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Involvement of Reactive Oxygen Species and Reactive Nitrogen Species in the Wound Response of *Dasycladus vermicularis*

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Summary

We investigated the signaling events involved in the wound response of the marine macroalga *Dasycladus vermicularis*, finding nitric oxide (NO) production in relation to injury. The addition of exogenous H_2O_2 to aliquots of injured algae accelerated the kinetics of NO production in the wounded region. Similarly, the addition of an NO donor caused an increase in detectable H_2O_2 around the site of injury. By wounding or incubating uninjured algae with an NO donor, peroxidase activity was enhanced. Based on the use of selected pharmacological probes, our results indicate that H_2O_2 production involves the upstream activation of signaling events similar to those observed in the physiology of higher plants.

Introduction

As for practically any other organism, the survival of marine algae relies upon chemical defense systems. Aside from the synthesis of secondary metabolites that may act as deterrents of predators, grazers, and pathogens [1–4], the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been shown to have a significant contribution toward the survival of organisms in a variety of contexts [5–9].

The oxidative burst has been defined as the rapid production of ROS in a defensive response to an external stimulus. It was first noted in the human immune system [10] and, much more recently, in higher plants [11, 12]. Such ROS may include superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). O_2^- and $^1O_2$ are relatively short-lived species (with a typical half-life in the microsecond range), depending on the environment [13]. Conversely, H_2O_2 is a longer-lasting species (half-life of hours or days in seawater) that is electrically neutral and able to pass through cell membranes. Due to its longevity, it may reach considerable distances from its site of formation [14].

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Recent years have provided a substantial amount of material recognizing the importance of ROS and the emerging role of RNS as defense response compounds, as well as signal transduction agents, in higher plants [5, 15, 16]. Within the last decade, the role of ROS in macroalgal biology, especially in the chemical defense against pathogens, has been emerging. Initially observed upon injury of the red alga *Eucheuma* [17], oxidative bursts have been reported in both red [18, 19] and brown [20] algae, typically in the defense against bacterial [18, 21] and eukaryotic [21, 22] pathogens. Although substantial genome responses and the activity of many enzymes are known to be affected by ROS and nitric oxide (NO), molecular and biochemical mechanisms of injury responses are poorly understood, and furthermore, many signaling pathways remain elusive.

Within the last decade, the role of NO in plant disease resistance [23], cell death, and DNA fragmentation in *Taxus* [24], tissue growth [25], stomatal closure [26, 27], and many other aspects of plant life have been described [28, 29]. Despite the wealth of knowledge that is accumulating in terrestrial animals and plants, little is known about the role of NO in marine organisms. Arumugam et al. [30] report NO production in blood cells of the mussel *Mytilus galloprovincialis* in response to various chemical triggers, including phorbol 12-myristate 13-acetate. Morall et al. [31] used the activity of the NO-generating enzyme, NO synthase (NOS), to develop a biomarker for the health of corals. NOS activity has been associated with thermal stress in populations of zooxanthellae isolated from scleractinian corals [32]. Except for the recent results with the marine diatom *Phaeodactylum* [33], nothing has been reported about the occurrence or function of NO in free-living marine algae.

Analysis of higher plant models has suggested that the onset of the oxidative burst is regulated by a series of signal transduction events involving an initial elicitation event where putative receptors are activated. In turn, a variety of downstream signaling events occur, involving GTP binding proteins (G proteins), adenylate cyclases, phospholipases, protein kinases, phosphatases, ion channel activation, and the final activation of an ROS enzymatic source.

Most of these findings have been obtained in higher plants, whereas signaling systems are virtually unexplored in marine chlorophytes. We have recently described evidence of a latent oxidative burst involved in the wound repair mechanism in the marine chlorophyte *Dasycladus vermicularis* [34]. Our previous work emphasized the pharmacological inhibition of a diphenylene iodonium (DPI-)-sensitive, putative NADPH oxidase that is believed to be the major contributor of ROS. We undertook this present study to backtrack systematically and identify the upstream steps involved in ROS production in *D. vermicularis* once injury is sustained. Furthermore, our additional aims were to investigate whether wounding would result in NO production, to identify the source of NO, to determine if NO had any relationship with ROS production, and, finally, to determine a functional role of NO in relation to injury.

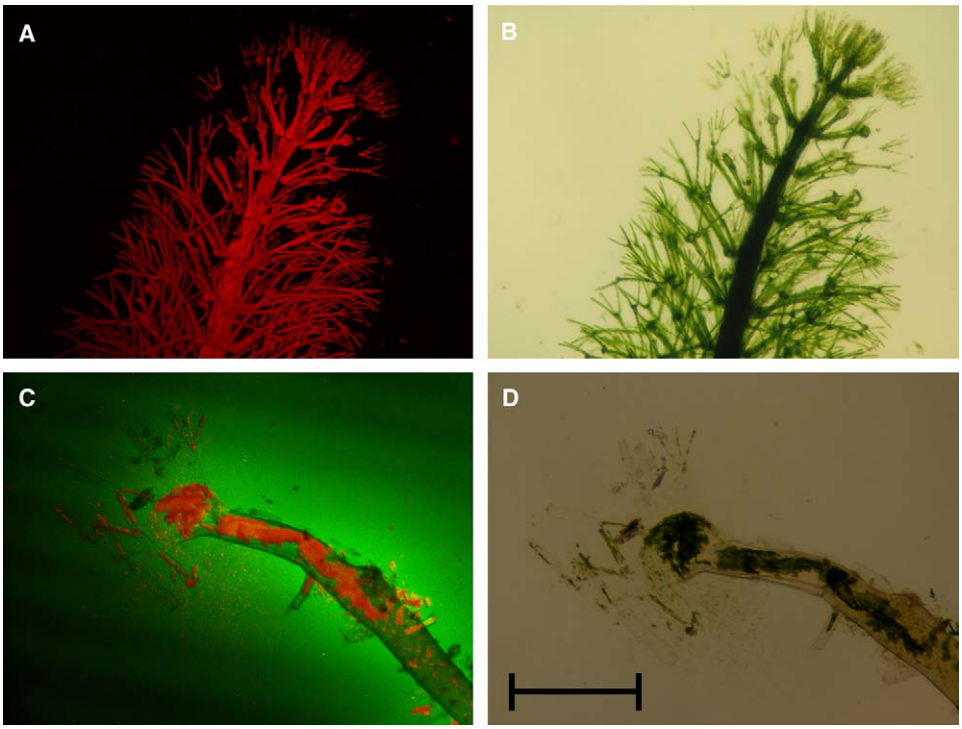

Figure 1. Detection of NO with DAF-FM Diacetate by Confocal Laser Scanning Microscopy

(A) Uninjured specimen with strong visualization of chloroplasts shown in red with no detection of NO (control).
(B) Uninjured specimen displayed under light microscopy (control).
(C) Injured specimen (25 min postinjury) displaying strong NO emission in green.
(D) Injured specimen displayed under light microscopy. Scale bar, 8 mm.

