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Identification of the pigment and its role in UV resistance in *Paecilomyces variotii*, a Chernobyl isolate, using genetic manipulation strategies

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A B S T R A C T

Fungi produce secondary metabolites that are not directly involved in their growth, but often contribute to their adaptation to extreme environmental stimuli and enable their survival. Conidial pigment or melanin is one of the secondary metabolites produced naturally by a polyketide synthesis (PKS) gene cluster in several filamentous fungi and is known to protect these fungi from extreme radiation conditions. Several pigmented or melanized fungi have been shown to grow under extreme radiation conditions at the Chernobyl nuclear accident site. Some of these fungi, including *Paecilomyces variotii*, were observed to grow towards the source of radiation. Therefore, in this study, we wanted to identify if the pigment produced by *P. variotii*, contributes to providing protection against radiation condition. We first identified the PKS gene responsible for synthesis of pigment in *P. variotii* and confirmed its role in providing protection against UV irradiation through CRISPR-Cas9 mediated gene deletion. This is the first report that describes the use of CRISPR methodology to create gene deletions in *P. variotii*. Further, we showed that the pigment produced by this fungus, was not inhibited by DHN-melanin pathway inhibitors, indicating that the fungus does not produce melanin. We then identified the pigment synthesized by the PKS gene of *P. variotii*, as a naptho-pyrone Ywa1, by heterologously expressing the gene in *Aspergillus nidulans*. The results obtained will further aid in understanding the mechanistic basis of radiation resistance.

1. Introduction

Paecilomyces variotii, an ascomycete, found ubiquitously in nature, has been isolated from soil, food, paint, textiles, and other organic matter (Mioso et al., 2015). It is a pigmented fungus, which produces several secondary metabolites (SMs) of economic importance such as viriditoxin, an anticancer compound (Urquhart et al., 2019), and cornexistin, a herbicide (Mioso et al., 2015). *P. variotii* IMV 00236 is one of the fungal strains that was isolated from the wall surface of unit-4 of the Chernobyl nuclear power plant (ChNPP) accident site (Zhdanova et al., 2000). The strain was initially identified as *Cladosporium cladosporioides*, however, it was later re-identified as *P. variotii* based on the analysis of genome sequence obtained thereafter (Singh et al., 2017).

The Chernobyl nuclear accident on April 25, 1986, was accompanied by the release of high levels of radioactivity that was detrimental to the surrounding environment making it inhospitable (Cardis et al., 2006). However, pigmented or melanized fungal communities were the first to replace and dominate existing soil fungi in the area within the failed reactor and beyond in the exclusion zone (Zhdanova et al., 1994). Since the nuclear accident in 1986, approximately 2000 strains belonging to 200 fungal species have been isolated from the nuclear accident site (Vember and Zhdanova, 2001; Zhdanova et al., 2004). Some of these fungi were recently shown to exhibit positive radiotropism, a previously unknown phenomenon of growing towards the radiation source (Dighton et al., 2008; Zhdanova et al., 2004). Some of the radiotrophic fungi, such as *Penicillium hirsutum* and *Cladosporium sphaerospermum,* could grow into and decompose hot particles or pieces of radioactive graphite from the reactor (Dadachova and Casadevall, 2008; Zhdanova et al., 2004). *C. cladosporioides* strain isolated from the 4th Block reactor room showed statistically significant growth directed towards ^{109}Cd

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source of radiation (Dadachova and Casadevall, 2008; Dighton et al., 2008; Zhdanova et al., 2004). Although not statistically significant, a trend of directional growth towards radiation was seen for *C. cladosporioides* strain isolated from uncontaminated soils (Dadachova and Casadevall, 2008). *C. cladosporioides* IMV 00236, re-named as *P. variotii* IMV 00236, was also shown to exhibit radiotropism (Blachowicz et al., 2019). Apart from fungi isolated from the Chernobyl site, many other melanized fungi, such as *Cryptococcus neoformans* (Jung et al., 2016), *Wangiella dermatitidis* (Robertson et al., 2012), *Aspergillus niger* (Singaravelan et al., 2008), and, *Aspergillus fumigatus* (Brakhage and Liebmann, 2005) are reported to be radioresistant. For example, LD_{10} value for *C. neoformans* is 4.3 kGy (Mirchink et al., 1972), which is greater than the standard dose (1 kGy) for food irradiation in the United States (Dadachova and Casadevall, 2008). A previous study reported that melanin protects fungi from both UV and ionizing radiations by scattering/trapping the photons and electrons, which are also utilized as energy source by the fungi, resulting in their increased growth compared to non-irradiated environment (Dadachova et al., 2007). Further on, another study found that melanized cells respond similarly to visible, UV and gamma radiation, that is, these cells exhibit reduced ATP levels (Bryan et al., 2011). It was hypothesized to be either due to oxidation of NADH by irradiated melanin, resulting in reduced ATP levels or due to activation of downstream processes like DNA repair in the presence of radiation that require ATP. These studies suggest that pigments like melanin play a role in imparting protection to fungi from extreme radiation conditions, including both ionizing and non-ionizing radiations.

