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Sclerotia formed by citric acid producing strains of *Aspergillus niger*: Induction and morphological analysis

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ABSTRACT

Some strains of *Aspergillus niger* have been previously reported to produce sclerotia under certain conditions. Sclerotia are aggregations of hyphae which can act either as survival or as sexual structures in species related to *A. niger*. In this study, we were able to induce the formation of sclerotia in the progenitor of the industrial citric acid producing strains of *A. niger*, ATCC 1015, and in *pyrG* mutants derived from it. Sclerotia can be stably formed by ATCC 1015 on malt extract agar medium supplemented with raisins, showing a spatial differentiation of the fungus dependent on the addition and on the position of the fruits into the medium. On other media, including malt extract agar, *pyrG* auxotrophs also form abundant sclerotia, while the complementation of this gene reverses this phenotype. Additionally, a macro- and microscopical analysis of the sclerotia is reported. Our results show that the sclerotia formed by *A. niger* are similar to those formed by other fungi, not only in their morphology but also in their ability to germinate and regenerate the organism.

1. Introduction

Sclerotia are described as resting structures that remain quiescent in the presence of adverse environmental conditions and are able to germinate when the conditions improve (Willetts and Bullock, 1992). They are formed by a wide range of fungal species (Smith et al., 2015), including some important plant pathogens like *Botrytis cinerea, Claviceps purpurea* and *Sclerotinia sclerotiorum*, and their initiation requires the aggregation of hyphae to form a multihyphal structure (Willetts and Bullock, 1992). Different members of the genus *Aspergillus*, including *Aspergillus sclerotioniger* (Varga et al., 2011) and *Aspergillus brasiliensis* (Frisvad et al., 2014) of section *Nigri* and *Aspergillus caelatus* of section *Flavi* (McAlpin, 2004), were already shown to produce sclerotia in particular conditions. Previous reports (McAlpin and Wicklow, 2005; Rai et al., 1967) showed that sclerotia production is dependent on different factors, such as the considered strain and the environmental conditions at which it is cultivated. However, no clear conclusions are applicable to all fungal species, since different species respond differently to the same environmental factors (Willetts and Bullock, 1992). Rai and colleagues observed the influence of various environmental factors, such as pH, temperature, light and oxygen level on the formation of sclerotia in different members of the genus *Aspergillus* (Rai et al., 1967). Also medium composition, and in particular the source and concentration of carbon and nitrogen, has a great influence on sclerotia, with some amino acids inducing or reducing the number of formed sclerotia (Agnihotri, 1968). In addition to their role as survival structures, sclerotia are also known to bear sexual structures for certain fungal species, including some *Aspergillus* species (Dyer and O'Gorman, 2011). Some examples are aspergilli belonging to the section *Flavi,* such as *Aspergillus flavus* (Horn et al., 2009a) and *Aspergillus parasiticus* (Horn et al., 2009b), and *Nigri*, such as *Aspergillus tubingensis* (Horn et al., 2013) and *Aspergillus japonicus* (Rajendran and Muthappa, 1980). Under the right, but still species and subgenus specific, environmental conditions, and in the presence of a proper mating partner, the

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sclerotia can mature and fertile ascospores derived by meiosis can be found inside them (Dyer and O'Gorman, 2012). Geiser suggested that, due to their morphology and phylogenetics, sclerotia should be considered ascomatal primordia (Geiser, 2009). However, it was more recently proposed (Dyer and O'Gorman, 2012) that the formation of mature sexual structures and of accessory tissues, such as sclerotia, might follow two independent developmental programs. A sexual cycle has not been discovered for all fungal species so far because, on commonly used synthetic media, fungi like *Aspergillus niger* only propagate asexually. Understanding the formation and the biology of sclerotia could be a first step towards the discovery of a sexual cycle in so far asexual fungi (Ellena, 2020).

The aim of this study is the investigation of conditions which favor the formation of sclerotia in the ancestor of citric acid producing *A. niger* strains, ATCC 1015, and their analysis with macro- and microscopical methods. Some UV mutagenized strains (Jørgensen et al, 2011, 2020) and some natural *A. niger* isolates (Frisvad et al., 2014) were already reported to form sclerotia. The latter being able to form these structures when cultivated on media supplemented with organic substrates, such as raisins or other fruits (Frisvad et al., 2014). However, the *A. niger* strain ATCC 1015, which is a progenitor of the industrial strains used for citric acid production and a genomic reference strain (Andersen et al., 2011), was so far not shown to produce sclerotia. Considered the important role of sclerotia in the propagation of the organism and in the sexual development of species closely related to *A. niger*, inducing the formation of these structures also in industrially important strains like *A. niger* ATCC 1015 would be of high interest.

In this study, we show the ability of the strain ATCC 1015 and of independent *pyrG* mutants derived from it to form sclerotia on different media compositions. Moreover, a detailed macro- and microscopic analysis of these structures is reported.

2. Materials and methods

2.1. Strains and culture conditions

A. niger strain ATCC 1015 was used as a parental strain. *PyrG* mutant strains (pyrG^{m}) derived from ATCC 1015 by a CRISPR/Cas9 strategy were previously described (Sarkari et al., 2017). The strain ATCC 1015 was maintained on minimal medium (MM) (Arentshorst et al., 2012), while pyrG^{m} was maintained on minimal medium supplemented with 10 mM uridine. Sclerotia formation was induced on malt extract agar (MEA), oatmeal (Hafer Getreidebrei, dm-drogerie markt GmbH, Karlsruhe, Germany) agar (OA) and mixed cereal (Bio7Korn Flocken, Milupa GmbH, Puch bei Hallein, Austria) agar (MCA). Malt extract agar was prepared with 30 g/L malt extract (AppliChem, Darmstadt, Germany) and 5 g/L peptone from casein (Merck KGaA, Darmstadt, Germany). Before autoclaving, 40 g/L oatmeal or 125 g/L mixed cereals were boiled for 45 min in deionized water (dH_2O). Agar was added at a final concentration of 15 g/L. These complete media were not supplemented with uridine, as they supported growth of all the strains. Malt extract agar was supplemented, when required, with either three whole raisins or macerated raisins (Sultanas, Reformhaus, Zarrentin, Germany), previously autoclaved for 20 min at 120 °C. A total amount of 10^3 conidia was four point inoculated on the tested media (4×10^3 total conidia on each plate). The plates were sealed with two layers of Parafilm and incubated at 30 °C in the dark.

