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RESEARCH ARTICLE

# Evidences of organic acids exudation in aluminium stress responses of two Madeiran wheat (*Triticum aestivum* L.) landraces

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**Abstract** Two wheat (*Triticum aestivum* L.) Madeiran landraces were subjected to 100 μM and 200 μM of aluminium (Al) in hydroponic culture, assessing the organic acid exudation role in plant's responses to this metal. Samples of initial landrace populations ($F_0$), $F_3$ and haplodiploid lines (DH) were evaluated using standard tests: eriochrome cyanine R staining, root elongation and callose accumulation in roots. Root exudates were obtained to determine if the accumulation of malic and citric acids in hydroponic medium was a response to Al exposure. Additionally, the presence of *ALMT1* gene was determined using five microsatellite markers. Standard tests confirmed that ISOP 76 was Al tolerant and ISOP 239, Al susceptible. ISOP 76, in the presence of 100 μM Al, exuded substantially more malic acid (12.87 to 43.33 mg/L), than ISOP 239 (3.65 to 7.72 mg/L). The levels of both organic acid exudation were substantially lower in ISOP 239 than in the ISOP 76. In the presence of 200 μM Al, ISOP 76 $F_0$ shows a higher root elongation ratio (better tolerates Al), but the DH line was the one that exuded higher content of malic acid. Different gene alleles and promoters were detected in both landraces. Molecular differences could explain the observed dissimilarity in organic acid exudation response to Al stress.

**Keywords** Haplodiploid lines · Aluminium tolerance · Eriochrome cyanine R staining · Organic acids exudation · *ALMT1* gene · Microsatellite markers

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## Introduction

Wheat is the third most harvested cereal worldwide (Shewry 2009). In many parts of the world, wheat is cultivated on acidic soils that affect 40% of arable land globally (Yang et al. 2012). Soil acidity is one of the main constrains of high wheat yield, with low pH (below 4.5) making aluminium cationic form to become bioavailable to plant roots, affecting their growth and capacity to uptake water and nutrients (Ma et al. 2001). Crop productivity due to aluminium (Al) toxicity can be reduced by 60% (Haug 1984). Therefore, screening of wheat genetic resources

under such edaphic conditions and understanding mechanisms of aluminium tolerance are vital. Different physiological tests have been developed to assess plants' Al tolerance, including measurements of root elongation, staining with eriochrome cyanide R and quantification of callose accumulation in root tips (Pinheiro de Carvalho et al. 2003, dos Santos et al. 2005).

The Archipelago of Madeira is an outmost Portuguese Region in the Atlantic Ocean, with volcanic origin where geochemical processes have contributed to the increase of soil acidity (Madeira et al. 1994, Pinheiro de Carvalho et al. 2003, Ganança et al. 2007). Wheat was the first crop introduced to Madeira by early settlers who arrived to the islands almost 600 years ago (Vieira 1988). Since then, the crop has developed a wide range of diversity (dos Santos et al. 2012) including landraces adapted to soil acidity and Al bioavailability (Pinheiro de Carvalho et al. 2003, Ganança et al. 2007).

Aniol (1990) hypothesized that wheat can develop several strategies to cope with Al stress. Importance of exclusion mechanisms in response to elevated Al in growing medium was emphasised by Rout et al. (2001). Generally, roots exude organic acids such as malic and citric acids, that are able to precipitate aluminium cations in soil (Ma et al. 2001; Ryan et al. 2009). Ryan et al. (2011) postulated the involvement of the protein *TaALMT1* (*Triticum aestivum* Aluminium-Activated Malate Transporter) in exudation of malic acid by forming an ion channel across the plasma membrane to the apoplast. The *ALMT1* gene is required to express this transporter protein and its structure includes a promoter, six exons and five introns (Raman et al. 2008). Several molecular markers are used to detect the *ALMT1* gene. The most common ones are SSR (Single Sequence Repeats) markers that encode small sequences located in the gene's introns and the CAPS (Cleavage Amplification Polymorphism Sequence) marker that is in an exon (Raman et al. 2008). Enzymatic digestion of CAPS amplified product allows to distinguish between two alleles, *ALMT1*-1 and *ALMT1*-2 (Jones et al. 2009). Finally, there are two other markers for the gene promoter, LPF (Long Promoter Fragment) and SPF (Short Promoter Fragment), which indicates what type of promoter is present in plant tissues. Promoter type I allows the lowest expression of the *ALMT1* gene, while promoters type VI, featuring triplicated sequence repeats, show the highest gene expression (Sasaki et al. 2006).

The overall goal of this study was to elucidate the early detection role of organic acids exudation in different Al tolerance level of two Madeira wheat landraces, ISOP 76 and ISOP 239. This was achieved by the comparison of landraces responses to Al exposure in nutrient solution and by correlation between Al tolerance, exudation of organic acids by the roots, and *ALTM1* gene presence and polymorphism.

