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ABSTRACT

Forty-eight genotypes representing wheat diversity from the Island of Madeira were screened for resistance to aluminum (Al) in nutrient solution. Seeds of wheat used in the experiments were obtained from local farmers. The soil pH and content of ionic Al of plots cultivated with

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wheat were analyzed. The pH of topsoils varied between 3.83 and 6.59. The amount of ionic Al in soil samples varied between 0.38 and 1.36 cmol Al^{3+} per kg of soil and was positively correlated with the altitude of a plot. Eriochrome cyanine staining was used to evaluate the effect of Al ions on the root elongation. Seventy-two hour exposure of 3-day-old seedlings to 100 and 200 μM Al in nutrient solution revealed a high number of Al resistant genotypes among wheat germplasm. After withdrawal of Al stress, survival and root regrowth was observed in 28 and 23 genotypes screened at 100 and 200 μM Al in nutrient solution, respectively. Enhanced resistance to Al among Madeiran genotypes was associated with the amount of ionic Al in the soils. Complexity and various patterns of responses of tested cultivars to Al stress may suggest that Madeiran germplasm could be a valuable source of genes controlling Al resistance for conventional breeding programs and for studies of molecular bases of mechanisms of Al resistance.

Key Words: Wheat; Genotypes; Aluminum resistance; Soil pH; Ionic aluminum.

INTRODUCTION

Aluminum (Al) toxicity is a main growth and yield limiting factor on mineral soils with pHs below 5.0.[1,2] Soil acidity is a serious agricultural problem throughout many parts of the world, affecting as much as 40% of the world's arable land.[3] Under acidic conditions, monomeric Al ions are released to soil solution from soil minerals and form polycationic, non-toxic Al complexes that exist at neutral pH. Once in soil solution, soluble Al ions can be taken up by roots and thus adversely affect plant growth. The first visual symptom of Al toxicity is a drastic reduction of root growth.[2,4] The ability of roots to continue elongation in the presence of Al ions in nutrient solution is often used to evaluate Al resistance of crops.[5] In recent years, a sensitive test measuring Al resistance that is based on eriochrome cyanine staining was developed.[6,7] This non-destructive test allows for the evaluation of the response of cultivated plants to Al at the seedling stage and to obtain progeny that can be further selected and tested for Al resistance.

During evolution, plants developed numerous mechanisms that allow survival in acid soils rich in available Al.[4,7–9] As result of selective pressure, inter- and intra-species differences in response to Al are widely observed in the plant kingdom. Cultivated species differ significantly in their response to Al toxicity. Among cereals, rye is considered to be the

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most Al resistant followed by oat, triticale, wheat, and barley.[7,10] Although wheat is regarded a sensitive species, dramatic differences in resistance of cultivars to Al have been reported.[11–13] The most Al resistant wheat forms are found among genotypes originated from Brazil where the intense selection pressure of acid soils containing high amounts of exchangeable Al resulted in the development of cultivars that are commonly used as standards of Al resistance.[13] These cultivars are often used as donors of genes controlling Al resistance in breeding programs around the world. However, the pool of suitable gene sources is limited and identification of new Al resistant genotypes could facilitate future selection efforts.

The Portuguese Archipelago of Madeira located on the Atlantic Ocean between latitudes 33°10 32°20N and longitudes 16°10 17°20 W, 630 km west off coast of North Africa consists of four islands. The main Island of Madeira with an area of 728 sq km (50 by 25 km) seems to be a good location to search for new forms of Al resistant wheats because of its volcanic origin, soil characteristics, geographical isolation and history of wheat cultivation. Several soil types are found on the Island of Madeira including Vertisols, Cambiosols, Phaeozems, Leptosols, and Andosols. Andosols derived from basaltic rocks and pyroclastic materials[14,15] predominate on the island and represent 60% of the total land area. Andosols are classified as acid or very acid soils with pH below 5.5 and with Al content ranging from 11 to 34 g per kg of soil.[14] The soil and climatic conditions of the island are strongly affected by elevation. While mean annual temperature decreases from about 19°C at the sea level to 9°C on mountainous plateau (about 1200 m a.s.l.), the mean annual precipitation increases from about 500 mm to 3000 mm, at low and high elevations, respectively.[13] These climatic conditions have a strong influence on agricultural practices and they have determined the evolution of local cultivars.

