Two isolates of Fusarium proliferatum from different habitats and global locations have similar abilities to degrade lignin

ARTICLE *in* FEMS MICROBIOLOGY LETTERS · SEPTEMBER 2005

Impact Factor: 2.12 · DOI: 10.1016/j.femsle.2005.06.014 · Source: PubMed

4 AUTHORS, INCLUDING:

Anne Anderson
Utah State University
177 PUBLICATIONS **4,693** CITATIONS
SEE PROFILE

Miguel Angel Falcón
Universidad de La Laguna
30 PUBLICATIONS **513** CITATIONS
SEE PROFILE

ELSEVIER

FEMS Microbiology Letters 249 (2005) 149–155

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Two isolates of *Fusarium proliferatum* from different habitats and global locations have similar abilities to degrade lignin

Anne J. Anderson [a], Sun-Il Kwon [a], Ana Carnicero [b], Miguel A. Falcón [b,*]

[a] *Biology Department, Utah State University, Logan, UT 84322-5305, USA*

[b] *Departamento de Microbiología y Biología Celular, Facultad de Farmacia, Universidad de La Laguna, 38206 La Laguna, Tenerife, Spain*

Received 29 March 2005; received in revised form 31 May 2005; accepted 7 June 2005

First published online 29 June 2005

Edited by B.A. Prior

Abstract

Two isolates of *Fusarium proliferatum* from different global locations and habitats mineralized several natural and synthetic lignins. MUCL 31970 was isolated from a forest soil whereas the second strain, NRRL 31071, was a wheat endophyte causing disease in stressed seedlings. Onset and the fastest rate of lignin mineralization occurred during logarithmic and early stationary-phase of culture. Reduction of glucose in the medium shortened log-growth phase and advanced the onset of mineralization for both isolates. Mineralization correlated with the detection of extracellular laccase and aryl alcohol oxidase activities. The carbon–nitrogen ratio in the medium influenced laccase isozyme production and secretion by both strains. These studies suggest that both *F. proliferatum* strains degrade lignin via comparable routes, despite their different habitats and saprophytic or endophytic strategies.

Keywords: *Fusarium*; Ligninolysis; Laccase; Aryl alcohol oxidase

1. Introduction

Fusarium proliferatum occupies many different habitats. One characterized isolate from forest soils in the Canary Islands (*F. proliferatum* MUCL 31970), termed the TF strain, is ligninolytic [1] and mineralizes lignin in media with a C/N ratio of 13, a ratio comparable to that of its habitat. This ligninolytic strain is taxonomically related to a second strain (*F. proliferatum* NRRL 31071 termed the UT strain) isolated as an endophyte from wheat kernels in the USA [2]. This isolate causes necrosis of young developing wheat seedlings grown under stressed conditions [3,4]. Pathogenicity of other strains of *F. proliferatum* has been reported, causing corn-ear rot in the field and in post-harvest storage in maize kernels [5,6], black point and tombstone in wheat [7,8], and rots affecting asparagus [9], bananas [10] and gladiolus corms [11].

Laccases are produced by the UT strain during infection of wheat seedlings [12]. These same isozymes are present in extracts from carbon-limited in vitro cultures of the fungus, while other isoforms are detected on low nitrogen or high nutrient media [12]. Apoplastic sugar levels are reduced by 70% in wheat when it was grown under stressed conditions. It is possible that the low carbon conditions in the stressed plants permitted laccase gene expression by the fungus [4]. Laccases, produced by the necrosis-causing fungus, *Botrytis cinerea*, during host infection, are proposed to be important in degradation of host toxic materials and in the limitation of lignification as a defense response during pathogenesis [13,14]. Laccases also are implicated in lignin degradation by

* Corresponding author. Tel.: +34 22 318479; fax: +34 22 318477.
E-mail address: mafalcon@ull.es (M.A. Falcón).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.06.014

anamorphic fungi isolated from soils, including the isolate of *F. proliferatum* MUCL 31970 [1,15].

In this paper, we address whether the wheat *F. proliferatum* isolate has a similar capacity to degrade lignin as the forest soil TF strain. To this end, both isolates were cultivated in media containing ^{14}C-labeled natural lignin and three lignin model substrates to determine the extent of mineralization. The activities for several enzymes, laccase, aryl alcohol oxidase and peroxidases, proposed in other systems to be involved in mineralization were examined over time courses to compare their presence with mineralization activity. Complex regulation of laccase production exists for the UT strain [12,16]. Similarly, transcript patterns for genes encoding several enzymes involved in wood digestion by *Phanerochaete chrysosporium* are dramatically different when the fungus colonizes wood chips, as opposed to those obtained in defined media [17,18]. Thus, the effect of nutrition on laccase isozyme production in TF was also compared between the wheat and forest soil isolates.

