

CAPSAICIN AS AN ORGANOPHOSPHATE SYNERGIST AGAINST COLORADO POTATO BEETLE (*LEPTINOTARSA DECEMLINEATA* SAY)

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Reserved: April 26, 2011

Accepted: August 2, 2011

Abstract: The aim of the presented study was to assess the applicability of capsaicin as a synergist for organophosphate insecticide against the Colorado potato beetle (*Leptinotarsa decemlineata* Say). We recorded the death rate, behavioral thermoregulation and metabolic rate of the examined insects after intoxication with capsaicin and organophosphate pesticide. The results obtained showed that the tested substances changed the thermal preferences of the potato beetle, causing the beetles to prefer a decrease in temperature in the thermal gradient system. We also observed an increase in the metabolic rate of the beetles intoxicated with capsaicin and organophosphate insecticide. The addition of capsaicin in a concentration of 10^{-7} M (dissolved in alcohol) to insecticide resulted in an increase in the death rate of the insects by 24% in 35°C and 73% in 15°C, in comparison to organophosphate alone. Our results show that capsaicin in low concentrations may be an efficient synergist for organophosphate insecticides against the Colorado potato beetle.

Key words: behavioral thermoregulation, capsaicin, Colorado potato beetle, organophosphate insecticide, metabolic rate

INTRODUCTION

Although the potato cultivation area in Poland has decreased over the last twenty years, the Colorado potato beetle is still an important potato pest (Sosnowska *et al.* 2009). The chemical treatment is so far the main and the most successful method used against the Colorado potato beetle (Węgorzek 2005). A great number of chemical insecticides was used against this pest. This insecticidal pressure on the pest populations resulted in the selection of resistant individuals. Growers rotate between insecticides with different modes of action, but the populations once exposed to some groups of chemicals remain to some extent resistant to it. After re-introduction of the insecticide the insects' resistance is likely to be invigorated (Alyokhin *et al.* 2008). For example, organophosphate insecticides are used against the Colorado potato beetle since 1970, and studies in Poland showed that these beetles demonstrate a higher tolerance to the organophosphorus substance – methidathion (Węgorzek 2005; Sosnowska *et al.* 2009). Resistance to organophosphates was also reported in other countries, such as Serbia and Croatia (Stanković *et al.* 2004; Barčić *et al.* 2006). The mechanism of action of these insecticides is connected with acetylcholine esterase inhibition, which leads to accumulation of acetylcholine at nerve synapses (Jokanović 2005). One of the reasons for pest resistance to organophosphates is a decreased acetylcholine esterase sensitivity to insecticides due to mutations in the enzyme (Alyokhin *et al.* 2008). Other reasons are connected with the reduced penetration of

organophosphates through the cuticle, or mutations in carboxylesterases responsible for enhanced degradation of insecticides (Yu 2008). Overcoming these problems requires using different chemical groups of insecticides or increasing doses of currently applied active substances. An alternative may consist in introducing an environmentally-friendly substance which would intensify the effect of active substances used against pests.

Thus, the problem of resistance to insecticides forces us to look for new opportunities in crop protection, such as natural substances used to control pests. Extracts from black pepper (*Piper nigrum*) were tested against Colorado potato beetle larvae and adults. The results indicate that piperamides reduce insect feeding on potato plants (Scott *et al.* 2003). The Colorado potato beetle feeding reduction was also obtained after applying aqueous extracts from such plants as: basil (*Ocimum basilicum* L.), marjoram (*Majorana hortensis* L.), common soapwort (*Saponaria officinalis* L.) and common thyme (*Thymus vulgaris* L.). Additionally, these extracts lowered the number of hatching larvae and limited oviposition (Wawrzyniak and Lamparski 2007). Extracts from common hop (*Humulus lupulus* L.) and cocklebur (*Xanthium strumarium* L.) also prevented Colorado potato beetles from feeding on potato leaves (Gökçe *et al.* 2006). In our study we tested another "hot" spice – capsaicin, which is a natural alkaloid responsible for the spicy taste of pepper. The mechanism of action of this substance is well known in mammals and is connected with its action on specific receptors, which induce