Discovered in the 15 century, the Island of Madeira was originally covered with natural *Laurisilva* (evergreen forest). Vast areas of the forest have been cut-down by early settlers to obtain timber for shipbuilding and agricultural land. Instead, it was introduced a great variety of plant species from different geographical regions including crops like wheat, corn, and sugar cane. The history of wheat cultivation begun with the islands colonization, when first varieties from Portuguese mainland have been introduced.[16] In the 16 and the 17 century several other major introductions of wheat occurred from the Canary Islands, Azores, North Africa and Southern and Northern Europe.[17,18]

The aim of the present study was to screen existing wheat collection of local cultivars, to identify Al resistant genotypes and to analyze factors determining this resistance among the genotypes.

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MATERIALS AND METHODS

Forty-eight Madeiran wheat producers and their plots were identified. Exact location and elevation of the plots was recorded using a GPS Magellan 320. Wheat plots were localized at altitudes ranging from 273 to 903 m a.s.l and thus reflected the variety of pedologic conditions found on the island. Samples of wheat seeds and the top 20 cm of soil were collected in the end of the growing season (Table 1). The seeds were stored until analysis at − 20°C in the ISOPlexis, Germplasm Bank at the University of Madeira, Funchal.

The pH and Al content of forty-eight soil samples, collected from plots corresponding to ISOP (wheat) entry, were analyzed. Soil was dried at 105°C for 24 hours. Ten grams of soil was suspended in 25 mL of 0.01 *M* KCl and shaken for 1 hour.[19] The pH of supernatant was measured using a WTW 320 pH meter. Ionic Al was extracted from 5 g of soil using sodium

Table 1. Localization of wheat plots on the Island of Madeira and corresponding ISOP Germplasm Bank numbers.

ISOP #	Plot location	Altitude (m a.s.l.)	ISOP #	Plot location	Altitude (m a.s.l.)
54	Santa Cruz	298	109	Calheta	635
73	Santana	470	110	Santa Cruz	698
76	Porto Moniz	447	111	Santana	420
77	Ribeira Brava	353	112	Santana	431
78	Ribeira Brava	310	114	Campanário	446
79	Porto Moniz	386	115	Ribeira Brava	390
80	Calheta	902	116	Ribeira Brava	517
82	Câmara de Lobos	437	117	Ribeira Brava	417
84	Santana	842	118	Ribeira Brava	837
85	Santana	842	119	Porto Moniz	580
89	Ponta do Sol	544	120	Machico	235
92	Santana	370	121	Ribeira Brava	838
93	Santana	425	122	Ribeira Brava	504
94	Santana	273	183	Ribeira Brava	429
95	Santana	525	235	Ribeira Brava	540
96	Ponta do Sol	373	238	Machico	300
97	Calheta	668	239	Calheta	519
98	Calheta	475	240	Santa Cruz	516
99	Calheta	619	241	Santa Cruz	835
100	Ponta do Sol	670	242	Ribeira Brava	447
102	Porto Moniz	447	243	Santana	273
103	Santana	431	245	Calheta	570
104	Santana	431	258	Ponta do Sol	670
105	Santana	425	263	Ribeira Brava	633

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acetate (Morgan reagent) while Al content was measured according to the modified hydroxylamine acid method.[20,21]

About 200 seeds were surface sterilized in 5% sodium hypochlorite and germinated overnight at 25°C. Sprouted seeds were placed on a raft floating on a surface of aerated nutrient solution containing (in mM) 2900 NO_3, 200 NH_4, 100 PO_4, 800 K, 1000 Ca, 300 Mg, 101 SO_4, 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, 0.1 Mo, and 10 EDTA and grown for 3 days in a growth chamber at 23°C. For Al exposure, seedlings were transferred for 72 h to fresh nutrient solution containing (in mM) 2900 NO_3, 200 NH_4, 1000 Ca, and 300 Mg, with 100 or 200 mM Al supplemented in form of $AlCl_3 \times 6H_2O$. After Al treatment, seedlings were transferred for 2 days to an Al free nutrient solution to determine the ability of roots to recover from Al stress. In all treatments, the pH of nutrient solutions was adjusted to 4.3 with 0.1 *N* HCl.

