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EVIDENCE OF A LATENT OXIDATIVE BURST IN RELATION TO WOUND REPAIR IN THE GIANT UNICELLULAR CHLOROPHYTE *DASYCLADUS VERMICULARIS*[1]

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We investigated the kinetics and composition of the second phase of the wound repair process of *Dasycladus vermicularis* ([Scropoli] Krasser) using fluorescent probes, chromatography, UV spectroscopy, and histochemistry. Our new evidence supports the hypothesis that the second phase of wound repair (initiated at approximately 35–45 min post-injury) is based on the activation of an oxidative burst that produces micromolar H_2O_2 levels. These results provide evidence of peroxidase activity at the wound site, real-time measurements of an oxidative burst, and catechol localization in wound plugs. Strong evidence is presented indicating that the biochemical machinery exists for oxidative cross-linking to ensue in the wound-healing process of *D. vermicularis*.

Key index words: **algae; coumarins; *Dasycladus vermicularis*; hydrogen peroxide; oxidative burst; peroxidase; wounding**

Abbreviations: **DCFH-DA, 2′,7′-dichloro-dihydrofluorescein diacetate; DDC, diethyldithiocarbamate; DPI, diphenyleneiodonium; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; RP, reversed phase**

Wound repair represents a vital process that is essential for the survival of sessile coenocytic macroalgae. Because no other cells assist or mediate in the organism's response to injury, survival is clearly based on the individual cell's ability to repair itself in an expeditious manner. Injury in a shallow water marine environment may arise from numerous causes, including strong surf action, sand abrasion, and grazing by a variety of predators, parasites, or epiphytes. Although multicellular organisms have the capability of producing regenerative tissue adjacent to a wound, unicellular algae must rely on an instantaneous sealing mechanism to avoid the loss of irreplaceable cytoplasmic contents.

In a previous report (Ross et al. 2005), we described the initial events involved in the wound-healing process in *Dasycladus vermicularis* using a series of biomolecular detection systems. The results support our hypothesis that the initial wound repair mechanism is based on a rapid gelling process (<1 min) that uses a complementary lectin–carbohydrate ligand system. In this report we provide evidence for the onset of an oxidative burst that occurs at the site of injury. This burst is coincident with the onset of browning and hardening of the wound plug.

Oxidative bursts have been described in a number of terrestrial plants (Doke 1983a,b, Lamb and Dixon 1997, Wojtaszek 1997) as well as in red, green, and brown algal model systems (Collen and Pedersen 1994, Bouarab et al. 1999, Weinberger and Friedlander 1999, Küpper et al. 2001, 2002, Choo et al. 2004).

Although there are no reports in the literature associating the oxidative burst with injury in a marine macrochlorophyte, injury in the form of mechanical stress has been shown to create reactive oxygen species (ROS) in groundnut (Epperlein et al. 1986), in the marine rhodophyte *Eucheuma platycladum* (Collen and Pedersen 1994), and in the leaves of other higher plant species (Orozco-Cardenas and Ryan 1999). In most cases involving terrestrial plants and different algal lineages, the oxidative burst has been implicated in disease resistance (Lamb and Dixon 1997, Küpper et al. 2002), whereas this reaction has only rarely been investigated in the context of injury (Orozco-Cardenas

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et al. 2001). The discovery of hydroxycoumarins in *Dasycladus* (Menzel et al. 1983) along with the localization of peroxidase and H_2O_2 described herein presents strong evidence that the biochemical machinery exists for oxidative cross-linking to ensue in the wound-healing process of *Dasycladus vermicularis*.

MATERIALS AND METHODS

Algal material. Juvenile and adult specimens of *D. vermicularis* (Scropoli) Krasser were collected live and cultured or flash frozen with liquid N_2 off Indian Key, Florida (USA) and Cay Sal Bank, Bahamas. For laboratory studies, a unialgal culture (strain LB 2685 *Dasycladus vermicularis*) was acquired from the Culture Collection of Algae at the University of Texas at Austin. Cultures were maintained using a 12:12-h light:dark photoperiod (at a photon flux density of 50–60 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and at 25° C). Cultures were grown in Muller's medium (Berger and Kaever 1992) that was filtered through 0.22-μm membranes (Millipore, SCGPT10RE Millipore, Bedford, MA, USA) before introduction of the algae.

Detection of browning and hardening. Dasycladus cells were injured via a complete mid-thallus cut in 2 mL of artificial seawater on a glass depression slide. Algal specimens were observed using a Zeiss Universal microscope (Zeiss, Jena, Germany) in conjunction with the Pixera 120es Application suite™ photo program (Pixera, Los Gatos, CA, USA). The degree of browning was qualitatively determined over a 24-h period. Photographs were selected from a minimum of 10 experimental replicates. Hardening of the plug was monitored by probing the wound plugs with microforceps.

In vitro simulation of browning. Isolated trihydroxy coumarin was dissolved in ethanol, and UV-Vis absorbance was subsequently measured on an Ultraspec 2000 (Pharmacia Biotech, Cambridge, UK) interfaced with Swift applications software (Pharmacia Biotech). *In vitro* oxidation was carried out by incubating a 5-mM solution of the isolated trihydroxy coumarin with 25 units of type VI-A horseradish peroxidase (Sigma, St. Louis, MO, USA) and 100 μM H_2O_2. The reaction was incubated in a 1-mL Eppendorf tube containing 10 mM sodium phosphate buffer, pH 6.8, at 30° C for 5 min.

To establish a relation between browning and the oxidation of trihydroxy coumarin, 5 mM of trihydroxy coumarin, 25 units of horseradish peroxidase, and increasing amounts of H_2O_2 were incubated as described above. One unit of type VI-A horseradish peroxidase will oxidize 1 μmol of 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) per min at 25° C at pH 5.0. The reaction mixture was scanned at 420 nm to establish an analytical correlation between H_2O_2 concentration and the formation of an oxidized product at 420 nm.

Effects of modified seawater on wound plug browning rates. To examine the effects of modified seawater on browning rates in *D. vermicularis* filtered seawater or Muller's medium was adjusted to a variety of pH values with HCl or NaOH and their effect on browning rate assessed relative to control preparations. Chemicals were purchased from Sigma and dissolved in aqueous stock solutions before addition to the algae.

Fluorescence microscopy. The 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) probe is a nonfluorescing nonpolar compound. When this compound reacts with cellular esterases, the diacetate group is cleaved off to yield the polar compound DCFH. Oxidation of DCFH by ROS yields the fluorescent product DCF (Collen and Davidson 1997, Rijstenbil et al. 2000).

