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The Role of Chemical Additives in Enhancing the Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* Against the Potato Tuber Moth *Phthorimaea operculella* (Zeller) (Lepidoptera:Gelechiidae)

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Abstract: Susceptibility of the potato tuber moth larvae *Phthorimaea operculella* to the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopilae* were detected. The LC_{50} of *B. bassiana* and *M. anisopliae* against *P. operculella* neonate larvae were 3.4x10 and 8.61x10^7 conidia/ml, respectively. The metabolic acids (oxalic and citric acids) scored the highest enhancement in the efficiency of *B. bassiana* and *M. anisopliae* against the first larval instar of *P. operculella*. Some chemical additives caused an increase in the efficiency of the tested fungi against the target insects.

Key words: *Beauveria bassiana, Metarhizium anisopliae, Phthorimaea operculella,* chemical additives, biological control

Introduction

The ubiquitous fungus *Beauveria bassiana* (Balsamo) vuillemin as well as *Metarhizium anisopliae* vuillemin (Metschnikoff) Sorokin causes common disease associated with dead moribund insects in nature (McCoy *et al.*, 1988) These fungi have been scrutinized world wide as microbial control agents of many insect pests (Hoffman *et al.*, 1993; Hoffman, 1997).

In Egypt, *Phthorimaea operculella*, is considered an important economic pest because its larvae causes severe damage to crops specially potato, tomato, egg plant and some Solanaceae plants (Abul-Nasr *et al.*, 1971; Salama *et al.*, 1972)

Effectiveness of *B. bassiana* and *M. anisopliae* against insect larvae in the laboratory has been enhanced by the addition of chemicals as organophosphates, carbamates and organochlorines such as DDT (Andersen *et al.*, 1995; Hassan *et al.*, 1989; Quintela and McCoy, 1997).

Fungi belongs to several major groups and ranged from obligate host specific parasites to omnivorous facultative saprophytic. The two most successful fungi for pest control were belonging to the fungal genera *Metarhizium* (Sorok.) and *Beauveria* (Vuill.) have been subjected to extensive studies and developed as commercial products to control specific insects on agricultural crops. The success in using *Metarhizium* lies in the selection of specific strains as indicated by Andersen *et al.* (1995). Taxonomic account of the genus *M. anisopliae* is a very effective entomopathogen. It attacks the target insects by penetrating its cuticle and invading the haemolymph.

When applied as a spray to infested crops, the entomopathogens invades and immobile the insect within two days, symptoms appeared within seven days. The mycoses tissues of the insects remain adhered to the crop and additional spores are released to maintain a high level of infective material on the crop (Andersen *et al.*, 1995; Samson *et al.*, 1988).

The entomopathogenic fungus *Metarhizium* spp. infects the insect by direct penetration through the insect cuticle. Members included both host specific and generalist strains, which have the potential for use as biocontrol agents are oxalic and citric acids which act as fungus metabolites (Bidochka and Khachatourians, 1990).

In present study 22 compounds that represents eight different groups, were used to determine which of these additive compounds can potentiate the toxicity of the fungus against the potato tuber moth *P. operculella*.

The entomopathological activity of the fungi against insects seems to be driven from several cuticle dissolving enzymes, chitinase and protease. In case of the fungus *M. anisopliae*, protease plays a major role in the early stage of innovation during penetration (Leger *et al.*, 1987, 1991, 1996). *Beauveria bassiana* and *M. anisopliae* produce a multiple extracellular chitinase isozymes (Havukkala *et al.*, 1993; McCoy, 1995)

This work aiming to determine which of the additives can potentiate the toxicity of the fungus against *P. operculella*.

Materials and Methods

Pathogens: The following entomopathogenic fungi were used:

1. A commercial formulation (Naturalis-L,Troy-Biosciences-Arizona) based on the fungus *Beauveria bassiana* with potency of 2.3 × 10^7 conidia/ml.
2. The fungus *Metarhizium anisopliae* was isolated from infested larvae. Isolates were identified and subcultured on nutrient PDA (potato dextrose agar medium). The spores of *M. anisopilae* were collected from the surface of mycelium growth and a spore suspension with Tween-80 (2 drops), was prepared. The preparations diluted in water and adjusted at concentration 4x10 conidia/ml.

