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Original article

Bioluminescence imaging in *Paracoccidioides* spp.: a tool to monitor the infectious processes

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ABSTRACT

The genus *Paracoccidioides* comprises the species complex causing paracoccidioidomycoses (PCM). These fungi are a serious public health problem due to the long-term drug therapy, follow-up treatment, and frequent sequelae generated by the infection, such as pulmonary fibrosis. In this sense, the objective of this work was to generate bioluminescent reporter strains of *Paracoccidioides* spp. harboring a thermostable, red-shifted luciferase gene under the control of different constitutive promoters. The strains were generated by the integration of a species-specific codon-optimized luciferase gene upon actin or enolase promoter's control. The insertion of the constructs in *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* yeast cells were performed through *Agrobacterium tumefaciens*-mediated transformation. The results demonstrated the presence of several transformants harboring the luciferase gene. These transformants were further confirmed by the expression of luciferase and by the presence of the hygromycin resistance gene. Moreover, the luciferase activity could be detected in *in vitro* bioluminescence assays and *in vivo* models of infection. In general, this work presents the methodology for the construction of bioluminescent strains of *Paracoccidioides* spp., highlighting potential promoters and proposing an *in vivo* model, in which those strains could be used for the systemic study of PCM.

Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by fungi belonging to the *Paracoccidioides* complex. The disease occurs through inhalation of infective propagules (hyphal or conidial forms). The propagules reach the host pulmonary alveoli and transit to multi-budding yeasts, which colonize human lungs and can disseminate to other organs [1–3]. The *Paracoccidioides* genus is composed of five species, as following: *Paracoccidioides brasiliensis*, *Paracoccidioides americana*, *Paracoccidioides restrepiensis*, *Paracoccidioides venezuelensis* and *Paracoccidioides lutzii* [4]. PCM is a neglected disease with a high impact on public health in Latin American countries [5,6]. Approximately 80% of PCM cases are reported in endemic regions in the Southeast and the Middle-West region of Brazil [7,8].

Bioluminescence imaging (BLI) is a non-invasive technique. It has been used for monitoring infection progression in living organisms, usually employing a luciferase reporter; the major advantages are high sensitivity and low background noise [9,10]. This tool has been applied to several opportunistic human fungal pathogens including *Candida albicans* [11,12], *Candida glabrata* [13], *Aspergillus fumigatus* [14–16], *Aspergillus terreus* [17], and *Cryptococcus neoformans* [18]. Moreover, BLI can be used to track the effectiveness of new therapeutic strategies and antifungal compounds, as previously reported for *C. albicans* [10,12], and *C. glabrata* [13].

Abbreviations: ATMT, *Agrobacterium tumefaciens*-mediated transformation.

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The *Agrobacterium tumefaciens*-mediated transformation (ATMT) system has been employed for functional genetic studies in *Paracoccidioides* species. The combination of ATMT and antisense RNA technology has been used to elicit the function of genes involved in metabolism and virulence traits of *Paracoccidioides* spp [19,20]. Fluorescent reporter genes, as red-fluorescent protein (MCherry) or the green fluorescent proteins (GFP), have been employed for genetic manipulation of *Paracoccidioides* spp., but no BLI system has been standardized yet. Such BLI tool could enhance our knowledge about how *Paracoccidioides* spp. develop inside living organisms and can be potentially used for the screening of new anti-paracoccidioidomycosis compounds, which are much needed [21,22]. Here we describe the generation of bioluminescent *Paracoccidioides* spp. strains. Although, the genetic toolbox of *Paracoccidioides* spp. is constantly expanding, the genus is still recalcitrant for large-scale genetic modification approaches. Thus, firstly, different promoters were screened in the model-species *Aspergillus niger*, and, subsequently, the most promising constructs were explored in *P. brasiliensis* and *P. lutzii*, for *in vivo* infection tracking through BLI.

1. Material and methods

1.1. Culture experimental conditions for Aspergillus and Paracoccidioides strains

The *A. niger* P2 Δ*pyrG*4.3 and A1144 Δ*pyrG* strains were generated through a *pyrG* deletion cassette, as previously described [23]. The strains were cultured at 28 °C in modified *Aspergillus* minimal medium (AMM) [24] supplemented with uridine (10 mM) and solidified by the addition of 2% (w/v) agar when required. For experimental procedures, *A. niger* conidia were harvested from AMM agar slants in phosphate-buffered saline (PBS) with 0.1% (v/v) Tween 20, filtered through 40 μm cell strainers, washed twice with PBS and counted using a haemocytometer. For DNA extraction and enzymatic activity determination, *A. niger* was cultivated for 16–20 hours (h) in liquid Yeast Extract Peptone Dextrose (YPD) medium. Yeast cells of *P. lutzii* (ATCC MYA-826) and *P. brasiliensis* (ATCC 32069) were maintained in Brain Heart Infusion (BHI) solid medium containing 3.7% (w/v) agar supplemented with glucose 1.1% (w/v) at 36 °C for 4 days. For all experiments, yeast cells were grown in liquid BHI for 72 h at 36 °C with an agitation of 150 rpm.

