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Research Article

Predation response of *Nabis pseudoferus* (Hemiptera: Nabidae) on untreated and *Metarhizium anisopliae*-treated larvae of *Tuta absoluta* (Lepidoptera: Gelechidae)

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Abstract: Functional response is an important behavioral characteristic of prey-predator interactions that can be utilized for assessing impact of natural enemies. In this research, the functional response of *Nabis pseudoferus* Remane females was examined to the third-instar larvae of *Tuta absoluta* (Meyrick) which were exposed to LC_{30} (2.03×10^4 conidia/ml) values of *Metarhizium anisopliae* (Metschnikoff) Sorokin isolate DEMI 001. Six densities of the prey (1, 2, 4, 8, 10 and 16) were exposed to the predator (0, 24, 48 and 72 h) after inoculation. *N. pseudoferus* exhibited a type II functional response to prey density in all treatments, indicating that predation increases asymptotically to a satiation level. The highest and the lowest attack rates (a) were 0.1052 ± 0.0440 and $0.0509 \pm 0.0133 h^{-1}$ for 48h and 72h post-infection treatments, respectively. Maximum theoretical predation rate (T/T_h) was estimated 10.96 in control. Our results suggest that *M. anisopliae* and *N. pseudoferus,* can be a useful combination in pest management of tomato leaf miner, although it must be confirmed in field condition.

Keywords: Functional response, Attack rate, *N. pseudoferus, T. absoluta, M. anisopliae*

Introduction

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechidae) is one of the most important pests of tomatoes both in field and greenhouse (Barrientos *et al*., 1998; Zappalà *et al*., 2013). Originating from South America (Desneux *et al*., 2010), this pest was recorded for the first time in Urmia, North West of Iran during November 2010 (Baniameri and Cheraghian, 2012). Subsequently, it was spread quickly and became one of the key pests of tomato in many regions in the country (Gharekhani and Salek-Ebrahimi, 2014; Ghaderi *et al.,* 2017). Larvae of *T. absoluta* attack all aerial parts of the plants including the apical buds, leaves, stems, flowers and fruits, causing up to 100% losses when management methods are not efficiently implemented (Biondi *et al*., 2018).

Recently, development of resistance by *T. absoluta* populations to traditional insecticides as well as other unfavorable side effects of the chemicals has encouraged safer methods of the pest control (Urbaneja *et al*., 2012; Ingegno *et al*., 2013). Thus, application of environmentally friendly tactics such as predators, parasitoids and entomopathogens is recommendable.

The damsel bug, *Nabis pseudoferus* Remane (Hemiptera: Nabidae) is a generalist and strong entomophagous predator that is commercially produced in Spain (Cabello *et al*., 2009; Gámez *et*

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al., 2012). This predator is able to feed on different life stage of *T. absoluta* including egg, larvae (in spite of being hidden inside the mines) and pupa (Cabello *et al*., 2009; Luna *et al.*, 2012; Zappala *et al*., 2013; Mahdavi and Madadi, 2015; Mahdavi and Madadi, 2017), unlike the *Nesediocoris teneuis* (Reuter) and *Macrolophus pygmeus* Rambur (Heteroptera: Miridae) that are predators of eggs and young larvae of tomato leaf miner. *N. pseudoferus* is a common species in Iran (Modarres Awal, 2008; Havaskary *et al*., 2012; Arbab *et al*., 2016) which may be used as a biological control agent in tomato crops (Cabello *et al*., 2009; Ghoneim, 2014; Mahdavi and Madadi, 2017). Furthermore, the muscardine fungus*, Metarhizium anisopliae* (Metschnikoff) is a virulent entomopathogenic fungus that attacks the eggs (Pires *et al*., 2009), larvae (Inanl and Oldargc, 2012; Tadele and Emana, 2017; Nozad-Bonab *et al*., 2017), and pupae (Contreras *et al*., 2014) of *T. absoluta*. The integration of *N. pseudoferus* and *M. anisopliae* can improve tomato leaf miner management; however, application of multiple biological control agents may act synergistically, additively or antagonistically (Roy and Pell, 2000). Accordingly, in order to make biocontrol programs more effective, multitrophic interactions among natural enemies, hosts and targeted plants need to be assessed. Alma *et al*. (2007) showed that utilization of entomopathogenic fungus, *Paecilomyces fumosoroseus* (Wize) to control *Trialeurodes vaporariorum* Westwood is compatible with simultaneous use of the whitefly predator *Dicyphus hesperus* Knight and these agents have additive effects. Similarly, Labbe´ *et al*. (2009) indicated that *Beauveria bassiana* (Balsamo), *D. hesperus* and *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) can be successfully combined for biological control of *T. vaporariorum*, in short-term in greenhouse tomato crops.

