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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1788065> since 2025-01-22T10:09:08Z

Published version:

DOI:10.1016/j.culher.2020.11.003

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1 **Hyphal morphology and substrate porosity -rather than melanization- drive penetration of**
2 **black fungi into carbonate substrates**

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28
29 **Abstract**

30 Due to their ability to penetrate, deteriorate and discolour marble/stone surfaces, rock-inhabiting
31 black fungi represent a remarkable issue for cultural heritage conservation.

32 Black microcolonial fungi (MCF) can also adapt to different environmental conditions, by
33 converting from yeast-like morphology to a peculiar meristematic development with swollen cells
34 (torulose hyphae, TH), to extremely thin structures (filamentous hyphae, FH). Furthermore,
35 black MCF produce protective pigments: melanin, dark pigment particularly evident on light stone
36 surfaces, and carotenoids. Black fungi produce melanin in critical, oligotrophic conditions as well
37 as constitutively. Melanin function is mostly related to stress resistance and the ability of fungi to
38 generate appressorial turgor to actively penetrate plant cells in pathogenic species. An involvement
39 of melanins in stone surface penetration has been suggested, but not experimentally proved.

40 In this work, we tested the role of hyphal melanisation in penetration mechanisms on the model
41 black fungus *Knufia petricola* A95 in lab conditions. The wild-type and three mutants with
42 introduced targeted mutations of polyketide-synthases (melanin production), and/or phytoene
43 dehydrogenase (carotenoid synthesis) were inoculated on artificial carbonate pellets (pressed
44 Carrara marble powder) of different porosity. After 5, 10, 17 and 27 weeks, hyphal penetration
45 depth and spread were quantified on periodic acid Schiff-stained cross-sections of the pellets,
46 collecting measurements separately for TH and FH. Droplet assay of the mutants on different media
47 were conducted to determine the role of nutrients in the development of different fungal
48 morphologies.

49 In our in vitro study, the hyphal penetration depth, never exceeding 200 µm, was proven to be
50 consistent with observed penetration patterns on stone heritage carbonate substrates. Pellet porosity
51 affected penetration patterns of TH, which developed in voids of the more porous pellets, instead
52 than actively opening new passageways. Oppositely, the thin diameter of FH allowed their

53 penetration independently of substrate porosity.
54 Instead, the long-hypothesized crucial role of melanin in black MCF hyphal penetration should be
55 rejected. TH were developed within the pellets also by melanin deficient strains, and melanized
56 strains showed an endolithic component of non-melanized TH. FH were non-melanized for all the
57 strains, but deeply penetrated all pellet types, with higher penetration depth probably related to their
58 potential exploratory (nutrient-seeking) role, while TH may be more related to a resistance to
59 surface stress factors. In the melanin deficient strains, the absence of melanin caused an increased
60 penetration rate of FH, hypothetically related to an earlier necessity to search for organic nutrients.

62 **Keywords**

63 biodeterioration, bioreceptivity, black microcolonial fungi, marble, stone cultural heritage, stress
64 tolerance

66 **1. Introduction**

67 Outdoor exposed stone cultural heritage is subject to various types of deterioration. Chemical and
68 physical deterioration, due to the action of the atmospheric agents, is the most evident, but in the
69 last decades the attention of the conservation scientists has been drawn towards the microorganisms
70 which can colonize stone surfaces and cause biodeterioration [1-5]. Although stone surfaces are
71 hostile due to the scarcity of nutrients, sun exposure and available water, and many physical
72 stresses, several microorganisms colonize these niches, often organized as sub-aerial biofilms
73 (SABs) [6].

74 Knowledge on the relationship between microorganisms and colonized substrate drives the
75 recognition of major threats to conservation and may address the definition of control strategies to
76 face biodeterioration [7]. However, the multiple factors involved, including the stone, the microbes,
77 and the surrounding environment, have often prevented a full comprehension of the mechanisms
78 underlying biodeterioration, which was mostly examined in the complexity of field conditions.

79 The physico-chemical properties of the stone substrate affect its bioreceptivity, which is the aptitude
80 of a material to be colonized by one or several groups of living organisms without necessarily
81 undergoing any biodeterioration [8]. The mineral composition and surface properties, as roughness,
82 control the microbial establishment [9]. Substrate porosity and texture were proved to regulate the
83 growth of biodeteriogens within the stone, as in the case of lichens [10]. Microbial growth on
84 monuments is mediated by several extrinsic factors, acting from a macro- (e.g. water regime,
85 relative humidity, solar radiation, temperature, wind, atmospheric pollution) to a micro- (e.g.
86 orientation, shading, permanent capillary humidity, etc.) scale [9, 11, 12].

87 Accordingly, protocols to test the bioreceptivity of stone materials in standardized environmental
88 conditions were proposed and applied to evaluate the establishment and growth on mineral surfaces
89 of stable phototrophic multi-species cultures [13-15]. However, laboratory approaches have still
90 been poorly used to examine the microbial-substrate interaction in terms of penetration and
91 dissolution patterns, and to explore the underlying mechanisms [10, 16-19].

92 Microcolonial fungi (MCF) are a major component of SABs on stone surfaces in extreme
93 environments, from Antarctica to hot deserts [20-23], and they often occur on stone cultural heritage
94 [24, 25]. Compact black microcolonies are equipped to withstand extreme environmental challenges
95 including desiccation, UV radiation, temperature and oxidative stress as well as biocide treatments.
96 They represent a remarkable issue for the conservation, due to their ubiquity, dark colour and the
97 ability to penetrate and modify stone surfaces [26-29]. Black MCF are a polyphyletic group of
98 Ascomycetes sharing morphological plasticity and functional adaptive traits which ensure their
99 survival in extreme environments [30, 31].

100 Since the early descriptions at the end of last century, it was recognized that rock-inhabiting black
101 fungi can drastically change morphology depending on environmental conditions, with conversions
102 from yeast-like cells, to a peculiar meristematic development, to extremely thin hyphae [21, 32].
103 Their growth by isodiametric enlargement and subsequent division of cell compartments
104 (meristeme-like), with thick and even multi-layered cell walls, gives rise to aggregated structures

105 and swollen, ‘torulose hyphae’ (sensu De Leo et al., [33]). These are recognized as a prominent
106 morphological trait of MCF, suitable to minimise the contact of the colonies with the atmosphere,
107 and thus evaporation [30, 31]. The co-development of thinner, ‘filamentous hyphae’ (sensu De Leo
108 et al. [33]) and their role has been poorly discussed yet. In particular, while the differentiation of
109 filamentous structures, called ‘pseudohyphae’, is well known for yeasts and has been related to
110 nutrient limitation [34], their presence has rarely been remarked for MCF [35]. For this reason, this
111 morphological form of growth has been poorly studied and it is still difficult to define if filamentous
112 hyphae of MCF are proper hyphae, pseudohyphae or if it is possible to distinguish the presence of
113 both these growth morphologies. The production of a variety of extracellular polymeric substances
114 (EPS), retaining water and inhibiting access of external agents, and the incrustation of the cell-wall
115 with melanins are additional adaptive traits to increase stress tolerance [6, 36, 37].

