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Barley mutants with increased tolerance to aluminium toxicity

M. Nawrot[1], I. Szarejko[1] & M. Maluszynski[2]
[1]*Department of Genetics, Silesian University, Jagiellonska 28, 40-132 Katowice, Poland;* [2]*Plant Breeding and Genetics Section, Joint FAO/IAEA Division, IAEA, Vienna, P.O. Box 200, 1400-Vienna, Austria*

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Summary

Acid soil and associated aluminium toxicity are considered as the number one abiotic factor limiting crop production. Over 2 billion hectares of acid soils exist world-wide, both in tropical and moderate climatic zones. In Poland acid soils represent up to 60% of arable land. At soil pH < 5.0 Al ions become soluble in water and toxic to plants. Genetic improvement of Al tolerance in crops is the only alternative to soil liming, a traditional but short term and expensive agricultural cure to raise soil pH. Of the various cereals, barley is the most sensitive to Al toxicity. The known sources of Al tolerance in barley are limited to old cultivars and landraces. While they represent multiple alleles of a single locus, there is no potential to improve Al tolerance through recombination of non-allelic additive genes. In the Department of Genetics, Silesian University we have employed induced mutations for rapid creation of variability for Al tolerance in barley. Thirteen mutants with increased levels of tolerance to Al toxicity have been selected in M_3 generation after mutagenic treatment of four barley varieties with N-methyl-N-nitroso urea (MNH) and sodium azide. Six further Al tolerant mutants were identified in the collection of semi-dwarf mutants of the Department. All selected mutants confirmed Al tolerance with the use of three different methods of screening, i.e., root re-growth, root tolerance index and hematoxylin staining. Fourteen mutants exhibited significant root re-growth after 48 hour incubation with 3 ppm Al^{+3} and two of them, namely RL819/2 and RL820/6 were tolerant even to 6 ppm Al^{+3}. Crosses of two selected mutants with their respective parent varieties indicated that Al tolerance in each mutant was controlled by a single recessive gene. Out of three methods tested, the root re-growth method facilitated by hematoxylin staining proved to be the most reliable technique for large scale testing. Double treatment with MNH or combined treatment with sodium azide and MNH and 6h inter-incubation germination between treatments were the most successful treatment combinations for induction of aluminium tolerance in barley.

Introduction

Plant sensitivity to toxic effects of aluminium ions is one of the major factors limiting crop production in acid soils which represent up to 40% of the world's arable land (Haug, 1984). Over 2 billion hectares of acid soils exist world-wide, both in tropical and moderate climatic zones. It is estimated that 391 million hectares in Europe are highly acidic. In Poland, about 60% of the cultivated land is classified as acid, with 24% having a pH below 4.5 and 36% with a pH between 4.5 and 5.5 (Aniol, 1989). Aluminium is the most abundant metal in earth's crust, and weathering of Al-containing minerals under acidic conditions at pH < 5.0 allows aluminium ions (Al^{+3}) to become mobilized, soluble in water and available to plants (Wright, 1989; Delhaize and Ryan, 1995). The content of soluble Al ions in soils ranges from 1–33 ppm, but seldom exceeds 4 ppm. Increased concentrations of aluminium (for many plant species as low as 1–2 ppm) inhibit root cell division and elongation, resulting in poor root development, reduction of water and nutrient uptake, drought susceptibility, and subsequently in a significant decrease in yield (Foy, 1983). These neg-

ative effects can be partly overcome by soil liming, a traditional agricultural practice used to raise the soil pH and causing the Al to become insoluble. Conventional liming methods treat only the topsoil, while the mobilized aluminium in the subsoil (>1 m depth) remains unaffected. Thus, a serious aluminium problem remains for any deeply-rooted crops, among them are the cereals.

An alternative way to lessen the effects of acid soil on crop production is the cultivation of Al tolerant crops that sustain high yields on these marginal soils, making soil amendments unnecessary or utilizing them more effectively. Species and genotypes within species differ widely in their tolerance to aluminium as various plants use different strategies to avoid toxic effects of aluminium ions (Foy, 1988; Aniol & Gustafson, 1990). The production of Al tolerant varieties provides a long-term solution that can be adapted to most regions as an economically and ecologically sound practice.

Of the various cereal species, barley is the most sensitive to Al toxicity and even micromolar concentrations of Al inhibit root growth (Aniol, 1989). The known sources of Al tolerance in barley are limited to old cultivars and landraces. A detailed study performed on 37 barley genotypes of diverse origin indicated that only 1 locus with multiple alleles was responsible for Al tolerance in barley (Minella & Sorrells, 1992). This study was in agreement with earlier results of Reid (1971) who found that increased Al tolerance in barley was inherited as a single dominant gene. Thus, the improvement of Al tolerance in barley can not be achieved through recombination of non-allelic additive genes. Minella & Sorrells (1992) concluded that the known genetic sources of Al tolerance had a limited value for barley breeding and a search for new genes/alleles was urgently needed. This search can be performed either through further screening of existing germplasm or through employment of mutation techniques. Since all known sources of Al tolerance are alleles of a single locus, induced mutations seem to be a choice for rapid creation of desired variability.

Mutation techniques have contributed significantly to world-wide plant improvement. According to the FAO/IAEA Mutant Varieties Database, more than 1840 mutant varieties involving 164 plant species have been officially released up to December 1997, and some of them have made an outstanding impact on the productivity of a particular crop (Maluszynski et al., 1995a; Maluszynski et al., 1999). In some countries, mutant varieties of economically important crops, e.g., barley, durum wheat and cotton, occupy the majority of cultivated areas. Semi-dwarfness and earliness are plant characters most often improved by mutagenic treatment, but there are also examples of successful mutagenic induction of tolerance to abiotic stresses, such as salt tolerance in barley (Forster et al., 1995) and rice (J. Deus, person. commun.), or cold tolerance in rice (Maluszynski et al., 1995b). Mutation techniques have also been successfully used for the induction of Al tolerance in wheat. After gamma irradiation of the Al sensitive cultivar 'Anahuak', 28 Al tolerant mutant lines were obtained and 3 of them performed better than a recently released variety, both in acid and limed soil (Camargo et al., 1995).

