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2 **Alkane-grown *Beauveria bassiana* produce mycelial pellets displaying peroxisome**  
3 **proliferation, oxidative stress, and cell surface alterations**

4

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16 **Abstract**

17           The entomopathogenic fungus *Beauveria bassiana* is able to grow on insect cuticle  
18 hydrocarbons as the sole carbon source, inducing several enzymes involved in alkane  
19 assimilation and concomitantly increasing virulence against insect hosts. In this study, we  
20 describe some physiological and molecular processes implicated in growth, nutritional  
21 stress response, and cellular alterations found in alkane-grown fungi. The fungal cytology  
22 was investigated using light and transmission electron microscopy (TEM) while the surface  
23 topography was examined using atomic force microscopy (AFM). Fungal hydrophobicity  
24 was also measured on the cell surface. Additionally, the expression pattern of several genes  
25 associated with oxidative stress, peroxisome biogenesis, and hydrophobicity were analysed  
26 by qPCR. We found a novel type of growth in alkane-cultured *B. bassiana* similar to  
27 mycelial pellets described in other alkane-free fungi, which were able to germinate and  
28 produce viable conidia in media without a carbon source and to be pathogenic against  
29 larvae of the beetles *Tenebrio molitor* and *Tribolium castaneum*. Optical microscopy and  
30 TEM showed that pellets were formed by hyphae cumulates with high peroxidase activity,  
31 exhibiting peroxisome proliferation and an apparent surface thickening. Alkane-grown  
32 conidia appeared to be more hydrophobic and cell surfaces displayed different topography  
33 than glucose-grown cells, as it was observed by AFM. We also found a significant  
34 induction in several genes encoding for peroxins, catalases, superoxide dismutases, and  
35 hydrophobins. These results show that both morphological and metabolic changes are  
36 triggered in mycelial pellets derived from alkane-grown *B. bassiana*.

37 **Keywords:** entomopathogenic fungi, hydrocarbon degradation, hydrophobicity, peroxins.

## 38 Introduction

39 Entomopathogenic fungi do not require any specialized mode of entry to invade  
40 their insect hosts; they usually start the infection cycle by penetrating through the insect  
41 epicuticle. This outermost cuticle layer is composed by lipids, mostly aliphatic  
42 hydrocarbons or alkanes with both straight-chain and methyl-branched, usually between 20  
43 to more than 40 carbons (Pedrini et al., 2007). The ability of entomopathogenic fungi to  
44 degrade insect hydrocarbons and utilize them for energy production and for the  
45 biosynthesis of cellular components was first shown in *Beauveria bassiana* and  
46 *Metarhizium anisopliae* (Napolitano and Juárez, 1997). In addition, alkane-grown *B.*  
47 *bassiana* was more virulent than glucose-grown fungi by producing either higher insect  
48 mortality or lesser mean lethal time against different hosts (Crespo et al. 2002; Pedrini et al.  
49 2009). Thus, alkane degradation by *B. bassiana* represents a key metabolic pathway related  
50 to the insect pathogenic nature of the fungus. However, growth on alkanes causes major  
51 changes in fungal metabolism (Crespo et al., 2000) and a scenario of oxidative stress is  
52 caused by the accumulation of reactive oxygen species, which is successfully overcome by  
53 the induction of antioxidant genes and enzymes (Huarte-Bonnet et al., 2015). Moreover, the  
54 spore yields in alkane-grown fungi are usually lower than those obtained in fungi grown in  
55 rich media (Napolitano and Juárez, 1997), impairing the achievement for acceptable mass  
56 production in industrial resources.

57 Filamentous fungi often grow in liquid cultures exhibiting a plethora of  
58 morphological structures, e.g. three-dimensional aggregates ranging from loose clumps of  
59 mycelia to dense pellets. Pellet growth seems favorable for the production of several  
60 biotechnological products, and the optimizations of pellets formation are constantly revised

61 (Wucherpfenning et al., 2010). In this regard, cell aggregation is dependent on several  
62 cultivation conditions like the initial particle concentration, the hydrodynamic conditions,  
63 and the pH value, among other factors (Grimm et al., 2005a). As far as we know, there are  
64 no reports about pellet formation in alkane-grown fungi. The aim of the current study was  
65 to characterise a novel type of cellular growth produced in alkane-cultured *B. bassiana*,  
66 similar to mycelial pellets described in other filamentous fungi. We described an oxidative  
67 stress scenario associated with peroxidase activity and peroxisome proliferation, the same  
68 as cell surface alterations in alkane-grown *B. bassiana*.

