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Effects of the nematicide imicyafos on soil nematode community structure and damage to radish caused by *Pratylenchus penetrans*

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Abstract: The effects of the non-fumigant nematicide imicyafos on soil nematode community structure and damage to radish caused by *Pratylenchus penetrans* were evaluated in two field experiments in consecutive years (2007 and 2008). Nematode densities in soil at 0 - 10 cm (the depth of nematicide incorporation) and 10 - 30 cm were measured. The application of imicyafos had a significant impact on the density of *P. penetrans* at 0 - 10 cm but had no effect on free-living nematode density. PCR-DGGE analysis conducted using extracted nematodes showed that the nematode community structure 12 d after application in 2007 was altered by the application of imicyafos at the 0 - 10 cm depth, but not at 10 - 30 cm. No significant differences were observed in the diversity of the nematode community at harvest (89 and 91 d after application) between the control and imicyafos treatments in both depths and both years. In both years, the damage to radish caused by *P. penetrans* was markedly suppressed by the nematicide. Overall, the nematicide imicyafos decreased populations of *P. penetrans* in soil and thereby decreased damage to radish, while having little impact on the soil nematode community.

Key words: free-living nematode, granular nematicide, imicyafos, lesion nematode, management, non-target effect, PCR-DGGE.

Miura, Kanagawa Prefecture, is a producing center of radish, with a total cultivation area of 779 ha, the second largest cultivation area of the Japanese cities. Damage caused by the root lesion nematode *Pratylenchus penetrans* is one of the obstacles in radish cultivation. The use of nematicides is one common control measure, either applied as a fumigant, such as 1,3-dichloropropene (1,3-D), or in granular form, such as fosthiazate and cadusafos. Fumigation of soil suppresses not only plant-parasitic nematodes but also a variety of non-target soil organisms, including bacteria and fungi (e.g., Ibekwe, 2004). Non-fumigant nematicides are generally thought to have little effect on organisms that do not have nervous systems because these compounds are cholinesterase inhibitors (Tomlin, 2009; Osaki and Fukuchi, 2010). For example, two organophosphorus nematicides, fosthiazate and imicyafos, had little impact on soil microbial community characteristics such as the number of nitrifying bacteria, soil microbial biomass, and cellulose decomposing activity (Wada and Toyota, 2008). The production of non-fumigant nematicides is readily increasing in Japan, while that of soil-fumigants is decreasing (Japanese Society of Plant Protection 2008). In cotton cultivation in the USA, non-fumigant nematicides have gained in popularity over soil fumigants due to their user-friendliness and cost (Starr et al., 2007).

Non-fumigant nematicides may have negative impacts on soil animals as well as the target, plant-parasitic nematodes. For example, oxamyl was toxic to a bacterial-feeding nematode *Bursilla* sp. (Bell et al., 2006). In contrast, aldicarb and fosthiazate had little or no suppressive effect on the number of free-living nematodes, although each effectively suppressed plant-parasitic nematodes (Sturz and Kimpinski, 1999; Cowgill et al., 2002; Kimpinski et al., 2005; Pankhurst et al., 2005). In our previous report, there were also no significant effects of fosthiazate and imicyafos on the number of free-living nematodes (Wada and Toyota, 2008). The impact of these nematicides on the soil nematode community structure and diversity was not examined, however. There are few data available of the effects of non-fumigant nematicides on the soil nematode community (Cadet et al., 2004), although it is well known that fumigants not only decrease nematode density but also affect the nematode community structure markedly (Okada et al., 2004; Sánchez-Moreno et al., 2010). If non-fumigant nematicides have a negligible effect on non-target nematodes, this will support the concept that non-fumigants are generally considered an effective and more environmentally friendly measure for the control of damage by nematodes. The objective of this study was to evaluate the effect of the recently registered nematicide imicyafos on the soil nematode community and the target nematode *P. penetrans* in field experiments.

Materials and Methods

Study site: Experiments were conducted in 2007 and 2008 in a farmer's field (Miura, Kanagawa Prefecture)

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that was naturally infested with the lesion nematode. Some properties of the soil (Andisol) were as follows: soil texture: loam (clay 6.0%, silt 62.2%, sand 31.8%), maximum water holding capacity 0.937 g g^{-1}, pH (H_2O) 6.2 (Min et al., 2007). There were two treatments (nematicide treated and untreated control), and each treatment had three plots (2 m x 2 m each). These two treatments were set in two different locations ca. 20 m away from each other depending on the year.