Additives: Chemicals used were

1. Inorganic salts: Ammonium phosphate dibasic, boric acid,, calcium carbonate, calcium oxide, calcium sulphate, copper carbonate, magnesium chloride, magnesium sulphate, potassium carbonate, potassium bicarbonate, sodium carbonate and zinc sulphate.
2. Tannin: Tannic acid and tween-80
3. Amino acids: Arginine and serine.
4. Organic acids: Calcium acetate.
5. Oxidized carbohydrate: Sorbic acid.
6. Aromatic compounds: Amino-salysalic acids.
7. Amide: Acetamide.
8. Fungus metabolites: Citric and oxalic acid.

The concentrations of the additives were adjusted in each combination at 0.05% concentration level.

Insect: Standard laboratory colony of potato tuber moth *P. operculella* was reared on potato tubers under laboratory conditions of about 26± 2°C and 70± 5% RH. Eggs were obtained from the stock culture and kept in Petri-dishes till hatching. The rearing technique by El–Sherif *et al.* (1978) was adopted. Pupae were individually kept in specimen tubes (1x3 cm^2) till adult emergence. Adult moths were kept in oviposition cages that consist of chimney glass, about 8 cm in diameter and 16 cm high, the lower rim of which rested on the bottom of a petri-dish lined with a disk of filter paper and the upper rim covered with muslin. Each cage was provided with a small plastic cover containing a piece of cotton soaked in 5% sugar solution. Eggs were obtained from the stock culture and kept in petri dishes till hatching. Groups of newly hatched larvae were transferred into petri dishes containing fresh pieces of potato. Larval development was allowed to continue until adult emergence.

Bioassay of *B. bassiana* and *M. anisopliae*: *Beauveria bassiana* and

M. anisopliae fungi were assayed against *P. operculella* larvae by dipping potato tubers in suspension containing the prescribed dose of the pathogens in distilled water to which, one drop of tween-80 was added as wetting agent. Excess liquid was removed from the tubers when allowed to dry at room temperature (26± 2°C). The tubers were then placed individually in plastic cups (15 x 4 cm^2). For each concentration, ten replicates were used. Each replicate comprised of 10 larvae placed on the tuber by means of a camel's hairbrush and reared at 26± 2°C and 70± 5% RH, for seven days. Control larvae were fed potato tubers treated only with water and the wetting agent (Tween-80). Mortalities were corrected according to Abbot (1925). Seven serial dilutions ranged from $16.5x10^7$ - $0.32x10^7$ conidia/ml of *B. bassiana* were tested in order to deduce the LC_{50} of *B. bassiana* against *P. operculella* larvae. *M. anisopliae* was assayed also using *P. operculella* neonate larvae by dipping potato tubers in seven serial suspension containing $4x10^7$, $2x10^7$, $1x10^7$, $0.5x10^7$, $0.25x10^7$, $0.125x10^7$ and $0.062x10^7$ conidia/ml of distilled water to which one drop of tween-80 was before. Excess liquid was shaken from the tubers, which were allowed to dry at room temperature (26± 2° C and 70± 5% RH).

Mortalities were corrected according to Abbott (1925). Experiments were carried out at 26± 2° C. In all testes, the larvae were examined after 6-7 days. The fungus suspensions at the LC_{50} concentration level was mixed with the prescribed amount of the additive that form 0.05% of the additive in the mixture.

