Efficacy of Six Aqueous Plant Extracts and Three Commercial Entomopathogenic Fungi Against the Corn Ground Beetle Larvae, *Zabrus tenebrioides* Under Laboratory Conditions

Sahand K. Khidr* **and Srwa M. Khalil**
Department of Plant Protection, College of Agricultural Engineering Sciences, Salahaddin University, Erbil, Kurdistan region, Iraq. *Email of the corresponding author: sahand.khidr@su.edu.krd

Abstract

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The efficacy of aqueous plant extracts and entomopathogenic fungi as an alternative to conventional pesticides to manage the corn ground beetle, *Zabrus tenebrioides* on wheat were investigated. The results obtained indicated that maximum mortality (100%) was achieved by both Eucalyptus, *Eucalyptus globulus* and Thuja, *Platycladus orientalis* aqueous extracts 5 days after treatment, followed by rosemary, *Salvia rosmarinus* and Neem, *Melia azedarach* seven and eight days, after treatment, respectively. In addition, the efficacy of aqueous plant extract types tested on the larval stage was influenced by the concentration applied, and higher doses were more toxic and caused more mortality in the insect population. On the other hand, the larvae were more resistant to the tested fungal bioinsecticides than the botanical insecticides utilized in the test. *Beauveria bassiana* and *Trichoderma harzianum* achieved maximum and minimum mortality rate of 100 and 71.7%, respectively, eight days after treatment and the mortality rate didn't differ significantly among the tested concentrations. The LC_{50} value of the aforementioned entomopathogenic fungi was 119960.37 and 566168.82 conidia/ml, respectively, during the last day of exposure. The positive control (Alphacypermethrin 10%) produced the highest mortality of 100% on the third day of exposure, whereas the negative control (distilled water) gave minimum mortality in all experiments.

Keywords: LC_{50}, *Beauveria bassiana*, *Metarhizium anisopliae*, *Eucalyptus globulus*, *Platyclaudus orientalis*, control.

Introduction

Wheat, (*Triticum aestivum* L.) is an economic staple crop that has been cultivated for over 10,000 years and the demand for increased wheat production around the world is as pressing as ever due to the rapid growth of the human population, which has resulted in an increase in average annual global wheat production from around 680 million tonnes in 2012 to 750 million tonnes in 2018 (Cole *et al.*, 2018; Grote *et al.*, 2021).

Agricultural insect pests cause significant losses to cereal crops both quantitatively and qualitatively during pre- and post-harvest. The cereal ground beetle, *Zabrus tenebrioides* (Goeze, 1777) (Coleoptera: Carabidae) represents one of the most important pests of wheat which is capable of causing yield losses of up to 100% when no management practices are applied. Many farmers either repeat sowing the same crop or plant another crop which increases the amount of imported wheat into the country (Ahmed *et al.*, 2017; Popov *et al.*, 1996). Larvae are the most damaging stage of the pest. They live in the soil and penetrate up to 40 cm deep as they feed on the root, in addition to dragging the plants in the soil and chewing them, especially when the plants are in the early vegetative stage (Georgescu *et al.*, 2017; Popov, 2002; Walczak, 2007).

Despite its broad distribution and significant economic impact on the cereal crop globally, there are limited methods and strategies that have been implemented to control the corn ground beetle, which can explain the insect population increase and its continuous spread in recent years. The traditional insecticides were the most commonly used either as a seed treatment or as systemic and foliar spray (Georgescu *et al.*, 2017; Nicosia *et al.*, 1996). On the other hand, entomopathogenic fungi have been utilized on a very small scale and mostly in a controlled environment to test their effect on the mortality of corn ground beetle (Haji & Ghazavi, 2011; Keller & Hülsewig, 2018) .The use of botanical insecticides, which are natural insecticidal compounds extracted from plants, was not documented against this pest.

In Iraq, surface and soil chemical pesticide applications have been used as control methods and scorched wheat was observed during the milk production stage. Therefore, due to the scarcity of research on the use of both biopesticides (aqueous plant extracts and entomopathogenic fungi) and chemical pesticides in the management of corn ground beetle, this study was carried out to explore the toxic impact of aqueous plant extracts from rosemary *Salvia rosmarinus* Spenn , thuja *Platycladus orientalis* (L.), eucalyptus *Eucalyptus globulus* Dehnh, bitter orange *Citrus aurantium* L., pomegranate *Punica granatum* L. and Iraqi neem *Melia azedarach* L. and the commercial formulations of microorganism *Beauveria bassiana* (Balsamo), *Metarhizium anisopliae* (Metsch), and *Trichoderma harzianum* Rifai, as potential biocontrol agents against corn ground beetle on wheat.