2. Materials and methods

2.1. Microorganisms, media and culture conditions

F. proliferatum NRRL 31071 (UT strain) was obtained from within surface-sterilized wheat kernels [15,18]. The strain was stored at −70 °C in 15% glycerol. *F. proliferatum* MUCL 31970 (TF strain; [1]) was isolated from pine forest soil by using a mineral medium solidified with agar containing the polymeric fraction (1 mg ml $^{-1}$) of Kraft lignin (PKL) (PKL, Indulin AT; Westvaco Co., Charleston, S.C.), purified as described by Rodríguez et al. [19] and used as the sole carbon and energy source. The TF strain was maintained at 4 °C on this medium. Inoculum for the studies in this paper were generated by transfer of 1 cm^2 plugs of mycelia on agar plates to the PKL medium modified with addition of 55.5 mM glucose.

Media used for lignin degradation studies contained different ^{14}C-labeled lignins as employed by Rodríguez et al. [19]. The uniformly ^{14}C-labeled milled wood lignin from wheat straw (^{14}C-MWL) had a specific activity (sa) of 0.009 μCi mg^{-1}. The synthetic lignins were dehydrogenative polymerizates of coniferyl alcohol (DHP), specifically labeled in the ^{14}C-β-side chain (^{14}C-β-DHP, sa: 0.126 μCi mg^{-1}), uniformly ^{14}C- labeled in the aromatic ring (^{14}C-ring-DHP, sa: 0.19 μCi mg^{-1}), or specifically labeled in the methoxy-groups (^{14}C-methoxy-DHP, sa: 0.083 μCi mg^{-1}). All these substrates were purchased from Forstbotanisches Institut der Universität Göttingen, Germany. These DHPs were added at 0.09 μCi per flask. Chromatography of both DHPs and natural lignin on Sephadex G-50 (45 × 2 cm) showed no low molecular weight materials. The products had a size range between 15.5 and 1.5 kDa. D-glucose was added as an easily metabolized carbon source at final concentrations of 5.5 or 55.5 mM together with nitrogen provided by 13.48 mM asparagine and 12.2 mM NH_4NO_3 to give C/N ratios of 1.287 and 12.87, respectively (referred to in this paper by the C/N ratios of 1.3 and 13). $^{14}CO_2$ evolved from the ^{14}C-labeled sources was trapped with 10% sodium hydroxide as used by Haider and Trojanowski [20], and radioactivity was measured by combining 1 ml of the NaOH sample with 1 ml of distilled water and 4 ml of scintillation liquid (Optiphase III; Kabi Pharmacia) in a liquid scintillation counter (LKB Wallace mod.1214). Lignin mineralization was expressed as the percentage of the labeled lignins recovered as $^{14}CO_2$. To determine the patterns of enzyme production, unlabeled DHP (1 mg ml^{-1}) was used.

All the assays described above were performed in triplicate in 250 ml Erlenmeyer flasks containing 25 ml of medium and incubated with shaking (50 strokes min^{-1}) at 28 °C for up to 30 days. Non-inoculated controls were incubated in duplicate but otherwise were treated identically.

2.2. Determination of the dry weight of mycelia

Spore suspensions were prepared by stirring a 1-cm^2 plug from a PKL-glucose agar plate into 5 ml of 0.01% Tween 80 in sterile water (vol/vol). One ml of this suspension (about 9×10^6 spores) was inoculated into 25 ml of media with C/N 1.3 or 13 supplemented with unlabeled DHP (1 mg ml^{-1}) contained in 250 ml Erlenmeyer flasks. Inoculated flasks were incubated at 28 °C with shaking for 10 days. Studies were run in triplicate and flasks were harvested daily. The cultures were filtered through pre-weighed Millipore filters (0.45 μm) and the retained mycelia were repeatedly washed with distilled water. Filters were maintained at 80 °C to reach a constant weight and the dry weights of the mycelia determined.

2.3. Enzyme assays

Enzyme activities from *Fusarium* strains were assayed periodically in cultures supplemented with unlabeled DHP. Cultures were harvested by centrifuging (9000 × g), discarding the pellet, and the extracellular fluids were evaluated for enzymatic activities. The activities were determined spectrophotometrically, all in final reaction volumes of 1 ml, using a UV–Vis Shimadzu 160 as described below. A unit (U) was defined as the amount of enzyme capable of transforming 1 μmol min^{-1} of its target substrate.

Laccase-like activities were tested by monitoring oxidation of ABTS using a modified assay as described by Wolfenden and Wilson [21]. The reaction mixture

contained 2 mM ABTS, sodium succinate and lactate buffers, 0.1 M at pH 4.5, and 50–200 μl of culture filtrate. Oxidation of ABTS was followed by an absorbance increase at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). Activity for oxidation of 2,6-dimethoxy phenol (DMP; $\varepsilon_{468} = 1 \times 10^4\ M^{-1}\ cm^{-1}$) was monitored using 2 mM DMP in 200 mM sodium tartrate pH 4.5, as previously described by Shuttleworth et al. [22]. Laccase isozymes composition was visualized after separation of the enzymes by non-denaturing polyacrylamide gel electrophoresis and staining for activity with ABTS by the method of Niku-Paavola et al.[23].

