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

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Abstract

Due to their ability to penetrate, deteriorate and discolour marble/stone surfaces, rock-inhabiting black fungi represent a remarkable issue for cultural heritage conservation.
Black microcolonial fungi (MCF) can also adapt to different environmental conditions, by converting from yeast-like morphology to a peculiar meristematic development with swollen cells (torulose hyphae, TH), to extremely thin structures (filamentous hyphae, FH). Furthermore, black MCF produce protective pigments: melanin, dark pigment particularly evident on light stone surfaces, and carotenoids. Black fungi produce melanin in critical, oligotrophic conditions as well as constitutively. Melanin function is mostly related to stress resistance and the ability of fungi to generate appressorial turgor to actively penetrate plant cells in pathogenic species. An involvement of melanins in stone surface penetration has been suggested, but not experimentally proved.
In this work, we tested the role of hyphal melanisation in penetration mechanisms on the model black fungus *Knufia petricola* A95 in lab conditions. The wild-type and three mutants with introduced targeted mutations of polyketide-synthases (melanin production), and/or phytoene dehydrogenase (carotenoid synthesis) were inoculated on artificial carbonate pellets (pressed Carrara marble powder) of different porosity. After 5, 10, 17 and 27 weeks, hyphal penetration depth and spread were quantified on periodic acid Schiff-stained cross-sections of the pellets, collecting measurements separately for TH and FH. Droplet assay of the mutants on different media were conducted to determine the role of nutrients in the development of different fungal morphologies.
In our in vitro study, the hyphal penetration depth, never exceeding 200 μm, was proven to be consistent with observed penetration patterns on stone heritage carbonate substrates. Pellet porosity affected penetration patterns of TH, which developed in voids of the more porous pellets, instead than actively opening new passageways. Oppositely, the thin diameter of FH allowed their

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

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

1. Introduction

Outdoor exposed stone cultural heritage is subject to various types of deterioration. Chemical and physical deterioration, due to the action of the atmospheric agents, is the most evident, but in the last decades the attention of the conservation scientists has been drawn towards the microorganisms which can colonize stone surfaces and cause biodeterioration [1-5]. Although stone surfaces are hostile due to the scarcity of nutrients, sun exposure and available water, and many physical stresses, several microorganisms colonize these niches, often organized as sub-aerial biofilms (SABs) [6].
Knowledge on the relationship between microorganisms and colonized substrate drives the recognition of major threats to conservation and may address the definition of control strategies to face biodeterioration [7]. However, the multiple factors involved, including the stone, the microbes, and the surrounding environment, have often prevented a full comprehension of the mechanisms underlying biodeterioration, which was mostly examined in the complexity of field conditions.
The physico-chemical properties of the stone substrate affect its bioreceptivity, which is the aptitude of a material to be colonized by one or several groups of living organisms without necessarily undergoing any biodeterioration [8]. The mineral composition and surface properties, as roughness, control the microbial establishment [9]. Substrate porosity and texture were proved to regulate the growth of biodeteriogens within the stone, as in the case of lichens [10]. Microbial growth on monuments is mediated by several extrinsic factors, acting from a macro- (e.g. water regime, relative humidity, solar radiation, temperature, wind, atmospheric pollution) to a micro- (e.g. orientation, shading, permanent capillary humidity, etc.) scale [9, 11, 12].
Accordingly, protocols to test the bioreceptivity of stone materials in standardized environmental conditions were proposed and applied to evaluate the establishment and growth on mineral surfaces of stable phototrophic multi-species cultures [13-15]. However, laboratory approaches have still been poorly used to examine the microbial-substrate interaction in terms of penetration and dissolution patterns, and to explore the underlying mechanisms [10, 16-19].
Microcolonial fungi (MCF) are a major component of SABs on stone surfaces in extreme environments, from Antarctica to hot deserts [20-23], and they often occur on stone cultural heritage [24, 25]. Compact black microcolonies are equipped to withstand extreme environmental challenges including desiccation, UV radiation, temperature and oxidative stress as well as biocide treatments. They represent a remarkable issue for the conservation, due to their ubiquity, dark colour and the ability to penetrate and modify stone surfaces [26-29]. Black MCF are a polyphyletic group of Ascomycetes sharing morphological plasticity and functional adaptive traits which ensure their survival in extreme environments [30, 31].
Since the early descriptions at the end of last century, it was recognized that rock-inhabiting black fungi can drastically change morphology depending on environmental conditions, with conversions from yeast-like cells, to a peculiar meristematic development, to extremely thin hyphae [21, 32]. Their growth by isodiametric enlargement and subsequent division of cell compartments (meristeme-like), with thick and even multi-layered cell walls, gives rise to aggregated structures

