Enhanced fungicide resistance in *Isaria fumosorosea* following ionizing radiation-induced mutagenesis

Shinobu Shinohara[1], Yuyun Fitriana[1], Katsuya Satoh[2], Issay Narumi[2] & Tsutomu Saito[1]

[1]Faculty of Agriculture, Shizuoka University, Shizuoka, Japan and [2]Quantum Beam Science Directorate, Japan Atomic Energy Agency, Gunma, Japan

Correspondence: Tsutomu Saito, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, Japan.
Tel.: +81 54 238 4790;
fax: +81 54 238 4790;
e-mail: atsaito@ipc.shizuoka.ac.jp

Present address: Issay Narumi, Faculty of Life Sciences, Toyo University, Gunma, Japan

Abstract

The application of entomopathogenic fungi such as *Isaria fumosorosea* to combat insect pests on plants is complicated by their sensitivity to commonly used fungicides. In this study, *I. fumosorosea* mutants with enhanced resistance to the fungicide benomyl were induced by irradiation using either ion beams or gamma rays, or a combination of the two. When grown on agar containing benomyl, mycelial growth was observed for five of the six mutant isolates at benomyl concentrations that were more than 2000-fold those observed for the wild-type isolate (EC_{50}: > 5000 mg L^{-1} c.f. EC_{50}: 2.5 mg L^{-1} for the wild-type isolate). The mutant isolates evaluated also showed enhanced resistance to other fungicides at recommended field application rates. No differences were observed at the β-tubulin locus between the wild-type and the mutant isolates, suggesting that the enhanced benomyl resistance was not attributable to mutations in that gene. Ion beams and gamma rays are thus potentially useful tools for inducing beneficial fungal mutations and thereby improving the potential for application of entomopathogenic fungi as microbial control agents.

Introduction

Entomopathogenic fungi, such as *Isaria fumosorosea*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Lecanicillium* spp., are all commercially available as microbial pesticides (Copping, 2009). However, entomopathogenic fungi can be negatively affected by fungicides, with the result that their biological control potential can be reduced if both products are used together (Clark *et al.*, 1982; Loria *et al.*, 1983; Saito & Yabuta, 1996; Pell *et al.*, 2010; D'Alessandro *et al.*, 2011). While the harmful effects of fungicides on entomopathogenic fungi are typically avoided by employing extended intervals between the application of each agent (Gardner *et al.*, 1984; Bruck, 2009), these intervals can complicate the effective and practical use of both products in the field. One way to overcome this problem has been to develop fungicide-resistant isolates of entomopathogenic fungi by selection on chemically amended media (Shapiro-Ilan *et al.*, 2002, 2011; Butters *et al.*, 2003), by transformation (Pfeifer & Khachatourians, 1992; Inglis

et al., 1999), or by exposure to mutagenic agents such as $NaNO_2$ (Zou *et al.*, 2006; Song *et al.*, 2011). Irradiation with ion beams is also a useful method for mutagenesis in microorganisms (Matuo *et al.*, 2006; Tanaka *et al.*, 2010; Toyoshima *et al.*, 2012). For example, exposure of the entomopathogenic fungus *Cordyceps militaris* to ion beams successfully generated a mutant isolate capable of enhanced production of cordycepin, a medicinal adenosine analogue (Das *et al.*, 2008, 2010). However, there is relatively little information in the literature on the use of ion-beam irradiation to induce mutations in entomopathogenic fungi and none considering induction of mutations conferring resistance to fungicides. Gamma-ray irradiation has also been demonstrated as a successful mutagenic agent in entomopathogenic fungi (Paccola-Meirelles & Azevedo, 1991; Kava-Cordeiro *et al.*, 1995), although there are no reports describing enhanced fungicide resistance induced by gamma-ray irradiation. In this study, an isolate of *I. fumosorosea* was subjected to ion-beam and/or gamma-ray irradiation to encourage the production of

mutants with the potential for increased resistance to benomyl as a model fungicide. Levels of benomyl resistance in the mutants and their responses to other fungicides at recommended field application rates were also examined. Moreover, the β-tubulin locus of the mutants was sequenced, as benomyl resistance in *B. bassiana* is known to be conferred by a mutation at this locus (Butters *et al.*, 2003; Zou *et al.*, 2006).

