Intrinsically High Capacity of Animal Cells From a Symbiotic Cnidarian to Deal With Pro-Oxidative Conditions

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The cnidarian-dinoflagellate symbiosis is a mutualistic intracellular association based on the photosynthetic activity of the endosymbiont. This relationship involves significant constraints and requires co-evolution processes, such as an extensive capacity of the holobiont to counteract pro-oxidative conditions induced by hyperoxia generated during photosynthesis. In this study, we analyzed the capacity of Anemonia viridis cells to deal with pro-oxidative conditions by in vivo and in vitro approaches. Whole specimens and animal primary cell cultures were submitted to 200 and 500 µM of H₂O₂ during 7 days. Then, we monitored global health parameters (symbiotic state, viability, and cell growth) and stress biomarkers (global antioxidant capacity, oxidative protein damages, and protein ubiquitination). In animal primary cell cultures, the intracellular reactive oxygen species (ROS) levels were also evaluated under H₂O₂ treatments. At the whole organism scale, both H₂O₂ concentrations didn’t affect the survival and animal tissues exhibited a high resistance to H₂O₂ treatments. Moreover, no bleaching has been observed, even at high H₂O₂ concentration and after long exposure (7 days). Although, the community has suggested the role of ROS as the cause of bleaching, our results indicating the absence of bleaching under high H₂O₂ concentration may exculpate this specific ROS from being involved in the molecular processes inducing bleaching. However, counterintuitively, the symbiont compartment appeared sensitive to an H₂O₂ burst as it displayed oxidative protein damages, despite an enhancement of antioxidant capacity. The in vitro assays allowed highlighting an intrinsic high capacity of isolated animal cells to deal with pro-oxidative conditions, although we observed differences on tolerance between H₂O₂ treatments. The 200 µM H₂O₂ concentration appeared to correspond to the tolerance threshold of animal cells. Indeed, no disequilibrium on redox state was observed and only a cell growth decrease was measured. Contrarily, the 500 µM H₂O₂ concentration induced a stress state, characterized by a cell viability decrease from 1 day and a drastic cell growth arrest after 7 days leading to an uncomplete recovery after treatment. In conclusion, this study highlights the overall high capacity of cnidarian cells to cope with H₂O₂ and opens new perspective to investigate the molecular mechanisms involved in this peculiar resistance.

Keywords: Anemonia viridis, in vitro cell cultures, oxidative stress, hydrogen peroxide, cnidarian
INTRODUCTION

The evolutionary success of symbiotic cnidarians is based on a mutualism with dinoflagellates of the family Symbiodiniaceae. The symbionts, living inside the gastrodermal host cells, find a protected and stable environment and benefit from inorganic compounds provided by the animal cells (e.g., nitrogen, phosphorus, and sulfate) for their photosynthetic activity. Conversely, the animal host benefits from the organic compounds produced by algal photosynthesis (e.g., glucose and subsequently amino-acids, lipids) and largely transferred from the alga to the animal cell (Davy et al., 2012). This partner cooperation allows autotrophy to the animal host, leading to the colonization of oligotrophic waters by the symbiotic holobiont.

Concomitantly with those advantages, some constraints appear, especially the photosynthetic-dependent production of oxygen in the animal tissue. Such oxygen production causes diurnal hyperoxia condition in a symbiotic cnidian, leading to a pro-oxidant state with reactive oxygen species (ROS) overproduction (Dykens et al., 1992; Richier et al., 2003; Saragosti et al., 2010; Shaked and Armoza-Zvuloni, 2013). Both partners have the pathways for cross-regulating the intracellular redox state, especially by ROS detoxication through a full suite of antioxidant enzymes to avoid cellular damages (Shick and Dykens, 1985; Richier et al., 2003, 2005; Plantivaux et al., 2004; Furla et al., 2005; Merle et al., 2007; Pey et al., 2017).

The study of ROS sensitivity in these organisms is also of environmental interest. Environmental perturbations (especially variations in temperature and UV radiation) induce oxidative stress that may lead in extreme cases to symbiosis breakdown, a process commonly called bleaching. Thus, under stressful oxidative conditions, Symbiodiniaceae can be eliminated from or exit the host through different cellular processes, like exocytosis, cell detachment, necrosis or apoptosis (see for review Suggett and Smith, 2020). Oxidative stress is known to induce specific cellular damages such as DNA modification (DNA adducts), lipid peroxidation and protein oxidation. In symbiotic cnidarians, several biochemical biomarkers (e.g., protein carbonylation, lipid peroxidation, and protein ubiquitination) were validated in studies following imbalances between ROS overproduction and antioxidant defenses during environmental stress, resulting in the disruption of the symbiotic association (e.g., Lesser and Farrell, 2004; Richier et al., 2006; Pey et al., 2011).

