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Influence of light on lignin-degrading activities of fungal genus *Polyporus* s. str.

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Six strains belonging to five species of *Polyporus* (*P. arcularius, P. arcularioides*, *P. tricholoma, P.* cfr. *tricholoma*, and *P. varius*), collected from an Atlantic Forest area in Misiones (Argentina), where species usually grow exposed to high temperatures and humidity, were identified by morphological and molecular analyses. *P. tricholoma* (BAFC 4536) and *P. arcularioides* (BAFC 4534) were selected by their lignin-degrading enzyme production, their ability to produce primordial of basidiomes under submerged fermentation, and the decrease in lignin content caused in Poplar wood (up to 29% after 45 days). Among several variables evaluated with a Plackett–Burman design (glucose, copper, vanillic acid and manganese concentration, incubation period, and light incidence), the most important factor affecting laccase and Mn-peroxidase (MnP) production by both strains, was light incidence. Light induced fruit body development but diminished laccase and MnP production. Moreover, a modified isoenzymatic laccase pattern was observed, showing additional isoenzymes when fungi were cultivated under darkness and differences in optimal temperature. Although the studied strains did not produce high laccase and MnP titers (uppermost detected 4230 and 90 U L^{-1}, respectively), their laccases showed thermal stability and optimal temperature above 70 °C, representing an interesting source in the search of thermo-tolerant enzymes for biotechnological applications.

KEYWORDS
light regulation, lignin-degrading enzyme production, *Polyporus*, wood degradation

1 | INTRODUCTION

Polyporus P. Micheli ex Adans is a genus of white rot fungi having worldwide distribution, characterized by its stipitate basidiomata, poroid hymenophore, dimitic hyphal system with skeletal-binding hyphae, and cylindrical to ellipsoid basidiospores [1]. Although this genus belongs to the core polyporoid clade [2] along with known lignin-degrading genera such as *Trametes*, *Pycnoporus*, and *Lentinus*; *Polyporus* have received scant attention. There are few studies of the lignin-degrading enzymes of *Polyporus* and their applications [3–6] mostly using strains from Europe, Asia, and North America. Moreover, different species of *Polyporus* are source of secondary metabolites, which are of medicinal interest as antibacterial compounds [7]. This genus is represented in subtropical climates [8], where species usually grow exposed to high temperatures and humidity. Therefore, they are potential fonts in the search of thermo-tolerant enzymes. There has been a growing interest in expanding the studies of lignin-degrading enzymes to other white rot genera in hopes of finding new robust enzymes to be utilized in biotechnological processes.

Copper, manganese, and aromatic compounds were widely applied to increase lignin-degrading enzyme production by white rot fungi [9]. The use of different statistical designs, among them Plackett–Burman [10], for medium

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optimization has been employed for lignin-degrading enzyme production by fungal cultures [11–13]. However, recent evidences suggested the role of light on lignin-degrading enzyme production [14–16]. Besides lignin degradation, fungal laccases have been linked to pigment production, pathogenesis, and fruiting body formation [17]. Nevertheless, the influence of light on basidiome production and its correlation with laccase production (if any) was poorly investigated yet.

The aim of this study was to isolate *Polyporus* fungi from subtropical forests in Oberá, Misiones, Argentina, and evaluate their ability to produce lignin-degrading enzymes in agar plates, in liquid cultures, and solid-state fermentation on Poplar wood chips. The effect of different chemical and physical factors on lignin-degrading enzyme production by different *Polyporus* isolates in submerged fermentation was investigated. In addition, the Plackett–Burman experimental design (PBED) was applied to evaluate the importance of copper, vanillic acid, manganese, light incidence, and incubation period on laccase and Mn-peroxidase (MnP) production by *Polyporus tricholoma* (BAFC 4536) and *Polyporus arcularioides* (BAFC 4534). These fungi were selected based on their lignin-degrading enzyme production and ability to produce primordial of basidiomes under submerged fermentation and considering the lignin content reduction they caused in poplar wood. The possible relation between laccase production, sexual fructification, and light incidence was also investigated.

2 | MATERIALS AND METHODS

2.1 | Collections, morphological, and molecular fungal identification

Specimens were collected in Oberá-Misiones (North East Argentina). Isolation into pure culture was undertaken from basidiocarps, directly after collection from the field sites according to the method of Stalpers [18]. Small pieces of inner layers of basidiocarp tissue were excised on to malt agar plates (malt extract 12.7 g L^{-1}, agar 20 g L^{-1}), supplemented with chloramphenicol (0.1 μg ml^{-1}) and benomyl (1 μg ml^{-1}) under sterile conditions. Isolation plates were incubated at 28 °C and axenic cultures were maintained on malt extract agar slants at 4 °C. Voucher specimens were deposited in BAFC (Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires). The macro and microscopically characterization of the basidiomes was carried out according to Borges da Silveira and Wright [8], Gilbertson and Ryvarden [19], Ryvarden and Gilbertson [20], Núñez and Ryvarden [21]. Microscopic observations were made from freehand cross sections of dried materials mounted in Melzer's reagent, 5% KOH, and/or 1% phloxine, lactophenol, cresyl blue or cotton blue. To observe the hyphal system, we followed the technique described by Decock et al. [22]. Basidiospore measurements were made in Melzer's reagent ($n = 40$). The protocol for molecular identification of this study was added as Supporting Information (Fig. S1).

