Article

Toxicity of *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) and boric acid against nosocomial cockroaches, *Blattella germanica*

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Received 13 April 2016; *Accepted 22 May 2016*; *Published online 1 September 2016*

Abstract

German cockroach is an important urban pest of worldwide distribution which harbors food poisoning and infectious organisms as well as allergens. In spite of the fact that insecticide application provides solution in severe cockroach infestation, it is associated with resistances development, pollution and economic lost. Integrated pest management (IPM) has been reported to be the best strategy for controlling such a nuisance pest. The main components of a successful IPM programme are biological agents and chemicals of reduced toxicity to non-target species. One of the biological agents which showed promising check on cockroaches is *Metarhizium anispoliae* (Metschnikoff) Sorokin. Also, boric acid has been traditionally used as a safe inorganic insecticide against many pest of agricultural and health importance. Boric acid and *M. anisopliae* showed not only compatibility, but also synergism in killing *Blattella germanica*, when applied as dust or liquid baits. However, incorporation and efficacy of both agents in semi-solid baits remains to be documented. This study revealed that boric acid incorporated at 8 gr/kg in semi-solid baits has no side effect on *M. anisopliae* but enhance its virulence causing higher mortality in adult males of German cockroaches. This study, also, showed that boric acid can be used at higher dosages without any harm to the fungal pathogen if allowed to be up-taken by the pest through cutaneous contacts and/or grooming.

Key words German cockroach; *Metarhizium anisopliae*; boric acid; synergism; biological control; probit analysis.

Arthropods
ISSN 2224-4255
URL: http://www.iaees.org/publications/journals/arthropods/online-version.asp
RSS: http://www.iaees.org/publications/journals/arthropods/rss.xml
E-mail: arthropods@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

The German cockroach, *Blatella germanica*, is a major structural pest of economic and health importance with worldwide distribution. Many studies indicated the role of this cockroach as a mechanical vector of a number of harmful and multi drug resistant microorganisms and parasites which cause household as well as nosocomial infections (Schal, 2009; Akinjogunla et al., 2012; Menasria et al., 2014). Also, its allergens may trigger allergic reactions in susceptible individuals such as dermatitis, itching, swelling of the eyelids and serious respiratory complications (Pomés et al., 2007; Wang et al., 2008). Given its high risks, the German cockroach has been subject to various control programmes mainly with synthetic insecticides. Extensive use of insecticides has enhanced development of significant resistance and even cross-resistance against various groups of chemicals particularly environmentally friendly ones such as pyrethroids, posing serious challenge to German cockroach management worldwide (Chai and Lee, 2010; Limoee et al., 2011; Moemenbellah-Fard et al, 2013). However, bait formulation was shown to enable selective delivery of lethal doses of insecticide to cockroach while reducing risks of resistance development inherent in sub-lethal dosages (Appel and Tanley, 2000). Despite growing interest in the use of baits due to easy and safe application, reports are emerging about cockroaches averting marketed gel baits (Srinivasan et al., 2005). The bait aversion, as a behavioral resistance exhibited by cockroaches and other pests, is believed to be triggered by both inert and toxic ingredients of gel baits (Wang et al, 2006; Butler et al., 2007; Wada-Katsumata, 2011). To the contrary of Rust et al. (1995) who concluded that German cockroach is repelled by most insecticides incorporated in bait formulations, Nasirian (2008) found that fipronil and imidacloprid gel baits were effective even against resistant ones. Durier and Rivault (1999) postulated that for a bait to be effective, it must be palatable, easy to consume with non-repellent and slow-acting insecticides. Nonetheless, bait performance remains to be dependent to several biotic and abiotic factors (Appel and Tanley, 2000).

