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On the nature and timing of oxygen radical production following exposure of *Arabidopsis thaliana* leaves to uranium, cadmium or a combination of both stressors

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Abstract. The toxicity and oxidative stress responses of 19-day old *Arabidopsis* seedlings induced by U (66 μM) and Cd (20 μM) alone or in a binary mixture set-up (equitoxic mixture) are studied in function of time. After 48h a significant decrease in root and shoot growth and a simultaneous increase in anthocyanin production was evident in all treated plants.
Production of O_2^- or H_2O_2 was visualized by staining freshly harvested leaves with nitrobluetetrazolium or diaminobezidine, respectively. With this method production of O_2^- was only significantly measurable after 168 h treatment which coincides with a significant decrease in biomass production and probably also plant cell death. For Cd treated plants a significant increase in H_2O_2 production was measurable from 24h onwards. In contrast, a similar H_2O_2 production could not be measured in U or U + Cd treated plants. Both water and lipophilic soluble antioxidants significantly increased in U treated plants after 48 h. These high antioxidant levels might detoxify potential H_2O_2 produced in the U treated plants. In contrast for Cd treated plants only after 168h a significant increase in water soluble antioxidants was measured whereas no difference in the lipophilic fraction was visible.

1. INTRODUCTION

As sessile organisms, plants are commonly exposed to a number of adverse conditions in their environment. Nuclear energy production encompasses a variety of industrial activities, from mining and milling through to power generation and waste management. These nuclear facilities potentially release low amounts of radioactive substances together with non-radioactive substances (e.g. heavy metals, inorganic and organic chemicals). In addition, increases in environmental concentrations of natural occurring radionuclides have arisen from industries such as metal mining and smelting and the phosphate industry. To understand and predict plant stress reactions to these different radionuclides and heavy metals it is needed to unravel plant stress responses to a combination of these different toxicants preferably mimicking field conditions. In the present study the effect of a combination of two toxic heavy metals Cd and the radionuclide U was studied on *Arabidopsis* plants. Both are non-essential elements present in the earth crust in an average concentration of 0.1–0.5 ppm for Cd and 1 ppm for U.

Here the toxicity and oxidative stress responses of *Arabidopsis* seedlings induced by U and Cd alone or in a binary mixture set-up are studied in function of time. Toxicity of Cd to plants has been extensively studied [for review see 1, 2]. Cd is a non-redox active element but induces oxidative stress by inducing an NADPH-oxidase regulated oxidative burst, lipid peroxidation [3, 4] and a subsequent cellular redox imbalance [1, 3]. Compared to Cd, information on U toxicity in plants is scant. However, the importance of oxidative stress in U toxicity has also been shown [5–7]. At a concentration of 100 μM U induced transcriptomic changes of NADPH-oxidases and Cu//ZnSOD, FeSOD and APX [7]. An increase in ascorbate levels to U exposure has been observed indicating the importance of the ascorbate/glutathione pathway in U induced defense reactions [7]. Recently Vanhoudt and co-workers [6] investigated the

toxicity of a 3-day exposure of *Arabidopsis* seedlings to a binary mixture of U (10 μM) and Cd (5 μM). In the experimental set-up used by Vanhoudt and co-workers [6] concentrations and exposure time of U and Cd were low and did not induce changes in rosette growth. However, under these experimental conditions Cd induced higher U uptake into the roots and changes in micronutrient profile.

To further unravel the oxidative stress responses induced by Cd and U in *Arabidopsis* seedlings the present study measures the production of H_2O_2 and O_2^- and changes in antioxidative metabolism induced by U, Cd or an equitoxic mixture of U and Cd applied in concentrations inducing 50% growth reduction (3days).