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a feeling of burning pain (Szallasi and Blumberg 1999). Capsaicin, a natural substance derived from pepper, is generally used as a pest repellent. It deters such insects as cotton pests, maize weevil (*Sitophilus zeamais*) or red flour beetle (*Tribolium castaneum*) (Spurr and McGregor 2003). Capsaicin was also effective as a repellent against *Arion lusitanicus*, an important pest of rapeseed (Kozłowski *et al.* 2008). Because we demonstrated earlier that capsaicin changes the thermoregulation and death rate of Colorado potato beetle larvae (Tęgowska *et al.* 2005), the aim of our study was to assess the effect of capsaicin and organophosphate insecticide on death rate, behavioral thermoregulation and metabolic rate of adult Colorado potato beetles. These data served us to evaluate the applicability of capsaicin as a synergist to organophosphate insecticides against this pest.

MATERIALS AND METHODS

Insects

Colorado potato beetle adults (*Leptinotarsa decemlineata* Say) were collected from a potato culture in Rzęczkowo near Toruń, Poland in July 2010, before the first crop spraying. During the experiments in the laboratory, the insects were kept in plastic containers filled with soil, at room temperature, and under natural photoperiod. Water and potato leaves were available at will.

Tested substances and death rate of the examined insects

The organophosphate insecticide used in our experiments was Actellic 500 EC, of which the active substance is pirimiphos-methyl (500 g in 1 l of insecticide formulation). Capsaicin (Sigma Aldrich) was tested in two concentrations: 10^{-4} M and 10^{-7} M, and with two different types of solvents – ethyl alcohol or Tween 80 (because a solvent may change the toxicity of tested substances). In two experimental groups, we examined the effect of these solvents alone. The control group was treated with water only. Insecticide solution (4.5 μ l in 1 ml of water) and capsaicin (2.5 μ l) were topically applied separately or together on the ventral side near the head with a pipette.

In each experimental series we observed 7 insects in 3 repetitions; different insects were used in each repetition. After application of the tested substances, the insects were put in glass containers at three different ambient temperatures: 15°C, 25°C and 35°C and under natural photoperiod, obtained in a laboratory incubator (ILW 115 STD, Pol-Eko Aparatura). The insects had open access to food which was potato leaves. The death rate was noted after the first, the second, and the third day from intoxication.

Measurements of thermal preferences

Thermal preferences of Colorado potato beetle were measured in the thermal gradient system. It is a long and narrow aluminum trough (60x5 cm). One end of the trough is cooled down to 10°C with cryostat, and the second end is heated up to 40°C by a thermostat (Fisherbrand FBH 612). This creates a temperature gradient inside the trough. The temperature gradient system was divided into 20 compartments of equal length. The com-

partments were not separated from one another. The behavior of the examined insects was filmed with a video camera (Sony HDR-XR 200VE), from which the image was sent to a computer. We recorded thermal preferences of animals (beetle position in the trough, every 3 minutes) in natural photoperiod (close to 16:8 L:D, depending on the natural length of day and night). Just after intoxication, the beetles were placed ($n = 6$) in the thermal gradient for 72 hours. Insect movements were not restricted and they had open access to food.

Measurements of metabolic rate

In order to determine the metabolic rate, we measured CO₂ production using an infrared CO₂ analyzer S157 (Qubit Systems Inc., Kingston, Canada). It was calibrated with a calibration gas (500 ppm CO₂). The analyzer was a part of a flow-through respirometry system. In this system, the air from the cylinder (21% O₂ in 79% N₂) was pulled through glass respirometry chambers, where the animals were placed, to a CO₂ analyzer with a pump P652 (Qubit Systems Inc., Kingston, Canada). The air was scrubbed of water vapour using magnesium perchlorate. The flow rate was set on the level of 150 ml/min. The beetles were deprived of food for about 2 hours before measurements to avoid any additional CO₂ release resulting from food absorption. Before each experiment, the insects were weighed on a precision balance (Radwag WTB 200). After the intoxication ($n = 8$ in every experimental series) the examined beetles were put into respirometry chambers (5 ml) for two hours. After every 15 minutes of measurements, the baselines (empty respirometry chamber) were recorded for 3 minutes. All measurements were made at room temperature (25±2°C).

Data analysis

All data presented are mean ±SD. Data were subjected to one-way analysis of variance. Post hoc Tukey's HSD (Honestly Significant Differences) test was used for separating treatment means. The results were considered statistically significant at $p < 0.05$.