There have been attempts to substitute insecticides in baits with inorganic or biological agents of favorable safety records such as boric acid and entomopathogenic fungi. Despite the early controversy over the use of boric acid in baits due to its assumed repellency to German cockroaches (Strong et al., 1993), other studies indicated no repellency when incorporated in liquid baits and dust (Gore and Schal, 2004). Boric acid was shown to be not only effective against cockroaches as a stand-alone insecticide in field trials (Gore and Schal, 2004), but also synergistic in combination with *M. anisopliae*in laboratory assays (Zurek et al., 2002). The mycopathogens such as *M. anisopliae* and *Baeuveria bassiana* have been evaluated as promising bioagents for controlling many insect pests including *B. germanica* and *Periplaneta americana* (Abedi and Dayer, 2006; Hernandez-Ramirez et al., 2008). However, there are few reports on the use of *M. anisopliae* together with boric acid incorporated in food base baits against German cockroaches and even fewer reports about the combination of both agents in solid or semi-solid baits (Gore and Schal, 2004; Zurek et al, 2002; Lopes and Alves, 2011). Also, there is no recorded study in Iran on the use of the fungal pathogen in baits against cockroaches (Nasirian, 2010).

This study was undertaken to evaluate the effect of boric acid and *M. anisopliae* in solid food-base baits as stand-alone as well as in combination against a hospital-collected strains of *B. germanica*. Also, the possibility was examined to use boric acid at higher dosages against the pest through its simultaneous application both as baits and as sprayed solution in test chambers.

2 Materials and Methods

2.1 Insect

The German cockroach used in this study was a hospital-collected strain raised in the insectarium of the Department of Parasitology and Medical Entomology of Tarbiat Modares University (TMU-Tehran). The colony was reared in ambient environment at 28±3 °C and 50±5 % RH, and photoperiod of about 12:12 h light: dark cycle, fed with white bread, rice and potato starches and banana peelings. Water was provided in small bird drinkers with cotton stoppers. Each glass cage (40 × 30 × 20 cm) was provided with a paper egg carton shelter. Newly emerged male adult (< 5 day old) were separated prior to experiments under CO_2 anesthesia from cohorts of synchronized cockroaches. The cockroaches were then starved for one scotophase (12 h) before being exposed to experimental baits.

2.2 Conidia

The fungal isolate of *M. anisopliae* var. *anisopliae* was obtained from the Stock of the Department of Parasitology and Medical Entomology (TMU-Tehran), cultured in Petri dishes on Sabouraud dextrose agar containing chloramphenicol (0.5 g/l) and cycloheximide (0.4 g/l) and incubated for 15 days at 35°C before the conidia were harvested for experimentation (Arthurs and Thomas, 2001). The conidia were scraped off with a sterile metal loop and suspended in sterile potassium- phosphate buffer (3mM) containing 0.05 % (v/v) triton X100 and subjected to 5 washing steps with the same buffer solution followed by centrifugation at 2800 g. The required spore concentration was determined using a haemocytometer.

2.3 Baits

The inert ingredients of the experimental baits were selected based on our preliminary assays (data not shown) and recommended food choices for rearing *B. germainca* (El-Sharabasy et al, 2014). Potato powder, rice flour, and wheat starch in equal amounts were blended and distilled water gradually added to make a consistent semi-solid food. In order to maintain the required moisture, 1% (v/w) tween 80 and 1% (v/w) glycerin were also added. The active ingredients were then mixed with food at required dosage as per experimental designs, before the baits were manually granulated into pellets and left to dry at room temperature for 24 h.

2.4 Experimental design

Boric Acid (Merck) was incorporated into the baits at 8 g/kg (0.8 % w/w), while, the conidia of *M. anisopliae* were added at a rate of 5.3×10^8 spore per 10 grams baits. The experimental cockroaches were divided into 5 groups; T_0, T_1, T_2, T_3 and T_4. The group T_0 was provided with untreated baits lacking active ingredients. Group T_1 was fed with baits containing both fungal spore and boric acid and confined in chambers previously contaminated with boric acid solution. These chambers were borated by rinsing in 8 g/l solution and dried at room temperature. Group T2 was provided with the same baits as Group T_1 but confined in non-contaminated chambers. Group $T_3$and GroupT_4included cockroaches fed with baits containing fungal conidia and boric acid alone respectively. The treated cockroaches were fed with fresh baits every 15 days and dried consumed baits were removed.

