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Screening and Identification of Aluminium Toxicity Tolerant Lentil (*Lens culinaris* Medik.) RILs based on Root Growth Studies and Organic Acid Exudation under Hydroponics

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ABSTRACT

Background: Aluminium (Al) stress is among the prime limitations of crop production including Lentil, in acidic soils as Al solubilizes into phytotoxic forms at low pH causing root growth inhibition, minimizing plant-vigor and yield. The current study was performed to identify the Al tolerant RILs based on root-regrowth studies, short term growth culture and organic acid exudation in reaction to Al toxicity under hydroponics.

Methods: The study involved screening for Al toxicity tolerance in recombinant inbred lines (RILs) population of lentil through root re-growth studies, short term growth response method under hydroponics treated with toxic concentration of Al (148 µM), organic acid exudation using HPLC and correlation analysis among the traits.

Result: ANOVA for root and shoot traits revealed presence of highly significant genotypic differences for all the traits. High GCV along with high H^2_{bs} and GA% were observed for the traits revealing the influence of additive genes and suggested reliable selection of these traits for Al toxicity tolerance. Citric acid was exudated in highest amount in all the genotypes and was positively and significantly correlated with RRG. Based on the hydroponics study and organic acid exudation, RILs identified as tolerant were LRIL-10, LRIL-37, LRIL-68, LRIL-96, LRIL-97, LRIL-113, LRIL-125, LRIL-133, LRIL-143, LRIL-144 and LRIL-148. With further evaluation, these RILs may serve as important Al toxicity tolerant varieties suitable for acidic soil conditions. Also, these lines may serve as donors of Al toxicity loci and mapping of Al tolerance genes.

Key words: Aluminium toxicity, Hydroponics, Lentil, Organic acid, Root re-growth.

INTRODUCTION

Lentil, (*Lens culinaris* Medik), which is among the most significant pulse crops consumed widely throughout the world, forms an essential component of traditional Indian meals. Being one of the earliest crop species to be domesticated, it is adaptable to a variety of soils and environments and is hardy in nature with minimal water requirement (Asakereh *et al*., 2010). Lentils constitute a nutrient-dense pulse crop, containing high protein, complex carbohydrates, vital minerals, vitamins and high energy (Eesha *et al*., 2024; Dhull *et al*., 2023; Noura *et al*., 2023; Joshi *et al*., 2017). Globally lentil is cultivated in over 40 countries with the leading lentil producing countries like India, Canada, Turkey, Syria, Australia, Nepal and United States contributing to a total lentil production of about 4.9 million tons. These countries have considerable land area under acidic soils along with a significant problem of aluminium (Al) toxicity (Singh *et al*., 2012). Aluminium (Al), which is among the major metals in the earth's crust, is regarded as the primary abiotic stress causing 25-80% yield reductions in crops cultivated on soils containing excessive Al (Singh *et al*., 2012). Al stress is responsible for decreased production in crops like lentil in acidic soils as Al solubilizes into phytotoxic forms at low pH, explicitly below 5.0, causing phosphorus deficiency, root growth inhibition, lowering plant vigor and yield. Among various Al toxicity symptoms, rapid inhibition of root growth, is the first visible symptom (Kochian *et al*., 2005; Minh *et al*., 2024) which has served as a biomarker to estimate Al sensitivity in crop plants, generally evaluated under hydroponics culture. To overcome the harmful consequences of Al toxicity, most plants express two different types of adaptive mechanisms (Singh *et al*., 2016). The first is the exclusion mechanism, which essentially modifies pH by blocking Al through the excretion of organic acids and chelating toxic Al^{3+} (Singh *et al*., 2016; Kinraide *et al*., 2005). In the second mechanism, Al is absorbed and detoxified by various proteins, phenolic compounds, organic acids and enzymes

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that attach to Al^{3+} and separate it into vacuoles, therefore reducing the total Al stress within the cell (Basu *et al*., 1994; Jones and Brasington, 1998).

