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Differential Physiological Responses of Portuguese Bread Wheat (*Triticum aestivum* L.) Genotypes under Aluminium Stress

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Abstract: The major limitation of cereal production in acidic soils is aluminium (Al) phytotoxicity which inhibits root growth. Recent evidence indicates that different genotypes within the same species have evolved different mechanisms to cope with this stress. With these facts in mind, root responses of two highly Al tolerant Portuguese bread wheat genotypes—Barbela 7/72/92 and Viloso mole—were investigated along with check genotype Anahuac (Al sensitive), using different physiological and histochemical assays. All the assays confirmed that Barbela 7/72/92 is much more tolerant to Al phytotoxicity than Viloso Mole. Our results demonstrate that the greater tolerance to Al phytotoxicity in Barbela 7/72/92 than in Viloso Mole relies on numerous factors, including higher levels of organic acid (OAs) efflux, particularly citrate efflux. This might be associated with the lower accumulation of Al in the root tips, restricting the Al-induced lipid peroxidation and the consequent plasma membrane integrity loss, thus allowing better root regrowth under Al stress conditions. Furthermore, the presence of root hairs in Barbela 7/72/92 might also help to circumvent Al toxicity by facilitating a more efficient uptake of water and nutrients, particularly under Al stress on acid soils. In conclusion, our findings confirmed that Portuguese bread wheat genotype Barbela 7/72/92 represents an alternative source of Al tolerance in bread wheat and could potentially be used to improve the wheat productivity in acidic soils.

Keywords: aluminium stress; bread wheat; phytotoxicity; physiological; histochemical assays

1. Introduction

Aluminium (Al) phytotoxicity has been recognized as one of the most detrimental factors for plant growth and productivity under acidic soil conditions which forms a large portion of the earth's land area [1]. Root tips have been identified as the primary target of Al phytotoxicity. In most of crops, Al phytotoxicity causes the disruption of cellular and molecular processes resulting in partial or complete inhibition of the main root growth and also restriction of lateral root as well as poor root hair development [2]. Prolonged Al exposure proceeds towards lower water and nutrient uptake, and subsequent shortening and stunting of the root system [3].

In both monocots and eudicots, the exclusion of Al through the efflux of organic acid anions from root apices has been demonstrated to be a main mechanism to overcome Al stress [4,5]. Phylogenetic closeness leads to the assumption that diverse genotypes within a species follow a similar suite of mechanisms. For instance, malate exudation is considered as the main mechanism of Al tolerance in bread wheat, whereas in other cereals such as rice, barley, oat, maize and sorghum, citrate exudation is mainly responsible for their Al tolerance. Nevertheless, some cereals such as rye and triticale, secrete both malate and citrate to cope with Al phytotoxicity [4,5]. Cumulative evidence shows that besides organic acids (OAs), other root traits such as susceptibility to Reactive Oxygen Species (ROS), callose, and rhizosheath size could also play a pivotal role in acidic soil where the roots of crop plants are exposed to toxic levels of Al [6,7]. The maintenance of longer root hairs is highly important for the efficient uptake of water and nutrients, including effective phosphorus acquisition in acid soils [8].

The majority of Portuguese soils are highly acidic in nature, which implies that plants experienced strong selection pressure of Al toxicity. Although substantial genetic variability has been reported for Al tolerance in Portuguese bread wheat germplasm, including landraces and obsolete cultivars [9,10], the mechanisms of Al tolerance are still far from being understood. In the past, the role of major genetic loci has been reported for Al tolerance in cereals. However, Al tolerance has only been partially transferred by marker-assisted selection which suggests the need for further identification of unexplored physiological variation associated with Al tolerance in elite genotypes [11–13]. Therefore, the aim of the present study was to investigate the mechanisms of Al tolerance in elite Portuguese bread wheat genotypes using different physiological and histochemical assays.

2. Experimental Section

2.1. Plant Material and Growth Conditions

Seeds of bread wheat genotypes used in the present study were obtained from plant germplasm unit, University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal. In order to obtain fresh seeds, each genotype was raised in pot and harvested manually. Prior to Al stress assays, seeds of all the genotypes were sterilized and kept on moist filter paper in petri plates for two days under dark conditions in a growth chamber. Germinated seeds with roots of about equal length were selected and placed on a plastic mesh floating on constantly aerated 2.5 L nutrient solution (0.4 mM $CaCl_2$, 0.65 mM KNO_3, 0.25 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $(NH_4)_2SO_4$ and 0.04 mM NH_4NO_3), and maintained in a controlled growth chamber, following previously described conditions [14]. The pH of nutritive solution was checked at 24 h intervals and adjusted to 4.0 with 1 M HCl or 0.1 M NaOH as needed. For all histological assays, a concentration of 74 µM Al in the form of $AlCl_3 \cdot 6H_2O$ was applied to the nutrient solution.

