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Research Article

Biorational management of maize fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) using *Bacillus thuringiensis* (Berliner) enriched with chemical additives

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Abstract
An invasive pest, fall armyworm, *Spodoptera frugiperda* (J.E.Smith) (Lepidoptera: Noctuidae**)** attacks maize at every stage of development, from seedling emergence up to cob formation. Early instar larvae were seen mostly on leaves of maize with characteristics pin or shot hole symptoms. Later instar larvae were confined to deep whorls, leaving typically ragged like appearance and fed on the reproductive stage of the crop especially tassels and developing cobs resulting in quality and quantity loss of maize produce. The effect of commercially available *Bacillus thuringiensis* subsp. *kurstaki* product, Dipel® against the second instar larvae of Fall Armyworm (FAW)was not promising under laboratory conditions. Hence, an effort was made to add an adjuvant along with *B. thuringiensis* to increase the virulence of commercially available *B. thuringiensis*.The Laboratory bioassays with *B. thuringiensis* and seven chemical additives (T1- *Bt* + Boric acid, T2- *Bt* + Zinc oxide, T3- *Bt* + Sodium nitrate, T4- *Bt* + Peptone, T5- *Bt* + Urea, T6- *Bt* + EDTA, T7- *Bt* + Citric acid & T8- *Bt* alone T9- Control) were tested against second instar larvae of *Spodoptera frugiperda* larvae. The results showed that *B. thuringiensis* plus sodium nitrate (T3) promoted maximum mortality 82.2 per cent with a minimum LC_{50} value of 54.620 mg/l. Sodium nitrate boosted *B. thuringiensis* activity at a concentration of 0.05 per cent by 2.128-fold than *B. thuringiensis* alone. Overall, sodium nitrate improved the efficacy of *B. thuringiensis* spray at the maximum level followed by boric acid, urea, EDTA and peptone.

Keywords: *Spodoptera frugiperda*, *B. thuringiensis*, Chemical additives, Synergistic interaction

INTRODUCTION

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae) is an exotic, polyphagous insect pest that originated in the Americas (Luginbill, 1928). Almost 100 plant species are affected by Fall Armyworm (FAW), including maize, sorghum, rice, soybean, cotton, wheat and sugarcane. In addition, Montezano *et al.* (2018) reported 353 hosts from 76 plant families with the Gramineae family having the

largest hosts with106 taxa, followed by Asteraceae and Fabaceae with 31 taxa each. Despite its ability to live in various host plants, the FAW is known to predominantly infest Maize (Nagoshi *et al.,* 2018). FAW was first recorded in West and Central Africa in 2016. Eventually, it also spreads to other continents of the world, wreaking havoc on maize production (Goergen *et al.,* 2016). *Bacillus thuringiensis* (Berliner) is a gram-positive, spore-forming bacteria is a most promising biopesticide used against lepidopteran pests (Baum *et al.,* 1999). Though it is used in commercial agriculture, forest pest management and mosquito control as an alternative to chemical pesticides, it has some drawbacks which limit its application, such as its limited scope of action and short duration in the field (Opisa *et al.,* 2020). As a result, several strategies to improve the potency of *B. thuringiensis* toxin are now being researched. Feeding stimulants, pesticides, allelochemicals, chemicals, other microbial pesticides, proteins such as serine protease inhibitors, chitinases, Cyt toxins, or cadherin fragments were used as additives to improve the efficacy of *B. thuringiensis* (Marzban *et al.,* 2009).

In this context, an effort was undertaken to enhance the toxic effect of *B. thuringiensis* by the addition of chemical substitutes. The chemical additives were integrated with the commercial formulation of *B. thuringiensis* sub sp. *Kurstaki* (Dipel®) and test were verified against second instar larvae of FAW that could considerably improve *B. thuringiensis* efficacy by broadening the range of the activity of *B. thuringiensis* formulation.

MATERIALS AND METHODS

Insect culture

The FAW culture was maintained using International Maize and Wheat Improvement Centre (CIMMYT) diet (Songa *et al.,*2004) at a temperature of 27±7°C and 70 per cent relative humidity at PG Entomology Laboratory of the Department of Plant Protection, Anbil Dharmalingam Agricultural College and Research Institute, Tiruchirappalli.