Aryl-alcohol oxidase (AAO) was assayed by monitoring the veratraldehyde ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$) formed on oxidation of veratryl alcohol following a method modified from Bourbonnais and Paice [24] to contain 2 mM veratryl alcohol, 0.05 M sodium tartrate buffer pH 4.5, and 500 μl aliquots of culture filtrate.

Several different potential substrates for oxidases that would generate hydrogen peroxide were used (methyl glyoxal, glyoxal, D-cellobiose, D-glucose, D-galactose and oxalic acid). Tests for hydrogen peroxide production used a peroxidase-coupled assay with phenol red as the substrate, as previously described by Kersten and Kirk [25]. Reaction mixtures contained 20 mM sodium 2,2-dimethyl succinate (pH 4.5), 10 mM oxidase substrate, 0.01% phenol red ($\varepsilon_{520} = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$), 10 μg of horse-radish peroxidase (Sigma Chemical Co), and up to 300 μl of culture fluid. Reactions were stopped by adding 50 μl of 2 N NaOH. Controls lacked addition of culture filtrate. Product formation was detected at 520 nm.

Lignin peroxidase (LiP) was assayed by the method of Tien and Kirk [26], monitoring oxidation of veratryl alcohol ($\varepsilon_{310} = 0.93 \times 10^4\ M^{-1}\ cm^{-1}$), after addition of hydrogen peroxide (0.4 mM). A second method described by Archibald [27] used azure B ($\varepsilon_{651} = 4.88 \times 10^4\ M^{-1}\ cm^{-1}$) as the substrate.

Manganese peroxidase (MnP) was determined according to the method previously described by Camarero et al. [28], following the oxidation of DMP in a reaction mixture with 100 mM Na tartrate (pH 5), 100 μM $MnSO_4$, 50–200 l of culture filtrate and starting the reaction by the addition of 50 μM hydrogen peroxide.

The protein content of extracts was determined spectrophotometrically at 595 nm with the Coomassie Blue method [29]. Bovine albumin fraction V was used as protein standard (Bio-Rad Protein Assay).

3. Results

3.1. The ligninolytic potential of F. proliferatum strain UT is comparable to that of TF

Both isolates caused mineralization of natural and synthetic lignins (^{14}C-MWL from wheat straw, $^{14}COH_3$-DHP, ^{14}C-β-DHP and ^{14}C-ring-DHP, Fig. 1A and B). After a 30-day incubation in C/N 13 medium, the UT isolate mineralized about 30% of the ^{14}C-MWL (Fig. 1A), a level that was comparable to that achieved by TF (Fig. 1B). Cumulative mineralization rates also were similar between UT and TF for the synthetic lignins: between 9% and 11% mineralization with ^{14}C-β-DHP, and up to 7% when $^{14}CH_3O$-DHP was used as substrate (Fig. 1A and B). However, mineralization of the aromatic-ring-labeled DHP by UT was about 75% higher than for TF (14% and 4%, respectively) in the 30-day period (Fig. 1A and B). The products obtained after TF cultures amended with either ^{14}C-MWL and ^{14}C-DHPs had a lower molecular size when compared with the starting substrates as determined by chromatography on Sephadex G-50 (data not shown). The rate of mineralization of ^{14}C-β-DHP by both strains in C/N medium 13 was higher during the initial time period of 3-to-7 days rather than 9-30 days (Fig. 2B and D). A time course in the C/N 13 amended with unlabeled DHP showed glucose was used by 72 h although maximum fungal biomass was not attained until four to five days (data not shown).

