Columella - Journal of Agricultural and Environmental Sciences Vol. 4, No. 2 (2017)

The influence of different substrates on the reproduction traits of Folsomia candida (Collembola) in an insecticide test

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Abstract: The standard OECD tests for *Folsomia candida* should be performed on the standard OECD soil. However, if it is necessary to measure more parameters than the juvenile number and mortality, the use of different substrates is necessary. But these substrates can modify the results of the experiments comparing to the standard OECD soil, resulting in different toxicity values. In our research, we tested the toxicity of an insecticide, Trebon® 10F to *F. candida* (Collembola). We tested the dose-dependent effect of Trebon® 10F on the egg number and the egg size on the substrates of (i) plaster of Paris mixed with activated charcoal and (ii) in standard OECD soil on two generation of collembolas.

Similarly to our previous study, the insecticide had a dose-dependent negative effect on the parent's egg size in the OECD soil, but on plaster of Paris, no response was found. The egg number did not show any response to the treatments. On the contrary, in the offspring generation, the egg size increased both in OECD and plaster of Paris tests. In the soil test, parent collembolas laid significantly more eggs, while the offspring laid much fewer, compared to the plaster of Paris test. Similar results were found in the case of egg size. In the soil test parent collembolas laid larger, while the offspring laid smaller eggs, compared to the plaster of Paris test.

In multigenerational tests (i) Trebon 10F could affect the different generations in the opposite way. The experiment performed on the plaster of Paris (ii) could underestimate toxicity compared to the OECD soil test. This should be taken into account when Ecotoxicologists interpret their data.

Keywords: springtail, reproduction, OECD soil, plaster of Paris, etofenprox

Introduction

Folsomia candida Willem, 1902 (Collembola, Isotomidae) is one of the most often used model animal in soil ecotoxicological tests. This species is distributed all over the world in the organic matter-rich soils. *Folsomia candida* is easy to rear in the laboratory because it is a parthenogenetic species (Fountain and Hopkin, 2005). Consequently, it is the subject of standard soil ecotoxicology tests (ISO, 1999, 2011; Canada, 2007; OECD, 2009).

Different properties of the soil, such as organic material content or pH are usually taken into consideration in ecotoxicological experiments. According to pore-water hypothesis, toxicity of xenobiotics is mainly influenced by their availability in the soil pore water, which depends primarily on soil pH and organic matter content (Van Gestel, 1997). Different environmental parameters can modify the toxicity of the xenobiotics significantly through changing their bioavailability. Bioavailability can not be described with the pore-water hypothesis in every case, while for example, springtails live in the air-filled pores and in the litter layer where they do not directly get into contact with pore-water (e.g., Pedersen *et al.*, 1997; Pedersen, 1999; Pedersen, Van Gestel and Elmegaard, 2000). A further problem may arise, namely that accurate measuring of pore-water concentration can be quite difficult (Pedersen, Van Gestel and Elmegaard, 2000). Organic matter influences toxicity not only by binding xenobiotics but through modifying microbial degradation (Shrestha *et al.*, 2015) and enhancing photodegradation (Amarathunga and Kazama, 2014).

Temperature could also affect toxicity in poikilothermic animals, like arthropods. The activity of animals decrease at a lower temperature, so they have reduced contact with the chemicals. Furthermore, because of the lower metabolism rate, detoxification rate will also decrease (Heimbach and Baloch, 1994; Martikainen and Rantalainen, 1999).

A standard method of keeping, rearing and testing

DOI: 10.18380/SZIE.COLUM.2017.4.2.33

collembola was developed by Goto (1960). According to his method, collembola is kept on the plaster of Paris mixed with activated charcoal. If pesticide test is performed on this substrate, chemicals could be bound by the activated charcoal (Li *et al.*, 2016). Crommentuijn et al. (1997) found that increasing organic matter content of artificial soil (2-10%) can decrease the solved cadmium content of the soil by 50%. Moreover, decreasing pH caused more available cadmium in the pore-water. Similar results were found in other studies as well (Van Gestel and Mol, 2003; Waalewijn-Kool *et al.*, 2014).