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Materials and methods

Collection and rearing of *Zabrus tenebrioides*

The laboratory experiment was carried out at the Biotechnology and High Education Laboratories of Agricultural Engineering Science College at Salahaddin University, Iraq. Larvae of *Zabrus tenebrioides* were collected regularly from Erbil Province, Kurdistan region, Iraq, either manually or via using pitfall traps (diameter 21 cm, height 17 cm) which is considered essential in ecological investigations of ground beetles. Larval instars of corn ground beetle were placed in cages (length 35 cm, width 20 cm, height 20cm) that contain soil and wheat plant parts (leaves, stems and roots) for feeding and covered with muslin cloth. Fresh plant parts were continuously provided for rearing and the soil moistened by spraying water (Gryuntal, 2008; Makarov & Matalin, 2009).

Preparation of aqueous extracts

Fresh leaves of rosemary, *Salvia rosmarinus* Spenn. (Sr), thuja, *Platycladus orientalis* (L.) (Po), eucalyptus, *Eucalyptus globulus* Dehnh (Eg), bitter orange, *Citrus aurantium* L., (Ca), pomegranate, *Punica granatum* L. (Pg) and the seeds of Neem, *Melia azedarach* L. (Ma) were collected from Erbil Minara Park, Shanadar Park and the nursery of the Directory of Parks Engineering in Hawler region.

Leaves and seeds of these plants were washed thoroughly with water, air dried under shade and then ground into fine powder with an electric grinder (DAMAI -China). A 10% stock of the aqueous plant extracts (APE) (100000 ppm) was prepared by mixing 100 g dry powder from each plant part (leaves or seeds) with 1000 ml distilled water and placed on the Magnetic Stirrer (China, INTLLAB) with Stirring Bar 3000 rpm prior to usage. The solution was kept overnight at room temperature, then filtered with muslin cloth and stored in the refrigerator (Abdulhay, 2012; Irié-N'guessan *et al.*, 2011). A five percent concentration of the extracts that corresponds to 50000 ppm was also prepared.

Extracts bioactivity assay

A sample size of 420 individuals of *Zabbrus tenebrioides* larvae was used for the experiment. The toxicity of the six aqueous extracts was tested on the 3^{rd} and 4^{th} larval instars. A Petri dish that contains small pieces of wheat plant parts (i.e., leaves and roots) lined with moistened filter paper to maintain the leaves and roots as fresh as possible. Three replicates each consisting of 10 larvae were sprayed with extract concentrations of 100000 and 50000 ppm using a hand amber glass Boston sprayer, and then covered with a muslin cloth. All treatments were kept at room temperature (25±2°C). A synthetic chemical insecticide, Alphacypermethrin 10% at 100 ppm and sterilized distilled water were used as positive (PC) and negative control (NC) and kept under the same conditions. For each concentration, larval mortality was recorded daily for eight consecutive days after exposure and larva was considered dead when the color changes and becomes motionless.

Preparation of microbial formulation

The commercial Entomopathogenic fungi formulation (EPF) (Rajan Labs, India) containing 1.87×10^8 conidia per gram powder was utilized in the assay. The EPF biopesticides were grown on potato dextrose agar (PDA) incubated at 25±2°C for 7 days to confirm the pathogen viability (Youssef, 2015). The entomopathogenic fungi used were *Beauveria bassiana* (B.b), *Metarhizium anisopliae* (M.a) and *Trichoderma harzianum* (T.h) (Rifai). Stock solution (0.187×10^7 spore/ml) for each EPF was prepared by mixing 1 g of the commercial powder with 100 ml distilled water containing 0.1% of Tween-80. The stock solution was then diluted to obtain concentrations of 0.935×107 spore/ml. The chemical insecticide (Alphacypermethrin 10%) (0.3ml/L) and sterilized distilled water were used as positive and negative controls, respectively.