The first and second instar of FAW was reared using maize leaves. FAW egg masses were placed in round plastic containers (24.5 cm dia. x 19.5 cm ht.) with filter paper at the bottom. Muslin cloth was used to cover the mouth of the round plastic containers. After hatching, the neonates were fed with tender maize leaves. The second instar larvae were reared individually on a small individual plastic container (5 cm dia. x 5 cm ht.) to prevent cannibalism of FAW and covered with gadda cloth. The pupae were collected and transferred to the rearing cages (30cm l x 30 cm b x 45 cm ht.) as and when pupated. Glass vials containing sterile absorbent cotton with 10 per cent adult diet served as adult food (Ashok *et al.,* 2020). The maize seedlings maintained in plastic trays (15 cm l x 7.5 cm b) served as oviposition substrate inside the oviposition cages (30cm l x 30 cm b x 45 cm ht.). The newly emerged adults were confined for oviposition on maize and the rearing continued as mentioned earlier.

Chemical additives

Chemical additives were tested to improve the efficacy of the *B. thuringiensis* subsp*. kurstaki* product Dipel® against second instar larvae of FAW. The seven chemical additives employed in this investigation were inorganic salts (boric acid and zinc oxide), nitrogenous chemicals (peptone, sodium nitrate), protein solubilizing agents (urea and ethylene diamine tetra acetate [EDTA]) and organic acids (citric acid). The safe and low-cost additives from SigmaAldrich® Chemical Company, USA were selected for the study to improve the virulence of *B. thuringiensis*. Dipel®8L, a commercial formulation of *B. thuringiensis* var kurstaki strain HB-1, serotype H3a,3b formulated as a 3.5% Emulsifiable Suspension (ES) with 17,600 IU/mg potency, obtained from Valent BioSciences, USA was utilized for the study.

Effects of additives on second instar larvae of FAW

Before testing with *B. thuringiensis* product, additives were tested individually against second instar larvae of FAW using leaf disc method. Two fresh young maize leaves collected from 2 to 3-week old seedlings of identical size (90 mm in dia.) were used for the bioassay. The fresh leaves were treated with ten ml of 0.05 per cent additive containing few drops of 0.05 per cent Triton X 100. After treatment, the leaves were air-dried for around 5 min. in a sterile laminar airflow chamber to eliminate any excess moisture. To maintain turgidity, the leaves were then placed over a moistened filter paper (110 x 60 mm) in a transparent 6 well tissue culture plates (HiMedia Laboratories *Pvt*. Ltd, Telangana).

Eight treatments *viz*., T1 (Boric acid), T2 (Zinc oxide), T3 (Sodium nitrate), T4 (Peptone), T5 (Urea), T6 (EDTA), T7 (Citric acid), T8 (Control) and three replications were maintained. Each replication contained 15 second instar larvae.The control plate was sprayed with sterilized distilled water. During the first 24 h, the larvae were fed with additive treated leaves and after that, fresh maize leaves was replenished as and when after cleaning. The larval mortality was recorded for a week at 24 h intervals. The experiment was carried out using a completely randomized design.

Bioassay with *B. thuringiensis* and chemical additives

The bioassay was carried out as described above with *B. thuringiensis* and additives, fresh leaves were treated with a mixture of 5 ml of 0.05 per cent of additive and 5 ml of 0.2 per cent Dipel® (*B. thuringiensis* subsp.

kurstaki). Nine treatments *viz*., T1 (0.2% *Bt* + 0.05% Boric acid), T2 (0.2% *Bt* + 0.05% Zinc oxide), T3 (0.2% *Bt* + 0.05% Sodium nitrate), T4 (0.2% *Bt* + 0.05% Peptone), T5 (0.2% *Bt* + 0.05% Urea), T6 (0.2% *Bt* + 0.05% EDTA), T7 (0.2% *Bt* + 0.05% Citric acid), T8 (0.2% *Bt* alone), T9 (Control) and three replications were maintained. Each replication contained 15 second instar larvae and the control plate was sprayed with sterilized distilled water.The chemical additives in combination with *B. thuringiensis* which produced less than 50 per cent larval mortality were not selected for probit analysis.

