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1 **_Cryptococcus neoformans_ can utilize the bacterial melanin precursor**
2 **homogentisic acid for fungal melanogenesis**

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26 **Abstract**

27 *Cryptococcus neoformans* melanizes in the environment and in
28 mammalian tissues, but the process of melanization in either venue is
29 mysterious given that this microbe produces melanin only from exogenous
30 substrates. Understanding the process of melanization is important because it is
31 believed to protect against various stresses in the environment including
32 ultraviolet radiation and pigment production is associated with virulence.
33 Melanization in *C. neoformans* requires the availability of diphenolic precursors.
34 In contrast, many bacteria synthesize melanin from homogentesic acid (HGA).
35 We report that *C. neoformans* strains representing all four serotypes can
36 produce a brown pigment from HGA. The brown pigment was acid resistant and
37 had the electron paramagnetic resonance spectrum of a stable free radical,
38 qualities that identified it as a melanin. Melanin 'ghost'-like particles obtained
39 from pigmented *C. neoformans* cells were hydrophobic, fluorescent under a
40 variety of irradiation wavelengths, negatively charged, insoluble in organics
41 solvents and alcohols, resistant to degradation by strong acids and vulnerable
42 to bleaching. HGA-melanization was laccase-dependent and repressed by high
43 concentrations of glucose. The ability of *C. neoformans* to utilize a bacterial
44 melanin precursor compounds suggests a new substrate source for
45 melanization in the environment.

46

47 **Introduction**

48 *Cryptococcus neoformans* is a free-living cosmopolitan organism that can
49 survive in a variety of environmental niches. This fungus is a major pathogen in

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50 inmuno-compromised hosts. Cases of cryptococcosis have been reported from
51 all regions of the world (1). *C. neoformans* strains have been divided into three
52 varieties based on biochemical and genetic differences, known as *neoformans*,
53 *grubii*, and *gattii,* and recent evidence suggests that the phylogenetic distances
54 between these varieties are sufficient for grouping as distinct species (2).
55 Variety *neoformans* and *grubii* strains are often recovered from soils
56 contaminated with bird excreta, while the major environmental habitat of variety
57 *gattii* is believed to be certain arboreal species (1,3). *C. neoformans* has several
58 virulence factors, such as capsule production, ability to grow at 37ºC and
59 melanin synthesis. Unlike other melanotic fungi, melanization in *C. neoformans*
60 requires exogenous substrates. Melanin synthesis in *C. neoformans* is
61 catalyzed by a laccase, which is considered a phenol oxidase or diphenol
62 oxidase because it produces pigment from phenolic compounds with two
63 hydroxyl groups, but not tyrosine (4,5,6). Although, *C. neoformans* cells in the
64 environment are melanized, the environmental source of melanin precursors is
65 unknown (7).
66 Melanins are negatively charged, hydrophobic macromolecules of high
67 molecular weight formed by the oxidative polymerization of phenolic and/or
68 indolic compounds (8). The resulting pigments are usually brown or black, but
69 other colors have also been observed, depending on the substrate used to
70 induce melanization. Melanin polymers are remarkable in that they have a
71 stable population of organic free radicals (9). Melanin pigments are
72 multifunctional polymers found in all biological kingdoms. For *C. neoformans*,
73 melanin synthesis confers reduced susceptibility to amoebae predators,
74 ultraviolet light, temperature extremes, oxidative fluxes and heavy metals.

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75 Although melanization may have developed in response to environmental
76 pressures, the ability to produce melanin is associated with virulence in
77 mammalian host (10).

78 Several types of melanins have been described in bacteria, plants,
79 animals and fungi: eumelanins, phaomelanins, allomelanins and pyomelanins:
80 Eumelanins are formed from quinines and free radicals. Phaeomelanins are
81 derived from tyrosine and cysteine. Allomelanins are synthesized from nitrogen
82 free precursors and pyomelanins are derived from the catabolism of tyrosine via
83 *p*hydroxyphenylpyruvate (HPP) and homogentisic acid (HGA) (8,11). HGA is
84 catabolite of phenolic metabolism in a wide variety of higher organisms,
85 including mammals, fish, birds, amphibians and plants. Pyomelanin formation is
86 correlated with HGA production in three disparate marine species: *Vibrio*
87 *cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana* (11*)*.

