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ORIGINAL ARTICLE

Formulation and application of the entomopathogenic fungus: *Zoophthora radicans* (Brefeld) Batko (Zygomycetes: Entomophthorales)

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Abstract

Aims: To isolate and formulate a native strain of *Zoophthora radicans* naturally infecting larvae of diamondback moth, *Plutella xylostella*, existing in South Australia and to provide evidence that formulation of the fungus is effective against *P. xylostella* larvae, and therefore, it could be used as a tool in pest management of this insect.

Methods and Results: Dose–response bioassays using formulated and unformulated forms of the fungus strain were carried out against third instar larvae of *P. xylostella*. Results obtained have indicated a significant increase in the larval mortality when higher concentrations of a formulated form of the fungus strain were applied compared to the treatments with the unformulated form (85·0 *vs* 57·5% of larval mortality, respectively, at the top concentration of 10^7 conidia/ml). The median lethal concentration (LC50) for a formulated form was 100 times less than that of the unformulated form when they were applied against the third instar larvae of *P. xylostella*. In addition, the formulation used in the present bioassays has preserved the viability of introduced fungus conidia for longer time in comparison with the unformulated conidia.

Conclusions: The effective application of a formulated fungus strain against *P. xylostella* larvae constitutes the first step towards its use in pest management of this insect.

Significance and Impact of the Study: The formulated fungus in inverted emulsion could be used as an alternative tool to insecticides in pest management of *P. xylostella* larvae because of the development of resistance to insecticides in the treated larvae.

Introduction

Diamondback moth (DBM), *Plutella xylostella*, is an important cosmopolitan insect pest of crucifers, such as, cabbage, cauliflower, radish, turnip, beet root, mustard and rape seed (Talekar and Shelton 1993; Sayyed *et al.* 2005). This insect pest can cause yield reduction in attacked crops varying from 30 to 100% (Lingappa *et al.* 2004). The larvae are considered the damaging form of this insect as they feed on the leaves of attacked plants creating large holes in the attacked leaves (Talekar and Shelton 1993; Lingappa *et al.* 2004).

Control of DBM is usually practiced by applying various synthetic chemical insecticides such as Emamectin benzoate (Proclaim®; Syngenta, Basel, Switzerland), Spinosad (Success®; Dow AgroSciences, Indianapolis, IN) and Indoxacarb (Avatar®; DuPont, Wilmington, DE), in addition to the large use of Bt-toxin (Dipel®; Valent Agricultural Products, Walnut Creek, CA) (Thompson *et al.* 2000; McCann *et al.* 2001; Zhao *et al.* 2002, 2006;

Liu *et al.* 2003; Syngenta 2004). However, frequent applications of increasingly larger doses of these insecticides against *P. xylostella* cause the development of resistance to these insecticides by the insect (Tabashnik *et al.* 1987, 2000; Zhao *et al.* 2002, 2006).

Alternative approaches to the conventional use of synthetic insecticides and Bt-toxins for the control of DBM are highly appreciated because of increasing importance of integrated management strategies relying on biocontrol possibly combined with selective insecticide application. Entomopathogenic fungi (EPF) are considered the most promising alternative approach because they are currently being developed as biocontrol agents of many insect species (Ibrahim and Low 1993; Padin *et al.* 1996, 1997, 2002; Selman *et al.* 1997; Hidalgo *et al.* 1998; Moino *et al.* 1998; Rice and Cogburn 1999; Odour *et al.* 2000; Toshio 2000; Dal-Bello *et al.* 2001; Sheeba *et al.* 2001). Recently, formulated forms of EPF have been used against various insect species, and inverted emulsion formulations (water-in-oil type) are used as a promising formulation for these entomopathogens when applied as biocontrol agents of insects (Batta 2003a,b, 2004, 2005, 2007, 2008; Batta and Abu-Safieh 2005; Michalaki *et al.* 2006). Some authors have reported the infection of DBM larvae with *Zoophthora radicans* in Australia (Furlong and Pell 2000, 2001; Vickers *et al.* 2004; CSIRO-Entomology 2008). This pathogen is found naturally infecting the DBM larvae in cabbage and cauliflower plantations, but its capacity to control the insect under field conditions is variable as the larval mortality attributed to fungus infection does not exceed 50% under the most favourable conditions (Pell and Wilding 1992, 1993, 1994; Pell *et al.* 1993; Furlong *et al.* 1995; Furlong and Pell 1996, 1997; Sarfraz *et al.* 2005). However, no investigations have been made to use this fungus as biocontrol agent against DBM larvae in any formulated form including inverted emulsion formulation. According to our experience regarding the formulation of other EPF like *Beauveria bassiana* and *Metarhizium anisopliae* (Batta 2003a,b, 2004, 2005, 2007, 2008; Batta and Abu-Safieh 2005), we think that the formulation of the present strain of *Z. radicans* in inverted emulsion has the potential to enhance its capacity for control of DBM larvae. Therefore, the objectives of the present research were the following: (i) to isolate a native strain of *Z. radicans* naturally infecting DBM larvae on cabbage fields in South Australia; (ii) to formulate the fungus strain using inverted emulsion as this formulation enhanced the efficacy of other EPF; (iii) to test the efficacy of treatment with the formulated fungus strain against DBM larvae; and (iv) to check the conidial viability of the fungus strain after introduction into the inverted emulsion used in the formulation.

