SUMMARY

Cercosporin is a perylenequinone pigment produced by fungi in the genus *Cercospora* which under light generates reactive oxygen species causing membrane damage and mortality of living cells. Our objectives were to demonstrate that fungal laccase, a lignolytic copper-containing enzyme, can degrade cercosporin and reduce cercosporin toxicity toward living cells. Cercosporin from *Cercospora beticola* and *Cercospora halyi* was treated with laccase from basidiomycete fungi (*Pleurotus ostreatus* and *Trametes versicolor*) in the dark and under constant light. Under these conditions the absorbance of the cercosporin decreased at 220, 279 and 295-500 nm within 10 min of reaction with laccase from either *P. ostreatus* or *T. versicolor*, indicating that basidiomycete laccase can induce changes in UV-visible spectra of cercosporin. The LIVE/DEAD® Bac Light™ VIABILITY kit and fluorescent microscopy showed more viable *E. coli* cells after incubation under light with cercosporin and laccase than with cercosporin alone. Lesions were apparent on sugar beet leaves exposed to cercosporin under light after 48 h, but leaves exposed to cercosporin and laccase showed visibly less damage. These data suggest that laccase from basidiomycete fungi can decrease the toxic effect of cercosporin toward microorganisms and plant tissue.

INTRODUCTION

Cercosporin (Kuyama and Tamura, 1957; Lynch and Geoghegan, 1979) is a perylenequinone pigment (Fig. 1) produced by fungi in the genus *Cercospora*, that belongs to a class of molecules called photosensitizers. Photosensitizers are characterized by the ability to be activated by light and to react with oxygen to produce highly toxic reactive oxygen species, such as superoxide anions (O$_2^-$) and singlet oxygen (1O$_2$) (Daub, 1982; Daub and Hangarter, 1983). These reactive molecules cause cell death of several organisms including mice, fungi and bacteria (Yamazaki et al., 1975; Macri and Vianello, 1979; Hartman et al., 1988; Daub et al., 1992).

*Cercospora* species appear to resist high concentrations of reactive oxygen species. Although the mechanisms providing cercosporin resistance to *Cercospora* is not fully understood yet, reports have demonstrated that *Cercospora* species were cercosporin-resistant organisms capable of protecting themselves against cercosporin and the damage of reactive oxygen species by the reduction and detoxification of cercosporin into an inactive form lacking photodynamic activity (Daub et al., 1992). Jenns et al. (1995) demonstrated that cercosporin-sensitive mutants of *Cercospora nicotianae* are unable to reduce cercosporin. Also, it has been reported that a protein similar to pyridine nucleotide reductase in the yeast *Saccharomyces cerevisiae* (Ververidis et al., 2001; Panagiotis et al., 2007) or an oxidoreductase produced by the bacterium *Xanthomonas campestris* pv. zinniae, can be used for cercosporin detoxification (Taylor et al., 2006).
Many fungi contain enzymes such as superoxide dismutase, peroxidases, catalase, and perhaps laccases and polyphenol oxidases that can help to remove or inactivate reactive oxygen species (Mayer et al., 2001). Laccase is a phenol oxidoreductase containing copper atoms which can oxidize organic and inorganic substrates, including both phenolic and non-phenolic compounds, to their corresponding quinones, with the concurrent reduction of molecular oxygen and release of water (Leonowicz et al., 1979; Thurston, 1994). Although little is yet known in the literature about the exact mechanisms by which laccases interact with reactive oxygen species in general, it was speculated that an intermediate reaction product of the activity of laccases, which is a free radical, could undergo non-enzymatic reactions with a variety of these damaging oxygen species (Ferrari et al., 1997; Duran and Esposito, 2000; Claus, 2004). It has been reported that white rot basidiomycete fungi (wood-decaying basidiomycetes) increase their extracellular discharge of laccase to detoxify their environment under culture conditions as a defense mechanism against oxidative stress conditioning factors, such as H$_2$O$_2$, (Cho et al., 2006) or paraquat which catalyzes the overproduction of superoxide anion radicals (O$_2^-$) and consequently other oxygen active species (Jaszek et al., 2006). Laccase has been proposed for a diverse range of applications, including waste detoxification and textile dye transformation (Rama et al., 1998, Abadulla et al., 2000; Fukuda et al., 2001), degradation of lignin (Bourbonnais et al., 1995) and humic acids (Hofrichter and Fritche, 1997) but little is known about the effects of laccase on cercosporin. In this preliminary study, we propose that laccase from basidiomycete fungi can decrease the toxicity of cercosporin toward living cells.