1.2. Cloning of Paracoccidioides spp. codon-optimized, red-shifted luciferase

All oligonucleotides utilized in this work are described in the Supplementary Table 1. A red-shifted luciferase was codon-optimized based on the codon usage of 50 highly expressed *P. brasiliensis* genes. The resulting gene (*PbLUC$_{OPT_red}$*; Genbank accession number MT978127) was synthesised by Genscript.

For all cloning steps, PCR products were amplified with Phusion Hot Start II polymerase (Thermo Scientific). Linearized plasmid and PCR products were purified by gel elution using Monarch® DNA gel extraction kit (NEB#T1020; New England Biolabs Inc). The *in vitro* assemblies were performed by using the InFusion HD cloning kit (Takara Bio Inc). The assembled plasmids were used to transform Stellar *Escherichia coli* cells (Takara Bio Inc) or 10-beta competent cells (New England Biolabs Inc) for constructs larger than 10 kb. The correctly assembled plasmids were identified by colony PCR using Phire Hot Start II DNA polymerase (Thermo Fisher Scientific) and plasmids were purified from overnight cultures, grown in LB medium, using the NucleoSpin Plasmid Miniprep kit (Macherey–Nagel).

To confirm the functionality of *PbLUC$_{OPT_red}$*, this gene was cloned in the *NcoI* site of the plasmid SM-Xpress_URA [23], to generate the plasmid *PbLUC$_{OPT_red}$*_SM-Xpress_URA. This vector contains an URA Blaster cassette (under regulation of a *gpdA* promoter from *A. nidulans*) for complementation of *pyrG* negative *Aspergillus* strains and expressed the luciferase under control of the *A. terreus terA* promoter [24]. In a second step, we selected two putative promoter regions from *P. brasiliensis* to compare their relative strength in driving gene expression of the *PbLUC$_{OPT_red}$* in *A. niger*. Approximately 1000 bp sequences (p*ACT1* and p*ENO1*), spanning the putative promoter region of the actin gene (*ACT1;* locus tag PAAG_03031) and enolase gene (*ENO1*; locus tag PAAG_11169), were amplified from genomic DNA (gDNA) of *P. brasiliensis* and cloned upstream of *PbLUC$_{OPT_red}$*. A 342 base pairs (bp) sequence (t*ENO1*), spanning the putative terminator region of *ENO1*, was used as a common putative terminator for both constructions. All fragments were assembled in the *PstI* site of the URA Blaster_pUC19 plasmid (PMID: 30527997), to generate the plasmids p*ACT1*:*PbLUC$_{OPT_red}$*:t*ENO1*_URABlaster_pUC19 and p*ENO1*:*PbLUC$_{OPT_red}$*:t*ENO1*_URABlaster_pUC19. *A. niger* strains were transformed with the generated plasmids employing the PEG-mediated protoplast transformation method described previously [25]. The transformants were selected based on uridine prototrophy. In addition, 0.4 mM of Beetle luciferin (D-luciferin) (Promega) was supplemented in the top agar of the transformation plates.

To generate the plasmids for *Paracoccidioides* spp. transformation, the expression cassettes p*ACT1*:*PbLUC$_{OPT_red}$*:t*ENO1* and p*ENO1*:*PbLUC$_{OPT_red}$*:t*ENO1* were PCR amplified. The PCR products were assembled in the *KpnI* site of the binary vector puR5750, to generate the plasmids p*ACT1*:*PbLUC$_{OPT_red}$*:t*ENO1*_puR5750 and p*ENO1*:*PbLUC$_{OPT_red}$*:t*ENO1*_puR5750. The hygromycin resistance gene (*hph*), under the control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (P*gpdh*) promoter region and the *trpC* (T*trpC*) terminator region, was used as selection marker [26]. The constructed binary vectors were introduced into *A. tumefaciens* LBA1100 ultracompetent cells by electroporation. ATMT was performed as described previously [26].

1.3. Southern blot analysis of A. niger and Paracoccidioides spp. transformants

Southern blot analyses were carried out on genomic DNA (gDNA) from bioluminescent *A. niger* transformants without suspicious morphological defects such as crippled growth or lack of conidiation to select for strains containing a single copy integration of the respective luciferase construct (Fig. 1). For gDNA extraction, the mycelium from overnight liquid culture of randomly selected *A. niger* luminescent transformants was pressed dry, frozen in liquid nitrogen, ground to a fine powder and extracted as described previously [27]. The gDNA was digested with *Pst*I for strains harbouring *PbLUC$_{OPT_red}$*_SM-Xpress_URA, with *Eco*RI for strains harbouring p*ACT1*:*PbLUC$_{OPT_red}$*:t*ENO1*_URABlaster_pUC19, and with *Sma*I for strains harbouring p*ENO1*:*PbLUC$_{OPT_red}$*:t*ENO1*_URA-Blaster_pUC19. The *PbLUC$_{OPT_red}$* gene was used as the probe. T-DNA integrations into *Paracoccidioides* spp. transformants were confirmed by Southern blot analysis using the hygromycin resistance gene (MH920367.1) as the probe. The gDNA of *P. lutzii* and *P. brasiliensis* was digested with *Kpn*I (Fig. 1).

