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ORIGINAL PAPER

Assessment of insecticidal activity of red pigment produced by the fungus *Beauveria bassiana*

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Abstract Insecticidal activity of the red pigment produced by a strain of the fungus *Beauveria bassiana* that was locally isolated from infected whitefly, *Bemisia tabaci* (Genn.) was assessed. The pigment is produced extracellularly and is a water-soluble. This makes it easy and simple to be recovered from fermentation broth and used in pathogenicity experiments. When applied alone to *Bm. tabaci* nymphs, mortality percentages of 18% was recorded. For nymphs treated with *Bv. bassiana* spore suspension, mortality was 60%. The best results were obtained when red pigment was combined with fungal spores with the mortality percentage being increased to up to 92%. The highest insecticidal activity against adults emerging later on from the surviving larvae of *Bm. tabaci* was recorded also with treatment combining pigment and fungal spores with the longest days to pupation.

Keywords Bioinsecticides · *Beauveria bassiana* · Red pigment · Whitefly · *Bemesia tabaci*

Introduction

The potential capabilities of the fungus *Bv. bassiana* as biological pest control agent was firstly recognized early nineteenth century (Van Driesche and Bellows 1996). Displaying a broad host range and being able to target a diverse number of arthropod species, strains of *Bv. bassiana* have been selected for control of insects and other arthropods that act as disease vectors, including mosquitoes and ticks (Clarkson and Charnley 1996; Kirkland et al. 2004), crop pests such as whiteflies, caterpillars and borers (Brownbridge et al. 2001) and even ecologically hazardous, invading pests such as fire ants and termites (Culliney and Grace 2000).

Species of the genus *Beauveria* have been reported to produce different extracellular toxins and enzymes (Takahashi et al. 1998). Unlike chemical pesticides and antibiotics used in agricultural production, and over a long period of field application, no reports of metabolites entering the food chain or accumulating in the environment have been reported as a result of *Bv. bassiana* metabolite secretion (Vey et al. 2001).

Recently, various types of pigments have been detected during cultivation of *Bv. bassiana*. Yellow *Bv. bassiana* pigments were identified as tenellin and bassianin (Strasser et al. 2000). A green pigmentation was reported to be a common characteristic of *Bv. bassiana,* at the mycelium base when the strain was cultured on Sabourauds Maltose Agar (Vey et al. 2001). A red pigment was also obtained from several mutant strains of *Bv. bassiana* (Eyal et al. 1994; Klingen et al. 2002; Quesada-Moraga and Vey 2004). Later on, this red pigment was identified as oosporein (Seger et al. 2005). Oosporein displays antibiotic activity towards Gram-positive, but not Gram-negative, bacteria, and appears to cause avian gout in chickens and turkeys (Pegram et al. 1982).

The present investigation was devoted towards assessment of a possible insecticidal activity of the red pigment produced by a locally isolated strain of *Bv. bassiana* (Narmen 1998). The whitefly, *Bemisia tabaci*, as one of the

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most agronomically important pest insects, was selected as the target organism.

Materials and methods

Microorganisms and media

The fungus *Bv. bassiana* was isolated from infested leaves of tomato with *Bm. tabaci* collected from various location at Fayoum Governorate, Egypt (Narmen 1998). It was maintained on Potato Dextrose Agar (PDA). The medium used for production of pigment contained glucose 40 g, yeast extract 5.0, $NaNO_3$ 1.0, KH_2PO_4 2.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5 and $FeSO_4 \cdot 7H_2O$ 0.02 g in 1000 ml of distilled water.

A stock suspension of fungal spores was prepared by harvesting fungus grown on PDA medium for 7 days in a Petri-dish with sterile distilled water. The suspension was cleared from mycelium debris by filtration using a sterile cheese cloth. As a wetting agent, Tween 40 was added to spore suspension at 0.05% (v/v). Spore concentration (spores/ml) was determined using a Neubauer haemocytometer according to the method of Hall (1985).

Time course for cell growth and pigment production in shake flasks

A set of 12 Erlenmeyer flasks (500 ml each) was charged with production medium (80 ml each) and inoculated with 4-day-old culture of the fungus at 10% (v/v). The flasks were incubated in a rotary shaker at 27°C, initial pH 6.0 and agitation speed 300 rev/min. At regular intervals, one flask was taken as sample and its content was analysed for fungal biomass, residual glucose and red pigment concentrations.

