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Evaluation of Nematicidal Activity of Fluensulfone against Non-Target Free-Living Nematodes under Field Conditions

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Abstract: The use of nematicides with reduced toxic side-effects against non-target free-living nematodes is a favorable option for farmers to control plant-parasitic nematodes. The nematicide fluensulfone was registered in several countries for the control of the root-knot nematodes, *Meloidogyne* spp. among other plant-parasitic nematodes. This study aimed to evaluate the nematicidal activity of fluensulfone against non-target nematode fauna in four field experiments, each under different conditions (soils types and plant hosts). Nematodes extracted from soil samples were classified and counted based on their morphological characters. Fluensulfone significantly reduced damage caused by root-knot nematodes to tomato and sweet potato plants, while overall non-target free-living nematode population densities were maintained at the same level as those in control. Different diversity indices (e.g., Shannon-Wiener H', Simpson's D, species richness, evenness J', maturity indices) and principal component analyses in the four experiments showed that fluensulfone treatment kept a similar diversity level of non-target free-living nematode fauna to that of the non-treated control. The results suggested that fluensulfone may have minimal impact to free-living nematode fauna in both population density and diversity when the nematicide was applied to control *Meloidogyne* spp.

Keywords: agroecosystem; biodiversity; fluensulfone; fosthiazate; free-living nematode; nematicide; non-target nematodes; 1,3-dichloropropene

1. Introduction

Soil biota consists of a wide variety of living organisms, both visible and microscopic; from plants, insects, amphibians, reptiles and mammals, to bacteria, fungi and nematodes. Farmlands are not an exception. Biodiversity on farmland is a precondition for sustainable farming [1]. Among soil organisms, nematodes play important roles in soil ecosystem function, such as nutrient cycling, decomposition and disease suppression [2]. Feeding activities of free-living nematodes generally lead to an increase in nutrient availability as a result of increased soil microbial activity and excretion of excess ingested nutrients [3], especially nitrogen mineralization [4,5]. Free-living nematodes also play significant roles in regulating soilborne pathogens (bacteria, fungi and plant-parasitic nematodes) [6]. Thus, nematode diversity in soil is a good indicator of soil health [7] and associated with sustainable agriculture [8]. Plant-parasitic nematodes, on the other hand, weaken plants, and reduce the yield and quality of harvests [9].

In order to enhance farmland productivity, fumigants, such as methyl bromide, chloropicrin and 1,3-dichloropropene, and also nematicides including organophosphates and carbamates have been

widely used to suppress plant-parasitic nematodes in farmlands [10]. Because of its ozone-depleting property, methyl bromide phased out under the Montreal Protocol [11]. Also, some fumigants and nematicides have been banned or restricted in some countries due to their toxicity to non-target organisms and impact on the environment. For example, a nematicide, carbofuran reduced both total nematode abundance and the number of taxa [12], while another nematicide, aldicarb, also decreased abundances of non-target free-living nematodes [13–15]. Effective chemical nematicides may be potentially harmful to non-target free-living nematodes because of their biocidal activity. Further, imicyafos, another widely used nematicide, did not affect the total numbers of non-target nematodes, but it altered overall nematode fauna evaluated by PCR-DGGE (denaturing gradient gel electrophoresis) [16]. Thus, nematicides with minimum impact on non-target free-living nematodes are desired for promoting farmland productivity by suppressing target plant-parasitic nematodes while keeping free-living nematodes' abundance and diversity.

Environmental conditions after nematicide application, such as temperature, soil moisture and pH, may affect the dissipation of nematicides [17,18]. While rapid dissipation is expected for nematicides, prolonged uses of the same nematicide has been a new problem for controlling plant-parasitic nematodes. This may be due to enhanced biodegradation by soil microbes [19]. Such phenomena have been reported in several nematicides (e.g., aldicarb, cadusafos, oxamyl and fosthiazate) [20–23]. Therefore, a variety of nematicides with different modes of action may be of great demand to avoid potential biodegradation of chemicals due to successive application of a single nematicide [24].

Fluensulfone (5-chloro-2-(3,4,4-trifluorobut-3-enylsulfonyl)-1,3-thiazole; CAS number 318290-98-1) is a heterocyclic fluoroalkenyl sulfone nematicide and its mode of action is different from that of the anticholinesterases and macrocyclic lactones [25]. The target nematodes cover three important plant-parasitic nematode groups, root-knot, root-lesion and cyst nematodes [9]. Efficacies of fluensulfone against the root-knot nematodes *Meloidogyne incognita* and *M. javanica* were shown in different experimental settings, including pot and field experiments using tomato and pepper and in a tomato-cucumber double cropping system, as well as in lima bean fields [26–31]. Fluensulfone was shown to be effective against *Pratylenchus* spp. (root-lesion nematodes) in pot experiments [32], so was against the potato cyst nematode, *Globodera pallida* [33] and sting nematode, *Belonolaimus longicaudatus* [34]. Fluensulfone also showed its efficacy on *Nacobbus aberrans* in pot experiments using tomato and cucumber [35]. Further, fluensulfone has less toxicity to *Caenorhabditis elegans*, a free-living nematode, than to *M. javanica* [25]. Recently, it was reported that fluensulfone had less impact to non-target nematodes in turfgrass, while its damage control of ground cover was limited [36]. For field crops, fluensulfone's efficacy on other free-living nematodes as well as *M. incognita* has not been tested until now.

In this study, we used tomato and sweet potato crops in fields to test the efficacy of fluensulfone on nematodes, since tomato and sweet potato are major hosts of *Meloidogyne* sp. [37,38], which reduced their yields by 20.6% and 10.2%, respectively [9]. In Japan, both tomato and sweet potato are very important agricultural products and their total productions in 2017 were ca 2.2 billion and ca 0.9 billion dollars, respectively [e-Stat: Portal Site of Official Statistics of Japan website (https://www.e-stat.go.jp/)].

Our hypothesis was that fluensulfone may be an efficient means to control root-knot nematodes in tomato and sweet potato. We further hypothesized that fluensulfone might not adversely affect the overall non-target free-living nematode fauna and population density. Therefore, the objectives of the current study were to confirm the efficacy of fluensulfone for root-knot nematodes and to explore the effects of fluensulfone on a broad range of non-target free-living nematodes, which are key players in sustainable crop production [7]. This study will provide in-depth insights into proper use of nematicides for farmers and researchers in the perspective not only from the efficacy on plant-parasitic nematodes but also from the impact on non-target free-living nematodes.

