Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 3052-3058
Received: 07-03-2019
Accepted: 09-04-2019

Lakidon Khonglah
Department of Fruit Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Lobsang Wangchu
Department of Fruit Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Siddhartha Singh
Department of Basic Sciences and Humanities, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Nesara Begane
Department of Fruit Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Longing Langstieh
Department of Fruit Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Athikho Kayia Alice
Department of Vegetable Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Ashok Chettri
Department of Fruit Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Correspondence
Lakidon Khonglah
Department of Fruit Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Aluminium toxicity effects on growth, pigments, lipid peroxidation and protein content in two citrus species

Lakidon Khonglah, Lobsang Wangchu, Siddhartha Singh, Nesara Begane, Longing Langstieh, Athikho Kayia Alice and Ashok Chettri

Abstract
The effects of aluminium toxicity on two citrus species – Rough lemon (*Citrus jambhiri*) and Samphola, a wild species, were evaluated in a sand culture experiment for 20 days. One month old seedlings were subjected to 3 concentrations of $AlCl_3.6H_2O$ *viz*., 600 µM, 1200 µM and 1800 µM with untreated pots monitored as control. After 20 days of exposure, aluminium induced toxicity decreased shoot and root length, dry weight, relative water content, chlorophylls and total soluble protein of both citrus species. However, alterations in these parameters were more distinct in higher Al doses wherein Rough lemon exhibited greater decline than compared to Samphola. The peroxidation of lipids, however, significantly elevated in both citrus species with Rough lemon exhibiting higher levels of lipid peroxidation than Samphola. Therefore, with respect to these parameters, our results show that Samphola is comparatively more tolerant to aluminium toxicity than Rough lemon.

Keywords: Acid soils, Aluminium toxicity, *Citrus* sp., chlorophylls, lipid peroxidation, relative water content, protein

Introduction
Abiotic stress poses major problems to agriculture and the understanding of the responses and mechanisms of plant stress tolerance is key to the successful selection of tolerant crops in growth areas affected by changes in the abiotic environment. The condition of the soil plays a major role in determining the success or failure of the crop grown. Soil fertility constraints, like soil salinity, soil acidity and nutrient deficient soils, are one of the critical components by which plants are subjected to abiotic stress. Under these circumstances, identification of tolerant plant species is fundamental to improve crop production and sustainability. In India, acidic soils are mainly confined in the Northeastern region and other parts including the Western Ghats with sporadic distribution in Jharkhand, Himachal Pradesh, Odhisha, West Bengal and Chattisgarh (Patiram *et al*., 2002) [26]. Acid soil infertility is a complex interaction of growth-limiting factors which can restrict growth by inducing stress on the plant. These factors may include low pH; Al or Mn toxicity; Ca, Mg, P or Mo deficiency; reduced mineralization, nitrification, nodulation and mycorrhizal infection (Foy, 1984) [7]. When soil pH is less than 5.0, toxic forms of Al (mainly Al^{3+}) are solubilised into the soil solution and accumulate to high concentration that rapidly inhibits plant root growth by damaging the roots functionally and structurally, subsequently decreasing nutrient and water uptake, eventually resulting in poor crop growth and productivity (Kochian *et al*., 2004, Yang *et al*., 2007) [16, 40]. Al is present in all soils, but Al toxicity is manifested only in acid conditions, in which the phytotoxic form Al^{3+} predominates. Research conducted on aluminium toxicity is extensive. However, different plant species exhibit more tolerance to different levels of aluminium tolerance.

Citrus fruits belong to the Rutaceae family and these fruit crops are evergreen subtropical trees which are cultivated in humid and subhumid of tropical, subtropical, and temperate regions of the world mainly on acidic soils. In India, the Northeastern region is considered as one of the primary gene centre and reservoir of various *Citrus* species (Ray and Deka, 2000) [28]. The extent of Citrus diversity in this region is possibly due to the soil and climatic factors agreeable to its growth (Hazarika, 2012) [12]. Apart from the cultivated Citrus crops, the Northeastern region is also home to numerous indigenous species that thrive in the wild forests of this region. Due to their adaptability, these species may hold a key that could solve certain bottlenecks that this region faces in terms of citrus fruit production. They may have potential to contribute healthy planting material and hardy rootstocks for the cultivated species.

Therefore, it is of vital importance to screen these wild species against biotic and abiotic stresses in order to aid in the improvement of fruit production.

