Ion-beam and gamma-ray irradiations induce thermotolerant mutants in the entomopathogenic fungus *Metarhizium anisopliae* s.l.

著者	Fitriana Yuyun, Satoh Katsuya, Narumi Issay, Saito Tsutomu
journal or publication title	Biocontrol Science and Technology
volume	24
number	9
page range	1052-1061
year	2014-06-16
出版者	Taylor & Francis
権利	This is an Accepted Manuscript of an article published by Taylor & Francis in Biocontrol Science and Technology on 16/06/2014, available online: http://www.tandfonline.com/10.1080/09583157.2014.918585 .
URL	http://hdl.handle.net/10297/8863

doi: 10.1080/09583157.2014.918585

1 *Y. Fitriana et al.*

2 Biocontrol Science and Technology

3
4
5 **RESEARCH ARTICLE**

6
7
8 **Ion-beam and gamma-ray irradiation induces thermotolerant mutants**
9 **in the entomopathogenic fungus *Metarhizium anisopliae* s.l.**

10
11 Yuyun Fitriana^a, Katusya Satoh^b, Issay Narumi^{b†} and

12 Tsutomu Saito^{a*}

13
14 ^a*Faculty of Agriculture, Shizuoka University, Shizuoka, Japan;* ^b*Quantum Beam Science*

15 *Directorate, Japan Atomic Energy Agency, Gunma, Japan*

16
17 *(Received 20 February, 2014; Returned 28 March; Accepted 23 April, 2014)*

18
19
20
21
22
23
24
25
26

***Corresponding author.** E-mail: atsaito@ipc.shizuoka.ac.jp

27 [†] Present address: Faculty of Life Sciences, Toyo University, Gunma, Japan

Entomopathogenic fungi, such as *Metarhizium anisopliae* (Metch.) Sorokin, are important agents for the biological control of insect pests. However, these fungi are not compatible with high temperatures. In this study, mutagenesis using ion beams or gamma rays was used to generate five potentially thermotolerant mutants from two wild-type isolates of *M. anisopliae* (two using ion beams and three using gamma rays). The mutant isolates had a higher upper thermal limit for vegetative growth compared to the wild types (by 2–3°C) and enhanced tolerance to wet-heat stress of 45°C for conidial germination. At 25 and 30°C, most mutants were as virulent to maize weevil adults as the wild-type, however, one mutant produced using ion beams almost lost virulence entirely. These results indicate that ion beams and gamma rays are useful tools for improving biological characteristics, such as thermotolerance, in entomopathogenic fungi, but that mutants must be carefully evaluated for unpredictable negative side effects.

Keywords: upper thermal limit; wet-heat stress; vegetative growth; conidial germination; virulence

1. Introduction

Entomopathogenic fungi, such as *Beauveria bassiana* (Bals.–Criv.) Vuill. (Hypocreales: Cordycipitaceae), *Isaria fumosorosea* Wize (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metch.) Sorokin (Hypocreales: Clavicipitaceae), are economically important agents for integrated pest management (IPM) programmes of insect pests (Butt, Jackson, and Magan 2001; Copping 2009). However, these fungi are affected by environmental factors, such as sunlight, humidity, temperature (Jaronski 2010; Zimmermann 2007) and artificial factors, such as fungicide applications (Pell, Hannam, and Steinkraus 2010). Negative effects of temperature, such as heat stress that exceeds 35°C, can have serious deleterious effects on conidial germination and persistence, vegetative growth, sporulation, and/or the infection process in these entomopathogenic fungi (e.g. Arthurs and Thomas 2001; Cabanillas and Jones 2009; Darbro, Graham, Kay, Ryan, and Thomas 2011; Davidson et al. 2003; Dimbi, Maniania, Lux, and Mueke 2004; Fargues, Goettel, Smits, Ouedrago, and Rougier 1997; Inglis, Johnson, Cheng, and Goettel 1997; Li and Feng 2009; Thomas and Jenkins 1997; Vidal, Fargues, and Lacey 1997). This may result in reduced effectiveness of these fungi in biological control, especially in hot seasons, tropical and subtropical regions, or in glasshouses with elevated temperatures.

