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The response of *Medicago sativa* to mobile aluminium toxicity at seedling stage

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Abstract

Aluminium (Al) toxicity is a major limiting factor of crop production in acidic soils. The objective of the current study was to evaluate the development of lucerne (*Medicago sativa* L.) cultivars and their response to mobile Al toxicity. Three screening methods: filter paper-based, hydroponic and soil-based, were used to assess 13 *M. sativa* cultivars for their tolerance of mobile Al. Major biological indicators used to analyse mobile Al tolerance were hypocotyl length, root length and root and hypocotyl tolerance index. Toxic effects of mobile Al manifested themselves on all the biological indicators tested, which was shown by the correlations between the biological indicators and screening methods. Under filter paper-based method, the root and hypocotyl tolerance index ranged from 0.0% to 23.2% and from 0.0% to 37.2%, respectively at 16 mM $AlCl_3$ (aluminium chloride). Greater differences in *M. sativa* cultivars' tolerance to mobile Al toxicity were identified between the hydroponic and soil-based methods. In the hydroponic method, the root and hypocotyl tolerance index ranged from 41.0% to 78.2% and from 62.1% to 90.2%, respectively at 50 μM $AlCl_3$. Under the soil-based method, hypocotyls were more damaged by Al toxicity compared with roots at soil pH 4.5 and 4.3. The root and hypocotyl tolerance index at pH 4.5 was 73.5% and 39.3%, at pH 4.3 – 35.6% and 26.3%, respectively. The cultivars 'Mriia odes'ka', 'Romagnola', 'Kunsmme' and 'Juurlu' were distinguished by the highest root and hypocotyl tolerance index under hydroponic and soil-based methods. 'Žydrūnė', 'Birutė', 'Magnat', 'Viktoria' and 'Magda' were distinguished by root and hypocotyl tolerance index under hydroponic method. The root tolerance index of these cultivars was the highest under filter paper-based method. These cultivars are considered as tolerant accessions and may be used as donors in breeding for Al toxicity tolerance.

Key words: lucerne, aluminium toxicity, hypocotyl, root, screening method, tolerance index.

Introduction

Lucerne (*Medicago sativa* L.) is the world's most important forage crop adapted to a wide range of environments. It is a deep-rooted perennial legume, grown as a high-quality animal feed on a wide range of soils, including acid soils, and is adapted to a wide range of ecological conditions (Bouton, 2012; Sabanci et al., 2013). *M. sativa* productivity is affected by reduced root growth due to soil acidity and mobile aluminium (Al) toxicity (Khu et al., 2012). Plants depend on their root systems for their survival in nature and for their yield and nutritional quality in agriculture. Root systems are complex, and a variety of traits have been identified over the past decade as contributing to adaptation to low fertility and toxic soils (Idupulapati et al., 2016). Plant root systems comprise a set of phenes, or traits, that interact with the environment, and phenes are the identifiable units of the plant phenotype (York et al., 2013). Phenotypic characterization of root adaptations to soils with low fertility and mobile Al toxicity is enabling plant breeders to develop cultivars that not only yield more but also contribute to yield stability and nutritional security in the face of climate variability (Idupulapati et al., 2016).

Approximately 40% of arable soils worldwide are acidic and rhizotoxicity of mobile Al is the primary limitation to crop and forage yields on most acid soils (Kochian et al., 2015). Recurrent selection based on field performance has been used to develop acid soil-tolerant germplasm (Khu et al., 2013). Field screening for mobile Al resistance would seem to be the most desirable approach, because it best approximates the intended cropping environment (Haling et al., 2011; Yang et al., 2013). However, in practice reliable ranking of accessions in the field has been difficult. This is

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mainly, because mobile Al levels are not uniform, and environmental factors may interact with soil mobile Al to mask the expression of Al resistance. Thus, it is necessary to combine field with greenhouse screening techniques based on physiological traits of mobile Al resistance (Rao, 2014).

Al tolerance in *M. sativa* can be evaluated using a callus bioassay and whole plant assay in media (Khu et al., 2012), hydroponic system (Narasimhamoorthy et al., 2007), soil-based evaluations comparing growth in unlimed and limed soil (Khu et al., 2012) and Petri dish method (Pan et al., 2008). The different phenotyping methods often give different results suggesting that multiple evaluations at the cell and whole plant level should be used to ascertain variation in the Al tolerance response (Narasimhamoorthy et al., 2007).

The callus bioassay is useful to determine mobile Al tolerance at the cell-based level, while the whole plant assay in media evaluates plant mobile Al tolerance responses and enables examination of root growth variation in response to different growing conditions. The whole plant assay in media is less time and labour consuming compared to previous soil-based assay (Khu et al., 2012). The whole plant assay in media enables the identification of phenotyping differences due to mobile Al exclusion mechanisms in the root area providing an advantage over hydroponic evaluation systems, in which molecules generated by a particular accession are freely diffused in the growth solution.

All screening methods of tolerance to mobile Al toxicity are associated with selection of tolerant accessions. However, cultivars respond differently to different screening methods for mobile Al toxicity. Cultivars developed in the countries with warm and hot climates are more tolerant of acidic soils and mobile Al. European *M. sativa* cultivars are more sensitive to exposure to acid soils and mobile Al, which causes a drastic decrease in crop density. Single surviving plants of these cultivars are considered the most resistant.

In current research, identification of the most promising cultivars tolerant of acid soils and mobile Al by applying selection methods was expected. Selection of Al-tolerant plants and their cultivation on a large scale have been envisioned as an alternative to overcome Al toxicity.

