Marine Environmental Research 83 (2013) 48–53

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Physiological response of the sea urchin *Paracentrotus lividus* fed with the seagrass *Posidonia oceanica* and the alien algae *Caulerpa racemosa* and *Lophocladia lallemandii*

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ARTICLE INFO

Article history:
Received 27 July 2012
Received in revised form
9 October 2012
Accepted 21 October 2012

Keywords:
Antioxidant response
Invasive algae
Mediterranean Sea
Oxidative stress

ABSTRACT

The aim was to determine the effects of alien algae feeding on biomarkers of oxidative stress in the sea urchin *Paracentrotus lividus*. Sea urchins were fed during three months with the native seagrass *Posidonia oceanica*, and the alien macroalgae *Caulerpa racemosa* and *Lophocladia lallemandii* and biochemical analysis were performed in the gonads. A control group was immediately processed after sampling from the sea. Antioxidant enzyme and glutathione *S*-transferase activities and GSH concentration were significantly higher in sea urchins fed with alien algae when compared with the control group and the one fed with *P. oceanica* group. This response was more intense in the group fed with *L. lallemandii* respect to the *C. racemosa* group. The concentration of MDA, protein carbonyl derivates and 8-OHdG reported no significant differences between treatments. In conclusion, the invasive algae *C. racemosa* and *L. lallemandii* induced an antioxidant response in *P. lividus* without evident oxidative damage.

1. Introduction

The presence of introduced species in marine ecosystems is an increasing problem around the world, directly related to the development of international shipping, aquaculture and the aquarium trade (Verlaque and Fritayre, 1994). An introduced species is considered as invasive when self-sustaining populations outside its native area spread and are able to modify the structure of the invaded ecosystems causing ecological and/or economic impact (Boudouresque and Verlaque, 2002; MacDougall and Turkington, 2005; Sakai et al., 2001). The Mediterranean Sea is one of the areas of the world most severely affected by introduced species (Cebrian and Rodriguez-Prieto, 2012; Zenetos et al., 2010), and among them, invasive macroalgae are of special concern as ecosystem engineers by being capable to alter both ecosystem structure and function (Boudouresque and Verlaque, 2002).

The invasive *Caulerpa racemosa* var *cylindracea* variety, originally from southwestern Australia (Verlaque et al., 2003), has rapidly spread throughout the western Mediterranean during the last 20 years (Verlaque et al., 2000). *C. racemosa* var *cylindracea* affects the development of seagrasses (Ceccherelli and Campo, 2002; Dumay et al., 2002), reduces species richness and diversity of macroalgae in the invaded communities (Balata et al., 2004; Piazzi et al., 2001), and changes benthic invertebrate communities (Argyrou et al., 1999; Buia et al., 2001; Deudero et al., 2011). The red macroalgae *Lophocladia lallemandii* (Montagne; F. Schmitz) was introduced through the Suez Canal and is widespread throughout the tropics and subtropics (Boudouresque and Verlaque, 2002). *L. lallemandii* grows over all types of substrates affecting the invertebrate community (Ballesteros, 2006; Patzner, 1998). *L. lallemandii* is an aggressive species which is able to colonise *Posidonia oceanica* meadows to such a degree that it completely covers great areas of the seagrass meadows (Ballesteros et al., 2007).

Generalist herbivores can feed on numerous species and consequently can contribute to invasion control (Parker et al., 2006). The edible *Paracentrotus lividus* (Lamarck) is the most abundant echinoid species in Mediterranean littoral communities. This urchin is one of the major macroherbivores in the Mediterranean Sea eating a range of red, green and brown algae in addition to seagrass (Boudouresque and Verlaque, 2001). However, *P. lividus* would choose *P. oceanica* rather due to the greater availability of shelter and food in the seagrass (Pinna et al., 2012) consuming all

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http://dx.doi.org/10.1016/j.marenvres.2012.10.008

parts of the seagrass as a "preferred" species for feeding during spring and summer (Boudouresque and Verlaque, 2001). In fact, the sea urchin is one of the main consumers of *P. oceanica* (Verlaque, 1987), avoiding other species that synthesize toxic or repellent secondary metabolites (Guerriero et al., 1992; Lemée et al., 1996). *P. lividus* is a key species that controls the dynamics of seaweeds and seagrasses, by eliminating, when at high densities, the erect stratum of algae and seagrasses (Sala and Zabala, 1996). However, several invasive macroalgae are capable to produce a rich variety of secondary metabolites that can function as deterrents against herbivores (Nylund et al., 2011). Macroalgae bioactive compounds are considered a possible source of reactive oxygen species (ROS) in marine organisms and can result in oxidative stress (Gross et al., 2006; Sureda et al., 2008). The antioxidant systems are capable to respond to stressful situations in order to protect cells against the deleterious effects of excess ROS and attenuating the damages related to their high reactivity. Several studies reported that sea urchins gonads possess significant antioxidant activity (Qin et al., 2011; Sheean et al., 2007).

