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Amorosia littoralis Mantle & D. Hawksw. survives as root endophyte of mangroves and produces water soluble melanin pigment

T.S. Suryanarayanan [a,*], M.B. Govindarajulu [a], T.S. Murali [b], J.P. Ravishankar [a], S. Bharathwaj [a], M.D. Kalaiarasan [a]

[a] Vivekananda Institute of Tropical Mycology, Ramakrishna Mission Vidyapith, Chennai, India
[b] Department of Public Health Genomics, Manipal School of Life Sciences, Manipal Academy of Higher Education (MAHE), Manipal, India

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ABSTRACT

Melanins are heterogenous biopolymers produced by many macro and microorganisms. They enhance the ecological fitness of the producer organisms by improving their virulence and protecting them from radiation, osmotic and heat stresses. Fungi synthesise either DOPA or DHN melanin and deposit them on their cell walls. Some fungal isolates produce water soluble melanin (pyomelanin) which is excerted out of the hyphae into the surrounding environment. Pyomelanin, a polymer of homogentisate, exhibits antimicrobial, UV screening, antioxidative, and anti-inflammatory activities and recent studies also show that pyomelanin could find use in space travel as radiation shield. However, scant literature is available on fungi with ability to produce pyomelanin. We report for the first time that *Amorosia littoralis* occurs as a root endophyte in mangrove species including *Avicennia marina*, *Bruguiera cylindrica* and *Bruguiera gymnorhiza* and produces pyomelanin. Considering the various technological uses of pyomelanin, this study underscores the need to explore fungi of different habitats to identify hyperproducers and to obtain chemically diverse pyomelanin.

1. Introduction

Melanins are high molecular weight dark-brown to black coloured pigments, and are products of phenolic compounds synthesized by oxidative polymerization (Eisenman and Casadevall, 2012). Though various groups of organisms are known to produce melanins including plants, animals, fungi, protozoans, and bacteria, their structure and chemical compositions are not fully known (Song et al., 2023). Fungal melanins are classified based on their precursor as eumelanin or DOPA-melanin which is synthesized from tyrosine, DHN-melanin whose precursor is 1,8-dihydroxynaphthalene (DHN), and water soluble pyomelanin which is also synthesized from tyrosine. Fungal DOPA-melanin and DHN-melanin are deposited in the outermost layer of their chitin cell wall of hyphae and spores while pyomelanin is excretd out. Melanins provide survival advantage to fungi by protecting them from microbial attack, harmful UV radiation, oxidative stress (Suthar et al., 2023), heat damage (Rajamani et al., 2021) and osmotic shock (Ravishanakar et al., 1995) and play a role in the virulence of pathogenic fungi (Jacobson, 2000), thus widening their competitive ability and ecological amplitude.

Pyomelanin is a polymer produced during oxidation of homogentisate (HGA) in the L-tyrosine pathway. Tyrosine is catabolized to acetoacetate and fumarate with the formation of 4-hydroxyphenylpyruvate which is reacted upon by 4-hydroxyphenylpyruvate dioxygenase (Hpd) to produce homogentisate (HGA). This is further converted to 4-maleylacetoacetate by the enzyme homogentisate 1,2-dioxygenase (Hgd) leading finally to the formation fumarate and acetoacetate. Hyperproduction of HGA or mutation leading to the absence or non-functional Hgd results in excretion of accumulated HGA from the cells (Kotob et al., 1995; Rodríguez-Rojas et al., 2009; Zeng et al., 2017a). Autooxidation and self-polymerization of the excreted HGA results in the production of brown coloured water soluble pyomelanin. Studies on microbial melanin have focused only on eumelanin (Tran-Ly et al., 2020) and fungal pyomelanin has not received significant attention (Lorquin et al., 2022). Considering the easily extractable nature and the various technological uses of pyomelanin, it is necessary to explore fungi assiduously as a source of natural pyomelanin. We observed for the first time that the fungus *Amorosia littoralis*, which is reported to occur in marine sediments (Mantle et al., 2006) and plant litter (Wijayawardene et al., 2022), survives as endophyte in the roots of *Avicennia marina*,

* Corresponding author.
E-mail address: t_sury2002@yahoo.com (T.S. Suryanarayanan).