Numerous studies on dose response relationships of Al have been investigated in many crop species over the years. Citrus species have also been under study in this aspect although only a few selected species have been studied particularly, *Citrus sinensis* (L) Osbeck and *Citrus grandis* (L) Osbeck (Chen *et al*., 2009; Yang *et al*., 2011; Jiang *et al*., 2015; Guo *et al*., 2018; Guo *et al*., 2017) [4, 39, 15, 10, 11], *Poncirus trifoliata* (Riaz *et al*., 2018) [30] and *Citrus volkermeriana* (Toan *et al*., 2003) [35]. On reviewing the current available literature, it is evident that studies of aluminium toxicity on citrus are confined to popular citrus species while wild species are neglected. Therefore, the purpose of this study was to screen the performance of a well-established citrus species with a wild species against the influence of aluminium toxicity with the objective to identify the tolerant species suitable for acidic soils. The determination of tolerance levels in this paper is based on the alterations in the physical growth, chlorophylls, protein content and lipid peroxidation of the citrus species.

A diverse range of citrus species is endemic to the Northeastern region of India and therefore this experiment was conveniently endowed in consequence to this. In this experiment, Rough lemon (*Citrus jambhiri*) and a citrus species known only by its local name, Samphola, was used for this study; both of which are found in the wild forests of Arunachal Pradesh, India. Rough lemon is a well established rootstock known to be exploited for its capabilities as a rootstock particularly for the propagation of Khasi mandarin (*Citrus reticulata*), a commercial species of the Northeastern region. The fruits of Samphola, on the other hand, have little or no market value; however, due to its prolific growth in the wild, it should be investigated further as a potential source of biotic or abiotic stress tolerance.

Materials and Methods

Well matured fruits of both Rough lemon and Samphola were collected from the East Siang district of Arunachal Pradesh. The seeds were extracted from the fruits, washed and separated from the pulp and treated with fungicidal solution. Then they were germinated hydroponically in a bamboo frame fitted with blotting paper in an incubator at a temperature of 25±2°C and 80% relative humidity. Once germinated, uniform sized seedlings were transplanted to clean imperforated plastic pots (1200 cc) containing sterilized river sand and was supplied with Hoagland's nutrient solution (Hoagland and Arnon, 1950) [14] with its pH maintained at 5.8 prior to its application. The seedlings were allowed to establish for 4 weeks in a polyhouse maintained in the College of Horticulture and Forestry, Pasighat, Arunachal Pradesh under natural photoperiod. Thereafter, the pots were treated with Hoagland's nutrient solution containing different concentrations of Aluminium chloride hexahydrate ($AlCl_3.6H_2O$), *viz*. 0 (control), 600 µM, 1200 µM and 1800 µM. The pH of the treated Hoagland nutrient solution was adjusted to 4.1 prior to its application while that for untreated plants remain at 5.8. Each treatment is replicated three times with three pots per treatment in a completely randomized design. Equal volumes of the treated Hoagland solution were applied to the pots until the sand was saturated and the solution was given after every three days. The plants subjected to the induced stress of aluminium toxicity were then uprooted at 20 days after treatment. Plants from each treatment were uprooted and separated into shoots and roots. The following physical growth parameters as well as their biochemical parameters were recorded from these samples.

Plant length, biomass and shoot relative water content

After 20 days of treatment with Al treatments, the length of shoot and root along with their corresponding fresh weights (FW) of 10 plants were recorded from each treatment. The weighed shoot and root samples were then placed in a hot air oven at 80°C and weighed after every 48 hours till constant dry weight (DW) was recorded.

The relative water content of shoot was measured according to the method given by Weatherley (1950) [37] with a few modifications. The fresh weight (FW) of shoot samples was measured as above. The samples were placed in water saturated jars and stored for 48 hours in dark at 4°C where their turgid weight (TW) was then recorded. The same samples were oven dried for 48 hours and weighed again to record the dry weight (DW). The relative water content of shoot was then calculated as, RWC (%) = [(FW-DW)/(TW-DW)] X 100

Leaf pigments

The estimation of chlorophylls and carotenoid content in leaves was estimated by the method proposed by Lichtenthaler (1987) [21]. Fresh leaf tissues were homogenized in ice cold 80% (v/v) acetone in darkness and then centrifuged at 4°C. The supernatant was then collected and used to determine the total chlorophyll, chlorophyll a, chlorophyll b and total carotenoids by recording the absorbance at 664, 648 and 470 nm using a UV-VIS spectrophotometer (Hitachi U-1900).

