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Original article (Orijinal araştırma)

Translocation of clothianidin to guttation fluid and its potential impact on honey bee, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae)[1]

Clothianidinin gutasyon sıvısında taşınımı ve bal arısı, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae) üzerindeki etkisinin belirlenmesi

Melis YALÇIN[2*] İlknur KAPIZ[2] Mustafa KÖSOĞLU[3] Yakup Onur KOCA[4] Nalan TURGUT[2] Erkan TOPAL[3] Perihan Binnur KURT KARAKUŞ[5] Levent ATATANIR[6] Serhan MERMER[7] Merve ÖZKALELİ AKÇETİN[5] Jens PISTORIUS[8] Cafer TURGUT[2]

Abstract

Honey bees, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae) forage water from guttation fluid so transported neonicotinoid insecticides in guttation fluid poses a risk to the bees. The first aim of this study was to determine the toxicity and risk of clothianidin to honey bees. In addition, the changes of clothianidin residue in the guttation fluid of maize plants in Turkey were determined in 2018 and 2019. Also, the toxicity of guttation fluid collected from the maize plants to bees was determined in ecotoxicological tests. The acute oral LD_{50} of clothianidin to honey bees in the first 24 h was 1.80 ng bee^{-1} and residue analysis demonstrated that honey bees were exposed to clothianidin concentration in guttation fluid ranging from 0.02 to 6.0 mg L^{-1} with mortality ranging between 80 and 100%. As the measured concentration of clothianidin in guttation fluid can lead to the mortality of honey bees, present study indicates that clothianidin, and possibly related pesticides in treated maize seed poses a risk to honey bees. Future studies are needed to determine the scale and distribution of this risk in Turkey.

Keywords: Clothianidin, exposure risk, guttation, honey bees, residue

Öz

Bal arılarının, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae) gutasyon sıvısından su ihtiyacını karşılaması sırasında gutasyon sıvısında tohum ilacı olarak kullanılan neonicotinoid insektisitlerin taşınması arılarda risk yaratmaktadır. Bu çalışmanın ilk amacı clothianidinin arılara toksisitesi ve riskinin belirlenmesidir. Bunun yanında 2018 ve 2019 yıllarında Türkiye'de mısır bitkisindeki gutasyon sıvısında clothianidin kalıntısının değişimleri belirlenmiştir. Ayrıca mısır bitkisinden toplanan gutasyon sıvılarının arılara toksisitesinde yapılan ekotoksikolojik testlerde tespit edilmiştir. Clothianidinin ilk 24 saatteki bal arılarına olan toksisitesi incelendiğinde ortalama akut oral LD_{50} değeri 1.80 ng arı$^{-1}$ olarak saptanmıştır. Bununla birlikte kalıntı analizleri sonucunda bal arılarının gutasyon sıvısında 0.02 mg L^{-1} ile 6 mg L^{-1} arasında değişen clothianidin konsantrasyonuna maruz kaldığı tespit edilmiş olup bal arılarının ölüm oranının %80 ile %100 arasında değiştiği gözlenmiştir. Gutasyon sıvısında ölçülen clothianidin konsantrasyonu bal arılarının ölümüne yol açabileceğinden, bu çalışma, mısır tohumlarına uygulanan clothianidin ve benzer grupta olan pestisitlerin, bal arıları için risk oluşturduğunu göstermektedir. Bu riskin Türkiye'deki miktarını ve dağılımını belirlemek için gelecekte yapılacak çalışmalara ihtiyaç vardır.

Anahtar sözcükler: Clothianidin, maruziyet riski, gutasyon, bal arıları, kalıntı

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[2] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Plant Protection, 09100 Aydın, Turkey
[3] Aegean Agricultural Research Institute, Apiculture R&D Innovation and Breeding Queen Bee Production Section, İzmir, Turkey
[4] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Field Crops, 09100 Aydın, Turkey
[5] Bursa Technical University, Architecture and Engineering, Faculty of Natural Sciences, Department of Environmental Engineering, 16310 Bursa, Turkey
[6] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition, 09100 Aydın, Turkey
[7] Oregon State University, Department of Horticulture, Corvallis, Oregon 09331, USA
[8] Julius Kuehn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Bee Protection, Messeweg11/12, Braunschweig, Germany
* Corresponding author (Sorumlu yazar) e-mail: melisusluy@adu.edu.tr
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Introduction

Seed coating with pesticides is widely used in agriculture to control and prevent pests and diseases. Pesticides used for seed coating also provide protection after emergence of the crop (Mancini & Romanazzi, 2014). Systemic insecticides are the most prevalent as a seed coating for agricultural crops. They are taken up from roots and transported to different parts of plants (e.g., stalk and leaves) (Karmakar et al., 2006; Huff Hartz et al., 2017). The high-water solubility, low use rates, broad spectrum activity and systemic movement in plants are desired properties for insecticides in pest management; however, their transportation leads to contaminated nectar, pollen and guttation fluid which can be a route of exposure for honey bees (Schmuck et al., 2001; Krupke et al., 2012; Cutler et al., 2014; Rundlöf et al., 2015; Alkassab & Kirchner, 2016; Schenke et al., 2018; Mörtl et al., 2020). Especially the neonicotinoids are the most significant systemic insecticides of the past few decades (Tomizawa & Casida, 2003). Their introduction was well-received by farmers, pesticide regulators and toxicologists due to the high efficacy against pests, selectivity and new modes of action (Matsuda et al., 2001; Jeschke & Nauen, 2008; Cutler et al., 2014). Due to the systemic properties, neonicotinoid insecticides have been widely applied to soil via irrigation, seed coatings, as well as foliar application. Neonicotinoids act as a competitive modulator of nicotinic acetylcholine receptor that causes immobility, abnormal behavior, excitation, and death of target and non-target insects (Jeschke & Nauen, 2008).

Several adverse effects of neonicotinoids are reported in honey bees including mortality, reduction in the hive fitness, increased susceptibility to pests and pathogens, long term colony viability, impaired learning, homing behavior, memory function, colony strength, reproductivity, flight dynamics, foraging behavior, cognitive functions, no production of new queens, and fewer total brood cells, and all these abnormalities can lead to colony disruption (Schneider et al., 2012; Di Prisco et al., 2013; Palmer et al., 2013; Sandrock et al., 2014; Scholer & Krischik, 2014; Williamson et al., 2014; Karahan et al., 2015; Larson et al., 2015; Alkassab & Kirchner, 2016; Brandt et al., 2016; Solomon & Stephenson, 2017; Woodcock et al., 2017; Çakmak, 2018; Abdelkader et al., 2021). There are lots of exposure routes of neonicotinoids to honey bees such as nectar of crops, accumulation of neonicotinoids on the plant parts such as flowers, transportation by the root system and contact exposure (soil and planter dust) (Krupke et al., 2012). In addition, guttation fluid that exudes from xylem pores on leaf margins can be an additional route of exposure for honey bees (Girolami et al., 2009; Thompson, 2010; Pistorius et al., 2012). Guttation is an active physiological process that occurs when leaves cannot transpire water because of the unfavorable conditions. In the humid and windless times, root pressure is so strong that plants exude xylem fluid (Shawki et al., 2006). In that situation, guttation fluid will be exude from xylem pores known as hydathodes. In general water demand of the beehive is high during spring and summer seasons, and honey bees use water to regulate the temperature and humidity of the beehive, to dilute thick honey and to feed the brood. Plants offering pollen and nectar will attract honey bees from long distances whereas water is gathered close to the colony (Pistorius et al., 2012).

Clothianidin, which breaks down slowly in the environment (US-EPA, 2005), is a neonicotinoid insecticide registered for seed coating (Tomlin, 2004) in various countries including Turkey. It is used for the management of stink bugs, planthoppers, whiteflies, aphids in vegetables, fruits and citrus. It is effective on various pests in the insect orders Coleoptera, Diptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera and Thysanoptera. The properties of clothianidin consists of broad insecticidal spectrum, low use rate, systemic action, high efficacy of control effect and variety of application method (Uneme, 2011). However, clothianidin belongs to nitro substitution group in neonicotinoids which is one of the most toxic insecticide to bees (Iwasa et al., 2004). The maximum residue limit of clothianidin in maize is 0.02 mg kg^{-1} (Anonymous, 2021). Clothianidin is used worldwide and there are many studies on its residue in pollen, wax syrup and honey (Tapparo et al., 2012; Scholer & Krischik, 2014; Codling et al., 2016; Sanchez-Hernandez et al., 2016; Çil et al., 2020), in guttation (Reetz et al., 2016; Marzaro et al., 2011; Tapparo et

al., 2011; Scmolke et al., 2018), in dust (Girolami et al., 2012; Krupke et al., 2012), and in bee bread and adult bees (Frommberger et al., 2011; Flores et al., 2021). In addition, many researchers have reported evidence of the toxic impacts of clothianidin on honey bees, bumble bees and other bees (Schneider et al., 2012; Palmer et al., 2013; Sandrock et al., 2014; Williamson et al., 2014; Scholer & Krischik, 2014; Larson et al., 2015; Çakmak, 2018; Abdelkader et al., 2021).