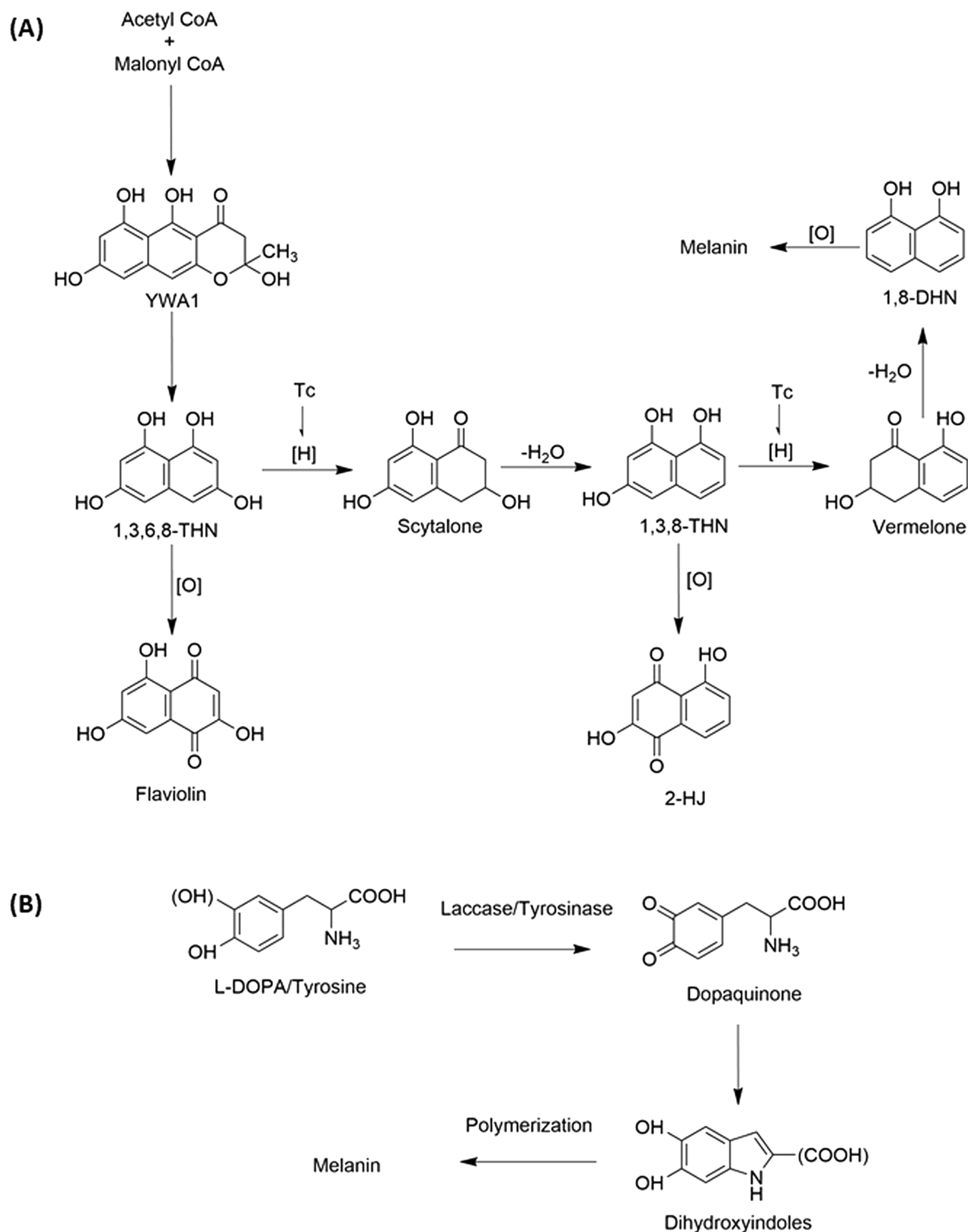

Fig. 1. The biosynthesis pathway of dihydroxynaphthalene (DHN)-melanin (A), and dihydroxyphenylalanine (DOPA)-melanin (B). Tricyclazole (Tc) (shown in A), and pyroquilon inhibit both THN reductase reactions in the DHN-melanin pathway.

Several pigments are produced by various fungi, and in some cases, fungal species contain multiple genes that impart different colors to the fungal conidia, depending on growth conditions (Chang et al., 2020). For instance, *A. fumigatus* contains multiple genes that encode for different enzymes resulting in varied conidial colors, including 1,3,6,8-trihydroxynaphthalene (THN) reductase and scylatone dehydratase enzymes responsible for reddish-pink color, and laccase resulting in brown pigmentation (Tsai et al., 1999). Additionally, polyketide synthase (PKS) genes in some fungi like *A. niger* and *Aspergillus carbonarius* encode for fawn pigment (Gerin et al., 2018; Jorgensen et al., 2011). Some fungi synthesize melanin, which can be of two types, that is, dihydroxynaphthalene (DHN)-melanin and the dihydroxyphenylalanine (DOPA)-melanin (Fig. 1), depending on the pathway utilized for its synthesis (Chang et al., 2020; Langfelder et al., 2003). Majority of fungi synthesize melanin via the DHN-melanin pathway, usually present in hyphae or conidia (Dadachova et al., 2007; Eisenman and Casadevall, 2012; Keller, 2019). In the DHN-melanin pathway, for instance, in *A. fumigatus*, acetyl CoA and malonyl CoA serve as precursors for a PKS gene to form a naptho-pyrone Ywa1, which is converted to THN. This is followed by a series of reduction and dehydration reactions to produce the intermediates scytalone, 1,3,8-THN, vermelone, and 1,8-DHN. DHN is then finally polymerized to form DHN-melanin (Chang et al., 2020; Eisenman and Casadevall, 2012; Langfelder et al., 2003; Tsai et al., 2001). However, some fungi can synthesize melanin through the L-3,4-DOPA melanin pathway, where L-DOPA or tyrosine are two possible precursor molecules. If tyrosine is the precursor, it is first converted to L-DOPA, which is further oxidized to dopaquinone by laccase. After several further enzymatic reactions, dihydroxyindoles are produced that polymerize to form melanin (Eisenman and Casadevall, 2012).

The aim of this study was to understand the mechanism that allows filamentous fungus *P. variotii* IMV 00236 to colonize high radiation environment such as the Chernobyl nuclear disaster site. Since the tools required for genetic manipulations in this fungus are lacking, the primary objective of this study was to utilize genetic tools for *P. variotii* IMV 00236 that will allow us to test the role of individual genes in protecting the organism from high radiation. Therefore, the use of CRISPR-Cas9 was established to create a genetic mutant in this fungus using the system available for various filamentous fungi (Nodvig et al., 2015). The role of the PKS gene responsible for pigmentation in this fungus, in UV radiation protection was thus tested using this tool. The PKS gene was also heterologously expressed in *Aspergillus nidulans* to identify the metabolite synthesized from the PKS enzyme. In addition to identifying the PKS gene product responsible for pigment production and its role in UV resistance, this is the first report where CRISPR-Cas9 was established to create genetic mutants in *P. variotii.*

2. Material and methods

2.1. Strains and growth conditions

The fungal strains used in the study are listed in Table 1.

The fungal strains were grown on potato dextrose agar (PDA) (4 g/L potato extract, 20 g/L dextrose, 15 g/L agar) at 26 °C for 5 days. The spore suspensions were prepared in 0.1% Tween 80 solution (prepared in 0.85% NaCl), and spore count was enumerated using a hemocytometer (Hausser Scientific, USA). The spore suspensions of all strains were stored in 15% glycerol at −80 °C.

Glucose minimal media (GMM) (6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4{\cdot}7H_2O$, 1.52 g/L KH_2PO_4, 10 g/L D-glucose, supplemented with 1 mL/L of Hutner's trace elements) with 1.5% agar, malt extract agar (MEA) (20 g/L malt extract, 1 g/L peptone, 20 g/L glucose) with 1.5% or 0.75% (half) agar, and lactose minimal media (LMM) (15 g/L lactose, 6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4{\cdot}7H_2O$, 1.52 g/L KH_2PO_4, 1 mL/L of Hutner's trace element) with 1.5% agar (when required), were used for some experiments as indicated.

Hutner's trace element (2.2 g $ZnSO_4{\cdot}7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.5 g $FeSO_4{\cdot}7H_2O$, 0.16 g $CoCl_2{\cdot}6H_2O$, 0.16 g $CuSO_4{\cdot}5H_2O$, 0.11 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 5.0 g Na_2EDTA) was prepared by adding the solids in the order mentioned to 80 mL of deionized water and dissolving each salt completely before adding the next. The pH was adjusted to 6.5 with 5.5 M KOH, and the volume adjusted to 100 mL with deionized water. The solution was autoclaved and stored at room temperature.

Escherichia coli DH5α was used for cloning experiments. It was grown in Lysogeny Broth (LB, Difco, USA) medium at 37 °C. 1.5% agar was added to the media to prepare plates. Ampicillin was added at a concentration of 100 μg/mL whenever required.

2.2. Primers

The list of primers used is mentioned in Table S1.

2.3. Buffers

The buffers used in the study are listed in Table 2.

Table 2
List of buffers used.

Buffer	Composition	Preparation
Reagents used for fungal transformation		
Citrate buffer	150 mM KCl, 580 mM NaCl, 50 mM sodium citrate	Dissolved all the components in water and adjusted the pH to 5.5 with citric acid. Autoclaved the solution and stored at room temperature.
STC1700 buffer	1.2 M sorbitol, 10 mM Tris-HCl (pH 5.5), 50 mM $CaCl_2$, 35 mM NaCl	Dissolved all the components in water and filtered under vacuum (0.22 μm). Stored at 4 °C.
PEG4000 buffer	60% (w/v) polyethylene glycol 4000 (PEG 4000), 50 mM $CaCl_2$	Added required amounts of PEG and $CaCl_2$, stirred until everything dissolved and then adjusted volume to 100 mL with H_2O. Autoclaved and stored at room temperature.

Table 1
List of fungal strains.