Among the potential ways to mitigate Al toxicity, the most feasible and environmentally friendly method is the breeding for Al tolerant varieties. Screening of genotypes for root growth studies under hydroponics culture along with the evaluation of organic acid secreted from roots under toxic levels of Al provides a reliable and effective method to identify Al toxicity tolerant genotypes (Silva *et al*., 2001; Singh *et al*., 2016) and are advantageous than field screening as Al concentrations may vary throughout the field and may get influenced due to interactions with other soil and environmental components masking the resulting expression of Al tolerance (Butare *et al*., 2011). In previous studies, genotypes were successfully differentiated into tolerant and susceptible to Al toxicity based on various growth parameters and root staining under hydroponics culture in legumes like pea, *Cicer*, common bean and lentil (Ansari *et al*., 2023; Vance *et al*., 2021; dos Santos Neto *et al*., 2020; Singh *et al*., 2016; Kulkarni *et al*., 2021). Exudation of different organic acids like malate, citrate, succinate oxalate and acetate from root zone in response to Al toxicity under hydroponics were observed in pea (Kichigina *et al*., 2017), soybean (Shi *et al*., 2020), chickpea (Sharma *et al*., 2016), alfalfa (Ma *et al*., 2020) and lentil (Singh *et al*., 2021), which was suggested as a principal mechanism of Al tolerance in these crops used to differentiate tolerant and susceptible genotypes. The present investigation was carried out to screen lentil genotypes for Al tolerance under hydroponics and identify tolerant genotypes based on root growth studies and organic acid exudation in response to toxic levels of Al.

MATERIALS AND METHODS

The present experiment was conducted in the Plant Breeding laboratory of School of Crop Improvement, CPGS-AS, Central Agricultural University (Imphal), Umiam, Meghalaya during *rabi* 2021-22 and 2022-23. The experimental material consisted of 154 genotypes including 150 RILs developed from cross between Al sensitive parent BM-4 and Al tolerant parent L-4602 with two checks (PDL-1 and DPL-62) and two parents (BM-4 and L-4602). All the genotypes were screened under hydroponic culture for root re-growth studies and short- term growth response studies for 7 days at toxic levels of Al (148 µM) under acidic conditions following the method of Singh *et al*. (2012). Further, the exudation of organic acids from the roots of lentil RILs in response to Al stress was also analyzed following Zhao *et al*., (2003) and Singh *et al*., (2016).

Short term growth response method

The seeds of the 154 genotypes were disinfected with 0.1% $HgCl_2$ for 2-3 minutes and germinated on moist germination paper. Seedlings were selected with uniform root lengths and transferred to nutrient solution containing a combination of macronutrients and micronutrients with 148 µM Al (Table 1) in plastic trays. The pH of the nutrient solution was maintained at pH 4.8 for all treatments using 1N NaOH or 1N HCl. The seedlings were evaluated in a completely randomized design with three replications and five seedlings were transferred for each replication. After 7 days, observations were recorded on various seedling traits like root length (RL), shoot length (SL), root fresh weight (RFW), root dry weight (RDW), shoot fresh weight (SFW) and shoot dry weight (SDW). The seedling roots were observed under Root Scanner (Biovis PSM R2000) and root morphology parameters *viz*., total root length (TRL) (cm), total root surface area (TSA) (cm^2) and root volume (RV) (cm^3) were recorded. The fresh weight of the roots and shoots of each seedling was recorded, followed by air drying and oven drying of the roots and shoots for 48 hours at 65°C. After complete drying, measurements of root and shoot weight of each seedling were recorded using electronic balance and average was worked out for each genotype. Root re-growth studies after Haematoxylin staining was performed following the procedure by Singh *et al.* (2012) as a reliable parameter to distinguish the genotypes for their Al toxicity tolerance. RRG was scored as sensitive (<0.5 cm), moderately tolerant (0.5-1 cm) and tolerant (>1 cm). Aluminium content in dried root samples was determined using Atomic Absorption Spectrophotometer (Model-Elico SL-194).

