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Mutant Screening of *Metarhizium lepidiotae* for Increased UV-Tolerance and Virulence

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WH, DY, SH, JX and PQ performed the experiments and analyzed the data. ZH and AS conceived and designed the experiments. ZH analyzed the data and wrote the article.

Key words
***Metarhizium lepidiotae*, Screen UV-induced mutant, Increased UV-tolerence, Increased virulence, Fungal secondary metabolites**

ABSTRACT

The ability of entomopathogenic fungi to be applied for pest control in field applications is often hampered by negatively active abiotic factors including high temperature, desiccation and UV irradiation. Selecting isolates with high UV tolerance and virulence is important in improving the efficacy and utility of fungal insect pathogens as insect biological control agents for use under field conditions. UV-irradiation of *Metarhizium lepidiotae,* coupled to growth selection, second metabolites change and insect bioassays using *Plutella xylostella* larvae as the host resulted in the isolation of a collection of mutants with increased virulence. One mutant, designated, MIUV-40b showed ~3.3-fold increase in virulence as compared to the wild type parent, with an LC_{50} = 0.3 ×10^5 conidia/ml versus 1.1 × 10^5 conidia/ml, respectively and LT_{50} = 92 and 123.0 h for the MIUV-40b and wild type, respectively. The MIUV-40b mutant displayed increased UV tolerance, but decreased total conidial production. In addition, alterations in the secretome were seen in the mutant. Contact insect toxicity of cell-free culture supernatants and the EthOAc extracts derived from the MIUV-40b mutant were 1.2-3 times more potent than that of the wild type. A simple approach coupling mutagenesis and growth & second metabolites were used to isolate strains with increased stress resistance and virulence. Increased virulence in some of the mutants correlated with increased insecticidal activity in cell-free extracts that could potentially be used directly for insect control.

INTRODUCTION

Broad host range insect pathogenic fungi, including *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria fumososea* have the capacity to control a wide variety of insect pests and used as biological control agent for instead of insecticides that were overused and caused to pesticide residue in agricultural production and environment (Glare *et al*., 2012; Lacy *et al*., 2015; Huang *et al*., 2016; Zhao *et al*., 2016). The efficacy of entomopathogenic fungi in insect pest control in field applications is often hampered by abiotic factors including, high temperature, desiccation exposure to UV irradiation (Bocias *et al*., 2018; Fernandes *et al*., 2015; Ortiz-Urquiza and Keyhani, 2015). A number of approaches have been used to increase resistance of insect pathogenic fungi to a wide range of abiotic stresses. These include genetic manipulation, manipulation of culture conditions, and addition of UV and other protectants (Ortiz-Urquiza and Keyhani, 2015; Inglis *et al*., 1995; Leland *et al*., 2005; St Leger and Wang, 2010; Behle *et al*., 2009). For example, transformation of the melanin biosynthesis genes containing a polyketide synthase, a scytalone dehydratase, and a 1, 3, 8-trihydroxynapthalnene reductase, into *M. anisopliae* enhanced resistance to UV irradiation and thermal stress (Tseng *et al*., 2011). Although molecular manipulation has opened a wide range of targets for fungal modification to enhance stress resistance (Ortiz-Urquiza and Keyhani, 2015; Tseng *et al*., 2011), significant obstacles remain particularly in acceptance or adoption by regulatory agencies, and the use of genetically modified strains are unlikely to occur in the near future. Several studies have assessed the effects of UV radiation on entomopathogenic fungi and have tried to isolate mutants with greater resistances, i.e. selection of *M. anisopliae* mutants with faster growth after UV irradiation resulted in a mutant with increased in UV tolerance as well as virulence (Zhao *et al*., 2016), and screens measuring UV-B susceptibility revealed significant variation amongst natural *M. anisopliae* isolates (Braga *et al*., 2001a, 2001b). It has also been shown that exposure to the two components of sunlight, i.e. solar UV-A and UV-B radiation, can directly reduce the survival of fungal conidia by inducing formation of

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cyclobutane pyrimidine dimers in the organisms genome (DNA) as well as toxicity due to the production of reactive oxygen species (Friedberg *et al*., 1995; Fargues *et al*., 1997; Griffiths *et al*., 1998; Hughes *et al*., 2003).

The susceptibility of entomopathogenic fungi to UV irradiation that can results in conidial inactivation, delayed conidial germination, and/or reduced mycelia growth-all important parameters for successful mycosis of insect targets, suggests that selecting isolates with based on these parameters linked to high UV tolerance, while maintaining virulence may be a feasible approach to improving the efficacy of these fungi as insect biological control agents for use under field conditions that have high solar UV exposure (Leland and Behle, 2005; Fargues *et al*., 1996; Huang and Feng, 2009; Keyer *et al*., 2014; Huang *et al*., 2010). Our objectives in this study were to develop a simple mutant selection and screening approach to identify fungal isolates with increased UV and virulence for use in insect pest management, using the more narrow host range species, *M. lepidiotea* and its diamondback moth host, the latter an important world-wide pest of vegetable crops including cabbage, collard (Huang *et al*., 2010).