Materials and methods

Fungal preparation

A wild-type isolate (PF-3110) of *I. fumosorosea* that had been isolated from the sweet potato whitefly *Bemisia tabaci* B biotype in 1990 was cultured in Petri dishes (90 mm diameter) containing Sabouraud's dextrose agar (SDA; Difco, BD Biosciences, NJ) at 23 ± 1 °C for 3 weeks in darkness. Conidial suspensions were prepared by scraping the conidia/mycelia into sterile 0.1% Tween 80 and then filtering the mixture through sterile cloth (0.2 mm mesh size) to provide a suspension of conidia. Before experimentation, the germination rate of conidia was determined on SDA at 23 ± 1 °C (> 95%).

Ion-beam and gamma-ray irradiation

Conidia of the wild-type isolate were irradiated with carbon-ion beams ($^{12}C^{5+}$, 121.8 keV μm^{-1}) accelerated by an azimuthally varying field cyclotron at the Takasaki Ion Accelerators for Advanced Radiation Application site (Gunma, Japan) and/or gamma rays (^{60}Co, 0.2 keV μm^{-1}) at the Food Irradiation Facility, Japan Atomic Energy Agency (Gunma, Japan), respectively.

Fungicides against which mutants were evaluated

Eight commercial fungicides were used: benomyl [50% wettable powder (WP), Sumitomo Chemical, Japan], thiophanate-methyl (70% WP, Nippon Soda, Japan), iprodione (50% WP, Nippon Soda, Japan), diethofencarb (25% WP, Sumitomo Chemical, Japan), chlorothalonil (40% WP, Kumiai Chemical Industry, Japan), polycarbamate (75% WP, Dow Chemical Japan, Japan), myclobutanil [25% emulsifiable concentrate (EC), Dow Chemical Japan, Japan] and triflumizole (15% EC, Ishihara Sangyo Kaisha, Japan). Each fungicide was added to autoclaved SDA once it had cooled to below 50 °C and mixed using a stirrer before pouring the agar and allowing it to set. The eight fungicides used in this experiment belong to different classes of chemical, except benomyl and thiophanate-methyl, which are both benzimidazoles.

Relationship between irradiation dose and survival rates

The relationship between irradiation dose and conidial survival rate was determined as follows. For ion-beam irradiation, 50 μL of the conidial suspension (2.5×10^3 conidia mL^{-1}) was spread on 20 mL SDA in each replicate plastic Petri dish (60 mm diameter). The dishes were covered with a polyimide film (Kapton 30EN, Du Pont-To-ray, Japan) and irradiated at a range of doses (0, 50, 100, 200, 300, 400, 500 and 600 Gy). For gamma-ray irradiation, 100 μL of the conidial suspension (2.0×10^3 conidia mL^{-1}) was used in each Petri dish (90 mm diameter), the dishes were covered with plastic lids, and the range of doses tested was 0, 30, 100, 300, 1000 and 3000 Gy. All dishes were incubated at 23 ± 1 °C in darkness, and five replicates were made for each dose. Survival rates were determined based on the number of colonies that grew on SDA after 3 days and used to select appropriate doses for the production of mutants.

Production of mutants with potential resistance to benomyl

Conidia from 3 mL of a suspension (1.0×10^8 conidia mL^{-1}) of the wild-type isolate of *I. fumosorosea* were transferred to each of three replicate 47-mm-diameter cellulose membrane filters with a pore size of 0.45 μm (Millipore, Merck Millipore, Germany) using filtration equipment (Swinnex, Merck Millipore, Germany). The filters were individually placed in plastic Petri dishes (60 mm diameter for ion-beam irradiation and 50 mm diameter for gamma-ray irradiation). Prior to ion-beam irradiation, the Petri dish lids were replaced with polyimide film.