Among ROS, hydrogen peroxide (H$_2$O$_2$) is a relatively stable chemical formed from O$_2$ and is naturally present in the aquatic systems (Ndungu et al., 2019) with concentrations ranging from 0.3 µM in the water column to 4 µM in intertidal areas (Abele-Oeschger et al., 1997). It originates from marine biota (Hansel and Díaz, 2021) or is carried by the rain (Cooper et al., 1987). In all the organisms, intracellular H$_2$O$_2$ levels can reach tens of micromolar and is generated during normal cellular metabolism (i.e., photosynthesis and respiration) playing crucial roles in the intracellular signaling such as hypoxic signal transduction, cell differentiation and proliferation as well as for immune responses (Halliwell et al., 2000; Apel and Hirt, 2004; Giorgio et al., 2007). At high production levels, the H$_2$O$_2$ effect can be mitigated by several antioxidant defenses including peroxidases, catalases, thioredoxin reductase, peroxiredoxins, and glutathione S-transferases family enzymes which can lead to rapidly decreasing intracellular H$_2$O$_2$ concentrations. However, if cellular redox homeostasis cannot be maintained, H$_2$O$_2$ leads to reversible and irreversible oxidative modifications of proteins (e.g., carbonylation), enhancing protein ubiquitination and subsequent proteasome activation. In addition, cell cycle arrest or apoptosis could also be observed (see for review Fulda et al., 2010). Although the impact of H$_2$O$_2$ has been more widely investigated in mammalian cells and particularly in tumor cells (see for review Lennicke et al., 2015), studies have shown a similar impact in marine invertebrates, such as bivalves or polychaetes (Abele-Oeschger et al., 1994; McDonagh and Sheehan, 2006, 2007; Da Rosa et al., 2008; Friedman et al., 2018; Nguyen, 2020).

In the coral symbiont, H$_2$O$_2$ has been shown to be a by-product of photosynthesis processes (Suggett et al., 2008; Armoza-Zvuloni and Shaked, 2014). Thanks to its cell-permeable properties, H$_2$O$_2$ may diffuse from algal to animal host cells. Interestingly, some studies reported a release of H$_2$O$_2$ from non-stressed corals (Armoza-Zvuloni and Shaked, 2014) and an extracellular production by the dynamics of the superoxide anion (Saragosti et al., 2010). Therefore, due to their symbiosis lifestyle, animal host cells are daily exposed to H$_2$O$_2$, raising the question of their intrinsic potential to resist a massive influx of H$_2$O$_2$. Nevertheless, in excess, ROS (including H$_2$O$_2$) cause negative impact (mostly on protein and lipids) on the symbiont, leading to photosynthesis impairment, even if no bleaching phenomenon is induced (Higuchi et al., 2009; Roberty et al., 2015, 2016).

*Anemonia viridis* is a temperate sea anemone deeply studied as biological model of the cnidarian-dinoflagellate symbiosis. Its enzymatic antioxidant properties, tissue distribution and regulation have been intensively investigated (Hawkridge et al., 2000; Richier et al., 2003, 2005; Plantivaux et al., 2004; Merle et al., 2007; Ganot et al., 2011; Pey et al., 2017). In addition, the sensitivity of *A. viridis* to thermal and UV stresses has been well described and some mechanisms of bleaching have been decrypted, including oxidative stress and apoptosis (Richier et al., 2006; Moya et al., 2012). Recently, we succeeded in the establishment of primary cell cultures from *A. viridis* exhibiting a gastrodermal signature (Barnay-Verdier et al., 2013; Ventura et al., 2018; Fricano et al., 2020). Thus, to test the hypothesis of an intrinsic resistance of animal cells to H$_2$O$_2$, we exposed *A. viridis* specimens and primary cell cultures at the same H$_2$O$_2$ concentrations (200 and 500 µM) during the same periods of time (24 h and 7 days), and compared the respective responses. For each treatment, we monitored global health parameters (symbiotic state, viability and cell growth) and stress biomarkers (global antioxidant capacity, oxidative protein damages, and protein ubiquitination). This allowed us to assess the cnidarian cell susceptibility to H$_2$O$_2$ exposure, highlighting the putative influence of the tissue organization or/and of the presence of symbionts.
MATERIALS AND METHODS