## Materials and methods

### Plant material

Two landraces of wheat, obtained from the ISOPlexis Gene Bank at the University of Madeira, *Serra* represented by ISOP 76 belonging to *Triticum aestivum* L. var. *erythrospermum* (Körn.) Mansf. and *Rapado branco*, ISOP 239, *Triticum aestivum* L. var. *milturum* (Alef.) Mansf. (dos Santos et al. 2009), were used. The initial populations ($F_0$), $F_3$ selected generations, and the haplodiploid lines (DH), obtained from $F_1$ plants of each landrace were compared. $F_3$ generations were selected for Al tolerance (ISOP 76) or susceptibility (ISOP 239) from the previously screened plants using the eriochrome cyanine R staining (Pinheiro de Carvalho et al. 2003). DH lines were developed at the Institute of Plant Breeding and Acclimatization, Radzikow, Poland, using the $F_1$ generation plants. This process started with the tillers harvest at the stage of the uninucleated microspore to two-nucleated pollen grain. After cold pre-treatment, the spikes were surface sterilised and washed in sterile water. Anthers were removed from the spike and put on solidified medium. They were subsequently cultured in Petri dishes in the dark at 26 ℃. Developing embryos and callus were transferred to regeneration media. Small green plantlets were transferred to the Erlenmeyer flasks containing MS medium (Murashige and Skoog 1962) for rooting. Plantlets were then planted into pots and grown in a greenhouse until maturity.

The Brazilian variety Maringá was also used as a standard for aluminium tolerance (Basu et al. 1994). Additionally, Chinese Spring, a moderately tolerant

variety, was used as a standard in the detection of SSRs molecular markers (Basu et al. 1994; Ma et al. 2006).

### Seed germination, screening and exudates collection

Random samples of $F_0$, $F_3$ and DH lines of each landrace were analysed. Eighty seeds without any symptoms of fungi contaminations were selected from each group. The seeds were surface sterilized for 30 min. with 0.1% Benlate (fungicide) solution and then transferred for 20 min to 10% sodium hypochlorite and for 3 min to 70% ethanol. Subsequently, the seeds were germinated for 3 days in Petri dishes containing solid MS medium to check for fungi contaminations. Thirty six non-contaminated seedlings were transferred to sterile hermetic vessels (3 per vessel) containing 30 mL of nutrient solution, pH 4.3. This solution contained 2.0 M calcium nitrate, 0.6 M magnesium nitrate, 0.6 M ammonia nitrate, 0.2 M dipotassium phosphate, 0.2 M potassium sulphate and 0.8 M potassium nitrate (Pinheiro de Carvalho et al. 2003). The seedlings were maintained in the gently stirred vessels for 48 h under a photoperiod of 9 h of light and 15 h of dark. Previously, Ganança et al. (2007) showed that 48 h incubation allows seedlings to overcome possible stressfully conditions, resulting from its transference from solid to liquid growth medium. Afterwards, the plantlets were divided into three subsamples and transferred to experimental solutions, pH 4.0, containing 1.0 mM calcium nitrate, 0.3 mM magnesium nitrate and 3.3 mM ammonia nitrate, added with 0 mM (control), 0.1 mM (aluminium I) or 0.2 mM (aluminium II) of aluminium chloride (Pinheiro de Carvalho et al. 2003), and grew for 9 days. During experimental period, the pH of growth solution has controlled and kept between 4.0 and 4.5 by addition of 1.0 N HCl or NaOH. The length of experiment was determined by the vessels volume. During this period seedlings were able to grow and exude enough organic acids to be quantified by the enzymatic kits. At the end of the experiment, both plants and the corresponding nutrient solutions were collected for further analysis.

### Screening tests

Length of the main root was manually measured, using a calibre digital pachymeter 150 mm, at the end of the experiment. The seedlings were tested for Al tolerance or sensitivity, using the eriochrome cyanine R staining method and the measurement of callose accumulation in root tips. Roots of 30 plants of each experimental variant of $F_0$, $F_3$ and DH lines were stained with the dye as described by Pinheiro de Carvalho et al. (2003) and the number of plants with irreversibly damaged root apex was computed.

Root apices harvested from a different set of 30 plants of each experimental variant of $F_0$, $F_3$ and DH lines were used to quantify callose accumulation as described by dos Santos et al. (2005).

### Exudates analysis and organic acids quantification

At the end of the assay, growth solutions containing exudates were recovered and screened for microbial contaminations through the incubation of a drop of solution onto universal growth medium. Contaminated growth solutions were discarded. To non-contaminated growth solution, was added 0.05% of sodium azide (v/w) and the solutions were stored at −20 ℃. Subsequently, growth solutions were lyophilized and the final pellet was suspended in 2 mL of ultrapure water. Lyophilisation at −50 ℃ allows to concentrate the organic acid avoiding its destruction by heating.

The enzymatic kits L-Malic Acid, Megazyme (Megazyme International Ireland 2012) and Citric Acid, Megazyme (Megazyme International Ireland 2014) were used to quantify malic and citric acids exuded by roots into growth solution.

### DNA extraction and molecular markers detection

Nine days old leaves of seedlings were collected and deeply frozen, with liquid nitrogen and stored at −20 ℃ for DNA extraction. DNA extraction from the tissues of landraces and standards was made according to Chao and Somers (2015). Molecular markers detection was performed using the iProof High-Fidelity Master Mix kit (Bio-Rad). The markers amplifications were performed in a thermocycler PxE 0.2, Thermohybaid using the programmes described in Table 1.