2.2 | Screening of enzymatic activity on agar plates

All the strains were inoculated on agar plates (90 mm in diameter, 20 ml medium/Petri dish) containing malt extract (12.7 g L^{-1}), glucose (10 g L^{-1}), and agar (20 g L^{-1}) (MEA), supplemented with DMP (2,6-dimethoxyphenol) 1 mM to determine laccase activity. Enzymatic activity was measured by the dark orange-colored halo produced by oxidation of DMP [23]. MnP activity was evaluated in MEA medium with $MnCl_2 . 4H_2O$ (1 mM*).* Enzymatic activity was detected by measuring the brownish-colored halo produced by the oxidation of Mn^{+2} ($MnCl_2$) to Mn^{+4} (MnO_2) [24]. Each plate was inoculated with a 0.5 cm^2 agar plug obtained from 7-day-old cultures pre-grown in MEA. The plates were incubated at 28 °C for 28 days. Growth was followed by measuring radial extension of the mycelium. Average growth rates (cm day^{-1}) were calculated. The data presented are the average of the results of three replicates with a standard error of less than 5%. Analysis of variance was tested by the software Infostat [25]. The significant differences between treatments were compared by Tukey's multiple comparison tests at 5% level of probability.

2.3 | Laccase and MnP production in submerged fermentation

2.3.1 | Culture media and conditions

Basal culture medium (GA) contained glucose, 20 g; L-asparagine monohydrate, 3 g; $MgSO_4 . 7H2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $MnCl_2 . 4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4 . H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; distilled water up to 1 l. The medium was sterilized at 121 °C for 20 min. The effect of three different chemical inducers on laccase and MnP activity was evaluated alternatively with $CuSO_4 . 5H_2O$ (650 μM), 4-hydroxy-3-methoxy-benzoic acid (vanillic acid) (500 μM) or $MnSO_4 . H_2O$ (333 μM). The initial pH of the medium was adjusted to 6.5 with NaOH 1 N. The effect of light on enzymatic activity was monitored at three different conditions: continuous light (L), 12 h light/12 h darkness (L/O) and continuous darkness

(O) in cultures supplemented with 1 mM copper sulfate. Incubation was carried out statically at 28 ± 1°C in 250 ml Erlenmeyer flasks with 25 ml of medium, which were inoculated with four 25-mm^2 surface agar plugs from a 7-day-old colony grown on Bacto-agar 2%. Cultures were harvested periodically, aliquots of the supernatant were collected aseptically, and at day 35, the last sampling day, whole cultures were filtered through a filter paper using a Büchner funnel, dried overnight at 70 °C, and weighed. Growth was estimated by measuring the biomass production. Culture supernatants were used as enzyme sources. All chemicals were of analytical grade and were used without further purification.

2.3.2 | Experimental design and statistical analysis

The PBED was used to evaluate the relative importance of glucose concentration in GA medium, supplements to the GA medium (Cu^{+2}, vanillic acid and Mn^{+2}), the cultivation period and light incidence on laccase and MnP production by *P. tricholoma* (BAFC 4536) and *P. arcularioides* (BAFC 4534) in submerged fermentation (Table 4). Each factor was tested at two levels (coded): high level (+1) and low level (−1). The rows in Table 4 represent the 12 different trials. Experiments were replicated three times. The PBED assumes that there are no interactions between the different factors, xi, in the range of variables under consideration. A linear approach is considered sufficient for screening. Where *Y* is the estimated target function and βi are the regression coefficients.

$$Y = \beta_0 + \Sigma\beta_{ixi}(i = 1,\ldots,k) \qquad (1)$$

where *Y* response (enzyme activity), β_0 model intercept, β_i estimated variable.

The PBED is a fractional factorial design and the main effect (the contrast coefficient, *b*) of such design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements made at the low level (−1). Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient either positive or negative indicates that a factor has a large impact on the response; while a coefficient close to zero means that a factor has little or no effect. The *p*-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low *p*-value indicates a “real” or significant effect. The significance of each variable was determined by applying the Student's *t*-test) [10,11]. The statistical analyses were performed by using multiple regressions and ANOVA with the software Minitab v 13.1. The experimental data were fitted according to Eq. (1), which includes the individual effects of each variable.