Screening for aluminium tolerance in acid soil in the field has many obvious limitations, among them a difficulty in dealing with large batches of soil with the same concentration of Al ions needed for large-scale selection. For this reason several protocols to screen for Al toxicity in nutrient media have been developed. While the primary effect of aluminium toxicity is the inhibition of root growth, most of these protocols are based on one of the three approaches: 1) the comparison of the root elongation in a nutrient solution with a toxic level of Al^{+3} to that in the same solution without aluminium (Baier et al., 1995; Khatiwada et al., 1996), 2) the ability of plants to re-start root growth after a brief period of exposure to the toxic concentration of Al^{+3} (Camargo, 1984; Aniol, 1991), and 3) the estimation of the damage caused by accumulation of Al^{+3} in the root tips based on their stainability with some histochemical indicators which form complexes with Al, such as hematoxylin, morin or Eriochrome cyianine R (Polle et al., 1978; Minella & Sorrells, 1992; Luo & Dvorak, 1996; Larsen et al., 1998). Additionally, the protocols proposed by authors for the same species often differed in: the content and concentration of macro- and micro-salts in the nutrient medium; the concentration of Al^{+3}; the duration of plant growth in a nutrient solution; the time of the exposure to Al stress; and the pH of the nutrient solution itself (pH 4.0 or pH 4.5). These differences make it difficult to compare the experimental results obtained with the use of one specific screening method with data published by other authors.

The objective of this study was to produce new sources of Al tolerance in barley utilizing induced mutations of commercial varieties cultivated in Poland and to identify a mode of inheritance of increased tolerance to Al toxicity in the selected barley mutants.

Table 1. Mutated populations used for selection of Al^{+3} tolerant mutants in barley

Variety	Treatment combination	No. of M_3 seedlings analysed	No. of corresponding M_2 plants	No. of corresponding M_1 plants
Dema	0.5 mM MNH – 6h iig – 0.5mM MNH	8510	851	100
	0.75 mM MNH – 6h iig –0.75 mM MNH	2810	281	50
	1.5 mM NaN_3 – 6h iig- 0.75 mM MNH	9180	918	100
Magda	0.5 mM MNH – 6h iig – 0.5 mM MNH	3070	307	50
	1.5 mM NaN_3 – 6h iig- 0.75 mM MNH	3620	362	201
Maresi	0.5 mM MNH	5850	585	100
	1.0 mM MNH	3790	379	100
	1.5 mM MNH	5600	560	62
	1.5 mM NaN_3 – 6h iig – 0.75 mM MNH	9180	918	100
Rudzik	0.5 mM MNH – 6h iig – 0.5 mM MNH	13260	1326	150
	1.5 mM NaN_3 – 6h iig- 0.75 mM MNH	3000	300	50
Total number of plants analysed		67870	6787	1063

Different methods of screening for Al tolerance in barley were examined in order to establish a protocol that allows one to analyse a large number of plants in a short time.

Material and methods

Plant material

For selection of Al^{+3} tolerant forms, M_3 seedlings from available mutated barley populations were used. The mutated material was derived from chemical mutagenesis of four spring barley varieties ('Dema', 'Magda', 'Maresi', 'Rudzik'). In total, 67,870 M_3 seedlings, representing more than 6,787 individual M_2 plants were examined for aluminium tolerance (Table 1). This material was derived from different mutagenic combinations, including double treatment (2×3 h) with different doses of N-methyl-N-nitrosourea (MNH), or combined treatment with sodium azide (NaN_3) and MNH, and 6h inter-incubation germination period between treatments (Maluszynska & Maluszynski, 1983; Szarejko & Maluszynski, 1999). In a separate study, 371 semi-dwarf (sd) mutants from the collection of the Department of Genetics, Silesian University were evaluated for Al tolerance. The sd mutants represented M_{15} – and further generations after mutagenic treatment with MNH, NaN_3, gamma rays and fast neutrons of 12 barley varieties ('Aramir', 'Delisa', 'Diva', 'Georgia', 'HDM', 'Julia', 'Karat', 'Mg 4170', 'Plena', 'Roland', 'Salka', 'Trumph'). Additionally, two barley lines (CB 240 and CB 245) selected as Al^{+3} tolerant in Brazil and provided by the courtesy of Dr. C.E. Camargo from the Agronomy Institute, Campinas, Sao Paulo were included in the experiments.

Screening methods for Al tolerance

The initial screening for barley mutants tolerant to aluminium was performed with the use of the root re-growth method proposed by Camargo (1984), modified at our Department (Protocol 1) through the addition of hematoxylin staining as proposed by Polle et al. (1978). The technique estimates the ability of plant roots to re-grow in a nutrient solution without Al^{+3} after a period of growth in the solution with toxic levels of aluminium. The staining with hematoxylin facilitates selection of the tolerant forms. The Al^{+3} concentration of 0.75 ppm was chosen for the first selection to ensure that all potentially tolerant forms can be detected.

Ten M_3 seedlings per M_2 plant were used for the initial screening with 0.75 ppm Al^{+3}. Mutant lines showing significantly increased tolerance were evaluated again at 1 ppm Al^{+3} and checked for homozygosity in M_4 generation.

The comparative study with two other methods used for assessing aluminium tolerance was performed for selected mutants:

1. Root tolerance index (RTI), the relative root elongation in the presence and absence of Al in hydro-

Table 2. Nutrient medium composition for the root re-growth method. Solution 'A' (pH 4.0 ± 0.05)

Components	Final concentration	Stock solution* 200 × (g/l)
Macro salts	mM	
$Ca(NO_3)_2 \times 4H_2O$	4.0	188,9
$MgSO_4 \times 7H_2O$	2.0	98.6
KNO_3	4.0	80.9
$(NH_4)_2SO_4$	0.435	11.6
KH_2PO_4	0.50	13.6
Micro salts	μM	
$MnSO_4 \times H_2O$	2.0	0.068
$CuSO_4 \times 5H_2O$	0.3	0.016
$ZnSO_4 \times 7H_2O$	0.8	0.046
NaCl	30.0	0.350
$Na_2MoO_4 \times 2H_2O$	0.1	0.0046
H_3BO_3	10.0	0.124
Iron source	μM	
$FeNa_2EDTA$	10.0	0.73

* Use 5 ml of stock solution per 1 l medium.