69

## 70 **Materials and methods**

### 71 *Cultivation of fungi*

#### 72 *Fungal cultivation and inoculants preparation*

73 *Beauveria bassiana* strain GHA was routinely cultured and maintained on potato  
74 dextrose agar (PDA) (BD Difco, Sparks, USA). Conidia harvested from this medium were  
75 suspended in 0.01% Tween 80 in sterile distilled water, vortexed for approximately 3 min,  
76 and filtered through a 75  $\mu\text{m}$  sieve to remove debris. These conidial suspensions were  
77 adjusted in a Neubauer chamber to  $1 \times 10^7$  conidia  $\text{ml}^{-1}$ , and were used to inoculate  
78 complete liquid medium (CM) flasks and incubated at 26°C for 2 days with aeration (180  
79 rpm). CM is composed by 0.4 g  $\text{KH}_2\text{PO}_4$ , 1.4 g  $\text{Na}_2\text{HPO}_4$ , 0.6 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g KCl,  
80 0.7 g  $\text{NH}_4\text{NO}_3 \cdot 7\text{H}_2\text{O}$ , 10 g glucose, and 5 g yeast extract in 1,000 ml of distilled water.  
81 Fungi were harvested by centrifugation for 20 min at 7200 $\times$ g, washed with sterile water,  
82 weighted and used as initial inoculums to grow under the same conditions for additional 3,

83 5 and 7 days in both CM and minimal liquid medium (MM) supplemented with *n*-  
84 hexadecane (MM-C16). MM is composed of CM without the glucose and yeast extract, and  
85 MM-C16 is MM supplemented with *n*-hexadecane (Sigma-Aldrich, USA) (C16, 1% final  
86 concentration) as previously described (Pedrini et al., 2010).

87 ~~Conidia suspensions were inoculated in CM flasks and incubated at 26°C for 2 days~~  
88 ~~with aeration (180 rpm). Fungi were harvested and grown under the same conditions for~~  
89 ~~additional 3 and 7 days in CM and MM-C16.~~ Mycelia and conidia (referred from now on as  
90 biomass) were obtained by pellet centrifugation for 20 min at 7200×g. The remaining  
91 fungal cells that were found in close contact with the alkane interface in the supernatant  
92 were isolated from the media culture by filtration and are referred from now on as mycelial  
93 pellets. All cell samples were washed with sterile water. At each time period, pH was  
94 measured on the remaining media, and humid biomass was weighted. Humid biomass ratio  
95 was calculated as humid final biomass/initial inoculum mass.

#### 96 ***Microbial adhesion to hydrocarbons (MATH) assay***

97 Cell surface hydrophobicity was determined as Holder et al. (2007). Briefly, conidia  
98 obtained in CM and MM-C16 after 3, 5, and 7 days were washed in PUM buffer (per litre:  
99 22.2 g K<sub>2</sub>HPO<sub>4</sub>, 7.26 g KH<sub>2</sub>PO<sub>4</sub>, 1.8 g urea, 0.2 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, final pH 7.1). Fungal cell  
100 suspensions were adjusted to OD<sub>470</sub> 0.4 and dispensed into acid-washed glass tubes.  
101 Hexadecane was then added to each tube and samples were vortexed three times for 30 s  
102 each. The tubes were then incubated at 4°C for 10 min and after removal of the *n*-  
103 hexadecane solid phase the density of the resultant cell suspension determined in a  
104 spectrophotometer at 470nm. The hydrophobic index was calculated using the following

105 equation:  $(A_{470, \text{ control}} - A_{470, \text{ hexadecane treated}}) / (A_{470, \text{ control}})$ . Four biological replicates with five  
106 repetitions each were measured.

### 107 *Microscopy images*

108 For optical and transmission electron microscopy (TEM) images, seven day cultures  
109 from CM, MM-C16, and mycelial pellets were used. Fresh samples were observed with a  
110 Nikon eclipse e200 optical microscope (Nikon Corp., Japan). For TEM, samples were  
111 washed and fixed in glutaraldehyde 2% for 2 h with soft vacuum, then washed three times  
112 with phosphate buffer (pH 7.2-7.4). Postfixation was performed with 1% osmium tetroxide  
113 at 4°C for 1 h, followed by dehydration with a series of alcohols in a vacuum chamber.  
114 Samples were finally infiltrated with epoxy resin and thin sections of approximately 70 nm  
115 were cut. TEM observation was made in a JEM 1200 EX II (JEOL Ltd., Japan). For 3,3-  
116 diaminobenzidine (DAB) (Sigma-Aldrich, USA) staining, an additional staining overnight  
117 with DAB was included in both fresh and fixated samples with glutaraldehyde 2%. In order  
118 to characterise the cell topography of fungi grown on CM and MM-C16, atomic force  
119 microscopy (AFM) images from 5 d conidia were obtained in air, using a MultiMode  
120 Scanning Probe Microscope (Veeco, USA) equipped with a Nanoscope V controller  
121 (Veeco). All measurements were obtained with Tapping® mode, using probes doped with  
122 silicon nitride (RTESP, Veeco with tip nominal radius of 8-12 nm, 271-311 kHz, force  
123 constant 20-80 N/m). Typical rate scanners were 1Hz. Fungal cells were placed on 0.22 µm  
124 pore-size Millipore filters and air-dried before examination.