Determination of organic acid exudation

Seven days old seedlings were selected from each genotype having almost similar lengths and placed into separate plastic containers containing 0.5 mM $CaCl_2$ solution and 148 µM Al ($AlCl_3.6H_2O$, pH 4.5) in 60 ml of doubled distilled water Root exudates were collected at 3 hours after the beginning of Al treatments as highest organic acid exudation was recorded at 3 hours of Al treatment (Singh *et al*., 2016). Root exudates (2 ml) were collected from each container and centrifuged at 20,000 rpm for 20 minutes and the supernatant was collected in

Table 1: Chemicals used for preparation of nutrient solution for short term growth response method.

Chemicals	Concentration
KNO_3	0.5 mM
$Ca(NO_3)_2.4H_2O$	0.5 mM
$MgSO_4.7H_2O$	0.2 mM
KH_2PO_4	0.1 mM
KCl	50 µM
H_3BO_3	46 µM
Fe-EDTA	20 µM
$MnCl_2.4H_2O$	2 µM
$ZnSO_4.7H_2O$	1 µM
$CuSO_4.5H_2O$	0.3 µM
$NaMoO_4.2H_2O$	0.5 µM
$AlCl_3.6H_20$	148 µM

separate tubes followed by purification of the root exudates using Nalgene Syringe Filter PTFE membrane with mesh of 0.45 µm (Thermo Fisher Scientific). Purified extract (20 µL) was injected into High performance liquid chromatography (HPLC) (Waters, 2489 UV/Visible Detector, Atlantis dC 18 5 µm, 4.6 × 160 mm Column) and run isocratically at 30°C using 20 mM phosphate buffer (NaH_2PO_4 + phosphoric acid), at pH 2.5 as the mobile phase at a flow rate 1 ml min^{-1}. The specific organic acids were detected using UV spectrophotometer at 210 nm wavelength based on the comparison of retention time of the samples with standards. Observations were recorded on the types and relative amounts of organic acid exudates released from RILs as a response to Al stress tolerance.

Formula used for conversion of area under the curve of HPLC Chromatogram as per Kupiec (2004):

$$\text{Conc.unknown} = \frac{\text{Area.unknown}}{\text{Area.known}} \times \text{Conc.known}$$

Statistical analysis

Analysis of variance (ANOVA) was performed for short term growth culture method. Mean performance and genetic parameters of variation were estimated for short term growth culture method and organic acid exudation. Correlation analysis between organic acid content and Al tolerance observed under hydroponics in terms of root growth and shoot growth parameters of the RILs was performed. GENES software was used for conducting all the statistical analysis.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA) and mean performance for root re-growth and short-term growth response method

Analysis of variance showed highly significant differences due to genotypes for all the nine traits *viz*., root length, shoot length, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, total root length, total root surface area and total root volume, suggesting the presence of sufficient variability in the genotypes under study for these traits (Table 2).

Considering the importance of root evaluation for the determination of Al-tolerant genotypes, in our study mean performance of various parameters of the root system and shoot system were evaluated in the genotypes (Fig 1). RRG in the genotypes ranged from 0.13- 2.38 cm and 54 RILs were identified as tolerant based on RRG estimates out of which LRIL-97, LRIL-136, LRIL-48, LRIL-37, LRIL-130, LRIL-143, LRIL-148, LRIL-116 and LRIL-125 and LRIL-139 were the genotypes identified with highest root regrowth. Singh *et al*. (2016) revealed that in lentil genotypes, RRG ranged from 1.20-1.60 cm in resistant cultivars, while it ranged from 0-0.47 cm in sensitive genotypes. Moderately resistant cultivars showed a range of 0.43-1.00 cm. Similarly, in chickpea genotypes evaluated for root regrowth, it was observed that tolerant parents had long root regrowth (3.45 and 2.58 cm) compared with sensitive parents that displayed shorter root regrowth (0.44 and 0.41 cm) (Singh and Raje, 2011). The longest primary

Table 2: Analysis of variance for root and shoot traits in 154 genotypes of lentil from root regrowth (RRG) studies and short-term growth experiments under hydroponics.

