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Title:

The halophyte *Cakile maritima* reduces phenanthrene phytotoxicity

Running head:

Cakile maritima reduces phenanthrene phytotoxicity

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Abstract

In a previous study, we showed that the halophyte plant model *Thellungiella salsuginea* was more tolerant to phenanthrene (Polycyclic Aromatic Hydrocarbon: PAH) than its relative glycophyte *Arabidopsis thaliana*. In the present work, we investigated the potential of another halophyte with higher biomass production, *Cakile maritma*, to reduce phenanthrene phytotoxicity. Sand was used instead of arable soil with the aim to avoid pollutant degradation by microorganisms or their interaction with the plant. After 6 weeks of treatment by 500 ppm phenanthrene (Phe), stressed plants showed a severe reduction (-73%) in their whole biomass, roots being more affected than leaves and stems. In parallel, Guaiacol peroxidase (GPX) activity was increased by 185 and 62% in leaves and roots, respectively. Non-enzymatic antioxidant capacity (assayed by ABTS test) was maintained unchanged in all plant organs. The model halophytic plant *Thellungiella salsuginea* was used as a biomarker of phenanthrene stress severity and was grown at 0 (control), 125, 250, and 375 ppm. *T. salsuginea* plants grown on the sand previously contaminated by 500 ppm Phe then treated by *C. maritma* culture (phytoremediation culture) showed similar biomass production as plants subjected to 125 ppm Phe. This suggests that the phytotoxic effects of phenanthrene were reduced by 75% by the 6-week treatment by *C. maritima.* Our findings indicate that *C. maritima* can constitute a potentially good candidate for PAH phytoremediation.

Keywords: antioxidant response, bioassay, PAH, *Thellungiella salsuginea*.

1. Introduction

Human activities drastically increased soil pollution that becomes a great challenge in the 21st century. Two major chemical families cause soil pollution: organic chemicals and heavy metals (Belden et al., 2004; Xia et al., 2009). They have in common low dose toxic properties that disturb environment. Polycyclic aromatic hydrocarbons (PAHs), a class of widely distributed organic carcinogenic pollutants, are of high priority for remediation efforts (Liu et al., 2009). Meudec et al. (2006) reported that their phytotoxicity varies with their concentration in soil. For instance, phenanthrene (Phe) represents a class of highly persistent PAHs that are poorly soluble in water and have the potential to accumulate in plants. Several works described the detrimental effects of PAHs on plants as well as plant responses to PAH-induced stress (e. g., Paková et al., 2006; Song et al., 2011; Weisman et al., 2010). However, little is known about plant efficiencies to extract these persistent pollutants from the soil (Liu, 2009; Alkio et al., 2005). Most studies addressed the analysis of the role of plants in stimulating microorganism activities by root exudates that help destroy PAH rings. The real plant role in phytoremediation without soil microorganism interactions is often neglected. In addition, the majority of these compounds have a common effect on plant cells: oxidant stress (Liu et al., 2009). Hence, (i) plants able to limit the disturbances caused by Reactive Oxygen Species (ROS) generated by a pollutant, (ii) to produce high biomasses in its presence at a relatively high level, and (iii) to accumulate and/or metabolize this pollutant are thought to be good candidates for its phytoremediation. This was shown in the comparative study between the two model plants *Arabidopsis thaliana* (glycophyte) and *Thellungiella salsuginea* (halophyte); the letter was more tolerant to phenanthrene-induced stress thanks to its more powerful antioxidant system (Shiri et al., 2014). It is therefore interesting to assess phytoremediation ability of extremophile plants, since along their evolution, they have likely developed molecular, biochemical, and

physiological mechanisms allowing them to develop and grow under stressful conditions. In the present work, the potential of *Cakile maritima* to reduce PAH phytotoxicity was studied using the model halophyte *T. salsuginea* as a biomarker.

