Advances in Crop Science and Technology
ISSN: 2329-8863

Research Article Open Access

Effects of Some Microbial Agents and Their Mixtures against the Terrestrial Snails *Eobania vermiculata* and *Monacha cartusiana*

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Abstract

The molluscicidal activity of *Bacillus subtilis* spore suspension and the commercial formulation of *Trichoderma album* (Biozeid) were tested alone and in binary mixtures against adults of *Eobania vermiculata* and *Monacha cartusiana* snails under laboratory and field conditions. The interaction patterns of the two bio-agents in the binary mixtures were wisely analyzed by calculating the co-toxicity factor to find combinations with the synergistic effect. The results revealed that the molluscicidal development of *B. subtilis* and Biozeid increased in the binary mixtures compared to when applied alone against both snail species in the laboratory and field. Moreover, the mortality of both species was increased by increasing the concentration of Biozeid combined with *B. subtilis*. The co-toxicity factor values of *B. subtilis* at the fixed concentration of 4×10^5 spores/ml mixed with each of 0.5×10^6, 1×10^6, and 1.5×10^6 spores/ml of Biozeid against *E. vermiculata* snails were respectively -12.03, - 9.08 and – 5.40%, which indicates an antagonism. While in the case of *M. cartusiana* snails, the same mixtures in the same order recorded an observed additive effect with co-toxicity factors of 11.29, 18.54, and 19.12%, respectively. It is worth noting that *M. cartusiana* snails were more susceptible to the tested binary mixtures than *E. vermiculata* snails under laboratory and field conditions. Generally, the results of the present study suggest that the combinations of the biological agents represent new effective molluscicides that can contribute to enhancing the control of land snails. Further studies are required in this regard.

Keywords: *Bacillus subtilis*; Biozeid; binary mixtures; *Eobania vermiculata*; *Monacha cartusiana*

Introduction

Land snails are the most severe pests attacking crops worldwide (Barker, 2002). The terrestrial snails *Eobania vermiculata* and *Monacha cartusiana* are particularly the most common and harmful snails infesting various plants in Egypt (Mahrous et al., 2002 and Gabr et al., 2006). Chemical control of these snails using synthetic organic molluscicides is costly, environmentally unfriendly, toxic to non-target organisms, and challenging to sustain (Mansour, 2005; Santos et al., 2010 and Nyandwaro, 2018). Consequently, alternative means or new strategies for controlling land snails have become necessary. Biological control of land snails by using microbial agents such as bacteria and fungi represents an alternative control method to pesticides that has recently gained greater attention (Genena and Mostafa, 2008). Bacillus spp. is a gram-negative bacteria that can produce crystalline spores and toxic chemicals for pests (Genena, 2010) [1]. *Bacillus megaterium* bacterium and *Trichoderma album* fungus have a significant molluscicidal effect against *E. vermiculata* and *Succinea putris* snails (Mona et al., 2017).

Additionally, these bio-agents are considered typical soil beneficial bio-fertilizers that increase plant growth and represent potential biocontrol agents against nematode, which dramatically impacts vegetables and crops (Affokpon et al., 2011). The binary mixtures of biological agents are considered an effective strategy for enhancing practical pest control (Qiu-Yang, 2020). Moreover, it is represented excellent, effective alternative to chemical pesticides for controlling pests (Narayanasamy, 2006) [2, 3].

The present study was conducted to assess the potential of *Bacillus subtilis* spore suspension and the commercial product Biozeid (*Trichoderma album*), applied alone and in binary mixtures against the terrestrial snails, *E. vermiculata* and *M. cartusiana* under laboratory and field conditions. The mixing patterns of these bio-agents in the binary mixtures were also analyzed to find combinations with synergistic effects that are of interest for practical applications of bio-agents [4].

Materials and Methods

Tested snails

Adults of the brown garden snail, *Eobania vermiculata* were collected from infested navel orange trees at Banadf village, Meniet El-Kamh district, Sharkia Governorate, Egypt. In contrast, adults of the glassy clover snail, Monacha cartusiana, were collected from infested clover field at Kafr al-Ashraf village, Zagazig district, Sharkia Governorate, Egypt. Adult individuals of each snail species were kept in muslin bags and transferred to the laboratory. There were kept in a glass container containing moist clay soil and covered with muslin cloth secured with a rubber band to prevent snails from escaping. Snails were supplied daily with fresh cabbage leaves for 14 days to acclimate [5, 6].

Tested bio-agents

Preparation of *Bacillus subtilis* inoculum

The identified isolate *Bacillus subtilis* was grown on a Nutrient broth medium (5 g/l bactopeptone, 5g/l beef extract, five g/l NaCl) (Ph 7.2) and incubated at 37°C. The concentration of 4×10^5 spores/ml of *B. subtilis* was prepared for the laboratory and field treatments in this study by adding the appropriate amount of sterilized distilled water for the bacterial spores [7].

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Received: 02-Oct-2023, Manuscript No: acst-23-115505, **Editor Assigned:** 05-Oct-2023, pre QC No: acst-23-115505 (PQ), **Reviewed:** 19-Oct-2023, QC No: acst-23-115505, **Revised:** 23-Oct-2023, Manuscript No: acst-23-115505 (R), **Published:** 30-Oct-2023, DOI: 10.4172/2329-8863.1000623

Citation: Ghareeb H (2023) Effects of Some Microbial Agents and Their Mixtures against the Terrestrial Snails *Eobania vermiculata* and *Monacha cartusiana.* Adv Crop Sci Tech 11: 623.