Nematicide treatment: A granular nematicide imicyafos (Nemakick, Agro-Kanesho Co., Ltd., Tokyo, Japan; 1.5% a. i.) was applied to the soil surface at the rate of 3 kg a. i./ha and plowed in 0 - 10 cm. In 2007, imicyafos was applied on 27 September, and radish (*Raphanus sativus* L.) seeds (cv. 'T465') were sown on 28 September with a 20-cm inter-plant distance and 40-cm inter-row space. In 2008, imicyafos was applied on 24 September, and radish (*R. sativus* L.) seeds (cv. 'T465') were sown on 24 September with a 20-cm inter-plant distance and 50-cm inter-row space. In both years, radish cultivation was carried out by the farmer using conventional techniques.

Soil sampling: Soil at 0 - 10 cm and 10 - 30 cm was collected from three locations within each plot using a root auger (4-cm diameter) and bulked to make a composite sample per plot for each depth. The first soil sampling was done before the nematicide treatment (20 September 2007 and 9 September 2008), and then soil sampling was done 12, 36, 70 and 89 (harvest) d after treatment in 2007. In 2008, soil sampling was done 91 (harvest) d after treatment.

Nematode number: Nematodes were extracted from 20 g of soil (fresh basis, in triplicate) by the Baermann funnel method for 48 hr at room temperature. The nematodes extracted from three Baermann funnels per plot were mixed to make a composite sample. The numbers of free-living nematodes and *P. penetrans* were separately counted under a microscope (x80).

Nematode community structure: We used PCR-DGGE (denaturing gradient gel electrophoresis) to evaluate the impact of the nematicide on nematode community structure, since this procedure allows assessment of the presence and relative abundance of different species and thus communities can be profiled in both a qualitative and a semi-quantitative way (Muyzer et al, 1993). PCR-DGGE is currently widely used to study soil microbial communities (Oros-Sichler et al., 2007). Recently, this technique has been applied to the analysis of nematode community structure (Sato and Toyota, 2006; Okada and Oba, 2008). Some limitations are known to the interpretation of PCR-DGGE results. For example, bands of different species may partially or completely overlap on gel (Oros-Sichler et al., 2007). Nonetheless, PCR-DGGE remains an effective technique for assessing whether changes in nematode community structure or diversity are associated with treatments.

A suspension of the nematodes extracted above was adjusted to a concentration of 200-250 individuals per 150-200 µl of distilled water and stored at -20°C until use. DNA was extracted from the nematode suspension by the method of Sato et al. (2009). One hundred fifty to two hundred microliters of a nematode suspension was put into a 2-ml tube with 0.2 g of zirconia beads (0.1 mm in diameter), and 20 µl of 10x TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) was then added. The nematode suspension was treated with bead beading at 2,770 x g for 90 sec two times (Bead-smash 12; Wakenyaku, Kyoto, Japan), and the tube was placed on ice. Fifty microliters of a skim-milk solution (200 mg/ml) was added to the tube, along with 40 µL of 3M sodium acetate, 200 µl of an extraction buffer (5M NaCl, 0.5M Tris, 0.5M EDTA, pH 8.0) and 500 µl of chloroform and mixed well. After centrifugation for 15 min at 4°C at 20,350 x g (3740; Kubota, Osaka, Japan), 400 µl of the supernatant was transferred to a new tube with 8 µl of a glycogen solution (5 mg/ml), 80 µl of 3M sodium acetate, and 600 µl of isopropanol. Four hundred microliters of distilled water was added to the tube, mixed well, and then centrifuged for 15 min at 4°C at 20,350 x g. Four hundred microliters of this supernatant was combined with the first 400 µl of the supernatant. The DNA was precipitated by centrifugation for 15 min at 4°C at 20,350 x g, washed with 70% ethanol once and air-dried. Finally, the DNA was dissolved in 200 µl of TE buffer.