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

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

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

2. Research aim

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

3. Materials and methods

3.1. Preparation of carbonate pellets with different porosity

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

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

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

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

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

3.2. Fungal material

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

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

3.3. Fungal inoculation on agar plates for droplet assays

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

3.4. Fungal inoculation on carbonate pellets

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

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

3.5. Microscopy observation of fungal growth

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

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

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

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

4. Results

4.1 Fungal growth in different nutrient conditions

The four strains of *Knufia petricola* cultured on the agarized media and the carbonate pellets macroscopically differed in colour and the colony morphology. The melanized wt and Δ*phd1* were black-brown and black-grey, respectively, while *Δpks1* and *Δpks1/Δphd1* were pink and white, respectively (Fig. 1 and Fig. 2A-D).

On nutrient-rich media ASM and MEA, the melanized strains grew on the agar surface, but also in the third dimension, becoming cerebriform with age. *Δpks1* and *Δpks1/Δphd1* were more splattered on the agar surface and displayed, with age, folded, membrane-like structures. The colonies were built by preponderant yeast cells, with torulose hyphae also widely observed for wt>*Δphd1*, *Δpks1*>*Δpks1/Δphd1* (Fig. 3A). On the nutrient-poor medium SDY all strains formed highly visible filamentous hyphae at the edge of the colony (Fig. 1, Fig. 3B), less to not developed on the nutrient rich media.

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

On the pellet surfaces, the mycelial growth was remarkable at the naked eye in the case of the wt and the *Δphd1* melanized mutant, already at the first time point and before the PAS-staining (Fig. 2A-B). Non-melanized mutants displayed a similar growth, but the mycelial development was mostly recognizable under microscopy observations. At t4, the mycelium of all the strains displayed a radial growth that extended on the pellet surface well beyond the diameter of the inoculum (up to 2 mm from its border).

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

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

4.2. Penetration patterns of torulose and filamentous hyphae

Throughout the assays, the hyphal penetration never affected more than three hundred microns from the pellet surface, and already reached its extension at t2 (approx. 20 μm growth per week from t0).

When considering altogether the four strains and the pellet types, filamentous hyphae showed higher NVS and HPD with respect to the torulose ones at all the time points (Fig. 4). Filamentous hyphae were already observable at t1 beneath the major part of the inoculum width (median NVS=20%), and extended well beyond the inoculum border at t2 (40%). NVS of torulose hyphae more slowly, gradually increased from t1 (5%) to t4 (35%). They were mostly observed beneath the inoculum, but also far beyond (Fig. S2). The median penetration depth of torulose hyphae at t1, t2, t3 was lower than 20 μm; at t4, the median hyphal penetration reached 40 μm, with a 95th percentile at approx. 200 μm. In the case of filamentous hyphae, the median penetration was already higher than 50 μm at t1, and stabilized at approx. 200 μm at the subsequent time points (with a trend similar to NVS), with a 95th percentile above 500 μm at t4.

