Research Article

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Occurrence of a population of the root-knot nematode, *Meloidogyne incognita*, with low sensitivity to two major nematicides, fosthiazate and fluopyram, in Japan

Aoto Fujikawa,[a] Roland N. Perry[b] and Koki Toyota[a*]

Abstract

BACKGROUND: The root-knot nematode (RKN), *Meloidogyne incognita*, affects food production globally and nematicides, such as fosthiazate and fluopyram, are frequently used in Japan to control damage caused by RKN. In aboveground pests, the emergence of a population with developed resistance is frequently found after the continuous use of the same pesticides; however, there are few studies on changes in the sensitivity of plant-parasitic nematodes, including RKN, to nematicides.

RESULTS: We compared the sensitivity of two populations of *M. incognita* to fosthiazate and fluopyram, one population with a history of exposure to fosthiazate and 1,3-dichloropropene (Ibaraki population) and the other without nematicide use for decades (Aichi population). A concentration of fosthiazate and fluopyram causing 50% mortality at 24 h post-treatment (LC_{50}) was markedly higher in the Ibaraki population (5.4 and 2.3 mg L^{-1}) than in the Aichi population (0.024 and 0.011 mg L^{-1} in fosthiazate and fluopyram, respectively), indicating the low sensitivity of the Ibaraki population to fosthiazate and fluopyram. Experiments using different enzyme inhibitors indicated the involvement of acetylcholinesterase (AChE), which is the target of fosthiazate, and glutathione S-transferase (GST), a typical enzyme related to detoxification, in the low sensitivity mechanism. The activity of AChE was 33-fold higher in the Ibaraki population than in the Aichi population and there were many differences in their nucleotide sequences. In addition, the gene expression level of GST was 239-fold higher in the Ibaraki population than in the Aichi population.

CONCLUSION: These results revealed differences in the sensitivity to nematicides among RKN populations. Two factors were identified as related to the mechanism of low sensitivity in the Ibaraki population. This is the first report showing the difference in the sensitivity to fluopyram between populations of *M. incognita*.

Keywords: acquired resistance; acetylcholinesterase; glutathione S-transferase; sensitivity; overexpression

1 INTRODUCTION

Plant-parasitic nematodes (PPNs) cause US$100 billion annual losses in global crops.[1] Effective control of PPNs is necessary for sustainable food production. Among PPNs, root-knot nematodes (RKNs) have a short generation time and a high proliferation capability resulting in rapid increases in populations causing over US $40 billion annual crop losses.[2,3] Among RKNs, *Meloidogyne incognita* is a major pest and causes damage and yield reduction to many host plants; a proper control strategy is needed before damage is serious.

In Japan, chemical methods with synthetic pesticides are generally used to control RKNs. Many farmers use fumigants such as 1,3-dichloropropene (1,3-D) and chloropicrin (CP). However, fumigants have environmental and human health risks because they are volatile and spread from the soil into the air. Fumigants have been used in Japan for a long time, but their use is banned or needs a special license in some countries. The Japanese government set a goal to decrease by 50% the usage of chemical pesticides by 2050. The use of fumigants will be more restricted in the near future. Thus, the use of non-fumigants is predicted to increase as an alternative nematicide. Currently, the Japanese government approves three non-fumigant types of nematicide: organophosphorus (fosthiazate and imicyafos), carbamate (oxamyl), and succinate dehydrogenase inhibitor (SDHI) (fluopyram).[4] Fluopyram is relatively new and was put on the market in 2018.[5,6] In Japan, fosthiazate and fluopyram are most widely used among

* *Correspondence to: K Toyota, Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan. E-mail: kokit@cc.tuat.ac.jp*

a *Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Tokyo, Japan*

b *School of Life and Medical Sciences, University of Hertfordshire, Hatfield, UK*

the non-fumigants and are predicted to remain as the major non-fumigant nematicides and play a key role in crop production.[7]

In aboveground pests, several studies have reported the appearance of resistance to insecticides, such as pyrethroids, and a knockdown resistance mutation has been identified as a major resistance mechanism.[8] However, there had been no report on the resistance of RKNs to nematicides until 2016, and a population of *M. incognita* with resistance to fosthiazate was reported in China.[9] The resistance mechanism was related to the change in the activity of acetylcholinesterase (AChE), the target enzyme of fosthiazate, and to mutations in the gene encoding the protein AChE. Since most RKNs are parthenogenetic,[10] this resistance will be inherited by the offspring if gene mutations are related to resistance. Despite the large impact of RKNs on crop production, further studies on the resistance of RKNs to nematicides are lacking.