Biological Material
Anemonea viridis Specimens
Specimens of A. viridis (Forskal 1775) were collected (prefectural authorization n107; 28 February 2019) from “Plage des ondes,” Antibes, France, (43°33′17″ N, 7°07′17″ E), and maintained in a closed-circuit aquarium with artificial seawater (ASW, Prodibio Expert Reef Salt) at 36–38°C in a thermo-regulated incubator (POL- et al. (2018) and Fricano et al. (2020). Briefly, cells were maintained as described in Ventura et al. (2018) and Fricano et al. (2020). Independent primary cell cultures were obtained from different individuals and maintained as described in Ventura et al. (2018) and Fricano et al. (2020). Cells were fed once a week with oysters.

Gastrodermal Primary Cell Cultures
Independent primary cell cultures were obtained from different A. viridis individuals and maintained as described in Ventura et al. (2018) and Fricano et al. (2020). Briefly, cells were cultured in the dark in a thermo-regulated incubator (POL-EKO-APARATURA, Poland) at 20.0 ± 0.5°C. The optimized culture medium was replaced weekly and consisted of 20% GMIM (Gibco, Carlsbad, CA, United States), 5% fetal bovine serum (FBS; PAA/GE Healthcare, Chicago, IL, United States), 1% kanamycin (100 µg/mL, Sigma-Aldrich), 1% amphotericin B (2.5 µg/mL; Interchim, Montluçon, France), 1% antibiotic antimycotic solution (Sigma-Aldrich), 1% L-glutamate (Sigma-Aldrich), and 71% of filtered ASW. The medium was adapted in respect to the Mediterranean Seawater characteristics (i.e., salinity 40 ppt and pH 8.1).

Hydrogen Peroxide Experimental Design
In vivo Experiments
Eight specimens of A. viridis were kept individually in 5 L tanks under controlled conditions. Six tentacles were cut from each specimen after 24 h and 7 days to assess the control condition. For 200 and 500 µM H₂O₂ treatments, a solution of H₂O₂ (Sigma-Aldrich) was added in each tank (200 µM for four of them and 500 µM for the four others). After 24 h and 7 days of treatment, six tentacles were cut from each specimen to assess the treatment condition. All tentacles from the different time points and conditions were longitudinally opened and the gastroderm (containing the dinoflagellates) was manually separated from the epidermis. A centrifugation at 1,000 × g for 5 min was used to separate the gastrodermal compartment from the dinoflagellates fraction (Richier et al., 2003).

In vitro Experiments
The cell response to H₂O₂ treatment was assessed on at least three independent primary cell cultures. Cells seeded in 12-well plates were exposed to H₂O₂ treatment (Sigma-Aldrich) starting with a dose-response experiment (between 0 and 2 mM) for 7 days. Another set of experiments was performed by incubating cells to 0 (control), 200 and 500 µM H₂O₂ for short term (1, 2, and 6 h) or long term (1 day or 7 days) exposure. We then conducted resilience experiments by reseeding the treated cells in their normal culture medium for 7 days to assess their recovery capacity. Three wells, for each condition and time point, were used for all analyses.

Symbiotic State Assessment of A. viridis Specimens
The density of the endosymbionts from the genus Philozoon sp. (LaJeunesse et al., 2021) was assessed according to Zamoun and Furla (2012). Briefly, two tentacles were cut from each animal before and after H₂O₂ treatment in each condition (0, 200, or 500 µM H₂O₂) and then put in 2 M solution of NaOH and incubated at 37°C for 1 h to dissolve all animal tissue. To determine symbiont density, three replicate samples were counted using a modified Neubauer hemocytometer (Sigma-Aldrich). The remaining extract was used to determine the protein content from which we normalized symbiont density.

Protein Extractions
Gastrodermal cells from primary cultures and the animal’s epidermal and gastrodermal tissues were all separately placed in 200 µL of lysis extraction buffer (HEPES 25 mM, MgCl₂ 5 mM, EDTA 5 mM, DTT 5 mM, and PMSE 2 mM) and homogenized. Tissue samples were potterized, and both tissue and cell samples were sonicated and centrifuged to obtain the cytosoluble protein content. The total protein concentration of each sample was then assessed by a Bradford assay (Bradford reagent; SIGMA-ALDRICH), using 0–2 µg/mL bovine serum albumin solutions as standard curve.