There are some important population parameters, that could not be measured in soil or artificial soil. For example, collembola eggs are not removable from the soil. Moreover, the effect of the xenobiotics is determined in standard tests at the end of the experiment (Fountain and Hopkin, 2001), so the measure of the exact time-response curve is problematic in the soil. Consequently, it is not possible involving egg parameters in the experiment if it is performed in the soil. This problem can be easily solved by using plaster of Paris instead of soil. Collembola can lay eggs on this flat surface, and the total number of eggs and any egg parameters are observable later on. One of the most studied parameters in ecotoxicological experiments with *F. candida* is the egg number, which is investigated from an ecotoxicological and evolution biological point of view. From the ecotoxicological point of view, the egg number decreases due to the effect of heavy metal contamination (Fountain and Hopkin, 2001; Smit *et al.*, 2004). If evolution biology of *F. candida* was in the focus of the study, difference between clonal lines (Stam, Leemkule and Ernsting, 1996), life-history traits (Tully and Ferrière, 2008; Hafer *et al.*, 2011), or *Wolbachia* symbiont presence (Timmermans and Ellers, 2009) were studied. Other parameters as the egg shape, the egg viability, and the egg size are rather involved in ecological and evolution biological studies not in ecotoxicological ones (Stam, Leemkule and Ernsting, 1996; Tully and Ferrière, 2008; Timmermans and Ellers, 2009; Hafer and Pike, 2010; Hafer *et al.*, 2011). For example, Tully and Ferrière (2008) measured egg size, egg number, clutch size and clutch number to reveal the different life-history strategies of *F. candida* reacting to different food availability and overcrowding. Another parameter is the growth of animals which is not measurable in experiments carried out in soil only in plaster of Paris. The growth of animals were estimated by weight (Stam, Leemkule and Ernsting, 1996; Smit *et al.*, 2004) or length (Fountain and Hopkin, 2001) of the animals. Smit *et al*. (2004) measured the dose-dependent effect of dietary zinc on *F. candida* by measuring the fresh weight of the animals. The weight of the animals was significantly lower in the treated group than in the control. The clutch size was decreased by zinc, and the juvenile period also became longer, as the hatching time, as well as, the hatching success also decreased significantly.

The aim of the study was to reveal whether different substrates, as artificial soil and plaster of Paris, will cause a difference (i) in the egg number or (ii) in the egg size of *F. candida* in a dose-response test with the pesticide Trebon® 10F? Moreover, it was questioned, whether (iii) a different effect is observable on parent and offspring generation in a two-generation test, and if so, (iv) is the substrate strengthening or weakening the effect?

Materials and Methods

F. candida was obtained from the stock population reared in the laboratory of the Szent István University, Department of Zoology and Animal Ecology for the past 20 years. Collembolas were kept in Petri dishes with a diameter of 9 cm and height of 1 cm based on the method of Goto (1960), so the dish was poured with plaster of Paris mixed with activated charcoal (10:1 volume ratio). The animals were kept at a temperature of 20 ± 0.2 °C, with constant humidity (~100%) and in total darkness. Petri dishes were watered, and the collembolas were fed with dry baker's yeast once per week ad libitum. During the breeding phase, they were aerated weekly. All phases of the experiment were performed at a temperature of 20 ± 0.2

DOI: 10.18380/SZIE.COLUM.2017.4.2.33

°C, with constant humidity (~100%) and in total darkness except when individuals were moved. Collembolas were transported with the aid of a small aspirator.

The Trebon® 10F (water soluble) is pyrethroid insecticide with the active ingredient, etofenprox. Etofenprox is an UV sensitive chemical. It degrades fast in homeotherm animals. It affects the sodium channels of the neurones inducing constant stimuli (FAO, 2006). It is not used in Europe anymore only in Asia, but it was applicable in orchards, cereals, maize, and forests. Nowadays Trebon® 30 EC (a.i. etofenprox) have been used instead, which contains aromatic hydrocarbons in order to enhance the dissolution. Recommended field concentration of Trebon® 10F in orchards and in forests is 0.883 ml/l. A reproduction test of *F. candida* was performed with the field concentration (0.883 ml/l) and its tenth diluted (0.0883 ml/l) and tenfold concentrated solution (8.83 ml/l), extended to parents and offspring.

Two experiments were performed. For the first experiment standard OECD artificial soil was used (OECD, 2009), which contains 74% sand, 20% kaolin clay, 5% sphagnum peak, and 1 % calcium carbonate, and the pH 7.29. In the test with the artificial soil (soil test hereafter) 10-12 days old synchronised individuals (n = 90 per treatment) were placed in plastic boxes (5 cm high, the basic diameter was 5.3 cm, and upper diameter was 6.6 cm). At this age, the size of the animals was 0.90 ± 0.11 mm. The box was filled with 24.5 g of dry soil and mixed with 5.5 ml of pesticide solution or tap water (60% water holding capacity). Four boxes were used, three for the treatments plus one for the control. After 20 days all of the animals were carefully separated from the dry soil. Thereafter, 30 out of the 90 individuals were chosen randomly from each treatment and the collembolas were placed individually in Petri dishes (9 cm in diameter) prepared in 10:1 volume ratio of plaster of Paris and activated charcoal. The new place induced egg laying in most cases. The boxes were opened for aeration, feeding, and cleaning of the mould if needed once every week. So, each animal was kept alone during the measurements and get a unique identification number (ID). The eggs were photographed on the 9th day, see in details later. At the start of the egg-laying phase, the animals were 1.99 ± 0.11 mm long. There were 30 replicate per concentration.

The second experiment in Petri dishes with plaster of Paris mixed with activated charcoal (plaster of Paris test hereafter) was performed. The collembolas were transferred to Petri dishes soaked with the concentrations mentioned before (30 dishes per concentration and 30 dishes as control). The animals were transferred to the Petri dishes at the age of 10-12 days, and they were left there for 20 days. The dishes were opened for aeration, feeding, and cleaning of the mould if needed once every week. Then egg-laying period began. The eggs were measured on the 9th day. Collembolas were kept individually during the experiment and get an ID. There were 30 replicate per concentration.

The egg clutches were spread carefully with a wet brush and a digital photo was taken of each (Olympus C7070 Wide zoom camera with Olympus C5060 ADL optic). The eggs were numbered in the photo manually. Thereafter, ten eggs were chosen randomly from each clutch with a random number generator. The first ten eggs with the smallest random number were chosen. The shortest and longest diameters of the eggs relative at a 90° angle to each other were measured with the aid of the ImageJ open source software (Schneider, Rasband and Eliceiri, 2012). A premade standard was used to calibrate the measurements at every magnification. The mean of five measurements was used as a final conversion rate from pixel to mm.

From the offspring generation, individuals were chosen randomly. In the soil test collembola was kept on artificial soil for 20 days as described above (different lineages were not mixed, the pedigree of the animals was recorded). In the plaster of Paris test, they get on Trebon® 10F soaked Petri dishes, and they were left there for 20 days. Then egg-laying period began. After 9 days the clutches were spread carefully with a wet brush and a digital photo was taken by each.

Thereafter, ten eggs were chosen randomly from each clutch with a random number generator as described above. The shortest and longest diameters of the eggs relative at a 90° angle to each other were measured. All of the statistical analyses were made with the R Statistical program 3.1.1 (R Core Team, 2012). The total number of laid eggs and the size of eggs were analysed with more type of models. The egg diameters were transformed so that the shortest and longest diameters were multiplied by each other and the square root of it was extracted. This kind of transformation results in a diameter as if the eggs were perfectly round. This is the geometrical mean of the two diameters which is less influenced by the shape of the egg than the mathematical mean. This measure is called egg size, hereafter. Respectively the logarithm of concentrations was inserted in the models to reduce the number of inner comparisons and the probability of the type I error.

The effect of concentration and test method on egg number were analysed with a linear model. The mixed effect model was applied for egg size analysis because the measurements on the eggs from the same clutch were not independent. Mixed effect model corrects the calculation accordingly with the so-called random effect (nlme package from R, (Pinheiro *et al.*, 2013). The identification numbers of the individuals were the random effect, which showed that the eggs had originated from the same dish. The total data set (which contains both tests) and concentrations were tested separately too. In models with total datasets including both tests interaction of the concentration and test type was included.

Results

Concentration does not have a significant effect on the total number of eggs either in soil or in the plaster of Paris test. In the soil test the egg size was significantly affected by concentration ($p < 0.001$, $t = -5.16$) (residual and standard deviation of the model were 0.018 and 0.0112, respectively). If Trebon® 10F concentration increase the egg size decreases, while there was no effect on egg size in the plaster of Paris test.

In the offspring generation, egg number was affected neither in the soil nor in the plaster of Paris test. The egg size was increased with Trebon® 10F concentration in both test types. Pesticide effect was significant in the soil test $p < 0.001$, $t = 4.57$ (residual and standard deviation of the model were 0.022 and 0.010) and in the plaster of Paris test $p = 0.021$, $t = 2.38$ (residual and standard deviation of the model were 0.017 and 0.0096) as well.

Statistical data are summarised in Table 1. In the soil test, parent animals laid significantly more eggs than in the plaster of Paris test, but offspring animals laid much fewer in the tenth concentration and in the field concentration. Similar results occurred in the case of egg size. In the parent generation animals in the soil test laid larger eggs in the control (14.4% larger) and in the tenth concentration (9.7% larger). By contrast, the offspring in the soil test laid smaller eggs in the control (8.3% smaller) and in the tenth concentration (11.9% smaller).

Discussion

In the case of the soil experiment, parents invested less into their offspring. In the plaster of Paris experiment, no such observable effect was found. This is consistent with the results of Mousseau and Fox (1998), who showed that trade-off between the reproduction and production is a common phenomenon in insect species. So our result could refer to poor environmental conditions (Fox, Thakar and Mousseau, 1997; Fox and Czesak, 2000), which was resulted in insecticide treatment. In the case of offspring, in both tests, egg size is increasing as a reaction to the insecticide treatment. Because of the constant egg number, the investment into the next generation increases, too. According to Tully and Ferriére (2008), the increase of the egg size improves the viability of the eggs of *Folsomia candida*. Similar trends were found with many herbivore insect species (Awmack and Leather, 2002). According to our results, it is plausible, that the parents can transmit

DOI: 10.18380/SZIE.COLUM.2017.4.2.33

Table 1. The statistical summary of Trebon 10F insecticide effects on *Folsomia candida*. The “model elements” refer to tested effects. The “difference” means the estimated difference to the effect of the model element to the total number of eggs (first column) and to the egg size (second column). The total number of eggs is the number of eggs laid during the experiments. The egg size is the geometrical mean of the shortest and longest diameter of the egg. The test type difference is the plaster of Paris test minus soil test. In the case of concentration model element is the continuous concentration effect. The difference between the different concentration levels (0.1; 1; 10) from the control are in the separate concentrations models. Total dataset models compare the plaster of Paris test to the soil test. The significant p-values are bolded.