MATERIALS AND METHODS

Preparation of fungal strains and insects

Metarhizium lepidiotae strain Ml03, isolated from Yunnan province, China, was identified by ITS sequencing using purified genomic DNA as the template and primer pairs ITS4/ITS5 (5'-TCCTCCGCTTATTGATATGC-3' /5'-GGAAGTAAAAGTCGTAACAAGG-3'). The resultant sequence (accession number AY646386.1) was analyzed via bioinformatics homology searching performed using BLAST algorithms against various databases (GenBank, http://www.ncbi.nlm.nih.gov/BLAST). Fungal isolates were cultured on potato dextrose agar (PDA) and aerial conidia were harvested by flooding the plate with sterile dH_2O + 0.1% Tween-80, 7-10 d after growth at 25 °C. The suspension was vigorously stirred and filtered through four layers of medical gauze (Lantian, Henan, China) to remove any mycelia after vigorously stirred 30 min on magnetic stirrer. Conidial suspensions were adjusted to concentrations as needed for insect bioassay use. *Plutella xylostella* were reared as described (Huang *et al*., 2010) and second instar *P. xylostella* was used in fungal virulence bioassays. *Brassica campestris* L. plants cultured in plastic pots were incubated at 26 ± 2°C. Intact plants were maintained in greenhouses until used.

UV mutagenesis of Metarhizium lepidiotae

Fungal mutants were isolated essentially as described (Zhao *et al*., 2016; Hughes *et al*., 2003). Briefly, a conidial suspension (5 ml, 1.0 × 10^6 conidia/ml) in potato dextrose broth (PDB) was incubated at 26 ± 1°C for 20- 24 h in a sterile Petri dish (Ø 9 cm). Germinated spores were exposed to UV-light (λ = 254 nm, 120 J/cm^2, Laminar Flow Cabinet, SJ-CJ-2FQ) for a series of exposure times, i.e. 0, 20, 40, and 60 min of exposure. After treatment, an aliquot from the cell suspension (100 l) was spread onto PDA (100 plates for each time point). Plates were incubated in the dark at 26 ± 1°C for 48 h and then for an additional 7-13 d at 26 ± 1°C using a light: dark cycle ratio equal to 14:10. Morphological observations led to the selection of ~90 of the fastest growing and second metabolites change (much different color in second metabolite observed from colony bottom view) colonies that were then streaked onto fresh PDA and Czapek-Dox agar (CZA) plates for further isolates screen.

Mutant phenotype characterization

The effect on UV exposure on fungal growth and second metabolite change were measured by plating of 10 µl aliquot of conidial suspensions (1×10^6 conidia/ml in 0.1% Tween-80) after treatment onto various media including PDA, CZA, Sabouraud dextrose agar (SDA) and SDA + 1% yeast extract (SDAY). Plates were incubated at 26±1°C for 12 days, with colony morphology and radial growth diameters were measured and second metabolite change was observed daily. Total conidial yield was determined as described (Zhao *et al*., 2016; Luo *et al*., 2014). Briefly, conidial suspensions (10 µl, 1×10^6 conidia/ml in 0.1% Tween-80) were spread onto PDA in 12 cm-diameter Petri dishes and the treated plates were incubated at 26 ± 1°C for 11, 15 and 20 days. Conidia were harvested and conidial concentrations determined as described above. Experiments were performed in triplicate, and each experiment repeated three times using different conidial batches.

Preparation of cell-free culture supernatants and ethyl acetate extracts

Conidia from the wild type (M103) and UV-mutant strain (UV-40b), (5 ml, 1×10^6 conidia ml^{-1}) were used to inoculate, a 1 L flask containing 200 ml of Czapek-Dox broth supplemented with 1% peptone (CZP). Flasks were incubated for 4 d (180 rpm at 26 ± 1°C), after which the growing culture was added to fresh CZP at 1: 9 ratios (v/v, 6000 ml total volume), and cultures were allowed to continue to grow (180 rpm at 26 ± 1°C) for 8 d. Samples (1000 ml) were harvested from the cultures after 3, 4, 5, 6, 7, and 8 d of fermentation, Aliquots were centrifugation at 12000 x g for 15 min to remove fungal cells, and the cell-free culture supernatant was filtered (Millipore 0.45um membrane, Qianhui Co. Guangzhou, China) and stored at

4 °C until use. Total protein from the cell-free supernatants (i.e. representing the "secretome" of the fungus) was extracted using trichloroacetic acid (TCA) and acetone as described (Zhao *et al*., 2016). Briefly, three volumes (6 ml) of pre-cooled 15% (w/v) TCA-acetone solution was added to the cell-free supernatant and after mixing, the precipitated proteins were harvested by centrifugation (3000xg, 10 min, 4 °C). The resulting protein pellets were washed three times with pre-chilled acetone (1 ml) and stored at 4°C until use. Precipitated proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, protein bands were visualized by silver nitrate staining (Ausubel *et al*., 1997).

Fungal metabolites from the cell-free culture supernatant were isolated via ethyl acetate (EthOAc) extraction as described (Zhao *et al*., 2016). Aliquots (6000 ml) derived from the cell-free culture supernatants were mixed with ethyl acetate (1: 1) and stirred vigorously and the organic phase was collected and concentrated. Samples were extracted three times and the organic phases pooled and concentrated. EthOAc-extracts were stored at -20°C until use. The sample of EthOAc extract for analysis was prepared by re-dissolved in MeOH and then filtered. One milliliter (saturated solution) of the UV-40b extract was analyzed by preparative HPLC using a C18 column (250 × 21.2 mm, 5 µm, Varian Dynamax, Elk Grove Village, IL, USA) eluted with a gradient system of methanol/water from 10:90 to 100:0 at a flow rate of 1 ml min^{-1} for 45 min.