Preparation of Biozeid (*Trichoderma album*) concentrations

Weights of 0.5, 1.0, and 1.5 grams of the commercial formulation of the fungus *Trichoderma album* were diluted in 10 ml sterilized distilled water for laboratory treatments. Other weights from the same biopesticide formulation; 50, 100, and 150 grams, were diluted in 1000 ml sterilized distilled water for the field application to get, in both cases of laboratory and field experiments, the desired concentrations of 0.5×10^6, 1×10^6 and 1.5×10^6 spores/ml, respectively (Mona et al., 2017) [8].

Preparation of bio-agents binary mixtures

The concentration of 4×10^5 spores/ml of *B. subtilis* was mixed with each prepared concentration of *T. album* formulation (0.5×10^6, 1×10^6, and 1.5×10^6 spores/ml) by dissolving the amount of *B. subtilis* bacterium required to obtain the appropriate concentration in the water amount which the fungal concentration prepared in it (Hend, 2013) [9].

Effect of the tested bio-agents used individually and as binary mixtures against both snail species under the laboratory conditions

The individual molluscicidal activity of *B. subtilis* spores suspension at the concentration 4×10^5 spores/ml and *T. album* formulation at three tested concentrations of 0.5×10^6, $1 \times 10^{6,}$ and 1.5×10^6 spores/ml were evaluated against *E. vermiculata* and *M. cartusiana* snails. The effect of binary mixing between *B. subtilis* spores suspension and each concentration of *T. album* was also investigated against the same snail's species under laboratory conditions [10].

This experiment was carried out using plastic boxes (3/4 kg capacity). Each box contained 1/2 kg of moistened sterilized clay soil. Ten adults of each snail species and ten discs of cabbage leaves were provided to each box on the soil surface. The spores suspension of *B. subtilis* and tested concentrations of *T. album* were sprayed separately on the soil and cabbage discs. The effect of the binary mixing between *B. subtilis* and each concentration of *T. album* against both snail species was also tested and sprayed in the same manner. Three replicates were prepared for the treatment with each bio-agent concentration and each binary mixture for the two snail species. The other three replicates were designed as the other boxes but without any treatment as a control. It is worth noting that each tested concentration of each bio-agent and mixture was sprayed at a rate of 10 ml for each replicate, and in the case of control, 10 ml of water only without any treatment was sprayed. All boxes were tightly covered with muslin cloth and secured with a rubber band to prevent snails from escaping. Mortality percentages of adults of each snail species at the treatment with bio-agents individually and as binary mixtures were recorded daily for a month and corrected by Abbott's formula (1925) [11, 12].

Synergism analysis of tested bio-agents against both snail species

The interaction between the two bio-agents in the mixture against *E. vermiculata* and *M. cartusiana* snails after 21 days of the experiment was expressed as the Co-Toxicity factor (C.F.) determined according to Sun and Johnson (1960) as follows:

C.F. =	Observed mortality % - Expected mortality %	× 100
	Expected mortality %	

Where the observed mortality was the visualized efficiency of the two bio-agents applied in combination, and the Expected mortality was the sum of the efficiency of each bio-agent used separately. It was considered potentiation if the Co-Toxicity factor was positive factor ≥ 20. A negative factor ≤ 20 means antagonism, while intermediate values between – 20 and + 20 indicate an additive effect [14].

Evaluation of the tested bio-agents used individually and as binary mixtures against both snail species under the field conditions

This field trial was conducted in March–2020 at two fields of navel orange trees heavily infested with each *E. vermiculata* and *M. cartusiana* snails. The first is at Banadf village, Meniet El-Kamh district, Sharkia Governorate, for *E. vermiculata* treatment, and the other field is located at El-Krakra village, Meniet El-Kamh district, Sharkia Governorate, for *M. cartusiana* treatment. Three infested trees were sprayed by each of *B. subtilis* spores suspension at 4×10^5 spores/ml, and *T. album* tested concentrations 0.5×10^6, 1×10^6, and 1.5×10^6 spores/ml individually for the two snail species. The binary mixing between *B. subtilis* and each concentration of *T. album* was also tested; three infested trees were sprayed with each mixture for both snail species. Each tested concentration of each bio-agent and mixture was poured at a rate of 1000 ml per tree, and in the case of control trees, 1000 ml of water without any treatment was sprayed per tree. Before the spraying of treatments, live snails were counted in four directions of 25×25 cm under each tested and control tree, at 1 m high of the tree trunk, and on five branches of the different directions of a tree. The number of living snails was counted in the same areas of control and tested trees after 1, 3, 7, 14, 21- and 30-days post-treatment. Reduction percentages were calculated by the formula of Henderson and Tilton (1955) [15, 16].

Statistical analysis

All data were statistically analyzed, and the treatment means were compared by the least significant difference (LSD) test at $P \leq 0.05$ levels according to the method of Costat's (2005) statistical program analysis, computer program software.