Statistical analysis

Mortality data obtained were converted to arc-sine values and subjected to Completely Randomised Design using Agres-agdata package. To correct the mortality in control, Abbott's formula was utilised and median lethal doses (LC_{50}) were calculated using probit analysis (Finney, 1971).

RESULTS AND DISCUSSION

Effects of additives on second instar larvae of FAW

The mortality caused by additives alone against second instar FAW larvae ranged from 08.88 per cent to 33.33 per cent, with sodium nitrate causing the highest mortality (33.33%) while zinc oxide caused the lowest mortality (08.88%) on 168 Hours After Treatment (HAT).

Efficacy of *B. thuringiensis* in combination with additives against second instar larvae of FAW

When each additive tested together with *B. thuringiensis* resulted in higher mortality than when they were used alone. The mortality caused by the combination of *B. thuringiensis* with additives against second instar larvae of FAW ranged from 82.22 per cent to 28.88 per cent, with sodium nitrate caused the highest mortality (82.22%) while zinc oxide produced the lowest mortality (13.33%) on 168 HAT (Table 1). The weight reduction of surviving larvae over control in 168 HAT was maximum in *B. thuringiensis* plus sodium nitrate (59.84%) followed by *B. thuringiensis* plus boric acid (59.55%), *B. thuringiensis* plus urea (57.68%), *B. thuringiensis* plus EDTA (54.38%), *B. thuringiensis* plus peptone (51.40%), *B. thuringiensis* plus citric acid (48.19%) and *B. thuringiensis* plus zinc oxide (41.98%) (Table 3)

Effects of *B. thuringiensis* and inorganic salts

The *B. thuringiensis* plus inorganic salts and the *B. thuringiensis* alone produced a significant difference in larval mortality. The *B. thuringiensis* plus boric acid mixture caused higher mortality, with the LC_{50} being 62.459 mg/l, compared to 116.239 mg/l for B. thuringiensis alone. The 26.67 percent survived larvae showed 59.55 per cent larval weight reduction in comparison over control (Table 3). Boric acid had shown to enhance the effectiveness of *B. thuringiensis* against

Table 1. Effects of additives against second instar larvae of FAW

Treatments (Chemical	Larval mortality (%)						
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT	144 HAT	168 HAT
T1- Boric acid (0.05%)	04.44±0.33 (12.17)^{a}	08.88±0.33 (17.35)^{b}	22.21±0.58 (28.13)^{ab}	22.21±0.58 (28.13)^{a}	22.21±0.58 (28.13)^{ab}	22.21±0.58 (28.13)^{ab}	22.21±0.58 (28.13)^{ab}
T2- Zinc oxide (0.05%)	0 (1.65)^{b}	0 (1.65)^{c}	0 (1.65)^{c}	0 (1.65)^{b}	02.22±0.33 (08.57)^{c}	6.66±0.67 (14.96)^{b}	8.88±0.33 (17.35)^{b}
T3- Sodium nitrate (0.05%)	06.67±0.33 (14.96)^{a}	31.11±0.33 (33.90)^{a}	33.33±0.33 (35.26)^{a}	33.33±0.33 (35.26)^{a}	33.33±0.33 (35.26)^{a}	33.33±0.33 (35.26)^{a}	33.33±0.33 (35.26)^{a}
T4-Peptone (0.05%)	0 (1.65)^{b}	0 (1.65)^{c}	0 (1.65)^{c}	02.22±0.33 (08.57)^{b}	11.11±0.33 (19.47)^{b}	15.55±0.33 (23.23)^{ab}	15.55±0.33 (23.23)^{ab}
T5- Urea (0.05%)	04.44±0.33 (12.17)^{a}	08.88±0.33 (17.35)^{b}	13.32±0.33 (21.42)^{b}	19.99±0.58 (26.57)^{a}	19.99±0.58 (26.57)^{ab}	19.99±0.58 (26.57)^{ab}	19.99±0.58 (26.57)^{ab}
T6- EDTA (0.05%)	0 (1.65)^{b}	0 (1.65)^{c}	0 (1.65)^{c}	02.22±0.33 (8.57)^{b}	13.33±0.33 (21.42)^{b}	17.77±0.33 (24.94)^{ab}	17.77±0.33 (24.94)^{ab}
T7- Citric acid (0.05%)	0 (1.65)^{b}	0 (1.65)^{c}	0 (1.65)^{c}	0 (1.65)^{b}	02.22±0.33 (8.57)^{c}	08.89±0.58 (17.35)^{b}	11.11±0.33 (19.47)^{b}
T8- Control (distilled water)	0 (1.65)^{b}	0 (1.65)^{c}	0 (1.65)^{c}	0 (1.65)^{b}	0 (1.65)^{bc}	0 (1.65)^{c}	0 (1.65)^{c}
SEd	1.02**	1.06**	1.22**	1.44**	1.19**	1.16**	1.02**
CD	2.20	2.29	2.62	3.09	2.56	2.43	2.21

