IONOME AND PROTEOME ASSISTED CHARACTERISATION OF ALUMINIUM TOLERANCE IN COWPEA (*VIGNA UNGUICULATA* (L.) WALP.)

A Thesis Submitted
To
Sikkim University

In Partial Fulfilment of the Requirement for the
Degree of Doctor of Philosophy

By

JITENDRA KUMAR KUSHWAHA

Department of Horticulture

School of Life Sciences

May, 2019

Date: 09/05/2019

DECLARATION

I, Mr. **Jitendra Kumar Kushwaha**, hereby declare that the research work embodied in the thesis titled "**Ionome and proteome assisted characterisation of aluminium tolerance in cowpea [*Vigna unguiculata* (L.) Walp.]** submitted to Sikkim University for the award of the degree of Doctor of Philosophy, is my original work. The thesis has not been submitted for any other degree of this university or any other university.

Jitendra

Signature of the Candidate
(Jitendra Kumar Kushwaha)
Registration No.: 14/Ph.D/HOR/02
Department of Horticulture
School of Life Sciences
Sikkim University
Gangtok

6 माइल, सामदुर, तादोंग -737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स -251067
वेबसाइट - www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

6th Mile, Samdur,Tadong -737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax: 251067
Website: www.cus.ac.in

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
(A central university established by an Act of Parliament of India in 2007 and accredited by NAAC in 2015)

Date: 09/05/2019

CERTIFICATE

This is to certify that the thesis titled "**Ionome and proteome assisted characterisation of aluminium tolerance in cowpea [*Vigna unguiculata* (L.) Walp.]**" submitted to the Sikkim University for partial fulfillment of the degree of Doctor of Philosophy in the Department of Horticulture, embodies the result of *bonafide* research work carried out by **Jitendra Kumar Kushwaha** under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, association and fellowship.

All the assistance and help received during the course of investigation have been duly acknowledged by him.

I recommend this thesis to be placed before the examiners for evaluation.

(S. Manivannan)
Supervisor
Department of Horticulture
School of Life Sciences
Sikkim University
Gangtok

6 माइल, सामदुर, तादोंग -737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स -251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur,Tadong -737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax: 251067
Website: www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
(A central university established by an Act of Parliament of India in 2007 and accredited by NAAC in 2015)

Date: 09/05/2019

CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"Ionome and proteome assisted characterisation of aluminium tolerance in cowpea (*Vigna unguiculata* (L.) Walp.)"** submitted to the Sikkim University, in partial fulfillment of the requirements of the award of the degree of **Doctor of Philosophy** in **Department of Horticulture,** embodies the result of *bonafide* research work carried out by **Mr. Jitendra Kumar Kushwaha** under the guidance and supervision of Dr. S. Manivannan. No part of the thesis has been submitted for any other degree, diploma, association and fellowship.

All the assistance and help received during the course of investigation have been duly acknowledged by him.

I recommend this thesis to be placed before the examiners for evaluation.

Niladri Bag 9.5.19
(Niladri Bag)
Head of the Department
Department of Horticulture
School of Life Sciences
Sikkim University
Gangtok

6 माइल, सामदुर, तादोंग -737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स -251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur,Tadong -737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax: 251067
Website: www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
(A central university established by an Act of Parliament of India in 2007 and accredited by NAAC in 2015)

Date: 09/05/2019

PLAGIARISM CHECK CERTIFICATE

This is to certify that plagiarism check has been carried out for the following Ph.D. thesis with the help of **URKUND SOFTWARE** and the result is **6 %** tolerance rate, within the permissible limit (below 10% tolerance rate) as per the norms of Sikkim University.

"Ionome and proteome assisted characterisation of aluminium tolerance in cowpea [*Vigna unguiculata* (L.) Walp.]"

submitted by **Jitendra Kumar Kushwaha** under the supervision **of Dr. S. Manivannan, Associate Professor, Department of Horticulture, School of Life Sciences, Sikkim University.**

.....

Jitendra Kumar Kushwaha

Signature of the Scholar

.....

Dr. S. Manivannan

Countersigned by Supervisor

.....

Vetted by Librarian

Affectionately

dedicated

To my beloved

Parents

ACKNOWLEDGEMENT

It is my immense pleasure to express heartfelt gratitude to my respected teacher and guide, ***Dr. S. Manivannan,*** *Associate Professor, Department of Horticulture, Sikkim University, Gangtok, Sikkim, for his able guidance and suggestion, sustained interest, kind encouragement and constructive criticism during the course of investigation. His sincerer monitoring and encouragement made the work complete with perfection. It has been my privilege to work under him. I am extremely indebted to him for being meticulous throughout the investigation and preparation of this manuscript. I will remain obliged to him in my personal thought and career.*

I avail to express my humble respect, deep sense of indebtedness to ***Dr. L. Sharma****, Associate Professor, Department of Horticulture and* ***Dr. Sujata Upadhyay****, Assistant Professor, Department of Horticulture for their valuable suggestion, inspiration and liberal help during the course of research.*

I express my profound gratitude to ***Dr. P. Umadevi****, Scientist, Department of Crop improvement and Biotechnology, Indian Institute of Spice Research, Kozhikode, Kerala for her valuable and kind help.*

I wish to record my deep sense and advent to ***Dr. Niladri Bag****, Associate Professor, Department of Horticulture,* ***Dr. Manju Rana****, Assistant Professor, Department of Horticulture,* ***Dr. K.D. Bhutia****, Assistant Professor and* ***Mr. Rajesh Kuamr****, Assistant Professor, Department of Horticulture for their valuable suggestion, inspiration and kind help rendered during the course of research.*

A full of gratitude and warm regards to ***Prof. (Dr.) J.P. Tamang,*** *Dean, School of Life Sciences, Sikkim University, Gangtok, Sikkim for his generosity in extending valuable support and needs.*

I gratefully acknowledge the funding agency, the University Grant Commission (UGC) of the Government of India, for providing financial support, to complete my thesis

work, in the form of National fellowship for students of Other Backward Classes to pursue Ph.D. degree. I acknowledge the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala for doing the proteomics analysis of my research samples.

I take this rare opportunity to record my heartfelt love and sincere thanks to my ever supporting father ***Shri Om Prakash Kushwaha*** *and my ever loving mother* ***Smt. Kusumlata Kushwaha,*** *younger sister* ***Manorama*** *and* ***Vasundhara*** *and younger brother* ***Pradeep*** *and* ***Vishal.***

I owe my special thanks to Mr. Dinesh Rai, lab attendant, Mr. Venkat Ramana Muddarsu and Ms. Uzma Khatoon, Department of Horticulture for their help during the present course of investigation.

Colorful blossoms would not have bloomed without the company of my lovely friends, I heartily thank to my friends particularly Bikas, Yamuna, Nadia, Trisang, Anjana, Dikki, Keisam Pradeep, Sangay, Suren, Kabita, Smriti, Doma, Vivek, Bahadur, Divya and Reymit for their love and kind co-operation.

The presentation that follows is the work assisted by many seen and unseen hands and minds. I am thankful to all of them.

Gangtok, 2019 ***(Jitendra Kumar Kushwaha)***

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATE

ABBREVIATIONS

MSL	Mean Sea Level
DM	Dry Matter
SDM	Shoot Dry matter
RDM	Root dry matter
PH1	Plant Height after one week of planting
PH2	Plant Height after two week of planting
PH3	Plant Height after three week of planting
PH4	Plant Height after four week of planting
PH5	Plant Height after five week of planting
PC	Principal Component
RL	Root length
BM	Biomass
RRL	Root regenerate length
ATP	Adenosine triphosphate
A0	Aluminium at 0 micromole level
A25	Aluminium at 25 micromole level
A50	Aluminium at 50 micromole level
A100	Aluminium at 100 micromole level
G	Genotype
G×Al	Genotype and aluminium interaction
meq	miliequivalent
@	at the rate of
h	hour
t	tonne
ha	hectare
g	gram
mg	milligram

kg	kilogram
cm	centimeter
%	Percentage
°C	Degree Centigrade
Da	Dalton
µg	Micro gram
µl	Micro litre
µM	Micro mole
hr	Hour
mm	Milimetre
mM	Milimole
nm	Nanometer
ml	Milliliter
v/v	Volume/volume
v/w	Volume /weight
$cmol_c\ kg^{-1}$	centimols of charge per kg
mRNA	messenger Ribonucleotide
PCA	Principal Component analysis
QTLs	quantitative trait loci
ICP-MS	Inductively coupled plasma mass spectrometry
LC/MS/MS	Liquid chromatography tandem mass spectrometry
Ag	Silver
Al	Aluminium
As	Arsenic
B	Boron
Ba	Barium
Be	Beryllium
Bi	Bismuth
C	Carbon
Ca	Calcium

Cd	Cadmium
Cl	Chlorine
Co	Cobalt
Cr	Chromium
Cu	Copper
Cu	Copper
Fe	Iron
Ga	Gallium
H	Hydrogen
K	Potassium
Li	Lithium
Mg	Magnesium
Mn	Manganese
Mo	Molybednum
N	Nitrogen
Na	Sodium
Ni	Nickel
O	Oxygen
P	Phosphorus
Pb	Lead
Pt	Platinum
Rb	Rubidium
S	Sulphur
Se	Selenium
Si	Silicon
Sn	Tin
Sr	Strontium
Zn	Zinc

CHAPTER- 1

INTRODUCTION

Chapter- 1

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp] is one of the legume vegetables, which belongs to family leguminosae (Fabaceae). It's a diploid species (2n = 2x = 22) and belongs to the section catiang, subspecies *unguiculata,* genus *Vigna*, tribe *Phaseoleae* (Marechal *et al*., 1978). It is originated from West Africa with center of domestication (Burkina Faso, Nigeria, Ghana and Niger) which shows rich diversity in their germplasm (Fawole *et al*., 2001). It is one among the oldest source of human food, most likely been used from Neolithic times (Kumar and Mahla, 2004). Cowpea is one of the main food legume crops in developing countries of the semi-arid tropics of Asia, southern Europe, Africa and Central and South America (Singh *et al.*, 1997). Apart from semi-arid tropics area, it is also grown in some temperate areas, including the Mediterranean region and the southern states of the USA (Pasquet, 2000). In Asia the main cowpea growing countries are India, Bangladesh, Sri Lanka, Pakistan, China, Myanmar, Indonesia, Korea, Nepal, Malaysia, Philippines and Thailand. India itself accounts for almost half of the total area of production.