**Table 1** Molecular markers and PCR programmes used in the detection of ALMT1 gene during the screening of sequences of $F_0$ and $F_3$ generations of wheat landraces and the DH lines

| Markers | Primer's sequences | PCR programmes |
|---|---|---|
| CAPS | F: 5′-GGA ATG GAA TTC AAC TGC TTT GGC G-3′<br>R: 5′-TCC TCA GTG GCC TTC GAA TTA AGG-3′ | **1** × (98 °C 1′), **40** × (98 °C 10″–67.5 °C 30″–72 °C 30″), **1** × (72 °C 10′) |
| SSR3a | F: 5′-CTC GTC ACA AAA GCC ACT CA-3′<br>R: 5′-GAC GCA ATC AAG GGG AAT AA-3′ | **1** × (98 °C 1′), **40** × (98 °C 10″–56.5 °C 30″–72 °C 30″), **1** × (72 °C 10′) |
| SSR3b | F: 5′-ATG CCA TTT CTT CTG TAC TGA CA-3′<br>R: 5′-AAA GAG TCC TCA GTG GCC TTC GAA-3′ | **1** × (98 °C 1′), **35** × (98 °C 10″–61.5 °C 30″–72 °C 30″), **1** × (72 °C 10′) |
| SPF | F: 5′-GCT CCT ACC ACT ATG GTT GCG-3′<br>R: 5′-CCA GGC CGA CTT TGA GCG AG-3′ | **1** × (98 °C 1′), **40** × (98 °C 10″–64 °C 30″–72 °C 30″), **1** × (72 °C 10′) |
| LPF | F: 5′-CCT GGT TTT CTT GAT GGG GGC ACA-3′<br>R: 5′-TGC CCA CCA TCT CGC CGT CGC TCT CTC T-3′ | **1** × (98 °C 1′), **35** × (98 °C 10″–70.5 °C 30″–72 °C 30″), **1** × (72 °C 10′) |

Amplification products were separated and detected on 1.4% (w/v) agarose gels. Additional enzyme restriction of PCR products to identify the CAPS alleles was performed, using enzyme XmnI (New England BioLabs) and separated on a 2.2% (w/v) agarose gel.

### Data treatment

Experiments were performed twice, using three replicates per experimental variant. Averages, standard deviations and graphics were made using Microsoft Excel, while statistical tests including One-Way and Two-Way ANOVA and Pearson correlations were determined using the software Statistical Package for the Social Sciences (SPSS ver. 22).

## Results

### Eriochrome cyanine R test

Root staining with eriochrome cyanide R allows to differentiate tolerant from susceptible plants by coloration of the root apex (Pinheiro de Carvalho et al. 2003). All individuals of ISOP 76 appeared to be tolerant (Fig. 1). The $F_0$ generation of ISOP 239 had more than 55% of susceptible individuals and this susceptibility increased with the selection (Fig. 1). In the Maringá population, 75% of plants were tolerant to aluminium, proving to be a good tolerance standard (Fig. 1).

### Root length

The elongation ratio derived by calculating the ratio of root length between experimental and control variants indexes to their Al tolerance. This ratio (data not showed) confirms that Maringá have smaller root growth, but exhibited the best root performance, regarding Al increasement in the growing medium (Fig. 2). The root measurements revealed that the $F_0$, $F_3$ and DH generations of ISOP 76, in all experimental variants, exhibited higher root length than the standard Maringá (Fig. 2). The Al bioavailability affects ISOP 76 root elongation in an increasing rate, between 26 and 36% in the presence of 200 μM Al. At the same time, $F_0$ of ISOP 239 displayed a higher root length in the absence of Al and at 100 μM of Al when compared to Maringá, but no significant differences were observed in the presence of 200 μM of Al. These differences become less evident in $F_3$ and the DH lines of ISOP 239 (Fig. 2). So, ISOP 239 suffers the highest impact of Al bioavailability in root elongation, between 63 ($F_0$) and 47% (DH) in the presence of 200 μM Al. Both landraces shown better root elongation in the $F_0$ and in presence 100 μM Al. However, results of root elongation and root elongation ratio are higher in the ISOP 76 than in ISOP 239, and even comparable with Maringá root elongation ratio.

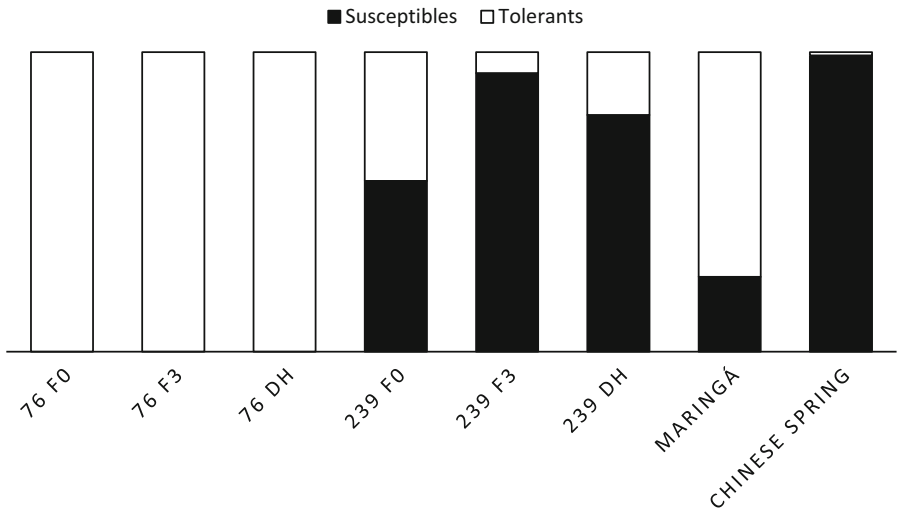


**Fig. 1** Evaluation of tolerance of the wheat lines from Madeira Island to 200 µM $AlCl_3$ in nutrient solution using the eriochrome cyanine R staining method. Three-day-old seedlings were exposed to aluminium for 6 days and subsequently placed in the dye. Root apex of the tolerant plants remained white, while susceptible plants exhibited orange root apex

