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Research Article
Toxic Effects of Neonicotinoid Insecticides on a Field Strain of Cotton Leafworm, *Spodoptera littoralis*

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Abstract
Background and Objective: The potency and selectivity of neonicotinoid insecticides against agricultural pests has lead to increase their use in control programmes. This study aimed to assess the status of indirect exposure on feeding mortality of *Spodoptera littoralis* to the recommended field rates of neonicotinoids, thiamethoxam (TMX), thiacloprid (TCD), imidacloprid (ICD) and acetamiprid (AMD) against 2nd and 4th larval instars of *S. littoralis* in two types of feeding trial in the laboratory. **Materials and Methods:** In the first trial, three different concentrations representing ½-(HFR), 1-(FR) and 2-(DFR) fold the recommended field application rate (FR) of each insecticide were separately mixed with artificial larval diet. Second and 4th instar larvae of *S. littoralis* were exposed to the treated diets for 1 day without further feeding (direct effect), 5 days (accumulative effect) or 1 day then on untreated artificial diet until pupation (transitional effect). In the second trial, tomato plants were sprayed with the recommended FR of each insecticide under field conditions. Leaves with different residual ages of each insecticide, together with leaves from unsprayed control plants were picked daily and fed to 2nd instar larvae in the laboratory for 5 successive days. **Results:** The TCD showed the highest non-significant direct toxic effect on 2nd instar larvae of *S. littoralis*, with shorter LT_{50} compared with other insecticides. The TMX was the most effective insecticide against 2nd instar (accumulative effect) and 4th instar (direct and accumulative effects) with a significantly shorter LT_{50} in 4th instars. In all trials, AMD had the least toxic effect compared other insecticides tested. The TMX, TCD and ICD significantly reduced pupation percentage but did not affect adult emergence compared with control suggesting low transitional toxic effect. Feeding 2nd instar larvae on tomato leaves treated with insecticides showed significant direct and accumulative mortality effects and a shorter LT_{50} with only TCD. **Conclusion:** Laboratory assays indicated that thiacloprid and thiamethoxam had the highest significant effects on *S. littoralis* which need to be confirmed in a larger-scale field trials.

Key words: *Spodoptera littoralis*, neonicotinoid insecticides, time-mortality, larval weight, feeding damage

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neonicotinoid insecticides used to protect various crops from a number of pests including whiteflies, aphids and leafhoppers[1-3]. They act as agonists of insect nicotinic acetylcholine receptors (nAChRs) in the central nervous system[4,5] resulting in excitation and paralysis followed by insect death. Neonicotinoids have strong insecticidal activities especially against homopteran pests and also used to control many coleopteran and some lepidopteran pest species[6].

The cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is a highly polyphagous pest of many cultivated crops. It is distributed in tropical and subtropical areas[7] that represent of most parts of Africa, Southern or Mediterranean Europe and Middle East[8]. *Spodoptera littoralis* attacks a wide range of important crops causing considerable feeding damage and economic crop losses. At least 87 plant species of more than 40 plant families including vegetable, fruit and ornamental crops can be attacked by this pest[9,10]. The overlapping of different crops that serve as hosts of *S. littoralis* throughout the annual cropping cycle facilitates high population densities during different seasons. Accordingly, control strategies of *S. littoralis* in Egypt rely on the intensive use of more than 40 non-selective chemical insecticides of organophosphates, carbamates, synthetic pyrethroids and other groups[7,11].

As neonicotinoid insecticides are increasingly used for the control of different pest species on a wide variety of crops, non-target spodopteran insects are indirectly exposed[12]. Also, due to the continuous presence of *S. littoralis* throughout the year on a wide range of host plants[13], it has been indirectly exposed to these insecticides as a non-target insect pest. Moreover, although the effects of various neonicotinoid insecticides on many insect pests were documented, their effects on *S. littoralis* had not been well studied. The work reported here thus aimed to characterize and compare the efficacy of four neonicotinoid insecticides (TMX, TCD, ICD and AMD) against *S. littoralis*. Direct, accumulative and transitional toxic effects of neonicotinoids were evaluated on a field strain of *S. littoralis*.