In India, cowpea is mainly grown in semi-arid regions and western, central and peninsular regions are the major contributors. It is cultivated by commercial and subsistence farmers for its long green pods as vegetable, seeds as pulse and foliage as vegetable, and as fodder. Cowpea is also known as China pea, black-eye pea, Kaffir pea and Southern pea, when it is grown for dry seeds production. The cultivars which are grown for the immature pods, variously known as yard-long bean, asparagus bean and snake bean (Bose *et al*., 1986). Cowpea is a warm weather crop and drought tolerant, which acclimates better to the dry areas of the tropics, where the other food

legumes are not performing well. It is also having the ability to fix the atmospheric nitrogen through its root nodules which enables it to develops well in poor soils with more than 85% sand, less than 0.2% of organic matter and less intensities of phosphorus (Singh, 2003). They are the good source of protein and thus nourishing the dietary balances of populations with low income (Singh *et al*., 1997).

A commonly used classification system sub-divides all the domesticated forms of cowpea into four cultivar groups which is principally based on characteristics of seed and pod (Westphal, 1974; Ng and Marechal, 1985). The first cultivar group is *Unguiculata* which is grown as a pulse. The second is known as *Biflora* (catjang) which is primarily used as a forage. Cowpea grown as a vegetable comes under *Sesquipedalis* group (yardlong or asparagus bean), and *Textilis* are cultivated for its long floral peduncles fibres. After that, Pasquet, (1998) proposed another cultivar group named as *Melanophthalmus* (black-eyed pea).

Growth habit of cowpea ranges from determinate, erect, non-branching type to prostate or climbing and indeterminate growth with profuse branching. It has robust tap root system along with numerous lateral roots. It's stem is cylindrical and slightly ribbed, with twisting, sometimes hollow and glabrous. Leaves are alternate arranged in trifoliate manner with one symmetrical terminal and two asymmetrical leaflets (Ram, 1998). The seeds of cowpea possess high nutritional value (Ehlers and Hall, 1997). The plants of cowpea are well acclimatized to grow under higher temperature and drought condition (Hall and Patel, 1985). They can tolerate the low level of soil fertility due to the high rate of nitrogen fixation ability (Eloward and Hall, 1987) and capability to form operational symbiotic mycorrhizae (Kwapata and Hall, 1985). Therefore, cowpea can perform a significant role in development of agriculture where the drought and salinity are prime limitation for crop production. Cowpea is used in various forms. The

green pods, green seeds and young leaves are used as vegetables; dried seeds are used in several food preparations; and the haulms are served to livestock as a nutritive supplement to cereal fodder.

A major limitation for production of cowpea is aluminium (Al), which becomes toxic on extremely weathered and leached soils in areas of humid tropics (Minella and Sorellis, 1992). In such soils acidity causes unproductiveness and limits crop production (Von Uexkull and Mutert, 1995). About 30% of the total land area of earth comprises of acid soils, and about 50% of the world's arable lands are acidic (Bot *et al*., 2000). Deficiencies of calcium (Ca), phosphorus (P) and magnesium (Mg) coupled with the presence of phyto-toxic matters are accountable for the productivity limitation of acid solids as intensified by the industrial pollution and nitrification. Al saturation is the main cause of poor growth in acidic soils (Akinrinde *et al*., 2004). A common symptom of toxicity of aluminium is deficiency symptom of phosphorus (Haynes, 1984; Huang *et al*., 1992). While estimating the soil constraint to plant growth in developing countries, it was found that on an average 23% of soils used were affected from aluminium toxicity (Anitzen and Ritter, 1984). Al toxicity had limited the spreading out of cowpea to important agricultural areas of the world (Alam, 1981). A tolerance at the genetic level is of great significance for toxic level of Al for crop production on acidic soils because increasing soil pH by lime application is very costly and it is limited to the surface layer (Alam, 1981; Foy, 1992). Restriction of crop growth by excess of aluminium could be due to the direct inhibition of nutrient uptake or disruption of root cell function (Kochian, 1995). A combination of sound management practices along with tolerance to Al is capable of ameliorating the adverse impact of acid stress on cowpea performance (Akinrinde *et al*., 2004). Several studies (Kadiata and Lumpungu, 2003; Oikeh *et al*., 2003; Hogh-Jensen and Pedersen, 2003) have

highlighted the fact that plant species and even varieties within species vary in their capability for biomass production and grain production under stress condition. So, identification of cowpea varieties that can stand excess Al in hydroponic condition would contribute in improving the yield of the crop. Thus, a major step in breeding of aluminium tolerant cultivar is to identify Al-tolerant cowpea genotypes.

After the screening of genotypes we have to also know the mode of aluminium tolerance and susceptibility as well as the effect of aluminium on uptake of various elements. So, for the study of effect on uptake of elements a high throughput elemental profiling technology named as 'Ionomics' is found to be very much helpful. Ionome can be defined as "the mineral nutrient and trace element composition of an organism, and represents the inorganic component of cellular and organismal system" (Salt *et al.* 2008). The study of the ionome known as Ionomics which involves the quantitative and concurrent measurement of the elemental composition of organisms or tissues. Any alterations in this composition in response to physiological processes needs the application of high-throughput elemental analysis technologies and their combination with both bio-informatics and genetic tools (Salt *et al*., 2008). Several latest studies related to high-throughput elemental profiling demonstrated that how the ionome responds to the environmental changes or changes the genetics that regulate the ionome (Baxter, 2009). Ionomics has capability to accurately capture information about the functional state of an organism under changed conditions. These conditions may either be determined by genetic differences, developmental differences, as well as the environmental effects including biotic and abiotic factors. Except carbon and oxygen, most of the other elemental composition of plant is acquired from soil (Baxter and Dilkes, 2012). Any variations related to soil will finally lead to the variation in plant ionome. Most of the preliminary ionomic studies ignored the impact of environmental

parameters until Baxter *et al*., (2008) found that either P deficient conditions produced significantly impact on the concentrations of B, As, Cu, Mn, Co, Zn, Mo and Cd. Although hydrology and topology of the field soil have large effects on the elements that are available for uptake, plants with altered elemental profiles in a field condition have the potential to shed light on soil and plant interactions which are not visible in controlled growth environments.

Identification and characterization of aluminium resistant genes or proteins will not only improve our understanding of aluminium resistant mechanisms, but more importantly, it will also provide the new molecular information that can be used to develop and improve the crop cultivars for cultivation on acid soils (Kochian, *et al.,* 2005).

Although the physiological aspects for Al tolerance have been comparatively well understood over the past decades but its molecular basis has been poorly known. Till now, the physiological studies have directed to propose two Al resistance mechanisms: one is to exclude Al from the root apex and the other that allows for the plant to tolerate Al accumulation in the root and shoot symplasm (Kochian, *et al.,* 2004). Accumulation and exudation of organic acids have been considered to play key roles in both internal detoxification and exclusion mechanisms (Kochian, *et al.,* 2004; Ryan *et al.,* 2001). More intriguingly, Al resistance may be an inducible process (Kochian, *et al.,* 2004; Kochian, *et al.,* 2005), which indicates the possibility that profiling of the aluminium responsive genes or proteins will lead to the identification of factors important for Al resistance (Kochian, *et al.,* 2004). Although transcriptome analysis might contributes very well to our understanding of Al stress responses but not all the data are functionally conclusive because the changes in mRNAs may not always keep pace with the alterations in protein levels and functions of the

corresponding proteins (Griffin *et al.*, 2002; Yan *et al.*, 2006). Since protein expression is controlled not only at transcriptional, but also at translational and post-transcriptional stages so, information at translational and post-translational stages can give more comprehensive understandings into the mature proteins and their functional interactions than genome-based prediction. Thus, proteome analysis may yield more precise and comprehensive information than what genomic studies can make available (Bae and Chen, 2004).

So, application of ionomics and proteomics can reveal the genetics and ionic interaction of aluminium tolerance in cowpea lines which may provide the complete understanding of mechanism of aluminium toxicity and its tolerance in cowpea for reducing their negative effects in future. Keeping these points in consideration the present investigation "Ionome and proteome assisted characterisation of aluminium tolerance in cowpea [*Vigna unguiculata* (L.) Walp.]" was executed with following objectives:-

1. To select tolerant and susceptible germplasm from collected cowpea accessions against aluminium toxicity in hydroponics culture.
2. To study the changes in morphophysiological character of cowpea lines against aluminium toxicity.
3. To elucidate profile of ions in the susceptible and tolerant germplasm lines of cowpea.
4. To bring out profile of proteins in the susceptible and tolerant germplasm lines of cowpea.
5. To correlate the morphophysiological characters, ionome and proteome profile of tolerant and susceptible cowpea germplasm lines grown in aluminium stress condition.

CHAPTER- 2

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

The available relevant literature on different aspects of present investigation has been reviewed underneath the following heads:

1. Acidic soil
2. Screening for aluminium tolerance
3. Ionome and multi-elemental profiling for stresses
4. Proteome profiling for aluminium tolerance

2.1 Acidic Soil

Soil acidity is one among the foremost vital factors that have an effect on crop production worldwide. Soil acidity affects more than 1.5 billion hectares worldwide.. In India, forty-nine million hectares of land are tormented by soil acidity out of which twenty-four million hectares have hydrogen ion concentration below 5.5 (Mandal, 1997). Within the north-eastern region of India, over 95% area is affected by soil acidity (Sharma and Singh, 2002). The productivity potential of acidic soils is calculated to vary from 25% to 80% less than normal soil. It may increase because of acid precipitation, nitrogen fertilization and natural weathering (Graham and Vance, 2000). The proton concentration per se, aluminium and manganese toxicity and deficiency of P, Mo or Ca all contributes to the problem (Graham, 1992). Crop productivity in acid soil is restricted as a result of the prevalence of multiple abiotic stresses. Aluminium is thought to be the largest contributor to upland soil acidity (Van Breeman and Moorman, 1978). Al-toxicity impairs productivity in soils having low pH (below 5.0). Free Al ions are solubilized at low pH. However, alternative factors, like Mn toxicity and deficiency of phosphorus,

calcium and magnesium, conjointly interplay in the manifestation of Al toxicity.