Results

NO Analysis

NO was detected in direct affiliation with injury to *D. vermicularis*. Uninjured specimens displayed no evidence of NO emission. Upon initial mechanical injury, a 5 min lag period occurred before NO could be detected in the wounded region. By 25 min post injury, over 0.15 μmol NO g^{-1} FW could be fluorimetrically detected in the surrounding media and visualized by fluorescence microscopy (Figures 1 and 2A). This localized event was more pronounced in adult specimens (25–30 cm) when compared to juveniles (8–10 cm). After 25 min postwounding there was no further increase in the fluorescence emission of the NO signal, suggesting that levels had saturated (~0.20 μmol NO g^{-1} FW from 25 to 60 min post injury).

To examine whether NO emission, produced upon injury, originated from NOS, specimens were preincubated with L-NMMA. This NOS-specific inhibitor had a negligible effect on decreasing the NO signal (Figure 2A). Conversely, preincubating healthy algae with the NO scavenger C-PTIO showed a complete elimination of the NO signal once the algae were injured (Figure 2A). In addition, preincubating the injured algae with 100 μM of the marine natural product pseudopterosin A resulted in complete elimination of the NO signal (data not shown). The elimination of NO had no visual effect on wound plug formation, as described previously [34, 35].

When healthy, noninjured algal specimens were preincubated for 25 min with sodium nitrite, detectible levels of NO were released into the surrounding seawater. Around 0.14 μmol NO g^{-1} FW of algae were recorded by 60 min (Figure 2B). By adding KCN prior to nitrite addition, NO production ceased. In addition, when the algae were preincubated with exogenous sodium nitrate, NO was released at concentrations roughly half of that observed when sodium nitrite was used as a substrate (Figure 2B).

Interrelation between NO and H_2O_2

Addition of 100 μM exogenous H_2O_2 to the injured algae accelerated the initial release of NO from 10 min to 5 min postinjury (0.05 μmol NO g^{-1} FW) in the surrounding media (Figure 3A). Not only was the NO signal initially expedited, but NO levels were consistently greater than those levels recorded without the addition of H_2O_2.

The addition of CPTIO (even in addition to the H_2O_2-supplemented experiments) was capable of completely inhibiting the detection of NO (Figure 3A). The preincubation of algae with 5 μM of the NADPH oxidase inhibitor DPI prior to injury (30 min) resulted in a reduced timeframe of NO production, as shown in Figure 3A.

We have previously reported the relationship between injury and H_2O_2 production in *D. vermicularis* [34]. Just as the addition of exogenous H_2O_2 had an intensifying effect on NO release, the addition of diethylamine NO (DEANO) donor amplified the H_2O_2 release (Figure 3B). Addition of 100 μM DEANO promoted the initial detection of H_2O_2 from ~25 min to 15 min postinjury. By 45 min postinjury, the DEANO-supplemented experiments displayed H_2O_2 concentrations greater than

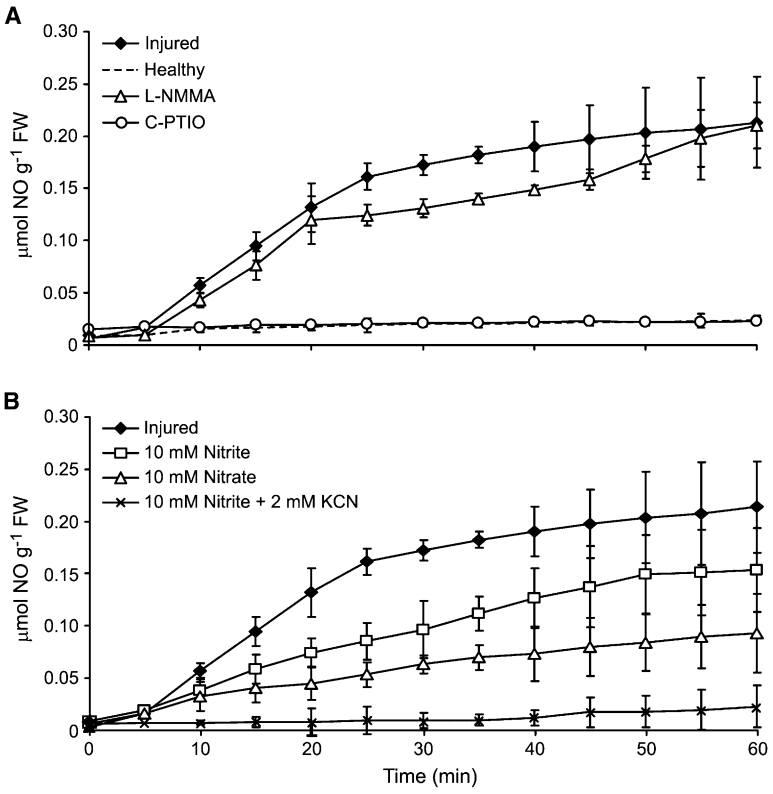

Figure 2. Fluorimetric Quantification of NO Generated Following Injury

The concentration of NO emitted into solution was calculated as µmol NO/g fresh weight as shown in the ordinate. Data points represent the mean ± 1 SEM.

(A) The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO) showed complete scavenging of detectible NO, while the NOS inhibitor N^G-methyl-L-arginine (L-NMMA) had a negligible inhibitory effect upon NO production (n = 10 individuals).

(B) The additions of 10 mM sodium nitrite and sodium nitrate were both capable as serving as substrates for the nitrate reductase-catalyzed production of NO. Preincubating the algae for 25 min with 2 mM potassium cyanide prior to the introduction of sodium nitrite inhibited the production of NO.