The first objective of this study was to measure the time evolution of the absorption spectrum of cercosporin from Cercospora beticola Sacc. C2 isolate and C. hayi L. Calpouzos with or without addition of laccase from two

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Treatments*</th>
<th>Number of viable cells (%)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>1h**</td>
</tr>
<tr>
<td>Dark</td>
<td>Cercorporin</td>
<td>96.51 A</td>
</tr>
<tr>
<td></td>
<td>Cercosporin + laccase</td>
<td>97.20 A</td>
</tr>
<tr>
<td></td>
<td>Laccase</td>
<td>97.28 A</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>96.51 A</td>
</tr>
<tr>
<td>Light</td>
<td>Cercosporin</td>
<td>38.79 B</td>
</tr>
<tr>
<td></td>
<td>Cercosporin + laccase</td>
<td>89.07 A</td>
</tr>
<tr>
<td></td>
<td>Laccase</td>
<td>95.32 A</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>97.06 A</td>
</tr>
</tbody>
</table>

*Controls contain neither cercosporin nor laccase; cercosporin and laccase concentration in LB medium are 100 µg ml$^{-1}$ and 100U ml$^{-1}$ respectively;  
**incubation time of bacteria after treatment.

Numbers followed by different letters within a column are significantly different by the Tukey-Kramer Honestly Significant Difference mean separation at $^*P \leq 0.05$.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dark</th>
<th>Light</th>
</tr>
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<tbody>
<tr>
<td>Cercosporin (20)*</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Cercosporin + laccase (24)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Laccase (20)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control (20)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Data were ranked on a visual basis with a scale of 1-5 to describe symptoms at the margins of the treated zones (1) no lesion; (2) dark brown lesions; (3) circular brown-yellow lesions; (4) circular light-yellow necrotic lesions (5) extended light-yellow, dry and crusty necrotic lesions.  
*Number of treated zones used for ranking.
white-rot basidiomycete fungi, *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. and *Trametes versicolor* (L.:Fr.) Pilát. We wanted to investigate the effects of cercosporin and/or laccase on living organisms, including bacteria and fresh sugar beet leaves. Although fungicides and appropriate cultural practices such as tillage and crop rotation (Ward et al., 1999; Windels et al., 1998) are successfully used to control diseases caused by *Cercospora* species, alternate methods of control would be desirable. This study seeks to develop insight for a possible approach to target cercosporin by antagonizing *C. beticola* with laccase-producing basidiomycete fungi to decrease *Cercospora* infection.

**MATERIALS AND METHODS**

**Strains, media and culture conditions.** *Cercospora beticola* isolate C2 (Whitney and Lewellen, 1976) was obtained from John J. Weiland, USDA, Fargo, ND. Prior to use, the fungus was purified by single conidia isolation. The isolate was maintained on solid potato dextrose agar (PDA, DIFCO, Detroit, MI) at 23°C with a 5 h photoperiod under fluorescent light (Universal/Hi-vision F32T8/TL735 tubes, Philips, NY, USA, light intensity 7,435±0.064 Rad [watt m⁻²]) for maximum production of cercosporin. Long-term stocks were maintained on PDA slants stored at 4°C.