In the current study, an attempt was made to determine the effect of toxic levels of mobile Al on *M. sativa* seedling characters and screen *M. sativa* cultivars for mobile Al toxicity at the seedling stage using root and hypocotyl tolerance index. The objective of this experiment was to evaluate the development of *M. sativa* cultivars exposed to mobile Al toxicity by using three screening methods.

Materials and methods

An experiment was carried out during 2017–2018 at Institute of Agriculture Lithuanian Research Centre of Agriculture and Forestry. Filter paper-based, hydroponic and soil-based assays were used to screen for aluminium (Al) tolerance in 13 lucerne (*Medicago sativa* L.) cultivars. The material subjected to Al resistance tests included cultivars of distinct country origin: 'Birutė' and 'Žydrūnė' from Lithuania, 'Ellerskie I' from Canada, 'Juurlu', 'Elda' and 'Kunsmme' from Estonia, 'Alina' and 'Magnat' from Romania, 'Mriia odes'ka' from Ukraine, 'Romagnola' from Italy and 'Viktoria' and 'Magda' from Czech Republic.

Filter paper-based screening method. The screening procedure was derived from Pan et al. (2008) with some modification. Well-developed *M. sativa* seeds of similar size were scarified, the surface sterilized in the solution of 10% NaClO (sodium hypochlorite) for 30 min and rinsed three times in distilled water. The seeds were sown in 90-mm sterile Petri dishes containing two pieces of sterilized filter paper and 7 ml of sterilized 50 mM $CaCl_2$ (pH 4.5) with five concentrations of $AlCl_3$ (aluminium chloride): 0, 2, 4, 8 and 16 mM. Thirty seeds were placed on the filter paper with three replicate dishes per treatment. The experiment was repeated twice. Petri dishes were incubated at 25°C temperature in the dark. After four days, the photoperiod was adjusted to 12/12h (day/night) at 25/20°C temperature, respectively. After three days, germinated seeds were counted, and root and hypocotyl lengths of the seedlings were measured. The percent of germination was calculated as a ratio of germinated seeds to not-germinated ones. The root and hypocotyl tolerance index was calculated as the maximum root and hypocotyl length in Al stress culture divided by the root and hypocotyl length in the control treatment at 0 mM $AlCl_3$.

Hydroponic screening method. The screening procedure was derived from Zhang et al. (2007) with some modification. Well-developed *M. sativa* seeds of similar size were scarified, and surface sterilized in solution of 3% NaClO for 7 min. After thorough rinsing with sterile distilled water, the seeds were imbibed in sterile water and kept in the dark overnight at 4°C temperature. The imbibed seeds were then cold treated by placing on moist filter paper in a Petri dish and incubating for at least 48 h at 4°C temperature. After cold treatment, the seeds were germinated in the dark at 24°C temperature for 24 h. Germinated seedlings were transferred to hydroponics in a growth room and grown under an 18/6 h light/dark regime at a constant temperature of 23°C. The hydroponic system consisted of multiple tanks with 25-L capacity. Twenty seedlings of each cultivar were placed into four plastic mesh-bottomed cups. Two cups of each cultivar were placed on a plastic rack that floated on the medium modified with Al and two cups on the medium without Al. The media were aerated with a small aquarium air pump for 15 min once per hour. The media were maintained at pH 4.3 by adding 1 M HCl (hydrochloric acid) as necessary throughout the experiment. After 5 days of growth in hydroponics with five mobile Al concentrations: 0, 5, 15, 25 and 50 µM $AlCl_3$, the seedlings were removed, and root and hypocotyl lengths were measured. The root and hypocotyl tolerance index of 13 cultivars was calculated as the maximum root and hypocotyl length in Al stress culture divided by the root and hypocotyl length in the control treatment at 0 µM $AlCl_3$. The experiment was repeated twice.

The Blade's medium was modified to contain: 0.5 mM $CaCl_2$, 1.8 mM KNO_3, 1.8 mM NH_4NO_3, 30.4 µM $MgSO_4 \times 7\ H_2O$, 22 µM KH_2PO_4, 13 µM $FeSO_4$, 31.7 µM $Ca(NO_3)_2$, 66 µM KCl, 26 µM NaCl, 26 µM H_3BO_3, 26 µM $MnSO_4 \times H_2O$, 5.2 µM $ZnSO_4 \times 7\ H_2O$ and 4.8 µM KJ.

Soil-based screening method in greenhouse conditions. The screening procedure was derived from Petcu et al. (2006). Physico-chemical properties of soil were: texture – loam, pH 7.2–7.5, P 87.6–117.7 mg kg^{-1} and K 83.8–145.2 mg kg^{-1}. The soil samples of *Endocalcari Epigleyic Cambisol* (WRB, 2014) and aluminium salt as aluminium sulphate ($Al_2(SO_4)_3$) were used for the research. The $Al_2(SO_4)_3$ is very soluble in water, therefore, an acidic solution was easily prepared. Using different concentrations of $Al_2(SO_4)_3$, soil treatments with different pH were obtained. The soil samples were analysed for mobile Al content, pH, P and K content under laboratory conditions (Table 1).