Cell Viability and Growth Rate of Cultivated Cells
Cell viability was assessed by evaluating the membrane integrity using the Evans blue method. Therefore, viable cells (unstained) and dead cells (stained) were identified and counted on a Neubauer improved hemocytometer (Sigma-Aldrich) using an optic microscope (Zeiss Axio Imager Z1). The cell viability was defined as the percentage of viable cells relative to total cells (i.e., viable and dead cells). Cell growth rate was assessed every week using the cells counts with Evans blue method, as previously described in Ventura et al. (2018) and Fricano et al. (2020). The following formula was then used to calculate the weekly growth rate:

\[
\text{Growth rate} = \frac{(\text{Viable cells (d + 7)} - \text{Viable cells (d)})}{\text{Viable cells (d)}} \times \frac{1}{d} \quad \text{(day)}
\]

Total Oxidative Scavenging Capacity Assay
The oxygen radical scavenging activities of protein samples were determined using fluorometric assay according to Naguib (2000). Protein samples are incubated with phosphate buffer (75 mM pH 7.5), in presence of 180 nM 6-carboxyfluorescein as fluorescent probe and 36 mM AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] as the peroxyl radical generator. In the assay, Trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] is used as antioxidant calibrator. Fluorescence recordings were performed in black microplates (96-wells Greiner Bio-One),
and fluorescence decay was measured by spectrofluorometer (Safas, Monaco) every minute for a total duration of 1 h at an excitation/emission wavelength of 520/495 nm. Relative antioxidant activities of protein samples (tested in duplicate) were measured by comparison with Trolox standard. Results were expressed in Trolox equivalents and represented in the figures as a ratio to the control condition.

**Protein Carbonylation Analysis**

Carbonyl content of the cytosolic extractions was measured using an ELISA assay and spectrophotometry, according to Buss et al. (1997). Protein derivatization was done by adding a dinitrophenylhydrazine (DNP) solution (10 mM in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer) to the protein samples. The ELISA assay used anti-DNP antibody produced in rabbit (1:500; Sigma-Aldrich) and anti-rabbit Ig (1:2000; Bio-Rad). 0–100% reduced bovine serum albumin (BSA) were used as standard curve. Carbonyl content of protein samples was expressed in nanomoles per milligram of protein and was then represented in the figures as a ratio to the control condition.

**Ubiquitin Conjugates Dot-Blot Analysis**

Ubiquitinated proteins were assayed, according to Haas and Bright (1985), by dot-blotting 3 µg of protein samples fixed to a nitrocellulose membrane, which was incubated with primary antibody (1:1000; Mono- and polyubiquitinylated conjugates recombinant monoclonal antibody; Enzo Life Sciences). The membrane was next incubated with the secondary antibody (1:5000; anti-mouse antibody; Sigma-Aldrich). After chemiluminescence revelation (ECL), levels of spot density were measured using image analysis on GeneTools (SynGene). Levels of ubiquitinated proteins were obtained after normalization by spot density measured after amido black solution staining. Results were represented in the figures as a ratio to the control condition.

**Detection of Intracellular Reactive Oxygen Species**

Intracellular ROS were detected in cultivated cells treated or not with H$_2$O$_2$ using a sensitive fluorescent probe that penetrates the cell and, when oxidized by intracellular free radicals, binds to DNA, emitting a more intense green fluorescence (CellROX$^\text{TM}$ Green Reagent, Invitrogen). We followed the manufacturer’s instructions. Briefly, 10 µL of a 500 µM solution of CellROX$^\text{TM}$ Green were added to each treated well during 30 min at room temperature in the dark. After rinsing, cells were resuspended in 200 µL of PBS 0.6 M. Samples were then analyzed by flow cytometry (CytoFLEX LX, Beckman Coulter), excited at 488 nm and detected at 515–530 nm. For data analysis, we selected the green-fluorescent cells (CellROX-positive cells) within the population of interest. The population of interest corresponds to the cell population from which the analysis has been conducted after hierarchical exclusion of debris and doublets. For each experiment, controls (i.e., untreated cells) allowed to evaluate the basal intracellular ROS level in the cell cultures.