Insect bioassays

Insect bioassays were used to evaluate the effect of UV irradiation on fungal virulence were performed using *P. xylostella* as the host as described (Huang *et al*., 2010). Second instar of *P. xylostella* larvae was treated topically by immersion in the conidial suspensions at different concentrations (i.e. 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7 conidia/mL) for 10 s, and then the larvae (30-35/replicate) were left to air dry before being placed on a dry filter paper inside clear Petri dishes. Control larvae were dipped in H_2O + 0.1% Tween-80 for 10 s. Petri dishes with treated insects were incubated at 26 ± 1 °C RH (relative humidity) > 80% and a photoperiod of 14:10 (L:D). Fresh 3x3-cm *B. campestris* leaf disks were supplied as food for the larvae. Insects were monitored daily for mortality and/or adult emergence. Dead insects were removed immediately and incubated separately on moistened filter paper in a clear Petri dish to observed development of fungal sporulation on the cadavers. Each treatment was performed in triplicated on different days using different batches of insects and fresh conidial suspensions. For bioassay of the EthOAc extracts, samples were dissolved in and then was diluted to a concentration of 400 mg/L using dH_2O with 0.1% Tween-80. Five different concentrations (12.5, 25, 50, 100, 200 mg/L) were prepared by serial dilution in dH_2O with 0.1% Tween-80. Excised leaves containing second instar *P. xylostella* were immersed in the test solutions for 10 s. Control leaves/larvae were treated with 0.1% Tween-80 in sterile distilled H_2O supplemented with equivalent concentrations of acetone as found in each test concentration mixture. Mortality was monitored daily. Treatments consisted of 30-35 leaves with 1-2 insects/leaf and with three technical replicates. The entire bioassay was repeated three different times with different batches of fungal extracts.

UV and stress tolerances of the UV-40b isolate

The UV-tolerance of fungal conidia was evaluated by exposing germinated spores suspensions (5 ml, 1 x 10^7) to UV irradiation (as above) for 0 and 40 minutes. After treatment, aliquots (5 ml) of the cell suspension were mixed with 45 ml CZ broth in a 250-ml flask. Flasks were then incubated with aeration (200 rpm) at 26±1°C. Conidial germination was monitored microscopically in aliquots (100 µl) taken from the growing cultures over a time course (17, 20, 25, and 30 h post-inoculation), with spore considered germinated when the length of the germ tube was equal or greater than the diameter of the spore. The percentage of germinated spore was calculated after examining at least 800 spore for each sample. All experiments were performed using three technical replicates and the entire experiment was repeated twice.

The effect of osmotic (NaCl, Sorbitol), cell wall (Congo Red), and oxidative (H_2O_2) stress agents on fungal growth were assessed using conidial suspensions (10 µl aliquot of 1×10^6 conidia/ml in 0.1% Tween-80) spotted in the center of PDA plates containing 0.5mol/L NaCl, 0.5mol/L Sorbitol, 500ug/ml Congo Red and 100mmol/L H_2O_2, respectively, and incubated at 28±1°C for 5 days. Colony morphology was examined visually and colony diameters were measured daily. Experiments were performed using three technical replicates, and experiments were repeated on different days with new batches of conidia.

Data analyses

Mortality data were arcsine square-root transformed prior to analysis. Mortality data were analyzed as follows; curves of (log concentration – Probit line (LC-p)) were calculated and validated using the chi-square test. The mean lethal concentration to kill 50% of treated hosts (LC_{50}), and median lethal time to kill (LT_{50}) were calculated by Probit analysis using SPSS (Statistical Package for Social Science) 8.0 for windows (SPSS,

1997). Phenoptyic analyses including vegetative growth, conidial germination, and conidial yield were analyzed by using one-way analysis of variance (ANOVA). Mean values were compared by Tukey's student range test (Tukey's HSD, a=0.05) (SASS, 2000).

RESULTS

Isolation of a hyper-virulent M. lepidiotae *UV-induced mutant*

Germinated spores of the *M. lepidiotae* (Ml03) wild type strain were UV-irradiated for 0, 20, 40, and 60 min and aliquots of spores were subsequently plated (~100 plates/treatment) on PDA medium as detailed in the methods section. For each treatment, initial plating resulted in > 5,000 colonies, with the number of the surviving colony forming units (CFUs) recovered on PDA plates declining with increasing exposure time. After 7-13 d growth, a total of 90 of the fastest growing colonies were selected and re-streaked on fresh PDA and CZA plates for further isolation screen. In the second cycle of isolates screen, a total of 63 (out of 90 isolates) of the fastest growing and second metabolites changed colonies derived from the 20, 40, and 60 min treatment time points were selected and re-streaked on fresh PDA and CZA plates. The process was repeated for 2 additional cycles resulting in a final set of 33, 19, and 11 isolates originally from the 20, 40, and 60 min time points, respectively. These isolates were selected based on rapid growth phenotypes and second metabolites changed, i.e. those displaying greater than wild type radial vegetative growth on PDA and CZA plates, and overall appearance (color, colony morphology, & robust conidiation) most similar to wild type; at the same time, those showing much different color in second metabolite observed from colony bottom view. All sixty-three original isolates were screened in a preliminary insect bioassay using a concentration of 1 x 10^7 conidia/ml and second instar *P. xylostella* larvae. From this screen, a total of twenty-one isolates corresponding to 9, 7, and 5 mutants, from the 20, 40, and 60 min UV-treatment time points, respectively, were identified as having greater mortality that the wild type strain 3 and 6 d post-inoculation (data not shown). From the twenty-one mutant pool that the mutants were maintained after 5 subculturing cycles in PDA and CZA medium, secondary growth experiments and insect bioassays for those displaying the highest virulence and fastest growth, one mutant from each time point, designated at MlUV-20a, MlUV-40b, and MlUV-60c, were further characterized.