Wekesa *et al*. (2007) demonstrated that the fungal pathogen *Neozygites floridana* (Weiser and Muma) could reduce egg predation rate of the predatory mite *Phytoseiulus longipes*, by increasing time and high energy invested grooming. In another study, population parameters of the predator *Eriopis connexa* (Coleoptera: Coccinellidae) were influenced by entomopathogenic fungus, *B. bassiana* (Scorsetti *et al*., 2017). Agboton *et al*. (2013) also reported a negative interaction between the predatory mite *Typhlodromalus aripo* and the entomopathogenic fungus, *N. tanajoae*.

The response of a predator to prey density is a key factor for the success of a biological control program (Barlow and Goldson, 1993; Lester *et al*., 1999). This behavior may determine if a natural enemy is able to regulate, stabilize or destabilize the target pest's population (Dick *et al*, 2013). The relationship between predation rate (i.e., number of prey consumption per predator in unit time) and prey density is termed ''functional response'' (Solomon, 1949). Holling (1959) categorized functional responses into three mathematical models, which he called types I, II, and III. These models are characterized by a hyperbolic curve: a linear rise in prey consumption with increasing prey densities to a plateau (type I), a decelerating curvilinear rise to a plateau (type II), or a sigmoidal shaped curve to a plateau (type III) (Holling, 1959).

Host-infection by an entomopathogen can affect natural enemies' performance and foraging behavior such as functional response (Wekesa *et al*., 2007; Labbe´ *et al*., 2006; Pourian *et al*., 2011; Seiedy *et al*., 2012; Rännbäck *et al*., 2015; Wu *et al*., 2015**;** Jarrahi and Safavi, 2016 a; Jarrahi and Safavi, 2016b). Hence, in this research, we investigated effect of sub-lethal concentration (LC_{30}) of *M. anisopliae* isolate DEMI 001 on functional response of *N. pseudoferus* to infected larvae of *T. absoluta.* Taking into account that a pathogen needs some time for developing inside the host body, we also included time intervals from 0 to 72h from infection to exposure.

Materials and Methods

Plant cultivation and insect rearing

Tomato plants (cultivar Super Luna), were used for rearing of *T. absoluta* while they were approximately 45 days old. A stock culture of *T. absoluta* was initiated by collecting infected leaves from tomato fields in Urmia, West-Azerbaijan Province, Iran. The insects were reared on tomato

plants in wooden cages (60 × 60 × 40cm) for two generations in a glasshouse at 25 ± 1 ºC, 16: 8 (L: D) h and 65 ± 5% R. H. Adults of *N. pseudoferus* were collected by sweeping the alfalfa plants in Urmia fields, West - Azerbaijan Province, Iran (N 57° 29 ′ 53″ E 45°, 3′,31″). Ten to 20 adult *N. pseudoferus* of both sexes were introduced into rearing cups (14 × 22cm), equipped with a cardboard panel as a shelter and covered by a fine mesh gauze at lid for ventilation. The predator was supplied by bean pods as an oviposition substrate, as well as moisture source. The bugs were fed by adults and nymphs of cotton aphid, *Aphis gossypii* Glover. A colony of the aphid was obtained from the culture collection of the Plant Protection Laboratory, Urmia University and reared on cucumber plants in a growth glasshouse at 25 ± 1 ºC, 65 ± 5% RH, 16:8 (L: D) h. The cups were checked in 24-h intervals, and the bean pods involving the predator eggs were transferred to new cups (8 × 12cm) in a growth chamber at 25 ± 1°C, 65 ± 10% RH, and a photoperiod of 16: 8 (L: D) h. As soon as the eggs were hatched, the nymphs were transferred individually into Petri dishes (6 cm in diameter) containing fresh bean pods as well as nymphs of the prey, *A. gossypii*. The dishes were renewed every day until adulthood. The predator was reared for a generation, prior to experiments.