Strain name	Genotype	Description	Reference(s)
Paecilomyces variotii IMV 00236*	Wild type	Isolated from Chernobyl disaster site	(Zhdanova et al., 2000)
Paecilomyces variotii IMV 00236 *PvBS090*_009107Δ	*PvBS090*_009107Δ; IMV 00236	*PvBS090_009107*, PKS gene from gene cluster 24, responsible for pigment production in *P. variotii* IMV 00236, was mutated using CRISPR-Cas9	This study
Aspergillus nidulans LO4389	*pyrG89; pyroA4; nkuA*::*argB; riboB2; stcA-W*Δ	It produces green conidia and is auxotrophic for pyridoxine, pyrimidine, and riboflavin.	(Ahuja et al., 2012)
Aspergillus nidulans LO4389-*PvBS090_009107*	*pyroA4; nkuA*::*argB; riboB2; stcA-W*Δ*; wA*::*pyrG-alcA(p)- PvBS090_009107*	*PvBS090_009107* from *P. variotii* IMV 00236, was integrated at *wA* locus in *A. nidulans* LO4889 under the control of inducible *alcA* promoter.	This study

* Initially identified as *C. cladosporioides*, however, re-identified as *P. variotii* based on genome sequence analysis. NCBI whole genome sequence accession number: MSJH00000000.

2.4. Antibiotic sensitivity test

The antibiotic sensitivity of *P. variotii* IMV 00236 was tested against hygromycin (Thermo Fisher Scientific, USA) to check if it can be used as an antibiotic for selection in fungal transformation. Hygromycin at following concentrations: 0 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1000 μg/mL, was added to 10 mL GMM agar in falcon tubes, mixed well, and poured to each well of the sterile, 6-well tissue culture plate (VWR, USA). 100 μL of 3.1×10^5 spores/μL spore suspension was spread evenly on the agar surface in each well. The plate was then incubated at 26 °C for 4 days. The effective concentration range of hygromycin was determined to be between 10 μg/mL − 100 μg/mL. Therefore, to determine the minimum inhibitory concentration, it was followed by another round of antibiotic sensitivity tests using protoplasts (spores with the digested cell wall used for fungal transformation). 100 μL of 3.1×10^5 protoplasts/μL suspension was plated on GMM containing 1.2 mM sorbitol, 0.7% agar and hygromycin at different concentrations, between 10 and 150 μg/mL (at intervals of 20 μg/mL). The plates were incubated at 26 °C for 4 days.

2.5. Fungal transformation

The transformation in *P. variotii* was performed using the standard polyethylene glycol (PEG) transformation method, as previously described with some modifications (Dümig and Krappmann, 2015). The fungal culture was set up at a starting spore concentration of 1×10^8 spores in 200 mL malt extract broth and incubated at 30 °C, 110 rpm for 16 h. After 16 h of incubation, the culture was filtered through a sterile Miracloth (EMD Millipore, USA) and washed three times with citrate buffer. Mycelia were gently collected and transferred to a sterile 125 mL Erlenmeyer flask using a sterile spatula. Simultaneously, 800 mg of VinoTaste Pro enzyme (Novozymes, Denmark) was dissolved in 20 mL of citrate buffer, filtered using a 0.22 μm syringe filter, and used to resuspend the collected mycelia. The mixture was then incubated at 30 °C, 100 rpm for 2 h to digest the cell wall, and obtain the protoplasts.

After sufficient amounts of protoplasts were obtained, the mixture was filtered through a new, sterile Miracloth into a 50 mL falcon tube placed on ice. 30 mL fresh, ice-cold STC1700 buffer was added to a maximum of 20 mL protoplast solution and left on ice for 10 min. The following mixture was centrifuged for 12 min at 1200 g, 4 °C, and the supernatant was discarded. The pellet was resuspended in ~300 μL leftover volume of the STC1700 buffer by flicking the tube gently. 30 mL of fresh, ice-cold STC1700 buffer was added again to the same falcon tube and centrifuged at 1200 g for 12 min, 4 °C. The supernatant was discarded, and the pellet was resuspended in the remaining ~300 μL STC1700 buffer.

5 μg of linearized plasmid DNA was added to the 150 μL protoplast-solution in a 15 mL falcon tube and incubated on ice for 30 min. 250 μL PEG4000 was slowly added to the mixture twice and mixed gently by rolling the tube. This was followed by the addition of 850 μL PEG4000 with gentle mixing until the mixture was homogeneous. The tube was incubated horizontally on ice for 20 min. 13–14 mL STC1700 buffer was added to the protoplast suspension and mixed gently by inverting the tube, followed by centrifugation for 15 min at 1200*g*, 4 °C. About ~500 μL of supernatant was left in the tube to resuspend the pellet, while the remaining supernatant was discarded.

The transformed protoplast suspension was plated and overlaid with a layer of agar as follows. For the bottom layer, 100 uL of prepared protoplasts and 70 μg/mL hygromycin were mixed with 20 mL of top agar (1.2 mM sorbitol, 0.7% agar in GMM) that was cooled to 55 °C in a falcon tube. The mix was then poured in an empty 15 cm petri dish (VWR, USA). The bottom layer was allowed to solidify. For the top layer, 70 μg/mL hygromycin was mixed with 20 mL of top agar in another falcon tube, poured to the same plate containing the bottom layer, and allowed to solidify. The plate was incubated at 26 °C for 5–7 days until colonies grew to the top layer of agar. The colonies obtained were restreaked three times on selection media for efficient cutting by Cas9. Positive transformants were selected based on the loss of pigmentation under selection conditions and were further confirmed by Sanger sequencing.

2.6. Construction of pigment encoding PKS gene mutant

The pigment encoding PKS gene mutant in *P. variotii* IMV 00236 was created using the CRISPR-Cas9 system developed by Mortensen lab (Nodvig et al., 2015). The 20 bp protospacer sequence 5′-TGA GAC CGG CGA GAT TAT CC-3′, followed by TGG targeting the PKS gene, was selected from the gene sequence (Figure S1A). The sgRNA (single chimeric guide RNA) targeting the PKS gene was constructed as follows. The sgRNA backbone sequence was amplified in two fragments using pFC334 (Nodvig et al., 2015) as a template such that the protospacer sequence overlapped between the two fragments. The protospacer sequence was incorporated in the reverse primer of the first fragment and forward primer of the second fragment. The two fragments were amplified using pFC334 as a template with custom primers: CRISPR Fw1 and CRISPR Rev1, CRISPR Fw2 and CRISPR Rev2. These fragments were then assembled in pFC332 (that contains Cas9 and hygromycin resistance gene for selection of fungal transformants) (Nodvig et al., 2015), using Gibson assembly mix (New England BioLabs (NEB), USA), after linearizing pFC332 plasmid with PacI (NEB, USA).

The recombined plasmid was then transformed into chemically competent *E. coli* DH5α (NEB, USA), and the colonies were selected on LB medium containing ampicillin. The plasmid was isolated from the colonies obtained using QIAprep Spin Miniprep Kit (Qiagen, Germany), and cloning was confirmed by diagnostic PCR using primers (listed in Table S1) that anneal outside the cloning site. The purified plasmid was linearized with PvuI (NEB, USA), and 5 μg of linearized plasmid was used for fungal transformation.

2.7. Ultraviolet-C (UV-C) exposure and survival evaluation

Strains were grown at 26 °C on MEA for 4 days, followed by which spore suspension was prepared in 0.1% Tween 80 solution. To evaluate UV-C sensitivity, approximately 1000 conidia were suspended in 50 mL MEA (half agar) cooled to 55 °C and 5 mL of this suspension (equivalent to 100 conidia per plate) was poured on top of 20 mL MEA in Petri plates (D = 10 cm). Each plate containing conidia was exposed to 0, 10, 15, 20, and 25 mJ/cm^2 dose of UV-C, respectively, using Hoefer UVC 500 crosslinker (Amersham Biosciences, UK) in triplicates. UV-C exposed, and unexposed (control) plates were incubated at 26 °C for 3 days. After 3 days, colony-forming units (CFUs) were counted, and percent survival (%) was calculated using the formula: $((N/N_0) * 100)$; where N- the number of CFUs obtained at a given dose and N_0- the number of CFUs in the control plate. Results from three biological replicates were averaged and used for statistical analysis with Welch's *t*-test. Lethal dose, 50% (LD_{50}) values for IMV 00236 wt, and its PKS gene mutant were calculated using the LD_{50} calculator from AAT Bioquest (https://www.aatbio.com/tools/ld50-calculator).