For all transformants, the digested DNA was electrophoresed in 0.8% agarose gels and transferred to a nylon membrane Hybond N+ (GE Healthcare). The probes were synthetized using the PCR DIG Probe Synthesis Kit (Sigma Aldrich, Germany). Hybridizations were performed using DIG Wash and Block Buffer Set (Sigma Aldrich, Germany) at 50 °C in Hybaid Micro-4 Hybridization Oven HBMCR4110 and the detection was conducted with anti-

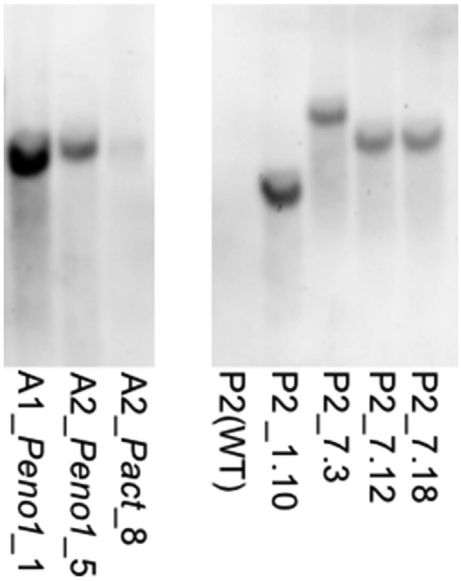

Fig. 1. Southern blot analyses showing the single-copy integration of luciferase constructs. Genomic DNA of selected transformants was restricted with *Sma*I for strains harbouring the luciferase under control of the enolase promoter (A1_P*eno1*_1 and A2_P*eno1*_5), with *Eco*RI for the strain with the luciferase under the actin promoter (A2_P*act*_8), and with *Pst*I for strains with the luciferase under control of the *terA* promoter (P2_1.10, P2_7.3, P2_7.12, P2_7.18). The luciferase negative *A. niger* expression platform strain P2 (wild type) served as control and a digoxygenin-labelled probe was used for hybridization. All selected transformants show a single copy integration of the luciferase into the genome.

Digoxigenin-AP Fab fragments (Sigma Aldrich, Germany) according to the manufacturer's instructions. The hybridization of the probe was detected by chemiluminescence using CDP-Star chemiluminescent substrate (Roche). The reaction was evaluated in a luminescent image analyzer Amersham Imager 600 (GE Healthcare, Uppsala, Sweden).

1.4. *Gene expression screening in* Paracoccidioides *spp. by RT-qPCR*

The total RNA from *P. lutzii* and *P. brasiliensis* was extracted using TRIzol (TRI Reagent, Sigma–Aldrich, USA) and mechanical cell rupture was realized in a bead beater apparatus (BioSpec, Oklahoma, USA). The total RNA was reverse transcribed using the Revertaid M-Mulv Reverse Transcriptase (Applied Biosystems, USA). Quantitative real time PCR (RT-qPCR) were performed using a *Power* SYBR® Green Master Mix (Applied Biosystems™) in a QuantStudio 3 Real-Time PCR System (Applied Biosystems™). As endogenous control the tubulin gene (PAAG_12506: Gene ID: 9093207 and PADG_08413, Gene ID: XP_010763621.1) was employed. A cDNA from sample was diluted serially at 1:5 and used to generate a relative standard curve. The relative expression levels of luciferase were obtained using the standard curve method for relative quantification [28]. Analysis of variance (one-way ANOVA) was applied in the statistical analysis and p-values ≤ 0.0001 were considered statistically significant.