The compound DCFH-DA was dissolved in DMSO in 10-mM aliquot stocks (stored at –80° C). Individual specimens of *D. vermicularis* were injured by a single transverse cut with a scalpel either apically or in the mid-thallus region. Forty-five minutes postinjury, the algae were transferred from 5 mL Muller's medium into an incubation mixture of 5 mL Muller's medium and 5 μL DCFH-DA stock. The algae were incubated in the dark for 15 min as described by Küpper et al. (2002). Before examination, the algal thallus was rinsed in Muller's medium and placed in 5 mL fresh media for 5 min. Imaging was taken using a Zeiss Universal microscope in conjunction with the Pixera 120es Application suite™ photo program.

Oxidative burst measurements. The concentration of hydrogen peroxide present in the medium around the algal specimens was determined from a protocol adapted from Weinberger and Friedlander (1999). Briefly, the H_2O_2 release was calculated by measuring the oxidation of DCFH-DA in the presence of esterase. Stock solutions of H_2DCF-DA (10 mM, see above) and esterase (E.C 3.1.1.1, Sigma 41 U/mL) were prepared in DMSO and Muller's medium, respectively. A total of 0.35 g ($n = 25$ cells) of cells was towel blotted dry and weighed. Each cell was then transversally cut in half to make 50 fragments (or 50 exposed injured surfaces). The 50 fragments were placed in a beaker of 50 mL Muller's medium and slowly mixed for a continuous exchange of reagents across the algal surface. The wavelengths of excitation and emission were 488 and 525 nm, respectively. Reaction mixtures included 1000 μL of algal extract, 0.82 U esterase, and 25 μM DCFH-DA for a total volume of 2000 μL. For calculating the concentration of H_2O_2 present in the samples, calibration with a standard curve was carried out at least once during any series of experiments. Standard curves were composed with known amounts of H_2O_2 in addition to 0.82 U esterase and 25 μM DCFH-DA for a total reaction volume of 2000 μL. The fluorometric quantification of H_2O_2 was analyzed for a 120-min time interval on an LS50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT, USA).

Origin of the oxidative burst. To identify the origin of the oxidative burst, selected inhibitors were assayed for their physiological selectivity on the signal transduction events leading up to the oxidative burst. Diphenyleneiodonium (DPI) is a suicide inhibitor of NADPH oxidases that binds irreversibly to the flavenoid group of the membrane-associated gp91-phox subunit (O'Donnell et al. 1993). A 1-mM stock solution was prepared in DMSO. Catalase (1000 U/mL stock; 1 unit being defined as the amount that decomposes 1 μmol $\cdot$ min^{-1} at a H_2O_2 concentration of 10 mM in pH 7 at 25° C) catalyzes the breakdown of H_2O_2. Sodium azide (NaN_3; 20 mM stock) targets a variety of redox enzymes, most notably heme-based peroxidases. Stock solutions of catalase and NaN_3 were prepared in Milli-Q H_2O (Millipore). Quinacrine targets flavin-dependent redox enzymes in particular oxidases. A 1-mM stock solution was prepared in ethanol.

Histochemical staining for O_2^- detection. Individual specimens of *D. vermicularis* were injured in the presence of diethyldithiocarbamate (DDC), a strong inhibitor of superoxide dismutase (Auh and Murphy 1995, Küpper et al. 2002). Algae were injured and immediately placed in 10 mM potassium phosphate buffer (pH 7.8) with varying amounts of DDC. After a 20-min incubation period, the algae were moved to a 10-mM solution of phosphate buffer (pH 7.8) with 0.1% nitroblue tetrazolium (NBT).

Localization and visualization of peroxidase. Individual cells were injured by a mid-thallus transverse cut, and wound plug formation was monitored under LM. The reaction mixture included o-dianisidine staining: 1 mM o-dianisidine, 1 mM H_2O_2 in artificial seawater (pH 7.8); 10 mM NaN_3 was added to the mixture for inhibitory studies. Photographs were selected from a minimum of 10 replicate experiments.

Peroxidase extract isolation and characterization. Partial isolation: Forty grams of flash frozen algae were pulverized into a homogeneous powder with liquid N_2, mortar and pestle. The

solid powder was subsequently mixed (4° C cold room) into 400 mL of 100 mM Tris HCl (pH 8.0) with the addition of 0.1% Triton X-100 and poly(vinylpolypyrrolidone) (6% weight/volume buffer). Extracts were centrifuged at 20,000*g* at 4° C for 10 min using a 5C-RB superspeed centrifuge (Sorvall, Asheville, NC, USA). Supernatants were collected and mixed with 100 mM $BaCl_2$ for 10 min. Upon centrifugation, under the prior conditions, the supernatant was collected and taken to 30% ammonium sulfate saturation. Upon centrifugation, the supernatant was collected and taken to 90% ammonium sulfate saturation. After the final centrifugation step, the peroxidase-active pellet was collected, resuspended in a minimal amount of Milli-Q water, and dialyzed overnight against several high volume changes of 1 mM Tris-HCl (pH 8.0) at 4° C.

The dialyzed extract was batch loaded onto a High Trap DEAE Sepharose FF 1 mL anion exchanger (Amersham Biosciences, Buckinghamshire, UK) using an AKTA FPLC system (Amersham Biosciences). A linear gradient was established using 100 mM Tris-HCl (pH 8.0) – 100 mM Tris-HCl (pH 8.0) + 1 M NaCl. Active fractions were pooled and subsequently desalted and concentrated using Centricon Plus-20 5000 NMWL polyethersulfone centrifugal filters (Millipore). Protein concentration was determined for the extract via Bradford, and specific activities were tested under a variety of buffer ranges and H_2O_2 concentrations.

pH optima: The specific activity of the partially purified peroxidase extract was studied in selected buffers. Buffers used were 100 mM Tris-sulfate (pH 8.0–7.5), 100 mM phosphate (pH 7.0–6.5), and 100 mM acetate (pH 6.0–3.0).

H_2O_2 effect: The effect of H_2O_2 on peroxidase activity was observed by incubating the partially purified peroxidase extract with o-dianisidine (5 mM) at various concentrations of H_2O_2 in acetate buffer (100 mM, pH 5.5).