**Statistical Analysis**

For all cellular and biochemical markers evaluated in this study, the effect of the different treatments was analyzed with a two-way ANOVA test or Kruskal–Wallis test followed by, respectively, a Tukey’s or Dunn post hoc, depending on the homoscedasticity of the data set. All experiments, i.e., in vivo and in vitro, were conducted on at least three independent biological replicates.

**RESULTS**

**Hydrogen Peroxide Effects on A. viridis Specimens**

To determine the susceptibility of A. viridis specimen to H$_2$O$_2$, we monitored the sign of bleaching, tissue necrosis and potential mortality during 7 days of exposure. No sign of tissue necrosis or mortality of the A. viridis specimens have been observed during H$_2$O$_2$ exposure. In addition, no differences in the symbiotic state have been measured since the quantity of symbionts, Philozoon sp. genus, per mg of protein remained the same after 7 days of treatment with both H$_2$O$_2$ concentrations (Figure 1). In addition, the sea anemones were also monitored several weeks after the treatment, and no signs of bleaching or disease were observed (data not shown).

**Hydrogen Peroxide Effects on Stress Biomarkers of A. viridis Tissues Under Treatments**

To analyze the impact of H$_2$O$_2$ at the tissue level, we assessed three biochemical stress markers (TOSC, carbonylation, and ubiquitination) known to be associated with oxidative stress conditions, in the three tissue compartments (epiderm, gastroderm, and symbiont) separately. In the epiderm, we only observed a slight but significant decrease of TOSC after 7 days of 200 µM H$_2$O$_2$ (Figures 2A–C). It is noteworthy...
that after 1 day of 200 µM H₂O₂, we observed a trend of increasing protein damages (carbonylation and ubiquitination), which totally disappeared after 7 days.

In the gastrodermal compartment (i.e., animal tissue), we only observed an effect of H₂O₂ treatments after 7 days of 500 µM H₂O₂, inducing a 34% increase of TOSC (Figure 2D) which is sufficient to avoid protein damages (Figures 2E,F). Finally, the analysis on the symbiont fraction showed an increase of TOSC (17% higher than the control), associated with an increase of protein carbonylation level (50% higher than the control) after 7 days at 500 µM H₂O₂ (Figures 2G,H). No impact on symbiont protein ubiquitination has been measured (Figure 2I). Taken together these data suggest a higher capacity of cnidarian animal cells to cope with H₂O₂ and prevent protein damages compared to the algal symbiont.

**Dose Response of Gastrodermal Cultivated Cells to Hydrogen Peroxide**

To gain more insight in the capacity of the animal gastrodermal compartment to cope with ROS we first assessed the overall toxicity of H₂O₂ using gastrodermal cell cultures from *A. viridis*. More precisely, we applied a range of H₂O₂ concentrations from 100 µM to 2 mM for 7 days and assessed cellular growth and viability (Figure 3). Without any treatment, the mean viability of cultivated cells was 93% ± 1.17 and the weekly growth rate was 18.7 ± 4.5. At 100 µM, no effect on viability and growth rate was observed. The first significant effects on both parameters appeared at 200 µM (10% viability and 42% growth rate decreases) and drastically exacerbated at 500 µM. With a concentration of 500 µM H₂O₂, the viability was decreased by 58% (EC₅₀ = 400.8 µM) and the growth was totally arrested. These results led us to direct further experiments toward the concentrations of 200 and 500 µM H₂O₂, where the response of the cells seemed pivotal and allowing the comparison with the in vivo experiments.

**Hydrogen Peroxide Effects on Oxidative Status of Gastrodermal Cultivated Cells**

To test whether H₂O₂ treatments induced an intracellular ROS increase, we stained cultivated cells with specific fluorescent
probe (CellROX™ Green) allowing to quantify the positive cells for CellROX™ Green. The level of intracellular ROS was thus correlated to the percentage of positive cells for CellROX™ Green. Interestingly, this analysis revealed that although an increasing trend, there was no significant difference between the control (no treatment) and the cells treated with 200 μM H₂O₂, whatever the time point (Figure 4). However, there was a significant increase of ROS-positive cells (2.7 times higher in average, 11.2% vs. 4.1%) within the cells treated with 500 μM H₂O₂ from 1 h until 7 days of treatment.