1.5. In vitro *and* in vivo *bioluminescence imaging*

A total of 500 conidia from *A. niger* transformants containing a single integration of the respective luciferase construct were spotted on AMM agar plates containing 0.4 mM of *D*-luciferin and plates were incubated for 2 days at 28 °C. Intensity of bioluminescence emitted by the different clones transformed with *PbLUC*$_{OPT\_red}$ constructs were assessed by acquiring images in a ChemiDoc XRS + system (BioRad). For measuring the luciferase activity in crude cell-free extracts, the conidia were cultivated overnight in 25 mL of YPD medium at 28 °C and 150 rpm. Mycelia were harvested, washed with sterile tap water, pressed dry, ground in the presence of liquid nitrogen into a fine powder and resuspended in Tris–HCl buffer containing glycerol (150 mM NaCl, 50 mM Tris–HCl, 10% (v/v) glycerol, pH 7.5). After two min centrifugation in a microcentrifuge at 14,000×*g* the protein content in the cell-free supernatant was determined by the Bradford micro assay (BioRad). The crude extracts were diluted two-folds in 5 serial two-fold dilutions and 100 μL transferred to wells of white 96-well plates. Subsequently, an equal amount of luciferase assay buffer (60 mM tricine, 15 mM $MgCl_2$, 10 mM DTT, 5 mM ATP, pH 7.8) was added to each well [14]. For *in vivo* luciferase assay, three different conidia concentrations ($2 \times$ x 10^4, $1 \times$ x 10^4 and 0.5 x $\times$ 10^4) were incubated at 28 °C for 16, 20 and 24 h in a white 96-wells plate containing 200 μL of liquid *Aspergillus* complete medium (ACM). The luciferase activity was determined as described previously [14]. In all assays, the light emission was measured after injection of 20 μL of 20 mM of D-luciferin in PBS by Glomax (Promega) with the following settings: heating: 30 °C; substrate injection: 20 μL with 300 μL/s; shaking: 5 s at 300 cycles/min; luminescence integration: 0.1 s; readings: 150 in intervals of 0.3 s.

The bioluminescence imaging of *Paracoccidioides* spp. was carried out using the Living Image 3.1 software implemented in the IVIS Lumina II equipment (PerkinElmer, UK). Yeast cells of *P. lutzii* and *P. brasiliensis* were cultured in liquid BHI for 72 h at 36 °C with an agitation of 150 rpm. Flat-bottom 96-well plates and *Galleria mellonella* larvae were used for *in vitro* and *in vivo* experiments, respectively. The inoculum standardization of *Paracoccidioides* spp. maintenance, and manipulation of *G. mellonella* larvae were performed as described previously [29]. *Paracoccidioides* spp. cells were counted in a hemocytometer and diluted to specified concentrations. D-D-luciferin (PerkinElmer, UK) substrate was prepared according to the manufacturer's instructions. For *in vitro* experiments, a total of 200 μL of PBS 1X per well harboring 10^5 and 10^6 *Paracoccidioides* spp. cells were used. DD-luciferin firefly substrate was prepared at 450 μg/mL and added per well. For *in vivo* assays, *G. mellonella* larvae were injected with 10 μL containing 2×10^6 cells of *Paracoccidioides* spp. and DD-luciferin firefly substrate was inoculated at 150 μg/mL final concentration. For the 0 h time-point, *G. mellonella* larvae were firstly inoculated with *Paracoccidioides* spp. and, subsequently, inoculated with D-luciferin, without further incubation. The IVIS equipment parameters were set to 30 s and 1 min exposure time for both *in vitro* and *in vivo* experiments.

2. Results

2.1. *Expression of* PbLUC$_{OPT_red}$ *in* A. niger

To analyse the functionality of the generated codon-optimised red-shifted luciferase for construction of bioluminescent strains, we first aimed in the expression of the synthetic luciferase gene in the a *pyrG* negative *A. niger* P2 Δ*pyrG*4.3 expression platform strain. This strain contains a genomic copy of the transcriptional regulator *TerR*, which drives gene expression from the *TerA* promoter. Accordingly, the *PbLUC*$_{OPT\_red}$ gene was cloned under the control of *TerA* promoter and the URA-blaster cassette was used as selection marker for transformation (Fig. 2A) [23,30]. Additionally, for selection of bioluminescent transformants selection, D-Luciferin was directly added to the transformation agar. After determination of single copy integration of selected bioluminescent strains by Southern blot analysis (Fig. 1), Five-hundred (500) conidia of the respective *A. niger* from positive single-copy transformants were