One potential method to overcome this problem is to enhance thermotolerance by artificial selection (de Crecy, Jaronski, Lyons, Lyons, and Keyhani 2009; Kim, Kassa, Skinner, Hata, and Parker 2011), transformation (Leng, Peng, Cao, and Xia 2011), or mutagenesis using UV-B (Rangel et al. 2006). Recently ion beams have been identified as potential tools for mutagenesis in microorganisms because of their characteristic point-like mutations, higher mutation frequency and broader mutation spectrum (Matuo et al. 2006; Shikazono et al. 2003, 2005; Tanaka, Shikazono, and Hase 2010; Toyoshima et al. 2012). In entomopathogenic fungi, the method was used to improve productivity of medical cordycepin in *Cordyceps*

militaris (L:Fr) Link (Hypocreales: Cordycipitaceae) (Das, Masuda, Hatashita, Sakurai, and Sakakibara 2008), and subsequently *I. fumosorosea* was successfully mutated to enhance tolerance to fungicides using ion beams (Shinohara, Fitriana, Satoh, Narumi, and Saito 2013). However, little is known about improving other characteristics of entomopathogenic fungi using ion beams. In this study, we attempted to enhance thermotolerance in a model entomopathogenic fungus, *M. anisopliae*, by mutagenesis using ion beams and evaluated the relative virulence of the resulting mutants compared with the wild-type isolates. Gamma rays were also used as a tool for mutagenesis because they had also not been used to enhance thermotolerance in entomopathogenic fungi.

2. Materials and Methods

2.1. Fungal preparation

Two isolates of *M. anisopliae* s.l., reference numbers AcMa5 and PaMa02, that originated from scarab larvae in Shizuoka, Japan, were used as the wild-type isolates. They were grown on Sabouraud's dextrose agar (SDA) (Difco BD Bioscience, USA) in 90 mm diameter Petri dishes and incubated at 23±1°C for 3 weeks in darkness prior to experimentation. Conidial suspensions for experiments were prepared by scraping the mycelium from plate cultures into sterile 0.1% Tween 80 and filtering the resulting suspension of conidia through a sterile cloth (0.2 mm mesh size) to remove mycelia. Conidial concentrations were determined using a Thoma haemocytometer and adjusted as required.

2.2. Induction of mutants

For each wild-type isolate, 3 mL samples from the conidial suspension (1×10^8 conidia mL⁻¹) were each passed through replicate 47 mm cellulose membrane filters (pore size 0.45 µm;

Millipore, Merck Millipore, Germany). The conidia laden filters (n =3 per treatment and irradiation dose) were placed individually in sterile 60 mm plastic Petri dishes and then irradiated with either carbon-ion beams ($^{12}\text{C}^{5+}$, 121.8 keV μm^{-1}) accelerated by an azimuthally varying field cyclotron at the Takasaki Ion Accelerators for Advanced Radiation Application site (Gunma, Japan), or gamma rays (^{60}Co , 0.2 keV μm^{-1}) at the Food Irradiation Facility, Japan Atomic Energy Agency (Gunma, Japan). Irradiation doses were 0, 100, 200, 300, 400, or 500 Gy for ion beams and 0, 30, 100, 300, 1,000, or 3,000 Gy for gamma rays as in our previous study using *I. fumosorosea* (Shinohara et al., 2013). Prior to ion-beam irradiation, the Petri dish lids were replaced with polyimide film (Kapton 30EN, Du Pont-To-ray, Japan). Each filter was then transferred to a vial containing 3 mL Sabouraud's dextrose broth (Difco BD Bioscience, USA) and agitated with a sterile glass rod to detach the conidia. The conidial suspensions were incubated at $20\pm 1^\circ\text{C}$ in darkness overnight to remove mutations that were unstable through cell division (germination), and then 200 μL of each suspension was spread onto 30 mL SDA in a 90 mm Petri dish and incubated at $38\pm 1^\circ\text{C}$ (a high enough temperature to prevent growth of both wild-type isolates) in darkness for 2 weeks to select for thermotolerant mutants. There were three replicate dishes for each suspension. After this incubation period, any colonies that had grown were assumed to be thermotolerant mutants and were isolated onto fresh SDA for further experiments.