Conidia collected on the membrane filters were irradiated either with ion beams or with gamma rays at a range of doses selected from the relationship between irradiation dose and survival rate determined in the previous experiment ($n = 3$ per dose). Each treated filter was then transferred to a vial containing 3 mL of sterile Sabouraud's dextrose broth and agitated with a sterile glass rod to detach the conidia from the filters. The resulting conidial suspensions were incubated at 20 ± 1 °C in darkness overnight to remove unstable mutations through cell division (germination). From each suspension, 200 μL was spread on to SDA into which 1000 mg L^{-1} benomyl had been incorporated, in each of five/six Petri dishes (90 mm diameter). The dishes were then incubated at 23 ± 1 °C in darkness for 7 days. Well-grown colonies were assumed to be benomyl-resistant mutants and

isolated onto fresh SDA for further evaluation; colonies that differed in shape and colour from the wild-type isolate were excluded.

In addition, the effectiveness of dual irradiation with both gamma rays and ion beams for inducing mutations was examined in the wild-type isolate; irradiation doses of 0, 50 or 500 Gy of gamma rays were followed by 0, 100 or 200 Gy of ion beams, respectively. After the conidia on the membrane filters were irradiated with gamma rays, they were placed in Petri dishes containing SDA at 23 ± 1 °C and incubated in darkness overnight to remove unstable mutations through cell division (germination). This was followed by ion-beam irradiation and subsequent treatment procedures were as described previously for single ion-beam irradiation.

Evaluation of levels of resistance to benomyl in mutants

The EC_{50} (effective concentration of benomyl to reduce mycelial growth by 50%) values were determined for wild-type and mutant isolates based on mycelial growth on SDA that contained different quantities of benomyl. Mycelial plugs (4 mm diameter) of each isolate were excised from the margins of colonies growing on SDA. The plugs were then placed individually into the centre of Petri dishes (90 mm diameter) containing 30 mL SDA to which benomyl had been added (0, 0.1, 0.3, 1, 3, 10 or 30 mg L^{-1} for the wild-type isolate and 0, 300, 1000, 3000 or 5000 mg L^{-1} for the mutant isolates; $n = 3$ per dose). Dishes were incubated at 25 ± 1 °C in darkness for 7 days and mean colony diameter was determined from two perpendicular measurements of each colony, excluding 4 mm to account for the diameter of the original inoculation plug. Each colony diameter was then divided by the mean colony diameter obtained for the same isolate when grown on SDA containing no fungicide, to provide relative growth rates. The EC_{50} value for each isolate was estimated using the growth rates obtained for each dose by linear regression using the software package SPSS Statistics (SPSS, 2009).

Resistance of mutants to other fungicides

Growth of benomyl-resistant mutants on SDA to which one of eight fungicides, including benomyl, had been incorporated at the recommended field application rate was evaluated. The methods were the same as described above, except that dishes were incubated for 8 days. Each isolate was also cultured in the absence of fungicides (control), and there were five replicate dishes per fungicide. The resistance ratio (RR) was obtained using the following formula: (mean colony diameter for fungicide/ mean colony diameter for control) mutant/(mean colony diameter for fungicide/mean colony diameter for control) wild type. For each mutant, the mean colony diameter for each fungicide was statistically compared to the mean colony diameter for the control using a Mann–Whitney's *U*-test (SPSS, 2009).

DNA sequencing of β-tubulin locus

After culturing the wild-type and mutant isolates on SDA at 20 °C for 3 days, genomic DNA was extracted from the conidia of each isolate using a FastDNA Spin Kit (MP Biomedicals, UK). To isolate the partial β-tubulin locus, PCR was conducted using a PrimeSTAR GXL DNA polymerase (Takara Bio, Japan), the purified genomic DNA as a template and two specific primers, Isaria_beta-tub-R and Metarhizium_beta-tub-F2 (Table 1), under the following conditions: 1 cycle of 98 °C for 20 s, 30 cycles of 98 °C for 10 s, 68 °C for 2 min, 1 cycle of 68 °C for 5 min and then storage at 4 °C. The PCR products were purified using a MinElute PCR Purification Kit (Qiagen, the Netherlands). To identify the mutation sites in the β-tubulin locus, DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, CA) and six specific primers (Table 1) with an ABI PRISM 377 DNA Sequencer (Life Technologies).