Glutathione S-transferases (GSTs) are one of the major detoxifying enzymes in eukaryotes and bacteria.[11–16] In addition, they have an important role in cell signaling and processes.[17] In a malaria vector mosquito, a high level of resistance to organochlorines and low level resistance to organophosphorus, carbamates and pyrethroids were observed after the combined use of different classes of insecticides.[18] The resistance to dichlorodiphenyltrichloroethane (DDT) was caused by an elevated level of GST activity. The fumigant 1,3-D has a similar structure, with a chlorine group, to DDT. A high GST activity is related with resistance to major types of insecticides.[19–22] Since the range of mode of actions is limited in nematicides, resistance related to GST will be a risk to sustainable nematode control.

Cytochrome P450s (CYPs) also have an important role as detoxification enzymes.[23] They are involved in the oxidative metabolism of endogenous and exogenous substrates including toxic factors. There are at least 27 CYP genes in *M. incognita* and its number of CYP genes is much less compared with *Caenorhabditis elegans* with 80 different CYP genes.[24] In an aboveground pest, the mosquito *Aedes aegypti*, CYP-mediated resistance to pyrethroid, an insecticide acting on the nervous system, is well known, and overexpression of CYP or mutations in the sequence coding CYPs impacts the metabolism of insecticides.[25]

To support sustainable food production with a proper use of nematicides, it is important to clarify the occurrence of resistance to nematicides and relating factors. As described earlier, Huang *et al*. reported different sensitivities of two RKN populations to fosthiazate and revealed the involvement of a low level of AChE in the resistance mechanism.[9] In addition, different sensitivities of different RKN species and populations to the nematicides fluazaindolizine and oxamyl and fluensulfone and fluopyram have been reported.[26,27] However, there is no report showing the appearance of resistant RKN population to the nematicides. The objectives of this study were: (i) to evaluate the sensitivity of two RKN populations, one with a history of fosthiazate and 1,3-D usage (Ibaraki population) and the other without a history of nematicide use for decades (Aichi population); (ii) to determine factors involved in different sensitivities.

2 MATERIALS AND METHODS

2.1 *Meloidogyne incognita* populations

RKN-infested soils were collected from two sites in Japan, Tahara city (tobacco crop), Aichi prefecture (34.382N, 137.151E) and Kamisu city (green pepper), Ibaraki prefecture (35.817N, 140.736E). Green pepper plants ('Kyomidori', TAKII & Co., Ltd, Kyoto, Japan) were grown in the soils for 2 to 3 months in a glasshouse, after which egg masses were collected from the roots by hand and placed in tap water for a few days at room temperature to obtain hatched second-stage juveniles (J2s). DNA was extracted from a single J2 according to the method of Iwahori *et al*. and they were identified as *M. incognita* based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis reported by Powers and Harris.[28,29]

As a pre-test, the mortality rates of six populations of *M. incognita* were compared. The result showed that sensitivities of the Aichi and Ibaraki populations showed the greatest difference, and histories of the nematicide usage were also clear. Thus, all the following experiments were performed with these two populations.

The Ibaraki population was collected from a field in Ibaraki Prefecture, which had a past history of fosthiazate use between 2000 and 2014. During the period, fosthiazate was applied one or twice per year (a total of 15 to 30 applications). Then, due to an emerged risk of the pesticide residue in crops, fosthiazata was been replaced by 1,3-D since 2013. In addition to 1,3-D, cadusafos has also been used one or twice per year since 2015. The Aichi population was collected from a field in Aichi Prefecture, which has no history of nematicide use for at least 30 years. Egg masses of each population were collected from the roots of green peppers grown in the soils for 3 months, kept in a moist Petri dish at 12 °C and used within 3 months of collection.

2.2 Bioassays of fosthiazate and fluopyram sensitivity in two populations

To obtain suspension of J2, egg masses of each population were placed in tap water for 3 days under dark conditions at 28 °C. Then, the J2 suspensions were centrifuged at 2900 × *g* for 5 min and concentrated to give 20 to 25 individuals per 20 μL. Two nematicide solutions were tested: fosthiazate [30% active substance (a.s.); ISK Bioscience, Tokyo, Japan] and fluopyram (41.7% a.s.; Bayer Crop Science, Cambridge, UK). Next, 20 μL of the J2 suspension and 20 μL of various concentrations of nematicide solutions were made to give final concentrations of 0, 0.1, 0.3, 1.0, 3.0 and 10 mg L^{-1}. The mixtures of nematicide and J2s were added in each well of a Violamo 96-well plate (AS ONE Co., Osaka, Japan) in four to five replicates. Wells containing fewer than ten J2s were excluded from the analysis. The plate was incubated under dark conditions for 24 h at 25 °C. After incubation, 4 μL of a 1 N sodium hydroxide (NaOH) solution was added to each well. After the exposure to the NaOH solution, if the J2s were alive, even previously non-mobile J2s started to move continuously for a short time. The number of live nematodes was counted.[30] These experiments were repeated five times.

