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Rye oxidative stress under long term Al exposure

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ABSTRACT

Aluminium (Al) toxicity decreases plant growth. *Secale cereale* L. is among the most Al-tolerant crop species. In order to study the response to Al-long term exposure, two rye genotypes with different Al sensitivity ('D. Zlote' and 'Riodeva') were exposed to 1.11 and 1.85 mM Al and the antioxidant responses were followed for 2 and 3 weeks in roots and leaves. Al toxicity signals, such as a severe decrease in root growth, occurred sooner in 'Riodeva.' The antioxidant response was dependent on the genotype, the organ, Al concentration and the exposure period. Al-exposed roots of 'D. Zlote' showed earlier enhancements of APX, SOD and G-POX activities than those of 'Riodeva.' 'D. Zlote' roots showed stimulation of the AsA–GSH cycle after the second week (when root growth inhibition was less severe), while later (when severe root growth inhibition was observed), oxidation of AsA and GSH pools was observed. In leaves of both genotypes, CAT, SOD and G-POX activities increased with Al exposure. In these leaves, the effect of AsA–GSH was time dependent, with maximum oxidation at the second week, followed by recovery. We confirmed that the oxidation state of AsA and GSH pools is involved in the detoxification of Al-induced oxidative stress. Moreover, our data demonstrate that the production of ROS does not correlate with the Al-induced root growth decrease. Finally, the differences observed over time indicate that long term exposure may provide additional information on rye sensitivity to Al, and contribute to a better understanding of this species' mechanisms of Al tolerance.

Introduction

In acidic environments, aluminium (Al) becomes available to plants, limiting plant growth and crop production. Acidification of soils is caused by common agricultural practices, such as removal of products from the farm, leaching of nitrogen below the plant root zone, inappropriate use of nitrogenous fertilizers and build up in organic matter. Therefore, it is imperative to fully understand the mechanisms used by Al-tolerant species to cope with Al toxicity. Cereal genotypes/cultivars that are able to grow and complete their life cycle in acidic soils may be exploited to increase production in those soils. Then, it is necessary to monitor genotype responses to continuous exposure to Al, and to decipher the long term mechanisms of their tolerance.

Rye (*Secale cereale*) is considered one of the most Al-tolerant species among cereals (Aniol and Gustafson, 1984), but research dealing with rye Al tolerance is limited (for review of Al toxicity see: Samac and Tesfaye, 2003; Panda and Matsumoto, 2007; Poschenrieder et al., 2008; Ryan et al., 2011). Within the different species (rye included), Al tolerance varies among genotypes and is often based on root growth/regrowth and callose deposition after short periods of Al exposure (e.g. Ma et al., 1997). Different mechanisms involved in Al resistance are described in the literature. One of the most mechanisms most often cited among species is related to efflux of organic acids, and several genes controlling this trait have been identified in different species (Ryan et al., 2011). The major Al resistance gene in wheat (*ALMT1*-aluminium-activated malate transporter) encodes an Al activated malate transporter protein (Motoda et al., 2007). Expression of *ALMT1* in different species (Delhaize et al., 2004; Sasaki et al., 2004; Hoekenga et al., 2006; Pereira et al., 2010), conferred an Al-activated efflux of malate, and in some, increased resistance to Al. Beyond the *ALMT1* genes, Al resistance in several crop species was related to the expression of some genes of the *MATE* (multidrug and toxic compound exudation) family based on Al-activated citrate exudation (Magalhaes, 2010).

The tolerance classification of rye (and other cereals) genotypes has been built mainly on short term assays (Wang et al., 2006; Li et al., 2008; Stass et al., 2008). For crop production, it is essential to evaluate whether short term tolerance reported in a rye genotype matches long term tolerance in the field. Furthermore, identifying whether the mechanisms that plants use against Al toxicity are the same under long term vs. short term exposure is also important. To the best of our knowledge, no study has been carried out considering rye behavior under long term Al exposure.