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Bruguiera cylindrica and *Bruguiera gymnorhiza*. Furthermore, we report that it produces the pyomelanin pigment.

2. Materials and methods

2.1. Isolation and characterization of endophytes

As a part of our ongoing research to study the diversity of endophytes of mangroves of different parts of India, we isolated root endophytes from *A. marina* (Ennore creek, 13.2319° N, 80.3263° E and Puducherry 11.9416° N, 79.8083° E, southern India), *B. cylindrica* (Puducherry) and *B. gymnorhiza* (Sundarbans, West Bengal, 21.9497° N, 89.1833° E, India). The fresh and healthy root samples were cleaned using tap water and 0.5 cm length segments were cut and surface sterilized by dipping in 75 % ethanol for 60 s, followed by sodium hypochlorite for 180 s (4 % available chlorine) and finally 75 % ethanol for 30 s (Fisher et al., 1993). The tissue segments were plated on Potato Dextrose Agar (PDA) medium amended with chloramphenicol (150 mg/l) and incubated for 4 weeks at 26 ± 1 °C. During this period, the cultures were placed in a near ultraviolet (NUV) light chamber with a 12 h light: 12 h dark cycle to induce sporulation (Suryanarayanan, 1992). The effectiveness of surface sterilization was confirmed by the method of Schulz et al. (1998). Fungi which sporulated in culture were identified based on spore structure and development. Four non-sporulating isolates were observed to release a dark brown pigment around their growth; of these, one which produced more of the pigment was taken up for further study.

2.2. Molecular characterisation of the fungal isolate

The genomic DNA from the fungal isolate was obtained as explained earlier (Sawmya et al., 2013). PCR amplification of ITS rRNA region was carried out in a total reaction volume of 25 μl and the reaction mixture contained 15 μl of filter sterilized MilliQ water, 2.5 μl of deoxynucleotide triphosphates mix (dNTPs– 4 mM), 2.5 μl of PCR buffer (10x), 1 μl of Magnesium chloride (50 mM), 1 μl each of forward and reverse primers (10 μM) ITS1 (5' - TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC), 1 μl Taq polymerase (1 Unit) and 1 μl of template DNA (~50 ng). DNA was amplified with the following cycling parameters: 95 °C for 10 min; 30 cycles of denaturation at 95 °C (1 min), annealing at 55 °C (1 min) and extension at 72 °C (1.5 min); followed by final extension at 72 °C for 10 min. Amplified PCR product was purified with the MagBio HighPrep PCR Clean-up System as per manufacturer's instructions and was sequenced with the Applied Biosystems 3130 Genetic analyser (ThermoFisher Scientific, USA) housed at Manipal School of Life Sciences, Manipal Academy of Higher Education, Manipal, India. The sequence has been deposited in GenBank with the accession number: PP049379. A phylogenetic tree using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) was constructed for *A. littoralis* 2328 isolated in the present study and other related sequences. A total of 10 nucleotide sequences were included for the final analysis. The branch support was calculated based on 500 bootstrap replications. *Angustimassarina acerina* was used as the outgroup. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

2.3. Extraction of melanin

The fungus was grown in liquid Czapek-Dox (CD) medium with XAD resin beads for 30 days at 26 ± 1 °C. One ml of XAD resin beads suspended in sterile water was added to 100 ml growth medium to adsorb the secondary metabolites released by the fungus which could interfere with pigment analysis. The culture filtrate was filtered and centrifuged and the supernatant was used for pigment analysis.

2.4. Qualitative assay for laccase activity (Rohrmann and Molitoris, 1992)

The fungus was grown in glucose yeast peptone (GYP) medium (pH 6.0) with 0.05 g 1-naphthol l^{-1}. The presence of laccase can be visualised when 1-naphthol gets oxidised, turning the colourless medium blue in colour.

