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Aluminium Toxicity: A Novel Approach for Identification of Tolerant and Susceptible Genotypes in Citrus

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ABSTRACT

Acidity of soils in India is steadily increasing due to some environmental problems as well as some farming practices along with acid rain. At mildly acidic or neutral soils, Aluminium (Al) occurs primarily as insoluble deposits and is essentially considered as biologically inactive. Citrus fruit production in acidic soils is restricted due to Aluminum (Al) toxicity and low availability of Phosphorous (P) content. Differential tolerance ability of Citrus genotypes to Aluminium stress is a promising approach for enhancing our understanding on Aluminium tolerance in different genotypes of Citrus. Studies on influence of Aluminium toxicities have been conducted over the years with little research focused on Citrus fruit crops. Also the significant effects of combinations of Aluminium with other heavy metals have received minor attention. In order to understand it thoroughly, efforts have been made to compare the relative sensitivity of various Citrus genotypes including micro and macro nutrients to Aluminium and its uptake in addition to transport of Aluminium are taken into account with respect to phytotoxicity and their interactions with nutrients. Therefore this manuscript is mainly focused to understand the knowledge of stress induced by Aluminium and other heavy metals on physiological and biochemical attributes of different genotypes of Citrus with an intention to screen degree of tolerance of each genotype as well as to compare their performance against each other. The current review primarily emphasizes on Aluminium toxicity and possible toxicity alleviation techniques in Citrus for identifying and differentiating tolerance ability of Aluminium toxicity among different genotypes of Citrus. Alleviation of Aluminium toxicity in Citrus could be overcome through increasing immobilization of Aluminium in roots and Phosphorous level in shoots rather than through increasing organic acid secretion.

Keywords Aluminium toxicity, Citrus, Tolerant genotypes, Soilless culture, Heavy metals.

INTRODUCTION

In neutral or slightly acidic soils, Al is primarily established in the form of insoluble deposits which is biologically inactive. In acidic solutions with a pH of less than 5.0, Al exists in the forms of $Al3^{+}$ and Al (OH) 2^{+}, which are soluble and easily available to the plants and because of the availability of micro-molar concentration of $Al3^{+}$ ion, it can rapidly inhibit root growth (Kinraide 1991). Al toxicity is a major fac-

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tor limiting crop productivity in many acidic soils through the tropics and subtropics. Over 50% of the world's potential arable lands are acidic in nature (Kochian 1995, Yang *et al.* 2013). Apart from it, acidity of the soils is gradually increasing due to some environmental problems, including acid deposition, improper application of chemical fertilizers, intensive agriculture and monoculture (Wu *et al*. 2013). Higher plants have evolved two main mechanisms of Al detoxification i.e., external and internal detoxification mechanisms that enable them to tolerate high levels of Al. Accumulation of Aluminium in plants is an important factor to comprehend the tolerance mechanism of plant species. Tolerance potentiality of plants to Aluminium toxicity is associated with not only to low Al uptake but also with little Al translocation from roots to shoots (Yang *et al*. 2011). But there are some complications that the molecular mechanisms for Al tolerance in higher plants are not fully understood as reported by the several researchers (Yang *et al.* 2013, Wang *et al*. 2015, Zhu *et al*. 2015). Soil acidification occurs naturally when basic cations involving calcium, magnesium and potassium are leached from soils. It is also accelerated by nitrogenous fertilizer and acid rain. Aluminium is the richest metal in earth's crust and constitutes approximately 8% by weight (Driscoll and Schecher 1990). Since Al is toxic to roots of higher plants, it inhibits root growth and uptake of water and nutrients resulting in retardation of plant growth leading to loss of crop production (Kochian 1995). Reason behind retardation of root growth is due to the sensitiveness of plant species to micro-molar concentrations of Al ions and low pH that enhances solubilization of Al. It has also been mentioned that Al ion has become toxic to plants and has been considered as a major factor in inhibition of plant growth and crop production in acidic soils. Root apex is the most sensitive zone, especially the distal transition zone, to Al stress because in this zone, a region behind the root tip measuring 1-3 mm, a transition occurs from cell division to cell elongation (Sivaguru *et al.* 1999 Sivaguru *et al*. 2013). In order to get rid of these problems, some special mechanisms have been evolved in particular for example, in case of detoxification of Al, Al-resistant plants have evolved some mechanisms by using organic acids to expel Al from the root apex or detoxify Al by chelating it with organic acids (Inostroza-Blancheteau *et al.* 2012). Exudation of organic acid anions, particularly citrate, oxalate and malate, enhances Al tolerance by forming stable complexes with Al (Ma *et al.* 2001). These organic acid anions also function in chelating Al in the cytosol. A suitable example for enhancement of Al due to an increasing use of organic acids is illustrated indicating over expression of citrate synthase in tobacco, canola and alfalfa; in addition, increase in malate synthesis in tobacco and alfalfa enhanced Al tolerance (Wang *et al.* 2010).

