ENHANCED PRODUCTION OF MELANIN THROUGH AN ENGINEERED *Trichoderma harzianum* STRAIN

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ABSTRACT

Melanins are a diverse group of biopolymers synthesized by various organisms, including fungi. These pigments play crucial roles in the biology and ecology of fungi, offering protection and contributing to their survival in diverse environments. There are broad biotechnological applications of these molecules, particularly in human health and agriculture. However, developing an efficient production system remains a challenge. The objective of this study was to optimize the production of a type of melanin through the genetic deletion of the *hmgA* gene in *T. harziaum*, using the CRISPR system through fungal transformation via protoplasts. We confirmed mutation through specific PCR (genotyping) and evaluated the development of color during mutant strain submerged fermentation (phenotyping). The strong dark color production meaning melanin biosynthesis was followed by a kinetics by measuring absorbance at 405 nm daily. This study presents the first evidence of the potential using a *Trichoderma harzianum* Δ*hmgA* mutant strain with improved biosynthesis of melanin, probably pyomelanin.

Keywords: 1. Melanin 2. *hmgA* 3. CRISPR.

1 INTRODUCTION

Melanins are a group of pigments present across nearly all biological kingdoms and can be classified into neuromelanin, pheomelanin, pyomelanin, eumelanin, and allomelanin (Bacurau et al., 2023). In filamentous fungi, melanins contribute to various functions, primarily related to the protection of hyphae and conidia from ultraviolet radiation, oxidants, heavy metals, and antifungal agents (Schmaler-Ripcke et al., 2009). Additionally, studies have shown that melanin also protects against attacks from host immune systems (Zhang et al., 2023; Wang et al., 2020). Pyomelanin is a type of melanin (brown pigment), notable for its thermostability and resistance to photodegradation, making it suitable for various applications. It is released extracellularly and has desirable properties compared to other melanins (via L-DOPA or DHN-melanin) (Lorquin et al., 2022). The molecule production in fungi occurs through the accumulation of tyrosine, which leads to the oxidation of intermediates such as homogentisic acid (HGA), subsequently polymerized into pyomelanin (Bacurau et al., 2023). In media supplemented with tyrosine *Aspergillus niger* and *Aspergillus fumigatus* pyomelanin production is observed, but in small quantities. However, deletion of *hmgA* resulted in significantly increase of pyomelanin, because the gene restricts its production (Koch et al., 2023, Schmaler-Ripcke et al., 2009). While pyomelanin biosynthetic pathway is well characterized in bacteria, notably in *Pseudomonas aeruginosa* (Yabuuchi and Ohyama, 1972), it remains poorly understood in economically important fungi. Our research group found a high homology of *hmgA* gene from *A. niger* in the biocontrol agent *Trichoderma harzianum* BRM 67436 genome (data not shown). Therefore, this study presents the first evidence of the potential for using a *T. harzianum ΔhmgA* mutant to enhance melanin biosynthesis, probably pyomelanin.

2 MATERIAL & METHODS

Vector propagation: CRISPR plasmid *pM33* and plasmid *pM31* carring dDNA (repair template) used in *hmgA* deletion were constructed for our group (data not shown) and were propagated in *E. coli* DH5a. Cells were growth in LB medium supplemented with 100 µg mL^{-1} of ampicillin, incubated under agitation at 150 rpm / 37ºC, overnight. Plasmids were purified using PureYield™ Plasmid Miniprep System Promega® and quantified for NanoDrop™ 2000/2000c.

Fungus transformation: transformation of *T. harzianum* BRM 67436 (WT) followed CÔRTES et al, (2023) protocol. *T. harzianum* (WT) was inoculated in YPD (1% yeast extract, 2% peptone and 2% dextrose) media at 72h, 25ºC and 150 rpm. After that the hyphae were washed with ultra-pure H20, followed by KCL (0.7 M, pH 5.8) and added in the falcon containing 10 ml of KCL and enzyme *Driselase* (12,5 mg mL^{-1}). For the transformation we added the protoplasts (1 $x10^{7}$), the plasmid DNA, we performed something like thermal shock, followed by a solution containing PEG 8000. Next, we added the transformation media, he uses sucrose for substitute glucose, furthermore the media was supplemented with Chlorimuron-ethyl at 60 µg mL-1. The plates were incubated in BOD at 25°C for seven days.

Mutants diagnostic PCR - genotyping: transformants were identified through diagnose PCR for detection of *hmgA* deletion. Specific primers were constructed and designed to detect the gene deletion in the targeted genome locus. Primers will be annealing of the external and internal region of the gene, and promote the change of model of bands observed in electrophoretic analysis.

Observation melanin production - phenotyping: *T. harzianum* WT and ∆*hmgA* was inoculated in minimal media agar. Was inoculated in minimal media broth and minimal medial with 0,5% of yeast extract broth, everyone was supplemented with 10mM of L-tyrosine, incubated at 25ºC for seven days. The absorbance was measured every day in wavelength of 405 nm for seven days.