Materials and methods

Strain of *Zoophthora radicans* used in the study

A native strain (designate as ZRYAC) of *Z. radicans* was used in this study. It was isolated and then characterized by our team. Isolation was performed from naturally infected DBM larvae infesting cabbage plantations in Wanilla Ayer Peninsula in South Australia in October 2008. Sabouraud dextrose agar (SDA) culture medium (Difco) amended with an antibiotic (chloramphenicole 250 g/l) was used in the isolation. Subculturing of the strain was performed on SDA medium plus 0·25% (w/v) yeast extract (Pell and Wilding 1993). The isolated strain is characterized by the production of a relatively long dense yellowish mycelium when subcultured on plates of SDA medium + 0·25% (w/v) yeast extract. It also produces small microscopic oval to elongate spores typical to *Z. radicans* called primary conidia. These conidia are different from those of Deuteromycotinous fungi, which usually produce true conidia when subcultured on a culture medium. Under certain environmental conditions especially at high humidity, the primary conidia of the fungus produced in the culture plates give smaller size secondary conidia with elongate shape called capilliconidia. Production of these conidia (capilliconidia) from primary conidia is typical to entomophthorales especially for *Z. radicans* (Furlong and Pell 1997; Griggs *et al.* 1999). Zygospores are also typical type of resting spores that can be produced by this fungus strain but their production requires certain environmental conditions that are not available in the culture plates. Among the above-mentioned types of spores produced by the fungus strain, the secondary conidia (infective type of conidia) were used for bioassays of this study. During the preliminary experiments, we have noticed that the fungus strain killed the infected DBM larvae within 4 days of treatment. Therefore, the assessment of treatment effects in bioassays of this study was performed within 96 h of treatment. When the treated larvae become infected by the fungus strain, they turn soft and become flattened and their colour changes first from emerald green to buff yellow then finally to black colour when larvae were completely killed by the fungus. Concentration of the secondary conidia (capilliconidia) used in the formulation of the fungus was standardized at 10^7 conidia/ml. Serial successive dilutions of this concentration was involved in bioassays of the study.

Formulation of ZRYAC strain of *Zoophthora radicans*

Formulation of the isolated fungus strain (*Z. radicans* ZRYAC) was carried out in inverted emulsion (water-in-oil formulation) because this formulation has been

