Efficacy of *Bacillus thuringiensis* and indigenous *Trichogramma turkistanica* for controlling lepidopterous pests on Taify pomegranate fruits

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The impact of *Bacillus thuringiensis* and native *Trichogramma turkistanica* on the infestation rates of two lepidopterous pests, *Virachola livia* and *Ectomyelois ceratoniae,* was assessed in field experiments conducted in four pomegranate farms distributed in the Taif region of Saudi Arabia. Pomegranate trees were sprayed with *B. thuringiensis* spores, and indigenous *T. turkistanica* was inundatively released during the pomegranate fruiting season from April to September of 2014. The highest infestation rates with *E. ceratoniae* and *V. livia* in control and treated trees gradually increased until the end of the season, reaching 79, 54 and 22 % for *E. ceratoniae*, and 22, 16 and 7 % for *V. livia* in control, *Bacillus*-treated trees, and *Trichogramma*-treated trees, respectively. The mean percentages of fruits infested with *E. ceratoniae* or *V. livia* were significantly different between the untreated trees and both the trees treated with *Trichogramma* and those treated with *Bacillus*. Moreover, the trees treated with *Trichogramma* had a lower infestation rate by both *E. ceratoniae* and *V. livia* compared to the trees treated with *Bacillus*. The number of larvae collected from infested fruits varied from one to two larvae per fruit for both *E. ceratoniae* and *V. livia*. Use of *Bacillus* or native *Trichogramma* to control these pests can achieve high yields of Taify pomegranate of better quality.

Key words: biological control, Lepidoptera, parasitoid, native species, *Punica granatum*.

INTRODUCTION

The pomegranate, *Punica granatum* L. (Lythraceae) is cultivated in many areas of Saudi Arabia, mainly the city Taif, where a special, well-known variety called the Taify pomegranate is cultivated, an important commercial fruit of Saudi Arabia (Al-Maiman & Ahmed 2002). Infestation with the larvae of two lepidopterous insects, namely the pomegranate butterfly, *Virachola livia* Klug, 1834 (Lepidoptera: Lycaenidae) and the carob moth, *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae) occurs during the pomegranate fruiting season in the Taif region (Al-Barty 2011; Elsayed & Bazaid 2011; Moawad *et al*. 2011). The carob moth is a polyphagous fruit pest that is widespread in many tropical and subtropical areas in world. It is known as a pest of citrus, almond, date, pistachio and fig (Warner *et al.* 1990; Nay & Perring 2005; Mehrnejad 2002).

While the pomegranate butterfly, *V. livia* is a serious pest of the pomegranate, the pomegranate is a secondary host, and the primary host of *V. livia* is *Acacia nilotica* pods (Ksentini *et al*. 2011). This pest has spread from the northeastern part of Africa up to the Arabian Peninsula and Iran (Katbeh-Bader *et al*. 2003).

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Usually the carob moth is controlled by spraying the trees with chemical insecticides, which are known for their health and environmental hazards. However, monitoring of this species in pomegranate cultivation has shown that insecticides seem to be inefficient due to its endophytic behaviour and the dangling position of the fruit on the pomegranate tree (Dhouibi *et al*. 2000). Therefore, there is a need for potent and safe bio-insecticides active against this pest (Marrone & MacInyosh 1993).

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium that forms parasporal crystal proteins during the stationary phase of its growth cycle (Theoduloz *et al*. 1997; Ito *et al*. 2004). *B. thuringiensis* has been used as a successful biological insecticide and is a uniquely specific, safe, and effective tool for the control of a wide variety of insect pests (Nester *et al.* 2002). While the crystal proteins studied so far are toxic to the larvae of certain insects of several orders, they are not pathogenic to mammals, birds, reptiles or amphibians (Feitelson *et al*. 1992; Hayakawa *et al*. 2004). Therefore, this bacterium has been widely used as a source of insecticidal proteins for direct use in formulations or in transgenic plants (Johnson & Bishop 1996: Bouillaut *et al*. 2005). The insecticidal

proteins are alternatives to synthetic chemical insecticides as they are considered to be friendlier to the environment than chemicals (Marrone & MacInyosh 1993).

