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Comparing the efficiency of fungal spores and their metabolites in some entomopathogenic fungi against cotton leafworm, *Spodoptera littoralis*

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Abstract
Spodoptera littoralis is the most destructive insect in Egypt and many other countries, where it attacks several economic crops, such as cotton and vegetables. Entomopathogenic fungi are widely used in controlling many pests. Usually, spores of them were used in the controlling process. In this study, intracellular and extracellular metabolites were extracted from broth cultures of four entomopathogenic fungi: *Beauveria bassiana*, *Metarhizium anisopliae*, *Cladosporium cladosporioides*, and *Verticillium lecanii*. The extracted metabolites and spores of fungal strains were evaluated to compare their efficiency against the 2nd instar larvae of *S. littoralis*. The results indicated that highly significant differences were found between the efficiency of intracellular and extracellular metabolites and fungal spores against the treated larvae, where the mortality percentage of *S. littoralis* larvae increased with the extracellular metabolites extract of *C. cladosporioides* and *V. lecanii* more than intracellular metabolites and spore suspension, while the intracellular metabolites extract of *M. anisopliae* was more effective compared with extracellular metabolites and spore suspension, but the spore suspension of *B. bassiana* caused significant mortality compared with intracellular and extracellular metabolites extracts. As a result, this study reported that it can be useful to use the metabolites of some entomopathogenic fungi in the control of *S. littoralis* because they may have more activity than fungal spores in some entomopathogenic fungi.

Keywords: Insecticidal activity, Entomopathogenic fungi, Intracellular, extracellular metabolites

Introduction
Spodoptera littoralis (Lepidoptera: Noctuidae) is considered a harmful polyphagous insect pest in Egypt and many other countries that attacks several crops such as cotton, okra, vegetables, and ornamental plants. Also, it is the main pest of economic importance in Egypt **(**Abdullah 2019) [2]. Entomopathogenic fungi are widely used in controlling many pests as one of biopesticides (Kim *et al*. 2013) [10]. There are several registered commercial biopesticides that contain entomopathogenic fungi. Among the common entomopathogenic fungi are *Beauveria bassiana*, *Metarhizium anisopliae*, *Cladosporium cladosporioides*, *Verticillium lecanii*, and others (de Faria and Wraight 2007) [5]. In Egypt, some of these entomopathogenic fungal strains are under registration as commercial formulations. Generally, the fungal spores of entomopathogens are used in the control process. These spores can cause direct killing of the pest and, in addition, secondary infection by mechanical transmission of spores from pest cadavers (Shan and Feng 2010) [16]. But, the spore growth of entomopathogenic fungi is affected by weather conditions, such as temperature and relative humidity that lead to a slow pest mortality process (Kim *et al*. 2013) [10]. Several metabolic compounds were produced by entomopathogenic fungi and toxic to many pests (Gurulingappa *et al*. 2011; Vey 2001). The metabolites of entomopathogenic fungi such as *Lecanicillium lecanii* and *B. bassiana* can reduce the aphid population (Kim *et al*. 2010; Khan *et al*. 2012) [11, 9] and anti-feeding to whitefly and larva of *Spodoptera littoralis* (Quesada-Moraga *et al*. 2006; Wang *et al*. 2007) [15, 18]. These metabolic compounds were secreted inside cells (intracellular metabolites) or outside cells (extracellular metabolites). As a result, culture extracts or filtrates may contain metabolic compounds having different insecticidal activity (Kim *et al*. 2010) [11]. Many researchers studied the use of secondary metabolites and found a high percentage of mortality in treated pests. Abdullah (2019) [2] extracted the metabolites from broth cultures of *B. bassiana* and *Trichoderma harzianum* with an ethyl acetate solvent and evaluated these metabolites against

S. littoralis and *Aphis gossypii*. His study recommended that the metabolites of both fungi can be used to control *A. gossypi*, but only the metabolites of *B. bassiana* can be used to control *S. littoralis*. Gurulingappa *et al*. (2011) [7] studied the effects of endophytic strains of *Lecanicillium lecanii* and *Beauveria bassiana* on the survival and reproduction of *A. gossypii*. They found that *A. gossypii* is affected by contact with both conidia and fungal metabolites. Mainly, the mode of action of entomopathogenic fungi involves the production of enzymes, toxic proteins, and bioactive metabolites to overcome the insect immune system or cause lysis in the insect body (Isaka *et al*. 2005; Ortiz-Urquiza and Keyhani 2013) [8, 14]. The aim of this study is to compare the efficiency of fungal spore suspension, intracellular, and extracellular metabolites of four entomopathogenic fungi against the second-instar larvae of the cotton leafworm, *Spodoptera littoralis.*

