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Co-occurring increases of calcium and organellar reactive oxygen species determine differential activation of antioxidant and defense enzymes in *Ulva compressa* (Chlorophyta) exposed to copper excess

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ABSTRACT

In order to analyse copper-induced calcium release and (reactive oxygen species) ROS accumulation and their role in antioxidant and defense enzymes activation, the marine alga *Ulva compressa* was exposed to 10 μM copper for 7 d. The level of calcium, extracellular hydrogen peroxide (eHP), intracellular hydrogen peroxide (iHP) and superoxide anions (SA) as well as the activities of ascorbate peroxidase (AP), glutathione reductase (GR), glutathione-S-transferase (GST), phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) were determined. Calcium release showed a triphasic pattern with peaks at 2, 3 and 12 h. The second peak was coincident with increases in eHP and iHP and the third peak with the second increase of iHP. A delayed wave of SA occurred after day 3 and was not accompanied by calcium release. The accumulation of iHP and SA was mainly inhibited by organellar electron transport chains inhibitors (OETCI), whereas calcium release was inhibited by ryanodine. AP activation ceased almost completely after the use of OETCI. On the other hand, GR and GST activities were partially inhibited, whereas defense enzymes were not inhibited. In contrast, PAL and LOX were inhibited by ryanodine, whereas AP was not inhibited. Thus, copper stress induces calcium release and organellar ROS accumulation that determine the differential activation of antioxidant and defense enzymes.

Key-words: antioxidant enzymes; calcium; marine alga; oxidative stress; ROS.

INTRODUCTION

Plants are constantly exposed to biotic and abiotic stresses which trigger common metabolic responses (Fujita *et al*. 2006). One of these common responses is the onset of an oxidative burst characterized by accumulation of reactive oxygen species (ROS) (Baker & Orlandi 1997; Wojtazsek 1997; Mittler *et al*. 2004; Van Breusegem, Bailey-Serres & Mittler 2008). ROS are a family of products derived from the sequential reduction of molecular oxygen and include superoxide anion (SA) ($.O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (.OH), among others (Wojtazsek 1997). During biotic stresses, ROS production is mainly due to the activation of a plasma membrane-bound NADPH oxidase (Desikan *et al*. 1996; Hammond-Kosack & Jones 1996; Torres & Dangl 2005). This enzyme produces SA in the apoplast which are dismutated to H_2O_2 by superoxide dismutase (SOD) and then it can diffuse through the cell wall to the extracellular medium or enters into the cell (Hammond-Kosack & Jones 1996). Another common response is the release of intracellular calcium induced by pathogen infection and elicitors (Lecourieux *et al*. 2002; Garcia-Brugger *et al*. 2006; Varnier *et al*. 2009). Elicitor-induced calcium release triggered the activation of the plasma membrane-bound NADPH oxidase due to the binding of calcium to the N-terminal region of this ROS-producing enzyme (Hu *et al*. 2007; Ogasawara *et al*. 2008; Van Breusegem *et al*. 2008). Calcium release from internal pools is probably determined by a calcium-induced calcium release mechanism involving calcium channels located in the plasma membrane (Tavernier *et al*. 1995; Kurusu *et al*. 2005).

The increase of intracellular hydrogen peroxide (iHP) triggers the activation of antioxidant and defense enzymes located in different cellular compartments in plants (Hammond-Kosack & Jones 1996; Jabs *et al*. 1997; Mittler *et al*. 2004). The antioxidant enzymes include SOD, catalase (CAT), glutathione peroxidase (GP) and those of the Haliwell-Asada cycle [ascorbate peroxidase (AP), dehydroascrobate reductase (DHAR) and glutathione reductase (GR)] and the antioxidant and detoxifying enzyme glutathione-S-transferase (GST). H_2O_2 production also activates defense enzymes which include the first and key regulatory enzyme of the phenylpropanoid pathway phenylalanine ammonia lyase (PAL) and the first and regulatory

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enzyme of the octadecanoid pathway lipoxygenase (LOX) (Bate *et al*. 1994; Howles *et al*. 1996; La Caméra *et al*. 2004). The phenylpropanoid pathway produces multiple families of phenolic compounds with antimicrobial, antioxidant and other biological activities (Dixon 2001) and the octadecanoid pathway leads to the synthesis of oxylipins with antipathogenic and regulatory activities (Howe & Schilmiller 2002; Blée 2004).

Regarding responses induced by abiotic stress in plants, ozone triggers a NADPH oxidase-dependent biphasic oxidative burst in *Arabidopsis thaliana* that activates antioxidant and defense enzymes (Schraudner *et al*. 1998; Mahalingam *et al*. 2006). On the other hand, ozone as well as absicic acid (ABA) treatment, dessication, cold, heat, salinity, ultraviolet (UV) light and anoxia induce intracellular calcium release and the activation of antioxidant enzymes (Knight, Trewavas & Knight 1997; Clayton *et al*. 1999; Lecourieux, Ranjeva & Pugin 2006; Hu *et al*. 2007). In this sense, the treatment of maize seedlings with ABA or H_2O_2 induces an increase in calcium which is required for the activation of SOD, AP and GR gene expression (Hu *et al*. 2007).

Regarding stress by heavy metals, cadmium induced a NADPH oxidase-dependent accumulation of extracellular hydrogen peroxide (eHP) in tobacco cells and lead promoted the production of SA in *Vicia faba* root segments (Olmos *et al*. 2003; Romero-Puertas *et al*. 2004; Garnier *et al*. 2006; Pourrut *et al*. 2008). In addition, a delayed wave of SA was triggered by cadmium in mitochondria of tobacco cells (Garnier *et al*. 2006). However, it has been recently shown that cadmium inhibits NADPH oxidase activity in purified plasma membranes and induces H_2O_2 and SA production in isolated mitochondria (Heyno, Klose & Krieger-Liszkay 2008). Thus, it is possible that ROS accumulation in response to cadmium may occur mainly in organelles. Regarding copper stress, it was shown that it triggers the activation of NADPH oxidase in isolated membranes of plant cells (Quartacci, Cosi & Navari-Izzo 2001; Raeymaekers *et al*. 2003), enhances the production of SA in yeast mitochondria (Liang & Zhou 2007) and induces the release of intracellular calcium in tobacco cells (Inoue *et al*. 2005). In addition, copper, unlike cadmium, produces. OH by reacting with H_2O_2 as part of the Fenton reaction. Until now, there is no information about coordinated responses of ROS and calcium induced by copper stress in plants, neither about their involvement in the activation of antioxidant and/or defense enzymes.

In macroalgae, much less is known on the biochemical responses to biotic and abiotic stresses. Some studies have addressed the responses to desiccation (Collén & Davison 1999; Burritt, Larkindale & Hurd 2002) and heavy metals (Collén, Pedersén & Colepicolo 2003; Ratkevicius, Correa & Moenne 2003; Contreras, Moenne & Correa 2005) in the field. Increases in ROS and activation of several antioxidant enzymes were detected in most cases (Burritt, Larkindale & Hurd 2002; Contreras *et al*. 2005). It was determined that copper induces in the brown seaweeds *Lessonia nigrescens* and *Scytosiphon lomentaria* a single peak of iHP at 2 h of copper exposure and only *L. nigrescens* showed SA accumulation (Contreras *et al*. 2009). On the other hand, it was reported that strontium induces calcium release in the green microalga *Eremosphaera viridis* (Bauer *et al*. 1998) as does osmotic stress in the zygote of the brown macroalga *Fucus serratus* (Coelho *et al*. 2002). Among marine macroalgae, the opportunistic species *Ulva compressa* (Chlorophyceae) is known for being highly tolerant to metals in general, and to copper in particular (Say, Burrows & Witton 1990; Ratkevicius *et al*. 2003). In fact, *U. compressa* from copper-enriched environments not only accumulated the metal but also activated the antioxidant enzyme AP and increased the synthesis of ascorbate (ASC), which was accumulated as dehydroascorbate (DHA) (Ratkevicius *et al*. 2003). As in the case of plants, there is no information about the effects of copper stress on calcium release and ROS production and their involvement in the activation of antioxidant and/or defense enzymes in marine algae.

