https://doi.org/10.17221/110/2021-CJGPB

Genetic variability for aluminium tolerance in sunflower (*Helianthus annuus* L.)

Vivek Kumar Singh[1], Subhash Chander[1], Ram Kumar Sheoran[1], Anu[1], Om Parkash Sheoran[2], Ana Luisa Garcia-Oliveira[3,4]*

[1]*Department of Genetics and Plant Breeding, College of Agriculture, CCS Haryana Agricultural University, Hisar, Haryana, India*
[2]*Department of Mathematics and Statistics, College of Basic Sciences and Humanities, CCS Haryana Agricultural University, Hisar, Haryana, India*
[3]*Excellence in Breeding (EiB), CIMMYT c/o ICRAF, Nairobi, Kenya*
[4]*Department of Molecular Biology, Biotechnology and Bioinformatics, College of Basic Sciences and Humanities, CCS Haryana Agricultural University, Hisar, Haryana, India*

**Corresponding author: subhash_chander@hau.ac.in*

Citation: Singh V.K., Chander S., Sheoran R.K., Anu, Sheoran O.P., Garcia-Oliveira A.L. (2022): Genetic variability for aluminium tolerance in sunflower (*Helianthus annuus* L.). Czech J. Genet. Plant Breed., 58: 201–209.

Abstract: Breeding for aluminium (Al) tolerance is a vital approach for enhancing the productivity of field crops in acidic soil regions where Al toxicity seems to be the most restraining factor for crop performance. Sunflower is generally considered extremely sensitive to Al toxicity; although no comprehensive information on the evaluation of sunflower genotypes for Al tolerance is available. In this study, 50 sunflower genotypes (set-I and set-II) were evaluated for Al tolerance at the seedling stage under hydroponic conditions. Substantial genetic variability in Al tolerance was observed among the studied genotypes. High estimates of heritability were obtained for both the total root length (TRL) and root regrowth (RRG), together with high estimates of genetic advance. A cluster analysis separated the genotypes into five different groups among the studied germplasm, the genotypes; NDLR-06 and EC-601861 were observed to be highly Al tolerant in terms of root regrowth under Al stress. In conclusion, the findings lreveal the complex mechanisms of Al tolerance in sunflower and may help to find new genetic resource for the improvement of Al tolerance in sunflower breeding.

Keywords: Al toxicity; characterization; stress; sunflower; variability

Aluminium (Al) is the third most abundant element in the Earth's crust, but very small quantities appear in soluble forms (Rengel 1992). Soil acidification enhances the Al dissolution in different ionic forms [$Al(OH)_2^+$, $Al(OH)^{2+}$ and $Al(H_2O)^{3+}$] of which Al^{3+} is considered potentially phytotoxic to plants (Kinraide 1997). Thus, Al toxicity is a critical obstacle for crop productivity on acidic soils which cover approximately 49% of the worldwide cultivated land (Waquil & Matzenbacher 2000). At the seedlings stage, Al ions rapidly inhibit the root elongation, and the absorption of water and nutrients, resulting in an inflated root apex and in the poor development of the root system (Delhaize & Ryan 1995; Ciamporova 2002). In acidic soils, a significant reduction in both crop production and its quality has been observed due to Al toxicity. There is considerable evidence to indicate that different plant species adopted external and/or internal mechanisms to detoxify the detrimental effects of Al toxicity (Garcia-Oliveira et al. 2016a). Among these mechanisms, the prominent role of organic acid exudates from the roots against Al toxicity has been well established (Ma et al. 2001; Kochian et al. 2005; Garcia-Oliveira

https://doi.org/10.17221/110/2021-CJGPB

et al. 2016a). Therefore, it is possible to find a broad range of Al tolerance among the genotypes selected from distinct gene pools in different geographical locations and genes conferring Al tolerance in crop plants can be utilised.

In the past, different phenotyping assays, such as hydroponic, soil and sand assays, have been devolved to assess the Al tolerance in plants, but a quick and efficient technique that can distinguish tolerant and sensitive genotypes is desirable from a breeding perspective. A hydroponic assay is considered the most useful phenotyping methodology that allows homogenous growth conditions with adequate accuracy and non-destructive measurements (Carver & Ownby 1995; Ma et al. 1997; Garcia-Oliveira et al. 2016b). Over the years, several histochemical techniques have been evolved to investigate the Al toxicity in plant tissues. Among these techniques, Eriochrome cyanine R staining has been extensively used for the measurement of root regrowth under Al stress that reveals if the root apical meristem is irreversibly damaged (Aniol 1995; Singh & Choudhary 2010; Singh 2012; Garcia-Oliveira et al. 2016b).