Source of variation	DF	RL	SL	RFW	SFW	RDW	SDW	TRL	SA	TV
Genotypes	153	13.50**	19.53**	0.00059**	0.00278**	0.000046**	0.000183**	6597.96**	4178.04**	2859.28**
Error	308	0.955	2.94	0.000006	0.000023	0.000005	0.000026	137.84	69.75	241.03
CV%		11.84	9.44	6.57	6.49	13.47	15.74	8.76	7.50	16.73

** = 1% level of significance.

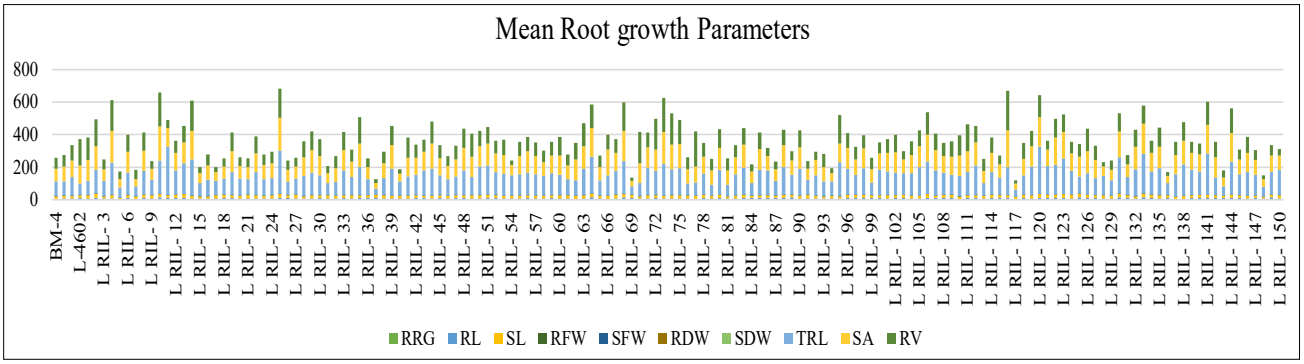

Fig 1: Mean Performance of RILS for various root growth parameters under short term hydroponic study.

roots were observed in the genotypes LRIL-149, LRIL-125, LRIL-133, LRIL-64 and LRIL-89 while the longest shoots were observed in the genotypes LRIL-2 and LRIL-133 followed by LRIL-25, LRIL-124 and LRIL-106. It was observed that not all the genotypes had longer roots and longer shoot length. Studies on lentil conducted by Singh *et al*. (2012) revealed that with gradual increase in levels of Al toxicity, there is progressive decline in root length and shoot length in lentil and suggested that the genotypes with highest root length and shoot length *viz*. ILL-6002 (RL-4.89 cm, SL-7.08 cm), L-7903 (RL-4.90 cm, SL-7.00 cm) and L-4602 (RL-4.90 cm, SL-6.73 cm) were tolerant.