2.2. Root Regrowth Measurement

Root regrowth after exposure to Al stress was assessed by eriochrome cyanine R (Sigma-Aldrich, Madrid, Spain) staining [10]. Briefly, four days old seedlings grown in nutrient solution at pH 4.0 were moved to fresh nutrient solutions with control (no Al) or Al stress treatments (50 µM and 74 µM Al) for 24 h. Subsequently, roots were stained with 0.1% eriochrome cyanine R for 10 min with gentle agitation and the excess dye was removed by thoroughly washing with distilled water for 2–3 min. Thereafter, the seedlings were again returned to a continuously aerated fresh control nutrient solution (without Al) for 48 h. For root regrowth length, the white part of the root (unstained) which grew after Al treatment was measured using a mm ruler. Root regrowth from ten seedlings per genotype was measured for each treatment and three biological replicates were independently carried out. Data analysis was performed using factorial analysis of variance. The results were expressed as mean values of measurement with replication ± standard error.

2.3. Determination of Al Accumulation in Roots

To measure the Al uptake, a hematoxylin staining assay was performed [15]. Control and Al treated roots from ten seedlings of each genotype were rinsed twice in fresh distilled water for 30 min. Roots were stained with hematoxylin solution [0.2% w/v hematoxylin and 0.01% w/v KIO_3 (Sigma-Aldrich, Madrid, Spain) for 30 min. Subsequently, the roots were washed with distilled water for an additional 30 min and root staining was observed under a light microscope. Similarly, for morin staining, control and Al treated roots were washed for 10 min in 5 mM solution of ammonium acetate (Sigma-Aldrich, Spain) buffer (pH 5.0), at room temperature and stained with 100 µM morin (Sigma-Aldrich, Seelze, Germany) in the same buffer for 1 h [16]. Morin fluorescence was visualized on the root surface of control and Al treated plants from each genotype using an Olympus inverted microscope (Olympus CX31, Tokyo, Japan) connected with a fluorescence camera (Olympus U-LH50HG, Olympus Optical, Tokyo, Japan).

2.4. Assessment of Plasma Membrane Integrity Loss and Cell Lipidic Peroxidation

For cell viability loss or cell death evaluation, freshly harvested roots from control and Al treated samples were stained with Evans Blue aqueous solution (0.25% v/v Evans Blue, Sigma-Aldrich, Spain) for 10–15 min, at room temperature followed by washing with $CaCl_2$ solution (100 µM; pH 5.6) three times and visualized under a light microscope [17]. For assessment of cell lipid peroxidation, one-quarter of the root length from control and Al-treated plants were left for 20 min in Schiff's reagent (Merck, Darmstadt, Germany) at room temperature. Subsequently, roots were cleaned in a solution of 0.5% $K_2S_2O_5$ and 0.05 M HCl and visualized under a light microscope [18].

2.5. Callose Detection

To detect callose formation, roots of ten seedlings from each genotype, treated with and without Al, were excised and stained with a few drops of aniline blue [0.1% w/v; Aniline blue di-ammonium salt (Sigma-Aldrich, Germany) diluted in 1 mL Gly/NaOH buffer (pH 9.5) [19]. After 15 min, root samples were observed under a fluorescent microscope (Olympus CX31).

2.6. Extraction and Quantification of OAs from Intact Roots

To investigate the effect of Al stress on OAs secretion, OAs efflux from intact roots of bread wheat was followed as previously described [20]. Briefly, both dose–response and time-course assays were performed on four days old seedlings grown under aseptic conditions. Before collection of root exudates, seedlings in groups of ten were transferred to a 100 mL conical flask containing 40 mL fresh nutrient solution at pH 4.0 under a laminar flow cabinet where they stayed overnight. Subsequently, the nutrient solution was replaced with fresh nutrient solutions with (Al treatment) or without Al (control) for dose–response and time-course assay. Flasks were kept under constant shaking at 100 rpm.

For the dose–response assay, root exudates were collected after 24 h from the seedlings exposed to 0 (control), 50 and 74 µM Al. For time-course assays, root exudates were collected at specific time points (0, 2, 6, and 12 h) from the flasks containing seedlings exposed to 0 or 50 µM Al. Three root exudate samples were collected for each genotype per treatment and time point, and the experiment was repeated twice.