Origin of acidic soils

Several factors are responsible for the originality of acidic soils. Generally climate, hydrologic cycle vegetation, parent rocks and human interference plays a significant role in origin and development of acid soils. Acid soils occur generally in humid regions where rainfall is regular and very heavy. Dry regions are devoid of acidic soils.

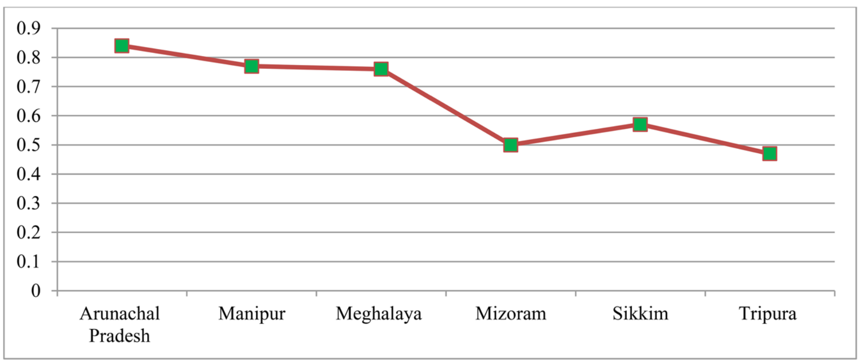

Fig. 1. Distribution of acidic soils in North Eastern Region of India.

Impact of plants on exposure to Aluminum toxicity in acidic soils

49 million hectares area in India is affected by soil acidity out of which 25 million hectares have a pH of less than 5.5. Such types of problematic soils are found in the regions of North Eastern states as well as some parts of Western Ghats. Soils of North Eastern states of India have a pH less than 5 and have been considered as strongly acidic in nature which constitutes about 84% soils of Arunachal Pradesh, Manipur (77%), Meghalaya (76%), Mizoram (50%), Sikkim (57%) and Tripura (47%) (Ishitani *et al.* 2004) (Fig.1). Acidic soils are mainly occurred in North Eastern as well as some other particular states of India such as Assam, Meghalaya, Arunachal Pradesh, Mizoram, Nagaland, Manipur, Tripura, West Bengal, Bihar, Uttar Pradesh, Himachal Pradesh, Jammu and Kashmir, Madhya Pradesh, Karnataka, Maharashtra, Tamil Nadu and Andhra Pradesh. Acidic nature of soils occurs predominantly in the areas where there is plenty of rainfall and can vary according to the landscape geology, clay mineralogy, soil texture and buffering capacity. Soil acidity is a natural phenomenon and is initiated and accelerated due to some agricultural practices. Probability of soil acidification is primarily due to the result of nitrate leaching. A term popularly known as 'Acid Soil Syndrome' is mainly reported in acidic soils covering almost 30% of the world's total land area (Chen *et al.* 2012). Constraints of acidic soil indicate a lower percentage of growth and survival of plants since roots of the plants are adversely affected if pH value exceeds limits of tolerance. Crops grown in acidic soils are likely to undergo several difficulties including toxicity of Aluminium (Al), Hydrogen (H), or Manganese (Mn), Potassium, Sulfur, Nitrogen, Boron, Copper and Zinc. It has been estimated that at lower pH, some elements are proved to be toxic to plants including Iron, Aluminium and Manganese and ultimately, Aluminium, Iron and Phosphorus combine to form insoluble compounds. Plant nutrients particularly Phosphorus, Calcium, Magnesium, Molybdenum, Iron, Manganese, Potassium, Sulfur, Nitrogen, Boron, Copper and Zinc available in natural form in plants decreases if the soil pH is in the range of 5-6.5. Maintaining ionic balance in soil-plant environment in agricultural lands requires crop plants to apparently absorb cations specifically K+, Ca+2 and Mg+2 leading to release in hydrogen ion. It is also obvious that addition of nitrogenous fertilizers may also release hydrogen ion through nitrification of ammonium (NH4+). Factors influencing major constraints in crop production, particularly in tropics and subtropics include soil acidification, a process associated with leaching of bases, high oxidative biological activities producing acids, high rainfall and low evaporation in addition to crop management practices etc. (Ishitani *et al.* 2004). Metal is considered to be the most widespread problem in acidic soils, where land use for agricultural purposes has been severely affected (Lilienfein *et al*. 2003). Al exists in a number of different forms in soil depending on pH (Wang *et al.* 2006). Al is solubilized into [Al (H_2O) 6]3+, generally referred to as Al3+, under acidic conditions, which proves to be highly toxic to many plant species (Éva *et al*. 2004). Majority of the plants are in a state of dilemma to withstand against Al toxicity since its common forms such as oxides and Alumino silicates are harmless to plants (Wang and Kao 2004). Under a condition of Al-stress, sensitive plants display a number of toxicity symptoms depending on species, variety and genotype. In some cases, increased susceptibility to drought stress, lodging and nutrient deficiencies are also reported from affected plants. Under this condition, crop species which are easily adapted on acidic soils gain increasing attention worldwide (Sun *et al.* 2010).