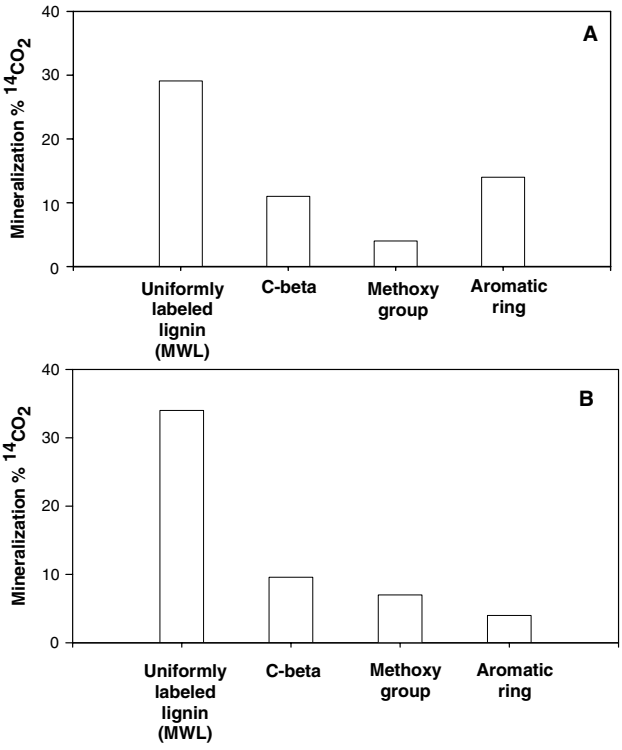

Fig. 1. Percentages of mineralization by *Fusarium proliferatum*, UT (A) and TF (B) strains, cultured in C/N 13 media with amendments of natural (^{14}C-lignin from wheat straw) or synthetic (^{14}C-DHP) lignins labeled in different positions. Mineralization, measured as release of $^{14}CO_2$, is shown after 30 days of incubation at 28 °C with shaking. Non-inoculated controls showed less than 0.2% mineralization. The standard errors were less than 10%. Data are the means of three trials.

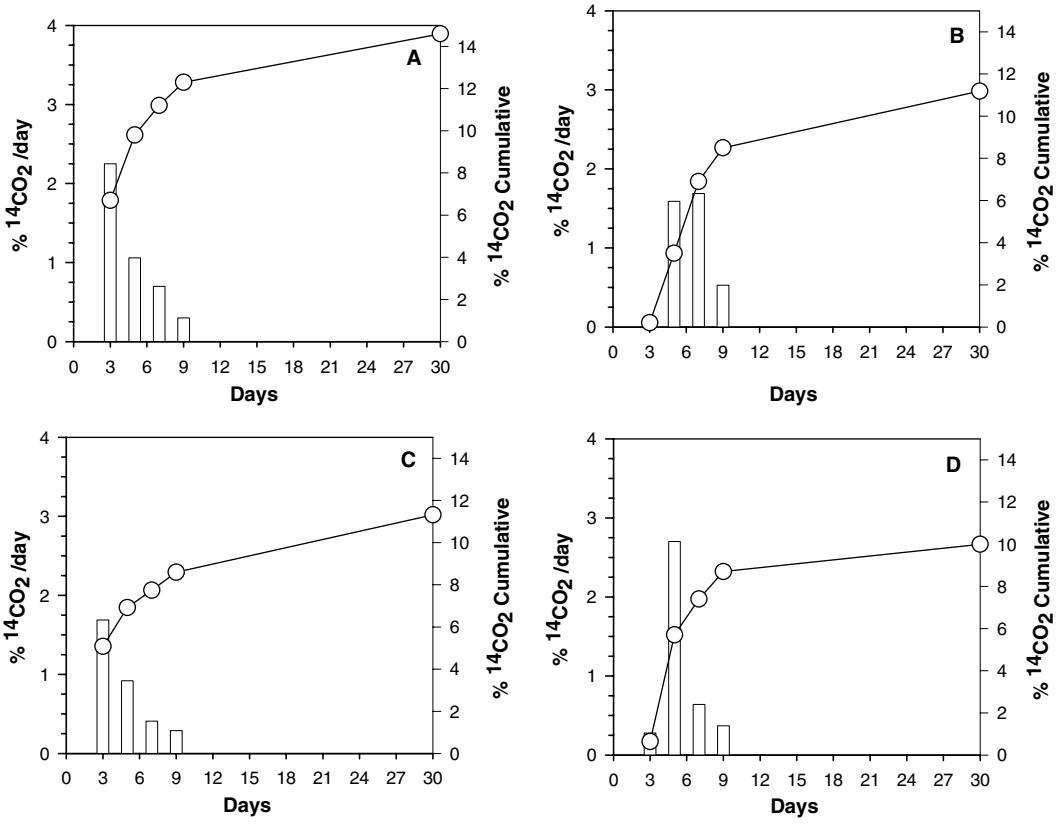

Fig. 2. Percentage of $^{14}CO_2$ released per day (bars) or cumulative (○) from ^{14}C-β-side chain labeled DHP by *Fusarium proliferatum* UT (A, B) and TF (C, D) in media with C/N 1.3 (A, C) and C/N 13 (B, D). Data are the mean values for three replicate cultures, the standard errors being less than 10% of the mean.

3.2. Effect of the C/N ratio on mineralization and production of enzyme activities by Fusarium strains of synthetic lignin

Mineralization of ^{14}C-β-side chain-labeled DHP occurred earlier in the C/N 1.3 medium (Fig. 2A and C) than the C/N 13 medium (Fig. 2B and D) for both strains. About 50% of total mineralization in C/N 1.3 medium occurred in the first three days of incubation, whereas five days were necessary in C/N 13 cultures (Fig. 2A–D). Mineralization occurred prior to the attainment of maximal mass. Mycelial mass was 4 and 12 mg (dry weight) for the UT and TF strains at 48 h, with a maximum of 20 mg for both strains at 72 h of incubation, in the C/N 1.3. The UT and TF strains, respectively, had masses of 4 and 20 mg at 48 h, both with a maximum of 80 mg, respectively, at 96 and 120 h of incubation, in the C/N 13. Glucose was catabolized by 48 h in C/N 1.3 and 72 h for C/N 13 (data not shown).