Note *Each value is a mean of three replications (Mean±SE); *Figures within parentheses are arcsine transformed values; *Means followed by common alphabets are not significantly different at 5% level by LSD; *HAT– Hours after treatment

Table 2. Efficacy of *Bacillus thuringiensis* (*Bt*) and additives against second instar larvae of FAW

Treatments with *Bt* + Chemical additives	Larval mortality (%)						
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT	144 HAT	168 HAT
T1- *Bt*(0.2%) + Boric acid (0.05%)	08.89±0.88 (17.35) [b]	26.67±0.66 (31.09) [b]	57.78±0.88 (49.47) [b]	68.89±0.33 (56.10) [ab]	73.33±0.33 (58.90) [a]	73.33±0.33 (58.90) [ab]	73.33±0.33 (58.90) [a]
T2- *Bt*(0.2%) + Zinc oxide (0.05%)	0 (1.65) [c]	0 (1.65) [d]	02.22±0.33 (8.57) [fg]	06.66±0.33 (14.96) [e]	11.10±0.33 (19.47) [f]	22.21±0.33 (28.12) [e]	28.88±0.33 (32.51) [d]
T3- *Bt*(0.2%) + Sodium nitrate (0.05%)	24.44±0.33 (29.63) [a]	64.44±0.58 (53.40) [a]	82.22±0.33 (65.06) [a]	82.22±0.33 (65.06) [a]	82.22±0.33 (65.06) [a]	82.22±0.33 (65.06) [a]	82.22±0.33 (65.06) [a]
T4- *Bt*(0.2%) + Peptone (0.05%)	0 (1.65) [c]	04.44±0.33 (12.17) [cd]	08.88±0.33 (17.35) [de]	19.99±0.33 (26.57) [cd]	39.99±0.58 (39.23) [cd]	53.32±0.58 (46.91) [bc]	62.21±0.33 (52.07) [ab]
T5- *Bt*(0.2%) + Urea (0.05%)	06.67±0.33 (14.96) [b]	22.23±0.33 (28.13) [b]	33.34±0.33 (35.26) [c]	57.78±0.33 (49.47) [b]	64.45±0.33 (53.40) [ab]	71.12±0.33 (57.49) [ab]	71.12±0.33 (57.49) [a]
T6- *Bt*(0.2%) + EDTA (0.05%)	0 (1.65) [c]	08.89±0.33 (17.35) [c]	15.56±0.33 (23.23) [d]	26.67±0.33 (31.09) [c]	48.89±0.33 (44.36) [bc]	64.45±0.33 (53.40) [ab]	68.89±0.33 (56.10) [ab]
T7- *Bt*(0.2%) + Citric acid (0.05%)	0 (1.65) [c]	02.22±0.33 (08.57) [d]	06.66±0.33 (14.96) [def]	13.33±0.33 (21.42) [de]	22.22±0.33 (28.13) [e]	28.89±0.58 (32.51) [de]	35.56±0.33 (36.60) [cd]
T8- *Bt* alone (0.2%)	0 (1.65) [c]	0 (1.65) [d]	04.44±0.33 (12.17) [efg]	11.11±0.33 (19.47) [de]	24.41±0.33 (29.63) [de]	39.97±0.33 (39.23) [cd]	48.86±0.33 (44.36) [bc]
T9- Control (distilled water)	0 (1.65) [c]	0 (1.65) [d]	0 (1.65) [fg]	0 (1.65) [f]	0 (1.65) [g]	0 (1.65) [f]	0 (1.65) [e]
SEd	1.16**	1.49**	1.57**	1.42**	1.63**	1.65**	1.56**
CD	2.43	3.14	3.29	3.00	3.43	3.46	3.27