Fungal pathogen

Metarhizium anisopliae isolate DEMI 001 was obtained from the culture collection of the Plant Protection Laboratory, Urmia University. After passage of the fungus through *T. absoluta* larvae, it was cultured on Sabouraud's dextrose agar with yeast extract (SDAY) for two weeks at 25 ± 1 ºC until sporulation. Fungal suspensions were prepared in distilled water containing 0.02% Tween-80 and spore concentration was determined using a Neubauer hemocytometer (Neubauer improved, Kavalier). The viability of the conidia was determined by inoculating plates of SDAY (four plates) with a conidial suspension (100µl of 10^{7} dilutions) which was then incubated for 24h at 25 ± 1 ºC. The conidia were considered viable when the germ tube lengths corresponded to the width (Inglis *et al*., 2012). The viability of conidia was assessed immediately before each experiment and just those conidia which their viability was above 95% were used in experiments.

Virulence of *M. anisopliae* (DEMI 001) against *T. absoluta* larvae

Bioassays were carried out using third instar larvae (L3) of *T. absoluta* (high exposure to predator and fungus). Separate batches of L3 larvae were immersed in five spore concentrations including 10^{3}, 10^{4}, 10^{5}, 10^{6}, 10^{7} conidia. mL^{-1} for 10s. Treated insects were transferred upon tomato leaves embedded in Petri dishes (10cm diameter) covered with a fine mesh gauze on lid for ventilation and fresh tomato leaves were provided daily. The control batch was treated by sterile distilled water plus 0.02% Tween-80. Mortality was monitored daily and dead larvae were removed. Larval cadavers were surface sterilized in 70% ethanol, followed by sterile distilled water and incubated on moist filter paper in Petri dishes (6cm diameter) to confirm infection by *M. anisopliae*. The experiment consisted of 4 replicates (15 insects per replicate) for each concentration.

Functional response experiment

Petri dishes (9cm diameter), with a meshed hole in the lid, were filled with a layer of 2% water agar. Excised tomato leaves were placed upside down onto the water agar. Six densities of *T. absoluta* L3 including 1, 2, 4, 8, 10 and 16 were exposed to sub-lethal (LC_{30}) concentration of 2.03×10^{4} of *M. anisopliae* isolate DEMI 001 conidia/ml and then put into each Petri dish. Subsequently the treated larvae, exposed immediately or after 24, 48 or 72h incubation to experimental unit containing an individual predator. A 7-day-old inseminated female *N. pseudoferus* starved for 24h was used in each experimental unit. After 24h, the predators were removed and the number of consumed prey individuals were determined by counting number of survived larvae and subtracting it from initial prey number. Prey individuals were not replaced during the experiment and each treatment was carried out in 10 replications. The experiments were conducted at 25 ± 1 °C, 65 ± 5% RH and a photoperiod of 16:8h (L: D).

Data analysis

The logistic regression model was used to determine the type of functional response by taking into consideration the proportion of prey eaten (N_a/N_0) as a function of prey offered (N_0) (Juliano, 2001):

$$\frac{N_a}{N_0} = \frac{\exp\left(P_0 + P_2 N_0 + P_2 N_0^2 + P_3 N_0^3\right)}{1 + \exp\left(P_0 + P_2 N_0 + P_2 N_0^2 + P_3 N_0^3\right)} \quad (1)$$

where N_a is the number of prey eaten, N_0 is the initial prey density, and P_0, P_1, P_2 and P_3 are the intercept, linear, quadratic, and cubic coefficients, respectively, estimated using the method of maximum likelihood (Juliano, 2001). The signs of the linear coefficients (i.e., P_1) from the regression can be used to distinguish the shape of the functional response (type II or III). A significant negative linear coefficient suggests a type II response, while a significant positive linear term indicates a type III response (Juliano, 2001).