Thus, in this work, we analysed the occurrence and kinetics of copper-induced intracellular calcium release and the nature, cellular location and kinetics of ROS accumulation as well as their involvement in the activation of antioxidant enzymes (AP, GR and GST) and defense enzymes (PAL and LOX) in the marine alga *U. compressa.*

MATERIALS AND METHODS

Algal and seawater sampling

U. compressa was collected in Cocholgüe (36°40′S), a non-impacted site of southern Chile (Ratkevicius *et al*. 2003), during spring 2008 and transported to the laboratory in sealed plastic bags in a cooler at 4 °C. Algal samples were rinsed three times in sterile filtered seawater and cleaned twice for 1 min using a Branson 3200 (Danbury, CT, USA) ultrasound bath to remove epiphytic bacteria and organic debris. Seawater was obtained from Quintay (33°12′S) in central Chile, filtered through 0.2 μm pore size membrane filters and stored in darkness at 4 °C.

In vitro cultures

U. compressa was cultured in 0.2 μm filtered seawater from Quintay, or in seawater containing 10 μM $CuCl_2$ (Merck, Darmstadt, Germany), which corresponds to 635 μg of copper L^{-1}. Culture medium was aerated and changed every 24 h. Triplicates of each treatment were cultured at 12 °C, 12:12 h photoperiod and 40–50 μmol m^{-2} s^{-1} irradiance. After sampling, material was treated with 100 mM Tris-HCl pH 8.0–10 mM ethylenediaminetetraacetic acid (EDTA) as standard rinsing step prior to different analyses.

Detection of calcium release

U. compressa (1 g of fresh weight) was incubated in 10 mL of filtered seawater (control) and 1 g of alga in 10 mL filtered seawater containing 100 μM ryanodine for 40 min. Half of the control algae were transferred to seawater

without copper, and half to seawater with 10 μM $CuCl_2$. Half of the ryanodine-treated lamina were transferred to seawater without copper and half to seawater with 10 μM $CuCl_2$ and cultivated for 0 to 24 h and 2 to 7 d. At each experimental time, three lamina of each sample were incubated with 20 μM Fluo-3AM (Molecular Probes, Invitrogen, Eugene, OR, USA) for 30 min and washed three times in filtered seawater to remove the excess of the fluorophor. The lamina were visualized by confocal microscopy using an Axiovert 100 confocal microscope (Carl Zeiss, Oberkochen, Germany), an emission wavelength of 488 nm from an argon laser and a filter of 505–550 nm for fluorescence detection. The intensity of fluorescence was quantified in 30 cells of each lamina using LSM510 software of the confocal microscope.

Detection of eHP

eHP levels were determined incubating 5 g of fresh weight of *U. compressa* in 100 mL of seawater without copper or with 10 μM $CuCl_2$ and incubated for 0 to 24 h and 2 to 7 d. At each experimental time, three samples of 1 mL of seawater was removed, added to 1 mL of 100 mM Tris–HCl pH 7.0 containing 100 μM Amplex Red (Invitrogen, Eugene, OR, USA) and 2 U mL^{-1} of horseradish peroxidase (Invitrogen) and incubated at room temperature for 5 min. The absorbance was determined at 570 nm using a Genesys 5 spectrophotometer (Spectronic, Milton Roy Co., Waltham, MA, USA). H_2O_2 level was calculated as nanomoles of resorufin using the extinction coefficient of resorufin ($\varepsilon = 54\ mM^{-1}\ cm^{-1}$) but was expressed as nanomoles of H_2O_2 which are equivalent to nanomoles of resorufin.

Detection of iHP

iHP levels were determined by cultivating 30 g fresh weight of *U. compressa* in 1 L of seawater without copper or with 10 μM copper for 0 to 24 h and 2 to 7 d, in triplicate. A sample of 1.5 g was incubated in 100 mL of Tris–HCl buffer pH 7.0 containing 10 μM 2′, 7′ dichlorohydrofluorescein diacetate (DCFH-DA, Calbiochem, San Diego, ca, USA) for 45 min at room temperature. Algal tissue was rinsed in seawater, blotted dry, weighed, frozen in liquid nitrogen and homogenized in a mortar with addition of 5 mL of 40 mM Tris–HCl buffer pH 7.0. The homogenate was centrifuged at 20 600 g for 15 min and the supernatant was recovered. Fluorescence of the clear extract was determined in a LS-5 spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. H_2O_2 level was expressed as nanomoles of 2′,7′ dichlorofluoresecein (DCF) using a calibration curve prepared with 0 to 500 nanomoles of DCF (Sigma, St. Louis, MO, USA).

Detection of intracellular SA

SA were determined by cultivating 30 g of fresh weight of *U. compressa* in 1 L of seawater without copper or with 10 μM copper for 0 to 24 h and 2 to 7 d, in triplicate. A sample of 1.5 g was incubated in 100 mL of Tris–HCl buffer pH 7.0 containing 100 μM hydroethidine (Molecular Probes, Invitrogen) for 1 h at room temperature. Algal tissue was rinsed in seawater, blotted dry, weighed, frozen in liquid nitrogen and homogenized in a mortar with addition of 5 mL of 40 mM Tris–HCl buffer pH 7.5. The homogenate was centrifuged at 20 600 g for 15 min and the supernatant was recovered. Fluorescence of the clear extract was determined in a LS-5 spectrofluorometer (Perkin-Elmer) using an excitation wavelength of 480 nm and an emission wavelength of 590 nm. SA level was expressed as nanomoles of 2-hydroxy ethidium (2OH-E) using the extinction coefficient of 2OH-E ($\xi = 9.4\ mM^{-1}\ cm^{-1}$).

Detection of antioxidant and defense enzymes

U. compressa (30 g of fresh weight) was cultivated in 1 L of seawater without copper (control) and with 10 μM copper, in triplicate. Samples were collected after 6, 12 and 24 h, and 2, 3, 4, 5, 6 and 7 d of exposure. Protein extracts from *U. compressa* were obtained as described by Ratkevicius *et al.* (2003) as well as CAT, GP, AP, DHAR and GR antioxidant enzymes activities detection.

GST activity dependent on 1-chloro 2, 4 dinitrobenzene (CDNB, Sigma) was determined in 1 mL of reaction mixture containing 100 mM phosphate buffer pH 7.0, 0.5 mM GSH, 1 mM CDNB and 50 μg of protein extract. The increase in absorbance at 340 nm due to GSH-CDNB adduct synthesis was monitored for 1 min. GST activity was calculated using the extinction coefficient of the GSH-CDNB adduct ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

PAL activity was determined in 1 mL of reaction mixture containing 100 mM phosphate buffer pH 7.0, 13 mM phenylalanine and 40–80 μg of protein extract at 40 °C. The increase in absorbance, due to cinnamic acid accumulation, was monitored at 290 nm for 45 min. PAL activity was calculated using the extinction coefficient of cinnamic acid ($\varepsilon = 17.4\ mM^{-1}\ cm^{-1}$).

LOX activity was determined in 1 mL of reaction mixture containing 100 mM phosphate buffer pH 7.0, 0.4 mM arachidonic acid and 25 μg of protein extract. The increase in absorbance due to the accumulation of conjugate dienes was monitored at 234 nm for 10 min. LOX activity was calculated using the extinction coefficient of conjugated dienes ($\varepsilon = 25\ mM^{-1}\ cm^{-1}$). Additional LOX assays were also conducted using 0.4 mM of linoleic and linolenic acids.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined in 1 mL reaction mixture containing 40 mM Tris–HCl pH 8.2, 4 mM glucose, 0.6 mM $NADP^+$, 5 mM $MgCl_2$ and 50 μg of protein extract. The increase in absorbance due to NADPH accumulation was monitored at 340 nm for 5 min. G6PDH activity was calculated using the extinction coefficient of NADPH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Treatment with ROS and calcium inhibitors

Diphenylene iodomium (DPI, Sigma) is an inhibitor of flavin-containing enzymes as plasma membrane NADPH

oxidase, mitochondrial complex I and II and chloroplast photosystem I ferredoxin:NADP oxidoreductase (Majander, Finel & Wikstrom 1994; Li & Trush 1998; Onda & Hase 2004; Ooyabu *et al*. 2008). Thus, for specific inhibition of organellar electron transport chains, the inhibitor of mitochondrial complex III, antimycin A (AA, Sigma), and the inhibitor of the photosystem II, 3-(2,3-dichlorophenyl)-1,1-dimethylurea (DCMU, Sigma-Aldrich, Milwaukee, WI, USA), were used.