Results and discussion

Production of benomyl-resistant mutants and fungal resistance levels

Ion-beam irradiation with a dose of 50 Gy was associated with high survival rates (64.6%), 500 Gy with very low survival rates (0.3%), and 600 Gy killed all the conidia (Table 2). Gamma-ray irradiation with doses below 300 Gy only slightly affected survival rates (> 78.1%), 1000 Gy was associated with very low survival rates

Table 1. Primers used in this study

Primer	Sequence (5′ to 3′)	Use
Isaria_beta-tub-R	TTACATGGGCTCCTCAGCCTCA	PCR and sequencing
Beauveria_beta-tub-R2	CTTCATGGCAACCTTACCAC	Sequencing
Beauveria_beta-tub-R3	GAACAACGTCGAGGACCTG	Sequencing
Metarhizium_beta-tub-F2	CCAAATTGGTGCTGCTTTCTGG	PCR and sequencing
Beauveria_beta-tub-F2	CAGGGTTTCCAGATCACCC	Sequencing
Beauveria_beta-tub-F3	GAGGACCAGATGCGTAATGTG	Sequencing

Table 2. Relationship between ion-beam irradiation doses and conidial survival rates in the wild-type isolate of *Isaria fumosorosea*

Dose (Gy)	No. colonies per Petri dish (mean ± SE)	Survival rate*
0 (control)	88.7 ± 4.3	100
50	57.3 ± 10.5	64.6
100	33.3 ± 2.4	37.5
200	12.0 ± 1.0	13.5
300	3.7 ± 0.3	4.2
400	1.7 ± 0.3	1.9
500	0.3 ± 0.3	0.3
600	0.3 ± 0.3	0.3
700	0	0

*Survival rate = (mean number of irradiated colonies/mean number of control colonies) × 100.

Table 3. Relationship between gamma-ray irradiation doses and conidial survival rates in the wild-type isolate of *Isaria fumosorosea*

Dose (Gy)	No. colonies per Petri dish (mean ± SE)	Survival rate*
0 (control)	140.0 ± 2.0	100
30	124.0 ± 9.0	88.6
100	115.7 ± 16.8	82.6
300	109.3 ± 5.9	78.1
1000	0.7 ± 0.3	0.5
3000	0	0

*Survival rate = (mean number of irradiated colonies/mean number of control colonies) × 100.

(0.5%), and 3000 Gy killed all the conidia (Table 3). In previous studies, the highest mutation frequency obtained by ion beam irradiation was associated with survival rates ranging from 1 to 10% in *Saccharomyces cerevisiae* (Matuo *et al.*, 2006) and *Aspergillus oryzae* (Toyoshima *et al.*, 2012). Consequently, we selected irradiation doses of 100, 200, 300, 400 and 500 Gy for ion-beam irradiation and 400, 600, 800 and 1000 Gy for gamma-ray irradiation when attempting to produce mutants.

Six benomyl-resistant isolates were established from colonies that appeared normal (Fig. 1); Ib-34 (200 Gy) and Ib-421 (300 Gy) from ion-beam irradiation, Gr-5 and Gr-22 (both 1000 Gy) from gamma-ray irradiation, and GrIb-8 and GrIb-9, both from dual irradiation by 500 Gy of gamma rays followed by 200 Gy of ion beams. The wild-type isolate had an EC_{50} value of 2.5 mg L^{-1}, while five mutant isolates, Ib-34, Ib-421, Gr-5, Gr-22 and GrIb-8, had EC_{50} values > 5000 mg L^{-1} (> 2000-fold more resistant than the wild type; Table 4). Another mutant isolate, GrIb-9, also had a high EC_{50} value of 1855 mg L^{-1} (742-fold more resistant than the wild type; Table 4). This considerably enhanced resistance in the mutant isolates was greater than that of *I. fumosorosea* benzimidazole-resistant isolates produced using other

Fig. 1. Benomyl-resistant mutant colony (indicated by arrow) derived from *Isaria fumosorosea* wild-type conidia irradiated with ion beams (400 Gy) plated on SDA to which benomyl had been added (1000 mg L^{-1}).