Effect on roots

Aluminium does not affect seed germination (Nosko *et al.*1988) but helps in new root development and seedling establishment but gradually it is observed that root growth inhibition could be detected immediately after 2-4 days of initiation of seed germination (Bennet *et al.* 1991). Plant species and ecotypes growing on acidic soils had become very resistant to inhibitory effects of Aluminium on root absorption and growth in course of time and phenological evolution (Vanpraag and Weissen 1985). Major Al toxicity symptoms observed in plants (Bennet *et al.* 1991, Delhaize and Ryan 1995, Foy *et al.* 1978, Kochian 1995, Marschner 1991, Ryan *et al.* 1993, Taylor 1988) indicates inhibition of root growth which has been literally reported by several researchers. Roots exhibit greater signs of cellular damage as compared to other parts of the plant (Rincon and

Gonzales,1992). Toxicity of Al is primarily observed in the root system particularly in root-tips and in lateral roots where its symptoms include thickening and browning of lateral roots (Roy *et al*. 1988). Root system as a whole is coralloid in appearance with many short lateral roots but lacks fine branching (Foy *et al*.1978). Toxicity appears to be determined by the availability of certain monomeric species of Al in plant roots (Blamey *et al.* 1983). Polymerization of Al leads to loss of phytoactive, monomeric Al which occurs due to increase in concentrations of Al and pH (Blamey *et al.* 1983) to make complex formation or chelation with phosphate and organic acids (Blamey *et al.* 1983). Aluminium is absorbed in large amounts in the tip portion of the root. In tip portion, Potassium content decreases with an increased amount of Al content, while concentration of Ca remains almost constant. An anisotropic growth response of cortical cells with an exposure to roots in Al for 20 hours were associated with disintegration of conducting tissue in addition to outer cells of the root (Bennet *et al.*1985). Inhibition of root growth is a typical symptom of Al and the extent of inhibition depends on both cultivar and Al concentration. Assessment of Al tolerance based on root growth and root tolerance index has been used extensively utilized in genetic and molecular studies (Somers *et al.*1996). Different genotypes have varying tolerance ability on exposure to varying Al concentrations. Toxic effects of Al on root growth with respect to length, spread and orientation in susceptible varieties are well-recognized (Doberman and Fairhurst 2000). Tolerance level of a genotype may not be always dependent on the number of primary roots and root length because both the parameters are likely to have similar results in stressed and stress-free environments. In this case, root vigour, root growth pattern, total root area, or total root mass of the genotypes under stressed and stress free environments have to be considered (Famoso *et al.* 2010).