116 The role of melanins in fungi, including in black MCF, has been related to the necessity of
117 photoprotection, resistance to oxidation, thermoprotection, energy harvesting and metal binding [24,
118 38, 39]. Moreover, for pathogens and phytopathogens, including taxa phylogenetically close to
119 rock-dwelling MCF, melanins have also been related to their ability to generate appressorial turgor
120 and thus actively penetrate cells [40]. Accordingly, the involvement of these pigments in the
121 penetration of stone surfaces has been suggested [18, 19, 25, 33], but not experimentally proved.

122 *Knufia petricola*, a species recognized as the model MCF to study the growth and interaction of
123 SABs with stone materials, offers the opportunity to experimentally evaluate the role of melanin in
124 MCF penetration. This species, belonging in *Chaetothyriales* [41], was first isolated from a marble
125 rock surface in Athens (Greece) and has subsequently been reported on both carbonate and silicate
126 rock materials, including cultural heritage surfaces [28, 42, 43]. A melanized strain was already
127 assayed *in vitro*, under controlled conditions, for its penetration patterns within different carbonate
128 and silicate rock coupons, showing a high penetration rate (after four months: from few hundreds of
129 microns to some millimetres, depending on the lithology), making it suitable for similar laboratory
130 experiments [16]. Moreover, the development of an efficient toolkit for the genetic modification of
131 *K. petricola*, including the deletion of genes regulating the production of protective pigments, as
132 melanins, but also carotenoids [44-46], has further enforced its suitability as a model organism to
133 understand biodeterioration processes on heritage surfaces and thus address conservation and
134 control strategies.

135

136 **2. Research aim**

137 In this work, we aimed to test the hypothesis that hyphal melanization is the adaptive trait which
138 allows the penetration of MCF structures within the stone substrates. To work on an experimental
139 system with a reduced number of factors involved, we developed an *in vitro* protocol including the
140 model *K. petricola* isolate A95 [31], constant incubation conditions and standardized mineral
141 substrates with defined porosity. In particular, the penetration of melanized and non-melanized
142 strains of *K. petricola* was compared in terms of hyphal penetration depth and spread within
143 carbonate pellets produced from pulverized Carrara marble. Measurements were separately
144 performed on torulose and filamentous hyphae (sensu De Leo et al. [33]) in order to address their
145 potentially different ecological significance. This is of particular interest for biodeterioration
146 scientists, allowing the evaluation of the biodeterioration potential of MCF on stones of interest for
147 conservation of cultural heritage and addressing suitable control strategies.

148 3. Materials and methods

149

150 3.1. Preparation of carbonate pellets with different porosity

151 Carrara marble was selected to produce standardized pellets, due to its light colour and the
152 consequent high susceptibility to the aesthetic damage caused by black fungi, and its widespread
153 use in buildings and statuary in the past as in the present.

154 White marble blocks were sampled in an ancient Roman quarry near Colonnata (Carrara, Italy;
155 UTM ED50 N4881839, E591489). The colonized and/or chemically altered volumes were removed
156 using a diamond saw. The blocks were then manually crushed to smaller fragments and pulverized
157 in an agate jar with agate balls, using a planetary ball mill (Planetary S100, Retsch, Germany). The
158 powder was sifted with a 100 µm sieve and the fine powder obtained was used to produce standard
159 circular pellets (weight: 850 mg; diameter: 12.7 mm), using a pelletizer IR-Pressé 25T (Maassen
160 GmbH, Germany). Two different pressures were applied for 60 seconds to produce pellets with
161 different porosity: 4 tons (4T pellets; 2.70 mm thick) or 7.5 tons (7.5T pellets; 2.55 mm thick).

162 The porosity of the two types of pellets was evaluated with analyses on images acquired in back-
163 scattered mode (BSE) with a scanning electron microscope JEOL JSM-IT300 (high vacuum/low
164 vacuum 10/650 Pa; 0.3-30 kV) and the software AZtec (v.3.3, Oxford Instruments, UK).
165 Representative pellets were cross-sectioned and semi-quantitative measurements of solids and voids
166 were performed along vertical transects with the software WinCAM2007d (Regent's Instrument,
167 Canada) in grey-scale mode, identifying the porosity as the black coloured areas in BSE following
168 the approach described by Favero-Longo et al. [10].

169 4T pellets showed a rather homogeneous porosity through the whole thickness, equivalent to
170 approx. 50% (Fig. S1A). 7.5T pellets showed a lower average porosity of approx. 12%, which
171 decreased to 5% in the 600 µm thick upper layer, which was in direct contact with the plunger of the
172 pelletizer during the pellet production (Fig. S1B).

173 The pellets were finally sterilized in autoclave at 120°C for 20 minutes to avoid possible biological
174 contaminations.

175

176 3.2. Fungal material

177 The model MCF *Knufia petricola* A95 was selected as fungal material [47]. The wild-type strain
178 (wt), isolated from a marble surface in Athens (Greece; [44]), and three genetically modified strains,
179 in which the gene of interest was replaced by a resistance cassette *via* homologous recombination,
180 were used. To investigate the influence of the *K. petricola* pigments on the substrate penetration, a
181 $\Delta pks1$ mutant with a deletion of the polyketide synthase gene within the melanin synthesis pathway,
182 the $\Delta phd1$ mutant with the deletion of the phytoene desaturase for the synthesis of carotenoids and
183 one pigmentless mutant ($\Delta pks1/\Delta phd1$) with both deletions were selected [46] (Fig. 1).

184 The four strains, stored at the Bundesanstalt für Materialforschung und -prüfung in cryopreservation
185 (-80°C), were transferred in Petri dishes on nutrient-rich medium (Malt Extract Agar, MEA) to the
186 University of Turin. Subcultures were produced and kept at the constant temperature of 20°C in a
187 dark environment for at least 2 months to allow the growth of fungal colonies with a diameter of at
188 least 2 cm and to proceed with inoculation on the carbonate pellets.

189

190 3.3. Fungal inoculation on agar plates for droplet assays

191 Cell numbers of suspensions of *K. petricola* wild-type and pigment mutants were determined with a
192 hemocytometer and 1000 cells in a 10µl droplet were inoculated on the nutrient-rich medium ASM
193 [31] and the poor medium SDY (0.17% (w/v) Difco™ Yeast Nitrogen Base without Amino Acids
194 and Ammonium Sulfate (BD Biosciences) + 0.1% yeast extract (Y). Plates were incubated for 14
195 days at 25°C in the dark until photo documentation.

