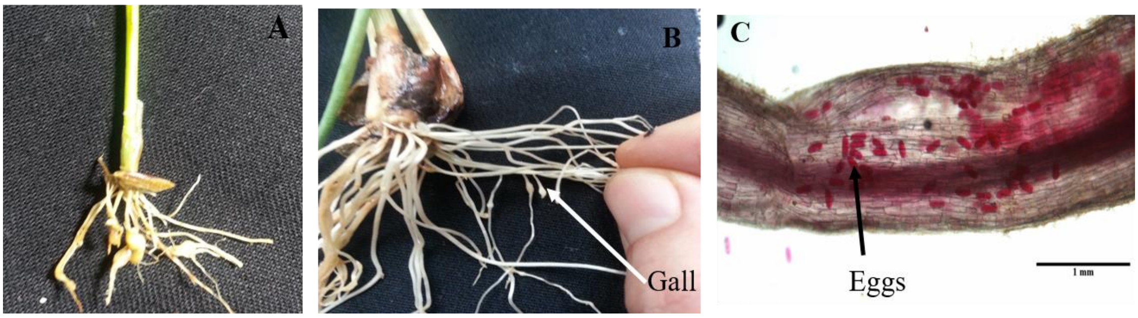

Figure 4. A: The typical root galls of Bac Thom rice variety in *M. graminicola* infested field. B: The terminal root galls of scallion bulbs after inoculated with *M. graminicola*. C: The eggs of *M. graminicola* (arrow) are released by the female directly in the root of scallion bulbs

After 27 dpi, the measurement of Rf revealed significant differences between IR64 and scallion plants (p-value < 0.001) with the Rf value in IR64 (19.25) being five times higher than that in scallions (3.96) (Fig. 4). Therefore, the varieties of rice and scallion used by farmers are susceptible to infection with *M. graminicola*.

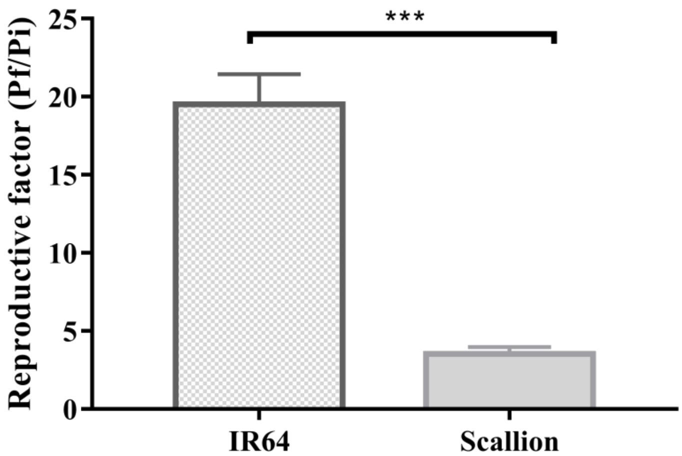

Figure 5. Reproduction factors of *M. graminicola* on *O. sativa* cv. IR64 and local scallion cv. The graph shows the average values of reproductive factor of scallion and IR64 in two repeat. The number of replicated plants is n = 5 for scallion and n = 10 for rice

DISCUSSION

Based on morphological observations and DNA barcoding, we showed that the 10 ha of the farm inspected in Hai Duong Province were severely infected with PPN, *M. graminicola*. This globally distributed species has become a serious pest in several tropical countries in Asia and notably in Vietnam in deep water and irrigated systems (Cuc & Prot, 1992; Cuc & Prot, 1995, Bridge et al., 2005; Bellafiore et al., 2015; Jain et al., 2012; Davide, 1988; Mantelin et al., 2017). However, to our knowledge, this is the first time that this species causes a massive infection in the country leading to almost 100% plant damage in a 10 ha field. Usually, *M. graminicola* infection in a field is limited to several small areas and the infection can be revealed by patch formation in the field where the plants are chlorotic and show a delay in their development (Mantelin et al., 2017). We therefore investigated the reasons that could explain this preliminary observation where *M. graminicola* could potentially devastate rice agriculture and farmer economy.

In the field, we systematically noticed that all plants with abnormal development were infected by PPN, which suggested that the selected varieties were highly susceptible to *M. graminicola* under natural growing conditions. In order to assess the aggressiveness of this specific Hai Duong *M. graminicola* population, we tested the infectivity of this population against IR64, an *Oryza sativa indica* species known to be a good host for *M. graminicola* (Soriano et al., 1999) and routinely used to study rice-nematode interactions. The aggressiveness of the Hai Duong *M. graminicola* on IR64 was similar to the results observed with other populations collected in Vietnam and in other countries. For instance, in Vietnam, 20 *M. graminicola* populations have been collected in 10 sites from different rice growing regions. After two life cycles, all Vietnamese *M. graminicola* populations were highly reproductive on rice cv. IR64 with a Rf value ranging from 11 to 19 (Bellafiore et al., 2015), similar to the isolate collected in Hai Duong (Rf of 19.25). This suggests that the high level of *M. graminicola* infection as observed in prospected Hai Duong fields is not due to the emergence/selection of a more aggressive host pathogen with a superior fitness but rather the plants becoming more susceptible to the infection due to exceptional agro-ecosystem conditions.