88 *C. neoformans* produces eumelanin from catecholamine precursors,
89 such as L-dopa, epinephrine and norepinephrine (12). However, unlike other
90 fungi, melanization in *C. neoformans* occurs only when the organism is exposed
91 to precursor compounds. Since *C. neoformans* in the environment is
92 melanized, initial human infection might occur with melanized organisms.
93 However, practically nothing is known about the sources of melanin precursors
94 in the environment. Recently, we showed that the interaction between a
95 bacterium, *Klebsiella aerogenes*, and *C. neoformans* resulted in fungal
96 melanization (13). The mechanism for that process involved production of
97 dopamine by the bacteria that was used as melanin precursor in *C.*
98 *neoformans*. That observation provided a precedent for microbial sources of
99 substrates for *C. neoformans* melanization in the environment (13). Now we

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100 demonstrate that *C. neoformans* can make melanin from HGA thus establishing
101 that bacteria and fungi can use the same precursor in melanization.
102
103 **Material and methods**
104
105 **Media**. SM media is composed of 10 g/l of glucose, 10 g/l of proteose peptone,
106 1 g/l of yeast extract, 1 g/l of $MgSO_4$, 1.9 g/l of KH_2PO_4 and 0.6 g/l of K_2HPO_4
107 in water. Minimal media consists of 15.0 mM glucose, 10.0 mM $MgSO_4$, 29.4
108 mM K_2HPO_4, 13.0 mM glycine and 3.0 mM thiamine, pH 5.5. Minimal media
109 agar was made by adding 20 g/l to this media.
110
111 **Fungal strains.** *C. neoformans* serotype A strain H99 was a gift from John
112 Perfect (Durham, NC). Serotype D strain B3501 was purchased from the ATCC
113 (Manassas, Va). Serotype AD strain MAS93-120 was provided by Mary Brandt
114 (Atlanta, Ga). Serotype B (NIH198) strain and serotype C strains 1343 and B
115 4546 were obtained from Thomas Mitchell (Durham, NC). In addition, we also
116 used the laccase negative mutant 2E-TU (URA5, Mel^-) of strain B3501 and its
117 laccase-complemented strain 2E-TUC (URA5, mel^+) (14).
118
119 **Agar assay for pigment induction.** To induce pigment production in *C.*
120 *neoformans*, H99 cells were grown in SM agar supplemented with 1 mM HGA
121 (Sigma Chemical Co, St. Louis, MO), at 22 ºC for 5 days. As a control for
122 spontaneous pigment production, SM agar with HGA without cells was
123 incubated in parallel for an equivalent time.
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125 **Role of laccase enzyme in pigment production.** The laccase-negative
126 mutant 2E-TU (URA5, Mel^-) of strain B3501, its laccase complemented strain
127 2E-TUC (URA5, mel^+) and the parental strain B3501 were grown in both liquid
128 and agar minimal media. Media was supplemented with different concentrations
129 of glucose (3, 10, 20 and 40 g/l) and 1 mM of HGA. Cells were incubated at
130 30ºC for 5 days.
131

132 **Isolation and study of biochemistry properties of melanin particles.**
133 *C. neoformans* cells pigmented by growth in minimal media supplemented with
134 1 mM of HGA at 22ºC for 5 days were suspended in phosphate-buffered saline
135 (PBS). Cells were collected by centrifugation at 3000 rpm for 10 minutes and
136 suspended in 1.0 M sorbitol-0.1 M sodium citrate (pH 5.5). Protoplasts were
137 generated by incubating cells at 30ºC in 10 mg/ml of cell wall-lysing enzymes
138 (*Trichoderma harzianum*, Sigma Chemical Co, St. Louis, MO) overnight. The
139 protoplasts were then collected by centrifugation, washed with PBS, and
140 incubated in 4.0 M guanidine thiocyanate for 12 hour at room temperature with
141 frequent vortexing. The resulting brown material was collected by centrifugation,
142 washed with PBS and digested with 1.0 mg/ml Proteinase K (Roche Molecular
143 Biochemicals, Indianapolis, IN). The resulting material was washed again in
144 PBS and boiled in 6.0 M HCl for 1 hour to hydrolyse cellular contaminants
145 associated with melanin. The particles were collected by centrifugation and
146 washed with PBS. Aliquots from each step of the sample preparation were
147 visualized by microscopy for size measurements after heat fixation on glass
148 slides using a Olympus AX 70 microscope (Melville,NY) equipped with
149 fluorescence filters. The resultant particles were then analyzed for their