2.3. Effect of temperature on vegetative growth of mutant and wild-type isolates

Resulting thermotolerant mutants and the two wild-type isolates were subcultured on SDA in 90 mm Petri dishes and grown for 4–5 d at $25\pm 1^\circ\text{C}$ in darkness. Mycelial plugs (4 mm diameter) from each isolate were then excised from the margins of these colonies, and each plug placed upside down at the center of a new 90 mm Petri dish containing 30 mL SDA. These plates were incubated at 15, 20, 25, 30, 33, 35, 36, 37, 38, or $39\pm 1^\circ\text{C}$ for 7 d in

darkness, after which time the mean colony diameter was determined from two perpendicular measurements of each colony, excluding 4 mm to account for the diameter of the inoculation plug. There were three replicate dishes per temperature for each isolate. The mean colony diameter of each mutant, at each temperature, was compared statistically to that of the wild-type from which it was derived by Mann-Whitney U test (SPSS 2009).

2.4. Effect of wet-heat stress on conidial germination of mutant and wild-type isolates

This experiment was done following the method of Leng et al. (2011) with some modification as described below. For each mutant and wild-type isolate, 20 mL samples of conidial suspension (1×10^7 conidia mL⁻¹), prepared as described previously, were placed into individual 50 mL flasks and incubated in a stirred water bath at 45±1°C. After 0, 0.5, 1, and 3 h, 50 µL samples were taken from each flask and inoculated at three positions in each of three 90 mm Petri dishes, each containing 30 mL SDA (i.e. nine 50 µL samples taken from each flask at each time). These plates were incubated at 25±1°C for 24 h, after which time the mean germination rate was determined for approximately 100 conidia from each position (total =approximately 300 per plate) under a microscope (Axio Imager 2, Zeiss, Germany). The mean germination rates for each treatment, at each incubation time, were statistically analysed using ANOVA followed by Tukey's HSD test and Student's *t*-test for multiple comparisons of means and single comparisons of means, respectively (SPSS 2009).

2.5. Virulence of mutant and wild-type isolates

Virulence of mutant and wild-type isolates were compared against maize weevils, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), that had been reared on rice grains at 25±1°C and a photoperiod 16L:8D. Groups of ten adults (3–7 d old) were placed in glass tubes (20 mm diameter, 30 mm high) from which the bottom had been removed and replaced

with gauze. Each group was dipped into a conidial suspension (1×10^7 conidia mL⁻¹), prepared as described previously, through the gauze for 10 s and then incubated on rice grains in a Petri dish (60 mm) with a moist filter paper (to maintain a high humidity) at 25, 30, or 35±1°C with a photoperiod 16L:8D. Five replicate groups of weevils were used for each isolate with an additional five control groups that were treated in the same way but only with 0.1% Tween 80 and no inoculum. Mortality was recorded daily for 10 d. Dead adults were transferred to Petri dishes lined with moist filter paper and incubated at 25±1°C in darkness for 5 d to allow fungal outgrowth to occur if it had been the cause of mortality. The percentage mortality due to fungal infection was arcsine square-root transformed and then the mean mortalities, at each day after inoculation, were statistically analysed using ANOVA followed by Tukey's HSD test and Student's *t*-test for multiple comparisons and single comparisons, respectively (SPSS 2009).