Honey bee colonies use water close to the hive and long-distant flight is avoided to save energy, therefore, the location of the bee hive and proximity to water sources such as guttation fluid determines the risk of insecticide exposure. Laboratory chronic exposure and long-term exposure tests have been used to clarify possible effects of clothianidin on bees (Schmuck & Keppler, 2003; Cutler & Scott-Dupree, 2007; Alkassab & Kirchner, 2016). Although the effects of clothianidin residues on honey bees have been studied under various meteorological conditions, there is insufficient information on the relationship between clothianidin residues in guttation fluid from seed treated maize and honey bee mortality in Turkey. Specifically, there is no information on the effects of clothianidin on *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae), which is the most common as well as economically and ecologically important honey bee race in Turkey (Akyol & Kaftanoğlu, 2001). Therefore, the main aim of this study was to determine whether under Turkish conditions, clothianidin used in seed coating is transformed into guttation fluid of maize and if this causes toxicity to *A. mellifera anatolica*. In addition, the toxicity of clothianidin in guttation fluid to *A. mellifera anatolica* was assessed over a range of concentrations in the laboratory test.

Materials and Methods

Bees

Worker honey bees, *A. mellifera anatoliaca* were collected from a single colony (disease free and queen-right) at the Aegean Agricultural Research Institute, Izmir, Turkey in 2018 and 2019. Honey bees were collected and transformed in stainless-steel test cages (8 × 6 × 4 cm) with aeration holes. Ten individuals (5-10 days old) were place in each cage and maintained in darkness at 25°C and under a controlled humidity of 50-70%. Sucrose solution and water were given together by syringe through the hole on the cages before starting the experiment and then replaced by guttation fluid collected with syringes from the field mixed with sucrose solution (1:1).

Field sites and planting

Maize fields of the Faculty of Agriculture Research Farm (37°45’32" N 27°45’35" E), Aydin Adnan Menderes University, Aydin, Turkey were uses in the experiments. Treatment plots consist of three field replicates (with clothianidin and control) and each experimental plot had an area of 250 m^2. The maize row and intra-row spacing was 70 and 18 cm, respectively.

Three plots were selected based on the randomized block design for the treatments. The maize sown was the hybrid Pioneer P2088 (Corteva Agriscience, Wilmington, DE, USA) with clothianidin applied to the seed as Poncho FS formulation (BASF, Ludwigshafen, Germany) provided by SeedEFE Ltd. (Aydın, Turkey) at 84 ml of product per 50,000 seed (Anonymous, 2021).

Collection of guttation fluid

Guttation fluid was collected from maize seedlings when the plants were at the 2-3 leaf-stage. Sampling was conducted throughout the growing period and the minimum time between any two sampling was 24 h. Sampling was done by using a Pasteur pipette from May until mid-June in 2018 and 2019. Approximately 10 ml of guttation fluid was collected from each row and field collections were conducted early in the morning (from 06:00 to 08:00 h). Samples were stored at -20ºC until the analyzed. Half of each sample was used for the residue analysis and the other half was used for the exposure experiment.

Chemicals

Acetonitrile (99.9% HPLC grade), acetic acid 96% and sodium acetate anhydrous for analysis were obtained from Merck (Darmstadt, Germany). $MgSO_4$ anhydrous for analysis was obtained from Sigma Aldrich (Merck). PSA (primary and secondary amines) bonded silica bulk was obtained from Supelco (Bellefonte, PA, USA). Hydrophilic PTFE, 0.20 µm pore size, 25 mm diameter syringe filters were obtained from Merck. Clothianidin (purity ≥98%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

LC-MS/MS analysis

Confirmation of the clothianidin results were provided by European Commission Guidelines (Pihlström, 2011). Experiments were performed with a Shimadzu 8030 (Tokyo, Japan) liquid chromatography (LC) tandem mass spectrometry (MS/MS) apparatus on a C18 Column (GL Services Luertsil OD54 - 3 µm 4.1 × 50 mm (UP), Cat No: 5020-04012 Merck. Mobile phase solvents were 0.1% formic acid and acetonitrile. The mobile phases were milli deionized water with of 0.03% of 5 mM ammonium formate (solvent A) and methanol with 0.03% ammonium formate (solvent B). The gradient elution program was: 0.0-6.5 min 95% B, 6.5-7.5 min 95% B, 7.5-8 min 5% B held for 12 min. The injection volume was 20 µL with a flow rate of 0.4 mL min^{-1}. Other operating conditions were: nebulizer gas flow 3 L min^{-1}, drying gas (N_2) flow rate 15 mL min^{-1} and drying gas temperature 400°C. The calibration curve was linear ($r^2 > 0.99$) in the range of 5-200 µg L^{-1}. Precursor ion (m/z) was 250 while product ions (m/z) were 169 and 132 for multiple reaction monitoring and related voltage was 12 and 16 V. Retention time was 3.7 min.