2. Materials and Methods

2.1. Experimental Fields

Four field experiments were conducted in *Meloidogyne* sp. infested fields. In experiments (1) and (2) tomato (*Solanum lycopersicum*) plants were grown in the summer of 2016 and the autumn of 2017 at Japan Plant Protection Association in Ushiku, Japan (35°57′44″ N, 140°10′23″ E), ca 70 km north east of Tokyo (Ushiku I and Ushiku II). In experiment (3), tomato plants were grown in the summer of 2018 at Japan Plant Protection Association in Miyazaki, Japan (32°00′01″ N, 131°27′23″ E), ca 870 km south west of Tokyo (Miyazaki). In experiment (4) sweet potato (*Ipomoea batatas*) plants were grown in the summer of 2018 at Chiba Prefectural Agriculture Research Center (35°32′40″ N, 140°11′29″ E), ca 50 km south east of Tokyo (Chiba). The soils were Ushiku: light clay (sand 40%, silt 26%, clay 33% with 31.6 mg C g^{-1}, 3.1 mg N g^{-1}, pH (H_2O) 6.2 and electric conductivity (EC) of 0.12 mS cm^{-1}), Miyazaki: silty clay loam (sand 17%, silt 45%, clay 38% with 61.0 mg C g^{-1}, 4.2 mg N g^{-1}, pH (H_2O) 4.9 and EC of 0.49 mS cm^{-1}) and Chiba: light clay (sand 35%, silt 40%, clay 25% with 54.4 mg C g^{-1}, 4.6 mg N g^{-1}, pH (H_2O) 5.9 and EC of 0.10 mS cm^{-1}). Trials consisted of (1) a tomato crop planted in June in 2016, (2) a tomato crop planted in September in 2017, (3) a tomato crop planted in May 2018, and (4) a sweet potato crop planted in May 2018. The sizes of an individual plot were (1) and (2) 3.6 m long and 1.8 m wide (6.5 m^2) with 18 tomato plants in two rows (nine plants/row), (3) 5.5 m long and 1.5 m wide (8.3 m^2) with 22 tomato plants in two rows (11 plants/row), and (4) 6 m long and 2 m wide (12 m^2) with 17 potato plants in a row.

2.2. Chemicals

The experimental plots of Ushiku I were treated with two nematicides, fluensulfone (in a granular form, 2% active ingredient; a.i.) supplied by ADAMA JAPAN K.K. (Tokyo, Japan) and fosthiazate (Nemathorin in a granular form, 1.5% a.i., Ishihara Sangyo Kaisha, Tokyo, Japan), and a fumigant (97.5% of 1,3-dichloropropene, DCP: Telone II in a liquid form, Dow AgroSciences, Tokyo, Japan) 2 weeks before crop planting, in triplicate. The experimental plots of Ushiku II, Miyazaki, and Chiba were treated with the two nematicides, fluensulfone and fosthiazate, separately just before crop planting, in triplicates. For each individual experiment, the surface 15 to 20 cm soil was tilled and each chemical was incorporated. Non-treated controls were also prepared in triplicates and each treatment was randomly arranged. Fluensulfone, fosthiazate and DCP were applied at 200 kg ha^{-1} in granular form (4 kg a.i. ha^{-1}), 200 kg ha^{-1} of Nemathorin (3 kg a.i. ha^{-1}), and 150 L ha^{-1} of Telone II (177 kg a.i. ha^{-1}), respectively. The plots applied with DCP after tillage were covered in plastic mulch until planting tomato seedlings.

2.3. Soils and Roots

Soils were collected at 0–15 cm depth, where chemicals were well mixed, at five randomly selected spots in each plot (3 replicates separately) just before chemical applications, 1- and 2-months after planting for all the experiments. The soils were passed through a 5 mm aperture sieve to remove rocks and debris, well mixed and kept at room temperature for no more than 2 days before nematode extraction. At the end of each experiment (2 months after planting for Ushiku I, Ushiku II, and Miyazaki, and 4 months after planting for Chiba), tomato roots and sweet potato tuberous roots were collected, and nematode-induced root galls were counted.

2.4. Nematodes

Nematodes were extracted in triplicate from 20 g subsample of each well mixed soil sample to evaluate nematode fauna using the Baermann funnel extraction method (room temperature, 72 h), and counted under a stereomicroscope (BX53, Olympus, Tokyo, Japan). The soils were confirmed to be infested by *M. incognita* by identifying the extracted root-knot nematodes with PCR-RFLP (restriction fragment length polymorphism) [39]. Occasional occurrences of *Pratylenchus penetrans*, *Paratylenchus*

sp., *Trichodorus* sp. and *Xiphinema* sp. were observed, but not considered in this study because of their relatively low populations. Free-living nematodes were counted in total and classified separately for the first 100 individuals or all, if total numbers were less than 100, based on their morphological characters [40] and general feeding habits [41]. The classification of free-living nematodes in the current study covered *Acrobeloides*, *Cephalobus*, *Rhabditis*, other genus in the Rhabditidae family, and the other bacterial feeders. The other feeding types including fungal feeders (*Aphelenchus*, *Aphelenchoides*, *Filenchus* and *Ditylenchus*) and mostly omnivorous nematodes in the Dorylaimida order were also recorded. The proportion of each nematode classification to the total free-living nematodes was shown in the Supplementary Table S1, in which the proportion of *Acrobeloides* and that of frugivorous and omnivorous nematodes, among other nematode groups, showed noticeable fluctuation after chemical treatments. Therefore, we further statistically analyzed the proportion of *Acrobeloides* and that of fungivorous and omnivorous nematodes.

2.5. Galls on Root Systems

Ten out of 18 (Ushiku I and Ushiku II), 22 (Miyazaki) tomato plants, and 17 sweet potato plants (Chiba) in each plot were randomly selected to evaluate root galling. Nematode damage to roots was assessed per plant using a 5-scale index system (0 = no galling, 4 = abundant galling) and was converted into disease index expressed as 0–100 based on the formula: disease index = (the number of infested plants in each index × each index scale)/(4 × total number of plants) [42,43].