Catechol extraction and chromatography. Previously frozen samples of *D. vermicularis* were exhaustively lyophilized and subsequently extracted with 90% MeOH/10% Milli-Q water with a drop of HCl for 1 h. The extract was subsequently centrifuged at 5000 rpm on a TJ-6 centrifuge (Beckman, Palo Alto, CA, USA). The supernatant was collected, and an equal volume of hexanes was added and mixed. The upper hexane layer was removed, and the lower aqueous layer was brought from 9:1 (MeOH/H_2O) to 1:1 (MeOH/H_2O). An equal volume of ethyl acetate was added and mixed. The upper ethyl acetate fraction was removed and the lower aqueous phase (1:1 MeOH/H_2O) was submitted to vacuum chromatography over an Avicel® cellulose column (4 × 7 cm, Fisher Scientific, Atlanta, GA, USA). The initial noncellulose binding aqueous flowthrough contained the most abundant fraction of coumarins of interest. Upon vacuum filtration through qualitative no. 1 Whatman filter paper, the aqueous flowthrough was subsequently chromatographed on an Affi-Gel™ 601 column (Biorad, Hercules, CA, USA). The gel was poured into a 1 × 8 cm column, washed with distilled water, and activated by washing with one volume of 10 mM NaOH solution. Excess NaOH was removed with a 0.75 column volume distilled water rinse. The aqueous sample was applied at this point. Bound contaminating molecules were washed with 2 M urea. The column was washed with several void volumes of 0.1 M mono/di basic phosphate buffer (pH 7.5) to remove residual urea. Bound cis-diol derivatives were eluted from the column with several washes of 0.025 M acetic acid.

The analytes released by elution with acetic acid from the Affi-Gel 601 were immediately run on a reversed-phase (RP)-HPLC system. The mobile phase was an isocratic solvent system consisting of 85% Milli-Q water + 0.2% acetic acid and 15% acetonitrile run at 0.5 $mL \cdot min^{-1}$. The column (stationary phase) was a Microsorb MV C18, 5 μm particle size, 100 Å pore size (4.6 × 250 mm, Varian, Walnut Creek, CA, USA). The instrument used was an L-6200 A Intelligent pump coupled to an L-4200 UV-Vis detector (Hitachi, San Jose, CA, USA). Eluting compounds were detected at 274 nm. The data analysis software package used was the D-7000 HSM (Hitachi).

Samples were acid hydrolyzed in 0.5 N HCl at 100° C for 30 min. The a-glycone form was extracted with ethyl acetate and dried down under a stream of N_2.

Fast-Blue BN salt for activated catechols. Fast-Blue BN salt (o-dianisidine [tetrazotized]) is a histochemical stain that reacts with activated phenolic moieties to turn a rust-like color (O'Brien and McCully 1981). Fast-Blue BN salt was dissolved in artificial seawater (1 $mg \cdot mL^{-1}$) to make a stock solution. One hundred microliters of this solution was added to injured algae on a depressed glass slide in 2 mL of artificial seawater.

UV difference spectroscopy of catecholic compounds. Aromatic dihydroxyl placement may be characterized by the fact that the UV absorbance spectrum of catechols is modified by complexation of the catechol with borate at high pH. The difference spectrum resulting from the subtraction of the absolute spectrum in 0.2 M HCl from that in 0.2 M sodium borate (pH 8.5) is characteristic of catecholic moieties and may be used as a diagnostic tool (Waite 1984).

Aliquots of N_2-dried RP-HPLC purified catechols were suspended in 0.1 mL deionized water. The stock solution of catechols was diluted 1:10 in two separate solutions: 1) 0.2 N HCl and 2) 0.2 M sodium borate (pH 8.5). λ_{max} values were compared between the HCl solution (using water as a reference), the borate solution (using water as a reference), and finally the borate solution (using the HCl solution as a reference).

RESULTS

Browning. To determine the time frame in which algal wound plugs undergo oxidatively induced browning, specimens were injured and viewed under LM for up to 24 h postinjury. Under controlled circumstances (algae injured in seawater with no experimental modifications), the algal wound plugs underwent a browning reaction that started 35 to 45 min postinjury and intensified as a function of time (Fig. 1). As can be seen from Figure 1, there is a marked increase in the spread of the natural browning that reached a maximum after 24 h in all preparations examined.

The factors preventing or promoting the rate of wound plug browning were studied using a variety of experimental treatments over a 24-h period (Table 1). The depletion of calcium or sulfate in the seawater media had no effect on the rate of browning. Modifying the seawater by doubling or halving the osmotic strength, the addition of EDTA, or KBr (supplement for bromoperoxidase) had no effect on the rate of browning either. When the pH of the seawater was 3 or 10, the onset of the browning was delayed from 45 min (control time) to 3 h. By adjusting the pH of the seawater to 5.5, the onset of browning was accelerated to 25 min. The optimal pH for the partially purified peroxidase extract was 5.5, suggesting involvement with the browning process. Interestingly, the time until browning was accelerated in the presence of 1 mM H_2O_2, whereas the addition of 1 mM ascorbic acid or 0.1% β-mercaptoethanol completely prevented the browning process. The addition of 0.1% SDS delayed

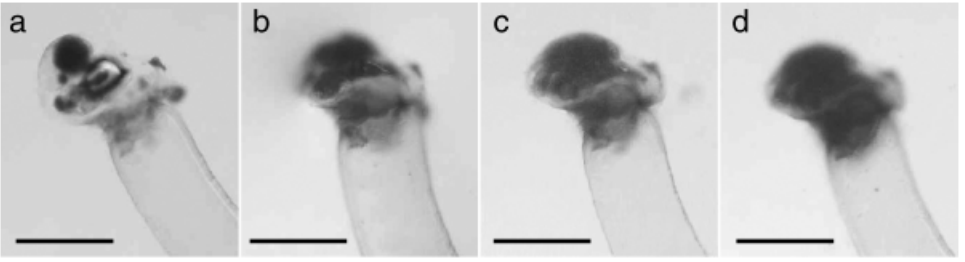

FIG. 1. Browning of plug as a function of time: (a) 1 h postinjury, (b) 2 h postinjury, (c) 3 h postinjury, (d) 24 h postinjury. Scale bar, 4 mm.

the browning response from 45 min to 10 h. The addition of 200 U catalase (in 5 mL seawater) or 1 mM NaN_3 significantly delayed the browning response to 24 h postinjury. Overall, we concluded that the browning is an oxidative process that can predictably be accelerated by promoting oxidation or delayed with antioxidants.