OAs were measured by high performance liquid chromatography (Shimadzu Corporation, Kyoto, Japan) connected to a Shimadzu SPD-10AV VP UV-vis detector set at 210 nm, an auto sampler SIL-10AF, a system control unit SCL-10A VP, equipped with software LC solution. Separation was achieved with a YHC-Pack ODS-A column (Ultrapure silica C18, 250 × 4.6 mm D. S-5 µm. 12 nm) (Waters, Milford, MA, USA) kept at 25–27 °C and attached to a guard-column. The mobile phase was 0.12% phosphoric acid and acetonitrile at pH 2.0–2.5 with a flow rate of 0.8 mL·min^{-1}. Six dilutions of each standard were used to make the standard curve. OAs in each sample were identified and quantified

by comparing their retention time and by use of external standards, respectively. All the standards were purchased from Sigma-Aldrich (Spain).

2.7. Statistical Analysis

Root regrowth and OAs exudates data from dose–response and time-course assays were analyzed using factorial ANOVA. Data were analyzed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Tukey's HSD (honest significance difference) test was applied to determine the significance of differences between the mean at $p \leq 0.05$. The standard error of each mean was calculated and represented as a vertical line on the graphs.

3. Results

3.1. Root Morphological Response to Al Exposure

The effect of Al toxicity was assessed on the roots of two Al tolerant Portuguese bread wheat genotypes—Barbela 7/72/92 and Viloso Mole—together with an Al sensitive genotype—Anahuac—as a reference control. These genotypes were characterised for Al tolerance based on root regrowth length using eriochrome cyanine R staining. Among the tested genotypes, the staining intensity was greater in the root apices (0–3 mm) of Anahuac which showed minimal root regrowth at a concentration of 50 µM Al and its root regrowth virtually stopped at 74 µM Al treatment. In contrast to what was expected, the Al tolerant genotypes showed high root regrowth basal to the stained region and root regrowth was inhibited less throughout the Al treatment range. Increasing the external Al concentration from 50 to 74 µM in the nutrient solution produced a significant inhibition in the mean root regrowth of Viloso Mole, whereas that of Barbela 7/72/92 was not significantly affected (Figure 1).

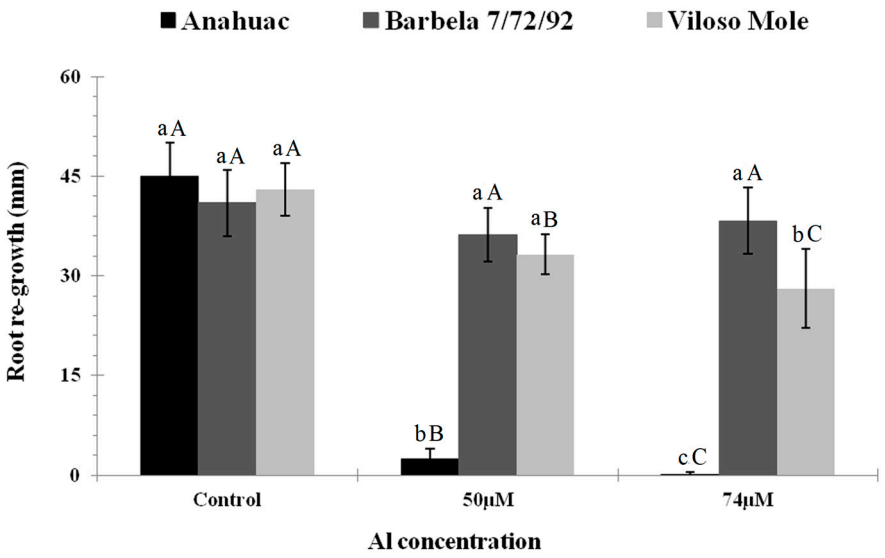

Figure 1. Root regrowth of three bread wheat genotypes (Barbela 7/72/92 and Viloso Mole, Al tolerant and Anahuac, Al sensitive) after exposure to different Al concentrations (0, 50 and 74 µM Al). Bars denote ± SE (n = 10). Means with different lowercase letters (a, b, c) represent significant differences among the genotypes in a particular Al treatment, whereas means with different capital letters (A, B, C) represent significant differences among the Al treatments with regard to a particular genotype at the $p \leq 0.05$, according to Tukey's test.