Results and Discussion

Molluscicidal effect of tested bio-agents when applied alone and as binary mixtures against *Eobania vermiculata* and *Monacha cartusiana* snails under the laboratory conditions

The individual and mixture effect of B. *subtilis* spores' suspension and *T. album* concentrations against *E. vermiculata* snails for a one-month exposure period is shown in (Table 1) (Figure 1) from which it can be seen that no mortality occurred after one day of exposure to each bio-agent individually. After three days of the experiment, *B. subtilis* at the tested concentration of 4×10^5 spores/ml recorded 53.33% mortality of snails, then slightly increased to 56.66% on the seventh day and then remained fixed until the end of the experiment. While in the case of *T. album*, its first molluscicidal effect was recorded on the seventh day of the investigation, with a 20% mortality of snails at the concentrations of 0.5×10^6, 1×10^6 spores/ml, and 23.33% at the concentration of 1.5×10^6 spores/ml. The mortality of snails increased with the increase in the tested concentrations and the experiment period, reached to its maximum value of 26.66, 30 and 40%, respectively, for 0.5×10^6, 1×10^6, and 1.5×10^6 spores/ml concentrations on day 21 of the experiment and after which no further increase in effect occurred until the end of the investigation [17, 18].

The molluscicidal effect of *B. subtilis* and *T. album* increased in all mixtures compared to when applied alone. The combination of *B. subtilis* with the highest concentration of *T. album* 1.5×10^6 spores/ml achieved the highest mortality of 70% after 21 days of the experiment.

Table 1: Molluscicidal effect of tested bio-agents used individually and as binary mixtures against *Eobania vermiculata* snails under the laboratory conditions.

Tested bio-agents	Conc. (spores/ml)	Mean of mortality % after indicated days						General mean
		1	3	7	14	21	30	
B. subtilis	4 × 10^5	0.00 ^{b}	53.33 ^{a}	56.66 ^{a}	56.66 ^{a}	56.66 ^{ab}	56.66 ^{ab}	46.66 ^{a}
T. album	0.5 × 10^6	0.00 ^{b}	0.00 ^{c}	20.00 ^{c}	26.66 ^{c}	26.66 ^{c}	26.66 ^{c}	16.66 ^{b}
	1.0 × 10^6	0.00 ^{b}	0.00 ^{c}	20.00 ^{c}	30.00 ^{bc}	30.00 ^{c}	30.00 ^{c}	18.33 ^{b}
	1.5 × 10^6	0.00 ^{b}	0.00 ^{c}	23.33 ^{c}	30.00 ^{bc}	40.00 ^{bc}	40.00 ^{bc}	22.22 ^{b}
B. subtilis **+** *T. album*	4 × 10^5 + 0.5 × 10^6	20.00 ^{a}	20.00 ^{b}	20.00 ^{c}	43.33 ^{ab}	60.00 ^{a}	60.00 ^{a}	37.22 ^{a}
B. subtilis **+** *T. album*	4 × 10^5 + 1.0 × 10^6	23.33 ^{a}	23.33 ^{b}	30.00 ^{bc}	46.66 ^{ab}	63.33 ^{a}	63.33 ^{a}	41.66 ^{a}
B. subtilis **+** *T. album*	4 × 10^5 + 1.5 × 10^6	23.33 ^{a}	23.33 ^{b}	43.33 ^{ab}	56.66 ^{a}	70.00 ^{a}	70.00 ^{a}	47.77 ^{a}
Control		0.00 ^{b}	0.00 ^{c}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{c}
P		.0000 ***	.0000 ***	.0000***	.0000 ***	.0000 ***	.0000 ***	.0000***
$LSD_{0.05}$		0.99	1.22	1.61	1.45	1.93	1.93	1.29

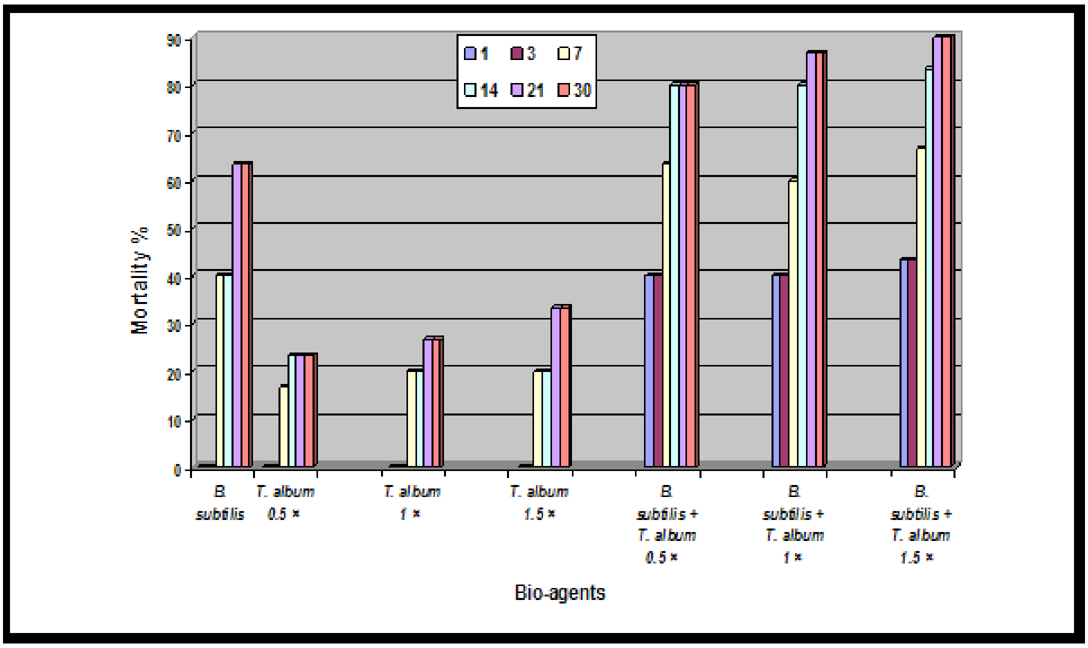

Figure 1: Molluscicidal effect of tested bio-agents used individually and as binary mixtures against *Eobania vermiculata* snails under laboratory conditions.