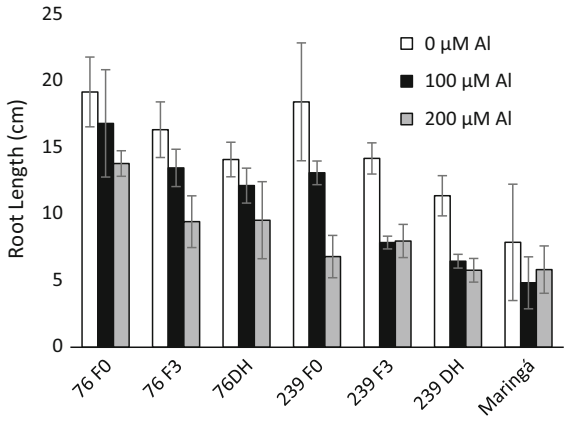


**Fig. 2** Root length of wheat lines, from Madeira Island, exposed for 9 days to 100 or 200 µM aluminium in nutrient solution. Data represents the mean ± SD

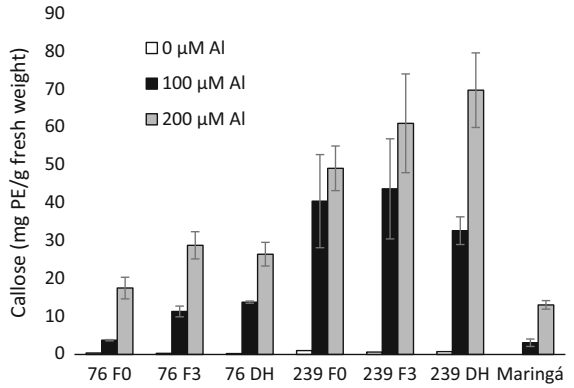


**Fig. 3** Callose content in roots tips of wheat lines, from Madeira Island, exposed for 24 h to 100 or 200 µM aluminium in nutrient solution. Data represents the mean ± SD of triplicate extractions

## Callose

Callose accumulation is a precise quantitative test displaying tissue's response to the presence of aluminium (Fig. 3). The quantity of callose and the ratio between control and experimental conditions (data not showed) indicated that the increase in Al concentration was positively correlated with the callose accumulation in every landrace's generation or line tested (Table 3). Accumulation of callose by the ISOP 76 lines closely resemble the pattern observed in the standard Maringá (Fig. 3), although overpasses its callose amount in 1.0 and 1.6 folds in the presence of 100 and 200 µM of Al, respectively. The comparison between $F_0$ of ISOP 239 and Maringá shows that callose accumulation has higher 19.5 and 4.1 folds in the presence of 100 and 200 µM of Al, respectively. Callose accumulation in the roots of $F_3$ and DH line of both landraces increases, although it is almost threefolds lower in the roots of the ISOP 76.

## Quantification of organic acids in exudates

In the absence of Al, ISOP 76 roots exuded lower quantity of malate when compared to roots grown in the presence of Al (Fig. 4a). The $F_0$ generation of ISOP 76 exuded four times more malic acid than Maringá, in the presence of 200 µM Al. In the presence of 100 µM Al, the highest malate exudation

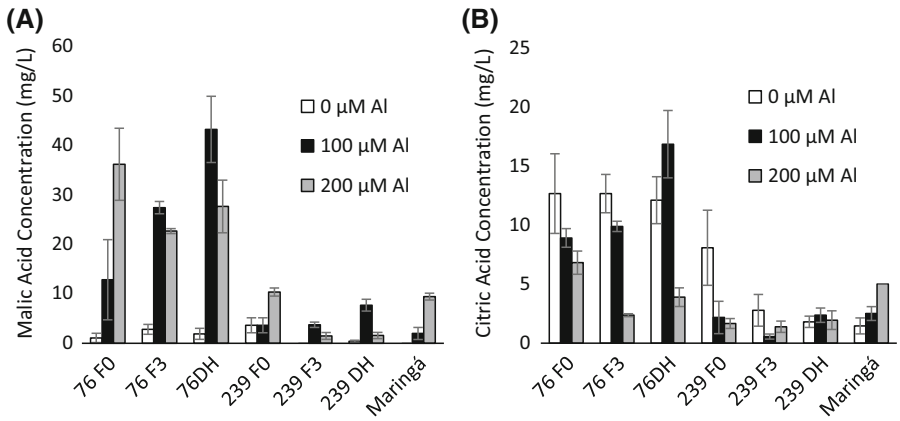


**Fig. 4** Malic acid (**a**) and Citric acid (**b**) exuded by wheat lines, from Madeira Island, exposed for 9 days to 100 or 200 µM aluminum in nutrient solution. Data represents the mean ± SD

was reported in the DH line followed by $F_3$ and $F_0$ while a slightly different trend ($F_0 < DH < F_3$) was observed in the presence of 200 µM Al (Fig. 4a). At the same time, malic acid exudation appeared to be unspecific or non-significant in ISOP 239 and absent in Maringá. The exudation of citric acid, in the absence of Al, was the highest in the ISOP 76, intermediate in the ISOP 239 and absent in Maringá (Fig. 4b). The citric acid exudation decreases, with the presence of Al, in all landraces lines, at exception of 76 DH line and Maringá in the presence of 200 µM Al,