Larvicidal effect under laboratory conditions

A total sample size of 300 mature larvae (3^{rd} and 4^{th} larval instars) of corn ground beetle with the tested microorganism was carried out by the spray-bioassay method. Three replicates each with ten insects were gently placed on filter papers inside a Petri dish that contained a fresh leaf and root for feeding purposes. Three filter paper discs were utilized to absorb excess suspension and preserve the freshness of wheat leaves and roots after exposure to the varied concentrations treatments (Migiro, 2010). Furthermore, in order to maintain sufficient moisture for the microorganism, the treatments were sprayed with water by hand sprayer whenever required. Larval mortality was recorded for eight days following spray with microbial suspension. To allow fungal overgrowth on the corpses of the deceased larvae, they were kept in the Petri plates for an extra three days to further confirm the cause of death.

Statistical analysis

Analysis of variance was used to check the significance of differences in mortality of the corn ground beetle when treated with various aqueous plant extracts and entomopathogenic microorganisms. The data were analysed using multi-factor analysis of variance (ANOVA) in Statgraphics Centurion XV followed by Fischer's least significant difference (LSD) test to determine statistical differences between means at $P \leq 0.05$. Abbott's algorithm (Abbott, 1925) was used to adjust mortality data to determine lethal concentration fifty (LC_{50}) values and associated statistics. Mortality data were subjected to the maximum likelihood program of Probit analysis using SPSS software version 20.

Results

Mortality rate of Zabrus tenebrioides larvae in reponse to treatment with plant extracts

The mortality rate of corn ground beetle larvae was significantly affected by the type of aqueous plant extracts utilized in the laboratory test ($F_{(7,383)} = 75.76$, $P < 0.001$). Eucalyptus extract was the most toxic extract among the six examined extracts, which gave 77.65% mortality rate, followed by thuja (74.17%), but there was no significant difference between them. However, the botanical extracts were significantly different from both chemical pesticide Alphacypermethrin (positive control) and standardized distilled water (negative control) which gave the highest and lowest mortality rate (87.46 and 9.63%), respectively (Figure 1-A). The mortality rate of corn ground beetle varied significantly depending on not only the plant extract concentration ($F_{(2,383)}$ = 54.56, P <0.001, Figure 1-B) but for how long the larvae were exposed to aqueous plant extracts ($F_{(7,383)}$ = 91.89, P <0.001, Figure 1-C). As a result, the maximum mortality (86.69%) occurred 8 days after treatment, without significance from 7 days after treatment, whereas as the least mortality rate (11%) occurred on day one following treatments (Figure 1-C). Likewise, the interaction between the plant extracts and the duration of larvae exposure had a significant impact on larvae survival ($F_{(49,383)}$ = 3.19, P <0.001). The top two toxic extracts (eucalyptus and thuja) attained absolute larval death (100%) 5 days after treatment, whereas the chemical insecticide achieved 100% insect mortality within the shortest period of exposure (three days, Figure 2-B). However, the amount of biopesticide applied on the larvae did not interact with the exposure duration ($F_{(7,383)}$ =2.34, P =0.91). Furthermore, the efficacy of aqueous plant extracts examined on the larval stage was influenced by the concentration applied (Figure 2-A).