Table 2: Molluscicidal effect of tested bio-agents used individually and as binary mixtures against *Monacha cartusiana* snails under the laboratory conditions.

Tested bio-agents	Conc. (spores/ml)	Mean of mortality % after indicated days						General mean
		1	3	7	14	21	30	
B. subtilis	4 × 10^5	0.00 ^{b}	0.00 ^{b}	40.00 ^{b}	40.00 ^{b}	63.33 ^{b}	63.33 ^{b}	34.44 ^{b}
T. album	0.5 × 10^6	0.00 ^{b}	0.00 ^{b}	16.66 ^{c}	23.33 ^{c}	23.33 ^{c}	23.33 ^{c}	14.44 ^{c}
	1.0 × 10^6	0.00 ^{b}	0.00 ^{b}	20.00 ^{c}	20.00 ^{c}	26.66 ^{c}	26.66 ^{c}	15.55 ^{c}
	1.5 × 10^6	0.00 ^{b}	0.00 ^{b}	20.00 ^{c}	20.00 ^{c}	33.33 ^{c}	33.33 ^{c}	17.77 ^{c}
B. subtilis + *T. album*	4 × 10^5 + 0.5 × 10^6	40.00 ^{a}	40.00 ^{a}	63.33 ^{a}	80.00 ^{a}	80.00 ^{a}	80.00 ^{a}	63.88 ^{a}
B. subtilis + *T. album*	4 × 10^5 + 1.0 × 10^6	40.00 ^{a}	40.00 ^{a}	60.00 ^{a}	80.00 ^{a}	86.66 ^{a}	86.66 ^{a}	65.55 ^{a}
B. subtilis + *T. album*	4 × 10^5 + 1.5 × 10^6	43.33 ^{a}	43.33 ^{a}	66.66 ^{a}	83.33 ^{a}	90.00 ^{a}	90.00 ^{a}	68.88 ^{a}
Control		0.00 ^{b}	0.00 ^{b}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}
P		.0000 ***	.0000 ***	.0000***	.0000 ***	.0000 ***	.0000 ***	.0000 ***
$LSD_{0.05}$		1.11	1.11	1.49	1.32	1.32	1.32	1.02

At the same trend, adding B. subtilis to the other tested concentrations of *T. album* 0.5 × 10^6 and 1 × 10^6 spores/ml recorded 60 and 63.33% mortality in the same experiment period, respectively. No other change occurred in the molluscicidal activity of all mixtures against snails until the end of the experiment. No mortalities were recorded in control at all. Moreover, it is strongly observed that there is a highly significant difference in the means of snail mortality compared to untreated snails [19].

The molluscicidal activity of *B. subtilis* and *T. album* was also investigated against *M. cartusiana* snails when applied separately and in mixtures. As illustrated in (Table 2) (Figure 2), no mortalities of snails were recorded by each bio-agent until the third day of the experiment. *B. subtilis* at the tested concentration of 4 × 10^5 spores/ml caused 40% mortality of snails on the seventh day of the investigation. The mortality increased up to 63.33% on day 21 of the treatment, after which no other increase in effects occurred until the end of the

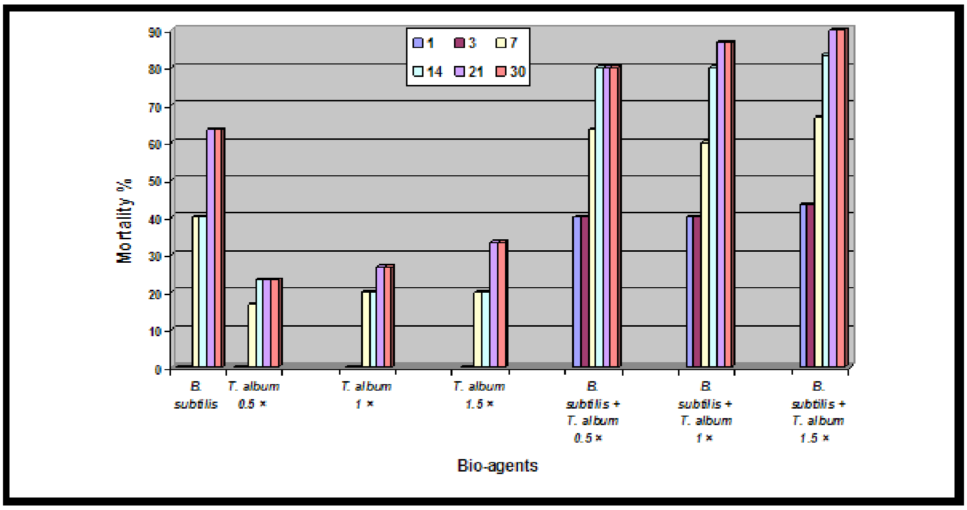

Figure 2: Molluscicidal effect of tested bio-agents used individually and as binary mixtures against *Monacha cartusiana* snails under laboratory conditions.

experiment. On the other hand, *T. album* showed only 16.66% mortality at the lowest concentration of 0.5×10^6 spores/ml on the seventh day of the experiment. While the other tested concentrations of 1×10^6 and 1.5×10^6 spores/ml recorded the same mortality of 20% after the same trial period [20, 21].