Lipid peroxidation

Lipid peroxidation was estimated from the levels of malondialdehyde and other aldehydes production using the thiobarbituric acid (TBA) method as described by Heath and Packer (1968) [13]. Fresh samples (Root and shoot) weighing 100 mg each were homogenized in 4 mL of 10% (w/v) trichloroacetic acid (TCA) containing 0.25% 2-thiobarbituric acid and heated at 95ºC for 25 min. Samples were incubated at 90°C for 30 min. Reaction was stopped immediately by an ice bath. The mixtures were centrifuged at 10,000 x g for 10 min and absorbance of the supernatant was recorded at 532 and corrected for unspecific turbidity by subtracting the absorbance of the same at 600 nm.

Total soluble protein

Proteins were extracted from 100 mg of both root and shoot samples using 100 mM Na-Phosphate buffer (pH 7.6) and the protein content was estimated according to the method of Bradford (1976) [2] using bovine serum albumin (BSA, Sigma) as standard.

Data Analysis

All the experiments including plant culture, treatment, growth and assessment of biochemical parameters, were performed based on completely randomized design (CRD). Each treatment was replicated thrice and comprised of 3 pots per replication. Results were determined using analysis of variance (ANOVA) and means were separated by least significant difference at $p < 0.05$. The results were presented in the form of mean±SE.

Results and Discussion

Plant length, biomass and shoot relative water content

The influence of the aluminium treatments on the physical parameters was found to be significantly altered after 20 days of exposure. Shoot length significantly declined with the increase in the aluminium concentration in both species with the decline more pronounced in Rough lemon as negative influence of Al treatments was observed even at the lowest dose (6.67% decline). Samphola, however, recorded an insignificant shoot length decline of 2.47% in the same treatment (Figure 1.A). The decline in shoot growth in this experiment is validated by the findings of Pereira *et al*. (2003) [27] in which the shoot length in the citrus rootstocks accompanied with total fresh matter and leaf area to leaf weight ratio decreased under the influence of Al toxicity. The findings of Larsen *et al.* (1997) [18] in *Arabidopsis* further support the above conclusion that Al inhibits shoot growth. However, due to the close relationship of root growth inhibition with aluminium toxicity, studies on the response of shoot growth to aluminium are given less attention. On the other hand, aluminium toxicity comprises of so many factors which work indirectly in derailing plant growth, it is suggested that shoot growth inhibition is a repercussion of restricted root growth under aluminium stress.

In this experiment, root length also followed a declining pattern similar to shoot length, although the decline in roots was more severe as compared to the shoots in both citrus species (Figure 1.B). Rough lemon recorded an 11.84% decline whereas Samphola root decreased by 6.61% at the lowest dose and the elongation of roots was further inhibited with the increase in Al doses in both citrus species. The highest dose of Al (1800 µM) recorded a 23% and 27.4% inhibition of root length in Samphola and Rough lemon, respectively. The inhibition of root elongation is one of the primary symptoms of Al toxicity and many theories have evolved behind its causes. Ryan *et al*. (1993) [31] suggested that out of the three components of a root apex i.e., root cap, meristem and elongation zone, the root cap and meristem are the most sensitive zones to aluminium toxicity. Thus, this leads to accumulation of more Al and inducing greater damage or even cell death in root tips. The injury to the root system, as a consequence of Al toxicity, often leads to obstruction in the uptake of minerals and nutrients thereby affecting the overall plant growth (Kochian *et al*., 2004) [16]. The reduced root growth may be related to the fact that Al alters cytoskeletal dynamics and signal transduction pathways which may eventually lead to inhibition of cell division and elongation (Frantzios *et al*., 2001) [8]. Thus, this finding suggests that Samphola roots were least inhibited to maximize water and nutrient uptake.