U. compressa (30 g fresh tissue) was incubated in 100 mL of filtered sea water containing 10 μM AA, 20 μM DCMU, 10 μM AA and 20 μM DCMU, 10 μM DPI or 100 μM ryanodine (Alexis Biochemicals, San Diego, CA, USA) for 60 min, in triplicate. Algae were transferred to 1 L of seawater without copper or with 10 μM $CuCl_2$ and incubated for 3 h to detect eHP, 3 and 12 h to detect iHP, 7 d to detect SA and 12 h or 7 d to detect antioxidant and defense enzyme activities.

Detection of cell viability

Three lamina of *U. compressa* were incubated in 500 μL of filtered seawater containing 5 μM of Scyto-13 (Molecular Probes, Invitrogen) for 45 min, a fluorophor that stains nuclei of viable cells. A lamina of the alga was visualized by confocal microscopy using an Axiovert 100 confocal microscope (Carl Zeiss), emission wavelengths of 488 nm from an argon laser and 525 nm from a neon laser and a filter of 505–530 nm for fluorescence detection.

Statistical analysis

Significant differences were determined by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (T). Differences between mean values were considered to be significant at a probability of 5% ($P < 0.05$) (Zar 1999).

RESULTS

Detection of cell viability after treatment with increasing concentrations of copper

Thalli of *U. compressa* cultivated with 0–50 μM of added copper for 7 d and then in seawater without copper addition for 7 additional days showed normal cell viability, as indicated by their green nuclei stained by Syto-13 fluorophor and chloroplasts normal in shape and fluorescence (Fig. 1a–e). In contrast, cells of thalli cultivated with 100 μM of added copper displayed collapsed nuclei and chloroplasts (Fig. 1f). Thus, copper additions of 0–50 μM are sub-lethal for *U. compressa*, and thus, 10 μM of added copper was selected for subsequent experiments.

Detection of copper-induced calcium release

Copper stress induced an increase in intracellular calcium in *U. compressa* which was inhibited by ryanodine (Fig. 2a–c). Calcium release showed a triphasic pattern with maximal

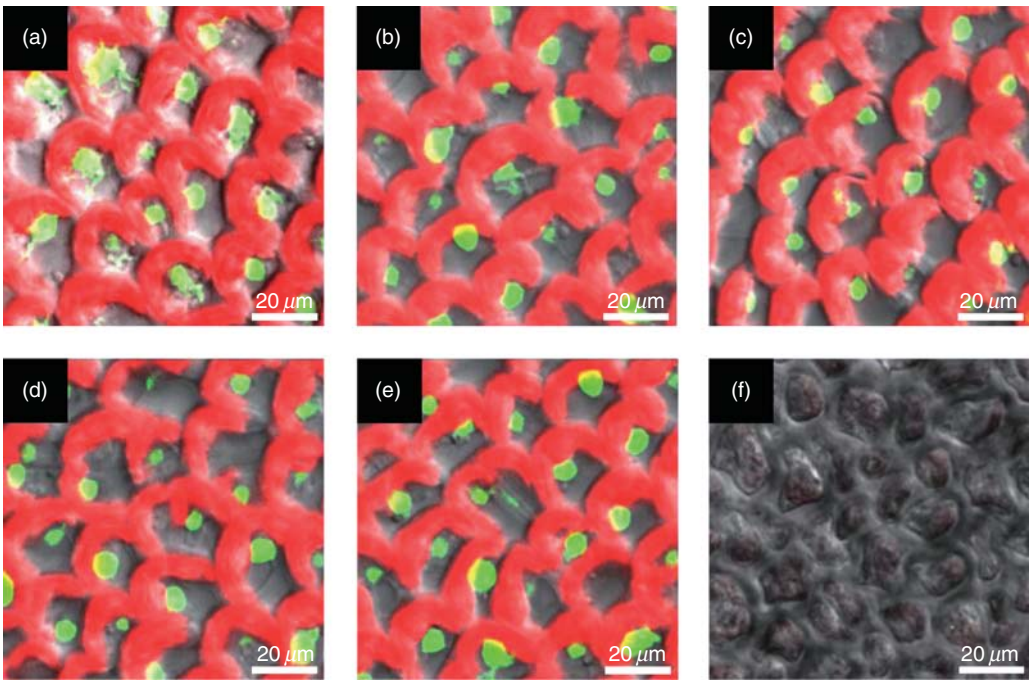

Figure 1. Visualization by confocal microscopy of cell viability in *Ulva compressa* cultivated in seawater without added copper (a), or with 5 μM (b), 10 μM (c), 25 μM (d), 50 μM (e) and 100 μM (f) of added copper for 7 d and then cultivated for 7 additional days in seawater without added copper. Green fluorescence of nuclei indicates cell viability and red fluorescence corresponds to chloroplast autofluorescence The white bar represents 20 μm.

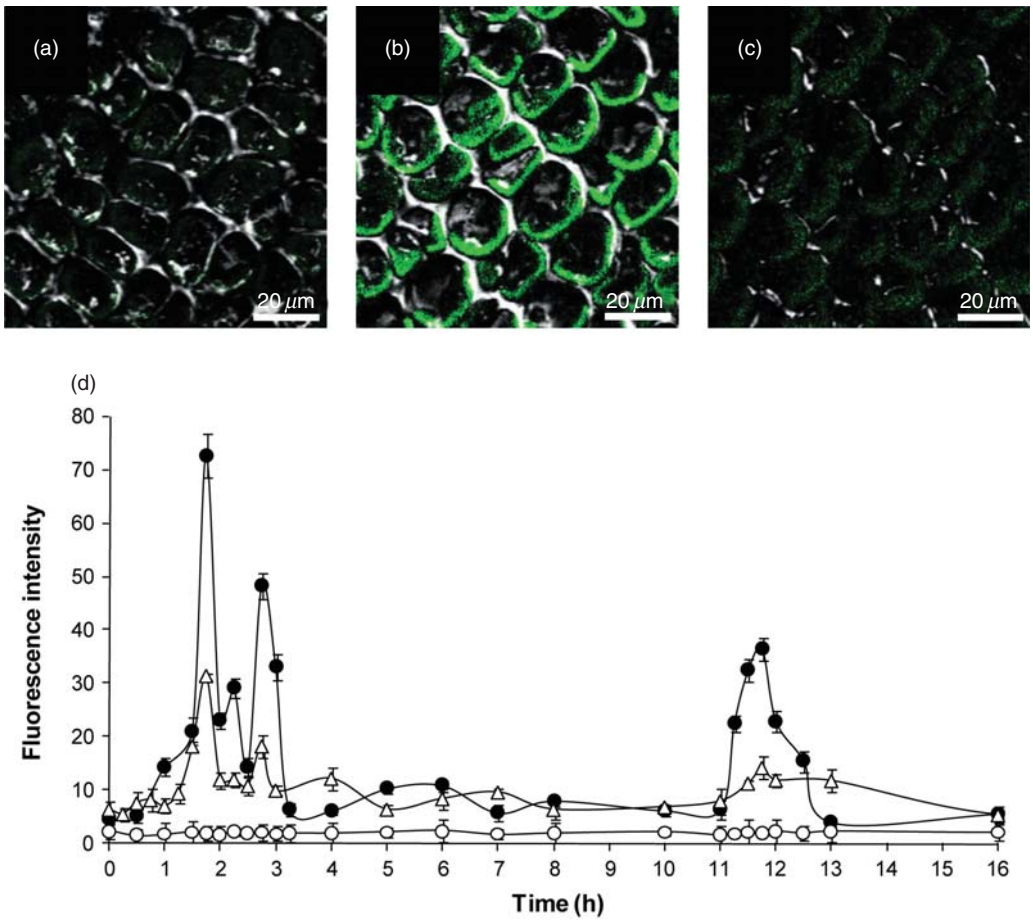

Figure 2. Visualization by confocal microscopy of intracellular calcium release in *Ulva compressa* incubated in seawater without copper addition (a), with 10 μM copper (b), treated 100 μM ryanodine and cultivated with 10 μM copper (c) for 12 h. Green fluorescence indicates calcium release (b). The level of intracellular calcium is expressed as fluorescence intensity (d) in samples incubated without copper (white circles), with 10 μM copper (black circles) and treated with 100 μM ryanodine and incubated with 10 μM copper (white triangles) for 0 to 16 h. Symbols represent mean values of three independent replicates ± standard deviation.

levels at 2, 3 and 12 h which decreased 57, 60 and 66%, respectively, with 100 μM ryanodine (Fig. 2d). Algae treated with ryanodine and cultivated without copper showed the same level of intracellular calcium as those cultivated without ryanodine and without copper (data not shown). The level of intracellular calcium reached the control level at 13 h of copper exposure and remained unchanged until day 7 (data not shown).