2. MATERIALS AND METHODS

2.1 Plant culture and uranium and cadmium contamination

Seeds of *Arabidopsis thaliana* (Columbia ecotype) were placed on moist filter paper at 4 °C for 3 days in order to synchronize germination. Afterwards, seeds were sown on plugs from 1.5 ml polyethylene centrifuge tubes filled with 2% agar (Difco) and plants were grown hydroponically in a modified Hoagland solution (macro-elements without phosphate: 1/10 diluted, phosphate solution: 1/20 diluted, micro-elements: 1/10 diluted and iron solution: 1/10 diluted) in a growth chamber (Microclima 1000E, Snijders Scientific B.V.) under a 14 h photoperiod (photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} at the leaf level, supplied by Sylvania BriteGro F36WT8/2084 and F36WT8/2023 lamps), with day/night temperatures of 22 °C/18°C and 65% relative humidity.

Cd was administered to the seedlings as $CdSO_4$ and U as $UO_2(NO_3)_2.6H_2O$ dissolved in 0.1N HCl, by adding it to the modified Hoagland solution. The pH was adjusted to $\pm$ 5.5 with NaOH. The concentration of U and Cd administered was the derived EC_{50} value for biomass accumulation in leaves of Cd (20 μM) or U (66 μM) or both stressors at half their EC_{50} value (Cd + U, 10 + 33 μM). EC_{50} values for inhibition of growth after 72 days for Cd and U were estimated based on the single dose-response curves (data not shown).

Plants were exposed to the different toxic conditions for a duration of 1h, 24h, 48h, 72h or 168h. At harvest fresh weight of roots and leaf rosettes determined and samples were snap-frozen in liquid nitrogen for further analysis.

2.2 Anthocyanin determination

Accumulation of anthocyanin pigments in leaf tissue was determined according to Porter et al. [8] by extracting leaf tissue (100 mg) in 1mL ice-cold ethanol supplemented with 1% (v/v) HCl in the dark. Chlorophyll was removed from the extracts as it might interfere with the spectrophotometrical anticyanin determination. Therefore the 1mL ethanol extract sample was diluted with 600 μL deionized water and subsequently 100 μL of chloroform was added and the solution was vortexed and centrifuged at 16.000g for 5 min and the chloroform layer was discarded [9]. Subsequently anthocyanin were determined spectrophotometrically at 535 nm.

2.3 Total antioxidative capacity

The total antioxidative capacity of the leaves is determined as the capacity of an aqueous or lipophilic extract to reduce ferric tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ). Fe^{2+}-TPTZ is bright blue colored and can be measured at 593 nm. The obtained absorptions were compared to a trolox standard curve and therefore antioxidative capacity is expressed as mol trolox equivalents/g FW [10]. The extraction of the leaf tissue in an aqueous extract (0.01N HCl, 1mM Na-EDTA) and a lipophilic fraction (80% (v/v) acetone) was performed as described by Kerchev and Ivanov [11]. The