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

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

4.3. Penetration patterns of wt and mutants

Within both the pellet types, the filamentous hyphae of the four strains showed different NVS at t1, while generally similar NVS (approx. 40-50%) at the subsequent time points (Fig. 6A). At t1, in particular, wt showed lower median values than the mutant strains, within both pellet types significantly different with respect to *Δpks1/Δphd1*. At the subsequent times, only wt at t3 significantly penetrated more than the other strains within 4T pellets, while similar high values were only exceptionally observed in the other study cases (at t3, one replicate for wt within 7.5T pellets; at t4, one replicate for *Δpks1/Δphd1* within 4T pellets).
In the case of torulose hyphae, from t1 to t3, each strain showed a higher NVS within 4T than within 7.5T. However, at t1 and t2, the values of the different strains within each type of pellet were similar. At t3, wt and *Δpks1/Δphd1* displayed some higher NVS than the other mutants, but the differences disappeared at t4 (Fig. 6B).
The HPD of torulose hyphae was also higher within the 4T pellets than within the 7.5T pellets at all the time points. However, as in the case of NVS, the penetration of the four strains within each pellet type was rather identical at t1 and t2, and showed slightly higher values for *Δpks1/Δphd1* within 4T and 7.5T pellets at t3 and t4, respectively (Fig. 6D). In the case of filamentous hyphae, HPD within 7.5T pellets was significantly higher for *Δpks1/Δphd1* than the wt and the other mutants at t1, higher for the mutants with respect to wt at t2, equal for all the strains at t3, and higher for *Δphd1* than *Δpks1/Δphd1* at t4. Within the 4T pellets, HPD of wt was lower than the mutants at t1 (as within 7.5T at t2), equal for all the strains at t2 (as within 7.5T at t3); at t3 and t4, *Δpks1/Δphd1* was higher than the other mutants, while the wt unexpectedly showed strongly lower values at t4 with respect to t3 (Fig. 6C). In general, peculiar cases of higher or lower growth for single replicate seem to characterize the t3 and t4 time points, affecting the overall variance of the datasets.

5. Discussion

The hyphal penetration of MCF was observed within different carbonate and silicate rocks, including the stone cultural heritage, and related to their physico-chemical deterioration, but the mechanisms underlying the penetration processes have still not been fully clarified [16, 21, 33, 43]. Our investigation on the model MCF *Knufia petricola* indicates that the long-hypothesized crucial role of melanin to allow hyphal penetration by increasing cell rigidity, and thus its mechanical force [29], should be rejected. Indeed, the melanin-deficient mutant strains *Δpks1* and *Δpks1/Δphd1* did not show significantly lower penetration with respect to the melanized wt and *Δphd1*, both in terms of penetration depth (parameter HPD) and spread (NVS). Some higher penetration of *Δpks1/Δphd1*, lacking melanin, but also the protective presence of carotenoids, rather suggests some positive relationship between higher stress susceptibility and higher penetration. These explanations are subsequently discussed at the light of the complexity of the investigated MCF-substrate systems, including morphologically distinct hyphae [33] and pellets with different porosity, a factor significantly affecting the penetration patterns [16].

5.1. Hyphal morphology

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

5.2. Hyphal penetration patterns and substrate porosity

The depth of hyphal penetration observed within the pellets, in the order of few hundreds microns, was generally consistent with that observed within natural carbonate substrates, including heritage objects [27, 33]. In particular, even at t4, HPD of torulose hyphae never exceeded 200 μm, in agreement with penetrations of 100-150 μm observed for *K. petricola* and other MCF incubated on carbonate rocks and cement slab, which are values remarkably lower than the millimetric penetration depths observed within acid lithologies [16, [53]. Beside the different ecophysiological role, the pellet porosity clearly affects the different penetration patterns of torulose and filamentous hyphae. This finding agrees with the influence of rock texture and structure on the penetration of MCF within different lithologies [16], a phenomenon mostly described until now for lichen-forming fungi [29]. Actually, the analogous HPD of filamentous hyphae within the different pellet types shows that their very thin diameters allow penetration along crystal boundaries without an influence of the different porosity. Oppositely, the development of torulose hyphae is limited in terms of HPD and NVS by the lower porosity of 7.5T with respect to 4T, and their presence seems more related to larger crevices and pores because of a sufficient void volume, in agreement with the ‘penetration

