European Journal of Plant Pathology **105:** 577–584, 1999.

Biological mode of action of the fungicide, flusulfamide, against *Plasmodiophora brassicae* (clubroot)

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Accepted 12 June 1999

Key words: Evan's blue staining, fungistatic effect, germination inhibition, resting spore

Abstract

Flusulfamide (2′, 4-dichloro-α,α,α-trifluoro-4′-nitro-*m*-toluenesulfonanilide) was investigated for its mode of action against *Plasmodiophora brassicae* Woronin. Seedlings of Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis*) were grown for 14 and 21 days in soil infested with *P. brassicae* and then transplanted into soil containing flusulfamide (0.9 µg a.i. g^{-1} dry soil). Clubroot was not suppressed by this treatment, indicating that the fungicide is ineffective against *P. brassicae* established within cortical cells of the host root. Where seedlings were grown in soil infested with resting spores which had previously been treated with flusulfamide, root-hair infection and club formation were suppressed. This indicates that flusulfamide directly acts against resting spores. When placed in root exudates of Chinese cabbage, untreated resting spores germinated at a high frequency while flusulfamide-treated resting spores hardly germinated at all. Use of the Evan's blue staining assay indicated that flusulfamide-treated resting spores remained viable. Flusulfamide was detected by high performance liquid chromatography on resting spores treated with flusulfamide for 30 min. This indicates that the chemical is adsorbed onto resting spores. These results suggest that flusulfamide suppresses clubroot disease by inhibiting germination of *P. brassicae* resting spores through adsorption onto their cell walls.

Introduction

Clubroot caused by *Plasmodiophora brassicae* Woronin is one of the most destructive soil-borne diseases of cruciferous crops worldwide. Although some soil fungicides are available for control of clubroot, these have limited efficacy where there is a high density of resting spores and highly virulent populations of *P. brassicae* (Akanuma et al., 1983; Tanaka et al., 1997). In Japan, quintozene (pentachloronitrobenzene), chlorothalonil, fluazinam and flusulfamide have been registered for clubroot control. Of these, flusulfamide has shown consistently high efficacy against clubroot at low doses (Inami et al., 1988; Tanaka et al., 1988, 1997; Shimotori et al., 1996). Flusulfamide has come to be used widely in cruciferous crop production in Japan (Tanaka, 1996). Knowledge of the mode of action allows more effective application of fungicides. However, the determination of the mode of action of fungicides against *P. brassicae* is made more difficult because it is an obligate pathogen and cannot be cultured axenically. Nevertheless, biological modes of action of some fungicides against *P. brassicae* have been postulated e.g. trichlamide and fluazinam may inhibit resting-spore germination, and primary or secondary infections (Naiki, 1985; Naiki and Dixon, 1987;

Suzuki et al., 1995). In contrast, quintozene possesses relatively low suppressive effects against resting-spore germination (Naiki and Dixon, 1987; Suzuki et al., 1995) but does act against *P. brassicae* established within host cortical tissues in a manner similar to benomyl (Naiki, 1985; Jacobsen and Williams, 1969; Dixon et al., 1972).

The research reported here provides evidence of the mode of action of flusulfamide against *P. brassicae* resting spores and their subsequent capacity to form primary zoospores.

Materials and methods

Plant and fungal populations

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) cv. Nozaki Ni-go, which is susceptible to most Japanese populations of *P. brassicae* (Tanaka et al., 1991), was used throughout this study. Prior to sowing, seeds were disinfected with sodium hypochloride (active chlorine concentration: 0.25%) for 30 min followed by washing with tap water for 1 h, except where specifically stated.

Resting spores of the highly virulent population (race 4; Williams, 1966) of *P. brassicae* were collected from Chinese cabbage at Hagi, Yamaguchi Prefecture (Tanaka et al., 1991) and used in all experiments. Unless otherwise described, resting spores were isolated from clubroot galls which were stored at $-40\,^{\circ}$C as previously described (Tanaka et al., 1990).

Fungicide and treatment of resting spores

Dust (0.3%) and wettable powder (50%) formulations of flusulfamide (2′, 4-dichloro-α,α,α-trifluoro-4′-nitro-*m*-toluenesulfonanilide) were used (Shimotori et al., 1996).

The dust formulation was applied at 0.9 µg a.i. g^{-1} dry soil, the wettable powder was used at 2.0 µg a.i. ml^{-1} in 0.1 M NaOH–potassium phosphate buffer (pH 6.0) for the direct treatment of resting spores. Flusulfamide is soluble upto 2.9 µg ml^{-1} in the buffer (Takazawa, 1993). The direct treatment was accomplished by incubating resting spores at 25 °C in 2.0 µg a.i. ml^{-1} flusulfamide with gentle agitation in 10 ml centrifuge tubes or in static cultures in 100 ml flasks. Flusulfamide-treated resting spores were then washed 3–5 times with the buffer; untreated resting spores were similarly washed with buffer.

Inoculation and effects on developmental stages in the life cycle

Three treatments were used to investigate the effects of flusulfamide on developmental stages of *P. brassicae*. Chinese cabbage seedlings were grown in pots of soil infested with *P. brassicae* for 7, 14 or 21 days after sowing in the greenhouse and then transplanted into uninfested soil containing flusulfamide (treatment A); or uninfested soil without flusulfamide (treatment B); or grown for 7, 14 or 21 days in infested soil containing flusulfamide and then transferred into uninfested soil containing flusulfamide (treatment C) (Table 1).

For this experiment, Kuroboku soil (volcanic black soil, pH 5.3) was infested with 1×10^7 resting spores g^{-1} dry soil following an application of flusulfamide dust at 0.9 µg a.i. g^{-1} dry soil. Chinese cabbage seedlings (10 plants per 10 cm Jiffy pot) were grown for the requisite number of days in the first pot in the greenhouse, uprooted, and washed carefully with neutral synthetic detergent followed by distilled water thereby removing resting spores which might adhere to root surfaces (Naiki and Dixon, 1987). Six seedlings were transferred to the second pot in the greenhouse. Six pot replicates were used for each treatment. At each time of transplant (7, 14 and 21 days after sowing), the developmental stages of *P. brassicae* were observed microscopically, following staining with 1% acetocarmine (Samuel and Garret, 1945), in additional seedlings grown in each pot. Forty days after sowing, the symptom severity was assessed and the data was converted to disease index (Seaman et al., 1966). The experiment was conducted once.

Inoculation and direct effects on resting spores

Direct effects of flusulfamide on resting spores was investigated in two ways. Firstly, six Chinese cabbage seedlings per pot (10 cm Jiffy pot) were grown in artificial soil (Yoshikawa et al., 1981) infested with either flusulfamide-treated or untreated resting spores at a density of 1×10^4 spores g^{-1} dry soil in the greenhouse. Treatment was given by incubating 2.1×10^9 resting spores for 2, 4 or 6 days in 7 ml phosphate buffer containing flusulfamide prepared from wettable powder. Six pot replicates were used for each treatment. Forty days after sowing, symptom severity was assessed and disease indices were obtained (Seaman et al., 1966). The experiment was repeated twice.

Table 1. Effect of flusulfamide on the developmental stages of *P. brassicae* in Chinese cabbage roots

Treatment	Soil		Days to transplanting[3]	Disease index
	Sowing[1]	Transplanting[2]		
A	Untreated[4]	Treated[5]	7	0
			14	98
			21	100
B	Untreated	Untreated	7	0
			14	98
			21	100
C	Treated	Treated	7	0
			14	0
			21	0

[1]Soil infested with *P. brassicae* at 1×10^7 spores g^{-1} dry soil.
[2]Uninfested soil.
[3]Days between sowing to transplanting.
[4]Soil without flusulfamide.
[5]Soil with flusulfamide at 0.9 µg a.i. g^{-1} dry soil.

Secondly, 40 Chinese cabbage seeds were sown on two layers of cheesecloth soaked with 3 ml of modified Hoagland's solution (Macfarlane, 1970) in a Petri-dish and incubated for 2 days at 25 °C in dark. Then the seedlings were inoculated with 2 ml of the modified Hoagland's solution containing 3×10^8 ml^{-1} flusulfamide-treated or untreated resting spores. The treatment was conducted by incubating 2.1×10^9 resting spores for 2 or 4 days in 7 ml buffer containing flusulfamide prepared from wettable powder. Inoculated seedlings were retained for 2 days in a sealed bell jar (Gas Pack 100™, Becton Dickinson and Co., Cockysville, MD, USA) to promote infection (Tanaka et al., unpublished data) and grown for 3 days, at 25 °C under continuous illumination (1000 lx) using Homolux fluorescent light for plant growth (National® FL20S-PG). Five days after inoculation, roots from these 20 seedlings per Petri-dish were washed with distilled water, sectioned and stained with 1% acetocarmine. The number of root-hair infections per plant, identified as secondary zoosporangial clusters, were counted by light microscopy. Three Petri-dish replicates were used for each treatment. The experiment was repeated twice.