PCR amplification for DGGE was performed using the template DNA extracted above in a 25 µl volume containing 5 µl template DNA, 5 µl of 5 × PrimeSTARTM buffer (Mg^{2+} plus; Takara, Otsu, Japan), 200 µM each dNTP, 0.5 µM each primer (SSU18A and SSU9RGC; Okada & Oba 2008) and 0.24 U of PrimeSTARTM HS DNA polymerase (Takara, Otsu, Japan). The temperature program included a denaturing step at 98°C for 3 min followed by 30 cycles of 98°C for 10 sec, 54°C for 15 sec, 72°C for 40 sec, and final extension step of 72°C for 10 min. The PCR products were loaded in an agarose gel (0.7%, w/v) and stained with ethidium bromide. Based on the band intensity determined visually post-PCR in an agarose gel, the volume of PCR product loaded onto the DGGE gel was adjusted (ca. 200 ng/lane). DGGE was carried out using a Bio-Rad DCode mutation analysis system (Bio-Rad Laboratories, Inc., Tokyo, Japan). Electrophoresis was done using a 6% (w/v) polyacrylamide gel in 1X TAE buffer (40 mmol/l Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.0) under 75V at 60°C for 16 hr. The polyacrylamide gels were made with parallel denaturing gradients 15 - 50%; 100% denaturant contained 7 M urea and 40% formamide (Sato et al., 2009). The marker lane consisted of known clones of soil fauna including nematodes. The fourth band from the top in the marker corresponds to *Pratylenchus* sp. (Okada and Oba, 2008). Band intensity was measured using a BioNumerics version 4.5 (Infocom Corporation, Tokyo, Japan), and the Shannon-Wiener index (*H'*) was calculated based on the intensity data. The primers used in this study (SSU18A and SSU9RGC) amplify protozoa as well as nematodes. In

our previous study (Sato and Toyota, unpub. data), results of random cloning of the PCR products showed that the clones with band positions below the second band of marker were all nematode species, while some of those above this band were nematode species and others were protozoan species. Therefore, to analyze the nematode community, we included only the bands below the second band of marker.

Estimation of damage to radish caused by P. penetrans: Ten to 16 radishes per plot were harvested, washed with running tap water, and damage caused by *P. penetrans* was evaluated based on 5 levels of 0 (no damage) to 4 (most severe) according to Mizukubo (2004).

Statistical analyses: Nematode density was compared between treatments with t-test. Two-way repeated measures ANOVA was also used to analyze the 2007 data. The Shannon-Wiener Index calculated from DGGE data was analyzed with t-test. Damage to radish caused by *P. penetrans* was analyzed with Mann-Whitney U-test. Excel statistics 2006 software for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for analysis of all data.

Results

Nematode density: In 2007, there were no significant differences in the density of free-living nematodes and *P. penetrans* before the application of nematicide between two treatments (Table 1A,B). After application, the number of *P. penetrans* in soil at 0 - 10 cm decreased compared to the control (no nematicide application), although the difference was only significant (t-test, $P < 0.05$) at 89 d after treatment (Table 1A). Two-way repeated measures ANOVA for a combined data set of 12, 36, 70 and 89 d after application showed that the application of imicyafos significantly decreased the density of *P. penetrans* in soil at 0 - 10 cm and 10 - 30 cm ($P < 0.01$) but not that of free-living nematodes at both depths.

In 2008, the density of *P. penetrans* 91 d after nematicide application was lower than the control, although the difference was only significant at 0 - 10 cm (t-test, $P < 0.05$, Table 2A). In contrast, there were no significant differences in the free-living nematode density at both depths before and after the application of nematicide between two treatments (Table 2B).

Soil nematode community structure evaluated by PCR-DGGE: Band numbers were lower for the imicyafos treatment, exemplified by the area X in Fig. 1A, at 0 - 10 cm 12 d after application in 2007 (Fig. 1A). Band P, corresponding to the band position of *P. penetrans*, disappeared in the imicyafos treatment (Fig. 1A). In contrast, slight differences were observed in the soil nematode community structure at 10 - 30 cm but were not as dramatic as at 0 - 10 cm (Figs. 1A; 2A). Diversity of the soil nematode community (Shannon-Wiener Index) was significantly lower in the imicyafos treatment at 0 - 10 cm ($P < 0.05$), but not at 10 - 30 cm (Table 3). At harvest (89 d after application), there were no apparent differences in nematode community structure between the treatments at both depths (Figs. 1B; 2B). There was also no significant difference in the diversity index 89 d after treatment at both depths (Table 3).

In 2008, the soil nematode community structure was only determined 91 d after nematicide application. One band (band Y) disappeared in the imicyafos treatment at 0 - 10 cm (Fig. 3A), while there were no differences at 10 - 30 cm between the treatments (Fig. 3B). There were no significant differences in the diversity index at both depths at 91 d after treatment (Table 3).

Damage to radish: In both years, damage to radish by *P. penetrans* was severe in the control, and the damage index averaged 4.0 ± 0.1 in 2007 and 2.0 ± 0.9 in 2008. It was lower in the imicyafos treatment (1.7 ± 1.1 and 0.2 ± 0.4)

Table 1. Densities of *Pratylenchus penetrans* and free-living nematodes in soils treated with imicyafos in 2007.

(A) *P. penetrans*

Soil depth	Treatment	Nematode density[a] (number per 20 g fresh soil)				
		Before treatment	12 DAT[b]	36 DAT	70 DAT	89 DAT
0-10 cm	Control	36 ± 13	18 ± 13	33 ± 11	20 ± 15	23 ± 10
	Imicyafos	37 ± 9	4 ± 3	18 ± 8	9 ± 6	6 ± 1*
10-30 cm	Control	107 ± 46	67 ± 29	78 ± 19	62 ± 26	52 ± 16
	Imicyafos	61 ± 18	34 ± 29	33 ± 19	48 ± 7	31 ± 16

(B) Free-living nematodes

Soil depth	Treatment	Nematode density (number per 20 g fresh soil)				
		Before treatment	12 DAT	36 DAT	70 DAT	89 DAT
0-10 cm	Control	185 ± 29	265 ± 137	241 ± 146	200 ± 143	269 ± 50
	Imicyafos	193 ± 36	212 ± 74	228 ± 95	157 ± 97	178 ± 31
10-30 cm	Control	365 ± 275	157 ± 26	202 ± 84	89 ± 6	210 ± 78
	Imicyafos	143 ± 16	128 ± 33	133 ± 50	128 ± 26	194 ± 29

[a] Each value shows mean ±S.D.
[b] Days after treatment.
*Indicates significant difference from control (t-test, $P \leq 0.05$).

TABLE 2. Densities of *Pratylenchus penetrans* and free-living nematode in soils treated with imicyafos in 2008.

(A) *P. penetrans*

Soil depth	Treatment	Nematode density[a] (number per 20 g fresh soil)	
		Before treatment	91 DAT [b]
0-10cm	Control	2.8 ± 2.3	1.8 ± 1.0
	Imicyafos	3.1 ± 0.5	0.1 ± 0.2*
10-30cm	Control	8.0 ± 4.9	5.1 ± 5.4
	Imicyafos	6.2 ± 4.5	0.7 ± 0.7

(B) free-living nematode

Soil depth	Treatment	Nematode density (per 20 g fresh soil)	
		Before treatment	91 DAT
0-10cm	Control	565 ± 101	536 ± 156
	Imicyafos	792 ± 306	482 ± 95
10-30cm	Control	393 ± 32	264 ± 93
	Imicyafos	410 ± 39	273 ± 66

[a] Each value shows mean ±S.D.
[b] Days after treatment.
*Indicates significant difference from control (t-test, $P \leq 0.05$).

in 2007 and 2008, respectively, and the differences were significant (n = 28 - 45, U-test, $P < 0.001$) in both years.

DISCUSSION

In this study over two consecutive years, the application of imicyafos decreased the density of *P. penetrans* in soil at 0 - 30 cm but not that of free-living nematodes. Similar results were reported for a pot experiment (Wada and Toyota, 2008), suggesting that imicyafos may have little impact on the density of non-target nematodes. In field studies, aldicarb decreased the density of potato cyst nematodes (Cowgill et al., 2002), but had no impact on free-living nematodes (Cowgill et al., 2002; Pankhurst et al., 2005). Similar results were reported for fosthiazate, which decreased the density of *P. penetrans*, but not that of bacterial-feeding nematodes (Sturz and Kimpinski, 1999). Consequently, it can be concluded that non-fumigant types of nematicides may have little impact on the density of free-living nematodes.

