ORIGINAL ARTICLE

Impact of ecological plant protection products on mortality and cocoon shell ratio of mulberry silkworms (*Bombyx mori* L.) – pilot studies

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Abstract

The paper describes the impact of two different plant protection products on silkworm (*Bombyx mori* L.) development. These products are commonly used in agrotechnical treatments and are officially allowed to use in ecological agriculture. They are also fungicides, which suggests lower negative impact on other groups of organisms. The two used products were Biosept Active Spray (grapefruit extract) and Miedzian 350 SC (copper oxychloride) which were sprayed on mulberry leaves used to feed silkworms from the beginning of the 4th instar. As to measure the level of impact, the mortality of larvae (percentage of dead specimens) and cocoon shell ratio (percentage of shell weight in whole cocoon) were checked. The highest mortality was recorded in the group treated with 0,7% Miedzian solution (92,5%) as well as the lowest shell ratio (12,06) comparing to the control group (mortality 7,5% and shell ratio 17,43). In the Biosept group, no significant mortality was recorded (comparing to the control group) but mean shell ratio showed a significant decrease in the cocoon quality. The study shows that one of the pesticides is highly effective against a non-target organism.

Keywords: agriculture, mulberry, pesticides, silkworm, sericulture

Introduction

An increasing awareness of human relationships to ecosystems and consequences that arise from unsustainable agriculture leads to changes in agrotechnical methods which attempt to reduce negative impacts on the natural environment. According to research, pesticides are second among the main factors of insect decline and intensive agriculture is first (Sánchez-Bayo and Wyckhuys 2019). Lepidoptera are the most vulnerable of all insect taxa because 50% of their biotope in Europe is farmlands (Erhardt and Thomas 1991). Silkworm (*Bombyx mori* L.) is one of the most widely bred insects, both for industrial purposes and as a research object. It is a monophagous insect, therefore the productivity and quality of rearing highly depends on the quality and quantity of mulberry leaves provided (Sunil Kumar *et al.* 2019). When mulberry pests attack only a small part of the crop, then it is better not to perform any chemical treatments that could negatively affect silkworm rearing. It is a species particularly sensitive to toxic compounds, resulting in very restrictive methods of cultivating mulberry (Yeshika *et al.* 2019). While agrotechnical practices within the cultivation area can be tightly controlled, chemicals used in adjacent areas can easily be transported through the air and even with the groundwater. Careless usage of pesticides is a common practice that can easily lead to mulberry crop contamination. This can further lead to severe damages resulting in larval mortality, and a decrease of cocoon yield and silk quality (Kuribayashi 1988). The possibility of rearing silkworms in a controlled environment makes it a perfect model species in toxicological research (Abdelli *et al.* 2018). Other features such as the well-known biology and pathogens of this insect, carefully developed rearing methods and relatively low cost contribute to the fact that it is adopted as an experimental tool in many laboratories and research facilities. Additionally, the genome of this insect has been fully sequenced, extending the array of possible research not only to *B. mori* but also facilitating studies on other Lepidoptera (Mita *et al.* 2004). Model organisms can make a significant contribution to understanding the effects of pesticides on various organisms. Silkworms are a good research object along with other insect species such as *Drosophila melanogaster* (Meigen, 1830) or *Tenebrio molitor* (Linnaeus, 1758), which are also widely studied for these reasons (Chudzińska *et al.* 2016; Spochacz *et al.* 2018). Numerous studies have evaluated the negative impact and toxicity of different pest management chemicals but only a few have investigated the topic of so called "green pesticides" (Kordy 2014; Sunil Kumar *et al.* 2019). Another issue worth mentioning is how pesticides impact non-target organisms from other trophic groups. The aims of this study were to check whether ecological plant protection products have a prominently negative impact on non-target organism and to determine if any of the examined products can be used safely in tested concentrations. Testing pesticides used in ecological agriculture can contribute to silk production in general, as strong pesticides used in conventional agriculture can result in the death of silkworms (Shanmugasundaram *et al.* 2015).

Materials and Methods

Insects

The experiment was conducted in the Department of Silkworms Breeding and Mulberry Cultivation (Institute of Natural Fibers and Medicinal Plants, Poznań, Poland) during the rearing season of 2018. The silkworm strain used throughout this study was BB. Larvae were randomly chosen and divided into four experimental groups. Each group contained 40 specimens and was raised under the same temperature (°C) and air humidity (%) conditions. The exact range of these conditions for each instar is shown in Table 1. Until the third molt the larvae were fed with non-sprayed white mulberry leaves. The leaf material came from the old Polish mulberry variety called "Żółwińska Wielkolistna", which was created in the 1950s in Poland for the purpose of silkworm rearing. It is characterized by rapid growth and large leaves (Łochyńska and Oleszak 2011). After the third molting, from the beginning of the fourth instar, the insects were fed only fungicide sprayed leaves, except for the control group which was fed water sprayed leaves. Each experimental group was kept in rearing boxes measuring 43 × 35 × 15 cm.