Note *Each value is a mean of three replications (Mean±SE); *Figures within parentheses are arcsine transformed values; *Means followed by common alphabets are not significantly different at 5% level by LSD; *HAT– Hours after treatment

tobacco cutworm *Spodoptera litura* (Govindarajan *et al.,*1976), which was consistent with our results. Boric acid is a stomach and contact poison. It damages the protective layer of the peritrophic membrane and gut lining, allowing *B. thuringiensis* toxin to reach the insect midgut epithelium and therefore increasing the effectiveness of toxicity (Govindarajan *et al.,*1976).

B. thuringiensis plus zinc oxide combination caused mortality of 28.88 per cent on the 168 HAT, which was much lower than the mortality produced by boric acid containing mixture with *B. thuringiensis* (73.3%) and more than 70 per cent survivors showed 41.98 per cent larval weight reduction. The zinc oxide proved ineffective at increasing the potential of *B. thuringiensis* and caused lower mortality than when *B. thuringiensis* was used alone. The failure of *B. thuringiensis* to potentiate against second instar of FAW larvae could be attributed to variances in the *B. thuringiensis* strain employed in this investigation and the heterogeneous nature lepidopteran insect species and their associated gut flora or microbiome. In contrast, Malaikozhundan *et al.* (2017) found that *B. thuringiensis* ZnO nanoparticles were particularly effective against *C. maculatus*, causing 100% mortality at a 25 g/ml concentration, which ultimately reduced the activity of midgutamylase, cysteine protease, glucosidase and glutathione Stransferase (GST).

Effects of *B. thuringiensis* and nitrogenous compounds

The sodium nitrate (0.05%) and peptone (0.05%), when applied in combination with *B. thuringiensis* (0.2%), produced the larval mortality of 82.22 per cent and 62.21 per cent on 168 HAT, respectively. *B. thuringiensis* plus sodium nitrate recorded maximum mortality (82.22%) among all additives with LC_{50} values of 54.620 mg/l and synergistically improved the activity of *B. thuringiensis* by 2.128-fold. The remaining 17.78 per cent of survivors showed 59.84 per cent larval weight inhibition in comparison over control. Sodium nitrate was the most efficient nitrogenous substance in suppressing the second instar of FAW larvae with *B. thuringiensis*, followed by peptone. This is similar to the results of Zhang *et al.*(2013), who reported that nitroge-

Table 3. Effects of *Bacillus thuringiensis* plus additives on sub-lethal effects on second instar larvae of FAW

Treatments with Bt + Chemical additives	Survival population[a]		Larval Weight (mg) 168 h		Larval weight inhibition in comparison over control (%)
	no.	%	Range	Mean±SD	
T1 - *Bt* + Boric acid	12	26.67	20-30	27.08 ± 2.75	59.55
T2 - *Bt* + Zinc oxide	32	71.12	34-44	38.84 ±2.10	41.98
T3 - *Bt* + Sodium nitrate	08	17.78	23-29	26.88±1.73	59.84
T4 - *Bt* + Peptone	17	37.79	30-36	32.53 ± 1.55	51.40
T5 - *Bt* + Urea	12	28.88	27-29	28.33±0.89	57.68
T6 - *Bt* + EDTA	13	31.11	30-32	30.54 ± 0.78	54.38
T7 - *Bt* + Citric acid	28	64.44	32-37	34.68 ± 1.06	48.19
T8 - *Bt* alone	23	51.14	32-42	37.96 ± 1.99	43.29
T9 - Control (distilled water)	-	-	55-74	66.94 ± 6.02	-

Note [a]A Total of 360 second instar larvae of FAW bio assayed in eight treatments with three replications. Each replication contains 15 larvae (15*8*3). Survivors from each treatmentwas collected and larval weight was recorded individually.