Toxicity tests

Dose response assessments was performed according to the OECD Guideline No 213/214 having a control and seven concentrations of clothianidin (OECD, 1998). Three replicate tests were performed for each test dose and totally 27 cages were used in the experiment. Each replicate group had 10 bees and dosed with each test concentration ranging from 0.04 to 2.7 ng mL^{-1}. During the experiment, honey bees were fed with 50% sucrose solution and incubated at 25°C (except during observation) under a controlled humidity of 50-70%. Before the test, honey bees were kept in stainless-steel test cages for at most 2 h without sucrose solution. Then 50% sucrose solution and different clothianidin doses (details given below) were added to each cage from weighed syringe feeders. For a calculation of exact dose uptake of the solution, the amount of consumed clothianidin with sucrose solution was determined from the weight difference of the syringes. Total of seven clothianidin doses according to the already established acute LD_{50} value (0.00379 µg bee^{-1}) (EFSA, 2013) of honey bees were used in the experiment. Mortality was assessed at 4 and 24 h intervals for up to 48 and 72 h after dosing. Honey bees were considered dead or moribund if they stopped moving. Mortality values were compared with values from positive control. The median lethal dose (LD_{50}) of clothianidin was established by using probit analysis using the POLO Plus program (LeOra Software, Berkeley, CA, USA).

Acute oral toxicity assessments were performed to determine the risks of guttation fluid to honey bees according to the draft OECD Guideline 213 (OECD, 1998). Honey bees were transferred to stainless-steel test cages in randomized groups of 10 individuals. The test conditions were same as the dose response assessments. The test solutions consisted of guttation fluid from 27 collection days with the addition of 50% w/v sucrose solution. The consumed quantity per cage was recorded after 4, 24 and 48 h by weighing the syringes at each time and also dead bees were recorded to determine mortality. Three replicate assessments were performed for each guttation fluid sample.

Clothianidin content in guttation fluid

Residues of clothianidin in guttation fluid were determined by the modified QuEChERS (quick, easy, cheap, effective, rugged and safe) method (Anastassiades et al., 2003). Ten ml acetonitrile acetic acid mixture (100:1 v/v) was added into 5 ml of guttation fluid. Sample was mixed for 15 s on a vortex mixer. $MgSO_4$ (4 g) and CH_3COONa (1 g) was then added to this mixture and mixed on a vortex mixer immediately for 1 min and centrifuged for 5 min at 4000 rpm. Two ml aliquot of supernatant was transferred into 15-ml centrifuge tubes by micropipette. $MgSO_4$ (0.3 g) and PSA (0.1 g) were added to the aliquot and mixed for 10 min, and then centrifuged for 2 min at 4000 rpm. The supernatant was transferred to glass centrifuge tubes and the volume of the extract was reduced to 0.25 ml by vacuum centrifugal evaporation filtered through into PTFE filters (25 mm x 0.22 µm) and transferred to the auto sampler vials for LC-MS/MS analysis. The extracts were stored at -20°C until analyzed.

Results

The toxicity (LD_{50}) of clothianidin to *A. mellifera anatoliaca* was 1.80 ng bee^{-1} (slope = 1.55; $\chi 2$ = 11.6) and the LD_{90} was 12.0 ng bee^{-1} (slope= 1.55; $\chi 2$ = 11.6).

The concentration of clothianidin in guttation fluid was slightly higher in 2018 than in 2019 with both residue levels comparably high. The highest concentrations of clothianidin in guttation fluid in 2018 and 2019 were 5.7 and 4.9 mg L^{-1}, respectively. In both years, the residues of the clothianidin were detected from the onset of guttation but declined by time. The residual toxicity of clothianidin in the guttation fluid collected in 2018 declined on day 12, and concentrations ranged between 0.5 and 5.7 mg L^{-1} (Figure 1). In 2019, clothianidin concentrations ranged from 0.02 to 4.9 mg L^{-1} (Figure 2) with the maximum concentration recorded on day 12 and the clothianidin residue followed a similar trend as in the first-year experiment (Figure 2). For guttation fluid the limit of detection for clothianidin was 0.001 mg L^{-1}, and the limit of quantitation was 0.005 mg L^{-1}.