2. Material and methods

2.1. Plant material and growth conditions

Cakile maritima seeds were collected in Soliman seashore (about 30 km N-E Tunis) in July 2012. Seeds were disinfected with a sodium hypochlorite solution then sown in pots (4 seeds per pot) filled with 1 kg sand and irrigated with distilled water until germination. Plantlets were then transferred onto sand contaminated or not with 500 ppm Phe (in eight replicates per treatment). The sand used in this study is pure (Sigma: 274739-5KG); it is composed of simple SiO_2 grains and does not contain organic matter or mineral nutrients. In addition, it was rinsed three times with distilled water, drained, and dried for several days to remove any chemical or biological activity. Additional pots were filled with sand contaminated (125, 250, and 375 ppm) or not (0 ppm) with Phe, left unplanted and irrigated similarly to the planted ones. The sand was contaminated using an ethanol solution of Phe. Ethanol was thereafter evaporated by agitating sand every 2 days during 6 days. Concentrations were chosen based on preliminary trials. After 6 weeks, plants were harvested, cut into three parts (leaves, stems, and roots), and weighed. Samples were freeze-dried then weighed and kept at -20°C until use. Planted and unplanted pots were used to cultivate *Thellungiella salsuginea* (Shandong wild-type) seedlings for 4 weeks under greenhouse conditions. At the harvest, plants were cut into shoots and roots and analyzed for fresh biomass production and pigment concentrations.

2.2. Enzymatic assays

The extraction of soluble proteins was performed on 100 mg samples of freeze-dried material chopped in micro-tubes using 1 ml buffer extraction (potassium phosphate buffer 50 mM, pH 7.5, EDTA 1 mM, PVPP 5%, 2-mercaptoethanol 4 mM, ascorbate 5 mM, and protease inhibitor cocktail Sigma P9599 to 5 µl. ml^{-1} added at the time of the extraction). The tubes were placed on a rotary shaker for 1 h at 4°C. After centrifugation for 20 min at 12,000 *g*, the supernatants were retrieved and kept at -80°C until the measurement of antioxidant enzyme activities. Protein concentration was determined in each enzyme extract according to the colorimetric method of Bradford (Bradford 1976) using albumin bovine serum as standard.

Guaiacol peroxidase (GPX) activity was determined from the maximum speed emergence of tetraguaiacol as described by Srivastava and van Huystee (1977) adapted to 96-well plates. Five or ten enzyme extract microlitres were deposited in each well then added with 30 µl phosphate buffer, 30 µl guaiacol, and 30 µl H_2O_2. Thereafter, the mixture was completed to 300 µl with ultrapure water to obtain a final reaction medium as follows: 100 mM potassium phosphate buffer (pH 6.5), 15 mM guaiacol, and 0.05% H_2O_2 (v/v). The reaction was started with the addition of H_2O_2 in the reaction mixture. GPX activity was followed as a function of time to the VERSA max spectrophotometer (Molecular Devices) by absorbance measurement at 470 nm (a measurement every 15 seconds for 5 min at 25°C).

Trolox Equivalent Antioxidant Capacity (TEAC) or $ABTS^+$ Decolorization Assay was also made for leaves. This test is based on the ability of an antioxidant to stabilize the radical cation $ABTS^+$ (blue-green) transforming it into ABTS (colorless). A comparison is made with the capacity of the Trolox to capture $ABTS^+$. The decay of the absorbance caused by the antioxidant reflects the capture capacity of free radicals. The antioxidant capacity, expressed as Trolox equivalent (TEAC), therefore corresponds to the concentration of Trolox having the

same activity as the test substance at a given concentration. The result is given in µM or mM Trolox equivalent per g of product and expressed as percentage of inhibition (Re et al., 1999).

3. Results and discussion

C. martima plants survived the extreme Phe concentration (500 ppm), but they showed a noticeable decrease in their size (Fig. 1). Their mean fresh weight was reduced by 73% in comparison with the control, roots being the most affected organs (Table 1). A previous study (Lee et al., 2008) reported that the toxic effects of Ph in the soil were detected at 90 ppm. Redondo-Gómez et al. (2011) showed that *Panicum bisulcatum* exhibited up to 70% of biomass reduction as grown on soil contaminated with 1000 ppm Phe. In *C. maritilma*, root growth was more decreased than that of leaves and stems. Indeed, Phe as other organic compounds can be fixed on lipid compounds of root cell membranes (Adam and Duncan, 1999). According to Van Overbeek and Blondeau (1954), PAHs may damage root membranes, increasing membrane permeability and disturbing plant ionic status.