2.2. pyrG *complementation*

The native *pyrG* gene was amplified from the gDNA of strain ATCC 1015 with Q5® High-Fidelity DNA Polymerase (New England Biolabs), according to the manufacturer's instructions and with primers pyrG_1 kb_fwd: AATACCGCCTAGTCATAGCA and pyrG_1 kb_rev: TCTTCTCATCCGCCATGTTA. This yielded a PCR product of 3217 bp containing the complete *pyrG* gene sequence and around 1000 bp sequence upstream and downstream of the gene, for homologous recombination. 1 and 4 μg of DNA were used to transform the protoplasts of the strain pyrG^{m}, according to the protocol of Arentshorst and colleagues (Arentshorst et al., 2012). Transformed protoplasts were plated on selective medium, without the addition of uridine. 5 independent clones were purified twice on minimal medium to confirm the successful complementation. Their growth and phenotype were observed on minimal medium, mixed cereal agar, oatmeal agar and malt extract agar and compared to the ones of the strains ATCC 1015 and pyrG^{m}.

2.3. Light and transmission electron microscopy of sclerotia

Sclerotia were hand sectioned in half with a scalpel and fixed overnight at 4 °C in 0.1 M cacodylate buffer pH 7.4 with 2.5% glutaraldehyde and 2% paraformaldehyde. Following primary fixation, the samples were washed three times with dH_2O and stained for 1 h on ice with 0.2% ruthenium red and 2% osmium tetroxide. After thorough washes with dH_2O, the samples were incubated in 1% thiocarbohydrazide (1h, RT), followed by a second incubation in 2% osmium tetroxide for 1 h at RT. The samples were washed multiple times with dH_2O and incubated overnight at 4 °C in 0.5% uranyl acetate, washed in dH_2O, incubated in Waltron's lead aspartate (30 min, 60 °C) and washed again with dH_2O. The samples were then dehydrated by 30 min incubations in cold ethanol series (30%, 50%, 70%, 90%, 100% and 100% ethanol) followed by two steps of pure ice-cold acetone. Finally, tissue pieces were progressively infiltrated in low viscosity resin (Agar low viscosity resin kit, Agar Scientific, Stansted, United Kingdom), embedded and polymerized at 60 °C. The samples were sectioned with a ultramicrotome (Leica Ultracut UCT) equipped with a diamond knife (diatome 45°). 1 μm sections of the samples were obtained for light microscopy and observed under the light microscope, either unstained or after staining with toluidine blue. For transmission electron microscopy 90 nm sections were obtained from the same resin blocks and mounted on a grid.

2.4. Sclerotia germination

Sclerotia formed by strain pyrG^{m} were picked with forceps from a malt extract agar plate and washed in 70% ethanol (v/v) by vortexing. After 1 min in ethanol, sclerotia were moved and vortexed in sterile 0.1% Tween 20 (v/v) for two times. Finally, they were placed on a fresh minimal medium plate, supplemented with uridine. A control experiment was performed by washing the sclerotia either with ethanol or with water and by plating then the residual conidia remained from the washes, confirming that ethanol efficiently kills the conidia and that the observed sclerotium germination was not due to the presence of residual conidia on the surface of the sclerotium. The plates containing the sclerotia were incubated at 30 °C. The germination of the sclerotia was monitored over a period of 4 days under the stereoscope.

3. Results and discussion

3.1. Induction of sclerotia formation in the citric acid producing strain ATCC 1015

Several natural isolates of *A. niger* were previously shown to form sclerotia on Czapek yeast autolysate agar medium supplemented with organic substrates, such as raisins or other plant parts (Frisvad et al., 2014). However, the reference strain for organic acid

production, ATCC 1015, did not show sclerotia formation on Czapek yeast autolysate supplemented with raisins (Frisvad et al., 2014). To find suitable cultivation conditions which induce the formation of sclerotia, the *A. niger* wild-type (WT) strain ATCC 1015 was cultivated on malt extract agar plates in the presence of raisins (Fig. 1). Abundant and dark brown conidia were formed on malt extract agar (Fig. 1a), close to the position of the raisins (Fig. 1b and c) and overall the plate in the presence of macerated raisins (Fig. 1d). Sclerotia were formed 7 days after inoculation when the raisins were placed, either whole or macerated, on one side of the plate (Fig. 1b and c) but not in the absence of the raisins (Fig. 1a) or when the raisins were distributed homogenously over the plate (Fig. 1d). Moreover, the formed sclerotia appeared in the area of the plate further away from the position of the raisins, while asexual conidiation was more abundant in close proximity to the raisins. Frisvad and colleagues observed a comparable effect when they cultivated another *A. niger* isolate in the presence of whole raisins, but they did not observe sclerotia formation when the raisins were macerated (Frisvad et al., 2014). Here we report that the formation of sclerotia is not dependent on the integrity of the raisins, but on their spatial distribution on the plate. Fungal mycelium is a highly differentiated structure, with differences in gene expression and, therefore, also phenotypes vary spatially depending on the considered zone of the colony (Vinck et al., 2005). It was previously shown that the heterogeneity of the medium accounts at least partly for this spatial differentiation (Levin et al., 2007). The presence of the raisins creates a chemical gradient in the plate. These differences in the medium composition determine the visible spatial differentiation of the colonies. Raisins contain various proteins, vitamins and minerals but around 60% of their weight consists of sugar, mainly glucose and fructose (Alnuwaiser, 2017; Ghrairi et al., 2013). In the edible mushroom *Morchella esculenta* sclerotia formation could be induced on a sugar heterogeneous medium obtained with the use of the split-plate (Amir et al., 1992). It is therefore possible that in *A. niger*, similarly to what observed in *M. esculenta*, the heterogeneous localization of sugar in the plate (due to the presence of the raisins) induces sclerotia formation and determines the extent of it. In order to check this, malt extract agar plates containing 30% or 60% of glucose, fructose or a mixture of the two sugars, either added to one side of the plate or homogenously distributed, were inoculated (Figure S1, Supplementary S1). Sclerotia formation could be induced in the presence of sugar, especially at higher concentrations (60%), indicating that sugar might play a role in the induction of these structures. However, the results were scarcely reproducible showing a variety of phenotypes and differences in sclerotia formation on different technical replicates, differently from the plates supplemented with raisins that always supported sclerotia formation. These findings suggest that the combined action of sugar and other compounds present in the raisins is responsible of sclerotia formation, more than the presence of a single component.