The aim of the present study was to determine the physiological response of a marine invertebrate to invasive macroalgae ingestion by measuring several biomarkers of oxidative stress in the gonads of the sea urchin *P. lividus* fed under aquarium conditions with the alien algae *C. racemosa* and *L. lallemandii* in comparison with the native seagrass *P. oceanica* for a three month period.

2. Materials and methods

2.1. Experimental procedure

All macrophytes and urchins used were collected around St Elm, Mallorca, Spain (39°34′44″N, 2°20′57″E) and were transported to the laboratory in aerated seawater tanks. The experiment was conducted in the Palma Aquarium installations, Palma de Mallorca (Spain), using a constant-temperature (21 °C) seawater flow-through system.

Sea urchins were divided in three homogeneous groups ($n = 8$ per group with a test diameter ranging from 5.5 to 6.7 cm, excluding the spines). Sea urchins were firstly acclimatized to laboratory conditions for 48 h before starting the experimental assay, during which they were fed freshly collected *Ulva* sp. The first group was fed with the native *P. oceanica* during three months whereas the second and third groups were fed with the invasive algae *C. racemosa* and *L. lallemandii* respectively. As a control group, sea urchins ($n = 8$) from *P. oceanica* meadows free of alien macroalgae species were sampled during the summer period and were immediately frozen to obtain reference data at the beginning of the experiment to discard captivity effects on biochemical parameters.

Sea urchins were randomly distributed in three independent large tanks (300 L) containing the correspondent feeding material. *P. lividus* were fed with 50 g wet weight of fresh material twice a week. After three months sea urchins from all tanks were collected and frozen.

2.2. Tissue extracts for biochemical analyses

In the laboratory, sea urchins were measured using callipers and dissected to obtain the gonads. Dissected gonads from each specimen ($n = 8$ for each treatment) were homogenized in 10 volumes (w/v) of 100 mM Tris–HCl buffer pH 7.5. Each homogenate was briefly sonicated (2–3 s) using an ultrasonic processor and centrifuged at 9000 × *g* at 4 °C for 15 min. After centrifugation, supernatants were collected and immediately used for biochemical analysis. All biochemical assays were performed in duplicate.

All results were referred to the total protein content of the samples (Biorad® Protein Assay) using bovine serum albumin as standard.

2.3. Enzymatic activities

CAT activity (K (s^{-1})/mg protein) was measured by the method of Aebi (Aebi, 1984) based on the decomposition of H_2O_2. SOD (pmol/min/mg protein) activity was determined by the degree of inhibition of the reduction of cytochrome C by superoxide anion generated by the xanthine oxidase/hypoxanthine system (Flohe and Otting, 1984). The activity was recorded at a wavelength of 550 nm. GPX activity (nmol/min/mg protein) was measured using an adaptation of the method of Flohé and Gunzler (Flohe and Gunzler, 1984) with H_2O_2 as substrate. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$, was indicative of GPX activity. GR activity (nmol/min/mg protein) was measured by a modification of the Goldberg and Spooner (1984) method, in which the rate of conversion of GSSG to GSH was estimated by monitoring oxidation of NADPH in the assay system at 340 nm. Glutathione *S*-transferase (GST) activity (nmol/min/mg protein) was determined at 314 nm using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Habig et al., 1974). All antioxidant enzyme activities and GST activity were determined with a ShimadzuUV-2100 spectrophotometer at 25 °C.

2.4. Reduced glutathione (GSH) levels

GSH (nmol/mg protein) was determined with a microtiter plate assay in the gonad homogenates of *P. lividus* by an adaptation of the method described by Vandeputte et al. (1994). Homogenate samples were deproteinised with 30% trichloroacetic acid containing 2 mM EDTA. After complete precipitation of the proteins, samples were centrifuged for 5 min at 15,000 × *g* at 4 °C. Supernatant aliquots (20 μl) were placed in a 96-well microtiter plate. 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) diluted in phosphate buffer (143 nmol/L; EDTA 6.3 mmol/L; pH 7.4) was added to each well (200 μl). The plate was placed in a microplate reader and the absorbance was measured at 415 nm. GSH was quantified using a standard curve of known concentration of GSH.

2.5. Malondialdehyde (MDA) determination

MDA concentration (mmol/mg protein), as a marker of lipid peroxidation, was analyzed by a specific colourimetric assay kit for MDA determination (Calbiochem®, San Diego, CA, USA) following the manufacturer's instructions. Briefly, samples or standard were placed in glass tubes containing n-methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1). HCl (12 N) was added and samples were incubated 1 h at 45 °C. The absorbance was measured at 586 nm. MDA concentration was calculated using a standard curve of known concentration.

2.6. Protein carbonyl determination

Protein carbonyl derivatives were determined by an immunological method using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International) following the manufacturer's details. Briefly, 10 μg of protein was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH). Once derivatised, samples were transferred onto a nitrocellulose membrane by the method of dot blot. Then, the membrane was incubated with primary antibody, specific to the DNP moiety of the proteins. This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit