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Original Research Article

Assessment of sensitivity level of honeybee (*Apis mellifera*) to neonicotinoid insecticides

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Abstract

Declining population of honeybee day by day is the major threat, and this decline is due to variety of stressors. As honeybees are considered most important pollinators of our managed and wild corps, we investigated the effect of neonicotinoids; acetamiprid, imidacloprid, thiacloprid and clothianidin on honeybees by ingestion and contact method. Finding of this study showed that among these neonicotinoids, imidacloprid and thiacloprid were most toxic insecticides with 100% mortality at realistic field doses by ingestion method with sugar solution as a substitute of nectar after the exposure of 3hrs. Honeybee's workers exposed with insecticides mixed pollen grains showed that imidacloprid and clothianidin were most toxic as compared to other insecticides at recommended field dose with 100% mortality and minimum mortality with 60% was observed at thiacloprid field dose after 3hrs exposure. The results of contact toxicity showed that all four insecticides were most toxic with wet filter paper with 100% mortality at recommend field dose after the exposure of 3hr, and with dry filter paper imidacloprid and thiacloprid showed 100% mortality and acetamiprid and clothianidin with 53.3% mortality after the exposure of 3hrs at field dose. Overall conclusion from the present study indicates that from these four insecticides imidacloprid was the most toxic as compared to all others on both ingestion and contact method at recommended field dose.

Keywords: Neonicotinoids, Insecticides, Sensitivity level, Honeybee, *Apis mellifera*

Introduction

Pollination is the fundamental requirement for healthier fruit set for more than 90% of flowering plant species in the hot and humid regions (Ollerton et al., 2011). Animal pollination is the demand of most plant species for their reproduction (Kremen et al., 2007). Honey bees are globally regarded as important crop pollinators. They have been introduced on an almost worldwide scale and are also valued for their honey production. Honeybees provide a fundamental role in the pollination of many field crops, fruit bearing plants and wild species (Winfree, 2008). Bees are known to pollinate among 71 most familiar crops out of hundred plant species that accounts for 90% of world's food supply (Gallaia et al., 2009). Honey bees have been widely and successfully used as pollinators in crop systems (Artz, et al., 2011; Morse and Calderone, 2000).

However, the population of honeybee is declining day by day due to intemperate uses of pesticides (Morse and Calderone, 2000). Generally, bee's colony parasitic mites and pathogens are controlled by using pesticides and also insecticides are sprayed to control insect pests of managed crops on which bees visit to collect pollen and nectar (Meixner, 2010). One possible cause of distressing bee mortality is the use of very active systemic insecticide called neonicotinoids (Wessler et al., 2016). Neonicotinoids are highly toxic to insects as compared to mammals and birds because they are unable to cross the blood-brain barrier due to the lack of a charged nitrogen atom at physiological pH.

Neonicotinoids are a class of highly selective neuro-active insecticides derived from the insecticide, nicotine which is accountable for bees' decline (Cresswell et al., 2012).

Many insecticides of neonicotinoid group have strong adversely effect on pollinators (Hassani et al., 2008) especially to the honeybee (*Apis mellifera*) causing behavioral disorder, homing ability and social behaviour (Maini et al., 2010; Desneux et al., 2007). In Pakistan these insecticides are recommended for the control of sucking pests, as they are most effective against thrips, jassid and whitefly (Bethke and Redak, 2008; Lopez et al., 2008).

Imidacloprid a neonicotinoid insecticide is the most widely spray and has drawn more attention to honeybee health problems than others (Chen et al., 2013). Exposure of neonicotinoids insecticides to honeybees can occur through inhalation, ingestion and contact (Girolami et al., 2009). There are many ways through which bees could be expected to expose to these insecticides, e.g. pollen colleting bees are different from those bees visiting for nectar. These bees just collect pollen and bring to their hive for young larvae and nurse bees hence the nurse bees and larvae exposed to neonicotinoids and their metabolites (Rortais et al., 2005).

