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Original research article

Longevity-enhancing Effects of Rosmarinic Acid Feeding on Honey bees (*Apis mellifera* L.) after Exposure to Some Pesticides Used in Strawberry Greenhouse

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Abstract Bee pollination plays important roles in increasing the yield, weight, and quality of strawberries grown in greenhouses. Use of pesticides is often included in the pest management strategy to control insects, fungi and weeds. However, these pesticides can negatively affect bees, compromising greenhouse pollination programs. In this study, we investigate the effect of rosmarinic acid (RA) feeding on the longevity of honey bees after oral and contact exposure of bees to some selected 14 different pesticides and G-3KM (commercial product) was used as a positive control. Among the 14 pesticides tested, six were found to be highly toxic to honey bee workers as indicated by their 48h-LD_{50} (μg/bee) values obtained from oral and spray bioassays; thiamethoxam (0.0002/0.0011), dinotefuran (0.005/0.018), emamectin benzoate (0.002/0.0002), spinetoram (0.018/0.001), sulfoxaflor (0.04/0.01), and cyantraniliprole (0.1/0.03), respectively. It was interesting to note that RA and G-3KM-supplemented feeding reduced honey bees mortality by 20–30% after they were exposed to high concentrations of the 6 highly toxic pesticides. The results indicate that RA could be used effectively in reducing honey bee mortality caused by pesticides.

Keywords Rosmarinic acid, G-3KM, *Apis mellifera*, Pesticides, Longevity

INTRODUCTION

Insect pollination for fruit set and seed development accounts 75% of agricultural crops of the world (Klein *et al*., 2007). Managed honey bees are used in greenhouse for pollinating vegetable crops due to their greater availability and low cost (Kalev *et al*., 2002) and the case of strawberry cultivation in greenhouse is not an exception. Increase in foraging activity and interval of visitation of honey bees (*Apis mellifera*) in strawberry greenhouse play a role for successful pollination which in turn enhances the quality of fruit (Begna *et al*., 2020). In this regards, managed species of honey bees like *A. mellifera* and *A. cerena* were recognized as essential pollinators for strawberry flower (Abrol *et al*., 2019). Pollination by these bee species enhances the quantity of strawberry fruit in Europe and Southern Asia (Sommeijer and Ruijter, 2000). Klatt *et al*. (2014) indicated that bee pollination plays an important role not only in increasing the fruit set, and weight, but also the quality of greenhouse-grown strawberries.

Strawberry are highly susceptible to pests. Among these, Western tarnished plant bug, *Lygus hesperus* Knight aphids (green peach aphid, *Myzus persicae* Sulzer, strawberry aphid, *Chaetosiphon fragaefolii* (Cockerell), greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) and Western flower thrips, *Frankliniella occidentalis* (Pergande) are economically important pests to strawberry (Zalom *et al*., 2014). These pests are causing a huge damage to strawberry, and they are

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responsible for strawberry production declines (Zalom *et al*., 2014; Dara, 2015). Pest management is important for producing high yields and aesthetic standard desired by consumers. The management of strawberry is mainly depended on chemical pesticides; generally limited to the rotation of pesticides of different modes of action (He *et al*., 2015; Dara, 2016). However, these pesticides can negatively affect bees, compromising greenhouse pollination programs (Gradish *et al*., 2010). For instance, abamectin, thiamethoxam, spinetoram and novaluron used to control mite, aphids, thrips, and caterpillars during strawberry crop production in Brazil affect stingless bees (Piovesan *et al*., 2020). Another study by Costa *et al*. (2014) showed that some commonly used pesticides (abamectin, acetamiprid, cartap chloride, chlorfenapyr, cyromazin, deltamethrin, thiamethoxam, flufenoxuron, and pyriproxyfen) in conventional melon production systems in Brazil had negative impacts on honey bees.