Table 4. Benomyl resistance in the wild-type and mutant isolates of *Isaria fumosorosea*

Isolate	EC_{50} (mg L^{-1})
Wild-type	2.5
Ib-34	> 5000
Ib-421	> 5000
Gr-5	> 5000
Gr-22	> 5000
GrIb-8	> 5000
GrIb-9	1855

methods. For example, carbendazim-resistant mutants generated using the mutagen $NaNO_2$ exhibited a maximum EC_{50} value of only > 1000 mg L^{-1}, which was still 830-fold more resistant than the wild type (Song *et al.*, 2011). Furthermore, benomyl-resistant transformants generated using a polyethylene glycol-mediated procedure had a minimum inhibitory concentration of only 20 mg L^{-1} compared with 7.5 mg L^{-1} for the wild type (Inglis *et al.*, 1999). Enhanced resistance in our mutants may be sufficient to avoid the negative effects resulting from benomyl application in the field (500 mg L^{-1} is the recommended application rate). This is the first study to demonstrate that ion-beam and gamma-ray irradiation are potentially useful tools for inducing mutations that enhance fungicide resistance in entomopathogenic fungi. As the increase in resistance obtained separately with either irradiation method alone was considerable, combined irradiation may be unnecessary. Ion-beam irradiation facilities are now available worldwide. This method creates different characteristics in entomopathogenic fungi, because it causes a high mutation frequency and a

broad mutation spectrum and creates point mutations in genes (Matuo et al., 2006; Zengliang, 2006; Tanaka et al., 2010; Toyoshima et al., 2012).

Resistance of mutants to other fungicides

The wild-type and mutant isolates produced significantly smaller colonies on most of the fungicides at a field application rate compared with the control ($P < 0.01$ or $P < 0.05$; Table 5). As expected, however, all the mutant isolates had large RR values (4.48–6.46) when grown on media containing benomyl. Similarly, the mutants also had comparatively large RR values (1.30–1.69) when grown on media containing thiophanate-methyl, which, like benomyl, is a benzimidazole fungicide. For the other fungicides, the mutant isolates generally had RR values around 1, which indicated they had similar resistance levels to the wild-type isolate, except for iprodione (RR: 1.05–2.05) and myclobutanil (RR: 1.16–4.99). These findings suggest that the mutant isolates may have multiple mechanisms conferring resistance to several fungicides including benzimidazoles.

Benzimidazole fungicides, such as benomyl, are negatively cross-resistant to N-phenylcarbamate fungicides, such as diethofencarb (Fujimura et al., 1992a; Ziogas & Girgis, 1993; Leroux et al., 1999). However, our benomyl-resistant isolates exhibited similar or little more sensitivity to diethofencarb (RR: 0.84–1.09) than the wild-type isolate. Regardless of this, all mutants should be screened for undesirable mutations that may have occurred alongside the desired mutation conferring benomyl resistance. For example, it will be important to compare the virulence of mutant and wild-type isolates against target pests to ensure that any benefits associated with benomyl resistance are not counteracted by any loss in virulence; these studies are currently under way in our laboratories.

Sequence of β-tubulin locus

The β-tubulin locus of the wild-type and six mutant isolates was amplified by PCR. Two of the mutant isolates, Ib-34 and Gr-5, produced very weak amplification signals and were excluded from further analysis. The β-tubulin sequence in I. fumosorosea is 1727 bp (Song et al., 2011), but only partial genomic DNA sequences of the β-tubulin gene (1424 bp at nucleotide position from 304 to 1727) were determined for the wild-type and four mutant isolates and the β-tubulin sequences in the four mutant isolates, Ib-421, Gr-22, GrIb-8 and GrIb-9, were identical to those of the wild type.