Morphological responses and tolerance efficiency of Citrus cultivars on toxicity to Aluminium

Citrus belongs to evergreen subtropical fruit trees and is known to be sensitive to Aluminium. Low pH and high Al concentration are the factors contributing to poor growth and shortened lifespan of citrus trees (Lin and Myhre 1990). Origin of the loose skinned mandarin has been reported to be found specifically in North Eastern states of India including Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Tripura and Sikkim as well as Darjeeling Hills of West Bengal. Entire production is through seedlings and not from the rootstocks. Locally cultivated mandarin varieties in these regions include "Khasi", Darjeeling and Sikkim oranges. Major constraints in these regions includes high rainfall (6-7 months), hills slope cultivation, eroded and heavily lead soils of acid reaction, malnutrition of nutrient deficient soils (both major and minor), high infestations of insect pests. In terms of occurance of disease particularly Phytophthora which is predominantly occurs in heavy clay soils of Assam is very destructive to Citrus plantation. Another major constraint in Citrus cultivation in the North Eastern region is prevalence of acidic soils that limits growth and production of better quality plants. During dry season, when Citrus is grown on slightly acid sulfate soils with a pH ranging from 4.2-5.5, there is a possibility of occurance of Aluminium toxicity because during this period, soil evaporation is very high and water transported from deep in the soil to the surface, contains sufficient levels of Aluminium to harm the root system which leads to serious yield loss. Although effects of Aluminium on mineral nutrient (Lin and Myhre 1991) and CO_2 assimilation (Chen *et al*. 2005a) of Citrus have been investigated by a few researchers, very little information is well-known in terms of effects of Aluminium on root system of Citrus fruits. Due to Aluminium (Al) toxicity and low availability of phosphorus (P) content, crop production is limited in acidic soils. Symptoms for example, inhibition of root elongation, photosynthesis and growth are occurred in Citrus due to Aluminium toxicity (Arunakumara *et al*. 2012). Emphasizing at toxicity mitigation, interaction between Boron (B) and Aluminium (Al) in addition to Phosphorus and Aluminium have been thoroughly discussed. Al toxicity in Citrus could be alleviated by Phosphorus through increasing immobilization of Al in roots and Phosphorus levels in shoots rather than through increasing organic acid secretion. Effects of Aluminium toxicity that induced alterations of protein profiles in Citrus leaves were examined and identified some new Al toxicity responsive proteins related to various biological processes (Li 2016). For conducting the experiment, seedlings of Aluminium tolerant "Xuegan" (*Citrus sinensis*) and Aluminium intolerant sour pummelo (*Citrus grandis*) were used. Seedlings of these two species were fertigated with nutrient solution containing 0 and 1.2Mm AlCl3.6H_2O for 18 weeks. It was observed that inhibition of photosynthesis as well as reduction of total soluble protein was occurred in leaves of *Citrus grandis* indicating *Citrus sinensis* had higher Aluminium tolerance than *Citrus grandis.* Aluminium toxicity related to RNA regulation, protein metabolism, cellular transport and signal transduction might also create a significant impact in higher Aluminium tolerance ability of *Citrus grandis*. Citrus is largely grown in acidic and strong acidic soils (Lin and Myhre 1990). Low pH and high Al are the factors responsible for causing poor growth and decrease in lifespan of Citrus trees. Effect of Al on growth of *Citrus limonia* Osbeck, *Citrus volkameriana* Hort. ex Tan, *Citrus reshni* Hort. ex Tan and *Citrus sunki* Hort. ex Tan in hydroponic culture was studied and was observed that there was a reduction in growth of shoot, leaf area ratio and leaf weight ratio under Al-stress (Pereira *et al.* 2003). However, at the initial stage of root growth, relative growth rate (RGR) of all the rootstocks was found to be increased even in presence of Al in nutrient solution, which might be due to initial increase in net photosynthesis rate (Pereira *et al.* 2000) which investigated gas exchange and chlorophyll fluorescence in four Citrus rootstocks under Al-stress conditions. Aluminium tolerance mechanism in yuzu (*Citrus junos* Sieb ex Tanaka) on the basis of root elongation was studied and was concluded that Yuzu was tolerant as compared to other plant species (Deng *et al.* 2009). Exposure of Yuzu to Al concentration had lead to the secretion of citrate from the roots. Thus mechanism of Aluminium tolerance in Yuzu involved Aluminium inducible increase in citrate released and increased expression level of mitochondrial synthase (CjCs) gene and enzyme activity in Yuzu. CjCs gene was cloned from Yuzu and over expressed in *Nicotiana benthamiana* using *Agrobacterium tumeficiens* mediated methods. Increased expression level of CjCs gene in addition to enhanced enzyme activity was observed in transgenic plants compared with wild type plants. Root growth experiments have indicated an enhanced level of Al tolerance in transgenic plants. Transgenic Nicotiana plants showed increased levels of citrate in roots compared to wild type plants. Exudation of citrate from roots of the transgenic plants significantly increased when exposed to varying concentrations of Aluminium. Results of transgenic plants suggest that over expression of mitochondrial CS can be a useful tool to achieve Al tolerance. A study was conducted to determine the interaction of Boron and Aluminium and its effect on growth, CO2 assimilation, ribulose 1-5 biphosphate, carboxylase/ oxygenase and photosynthetic electron transport of *Citrus grandis* seedlings (Jiang *et al*. 2009b). It was observed that an adverse effects of Al inhibited photosynthesis in plants as well as inhibition of growth. Considering the response of Boron, decrease in growth of stem and leaf with varying concentrations of Al under Al stress condition was observed. Furthermore, Phosphoros is also reported to alleviate Al toxicity in Citrus by increasing immobilization of Al in roots (Yang *et al.* 2011). Soil acidification has been occurring rapidly in pummelo orchards since last few years. Therefore, understanding such mechanisms of Al toxicity and Al tolerance in Citrus plants is very important for Citrus production. During 1998-1999 pH of 200 soil samples from Pinghe pummelo orchards were examined. The pH ranged from 3.57 to 7.25 with an average value of 4.63. 