Sclerotia can act as sexual structures in some aspergilli, including some belonging to the same section *Nigri* of *A. niger* (Horn et al., 2013; Rajendran and Muthappa, 1980). In general, the induction of sexual structures and, ultimately, of a sexual cycle in filamentous fungi often requires specific conditions, which mimic the natural environment of the fungus and are normally not found in a laboratory setting (Houbraken and Dyer, 2015). Therefore, the results obtained in this study suggest that the presence of raisins induces the formation of pre-mature sexual structures in *A. niger* by mimicking the natural environment of this species: decaying vegetation and various organic substrates (Bennett, 2009).

The formation of sclerotia in the section *Nigri* of the genus *Aspergillus* was previously linked to the production of secondary metabolites (Frisvad et al., 2014). Therefore, the possibility to induce sclerotia formation in the strain ATCC 1015 might open new strategies for the induction of novel secondary metabolites from previously silent gene clusters.

3.2. *Inactivation of* pyrG *induces sclerotia formation in the strain ATCC 1015*

In order to induce higher amounts of sclerotia, additional media compositions were tested. In addition to the WT, three *pyrG* mutants, independently obtained with CRISPR/Cas9 in the WT strain, were tested and compared to the WT strain from which they were derived. *PyrG* mutants were included in this analysis because they are often used as selection marker strains and can, therefore, provide an advantage for genetic engineering, especially in the context of industrially relevant strains. Results are shown for one of this *pyrG* mutant strains: pyrG^{m} (Fig. 2), although the same results have been obtained for two other biological replicates and for a *pyrG* mutant obtained by gene disruption through the integration of a resistance cassette. Moreover, the same phenotype observed in our *pyrG* mutants on malt extract agar medium was also observed for the *pyrG* mutant AB4.1 strain obtained in the N402 background (van Hartingsveldt et al., 1987) (data not shown). The tested media were minimal medium (MM), mixed cereal agar (MCA), oatmeal agar (OA) and malt extract agar (MEA). On minimal medium supplemented with uridine pyrG^{m} showed a phenotype comparable to the WT: both strains were characterized by hyphal growth and abundant formation of black asexual conidia.

On the tested complex media the strain pyrG^{m} was able to form sclerotia. Few sclerotia (<50) were formed on mixed cereal agar and oatmeal agar. When sclerotia were formed by ATCC 1015 on mixed cereal agar, these were covered by black conidia and, therefore, not

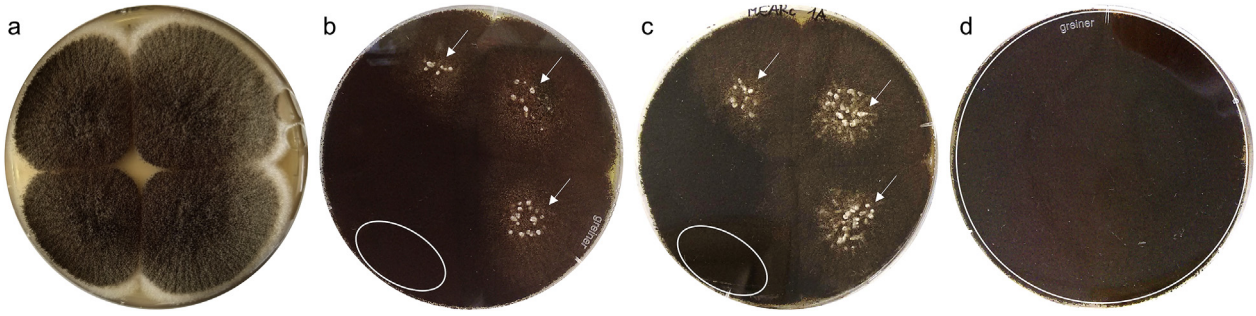

Fig. 1. ATCC 1015 strain growing for 7 days on malt extract agar (a) or malt extract agar supplemented with either three whole pieces of raisins (b) or macerated raisins, either on one side of the plate (c) or homogenously distributed on the plate (d). The position of the raisins is indicated by white ellipses. White arrows point to the areas where sclerotia were formed. Representative pictures of one replicate, other two replicates showed the same phenotype.