3.2. Al Accumulation

Al treated roots of Barbela 7/72/92 and Viloso Mole exhibited a typical hematoxylin staining pattern with minimal staining of the root tip, whereas Anahuac roots could be easily differentiated by

their intensive staining of a large root extension (Figure 2). Similarly, the roots of all the genotypes were compared for the intensity of their morin signal under control and Al stress conditions. Figure 2 illustrates that Al is distributed uniformly in all the root structures of the sensitive genotype Anahuac, whereas the Al tolerant genotypes—Barbela 7/72/92 and Viloso Mole—presented less Al accumulation and retained staining only in the root tip. By contrast, under control conditions, no hematoxylin or fluorescent dyes were present in the roots of all the studied genotypes.

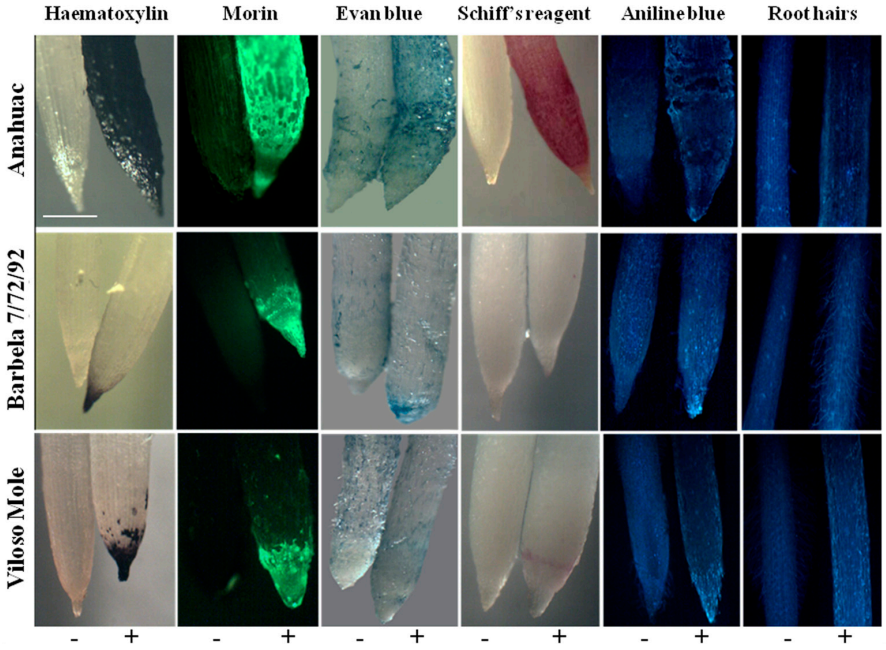

Figure 2. Physiological responses of the roots of Al sensitive (Anahuac) and Al-tolerant (Barbela 7/72/92 and Viloso Mole) bread wheat genotypes under control [0 μM Al (−)] and Al stress [74 μM Al (+)] conditions. Note: These genotypes were characterized for their response to Al stress by localization of root Al (hematoxylin), Al accumulation (Morin assay), plasma membrane integrity loss (Evans blue), lipid peroxidation (Schiff's reagent), callose formation (Aniline blue) and root hair development. Horizontal scale bar indicates 250 μm.

3.3. Plasma Membrane Integrity

The loss of plasma membrane integrity in roots under Al stress was visualized by the Evans blue staining technique. Roots grown in control conditions (without Al) exhibited almost no Evans blue staining and the root periphery was smooth, suggesting better plasma membrane integrity (Figure 2). Following the Al exposure, roots of the Al sensitive genotype Anahuac exhibited symptoms of a high degree of Al-induced injuries, confirmed by the strong Evan blue staining indicating cell ruptures and cell death in both the differentiation and elongation zones of the roots. Contrarily, the Al tolerant genotypes—Barbela 7/72/92 and Viloso Mole—did not present Evan blue staining, suggesting that the cell membranes were not affected by the Al treatment (Figure 2).

3.4. Lipid Peroxidation and Callose Formation under Al Accumulation

In plants, the Al-induced lipid peroxidation has been widely assessed by Schiff's reagent staining technique based on the intensity of the pink colour development. Under control conditions, no pink colour was detected in any of the genotypes. Upon Al exposure, the level of lipid peroxidation was remarkably higher in the roots of Anahuac, exhibiting roots with intense pink staining (Figure 2). On the other hand, the roots of Barbela 7/72/92 did not show Schiff's reagent staining, indicating that Al induced lipid peroxidation is almost negligible in this variety, whereas a moderate staining in the root apices of Viloso Mole was detected, indicating that it has a weaker capacity to avoid

Al-induced ROS damage to the plasma membrane. According to aniline blue staining, Al-treated roots of Barbela 7/72/92 and Viloso Mole showed higher callose accumulation upon Al exposure than the corresponding control plants, while, Anahuac presented a similar fluorescence signal in the control and Al-treated samples (Figure 2).