Table 3. Nutrient medium composition for the root re-growth method. Solution 'B' (pH 4.0 ± 0.05; Al^{3+} is added as $AlCl_3 \times 6\,H_2O$)

Components	Final concentration	Stock solution* 200 × (g/l)
Macro salts	mM	
$Ca(NO_3)_2 \times 4H_2O$	0.4	188.9
$MgSO_4 \times 7H_2O$	0.2	98.6
KNO_3	0.4	80.9
$(NH_4)_2SO_4$	0.0435	11.6
Micro salts	μM	
$MnSO_4 \times H_2O$	0.2	0.068
$CuSO_4 \times 5H_2O$	0.03	0.016
$ZnSO_4 \times 7H_2O$	0.08	0.046
NaCl	3.0	0.350
$Na_2MoO_4 \times 2H_2O$	0.01	0.0046
H_3BO_3	1.0	0.124
Iron source	μM	
$FeNa_2EDTA$	1.0	0.73

* Use 5 ml of stock solution per 1 l medium.

ponic nutrient solutions (Baieret al., 1995), with modifications (Protocol 2).

2. Hematoxylin staining (Polle et al., 1978; Minella & Sorrells, 1992) (Protocol 3). This method evaluates the level of Al^{+3} sensitivity/tolerance on the basis of hematoxylin stainability of roots after growth of seedlings in three concentrations of Al^{+3}: 0.03 mM, 0.06 mM and 0.09 mM (equivalent to 0.81 ppm, 1.62 ppm, 2.43 ppm Al^{+3}).

M_4 mutants or sd mutant lines from the collection showing significant tolerance to 1 ppm Al^{+3} in all three applied methods were evaluated again with the use of higher concentrations of Al^{+3} (2–4 ppm). Additionally, tolerance to 5 and 6 ppm concentrations of Al^{+3} ions was evaluated for mutants indicating root re-growth at 4 ppm Al^{+3}. In all experiments 3 replications were used with 10 seedlings per replication. Protocols for three methods applied in the experiments and composition of nutrient media are presented below.

Protocol 1

Modified root re-growth method of screening for Al^{3+}tolerance (Camargo, 1984; Polle et al., 1978)

1. Seeds are pre-germinated in Petri dishes with wet filter paper (3–5 layers), first in a refrigerator for 48 hours and then at room temperature for the next 5–6 hours, until the coleorhiza appears.
2. Germinated seeds are plated into floating trays, covered with black plastic film and placed in containers with aerated nutrient solution 'A' (Table 1). About 30 ml of solution per seedling is used. The containers are placed in a growth chamber at 22–24 °C, 3000 lux, 16 h photoperiod.
3. After 48 hours, the floating trays are moved into Al^{3+} containing nutrient solution 'B' (Table 2). It is 10x less concentrated than solution 'A' and because of amelioration of aluminium toxicity by phosphorus, there is no KH_2PO_4 in this solution. The nutrient medium 'A' used in the first part of experiment is stored for the further use.
4. After 48 hours of growth in the Al^{+3} containing nutrient solution 'B", roots are rinsed with distilled water and floating trays are transferred back into nutrient solution 'A'.
5. After the next 72 hours, roots are rinsed again with distilled water and stained with 0.2% hematoxylin solution. To prepare 1 l of hematoxylin solution, 2g of hematoxylin and 0.2 g $NaIO_3$ are dissolved in 1000 ml distilled water.
6. Results of staining: parts of the roots that accumulated Al^{3+} have a dark navy-blue colour. Plants tolerant to aluminium continue growth and below the stained part of the root, white re-growth of the root tip is visible.

Table 4. Nutrient solution for the root tolerance index method (pH 4.0 $\pm$ 0.05; Al^{3+} is added as $AlCl_3 \times 6\ H_2O$)

Components	Final concentration (μM)	Stock solution* 1000 × (g/l)
$CaCl_2 \times 2H_2O$	400	44.4
KNO_3	650	65.7
$MgCl_2$	250	21.8
$(NH_4)_2SO_4$	10	1.32
NH_4NO_3	40	3.2

* Use 1 ml of stock solution per 1 l medium.

Table 5. Nutrient solution for the hematoxylin staining method (pH 4.0 $\pm$ 0.05; Al^{3+} is added as $AlCl_3 \times 6H_2O$)

Components	Final concentration (mM)	Stock solution* 100 × (g/l)
$CaCl_2$	4.0 mM	44.40
KNO_3	6.5 mM	65.70
$MgCl_2$	2.5 mM	23.80
$(NH_4)_2SO_4$	0.1 mM	1.32
NH_4NO_3	0.4 mM	3.20

* Use 1 ml of stock solution per 1 l medium.

Protocol 2

Modified root tolerance index (RTI) method of screening for Al^{3+} tolerance (Baier et al., 1995)

1. Seeds are pre-germinated in Petri dishes with wet filter paper (3–5 layers), first in a refrigerator for 24 hours and then in an incubator at 24 °C for 12 hours.
2. Germinated seeds with seminal roots about 3 mm long are transferred into floating trays. Trays are placed in containers with aerated low ionic strength hydroponic medium, containing Al^{+3} (Table 4). One seedling requires $\sim$ 50 ml of solution. The control without Al^{+3} is included in each experiment. Containers are put in a growth chamber at 22–24 °C, 3000 lux, 16 h photoperiod.
3. During the whole experiment, the pH must be constant 4.0 $\pm$ 0.05. pH should be checked every day and adjusted, if necessary to 4.0 $\pm$ 0.05 with HCl.
4. After 4 days of plant growth, the length of the two longest roots from each seedling is measured.
5. Root tolerance index (RTI) is calculated as the ratio of average root length in each Al^{+3} concentration to the average root length of the same genotype grown in the solution without Al^{3+}.

Table 6. Hematoxylin stainability of barley root tips related to Al tolerance (Minella & Sorrels, 1992)

Hematoxylin stainability scores*	Al tolerance level
CCC	Very sensitive (VS)
NCC, PCC	Moderately sensitive (MS)
NPC, PPC	Moderately tolerant (MT)
NNN, NNP, NPP, PPP	Tolerant (T)

* C – complete staining; P – partial staining; N – no staining.