2.6. Statistical Analysis

The Shannon-Wiener index (H') was used to evaluate diversity of soil free-living nematode fauna and the Simpson's D was applied to assess dominance of abundant taxa [44]. Species richness (Margalef index) and evenness J' was evaluated using genus and family levels as taxa classification [44]. Maturity index [45,46], maturity index (colonizer-persister (*cp*) value 2–5) [47] and maturity index (Cephalobidae adjusted; Cephalobidae's *cp* adjusted from 2 to 1 to reflect the fact that Cephalobidae can be the first colonizer in the experimental fields) were also used to gauge the condition of the soil ecosystem. All the indices were calculated primarily following Yeates and Bongers [7]. Values of each indicator before chemical application were not significantly different ($p > 0.183$) among treatments. At first, the statistical difference of the values in each experiment was analyzed by ANOVA followed by Dunnett's tests, and the analyses were conducted using Microsoft Excel add-in software Statcel (3rd ed.; OMS, Tokyo, Japan). Then, results from the field experiments (combined, excluding the DCP treatment) were also analyzed in R v.3.6.1 [48] using linear mixed-effects models (LMM; lmer library of lme4 package; [49]) for all variables (square-rooted). Chemical treatment was fitted as the categorical explanatory variable and experiment (different soils, hosts and seasons) was treated as a random effect to control for variation in disease index, species abundance, nematode diversity and maturity indices among experiments. To analyze the response of subgroups within nematode fauna a principal component analysis (PCA) was carried out using 9 to 11 nematode species or groups that were present in the samples. PCAs were conducted using Microsoft Excel add-in software Mulcel (OMS, Tokyo, Japan).

3. Results

3.1. Galling on Tomato and Sweet Potato Roots

The disease index in fluensulfone in Ushiku I was just less than 30% (not statistically significant) of that in the non-treated control, and those in Ushiku II, Miyazaki, and Chiba were significantly ($p < 0.01$) lower in fluensulfone treatments than those in the non-treated control (Figure 1). The disease index in DCP (Ushiku I) was significantly ($p < 0.05$) lower than that in the non-treated control, so ($p < 0.01$) was that in a fosthiazate treatment in Chiba (Figure 1). A mixed-effect model among the four experiments

showed that the disease index was significantly ($p < 0.001$) lower in fluensulfone treatments than in the non-treated control but that in fosthiazate treatments was not ($p = 0.210$).

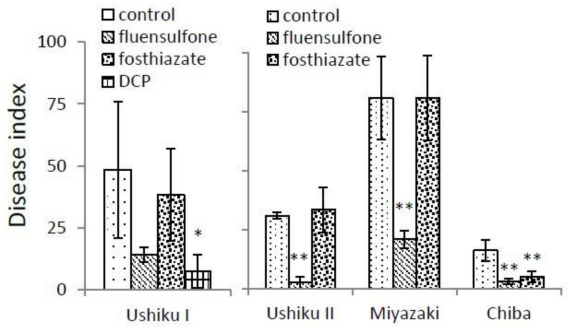

Figure 1. Disease index in Ushiku I, Ushiku II and Miyazaki at 2-months after planting, and Chiba at 4-months after planting. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}), and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control in triplicates. Each value is the mean of three replicates ± standard deviation (** and *: Dunnett's test, $p < 0.01$ and $p < 0.05$, respectively).

3.2. Free-Living Nematode Assemblage

At 1-month after planting, the number of free-living nematodes was significantly ($p < 0.05$) lower in DCP than that in the non-treated control, but not in the other treatments (Figure 2A). At 2-months after planting, the free-living nematode numbers were similar among treatments (Figure 2B). A mixed-effect model at 1- and 2-months among the four experiments did not show significant ($p > 0.082$) difference among treatments.

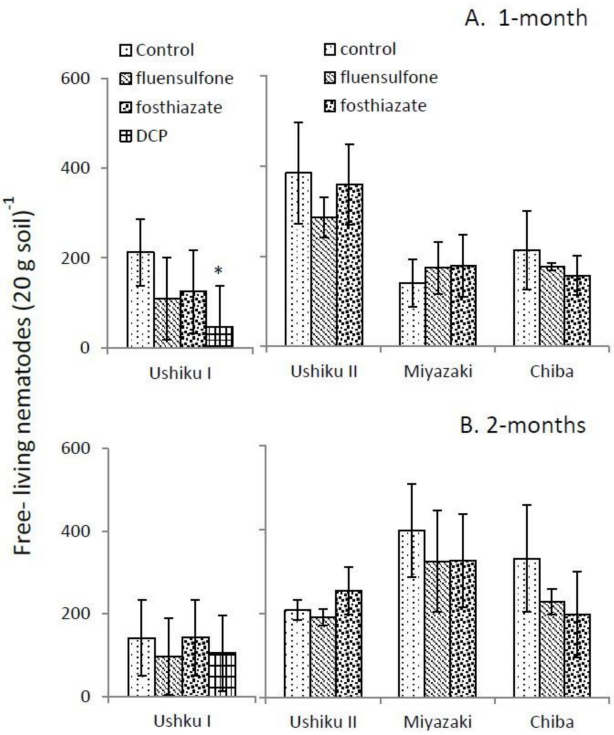

Figure 2. Free-living nematode density (20 g soil)$^{-1}$ in Ushiku I, Ushiku II, Miyazaki and Chiba at (**A**): 1- and (**B**): 2-months after planting. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}), and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control in triplicates. Each value is the mean of three replicates ± standard deviation (*: Dunnett's test, $p < 0.05$).

3.3. Diversity of Free-Living Nematodes

3.3.1. Shannon-Wiener Index (H') and Simpson's D

For the entire period (just before chemical applications to 2 months after planting), the values of Shannon-Wiener index (H') in fluensulfone treatments were not significantly different from those in the non-treated control for each individual experiment (Figure 3A,B). The values of H' in fosthiazate in Ushiku II and Chiba at 1-month after planting and in Ushiku I at 2-months after planting were significantly ($p < 0.05$) lower than that in the non-treated control, so were the values in DCP at 1- and 2-months after planting. A mixed-effect model among the four experiments at 1- and 2-months after planting showed that the values of H' were significantly ($p < 0.001$) lower in fosthiazate treatments than in the non-treated control, but those in fluensulfone were not ($p > 0.447$). The values of Simpson's D in Ushiku II and Chiba at 1-month after planting were significantly ($p < 0.05$) higher than those in the non-treated control, so were in DCP at 1- and 2-months after planting (Figure 3C,D). A mixed-effect model among the four experiments at 1- and 2-months after planting showed that D was significantly ($p < 0.01$) higher in fosthiazate treatments than in the non-treated control but those in fluensulfone were not ($p > 0.467$).

