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Effectivity test of the fungi *Trichoderma viride* and *Metarhizium anisopliae* as biocontrol agents against cow ticks *Rhipicephalus microplus*

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Abstract. Ticks are usually common parasites that disturb and destroy some livestocks of sheep, cows and others. That is why, most agricultural and livestock sectors depend on using chemical pesticide or acaricide to terminate these ticks. Most of the pesticidal users are unaware of the impacts of these chemicals to their health and environment if used continually. Ticks maybe able to adapt resistance from these acaricides and may lead to an increase of dosage of chemicals same as its price. Finding a solution to this using biological control agent (BCA) is recommended. This study was conducted to test the biocontrol agent of the available fungi in the Department of Agriculture-General Santos City, against the increasing number of cow ticks, *Rhipicepalus microplus* within the study area. Two fungi were used in the treatment, the *Trichoderma viride* and *Metarhizium anisopliae.* The three stages of ticks were treated with these fungi in direct application using suspension method. The treated ticks were then placed to their designated Petri dishes. Significant results and observations were then recorded and total number of dead specimens was counted. Graphical representation and statistical analysis using PAST and Microsoft Excel software were provided for better and simplified discussion of the result. It was shown that *M. anisopliae* exhibits a higher effectivity compared to the *T. viride* in killing cows ticks. This fungus can be an alternative pesticide against the said parasite.

Key Words: BCA, entomopathogenic fungus, alternative biopesticide, environmental health.

Introduction. Ticks are small animals that feed on the blood of mammals, birds, reptiles and amphibians. Although they resemble small insects, ticks belong to a group of animals called arachnids. They are pest that contaminate largely on the livestock of cows, sheep and others. Farmers today are still dependent on using chemical pesticides or acaricides in terminating ticks that have disadvantages. Ticks could have grown resistant of these acaricides that could only lead to an endlessly increasing of dosage of chemicals as well as its price.

An alternative way than using acaricide is the wise use of biological control agent (BCA) (Pourseyed et al 2010). BCA species consumes target pest organisms via insectivorous birds or predators, (Barre et al 1991; Kok & Petney 1993; Samish & Alexseev 2001); nematodes (Samish et al 2000a,b; Samish & Glazer 2001), viruses and bacterial pathogens (Lipa 1971; Hoogstraal 1977; Chandler et al 2000). These agents are typically nontoxic to humans and non-target organisms. They apply high efficacy against insect pests and low virulence for the non-target insects (Zimmerman 1993). According to the US Environmental Protection Agency (USEPA), a kind of pesticide known as biopesticides are basically pesticides that come from natural materials such as animals, plants, bacteria and minerals (USEPA 2008). These also include those living organisms that destroy agricultural pests which were the cause of failure in the crop production of the farmers that is the main source of their living. Biochemical pesticides are distinguished based on conventional pesticide both by their structure and mode of action (O' Brein et al 2009). Another kind of pesticide is the microbial pesticide also known as BCA. It gives us advantages of higher selectivity and lower or no toxicity compared to conventional pesticides (MacGregor 2006). The active ingredient of a microbial pesticide is typically the microorganism. The microbial ingredient can either be the spores or the organism themselves. Entomopathogenic fungi such as *Metarhizium anisopliae* is a well known controlling agent and has been developed worldwide (Lopez et al 1998). Along with other fungal pathogens, it does not need to be consumed but infect the insect with contact. *M. anisopliaie* is a biocontrol agent against a range of several ticks under laboratory and field conditions (Kaaya 2000). Another amazing BCA is the *Trichoderma viride*. *Trichoderma* genus contains very versatile mold species, sometimes irritating agents for humans. However, it is a useful fungus for industry and biocontrol, and a threat to other fungi (Khandelwal et al 2012). Because of the presence of chitinases in its biochemical structure, it can act as a parasite against other fungi and to those chitin made organism such as arachnids. Basically, this fungus is well-known as a microbial pesticide presenting an important advantage because of its lower toxicity compared to the chemical ones. Trichoderma viride is identified to be a potential antagonistic fungus that protects the crops against a wide range of diseases (Chet et al 1981; Papvizas 1985; Kumar & Mukerji 1996).