Materials and methods

Rearing procedure of *Spodoptera littoralis*

Egg clusters of *S. littoralis*, a laboratory strain, were kindly obtained from the Cotton Leafworm Research Department, Plant Protection Research Institute, Agriculture Research Center. The egg clusters were incubated in climatic control chamber at 25 °C, 70% RH, and 14L: 10D photoperiod till hatching. The produced larvae were fed pieces of castor leaves to obtain the 2nd instar larvae that were used in the bioassay experiment.

Fungal strains

Four entomopathogenic fungal strains were used in this study and kindly obtained from Assiut University, Mycological Centre (AUMC), Assuit, Egypt. The fungal strains were *Beauveria bassiana (Bb), Metarhizium anisopliae (Ma), Cladosporium cladosporioides (Cc),* and *Verticillium lecanii (Vl).* All fungal strains were cultured and maintained on sabouraud dextrose agar (SDA) medium. Sabouraud dextrose agar typically contains 40 g/l dextrose, 10g/l peptone, and 20 g/l agar, pH 5.6.

Preparing the fungal spore suspension

All fungal strains were cultured on SDA medium and incubated at 25 ˚C for 14 days to obtain the fungal spores. After the incubation period, the spores were taken with a sterilised spatula and put in a 100-ml sterile saline solution. The hemocytometer slide was used to calculate the spores count in ml.

Extraction of the intracellular metabolites of fungal strains

All fungal strains were cultured separately in 400 ml of sterilized sabouraud dextrose broth medium (SDB) in a 1L conical flask and incubated at 25 °C for 21 days. After the incubation period, all fungal cultures were filtered to separate the mycelial mats and mixed with methanol solvent to extract the intracellular metabolites. The mixture of mycelial mats and methanol was blended very well in the blender or by a glass rod, then left overnight. The mixture was filtered, and the mycelial mats were washed twice with methanol. The methanol extract was concentrated in a rotary evaporator system to obtain the crude extract of intracellular metabolites of fungal strains.

Extraction of the extracellular metabolites of fungal strains

All fungal strains were cultured separately in 400 ml of sterilized sabouraud dextrose broth medium (SDB) in a 1L conical flask and incubated at 25 °C for 21 days. After the incubation period, all fungal cultures were filtered. The supernatant of each fungal culture was mixed with ethyl acetate and put in a separating funnel to extract the extracellular metabolites. The mixture was mixed very well and left to separate into two layers. The upper layer was taken and concentrated in a rotary evaporator system to obtain the crude extract of extracellular metabolites from fungal strains.

Bioassay procedure

Twenty larvae of the 2nd instar of *S. littoralis* were put in a plastic jar and left for two hours without feeding. Three concentrations were prepared from intracellular metabolites extract (500, 1000, and 1500 ppm), extracellular metabolites extract (500, 1000, and 1500 ppm), and fungal spore suspension ($1x10^8$, $2x10^8$, and $3x10^8$ spores/ml) of each fungal strain. Equal pieces of castor leaves were dipped in each concentration for 30 seconds then allowed to air dry. The treated castor leaves were put on the larvae in the plastic jars and covered by muslin. The dead larvae were recorded, and the mortality percentages were calculated after the first, third, and fifth days post-treatment.

Statistical analysis

Mortality percentages were corrected by Abbot's formula (Abbot 1925) [1]. CoHort Software (2004) [4] was used to calculate the analysis of variance (ANOVA). A three-way completely randomized design was applied to the bioassay experiment's results against the cotton leafworm.

Results

The data in Table 1 showed that all investigated entomopathogenic fungi had various levels of toxicity against *S. littoralis* treated with fungal spores as well as intracellular and extracellular metabolite extracts. In addition, the treatments with the fungal spore solution did not cause any mortality throughout the first three days of treatment, while other treatments caused mortality percentages after one day of treatment. Table 2 shows the analysis of variance (ANOVA) for the experimental results of the bioassay test. The data referred to highly significant differences found among fungal strains, extracts from each fungus, and concentrations of each extract. In addition, highly significant differences were found between interactions, as shown in Table 2.