To examine whether the delay in browning in *D. vermicularis* is due to a transcriptional or translational regulation process, individual specimens were cut in half. Because this algal species has a single nucleus located in the rhizoidal region, this type of injury results in a basal section containing the rhizoidal nucleus and the distal section containing an anucleate apical fragment. It was observed that both sections were still capable of browning. The addition of cycloheximide and actinomycin D showed no inhibitory effect upon browning (Table 1). It seems that the peroxidase enzyme is probably already present yet has a significant latency period.

In vitro simulation of oxidative browning. Isolated trihydroxy coumarin dissolved in ethanol displayed a UV-Vis spectrum that was identical to the report made by Menzel et al. (1983). Absorption maxima were observed at 215, 265, and 344 nm (Fig. 2). The reduced trihydroxy coumarin had a coupled resonance effect that is indicative of well-defined absorbance peaks.

Upon enzymatic oxidation of the coumarins with horseradish peroxidase and hydrogen peroxide, a loss of the three peaks was noted. Conversely, an absorption increase in the range of 400–450 nm was noted, typical of melanin-like products (Fig. 2). We believe the shifts in absorbance maxima may be attributed to electron rearrangement via oxidation resulting in quinone formation.

To establish a relationship between browning and the oxidation of the trihydroxy coumarin, 5 mM of trihydroxy coumarin, 25 units of horseradish peroxidase, and increasing amounts of H_2O_2 were incubated to simulate the oxidative burst in *D. vermicularis*. As can be seen in Figure 2 (inset), increasing concentrations of H_2O_2 produced an increased absorbance at 420 nm. The concentration curve was typical of an enzymatic oxidation reaction that saturated at about 300 μM H_2O_2. The apparent K_D of H_2O_2 was estimated to be 80 μM. The omission of peroxidase or either cosubstrate (trihydroxy coumarin or H_2O_2) resulted in the failure to observe a browning reaction.

Oxidative burst. Figure 3 depicts the fluorescence microscopy results of incubating injured algae with DCFH-DA. Healthy (uninjured) specimens chronically release low levels of H_2O_2 as a byproduct of

TABLE 1. Treatments used in determining the effects of enzymatic browning in *Dasycladus vermicularis* ($n = 10$ individuals per treatment)

Treatment	Average Time Until Onset of Browning
None (ASW pH 7.8)	40 ± 10 min
ASW (no Ca^{2+})	40 ± 10 min
ASW (no SO_4^{2-})	40 ± 10 min
ASW + 1 mM EDTA	40 ± 10 min
ASW + 100 mM KBr	40 ± 10 min
1/2 strength ASW	40 ± 10 min
2× strength ASW	40 ± 10 min
ASW + cycloheximide (30 $\mu g \cdot mL^{-1}$)	40 ± 10 min
ASW + actinomycin D (20 $\mu g \cdot mL^{-1}$)	40 ± 10 min
ASW (pH 3.0)	3.0 ± 0.5 h
ASW (pH 10.0)	3.0 ± 0.5 h
ASW (pH 5.5)	25 ± 10 min
ASW + 1 mM H_2O_2	25 ± 10 min
ASW + 1 mM ascorbate	No browning
ASW + 0.1% 2-mercaptoethanol	No browning
ASW + 0.1% SDS	10 ± 1 h
ASW + 100 mM NaN_3	> 24 h
ASW + 200 U catalase	> 24 h

Values are means ±SE. ASW, artificial seawater.

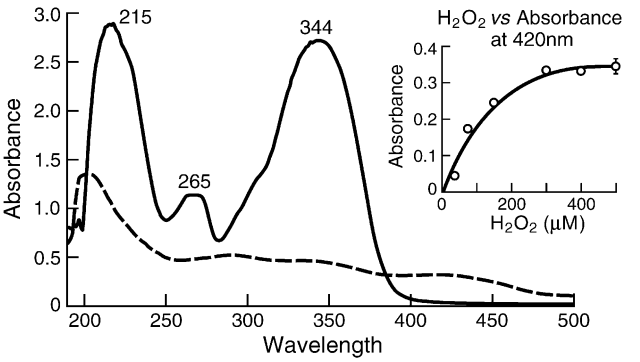

FIG. 2. *In vitro* simulation of browning. UV-Vis absorbance of reduced and oxidized trihydroxy coumarin. The solid line represents the coumarin in the reduced state, whereas the dashed line represents the oxidized molecule in the presence of peroxidase and hydrogen peroxide. Inset shows oxidation of trihydroxy coumarin (monitored at 420 nm) with increasing concentrations of H_2O_2.

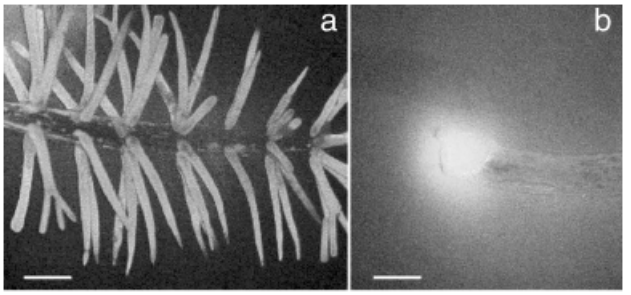

FIG. 3. Detection of H_2O_2 with DCFH-DA. (a) Uninjured specimen (control). (b) Injured specimen (45 min postinjury) displaying strong reaction with probe during oxidative burst. Scale bar, 4 mm.

respiration and photosynthesis (Fig. 3a, depicted as a lighter shade of gray surrounding the algal siphon). Upon initial injury there was a moderate increase in H_2O_2. However, by 40 min postinjury a sharp increase in fluorescence was observed (Fig. 3b), reflecting the sudden release of H_2O_2 into solution.

Kinetics of the origin of the oxidative burst. The concentration of H_2O_2 emitted into solution was calculated as a function of algal fresh weight. Using the fluorometric quantification of H_2O_2 as a function of injured algal mass (as described above), Figure 4 shows the production of H_2O_2 as a function of time postinjury. After a 40-min lag time postinjury, a sudden increase in H_2O_2 was observed in injured specimens. Repeated experiments in which 1-min samples were sequentially analyzed over time after injury demonstrated a gradual increase in the rate of appearance of H_2O_2 from the time of injury. At 40 min the rate of H_2O_2 formation reached about half maximum (Fig. 4). At 90 min a steady-state rate is seen. In all succeeding studies we used rate values corresponding to the 45-min level ($\pm$ 0.875 $\mu mol \cdot g\ FW^{-1} \cdot min^{-1}$).