RESULTS

Death rate after application of the tested substances

Capsaicin in the tested concentrations was not toxic to Colorado adult potato beetles; it caused the death of only a few individuals. These results show that this substance does not possess insecticidal properties. Organophosphate insecticide demonstrated the lowest insecticidal activity against the examined insects at 15°C, whereas at 35°C its effectiveness was the highest, causing the death of 72% of the insects. A similar effect was observed after using insecticide simultaneously with Tween. The addition of alcohol to the insecticide, at 15°C, increased the death rate to 63%. Capsaicin (dissolved in alcohol) 10^{-4} M added to organophosphate, increased insecticide toxicity only at 15°C (but the effectiveness was lower than in organophosphate and alcohol). Capsaicin (dissolved in Tween) 10^{-4} M lowered the toxicity of insecticide at 25°C, but raised it at 35°C, where a 90% death rate was

observed. After using organophosphate with capsaicin (dissolved in Tween) 10^{-7} M similar effects, as to the ones when insecticide was applied alone, were observed. The best effect was noted after using capsaicin (dissolved in alcohol) 10^{-7} M as an additional substance to organophosphate insecticide: 88% of the beetles died at 15°C and 95% at 35°C , but only 19% at 25°C (Fig. 1).

Thermal preferences of the Colorado potato beetle

The control insects preferred to stay at a mean temperature of 21.8°C . When intoxicated with capsaicin, they chose cooler places – 18.3°C for capsaicin (dissolved in alcohol) 10^{-4} M and 17.5°C for capsaicin (dissolved in alcohol) 10^{-7} M. Although the insects stayed at cooler parts of the gradient, their movements were not affected. Their ac-

tivity and feeding remained unchanged. Applying Tween also had a significant effect on thermal preferences. Colorado potato beetle preferred lower temperatures after intoxication with Tween alone, and capsaicin dissolved in Tween, in both concentrations (Fig. 2). Despite this fact, the substances used did not have a toxic effect on the insects. Only Tween caused the death of 17% of the insects in thermal gradient. Survival rate after capsaicin in both solvents was 100% (Fig. 3). Application of organophosphate insecticide also lowered temperature preference by the Colorado potato beetle to the preferred 19.5°C . Application of all tested substances in combination with the insecticide resulted in a significant decrease of preferred temperatures in comparison to the control group. However, these changes were not significant when compared

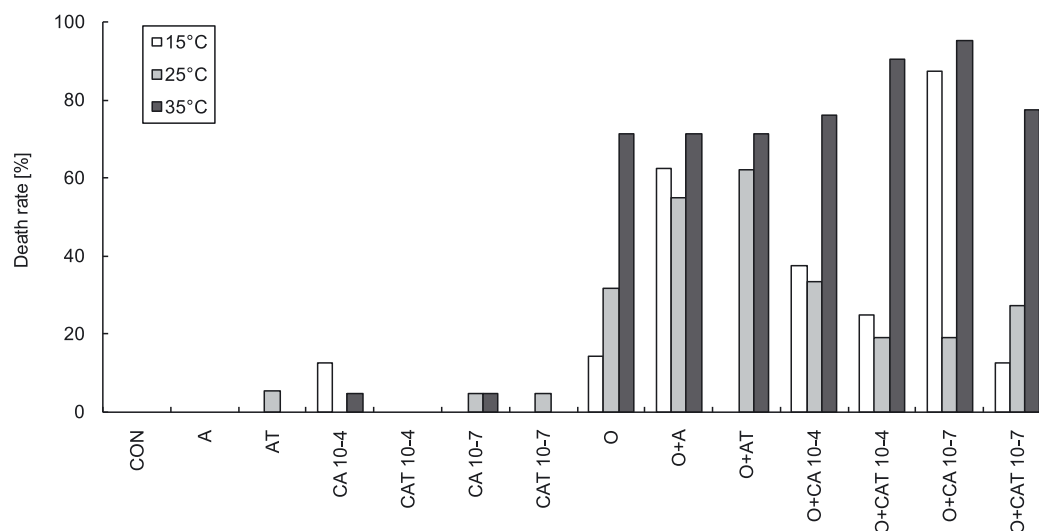


Fig. 1. The death rate (%) of the Colorado potato beetle intoxicated with capsaicin and organophosphate. Abbreviations: CON – control, A – alcohol, AT – Tween dissolved in alcohol in a concentration of 10^{-2} M, CA – capsaicin dissolved in alcohol (two concentrations: 10^{-4} and 10^{-7} M), CAT – capsaicin dissolved in Tween (two concentrations: 10^{-4} and 10^{-7} M), O – organophosphate insecticide