80 µmol H_2O_2 g^{-1} FW (Figure 3B). By preincubating the algae for 30 min with 5 µM DPI prior to injury and subsequently adding DEANO, the H_2O_2 production was drastically reduced (Figure 3B). The addition of catalase (even in the presence of the DEANO-supplemented experiments) was capable of completely eliminating the

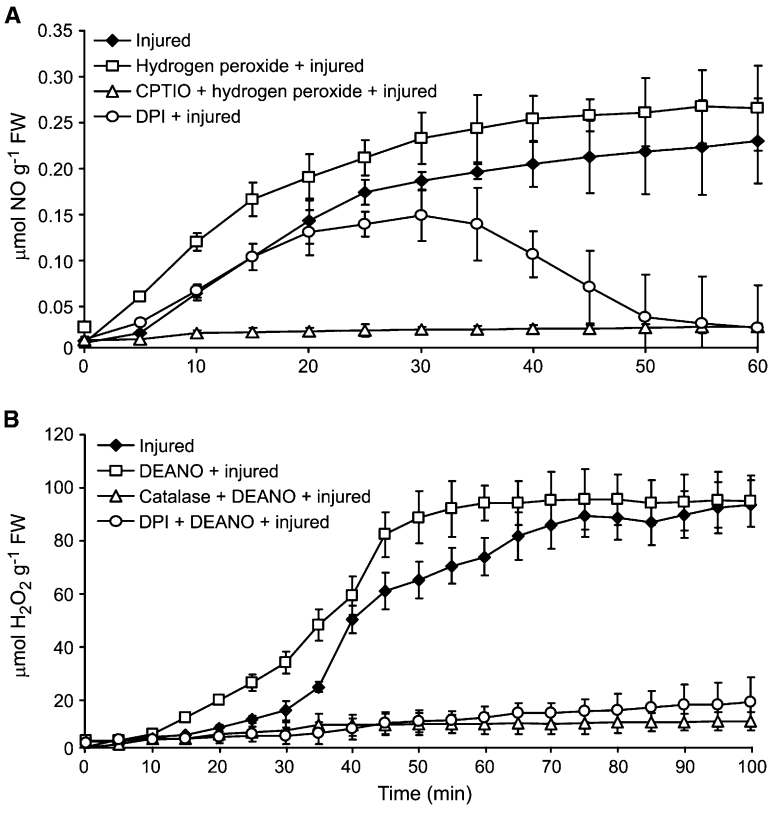

Figure 3. Interrelation between NO and H_2O_2

(A) Upregulation of NO generated by the addition of 100 µM exogenous H_2O_2. The concentration of NO emitted into solution was calculated as µmol NO/g fresh weight. Injured algae supplemented with exogenous H_2O_2 showed an increase in NO production. The addition of CPTIO was capable of completely inhibiting the reaction. Preincubating the algae with DPI prior to injury reduced the time frame of NO production.

(B) Fluorimetric quantification of H_2O_2 generated following the addition of the NO donor DEANO. The concentration of H_2O_2 emitted into solution was calculated as µmol H_2O_2/g fresh weight. DEANO was capable of increasing the signal, yet the addition of catalase or DPI completely eliminated the H_2O_2 signal.

Data points represent the mean ± 1 SEM (n = 10 individuals).

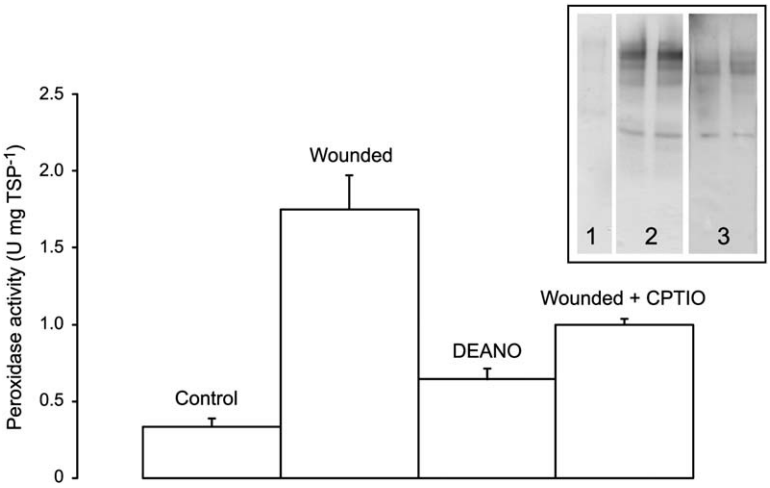

Figure 4. Addition of the NO Donor DEANO Leads to the Upregulation of Peroxidase Activity, Exhibiting a Similar Effect in this Respect as Wounding

Specimens of *D. vermicularis* were untouched (control), crushed with forceps (wounded), incubated with DEANO for 2 hr, or preincubated with 100 μM carboxy-PTIO for 30 min prior to injury (wounded + CPTIO). Partially purified extracts were assayed for peroxidase activity using the oxidation of o-dianisidine. Data points represent the mean ± 1 SEM (n = 3 groups). Units of peroxidase activity are expressed as U/mg TSP (total soluble protein). The same extracts are shown on an activity gel stained with o-dianisidine. Lane 1 represents uninjured algae. Group 2 represents crushed algae (run in duplicate). Group 3 represents uninjured algae incubated with DEANO (in duplicate).

H_2O_2 signal as well (Figure 3B), yet had no effect on NO synthesis.

Activation of Peroxidase Activity by Wounding or NO Supplementation

By wounding the algae or preincubating specimens with DEANO, an increase in peroxidase activity was observed (Figure 4). In spectrophotometric assays by the peroxidase-catalyzed oxidation of o-dianisidine to bis (3,3′-dimethoxy-4-amino) azobiphenyl, wounding caused a 10-fold intensification of detectible peroxidase activity by 300 min, when compared to uninjured algae. The addition of 100 μM DEANO caused a doubling of peroxidase activity when compared to uninjured algae. Gel activity assays showed the activation of several peroxidase-reactive bands when algae were wounded or when pretreated with DEANO (Figure 4).

Signal Transduction Events Leading to H_2O_2 Release

In this study, we carried out a more detailed investigation of the signaling events that ultimately give rise to an oxidative burst at 40 min postinjury. The protein kinase-c activator phorbol 12-myristate 13-acetate (PMA) was ineffective at enhancing H_2O_2 levels (at the level of accuracy that could be achieved in these experiments) when algae were preincubated in concentrations ranging from 10 to 20 μM (Figure 5A). However, by preincubating the algae with 50 μM PMA (prior to wounding), a 25% increase in H_2O_2 production could be detected. Incubating the algae with concentrations of 100 μM or more resulted in a saturation of detectible H_2O_2 levels (~25% increase above standard levels normally detected after wounding).

To study the possible role of protein phosphorylation in the process of injury recognition and subsequent signal transduction in *D. vermicularis*, we applied staurosporine, calyculin A, and cantharadin. Staurosporine is a potent inhibitor of serine and threonine kinases in animal cells [36]. In *D. vermicularis*, staurosporine was capable of blocking H_2O_2 production with an IC_{50} of 15.5 μM (Figure 5B). The protein phosphatase inhibitors calyculin A and cantharidin [37, 38] had no effect on H_2O_2 production.

The anion channel blocker NPPB was capable of partially inhibiting the generation of ROS. Preincubation with 100 μM NPPB was needed to cause a 15% reduction in ROS detection (Figure 5C).