The handling times and attack coefficients of a type II response were estimated as Royama (1971):

$$N_a = N_0\{1 - \exp(a(T_h N_a - T))\} \quad (2)$$

where N_a and N_0 are described in equation (1), T is the time available for searching during the experiment (24h in this experiment), a is the instantaneous attack rate and T_h is per capita handling time. Parameters at various treatments were compared based on 95% confidence limits (CI). The coefficient of determination was calculated as R^2 = 1-residual sum of squares/corrected total sum of squares. The functional response analyses were done using SAS PROC NLIN (SAS Institute, 2003). Curves were drawn by Excel (2016).

Results

Bioassays of *M. anisopliae* (isolate DEMI 001) on *T. absoluta* L3 resulted in LC_{30} value of 2.03×10^4 (CI 95% = 9.35×10^3-3.81×10^4) conidia.ml^{-1}. In all treatments, the linear parameter of the polynomial regression of the proportion of prey consumed versus initial density was negative (Table 1).

Table 1 Maximum likelihood estimates of logistic regression parameters drawing of *Tuta absoluta* larvae eaten by female *Nabis pseudoferus* as a function of initial prey densities at 0, 24, 48 and 72h post-inoculation with LC_{30} of *Metarhizium anisopliae*.

Treatments	Parameters[1]	Estimate ± SE	χ^2	P-value
Control	Intercept	3.4309 ± 1.3005	6.96	0.0083
	N_0	-0.7787 ± 0.5817	1.79	0.1807
	N_0^2	0.0656 ± 0.0725	0.82	0.3660
	N_0^3	-0.0019 ± 0.0026	0.56	0.4545
0h	Intercept	2.9714 ± 1.1175	7.07	0.0078
	N_0	-0.8298 ± 0.5168	2.58	0.1084
	N_0^2	0.0826 ± 0.0655	1.59	0.2071
	N_0^3	-0.00275 ± 0.0023	1.37	0.2424
24h	Intercept	4.2802 ± 1.4740	8.43	0.0037
	N_0	-1.1128 ± 0.6372	3.05	0.0808
	N_0^2	0.0934 ± 0.0780	1.43	0.2311
	N_0^3	-0.0038 ± 0.0025	0.94	0.3332
48h	Intercept	3.7191 ± 1.2754	8.50	0.0035
	N_0	-1.0828 ± 0.5709	3.60	0.0579
	N_0^2	0.1117 ± 0.0711	2.46	0.1164
	N_0^3	-0.00380 ± 0.0025	2.26	0.1330
72h	Intercept	3.7456 ± 1.1413	10.77	0.0010
	N_0	-1.4324 ± 0.5244	7.46	0.0063
	N_0^2	0.1555 ± 0.0660	5.55	0.0185
	N_0^3	-0.00524 ± 0.2360	4.91	0.0267

[1] N_0, N_0^2 and N_0^3 are linear, quadratic and cubic coefficients, respectively.

Fitting the polynomial logistic regression (equation 1) to the data suggested that adult females of *N. pseudoferus* exhibited type II functional response to *T. absoluta* L3 whether treated or untreated with *M. anisopliae* (DEMI 001) (Fig. 1). Moreover, in all post-inoculation intervals (0, 24, 48 and 72h treatments), the proportion of the consumed prey declined with increasing the prey densities (Fig. 2). Table 2 represents the coefficient of attack rates (*a*) and handling times (T_h) of the predator. The attack rate coefficient and handling time of *N. pseudoferus* were significantly different from 0 as the asymptotic 95% confidence interval overlapped these values (Table 2). Maximum attack rate was estimated at 48h treatment ($0.1052 \pm 0.0440h^{-1}$). The lowest and highest handling time were observed in control (2.1899h) and 72h (3.3760h) post-inoculation treatments, respectively.