**Cercosporin extraction.** Cercosporin from *C. beticola* isolate C2 was extracted following a modified procedure of Daub (1982). Briefly, potato dextrose agar (PDA, DIFCO, Detroit, MI) was inoculated with *C. beticola* conidia and incubated for 2 weeks at 25°C. The mycelial mat was then harvested, air-dried for 2-3 days, and ground in a spice blender for 30 sec. Ten mycelial mats (ca 10 g dry weight) were usually extracted at a time. Dried cultures were finely ground in a spice blender for 30 sec. Then materials were extracted in 300 ml ethyl acetate (CCI, USA) with agitation for 1 h. After decanting the solvent, the procedure was repeated four times until no further red pigment was extracted from the mycelia. The red pigment formed in culture is indicative of cercosporin. Long-term stocks were maintained from either *P. ostreatus* or *T. versicolor* in buffer without laccase.

**Bioassays on living microorganisms.** Bioassays on bacteria and fresh sugar beet leaves were developed to demonstrate the effects of cercosporin and laccase on living cells. Experiments on bacteria and fresh sugar beet leaves consisted of 4 treatments and 2 incubation conditions. The treatments were a (1) control, (2) cercosporin at 100 µg ml⁻¹, (3) cercosporin at 100 µg ml⁻¹ and laccase at 100 U ml⁻¹, and (4) laccase at 100 U ml⁻¹. Incubation conditions were in the dark or under constant fluorescent light (as described above in the Materials and Methods). MOPS buffer was used to dilute the chemicals. Only cercosporin from *C. beticola* C2 and *P. ostreatus* laccase were used for the bioassay on bacteria and on fresh leaves.

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**Assays on living microorganisms.** Bioassays on bacteria and fresh sugar beet leaves were developed to demonstrate the effects of cercosporin and laccase on living cells. Experiments on bacteria and fresh sugar beet leaves consisted of 4 treatments and 2 incubation conditions. The treatments were a (1) control, (2) cercosporin at 100 µg ml⁻¹, (3) cercosporin at 100 µg ml⁻¹ and laccase at 100 U ml⁻¹, and (4) laccase at 100 U ml⁻¹. Incubation conditions were in the dark or under constant fluorescent light (as described above in the Materials and Methods). MOPS buffer was used to dilute the chemicals. Only cercosporin from *C. beticola* C2 and *P. ostreatus* laccase were used for the bioassay on bacteria and on fresh leaves.

**Spectrophotometric conditions.** Laccase from the basidiomycetes *P. ostreatus* and *T. versicolor* was purchased from a commercial source (Tienzyme Inc. USA). Cercosporin used in the experiment was extracted from *C. beticola* C2 isolate (stock solution: 1 mg ml⁻¹) and from *C. bayi*, the latter commercially purchased (Sigma, USA; stock solution: 3.08 mg ml⁻¹). Laccase (50 U ml⁻¹) from either *P. ostreatus* or *T. versicolor* was added to cercosporin (50 µg ml⁻¹) in MOPS buffer (3-morpholino propane sulfonic acid, 20 mM, pH 6, Sigma-Aldrich, USA) and the mixture was incubated for 0, 2, 4, 6, 8, and 10 min at 25°C in the dark. UV-visible absorption spectrum from 200 to 600 nm was determined using a spectrophotometer (Lambda 20, Perkin-Elmer, USA) with a scanning speed of 960 nm min⁻¹. Controls were cercosporin in buffer without laccase.