3. Results and Discussion

Thermotolerant mutants were successfully isolated by screening at 38°C which completely inhibited vegetative growth of the wild-type isolates (Table 1). Four mutants were isolated from the wild-type isolate, AcMa5; one mutant (AcMa5-ib) was from ion-beam irradiation and three mutants (AcMa5-gr-1, AcMa5-gr-2, and AcMa5-gr-3) were from gamma-ray irradiation. From another wild-type isolate, PaMa02, one mutant (PaMa02-ib) was isolated as a result of ion-beam irradiation and no mutants were obtained from gamma-ray irradiation.

Vegetative growth of the mutant isolates at different temperatures was compared to that of the wild-type isolates (Table 2). All the mutant isolates derived from wild-type isolate AcMa5 had an upper thermal limit of 38°C which was higher than that (36°C) of the wild-type isolate by 2°C. Of the mutants, at each temperature from 25 to 36°C, three mutants (AcMa5-ib, AcMa5-gr-2, and AcMa5-gr-3) also grew significantly faster than the

wild-type ($P < 0.05$, Mann-Whitney U test). In contrast, mutant isolate PaMa02-ib derived from wild-type isolate PaMa02 had an upper thermal limit of 39°C which was higher than that (36°C) of the wild-type by 3°C, although this mutant grew significantly slower than the wild-type at 25°C which was the optimal temperature for vegetative growth of both mutant and wild-type isolates ($P < 0.05$, Mann-Whitney U test). de Crecy et al. (2009) reported that a *M. anisopliae* variant generated by natural selection-adaptation was able to germinate and grow well at 37°C; in contrast, while the wild-type from which it was derived was able to germinate at 37°C, it failed to subsequently grow. Their findings are similar to our results for vegetative growth in mutant and wild-type isolates.

From observations of the colonies growing at each temperature it was notable that, at 35°C, neither the wild-type isolates nor mutant isolate PaMa02-ib produced any conidia. In contrast, all the mutants derived from wild-type AcMa5 produced characteristic green conidia at temperatures of 35°C and below, suggesting that these mutants were better adapted to high temperatures in respect of their sporulation capacity than the wild-type.

In a study by Leng et al. (2011), mutant isolates of *Metarhizium acridum* (Driver and Milner) Bischoff, Rehner and Humber (Hypocreales: Clavicipitaceae) generated by neutral trehalase gene RNA interference transformations, were significantly more tolerant to wet-heat stress at 45°C for 1, 1.5, 2, 2.5 and 3 h exposure than the wild-type isolate from which they were derived. Using their methods, similar results were obtained in our study (Table 3). Conidia of the wild-type AcMa5 were unable to germinate after 1 h exposure, whereas all the mutants derived from it germinated (5.6–19.3%). Of these mutants, AcMa5-ib had significantly greater germination rates than other mutants after both 0.5 h ($F = 34.9$; $df = 4, 10$; $P < 0.05$, Tukey's HSD test) and 1 h exposure ($F = 50.0$; $df = 4, 10$; $P < 0.05$, Tukey's HSD test). Thus, mutant isolates derived from AcMa5 had enhanced thermotolerance in respect to conidial germination, though no mutant isolates survived the longest exposure period (3 h). In the study of Leng et al. (2011) the mutant isolates of *M. acridum* remained able to

germinate at low levels (<20%) after 3 h, which was significantly greater than the wild-type isolate and in contrast to our findings after 3 h exposure. By contrast, the mutant PaMa02-ib derived from wild-type isolate PaMa02 was, like the wild-type, unable to germinate after 1 h exposure, and also, its germination rate was not significantly different to the wild-type after 0.5 h exposure ($P > 0.05$, Student's t -test). As mentioned above, some mutants of *M. anisopliae* have enhanced tolerance to heat stress in vegetative growth, sporulation, and germination. This is the first report of enhanced thermotolerance induced by ion beams or gamma rays in an entomopathogenic fungus.