shown to be the most appropriate for other EPF like *B. bassiana* and *M. anisopliae*. For the preparation of this formulation, the ingredients of each one of the two phases constituting the formulation were first mixed separately and then the two phases were combined in a 50 : 50% ratio by adding the aqueous phase onto the oil phase to obtain water-in-oil formulation. The mixture of the two phases was then homogenized mechanically using a high-speed homogenizer (model: Gastro 350, Bamix®, Basel, Switzerland). The speed and duration of homogenization were adjusted at 21 000 rev min^{-1} for 1·5 min. The resulted inverted emulsion should be stable (no separation of the two phases comprising the emulsion for at least 24 h after preparation), so it becomes appropriate for the introduction of the fungus conidia. The aqueous phase of the formulation was consisted of a mixture of sterile de-ionized water (45·25% w/w), glycerine (4·00% w/w) and water-soluble emulsifier (Dehymuls® LS: 0·75% w/w; Carechemicals, Cognis, http://www.cognis.com, Germany). Before adding to the formulation, the water-soluble emulsifier 'Dehymuls® LS' was first melted in sterile de-ionized water at 75°C using water bath for 10 min. Glycerine was added to this mixture because it acts as a depressor of water molecule activity through reduction in water evaporation during application. The oil phase of the formulation consists of a mixture of two oils of plant origin containing a high content of monounsaturated fat (Rape seed oil known commercially as Canola oil: 24·00% w/w and Camelia oil known commercially as Tea oil: 24·00% w/w) and oil-soluble emulsifier: Tween 20 (2·00% w/w).

Introduction of conidia of ZRYAC strain of *Zoophthora radicans* into the formulation

The strain ZRYAC of *Z. radicans* was first subcultured on SDA medium plus 0·25% (w/v) yeast extract and then incubated at 25 ± 1·0°C for 4 days before the primary conidia of the fungus were produced. The secondary conidia (capilliconidia) of the fungus were then produced from the primary conidia within 2 days following the appearance of the primary conidia. The capilliconidia (infective form of the fungus conidia) were harvested and then used for fungus formulation. Conidial harvesting was carried out by scraping the conidia from the surface of culture medium using sterile scalpel blades and then suspended in sterile de-ionized water. Suspended conidia were then separated from fragments of fungus mycelium by sieving through a 75-μm mesh. The conidial suspension was then standardized at $4{\cdot}0 \times 10^7$ conidia/ml to get a final conidial concentration of $1{\cdot}0 \times 10^7$ conidia/ml in the prepared formulation. Introduction of standardized conidial suspension into emulsion was performed during preparation of the ingredients by mixing them first with sterile de-ionized water which comprised 45·25% w/w of total volume of the emulsion. The part of sterile de-ionized water was also used to melt the water-soluble emulsifier Dehymuls® LS (0·75% w/w of the total volume of the emulsion) as described in the previous section on the formulation of the strain. Mixing and homogenization of this phase with the oil phase of the emulsion was carried out as described in the previous section on formulation of the strain. The concentration of the fungus conidia in the final emulsion was fixed at $1{\cdot}0 \times 10^7$ conidia/ml. This is because the introduced conidial suspension in the aqueous phase was standardized at $4{\cdot}0 \times 10^7$ conidia/ml as determined by a haemocytometer. The prepared emulsion was held in screw-caped glass bottles (500-ml capacity) and stored at 25 ± 1·0°C for the whole duration of bioassays.

Effect of formulation on conidial viability of ZRYAC strain of *Zoophthora radicans*

Two techniques were used to assess the conidial viability of *Z. radicans* strain incorporated into emulsion over time by taking two samples of the emulsion (100 μl each) at weekly intervals. One sample was spread on the surface of plates containing SDA medium plus 0·25% (w/v) yeast extract and then incubated at 25 ± 1·0°C for 6 days, at which time plates were examined for the appearance of typical *Z. radicans* colonies. This indicates that the conidia have remained viable in the emulsion. The second sample was spread in a thin layer on the surface of glass slides then transferred to a Petri dish with moistened filter paper at the bottom to secure humid conditions needed for germination. Petri dishes were then incubated at 25 ± 1·0°C. Conidial germination was assessed 24 h later under the microscope, and average per cent germination was calculated for each sampling time. Ten replicates were set up for each method of assessment at each sampling time. To determine whether the formulation prompted conidial viability or was detrimental to it, viability of the unformulated fungal conidia was also assessed over the same experimental period as in formulated conidia by suspending them in sterile de-ionized water and storing them under the same conditions as in the formulated conidia. The same techniques described previously for formulated conidia were used during the assessment of unformulated conidial viability.