To attempt to understand this effect of nutrition on enzyme activities potentially associated with lignin degradation, culture filtrates of C/N 1.3 and 13 media supplemented with unlabeled DHP were examined. No H_2O_2-generating oxidases that used methyl glyoxal, glyoxal, D-cellobiose, D-glucose, D-galactose or oxalic acid as substrates were detected. No Lip activities were detected when either veratryl alcohol or the dye azure B were used as substrates nor was MnP activity observed with DMP. However, the extracellular medium from both cultures showed laccase and aryl alcohol oxidase (AAO) activities (Figs. 3 and 4). For both strains extracellular laccase detected with ABTS was present early in culture and reached its highest levels during growth on the C/N 1.3 medium but was negligible in C/N 13 medium during the experimental period. DMP-detected laccase activity peaked much higher and earlier in the C/N 13 medium than the C/N 1.3 medium for the UT strain. For the TF strain, DMP-laccase activity increased as the cultures aged, thus exhibiting a different temporal pattern than for the UT strain. AAO activity patterns were similar for both strains in the C/N 1.3 medium and resembled those for the ABTS-laccase acitivity. In the C/N 13 medium, the patterns for AAO activity were again different between strains, being lower for the UT strain than the TF strain. Low levels of both AAO and laccase were present prior to the peak of mineralization measured with ^{14}C-β-side chain labeled DHP (Figs. 2–4).

3.3. Laccase isozyme production is similar in TF and UT

Previous studies by Kwon and Anderson with UT [12] demonstrated that laccase activity as detected by

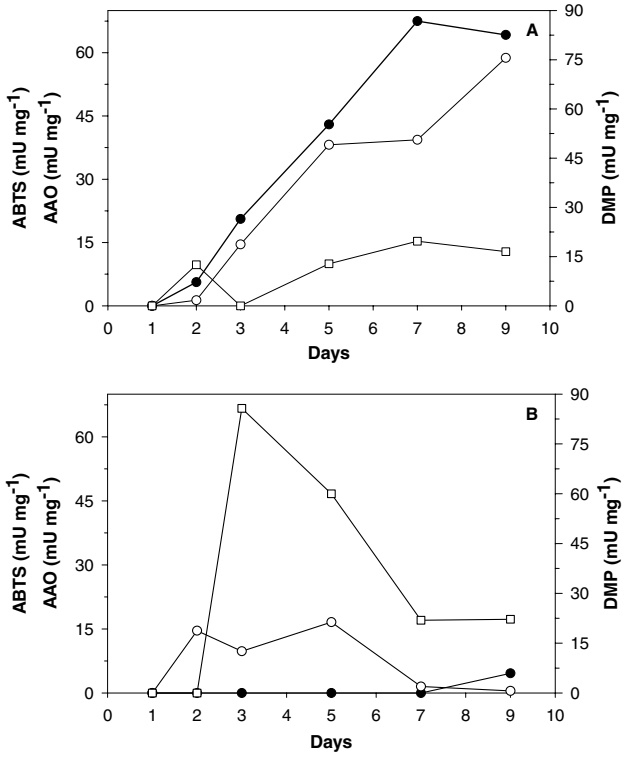

Fig. 3. Activities detected in the supernatant from *Fusarium proliferatum* UT cultured in media with C/N 1.3 (A) and C/N 13 (B) containing unlabeled DHP (1 mg ml^{-1}) during 10 days of incubation at 28 °C with shaking. Activities: aryl alcohol oxidase activity (AAO) (○); and laccase activity with ABTS (●) and DMP (□) as substrates. Data are the means of three replicates and standard errors were less than 10%.