In our study, the maximum root fresh weight was observed in the genotypes LRIL-128 and LRIL-133 followed by LRIL-126 and LRIL-134 which were on par with check PDL-1 and tolerant parent L-4602, while maximum root dry weight was recorded in the genotypes LRIL-21, LRIL-128, LRIL-64 and LRIL-133 which were on par with the tolerant parent L-4602. Shoot fresh weight was recorded to be the highest in the check, PDL-1, followed by LRIL-128 which was at par with tolerant parent L-4602, followed by LRIL-133 which was on par with check DPL-62, followed by LRIL-126. Maximum shoot dry weight was recorded in the Al tolerant parent, L-4602 followed by the genotypes LRIL-128, LRIL-64, LRIL-133 and LRIL-21. Brhane *et al*. (2018) screened accessions of finger millet under hydroponics for Al toxicity tolerance and observed symptoms of significant Al stress in root length, fresh weight, while no distinct and visible symptom were observed in shoot growth. Roy and Bhadra (2014) reported that toxic levels of Al in nutrient solution significantly decreased seedling root growth, number of primary roots, seedling shoot length, number of leaves per seedling, seedling fresh weight and seedling dry weight in rice. Aluminium content in the roots of genotypes screened under hydroponics with Al treatment ranged from 0.678-1.32 mg/g (Fig 2). Based on the higher expression of the root re-growth, root architectural traits and shoot traits in our study, RILs identified as tolerant were LRIL- 4 LRIL- 10, LRIL- 11, LRIL- 13, LRIL- 18, LRIL-22, LRIL- 37, LRIL-43, LRIL-48, LRIL- 63, LRIL- 68, LRIL-80, LRIL-86, LRIL- 96, LRIL- 97, LRIL-99, LRIL-106, LRIL-113, LRIL-116, LRIL-125, LRIL-127, LRIL- 130, LRIL-133, LRIL-143, LRIL- 144 and LRIL-148. It can be suggested that the favourable alleles are expressed fully under Al stress conditions in these genotypes that helped in better expression of these traits resulting in Al tolerance in the corresponding genotypes.

Genetic parameters of variability for RRG and short-term growth response method

GCV, PCV, H^2_{bs} and GA% were estimated for the traits evaluated under hydroponics (Table 3). GCV estimates were recorded as high for all the traits except shoot length for which it was moderate. GCV was highest for the trait shoot fresh weight (40.96%) followed by root regrowth (40.75%), root fresh weight (38.18%) and total root length (34.64%). PCV estimates were higher than GCV for all the traits, emphasising the influence of environment for all the traits. Heritability (H^2_{bs}) estimates were high for all the traits studied and the highest heritability was observed for SFW (97.55%), followed by RFW (96.01%) and TRA (95.15%). Genetic advance estimates were also high for all the traits, where highest GA was estimated for SFW (83.38%) followed by RRG (78.33%) and RFW (77.48%). High heritability coupled with high genetic advance were observed for all the traits suggests that the additive gene effects are most probably responsible in the inheritance of these traits and thus direct selection for these traits would be effective for selecting Al toxicity tolerant genotypes. Similar results were reported by Ambachew and Blair, (2021) in common bean genotypes screened under Al toxic hydroponics system.

Correlation analysis between RRG, root and shoot traits under short term growth culture and Al content

Root length was found to be significantly correlated with shoot length (0.46***) (Fig 3). A highly significant and highly positive correlation was observed between root dry weight and shoot dry weight (0.87***), while shoot fresh weight was significantly correlated with root dry weight (0.39***) and shoot dry weight (0.45***). Root fresh weight was highly positively and highly significantly correlated with shoot