Fermentation and pigment production in bioreactor

A double-walled glass bioreactor with a working volume of 800 ml was charged with the production medium, except glucose, and autoclaved at 120°C for 20 min. Glucose was sterilized separately and added aseptically to the bioreactor. The inlet and outlet of the double wall section were connected to a water bath (Shelden, model W20M-2E, U.S.A.) equipped with a temperature control unit (Techne, model Te-10D tempette, U.K.). Then the bioreactor was inoculated with 10% (v/v) of a 4-day old culture of *Bv. bassiana* and fermentation started. The pH was adjusted at 6.0, temperature at 27°C and aeration rate at 1 volume of air/volume of culture/min. Samples from the fermentation broth was taken and analysed for glucose and red pigment. Fungal biomass was determined at the end of cultivation period.

Extraction of the red pigment

The method described by Eyal et al. (1994) was used after adaptation. At the end of logarithmic phase, aeration and/or agitation was stopped and cultivation continued for additional 2 days in order to maximize pigment excretion. Then, fermentation broth (800 ml) was centrifuged at 4383*g* for 20 min and filtered through a filter paper Whatman No.2 in order to remove mycelium and cell debris. Acetic acid was added to the spent broth to bring the pH down to 2.0. Then, the red pigment was extracted from the broth using ethyl acetate at a ratio of 1:2 (v/v). The resulting red extract was evaporated using a rotary vacuum evaporator to dryness. The dry pigment was used in toxicity tests and throughout execution of calibration curve between pigment concentration and absorbance measurements. A spectrophotometer, model Genesys 10vis, U.S.A. was used.

Pathogenicity assessment

Stock culture of Bm. tabaci

Stock culture of *Bm. tabaci* was established under the laboratory conditions (13–36°C and 55–85% RH). Tomato leaves, highly infested with nymphs of *Bm. tabaci* were collected from the Faculty of Agriculture Research Farm, and transferred to the laboratory, kept in woody cages with fine wire screen sides and provided with pieces of cotton soaked with water in half Petri dishes. The newly emerged adults of the whitefly were collected and introduced in other cages (oviposition cages) which contained castor bean seedlings grown in plastic pots of 15 cm diameter and 16 cm height.

Toxicity

Newly emerged adults of *Bm. tabaci* were collected from the stock culture and introduced, for 24 h, onto castor seedlings in pots covered with chimney glasses, which were used as oviposition cages, with a rate of 20 pairs/cage. The upper openings of the cages were covered with muslin held in position by rubber bands. Newly deposited egg masses were inspected daily until the second nymphal instar was reached and they were then used for the pathological experiments under room conditions.

Three different preparations, each supplied with 0.05% Tween 40, were tested. They were: a 4.6×10^6 spores/ml spore suspension alone, the same spore concentration plus 100 mg red pigment/l and the pigment solution alone. Distilled water containing 0.05% Tween 40 was used as control. For each treatment, five pots (each containing a castor seedling with 5 leaves infested with certain numbers

of the second nymphal instar of *Bm. tabaci*) were chosen and sprayed completely with the respective preparation. Then, they were left to dry at room temperature for 10 days. At 2-day regular intervals, leaves were examined under binocular stereo-microscope where dead and living nymphs were counted in order to calculate percentages of mortality which were corrected by Abbot's formula (Amin et al. 2008a).

Latent effect

For each treatment including control, the dead nymphs were excluded, while the living ones were followed until the appearance of the next generation. Periods of nymphal stage and adult and female reproductive potential were recorded. Significance of differences between data obtained on toxicity and latent effects were determined using L.S.D. at level of 0.05% (Snedector 1956).

Analytical methods

Dry fungal biomass was determined after centrifugation of fermentation broth at 4383*g* for 20 min. The biomass obtained was dried in an oven at 80°C until constant weight. Residual glucose in supernatants was analysed using the enzymatic method employing glucose oxidase (Amin et al. 2008b). The red pigment was determined using absorbance measurements at 530 nm which were converted into concentration (ppm) using a calibration curve.