Roots were stained for 10 minutes with a 0.1 solution (w/v) of eriochrome cyanine R_{250} (Sigma), which facilitated the visualization of root survival and regrowth.[22] After staining, the roots were extensively rinsed under tap water to remove the excess of dye. Root tips of plants that were able to continue growth after exposure to Al remained white, while roots with irreversibly damaged apical meristems were dark purple what indicated the absence of regrowth even after transfer to Al free medium.

All experiments were run in two duplicates for each experimental variant. Experimental results represent the mean values of these duplicates. The experimental standard deviation of performed measurements was lower than 15%. Pearson correlation between variables, e.a., soil pH, ionic Al content, altitudes, and Al resistance at 100 and 200 μM was determined, as well as their statistical significance. Correlations were considered significant at P values below 0.05. All statistical analyses and data treatment were preformed using software program Excel and SPSS 10.0 for Windows.

RESULTS

Farmer plots cultivated with wheat were found at altitudes ranging from 243 to 902 m a.s.l. The average altitude of all examined plots was 513.8 m. These plots had specific soil characteristics. Eighteen out of 48 soil plots belong to the Andosols, the most common soil class on the island, 12 soils are Cambiosols, 11 Leptosols, 4 Rocky soils and 3 Phaeozems. The analysis of pH of soil samples collected from 48 plots cultivated with wheat yielded values ranging from 3.83 to 6.59, in the Ribeira Brava and Calheta locations, respectively. Thirty-one plots showed acidic pH values below 5.0 (Figure 1). Normal-like frequency distribution of soil pH was noticeably shifted towards acid values with 18 plots exhibiting pH between 4.01 and

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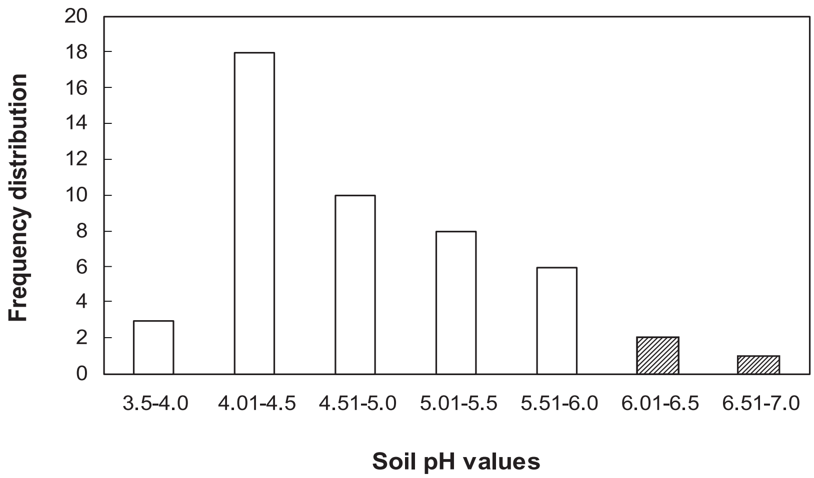

Figure 1. The frequency distribution of pH of topsoil collected from 48 farmer plots cultivated with wheat on the Island of Madeira.

4.50. The average pH value of all examined plots was 4.8. The content of ionic Al in soil sampled from tested plots was measured (Figure 2). Madeiran topsoils contained between 0.38 and 1.36 cmol Al^{3+} per kg of soil, in the Ribeira Brava and Santana locations, respectively. The analysis of frequency distribution of Al content indicated that the majority of plots

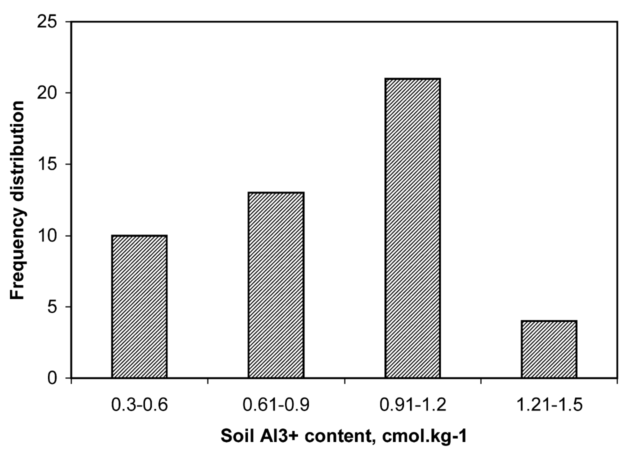

Figure 2. The frequency distribution of ionic aluminum content in topsoil samples collected from 48 farmer plots cultivated with wheat on the Island of Madeira.

