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**Original Article**

# Tests for Evaluating the Side Effects of Chlorothalonil (TPN) and Spinosad on the Parasitic Wasp (*Aphidius colemani*)

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To better understand the side-effects of pesticides on nontarget insects, exposure and mortality assessment tests were performed with the parasitic wasp, *Aphidius colemani*. Quantities of chlorothalonil (TPN) and spinosad in individual wasps were determined using commercially available ELISA kits. TPN showed no side-effect on contact with dry film or oral administration. The lethal dosage of spinosad on plant leaves was estimated at ca. 0.2 $\mu$g/cm$^2$ for individual wasps, and was supported by the results of residual activity tests. Furthermore, a minimum of around 0.1 ng of spinosad was observed in dead individuals exposed to plant leaves treated with a lethal dosage of the posticide. 

*Keywords*: *Aphidius colemani*, contact exposures, mortality, ELISA, spinosad, chlorothalonil (TPN).

## INTRODUCTION

Many pesticides affect nontarget species in aquatic and terrestrial ecosystems. In aquatic ecosystem, the effects mainly depend on pesticide concentrations in water. However, the situation seems to be more complicated in terrestrial ecosystems. Migrant insects may move into agricultural fields and be exposed directly or indirectly to applied pesticides through contact or oral intake. Resident insects nearby agricultural fields may be exposed through spray drifts. Methods for laboratory tests, such as contact with dry films on glass plates and residue on leaves, have been developed for examining indirect exposure.[1–3] To detect the quantities of applied pesticides on individual aphids, an enzyme-liked immunosorbent assay (ELISA) has been employed.[4,5] The ELISA is more maneuverable than conventional methods, due to advantages such as rapid testing, small volume of assay samples and simplicity without any purification process. Some commercially available ELISA kits have been used for screening pesticide residues in crops,[6] for the evaluation of pesticide applications[4,7,8] and pesticide distributions in field environments.[9,10]

In this study, two pesticides on individuals of the parasitic wasp, *Aphidius colemani* (Hymenoptera: Braconidae; Aphidinae), were analyzed using commercially available ELISA kits, to better understand the side-effects of pesticides on the nontarget insects. Chlorothalonil (TPN), a widely used fungicide, was employed to investigate exposure-dosage without killing. Spinosad, a microbial insecticide, was used for examining side-effects on the wasps. Parasitic wasps were exposed to both pesticides by dipping or spraying, indirect contact with dry films, and oral administration. Furthermore, the lethal dosage of spinosad on plant leaves for *A. colemani* was also determined by ELISA and mortality assessment tests.

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## MATERIALS AND METHODS

### 1. Insects and Pesticides

Commercially available *Aphidius colemani* (Aphipar, Arysta Life Science Corp., Tokyo) was used in this study. Adults of *A. colemani* were removed from an Aphipar bottle, and newly emerged individuals were obtained after incubation at 23°C. Adult individuals 24 hr old were used for the tests.

Chlorothalonil (TPN) flowable (a.i. 40%, Kumiai Chemical Industry Co., LTD., Tokyo, Japan), which is a widely used fungicide, and spinosad WP (a.i. 25%, Kumiai Chemical Industry Co., LTD., Tokyo, Japan), which is a microbial insecticide composed primarily of spinosyn A and spinosyn D, as insect control agents, were used for exposure tests. To use registered applicative concentrations, pesticides were diluted to 1 : 1000 (400 mg/l) for TPN and to 1 : 2500 (100 mg/l) for spinosad with distilled water. Further diluted solutions were also used in mortality assessment tests.

## 2. Exposure and Mortality Assessment Tests

### 2.1. Dipping and spraying

A cohort (approx. 50 individuals) of *A. colemani* was dipped into TPN solution (400 mg/l) for 10 sec. After drying, each individual was stored in a micro tube at −20°C for ELISA.

The TPN solution (400 mg/l) and spinosad solution (100 mg/l) were sprayed directly on a cohort (approx. 50 individuals) of *A. colemani* at a rate of 20 l/10a, using box-type (0.3 $m^2$ : 0.55 m×0.55 m) spray equipment (DIK-7321, Daiki Rika Kogyo Co., Ltd, Tokyo, Japan) with an air pressure of 0.3 $kgf/cm^2$ and a turn table speed of 7 rpm. After drying, the wasps were also stored individually in micro tubes at −20°C for ELISA.