For each group, three replications containing 30 newly emerged adults were conducted. Mortality was recorded at 5 day intervals until 60 days after the beginning of experiments and corrected using Abbott's formula. Samples of fungus exposed cadavers were externally disinfected by dipping in 2.5 % sodium hypochlorite and washing with distilled water (3 times), before being placed in sterile Petri dishes lined with wet filter papers and covered with parafilm. The cadavers were then incubated at room temperature (28±3 °C) and examined for fungal sporulation 15 days post-mortem. Bioassay data were subjected to probit analysis using SPSS 16.0 for Windows (2007-SPSS Inc.) and estimated LT50 and their fiducial limits were determined. The correlation between doses and duration of exposition was measured using the Pearson goodness-of-fit test.

The equality of the regression slopes (parallelism) were tested using Student’s t in a fashion analogous to that of testing for difference between two population means (Zar, 1974). The test statistic was

$$t = \frac{b_1 - b_2}{S_{b_1 - b_2}}$$

where: b1 and b2 were the slopes of the line to be compared and

$$S_{b_1 - b_2} = \sqrt{\frac{(S^2_{Y.X})_P}{(\sum x^2)_1} + \frac{(S^2_{Y.X})_P}{(\sum x^2)_2}}$$

The pooled residual mean square was calculated as

$$(S_{Y.X}^2)_P = \frac{(residual\,SS)_1 + (residual\,SS)_2}{(residual\,DF)_1 + (residual\,DF)_2}$$

The subscripts 1 and 2 refer to the two regression lines being analyzed. The critical value of t for this test had (n1 – 2) + (n2 – 2) degrees of freedom.

3 Results

The adults of German cockroach fed with baits containing fungal spores and boric acid and confined in borated chambers (T1) showed 100 % mortality after 40 days post treatment, whereas those fed similar baits but confined in clean chamber (T2) resisted 5 more days before succumbing to the same mortality rate (Fig 1). We observed no external fungal growth on incubated cockroach cadavers which died in the first 10 days after the onset of treatments involving *M. anisopliae* spores. In fact, the re-growth of fungal mycelia was observed only on insect cadavers died at day 15 post treatments (Table 1).

Table 1 Mycelial growth of *Metarhizium anisopliae* on cockroach cadavers incubated at 5 day interval.

Treatment (active ingredients)	Day 5	Day 10	Day 15
T1 (*M. anisopliae* +Boric acid + borated chambers)	-	-	+
T2 (*M. anisopliae* +Boric acid)	-	-	+
T3 (*M. anisopliae*)	-	-	+
T4 (Boric acid)	-	-	-
T0 (control)	-	-	-