196

197 3.4. Fungal inoculation on carbonate pellets

198 The 4T and 7.5T pellets were placed inside Petri dishes over a thin layer of agar-water terrain (15g
199 agar per litre), necessary to keep in position the samples and to maintain humidity. For each pellet, a

200 circular fragment (\varnothing 4 mm) of mycelium was cut from a *K. petricola* subculture, cleared from MEA
201 residues, and inoculated in the centre of the pellet surface (Fig. 2A-D). Each sample (pellet + *K.*
202 *petricola* inoculum) was incubated at 20°C in a dark environment until its planned observation. For
203 each type of pellet (4T and 7.5T), groups of five (wt) and four ($\Delta pks1$, $\Delta phd1$, $\Delta pks1/\Delta phd1$)
204 replicates were examined at each of the following time points: 5 weeks (t1), 10 weeks (t2), 17
205 weeks (t3) and 27 weeks (t4) after the inoculation. Triplicate controls (C) with no inoculation were
206 also prepared and checked for contamination at t1, t2 and t3.

207

208 3.5. Microscopy observation of fungal growth

209 At the planned time points, each sample was preliminary observed with a stereomicroscope
210 Olympus SZH10 to evaluate the growth of mycelium on the pellet surface. The sample was then
211 taken from the Petri dish and the fungal inoculum was gently removed from the pellet surface using
212 a lancet. Each pellet was embedded in a polyester resin (R44 Politex-P fast, ICR S.p.A, Italy) and
213 then cut in two halves, using a drill with polycrystalline diamond cutters (Micromot 50/E, Proxxon,
214 Niersdorf, Germany) and perfecting the sectioned surface with a mini hand saw. One of the two
215 cross-sectioned pellets was stained using the periodic acid - Schiff's reagent method (PAS; [48]) to
216 highlight hyphal growth within the mineral substrate.

217 Each PAS-stained cross-section was examined under reflected light using an Olympus SZH10
218 stereomicroscope equipped with a digital camera to acquire images (300 ppi) of the whole sectioned
219 surface. The images of each section were assembled (n from approx. 30 to 70, depending on the
220 section and the time point) using GIMP 2.10.10, and a grid 100 μ m wide and 20 μ m high was
221 overlapped to define vertical sectors and support the measurement of hyphal depth, respectively
222 (n=125-130 vertical sectors, 38-42 of which covered by the inoculum; Fig. S2).

223 Two parameters were measured per each section: *i.* the hyphal penetration depth (HPD), obtained
224 by averaging the maximum penetration depth per vertical sector, and *ii.* the percentage of vertical
225 sectors (NVS) in which the hyphal penetration was observed, as informative of hyphal spread.
226 These two parameters were separately measured for the torulose hyphae (TH) and the filamentous
227 hyphae (FH) (*sensu* De Leo et al. [33]). The data collection for each section took 1-2 hrs depending
228 on the spread of hyphal penetration.

229 At each time point, differences between (a) HPD and NVS of torulose and filamentous hyphae,
230 considering strains and pellet types together; (b) HPD and NVS within 4T and 7.5T pellets,
231 considering torulose and filamentous hyphae separately, and the four strains together; (c) HPD and
232 NVS of the four strains, considering the pellet types and the torulose and filamentous hyphae
233 separately, were analysed by means of ANOVA with *post-hoc* t- or Tukey's test ($P < 0.05$ as
234 significant), using SYSTAT 10.2. Data are shown through box-plots generated with Origin 6.1.

235 4. Results

236

237 4.1 Fungal growth in different nutrient conditions

238 The four strains of *Knufia petricola* cultured on the agarized media and the carbonate pellets
239 macroscopically differed in colour and the colony morphology. The melanized wt and $\Delta phd1$ were
240 black-brown and black-grey, respectively, while $\Delta pks1$ and $\Delta pks1/\Delta phd1$ were pink and white,
241 respectively (Fig. 1 and Fig. 2A-D).

242 On nutrient-rich media ASM and MEA, the melanized strains grew on the agar surface, but also in
243 the third dimension, becoming cerebriform with age. $\Delta pks1$ and $\Delta pks1/\Delta phd1$ were more splattered
244 on the agar surface and displayed, with age, folded, membrane-like structures. The colonies were
245 built by preponderant yeast cells, with torulose hyphae also widely observed for wt $> \Delta phd1$,
246 $\Delta pks1 > \Delta pks1/\Delta phd1$ (Fig. 3A). On the nutrient-poor medium SDY all strains formed highly visible
247 filamentous hyphae at the edge of the colony (Fig. 1, Fig. 3B), less to not developed on the nutrient
248 rich media.

249 The four strains exhibited mycelial growth on and within the two pellet types, but different patterns
250 were recognizable, as detailed below.

251 On the pellet surfaces, the mycelial growth was remarkable at the naked eye in the case of the wt
252 and the $\Delta phd1$ melanized mutant, already at the first time point and before the PAS-staining (Fig.
253 2A-B). Non-melanized mutants displayed a similar growth, but the mycelial development was
254 mostly recognizable under microscopy observations. At t4, the mycelium of all the strains displayed
255 a radial growth that extended on the pellet surface well beyond the diameter of the inoculum (up to
256 2 mm from its border).

257 Almost in every sample observed (with the exception of two samples at t1), the mycelium
258 penetrated from the colonized surface of the pellet within its interior. Hyphal penetration started
259 from the region beneath the inoculum and then developed also beyond the border of the inoculum.
260 The hyphal growth was remarkably visible within large discontinuities, but it was also evident
261 along intergranular borders, strictly bond to the crystal surfaces (Fig. 2E-F).

262 Both torulose and filamentous hyphae were observed on the pellet surface and within its interior.
263 The melanized torulose hyphae were observable even before PAS staining (Fig. 2E). They were
264 characterised by swelling hyphae, with a diameter of 3-5 μm and meristematic growth, typical of
265 MCF (Fig. 3A). Next to them, the co-occurrence of filamentous hyphae with a diameter $< 3 \mu\text{m}$,
266 hyaline and elongated in the case of all strains, was only highlighted with the PAS-staining (Fig.
267 2F). Moreover, the PAS-staining highlighted extracellular polymeric substances (stained in pink),
268 diffused in the volume interested by the hyphal penetration (Fig. S2).

269

270 4.2. Penetration patterns of torulose and filamentous hyphae

271 Throughout the assays, the hyphal penetration never affected more than three hundred microns from
272 the pellet surface, and already reached its extension at t2 (approx. 20 μm growth per week from t0).
273 When considering altogether the four strains and the pellet types, filamentous hyphae showed
274 higher NVS and HPD with respect to the torulose ones at all the time points (Fig. 4). Filamentous
275 hyphae were already observable at t1 beneath the major part of the inoculum width (median
276 NVS=20%), and extended well beyond the inoculum border at t2 (40%). NVS of torulose hyphae
277 more slowly, gradually increased from t1 (5%) to t4 (35%). They were mostly observed beneath the
278 inoculum, but also far beyond (Fig. S2). The median penetration depth of torulose hyphae at t1, t2,
279 t3 was lower than 20 μm ; at t4, the median hyphal penetration reached 40 μm , with a 95th
280 percentile at approx. 200 μm . In the case of filamentous hyphae, the median penetration was already
281 higher than 50 μm at t1, and stabilized at approx. 200 μm at the subsequent time points (with a trend
282 similar to NVS), with a 95th percentile above 500 μm at t4.