3.3.2. Species Richness and Evenness J'

The values of species richness (Margalef index) in fluensulfone were not different from those in the non-treated control for each individual experiment (Figure 4A,B). Those in fosthiazate in Miyazaki at 1-month after planting and in Ushiku II at 2-months after planting were significantly ($p < 0.01$) lower than that in the non-treated control, so was DCP at 2-months after planting (Figure 4B). A mixed-effect model among the four experiments showed that species richness was significantly ($p < 0.05$) lower in fosthiazate treatment than in the non-treated control at 1- and 2-months after planting. A similar and clearer trend was seen in evenness J'. The values in fosthiazate at 2-months after planting and DCP at 1- and 2-months after planting in Ushiku I were significantly ($p < 0.05$) lower than those in the non-treated control (Figure 4C,D). The values of J' in fosthiazate treatments in Ushiku II and Chiba at 1-month after planting were also significantly ($p < 0.05$) lower than those in the non-treated control. A mixed-effect model among the four experiments showed that the values of J' were significantly ($p < 0.001$) lower in the fosthiazate treatments than in the non-treated control at 1- and 2-months after planting, but those in fluensulfone treatments were not ($p > 0.448$).

3.3.3. Maturity Indices

The values of maturity index were not significantly different among all the treatments in the four experiments (Figure 5A,B). In contrast, the values of maturity index (cp2–5) in fosthiazate treatments in Ushiku I at 2-months after planting and Miyazaki at 1- and 2-months after planting were significantly ($p < 0.05$) lower than those in the non-treated control (Figure 5C,D). Those in DCP at 1- and 2-months after planting were significantly ($p < 0.05$) lower than those in the non-treated control. A mixed-effect model among the four experiments showed that maturity index (cp2–5) was significantly ($p < 0.05$) lower in a fosthiazate treatment than the non-treated control at 1-month and 2-months after planting, so was that in a fluensulfone treatment but only in 2-months. A mixed-effect model among the four treatments showed that the values of maturity index (Cephalobidae adjusted) in fosthiazate treatments at 1- and 2-months after planting were significantly ($p < 0.01$) lower than in the non-treated control, but those in fluensulfone treatments were not ($p > 0.101$).

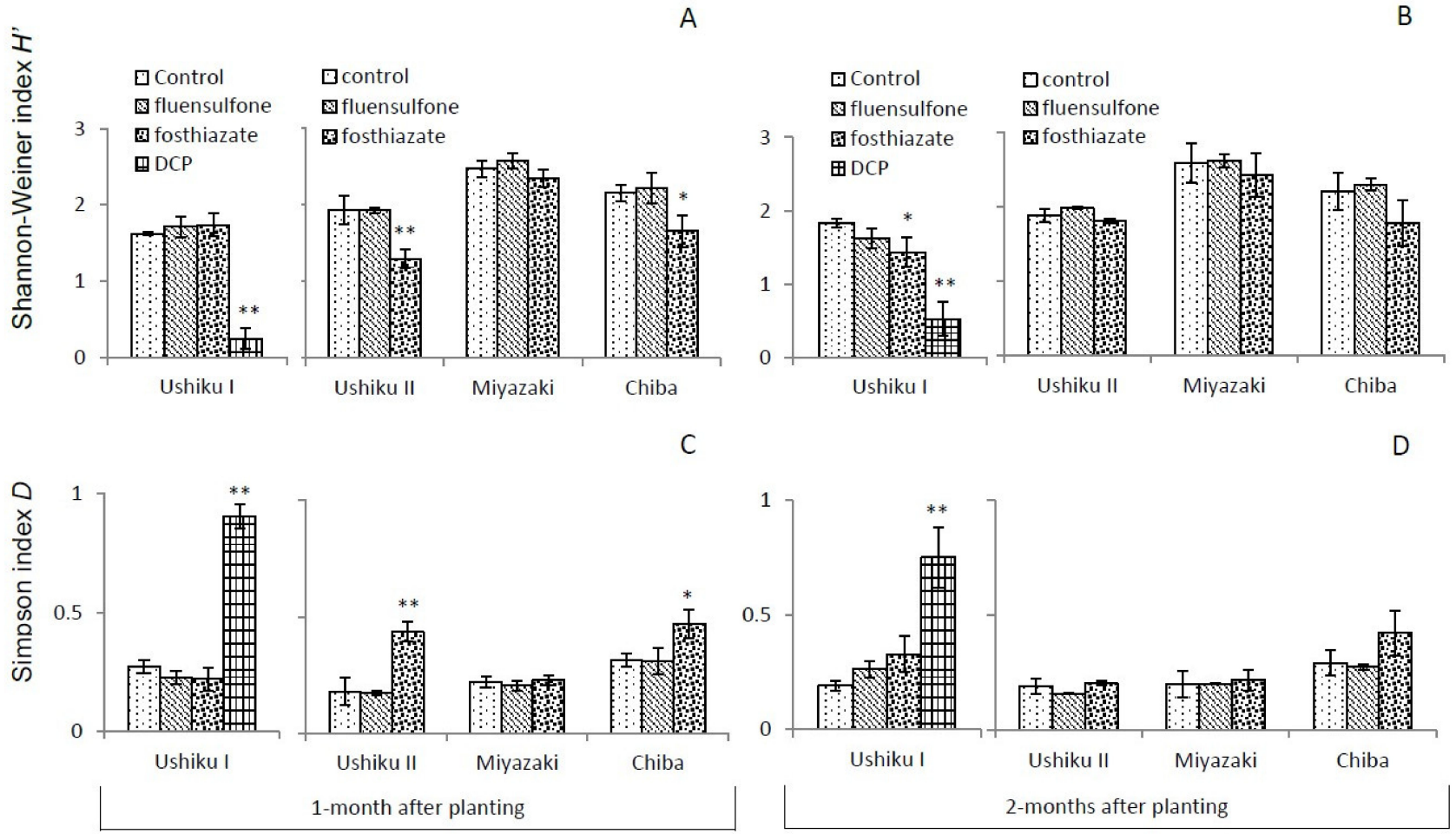

Figure 3. Shannon-Wiener index *H'* (**A**,**B**) and Simpson index *D* (**C**,**D**) in Ushiku I, Ushiku II, Miyazaki and Chiba at 1- (**A**,**C**) and 2-months (**B**,**D**) after planting. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}), and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control. Each value is the mean of three replicates ± standard deviation (** and *: Dunnett's test, $p < 0.01$ and $p < 0.05$, respectively).

