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SON, Tae-Kwon
Department of Farm Management, College of Agriculture and Life Sciences, Kyungpook National University

HWANG, Hwal-Su
School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University

Md Munir MOSTAFIZ
School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University

OZAKI, Yukio
Faculty of Agriculture, Kyusuh University

他

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Effects of Eco–Friendly Flower Thinning Formulations on a Pollination Insect, *Apis mellifera*

Tae–Kwon SON[1], Hwal–Su HWANG[2], Md Munir MOSTAFIZ[2], Yukio OZAKI and Kyeong–Yeoll LEE[2, 3]*

Laboratory of Horticultural Science, Division of Agrobiological Sciences, Department of Bioresource Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 819–0395, Japan
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Flower thinning is necessary for crop production in various orchards. The effects of various ingredients of Koduri–Plus, an eco–friendly flower thinning formulation (FTF), were determined on the major pollination insect *Apis mellifera*. Three different FTFs, a mixture of 0.7% zinc and 1.5% manganese (A), a mixture of 0.7% zinc and 2.0% boron (B), a mixture of 2.0% seaweed extract (C), and lime sulfur solution were examined by measuring the contact and oral toxicities against adult worker bees. Both direct spray and oral ingestion of all three 1% FTF solutions did not cause any lethal effects for workers based on 72 h observation, but treatment with 1% lime sulfur solution increased worker mortality. Oral ingestion of FTF A and FTF C did not inhibit acetylcholinesterase (AChE) activity of workers at 24 h after treatment, but was slightly decreased by FTF B treatment. However, oral ingestion of the organophosphate pesticide dichlorvos or lime sulfur solutions significantly inhibited AChE activities. Our results suggest that manganese and seaweed extract of FTFs were not toxic for honeybees, in terms of contact and ingestion. Therefore, newly developed FTFs can be used to improve flower thinning activity without any detrimental effects on pollinating insects.

Key words: acetylcholinesterase, flower thinning, insecticidal activity, pollination insects

INTRODUCTION

Flower or fruit thinning improves fruit quality of many orchard plants (Link, 2000). Although thinning is conventionally conducted manually, various chemical and mechanical thinning methods have been developed to reduce crop load (Williams, 1994). Both ammonium and potassium thiosulfates have been commercially and experimentally used to thin apple orchards in North America and Europe (Dennis, 2000; Milic *et al*., 2011). Both naphthalene acetic acid and benzyladenine are efficient and reliable thinning agents (Batjer and Billingsley, 1964; Basak, 1996; Stopar *et al*., 2009). The derivative sulfcarbamide is used as a blossom thinning agent for apples in the United States (Williams, 1993). Even though chemical thinning agents have been developed, their safety for pollination insects is uncertain. For example, carbaryl (1–naphthyl–*N*–methylcarbamate) has fruit thinning activity on apple trees (Batjer and Westwood, 1960; Williams, 1993). However, carbaryl is a highly toxic carbamate pesticide and its use has been reduced due to its harmful effects on pollinating insects (Dennis, 2000). Therefore, it is necessary to develop eco–friendly thinning agents that are safe for pollination insects as well as the environment.

Recently, an eco–friendly thinning agent, Koduri, has been developed by using various organic compounds and minerals, and its safety on honeybees was demonstrated (Jahan *et al*., 2014). We hypothesized that flower thinning effects can be influenced by the presence and quantity of minerals in the formulation. The present study demonstrated the effects of various mineral ingredients such as manganese, boron, or seaweed extract in flower thinning formulations on the toxicity of the European honeybee *Apis mellifera* by determining contact and oral toxicities and acetylcholinesterase activity.

MATERIALS AND METHODS

Treatment with flower thinning formulations, sulfur, and pesticides

Three different kinds of flower thinning formulations (FTFs) (Apple Co. Ltd., Daegu, Korea) were prepared as shown in Table 1. These formulations were diluted to 0.1% and 1% concentrations. The commercial product S–lime sulfur containing 26.04% sulfur and 13.02% lime (Seoul Environment Industry, Seoul, Korea) was also diluted to 0.1% and 1% final concentrations of sulfur. The organophosphate insecticide containing 10% dichlorvos (Dongbu Farm; Hannong, Seoul, Korea) was diluted to a 0.1% final concentration.