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The major morphological aspect of Al toxicity is the inhibition of root elongation. This effect may be related to oxidative stress and changes in cell wall properties as a consequence of Al toxicity (Yamamoto et al., 2003; Zheng and Yang, 2005). Supporting this hypothesis, Al exposure has been reported to induce membrane damage by lipid peroxidation in many species, mostly in sensitive genotypes (e.g. Tabaldi et al., 2009; Schuch et al., 2010; Yin et al., 2010).

Among the several antioxidant stress pathways, it remains unclear how Al affects the AsA–GSH (ascorbate–glutathione) cycle (Li et al., 2010), which scavenges H_2O_2 and regulates ascorbate/dehydroascorbate (AsA/DHA), reducing glutathione/oxidized glutathione (reduced GSH/GSSG) and NAD(P)H/NAD(P) ratios. AsA is present in most cellular compartments (Chen and Gallie, 2006), serves as an electron donor and reacts with ROS. It plays multiple roles in plants (e.g. as a cell division regulator and signal transduction molecule) (Green and Fry, 2005). In rice, treatments with AsA alleviated Al-induced inhibition of root elongation (Guo et al., 2005; Wang and Kao, 2007). GSH is an important antioxidant and redox buffer (Noctor and Foyer, 1998), is implicated in cell division (Foyer et al., 2005) and is also involved in gene expression regulation (Grene, 2002). Its accumulation has been correlated with enhanced Al tolerance in tobacco (Devi et al., 2003). In rice, Al exposure for 20 days led to imbalances in the AsA–GSH cycle, namely in enzyme activities (Sharma and Dubey, 2007) and in non-enzymatic antioxidant contents (Wang and Kao, 2007; Sharma and Dubey, 2007). Further, these Al-induced alterations were dependent on the Al concentration and time of exposure (Sharma and Dubey, 2007).

In addition to these two non-enzymatic antioxidant molecules, there are four key enzymes in this cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer, 1998; Li et al., 2010).

However, other antioxidant enzymes, apart from the AsA–GSH cycle, have important roles. These include catalase (CAT), guaiacol peroxidase (G-POX) and superoxide dismutase (SOD). SOD represents a first line of defense against ROS, being responsible for the scavenging of O_2^- by the dismutation of O_2^- to H_2O_2 (Blokhina et al., 2003). Produced H_2O_2 is then used by the AsA–GSH cycle or by CAT and G-POX, which contribute to regulating the intracellular level of H_2O_2. Al-induced unpaired activities in these enzymes have been identified for many species (Ghanati et al., 2005; Liu et al., 2008a,b; Tabaldi et al., 2009; Yin et al., 2010), although very little is known with respect to rye. Milla et al. (2002) reported that Al induced transcripts encoding a glutathione peroxidase and down-regulated APX in a rye cultivar after a short period exposure (up to 48 h).

We hypothesized here that long term exposure to Al would differentially affect the oxidant status of rye tolerant and sensitive genotypes, due to different internal/external detoxification systems, and may lead to plant morpho-physiological changes. In this comprehensive study, we analyzed the Al effect in ROS (H_2O_2), antioxidant enzymes from AsA–GSH cycle (APX, GR, MDHAR), non-enzymatic antioxidant contents (AsA, GSH) and other pathways (CAT, SOD, G-POX) using two genotypes with different Al sensitivities.

Material and methods

Plant material selection, growth and exposure to aluminium (Al)