283 When considering the four strains altogether, but the pellet types separately, torulose and
284 filamentous hyphae showed different penetration patterns. The NVS of filamentous hyphae was
285 similar within the two pellet types until t3, and it showed a higher increase within 4T pellets only at
286 t4 (Fig. 5A). By contrast, torulose hyphae displayed higher NVS within 4T pellets from t1 to t3,

287 while similar values were observed for 4T and 7.5T pellets at t4 (Fig. 5B).
288 At all the time points, the median penetration depth of torulose hyphae was significantly higher in
289 the 4T pellets with respect to the denser 7.5T (Fig. 5D). In particular, at t1, t2 and t3, the penetration
290 of torulose hyphae within the 7.5T pellets was negligible, while it reached 10-50 μm in the case of
291 4T pellets. Only at t4, the median penetration depth of torulose hyphae within 7.5T pellets was 25
292 μm , and the 95th percentile reached 75 μm . By contrast, the penetration depth of filamentous
293 hyphae did not show significant differences between 4T and 7.5T pellets, displaying a gradual
294 increase of penetration values from t1 to t4 (Fig. 5C).

295

296 4.3. Penetration patterns of wt and mutants

297 Within both the pellet types, the filamentous hyphae of the four strains showed different NVS at t1,
298 while generally similar NVS (approx. 40-50%) at the subsequent time points (Fig. 6A). At t1, in
299 particular, wt showed lower median values than the mutant strains, within both pellet types
300 significantly different with respect to $\Delta pks1/\Delta phd1$. At the subsequent times, only wt at t3
301 significantly penetrated more than the other strains within 4T pellets, while similar high values were
302 only exceptionally observed in the other study cases (at t3, one replicate for wt within 7.5T pellets;
303 at t4, one replicate for $\Delta pks1/\Delta phd1$ within 4T pellets).

304 In the case of torulose hyphae, from t1 to t3, each strain showed a higher NVS within 4T than
305 within 7.5T. However, at t1 and t2, the values of the different strains within each type of pellet were
306 similar. At t3, wt and $\Delta pks1/\Delta phd1$ displayed some higher NVS than the other mutants, but the
307 differences disappeared at t4 (Fig. 6B).

308 The HPD of torulose hyphae was also higher within the 4T pellets than within the 7.5T pellets at all
309 the time points. However, as in the case of NVS, the penetration of the four strains within each
310 pellet type was rather identical at t1 and t2, and showed slightly higher values for $\Delta pks1/\Delta phd1$
311 within 4T and 7.5T pellets at t3 and t4, respectively (Fig. 6D). In the case of filamentous hyphae,
312 HPD within 7.5T pellets was significantly higher for $\Delta pks1/\Delta phd1$ than the wt and the other mutants
313 at t1, higher for the mutants with respect to wt at t2, equal for all the strains at t3, and higher for
314 $\Delta phd1$ than $\Delta pks1/\Delta phd1$ at t4. Within the 4T pellets, HPD of wt was lower than the mutants at t1
315 (as within 7.5T at t2), equal for all the strains at t2 (as within 7.5T at t3); at t3 and t4, $\Delta pks1/\Delta phd1$
316 was higher than the other mutants, while the wt unexpectedly showed strongly lower values at t4
317 with respect to t3 (Fig. 6C). In general, peculiar cases of higher or lower growth for single replicate
318 seem to characterize the t3 and t4 time points, affecting the overall variance of the datasets.

319

320 5. Discussion

321 The hyphal penetration of MCF was observed within different carbonate and silicate rocks,
322 including the stone cultural heritage, and related to their physico-chemical deterioration, but the
323 mechanisms underlying the penetration processes have still not been fully clarified [16, 21, 33, 43].
324 Our investigation on the model MCF *Knufia petricola* indicates that the long-hypothesized crucial
325 role of melanin to allow hyphal penetration by increasing cell rigidity, and thus its mechanical force
326 [29], should be rejected. Indeed, the melanin-deficient mutant strains $\Delta pks1$ and $\Delta pks1/\Delta phd1$ did
327 not show significantly lower penetration with respect to the melanized wt and $\Delta phd1$, both in terms
328 of penetration depth (parameter HPD) and spread (NVS). Some higher penetration of $\Delta pks1/\Delta phd1$,
329 lacking melanin, but also the protective presence of carotenoids, rather suggests some positive
330 relationship between higher stress susceptibility and higher penetration. These explanations are
331 subsequently discussed at the light of the complexity of the investigated MCF-substrate systems,
332 including morphologically distinct hyphae [33] and pellets with different porosity, a factor
333 significantly affecting the penetration patterns [16].

334

335 5.1. Hyphal morphology

336 The morphological plasticity of MCF is well-known and described for several species [42, 49, 50],
337 and drastic morphological shifts following changes in microenvironmental conditions were
338 observed, with meristematic hyphae appearing in response to temperature and desiccation stress
339 [30, 51]. A conversion from yeast-like to meristematic growth was also considered in the
340 description of *K. petricola*, and the meristematic growth and penetration of torulose hyphae was
341 associated to stone biodeterioration [32, 52]. We similarly observed yeast-like and meristematic
342 growth patterns for the different strains cultured on nutrient-rich media (ASM, MEA), in which
343 absence, or a strongly subordinate presence, of filamentous hyphae occurred. These latter, however,
344 were remarkable on the nutrient-poor SDY and within the stone pellets, according to previous
345 observations for MCF of extremely thin hyphae penetrating fissures and pores of rock substrates
346 [21]. In particular, the co-presence of torulose and filamentous hyphae within the pellets fully
347 mimics that recently observed by the resin-casting techniques within marble statues colonized by
348 MCF, including *K. petricola* [33]. A higher penetration depth of the filamentous hyphae agrees with
349 their potential explorative (nutrient-seeking) role within the bare rock material [36]. The growth of
350 the torulose hyphae closer to the pellet surface may be related with the higher distance from the
351 agar-water terrain on which the pellet lain within the Petri-dishes, and thus a higher desiccation
352 stress over time [30]. This first level of observation already suggests that the torulose hyphae,
353 usually recognized as melanized penetrating structures of MCF, may be more related to a resistance
354 to surface stress factors than to a penetration role, which is mostly exerted by the filamentous
355 hyphae.