A comprehensive summary of the tolerance tests and organic acid exudation for $F_0$ generations is provided (Table 2). ISOP 76, in the presence of 100 µM Al, had the highest number of tolerant individuals and malic acid exudation, whereas Maringá shows the biggest root length. In the same experimental variant, ISOP 239 had the highest callose deposition. The two-way ANOVA (Table 3) shows significant influence ($p > 0.01$), of both generations and experimental variants (presence of Al), in the variation of root length or callose accumulation (tolerance or sensitivity) or in the malic or citric exudation (mitigation strategy). The values of Z coefficient shows that presence of Al in growth medium was a key factor in the observed results, namely the root elongation, callose elongation and citric acid exudation. However, in the variation of malic acid exudation, the generation factor had more weight. The one way ANOVA (data not shown) demonstrated that Al presence had a significant influence in the variation of malic acid exudation in the $F_0$, $F_3$ and DH of the ISOP 76. In the case of ISOP 239, this influence was observed only in the $F_3$ and DH lines. Statistical bivariate analysis (Tables 4, 5), using Pearson correlation coefficient shows that, malic acid exudation appears negatively correlated ($p > 0.05$) with root elongation and positively correlated with callose accumulation ($p > 0.05$ and $p > 0.01$) in $F_0$ e $F_3$ of ISOP76. At the same time, in the ISOP 239 no significant correlations of malic acid with the other parameters was observed, and root elongation is the unique parameter that had

**Table 2** Summary of screening tests results assessing performance of Maringá (benchmark) and $F_0$ generations of the Madeiran landraces in the presence of 100 µM $AlCl_3$

| Tests | Maringá | ISOP 76 | ISOP 239 |
|---|---|---|---|
| Eriochrome cyanide R | ± | + | − |
| Root length | + | ± | − |
| Callose deposition | − | ± | + |
| Malate exudation | − | + | − |
| Citrate exudation | + | + | − |

+ High pronunciation; ± medium pronunciation; − low pronunciation

**Table 3** Statistical analyses of the screening tests using *Two-way* ANOVA

| Source | Root length | | Callose | | Malic acid | | Citric acid | |
|---|---|---|---|---|---|---|---|---|
| | Z | Sig. | Z | Sig. | Z | Sig. | Z | Sig. |
| Generation | 54.642* | 0.000 | 79.585* | 0.000 | 19.550* | 0.000 | 8.121* | 0.001 |
| Experimental variant | 116.870* | 0.000 | 470.610* | 0.000 | 9.559* | 0.000 | 11.559* | 0.000 |
| Generation × experimental variant | 4.942* | 0.000 | 27.725* | 0.000 | 3.685* | 0.001 | 2.626** | 0.011 |

*The correlation is significant at the 0.01 level

**The correlation is significant at the 0.05 level

**Table 4** Pearson correlations (PC) between screening tests for ISOP 76: root length (RL), malic acid exudation (MA), citric acid exudation (CA) and callose accumulation (C)

| | $F_0$ | | | | $F_3$ | | | | DH | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RL | MA | CA | C | RL | MA | CA | C | RL | MA | CA | C |
| RL | | | | | | | | | | | | |
| PC | 1.000 | −0.722* | 0.691* | 0.677* | 1.000 | −0.747* | 0.874** | −0.895** | 1.000 | −0.157 | 0.534 | −0.694* |
| Sig. | – | 0.028 | 0.039 | 0.022 | – | 0.021 | 0.002 | 0.000 | – | 0.687 | 0.139 | 0.012 |
| MA | | | | | | | | | | | | |
| PC | −0.722* | 1.000 | −0.352 | 0.947** | −0.747* | 1.000 | 0.673* | 0.673* | −0.157 | 1.000 | 0.350 | 0.602 |
| Sig. | 0.028 | – | 0.353 | 0.000 | 0.021 | – | 0.047 | 0.047 | 0.687 | – | 0.355 | 0.087 |
| CA | | | | | | | | | | | | |
| PC | 0.691* | −0.352 | 1.000 | −0.481 | 0.874** | −0.509 | 1.000 | −0.921** | 0.534 | 0.350 | 1.000 | −0.504 |
| Sig. | 0.039 | 0.353 | – | 0.227 | 0.002 | 0.162 | – | 0.000 | 0.139 | 0.355 | – | 0.167 |
| C | | | | | | | | | | | | |
| PC | −0.677* | 0.947** | −0.481 | 1.000 | −0.895** | 0.673* | −0.921** | 1.000 | −0.694* | 0.602 | −0.504 | 1.000 |
| Sig. | 0.022 | 0.000 | 0.227 | – | 0.000 | 0.047 | 0.000 | – | 0.012 | 0.087 | 0.167 | – |

*The correlation is significant at the 0.05 level

**The correlation is significant at the 0.01 level

significant negative correlations with callose accumulation. These findings confirm that malic acid play an important role in ISOP 76 Al tolerance, but the same was not observed in ISOP 239.

## Molecular markers detection

Plants obtained from hydroponic culture were screened using specific molecular markers. The results of molecular screen are summarized in Table 6 and Fig. 5. The products of CAPS marker amplification resulting from enzymatic digestion allow to discriminate between two *ALMT1* alleles (Sasaki et al. 2004). Our data indicate that ISOP 76, Maringá and Chinese Spring possess *ALMT1*-2, while ISOP 239 had *ALMT1*-1 allele.