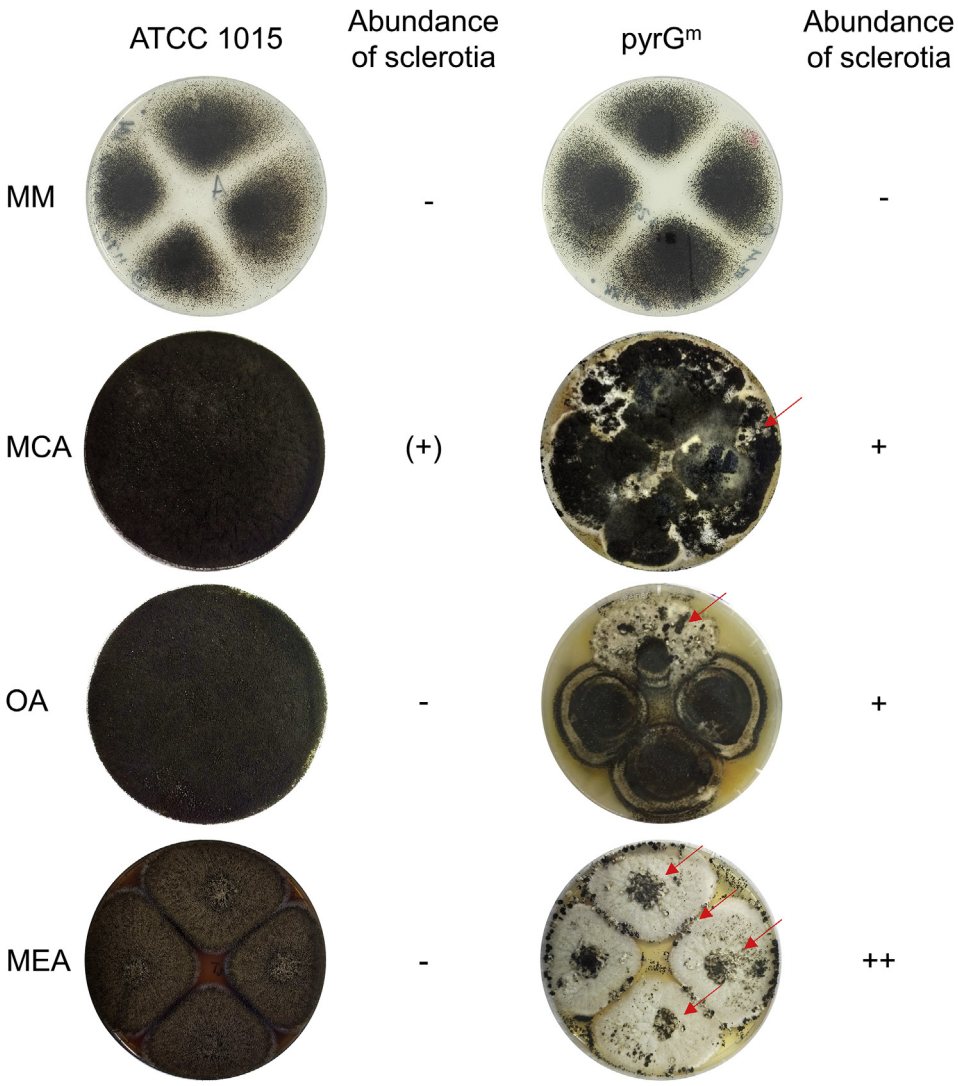

Fig. 2. Representative pictures, taken after 30 days of incubation, of one out of at least three replicates showing the phenotypic differences between the WT strain ATCC 1015 and a *pyrG* mutant (pyrG^{m}) obtained from it. Growth and phenotypes of these strains were monitored on different media: minimal medium (MM), mixed cereal agar (MCA), oatmeal agar (OA) and malt extract agar (MEA). Only the minimal medium plates for pyrG^{m} were supplemented with uridine. While on minimal medium none of the two strains produced sclerotia, on mixed cereal agar, oatmeal agar and malt extract agar the formation of sclerotia could be observed in the *pyrG* mutant strain (sclerotia are indicated with the red arrows), while the WT was characterized by the usual strong black conidiation. -: no sclerotia; +: <50 sclerotia per 8,5 cm Petri dish; ++: >50 sclerotia per 8,5 cm Petri dish (+): sclerotia formed in ¼ of the replicates. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

visible in the picture. Formation of sclerotia by the WT strain was not observed on oatmeal agar. It occurred, however, occasionally, on mixed cereal agar, in a minority of the inoculated plates. The largest amount of sclerotia formed by pyrG^{m} could be observed on malt extract agar where the conidiation level was also reduced, compared to the WT. The conidiation level was lower also on oatmeal agar but just on the colony forming sclerotia, suggesting a trade-off between the asexual conidiation and the formation of sclerotia. In order to establish whether sclerotia production could be further induced in pyrG^{m}, this strain was inoculated on MEA supplemented with raisins (Fig. 3).

In the presence of raisins, pyrG^{m} formed sclerotia only in the center of the colony (Fig. 3b, c and d) and not at the intersection between the colonies as on malt extract agar without raisins (Fig. 3a). The presence of the raisins induced conidiation either in the area closer to the raisins (Fig. 3b and c) or all over the plate where raisins were crushed and homogeneously distributed (Fig. 3d). In the presence of raisins abundant conidiation could also be observed at the intersection of the colonies. The conidiation pattern observed in the presence of raisins suggests again a gradient effect induced by the addition of these fruits, which influences fungal development. However, the addition of raisins did not further induce the sclerotia forming phenotype of strain pyrG^{m}.

In a previous study in *A. niger* two mutants generated by UV radiation and derived from a *fwnA* mutant of the N402 strain gained the ability to form sclerotia while being also strongly affected in their asexual conidiation (Jørgensen et al., 2011). A characterization of one of these mutants was recently performed, leading to the identification of the transcription factor *sclB* (An08g07710), which acts both as positive regulator of asexual growth and conidiation and as negative regulator of sclerotial development in *A. niger* (Jørgensen et al., 2020). Besides being survival structures, sclerotia can act as ascospores containing fruiting bodies in some species. Moreover, the asexual and the sexual development are reported to inhibit each other in the aspergilli (Dyer and O'Gorman, 2012). Considered from this point of view, the formation of sclerotia

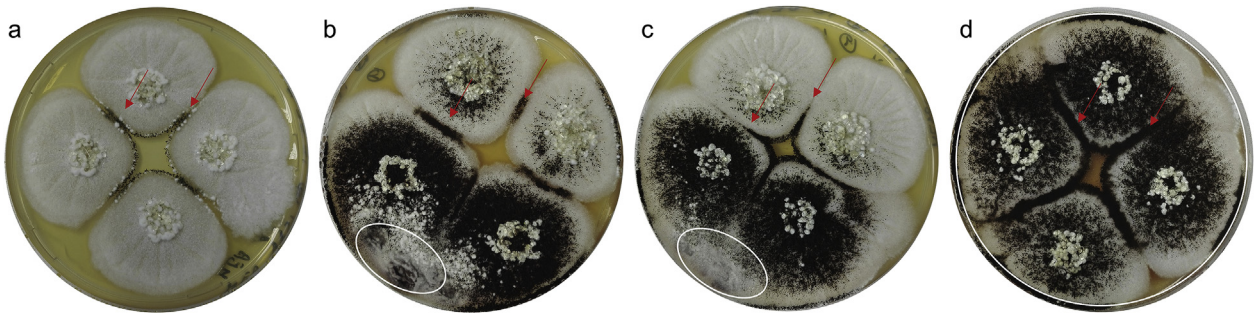

Fig. 3. pyrG^{m} strain growing for 7 days on malt extract agar (a) or malt extract agar supplemented with either three whole pieces of raisins (b) or macerated raisins, either on one side of the plate (c) or homogenously distributed on the plate (d). The position of the raisins is indicated by white ellipses. Red arrows point to the intersection zones between the colonies where either sclerotia (a) or conidia (b, c, d) are formed. Representative pictures of one replicate, other two replicates showed the same phenotype. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

accompanied by a reduction of the asexual sporulation might indicate a potential for sexual development in a fungus so far believed to be asexual.