### 2.2. Exposure to dry films on glass plates

Dry films on glass plates (13 cm×13 cm) were prepared by spraying the TPN solution (400 mg/l) and spinosad solution (100 mg/l) at a rate of 20 l/10a. Furthermore, to analyze pesticide deposits on glass plates as dry films, five slide-glasses (2.6 cm×7.6 cm) were sprayed with TPN solutions (400 mg/l and 40 mg/l) and spinosad solutions (100 mg/l and 10 mg/l) at a rate of 20 l/10a (2 $\mu l/cm^2$). Water without pesticide was also sprayed onto the slide-glasses, and then the amount of water deposited was calculated from measurements of weight.

For exposing individual insects to pesticides, contact treatment with the dry films on glass plates[3] was performed. An aluminum frame (1 cm thick) was sandwiched with two glass plates whose surfaces were sprayed facing inwards.[2,3] Ten wasps were put into each test unit with TPN (400 mg/l) or spinosad (100 mg/l) dry films, respectively. Three test units were used as one test plot. All test plots were incubated at 23°C with 16L–8D. After 24-hr exposure, individual wasps were collected and stored in micro tubes at −20°C for ELISA.

Mortalities[3] of *A. colemani* were determined after 24-hr exposure. Mortality was corrected according to Abbott.[11]

### 2.3. Oral administration

Oral treatment of *A. colemani* was conducted with the TPN solution (400 mg/l) and spinosad solution (100 mg/l). The test unit used was the same as described above except for dry films. A glass tube containing a pesticide solution without any additives was attached to a hole of an aluminum frame. Ten wasps were put into each test unit in triplicate.

Furthermore, *A. colemani* were treated via both oral administration and exposure to dry films on glass plates in the test units with dry films and the pesticide solutions of TPN (400 mg/l) and spinosad (100 mg/l). All test plots were incubated at 23°C with 16L–8D. Wasps exposed for 24 hr were collected and stored individually in micro tubes at −20°C for ELISA.

Mortalities[3] of *A. colemani* were determined after 24-hr exposure. Mortality was corrected according to Abbott.[11]

### 2.4. Exposure to sprayed plant leaves

Strawberry (cv. Nyoho) plants with 9 leaves, kidney bean (cv. Kintoki) plants with a second true leaf and cucumber (cv. Satsukimidori) plants with 5 leaves planted in vinyl pots were sprayed with each solution of spinosad (0.8–100 mg/l) by using hand sprayers. Application rates were 33 ml/pot (approx. 240 l/10a) for strawberry, 16 ml/pot (approx. 267 l/10a) for kidney bean and 30 ml/pot (approx. 83 l/10a) for cucumber. After drying, uniformly sprayed plant leaves were collected for exposure tests. Each leaf and more than 10 wasps were put into a test unit such as an acrylic pipe (9 cm in diameter and 3.5 cm height) sealed at both ends with glass disks. Five test units were used as one test plot. All test plots were incubated at 23°C with 16L–8D. Mortalities of the wasps were recorded after 24-hr and 48-hr exposure. Dead and/or living individuals were collected from the test plots after the exposure on cucumber leaves and stored in micro tubes at −20°C for ELISA. Furthermore, about 10–20 leaf disks (1.5 cm in diameter) were collected from plants sprayed with each concentration. Leaf disks were stored in plastic bags at −20°C for ELISA.

### 2.5. Residual activity

Sets of strawberry (cv. Nyoho) plants in a vinyl house were sprayed with a 50 mg/l spinosad solution at a rate of 134 l/10a using a handy type air-compressing sprayer. Compound leaves were collected from 5, 10, 20, 30 and 40 days after the application. Mortalities of *A. colemani* were examined using the collected leaves. An aluminum frame (1 cm thick) sandwiched with glass plates (13 cm×13 cm) was used as a test unit. Ten individuals and one compound leaf were put into the test unit. Five test units were prepared as one test plot. All test plots were incubated for 48 hr at 23°C with 16L–8D. Mortalities of the wasps were determined after 24-hr and 48-hr exposure as described above.

Furthermore, pesticide residues in the leaves collected from 5, 10, 20, 30 and 40 days after the application were analyzed with LC-MS by a contract laboratory (Japan Analytical Chemistry Consultants Co., LTD). Though spinosad is a mixture of spinosyn A and spinosyn D, only spinosyn A was analyzed as the major active ingredient for spinosad. A 92.7% pure spinosyn A (Hayashi Pure Chemicals, Osaka, Japan) was used as a standard in the analysis.