**Cercosporin used in the experiment was extracted from *C. bayi* (Sigma-Aldrich, USA) was also used in this study. Both sources of cercosporin were dissolved in 100% ethanol, filtered through a 0.25 µm pore size acrodisc, and stored in RNase/DNase-free pyrogen tubes at -80°C.
and incubated for 1 h in the dark before adding to the bacterial suspension having a cell concentration of $10^6$ cells ml$^{-1}$. Treated samples and the controls were incubated 1 h on a shaker (200 rpm) at 25°C in the dark or under constant fluorescent light. The LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, USA) which provides two different nucleic acid stains, the SYTO 9 dye (green fluorescence) and propidium iodide (red fluorescence), to rapidly distinguish live bacteria with intact plasma membranes from dead bacteria with compromised membranes respectively, was used according to the manufacturer recommendations. After staining, the cells were rinsed for a few seconds with water then mounted on microscope slides using a medium (Vectashield, Vector Inc., USA) to prevent bleaching of fluorescence. Samples were observed with a confocal scanning laser microscope (Zeiss LSM 410) equipped with a 488 nm internal laser and two external lasers (633 nm and 543 nm) and connected to a Zeiss Axiocine S100 TV. SYTO 9 dye was detected with a 520 nm filter and propidium iodide with a 635 nm filter. A fluorescent oil immersion 100X Plan apochromat (N.A. 1.4) objective was used. Images were scanned to 512 x 512 pixels in each axis. Five images were scanned for each treatment. Phenix software (Microcosm, USA) was used to count dead and viable cells. Experiments were repeated at least 3 times. The Tukey-Kramer Honesty Significant Difference procedure, standard ANOVA was performed on counts of bacteria with statistical differences evaluated at $P \leq 0.05$ using the JMP statistical software package (version 6.0, 2005, SAS Institute, USA).

Fresh sugar beet (Crystal™ ACH 927 variety) leaves were collected from the field and used to test the effects of cercosporin and/or laccase on plant tissue. Delivery of cercosporin and laccase solutions was performed on sugar beet leaves by lightly touching the surface of the tissue using a syringe (1 ml) and needle (Precision Glide #23G1, Becton Dickinson, NJ, USA). The leaves were placed in a tray layered with wet tissue to prevent desiccation and incubated for 48 h either in the dark or under constant fluorescent light. After incubation, the morphology of the treated areas was assessed visually for each treatment. To rank the various treatments according to the morphology of the lesions, a scale of 0-4 (no lesion to heavy necrotic lesions) was used. Images were captured using a digital camera (Olympus Camedia C-4040 ZOOM). Three to four leaves were used for each treatment and the experiment was repeated twice.

**Fig. 2.** Decolorization of poly B-411 dye by *P. ostreatus* and *T. versicolor* laccases. Squares indicate reactions in which *Pleurotus ostreatus* laccase was added; circles, reactions where *Trametes versicolor* laccase was added; diamonds, control (no laccase added); results shown are averages oftriplicates.

**Fig. 3.** Effect of *P. ostreatus* and *T. versicolor* laccase on the time evolution of UV-visible absorption spectrum (200-600 nm) of cercosporin from *C. beticola* (isolate C2) in MOPS buffer at 25°C in the dark. Numbers indicate incubation time intervals in minutes; arrows show decrease in absorbance of cercosporin with time. Control is cercosporin without laccase.
RESULTS AND DISCUSSION

Spectrophotometric studies of laccase-treated cercosporin. Decolorization of pigments or dyes has been used by others as a standard assay to rapidly assess the oxidative activity by ring opening of ligninolytic enzymes of white-rot basidiomycetes, including laccases and peroxidases (Gold et al., 1988, Rodriguez et al., 1999; Claus et al., 2002). In our case, P. ostreatus and T. versicolor laccases used in this study were able to decolorize (data not shown) and decrease the absorbance of the pigment Poly B-411 (Fig. 2). After 5 min of reaction, a more drastic decrease in the absorbance of Poly B-411 was found in preparation with P. ostreatus laccase (OD 2.47 ± 0.09) than with T. versicolor laccase (OD 5.63 ± 0.12) when compared to the control without laccase added (OD 6.4 ± 0.10). No significant changes in the enzyme activity were found after the first 5 min. These results indicating that both laccases have oxidative activity potential are in accordance with Platt et al. (1985) who have demonstrated that P. ostreatus laccase can oxidize the pigment Poly B-411 in both liquid and solid medium and with Wong and Yu (1999) who have showed that T. versicolor laccase can degrade synthetic dyes with typical chromophores (anthraquinone, azo and indigo).