The percentage of reduction in LC_{50} was calculated according to the following equation:

$$\text{The percentage of } LC_{50} \text{ reduction} = \frac{LC_{50} \text{ of the fungus alone} - LC_{50} \text{ of fungus after added additives}}{LC_{50} \text{ of the fungus alone}} \times 100$$

Results and discussion

Inorganic salts: The data presented in Table (1) show that the LC_{50}`s of *B. bassiana* against larvae of *P. operculella* was $3.40x10^7$. When some inorganic salts (at 0.05% concentration level) as ammonium phosphate dibasic, boric acid, calcium carbonate, calcium oxide, calcium sulphate, copper carbonate, magnesium chloride, magnesium sulphate, potassium carbonate, potassium bicarbonate, sodium carbonate and zinc sulphate was combined with LC_{50} of *B. bassiana*, they caused a variously reduction to LC_{50} by 70, 73, 79, 57, 52, 26, 67, 55, 21, 48, 40 and 56%, respectively (Table 1).

This indicated that the addition of calcium carbonate, boric acid and ammonium phosphate dibasic to *B. bassiana* scored the potentiation of activity followed by magnesium chloride, calcium oxide, zinc sulphate, magnesium sulphate and calcium sulphate. These salts significantly affected as indicated by non overlapping the corresponding 95% confidence limits, according to Finney (1964).

While copper carbonate, potassium carbonate, sodium carbonate and potassium bicarbonate caused less than 50% reduction in LC_{50} value.

Regarding the fungus *M. anisopliae*, the LC_{50} of *M. anisopliae* against the neonate larvae of *P. operculella* was $8.61x10^7$ conidia/ml. The addition of small amount (0.05%) of some inorganic salts had greatly potentiated the activity of the pathogen and the LC_{50} value of *M. anisopliae* was declined the reduction reached about, 25, 57, 76, 45, 49, 52, 74, 71, 41, 53, 60 and 65%, of its value, when ammonium phosphate dibasic, boric acid, calcium carbonate, calcium oxide, calcium sulphate, copper carbonate, magnesium chloride, magnesium sulphate, potassium carbonate, potassium bicarbonate, sodium carbonate and zinc sulphate were added at the 0.05% level, respectively, (Table 2). Knowing that additives at this concentration level caused negligible mortalities among treated larvae as compared with natural mortalities (Table 2).

Table 1: Effect of additives on the LC_{50} of *B. bassiana* against *P. operculella* larvae

Additives at (0.05%)	$LC_{50}x10^7$ condia/ml	% of reduction	S	V	95% confidence limits
B. bassiana alone (no additive)	3.40		1.00	3.5	2.11-4.51
Inorganic salts					
Ammonium phosphate dibasic	1.02	70	1.4	1.40	0.11-4.64
Boric acid	0.90	73	1.50	6.59	0.03-1.53
Calcium carbonate	0.71	79	1.60	1.19	0.12-2.10
Calcium oxide	1.61	52	1.70	2.40	1.09-3.08
Calcium sulphate	1.46	57	1.76	4.61	0.86-6.57
Copper carbonate	2.50	26	2.87	3.11	0.71-6.89
Magnesium chloride	1.10	67	1.10	0.26	0.51-5.86
Magnesium sulphate	1.50	55	1.90	2.68	0.98-3.38
Potassium carbonate	2.68	21	1.74	0.49	1.86-5.49
Potassium bicarbonate	1.75	48	1.91	4.37	0.01-3.29
Sodium carbonate	2.01	40	1.01	2.89	1.17-6.89
Zinc sulphate	1.49	56	1.99	3.49	0.29-3.53
Tanin					
Tannic acid	1.01	70	2.78	0.10	0.88-4.40
Tween -80	2.32	31	1.96	1.13	1.43-6.95
Amino acids					
Arginine	1.80	47	1.21	3.60	0.85-6.30
Serine	1.70	50	1.50	1.50	0.12-6.78
Organic acids					
Calcium acetate	1.68	50	1.51	0.33	0.67-5.21
Oxidized carbohydrate					
Sorbic acid	1.05	69	1.31	3.32	0.99-5.99
Aromatic compounds					
Amino-salysalic acid	2.08	38	0.99	4.30	1.09-6.87
Amide					
Acetamide	1.30	76	0.90	2.41	0.75-5.84
Fungus metabolite					
Citric acid	0.80	82	1.20	1.20	0.001-2.00
Oxalic acid	0.60	0.90	1.02	1.40	0.09-1.01