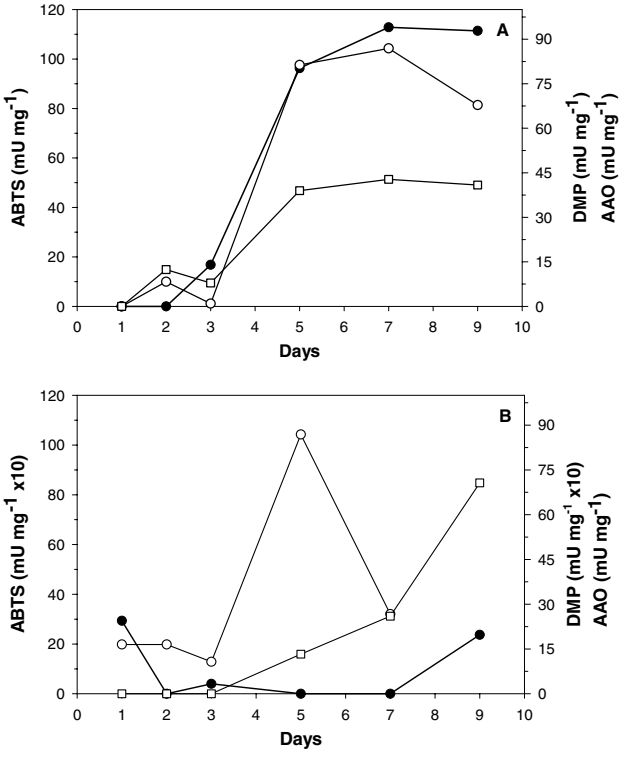

Fig. 4. Activities detected in the supernatant from *Fusarium proliferatum* TF cultured in media with C/N 1.3 (A) and C/N 13 (B) containing unlabeled DHP (1 mg ml^{-1}) during 10 days of incubation at 28 °C with shaking. Activities: aryl alcohol oxidase activity (AAO) (○); and laccase activity with ABTS (●) and DMP (□) as substrates. Data represent means of three replicates and standard errors were less than 10%.

ABTS oxidase activity resulted from different isozymes. Isolate TF also produced different laccase isozymes (Fig. 5) that were detected in both mycelial extracts and extracellular culture filtrates in a manner similar to that for the UT strain. Production of the laccases was affected by the C/N ratio, occurring earlier in the C/N 1.3 medium than the C/N 13 medium. In addition, the production of the laccase isozymes was repressed temporally in the C/N 13 medium, in agreement with the spectrophotometric analyses of ABTS laccase activities (Figs. 3 and 4). The faster moving intracellular isozymes for the TF isolate were expressed extracellularly to a lesser extent in comparison to the UT strain.

4. Discussion

Two *F. proliferatum* isolates from different global locations and ecological sites, strain TF from a forest soil in the Canary Islands, and strain UT, an opportunistic endophytic wheat pathogen from the USA, displayed similar abilities to degrade natural lignin from wheat (^{14}C-MWL) and synthetic polymers. The maximal rates of mineralization were observed early in

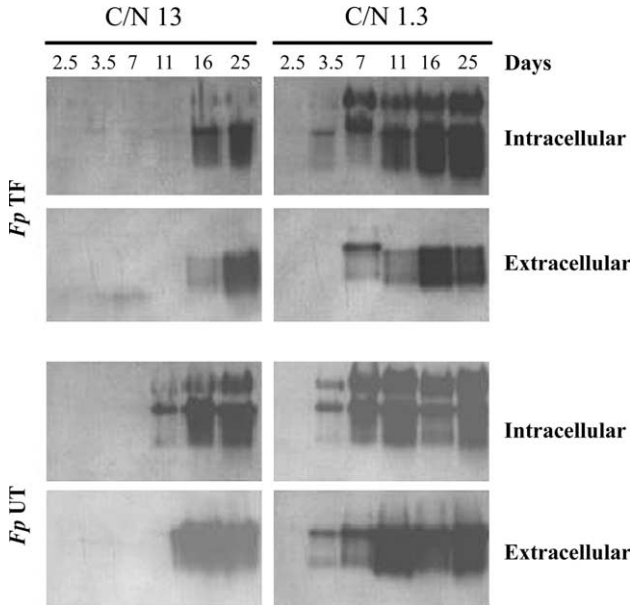

Fig. 5. Intracellular and extracellular laccases isozymes from *Fusarium proliferatum* strains TF and UT separated by anionic non-denaturing PAGE and stained with ABTS. Mycelia were grown either in C/N 13 or C/N 1.3 media without phenolic addition. Data are from one of three studies with the same results.

growth. This finding agrees with reports that actinomycetes solubilized and degraded lignin during their primary growth [30] but differs from mineralization by the white-rot fungi, like *P. chrysosporium*, where it is associated with late stationary-phase growth [31,32].

Differences were detected between the strains in mineralization of aromatic-ring-labeled DHP with the UT strain having the higher activity. The UT and TF strains also differed in the relative production of AAO and DMP-laccase activities in C/N 13 medium, in which the UT strain had higher DMP-laccase and lower AAO activies than the TF strain. Whether this difference relates to the more extensive degradation of the aromatic-ring-labeled DHP requires additional investigation.

Only laccase and AAO activities were found in the supernatant of both strains after searching for other oxidases and peroxidases associated with lignin degradation. Cooperative action between laccase and AAO or other oxidases (e.g., glucose oxidase) has been described for basidiomycetes when degrading lignin derivatives and soluble lignosulphonate [33,34]. The peak activities of the ABTS and DMP laccases did not correspond to the timing of maximum mineralization. However, only low levels of laccase activity were required for the ligninolytic system of *Petriellidium fusoideum*, where the activity was correlated with production of ·hydroxyl radicals [15].