### 125 *Mycelial pellets viability and pathogenicity*

126 Sporulation and viability of 7 d mycelial pellets were assessed as follows: fungal  
127 propagules were placed in Petri plates and held for 5 d at 4°C in a desiccator with activated  
128 silica gel until moisture content was reduced to  $\leq 5\%$  (w/w). Then, 30 mg of dried pellets  
129 were cultured in agar-agar media with ampicillin for 14 d at 26°C, growth was monitored  
130 daily and conidia produced were harvested with sterile water. Conidial production from the  
131 suspension was determined with a Neubauer chamber, calculated as total conidia/ initial  
132 dried pellet mass. To establish conidial viability, the suspension was also used to inoculate  
133 CM-agar plates and germination was monitored and calculated after 24 h at 26°C. For each  
134 replicate, 300 conidia were studied, and germination was calculated as  $100 \times$  germinated  
135 conidia/total conidia. For these tests, five replicates were done.

136 For pathogenicity assays, three groups of 10 larvae of each *T. castaneum* and *T.*  
137 *molitor* were placed in sterile plates containing a mixture of fresh 7 d mycelial pellets and  
138 humid vermiculite, prepared as follows: 0.325 g of mycelial pellets were placed in a sterile  
139 plate with 1.5 ml of sterile water, then 1.75 g of sterile vermiculite was added and mixed  
140 gently with a sterile spatula. Control plates were similarly prepared without the mycelial  
141 pellets. Beetles were maintained at 26°C and 70% RH. Mortality was checked every three  
142 days and all dead larvae were removed after each count. Afterward, dead beetles were  
143 washed in 70% ethanol for 30 s, rinsed in sterile distilled water for 2 min, allowed to dry,  
144 and then placed in individual humid chambers at 25°C to confirm fungal infection. The  
145 experiment was repeated two more times. Mortality data were corrected for control  
146 mortality using the Abbott's equation (Abbott, 1925).

147 ***Gene expression analysis***



148 Total RNA was extracted employing the RNAeasy Plant Mini kit (Qiagen,  
149 Germany), including an on-column DNA digestion step (Qiagen). RNA samples were  
150 quantified with a Nanodrop spectrophotometer (Thermo, USA), and the integrity was  
151 assessed on a 1% (w/v) agarose gel. Two-step real-time polymerase chain reaction (RT-  
152 PCR) was carried out with iScript cDNA Synthesis Kit and iQ SYBR Green Supermix  
153 (Bio-Rad, USA). Amplification was performed in an StepOne Plus equipment (Applied  
154 Biosystems, USA) employing 20 ng reverse transcribed total RNA for each sample.  
155 Primers corresponding to oxidative stress markers *Bbsod1*, *Bbsod2*, *Bbsod3*, *Bbcata*,  
156 *BbcataB*, *BbcataC*, *BbcataD*, *BbcataP*, *Bbgpx* and *Bbgst*, peroxisome biogenesis genes *Bbpex5*,  
157 *Bbpex7*, *Bbpex14/17* and *Bbpex19*, and hydrophobicity related genes *Bbhyd1* and *Bbhyd2*  
158 were designed using Gene Runner program. In order to confirm that only single products  
159 were amplified, a temperature-melting step was then performed. The calibration curve  
160 method was used for the analysis of data obtained from the RT-PCR system, with gamma  
161 actin (*Bbact*) as the housekeeping gene. This gene was selected after a validation test with  
162 geNorm algorithm for several commonly used housekeeping genes; i.e., *Bb28*, *BbcypA*,  
163 *Bbtub*, *BbCrza*, *Bbact* and *Bbgpd* using Qbaseplus software (<https://www.qbaseplus.com>).  
164 Four independent biological replicates were tested, with technical duplicates for each  
165 sample. The relative expression ratio of each target gene was calculated with  $\Delta\Delta C_t$   
166 approach in MM-C16 cells and mycelial pellets, using CM cells as control. Primers used,  
167 PCR efficiencies, and putative functions of the proteins encoded are listed in Table 1.

#### 168 ***Statistical analysis***

169 Differences among means were determined by two-way analysis of variance  
170 (ANOVA) followed by the Tukey posttest, using GraphPad Prism (GraphPad Software  
171 Inc., San Diego, CA).