Detection of copper-induced eHP accumulation

The accumulation of eHP showed a monophasic pattern in *U. compressa* cultivated with copper reaching a maximal level of 12.4 nmoles g^{-1} of fresh tissue after 3 h of culture compared with the control (Fig. 3a). eHP accumulation decreased to reach the control level at 6 h of copper exposure and remained unchanged until day 7 (data not shown). The accumulation of eHP was completely inhibited by DPI (Fig. 3a). Mean values of eHP at 3 h of copper exposure decreased 36% with AA, 40% with DCMU, 58% with the mixture of both organellar inhibitors and was completely inhibited by DPI (72%) reaching control level (Fig. 3b). Algae treated with ROS inhibitors and cultivated without copper showed the same level of eHP as those cultivated without inhibitors and without copper (data not shown). This shows that copper-induced eHP is produced mainly in organelles and it is also dependent on NADPH oxidase activity.

Detection of copper-induced iHP accumulation

The accumulation of iHP showed a biphasic pattern during the first 12 h of exposure to copper, with a first peak of 1 nmoles g^{-1} of fresh tissue after 3 h followed by a second peak of 1.3 nmoles g^{-1} fresh tissue after 12 h (Fig. 4a). A sharp decline was recorded after 14 h of exposure, although the lowest level was still twice the basal level. Subsequent decline was gradual and continued until the basal level was reached after 48 h of exposure (Fig. 4a), a condition that persisted to the end of the experiment (day 7, data not shown). Incubation of *U. compressa* with DPI resulted in complete inhibition of both peaks (Fig. 4a). Mean values of

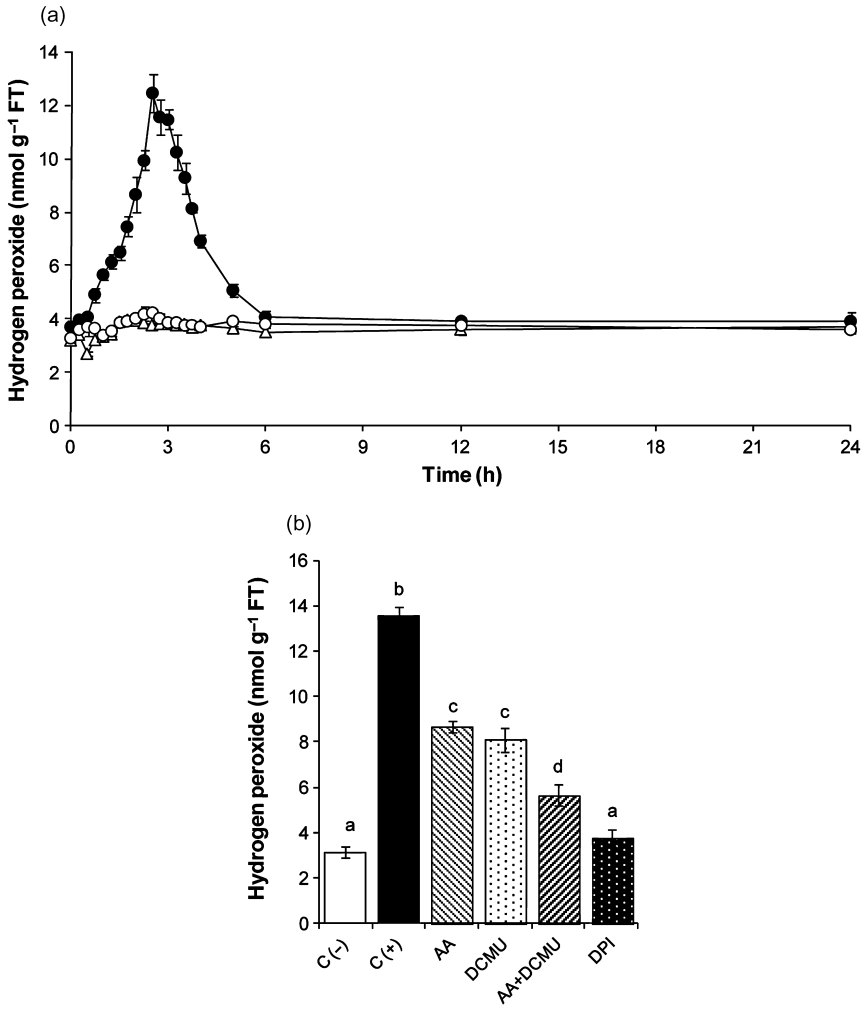

Figure 3. Levels of extracellular hydrogen peroxide in *Ulva compressa* cultivated in seawater without copper addition (white circle), with 10 μM copper (black circle), treated with 10 μM diphenylene iodomium (DPI) and cultivated with 10 μM copper (white triangle) for 0 to 24 h (a). The level of hydrogen peroxide was determined in the alga cultivated without copper (C–), with 10 μM copper (C+) and treated with 10 μM antimycin A (AA), 20 μM 3-(2,3-dichlorophenyl)-1,1-dimethylurea (DCMU), the mixture of the latter inhibitors (AA + DCMU) or 10 μM DPI and cultivated with 10 μM copper for 3 h (b). The level of hydrogen peroxide is expressed in nanomoles per gram of fresh tissue (FT). Bars and symbols represent mean values of three independent replicates ± standard deviation. Different letters indicate significant differences ($P < 0.05$).

H_2O_2 at 3 h of copper exposure decreased 41% with AA, 52% with DCMU, 66% with the mixture of organellar inhibitors and was completely with DPI (88%) reaching control level (Fig. 4b). In addition, mean values of H_2O_2 at 12 h of copper exposure decreased 48% with AA, 51% with DCMU, 67% with the mixture of organellar inhibitors and was completely by DPI (69%) reaching control level (Fig. 4c). Algae treated with ROS inhibitors and cultivated without copper showed the same level of iHP as those cultivated without inhibitors and without copper (data shown). Thus, copper-induced iHP is produced exclusively in organelles.

Detection of copper-induced SA accumulation

The accumulation of SA did not increase during the first 3 d (Fig. 5a), but from day 3 onwards, they increased rapid and significantly, with no evidence of reaching a plateau at the end of the experimental period (Fig. 5a). DPI almost completely inhibited SA accumulation (Fig. 5a). The accumulation of SA at day 7 of copper treatment decreased 54% with AA, 60% with DCMU, 69% with a mixture of organellar inhibitors and was not completely inhibited with DPI (84%) (Fig. 5b). Algae treated with ROS inhibitors and cultivated without copper showed the same level of SA as those cultivated without inhibitors and without copper (data not shown). Thus, a small fraction of SA is due to NADPH oxidase activity (15%) and another small fraction originates via Fenton reaction (7%).