Egg parasitoids of the genus *Trichogramma* have been used successfully as inundative biological control agents against the eggs of a large number of important lepidopteran pests and are the most widely used natural enemies in biological control worldwide (Li *et al*. 1994; Dang *et al*. 2005). It is considered as the most described parasitoid genus, and consists of approximately 180 species (Rohi & Pintureau 2003). The augmentative release of mass-reared trichogrammatid egg parasitoids has been identified as a promising method to reduce both egg hatching and subsequent damage due to larval feeding (Smith 1996). Additionally, the diversity in the host preference of *Trichogramma* species has been fully recognized (Hassan 1993). Success or failure in the use of egg parasitoids in biological control will depend mainly on the choice of the species (Hassan 1994). For this reason, growers need information on which species is best for release against any given host (Losey & Calvin 1995).

Conservation control refers to the use of indigenous predators and parasitoids, usually against native pests (Gurr *et al*. 2000). The introduced pest-controlling agent will only be established successfully if the environmental pressure factors are matched by their corresponding support capacities (Morales & Freitas 2010). Climatic conditions play an important role in tritrophic interactions among poikilotherms, as they influence the level of control that natural enemies exert (Huffaker *et al*. 1971). Biological pest control schemes based on the collection of natural enemies of the pest from its country of origin may be more successful, or maintain control for longer periods of time, than other strategies (Hokkanen & Pimentel 1984). It is important to use *B. thuringiensis* and *Trichogramma* separately because the use of both bioagents together could be harmful for some species of *Trichogramma* (Brunner *et al*. 2001; Ksentini *et al*. 2010).

This study aimed to evaluate a commercial strain of *B. thuringiensis* and an indigenous strain to Saudi Arabia, *T. turkistanica* (Sayed *et al*. 2011), for controlling two important lepidopterous pests of pomegranate fruits, *i.e. E. ceratoniae* and *V. livia*, through field applications in Taif, Saudi Arabia during the fruiting season of 2014.

MATERIAL AND METHODS

Study site and experimental design

Four pomegranate farms were selected for these experiments in 2014 (Table 1). Each farm had an area of approximately 3 ha, with trees spaced at 5 m within and between rows, totalling around 40 trees/ha. Each farm was divided into three regions (Fig. 1); the first was for *Trichogramma* release, the second was a separation area, and the third was for *B. thuringiensis* treatment and the controls. The separation area had a length of about 100 m to make sure *Trichogramma* did not reach the control and *B. thuringiensis* treatment areas. The positions of these areas on each farm are also indicated in Fig. 1.

Bacterium propagation and sporulation

The *B. thuringiensis* used in the present study was a commercially available product (Dipel 2X 6.4 % WP), which contained the subspecies *kurstaki*. In order to propagate its spores, the suspension was cultured on Luria-Bertani (LB) agar. The colonies were then transferred to multiple flasks containing LB liquid media (1 l) for 3 days at 37 °C. The suspensions were then transferred to conical centrifuge tubes (15 ml) and centrifuged at 8000 rpm for 2 min. The pellets were cultured in flasks with 2 l of sporulation liquid medium containing 0.016 % glutamate (Kenneth *et al*. 1974) for 3 days at 37 °C. The suspension was heated to 100 °C for 10 min to kill the vegetative cells, allowing the counting of the number of spores per ml. The suspension was next diluted and transferred to plates containing LB agar media for 2 days of incubation at 37 °C. The colonies were then counted. Harvested cultures were washed twice with distilled water.

Application of B. thuringiensis *spores in fields*

Bacillus thuringiensis spores were diluted to 3×10^5 spores/ml of distilled water. The spore suspen-

Table 1. Locations of four pomegranate farms in Taif, Saudi Arabia.