Corrected for natural mortality by Abbott (1925) formula, S: Slope, V: variance

Table 2: Effect of additives on the LC_{50} of *M. anisopliae against P. operculella*

Addition at 0.05%	$LC_{50}x10^7$ conidia/ml	% of reduction	S	V	95% confidence limits
M. anisopliae alone (no additive).	8.61		1.90	1.2	6.01-12.12
Inorganic salts					
Ammonium phosphate dibasic	6.42	25.00	3.81	1.20	3.00-11.93
Boric acid	2.01	76.00	3.70	1.80	0.97-10.07
Calcium oxide	4.67	57.00	1.70	1.20	1.90-9.09
Calcium sulphate	4.33	49.00	2.78	1.80	1.54-9.22
Copper carbonate	4.09	52.00	1.07	1.30	1.67-5.98
Magnesium chloride	2.21	74.00	3.77	2.34	1.53-5.99
Magnesium sulphate	2.43	71.00	3.70	3.69	1.98-5.78
Potassium carbonate	5.01	41.00	2.89	3.20	2.02-9.87
Potassium bicarbonate	4.01	53.00	1.70	3.80	2.98-7.77
Sodium carbonate	3.44	60.00	1.71	3.67	1.01-4.01
Zinc sulphate	3.01	65.00	1.70	2.65	1.02-5.97
Tanin					
Tannic acid	8.55	0.69	178.00	1.23	5.34-12.04
Tween-80	8.32	3.30	1.66	3.60	3.09-12.88
Amino acids					
Arginine	3.62	57.00	2.78	3.87	0.23-4.65
Serine	2.25	73.00	1.77	4.06	1.23-4.65
Organic acid					
Calcium acetate	5.68	34.00	2.53	4.33	2.09-9.09
Oxidized carbohydrate					
Sorbic acid	1.85	78.00	1.20	1.21	0.55-4.07
Aromatic compounds					
Amino salysalic acid	5.81	32.00	1.52	2.44	3.98-10.32
Amide					
Acetamide	6.43	1.30	1.34	3.03	4.32-9.11
fungus metabolite					
Citric acid	1.30	1.20	1.20	2.20	0.45-3.21
Oxalic acid	1.20	1.31	1.31	3.32	0.32-3.97

Mortalities corrected according to Abbott (1925) formula, S: Slope V: variance

it is reported that a similar enhancement in microbial control agents, bacteria *B. thuringiensis* with boric acid against larvae of *Lymantria dispar*. The role of boric acid is not clear, but is known to be an abrasive when used as a constant insecticide. Boric acid is also used as stomach poison for insect species (Metealf and Flint, 1951). Assuming that the same effect occurs in *P. operculella*, it may exert some damage on the peritrophic membrane, there by facilitating the diffusion of *B. bassiana* toxin to the insect midgut epithelium. It may also directly damage the midgut epithelium, thus allowing more rapid acidification of the gut contents and enhanced spore germination and septicemia. Govindarajan *et al.* (1976) provided some evidence for this interpretation by reporting that the addition of 0.05 to 0.01% boric acid markedly decreased the lethal time of the pathogen in *Spodoptera litura* larvae. Similar results were obtained when *B. thuringiensis* was added to the same additives against *S. littoralis* and *Agrotis ypsilon* (Salama *et al.*, 1985; 1990 a,b).