Population of DBM used in bioassays

A susceptible population of the DBM known as 'Waite susceptible' has been used in bioassays. This population has been left for reproduction for many generations and for long time (>10 years) without being exposed to any

type of insecticides and/or natural enemies including pathogens. During this period, it has been maintained on cabbage seedlings (*Brassica oleracae* var. capitata cultivar Green Coronet) grown under the insectary conditions (25 ± 1·0°C, 14/10 h, L/D photoperiod) in separately caged cultures at South Australian Research and Development Institute (SARDI)-Entomology Unit, Waite Campus, South Australia, Australia. The cabbage seedlings were produced in the laboratory by planting the seeds in 500 ml Plaspak® (St Arndell Park, NSW, Australia) plastic pots with compost and vermiculite (3 : 1 ratio). A thin layer of honey on masking tape and 10% honey solution with 0·1% sorbic acid were provided as food source for DBM adults during rearing. Third stage larvae produced during rearing of the susceptible population were collected then used for bioassays.

Bioassays using formulated and unformulated conidia of *Zoophthora radicans* (ZRYAC strain)

Cabbage leaf discs of 90 mm diameter were cut from washed cabbage leaves taken from healthy 8-week-old plants grown under the glass house conditions at SARDI-Entomology Unit, Waite Campus, South Australia, Australia. The leaf discs were then embedded into agar that has been poured into 90 mm diameter Petri dishes with the underside of leaf discs facing upwards. Before formal bioassays, preliminary assays were conducted using a broad range of concentrations of the fungus preparation containing known conidial concentration to determine the proper concentrations for the formal assays. Each formal bioassay included eight concentrations of the fungus preparation containing the fungus conidia (capilliconidia) starting from 10^7 to 0 conidia/ml (0 concentration was sterile de-ionized water used as a control). Therefore, successive dilutions of ten times each were performed using the highest concentration (10^7 conidia/ml) until reaching the lowest one (0 conidia/ml). Four leaf discs representing four replicates were used for each concentration. The successive dilutions were made up in sterile de-ionized water to obtain the specific concentrations using volumetric flasks. A precise deposit of each concentration (4·0 ml) was administered using a Potter precision laboratory spray tower (Burkard manufacturing Co Ltd, Rickmansworth Herts, UK). Eight to ten-third instar larvae of DBM were placed on each leaf disc in a Petri dish, and then each Petri dish was sprayed with 4·0 ml of each concentration using the Potter spray tower. Once removed from the tower, the dishes were covered with a plastic film that was secured with a rubber band, and then about 200–250 tiny holes were then punched into the plastic film using a very fine needle to allow the exchange of air. The tower was calibrated before and after each trial allotment, and rinsed three times with ethyl alcohol (70%) and sterile de-ionized water between each change in treatment. The treated Petri dishes were then kept in an incubator at 25 ± 0·5°C (14/10 h, L/D photoperiod). The treatment effects at the different concentrations were assessed 96 h after the treatment. Assessment of the treatment effect was performed by counting dead and living larvae in each Petri dish and then calculating the per cent mortality of treated larvae. The mean per cent mortality was calculated for each concentration. Each type of the aforementioned bioassays was repeated three times for the confirmation of the results and for obtaining more reliable data set.

Statistical analysis

The data obtained on percentage of larval mortality were statistically analysed using one- or two-way ANOVA to compare mortality caused by the application of concentrations of serial successive dilutions within a treatment and between treatments. Tukey's HSD was used to separate means when significant treatment effects were detected (JMP version 8 was used). Probit analysis was used to determine the median lethal concentration (LC50) of a formulated and an unformulated fungus strain for the comparison of these treatment effects.

Results

Treatment effect of DBM larvae with a formulated versus unformulated conidia of *Zoophthora radicans*

Results obtained from bioassays compared the larval mortality caused by the application of different concentrations of serial successive dilutions within a treatment and between the treatments.

Comparison of larval mortality within a treatment

Results indicate that significant differences (at $P = 0{\cdot}05$) were obtained between the means of per cent mortality of treated larvae with a formulated conidia of fungus strain (ZRYAC) for the first two highest concentrations of the formulation (10^7 and 10^6 conidia/ml) and the other concentrations used in bioassays (Table 1). Similar significant differences were obtained for the medium concentrations of the formulated fungus conidia used in the treatment: 10^5 and 10^4 conidia/ml (Table 1). Overall, the treatment effect with a formulated fungus was the most effective at higher concentrations that exceeded 10^4 conidia/ml. Significant differences (at $P = 0{\cdot}05$) were also obtained between the means of per cent mortality of treated larvae with an unformulated conidia of fungus strain for the first two highest concentrations of the fungus conidial