Thermotolerance in conidia of the mutants may represent a considerable advantage for their practical use in biological control because conidial germination is the first step in the infection process of host insects (Castrillo, Roberts, and Vandenberg 2005). In this study, each mutant isolate was also evaluated for virulence to weevil adults at a standard temperature (25°C) and at higher temperatures (30 and 35°C). At 25°C, all the mutants derived from wild-type isolate AcMa5 caused high mortalities (86–100%) at Day 10, which were not significantly different from the mortality (90%) caused by the wild-type ($F = 1.5$; $df = 4, 20$; $P > 0.05$, Tukey's HSD test) (Figure 1A). By contrast, the mutant PaMa02-ib derived from wild-type isolate PaMa02 caused significantly less mortality (4%) at Day 10 than the wild-type (94%) ($P < 0.05$, Student's t -test) (Figure 1B). At 30°C, there was no significant difference in mortality at Day 10 among wild-type isolate AcMa5 and the four mutants derived from it ($F = 2.1$; $df = 4, 20$; $P > 0.05$, Tukey's HSD test), though there was a trend for AcMa5-ib to cause higher mortality compared to the wild-type throughout the experiment (e.g. 84% mortality compared with 58%, respectively) (Figure 2A). Mutant isolate PaMa02-ib derived from wild-type isolate PaMa02 caused significantly less mortality (6%) at Day 10 than the wild-type (30%) ($P < 0.05$, Student's t -test) (Figure 2B). Throughout the bioassays at 25 and 30°C, no mortality was observed in control weevils. At 35°C, all mutant and wild-type isolates caused some fungal-induced mortality, however, the weevils lost their

appetite and a number died without obvious signs of fungal infection in both treatments (18–32%) and the control (24%). Since such high temperatures were harmful to the weevils, we did not use data from the 35°C treatment in the analysis.

The virulence tests showed that most mutant isolates retained high levels of virulence at the standard temperature (25°C), and one mutant (AcMa5-ib) generated by ion beams was apparently more virulent than the wild-type at a higher temperature (30°C). The results suggest that ion beams are a useful tool for improving thermotolerance in *M. anisopliae* and thereby increasing the potential for application of entomopathogenic fungi as microbial control agents. However, we also found that some thermotolerant mutants generated by ion beams, including mutant isolate AcMa5-ib, almost lost their virulence entirely. This finding suggests that thermotolerance does not always promise higher virulence at a high temperature range. The resulting mutant isolates should be carefully evaluated for unpredictable negative effects before practical use is possible (Shapiro-Ilan, Reilly, and Hotchkiss 2011; Shinohara et al. 2013).

The mechanisms for enhanced thermotolerance in the mutants are unknown although initial studies (data not shown) suggest there were no mutations in the neutral trehalase gene, β -tubulin gene or the ABC transporter gene (*ifT1*) previously associated with thermotolerance and fungicide tolerance in entomopathogenic fungi (Fitriana et al. unpublished; Leng et al. 2011; Song, Ying, and Feng 2011; Zou, Ying, Shen, and Feng 2006). This requires further research.

Acknowledgements

This work was supported by the JSPS KAKENHI under Grant (number 24510121), and the Programme of the Directorate General of Higher Education of Indonesia. We thank J. K. Pell Consulting for editing the manuscript.