Morris *et al.* (1995), mentioned that ammonium salts in the current tests did not cause material enhancement in activating the pathogen. Concerning studies on the physiological effects of magnesium salts on lepidopterous larvae. Shaplay and Bradley (1982) reported adverse effect of magnesium sulphate on egg hatch and survival of mosquitoes. Potassium carbonate at 0.05% was effective. The calcium salts showed only weak enhancement of *B. bassiana*. Calcium is a potassium channel blocker and is known to inhibit the effect of *B. bassiana* on the midgut apical membrane of insect. Calcium and magnesium sulphate have less efficient enhancements than other compounds. These results are partly consistent with those of Salama *et al.* (1990a, b), who reported that calcium carbonate and potassium carbonate enhanced the effectiveness of *B. thuringiensis* against *S. littoralis* and *A. ypsilon* on soybean and various vegetable crops. In the concern tests, the additives did not appear to reduce feeding activity on plants compared with the pathogen alone.

The calcium and potassium carbonates tested were effective as *B. bassiana* was enhanced in diet.

Tanin: The addition of tannic acid and Tween-80, at 0.05% concentration level reduced LC_{50} of *B. bassiana* against *P. operculella* larvae to about one third and one fourth, respectively (Table 1).

When the larvae treated with the combination of tannic acid and *M. anisopliae*, at the concentrations 0.05%, very slight reduction in LC_{50} (0.69 and 3.3%) was obtained, respectively (Table 2). The results agree with Klocke and Chan (1982), who reported that tannin allelochemicals tannic acid, which at 0.05%, did not reduce LC_{50} of the pathogen. Tannin inhibits the growth of lepidopterous larvae by depressing the midgut digestive enzymes (Klocke and Chan, 1982). Schutz (1983) suggested that an insect adapted to feeding on a light diet would have a higher gut pH, which would make it more susceptible to *B. thuringiensis* ssp. *Kurstaki*. The ineffectiveness of tannic acid in enhancing a pathogen as *B. thuringiensis* in *S. littoralis* was mentioned.

Lipid emulsifying agent: Tween-80, increased the infectivity of the pathogen. Salama *et al.* (1985), reported 3 and 5 fold increases in toxicity in *S. littoralis* larvae fed a diet supplemented with microbial agents *B. thuringiensis* ssp. *Kurstaki* with Tween-60 and Tween-80 at 0.05% respectively.

Amino acids: Addition of the amino acid, arginine and serine reduced the LC_{50} of *B. bassiana* alone against the larvae of *P. operculella* to 47 and 50%, of its value, respectively (Table 1).

Addition of these amino acids, to *M. anisopliae* caused 57 and 73% reduction in LC_{50} values of the pathogen alone.

The amino acids at 0.05%, arginine was an effective enhance of *B. bassiana* activity at LC_{50}. Serine had low activity. Wolfersberger (1990) hypothesize that ingested amino acids have an effect on the potassium and sodium ion transport in the insect gut, this effect could in turn affect the effectiveness of the ingested microbial agents. Wigglesworth (1972) also noted that most of the nitrogen in insect haemolymph is in the form of amino acids. The

alteration of these amino acids would interfere with normal physiological processes, thereby, increasing insect susceptibility to the fungus *B. bassiana*. Such physiological interference has been noted in locust (Rafaei and Applebaum, 1981), tobacco hornworm, *Protoparce sexta* (Johanssen) and *Rhodnius* sp. (Barrette and Friend, 1972). Our research indicated that amino acids enhanced the *B. bassiana* effectiveness.

Organic acids: The increased activity due to the addition of organic acid calcium acetate at 0.05% was more pronounced in case of *B. bassiana* than that of *M. anisopliae*. The LC_{50} reduced by 50 and 34% of its value, respectively (Tables 1, 2). Agree with Salama *et al.* (1990a, b), who, reported 3.7, 3.4 and 3.7 folds increase in potency of Dipel 2X in *A. ypsilon* feed with a diet containing *B. thuringiensis* and calcium acetate, sodium thioglycolate and malic acid at 0.05%, respectively.

Oxidized carbohydrates: The oxidized carbohydrates examplified by sorbic acid at a concentration of 0.05% caused 69 and 78% reduction in LC_{50} of *B. bassiana* and *M. anisopliae* against *P. operculella*, respectively (Tables 1, 2).
Supraoptimal levels of sorbic acid in diet reduced the numbers of circulating haemocytes and phagocytes in *Lasperyresia pomonella* L. and increased the susceptibility of the larvae to *B. thuringiensis*. (Pristavko and Dovzhenok, 1974).