MATERIALS AND METHODS

Insect rearing: Egg masses of a field colony of *S. littoralis* were collected from cotton fields of Sharkia Governorate in North-East Egypt. Hatched larvae were reared on an artificial diet or on clean, fresh tomato leaves in the laboratory at 27±2°C, 60-70% RH and 16 L:8 D photoperiod. The diet was prepared according to the method described by El-Sheikh[7]. To get the required larvae for experiments, 50-60 pupae were placed in 5 L glass containers until adult emergence. The emerged adults were provided with clean branches of Tafla (*Nerium oleander*) for oviposition and supplied with 10% (W/V) of honey solution. Leaves with freshly deposited eggs were collected daily and placed in plastic containers (12×18×5 cm) provided with artificial diet (for use in experiments with artificial diet) or clean and fresh tomato leaves (for use in experiments).

Insecticides used: The following neonicotinoid insecticides were used: Thiamethoxam (TMX; Thiamex 25% WG; MAC-GmbH, AlteLandst., Germany), thiacloprid (TCD; Blanch 48% SC; Jiangsu Flag Chemicals Industry Co., Ltd., Nanjing, China), imidacloprid (ICD; Imidazed 20% SC; United Phosphorus Ltd., Mumbai, India) and acetamiprid (AMD; Mospilan 20% SG; Nippon Soda Co., Ltd, Tokyo, Japan).

Bioassay experiments

Insecticide-containing diet assay: Direct and accumulative toxicity of TMX, TCD, ICD and AMD were investigated by mixing different field rates (HFR, half field rate; FR, field rate and DFR, double field rate) of the tested insecticides with semi-synthetic larval diet. Contaminated diets were prepared by mixing insecticides-concentrations individually with diet at ~35°C to give the tested field rate of each insecticide. Concentrations representing HFR, FR and DFR were 100, 200 and 400 μg mL^{-1} diet (TMX), 288, 576 and 1152 μg mL^{-1} diet (TCD), 125, 250 and 500 μg mL^{-1} diet (ICD) and 25, 50 and 100 μg mL^{-1} diet (AMD), respectively. Ten 2nd or 4th instar larvae were transferred into 9 cm glass Petri-dishes containing cubes of clean diet (control) or contaminated diet (treatments) in triplicates. Larval mortality was recorded after 24 h of exposure (direct toxicity) and daily for 5 days of exposure (accumulative toxicity).

Time-mortality response assay: To calculate the median lethal time (LT_{50}) of the tested insecticides, 2nd and 4th instar larvae of *S. littoralis* were exposed to the FR of TMX, TCD, ICD or AMD mixed with artificial diet. Thirty larvae of each instar were separately and continuously exposed to diet containing previously mentioned FR of each insecticide in 9 cm glass Petri-dishes. Larval mortality was recorded at 12 h intervals until 100% mortality or pupation with repeating experiments three times. The LT_{50} was calculated for each insecticide using the ViStat 2.1 program[14].

Biological effects: To investigate the effect of neonicotinoid insecticides on the development of *S. littoralis*, 4th instar larvae were allowed to feed on artificial larval diet containing the FR of each insecticide for 24 h. Following this, any live larvae were transferred onto clean diet and observed for mortality or development to adult emergence. Fifty larvae were used in five replicates for each insecticide treatment and for untreated controls. Mortality was recorded every 24 h after exposure and then daily until pupation. Larval and pupal durations, pupal weights, percentage pupation and adult emergence were also recorded.

Field application and effects on development and feeding: To investigate the effect of field applications of the neonicotinoid insecticides on larval development and feeding, tomato plants were grown, treated with the four neonicotinoids and leaves fed to *S. littoralis* larvae in the laboratory. Super strain B tomato seedlings were grown at the faculty farm in a rate of 10 seedlings/m^2 and given irrigation and fertilization as recommended. After about 10 weeks an area of 4 m^2 of tomato plants was selected for the treatments. The FR of each insecticide was prepared in 1 L of tap water and sprayed on 4 m^2 of tomato plants using a pre-compression sprayer (MATABI, KIMA-6). One hour after application, leaves of treated and untreated plots were collected at random from each treatment and placed separately in 10 glass jars containing 10th and 2nd instar larvae/each jar and covered with a small piece of fine cloth. New leaves were collected from treated and untreated plots every day and transferred to the corresponding treatments for a period of 5 days.