Workers (10–20 days old) of *A. mellifera* were obtained from an apiculture farmer in Chilgok, Korea and placed in a plastic box ($10 \times 10 \times 5\,cm^3$). For the contact toxicity experiment, diluted solutions were sprayed onto honeybees (n=10) within the cage. For oral toxicity experiments, all FTF solutions were pre-

[1] Department of Farm Management, College of Agriculture and Life Sciences, Kyungpook National University, 80, Daehak–ro, Buk–gu, Daegu, 41566, Republic of Korea
[2] School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, 80, Daehak–ro, Buk–gu, Daegu, 41566, Republic of Korea
[3] Institute of Agricultural Science and Technology, Kyungpook National University, 80, Daehak–ro, Buk–gu, Daegu, 41566, Republic of Korea
* Corresponding author (E–mail: leeky@knu.ac.kr)

Table 1. Major compounds in three different kinds of flower thinning formulations

Contents	Flower thinning formulation (FTFs)		
	A	B	C
Water–soluble zinc	0.7%	0.7%	–
Water–soluble manganese	1.5%	–	–
Water–soluble boron	–	2.0%	–
Seaweed extract	–	–	2.0%
Glucose	140 mg/L	140 mg/L	140 mg/L
Mannitol	50 mg/L	50 mg/L	50 mg/L
Glycine	1.5 mg/L	1.5 mg/L	1.5 mg/L
Glutamic acid	1.2 mg/L	1.2 mg/L	1.2 mg/L

pared into solutions with 50% sugar content and soaked into cotton balls. Bees were allowed to feed on the sugar solution–soaked cotton balls for four days at 25°C. Mortality was observed until 72 h after treatments. Corrected mortality (%) was calculated by Abbott's formula as follows: (1 – n in T after treatment / n in Co after treatment) × 100, where n = worker population, T = treated, and Co = control.

Extraction of honeybee proteins

A single worker of *A. mellifera* was allowed to ingest liquid from cotton balls soaked with various kinds of 1% FTF solutions and bodies collected at 1 h after exposure. Honeybee samples in 0.7 mL of 0.1 M phosphate buffer, pH 7.4 (PBS) were homogenized by spinning at 12,000 rpm for 20 min at 4°C, and the supernatants transferred to new tubes. The protein concentrations in the samples were determined using a Bradford assay (Bradford, 1976) with bovine serum albumin fraction V (Sigma, USA) as the standard.

Acetylcholinesterase enzyme activity assay

Acetylcholinesterase (AChE) activity was measured using acetylthiocholine iodide as substrate by the method of Ellman *et al.* (1961). A solution of 0.01 mL of 75 mM acetylthiocholine iodide and 0.01 mL of dithionitrobenzoic acid was prepared, followed by the addition of 0.02 mL of enzyme and 0.16 mL of PBS. The mixture was incubated with shaking for 15 min at 27°C. Enzyme activity was measured using a microplate reader at 412 nm at 25°C using the kinetic mode for 15 min. The results are expressed as nmol/min/mg protein.

Statistical analysis

Statistical analysis of the data was conducted using the SPSS version 12.0 program (SPSS Inc., 2004) for Windows. Data were analyzed using a one–way ANOVA or Student's *t*–test. Data that were not normally distributed were analyzed using Tukey's method ($p < 0.05$).

RESULTS AND DISCUSSION

Koduri–Plus, as an eco–friendly FTF, enhanced flower thinning activity in apple orchards (Fig. 1). It consists of various organic compounds and a small amount of minerals (Table 1). Toxicities of three different kinds of FTFs on pollination insects were compared using honeybees *A. mellifera*. Each FTF used in this study differentially contained 1.5% manganese (FTF A), 2.0% boron (FTF B) or 2.0% seaweed extract (FTF C). Our results showed that all three FTFs did not have any contact and oral toxicities at 0.1% and 1% concentrations in 72 h after treatment (Figs. 2, 3). Otherwise, both body spray and oral ingestion of lime sulfur solutions as a control at a concentration of 1%, but not 0.1%, significantly increased mortality of worker honeybees. In addition, our results showed that AChE activity of *A. mellifera* was not changed by treatment with FTF A or FTA C, but was slightly inhibited by FTF B solution. In contrast, AChE activity was strongly decreased by treatments with the organophosphate pesticide dichlorvos and lime sulfur solutions at a concentration of 0.1% (Fig. 4).