Table 1 Mean (±SE) mortality (%)† of diamondback moth third larval instar treated with unformulated (aqueous suspension) and formulated (inverted emulsion) conidia of *Zoophthora radicans* (strain ZRYAC) at different concentrations (four replicates per treatment, n = 8–10 larvae per replicate)

	Comparison of larval response to serial dilution concentrations within a treatment*			Comparison of larval response to serial dilution concentrations between the treatments*		
Concentration (conidia/ml)	Unformulated fungus conidia	Formulated fungus conidia in invert emulsion	Blank formulation and its successive dilutions	Unformulated fungus conidia	Formulated fungus conidia in invert emulsion	Blank formulation and its successive dilutions
10^7	57·5 ± 4·8 e	85 ± 2·9 e	35 ± 2·9 d (undiluted)	57·5 ± 4·8 b	85 ± 2·9 c	35 ± 2·9 a (undiluted)
10^6	45 ± 2·9 d	77·5 ± 2·5 e	22·5 ± 2·5 c (10^{-1} dilution)	45 ± 2·9 b	77·5 ± 2·5 c	22·5 ± 2·5 a (10^{-1} dilution)
10^5	37·5 ± 4·9 cd	57·5 ± 2·5 d	12·5 ± 2·4 b (10^{-2} dilution)	37·5 ± 4·9 b	57·5 ± 2·5 c	12·5 ± 2·4 a (10^{-2} dilution)
10^4	27·5 ± 4·8 c	32·5 ± 2·4 c	5 ± 2·8 ab (10^{-3} dilution)	27·5 ± 4·8 b	32·5 ± 2·4 b	5 ± 2·8 a (10^{-3} dilution)
10^3	22·5 ± 2·5 bc	25 ± 2·8 bc	2·5 ± 2·5 a (10^{-4} dilution)	22·5 ± 2·5ab	25 ± 2·8 b	2·5 ± 2·5 a (10^{-4} dilution)
10^2	15 ± 2·9 b	17·5 ± 2·3 b	0 ± 0 a (10^{-5} dilution)	15 ± 2·9 ab	17·5 ± 2·3 b	0 ± 0 a (10^{-5} dilution)
10	5 ± 2·8 ab	7·5 ± 2·2 a	0 ± 0 a (10^{-6} dilution)	5 ± 2·8 a	7·5 ± 2·2 a	0 ± 0 a (10^{-6} dilution)
0 (control)	2·5 ± 2·5 a	0 ± 0 a	0 ± 0 a (10^{-7} dilution)	2·5 ± 2·5 a	0 ± 0 a	0 ± 0 a (10^{-7} dilution)

*Means of larval mortality caused by concentrations of serial dilutions within a treatment and those caused by concentrations of serial dilutions between the treatments followed by the same letter are not significantly different at P = 0·05.
†Statistical analysis using ANOVA and Tukey's HSD test for means separation was performed for the data.

suspension: 10^7 and 10^6 conidia/ml (Table 1) but no significant differences were obtained between the means of per cent mortality of treated larvae with the fungus strain for the concentrations of the fungus: 10^5, 10^4, 10^3 and 10^2 conidia/ml (Table 1). Overall, the treatment with unformulated fungus conidia was the most effective at higher concentrations that exceeded 10^5 conidia/ml.

Comparison of larval mortality between the treatments

Results indicate that significant differences (at P = 0·05) were obtained between the means of per cent mortality of treated larvae with a formulated and an unformulated conidia of fungus strain (ZRYAC) for the first three highest concentrations of these treatments (10^7, 10^6 and 10^5 conidia/ml) (Table 1). However, no significant differences were obtained for the other concentrations of a formulated and unformulated fungus conidia used in these treatments: 10^4, 10^3, 10^2, 10 and 0 conidia/ml (Table 1). Overall, the treatment effect with a formulated and an unformulated fungus was most effective at higher concentrations that exceeded 10^4 conidia/ml. These values of conidial concentration are similar to those that have been obtained from comparisons within a treatment for the formulated and unformulated forms of the fungus.

