Stress responses of duckweed (*Lemna minor* L.) and water velvet (*Azolla filiculoides* Lam.) to anionic surfactant sodium-dodecyl-sulphate (SDS)

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**Article info**

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**Abstract**

Surfactants are used for several purposes and recently they have attracted the attention for their ability to modify the behavior of other preexistent or co-disposed contaminants, although their use or discharge in wastewaters can represent a real or potential risk for the environment. *Lemna minor* L. and *Azolla filiculoides* Lam. are floating aquatic macrophytes, very effective in accumulating several pollutants including sodium dodecyl sulphate (SDS). In this work we evaluated the effects of SDS on these species by determining the stress ethylene production via laser-based trace gas detection, and the activities of enzymes involved in stress response, such as guaiacol peroxidase (G-POD), phenylalanine ammonia-lyase (PAL) and polyphenol-oxidase (PPO). Phenolics content was also determined. The macrophytes were treated with different concentrations of SDS for one week. SDS affected duckweed enzymatic activities and phenol content. While in the fern phenolics amount, PAL, G-POD and PPO activities were not affected by SDS except for 100 ppm SDS, the only concentration that was taken up and not completely degraded. Stress ethylene production was induced only in the fern treated with 50 and 100 ppm SDS.

1. Introduction

Numerous man made chemicals are discharged in the waters (Dhote and Dixit, 2009), and among them several classes of surfactants. The latter are an important source of pollution, causing adverse effects to the aquatic organisms (Tabor and Barber, 1996; Shang et al., 1999; Ying, 2006). Anionic surfactants are amphiphilic compounds, of which hydrophobic and hydrophilic parts readily interact with polar and apolar molecules and macromolecules such as starch (Merta and Stenius, 1999), proteins (Nielsen et al., 2000) and DNA (Marques et al., 2000) or causing misfunction of phospholipid membranes (Heerklotz, 2008). Due to their physicochemical characteristics, anionic surfactants have been widely used in biotechnological as well as other industrial processes (Reich and Robbins, 1993; Tong et al., 2000).

Concerns about the ecotoxicity of surfactants arise not only from their tremendous exploitation in everyday life, but also from the particular focus addressed to the potential of these molecules to remediate contaminated environments. Recently, it was found that some surfactants have the ability to modify the behavior of other contaminants. This depends on the type and concentration of the surfactant and charges of the sorbent surface and co-contaminant. The latter may exhibit either enhanced sorption or solubilisation (Hari et al., 2005; Jones-Hughes and Turner, 2005; Karapanagioti et al., 2005; Yang et al., 2002; Zhang and Zhou, 2005). The application of either non-ionic or ionic surfactants, in amounts above their critical micelle concentrations (CMCs), has been exploited to release neutral chemicals from aquifers and soils (Grimberg et al., 1996; Ko and Schlautman, 1998) as well as to enhance heavy metals removal (Turner and Xu, 2008).

The amphoteric character of anionic surfactants facilitates their accumulation in living organisms where they may exhibit toxic effects (Garcia et al., 2001; Guillemine et al., 2000; Ivanović and Hrenović, 2010; Ying, 2006). Thus, their use to remove pollutants has to be considered with care.

Plant exposure to both biotic and abiotic stressors mobilizes or generates, directly or indirectly, diverse signaling molecules and regulates many processes that amplify and specify the physiological response through transcriptional and metabolic changes (Zhao et al., 2005). Among the various mechanisms by which plants react to stresses, ethylene plays an important role since it is one of the major modulators in the first phases of plant response to pathogens or pathogen-derived elicitors (Boller, 1991). Ethylene is also assumed to be involved in the defense against abiotic stresses (Arteca and Arteca, 2007 and references therein; Francia et al., 2007; Sanità di Toppi and Gabrielli, 1999). It has been
shown that ethylene induces formation of phytoalexins, lignin and other phenolic compounds, and affects the activities of enzymes like chitinases, β-1,3-glucanases, phenylalanine-ammonia-lyase, polyphenol oxidase, peroxidase, and other defense related proteins (Almagro et al., 2009 and references therein; Broekaert et al., 2006; Newman et al., 2011 and references therein).