Germination of resting spores

The germinability of flusulfamide-treated and untreated resting spores of *P. brassicae* was investigated using root exudates from Chinese cabbage. Resting spores were taken from clubroot galls decayed for 20 days at room temperature since such spores are preferred for germination tests *in vitro* (Suzuki et al., 1992). Resting spores (3.5×10^9) were treated for 2 days with 7 ml phosphate buffer either with or without flusulfamide prepared from wettable powder. Root exudates of Chinese cabbage were prepared according to Suzuki et al. (1992). Seeds were disinfected with 70% ethanol for 5 min followed by sodium hypochloride (active chlorine concentration: 0.5%) for 30 min and rinsed four times in sterile distilled water, and incubated at 25 °C overnight in dark. Fifty germinated seeds were sown on nylon sheet mesh fixed to a styrofoam ring. This was floated on 50 ml modified Hoagland's solution in a deep Petri-dish. The seedlings were grown for 7 days at 25 °C with 8 h dark and 16 h light photoperiods at 1000 lx under Homolux fluorescent light for plant growth (National® FL20S-PG). The culture solution was recovered, filtered (Millipore Co., 0.45 µm in pore size), and adjusted to pH 6.0 using KOH-MES (2-(N-morpholino)-ethanesulfonic acid) buffer (final concentration of MES in culture solution: 10 mM). Each of the flusulfamide-treated and untreated resting-spore samples was suspended at 5×10^7 ml^{-1} in root exudates and incubated at 25 °C in darkness. Percentages of germinated resting spores (empty spores) in the exudates were recorded for 9 days in the first experiment and 14 days in the second experiment using a differential interference contrast microscope. The experiment was repeated twice.

Evan's blue staining assay of resting spores

The effects of flusulfamide on resting spore cells was investigated by the Evan's blue staining assay, which Newcombe and Thomas (1990) used to determine the fungicidal effects of carboxin on sporelings of *Usatilago nuda*. Evan's blue (Merck, Darmstadt, Germany) was dissolved in distilled water at 20 mg ml^{-1}. Resting spores (3.5×10^9) were treated for 2 days with 7 ml phosphate buffer either with or without flusulfamide prepared from wettable powder. Ten microliters of buffer containing 1×10^9 ml^{-1} flusulfamide-treated or untreated resting spores was mixed with equivalent volume of the Evan's blue stain. After incubation for 10–30 min at room temperature, the mixture was diluted four-fold with distilled water and resting spores were examined by light microscopy. Resting spores with and without stained cytoplasm (Figure 1) were identified as dead and viable, respectively (Newcombe and Thomas, 1990). The experiment was repeated twice.

High performance liquid chromatography (HPLC)

Adsorption of flusulfamide to resting spores was examined quantitatively by HPLC. Resting spores (3.5×10^9) were treated with 7 ml phosphate buffer containing flusulfamide prepared from wettable powder for 30 min to 48 h, and then washed five times with buffer. Adsorbed flusulfamide was recovered from each sample of resting spores by shaking with 100% acetone (HPLC grade, Kanto Chem. Co., Inc., Tokyo, Japan) for 10 min. Resting spores were removed by centrifugation and the supernatants were dried under reduced pressure. Each dried sample was dissolved in 100% methanol (HPLC grade, Kanto Chem. Co., Inc.) and subjected to HPLC following filtration (Millipore Co., 0.45 µm in pore size). HPLC used a Cica-Merk RP-18 column (Kanto Chem. Co., Inc., Tokyo), eluted with a mixture of 0.5% formic acid and 100% methanol (1 : 4 in volume ratio) at a flow rate of 0.8 ml min^{-1}. The elution profile of flusulfamide at 285 nm is shown in Figure 2. The experiment was repeated three times.

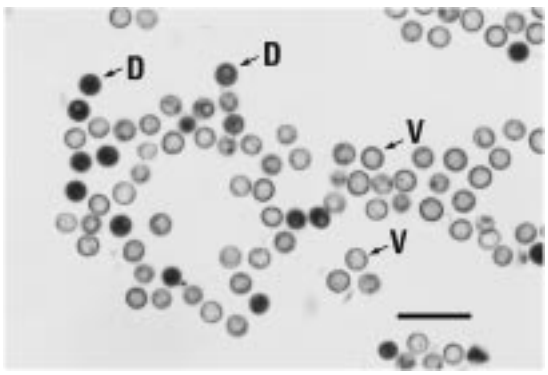

Figure 1. Evan's blue staining of flusulfamide-treated resting spores of *P. brassicae*. Resting spores with and without stained cytoplasm represented as dead (D) and viable (V) ones, respectively. Scale bar = 10 µm.