Farm no.	Location in Taif	Latitude	Longitude
1	Qiaa	21°11′52″	40°38′28″
2	Qarua	21°16′29″	40°22′26″
3	Qarua	21°16′46″	40°22′25″
4	Alhada	21°22′42″	40°20′38″

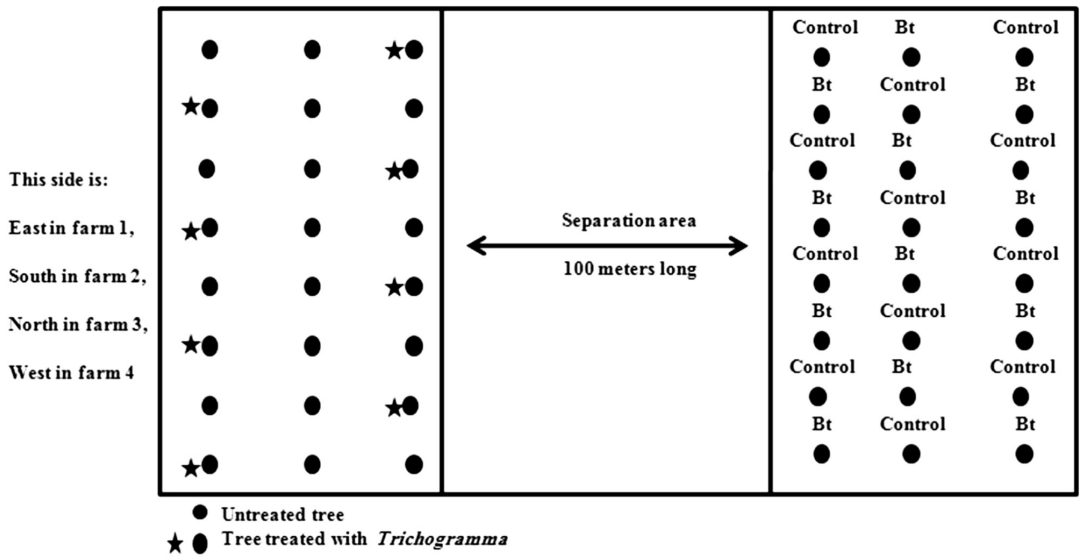

Fig. 1. Experimental design of treatments on all four pomegranate farms.

sion was sprayed directly on the fruits once their setting began. Tree spraying was continued weekly for six months, from the beginning of April to 15 September, totalling 23 sprays.

Trichogramma *mass production*

The indigenous strain of *T. turkistanica,* TaifKSA, was previously collected from the Taif governorate, Saudi Arabia, by Sayed *et al.* (2011). Rearing of the parasitoid was performed on eggs of the factitious host *Ephestia kuehniella*, which were routinely reared in the laboratory at 25 ± 1 °C, 60 ± 10 % relative humidity, and a 16:8 (light:dark) photoperiod, following the standard rearing procedure of Wührer & Hassan (1993).

Trichogramma *releasing technique*

Cards containing the parasitized eggs were divided into small pieces (3 × 3 cm) containing about 2100 to 2400 parasitized eggs each. Each piece was kept in a carton-paper envelope (6 × 4.5 cm) with small holes to allow the wasps to emerge and prevent any predators from entering. The envelopes were transferred to pomegranate orchards in ice boxes, and hung on the trees spaced at a distance of 10 m for a total of about 90 000–100 000 wasps/acre/release. Releases of *Trichogramma* were carried out weekly in all four pomegranate orchards. *Trichogramma* release was started at the beginning of April 2014 and continued until 15 September, totalling 23 releases.

