Journal of Photochemistry and Photobiology B: Biology 140 (2014) 228–239

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

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

Unveiling Zn hyperaccumulation in *Juncus acutus*: Implications on the electronic energy fluxes and on oxidative stress with emphasis on non-functional Zn-chlorophylls

D. Santos, B. Duarte [*], I. Caçador

Centre of Oceanography of the Faculty of Sciences of the University of Lisbon (CO), Campo Grande, 1749-016 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form 30 June 2014
Accepted 29 July 2014
Available online 19 August 2014

Keywords:
Hyperaccumulator
Zinc-chlorophylls
Photochemistry
Electronic transport
Oxidative stress

ABSTRACT

Juncus acutus arises as possible hyperaccumulator specie, tolerating exogenous Zn concentrations as high as 60 mM. Zinc concentrations here detected in seedlings germinated in the presence high Zn concentrations, were above the described upper toxic levels for higher plants. Even at the highest Zn concentration, growth inhibition only accounted to approximately 30% of control seedlings biomass, presenting an EC_{50} value in the range of 10–20 mM of metal. PSII quantum yields showed a marked decline, reflection of changes in the thylakoid structure on the PSII electron donor sites. In fact, the electron transport rate was severely affected by Zn in seedlings exposed to higher Zn concentrations leading to a decrease in their maximum electronic transport rate and consequently presenting lower light saturation and lower photosynthetic efficiencies. Although light absorption capacity was not affected by Zn exposure and uptake, energy trapping flux in the photosynthetic apparatus and transport throughout the electronic chain was severely impaired. This lack of efficiency is related with non-functional Zn-chlorophylls formation. There was a strong linear correlation between exogenous Zn concentration applied and the concentration actually verified in the seedlings tissue with the concentration of both ZnChl a and b. There was also a gradual loss of connectivity between the antennae of the PSII units being this more evident at the higher Zn concentrations and thus impairing the energetic transport. The reduction in light harvesting efficiency, leads inevitably to the accumulation of redox energy inside the cells. To counteract ROS generation, all anti-oxidant enzymatic activities (except catalase) showed a proportional response to exogenous and *in vivo* Zn concentrations. Not only this plant appears to be highly tolerant to high Zn concentrations, but also it can overcome efficiently the damage produced during this uptake by efficiently dissipating the excessive cellular redox potential accumulated, essentially due to Zn incorporation into the chlorophyll molecule.

1. Introduction

Salt marsh ecosystems, are of great ecological importance. *Sarcocornia perennis*, *Sacocornia fruticosa*, *Spartina maritima*, *Halimione portulacoides* [5] and *Juncus acutus* [31,4] are some of the halophyte species commonly inhabitant in this environments, in Mediterranean marshes. Due to their location near or alongside estuaries, these ecosystems are often exposed to high anthropogenic pressures and considered as natural sink of heavy metals from industrial and urban sewage [38,5]. Despite the fact that some halophyte species from salt marshes can withstand some degree of contamination, excessive concentration of metals in the soil, driven from long-term accumulation or toxic discharges, can not only cause damages in the plants, but also be potentially harmful to human health, through food chain.

Metals like zinc (Zn), iron (Fe) and nickel (Ni) are essential to plant metabolism at lower concentrations, however in higher concentrations can cause disorders. Zinc (Zn), as other essential metals like copper (Cu), can become toxic to plants whenever their concentrations are above their nutritional threshold [29]. At organismal level, excess Zn inhibits seed germination, plant growth [36] root development [26] and causes leaf chlorosis [16]. At cellular level, excess Zn can significantly alter mitotic activity [45], affect membrane integrity and permeability [48] and even induce cell death [7]. Excessive Zn concentrations can also cause imbalances between production and scavenging of reactive oxygen species, inducing oxidative stress conditions. To counteract the effect of free radicals, plants developed a battery of defences that includes

---

[*] Corresponding author. Tel.: +351 217 500 009.
E-mail address: baduarte@fc.ul.pt (B. Duarte).

http://dx.doi.org/10.1016/j.jphotobiol.2014.07.019
1011-1344/© 2014 Elsevier B.V. All rights reserved.

some antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidise (GPx) and ascorbate peroxidise (APx). These enzymes have a fundamental role in the O_2 and H_2O_2 production by ROS catalysis. These oxygen forms are far less harmful to the organism and can result for example from the dismutation of superoxide ion (O^{2-}), preventing serious cellular damage. Previous works have shown that excess Zn induces oxidative stress and lipid peroxidation [43,27]. Several recent studies [27,9,8,56,52] have demonstrated the effects of Zn stress on the activity of many antioxidative enzymes (APx, SOD, POD and CAT) and antioxidant contents (ascorbate and GSH) in plants.

Nevertheless, ROS generation is a rather transversal effect from almost all excessive cations in cellular compartments. In fact, Zn has another a potentially more damaging effect in plant bioenergetics, by substituting the Mg atom at the chlorophyll reaction center. Hence, the formation of minor proportions of [Zn]-Chl relative to the total Chl may already inhibit photosynthesis completely, affecting the overall cell bioenergetics and redox homeostasis [25]. This substitution effect is rather well known and was already described in algae [25] and in higher plants [37].

Juncus acutus Lam. (*J. acutus*) is highly common halophyte specie in Portuguese marshes, found in several kinds of sediments from sandy to muddy, gathering this way an increased colonization potential in all sorts of salt marshes [4]. Alongside, Zn is highly abundant in the Earth's crust and has a widespread role in industrial processes, with inevitable inputs to the surrounding environment, especially in estuarine systems [11]. Therefore, *J. acutus* widespread presence and wide range of habitat physical attributes, makes this halophyte ideal for testing the effects, that a highly abundant metal such as Zn, can have in the establishment of a halophytic specie independently the marsh type [4].