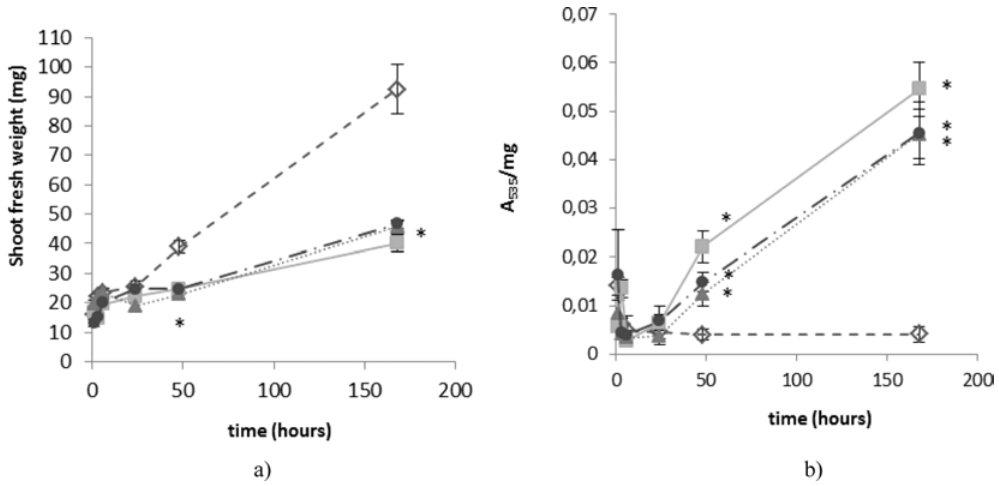

Figure 1. Biomass accumulation (a) and anthocyanin production in *Arabidopsis* plants exposed to control conditions (- - - -, ◊), 66 μM U (——, ■), 20 μM Cd (....., ▲) or a combination of 33 μM U + 10 μM Cd (— · —, •). n = 10 for biomass accumulation and n = 3 for anthocyanin production, mean ± SE is depicted. * indicates significant differences compared to the control ($P < 0.05$). For shoot fresh weight (a) at 48 h and 168 h, all 3 treatments were significantly different from the control. However, for the clarity of the figure only one ∗ was added.

aqueous extract typically contains antioxidants like ascorbate and glutathione whereas tocopheroles are the major contributors to the lipophilic fraction.

2.4 Hydrogen peroxide and superoxide determination

The production of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$)in the leaves was determined by submerging the leaves in 0.1% (w/v) diaminobenzidine (DAB) in 10 mM MES-buffer pH 6.5 for 10 min or in 0.1% (w/v) nirtobluetetrazolium (NBT) in 50 mM K_2PO_4 buffer pH 6.4 supplemented with 10 mM Na-azide for 20 min as described by Romero-Puertas and co-workers [12]. To facilitate the entrance of the dyes into the apoplast a light vacuum was administered at the beginning of the staining procedure. Staining was stopped and leaves chlorophyll was removed from the leaves by washing them twice in water and submerging them for 10 min in 95% (v/v) ethanol at 70°C. In order to quantify the production of the reactive oxygen species (ROS) pictures from the stained leaves were analyzed with ImageJ 1.41 software that enabled the expression of the colored area as % of the total leaf area.

2.5 Statistics

Data are depicted as mean-values ± standard error with n ≥ 4 were evaluated by means of an two-way ANOVA followed by a Duncan-test to identify statistically difference compared to the control.

3. RESULTS AND DISCUSSION

Plants were exposed to Cd (20 μM) or U (66 μM) or in an equitoxic mixture of Cd (10 μM) + U (33 μM) for 1h to 168h. Biomass accumulation was measured over time (Fig. 1a). The equitoxic mixture of U and Cd reduced growth over time to the same extent than the toxicants alone indicating that growth was reduced in the equitoxic mixture according to concentration addition.

Plants responded to the applied stress conditions by an increased anthocyanins biosynthesis. Anthocyanins are secondary metabolites and flavonoids that have been linked to plant stress tolerance