172

## 173 **Results**

### 174 *Fungal growth on hydrocarbons*

175 After three days of culture with vigorous agitation, several macroscopic, spherical,  
176 non-uniform size aggregates appeared in C16-added MM (Fig. 1A). These aggregates were  
177 found in close contact with the hydrocarbon phase (they could not be isolated from the  
178 aqueous phase by centrifugation) but were not found in CM in the same growing  
179 conditions. Humid biomass (fungal cells excluding the mycelial pellets) and pH of the  
180 media were monitored and results are shown in Table 2. In all cases, biomass ratio  
181 (~~calculated as humid final mass/initial inoculum mass~~) was significantly lower ( $p < 0.05$ ) in  
182 MM-C16 than in CM. pH behavior appeared to be inverted for minimal and complete  
183 cultures; cell-free MM-C16 had a decreased but not significant tendency in pH over days,  
184 whereas cell-free CM increased pH significantly ( $p < 0.01$ ).

### 185 *Microbial adhesion to hydrocarbons (MATH) assay*

186 Conidia from CM and MM-C16 cultures were isolated after 3, 5, and 7 days. The  
187 hydrophobic index (HI) for all samples is shown in Fig. 2. The HI from CM conidia  
188 decreased from 0.62 (day 3) to 0.25 (day 7) ( $p < 0.001$ ). On day 3, the HI was significantly

189 higher ( $p < 0.05$ ) in CM conidia than in MM-C16 conidia. On the contrary, on day 5 the HI  
190 was significantly higher ( $p < 0.01$ ) in MM-C16 conidia than in CM conidia.

### 191 ***Microscopy images***

192 ~~After observation by optical microscopy, the spherical aggregates showed to be~~  
193 ~~formed by hyphae cumulate.~~ Optical microscopy showed that the spherical aggregates are  
194 formed by hyphal cumulates (Fig. 1B). Visible *n*-hexadecane droplets were found  
195 surrounding the pellet surface (Fig. 1C). Alkane-grown mycelial pellets were also stained  
196 with DAB, a chemical used for determining peroxidase activity, which is usually employed  
197 as peroxisome marker (Fahimi, 2017). The hyphal cumulates appeared strongly stained,  
198 different from hyphae close to the aggregate borders, which showed lower staining  
199 reaction. (Figs. 1D, E).

200 Alkane-grown mycelial pellets were also processed and observed by TEM, the same  
201 as fungal conidia and mycelia. The preparative section cuts from mycelial pellets were  
202 previously analysed by optical microscopy (Fig. 3A), where spheres looked like low dense  
203 clusters of fungal cells that might contain other compounds, like hydrocarbons, immersed  
204 in their structure. In the fungal cells forming the hyphal aggregates, TEM images showed  
205 surfaces with an irregular comb-like form facing the cytoplasm and small vesicles (Fig.  
206 3B), the same as an apparent surface thickening ( $\sim 0.36 \mu\text{m}$ ) compared to MM-C16 ( $\sim 0.15$   
207  $\mu\text{m}$ ) (Fig. 3C) and CM cells ( $\sim 0.11 \mu\text{m}$ ) (Fig. 3D). Images from DAB stained mycelial  
208 pellets revealed higher peroxidase activity, visualized as small black dots due to DAB  
209 reaction with  $\text{H}_2\text{O}_2$ , inside the cells and also in cellular interconnections; several  
210 peroxisomes and clear hairpin-like structures in the cell surface were also observed (Figs.

211 3E, F). On the contrary, DAB staining CM cells revealed lower peroxidase activity, mainly  
212 found in vacuoles, fewer or not clear mature peroxisomes and smooth cell surfaces (Figs.  
213 3G, H).

214 AFM images were obtained for CM and MM-C16 conidia from 5-day cultures,  
215 showing distinctive differences on cell surfaces. CM conidia displayed irregular forms and  
216 different and variable diameters (between 1.5 and 5.4  $\mu\text{m}$ ) (Figs. 4A, C). On the contrary,  
217 MM-C16 conidia looked like a spherical form, smaller, and homogeneous (between 0.9 and  
218 2.5  $\mu\text{m}$ ) (Figs. 4B, D). Also, CM conidia showed distinctive topographical characteristics  
219 with deep and rough edges, whereas MM conidia showed a more uniform structure (Figs.  
220 4E, F).

#### 221 *Mycelial pellets viability and pathogenicity*

222 Dried mycelial pellets were cultured in agar-agar plates. Hyphal growth was visible  
223 after two days and sporulation started on day 6. At day 14, conidia were harvested and  
224 counted. Conidial production varied from  $1 \times 10^9$  to  $5 \times 10^9$  conidia per gram of dried pellet  
225 and germination in CM plates was 99.7%. Pathogenicity bioassays were done with fresh 7  
226 d mycelial pellets and results are shown in Table 3. At day 8, *T. castaneum* mortality was  
227  $67.3 \pm 13.1$  % mortality in all replicates, whereas *T. molitor* reached  $96.7 \pm 1.9$  % at day 8.  
228 These results showed that mycelial pellets are formed by active fungal cells, pathogenic  
229 against beetle larvae, which can grow without an external carbon source producing viable  
230 conidia.