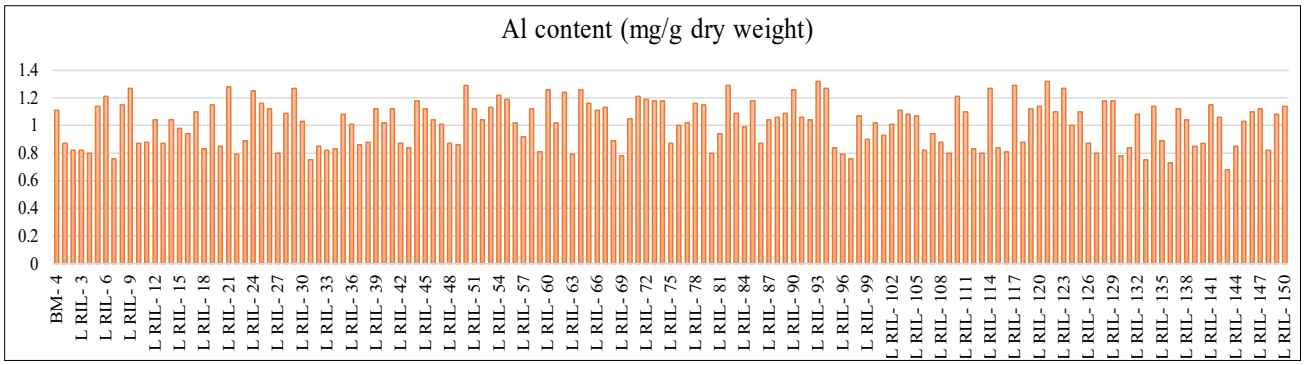

Fig 2: Frequency distribution graph of Aluminium content (mg/g dry weight) in the roots of RILs screened under hydroponics treated with 148 µM Al.

fresh weight (0.98***) and significantly correlated with shoot dry weight and root dry weight. Highly significant and highly positive correlation observed between most of the root and shoot growth parameters infers that selection for these traits will facilitate in the simultaneous selection of the associated traits facilitating in selection of Al toxicity tolerant genotypes. A highly significant negative correlation between root regrowth and Al content (-0.796**) in lentil roots suggested selection of plants with higher root re-growth which will lead to simultaneous selection of tolerant genotypes having lower Al content in their roots. Singh *et al*. (2012) reported that root regrowth after staining showed significant correlation with root and shoot length, dry weight of roots and shoots and pods per plant in lentil. Correlation analysis among six root traits in chickpea genotypes grown in Al solution revealed significant positive correlations among all traits, except the non-significant association observed among root length and shoot length with root weight measurements (Negusse *et al*., 2022). Aluminium concentrations showed a significant negative correlation with root re-growth in lentil genotypes (Singh *et al*., 2012).

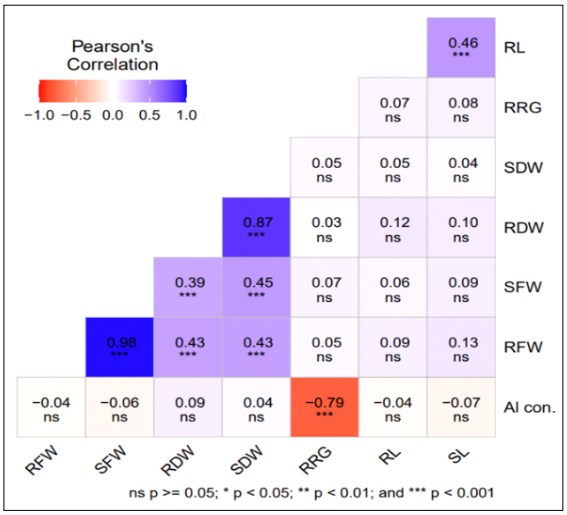

Fig 3: Correlation analysis between root and shoot traits in lentil RILs from root regrowth (RRG) studies and short-term growth experiments.

Organic acid evaluation

It was observed that four important organic acids *viz*. oxalic acid, citric acid, malic acid and fumaric acid were exudated by the lentil roots, out of which citric acid (93.8%) was exudated in the highest amounts followed by malic acid (4.46%), oxalic acid (1.66%) while fumaric acid (0.067%) was exudated in negligible amounts (Fig 4). Most of the RILs identified as tolerant from hydroponics screening exudated higher amounts of organic acids as compared to the sensitive RILs.