Plant biomass is a good indicator of metal toxicity (Baker and Walker, 1990) [1] and in this experiment; plant dry weight (shoot + root) revealed a negative impact with respect to the increase in aluminium concentrations. Similar to plant length, the pattern of dry weight reduction of shoots was less severe as compared to roots. The effects of the aluminium treatments compromised the growth of the seedlings wherein both citrus species showed comparable decline in dry weight in the lowest dose of Al (Figure 2). At 600 µM Al, shoot DW decreased by 2.41% and 5.67% whereas root DW decreased by 14% and 17% in Samphola and Rough lemon, respectively. However, the increase in treatment concentration, interestingly, created a more pronounced decline in Rough lemon. At 1800 µM Al, shoot DW decreased by 28% and 32.2% while root DW decreased by 30.5% and 34.7% in Samphola and Rough lemon, respectively. Decline in dry weight under Al toxicity was associated with lower tolerance as reported in *Citrus grandis* seedlings when compared with *Citrus sinensis* (Yang *et al*., 2011; Li *et al*., 2016; Guo *et al*., 2017; Guo *et al*., 2018) [40, 11, 10]. The decline in dry matter was associated with Al-sensitive *Citrus grandis* as changes in root dry weight of Al-tolerant *Citrus sinensis* was insignificant (Jiang *et al*., 2015) [15].

The reduction in relative water content (RWC) of the citrus shoots observed a higher decline with the increase in treatment concentration (Figure 3). Guo *et al*. (2018) [10] also corroborated these findings in their experiment that revealed a decline in RWC in *Citrus grandis* roots. In this experiment, Samphola recorded lower decline in shoot RWC as compared to Rough lemon in all the treatment concentrations. Moreover, it was observed that RWC of Samphola at the lowest dose was at par with control (0.3% decline), thus showing a higher endurance to the stress as compared to Rough lemon (6.02% decline). The ability to maintain the relative water content suggests that photosynthetic and respiration rates were least altered (González and González-Vilar, 2001) [9]. Tang *et al.* (2002) suggested that Al-tolerant species wield in more water when grown in acidic soils because of their ability to anchor their roots deep into the soil thereby bringing about higher root length and significant shoot growth than their sensitive counterparts.

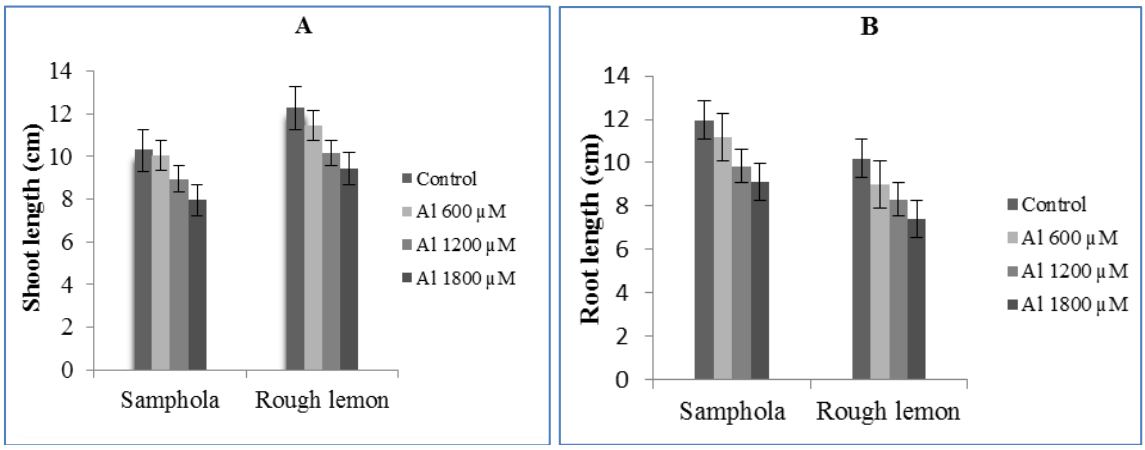

Fig 1: Root (A) and shoot (B) length of citrus species at different Al treatments. Values represent the mean ± SE (n=3)