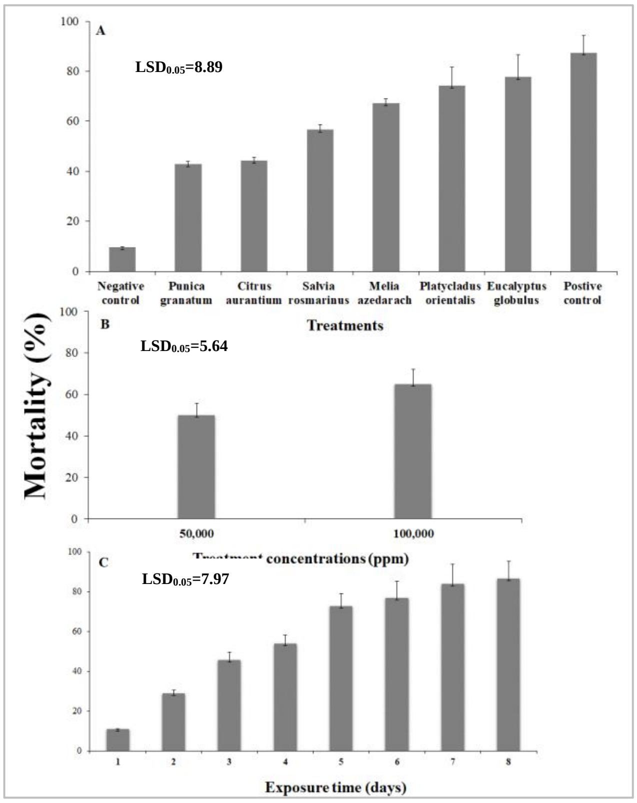

Figure 1. Mortality rate of *Zabrus Tenebrioides* larvae in response to treatment types (A) extract concentrations (B), and exposure time (C).

Determination of LC_{50} values

The toxicity of various aqueous plant extracts was evaluated by the determination of the LC_{50} values on the mature larval stage of *Zabrus tenebroides* (Table 1). The LC_{50} values revealed that, among the six examined botanical insecticides, eucalyptus extract was the most toxic and the bitter citrus was the least toxic. Furthermore, corn ground beetle showed variation and consistent decrease in LC_{50} values whenever the larvae were subjected to the biopesticides for longer periods of time, regardless of the plant extract type (Table 1).

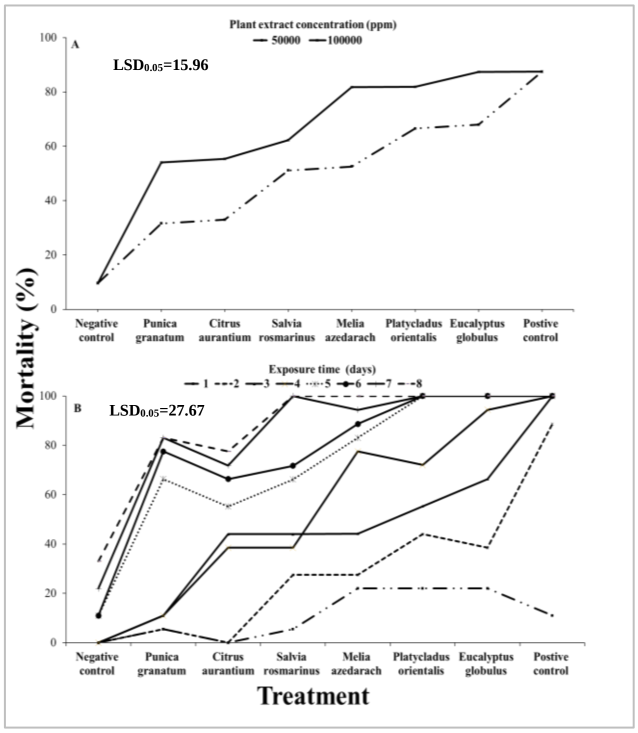

Figure 2. Mortality rate of corn ground beetle in relation to interaction between treatment types (A) and plant extract concentrations treatment types and exposure times (B).

Effect of entomopathogenic fungi on the mortality rate of *Zabrus tenebrioides* larvae

The current study revealed that using different entomopathogenic fungi in a controlled environment had a substantial impact on the mortality rate of *Zabrus tenebroides* larvae ($F_{(4,239)}$ =227.54., P <0.001, Figure 3-A). Among the entomopathogens, *Beauveria bassiana* achieved the highest mortality rate, followed by *Metarhizium anisopliae* and *Trichoderma harzianum*, respectively. On the other hand, both the positive and negative controls (Alphacypermethrin and sterile distilled water) attained the highest and lowest mortality rate (80.50 and 6.25%, respectively (Figure 3-A).

Likewise, the exposure duration had a significant effect on the larvae mortality rate ($F_{(4,239)}$ =227.54, P <0.001), with maximum mortality rate (57.08%) 8 days after treatment and 0% mortality one day after treatment (Figure 3-B). In addition, the interaction between the exposure time and treatment types was significant ($F_{(28,239)}$ =13.98., P <0.001) (Figure 3-C). The corn ground beetle was neither influenced by the bioagent concentration alone ($F_{(1,239)}$ =2.02., P <0.001) nor by the interaction between the concentration and bioagent type ($F_{(4,239)}$ =0.49., P = 0.81) or concentration and exposure time ($F_{(7,239)}$ =0.98., P =0.099).