Detection of antioxidant enzymes activities and effect of ROS and calcium inhibitors

AP activity increased during the first 3 d from 0.04 to 13 μmoles min^{-1} mg^{-1} protein, which represents a 325 times increase, to then undergo an exponential increase, reaching the exceptional activity of 654 μmoles min^{-1} mg^{-1} protein (16 350 times increase) at the end of the experiment (Fig. 6a). AP activity was almost completely inhibited by DPI at day 7 of copper treatment (Fig. 6a). Mean values of AP activity was 61% inhibited by antimycin A, 71% by DCMU, 75% by the mixture of organellar inhibitors, 90% by DPI and was not inhibited by ryanodine after 7 d of copper treatment (Fig. 6b). GR activity, on the other hand, showed a sustained increase from day 1, reaching

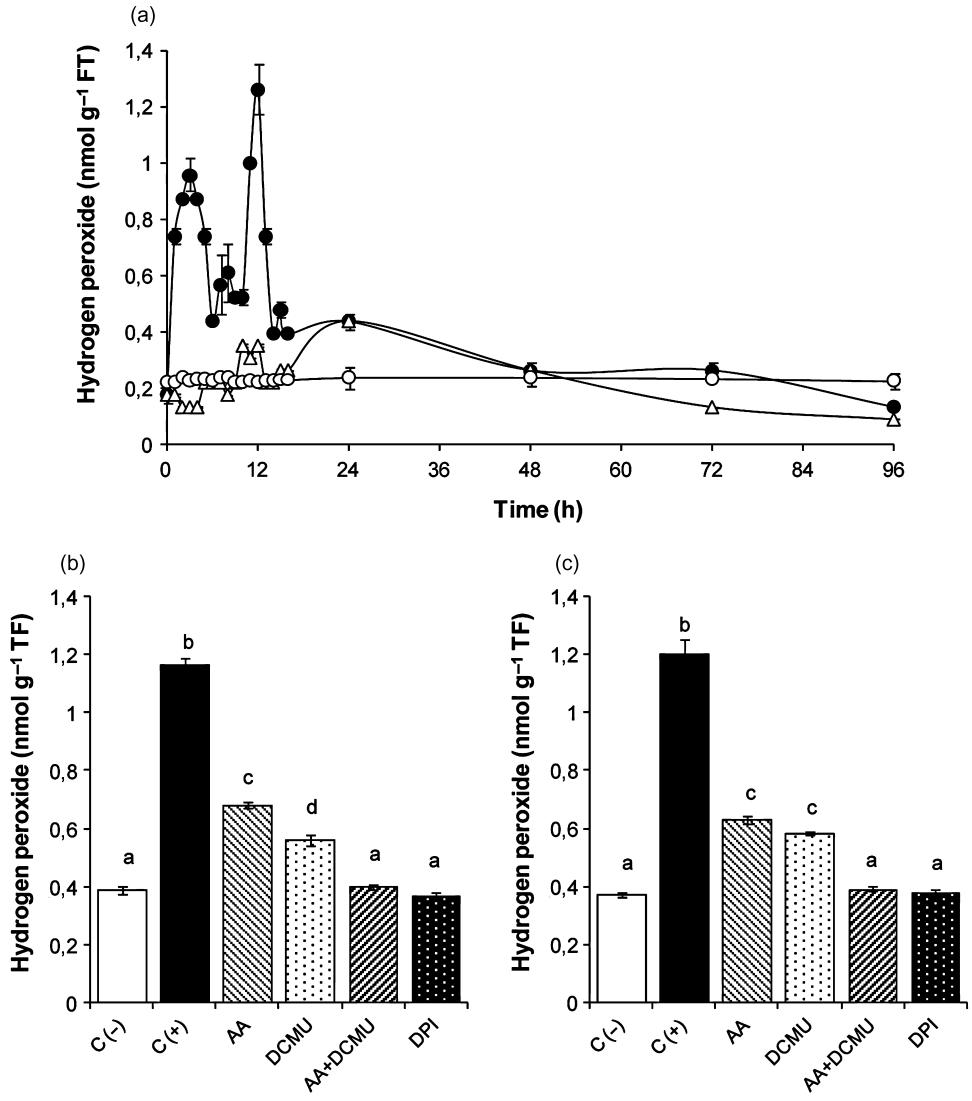

Figure 4. Levels of intracellular hydrogen peroxide peroxide in *Ulva compressa* cultivated in seawater without copper addition (white circle), with 10 μM copper (black circle), treated with 10 μM diphenylene iodomium (DPI) and cultivated with 10 μM copper (white triangle) for 0 to 96 h (a). The level of hydrogen peroxide was determined in the alga cultivated without copper (C–), with 10 μM copper (C+) and treated with 10 μM antimycin A (AA), 20 μM 3-(2,3-dichlorophenyl)-1,1-dimethylurea (DCMU), the mixture of the latter inhibitors (AA + DCMU) or 10 μM DPI and cultivated with 10 μM copper for 3 h (b) or 12 h (c). The level of hydrogen peroxide is expressed in nanomoles per gram of fresh tissue (FT). Bars and symbols represent mean values of three independent replicates ± standard deviation. Different letters indicate significant differences ($P < 0.05$).

0.85 μmoles min^{-1} mg^{-1} of protein extract (14 times increase) at day 7 (Fig. 6c) and was moderately inhibited by DPI (Fig. 6c). GR activity was 22% inhibited by antimycinA, 32% by DCMU, 44% by the mixture of organellar inhibitors, 64% by DPI and 13% by ryanodine after 7 d of copper treatment (Fig. 6d). GST activity, on the other hand, increased from 0.03 to 0.34 μmoles min^{-1} mg^{-1} of protein extract after 24 h exposure (11 times increase) decreasing to the basal level after 5 d to remain stable to the end of the experiment (Fig. 6e). This activity was moderately inhibited by DPI (Fig. 6e). GST activity was 42% inhibited by antimycinA, 50% by DCMU, 58% by the mixture of organellar inhibitors, 54% by DPI and 27% by ryanodine after 12 h of copper treatment (Fig. 6f).

Detection of defense enzymes activities and effect of ROS and calcium inhibitors

PAL activity showed a constant increase in individuals of *U. compressa* cultivated with copper (Fig. 7a) from 2.9 to 40 nmoles min^{-1} mg^{-1} of protein extract after 7 d of exposure (14 times increase) and was not inhibited by DPI (Fig. 7a). PAL activity was not inhibited by AA, DCMU, the mixture of organellar inhibitors or DPI but was 38% inhibited