2.3 Effects of enzymes, glutathione S-transferase and carboxyl cholinesterase including acetylcholinesterase, to nematicide sensitivity

To evaluate whether detoxifying enzymes might play a role in the low sensitivity in the Ibaraki population to fosthiazate and fluopyram, three inhibitors were used; tribufos (FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan), diethyl maleate (DEM; FUJIFILM Wako Pure Chemical Corp.), and piperonyl butoxide (PBO; FUJIFILM Wako Pure Chemical Corp.).[31,32] Tribufos is an inhibitor of carboxyl/cholinesterase (CCE) that includes the target enzyme, AChE, of fosthiazate. DEM is an inhibitor of GST and PBO is an inhibitor of CYP. Since the Ibaraki population showed low sensitivity to fosthiazate and fluopyram, the involvement of these enzymes was evaluated. Thus, 100 μL of J2 suspension (including 500 J2s) within 2 days after hatching and 100 μL of inhibitor

solution (final concentration of 100 mg L^{-1} for tribufos and DEM, 50 mg L^{-1}) for PBO were mixed into a cell culture dish (40 mm diameter) and incubated under dark conditions at 28 °C for 24 h. After incubation, the same procedures as for the bioassay of fosthiazate and fluopyram sensitivity, described earlier, were performed.[31,32] In a preliminary test, we used three inhibitors and different concentrations (final concentrations 0, 0.1, 0.3 and 1 mg L^{-1}) of fosthiazate and fluopyram in five replicates. Since the mortality rate of the nematicides was not impacted by PBO, we excluded it from the further experiments and only included the inhibitors tribufos and DEM. To evaluate the effect of the inhibitors, the mortality rates compared between with and without the inhibitors in the nematicide 0 ppm treatment were collected by the Mann–Whitney U test using SPSS (IBM, Armonk, NY, USA). These experiments were repeated five times.

2.4 Acetylcholinesterase (AChE) activity

The two populations were used in this experiment. Thus, 100 µL of J2 suspension (350 ± 15 J2 per 100 µL) was mixed with 1.0 mL of 20 mM Tris–HCl buffer (pH 8.0), including 0.1 mg L^{-1} of bacitracin solution (Funakoshi, Tokyo, Japan) and 1 mM benzamidine (FUJIFILM Wako Pure Chemical Corp.) in a 2 mL microtube.[9] After centrifugation for 1 h at 4 °C at 12 000 × *g*, 200 µL of the supernatant was taken and used as a solution for the AChE assay. The supernatant (200 µL), 100 µL of 0.12 M sodium phosphate buffer (pH 7.2), and 100 µL of 2 mM acetylcholinesterase iodide were mixed in a new 2 mL microtube. After pipetting, the mixed solution was incubated in a water bath at 35 °C for 30 min. After incubation, 500 µL of 4% sodium dodecyl sulfate (SDS) solution and 1 mL of 0.12 M sodium phosphate buffer were added in the microtube and mixed. Then, 100 µL of the solution was added in each well of a 96-well microplate with three replicates. Absorbance was measured using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA) at 415 nm for 10 min. The values of AChE activity were calculated using the Ellman method.[33]

2.5 Nucleotide sequence analysis

DNA was extracted from 30 egg masses or over 1000 J2s using a NucleoSpin Tissue® kit (Takara Bio, Kusatsu, Japan) following the manufacturer's protocol. The reaction solution for PCR was mixed with Takara Ex Premier™ DNA Polymerase (Takara Bio) following the manufacturer's protocol. Based on the sequences of the reference population of *M. incognita* from the National Center for Biotechnology Information (NCBI) [ANH21152.1 (acetylcholinesterase, ace2), ABN64198.1 (glutathione S-transferase, gsts1)], primers including the active sites of 252S, 387Val and 514His in ace2 were designed using the software Primer 3 (Table 1). The reaction program included one cycle of 94 °C for 1 min followed by 40 cycles of 98 °C for 10 s, 67 °C for 15 s, 68 °C for 40 s using Takara Thermal Cycler Dice Touch (Takara Bio). The annealing temperature was changed depending upon the information on melting temperature (T_m) values of primers. The quality of the PCR products was checked by electrophoresis, and they were purified using a FavorPrepTM GEL/PCR Purification Kit (FAVORGEN BIOTECH Corp., Ping Tung, Taiwan) following the manufacturer's protocol. Sequence data of the PCR products were compared with the NCBI database using BLAST [GenBank: ANH21152.1 (ace2), ABN64198.1 (gsts1)].