Protocol 3

Hematoxylin staining method of screening for Al^{3+} tolerance (Polle et al., 1978; Minella & Sorrells, 1992)

1. Seeds are pre-germinated in Petri dishes with wet filter paper (3–5 layers), first in a refrigerator for 48 hours and then at room temperature for the next 5–6 hours, untill coleorhiza appears.
2. Germinated seeds are placed into floating trays. They are covered with black plastic film and placed in containers with aerated nutrient solution (Table 5). One seedling requires 30 ml of solution. The containers are placed in a growth chamber at 22–24 °C, 3000 lux, 16 h photoperiod. After 24 hours of growth, pH is adjusted to 4.0 $\pm$ 0.05 with HCl. Seedlings are grown in the nutrient solution without Al^{+3} for the next 30 hours.
3. After 30h of growth, Al^{+3} is added to the nutrient solution. Each genotype should be evaluated at 0.03 mM, 0.06 mM and 0.09 mM concentration of Al^{+3}. Seedlings are grown in Al^{+3} containing solutions for 17 hours.
4. After 17 hours of growth in Al^{+3} solution, the seedlings are rinsed and kept in distilled water for 60 min.
5. Roots are stained with hematoxylin staining solution for 15 min.
6. After staining, roots are washed briefly and returned to distilled water for 30 min.
7. Root tips are scored for the degree of hematoxylin staining at the 0.03, 0.06, 0.09 mM Al^{3+} (0.81, 1.62 and 2.43 ppm). Those parts of roots that accumulated Al^{3+} have a dark navy-blue colour. At each Al concentration approximately 5 mm of root tip of a genotype is scored as: completely stained (C), partially stained (P), or non staining (N). Additionally, the distance of the not stained part of the root can be measured under stereo-microscope. The scores of root stainability corresponding to the

Table 7. Results of screening for Al^{+3} tolerant M_3 mutant lines with the use of modified root re-growth method – Protocol 1

Variety/Mutant	Root re-growth length (mm) at different Al^{3+} concentration			Variety/Mutant	Root re-growth length (mm) at different Al^{3+} concentration		
	0.75 ppm	1.0 ppm	1.0 ppm		0.75 ppm	1.0 ppm	1.0 ppm
	M_3	M_3	M_4		M_3	M_3	M_4
Dema	0.5 ± 0.4	0.3 ± 0.3	0.3 ± 0.3	Rudzik	0.6 ± 0.3	0.5 ± 0.7	0.2 ± 0.2
DM170/8	4.5 ± 1.0*	0.9 ± 0.2*	0.7 ± 0.3*	RD65/6	5.2 ± 3.7*	0.0 ± 0.0	0.0 ± 0.0
DM184/7	5.8 ± 3.0*	0.3 ± 0.4	0.3 ± 0.6	RD65/7	5.8 ± 2.5*	0.1 ± 0.1	0.0 ± 0.0
DM185/7	3.8 ± 3.0*	0.0 ± 0.0	0.0 ± 0.0	RD65/8	4.9 ± 3.5*	0.1 ± 0.1	0.0 ± 0.0
DM185/8	3.3 ± 1.0*	0.0 ± 0.0	0.0 ± 0.0	RD66/2	6.8 ± 3.5*	0.0 ± 0.0	0.0 ± 0.0
DM186/1	11.5 ± 3.1*	3.0 ± 2.8*	1.2 ± 0.8*	RD85/6	5.8 ± 1.0*	0.5 ± 0.7	0.0 ± 0.0
DM186/8	8.0 ± 2.2*	2.0 ± 1.4*	2.7 ± 1.5*	RD114/1	6.0 ± 2.9*	0.8 ± 0.4	1.5 ± 1.3*
DM189/6	9.9 ± 2.3*	1.5 ± 1.5	1.7 ± 1.2*	RD119/5	4.5 ± 1.0*	0.5 ± 0.6	0.0 ± 0.1
DM206/9	4.3 ± 1.5*	0.0 ± 0.0	0.0 ± 0.0	RD128/1	6.5 ± 2.4*	0.0 ± 0.0	0.0 ± 0.0
DM214/6	3.5 ± 1.0*	0.0 ± 0.0	0.0 ± 0.0	RD138/1	6.2 ± 2.5*	0.5 ± 0.7	0.3 ± 0.6
DM215/4	2.3 ± 0.5*	0.0 ± 0.0	0.0 ± 0.0	RD139/2	6.8 ± 4.3*	0.3 ± 0.4	0.3 ± 0.6
DM218/5	4.8 ± 2.9*	0.0 ± 0.0	0.0 ± 0.0	RD145/8	7.8 ± 3.8*	1.5 ± 0.7*	2.7 ± 2.1*
DM221/2	9.5 ± 1.3*	1.7 ± 1.8	1.3 ± 0.6*	RD167/3	6.0 ± 3.9*	2.0 ± 1.4*	1.3 ± 0.6*
Maresi	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.3	RD167/6	10.7 ± 4.0*	1.0 ± 0.0*	1.3 ± 0.6*
MR211/5	3.0 ± 1.4*	0.0 ± 0.0	0.0 ± 0.0	RD169/4	11.0 ± 3.9*	3.5 ± 2.1*	1.3 ± 0.6*
MR211/7	3.5 ± 1.9*	0.0 ± 0.0	0.0 ± 0.0	RD170/3	6.8 ± 1.7*	1.0 ± 1.4	0.3 ± 0.6
MR213/13	3.1 ± 2.3*	0.0 ± 0.0	0.0 ± 0.0	RD170/8	4.3 ± 1.5*	0.0 ± 0.0	0.0 ± 0.0
MR218/1	3.5 ± 1.0*	0.0 ± 0.0	0.0 ± 0.0	RD177/6	6.5 ± 1.3*	0.0 ± 0.0	0.0 ± 0.0
MR220/1	4.8 ± 0.5*	0.0 ± 0.0	0.0 ± 0.0	RD177/9	11.0 ± 1.8*	2.0 ± 1.4*	1.3 ± 0.6*
MR250/13	6.0 ± 2.9*	0.0 ± 0.0	0.0 ± 0.0	RD178/1	5.8 ± 1.5*	0.3 ± 0.4	0.3 ± 0.6
Magda	0.4 ± 0.4	0.1 ± 0.1	0.1 ± 0.1	RD180/6	9.5 ± 3.7*	1.5 ± 0.7*	1.7 ± 1.1*
MD134/9	3.8 ± 1.0*	0.0 ± 0.0	0.0 ± 0.0	RD180/12	6.8 ± 3.3*	0.5 ± 0.7	0.3 ± 0.6
				RD190/6	8.3 ± 2.5*	2.5 ± 2.1*	2.3 ± 2.3*

* – significantly better than parent variety at $p = 0.05$.

level of Al^{+3} tolerance, according to Minella and Sorrels (1992) are presented in Table 6.