Laccase activity in both isolates was derived from different isozymes. The lower glucose concentration in the C/N 1.3 medium promoted a much earlier production of both the mycelial and extracellular activities. These findings confirmed studies with the UT strain where carbon and nitrogen levels were shown to regulate laccase isozyme production [12]. The regulation of mineralization and laccase activities by nutrient availability may be relevant to the natural habitats of these *F. proliferatum* strains. Similar nutritional regulation was demonstrated for *P. chrysosporium* where lignin peroxidase activity was enhanced by the low nitrogen conditions thought to exist in woody materials [35]. In non-living woody material, carbon is sequestered in polymeric forms until ligninolysis has proceeded. Thus, an ability to initiate mineralization under conditions of low carbon could be an advantage for a forest soil isolate. Maintenance of mineralization as carbon level increases also could be advantageous for the TF isolate. For the opportunistic pathogen, the ability to degrade lignin may be exploited when the *F. proliferatum* is growing as a pathogen rather than an endophyte. As discussed by Kwon and Anderson [4], the switch to the pathogenic state may have occurred when the supply of low molecular weight sugars in the wheat apoplast was low, as conditioned by plant growth under stress. Degradation of phenolic modifications to the plant cell wall may then promote pathogenic growth of the *F. proliferatum*. Indeed, the full complement of anionic laccases and expression of transcripts corresponding to *lac-1* was detected in wheat showing symptoms of necrosis upon infection with UT strain [12]. Continued studies of the mechanisms of lignin mineralization and the regulation of specific enzymes related to phenolic modification may help reveal the subtleties in the genomes of these *F. proliferatum* strains that enable them to occupy different habitats in nature.

Acknowledgements

This research has been partially funded by the Projects PI 2002/064 (Gobierno Autónomo de Canarias) and REN 2002-02732/TECNO (Ministerio de Ciencia y Tecnología) and Utah Agricultural Experiment Station funding to AJA.

References

[1] Regalado, V., Rodríguez, A., Perestelo, F., Carnicero, A., DeLa Fuente, L.G. and Falcón, M.A. (1997) Lignin degradation and modification by the soil-inhabiting fungus *Fusarium proliferatum*. Appl. Environ. Microbiol. 63, 3716–3718.

[2] Kwon, S.-I., von Dohlen, C. and Anderson, A.J. (2001) Gene sequence analysis of an opportunistic wheat pathogen, an isolate of *Fusarium proliferatum*. Can. J. Bot. 79, 1115–1121.

[3] Bishop, D.L., Levine, H.G., Kropp, B.R. and Anderson, A.J. (1997) Seed borne fungal contamination: consequences in space-grown wheat. Phytopathol. 87, 1125–1133.

[4] Kwon, S.-I. and Anderson, A.J. (2001) Differential production of superoxide dismutase and catalase isozymes during infection of wheat by a *Fusarium proliferatum*-like fungal isolate. Physiol. Mol. Plant Pathol. 58, 73–81.

[5] Abbas, I.K., Mirocha, C.J., Meronuck, R.A., Pokorny, J.D., Gould, S.L. and Kommedahl, T. (1988) Mycotoxins and *Fusarium* spp. associated with infected ears of corn in Minnesota. Appl. Environ. Microbiol. 54, 1930–1933.

[6] Kedera, C.J., Plattner, R.D. and Desjardins, A.E. (1999) Incidence of *Fusarium* spp. and levels of fumonicin B1 in maize in western Kenya. Appl. Environ. Microbiol. 65, 41–44.

[7] Clear, R.M. and Patrick, S.K. (1990) *Fusarium* species isolated from wheat samples containing tombstone (SCAB) kernels from Ontario, Minnesota and Saskatchewan. Can. J. Plant Sci. 70, 1057–1069.

[8] Conner, R.L., Hwang, S.F. and Stevens, R.R. (1996) *Fusarium proliferatum*: a new causal agent of black point in wheat. Can. J. Plant Pathol. 18, 419–423.

[9] Elmer, W.H. (1995) Association between Mn-reducing root bacteria and NaCl applications in suppression of *Fusarium* crown and root rot of asparagus. Phytopathology 85, 1461–1467.

[10] Jiménez, M., Logrieco, A. and Bottalico, A. (1993) Occurrence and pathogenicity of *Fusarium* species in banana fruits. J. Phytopathol. 137, 214–220.

[11] Viljoen, A., Wingfield, M.J., Marasas, W.F.O. and Coutinho, T.A. (1995) Characterization of *Fusarium* isolates from gladiolus corms pathogenic to pines. Plant Dis. 79, 1240–1244.

[12] Kwon, S.-I. and Anderson, A.J. (2001) Laccase isozyme production by an opportunistic pathogen, a *Fusarium proliferatum* isolate from wheat. Physiol. Mol. Plant Pathol. 59, 235–242.