2.6 Analysis of expression levels of glutathione S-transferase in *Meloidogyne incognita*

To compare the expression levels of glutathione S-transferase (*gsts1*) between the Aichi and Ibaraki populations, J2 suspensions (≈ 10 000 J2s) of each population were prepared within 3 days of hatching. Total RNA was extracted using a NucleoSpin® RNA kit (Takara Bio) following the manufacturer's protocol. Then, the total RNA was used as a template to synthesize complementary DNA (cDNA). The reaction of the reverse transcription was done using a ReverTra Ace™ qPCR RT Master Mix with gDNA Remover kit (TOYOBO Co. Ltd, Osaka, Japan) following the manufacturer's protocol. As a housekeeping gene, 18S rRNA in *M. incognita* (F-5′-ACCGTGGCCAGACAAACT, R-5′-GATCGCTAGTTGGCATCGTT) was used.[34] To check the expression levels, a forward primer (F-5′- GGCAGAGCTGAAGCAATTCG) was designed using the software Primer 3, and the reverse primer (R-5′-CTTCTAACACTGGAACTTGTC) was used from Dubreuil *et al.*[34] Briefly, 10 µL of a reaction solution, including 5 µL of Fast SYBR™ Green Master Mix (Thermo Fisher Scientific, Tokyo, Japan), 0.4 µL each of forward and reverse primers (10 µM), 2 µL of cDNA and 2.2 µL of sterilized water, were mixed in a PCR reaction tube. Quantitative PCR was done using a step One System (Thermo Fisher Scientific) following the program, which included one cycle of 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method and there were four replicates in each population.[35]

2.7 Statistics

Statistical comparisons between Aichi and Ibaraki populations were made by the Mann–Whitney U test using SPSS (IBM). Each data set in the experiment evaluating the sensitivity to fosthiazate and fluopyram to calculate the value causing 50% mortality at 24 h post-treatment (LC_{50}) was corrected by the Abbott's formula

Table 1. Primer information for partial DNA sequencing and quantitative polymerase chain reaction (qPCR) of the acetylcholinesterase 2 (*ace2*) and the glutathione S-transferase (*gsts*)

Name	Sequence (5′ → 3′)	T_m (°C)	Annealing temperature (deg)	Reference
ace2a-252F	CCTGTTGCTCTCACGGATGTTC	52.9	54	This study
ace2a-252R	GAGTAACACGCCGAGGATCAC	52.5		This study
ace2b-387F	TCGGGCTCTCCTTCTCTTGATTTATATG	54.7	58	This study
ace2b-387R	GCAACACCATCCCGCCATCTTTGTTC	57.3		This study
ace2c-514F	AAAGATGGCGGGATGGTGTT	48.0	52	This study
ace2c-514R	TAACTGTAGTTGCCCGACCG	50.0		This study
gsts1a-F	GGAAATGTGGAGCAACTTCGA	48.6	51	This study
gsts1a-R	TCATTCCCGTAAAGTGAGCAAC	49.2		This study
gsts1b-F	GAGGATTGGCCAACAATTAAATCGA	50.6	50	This study
gsts1b-R	CTTCTAACACTGGAACTTGTC	46.6		Dubreuil *et al.*[34]

to eliminate differences in the mortality in the control treatment among repeated experiments.[36,37] Then, LC_{50} value and 95% confidence interval of each population were calculated by probit analysis. Some outliers that exceeded the range of first quartile – [1.5 × interquartile range (IQR)] or third quartile + (1.5 × IQR) were excluded from calculations of mortality rates and LC_{50} values. Analyses were done using SPSS (IBM).

3 RESULTS

3.1 Differences in sensitivity to fosthiazate and fluopyram between two *Meloidogyne incognita* populations

The differences in sensitivity to the two nematicides tested were clearly observed between Aichi and Ibaraki populations (Fig. 1). Results for the Aichi population indicated high mortality to each nematicide even under low nematicide concentration (0.1 mg L^{-1}). The LC_{50} values of the Aichi population were 0.024 mg L^{-1} for the fosthiazate treatment and 0.011 mg L^{-1} for the fluopyram treatment (Table 2). By contrast, the Ibaraki population showed low mortality in all ranges of concentrations tested. In other words, the Ibaraki population had low sensitivity to fosthiazate and fluopyram. For both nematicides, the LC_{50} values of the Ibaraki population were significantly ($P < 0.01$) higher than those of the Aichi population. The LC_{50} values of the Ibaraki population were 5.4 mg L^{-1} for the fosthiazate treatment and 2.3 mg L^{-1} for the fluopyram treatment (Table 2).

3.2 Effect of the inhibitor of enzymes, glutathione S-transferase and carboxyl cholinesterase in the Ibaraki population with low sensitivity to nematicides

The average rates of mortality in the inhibitors only were 13% with DEM and 14% with tribufos. The average rate in tap water without