C. maritima showed a high GPX activity when grown under high Phe concentration conditions; values were 281 and 162% of the control respectively in leaves and roots (Table 1). However, the percentage of inhibition of $ABTS^{+}$ experienced no variation with treatment in both organs. The increase in GPX activity under Phe stress conditions indicates the involvement of high level scavenging system of Phe-generated ROS in plant cells. The enhancement was more pronounced in leaves than in roots, which suggests that stress severity and ROS production were higher in leaves. We can speculate that Phe was internalized and transported from roots to leaves where it was probably metabolized or volatilized. The contribution of plant uptake and accumulation to the dissipation enhancement of Phe is often neglected, as reported in previous studies (Gao and Zhu, 2004; Reilley et al., 1996). Cheema et al. (2010) showed that enhanced dissipation of Phe in planted soil was mainly due to plant-

promoted biodegradation. Plants may contribute to the biodegradation of organic compounds by an increase in microbial communities (Muratova, et al., 2003; Reilley et al., 1996), a promotion in microbial activity, and a modification in microbial community in rizosphere (Joner et al., 2002). For this reason, we used sand in this work to avoid any co-metabolization by bacteria or fungi. In this context, the model halophyte *T. salsuginea* was used as biomarker by growing it on a range of Phe concentrations. This concept can give two data: i) the response of this model plant to Phe stress and to identify its tolerance threshold and ii) to estimate the potential of *C. maritima* to reduce Phe phytotoxicity.

After 4 weeks of treatment (0, 125, 250, and 375 ppm), *T. salsuginea* plants expressed a significant decrease in size with the increasing Phe level up to 250 ppm (Fig. 2). However, no difference was detected between the two most severe treatments (250 and 375 ppm Phe). Leaf biomass was diminished by 21, 67, and 66% at 125, 250, and 375 ppm Phe, respectively. The effect was more pronounced in roots; their fresh weight was respectively decreased by 40, 70, and 71%. Interestingly, plants grown on the soil previously treated by *C. maritima* culture exhibited similar leaf and root growth as those subjected to 125 ppm Phe. This indicates that *C. maritima* has a high potential of reducing Phe phytotoxicity.

In conclusion, the halophyte *C. maritima* is able to overcome PAH-induced oxidant stress, to produce high shoot biomass, and to decrease PAH phytotoxicity in less tolerant plants such as *T. salsuginea* if subsequently grown on the same substrate. This supports further inquiry into *C. maritima* phytoremediation potential.

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Table. Biomass production (n = 9) and antioxidant responses (n = 3) of *C. maritima* plants grown for 6 weeks on sand contaminated or not with 500 ppm phenanthrene. Means followed by different letters are significantly different according to Student's t test at 5%. FW: fresh weight, GPX: guaiacol peroxidase.

	Control	**Treated**
Leaf FW (g)	5.52b	1.59a
Stem FW (g)	4.04b	1.16a
Root FW (g)	1.36b	0.12a
Whole plant FW (g)	10.91b	2.88a
Leaf GPX activity (nkat. mg^{-1} proteins)	3.02a	8.61b
Root GPX activity (nkat. mg^{-1} proteins)	7.19a	11.70b
Leaf percentage of inhibition (%)	70.17a	62.93a
Root percentage of inhibition (%)	70.35a	64.80a

Figure legends:



Figure 1. Phenotypical changes of *C. maritima* plants grown for 6 weeks on sand contaminated or not with 500 ppm phenanthrene.