Most of the molecular studies on benzimidazole-resistant phytopathogenic fungi have focused on the replacement of amino acids at codon 198 and/or 200 in the β-tubulin locus

Table 5. Colony diameters (mm, mean ± SE) of the wild-type and mutant isolates of Isaria fumosorosea on SDA containing each fungicide at the recommended application rate and the RR† in parentheses

Isolate	Benomyl (500 mg L⁻¹)	Thiophanate-methyl (700 mg L⁻¹)	Iprodione (500 mg L⁻¹)	Diethofencarb (250 mg L⁻¹)	Chlorothalonil (400 mg L⁻¹)	Polycarbamate (937 mg L⁻¹)	Myclobutanil (625 mg L⁻¹)	Triflumizole (150 mg L⁻¹)	Control (No fungicide)
Wild-type	3.4 ± 0.1** (1)	14.0 ± 0.2** (1)	6.2 ± 0.3** (1)	21.6 ± 1.2* (1)	16.3 ± 0.5** (1)	9.6 ± 0.5** (1)	2.0 ± 1.0** (1)	0.6 ± 0.1** (1)	25.4 ± 0.4
Ib-34	7.7 ± 0.3** (5.70)	9.4 ± 0.4 (1.69)	4.3 ± 0.2** (1.74)	7.2 ± 0.2** (0.84)	9.4 ± 0.2 (1.45)	5.6 ± 0.4** (1.47)	3.3 ± 0.2** (4.15)	0** (0)	10.1 ± 0.4
Ib-421	12.7 ± 0.9** (4.70)	14.5 ± 0.6** (1.30)	5.2 ± 0.2** (1.05)	18.7 ± 0.6 (1.09)	13.2 ± 1.0** (1.02)	9.1 ± 0.2** (1.19)	4.9 ± 1.2** (3.08)	0.1 ± 0.1** (0.21)	20.2 ± 0.1
Gr-5	17.9 ± 0.2* (5.31)	22.4 ± 0.2** (1.61)	9.2 ± 0.7** (1.50)	19.1 ± 0.2** (0.89)	16.7 ± 0.4** (1.03)	7.9 ± 0.2** (0.83)	2.3 ± 0.2** (1.16)	0.7 ± 0.2** (1.18)	25.2 ± 0.1
Gr-22	11.4 ± 0.4** (4.48)	16.1 ± 0.2** (1.54)	6.0 ± 0.3** (1.29)	15.9 ± 0.2** (0.98)	11.9 ± 0.2** (0.98)	6.2 ± 0.1** (0.86)	6.2 ± 0.7** (4.14)	0.2 ± 0.2** (0.45)	19.0 ± 0.1
GrIb-8	7.7 ± 0.4* (6.46)	7.9 ± 0.2* (1.61)	3.7 ± 0.3** (1.70)	7.3 ± 0.2* (0.96)	7.7 ± 0.3* (1.35)	4.7 ± 0.2** (1.40)	3.5 ± 0.2** (4.99)	0** (0)	8.9 ± 0.2
GrIb-9	6.2 ± 0.2** (4.73)	8.5 ± 0.4* (1.57)	4.9 ± 0.3** (2.05)	7.4 ± 0.1** (0.89)	8.6 ± 0.3* (1.37)	4.6 ± 0.2** (1.24)	3.2 ± 0.3** (4.15)	0** (0)	9.8 ± 0.3

* and ** in each isolate indicate significant differences from control at $P = 0.05$ and $P = 0.01$, respectively.

†RR = (mean colony diameter for fungicide/mean colony diameter for control) mutant/(mean colony diameter for fungicide/mean colony diameter for control) wild-type.

(Fujimura *et al.*, 1992b; Koenraadt & Jones, 1993; Yarden & Katan, 1993; Davidson *et al.*, 2006; Schmidt *et al.*, 2006; Kongtragoul *et al.*, 2011). A similar mutation at codon 198 has been reported in NaNO$_2$-induced benomyl-resistant mutants of *B. bassiana* (Butters *et al.*, 2003; Zou *et al.*, 2006). However, none of these mutations in the β-tubulin locus were detected in our mutant isolates, suggesting that other mechanisms may be responsible for the observed enhancement in benomyl resistance. In another study, Song *et al.* (2011) showed that, although benomyl-resistant *I. fumosorosea* mutants did not possess any mutations at the β-tubulin locus, mutations were observed in the promoter region of the ABC transporter gene (*ifT1*). It is thus possible that similar mutations may have occurred in our benomyl-resistant mutant isolates. These findings indicate that different mechanisms are responsible for conferring benomyl resistance in *B. bassiana* and *I. fumosorosea*. Future studies will examine the possibility of mutations in other genes including *ifT1* in our mutants.