Two rye genotypes, 'Dankowskie Zlote' (Polish cultivar) and 'Riodeva' (Spanish inbred line) were provided by University of Trás-os-Montes and Alto Douro (UTAD/Vila Real, Portugal). 'D. Zlote' was classified as an Al-tolerant cultivar (Pinto-Carnide and Guedes-Pinto, 1999, 2000; Niedziela et al., 2012) and 'Riodeva' as Al-sensitive (Gallego and Benito, 1997). Seeds were disinfected, rinsed in distilled water and germinated (in the dark at 24 °C) in Petri dishes. Germinated seeds were transferred for 4 days to a modified Hoagland's solution: KNO_3 303 mg L^{-1}; $Ca(NO_3)_2{\cdot}4H_2O$ 470 mg L^{-1}; $MgSO_4{\cdot}7H_2O$ 123 mg L^{-1}; $NH_4H_2PO_4$ 14.4 mg L^{-1}, and the following micronutrients: Fe-tartrate 2.65 mg L^{-1}; H_3BO_3 1.43 mg L^{-1}; $MnCl_2$ 0.905 mg L^{-1}; $CuSO_4{\cdot}5H_2O$ 0.04 mg L^{-1}; $ZnSO_4{\cdot}7H_2O$ 0.11 mg L^{-1}; H_3Mo 0.008 mg L^{-1} (RW medium).

For Al exposure, plants were transferred to the same nutritive solution containing 1.11 mM or 1.85 mM of $AlCl_3{\cdot}6H_2O$, corresponding to 0.26 mM and 0.42 mM of Al activity, respectively (Al activity was estimated by Geochem-EZ). Plants were grown for 3 weeks on nutritive solution containing Al. A control group was maintained in the nutritive solution without Al. Plants were kept in a culture room at 24 °C with a 16/8 h photoperiod. The nutrient solution was continuously aerated and renewed every 3 days. The pH was maintained at 4.0 throughout the assay (e.g. Silva et al., 2010).

Roots and leaves were measured 2 and 3 weeks after the beginning of Al exposure. Except for growth analysis (5 plants), at least 4 replicates were analyzed per assessed parameter. Each replicate consisted of leaf or root pools from 7 to 10 plants. The samples were frozen in liquid nitrogen and stored at −80 °C until analyzed.

Growth analysis

Plant (roots and leaves) and root length were measured. For fresh biomass determinations, plant (roots and leaves) and root fresh weight were used.

Concentration of H_2O_2, MDA and total soluble proteins

Hydrogen peroxide was quantified as described by Zhou et al. (2006). Samples were homogenized with trichloroacetic acid (TCA) and centrifuged. The supernatant was adjusted to pH 8.4 and the extract was divided into two aliquots. 8 μg of catalase (CAT; 1.11.1.6) were added just to one of the aliquots but to both aliquots were added 0.5 mL of colorimetric reagent. The reaction solution was incubated for 10 min at 30 °C and the absorbance was read at 505 nm. The amount of H_2O_2 was obtained against a H_2O_2 standard curve ($R^2 = 0.93$).

Lipid peroxidation was determined by quantifying malondialdehyde (MDA) content according to Santos et al. (2001).

Soluble proteins were determined by the Bradford (1976) method using the Total Protein Kit, Micro (Sigma–Aldrich, USA).

Enzymatic activity of AsA–GSH cycle enzymes

Enzyme extracts of L-ascorbate peroxidase (APX; 1.11.1.11) were prepared by grinding frozen samples with chilled potassium phosphate buffer (pH 7.5) containing ethylenediamine tetraacetic acid (EDTA) and ascorbic acid (AsA). The homogenate was centrifuged and the activity was assayed according to the method of Nakano and Asada (1981) by recording the decrease in AsA content at 290 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 0.5 mM AsA, 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.1 mL enzyme in a total volume of 1.5 mL, and the reaction was started with the addition of H_2O_2. The specific activity was calculated using the 2.8 mM^{-1} cm^{-1} molar extinction coefficient.

For glutathione reductase (GR; 1.6.4.2) activity, each frozen sample was homogenized with extraction buffer and then centrifuged. The reaction mixture containing 0.2 M potassium phosphate buffer (pH 7.5), 0.2 mM EDTA, 1.5 mM magnesium chloride, 0.25 mM oxidized glutathione (GSSG), 25 μM nicotinamide adenine dinucleotide phosphate (NADPH) and 50 μL enzyme extract (Loggini et al., 1999) was initiated by the addition of NADPH. The absorbance was