#### 231 *Gene expression analysis*

232 Antioxidant stress-marker genes displayed different expression patterns in alkane-  
233 grown mycelial pellets (Fig. 5A). For the superoxide dismutase family, *Bbsod1* was  
234 induced on day 3 (6.1-fold induction), but not as significantly as on day 7. In contrast,  
235 *Bbsod3* was induced on day 3 and day 7 (1.7- and 4.9-fold induction, respectively).  
236 However, *Bbsod2* was not induced in the conditions tested. For the glutathione system,  
237 *Bbgst* showed little induction (3.4- and 1.8-fold on day 3 and day 7, respectively), and  
238 *Bbgpx* expression was reduced on day 3 (0.7-fold) and induced at day 7 (2.5-fold). For the  
239 catalase family, *Bbcacb* (4.9-fold expression at day 3), *Bbcacg* (4.2- and 4.7-fold on day 3  
240 and day 7, respectively) and *Bbcacp* (14.8-fold and 4.3-fold on day 3 and day 7,  
241 respectively) were induced in alkane cultures. Moreover, the peroxisomal-protein encoding  
242 gene *Bbcacp* was significantly induced ( $p < 0.01$ ) on day 3 compared with day 7.

243 In order to study the expression pattern of genes involved in the peroxisomes  
244 biosynthesis pathway, several genes encoding for PEX proteins were also measured by  
245 qPCR (Fig. 5B). Some *pex* genes were induced in mycelial pellets, as follows: *Bbpex7* (2.5-  
246 fold expression on day 3), *Bbpex14/17* (1.9-fold and 4.5-fold on day 3 and 7, respectively)  
247 and *Bbpex19* (2.8-fold and 3.9-fold on day 3 and 7, respectively). In contrast, *Bbpex7* and  
248 *Bbpex5* were not induced on day 7, and at both time periods, respectively.

249 To study candidate genes involved in cell surface hydrophobicity, hydrophobin  
250 genes were measured in mycelial pellets and MM-C16 biomass, using CM biomass as  
251 control (Fig. 5C). Although *Bbhyd1* and *Bbhyd2* genes were not induced in mycelial pellets,  
252 both genes were strongly induced on day 3 and day 7 in MM-C16 biomass. The transcripts  
253 levels of *Bbhyd1* were 22.8-fold induction and 5.8-fold induction on day 3 and day 7,  
254 respectively. For *Bbhyd2*, the expression level was 74.1-fold induction on day 3, and 13.9-

255 fold induction on day 7. Thus, gene expression for *Bbhyd2* at day 3 was significantly higher  
256 ( $p < 0.001$ ) than on day 7.

257

## 258 **Discussion**

259 A novel type of growth was described in *B. bassiana* cultured in hexadecane-  
260 supplemented liquid minimal media, consisting in hyphal aggregates similar to mycelial  
261 pellets found in other filamentous fungi (Grimm et al., 2005a; Metz and Kossen, 1977).  
262 However, *B. bassiana* pellets were found only in alkane-grown fungi but not in glucose-  
263 grown cells. These complex structures resulted to be stable, spherical aggregates, formed  
264 by branched and partially intertwined hyphae networks that were able to germinate and  
265 produce viable conidia without an external carbon source. Preliminary pathogenicity assays  
266 showed that after one week, these propagules caused the death of mealworms and red flour  
267 beetle larvae. Apart from the mycelial pellets development, no significant increment was  
268 recorded in MM-C16 biomass during the entire incubation period; in contrast to CM  
269 cultures, where biomass was more than 4 times higher than the initial inoculums. It has  
270 been already reported that *B. bassiana* is capable of growing on media supplemented with  
271 hydrocarbons; moreover, it was established that *n*-hexadecane was the preferred substrate  
272 among several hydrocarbons tested (Huarte-Bonnet et al., 2017; Pedrini et al., 2010). Thus,  
273 these results indicate that alkane-grown *B. bassiana* is actually under active division and  
274 growth, but these new cells are forming mycelial pellets in the alkane interface and are not  
275 part of the initial biomass.

276 Differences in both hydrophobic cell surface indexes and pH pattern were found in  
277 conidial cells isolated from CM and MM-C16 biomass. AFM images also showed  
278 topographical differences between CM and MM-C16 conidia. It is clear that the presence of  
279 *n*-hexadecane is triggering different cell responses in order to uptake this hydrophobic  
280 substrate. Although it is well established how *B. bassiana* degrade cuticular hydrocarbons  
281 (Pedrini et al., 2007; 2010; 2013), little is known about the mechanisms involved in the  
282 uptake of alkanes by entomopathogenic fungi. It is believed that different compounds are  
283 secreted or anchored to the fungal cell surface to internalize those carbon sources, as shown  
284 by TEM images in both conidia (Pedrini et al., 2007) and mycelial pellets (this study).