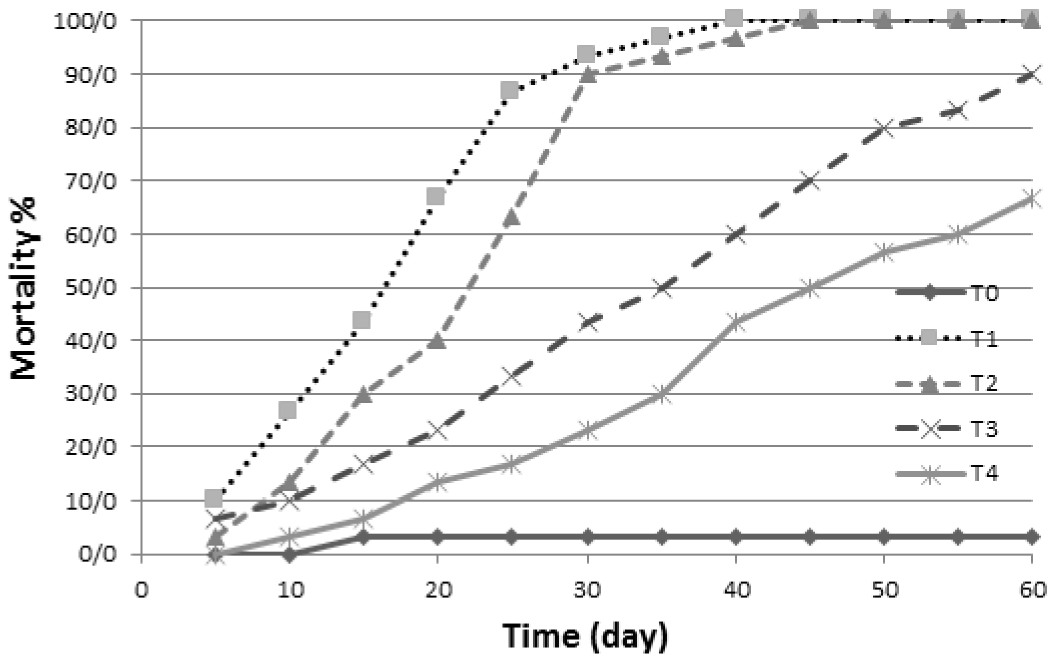

Fig. 1 Accumulated mortality rate of German cockroach fed with baits containing fungal spore and boric acid in borated chambers (T1), the same baits in clean chambers (T2), baits containing fungal spores (T3), baits containing boric acid (T4) and control baits void of active ingredients (T0).

However, early mortality was recorded during the first 5 days post-treatment amongst cockroaches treated with baits containing boric acid. This indicates a lag phase between the functions exerted by the fungus and that of boric acid; the latter being faster to cause insect death. While, the use of boric acid baits induced about 25 % mortality in one month time, the fortified baits with *M. anisopliae* killed 90 % of cockroaches in the same period (Fig. 1). Regardless of the type of active ingredients, the baits were all visited and consumed by German cockroaches during this time and no sign of repellency or avoidance was observed.

Table 2 summarizes probit analyses of mortality data, indicating LT50 values equal to 16 days for T1 and 27 days for T2. The LT50 value of treatment with baits containing fungal spores was 35 days. The cockroaches treated with boric acid at the applied concentration (0.8 % w/w) showed steady mortality reaching 42 % mortality on day 40 post treatment with an LT50 equal to 47 days. Probit analysis of the pooled mortality data obtained from treatments with baits containing fungal spore (T3) and boric acid (T4) as standalone active ingredients resulted in LT50 value equal to 41 days. Comparison of the probit regression lines of LT50 resulting from mortality data induced by T2 baits (mixed fungal spore and boric acid) and that obtained from pooled mortality of both T3 baits and T4 baits revealed significant potency of T2 baits for its reduced value of LT50 by 20 days. This is a remarkable sign of synergism effect of both active ingredients when incorporated in one bait formulation rather than as standalone agents.

Table 2 LT50 values of different treatments on *Blattella germanica* adults and slopes of regression lines.

Treatment	**LT50 (95% CI) day**	**Slop ± SE**	x^2	*p*
T1 (mixed Ma and Ba in borated chamber)	16 (12.3-19.2)	0.116 ± 0.016	2.114	0.005
T2 (mixed Ma and Ba)	21 (17.6-23.5)	0.112 ± 0.015	2.772	0.004
T3 (Ma only)	35 (28.1-39.3)	0.052 ± 0.007	1.568	0.001
T4 (Ba only)	47 (41.5-51.9)	0.046 ± 0.007	3.123	0.022
T3+T4 = cumulative effects	41 (36.7-439)	0.047 ± 0.005	3.734	0.041