Table 1. Properties of soil samples with different mobile Al concentrations

$Al_2(SO_4)_3$ content g	pH	P	K	Al
		mg kg^{-1}		
0.0	7.2	113.4	141.9	0.0
3.5	6.6	109.4	142.8	0.0
5.0	6.0	79.8	132.8	0.0
11.9	4.5	74.1	53.9	732.5
23.8	4.3	52.3	37.3	4594

This experiment was conducted in greenhouse and under laboratory conditions with *M. sativa* plants grown in alkaline (pH 7.2), neutral (pH 6.6), slightly acidic (pH 6.0) and highly acidic (pH 4.5 and 4.3) soil. Two rows and 15 to 20 seeds were sown in each cup and covered with 60 g of sand. One cup was one replication. Three replications were used in the experiment. The cups were watered by weight of 70% field capacity with distilled water every 3 to 4 days and kept under laboratory conditions. One week after emergence, the seedlings were randomly thinned to 10 per row and grown in the greenhouse. Plants were harvested 35 days after sowing, roots were rinsed with tap water, and length of the main root and hypocotyls was measured.

Root and hypocotyl tolerance index was measured: (the root and hypocotyl length in acid soil / the root and hypocotyl length in neutral soil) × 100 was scored. The root and hypocotyl length on each Al concentration was compared with root and hypocotyl length of the control soil pH 7.2.

Soil agrochemical characteristics were determined by the following methods: the soil pH_{KCl} was measured by the potentiometric method in the extraction of 1 M KCl according to ISO 10390:2005 (Soil quality - Determination of pH); P and K contents were calculated by multiplying mobile P_2O_5 and mobile K_2O contents by 0.436 and 0.830 coefficients, respectively. Mobile P_2O_5 and K_2O contents in the soil were determined using the Egner-Riehm-Domingo (A-L) method. Exchangeable Al content in the soil was determined by the ISO 14254:2018 (Soil quality - Determination of exchangeable acidity using barium chloride solution as extractant).

Statistical analysis. The length of roots and hypocotyls of *M. sativa* cultivars depending on the differences in Al concentrations was calculated, one-way analysis of variance (ANOVA) was used followed by Fisher's least significant difference tests; *P*-values <0.05 were considered significant. A two-way ANOVA was performed to determine the effects of factors in two separate pairs (cultivars × Al concentration of root length and cultivars × Al concentration of hypocotyl length). All data presented are the mean values of three independent sets of experiments (± bars denote standard error, SE). The correlations between experimental data were investigated using liner regression analysis with the statistical program *SAS Enterprise Guide*, version 7.13 (SAS Institute Inc., USA).

Results and discussion

The classic symptoms of Al toxicity are inhibition of root growth and reduction in root penetration and branching, which were observed in the study conducted by Scheffer-Basso and Priori (2015). The analysis of variance (ANOVA) revealed significant differences in all screening methods. A significant difference between cultivars was determined using filter paper-based method, concentrations of mobile Al and an interaction between Al levels and cultivars for root and hypocotyl lengths (Table 2). Also, significant differences were revealed between cultivars, concentrations and an interaction between Al concentrations and root length of cultivars. However, the interaction between mobile Al levels and cultivars was not found for hypocotyl length when using hydroponic method. Significant differences between pH of soils for root lengths were determined under soil-based method. Also, significant differences were determined between cultivars and soil pH for hypocotyl length.

The root and hypocotyl growth was very slow at the highest mobile Al concentration under filter paper-based and hydroponic methods. The development of roots and hypocotyls was most affected by the highest mobile Al concentration: compared with the control treatment (0 mM and 0 μM $AlCl_3$), it was 11.5 and 11.8, and 2.0 and 1.4 times lower, respectively. The elongation

Table 2. Analysis of variance (ANOVA) of *Medicago sativa* cultivars' response to mobile Al toxicity

	Filter paper-based screening method					
	root length			hypocotyl length		
	cultivar	Al	cultivar × Al	cultivar	Al	cultivar × Al
Sum of squares	365.8	6746.8	703.9	852.3	3435.1	527.7
Degree of freedom	12	4	48	12	4	48
Mean square	30.5	1686.7	14.7	71.0	858.8	11.0
F-ratio	4.3	239.3	2.1	21.7	262.6	3.4
P-value	0.0001	0.0000	0.0030	0.0000	0.0000	0.0000
	Hydroponic screening method					
	root length			hypocotyl length		
	cultivar	Al	cultivar × Al	cultivar	Al	cultivar × Al
Sum of squares	2144.7	7184.05	1067.1	1764.8	888.9	258.3
Degree of freedom	12	4	48	12	4	48
Mean square	178.7	1796.0	22.2	147.1	222.2	5.4
F-ratio	20.1	201.5	2.5	24.1	36.4	0.9
P-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.6858
	Soil-based screening method					
	root length			hypocotyl length		
	cultivar	pH	cultivar × pH	cultivar	pH	cultivar × pH
Sum of squares	36.2	1282.69	96.0	163.1	1528.8	169.0
Degree of freedom	12	4	48	12	4	48
Mean square	3.0	320.7	20.0	13.6	382.2	3.5
F-ratio	1.5	155.4	1.0	4	115.1	1.1
P-value	0.1628	0.0000	0.5401	0.001	0.0000	0.4091

of roots and hypocotyls in acid soil (pH 4.3) was 2.8 and 3.9 times lower, respectively compared with the control treatment (pH 7.2) (Table 3).