2.8. DHN-melanin biosynthesis pathway inhibitor test

Pyroquilon ($C_{11}H_{11}NO$) (Sigma-Aldrich, USA), an inhibitor that targets the reduction steps of 1,3,6,8-THN and 1,3,8- THN to scytalone and vermelone, respectively in the DHN-melanin biosynthesis pathway, was used to examine its effect on pigment synthesis. The stock solution of pyroquilon was prepared in ethanol (IBI Scientific, USA) at a concentration of 10 mg/mL. In different tubes, 20 mL PDA half agar was mixed with 0, 8, 16, 32, 64, 128, and 256 μg/mL pyroquilon, and ethanol (volume equivalent to the highest concentration of pyroquilon), and 5×10^6 spores were added to each tube. Media mixed with spore suspension in each tube was poured in Petri plates (D = 10 cm) and allowed to solidify. The plates were incubated for 7 days at 26 °C. On

day 7, three agar plugs (~8 mm in diameter, each) were collected from the plates under sterile condition. The secondary metabolites were extracted from the agar plugs and analyzed using HPLC-DAD-MS (high-performance liquid chromatography-photodiode array detection-mass spectroscopy).

2.9. Heterologous expression of *P. variotii* pigment encoding PKS gene in *A. nidulans*

The PKS gene responsible for synthesis of pigment in *P. variotii* was heterologously expressed in *A. nidulans* via integration at *wA* locus. The cassette used for integration was constructed by fusion PCR as described previously with some modifications (Chiang et al., 2013; Szewczyk et al., 2006). Different fragments required for the construction of cassette i.e., nearly 1 kb upstream flanking region of the integration site *wA*, *A. fumigatus pyrG* as a selection marker, *A. nidulans alcA* promoter, *P. variotii* PKS gene coding sequence (CDS) along with its terminator, and nearly 1 kb downstream flanking region of the integration site *wA*, were amplified individually. The cassette was constructed in two fragments (Fig. 2) known as split markers such that both the fragments contained an overlap region of nearly 1 kb within the PKS CDS region. The first fragment was constructed by fusing *wA* upstream flanking region, *pyrG* selection marker, *alcA* promoter, and 5′ end of PKS CDS by PCR. Similarly, the second fragment was constructed by fusing 3′ end of PKS CDS + terminator with *wA* downstream flanking homology. 0.5 pmole of each fragment was simultaneously used for transformation in *A. nidulans*.

The fungal transformation was done as previously described (Chiang et al., 2013; Szewczyk et al., 2006). The transformants were selected on GMM supplemented with pyridoxine (0.5 mg/L) and riboflavin (2.5 mg/L). The transformation plates were incubated at 37 °C for 3 days and the transformants were re-streaked twice to obtain single colonies with the desired mutation.

The genomic DNA was isolated from the transformants as previously described (Hervas-Aguilar et al., 2007). The positive transformants were confirmed for integration of the construct using diagnostic PCR with primers flanking the integration site.

For *alcA*(p) induction, 3×10^7 spores were inoculated in 30 mL LMM in 125 mL flasks and incubated at 37 °C, 180 rpm for 42 h. LMM was supplemented with pyridoxine (0.5 mg/L) and riboflavin (2.5 mg/L) for the PKS expressing strain; and with pyridoxine (0.5 mg/L), riboflavin (2.5 mg/L), uracil (1 mg/mL) and uridine (10 mM) for *A. nidulans* parent strain. After 42 h of growth, 2-butanone was added to the media at a final concentration of 50 mM for inducing the PKS gene expression from *alcA*(p). The culture medium was filtered after 72 h of induction, for the extraction of secondary metabolites (SMs) from the culture filtrate.

2.10. Secondary metabolite extraction and analysis

To examine the SMs produced on agar in the DHN-melanin pathway inhibitor test, they were extracted with 3 mL methanol (MeOH) followed by 3 mL of 1:1 methanol/dichloromethane by sonication for 1 h, each time. The solvent was evaporated in TurboVap LV (Caliper LifeSciences, USA), and the crude extracts were resuspended in 3 mL water followed by extraction with 3 mL of ethyl acetate. This step was repeated twice. The ethyl acetate layer was transferred to a fresh tube and evaporated in TurboVap LV. The SMs residue obtained after drying the solvent was resuspended in 5% dimethyl sulfoxide (DMSO)/MeOH, such that the final concentration of the extract is 1 mg/mL. 10 μL of the extract was injected for HPLC-DAD-MS (High performance liquid chromatography-diode array detection-mass spectrometry) analysis.

To examine the SMs produced and secreted in culture filtrate during heterologous expression, they were extracted using equal volume of ethyl acetate twice. The ethyl acetate layer was transferred to a fresh tube and evaporated in TurboVap LV. The residue obtained after drying the solvent was resuspended in MeOH at a final concentration of 1 mg/ml, and 10 μL of the extract was injected for HPLC-DAD-MS analysis.

HPLC-DAD-MS was carried out using Thermo Finnigan LCQ Advantage ion trap mass spectrometer with an RP C18 column (Alltech Prevail C18 3 mm 2.1 × 100 mm), flow rate 125 μL/min. The solvent gradient for liquid chromatography/mass spectrometry (LC/MS) was 95% acetonitrile (MeCN)/H_2O (solvent B) in 5% MeCN/H_2O (solvent A), both containing 0.05% formic acid, as follows: 0% solvent B from 0 to 5 min, 0 to 100% solvent B from 5 min to 30 min, 100% solvent B from 30 to 45 min, 100 to 0% solvent B from 45 to 45.10 min, and re-equilibration with 0% solvent B from 45.10 to 50 min.

High-resolution MS was carried out Thermo Q Exactive Orbitrap mass spectrometer at a flow rate of 10 μL/min. The sample dissolved in MeOH, was diluted in 50% MeCN containing 0.1% formic acid and directly injected to the MS.

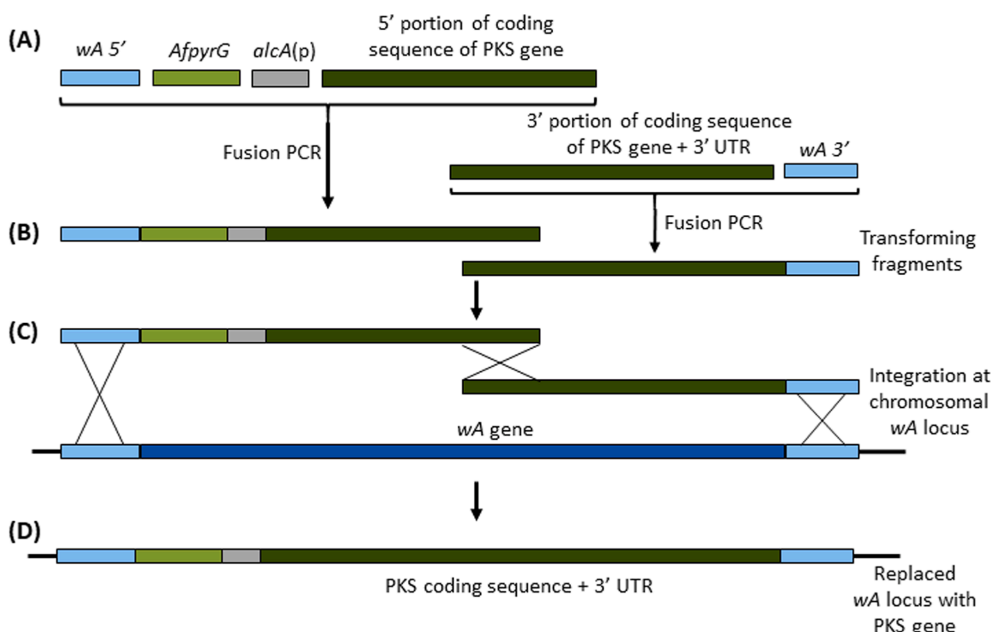

Fig. 2. Steps for construction of cassette for heterologous expression of *P. variotii* pigment encoding PKS gene in *A. nidulans*. (A) Amplification of individual fragments with primers containing overlap to the adjoining fragment. (B) The fusion of fragments by PCR using end primers to construct two fragments that contain nearly 1 kb of the overlapping region. (C) Transformation of the two fragments in *A. nidulans* is combined by homologous recombination within the overlap region, followed by integration at the *wA* locus in the genome by homologous recombination at the ends. (D) Replacement of *wA* locus with *the P. variotii* PKS gene.