The sunflower (*Helianthus annuus* L.) is one of the main edible oilseed crops that is widely grown across the globe and ranks third in both the oilseed produced and oilseed meal among protein feed sources. Similarly, it also occupies a prominent position among edible oilseed crops in India, covering an area of 0.26 million ha with a productivity of 825 kg per ha (FAOSTAT 2019). While genetic variability for Al tolerance is well studied in different field crops, in oilseeds crops, especially the sunflower, limited information is available thus far. Being an economic and nutritionally important crop, it is essential to understand the existence of the genetic variation for Al toxicity in this crop. Despite the genetic variability, knowledge of the coefficient of variability along with the heritability is also necessary for the estimation of the genetic gain for the trait of interest, as the selection efficiency depends on the heritable variations being available (Chander et al. 2008; Singh et al. 2019). Thus, the main objective of present study was to carry out a comprehensive screening and to assess the genetic variability for Al tolerance in sunflower.

MATERIAL AND METHODS

Experimental materials and growth conditions. The experiment was established with two sets of sunflower inbred lines (Table 1), comprising 36 indigenous collections (set-I) and 14 exotic collections (set-II). The seeds of all the genotypes were obtained from the Oilseeds Section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar. Following surface sterilisation (10% C_2H_5OH and 0.1% Hg_2Cl_2) of 25 seeds of each genotype for 2–3 min, the seeds were rinsed thoroughly with ddH_2O and, subsequently, placed between wet filter paper in petri-plates for germination at room temperature (25–27 °C) for 24 h in dark chamber. Thereafter, fifteen uniform seedlings representing each genotype were selected and a single seedling was transferred per hole on Styrofoam blocks stitched with muslin cloth in a glass chamber containing a 5L nutrient solution (Garcia-Oliveira et al. 2013) with pH 4.5. The seedlings were raised in an aerated nutrient solution for 48 h under a 12 h light and 12 h dark regime at room temperature. The pH of the nutrient solution was checked at intervals of 24 h and adjusted to 4.5 with 1 M HCl or 0.1 M NaOH as needed.

Experimental design and Al stress treatments. The experiment was performed in a two-way factorial design as described by Romheld et al. (1984). The genotypes, as subplots, were randomised uniformly for each replication. Five seedlings per replication were evaluated to determine the Al tolerance at 0, 0.5, 1.0 and 2.0 ppm Al levels.

Table 1. List of the sunflower genotypes used in present study

Genotypes	No.	Name of genotypes
Set-I	36	MR–1, MSF–1–7, MR–6, AH–14, RHA–271, IHT–201, RHA–265, IHT–298, RHA–274, HB–15, RHA–856, NDR–2, HRHA–5–3, DRSF–160, HRHA–271–P3, RHA–298, RHA–297–P3, GPB–07, RHA–297–P2, GPB–51, RHA–298–P3, GPB–61, RHA–3, 1–OH–04–29, Nandyal–1, 1–OH–07–41, IB–4, 1–OH–07–45, IB–43, CSFI–5304, ACC–350–2, RCR–39, MSF–2–16, LSF–902, MSF–1–4 and NDLR–06
Set-II	14	EC–152673, EC–512681, EC–512684, EC–512686, EC–512687, EC–601746, EC–601747, EC–601751, EC–601755, EC–601800, EC–601820, EC–601861, EC–601874 and EC–601875

EC – exotic collection

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For the Al treatments, three-day-old seedlings were transferred to a fresh nutrient solution having 0 (control), 0.5, 1.0 and 2.0 ppm aluminium ($AlCl_3 \cdot 6H_2O$) levels for 24 h. To remove the excess amount of Al present on the root surface after incubation of 24 h, the roots of the seedlings were immersed in ddH_2O and rinsed for 15–30 min. Subsequently, the seedlings were stained with a 0.1% Eriochrome Cyanine R solution for 15 min followed by washing with ddH_2O for 10 min. Finally, the stained seedlings were raised in a fresh nutrient solution devoid of Al for another 24 h.