Table 1. The LC_{50} values (ppm) of aqueous plant extracts on corn ground beetle larvae following different exposure periods.

Plant Extract	Period (days)	LC_{50} (ppm)	Average
Eucalyptus globulus	1	103332.96	41972.42
	2	85686.59	
	3	59938.90	
	4	31890.76	
	5	18094.48	
	6	18907.79	
	7	12604.75	
	8	5323.14	
Platyclaudus orientalis	1	106241.89	45865.93
	2	80152.84	
	3	70687.49	
	4	52886.41	
	5	18616.77	
	6	18607.54	
	7	12773.68	
	8	6960.76	
Melia azedarach	1	106292.42	53592.93
	2	97669.47	
	3	8132353	
	4	49994.61	
	5	36232.91	
	6	30833.71	
	7	19445.91	
	8	6951.15	
Salvia rosmarinus	1	155970.17	70848.74
	2	106651.15	
	3	84463.07	
	4	91001.58	
	5	54200.87	
	6	48938.19	
	7	17241.76	
	8	8323.024	
Punica granatum	1	152169.96	88922.43
	2	152232.57	
	3	130495.82	
	4	130529.04	
	5	53085.91	
	6	40810.93	
	7	28983.58	
	8	23071.52	
Citrus aurantium	1	213151.68	98169.46
	2	203556.20	
	3	86877.45	
	4	94371.72	
	5	66007.44	
	6	53237.62	
	7	41164.13	
	8	26989.43	

Determination of LC_{50} values

The toxicity of different entomopathogenic fungi indicated significant variation in the LC_{50} values as the larvae were more tolerant to *T. harzianum* than the other two bioagents and consequently displayed the lowest insecticidal activity and higher doses must be utilized in order to induce 50% mortality (Table 2). In addition, the exposure duration played an efficient role in fluctuating the LC_{50} values, for instance, larvae treated with *B. bassiana* required 3225958.12 conidia/ml to achieve 50 percent mortality during the first day of exposure while this value has decreased to only 119960.37 conidia/ml on the last day of subjecting larvae to the agent (Table 2).

Discussion

The corn ground beetle varied in its vulnerability towards the bioagents tested utilized in the experiment. The entomopathogenic fungi might have infected the corn ground beetle larvae either via the integument by deploying mechanical and enzymatic pressure or it might have been ingested by the insect. Hence, the presence of various hydrolytic enzymes (chitinases, proteases, and lipases) might encourage the fungus germination and eventual hyphae insertion into the cuticle, in addition, the toxins produced by the fungi can contribute further towards the rapid death of the insect species (Ortiz-Urquiza & Keyhani, 2013). Contrary to expectation, the entomopathogenic fungi did not produce significant impact on *Z. tenebrioides* compared to the efficacy of the standard chemical pesticide, however, their effectiveness increased with increasing exposure time.

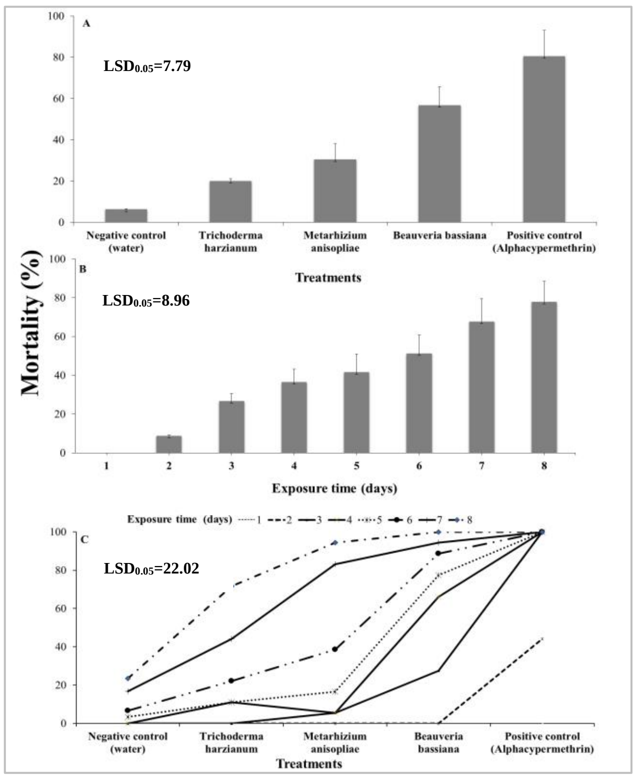

Figure 3. Mortality rate of *Z. tenebrioides* larvae, as influenced by (A) entomopathogen species, (B) exposure time, and (C) interaction between entomopathogen species and exposure time.