285 The present study also showed different pH patterns between CM and MM-C16  
286 remaining liquid media. In this regard, secretion of acid compounds could be responsible  
287 for the acidification of the media observed in MM-C16 cultures. Oxalic acid was reported  
288 to be secreted by *B. bassiana* as a virulence factor against ticks, and its secretion was  
289 dependent on the media used (Kirkland et al., 2005). Also, alkane-assimilating  
290 microorganisms are known to secrete acid biosurfactants to facilitate hydrocarbon uptake;  
291 e.g., acidic sophorolipids, free fatty acids, among others (Kitamoto et al., 2002). Thus, it  
292 could be expected that similar compounds might be found in the remaining media of  
293 alkane-grown *B. bassiana*. ~~Differential pH in the remaining media and cell surface~~  
294 ~~hydrophobicity might indirectly shed light to possible mechanisms of secretion and cell~~  
295 ~~surface adaptation to hydrocarbon growth that is still to be discovered.~~ It was also  
296 previously shown that *B. bassiana*, neither *M. anisopliae* nor *Aspergillus niger*, strongly  
297 acidified the liquid minimal media supplemented with alkanes (Huarte-Bonnet et al., 2017).  
298 As pellet formation is highly regulated by pH in other filamentous fungi (Dynesen and

299 Nielsen, 2003; Glazebrook et al., 1992; Grimm et al., 2005b; Ryoo and Choi, 1999), pH  
300 values might be either the cause or the consequence to pellet formation in alkane-grown *B.*  
301 *bassiana*. Also, hydrophobins might play a role in hydrocarbons uptake. In this regard, we  
302 found that though no induction was observed for *hyd* genes in mycelial pellets, MM-C16  
303 biomass showed high expression of both *Bbhyd1* and *Bbhyd2*. However, hydrophobins play  
304 a key role in pellet formation in *A. niger*, and hydrophobic interactions in the cell surface  
305 were reported to favor the formation and stability of these aggregates (Dynesen and  
306 Nielsen, 2003). These results suggest that although *hyd* genes are highly induced in *B.*  
307 *bassiana* biomass immediately after contact with hydrocarbons, the mycelial pellets derived  
308 from those cells did not express these genes since they might already have these proteins in  
309 their surfaces. However, further assays to specifically detect hydrophobins in both fungal  
310 cells are required to confirm this hypothesis.

311 An antistress response was triggered in mycelial pellets during growth on alkanes,  
312 in coincidence with previous results obtained for MM-C16 biomass grown in similar  
313 culture conditions (Huarte-Bonnet et al., 2015). In mycelial pellets, at least one gene of  
314 each antistress response family was up-regulated at each time incubation period. Thus,  
315 catalase and superoxide dismutase gene induction could also be used as clues for reactive  
316 oxygen species localization, i.e., *Bbsod1* encodes for a Cu/Zn-dependent superoxide  
317 dismutase localized in the cytoplasm (Xie et al., 2010) and *Bbsod3* encodes for a  
318 mitochondrial Mn-isoform (Xie et al., 2012), whereas *Bbcatb*, *Bbcatc*, and *Bbcatp* encode  
319 for cytoplasmic, secreted, and peroxisomal catalases, respectively (Wang et al., 2013). In  
320 fact, the peroxisomal isoform was the most induced gene from the antistress response  
321 system in mycelial pellets. However, gene expression data is not sufficient to fully



322 comprehend if the observed upregulation is useful to protect cells against potential  
323 antagonists (both biotic and abiotic) or if it is a result to cells differentiation into mycelial  
324 pellets, or both. Targeted single-gene knockout strategies and/or enzymatic studies in  
325 subcellular fractions will be needed to confirm this point.

326 Peroxisomes are known to be the organelles where the last hydrocarbon degradation  
327 reactions take place in *B. bassiana* (Pedrini et al., 2007). The biogenesis of peroxisomes  
328 involves the action of several proteins, named peroxins, which are encoded by *pex* genes  
329 (Li et al., 2016; Smith and Aitchison, 2013). In this study, *Bbpex7*, *Bbpex14/17*, and  
330 *Bbpex19* were induced after 3 and/or 7 days of culture. In addition to gene expression  
331 patterns, mycelial pellets staining reaction with DAB gave strongly brown stained cells  
332 inside the pellet structure, but also inside the individual cells, indicating high  
333 catalase/oxidase activity and peroxisomal proliferation. Moreover, the high number of  
334 small black dots found inside alkane-grown cells and the induction of *pex* genes are  
335 suggesting that the peroxisome biogenesis pathway is being activated under this growth  
336 condition, as it was reported for alkane-grown yeast (Fukui and Tanaka, 1979; Monosov et  
337 al., 1996; Smith and Aitchison, 2013; Tanaka and Ueda, 1993).