Trait measurements. The root tip of the seedlings with the injured apical meristem by the imposed Al stress were extensively stained (purple). The unstained portion of the root, which was regenerated after the Al treatment, was measured in mm and considered as root-regrowth (Figure 1). The total root length (TRL) was measured from the base of the stem to the root tip. The growth inhibition rate (GR_{50}) was estimated from the Al level causing a 50% root regrowth inhibition rate using a dose response curve (Horsfall 1956; Sagers et al. 2017). The percent inhibition values were transformed to probit values by reading the corresponding probit units and plotted against the log dose. Thus, the GR_{50} was estimated graphically using the probit value that corresponds to probit 5 or 50%. The root tolerance index (RTI) was calculated as the maximum root regrowth in the Al stressed medium divided by the maximum root regrowth in the control.

Based on the GR_{50} values, the subordinate function values for the Al tolerance (Fi) were determined using the Gower (1971) formula as follows:

$$F_i = (X_i - X_{min})/(X_{max} - X_{min})$$

where:

X_i – estimations of the trait of the selected genotypes;

X_{min} – lower limit of the trait between the evaluated genotypes;

X_{max} – upper limit of the trait between the evaluated genotypes.

Statistical analysis. A nested analysis of variance (ANOVA) was carried out to estimate the significant differences among the genotypes, Al treatments, and their interaction effects. Duncan's test ($P < 0.05$) was performed using the R programme (R Core Team 2020) to estimate the difference among the genotypes/treatments. The phenotypic variance, genotypic variance and coefficient of variations, heritability estimated in a broad sense, and genetic advance were analysed using the software TNAUSTAT (Manivannan 2014). The dendrogram was generated by an unweighted pair group method with an arithmetic mean (UPGMA) using the Manhattan dissimilarity coefficients in DARwin (Ver. 6.0) software (Perrier & Jacquemoud-Collet 2006).

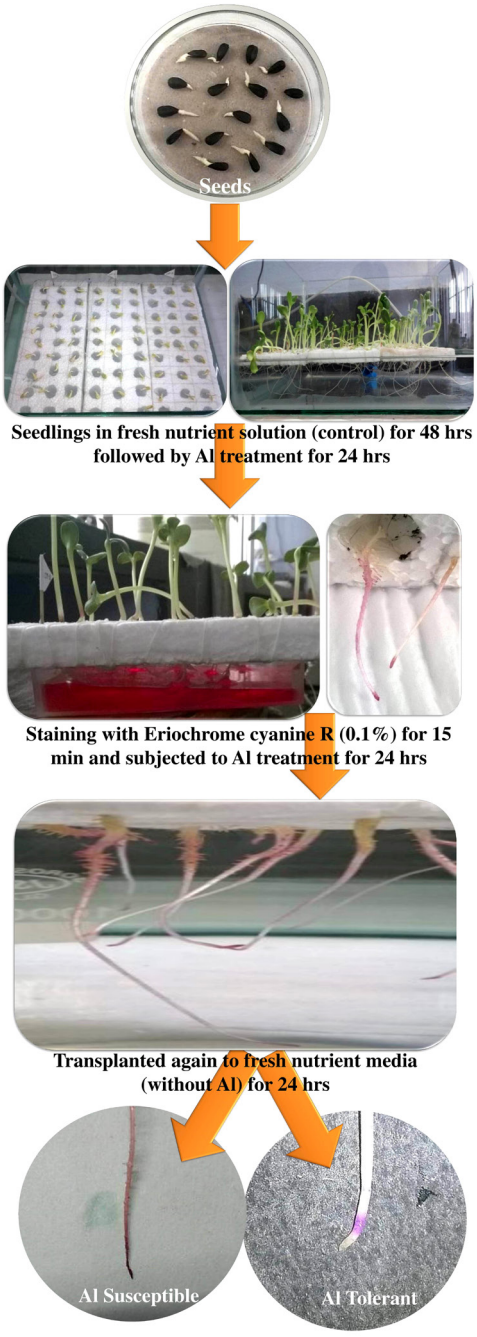

Figure 1. Screening of sunflower genotypes for Al tolerance under hydroponic conditions