3.5. Root Hair Development

Bread wheat genotypes used in the present study clearly showed genotypic differences in their root hair development in response to Al exposure (Figure 2). Interestingly, the Al tolerant genotype Barbela 7/72/92 exhibited dense root hairs under Al stress conditions, indicating that Al stress has positive effects on root hair development, whereas an opposite effect was noticed in the genotype Viloso Mole, where the abundance of root hairs was inhibited by Al stress. On the other hand, there was no obvious relation between root hair development and Al treatment in the Al sensitive genotype Anahuac (Figure 2).

3.6. Pattern and Level of OAs in Root Exudates

Based on the root regrowth data and different histological results, it is evident that Barbela 7/72/92 exhibits a higher tolerance to Al phytotoxicity than Viloso Mole. Therefore, in order to understand the role of OAs exudation in Al tolerance in Portuguese bread wheat genotypes, the OAs exudation was evaluated using intact roots of all the three genotypes in response to the different doses of Al. Total OAs exudates contained a sum of citrate, malate, oxalate, tartrate and succinate. Interestingly, the highest OAs secretion was detected from the roots of Barbela 7/72/92, followed by Viloso Mole and Anahuac, under control and Al stress conditions (Figure 3). OAs secretion in Anahuac was extremely low under control and Al stress conditions, consistent with its highly sensitive nature to Al phytotoxicity. On the other hand, total OAs in the root exudates of Barbela 7/72/92 remained unchanged both under control and 50 µM Al treatment. Exposure to 74 µM Al was required for enhanced OAs exudation. Interestingly, a substantial induction (about 2.5 fold) in the level of total OAs secretion from the roots of Viloso Mole was noticed in response to Al treatment (50 µM Al), subsequently, an inhibition in the OAs secretion was observed with increasing external Al concentration (Figure 3).

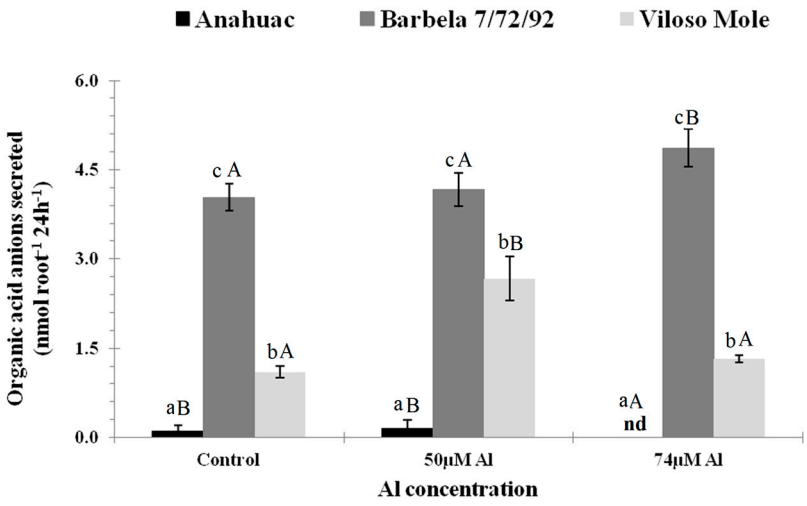

Figure 3. Effect of Al on the organic acids (OAs) secretion from the intact roots of bread wheat genotypes: Anahuac, Barbela 7/72/92 and Viloso Mole. Roots were exposed in nutrient solution (pH 4.0) containing 0, 50 and 74 µM Al for 24 h. Values are means of two replicates per time sample and treatment. Bar denotes ± SE (n = 6) and nd stands for non-detectable. Means with different lowercase letters (a, b, c) represent significant differences among the genotypes in a particular Al treatment, whereas means with different capital letters (A, B) represent significant differences among the Al treatments with regard to a particular genotype at the $p \leq 0.05$, according to Tukey's test.