356

357 5.2. Hyphal penetration patterns and substrate porosity

358 The depth of hyphal penetration observed within the pellets, in the order of few hundreds microns,
359 was generally consistent with that observed within natural carbonate substrates, including heritage
360 objects [27, 33]. In particular, even at t4, HPD of torulose hyphae never exceeded 200 μm , in
361 agreement with penetrations of 100-150 μm observed for *K. petricola* and other MCF incubated on
362 carbonate rocks and cement slab, which are values remarkably lower than the millimetric
363 penetration depths observed within acid lithologies [16, 53]. Beside the different ecophysiological
364 role, the pellet porosity clearly affects the different penetration patterns of torulose and filamentous
365 hyphae. This finding agrees with the influence of rock texture and structure on the penetration of
366 MCF within different lithologies [16], a phenomenon mostly described until now for lichen-forming
367 fungi [29]. Actually, the analogous HPD of filamentous hyphae within the different pellet types
368 shows that their very thin diameters allow penetration along crystal boundaries without an influence
369 of the different porosity. Oppositely, the development of torulose hyphae is limited in terms of HPD
370 and NVS by the lower porosity of 7.5T with respect to 4T, and their presence seems more related to
371 larger crevices and pores because of a sufficient void volume, in agreement with the 'penetration

372 stage' described by Sterflinger and Krumbein [27] for MCF within marble. Similar trends observed
373 for HPD and NVS particularly remark that torulose hyphae need available voids for their
374 development, not only with respect to their growth in depth, but also with regard to their spread
375 immediately below the surface. Accordingly, in the months-long monitored time, the exploitation of
376 existing discontinuities seemed to prevail on an active opening by torulose hyphae of new
377 passageways [54]. However, in a longer term, the chemical action of EPS [37], also highlighted
378 within the pellets, may couple with the mechanical forces of hyphae and determine an active
379 increase of rock discontinuities, as long supposed for MCF [6, 30].

380

381 5.3. Hyphal penetration patterns, melanization and stress tolerance

382 Multiple ecophysiological functions of melanin in fungi have been widely characterized [39]. In
383 particular, melanin incrustation of thick-walled yeast-like cells and meristematic torulose hyphae of
384 MCF was related with their tolerance of stress-conditions in temperature, irradiation, salt and water
385 availability [30] and their penetration [29]. Our investigation showed the presence of torulose
386 hyphae also in the case of melanin deficient strains, and their penetration within the substrate.
387 Accordingly, melanin does not result the key factor driving the penetration of torulose hyphae, in
388 agreement with early insights by Diakumaku [55] on a scarce effect of the inhibition of melanin
389 synthesis by tricyclazole on the MCF ability to colonize marble [56]. Filamentous hyphae were
390 always hyaline for all the strains and, nevertheless, they deeply penetrated both the pellet types. The
391 absence of melanin prevented their observation before the staining, possibly explaining why their
392 development and penetration was less reported than that of torulose ones in observations of MCF on
393 cultural heritage, where their presence within stone was detected with SEM observations [33].

394 Although the absence of melanization did not reduce the penetration ability of *K. petricola*, it
395 seemed to affect the penetration rate of filamentous hyphae. Higher penetration of these latter
396 observed at t1 and t4 in the case of $\Delta pks1/\Delta phd1$ may be hypothetically related to an earlier
397 necessity to explore the substrate and find organic nutrients and/or a different stress tolerance,
398 respectively. The degradation of melanin in the own cell walls was indeed suggested as a way of
399 supporting the MCF metabolism in the absence of nutrients on bare rock [31]. Since this was not
400 possible for melanin deficient strains, the explorative filamentous hyphae may have early been
401 produced. On the other hand, the melanin deficient strains, and in particular the $\Delta pks1/\Delta phd1$ which
402 also lacks carotenoids, should less cope with stresses [44] which may affect the colonies after the
403 long incubation. However, the incubation at 20°C and in the dark should have mostly reduced any
404 stress factor acting on the colonies. Just the mild conditions may have generally favoured the
405 development of filamentous hyphae, which could be instead less adapted to tolerate the stress of the
406 life on rocks in the environment. This is an additional, alternative hypothesis which may justify the
407 lower records in field samples of MCF of this hyphal morphology, which may only appear as a
408 response to certain conditions, and certainly needs further investigations to solve its ecological
409 significance.

410

411 5.4. Hyphal penetration patterns and cultural heritage

412 The ability of MCF to cope with environmental stress factors was often related to the difficulty
413 encountered in their removal from the cultural heritage surfaces, and associated to their resistance to
414 biocides and other anti-microbial treatments [24, 28]. However, the inhibition of their growth by
415 widely-used biocidal active principles, as benzalkonium chloride, was shown *in vitro* for some
416 species [57]. Their resilience was also related to their ability to grow inside the rock substrate [58]
417 and this hypothesis seems supported by our observation of a spread hyphal penetration. In
418 particular, beside the evident melanized torulose hyphae, the filamentous ones may be less reached
419 by control treatments because of the deeper penetration. Such finding remarks the importance of
420 selecting control treatments which are efficient not only directly on the rock surface, but also
421 against the endolithic component [59].

422 The recognition of porosity as crucial factor of substrate susceptibility to MCF spread suggests the
423 potential opportunity to reduce porosity or limit the hyphal access to porosity to contain hyphal

424 penetration and favour a MCF control at the surface. However, MCF are able to exploit most of
425 organic compounds as nutrients, including protective coatings and consolidants [60], and non-
426 traditional approaches, as mineral (bio-)precipitation [61] may be more suitable against the MCF
427 threat. Moreover, less porous lithologies should be used as new materials or for integrations where
428 MCF are recognized as a conservation issue.

429 In this framework, the incubation of MCF on mineral slabs/coupons in controlled conditions is here
430 confirmed as a suitable approach to investigate their biology and unveil their impact on cultural
431 heritage, by limiting the influence of multiple macro- and micro environmental factors [16, 17, 27].
432 In particular, we highlight the suitability of standardized mineral pellets to focus on the influence of
433 selected intrinsic properties of rock material, as porosity, on their interactions with lithobionts. Its
434 combination with the use of mutant strains is particularly promising for future researches to further
435 unveil the colonization mechanisms of MCF and their impact on stone durability, and to address
436 prevention and control strategies.

437

438 **6. Conclusive remarks**

439 The wild-type and three mutants of *Knufia petricola* A95 developed different mycelial structures on
440 media with different nutrient content. Morphological flexibility of black MCF involves exploratory
441 nutrient-seeking thin filamentous hyphae combined with torulose hyphae (and microcolonies) that
442 donate sufficient resistance to surface stress factors. This growth form combination along with
443 physiological protective measures (pigments, compatible solutes) makes MCF a versatile group
444 ubiquitously present and successfully colonising porous and vulnerable stone monuments.

445 On marble pellets fungal structures of all tested strains were able to penetrate into the depth (up to
446 200 μm) of carbonate substrates, independently of their ability to synthesise protective pigments,
447 thus rejecting the hypothesis that hyphal melanization is the adaptive trait which allows the MCF
448 penetration. Rather, substrate porosity significantly drives the penetration patterns of different
449 hyphal morphologies. The fact that development of penetrative filamentous hyphae by MCF also
450 correlates with nutrient conditions makes preliminary testing of planned conservation treatments
451 necessary. As any external input (e.g. an impregnation or consolidating solution) is a potential
452 source of nutrients for microbial growth, treatments may increase biodeterioration phenomena.