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

5.3. Hyphal penetration patterns, melanization and stress tolerance

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

Although the absence of melanization did not reduce the penetration ability of *K. petricola*, it seemed to affect the penetration rate of filamentous hyphae. Higher penetration of these latter observed at t1 and t4 in the case of *Δpks1/Δphd1* may be hypothetically related to an earlier necessity to explore the substrate and find organic nutrients and/or a different stress tolerance, respectively. The degradation of melanin in the own cell walls was indeed suggested as a way of supporting the MCF metabolism in the absence of nutrients on bare rock [31]. Since this was not possible for melanin deficient strains, the explorative filamentous hyphae may have early been produced. On the other hand, the melanin deficient strains, and in particular the *Δpks1/Δphd1* which also lacks carotenoids, should less cope with stresses [44] which may affect the colonies after the long incubation. However, the incubation at 20°C and in the dark should have mostly reduced any stress factor acting on the colonies. Just the mild conditions may have generally favoured the development of filamentous hyphae, which could be instead less adapted to tolerate the stress of the life on rocks in the environment. This is an additional, alternative hypothesis which may justify the lower records in field samples of MCF of this hyphal morphology, which may only appear as a response to certain conditions, and certainly needs further investigations to solve its ecological significance.

5.4. Hyphal penetration patterns and cultural heritage

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

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

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

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

6. Conclusive remarks

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

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

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

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Captions for figures

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

Fig. 2. Growth of *K. petricola* on (A-D) and within (E-F) the marble pellets. Wildtype A95 (A); Δphd1 (B); Δpks1 (C); Δpks1/Δphd1 (C); melanized torulose hyphae developed by Δphd1 within the pellet (cross-sectioned pellet before PAS-staining; E); hyphal growth within large porosities (#) and along intergranular borders (arrows) (PAS-stained, cross-sectioned pellet, F). Scales: pellet diameter = 1.27 cm (A-D); bars: 100 μm (E-F).

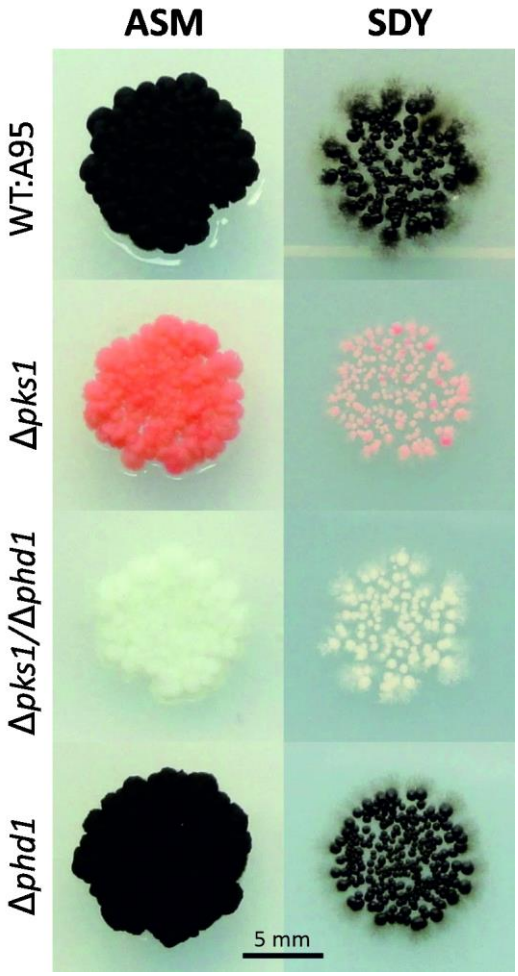
Fig. 3. Hyphal morphology on agar and marble surfaces. Torulose hyphae on agar surface, composed by swelling cells (A); filamentous hyphae (arrows) on agar surface, evidently composed by elongated, thinner cells (B); growth of torulose (C) and filamentous (D) hyphae strictly bond to the crystal surface.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

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

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

Fig. 6. Percentage of vertical sectors (NVS) in which the penetration was observed (A,B) and hyphal penetration depth (HPD; C,D) of filamentous (FH; A,C) and torulose (TH; B,D) hyphae within 7.5T (grey box-plots) and 4T (white box-plots) pellets, after 5 (t1), 10(t2), 17 (t3) and 27 (t4) weeks, considering separately the four strains: wt, Δpks1 (Δm), Δphd1 (Δc), Δpks1/Δphd1 (Δ/Δ). At each time point, the box-plots of the different strains which do not share any letter are statistically different (ANOVA, Tukey's test; $P<0.05$).