It is a proven fact that plant species or cultivars among the identical species considerably differ in their tolerance array to aluminium stress (Khatiwada *et al*., 1996). Hydrogen ion toxicity might have an effect on some nodulating legumes and grasses susceptible to soil acidity (Scott *et al*., 1992).

In acid soils, reduced crop productivity and low soil fertility are principal factor because of the amalgamated impact of toxicity of aluminium and manganese including deficiencies of P, Ca, Mg and K. Among these issues, aluminium toxicity has been known as a serious growth limiting factor in acidic soils which are restraining crop production on 67% area of the total acid soil area within the world (Eswaran *et al*., 1997). Aluminium is the most abundant metal and therefore the third most common element within the earth's crust (Delhaize and Ryan, 1995; Vitorello *et al*., 2005). In soils, it principally exists as a structural constituent of primary and secondary minerals, particularly of the aluminosilicates. However, despite its abundance, Al is not identified to be utilized in any living organisms (Vitorello *et al*., 2005). Though Al is a major constituent of most of the soils, it can affect plants only when it moves into the soluble or exchangeable form. Aluminium toxicity is a significant issue in low hydrogen ion concentration acidic soils (below 5.5). Exchangeable aluminium values could be high in soils with a pH below 5.5 but may occur at pH values as high as 6.0 in heavy textured soils (Matsumoto *et al*., 2001).

The critical level of soil pH, at that aluminium becomes exchangeable in cytotoxic concentration, depends on several factors, together with the predominant clay minerals, organic matter level, and concentrations of different cations, anions and total

salts as well as the species or cultivar of the plant being considered. Al is primarily in form of insoluble oxides, $Al(OH)_3$, at neutral pH as the soil gets acidic, the silicon will be leached leaving aluminium in the solid forms as aluminium oxyhydroxides, such as boehmite and gibbsite. These forms unleash the phytotoxic aluminium species, Al^{3+} conjointly called $Al(H_2O)_6^{3+}$ into the soil solution (Abebe, 2007; Miyasaka *et al*., 2007). Although, there are many types of aluminium species within the soil, Al^{3+} and monomeric Al-hydroxyl species ($Al(OH)^{2+}$ and $Al(OH)_2^{+}$) are the foremost phytotoxic ones (Miyasaka *et al*., 2007). The trivalent Al^{3+} is dominant in soil solutions once the soil pH is less than five. The problem is exacerbated by the employment of ammonium ion fertilizers and acid precipitation (Beebe *et al*., 2008). Balkrishna, (2005) reported that soil pH was the major issue that controls Al^{3+} availableness and uptake of Al from the soil into tea plants. The Al^{3+} ion are dominant once the soil pH is less than 5.0. Different types of Al occur in soil solution *viz.* $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ at pH four to five, Al^{3+} at pH 5.5 to 7.0 and $Al(OH)4^{-}$ at pH 7.0 to 8.0 (Wenzl *et al*., 2002).

The diagnosis of aluminium toxicity from visual signs in plants is unreliable (Matsumoto *et al*., 2001), and critical plant concentrations of aluminium don't seem to be well outlined. The aluminium concentration in leaves of Lucerne is of very little worth in deciding toxicity (Pineros *et al*., 2002). A value higher than 150 mg Al/kg DM in sub clover leaves could indicate toxicity (Ma, 2000). Soil exchangeable aluminium concentration is employed as a guide to the probability of aluminium toxicity. Aluminium levels higher than 15 mg kg^{-1} could also be a retardant and higher than 50 mg kg^{-1} cytotoxic, in which case the economics of liming ought to be thought of to overcome this drawback (Wenzl *et al*., 2002). Testing for Al tolerance within the field encompasses

a variety of disadvantages some of that include the presence of different cytotoxic elements and variability within the Al content throughout the field. This constraint can be alleviated by exposing seedlings to known levels of Al in the nutrient solution.

The foremost common and immediate cytotoxic impact of Al^{3+} in plants is inhibition of root growth that happens within a few minutes to many hours once exposure to micromolar concentrations of Al (Barcelo and Poschenrieder, 2002). Root inhibition could be exhibited on primary and lateral root apexes, and such roots become thick, stubby, dark coloured, brittle, poorly branched and rubberized with a reduced root length and volume resulting in poor development of the root system, susceptibility to moisture stress and nutrient deficiencies (Nguyen *et al*., 2003; Claudio *et al*., 2008; Vitorello *et al*., 2005; Wang *et al*., 2006). Supersession and abnormal root morphology directly hinder nutrient uptake and water absorption also. Consequently, plants show underdeveloped growth and become vulnerable to drought (Vitorello *et al*., 2005; Miyasaka *et al*., 2007; Wang *et al*., 2006). These symptoms become evident after a few minutes or hours of the plants being exposed to micromolar concentrations of Al in hydroponic solutions (Rengel and Zhang, 2003).

The yield loss related to Al-toxicity varies depending on soil Al saturation, the crop species and the specific variety used. For instance, Al-tolerant maize genotype gave 61% higher grain yield than the Al-sensitive genotype, and with lime treatment, there was yield increment of 20% and 82% were obtained for Al-sensitive and Al-tolerant genotypes of maize, respectively (The *et al*., 2006).

Applications of lime, manure, compost, and use of tolerant crop species or varieties are the most common methods used to reduce the effect of Al-toxicity. Lime has

been extensively used to ameliorate acid soils of temperate areas. In these areas, soil acidity develops principally as a consequence of the heavy use of chemical fertilizers and environmental pollution (Rao *et al*., 1993). Within the tropics, many experimental reports also indicate significant yield increment with the application of lime (The *et al*., 2006). However, the extremely acidic soils of this region have strong buffering capability against an amendment by lime. Such soils demand higher dose and wants deeper incorporation to ameliorate the subsurface acidity. Most of the resource-poor farmers with in the tropics, however, are helpless due to unavailability, transport and high price of this much bulky dose (Rao *et al*., 1993). In addition, since lime amalgamation to the subsoil is very tedious job, even when the surface soil is neutralized, the problem of ameliorating the subsoil hinders the root growth of plants to surface soil and make them vulnerable to drought (Little, 1989; Foy, 1992). Runoff pollution and adverse effects of lime on rotation crops also are different side effects of lime application (Wang *et al*., 2006).

Use of organic matter appears an applicable strategy to resource-poor farmers of the tropics who cannot afford the purchase of a large volume of lime and fertilizers. However, regular and high volume application of manure and compost to the extremely acidic soils is limited by competing uses of organic matter sources for fuel, animal feed and construction (IFPRI, 2010; Schlede, 1989; Buresh *et al*., 1996). On the contrary, with in the tropics, the addition of acid forming fertilizers on cultivated land and enlargement of crop production to forest areas accelerate the development of soil acidity and Al-toxicity (Giller *et al*., 1997).

The most effective approach of solving this problem is to develop aluminium

tolerant crop cultivars with increased aluminium tolerance. Three basic approaches are being used to enhance the stress tolerance in any crop, viz. (i) exploitation of natural genetic variation present in germplasm through direct selection in aluminium stress environments, (ii) mapping of quantitative trait loci (QTLs) followed by marker-assisted selection, and (iii) the development of transgenic plants to introduce novel genes or to change the tolerance levels of existing genes.

Several native crop species exhibit significant genetic-based variability in their responses to Al toxicity. This variability is useful to plant breeders for the production of Al-tolerant crops. Selection and breeding of crops for Al tolerance is a useful approach to increase production on acid soils. For a selection of genotypes tolerant to Al, a precise screening technique to evaluate the sensitivity of plants to Al is needed. This requires a rapid and reliable system to discriminate between Al-tolerant and Al-sensitive genotypes (Ma *et al*., 1997b).

2.2 Screening for aluminium tolerance

A reliable screening procedure for Al stress is one of the most important tools required to effectively develop Al-tolerant cultivars. Each screening technique has distinct advantages and disadvantages and techniques also vary widely in their ease of use for screening large numbers of entries for breeding programs. With the identification of molecular markers linked with Al tolerance genes, future screening for Al tolerance may be possible based on genotype or a combination of genotype and phenotype.

Generally, the Al-screening technique can be classified into laboratory screening and field screening. Laboratory screening methods include a screening of plants with solution-soaked paper and solution culture methods (Naserian *et al*., 2007), soil-petri dish

method (Stass *et al*., 2007), and screening in pots in a greenhouse (Tazeen *et al*., 2009).

For cowpea, screening in the field and in pots or nutrient solution is commonly used for selection of Al-tolerant genotypes. A rapid screening method is needed to select a large number of new genotypes or new inbred lines in plant breeding, such as solution-soaked paper, solution culture and soil-petri dish methods used to evaluate Al-tolerant cowpea. All of these rapid screening techniques use the response to Al of the rate of seedling germination and root development. However, the method using such growth responses would curtail the accuracy of screening (Yoshida and Yoshida, 2000). Detection systems not dependent on the rate of seedling or root development would greatly improve the success of the screening procedure (Abdel-Hady, 2006).

2.2.1 Laboratory Screening

Al tolerance screening is typically conducted by comparing the root growth of seedlings in hydroponic solutions, with and without Al. Solution culture assays with, or without staining procedures are efficient methods for identifying tolerance to Al. Nevertheless, in only a few cases has Al tolerance observed in solution cultures been correlated with Al tolerance in acidic soil (Sasaki *et al*., 2004). Chaudhary *et al*. (2011) studied the consequences of 5 aluminium concentrations (0, 10, 20, 30 and 50 ppm Al) on thirty two genotypes of pigeon pea in hydroponic and sand assays. Responses of genotypes were similar for the two screening methods which suggest that any one of the two could be used for evaluating the genotypes for Al tolerance. Root and shoot aluminium contents were significantly lower in tolerant (‘IPA 7-10' and ‘T 7’) genotype than the sensitive one (‘Bahar’ and ‘Pusa 9’). It indicates that aluminium tolerance mechanism is due to aluminium exclusion in the tolerant genotypes.