256

257 **References**

- 258 Arthurs, S., and Thomas, M.B. (2001), ‘Effects of temperature and relative humidity on
259 sporulation of *Metarhizium anisopliae* var. *acridum* in mycosed cadavers of *Schistocerca*
260 *gregaria*’, *Journal of Invertebrate Pathology*, 78, 59–65.
- 261 Butt, T.M., Jackson C., and Magan, N. (2001), ‘Introduction–Fungal Biological Control
262 Agents, Progress, Problems and Potential’, in *Fungi as Biological Control Agents:*
263 *Progress, Problems and Potential*, eds. T.M. Butt, C. Jackson and N. Magan, Oxon:
264 CABI Publishing, pp. 1–8.
- 265 Cabanillas, H.E., and Jones, W.A. (2009), ‘Effects of temperature and culture media on
266 vegetative growth of an entomopathogenic fungus *Isaria* sp. (Hypocreales:
267 Clavicipitaceae) naturally affecting the whitefly, *Bemisia tabaci* in Texas’,
268 *Mycopathologia*, 176, 263–271.
- 269 Castrillo, L.A., Roberts, D.W., and Vandenberg, J.D. (2005), ‘The fungal past, present, and
270 future: Germination, ramification, and reproduction’, *Journal of Invertebrate Pathology*,
271 89, 46–56.
- 272 de Crecy, E., Jaronski, S., Lyons, B., Lyons, T.J., and Keyhani, N.O. (2009), ‘Directed
273 evolution of a filamentous fungus for thermotolerance’, *BMC Biotechnology*, 9, 74.
- 274 Copping, L.G. (2009), *The Manual of Biocontrol Agents, 4th edition*, Hampshire: BCPC
275 Publications.
- 276 Darbro, J.M., Graham, R.I., Kay, B.H., Ryan, P.A., and Thomas, M.B. (2011), ‘Evaluation of
277 entomopathogenic fungi as potential biological control against of the dengue mosquito,
278 *Aedes aegypti* (Diptera: Culicidae)’, *Biocontrol Science and Technology*, 21, 1027–1047.
- 279 Das, S.K., Masuda, M., Hatashita, M., Sakurai, A., and Sakakibara, M. (2008), ‘A new
280 approach for improving cordycepin productivity in surface liquid culture of *Cordyceps*

militaris using high-energy ion beam irradiation', *Letters in Applied Microbiology*, 47, 534–538.

Davidson, G., Phelps, K., Sunderland, K.D., Pell, J.K., Ball, B.V., Shaw, K.E., and Chandler, D. (2003), 'Study of temperature–growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development', *Journal of Applied Microbiology*, 94, 816–825.

Dimbi, S., Maniania, N.K., Lux, S.A., and Mueke, J.M. (2004), 'Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies', *BioControl*, 49, 83–94.

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A., and Rougier, M. (1997), 'Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins', *Mycologia*, 89, 383–392.

Inglis, G.D., Johnson, D.L., Cheng, K.J., and Goettel, M.S. (1997), 'Use of pathogen combinations to overcome the constraints of temperature on entomopathogenic Hyphomycetes against grasshoppers', *Biological Control*, 8, 143–152.

Jaronski, S.T. (2010), 'Ecological factors in the inundative use of fungal entomopathogens', in *The ecology of fungal entomopathogens*, eds. H.E. Roy, F.E. Vega, D. Chandler, M.S. Goettel, J.K. Pell and E. Wajnberg, Dordrecht: Springer, pp. 159–185.

Kim, J.S., Kassa, A., Skinner, M., Hata, T., and Parker, B.L. (2011), 'Production of thermotolerant entomopathogenic fungal conidia on millet grain', *Journal of Industrial Microbiology and Biotechnology*, 38, 697–704.

Leng, Y., Peng, G., Cao, Y., and Xia, Y. (2011), 'Genetically altering the expression of neutral trehalase gene affects conidiophore thermotolerance of the entomopathogenic fungus *Metarhizium acridum*', *BMC Microbiology*, 11, 32.

Li, J., and Feng, M.G. (2009), 'Intraspecific tolerance of *Metarhizium anisopliae* conidia to the upper thermal limits of summer with a description of a quantitative assay system',

307 *Mycological Research*, 113, 93–99.

308 Matuo, Y., Nishijima, S., Hase, Y., Sakamoto, A., Tanaka, A., and Shimizu, K. (2006),
 309 ‘Specificity of mutations induced by carbon ions in budding yeast *Saccharomyces*
 310 *cerevisiae*’, *Mutation Research*, 602, 7–13.