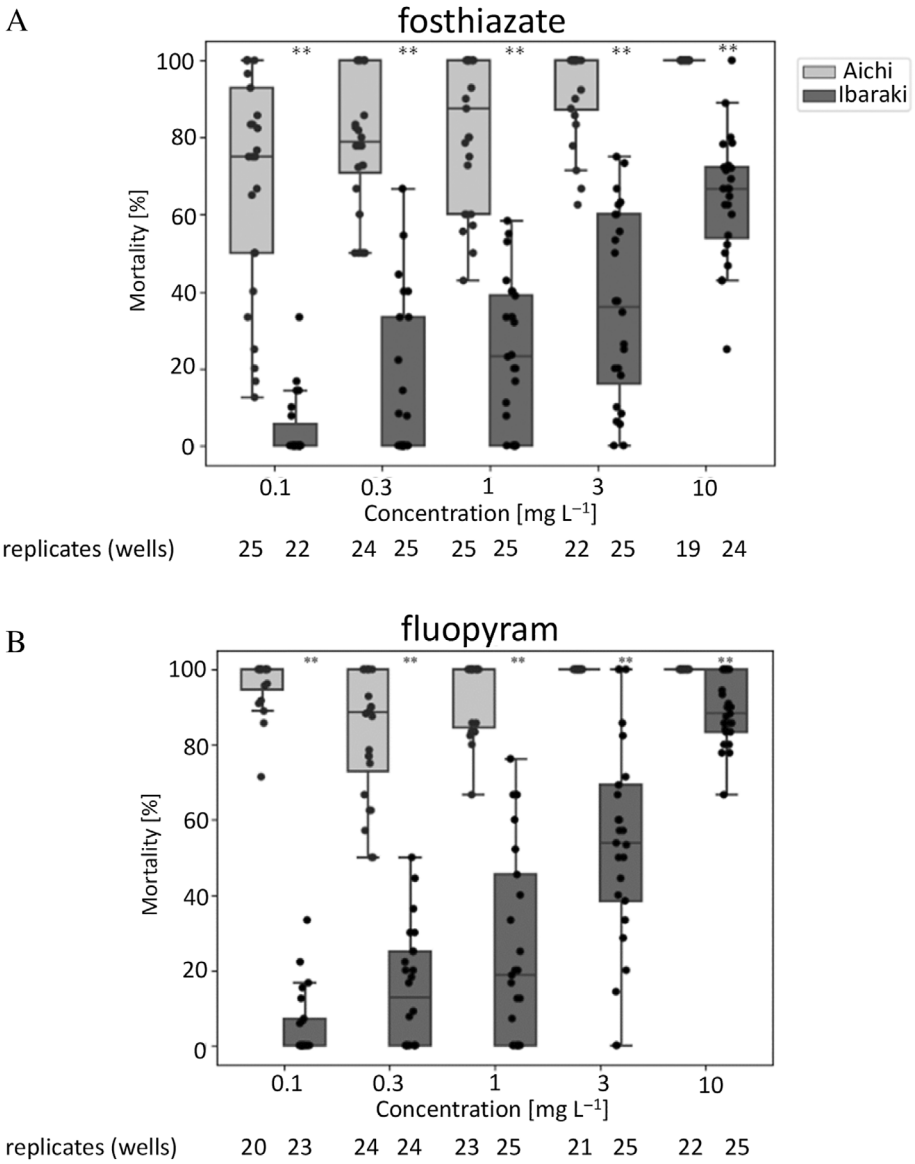

Figure 1. Differences in the mortality of two *Meloidogyne incognita* populations in different concentrations of fosthiazate (A) and fluopyram (B). The boxplot indicates the median and range of each mortality, symbols (**) show significant difference ($P < 0.01$) by Mann–Whitney U test between Aichi and Ibaraki populations in the same nematicide concentrations. This figure is drawn after excluding outliers from the initial data. The replicates after excluding outliers are indicated below the figures.

Table 2. Comparison of the LC_{50} values with fosthiazate or fluopyram between two *Meloidogyne incognita* populations (Aichi and Ibaraki) and effects of two enzyme inhibitors [diethyl maleate (DEM) and tribufos] on the LC_{50} values with fosthiazate or fluopyram

	Fosthiazate (an organophosphorus inhibitor)		Fluopyram (a succinate dehydrogenase inhibitor)	
Population/ treatment	LC_{50} (95% CL) (mg L^{-1})	Slope (±SE)	LC_{50} (95% CL) (mg L^{-1})	Slope (±SE)
Aichi	0.024 (0.00031–0.088)	0.71 ± 0.051	0.011 (0.000015–0.051)	0.82 ± 0.068
Ibaraki	5.4 (3.4–11)	0.97 ± 0.046	2.3 (1.7–3.2)	1.6 ± 0.056
Ibaraki + DEM	<0.001	—	<0.001	—
Ibaraki + Tribufos	<0.001	—	<0.001	—

Note: Since Ibaraki population showed low sensitivity to both nematicides, the contribution of two detoxifying enzymes, glutathione S-transferase and carboxyl cholinesterase to the low sensitivity was evaluated using two enzyme inhibitors.
LC_{50}, concentrations of fosthiazate or fluopyram causing 50% mortality at 24 h post-treatment. The LC_{50} values were calculated based on the results of five repeated experiments per concentration and each experiment was conducted in four to five replicates; CL, 95% confidence limit; SE, standard error.