To determine the relationship between Ca^{2+} channel blocking and H_2O_2 generation, *D. vermicularis* cells were incubated with methoxyverapamil prior to injury. A 20% decrease in H_2O_2 levels occurred in the presence of 100 μM methoxyverapamil as noted in Figure 5D. Increasing concentrations of methoxyverapamil did not cause an additional decrease in H_2O_2 levels. Concentrations less than 100 μM had negligible effects.

Mastoparan is an amphiphilic wasp venom tetradecapeptide capable of directly activating pertussis toxin-sensitive G proteins by a mechanism analogous to that of G protein-coupled receptors. Mastoparan had no effect on H_2O_2 generation. However, pertussis toxin had a significant inhibitory effect on the oxidative burst, showing an IC_{50} of 0.17 μg ml^{-1} (data not shown).

Antioxidants were capable of completely quenching the detectible H_2O_2 levels. Ascorbic acid was capable of 100% inhibition of the ROS signal, with an IC_{50} of 20 μM. Two coumarins with known antioxidant effects were also capable of eliminating the H_2O_2 signal [39, 40]. Umbelliferone (7-hydroxycoumarin) had an IC_{50} of 50 μM and esculetin (6,7-dihydroxycoumarin) had an IC_{50} of 65 μM (data not shown). Both compounds showed a significant decrease in H_2O_2 production when compared to injured algae incubated without any added compound.

Ion flux regulation, in response to injury, was investigated by using a series of specific ion channel blockers, as well as ionophores, to examine their impact on ROS generation. To determine the relationship between Ca^{2+} and H_2O_2 generation, *D. vermicularis* cells were incubated with the Ca^{2+} ionophore A23187 prior to injury. A23187 was capable of enhancing H_2O_2 levels (postinjury) by 35%, as seen in Figure 6. Concentrations ranging from 5 to 50 μM showed a steady percent increase in H_2O_2 levels. By 50–75 μM A23187 the effects were clearly saturated.

The K^+ ionophore, valinomycin, was capable of enhancing H_2O_2 levels (postinjury) from 5 to 75 μM (Figure 6). By applying 100 μM, a 10% increase in H_2O_2

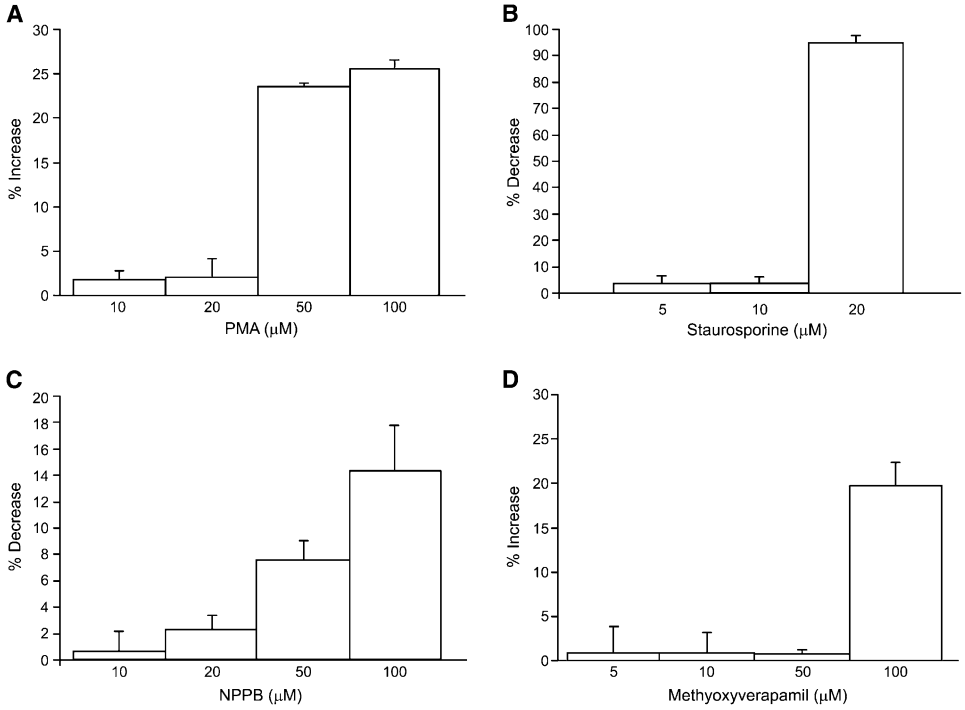

Figure 5. Enhanced or Reduced Production of H_2O_2

Relative percent change of H_2O_2 generated when injured algae were preincubated with (A) the protein kinase-c activator Phorbol 12-myristate 13-acetate (PMA); (B) the protein kinase inhibitor staurosporine; (C) the anion channel blocker NPPB; and (D) the calcium channel blocker methoxyverapamil (n = 10 individuals). Data points represent the mean ± 1 SEM.

production could be observed. No further percent increase in ROS production was noted with increasing valinomycin concentrations greater than 100 μM.

The Na^+ ionophore monensin was capable of enhancing H_2O_2 levels (postinjury) from 5 to 50 μM (Figure 6). By 50 μM, a 20% increase in H_2O_2 production could be observed, with the increase leveling off at monensin concentrations around 50 μM.

O_2 Measurements

Oxygen consumption was monitored for 50 min for healthy (basal respiration) and injured cells. As shown in Figure 7, the increase in oxygen consumption was linear as a function of time postinjury. By 20 min postwounding, the injured algae consumed twice as much O_2 as uninjured cells. By 50 min postwounding, injured cells showed a relative O_2 consumption rate four times that of uninjured cells.

Discussion

Despite the paramount role of NO in medicine and animal and higher plant physiology, reports of its occurrence in marine algae are scarce and its role remains poorly understood. To our knowledge, this study represents the first finding of NO production in a macroalga and is, together with the recent findings of NO in the marine diatom *Phaeodactylum* [33], among the first in any marine algal lineage.