338 We conclude that two cellular populations are present in fungi grown in liquid  
339 minimal media supplemented with *n*-hexadecane. Both fungal propagules have different  
340 molecular and physiological characteristics between them and also compared to cells grown  
341 in rich media. In this sense, several unknown direct or indirect responses to hydrocarbon  
342 supplementation might be acting as triggers to initiate pellet formation since they are not  
343 present in rich media in the same incubation conditions. For other filamentous fungi, pellets  
344 formation in liquid cultures are preferred for the non-viscous rheology of the broth, better

345 mass transfer and easier pellet separation than with mycelial cultures (Wucherpfenning et  
346 al., 2010). In alkane-grown fungi, hydrocarbon droplets in liquid media might be acting as  
347 initial nucleation for pellets formation since these structures might assure fungal cells of an  
348 easy access to the hydrophobic carbon source in the interface. However, additional studies  
349 are needed to better understand the relationship between fungal metabolic adaptations in  
350 hydrocarbon-supplemented cultures and pellet formation.

351

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365

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- 449
- 450

451 **Figure legends**

452 **Fig. 1.** A) Mycelial pellets in aqueous solution in a test tube. B) Phase-contrast microscopy  
453 of an isolated pellet formed by hyphae cumulates (40× magnification). C) Hexadecane  
454 droplets surrounding the mycelial pellet (10× magnification). D) Mycelial pellet stained  
455 with DAB (10× magnification). E) Mycelial pellet stained with DAB (20× magnification).

456

457 **Fig. 2.** Cell surface hydrophobicity of CM and MM-C16 conidia after 3, 5 and 7 days of  
458 culture. \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ).

459

460 **Fig. 3.** A) Optical observation of a semi-thin section of a mycelial pellet showing the  
461 hyphal cumulate structure (40× magnification). B) Ultrastructure of mycelial pellets at  
462 50,000× magnification. C) Ultrastructure of MM-C16 biomass at 30,000× magnification.  
463 D) Ultrastructure of CM biomass at 30,000× magnification. E), F) Ultrastructure of  
464 mycelial pellets at 30,000× magnification stained with DAB. The reaction appears as black  
465 dots (white arrow), and several peroxisomes are visible. Hairpin-like structures are found in  
466 the cell surface (black arrow). G), H) Ultrastructure of CM biomass at 30,000×  
467 magnification stained with DAB. The reaction is positive mainly in vacuoles, and fewer or  
468 none peroxisomes are visible. V: vacuole. N: nucleus. M: mitochondrion. P: peroxisome.

469

470 **Fig. 4.** Atomic force microscopy images of MM-C16 conidia (A, C, E) and CM conidia  
471 (B, D, F) obtained in tapping mode. Images have a resolution of 512 x 512 pixels. The  
472 height is expressed in color scale (right bar).

473

474 **Fig. 5.** Gene expression ratios of 3 days and 7 days of s of *B. bassiana* grown in MM-C16  
475 or CM (control). A) Oxidative stress marker genes in mycelial pellets. B) Peroxin genes in  
476 mycelial pellets. C) Hydrophobin genes in mycelial pellets (MP) and MM-C16 biomass.  
477 Error bars represent standard errors of four independent assays. Dashed line showed a  
478 relative expression ratio = 1. \*\*( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ).