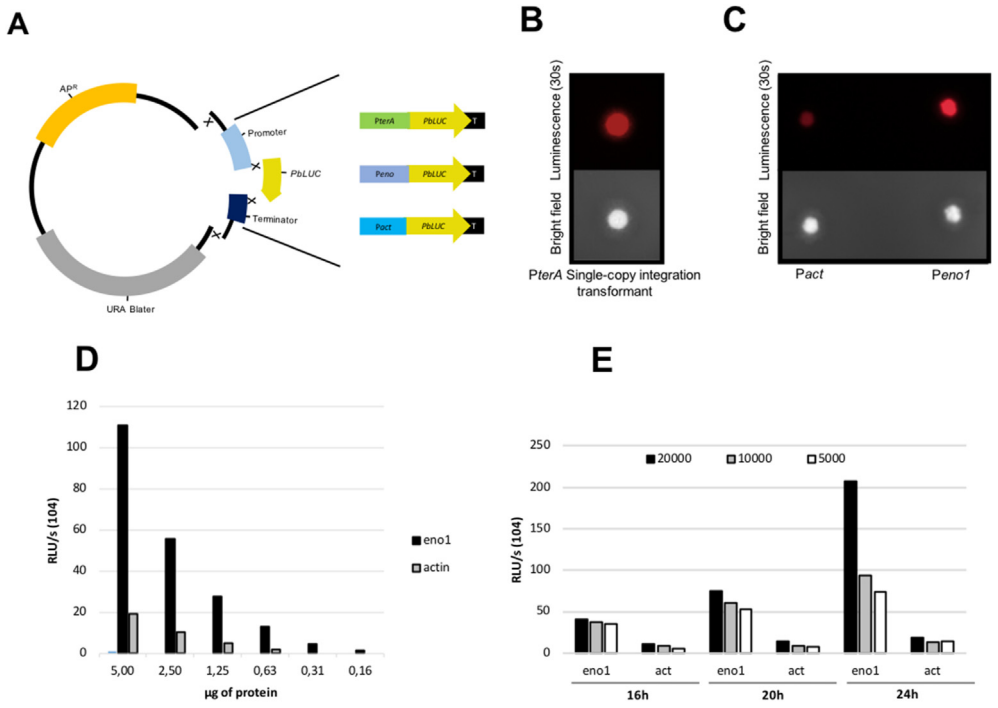

Fig. 2. Construction scheme of *Aspergillus niger* strains containing the luciferase constructs with the actin and enolase promoters (A) Schematic representation of plasmid: The PbLucOPT_red gene was cloned under the control of terA, act and eno1 promoters, using URA-blaster cassette as selection marker. **(B)** Bioluminescent imaging of *Aspergillus niger* colonies containing a single copy integration of the luciferase construct under the control of terA promoter or **(C)** act and eno1 promoters. 500 spores were spotted in GG10 agar plates containing 0.4 mM of D-luciferin and the images acquired after two days of culture. The luciferase assay was performed with cell-free extracts **(D)** and during spore germination **(E)** to confirm the higher level of light emission from the eno1 promoter.

spotted onto D-luciferin-containing AMM agar plates and light emission was recorded after 48 h of growth using a ChemiDoc XRS + system. The results clearly show that the synthetic construct is functional, since positive bioluminescence signals could be detected at least when expressed in *A. niger* (Fig. 2B).

Next, we explored the expression of the luciferase gene in the heterologous host under the control of *P. brasiliensis* constitutive promoters (Fig. 2C). This screening was performed in *A. niger*, since genetic modification is more easily achieved in this species when compared to *Paracoccidioides* spp. The explored putative promoters included the upstream region of the actin (p*ACT*) and enolase (p*ENO1*) genes. Moreover, the putative terminator region of the enolase gene (t*ENO1*) was used in all constructs and the URA-blaster cassette was employed as selection marker of a *pyrG* negative derivative of the *A. niger* wild-type strain A1144. Imaging of cultures on plates implied that the enolase promoter supported a higher level of bioluminescence (Fig. 2C).

To quantify and confirm the higher level of light emission from the *ENO1* promoter constructs, luciferase activity was determined from cell-free extracts [14]. In accordance with colony imaging, light emission was more intense in p*ENO1*:*PbLUC*$_{OPT\_red}$ transformants compared to p*ACT1*:*PbLUC*$_{OPT\_red}$ clones (Fig. 2D). To confirm this result further, we also inoculated between 5000 and 20000 conidia of each strain in wells of a white 96-well plate and determined the light emission after different time points of growth. As expected, the light emission was stronger in p*ENO1* transformants and is correlated to the number of spores inoculated per well (Fig. 2E). These results confirm that the synthetic luciferase construct not only is functional, but can also be expressed under control of promoters from *P. brasiliensis* in the heterologous host *A. niger*. However, at this stage we were not able to exclude if recognition of the individual promoters in the native host differs. Therefore, the actin promoter cannot be excluded as a suitable promoter for the construction of bioluminescent *Paracoccidioides* strains. In this regard, the promoter p*ENO1* and the weakest p*ACT1* promoter were used in subsequent experiments for the construction of *Paracoccidioides* spp. reporter strains.