## 3. ELISA

Fifty microliters of methanol was put into a micro tube containing an individual wasp. The micro tube was vortexed for 1 min, and then 0.95 ml of attached diluent in the ELISA kit was added as an extract. The extract was subjected to analysis.

Deposits of pesticides on plant leaves were also analyzed. A 10 ml volume of methanol was put into a plastic bag containing leaf disks. The bag was shaken vigorously by hand for 1 min, and then the extract was collected for analysis.

Furthermore, to analyze the pesticide quantity of dry film on glass plate, each slide-glass (19.76 $cm^2$) with dry film was put into a 50 ml tube, and then 10 ml of methanol was added. After approx. 1 min shaking, the extract was collected for

analysis.

The extracts were analyzed using ELISA kits (RaPID assay kit, Strategic Diagnostics Inc., USA). The detection limit of the TPN assay kit is 0.07 $\mu$g/l. As the spinosad assay kit does not differentiate between the compounds in spinosad, the detection limits are 0.02 $\mu$g/l for spinosyn A, 0.07 $\mu$g/l for spinosyn D and 0.03 $\mu$g/l for spinosad (mixture of spinosyn A and spinosyn D). The working ranges of kits were 0.1–5.0 $\mu$g/l for TPN and 0.05–1.0 $\mu$g/l for spinosad. The extracts were diluted with diluents of the kits into the working ranges. The assays were performed according to the protocols supplied. The determination limit for an individual wasp was 0.3 ng for TPN and 0.05 ng for spinosad. Furthermore, the determination limit of spinosad on plant leaves was 0.3 ng per $cm^2$ for strawberry and 0.2 ng per $cm^2$ for bean and cucumber. At least two diluted extracts were assayed. When one nanogram of TPN and 0.2 ng of spinosyn A were added to individual wasps, recoveries of the pesticides by the kits were 115% for TPN and 99% for spinosyn A.

## RESULTS

### 1. *Direct Exposures through Dipping and Spraying*

Quantities of TPN and spinosad on individuals of *A. colemani* exposed directly by dipping and spraying are shown (No. 1, 2 and 6) in Fig. 1. When the results of direct exposure were compared, the average quantity of TPN on the wasps was found to be larger for spraying (No. 2) than dipping (No. 1). The minimum quantities on the individuals were 5.5 ng of TPN by dipping (No. 1), 25.1 ng of TPN by spraying (No. 2) and 3.63 ng of spinosad by spraying (No. 6).

### 2. *Deposits on Glass Plates as Dry Films*

Pesticide deposits on glass plates applied by spraying were examined first. When water was applied to slide-glasses at a rate of 2 $\mu$l/$cm^2$, the water deposit was ca. 2 mg/$cm^2$. This was the same value as the theoretical one. However, TPN was 1.6 (0.125 $\mu$g/$cm^2$) and 2.5 (2.024 $\mu$g/$cm^2$) times higher than theoretical values (0.08 and 0.80 $\mu$g/$cm^2$) and spinosad was 1.4 (0.028 $\mu$g/$cm^2$) and 2.0 (0.395 $\mu$g/$cm^2$) times higher than theoretical levels (0.02 and 0.20 $\mu$g/$cm^2$), respectively, when TPN solutions (40 and 400 mg/l) and spinosad solutions (10 and 100 mg/l) were sprayed at the same rate on the glass. Thus, quantities of both dry films used for contact exposure tests were determined as ca. 2.0 (TPN) and ca. 0.4 $\mu$g/$cm^2$ (spinosad).

### 3. *Contact with Dry Films and Oral Administration*

Mortalities of *A. colemani* after exposure to dry films (TPN: ca. 2.0 $\mu$g/$cm^2$, spinosad: ca. 0.4 $\mu$g/$cm^2$) on glass plates, oral administration and both treatments are shown in Table 1. A low rate of mortality was observed in the test plots for not only TPN treatments but also controls. Thus, TPN seemed to have almost no toxic effect on *A. colemani* after 24-hr exposure. On the other hand, spinosad had a marked effect on the wasps with all treatments at 24 hr.