**Table 1.** Oligonucleotides used in this study.

Name	Forward primer	Reverse primer	Efficiency (%)	R <sup>2</sup>	Name/Function	Reference
<i>Bbact</i>	ATGGAGGAAGAAGTTGCTGC	ACACGGAGCTCGTTGTAGAA	112.7	0.992	$\gamma$ -Actin	Zhou et. al. (2012)
<i>Bbsod1</i>	ACAACACCAACGGCTGCACC	ACGGCCAACAACGCTGTGAG	116	0.997	Cu/Zn- superoxide dismutase / O <sub>2</sub> <sup>-</sup> scavenging	Forlani et. al. (2014)
<i>Bbsod2</i>	CCAGTGTTTGGCATTGACATG	TCAGCCGTCTTCCAGTTGATG	105.6	0.996	Mn-superoxide dismutase / O <sub>2</sub> <sup>-</sup> scavenging	Forlani et. al. (2014)
<i>Bbsod3</i>	ACATCAATCACACTCTCTTCTG	GCGTTGGTCTGCTTCTTG	103.1	0.992	Mn-superoxide dismutase / O <sub>2</sub> <sup>-</sup> scavenging	Forlani et. al. (2014)
<i>Bbgpx</i>	CAAGGTCGTCCTCGTCGTC AAC	CTTGTCGCCATTGACCTCCACC	122.5	0.994	Glutathione peroxidase / GSH protection system	Forlani et. al. (2014)
<i>Bbgst</i>	TCTTG TAGCCAGCCCTCCATCG	AGAGATGTGGTCGCGGAACGA	115.5	0.969	Glutathione-S-transferase / GSH protection system	Forlani et. al. (2014)
<i>Bbcata</i>	GAAAGCCGCGCAAGTGAAAG	TCTCTGGCAAAGACATCCAG	107.2	0.993	Spore-specific catalase/ H <sub>2</sub> O <sub>2</sub> scavenging	Forlani et. al. (2014)
<i>Bbcatb</i>	GAAGACGCCCATGTTTGTTCG	AAAGTTGCCCTCATCGGTATAGC	117.3	0.987	Secreted catalase/ H <sub>2</sub> O <sub>2</sub> scavenging	Forlani et. al. (2014)
<i>Bbcate</i>	TGCTGGACGATGTGTCTGAC	CACGCACCGTATCGCTAGAG	108.6	0.991	Cytoplasmic catalase/ H <sub>2</sub> O <sub>2</sub> scavenging	Forlani et. al. (2014)
<i>Bbcatd</i>	GCGCTCGCAGTGACTGTAC	CTAGCACGGCCCTGTATAATGG	113.3	0.998	Secreted peroxidase/catalase / H <sub>2</sub> O <sub>2</sub> scavenging	Forlani et. al. (2014)
<i>Bbcatp</i>	TGTA CTGGGGCTCCGAACC	ATGAGACCTGTGTAGCGTTAGC	105.7	0.967	Peroxisomal catalase/ $\beta$ -oxidation pathway	Forlani et. al. (2014)
<i>Bbpex5</i>	AATGCCGGGCCGAATATGC	CAGGCTGGCTGTTGAAATCGTG	130.2	0.991	Peroxisomal	This study

					biogenesis factor 5/ PTS1 import receptor	
<i>Bbpex7</i>	TCGCTTCGGCTGCCAATTC	TGCGACAATGAGCTGGTTCTCG	111.1	0.993	Peroxisomal biogenesis factor 7/ PTS2 import receptor	This study
<i>Bbpex14/ 17</i>	TCGCCAACCTCGTCAGACACTG	CCTCGACGCCCTTTGACTTGAG	114.3	0.991	Peroxisomal biogenesis factor 14/17/ Receptor docking complex	This study
<i>Bbpex19</i>	AAGTTCCTGTCTGGCTGTCGG	CCGCAAAGGCTTCTTGTGC	114.3	0.993	Peroxisomal biogenesis factor 19/ soluble chaperone and receptor	This study
<i>Bbhyd1</i>	CACCATGGTGGAAAGGATCTGCAC	CCGAGAAGGTGGGAAAGAAGACCA	108.5	0.996	Hydrophobin 1 / cell surface hydrophobicity	This study
<i>Bbhyd2</i>	TGTCAAGACTGGCGACATTTGCG	TCGATGGGGACAAGCTGGTTGA	117.7	0.985	Hydrophobin 2 / cell surface hydrophobicity	This study

**Table 2.** Determination of humid biomass ratio and pH of the remaining media at different time periods. For both assays, values in each column followed by different lowercase letters and values in each line followed by different uppercase letters indicate significant differences ( $p < 0.05$ ).

Day	Biomass ratio		pH	
	MM-C16	CM	MM-C16	CM
3	$1.2 \pm 0.2aA$	$6.1 \pm 0.8aB$	$6.1 \pm 0.4aA$	$3.6 \pm 0.1aB$
5	$1.2 \pm 0.2aA$	$7.4 \pm 1.4aB$	$5.4 \pm 0.7aA$	$6.5 \pm 0.1bA$
7	$1.1 \pm 0.3aA$	$5.4 \pm 1.9aB$	$4.9 \pm 0.5aA$	$7.1 \pm 0.1cB$

**Table 3.** Pathogenicity bioassay. Cumulative percentage mortality  $\pm$  SEM of *Tribolium castaneum* and *Tenebrio molitor* larvae treated with *Beauveria bassiana* mycelial pellets and corrected for control mortality using the Abbott's formula. For both insects, different letters indicate significant differences ( $p < 0.05$ ).

Day	Mortality (%)	
	<i>Tribolium castaneum</i>	<i>Tenebrio molitor</i>
0	0 $\pm$ 0a	0 $\pm$ 0a
2	0 $\pm$ 0a	0 $\pm$ 0a
5	70.2 $\pm$ 11.9b	53.3 $\pm$ 10.7b
8	67.3 $\pm$ 13.1b	96.7 $\pm$ 1.9c