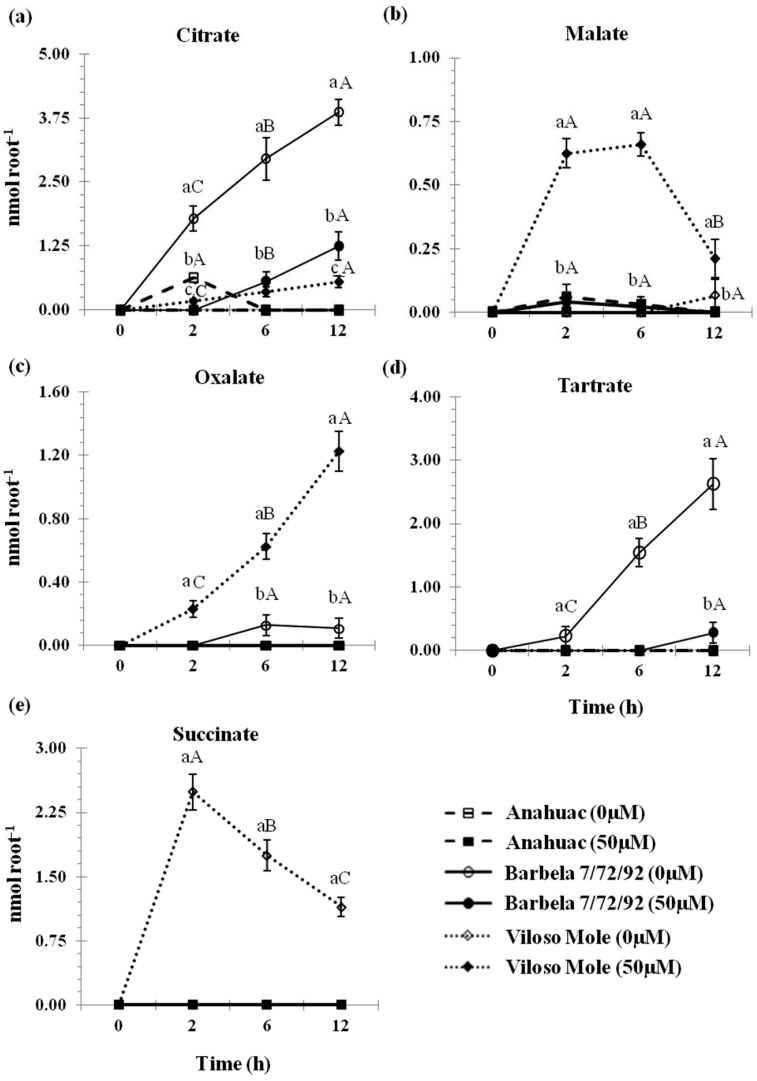

Figure 4. Pattern of OAs secretion from the intact roots of bread wheat genotypes; Anahuac, Barbela 7/72/92 and Viloso Mole. Roots were exposed to control (0 µM Al) or Al stress (50 µM Al). Root exudates were collected at specific intervals (0, 2, 6 and 12 h) for the determination of individual OAs. Vertical lines on the graph indicate mean ± SE (n = 6). (**a**) Citrate; (**b**) Malate; (**c**) Oxalate; (**d**) Tartrate; (**e**) Succinate. Means with different lowercase letters (a, b, c) represent significant differences among the treatments at specific time point whereas means with different capital letters (A, B, C) represent significant differences among the time point intervals at the $p \leq 0.05$, according to Tukey's test.

In conjunction with the dose–response assay, we performed a time-course experiment to investigate the pattern and profile of OAs secretion from the intact roots of the studied genotypes. The time-course assay revealed that secretion of citrate and tartrate from the Barbela 7/72/92 roots increased with the duration of the treatment in the absence or presence of Al (Figure 4a,d). Under control conditions, roots of the Barbela 7/72/92 exhibited a constitutively high exudation of citrate over the assay period (2–12 h), whereas under Al exposure, detectable citrate exudation was found only 6 h after the start of Al exposure; the citrate concentration increased to 1.25 nmol root^{-1} by 12 h exposure. Similarly, a constitutively high exudation of tartrate from Barbela 7/72/92 roots was observed under control conditions (0.2–2.6 nmol root^{-1}), while tartrate was not detected until 6 h after Al was imposed. Oxalate efflux from Barbela 7/72/92 roots was also noticed at 6 h and constitutively maintained thereafter under control conditions, but it was not detected under Al stress conditions

(Figure 4c). Surprisingly, the level of malate exudation was extremely low, and only detectable in root exudates after 2 h under Al treatment.

On the other hand, the pattern and level of OAs exudation by Viloso Mole roots was different (Figure 4). The Al induced citrate exudation from the roots of Viloso Mole was detected at a slightly lower level in the first 2 h and began to increase slowly to 0.2–0.6 nmol root^{-1} over the assay period (2–12 h), whereas the oxalate exudation was significantly higher in the Al exposed roots and its up-regulation was more pronounced with the increase in the exposure to Al stress (0.2–1.3 nmol root^{-1} in 2–12 h). Interestingly, no exudation of citrate and oxalate was detectable when root exudates were collected from the Viloso Mole grown in the absence of Al (Figure 4a,c). Contrarily, Al treatment triggered malate exudation from the roots of Viloso Mole and reached at maximum level within 6 h followed by a sharp decline (Figure 4b). No malate exudation was detected in the root exudates of Viloso Mole collected from the control condition (0 μM Al) except at 12 h. No Al-induced secretion of succinate from the roots of Viloso Mole was detected, while succinate was detected in controls after 2 h, followed by a continuous decline within the following hours (Figure 4e).

