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Do neonicotinoid insecticides impaired olfactory learning behavior in *Apis mellifera*?

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Abstract

Bee's population is declining and disappearing at alarming rate. There are many factors responsible for declining the population of bees including diseases, natural enemies, environmental conditions and pesticides. Insecticides play its role dramatically for their population decline and neonicotinoid insecticides are critically important due to their wide application for pest control. Keeping in view of above problem, effect of neonicotinoid insecticides on olfactory learning behavior in *Apis mellifera* was observed using Proboscis Extension Reflex (PER) method. In this method, bees were harnessed in centrifuges tubes and feed on insecticides mixed sugar solution after three hours hunger. Bees were checked by feeding on non-treated sugar solution to observe PER response. Minimum proboscis extension was observed for acetamiprid and imidacloprid with 26% and 20% respectively at their recommend field doses while it was maximum for dinotefuran and thiamethoxam with 73% and 60% respectively. Only 40% bees showed response when exposed at 1/10 concentration of field dose for imidacloprid and the least at 1/100 of field dose. At control (Sugar solution) about 90% bees showed PER response. Among these neonicotinoid insecticides tested, imidacloprid and acetamiprid were the most damaging which impaired the olfactory learning performance in *Apis mellifera*.

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Introduction

Pollinators, specifically bees, are very essential creatures supporting biodiversity by providing vital pollination services for wild and managed crops. Food we eat depends on pollination and about 70% of wild and managed crops are pollinated by bees including honeybee and other pollinators (Klatt *et al*., 2013; Imran *et al.,* 2017). Despite their vital importance, population of honeybees is declining throughout the world (Dainat *et al*., 2012). Beekeepers have recorded continuous weakening of *Apis mellifera* fitness and increased colony losses since 1990. Though, it was not until the sudden rise of colony collapse disorder in the US in 2006 that has raised the concern of decreasing this vital pollinator (Van Engelsdorp *et al*., 2008). This colony collapse disorder results sudden disappearance of honeybees from the hive containing sufficient amount of pollen and nectar. At first it

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was considered that disappearing of bees in winter is common in beekeeping, but latter it was investigated to be associated with a group of insecticides (Van Engelsdorp *et al*., 2007).

Uses of Insecticides on agricultural crops are common and also important to control the insect pests (Mpumi *et al*., 2016). These insecticides have adverse effect on physiological and behavioral activities of honeybees and ultimately decreasing the population of bees (Henry *et al*., 2012). Among these, neonicotinoid got more attention because it impaired the colony health at even sub-lethal levels (Goulson, 2013). This neonicotinoid group is systemic in nature, when applied on plants to control insect pest of crops it absorbed and spreads throughout the plants parts and also in pollens and nectar. Among neonicotinoid, imidacloprid is the largest used insecticides throughout the world and play role in disappearing the bees with very little quantity in bee food sources (Goulson, 2013; Johnson and Pettis, 2014).

Long-lasting oral toxicity changes the gustatory receptiveness of adult honeybees and effect on their olfactory learning performances (Imran *et al*., 2018). Proboscis extension response studies showed that these insecticides when administered to bees affected their learning behavior (Goñalons and Farina, 2015). Honeybee larvae with on 0.04 ng of imidacloprid showed decreased synaptic density and later impaired olfactory learning performance at adult stage (Peng and Yang, 2016). When a worker honeybee leaves the hive, she take some amount of honey from its hive that provide energy during flight activity, the amount of honey taken depend upon the distance between the food source and hive (Dively *et al*., 2015). Residues present in the honey contaminate the bees and damage its orientation phenomena to reach their food source and return (Maxim and Sluijs, 2013). Crops sprayed also contaminate these foragers and ultimately damage the whole colony that lead to colony collapsed disorder (CCD) (Blacquière *et al*., 2012; Genersch *et al*., 2010). Beehives made up of trees treated with neonicotinoids insecticides could have residues which may lead to colony damage (Beekman and Ratnieks, 2000).

Many insect pests of major corps in Pakistan and other Asian countries are managed by using various types of systemic insecticides and one of them is neonicotinoid group. Forager bees visiting these crops for collecting pollen and nectar get exposed to their residues which ultimately decrease the population of pollinators (Khan *et al*., 2013). Due to lack of suitable regulatory measures and agricultural community knowledge, these insecticides in Pakistan are not used properly to get benefit. A very few toxicological studies have been conducted to check the susceptibility level of honey bees against neonicotinoids. Present study aims to explore the effect of neonicotinoid insecticides on olfactory learning behavior in honeybee, *Apis mellifera*.

Materials and Methods

The research study was carried out to explore the effect of neonicotinoid on olfactory learning behavior in *Apis mellifera* under controlled laboratory condition in the Department of Entomology, The University of Poonch Rawalakot, Azad Jammu and Kashmir during 2017-2018.

Source of experimental insect

The study was conducted *Apis mellifera* workers. Honeybee colony was obtained from professional beekeeper apiary on. Before selection, colony was checked thoroughly to minimize the risk of pests and diseases. Personal protective measures (bee veils and gloves) were used during collection from the hive with the help of 50 ml falcon tubes.

Experimental laboratory conditions

All the experimental equipment used during the bioassay were cleaned with 75% ethyl alcohol to minimize the disease risk on bees. The study was conducted under controlled laboratory condition maintained at 25±2^{o}C temperature and 60-70% relative humidity.

Insecticides and their concentrations

Technical grade insecticides of neonicotinoid group i.e., acetamiprid, nitenpyram, thiacloprid, dinotefuran, thiamethoxam, clothianidin and imidacloprid were obtained from pesticide market. Olfactory learning behavior was tested on three concentrations of each insecticides i.e., FD, FD/10 and FD/100 (FD= field dose) by exposing fifteen bees on each concentration.

Proboscis extension reflex test

We used olfactory learning performance of individual honeybees by using Proboscis Extension Reflex (PER). This

method is commonly used for testing learning performance and memory ability of living organism mostly in bees (Giurfa and Sandoz, 2012). In this experiment, 1ml centrifuge tubes were used for fixing bees. Honeybee's workers were collected from the bee colony with the help of 50ml falcon tubes. These were placed in refrigerator for some time to slow down their flying activity. Centrifuge tubes were cut from bottom and a bee was fixed in each tube and pushed upward until its head come out and these bees were fixed with the help of sticking tape. After placing bees in harness, individual bee was feed with 50% fresh sugar solution with the help of straw to check their proboscis extension response. Three concentrations of each insecticide i.e., at FD, FD/10 and FD/100 were mixed with sugar solution and used as nectar for feeding. At each concentration of insecticide, fifteen bees were tested. After feeding with insecticides mixed sugar solution all bees were starved for three hours to check insecticides effects. Proboscis extension response was checked after three hours starving by feeding each bee with untreated sugar solution. Bees that extend their proboscis with untreated sugar solution were considered healthy and considered had no effect on their learning performance. Those bees that showed no response on feeding with untreated sugar solution were considered affected learning performance and for statistical analysis only percentage PER was calculated PER response

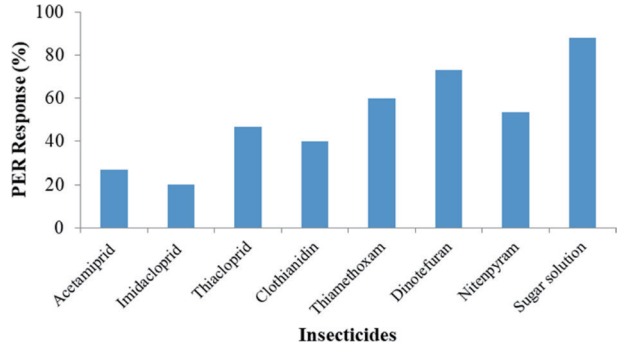

Fig. 1. Proboscis Extension Reflex (PER) response of honeybees at field dose rates FD*of neonicotinoid insecticides when mixed with sugar solution.
*Field Dose

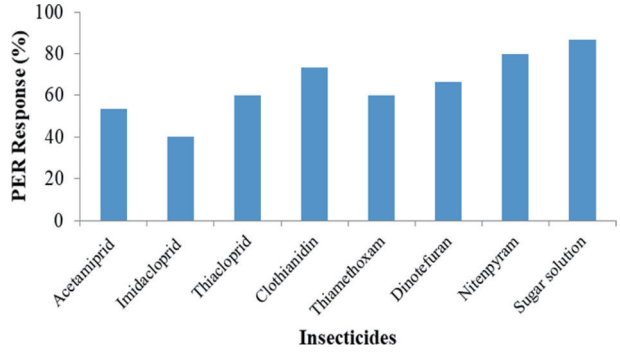

Fig. 2. Proboscis Extension Reflex (PER) response of honeybees at field dose rates FD/10*of neonicotinoid insecticides when mixed with sugar solution.