Source of ROS production. Inhibition of the oxidative burst by DPI (suicide inhibitor of mammalian neutrophil and plant NADPH oxidases) suggests that *D. vermicularis* may possess a flavoprotein-containing

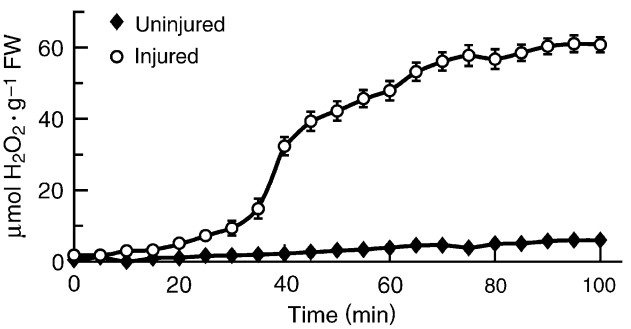

FIG. 4. Fluorometric quantification of H_2O_2 generated after injury. The concentration of H_2O_2 emitted into solution was calculated as $\mu mol\ H_2O_2 \cdot g\ FW^{-1}$ as shown in the ordinate ($n = 10$ individuals).

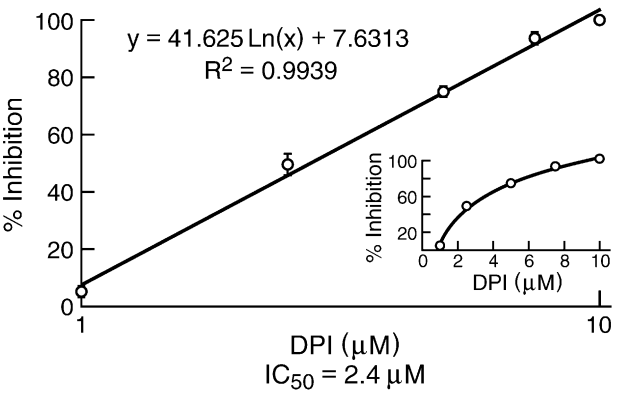

FIG. 5. Inhibition of H_2O_2 synthesis in injured *Dasycladus vermicularis* treated with DPI on a semi-log scale. Inset is the arithmetic plot showing an exponential increase in inhibition with increased concentrations of DPI.

NADPH oxidase complex that is analogous to those involved in the production of ROS in animals and higher plants (Groom et al. 1996, Jabs et al. 1997). Diphenyleneiodonium was very selective in the inhibition of H_2O_2 formation as noted by the IC_{50} of 2.4 uM (Fig. 5). Diphenyleneiodonium is an irreversible inhibitor in which the inhibition is linear throughout the range of the concentrations tested (there is an apparent single rate constant, $[A] + [B] \rightarrow [AB]$, that reached 100% at 10 μM). Hence, complete inactivation occurred within a 10-fold concentration range. Our data show that DPI acted within a narrow range and was highly reproducible ($r^2 = 0.9939$), suggesting that a uniform population of binding sites for the drug are present, which we assume to be an NADPH oxidase complex. Quinacrine, an inhibitor of flavin dependent redox enzymes (Auh and Murphy 1995), gave an IC_{50} value of 5 μM (data not shown). This may indicate specificity toward the flavenoid group of the membrane-associated gp91-phox subunit of the NADPH oxidase complex.

Injured algae controls produced 36 $\mu mol\ H_2O_2 \cdot g\ FW^{-1}$ at 45 min postinjury. The addition of increasing concentrations of catalase (7.3–29.3 $U \cdot mL^{-1}$) resulted in an exponential decrease in H_2O_2 detection (Fig. 6). This linear concentration-dependent decomposition of H_2O_2 was complete at concentrations greater than 30 $U \cdot mL^{-1}$. The IC_{50} was calculated to be 13.3 $U \cdot mL^{-1}$. The highly reproducible concentration curve ($r^2 = 0.98$) suggested the substrate (H_2O_2) was homogeneous in nature, and thus other ROS were probably not being produced at detectible levels.

Although sodium azide (NaN_3) proved to partially inhibit ROS formation (Fig. 7a), it proved to be nonselective (IC_{50}, 483 uM) (Fig. 7b) when compared with DPI. Sodium azide has been noted to act as a reductant against the highly oxidizing redox pair (H_2O_2/H_2O) in acidic solutions (Barcelo 1998). Our studies (conducted in seawater, pH 7.8) did not show any change in

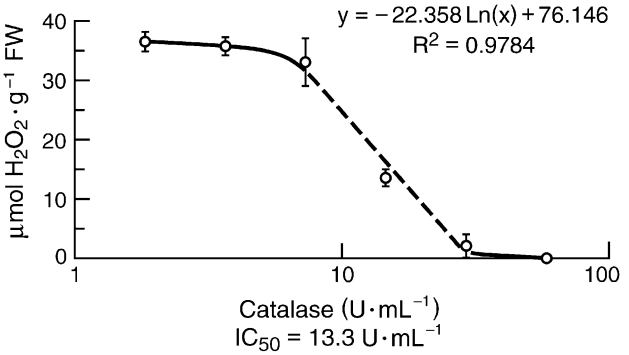

FIG. 6. Semi-log dose–response curve for the enzymatic decomposition of H_2O_2 by catalase. Algae were injured and incubated for 45 min with selected concentrations of catalase.

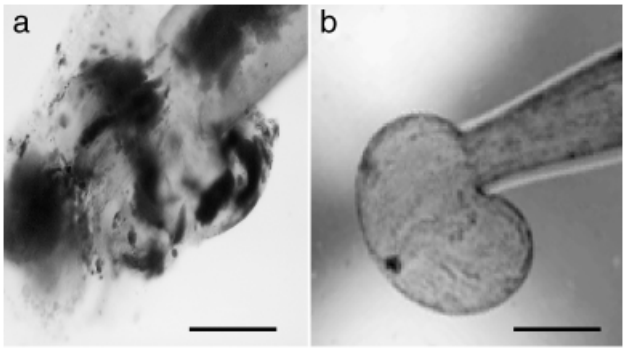

FIG. 8. Localization of peroxidase in wound plugs. Reaction mixture includes 1 mM o-dianisidine, 1 mM H_2O_2, and artificial seawater, pH 7.8. Stained 45 min postinjury. (a) Peroxidase histochemically localized by o-dianisidine. (b) Inhibition of staining with addition of 10 mM NaN_3. Scale bar, 4 mm.

H_2O_2 concentrations when incubated with 0.01–0.5 mM NaN_3. It is therefore believed that any decrease in H_2O_2 concentrations in the presence of NaN_3 may be attributed to a nonselective enzymatic inhibitory process.