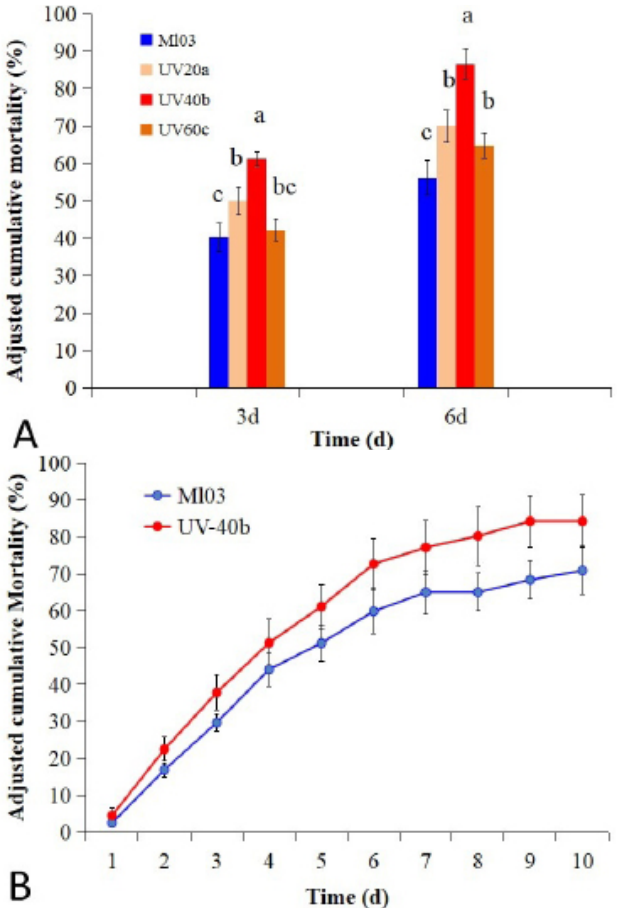

Fig. 1. A, Virulence of wild-type strain (Ml03) and fast growing UV-induced mutants UV-20a, UV-40b, and UV-60c; B, the percent mortality is given on 3 and 6 d post-infection. (B) Adjusted accumulative mortality of wild-type strain (Ml03) and MlUV-40b mutant (UV-40b) tested against 2nd instar *P. xylostella*. The percent mortality is given at concentration of 1.0x106 conidia/ml. Data are presented as Mean ± SE.

Insect bioassays of selected UV-mutants

The wild type and mutant *M. lepidiotae* strains, MlUV-20a, MlUV-40b and MlUV-60c, were tested against *P. xylostella* larvae as detailed in the Methods section. A general trend in increased virulence was seen in the mutants as compared to the wild type strain (Fig. 1A). In particular, the MlUV-40b mutant showed significant ($P < 0.05$) increase mortality, with a sharp decrease in the LT_{50}. These data showed an ~61% and 87% mortality at 3 and 6 d post-infection, respectively, for the Uv-40b strain. Mortality was increased by ~51% and 55% as compared to the wild type strain that had a mortality rate of ~40% and 56% at 3 and 6 d, respectively (Fig. 1A). Overall, MlUV-40b conidia showed significantly higher ($P < 0.05$) insecticidal activity than the wild type strain throughout the experiment (Fig.

1B). The calculated LC_{50} values to kill *P. xylostella* larvae at 5 and 10 d post-treatment was 7.25 x 10^5 and 1.08 x 10^5 conidia/ml for the wild type strain, but only 2.35 x 10^5 and 0.29 x 10^5 conidia/ml for the mutant, indicating a ~ 3.1 and 3.6-fold increase in virulence (Table IA). The LT_{50} values at the concentration of 1.0 x 10^6 conidia/mL post-treatment was 123.0 h for the wild type, and 91.9 h for the MlUV-40b mutant, respectively, indicating an ~1.3-fold change (increase) in virulence (using the relative potency test) (Table IB).

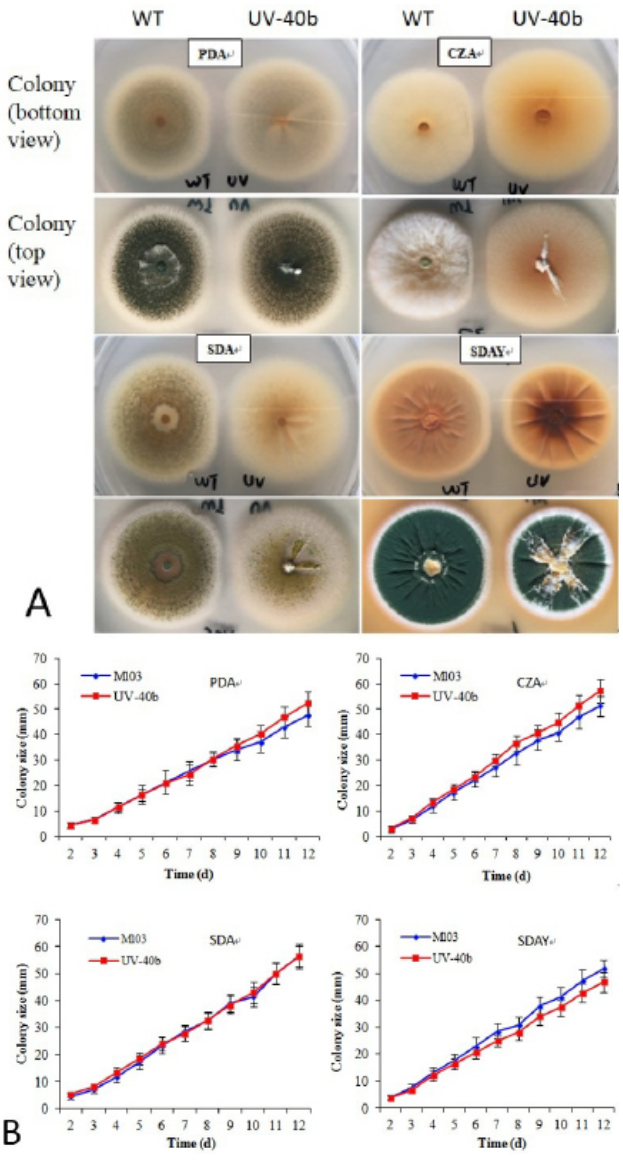

Fig. 2. A, morphological features of wild-type strain (Ml03) and MlUV-40b mutant on PDA, CZA, SDA and SDAY (12 d). B, Vegetative growth of wild-type strain (Ml03) and MlUV-40b (UV-40b) mutant on CZA, PDA, SDA and SDAY. Data are presented as mean ± SE.