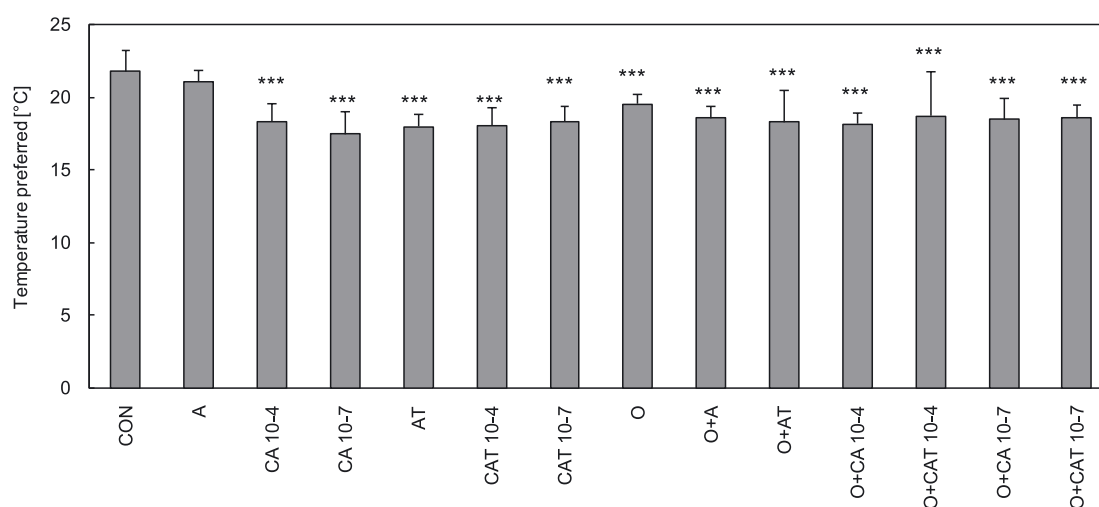


Fig. 2. Thermal preferences [$^{\circ}\text{C}$] of the Colorado potato beetle intoxicated with capsaicin and organophosphate (mean \pm SD). Abbreviations: CON – control, A – alcohol, AT – Tween dissolved in alcohol at a concentration of 10^{-2} M, CA – capsaicin dissolved in alcohol (two concentrations: 10^{-4} and 10^{-7} M), CAT – capsaicin dissolved in Tween (two concentrations: 10^{-4} and 10^{-7} M), O – organophosphate insecticide
indicates values statistically significant compared to the control insects ()

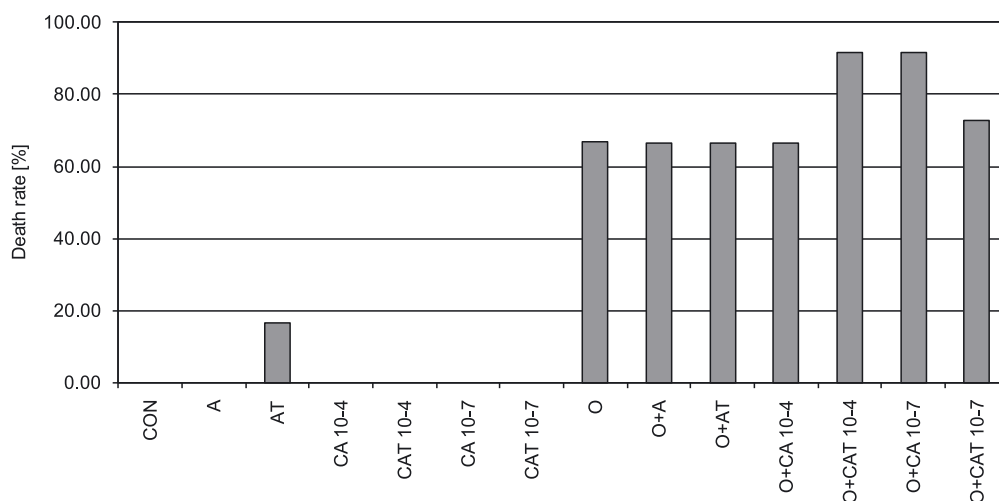


Fig. 3. The death rate [%] of the Colorado potato beetle intoxicated with capsaicin and organophosphate placed in a thermal gradient system.