**Hydrogen Peroxide Effects on Cellular Parameters Under Treatments and Resilience Phase**

We next wondered whether H₂O₂ exposures induce a stress at the cellular level by monitoring cellular parameters during treatments. In addition, we also evaluated the resilience capacity of treated cells by following the same parameters after a week-recovery phase. Firstly, the analysis of the cell viability, during 1–6 h of exposure, showed no impact of 200 or 500 μM H₂O₂ treatments (see Supplementary Figure 1). At 200 μM, there was only a light significant decrease in cell viability (9%) after 7 days of exposure, which was totally recovered after the resilience period (Figure 5A). At 500 μM, a 28% decrease was observed at 1 day that worsened after 7 days (58% of decrease, Figure 5A), confirming our results obtained during the dose-response assay. After a resilience period, only a partial recovery was observed (71% of the control condition), suggesting that not all the cells that survived may be able to compensate the H₂O₂-induced stress. In line with this hypothesis, the analysis of the cell population by flow-cytometry showed, after 7 days of 500 μM H₂O₂, a modification of cell-population pattern associated with a significant increase of autofluorescence (in average: 3.86% vs. 0.53% for the control condition; p-value = 0.021; Figure 6), which could be reflecting cell apoptosis.

The growth rate measurements showed a significant impact of both treatments, 200 and 500 μM H₂O₂, with a dose-dependent response as we observed a decrease of 38 and 97%, respectively (Figure 5B). This H₂O₂ dose-dependent response was maintained after the resilience period with a partial growth rate recovery for both treatments (75 and 39% of control, respectively, for 200 and 500 μM H₂O₂). Taken together these data suggested that 200 μM H₂O₂ can be considered as the tolerance threshold of cultivated cnidarian cells while 500 μM H₂O₂ can represent a critical concentration inducing deleterious cellular injuries.

**Hydrogen Peroxide Effects on Stress Biomarkers Under Treatments and Resilience Phase**

To determine the cellular response induced by H₂O₂ treatments on cultivated cells we analyzed biochemical stress markers (TOSC, carbonylation, and ubiquitination). After 1–6 h of treatment, we observed no significant effect on any of the three biomarkers (Supplementary Figure 2). However, after 24 h of treatment TOSC was impaired at both 200 and 500 μM H₂O₂. A full recovery was observed after 7 days of treatment and maintained during the resilience phase (Figure 7A), suggesting an acclimation of the cells to the pro-oxidant conditions. No signs of protein damage (protein carbonylation and ubiquitination) were measured after 24 h. However, the 200 μM H₂O₂ treatment induced an increase of ubiquitinated proteins after 7 days (Figure 7C). This effect was totally abolished after the resilience period (Figure 7C). These data suggest that H₂O₂ exposures, even at 500 μM and after 7 days, didn’t
lead to persistent oxidative damages on proteins in cnidarian cultivated cells.

**DISCUSSION**

**Extended Tolerance of Symbiotic Cnidarians to Hydrogen Peroxide**

In this study, we used H$_2$O$_2$ to induce pro-oxidative condition and to investigate the stress response in the symbiotic sea anemone *A. viridis* at both whole animal and cellular scales. The H$_2$O$_2$ concentrations used in the present study correspond to extremely high levels never measured in the seawater. However, rainwater can temporally induced increase of H$_2$O$_2$ to tens of micromolar (Ndungu et al., 2019; Jones and Lee, 2020). Benthic marine organisms from coastal areas are therefore regularly facing H$_2$O$_2$, that could lead to oxidative stress (Abele-Oeschger et al., 1997). Indeed, among ROS, H$_2$O$_2$ is the most abundant and long-lived in sea water and contrary to other ROS, H$_2$O$_2$...
could rapidly diffuse across membranes (see for review Halliwell and Gutteridge, 2015).

In our study model, at both whole animal and cellular scales, 200 μM of H$_2$O$_2$ did not create a condition of stress, since very weak impact was observed in global health: no sign of mortality or bleaching on specimens (Figure 1), no intracellular ROS accumulation (Figure 4) and no effect was observed on any stress biomarkers we tested (Figures 2, 7). In cell cultures, we nevertheless measured a significant cellular growth arrest (Figure 5), reflecting a common feature of stress response and could be interpreted as an usual resistance mechanism (see for review Davies, 1999, 2000).