In the Al sensitive genotype, Anahuac, pattern and level of organic acids exudation was too small to be detectable, except after 2 h of Al exposure where 0.6 nmol root^{-1} of citrate was found in the reaction medium (Figure 4a). These results indicate that tolerant lines were able to exudate higher amounts of OAs compared to Al-sensitive line Anahuac.

4. Discussion

Considerable inter- and intra-specific variation for Al tolerance has been reported in different plant species, including members of the *Poaceae* family [7]. Nevertheless, numerous aspects of Al toxicity are still to be clarified, especially, the understanding of physiological mechanisms responsible for Al tolerance in genotypes within a species exhibiting similar or different responses to Al phytotoxicity. In the present work, we illustrated how Portuguese bread wheat genotypes, especially landrace Barbela, cope with Al phytotoxicity by using different physiological and histochemical assays.

Eriochrome cyanine R assay clearly re-confirmed that Barbela 7/72/92 and Viloso Mole genotypes are tolerant to Al phytotoxicity. However, the root regrowth of Viloso Mole was inhibited at a high external Al concentration that did not affect the root regrowth of Barbela 7/72/92, indicating that Al tolerance of Barbela 7/72/9 is markedly superior to that of Viloso Mole (Figure 1). Furthermore, hematoxylin and morin staining assays also revealed that Barbela 7/72/92 and Viloso Mole accumulate less Al in their root tips when compared with Anahuac (Figure 2). However, the presence of morin stain in the root tips of Al tolerant Portuguese bread wheat genotypes—Barbela 7/72/92 and Viloso Mole—point out that both genotypes may somehow allocate Al in a better way in their roots without suffering further damages. Evans blue and Schiff's staining's assays indicated that Al induces membrane damage which might be due to the mechanical disruption of the cells, particularly in the case of the Al sensitive genotype Anahuac (Figure 2). A paramount question that remains to be answered is whether this is due to necrosis or apoptosis, but there is still also the possibility that Al may cause a minor alteration of the plasma membrane permeability [21]. Nonetheless, in the case of Al sensitive genotypes, it might be possible that a high level of root injury allows the direct entering of Al into the roots [22].

It has been speculated that in acidic soils, when the growing roots enter into a region containing toxic levels of Al, a mechanism could be activated to destroy the root tip cells. This breaks the root apical dominance, and thus induces the formation of secondary roots which may allow the exploration of other soil portions with lower Al levels [23]. Such a change in root tip cell patterning has been previously demonstrated in maize [24]. Similarly, the development of root hairs close to the tip has been also observed in a Brazilian bread wheat cultivar Fronteira, where a strong correlation was detected between Al tolerance and rhizosheaths size in acid soils [6]. Recently, the concentration of chlorogenic acid, a key factor in the induction of root hair development, was found to increase two-fold in response to Al supply in roots of the Al hyperresistant signalgrass (*B. decumbens*) [25,26]. It is noteworthy that

Al exposure appeared to stimulate the root hairs development in Barbela 7/72/92 (Figure 2) which can be explained by an alleviation of proton toxicity by Al under low pH conditions and may contribute to both more efficient uptake of essential nutrients, such as P, and a controlled intake of Al via the symplastic pathway in the root zone where the development of a suberized hypodermis may restrict the apoplastic Al transport [26,27].

During the past quarter of a century, it has been widely accepted that Al-chelating OA anions play a crucial role in the detoxification of Al in different plant species, including cereals [5,28]. The Al-detoxifying capacity of OAs depends on the stability constants of the Al-organic acid complexes. The different Al-detoxifying capacity of organic acids results from their structural configurations (relative positions of OH/COOH groups on the main carbon chain) [29]. The main organic acids known to effectively detoxify Al are citrate, oxalate, and tartrate followed by malate, malonate and lactate [30]. The most effective detoxifying acids have either two pairs of OH/COOH attached to two adjacent carbons (citric and tartaric) or two COOHs directly connected (oxalic), forming stable 5- or 6-bond ring structures with Al [31].