Despite their position as primary producers in the food chain in aquatic ecosystems, the macrophytes are among the first organisms reached by pollutants in these environments. Such plants are used as in situ biomonitors of water pollution because of their abundance and limited mobility (Roy and Hanninen, 1992). Among them, duckweed (Lemna minor L.) and water velvets (Azolla filiculoides Lam.) have shown a remarkable effectiveness in phytoremediation (Mashkani and Ghazvini, 2009; Tel-Or and Forni, 2011 and references therein). Moreover, Lemma is regularly used as model plant in ecotoxicological studies (Tel-Or and Forni, 2011 and references therein). Previously, the uptake and the effects on growth and chlorophyll contents of the anionic surfactant sodium dodecyl sulphate (SDS) have been determined on these floating macrophytes (Forni et al., 2008). The results showed that growth rates and chlorophyll content of plants were affected by SDS, although both species were able to remove the surfactant. Due to the low availability of data on the toxic effect of SDS on these aquatic species (Dilrigen and Ince, 1995; Forni et al., 2008), we carried out further studies to investigate the stress response induced by SDS. In fact, although aquatic plants contain a whole set of detoxification enzymes, little is known about their detoxication capacity (Pflugmacher, 2002). Thus, in this paper we investigated the stress response by determining changes in ethylene production and some enzymes activities, used as toxicity stress marker, i.e. guaiacol peroxidase (G-POD) (Horvat et al., 2007), or involved in the response, i.e. phenylalanine ammonia-lyase (PAL) and polyphenol-oxidase (PPO). Phenolics content was also determined as potential markers of non-visible plant damage (Pasqualini et al., 2003), since they play an important role in defending plants against biotic and abiotic stresses (Grace, 2005; Lavid et al., 2001).

2. Materials and methods

2.1. Reagents and working solutions

All reagents were purchased from Merck or Sigma–Aldrich and were of analytical grade or equivalent. In each set of experiments working solutions were prepared from stock reagents immediately before use. Sodium dodecyl sulphate (SDS) (CH\textsubscript{12}CH\textsubscript{2})\textsubscript{11}OSO\textsubscript{4}Na; relative molar mass 288.4 g/mol). The octanol–water partition coefficient (K\textsubscript{ow}) for SDS is about 50 (Jones-Hughes and Turner, 2005), and its CMC is about 10 mM (Otto et al., 2003). For the experiments we used the following SDS concentrations: 0, 2.5, 25, 50 and 100 ppm.

2.2. Plant growth conditions

A. filiculoides and L. minor, were maintained under environmental conditions in IRRI medium, pH 6.8 (Watanabe et al., 1977); the medium was N-free for Azolla and added with KNO\textsubscript{3} for Lemma (Forni et al., 2001a,b). To test the effect of SDS on plants, 5 g/l of Azolla and 4 g/l of Lemma, corresponding to 80% of surface covered (Forni et al., 2008), were inoculated in plastic bowls containing 1000 ml of medium, added or not with SDS. Growth media were not renewed during the experiments. Plants were kept indoor under environmental conditions. Basing on previous work (Forni et al., 2008), we applied short-term tests of one week to detect the tolerance of macrophytes to SDS.

A randomized block design was applied in the experiment; each treatment was performed in triplicate and the experiments were repeated three times. For the analyses, plants were sampled after 3 and 7 days of treatments and immediately frozen in liquid nitrogen.

2.3. Laser-based photoacoustic detection of ethylene

Ethylene production was measured with a commercial laser-based ethylene detector (type ETD-300, Sensor Sense B.V., Nijmegen, the Netherlands) in combination with a gas handling system (Clarke et al., 2009). The ETD-300 is a state-of-the-art ethylene detector based on laser photoacoustic spectroscopy that is able to detect on-line about 300 pptv (pptv = parts-per-trillion volume, 1:10\textsuperscript{12}) of ethylene within a 5 s time scale (Cristescu et al., 2008).

The gas handling is performed by a valve controller (type VC-6, Sensor Sense B.V., Nijmegen, the Netherlands), designed for controlling the flow to and from sampling cuvettes. The laser-based ethylene detector and the valve controller are operated fully automatically with a user-friendly interface for processing and displaying the data during the experiment. The plants were placed into closed glass cuvettes (100 ml volume) equipped with inlet and outlet ports. The VC-6 sampling system operates in two modes: continuous flow and stop and flow, respectively. As the ethylene production from the plants was expected to be very low, a stop-and-flow mode was chosen. In this way, ethylene accumulates in the headspace for 125 min in an enclosed environment, before it is transported to the ethylene detector with a low flow rate of 1 l/h for a period of 25 min. Up to 6 cuvettes are sampled in series in this way. While one is measured, the cuvettes are accumulating, hence optimizing the measurement efficiency. A scrubber with KOH was used to remove the CO\textsubscript{2} concentrations in the flow from the cuvettes to the detector. In addition, a tube filled with CaCl\textsubscript{2} was placed directly after the CO\textsubscript{2} scrubber to decrease the water content in the gas sampling flow. Both gasses needed to be removed to obtain accurate ethylene concentration data. Ethylene emission values are collected continuously during the seven days of SDS treatment. The ethylene emission from media with and without SDS was also tested, with negative results.