2.3.3 | Enzymatic determinations

Laccase activity (E.C:1.10.3.2) was measured using DMP 5 mM as substrate in 0.1 M sodium acetate buffer at pH 3.6. The reaction was carried out in test tubes containing 1.5 ml of substrate solution and incubated for 1 min at 30 °C. An aliquot of supernatant was added (5–20 µl), and absorbance of the product (coerulignone) was recorded at 469 nm ($\varepsilon469 = 27.5\ mM^{-1}\ cm^{-1}$) [26]. MnP activity (E.C:1.11.1.13) was determined using phenol red as the substrate in 0.1 M sodium dimethylsuccinate buffer (pH 4.5) at 30 °C ($\varepsilon610 = 22\ mM^{-1}\ cm^{-1}$) [27]. The reagents without supernatant were used as baseline for absorbance measurements. International enzymatic units (U) were used (µmol product min^{-1}). Enzyme activity was expressed as U ml^{-1} of culture filtrate. To test the effect of pH on laccase activity, its substrate was dissolved in 0.1 M sodium citrate-phosphate buffers of different pHs (3–6). In the experiments testing the effect of temperature, the incubation temperature was in the range 30–70 °C. The effect of pH on the enzyme stability was investigated measuring the activity remaining after incubation for up to 24 h at 30 °C in 0.1 M sodium citrate-phosphate buffers of different pHs (3–6). Aliquots of the culture filtrate were incubated at different temperatures (30–70 °C) for up to 48 h for temperature stability assays, and laccase residual activity was measured afterwards.

2.3.4 | Polyacrylamide gel electrophoresis (PAGE) and activity staining of gels

Electrophoretic separation was performed in 9% polyacrylamide gels using Novex® Sharp Pre-Stained Protein Standard (Life Technologies, USA) as molecular weight markers. Thirty microliter of culture filtrates from different species and culture conditions (water-diluted in some cases, to render a laccase activity of approx. 0.01 U ml^{-1}) were loaded onto the gel and electrophoresed with Tris-glycine buffer (pH 8.3) at 120 V. The buffer solution for the separating gel was Tris-HCl 50 mM (pH 8.8). After electrophoresis the gel was fixed in a mixture of methanol: acetic acid: water (1:1:1) for 5 min, then immersed in a solution of 5 mM DMP in acetate buffer pH 3.6 and incubated at room temperature (25 °C) until laccase activity bands appeared.

2.4 | Wood decay assay

In vitro weight losses of wood samples were determined according to the methodology employed by Robles et al. [28]. Blocks of 3.5 × 0.6 × 1 cm of sound wood from *Populus nigra* were used to estimate after 3 months the degradation abilities of *Polyporus arcularius* (BAFC 4535)*, P. tricholoma* (BAFC 4536 and BAFC 4554)*, Polyporus varius* (BAFC 4556)*, P. arcularioides* (BAFC 4534), and *P.* cfr. *tricholoma* (BAFC

862). Blocks were dried at 70 °C for 48 h, to determine the initial dry weight. Afterwards, each block was saturated by immersion in distilled water for 48 h and sterilized in autoclave at 121 °C for 20 min. MEA slopes in 19 × 2 cm test tubes were inoculated with mycelial plugs (25-mm^2 surface) of either one of the fungi assayed and then incubated at 28 °C. Once the mycelium covered the surface of the agar, one sterilized wood block was introduced into each test tube. Uninoculated wood blocks were used as controls. The fungal wood decay proceeded at 28 °C in darkness. At 45 and 90 days of incubation, six independent samples were collected to evaluate dry weight loss and lignin content. Wood chips were dried up to constant weight at 70 °C after gently removing the superficial mycelium. Weight losses were determined based on the initial and final dry weights. *P. nigra* wood contained 29% of lignin. Lignin decrease was measured as percentage. Klason lignin was quantified according to the TAPPI Official Test Method T222 om-83 [29]. To determine acid-insoluble lignin in wood and pulp, the carbohydrates in wood were hydrolyzed and solubilized with sulfuric acid (72%); the acid-insoluble lignin was filtered off, dried, and weighed. For the extraction of extracellular proteins, three pieces (1 g each) of the colonized chips were cut in small pieces with a knife and stirred at 120 rpm and 25 °C 24 h with 2.5 ml of sodium acetate buffer pH 3.6, followed by centrifugation and filtration. Supernatants were stored at 4 °C until needed for assays. Laccase enzymatic units were expressed per kg of wood dry weight.

3 | RESULTS

3.1 | Enzyme production in agar plates and under submerged fermentation

The extracellular production of the lignin-degrading enzymes laccase and MnP by different *Polyporus* species from Argentina was investigated. Six strains were screened for enzyme activity on agar plates. Five were new field isolates and one was kept at BAFC Culture Collection.

We generated sequences of internal transcribed spacers (ITS) to corroborate their morphological identification, as *P. arcularius* (BAFC 4535), *P. arcularioides* (BAFC 4534), *P. tricholoma* (BAFC 4554 and BAFC 4536), and *P. varius* (BAFC 4556). The isolate BAFC 862 previously identified as *P. brumalis* should be renamed, considering that the molecular identification placed it far away from the clade *P. brumalis*, and in a closer relationship with the clade *P. tricholoma*. We referred to it as *P.* cfr. *tricholoma.*

Enzyme activity was assessed on solid medium supplemented with DMP for laccase activity and $MnCl_2$ to detect MnP (Table 1). Five out of six the strains produced laccase in plate cultures, but only three out of six also produced MnP. Neither laccase nor MnP activity was detected in cultures of *P. varius* (BAFC 4556) after 28 days of incubation. Best results among MnP producers were obtained with *P. arcularioides* (BAFC 4534). Laccase activity evaluated by DMP oxidation rate exceeded in all the strains 1 cm day^{-1}.