The lethal effect of *T. album* at all tested concentrations increased gradually, reached to 23.33 and 26.66% mortality, respectively, for 0.5×10^6 and 1×10^6 spores/ml after 21 days of the experiment. A rather observed increase in the molluscicidal effect of the highest concentration, 1.5×10^6 spores/ml, occurred by achieving 33.33% mortality after the same trial period. Concerning mixing *B. subtilis* with each tested concentration of *T. album*, adding *B. subtilis* to 0.5×10^6 or 1×10^6 spores/ml of *T. album* recorded the same mortality of 40% on the first day of the experiment. At the same time, it recorded 43.33% mortality in combination with the highest concentration of *T. album* 1.5×10^6 spores/ml during the same experiment period. Subsequently, the molluscicidal activity of all mixtures significantly increased by record 80, 86.66 and 90% at the combination of *B. subtilis* with each of 0.5×10^6, 1×10^6, and 1.5×10^6 spores/ml of *T. album* on day 21 of the trial, respectively. The lethal effect of all mixtures remained constant until the end of the experiment. On the other hand, no mortalities have been achieved in control. All the tested treatments showed a significant difference in the mortality of snails compared to the untreated individuals [22, 23].

The obtained results in (Tables 1-2). are strongly corroborated by *Heiba et al.* (2002) reporting that snails' mortality rates increased with increased exposure to molluscicides. In this trend, *Mona et al.* (2017 showed that the mortality of *E. vermiculata* and snails caused by Bioarc (*Bacillus megaterium*) and Biozeid (*Trichoderma album*) was increased with an increase in the exposure period to these biopesticides. The same authors added that the molluscicidal activity of Bioarc was more than Biozeid against both snails' species. Bioarc caused 20 and 26.66% mortality, respectively for *E. vermiculata* and *S. putris* snails after 21 days of treatment compared with only 6.66 and 10% mortality exhibited by Biozeid against the two snail's species after the same period of the experiment, respectively. Ghamry (1997) also stated that *Helicella* sp. is more susceptible to *Bacillus thuringiensis* bacterium, followed by *Monacha* sp. and *Eobania* sp. In a similar study, Genena and Mostafa (2008) demonstrated that *B. thuringiensis* at the tested concentration 7×10^6 cfu/ml was more effective against *Monacha cantiana* than *E. vermiculata* snail, it was recorded 86.6% and 53% mortality of the two snails after four weeks of the treatment, respectively. On the other hand, Arafa (2006) reported that *B. thuringiensis* failed to record any molluscicidal effect against *E. vermiculata* snail [24, 25].

According to the report of *Durel et al.* (2015), mixtures of compounds used for pests control can contribute to preserve the effectiveness of existing these compounds against pests as long as possible. Little information has been published about mixing bio-agents for use in the control of land snails. But in a related study, Hend (2013) showed that *E. vermiculata* adult snails were more susceptible to mixing *Paecilomyces variotii* with ammonium nitrate at the concentration 1.5%, *Paecilomyces lilacinus* with 1% of urea and *T. album* with ammonium nitrate at 2%. All these mixtures caused complete mortality of snails after 5, 6 and 8 weeks of the experiment, respectively. The same effect was achieved against *M. cartusiana* adults by combining *P. lilacinus* with each urea and ammonium nitrate at the concentration 1% after 5 and 6 weeks of the treatment, respectively [26].

On the other hand, mixing *P. variotii* with urea at 0.5% and *T. album* with each of ammonium nitrate and urea at 0.5% exhibited the lowest molluscicidal effect against the same snail species with record 60% mortality after 5 weeks of the trial. The same mortality percentage was recorded against *M. cartusiana* snails by the mixture of *P. variotii* with 0.5% ammonium nitrate after 8 weeks of the treatment. Moreover, the binary combination of urea with each of *P. lilacinus* and *Trichoderma harzianum* caused significant reduction in *Meloidogyne incognita* multiplication (Siddiqui and Shakeel, 2009). Selecting the relevant incision in the binary mixing is necessary for effective pest control (Levchenko and Silivanova, 2019) [27].

Synergism analysis of tested bio-agents against *Eobania vermiculata* and *Monacha cartusiana* snails

The joint action between *B. subtilis* and each tested concentration of *T. album* against *E. vermiculata* snails was determined. As cleared in (Table 3) after 21 days of application, the all mixtures showed an antagonistic effect against *E. vermiculata* snails with co-toxicity factors - 12.03, - 9.08 and – 5.40% for the combination of 4×10^5 spores/ml of *B. subtilis* with each of 0.5×10^6, 1×10^6 and 1.5×10^6 spores/ml of *T. album*, respectively. The joint action of B. subtilis with each tested concentration of *T. album* after 21 days of the application against *M. cartusiana* snails was also investigated. The obtained results in (Table 4)

Table 3: Synergism analysis of tested bio-agents against *Eobania vermiculata* snails.

Treatments(spores/ml)	Measurement	21 days Mortality	Co-toxicityfactor
B. subtilis (4×10^5) + *T. album* (0.5×10^6)	Observed Expected	60 68.21	- 12.03 a
B. subtilis (4×10^5) + *T. album* (1×10^6)	Observed Expected	63.33 69.66	- 9.08 a
B. subtilis (4×10^5) + *T. album* (1.5×10^6)	Observed Expected	70 74	- 5.40 a

Observed = visualized efficiency of the two bio-agents applied in combination
Expected= sum of the efficiency of each bio-agent applied separately
a = antagonism

Table 4: Synergism analysis of tested bio-agents against *Monacha cartusiana* snails.