Results and Discussion

Results of neonicotinoid insecticides i.e., acetamiprid, imidacloprid, thiacloprid, clothianidin, thiamethoxam, dinotefuran and nitenpyram at their recommended field doses on olfactory learning behavior of *A. mellifera* showed acetamiprid and imidacloprid most toxic insecticides as compared to others tested insecticides. Bees exposed on these insecticides had their different proboscis extension reflex (PER) responses. It was minimum for acetamiprid and imidacloprid with 26% and 20%, respectively at recommended field dose (Fig. 1) which showed that these two insecticides impaired the learning ability of bees. Maximum proboscis extension reflex response was observed at dinotefuran with 73% indicate less toxicity and lest damaging effect on learning ability of honeybee. At control treatment (only used 50% sugar solution) 88% bees showed PER response. (Fig. 1).

At recommended field dose/10 (FD/10), imidacloprid was the most toxic insecticide as compared to all others neonicotinoid. Only 40% bees showed proboscis extension reflex (PER) response when feed on imidacloprid at FD/10, showing that this concentration also impaired the learning ability of honeybee (Fig. 2). Maximum PER response was observed at nitenpyram with 80% and control with 86.66% showed that maximum bees showed their proboscis extension response (Fig. 2). At recommend field dose/100 (FD/100), imidacloprid impaired the memory of honeybee even at this sublethal level. Maximum proboscis extension response was observed at control and thiamethoxam and minimum for imidacloprid with 93.33%, 86.66% and 66.66%, respectively (Fig. 3).

Insecticides are responsible for declining of bee's population (Whitehorn *et al*., 2012). This group of insecticides not only damaged physiological activity but also disturbed behavioral activities of honeybees (Tavares *et al*., 2015). Imidacloprid the most toxic insecticides that impaired the olfactory learning behavior in honeybee which have negative effect on immune response with strong effect on haemocyte counts in honeybee

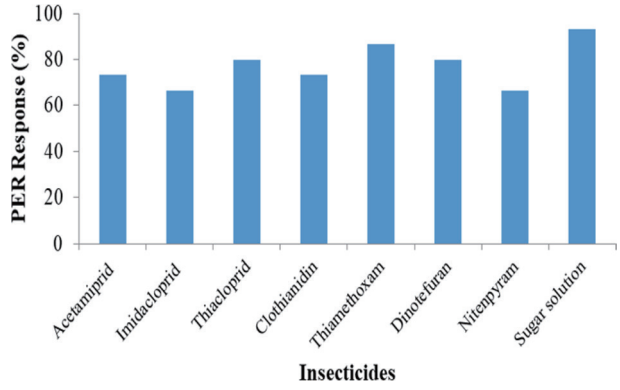

Fig. 3. Proboscis Extension Reflex (PER) response of honeybees at field dose rates FD/100*of neonicotinoid insecticides when mixed with sugar solution.

(Brandt *et al*., 2016).

Insecticides of this group are referred as systemic in nature because when applied on plant, absorbed by plants roots and then spread to all parts including nectar and pollen (Elbert *el al*., 2008). When these insecticides were mixed with pollen, they showed Imidacloprid and clothianidin most toxic at their recommended realistic field doses than other neonicotinoids. Honeybee workers can be exposed to neonicotinoid insecticides with other way as during pollen collection which impact on bees returning to hive and ultimately damage colony health (Dively *et al*., 2015). Larval stage of *Apis cerana* when exposed with imidacloprid showed that its 0.24ng/bee had significant effect on larval stage and olfactory learning ability of adult bee (Tan *et al*., 2015). Imidacloprid was the most toxic insecticide among all other tested neonicotinoid insecticides with minimum proboscis extension response at its recommended field dose. In European honeybee, imidacloprid impaired learning ability when exposed short term with 59ug/L (Ramirez-Romero *et al*., 2008).

Brood of controlled European honeybee *Apis mellifera* when were treated with neonicotinoid insecticides showed lower learning acquisition as compared to treated on adults workers. After the emergence of new worker that was treated at larval stage exhibit poor olfactory learning performance as compared when treated at adult's stage of worker (Behrends *et al*., 2008). Similar finding on *Apis mellifera* when exposed on neonicotinoid insecticides by using ingestion method both by pollen and nectar was found, i.e. maximum mortality was at imidacloprid as compared to others neonicotinoid insecticides (Imran *et al*., 2018). A same result was found in our finding i.e. minimum proboscis extension reflex PER response was observed when bees were exposed on imidacloprid.

Conclusion

Finding of this study clearly showed that neonicotinoid insecticides at recommended field dose and on its dilution had significantly effect on olfactory learning performance in honeybee *Apis mellifera*. Imidacloprid was found the most toxic insecticide that damaged olfactory learning performance when compared to all other neonicotinoid insecticides with less PER response. Still research is needed to find out the sensitivity mechanism that impaired learning performance.

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Authors' Contributions

All authors contribute equally for collection, conducting experiment, data collection, and analyses and in write-up of this manuscript. .

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