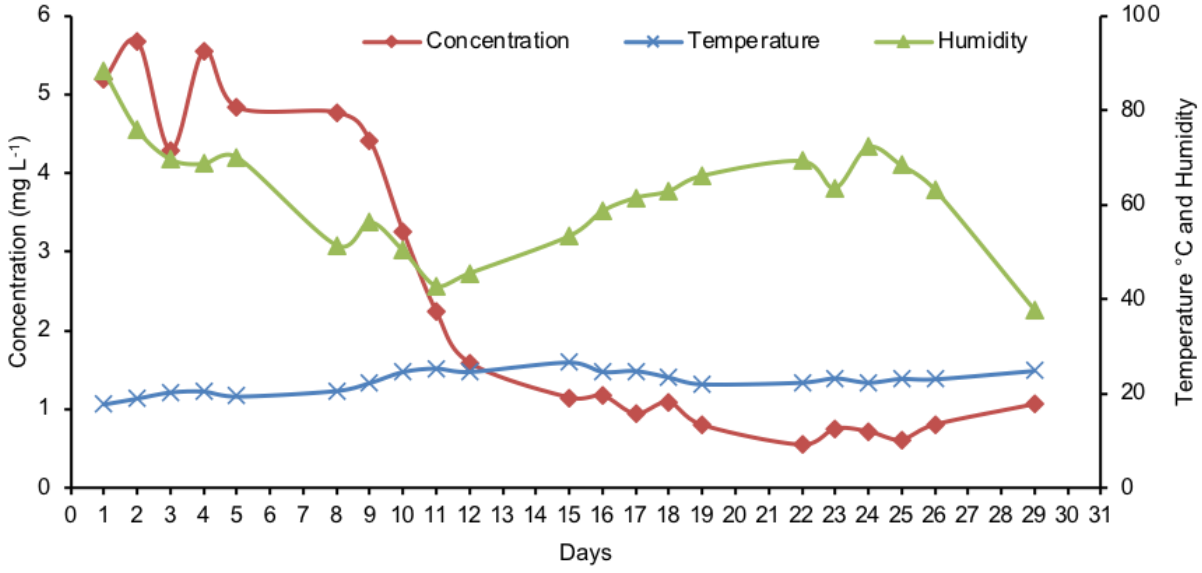

Figure 1. Concentration of clothianidin in sampled guttation fluid in maize by days in 2018.

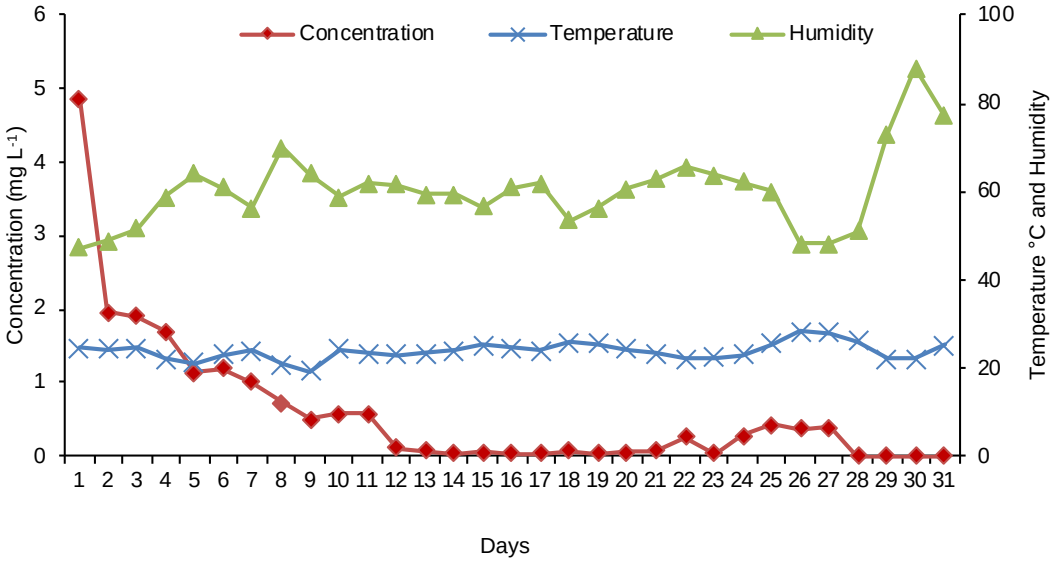

Figure 2. Concentration of clothianidin in sampled guttation fluid in maize by days in 2019.