Table 2. The LC_{50} values of the entomopathogens (conidia/ml) against the larva of *Z. tenebrioides following* different exposure periods.

Entomopathogenic Fungi	Exposure time (days)	LC_{50} conidia/ml	Average
Beauveria bassiana	1	3225958.12	1443941.74
	2	3214619.45	
	3	1889615.56	
	4	114288.06	
	5	900941.19	
	6	666729.28	
	7	390921.86	
	8	119960.37	
Metarhizium anisopliae	1	4425599.29	2647167.99
	2	4040029.60	
	3	3656023.02	
	4	3540843.11	
	5	1909477.14	
	6	2732169.77	
	7	546871.27	
	8	326330.76	
Trichoderma harzianum	1	6064716.07	4146814.61
	2	6389258.09	
	3	6667756.49	
	4	4443985.77	
	5	4186016.47	
	6	3172656.17	
	7	1683959.01	
	8	566168.82	

Thus, there was a significant increase in the larvae mortality rate by the microbial agents, especially following the third day of treatment. The entomopathogenic fungi ability to infect the larval stage and the delay in mortality after treatment suggest that these entomopathogens might have a chance for dissemination throughout the pest population.

A similar finding was reported earlier (Lawrence *et al.*, 1995) when both *B. bassiana* and *M. anisopliae* exhibited a sharp increase in mortality rate of Japanese beetle after the third day of treatment, followed by a decrease in the LC_{50} value. In this study, *B. bassiana* was the most toxic species against corn ground beetle followed by *M. anisopliae* and *T. harzianum*, respectively. Results obtained in this study were in agreement with prior investigation performed using various doses of both *B. bassiana* and *M. anisopliae* to manage *Z. tenebrioides* in a laboratory experiment with moderate effects on the ground beetle (Haj et al., 2010). Many studies have pointed out that *B. bassiana* was more effective than *M. anisopliae* in the management of the different types of beetles (Erler & Ates, 2015; Lawrence *et al.*, 1995). Moreover, Sharma *et al.* (1994), in an investigation on the conidia of both species revealed that *B. bassiana* released 10–200 times more conidia than *M. anisopliae* from infected adults of pecan weevils (Gottwald & Tedders, 1982). This is probably the reason why almost 40% of the total insecticides in the market were *Beauvaria*-based biopesticides (de Faria & Wraight, 2007). On the other hand, the aqueous botanical extracts may have affected the larvae directly through the cuticle and respiratory activities, or indirectly by influencing the insects' ability to either eat or avoid feeding on the treated wheat leaves with the plant extracts. Furthermore, the aqueous extracts exhibited a significant influence on the corn ground beetle.

Thus, the presence of several phytochemicals (i.e., tannins, glycosides, steroids, flavonoids and saponins) in low or high concentrations either in the aqueous or alcoholic leaf extract of eucalyptus might be responsible for the plant's antimicrobial and insecticidal properties (Chuku *et al.*, 2016; Soni *et al.*, 2014). Likewise, the seeds of *Melia azedarach* contain triterpenoids and limonoids in large quantities that exhibit antifeedant and cytotoxic activities on various insects (Carpinella *et al.*, 2002; Hammad *et al.*, 2000a, Liu *et al.*, 2011). Furthermore, the mortality rate increased significantly with increasing the exposure time and concentration. Similar outcome was observed when the chinaberry seed extract exhibited a lethal impact on Khabra beetle (*Trogoderma granarium* Everts) (Ahmad *et al.*, 2022). Nonetheless, a decline in *M. azedarach* effectiveness was recorded whenever the leaf miner pest *(Liriomyza huidobrensis)* was exposed for a longer period of time (e.g., one week) in the field. The possible reason behind decrease in mortality rate might be due to open field environment and the phytochemical degraded more rapidly in comparison with controlled microclimate conditions (Banchio *et al.*, 2003; Hammad *et al.*, 2000b).