The SPF and LPF markers (Fig. 5) allow the identification of the type of promoter present in the gene *ALMT1* (Sasaki et al. 2006). In the $F_0$ and $F_3$ generations of ISOP 76, type IV promoter was detected, but it could not be detected in the DH line. ISOP 239 $F_0$ generation showed the presence of type V promoter while $F_3$ generation had type I promoter. No promoter was detected in the DH line of ISOP 239. As expected, the standard varieties showed the presence of the promoters type VI for Maringá and type I and III for Chinese Spring.

The SSR3 markers signalize repeated sequences in the intron 3 (Raman et al. 2006). The SSR3a marker

**Table 5** Pearson correlations (PC) between screening tests for ISOP 239: root length (RL), malic acid exudation (MA), citric acid exudation (CA) and callose accumulation (C)

| | $F_0$ | | | | $F_3$ | | | | DH | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RL | MA | CA | C | RL | MA | CA | C | RL | MA | CA | C |
| RL | | | | | | | | | | | | |
| PC | 1.000 | −0.023 | 0.447 | −0.768** | 1.000 | −0.631 | 0.497 | −0.940** | 1.000 | −0.414 | −0.045 | −0.896** |
| Sig. | – | 0.953 | 0.228 | 0.004 | – | 0.068 | 0.173 | 0.000 | – | 0.268 | 0.909 | 0.000 |
| MA | | | | | | | | | | | | |
| PC | −0.023 | 1.000 | 0.027 | 0.057 | −0.631 | 1.000 | −0.340 | 0.403 | −0.414 | 1.000 | 0.331 | 0.070 |
| Sig. | 0.953 | – | 0.946 | 0.884 | 0.068 | – | 0.371 | 0.282 | 0.268 | – | 0.384 | 0.857 |
| CA | | | | | | | | | | | | |
| PC | 0.447 | 0.027 | 1.000 | −0.551 | 0.497 | −0.340 | 1.000 | −0.505 | −0.045 | 0.331 | 1.000 | −0.001 |
| Sig. | 0.228 | 0.946 | – | 0.124 | 0.173 | 0.371 | – | 0.166 | 0.909 | 0.384 | – | 0.998 |
| C | | | | | | | | | | | | |
| PC | −0.728** | 0.057 | −0.551 | 1.000 | −0.940** | 0.403 | −0.505 | 1.000 | −0.896** | 0.070 | −0.001 | 1.000 |
| Sig. | 0.004 | 0.884 | 0.124 | – | 0.000 | 0.282 | 0.166 | – | 0.000 | 0.857 | 0.998 | – |

*The correlation is significant at the 0.05 level

**The correlation is significant at the 0.01 level

**Table 6** Summary results of specific ALMT1 molecular markers amplification, consequent promoter type and allele, and expected gene expression (EGE) based on promoter type and observed malic acid exudation (OME)

| Variety | CAPS (bp) | Allele[a] | SPF (bp) | LPF (bp) | Promoter type[b] | SSR3a (bp) | SSR3b (bp) | EGE[c] | OME (mg/L)[d] |
|---|---|---|---|---|---|---|---|---|---|
| Chinese Spring | 50; 57 | ALMT1-2 | 612 | 1190; 1993 | I and III | 233 | – | Low/medium | – |
| Maringá | 50; 57 | ALMT1-2 | – | 1600 | VI | – | – | High | 1.99 |
| $76F_0$ | 50; 57 | ALMT1-2 | – | 1470 | IV | 233 | – | Medium/high | 12.87 |
| $76F_3$ | 50; 57 | ALMT1-2 | – | 1470 | IV | – | – | Medium/high | 27.48 |
| 76DH | 50; 57 | ALMT1-2 | – | – | – | – | – | – | 43.33 |
| $239F_0$ | 107 | ALMT1-1 | – | 1750 | V | 233 | 173 | High | 3.65 |
| $239F_3$ | 107 | ALMT1-1 | 612 | 1190 | I | – | 173 | Low | 3.74 |
| 239DH | 107 | ALMT1-1 | – | – | – | – | – | – | 7.72 |

[a] Determined with the molecular marker CAPS

[b] Determined with the molecular markers SPF and LPF. (–) absence of amplification and detection of the markers

[c] Based on bibliographic data

[d] These values were obtained for 100 μM of $AlCl_3$

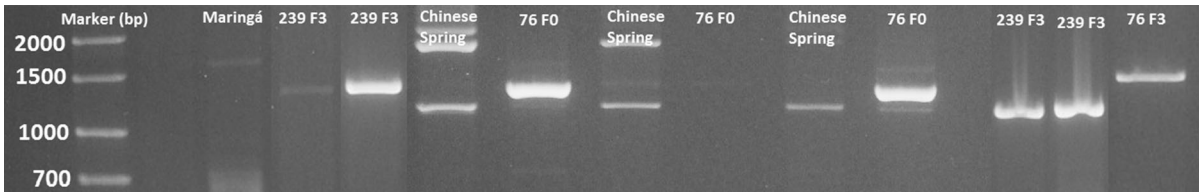


**Fig. 5** Detection of LPF marker amplifications products on 1.4% (w/v) agarose gel

was detected in Chinese Spring and in $F_0$ generations of both wheat landraces. On the other hand, SSR3b marker was only detected in $F_0$ and $F_3$ generations of ISOP 239.