The aim of this study is to test the pesticidal efficiency of *Trichoderma viride* and *M. anisopliae* against the cow ticks *Rhipicephalus microplus*.

Material and Method

Specimen collection. In order to obtain the required ticks, the researchers collected specimens from five cows owned by the Banisil family located at this GPS points 6°04' 23.78" N; 125°08'13.36" E Brgy, Fatima, General Santos City. The ticks were placed in vials where they can be reared and comfortable maintained. These containers were stored properly at the room temperature. The ticks were divided equally in three groups, two experimental, and one control. Samples of all three groups were placed in Petri dishes. The adult ticks were separated by the nymphs and larvae.
Same methods were performed for all the groups. The ticks were feeded with honey syrup as an alternative source of their food. The observation and data recording were conducted for 7 days. Observations of the ticks where then recorded.

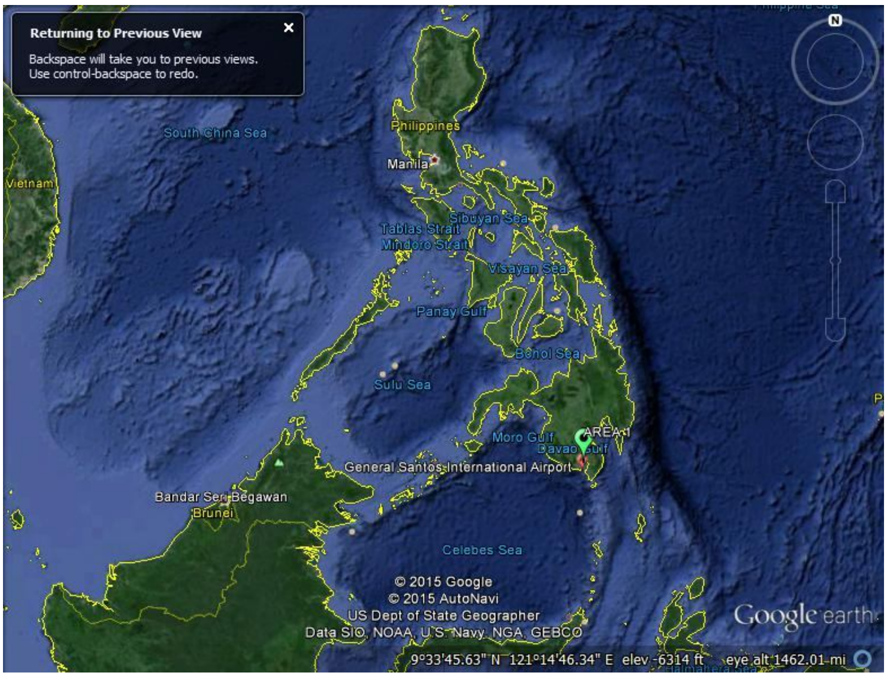


Figure 1. The Philippine map.