Fluorescent probes, such as 4,5-diaminofluorescein diacetate (DAF-2 DA) or 2′7′-difluorofluorescein diacetate (DAF-FM DA), have become strong analytical tools for the detection and quantification of NO in higher

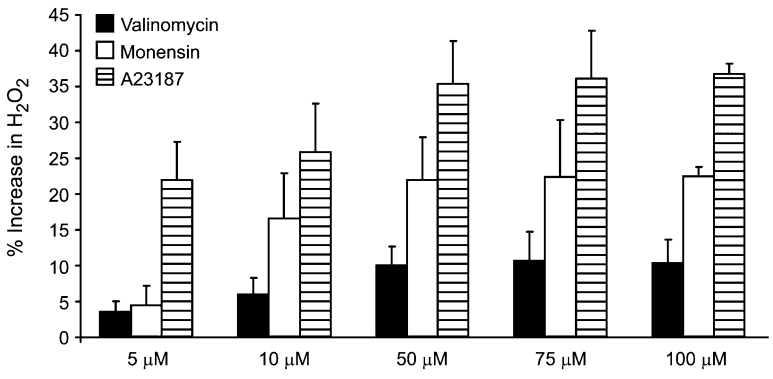

Figure 6. Relative Percent Increase of H_2O_2 Generated when Injured Algae Were Preincubated with the K^+ Ionophore Valinomycin, the Na^+ Ionophore Monensin, and the Ca^{2+} Ionophore A23187

Data points represent the mean ± 1 SEM (n = 10 individuals).

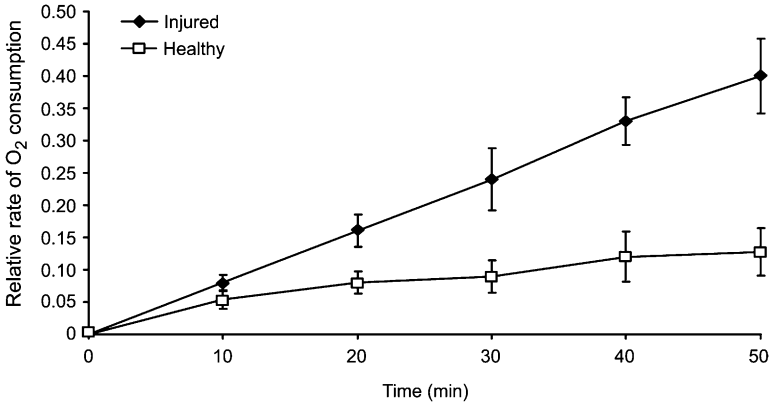

Figure 7. The Relative Rate of Oxygen Consumption per Cell in Healthy and Injured Specimens

Relative rate of oxygen consumption per cell was compared between healthy (uninjured) and injured specimens over a 50 min time course. Data points represent the mean ± 1 SEM (n = 10 individuals per series).

plants and mammals [41, 42]. This study highlights their usefulness in marine models as well. In mammalian systems, the generation of NO plays a key role in inflammation and tissue repair [43]. It is capable of acting as a cytostatic, chemotactic, and vasodilatory agent during the early wound response; and it mediates cell proliferation, collagen deposition, and angiogenesis, leading to wound closure [44]. The multifunctional roles of NO are ascertained by a series of biochemically diverse redox and additive interactions [45]. We believe that there are strong parallels between the function of NO in algal lineages and that in animals and terrestrial plants.

More and more evidence is supporting the multisignaling functions of H_2O_2 and NO in higher plants. NO can be synthesized during wound responses concomitantly with H_2O_2 production [46, 47]. For example, NO has been shown to act synergistically with reactive oxygen intermediates to increase oxidative cell death in soybean cells [28]. In addition, NO has been shown to inhibit wound-inducible H_2O_2 generation as well as the downregulation of wound-inducible defense genes in tomato [48, 49]. NO synthesized at the same time as H_2O_2 in response to pathogen attack was found to mediate defense responses similar to those seen following H_2O_2 generation [46]. We used *D. vermicularis* as a model organism to investigate whether wound-induced NO formation is a conserved response in lower chlorophytes. Additionally, it was of interest to determine whether the production of NO was involved in any synergistic crosstalk with H_2O_2 or had a pertinent functional role with respect to mechanical injury.

In this report, we demonstrate that NO and H_2O_2 are indeed both emitted upon injury to *D. vermicularis*, and that their respective activation appears to be based upon partly coregulated processes. NO emission precedes the oxidative burst (~25 and 45 min, respectively), and furthermore, the two reactive species seem to have an inductive relationship with each other. Reactive oxygen intermediates and NO have been previously shown to have mutually potentiating effects in higher plant cell systems [23]. The addition of the NO donor, DEANO, to incubations of injured *D. vermicularis* caused an increase in H_2O_2 levels and, conversely, adding exogenous H_2O_2 to an analogous experimental setup increased the NO signal detected in wounded *D. vermicularis*.

The detection of low micromolar concentrations of NO is in agreement with relative concentrations detected in soybean suspensions [23] and in tobacco leaves [47]. We stress the term relative, since NO is short-lived due to its high reactivity with many biomolecules. In addition, the ability of a fluorescent probe to bind to released NO does not take into account the NO that is actually trapped within cellular organelles. While the addition of an NO donor may expedite the wound healing process (based upon the promotion of H_2O_2 generation and the upregulation of peroxidase activities), the inhibition of NO formation appears to have no deleterious effect on the immediate wound sealing process; yet we can not exclude that long-term responses may be impacted.

Cytofluorometric analysis of NO and H_2O_2 in oat (*Avena sativa*) noted an inhibitory effect between the two compounds [48]. Other higher plant studies have noted the mediating role of NO in relation to decrease in catalase and ascorbate peroxidase activity [49]. Our results would support the existing evidence suggesting that a signaling relationship probably exists between NO and H_2O_2 [50, 51]. Our data show that the addition of exogenous NO donor, DEANO, was capable of mimicking the wound response by causing an upregulation in peroxidase activity (Figure 4), suggesting that NO may contribute to coordinating the secondary hardening of the wound plug and other, relatively late, events. The exogenous addition of CPTIO (even when algal samples were wounded) was capable of reducing peroxidase activity when compared to DEANO-treated or injured algae samples, suggesting the involvement of NO in peroxidase upregulation. Heme-based enzymes, such as peroxidase, catalase, dioxygenase, and cyclooxygenases, have metal- and thiol-containing prosthetic groups capable of serving as reaction sites for NO [45, 52]. In addition, nitrogenous compounds, including ammonium ion, nitrosonium ion, and NO free radicals, have been reported to increase heme-based peroxidase activity several-fold in radish [53, 54], human leukocytes [55], and human thyrocytes [56].

In *D. vermicularis*, it is doubtful whether NO synthesis can be attributed to NOS activity, due to the lack of inhibition by L-NMMA. A similar response was noted in tobacco, where the partial inhibition of DAF-2DA fluorescence, by an NOS inhibitor, could only be attributed to the high specificity for an individual NOS isoform [57]. The individual forms of animal and plant NOS activity differ in their degree of sensitivity to NOS inhibitors [58, 59].