Genetic analysis of Al tolerant mutants

In order to identify the genetic mechanism controlling increased tolerance to Al toxicity, the selected barley mutants were crossed with the parent variety and F_1, F_2, F_3 generations were produced. F_3 seedlings derived from individual F_2 plants (30 seedlings/F_2 plant) were evaluated for Al tolerance together with the mutant, parent variety and F_1 generation. The segregation ratios of Al tolerant to sensitive plants in F_2 generation were calculated on the basis of F_3 screening. Using this approach it was possible to score each F_2 plant as homozygous or heterozygous for Al tolerance/sensitivity. The screening was performed with the root re-growth method, at 1 ppm Al^{+3}. Three replications were used with ten seedlings per replication for each evaluated plant.

Results and discussion

In total, 41 M_3 putative mutant lines derived from 4 barley varieties showed increased tolerance to 0.75 ppm Al^{+3} in the initial screening with the root re-growth method. Thirteen of these mutants were confirmed to have increased tolerance to the higher level, 1 ppm of Al^{+3} ions, in both M_3 and M_4 progeny (Table 7). These mutant lines were selected in M_3 progeny of two modern Polish varieties: Dema (5 mutants) and Rudzik (8 mutants) after mutagenic treatment with chemical mutagens (MNH and sodium azide). The mutants were found in the M_3 progeny of 1032 of Dema and 1626 of Rudzik M_2 plants (Table 1), indicating a relatively high frequency of M_1 plants carrying mutation(s) responsible for increased Al^{+3} tolerance. On the other hand, none of the putative mutants from varieties Maresi and Magda, selected as tolerant to 0.75 ppm Al^{+3} in M_3 generation, were confirmed as tolerant to the higher level (1 ppm) of

Al^{+3} ions, neither in the same nor the next generation (Table 7). This result shows high genomic specificity of barley varieties to mutagenic action of the applied chemical agents, in relation to this character. High mutagenic potential of N-mehtyl-N-nitrosourea and sodium azide has been proven many times by several authors (Warner et al., 1977; Maluszynski et al., 1988; Ullrich & Aydin, 1988; Kaushik & Khush, 1991; Jende-Strid, 1993; Satoh et al., 1997). At the Department of Genetics, Silesian University, Katowice, both MNH and sodium azide have been routinely used for mutation induction in barley. Mutants exhibiting different changes in root system development were found in mutated barley generations with the similar high frequency of 3.3% (Maluszynski, 1999).

Two mutants from variety Dema (DM186/1 and DM186/8) originated from the same M_1 plant what suggests that they could be derived from the same mutagenic event. That was also the case of two mutants from variety Rudzik namely RD167/3 and RD167/6. All other mutants were derived from different M_1 plants.

Among semi-dwarf mutants from the collection of the Department of Genetics, 17 mutants expressed higher levels of tolerance to 0.75 ppm Al^{+3} than the parent variety and 8 of these forms were also tolerant to 1 ppm Al^{+3} (Table 8). The highest number of sd mutants with increased tolerance to Al^{+3} was found among mutants originating form the variety Roland. It should be noted, however, that Roland itself expressed increased tolerance to Al toxicity, as compared to other varieties analysed in this study. Four mutants derived from the variety Roland, namely RL806/9, RL807/8, RL808/5 and RL809/5 were obtained after gamma radiation and two mutants (RL819/2 and RL820/6) after chemical mutagenesis.

Tolerance to the higher concentration of Al^{+3} ions (2–6 ppm) was evaluated for the selected 19 mutants with the use of Protocols 1 and 2. All mutants derived form variety Dema and three mutants originating from variety Rudzik showed significant root re-growth up to the concentration of 3 ppm Al^{+3} (Table 9). Root tolerance index was significantly higher than in the parent variety even at a concentration of 4 ppm of Al^{+3} ions for all, except one, mutants of variety Rudzik and three mutants of variety Dema (Table 10). The highest level of Al tolerance was expressed by mutants derived from the variety Roland. All Roland mutants exhibited root re-growth at 4 ppm Al^{+3} and three of them were able to re-start root growth even at 6 ppm Al^{+3}, the highest concentration tested (Table 9).

Table 8. Results of screening for Al^{+3} tolerant sd mutants by the modified root re-growth method – Protocol 1

Variety/Mutant	Root re-growth length (mm) at different Al^{3+} concentration	
	0.75 ppm	1.0 ppm
Aramir	0.8 ± 0.4	0.1 ± 0.1
043AR	4.3 ± 1.0*	0.0 ± 0.0
Diva	1.1 ± 1.2	0.3 ± 0.4
200DV	9.0 ± 1.4*	0.0 ± 0.0
203DV	6.5 ± 2.1*	0.0 ± 0.0
216DV	6.5 ± 2.1*	0.0 ± 0.0
225DV	6.0 ± 1.4*	2.5 ± 0.7*
237DV	4.0 ± 1.4*	0.0 ± 0.0
HDM	0.8 ± 0.4	0.2 ± 0.2
358HD	3.5 ± 2.1*	0.0 ± 0.0
Karat	0.7 ± 0.5	0.0 ± 0.0
678Q	3.5 ± 2.1*	0.9 ± 0.2*
709Q	3.0 ± 1.4*	0.5 ± 0.4
842Q	6.5 ± 0.7*	0.8 ± 0.4
Roland	2.3 ± 0.4	1.3 ± 0.4
RL806/9	4.4 ± 0.6*	3.4 ± 1.3*
RL807/8	9.0 ± 1.4*	4.3 ± 0.4*
RL808/5	9.3 ± 1.0*	4.1 ± 0.8*
RL809/5	4.6 ± 0.6*	3.1 ± 0.8*
RL819/2	11.5 ± 2.1*	5.4 ± 0.6*
RL820/3	4.5 ± 0.9*	0.4 ± 0.3
RL820/6	8.8 ± 1.1*	6.0 ± 0.5*

* – significantly better than parent variety at $p = 0.05$.