In 2018 (Figure 3), mortality decreased irregularly, especially in the 4 h observations, and mortality in the 24 and 48 h observations were significantly higher than after 4 h. Mortality varied between 70 and 100% after 4 h due to the high concentration of clothianidin in guttation fluid until day 12. From day 12, mortality decreased unevenly. After 24 h, 100% mortality was observed for the first 15 days then it decreased in parallel with diminished clothianidin concentrations. After 48 h, 100% mortality continued for 22 days and decreased to 37% by day 29. In 2019 (Figure 4), up to day 13, mortality ranged from 50 to 100% after 4 h and 100% after 24 and 48 h. After day 13, mortality decreased irregularly after 4, 24 and 48 h. The concentration of clothianidin in guttation fluid in 2019 decreased to day 12 (Figure 2), so the decreased mortality observed was consistent with this.

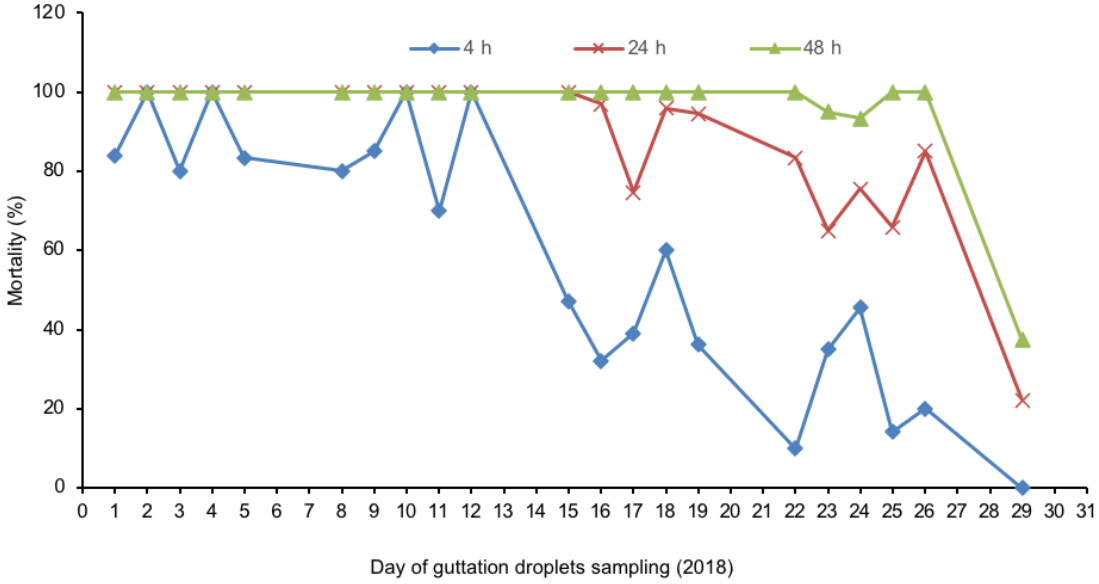

Figure 3. Bee mortality observed at 4, 24, 48 h after oral application of guttation fluid collected from different days from maize plants in year 2018.

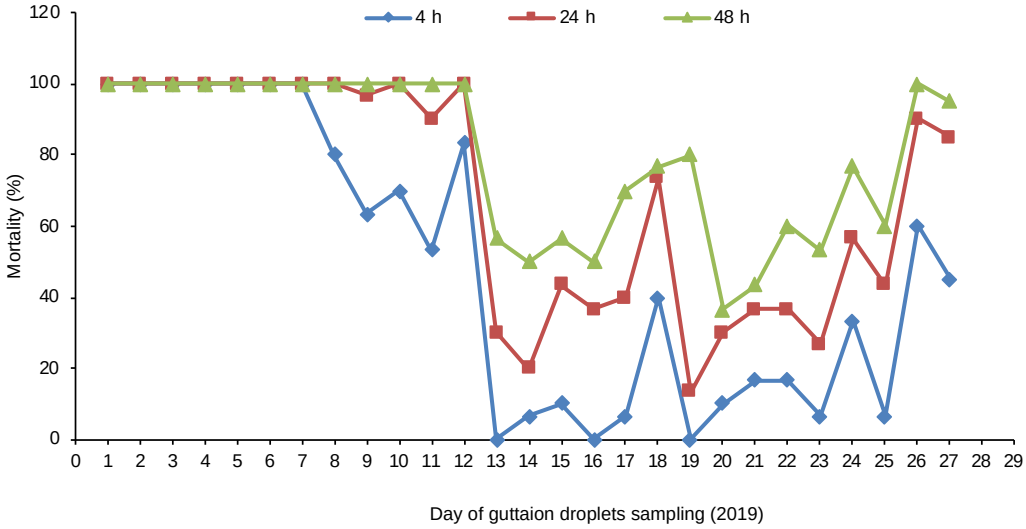

Figure 4. Bee mortality observed at 4, 24, 48 h after oral application of guttation fluid collected from different days from maize plants in year 2019.