Although the *pyrG* gene was not previously associated with this process, it can be observed that its inactivation is related to the formation of sclerotia in an industrially important strain of *A. niger*. Sclerotia in *A. niger*, similarly to closely related strains, are likely to be pre-mature sexual structures, needed by the fungus to undergo sexual development. In a previous study on the model organism *Aspergillus nidulans* (Sun et al., 2013), a correlation between the mutation of the *pyrG* gene and the sexual cycle was drawn. A *pyrG* mutant derived from the *A. nidulans* strain FGSC A773 grown in excess of uridine and uracil was shown to undergo sexual reproduction while having inhibited asexual conidiation, differently from the parental strain grown under the same conditions. It was suggested that an abnormal level of metabolites, such as uracil, might induce a stress in the impaired *pyrG* mutant strain, thus leading to sexual development. Based on these previous findings in *A. nidulans*, the results presented here for *A. niger* ATCC 1015 suggest that the inactivation of the *pyrG* gene leads to a metabolic stress which induces the formation of sclerotia and which acts independently of the presence of raisins in the medium. We tested if other auxotrophies can also promote the formation of sclerotia and inactivated the genes *adeA*, *argB* or *nicB* and tested the growth and development on minimal medium, mixed cereal agar, oatmeal agar and malt extract agar plates. However, no sclerotia formation was observed in these deletion strains (data not shown). This indicates that sclerotia production is promoted by the impairment of *pyrG*. To further confirm that the formation of sclerotia depends on the inactivation of the *pyrG* gene, a complementation experiment was performed. In this experiment, pyrG^{m} was transformed with the native *pyrG* gene of ATCC 1015 flanked by 1 kb sequences up- and downstream. Transformants were selected on medium without uridine and purified two times on minimal medium, to confirm their restored prototrophy. 5 independent biological replicates were then tested for growth and sclerotia formation on minimal medium, mixed cereal agar, oatmeal agar and malt extract agar. In all the replicates the wild-type phenotype was restored and sclerotia formation was not observed (Fig. 4).

Besides *pyrG* and *sclB*, the genetic basis of sclerotia formation in *A. niger* can be studied by selecting additional genes. For instance, the gene *sclR* (AO090011000215) promotes sclerotial development in *Aspergillus oryzae* (Jin et al., 2011). Published transcription data (Andersen et al., 2011) showed that the *A. niger* homologue of this gene (Aspni7|1181156) is expressed in the strain ATCC 1015, making it an interesting target for genetic engineering.

The discovery that the impairment of *pyrG* is related to the formation of sclerotia in *A. niger* has important implications, considering that many gene targeting and selection methods upon transformation are based on this gene (Arentshorst et al., 2015). Therefore, the use of the *pyrG* locus as marker gene and integration locus should be carefully evaluated.

3.3. Macroscopical analysis of sclerotia

The formation and the maturation of the sclerotia formed by the strain pyrG^{m} was followed by taking pictures over time. While sclerotia on malt extract agar were formed 7 days after inoculation, on oatmeal agar and mixed cereal agar they started to form after approximately 14 days. However, also the mycelial growth was slower on these two media as compared to the one on malt extract (data not shown). Due to the highest amount of sclerotia produced on malt extract agar, this medium provided the best conditions to observe the maturation of sclerotia over time. In Fig. 5a and b it is possible to observe that sclerotia were completely white when they were formed and were often associated with liquid droplets, which is a typical feature of these structures. Already after 14 days the sclerotia became darker and the liquid droplets disappeared (Fig. 5a and c), probably due to evaporation, as suggested also by Willetts and Bullock (1992).

Sclerotia appeared as hard, elongated structures of irregular shape, with a length ranging from 1 mm to 5 mm. When sectioned they revealed an inner compact composition of cream-white colour (Fig. 5d). They formed both around the inoculation point and terminal to the mycelial colony. The solid cultures were monitored for up to 5 months, showing that the sclerotia appearance did not change after 30 days. Around 2 months after inoculation, sclerotia detached from the mycelium, suggesting complete maturation.

3.4. Morphological analysis of sclerotia by light and transmission electron microscopy

Semithin sections (1 μm) of mature sclerotia from representative samples were observed under the light microscope (Fig. 6a and b). Images of 90 nm sections of mature sclerotia were taken with a transmission electron microscope (TEM) (Fig. 6c, d, e and f). Fig. 6c is a composition of several pictures taken with the TEM in order to show the overall morphology of the sclerotium.

All samples showed a common morphology, independently of the strain that produced them.

In general, sclerotia are constituted by three different layers, formed by cells with different morphologies and different reaction to the toluidine blue staining, which selectively stains acidic