The UV-visible absorption spectra of cercosporin extracted from either C. beticola C2 or C. hayi in the course of incubation with P. ostreatus or T. versicolor laccase in the dark were investigated in this study. Figure 3 illustrates the UV-visible absorption spectrum of C. beticola C2 cercosporin. The maximum absorption of cercosporin from the two sources, at 220, 279, and 495-500 nm, was in good agreement with spectra reported previously by Yamazaki and Ogawa (1972) in their study of the chemistry and stereochemistry of cercosporin from Cercospora kikuchii Matsumoto & Tomoy. Cercosporin from C2 and C. hayi (data not shown) presented similar visible decreases in absorbance at 220, 279 and 495-500 nm within 10 min of reaction with P. ostreatus or T. versicolor laccase compared to the controls without laccase added. For example, cercosporin from C2 showed decreases in absorbance of 48.61%, 47.97%, and 53.13% at 220, 279, and 500 nm respectively with P. ostreatus laccase, and 35.50%, 35.09%, and 41.79% with T. versicolor laccase, and a peak shift of 21 nm at 500 nm. A gradual decolorization of cercosporin was noted from deep red to light pink for both sources of cercosporin during the enzymatic reaction. These results indicate that laccase from basidiomycetes can degrade C. beticola and C. hayi cercosporin. Since the enzymatic reaction was performed in the dark where no reactive oxygen species are generated, we can assume that the degradation of cercosporin was due only to the activity of the basidiomycete laccase.

Although a direct comparison cannot be made, these results indicate that both P. ostreatus and T. versicolor laccases react not only with a perylenequione pigment (cercosporin) but also with an anthraquinone-based pigment (Poly B-411). Edens et al. (1999) have shown that laccase from Gaeumannomyces graminis (Sacc.) Arx & D.L. Olivier, a fungus causing take-all disease in wheat and barley can decolorize Poly B-411, an effect suggesting a role in lignin degradation. Anthraquinone is an organic compound whose structure serves as a basic building block for a number of naturally occurring pigments. Interestingly, other pigments produced by C. beticola are polymeric anthraquinone-based pigments, such as cebelin (Jalal et al., 1992) and beticolin (Goudet et al., 2000) which cause a broad range of cytotoxic effects on plants and animals similar to cercosporin. It would be informative to investigate whether or not ligninolytic enzymes, including laccases and peroxidases can affect the toxicity of the anthraquinone-based pigments produced by C. beticola.

Bacteria and plant tissue response to cercosporin and laccase. Table 1 shows the effects of cercosporin from isolate C2) and/or laccase from P. ostreatus on bacterial cells after incubation for 1, 2 and 3 h in the dark and under constant light. The percentage of the total number of viable cells between treatments was not significantly different in the dark but was significantly the lowest when cells were exposed to cercosporin for 1, 2, and 3 h under constant light. For example after 1, 2, and 3 h under light, the number of viable cells was respectively 38.79%, 54.44% and 53.28% in cercosporin treatment and 97.06%, 97.67% and 97.22% in the controls without cercosporin added. This indicates that cercosporin was toxic to E. coli bacteria under constant light. This is probably due to the production of toxic oxygen radicals by cercosporin known to cause oxidation of membrane fatty acids, proteins, carbohydrates and nucleic acids leading to cell death. However, the number of viable cells exposed under light to the mixture of cercosporin and laccase was significantly higher than when cells were exposed to cercosporin alone. For example, after 1 h under light, the number of viable cells was 89.07% in the cercosporin-laccase treatment, compared to 38.79 % in the treatment with cercosporin alone. This suggests that laccase from P. ostreatus basidiomycete reduced the toxicity of cercosporin extracted from C2 isolate to E. coli.