85.5% of the soils had a lower pH than 5.0 (Huang *et al.* 2001). Growth parameters such as leaf, stem and root (fresh) and dry mass of Citrus plants (Citrus reshni Hort. Ex Tan) showed marked reductions compared to control. Furthermore, it has been noticed that reductions in leaf and fresh and dry mass of stem were greater than those of the root in response to Al (Chen *et al*. 2005b). A reduction in shoot dry mass in Citrus grandis (L.) seedlings irrigated for 5 months with nutrient solution containing different concentrations (0.2, 0.6 or 1.6mM) of Al was observed (Jiang *et al*. 2008. Chen *et al.* 2009). However, no significant decrease in root dry mass was being observed up to 0.6 mM Al in the nutrient solution. C. sinensis roots secretes more malate and citrate than C. grandis in response to Al toxicity (Yang *et al.* 2011) however ,it was also prominent that Al-induced secretion of malate and citrate decreased with increasing supply of Phosphorous. Involvement of Phosphorous in alleviating Al toxicity through increasing immobilization of Al in roots is much stronger than toxicity reduction through increased secretion of organic acid anion. Furthermore, probability of higher Al-tolerance percent observed in *C. sinensis* might be due to the secretion of organic acids and precipitation of Al by Phosphorus in roots. Al-induced secretion of malate and citrate was observed in *C. grandis* indicating concentrations of malate and citrate were less affected by the interaction of Phosphorus and Al in roots compared to leaves (Chen *et al.* 2009). This might be due to the concentrations of both organic acids which were higher in shoots with Al than without Al, whereas on the other hand in roots, both the components were lower with Al than without Al. Therefore decreased concentrations of malate and citrate in roots in response to Al could be due to Al-induced exudation of organic acids under Phosphorus (Chen *et al*. 2005). In *Citrus reshni*, in terms of CO_2 assimilation, non-photochemical quenching (NPQ), photochemical quenching (qP), effective quantum yield of PSII and maximum quantum yield of PSII (Fv/Fm) were decreased due to the Al toxicity (Chen *et al*. 2005, Martins 2013). In Al-sensitive *Citrus grandis,* a decrease in total soluble protein in leaves was reported under Al toxicity, whereas no change occurred in the Al-tolerant species *C. sinensis* (Li 2016). Health risk assessment of Citrus contaminated with heavy metals and the potential risk of Aluminium and Copper were studied (Raja *et al.* 2016) which further indicated that there was no potential risk for children and adult consuming those experimented Citrus but as a result of the increased utilization of agricultural inputs (metal based fertilizers and pesticides, sewage, sludge and waste water) by farmers and orchardists, regular periodic monitoring of chemical pollutants content in foodstuffs are recommended for food safety. Determination of a relationship between different concentrations of Aluminium and mineral concentrations in Citrus seedlings were studied where six months old seedlings of five Citrus root stocks were used and were grown for 60 days in supernatant nutrient solutions of Aluminium, Phosphorus and other nutrients (Lin and Myhre, 1991). The solution consisted of seven different concentrations of Aluminium ranging from 4-1655µM and similar Phosphorus concentrations of 28µM Phosphorus. Aluminium concentrations in roots and shoots increased with increasing Aluminium concentration in nutrient solution. Interestingly, it was further noticed that Aluminium concentrations in roots of Aluminium tolerant rootstocks were higher than those of Aluminium sensitive rootstocks. It was also observed that correlations of minerals were different at varying Aluminium concentrations in nutrient solutions in addition to K, Mg and P concentrations and the K and P levels in shoots increased at 4-178 µM. However concentration of Ca, Zn, Mn and Fe in the shoots had gradually decreased. Higher concentrations of Fe were found in roots of more tolerant rootstocks as compared to lesser tolerant ones when Al concentrations in solutions were lower than 308µM. Considering the importance of other nutrient elements in response to shoot and root growth of Citrus rootstocks, concentrations of other elements (Ca, K, Mn, Zn and Mg) in roots or shoots exhibited no apparent relationship to Al tolerance for roots or shoot growth of the rootstocks. Conversely, Ca, K, Zn, Mn and Fe concentrations in roots in addition to Mg and K concentrations in shoots of all five rootstocks seedlings have shown significant negative correlations with Al concentrations in corresponding roots or shoots. Response of two Citrus species with respect to Aluminium toxicity were studied where young seedlings of *Citrus sinensis* and *Citrus grandis* were treated with a nutrient solution containing 0 (control) and 1.2 mM $AlCl3.6H_20$ for 18 weeks (Jiang *et al*.2015). Due to the effect of concentrations of Aluminium, it gradually inhibited shoot growth of *Citrus grandis* but had no significant influence on shoot growth of *Citrus sinensis,* whereas in case of root, Al concentration did not differ between two Citrus species. In addition, it was also observed that inhibition of shoot growth on exposure of Al toxicity was less severe in seedlings of *Citrus sinensis* compared to *Citrus grandis*. Potentiality of higher Al tolerance ability of *Citrus sinensis* is significantly related to several factors such as activation of Sulphur metabolism, which helps in improving total ability of anti-oxidation and detoxification, upregulation of carbohydrates and energy metabolism enhancing cell transport, decreased (increased) abundance of proteins synthesis (proteolysis) keeping a better balance between protein phosphorylation and de-phosphorylation. A hydroponic approach was experimented in order to discuss consequences of Aluminium on *Citrus volkemeriana, Citrus nobilis* var. *microcarpa* and three varieties of *Citrus grandis* (Toan *et al.* 2003). Seedlings of these particular Citrus species were treated with seven concentrations of AlCl3 viz., 0 Mm, 50µM, 100µM, 300µM, 500µM 1000µM, and 2000µM. After 27 days of treatment, root growth was completely inhibited by an exposure of Aluminium concentrations between 1000µM-2000µM. In addition, a negative correlation between root elongation and higher concentrations of Aluminium were observed.