The isolates were screened for their ability to produce lignin-degrading enzymes in a synthetic liquid medium with glucose and asparagine as carbon and nitrogen sources, respectively, with the alternatively addition of copper sulfate (650 µM), manganese sulfate (333 µM), or vanillic acid (500 µM) (Table 2). Highest laccase and MnP titers were secreted by *P. arcularius* (BAFC 4535) with copper addition (respectively, 1510 and 38.44 U L^{-1}). Vanillic acid increased lignin-degrading secretion by *P. arcularioides* (BAFC 4534) attaining high values as well (970 and 36.08 U L^{-1} of, respectively, laccase and MnP). Laccase secretion significantly increased in all *P. tricholoma* (BAFC 4536) cultures when the fungus was incubated in darkness, but the highest titers were detected with copper supplementation (990 U L^{-1}).

TABLE 1 Growth and solid-plate ligninolytic production on MEA medium supplemented, respectively, either with DMP or $MnCl_2$ to detect laccase or MnP

Fungal name (strain number)	Subgenus	Lacasse (cm day^{-1})	MnP (cm day^{-1})	Growth category
Polyporus arcularius (BAFC 4535)	Polyporellus	1.48^a	1.2^a	Medium
Polyporus arcularioides (BAFC 4534)	Polyporellus	1.14^b	0.92^b	Medium
Polyporus tricholoma (BAFC 4536)	Polyporellus	1.54^a	ND^d	Fast
Polyporus tricholoma (BAFC 4554)	Polyporellus	1.48^a	ND^d	Medium
Polyporus cfr *tricholoma* (BAFC 862)	Polyporellus	1.56^a	0.68^c	Fast
Polyporus varius (BAFC 4556)	Melanopus	ND^c	ND^d	Medium

ND, not detected. The plates were incubated for 28 days at 28 °C. The values are the mean of three replications, SEM <5%. Means superscripted with the same letter are not significantly different ($p < 0.05$). Growth rate categories: slow <0.6 cm/day; Medium: 0.6–1.2 cm/day; Fast: 1.2–1.8 cm/day.

TABLE 2 Laccase, Mn-peroxidase (MnP), and biomass attained under submerged fermentation in GA medium alternatively amended with copper sulfate (650 µM), manganese sulfate (333 µM), or vanillic acid (500 µM)

Fungal name (strain number)	Control[a,b]			$CuSO_4$[b]			$MnSO_4$[b]			Vanillic acid[b]		
	Lac (U/L	MnP (U/L)	Biomass (g/L)	Lac (U/L)	MnP (U/L)	Biomass (g/L)	Lac (U/L)	MnP (U/L)	Biomass (g/L)	Lac (U/L)	MnP (U/L)	Biomass (g/L)
P. arcularius (4535)	$390^{(28)}$	$7.9^{(28)}$	6.8	$1510^{(21)}$	$38.4^{(28)}$	7.1	$360^{(21)}$	$13.7^{(28)}$	6.9	$740^{(28)}$	$17.3^{(28)}$	6.1
P. arcularioides (4534)	$710^{(35)}$	ND[c]	5.5	$870^{(28)}$	ND	5.6	$270^{(28)}$	$16.5^{(28)}$	4.7	$970^{(21)}$	$36.1^{(28)}$	2.3
P. tricholoma (4536) Light	$50^{(35)}$	ND	7.0	$60^{(28)}$	$0.2^{(28)}$	7.9	$270^{(21)}$	$0.3^{(28)}$	6.6	$140^{(28)}$	ND	6.7
P. tricholoma (4536) Dark	$330^{(35)}$	ND	7.1	$990^{(35)}$	$0.3^{(21)}$	7.1	$420^{(35)}$	$0.4^{(21)}$	6.4	$630^{(21)}$	ND	6.1
P. tricholoma (4554)	$50^{(28)}$	ND	6.3	$400^{(21)}$	$0.6^{(28)}$	6.5	$70^{(28)}$	ND	6	$30^{(28)}$	ND	5.9
P. cf. *tricholoma* (862)	$170^{(28)}$	ND	5.9	$280^{(21)}$	$0.6^{(28)}$	6.2	$770^{(28)}$	ND	5.7	$300^{(21)}$	$0.3^{(28)}$	5.7
P. varius (4556)	$80^{(21)}$	$31.7^{(28)}$	3.4	$130^{(21)}$	ND	3.3	$180^{(35)}$	$3.2^{(28)}$	3.6	$270^{(21)}$	$3.2^{(28)}$	2.4

The values shown correspond to the peak of enzyme production. The numbers above the columns indicate the day these maxima were achieved. Biomass was determined at 35 days of incubation.

[a]Control: non-supplemented GA.

[b]Values represent the mean of three replicates, SEM <5%.

[c]ND: not detected.

3.2 | Wood degradation by *Polyporus* isolates and laccase enzyme production

After 12 weeks, mass losses caused on poplar wood by five out of six *Polyporus* isolates were between 4.4–10.8% (Table 3). *P. arcularius* (BAFC 4535) decay resulted in a higher mass loss (18.5%). Highest lignin content reduction after 45 days resulted from *P. arcularioides* (BAFC 4534) decay (29.2%), but after 12 weeks lignin content decreased 31–34% in poplar wood degraded by five out of six *Polyporus* isolates. Laccase activity extracted from wood chips did not correlate with lignin decrease. MnP was not detected. The extraction procedure may have not been appropriate to guarantee the recovery of the enzymes adsorbed on the wood substrate. Highest activities 687.5 and 327.8 $U\,kg^{-1}$ dry wood were detected in chips colonized respectively by *P. arcularioides* (BAFC 4534) and *P. tricholoma* (BAFC 4536). The first fungus also produced high titers of laccase and MnP in liquid cultures, both isolates were capable of producing primordial basidiomes under submerged fermentation and laccase production by *P. arcularioides* (BAFC 4534) proved to be light sensitive, thus both fungi were selected for further attempting to improve their lignin-degrading enzyme production applying a PBED.

3.3 | Plackett–Burman experimental design

The factors tested included different concentrations of the medium components: glucose; Cu^{2+}; Mn^{2+}, and vanillic acid; light incidence and incubation period. A summary of the variables and their variation level is given in Table 4 along with the design of the experiment and the measured responses: fungal growth (dry weight), laccase, and MnP titers ($U\,L^{-1}$) obtained with the different conditions assayed. The data in Table 4 indicate that, there was a wide variation in laccase and MnP production during the 12 runs. This variation reflects the importance of medium optimization. The incorporation of the most suitable conditions and supplements to the medium (First trial run) resulted in a noteworthy increment in laccase and MnP production. Up to 3950 and 90 $U\,L^{-1}$ were obtained by *P. arcularioides* and 4230 and 81 $U\,L^{-1}$ by *P. tricholoma* (in an optimized medium with 10 $g\,L^{-1}$ glucose, 650 µM $CuSO_4 . 5H_2O$, vanillic acid 500 and 333 µM $MnSO_4 . H_2O$ after 28 days incubation under darkness). The *p*-value was used to evaluate the significant factors when confidence levels were greater than 95%. As witnessed from Table 5 the most important variable affecting laccase and MnP production was light incidence. PBED results showed that laccase and MnP production decreased with light exposition in both species. Laccase of *P. tricholoma* as well as MnP of *P. tricholoma* and

TABLE 3 Poplar wood degradation by different *Polyporus* isolates (dry weight and lignin loss) and laccase production on poplar wood chips

		Dry weight loss (%)[b]		Lignin loss (%)[b]	
Fungal name (strain number)	**Laccase (U/kg)(day)[a,b]**	**45 days**	**90 days**	**45 days**	**90 days**
P. arcularius (BAFC 4535)	53.9(45)	6.34	18.5	18.8	33.8
P. arcularioides (BAFC 4534)	687.5(45)	4.77	10.4	29.2	33.0
P. tricholoma (BAFC 4536)	327.8(45)	6.46	7.2	16.7	32.2
P. tricholoma (BAFC 4554)	91.6(90)	10.01	10.8	15.8	32.6
P. cf. *tricholoma* (BAFC 862)	278.7(45)	2.27	5.6	19.8	31.1
P. varius (BAFC 4556)	89.5(45)	2.77	4.4	7.2	21.6

[a]The values shown correspond to the peak of enzyme production. The numbers above the columns indicate the day these maxima were achieved.
[b]Values represent the mean of three replicates, SEM < 5%.

P. arcularioides increased with the higher glucose concentrations assayed, probably due to the stimulatory effect of glucose on biomass yield. Mn^{+2} supplementation as well as longer incubation periods increased laccase production by *P. tricholoma*, while vanillic acid addition increased MnP production by *P. arcularioides.* The optimized medium was efficient in increasing lignin-degrading secretion by another strain of *Polyporus*, not assayed in the PBED: *P. arcularius* (BAFC 4535), maximal titers registered 1800 and 65 U L^{-1}, respectively for laccase and MnP.

As depicted in Fig. 1 A and B light incidence was required for *P. tricholoma* fructification, but laccase secretion diminished up to undetectable levels if ambient conditions favored fungal basidiome development. In addition, a modified isoenzyme laccase pattern was observed, showing additional isoenzymes when fungi were cultivated under darkness (Fig. 2). Furthermore, light influenced optimal laccase temperature. The effects of pH and temperature on laccase activity and stability of *P. tricholoma* and *P. arcularioides* were evaluated in the crude extracellular fluids. The optimum pH for laccase activity was in the range of 3.0–4.0 for both fungi, at pH 5 *P. tricholoma* and *P. arcularioides* laccases retained 55 and 30% of their activities, but both activities were substantially reduced beyond this pH range. Optimal temperatures were respectively 70 and 75 °C for laccase activities of *P. arcularioides* and *P. trichloma* from dark incubated cultures, but 75 °C was the optimum in *P. arcularioides* light incubated cultures. The enzymes incubated at 30 °C retained more than 80% of their activities at pHs 3–5 for 48 h, but *P. arcularioides* and *P. tricholoma* laccases lost, respectively, 40 and 60% of their activities after 24 h incubation at pH 6. Fungal laccases usually rapidly decrease their activity at temperatures above 60 °C. Nevertheless, the activity half-lives (T1/2) of *P. arcularioides* and *P. tricholoma* laccases were, respectively, 2 and 6 h at 60 °C, retaining 55–60% of their activities after 48 h incubation at 50 °C.

4 | DISCUSSION

Although *Polyporus* belongs to Polyporaceae along with known lignin-degrading genera, it has received scarce attention. In this work, laccase and MnP production by different species was evaluated in agar plates and under submerged and solid state fermentation on poplar wood chips. Additionally, a PBED was applied to evaluate the relative importance of glucose, copper, vanillic acid, and manganese concentration in the medium as well as light incidence and incubation period on laccase and MnP production by *P. tricholoma* (BAFC 4536) and *P. arcularioides* (BAFC 4534). Copper has been reported to be a strong laccase inducer in several white rot fungi, among them *Trametes* and *Polyporus* species [30,4]. It is known that copper induces both laccase transcription and activity [31]. In *Trametes trogii*, copper addition increased MnP activity as well [30]. Moreover, in *Trametes* species copper 1 mM addition decreased extracellular proteolytic activity, thus diminishing laccase degradation [32]. Phenolic compounds such as vanillic acid were also applied to stimulate laccase production by *Trametes versicolor* [33]. Vanillic acid increased laccase but also MnP secretion by *Cerrena unicolor* [34]. Manganese proved to increase MnP production long time ago [35] although repressive effects such as those found in *P. varius* (BAFC 4556), have also been reported [36].

Light incidence had a significant negative influence on laccase and MnP production in both *Polyporus* strains evaluated, but induced fruit body development. As far as we know, in our work light incidence was for the first time incorporated in a PBDE to evaluate its relevance in laccase as well as MnP secretion. Moreover, laccase activity has been related with fruit body formation [37], being light one of the main factors involved in basidiome development initiation [38] but the possible relation among laccase production, sexual fructification, and light incidence was scarcely investigated yet. Light governs many developmental

TABLE 4 Plackett–Burman experimental design applied to screen the key factors for the production of Laccase and MnP by two *Polyporus* species

Column	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	Variables												*P. tricholoma* BAFC 4536			*P. arculariodes* BAFC 4534		
Trial number	$CuSO_4$ (µM)		Vanillic acid (µM)		$MnSO_4$ (µM)		Glucose (g/L)		Light		Days		Laccase (U/L)	MnP (U/L)	Biomass (g/L)	Laccase (U/L)	MnP (U/L)	Biomass (g/L)
1	(+1)	**650**	(+1)	**500**	(+1)	**333**	(+1)	**10**	(−1)	**No**	(+1)	**35**	2700	74	7.6	2410	87	3.2
2	(−1)	**150**	(−1)	**0**	(+1)	**333**	(+1)	**10**	(−1)	**No**	(+1)	**35**	1700	59	6.4	1540	68	3.2
3	(+1)	**650**	(−1)	**0**	(−1)	**50**	(+1)	**10**	(−1)	**No**	(−1)	**21**	760	49	8.4	840	77	5.6
4	(−1)	**150**	(−1)	**0**	(−1)	**50**	(−1)	**5**	(−1)	**No**	(+1)	**35**	420	18	3.6	600	51	1.6
5	(−1)	**150**	(+1)	**500**	(−1)	**50**	(−1)	**5**	(−1)	**No**	(−1)	**21**	340	33	4.4	1180	84	2.8
6	(+1)	**650**	(+1)	**500**	(+1)	**333**	(−1)	**5**	(−1)	**No**	(−1)	**21**	360	20	6.8	1000	73	3.6
7	(+1)	**650**	(−1)	**0**	(−1)	**50**	(+1)	**10**	(+1)	**Yes**	(−1)	**21**	230	26	8.0	540	54	4.8
8	(+1)	**650**	(−1)	**0**	(+1)	**333**	(−1)	**5**	(+1)	**Yes**	(+1)	**35**	190	3	2.8	340	27	1.6
9	(−1)	**150**	(+1)	**500**	(+1)	**333**	(+1)	**10**	(+1)	**Yes**	(−1)	**21**	110	6	9.6	330	52	4.8
10	(−1)	**150**	(−1)	**0**	(+1)	**333**	(−1)	**5**	(+1)	**Yes**	(−1)	**21**	40	3	4.4	680	52	2.4
11	(+1)	**650**	(+1)	**500**	(−1)	**50**	(−1)	**5**	(+1)	**Yes**	(+1)	**35**	10	1	3.2	680	52	2.0
12	(−1)	**150**	(+1)	**500**	(-1)	**50**	(+1)	**10**	(+1)	**Yes**	(+1)	**35**	20	1	6.8	1090	58	2.0