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Table 2. Nested analysis of variance of the total root length (TRL) and root regrowth (RRG) in response to the Al stresses under the nutrient solution in sunflower (in mm)

Source of variation	df	TRL		RRG	
		MSS	*F*-value	MSS	*F*-value
Germplasm set-I					
Replication	2	6.00	3.52	0.06	1.65
Aluminium level (Al)	3	49 170.00**	27 877.75	775.65**	21 630.46
Replication × Al level (error A)	6	2.00		0.04	
Genotype (G)	35	1 667.00**	277.56	48.50**	1 948.26
Al × G	105	70.00**	11.74	3.19**	128.07
Residual (error B)	280	6.00		0.02	
Germplasm set-II					
Replication	2	15.10	4.24	0.04	1.48
Aluminium level (Al)	3	17 968.50**	5 027.14	357.75**	11 810.39
Replication × Al level (error A)	6	3.60		0.03	
Genotype (G)	13	4 347.10**	977.47	14.52**	856.50
Al × G	39	53.50**	12.03	1.59**	93.59
Residual (error B)	104	4.40		0.02	

MSS – mean sum of squares; df – degree of freedom; **significant at $P \leq 0.001$

RESULTS

For both the TRL and RRG traits, the deviation within each set and among the Al levels, genotypes and their interactions (genotype × Al level) were highly significant ($P < 0.01$) indicating the presence of substantial genetic variability among the genotypes in both sets of germplasm (Table 2). Overall, all the genotypes exhibited gradual reductions in the average root growth with an increasing Al stress level (Figure 2).

Root growth response to Al exposure. Both sets of germplasm lines (set-I and set-II) presented substantial variation for the RRG as well as the TRL (Figure 3). The average RRG and TRL was significantly higher ($P < 0.05$) in the control (0 ppm Al) when compared to the Al stress (0.5, 1.0 and 2.0 ppm level of Al). The TRL ranged from 60.8 to 111.8 mm and 70.2 to 137.7 mm with an average of 89.7 and 96.0 mm in set-I and set-II, respectively (Table 3). On the other hand, the RRG varied from 0.33 to 10.51 mm and 2.73 to 7.05 mm with a mean value of 4.21 and 4.37 mm in set-I and set-II, respectively. At 0.5 ppm Al, the genotypes MR–06 and RHA–298 in set-I showed no RRG and were found to be most sensitive to Al toxicity (Figure 3B).

At a higher Al stress level (2.0 ppm), both the RRG and TRL were reduced significantly ($P < 0.05$) in all the genotypes of set-I as well as set-II. Among the 50 genotypes, eleven genotypes showed an RRG greater than the grand mean values, demonstrating that they were least affected at an Al stress of 2.0 ppm. Across the Al levels, the genotypes NDLR–06 and EC–601861 exhibited the maximum RRG (10.51 and 7.05 mm, respectively) with the least percent reduction, indicated as highly Al tolerant genotypes in terms of the root development (Table S1 in the Electronic Supplementary Material (ESM)). While MR–06 and RHA–298 exhibited a minimum RRG with a maximum percent reduction, demonstrated as highly Al susceptible genotypes.

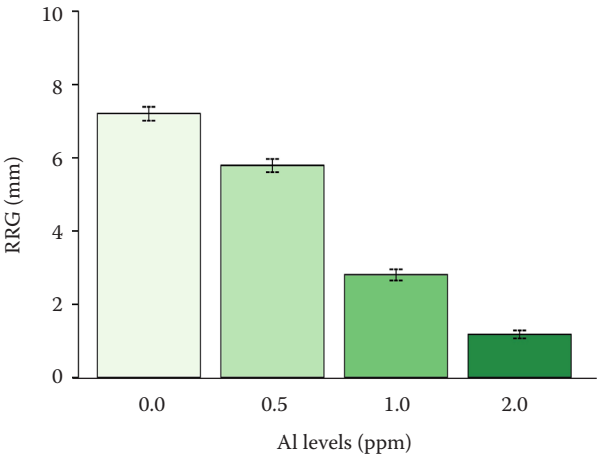

Figure 2. Effect of the different Al levels on the mean root regrowth (RRG) of the sunflower genotypes

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Genetic parameters of variability for Al-tolerance response. The variance components indicated that the variability for RRG and TRL was mainly due to genotypic variance (Table 3). As expected, the values of the phenotypic coefficient of variation (PCV) for both traits (TRL and RRG) were slightly higher than their corresponding genotypic coefficient of variation (GCV). Furthermore, these traits also had a very high level of heritability estimates in a broad sense (h^2bs) in both sets of germplasms (> 97%). As a result, a genetic advance of 23.69 mm (set-I) to 38.98 mm (set-II) and 4.13 mm (set-I) to 2.34 mm (set-II) might be expected for the TRL and RRG, respectively.