Phenotypic characterization of UV-40b

The wild type and MlUV-40b mutant colonies were grown on PDA, CZA, SDA and SDAY for 12 d, with the two isolates differing color, colony morphology (circular versus more longitudinal growth), and changes in the appearance of the fluffy white mycelial growth across the surface of the colony that occurs before conidiation (Fig. 2A). The MlUV-40b isolated displayed similar vegetative growth on PDA, CZA, SDA and SDAY medium over the time course of the experiment (12 d, Fig. 2B). Overall conidial production by the MlUV-40b mutant and the wild type parent was significantly different ($P < 0.05$) over the time course examined (11, 15 and 20 d post-inoculation, Table II). When normalized to total conidial production in terms of colony (conidia/colony), the number of conidia produced by the MlUV-40b mutant in PDA plates after 11, 15 and 20 d of growth was reduced by 17 to 39% as compared to the wild type, i.e. 2.5×10^{10} conidia / colony for the MlUV-40b mutant versus 2.1×10^{10} conidia / colony for the wild type strain after 20 d of growth (on PDA). In contrast, on CZA, conidial production of the MlUV-40b mutant was sharply reduced, from 88-91% of wild type levels.

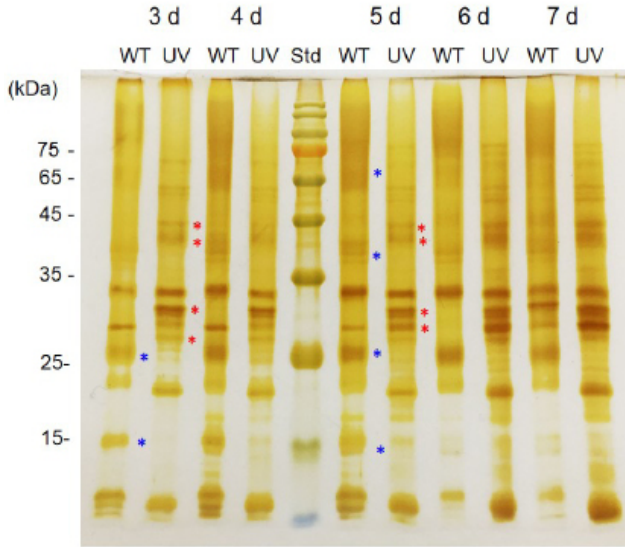

Fig. 3. Secreted protein profiles of *M. lepidiotae* wild type strain (WT) and MlUV-40b mutant (UV). Cell free culture supernatants were isolated and analyzed by SDS- PAGE after 3, 4, 5, 6 and 7 d of culture as detailed in the methods section. Std, molecular weight standards.

As virulence in enotomopathgenic fungi is considered to be significantly impacted by the secretion of cuticle-degrading and other proteins, e.g. lipases, proteins, and potential peptide toxins and effectors. The secretome profile of the wild type *M. lepidiotae* and MlUV-40b

Table IA. Median lethal time (LC_{50}) for conidia of wild type Ml03 and MlUV-40b mutant strain of *M. lepidiotae* against *P. xylostella*.

Time	strains	Regression equation	LC_{50}+SE (10^5conidia/ml)	95% Fiducial limit	$\chi 2$
5d	Ml03	Y=2.3973+0.4441x	7.25+2.74 *	$3.46x10^5$, $1.52x10^6$	0.3240
	MlUV-40b	Y=3.0453+0.3640x	2.35+0.91	$1.10x10^5$, $5.00x10^5$	0.2140
10d	Ml03	Y=2.1470+0.5667x	1.08+0.27 *	$0.66x10^5$, $1.78 x10^5$	0.0521
	MlUV-40b	Y=2.3744+0.5866x	0.29+0.08	$0.18 x10^5$, $0.51 x10^5$	0.0515

Means in the same column followed by * are significantly different (Turkey's HSD test, a=0.05).

Table IB. Median lethal time ($LT_{50,}$ h) for conidia (10^6 conidia/ml) of wild type and MlUV-40b mutant strain of *M. lepidiotae* against *P. xylostella*.

Strains	Regression equation	LT_{50}+SE (h)	95% Fiducial limit	$\chi 2$
Ml03	Y=0.4793+2.1629x	123.04+7.72 *	108.80, 139.15	4.9989
MlUV-40b	Y=-0.0993+2.5970x	91.94+5.75	81.34, 103.93	7.0741

Means in the same column followed by * are significantly different (Turkey's HSD test, a=0.05).