Abbreviations: CON – control, A – alcohol, AT – Tween dissolved in alcohol at a concentration of 10^{-2} M, CA – capsaicin dissolved in alcohol (two concentrations: 10^{-4} and 10^{-7} M), CAT – capsaicin dissolved in Tween (two concentrations: 10^{-4} and 10^{-7} M), O – organophosphate insecticide

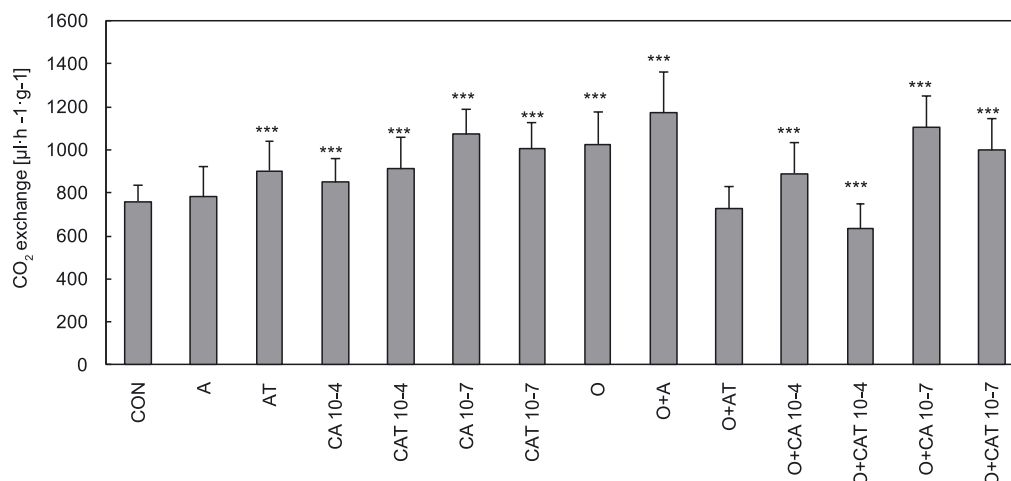


Fig. 4. CO₂ release (µl/g/h) (mean ±SD) of Colorado potato beetle intoxicated with capsaicin and organophosphate.

Abbreviations: CON – control, A – alcohol, AT – Tween dissolved in alcohol at a concentration of 10^{-2} M, CA – capsaicin dissolved in alcohol (two concentrations: 10^{-4} and 10^{-7} M), CAT – capsaicin dissolved in Tween (two concentrations: 10^{-4} and 10^{-7} M), O – organophosphate insecticide

*indicates values statistically significant compared to the control insects (***) $p < 0.001$

to an application of organophosphate alone. Although the addition of the tested substances to the insecticide changed the behavioral thermoregulation of Colorado potato beetle only slightly, we observed an increased death rate in the thermal gradient system. Application of organophosphate alone caused the death of 66.7% of the insects placed in the thermal gradient. After the addition of capsaicin dissolved in alcohol 10^{-7} M and capsaicin dissolved in Tween 10^{-4} M, the death rate increased to 91.7%. It is very interesting, that these results are distinct from those obtained when the insects were exposed to constant temperatures. The insects after intoxication with insecticide alone, and with capsaicin jointly, when placed in the thermal gradient system preferred to stay between 18.5–19.5°C, but when they stayed at the constant temperature of 25°C, the death rate was only 20–30%.