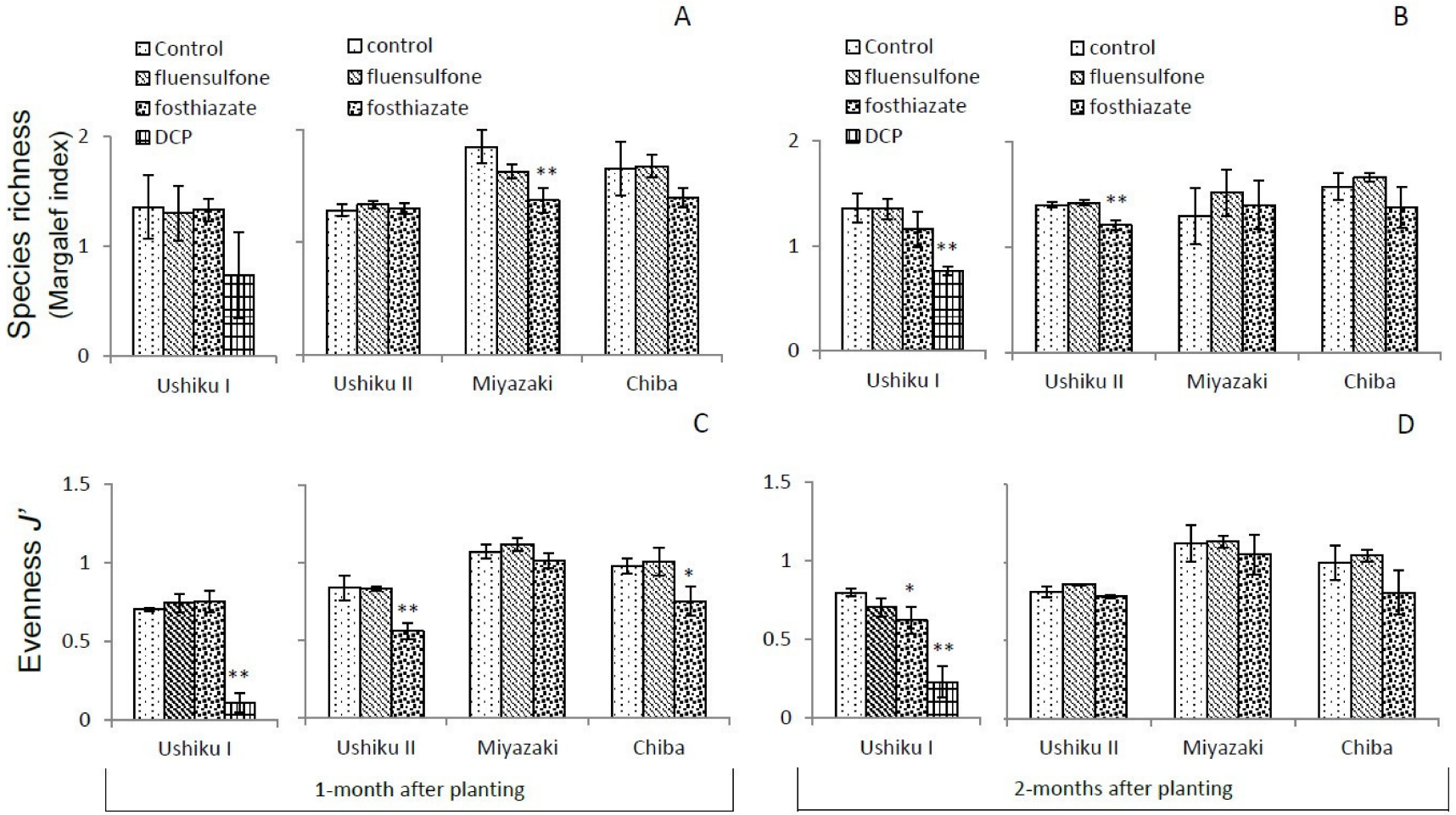

Figure 4. Species richness (Margalef index; (**A**,**B**)) and Evenness J' (**C**,**D**) in Ushiku I, Ushiku II, Miyazaki and Chiba at 1- (**A**,**C**) and 2-months (**B**,**D**) after planting. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}), and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control. Each value is the mean of three replicates ± standard deviation (** and *: Dunnett's test, $p < 0.01$ and $p < 0.05$, respectively).