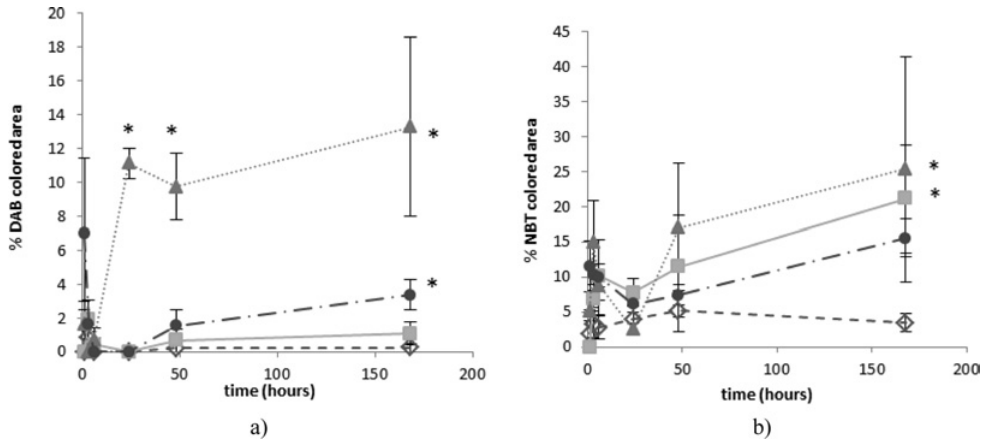

Figure 2. H_2O_2 (a) and $O_2^{\cdot-}$ (b) production in *Arabidopsis* plants exposed to control conditions (- - - -, ◊), 66 μM U (——, ■), 20 μM Cd (....., ▲) or a combination of 33 μM U + 10 μM Cd (— · —, ●). The production is expressed as colored leaf area relative to the total leaf area. n = 4, mean ± SE is depicted. * indicates significant differences compared to the control ($P < 0.05$).

[13]. Both U and Cd induced anthocyanins to the same extent from 48h onwards (Fig. 1b). The mixture was indistinguishable from the single toxicants indicating that for anthocyanin production, similar to biomass accumulation, the equitoxic mixture behaved according to concentration addition.

Both U and Cd have been shown to induce oxidative stress in plants [3–7]. Increased oxidative stress can originate from various sources including interaction with the antioxidative defense system, induction of lipid peroxidation, disruption of electron transport chains or the H_2O_2 production via the activation of $O_2^{\cdot-}$ producing NADPHoxidase and superoxide dismutase enzymes. Cd rapidly induced the transcription of genes encoding for NADPoxidases [3, 4, 14] giving rise to an increased level of H_2O_2 [3, 14]. In addition Cd triggers lipid peroxidation and specifically induces lipoxygenase genes (*LOX1*) in both roots and shoots of *Arabidopsis* [3, 15]. The response of plants to U treatment was previously shown to involve the differential expression of certain NADPHoxidase genes mainly in the roots [16] whereas *LOX* genes were differentially expressed in roots (*LOX1*) and shoots (*LOX2*) [17]. Here, the production of ROS in the leaves was measured *in situ* by staining leaves at different time points after exposure to U, Cd or the equitoxic mixture with DAB or NBT. DAB will form brown spots when it reacts with H_2O_2 whereas NBT forms dark blue precipitates when interacting with $O_2^{\cdot-}$.

Using this method Cd showed significant H_2O_2 production compared to non-stressed control plants that started after 24h and sustained for the duration of the experiment (Fig. 2a). For the equitoxic mixture H_2O_2 production only increased significantly after 168 h. In contrast, no H_2O_2 production was measured in the U exposed leaves (Fig. 2a), which indicates that although U induced gene expression of oxidative stress enzymes such as lipid peroxidases [17] the leaves were able to keep the production of ROS under control. The data of Cd treated plants are in accordance with previous results showing a rapid and high oxidative burst of H_2O_2 as part of the stress signaling pathway [3]. Variation between the leaves of the biological replicates stained for the presence of superoxide was more pronounced which rendered superoxide quantification more difficult. However, at 168 h both Cd and U showed increased $O_2^{\cdot-}$ levels (Fig. 2b). The mixture followed the same tendency but due to the large variation observed $O_2^{\cdot-}$ production was not significantly different from the control. The higher $O_2^{\cdot-}$ levels at the end of the experiment possibly indicate cell death.