Metabolic rate of Colorado potato beetle

Intoxication with capsaicin induced an increase in metabolic rate. The greatest effect was observed after application of capsaicin dissolved in alcohol at a concentration of 10^{-7} M, – the CO₂ released increased above 40% compared to the control group. The examined beetles also showed an increased metabolic rate after intoxication with organophosphate as well as the insecticide with alcohol. However, paradoxically, the insects exposed to both insecticide and capsaicin did not show any increase in CO₂ release. Organophosphate applied jointly with capsaicin induced a decrease in metabolic rate in comparison to the insecticide applied alone, but most of the values were significantly higher than these observed in the control beetles. This was true except for the insecticide applied together with capsaicin dissolved in Tween

at a concentration of 10^{-4} M, where the CO_2 release was even lower than in the control group (Fig. 4).

DISCUSSION

The aim of our study was to assess the applicability of capsaicin as a synergist for organophosphate insecticide against the Colorado potato beetle (*Leptinotarsa decemlineata* Say). Recent research showed that capsaicin inhibits acetylcholinesterase action *in vitro* (Orhan *et al.* 2007). This suggests that capsaicin could strengthen the action of organophosphate insecticides *in vivo* and may be used as an efficient synergist against the pest.

In one of our previous studies, we demonstrated that capsaicin has an influence on insect thermoregulation and that capsaicin causes changes in temperature preferences of intoxicated insects (Olszewska *et al.* 2010). Tęgowska *et al.* (2005) revealed that Colorado potato beetle larvae fed on red pepper chose warmer environments in the thermal gradient system when compared to larvae fed on potato. It is commonly known that the effectiveness of insecticides depends on ambient temperature. Insecticides also change the behavioral thermoregulation of insects. For example, the American cockroach (*Periplaneta americana*) intoxicated with pyrethroid chooses high ambient temperatures (higher than the insects not exposed to xenobiotic), where the activity of this insecticide is the lowest (Grajpel *et al.* 2001). In the presented study, we tried to assess how capsaicin changes the thermal preferences of the Colorado potato beetle, and how joint application of capsaicin and organophosphate insecticide changes the preferred temperature of the intoxicated beetles. Our studies revealed that application of capsaicin alone induced seeking lower temperatures than what the control insects sought. It is therefore, a distinct reaction from that observed in the Colorado potato beetle larvae (Tęgowska *et al.* 2005). Admittedly, larvae cuticle is slightly more permeable than the cuticle of adults, so the intoxication of larvae could have been stronger. The different response of larvae to capsaicin, however, might have had other causes, for example, alterations in channel functioning. Changes in membrane potential genesis have been known since the seventies and were observed *inter alia* during amphibian development (Spitzer and Lamborghini 1976). Change in receptor functioning was observed in the case of GABA receptor. In the immature brain GABA acts excitatory and then during development it shifts to an inhibitory function (Ben-Ari 2002). It is possible that a similar phenomenon occurs during the ontogenesis of the Colorado potato beetle and cause differences in reactions to capsaicin of the intoxicated larvae and imago.

The Colorado potato beetle's choosing of lower temperatures after capsaicin application is similar to the behavior of other insects, such as the mealworm *Tenebrio molitor* (data not published) observed at our laboratory. Although the mechanism of capsaicin action on insect thermoregulation is still not known, we suppose that this substance may act on specific receptors similar to vanilloid receptors (TRPV1) in mammals. TRPV1 is a mammalian nociceptor, which activated by capsaicin induces a feeling of heat and pain, which leads to reactions induc-

ing hypothermia (Kobayashi *et al.* 1998). The research in comparison of TRP receptors in mammals and insects are discussed in Olszewska (2010) and the outcome suggests the existence of insects' TRP receptors sensitive to capsaicin. The observed behavior of the Colorado potato beetle in a thermal gradient indicates that capsaicin may activate some kind of receptors and induce feeling of heat, which force them to look for places with a lower ambient temperature.

Another possible explanation is that capsaicin changes the lipid bilayer fluidity. Lundbæk *et al.* (2005) demonstrated that capsaicin activates vanilloid receptor only at submicromolar concentrations. At higher concentrations (micro- to milimolar), this substance decreases membrane stiffness, modulating function of many membrane proteins. When the lipid bilayer fluidity is increased, the animal may look for low ambient temperatures to increase its stiffness. Capsaicin was dissolved in alcohol and Tween, which also increase bilayer fluidity. The intoxicated beetles, in order to avoid the consequences of increased membrane fluidity, stayed at cooler parts of the thermal gradient, in comparison to the control group.