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150 solubility in bleach and various organic solvents (1-butanol, methanol,
151 chloroform, 1-butanol:chloroform (1:1 v/v), methanol:chloroform (1:1 v/v),
152 acetone, carbon tetrachloride, NN-dimethyl formamide and toluene).
153 Additionally, the particles recovered from brown cells after treatment with
154 enzymes, denaturant, and hot acid were visualized using fluorescence
155 microscopy. Melanin particles obtained from *C. neoformans* cells grown with L-
156 DOPA were used as control.
157
158 **High Performance Liquid Chromatography (HPLC) of oxidized melanins**
159 **and culture supernatants**. The melanin particles derived from H99 cells grown
160 with 1 mM of HGA at 22 ºC for 5 days and L-dopa were oxidized with
161 permanganate and analyzed by HPLC. Samples were dried at 50°C and then
162 oxidized with acidic permanganate. Pyrrole-2,3,5-tricarboxylic acid (PTCA),
163 pyrrole-2,3-dicarboxylic acid (PDCA), 1,3-thiazole-2,4,5-tricarboxylic acid
164 (TTCA) and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) were used as standard
165 compounds of melanin degradation products (gifts from K. Wakamatsu,
166 Toyoake, Japan). Chromatograms of PDCA, TDCA, TTCA and PTCA standards
167 yielded peaks at 5.1, 6.1, 7.1 and 11.0 min, respectively. The oxidation products
168 were analysed by HPLC using a Shimadzu LC-600 liquid chromatograph (NY,
169 NY), Hamilton PRP-1 C_{18} column (250 x 4.1 mm dimensions, 7 μm particle
170 size), and Shimadzu SPD-6AV UV detector. The mobile phase was 0.1%
171 trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in
172 acetonitrile (solvent B). At 1.0 mL/min, the elution gradient was (min, %B): 0, 0;
173 1, 0; 12, 25; 14, 25; 16, 0. The UV detector was set at a 255 nm absorbance.
174 A filtered supernatant from a *C. neoformans* culture was analysed for 7 days by

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175 HPLC using a Waters 600 liquid chromatograph, Alltech C18 column (250 x 4.6
176 mm dimensions, 5 um particle size), and Waters 486 detector measuring
177 absorbance at 270 nm. The mobile phase was 25 mM ammonium bicarbonate
178 buffer pH 7.9. The flow rate was 1.0 mL/min. Standards consisted of 1-50 mM
179 solutions of HGA.

180

181 **Zeta potential**. A suspension of 10^7 cells/ml from *C. neoformans* H99 grown in
182 SM media supplemented with 1 mM HGA for 5 days at 22ºC was suspended in
183 10 mM KCl and the cell zeta potential was measured with a Z potential analyzer
184 (Brookhaven Instrument Corporation, Moltsville, NY). The zeta potential, or
185 surface charge, of the particles was determined by applying an electric field to
186 the particles in suspension and then determining the direction and velocity of
187 the particles movement by measuring light scattering of a laser beam passed
188 through the sample. Each value reported represents the average of twenty
189 measurements.

190

191 **Electron paramagnetic resonance (EPR).** Particles recovered from
192 pigmented cells grown in media supplemented with HGA were analysed by
193 electron paramagnetic resonance (EPR). EPR spectra were obtained with a
194 Varian E112X- Band Spectrometer model. Parameters for EPR were as follows:
195 modulation frequency, 9.07 GHz; modulation amplitude, 1633.0 G, centre field,
196 3240.0 G, sweep width 80.0 G, microwave frequency, 9.2995 GHz, microwave
197 power, 0.20 mW, and temperature, 77 K. As controls we used cells grown in
198 media without HGA (non-pigmented cells) and L-dopa melanin particles.

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200 **Transmission electron microscopy (TEM).** Particles from pigmented cells 201 were fixed in 2% v/v glutaraldehyde in 0.1 M cacodylate at room temperature for 202 2 hours, followed by overnight incubation in 4% v/v formaldehyde, 1% v/v 203 glutaraldehyde and 0.1% v/v PBS. The samples were subjected to 90 minutes 204 post-fixation in 2% w/v osmium tetraoxide, serially dehydrated in ethanol, and 205 embedded in Spuris epoxy resin. Sections (70-80 nm) were cut on a Reichert 206 Ultracut UCT and stained with 0.5% w/v uranyl acetate and 0.5% w/v lead 207 citrate. Samples were viewed in a JEOL 1200EX transmission electron 208 microscope at 80KV. The diameter size and thickness of "ghost" shells from 209 200 particles recovered from cells pigmented with L-dopa and HGA were 210 measured. The average, standard deviation and p-value were calculated.