In addition to assessing the impact of imicyafos on the density of free-living nematodes, the structure of the soil nematode community was evaluated by PCR-DGGE. The results showed that some bands disappeared in the surface soil at 0 - 10 cm with nematicide treatment.

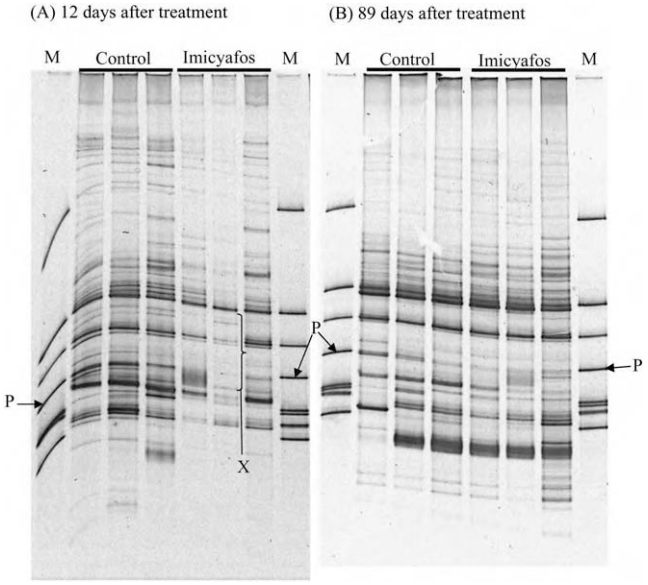

FIG. 1. PCR-DGGE analysis of the amplified 18S rDNA from nematodes from the upper layer of soil (0 - 10 cm depth) 12 and 89 d after treatment, 2007. M: marker lane. P: a band corresponding to *Pratylenchus penetrans*. X: bands that disappeared or became faint in the imicyafos treatment.

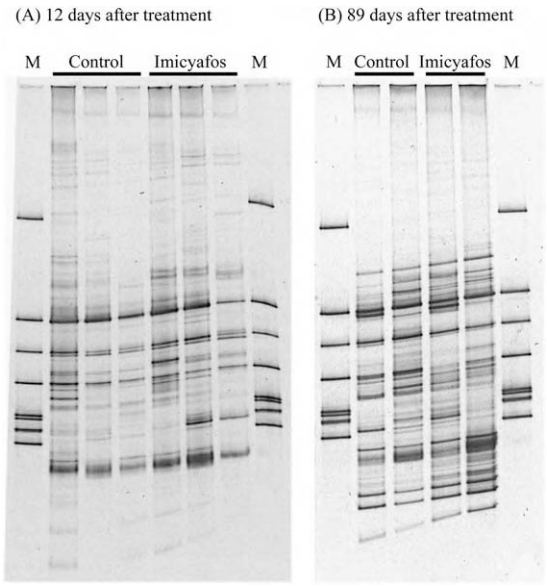

FIG. 2. PCR-DGGE analysis of the amplified 18S rDNA from nematodes from the lower layer of soil (10 - 30 cm depth) 12 and 89 d after treatment, 2007. M: marker lane.

TABLE 3. Calculated Shannon-Wiener index (*H'*) from the PCR-DGGE patterns.

		Shannon-Wiener index (*H'*) [a]		
		2007		2008
Soil depth	Treatment	12 DAT[b]	89 DAT	91 DAT
0-10 cm	Control	3.09 ± 0.09	2.33 ± 0.06	2.37 ± 0.15
	Imicyafos	2.81 ± 0.11*	2.22 ± 0.37	2.28 ± 0.18
10-30 cm	Control	2.79 ± 0.20	3.11 ± 0.27	2.09 ± 0.10
	Imicyafos	2.84 ± 0.07	3.18 ± 0.03	2.22 ± 0.33

[a] Each value shows mean ±S.D.
[b] Days after treatment.
*Indicates significant difference from control (t-test, $P \leq 0.05$).