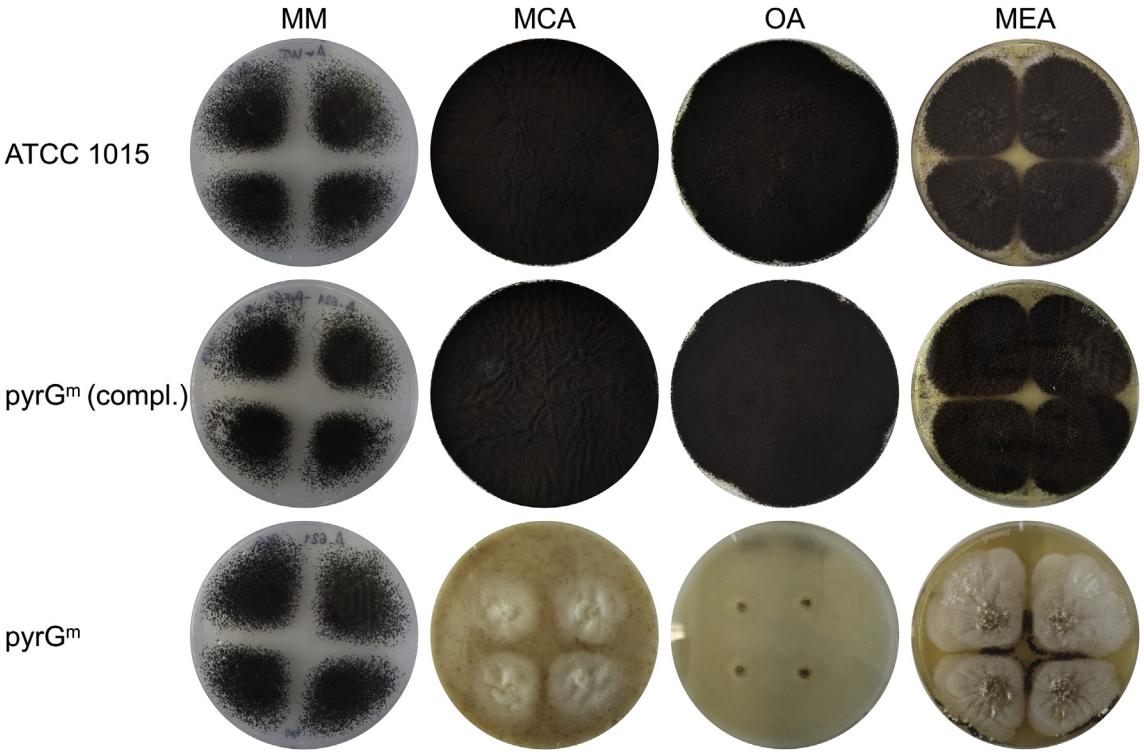

Fig. 4. Phenotypic analysis of the strain complemented in *pyrG* (pyrG^m (compl.)) compared to ATCC 1015 and pyrG^m, grown for 9 days on minimal medium (MM), mixed cereal agar (MCA), oatmeal agar (OA) and malt extract agar (MEA). The strains pyrG^m (compl.) restored the wild-type phenotype and did not show formation of sclerotia, contrary to the parental strain with an inactivated *pyrG* (pyrG^m).

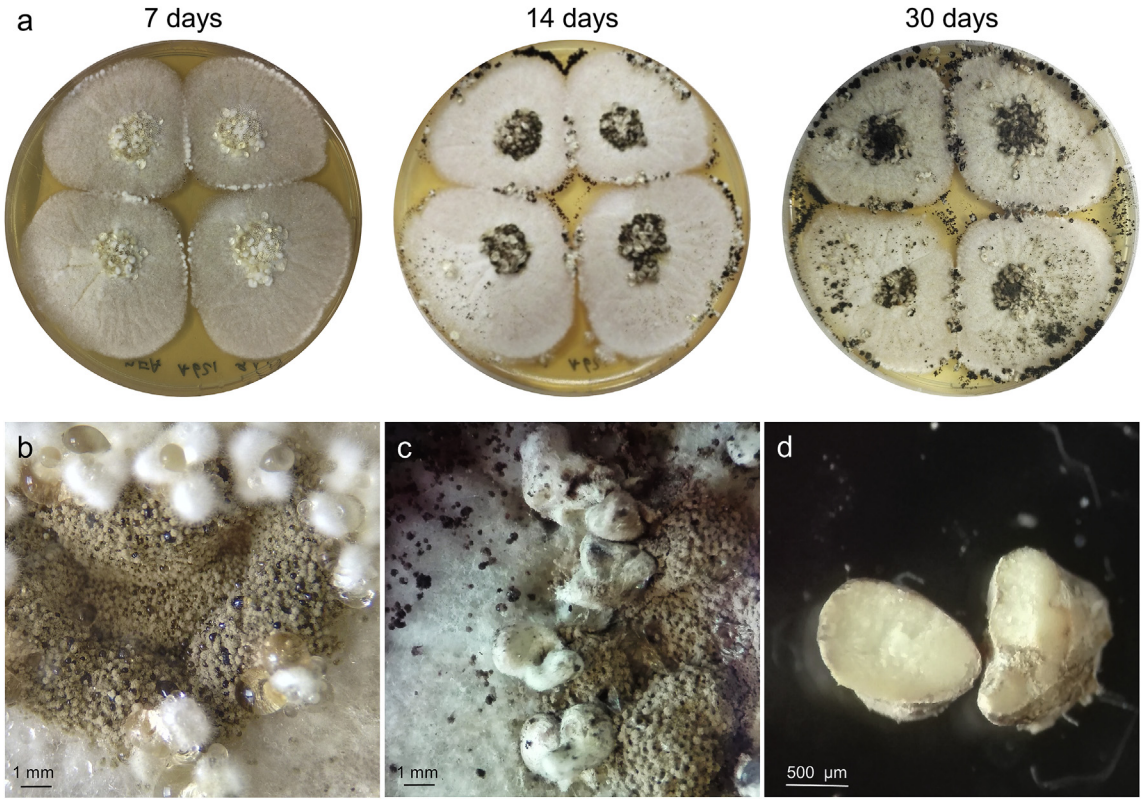

Fig. 5. a: Formation and maturation of sclerotia formed by the strain pyrG^m on malt extract agar medium over time. Pictures were taken 7, 14 and 30 days after inoculation. b–c: Stereoscope images of sclerotia formed by pyrG^m growing for 7 days (b) and 30 days (c) on malt extract agar. d: Hand sectioned sclerotium observed under the stereoscope.