Honeybee's foragers take some amount of honey before leaving hive, and this amount depend upon the distance between field and colony, therefore they can take more or less residues of insecticides (Maxim and Sluijs, 2013). Forager bees become contaminated when contact with insecticides spray crop and these residues ultimately contaminate the whole colony where they are normally observed in honey and bee bread (Blacquière et al., 2012; Genersch et al., 2010). Bee hives made up of trees treated with neonicotinoids could have residues which may cause trouble for bees (Beekman and Ratnieks, 2000).

In Pakistan many of these insecticides are used on many agriculture and fruit crops which ultimately decrease the pollinator's population. 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 manage honeybees therefore it is necessary to check the susceptibility level of honey bees against neonicotinoids. Our aim is to explore the effect of lethal and sub-lethal level of neonicotinoid and find out realistic field dose of neonicotinoids on honeybees.

Material and Methods

This research was carried out to find the sensitivity level of honeybee to neonicotinoid toxicity under controlled laboratory condition in the Department of Entomology, The University of Poonch Rawalakot, Azad Jammu and Kashmir during 2017-2018. All handling in the laboratory was made in red light to minimize the risk of honeybee flight and stinging.

Experimental insect and collection

The experimentation was carried out on worker honey bees *A. mellifera* (Hymenoptera: Apidae). A colony of honeybee was obtained from farmer honeybee apiary. To minimize the risk of disease and pest attack on bees, colony was checked thoroughly before selecting. To keep the bees' calm smoker was used and then bees were collected from the colony with the help 50ml falcon tubes. Bee veils and gloves were used for personal protection.

Laboratory condition

To minimize the risk of disease attack on experimental bees, all the experimental equipment was cleaned with a piece of cotton soaked with 75% ethyl alcohol. The laboratory conditions were maintained at 25±2ºC and 60-70% relative humidity.

Bioassay and insecticide

Four insecticides of neonicotinoid groups i.e. acetamiprid, imidacloprid, thiacloprid and clothianidin (125µl/100ml, 200µl/100ml, 200µl/100ml and 200µl/100ml, respectively) obtained from commercial chemical supplier were used to test their toxicity on honeybee. For each treatment, six to eight serial concentration levels were prepared with control to compare the toxicity. Insecticides stock solution was prepared by using recommended field dose of insecticide.

Test procedure

For experiment small plastic boxes were used. Two different methods ingestion and contact were used to test these insecticides. The first part of experiment was ingestion method; in this method different concentrations of these four insecticides were mixed in 50% sugar solution and used as a nectar substitute.

These insecticides mixed sugar solution was be filled in 15ml falcon tubes having a small hole on bottom and used as a bee's feeder. In second ingestion method honeybee pollen were treated with these different

concentrated insecticides solution and used for bees feeding. In the second part of experiment, contact method was used to test toxicity of these four insecticides. Dip method was used, in this method filter papers were cut with the size of plastic box and dip into different insecticidal concentration solution and these filler papers were placed in plastic boxes. Bees were released in each box with non-treated sugar solution and pollen. Five bees were released in each box with three replications (Fig. 1).

Data recording and analysis

Mortality data were recorded after 3hr, 6hr, 24 and 48 hours of the treatment. Bees were considered as dead when they failed to show any movement with gentle touch with blunt needle and percentage mortality was calculated.

Results and Discussion

Ingestion method

Comparison toxicity of four neonicotinoid insecticides i.e. acetamiprid, imidacloprid, thiacloprid and clothianidin against honeybee *A. mellifera* by ingestion method with sugar solution showed that imidacloprid, thiacloprid and clothianidin were most toxic insecticides with 100% mortality after 3hrs, 100% after 6hrs and 100% after 6hrs, respectively at recommended field dose (Table 1). The results showed that maximum mortality was observed at field dose and mortality level decrease with serial dilution of field doses (FD, FD/10, FD/100, FD/1000 and FD/10000) (Table 1). Acetamiprid showed less toxicity at recommended field dose with 86.6% mortality and this level decreased with decrease of serial dilution concentrations after 3hr exposure (Table 1).