In addition to ROS production, total antioxidative capacity of leaves was determined (Fig. 3). As both aqueous and lipophilic extracts were prepared we were able to distinguish between water soluble antioxidants and lipid soluble ones. Major contributors to the water soluble fraction are ascorbate

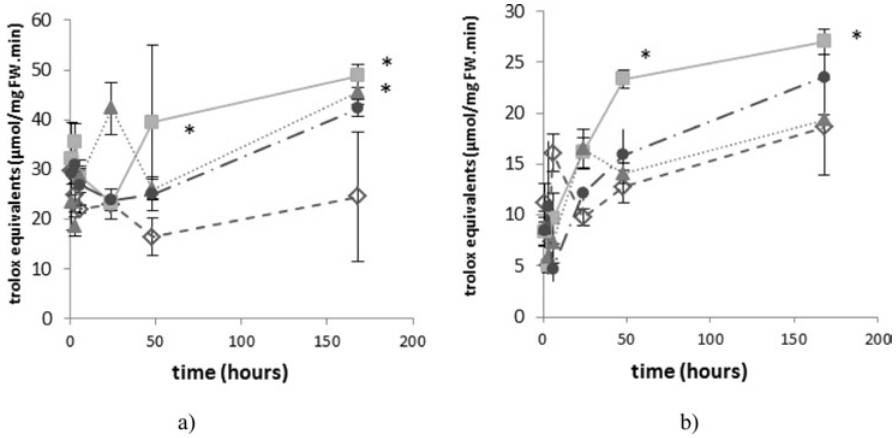

Figure 3. Total antioxidative capacity expressed as trolox equivalents of water (a) or acetone soluble leaf extracts of *Arabidopsis* plants exposed to control conditions (- - - -, ◊), 66 μM U (——, ■), 20 μM Cd (....., ▲) or a combination of 33 μM U + 10 μM Cd (— · —, ●). n = 4, mean ± SE is depicted. * indicates significant differences compared to the control ($P < 0.05$).

and glutathione whereas tocopherols are predominantly present as lipophilic antioxidants. However, measuring the antioxidants as a total water or lipid soluble antioxidative capacity has the advantage that the contribution of unknown or less apparent antioxidants is not missed. In the aqueous leaf extracts an increase in antioxidative capacity was observed in the U-treated plants from 48 h onwards (Fig. 3a). An increase in the ascorbate and glutathione pool and redox status was previously observed in *Arabidopsis* plants treated for 7 days with 100μM U [17]. As ascorbate and glutathione have been indicated as the major water soluble antioxidants present the increase in water soluble antioxidative capacity in the plants after U treatment could be explained by a rise in ascorbate and glutathione. After 168 h both U and Cd induced significant higher water soluble antioxidants (Fig. 3a). The mixture followed the same tendency although not significant. For U, a significant and sustainable increase in lipid soluble antioxidants was observable from 48 h onwards indicating the importance of lipid soluble antioxidants in the response of plants to U (Fig. 3b). To our knowledge this is the first time that the induction of lipid soluble antioxidants has been investigated in U-treated plants. In contrast, in Cd treated leaves no changes in the lipid soluble antioxidants were measured (Fig. 3b). From these results it can be concluded the induction of antioxidants is a major contributor to the response of plants to U treatment. This increased antioxidative capacity can actively scavenge ROS and consequently possibly explain the low levels of ROS production observed in the U treated leaves compared to the Cd ones.

4. CONCLUSION

In conclusion it is shown here that both U and Cd adversely affect growth of *Arabidopsis* seedlings and that this is accompanied by an oxidative stress response. Whereas for Cd an increased H_2O_2 production was detectable after 24 h a similar oxidative burst in U treated plants might be balanced by the presence of a higher antioxidative capacity. It was shown for the first time that not only water soluble antioxidants but also lipid soluble ones contribute to the antioxidative response from plants to U exposure. U has been shown to induce *LOX* genes in plants. Lipoxygenases facilitate lipid peroxidation leading to ROS formation, lipid damage but also the production of poly unsaturated fatty acids. The latter are used for the biosynthesis of lipoxins like jasmonic acid that in turn are suggested to be important signaling molecules in abiotic stress reactions [18, 19]. Therefore the observed increased lipid soluble antioxidative capacity

might be important at keeping ROS production under control but at the same time enabling the formation of lipoxins as signaling molecules. Further research is needed to confirm this hypothesis.

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