Cluster analysis. Based on the root regrowth (RRG) data, the root tolerance index (RTI) and growth inhibition rate (GR_{50}) were calculated for each genotype with their corresponding control values (Table S1 in the ESM). The RTI and GR_{50} values ranged from 0.00 to 0.65 and 0.00 to 1.93 with a coefficient of variance (*CV*) of 32.56% and 36.55%, respectively. Based on the UPGMA, all the genotypes were classified into five major clusters after a hierarchical clustering analysis of RRG across the Al-levels, RTI and GR_{50} values (Table S1 in the ESM and Figure 4). Cluster I comprised of five genotypes representing 10% of the germplasm lines with a group mean of 0.159, which showed below average Al sensitivity. While the highest group *Fi* mean (0.720) was observed for cluster II, suggesting high Al tolerance. This cluster (II) contained seven genotypes (14% lines) and exhibited maximum values for the studied traits. The highly

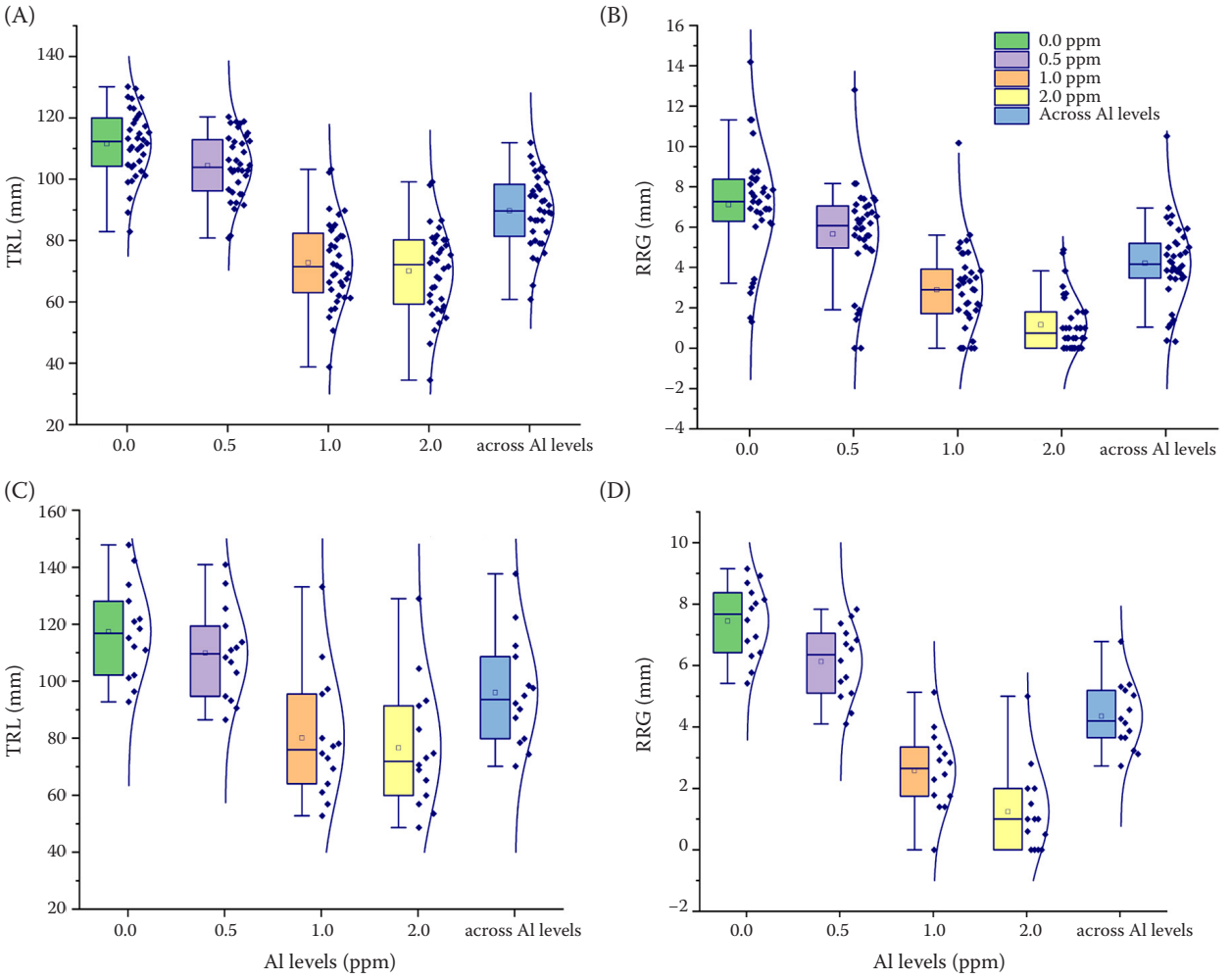

Figure 3. Box and whisker plots of the sunflower germplasms evaluated in set-I (A, B) and set-II (C, D) for the total root length (TRL) and root regrowth (RRG) at the different Al levels