H$_2$O$_2$ usually represents a threat for most organisms. Indeed, in diverse mammalian cell lines, a cytotoxic effect of H$_2$O$_2$ could be observed from 60 μM (Coyle et al., 2006) with drastic and irreversible impacts (i.e., apoptosis and necrosis) induced at 400 μM H$_2$O$_2$ (Xiang et al., 2016). Some studies performed on marine invertebrates showed that micromolar concentrations of H$_2$O$_2$, ranging from 0.5 to 20 μM, could impact the metabolism of the whole animal. For example, a 40% drop in O$_2$ consumption was observed in the Polychaete Nereis diversicolor under 5 μM of H$_2$O$_2$ (Abele-Oeschger et al., 1994), while higher level of H$_2$O$_2$ (50 μM) can cause oxidative damages (i.e., lipid peroxidation), as observed in another Polychaete species, Laenoeireis acuta (Da Rosa et al., 2008).

Contrasting with those results, our study highlighted an extended tolerance of symbiotic cnidarian facing even greater H$_2$O$_2$ concentrations. This resistance to H$_2$O$_2$ is, however, not a general cnidarian feature as it has been shown that concentrations exceeding 163 μM H$_2$O$_2$ caused mortality of the non-symbiotic sea anemone Nematostella vectensis (Friedman et al., 2018). Therefore, these results reinforced the hypothesis of adaptation of symbiotic cnidarians to pro-oxidative conditions, due to their lifestyle with a photosynthetic symbiont. Indeed, it has been already highlighted that symbiotic cnidarians exhibited a wide diversity of biochemical antioxidant actors, compared to non-symbiotic species (Furla et al., 2005). For instance, higher number of superoxide dismutase (SOD) isoforms was identified in the symbiotic cnidarian A. viridis compared to the non-symbiotic one Actinia schmidti. In complement, another comparative analysis showed that the glutathione peroxidase (GPx) isoforms were less numerous in the non-symbiotic sea anemone N. vectensis than in the symbiotic sea anemone A. viridis (Pey et al., 2017). To confirm the hypothesis of the adaptive process, it will be required to extend the comparison of the antioxidant battlefield between symbiotic and non-symbiotic cnidarians at multiple scales, even by including the non-enzymatic actors.

**Importance of Host Cells to Hydrogen Peroxide Defense**

In the present study, H$_2$O$_2$ exposure on whole organism affected mainly the endosymbiont, Philozoon sp. genus (LaJeunesse et al., 2021), rather than the animal host tissues. Although antioxidant defenses were stimulated after 7 days, an increase of protein carbonylation was measured in the symbiont fraction, whereas no increase was observed in the animal compartments (Figure 2). The susceptibility of free-living algae to H$_2$O$_2$ is well documented and highlighted an important heterogeneity in H$_2$O$_2$ response. For example, although the cyanobacterium Synechococcus aeruginosus tolerated until 2 mM H$_2$O$_2$, another cyanobacterium species, Microcystis aeruginosa, was affected by around 20 μM (EC50) and the diatoms Navicula seminulum by 200 μM (Drábková et al., 2007). Few studies have been performed on Symbiodiniaceae sensitivity to ROS and again they highlighted species-specific impacts on photosynthesis (Wietheger et al., 2015; Roberty et al., 2016). For example, cultured Symbiodinium microadriaticum strain showed high resistance to 1 mM H$_2$O$_2$, compared to Fugacium kawagutii showing drastic damage to photosystem function at the same H$_2$O$_2$ concentration (Wietheger et al., 2015). In addition, light exposure increased the photosynthesis impairment of cultured
Symbiodiniaceae from 30 min of >1 mM H$_2$O$_2$ exposure (Wiethegar et al., 2015).

Protected inside the gastrodermal host cell, the endosymbiont would not have shown any signs of oxidative stress under H$_2$O$_2$ exposure, but the sensitivity assessed in our study did not confirm this assumption. This sensitivity to H$_2$O$_2$ could, however, be correlated to previous studies demonstrating that Symbiodiniaceae living in hospite present a reduction of the antioxidant enzymatic defenses (i.e., SOD, catalases or peroxidases) compared to the free-living condition (Lesser and Shick, 1989; Richier et al., 2005; Pey et al., 2017). Nevertheless, it has been shown that in hospite, Symbiodiniaceae harbor a higher surface volume of thylakoid lamellae (Lesser and Shick, 1990), increasing definitely the photosystem density and consequently the source of ROS associated with electron chain transports (Saragosti et al., 2010).