Results and discussion

Time course for cell growth and pigment production by *Bv. bassiana*

As shown in Fig. 1, synthesis of red pigment by *Bv. bassiana* seemed to be a non-growth associated process. The fungus grew rapidly and produced more than 90% of final biomass concentration in 3 days with almost no pigment synthesis. On day 4, red pigment was detected in fermentation broth and its concentration increased as fermentation progressed to reach its maximum value of 480 mg/l on day 7. The final biomass concentration was 5.4 g l^{-1}. Similarly, several secondary metabolites were reported as non-growth associated products of microorganisms. Among them, the crystal protein toxin produced by the bacterium *Bacillus thuringiensis* (Amin et al. 2008a), and the non-toxic red pigment produced by the fungus *Monascus* spp. (Juslová et al. 1996; Hamano et al. 2005). The red pigment is produced extracellularly and is totally soluble in water (Fig. 2). This represents a great advantage when considered for large scale production because both downstream processes for its recovery from fermentation broth and field application are much cheaper and easier compared to similar metabolites produced intracellularly. The red pigment from *Bv. bassiana* was identified and documented by several investigators as oosporein (El-Basyouni and Vining 1966; Eyal et al. 1994; Klingen et al. 2002).

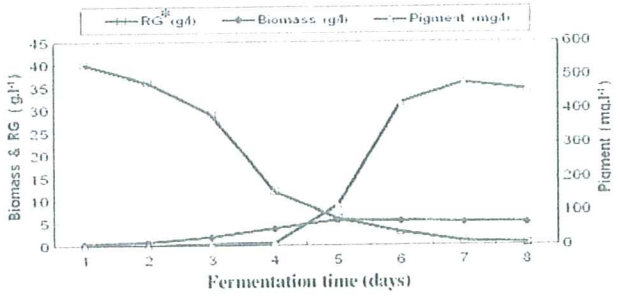

Fig. 1 Time course of cell growth and pigment production by *Beauveria bassiana*. *, RG: Residual Glucose

Toxicity of spore suspension and red pigment of *Bv. bassiana* on *Bm. tabaci*

In a cumulative study, nymphs of *Bm. tabaci* (in the second instar) sprayed with *Bv. bassiana* red pigment, fungal spore suspension and a combination of both as described in Sect. "Materials and Methods". Control was also included using distilled water supplied with 0.05% Tween 40. Accumulative mortality percentages were recorded over 10 days after treatment (Table 1). Obviously, the red pigment treatment at 100 mg/l exhibited a significant insecticidal activity on nymphs of *Bm. tabaci* compared to control. With treatment receiving fungal spores, the mortality percentage recorded 60.3%. When red pigment was combined with fungal spores, a tremendous increase in toxicity was observed. The mortality percentages increased to up to 92.2% with the highest calculated value of L.C.D. This might be explained by a possible synergistic action. An antibacterial activity was reported for red pigment produced by another strain of *Bv. bassiana* (Pegram et al. 1982; Eyal et al. 1994) Therefore, it could be postulated that upon introduction into the host insect, the red pigment may act as an antagonist to the intestinal bacteria, allowing the fungal spores to germinate and proliferate leading to much higher and faster lethal effect on pest insects.

Pathogenicity assessment

Performances of the surviving second instar nymphs after treatment

As could be seen from Table 1, some treated nymphs could tolerate infection and survived after 10 days of treatments.