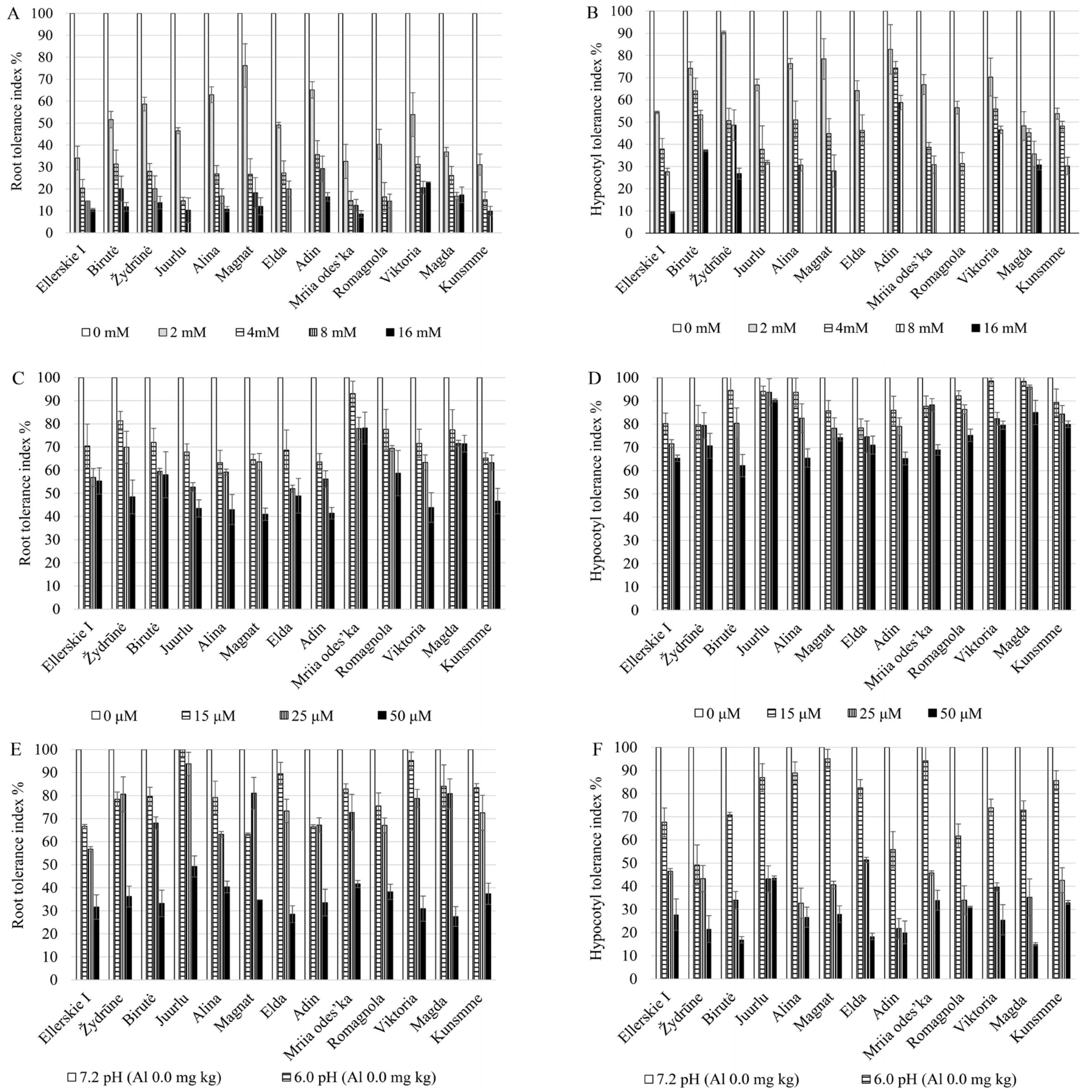
Table 3. The root and hypocotyl length (mm) of *Medicago sativa* using three screening methods

Filter paper-based method			Hydroponic method			Soil-based method		
$AlCl_3$ mM concentration	root length	hypocotyl length	$AlCl_3$ µM concentration	root length	hypocotyl length	pH	root length	hypocotyl length
0	21.8 d	16.5 e	0	34.5 e	22.6 d	7.2	14.5 d	12.2 e
2	10.2 c	11.2 d	5	30.3 d	21.2 cd	6.6	12.5 c	8.9 c
4	5.0 b	7.7 c	15	24.6 c	20.1 bc	6.0	11.8 c	10.4 d
8	3.6 ab	5.2 b	25	21.4 b	18.6 b	4.5	10.6 b	4.7 b
16	1.9 a	1.4 a	50	17.6 a	16.4 a	4.3	5.2 a	3.1 a

Note. Values within a column followed by the same letter are not significantly different ($P > 0.05$).

Filter paper-based screening method. The Al tolerance of plants is commonly evaluated during seedling stage, which might be more critical than later stages of growth (Meriño-Gergichevich et al., 2010). The Al toxicity is associated with severe changes in root morphology. Briefly, it results in curved, swollen, cracked, brownish, stubby and stiff root apices. The criteria most often utilized for evaluating Al toxicity are measurements of the growth, number, colour and branching pattern of the root (Scheffer-Basso, Priori, 2015). In our experiment, it was clear that Al inhibited root and hypocotyl elongation of *M. sativa* (Fig.). Those effects become much more evident with increased Al concentrations (Pan et al., 2008).

Root and hypocotyl length of the control treatment ranged between 11.8–35.0 and 10.8–24.3 cm, respectively. At the low (0, 2 and 4 mM $AlCl_3$) Al



Note. ± bars denote SE of the mean.

Figure. The root and hypocotyl tolerance index of *Medicago sativa* using three screening methods: filter paper-based (A, B), hydroponic (C, D) and soil-based (E, F) concentrations, the hypocotyl and root length significantly differed (Table 3). The root lengths of the cultivars were more similar than hypocotyl lengths at 0 mM $AlCl_3$. Among the tested cultivars, root and hypocotyl growth was more inhibited at 8 and 16 mM $AlCl_3$ (Table 4).

