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Analysis of cellulase and polyphenol oxidase production by southern pine beetle associated fungi

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Abstract
In this study, the production of extracellular enzymes by fungi associated with southern pine beetle was investigated for the first time. Cellulase and polyphenol oxidase production were analyzed for three beetle associated fungi. Only the mutualistic symbiont *Entomocorticium* sp. A was found to produce cellulases and polyphenol oxidase. In time course analyses of cellulase production in batch cultures, *Entomocorticium* sp. A showed maximum activity of 0.109 U/ml and 0.141 U/ml for total cellulase and endoglucanase activity respectively. Polyphenol oxidase production was simultaneous with fungal growth. Characterization of polyphenol oxidase by activity staining suggests that the enzyme is a tyrosinase/catechol oxidase. Enzyme assays in the presence of polyphenol oxidase inhibitors support the results of the activity staining.

Keywords: *Ceratocystiopsis*, *Entomocorticium*, *Ophiostoma,* cellulase, tyrosinase/catechol oxidase

1. Introduction

The southern pine beetle (SPB) *Dendroctonus frontalis* Zimmermann is among the most damaging of North American forest insects (Thatcher et al., 1980). Considered a primary bark beetle, in that it is essentially an obligate parasite (Raffa et al., 1993), it attacks and kills healthy living trees through mass colonization (Paine et al., 1997). Female beetles initiate attacks on host trees by boring entrance holes through the outer bark, creating a nuptual chamber and releasing a pheromone to attract more beetles to the tree (Kinzer et al., 1969). Once the female beetle has mated, she begins chewing ovipositional galleries within the inner bark and phloem of the tree (Thatcher et al., 1960), and inoculates fungi into the phloem tissue (Bramble and Holst, 1940). After the eggs hatch the early instar larvae feed, constructing, sinuous galleries which terminate in obovate "feeding chambers" (Payne et al., 1983). Within these chambers can be found growth of SPB associated, nutritionally mutualistic fungi (Coppedge et al., 1995). Although many fungi have been associated with galleries of SPB in pine phloem, three have been the focus of most SPB-fungal research, and appear to have the most significant impacts on the SPB life cycle. *Ceratocystiopsis ranaculosus* Hausner (Jacobs and Kirisits, 2003) and *Entomocorticium* sp. A (an undescribed basidiomycete, Hsiau and Harrington, 2003) are both considered to be nutritional mutualists of the SPB (though *Entomocorticium* sp. A appears to be more beneficial) and are carried within specialized structures called mycangia, in the beetle. *Ophiostoma minus* (Hedgcock) H. and P. Sydow is a so-called bluestain fungus which aids the beetle in depleting host defences (Klepzig et al., 2005), but later acts antagonistically, competing with the mycangial fungi and larvae for uncolonized phloem (Barras, 1970; Klepzig and Wilkens, 1997).

The SPB life strategy of development within phloem [a relatively nitrogen poor substrate (Ayres et al., 2000)] poses challenges. Southern pine beetles, in particular, seem to use their symbiotic fungi as nutritional intermediaries, possibly providing nitrogen and sterols (Ayres et al., 2000; Klepzig and Six, 2004). To provide these benefits, the fungi must be able to digest and/or convert the lignocellulosic compounds within, likely via the production of extracellular enzymes (Rosch et al., 1969; Przybyl et al., 2006). The efficient hydrolysis of cellulose requires the coordinated action of several enzymes. Endoglucanases (EC 3.2.1.4) are responsible for the cleavage of the internal bonds in the glucose chains, producing new ends at random. Exo-

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glucanases (cellobiohydrolases, EC 3.2.1.91) split off cellobiose units from such free ends (Tomme et al., 1995).

Most cellulolytic microorganisms produce a group of cellulases which act synergistically to degrade cellulose (Sánchez et al., 2004). In addition, polyphenol oxidase (EC 1.14.18.1) may be important in this system. Polyphenol oxidase is a copper-containing enzyme, widely distributed throughout microorganisms, plants and animals. The enzyme uses the copper ion as a prosthetic group which participates in several redox reactions and has a crucial role in melanin synthesis. Melanin is correlated with spore formation and virulence of some pathogenic fungi (Miranda et al., 1997) may be involved in cell defence (Score et al., 1997), and likely plays a key role in fungal interactions in the SPB system (Klepzig, 2006).