The median is the horizontal line within the box, while the small dot within the box indicates the mean; the whiskers represent the maximum and minimum range of the traits in the studied genotypes

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Table 3. Estimates of the variance components for the total root length (TRL) and root regrowth (RRG) in sunflower (set-I and set-II) under the Al stress

Genetic parameter	Set-I (indigenous collection)		Set-II (exotic collection)	
	TRL	RRG	TRL	RRG
Mean	89.72 ± 0.98	4.21 ± 0.50	96.04 ± 5.44	4.37 ± 0.38
Range	60.80–111.88	0.33–10.51	70.17–137.70	2.73–7.05
σ^2e	2.88	0.01	1.84	0.01
σ^2g	137.89	4.04	361.59	1.31
σ^2p	140.77	4.05	363.43	1.32
GCV (%)	13.09	47.74	19.80	26.19
PCV (%)	13.22	47.80	19.85	26.29
h^2bs (%)	97.95	99.75	99.50	99.24
GA	23.69	4.13	38.98	2.34
GG (%)	26.40	98.10	40.59	53.55
LSD	1.97	0.127	1.707	0.105
SD	11.74	2.01	19.02	1.14
Criteria	narrow	broad	narrow	broad

σ^2g – genotypic variance; σ^2e – environmental variance; σ^2p – phenotypic variance; GCV – genotypic coefficient of variation; PCV – phenotypic coefficient of variation; h^2bs – heritability in a broad sense; GA – genetic advance; GG – genetic gain; SD – standard deviation