[13] Bar-Nun, N., al Lev, A.T., Hared, E. and Mayer, A.M. (1988) Repression of laccase formation in *Botrytis cinerea* and its

possible relation to phytopathogenicity. Phytochem. 27, 2505–2509.
[14] Sbaghi, F., Jeander, P., Bessis, R. and Leroux, P. (1996) Degradation of stilbene-type phytoalexins in relation to the pathogenicity of *Botrytis cinerea* to grape vines. Plant Pathol. 45, 139–144.
[15] Gonzales, L., Hernández, J.R., Perestelo, F., Carnicero, A. and Falcón, M.A. (2002) Relationship between mineralization of synthetic lignins and the generation of hydroxyl radicals by laccase and low molecular weight substances produced by *Petriellidium fusoideum*. Enzyme Microb. Technol. 30, 474–481.
[16] Kwon, S.-I. (2000) Ph.D.thesis. Molecular and morphological identification of an endophytic Fusarium isolated from wheat and characterization of its laccases in comparison to affiliated Fusarium isolates. Utah State University, Logan, UT, USA.
[17] Janse, B.J.H., Gaskell, J., Masood, A. and Cullen, D. (1998) Expression of *Phanerochaete chrysosporium* genes encoding lignin peroxidases, manganese peroxidases, and glyoxal oxidase in wood. Appl. Environ. Microb. 64, 3536–3538.
[18] Vallim, M.A., Janse, B.J.H., Gaskell, J., Pizzirani-Kleiner, A.A. and Cullen, D. (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. Appl. Environ. Microb. 64, 1924–1928.
[19] Rodríguez, A., Carnicero, A., Perestelo, F., De la Fuente, G., Milstein, O. and Falcón, M.A. (1994) Effect of *Penicillium chrysogenum* on lignin transformation. Appl. Environ. Microb. 60, 2971–2976.
[20] Haider, K. and Trojanowski, J. (1975) Decomposition of specifically ^{14}C-labelled phenols and dehydropolymers of coniferyl alcohol. Arch. Microb. 105, 33–41.
[21] Wolfenden, B.S. and Wilson, R.L. (1982) Radical-cations as reference chromogens in kinetic studies of Cro-electron transfer reactions: pulse radiolysis studies of 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate). J. Chem. Soc., Perkin Trans. II, 805–812.
[22] Shuttleworth, K.L., Posti, L. and Bollag, J.-M. (1986) Production of induced laccase by the fungus *Rhizoctonia praticola*. Can. J. Microbiol. 32, 867–870.
[23] Niku-Paavola, M.L., Raaska, L. and Itävaara, M. (1990) Detection of white-rot fungi by a non-toxic stain. Mycol. Res. 94, 27–31.
[24] Bourbonnais, R. and Paice, M.G. (1988) Veratryl alcohol oxidase from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. Biochem. J. 255, 445–450.
[25] Kersten, P.J. and Kirk, T.K. (1987) Involvement of a new enzyme, glyoxal oxidase in intracellular H_2O_2 production by *Phanerochaete chrysosporium*. J. Bacteriol. 169, 2195–2201.
[26] Tien, M. and Kirk, T.K. (1984) Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA 81, 2280–2284.
[27] Archibald, F.S. (1992) A new assay for lignin-type peroxidases employing the dye azure B. Appl. Environ. Microb. 58, 3110–3116.
[28] Camarero, S., Savkar, S., Ruiz-Dueñas, F.J., Martínez, M.J. and Martinez, A.T. (1999) Description of a versatile peroxidase envolved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrates interaction sites. J. Biol. Chem. 274, 10324–10330.
[29] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
[30] Hernández, M., Hernández-Coronado, M.J., Ball, A.S. and Arias, M.E. (2001) Degradation of alkali-lignin residues from solid-state fermentation of wheat straw by streptomycetes. Biodegradation 21, 219–223.
[31] Kirk, T.D. and Farrell, R. (1987) Enzymatic "combustion": the microbial degradation of lignin. Ann. Rev. Microbiol. 41, 465–505.
[32] Eriksson, K-E., Blanchette, R.A. and Ander, P. (1990) In: Microbial and enzymatic degradation of wood and wood components (Timell, T.E., Ed.). Springer, Berlin.
[33] Szklarz, G. and Leonowicz, A. (1986) Cooperation between fungal laccase and glucose oxidase in the degradation of lignin derivatives. Phytochemistry 25, 2537–2539.
[34] Marzullo, L., Cannio, R., Giardina, P., Palmieri, G., Aurilia, E. and Sannia, G. (1996) Cooperative action of *Pleurotus ostreatus* enzymes in lignin biodegradation In: Biotechnology in the Pulp and Paper Industry. Recent Advances in Applied and Fundamental Research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry (Srebotnik, E. and Messner, K., Eds.), pp. 51–356. Facultas-Universitätverlag, Vienna, Austria.
[35] Fenn, P. and Kirk, T.K. (1981) Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium*. Arch. Microbiol. 130, 59–63.