Because extracellular cellulase and polyphenol oxidase are produced by most wood-decaying fungi, and because enzyme production by SPB-associated fungi is relatively unexplored (though see Delalibera et al., 2005), the main objective of this study was to analyze cellulase and polyphenol oxidase production by SPB-associated fungi in batch cultures in order to better understand their biological roles in SPB development.

2. Materials and Methods

Chemicals and microorganisms

Catechol and gallic acid were obtained from Sigma Chem Ltd., (USA). Yeast extract and Avicel® (microcrystalline cellulose) were purchased from Merck Ltd.. Acrylamide and bisacrylamide were obtained from AppliChem Ltd. All other chemicals used were analytical grade from Sigma (USA) and Merck (Germany).

The SPB associated fungi *O. minus*, *C. ranaculosus* and *Entomocorticium* sp. A were isolated from infestations on the Homochitto National Forest in Mississippi, USA. All fungi were isolated using established methods (Klepzig and Wilkens, 1997).

Microbial cultivations and biomass determination

All cultures were inoculated onto malt extract agar (MEA) or potato dextrose agar (PDA) slants and incubated at 30°C for 6–7 days and stored at 4°C. Spores from stock cultures were inoculated into 20 ml of preculture which consisted of modified yeast protein soluble starch (YpSs) broth (4 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, and 20 g glucose in 1 liter distilled water) containing glucose instead of starch as a carbon source. After 24 hours of incubation at 30°C, the preculture was transferred into 1 liter of YpSs broth. For polyphenol oxidase production, the main culture contained 2% (w/v) glucose and for cellulase production it contained 2% (w/v) Avicel® (microcrystalline cellulose) (Merck) as a carbon source. Cultures were incubated in a shaker incubator at 30°C and 155 rpm.

Culture supernatant was removed by filtration through a Whatman® No.1 filter paper. Separated cell biomass was dried 24 h at 60°C for the determination of fungal biomass.

Enzyme assays

Qualitative cellulase assay

Screening of SPB associated fungi was conducted using the Congo red test as a preliminary study for identifying cellulase producers (Ruijssenaars and Hartsmans, 2000). A total of 3 strains of SPB associated fungi were grown on carboxymethyl cellulose (CMC) agar (malt extract 30 g, peptone from soymeal 3 g, CMC 1 g, and agar 15 g in 1 liter distilled water). The CMC agar plates were incubated at 30°C for 7 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of 1% (w/v) Congo red for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation.

Qualitative polyphenol oxidase assay

For this purpose modified Bavendamm (Bavendamm et al., 1928) test for extracellular oxidases was used**.** Bavendamm's medium contained 2% Difco® malt extract, 2% Difco bacto agar, amended with either 0.5% tannic acid or 0.5 % gallic acid, adjusted to pH 4.5 with 1 N sodium hydroxide before autoclaving. Plates were inoculated with test isolates from stock culture and Bavendamm's medium without gallic or tannic acid used as a negative control. Plates were incubated at 30°C until fungal growth covered the non-amended control. Any change in color due to phenol oxidase reaction or gallic/tannic acid oxidation and its effect on fungal growth were recorded.

Quantitative cellulase assay

All enzyme assays were carried out in 100 mM sodium acetate buffer (pH 6.0) at 35°C (previously determined as optimal). Total cellulase activity was measured as filter paper activity. A reaction mixture was prepared using a strip of filter paper (Whatman® No: 1, 1 × 6 cm), 0.5 ml of buffer and 0.5 ml appropriately diluted supernatant. The mixture was then incubated at the stated temperature for 30 min. The reducing sugar content was measured by the Nelson-Somogyi method (Wood and Bhat, 1988) using glucose as standard. Endoglucanase (EG) activity was determined on carboxymethyl cellulose (CMC). One milliliter of 1% (w/v) CMC solution, 0.5 ml of an appropriate buffer and 0.5 ml of appropriately diluted supernatant were mixed and incubated at the stated temperature. After 30 min of incubation, the reducing sugar content was estimated by the Nelson-Somogyi method.

Dilutions of culture supernatants were carried out in the same buffer as that used for the enzyme assay. Experiments were performed in duplicate to ensure reproducibility. Mean results were recorded for each assay. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute under the assay conditions.