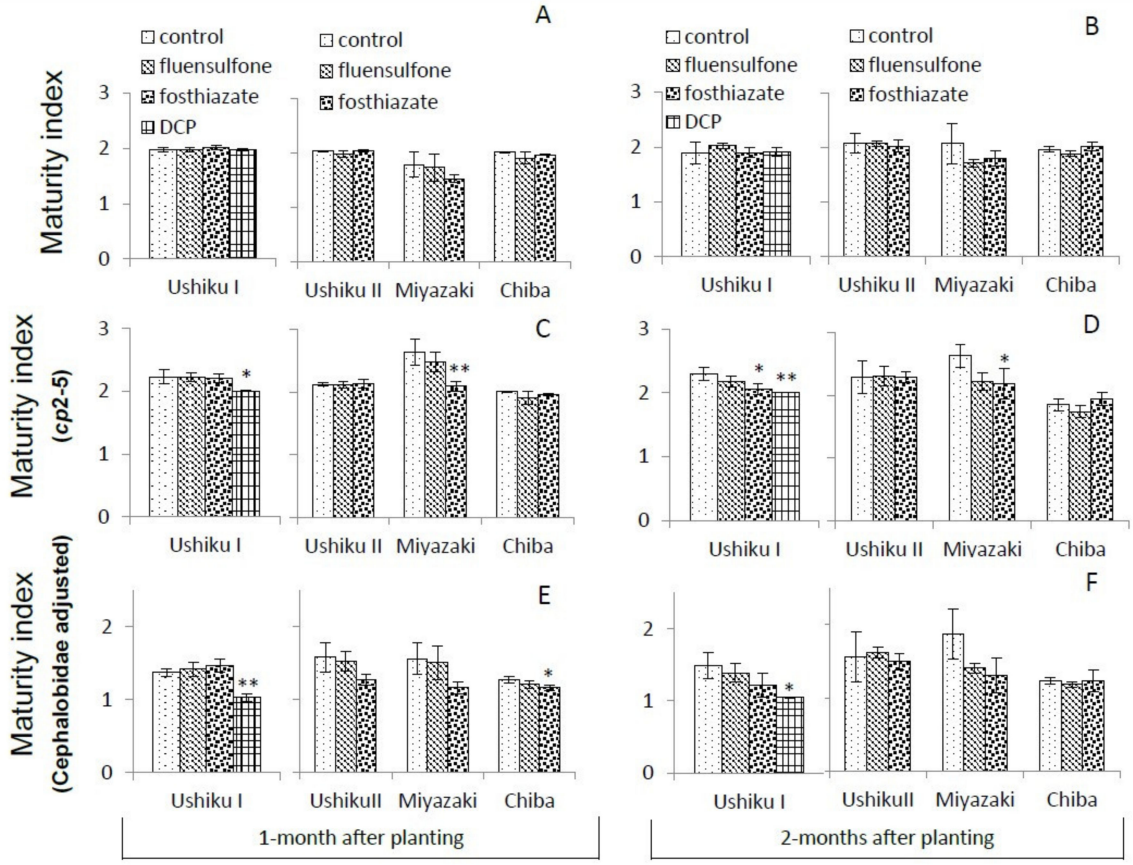

Figure 5. Maturity index (**A**,**B**), Maturity index (*cp*2–5; (**C**,**D**)) and Maturity index (Cephalobidae adjusted; (**E**,**F**)) in Ushiku I, Ushiku II, Miyazaki and Chiba at 1- (**A**,**C**,**E**) and 2-months (**B**,**D**,**F**) after planting. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}), and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control. Each value is the mean of three replicates ± standard deviation (** and *: Dunnett's test, $p < 0.01$ and $p < 0.05$, respectively).

3.3.4. *Acrobeloides* sp., and Fungivorous and Omnivorous Nematodes

For each individual experiment, the proportions of *Acrobeloides* sp. to the total free-living nematodes in fluensulfone were not different from those in the non-treated control (Figure 6A,B). Those in fosthiazate were significantly ($p < 0.05$) higher in Ushiku II, Miyazaki and Chiba at 1-month after planting and in Ushiku I, Miyazaki and Chiba at 2-months after planting. Those in DCP were significantly ($p < 0.01$) higher than those in the non-treated control at 1- and 2-months after planting (Figure 6A,B). A mixed-effect model among the four experiments showed that fosthiazate treatments at 1- and 2-months after planting were significantly ($p < 0.001$) higher in the value than the non-treated control, so was fluensulfone treatment at 2-months ($p < 0.05$) but not at 1-month ($p = 0.958$). Fungivorous and omnivorous nematodes' proportions to the total free-living nematodes in fluensulfone and the non-treated control were not statistically different in each individual experiment (Figure 6C,D). The proportions in DCP at 1- and 2-months after planting were significantly ($p < 0.01$) lower than those of the non-treated control. A mixed-effect model among the four experiments showed that fosthiazate treatments at 1- and 2-months after planting were significantly ($p < 0.001$) lower in the value than the non-treated control, but fluensulfone treatments were not ($p > 0.427$).

3.3.5. Principal Component Analysis

The result of PCA analysis showed that DCP in Ushiku I, and fosthiazate in Ushiku II and Chiba after chemical treatments were seen in the dotted circles (Figure 7), which were remote from the other

treatments. Among the loading factors (LF) for Ushiku I, Ushiku II and Chiba, the values of *Acrobeloides* sp. were over 0.98, while most of the other LF values were negative. *Rhabditis* sp. was the most important LF value (0.99) for Miyazaki (Table 1).

Table 1. Principal component analysis.

	Ushiku I		Ushiku II		Miyazaki		Chiba	
	Principal Components		Principal Components		Principal Components		Principal Components	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Component ratio	0.81	0.09	0.69	0.16	0.52	0.20	0.52	0.34
Cumulative component ratio	0.81	0.91	0.69	0.86	0.52	0.72	0.52	0.86
Loading factor								
Acrobeloides sp.	1.00	0.03	1.00	0.05	−0.50	−0.41	0.98	0.17
Other Cephalobidae	−0.64	0.76	−0.44	0.19	−0.68	0.06	−0.45	0.89
Rhabditis sp.	−0.74	−0.42	−0.28	−0.84	0.99	0.04	−0.39	−0.77
Other Rhabditidae	−0.61	−0.24	−0.27	−0.54	0.00	−0.80	−0.29	−0.30
Other bacterivore	−0.59	−0.41	−0.67	−0.51	−0.47	0.18	−0.28	−0.63
Aphelenchus sp.	−0.45	−0.07	−0.37	0.57	0.43	−0.31	−0.41	−0.57
Aphelenchoides sp.	−0.09	−0.27	−0.60	0.70	0.31	0.06	−0.20	−0.41
Filenchus sp.	−0.30	−0.08	−0.53	0.52	−0.53	0.39	−0.32	0.01
Ditylenchus sp.	−0.08	−0.11	0.23	0.16	−0.40	0.22	-	-
Dorylaimida	−0.61	−0.25	−0.34	−0.82	−0.20	0.81	−0.31	−0.26
Other	-	-	-	-	0.36	−0.13	-	-