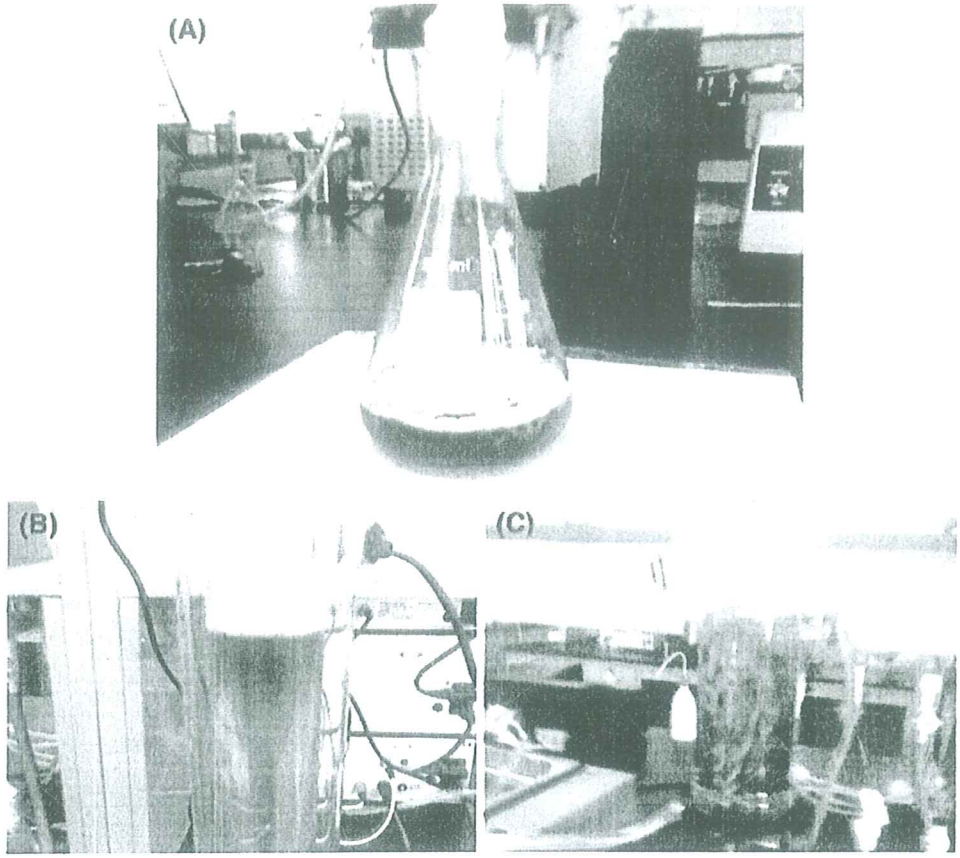

Fig. 2 Red pigment synthesis by *B. bassiana* in Erlenmeyer shaken flasks (**a**) and vertical column bioreactor, at the top section (**b**) and after mixing of bioreactor content (**c**)

Table 1 Accumulative mortality percentages of *B. tabaci* nymphs (second instar) recorded after treatment with spore suspension and red pigment of *B. bassiana*

Treatments	Accumulative mortality (%)					L.S.D.
	Days after treatment					
	2	4	6	8	10	
300 mg red pigment/l	3.6 d	10.2 c	13.5 c	17.8 c	18.3 d	3.19
4.6×10^6 spore/ml	10.1 bc	27.6 b	42.5 b	46.2 b	60.3 c	7.34
300 mg red pigment/l + 4.6×10^6 spore/ml	20.6 a	54.4 a	78.0 a	85.5 a	92.2 a	7.56
L.S.D.	5.45	12.5	8.76	9.15	9.39	–

Vertical data with the same letter differed insignificantly

Therefore, it was attempted to follow up their performances over the successive life stages until adulthood. Table 2 shows the results obtained. Generally, in all treatments including the control, recorded periods of nymphs and pupae stages coincided with the respective mortality percentages. Compared to the control, both durations and mortality over the two stages and their total increased significantly, except those treated with red pigment alone which differed insignificantly. This was more pronounced with the nymphal stage. However, in the treatment that received both red pigment and spore suspension, the extra lethal effect of red pigment was noticeable. Total duration and mortality recorded an increase of >50 and 26%, respectively compared to those obtained in the absence of red pigment. This represents a tremendous surplus in insecticidal activity against pest insect that could not be ignored, particularly when this process is to be considered for large scale application.