Table 4. Dosage - mortality response of *Bacillus thuringiensis* plus additives on second instar larvae of FAW

Treatments with *Bt* + Chemical additives	LC_{50} (mg/l)	Fold increase	Fiducial Limit (95%)		Y=bx+a	R^2	χ^2
			Lower limit	Upper limit			
T8 - *Bt* alone	116.239	-	21.899	616.978	Y=0.496x+3.977	0.875	0.985
T3 - *Bt* + Sodium nitrate	54.620	2.128	16.565	180.106	Y=0.815x+3.574	0.910	0.999
T1 - *Bt* + Boric acid	62.459	1.861	14.728	264.871	Y=0.605x+3.883	0.854	0.999
T5 - *Bt* + Urea	75.694	1.535	13.603	421.191	Y=0.490x+4.052	0.847	0.998
T6 - *Bt* + EDTA	85.932	1.353	15.366	480.572	Y=0.489x+4.054	0.809	0.997
T4 - *Bt* + Peptone	93.464	1.244	10.910	800.697	Y=0.384x+4.244	0.936	0.996

Note *Dosage - mortality response was workout for treatments having mortalities >50%; *Fold increase is LC_{50} value of *B. thuringiensis* alone divided by the LC_{50} value of each *B. thuringiensis* + additive mixture. ± 95% fiducially limit (FL)

nous compounds such as peptone, sodium nitrate, and ammonium nitrate enhanced the activity of *B. thuringiensis* 1.62, 1.32 and 1.37 fold, respectively. Wigglesworth (1977) linked the synergistic effects of nitrogenous substances and *B. thuringiensis* in the regulation of lepidopteran larvae to changes in the physiology of haemolymph generated by leakage through the *B. thuringiensis* treated larval midgut cells.

Effects of *B. thuringiensis* and protein solubilizing agents

With an LC_{50} value of 75.694 mg/l, the synergistic effect of the*B. thuringiensis* plus urea mixture enhanced the mortality of second instar larvae of FAW from 48.86 per cent (*Bt* alone) to 71.12 per cent on 168 HAT with 57.68 per cent survivors showed 51.4 per cent larval weight reduction in comparison over control. The *Bt* plus EDTA mixture increased the mortality from 43.29 per cent (*B. thuringiensis* alone) to 68.89 per cent on 168 HAT with an LC_{50} value of 85.932 mg/l (Table 4). The two protein solubilizing agents such as urea and EDTA improved the efficacy when combined with *B. thuringiensis.*This result was correlated with the findings of Zhang *et al.*(2013), who reported when *B. thuringiensis* was combined with EDTA (0.2 per cent) and EDTA-Na_2 (0.1 per cent) enhanced the efficiency by 1.62- and 2.31-fold increase in *B. thuringiensis* activity against the *Plutella xylostella*respectively.The urea denatured the proteins that reduce the disulphide bonds of protein molecules to sulfhydryl groups, thus increasing the dissolution of the endotoxin in the insect gut, which results in more than 50% mortality of the second instar of FAW larvae (Nickerson, 1980). In contrast, Salama *et al.*(1985) observed antagonistic effects of EDTA when coupled with *B. thuringiensis* against the cotton leafworm *S. littoralis*.

Effects of *B. thuringiensis* and organic acids

The efficiency of *B. thuringiensis* plus citric acid mixtures was much lower (35.56±0.33%) on 168 HAT than *B. thuringiensis* alone. More than 60 per cent of survivors showed 48.19 per cent larval weight reduction in

comparison over control. The mortality produced with the organic salt (citric acid) alone (11.11%) was also much lower than the cumulative effects with *B. thuringiensis* alone (Table 1). Citric acid failed in improving the efficacy of *B. thuringiensis* when used in a combinationat a concentration of 0.05 percent. Similar results were obtained by Salama *et al.* (1989) against *A. ipsilon*. Morris *et al.*(1995) also found that there was no effect of adding any of four organic acids, namely calcium acetate, lauric acid, sodium thioglycolate and malic acid, to *B. thuringiensis* against the fourth instar *M. configurata* larvae. In contrast, the activity of *B. thuringiensis* against the diamondback moth, *P. xylostella*, was found to be enhanced by malic acid and citric acid (Zhang *et al.,* 2013).

Conclusion

According to the present study, sodium nitrate promoted maximum synergistic interaction with *B. thuringiensis* sub sp *kurstaki* product Dipel® followed by boric acid, urea, EDTA and peptone against second instar FAW larvae and so significantly (5%) contributed to pest control. However, more field tests with these effective chemical adjuvants in combination with the *B. thuringiensis* product are required for further validation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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