Relationship of peroxidase in the browning process. To better understand the basis of the browning mechanism, we assayed for the presence of peroxidase enzymes. Peroxidase activity was detected in wound plugs by using o-dianisidine and H_2O_2 as cosubstrates (Fig. 8a). Algal specimens were wounded and left undisturbed for 45 min. Before this time, peroxidase activity was not detectable. Peroxidase could be detected at 35–45 min postinjury (simultaneous with the elevated H_2O_2 burst as described above). Activity staining could be inhibited with the addition of 10 mM NaN_3 (Fig. 8b).

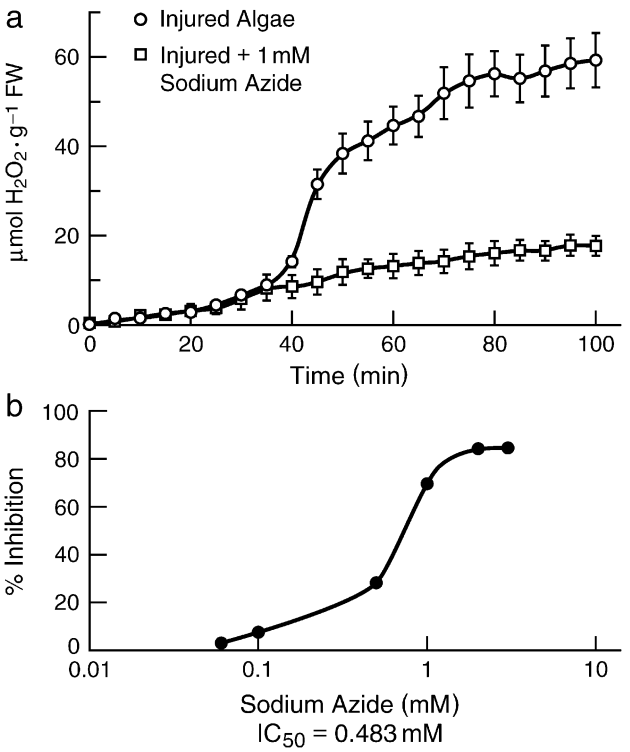

FIG. 7. Inhibition of H_2O_2 synthesis in injured *Dasycladus vermicularis* treated with sodium azide (NaN_3). (a) Fluorometric quantification of H_2O_2 generated after injury. The concentration of H_2O_2 emitted into solution was calculated as μmol $H_2O_2 \cdot$ g FW^{-1} of algae as shown in the ordinate ($n = 10$ individuals). (b) Dose–response curve.

Partial peroxidase purification and characterization. Peroxidase activity was studied in attempt to establish the relationship of the components of the oxidative browning system (hydrogen peroxide, peroxidase, and catechols). A peroxidase enzyme was partially purified using a DEAE Sepharose fast-flow anion exchanger with a linear gradient (A) 100 mM Tris (pH 8.0) – (B) 100 mM Tris (pH 8.0) + 1 M NaCl. Optimal pH activity of the partially purified peroxidase extract was determined to be pH 5.5. Figure 9a shows that a pH of 5.5 (100 mM acetate buffer) correlates with an enhanced specific activity of 5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$. The effect of H_2O_2 on peroxidase activity was studied in attempt to determine the optimal concentration of H_2O_2 needed for maximal peroxidase activity. The partially purified extract of peroxidase (1.032 $mg \cdot mL^{-1}$) was incubated with o-dianisidine (5 mM) at various concentrations of H_2O_2 in acetate buffer (100 mM, pH 5.5). Figure 9b shows that approximately 0.5 mM H_2O_2 resulted in the highest level of specific activity at the optimal pH of 5.5.

Catechol isolation and characterization. Algae were injured and immediately incubated in a Fast Blue BN salt solution that stains activated phenolic compounds a rust color. Immediately upon injury, the internal contents of the algae exuded into solution and were found to be reactive with the histological dye.

Coumarin containing extracts were chromatographed on an Affi-Gel 601 column (BioRad) that binds cis-diol derivatives. The rationale for using this column is that the boronate-derivatized polyacryl-

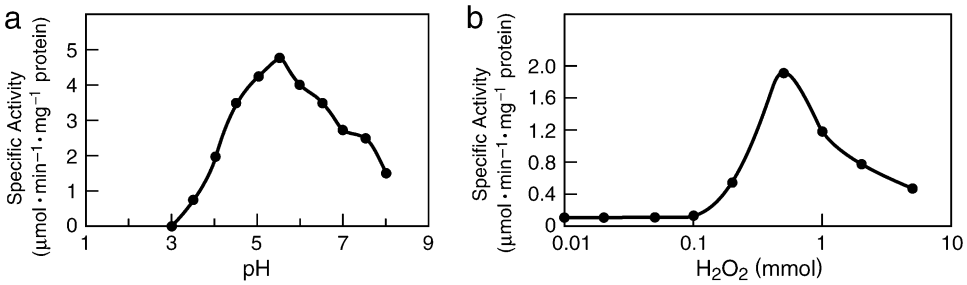

FIG. 9. (a) pH profile of partially purified peroxidase extract (32 $\mu g \cdot mL^{-1}$) isolated from DEAE Sepharose anion exchanger. Extract was incubated at various pH values with o-dianisidine (5 mM) + H_2O_2 (1 mM). The pH of 5.5 showed optimal specific activity. Buffers used were 100 mM Tris-sulfate (pH 8.0–7.5), 100 mM phosphate (pH 7.0–6.5), and 100 mM acetate (pH 6.0–3.0). (b) Effect of H_2O_2 on peroxidase activity. Partially purified extract (1.032 $mg \cdot mL^{-1}$) was incubated with o-dianisidine (5 mM) at various concentrations of H_2O_2 in acetate buffer (100 mM, pH 5.5).

amide gel has an affinity for compounds having adjacent hydroxyl groups such as saccharides, diphenolics, and ascorbate. The 280-nm absorbing peak eluted from the Affi-Gel 601 with acetic acid was immediately run on an RP-HPLC system. This produced a major peak, which was collected, acid hydrolyzed, and further analyzed for its dihydroxy configuration. Evidence in favor of an aromatic orthodihydroxy placement is suggested by UV absorbance in borate at high pH. The difference spectrum resulting from the subtraction of the absolute spectrum in 0.2 M HCl from that in 0.2 M sodium borate (pH 8.5) is characteristic of catecholic moieties and may be used as a diagnostic tool (Waite 1984). Using this method we detected a shift (275–287 nm in a complexed state) signifying the dihydroxy functionality of catechol complexed with borate.