The LC50 of this formulated strain was 34 928 conidia/ml ($3{\cdot}5 \times 10^4$ conidia/ml) compared to a higher LC50 of the unformulated strain 4778 694 conidia/ml ($4{\cdot}78 \times 10^6$ conidia/ml), which means that this LC50 was 100 times less than that of the unformulated form of the fungus strain (Table 2). Moreover, the LC90 of the formulated strain was 38 704 932 conidia/ml ($3{\cdot}9 \times 10^7$ conidia/ml) compared to an extremely high LC90 for the unformulated fungus form (Table 2).

Results obtained on the treatment effect with the blank formulation of inverted emulsion as a control treatment of the formulated form of the fungus strain indicate that means of per cent mortality of treated larvae decreased significantly from 35 to 5% when the blank formulation was diluted into three successive dilutions of ten times each (Table 1). Further successive dilutions of ten times each did not significantly decrease the mean of per cent larval mortality (Table 1). It is important to note that significant differences (at P = 0·05) were obtained between the formulated form of the fungus and the blank formulation at all concentrations used except for the lowest one (10 conidia/ml) (Table 1).

Viability of formulated conidia of *Zoophthora radicans* in inverted emulsion

Conidial viability of the fungus strain ZRYAC formulated in inverted emulsion has not significantly reduced during a four-week period of storage at 25 ± 1°C following preparation of emulsion and subsequent introduction of the conidia. The total values of reduction in conidial viability did not exceed 3% (reduced from 81·8 to 79·0%) (Table 3). In comparison with the conidial viability of the same strain in aqueous suspension stored at the same conditions (25 ± 1°C), the fungus has lost its viability because of the germination of its conidia within one week of their suspension in water and storage. The loss may be also attributed to subsequent formation of dense mycelium within 1 week of conidial germination so that they could not be stored for long time compared with the unformulated conidia. Moreover, the direct effect of preparation technique of the emulsion especially using

Table 2 Probit analysis of the treatment effect of diamondback moth third instar larvae with a formulated and an unformulated conidia of *Zoophthora radicans* (strain ZRYAC) (four replicates per treatment, n = 8–10 larvae per replicate)

Results of Probit analysis	Treatment with a formulated fungus conidia	Treatment with an unformulated fungus conidia
LC50 (conidia/ml)	34 928·302 ($3{\cdot}5 \times 10^4$)	47 78 694·888 ($4{\cdot}78 \times 10^6$)
95% CL (conidia/ml) for the LC50	14 035·473 – 93 736·177 ($1{\cdot}40 \times 10^4$ – $9{\cdot}37 \times 10^4$)	541 735·419 – 314 954 493·741 ($5{\cdot}42 \times 10^5$ – $3{\cdot}15 \times 10^8$)
LC90 (conidia/ml)	38 704 932·691 ($3{\cdot}9 \times 10^7$)	0·000 (extremely high)
95% CL (conidia/ml) for the LC90	7614 140·476 – 432 258 684·054 ($7{\cdot}61 \times 10^6$ to $4{\cdot}32 \times 10^8$	–

Table 3 Mean (±SE) conidial germination (%) of a formulated and an unformulated strain (ZRYAC) of *Zoophthora radicans* stored at 25 ± 1°C. Ten replicates representing ten samples taken from unformulated and formulated conidia per sampling time

Sampling time (in weeks) after formulation of the fungus	Conidial germination (%) of a formulated fungus	Re-isolation of a formulated fungus	Conidial germination (%) of an unformulated fungus	Re-isolation of an unformulated fungus
0‡	81·8 ± 4·2	+†	85·2 ± 5·1	+
1	80·1 ± 3·8	+	*	–†
2	79·8 ± 4·6	+	*	–
3	79·0 ± 4·9	+	*	–
4	79·0 ± 3·7	+	*	–

*Conidial germination % is difficult to be assigned because of a previous germination and/or subsequent formation of a dense mycelium.
†The sign + (positive test) or – (negative test) indicates whether the fungus was recovered from the one hundred microliters of the formulation or conidial suspension spread on the plate's surface of Sabouraud agar culture medium (SDA) + 0·25% (w/v) yeast extract. Negative test (– sign) for re-isolation of the fungus in the aqueous suspension may be attributed to its previous germination and/or subsequent formation of a dense mycelium.
‡Week 0 indicates the day on which the invert emulsion was prepared and the fungus strain was introduced.

high speed for homogenization (21 000 rev min^{-1} for 1·5 min) on conidial viability was so small because the reduction in conidial viability was <3·5% (reduced from 85·2 to 81·8%) (Table 3).