These data, in addition with measurements of H$_2$O$_2$ diffusion from the symbiont (Suggett et al., 2008; Armoza-Zvuloni and Shaked, 2014), support the conclusion that, in hospite, the redox homeostasis of the symbiont is bolstered by the antioxidant defenses of the animal host cells. For example, in A. viridis it has been frequently observed that the animal compartment constitutes the major contributor to the holobiont antioxidant potential, with higher amount of antioxidant defenses compared to the symbiont fraction (Richier et al., 2003, 2005; Plantivaux et al., 2004; Merle et al., 2007; Pey et al., 2017). This agrees with studies performed on other symbiotic cnidarian species (Yakovleva et al., 2004; Levy et al., 2006; Krueger et al., 2015). By consequence, an experimental burst of H$_2$O$_2$ leads to more deleterious effects in the endosymbiont than in animal cells, illustrating their capacity to cope with H$_2$O$_2$.

**No Bleaching Induction by Hydrogen Peroxide**

Even at highest H$_2$O$_2$ exposure, no bleaching was observed neither during, nor after the exposure period in the treated A. viridis specimens (Figure 1). Interestingly, despite the protein damages observed in the symbiont, the equilibrium of the symbiosis was maintained. It has been largely documented in symbiotic cnidarians that stress-induced bleaching (e.g., thermal stress) is linked with the over-production of ROS by the endosymbiont, leading to significant oxidative damages in the host cells (see for review Suggett and Smith, 2020). Due to its permeable properties and its overproduction in several strains of Symbiodiniaceae exposed to thermal stress (Lesser, 1996; Suggett et al., 2008; Roberty et al., 2015), this ROS has then been suggested to be responsible of oxidative stress occurring in host cells during bleaching events. However, in this study, the absence of bleaching and of oxidative damages in host cells under H$_2$O$_2$ exposures can support the conclusion that H$_2$O$_2$ may not be the most important ROS associated with coral bleaching.

**Limits of the Resistance**

At cellular level, pro-oxidative condition can elicit a broad spectrum of responses from proliferation to growth arrest, or senescence and cell death, depending on the cell capacity to overcome the stress by repairing or removing damaged molecules. The observed effect reflects the balance between intracellular pathways activated in response to the oxidative injury and can vary significantly with the concentration of the oxidant agent and the treatment exposure. In our *in vitro* study model, 500 µM of H$_2$O$_2$ induced a decrease of cell viability, particularly pronounced after 7 days of treatment and associated with a drastic growth arrest (Figure 5).

This response could be explained by a strategy of the “sacrifice” signaling pathway set up by the cells to eliminate the most damaged cell population (Davies, 2000). Indeed, a change in the cell population pattern was highlighted after 7 days at 500 µM H$_2$O$_2$ (Figure 6) and no protein damages were observed in these surviving cells (Figure 7). Nevertheless, the increase of auto fluorescence measured in surviving cells after 7 days at 500 µM H$_2$O$_2$ (Figure 6) might be correlated with changes in metabolic activity of mitochondria that cells undergo during apoptosis, as it has been previously observed in mammalian cells (Levitt et al., 2006). In addition, the partial recovery of cell growth after the resilience period (Figure 5) suggested a non-reversible impact of 500 µM H$_2$O$_2$ injury on surviving cells, whose mechanisms should be deeply addressed in the future.

Finally, comparing the *in vitro* and the *in vivo* approaches, we highlighted that the sensitivity of A. viridis gastrodermal cells at 500 µM of H$_2$O$_2$ exposure is less pronounced in the gastrodermal tissue than in the isolated cultivated cells (Figures 2, 7). This is likely due to the contribution of the tissue organization thanks to host cell/cell communication, more efficient turnover of damaged cells and/or by a signaling pathway linked to the presence of the symbiont.

Moving forward thanks to the *in vitro* cnidarian cell culture, an ambitious perspective of this study will be to disentangle the mechanisms of H$_2$O$_2$ resistance of cnidarian cells and more specifically to assess the impact of other ROS (as the superoxide anion and the hydroxyl radical), thus contributing to decipher the adaptive tools that have evolved for a successful symbiosis stability and conversely to better understand the bleaching processes.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

PC did all the investigations (experimental work) and statistical analyses. CF contributed to the obtaining of cell cultures and their maintenance. CF and GT initialized the H$_2$O$_2$ experiments on cell cultures. PF and SB-V designed and supervised the research. PC, ER, SB-V, and PF wrote the manuscript. All authors read and agreed to the published version of the manuscript.
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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.819111/full#supplementary-material

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