The UV spectral properties of the peak collected from the RP-HPLC setup was similar to the initial report of 3,6,7-trihydroxycoumarin (Menzel et al. 1983). Two major peaks were displayed at 220 and 348 nm, whereas a smaller peak was shown at 261 nm.

DISCUSSION

Browning. In this report we demonstrate that browning and hardening of the plugs in *D. vermicularis* initiates approximately 40 min after wounding and intensifies throughout the next 24 h as an oxidative process. Natural browning of the plugs in *D. vermicularis* may be analogous to the deposition of phenolics as reported in higher plants and fungi (Lagrimini 1991, Gandia-Herrero et al. 2003, Rajarathnam et al. 2003). The deposition of polyphenolic acids can promote cross-linking of proteins or cell wall constituents via a peroxidase-mediated reaction (Bernards and Fawzi 2001, Oudgenoeg et al. 2001).

At first glance, it appears the browning in *D. vermicularis* originates directly from the injured cell wall and spreads into the gelled plug as a function of time. Yet in other experiments, we found that when wound plugs were immediately removed from the injured thallus they were still capable of browning, suggesting the cell wall is not the only point of origin (data not shown). During the initial formation of a wound plug, the extruded material consisted strictly of vacuolar–cytoplasmic origin and did not contain cell wall fragments. This supports the hypothesis that the peroxidase source is in at least two forms, a cell wall form and a cytoplasmic peroxidase form; hence, the oxidative cross-linking occurs within the cell wall as well as within cytoplasmic/vacuolar gelled areas upon injury (Barcelo and Aznar-Asensio 1999, Henriksen et al. 2001).

Latency of browning. A unique feature seen in *D. vermicularis* is that upon injury, a 40-min delay occurs before browning initiates. Pith tissues from tobacco expressing high levels of peroxidase were noted to brown within 24 h after wounding, whereas tissues from control plants with normal peroxidase levels did not brown until 7 days after wounding (Lagrimini 1991). This author did not determine whether the relatively slow induction of peroxidase activity (browning) in response to wounding is an effect of transcription rate, mRNA stability, translational control, or related to heme availability. The 40-min latency in browning, seen in our experiments with *D. vermicularis*, warranted an investigation in these issues.

Roberts et al. (1988) reported on the steady-state mRNA levels of suberin-associated peroxidase in wounded potato and tomato. They showed peroxidase mRNA levels peaking 4 days after wounding, suggesting either transcriptional control or regulation of mRNA stability accounted for this delay. The addition of cycloheximide and actinomycin D (inhibitors of protein synthesis and total cellular mRNA, respectively) showed no inhibitory effect upon browning in our studies. Hence, the 40-min latency of browning (upon injury) did not appear to be dependent on proteins transcribed or translated *de novo*.

It appears that a peroxidase enzyme is already present in some form yet has a significant latency period. A study by Vandendriessche et al. (1984) noted a similar delay in enzyme activation in *Acetabularia* (Dasycladales); the possibility of the presence of an inhibitor was raised. The use of polyvinylpyrrolidone

(phenolic binding agent) in the peroxidase preparation enhanced the activity and shortened the latency period. We observed similar effects in our studies on the peroxidase extracts of *D. vermicularis*. Presumably, this treatment removes the endemic phenolics that are capable of competing for the peroxidase active site (Vreeland et al. 1998).

Oxidative burst and browning. The biological function of the oxidative burst in *Dasycladus* is remarkably different from what has been documented in most higher plants and most algal models. Only in the case of the red alga *Eucheuma* does the oxidative burst seem to be related to a mechanical injury (Collen and Pedersen 1994), whereas in all other models, chemical stimuli (i.e. systemin; Ryan 2000, Orozco-Cardenas et al. 2001) trigger this response. In most documented cases, the oxidative burst serves a defense function, either directly by the toxic effects of the generated activated oxygen species (Peng and Kuc 1992, Küpper et al. 2001, 2002), by coordinating an up-regulation of defense genes mediated by the easily diffusible H_2O_2 (Orozco-Cardenas et al. 2001), or even by regulation of calcium signaling in response to osmotic stress (Coelho et al. 2002).

An involvement of the oxidative burst in the cross-linking of cell wall proteins has been shown by Brisson et al. (1994) and Otte and Barz (1996), with both studies supporting a direct chemical role of hydrogen peroxide in the cross-linking reaction of phenolic amino acid residues. Similarly to the latter two studies, in *Dasycladus* the biological significance of the oxidative burst could be to provide sufficient H_2O_2 for the oxidative cross-linking reaction of catechols during wound plug formation.

Studies in the Rhodophyte *Chondrus crispus* (Bouarab et al. 1999) and the Phaeophyte *Laminaria digitata* (Küpper et al. 2001) have shown that upon surface elicitation, peak hydrogen peroxide concentrations may reach millimolar levels, which is similar to values reported for the oxidative burst in higher plants (Legendre et al. 1993). In the present study, partially purified peroxidase extracts of *D. vermicularis* displayed maximal activity when incubated with H_2O_2 levels ranging from 0.5 to 2 mM. The induction event (mechanical injury in this case) provides adequate substrate levels for a peroxidase-based cross-linking reaction to ensue if all the essential constituents are present (H_2O_2, catechols, and peroxidase).

Origin of the oxidative burst. The results obtained from the fluorometric studies using DCFH-DA with DPI support the premise that the oxidative burst in *D. vermicularis* originates from a flavoprotein-containing NADPH oxidase complex that is analogous to those involved in ROS production in animals (Segal and Abo 1993) and higher plants (Groom et al. 1996, Jabs et al. 1997, Torres et al. 1998). The quinacrine inhibition of the burst supports an involvement of a flavonoid group reminiscent of the membrane-associated gp91-phox subunit. It should be noted that the ability of NaN_3 to inhibit the production of H_2O_2 (at a high concentration) promotes speculation as to exactly where H_2O_2 is being produced. Azide can inhibit heme-based peroxidases (Murphy and O'hEocha 1973, Menzel and Grant 1982, Murphy et al. 2000, Verdel et al. 2000). Additionally, DPI has been noted to inhibit the peroxidase-mediated production of H_2O_2 in higher plants (Bolwell et al. 1998). Frahry and Schopfer (1998) noted that DPI is clearly an effective inhibitor of the H_2O_2-producing catalytic activity of the heme-based peroxidase from horseradish, thus suggesting that DPI is not the most selective inhibitor when distinguishing between mammalian-type NAD(P)H oxidase and peroxidase in higher plants in the context of ROS production. Doussiere et al. (1999) noted that DPI has had inhibitory effects of the heme component of the neutrophil NADPH oxidase complex. DPI can inhibit the O_2-reducing activity of heme prosthetic groups by reacting with the Fe^{2+} state (compound III) formed in the presence of a reductant.