Larval mortality in the treatments and control was recorded daily for 5 days. Larval weight was recorded at the beginning of experiments and daily for all live larvae for 5 days. To determine the amount of feeding damage, treated and untreated leaves were weighed at the beginning of experiment and after 24 h of larval feeding. Three leaves which had not been used in the feeding experiments were subjected to estimate the percentage of normal weight decrease in leaves after 24 h at the same experimental conditions. The percentage decrease in leaf weight and increase in larval weight were calculated as follow:

$$\text{Decrease (normal+feeding) (\%)} = \frac{\text{FWB-FWE}}{\text{FWB}} \times 100 \qquad \text{(A)}$$

$$\text{Decrease (normal) (\%)} = \frac{\text{FWB-FWN}}{\text{FWB}} \times 100 \qquad \text{(B)}$$

$$\text{Decrease (feeding) (\%)} = \text{A-B}$$

where, FWB, FWE and FWN are leaf weight in the beginning, leaf weight after 24 h of exposure and leaf weight after 24 h with no larval feeding, respectively[7]:

$$\text{Increase (larval weight) (\%)} = \frac{\text{LWE - LWB}}{\text{LWE}} \times 100$$

where, LWE and LWB are larval weight after 24, 48, 72, 96 or 120 h of exposure to treated or untreated leaves and larval weight in the beginning of treatment, respectively[7]. For calculating increase percentage in larval weight, value of larval weight in the beginning was subtracted from values of larval weight after 24, 48, 72, 96 or 120 h divided by the same corresponding value after 24, 48, 72, 96 or 120 h.

Statistical analysis: The SPSS 14.0 for Windows software package was used to statistically analyze the differences in larval mortality, biological aspects, LT_{50}, percentage increase in larval weight and percentage decrease in leaves weight using least significant difference (LSD) of one-way ANOVA at $p<0.05$.

RESULTS

Toxicity of insecticides: Direct and accumulative toxicity of the tested insecticides on 2nd and 4th larval instars of *S. littoralis* are shown in Table 1 and 2, respectively. Data showed that although TCD is the most effective insecticide, the differences were not significant between this insecticide and TMX or ICD in direct effect. In accumulative toxicity, TMX showed the most effective insecticide followed by TCD with no significant differences between them in all field rates tested (Table 1). Toxicity of insecticides on 4th larval instars showed that TMX and TCD were the most effective insecticides with no significant differences between them in direct toxicity. While, TMX, TCD and ICD showed the almost same effect in accumulative toxicity as no statistical differences were noted among them (Table 2). In all cases, AMD showed the lowest significant ($p<0.05$) toxicity compared with other insecticides in both direct and accumulative effects on 2nd or 4th instar larvae of *S. littoralis*.

Time-mortality relationship: Time-mortality response was determined on 2nd and 4th instar larvae of *S. littoralis* using a FR of each insecticide (Table 3). Time required for 50% mortality was longer in 4th instar compared to 2nd instar larvae of each insecticide. The LT_{50} of AMD on 2nd or 4th

Table 1: Direct and accumulative toxicity of neonicotinoid insecticides to 2nd instar larvae of *S. littoralis* using insecticide-contaminated larval diet

		Field rates**		
Insecticides	n*	HFR	FR	DFR
Direct toxicity				
Thiamethoxam	30	33.0±10[a]	59.0±9[a]	71.0±15[ab]
Thiacloprid	30	41.0±18[a]	63.0±10[a]	84.0±4[a]
Imidacloprid	30	35.0±8[a]	59.0±11[a]	70.0±15[ab]
Acetamiprid	30	0.0±0[b]	36.0±8[b]	55.0±12[b]
Accumulative toxicity				
Thiamethoxam	30	97.0±3[a]	98.0±2[a]	100.0±0[a]
Thiacloprid	30	87.0±5[a]	93.0±3[ab]	99.0±1[a]
Imidacloprid	30	61.0±6[b]	87.0±6[b]	100.0±0[a]
Acetamiprid	30	47.0±11[c]	76.0±9[c]	91.0±2[b]

*Number of larvae (n) used for each field rate/experiment, Mean±SD of three separate experiments. Values followed by different letters in the same column (direct and accumulative toxicity independently) are statistically different at p<0.05

Table 2: Direct and accumulative toxicity of neonicotinoid insecticides to 4th instar larvae of *S. littoralis* using insecticide-contaminated larval diet

		Field rates**		
Insecticides	n*	HFR	FR	DFR
Direct toxicity				
Thiamethoxam	30	34.0±5[a]	49.0±7[a]	62.0±7[a]
Thiacloprid	30	34.0±11[a]	42.0±15[a]	53.0±10[a]
Imidacloprid	30	11.0±6[b]	16.0±6[b]	30.0±5[b]
Acetamiprid	30	0.0±0[c]	11.0±6[b]	27.0±8[b]
Accumulative toxicity				
Thiamethoxam	30	89.0±10[a]	96.0±4[a]	100.0±0[a]
Thiacloprid	30	75.0±9[a]	84.0±9[ab]	91.0±8[ab]
Imidacloprid	30	81.0±19[a]	89.0±10[ab]	93.0±7[ab]
Acetamiprid	30	40.0±7[b]	78.0±7[b]	88.0±10[b]