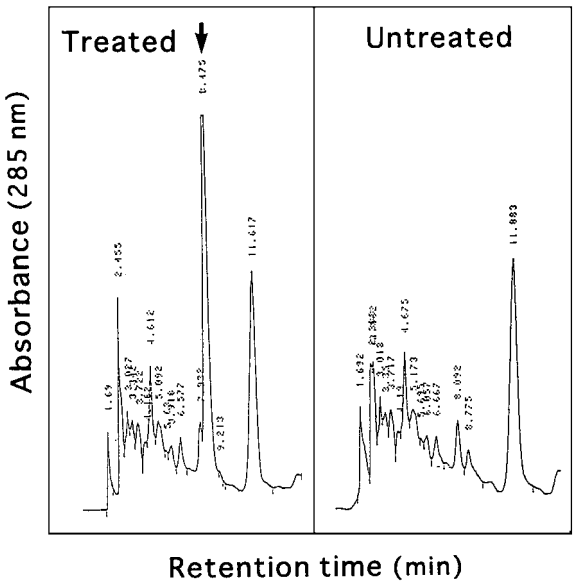

Figure 2. Detection profile of flusulfamide by HPLC. Arrow represents the flusulfamide peak.

Results

Effect of flusulfamide on the life cycle of P. brassicae

In treatments A and B (Table 1), root hairs of Chinese cabbage usually contained numerous mature (unevacuated) secondary zoosporangia after sowing in the 7 days treatment and partially or completely evacuated zoosporangia in the 14 days treatment. Slight swelling of roots was observed in the 21 days treatment. By 40 days after sowing, club formation was recorded for each treatment (Table 1). In treatment A, seedlings transplanted at 7 days after sowing were not affected by symptoms of clubroot. Seedlings transplanted at 7 days in treatment B were also not affected by the

disease. It was not possible to determine whether the suppression of clubroot in treatment A resulted from the presence of flusulfamide. Seedlings transplanted at 14 and 21 days in treatments A and B were severely affected by clubroot symptoms. Those seedlings grown continuously in soil containing flusulfamide were unaffected by the disease regardless of the time of transplant (treatment C).

Direct effect on resting spores

When Chinese cabbage seedlings were grown in soil containing resting spores treated directly with flusulfamide for either 2, 4 or 6 days, clubroot symptoms were completely suppressed (Table 2). Seedlings grown in soil containing resting spores untreated with flusulfamide were severely or moderately affected with clubroot.

Large numbers of root-hair infections were found in Chinese cabbage seedlings grown in solutions containing resting spores untreated with flusulfamide, while these were hardly observed in seedlings grown in solutions containing resting spores treated with flusulfamide for 2 or 4 days (Figure 3).

Germination tests of resting spores

When resting spores untreated with flusulfamide were incubated in root exudates of Chinese cabbage, the percentage of spore germination increased gradually and reached 27% at 9 days in the first experiment, and 26% at 14 days in the second experiment after incubation (Figure 4). Swimming primary zoospores were also observed. When flusulfamide-treated resting spores were incubated in exudates, the percentage of spore germination remained static for 9 days or 14 days after incubation and swimming zoospores were absent.

Table 2. Disease indices of Chinese cabbage grown in soil infected with flusulfamide-treated resting spores of *P. brassicae*[1]

Treatment	Days of treatment		
	2	4	6
First experiment			
Flusulfamide[2]	0	0	0
Untreated (buffer)[3]	67	35	58
Second experiment			
Flusulfamide	0	0	0
Untreated (buffer)	100	100	100

[1]Inoculum density: 1×10^4 spores g^{-1} dry soil.
[2]Resting spores at 3.5×10^9, treated with 7.0 ml buffer containing 2.0 µg ml^{-1} flusulfamide.
[3]Resting spores at 3.5×10^9, treated with 7.0 ml buffer without flusulfamide.