The mortality percentage of treated larvae by intracellular metabolites extract of *M. anisopliae* (Ma) was higher than the treatments with extracellular metabolites extract and fungal spores in the same fungus after five days of treatment, as shown in Fig. 1 Ma. On the other hand, the mortality percentage of treated larvae by fungal spores of *B. bassiana* (Bb) was the highest, followed by extracellular metabolites and then intracellular metabolites extracts in the same fungus after five days of treatment, as shown in Fig. 1 Bb. But in the case of *C. cladosporioides* (Cc), the fungal spores did not cause any mortality percentage in treated larvae, while extracellular metabolites extract caused a high mortality percentage, followed by intracellular metabolites extract in the treated larvae after five days of treatment, as shown in Fig. 1 Cc. Only extracellular metabolite extract in *V. lecanii* caused the mortality percentage of treated larvae, while other treatments in the same fungus did not cause any mortality percentage after five days of treatment, as shown in Fig. 1 Vl.

Table 1: Mortality percentages of treated *S. littoralis* by fungal spores and metabolites of tested entomopathogenic fungi under laboratory conditions

Treatments	Concentrations	Mortality % of *S. littoralis*											
		After 1 day				After 3 days				After 5 days			
		Ma	*Bb*	*Cc*	*Vl*	*Ma*	*Bb*	*Cc*	*Vl*	*Ma*	*Bb*	*Cc*	*Vl*
Meth. Extract	500 ppm	00	00	00	00	00	8.0	00	00	8.0	8.0	00	00
	1000 ppm	17	00	00	00	17	8.0	00	00	17	8.0	00	00
	1500 ppm	58	8.0	17	00	75	17	25	00	75	17	25	00
Ethyl acetate extract	500 ppm	00	8.0	00	00	00	8.0	8.0	00	8.0	8.0	8.0	00
	1000 ppm	8.0	17	8.0	00	8.0	25	17	00	17	33	33	8.0
	1500 ppm	25	33	50	50	25	33	50	50	25	42	50	50
Spores	$1x10^8$ Spores/ml	00	00	00	00	00	00	00	00	00	8.0	00	00
	$2x10^8$ Spores/ml	00	00	00	00	00	00	00	00	8.0	25	00	00
	$3x10^8$ Spores/ml	00	00	00	00	8.0	8.0	00	00	25	58	00	00

Ma: *Metarhizium anisopliae*; *Bb*: *Beauveria bassiana; Cc: Cladosporium cladosporioides; Vl: Verticillium lecanii.*

Table 2: Analysis of variance (ANOVA) for the results of the bioassay experiment.

Source of variation	Degree of freedom	Sum of Squares (Type III SS)	Mean Squares (MS)	F. Value	P. Value	Significant
Main effects						
Fungi	3	4544.67	1514.89	2.7121e15	0.0000	***
Extracts	2	3458	1729	3.0954e15	0.0000	***
Concentrations	2	13290.5	6645.25	1.1897e16	0.0000	***
Interactions						
Fungi * Extract	6	7607.33	1267.89	2.2699e15	0.0000	***
Fungi * Concentration	6	1264.83	210.81	3.7741e14	0.0000	***
Extract * Concentration	4	1262	315.5	5.6484e14	0.0000	***
Fungi * Extract* Concentration	12	7686.67	640.56	1.1468e15	0.0000	***
Error	72	4.02167e-11	5.586e-13<-			
Total	107	39114				