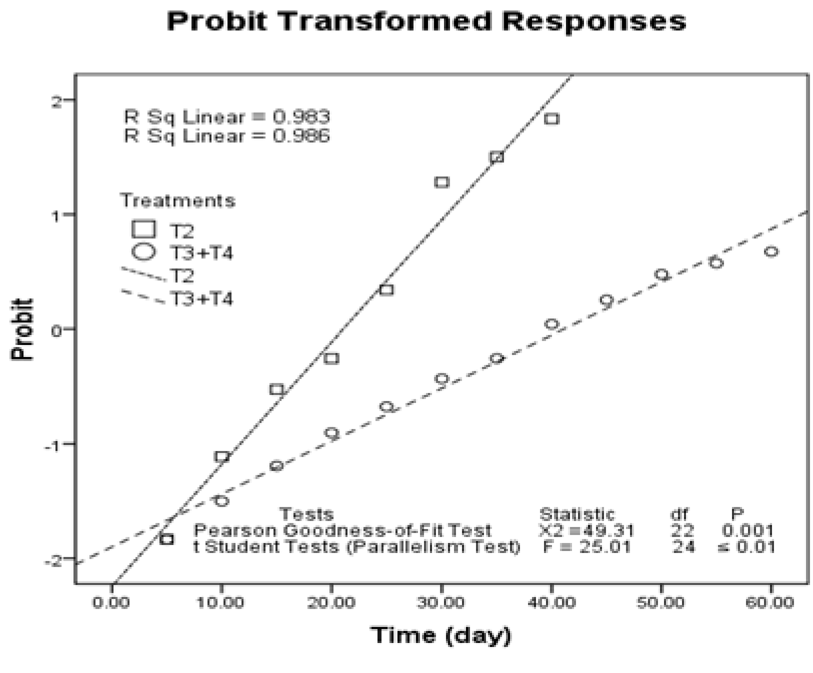

Fig. 2 Comparison between the probit regression lines of responses of German cockroach to T2 (mixed Ma and Ba) and pooled T3 (Ma only) and T4 (Ba only).

Fig. 2 depicts the synergized mortality rate of German cockroaches fed on baits containing mixed *M. anisopliae* conidia and boric acid compared to the pooled mortality caused by baits containing fungal spores and boric acid as standalone insecticides. There is significant difference between the resulted LT50s; 21 days for T2 and 41 days for T5 (pooled T3+T4). Also, there exists a significant difference between the slope of the regression lines of both treatments. This provides another evidence for synergistic effect that both agents produce when mixed together in a semi-solid baits.

However, Fig. 3 shows the comparison between the slopes of the regression lines resulted from treatments which were applied to two groups of *B. germanica* adults using similar baits impregnated with fungal spores and boric acid. A group of pests were confined in borated chambers; T1 and the other placed in clean chambers; T2. Carrying out the t test for parallelism indicated no significant difference between the slopes of the regression lines of T1 and T2 (Fig. 3), although the corresponding LT50 values showed difference of 5 days from 16 days for T1 to 21 days for T2.