2.2. Paracoccidioides *spp. bioluminescent strains*

To generate *Paracoccidioides* spp. bioluminescent strains, firstly, the expression cassettes (p*ACT1*:*PbLUC*$_{OPT\_red}$:t*ENO1* and p*ENO1*:*PbLUC*$_{OPT\_red}$:t*ENO1*) were introduced in the shuttle plasmids for *A. tumefaciens*-mediated transformation pUR5750:p*GPDA*:*hph*:t*TRPC*, (i.e., which contains an hygromycin selection marker) resulting in the plasmids pUR5750:p*GPDA*:*hph*:t*TRPC*_: p*ACT1*:*PbLUC*$_{OPT\_red}$:t*ENO1* and pUR5750:p*GPDA*:*hph*:t*TRPC*_: p*ENO1*:*PbLUC*$_{OPT\_red}$:t*ENO1*) (Supplementary Fig. 1 A and B). The obtained plasmids were then introduced in *P. lutzii* and *P. brasiliensis* through ATMT. To explore the expression of the luciferase gene in the isolated strains, RT-qPCR was employed, and at least 4 transformants were inspected (Fig. 3). As expected, in agreement with the ectopic integration of T-DNA in *Paracoccidioides* strains and the accompanied positioning effects, different levels of gene expression were observed (Fig. 3). However, we used Southern blotting to further confirm the integration of the expression cassette in the strains that displayed the higher levels of luciferase expression and confirmed a single copy integration into the genome (Supplementary Fig. 2).

2.3. In vitro *bioluminescence assay*

The bioluminescence of PbLUC OPT_red expressing strains, was analyzed in triplicate assay using cells in 96-well plates (Fig. 4). For this experiment, we used the previous selected *Paracoccidioides* spp. strains which presented higher levels of luciferase expression

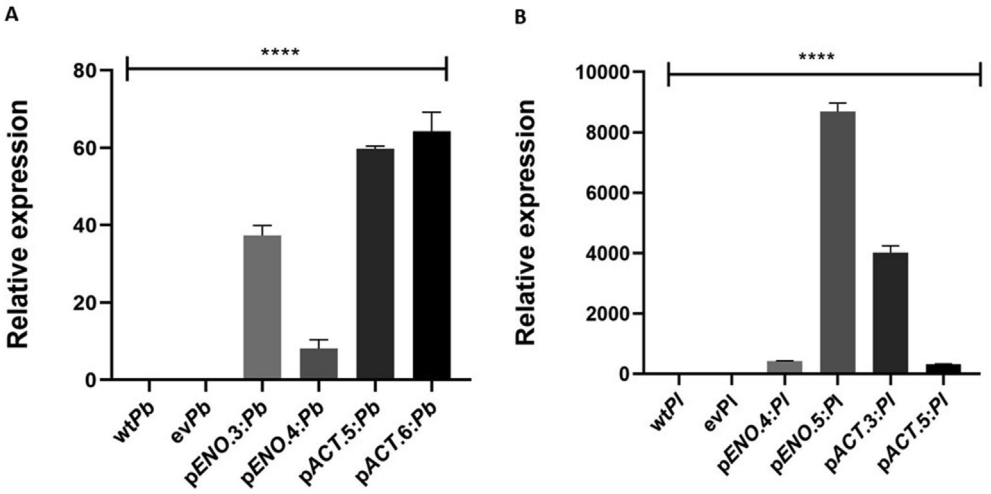

Fig. 3. Detection of transcriptional levels of luciferase in LUCOPT_red *P. lutzii* and *P. brasiliensis* strains. A - Yeast cells of *P. brasiliensis;* B −*P. lutzii*, were grown in BHI liquid medium for 72 h. The cDNA of both species. was synthetized by reverse transcription using OligodT and relative expression of luciferase was obtained by qPCR. The data was normalized using tubulin gene (PAAG_12506: Gene ID: 9093207/and PADG_08413, Gene ID: XP_010763621.1) and were expressed as the mean ± standard deviation of the triplicates of independent experiments. wt: wild type strains; ev: strains harboring the empty vector. *Statistically significant data as determined by one-way ANOVA ($p \leq 0.0001$).