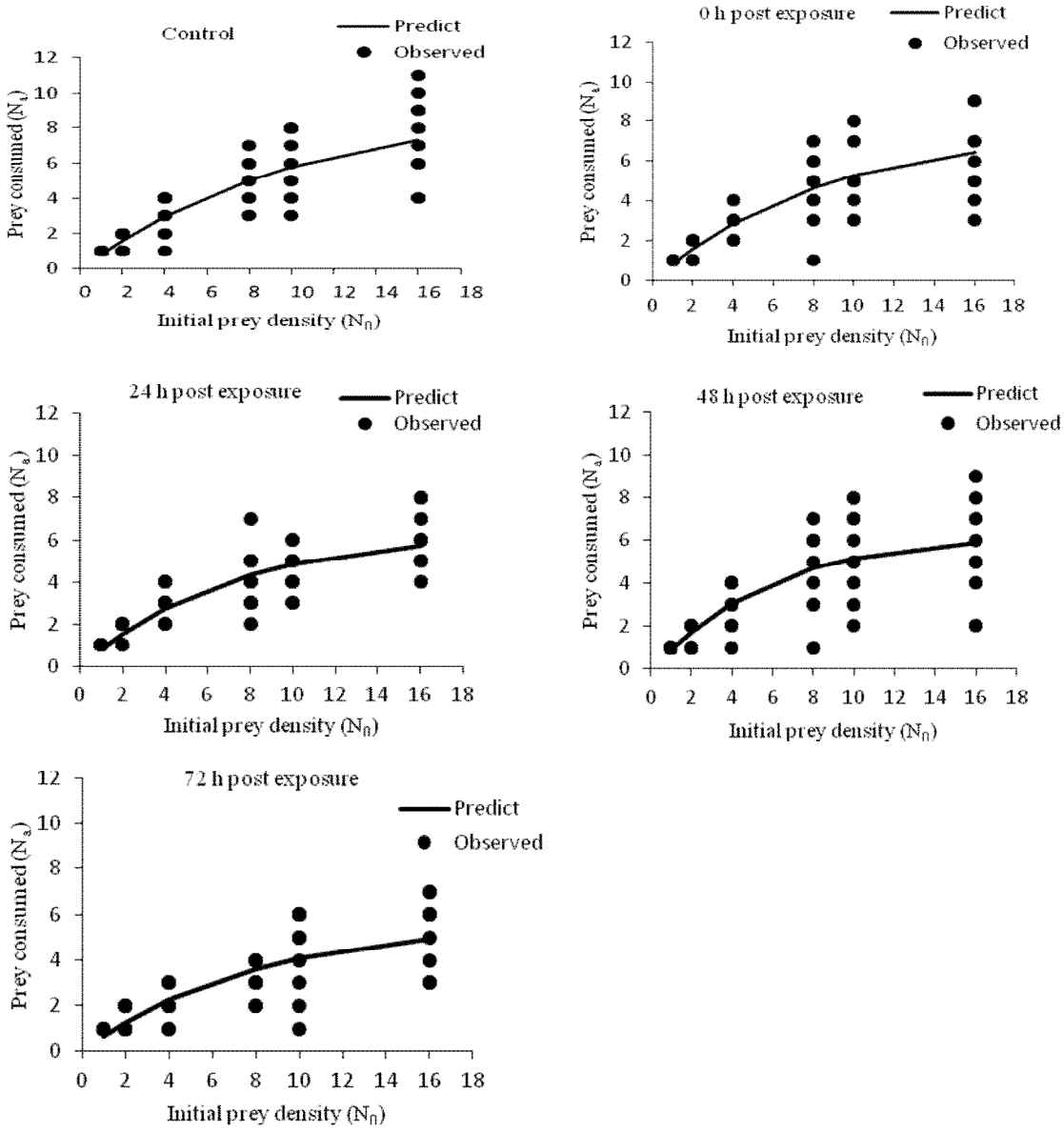

Figure 1 Functional response of *Nabis pseudoferus* to *Tuta absoluta* L3 at different post-exposure treatments to *Metarhizium anisopliae*.

Table 2 Parameters estimates (mean ± SE) of functional response of *Nabis pseudoferus* to *Tuta absoluta* larvae at different inoculation intervals to *Metarhizium anisopliae*.