453 Marble pellets that are produced by compaction possess defined chemistry and porosity and are
454 particularly suitable for accelerated testing and selecting conservation treatments in lab
455 bioreceptivity tests. In combination with a model rock-inhabiting black fungus and quantification
456 analyses, this test can be established in conservation praxis when selecting restoration treatments for
457 monument protection.

458

459 **Acknowledgements**

460 This work was supported by local funding by the University of Turin.

461 The authors would like to thank Nicole Knabe for providing the strains, Federica Consogno for the
462 technical support and Ruben Gerrits, Julia Schumacher and Enrica Matteucci for the fruitful
463 discussions that greatly helped the overall outcome of this work.

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711 **Captions for figures**

712

713 Fig. 1. Colony morphology of *K. petricola* on agar with different nutrient concentration. Droplets of
714 cell suspensions of *K. petricola* wild-type and pigment mutants were inoculated on nutrient rich
715 medium (ASM) and on poor medium (SDY). On ASM no hyphae were observed whereas on SDY
716 all strains formed hyphae at the border of the colonies.

717

718 Fig. 2. Growth of *K. petricola* on (A-D) and within (E-F) the marble pellets. Wildtype A95 (A);
719 Δ pks1 (B); Δ pks1 (C); Δ pks1/ Δ pks1 (C); melanized torulose hyphae developed by Δ pks1 within
720 the pellet (cross-sectioned pellet before PAS-staining; E); hyphal growth within large porosities (#)
721 and along intergranular borders (arrows) (PAS-stained, cross-sectioned pellet, F). Scales: pellet
722 diameter = 1.27 cm (A-D); bars: 100 μ m (E-F).

723

724 Fig. 3. Hyphal morphology on agar and marble surfaces. Torulose hyphae on agar surface,
725 composed by swelling cells (A); filamentous hyphae (arrows) on agar surface, evidently composed
726 by elongated, thinner cells (B); growth of torulose (C) and filamentous (D) hyphae strictly bond to
727 the crystal surface. Scale bars: 20 μ m (A), 25 μ m (B,C), 10 μ m (D).

728

729 Fig. 4. Percentage of vertical sectors (NVS) in which the penetration of torulose (TH, black box
730 plots) and filamentous (FH, grey) hyphae was observed (A), and their penetration depth (HPD)
731 within the pellets (B), after 5 (t1), 10(t2), 17 (t3) and 27 (t4) weeks, considering altogether the pellet
732 types and the four strains. At each time point, box-plots marked with different letters are statistically
733 different (ANOVA, t-test; $P < 0.05$).

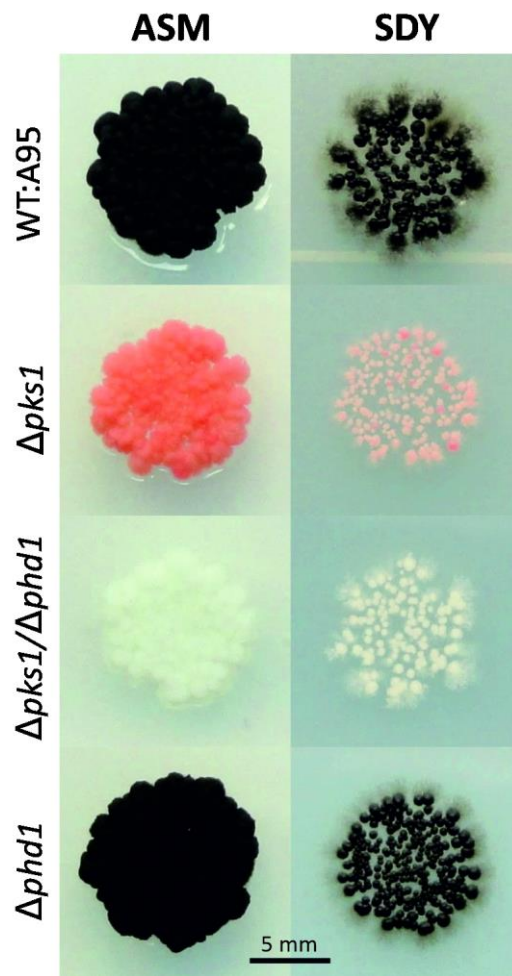
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735 Fig. 5. Percentage of vertical sectors (NVS) in which the penetration was observed (A,B) and
736 hyphal penetration depth (HPD; C,D) of filamentous (FH; A,C) and torulose (TH; B,D) hyphae
737 within 7.5T (grey box-plots) and 4T (white box-plots) pellets, after 5 (t1), 10(t2), 17 (t3) and 27 (t4)
738 weeks, considering altogether the four strains. At each time point, box-plots marked with different
739 letters are statistically different (ANOVA, t-test; $P < 0.05$).

740

741 Fig. 6. Percentage of vertical sectors (NVS) in which the penetration was observed (A,B) and
742 hyphal penetration depth (HPD; C,D) of filamentous (FH; A,C) and torulose (TH; B,D) hyphae
743 within 7.5T (grey box-plots) and 4T (white box-plots) pellets, after 5 (t1), 10(t2), 17 (t3) and 27 (t4)
744 weeks, considering separately the four strains: wt, Δ pks1 (Δ m), Δ pks1 (Δ c), Δ pks1/ Δ pks1 (Δ/Δ). At
745 each time point, the box-plots of the different strains which do not share any letter are statistically
746 different (ANOVA, Tukey's test; $P < 0.05$).