Treatments (spores/ml)	Measurement	21 days Mortality	Co-toxicityfactor
B. subtilis (4×10^5) + *T. album* (0.5×10^6)	Observed Expected	80 71.88	11.29 d
B. subtilis (4×10^5) + *T. album* (1×10^6)	Observed Expected	86.66 73.1	18.54 d
B. subtilis (4×10^5) + *T. album* (1.5×10^6)	Observed Expected	90 75.55	19.12 d

Observed = visualized efficiency of the two bio-agents applied in combination
Expected = sum of the efficiency of each bio-agent applied separately
d = additive

indicated that all mixtures of *B. subtilis* with *T. album* concentrations showed an observed additive effect against snails with co-toxicity factors 11.29, 18.54 and 19.12% for the mixing of 4×10^5 spores/ml of *B. subtilis* with *T. album* at each of 0.5×10^6, 1×10^6 and 1.5×10^6 spores/ml, consecutively [28].

The illustrated results in (Tables 3- 4) follow those obtained by Hend (2013) and showed that at the mixing of each of *P. lilacinus*, *P. variotii* and *T. album* with ammonium nitrate at the concentration 0.5% against *E. vermiculata* snails, there is an antagonistic effect was achieved with co-toxicity factors –76.47, -51.72 and -69.56%, respectively. The same effect of the same mixtures was also recorded against *M. cartusiana* snails with co-toxicity factors -75.67, -81.24 and -69.38%, respectively. On the contrary, an additive effect was recorded at the combination of *P. variotii* with urea at the concentration 1% against *E. vermiculata* snails with co-toxicity factor 15.42%. While, the variety of the same fungus with the same fertilizer at the concentration 0.5% showed an observed potentiation activity with co-toxicity factor 400.45% against the same snail species. In the same aspect, Guruswamy et al. (2017) recorded a substantial synergistic effect from mixing nerium extract with each tobacco, neem and piper extracts against *Pomacea maculata* snail. Similarly, a synergistic action was also determined at the combination of potassium sulphate with each copper sulphate, niclosamide and mollutox against *Lymnaea natalensis* snails [29].

Moreover, the mixing of urea with each of copper sulphate, niclosamide and mollutox also caused significant reduction on the growth rate and survival of this snail (Ragab and Shoukry, 2006). On another related study, Hend (2018) reported that an additive effect was achieved at the binary mixing of *citrus limon* peel juice with each of *citrus limon* fruit and *Mentha spicata* juices against *M. cartusiana* snails with co-toxicity factors 7.92 and 9.43%, respectively. While, the binary mixing of *M. spicata* with *Coriandrum sativum* recorded an observed potentiating activity against the same snail species with a co-toxicity factor 53.26%. At the same trend, Rao and Singh (2001) confirmed that the synergistic action of *Acalypha indica* and *Cedrus deodara* oil combination was more toxic to *Lymnaea acuminata* snail than the single treatment with each one [30].

Molluscicidal activity of tested bio-agents when applied individually and as binary mixtures against *Eobania vermiculata* and *Monacha cartusiana* snails under field conditions

The molluscicidal effect of both bio-agents *B. subtilis* and *T. album* was evaluated individually and as a binary mixture against *E. vermiculata* and *M. cartusiana* snails in March 2020 under the field conditions. As shown in (Table 5) (Figure 3) the molluscicidal effect of both bio-agents when combined is greater than the effect of each one alone against the *E. vermiculata* snails. At the individual treatment with each bio-agent, *T. album* recorded the highest initial effect against snails at the highest concentration, 1.5×10^6 spores/ml, achieving 21.12% reduction of snails. It was followed by *B. subtilis* at its only tested concentration 4×10^5 spores/ml which caused 20.03% reduction of snails then 18.52 and 13.42% reduction of snails which recorded by 1×10^6 and 0.5×10^6 spores/ml of *T. album*, respectively. The decrease in snails increased for all treatments with the trial period, reaching 51.90% reduction of snails, representing the highest residual effect exhibited by 1.5×10^6 spores/ml of *T. album* followed by 50.45% reduction of snails achieved by *B. subtilis*. The lowest residual effect was recorded by 1×10^6 and 0.5×10^6 spores/ml of *T. album* by gave 37.04 and 26.19% reduction of snails, respectively. On the other hand, at the treatment of snails with the binary mixtures of both bio-agents, the mixing of *B. subtilis* with the highest concentration of *T. album* 1.5×10^6 spores/ml achieved the highest initial effect against snails by record 53.61% reduction followed by the binary mixing of *B. subtilis* with each of 1×10^6 and 0.5×10^6 spores/ml of *T. album* which gave 50.26 and 43.71% reduction of snails, respectively.

These same mixtures in the same order achieved a noticeable effect against snails with increasing the experiment period by record 77.95, 72.63 and 67.21% reduction of snails as a residual effect, respectively. The obtained data also showed a significant difference in the decrease in snails treated with all tested treatments compared with the untreated snails in control.

At studying the molluscicidal activity of the same two bio-agents

Table 5: Molluscicidal effect of tested bio-agents used alone and as binary mixtures against *E. vermiculata* snails under field conditions.