An additional point of interest regarding the source of ROS production deals with the staining of functional redox centers with NBT. According to most models, molecular oxygen is converted to superoxide anion (O_2^-) by the NADPH oxidase complex. Subsequently, superoxide dismutase catalyzes the transformation of O_2^- to H_2O_2.

Nitroblue tetrazolium has been used to indicate (resulting in a blue precipitate in the presence of superoxide anion) that superoxide anions are formed during the oxidative burst via superoxide dismutase (Küpper et al. 2001). Staining *D. vermicularis* with NBT upon injury did not result in a blue precipitate. Even with the addition of the superoxide dismutase inhibitor DDC (which should promote a buildup of superoxide anion), no detectible levels of superoxide anion were observed via NBT histology.

In *D. vermicularis*, the lack of detectable O_2^- upon injury promotes an interesting viewpoint. Although used in the literature as an indicator of superoxide anion, NBT is certainly less specific than electron paramagnetic resonance. In addition, DDC has been noted to be a strong scavenger of H_2O_2. Keeping in mind the controversies of the use of inhibitors to identify ROS sources (Barcelo 1998), one must use caution when interpreting these types of results.

Inhibition of the oxidative burst by DPI suggests that *D. vermicularis* may possess a flavoprotein-containing NADPH oxidase complex that is analogous to those involved in the production of ROS in animals and higher plants (Groom et al. 1996, Jabs et al. 1997). The high degree of selectivity of DPI ($IC_{50} = 2.4\ \mu M$) certainly implies that an NADPH oxidase complex is most likely involved.

Peroxidase role in browning. In the studies reported here, histochemical staining (o-dianisidine) of the plug has corroborated the presence of peroxidase 35–45 min postinjury. Natural browning, as well as histochemical staining of peroxidase, is eliminated or drastically reduced by the addition of exogenous

antioxidants or catalase. These observations show a direct correlation with the massive production of ROS described above.

The location and activity of peroxidase has been investigated by cytochemical techniques in 14 species of Dasycladales and in members of the genera *Bryopsis, Derbesia,* and *Halicystis* (Caulerpales) (Menzel 1979, 1980). In each species examined, the peroxidase was either located in wound plugs or in plugs associated with gametangial and sporangial formations. We partially purified a peroxidase from *D. vermicularis* that shows a similar specific activity and pH optima quite similar to what has been reported in other members of the Dasycladales (*Batophora* and *Cymopolia* from Menzel and Grant 1982).

Peroxidases have been noted to undergo modified ping-pong reaction kinetics where H_2O_2 binds to the enzyme and water is released before an electron donor can be oxidized. Because of this behavior, the K_M cannot be calculated. In our specific case we also could not get first-order kinetics and estimate the affinity for the enzyme extract. The curve was biphasic in nature, with H_2O_2 inhibition being observed at concentrations above 0.5 mM. Verdel et al. (2000) noted this type of substrate inhibition when peroxide concentrations exceeded 3.8 mM. Baden and Corbett (1980) noted that peroxide concentrations above 2 mM completely inhibit peroxidase activity of *Penicillus capitus* (Chlorophyta). Murphy et al. (2000) observed inhibition of peroxidase activity when high concentrations of hydrogen peroxide were present (this type of inhibition is characteristic of heme-based peroxidases). The inhibition by NaN_3 in *D. vermicularis* might indicate the presence of a heme-based prosthetic group (Murphy and O'hEocha 1973).

Presence of catechols and their function in browning. There are numerous precedents established in biological systems using a peroxidase–phenylpropanoid interaction giving rise to cross-linked polymers (Wallace and Fry 1995, Hasson and Sugumaran 1987, Oudgenoeg et al. 2001). Edens et al. (2001) confirmed the presence of a bifunctional enzyme, termed Duox, from *Homo sapiens* and *Caenorhabditis elegans*. This multidomain enzyme consists of a COOH-terminal flavoprotein domain containing an NADPH-binding site as well as a NH_2-terminal region that is homologous to peroxidases. By integrating two catalytic systems into one protein, the hydrogen peroxide generated from a gp91phox-homology domain could subsequently serve as a substrate for the peroxidase domain. Functionally, protein containing tyrosyl radicals would generate di- and trityrosine, which would form the basis of cross-linked nematode cuticle (Edens et al. 2001). With the discovery of trihydroxylated coumarins in *D. vermicularis* (Menzel 1983) and the verification that these molecules are capable of serving as electron donors/acceptors in a peroxidase mediated system (Perez-Rodriguez et al. 2001), it would be of interest to see whether an analogous dual-enzymatic moiety serves as the catalytic basis for reactive phenolics to engage in cross-linking in *D. vermicularis*.

Our preliminary studies have confirmed the presence of a trihydroxycoumarin with UV-Vis spectroscopy and ESI-MS (upon acid hydrolysis of chromatographic extracts of wound plugs of *D. vermicularis*). Preliminary mass spectral identification of fractions were performed by comparisons with the mass spectra database (NIST, NISTREP, and WILEY). Our extracted compound yielded a very similar match to 3,5,7-trihydroxy coumarin. However, upon ^{1}H-NMR examination, Menzel et al. (1983) suggested a 3,6,7-trihydroxy configuration in extracts of *D. vermicularis*. We have not yet determined whether 3,6,7-trihydroxycoumarin is the actual constituent of the plug or whether other unidentified phenolics are serving as cross-linkers. Coumarins are naturally present as glycosides, whereas Menzel (1983) isolated the 3,6,7-trihydroxy compound after acid hydrolysis. We are currently investigating this discrepancy by characterizing the biochemical structure and cross-linking potential of the glycone and aglycone coumarins from *D. vermicularis*.

In summary, we demonstrated that a browning process begins at about 35–45 min after mechanical injury and is coincident with a marked production of H_2O_2 in micromolar concentrations. In histochemical studies, we found evidence of a peroxidase that is activated with the onset of hydrogen peroxide synthesis. In other experiments we showed the presence of phenolics, which gives support that the biochemical machinery is present to drive a cross-linking reaction in damaged tissue.

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