Screening by using hematoxylin staining of seedling roots (hematoxylin staining method) which requires less time and simpler pH management than the other methods, is very useful for selection or screening a relatively large population in a breeding program. Measurement of Al tolerance is based on the staining pattern of the root. The hematoxylin staining method is a very common technique for the evaluation of Al-tolerance in wheat (Kashif and Khaliq, 2004) and barley (Shahinnia *et al*., 2005). 36 genotypes of common bean evaluated under hydroponic conditions by Blair *et al*. (2009) to analyse root morphological traits that could be related with Al resistance. A total of 5 root traits (total root length, elongation rate of the primary root, average root diameter, root biomass and specific root length) were observed using a nutrient solution with or without Al (20 µM) over a 48 hours of growth period in five replications. Their study revealed under these conditions, genotypes from the Andean gene pool were more resistant to Al than Mesoamerican genotypes, depending on a smaller decrease in the total root length, elongation rate of the primary root, specific root length and a smaller increase in root diameter in the presence of Al in the nutrient solution. These root traits but not root biomass can serve as selection criteria to differentiate between Al-resistant and Al-sensitive. Rao *et al*. (2008) also compared 53 common bean genotypes with differing levels of adaptation to acid soils to identify Al-resistant genotypes using a hydroponic screening method. They found four different root traits (percent inhibition of root elongation, percent increase of average root diameter, total root length per plant and a total number of root tips per plant) as useful indicators for aluminium resistance. Application of this method to 30 accessions of scarlet runner bean (*P. coccineus* L.) identified three aluminium resistant genotypes that could be used for introgressing

aluminium resistance into cultivated germplasm. Screening of 173 landraces of bread wheat in a hydroponic method gives a result that there are significant diversities on root regenerate length (RRL) (Dai *et al*., 2009). The RRL of a large of landraces were more than 7.00 cm in pH 7 (58.38%) and pH 4.5 (66.47%), but shorter than 5.00 cm in pH 4.5 + 50 µM Al^{3+} (80.93%). This shows that low pH either promote or restrain root RRL which depend on landraces, but Al toxicity under low pH surely had restraining effects on root elongation.

Haematoxylin and root growth method was used by Hede *et al*. (2002) to evaluate 63 rye accessions which showed there are significantly higher levels of Al tolerance in rye than in the Al-tolerant bread wheat cultivar `Maringa'. Under the supposition that the roots ability to grow under Al stress is a combination of root vigour and Al tolerance, a hypothesis permitting for the differentiation of five genotype classes was suggested. This study validated that the haematoxylin method and the root growth parameter identified genotypes with long root growth under Al stress, but failed to detect Al tolerance in a genotypes with poor root vigour. These genotypes can only be identified using the root tolerance index parameter. Mugai *et al*. (2002) investigated four beans (*Phaseolus vulgaris*) varieties for their aluminium tolerance by root elongation and staining method. Using the hydroponic system, 3-day old seedlings were exposed to aluminium treatments followed by root elongation studies and staining by Eriochrome cyanine R. Root elongation resulted in superior differential rating in evaluating for aluminium toxicity in the beans. On the other hand, Eriochrome cyanine R staining lacked clear differentiation, particularly where marginal differences of Al tolerance is present. It showed that screening for aluminium tolerance in common beans can be performed by the staining

technique procedure followed by root elongation method under circumstances of ambiguity or where the difference in the tolerance are not distinguishable through the previous one. In another study, five common beans (*Phaseolus vulgaris*) cultivars were evaluated in a nutrient solution containing aluminium. The cultivars were assessed according to a tolerance index, estimated from root and aerial part length and dry weight. These traits were measured at 10 and 20 days after the addition of aluminium to the solution. The aerial part length was the least able to discriminate tolerant and sensitive cultivars. In general, Ouro was the most sensitive to aluminium. In last, they concluded that the nutrient solution technique may not efficiently describe the reaction of genotypes to actual cerrado soil conditions (Santos *et al*., 1997).

2.2.2 Field Screening

In the field screening, the symptoms of aluminium toxicity are not simply distinctive. The foliar symptoms may be small, dark green and stunting leaves with late maturity (Fukrei *et al*., 2011), purpling of stems, leaves, and leaf veins which resemble to the symptom of phosphorous deficiency. In some cases, rolling or curling of young leaves and dying growing points or petioles are observed which confused with calcium deficiency. Excess Al induces iron deficiency symptoms in wheat, rice and sorghum. Aluminium does not affects the seed germination but it helps in the development of new root and seedling establishment. Root growth inhibition was detected 2 to 4 days after the initiation of seed germination (Sasaki *et al*., 2004).

In a sand culture, Ojo and Ayuba (2012) raised 15 genotypes of soybean at 8 levels of aluminium activity and reported that aluminium activity at 300 µM Al^{3+} level along with the control was appropriate for root dry weight, while the 450 µM Al^{3+} level

along with the control was found to be appropriate for shoot dry weight and relative root surface area. It was concluded that the relative root surface area was the most profound character in discriminating between levels of aluminium activity and it should be preferred for selection programme. Ezeh *et al*., (2007) while evaluating Al tolerance potentials of eight cowpea cultivars with 0, 20 and 50 µM $AlCl_3$ applied to 2 kg alfisol samples. Genotypic (G) and G×A1 effects were significant for the growth and yield parameters. Akinrinde and Neumann, (2006) evaluated eight cowpea genotypes for their differential tolerance to 0, 20 and 50 µM $AlCl_3$ applied prior sowing in an alfisol (Typic Paleudalf). Except at two weeks after planting, Al effect was insignificant in case of plant height, though extractable- Al differed greatly ($p<0.01$) among soil sampled after cropping, which suggested to test higher rates and/or continuous application through irrigation water. On the contrary, G and G X Al interaction significantly affected plant height, yield, soil pH, P-availability and Al tolerance potential.

Cowpea lines were evaluated to study the tolerance to aluminium (Al) application and the effect of phosphorus. Fourteen out of the fifteen tested lines showed decrease in root biomass (ranges from 19-81% reduction) with Al addition, fewer lines showed decreased shoot biomass and grain yield with Al application. Al application changes little in nodule number but, there was a significant decrease in nodule weight (ranges from 24-53% reduction) for almost all lines. Al-tolerant lines showed a higher response in the shoot and root biomass and nodulation to P fertilization than Al-sensitive lines. P fertilization increased shoot dry weight from 64 to 107% in Al-tolerant lines and from 44 to 48% in Al-sensitive lines while, increase in root dry weight was 46-86% for the Al-tolerant lines and from 7-42% for the Al-sensitive lines (Kolawole *et al*., 2000). Nine

soybean genotypes for aluminium (Al) tolerance were evaluated by growing them for 21 days in greenhouse pots of acid, Al-toxic, unlimed Tatum (Typic Hapludult) subsoil at pH 4.0 and in the limed subsoil at pH 5.1. Based on absolute dry shoot weights at pH 4.0, Giessener, St.-59 (USSR), Brunatna and Biloxi (USA) were the most tolerant; least tolerant entries included Yantarnaya and Smena (USSR) and Davis (USA). Based on relative shoot dry weights (pH 4.0/pH 5.1%), Giessener, Brunatna and St.-59 were among the most tolerant, Essex, Bossier, Biloxi and Perry were intermediate, and Salute 216 (USSR), Chief (USA) and Santa Rosa and IAC9 (Brazil) were sensitive to the acid soil. Based on absolute root dry weights, Giessener, St.-59 and Biloxi were among the most tolerant and Smena, Yantarnaya, Salute 216 and Chief were most sensitive. Based on relative root dry weights (pH 4.0/pH 5.1%), Giessener was the most tolerant and Smena and Salute 216 least tolerant (Foy *et al*., 1993).

Horst, (1985) reported growth inhibition of cowpea plants within 7 days when grown in soil containing 2.2 meq Al/100 g soils. 783 genotypes showed a wide range of Al tolerance, however, no significant correlation could be establish between reduction by Al in seedling growth and grain yield of the genotypes, the same genotypes were categorized as most tolerant and most sensitive in both cases. The results showed that the simple and quick screening method using Al-treated soil allows the identification of genotypes adapted to soils with high Al supply. Field screening for Al tolerance is considered much appropriate for selecting Al-tolerant genotypes. In practice, however, a reliable ranking of tolerance in the field screening is difficult because the Al concentration in soil may not be uniform and because environmental factors interact with soil Al to mask the expression of Al tolerance. Screening by using the growth response to

Al added to the soil in pots at in a greenhouse (referred to as growth-response method hereafter) may be superior in this respect (Naserian *et al*., 2007). Edward *et al*., (1981) studied the differential response of six cowpeas cultivars to liming assessed in a greenhouse trial using an Ultisol (Typic Paleudult) from southeastern Nigeria. Lime rates from 0 to 5.0 t/ha were applied to give a range of soil pH, determined in 1:1 soil/water from 4.25 to about 7.0. Without application of lime, relative dry matter yields of tops ranged between 46.6-76.8%. Significant yield responses were observed to the first lime increment (0.5 t/ha) predominantly in the less tolerant cultivars and highest yields were obtained with application of 1.6-2.5 t/ha lime. Among the six cultivars, Ife Brown and TVu 4557 were most tolerant, Vita-3 and Vita-1 were moderately tolerant, and TVu 1977-OD and TVu 4552 were least tolerant to soil acidity. The latter two cultivars were the very less nodulated when grown in the soil with no lime application. The first lime increment facilitated excellent nodulation in all cultivars. Further, they concluded that aluminium toxicity is the major growth-limiting factor for cowpeas in this soil.