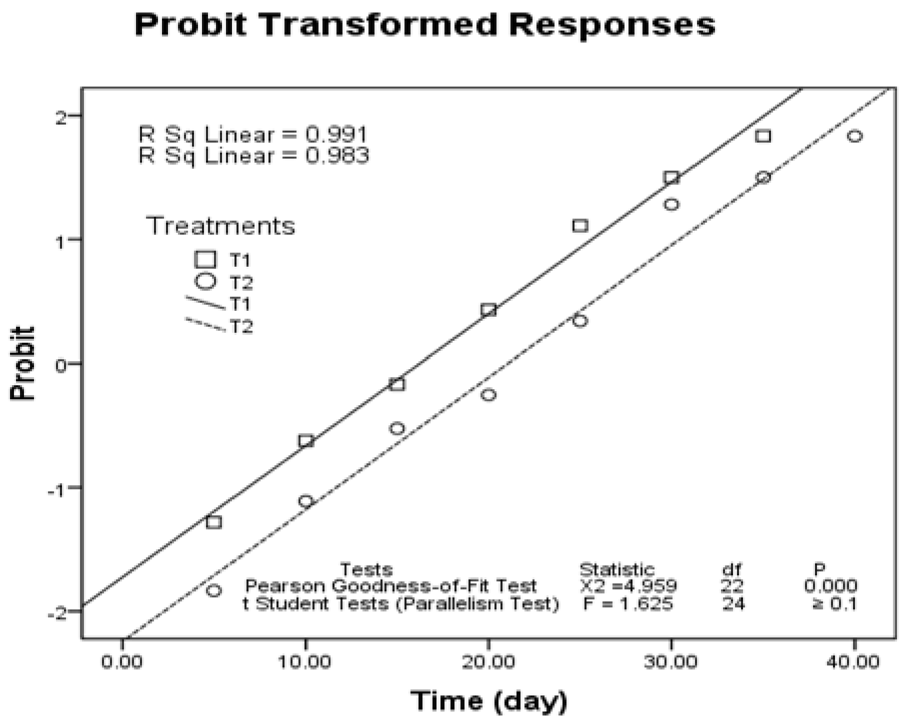

Fig. 3 The parallelism of probit regression lines of responses of German cockroaches to T1 and T2 treatments.

4 Discussion

In this study, adult males of German cockroaches were used to test the efficacy of different formulation of *M. anisopliae* conidia and boric acid in inert baits as standalone and integrated active ingredients. The adult males being the most active foragers (Metzger, 1995), can play important role in translocating the baits to roach shelters and activate coprophagy, necrophagy and/or cannibalism as secondary kill mechanisms in the population (Kopanic and Schal, 1999). It has, also, been shown that allergen Blag 4 is produced only by accessory reproductive glands in adult male and not in female (Fan et al., 2005). Therefore, formulating and using baits which primarily target adult males of *B. germanica* may not only reduce complications of male infestation by direct kill, but also trigger secondary kill mechanisms in the whole population.

Apart from active ingredients, inert portion, including a food base, is the most important fraction of bait formulation. It is intended not only to lure roaches to toxic baits, but also to ensure maximum viability and delivery of bioagents to target pests against harsh environmental conditions (Nalyanya, 2001; Davari et al., 2015). While white bread was reported by a number of authors to be an attractive food to German cockroaches, others found carbohydrate and protein foods such as biscuit and cheese to be more attractive (Nalyanya et al., 2001; Wang and Bennett, 2006; El-Sharabasy et al., 2014). However, in our experiments, baits composed of wheat starch, rice flour and potato powder was very attractive to German cockroaches. Also, addition of fungal spores and boric acid to our experimental baits exerted no repellency effects on the pest at the applied dosage. Schal (2009) postulated that gel bait formulation is of advantages in field conditions, because it provides moisture, increases efficacy and enables easy dispense in cockroaches' shelters. In our laboratory trials, the incorporation of tween 80 and glycerin in baits conferred the same traits and confirmed that moist baits are more attractive to cockroaches than dry ones. In fact, semi-solid texture of our baits not only maintained palatability to cockroaches but also supported fungal spore viability for 15 days after application at room conditions.

The rapid development of genetic and behavioral resistances by German cockroaches including aversion to baits impregnated with certain insecticides represents a challenging setback to roaches chemical control (Wang et al., 2006). This problematic may not be circumvented by simply rotating baits of different chemical composition (Limoee et al., 2011). Therefore, searching for alternatives amongst bioagents and chemicals of different mode of actions is a must for roach control. The entomopathgenic fungus, *M. anisopliae* has shown to be a promising bioagent against *B. germanica* both in laboratory and field trials (Wakil et al., 2012; Davari et al., 2015). Gutierrez et al. (2014) reported that topical application of *M. anisopliae* produced 93.3% mortality in adults of *B. germanica* after 10 days. Quesada-Moraga et al. (2004) showed that horizontal transmission of *M. anisopliae* between infected and healthy adults of *B. germanica* resulted in 87.5% mortality rate with an LT50 value of 12.2 days. Nonetheless, for the fungus to gain satisfactory action compared with synthetic chemicals, it must be incorporated with sub-lethal doses of insecticides (Wakil et al., 2012).