Metabolic detoxification is a major mechanism accounting for insect resistance to xenobiotics, including insecticides (Aupinel *et al*., 2007). Cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and carboxylesterases (COEs) are three major enzyme that play a role in detoxification in mammals (Li *et al*., 2007). RA has been reported to possesses several biological activities, including health enhancing activities (Chun *et al*., 2014; Teruel *et al*., 2015), immune responses, anti-inflammatory, and antioxidant activities (Kim *et al*., 2013; Alagawany and Abd El-Hack, 2015; Alagawany *et al*., 2017). In the present study, we first investigate the oral and spray toxicities of different pesticides on honey bee worker, and followed by the effect of RA-supplemented feeding in reducing honey bee mortality after honey bees were intoxicated with highly toxic pesticides.

MATERIALS AND METHODS

1. Worker bees

Adult worker bees *A. mellifera* were collected from the healthy colonies from the Experimental Apiary of Andong National University, Korea by using a small amount of smoke, brushing them from the combs and transferring them into plastic cups.

2. Chemicals

Commercial formulations of pesticides used in this study are listed below in Table 1. Additionally, rosmarinic acid (purity > 96%, CAS-No. 20283-92-5; Sigma-Aldrich, Korea), acetone (purity 99.5%, CAS No. 67-64-1; Daejung, South Korea) and G-3KM detoxicant (Dogo Medical Company Seoul, South Korea) were purchased and kept in the refrigerator prior to use.

3. Rosmarinic acid treatment

Prior to the treatment, we prepared 10 bees in each cage of treatment groups by using CO_2 gas as an anesthetic and kept in the experimental room at 25°C prior to the test. All pesticides were diluted in sugar syrup for oral test or pure water for spray test to prepare the test concentrations (from the producer's recommended concentration to 10^{-6} times dilution). Each pesticides had six concentrations and each concentration replicated five times. Ten honey bees were used per replicate (cage). Mortality was observed and recorded at 48 hours after the treatment.

For LD_{50} estimation, we measured feeders with the treated sugar solution before and after pesticides exposure for oral test and bees (n = 10) weighed before and immediately after being sprayed with pesticides in spray bioassay.

1) Oral test

Caged bees (10 individuals per cage) were starved for 2 hrs at room temperature (25°C), RH 50−70% prior to the test. Bees were fed sugar solution (50%) with different concentrations of pesticides for 1 hour (Laurino *et al*., 2011) using feeder and weighed (FX-200i, A&D, Korea) to estimate the amount of pesticides taken up by bees. Control bees were fed only sugar solution (50%). Followed starvation, the worker bees were fed with syrup adulterated with different pesticides using a feeder at the bottom of the cup for 1 hr. Subsequently, the worker bees were fed with sugar syrup supplemented with rosmarinic acid (100 μg/mL) for 48 hrs, and this procedure was repeated five times for each treatment. The feeder unit containing syrup alone was considered as control. The numbers of dead bees were recorded 48 hrs after feeding with syrup supplemented with rosmarinic acid. Worker bees were considered dead if they did not move

Table 1. Pesticide name, manufacturer, percentage of active ingredient, commercial formulation type, field concentration and mode of action

No	Pesticides	IRAC group	MoA[1]	Sub-groups	a.i.[2] (%)	RC[3] (a.i.ppm)
1	Imicyafos	1b	(AChE) inhibitors	Organophosphates	30	75
2	Thiacloprid	4a	(nAChR) competitive modulators	Neonicotinoids	10	50
3	Acetamiprid	4a		Neonicotinoids	8	40
4	Dinotefuran	4a		Neonicotinoids	20	100
5	Thiamethoxam	4a		Neonicotinoids	10	50
6	Sulfoxaflor	4c		Sulfoximines	7	35
7	Spinetoram	5	(nAChR) allosteric modulators Site I	Spinosyns	5	25
8	Emamectin benzoate	6	(GluCl) allosteric modulators	Avermectin	2.15	10.75
9	Lufenuron	15	Inhibitors of chitin biosynthesis affecting CHS1	Benzoylureas	5	25
10	Acequinocyl	20b	Mitochondrial complex III electron transport inhibitors	Acequinocyl	15	150
11	Metaflumizone	22b	Voltage-dependent sodium channel blockers	Semicarbazones	20	100
12	Cyflumetofen	25a	Mitochondrial complex II electron transport inhibitors	*Beta*-ketonitrile derivatives	20	100
13	Cyantraniliprole	28	Ryanodine receptor modulators	Diamides	5	50
14	Fluxametamide	30	GABA-gated chloride channel allosteric modulators	Isoxazolines	9	45