Total number of eggs				**Egg size**			
Model elements	p-value	t-value	difference	Model elements	p-value	t-value	difference
Parents’ total dataset				Parents’ total dataset			
Test type	**<0.001**	4.731	51.93	Test type	**0**	4.618	0.0145
Concentration	0.245	-1.168	-1.95	Concentration	0.883	0.147	0.0008
Interaction	0.091	1.704	3.57	Interaction	**0.004**	-2.956	-0.0192
Parents’ test type difference in separate concentrations				Parents’ test type difference in separate concentrations			
Control	**0.001**	3.720	65.60	Control	**<0.001**	4.048	0.0187
0.1	**0.033**	2.242	41.88	0.1	**0.005**	3.056	0.0123
1	**0.010**	2.771	51.69	1	0.560	0.589	0.0035
10	**<0.001**	4.814	88.29	10	0.488	-0.706	-0.0039
Offspring’s total dataset				Offspring’s total dataset			
Test type	**<0.001**	-4.856	-107.90	Test type	**0**	-4.385	-0.0112
Concentration	**0.001**	-3.290	-12.00	Concentration	0.099	1.660	0.0064
Interaction	**0.020**	2.352	10.53	Interaction	0.134	1.506	0.0073
Offspring’s test type difference in separate concentrations				Offspring’s test type difference in separate concentrations			
Control	0.138	-1.520	-52.64	Control	**0.001**	-3.532	-0.0099
0.1	**0.001**	-3.683	-146.20	0.1	**<0.001**	-3.925	-0.0141
1	**0.018**	-2.480	-113.56	1	0.170	-1.400	-0.0063
10	0.984	-0.021	-0.53	10	0.389	-0.873	-0.0044

information about the unfavourable environment to their offspring. The transmission could happen through the egg content or through epigenetic variation. Further explanation of this hypothesis is to be found in Szabó and Bakonyi (2017).

The comparison of the two substrates shows, that parents lay more and larger eggs in a soil test, however offspring lay larger eggs and with twice more eggs in the plaster of Paris test. So in general in the soil test parents invest more in their offspring, but offspring invest much less in their eggs than in the plaster of Paris test. One explanation of this result is that probably in the soil the collembola has a contact with the pesticide on a much larger surface. So collembolas can take up Trebon® 10F not only through the legs, but through the whole body surface, and this fact could cause higher toxicity. According to Smit *et al*. (2004), this toxicity is caused by the more intensive contact with the pore-water. The activated charcoal mixed in the plaster of Paris could bind the compound permanently (Lebo *et al.*, 2003; Yu *et al.*, 2011). That is why in experiments carried out with activated charcoal effective concentration of a pesticide is less than the nominal one. (Crommentuijn, Doornekamp and Van Gestel, 1997; Van Gestel and Mol, 2003; Waalewijn-Kool *et al.*, 2014; Li *et al.*, 2016). Crommentuijn *et al*. (1997) found that increasing organic matter content of artificial soil significantly decreased the cadmium content of the pore water. Kang et al. (2001) found similar

DOI: 10.18380/SZIE.COLUM.2017.4.2.33

results as ours in the case of *Paronychiurus kimi* (Collembola), where the glufosinate-ammonium was less toxic to springtails on the plaster of Paris than in artificial soil.

We can assume, that the binding effect was not linear in the present experiment. While the organic matter content decreases the effect of the Trebon® 10F on the egg number approximately to the half in the artificial soil, but the effect is not halved in the case of the egg size. This fact supports the hypothesis that egg size is more sensitive or more strictly regulated parameter compared to the egg number. Consequently, the substrate is a key factor in soil ecotoxicological studies with collembola.

In conclusion, the following hypothesis could be formulated: (i) Trebon 10F could affect different generations in the opposite way. The experiment performed on the plaster of Paris (ii) could underestimate toxicity compared to the OECD soil test. Further studies should be carried out with different materials in the plaster of Paris (e.g. graphite instead of activated charcoal), to find the substrate in which soil toxicity can be reliably estimated together with measuring egg and growth parameters.

Acknowledgement

The authors would like to thank I. Ványiné Surman for her continuous help in the laboratory and Zs. Bakonyi for polishing the English.

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