The results of the present study suggest that the Al tolerance of Barbela 7/72/92 is clearly outstanding compared to Viloso Mole, probably due to the difference in both level as well as kind of OAs secreted by its roots (Figures 3 and 4). In the past, most studies advocated that Al-activated malate efflux from root apices is responsible for Al tolerance in wheat, even though some Al tolerant Brazilian bread wheat genotypes, particularly cultivar Carazinho, presented a constitutive citrate efflux from their root apex and was identified as an alternate and rare mechanism to cope with Al phytotoxicity [32–34]. More recently, our group has also established that, at the molecular level, the mechanism of citrate efflux also operates in Al tolerant Portuguese bread wheat genotypes [35]. In our previous study, we found that Al-tolerant bread wheat genotypes from Portugal, including Barbela 7/72/92 and Viloso Mole, also contained Sukkula-like transposon in their promoter of *TaMATE1-4B*, the gene encoding the citrate transporter in wheat which has been shown to be responsible for the constitutively higher expression of *TaMATE1* in bread wheat [35,36]. The present results suggest that the molecular mechanism of Al exclusion via root OAs exudation is much more complex in wheat; while the most Al tolerant variety—Barbela 7/72/92—displays a constitutively strong citrate exudation, the moderately Al tolerant Viloso Mole secretes citrate only in response to Al treatment (Figure 4). The results of the time-course experiment indicate that besides Sukkula-like transposon in the promoter of *TaMATE1-4B* in Barbela 7/72/92, there is a possibility of the presence of additional either cis and/or trans elements, as yet unidentified, that might play an important role in the constitutive exudation of high levels of citrate efflux from the Barbela 7/72/92 roots. Similar to the case in wheat, incomplete transfer of Al tolerance was observed in sorghum [13], indicating the occurrence of strong genetic background effects related to Alt_{SB} locus which harbour *SbMATE* in this cereal [37].

Al-activated malate efflux is a common characteristic in most of Al-resistant genotypes of wheat [34]. In accordance with previous studies, Al treatment also triggered malate exudation from the roots of Viloso Mole followed by continuous decline (Figure 4b). It is tentative to speculate that a reduction in Al-activated malate efflux can be a result of reabsorption of OAs by roots or enzymatic transformation occurring in the roots of Viloso Mole, because malate accumulation in the apoplastic compartment and a reverse reaction (an oxidation of malate by the cell wall associated NAD-dependent malate dehydrogenase), could play an important role [38]. In addition, it is also possible that at least in Viloso Mole, peroxidases may play a role in influencing the OAs efflux from the roots under Al stress. Nevertheless, the mechanisms by which Viloso Mole achieves a preferential accumulation of oxalate and the reduction in malate after 6 h under Al stress remains to be established.

In our previous study, we analyzed the promoter of *TaALMT1*, the gene controlling malate efflux in bread wheat [39], in the selected Portuguese bread wheat genotypes. We found that both Al tolerant genotypes—Barbela 7/72/92 and Viloso Mole—contain the type VI promoter which is associated with the higher basal expression of TaALMT1 transcript in wheat, whereas the genotype Anahuac with the type I promoter showed the lowest level of expression and Al tolerance [35,40]. It is noteworthy that

both Al tolerant Portuguese bread wheat genotypes have the type VI promoter, but the mechanism of Al responsive high malate efflux only operates in Viloso Mole (Figure 4). Analogous to our results, some Japanese lines with the type VI promoter were found to be significantly more sensitive to Al than the non-Japanese lines with a type VI pattern despite expressing ALMT1 to similar levels [40].

5. Conclusions

This study clearly shows that within a species, different genotypes can evolve specific mechanisms to cope with Al phytotoxicity. For instance, the mechanism of constitutive efflux of citrate at a higher level seems to play a pivotal role in the Al tolerance of Barbela 7/72/92, whereas the Al responsive exudation of malate, citrate and oxalate appeared as important factors for Al tolerance of Viloso Mole. The higher level of tolerance towards Al phytotoxicity in Barbela 7/72/9 over Viloso Mole might be possible due to the high amount of OAs secretion, especially citrate exudation, that can avoid Al toxicity more efficiently than the malate and oxalate efflux, because citrate has the highest Al chelating activity, followed by oxalate and malate. Additionally, root hairs stimulation in response to Al treatment in Barbela 7/7292 reinforces the existence of multiple physiological mechanisms present in this genotype to cope with Al phytotoxicity. Finally, our study demonstrates that Barbela 7/72/92 is a useful experimental material to further elucidate the complex mechanisms of Al phytotoxicity at the molecular level and could be utilized to develop improved cultivars in bread wheat for acidic regions.

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