CONCLUSION

Aluminium toxicity is an imperative growth-limiting factor for plants in many acidic soils, particularly in pH of 5.0 or below. Aluminium toxicity in plants is often clearly identifiable through morphological and physiological symptoms. Differential tolerances to Al toxicity involve differences in the structure and function of roots. An adverse effect of Aluminium toxicity on various genotypes of Citrus indicates interference with cell division in roots, decreases root respiration and uptake and use of water and nutrients, particularly Calcium and Phosphorus along with metabolic pathway. Other promising approaches to study metal toxicity in tolerant and susceptible genotypes of Citrus are to determine the metal uptake and transportation in various plant parts, mechanism behind interaction with mineral nutrients, specific genes responsible for tolerance, levels and kinds of organic and amino acids which acts as metal chelators and detoxifiers, level and forms of enzymes and changes in root permeability to ions and molecules along with its mechanisms. In this manuscript, as already mentioned above, though Citrus is sensitive to Al toxicity, ability of these genotypes to withstand against adverse impacts of Al toxicity through several mechanisms is impressive. Here, justifications of findings laid out by several researchers have been reviewed with an objective to increase our understanding of the mechanism of Citrus on tolerance to Al toxicity prevalent in acidic soils. Moreover this manuscript has shed lighted on new information that could aid in identification of suitable rootstocks for Citrus production in acidic soils.

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