Similarly, the results of these four neonicotinoid insecticides against honeybee workers by ingestion method with pollen showed that imidacloprid and clothianidin were most toxic for honeybee workers at field dose with 100% mortality when mixed with pollen. All the bees died at recommended field dose after 3hr of exposure and mortality level decrease with decrease the concentration level. Minimum mortality was observed at thiacloprid at FD with 60%, 33% at FD/10 and 0.0% at FD/100 (Table 2).

Contact method

In contact method filter paper was use as a contact surface and dip with these four neonicotinoid insecticides. Two methods were used in this; one was wet filter method and other dry filter paper. The results of wet filter paper showed that all four insecticides were most toxic at recommended field dose with 100% mortality. All the bees died when came in contact with wet filter paper dip at recommended field dose of these insecticides after the exposure of 3hr (Table 3). So the maximum mortality was observed at field dose and this mortality decreased with the serial dilution concentration. The results showed that even maximum mortality was observed at thiacloprid with 100% mortality when bees were exposed with wet filter paper at FD/10,000 (Table 3).

The toxicity results of these four insecticides with filter paper impregnation method showed that imidacloprid and thiacloprid were most toxic insecticides against honeybee workers. Maximum mortality (100%) was observed at imidacloprid at recommended field dose after the exposure of 3hrs. Minimum mortality at field dose (53.3 %) was observed at both acetamiprid and clothianidin after 3hrs interval and this mortality level decreased with the decrease of concentration levels (Table 4).

Overall finding of these four neonicotinoids showed that imidacloprid and thiacloprid were the most toxic insecticides with maximum mortality at realistic recommended field dose against honeybee workers by ingestion method with sugar solution (Table 1). When these insecticides mixed with pollen the results showed that 100% mortality was observed at Imidacloprid and clothianidin at recommended field dose (Table 2). Contact toxicity results showed that these all four insecticides were most toxic with wet filter paper at recommend field dose after the exposure of 3hr, and with dry filter paper imidacloprid and thiacloprid showed maximum mortality at field dose (Table 3, 4)

Honeybees are worldwide regarded as vital crop pollinators; provide a fundamental role in the pollination of many field crops, fruit bearing plants and wild species (Winfree, 2008). These neonicotinoids pose the largest risk to honeybees at a global agriculture and ecosystem (Whitehorn et al., 2012). Previous research indicates that these neonicotinoids not only affect the physiology but also behaviour of bees (Tavares et al., 2015). Due to wide use of neonicotinoids in Pakistan, it is very necessary to evaluate the effect of neonicotinoids on *Apis mellifera* called European honeybee.

Our results showed that among these four insecticides imidacloprid has the most toxic effect on bee's workers at recommended field dose by ingestion method with sugar solution as a substitute of nectar but also toxic at

FD/10 concentration. Similar results was found by researcher in University of Hertfordshire in 2013; they found that acute oral toxicity of Imidacloprid is much higher than thiacloprid acute oral toxicity against honeybee (LD_{50}48h = 17.32 ug/bee for thiacloprid and oral acute LD_{50}48h = 0.0037 ug/bee for imidacloprid). Research conducted on haemocyte counts in honeybee show that Imidacloprid have strong effects on the total hemocyte counts as compare to other neonicotinoids (Brandt et al., 2016). Our findings, when these insecticides were mixed with pollen showed that imidacloprid and clothianidin were most toxic at recommended realistic field dose as compared to other neonicotinoids. As previous research showed that honeybees workers can be also exposed to neonicotinoid insecticides with other way such as during pollen collection and this way of exposure also impact on bee's colony health (Dively et al., 2015).