Treatments	Functional response type	$a \pm SE$ $(h^{-1})^1$ (95% CI)[2]	$T_h \pm SE$	T/T_h	R^2
Control	II	0.0756 ± 0.0199 (0.0358 - 0.1153)	2.1899 ± 0.4293 (1.3306 - 3.0492)	10.96	0.92
0h	II	0.0744 ± 0.0215 (0.0315 - 0.1174)	2.6667 ± 0.4842 (1.6972 - 3.6359)	9.00	0.90
24h	II	0.0772 ± 0.0182 (0.0407 - 0.1137)	3.2005 ± 0.3942 (2.4114 - 3.9895)	7.50	0.94
48h	II	0.1052 ± 0.0440 (0.0170 - 0.1933)	3.3227 ± 0.5462 (2.2294 - 4.4160)	7.44	0.87
72h	II	0.0509 ± 0.0133 (0.0244 - 0.0774)	3.3760 ± 0.6102 (2.1547 - 4.5974)	7.11	0.90

[1] a, T_h and T/T_h are attack rate, handling time and maximum theoretical predation rate of predator, respectively.
[2] 95% confidence intervals.

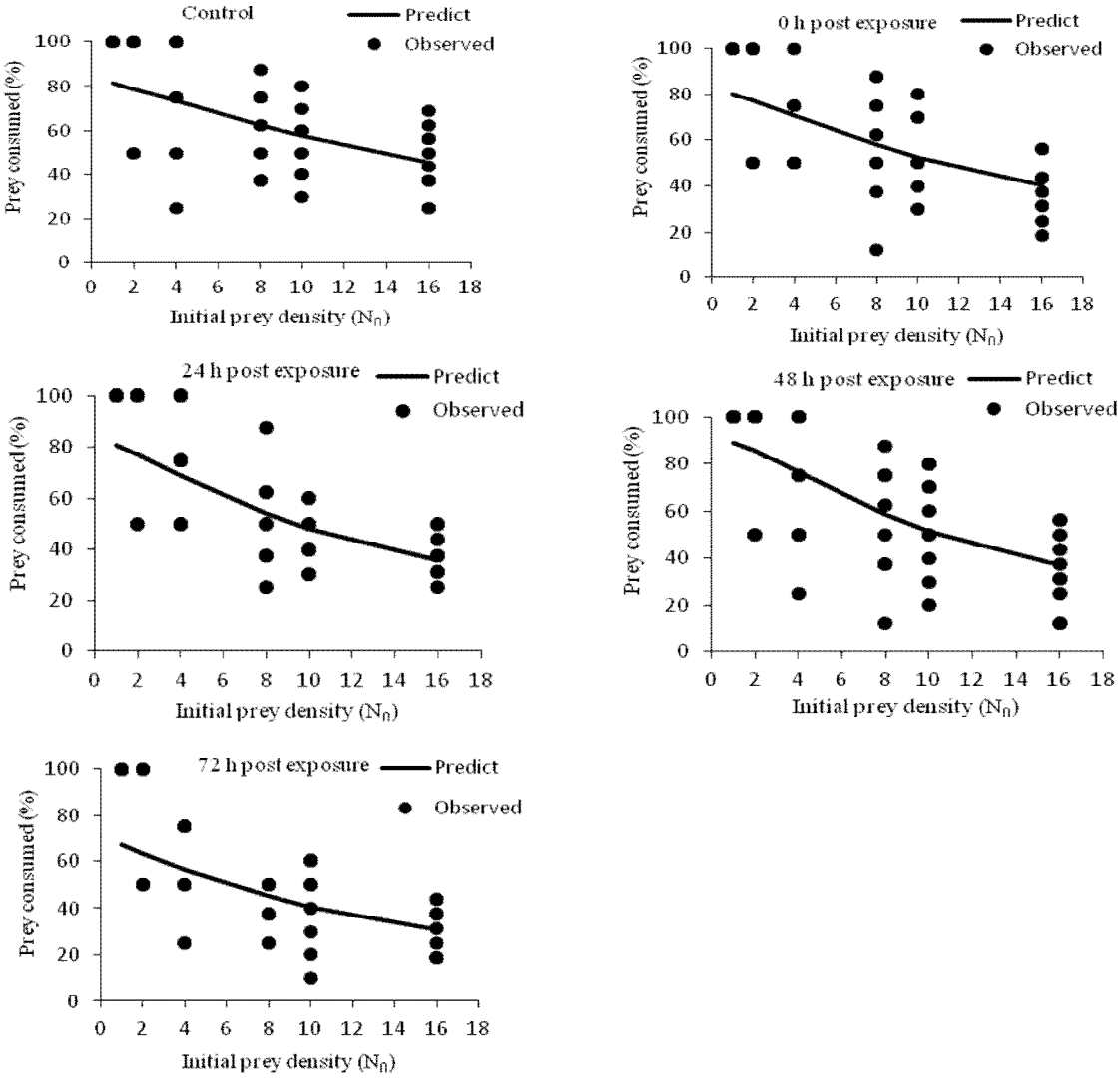

Figure 2 The percentage of consumed *Tuta absoluta* L3 by *Nabis pseudoferus* in different post-exposure treatments to *Metarhizium anisopliae*.

Discussion

The third instar larvae of *T. absoluta* tend to spread among tomato plant to avoid competition (Miranda, 1998; Cuthbertson, 2013). This behavior can increase exposure to external mortality factors such as natural enemies and insecticides (Miranda, 1998; Cuthbertson, 2013). As a result of this study, infection of *T. absoluta* third-instar larvae with a sub-lethal concentration (LC_{30}) of *M. anisopliae* (isolate DEMI 001) at different time intervals (0, 24, 48 and 72h) had no effect on the type of *N. pseudoferus* functional response. According to our results, the female predator exhibited type II functional response in all the treatments. A type II functional response indicates that the proportion of time a *N. pseudoferus* spends in handling prey increases as the density of *T. absoluta* larvae (treated or untreated) increases. These results agree with some studies in which fungus treatment of host has no effect on functional response type of some parasitoids (Jarrahi and Safavi, 2016a; Jarrahi and Safavi, 2016b) and predators (Seiedy *et al*., 2012; Wu *et al*., 2015). Furthermore, *Nabis* species commonly display type II