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contained between 0.90 and 1.36 cmol Al^{3+} per kg of soil. The average ionic Al content in analyzed soils was 0.86 cmol per kg of soil. Variations in the soil ionic Al of analyzed top soil shown a negative, non-significant Pearson correlation ($R = -0.177$, $P = 0.229$), with soil pH, and a positive correlation with altitude, $R = 0.161$, $P = 0.275$ (Figure 3).

Wheat genotypes collected from 48 farmer plots were screened for Al resistance using eriochrome cyanine test. Al resistance of the genotypes was expressed as a capability of roots to growth after 72 hours exposure in the presence of 100 or 200 μM Al. Two major types of response to Al stress were identified (Figure 4). Plants were either highly resistant to Al, where over 80% of seedlings within tested genotype did not show symptoms of Al injury, or very sensitive where only less than 20% of seedlings were able to continue growth after exposure to Al. It is worth to note, that 58 and 48% of the genotypes were classified as very resistant, showing over 80% of root survival and regrowth at 100 and 200 μM Al, respectively.

This study has attempted to identify possible contribution of environmental factors such as altitude, soil pH and Al content in soil to the development of these two distinct groups of wheat genotypes in response to Al stress. For ranking purposes, a genotype was considered as an Al resistant when over 50% of tested seedlings exhibited root regrowth after exposure to Al in nutrient solution. It was found that the enhanced resistance of Madeiran genotypes was not significantly correlated with altitude and soil pH (data not shown). At the same time the enhanced resistance at 100 or 200 μM Al was significantly correlated with ionic Al

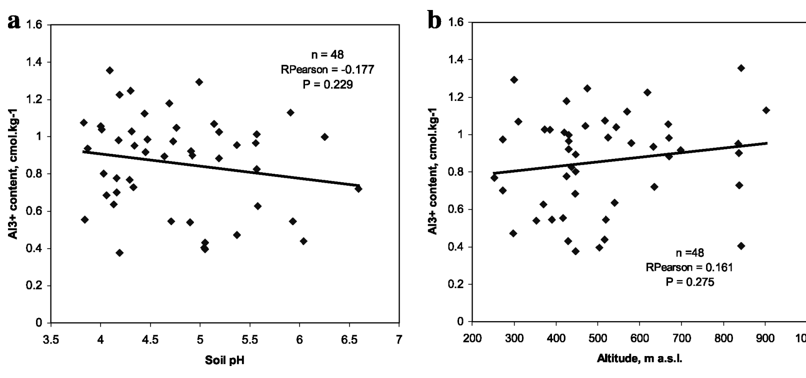

Figure 3. The correlation between ionic aluminum content and soil pH (a) or altitudes (b) of wheat plots on the Island of Madeira. Ionic aluminum content was determined using the hydroxylamine acid method[20] and soil pH was measured according to the Forster method. (From Ref. [19].)