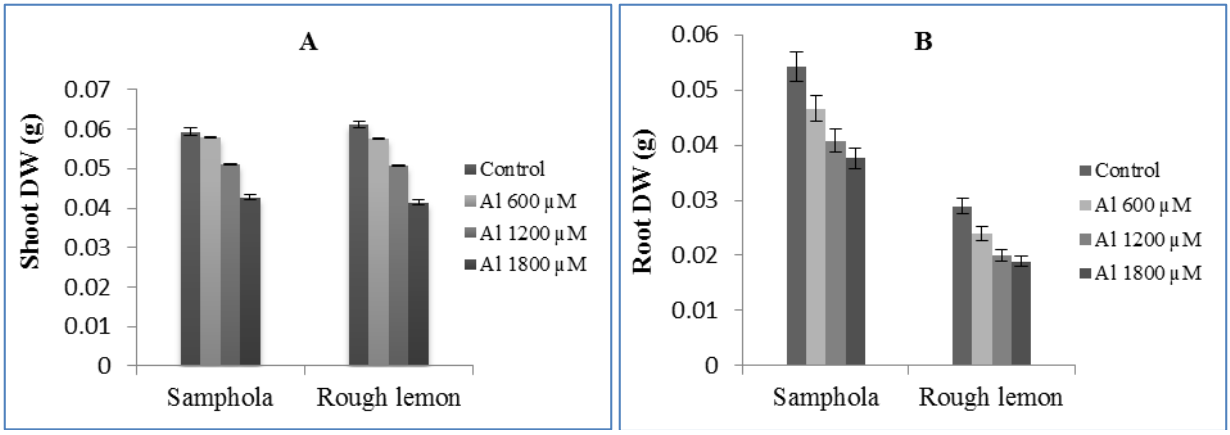

Fig 2: Dry weight (DW) of shoot (A) and root (B) of the citrus species subjected to Al treatments. Values represent the mean ± SE (n=3)

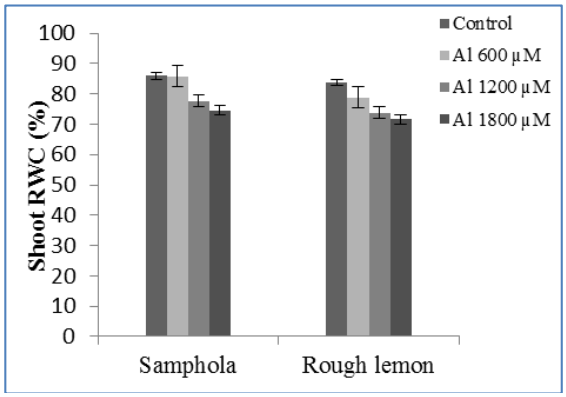

Fig 3: Changes in the relative water content (RWC) of citrus shoots (%) subjected to Al treatments. Values represent the mean ± SE (n=3)

Pigments

Leaf chlorophyll concentration is an important parameter that is regularly measured as an indicator of chloroplast content, photosynthetic mechanism and of plant metabolism. It often indicates the impact of environmental stress, as changes in pigment content are linked to visual symptoms of plant illness and photosynthetic productivity (Parekh, 1990) [25]. In this experiment, it was observed that the total chlorophyll content of the citrus leaves under Al toxicity decreased with the increase in the treatment concentration (Table 1). Yet it is interesting to observe that chlorophyll in Samphola at 600 µM concentration and was at par with control whereas total chlorophyll in Rough lemon declined by 8.87%. Higher Al doses caused more decline in total chlorophyll content in both citrus species with Samphola showing lower susceptibility to the treatments than Rough lemon. Chlorophylls a and b also declined with the increase in the treatment concentrations in both citrus species with chlorophyll a recording higher decline than chlorophyll b in both citrus species validating that Al toxicity causes a more deleterious effect on chlorophyll a than b in citrus. The decline in chlorophylls a and b could be attributed to decreased Mg concentrations leading to decreased photosynthetic activity (Zhang *et al*., 2007) [40]. It is also suggested that decline in chlorophyll content is a defense mechanism by reducing light absorbance thereby reducing the chances of destruction of the photosynthetic system by reactive oxygen species that are induced as a consequence of stress (Munné-Bosch and Alegre, 2000; Kranner *et al*., 2002; Elsheery and Cao, 2008) [23, 17, 5].

