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Research article

Physiological and biochemical mechanisms preventing Cd-toxicity in the hyperaccumulator *Atriplex halimus* L.

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ABSTRACT

The xero-halophyte *Atriplex halimus* L., recently described as Cd-hyperaccumulator, was examined to determine Cd toxicity threshold and the physiological mechanisms involved in Cd tolerance. An experiment was conducted to investigate the effect of cadmium from 0 to 1350 μM on chlorophyll fluorescence parameters, gas exchange, photosynthetic pigment concentrations and antioxidative enzyme activities of *A. halimus*. Cadmium, calcium, iron, manganese, magnesium, potassium, phosphorous, sodium and zinc concentrations were also analyzed. Plants of *A. halimus* were not able to survive at 1350 μM Cd and the upper tolerance limit was recorded at 650 μM Cd; although chlorosis was observed from 200 μM Cd. Cadmium accumulation increased with increase in Cd supply, reaching maxima of 0.77 and 4.65 mg g^{-1} dry weight in shoots and roots, respectively, at 650 μM Cd. Dry mass, shoot length, specific leaf area, relative growth rate, net photosynthetic rate, stomatal conductance, pigments contents and chlorophyll fluorescence were significantly reduced by increasing Cd concentration. However, the activities of superoxide dismutase (SOD; EC1.15.1.1), catalase (CAT; EC1.11.1.6) and guaiacol peroxidase (GPx; EC1.11.1.7) were significantly induced by Cd. Exposures to Cd caused also a significant decrease in P contents in roots, Mg and Mn contents in shoots and Fe and K contents in roots and shoots and had no effect on Ca, Na and Zn contents. The tolerance of *A. halimus* to Cd stress might be related with its capacity to avoid the translocation of great amounts of Cd in its aboveground tissues and higher activities of enzymatic antioxidants in the leaf.

1. Introduction

Heavy metal pollution has become an important environmental problem today and increasing continuously as a result of growing industrialization and the massive use of fertilizers. Cadmium is one of the most phytotoxic elements, because of the high water solubility, relative mobility and long biological half-life (Wang et al., 2014). It is not an essential nutrient in higher plants, and the exposure to relatively low concentrations results in high toxicity to plant and animal (Redondo-Gómez et al., 2010). Several studies have shown that plant metabolism is affected by Cd in different ways, and the photosynthetic process appears to be particularly sensitive to this metal (Ci et al., 2010). In particular, Cd causes chlorosis and growth reduction (Zemanová et al., 2015). Cd can interact with the plant water balance (Costa and Morel, 1994), disturb nutrient uptake (Redondo-Gómez et al., 2010), inhibits stomatal opening (Perfus-Barbeoch et al., 2002), provokes damages to the photosystems I and II (Küpper et al., 2007; Li et al., 2015), and inhibits some of the enzymes of the Calvin cycle (Kabata-Pendias and Pendias, 2001). High concentration of Cd induces increased respiration and activities of tricarboxylic acid cycle as well as other pathways of carbohydrate utilization (Liphadzi and Kirkham, 2005).

Abbreviations: A_N, net photosynthetic rate; CAT, catalase; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; C_i, intercellular CO_2 concentration; *Cx* + *c*, carotenoids; GPx, guaiacol peroxidase; F_0, minimal fluorescence level in the dark-adapted state; F_m, maximal fluorescence level in the dark-adapted state; F_s, steady state fluorescence yield; F_v, variable fluorescence level in the dark-adapted state; F_v/F_m, maximum quantum efficiency of PSII photochemistry; Φ_{PSII}; actual quantum yield of PSII photochemistry, g_s; stomatal conductance, Φ_{NPQ}, quantum yield of non-photochemical quenching; ROS, reactive oxygen species; RGR, relative growth rate; SOD, superoxide dismutase; SLA, specific leaf area; iWUE, intrinsic water use efficiency.

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It has also been reported that Cd can increase the production of reactive oxygen species (ROS) in plants cells, which can cause peroxidative damages, such as cell membrane lipids peroxidation and protein carbonylation (Gill et al., 2012). To cope with oxidative damage, plants possess an efficient ROS scavenging system composed of enzymatic and non-enzymatic antioxidants. Among antioxidative enzymes, superoxide dismutase (SOD), guaiacol peroxidase (GPx) and catalase (CAT) play an important role in controlling the level of ROS (Qiu et al., 2008).

Atriplex halimus L. is a xero-halophyte which is perennial and native in arid and semi-arid Mediterranean regions. This species, that is present as a natural invading shrub in several mining areas of northern Africa and southern Europe (Pérez-Esteban et al., 2013), is known to grow rapidly and densely on degraded soils and tolerates extreme environmental conditions such as drought (Martínez et al., 2005), salinity (Bajji et al., 1998) and light stress (Streb et al., 1997). Recent work with *A. halimus* showed that it is hypertolerant to high concentrations of Cd up to 400 μM (Lefèvre et al., 2010; Nedjimi and Daoud, 2009) and Pérez-Esteban et al. (2013) described it as appropriate species for the phytostabilization of metals in mine soils. However, no studies are available concerning the Cd toxicity threshold in *A. halimus* and its physiological and biochemical mechanisms to prevent Cd toxicity. Although it is important to identify interesting hypertolerant species for phytoremediation, it is equally important to know the mechanisms underlying tolerance of these species. Therefore, this work is aimed to: (1) elucidate the Cd phytotoxicity thresholds in *A. halimus* by examining its growth response to different Cd levels; (2) determine the effect of Cd on the fluorescence parameters, gas exchange characteristics, anti-oxidative enzymes system (CAT, GPx and SOD) and photosynthetic pigments; and (3) examine the effect of Cd on nutrient status. Results obtained from this study may be useful for understanding the mechanisms of Cd tolerance in *A. halimus*.