[1]Mode of action, [2]Active ingredient, [3]Recommended concentration

after being touched with the fine-tipped brush.

2) Spray test

After transferring 10 individual honey bees onto the Petri dish (15 cm in diameter), we sprayed (from 15 cm distance, 10 times) the pesticide solution with 600 mL hand sprayers (KOMAX G600, Sansoo Co., LTD, Korea). Control bees were sprayed only pure water. Then those bees were transferred into testing plastic cages. One group were fed with 50% sugar solution (control group) and the other group (treatment group) were fed with 50% sugar solution with RA or G-3KM. The number of bee mortality were counted 48 hr after the treatment.

3) Statistical analysis

The LD_{50} values of 48 hr post exposure to 14 different pesticides against honey bee workers as well as those of 48 hr post exposure to sugar, RA or G-3KM with 6 more toxic pesticides were calculated using the Probit analysis in SPSS version 26 (IBM Corp., 2011) to determine the dose-mortality response curves. LC_{50} values of contact (spray) exposure were also calculated by Probit analysis considering 4.52 μL amount of pesticides deposited on each honey bees body during spray bioassay, the spray LD_{50} was presumed from LC_{50}. We used the LD_{50}, when calculating Hazard Quotients (HQ). HQ = field application rate/oral or contact (LD_{50}) relative to the field application adopted for field concentration determination (Halm *et al*., 2006; Stoner and Eitzer, 2013; Abdu-Allah and Pittendrigh, 2018). If the $HQ < 50$: harmless; $50 < HQ < 2500$: slight to moderately toxic; $HQ > 2500$: dangerous for bees (Villa *et al*., 2000).

A two-way analysis of variance (ANOVA) was conducted, followed by Tukey multiple-range tests at $p < 0.05$ for mean separation, using Embedded on SPSS Statistics 26 (IBM Corp., 2011) to compare the statistical significance of differences in mortality between control, sugar, RA and G-3KM groups.

RESULTS

1. Oral and spray toxicity (48 h-LD_{50}/LC_{50})

Honey bees (*A. mellifera* L.) exhibited different levels of susceptibility to 14 tested pesticides as shown in Table 2. The results of toxicity of 14 pesticides, belonging to different classes, to honey bees (*A. mellifera* L.) are summarized in Table 2 for the oral and spray bioassays. As indicated in Table 2 for the oral application, thiamethoxam was found to be the most toxic to honey bees (48h-LD_{50} = 0.0002 μg/bee), followed by emamectin benzoate (48h-LD_{50} = 0.002 μg/bee). Five of the tested pesticides (acequinocyl, cyflumetofen, fluxametamide, lufenuron, metaflumizone) showed the lowest toxicity to honey bees (LD_{50} > 100 μg/bee) on oral toxicity assay. When honey bees were subjected to 14 pesticides on spray toxicity assay, we observed similar toxicity profile as of the oral toxicity tests as indicated in Table 2.

Among the 14 tested pesticides, six (thiamethoxam, dinotefuran, emamectin benzoate, spinetoram, sulfoxaflor, and cyantraniliprole) were highly toxic to honey bees. Especially, thiamethoxam, emamectin benzoate and spinetoram showed high risk (HQ > 2500) against *A. mellifera* (Table 2). The imicyafos and acetamiprid were moderately toxic in oral bioassay, and the other pesticides were non-toxic (> 100 μg/bee) for both tests to *A. mellifera* adults (Table 2). In general, our data showed that oral application of the pesticides was more toxic than that of spray except for the cases of imicyfos, spinetoram, and emamectin benzoate. The toxicity of pesticides after 48 h of oral treatment followed the order of: thiamethoxam > emamectin benzoate > dinotefuran > spinetoram > sulfoxaflor > cyantraniliprole > imicyafos > acetamiprid.