2.4. Determination of total phenolics

Phenols were extracted from frozen samples, grounded with a pestel in a mortar and suspended in 0.1 N HCl (Legrand, 1977). Samples were incubated overnight at 4 °C in the dark in orbital shaker. The amount of total phenolics was measured by means of Folin – Ciocalteu method (Booker and Miller, 1998), using chlorogenic acid (CA) as standard, for which a calibration curve was carried out with solutions of 1, 2, 5, 10, and 20 μg/l of this compound (y = 0.0659x – 0.013, R\textsuperscript{2} = 0.9997). Results are expressed as micrograms of CA equivalents per g of fresh weight of plants. The data are expressed as mean values ± SE.

2.5. Enzymatic activities

All enzymatic activities were determined in plants treated with SDS for 3 and 7 days. Protein concentration was determined by Bradford protein assay (Bradford, 1976), using bovine serum albumin as standard. The possible interference of SDS with Bradford reagent has been checked by calibration curve, no significant changes of absorbance were detected in the presence of SDS.

PAL. Phenylalanine ammonia-lyase (PAL) activity was determined according to the method described by Forni et al. (1999) and Conte et al. (2005). The activity is expressed as μg t-cinnamic acid/h/μg protein.

G-POD. Guaiacol peroxidase (G-POD) activity determination was performed according to the method of Forni et al. (2001a) modified

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as follows: aliquots (50 mg) of freeze-dried powder were transferred into 5 ml of extraction buffer (0.2 M sodium phosphate buffer containing 1%, w/v insoluble polyvinyl polypyrrolidone (PVPP), 0.1%, w/v SDS and protease inhibitor, pH 7.0) and homogenized for 1 h at 4 °C. This extraction buffer was chosen to prevent protein precipitation by tannins during the extraction. The homogenates were centrifuged for 30 min at 17,000 g at 4 °C and the supernatants were recovered. These crude extracts were used immediately for spectrophotometric determination of enzymatic activities. Three ml reaction mixture containing sodium phosphate buffer (0.2 M; pH 6.5) 100 μl enzyme extract, 5.3 mM guaiacol, 30 mM H2O2; pH 6.5. Enzymatic activity unit (U) was defined as the amount of enzyme required to increase 0.001 OD unit/min at test conditions. Increases in absorbance at 470 nm were monitored in a Varian 50 spectrophotometer. Blanks were recorded in the absence of either substrates or enzyme extract, or with boiled crude extracts; in both cases, activity was negligible.

PPO. Polyphenol oxidase activity was assayed according to Lavid et al. (2001). Crude enzyme extracts (100 μl), obtained as above described, were added to reaction mixtures containing 2.75 ml sodium phosphate buffer (50 mM; pH 6.5), and 50 mM catechol. Increases in absorbance at 420 nm were monitored in a Varian 50 spectrophotometer. Enzyme activities were determined by measuring the initial rate of the reaction. Blank rates recorded in the absence of either substrates or enzyme extracts, were negligible. The data are expressed as mean values ± SE.

2.6. Statistical analysis

The results were obtained from three independent experiments. Data are the means values ± standard errors (SE). Statistical analysis was performed by computer software GraphPad Prism, Version 5.0 and Microsoft Excel 2003. The significance of the differences between treated and untreated plants at the same time of exposure was determined by analysis of variance (ANOVA). The differences were considered significant at p values lower than 0.05 (p < 0.05).

3. Results

3.1. Morphological analysis

No stress symptoms were present in Lemma plants exposed to the lowest SDS concentrations. While at higher concentration the leaves were partially yellowed and dehydrated, the effect was most evident at 100 ppm. The progressive yellowing of the leaves started from the third day of treatment; after seven days plants had 50% and at least 80% of the yellow leaves respectively at 50 and 100 ppm SDS. Moreover during the last two days of treatment, in Lemma exposed to these concentrations the roots were detached.

No stress symptoms were detected in Azolla plants treated with 2.5 ppm SDS; but from 10 ppm up to 100 ppm SDS the leaves were gradually smaller and brown. Plants exposed to 10 and 25 ppm SDS showed signs of browning after the first three days of treatment and at the end of the experiments about 10% of the plants had brown leaves; similarly the plants exposed to 50 and 100 ppm SDS showed signs of leaf browning from the third day, and at the seventh day they had respectively about 70% and 80% of the abaxial side of the leaves strongly dehydrated with brown basal portion. Fern roots too were detached in the last two days of the experiments.

3.2. Ethylene production

No changes in ethylene emission were detected in both macrophytes treated with the lower detergent concentrations, i.e. 2.5, 10 and 25 ppm SDS (data not shown). In Lemma the ethylene production seemed to be inhibited at 50 and 100 ppm SDS. In fact, after 36 h of treatment ethylene emission was 0% (Fig. 1A). Vice versa in Azolla increased ethylene production was observed within the first 3 days after treatment in plants treated with higher SDS concentrations, i.e. 50 ppm and 100 ppm (Fig. 1B). Compared to the control plant a maximum observed increase of 30% was detected during the second day after the start of the treatment, with 100 ppm SDS. After the 2nd day for Azolla and the 3rd day for Lemma the ethylene production reached similar values as the control; in both macrophytes the changes in hormone synthesis may be considered an early response to SDS.

3.3. Total phenolics content

In the first three days of treatment, SDS concentrations up to 50 ppm induced a higher production of phenols in Lemma. While, after seven days of exposure to 25 ppm SDS, phenols concentration was lower than that of control plants (Fig. 2).

Phenols concentrations in Azolla did not show significant differences (p < 0.05) till 50 ppm SDS (Fig. 2). The major changes in phenols concentration were detected at 100 ppm SDS, i.e. at the third day of treatment phenols concentration was increased (32.7%) respect to untreated plants, while at the end of treatment phenols were 27.3% lower than the control values.

3.4. Enzymatic activities

3.4.1. PAL activity

SDS effect on PAL activity was different in duckweed and fern (Fig. 3). In Lemma after three days of exposure, enzyme activity increased in a concentration dependent manner, reaching a maximum at 50 ppm SDS. At the end of the treatment an enhancement of activity was detected up to 10 ppm, after that the values decreased and were negatively related to the concentrations of surfactant. In Azolla treated plants the PAL activity was almost constant during the experiment. A significant enhancement (p < 0.01) was detected only in plants treated for 3 days with 100 ppm.

3.4.2. G-POD activity

In Azolla G-POD activity, similarly to PAL, was not affected by SDS except for 100 ppm, i.e. the enzyme showed a peak of activity (279.7%) at the third day (Fig. 4). Duckweed plants treated for three days showed a significant enhancement of the enzyme activity, which decreased at the end of the experiment only in plants exposed to surfactant concentrations higher than 25 ppm.

3.4.3. PPO activity

In both plants after three days of exposure to SDS PPO activity was not affected (Fig. 5). At the end of the experiment, in Lemma the activity increased in a concentration dependent manner reaching a maximum at 100 ppm SDS. While Azolla after seven days of treatment showed an increase of the enzyme activity only at 100 ppm SDS.

4. Discussion

The amphoterics character of the surfactants facilitates their accumulation in living organisms, as already reported also for SDS and the macrophytes object of this study (Forni et al., 2008), causing both marked environmental pollution and toxicity. Previously, bioaccumulation of the detergent was detected in duckweed plants grown for 3 days in medium added with 50 and 100 ppm SDS and after 7 days in plants exposed to 10 (4.4 μg SDS/g, fresh weight) up to 100 ppm (2607 μg SDS/g, fresh weight) (Forni et al., 2008). In Azolla, bioaccumulation was detected in plants treated for 3 days with 50 and 100 ppm, but at the 7th day a biodegradation of SDS, was shown, except for 100 ppm SDS (248.8 μg SDS/g, fresh weight).
Fig. 1. Stress ethylene production in *Lemna* (A) and *Azolla* (B) treated with 50 and 100 ppm SDS. The data were expressed as percentage respect to the control.