Discussion

This study showed that clothianidin was significantly toxic to honey bees with LD_{50} of 1.80 ng bee^{-1} at 24 h. According to the Insecticide Classification of WSDA (2010) pesticides, LD_{50} <2 µg bee^{-1} is classified as significantly toxic to honey bees. Also, Laurino et al. (2013) reported that mean acute oral toxicity LD_{50} of clothianidin at 24 h was 3.53 ng bee^{-1} and at 48 and 72 h was 3.35 and 3.28 ng bee^{-1}, respectively. Pistorius et al. (2012) reported that after consuming 3.7 µL water containing 1 ng µL^{-1} clothianidin, the LD_{50} value of clothianidin in honey bee was 3.7 ng bee^{-1} would be reached. European Commission also reports that for the *A. mellifera* acute oral LD_{50} for clothianidin is 3.79 ng bee^{-1}. When published LD_{50} values are compared to the LD_{50} value detected in the present study, European Commission's value and Pistorius et al. (2012) results are less toxic. Suchail et al. (2001) reported that imidacloprid, which is in the same group with clothianidin (Group 4 in IRAC modes of action), has an LD_{50} of 60 ng bee^{-1} at 48 h and 40 ng bee^{-1} at 72 and 96 h, whereas Laurino et al. (2013) reported an LD_{50} of 90.1 ng bee^{-1} at 48 h and 69.7 ng bee^{-1} at 72 h. These contrasting results of the present study would likely be due to the particular features of pesticide toxicology or variation in the amounts ingested (Nauen et al., 2001; Suchail et al., 2001) or the differences could also be due to the different bee genotypes. For example, the acute oral LD_{50} of clothianidin in *Apis mellifera ligustica* Spinola, 1806 (Hymenoptera: Apidae) was 2.0 ng bee^{-1} whereas in *Apis cerana cerana* Fabricus, 1793 (Hymenoptera: Apidae) it was 0.5 ng bee^{-1} (Li et al., 2017), with the latter much lower.

In this study, the clothianidin residues in guttation fluid of maize was found to be between 0.02 to 6 mg L^{-1}. Similar clothianidin residue concentrations (up to 8 µg mL^{-1}) were reported in Germany for treated maize guttation fluid and this remained detectable over several weeks (Reetz et al., 2011). Nikolakis et al. (2014) who reported that guttation fluid collected in the early stages of the crop were more harmful due to the peak residue levels of neonicotinoids. Peak residue levels which were 8.5 mg L^{-1} for clothianidin and 6.7 mg L^{-1} for imidacloprid in winter barley (Nikolakis et al., 2014). The concentrations of three different neonicotinoids in guttation fluid of maize were: imidacloprid from 8.2 to 346 mg L^{-1}, clothianidin from 7.3 mg L^{-1} to 102 mg L^{-1} and thiamethoxam from 2.9 to 40.8 mg L^{-1} (Tapparo et al., 2011). The residue of clothianidin in guttation fluid of sugar beet was reported as 9.04 mg L^{-1} (Wirtz et al., 2018). In combination, these results suggest that neonicotinoid concentrations in guttation fluid are highly variable. Residues of clothianidin used as seed coating have been reported in guttation fluid at lower concentrations in oilseed rape than in maize (Nikolakis et al., 2014). Similar results were found in Germany where honey bees exposed to up to 130 µg L^{-1} clothianidin in guttation fluid of oilseed rape. It is reported that rapid growth of the plants parallels

a rapid decrease in pesticide residues in the oilseed rape guttation fluid (Reetz et al., 2016). Girolami et al. (2009) found that the high concentrations of clothianidin (23.3 ± 4.2 mg L^{-1}) occurred in guttation fluid collected from potted plants in the laboratory. Similar clothianidin concentrations were measured in some studies (Reetz et al., 2011; Nikolakis et al., 2014) and higher in others (Girolami, 2009; Tapparo, 2011; Schenke, 2018; Mörtl et al, 2020). The higher concentrations of clothianidin can be more variable due to various factors including amount of water evaporation, the rate of seedling emergence and time of the day of sample collection (Girolami et al., 2009; Sing et al., 2013; Mörtl et al., 2020). In the present study, sampling time and the time interval for collecting guttation fluid were constant and water evaporation was assessed for each replicate. Also, humidity and temperature can affect the guttation fluid quantity and residue concentrations. Marzaro et al. (2011) reported that highly concentrated insecticide sufficient to kill bees was related to variation in humidity in the field. Also, Wirtz et al. (2018) reported that increased humidity caused the highest occurrence of guttation and Nikolakis et al. (2014) found seasonal difference in residue levels in guttation water. These findings are consistent with the present study. High mortality was observed especially at the beginning of the present experiment, which could be linked to the humidity. At the commitment of the study in 2018, the humidity was about 88% and at about 17°C (Figure 1) whereas in 2019, it was 47% and 24°C, respectively (Figure 2). This indicates that humidity and other environmental factors can influence the pesticide concentration in guttation fluid.