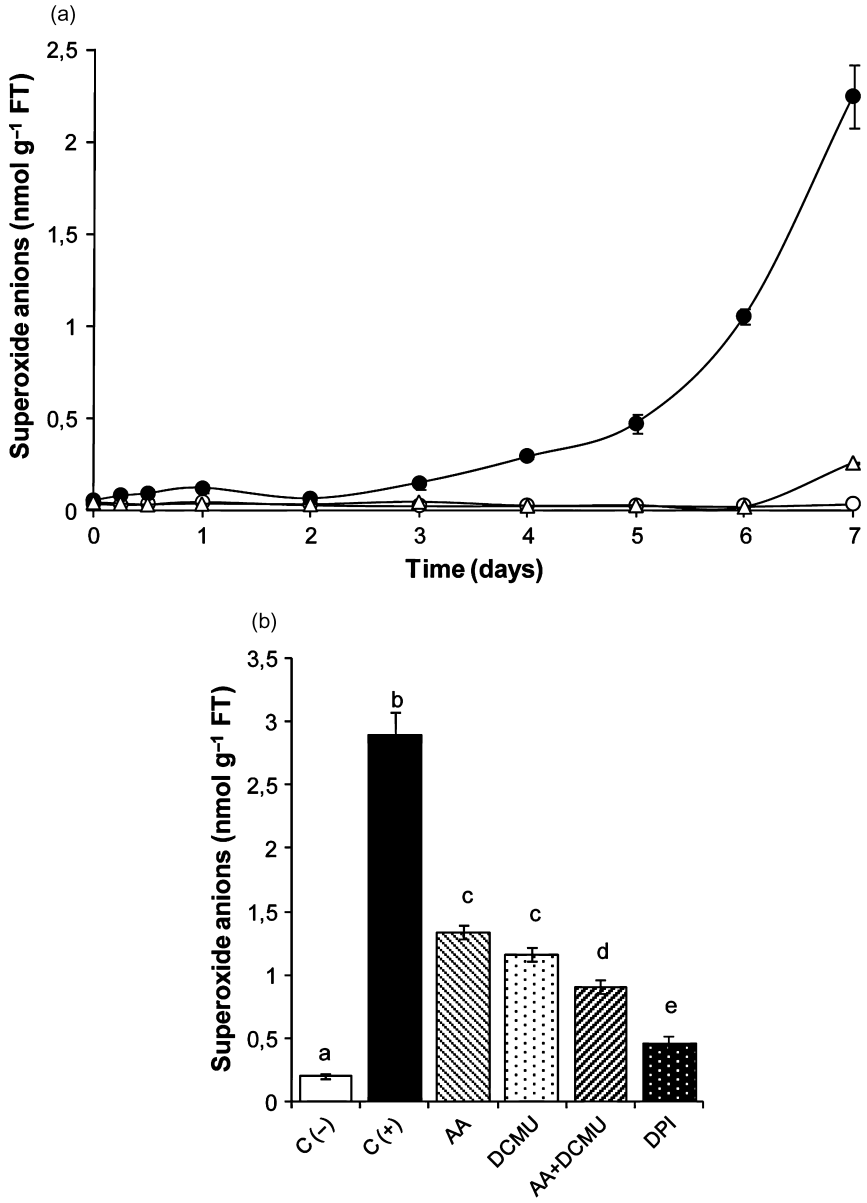

Figure 5. Levels of intracellular superoxide anions in *Ulva compressa* cultivated in seawater without copper addition (white circle), with 10 μM copper (black circle), treated with 10 μM diphenylene iodomium (DPI) and cultivated with 10 μM copper (white triangle) for 0 to 7 d (a). Level of superoxide anions was determined in the alga cultivated without copper (C–), with 10 μM copper (C+) and treated with 10 μM antimycin A (AA), 20 μM 3-(2,3-dichlorophenyl)-1,1-dimethylurea (DCMU), the mixture of the latter inhibitors (AA + DCMU) or 10 μM DPI and cultivated with 10 μM copper for 7 d (b). The level of superoxide anions is expressed in nanomoles per gram of fresh tissue (FT). Bars and symbols represent mean values of three independent replicates ± standard deviation. Different letters indicate significant differences ($P < 0.05$).

by ryanodine after 7 d of copper treatment (Fig. 7b). LOX activity, on the other hand, was detected only with arachidonic acid as substrate (Fig. 7c). The arachidonic acid-dependent LOX activity increased from 4.4 to 20 μmoles min^{-1} mg^{-1} of protein extract after 12 h of exposure (4.5 times increase) and showed a sharp decline after 12 h to reach the basal level at day 5 and it was not inhibited by DPI (Fig. 7c). LOX activity was not inhibited by AA, DCMU, the mixture of organellar inhibitors or DPI but was 34% inhibited by ryanodine after 12 h of copper treatment (Fig. 7d).

Detection of cell viability after treatment with inhibitors and G6PDH activity

Cell viability of *U. compressa* was analysed after 5 d of culture in control conditions (Fig. 8a), with copper only

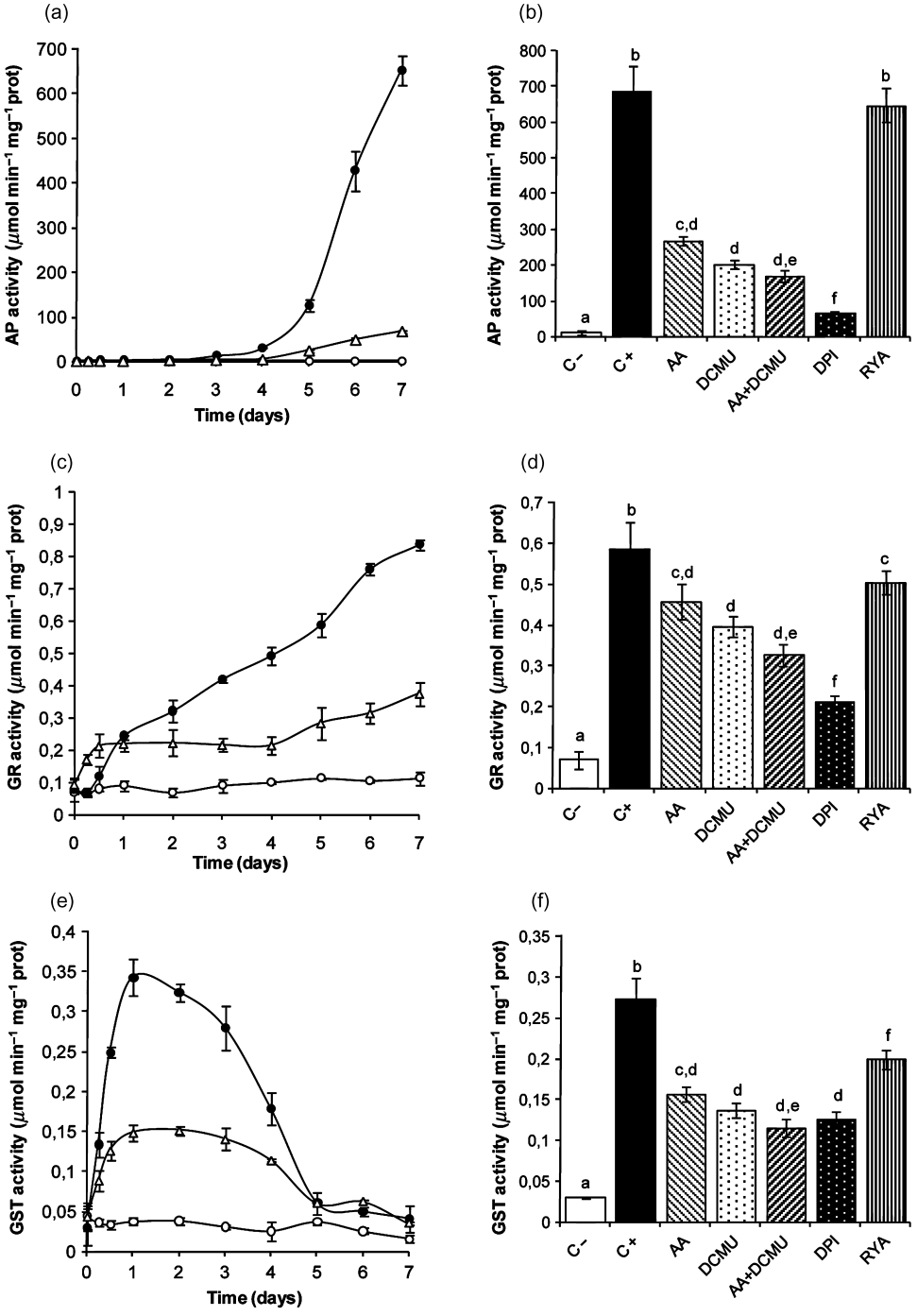

Figure 6. Activities of antioxidant enzymes ascorbate peroxidase (AP) (a), glutathione reductase (GR) (c) and glutathione-S-transferase (GST) (e) in *Ulva compressa* cultivated without copper (white circles), with 10 μM copper (black circles), treated with 10 μM diphenylene iodomium (DPI) and cultivated with 10 μM copper (white triangles) for 0 to 7 d. Activities of AP (b), GR (d) and GST (f) were determined in the alga cultivated without copper (C–), with 10 μM copper (C+) and treated with 10 μM antimycin A (AA), 20 μM 3-(2,3-dichlorophenyl)-1,1-dimethylurea (DCMU), the mixture of the latter inhibitors (AA + DCMU), 10 μM DPI or 100 μM ryanodine (RYA) and cultivated with 10 μM copper for 7 d (b,d) or 12 h (f). Activities of AP, GR and GST are expressed as micromoles per minute per milligram of protein. Bars and symbols represent mean values of three independent replicates ± standard deviation. Different letters indicate significant differences ($P < 0.05$).