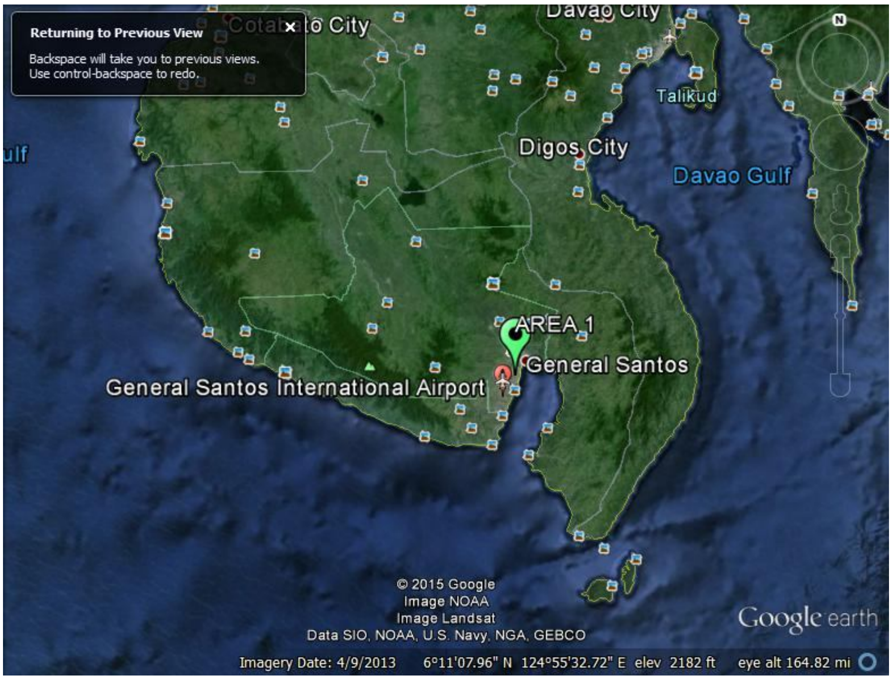


Figure 2. Southern Mindanao.

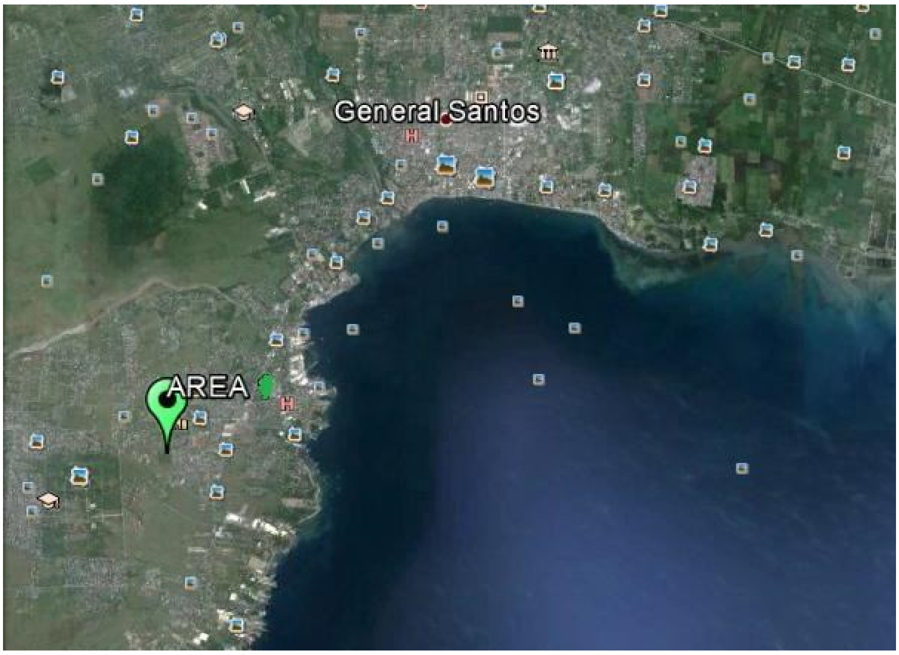


Figure 3. Map of the the study area marked with green arrow where *Rhipicephalus microplus* were obtained.

Fungi rearing. The biological control agents *Trichoderma viride* and *Metarhizium anisopliae* were originally cultured by the Department of Agriculture. They were cultured for agricultural purposes such as pesticide and fungicide. The fungal species *T. viride* was isolated from soil samples using a potato dextrose agar (PDA) medium. Samples were inoculated over plates by multiple tube dilation technique (MTDT) and the plates were incubated at 26°C for 4 days (Abdel-Fattah et al 2007). The Department of Agriculture was able to produce and culture *M. anisopliae* using corn plants that were boiled and stored for some months in order to produce pure conidia.