747 Fig.1



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754 Fig.2

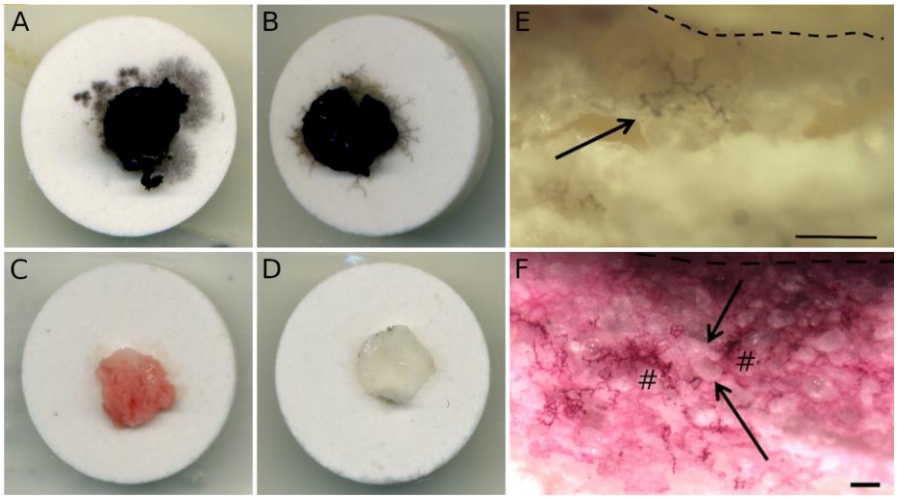