2.11. Software used

ChemDraw Professional v 17.1 was used to generate Fig. 1.

3. Results

3.1. Identification and construction of pigment encoding PKS gene mutant using CRISPR-Cas9

The SM gene clusters in *P. variotii* IMV 00236 were predicted using the antibiotics and secondary metabolite analysis (antiSMASH) pipeline (Medema et al., 2011). This analysis revealed a total of 31 putative SM gene clusters, including 6 terpenes, 6 non-ribosomal peptide synthetase (NRPS), 7 PKS, 10 NRPS-like, and 2 PKS-NRPS. Since the pigment, melanin, is known to be produced by a PKS gene cluster in many fungi (Tsai et al., 1999; Zhang et al., 2017), the analysis was narrowed to the 7 PKS gene clusters identified in *P. variotii* IMV 00236. The annotated protein sequence of the PKS gene *PvBS090_009107* (Figure S1B) of gene cluster 24, obtained from antiSMASH, aligned to several pigment encoding PKS genes from other fungal species. Figure S2 represents the organization of gene cluster 24, which contains the core biosynthetic PKS gene *PvBS090_009107* and antiSMASH analysis showed that this gene contains a ketosynthase, acyltransferase, thioesterase domains and sequences encoding acyl carrier proteins. The protein BLAST analysis showed this gene to be 60–70% identical to polyketide synthetase PksP and conidial pigment polyketide synthase PksP/Alb1 of several *Aspergillus* and *Penicillium* species, such as *Aspergillus flavus*, and *Penicillium brasilianum*. The identity of other genes in the gene cluster 24 was also determined based on protein BLAST analysis and several regulatory genes were identified (Table S2). All the genes of gene cluster 24 showed highest identity to the orthologous genes of *Aspergillus* species. Thus, we hypothesized that the PKS gene *PvBS090_009107* of gene cluster 24 might be responsible for pigment or melanin production in *P. variotii* IMV 00236.

The CRISPR-Cas9 system was used to disrupt the PKS gene of identified gene cluster 24. Prior to fungal transformation of the constructed plasmid, the effective hygromycin concentration for selection of transformants was determined to be 70 μg/mL (Figure S3). Furthermore, it was observed that linearized plasmid resulted in a higher transformation efficiency compared to circular plasmid in *P. variotii* (data not shown). Therefore, the linearized CRISPR plasmid containing hygromycin resistance gene, *cas9* gene, and sgRNA with protospacer for site-directed mutagenesis in the PKS gene, was transformed in *P. variotii*. Following incubation for 5–7 days, the loss of typical pigmentation was observed in IMV 00236 transformants obtained on the selection plate. The six transformants obtained, all of which exhibited loss of pigmentation, were re-streaked three times on selection plates followed by a non-selective plate to evict the plasmid. To confirm the deletion within the targeted PKS gene, genomic DNA was extracted from both the wild type (WT) and two selected transformants. The region flanking the protospacer was amplified using primers Seq Fw and Seq Rev (Table S1), and the PCR product was sequenced by Sanger sequencing. The sequencing result revealed a 14 bp deletion within the 20 bp targeted protospacer sequence in the pigment/melanin PKS gene (Figure S4) in both the transformants, thus, confirming the mutation in the gene.

Further, both *P. variotii* IMV 00236 wt and mutant strain were point inoculated on PDA plates and grown for three days at 26 °C, to confirm their morphology and pigmentation pattern. After three days of growth, loss of pigmentation was observed in the mutant strain compared to the parent strain (Fig. 3). These results thus established the use of CRISPR-Cas9 to successfully create gene deletions in *P. variotii*; and disruption of the PKS gene *PvBS090_009107* (gene cluster 24) further validated its role in pigment production in *P. variotii*.

3.2. P. variotii PKS mutant strain displays sensitivity to UV-C compared to the parent strain

Further, to determine if the pigment encoding PKS gene plays a role in protection from UV-irradiation, *P. variotii* IMV 00236 wt, and its PKS gene mutant strains were exposed to varying doses of UV-C (0, 10, 15, 20, 25 mJ/cm^2). The results obtained from the exposure experiment revealed that the PKS gene mutant was significantly more UV-C sensitive compared to the WT strain at UV-C dose of 25 mJ/cm^2 ($p = 0.0354$, $p \leq 0.05$), while both the strains exhibited dose-dependent UV-C sensitivity (Fig. 4). Also, LD_{50}, the dose required to kill 50% of the population, for PKS gene mutant strain was calculated to be nearly 1.4 times lower compared to the WT strain (10.92 mJ/cm^2 versus 14.75 mJ/cm^2). These

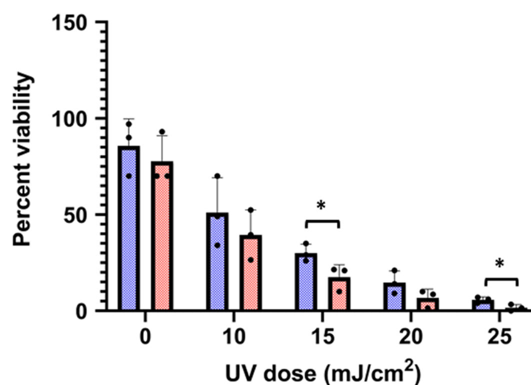

Fig. 4. UV-C sensitivity test of *P. variotii* IMV 00236 wt (blue) and the mutant strains (red). The histogram represents the percent viability following the exposure to varying doses of UV-C radiation. Statistical significance was determined by Welch's corrected *t*-test and is marked with * ($p \leq 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

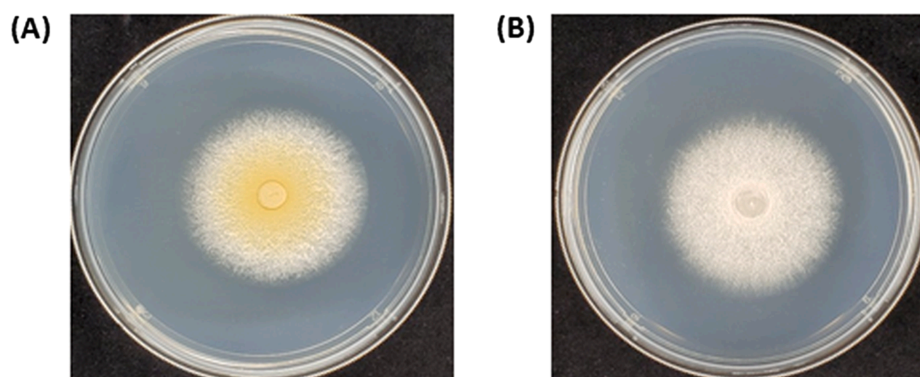

Fig. 3. Growth of *P. variotii* IMV 00236 wt (A) and the PKS gene mutant strain (B) on PDA at 26 °C for 3 days, showing colony morphology and pigmentation.

results suggest that pigment does play a role in protecting *P. variotii* IMV 00236 from UV-C radiation.