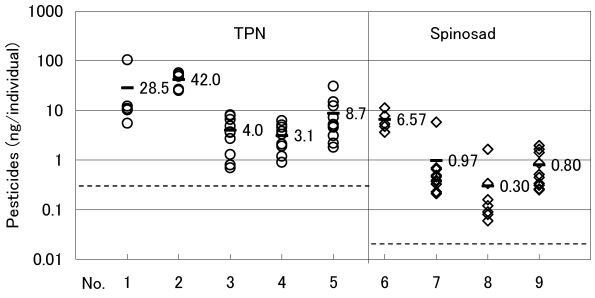


**Fig. 1.** Quantities of TPN (○, No. 1–5) and spinosad (◇, No. 6–9) on individuals of *A. colemani* exposed by dipping (No. 1, n=5), spraying (No. 2 and No.6, n=5), contact with dry films (No. 3 and No. 7, n=10), oral administration (No. 4 and No. 8, n=10) and both treatments (No. 5 and No. 9, n=10). Each of horizontal bars (–) indicates an average quantity (ng) of pesticide on individuals. A TPN solution (400 mg/l) and spinosad solution (100 mg/l) were used in the tests. Broken lines indicate the determination limit (<0.3 ng for TPN and <0.05 ng for spinosad) in ELISA.

**Table 1.** Mortalities of *A. colemani* after contact exposure to dry films on glass plates, oral administration and both treatments with TPN and spinosad

| Pesticide | Concentration used (mg/l) | Treatment[a)] | Mortality at 24 hr exposure (%) | Corrected[b)] mortality (%) |
|---|---|---|---|---|
| TPN | 400 | Contact | 30.0 | 8.7 |
| | | Oral | 0.0 | 0.0 |
| | | Both | 3.3 | 0.0 |
| Spinosad | 100 | Contact | 100.0 | 100.0 |
| | | Oral | 93.3 | 91.3 |
| | | Both | 100.0 | 100.0 |
| Control | – | – | 23.3 | 0.0 |

[a)] Approx. 2.0 $\mu$g/$cm^2$ of TPN and 0.4 $\mu$g/$cm^2$ of spinosad were determined on the dry films of each pesticide.
[b)] Mortalities (n=30) were corrected with control values according to Abbott.[11)]

Quantities of TPN and spinosad on the individuals of *A. colemani* after contact with the dry films on glass plates, oral administration and both treatments are also shown (No. 3–5 and 7–9) in Fig. 1. The minimum quantities of TPN on individuals were 0.7 ng by contact exposure (No. 3), 0.9 ng by oral exposure (No. 4) and 1.8 ng by both treatments (No. 5). Minimum quantities of spinosad on individuals were 0.21 ng by contact exposure (No. 7), 0.06 ng by oral exposure (No. 8) and 0.25 ng by both treatments (No. 9). Thus, the minimum quantities by contact and oral exposure amounted to almost the same level after both treatments. Furthermore, average

**Table 2.** Mortalities of *A. colemani* after contact exposure to plant leaves sprayed with spinosad

| Plant leaf | Applied concentration (mg/l) | Deposit of spinosad on leaf ($\mu$g/cm$^2$) | Corrected[a] mortality at 24 hr (%) | Corrected[a] mortality at 48 hr (%) |
|---|---|---|---|---|
| Strawberry | 100 | 1.145 | 95.8 | 100.0 |
| | 5 | 0.022 | 3.3 | 17.2 |
| | 1 | 0.003 | 0.0 | 0.0 |
| | 0 | <0.0003 | 0.0 (0.0)[b] | 0.0 (6.7 )[b] |
| Kidney bean | 83.3 | 2.108 | 96.9 | 100.0 |
| | 8.3 | 0.219 | 90.0 | 98.2 |
| | 0.8 | 0.023 | 0.0 | 8.9 |
| | 0 | <0.0002 | 0.0 (3.3)[b] | 0.0 (10.0)[b] |
| Kidney bean | 100 | 2.370 | 90.0 | 97.8 |
| | 5 | 0.025 | 4.0 | 0.0 |
| | 1 | 0.004 | 0.0 | 21.4 |
| | 0 | <0.0002 | 0.0 (0.0)[b] | 0.0 (10.9)[b] |
| Cucumber | 100 | 0.930 | 100.0 | 100.0 |
| | 20 | 0.177 | 48.3 | 86.7 |
| | 10 | 0.044 | 3.3 | 10.0 |
| | 5 | 0.022 | 3.3 | 0.0 |
| | 0 | <0.0002 | 0.0 (0.0)[b] | 0.0 (10.0)[b] |

[a] Mortalities (n=30–72/test plot) were corrected with control values according to Abbott.[11]
[b] Each number in parentheses indicates control mortality (%).

quantities by contact and oral exposure were also roughly similar to the results of both treatments, respectively (Fig. 1, No. 3–5 and No. 7–9).