2.5. UV, FTIR tests and NMR for structural elucidation

UV–Visible spectrum of the culture supernatant was measured in a UNICO double beam spectrophotometer. Chemical composition of the pyomelanin pigment was recorded with an ATR - FTIR (Attenuated Total Reflectance - Fourier Transform Infrared) spectrometer (Alpha II, Bruker, USA) that allowed for direct analysis of the lyophilized samples. Spectrum was acquired in the wavenumber range of 500–4000 cm^{-1} at a scanning number of 40 with 4 cm^{-1} resolution. The resulting spectrum was analyzed for changes in various chemical entities within the sample. The ^{1}H 1D NMR spectrum of pyomelanin was acquired at 298 K on a 16.45 T (700 MHz ^{1}H Larmor frequency) Bruker Avance Neo NMR spectrometer equipped with a room-temperature QXI probe. Approximately 0.5 g of lyophilized pyomelanin was dissolved in D_2O and the sample was centrifuged to remove insoluble material. ^{1}H 1D NMR data was collected on the supernatant using a *zgesgp* Bruker pulse sequence which incorporates an excitation sculpting module (Hwang and Shaka, 1995) for suppressing the residual H_2O signal from the sample. The spectrum was referenced to the water chemical shift at 298 K.

2.6. Melanin synthesis inhibitors

CD liquid medium (100 ml) was inoculated with a plug of the mycelium (5 mm dia) grown on CD agar medium. The medium was amended with 1, 10, 100 or 1000 μg of Tricyclazole (inhibitor of DHN melanin syntheis) or 1, 10, 100, 1000 μg of Kojic acid (inhibitor of DOPA melanin synthesis) (Suryanarayanan et al., 2004), or 125, 250, 500, 1000 μM of NTBC (inhibitor of Pyomelanin synthesis) (Ketelboeter et al., 2014). The cultures were observed periodically for pigment inhibition for 30 days. Fungi grown in medium without the presence of inhibitor acted as control. All experiments were performed in triplicates and were repeated thrice.

3. Results

On PD agar medium, two hundred and twelve endophyte isolates were obtained from the root sample. Of these, 4 sterile forms produced a dark brown extracellular pigment. Of these isolates, one endophyte from the root of *A. marina* occurring in Puducherry was studied in detail since it appeared to produce more of the pigment under defined culture conditions, as revealed by the intense dark brown colour of the culture medium. The fungus remained sterile in PD and CZ agar media even under NUV illumination which is known to induce sporulation in many fungi (Suryanarayanan, 1992). Hence, it was sequenced and the DNA fragment was aligned with the standard nucleotide databases at GenBank using BlastN with default parameters. The sequenced product showed the highest similarity with *A. littoralis* (OL826791.1) at 98.7 % identity with 96 % query coverage. A phylogenetic analysis based on maximum-likelihood approach showed that our isolate formed a distinct clade with other *A. littoralis* isolates with good clade support (Fig. 1). This initial classification of the fungus should be verified using additional primers. The isolate is henceforth referred to as *Amorosia* cv. *littoralis* 2328.

The fungus produced pigmented colonies when grown on CD agar medium. Though the growth could be observed within 48 h of inoculation, pigment production was observed only after 10–15 days of colony growth. In liquid CD medium, the pigment was excreted after 16–20 days of growth. The excreted brown pigment was positive for the

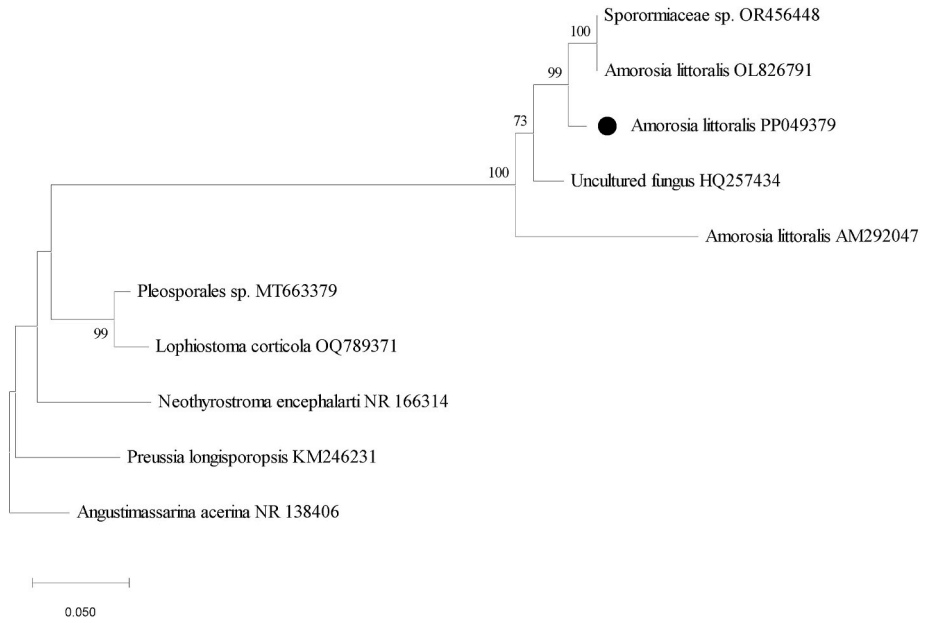

Fig. 1. Phylogenetic analysis showing the highest log likelihood tree for *A.* cv. *littoralis* 2328 constructed using Maximum-likelihood approach. The sequence obtained in the present study is marked with a filled circle. Numbers next to branches indicate the percentage of trees in which the associated taxa clustered together. *Angustimassarina acerina* was used as the outgroup.

chemical tests specific to melanins including pyomelanin; it was soluble in 1 M KOH, precipitated with 3 N HCl (Song et al., 2023), was insoluble in organic solvents, did not decolourise with H_2O_2 (Ahmad et al., 2016; Lorquin et al., 2022) and formed a brown precipitate with $FeCl_3$ (Gadd, 1982; Suryanarayanan et al., 2004; Koch et al., 2023) indicating that the fungus produces pyomelanin.

The absorption spectrum of the pigment was characteristic of melanin with the maximum absorption occurring in the UV region and the absorbance decreasing with increasing wavelength beyond approximately 275 nm (Bell and Wheeler, 1986) (Fig. 2A). Such an absorption spectrum is displayed by melanins including pyomelanin (Li et al., 2017; Bayram, 2021; Koch et al., 2023; Song et al., 2023). When log of the absorbance is plotted against the wavelength, melanin pigments show a characteristic linear curve with negative slope (Ellis and Griffiths, 1974; Ravishankar et al., 1995; Suryanarayanan et al., 2004) as is the case with pyomelanins of fungi (Koch et al., 2023). The water soluble melanin of *A.* cv. *littoralis* 2328 also exhibited this trend (slope value −0.0055) (Fig. 2B). The FTIR spectrum further confirmed that the pigment was pyomelanin with characteristic peaks between 3600 and 2900 cm^{-1} (hydroxyl stretching) and 1400–1300 cm^{-1} (Li et al., 2021; Koch et al., 2023) (Fig. 3). It showed a strong absorption peak at 3276.47 cm^{-1} which was very similar to pyomelanin from bacteria (Bayram, 2021). The ^{1}H 1D NMR spectrum of the pigment showed intense resonances between 3.0 and 4.2 ppm which is characteristic of fungal pyomelanin (Li et al., 2021) (Fig. 4).

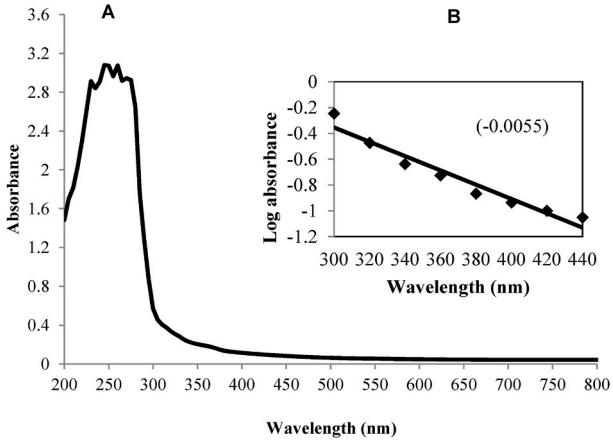

Fig. 2. A. UV–Vis absorption spectrum of excreted melanin pigment of *A.* cv. *littoralis* 2328.**2B.** Plot of log of optical density against wavelength.

A qualitative assay revealed that the fungus elaborates extracellular laccase enzyme which plays a role in the production of pyomelanin (Lorquin et al., 2021). Furthermore, synthesis of the pyomelanin is inhibited by the herbicide NTBC (2-(2-nitro-4--trifluoromethylbenzoyl)-1,3-cyclohexanedione) (Ketelboeter et al., 2014; Ketelboeter and Bardy, 2015). We observed that addition of NTBC (1000 μM) to the culture medium inhibited the excretion of the brown pigment confirming that it was pyomelanin (Fig. 5).

4. Discussion

A. littoralis occurs in marine sediments (Mantle et al., 2006) and rhizosphere of mangrove plants (Sanka Loganathachetti et al., 2017; Vanegas et al., 2019). We isolated it as an endophyte from the roots of mangroves and observed that it produced a pigment which we identified as pyomelanin. Pyomelanin production has been observed in bacteria (Song et al., 2023) and has been recorded from a few fungi such as *Aspergillus fumigatus* (Schmaler-Ripcke et al., 2009), *Aspergillus niger* (Koch et al., 2023; Keller et al., 2011), *Penicillium chrysogenum* (Vasanthakumar et al., 2015), *Yarrowia* (Carreira et al., 2001), *Sporothrix* (Almeida-Paes et al., 2012), and *Ionotus hispidus* (Li et al., 2022). The chemical tests and UV–Vis and FTIR spectral properties of the excreted pigment of *A.* cv. *littoralis* 2328 agreed with those of pyomelanin. It showed high maximum absorbance between 250 and 280 nm wavelengths in the UV region coinciding with the results published for pyomelanin (Li et al., 2017; Bayram, 2021). Melanin shows high absorption in the UV range but the absorption decreases with increased wavelength. This results in a linear curve with negative slope when the log of absorbance is plotted against the wavelength and is used as a criterion for the identification of melanin (Suryanarayanan et al., 2004). The negative slope value for different melanins vary from −0015 to −0.2646 (Pralea et al., 2019); it was −0.0055 for *A.* cv. *littoralis* 2328. The FTIR spectrum was very similar to that reported for pyomelanin (Li et al.,

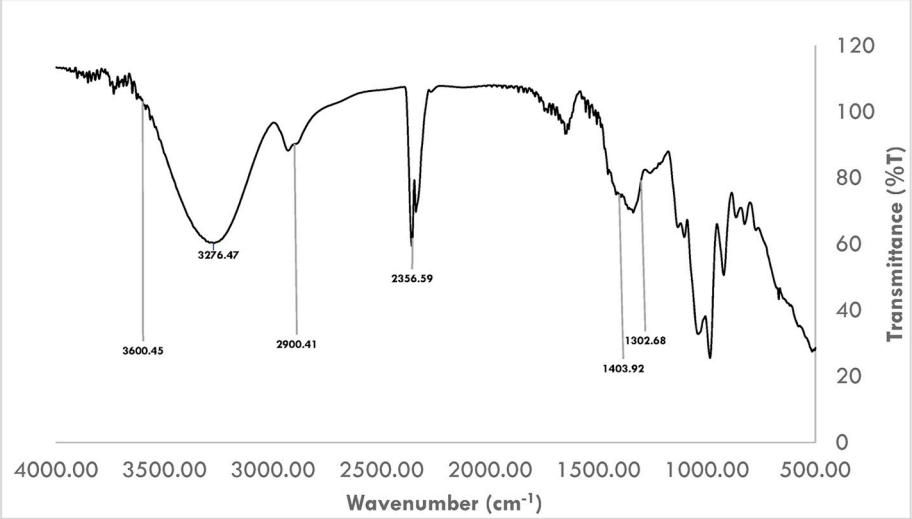

Fig. 3. FTIR spectrum of pyomelanin pigment isolated from *A.* cv. *littoralis* 2328.