At 8 mM $AlCl_3$ concentration, the root and hypocotyl length was 6.0 and 3.2 times lower, respectively compared with the control (0 mM $AlCl_3$); at 16 mM $AlCl_3$ concentration it was 11.4 and 11.7 times lower, respectively compared with the control (0 mM $AlCl_3$) (Table 3). The decrease in root and hypocotyl growth may be due to the role of Al in causing plant etiolation and reducing bicipital auxin flow in distal part of the transition zone (Zhang et al., 2014). As a part of natural selection, plants have evolved some specific mechanism to cope with Al toxicity (Arunakumara et al., 2013).

The resistant cultivars 'Birutė', 'Žydrūnė', 'Adin', 'Magnat', 'Viktoria' and 'Magda' showed the best results of root tolerance index at 16 mM $AlCl_3$: it was 11.9, 13.8, 16.4, 12.1, 23.2 and 17.3 %, respectively (Fig. A). However, 'Birutė', 'Žydrūnė' and 'Magda' showed the best hypocotyl tolerance index at 16 mM $AlCl_3$. The hypocotyl tolerance index of these cultivars ranged from 26.8% to 37.2% at 16 mM $AlCl_3$ (Fig. B). The sensitive cultivars 'Elda' and 'Romagnola' did not elongate hypocotyls at 8 and 16 mM $AlCl_3$. Also, the roots did not elongate at 16 mM $AlCl_3$. The 'Juurlu' did not elongate hypocotyls and roots at 16 mM $AlCl_3$ (Table 4).

Table 4. The root and hypocotyl length (mm) of *Medicago sativa* using filter paper-based screening method

Cultivar	Country of origin	$AlCl_3$ mM concentration					
		0	8	16	0	8	16
		root length			hypocotyl length		
Ellerskie I	Canada	24.9 bc	3.6 a-e	2.6 bcd	20.4 cd	5.6 bcd	1.9 b
Birutė	Lithuania	24.5 bc	4.9 de	2.9 cde	18.7 bc	10.0 e	7.0 e
Žydrūnė	Lithuania	25.4 bc	5.1 e	3.5 ef	20.9 cd	10.2 e	5.6 d
Juurlu	Estonia	21.8 ab	2.3 a	0.0 a	12.5 a	4.0 b	0.0 a
Alina	Romania	16.5 ab	2.8 ab	1.8 b	14.4 ab	4.4 bc	0.0 a
Magnat	Romania	17.7 ab	3.2 a-c	2.1 bc	20.3 cd	5.7 bcd	0.0 a
Elda	Estonia	18.6 ab	3.7 a-e	0.0 a	12.7 a	0.0 a	0.0 a
Adin	Romania	11.8 a	3.5 a-d	1.9 b	10.8 a	6.3 cd	0.0 a
Mriia odes'ka	Ukraine	35.0 c	4.4 def	3.0 de	24.3 d	7.5 d	0.0 a
Romagnola	Italy	26.7 bc	3.9 b-e	0.0 a	21.6 cd	0.0 a	0.0 a
Viktoria	Czech Republic	17.3 ab	3.6 a-e	4.0 f	11.7 a	5.4 bcd	0.0 a
Magda	Czech Republic	19.6 ab	3.3 abc	3.4 def	13.7 ab	4.9 bc	4.2 c
Kunsmme	Estonia	23.6 b	2.3 ab	0.0 a	12.0 a	3.6 b	0.0 a

Note. Values within a column followed by the same letter are not significantly different ($P > 0.05$).

Hydroponic screening method. A significant cultivar and Al interaction was detected for the variable root length in the analysis of variance (ANOVA). It is clear that all cultivars exhibited a very similar behaviour in response to the variation of Al concentrations. In a more accurate analysis, it can be viewed through plotting of the mean values of root length that differed most at 0 and 50 μM concentrations of Al. The root length significantly differed at other concentrations. However, the hypocotyl length differed less at 0 and 5 μM $AlCl_3$. Nevertheless, significant differences between hypocotyl lengths were determined at all concentrations (Table 3).

The differences in root and hypocotyl lengths at the different concentrations allow the diversity between various cultivars to be assessed. It can be inferred that the concentrations of 25 and 50 μM $AlCl_3$ were very toxic, resulting in very noticeable damage to the root and hypocotyl length of all cultivars. The root and hypocotyl lengths significantly differed at 25 μM $AlCl_3$, they were 1.6 and 1.2 times lower, respectively, and 2.0 and 1.4 times lower at 50 μM $AlCl_3$ compared with the control treatment (0 μM $AlCl_3$) (Table 3).

The highest 50 μM $AlCl_3$ concentration was the most effective for selection of cultivars. Some cultivars appeared to be less affected by the highest Al concentration. The growth of 'Ellerskie I', 'Elda' and 'Mriia odes'ka' was least impacted at 50 μM $AlCl_3$, their root length was 21.2, 19.6 and 21.7 mm, respectively. The hypocotyl length of 'Žydrūnė', 'Romagnola' and 'Viktoria' was the longest – 20.7, 18.4 and 19.7 mm, respectively, and least affected by Al at 50 μM $AlCl_3$ (Table 5).