3.3. Identification of pigment Ywa1 by heterologously expressing *P. variotii* PKS gene in *A. nidulans*

Since *P. variotii* PKS gene showed identity to PKS genes of the DHN-melanin pathway in other fungal species, the next aim of the study was to identify if the pigment produced by *P. variotii* is DHN-melanin. It is known that inhibitors tricyclazole or pyroquilon target the reduction steps of 1,3,6,8-THN and 1,3,8- THN to scytalone and vermelone, respectively, in the DHN-melanin pathway, resulting in the formation of intermediates, flaviolin, and 2-hydroxyjuglone (HJ) (Fig. 1A). Therefore, we cultured *P. variotii* IMV 00236 on PDA (with 0.7% agar) in the absence or presence of different concentrations of the inhibitor pyroquilon. After seven days of growth, SMs were extracted and analyzed to identify the intermediates, revealing a similar profile for the strain in the absence or presence of inhibitor. Furthermore, it did not show the existence of any intermediates in the presence of inhibitor even when added at a very high concentration of 256 μg/ml (Figure S5). These results indicated the possibility that the genes for synthesis of DHN-melanin, required to convert the PKS gene product to DHN-melanin are absent in *P. variotii* IMV 00236.

Therefore, to further confirm and identify the pigment synthesized by the PKS gene in *P. variotii*, we heterologously expressed the PKS gene in *A. nidulans* under the control of an inducible promoter *alcA*. *A. nidulans* LO4389 was used since several major secondary metabolite gene clusters have been deleted in this strain, resulting in a clean background and no interference from its inherent metabolites (Ahuja et al., 2012). The construct for transformation was designed as described in methods and was used to replace the disrupted *wA* (pigment) locus of *A. nidulans* by protoplast transformation. The transformants obtained were checked for correct integration by diagnostic PCR using primers sets Check-Fw and Fuse-1-Rev, and Fuse-2-Fw and Check-Rev (Table S1), among which one of the primers anneal outside the integration site and second primer anneals within the construct.

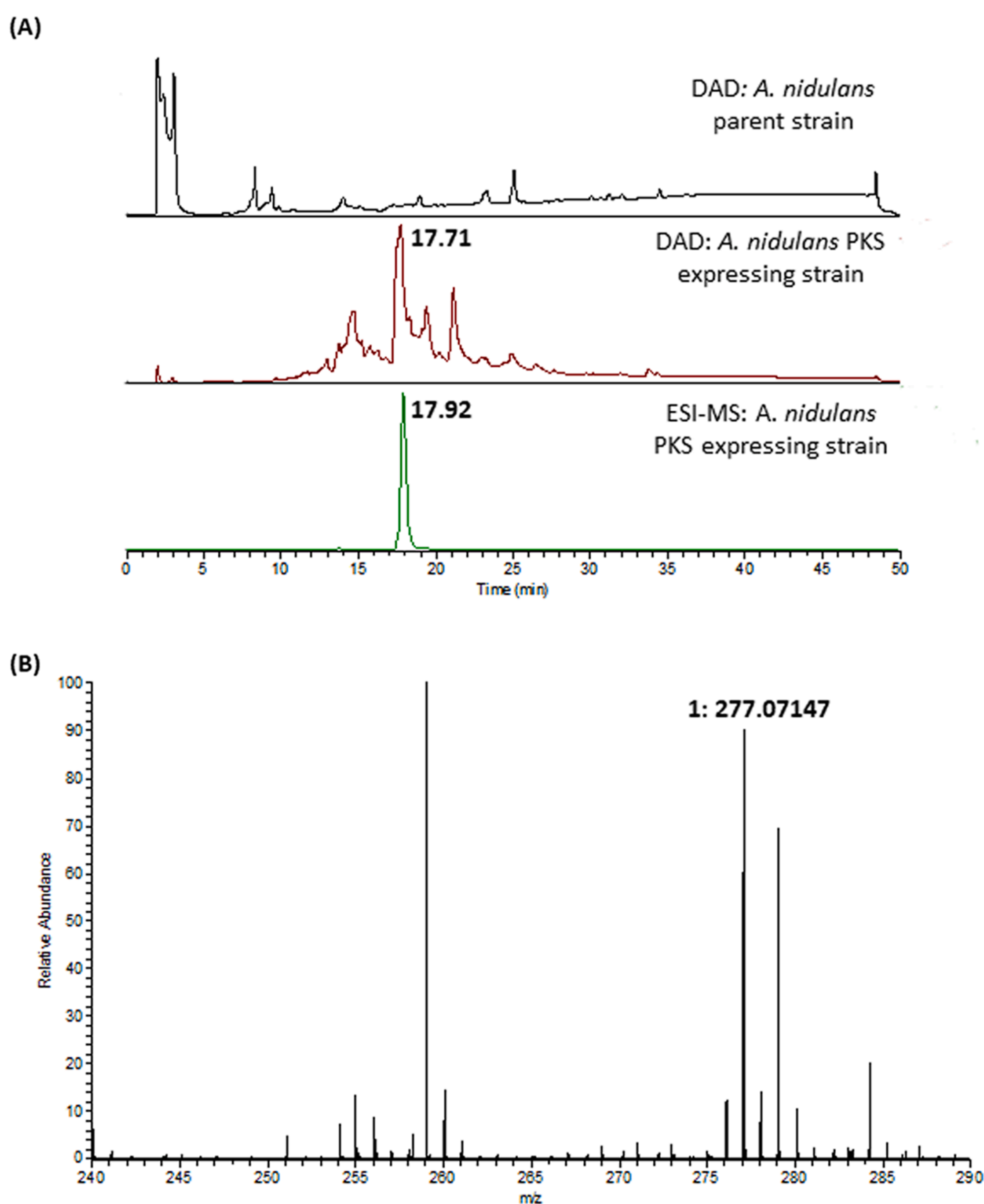

Fig. 5. (A) DAD profile of *A. nidulans* LO4389, DAD and ESI-MS profile of PKS expressing strain after growth in LMM under promoter inducing conditions. The DAD and MS profile show the retention time of PKS gene product, Ywa1 to be 17.71 min and 17.92 min, respectively. (B) High-resolution ESI-MS data for Ywa1 (1) in positive mode (M + H = 277.07147; M = 276.06339) that corresponds to the molecular weight of heptaketide naphthopyrone Ywa1.

The positive transformants and *A. nidulans* LO4389 were grown in LMM under *alcA* promoter inducing conditions such that the expression of integrated PKS gene is induced. Followed by their growth under inducing conditions, *A. nidulans* strain expressing *P. variotii* PKS gene resulted in the production of a brownish-black pigment, compared to *A. nidulans* LO4389 (Figure S6). SMs were extracted and analyzed from both the *A. nidulans* PKS expressing strain and *A. nidulans* LO4389, to identify the product of the PKS gene. The results obtained showed that the PKS expressing strain generated a profile that was different from the parent strain and produced several metabolites under promoter inducing conditions (Fig. 5A). The mass spectra obtained for the PKS expressing strain was analyzed for different pigment masses, from which we identified the compound of interest to be heptaketide naphtho-pyrone Ywa1. The PKS gene product was further confirmed using high-resolution MS, which predicted the formula of the compound to be $C_{14}H_{12}O_6$ (MW = 276.06339) that corresponds to Ywa1 (Fig. 5B). Therefore, we conclude that the PKS gene is indeed responsible for the biosynthesis of a yellow pigment Ywa1.

4. Discussion and conclusion

Fungi exhibit diverse mechanisms to adapt to extreme radiation conditions; these include production of pigments like melanin or other UV-absorbing metabolites, enzymatic and non-enzymatic antioxidants, metabolites like polyols, and activation of DNA repair systems (reviewed in Braga et al., 2015). Several fungal including *Paecilomyces* sp., *Cladosporium* sp., *Aspergillus* sp., and *Penicillium* sp. colonized Chernobyl disaster site despite the presence of harmful radioactivity (Zhdanova et al., 2004; Zhdanova et al., 2000), some of which also exhibited radiotropism (Dighton et al., 2008; Zhdanova et al., 2004). Therefore, this study aimed to identify the molecular mechanism exhibited by the Chernobyl isolate, *P. variotii* IMV 00236, towards protection from UV-C radiation. The first step of the study required identifying the PKS gene that might be responsible for pigment production in this fungus. The initial *in silico* analysis resulted in identifying a PKS gene *PvBS090_009107* (gene cluster 24), the product of which showed a 60–70% identity to PKS gene products involved in pigment production in closely related fungal species. The role of this PKS gene in pigment production was then confirmed by the loss of pigmentation in the PKS gene mutant strain constructed using CRISP-Cas9 (Fig. 3). In addition to identifying pigment-encoding PKS gene in the Chernobyl isolate *P. variotii*, this is the first report where the use of CRISPR-Cas9 was established for creating genetic manipulations in this fungus. Noteworthy, another recent study identified a PKS gene *pvpP* in a different strain of *P. variotii*, the deletion of which resulted in the loss of pigmentation (Urquhart et al., 2019). The PKS gene product of *P. variotii* IMV 00236 identified in our study is nearly 99% identical (query coverage: 98%, E value: 0.0) to *PvpP*, thus further supporting the role of the identified PKS gene in pigment production.

Further, to determine if production of pigments imparts resistance to radiation, the WT and the PKS mutant strain were exposed to different doses of UV-C. The results obtained led to the conclusion that pigment production plays a role in protecting against UV-C radiation (Fig. 4), consistent with that seen in other pigmented or melanized fungal species (Brakhage and Liebmann, 2005; Jung et al., 2016; Robertson et al., 2012; Singaravelan et al., 2008). The pigments produced by different fungi contain aromatic rings that allow energy transfer in the cells (Cordero and Casadevall, 2017). It was hypothesized previously that pigments like melanin protect fungi against radiations via quenching the free radicals generated, thereby preventing DNA damage (Dadachova et al., 2008). Also, it has been observed that melanized fungi exhibit increased growth in the presence of radiation as compared to non-melanized fungi, raising a possibility of energy capture and utilization by melanin, thereby resulting in increased growth (Dadachova et al., 2007). The results obtained from our study suggest a similar mechanism occurring in the Chernobyl isolate *P. variotii* IMV 00236. However, since the effect of UV-C radiation was observed to be significant only at a higher dose of UV-C (25 mJ/cm^2), we cannot rule out the possibility of other factors (like DNA repair pathways) that might also contribute to radiation resistance mechanism.

Next, since it is known that the orthologous PKS gene in several *Aspergillus* species is the core gene responsible for the synthesis of DHN-melanin (Jahn et al., 1997; Langfelder et al., 1998; Tsai et al., 1998) as shown in Fig. 1A, the study aimed to identify if the PKS gene product in *P. variotii* is eventually converted to DHN-melanin. To test this hypothesis, pyroquilon, which inhibits the DHN-melanin pathway, was used. It acts by targeting the reduction steps of 1,3,6,8-THN and 1,3,8-THN to scytalone and vermelone, respectively (Fig. 1A), and results in the production of intermediates, flaviolin, and 2-HJ (Chang et al., 2020; Tsai et al., 1998; Wheeler and Klich, 1995). However, analysis of SMs did not reveal the intermediates, flaviolin and 2-HJ in the presence of pyroquilon (Figure S4), indicating that the reduction steps resulting in the production of DHN-melanin do not occur in *P. variotii*. The observed results are further supported by another study where it was shown that *P. variotii* does not contain other genes that are required to convert PKS gene product *PvpP* to DHN-melanin, based on genomic comparison with *A. fumigatus* (Urquhart et al., 2019). The yellow pigment produced by *P. variotii* is due to the PKS or *pvpP* gene product, Ywa1. In addition to *A. fumigatus*, other downstream genes that convert Ywa1 to DHN-melanin are also found in *A. nidulans*, *A. oryzae*, *A. flavus*, and *A. niger* (Baker, 2008). However, it has been reported that the use of the DHN-melanin pathway inhibitor did not result in a loss of pigmentation in *A. niger* (Jorgensen et al., 2011). Similarly, disruption of *ayg1* ortholog, which converts Ywa1 to 1,3,6,8-THN in the DHN-melanin pathway, did not result in pigmentation loss in *A. flavus* (Chang et al., 2010; Saitoh et al., 2012; Tsai et al., 1997). These studies suggested that not all fungi that produce the precursor Ywa1 convert it to DHN-melanin.

Lastly, to confirm that the PKS gene product produced in *P. variotii* IMV 00236 is Ywa1, which is responsible for yellow pigmentation, we heterologously expressed the gene in *A. nidulans* under inducible *alcA* promoter. Analysis of SMs produced by the PKS expressing *A. nidulans* strain under inducing conditions, led to the identification of a compound with molecular weight ~276 g/mol, which corresponds to heptaketide naphtho-pyrone Ywa1 (yellow pigment). High-resolution mass spectrometry further predicted the formula of the compound to be $C_{14}H_{12}O_6$, which corresponds to Ywa1. The heptaketide napthopyrone Ywa1 was first identified by NMR analysis by heterologously expressing the *A. nidulans* PKS gene *wA* in *Aspergillus* oryzae (Watanabe et al., 1999). The expression of *wA* in *A. oryzae* resulted in the formation of colonies that exhibited yellow pigmentation on agar plate. Thereafter, it was also identified to be encoded by a PKS gene *alb1* in *A. fumigatus* by heterologously expressing the gene in *A. oryzae* (Watanabe et al., 2000). Expression of *alb1* in *A. oryzae* resulted in yellow pigment formation, and its physico-chemical analysis identified it to be Ywa1 (Watanabe et al., 2000). However, in our study, heterologous expression of *P. variotii* IMV 00236 PKS gene in *A. nidulans* resulted in the formation of a brownish-black pigment (Figure S6), which is also different from the yellow pigment produced in *P. variotii* IMV 00236. A possible explanation for altered pigmentation in *A. nidulans* can be the differences in downstream gene products that might act on Ywa1, thereby resulting in the formation of different products or pigments in addition to Ywa1. Though the genes required to convert Ywa1 to DHN-melanin have been identified in *A. nidulans*, however, their functions have not been characterized so far (Chang et al., 2020). Moreover, addition of tricyclazole, did not inhibit the production of melanin (Pal et al., 2014), indicating DHN-melanin is not produced by this fungus. Therefore, the altered pigmentation is probably not due to production of DHN-melanin, instead it can be due to the production of some other pigment(s), the identification of which warrants investigation in future.

Overall, the use of CRISPR-Cas9 tool was established to identify the PKS gene that encodes for the production of yellow pigment Ywa1 in the Chernobyl isolate *P. variotii* IMV 00236, and its role in imparting

resistance to UV-C radiation. Thus, we hypothesize that the aromatic structure of Ywa1 protects the pigmented fungi from both ionizing and non-ionizing radiations based on a mechanism similar to melanin, that is, free radical quenching and spherical spatial arrangement (Dadachova et al., 2008; Schweitzer et al., 2009). These results can be further extrapolated to understand the mechanistic basis of how fungi adapt to other environments with extreme radiation conditions. Besides, the identification of fungi capable of producing such pigments can provide a natural source for production of safe sunscreen to protect humans from harmful radiation.

5. Author's contributions

SL and SB conducted experiments, analyzed data and drafted the manuscript. SB and AB designed experiments. AB contributed to UV-C sensitivity experiment and data analysis, and YC helped in the heterologous expression experiments. ML contributed to the HPLC-DAD-MS data analysis. TT provided the Chernobyl isolate and reviewed the manuscript. KV critically reviewed the manuscript. CCCW designed the study, interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2021.103567.

References

Ahuja, M., et al., 2012. Illuminating the diversity of aromatic polyketide synthases in *Aspergillus nidulans*. J. Am. Chem. Soc. 134, 8212–8221.
Baker, S.E., 2008. Aspergillus genomics and DHN-melanin conidial pigmentation. In: Varga, J., Samson, R.A. (Eds.), Aspergillus in the Genomic Era. Wageningen Academic Publishers, The Netherlands.
Blachowicz, A., et al., 2019. Proteomic and metabolomic characteristics of extremophilic fungi under simulated mars conditions. Front Microbiol. 10, 1013.
Braga, G.U., et al., 2015. Molecular and physiological effects of environmental UV radiation on fungal conidia. Curr. Genet. 61, 405–425.
Brakhage, A.A., Liebmann, B., 2005. *Aspergillus fumigatus* conidial pigment and cAMP signal transduction: significance for virulence. Med. Mycol. 43 (Suppl 1), S75–S82.
Bryan, R., et al., 2011. The effects of gamma radiation, UV and visible light on ATP levels in yeast cells depend on cellular melanization. Fungal Biol. 115, 945–949.
Cardis, E., et al., 2006. Estimates of the cancer burden in Europe from radioactive fallout from the Chernobyl accident. Int. J. Cancer 119, 1224–1235.
Chang, P.K., et al., 2020. Biosynthesis of conidial and sclerotial pigments in *Aspergillus* species. Appl. Microbiol. Biotechnol. 104, 2277–2286.
Chang, P.K., et al., 2010. Development and refinement of a high-efficiency gene-targeting system for *Aspergillus flavus*. J. Microbiol. Methods 81, 240–246.
Chiang, Y.M., et al., 2013. An efficient system for heterologous expression of secondary metabolite genes in *Aspergillus nidulans*. J. Am. Chem. Soc. 135, 7720–7731.
Cordero, R.J., Casadevall, A., 2017. Functions of fungal melanin beyond virulence. Fungal Biol. Rev. 31, 99–112.
Dadachova, E., et al., 2008. The radioprotective properties of fungal melanin are a function of its chemical composition, stable radical presence and spatial arrangement. Pigment Cell Melanoma Res. 21, 192–199.
Dadachova, E., et al., 2007. Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. PLoS One 2, e457.
Dadachova, E., Casadevall, A., 2008. Ionizing radiation: how fungi cope, adapt, and exploit with the help of melanin. Curr. Opin. Microbiol. 11, 525–531.
Dighton, J., et al., 2008. Fungi and ionizing radiation from radionuclides. FEMS Microbiol. Lett. 281, 109–120.
Dümig, M., Krappmann, S., 2015. Controlling fungal gene expression using the doxycycline-dependent Tet-ON system in Aspergillus fumigatus. In: van den Berg, M., a. M.K. (Eds.), Genetic Transformation Systems in Fungi. Springer, Cham, Switzerland, pp. 131–138.
Eisenman, H.C., Casadevall, A., 2012. Synthesis and assembly of fungal melanin. Appl. Microbiol. Biotechnol. 93, 931–940.
Gerin, D., et al., 2018. Functional Characterization of the *alb1* Orthologue Gene in the Ochratoxigenic Fungus *Aspergillus carbonarius* (AC49 strain). Toxins (Basel) 10.
Hervas-Aguilar, A., et al., 2007. Evidence for the direct involvement of the proteasome in the proteolytic processing of the *Aspergillus nidulans* zinc finger transcription factor PacC. J. Biol. Chem. 282, 34735–34747.
Jahn, B., et al., 1997. Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. Infect. Immun. 65, 5110–5117.
Jorgensen, T.R., et al., 2011. The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. Fungal Genet. Biol. 48, 544–553.
Jung, K.W., et al., 2016. Unraveling Fungal Radiation Resistance Regulatory Networks through the Genome-Wide Transcriptome and Genetic Analyses of *Cryptococcus neoformans*. mBio 7.
Keller, N.P., 2019. Fungal secondary metabolism: regulation, function and drug discovery. Nat. Rev. Microbiol. 17, 167–180.
Langfelder, K., et al., 1998. Identification of a polyketide synthase gene (*pksP*) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. Med. Microbiol. Immunol. 187, 79–89.
Langfelder, K., et al., 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet. Biol. 38, 143–158.
Medema, M.H., et al., 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res. 39, W339–W346.
Mioso, R., et al., 2015. The chemical diversity of the Ascomycete Fungus *Paecilomyces variotii*. Appl. Biochem. Biotechnol. 177, 781–791.
Mirchink, T.G., et al., 1972. Resistance of fungi with different pigments to -radiation. Mikrobiologiia 41, 83–86.
Nodvig, C.S., et al., 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS One 10, e0133085.
Pal, A.K., et al., 2014. DOPA and DHN pathway orchestrate melanin synthesis in *Aspergillus* species. Med. Mycol. 52, 10–18.
Robertson, K.L., et al., 2012. Adaptation of the black yeast *Wangiella dermatitidis* to ionizing radiation: molecular and cellular mechanisms. PLoS One 7, e48674.
Saitoh, Y., et al., 2012. Cloning of *sal1*, a scytalone dehydratase gene involved in melanin biosynthesis in *Cochliobolus heterostrophus*. Mycoscience 53, 330–334.
Schweitzer, A.D., et al., 2009. Physico-chemical evaluation of rationally designed melanins as novel nature-inspired radioprotectors. PLoS One 4, e7229.
Singaravelan, N., et al., 2008. Adaptive melanin response of the soil fungus *Aspergillus niger* to UV radiation stress at "Evolution Canyon", Mount Carmel, Israel. PLoS One 3, e2993.
Singh, N.K., et al., 2017. Draft genome sequences of several fungal strains selected for exposure to microgravity at the international space station. Genome Announcements 5.
Szewczyk, E., et al., 2006. Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Protoc. 1, 3111–3120.
Tsai, H.F., et al., 1998. The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. J. Bacteriol. 180, 3031–3038.
Tsai, H.F., et al., 2001. Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. J. Biol. Chem. 276, 29292–29298.
Tsai, H.F., et al., 1997. *Aspergillus fumigatus arp1* modulates conidial pigmentation and complement deposition. Mol. Microbiol. 26, 175–183.
Tsai, H.F., et al., 1999. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J. Bacteriol. 181, 6469–6477.
Urquhart, A.S., et al., 2019. The fungal gene cluster for biosynthesis of the antibacterial agent viriditoxin. Fungal Biol. Biotechnol. 6, 2.

Vember, V.V., Zhdanova, N.N., 2001. Peculiarities of linear growth of the melanin-containing fungi *Cladosporium sphaerospermum* Penz. and *Alternaria alternata* (Fr.) Keissler. Mikrobiol Z. 63, 3–12.
Watanabe, A., et al., 2000. *Aspergillus fumigatus alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. FEMS Microbiol. Lett. 192, 39–44.
Watanabe, A., et al., 1999. Re-identification of *Aspergillus nidulans wA* Gene to Code for a Polyketide Synthase of Naphthopyrone. Tetrahedron Lett. 40, 91–94.
Wheeler, M.H., Klich, M.A., 1995. The effects of tricyclazole, pyroquilon, phthalide, and related fungicides on the production of conidial wall pigments by *Penicillium* and *Aspergillus* species. Pesticide Biochem. Physiol. 52, 125–136.
Zhang, P., et al., 2017. A cryptic pigment biosynthetic pathway uncovered by heterologous expression is essential for conidial development in *Pestalotiopsis fici*. Mol. Microbiol. 105, 469–483.
Zhdanova, N.N., et al., 2004. Ionizing radiation attracts soil fungi. Mycol. Res. 108, 1089–1096.
Zhdanova, N.N., et al., 1994. Changes in micromycete communities in soil in response to pollution by long-lived radionuclides emitted in the Chernobyl accident. Mycol. Res. 98, 789–795.
Zhdanova, N.N., et al., 2000. Fungi from Chernobyl: mycobiota of the inner regions of thecontainment structures of the damaged nuclear reactor. Mycol. Res. 104, 1421–1426.