### 4. *Exposure to Spinosad Sprayed Leaves*

Corrected mortalities of *A. colemani* 24 and 48 hr after exposure to spinosad sprayed leaves and the deposits of spinosad on the leaves are shown in Table 2.

Quantities of spinosad on individuals of *A. colemani* after exposure to applied cucumber leaves are shown in Fig. 2. Quantities of spinosad on dead individuals after 24-hr exposure on the leaves sprayed with the 100 mg/l solution were 1.93 (average) and 0.19 ng/individual (minimum). Dead individuals obtained after 24-hr and 48-hr exposure on leaves which were sprayed with the 20 mg/l solution showed almost the same averages (0.25 and 0.24 ng/individual) and minimum quantities (0.08 and 0.1 ng/individual). The average and minimum quantities on dead individuals after 48-hr exposure on the leaves sprayed with the 10 mg/l solution were 0.15 and 0.08 ng/individual, and all living individuals showed <0.05 ng/individual. Both living and dead individuals, which were exposed for 48 hr on leaves sprayed with the 5 mg/l solution and non-treated leaves as a control, all showed <0.05 ng of

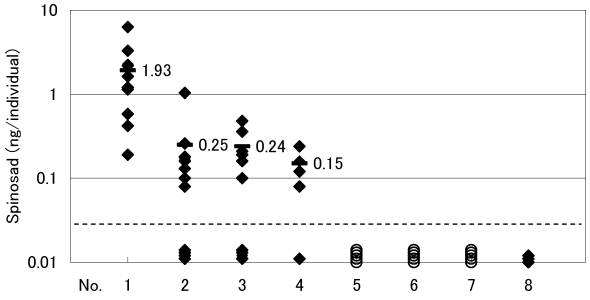


**Fig. 2.** Quantities of spinosad on individuals of *A. colemani* after contact exposure to sprayed cucumber leaves. Dead individuals after 24 hr on leaves sprayed with the 100 mg/l solution (No. 1: ◆, n=10) and 20 mg/l solution (No. 2: ◆, n=13). Dead individuals after 48 hr on leaves sprayed with the 20 mg/l solution (No. 3: ◆, n=11). Dead (No. 4: ◆, n=5) and living (No. 5: ○, n=5) individuals after 48 hr on leaves sprayed with the 10 mg/l solution. Individuals alive after 48 hr on leaves sprayed with the 5 mg/l solution (No. 6: ○, n=5). Alive (No. 7: ○, n=5) and dead (No. 8: ◆, n=3) after 48 hr on control leaves. Each of horizontal bars (−) indicates an average quantity (ng) of spinosad on individuals. A broken line indicates the determination limit (<0.05 ng/individual) in ELISA.

**Table 3.** Residual toxicity to *A. colemani* on strawberry leaves collected after spinosad application

| Days after application[a)] | Corrected[b)] mortality at 24 hr (%) | Corrected[b)] mortality at 48 hr (%) | Residue of spinosyn A ($\mu$g/g) | Estimation[c)] of deposit on leaf surface ($\mu$g/cm$^2$) |
|---|---|---|---|---|
| 5 | 74.4 | 95.1 | 38.8 | 0.194 |
| 10 | 62.0 | 93.6 | 28.8 | 0.144 |
| 20 | 27.7 | 55.9 | 23.0 | 0.115 |
| 30 | 4.3 | 0.0 | 11.4 | 0.057 |
| 40 | 4.0 | 0.0 | 9.2 | 0.046 |

[a)] Strawberry leaves collected after the application of spinosad (50 mg/l) were used for each test plot.
[b)] Mortalities (n=50) were corrected with control values according to Abbott.[11)]
[c)] Residue of spinosyn A in strawberry leaves ($\mu$g/g) was converted to $\mu$g/cm$^2$, as the average weight of a leaf was 0.01 g/cm$^2$.

spinosad.

*5. Residual Activity of Spinosad to A. colemani*

Corrected mortalities of *A. colemani* after 24-hr and 48-hr exposure on the strawberry leaves are shown in Table 3. The residues of spinosyn A in the leaves 5–40 days after the spinosad application are also shown in Table 3. Furthermore, deposits of spinosyn A on one side of the leaf ($\mu$g/cm$^2$) were estimated by calculations with the average weight of leaf (0.01 g/cm$^2$) and the results of residue in strawberry leaves ($\mu$g/g).

