

## **Aged-residue method for evaluating toxicity of plant protection products to *Stethorus punctillum* (Weise) (Coleoptera: Coccinellidae)**

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**Abstract:** *Stethorus punctillum* (Weise) (Coleoptera: Coccinellidae) is known as an obligate predator of spider mites. Currently there are no widely recognised laboratory methods for testing the effects of plant protection products (PPP) to this species. Here we present a method for evaluating the toxicity of PPP under extended laboratory conditions or as a persistence (aged residue) study, combining field applications with laboratory bioassays. *S. punctillum* larvae were exposed to treated apple leaf disks and their development through to pupation and adult emergence monitored. An assessment of reproduction was also performed. Example data corresponding to control, methoxyfenozide and fenoxycarb treatments are presented.

**Keywords:** *Stethorus punctillum*, potential effects of plant protection products

### **Introduction**

All known species of the genus *Stethorus* (Weise) (Coleoptera: Coccinellidae) are predators of tetranychid spider mites (Scriven and Fleschner, 1960, Rott and Ponsonby, 2000). *Stethorus punctillum* is known as spider mite natural enemy in fruit orchards (Ivancich, 1974) and commercially available as biological control agent.

Currently there are no widely recognised laboratory methods for testing the effects of plant protection products (PPP) to this species. Here we present a method to assess potential negative effects of plant protection products to *Stethorus* sp. under laboratory conditions. The presented method is based on the IOBC-method established for *Coccinella septempunctata* by Schmuck *et al.* (2000), which is used for regulatory purposes in the EU (Commission Directive 96/12/EC amending Council Directive 91/414/EEC, Barrett *et al.* 1994, Candolfi *et al.* 2001).

### **Materials and methods**

We determined the potential effects of Intrepid<sup>®</sup> 2F (trademark of Dow AgroSciences LLC, active ingredient methoxyfenozide 23.7 % w/w) and Insegar<sup>®</sup> DG (trademark of Syngenta, active ingredient fenoxycarb 25 % w/w) applied on a natural substrate (apple trees) on *Stethorus punctillum* (Weise) (Coleoptera: Coccinellidae). Potted apple trees (*Malus domestica* Borkh. var. Spartam) were held and treated under field conditions, and thereafter placed under a rain protection in the field. Bioassays exposing *Stethorus punctillum* under laboratory conditions to treated apple tree leaves were started immediately and 7 days after treatment application. Pre-imaginal mortality and the effect on reproduction were used as toxic endpoints.

### ***Stethorus punctillum* culture**

*S. punctillum* beetles were obtained from Benfried International b.v., Den Hoorn, The Netherlands, and cultured as described by Walters (1974) providing *Tetranychus urticae* ad libitum as food. *T. urticae* was reared in a separate room on bean plants (*Phaseolus vulgaris* L.) at approximately 25 °C. Fresh bean plants were added to the culture 2 to 3 times per week in order to have a continuous supply of spider mites.

### **Treatment application**

The present study was designed as an aged residue test. The treatments were applied under field conditions to potted apple trees (*Malus domestica* Borkh., var Spartan) held under field conditions and watered regularly until test start. No chemical plant protection measures were carried out since start of the growing season, i.e. for 3 months before experimental starting. At treatment application the trees were at BBCH 74.

The treatments performed were a control (deionised water), methoxyfenozide at 48 g a.i./ha (10% of the maximum use rate), methoxyfenozide at 480 g a.i./ha (100% of the maximum use rate), methoxyfenozide at 960 g a.i./ha (200% of the maximum use rate), and fenoxycarb at the recommended field rate of 480 g /ha (equivalent to 120 g a.i./ha). Each treatment was applied in deionised water on a tree row of 7 potted trees of approximately 1.65 m height. The trees were located simulating a continuous canopy at 0.6 m distance each from the other (row length of 4.2 m). A 5 m distance between the rows was assumed for calculation of the application rate. The applications were performed with a Birchmeier M125 back-pack-sprayer. The application volume was determined prior to application to be 428.6 L/ha, guaranteeing an optimal wetting of the foliage but avoiding run-off. Each tree row was sprayed twice (once per row side). After treatment application and until the start of the 2<sup>nd</sup> bioassay, the apple trees were held under rain cover. During this period, temperature ranged from 16.0 to 32.5 °C and the relative air humidity from 36 to 98 %.

### **Bioassays**

Bioassays consisted of an exposure and a reproduction phase and were started immediately and 7 days after treatment application. After application of the apple trees, treated leaves were detached immediately after drying of the applied solutions and used as substrate for the exposure of beetle larvae during the 1<sup>st</sup> bioassay. For the 2<sup>nd</sup> bioassay, treated leaves were detached 7 days after application and used immediately for the corresponding exposure.