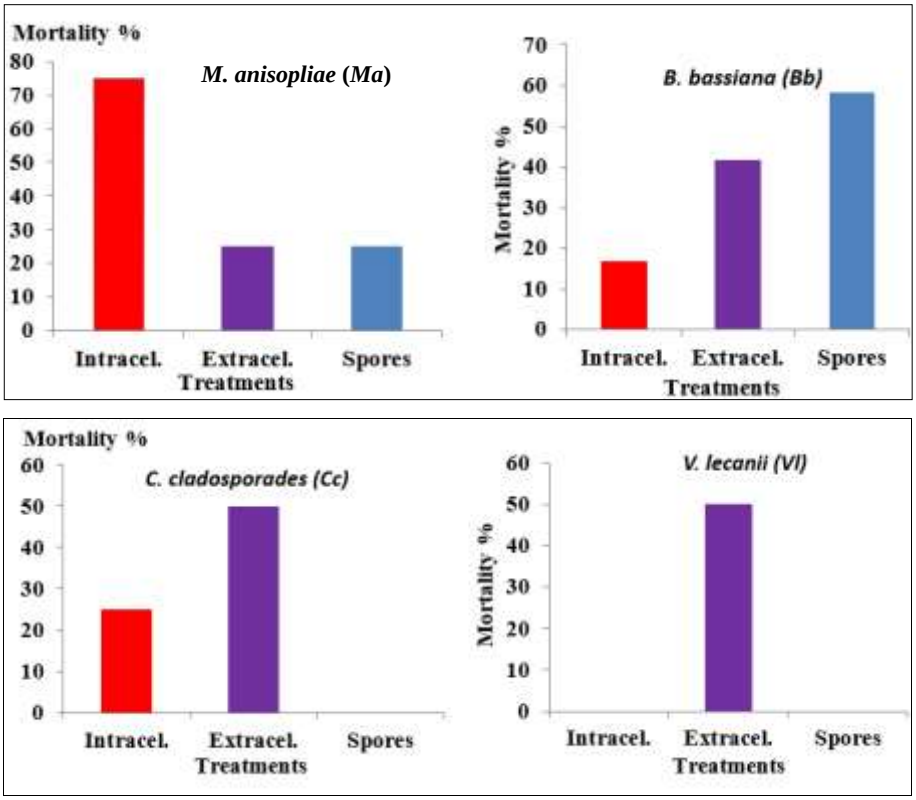

Fig 1: Comparison of the effectiveness of fungal spores and intracellular and extracellular metabolites in each fungal strain against *S. littoralis*

On the other hand, the results in Table 1 and Fig. 2 illustrated that the treated larvae using the intracellular metabolites of *M. anisopliae* were more susceptible compared with other entomopathogenic fungi in the same treatment. While the intracellular metabolites of *B. bassiana* and *C. cladosporioides* were less effective against the treated larvae. But the treated larvae using the intracellular metabolites of *V. lecanii* were not affected. As shown in Fig. 2, the extracellular metabolites extracted from all tested entomopathogenic fungi were effective against treated larvae, with *C. cladosporioides* and *V. lecanii* being more toxic, followed by *B. bassiana* and *M. anisopliae*. In addition, the spore suspension was the most

effective treatment in the cases of *B. bassiana* and *M. anisopliae*, but it was not effective in the cases of *C. cladosporioides* and *V. lecanii*, as shown in Fig. 2.