Our results suggest that the manganese and seaweed extract content in FTFs are not toxic for vitality as well as for AChE activity of honeybees but boron is slightly toxic at a biochemical level. Seaweeds contain high amounts of carbohydrates and proteins as well as various minerals. Marine seaweeds contain both macro–

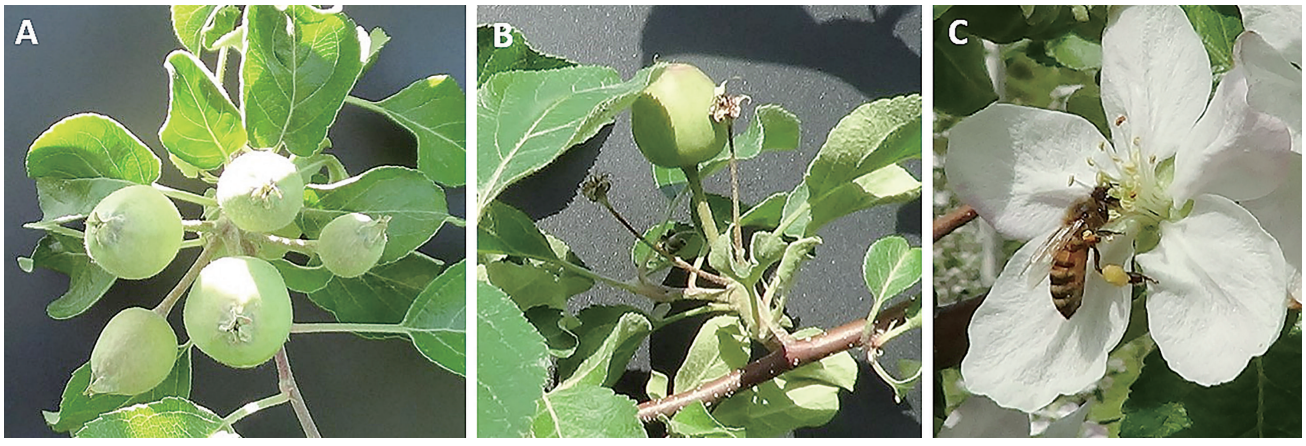

Fig. 1. Effects of a flower thinning formulation, Koduri–Plus, in apple orchards. Apple formation without (A) or with (B) flower thinning formulation treatment. The honeybee *Apis mellifera* as a major pollination insect in the apple flower (C).

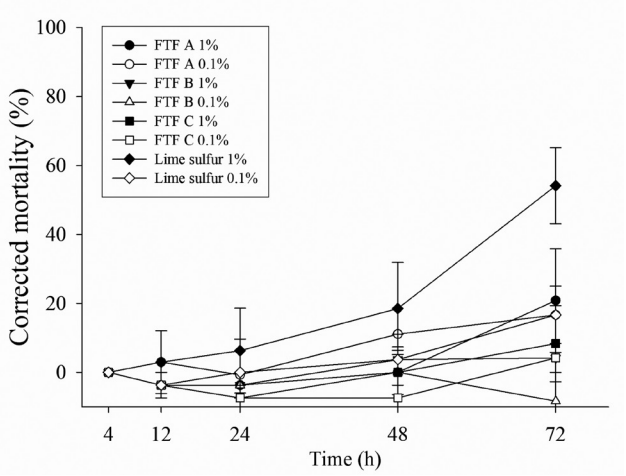

Fig. 2. Contact toxicity of various flower thinning formulations on *Apis mellifera*. Adult workers (n=10) were sprayed with three different kinds of FTFs and lime sulfur solutions at 0.1% or 1% concentrations. Non–treated honeybees were used as a control. Mortality was assessed at 4, 12, 24, 48, and 72 h of exposure. The corrected mortalities of adult workers of *A. mellifera* were determined at 72 h after treatments. Values shown are the means of five replications.

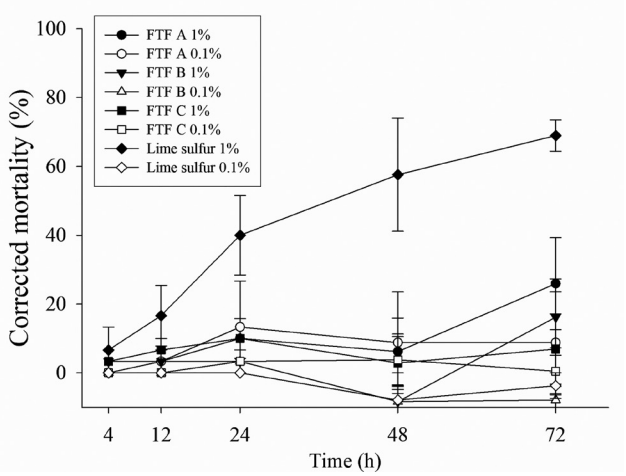

Fig. 3. Oral toxicity of various flower thinning formulations on *Apis mellifera*. Adult workers (n=10) were allowed to ingest three different kinds of FTFs and lime sulfur solutions at 0.1% or 1% concentrations. Non–treated honeybees were used as a control. Mortality was assessed at 4, 12, 24, 48, and 72 h of exposure. The corrected mortalities of adult workers of *A. mellifera* were determined at 72 h after treatments. Values shown are the means of five replications.