## DISCUSSION

Quantities of pesticides on individuals of *A. colemani* mainly depended on the concentrations that the wasps were exposed to. Ratios of average quantities of TPN and spinosad on the wasps were 6 : 1 (42.0 : 6.57 ng/individual) for spraying (Fig. 1, No. 2 and No. 6) and 4 : 1 (4.0 : 0.97 ng/individual) for contact with dry films (Fig. 1, No. 3 and No. 7). These values were similar to the ratio (4 : 1) of the applied concentrations (TPN: 400 mg/l and spinosad: 100 mg/l). The results indicate that almost the same quantities of pesticide were obtained from individuals of *A. colemani* even if other pesticides were applied at the same volume and concentrations. On the other hand, ratios were approx. 10 : 1 for oral intake (3.1 : 0.30 ng/individual) (Fig. 1, No. 4 and No. 8) and two treatments such as contact and oral administration (8.7 : 0.80 ng/individual) (Fig. 1, No. 5 and No. 9). This difference may depend on the toxic activity of the pesticide. That is, the insecticide killed *A. colemani* before enough was ingested or the wasps evaded the insecticide.

Based on the mortality assessment tests, more than 90% of individuals were dead on the plant leaves with approx. 0.2 $\mu$g/cm$^2$ of spinosad (Table 2). Therefore, the lethal dosage of spinosad on plant leaves was estimated as more than 0.2 $\mu$g/cm$^2$ for *A. colemani*. This level was 1/5 to 1/10 the amount in ordinary use (ca. 1–2 $\mu$g/cm$^2$) (Table 2). On the other hand, the minimum quantity of spinosad on individual wasps after contact with plant leaves with a lethal dosage of spinosad was around 0.1 ng (Fig. 2). Thus, the lethal dosage of spinosad on individuals was estimated at more than 0.1 ng for *A. colemani*. The lethal dosages on plant leaves and on the wasps may change in accordance with the toxic activity of the pesticide used.

Furthermore, only around 0–10% of individual wasps were dead on the leaves with 0.02–0.04 $\mu$g/cm$^2$ of spinosad (Table 2). On the other hand, in contact tests with dry films on glass plates, over 50% of individuals were killed by around 0.008 $\mu$g/cm$^2$ of spinosad (sprayed with 4.02 mg/l solution for dry films).[12)] The ratio of dry film area against the inside surface of the test unit is approx. 73%, while the ratio of plant leaves per total surface of the test unit inside is approx. 34% for strawberry and kidney bean and 40% for cucumber. This may be why in the contact test, individuals on the leaves were more tolerant than those on the dry films. As the plants were sprayed with a pesticide solution of ordinary concentration, the contact exposure test on plant leaves seemed to be more practical for the evaluation of side-effects on the wasps.

Based on the residual activity tests with spinosad, the activity toward *A. colemani* on strawberry leaves was preserved for over 20 days but less than 30 days after the application (Table 3). Though 30-day and 40-day leaf samples had approx. 10 $\mu$g/g of spinosyn A, the leaves lost the toxic activity. Assuming that residue of spinosyn A existed on both sides of the leaf, the estimated deposit was maximally 0.05 $\mu$g/cm$^2$ on one surface. The amount of spinosyn A, however, might be too small to have a toxic effect on the wasps. The spinosad residues in 5-day and 10-day samples killed more than 90% of individual wasps. At that time, estimated deposits of spinosyn A were around 0.19 and 0.14 $\mu$g/cm$^2$ on the one side of the leaf. This also supported that the lethal dosage of spinosad on plant leaves is more than 0.2 $\mu$g per cm$^2$ for individuals of *A. colemani*.

In conclusion, the fungicide TPN had no effect on *A. colemani*. In contrast, the insecticide spinosad, at concentrations of more than 0.2 $\mu$g/cm$^2$ on plant leaves, appeared to have a

potential side-effect on the wasps. However, only individuals that came into contact with the pesticide on the leaves were affected. In the semi-field test, almost no effect in a cohort of *A. colemani* was observed on young plants of cucumber after application of spinosad (100 mg/l) in a vinyl house (Izono, personal communication). Some individuals may have died from a lethal dosage of spinosad, but most of the cohort avoided the lethal effect and/or survived on new leaves. That is, the population of wasps in the field test may be more tolerant to the potential side-effects than the individuals in laboratory tests. However, determination of the lethal dosage of a pesticide on plant leaves is useful as a first step to evaluating the side-effects of pesticides on non-target species.

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