(Fig. 8b) and with copper using individuals treated with the inhibitors AA (Fig. 8c), DCMU (Fig. 8d), the mixture of organellar inhibitors (Fig. 8e) or DPI (Fig. 8f). Algal cells were viable in all cases as their nuclei were green-stained by Syto-13 fluorophor. In addition, the activity of the NADPH-producing enzyme G6PDH involved in basal metabolism (i.e. ribose synthesis) showed a constant activity of 0.35 μmoles min^{-1} mg^{-1} of protein extract that fluctuated

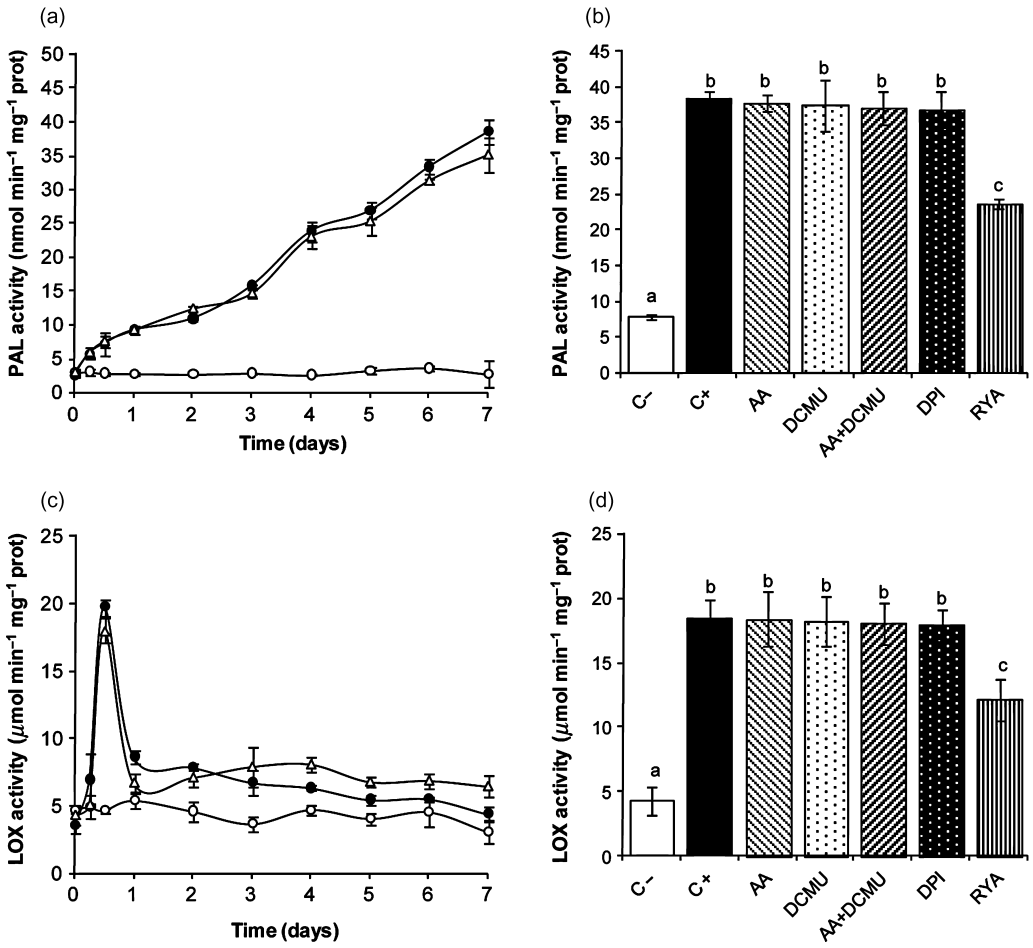

Figure 7. Activities of phenylalanine ammonia lyase (PAL) (a) and lipoxygenase (LOX) (c) in *Ulva compressa* cultivated without copper (white circles), with 10 μM copper (black circles), treated with 10 μM diphenylene iodomium (DPI) and cultivated with 10 μM copper (white triangles) for 0 to 7 d. Activities of PAL (b) and LOX (d) were determined in the alga cultivated without copper (C–), with 10 μM copper (C+) and pre-treated with 10 μM antimycin A (AA), 20 μM 3-(2,3-dichlorophenyl)-1,1-dimethylurea (DCMU), the mixture of the latter inhibitors (AA + DCMU), 10 μM DPI or 100 μM ryanodine (RYA) for 7 d (b) or 12 h (d). Activity of PAL is expressed in nanomoles per minute per milligram of protein, whereas that of LOX is expressed in micromoles per minute per milligram of protein. Bars and symbols represent mean values of three independent replicates ± standard deviation. Different letters indicate significant differences ($P < 0.05$).

only marginally during 7 d of copper exposure and was not inhibited by DPI (data not shown).

DISCUSSION

Calcium release co-occurs with H_2O_2 accumulation

This work shows that copper stress induced a triphasic release of intracellular calcium which was inhibited by ryanodine and co-occurred with the monophasic accumulation of eHP and the biphasic accumulation of iHP. The co-occurrence of calcium release and organellar H_2O_2 accumulation suggests that both responses might be coordinated and that calcium release may originate in organelles. In this sense, it is known that calcium is stored in mitochondria of animal cells, as in endoplasmic reticulum, and that there is a cross-talk between calcium and ROS since an enhanced accumulation of mitochondrial ROS increases calcium release (Feissner *et al.* 2009). Thus, it is possible that a cross-talk between ROS and calcium could take also place in mitochondria and/or chloroplasts of *U. compressa*. The inhibition of calcium release in *U. compressa* by ryanodine indicates that copper-induced calcium release involves ryanodine-sensitive channels, mainly located in the endoplasmic reticulum, and a calcium-induced calcium release mechanism. It is important to point out that calcium release was only partially inhibited by ryanodine indicating that another type of calcium channels may also participate in copper-induced calcium release in our algal model (i.e. inositol triphosphate-sensitive channels, among others). Another interesting point is that the first peak of calcium did not coincide with those of H_2O_2, suggesting that this initial increase in calcium could participate in the activation of NADPH oxidase that is partially responsible for eHP accumulation and SA (see below).

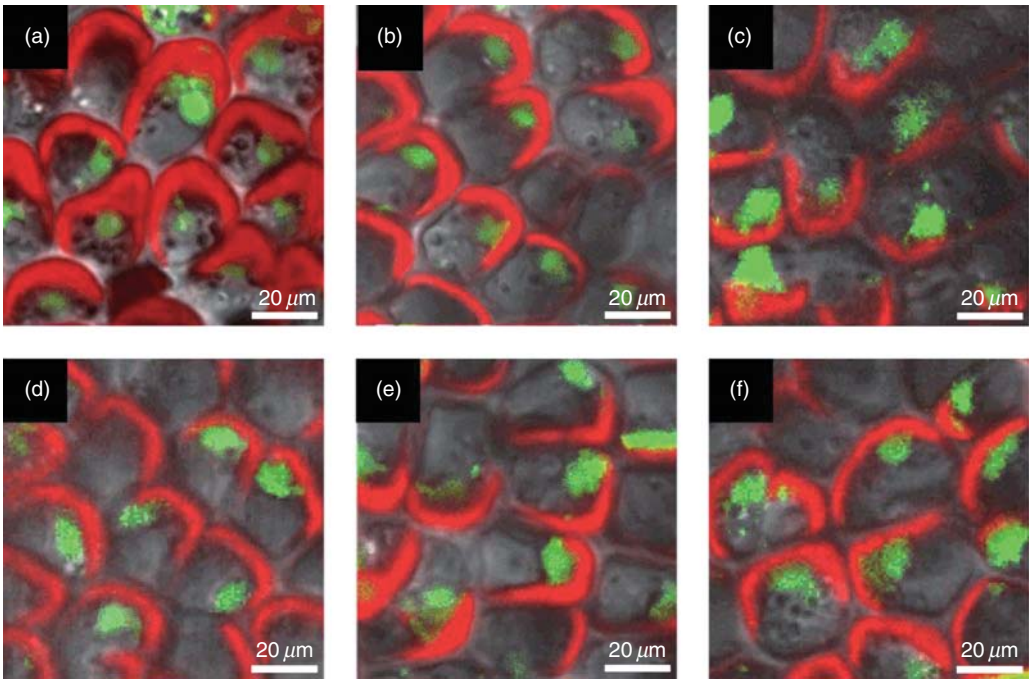

Figure 8. Visualization by confocal microscopy of cell viability in *Ulva compressa* cultivated in seawater without copper (a), with 10 μM copper (b), treated with 10 μM antimycin A (c), 20 μM 3-(2,3-dichlorophenyl)-1,1-dimethylurea (d), the mixture of the latter inhibitors (e) or 100 μM ryanodine (f) and cultivated with 10 μM copper for 5 d. Green fluorescence of nuclei indicates cell viability and red fluorescence corresponds to autofluorescence of chloroplasts. The white bar represents 20 μm.