Figure 4 shows the necrotic symptoms of sugar beet leaves induced by C. beticola isolate C2 cercosporin and/or P. ostreatus laccase. After 48 h of incubation in the dark, no striking difference in the necrosis of the treated areas was detected compared to the controls, except that in the controls the dark spots observed in all treatments were caused by cell injury during delivery of the compounds using the tip of a sharp needle. However, incubation under constant light of cercosporin-treat-
ed samples showed dark spots surrounded by circular to irregular yellow-brown margins which appear dry and crusty. These necrotic lesions become enlarged and occasionally extended to the whole treated area at the end of the incubation period. However, when samples were treated with the cercosporin-laccase mixture, necrotic zones were greatly reduced, compared to the cercosporin-treated samples. No lesions were found on leaf surfaces exposed to laccase alone or in the controls under light. After 48h of incubation under light, the rank of the data based on visual basis was cercosporin > cercosporin-laccase mixture > laccase, control (Table 2). This suggested that the toxicity of cercosporin toward sugar beet leaf tissue can be reduced by basidiomycete laccase. The damaging effect of extracted cercosporin on sugar beet leaves was previously reported (Steinkamp et al., 1981) using electron microscopy to describe the disruption of the plasmalemma, tonoplast, and chloroplast membranes. More recently a similar study involved tobacco leaves (Panagiotis et al., 2007).

Studies have indicated that soil is a source of primary inocula of leaf spot disease of sugar beet (Nagel, 1938; Giannopolitis, 1978). Crop debris has been demonstrated to serve as a source of inoculum of C. beticola for at least two years (Jones and Windels, 1991; McKay and Pool, 1918). A recent 3 year study (Khan et al., 2008) reported that inocula of C. beticola as infested beet residue survived longest (22 months) on the soil surface, with inocula buried 10 or 20 cm deep surviving for 10 months. Thus, if the infested debris is incorporated, the chances of the pathogen to survive are decreased but such debris remaining on the soil surface would serve as the source of primary inoculum. To target the primary inoculum, we thus propose the use of an enzymatic approach to diminish the chances of the pathogen to persist in leaf debris. Since saprophytic basidiomycete fungi which produce large amount of lignolytic enzymes, such as laccases, peroxidases, and lignin peroxidases, are important decomposers of crop residue (Thorn et al., 1996; Stubbis et al., 2004), the use of laccase-producing basidiomycete fungi as antagonists to control C. beticola in soil is an approach which we are currently investigating. The question arises as to whether adding laccase as an active catalyst on C. beticola-infested plant debris could contribute to ultimately decreasing the inoculum of the pathogen in soil. Considering that cercosporin helps the pathogen to protect itself against antagonists on plant tissue, if the mode of action of laccase on cercosporin can attenuate the defense mechanisms of the pathogen, this might offer a new approach for the development of supervised control systems. Surface-borne inoculum could thus be the target of laccases that could both degrade cercosporin being produced for antibiosis bystromata on the soil surface against antagonists and decompose beet leaf tissue bearing stromata. Immunological methods could be used to detect and quantify C. beticola in natural soil to monitor its inoculum (Caesar-TonThat et al., 2007). Extracellular enzymes such as laccase are inactivated by absorption (particularly to clay), denatured by chemical factors in soil (e.g., pH, ionic composition of soil solution), or serve as substrates for proteolytic microorganisms. To improve the chance of laccase to persist long enough to degrade cercosporin, laccase activity could be improved and stabilized by immobilization of the enzyme (Leonowicz et al., 1988; Palmieri et al., 1994; Sarkar et al., 1989) on solid supports (e.g. kaolinite) to increase its resistance to degradation by proteases and protect it against changes in pH, temperature, or fluctuations of ionic strength. However, large-scale production of laccase may not be economically feasible, and thus laccase-producing basidiomycetes adapted to the soils involved may be the best approach.

ACKNOWLEDGEMENTS

We thank M. E. Daub, R. G. Thorn, R. J. Kremer, and B. Iversen for reviewing the manuscript and for their valuable advice.

REFERENCES


Fig. 4. Necrotic symptoms of fresh sugar beet leaf surfaces induced by cercosporin (100 µg ml⁻¹) from *C. beticola* (C2) and/or laccase (100U ml⁻¹) from *P. ostreatus*. Incubation was for 48 h at 25°C in the dark or under constant light.
Effects of laccase on cercosporin