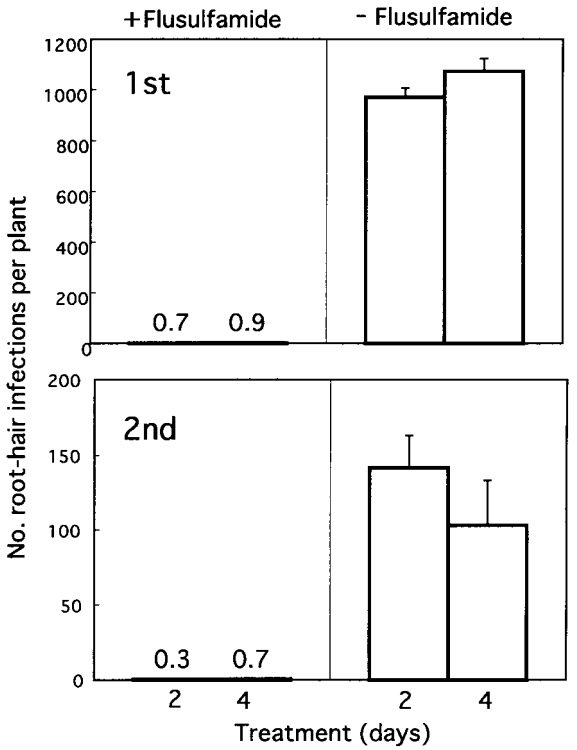

Figure 3. Numbers of root-hair infections in Chinese cabbage seedlings grown in culture solutions containing either flusulfamide-treated or untreated resting spores of *P. brassicae*. Resting spores were treated with flusulfamide or phosphate buffer for either 2 or 4 days.

Evan's blue staining assay

The percentages of resting spores with blue-stained cytoplasm (dead resting spores) were between 27% and 28% in both flusulfamide-treated and untreated suspensions, indicating that there were no differences between these two treatments (Table 3).

HPLC analysis

Flusulfamide, 3.9 µg, was obtained from resting spores (3.5×10^9) treated for 30 min (Figure 5). The amount of flusulfamide detected from resting spores increased with time of treatment, reaching a maximum (6.2 µg)

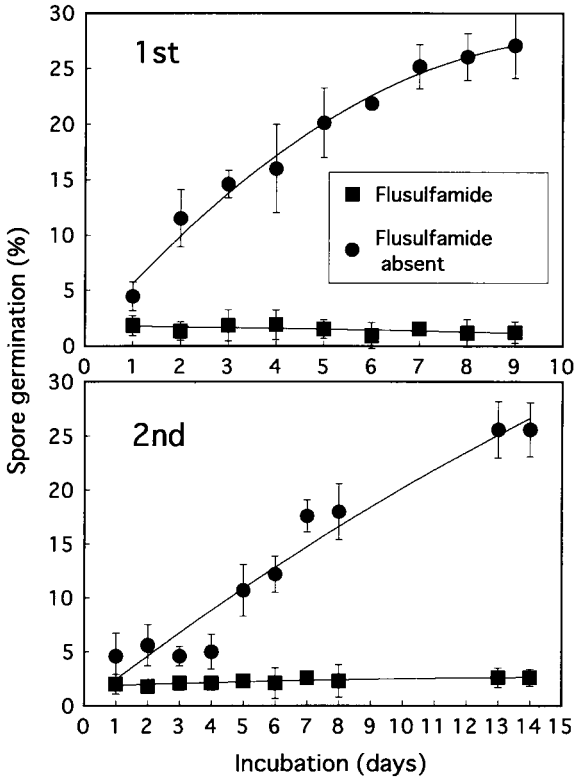

Figure 4. Percentage germination in flusulfamide-treated and untreated resting spores incubated for 1–9 or 1–14 days in root exudates from Chinese cabbage seedlings. Resting spores were treated with either flusulfamide or phosphate buffer for 2 days and then incubated in the root exudates.

Table 3. Viability of resting spores treated with flusulfamide

Treatment[1]	Dead spores (%)[2]
Flusulfamide	28.1 ± 2.2
Untreated buffer only	27.3 ± 2.3

[1]Resting spores at 3.5×10^9, treated with 7 ml buffer containing 2 µg ml^{-1} flusulfamide for 2 days or retained in buffer alone.
[2] Mean ± standard deviation.

after 24 h and thereafter remaining constant. These results showed that 63% of flusulfamide was adsorbed onto resting spores during the first 30 min. On the basis of these results, the maximum amount of flusulfamide which can be adsorbed per resting spore was calculated as 1.8×10^{-9} µg.