the inhibitor was 5%. The average rates with the inhibitors were not significantly different compared those in the treatment without the inhibitors [$P = 0.548$ (DEM) and 0.151 (tribufos)]. The mortality rates by the concentrations provided in Table 2 and Figs 1 and 2 show the effect of the inhibitors after using the Abbott formula. There was no significant difference [$P = 0.235$ (fosthiazate) and $P = 0.682$ (fluopyram)] in the mortality rates in all concentrations of two nematicides between with and without PBO (data not shown). By contrast, mortality rates with DEM, an inhibitor of GST, were significantly ($P < 0.01$) increased under low concentrations of both nematicides (Fig. 2). The LC_{50} value with DEM was decreased to less than 0.001 mg L^{-1} for the two nematicides. Also, mortality rates in the treatments of each concentration with tribufos, an inhibitor CCE, were increased and the LC_{50} value was decreased to less than 0.001 mg L^{-1} for the two nematicides. Significant ($P < 0.01$) differences were observed in all concentrations of fosthiazate and fluopyram, except between the with and without tribufos at the 10 mg L^{-1} concentration.

3.3 Acetylcholinesterase (AChE) activity

The AChE activity was 33 times higher in the Ibaraki population ($6.04 \pm 0.17 \times 10^{-7}$ mol L min^{-1}) than in the Aichi population ($0.18 \pm 0.05 \times 10^{-7}$ mol L min^{-1}).

3.4 Nucleotide sequences of *ace2* encoding acetylcholinesterase and *gsts1* encoding glutathione S-transferase

In *ace2*, there was no substitution in the active sites in the Aichi population compared with the reference sequence deposited on NCBI (accession: ANH21152.1). In the Aichi population, many parts between Leu61 to Leu577 in the domain region matched with the reference sequences. In the Ibaraki population, there was also no substitution in the active sites. However, an amino acid insert was observed near the active site and multiple substitutions were observed in the domain region between Val155 to Asn199 (Fig. 3(A)).

In *gsts1*, there was a substitution Trp83Leu in the active sites in the Aichi population. This substitution matched with that in the Ibaraki population. Some mutations were observed at the active sites in the Ibaraki population, such as the glutathione (GSH) binding site (G-site) (chemical binding), C-terminal domain interface (polypeptide binding) and dimer interface (polypeptide binding), compared with their reference sequences on NCBI (ABN64198.1). Some of the mutated amino acids in the Ibaraki population matched with those in other nematodes, *Caenorhabditis elegans* or *Meloidogyne graminicola*, such as Ala64Ser and Gly72Ala (Fig. 3(B)).

3.5 Expression level of *gsts1* encoding glutathione S-transferase

When the gene expression level of *gsts1* in the Aichi population was regarded as 1, it was 239 ± 16 times higher in the Ibaraki population. Thus, considerable overexpression of *gsts1* in the Ibaraki population was observed.

4 DISCUSSION

The LC_{50} values after exposure to two nematicides of the *M. incognita* Ibaraki population, which was obtained from a field with a history of fosthiazate use and recent use of a fumigant 1,3-D, were markedly higher than the LC_{50} values of Aichi population. This result indicated that the Ibaraki population has low sensitivity to fosthiazate and fluopyram. A population of *M. incognita* with low sensitivity to fosthiazate was reported by Huang *et al*., in which the LC_{50} value was 2.7 times higher than that of a susceptible population.[9] As far as we know in the literature, the Ibaraki population may be the most resistant *M. incognita* population to fosthiazate, because it showed 225 times higher LC_{50} value than a susceptible population. Also, the half maximal effective concentration (EC_{50}) value of fluopyram of *M. incognita* was 0.071 mg L^{-1}.[37] This value is similar to LC_{50} of the Aichi population. This is the first report of *M. incognita* population that has low sensitivity to fluopyram, indicating acquired resistance to this nematicide.

In the field from where the Ibaraki population was obtained, a fumigant 1,3-D has been used for more than the last 10 years. The nematicide has been used once a year before transplanting. In this field, fosthiazate was changed to the fumigant due to an emerging risk of the pesticide residue. Unlike fosthiazate and fluopyram that have specific target enzymes, fumigants act on multiple enzymes that have a sulfanyl group (–SH).[38,39] A target enzyme is GST, which is involved in glutathione metabolism and plays an important role in redox homeostasis. We hypothesized that GST is one of the target enzymes of fumigant 1,3-D.[40] Indeed, in the experiment using inhibitors of detoxifying enzymes, the mortality rate increased markedly in the presence of tribufos inhibiting CCE, including AChE, and DEM inhibiting GST. These results suggest that both CCE and GST may be responsible for the low sensitivity of the Ibaraki population to fosthiazate and GST to