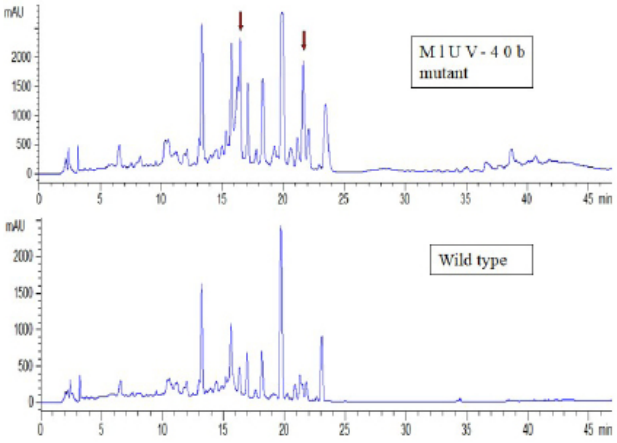

Fig. 4. HPLC analysis of secondary metabolites produced by wild type strain and MlUV-40b mutant in liquid culture.

mutant were examined over time during growth in CZB (Fig. 3). SDS-Page analyses of secreted protein revealed distinct patterns of protein bands between the mutant and wild type parent. These analyses revealed the expression of at least two proteins, one between15-25 kDa and another between 40-75 kDa (Fig. 3, blue asterisks) present in the wild type (at the 3 and 5 d time points) that were not found in similar MlUV-40b extracts. Several moderately expressed proteins of estimate molecular masses between 25-45 kDa, (Fig. 3, red asterisks) were observed in the MlUV-40b mutant that were less visible in the wild type. Virulence is also considered to be impacted by the secretion of fungal toxins. Second metabolite production was evaluated in ethyl acetate extractions of wild type and MlUV-40b mutant culture supernatants. HPLC analysis of fungal metabolite toxins showed many different peaks between Ml03 and MlUV-40b (Fig. 4). Two sharply defined peaks (retention tome between 16 ~ 17 min and 21~22 min) were seen in the mutant (Fig. 4, red arrow) and largely absent in the wild type strain.

UV and stress resistances of MlUV-40b

Conidia derived from *M. lepidiotae* wild type and the MlUV-40b isolated were examined for their susceptibility to UV-exposure (Fig. 5). Exposure of harvested conidia to UV-irradiation for 40 min resulted in a reduction of germination (at 17 h post-treatment incubation in PBD) from ~18% to ~11% for the wild type, but remained unaffected in the mutant where germination was between 16-17% irrespective of UV-treatment. Germination remained reduced for the wild type reaching only 39% after 30 h recovery, representing a 40-60% reduction in germination as compared to untreated wild type cells. In contrast, overall germination for the MlUV-40b mutant was similar for both untreated and UV-exposed cells, over the post-recovery time course.

The sensitivity of the wild type and MlUV-40b isolates to NaCl, Sorbitol, Congo Red and H_2O_2 stress was examined (Fig. 6). The colony diameter of UV-40b mutant was 1.4 times larger than that of the wild type stain when growth on PDA supplemented with 0.5 M NaCl. However, growth of MlUV-40b reduced (10-20%, $P < 0.05$) compared to the wild type stain when cultured on PDA medium amended with sorbitol (0.5 mM) or H_2O_2 (100 mM). The MlUV-40b mutant produced more conidia than the wild type strain (increased 41%, $P<0.05$) when

grown on media containing Congo Red, however, reduced conidial yields were seen for the UV-40b mutant as compared to wild type strain during growth on sorbitol or H_2O_2 (reduced 29% or 32%, respectively, P<0.05).

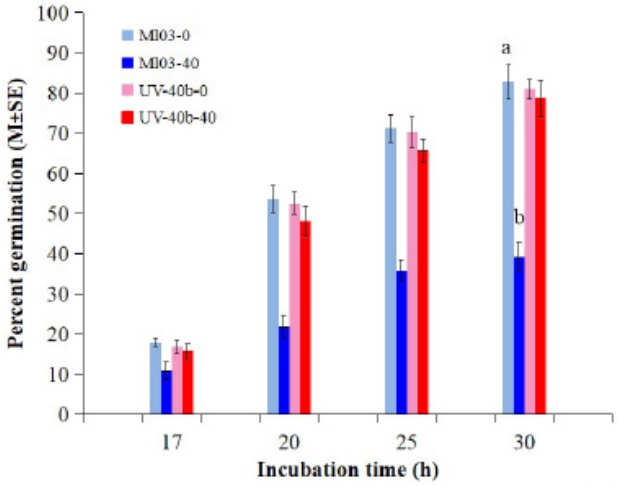

Fig. 5. Effect of UV exposure on germination of *M. lepidiotae* Ml03 wild-type strain and MlUV-40b mutant. Conidial germination was determined over the indicated time course for untreated (Ml03-0 and UV-40b-0) and cells exposed to UV radiation for 40 min (Ml03-40 and UV-40b-40) (as described in the "Materials and Methods" section). Data are presented as mean ± SE.

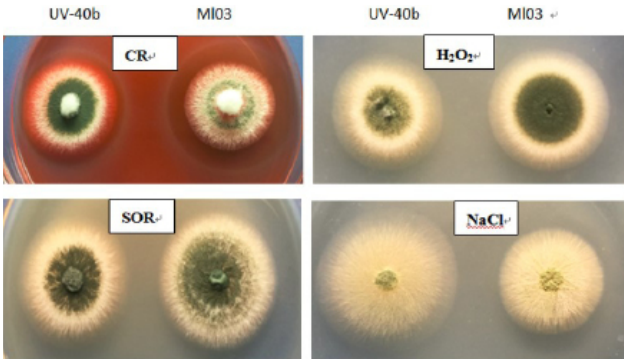

Fig. 6. Morphological features of wild-type strain (Ml03) and MlUV-40b (UV-40b) mutant on PDA medium plus NaCl, Sorbitol (SOR), Congo Red (CR) and H_2O_2.