Exposure phase test units consisted of a leaf disk (approximate diameter: 30 mm) cut from a treated leaf, resting upside-up on agar (1 % w/v) in a transparent, circular plastic box of Polystyrol (diameter 3.9 cm, height 3.0 cm). Into each test unit, one *Stethorus punctillum* larva hatched no more than 48 hours ago was transferred together with food (*Tetranychus urticae*). The test units were then placed top-side down over a mesh fixed on a frame. For each treatment and bioassay, 40 replicates were set up. The status (normal, moribund, missing, pupae, or dead) of the individuals was inspected daily until day 15 of the corresponding bioassay or until hatching of adults. After day 15, the observations were done every 2 to 3 days. The hatched beetles from each treatment were pooled in transparent 1.3 L plastic containers and fed regularly every 2 to 3 days until starting of the reproduction phase.

Reproduction phase test units consisted of a circular plastic box of Polystyrol (diameter 3.3 cm, height 1.0 cm). Into each test unit, one *Stethorus punctillum* adult was transferred together with food (*T. urticae*). During one week, beetles were transferred every 2 to 3 days to new boxes with fresh food, so that a total of 3 consecutive egg laying samples were obtained by beetle. After removal of the beetles, the eggs in each box were counted. After beginning of larvae hatching, i.e. 2 to 3 days after egg laying, the number of larvae hatched per box was counted during 3 consecutive days removing the hatched larvae on each observation day.

After the reproduction phase, all adult beetles which did not lay eggs were sexed (beetles which laid eggs were obviously females).

During the bioassays, temperatures ranged from  $25 \pm 2$  °C and the relative air humidity from  $75 \pm 15$  %. The light intensity was 1500 to 3000 lux with a photoperiod of 16L: 8D.

Cumulative pre-imaginal mortality was determined for the exposure phase. For the reproductive phase, the average number of eggs per female per day and the hatching rate of eggs were calculated. Mortality was analysed by Fisher's Exact tests. Eggs laid per female and hatching rate were analysed by ANOVA followed by Dunnett t-test.

## Results

Control mortality in the present study was 2.5 and 7.5 %. Pre-imaginal mortality in the fenoxycarb treatment was 80 % and statistically significant different from the control (Fisher Exact test:  $p < 0.001$ ). In this treatment, typical IGR effects were also observed with 20 % of crippled (abnormal) larvae (Table 1).

The pre-imaginal mortality in the methoxyfenozide treatments during the 1st bioassay varied between 22.5 and 51.3 % (48 and 960 g a.i./ha methoxyfenozide treatments, respectively) and was statistically significantly different to the control at all the application rates (Fisher Exact Tests:  $p < 0.05$ ). During the 2nd bioassay, after 7 days of ageing, mortality varied between 4.8 and 45.0 % (48 and 960 g a.i./ha treatments, respectively). A statistically significant difference to the control was observed at the application rate of 960 g a.i. methoxyfenozide/ha (Fisher Exact Test:  $p < 0.001$ ) but not at 48 and 480 g a.i. methoxyfenozide/ha (Fisher Exact Tests:  $p = 0.477$  and  $p = 0.662$ , respectively) (Table 1).

Table 1. Hatching of adults and pre-imaginal mortality in the 1<sup>st</sup> and 2<sup>nd</sup> bioassay exposing *Stethorus punctillum* to methoxyfenozide and fenoxycarb.

| Treatment                     | Bioassay 1<br>(0 days after<br>treatment application) |                           |                  |    | Bioassay 2<br>(7 days after<br>treatment application) |                  |    |
|-------------------------------|---|---------------------------|------------------|----|---|------------------|----|
|                               | Hatched<br>adults<br>(%)                              | Crippled<br>larvae<br>(%) | Mortality<br>(%) | n  | Hatched<br>adults<br>(%)                              | Mortality<br>(%) | n  |
| Control                       | 97.5  | 0.0                       | 2.5              | 40 | 92.5  | 7.5              | 40 |
| 48 g a.i./ha methoxyfenozide  | 77.5  | 0.0                       | 22.5 *           | 40 | 95.2  | 4.8              | 42 |
| 480 g a.i./ha methoxyfenozide | 82.9  | 0.0                       | 17.1 *           | 41 | 92.5  | 7.5              | 40 |
| 960 g a.i./ha methoxyfenozide | 48.7  | 0.0                       | 51.3 *           | 39 | 55.0  | 45.0 *           | 40 |
| 120 g a.i./ha fenoxycarb      | 0   | 20.0                      | 80.0 *           | 40 | -   | -                | -  |

\* Statistically significant different from the control (Fisher Exact Test:  $p < 0.05$ )

Table 2. Mean number of eggs laid per female during the reproduction phase of the 1<sup>st</sup> bioassay exposing *Stethorus punctillum* to methoxyfenozide (A, B, C refers to egg laying periods of 2 to 3 days respectively).