Aromatic compounds: The amino salycilic acid at 0.05% decreased LC_{50} of *B. bassiana* and *M. anisopliae* against the neonate larvae of *P. operculella* by 38 and 32% respectively (Tables 1, 2). Morris *et al.* (1995), considered that the aromatic compounds at the concentration of 0.05% increased the toxicity of *B. thuringiensis* against bertha armyworm by 1.5 to 1.6 folds. P-amino salysalic acid at 0.02 to 0.1% enhanced the *B. thuringiensis* against *Galleria mellonella* 2.9-folds.

Amides: Acetamide at 0.05% concentration level, enhanced the activity of two fungi against the larvae of *P. operculella.* The LC_{50} of *B. bassiana* and *M. anisopliae* without additives was, 3.41 and 8.61×10^7 conidia/ml which reduced by 61 and 25% of its value, after addition to acetamide, respectively (Tables 1, 2).
Compounds in this group are known to reduce feeding and fecundity in lepidopterous larvae and have suggested as synergists for chemical pesticides (Giles and Rothwell, 1983). Morris *et al.* (1995) found that acetamide at 0.05% enhanced the toxicity of *B. thuringiensis* against *Mamestra configurata,* which agreed with the obtained results.

Metabolite production by fungi: Citric and oxalic acids as *B. bassiana* metabolite reduced the LC_{50} of *B. bassiana* by 76 and 82% of its value, respectively (Table 1). In case of *M. anisopliae* the reduction reached to 84 and 86%, respectively (Table 2).
Pathogenic fungi posses an intriguing array of mechanisms that permit them to break down and assimilate host materials while overcoming mechanisms host-resistance mechanisms. For the most part, the fungal metabolites assist the pathogen with physical aspects of ingress, e.g. cuticle-degrading enzymes that destroy activity or modify the structural integrity of the host, inhibition of selective processes or enzymes of the host and interference with the regulatory systems of the host. Such damage, associated with disease symptoms, may be produced both by the pathogen enzymes and by its low molecular weight metabolites (toxin).
Undoubtedly, many pathogen enzymes are important determinates of virulence because they enable the pathogen to co-exist with the changing metabolic processes associated with the host's diseased state. Once fungi invade the hemocoel, the host may be killed by some combination of mechanical damage produced by fungal growth, nutrient exhaustion and toxicosis. The relative importance of these mechanisms varies with the specific fungal isolate or host. Many entomopathogenic fungi produce toxins, but although some toxins are fully described chemically. Destruxins and other toxins include the cyclic depsipeptides beauvericin (*Verticillium lecanii*) and bassianolide (*Beauveria bassiana*), which may function principally as endocellular ionophores, leucinostatins and efrapeptins (linear peptides from *Paecilomyces* and *Tolypocladium* spp., respectively) with antimicrobial activity and cytochalasins (*Metarhizium anisopliae*), which may paralyze host cells.
At a given concentration of tested pathogen, *B. bassiana* the mortality response data are usually characteristic for the pathogenic potency against the tested worm *P. operculella*. LC_{50} of *B. bassiana* against *P. operculella* neonate larvae was 2.3×10^7 conidia/ml. Evaluations of the effect of metabolic acids of *B. bassiana* (oxalic acid, citric acid) and slight effect (additives) with oxalic acid against *P. operculella* larvae. Investigations carried out by Bidochke and Khachatourians (1990) revealed that the relationship between oxalic acid and citric acid together with *B. bassiana* conidia in grasshopper mortality was marked by synergistic. They suggested that acid metabolites produced by *B. bassiana* might play an important role in cuticle solubilization and subsequent hyphal penetration.
The use of the chemical additives can enhance the efficacy of the fungi *B. bassiana* and *M. anisopliae*, especially the metabolic acids (oxalic and citric acids).

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