Figure 4. *Metarhizium anisopliae* (www.google.com.ph/metarhizium anisopliae).

Figure 5. *Trichoderma viride* (original).

Application. The study conducted two experimental set ups named Treatment 2 (T2) and Treatment 3 (T3) and one control set up named Treatment 1 (T1). The T2 was purely composed of the formulation of *T. viride* fungi. The T3 was formulated using the fungi *M. anisopliae.* The T1 served as a control group. The ticks were separated in adults, nymphs and larvae, and were divided equally in three for the three treatments (90 adults, 90 nymphs and 90 larvae). The cow ticks were then placed in three Petri dishes fairly and treated with their designated treatments. Each of them was treated individually using the pure conidia of each fungi. The method of application used to ticks was the direct contact with the fungi through dipping or suspension for about 20 seconds. The ticks in T1 remained untreated. The performed treatment had three total replications using the same methods. Observations and data recordings were conducted for 7 days.

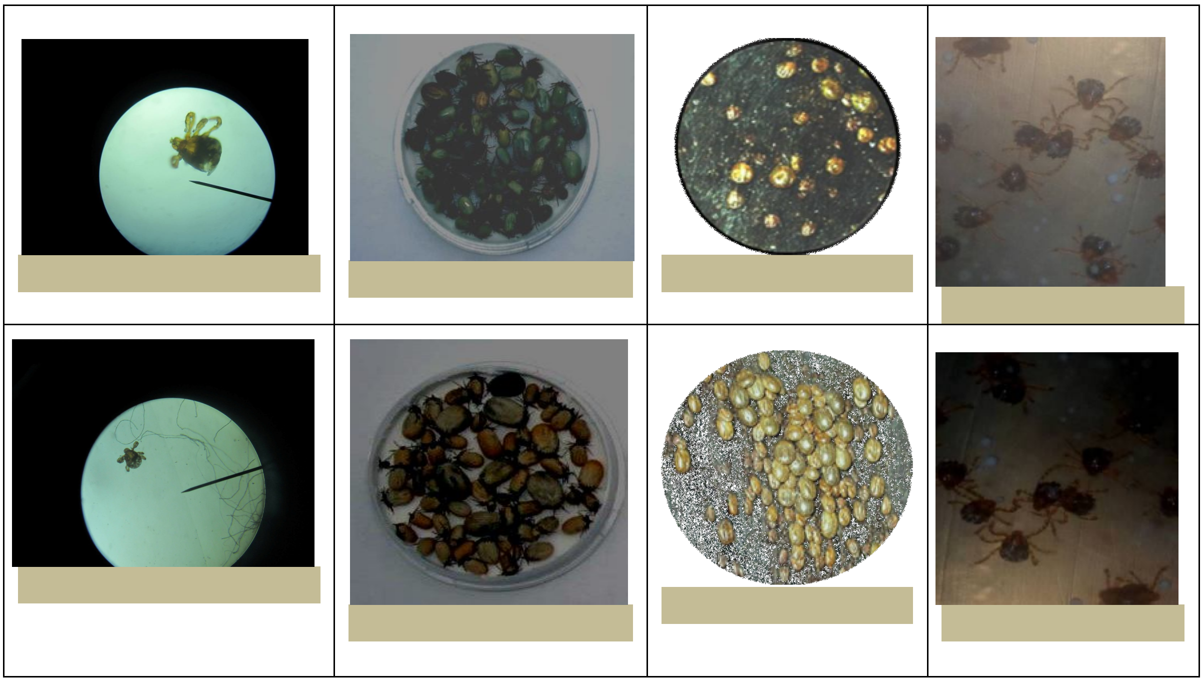


Figure 6. Photos showing the images of ticks (*Rhipicephalus microplus)* obtained from cows as well as the samples placed in a petri dish for preparation of treatment application (A, E, D, H original; B, C, F, G, www.google.com.ph/Rhipicephalus_microplus photos).