In the present work, the authors aimed to understand zinc effects in *J. acutus* seed germination, growth, photosynthetic apparatus as well as in photosynthetic pigments (including Zn substituted chlorophylls) and seedlings antioxidant response to acute metal stress, for use as potential biomarkers. This approach aims to unveil the Zn hyperaccumulation ability in this specie, understanding the effect of this specific metal on the seedling survival and its implications on cellular bioenergetics and redox homeostasis.

2. Materials and methods

2.1. Seed harvest and incubations

Juncus acutus seeds were harvested in November 2011 in an undisturbed salt marsh of Tagus estuary within the old World Exposition 98' site. All the collected flowers were brought to the laboratory and kept in dry conditions. Seeds were incubated in ¼ Hoagland solution supplemented with $ZnSO_4$ (Sigma–Aldrich Ultra-Pure) in order to make the desired concentrations (10, 20, 40 and 60 mM). Zinc treatments were established by supplementing ¼ Hoagland´s solution with $ZnSO_4{\cdot}7H_2O$ to achieve the target concentrations comparable to previous studies with halophytes using acute heavy metal concentrations in salt marshes [6,32]. The control, 0 mM Zn treatment, had exactly 0.002 mM Zn, as Hoagland´s solution contains a small amount of Zn as essential nutrient. Approximately 20 seeds were placed in each petri dish with a Whatman GF/C filter as solid substrate. Each treatment ($n = 3$ petri dish, approx. 60 seeds) was soaked with 800 µL of the correspondent Zn concentration solution. The petri dishes were sealed with Parafilm and placed in a FytoScope Chamber (Photon System Instruments, Czech Republic) in the dark at 25 °C. Every three days, germinated seeds were counted until a maximum of 15 days. After germination (approximately after 6 days of incubation), the petri dishes were placed in light conditions (150 µmol photons $m^{-2}\,s^{-1}$) in a 16 h/8 h day–night regime using a sin function to simulate sunrise–midday–sunset conditions. At the end of the experiment, seedlings were collected and used for analysis. All seedlings for biochemical analysis were flash-frozen in liquid-N_2. Seedlings were also weighted and measured for total length. The Germination Index (GI) and germination rates were calculated by the following formulas:

$$GR = \frac{\sum(\text{Number of germinated seed in n days} \times \text{number of days})}{\text{total number of days}}$$

$$GI = \sum\frac{\text{Number of germinated seeds}}{\text{Days since first count}}$$

2.2. Metal content analysis

Dry homogenized material was digested by adding 2 ml $HNO_3/HClO_4$ (7:1, v/v) in a Teflon reactor and heated at 110 °C for 6 h. After cooling overnight, the digestion products were filtered through Whatman No. 42 (2.5 µm of pore diameter) filters and diluted with distilled water to a total volume of 10 ml. Zinc concentrations in the extracts were determined by atomic absorption spectrometry (Perkin–Elmer A Analyst 100). International certified reference materials (CRM 145, CRM 146 and BCR 62) were used to ensure accuracy and precision of the extraction and analytical. The concentrations in the reference materials determined by FAAS were not statistically different from their certified ones (*t* student; $a = 0.05$).

2.3. PAM fluorometry

Modulated chlorophyll fluorescence measurements were made in attached leaves in field with a FluoroPen FP100 PAM (Photo System Instruments, Czech Republic). All the measurements in the dark-adapted state were made after darkening of the leaves for at least 30 min. The minimal fluorescence (F_0) in dark-adapted state was measured by the measuring modulated light, which was sufficiently low (<0.1 µmol $m^{-2}\,s^{-1}$) not to induce any significant variation in fluorescence. The maximal fluorescence level (F_M) in dark-adapted state was measured by a 0.8 s saturating pulse at 8000 µmol $m^{-2}\,s^{-1}$. The maximum photochemical efficiency was assessed as $(F_M - F_0)/F_M$. The same parameters were also measured in light-adapted leaves, being F'_0 the minimum fluorescence, F'_M the maximum fluorescence and the operational photochemical efficiency. Rapid light curves (RLC) measurements, in dark-adapted leaves, were attained using the pre-programed LC1 protocol of the FluoroPen, consisting in a sequence of pulses from 0 to 500 µmol $m^{-2}\,s^{-1}$. During this protocol the F_0 and F_M as well as the maximum photochemical efficiency were measured. Each Φ_{PSII} measurement was used to calculate the electron transport rate (ETR) through photosystem II using the following equation: $ETR = \Phi_{PSII} \times PAR \times 0.5$, where PAR is the actinic photosynthetically active radiation generated by the FluoroPen and 0.5 assumes that the photons absorbed are equally partitioned between PSII and PSI [18]. Without knowledge of the actual amount of light being absorbed, fluorescence measurements can only be used as an approximation for electron transport [1,2] and [46]. Rapid light curves (RLC) were generated from the calculated ETRs and the irradiances applied during the rapid light curve steps. Each RLC was fitted to a double exponential decay function in order to quantify the characteristic parameters, alpha and ETR_{max} [42]. The initial slope of the RLC (α) is a measure of the light harvesting efficiency of photosynthesis and the asymptote of the curve, the maximum rate of photosynthesis (ETR_{max}), is a measure of the capacity of the photosystems to utilize the absorbed light energy [30]. The onset of light