eHP and iHP is mainly produced in organelles

The monophasic increase of eHP was mainly due to organellar H_2O_2 accumulation, but it was also a consequence of NADPH oxidase activity. This contrasts with results showing that cadmium-induced eHP is derived from NADPH oxidase activation in tobacco cells and alfalfa seedlings (Olmos *et al*. 2003; Garnier *et al*. 2006; Ortega-Villasante *et al*. 2007). However, it was recently reported that cadmium inhibits NADPH oxidase activity in isolated soybean plasma membranes and induces the production of H_2O_2 in purified potato mitochondria (Heyno *et al*. 2008) which is in accord with our results in *U. compressa*. It is possible that organellar H_2O_2 may reach the extracellular medium using aquaporin channels that are present in organelle and plasma membranes (Bienert *et al*. 2007).

The biphasic increase of iHP was exclusively due to organellar H_2O_2 accumulation. This biphasic behaviour is similar to that registered in ozone-treated *Arabidopsis* plants where maximal levels were also registered at 3 and 12 h, but in this case, H_2O_2 accumulation was due to NADPH oxidase activity (Clayton *et al*. 1999). The biphasic behaviour of iHP accumulation in *U. compressa* contrasts with the monophasic increase of iHP in the brown macroalgae *S. lomentaria* and *L. nigrescens* cultivated with 100 μg L^{-1} of copper for 4 d where maximal levels of iHP were registered at 2 h of copper exposure (Contreras *et al*. 2009). An intriguing point related with H_2O_2 accumulation in *U. compressa* is that the effect of mitochondrial and chloroplast inhibitors is not additive indicating that mitochondrial ETC inhibition induces partial inhibition of chloroplast ETC and vice versa. This suggests the occurrence of a metabolic communication between both compartments and the participation of molecule(s) produced in one compartment that can reach the other. In this sense, it has been shown that chloroplast and mitochondria can communicate through the malate:oxaloacetate shuttle (Yoshida, Terashima & Noguchi 2007). In chloroplasts, the excess of reducing power (NADPH) produced in response to high light is used as substrate by NADP-malate dehydrogenase to synthesize malate from oxaloacetate. Malate is then transported from the chloroplast to the mitochondria by the malate:oxaloacetate shuttle and used by NAD-malate dehydrogenase to synthesize NADH which is incorporated in mitochondrial complex I. In this way, the excess of reducing power produced in the chloroplast is transferred to mitochondria. Such communication may also occur in the opposite direction (i.e. from mitochondria to chloroplasts) explaining that a reduction in electron transport in one compartment can reduce electron transport in the other. Then, it is possible that this organellar communication may also occur in *U. compressa*.

A retarded wave of SA is mainly produced in organelles

Copper stress also induced a retarded wave of SA in *U. compressa* which was mainly due to organellar SA accumulation and only a minor fraction originated from NADPH oxidase activity and Fenton reaction. The temporal

accumulation of SA differs from that of eHP and iHP since it began at day 3 and increased until day 7. Interestingly, the accumulation of SA is temporally coincident with the increase of lipoperoxides (data not shown) which suggests that SA increase may be involved in lipoperoxides accumulation. The accumulation of SA in *U. compressa* may be due to the overwhelmingness of mitochondrial and chloroplast SOD which cannot completely detoxify SA that are mainly produced in mitochondrial complex I and III and chloroplast photosystem I (Siraki *et al*. 2002; Asada 2006).

Activation of antioxidant enzymes is differentially triggered by ROS and calcium

In *U. compressa*, the activation of antioxidant enzymes is dependent on ROS increase and, in some cases, on intracellular calcium release. In particular, the increase in AP activation is exclusively ROS dependent since its activation was almost completely inhibited by DPI and not inhibited by ryanodine. This contrasts with results obtained in maize leaves where increase in intracellular calcium induced by ABA is required for the activation of AP gene expression (Hu *et al*. 2007). In addition, GR activity is dependent on ROS increase production and calcium release in *U. compressa* which is in accord with the calcium-dependent activation of GR gene expression in maize leaves treated with ABA and H_2O_2 (Hu *et al*. 2007). Furthermore, GST activity is also dependent on ROS increase and calcium release in *U. compressa* which is in agreement with the requirement of calcium for the activation of GST gene expression in *A. thaliana* seedlings treated with ozone (Clayton *et al*. 1999).

Activation of defense enzymes is triggered by calcium

In addition, the activation of defense enzymes PAL and LOX depends exclusively on calcium release in *U. compressa*. PAL activity has been detected in the red macroalga *Chondrus crispus* (Bouarab *et al*. 2004) and in a green macroalgae (this work) indicating that this enzyme is present in marine macroalgae. This contrasts with genomic data suggesting that PAL encoding gene may not be present in algae since it was not detected in green and red microalgae genomes (Emiliani *et al*. 2009). Regarding calcium release and PAL activation in terrestrial plants, it was recently shown that methyl jasmonate induces calcium release in grapevine cells and that calcium is involved in the activation of PAL gene expression (Faurie, Cluzet & Mérillon 2009). In *U. compressa*, copper stress induced a sustained activation of PAL which is similar to that reported in potatoes (Reyes & Cisneros-Zeballos 2003) and lettuces (Kim *et al*. 2007) where wounding and exposure to methyl jasmonate also resulted in a persistent increase in this enzyme activity. In the latter studies, it was additionally demonstrated that the steady increase in PAL activity led to the accumulation of phenolic compounds with antioxidant capacity (Reyes & Cisneros-Zeballos 2003; Kim *et al*. 2007). This is consistent with the accumulation of phenolic compounds detected in *U. compressa* (data not shown) which are probably involved in buffering oxidative stress induced by copper stress. In this context, it has been shown that some phenolic compounds and derived polyphenols have antioxidant capacity (i.e. Grace & Logan 2000; Moore *et al*. 2005) and metal-binding properties (i.e. Lavid *et al*. 2001). On the other hand, the transient increase in LOX activity was dependent on calcium release and not on ROS accumulation. The transient activation of LOX in *U. compressa* contrasts with the situation found in brown macroalgae where copper induces a sustained activation of LOX in *L. nigrescens* and a LOX activity that reached a constant level in *S. lomentaria* (Contreras *et al*. 2009). Interestingly, LOX in *U. compressa* only uses arachidonic acid as substrate, as it was shown in the brown macroalgae *L. nigrescens* and *S. lomentaria* (Contreras *et al*. 2009). This contrasts, with results in the red macroalga *C. crispus* where LOX also uses linolelic/linoleic acid as substrates, as in terrestrial plants (Bouarab *et al*. 2004). Finally, in *U. compressa*, the activity of G6PDH which is involved in ribose synthesis and produces NADPH did not change in response to copper stress or during the treatment with DPI, indicating that basal metabolism was probably not affected by copper stress in this alga.

CONCLUSIONS

This work shows that copper stress triggers a triphasic release of intracellular calcium which co-occurs with a monophasic increase of eHP and a biphasic accumulation iHP. In addition, copper induces a retarded wave of SA which is not accompanied by calcium release. The accumulation of eHP occurred mainly in organelles but was also due to NADPH oxidase activity, that of iHP occurred exclusively in organelles and that of SA occurred mainly in organelles but was also due to NADPH activity and to the Fenton reaction. ROS accumulation and calcium release determined the differential activation of antioxidant and defense enzymes. In particular, AP activation was only ROS-dependent, PAL and LOX activities were exclusively calcium dependent and GR and GST required calcium and ROS as triggering signals.

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