The mortality of honey bees paralleled the residues of clothianidin in guttation fluid of treated maize in the field. Laurino et al. (2013) reported that according to the acute oral toxicity of clothianidin to honey bees, 24 and 48 h results were found similar because most mortality was observed in the first 24 h, as observed in the present study. In contrast, in a field experiment on colony health instead of individual mortality, it was reported that number of adults per colony did not change when they were exposed to clothianidin seed treated canola and exposure time did not affect the honey bee mortality. However, it was observed that queens ceased depositing egg and overwintering was reduced (Cutler et al., 2014). Similarly, in a semi-artificial study, homing flights of honey bees decreased with the exposure to one tenth of median lethal dose of clothianidin (0.002 µg bee^{-1}) (Matsumoto, 2013). Also, honey bees exposed to 10 µg L^{-1} clothianidin had increase hemocyte density than bees exposed to 50 µg L^{-1} (Brandt et al., 2017). Although, in some cases, clothianidin residues in guttation fluid decreased over time, the amount of pesticides required to cause mortality in honey bees decreases and mortality increases (Suchail et al., 2001; Moncharmont et al., 2003; Alkassab & Kirchner, 2016). According to Pistorius et al. (2012), honey bee mortality and colony health were normal but although dead bees were observed, but there was no increase in the mortality and results indicated that single bees came in contact with clothianidin but this did not lead to increase of mortality.

In two experiments, with treated seed and untreated maize crops under semi field conditions, the effects of clothianidin to *A. mellifera* were examined. In the first experiment two treatment variants were used. In the first one, none of the colonies had additional water source while in the second one, all colonies had, uncontaminated water source as an artificial water source. As a result, in the variant with no additional water source high adult bee mortality was observed and the mortality increased in time while no increase in mortality was observed in colonies with alternative water source. Thus, in the extreme semi field scenario it could be demonstrated that bees may use guttation water as a water source; in this scenario no additional water was available and high mortality of foragers and also effects on the colony level were observed in contrast to semi field tunnels in which an uncontaminated water source or uncontaminated guttation fluid were available (Frommberger et al., 2011). This highlights the need to investigate if, and to what extent, honey bees may use guttation fluid in different climate zones and environmental conditions, to exclude that such scenarios as found by Frommberger et al. (2011) might take place in a field-realistic scenario. In the field experiment conducted by Nikolakis et al. (2014) maize seeds treated with clothianidin at a rate of 0.5 mg $seed^{-1}$. The mean numbers of dead bees were 12.7, 46.3 and 38.4 after exposure of 48, 43 and 32

days, respectively. Based on the results observed for control group, mortality cases were uncommon, however, it was concluded that clothianidin did not affect the bee health, overwintering success and honey production. In contrast one of the surveys which compared mortality rate of honey bees located in maize-dominated and maize-free contexts showed that colonies located in maize-dominated areas had 3.51 times higher daily mortality counts compared to those found in maize-free areas (Samson-Robert et al., 2017).

In conclusion, this study demonstrated that clothianidin was transported from seed coated maize and exuded via guttation under Turkish environmental conditions. The concentration of clothianidin in guttation fluid declined over time to a low level for both years. The LD_{50} for clothianidin was lower than reported to published literature; therefore, clothianidin can be classified as one of the most potent insecticide to honey bees *A. mellifera anatoliaca*. In the laboratory experiments, honey bees exposed to clothianidin from guttation fluid from treated maize crop had a wide range of acute oral toxicity and peak residue concentrations coincided with high honey bee mortality indicating the relation between residue concentration and mortality. Also, in the laboratory test clothianidin toxicity increased with exposure time, varying about twofold from 4 to 48 h in both years. Thus, the present study documented clothianidin residues in guttation fluid collected from maize in the field and their effects on the mortality of honey bees in the laboratory. However, the effects of field conditions need to be investigated in future work since the effects of environmental stress factors and realistic water consumption behavior are not addressed in the laboratory tests. Also, winter survival of colonies near clothianidin treated field should be investigated for since bees will consume clothianidin deposited pollen and honey in the hive. In addition, future investigations should consider the effects of other insecticides applied to seed both individually and in a combination.

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