Quantitative polyphenol oxidase assay

The activity of polyphenol oxidase was assayed spectrophotometrically by measuring the initial rate of increase in absorbance at 420 nm at 30°C. Culture supernatant was used as crude enzyme source. The enzyme and substrate solutions were preincubated separately for 5 minutes at 30°C before activity measurement. The reaction mixture contained 0.5 ml of 0.1 M catechol solution in 0.1 M phosphate buffer, pH 8.0, 1 ml of buffer and 0.5 ml enzyme solution. The reference cuvette contained only enzyme solution and buffer. Experiments with inhibitors were conducted in the same manner, but in the presence of the inhibitor dissolved at stated concentrations in reaction buffer. The initial line of the progress curve was used to express enzyme activity. One unit of enzyme activity was defined as a change in optical density at 420 nm of 0.01/min under the stated assay conditions.

Activity staining

An analytical zymogram technique, allowing the differentiation of the three polyphenol oxidase enzymes (laccase, peroxidase and tyrosinase) on the same polyacrylamide gel electrophoresis, was used to visualize the polyphenol oxidase bands (Rescigno et al., 1997). In this method enzyme remained active under non-denaturating conditions (without sodium dodecylsulfate). Electrophoresis was carried out by using 4% polyacrylamide stacking gel and 7.5% polyacrylamide separating gels containing no anionic detergent, SDS. β-mercaptoethanol was also excluded from sample application buffer. Separation was carried out at a constant voltage of 80 V for 4–5 hours. After electrophoresis, the gel was stained for different polyphenol oxidase activities (modified from Rescigno 1997): staining with 4-amino-*N,N*-diethylaniline (ADA) for laccase activity, with hydrogen peroxide (H_2O_2) for peroxidase activity and with 4-tert-butyl catechol (*t*BC) for tyrosinase activity.

3. Results

Cellulase and polyphenol oxidase production by SPB associated fungi

All three strains of fungi showed signs of growth on CMC agar after 7 days of incubation. Only *Entomocorticium* sp. A cultures formed the clear zones associated with cellulose degradation. Similarly, screening fungi for polyphenol oxidase activity showed production only by *Entomocorticium* sp. A.

Time course of cellulase and polyphenol oxidase production

The patterns of enzyme production were similar for both types of cellulases. Total cellulase and endoglucanase activities increased during the first four days, reached the maximum level and then decreased until the end of cultivation. *Entomocorticium* sp. A showed maximum total cellulase production (0.109 U/ml) and maximum endoglucanase production (0.141 U/ml) both on day 4 (Figs. 1A, B).

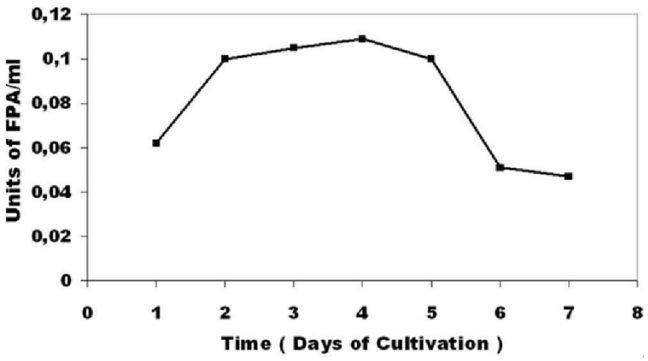

Figure 1A. Time course of total cellulase production by *Entomocorticium* sp. A on 2% (w/v) Avicel (microcrystalline cellulose) as the main source of carbon and at 30°C. Total cellulase activity was measured as filter paper activity (FPA) using a string of filter paper (Whatman No: 1, 1 × 6 cm) as a substrate. Experiments were performed in duplicates to ensure reproducibility, and results were taken as the mean of the data.