In our study we also find out the effect of these four neonicotinoid insecticides on honeybee by contact method by using dry and wet filter paper as a surface. Finding showed that wet surface killed all the bees at recommended field dose after the exposure of 3hr. This finding may be due to the direct body contact of honeybee with wet surface of filter paper because this concentration contains maximum toxicity and has knockdown effect. Neonicotinoids not only increased the bee mortality but also decreased the health of queen and lead colony to queenlessness over time. This acute toxicity to bees become double when mixed with fungicide and reduces honeybee health (Tsvetkov et al., 2017). Low toxic results was appeared with dry filter paper; showed that Imidacloprid and thiacloprid was most toxic insecticides against honeybee as compared to others. Previous finding showed that forager bee population reduced returning to their hive when exposed with a single dose of thiamethoxam a neonicotinoid insecticide (Henry et al., 2012)

Neonicotinoids (imidacloprid and clothianidin) also effect on foraging activity of bees and longtime flight ability of forager even with sub-lethal doses (Schneider et al., 2012). Colony collapse disorder called CCD is also associated with neonicotinoids. Research showed that thiamethoxam can detract foragers and reduce their ability to return their hives. This can lead high mortality and put colony at CCD danger (Henry et al., 2012). Most widely used insecticide from this group is imidacloprid and is associated for the reduction of vitellogenin called egg yolks that regulate bee's development (Abbo et al., 2017). Examination of different body parts of honeybee after exposure with imidacloprid showed that residues found in various body parts midgut, head abdomen etc. of honeybee (Suchail et al., 2004). Previous study also showed that neonicotinoids especially imidacloprid effect on memorizing ability of honeybees and this lead abnormal foraging ability during flower visiting (Decourtye et al., 2004; Yang et al., 2008).

Figure 1. Picture representing the contact and ingestion bioassay method using small plastic boxes at recommended field dose of neonicotinoid insecticides

Table-1: Toxicity of four neonicotinoid insecticides against *Apis mellifera* at recommended field dose and its serial dilation by ingestion method with sugar solution

Insecticides	Time	Expose bees	FD	FD/10	FD/100	FD/1000	FD/10000	Control
Acetamiprid	3hrs	15	86.7	20.0	0.0	0.0	0.0	0.0
	6hrs	15	93.3	33.3	0.0	0.0	0.0	0.0
	24hr	15	100.0	60.0	53.3	46.7	20.0	33.3
	48hr	15	100.0	100.0	80.0	53.3	33.3	33.3
Imidacloprid	3hrs	15	100.0	100.0	66.7	26.7	6.7	0.0
	6hrs	15	100.0	100.0	73.3	40.0	13.3	0.0
	24hr	15	100.0	100.0	86.7	46.7	60.0	0.0
	48hr	15	100.0	100.0	100.0	100.0	80.0	26.7
Thiacloprid	3hrs	15	80.0	40.0	6.7	13.3	40.0	0.0
	6hrs	15	100.0	40.0	6.7	20.0	40.0	0.0
	24hr	15	100.0	73.3	20.0	60.0	60.0	0.0
	48hr	15	100.0	80.0	73.3	73.3	80.0	26.7
Clothianidin	3hrs	15	80.0	40.0	6.7	13.3	40.0	0.0
	6hrs	15	100.0	40.0	6.7	20.0	40.0	0.0
	24hr	15	100.0	73.3	20.0	60.0	60.0	0.0
	48hr	15	100.0	80.0	73.3	73.3	80.0	26.7

Table-2: Toxicity of four neonicotinoid insecticides against *Apis mellifera* at recommended field dose and its serial dilation by ingestion method with pollen

Insecticides	Time	Expose bees	FD	FD/10	FD/100	FD/1000	FD/10000	Control
Acetamiprid	3hrs	15	86.7	20.0	0.0	0.0	0.0	0.0
	6hrs	15	93.3	33.3	0.0	0.0	0.0	0.0
	24hr	15	100.0	46.7	40.0	33.3	13.3	6.7
	48hr	15	100.0	66.7	46.7	40.0	13.3	13.3
Imidacloprid	3hrs	15	100.0	80.0	53.3	6.7	0.0	0.0
	6hrs	15	100.0	80.0	60.0	13.3	13.3	0.0
	24hr	15	100.0	86.7	66.7	33.3	26.7	0.0
	48hr	15	100.0	93.3	86.7	40.0	40.0	20.0
Thiacloprid	3hrs	15	60.0	33.3	0.0	0.0	0.0	0.0
	6hrs	15	73.3	46.7	6.7	6.7	13.3	0.0
	24hr	15	80.0	53.3	20.0	26.7	26.7	0.0
	48hr	15	80.0	66.7	40.0	40.0	46.7	13.3
Clothianidin	3hrs	15	100.0	80.0	53.3	6.7	0.0	0.0
	6hrs	15	100.0	80.0	60.0	13.3	13.3	0.0
	24hr	15	100.0	86.7	66.7	33.3	26.7	0.0
	48hr	15	100.0	93.3	86.7	40.0	40.0	20.0