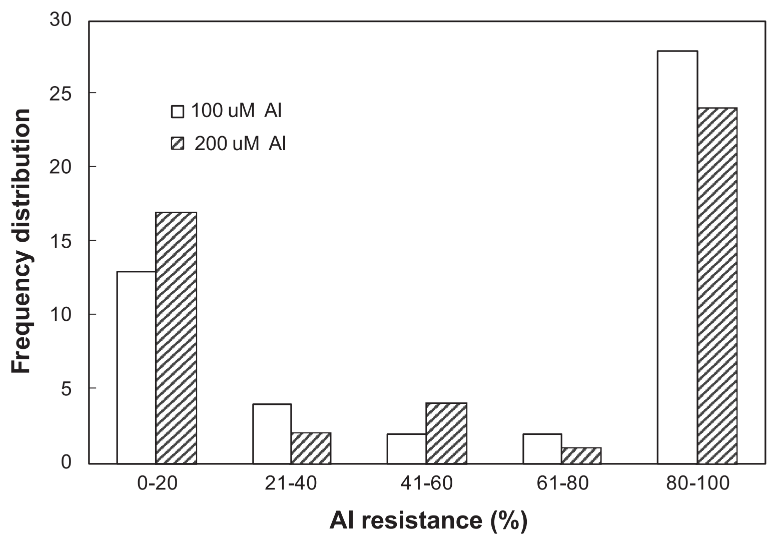

Figure 4. The frequency distribution of Al resistance of wheat cultivars collected from 48 farmer plots on the Island of Madeira. Cultivar was considered Al resistant if more than 50% of roots was able to continue growth after exposure to 100 and 200 μM Al for 72 h. Wheat cultivars were collected from 48 farmer plots on the Island of Madeira.

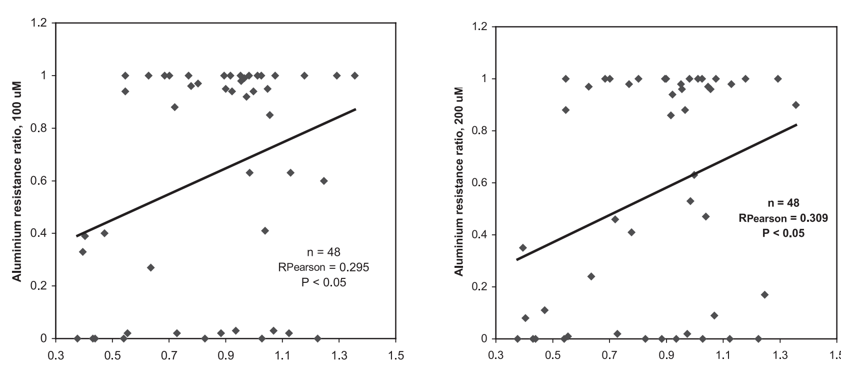

Figure 5. The correlation between soil ionic aluminum and wheat aluminum resistance to 100 (a) and 200 μM Al (b). The aluminum resistance was determined using the eriochrome cyanine staining method. (From Ref. [5].)

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content in soil (Figure 5a, b). The degree of response of the Madeiran wheat genotypes to Al stress imposed in nutrient solution significantly correlated with the amount of ionic Al in soils.

No significant correlation between environmental factors and the enhanced Al resistance was found when wheat genotypes were analyzed with respect to the soil type on which they were cultivated, e.a., Andosols, Cambiosols and Phaeozems, Leptosols and rocky soils (data not shown). These data suggest that none of the major Madeiran soil class acted as a specific source of enhancement of Al resistance among wheat genotypes.

Although the majority of wheat cultivars were Al resistant and the two major types of genotypes could be distinguished, a detailed analysis of Al resistance revealed a variety of complex and intermediate responses to Al among Madeiran cultivars. ISOP accessions exhibiting various patterns of response to different levels of Al stress were identified. These include genotypes totally sensitive to Al (ISOP 183), moderately sensitive (ISOP 122), and very resistant (ISOP 115). Some genotypes, however, appeared to be resistant to 100 μM Al while they were sensitive to this metal at higher concentration (ISOP 105) and others where the full expression of resistance was triggered by exposure to 200 μM Al (ISOP 76 and 80) (Table 1).

DISCUSSION

The first prerequisite for achieving success in conventional breeding program aimed on a development of the Al resistant cultivar is identification of genotypes that could be used as potential sources of genes controlling this feature. The genetic variability of wheat cultivars originated from geographical regions with highly Al toxic soils that favors natural selection towards Al resistance has been extensively studied and the genotypes suitable for breeding programs have been identified. This paper reports the results of our search for genetic variability in wheat on the Archipelago of Madeira. To our best knowledge this location has not been explored for Al resistance in wheat so far. Using the rapid eriochrome cyanine screening technique that has been proven to work effectively in selection of the Al resistant genotypes in several breeding programs,[7,12] this study found that more than half of 48 tested cultivars were resistant to Al (Figure 4). In nutrient solution culture, to obtain separation between cultivars in response to Al we had to subject wheat seedlings to the very stringent stress conditions of 72 h exposure at 100 and 200 μM Al. Shorter times of exposure (i.e., 24 h), commonly used in screening of wheat populations in breeding programs,[6,7] were not effective since the majority of genotypes exhibited resistance to Al. Our preliminary data indicated that