*Number of larvae (n) used for each field rate/experiment, Mean±SD of three separate experiments. Values followed by different letters in the same column (direct and accumulative toxicity independently) are statistically different at p<0.05

Table 3: Time-mortality response of 2nd and 4th instars of *S. littoralis* following continuous exposure to field application rates of neonicotinoid insecticides for 5 days using insecticide-contaminated larval diet

			Confidence limits	
Insecticides	n*	LT_{50} (h)	Lower	Upper
2nd instar				
Thiamethoxam	90	20.2[b]	15.0	25.0
Thiacloprid	90	17.2[b]	11.9	21.8
Imidacloprid	90	20.1[b]	13.4	26.6
Acetamiprid	90	47.3[a]	40.1	54.7
4th instar				
Thiamethoxam	90	25.0[c]	19.3	29.5
Thiacloprid	90	38.1[b]	32.2	43.4
Imidacloprid	90	47.5[b]	42.9	52.1
Acetamiprid	90	61.2[a]	57.0	65.6

*Number of larvae (n) used for each insecticide, LT_{50} values were considered significantly different when their CLs did not overlap, Source: El-Sheikh and Aamir[10] and El-Sheikh[7]

instars was significantly (p<0.05) longer compared to TMX, TCD and ICD. No marked differences were found among TMX, TCD and ICD on 2nd instar, while TMX was killing 50% of 4th instar significantly (p<0.05) faster (25 h) than TCD, ICD and AMD (38.1, 47.5 and 61.2 h, respectively).

Transitional effects of insecticides

Effect on the development: Transitional effects of neonicotinoid insecticides on the biological aspects (Table 4) showed that mortality percentage in 4th larval instar exposed to TMX and TCD was significantly (p<0.05) higher than ICD, AMD and control. The TMX caused 37% mortality by the end of larval stage comparing to 9% in the case of AMD. Subsequently, pupation (%) was significantly (p<0.05) lower in TMX, TCD and ICD compared to AMD and control, which showed 63, 68 and 86% pupation, respectively compared to 91 and 100% pupation in AMD and control. All the tested insecticides did not show significant differences in larval or pupal duration, pupal weight or percentage of adult emergence compared with control.

Effect on larval weight and feeding: The TCD insecticide was found to cause significant (p<0.05) direct or accumulative mortality against 2nd instar compared with other tested insecticides when exposed daily for 5 days to tomato leaves treated by FR. Time required for 50% death was significantly (p<0.05) shorter in TCD compared with other neonicotinoids (Table 5), which showed 67.2 h against 105.3, 114.2 and >120 h for TMX, ICD and AMD, respectively. The larval weight of 2nd instar decreased significantly (p<0.05) after 48 and 72 h of feeding on the tomato leaves treated by TMX and TCD and the marked decrease still noted with TCD only after 96 and 120 h compared to the rest insecticides tested or control (Fig. 1). Feeding damage due to 2nd larval instar feeding on insecticides-treated and untreated tomato leaves is shown in Fig. 2. Data showed that TCD treatment significantly (p<0.05) reduced feeding damage, followed by leaves treated with TMX and ICD with no statistical differences between them. Percentage of reduction in leaves weight treated with AMD did not show significant differences compared with control.

DISCUSSION

Neonicotinoid insecticides comprise 7 commercially marketed active ingredients of ICD, TMX, TCD, AMD, nitenpyram, clothianidin and dinotefuran[15]. They were commercialized to control many pests of different orders such