211

212 **Susceptibility of L-dopa- and HGA-melanized and nonmelanized *C.*** 213 ***neoformans* cells to UV radiation.** Eight day-old *C. neoformans* melanized 214 (HGA and L-dopa) and nonmelanized cells were plated in equal number ($1x10^4$) 215 on Sabouraud dextrose agar (Difco, Detroit, Michigan). Plates were exposed to 216 UV light (254 nm) generated in a Stratalinker 1800 (Stratagene, La Jolla, 217 California) at 10,000 µJoules/cm^2. The percent of cells surviving were 218 measured. The average, standard deviation and p-value were calculated from 5 219 measurements.

220

221 **Phagocytosis experiments.** Macrophage assays were done with *C.* 222 *neoformans* cells grown in minimal medium for 6 days in the presence 223 (melanized) or absence (nonmelanized) of 1 mM of L-dopa and 1 mM of HGA. 224 The ratio of macrophages (J774) to *C. neoformans* was 1:1. The assay was

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225 done in the presence and absence of antibody 18B7. The results were
226 expressed as the phagocytic index, which was defined as the number of
227 attached and internalized yeast per number of macrophages X 100. The
228 average, standard deviation and p-value were calculated.

229

230 **Statistical analysis.**

231 The statistical analysis was analyzed using Prism version 4 (GraphPad
232 Software). Results were compared by *t* test.

233

234 **Results**

235 ***C. neoformans* brown pigment production from homogentisic acid is**
236 **laccase-dependent.** *C. neoformans* yeast cells (wild type B3501) grown in
237 media supplemented with homogentisic acid were brown (figure 1A). Since
238 melanization in *C. neoformans* is catalyzed by laccase we investigated whether
239 this enzyme was involved in HGA-derived pigment production. No pigment
240 production was observed after incubation of the *C. neoformans* 2E-TU Lac-
241 mutant in minimal media supplemented with HGA (figure 1B). In contrast,
242 incubation of the *C. neoformans* 2E-TU mutant complemented with laccase (2E-
243 TUC) or the wild type strain resulted in brown pigment production (figure 1A,C).
244 Hence, brown pigment production was dependent on fungal laccase and, since
245 laccase expression is repressed by glucose, we hypothesized that the glucose
246 concentration in the medium would affect pigment induction. Consistent with
247 this premise, pigment production was inhibited at glucose concentrations above
248 20 g/l (figure 1). The same results were observed irrespective of whether the
249 experiment was done in liquid or on agar plates.

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250

251 Pigment production required live *C. neoformans* cells (data not shown). No
252 differences in pigment induction were observed between different serotypes of
253 *C. neoformans* (data no shown). Finally, pigment was observed not only in the
254 yeast cells, but also in culture medium (figure 2). To test if the HGA precursor
255 *p*-hydroxyphenylpyruvate induced brown pigmentation in *C. neoformans*, yeast
256 cells were grown in minimal media supplemented with either 1, 10, or 50 mM *p*-
257 hydroxyphenylpyruvate at 22 ºC for 5 days. No pigment induction was observed
258 when *C. neoformans* cells were grown with this compound (data not shown).

259

260 **The brown pigment is a melanin**. Characteristics of melanin are acid
261 resistance and insolubility in aqueous solvents. Since melanin particles can be
262 recovered from melanized cells with L-dopa by a method that involves digestion
263 with cell wall lytic enzymes, denaturant extraction and hot acid treatment, we
264 evaluated whether similar particles could be recovered from brown cells
265 induced by growth in minimal media supplemented with HGA. Treatment of
266 brown cells sequentially with enzymes, guanidinium isothiocyanate, and boiling
267 6 M HCl led to the recovery of spherical-particles similar to those recovered
268 from L-dopa melanized cells, although the particles from the brown cells were
269 significantly smaller (figure 3).