functional responses (Fernandez-Maldonado *et al*., 2017). Similarly, Ma *et al*. (2005) demonstrated that functional response of adult damsel bug, *N. kinbergii* Reuter (Hemiptera: Nabidae) to *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) was type II. Moreover, Propp (1982) illustrated that *N. americoferus* displayed type II response to *Spodoptera exigua* (Hübner) and *Lygus hesperusl* Knight. Furthermore, Fathipour and Jafari (2003) demonstrated that functional response of *N. capsiformis* to second instar nymphs of *Creontiades pallidus* (Rambur) was type II. Contrarywise, Fernandez-Maldonado *et al*. (2017) found that functional response of *N. pseudoferus* females was type I. These authors used dead larvae of *S. exigua*, as prey to remove fighting. This can be a reason for the difference in functional response patterns in the two studies.

Based on our data, the highest coefficient of predator successful attack rate (a) is observed at 48h treatment. Usually, host locomotion declines at developed stages of infection (Roy *et al*., 1999). Faster movement lets a prey more frequently dose escape from predators (van den Meiracker and Sabelis, 1999). Hence, it can be concluded that lower mobility of larvae in 48h treatment might have been the cause for predator to overcome the prey faster both due to lower energy investment in fighting and higher speed of moving proportional to the prey. On the other hand, the slowest attack rate and the longest handling time both were observed in 72h treatment. Presence of hyphal bodies or fungal metabolites in the hemolymph of the diseased host, may act as an inhibitory factor against the predator which may in turn reduce intake rate of the predator. Reduced attack rate also may be due to ceased mobility of the prey that may cause visual detection by the predator became more difficult. These results are consistent with some results reported in the literature (Madurappulige, 2005; Wekesa *et al*., 2007; Seiedy *et al*., 2012; Wu *et al*, 2016).