Fig. 2. Total phenolics content in plants treated with SDS. Data represent the average ± SE of three different determinations and differences between treated plants and control were considered significant when *p* < 0.01 (ANOVA).
The exposure to SDS induces changes mostly within the first three days. In this period the first morphological changes become evident together with the induction of mechanisms of stress response, like the enhancement of levels of antioxidant enzymes, important for ROS detoxification (Gill and Tuteja, 2010), as well as phenolics production. Peroxidases were reported to reduce H₂O₂ using phenolic compounds or flavonoids as donors of electron (Teisseire and Vernet, 2000; Yamasaki et al., 1997), they are also involved in lignifications and in metabolism of phenolics (Schopfer, ...
The activity of G-POD varies considerably depending upon plant species and stress conditions (Gill and Tuteja, 2010 and references therein). Higher peroxidase activities in water plants have been related to the tolerance to the pollutants (Lavid et al., 2001; Roy et al., 1992), although controversy exists in Lemma minor treated with xenobiotic, e.g. pesticides, G-POD activity was not stimulated (Mitsou et al., 2006; Teisseire and Vernet, 2001) or transient induction was detected (Teisseire and Vernet, 2000). In our experiments, this enzyme plays a role mainly in duckweeds treated with 10 up to 100 ppm for three days and only in Azolla treated with 100 ppm for the same time, i.e. in the latter the enzyme showed a peak of activity (279.7%). These findings are in agreement with the literature which shows that G-POD up-regulation is strongly induced at the beginning of an event, and slowly decrease within time (Passardi et al., 2005 and references therein).

In Lemma, significant changes in phenolics production as well as up-regulation of PAL and PPO were also detected. PAL is the key enzyme in the shikimic acid pathway, and PPO utilizes phenolics, the end products from the phenylpropanoid biosynthetic pathway, as substrates for oxidation. PAL activity and phenolics production were positively related. These responses were again detected mostly within the 3rd day, when the beginning of surfactant taken up was detected. At higher concentrations of SDS the enzymatic activity decreased at the end of experiment, because of the toxicity of SDS accumulated in the plants (Forni et al., 2008). In the fern phenolics content, PAL and PPO activities were not affected by SDS except for 100 ppm SDS, the only concentration that was taken up and not completely degraded (Forni et al., 2008).

The enhancement of phenolics concentrations in SDS stressed plants can improve their antioxidant capacities, since in such systems, phytophenolics can act as antioxidants by donating electrons to guaiacol-type peroxidases for the detoxification of H$_2$O$_2$ produced under stress (Sakihama et al., 2002 and references therein). Under conditions of severe stress it has been proposed that phytophenolics, particularly polyphenols, function as antioxidants to support the primary ascorbate-dependent detoxification system as a backup defense mechanism of vascular plants (Sakihama et al., 2002).

Like the enzymes involved in general phenylpropanoid metabolism, PPOs are present in plants under normal conditions, and several studies have demonstrated that the PPO family is spatially and temporally regulated and inducible following wounding, pathogen attack, and ethylene application (Newman et al., 2011 and references therein). Furthermore, in higher plants, PPOs are ubiquitous plastid located enzymes (Newman et al., 2011), that have one intriguing characteristic such as the ability to exist in an inactive or latent state, that can be activated by SDS (Moore and Flurkey, 1999; Saedian et al., 2007). Differently from literature data (Newman et al., 2011 and reference therein), in the fern and duckweed ethylene is not involved in PPO induction. In fact PPO was always activated in the duckweed, while in the fern 100 ppm SDS was the only concentration affecting the activity, suggesting that is SDS accumulation within the plants rather than ethylene stimulation that affects PPO activity. In the macrophytes the molecule may damage chloroplast membranes exposing PPO directly to its action.

Plant-emitted ethylene has been shown to be involved in the response to environmental stresses in several species (Larkindale and Knight, 2002; Weyers and Paterson, 2001). This hormone acts as signaling molecule at low concentrations, and in cooperation with other signaling compounds, such as abscisic acid, salicylic acid and calcium. In both macrophytes ethylene emission can be considered as an early stress response, since the production was changed within the first days of treatments; furthermore, only at 50 and 100 ppm we detected differences in the ethylene synthesis as compared to the untreated plants. Macrophyte behavior was once more different, i.e. SDS exposure can lead either to a promotion of ethylene production in the fern or to an inhibition of the synthesis in the duckweed. The different role of ethylene in plant defense response of Azolla and Lemma may be due to different signaling pathways that plants use to cope with this abiotic stress. As already reported above for PPO, and differently from the literature, in these macrophytes the increase of G-POD activity is not associated to ethylene production. Further investigation is necessary to provide a better insight into the role of ethylene and phenolics/enzyme: exogenously treatment with ethylene or ethephon in these two plants to see whether the phenolic production and enzyme activity increase are caused by ethylene are two options to be considered.

To our knowledge, the emission of ethylene by Lemma and Azolla has not been studied so far and this paper is the first report on ethylene production by macrophytes exposed to SDS.

5. Conclusion

Our results have shown that the SDS exposure induces an enhancement of levels of antioxidant enzymes and phenolics involved in the detoxification system of water macrophytes, thus improving their tolerance. Such responses are strongly induced in the first days of the treatments.

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