Table 1. Rearing conditions in various stages of development of the experimental groups (Łochyńska 2019)

Instar	Temperature [°C]	Air humidity [%]
First	26–27	60–70
Second	26–27	60–70
Third	24	60–70
Fourth	24	60–70
Fifth	23	60–70
Cocoon spinning	22–23	50–60
Imago	22–23	50–60

Chemicals

For the purposes of this study, two commercially used ecological plant protection products were selected. The first was Miedzian 350 SC (fungicide), the active ingredient of which is copper oxychloride ($Cu_2Cl(OH)_3$). Copper oxychloride is a widely used pesticide known for its antifungal properties (López-Prieto *et al.* 2020). The second was Biosept Active Spray (fungicide, bactericide and growth stimulant), the active ingredient of which is 33% grapefruit extract. Some studies show that 33% grapefruit extract exerts a potent activity against various species of fungi (Krajewska-Kułak *et al.* 2001). Both chemicals were diluted in water.

Bioassays

Fourth instar silkworm larvae were randomly selected and divided into four experimental groups, including three treatment groups and one control group. The control group was fed water sprayed white mulberry leaves. The treatment groups were as follows: 3 ml/l/10 m^2 Miedzian 350 SC solution sprayed leaves ("M 0.3%" group); 7 ml/l/10 m^2 Miedzian 350 SC solution sprayed leaves ("M 0.7%" group); and 1 l/10 m^2 Biosept Active Spray sprayed leaves ("Biosept" group). The 0.7% Miedzian solution is a common concentration recommended by producers for fruit tree protection. The 0.3% Miedzian solution is a relatively low concentration. Biosept Active Spray is a 0.1% solution made from 33% grapefruit extract, already prepared by the producer for direct application. All treatments were carried out in the mulberry field and administered using a hand pressure sprayer. They were performed twice, with a 7-day interval. Preventive measures were taken to avoid pesticide contamination between experimental groups by: keeping several non-sprayed mulberry bushes between sprayed ones as a buffer zone, gathering the leaves into separate boxes, changing/washing gloves between interaction with leaves from different bioassays, using different hand sprayers to administrate treatments as well as different containers for the preparation of solutions. A pre-harvesting period was not taken into account in order to determine what effect the use of pesticides on organisms in a non-experimental environment could have. On the other hand, from the perspective of silkworm farmers, the information about toxicity of a certain pesticide on larvae is also important, as many unexpected external factors can negatively affect rearing.

Shell ratio and mortality measurements

Mortality was measured in each group as well as the cocoon shell ratio of each individual which completed cocooning. In order to obtain the data needed to calculate the shell ratio, the whole cocoon (cocoon with pupa) and the cocoon shell (cocoon without pupa) were weighed separately for each individual, using a laboratory scale. The shell ratio was measured to estimate the quality of each individual based on the raw silk content in its cocoon which was calculated using the following formula:

$$Z = (X \times 100)/Y,$$

where: Z – shell ratio [%]; X – weight of cocoon shell [g]; Y – weight of whole cocoon (cocoon shell with pupa) [g].

The mortality was calculated using the following formula:

$$M = ND/P \times 100,$$

where: M – mortality [%]; ND – number of deaths; P – population size (number of individuals).

Statistical analysis

In order to verify the differences between the groups in terms of the shell ratio and mortality, a non-parametric analysis with the Kruskal-Wallis test was performed, as well as a series of non-parametric analyses with the Mann-Whitney U test to compare groups. *P*-values were calculated to test if the differences between the experimental groups were statistically significant. Data analysis was performed using Jamovi software.

Results

Shell ratio and mortality

The overall results are presented in Table 4. When compared to the control group, there were visible differences in both the shell ratio and mortality in each of the experimental groups. In the Biosept group, a low level of mortality was recorded, however, a significant decrease in the shell ratio may suggest a negative effect of grapefruit extract on the development of silkworms. In the M 0.3% group, more than half of the larvae died, and a large drop in the shell ratio was also noted, which shows the strong toxicity of even low concentrations of copper oxychloride on silkworms. Almost 100% mortality was recorded in the M 0.7% group, as well as the lowest shell ratio among all experimental groups. This shows a very strong negative impact of the fungicide on a non-target organism. It is worth noting that the mortality recorded in the control group did not exceed the usual mortality levels in silkworm rearing. The average weights of whole cocoons and cocoon shells of each experimental group are provided in Table 2 as well as in Table 3 where they are divided into males and females.