Mean performance of the genotypes for release of different organic acids

Oxalic acid exudation in the lentil roots ranged from 0.062-0.601 µg/ml. The RILS exhibiting the highest exudation of OA were LRIL-86 (0.601 µg/ml), LRIL-107 (0.456 µg/ml), LRIL-56 (0.4465 µg/ml), LRIL-42 (0.4374 µg/ml) and LRIL-96 (0.4357 µg/ml). The exudation of citric acid was the highest in response to Al stress, among all the four organic acids and it ranged from 0.622 to 93.01 µg/ml. The genotypes exhibiting the highest exudation of CA were-LRIL-144 (93.010 µg/ml), LRIL-143 (46.377 µg/ml), LRIL-113 (41.655 µg/ml), LRIL-124 (41.586 µg/ml) and LRIL-92 (39.289 µg/ml). The exudation of malic acid ranged from 0 to 11.73 µg/ml. The maximum exudation was observed in LRIL-3 (11.738 µg/ml) followed by, LRIL-10 (7.55 µg/ml), LRIL-144 (4.27 µg/ml), LRIL-119 (2.62 µg/ml) *etc*. Fumaric acid was exudated in negligible amounts in most of the RILs (Fig 4). In soybean genotypes treated with Al, citrate and malate efflux increased in all genotypes initially, but

Table 3: Estimates of genetic parameters of variability for root and shoot traits in 154 genotypes of lentil from root regrowth (RRG) studies and short-term growth experiments under hydroponics.

Characters	Mean	Min	Max	Range	GCV	PCV	Heritability (H^2_{BS})	Genetic advance as per cent of mean
RRG	0.90	0.13	2.38	2.25	40.75	43.68	87.03	78.33
RL	8.25	1.97	12.83	10.86	24.78	27.46	81.40	46.06
SL	18.16	9.40	28.3	18.9	12.95	16.02	65.26	21.54
RFW	0.03	0.01	0.09	0.071	38.18	38.75	96.01	77.48
SFW	0.07	0.02	0.20	0.172	40.96	41.48	97.55	83.38
RDW	0.01	0.007	0.03	0.021	22.99	26.86	73.21	40.52
SDW	0.03	0.015	0.07	0.053	22.53	27.57	66.81	37.94
TRL	133.95	37.54	297.02	259.48	34.64	35.73	93.98	69.18
RA	111.34	31.46	224.10	192.64	33.23	34.07	95.15	66.78
RV	92.80	19.08	243.48	224.4	31.83	35.96	78.35	58.05

Abbreviations: RRG- Root re-growth (cm), RL- Root length (cm), SL- Shoot length (cm), RFW- Root fresh weight (g), SFW- Shoot fresh weight (g), RDW- Root dry weight (g), SDW- Shoot dry weight (g), TRL- Total root length (cm), SA- Root surface area (cm^2), RV- Root volume (cm^3).

Table 4: Correlation analysis between different organic acids exudated from lentil roots and root regrowth in response to Al toxicity stress.

	Oxalic acid (µg/ml)	Citric acid (µg/ml)	Malic acid (µg/ml)	Fumaric acid (µg/ml)	Root re-growth (cm)
Oxalic acid (µg/ml)	1.00	0.120	0.153	0.151	-0.076
Citric acid (µg/ml)		1.000	0.378**	0.058	0.452**
Malic acid (µg/ml)			1.000	0.342**	0.098
Fumaric acid (µg/ml)				1.000	-0.050
Root re-growth (cm)					1.000