Each factor was tested at two levels (coded): high level (+1) and low level (−1). The numbers in columns 2–13 correspond to the coded (in brackets) and real values (in bold) of the variables assayed. For example for copper the real value is the concentration of copper in the medium and is expressed in µM. Measured responses are depicted in columns 14–19. Values represent the mean of three replicates, SEM <5%

TABLE 5 Degree of positive or negative effects of various medium components: CuSO4; vanillic acid (VA); MnSO4; glucose; as well as incubation period and light incidence; on growth, laccase, and MnP enzyme production by two *Polyporus* species: *P. arcularioides* (BAFC 4534) and *P. tricholoma* (BAFC 4536), according to the Plackett–Burman experimental design

	Laccase activity *P. arcularioides*		Laccase activity *P. tricholoma*		MnP activity *P. arcularioides*		MnP activity *P. tricholoma*		Biomass *P. arcularioides*		Biomass *P. tricholoma*	
Factor	***b***	***p*-value**	***b***	***p*-value**	***b***	***p*-value**	***b***	***p*-value**	***b***	***p*-value**	***b***	***p*-value**
$CuSO_4$	0.03	NS[a]	0.54	NS	0.01	NS	0.02	NS	0.001	0.004	0.001	NS
VA	456.00	NS	−22.00	NS	32.81	<0.0001	2.61	NS	−0.12	NS	−0.60	NS
$MnSO_4$	−0.16	NS	1.67	0.002	−0.02	NS	0.02	NS	−0.00	NS	0.002	NS
Glucose	39.20	NS	138.71	<0.0001	2.67	0.001	4.59	<0.0001	0.31	<0.0001	0.72	<0.0001
Days	9.95	NS	38.13	0.003	−0.32	NS	0.24	NS	−0.12	<0.0001	−0.13	<0.0001
Light	−689.02	<0.0001[b]	−945.04	<0.0001	−27.82	<0.0001	−35.67	<0.0001	−0.41	0.039	−0.46	NS

Main effects (b) (laccase activity ($U\,ml^{-1}$), MnP activity ($U\,ml^{-1}$), and dry weight ($g\,L^{-1}$). Significance levels (*p* values) of the experimental variables.

[a]NS, non-significant.

[b]Indicates a significant effect (positive or negative) $p < 0.05$.