On the other hand, boric acid was shown to be an inorganic insecticide of choice. This is because it is not only safe to man and environment but also it may suppress cockroach population via neurotoxic action, abrasion of cuticle and midgut and disruption of vitellogenesis (Habes et al., 2013). Boric acid can synergistically accelerate mycosis of *B. germanica* by *M. anisopliae* conidia when applied either as dust or in drinking water (0.1 % w/v), arriving at 100 % mortality in 10 days (Zureck et al., 2002 and 2003). However, there have been few reports on boric acid being tested in semi-solid baits as a standalone or incorporated with the fungus against German cockroaches. This work provided new evidence that boric acid may be successfully incorporated with conidia of *M. anispoliae* in semi-solid baits against the cockroaches. It, also, indicated that the combination of both agents result in synergized mortality of *B. germanica*, accelerating the mortality by 20 days (Table 2). Although, the addition of boric acid to bait enhances the virulence of the fungal spores, but an elevated concentration of boric action may be detrimental to the fungus. Zureck et al. (2002) showed that boric acid causes 100 % mortality in adults German cockroaches at 0.2 g dust/m2, while lower concentration failed to do the same even after 28 days. Hernandez-Ramirez et al. (2008) reported that addition of boric acid at 3 and 5% to food containing *Beauveria bassiana* produced a synergic effect killing 92% of American cockroach. In this work, we found that addition of boric acid at 0.8 % w/w to bait was synergistic to the performance of the fungal spores. Based on this finding, we may hypothesize that synergistic interaction of *M. anisopliae* and boric acid is independent of formulation. This is true as we showed that additional boric acid dosages may be concomitantly used with borated baits to control German cockroaches without compromising conidia viability and growth. This was possible when boric acid solution (8 g/l) was topically applied to experimental chambers of cockroaches before confining the cockroaches and feeding them the borated baits. This enabled additional uptake of boric acid dosages via cuticle and/or grooming which leads to destruction of midgut epithelial cells, reduced activity of acetylcholinesterase and eventually death of *B. germanica* (Habes et al., 2006). The resulted additional mortality rate has no effect of the slope of regression line of LT50s as depicted in Fig 3, confirming additive but not synergistic effects by additional boric acid dosages.

The emergence of fungal conidia was observed only from roach cadavers which were treated with baits containing fungal conidia with or without boric acid and died within the third interval of mortality record post treatment (10<day< 15). In fact, the incorporated boric acid in the ingested baits or that up-taken additionally from borated chambers, via positive thigmotropism and subsequent grooming by cockroaches, did not accelerate the penetration process of *M. anisopliae* into the hemocoel of the pest (Table 1). This is because the fungus could not be retrieved from cadavers which died earlier due to T1 or T2 treatments. This is in contrast to what Zurek et al. (2002) suggested as if boric acid facilitates the penetration of *M. anisopliae* by destroying the cellular lining of the roach foregut producing synergistic effects. Instead, it seems that true synergistic interactions between boric acid and fungus start after the fungus gain entrance to the pest hemocoel. For more clarification, the nature of interaction between *M. anisopliae* and boric acid needs further investigation. However, given the sociality of *B. germanica* as mixed family herds, one may infer that such boric acid-fungus interactions favour initiation of epizootics in cockroach populations, hence increase translocation of the active agents to their shelter, debilitate their pest status and suppress them in longer terms(Quesada-Moraga et al., 2004; Lihoreau et al., 2012).

We may conclude that *M. anisopliae* virulence to German cockroaches can be synergistically accelerated in semi-solid baits containing boric acid. Being sound and safe to human and other mammalian animals, both agents may be applied as baits at households and animal farms to offer a relative long terms and environmentally friendly control of *B. germanica*. Although, the concentration of boric acid in fungal bait formulation is limited to the extent that does not compromise the bioagent viability, additional boric acid can separately be applied to maximize the mortality of *B. germanica*.

Acknowledgments

The authors would like to acknowledge the support of University of Tarbiat Modares and invaluable assistance of the Department of Medical Parasitology and Entomology in carrying out this study.

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