Estimation of field infestation

Five trees were selected from each farm using a zigzag sampling technique during each treatment (every 15 d). Each tree was circled, and one fruit was collected from the north, south, east, west and middle of the tree at an intermediate height, totalling five fruits/tree (Nava *et al.* 2005). The fruits were dissected in the laboratory to evaluate the infestation rates of *E. ceratoniae* and *V. livia*. Also, the number of larvae per infested fruit throughout the production cycle (April–September) was determined. Collected fruits of each sampling were kept in cages covered by white tissue (60 × 60 × 60 cm) under laboratory conditions (25 ± 2 °C) for one week in order for larval development to be easily observed.

Statistical analysis

Means and standard deviations were calculated and the data were compared using one-way ANOVA and the significance among means within each row and column were compared by using Duncan's multiple range test at $P < 0.05$ (SPSS 2002).

RESULTS AND DISCUSSION

In all the locations of this study, the pomegranate fruits were infested with *E. ceratoniae* and *V. livia*. For both pests, the infestation rates increased overtime (Tables 2, 3). The mean percentage of fruits infested by *E. ceratoniae* is shown in Table 2. The trees

Table 2. Mean infestation percentages of *Ectomyelois ceratoniae* on pomegranate fruits.

Date	Infestation (mean ± S.D.)			*F*-value	*P*
	With *T. turkestanica*	With *B. thuringiensis*	Control		
15 Apr	$8 \pm 3.27_{a}$	$11 \pm 6.00_{a}$	$11 \pm 3.83_{a}$	0.587	0.576
01 May	$9 \pm 3.83^{A}{}_{a}$	$15 \pm 5.03^{AB}{}_{a}$	$19 \pm 3.83^{B}{}_{a}$	5.561	0.027
15 May	$12 \pm 3.26^{A}{}_{ab}$	$20 \pm 4.62^{A}{}_{a}$	$39 \pm 7.57^{B}{}_{b}$	25.836	<0.001
30 May	$19 \pm 6.83^{A}{}_{bc}$	$37 \pm 6.83^{B}{}_{b}$	$50 \pm 5.16^{C}{}_{c}$	24.233	<0.001
15 Jun	$20 \pm 3.27^{A}{}_{bc}$	$38 \pm 5.16^{B}{}_{b}$	$56 \pm 8.64^{C}{}_{cd}$	34.714	<0.001
30 Jun	$20 \pm 5.66^{A}{}_{bc}$	$43 \pm 10.5^{B}{}_{bc}$	$52 \pm 3.27^{B}{}_{c}$	21.313	<0.001
15 Jul	$20 \pm 8.64^{A}{}_{bc}$	$52 \pm 6.53^{B}{}_{c}$	$62 \pm 9.52^{B}{}_{de}$	27.769	<0.001
30 Jul	$18 \pm 5.16^{A}{}_{bc}$	$48 \pm 3.27^{B}{}_{bc}$	$68 \pm 6.53^{C}{}_{ef}$	95.000	<0.001
15 Aug	$24 \pm 8.64^{A}{}_{c}$	$46 \pm 9.52^{B}{}_{bc}$	$69 \pm 5.03^{C}{}_{efg}$	31.867	<0.001
30 Aug	$21 \pm 5.03^{A}{}_{bc}$	$47 \pm 10.0^{B}{}_{bc}$	$70 \pm 6.93^{C}{}_{efg}$	41.608	<0.001
15 Sep	$21 \pm 3.83^{A}{}_{bc}$	$47 \pm 6.00^{B}{}_{bc}$	$76 \pm 7.30^{C}{}_{fg}$	87.346	<0.001
30 Sep	$22 \pm 7.66^{A}{}_{c}$	$54 \pm 9.52^{B}{}_{c}$	$79 \pm 6.83^{C}{}_{g}$	49.980	<0.001
F-value	3.269	16.334	44.663		
P	0.003	<0.001	<0.001		

Values followed by the same letter are not statistically different. Upper case letters following the values represent comparisons within a row and lower case letters represent comparisons within a column (Duncan's test; $P < 0.05$).