Table-3: Toxicity of four neonicotinoid insecticides against *Apis mellifera* at recommended field dose and its serial dilation by contact method with wet filter paper

Insecticides	Time	Expose bees	FD	FD/10	FD/100	FD/1000	FD/10000	Control
Acetamiprid	3hrs	15	100.0	40.0	33.3	40.0	6.7	20.0
	6hrs	15	100.0	73.3	66.7	46.7	46.7	26.7
	24hr	15	100.0	93.3	80.0	66.7	60.0	26.7
	48hr	15	100.0	100.0	86.7	86.7	86.7	33.3
Imidacloprid	3hrs	15	86.7	80.0	0.0	33.3	20.0	0.0
	6hrs	15	100.0	86.7	40.0	53.3	33.3	0.0
	24hr	15	100.0	100.0	60.0	86.7	80.0	0.0
	48hr	15	100.0	100.0	93.3	93.3	93.3	20.0
Thiacloprid	3hrs	15	80.0	53.3	53.3	40.0	86.7	0.0
	6hrs	15	100.0	80.0	60.0	40.0	86.7	0.0
	24hr	15	100.0	100.0	100.0	93.3	100.0	66.7
	48hr	15	100.0	100.0	100.0	100.0	100.0	80.0
Clothianidin	3hrs	15	100.0	93.3	93.3	40.0	60.0	6.7
	6hrs	15	100.0	93.3	93.3	53.3	73.3	13.3
	24hr	15	100.0	100.0	100.0	100.0	66.7	26.7
	48hr	15	100.0	100.0	100.0	100.0	93.3	40.0

Table-4: Toxicity of four neonicotinoid insecticides against *Apis mellifera* at recommended field dose and its serial dilation by contact method with dry filter paper

Insecticides	Time	Expose bees	FD	FD/10	FD/100	FD/1000	FD/10000	Control
Acetamiprid	3hrs	15	53.3	33.3	26.7	33.3	33.3	20.0
	6hrs	15	53.3	40.0	40.0	40.0	60.0	20.0
	24hr	15	53.3	53.3	60.0	46.7	93.3	20.0
	48hr	15	100.0	93.3	86.7	73.3	93.3	0.0
Imidacloprid	3hrs	15	93.3	66.7	26.7	20.0	40.0	0.0
	6hrs	15	100.0	80.0	40.0	46.7	53.3	0.0
	24hr	15	100.0	100.0	60.0	93.3	80.0	0.0
	48hr	15	100.0	100.0	80.0	93.3	93.3	13.3
Thiacloprid	3hrs	15	100.0	73.3	66.7	80.0	60.0	0.0
	6hrs	15	100.0	73.3	66.7	80.0	66.7	0.0
	24hr	15	100.0	80.0	66.7	86.7	66.7	0.0
	48hr	15	100.0	80.0	100.0	86.7	73.3	33.3
Clothianidin	3hrs	15	53.3	40.0	46.7	33.3	20.0	6.7
	6hrs	15	66.7	53.3	60.0	60.0	26.7	6.7
	24hr	15	86.7	80.0	80.0	73.3	40.0	6.7
	48hr	15	100.0	93.3	100.0	93.3	46.7	33.3

Conclusion

This research shows a clear impact of neonicotinoids at recommended realistic field doses on honeybees. Overall results showed that among these four neonicotinoids, insecticides imidacloprid was the most toxic against bees during ingestion method with sugar and honey and also during contact method with maximum mortality as compared to others. But further study with molecular biology techniques is needed to find out the sensitivity mechanism. In conclusion we also request to all farmers and researchers please find ways to kill pests not honeybees.

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