** = 1% level of significance; * = 5% level of significance.

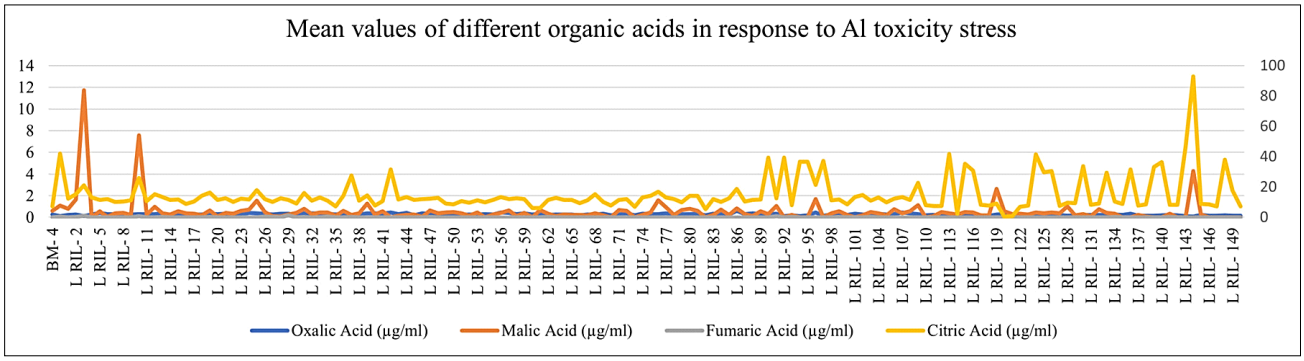

Fig 4: Mean values of different organic acids exudated from lentil roots in response to Al toxicity stress.

only efflux of citrate in the Al tolerant genotypes was observed for an extended period along with lesser Al accumulation in the root tips as compared to the sensitive genotypes (Silva *et al*., 2001). In a previous study performed in lentil, both malate and citrate exudation were found to be significantly higher in the resistant genotypes, as compared to the sensitive ones. However, malate was produced in relatively higher amounts than citric acid in all the genotypes (Singh *et al*., 2021). Similarly, exudation of citrate was found to be associated with increased tolerance to Al in chickpea genotypes (Sharma *et al*., 2015).

Most of the of the RILs identified as tolerant from hydroponics screening exudated higher amounts of organic acids as compared to the sensitive RILs, although some of the genotypes identified as tolerant from hydroponics screening had moderate exudation of organic acids which suggests the presence of other mechanisms besides the release of organic acids that accounted for the tolerance observed in the lentil RILs.

Correlation analysis between different organic acids exudated from lentil roots and root re-growth in response to Al toxicity stress

Citric acid was positively and highly significantly correlated with malic acid exudation (0.3783**) and RRG (0.4519**), while malic acid was positively and highly significantly correlated with fumaric acid (0.3425**) (Table 4). It can be suggested that exudation of organic acids in response to Al toxicity stress is associated with Al tolerance observed in terms of higher root re-growth under hydroponics. Zhao *et al*. (2003) observed a positive correlation between citrate secretion and Al resistance [(root elongation with Al)/(root elongation without Al)] and a negative correlation between citrate secretion and Al content of root apices, suggesting that citrate secretion from the root apices plays an important role in excluding Al and thereby detoxifying Al. Based on the above screening methods *viz*., root re-growth, root and shoot growth parameters of short-term growth method and organic acid exudation in response to Al toxicity stress, RILS identified as Al toxicity tolerant genotypes are LRIL-10, LRIL-37, LRIL-68, LRIL-96, LRIL-97, LRIL-113, LRIL-125, LRIL-133, LRIL-143, LRIL-144 and LRIL-148 (Table 4).

CONCLUSION

Our results demonstrated presence of high variability for the various parameters screened under short term growth method. High heritability coupled with high genetic advance observed for the root growth and shoot growth traits in response to Al toxicity under hydroponics suggests direct selection for these traits would be effective for selecting Al toxicity tolerant genotypes. High correlation observed between various traits with root-re growth suggested the possibility of simultaneous selection of the traits for Al toxicity tolerance. High exudation of citric acid in the tolerant genotypes and high correlation with root re-growth suggested its influence on Al tolerance observed in the lentil RILs. The lentil RILs identified as tolerant to Al toxicity based on root-regrowth, short term growth method and organic acid exudation studies may serve as potential varieties for Al toxicity prone acidic soils and may also be used for dissecting Al tolerance loci and act as potential donors of Al tolerant genes in hybridization programmes.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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