Table 3. Mean of infestation percentages of *Virachola livia* on pomegranate fruits.

Date	Infestation (mean ± S.D.)			*F*-value	*P*
	With *T. turkestanica*	With *B. thuringiensis*	Control		
15 Apr	0.00	0.00	0.00		
01 May	$1 \pm 2_{ab}$	$2 \pm 4_{ab}$	$2 \pm 2.31_{a}$	0.158	0.856
15 May	$2 \pm 2.31_{abc}$	$1 \pm 2_{a}$	$2 \pm 4_{a}$	0.158	0.856
30 May	$3 \pm 2_{abc}$	$2 \pm 2.31_{ab}$	$3 \pm 3.83_{ab}$	0.167	0.849
15 Jun	$3 \pm 3.83_{abc}$	$4 \pm 5.66_{abc}$	$5 \pm 6_{ab}$	0.145	0.867
30 Jun	$4 \pm 3.27_{abc}$	$6 \pm 2.31_{abcd}$	$6 \pm 2.31_{ab}$	0.750	0.500
15 Jul	$5 \pm 3.83_{abcd}$	$8 \pm 5.66_{bcd}$	$11 \pm 3.83_{bc}$	1.761	0.226
30 Jul	$5 \pm 5.03^{A}{}_{abcd}$	$10 \pm 4.0^{A}{}_{cde}$	$19 \pm 3.83^{B}{}_{d}$	10.786	0.004
15 Aug	$7 \pm 5.03^{A}{}_{bcd}$	$11 \pm 3.83^{AB}{}_{de}$	$18 \pm 5.16^{B}{}_{cd}$	5.580	0.027
30 Aug	$8 \pm 5.66^{A}{}_{cd}$	$15 \pm 3.83^{AB}{}_{e}$	$20 \pm 8.64^{B}{}_{d}$	3.593	0.071
15 Sep	$10 \pm 2.31^{A}{}_{d}$	$15 \pm 3.83^{AB}{}_{e}$	$21 \pm 6.0^{B}{}_{d}$	6.500	0.018
30-Sep	$7 \pm 3.83^{A}{}_{bcd}$	$16 \pm 6.53^{AB}{}_{e}$	$22 \pm 9.52^{B}{}_{d}$	4.622	0.042
F-value	2.767	8.337	10.721		
P	0.010	<0.001	<0.001		

Values followed by the same letter are not statistically different. Upper case letters following the values represent comparisons within a row and lower case letters represent comparisons within a column (Duncan's test; $P < 0.05$).

treated with *Trichogramma* were significantly less infested by *E. ceratoniae* than the trees treated with *Bacillus* during all periods of the season except the first month of fruiting. Trees treated with *Trichogramma* and *Bacillus* were significantly less infested than untreated trees. At the beginning of the fruiting season (April) the infestation rates of the treatments and control were not significantly different. It was 8 % for the trees treated with *Trichogramma* and 11 % for trees treated with *Bacillus* and the control trees. The infestation rate in control and treated trees increased gradually until the end of the season, and reached a maximum of 79, 54 and 22 % for the control trees, trees treated with *Bacillus* and trees treated with *Trichogramma*, respectively. This result conforms to those of Shakeri (2004) who indicated that pomegranate is the main host of *E. ceratoniae* in Iran, and that this

pest causes up to 80 % damage during the growing season. Elsayed & Bazaid (2011) also recorded that the highest rate of infestation with *E. ceratoniae* on pomegranate in the Taif region in 2008 was 70 % in August and the lowest infestation rate (35 %) in the first month of the survey (May). Moreover, Mediouni & Dhoubi (2007) indicated that in Tunisia *E. ceratoniae* causes staggering economic losses with infestation rates as high as 90 % in pomegranates. Vreysen *et al*. (2006) indicated that the spraying with *Bacillus thuringiensis* is an effective control method for *E. ceratoniae* on different crops.