Carboxy-PTIO is a highly specific scavenger for NO that is not only membrane-permeable, but capable of

reacting in a stoichiometric manner [60]. Since carboxy-PTIO does not react with ROS, it has been specifically utilized as an inhibitor for NO-related studies. For example, it has been reported to block NO production as well as NO-dependent cell death and defense gene activation in tobacco, soybean, *Arabidopsis*, and barley [23, 57, 58]. While carboxy-PTIO is a useful control to specify NO accumulation, it does not give any indication as to its origin.

Several sources of NO have been described in higher plants. NO may arise from NOS, nitrate reductase, or nonenzymatic sources. NOS catalyzes the NADPH- and O_2-dependent oxidation of L-arginine to NO and L-citrulline [61, 62]. The L-arginine analog, L-NMMA, inhibits NOS in a variety of cells, including neurons, endothelial cells, and macrophages [63]. Several plant proteins have been shown to cross-react with antibodies directed against mammalian NOS [64]; however, only one NOS plant gene (*AtNOS*1) has been identified to date [65].

Studies describing a nitrate reductase-based production of NO have been reported in a diverse set of photosynthetic organisms, including *Helianthus annuus* (sunflower), *Spinacia oleracea* (spinach) [66], and the freshwater microalga *Chlamydomonas reinhardtii* [67]. These reports prompted our investigation to see if this process was conserved in marine green algae.

Studies using *C. reinhardtii* previously demonstrated that the exogenous addition of sodium nitrite in the presence of nitrate reductase could promote the one-electron reduction to NO [67]. Our results might suggest that a similar process is occurring in *D. vermicularis*. The enzymatic production of NO was inhibited with potassium cyanide. Although this inhibitor is fairly unspecific, it has been used as a nitrate reductase inhibitor in other biological systems [67, 68]. Interestingly, *C. reinhardtii* was not capable of using sodium nitrate as a substrate, unlike *D. vermicularis*. The two-step electron reduction may be more feasible in *D. vermicularis*, given the high concentration of coumarins that may serve as reducing equivalents. Phenolic metabolites in *Hordeum vulgare* (barley) have been shown to serve as reducing agents capable of accelerating NO formation [69]. The nonenzymatic production of NO in *D. vermicularis* should not be completely overlooked. While this type of NO production requires acidic conditions, physical injury results in a mixing of cellular components that may result in conducive physiological conditions (nitrite-cytosol, acidic pH-vacuole, and reductants-chloroplasts) that may contribute to NO formation [70].

While experimental evidence is increasingly identifying the components of higher plant signaling pathways, the reports of signal transduction systems in macrochlorophytes presently remain scarce. To add to the complexity, the activation of different signaling systems may vary based upon the origin of the sensor and on the nature of the stress event [71–74]. As described in a recent review of abiotic plant stressors, it is currently accepted that there are two groups of membrane-dependent stress-sensing systems in higher plant cells [74], which we would consider as potential sensors for injury in *Dasycladus*. One group consists of the redox/H_2O_2-dependent systems localized in mitochondria, chloroplasts, or peroxisomes, and the other group is based on cell wall-plasma membrane interactions. In regard to the latter, mechanotransduction (physical injury) has a profound effect on the signaling relationships between the cell wall and plasma membrane.

The most widely recognized model for plasma membrane-based ROS production is the NADPH oxidase system. The use of specific inhibitors of NADPH oxidase in plant and animal systems, especially DPI [37, 75], has confirmed the presence of an NADPH oxidase system in various algal lineages [20, 34]. According to the NADPH oxidase model, an elicitor molecule or mechanical pressure is detected by a receptor located on the plasma membrane. In the case of mechanical injury, it appears that a mechanical transducer detects cell wall-plasma membrane perturbations and, in turn, a signal transduction cascade becomes activated. Data available from higher plants support the involvement of GTP binding proteins, ion channels (especially calcium), protein kinases, protein phosphatases, phospholipases A and C, and cyclic AMP. This pathway ultimately gives rise to the activation of an NADPH oxidase complex, which produces superoxide anion. The basis for this reaction is the reduction of molecular oxygen to superoxide anion. This molecule is rapidly dismutated to H_2O_2 in the presence of superoxide dismutase. Our data show that a variety of identifiable signal transduction steps (homologous to what has been reported in higher plants) are believed to be involved in ROS production in *D. vermicularis* once injury is sustained [14].

Our O_2 consumption data provide evidence that there is a notable uptake of O_2 from the ambient seawater, which would clearly provide adequate levels of oxygen as a substrate for the enzymatically based turnover of O_2 to H_2O_2. DPI was shown to be very potent in the inhibition of H_2O_2 formation in *D. vermicularis*, as evidenced by the IC_{50} of 2.4 μM [34]. Taken together, this inhibition and the finding of increased O_2 uptake during the oxidative burst suggest that *D. vermicularis* may possess a flavoprotein-containing NADPH oxidase complex that is homologous to those involved in the production of AOS in animals and higher plants, and that would reduce molecular oxygen to generate ROS [76, 38].

We have previously reported that quinacrine, an inhibitor of flavin-dependent redox enzymes [77], yielded an IC_{50} value of 5 μM when H_2O_2 levels were measured in injured *D. vermicularis* [34]. This may indicate specificity toward the flavonoid group of the membrane-associated $gp91^{PHOX}$ subunit of the NADPH-oxidase complex.

The reversible phosphorylation of moieties by kinases and phosphatases is considered to be an integral part of the basic regulatory mechanisms. The general inhibitor of protein kinases, staurosporine, was reported to block the protein phosphorylation events involved in induction of defense responses in tobacco cells upon elicitation by cryptogein [78] or oligogalacturonides [79]. Similar inhibition observations were reported with the inhibition of oligoguluronate-induced oxidative bursts in the brown algal kelp *Laminaria digitata* [20]. Staurosporine did prove to be an effective inhibitor in the *D. vermicularis* system, suggesting that serine and threonine kinases are involved in an upstream stage of signal transduction in *D. vermicularis*. The protein phosphatase inhibitors calyculin A and cantharidin [37, 38] had no effect on the release of H_2O_2 in this system.

Heterotrimeric G proteins play a key role in signal transduction by integrating cell surface receptors to effector systems. Bacterial toxins have been proven to be extremely useful tools for identifying and studying the role of G proteins [80, 81]. The participation of G proteins has been analyzed using cholera toxin in French beans [82]. This same toxin, as well as mastoparan and PMA, was shown (in the bloodroot flower *Sanguinaria canadensis*) to involve the participation of G proteins and protein kinases, respectively, in the signal transduction for the synthesis of alkaloids induced by fungal elicitors [83]. Pertussis toxin covalently modifies the α subunits of numerous G proteins by ADP-ribosylating specific amino acid residues [84]. As in *L. digitata* [20], mastoparan (G protein activator) failed to influence ROS production in *D. vermicularis*. However, a potent G protein inhibitor, pertussis toxin, proved to strongly inhibit ROS production.