270

271 Treatment of brown pigmented *C. neoformans* cells with 100% and 10% bleach
272 led to the complete disappearance of color. However, *C. neoformans* cells
273 melanized with HGA were resistant to degradation by organic solvents and
274 alcohols, such as, 1-butanol, methanol, chloroform, 1-butanol:chloroform (1:1

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275 v/v), methanol:chloroform (1:1 v/v), acetone, carbon tetrachloride, NN-dimethyl
276 formamide and toluene. On the basis of the susceptibility of the brown pigment
277 to bleaching, its resistance to acid and its insolubility in aqueous and organic
278 solvents we tentatively identified the HGA-derived brown pigment as a melanin.
279

280 To explore the biochemical composition of the brown pigment, the material was
281 oxidized with acidic permanganate and the products analyzed by HPLC. The
282 chromatogram of oxidized products from brown cells revealed three peaks,
283 eluting at 6.1, 7.1 and 11.0 minutes with retention times that matched those of
284 TDCA, TTCA and PTCA respectively (figure 4A). The chromatographic results
285 are consistent with the presence of melanin degradation products in
286 permanganate-oxidized particles. In contrast, oxidation of melanin particles
287 derived from L-dopa melanized *C. neoformans* cells revealed the presence only
288 of a PTCA component characteristic of eumelanin (figure 4B).
289

290 Since melanization is known to impart an additional negative charge to *C.*
291 *neoformans* cells, we evaluated the zeta potential of brown-pigmented
292 cryptococcal cells and particles. Melanin particles from cells grown in minimal
293 media supplemented with HGA manifested a significantly higher negative
294 charge (-48.90 ± 1.63 mV) than melanin particles from cells grown in minimal
295 media supplemented with L-dopa (-35.93 ± 0.93 mV) ($p < 0.05$).

296 **Fluorescence of HGA-derived melanins.** Melanin particles recovered
297 from brown cells were evaluated for their fluorescence properties under
298 different wavelengths. Fluorescence was observed when the particles
299 were irradiated with light of 420, 535 and 610 nm (figure 5). In contrast,

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300 melanin particles from cells melanized by growth in L-dopa did not
301 fluoresce (data not shown). Additionally, intact pigmented *C. neoformans*
302 cells grown in minimal media with HGA did not fluoresce (data not shown)
303

304 ***C. neoformans* consumes HGA to produce melanin.** HPLC analysis of *C.*
305 *neoformans* culture supernatants in minimal media supplemented with HGA
306 was used to establish whether there was a correlation between the metabolism
307 of HGA in solution and the appearance of brown pigmentation in *C.*
308 *neoformans*. The chromatograms of HGA standard revealed a main peak
309 eluting at 9.76 minutes. As culture incubation times increased, the HPLC peak
310 corresponding to HGA diminished in size, which was consistent with utilization
311 of the compound by the melanizing fungal cells. (figure 6).
312

313 ***C. neoformans* melanin particles induced by HGA showed the presence of**
314 **stable free radicals.** The acid-resistant melanin particles isolated from *C.*
315 *neoformans* cells growth in minimal medium supplemented with HGA produced
316 a signal indicative of a stable free-radical population (figure 7). L-dopa melanin
317 particles used as positive controls showed the same signal as the melanin
318 particles induced by HGA. Non-melanized cells used as a negative control did
319 not produce signal (data not shown).
320

321 **Visualization, measurement and statistical analysis of melanin particles.**
322 Melanin particles made from L-dopa and HGA were sectioned and viewed by
323 transmission electron microscopy (figure 8A and B). The particle diameter and
324 thickness of the ghost shell were measured. Ghost particles from L-dopa had a

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diameter similar to the parental cells, but the HGA particles showed a significant reduction in size ($p<0.05$) (figure 8C). The thickness of the shell in the HGA particles was significantly thinner than that of L-dopa ghost particles ($p< 0.05$) (figure 8D).

Susceptibility of melanized and nonmelanized cells to UV radiation. To study whether melanized cells with L-dopa and HGA were susceptible to UV light, 8 day old melanized cells were exposed to UV radiation (figure 9). *C. neoformans* cells grown in the presence of HGA were significantly less susceptible to the fungicidal effect of UV radiation than were non-melanized cells ($p< 0.05$). The differences in susceptibility between L-dopa- and HGA-melanized cells were not significant ($p=0.06$).

Effect of melanin on macrophage phagocytosis of *C. neoformans*. To study the effect of melanin on phagocytosis, J744 macrophages were incubated with melanized (HGA and L-dopa) and non-melanized *C. neoformans* cells in the presence and absence of capsule-binding monoclonal antibody 18B7. In the absence of 18B7, there was no phagocytosis or difference in the phagocytic index (data not shown). Phagocytosis of HGA-melanized cells in the presence of 18B7 was significantly less than that of non-melanized cells ($p< 0.05$) (figure 10). There were no significant differences in the phagocytosis of L-dopa and HGA melanized cells ($p=0.3$).