Comparison of two protocols of screening for Al tolerance in barley, namely root re-growth and RTI methods, showed that the latter technique was less selective than the root re-growth method. For example, with the use of the RTI protocol, all 6 mutants from the variety Roland showed a significantly increased tolerance over the parent variety at 6 ppm Al, while only three of them (RL807/8, RL819/2 and RL820/6) were able to re-start their root growth at this concentration (Table 9 and 10). The RTI method, although shorter than the root re-growth procedure (4 and 7 days, respectively), requires much more labour in measurements and data evaluation. This could be a limitation in fast screening of tolerant individuals. The root re-growth method also allows for the immediate transfer to soil of recovered mutants.

In order to compare the level of Al tolerance of barley mutants selected in this study with literature

Table 9. Tolerance of selected barley mutants to different concentrations of Al^{+3} ions according to the modified root re-growth method – Protocol 1

Genotype	Root re-growth lenght (mm) at different Al^{3+} concentration					
	1 ppm	2 ppm	3 ppm	4 ppm	5 ppm	6 ppm
Dema	0.26 ± 0.05	0.09 ± 0.02	0.04 ± 0.02	0.0 ± 0.0	–	
DM170/8	0.91 ± 0.17*	0.23 ± 0.07*	0.13 ± 0.06*	0.03 ± 0.02*	-	
DM186/1	0.88 ± 0.05*	0.17 ± 0.02*	0.19 ± 0.02*	0.02 ± 0.01	–	-
DM186/8	1.08 ± 0.08*	0.19 ± 0.06*	0.13 ± 0.03*	0.02 ± 0.02	–	-
DM189/6	1.24 ± 0.14*	0.20 ± 0.03*	0.15 ± 0.05*	0.02 ± 0.01	–	-
DM221/2	1.08 ± 0.18*	0.16 ± 0.05*	0.15 ± 0.04*	0.01 ± 0.01	–	-
Rudzik	0.19 ± 0.08	0.02 ± 0.01	0.03 ± 0.06	0.0 ± 0.0	–	-
RD114/1	1.01 ± 0.14*	0.18 ± 0.02	0.17 ± 0.08	0.0 ± 0.0	–	-
RD145/8	1.01 ± 0.22*	0.77 ± 0.10*	0.59 ± 0.13*	0.0 ± 0.0	–	–
RD167/3	1.24 ± 0.14*	0.76 ± 0.15*	0.75 ± 0.15*	0.0 ± 0.0	–	–
RD167/6	0.84 ± 0.20*	0.21 ± 0.10*	0.10 ± 0.09	0.0 ± 0.0	–	–
RD169/4	1.02 ± 0.16*	0.51 ± 0.20*	0.43 ± 0.12*	0.0 ± 0.0	–	–
RD177/9	0.81 ± 0.07*	0.36 ± 0.03*	0.17 ± 0.05	0.0 ± 0.0	–	–
RD180/6	1.03 ± 0.15*	0.48 ± 0.04*	0.11 ± 0.02	0.0 ± 0.0	–	–
RD190/6	1.04 ± 0.1*	0.53 ± 0.15*	0.17 ± 0.06	0.0 ± 0.0	–	–
Roland	1.26 ± 0.49	0.09 ± 0.10	0.02 ± 0.02	0.01 ± 0.01	0.0 ± 0.01	0.0 ± 0.0
RL806/9	4.37 ± 0.72*	1.71 ± 0.70*	0.48 ± 0.18*	0.34 ± 0.26*	0.09 ± 0.04	0.03 ± 0.02
RL807/8	3.72 ± 0.79*	1.45 ± 0.61*	0.36 ± 0.09*	0.29 ± 0.10*	0.11 ± 0.02	0.10 ± 0.04*
RL808/5	3.60 ± 1.59*	1.54 ± 0.58*	0.30 ± 0.11*	0.21 ± 0.13*	0.19 ± 0.09*	0.0 ± 0.0
RL809/5	3.26 ± 1.19*	1.52 ± 0.51*	0.65 ± 0.15*	0.39 ± 0.25*	0.22 ± 0.17*	0.0 ± 0.0
RL819/2	5.53 ± 1.74*	2.79 ± 1.24*	0.67 ± 0.29*	0.40 ± 0.13*	0.43 ± 0.07*	0.09 ± 0.04*
RL820/6	7.66 ± 2.32*	4.96 ± 0.70*	1.87 ± 0.29*	1.68 ± 0.24*	1.44 ± 0.17*	0.38 ± 0.13*
CB 240	6.50 ± 1.21*	0.04 ± 0.04	0.05 ± 0.05	0.09 ± 0.07	0.01 ± 0.01	0.0 ± 0.0
CB 245	1.33 ± 0.47	0.69 ± 0.13	0.15 ± 0.05	0.14 ± 0.15	0.01 ± 0.01	0.0 ± 0.0

* – significantly better than parent variety at $p = 0.05$; lines CB 240 and CB 245 were compared to the variety Roland.

references, the third method of evaluation was applied. This technique is based on hematoxylin staining ability of plant roots grown for 17 hours in solution with three different doses of Al, namely: 0.03 mM (0.81 ppm), 0.06 mM (1.62 ppm) and 0.09 mM (2.43 ppm). These are the doses recommended for evaluation of barley germplasm by Minella and Sorrells (1992). All mutants selected in the presented experiments can be scored as tolerant or moderately tolerant, according to the scale presented by the authors (Table 11). Among forms scored as tolerant, there were six mutants selected from variety Roland, five mutants derived from variety Rudzik and two mutants from variety Dema. Roots of these mutants exhibited no hematoxylin stainability at the 0.03 mM Al^{+3}, no or partial stainability at 0.06 mM Al^{+3}, and partial stainability at 0.09 mM Al^{+3}. These results correspond to the tolerance of known sources of aluminium tolerance in barley examined by Minella & Sorrels (1992). Mutants derived from varieties Rudzik and Roland and two barley lines, CB 240 and CB 245 selected as aluminium tolerant in Brazil, were additionally checked for hematoxylin stainability at 0.12 mM Al (3.24 ppm). Also at this higher concentration of Al ions, four mutants (two from Rudzik: RD180/6, RD190/6 and two from Roland: RL819/2 and RL820/6) were scored as partially stained. Two of them, RL819/2 and RL820/6 exhibited also the highest level of tolerance during evaluation with the two other methods used in this study, i.e., root re-growth and RTI (Table 9 and 10). At the same concentration 0.12 mM Al, both barley lines from Brazil were evaluated as completely hematoxylin stained, so they appear to be less tolerant than mutants RL819/2 and RL820/6. Out of three methods of selection for Al tolerance in barley, the root re-growth method facilitated by the hem-