Statistical analysis. The statistical tool used to test the significance of the study is the ANOVA. This tool performs a simple analysis of variance on data for two or more samples. PAST software version 1.34 (Hammer et al 2001) and Microsoft Excel were also used in solving and graphing the results.

Results and Discussion

During the study, immobility of adult ticks in T3 was observed after 7 days which indicated the effect of the treatment. Sporulation in the ticks was observed in a minimal amount. Unexpected events like laying eggs of the female ticks were also observed.

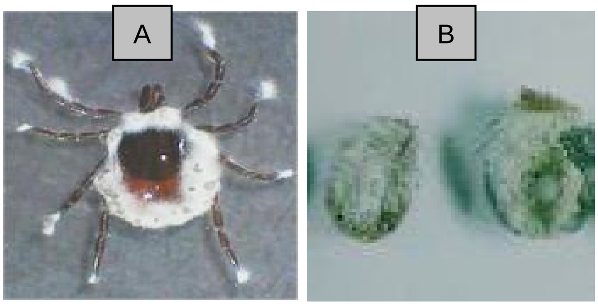


Figure 7. Images of ticks infected by the fungi *Metarhizium anisopliae* (Photo source: A. www.google.com.ph/Boophilus_microplus; B. www.google.com.ph/url.-parasitipedia.net).

Table 1 shows the quantitative results of the dead samples. The recorded data represent the exact number of ticks died during the conduct of the study. Graphs of mortality were also presented.

Table 1

List of total number of dead Rhipicephalus microplus in the 3 reproductive stages

Replications	*Treatment 1*			*Treatment 2*			*Treatment 3*		
	Larvae	*Nymphs*	*Adults*	*Larvae*	*Nymphs*	*Adults*	*Larvae*	*Nymphs*	*Adults*
Replication 1	10	10	3	15	10	5	29	26	24
Replication 2	15	9	6	14	7	4	30	29	26
Replication 3	11	8	4	17	10	4	29	27	25

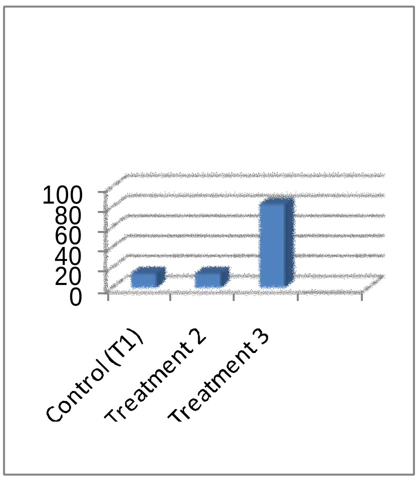


Figure 8. Percentage of dead adult ticks.

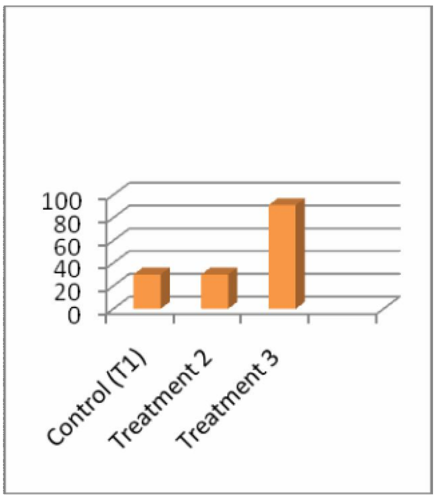


Figure 9. Percentage of dead nymphs.

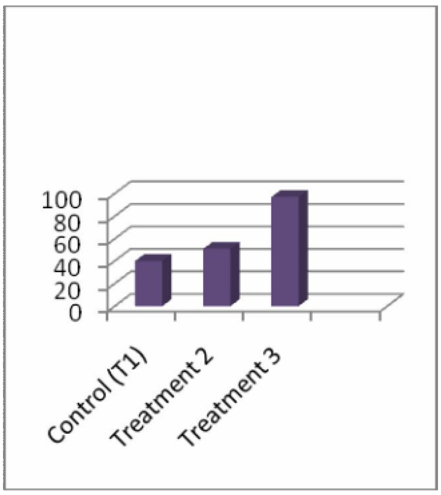


Figure 10. Percentage of dead larvae.