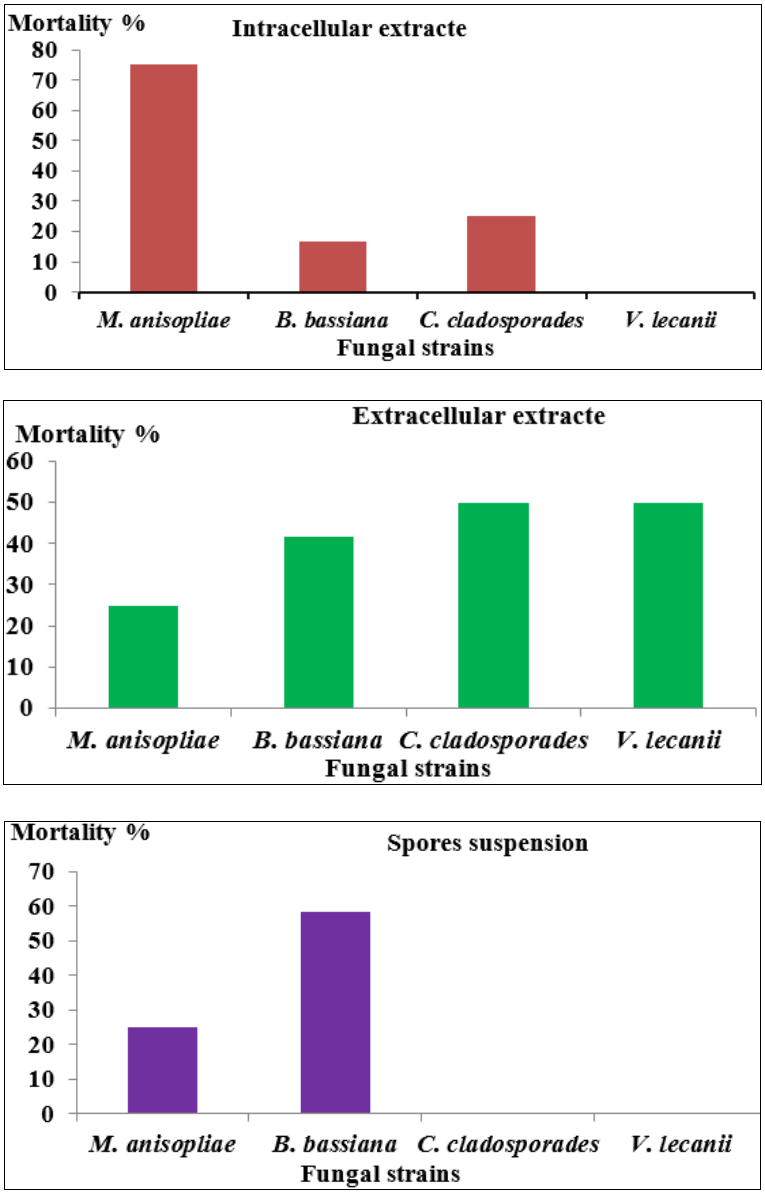

Fig 2: Comparison of the effectiveness of fungal strains at different treatments (fungal spores and intracellular and extracellular metabolites) against *S. littoralis*

Discussion

This study indicated that the different toxic levels of entomopathogenic fungi were found with their different treatments, such as fungal spores and metabolite extracts, against the cotton leafworm *S. littoralis*. Quesada-Moraga *et al*. (2006) [15] reported that the fungal spores or metabolites of entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* have shown different effects against moth larvae. Also, Gurulingappa *et al*. (2011) [7] studied the effects of the culture filtrate and mycelia of endophytic fungi, *L. lecanii* and *B. bassiana*, on the survival of *A. gossypii*. They reported that the ethyl acetate and methanolic fractions of the culture filtrate and mycelia of both fungi caused significant mortality in *A. gossypii.* Also, they found that the ethyl acetate extract of *L. lecanii* culture was more toxic against *A. gossypii* than the methanol extract of the same fungus. Similarly, Wang *et al*. (2000) [19] mentioned that whiteflies and aphids' survival was decreased in a laboratory study by feeding on *L. lecanii* culture filtrate. Anderson *et al*. (2007) [3] reported that the two most likely causes of pest deaths seem to be insect afflictions with either the endophytic entomopathogen's spores or metabolites produced by fungus in plants or as liquid films on leaf surfaces.

Additionally, according to McGee (2002) [13], the methanol extracts of four morphospecies of fungi that were isolated from cotton plant leaves reduced the growth rates of the larvae of *Helicoverpa armigera* (Hubner) and *Helicoverpa punctigera* (Wallengren). Furthermore, *Spodoptera littoralis* larvae were significantly reduced by the crude soluble protein extracts of *B. bassiana* when applied to alfalfa leaf discs or included in artificial diet (Quesada-Moraga *et al*. 2006) [15]. According to Leckie *et al*. (2008) [12], the toxicity of mycelia or fungal metabolites may be responsible for insect mortality and delayed growth. Elbanhawy *et al*. (2019) [6] investigated the influence of methanol and ethyl acetate extracts of four entomopathogenic fungi, *Metarhizium anisopliae*, *Trichoderma longibrachiatum*, *Cladosporium cladosporioides* and *Purpureocillium lilacinum*, on the

mortality percentage of cotton aphids, *Aphis gossypii*. They found that the methanol extracts (intracellular metabolites) of *P. lilacinum* and *C. cladosporioides* were more toxic against *A. gossypii* compared with other extracts. So, some of the entomopathogenic fungi are able to produce some active compounds in enough amounts to kill the insect pests quickly compared with their spores. But some other entomopathogenic fungi can't produce active compounds in enough amounts to kill the insect pests, so they used the insect body for growth and development that led to insect death slowly.

Conclusion

Hence, this study investigated the survival of *S. littoralis* following treatment with fungal spores, intracellular and extracellular metabolites expressed by four entomopathogenic fungi: *M. anisopliae, B. bassiana, C. cladosporioides*, and *V. lecanii*. Generally, the results in this study illustrated that the mortality percentage of *S. littoralis* larvae increased with the extracellular metabolites extract of *C. cladosporioides* and *V. lecanii* more than intracellular metabolites and spore suspension, while the intracellular metabolites extract of *M. anisopliae* was more effective compared with extracellular metabolites and spore suspension, but the spore suspension of *B. bassiana* caused significant mortality compared with intracellular and extracellular metabolites extracts. As a result, this study reported that it can be useful to use the metabolites of some entomopathogenic fungi in the control of *S. littoralis* because they may have more activity than fungal spores in some entomopathogenic fungi.

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