According to Leônidas et al. (2012), the main Al damage to plant development is primarily related to cell death of the tissues in direct contact with the toxic element, in this case roots, which, therefore, should exhibit more pronounced symptoms of toxicity of this metal. The slower growth of the seedling aerial part occurs due to side effects related to the observed lower root development (Zhang et al., 2007). When comparing

Table 5. The root and hypocotyl length (mm) of *Medicago sativa* using hydroponic screening method

Cultivar	Country of origin	$AlCl_3$ μM concentration					
		0	25	50	0	25	50
		root length			hypocotyl length		
Ellerskie I	Canada	38.4 def	21.8 b-e	21.2 cd	26.9 ef	19.3 bcd	17.6 bcd
Birutė	Lithuania	31.9 bcd	19.0 ab	18.5 bcd	27.3 ef	22.0 de	17.0 bcd
Žydrūnė	Lithuania	35.1 cde	24.5 de	17.0 bc	29.2 f	23.2 e	20.7 d
Juurlu	Estonia	38.5 def	20.3 a-d	16.7 bc	16.6 a	15.6 a	15.0 ab
Alina	Romania	43.7 f	25.8 e	18.8 cd	17.5 a	14.4 a	11.4 a
Magnat	Romania	41.3 ef	26.3 e	16.9 bc	20.6 abc	16.1 ab	15.3 abc
Elda	Estonia	40.0 ef	20.8 a-d	19.6 cd	21.6 bcd	16.1 ab	15.3 abc
Adin	Romania	28.9 abc	16.2 a	11.9 a	22.0 bcd	17.4 abc	14.4 ab
Mriia odes'ka	Ukraine	27.8 ab	21.7 b-e	21.7 d	24.8 de	21.9 de	17.1 bcd
Romagnola	Italy	28.6 abc	19.9 abc	16.8 bc	24.4 cde	21.1 de	18.4 bcd
Viktoria	Czech Republic	32.1 bcd	20.3 a-d	14.1 ab	24.7 de	20.3 cde	19.7 cd
Magda	Czech Republic	24.6 a	17.6 ab	17.6 bcd	18.3 ab	17.5 abc	15.5 abc
Kunsmme	Estonia	37.7 def	23.8 cde	17.6 bcd	20.4 ab	17.2 abc	16.3 bcd

Note. Values within a column followed by the same letter are not significantly different ($P > 0.05$).

the results of root and hypocotyl elongation among all cultivars in the absence of Al stress, 'Birutė', 'Mriia odes'ka', 'Romagnola' and 'Magda' showed the best performance. The root tolerance index of these cultivars was 58.0, 78.2, 58.7 and 71.4 %, respectively at 50 μM $AlCl_3$ compared with the control treatment (0 μM $AlCl_3$) (Fig. C). However, the hypocotyl length was very similar and ranged from 11.4 to 20.7 mm at 50 μM $AlCl_3$ (Table 5). Cultivars 'Žydrūnė', 'Juurlu', 'Magnat', 'Romagnola', 'Viktoria', 'Magda' and 'Kunsmme' showed the best performance for hypocotyl length. The hypocotyl tolerance index of these cultivars ranged from 70.7% to 85.0% compared with the control treatment (0 μM $AlCl_3$) (Fig. D).

Soil-based screening method in greenhouse conditions. Acid soils affect the plant growth by altering various physiological processes (Skuodienė et al., 2017; Tomchuk, 2018). External pH affects the root growth by causing a severe reduction in shoot and root growth of *M. sativa* and other plants. According to Zhou et al. (2016), with respect to root growth three responses can be observed depending on the concentration of Al. The Al concentrations in the soil with pH 4.5 and pH 4.3 were very toxic for root and hypocotyl growth (Table 1). The length of root and hypocotyl was significantly 1.4 and 2.6 times lower at pH 4.5, respectively compared with the control treatment at pH 7.2. Also, there were determined differences between lengths of roots and hypocotyls (2.8 and 3.9 times lower, respectively) at pH 4.3 compared with the control soil pH 7.2 (Table 3).

The hypocotyls of cultivars were more inhibited by Al toxicity, hypocotyl length ranged from 2.0 to 3.8 cm at pH 4.3. However, hypocotyl length significant differed at pH 4.5, it ranged from 2.9 to 7.2 cm. The significant differences in hypocotyl length among the tested *M. sativa* cultivars are shown in Table 6. The hypocotyl growth depended on growth and development of roots and cultivars' reaction to Al toxicity.

In acid soils, the toxicity of Al affected growth and development of roots. The growth and development of hypocotyls depended on root development. At the same time Al toxicity affected the length of hypocotyls. The root and hypocotyl lengths were significantly 2.0 and 1.5 times lower, respectively at pH 4.3, compared with pH 4.5 (Table 3). The cultivar and Al interaction showed that the lengths of roots and hypocotyls depended more on the cultivars' reaction to Al toxicity under different soil pH levels (Table 2).

The results of the current experiment also demonstrated the variation in *M. sativa* cultivars for tolerance to soil pH. Clearly lower response to Al toxicity was detected in 'Žydrūnė' and 'Magnat' – the roots were the longest (12.5 cm) at 4.5 pH compared with the other cultivars tested. A lower response to Al toxicity was also observed in 'Ellerskie', 'Žydrūnė', 'Alina', 'Magnat', 'Mriia odes'ka', 'Romagnola' and 'Kunsmme'. The root length of these cultivars was similar – it ranged from 5.4 to 5.9 cm at pH 4.3 (Table 6). However, stronger Al toxicity was seen at pH 4.5 (Table 6). About 69.2% of cultivars were more damaged by Al toxicity at pH 4.5.