Discussion

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349 In this study, we analyzed the effect of homogentisic acid on pigment
350 production in *C. neoformans*, since this substrate produces melanin pigment in
351 a wide variety of organisms (11). Growth of *C. neoformans* in media
352 supplemented with HGA resulted in brown pigmentation of fungal colonies. This
353 phenomenon was observed with *C. neoformans* strains representing all
354 serotypes and required viability of fungal cells. Pigment production required
355 expression of *C. neoformans* laccase and was inhibited by glucose
356 concentrations higher than 20 g/l, consistent with the fact that laccase
357 expression is glucose-repressible (15). Since *C. neoformans* can produce
358 melanin from diverse compounds, including catecholamines such as L-dopa,
359 dopamine, epinephrine and norepinephrine, but not tyrosine, we investigated
360 whether or not *C. neoformans* could produce pigment from an HGA precursor
361 (*p*-hydroxyphenylpyruvate). Hence, like the situation for the catecholamines, the
362 precursor compound for HGA is unable to sustain melanogenesis.
363 HGA is a precursor of melanin for many types of melanogenic bacteria.
364 Several lines of evidence indicate that the brown pigment synthesized by *C.*
365 *neoformans* from HGA is a melanin. First, brown pigment particles were
366 insoluble in aqueous or acidic solutions, yet were susceptible to oxidation by
367 permanganate. Second, HPLC analysis of permanganate oxidation products
368 revealed the compounds with chromatographic migration matched that of the
369 melanin degradation products TDCA, TTCA and PTCA. Third, pigment
370 production required the presence of laccase, which catalyzes the first step of
371 melanin production in *C. neoformans*. Fourth, EPR spectrum revealed that the
372 brown pigment showed a signal indicative of a stable free radical population that

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373 is characteristic of melanins. Hence, we conclude that HGA can also serve as a
374 precursor for melanin synthesis in fungi.

375 Nevertheless, there were significant differences between melanin
376 produced from HGA and the classic black pigments produced from L-dopa and
377 other catecholamines. The finding of compounds with chromatographic
378 properties similar to TDCA, TTCA and PTCA among HGA oxidative degradation
379 products contrasts with the solitary presence of PTCA in L-dopa derived
380 melanin. Although one cannot infer the presence of these compounds in HGA
381 oxidative generation products on the basis of chromatographic migration alone
382 the presence of additional oxidative products implies a different molecular
383 structure for HGA and L-dopa derived melanins. The diameter of the particles
384 recovered from the HGA-pigmented cells and the thickness of the shell were
385 smaller and more negatively charged than were L-dopa derived particles.
386 Furthermore, HGA-derived particles were fluorescent when illuminated with
387 several wavelengths, while no fluorescence is produced by similarly irradiated
388 L-dopa particles. Since melanization in *C. neoformans* is dependent on
389 exogenous substrate and the type of melanin formed reflects the chemical
390 structure of the substrate (12), the differences between brown-pigmented
391 particles derived from cells grown with HGA and the black particles derived from
392 cells grown in L-dopa implied significant differences in the chemical structures.
393 These differences probably reflect variances in the precursor compounds, but
394 do not result in different effects on phagocytosis or susceptibility to UV
395 radiation.

396 Despite the biochemical and biophysical differences apparent from the
397 comparison of the HGA- and L-dopa-derived ghosts, HGA- and L-dopa-derived

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398 pigment conferred remarkably similar photoresistant and anti-phagocytic
399 properties to *C. neoformans* cells. Hence, the HGA- and L-dopa-derived
400 pigments appeared to be functionally similar and suggest that precursor
401 compounds for melanogenesis are relatively interchangeable with regard to the
402 function of the final product.

403 The fact that *C. neoformans* can utilize HGA for melanin synthesis
404 indicates that this compound can be used as a precursor of melanization in both
405 eukaryotes and prokaryotes. Given that HGA is involved in bacterial, animal,
406 and plant metabolism, this result implies a new source for *C. neoformans*
407 melanization substrate in both the environment and during animal infection.
408 From the vantage point of *C. neoformans* biology, the finding that HGA is a
409 precursor for melanization significantly could help to explain the paradox that
410 this organism has the machinery for melanization and for remodeling its cell wall
411 in the melanized state, yet is unable to make melanin unless provided with
412 certain substrates. Although some Aspergillus spp. can synthesize HGA (16),
413 there is no evidence that *C. neoformans* can use endogenous compounds for
414 melanization, even if they were available, since the fungus makes no pigments
415 without exogenous substrates. For *C. neoformans* the capacity for scavenging
416 chemical compounds made by other microbes suggests that this organism
417 gains the benefit of melanization by having the machinery for melanin synthesis
418 and remodeling without having to invest in the biosynthetic pathways required to
419 generate precursor compounds.