747 Fig.1



748

749

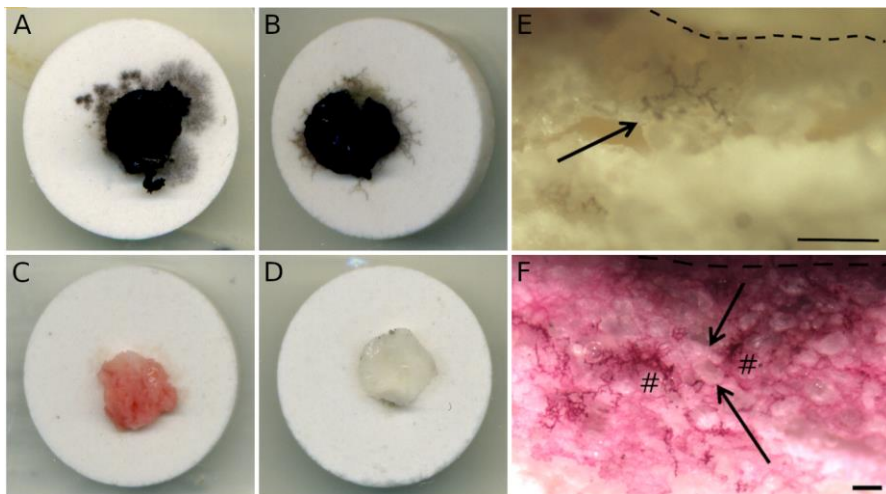
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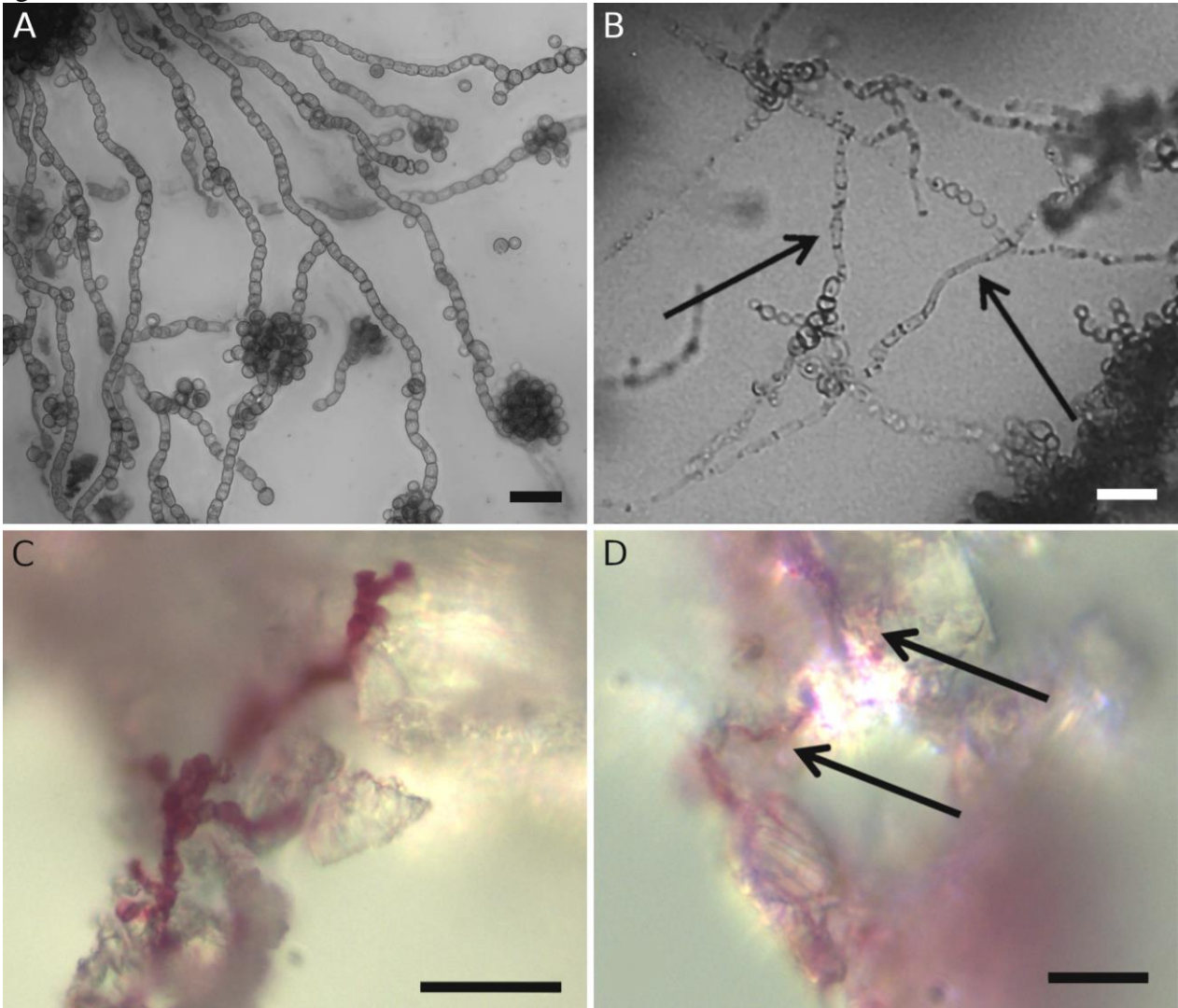
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754 Fig.2