Cell-free extracts derived from MlUV-40b display increased insecticidal activity

Cell-free culture supernatants were isolated from *M. lepidoitea* wild type and MlUV-40b mutant strains after 4, 6, 8 d fermentation as detailed in the Methods section and tested for insect toxicity using *P. xylostella* larvae as the host. TCA (protein) precipitated culture supernatants derived from MlUV-40b showed ~1.2-2.1 fold higher insecticidal activity than wild type extracts (Table III). Cell-free culture supernatants were also extracted by ethyl acetate as indicated in the Methods section. Ethyl acetate extracts of the wild type and MlUV-40b strains showed time- and dose-dependent toxicity towards *P. xylostella* larvae (Fig. 7). The ethyl acetate extract derived from the UV-40b mutant showed ~3.2-fold higher insecticidal activity than the wild type (parallelism test). The LC_{50} values for the wild type and MlUV-40b extracts at 96 and 120 h post-treatment were 107.9 mg/L and 86.5 mg/L, and 30.9mg/L and 26.6 mg/L, respectively (Table IV).

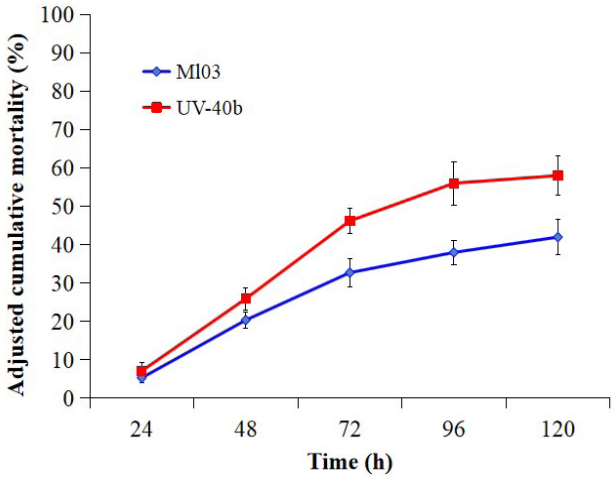

Fig. 7. Adjusted cumulative mortality of ethyl acetate extracts from wild-type strain (Ml03) and MlUV-40b (UV-40b) mutant tested against 2nd instar *P. xylostella*. The percent mortality is given at concentration of 50 mg/L. Data are presented as Mean± SE.

Table II. Conidial production by the *M. lepidiotae* Ml03 wild type and mutant MlUV-40b strains on different media (conidia/colony, x10^7).

Media	Strain	11 d	15 d	20 d
PDA	Ml03	341.3±29.7*	1119.3±87.2*	2549.2±103.6*
	MlUV-40b	207.9±13.5	733.1±45.6	2103.8±113.7
CZA	Ml03	145.3±11.2*	347.2±21.8*	501.7±33.6*
	MlUV-40b	11.9±2.4	30.4±3.5	56.4±4.1

Means in the same column followed by * are significantly different (Turkey's HSD test, a=0.05).

DISCUSSION

There is significant interest in the application of integrated management practices for the control of *P. xylostella*, a pest of cruciferous vegetables, worldwide, that

Table III. Adjusted accumulative mortality of cell free culture supernatants from wild-type strain (Ml03) and MlUV-40b mutant tested against *P. xylostella*.

Strains	4 d fermentation		6 d fermentation		8 d fermentation	
	3d	6d	3d	6d	3d	6d
Ml03	11.8±0.9	26.7±1.7	29.4±1.6	33.3±2.9	35.3±2.3	46.7±3.1
MlUV-40b	23.5±1.9*	40.0±2.4*	41.2±2.1*	53.3±4.5*	47.1±2.8*	60.0±4.1*

Means in the same column followed by * are significantly different (Turkey's HSD test, a=0.05).

Table IV. Median lethal concentrations (LC_{50} mg/L) for the ethyl acetate extracts of wild type and MlUV-40b mutant of *M. lepidiotae*strain against *P. xylostella.*

Strains	Time	Regression equation	LC_{50}(95% Fiducial limit)	χ^2
Ml03	48h	Y=2.9279+0.7161x	782.5+359.1(318.3, 1923.5)	2.2177
	72h	Y=3..0658+0.8425x	197.6+38.4(135.1,289.1)	3.9048
	96h	y=2.9654+1.0007x	107.9+14.4(83.1, 140.2)	5.0427
	120h	y=2.9794+1.0431x	86.5+11.2(67.1, 111.6)	5.6142
MlUV-40b	48h	Y=3..3943+0.5809x	581.2+377.9(162.5, 2078.7)	3.3441
	72h	Y=3.6286+0.7608x	61.6+10.9 (43.5, 87.2)	6.4275
	96h	y=3.4343+1.0510x	30.9+4.4(23.3, 40.9)	8.4442
	120h	y=3.2233+1.2472x	26.6+3.6(20.5, 34.5)	9.4011