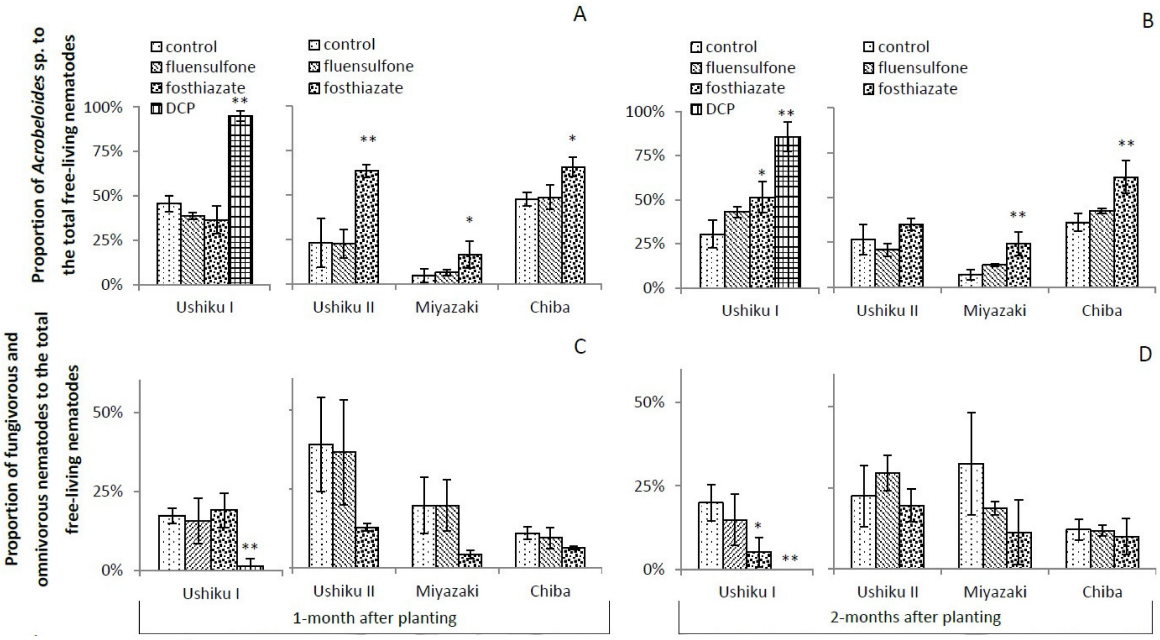

Figure 6. Proportion of *Acrobeloides* sp. (**A**,**B**) and that of fungivorous and omnivorous nematodes (**C**,**D**) to the total free-living nematodes in Ushiku I, Ushiku II, Miyazaki and Chiba at 1- (A,**C**) and 2-months (**B**,**D**) after planting. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}), and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control. Each value is the mean of three replicates ± standard deviation (** and *: Dunnett's test, $p < 0.01$ and $p < 0.05$, respectively).

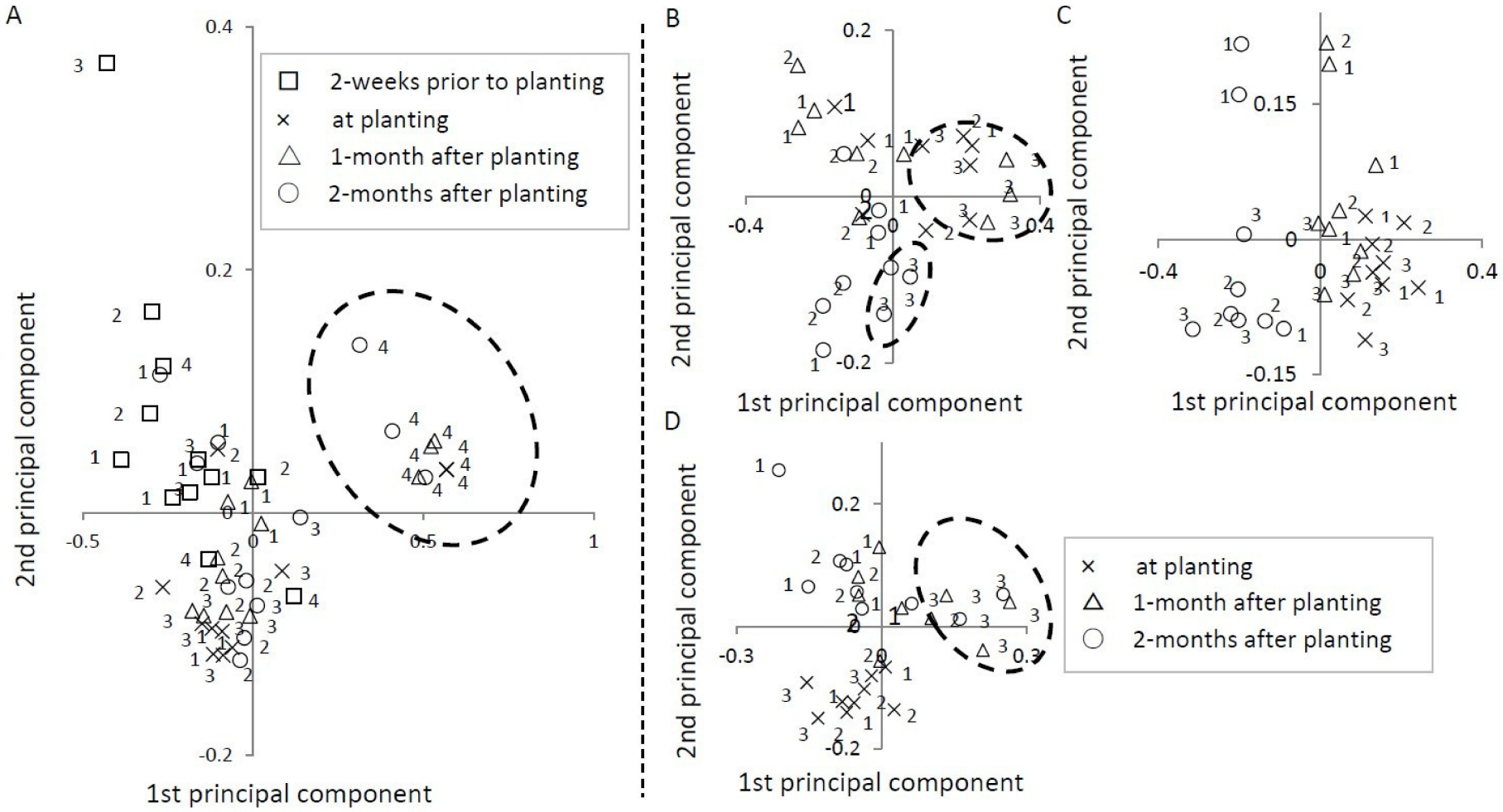

Figure 7. Principal component analysis for (**A**): Ushiku I, (**B**): Ushiku II, (**C**): Miyazaki and (**D**): Chiba. Experimental plots were treated with fluensulfone (4 kg active ingredient (a.i.) ha^{-1}), fosthiazate (3 kg a.i. ha^{-1}) and 1,3-dichloropropene (DCP, 177 kg a.i. ha^{-1}), or non-treated as a control in triplicates. Numbers in the graphs indicate 1: non-treated control, 2: fluensulfone, 3: fosthiazate and 4: DCP.

4. Discussion

The disease index analysis results confirmed the effectiveness of fluensulfone against root-knot nematodes [26–31], though the main focus of this study was to evaluate fluensulfone's nematicidal activity against non-target free-living nematodes. The present study revealed that fluensulfone is a nematicide with little impact on non-target free-living nematodes. The fluensulfone treatment did not affect the total number of free-living nematodes throughout the experimental period in each individual experiment conducted in three locations and in different hosts and seasons. The nematode diversity level in the fluensulfone treatment, shown by Shannon-Wiener index (H'), an important indicator of biodiversity in soil [7], was also consistently similar to that in the non-treated control. As indicated by Simpson's D, which generally amplifies the impact of high density species [50], nematode diversity in a fluensulfone treatment did not heavily rely on high density species. The results observed in H' and D were supported by species richness (Margalef index), evenness J' and maturity indices as they were the same levels between the fluensulfone treatment and the non-treated control. Further, the proportion of *Acrobeloides* sp. and that of fungivorous and omnivorous nematodes to the total free-living nematodes were consistently at very similar levels in both the fluensulfone treatment and the non-treated control for each individual experiment over the experimental periods. This was supported by PCA analysis, which also showed consistently similar results of fluensulfone treatments to those of the non-treated control. These results concluded that fluensulfone has very little effect on the free-living nematode fauna in soil. As previous studies reported [4,5], diverse free-living nematodes play important roles in soil ecosystem function, such as nutrient cycling, decomposition and disease suppression [2]. Fluensulfone may serve well for maintaining a diverse free-living nematode community while suppressing root-knot nematodes.