Although capsaicin exerts little toxic effect on intoxicated beetles, it lowers the insects' thermal preferences, which may be important. Temperature essentially affects Colorado potato beetle development and reproduction. There are studies showing that at 15°C females do not oviposit, and the percentage of ovipositing females increases 100% when the temperature is at 24°C (Tauber 1988). At low temperatures (15–18°C), the development from eggs to pupa lasted remarkably longer than in higher temperatures (24–26°C), and the mortality of instars was higher (59% in 15.6°C) (Tauber 1988).

Organophosphate insecticide affected behavioral thermoregulation of the examined beetles in a way similar to capsaicin – we observed a decrease of preferred temperature. Organophosphate insecticides not only block the acetylcholinesterase action, leading to accumulation of acetylcholine in synapses. Błasiak (1996) demonstrated that these insecticides also affect fluidity and functioning of biological membranes. Such modifications in membrane performance may lead to changes in the behavioral thermoregulation of the insects. The addition of capsaicin to organophosphate insecticide did not further change the temperature preferred by the examined beetles. This may suggest, that both substances given simultaneously did not lead to any additional changes in membrane fluidity. The decreased stiffness induced by the tested substances was overcome by staying at cool parts of the gradient.

It is very interesting, that although the examined insects preferred temperatures at which the insecticidal efficacy is lower (when we examine the toxicity of the insecticide in glass containers – figure 1), we observed a high death rate in the thermal gradient system (Fig. 3). It is worth mentioning however, that during the thermal gradient experiments we observed a high death rate mainly in the case of insects which stayed at warmer parts of the gradient (at least 20°C). The insects which chose the cold end of the thermal gradient, in most cases survived. The observed differences between the death rate in constant

temperatures (glass containers) and death rate in the thermal gradient, call into question the methods used to examine the death rate after intoxication. Although testing the toxicity of different ambient temperatures in containers is a standard procedure, the behavior of insects in the field, and thus the results of toxicity, is rather similar to the one observed in the thermal gradient. We observed differences in the death rates of beetles in constant temperatures and using the thermal gradient system. It would be interesting to do similar experiments in the field.

The results of investigation on the metabolic rate of the Colorado potato beetle after intoxication are also interesting. Application of organophosphate alone caused a 35% increase in CO₂ release. This shows that the insects activated their detoxifying systems to combat the applied xenobiotic. Nath (2003) showed that organophosphate insecticides induce a glycogen decrease in the body fat of silkworm (*Bombyx mori*). After application of fenitrothion and ethion, an increase of trehalose and glucose levels in hemolymph, and a decrease in their body fat was observed. This means that the intoxicated silkworms use reserves from body fat to meet higher energy demands caused by detoxification processes. The high metabolic rate of the Colorado potato beetle after insecticide application implies that insects cope with intoxication. As capsaicin is dissolved in alcohol or Tween, both solvents were used. The change of solvent proved to affect the toxicity and metabolic rate. However, after simultaneous application of insecticide and Tween, and capsaicin at a higher concentration (10⁻⁴ M), we observed a decrease of CO₂ release in comparison to the group intoxicated with organophosphate alone. Tween and capsaicin may increase the lipid bilayer fluidity, which may increase insecticide penetration. Therefore, we suppose the high toxicity of both substances given together may lead to suppression of metabolism. After the application of organophosphate with capsaicin 10⁻⁴ M (dissolved in Tween) the death rate increased to 91.7%. In the case of organophosphate with Tween and organophosphate with capsaicin 10⁻⁴ M (dissolved in alcohol), the death rate of the beetles in the thermal gradient did not increase compared to the use of insecticide alone.

It seems that the best synergist of all the tested combinations for organophosphate insecticide against Colorado potato beetle is capsaicin 10⁻⁷ M dissolved in alcohol. The insecticidal action of this combination is very effective in both low and high temperatures, causing the death of 88% of the beetles at 15°C, and 95% at 35°C. Although after application of these substances the examined insects stayed at temperatures where insecticidal activity of organophosphates is lower (investigated in constant temperatures), the death rate in the thermal gradient of the beetles was very high – 91.7%. This result may also be obtained under field conditions, and it is worth checking it in the field.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Science and Higher Education grant number 3039/B/P01/2008/34.

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