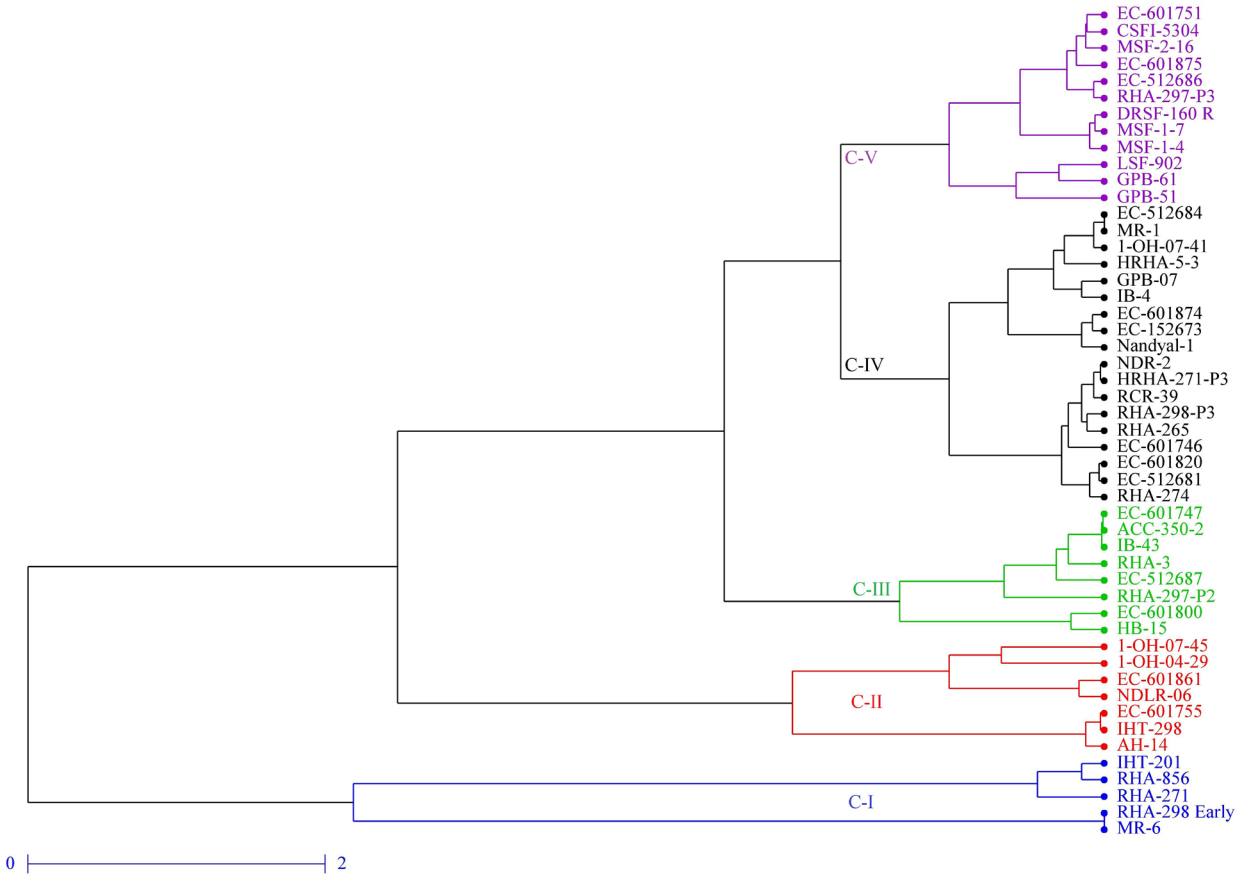

Figure 4. Clustering of the sunflower genotypes based on their response to the Al toxicity

https://doi.org/10.17221/110/2021-CJGPB

Al susceptible genotypes MR-06 and RHA-298 (*Fi* = 0.000) belonged to cluster I whereas most tolerant genotypes NDLR-06 and EC-601861 (*Fi* = 0.798 and 1.000, respectively) were grouped in cluster II. Cluster III containing eight genotypes (16% lines) had an average *Fi* value of 0.355 (0.306–0.378), indicating the fact that the Al tolerance of this group was below average. Cluster IV had the largest germplasm lines (18 genotypes) with an *Fi* mean value of 0.440 (0.394–0.606) indicating a moderate level of Al tolerance. While the remaining eleven genotypes were grouped in cluster V (representing 22% of the lines). The average group *Fi* value of cluster V for the Al tolerance was 0.533 (0.466–0.694), designating that the Al tolerance of this group was above the average.

Ranking of sunflower genotypes based on the natural variation for Al tolerance. Based on the cluster analysis and subordinate function values (*Fi*), all the genotypes were ordered into five ranks for their response to Al toxicity as: rank 1, highly tolerant ($Fi \geq 0.720$); rank 2, tolerant ($0.480 \leq Fi < 0.720$); rank 3, moderately tolerant ($0.360 \leq Fi < 0.480$); rank 4, susceptible ($0.160 \leq Fi < 0.360$); and rank 5, highly susceptible ($Fi < 0.160$). Of the 50 sunflower genotypes, 39 genotypes (78%) belong to the tolerant to moderately tolerant ranked within levels 2–3 and seven genotypes belong to the Al susceptible ranked within level 4 (Table S1 in the ESM). While two genotypes belong to highly Al tolerant (HT) and susceptible (HS) ranked within level 1 and 5, respectively.