Our results showed that the handling time of the predator increased on treated preys, resulting in a reduction in mean number of prey consumed. Seiedy *et al*. (2012) also obtained similar results in *P. persimilis*-*Tetranychus urticae* predator-prey system whether treated or untreated by *B. bassiana*. Moreover, handling time of predatory mite, *Neoseiulus barkeri* Hughes increased with longer incubation of *B. bassiana*-exposed adult *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), while the feeding rate decreased (Wu *et al*., 2015). Also, predation rate of *Orius albidipennis* (Reuter) decreased on *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) larvae treated by *M. anisopliae*, and predator was able to detect the treated larvae (Pourian *et al*., 2011).

Time interval between prey infection and subsequent access of predator affects acceptance or rejection of prey by predator (Labbe´ *et al*., 2006). Likewise, in our study incubation time increased from 0 to 72h, which in turn, caused daily per capita prey consumption rate to decrease from 9 to 7.11.

Our laboratory results showed that long-time exposure to the entomopathogenic fungus

causes partial mal-effects on functional response parameters of the predator. However it is not an absolute effect and the predator can successfully attack and kill the treated hosts in high numbers enough to insure that the predator increases host mortality by the fungus. For example, 30% mortality by the fungus corresponds to ≈ 4.8 preys, plus 7.11 killed by the predator exceeds that of the control (4.8 + 7.11 = 11.91 > 10.96). However, studying other aspects of such an integration is necessary for example, effects of environmental factors (temperature and humidity) on prey, predator and fungus; application sequence of the predator and the pathogen, prey preference of the predator, numerical response, ovipositional behavior and inter-specific competition by the predator individuals.

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Statement of conflicting interest

The authors state that there is no conflict of interest.

Author contribution

All authors contribute equally in this research.

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واکنش شکارگری سن *Nabis pseudoferus* (Hemiptera: Nabidae) در تغذیه از لارو *Tuta absoluta* (Lepidoptera: Gelechidae) سالم و تیمار شده با قارچ بیمارگر *Metarhizium anisopliae*

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چکیده: واکنش تابعی یک ویژگی مهم رفتاری در برهم‌کنش بین شکار و شکارگر می‌باشد که می‌تواند در ارزیابی تأثیر دشمنان طبیعی مورد استفاده قرار گیرد. در این پژوهش واکنش تابعی حشرات ماده سن شکارگر *Nabis pseudoferus* Remane نسبت به لارو سن سوم (Meyrick) *Tuta absoluta* تیمار شده با غلظت LC_{30} ($۲/۰۳ \times ۱۰^{۴}$ کنیدی در میلی‌لیتر) قارچ بیمارگر *Metarhizium anisopliae* (Metschnikoff) Sorokin، جدایه DEMI 001 مورد بررسی قرار گرفت. شش تراکم طعمه (۱، ۲، ۴، ۸، ۱۰ و ۱۶) در چهار بازه زمانی (صفر، ۲۴، ۴۸ و ۷۲ ساعت) پس از تیمار در معرض سن شکارگر قرار گرفت. واکنش تابعی سن *N. pseudoferus* نسبت به تراکم‌های طعمه در تمام تیمارها از نوع دوم به‌دست آمد که نشان‌دهنده افزایش نرخ شکارگری تا رسیدن به آستانه سیری است. بیش‌ترین و کم‌ترین مقدار نرخ حمله (a) به‌ترتیب ۰/۰۴۴۰ ± ۰/۱۰۵۲ و ۰/۰۱۳۳ ± ۰/۰۵۰۹ بر ساعت در ۴۸ و ۷۲ ساعت پس از تیمار به‌دست آمد. بیشینه نرخ حمله تئوریکی (T/T_h) در شاهد و برابر با ۱۰/۹۶ بود. نتایج نشان داد ترکیب قارچ *M. anisopliae* و سن شکارگر *N. pseudoferus* می‌تواند یک ترکیب مفید در مدیریت تلفیقی مینوز گوجه‌فرنگی باشد، با این حال این نتایج باید در شرایط کاربردی نیز مورد تأیید قرار گیرد.

واژگان کلیدی: واکنش تابعی، نرخ حمله، *N. pseudoferus*، *T. absoluta*، *M. anisopliae*