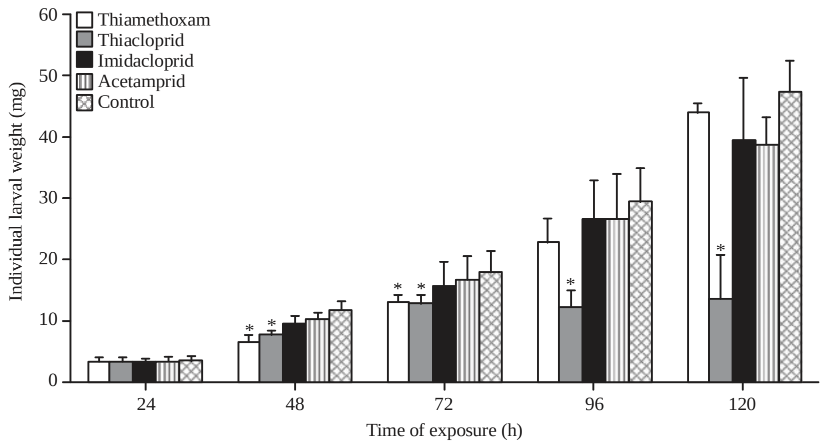

Fig. 1: Changes in weight of 2nd instar larvae of *S. littoralis* after exposure to tomato leaves treated with a field rate of neonicotinoid insecticides. Bars for each time period with an asterisk are significantly different to the untreated control at $p<0.05$

Table 4: Transitional effects of neonicotinoid insecticides on the development of 4th instar larvae of *S. littoralis*

Insecticide	n*	Larval stage: Mortality (%)	Larval stage: Duration (day)	Pupal stage: Pupation (%)	Pupal stage: Duration (day)	Pupal stage: Weight (mg)	Adult emergence (%)
Thiamethoxam	50	37 ± 5^a	20.6 ± 0.5^a	63.0 ± 10^b	7.5 ± 0.4^a	248 ± 23^a	98 ± 2^a
Thiacloprid	50	34 ± 2^a	21.1 ± 1.1^a	68.0 ± 8^b	7.3 ± 0.5^a	254 ± 13^a	91 ± 8^a
Imidacloprid	50	15 ± 3^b	20.8 ± 0.8^a	86.0 ± 7^b	7.4 ± 0.5^a	260 ± 22^a	96 ± 4^a
Acetamiprid	50	9 ± 3^c	20.9 ± 0.9^a	91.0 ± 8^a	7.6 ± 0.5^a	255 ± 15^a	92 ± 8^a
Control	50	0 ± 0^d	20.3 ± 0.9^a	100.0 ± 0^a	6.6 ± 0.5^a	254 ± 16^a	100 ± 0^a

*Number of larvae (n) used for each insecticide/experiment, Data are Mean±SD of three separate experiments. Mean values followed by different letters in the same column are statistically different at $p<0.05$

Table 5: Mortality and median lethal times (LT_{50}s) of 2nd instar larvae of *S. littoralis* following feeding on leaves of tomato plants treated with field rates of neonicotinoid insecticides

Insecticides	n*	Mortality±SD: Direct	Mortality±SD: Accumulative	LT_{50} (h)
Thiamethoxam	30	0.0 ± 0.0^b	60.0 ± 6.4^b	105.3^b
Thiacloprid	30	5.0 ± 1.6^a	91.5 ± 8.1^a	67.2^c
Imidacloprid	30	0.0 ± 0.0^b	51.5 ± 9.0^b	114.2^b
Acetamiprid	30	0.0 ± 0.0^b	26.5 ± 4.9^c	$>120.0^a$

*Number of larvae (n) used for each insecticide/experiment, Morality data are Mean±SD of three separate experiments. Values followed by different letters in the same column are statistically different at $p<0.05$

as Hemiptera, Coleoptera and Lepidoptera[6]. In Egypt, sucking pests are infesting different field and vegetable crops which are in the same time hosts of *S. littoralis*. Accordingly, *S. littoralis* indirectly exposes to different registered and used neonicotinoid formulations during field application. Hence, the aim behind this work was to evaluate different toxic effects of neonicotinoid formulations against *S. littoralis*. The TMX and TCD showed the most effective insecticides against *S. littoralis* larvae compared to ICD or AMD as obtained from insecticide-diet mixture or tomato application experiments. Although there is lack of review on the effects of neonicotinoids against *S. littoralis*, in agreement with current findings. *Ostrinia nubilalis* (Hübner), *Plodia interpunctella* (Hübner) and *S. litura* (Fabricius) showed high susceptibility to TMX compared to ICD, cyantraniliprole or spinosad[16,17]. Also, TMX showed an efficient, long-lasting activity against wireworms and false wireworms (*Agrotes* sp., *Melanotus* spp., *Somaticus* spp.)[18] and effectively suppress the key pest of soybean[19]. Although ICD did not show high toxicity against *S. littoralis* larvae in the current study compared to TMX or TCD. Lagadic *et al.*[20] reported that it exhibits a good ovicidal activity against *Heliothis virescens* (Fabricius) and *S. frugiperda* (Smith). Also, data of the present study showed that toxic effects of the tested neonicotinoid insecticides when incorporated into artificial diet (Table 1, 2) were higher than that of field application on 2nd instars. The different effects may be due to the fact that neonicotinoid residues on or inside the tomato leaves were much lower compared to the amount of insecticides incorporated in diet that could be consumed by each larva in the same time unit. These findings were consistent with Brunner *et al.*[21] who reported that TCD was less active against *Cydia pomonella* (L.) eggs in the laboratory when tested using apple-dip assay compared to the field application.