DISCUSSION

Al toxicity is considered a key factor limiting the yield potential of crop plants grown in acidic soils (Ma et al. 2001). Being an important oilseed crop, the evaluation of the sunflower germplasm for Al tolerance is essential to provide fundamental information toward breeding for Al tolerance, but such information is very limited in this crop (Arsintescu et al. 2001; Jesus et al. 2016). In the present study, a total of 50 sunflower germplasm lines consisting of two sets, were screened for Al tolerance in a nutrient solution, because the hydroponic method provides easy contact to the root system, controlled nutrient availability and pH (Carver & Ownby 1995; Ma et al. 1997; Garcia-Oliveira et al. 2016b).

A highly significant genotype by the Al interaction was noticed, indicating genotypic discrepancy in response to the imposition of Al stress (Martins-Lopes et al. 2009). Expectedly, the decline in the both the TRL and RRG was dose dependent across the sunflower genotypes as described earlier in saffron (Chen et al. 2008), lentils (Singh et al. 2012), rice (Roy & Bhadra 2014) and wheat (Garcia-Oliveira et al. 2016a). Nonetheless, significant differences were recorded among the genotypes in response to the Al levels (0.5–2.0 ppm), suggesting the existence of Al induced differential response in the sunflower genotypes. Noble and Sumner (1988) suggested that inhibition of the root growth is a primary sign of Al toxicity in a short-term experiment. The use of Eriochrome Cyanine R staining enables one to visualise the detrimental effect of Al toxicity on the RRG after the Al treatment (Aniol 1995; Ma et al. 1997; Pinheiro et al. 2003; Martins-Lopes et al. 2009; Garcia-Oliveira et al. 2016a). Highly susceptible genotypes, such as MR-06 and RHA-298, did not exhibit RRG following the exposure to the Al stress, indicating irreversible damage to the root tip structure (de Jesus & de Azevedo Neto 2013). On the other hand, the Al tolerant genotypes did not abolish the root apical meristem, the portion of the root that regenerated after the Al stress remained unstained. This effect may be due to the fact that genotypes which continuously grew in the Al solution would have the capability to exclude the Al through the efflux of organic acid anions from their root apices (Garcia-Oliveira et al. 2016a). Arsintescu et al. (2001) observed an improvement in the relative elongation rate of the roots in sunflowers following the addition of citric acid in the Al stress solutions.

In the present study, the substantial genetic variability observed for the studied traits is crucial for the further improvement of Al tolerance in sunflower breeding programmes. The closer the PCV and GCV values for both TRL and RRG indicates the comparatively lower influence of environment in the expression of the characters (Richard et al. 2015; Kuswantoro 2017). Similarly, h^2bs offers statistics on the comparative extent of the genetic and environmental variation, but estimation of h^2bs alone is inadequate to regulate the selection (Chander et al. 2008). High heritability accompanied with high genetic gain for both the TRL and RRG indicate the superiority of the additive gene effects and could be used for selection of Al tolerant genotypes (Richard et al. 2015).

The subordinate function analysis has been widely used in plant resistance evaluations that overcome the shortage of a few indicators to evaluate and draw conclusions more accurately (Richard et al. 2015; Huang et al. 2017; Xu et al. 2017; Zhao et al. 2022).

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In the present work, the *Fi* was estimated for the GR_{50} values; the higher the mean *Fi* indicated the higher the tolerance to Al toxicity (Huang et al. 2017). Based on the cluster analysis and average subordinate function values (*Fi*), all the genotypes were classified into five categories. The highly tolerant and highly susceptible genotypes were clearly separated into different clusters. All the genotypes including NDLR-06 and EC-601861 in cluster II exhibited natural tolerance with respect to the Al toxicity and had a maximum mean value for GR_{50}, RRG and *Fi*, indicating the least reduction in RRG with an increased Al level. This is consistent with previous findings which characterised germplasm lines for abiotic stresses including Al toxicity and identified the most tolerant material based on a cluster analysis and ranking (Huang et al. 2017; Zhao et al. 2022).

CONCLUSION

In the present study, substantial genetic variability was observed among the tested sunflower genotypes. The genotypes NDLR-06 and EC-601861 were identified as highly tolerant to Al toxicity and could be used to further study the genetic basis of the Al tolerance in sunflowers which depends on several complex mechanisms including the organic acid (OA) efflux. Finally, the utilisation of the novel genetic resources identified in the present study may help the development of suitable sunflower cultivars to cope with Al toxicity.

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Received: November 12, 2021
Accepted: February 24, 2022
Published online: April 6, 2022