Discussion

The frequent isolation of the fungus strain (ZRYAC of *Z. radicans*) from naturally infected DBM larvae in cabbage plantations in South Australia indicates that this entomopathogenic fungus is a native pathogen of the insect in South Australia. This is confirmed by other researchers who have reported that the fungus is a native pathogen of DBM larvae in other Australian states and elsewhere in the world (Furlong and Pell 2000, 2001; Vickers *et al.* 2004; CSIRO-Entomology 2008). Moreover, the characteristics of this native strain (e.g. morphological and entomopathological) were described and studied in the present research. These characteristics are typical to the fungus *Z. radicans* and conform to the characteristics of other fungus strains reported by other authors (Glare *et al.* 1987; Furlong and Pell 1997; Griggs *et al.* 1999).

The present research is considered the first attempt to develop an effective formulation of this entomophthoralean fungus. The formulation developed in this research has improved the efficacy of the fungus against the DBM larvae, and the results obtained in this respect would be a very important advance in this field of research. The results have also confirmed the effectiveness of the developed formulation through conduction of bioassays for reaching such important finding.

The fungus strain (ZRYAC) has shown a moderate efficacy when an unformulated form of the fungus strain has been applied using the top concentration of 10^7 conidia/ml (57·5% of larval mortality), but the efficacy has significantly increased (reached at 85% of larval mortality) when a formulated form of the same strain has been applied using the same concentration. This significant increase in efficacy could be attributed to the effect of inverted emulsion formulation used in bioassays because this formulation has maintained the viability of introduced fungus conidia and enhanced their effect. Moreover, the present formulation may offer the following additional advantages when used: (i) ingredients of the

present formulation are cheap and easy to obtain from local commercial sources; (ii) the technique used for the preparation of the formulation is simple and does not need sophisticated tools; (iii) the formulation could be applied in liquid formulation (spray form) with or without dilution in water at the time of application especially under field conditions; and (iv) although we did not perform any toxicological assay, the ingredients are not likely to be toxic as all of them are used as food additives or in manufacturing of cosmetics.

In this study, the median lethal concentration (LC50) of a formulated form of the fungus strain was 100 times less than that of the unformulated form ($3{\cdot}5 \times 10^4$ *vs* $4{\cdot}78 \times 10^6$ conidia/ml, respectively). This means that the fungus strain was much more effective when applied in a formulated form against DBM larvae than when applied in an unformulated form. The earlier discussion relating to the higher efficacy of a formulated form of the strain suggests the possibility of using it as biocontrol agent in pest management strategies of this serious insect pest of crucifers under field conditions but further studies are needed to be performed before conducting any field experiment.

It is important to note that the effect of formulation ingredients on the conidial viability of the introduced fungus strain compared to the unformulated form was small (79 *vs* 85·2% of conidial germination during a 4-week period of storage at 25 ± 1°C; respectively). In contrast, the conidial viability of the unformulated strain in form of conidial suspension in water was completely lost within 1 week of storage at the same conditions because of early conidial germination and subsequent formation of mycelium. This will make the fungus unsuitable for storage in water for long time until application. These results are similar to previous results that have been obtained when another entomopathogenic fungus *M. anisopliae* was formulated in inverted emulsion (Batta 2003a), but no previous studies have been reported on measuring the viability of *Z. radicans* formulated in inverted emulsion. Therefore, these results represent the first attempt to investigate the viability of *Z. radicans* formulated in invert emulsion by measuring its conidial viability in the formulation over time, but further studies on conidial viability of the fungus are recommended to be carried out at longer term (e.g. 6 months or more).

In conclusion, the present research constitutes the first investigations into the formulation and application of this fungus against the larvae of *P. xylostella*. However, further research studies are needed in this respect especially under field conditions before using this fungus in pest management of *P. xylostella* or of other related insect species that may be infected with the fungus.

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