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Fig.3

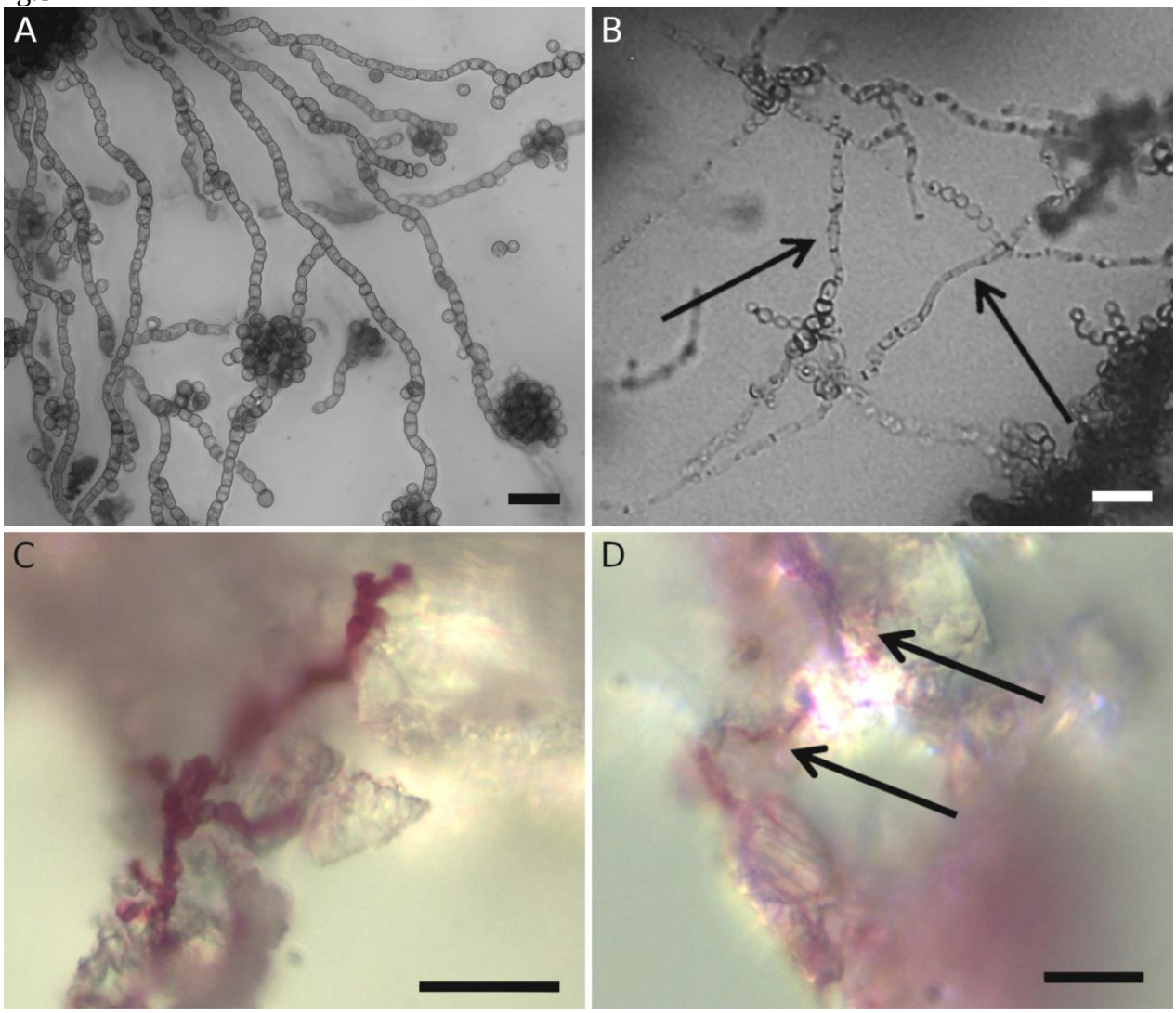