Performance of *Bm. tabaci* adults emerging from nymphs surviving after treatment was also followed. As

Table 2 Duration (days) of *Bm. tabaci* nymphs and pupae survived after 10 days of treatment with spore suspension and red pigment of *Bv. bassiana*

Treatments	Duration (days)		
	Nymphs	Pupae	Total
300 ppm red pigment	8.3 ± 0.02	6.48 ± 0.03	d
	(8–9)	(6–7)	14.78 ± 0.04
	5.8*	5.19*	(14–16)
			10.7*
			c
4.6×10^6 spore/ml	10.98 ± 0.08	6.43 ± 0.04	17.29 ± 0.1
	(9–13)	(6–7)	(15–20)
	15.7*	21.19*	33.57*
			a
300 ppm red pigment + 4.6×10^6 spore/ml	15.16 ± 0.17	6.83 ± 0.14	21.93 ± 0.31
	(14–17)	(6–8)	(20–25)
	27.9*	29.5*	49.1*
			d
Control	8.43 ± 0.02	6.08 ± 0.03	14.5 ± 0.04
	(8–9)	(5–7)	(13–16)
	2.49*	3.54*	5.94*
L.S.D.	–	–	1.41

Data in brackets represent ranges

Asterisked data represent mortality percentages

Data having the same letter differed insignificantly

shown in Table 3, periods of pre- and post-oviposition were prolonged and those of oviposition and adult longevity were reduced. Again, the most detrimental effect was found with treatment in which red pigment was combined with fungal spores. Differences between data recorded for the other two treatments were insignificant.

With respect to adult's potential for reproduction, all three treatments exhibited reduction in counts of eggs

Table 3 Behavior of *Bm. tabaci* adults treated during the second nymphal instar with fungal spores and red pigment of *Bv. bassiana* over the remaining stages of their life cycle after treatment

Stages of insect life cycle	Duration (days) at the different treatments				
	300 ppm red pigment	4.6×10^6 spore/ml	4.6×10^6 spore/ml + 300 ppm RP	Control	L.S.D.
Pre-oviposition	d	c	a	d	0.38
	1.8 ± 0.2	2.6 ± 0.4	4.2 ± 0.2	1.6 ± 0.1	
	(1–2)	(2–4)	(4–5)	(1–2)	
Oviposition	ab	abc	c	a	7.72
	21.0 ± 3.8	16.6 ± 2.4	9.0 ± 1.7	23.2 ± 4.42	
	(10–34)	(9–24)	(5–15)	(9–36)	
Post-oviposition	c	b	a	c	1.04
	2.0 ± 0.32	2.4 ± 0.2	3.6 ± 0.51	1.8 ± 37	
	(1–3)	(2–3)	(2–5)	(1–3)	
Longevity	24.8 ± 3.7	21.6 ± 2.6	16.8 ± 1.4	27.0 ± 4.4	10.7
	(13–38)	(13–29)	(13–21)	(13–40)	
Mean no, eggs/5adults	ab	cd	d	a	318.65
	729.0	387	135	831.8	
% Egg reduction	12.36	53.47	83.77	–	–

deposited/5 adults. This reduction was significant with treatments containing fungal spores, whether alone or in combination with red pigment, with the latter having the highest percentages of egg reduction compared to the control. Again, treatment with red pigment alone recorded a reduction in deposited eggs, but differences with the data of the control were insignificant.

Comparing the results obtained to those given by Eyal et al. (1994) working with red pigment or spore suspension of *Bv. bassiana* against whiteflies mealy bugs, Narmen (1998) working with *Bv. bassiana* spore suspension on *Bm. tabaci* and Leckie et al. (2008) working with spent fermentation broth or mycelia of *Bv. bassiana* against *Helicoverpa zea*, it is clear that the approach utilizing a combination of red pigment and fungal spores offers excellent perspectives for biological control of the pest insect.

Conclusion

The proposed approach for biocontrol of whitefly, *Bm. tabaci* with a combination of red pigment and spores of the fungus *Bv. bassiana* has many advantages. In addition to its insecticidal activity, the solubility of the red pigment in water guarantees cheaper and much easier downstream and field application processes. Upon penetrating the host insect and being with fungal spores at the right time and place, red pigment with its antibacterial activity could suppress local bacterial flora and allows a rapid spore germination and proliferation. Moreover, such antibacterial activity provides protection to fungal spore formulations during storage and in soil upon application as well.

However, pigment yield obtained throughout this study was relatively low. Work is under way in order to optimize fermentation parameters and hopefully increase pigment yield to the level that could satisfy a successful competition with currently employed field application techniques.

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