Changes in membrane permeability and the resulting ion fluxes, mainly Ca^{2+} and H^+ influx and K^+ and Cl^- efflux, are among the most rapid responses of plant cells to elicitation [85, 86]. Ion flux regulation in response to stress changes involves the activation of K^+ and Cl^- channels in motor cells in leaves of higher plants [87, 88], as well as the light-dependent ionic movements dictating turgor adaptation involving stomatal function. The multifunctional roles of ion fluxes in plant systems prompted our investigation into the utilization of ion channels as part of the signal transduction process in response to injury in *D. vermicularis*.

An increasing amount of evidence is demonstrating the interrelationship between dependence of ROS production on ion fluxes and the activation of plasma membrane bound Ca^{2+} channels by H_2O_2 [89, 90]. The calcium ionophore A23187 has been used to establish the participation of calcium in the abscisic acid (ABA) transduction system [91], as well as in the hypersensitive response developed in lemon seedlings [92]. Elicitors promote both cytosolic Ca^{2+} increases and ROS production. In some cases, and depending on the species investigated, Ca^{2+} elevations have been reported both upstream and downstream of ROS production [93], indicating complex spatiotemporal Ca^{2+} elevation mechanisms.

The importance of the presence of Ca^{2+} fluxes has been demonstrated in isolated plasma membrane-rich fractions of potato tuber [94]. In this report, activation of the NADPH-dependent O_2^--generating reaction was strictly dependent upon the presence of Ca^{2+} ions. Similarly, the emission of H_2O_2 required the increased cytosolic presence of calcium ions in injured *D. vermicularis.* This was demonstrated by the enhancing effect of the Ca^{2+} ionophore A23187 and the inhibitory effects of the antagonists of Ca^{2+} channels, such as methoxyverapamil. In *Fucus* rhizoid cells, DPI inhibited both tip growth and the tip-localized Ca^{2+} gradient [95]. This infers that the ROS-Ca^{2+} relationship may represent a more widely used, signal codependence, aside from what has been observed in higher plant physiology. Our findings support the notion that the Ca^{2+}-ROS relationship has a strong signaling role in the wound response in *D. vermicularis*.

Current knowledge emphasizes the multifunctional roles of ROS and RNS in higher plants and mammalian systems. Marine algae cover evolutionary lineages that diverged approximately one billion years ago [96]. We have demonstrated that wounding in *D. vermicularis* elicits homologous signal transduction events, suggesting that the cellular machinery to respond to wounding developed early on in the course of evolution.

Significance

These results provide evidence for the presence of nitric oxide (NO) in a marine macroalga, and suggest a role in the rapid wound-healing response in controlling peroxidase activities likely involved in the wound-healing process. Real-time measurements of these compounds can be acquired with fluorescent probes for reactive oxygen species (ROS) and NO. Additionally, pharmacological agonists and specific metabolic inhibitors have been utilized to demonstrate the signal transduction and molecular crosstalk amongst signaling pathways involved in the production of ROS and NO. This experimental evidence clearly demonstrates that *Dasycladus vermicularis* produces NO in response to wounding, and that there is a potential for feedback amplification between ROS and NO levels that may lead to an accelerated wound-healing response. The initial production of ROS and NO, and potentially the crosstalk between them, rely on signal transduction mechanisms that involve GTP binding proteins, protein kinases, and ion channels. These mechanisms share similarities with signaling pathways in higher plant systems, but they have never before been demonstrated in a marine chlorophyte or other macroalgae, suggesting that key elements in wounding and signal transduction responses are evolutionarily conserved.

Experimental Procedures

Algal Material

Juvenile and adult specimens of *D. vermicularis* (Scropoli) Krasser were collected live off Indian Key, Florida, and Cay Sal Bank, The Bahamas. For laboratory studies, a unialgal culture (strain LB 2685 *D. vermicularis*) was ascertained from the Culture Collection of Algae at the University of Texas at Austin. Cultures were maintained with a photoperiod of 12 hr, with 12 hr of darkness at a photon flux density of 50–60 μmol m^{-2} s^{-1} and at a constant temperature of 25°C. Muller's medium was utilized in all experiments, and was filtered through 0.22 μm membranes (no. SCGPT10RE; Millipore, Bedford, MA) prior to introduction of the algae.

Light Microscopy

Wounded and noninjured specimens were visualized with a Leica DMLB compound microscope (Leica Microsystems, Bannockburn, IL) outfitted with a Nikon Cool Pix 8700 camera (Nikon, Melville, NY).

NO Detection

Confocal Laser Scanning Microscopy

DAF-FM DA (Molecular Probes, Eugene, OR) is a cell-permeable probe that passively diffuses across cellular membranes. Once inside the cell, it is deacetylated by intracellular esterases to become DAF-FM. Oxidation of DAF-FM by NO yields a fluorescent benzotriazole derivative [83]. The protocols used in this study were kindly provided by C. Bowler and A. Vardi (Ecole Normale Superieure, Paris): DAF-FM DA was dissolved in DMSO in 5 mM aliquot stocks (stored at −80°C). Individual specimens of *D. vermicularis* were transferred into an incubation mixture of 2 ml filtered seawater and 20 μl DAF-FM DA (final concentration of 20 μM DAF-FM DA). The algae were incubated in the dark for 20 min. In order to detect NO production via

physical wounding, postincubation, the algae were subsequently injured by a single transverse cut with a scalpel and crushed with forceps. The specimens were then washed in 5 ml fresh seawater and subsequently imaged. Confocal laser scanning microscopy (CLSM) was performed with a Nikon Eclipse E800 compound microscope (Nikon Instruments, Kanagawa, Japan) equipped with a Bio-Rad Radiance 2000 laser system (Bio-Rad, Hercules, CA). Laser power was set at 20%, with an excitation of 488 nm and an emission of 525 nm (channel 1, green) or 580 nm (channel 2, red). Series of 0.2 μm optical sections with maximum intensity projection along the z axis were made into one 2D image with greater focal depth. Bio-Rad images were imported into Confocal Assistant 4.02 and converted into TIF files.