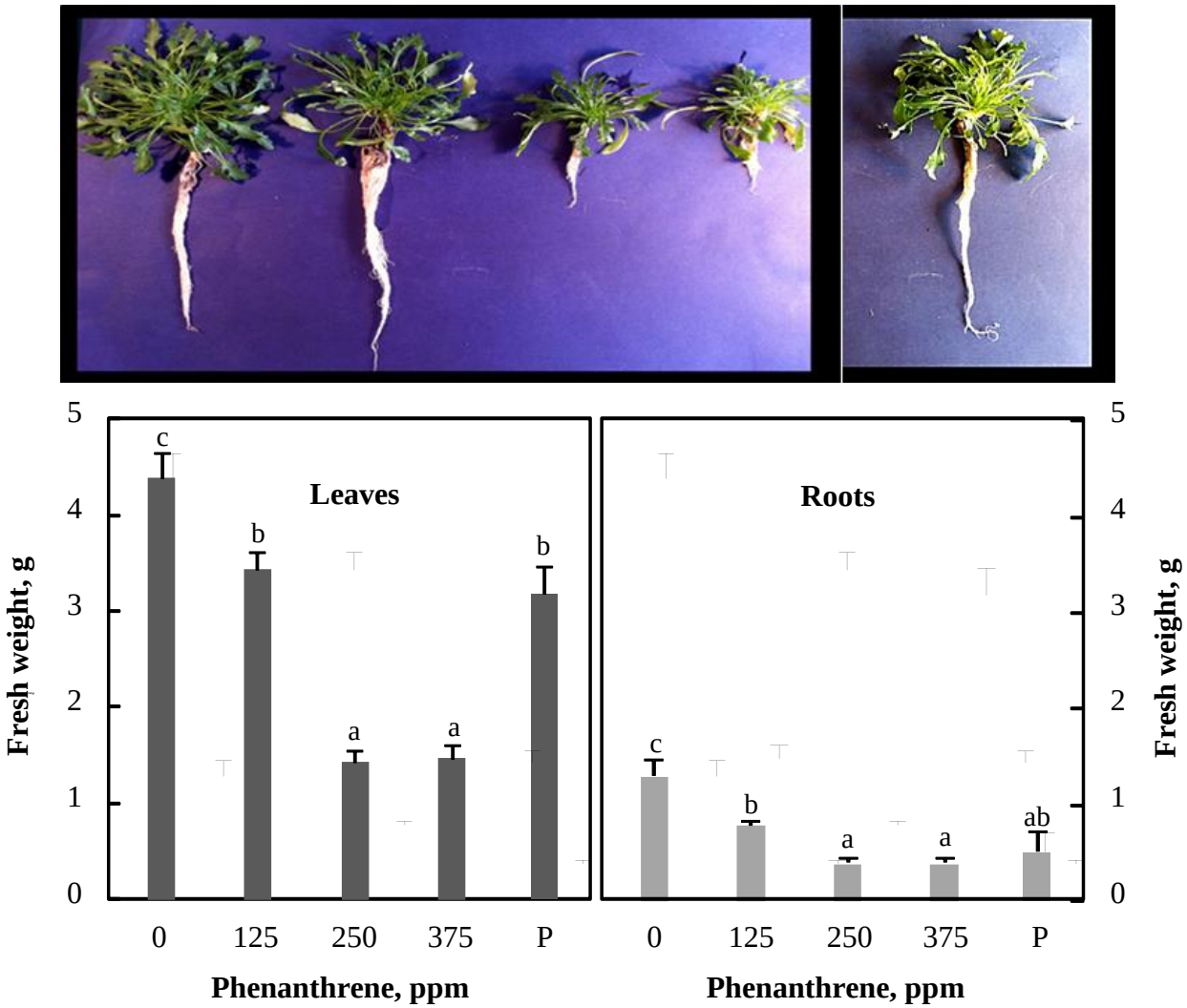


Figure 2. Comparison of morphological aspects and fresh weights between *T. salsuginea* plants grown for 4 weeks on sand added with 0, 125, 250, or 375 ppm phenanthrene and those grown on *C. maritima*-phytoremediated sand (P). For fresh weights, bars with different letters are significantly different according to Duncan's test at $P \leq 0.05$.