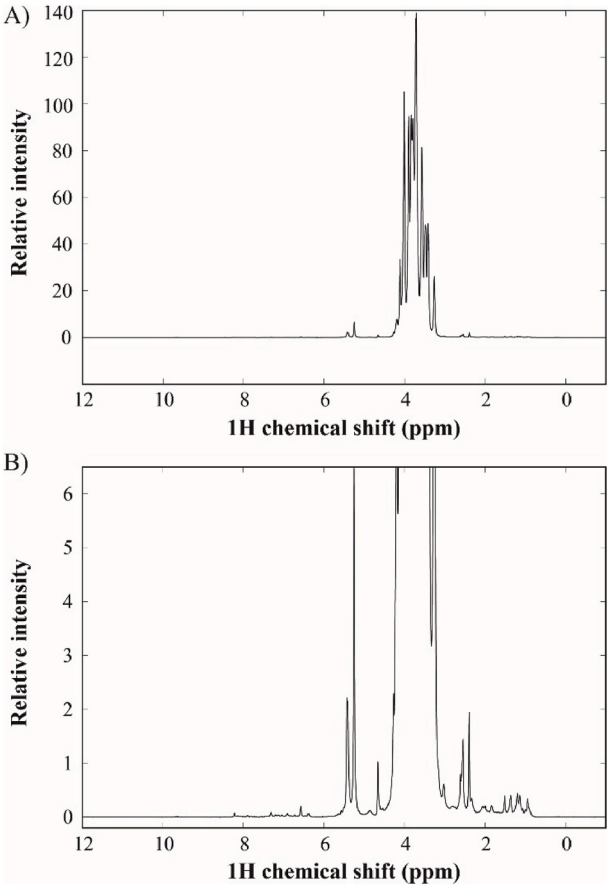

Fig. 4. A. ^{1} H 1D NMR spectrum of water soluble pyomelanin of *A.* cv. *littoralis* 2328.**4B.** Expanded version of the ^{1} H 1D NMR spectrum showing peaks at lower intensities.

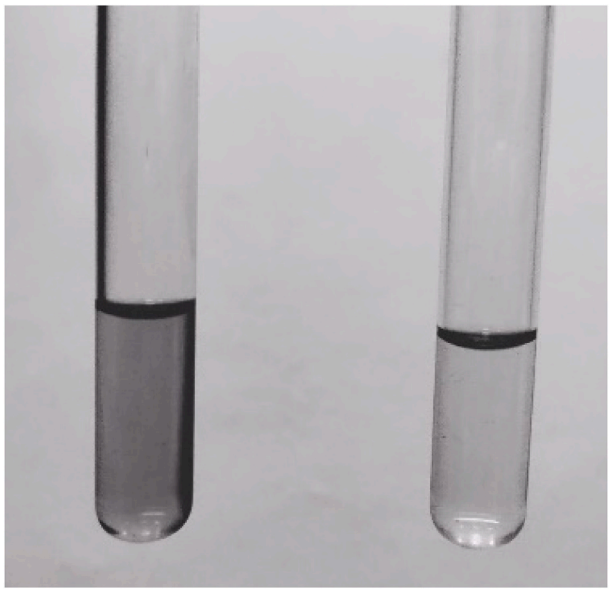

Fig. 5. Culture filtrate of 30-day old culture of *A.* cv. *littoralis* 2328 in CD medium. **A.** Control, **B.** with NTBC (1000 μM).

2022; Koch et al., 2023); the pigment showed a strong absorption peak at 3276.47 cm-1, which was very similar to that of bacterial pyomelanin (Bayram, 2021). However, a few differences in the FTIR, especially in the 1600-1500 cm^{-1} region and the presence of a peak around 2300 cm^{-1} in our sample need to be explored further to confirm if the pyomelanin of *A.* cv. *littoralis* 2328 has subtle structural differences compared to the reported ones. In support of this hypothesis, differences in peak intensities are also observed in the region between 0.5 and 3.0 ppm of the ^{1}H 1D NMR spectrum of *A.* cv. *littoralis* 2328 pyomelanin and the ^{1}H 1D NMR spectrum of *Inonotus hispidus* fungal pyomelanin (Li et al., 2021).