It is important to compare the laboratory screening methods with field screening methods. Sorghum plants that showed a severe reduction of shoot or root weight in a greenhouse also had low grain yield in the field (Abdel-Hady, 2006). There was also a similar genotype response to Al-induced stress in nutrient solution and to acid-soil stress in the field (Shahinnia *et al*., 2005). So, from the above discussion on the application of lab and field-based screening methods, it may be concluded that various methods could be adapted for different purposes.

2.3 Ionome profiling for the screening of abiotic stresses

The ionome is a vibrant network of elements which are regulated by biochemistry

and physiology of plant, which is ultimately governed by genome, in response to the environment. Ionomics is study of chemical elements accumulation in the living systems using high-throughput technology for elemental profiling (Salt, 2008).

92 elements are known on earth and 17 of these are known essential to plants. Essential elements mandatory in relatively high amount (>0.1% of dry weight) are called macronutrients (C, H, O, N, S, P, Ca, K and Mg). Elements which are required in smaller amounts (<0.01% of dry weight) referred as micronutrients or trace elements (Ni, Mo, Cu, Zn, Mn, B, Fe and Cl). Plant growth and development is a dependent factor of balanced supply of all the essential elements and therefore plant has a variety of homeostatic mechanisms functioning to make sure that balanced supply of all the essential nutrients are maintained. Beneficial elements are the elements which promote growth and are essential to some taxon, includes Na, Co, Al, Se and Si. Elements like heavy metal Cd and metalloid As don't have any demonstrated biological utility in plants, however though taken up and causes severe toxicity almost in all plant species. To fulfill the requirements for all metabolic processes and for minimizing deleterious effects of excess and deficit of nutrients in environment, plants developed flexible and adaptive approaches *viz.* a) achieve adequate quantities of all essential elements, b) elude excessive accumulation which may be toxic, and c) cope with toxic effects of nonessential elements.

A landmark study in development of ionomics in plants was executed in the genetic model plant *i.e. Arabidopsis thaliana* (Lahner *et al*., 2003; Hirschi, 2003; Rea, 2003). This study utilizes the inductively coupled plasma mass spectrometry (ICP-MS) technology to analyse the ionome profile of leaves of thousands of plants which paves the

way to identification of several ionomic mutants. But remarkably only 11% of identified mutants showed single element alterations which support concept that ionomic linkages in plants are controlled coordinately and essentially should be viewed as whole. This concept was further strengthened by the finding of ionomic regulatory networks involved in both iron and phosphorus homeostasis (Baxter *et al*., 2008). This concept was recently reviewed by Baxter, (2015). In case of salt, tolerance ionomics has been applied in understanding the mechanisms. Currently, it is improving in combination with the other platforms like transcriptomics, proteomics and metabolomics which are applied frequently in understanding numerous physiological processes in the plants (Salt *et al*., 2008). A better strategy to overcome Al toxicity is to develop crop varieties that are resistant to Al stress. Fortunately, plants have developed resistant mechanisms which also enabled them to resist the toxic levels of aluminium, such that several species or genotypes shows extensive variations in their capability to manage the Al toxicity.

There is strong interest in utilizing such molecular signatures as a tool of data reduction for screening of large multivariable datasets to identify molecular networks that connect particular physiology to genes that control it. To be practical, these molecular signatures should closely linked to particular physiology of the interest, and there is growing consensus that these signatures composed of the multiple components are possible to be utmost useful (Rifai *et al*., 2006). In recent years there was explosion in high-throughput profiling experiments, impartial discovery and authentication of molecular signatures are frequently hampered by limited availability of the biological samples, difficulties for handling large datasets, heterogeneous sources of variation. The shoot ionome of plant signifies its mineral nutrient and also trace element content (Salt *et*

al., 2008), that is controlled by numerous physiological processes which starts in rhizosphere, and ends with the evapotranspiration and phloem recycling. Alterations of any processes that transport inorganic ions from soil solution to aboveground part could potentially affect shoot ionome. Because of this, shoot ionome is very sensitive to physiological state of plant, with different ionome signatures being reflective for different physiological states. Since the shoot is much more available tissue than roots for profiling, such shoot ionome signatures could be useful like markers for particular physiological state with which that was associated. Using high-throughput technology of elemental profiling and data management pipeline to rapidly investigate shoot elemental composition for thousands of *Arabidopsis* plants, Salt *et al*., (2008) identified and used a multivariable ionomic signatures that are diagnostic to plants response for reduced Fe or P nutrition.

Upon exposure to excessive concentrations of soluble aluminium (Al) in acidic soil (pH < 5.0), plants often develop a stunted and stubby root system due to toxic effects of Al^{3+} ions on root elongation and lateral root development (Ciamporova, 2002 and Ryan *et al*., 1992). These mal-developed root systems are not as efficient in the selective uptake and translocation of essential mineral nutrients and water, thus making the plants highly susceptible to additional suboptimal soil conditions, such as toxic metals, drought, and salinity (Yang *et al*., 2013). As a consequence, crops are grown in this type of soil usually produce very low yields in total biomass and grain production (Kochian, 1995; Samac and Tsefsye, 2003). Previous studies have shown that some plants can grow in presence of high Al^{3+} ion concentrations by activating molecular and cellular activities that would help reduce or prevent absorption of Al^{3+} into root cells (the Al^{3+} exclusion

mechanism) and those that facilitate plants to tolerate Al^{3+} ions once they have entered into roots and shoots symplast (the Al^{3+} tolerance mechanisms) (Brunner and Sperisen, 2013). In the former mechanism, the exudation of root malate and other organic acids into the surrounding rhizospheric area functions to bind Al^{3+} in the soil, thus reducing the number of ions entering the root system (Kochian, 1995 and Liang *et al*., 2013). The second mechanism involves Al tolerance, which is achieved by the removal of the toxic ions from cell walls followed by sequestration of the internalized ions into vacuoles and detoxification in the forms of Al-oxalate or Al-citrate complexes (Ma, *et al*., 1997a &1997b).

Shibuya *et al*., (2015) performed ionomic analysis in three diverse fruit in order to illustrate element concentration in edible parts and for comparison of elemental concentrations between edible part and vegetative organ and between species. 19 elements, including elements that are essential for human's health, toxic elements and element with radioisotope from nuclear reactor were studied in apple, Japanese pear, eggplant and soyabean. The concentration of elements were presented as basic information needs for bio fortification and a high mobility depending on species were found in some elements whose mobilities are low. The classification of element profiles into leaf and other organs comprising edible parts, except calyx, by principal component analysis discovered similarity in element concentrations between species. Distribution of plant macronutrient in podzolized sands of Amazon caatinga has studied in several studies, however, distribution of micronutrients was not assessed (Sobrado, 2013). Availability of soil micronutrient was hypothesized for reflecting contrast habitat features and fundamental dissimilarities in substrate also, and micronutrient composition of leaf

may reflect macronutrient content required to maintain equilibrium for functions of leaf cell. Sobrado, (2013) analysed soil micronutrient and macronutrients and total leaf content by ICP-atomic emission spectrometer and MS obtained in a topographical sequence (valley, slope and mound). Available soil B, Zn and Cu levels were extremely low. Soil Mn was lower in valleys and slopes, but higher in mound. Soil Fe was adequately above critical level in all the habitats. Leaf micronutrients Copper, B, Zinc and Fe were below critical levels for tropical crops. Leaf Mn and Al were below accumulator's level. A sturdy relationship between leaf micronutrient and macronutrients suggested that maintenance of homeostatic composition of elements, which favours photosynthetic function. Therefore, species local distribution might be shaped by their abilities of maintaining balance of micronutrient accumulated through roots in critically low concentration of available Zn, boron and copper while excluding potentially harmful ions of Mn, Fe and Al.

For providing new insight into response of plants towards abiotic stresses, ionomic profiling of *Nicotiana langsdorffii* specimens has been compared before and after introduction to toxic metals e.g. chromium or drought conditions. Elemental profiles were acquired by applying analytical procedures based upon inductively coupled plasma-atomic emission and mass spectrometry (ICP–AES/MS). The combined use of ICP atomic emission and mass spectrometry enabled the analysis of 29 major and trace elements (Bi, Ba, Ca, Co, Cd, Cr, Eu, Cu, Fe, Ga, Li, K, Mg, Mn, Mo, Na, Pb, P, Pt, S, Sb, Rb, Sn, Sr, W, Te, Y, V and Zn) in different parts like roots, stems and the leaves, with higher accuracy and precision. Multivariate data processing and element distribution patterns study provided new evidences about the ionome responses of target organisms to

chemical treatments or water stress. A genetic modification chiefly affected distribution of Mo, Bi, Cr, S and Na which indicates involvement of these elements in biochemical processes that were controlled by GR or rolC genes. Chemical stress strongly exaggerated accumulation of numerous elements (Ba, Ca, Ga, Fe, K, Li, Mo, Mn, Na, P, Rb, Pb, S, Sn, V, Te and Zn) in diverse ways; for Ca, K, Fe, Mn, P, Na the effect was quite analogous to that perceived in other experiments after treatment with other transition (Cu and Cd) elements. The effects of drought was less marked, mainly comprising of a decrease in Ba, Cr, Sr and Na concentration in roots (Ardini *et al*., 2013).

Nutritional imbalances under water-deficit circumstances depresses plant development by affecting mineral nutrient uptake, transportation and distribution. Sanchez-Rodriguez *et al*., (2010) analyzed the differences in foliar concentrations of macronutrient and micronutrients, and also the transport of these elements in five cultivars of cherry tomato under adequate and moderate water stressed situations with aim of establishing that whether ionome of the plants is relating to the degree of susceptibility or tolerance to this type of stress. The results showed a reduction in growth along with a lower content and uptake in both of macronutrient as well as micronutrients in all cultivars studied, except for cv. Zarina that showed better growth and also increase in concentration and uptake of nitrogen, magnesium, phosphorus, chloride and potassium with respect to the control plants. Beside the phytochemical characters, ionomic fingerprinting represents inorganic trace element concentration of cellular and organismal constituent. Akundabweni *et al*., (2010) applied high-throughput technologies for elemental analysis, such as X-Ray Fluorescence (XRF), for ionomic analysis while phytochemical analysis tend to be *in vitro*. Both could contribute for insights on ionomic

phytochem micronutrient composition, genetic diversity variant discrimination among the accessions to allow simple grouping; rationalization of core and/or reserve collections, integration of bioinformatics and genetic tools and micronutrient dense varietal improvement and/or cropping decisions. They showed that for primary data mining, XRF could be utilized as first sequence of action for large sized ionomic screening that can be rationalized into Core and Reserve collections to lead phytochemical screening for conservation and/or utilization.