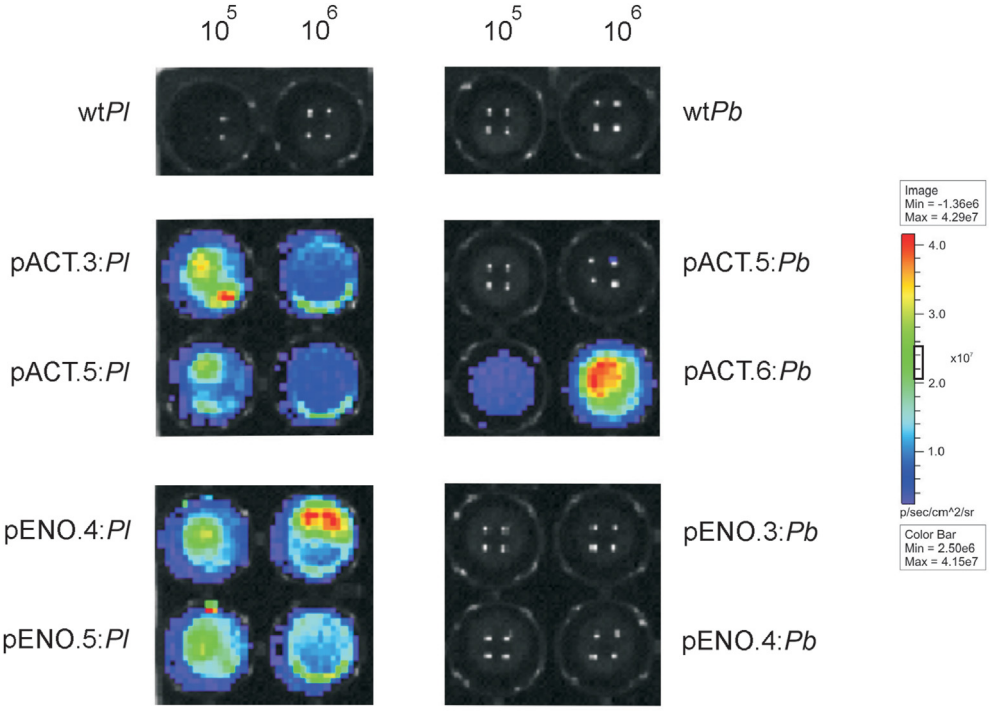

Fig. 4. *In vitro* bioluminescence assays of *P. lutzii* and *P. brasiliensis* producing luciferase. Different yeast cells concentrations (105 and 106) of the wild-type strains of both species and of the transformants containing the luciferase gene under control of the ACT or ENO promoters were distributed in a 96-well plate. The light emission was induced by the addition of D-luciferin to the well. The equipment used was IVIS Lumina II and the images were acquired by Living Image 3.1 software (PerkinElmer, UK).

compared to controls. Four strains of *P. lutzii* harboring actin or enolase promoters showed bioluminescence for both concentrations employed (Fig. 4). However, in *P. brasiliensis*, only one transformant (i. e., harboring the p*ACT1*:*PbLUC*$_{OPT\_red}$:t*ENO1* construct) displayed bioluminescence, whereas the strains expressing the luciferase from the enolase promoter unexpectedly failed to produce bioluminescence. In addition, it is known that in random gene integrations a positioning effect can occur which bases on the integration into a euchromatin or heterochromatin structure. Thus, while he transformants were checked for a single copy integration by Southern blot analysis, it is likely that this difference in BLI is a result from the site of genomic integration.

2.4. In vivo *infection of* G. mellonella *with bioluminescent strains of* Paracoccidioides *spp.*

For the *in vivo* assay with *G. mellonella* larvae, two bioluminescent strains of *P. lutzii*, one harboring the p*ACT1*:*PbLUC*$_{OPT\_red}$:t*ENO1* construct (i. e., named p.*ACT1.3:Pl*) and one harboring the p*ENO1*:*PbLUC*$_{OPT\_red}$:t*ENO1* construct (i. e., named p.*ENO1.5:Pl)*, as