Tested bio-agents	Conc. (spores/ml)	Reduction % after indicated days								General mean
		1	3	7	Initial effect	14	21	30	Residual effect	
B. subtilis	4×10^5	0.00 c	26.87 d	33.23 c	20.03 d	45.95 d	52.71e	52.71 d	50.45 e	35.24 e
T. album	0.5×10^6	0.00 c	18.73 f	21.53d	13.42 f	24.32 f	24.32g	29.94 f	26.19 g	19.80 g
	1.0×10^6	0.00 c	22.23 e	33.34 c	18.52 e	37.04 e	37.04 f	37.04 e	37.04 f	27.78 f
	1.5×10^6	0.00 c	27.50 d	35.87b	21.12 d	46.56 d	54.57d	54.57 d	51.90 d	36.51 d
B. subtilis + *T. album*	$4 \times 10^5 + 0.5 \times 10^6$	34.29b	41.56 c	55.28 a	43.71 c	67.21 c	67.21 c	67.21 c	67.21 c	55.46 c
B. subtilis + *T. album*	$4 \times 10^5 + 1.0 \times 10^6$	45.84a	48.14 b	56.80 a	50.26 b	69.75 b	74.07b	74.07 b	72.63 b	61.44 b
B. subtilis + *T. album*	$4 \times 10^5 + 1.5 \times 10^6$	47.23a	56.81 a	56.81 a	53.61 a	76.02 a	76.02 a	81.82 a	77.95 a	65.78 a
P		.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***
$LSD_{0.05}$		1.55	2.47	1.69	1.24	2.03	1.54	1.91	1.4	0.87

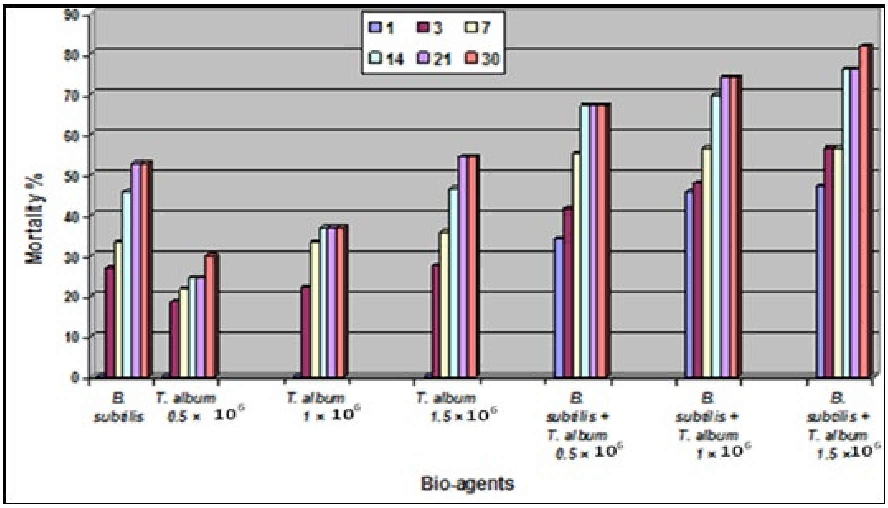

Figure 3: Molluscicidal effect of tested bio-agents used alone and as binary mixtures against *E. vermiculata* snails under field conditions.

Table 6: Molluscicidal effect of tested bio-agents used alone and as binary mixtures against M. cartusiana snails under field conditions.

Tested bio-agents	Conc. (spores/ml)	Reduction % after indicated days								General mean
		1	3	7	Initial effect	14	21	30	Residual effect	
B. subtilis	4×10^5	0.00 c	17.39 e	21.73 e	13.04 e	34.78 d	34.78ef	47.82 d	39.12 d	26.08 d
T. album	0.5×10^6	0.00 c	13.51 f	18.91 f	10.80 f	27.02 e	35.13 e	35.13 f	32.42 f	21.61 f
	1.0×10^6	0.00 c	16.66 e	20.83 e	12.49 e	33.33 d	37.50 d	37.50 e	36.11 e	24.30 e
	1.5×10^6	0.00 c	19.04 d	23.80 d	14.28 d	28.57 e	33.33 f	47.61 d	36.50 e	25.39 de
B. subtilis + *T. album*	$4 \times 10^5 + 0.5 \times 10^6$	47.22b	52.77 c	61.11 c	53.70 c	72.22 c	83.33 c	83.33 c	79.62 c	66.66 c
B. subtilis + *T. album*	$4 \times 10^5 + 1.0 \times 10^6$	52.38a	54.76 b	66.66 b	57.93 b	76.19 b	88.09 b	88.09 b	84.12 b	71.02 b
B. subtilis + *T. album*	$4 \times 10^5 + 1.5 \times 10^6$	52.63a	57.89 a	68.42 a	59.64 a	78.94 a	94.73 a	94.73 a	89.46 a	74.55 a
P		.0000***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***	.0000 ***
$LSD_{0.05}$		1.18	1.59	1.53	1.07	2.04	1.6	1.77	1.4	1.11

also separately and as binary mixtures against *M. cartusiana* snails under the field conditions, the results in (Table 6) (Figure 4) indicated that the effect of *B. subtilis* against snails at its only tested concentration 4×10^5 spores/ml was more than *T. album* effect at the all concentrations [31].