Figures 8, 9 and 10 represent graphic expression of the percentage mortality rate of *R. microplus* in adults, nymphs and larval stages. Figure 8 show that T3 had the highest percentage of mortality for the adults, respectively an 83%, being the highest percentage value. The T2 did not demonstrate however an impact in diminishing the number of ticks. Figure 9 represents the bar chart percentage that shows the mortality of the nymphs. The highest rate is given to T3 that produced a big difference compared to others. In Figure 10 can be seen that T3 demonstrated the highest mortality for larvae (97%) followed by T2 (51%).

Statistical analysis. The Table 2 shows the computed values that will test if there is a significant in the experiment conducted. This ANOVA statistics indicates assumptions that the subjects are randomly assigned to one of n groups. The distributions of the means by group are normal with equal variances. Sample sizes between groups do not have to be equal, but large differences in sample sizes by group may affect the outcome of the multiple comparisons tests. In this statistical test is rejected the null hypothesis if the calculated value is greater than or equal to the critical value ($F \geq F_{critical}$). This means that there is a significant result. On the other hand accept the null hypothesis if the calculated value is less than the critical value ($F < F_{critical}$) which signifies that there is a non-significant result. For the adult stage, the F–value is 349.4545. It has a higher value compared to the F-crit which means that there is a significant result for this stage. Larval and nymph stages present greater F-values than the F-critical that give us significant results (Table 2).

Table 2

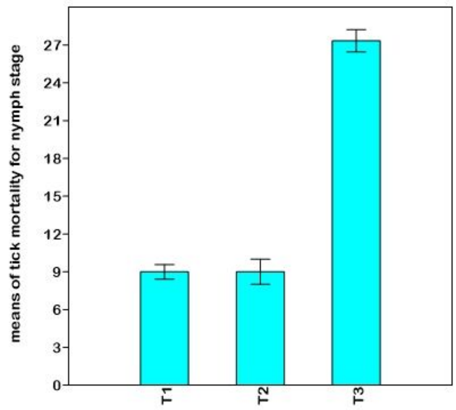
Level of significance of dead *Rhipicephalus microplus* in the three stages using the F-distribution in ANOVA

Stages	*P-value*	*F-crit*	*F*	*P*	*Remarks*
Adults	6.17E-07	5.143253	349.4545	P< 0.05	Highly significant
Nymphs	6.33E-06	5.143253	159.2105	P< 0.05	Highly significant
Larvae	4.94E-05	5.143253	78.75862	P< 0.05	Highly significant

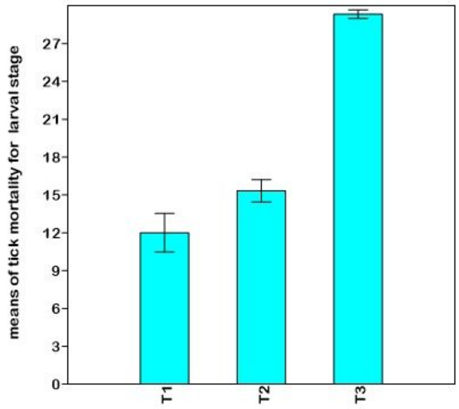
These obtained results using one-way ANOVA offers evidence supported by computations that there are differences in the means among the dead samples treated. The underlying probability distributions are not the same for all samples. The alpha level was used in this analysis. This is a significance level that is related to the probability of having a type I error (rejecting a true hypothesis). The null hypothesis for the adult and nymph stage is rejected same as for the larval stage. All the P-values for the three reproductive stages give us a highly significant remarks ($p<0.05$) tested in the value 0.05 in ANOVA table.