The essential oil in thuja leaf extract is mainly rich in monoterpene hydrocarbons, especially the α-pinene compound which possesses a toxic activity on the target insect species (Jain & Sharma, 2017; Ojimelukwe & Adler, 1999). Thus, foliar application of *T. orientalis* extract on maize crop proved particularly poisonous to the lepidopterous stem borers, *Chilo partellus*, (Bhatnagar & Sharma, 1999). Similar results were observed by others (Hashemi & Safavi, 2012) during a laboratory experiment to test thuja leaf extract on coleopteran pests inside stored products, where not only the mortality increased but also the LC_{50} value decreased when the insects were exposed to the extract for a longer period.

It can be concluded from this study that extracts from *E. globulus, P. orientalis and B. bassiana* were very successful in the management of corn ground beetle under controlled environment. Hence, constructing a control program that either relies only on biological control or combines this strategy with other control measures in order to lessen the use of conventional pesticides is highly recommended. Additional experiments on large-scale (wheat field trial) are required to further investigate the effectiveness of these biopesticides under farmers field conditions.

الملخص

خضر، ساهند ك. وسروة م. خليل. 2024. فعالية ستة مستخلصات نباتية مائية وثلاثة فطور تجارية ممرضة للحشرات ضد يرقات ماضغة بادرات الحبوب/خنفساء الحبوب الأرضية (*Zabrus tenebrioides*) تحت ظروف المختبر. مجلة وقاية النبات العربية، 42(1): 120-127.
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هدفت هذه الدراسة لإجراء تجربة مختبرية للتحقق من فعالية العديد من المبيدات الحيوية (مستخلصات نباتية مائية وكائنات دقيقة ممرضة للحشرات) كبديل للمبيدات التقليدية في إدارة ماضغة بادرات الحبوب/خنفساء الحبوب الأرضية (*Zabrus tenebrioides*). دلّت النتائج على تباين نسبة موت اليرقات بعد الرش بالمبيدات الحشرية النباتية الستة المختبرة مع مدد تعرض مختلفة. تحقّق الحدّ الأقصى للموت (100%) عند استخدام كلٍّ من نبات الكافور (*Eucalyptus globulus*) والعفص الشرقي (*Platycladus orientalis*) بعد اليوم الخامس من رشّ اليرقات بالمستخلصات المائية، يليها إكليل الجبل (*Salvia rosmarinus*) ونبات النيم (*Melia azedarach*) بعد اليوم السابع والثامن من الرش، على التوالي. بالإضافة إلى ذلك، فإن فعالية أنواع المستخلصات النباتية المائية التي تم فحصها على مرحلة اليرقات قد تأثرت بالتركيز المستخدم وكانت الجرعات العالية أكثر سمية وتسببت في مزيد من الموت في مجتمع الحشرة. من ناحية أخرى، كانت اليرقات أكثر مقاومة للمبيدات الحيوية الحشرية الفطرية المختبرة من المستخلصات النباتية المستخدمة. حقّقت العوامل الميكروبية *Beauveria bassiana* و *Trichoderma harzianum* الحدّ الأقصى والأدنى في موت الحشرة (100 و 71.7%)، بعد ثمانية أيام من التعرض للعامل الميكروبي، على التوالي، ولم تختلف النسبة المئوية للموت بشكل معنوي بين التراكيز المستخدمة. كانت قيمة التركيز المميت النصفي للفطريات الممرضة للحشرات المذكورة أعلاه هي: 119960.37 و 566168.82 بوغ/مل، على التوالي، بعد اليوم الثامن من التعرض. حقّق الشاهد الإيجابي (Alphacypermethrin 10) أعلى معدل قتل بنسبة 100% بعد اليوم الثالث من التعرض، بينما سجل الشاهد السلبي (الماء المقطر) أدنى معدل خلال التجارب.

كلمات مفتاحية: التركيز النصفي، *Meterhizium anisopliae*، *Beauveria bassiana*، *Platyclaudaus orientalis*، *Eucalyptus globulus*، طرائق المكافحة.

عناوين الباحثين: ساهند ك. خضر* وسروة م. خليل. قسم وقاية النبات، كلية علوم الهندسة الزراعية، جامعة صلاح الدين، إربيل، إقليم كردستان، العراق. *البريد الإلكتروني للباحث المراسل: sahand.khidr@su.edu.krd

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