2. Materials and methods

2.1. Plant material and Cd treatments

Seeds of *A. halimux* were collected in November 2013 from wild population grow at the arid salty area El-Outaya located in the province of Biskra, southeast of Algeria (34°55′42″N 5°38′58″E, and 198 m elevation). Seeds were removed from the bracts then stored at room temperature until use.

Seeds were placed in a germinator at 12 °C and photoperiod of 16 h of light for a month. The seedlings were then transferred separately into perlite filled pots (one plant per pot) and placed in a glasshouse with controlled temperature of 21–25 °C, 40–60% relative humidity and natural daylight (maximum light flux: 1000 μmol m^{-2} s^{-1}). Pots were irrigated with 20% Hoagland nutrient solution (pH 6.2) (Hoagland and Arnon, 1950).

After 70 days of seedling culture, the plants (n = 7) were exposed to six Cd treatments: 0, 50, 200, 400, 650 and 1350 μM Cd (supplied as $CdCl_2 \cdot 5/2H_2O$). Each treatment was allocated in one tray, each containing seven pots. During the treatment, the nutrient solution level in the trays was controlled every two days, and if necessary, 20% of Hoagland's solution was added to keep the level constant. The entire nutrient solutions were replaced every week to prevent nutrient and metal depletion. Cd concentrations were selected to cover variations recorded by Pérez-Sirvent et al. (2008) and Redondo-Gómez et al. (2009) in the salt marshes of southern Iberian Peninsula, where *A. halimus* is widely distributed. All measurements were carried out after 22 day of Cd treatment.

2.2. Sample collection and growth analysis

At the end of the experiment, plants (n = 7) were harvested, divided into shoots and roots and rinsed with distilled water to remove any perlite particles attached to plant surfaces. Growth parameters (shoots lengths, shoots and root dry mass) were measured. Specific leaf area (SLA) was calculated as leaf area of the sampled leaves divided by their dry mass. The dry mass was determined after drying shoots and roots at 80 °C for 48 h.

The relative growth rate (RGR) of whole plants was calculated as: RGR = (DMf - DMi) × D^{-1} (gg^{-1} day^{-1}), where DMf = final dry mass, DMi = initial dry mass and D = duration of experiment (days).

The symptoms of Cd toxicity (leaf senescence, and chlorosis) were registered by visual observation during the experiment. At the end of treatment, plant survival was recorded: plant was considered as dead if all leaves were not green.

2.3. Gas exchange and chlorophyll fluorescence analysis

Gas exchange and chlorophyll fluorescence measurements were carried out on fully expanded leaves before harvesting (n = 7, one measurement per plant).

Net photosynthetic rate (A_N), intercellular CO_2 concentration (C_i) and stomatal conductance (g_s) were determined according Mateos-Naranjo et al. (2013) using an open infrared gas analyzer system (Li-6400-40, Li-COR Inc., Lincoln, NE, USA). Intrinsic water use efficiency (iWUE) was calculated as the ratio between A_N and gs.

Chlorophyll fluorescence measurements were made using a portable modulated fluorimeter (FMS-2, Hansatech Instruments Ltd., UK). Light- and dark-adapted fluorescence were taken at dawn (stable, 50 μmol m^{-2} s^{-1} ambient light) and at midday (1600 μmol m^{-2} s^{-1}) as described by Mateos-Naranjo et al. (2013) to investigate the effect of Cd stress on the sensitivity of plants to photoinhibition.

Using fluorescence parameters determined in both light- and dark-adapted states, the following were calculated: actual quantum yield of PSII photochemistry [$\Phi_{PSII} = (F_m' - F_s)/F_m'$] (Genty et al., 1989) and quantum yield of non-photochemical quenching, which is the regulatory light-induced non-photochemical quenching [$\Phi_{NPQ} = (F_s/F_m') - (F_s/F_m)$] (Lazár, 2015). Φ_{PSII} relates to achieved efficiency in a plant under a given treatment and indicates the proportion of absorbed energy being used in photochemistry, while Φ_{NPQ} provides an indication of the amount of energy that is dissipated in the form of heat (Maxwell and Johnson, 2000).

Chronic (PI_{chr}) and dynamic (PI_{dyn}) photoinhibition were calculated according to Werner et al. (2002) as:

$$PI_{chr} = \left[(F_v/F_m)_{max} - (F_v/F_m)_d\right](F_v/F_m)_{max} \times 100$$

$$PI_{dyn} = \left[(F_v/F_m)_d - (F_v/F_m)_{mid}\right](F_v/F_m)_{max} \times 100$$

where $(F_v/F_m)_d$ and $(F_v/F_m)_{mid}$ are dawn and midday F_v/F_m values, respectively. $(F_v/F_m)_{max}$ is the maximum F_v/F_m value, which was calculated as the average of dawn measurements of the control one day after imposing Cd treatments.

2.4. Photosynthetic pigments

Photosynthetic pigments of five shoots per treatment were extracted using 0.1 g of fresh material in 5 ml of 80% aqueous acetone. After centrifuging, 1 ml of the suspension was diluted with a further 2 ml of acetone and chlorophyll *a* (Chl *a*), chlorophyll *b*