Generally, specific inhibitors of different forms of melanin synthesis are used to confirm the type of the pigment. In our study, the synthesis of the pigment was not inhibited by tricyclazole which inhibits DHN melanin synthesis or by kojic acid which inhibits DOPA-melanin synthesis confirming that it was not a derivative of DHN or DOPA melanin (Schmaler-Ripcke et al., 2009; Lorquin et al., 2022). In bacteria, NTBC inhibits pyomelanin production by binding irreversibly to the enzyme to Hpd thus inhibiting the conversion of 4-hydroxyphenylpyruvate to homogentisate (Kavana and Moran, 2003; Ketelboeter et al., 2014). In the present study also, NTBC inhibited pigment formation by the fungus confirming that the excreted melanin was pyomelanin (Ketelboeter et al., 2014; Ketelboeter and Bardy, 2015). Since the shikimate pathway generates tyrosine, exogenous tyrosine is not necessary to induce pyomelanin synthesis. However, some bacteria (Pavan et al., 2015) and

fungi fail to produce pyomelanin in the absence tyrosine in the culture medium (Schmaler-Ripcke et al., 2009; Almeida-Paes et al., 2018; Fernandes et al., 2021). *A.* cv. *littoralis* 2328 produced pyomelanin even in the absence of tyrosine in synthetic medium (CD). The need for external supply of phenolic substrate for melanin synthesis is intriguing since yeast cells of *Histoplasma capsulatum* require it for melanin production while the conidia synthesise the pigment in the absence of it (Nosanchuk et al., 2002). Moreover, *A.* cv. *littoralis* 2328 elaborated laccase enzyme which contributes to the synthesis of gentisyl alcohol and gentisaldehyde which are parts of pyomelanin (Lorquin et al., 2021). Since pyomelanin is a complex molecule exhibiting many structural complexities (Koch et al., 2023), focused study is needed to know if *A. littoralis* uses a unique pathway to produce the pigment.

Pyomelanin provides survival benefits by scavenging free radicals, binding to different drugs, resisting radiation and reactive oxygen species and aids in iron accumulation and extracellular electron transfer (Ketelboeter et al., 2014). Pyomelanin enhances fitness of bacteria by protecting them from heat stress (Zeng et al., 2017b), increasing iron uptake under iron-limiting conditions (Zheng et al., 2013), enhancing their virulence (Valeru et al., 2009), and providing resistance against antibacterial pyocins (Hocquet et al., 2016). Extending these results, pyomelanin producing fungi arguably should exhibit enhanced fitness enabling them to interact more efficiently with co-occurring competitors. It would of interest to study the role of pyomelanin in intra and interspecific competition among endophytic fungi.

Melanins, apart from being biodegradable and highly biocompatible, possess properties which could find use in cosmetics, biomedicine, material science, medical imaging, drug delivery, optoelectronics, and organic electronic devices (Tran-Ly et al., 2020; Bayram, 2021). Pyomelanin is superior to L-DOPA or DHN-melanin since it is not deposited in the cell wall but is secreted out of the cell thereby reducing the cost of production by eliminating complex extraction and purification steps (Koch et al., 2023). Apart from its antimicrobial, UV screening, antioxidant and anti-inflammatory properties (Lorquin et al., 2022), Koch et al. (2023) predict that pyomelanin has great potential for use in space travel by serving as radiation shield. Compared to bacteria, relatively fewer studies have been done on fungi for their pyomelanin production (Narayanan et al., 2020; Lorquin et al., 2021; Beeson et al., 2023). Most of these investigations are focused on opportunistic human pathogenic fungi (Schmaler-Ripcke et al., 2009; Keller et al., 2011; Heinekamp et al., 2013; Perez-Cuesta et al., 2020). Our study shows that fungi, including endophytes, should be screened for pyomelanin production to identify hyper producing isolates and those that produce chemically heterogenous pyomelanin to widen the spectrum of their technological use. This is essential since pyomelanin production by a given fungal species could vary depending on the strain (Almeida-Paes et al., 2018) as also ascertained by our observation that four isolates of a species produced different quantities of the pigment which could result in the evolution of ecotypes.

Conflict of interest statement

As the corresponding author of the paper titled '*A. littoralis* Mantle & D. Hawksw. survives as root endophyte of mangroves and produces water soluble melanin pigment', I declare that there is no conflict of interest.

CRediT authorship contribution statement

T.S. Suryanarayanan: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **M.B. Govindarajulu:** Supervision, Investigation, Data curation. **T.S. Murali:** Methodology, Investigation, Formal analysis, Data curation. **J.P. Ravishankar:** Supervision, Methodology, Conceptualization. **S. Bharathwaj:** Methodology, Investigation, Data curation. **M.D. Kalaiarasan:** Investigation, Formal analysis, Data curation.

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