Table 10. Tolerance of selected barley mutants to different concentrations of Al^{+3} ions according to the modified root tolerance index (RTI) method – Protocol 2

Genotype	Root Tolerance Index at different Al^{3+} concentration					
	1 ppm	2 ppm	3 ppm	4 ppm	5 ppm	6 ppm
Dema	0.33 ± 0.1	0.28 ± 0.03	0.31 ± 0.05	0.28 ± 0.04	–	–
DM170/8	0.39 ± 0.04	0.30 ± 0.04	0.34 ± 0.05	0.30 ± 0.04	–	–
DM186/1	0.44 ± 0.01*	0.40 ± 0.02*	0.44 ± 0.06*	0.34 ± 0.07	–	–
DM186/8	0.42 ± 0.03	0.33 ± 0.07	0.46 ± 0.04*	0.41 ± 0.04*	-	-
DM189/6	0.53 ± 0.02*	0.44 ± 0.02*	0.51 ± 0.03*	0.48 ± 0.04*	–	–
DM221/2	0.42 ± 0.04	0.35 ± 0.04	0.45 ± 0.06*	0.40 ± 0.05*	–	–
Rudzik	0.33 ± 0.04	0.23 ± 0.03	0.26 ± 0.02	0.17 ± 0.001	–	–
RD114/1	0.41 ± 0.07	0.25 ± 0.03	0.37 ± 0.02*	0.22 ± 0.001	–	–
RD145/8	0.47 ± 0.05*	0.31 ± 0.06	0.39 ± 0.06*	0.32 ± 0.03*	–	–
RD167/3	0.67 ± 0.03*	0.50 ± 0.06*	0.46 ± 0.05*	0.35 ± 0.06*	–	–
RD167/6	0.50 ± 0.03*	0.40 ± 0.04*	0.37 ± 0.02*	0.38 ± 0.02*	–	–
RD169/4	0.54 ± 0.09*	0.50 ± 0.10*	0.47 ± 0.13*	0.28 ± 0.05*	–	–
RD177/9	0.56 ± 0.06*	0.41 ± 0.03*	0.37 ± 0.07*	0.28 ± 0.02*	–	–
RD180/6	0.57 ± 0.13*	0.42 ± 0.02*	0.34 ± 0.01	0.33 ± 0.03*	–	–
RD190/6	0.64 ± 0.01*	0.56 ± 0.09*	0.51 ± 0.01*	0.46 ± 0.02*	–	–
Roland	0.29 ± 0.05	0.25 ± 0.04	0.22 ± 0.05	0.27 ± 0.02	0.16 ± 0.02	0.10 ± 0.02
RL806/9	0.64 ± 0.08*	0.47 ± 0.08*	0.57 ± 0.15*	0.49 ± 0.07*	0.52 ± 0.08*	0.63 ± 0.04*
RL807/8	0.60 ± 0.09*	0.51 ± 0.04*	0.37 ± 0.06*	0.37 ± 0.05*	0.40 ± 0.05*	0.39 ± 0.06*
RL808/5	0.54 ± 0.07*	0.47 ± 0.07*	0.36 ± 0.04*	0.36 ± 0.07*	0.31 ± 0.04*	0.32 ± 0.09*
RL809/5	0.59 ± 0.09*	0.55 ± 0.09*	0.46 ± 0.04*	0.42 ± 0.07*	0.31 ± 0.04*	0.29 ± 0.01*
RL819/2	0.68 ± 0.08*	0.54 ± 0.04*	0.49 ± 0.13*	0.51 ± 0.10	0.50 ± 0.07*	0.52 ± 0.00*
RL820/6	0.71 ± 0.04*	0.65 ± 0.08*	0.55 ± 0.03*	0.52 ± 0.03*	0.47 ± 0.07*	0.50 ± 0.04*
CB 240	0.52 ± 0.03*	0.49 ± 0.13*	0.36 ± 0.05	0.22 ± 0.02	0.30 ± 0.02*	0.34 ± 0.01*
CB 245	0.31 ± 0.02	0.25 ± 0.01	0.31 ± 0.05*	0.27 ± 0.04	0.30 ±0.05*	0.40 ± 0.04*

* – significantly better than parent variety at $p = 0.05$; lines CB 240 and CB 245 were compared to the variety Roland.

atoxylin staining can be recommended as the simplest and most reliable technique for large scale screening. A similar approach but with the use of different nutrient media and staining was used by Aniol (1991) for investigation of Al tolerance in wheat, rye and triticale.

Even though the methodology and Al^{+3} doses applied in this experiment were the same as described by Minella & Sorrells (1992), it is difficult to relate directly results of our study to the evaluation of barley germplasm presented by those authors. The most tolerant forms reported by them were scored as partially stained at 0.09 mM Al^{+3} but all exhibited complete staining at 0.12 mM. It is likely that mutants RL819/2 and RL820/6 which were scored as partially stained at 0.12 mM Al^{+3} in our study, exhibit higher levels of aluminium tolerance than tolerant forms described up to now in barley, but this conclusion remains to be checked in a separate experiment.

Genetic analysis of Al tolerance was performed for two selected mutants: RL819/2 from the variety Roland and RD177/9 from variety Rudzik, using the root re-growth method at 1 ppm Al^{+3} in the nutrient solution. The F_1 generation of crosses between mutants and their respective parent varieties showed that aluminium tolerance in both mutants was inherited as a recessive trait (Table 12). The segregation for Al tolerant and Al sensitive plants in the F_2 generation were done on the basis of F_3 progeny tests that allowed easy identification of all F_2 genotypes. The data presented in Table 13 indicate that increased level of Al tolerance in both mutants was controlled by a single recessive gene. Therefore, these new genetic sources of Al tolerance appear to be different from those previously described as dominant at the analysed concentration of Al^{+3} by Minella & Sorrells (1992).