Sha *et al*., (2012) conducted two field experiments to study effects of previous farming of an arbuscular mycorrhizal (AM) host plant and manure application on concentration of 19 elements in seed of soybean cv. Tsurumusume. On the basis of two experiments, manure application significantly increases available potassium and decreased available iron and cesium in the soil. Higher concentrations of cadmium, barium and low concentration of Cs in the seed were prompted by application of manure. Cd levels in seed were reduced by prior husbandry with AM host plant. They concluded that identity of the prior harvest and manure application altered the mineral profile of soybean seed and suggested a connection between the environmental factors and the food safety. Shamsi *et al*., (2007) investigated the effect of aluminium and cadmium on growth, photosynthesis and accumulation of aluminium, cadmium and plant nutrient in two soybean genotypes using hydroponic culture. Low pH (4.0) and aluminum treatments caused significant reduction in shoot height, root length, chlorophyll content (SPAD value), photosynthetic rate and dry weight. Aluminium sensitive cv. Zhechun 2 accumulated reasonably more aluminum and cadmium in plants than aluminum tolerant cv. Liao 1. In comparison with pH 6.5, pH 4.0 caused a significant upsurge in cadmium

and aluminum concentration in plants. A combined application of cadmium and aluminium enhanced their concentration in roots but reduced concentration in shoots. The concentration of all the 10 nutrients (K, P, Ca, Mg, Mn, Fe, Cu, B and Zn), except Mo, were increased in plants exposed to pH lower than 6.5. Addition of aluminium caused reduction in accumulation of most of the nutrients in the plant roots and shoots, but concentration of K, Mn and Zn were increased in roots. Treatments of cadmium alone or in combination with aluminium reduced concentrations of all nutrients in plants. Al-sensitive genotype Zhechun 2 had lower mineral concentration than aluminium tolerant genotype Liao 1. Their work showed that aluminum and cadmium effects were synergistic on the plant growth, nutrient uptake and physiological traits.

Toxicity of aluminium and drought stress both are major constraints in crop production particularly in tropics. The variation of rainfall distribution and lengthier dry spells in all the tropics during main growing stage of crops are fetching increasingly important yield restraining factors with global climate change. So crop genotypes that are tolerant towards both drought and aluminium toxicity needs to be developed (Yang *et al*., 2013). A significant progress has been made in understanding physiology and molecular biology of interaction between aluminum toxicity and drought stress in common bean (*Phaseolus vulgaris* L.) in hydroponics and in an aluminium toxic soil. Crops grown in acid soils gives yield lesser than potential yield because they have poorly developed root structure which limits water and mineral uptake. The breeding for resistance towards drought condition must be combined with aluminium resistance, to ensure that drought resistance is expressed adequately in crops grown on soils with acid aluminium toxic subsoils. Bityutskii *et al*., (2017) investigated the significance of iron and silicon nutrition

under aluminium stress conditions and to determine the effects induced by iron and silicon for limitation of aluminium moving via xylem in cucumber (*Cucumis sativus* L.). Cucumber cv. Solovei and Phoenix plants were grown in hydroponics in a complete nutrient solution at pH 4.0, either with Fe-free (-Fe) or in +Fe nutrient solution), with (+Si) or without (-Si) supply of Si, with (+Al) or without (-Al) exposure of Al and in soil. Concentrations of Al, iron and silicon in xylem sap were measured. To characterize pattern of transport of aluminium and iron in xylem sap, root tissues metabolomic changes were investigated. The growth of plants was not significantly affected by Al^{3+} (Al-tolerant), Al exposure decreased xylem sap Fe (+Fe plants) and increased ferric chelate reductase (FC-R) activity of roots (-Fe plants). On the other hand, Fe supply greatly mitigated the Al-induced increase in xylem sap Al. The ameliorative effect of iron was dependent on plant genotypes and more pronounced in more Fe-efficient cultivar Phoenix, which presented highest concentration of xylem sap iron. Xylem sap iron was correlated positively with root serine, fumaric and succinic acids, suggesting that the probable causal mechanism of aluminium tolerance might involve chelation of iron by biosynthesis of chelating compounds. The Si-modulated root succinate increase appears to be of great importance for facilitating long-distance transport of Fe, thereby hindering aluminium transport from roots to shoots. The results highlight the importance of both iron and silicon supply in the plant exclusion of aluminium under acidic conditions.

2.4 Proteomics profiling for aluminium tolerance

Inhibition of plant development and considerable reduction in yield of aluminium sensitive crops caused by lethal levels of aluminium present in soil. The first line of the protection against the aluminium toxicity lies in the root structure of the plant because it

controls the absorption and transportation of all the toxic and non-toxic elements to the above-ground tissues (Zhou *et al*., 2009). All physiological, biochemical and cellular disorders prompted by Al stress hinder root root growth and development (Kochian, 1995; Yamamoto *et al*., 2002). These changes are regulated by alterations in gene expression at transcriptional, post-transcriptional, translational, and post-translational levels. Comparative proteomics analysis alongwith with bioinformatics techniques allows for the identification of expressed proteins under specific stress conditions (Pandey and Mann, 2000; Qureshi *et al*., 2007). Studies with an aluminium resistant soybean cultivar revealed that Al-induced the generation of chalcone-related synthetase, ATP binding protein, GTP-binding protein, glutathione S-transferase, ABC transporter, heat shock proteins (Zhen *et al*., 2007), S-adenosyl methionine synthetase, copper/zinc superoxide dismutase, 1-aminocyclopropane-1-carboxylate oxidase, cysteine synthase and other abiotic and biotic stress-induced proteins (Yang *et al*., 2007). The impact of aluminium stress on the development of roots can be divided into two stages, the instant inhibition of elongation of root cell that happens within 30–60 minute after exposure of aluminium (Horst, 1995), and decrease in the root tip cell proliferation that happens in hours to days after exposure of aluminium (Doncheva *et al*., 2005). Analysis of proteome configuration of the embryogenic cell suspensions leads to the resolution of 550 proteins, among that 128 were isolated by trypsin digestion. Sixty-seven different proteins involved in many biological processes like metabolism, hormone response, cell growth-division, transport, cytoskeleton composition, protein synthesis and processing, regulation and signal transduction, disease, defense and stress response were identified. Most abundant proteins among these are ribonuclease and chitinase belonging to family of PR-10 and

PR- 4 proteins, respectively (Nogueira *et al*., 2007). Also, proteins of wider range was synthesized during manganese toxicity like acidic apoplastic peroxidases and proteins related to pathogenesis such as glucanase, chitinase and thaumatin-like proteins (Fecht-Christoffers *et al*., 2003b).

Yang *et al*., (2007) identified responsive proteins for aluminium stress in rice, on the basis of indication that aluminium resistance is inducible process. A total of seventeen aluminium responsive proteins were identified, with twelve of those were upregulated and 5 downregulated. Among the upregulated proteins copper/ zinc superoxide dismutase (Cu-Zn SOD), S-adenosylmethionine synthetase 2 and GST, were found, which are the steadily known aluminium induced enzymes earlier detected at transcriptional level in other plants. More importantly, various other identified proteins which includes G protein b subunit-like protein, cysteine synthase, abscisic acid and stress-induced protein,1-aminocyclopropane-1-carboxylate oxidase, 33 kDa secretory protein and putative Avr9/Cf-9 rapidly elicited protein 141 was novel aluminium induced proteins. Most of these proteins were functionally related with signaling transduction, detoxification and antioxidation. CS, as steadily detected in both aluminium stress systems, was again validated by CS activity assays and western blot. Moreover, CS catalysis's metabolic products, the reduced glutathione and total glutathione pool, were also significantly amplified in response to aluminium stress. In a broad way, results suggested that detoxification and antioxidation ultimately related to sulphur metabolism, chiefly to CS might play a useful role for aluminium adaptation in rice.

Aluminium toxicity induced inhibition of photosynthesis, reduction of total soluble protein occurred only in *C. grandis* leaves, proves that *Citrus sinensis* had higher

aluminium tolerance than *Citrus grandis* (Li *et al*., 2016). Using isobaric tags for relative and absolute quantification (iTRAQ), found more aluminium toxicity responsive proteins from *Citrus sinensis* than from *Citrus grandis* leaves, which may be accountable for the higher aluminium tolerance of *Citrus sinensis*. The features that might contribute to Al tolerance of *Citrus sinensis* are better preservation of energy balance and photosynthesis by increasing energy and photosynthesis-related proteins, less increased necessity for detoxification of ROS and various other toxic compounds, great reclamation of total capability of detoxification and upregulation of low-phosphorus-responsive proteins. Al toxicity responding proteins related to protein metabolism, RNA regulation, cellular transport, signal transduction may also impart for higher aluminum tolerance of *Citrus sinensis*. Duressa *et al*., (2011) conducted a proteomic analysis in roots of Al-tolerant and Al-sensitive soybean genotypes under aluminium stress using tandem combination of 2-dimensional DIGE followed by mass spectrometry and bioinformatics tools at 6, 51 and 72 hour of aluminium treatment. Comparison of changes in protein profile revealed that aluminium induced aluminium tolerance related protein and enzymes in aluminium tolerant genotype but aroused proteins associated to common stress response in aluminium sensitive genotype. Specifically, aluminium upregulated: malate oxidoreductase, enolase, pyruvate dehydrogenase, and malate dehydrogenase, in Al tolerant but not in Al sensitive. These enzymes gives increased production of citrate which is key organic acid engaged in aluminium detoxification. They assumed that concurrent transgenic overexpression of many of the enzymes would be a vigorous genetic engineering approach for developing aluminium tolerant crops.