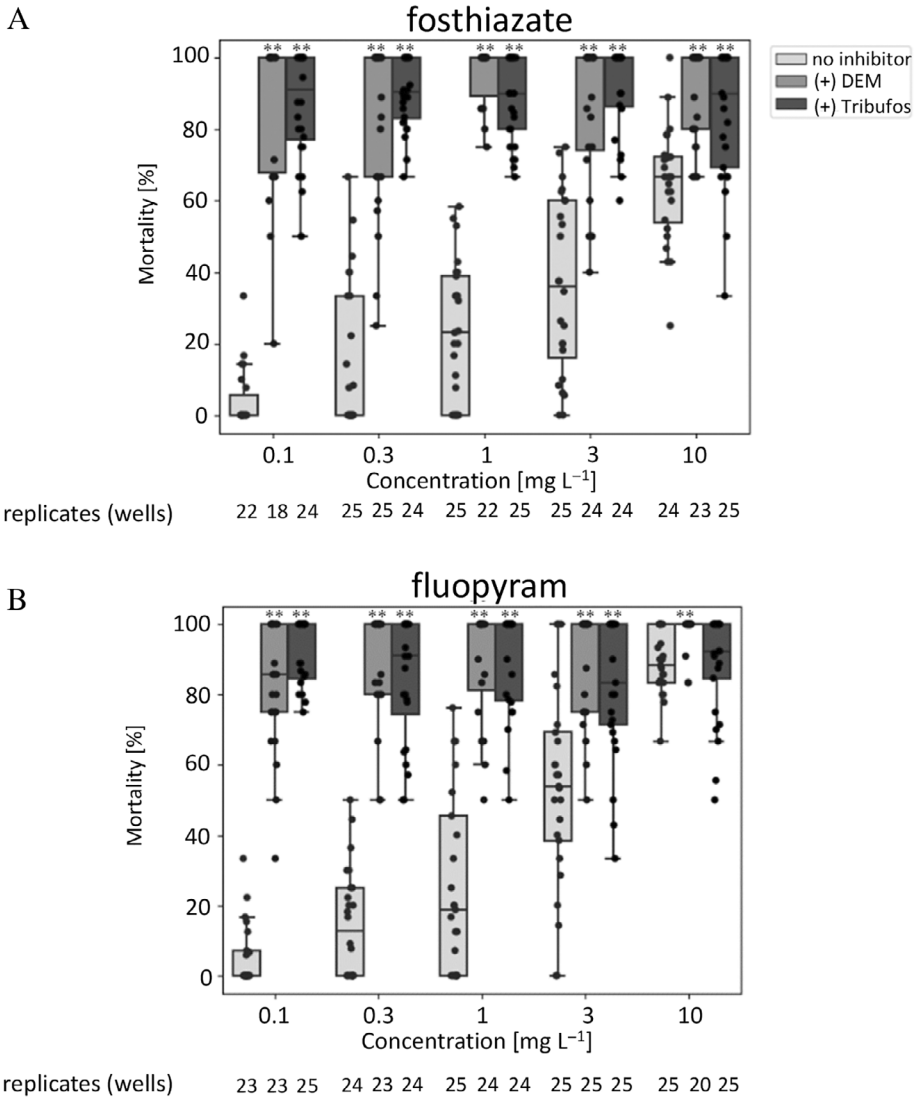

Figure 2. Effect of two inhibitors [diethyl maleate (DEM) and tribufos] on the mortality of the *Meloidogyne incognita* Ibaraki population in different concentrations of fosthiazate (A) and fluopyram (B). The boxplot indicates the median and range of each mortality, symbols (**) show significant difference ($P < 0.01$) by Mann–Whitney U test between with and without DEM or tribufos. Data in no inhibitor of the Ibaraki population were the same as Fig. 1. This figure is drawn after excluding outliers from the initial data. The replicates after excluding outliers are indicated below the figures.

fluopyram. However, the mortality rate did not change in the presence of PBO inhibiting CYP, suggesting that CYP may not be related with the low sensitivity of the Ibaraki population to fosthiazate and fluopyram.

In a study focusing on bacteria and fungi, Kantachote *et al.* reported that microbes developed resistance to the organochlorine insecticide DDT under long-time exposure to DDT.[41] Since 1,3-D also has a chlorine group, the use of 1,3-D might cause changes in the GST activity of *M. incognita*. Hemingway *et al.* reported that elevated levels of GST activity conferred resistance in *Anopheles albimanus* to DDT. In addition, GST offered protection against the organophosphorus insecticides parathion and paraoxon in the yellow mealworm *Tenebrio molitor*.[18,42] These results support the indication from the present study that resistance to fosthiazate of the *M. incognita* Ibaraki population may be developed via enhanced GST activity induced by continual fumigant use.

According to Huang *et al.*, mutations in acetylcholinesterase 2 were involved in the resistance mechanism of the resistant population of *M. incognita*.[9] In addition, the number of mutations in the acetylcholinesterase gene has a key role in the resistance to organophosphorus of the Australian sheep blowfly *Lucilia cuprina*.[43] Therefore, we compared the activity and amino acid sequences of the acetylcholinesterase gene between the Aichi and Ibaraki populations.