420 In summary, we report that HGA can induce melanization of *C.*
421 *neoformans* by a fungal laccase. Although the effect was observed only in
422 laboratory conditions its demonstration establishes a precedent for a new

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423 substrate for melanization in *C. neoformans* that could provide a new source for
424 melanin substrates in the environment, since HGA is a ubiquitous microbial
425 product that is also found in arboreal species and animals.
426

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FIGURE 1: *C. neoformans* cells grown in minimal media supplemented with 1 mM HGA incubated at 30ºC for 5 days. A.- B3501 parental strain. B.- 2E-TU lac- mutant. C.- 2E-TUC mutant complemented with laccase gene. I.- Minimal media supplemented with 40 g/l of glucose. II.- Minimal media supplemented with 20 g/l of glucose. III. - Minimal media supplemented with 10 g/l. IV.- Minimal media supplemented with 3 g/l of glucose.

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494 **FIGURE 2:** Supernatants obtained from a culture of *C. neoformans* in SM
495 media supplemented with 1 mM HGA. A. B3501 parental strain. B. 2E-TU lac-
496 mutant. C. 2E-TUC mutant complemented with laccase gene.
497
498 **FIGURE 3:** Brown particles from *C. neoformans* H99 cells after treatment with
499 HCl. Panel A. Positive control. Particles recovered from H99 cells grown in SM
500 media with L-dopa. Panel B. Particles recovered from H99 cells grown in SM
501 media supplemented with 1 mM of HGA. Scale bar represents 10 μm for both
502 panels.
503 **FIGURE 4:** HPLC Chromatogram of *C. neoformans* HGA (panel A) and L-dopa
504 (panel B) particles after treatment with HCl. The results show the presence of
505 peaks with retention times of TDCA, TTCA and PTCA in HGA melanin particles
506 and an exclusive PTCA product in L-dopa melanin.
507
508 **FIGURE 5:** Fluorescence of particles recovered after HCl treatment from *C.*
509 *neoformans* H99 cells grown in SM media supplemented with 1 mM of HGA. A.
510 Light microscopy. B. Fluorescence under DAPI filter. C. Fluorescence under
511 Rhodamine filter. D.Fluorescence under FITC filter.
512
513 **FIGURE 6**: Analysis by HPLC of *C. neoformans* supernatant grown in SM
514 media supplemented with 1 mM of HGA. The panel showed the presence of
515 HGA at 9.76 minutes, which is consumed with the passage of time.
516
517 **FIGURE 7:** Electron paramagnetic resonance spectrum. The results detect a
518 signal at 3240 G, showing the presence of stable free radical population.

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519

520 **FIGURE 8:** Transmission electron microscopy of melanin particles obtained
521 from H99 cells of *C. neoformans* grown in minimal medium supplemented with 1
522 mM of HGA (A and B). Measurement of the diameter (C) and the thickness of
523 the shell (D) of melanin particles. In black HGA melanized cells. In white L-dopa
524 melanized cells.

525

526 **FIGURE 9:** Percent survival of *C. neoformans* melanized with HGA (gray, filled)
527 and L-dopa (black, filled) and nonmelanized cells (open bars) after UV radiation.
528 *, indicates $p<0.05$ compare with melanized cells.

529

530 **FIGURE 10:** Phagocytosis index of *C. neoformans* melanized with HGA (gray,
531 filled), L-dopa (black, filled) and nonmelanized cells (open bars) by J774.16
532 cells. *, indicates $p<0.05$ compare with melanized cells.

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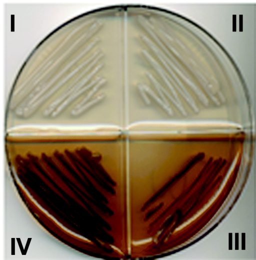

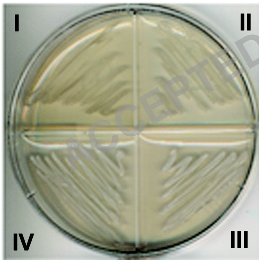

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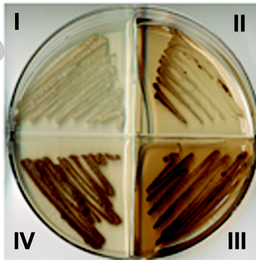