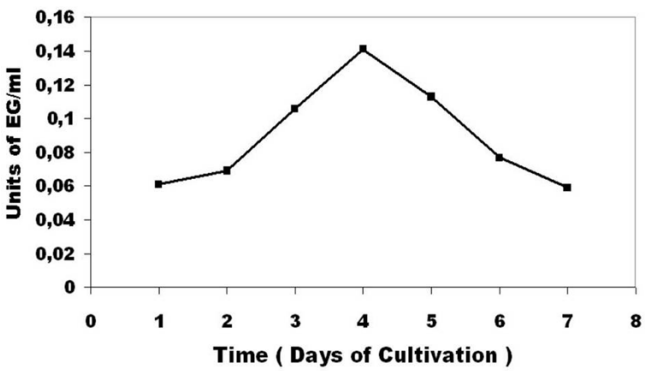

Figure 1B. Time course of endoglucanase (EG) production by *Entomocorticium* sp. A on 2% (w/v) Avicel (microcrystalline cellulose) as the main source of carbon and at 30°C. Endoglucanase (EG) activity was determined using carboxymethyl cellulose (CMC) as a substrate. Experiments were performed in duplicates to ensure reproducibility, and results were taken as the mean of the data

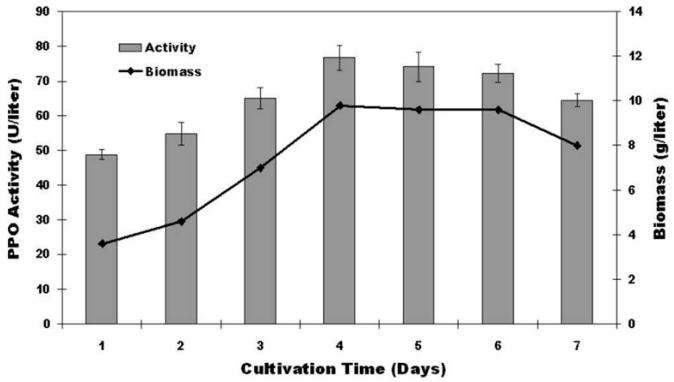

Figure 2. Time course of polyphenol oxidase (PPO) production by *Entomocorticium* sp. A with respect to biomass generation at 30°C on 2% glucose as the sole carbon source. Error bars indicate standard deviations of two parallel experiments.

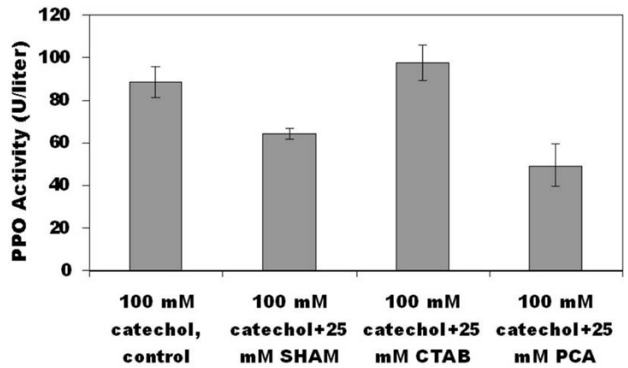

Figure 3. Effect of different inhibitors on polyphenol oxidase (PPO) activity of *Entomocorticium* sp. A. Samples were from fourth day culture supernatants grown on 2% glucose. Error bars indicate standard deviations of two parallel experiments. SHAM: Salicyl hydroxamic acid; PCA: *p*-coumaric acid; CTAB: cetyltrimethylammonium bromide.

Entomocorticium sp. A extracellular polyphenol oxidase production and fungal biomass generation was analysed over 7 days of cultivation on 2% glucose-containing modified YpSs medium with gallic acid as an inducer in batch cultures (Fig. 2). The exponential phase continued until day 4 followed by stationary phase until day 6 and death phase. Phenol oxidase production was simultaneous to fungal growth, indicating growth-associated production of the enzyme, rather than a secondary metabolite. Accordingly, maximum activity (76.78 U/liter) was reached on day 4.

Effect of different inhibitors on polyphenol oxidase activity

The effect of a number of polyphenol oxidase (PPO) inhibitors on the activity of PPO in crude extracts of *Entomocorticium* sp. A was analysed with catechol as the substrate. We found inhibition in the presence of the copper-chelator salicyl hydroxamic acid (SHAM) and *p*-coumaric acid (PCA) (Fig. 3), both inhibitors of tyrosinase and catechol oxidase (Rescigno et al., 2002). In culture supernatants SHAM and PCA reduced enzyme activity to nearly half that of the control. Activity was enhanced by the non-ionic detergent cetyltrimethylammonium bromide (CTAB), reported as an inhibitor of laccase (Walker and McCallion, 1980).