Physical changes in the soil are known to affect nematode behaviors (Oka Y., 2010). We analyzed the soil physical and chemical properties of the infested fields but only the content of P was relatively high and all the other parameters were in an optimum range for rice production. The high P value could be due to massive use of phosphorus fertilizers by farmers for intensive rice and scallion production. There are three main form of phosphorous in the soil: active P, fixed P and soluble P. Plants will firstly uptake soluble P which contains a mix of inorganic P and organic P with inorganic P being the major type, followed by active and fixed P (Pagliari et al., 2017; Nishigaki et al., 2019). Continuous addition of more P in the soil could increase more fertility in the soil but P could also be fixed and become unavailable (Pagliari et al., 2017), resulting in environmental pollution (Choudhury et al., 2007). However, high levels of phosphate will not negatively impact the crops and no correlation between P abundance and nematode infection has been previously reported. Therefore, the physical and chemical properties did not reflect any major characteristic that could explain the abundance of *M. graminicola*.

We, therefore, investigated if farmers applied a specific agricultural practice that could explain the high infection level. In Asia, farmers mainly use wet direct seeding method to cultivate rice by broadcasting or drilling into drained, well-puddled seedbeds or into shallow standing water (Balasubramanian et al., 2002) in which the two first seedbed types might be convenient conditions for *M. graminicola* infectivity. Indeed, this nematode can quickly invade the young rice roots when infested soils are drained (Manser, 1968). Direct seeding methods have many benefits such as reduction of labor work but also have side effects, such as promoting weed development and in some conditions, disease and pest infections (Farooq et al., 2011). According to Farooq et al. (2011), grain yields in direct seeding field were lower than this in transplanting field, whereas others reported that the rice yields of direct seeding under good management control was equal to, or even higher than those of transplanted rice (Huang et al., 2011; Liu et al., 2015). Because *M. graminicola* has a wide range of hosts which include many common weeds in the rice field, direct seeding methods could create favorable conditions for *M. graminicola* proliferation on the weeds which continue to infect rice in the next season (De Waele & Elsen, 2007; MacGowan & Landon, 1989). In the prospected area of the Hai Duong Province, several farmers modified their agricultural practices from traditional transplanting to direct seeding method. We observed that the farmers that had shifted to the direct seeding method suffered severe damage due to a massive *M. graminicola* infection.

Finally, we showed that the scallion used by farmers in the crop rotation sequence was a variety susceptible to *M. graminicola*. Consequently, it helped maintain a significant *M. graminicola* population in the soil during the winter season before planting rice. Scallion was previously reported as a good host of *M. graminicola*. The growth and yields of the Yellow Granex scallion variety grown in a cropping sequence with rice in the Philippines, was severely reduced due to *M. graminicola* infection (Gergon et al., 2002). Therefore, *M. graminicola* infection is not only reducing the expected income from rice cultivation but also from scallion. Although crop rotation is an important practice that can help farmers to limit nematode occurrence in a field (Mantelin et al., 2017, Védie et al., 2014), a wrong combination of plants can have the opposite effect of contributing to the proliferation of the pest followed by severe damage to the cultivated plants. A solution for the farmers should be using resistant rice varieties (Dimkpa et al., 2016; Thi Phan et al., 2017) and/or to grow non-susceptible plants instead of scallion. If the same cropping system persisted and no nematode control strategies were implemented, a strongly increasing number of *M. graminicola* would be expected in the field year by year.

In order to reduce the negative impact of this pest on rice production, it is critical to increase the farmer's awareness on the risk of plant parasitic nematode infection as too many severe nematode infections on rice are being mis-identified. Indeed, due to limited root development caused by the nematode infection, parasitized plants can present the same leaf symptoms as nutrient starvation and water stress. The infected plants can also present other sickness symptoms that are originally due to *M. graminicola*, as this nematode causes its host to be more susceptible to other pathogens (Kyndt et al., 2017). Fortunately, symptoms of infected roots are easily identifiable and farmers can quickly be aware of the presence of *M. graminicola* when they inspect carefully the rice root system.

CONCLUSION

The RKN found in Hai Duong fields were morphologically and molecularly identified as *M. graminicola*-a serious pathogenic species in rice. For the first time in Vietnam, our experiment showed that *M. graminicola* could parasitize and propagate in scallions of Vietnam although this plant is annually cultivated on a paddy field for crop rotation. A combination of three factors (wrong crop choice for rotation, rice variety susceptible to *M. graminicola* and direct seeding) obviously favored the nematode infection and its high proliferation in the surveyed fields. The results of this study suggested some recommendations: 1. Using a crop rotation system with at least one plant not susceptible to *M. graminicola*. If planting two susceptible crops (e.g. scallion and rice) is vital for the farmers, then a precise water management system is required to flood the field to limit the nematode infection. 2. Using rice varieties less or not susceptible to nematode infection. If no specific nematode control is planned (soil solarization, use of resistant cultivars…), avoid direct seeding and irrigation delay as both are favorable to *M. graminicola* infection. We recommend transplanting young rice plants from a non-infected nursery in a flooded field. Under flooding conditions, RKN like *M. graminicola* are unable to penetrate the root system and cause significant rice yield loss.

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