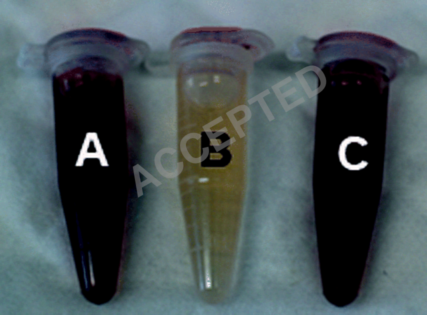

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A

B

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L-DOPA

Homogentisic acid

A

Absorbance, arbitrary units

TDCA

TTCA

PTCA

0 5 10 15 20

Elution time, min

B

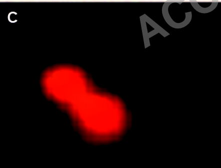
Absorbance, arbitrary units

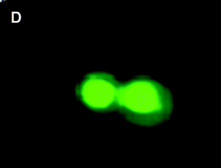
PTCA

0 10 20 30

Elution time, min

ACCEPTED





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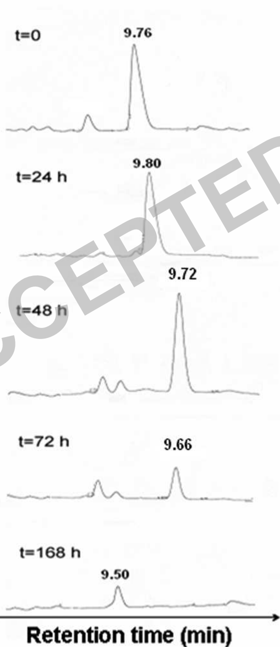

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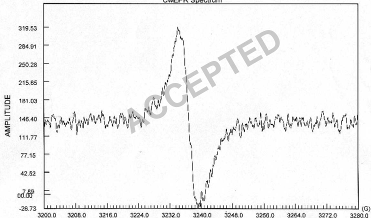
CwEPR Spectrum
AMPLITUDE
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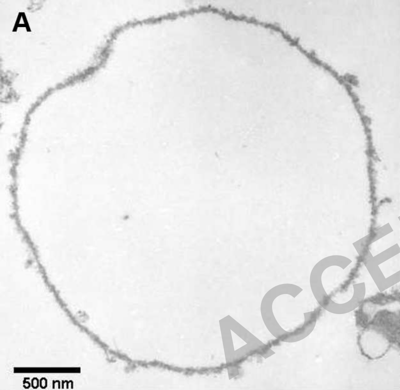
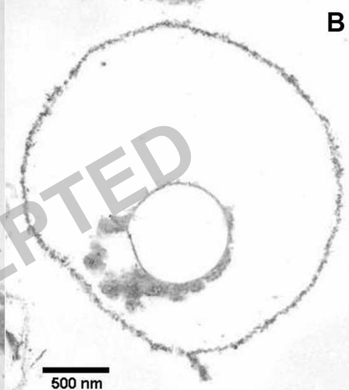
284.91
250.28
215.65
181.03
146.40
111.77
77.15
42.52
7.89
00.00
-26.73
3200.0
3208.0
3216.0
3224.0
3232.0
3240.0
3248.0
3256.0
3264.0
3272.0
3280.0
(G)
ACCEPTED





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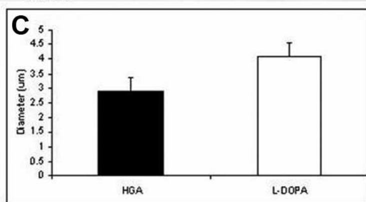

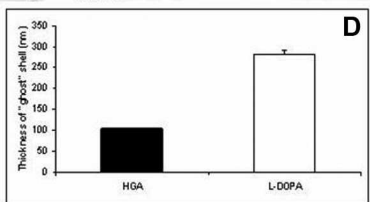

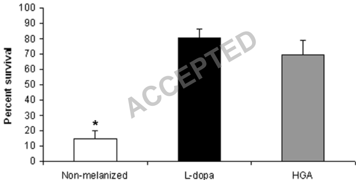
100
90
80
70
60
50
40
30
20
10
0
Percent survival
*
Non-melanized
L-dopa
HGA
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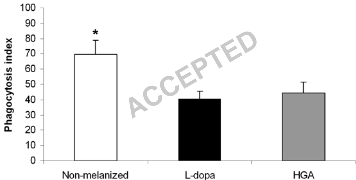
100
90
80
70
60
50
40
30
20
10
0
Phagocytosis index
*
Non-melanized
L-dopa
HGA
ACCEPTED