B. subtilis recorded the highest residual effect 39.12% reduction of snails followed by 36.50, 36.11 and 32.42% reduction which was recorded by 1.5×10^6, 1×10^6 and 0.5×10^6 spores/ml of *T. album*, respectively. The binary mixing of *B. subtilis* with the highest concentration of *T. album* 1.5×10^6 spores/ml caused the highest residual effect against snails by recording 89.46% reduction of snails. It was followed by the binary combination of *B. subtilis* with 1×10^6 and 0.5×10^6 spores/ml of *T. album* by record 84.12 and 79.62% reduction of snails, respectively. That is a significant difference in the decrease in treated snails with the different tested treatments compared to the untreated individuals. From the previous results, it could be concluded that the molluscicidal activity of *B. subtilis* and *T. album* as binary mixtures against both snail species is more than their effect separately and *M. cartusiana* snails were highly explicitly affected by the tested binary mixtures than the snails of *E. vermiculata* [32].

The obtained results in (Tables 5-6) are in harmony with those reported by Hend (2013) demonstrating that the microbial molluscicides were effective when sprayed on the healthy snails. *T. album* didn't record observable mortality of *E. vermiculata* and *M. cartusiana* snails

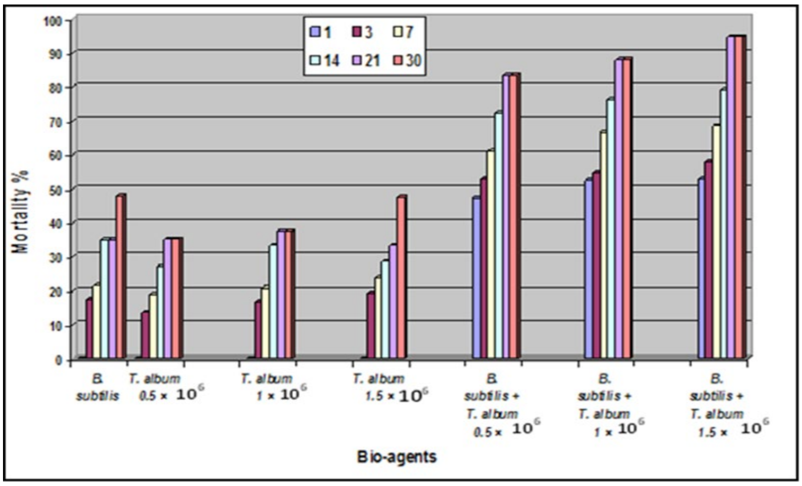

Figure 4: Molluscicidal effect of tested bio-agents used alone and as binary mixtures against *M. cartusiana* snails under field conditions.

at all tested concentrations during the field treatment's first period. But at the second week, the reduction of snails started to increase. It was recorded 16.66, 20 and 41.66% reduction of *E. vermiculata* snails at 1×10^3, 2×10^3 and 4×10^3 spores/ml concentrations after 21 days of the field treatment, consecutively. While, after the same period the same fungus at the same concentrations caused 10, 11.66 and 23.33% reduction of *M. cartusiana* snails, respectively. The inability of the fungus to achieve any lethal effect against pests at the beginning of the treatment is due to a few days between spraying the fungus and kill and continued of the pest in feeding (Abd El-Fattah, 2005). On the other hand, *Raul et al.* (2008) explained that *Trichoderma* species are successful fungi in field trials to control pests in general because it produced many secondary metabolites and various degradative enzymes such as chitinase. *Trichoderma viride* achieved a significant reduction of land snails under the field conditions (Babou and Jayakumar, 2009). At the same time, Ghamry (1997) reported that the suspension of *Bacillus thuringiensis* bacterium recorded higher molluscicidal impact against *Helicella vestalis* snail than *E. vermiculata* in the field. Moreover, *Zedan et al.* (1999) added that the formulation of the same bacterium was the more effective against *Monacha obstructa* snail. At a pea field highly infested with land snails, this bacterium formulation reduced 23.54% of *M. cartusiana* snails after one week of the treatment. The reduction of snails increased by increasing the application period to 28.03% after 21 days of the trial (*Mortada et al.*, 2012). There is no published paper or studies conducted to support the effect of combining two microorganisms against terrestrial snails. But in this trend, Godan (1983) published that the effectiveness of fungi against terrestrial pulmonates can be increase by using other active ingredients in combination. The mixing of *T. album* with ammonium nitrate at the concentration of 2% achieved the highest molluscicidal activity against *E. vermiculata* snails by recording 53.33% reduction after 21 days of the field experiment. This mixture was followed by the combination of *Paecilomyces variotii* with ammonium nitrate at the concentration of 1.5% and *Paecilomyces lilacinus* with urea at 1% by record 37.50 and 20.83% reduction of snails after the same period of application, respectively. Whereas, at testing an impact of the same mixtures against *M. cartusiana* snails also in the field, the highest molluscicidal effect was recorded by *P. variotii* mixed with ammonium nitrate at 1.5% which caused 82.50% reduction of snails after 21 days of the application. After the same period, the combination of *T. album* with ammonium nitrate at 2% and *P. lilacinus* with urea at 1% achieve a 73.33% and 71.66% reduction in snails, respectively (Hend, 2013) [33].

Conclusion

The binary mixing of the spore suspension of *B. subtilis* with the biopesticide formulation Biozeid (*T. album*) has a strong effect against *E. vermiculata* and *M. cartusiana* snails more than the use of each bio-agent individually. *M. cartusiana* snails were more affected by the tested mixtures than the snails of *E. vermiculata* under laboratory and field conditions. Mixing these bio-agents is considered a natural, effective, inexpensive molluscicide with lower environmental toxicity than the other commonly used molluscicides. Thus, its production offers a possible alternative strategy for controlling the field populations of *E. vermiculata* and *M. cartusiana* intending to design a new biopesticide formulation.

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