The AChE activity was markedly higher in the Ibaraki population than in the Aichi population. In the Chinese resistant population, Huang *et al.* found that the AChE activity was decreased compared with that in the susceptible population, unlike data from this study.[9] A possible reason for the different results might be the difference in the LC_{50} value as described earlier. In other organisms, such as the medfly *Ceratitis capitata* and the Colorado beetle *Leptinotarsa decemlineata*, the increase of the AChE activity was related to the resistance to organophosphorus

Figure 3. Amino acid sequences of the acetylcholinesterase 2 (A) and the glutathione S-transferase (B) of *Meloidogyne incognita* Aichi and Ibaraki populations. (A) Homology with the reference sequence of *M. incognita* acetylcholinesterase 2 (ANH21152.1). Several active sites in acetylcholinesterase 2 are indicated using symbols: the oxyanion hole with inverted triangle (▽), catalytic triad residues with circle (○), and choline-binding site with triangle (△).[9] An insert of amino acid was observed between 251Asn and 252Ser in the Ibaraki population. (B) Homology with the reference sequences of *M. incognita* (ABN64198.1), *Meloidogyne graminicola* (KAF7638000.1) and *Caenorhabditis elegans* (NP_001254267.1). The following symbols indicates the active sites in GST: GSH binding sites (G-site) (chemical binding) with black circle (▲), C-terminal domain interfaces (polypeptide binding) with black inverted triangle (▼), dimer interfaces (polypeptide binding) with black circle (●), N-terminal domain interfaces (polypeptide binding) with black triangle (■) and substrate binding pocket (H-site) (chemical binding) black star (★). The underlines indicate the mutations of amino acids in the Ibaraki population compared with the reference sequence of *M. incognita* (ABN64198.1). The alignment was prepared using Clustal Omega. The information of the conserved domain database (CDD) was cited from Wang *et al.*[51]

insecticides.[44,45] These results suggest that the increase in AChE activity may also contribute to the development of the low sensitivity in the *M. incognita* Ibaraki population.

In the partial analysis of nucleotide sequences in the gene *ace2* encoding AChE, there was no substitution at the active sites in both populations. However, in the Ibaraki population, there was an insertion of the amino acid glutamic acid (Glu) between catalytic triad residues (the active site) and the oxyanion hole. In an elapid snake, *Bungarus fasciatus*, the mutation in the oxyanion hole was related to the resistance to organophosphorus pesticides.[46] In addition, the formation of the oxyanion hole was related to the variation of the enzyme activity in a species of the bed bugs *Cimex lectularius*.[47] These results support the suggestion that substitutions in *ace2* might be related to the increase in the AChE activity in the Ibaraki population of *M. incognita*. In addition, the partial nucleotide sequences in the *gsts1* encoding GST revealed some mutations of amino acids at the active sites in the Ibaraki population. In particular, there were two mutations at the dimer interface. GTS is a dimer enzyme, and the dimer interface is important for its function. Actually, some mutations of the important region related with the dimer interface changed the enzyme activity.[48] There were two mutations at the active sites of the C-terminal domain interface, Gln69Asn and Gly72Ala. GST has two domains, C-terminal domain and N-terminal domain, and these domains have important roles in the enzyme activity.[49] The mutations around the domain–domain interface impact the enzyme activity and its stability.[50] Most mutations excluding Ala64Ser in the Ibaraki population were arranged differently from the Aichi population and related species (*M. graminicola and Caenorhabditis elegans*). These mutations indicate that the Ibaraki population may exhibit unusual characteristics. Indeed, some mutations in the Ibaraki population were observed around the active sites of the domain–domain interface, and the gene expression level of *gsts1* was 239 times higher in the Ibaraki population than in the Aichi population. Thus, these mutations observed in the Ibaraki population indicate an increase in the GST activity and the low sensitivity to fluopyram and fosthiazate.

In Japan, only three types of nematicides are approved: organophosphorus (e.g., fosthiazate and imicyafos), carbamate (oxamyl) and SDHI (fluopyram). Organophosphorus and carbamate have the same target and the same mode of action. The low sensitivity to fosthiazate and fluopyram, which was observed in the Ibaraki population, might indicate potential resistance to imicyafos and oxamyl. This risk should be confirmed in the near future. Until now, there have been few studies on the resistance of *M. incognita* but the occurrence of resistant populations could have a major impact on management strategies for stable and sustainable food production. Thus, it is necessary to survey the possible occurrence of resistant populations in other fields where nematicides have been used continuously.

5 CONCLUSIONS

Low sensitivity of the *M. incognita* Ibaraki population to fosthiazate and fluopyram was found in this study. Two enzymes, AChE and GST, were identified that relate to the low sensitivity. GST is a basic enzyme of the detoxifying pathway and is associated with low sensitivity to different types of nematicides. From the previous history of nematicide use, the factor causing the change in GST activity and mutations in the gene was most likely the continuous use of 1,3-D.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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