Acknowledgement

References

Bruck DJ (2009) Impact of fungicides on *Metarhizium anisopliae* in the rhizosphere, bulk soil and *in vitro*. *Biocontrol* **54**: 597–606.

Butters JA, Devi KU, Mohan CM & Sridevi V (2003) Screening for tolerance to bavistin, a benzimidazole fungicide containing methyl benzimidazol-2-yl carbamate (MBC), in *Beauveria bassiana*: sequence analysis of the β-tubulin gene to identify mutations conferring tolerance. *Mycol Res* **107**: 260–266.

Clark RA, Casagrande RA & Wallace DB (1982) Influence of pesticides on *Beauveria bassiana*, a pathogen of the Colorado potato beetle. *Environ Entomol* **11**: 67–70.

Copping LG (2009) *The Manual of Biocontrol Agents*. BCPC Publications, Hampshire.

D'Alessandro CP, Padin S, Urrutia MI & López Lastra CC (2011) Interaction of fungicides with the entomopathogenic fungus *Isaria fumosorosea*. *Biocontrol Sci Technol* **21**: 189–197.

Das SK, Masuda M, Hatashita M, Sakurai A & Sakakibara M (2008) A new approach for improving cordycepin productivity in surface liquid culture of *Cordyceps militaris* using high-energy ion beam irradiation. *Lett Appl Microbiol* **47**: 534–538.

Das SK, Masuda M, Hatashita M, Sakurai A & Sakakibara M (2010) Optimization of culture medium for cordycepin production using *Cordyceps militaris* mutant obtained by ion beam irradiation. *Process Biochem* **45**: 129–132.

Davidson RM, Hanson LE, Franc GD & Panella L (2006) Analysis of β-tubulin gene fragments from benzimidazole-sensitive and -tolerant *Cercospora beticola*. *J Phytopathol* **154**: 321–328.

Fujimura M, Kamakura T & Yamaguchi I (1992a) Action mechanism of diethofencarb to a benzimidazole-resistant mutant in *Neurospora crassa*. *J Pesticide Sci* **17**: 237–242.

Fujimura M, Oeda K, Inoue H & Kato T (1992b) A single amino-acid substitution in the beta-tubulin gene of *Neurospora* confers both carbendazim resistance and diethofencarb sensitivity. *Curr Genet* **21**: 399–404.

Gardner WA, Oetting RD & Storey GK (1984) Scheduling of *Verticillium lecanii* and benomyl applications to maintain aphid (Homoptera: Aphididae) control on chrysanthemums in greenhouses. *J Econ Entomol* **77**: 514–518.

Inglis PW, Tigano MS & Valadares-Inglis M (1999) Transformation of the entomopathogenic fungi, *Paecilomyces fumosoroseus* and *paecilomyces lilacinus* (Deuteromycotina: Hyphomycetes) to benomyl resistance. *Genet Mol Biol* **22**: 119–123.

Kava-Cordeiro V, Luna-Alves-Lima EA & Azevedo JL (1995) Survival and mutant production induced by mutagenic agents in *Metarhizium anisopliae*. *Sci Agric* **52**: 548–554.

Koenraadt H & Jones AL (1993) Resistance to benomyl conferred by mutations in codon 198 or 200 of the beta-tubulin gene of *Neurospora crassa* and sensitivity to diethofencarb conferred by codon 198. *Phytopathology* **83**: 850–854.

Kongtragoul P, Nalumpang S, Miyamoto Y, Izumi Y & Akimitsu K (2011) Mutation at codon 198 of *TUB2* gene for carbendazim resistance in *Colletotrichum gloeosporioides* causing mango anthracnose in Thailand. *J Plant Prot Res* **51**: 377–384.