Table 6. The root and hypocotyl length (cm) of *Medicago sativa* under soil-based screening method in greenhouse conditions

Cultivar	Country of origin	pH of soil					
		7.2	4.5	4.3	7.2	4.5	4.3
		root length			hypocotyl length		
Ellerskie I	Canada	17.2 b	9.7 ab	5.4 cd	13.6 bcd	6.3 de	3.8 b
Birutė	Lithuania	14.8 ab	10.1 ab	4.9 bc	14.6 d	4.9 bcd	2.5 ab
Žydrūnė	Lithuania	15.5 ab	12.5 d	5.6 cd	12.7 abcd	5.5 cde	2.7 ab
Juurlu	Estonia	12.7 a	11.9 cd	6.3 d	7.8 a	3.4 ab	3.4 ab
Alina	Romania	14.2 ab	8.9 a	5.7 cd	12.8 bcd	4.2 abc	3.4 ab
Magnat	Romania	15.4 ab	12.5 d	5.4 cd	11.6 abcd	4.7 abcd	3.3 ab
Elda	Estonia	14.0 ab	10.3 ab	4.0 ab	13.9 bcd	7.2 e	2.6 ab
Adin	Romania	14.8 ab	9.9 ab	5.0 bc	13.5 bcd	2.9 a	2.7 ab
Mriia odes'ka	Ukraine	14.1 ab	10.3 ab	5.9 cd	9.5 abc	4.3 abc	3.2 ab
Romagnola	Italy	14.3 ab	9.6 ab	5.5 cd	11.6 abcd	3.9 abc	3.6 ab
Viktoria	Czech Republic	13.8 ab	10.8 bc	4.3 ab	14.3 cd	5.7 cde	3.7 b
Magda	Czech Republic	13.2 a	10.7 bc	3.6 a	13.2 bcd	4.6 abcd	2.0 a
Kunsmme	Estonia	15.1 ab	10.9 bc	5.6 cd	9.3 ab	4.0 abc	3.1 ab

Note. Within a column values followed by the same letter are not significantly different ($P > 0.05$).

The hypocotyls of the 'Ellerskie I', 'Žydrūnė', 'Elda' and 'Viktoria' were significantly 1.5 times longer compared with those of the other cultivars. The hypocotyl length of these cultivars ranged from 5.5 to 7.2 cm at pH 4.5 and from 2.5 to 3.8 cm at pH 4.3. However, the hypocotyl length of these cultivars significantly differed from that of the cultivar 'Magda' (Table 6). The root and hypocotyl lengths were similar (14.5 and 12.2 cm, respectively) at pH 7.2 (Table 3).

The root lengths of cultivars less differed by resistance for Al toxicity at pH 4.5. Therefore, selection of the most resistant cultivars at pH 4.5 was very difficult, because the root tolerance index was very similar and high, and ranged from 56.7% to 93.8%. The cultivars were more exposed to Al toxicity stress at pH 4.3. The 'Juurlu', 'Alina', 'Mriia odes'ka', 'Romagnola' and 'Kunsmme' were characterised by the root tolerance index. The root tolerance index of these cultivars was similar and ranged from 37.40% to 49.22% (Fig. E). The 'Juurlu', 'Mriia odes'ka', 'Romagnola' and 'Kunsmme' showed the best hypocotyl tolerance index ranging from 31.0% to 43.6% (Fig. F).

The Al and cultivar interaction revealed that Al influenced the growth of roots and hypocotyls of the *M. sativa* cultivars and showed that these traits can be used in selection. It has been demonstrated that cultivars selected for superior root and hypocotyl growth using filter paper-based, hydroponic and soil-based methods, also confer significant increases in root growth in highly acidic soil that is high in mobile Al (Choudhary et al., 2011).

The most resistant seedlings at early plant stage of development can be screened quickly using a filter paper-based method. Significant correlations between hypocotyl lengths at different concentrations of Al ($r = 0.575$–0.726, $P < 0.01$) showed strongly inhibited hypocotyl growth in the filter-paper based method. Also, the growth of roots was strongly affected by the toxicity of Al using this method. This was indicated by significant correlations between root lengths at different Al concentrations ($r = 0.555$–0.8, $P < 0.01$). The effect of Al toxicity on root and hypocotyl growth was seen using the hydroponics method. Correlation coefficients between roots and between hypocotyls were determined at different concentrations of Al ($r = 0.582$–0.872, $P < 0.01$, and $r = 0.586$– 0.918, $P < 0.01$, respectively). The effect of Al toxicity was seen using the soil-based method. This was shown by the mean correlation between root and hypocotyl lengths ($r = 0.573$, $P < 0.01$) at pH 4.3.

The root and hypocotyl length and other parameters could be used to assess Al tolerance, but selection of resistant roots and hypocotyls was slow in acidic soils. The processes taking place in the root react very strongly to the toxicity of Al during growth. The process of root development takes a long time by studying resistance under a soil-based method. The development of roots depended on successful nodulation and rhizobium in acidic soil conditions. Therefore, improved *M. sativa* nodulation will most likely be achieved thought the selection of compatible rhizobium in acidic soil and selection of *M. sativa* plants for increased capacity to nodulate in acidic conditions (Charman et al., 2008; Hayes et al., 2011). Root development (elongation and absorption), translocation and utilization of nutrients may make major differences in tolerant and intolerant cultivars to acid soils. Cultivars that are efficient nutrient utilizers might be useful in breeding for more efficient cultivars for mineral-stressed-acid soils (Gupta et al., 2013; Bose et al., 2015).

The screening methods tested demonstrated that the Al toxicity influenced development and growth of roots and hypocotyls of *M. sativa*. Different root and hypocotyl lengths obtained by studying different methods allowed the selection of resistant material as described in the literature (Pan et al., 2008; Hayes et al., 2011; Khu et al., 2012). However, the hydroponic method was effective in that it was possible to estimate seedling length as well as adult seedling growth in other methods (Köpp et al., 2011). The latter methods, especially soil-based screening, are hardly suitable for screening of thousands of seeds per cultivar. However, these methods could be successfully applied for the final screening steps, when several populations have been selected after multiple cycles of recurrent selection.