These results, along with the outcome of the activity staining experiment, indicate that the enzyme in question is likely to be tyrosinase/catechol oxidase.

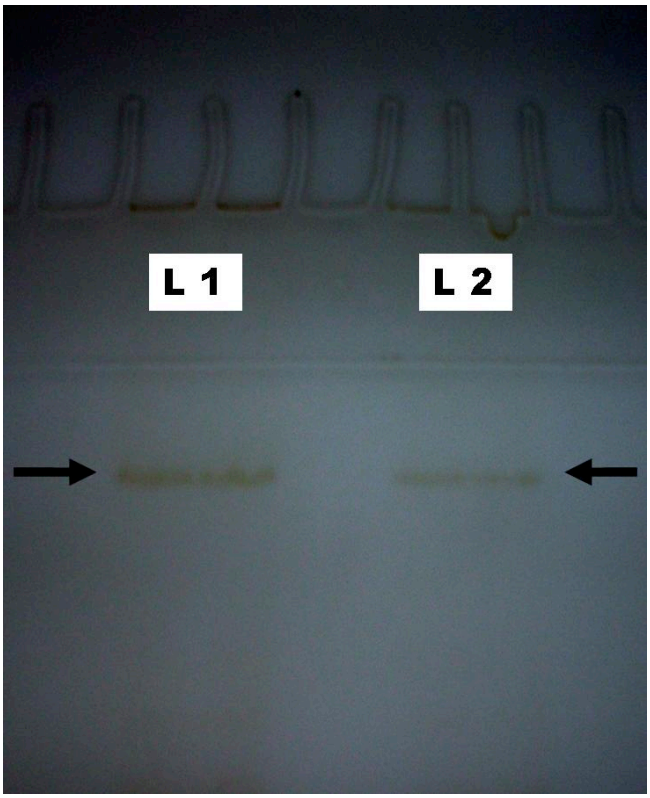

Figure 4. Activity staining of *Entomocorticium* sp. A concentrated culture supernatants for laccase, peroxidase and tyrosinase activities using 4-amino-*N,N*-diethylaniline (ADA), hydrogen peroxide (H_2O_2) and 4-tert-butyl catechol (*t*BC). L 1: Activity staining of 30-fold concentrated supernatant, L 2: Activity staining of 15-fold concentrated supernatant.

Determination of the type of polyphenol oxidase(s) by activity staining on polyacrylamide gel

Native-polyacrylamide gel electrophoresis (PAGE) gel staining for different types of PPO activities showed the following results. Staining with ADA, revealed no visible bands, indicating that the observed PPO activity was not

due to laccase. The addition of H_2O_2 also showed no band development, indicating the absence of peroxidase activity. Further staining with *t*BC, a visible band (Fig. 4) appeared indicating the presence of tyrosinase/catechol oxidase activity. Further analysis using L-tyrosine revealed a lack of monophenolase activity but the presence of only diphenolase (catecholase) activity.

4. Discussion

The SPB associated fungus *Entomocorticium* sp. A produces lignocellulose-degrading enzymes including cellulase and polyphenol oxidase. Previous studies have indicated that SPB larvae feed on fungal hyphae and spores, although none have been able to explicitly demonstrate this (Coppedge et al., 1995; Klepzig and Wilkens, 1997; among others). Guts of SPB appear to contain little in the way of either undigested cellulose or cellulolytic bacteria (Delalibera et al., 2005). Given this, and our results indicating extracellullar cellulase and polyphenol oxidase activities by a fungal symbiont, it appears increasingly likely that larval SPB get the majority of their nutrition from the fungal growth within their feeding chambers rather than from the phloem itself (Klepzig and Six, 2004). This further supports the importance of *Entomocorticium* sp. A as a key fungal symbiont in this system.

According to our results cellulase production was low as compared to other recent results with different fungi, such as *Penicillium echinulatum* (maximum total cellulase activity of 0.27 U/ml and maximum endoglucanase activity of 1.53 U/ml) (Martins et al., 2008). As for the polyphenol oxidase activity, results were found to be similar to those observed in other fungi (Ogel et al., 2006). Since polyphenol oxidases have an important role in cell defence via melanin synthesis, the results indicate that *Entomocorticium* sp. A not only nourishes larvae of SPB but also protects it against antagonistic fungi.

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