Data regarding the infestation rate of *V. livia* on pomegranates are shown in Table 3. Infestation rates were significantly lower across the period at the end of the season (August and September) for the trees treated with *Trichogramma* compared to the control trees, while no significant differences were found between the trees treated with *Bacillus* and the control trees. This result may be because the infestation rate of this pest was very low, as indicated in the control. The infestation rate of *V. livia* in both control and treated trees increased gradually throughout the season and reached a maximum of 22, 16, and 7 % for control trees, trees treated with *Bacillus*, and trees treated with *Trichogramma*, respectively. This finding for control trees approximates that recorded by Elsayed & Bazaid (2011), who indicated that the infestation rate of *V. livia* at Taif was 5 % at the beginning of the pomegranate fruiting season in 2008 and was higher in August (17.5 %). In Tunisia, Ksentini *et al*. (2011) reported that the damage caused by *V. livia* in pomegranate ranged between 5.2 and 52 % depending on the pomegranate varieties. This was hypothesized to be due to female preference in choosing to lay its eggs more frequently on certain varieties. This choice might be a response to different variety characteristics such as pomegranate tree chemical cues.

Different investigations for controlling *V. livia* on pomegranates were performed by Kahramanoglu & Usanmaz (2013), who found that the most important cause of damage on pomegranate fruits in Cyprus was *V. livia*. Damage totaled 14.63 % in 2011 and 15.57 % in 2012 in the untreated control trees, while the damage decreased to below 5 % in these two years when treated with *B. thuringiensis*. Additionally, Blumenfeld *et al*. (2000) reported that applying *B. thuringiensis* was the best way of controlling *V. livia* damage during ripening or in storage. Singh & Singh (2000) also reported that *B. thuringiensis* is an effective control agent for *V. livia*.

Different native species of *Trichogramma* have been evaluated for controlling the carob moth in Iranian pomegranate orchards, and some of these native strains were effective in the control of *V. livia* (Moezipour 2006). Generally, biological control programmes of several exotic pests demonstrated the importance of indigenous biocontrol agents in the regulation of pest populations (Pimentel 2000; Viggiani 2000; El-Arnaouty *et al*. 2014).

The number of larvae collected from fruits in this investigation varied from one to two larvae per fruit for both *E. ceratoniae* and *V. livia* (Tables 4, 5). There was a range of different ages of larvae and pupae in infested fruits. At the end of the pomegranate fruiting season, the numbers of *E. ceratoniae* showed significant differences between the trees treated with *Trichogramma* (1.21 larvae/fruit) and the control trees (1.64 larvae/fruit), while there were no significant differences between the trees treated with *Bacillus* (1.4 larvae/fruit) and the control trees (Table 4). In contrast, there were no significant differences between the numbers of *V. livia* in the untreated trees and the trees treated with *Trichogramma* or *Bacillus* at any period of the fruiting season (Table 5). In our opinion, this finding is due to the low rates of infestation by this pest.

CONCLUSION

The mean percentages of fruits infested by *E. ceratoniae* or *V. livia* in untreated trees were significantly higher than those in the trees treated with *Trichogramma* and *Bacillus*. Moreover, the trees treated with *Trichogramma* had a lower infestation rate for both *E. ceratoniae* and *V. livia* compared to the trees treated with *Bacillus*. Thus, the use of *Bacillus* or *Trichogramma* to control these pests can result in higher yields of Taify pomegranate with better quality. Native *Trichogramma* might be preferred for its high efficacy and for its matching with Taify environmental conditions. The effects of *B. thuringiensis* can possibly be improved by increasing the number of applications or its concentration. Furthermore, since many investigators have reported that indigenous *B. thuringiensis* isolates are effective in the control of native lepidopterous pests (Whitlock *et al*. 1991). The efficacy of *B. thuringiensis* can probably be increased by using native strains.