has in several instances developed resistance to various conventional insecticides (Xu *et al*., 2004; Guo *et al*., 2013; Zalucki *et al*., 2012; Sun *et al*., 2012). Although many strains of entomopathogenic fungi have been reported to have the potential to control *P. xylostella* larvae under laboratory conditions, various abiotic factors have limited their use in field. Prominent among these include solar radiation, high temperature and low humidity (Fernandes *et al*., 2015; Tseng *et al*., 2011; Braga *et al*., 2001a; Zimerman, 1982; Fang *et al*., 2012). It is now recognized that for insect pathogenic fungi to survive and persist long enough to have an effect in the insect habitat knowledge concerning the insect life-style coupled to high UV and thermal stress tolerances of the infectious conidia are needed (de Crecy *et al*., 2009). Studies on fungal genetic factors involved in conidial viability, regulation of stress tolerance, and membrane stability have been shown to be critical for virulence (Qin *et al*., 2014; He *et al*., 2015). Many researchers have evaluated the effects of UV irradiation on *M. anisopliae* virulence (Zhao *et al*., 2016; Aidroos and Seifert, 1980; Alston *et al*., 2005) and UV tolerances in *Beauveria* spp. (Fernandes *et al*., 2007). It is well known that conidial pigmentation may confer protection against UV-irradiation owing to ability to absorb energy in the UV region of the spectrum (Ortiz-Urquiza and Keyhani, 2015; St Leger and Wang, 2010; Braga *et al*., 2006; Rangel *et al*., 2006). Transformation of pigmentation related gene in to *M. anisopliae* resulted in production of melanin mutant strain with increased tolerance to UV-irradiation as well as virulence (Behle *et al*., 2009). Approaches to isolate more vigorous strains that do not rely on genetic manipulation, typically rely on either screening of environmental and/or mutant isolates with desirable phenotypes, e.g. increased virulence and UV-resistance. A previous study used UV-irradiation to isolate mutants of a strain of the broad host range entomopathogen, *M. anisopliae*, resulted in mutants with increased virulence (Zhao *et al*., 2016). It was, however, unclear, to what extent this approach was more broadly applicable, especially using a narrower host range species.

Our data indicate that UV-irradation followed by growth and colony morphology screening represents a simple means of isolating mutants with both increased UV-tolerance and virulence. Our data show that mutant MlUV-40b displayed significantly increased UV tolerance as compared to the wild type parent, retaining greater than 80% conidial germination rates after 40 min exposure to UV radiation, as opposed to only ~40% germination seen for the wild type (30 h post-recovery in nutrient media). Similar results were obtained by Zhao *et al*. (2016), who isolated a mutant from *M. anisopliae* that showed greater UV tolerance with conidial germination unaffected by UV-irradiation as compared to the wild type parent.

After UV-irradiation > 5000 *M. lepidoitae* mutants were generated. As expected, overall survival during the mutagenesis protocol decreased with increased time of

exposure to UV irradiation (range tested from 20 to 60 min). Three rounds of successive screening of colonies were subsequently performed by selecting those showing the fastest vegetative (colony) growth resulting in a pool of ~61 mutant isolates. These mutants were then assayed for any changes in virulence using *P. xylostella* larvae and for any alterations to UV tolerance. A total of 21 colonies were obtained meeting our criteria that included increased virulence, faster vegetative colony growth and higher UV tolerance. One colony selected from the 40 min UV irradiation exposure, designated as MlUV-40b with the highest virulence and UV tolerance in preliminary bioassays, was chosen for further study. The MlUV-40b mutant showed an approximate 3-fold decreased in the LC_{50} as compared to the wild type parent indicating that less spores were needed for similar levels of control. Similarly, MaUV40.1 mutant revealed sharp increase in virulence as having ~2-fold decrease in the LT_{50} as compared to its parental strain (Zhao *et al*., 2016). It is interesting to note that in both instances, the major trade-off appeared to be decreased conidiation, ~20-30% in standard media (PDA), but a 80-90% decrease in more minimal media (CZA). These data suggest that conidiation acts as a significant barrier limiting aspects of the physiology of the fungus, including stress responses and virulence.

Secreted proteins, e.g. chitinases, proteinases, lipases, and other cuticle degrading enzymes, are considered important fungal virulence factors that are essential to the ability of the fungi to penetrate the insect exoskeleton. In order to test whether alterations in the secreted protein repertoire of *M. lepidoitae* was seen in the MlUV40b mutant, cell-free culture supernatants were generated. SDS-PAGE analyses of the "secretome" revealed changes, with both the expression of additional (new) proteins and decreased expression of several proteins seen when comparing the mutant to the wild type parent. Application of the TCA precipitated secreted proteins revealed increased contact toxicity of mutant (MlUV-40b) as compared to the parent. In addition, ethyl acetate extraction of the cell-free culture supernatant which would enrich for small molecular weight fungal metabolites also showed increased contact toxicity for the mutant as compared to the wild type parent. It is well known that fungal culture supernatants (from entomopathogenic fungi) can contain a variety of insecticidal components (Lozano-Tovar *et al*., 2015), including cuticle degrading enzymes, proteinaceous insect toxins, and insect toxic fungal secondary metabolites (Gibson *et al*., 2014; Kirkland *et al*., 2005; Ortiz-Urquiza and Keyhani, 2013; Ortiz-Urquiza *et al*., 2015; Pedrini *et al*., 2013; Molnar *et al*., 2010; Xu *et al*., 2008; Xu *et al*., 2009). The alteration in secondary metabolite production that included the production of the insect toxin, destruxin A, was reported to be a contributing factor in the increased virulence seen in a *M. anisopliae* mutant. Manipulation of secreted metabolites, including oxalic acid have also been shown to be linked to virulence in *B. bassiana* (Kirkland *et al*., 2005; Luo *et al*., 2015). Our data suggest that both protein constituents and secondary metabolites may be contributing to the increased virulence seen for the MlUV40.b mutant. This coupled to the faster germination and growth rate, likely provides the simplest explanations for the phenotype of the mutant. Our study confirms that mutant generation via UV irradiation coupled to screening for maintenance/enhancement of vigorous vegetative growth can be a simple and powerful approach towards selecting isolates with enhanced virulence. In addition, one benefit of using UV-irradiation as the mutagenic agent may be the potential to isolate mutants with increased UV-tolerance. These data also show that both protein and secondary metabolite enriched cell-free extracts may be used directly as contact pesticides, that can potentially be used in conjunction to and/or instead of the fungus.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

All authors declare that they have no conflict of interest.

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