311 Pell, J.K., Hannam, J.J., and Steinkraus, D.C. (2010), ‘Conservation biological control using
 312 fungal entomopathogens’, in *The ecology of fungal entomopathogens*, eds. H.E. Roy, F.E.
 313 Vega, D. Chandler, M.S. Goettel, J.K. Pell and E. Wajnberg, Dordrecht: Springer, pp.
 314 187–198.

315 Rangel, D.E.N., Butler, M.J., Torabinejad, J., Anderson, A.J., Braga, G.U.L., Day, A.W., and
 316 Roberts, D.W. (2006), ‘Mutants and isolates of *Metarhizium anisopliae* are diverse in
 317 their relationships between conidial pigmentation and stress tolerance’, *Journal of*
 318 *Invertebrate Pathology*, 93, 170–182.

319 Shapiro-Ilan, D.I., Reilly, C.C., and Hotchkiss, M.W. (2011), ‘Comparative impact of
 320 artificial selection for fungicide resistance on *Beauveria bassiana* and *Metarhizium*
 321 *brunneum*’, *Environmental Entomology*, 40, 59–65.

322 Shikazono, N., Yokota, Y., Kitamura, S., Suzuki, C., Watanabe, H., Tano, S. and Tanaka, A.
 323 (2003), ‘Mutation rate and novel *tt* mutants of *Arabidopsis thaliana* induced by carbon
 324 ions’, *Genetics*, 163, 1449–1455.

325 Shikazono, N., Suzuki, C., Kitamura, S., Watanabe, H., Tano, S., and Tanaka, A. (2005),
 326 ‘Analysis of mutations induced by carbon ions in *Arabidopsis thaliana*’, *Journal of*
 327 *Experimental Botany*, 56, 587–596.

328 Shinohara, S., Fitriana, Y., Satoh, K., Narumi, I., and Saito, T. (2013), ‘Enhanced fungicide
 329 resistance in *Isaria fumosorosea* following ionizing radiation-induced mutagenesis’,
 330 *FEMS Microbiology Letters*, 349, 54–60.

331 Song, T.T., Ying, S.H., and Feng, M.G. (2011), ‘High resistance of *Isaria fumosorosea* to
 332 carbendazim arises from the overexpression of an ATP-binding cassette transporter (ifT1)

rather than tubulin mutation', *Journal of Applied Microbiology*, 112, 175–184.

SPSS (2009), PASW Statistics 18. Chicago: SPSS Inc.

Tanaka, A., Shikazono, N., and 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', *Journal of Radiation Research*, 51, 223–233.

Thomas, M.B., and Jenkins, N.E. (1997), 'Effects of temperature on growth of *Metarhizium flavoviride* and virulence to the variegated grasshopper, *Zonocerus variegatus*', *Mycological Research*, 101, 1469–1474.

Toyoshima, Y., Takahashi, A., Tanaka, H., Watanabe, J., Mogi, Y., Yamazaki, T., Hamada, R., Iwashita, K., Satoh, K., and Narumi, I. (2012), 'Lethal and mutagenic effects of ion beams and γ -rays in *Aspergillus oryzae*', *Mutation Research*, 740, 43–49.

Vidal, C., Fargues, J., and Lacey, L.A. (1997), 'Intraspecific variability of *Paecilomyces fumosoroseus*: effect of temperature on vegetative growth', *Journal of Invertebrate Pathology*, 70, 18–26.

Zimmermann, G. (2007), 'Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*', *Biocontrol Science and Technology*, 17, 553–596.

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

Table 1. Origin of thermotolerant *M. anisopliae* mutants developed by ion-beam or gamma-ray irradiation.

Wild-type isolate	Mutant isolate	Irradiation (dose, Gy)
AcMa5	AcMa5-ib	Ion-beam (300)
	AcMa5-gr-1	Gamma-ray (100)
	AcMa5-gr-2	Gamma-ray (100)
	AcMa5-gr-3	Gamma-ray (1,000)
PaMa02	PaMa02-ib	Ion-beam (100)

Table 2. Colony diameter (mm, mean±SD) of wild-type and mutant isolates of *M. anisopliae* on SDA at different temperatures.