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several Madeiran cultivars seem to be more Al resistant than cvs. Maringa and Atlas 66, worldwide accepted standards of Al resistance among wheat.[5] It is hypothesized that a high number of Al resistant genotypes found on a small island could be a result of pedologic and historical conditions. The soils of volcanic origin are classified as very acid.[14,15] The mean pH of topsoil sampled from wheat plots was 4.8. Madeiran topsoils were found to contain high amounts of total Al ranging 11 to 34 g per kg of soil.[14] The average ionic Al contents in topsoil sampled from the wheat plots was 0.86 cmol Al^{3+} per kg of soil. As expected, we found that the increase in soil ionic Al content was negatively correlated with the soil pH and positively correlated with the altitudes (Figure 3). Low soil pH was apparently contributing to the development of the Al resistant wheat forms since genotypes showing resistance were predominantly found on the acidic plots. Strongly acidic conditions increase Al solubility and the pool of exchangeable Al (monomeric hexaaqua ions ($pH < 4.0$) or hydroxo-Al mononuclear complexes ($pH < 6.0$)) in mineral soils.[23] It seems that the ionic Al in tested topsoil had a direct effect on the development of Al resistant cultivars on the island (Figure 5). At the same time, a large number of Al resistant genotypes originated from the plots having low level of ionic Al and high pH values. It cannot be excluded that on Madeira the process of adaptation of wheats to unique soil conditions of the island occurred in the past and current correlation between resistance and the soil level of ionic Al do not reflect selective potential of this ion. It is also possible that the Al resistance of the Maderian wheats might be associated with the effectiveness of nitrogen uptake and metabolism as the Al resistant cultivars were found in regions not experiencing Al toxicity.[13] The nitrogen use efficiency of the Al resistant genotypes from the island should be warranted.

A preliminary evaluation of wheat genotypes collected on the island and preserved in the local ISOPlexis Germplasm Bank revealed the presence of at least 16 botanical varieties of genus *Triticum*[24,25] and species or subspecies of *Triticum aestivum* subs. *aestivum*, *T. aestivum* subs. *compactum* (Host) Mackey, *T. turgidum* subs. *turgidum* and *T. turgidum* subs. *durum* (Desf.) Husn. High biodiversity of the Madeiran wheats was established as a result of introduction of cultivars by early Portuguese explorers from various phytogeographical locations around the world as early as in the 15 century.[16,24] At least 3 major wheat introductions to the Archipelago of Madeira were reported, namely from Portuguese mainland, during the fifteen-century,[16,24] from the Azores and the Canary Archipelagos and North of Africa in the sixteen century[17] and from Northern and Southern Europe during the 17 century.[17,18] Before cultivation on the Portuguese mainland or other Portuguese possessions,

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new cultivars were tested and quarantined on the island. Those exhibiting the capability of yielding on highly Al-toxic soils survived and were retained by farmers for local cultivation. Local farmers, who often operated on small plots located on terraces ploughed in on steep slopes of remote and isolated mountain valleys, have been using for decades own stock of wheat seeds that supposedly were introduced to the island centuries ago. Recently, the low profitability of traditional farming urged farmers, particularly those operating on small, hardly accessible plots to abandon wheat production. Fortunately, we were able to collect and to preserve existing wheat germplasm before its extinction.

Aluminum resistance in wheat is a complex feature, controlled by different cellular mechanisms.[7,25–28] The observed complexity of responses of tested cultivars to Al stress may indicate the importance of the identified Al tolerant genotypes in Madeiran germplasm as a source of genetic variability. Further analyses are being currently undertaken to evaluate suitability of identified sources of Al resistance for breeding programs and to identify physiological bases of Al resistance in wheat.

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