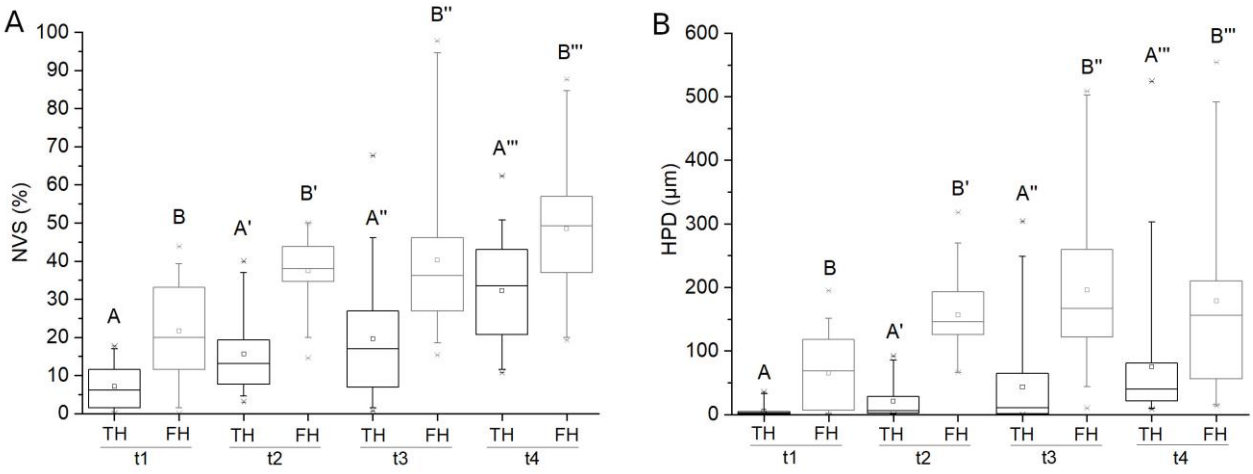
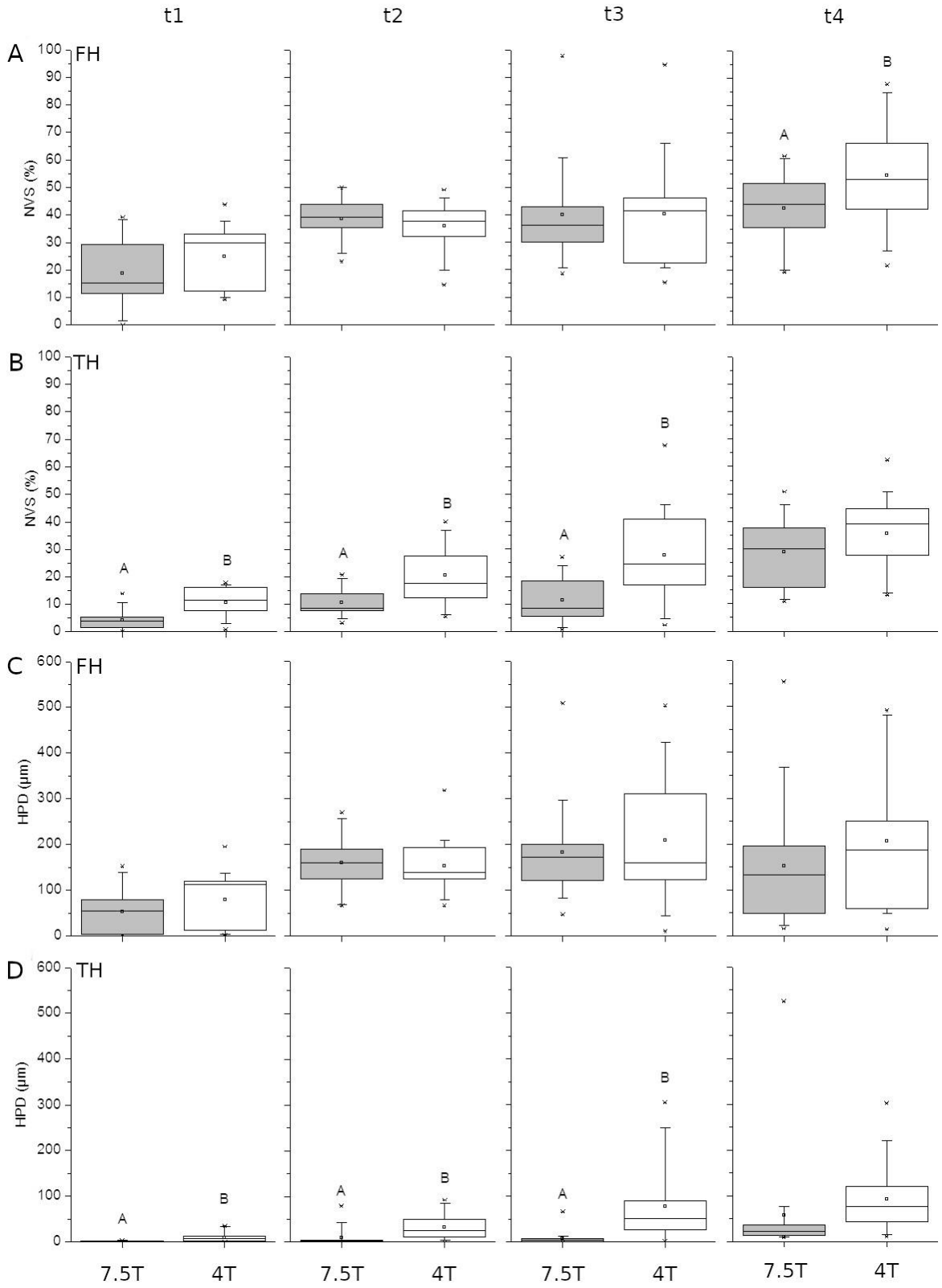