Quantitative NO Measurements

The NO release from injured cells was calculated by measuring the oxidation of DAF-FM DA in the presence of esterase (E.C 3.1.1.1; Sigma, St. Louis, MO; 40 U ml^{-1}). Reaction mixtures included 1000 μl algal extract, 0.40 U esterase, and 20 μM DAF-FM, for a total volume of 2000 μl. For NO inhibition or promotion studies, algal samples were preincubated for 30 min with either 100 μM carboxy-PTIO, L-NMMA, 2 mM potassium cyanide, or 100 μM DEANO (all stored at −80ºC as 100 mM stock solutions dissolved in H_2O). For nitrate reductase detection, solutions of 10 mM sodium nitrate or 10 mM sodium nitrite were preincubated with the algae for 25 min prior to NO analysis. For calculating the concentration of NO 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 DEANO sodium salt in addition to 0.40 U esterase and 20 μM DAF-FM DA, for a total reaction volume of 2000 μl. The fluorimetric quantification of NO was analyzed for a 120 min time interval on a Bio-Rad VersaFluor fluorometer.

NO Upregulates Peroxidase Activity

A total of 100 g fresh algae was divided into four treatments: 25 g was crushed with forceps and left undisturbed for 1 hr; 25 g of uninjured algae was incubated with 1 mM of the NO donor, DEANO, for 1 hr; an additional 25 g was preincubated for 30 min with 100 μM carboxy-PTIO and subsequently crushed with forceps; and, as a control, 25 g of algae was left undisturbed for the same 30 min time frame.

Partial Peroxidase Purification

A partially purified extract of peroxidase was obtained as reported by Ross et al. [34] with slight modification. After wounding, or incubation with an NO donor, 25 g of algae was pulverized into a homogeneous powder with liquid N_2, mortar, and pestle. The solid powder was subsequently mixed (4ºC cold room) into 400 ml of 100 mM Tris HCl (pH 8.0) with the addition of 0.1% Triton X-100 and PVPP (6% w/v buffer).

Extracts were centrifuged at 7000 rpm for 20 min at 4ºC on a Beckman TJ-6 centrifuge. 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 4ºC.

The dialyzed extract was batch loaded onto a High Trap DEAE Sepharose FF 1 ml anion exchanger (Amersham Biosciences) using a Bio-Rad Econopump (Bio-Rad). A step gradient of elution buffers was used, ranging from 100 mM Tris-HCl (pH 8.0) to 100 mM Tris-HCl (pH 8.0) + 1 M NaCl (200 mM increments). Active fractions were pooled, subsequently desalted via dialysis, and then equilibrated against 100 mM acetate buffer (pH 5.5). Samples were concentrated using Centricon Plus-20 5000 NMWL polyethersulfone centrifugal filters (Millipore). Total soluble protein was quantified with the Quick Start Bradford Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

Peroxidase Activity

Peroxidase extracts of healthy, wounded, and DEANO-supplemented (1 mM) *D. vermicularis* were normalized by protein concentration and assayed for peroxidase activity. Peroxidase extracts (1.45 mg ml^{-1}) were mixed with 5 mM o-dianisidine and 0.5 mM H_2O_2 in acetate buffer (100 mM, pH 5.5), for a total volume of 2 ml. Samples were immediately added to a cuvette and spectrophotometrically assayed for the peroxidase-catalyzed oxidation of o-dianisidine at 460 nm (ϵ_{460} = 11.3 mM^{-1} cm^{-1}) with a Shimadzu UV-265 spectrophotometer (Shimadzu Biotech, Columbia, MD).

Thirty microliters of normalized samples (1.032 mg ml^{-1}) were run under nondenaturing SDS-PAGE conditions (10% Tris-HCl Ready Gels; Bio-Rad). Peroxidase activity was detected with a staining solution of 5 mM o-dianisidine and 0.5 mM H_2O_2 in 100 mM acetate buffer (pH 5.5).

Oxidative Burst Measurements

The concentration of H_2O_2 present in the medium around the algal specimens was determined from a protocol previously reported by Ross et al. [34]. 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 (Molecular Probes; 10 mM) and esterase (E.C 3.1.1.1; Sigma; 41 U ml^{-1}) were prepared in DMSO and Muller's medium, respectively. A total of 350 mg (n = 25 cells) of cells was towel-blotted dry and weighed. Each cell was then transversely 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. For inhibitory experiments, catalase (E.C 1.11.1.6; Sigma; 25 U ml^{-1}) was preincubated with the algae for 30 min prior to injury. Excitation and emission wavelengths were 488 and 525 nm, respectively. Reaction mixtures included 1000 μl 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 fluorimetric quantification of H_2O_2 was analyzed for a 120 min time interval on a Perkin Elmer LS50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT).

Inhibitors and Activators of H_2O_2 and NO Production

In order to identify upstream signal transduction mechanisms involved in the oxidative burst, selected inhibitors and activators were assayed for their physiological activity. Drugs were preincubated with healthy algae for 30–45 min prior to injury. Diphenyleneiodonium (Sigma) is a suicide inhibitor of NADPH oxidases that binds irreversibly to the flavonoid group of the membrane-associated $gp91^{phox}$ subunit. A 1 mM stock solution was prepared in dimethyl sulfoxide (DMSO). Methoxyverapamil (a Ca^{2+} channel blocker widely used in medicine, which has also been utilized in plant studies), staurosporine (a protein kinase inhibitor) and NPPB (5-nitro-2-[3-phenylpropylamino]-benzoic acid; an anion channel blocker) were dissolved in ethanol (all from Sigma). The ionophores valinomycin (K^+), monensin (Na^+), and A23187 (Ca^{2+}) (Sigma) were prepared from stocks in ethanol as well. Ascorbic acid, sodium nitrate, sodium nitrite, and potassium cyanide (Sigma) were dissolved in Milli-Q water. The coumarins esculetin (6,7-dihydroxycoumarin; Indofine, Hillsborough, NJ) and umbelliferone (7-hydroxycoumarin; Indofine) were diluted from stocks dissolved in ethanol. Calyculin A, cantharidin (both target protein phosphatases; Sigma), and phorbol 12-myristate 13-acetate (protein kinase c activator; Sigma) were from stocks dissolved in DMSO. Pertussis toxin (a G protein inhibitor, specifically used in the study of adenylate cyclase regulation and the role of G_i proteins; Sigma) was prepared from a water soluble (2 mg/ml) stock solution. Pseudopterosin A (G protein inhibitor) was obtained from our laboratory [67]. The NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO), the NO generator (diethylamine NO), and the NOS inhibitor N^G-methyl-L-arginine (L-NMMA) were purchased from Molecular Probes.

O_2 Measurements

Oxygen consumption was measured in healthy and injured specimens with a YSI 5300 Biological Oxygen Monitor (Scientific Division, Yellow Springs Instrument Co., Inc.). Analyzer chambers were filled with 10 ml filtered seawater. A total of 25 uninjured algal cells (~0.35 g) were placed in the chamber. O_2 consumption was monitored for 50 min. The same procedure was repeated for injured cells

(same number, with a transverse cut completely through the middle of the siphon). The rate of O_2 utilization was measured as the relative O_2 consumption per cell.

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