Isolate	Temperature (°C)									
	15	20	25	30	33	35	36	37	38	39
AcMa5 (wild-type)	4.3±0.6	15.0±0.7	23.7±0.3	22.0±0.7	16.1±0.2	5.4±0.5	2.1±1.0	0	0	0
AcMa5-ib	2.7±0.6 *	19.3±1.3	34.2±0.5 *	27.9±1.0 *	18.9±0.7 *	12.2±0.4 *	4.9±0.2 *	1.0±0.0 *	0.7±0.6	0
AcMa5-gr-1	4.1±0.2	19.1±1.0	24.7±0.7	22.3±0.3	18.7±0.7 *	11.0±0.0 *	6.8±0.2 *	3.2±0.4 *	1.7±0.6 *	0
AcMa5-gr-2	3.4±0.5	18.7±0.0 *	26.3±0.6 *	25.2±0.4 *	16.0±1.5	10.8±0.2 *	6.1±0.8 *	3.1±0.2 *	2.3±0.9 *	0
AcMa5-gr-3	2.9±0.7	18.6±0.5 *	27.1±0.2 *	25.5±0.4 *	19.1±0.2 *	11.7±1.2 *	5.6±0.2 *	2.9±0.5 *	2.3±0.6 *	0
PaMa02 (wild-type)	9.9±1.0	19.1±0.2	32.7±1.2	26.0±1.0	15.7±0.6	3.0±0.0	0	0	0	0
PaMa02-ib	8.7±0.6	14.8±0.2 *	23.0±0.0 *	23.0±1.0	15.3±0.7	6.6±1.0 *	2.6±0.5 *	1.1±0.2 *	1.0±0.0 *	0

* indicates significant difference in colony diameter between wild-type and each mutant isolates ($P<0.05$, Mann-Whitney U test).

Table 3. Percent germination (mean $\pm$ SD) of wild-type isolates of *M. anisopliae* and the mutants derived from them after exposure to wet-heat stress of 45°C.

Isolate	Hours of exposure to wet-heat stress			
	0	0.5	1	3
AcMa5 (wild type)	92.3 $\pm$ 0.4 a	18.7 $\pm$ 3.5 a	0 a	0
AcMa5-ib	93.0 $\pm$ 1.8 a	66.1 $\pm$ 6.9 d	19.3 $\pm$ 1.8 c	0
AcMa5-gr-1	94.3 $\pm$ 2.3 a	22.9 $\pm$ 3.2 ab	7.5 $\pm$ 3.2 b	0
AcMa5-gr-2	96.0 $\pm$ 1.3 a	45.9 $\pm$ 7.4 c	5.6 $\pm$ 1.4 b	0
AcMa5-gr-3	92.4 $\pm$ 1.9 a	35.0 $\pm$ 5.5 bc	8.8 $\pm$ 2.7 b	0
PaMa02 (wild type)	91.6 $\pm$ 3.0	16.0 $\pm$ 2.3	0	0
PaMa02-ib	91.0 $\pm$ 0.5	12.2 $\pm$ 2.0	0	0

In AcMa5 and the mutants derived from it, means followed by different letters in each column are significantly different from each other ($P < 0.05$, Tukey's HSD test). There was no significant difference between PaMa02 and the mutant derived from it ($P > 0.05$, Student's *t*-test).

Figure legends

Figure 1. Virulence of wild-type and mutant isolates of *M. anisopliae s.l.* against rice weevil adults at 25°C. A. Wild-type isolate AcMa5 and the mutants derived from it. B. Wild-type isolate PaMa02 and the mutant derived from it.

Figure 2. Virulence of wild-type and mutant isolates of *M. anisopliae s.l.* against rice weevil adults at 30°C. A. Wild-type isolate AcMa5 and the mutants derived from it. B. Wild-type isolate PaMa02 and the mutant derived from it.

A

B