Development of *M. sativa* breeding populations requires evaluating and selecting several hundreds of plants with desired agronomic traits. Cultivars originating in the countries with hot and dry climates are usually crossed with that originating in Europe. The results of the experiment revealed that 'Mriia odes'ka' and 'Romagnola' are more suitable for crossbreeding with other cultivars from Lithuania, Estonia, Czech Republic and Romania.

Conclusions

1. The results of the experiment suggested that root and hypocotyl length of *Medicago sativa* was most influenced by aluminium (Al) toxicity at 8 and 16 mM $AlCl_3$ (aluminium chloride) using the filter paper-based screening method. The root and hypocotyl tolerance index was 17.2% and 32.5% lower at 8 mM $AlCl_3$ and 9.6% and 8.0% lower at 16 mM $AlCl_3$ compared with the control treatment (0 mM $AlCl_3$). Of the 13 investigated cultivars, 'Birutė', 'Žydrūnė' and 'Magda' were found to be the most tolerant to Al toxicity at 16 mM $AlCl_3$.

2. The root growth and elongation were more inhibited by Al toxicity compared with hypocotyl growth at 50 µM $AlCl_3$ using the hydroponic screening method. The root and hypocotyl tolerance index was 52.2% and 73.3% lower, respectively at 50 µM $AlCl_3$ compared with the control treatment (0 µM $AlCl_3$). Cultivar 'Magda' was distinguished by the longest hypocotyl and root lengths at 50 µM $AlCl_3$.

3. The growth of hypocotyls was by 1.4 times lower compared with root growth at soil pH 4.3 using the soil-based screening method. The root and hypocotyl tolerance index was 73.5% and 39.3% at pH 4.5, and 35.6% and 26.3% at pH 4.3 compared with pH 7.2. The cultivars 'Juurlu', 'Mriia odes'ka', 'Romagnola' and 'Kunsmme' were distinguished by the longest hypocotyl and root length at pH 4.3.

4. The results of this experiment showed that the filter paper-based and hydroponic methods can be used at early stage of development of seedlings to focus selection on cultivars that are responding specially to Al toxicity. The soil-based method is more stringent than either the filter-paper based or hydroponic methods for *M. sativa*. The most tolerant to Al toxicity cultivars 'Birutė', 'Magda', 'Magnat', 'Romagnola', 'Juurlu' and 'Mriia odes'ka' under laboratory conditions could grow in acid soil under natural conditions. A large percentage of plants resistant to acid soils of these cultivars can be used for the development of new accessions.

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Medicago sativa atsakas į judriojo aliuminio toksiškumą daigelių tarpsniu

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Santrauka

Rūgščiuose dirvožemiuose pasėlių produktyvumą labiausiai riboja judriojo aliuminio (Al) toksiškumas. Tyrimo tikslas – įvertinti mėlynžiedės ir margažiedės liucernų (*Medicago sativa* L.) veislių vystymąsi ir atsaką į judriojo Al toksiškumą. Tyrimo metu, taikant tris atrankos metodus: Petri lėkštelių, hidroponikos ir vegetacinių bandymų, buvo vertinta 13 veislių *M. sativa* tolerancija judriajam Al. Analizuoti biologiniai rodikliai: hipokotilių ilgis, šaknų ilgis ir hipokotilių bei šaknų ilgių tolerantiškumo indeksai. Toksinis judriojo Al poveikis pasireiškė visiems *M. sativa* biologiniams rodikliams; tai rodo koreliaciniai ryšiai tarp biologinių rodiklių ir atrankos metodų. Taikant Petri lėkštelių atrankos metodą (16 mM $AlCl_3$), šaknų ir hipokotilių ilgių tolerantiškumo indeksai svyravo atitinkamai nuo 0,0 iki 23,2 % ir nuo 0,0 iki 37,2 %. Hidroponikos ir vegetaciniuose bandymuose rūgščiame dirvožemyje *M. sativa* veislės labiau skyrėsi tolerantiškumu judriojo Al toksiškumui. Taikant hidroponikos metodą (50 µM $AlCl_3$), hipokotilių ir šaknų ilgių tolerantiškumo indeksai pakilo nuo 41,0 iki 78,2 % ir nuo 62,1 iki 90,2 %. Rūgščiame dirvožemyje, kurio pH 4,5 ir 4,3, hipokotiliai buvo labiau paveikti Al toksiškumo nei šaknys. Rūgščiame dirvožemyje, kurio pH 4,5, šaknų ir hipokotilių ilgių tolerantiškumo indeksai buvo 73,5 ir 39,3 %, o dirvožemyje, kurio pH 4,3 – 35,6 ir 26,3 %. Veislės ‘Mriia odes’ka’, ‘Romagnola’, ‘Kunsmme’ ir ‘Juurlu’ išsiskyrė hipokotilių ir šaknų tolerantiškumo indeksais hidroponikos bei vegetaciniuose bandymuose rūgščiame dirvožemyje.

Taikant Petri lėkštelių ir hidroponikos atrankos metodus, veislės ‘Žydrūnė’, ‘Birutė’, ‘Magnat’, ‘Viktoria’ bei ‘Magda’ išsiskyrė hipokotilių ir šaknų tolerantiškumo indeksais. Šios veislės laikomos tolerantiškomis ir gali būti panaudotos kaip donorai kuriant judriajam Al atsparius genotipus ir vykdant tolesnę *M. sativa* atsparumo judriajam Al selekciją.

Reikšminiai žodžiai: liucerna, aliuminio toksiškumas, hipokotilis, šaknis, atrankos metodas, tolerancijos indeksas.