2. RA in reducing honey bee mortality

Six highly toxic pesticides of different mode of action groups were selected for further investigation of RA-supplemented feeding in reducing honey bee mortality after oral and contact pesticide intoxication. The results of RA-supplemented feeding in reducing honey bee mortality are summarized in Table 3. It was interesting to note that RA-supplemented feeding responses to worker bees intoxicated with imicyafos and cyantraniliprole were found to be higher than G-3KM, and sugar (control) treatments ($p = 0.03$ and $p = 0.09$, respectively) in oral bioassays (Table 3). Both RA and G-3KM were found to be significantly effective with sulfoxaflor and emamectin benzoate in spray test ($p < 0.005$).

It was also noted that RA and G-3KM-supplemented feeding reduced honey bees mortality by 20–30% after they were exposed to high concentrations of the 6 highly

Table 2. Lethal concentration (LC_{50}), lethal dose (LD_{50}) and hazard quotient (HQ) of used pesticides to honey bees, for the oral and spray toxicity, at 48 hr

No	Pesticides	48 hr-LC_{50} (μg/mL)		48 hr-LD_{50} (μg/bee)		HQ	
		Oral	Spray	Oral	Spray	Oral	Spray
1	Imicyafos	157.7	55.53	2.7	0.3	5.0	45.0
2	Thiacloprid	> 10,000	696.7	–	3.177	–	0.46
3	Acetamiprid	337.8	5741.8	6.756	26.183	0.11	0.03
4	Dinotefuran	0.35	3.93	0.005	0.018	30.0	16.7
5	Thiamethoxam	0.01	0.248	0.0002	0.0011	5000	909.09
6	Sulfoxaflor	1.45	1.55	0.04	0.01	17.5	70.0
7	Spinetoram	0.93	0.28	0.018	0.001	32.5	650.0
8	Emamectin benzoate	0.1	0.04	0.002	0.0002	344.0	3440.0
9	Lufenuron	> 10,000	> 10,000	> 100	> 100	< 1	< 1
10	Acequinocyl	> 10,000	> 10,000	> 100	> 100	< 1	< 1
11	Metaflumizone	> 10,000	> 10,000	> 100	> 100	< 1	< 1
12	Cyflumetofen	> 10,000	> 10,000	> 100	> 100	< 1	< 1
13	Cyantraniliprole	5.43	7.4	0.1	0.03	10	33.3
14	Fluxametamide	> 10,000	> 10,000	> 100	> 100	< 1	< 1

Table 3. 48h-LD_{50} and Hazard Quotient (HQ) estimated for each group (sugar = control, RA and G-3KM) in the honey bees with pesticides

No	Pesticides	Groups	48 hr-LD_{50} (μg/bee)		HQ	
			Oral*	Spray*	Oral	Spray
1	Imicyafos	Sugar	2.7[a]	0.3[a]	5.0	45.0
		RA	33.75[b]	0.9[a]	0.004	15.0
		G-3KM	24.4[b]	13.7[b]	0.6	1.0
2	Dinotefuran	Sugar	0.01[a]	0.018[a]	30.0	16.7
		RA	0.01[a]	0.025[a]	30.0	12.0
		G-3KM	0.02[a]	0.015[a]	15.0	20.0
3	Sulfoxaflor	Sugar	0.04[a]	0.01[a]	17.5	70.0
		RA	0.07[b]	0.02[a]	10.0	35.0
		G-3KM	0.07[b]	0.12[b]	10.0	5.8
4	Spinetoram	Sugar	0.02[a]	0.001[a]	32.5	650.0
		RA	0.02[a]	0.002[b]	32.5	325.0
		G-3KM	0.04[b]	0.003[b]	16.3	216.7
5	Emamectin benzoate	Sugar	0.002[a]	0.0002[a]	344.0	3440.0
		RA	0.003[a]	0.0005[b]	229.3	1376.0
		G-3KM	0.01[b]	0.0007[b]	68.8	982.9
6	Cyantraniliprole	Sugar	0.1[a]	0.03[a]	9.5	31.7
		RA	3.1[b]	0.05[b]	0.3	19.0
		G-3	0.5[a]	0.07[b]	1.9	13.6