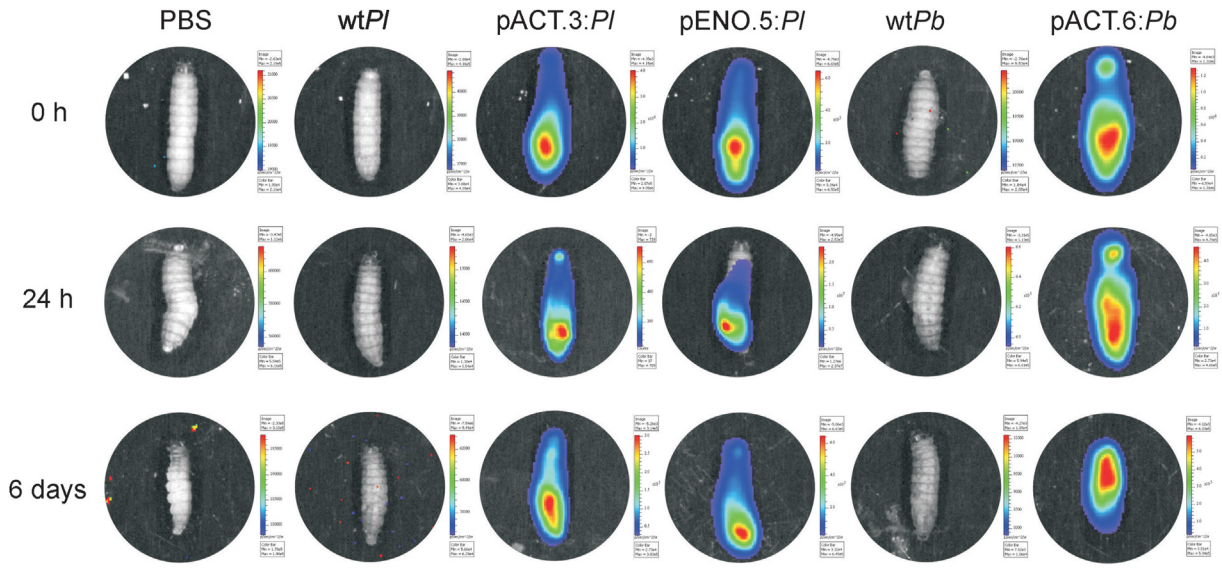

Fig. 5. Infection *in vivo* in *G. mellonella* larvae with bioluminescent strains of *P. brasiliensis* and *P. lutzii*. G. *mellonella* larvae were infected with 2x106 wild type cells of *P. lutzii*, *P. brasiliensis* or of the transformants containing the luciferase gene under control of the ACT or ENO promoters. The light emission was induced by the addition of D-luciferin to the well or inoculated in *G. mellonella*. Photons were collected for 30 s and 1 min, respectively, in a period of 0 h for in vitro and 0 h, 24 h and 6 days for in vivo assay. As a control, G. mellonella larvae were inoculated with PBS. The equipment used was IVIS Lumina II and the images were acquired by Living Image 3.1 software (PerkinElmer, UK).

well as the only bioluminescent transformant of *P. brasiliensis* (i. e., named p.*ACT1*.6:*Pb*) were explored. Moreover, the wild-type strains of *P. lutzii* and *P. brasiliensis* were used as controls, as well as the vehicle (PBS 1x) (Fig. 5). Notably, infected larvae, with all the *Paracoccidioides* spp. strains inspected, displayed bioluminescent signals in at three different time-points explored. Together, these results show that the *ACT* and *ENO* promoters are efficient driving luciferase expression and that the transformation mediated by *A. tumefaciens* was efficient in obtaining bioluminescent strains of *Paracoccidioides* spp.

3. Discussion

In this work, we developed bioluminescent reporter strains of the genus *Paracoccidioides* and explored the usability of these isolates to detect bioluminescence signals *in vitro* and in *vivo.* This strategy has already been applied to the study of different mycoses such as aspergillosis [10], cryptococcosis [18], and candidiasis [13]. However, until now, there has been a lack of similar tools for PCM study. Fluorescent reporters have previously been generated in *Paracoccidioides* strains [22] but fluorescent reporters are mainly used to visualize cellular and subcellular processes rather than allowing the non-invasive real-time imaging of fungal infections in temporal and spatial resolution. By contrast, bioluminescence imaging (BLI) by exploitation of a luciferase is a non-invasive technique and, combined in a multimodal imaging approach with micro-computed tomography (micro-CT) and magnetic resonance imaging (MRI), can provide information on the pathogen load and state of tissue lesions in individual animals over a period of days, weeks or even months [31]. When applied to murine infection models, this not only allows to follow disease progression or treatment efficacy in individual animals, but also reduces the number of animals required to draw conclusions on the significance of the experiments.

Accordingly, studies regarding the development of diseases caused by fungi have been approached using bioluminescence imaging, as described for cryptococcosis and aspergillosis, as examples [16,32]. The system of firefly luciferase bioreporters based on the reaction of luciferase and luciferin, has been an important tool for monitoring in real time diseases caused by microorganisms [33]. In this work we utilized the gene reporter of a red-shifted codon optimised luciferase. The development of this reporter can allow experimental designs in higher eukaryotic organisms, due to the reduced light absorption by hemoglobin, process related in *in vivo* studies with *C. albicans* [34] and *C. neoformans* [18].

An important factor in bioimaging is the amount of the functionally produced reporter protein, which is, at least partially, dependent on the selected promoters. The initial approaches utilizing luciferase as a reporter gene fluorescent in *Saccharomyces cerevisiae* had employed the promoter for alcohol dehydrogenase which resulted in low expression of luciferase and pointed for the relevance of using strong promoters in constructions for bioimaging [35]. In this way, *in C. albicans* using promoters of constitutively expressed genes allowed bioluminescence levels suitable for following vulvovaginal infections when luciferin was applied in the lumen of the affected organ [36] or, when using an optimized luciferase, disseminated infection from intraperitoneal injection of luciferin [11]. ACT1 and ENO1 are constitutively expressed at high levels in members of the *Paracoccidioides* complex [37,38] and employing them in to our experiments allowed give high levels of light emission and sensitive detection of the pathogen in *Galleria mellonela*.

4. Conclusions

In the present work we used a two step-approach to develop bioluminescent strains of *Paracoccidioides* spp. Through heterologous expression in *A. niger*, putative promoter sequences of *Paracoccidioides* spp. were selected, that were posteriorly transformed in yeast cells of the human pathogen. The constructions were functional and the generated bioluminescent signal could be detected *in vitro* and in an insect animal model of infection. Those findings reinforce that the bioluminescence strategy can also be useful to perform studies aiming to investigate new compounds for therapy coupled with infection tracking, further increasing the efficiency of these approaches.

Declaration of competing interest

The authors declare that they have 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.micinf.2022.104975.

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