The Table 1 reveals the quantitative data of the dead ticks, larvae, nymphs and adult ones. Research showed that entomopathogenic fungi *M. anisopliae* has the capacity to kill parasites like ticks and they can act as an effective biocontrol agent against them (Ostfield et al 2006; Benjamin et al 2002; Hornbostel et al 2005). This is already proven by other researches conducted that showed its capability to being a BCA (Kaaya et al 1996; Norval et al 1992; Thungrabeab & Tongma 2007; Kaaya & Hassan 2000; Pirali-Kheirabadi et al 2007; Tavassoli et al 2012). In addition, the entomopathogenic fungi *M. anisopliae* is used as a substitute instead of acaricide. It was revealed that acaricide mixed with the fungi doesn't have a damaging effect on the fungi's effectivity (Kaaya et al 1996). Thus, it is a good option to use the fungi mixed with acaricide for those acaricide resistant ticks. However, the fungus could be quite specific in certain type of host it infects supported by previous studies (Broza et al 2001; Dromph & Vestergaard 2002). In this study, its capacity is highlighted because of the demonstrated effect in the number of died *R. microplus* in all stages. The *M. anisopliae* has an undeniably high level of mortality compared to the T2 *(T. viride*). The formulation of a certain fungus can influence the capability of the fungus to be an effective BCA or biopesticide. The speed of infection and invasion of the fungi may be increased by adding that amount that can facilitate the attachment of the spores to the cuticle (Frazzon et al 2000). In this study, a large amount of the conidial suspension were applied (10^9 conidia/mL) to the ticks for a faster effect as suggested in the study of Kaaya et al (1996) instead of having 10^8 conidia/mL. However T2 did not exhibit a reliable effect.

Figure 11 shows the means of the three treatments implicating the significance of the experiment in the mortality of each reproductive stage of ticks.



A



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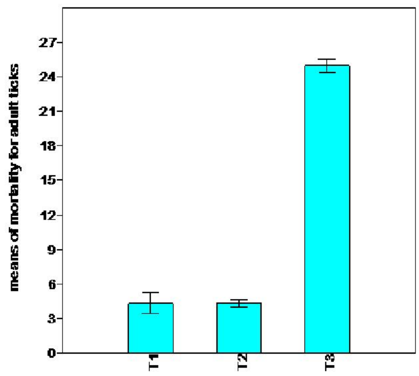

C

Figure 11. Means of *R. microplus* mortality for nymphs (A) ,larvae (B), and adults (C) for each treatment (T1, T2, T3), arranged respectively with confidence intervals, or quartile box with medians.

The method of application of fungal formulation on the host is considered to be relevant in every study. There are suspension or dipping method, immersion and spray methods used by the scientists. The performed study used direct method by means of suspension that is more convenient than immersion method used by Cradock (2005). In a research study, *Trichoderma* was used and proven to be a potential biological control agent against plant pathogenic fungi. It showed that the genus has a promising effect against such plant pathogens (Bailey & Gilligan 2004). It is widely distributed as biopesticide that is used mainly in agricultural purposes. The said the fungus that was used in T2 of the study did not exhibit its potentiality as BCA against ticks. Based on the study of Khandelwal et al (2012), the success and effectivity of biocontrol is dependent not only in the isolation, characterization and pathogenicity but also on the successful mass production of the fungal agents. For successful integrated pest management programme, the pathogenic fungi like *T. viride* should be amenable to easy and cheap mass production as sugested and to avoid unfavourable effect to the said BCA.

Conclusions. The obtained results demonstrated that, *M. anisoplaie* exhibits the higher acaricidal capacity against cow ticks *R. microplus* than *T. viride*. As such, it is considered that this entomopathogenic fungus can be used as biopesticide against cow ticks. It can be applied as an alternative for acaricide or chemical pesticide that exhibits negative impacts to humans, environment and even on the hosts of ticks.

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