Proteomic analysis of the primary root tissues which were grown in aluminium

amended and without aluminium liquid cultures was executed by Zhou *et al*., (2009). DIGE-SDS-MALDI-TOF-TOF analysis of these tissues has given rise to identification of 49 proteins that were differentially expressed. Catalase enzymes, Dehydroascorbate reductase and glutathione reductase linked with antioxidant activities were prompted in Al-treated roots. Induced enzyme proteins related to detoxification were, catechol oxidase, mitochondrial aldehyde dehydrogenase, lactoylglutathione lyase and quinone reductase. The germin-like proteins, wali7, malate dehydrogenase and heavy-metal related domain containing proteins were downregulated. VHA-ATP that translates for catalytic subunit A of vacuolar ATP synthase was induced and two ATPase subunit 1 isoform were downregulated. Numerous proteins in active methyl cycle, comprising quercetin 3-O-methyltransferase, SAMS and AdoHcyase, were upregulated by Al stress. Other induced proteins were GDSL motif lipase hydrolase family protein and isovaleryl-CoA dehydrogenase. b-hydroxy acyl-ACP dehydratase and NADPH-dependent flavin reductase were suppressed.

To identify the aluminium-induced proteomes in *Solanum lycopersicum* variety "Micro-Tom" after long term exposure to stress factor Zhou *et al*., (2016) identified proteins using an iTRAQ labelling strategy followed by a 2-D (high and low pH) chromatographic separation and tandem mass spectrometry (MS/MS) spectra on an LTQ-Orbitrap Elite mass spectrometer. The PCA revealed that Al-treatment had prompted systemic changes in proteomes from leaves and roots but not from seed tissues. The significantly altered root proteins were having putative functions in aluminium ion uptake, transport, root development and gathering of additional cellular processes. Variations in the leaf proteome directed that the light reaction centers of photosynthetic

machinery were the chief targets of Al-induced stress. Tissues of embryo and seed-coat derivative from aluminium treated plants were augmented with stress proteins. The biological processes concerning these aluminium induced proteins coincide with the morphological changes and physiological, like the disruption of mineral homeostasis (increased concentration of aluminum, iron and phosphorus and reduced concentration of sulfur, manganese and zinc in aluminium treated compared to non-treated plant) in root and reduced sizes of roots and thereof the whole plants. More significantly, the recognized significant proteins may characterize molecular mechanism for plants to improve toward establishing the aluminium tolerance and adaptation mechanism over prolonged period of stress treatment.

CHAPTER- 3

MATERIALS AND METHODS

Chapter-3

MATERIALS AND METHODS

The present investigation entitled "Ionome and proteome assisted characterization of aluminium tolerance in Cowpea [*Vigna unguiculata* (L.) Walp]" was carried out during kharif season of 2015-16 at Green house of Department of Horticulture, Sikkim University, Gangtok, Sikkim. The details of materials used and methods employed during the present investigation are described below:

3.1 EXPERIMENTAL LAYOUT AND DESIGN

The experiment was laid out in Complete Randomized Design (CRD) in factorial concept. The details of experimental plan are given below:

Replication	: 03
No. of genotypes (Factor 1)	: 15
Treatments of Aluminium (Factor 2)	: 4 levels (0, 25, 50, 100 µM)

3.2 EXPERIMENTAL MATERIALS

The experimental material for the present study was comprised of 15 genotypes of Cowpea [*Vigna unguiculata* (L.) Walp]" the list of genotypes along with their sources is given in Table 3.1. First four genotypes were released varieties from Indian Institute of Vegetable Research (IIVR), Varanasi which are cultivated over the large geographical area with high yield and resistant to the Golden mosaic virus. Other genotypes were the indigenous and exogenous collection of germplasm having various desirable traits and are maintained at IIVR, Varanasi. So, we were trying to

find out the possible genetic resource of cowpea which will be having tolerance towards aluminium and acidic soil.

Table 3.1: List of Cowpea genotypes with source

Sl. No	Genotype	Variety (Source)
1	G-1	Kashi Unnati (IIVR, Varanasi)
2	G-2	Kashi Shyamal (IIVR, Varanasi)
3	G-3	Kashi Gauri (IIVR, Varanasi)
4	G-4	Kashi Kanchan (IIVR, Varanasi)
5	G-5	EC-9738 (IIVR, Varanasi)
6	G-6	EC-9736 (IIVR, Varanasi)
7	G-7	IC-202786 (IIVR, Varanasi)
8	G-8	IC-249588 (IIVR, Varanasi)
9	G-9	IC-201098 (IIVR, Varanasi)
10	G-10	IC-33922 (IIVR, Varanasi)
11	G-11	EC-19736 (IIVR, Varanasi)
12	G-12	IC-201081 (IIVR, Varanasi)
13	G-13	IC-559386 (IIVR, Varanasi)
14	G-14	IC-559397 (IIVR, Varanasi)
15	G-15	IC-259063 (IIVR, Varanasi)

There were four levels of aluminium *i.e.* A0 (0 µM), A25 (25 µM), A50 (50 µM) and A100 (100 µM). Each genotype was treated with all four levels of aluminium and replicated thrice.

3.3 EXPERIMENTAL METHOD

Seeds of cowpea genotypes were disinfected with 1% sodium hypochlorite and then germinated in germination paper for seven days for reaching length of 4-6 cm. After that seedlings were transferred to dilute nutrient solution *i.e.* Hoagland solution (Simon *et al.*, 1994) having 0 µM (control), 25 µM, 50 µM and 100 µM

aluminium solution. Macronutrients were added as PO_4^{2-} (KH_2PO_4), 3.38 $cmol_c$ kg^{-1}; K^+ (KNO_3), 9.00 $cmol_c$ kg^{-1}; Ca^{2+} [$Ca(NO_3)_2.H_2O$], 10.13 $cmol_c$ kg^{-1}; Mg^{2+} ($MgSO_4.7H_2O$), 3.38 $cmol_c$ kg^{-1} while, the micronutrients were added as Fe^{2+} ($FeCl_3.6H_2O$), 1.6 mg L^{-1}; Na^+ ($Na_2EDTA.H_2O$), 1.43 mg L^{-1}; Mn^{3+} ($MnSO_4.4H_2O$), 0.25 mg L^{-1}; MoO_4 [$(NH_4)_6$ $Mo_7O_{24}.4H_2O$], 0.006 mg L^{-1}; B (H_3BO_3), 0.37 mg L^{-1}; Zn^{2+} ($ZnSO_4.7H_2O$), 0.12 mg L^{-1} and Cu^{2+} ($CuSO_4.5H_2O$), 0.03 mgL^{-1} (Ogbonnaya *et al.*, 2003). The aluminium treatment were supplied as Aluminium sulphate ($Al_2(SO_4)_3.16H_2O$). The pH of the nutrient solution was maintained at 4.5 for all the treatments using 1M HCl and examined regularly at 5 days interval. The solution was regularly aerated by using aquarium pump and replaced after every 4 days in order to maintain the proper nutrient and aluminium concentration. Ten plants of each genotype per replication were grown in each treatment. After 8 weeks of growth, the root and shoots were harvested and rinsed with distilled water for 20 seconds in order to remove surface contamination followed by blotting paper to remove moisture. They were dried at 70^0C for 72 hr to determine dry weight of roots and shoot.

3.4 OBSERVATION RECORDED

3.4.1 Plant height at weekly interval up to 5 weeks

Plant height of all the plants of each replication were taken at weekly interval for 1^{st} -5^{th} week of growth and their average was worked out and expressed in cm.

3.4.2 Biomass

All the plants from each replication were harvested, their fresh weight was recorded and the average was calculated. The biomass expressed in g $plant^{-1}$.

3.4.3 Root length

Root length of each harvested plant was measured, their average was calculated and expressed in cm. Root length was measured from the base of the cotyledon to the tip of the roots for each plant in each treatment.

3.4.4 Root dry matter

Roots of all the harvested plants were washed and kept in hot air oven for 24 hours, thereafter dry matter weight per plant was recorded and expressed in g plant^{-1}.

3.4.5 Shoot dry matter

Shoots of all the harvested plants were washed and kept in hot air oven for 24 hours, thereafter dry matter of per plant was recorded and expressed in g plant^{-1}.

3.5 Ionomics profiling of cowpea genotypes

Multi-elemental analysis was carried out by employing Inductively Coupled Plasma mass Spectroscopy (ICPMS make: Perkin Elmer Nex ION 300X, USA).

3.5.1 Preparation of plant samples

Fully matured leaves were collected after 25 days from the top of the plant and washed with running water for 20 minutes and then with distilled water. Further washed with 0.1 N HCl then 20% teepol and finally thrice with distilled water. The leaves were spread for 30 minutes on the blotting paper to remove the extra moisture. Then leaves were put into the paper bag with proper labelling and dried in the hot air oven at 60^{0}C for 48 hrs. After drying, leaves were powdered using Willey's mill (SNS- WM-1, India). The fine powder was transferred into the air tight plant sample container. The same process was followed in case of roots.

3.5.2 Preparation of acid digested plant samples

The di-acid digestion method has been followed for the analysis of inorganic constituents to achieve a clear and colorless solution. Oven dried powdered plant sample was transferred to 150 ml clean borosil conical flask and to that 15 ml of di-acid mixture (Concentrated Nitric acid and Perchloric acid) in the ratio of 9 ml: 5ml was added. It was covered with watch glass and kept for an hour till the primary reactions subsided. Then, it was then heated strongly on the hot plate until the solution became colourless and reduced to about 2-3 ml. While heating, the solution was not allowed to dry. After cooling, the extracts were filtered through Whatman No. 44 (Ashless) filter paper and then it was transferred to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and stored properly in the narrow mouth bottle for subsequent use.

3.5.3 Analysis by ICP-MS

Analysis of the sample was carried out by ICPMS with cross nebulizer. The instrument was calibrated using standard reference material (Peach leaves- NIST, 1547, USA). The instrument was standardised using the Multi-elemental standard solution no.1, 2 and 5 supplied by Perkin-Elmer containing elements Ag, Al, B, Ba, Be, Ca, Co, Cu, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Rb and Zn. Then the samples were analyzed for the multi elemental profiling.