Carotenoids are light accessory pigments that function as antioxidants that protect plants from photooxidative damage by reactive oxygen species (Young and Britton, 1990; Verma and Mishra, 2005) [42, 36]. The increase in carotenoid content under metal stress has been recorded in certain plant species (Reyes-Diaz *et al*., 2009) [29]. However, in this experiment, the total carotenoid content of the citrus species were significantly reduced under Al treatments (Table 1). At the lowest dose of Al (600 µM), total carotenoid content declined in Rough lemon by 14.72% whereas no significant decline was observed in Samphola. Al 1200 µM further declined carotenoid content in both Samphola and Rough lemon with 20.51% and 39.79%, respectively. The decline in carotenoids is in agreement with the findings of Sai Kachout *et al*. (2015) [32] in *Atriplex* sp., Zengin (2013) [43] in beans and Lau *et al*. (2006) [19] in maize under the influence of metal. Considering the conflicting reports, it is therefore suggested that metal toxicity can elevate or reduce carotenoid content depending on metal types (Singh and Tewari, 2003) [33]. The findings in this experiment suggest that carotenoids only play a secondary role in the contribution to the defense mechanism of citrus species against Al toxicity.

Table 1: Changes in chlorophylls a, b, total and total carotenoids of Rough lemon (Ro) and Samphola (Sa) under the influences of Al treatments. Values represent the mean ± SE (n=3)

$AlCl_3.6H_2O$ (mM)	Total chlorophylls (mg/g leaf tissue)		Chlorophyll a (mg/g leaf tissue)		Chlorophyll b (mg/g leaf tissue)		Total carotenoids (mg/g leaf tissue)	
	Sa	Ro	Sa	Ro	Sa	Ro	Sa	Ro
0	1.888	2.929	1.230	2.042	0.658	0.887	0.430	0.526
600	1.881	2.669	1.203	1.841	0.678	0.828	0.433	0.448

1200	1.559	2.168	0.984	1.482	0.575	0.685	0.342	0.316
1800	1.449	1.978	0.914	1.340	0.534	0.638	0.272	0.265
CD *(p<0.05)*	0.062	0.094	0.039	0.070	0.029	0.034	0.017	0.028
SE(m)	0.019	0.029	0.012	0.021	0.008	0.010	0.005	0.008

Lipid peroxidation and total soluble protein

Lipid peroxidation is one of the most common features initiated by a range of abiotic and biotic stress factors and thus plays an indicating role when plants are under stress. Cakmark and Horst (1991) [3] suggested that peroxidation of lipid membranes occur due to the stress related increase in the production of free radicals or reactive oxygen species (ROS). It is suggested that lipid peroxidation is an early response to Al that is associated with Al-triggered inhibition in root elongation (Xu *et al*., 2012) [38S]. The oxidative damage to lipids of both the citrus species under the influence of aluminium significantly elevated with the corresponding increase in Al concentration with higher elevation seen in shoots than in roots of both citrus. The lipid peroxidation levels at the lowest Al dose recorded comparable elevations between the two species with 10.52% and 12.88% increase in shoots, and 5.31% and 7.42% increase in roots recorded in Samphola and Rough lemon, respectively. However, in the higher doses, the increase in lipid peroxidation levels was observed to be distinctly higher in Rough lemon in both shoots and roots as seen in Figure 4. It is therefore suggested that the higher oxidation of lipids in shoots of these two citrus species maybe due to low antioxidant activity to neutralize the effects of reactive oxygen species on the peroxidation of lipids. Higher level of lipid peroxidation in sensitive cultivar was also reported in rice (Pandey *et al*., 2013) which led to a consequential increase in electrolytic leakage and oxidative damage ultimately leading to reduced growth of the rice seedlings.

Alterations of plant proteins are an inevitable process in response to toxic heavy ions (Fecht-Chritoffers *et al*., 2003) [6]. In this experiment, total soluble protein content also significantly declined with the increase in the aluminium concentrations. Protein content in shoots was higher than in roots. However, under the influence of aluminium, higher decline was recorded in shoot protein than compared to roots. On comparing the citrus species at the lowest dose, Samphola recorded at par results with control in shoot protein (0.377% decline) and a slight insignificant increase in root protein (3.21% increase). On the other hand, Rough lemon recorded higher decline in protein content of both root and shoot and was observed to be more pronounced in the higher doses (Figure 5). The increase in total soluble protein in Samphola roots may be due to the expression of new stress proteins as suggested by Mohammadkhani and Heidari (2008) [22]. You *et al*. (2014) [41SS] also reported protein content decline of Citrus in which the 'sensitive species' *Citrus grandis* recorded higher decline than 'tolerant species' *Citrus sinensis*.