A
B
C
D

Fig.4


A
NVS (%)
100
90
80
70
60
50
40
30
20
10
0
A
B
A'
B'
A''
B''
A'''
B'''
TH
FH
t1
t2
t3
t4
B
HPD (µm)
600
500
400
300
200
100
0
A
B
A'
B'
A''
B''
A'''
B'''
TH
FH
t1
t2
t3
t4

763 Fig.5



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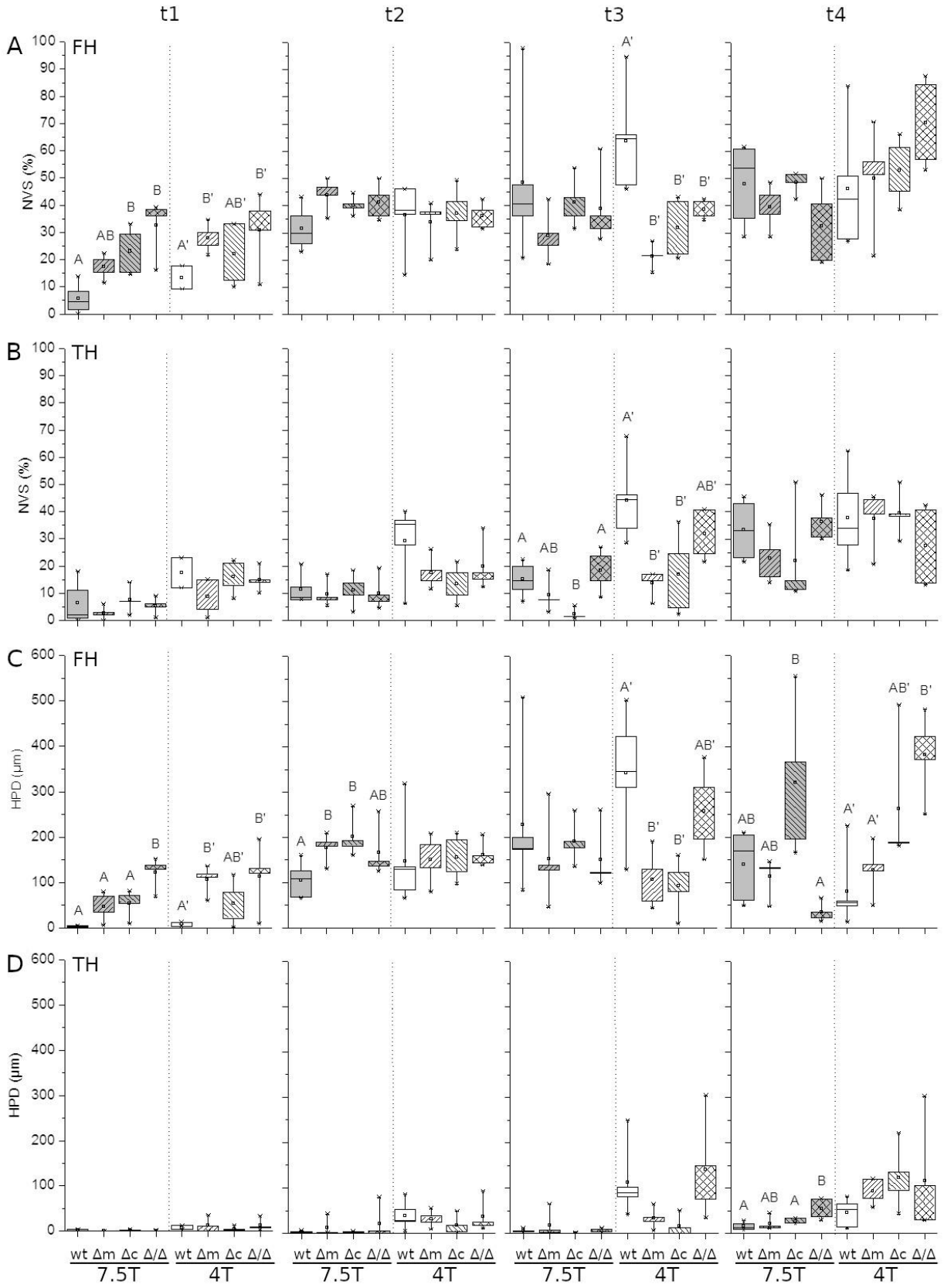
767 Fig.6



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