minerals (sodium, potassium, calcium, and magnesium) at 8.0–17.9 mg/100 g and trace elements (iron, zinc, manganese, and copper) at 5.1–15.2 mg/100 g (Ruperez, 2002). The addition of seaweed extract to FTFs has improved thinning activity in apple orchards (unpublished observations). Treatment with 2% seaweed extract did not affect the vitality of honeybees.

Boron is an essential mineral for survival yet becomes toxic at concentrations higher than those required for normal physiological conditions (Woods, 1994; Rainey *et al*., 1999). Our results suggest that neither treatment with body spray nor oral ingestion of FTF

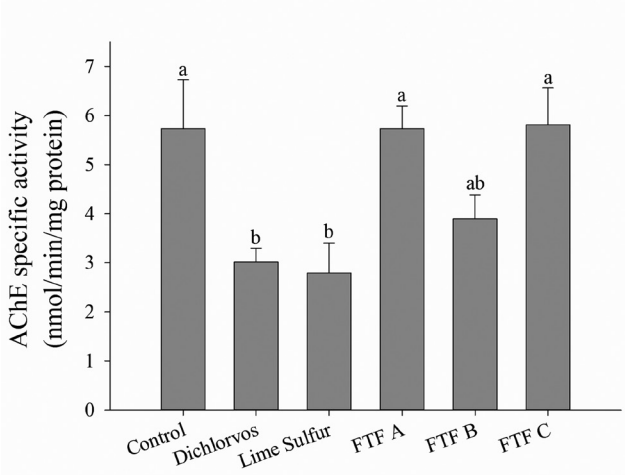

Fig. 4. Effects of various flower thinning formulations on acetylcholinesterase (AChE) activities of *Apis mellifera*. Adult workers (n=10) were allowed to ingest three different kinds of FTFs (A, B, and C), dichlorvos, and lime sulfur solutions at 0.1% concentrations for 1 h. Honeybees that ingested only 50% sugar solution were used as a control. The specific activity of AChE was determined as nmol/min/mg protein of whole body of the worker bees. Values shown are the means of five replications.

B containing 1% boron was lethal to honeybees but ingestion of FTF B with 0.1% boron slightly inhibited AChE activity. In practical use, boron is used as a wood preservative to prevent attack by decay fungi and certain insects including termites and wood–boring beetles, as well as in insecticide formulations against urban insects, such as cockroaches and fleas. Various boron formulations alter behavioral and physiological responses of the termite *Coptotermes formosanus* (Gentz and Grace, 2006). For example, termite damage is lower in borate–treated lumber than in untreated wood (Grace *et al*., 2006; Tsuboda *et al*., 2006). Thus, significant amounts of boron are not recommended in FTF formulations.

Sulfur is non–toxic to bees (Farm Chemical Handbook, 1994). Sulfur formulations such as 98% dust and 92% wettable powders are low in contact and oral toxicities for honeybees (USEPA, 1991). However, our previous study (Jahan *et al*., 2014) suggested that formulations containing sulfur compounds inhibit midgut proteolytic enzyme activities within 24 h after treatment, although it did not disturb the behavior of the honeybees. Our results suggest that the sulfur formulation is lethal to honeybees after exposure for several days.

In conclusion, we found that minerals such as zinc, manganese, and seaweed extract did not harm honeybees, but high amounts of boron should be avoided in FTF. This study provides important information that can be used for the formulation of non–toxic flower thinning agents against pollination insects such as honeybees.

AUTHOR CONTRIBUTIONS

Tae–Kwon SON perfeomed substantial contribution to the concept and design on this paper. Hwal–Su HWANG and Md Munir MOSTAFIZ carried out analysis

and interpretation of the data. Yukio OZAKI verified the data. Kyeong–Yeoll LEE supervised the experiments and contributed to the manuscript writing.

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