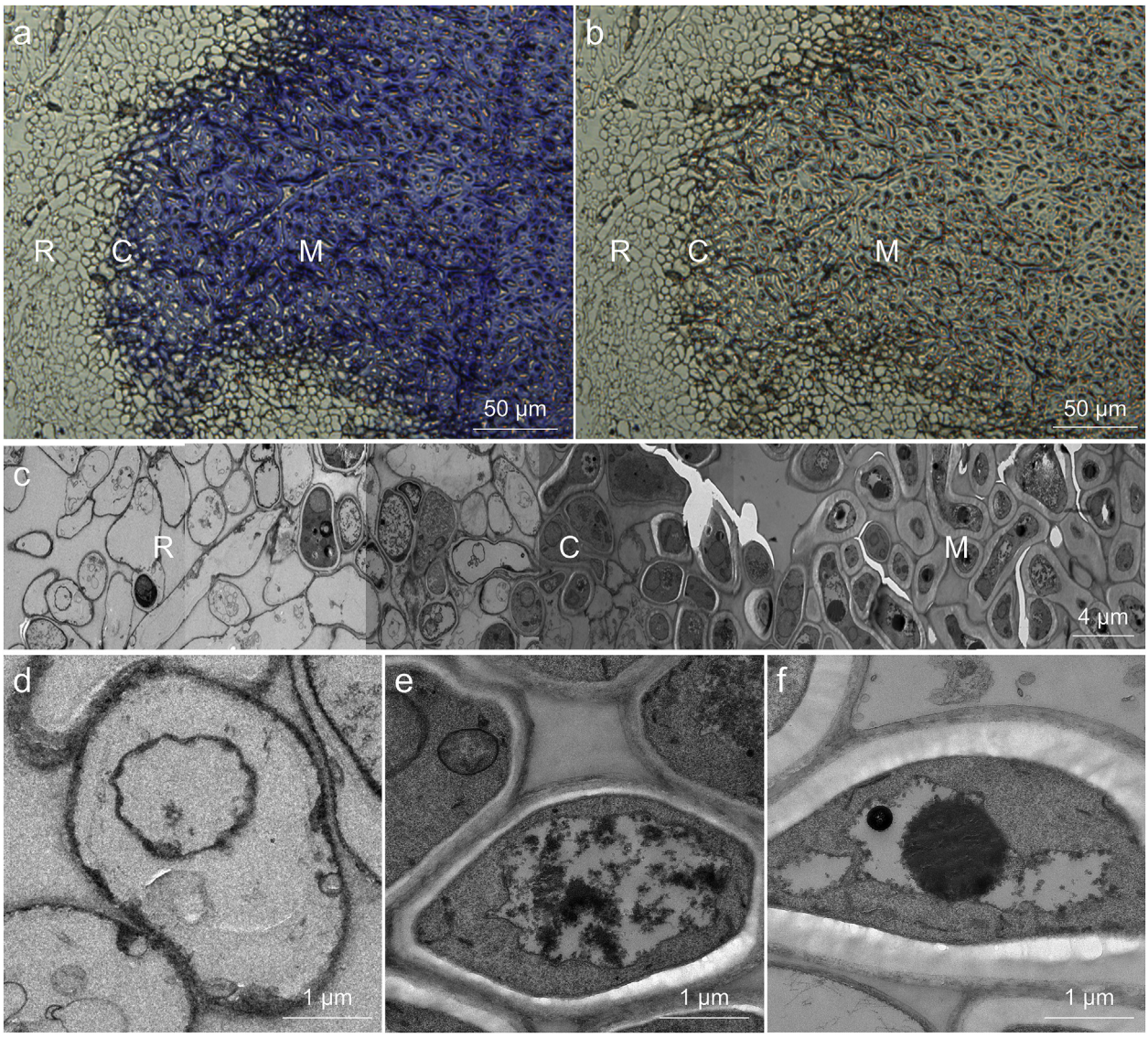

Fig. 6. a, b: Representative light micrographs of a sclerotium of *A. niger* stained with toluidine blue (a) or unstained (b), showing an outer rind (R), a thin cortex (C) beneath it and a large central medulla (M). c: Representative transmission electron micrograph of a sclerotium of *A. niger* showing an outer rind (R), a thin cortex (C) beneath it and a large central medulla (M). d, e, f: Transmission electron micrographs of the three different cell types constituting the sclerotium of *A. niger*: rind cell (d), cortical cell (e) and medulla cell (f). (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

components in blue and polysaccharides in violet. The outer layer, termed rind, is composed by irregularly shaped cells, not stained by toluidine blue, that are empty or in the process of losing organelles (Fig. 6a, c and d). A thin cortical layer, made up of a few rows of smaller and rounder cells can be observed just beneath the rind (Fig. 6a, c and e). The cortical cells are densely stained by toluidine blue, marking the separation between the other two layers. The cortical region appeared pigmented also in the absence of toluidine blue (Fig. 6b). This explains the more intense staining of this region in the presence of the dye. The cell wall of cortical cells is composed by an outer layer of mid electron-dense, fibrillar material and an inner layer of electron transparent, uniform nature. Moreover, large vacuoles containing electron-dense, flocular material were often observed (Fig. 6c and e). The inner and largest region of the sclerotium is constituted by the medulla. The medullar cells showed toluidine blue staining, less intense than in the cortical region and mainly at the cell wall. Moreover, large vacuoles are present inside the cells, mainly containing large inclusions of a material of similar electron-density as the one observed in the vacuoles of the cortical cells (Fig. 6c, e and f). Additionally, the cells constituting the medulla show very thick cell walls, in which the inner electron-transparent cell wall, also observed in the cortical cells, is much larger. The fibrillar layer of these cell walls was described in other fungi, such as *Sclerotinia minor*, as a secreted extracellular matrix (Willetts and Bullock, 1992). Between the medullar cells some interhyphal spaces, termed lacunae, can be observed (Fig. 6c). The inclusions present in the medullar cells might be storage bodies, as indicated in previous works (Bullock et al., 1980; Willetts and Bullock, 1992), needed for survival and subsequent germination. The morphology of *A. niger* sclerotia is comparable to those produced by other fungi, including *S. minor* (Bullock et al., 1980), *S. sclerotiorum* (Insell et al., 1985) and *Sclerotium rolfsii* (Chet et al., 1969; Insell et al., 1985). The presence of empty cells in the outer rind of the sclerotia was previously observed. In a study on *Aspergillus alliaceus*, which undergoes sexual development by forming ascospores inside sclerotia (Fennell and Warcup, 1959), Tewari

showed that autolysis of the cells occurs during the maturation of stromata (Tewari, 1983). This process involves the vesiculation of the membranes and the lysis of the cell walls and it is not synchronized for all the cells. For this reason, microscopy images showed that *A. alliaceus* stromata are composed of empty cells interspersed between cells full of content, similarly to what observed in the rind cells of *A. niger* sclerotia. The author suggested that cell autolysis might be necessary to initiate the sexual cycle, by providing a readily available carbon source for the process. In a previous review, the cortex was described as a region where reserve materials are stored and accumulated (Willetts and Bullock, 1992). However, not all the species form sclerotia with a discernible cortical region, such as in the case of several species belonging to the genus *Aspergillus* (Abu El-Souod et al., 2017). The presence of reserves and storage bodies in the inner cells of the sclerotium is in accordance with the role played by these structures in the dormancy during adverse environmental conditions. The heavy staining of the matrix surrounding the cells in the medulla suggests that this might be composed of carbohydrates, as reported by Willetts and Bullock (1992), although similar experiments performed on sclerotia formed by other species did not show toluidine blue staining of the extracellular matrix. This region of the sclerotium might have a role as energy source during sclerotial germination.