3 RESULTS & DISCUSSION

According to our results, we constructed a *T. harzianum ΔhmgA* with increased melanin production. Figure 1A shows genotyping data, the first evidence of *hmgA* deletion in *T. harzianum* via a marker-free CRISPR/Cas9 system. The phenotyping (Figure 1B) also presents clear evidence of increased melanin production in both *ΔhmgA* H3 and H7 mutants, due to the change in the color of the medium supplemented with tyrosine, showing that the wild-type strain is not capable of producing melanin. As well as evaluating growth and production of H3 and WT, in different broth media (Figure 1C) and moreover we quantified pigment production by absorption measurements at 405 nm (according to Schmaler-Ripcke et al., 2009) (Figure 1D) in which pigment formation is reflected by the increase in absorption at the defined wavelength. Therefore, our results showed that both analyzed media can be used for the possible production of melanin.

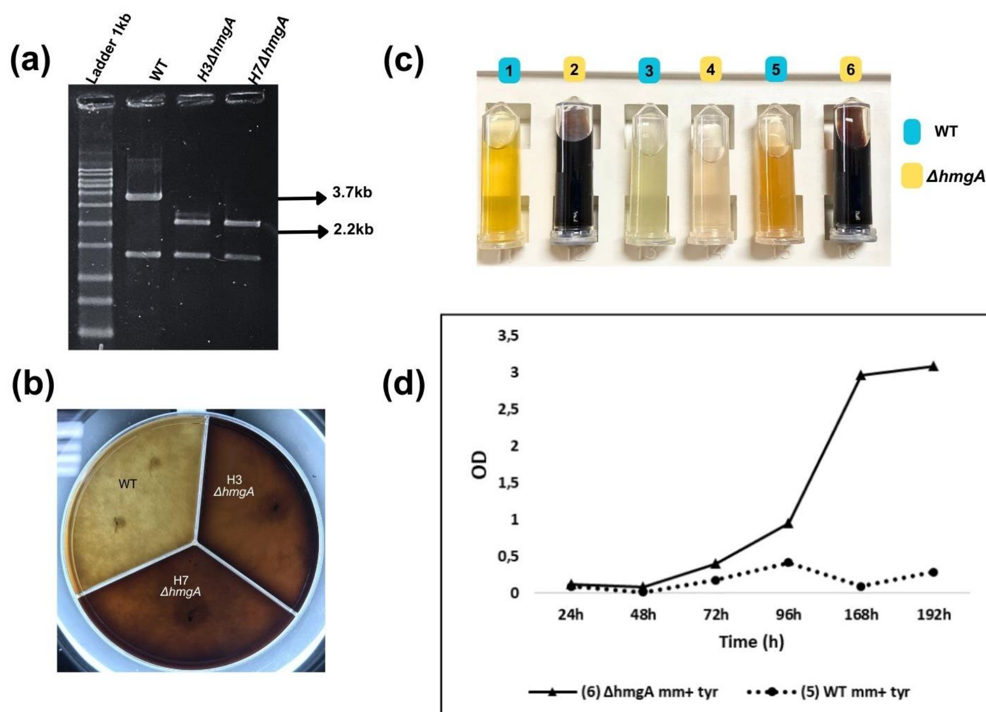

Figura 1 – Melanin enhanced production throgh improved *T. harzianum* strain. A) Genotyping - diagnostic PCR of ∆*hmgA* H3; B) Phenotyping - back of Petri dish with *T.harzianum* WT, ∆*hmgA* H3 and ∆*hmgA* H7 in minimal medium suplemented with L-tyrosine after seven days; C) Melanin production in different media supplemented with 10 mM L-tirosin: (1-2) MM + yeast extract, (3-4) MM no glucose (5-6) MM; D) Melanin quantification in MM (5, 6), supplemmented with 10 mM L-tyrosine after seven days.

According to the literature, the dark pigment produced by *T. harzianum ΔhmgA* is probably pyomelanin. This is explained by the exacerbate expression of the dark pigment after supplementation with tyrosine, which contributes to the increased biosynthesis of this type of melanin, a consequence of the accumulation of HGA provided by the genetic deletion of *hmgA*. Moreover, we observed that production occurs in more than one medium composition, something that can be further studied in order to optimize time and costs. However, additional characterization of the molecule and mutant sequencing are necessary to confirm our findings.

4 CONCLUSION

This study presents the first evidence of the potential for using a *Trichoderma harzianum ΔhmgA* mutant to enhance melanin biosynthesis, probably pyomlanin. Further studies are necessary to characterize the produced melanin, optimize the production process trough submerged fermentation and its application.

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ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Agriculture, Livestock and Supply (MAPA) and the Brazilian Agricultural Research Corporation - Embrapa Rice & Beans.