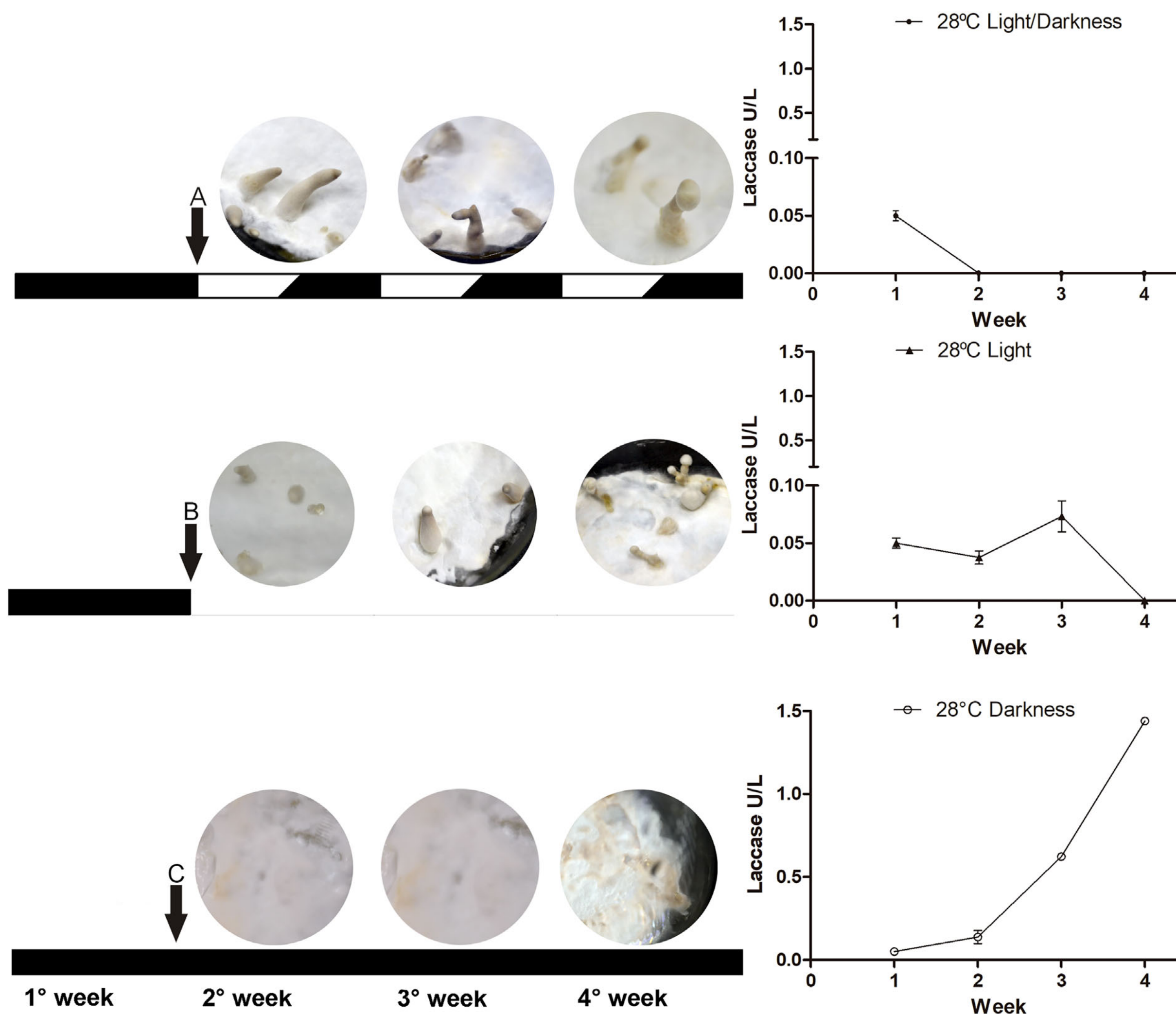

FIGURE 1 Effect of light on *in vitro* basidiome primordial initiation and laccase production by *P. tricholoma* (BAFC 4536): 12 h light/12 h darkness (A) continuous light (B), and continuous darkness (C). All treatments started with 1 week under darkness

transitions and accompanying expression of genes associated with regulation in the production of exoenzymes and with various bioprocesses in fungi such as sporulation, fruit body development, and metabolite production [37]. Laccase activity by *Pleurotus eringyii* [39] was not significantly affected by the light conditions tested, but light stimulated its aryl-alcohol oxidase activity. On the contrary, Hernández et al. [15] found out that short wavelengths (green, blue, and white) strongly inhibited laccase production by *Pycnoporus sanguineus* whereas maximum activity was induced in darkness. This can be explained as an ecological mechanism of environmental recognition, wood rot fungi develop in darkness inside lignocellulose structures. Light presence would indicate them, that they are in an external environment, without lignin, and hence without need to secrete laccase. This possible new regulation in laccase production has important biotechnological implications, for it would be possible to control its production using light stimuli [15]. In *Schizophyllum commune*, the condition for maximum laccase synthesis was cultivation at 30 °C in darkness, whereas fruit body formation was greatest at 25 °C in the light [40]. In *Agaricus bisporus*, laccase activity accumulated during vegetative growth, but suffered rapid inactivation shortly after the onset of fruit body formation [41]. The most widespread and conserved photosensory protein in fungi is White collar 1 (WC-1), a flavin-binding photoreceptor that functions with WC-2 as a transcription factor complex. Other photosensory proteins in fungi include opsins, phytochromes, and cryptochromes whose roles in fungal photobiology are not fully resolved [42]. Thirty-one fungal genomes were analyzed for the presence of different photoreceptors and related proteins [43]. In the genomes of several Agaricomycotina such as *Phlebia brevispora, C. unicolor*, *Phanerochaete chrysosporium*, *Schyzophylum commune, Coprinopsis cinerea*, *Laccaria bicolor*, and *Pleurotus ostreatus* photosensory proteins may be found, among them: the blue light receptor complex WC-1/2, opsins, cryptochromes, and phytochromes [16,42–44].

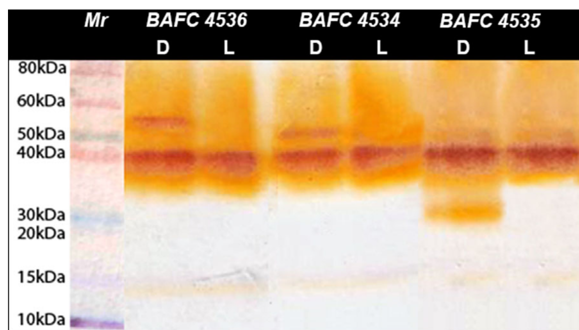

FIGURE 2 Native-PAGE separation of laccase isoenzymes eluted from cultures of *P. tricholoma* (BAFC 4536) (lanes 2 and 3), *P. arcularioides* (BAFC 4534) (lanes 4 and 5), and *P. arcularius* (BAFC 4535) (lanes 6 and 7) grown under darkness (D) or with light (L). Lane 1: pre-stained molecular weight protein markers

As depicted in Fig. 1 A and B light incidence was required for *P. tricholoma* fructification, but laccase secretion diminished up to undetectable levels if ambient conditions favored fungal basidiome development. In addition, a modified isoenzyme laccase pattern was observed, showing additional isoenzymes (Fig. 2) when fungi were cultivated under darkness and differences in optimal temperature. In order to enable more effective biocatalytic process applications, there is a need for laccases with improved biochemical properties, such as thermostability and thermotolerance [45]. Thermal stability of laccases differs considerably and correlates with the temperature range of the growth of the source organism [45]. Although the studied strains did not produce high laccase and MnP titers (uppermost detected 4230 and 90 U L^{-1} in an optimized synthetic medium after 28 days incubation under darkness), their laccases showed thermal stability and optimal temperature above 70 °C.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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