Leroux P, Chapeland F, Desbrosses D & Gredt M (1999) Patterns of cross-resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards *Botryotinia*. *Crop Prot* **18**: 687–697.

Loria R, Galaini S & Roberts DW (1983) Survival of inoculum of the entomopathogenic fungus *Beauveria bassiana* as influenced by fungicides. *Environ Entomol* **12**: 1724–1726.

Matuo Y, Nishijima S, Hase Y, Sakamoto A, Tanaka A & Shimizu K (2006) Specificity of mutations induced by carbon ions in budding yeast *Saccharomyces cerevisiae*. *Mutat Res* **602**: 7–13.

Paccola-Meirelles LD & Azevedo JL (1991) Parasexuality in *Beauveria bassiana*. *J Invertebr Pathol* **57**: 172–176.

Pell JK, Hannam JJ & Steinkraus DC (2010) Conservation biological control using fungal entomopathogens. *The Ecology of Fungal Entomopathogens* (Roy HE, Vega FE, Chander D, Goettel MS, Pell JK & Wajnberg E, eds), pp. 187–198. Springer, London.

Pfeifer TA & Khachatourians GG (1992) *Beauveria bassiana* protoplast regeneration and transformation using electroporation. *Appl Microbiol Biotechnol* **38**: 376–381.

Saito T & Yabuta M (1996) Laboratory studies on effect of pesticides on entomopathogenic fungus, *Verticillium lecanii*. *Jpn J Appl Entomol Zool* **40**: 71–76.

Schmidt LS, Ghosoph JM, Margosan DA & Smilanick JL (2006) Mutation at β-tubulin codon 200 indicated thiabendazole resistance in *Penicillium digitatum* collected from California citrus packinghouses. *Plant Dis* **90**: 765–770.

Shapiro-Ilan DI, Reilly CC, Hotchkiss MW & Wood BW (2002) The potential for enhanced fungicide resistance in *Beauveria bassiana* through artificial selection. *J Invertebr Pathol* **81**: 86–93.

Shapiro-Ilan DI, Reilly CC & Hotchkiss MW (2011) Comparative impact of artificial selection for fungicide resistance on *Beauveria bassiana* and *Metarhizium brunneum*. *Environ Entomol* **40**: 59–65.

Song T-T, Ying S-H & Feng M-G (2011) High resistance of *Isaria fumosorosea* to carbendazim arises from the overexpression of an ATP-binding cassette transporter (ifT1) rather than tubulin mutation. *J Appl Microbiol* **112**: 175–184.

SPSS (2009) *PASW Statistics 18*. SPSS Inc., Chicago, IL.

Tanaka A, Shikazono N & Hase Y (2010) Studies on biological effects of ion beams on lethality, molecular nature of mutation, mutation rate, and spectrum of mutation phenotype for mutation breeding in higher plants. *J Radiat Res* **51**: 223–233.

Toyoshima Y, Takahashi A, Tanaka H, Watanabe J, Mogi Y, Yamazaki T, Hamada R, Iwashita K, Satoh K & Narumi I (2012) Lethal and mutagenic effects of ion beams and γ-rays in *Aspergillus oryzae*. *Mutat Res* **740**: 43–49.

Yarden O & Katan T (1993) Mutations leading to substitutions at amino acids 198 and 200 of beta-tubulin that correlate with benomyl-resistance phenotypes of field strains of *Botrytis cinerea*. *Phytopathology* **83**: 1478–1483.

Zengliang Y (2006) *Introduction to Ion Beam Biotechnology*. Springer, New York, NY.

Ziogas BN & Girgis SM (1993) Cross-resistance relationships between benzimidazole fungicides and diethofencarb in *Botrytis cinerea* and their genetical basis in *Ustilago maydis*. *Pestic Sci* **39**: 199–205.

Zou G, Ying S-H, Shen Z-C & Feng M-G (2006) Multi-sited mutations of beta-tubulin are involved in benzimidazole resistance and thermotolerance of fungal biocontrol agent *Beauveria bassiana*. *Environ Microbiol* **8**: 2096–2105.