## Discussion

### Screening tests of Al tolerance

The eriochrome cyanide R staining is a reliable qualitative method allowing to differentiate between tolerant and susceptible plants within a sample or a population. However, this method does not allow weighting the plant's answer to aluminium among individuals or plants sets in the population. In this study the eriochrome cyanide R staining was used to assess the level of Al tolerance of the generations of wheat landraces. According to Ma et al. (2001) the exposure to cationic aluminium affects root development and its elongation, can give an evidence that plant can cope with this metal presence. Overall root elongation (Fig. 2) and elongation ratio data confirm that ISOP 76 have Al tolerance and its performance is equal or even better than Maringá. However, the root performance of Maringá improved with Al increasement in the growing medium (Fig. 2). At the same time, ISOP 239 suffers the highest impact of Al bioavailability in root elongation, confirming that this landrace has an Al susceptible. In both cases, $F_0$ shown better root elongation in the presence of 100 μM Al. The elongation ratio decreased along the series $F_0 > F_3 > DH$ lines, which could mean that both landraces are losing their ability to cope with bioavailable aluminum, possibly due to the loss of some genetic variability. Nevertheless, the elongation ratio decrease was less than 10 and 14% for the ISOP 76, in the presence of 100 and 200 μM Al, respectively, showing that landraces generations kept significant part of its Al tolerance. The findings about Maringá combined with the increase of root performance and the low organic acid exudation, possibly mean that aluminium triggers different tolerance mechanisms, for instance the internal Al neutralization, avoiding organic acid exudation.

The accumulation of callose in roots is a typical plant response to stress, including the one caused by aluminium (Zhang et al. 1994). In this case, accumulation of this polysaccharide is an evidence of plant susceptibility. Callose accumulation demonstrated to be greatly correlated with the experimental variants and generations (Table 3), being its amounts higher when Al increases. Among the landraces, ISOP 239 shown a higher callose deposition, indicating that it is less tolerant to the presence of aluminium. At the same time, the initial population of ISOP 76 produces amounts of callose that resemble Maringá behaviour. The deposition of callose in $F_3$ and DH lines of ISOP 76 increases in the presence of Al, reflecting some difficulties to cope with metal bioavailability.

Overall, the performed screening tests (Table 2) confirmed the Al tolerance of ISOP 76 landrace, which offers some features comparable to Maringá, such as high root length and relative lower callose accumulation. At the same time, ISOP 239 landraces confirm to be susceptible to Al, showing opposite features: more than 55% of susceptible individuals, lower root elongation ratio and the highest callose deposition.

### Quantification of organic acids in exudates

Malate exudation was acknowledged as the main mechanism responsible for wheat tolerance to Al (Ma et al. 2001; Ryan et al. 2009). Malic acid was also implicated in the plant responses to different abiotic stresses (Lance and Rustin 1984; Pinheiro de Carvalho et al. 1991). This account also appears to be true for ISOP 76, where malate was actively exuded

in the presence of Al (Fig. 4a), as previously documented by Sharma et al. (2016). The malic acid exudation has positive and significantly influenced by experimental conditions (100 and 200 μM Al) and generations ($F_0$, $F_3$, DH) variants (Table 3). Organic acid exudation was also significantly correlated with root length (negative correlation) and callose (positive) for ISOP 76 (Tables 4, 5). Our observation suggests that malate exudation is probably the main mechanism for Al neutralization resulting in Al tolerance of ISOP 76. Lack of enhanced malate exudation in Al tolerant Maringá may imply that this cultivar features a different Al coping mechanism(s).

In contrast, citrate is continuously exuded by wheat under stress-free conditions, which was corroborated by our observations of ISOP 76 and by Ryan et al. (2009). The highest exudation of citric acid by ISOP 76 was reported in Al free medium, while it dropped in the presence of Al, both in the $F_0$ and $F_3$ generations. An increase of citric acid exudation is observed in $F_3$ and DH line in the presence of 100 μM Al. Wang et al. (2006) demonstrated that in the presence of aluminium, citrate exudation in some wheat genotypes may be inhibited. In the case of ISOP 76 this inhibition was not total, occurring citric acid exudation even in the presence of 200 μM Al, although a significant switch to malate exudation occurs in the roots. No significant changes in citric acid exudation in response to Al exposure were detected in Al susceptible ISOP 239. One can hypothesize that through Al-triggered activation of *ALMT1* the usually citrate exudation in some plants can be switch to malate exudation resulting in an increase of plant tolerance to the stress. Chelating capacity of citrate is rather higher than malate because of the presence of three instead of two carboxylic groups (Yang et al. 2013). However, we hypothesize that malic acid could be more effective than citric acid in Al neutralization since its cellular pool is higher and can be supplied by different metabolic pathways, reaching in some tissues up to 10 mM (Lance and Rustin 1984; Pinheiro de Carvalho et al. 1991). At the same time, citric acid supply depends mainly from Krebs cycle and root cellular pool of this acid is always lower (Popova and Pinheiro de Carvalho 1998). There is also the fact that malate exudation is specifically promoted by the presence of the specific *TaALMT1* transporter, controlled by the appropriate *ALMT1* gene alleles and promoters.

## Molecular markers detection

The data of organic acid exudation point out to the probable presence of *TaALMT1* transporter protein coded by *ALMT1* gene in ISOP 76 landrace, triggering malic acid exudation, leading to precipitation of cationic aluminium.