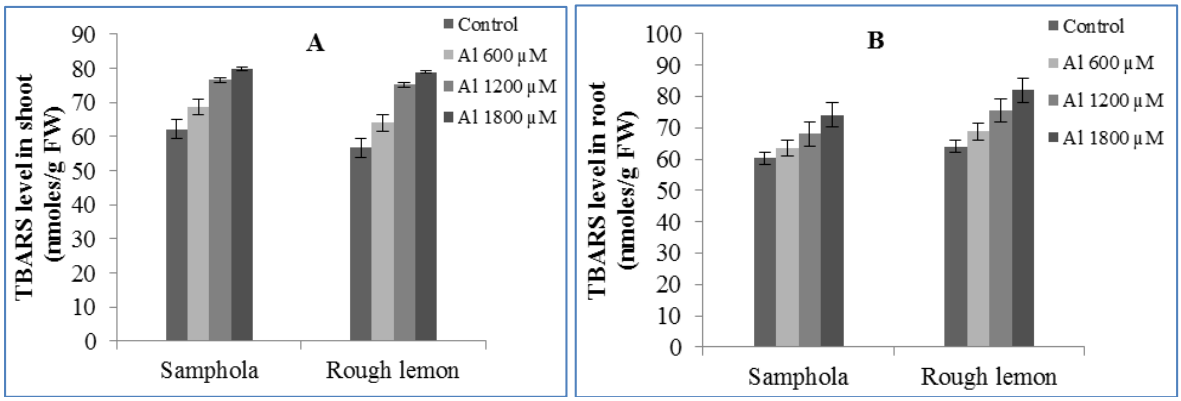

Fig 4: Elevation of lipid peroxidation levels in shoots (A) and roots (B) in the citrus species under the influence of Al treatment. Values represent the mean ± SE (n=3)

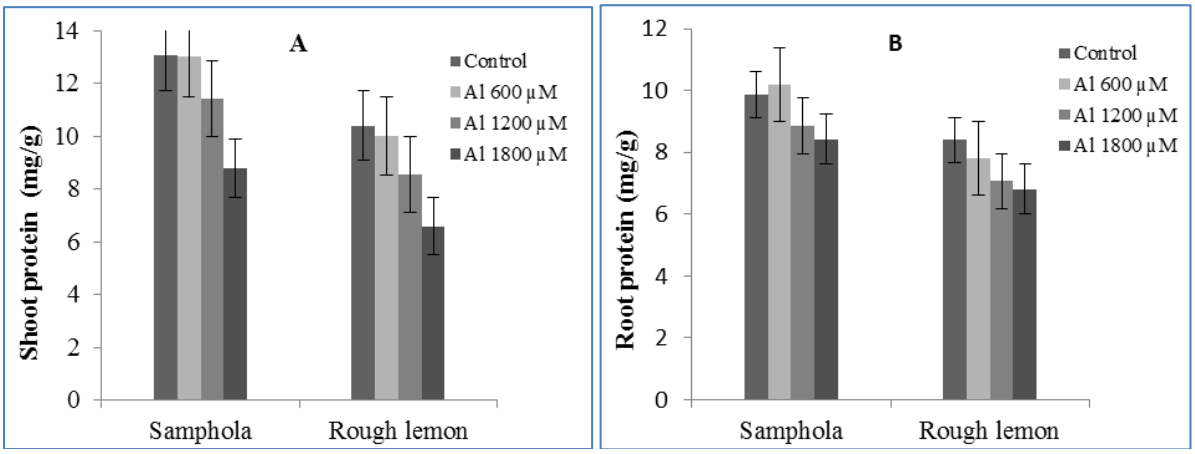

Fig 5: Changes in the total soluble protein content in shoots (A) and roots (B) in the citrus species under the influence of Al treatment. Values represent the mean ± SE (n=3)

Conclusion

In summary, the results of this study revealed distinct differences in the tolerance levels of Rough lemon and Samphola to the Al treatments which became more apparent in the higher doses. Our study shows that root growth inhibition as well as peroxidation of lipids is the prime symptom of Al toxicity in citrus. Reduction in total carotenoid content suggests that these pigments do not efficiently help in the detoxification of reactive oxygen species brought about by Al stress. The main outcome of this study, therefore, sheds light on the importance of wild citrus species and their contribution of tolerance to abiotic stress. In conclusion, Samphola emerges as a more efficient citrus species suitable for acid soils and thus have the potential to be used as a rootstock for cultivated species.

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