755

756 Fig.3



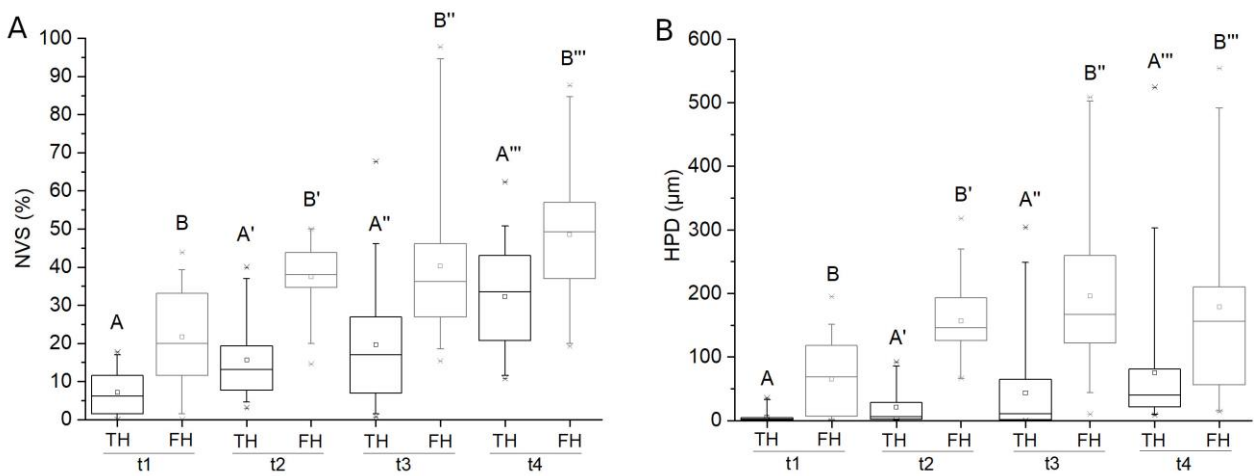
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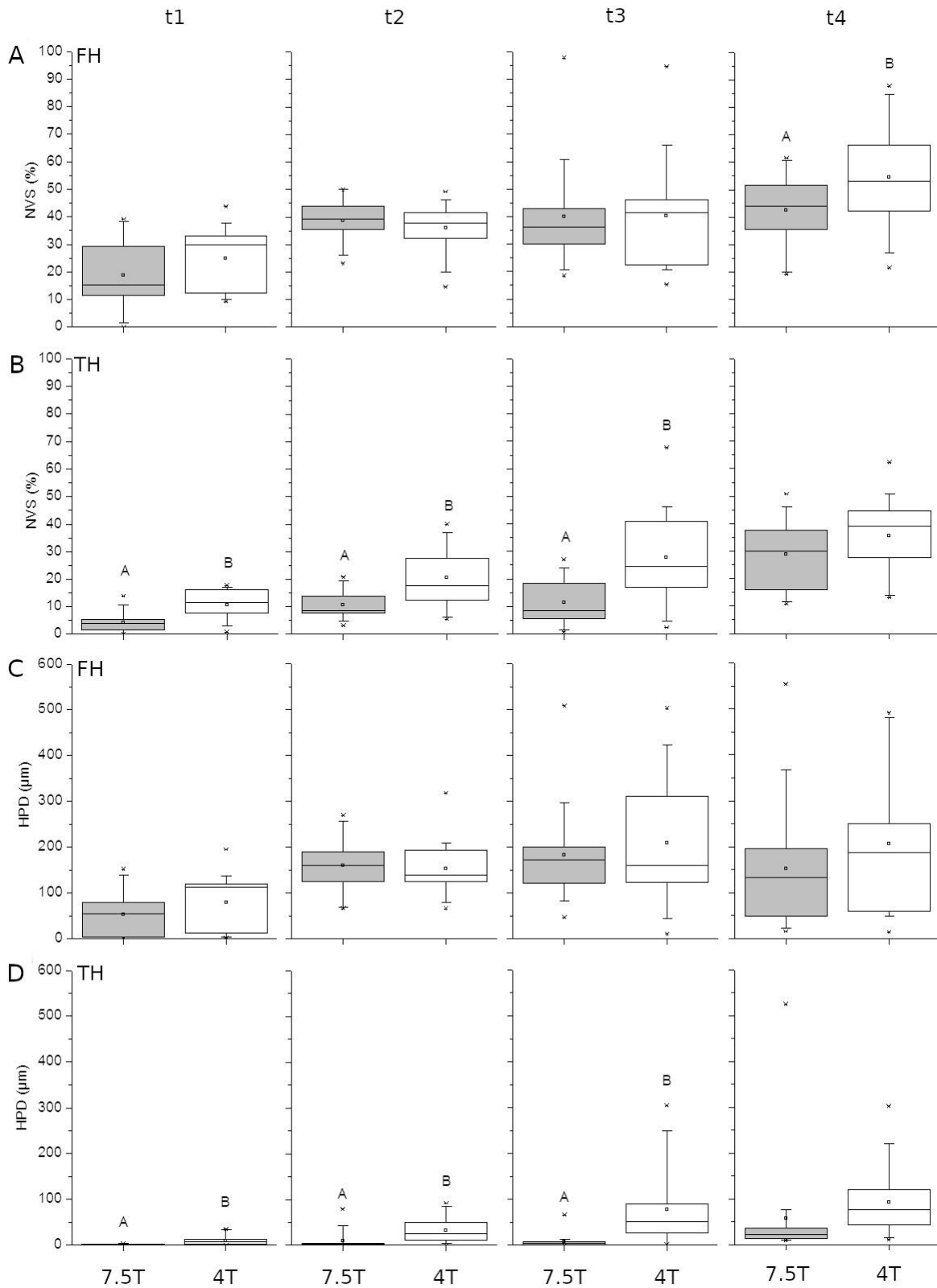
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761 Fig.4



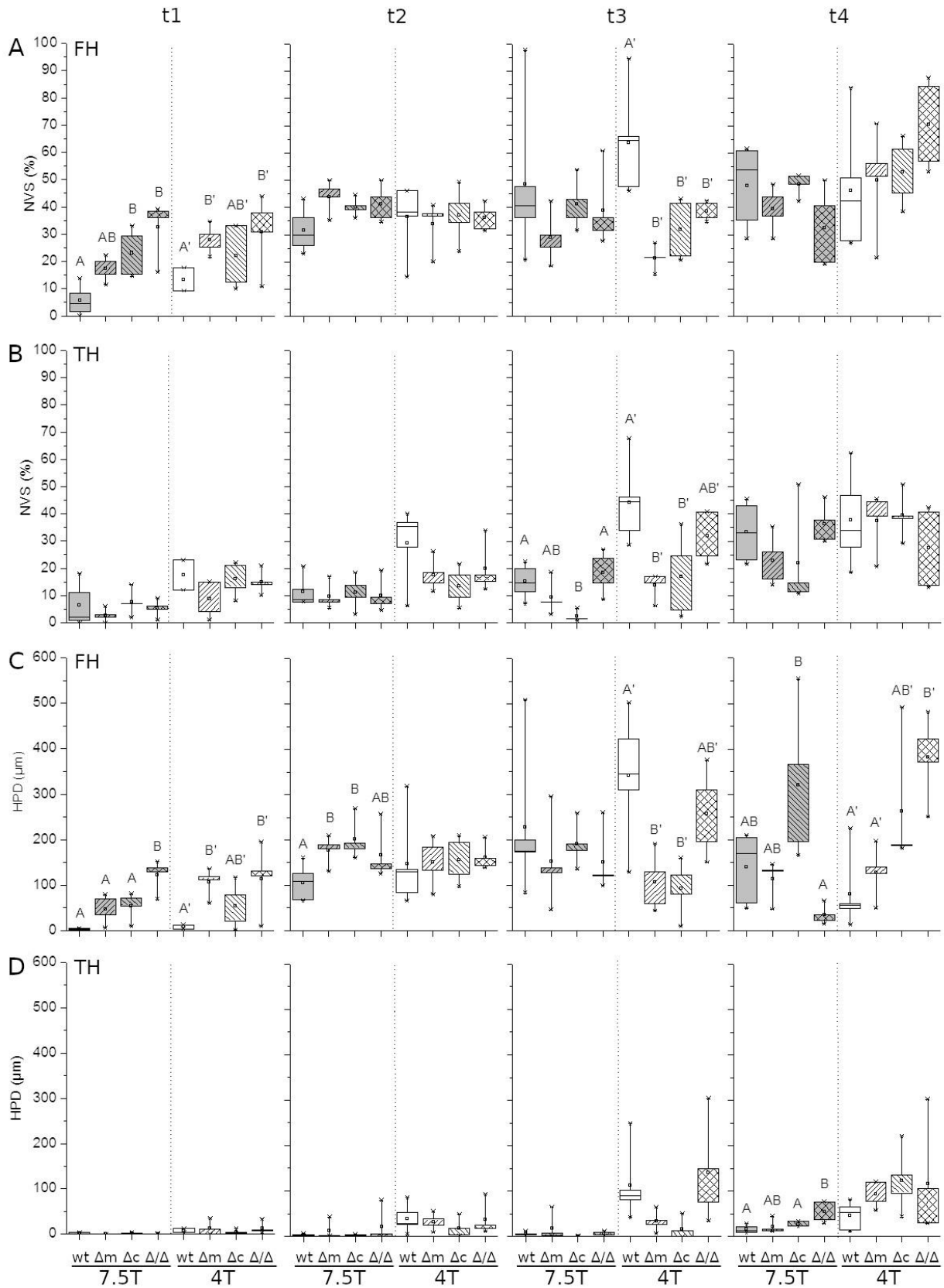
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763 Fig.5



764
765
766

767 Fig.6



768
769