| Treatment                     |           | Eggs A | Eggs B | Eggs C | Eggs per female per day <sup>ns</sup> |
|-------------------------------|-----------|--------|--------|--------|---------------------------------------|
| Control                       | Mean      | 20.4   | 22.2   | 31.2   | 10.5                                  |
|                               | N         | 21     | 21     | 21     | 21                                    |
|                               | Std. Dev. | 8.0    | 6.3    | 8.1    | 1.5                                   |
| 48 g a.i./ha methoxyfenozide  | Mean      | 17.6   | 19.8   | 26.8   | 9.2                                   |
|                               | N         | 14     | 14     | 14     | 14                                    |
|                               | Std. Dev. | 8.6    | 9.1    | 11.2   | 3.2                                   |
| 480 g a.i./ha methoxyfenozide | Mean      | 17.5   | 16.9   | 24.9   | 8.5                                   |
|                               | N         | 17     | 17     | 17     | 17                                    |
|                               | Std. Dev. | 10.6   | 8.3    | 11.7   | 3.6                                   |
| 960 g a.i./ha methoxyfenozide | Mean      | 18.3   | 18.4   | 29.4   | 9.4                                   |
|                               | N         | 10     | 10     | 10     | 10                                    |
|                               | Std. Dev. | 7.0    | 12.4   | 6.8    | 2.3                                   |

ns – no statistically significant differences between the treatments (ANOVA:  $p = 0.145$ )

Table 3. Hatching rate of larvae from eggs laid during periods A, B, C, of the reproduction phase of the 1<sup>st</sup> bioassay exposing *Stethorus punctillum* to methoxyfenozide.

| Treatment                     |           | Hatching A | Hatching B | Hatching C | Hatching (mean) |
|-------------------------------|-----------|------------|------------|------------|-----------------|
| Control                       | Mean      | 0.72       | 0.84       | 0.84       | 0.80            |
|                               | N         | 21         | 21         | 21         | 21              |
|                               | Std. Dev. | 0.22       | 0.20       | 0.23       | 0.14            |
| 48 g a.i./ha methoxyfenozide  | Mean      | 0.81       | 0.92       | 0.79       | 0.84            |
|                               | N         | 14         | 14         | 14         | 14              |
|                               | Std. Dev. | 0.16       | 0.38       | 0.27       | 0.17            |
| 480 g a.i./ha methoxyfenozide | Mean      | 0.73       | 0.72       | 0.81       | 0.76            |
|                               | N         | 15         | 16         | 15         | 14              |
|                               | Std. Dev. | 0.22       | 0.32       | 0.29       | 0.21            |
| 960 g a.i./ha methoxyfenozide | Mean      | 0.91       | 0.93       | 1.00       | 0.97            |
|                               | N         | 10         | 9          | 10         | 9               |
|                               | Std. Dev. | 0.23       | 0.17       | 0.13       | 0.13            |

ns – no statistically significant differences between the treatments (ANOVA:  $p = 0.033$  / Dunnett t tests between each test item treatment and control  $p > 0.05$ )

The reproduction was assessed for the beetles hatched in the control and methoxyfenozide treatments during the 1<sup>st</sup> bioassay. A mean of 10.5, 9.2, 8.5, and 9.4 eggs per female per day were observed in the control and the 48, 480 and 960 g a.i./ha methoxyfenozide treatments (Table 2) with no statistically significant differences between these treatments (ANOVA:  $p = 0.145$ ).

The hatching rate was 80, 84, 76, and 97 % in the control and the 48, 480 and 960 g a.i./ha methoxyfenozide treatments (Table 3). No statistically significant differences were observed between these treatments (ANOVA:  $p = 0.033$  / Dunnett t-tests between each methoxyfenozide treatment and control  $p > 0.05$ ).

## Discussion

Control mortality in the present study was below 10 %. Comparing these mortality values with the trigger value of 30 % control mortality stated in the IOBC guideline for *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (Schmuck *et al.*, 2000), the presented method can be considered appropriate.

Mortality in the fenoxycarb treatment was above 50 % and statistically significant different to the mortality in the control treatment. Specific IGR effects (crippled / abnormal larvae) were also observed. These effects are typical for the mode of action as non-neurotoxic insect growth regulator with contact and stomach action which inhibits metamorphosis to the adult stage and interferes with moulting of early instar larvae (Tomlin, 1994). As such fenoxycarb demonstrated the sensitivity of the test method and the suitability of the route of exposure, and could be used as positive control (toxic standard) for further tests.

Immediately after application of methoxyfenozide, statistically significant effects on preimaginal mortality of *Stethorus punctillum* exposed under laboratory conditions to the applied apple tree leaves were observed from 48 g a.i./ha onwards. The mortality results obtained with methoxyfenozide showed dose responsiveness. Decay of toxicity with time was observed since after 7 days of residue ageing, statistically significant effects on preimaginal mortality were only observed at 960 g a.i./ha while no effects were observed up to 480 g a.i./ha.

Concerning the assessment of reproduction (eggs laid per female per day / hatching rate of larvae) the chosen method also was proven suitable leading to mean values of  $9.5 \pm 2.8$  eggs / female / day and  $83 \pm 18$  % hatching of eggs. No effects were observed on the reproduction of adult beetles exposed as larvae to up to 960 g a.i./ha methoxyfenozide.

The method presented in this manuscript gives A) natural mortality values similar to the values stated in guidelines of closely related species (Schmuck *et al.*, 2000), B) reasonable reproduction values, and C) shows sensitivity for different products and application rates. Therefore, we consider it as suitable for detecting side effects of pesticides under laboratory and aged-residue designs.

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