The microscopical analysis performed in this study sets the basis for a better understanding of the structural and morphological characterization of the sclerotia formed by *A. niger*.

3.5. Sclerotia germination

Sclerotia are dormant structures, generally formed in order to persist adverse environmental conditions but able to germinate and form mycelium, conidia and, in some cases, ascospores (Coley-Smith and Cooke, 1971). In this study, the ability of the sclerotia formed by the strain pyrG^{m} to germinate was analyzed.

Sclerotia picked from plates were washed for 1 min in 70% ethanol to kill the conidia present on their outer surface and remove them by subsequent washing with Tween solution. In a control experiment the washing solution was plated and incubated to check for the presence of viable conidia. No growth was observed on these control plates suggesting that all conidia were killed. In contrast, we observed that sclerotia were able to germinate when treated up to 10 min with ethanol while an incubation of 1 h in ethanol prevented subsequent sclerotia germination. This suggests that ethanol can diffuse in the inner part of the sclerotium, killing the cells that constitute it.

The germination of single sclerotia was followed over a period of 4 days by taking pictures through a stereoscope. In Fig. 7 the germination of a sclerotium washed for 1 min in ethanol is shown. 24 h after inoculation no germination was visible. After 48 h the mycelium started to grow out of the sclerotium itself. The formation of conidiophores was visible after 72 h and these structures covered almost the entire sclerotium after 4 days.

Being resting structures able to withstand adverse environmental conditions, the sclerotia have to germinate and regenerate the organism when the conditions improve. Previous studies showed that germination of sclerotia depends on different factors, both related to the structure of the sclerotium itself and to the environmental conditions (Coley-Smith and Cooke, 1971). In particular, the size of the sclerotium and the presence and integrity of the rind might influence its germination. Sclerotia are often formed by plant pathogens and they require a minimum size to be effectively pathogenic and thus germinate. The results presented here show the ability of sclerotia formed by *A. niger* to germinate and regenerate the organism when washed with ethanol and placed on fresh medium, similarly to what previously observed by Jørgensen and colleagues when sclerotia were washed with a solution of 0.9% NaCl (Jørgensen et al., 2020).

4. Conclusions

The presence of raisins in the medium not only induces the formation of sclerotia in the industrial ancestor strain of *A. niger*, ATCC 1015, but also shows that this formation is due to diffusible chemicals, leading to a clear spatial differentiation in the fungal colony. Formation of sclerotia can be also obtained on different complex media (mixed cereal agar, oatmeal agar and malt extract agar) for *pyrG* mutants strains derived in ATCC 1015. This indicates the negative role played by the *pyrG* gene in sclerotial formation, showing a connection between *pyrG* metabolism and fungal development. The macro- and microscopical analysis of sclerotia showed that these structures share some characteristics with sclerotia formed by other fungal species, such as the presence of three different cell types constituting the rind, the cortex and the medulla. The sclerotia of *A. niger* were also shown to be able to germinate when washed with ethanol and plated on fresh medium. These results show the capability of sclerotia to regenerate the organism, according to their role as quiescent structures. The possibility of inducing sclerotia formation in *A. niger* ATCC 1015, which are known to act also as sexual structures in related organisms, might be a step forward towards the discovery of a sexual cycle in this important industrial species. In order to achieve this important goal and given the likely heterothallic nature of *A. niger*, further research should focus on the discovery of the opposite mating-type locus in this species and on the combination of opposite mating-type strains on media shown to induce sclerotia formation.

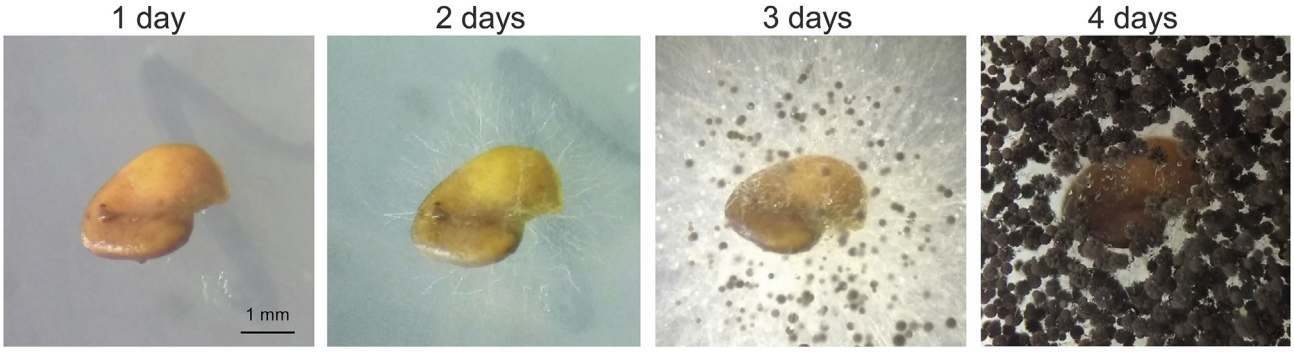

Fig. 7. Germination of a sclerotium of *A. niger* washed with ethanol and followed over a period of 4 days. The same germination pattern was observed by analyzing multiple (>6) sclerotia.

Authors' contributions

MS and MGS conceived the study. VE, MS and MGS designed the experiments. VE, DB and EA performed the experiments. VE prepared the manuscript and all authors contributed to the writing and provided critical feedback. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this published article.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2021.01.008.

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