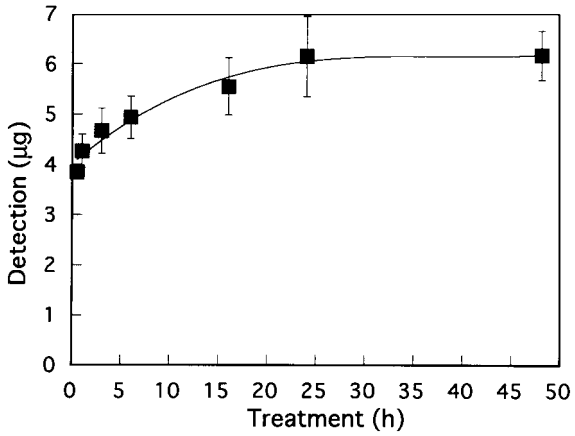

Figure 5. Quantitative detection of flusulfamide adsorbed onto resting spores by HPLC. Resting spores at 3.5×10^9 ml^{-1} were treated with 7 ml of buffer containing 2 µg ml^{-1} flusulfamide for 0.5–48 h.

Discussion

Chinese cabbage seedlings, grown in soil infested with *P. brassicae*, contained partially and completely evacuated secondary zoospores within their root hairs at 14 days after sowing, and often showed slight root swelling at 21 days, indicating that the pathogen established an infection in host cortical cells (Naiki and Dixon, 1987). When seedlings were transplanted into flusulfamide-treated soil (Table 1, treatment A), clubroot was not suppressed, suggesting that flusulfamide is ineffective against *P. brassicae* established in cortical cells. In the present work, it was not possible to elucidate whether flusulfamide had an effect on secondary zoosporangia within root hairs, or against secondary zoospores released into the soil. However, when Chinese cabbage was grown first in soil containing flusulfamide, clubroot was completely suppressed regardless of the time at which these seedlings were transplanted (Table 1, treatment C). These results suggest that *P. brassicae* is affected by flusulfamide at early stages in the life cycle.

The present study concentrated on the effects of flusulfamide on resting spores and their germination. The results obtained showed that prior treatment of resting spores with flusulfamide suppressed both root-hair infection and club formation in Chinese cabbage, indicating that flusulfamide acts directly on resting spores (Table 2, Figure 3). Additionally flusulfamide inhibited the germination of resting spores in root exudates of Chinese cabbage (Figure 4). The suppressive effect of

flusulfamide on root-hair infection and club formation possibly contributes to the inhibition of resting-spore germination. Inhibition of resting-spore germination may be the most critical effect of flusulfamide in the control of clubroot disease.

The action of flusulfamide on the viability of resting spores was investigated using Evan's blue staining assay. This assay tests the functional efficiency of plasma membranes and has been used successfully to assess plant cell survival after exposure to salt stress (Gaff and O'kong'o-ogola 1971; Taylor and West 1980) and the effects of fungicides on sporelings of *Ustilago nuda* (Newcombe and Thomas 1990). There are no previous reports on the application of this assay to study viability of *P. brassicae* resting spores. Results reported here confirmed that resting spores containing cytoplasm stained with Evan's blue clearly increased after heat treatment and repeated freezing and thawing in the preliminary tests (Tanaka et al., unpublished data). The staining assay showed that there were no differences in the percentage of dead cells in flusulfamide-treated and untreated suspensions of *P. brassicae* resting spores (Table 3). Furthermore flusulfamide caused no disruption of resting-spore integrity and possibly acts fungistatically.

Moderate residual activity in soil is an important property of soil-applied fungicides. Naiki and Dixon (1987) reported that residual activity was consistently strong for trichlamide and benomyl, but decreased with time for quintozene during the 2 weeks after application to sandy loam soil. Flusulfamide retains high efficacy against clubroot for at least 8 weeks after application in Kuroboku soil (volcanic black soil) (Takazawa, 1993). In the present work, HPLC analyses identified that flusulfamide was rapidly adsorbed and retained by resting spores (Figure 5). This adsorption property may relate to the mode of action of flusulfamide against *P. brassicae* in soil. Flusulfamide is firmly adsorbed to both soil particles (Takazawa, 1993) and to resting spores. Presumably, flusulfamide inhibits subsequent resting-spore germination or the viability of free swimming primary zoospores liberated from the resting spore. The entire fate of resting spores exposed to flusulfamide in soil is unknown. Further studies on the mechanisms by which flusulfamide inhibits germination of resting spores of *P. brassicae* are required in order that its full potential as a fungicide may be exploited.

Acknowledgements

The authors are grateful to Dr. H. Shimotori (Mitsui Chemical Co., Tokyo, Japan) for providing dust and wettable powder of flusulfamide.

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