Table 11. Tolerance of selected barley mutants to different concentrations of Al^{+3} ions according to the hematoxylin staining method – Protocol 3

Genotype	Hematoxylin stainability scores at different Al^{3+} concentration				Length of non stained part of root (mm) at different Al^{3+} concentration				Result*
	0.03 mM	0.06 mM	0.09 mM	0.12 mM	0.03 mM	0.06 mM	0.09 mM	0.12 mM	
Dema	N	C	C		0.93 ± 0.21	0.32 ± 0.29	0.05 ± 0.08		MS
DM170/8	N	P	C		1.66 ± 0.50	0.38 ± 0.15	0.10 ± 0.09		MT
DM186/1	N	P	P		1.10 ± 0.50	0.47 ± 0.06	0.44 ± 0.13		T
DM186/8	N	P	C		1.25 ± 0.38	0.80 ± 0.24	0.22 ± 0.26		MT
DM189/6	N	N	P		1.26 ± 0.43	0.86 ± 0.10	0.43 ± 0.10		T
DM221/2	N	P	C		1.72 ± 0.60	0.83 ± 0.33	0.13 ± 0.10		MT
Rudzik	P	P	C	C	3.17 ± 0.74	0.28 ± 0.15	0.10 ± 0.10	0.0 ± 0.0	MS
RD114/1	P	P	C	C	2.75 ± 0.96	0.94 ± 0.56	0.27 ± 0.39	0.17 ± 0.41	MS
RD145/8	N	P	C	C	3.25 ± 0.42	2.20 ± 0.45	0.79 ± 0.36	0.36 ± 0.47	MT
RD167/3	P	P	C	C	3.10 ± 0.82	1.88 ± 0.35	0.71 ± 0.49	0.55 ± 0.46	MT
RD167/6	N	P	P	C	4.36 ± 0.75	0.64 ± 0.27	0.86 ± 0.24	0.13 ± 0.22	T
RD169/4	N	N	P	C	2.80 ± 0.84	1.56 ± 0.86	1.16 ± 0.29	0.76 ± 0.31	T
RD177/9	N	N	P	C	5.60 ± 0.49	1.21 ± 0.57	1.00 ± 0.50	0.57 ± 0.35	T
RD180/6	N	N	P	P	4.67 ± 1.03	1.67 ± 0.41	1.12 ± 0.36	0.76 ± 0.25	T
RD190/6	N	N	P	P	5.00 ± 0.89	1.50 ± 0.64	0.94 ± 0.50	1.13 ± 0.38	T
Roland	N	P	C	C	1.63 ± 0.48	0.79 ± 0.27	0.71 ± 0.42	0.09 ± 0.12	MT
RL806/9	N	N	P	C	4.00 ± 0.93	4.25 ± 0.46	0.86 ± 0.24	0.40 ± 0.35	T
RL807/8	N	P	P	C	5.70 ± 1.16	3.00 ± 0.70	2.63 ± 0.91	0.19 ± 0.19	T
RL808/5	N	P	P	C	4.50 ± 0.76	3.50 ± 0.72	1.50 ± 0.53	0.10 ± 0.14	T
RL809/5	N	N	P	C	4.20 ± 1.08	3.30 ± 1.30	1.88 ± 1.11	0.29 ± 0.37	T
RL819/2	N	N	P	P	5.50 ± 0.54	5.50 ± 0.55	1.83 ± 0.40	0.88 ± 0.25	T
RL820/6	N	N	P	P	6.80 ± 1.50	5.20 ± 0.79	1.57 ± 0.53	0.78 ± 0.30	T
CB 240	N	P	P	C	2.60 ± 0.99	1.80 ± 0.94	1.14 ± 0.38	0.96 ± 0.46	T
CB 245	N	N	P	C	2.20 ± 0.83	2.45 ± 1.46	1.13 ± 0.35	1.20 ± 0.58	T

* MS – moderately sensitive; MT – moderately tolerant; T – tolerant.
Al^{3+} doses: 0.03 mM = 0.81ppm; 0.06 mM = 1.62 ppm; 0.09 mM = 2.43 ppm; 0,12 mM = 3,24 ppm.

Table 12. Al tolerance of barley mutants RL819/2, RD177/9, their parent varieties Roland and Rudzik and F_1 progeny of the crosses between parents and mutants, according to the root re-growth method

Genotype	Root re-growth length (mm)	Comments
Roland	0.87 ± 0.36	
RL819/2	3.60 ± 0.22	
F_1 Roland × RL819/2	0.95 ± 0.29	as Roland
Rudzik	0.21 ± 0.19	
RD177/9	1.90 ± 0.27	
F_1 Rudzik × RD177/9	0.15 ± 0.08	as Rudzik

Table 13. Segregation for Al^{3+} sensitive and tolerant plants in F_2 generation of the crosses Roland × RL819/2 and Rudzik × RD177/9 (on the basis of root re-growth of F_3 seedlings)

Cross-combination	F_2 genotype	No. of F_2 plants	Root re-growth length in F_3(mm)	$\chi^2_{1:2:1}$
Roland × RL819/2	Homozygous sensitive	22	0.85 ± 0.38	0.613
	Heterozygous	47		
	Homozygous tolerant	19	3.80 ± 0.45	
Rudzik × RD177/9	Homozygous sensitive	78	0.18 ± 0.11	3.882
	Heterozygous	120		
	Homozygous tolerant	74	2.30 ± 0.25	

$p = 0.05\ \chi^2_{1:2:1} = 5.991$.

Table 14. Successful mutagenic treatment combinations in developing Al tolerant mutants in barley

Mutant	Treatment combination
DM170/8, DM186/1, DM186/8, DM189/6, RD114/1, RD145/8	0.5 mM MNH – 6h iig – 0.5 MNH
DM221/2, RD167/3, RD167/6, RD169/4, RD177/9, RD180/6, RD190/6	1.5 mM NaN_3 – 6h iig – 0.75 mM MNH
RL819/2, RL820/6	0.7 mM MNH – 6h iig – 0.7 mM MNH
RL806/9, RL807/8, RL808/5, RL809/5	120 Gy gamma rays

Al tolerant barley mutants in our study were obtained both in chemical and physical mutagenesis (Table 14). The most successful mutagenic combinations include double treatment with the same dose (0.5 or 0.7 mM MNH) and 6 hours of inter-incubation germination between treatments (Maluszynska & Maluszynski, 1983). Two mutants expressing the highest level of Al tolerance, namely RL819/2 and RL820/6 were obtained after double applications of 0.7 mM MNH. Also combined treatments with sodium azide and MNH gave good results as 7 mutants from different varieties resulted from this combination.

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