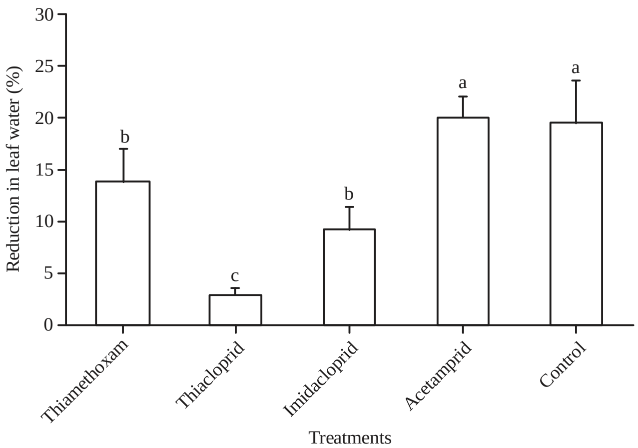

Fig. 2: Reduction (%) in tomato leaf weights treated with field rates of neonicotinoid insecticides. Bars with different letters indicate that the corresponding insecticides are statistically different at $p<0.05$

The TMX kills 50% of 4th instar larvae of *S. littoralis* significantly faster than other insecticides (Table 3) when incorporated with diet, while TCD was faster than TMX and other tested neonicotinoid insecticides in killing 50% of 2nd larval instar (Table 5) when sprayed on tomato plants. This explained that TCD binds fast to insect nicotinic receptors in 2nd instar, while 4th instar larvae may be able to convert TMX to a more potent derivative of clothianidin compared to 2nd instar that show high affinity for binding nAChRs[6] and produced largely in 5th instar larvae of *S. frugiperda* within minutes of feeding on TMX-treated discs. Similar effect was observed in *S. littoralis* and *H. virescens* larvae when exposed to ICD and cyfluthrin as reported by Lagadic *et al.*[20]. They showed that mortality caused by both insecticides occurred more slowly in dietary exposure than in topical application. The aforementioned effects of slow acting were observed in transitional effects of neonicotinoids on biological aspects of *S. littoralis*. The tested insecticides showed low transitional effects on percentage of pupation but not on larval or pupal durations, pupal weight and adult emergence (Table 4) as TMX showed more than 90% mortality of *Drosophila suzukii* adults in European cherry orchards[22]. The low transitional effects of the tested neonicotinoids might be due to reduction in feed consumption of the treated leaves during the first 24 h. Subsequently, a temporary repellant/anti-feedant effect and a marked feeding inhibition leading to reduction in larval weight (Fig. 1) resulting from knock-down might occur as noted with larvae treated with TCD in the current study. The same effect was reported in *S. littoralis* and *H. virescens* exposed to diet containing cyfluthrin[20]. Low consumption especially with TCD (Fig. 2) on treated leaves of tomato plants might indicate that *S. littoralis* ceased feeding but not show true knock-down, as they remained able to move very slowly and no signs of hyper-excitation were observed. Such a hypothesis was proposed as an explanation for decreased food intake in the gypsy moth exposed to abamectin and milbernycin[23] and in *S. littoralis* exposed to emamectin benzoate[7].

CONCLUSION

The TMX and TCD were the most effective neonicotinoid insecticides in killing and reducing larval weight of 2nd instar due to feeding on the treated tomato leaves. The tested neonicotinoids showed low transitional effects as their toxic effects did not extend to adult emergence. Consecutively, field studies might be useful in giving broadening idea about their efficacy against *S. littoralis* under field conditions.

SIGNIFICANCE STATEMENT

This study discovered the most effective insecticide of neonicotinoids against *S. littoralis* when feed on artificial diet or tomato plants that can be beneficial for designing integrated control protocols. This study will help the researchers to cover the critical areas of indirect exposure of *S. littoralis* into neonicotinoides. Thus a new theory on investigating indirect exposure to insecticides might be considered during designing programs for pest control.

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