Note:*Values in the same column for the same pesticide followed by different lowercase letters are significantly different ($p < 0.05$).

toxic pesticides. The results indicated that RA was effective in reducing the mortality of honey bees caused by pesticides.

DISCUSSION

A total fourteen pesticides of different groups tested on *A. mellifera* adults by the oral and spray bioassays. The order of pesticides toxicity was thiamethoxam > emamectin benzoate > dinotefuran > spinetoram > sulfoxaflor > cyantraniliprole > imicyafos > acetamiprid. Thiamethoxam and dinotefuran (nitro-neonicotinoid), emamectin benzoate (Avermectin), spinetoram (Spinosyns), sulfoxaflor (Sulfoximine), and cyantraniliprole (Diamide) pesticides were found to be highly toxic (< 2 μg/bee), for both of oral and spray bioassays. Imicyafos (organophosphate) and acetamiprid (cyano-neonicotinoid) were moderately toxic (2 < 11 μg/bee) in oral bioassay test, and the other pesticides were non-toxic (> 11 μg/bee) for both bioassay tests to *A. mellifera* adults (Table 2) which is in agreement to the previous studies (Hardstone and Scott, 2010). Thiamethoxam, emamectin benzoate and spinetoram were dangerous (HQ > 2500) for *A. mellifera* adults.

The nitro-neonicotinoids (thiamethoxam and dinotefuran) are highly toxic to bees, with acute LD_{50} from 0.004 to 0.075 μg/bee (Iwasa *et al.*, 2004; Cresswell, 2011). When thiamethoxam orally administrated, the 48h-LD_{50} value was 5 ng, and 24h-LD_{50} for contact administration was reported to be 29 ng (Decourtye *et al.*, 2005). In our findings, the cyano-neonicotinoids (thiacloprid and acetamiprid) are much less toxic than the other pesticides, which is consistent with a similar report by Iwasa *et al.* (2004). Abdu-Allah and Pittendrigh (2018) reported that macrocyclic lactone-class pesticides, such as emamectin benzoate and spinetoram were more toxic topically (LD_{50} = 0.0006 and 0.0023 μg/bee, respectively) and orally (LD_{50} = 0.66 and 4.99 μg/bee, respectively) for honey bees. As shown in Table 3, emamectin benzoate

was the least safest to honey bees as indicated by the highest Hazard Quotient value, which was also supported by several other similar investigations with bees and other insects (Lumaret *et al*., 2012; Abdu-Allah and Pittendrigh, 2018). However, lufenuron, acequinocyl, metaflumizone, cyflumetofen and fluxametamide were non-toxic (>100 μg/bee) for both bioassay tests to *A. mellifera* adults.

RA-supplemented feeding reduced honey bees mortality by 20−30% after they were exposed to toxic pesticides. This reduction in mortality might be due to the induction of detoxification or antioxidant mechanism in honey bees. Honey bees activate detoxification and antioxidant mechanisms when they are exposed to toxic pesticides (Johnson *et al*., 2010). Enhancing the production level of acetylcholinesterase (AChE) is one of the main mechanisms when individuals are exposed to pesticides such as organophosphates (Walker *et al*., 2005) and neonicotinoids (Boily *et al*., 2013). The mortality of *A. mellifera* workers supplemented with RA and G-3KM was reduced. Bisrat *et al*. (2020) also reported that G-3KM treatment was effective in reducing the mortality of honey bees when they were first intoxicated with pesticides.

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