Molecular analyses revealed that ISOP 76, Maringá and Chinese Spring share the same *ALMT1*-2 allele, while ISOP 239 had the *ALMT1*-1 allele (Table 6). The *ALMT1*-2 allele can be detected both in tolerant and susceptible plants and its presence depends of geographical origin of wheat (Raman et al. 2008). Initially, this allele was predominantly reported in the susceptible and moderately tolerant plants (Zhou et al. 2007), such as Chinese Spring and Scout 66 (Sasaki et al. 2006). On the other hand, the presence of *ALMT1*-1 allele was detected in Al tolerant Atlas 66 (Sasaki et al. 2006). The *ALMT1*-1 and *ALMT1*-2 alleles' expression in Scout 66 and Atlas 66 was constitutive and not induced by Al. Tolerance screening tests and measurements of organic acid exudation showed that ISOP 239 was susceptible to Al and exuded low levels of malic acid despite the presence of *ALMT1*-1 allele. However, in ISOP 76, owning the *ALMT1*-2 allele, high levels of malic exudation are in agree with Al tolerance and citric acid exudation under control conditions.

In the $F_0$ and $F_3$ generations of the tolerant ISOP 76, the type IV promoter of *ALMT1*-2 allele that endorses high to moderate expression of the *ALMT1* gene was detected. Interestingly, gene promoters were not detected in the DH lines. All ISOP 76 lines appeared to be Al tolerant in presence of 100 μM Al, which agrees with the increase of acid malic exudation (Table 6). These results seem to indicate that allele gene expression do not determine the rates of malic acid exudation, although the promoter type IV is correlated with Al tolerance (Raman et al. 2008) and malic acid exudation. This finding was not supported by the presence of *ALMT1*-1 type V promoters in ISOP 239 $F_0$, which convey the highest gene expression (Sasaki et al. 2006), but do not result in the increase of malate exudation when roots were exposed to aluminium. This observation also indirectly suggests that Al tolerance in Maringá is

achieved not due to the activation of the *ALMT1* gene, and the Maringá's mechanism of Al tolerance is not present in ISOP 239 landrace nor it is responsible for Al tolerance of the ISOP 76 landrace. In addition, $F_3$ of ISOP 239 had the *ALMT1*-1 type I promoter and the DH line did not exhibit the presence of any promotors. The type I promoter variant transmitted low gene expression, which corresponds to the lowest malate exudation and lack of Al tolerance. The variation in ISOP 239 *ALMT1*-1 promoters can be explained by the heterogeneity of initial landrace population and the selection process for the Al susceptibility.

Our findings concerning the SSR markers obtained for Chinese Spring and Maringá remain in agreement with Raman et al. (2006). Presence of SSR3a in $F_0$ of both landraces and absence in $F_3$ and the DH lines may be explained by the fact that these repeating sequences were lost during the selection process. The presence of SSR3b marker in $F_0$ and $F_3$, but its lack in the DH line of ISOP 239 could indicate that these repeated sequences are specific to *ALMT1*-1 and vanished during the process of production of the DH lines from male $F_1$ progeny.

Overall, our results seem to indicate that malate exudation and *ALTM1*-2 gene allele are probably involved in the key tolerance mechanism of ISOP 76 landrace to Al toxicity. The Al tolerance of ISOP 76 landraces measured by root elongation slightly decreased along with the selection process $F_0 > DH > F_3$ (100 μM Al) and $F_0 > DH > F_3$ (200 μM Al). This variation of root elongation was almost inverse to the increase of malate exudation, that occurs along the series $F_0 < F_3 < DH$ (100 μM Al) and $F_3 < DH < F_0$ (200 μM Al). The variation in malate exudation in the roots of ISOP 76 could be explained by the detected polymorphism of the *ALMT1*, using molecular markers (Table 6). These results seems to point out that malate exudation is an important mechanism that plants activate to neutralize metal and mitigate its toxic action in roots. This fact explains the observed negative correlation with root elongation and positive correlation with callose accumulation. These observations also point out to the polygenic nature of Al tolerance and support our previous findings, reporting high variability and heterogeneity of Al tolerance among the Madeiran wheat landraces (Pinheiro de Carvalho et al. 2003, Ganança et al. 2007). It implies a high coexistence probability of different tolerance mechanism (Kochian et al. 2005; Sharma et al. 2016) that could be differentially engaged depending on the level of Al bioavailability. One can hypothesize that promoter type IV of *ALMT1* is heritably transmitted by the female progenitor, which explain its disappearance from the DH lines, while these lines still maintained ability to exude malate. It might also explain the observation that the response to aluminium of some lines is lower in the presence of 200 μM Al than in 100 μM Al.

## Conclusions

In conclusion, both ISOP 76 and ISOP 239 showed typical tolerance and susceptibility to Al stress responses, respectively. ISOP 76 demonstrated a superior Al tolerance that appears to be in part related with malic acid exudation. Malic acid exudation depends on the presence of *ALMT1* gene that was detected in both landraces. However, only ISOP 76 exude malic acid in significant quantities. This difference between ISOP 76 and ISOP 239 can be related with the presence of different genes alleles, *ALMT1*-2 and *ALMT1*-1, respectively, and thus exhibited different effectiveness in the landraces' response to the Al stress. However, different gene alleles and promoter types could only partially explain dissimilarities observed among the original landraces, their advanced generations and the DH lines, in response to Al toxicity.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights** No human participants or animals were involved in this research.

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