Statistical analysis

The analysis by the Kruskal-Wallis test showed that there were significant differences between the groups in terms of the intensity of the shell ratio χ^2 (11) = 38.61; $p < 0.001$, as well as mortality χ^2 (11) = 80.88; $p < 0.001$. Detailed analyses with the Mann-Whitney U test showed that the shell ratio was the highest in the control group. It was significantly lower in the Biosept group ($p < 0.004$), and significantly the lowest in the M 0.3% ($p < 0.006$) and M 0.7% ($p < 0.041$) groups. The results of the analyses are shown in Figure 1. The analysis performed for mortality showed that the mortality was the lowest in the control group. It was not significantly higher in the Biosept group ($p < 0.694$). It was

Table 2. Weight of whole cocoon and weight of cocoon shell in each experimental group (values expressed in mean ± standard deviation)

Experimental group	Whole cocoon [g]	Cocoon shell [g]
Control	1.00 ± 0.19	0.17 ± 0.03
Biosept	0.93 ± 0.11	0.15 ± 0.02
M 0.3%	0.73 ± 0.15	0.11 ± 0.04
M 0.7%	0.68 ± 0.12	0.08 ± 0.01

Table 3. Weight of whole cocoon and weight of cocoon shell in each experimental group, divided into males and females (values expressed in mean ± standard deviation)

Experimental group	Whole cocoon [g]	Cocoon shell [g]
Control		
Male	0.90 ± 0.12	0.16 ± 0.02
Female	1.12 ± 0.18	0.19 ± 0.04
Biosept		
Male	0.86 ± 0.07	0.15 ± 0.02
Female	0.99 ± 0.12	0.14 ± 0.03
M 0.3%		
Male	0.69 ± 0.14	0.11 ± 0.04
Female	0.84 ± 0.19	0.13 ± 0.03
M 0.7%		
Male	0.62 ± 0.007	0.09 ± 0.007
Female	0.82 ± 0.00	0.07 ± 0.00

Table 4. Shell ratio and mortality in each experimental group (values expressed in mean ± standard deviation)

Experimental group	Shell ratio [%]	Mortality [%]
Control	17.43 ± 1.89	7.5 ± 0.27
Biosept	15.77 ± 2.22	10 ± 0.30
M 0.3%	14.88 ± 2.80	55 ± 0.50
M 0.7%	12.06 ± 3.19	92.5 ± 0.27

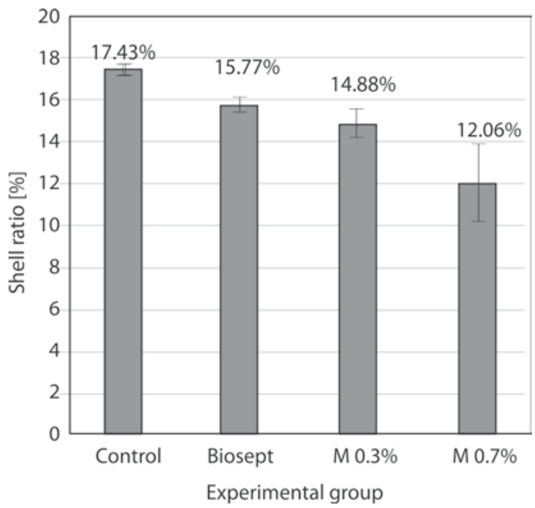

Fig. 1. Differences between groups in terms of the shell ratio. Standard error whiskers represent the standard error of the mean

significantly higher in the M 0.3% group ($p < 0.001$) and significantly the highest in the M 0.7% group ($p < 0.001$). The results of this analysis are shown in Figure 2.

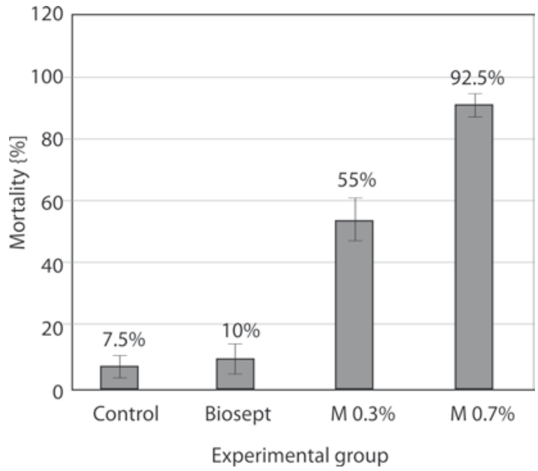

Fig. 2. Differences between groups in terms of the mortality. Standard error whiskers represent the standard error of the mean