Table 4. Mean number of larvae per pomegranate fruit infested with *Ectomyelois ceratoniae.*

Date	No. of larvae per infested fruit (mean ± S.D.)			*F*-value	*P*
	With *T. turkestanica*	With *B. thuringiensis*	Control		
15 Apr	1.25 ± 0.289	1.19 ± 0.239	$1.42 \pm 0.290_{a}$	0.756	0.497
01 May	1.19 ± 0.239	1.19 ± 0.239	$1.27 \pm 0.961_{ab}$	0.207	0.817
15 May	1.19 ± 0.239	1.21 ± 0.143	$1.38 \pm 0.881_{ab}$	1.528	0.268
30 May	1.23 ± 0.270	1.23 ± 0.050	$1.38 \pm 0.292_{ab}$	0.571	0.584
15 Jun	1.25 ± 0.300	1.22 ± 0.130	$1.41 \pm 0.562_{ab}$	1.107	0.372
30 Jun	1.22 ± 0.211	1.24 ± 0.140	$1.42 \pm 0.180_{ab}$	1.509	0.272
15 Jul	1.08 ± 0.090^{A}	1.31 ± 0.102^{B}	$1.48 \pm 0.440^{C}_{ab}$	23.908	0.000
30 Jul	1.25 ± 0.214	1.31 ± 0.160	$1.40 \pm 0.172_{ab}$	0.654	0.543
15 Aug	1.30 ± 0.276^{A}	1.40 ± 0.216^{AB}	$1.66 \pm 0.097^{B}_{b}$	2.997	0.101
30 Aug	1.19 ± 0.166^{A}	1.27 ± 0.196^{A}	$1.61 \pm 0.162^{B}_{b}$	6.517	0.018
15 Sep	1.21 ± 0.209^{A}	1.26 ± 0.157^{AB}	$1.55 \pm 0.190^{B}_{ab}$	3.832	0.063
30 Sep	1.21 ± 0.143^{A}	1.40 ± 0.308^{AB}	$1.64 \pm 0.168^{B}_{b}$	3.971	0.058
F-value	0.230	0.631	1.986		
P	0.994	0.790	0.060		

Values followed by the same letter are not statistically different. Upper case letters following the values represent comparisons within a row and lower case letters represent comparisons within a column (Duncan's test; $P < 0.05$).

Table 5. Mean number of larvae per pomegranate fruit infested with *Virachola livia*.

Date	With *T. turkestanica*	With *B. thuringiensis*	Control	*F*-value	*P*
01 May	1.00	1.00	1.00 ± 0.0		
15 May	1.00 ± 0.0	1.00	1.00		
30 May	1.00 ± 0.0	1.00 ± 0.0	1.50 ± 0.707	1.429	0.340
15 Jun	1.00 ± 0.0	1.00 ± 0.0	1.34 ± 0.474	1.00	0.465
30 Jun	1.17 ± 0.289	1.13 ± 0.25	1.38 ± 0.479	0.538	0.604
15 Jul	1.17 ± 0.289	1.08 ± 0.144	1.42 ± 0.290	1.642	0.260
30 Jul	1.22 ± 0.387	1.19 ± 0.239	1.21 ± 0.249	0.482	0.637
15 Aug	1.28 ± 0.254	1.13 ± 0.25	1.25 ± 0.174	0.438	0.615
30 Aug	1.13 ± 0.250	1.13 ± 0.162	1.27 ± 0.253	0.487	0.630
15 Sep	1.29 ± 0.345	1.16 ± 0.197	1.18 ± 0.128	0.348	0.715
30 Sep	1.38 ± 0.479	1.13 ± 0.250	1.24 ± 0.219	0.553	0.594
F-value	0.536	0.259	0.536		
P	0.846	0.984	0.848		

Values within each row and within each column do not differ significantly (Duncan's test; $P < 0.05$).

ACKNOWLEDGEMENT

This work was supported by a project No. 3349-435-1 from Taif University, Saudi Arabia.

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Accepted 18 March 2015