Fosthiazate treatments, except in Chiba, were not effective against root-knot nematodes, unlike previous studies [51–53], yet, the exact reason for this is uncertain. Fosthiazate treatments did not affect the total free-living nematodes density for each individual experiment, and the result of this non-response is consistent with findings of a previous study [54]. Diversity of free-living nematodes, however, was affected by fosthiazate treatments, as presented in H', D, species richness, J', maturity index (Cephalobidae adjusted), all of which indicated significantly different results of fosthiazate treatments from the non-treated control. PCA also implied that fosthiazate affected the free-living nematode fauna. As indicated in the proportions of *Acrobeloides* sp., and fungivorous and omnivorous nematodes to the total free-living nematodes, fosthiazate did not reduce the population of *Acrobeloides*, but reduced the populations of fungivorous and omnivorous nematodes. On this point, Sturz and Kimpinski [55] showed that fosthiazate did not affect bacterivorous nematodes, to which *Acrobeloides* belongs.

DCP expelled most of nematodes in the soils, including both root-knot and free-living nematodes. Since DCP treatment killed the nematodes almost completely, nematode diversity was lost. Even at 2-months after planting, the level of diversity was very low and heavily relied on limited species as indicated by H', D, species richness, J', and maturity indices (*cp*2–5 and Cephalobidae adjusted). The overall results were consistent with previous studies [56–61]. The species recovered first in the DCP treatment was *Acrobeloides* sp., while fungivorous and omnivorous nematodes did not recover until the end of the experiment. The results are consistent with those of Okada et al. [62] who showed that Cephalobidae nematodes, to which *Acrobeloides* sp. belongs, increased greatly in the first 2 months after fumigation. Though this study did not cover the long-term effect, Sánchez-Moreno et al. [59] and Timper et al. [60] reported recovery of omnivorous nematodes in 22 weeks (by treating DCP plus chloropicrin) and by the following season (by treating DCP plus aldicarb), respectively.

Maturity index in each chemical treatment was not different from that in the non-treated control. This may be due to the simple colonizer-persister (*cp*) value appointment for each nematode species. Except Miyazaki, 75% or more of the free-living nematodes in each experimental location was in the category of *cp*2 on average. Also, especially in the non-treated control, Dorylaimida with a high *cp* value (*cp*4) and Rhabditidae with a low *cp* value (*cp*1), may be offset. Dorylaimida and Rhabditidae at

2-months after planting in DCP were 0% and very limited (< 2%), respectively. As a result of offsetting high and low *cp* value nematode classifications, the end maturity index levels converged to ca 2 due to relatively abundant Cephalobidae (*cp*2) including *Acrobeloides* sp. Since the proportion of *cp*1 and *cp*4 nematodes in Miyazaki were relatively higher than the other experimental locations, maturity index (*cp*2–5) may be more sensible than maturity index for the difference among the treatments. On this point, Yeates [63] indicated that the dominant nematode species may be different depending on resource and soil texture. Okada et al. [62] further discussed that depending on soil types Cephalobidae may be the first colonizer and increase rapidly. Using another maturity index (*cp*2–5) which excludes enrichment opportunists (*cp*1), the consistent conditions of the soil nematode fauna with the other indices of ecological status were observed to a certain extent. Further, since Cephalobidae may play as the first colonizer in the fields of this study, maturity index (Cephalobidae adjusted) was tested by reassigning the *cp* value of Cephalobidae from 2 to 1 as an alternative indicator in this particular environment. Though maturity index (Cephalobidae adjusted) may be more capable to highlight the difference among treatments than the other maturity indices in this study, further analysis in a variety of field environments may be desired. As Okada et al. [62] indicated, due care for using maturity indices may be essential especially in case of the presence of dominant Cephalobidae, yet maturity index (*cp*2–5) and even maturity index (Cephalobidae adjusted) may be useful in evaluating the condition of free-living nematode fauna.

PCA reinforced the discussion made on the several different indices by highlighting a certain nematode classification as an important factor in the four experiments. Depending on the experimental locations, there were certain differences in the importance of the 1st and 2nd component ratios and the loading factors (LF). *Acrobeloides* sp. was the most important LF (more than 0.75) except Miyazaki, where *Acrobeloides* sp. was still one of the important LF (−0.50; the most important LF in Miyazaki was *Rhabditis* sp.: 0.99). This is consistent with the discussion for the proportion of *Acrobeloides* sp. to the total free-living nematodes. In all the experiments, PCA showed that fluensulfone treatments were not different from the non-treated control, while fosthiazate in Ushiku II and Chiba and DCP treatments were in different positions. DCP demonstrated an obvious difference shown in the remote plots in the PCA graphs. In Ushiku II and Chiba, PCA also revealed that fosthiazate treatments were in separate areas from the non-treated control and fluensulfone treatments to some extent.

The current study used nine to 11 different nematode classifications to measure nematode diversity, though Stirling and Wilsey [64] discussed richness of > 10 and < 100 species fits for modeling biodiversity using H'. There were possibly more species existed in the tested fields, however, using this level of classification may be an empirically feasible approach for field studies considering the robust process for nematode identification and quantification. Also, the present study applied several different measurements including H', D, J', maturity indices and PCA to figure out the status of nematode diversity in each experiment. As Bardgett and van der Putten [65] mentioned, belowground communities are remarkably diverse and the theoretical models to explain patterns of belowground community organization are still under development.

5. Conclusions

As a conclusion, fluensulfone was an effective nematicide against galling on tomato and sweet potato roots by root-knot nematodes with very limited impact on the soil free-living nematode fauna. This is the first report of fluensulfone's nematicidal activity against *M. incognita*, but less activity against non-target free-living nematode fauna in field crops.

Supplementary Materials: For the details of each experiment, the following is available online at http://www.mdpi.com/2073-4395/9/12/853/s1, Table S1: Proportion of each nematode classification to the total free-living nematodes.

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