Discussion

The results show that the tested fungicides had a significantly negative impact on mulberry silkworms, either by greatly increasing the population's mortality (Miedzian) or by reducing the mean shell ratio of a population (Miedzian and Biosept) leading to an overall reduction in the quality of the specimens. Moreover, the study showed that copper compounds such as copper oxychloride in concentrations widely used for fungal pest control, have a potential of killing all the silkworms exposed to it. It is concerning how strong the anti-insect effect was of the pesticide designed to eliminate phytopathogenic fungi. There are not many studies that describe the effects of copper oxychloride on non-target organisms, but from those that are available it can be concluded that the negative effect is due to histopathological changes in exposed individuals, especially in tissues where it accumulates the most. In one of the studies it was reported that copper oxychloride accumulated the most in the digestive gland of *Cornu aspersum* (formerly *Helix aspersa*) resulting in a significant decrease of digestive gland epithelium height and area (Snyman *et al.* 2003). It is possible that a similar situation occurred in silkworms, where the fungicide ingested with food caused histopathological changes mainly in the digestive system, causing the decrease in survivability. More research is needed in this regard.

In certain cases, the use of plant protection products is unavoidable, especially when a certain pathogen threatens a mulberry plantation to such an extent that it would be impossible to rear silkworms. This is exceptionally important in the case of a monophagous insect where no substitute food source can be provided, which differs it from other industrially reared silkworm species like *Samia cynthia ricini* (Drury, 1773), which have several host plants (Shifa *et al.* 2014). When most or all of a mulberry plantation area has been attacked by a pathogen during silkworm rearing already in progress, certain chemical treatments should be performed if it is not possible to obtain the food base from other sources. When faced with such a situation, the pre-harvesting period cannot always be applied. Moreover, it should be noted that one of the aims of this experiment was also to assess the general impact of the pesticide on a non-target organism, and in this case the silkworm served as a model organism. Beneficial insects occurring in nature, such as pollinators, are highly exposed to the effects of pesticides, and naturally, pre-harvesting periods are not applicable to them. However, when pest management on mulberry crop is necessary, it is important to use chemicals with little or no negative effect on silkworms. This study clearly showed that grapefruit extract had a relatively low impact on rearing performance in comparison to copper oxychloride but more research is needed to verify this subject. Biopesticides could be a safer solution in interaction with *B. mori* primarily because various types of plant extracts are more biodegradable and more compatible with environmental components (Bora *et al.* 2012). A Bordeaux wine mixture even showed positive results in reducing diseases both in mulberry and silkworms (Fernandes *et al*. 2016). It should be noted, however, that the organic origin of a substance does not mean that it is less toxic or more selective. Some organic substances, such as neem oil have shown positive results in reducing diseases in mulberry but on the other hand, it caused decreased fecundity and a reduction in the hatching percentage of silkworms (Kumutha *et al.* 2009). Azadirachtin, a chemical compound extracted from neem has been shown to possibly significantly inhibit cocooning behavior and development of silkworms (Zhang *et al.* 2017). Some organic compounds like pyrethrins can be as harmful as their inorganic counterparts, and have extremely low selectivity, harming many groups of organisms (Soni and Anjikar 2014). Each substance before being put into use, should be thoroughly tested in terms of its impact on the environment and non-target organisms.

Growing mulberry in close proximity to plantations where plant protection products are used always involves the risk of chemical transfer and leaf contamination. Accumulation of pesticides and problems with their degradability are some of the main issues of using them and consequences of chemical treatments can last for a long period of time. Some studies show that chemical treatments performed long before mulberry plantations were established in a given place resulted in long-term accumulation of toxins in the soil. The effects of those accumulations affected larval mortality up to 36 months after sprayings (Jyothi *et al.* 2019). Even small amounts of pesticides can have a significant impact on different aspects of development and rearing performance of silkworms. Thus many studies have been conducted on this subject. Sublethal doses of pesticides like parathion or dichlorvos have negative effects on silkworm longevity and reproductive capabilities, reducing the number of eggs laid and the number of fertilized eggs (Kuribayashi 1981; Kumutha *et al.* 2013). Other chemicals may have different effects like inhibition of the development of preimaginal stages (fenoxycarb) (Dedos and Fugo 1996), hinderance of posterior silk glands growth (Lu *et al.* 2021), histopathological changes in silk glands, impairment of energetic mitochondrial metabolism (Nicodemo *et al.* 2018) or even changes in expression of silk protein synthesis-related genes (Zhao *et al.* 2020).

Pesticides sprayed on plants affect not only targeted pest species but also a wide array of other trophic groups including beneficial species such as pollinators and even pest predators or parasitoids (Stanley and Preetha 2016). The presented impact of copper based fungicides on silkworms shows that more complex studies should be conducted (as well as stricter standards for introduction into general use) to evaluate the effect of certain pesticides on other groups of organisms, especially those plant protection products that are allowed in ecological agriculture.

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