In addition, the diversity of the soil nematode community, expressed as the Shannon-Wiener Index and based on DGGE banding patterns and their intensity, was lower in the nematicide treatment 12 d after application. PCR-DGGE patterns also showed a few differences between the control and imicyafos treatment in soil at 10 - 30 cm as well as 0 - 10 cm 12 d after application, although the diversity index was not affected. These results may suggest that imicyafos had a negative impact on certain species of free-living nematodes, although overall free-living nematode density was not impacted.

The negative impact of imicyafos on the nematode community that was apparent from DGGE disappeared at harvest, when no differences were observed in the soil nematode community structure or the diversity index between the treatments both in 2007 and 2008. It has been previously reported that imicyafos had few impacts on the soil microbial community, evaluated by soil microbial biomass, cellulose decomposing activity, and the density of nitrifying bacteria, as well as the number of free-living nematodes (Wada and Toyota, 2008). This study further demonstrates that the effect of imicyafos on the soil nematode community structure may be transient, with recovery apparent by harvest. Cadet et al. (2004) also reported that the effect of aldicarb on the nematode community was not evident for nine months after application. Studies that have evaluated the effects of non-fumigant nematicides on soil nematode community are limited, and it is clear that further studies using other non-fumigant nematicides are needed to fully understand their effects on free-living nematodes and other non-target organisms.

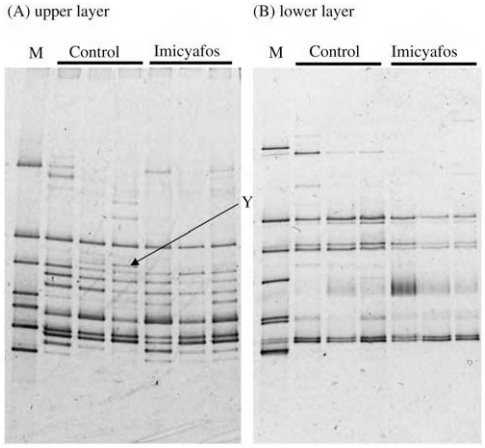

FIG. 3. PCR-DGGE analysis of the amplified 18S rDNA of nematodes from upper (0 - 10 cm depth) and lower layer (10 - 30 cm depth) of soil 91 d after treatment, 2008. M: marker lane. Y: band that disappeared in the imicyafos treatment.

It is well known that DGGE does not detect minor species [i.e., less than 0.1-1% of total DNA (Muyzer and Smalla, 1998; Gelsomino et al., 1999)], and therefore, we cannot evaluate the effect of imicyafos on rare nematode species. Nevertheless, these results suggest that the influence of imicyafos application on the soil nematode community is mainly limited to the surface soil, where the nematicide application is concentrated, and that the effects are transient, unlike soil fumigant-type nematicides. For example, chloropicrin fumigation altered the soil nematode density and community structure in a soybean field, and its effect lasted for 172 d (Okada et al., 2004). Fumigation with 1,3-D altered the composition of the soil nematode assemblage in strawberry farms, and changes were observed even 31 wk after treatment (Sánchez-Moreno et al., 2010).

In contrast with the lack of effects on soil microbial and nematode communities, imicyafos consistently suppressed the density of its target nematode, *P. penetrans*, throughout the cultivation period and successfully controlled the damage to radish at harvest. It has been reported that root populations of root-knot nematode also can be reduced, along with disease, although the soil population density may remain stable (Kokalis-Burelle et al., 2010). Systemic nematicides are known to affect plant-parasitic nematodes within roots more than they impact those in soil (Kimpinski et al., 2005). Imicyafos application resulted in effective control in radish in this study, as even the soil populations of *P. penetrans* were reduced. A difference in sensitivity to nematicides (aldicarb and fosthiazate) between plant-parasitic nematodes and free-living nematodes was previously reported (Kimpinski et al., 2005), and sensitivity to the antibiotic 2,4-diacetylphloroglucinol was different even among plant-parasitic nematodes (Meyer et al., 2009).

In conclusion, the nematicide imicyafos had little impact on soil nematode communities, yet effectively suppressed *P. penetrans*. The reason that imicyafos was toxic to *P. penetrans* and not to free-living nematodes remains to be determined.

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