3.6 Proteomics profiling of Susceptible and tolerant genotypes

- To find out responsible proteins for aluminium tolerance label free protein quantification was done.

- For label free protein quantification root samples of aluminium tolerant and susceptible cultivars were collected from three biological replications and they were pooled for further analysis.

- Equal amounts from each protein sample were subjected to in-solution digestion with trypsin to make peptides.

- The extracted peptides were analysed by liquid chromatography tandem mass spectrometry (LC/MS/MS) using Synapt G2 HDMS (Waters) connected online through nano ACQUITY UPLC (Waters).

- Protein identification and expression analysis of the post-MS data were performed by Progenesis using UniProt database.

  (The entire protein analysis was carried out at Rajiv Gandhi Centre for Biotechnology, Tiruvananthapuram, Kerala.)

3.7 STATISTICAL ANALYSIS

Statistical analysis of the data was carried out by using Statistical package for Agricultural Research (SPAR 2.0) and Indostat.

The data obtained from different observations during field experimentation and laboratory analysis were subjected to the analysis of variance by Complete Randomized Design with factorial concept. Significance and non-significance of the variance due to the different treatments were determined by calculating the respective 'F' values (Panse and Sukhatme, 1985).

3.7.1 Analysis of variance

Analysis of variance (ANOVA) and components of variance for individual character was carried out as per procedure described here.

Table 3.2 Structure of ANOVA table

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**Expected value of MS**	**Calculated F**
Genotypes (g)	(g-1)	SSg	$MSg = \frac{GSS}{g-1}$	$\sigma_e^2 + r\,\sigma^2 g$	MSg/MSe
Aluminium(al)	(al-1)	SSal	$MSal = \frac{alSS}{al-1}$	$\sigma_e^2 + r\,\sigma^2 al$	MSal/MSe
Genotypes x aluminium (gxal)	(g-1)(al-1)	SS(gxal)	$MSgxal = \frac{(gxal)SS}{(g-1)(al-1)}$	$\sigma_e^2 + r\,\sigma^2(gxal)$	$\frac{MS(gxal)}{MSe}$
Errors (e)	gal(r-1)	SSe	$MSe = \frac{ESS}{gal(r-1)}$	σ_e^2	
Total	(galr – 1)				

Where,

r = Number of replications

g = Number of genotype

al = number of aluminium treatment

SSg = Sum of squares due to genotypes

SSal = Sum of squares due to aluminium treatments

SS (gxal) = Sum of squares due to interaction of genotypes and aluminium treatment

SS_e = Sum of squares due to errors

MSg = Mean sum of squares due to genotypes

MS al = Mean sum of squares due to aluminium treatment

MS (gxal) = Mean sum of squares due to genotype and aluminium interaction effect

MSe = Mean sum of squares due to errors

σ_g^2 = Genotypic variance

σ_{al}^2 = Aluminium treatment variance

$\sigma_{(gxal)}^2$ = Genotypic and aluminium interaction variance

σ_e^2 = Error variance

The standard error of mean ($SE_m\pm$), Standard error ($SE_d\pm$) for genotypes and critical difference (CD) for comparing the means of genotypes were computed as follows:

$SE_m \pm = \sqrt{MSe/r}$

$SE_d \pm = \sqrt{2MSe/r}$

CD at 5% = $SE_{(d)}$ x t value at error degree of freedom at 5% level of significance.

The calculated F value were compared with the tabulated F value at P=0.05 and P=0.01, If the calculated F value was found higher than the tabulated, it was considered to be significant.

3.7.2 Principal Component Analysis (PCA) and two way cluster analysis

PCA and two way cluster analysis were done for physiological parameter and ionomics profile of the cowpea genotypes with the help of JMP 11.

CHAPTER- 4

EXPERIMENTAL RESULTS

Chapter- 4

Experimental Results

The present investigation entitled "Ionome and Proteome Assisted Characterisation of Aluminium Tolerance in Cowpea [*Vigna unguiculata* (L.) Walp.]" was carried out at Department of Horticulture, Sikkim University, Gangtok, Sikkim to screen the fifteen Cowpea genotypes for their aluminium tolerance and to find out the ions and proteins responsible for the aluminium tolerance in Cowpea. The experimental results are presented in this chapter after subjecting the data to statistical analysis for precise interpretation with respective tables and figures under the following sub heads:

i) Screening of cowpea genotypes for aluminium tolerance

ii) Ionome profiling of the cowpea genotypes for aluminium tolerance

iii) Proteome profiling of the cowpea genotypes for aluminium tolerance

4.1 Screening of cowpea genotypes for Aluminium tolerance

Fifteen genotypes of Cowpea were screened for aluminium tolerance in hydroponics system and data were recorded for plant height at weekly interval up to 5 weeks, root length, total biomass, root dry matter and shoot dry matter.

4.1.1 Plant height at 1st week after planting (cm)

The plant height was found to be significantly influenced by different aluminium concentration and genotypes (Table 4.1). The mean plant height of all treatments combined was found to be highest in G2 (19.82 cm) which was significantly at par with genotypes G13 (19.48 cm), G3 (18.98 cm), G4 (18.39 cm), G15 (17.70 cm)

and G5 (17.62 cm). The lowest plant height was recorded in G6 (13.51 cm) which was significantly at par with G8 (15.20 cm) and G14 (15.25 cm).

Out of four levels of aluminium, the minimum height was recorded at the moderate concentration of aluminium @ 50 µM (15.29 cm). The maximum height (18.38 cm) was recorded under aluminium concentration of 100 µM. As the concentration of aluminium had increased from 25 to 50 µM there was gradual decrease in the plant height of cowpea. In comparison to control, there was increase in plant height at 25 µM (2.33 cm) and 100 µM (0.81 cm) and decreased at 50 µM (-0.76 cm) (Table 4.10). Interaction effect between genotypes and aluminium concentration were also found to be significant. Maximum height was found in G3 (28.13 cm) at 25 µM aluminium concentration which was at par with G2 (23.53 cm) at 25 µM. Whereas, the lowest plant height at first week was found in G1 (11.00 cm) at control. In most of the genotypes, there was a decrease in the plant height as the aluminium concentration increased and maximum height was found at lower concentration of aluminium.

4.1.2 Plant height at 2nd week after planting (cm)

The data regarding plant height at 2nd week after planting as influenced by different genotypes and aluminium concentration were found significant (Table 4.2). The maximum plant height was recorded in genotype G2 (28.45 cm) which was at par with G5 (27.88 cm), G3 (27.39 cm), G13 (26.94 cm), G15 (26.45 cm) and G10 (25.29 cm). The minimum plant height was observed in G6 (19.68 cm) which was statistically at par with G11 (20.20 cm), G1 (20.63 cm), G7 (21.38 cm), G14 (21.57 cm), G9 (22.17 cm) and G8 (22.89 cm).

There was gradual increase in plant height in all aluminium concentrations compared to control but increase in plant height at 50 µM aluminium concentration was

Table 4.1 Effect of aluminium concentration on plant height (cm) of cowpea genotypes after one week of planting.

Genotype	A0	A25	A50	A100	Mean G
G1	11.00	22.52	14.76	16.47	**16.19**
G2	17.72	23.53	17.95	20.09	**19.82**
G3	14.25	28.13	17.18	16.35	**18.98**
G4	16.62	21.47	16.82	18.66	**18.39**
G5	18.54	19.90	14.53	17.50	**17.62**
G6	13.53	13.39	13.22	13.93	**13.51**
G7	16.86	12.79	17.87	16.71	**16.06**
G8	12.48	18.24	15.94	14.15	**15.20**
G9	16.18	15.40	13.58	19.11	**16.07**
G10	15.86	15.93	15.55	17.26	**16.15**
G11	12.14	13.44	13.08	14.98	**13.41**
G12	18.01	13.17	14.58	17.93	**15.92**
G13	21.50	20.53	16.24	19.66	**19.48**
G14	16.40	16.89	15.11	12.59	**15.25**
G15	19.71	20.47	13.03	17.59	**17.70**
Mean A	**16.05**	**18.38**	**15.29**	**16.86**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	2.38	1.19	0.84		
Factor (A)	1.23	0.61	0.43		
Factor (GxA)	4.76	2.38	1.68		

Table 4.2 Effect of aluminium concentration on plant height (cm) of cowpea genotypes after two weeks of planting.

Genotype	A0	A25	A50	A100	Mean G
G1	17.04	25.67	18.52	21.31	**20.63**
G2	24.52	29.71	27.76	31.80	**28.45**
G3	21.50	32.41	28.72	26.95	**27.39**
G4	22.09	27.43	26.63	23.93	**25.02**
G5	27.33	27.62	26.72	29.86	**27.88**
G6	24.45	19.39	16.22	18.67	**19.68**
G7	20.55	15.23	27.82	21.92	**21.38**
G8	19.90	21.17	26.26	24.24	**22.89**
G9	23.41	22.48	20.27	22.53	**22.17**
G10	20.68	28.18	24.78	27.52	**25.29**
G11	21.83	19.81	17.35	21.81	**20.20**
G12	22.05	27.35	18.61	30.34	**24.58**
G13	28.83	26.73	23.73	28.46	**26.94**
G14	22.94	25.72	21.65	16.00	**21.57**
G15	28.63	26.47	23.69	26.99	**26.45**
Mean A	**23.05**	**25.02**	**23.25**	**24.82**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	3.32	1.66	1.17		
Factor (A)	1.71	0.86	0.61		
Factor (GxA)	6.64	3.32	2.35		

very less compared to control (Table 4.10). The minimum height (23.05 cm) was recorded at control which was statistically at par with A50 (23.25 cm). Whereas, maximum plant height was measured (25.02 cm) at 25 µM which was significantly superior than the control and A50.

The interaction effect between genotypes and aluminium effect was also found to be statistically significant. The minimum plant height was measured in G7 (15.23 cm) at 25 µM aluminium level while maximum plant height at 2nd week was observed in G3 (32.41 cm) at 25 µM aluminium level.

4.1.3 Plant height at 3rd week after planting (cm)

Plant height at 3rd week after planting as influenced by genotypes was significant (Table 4.3). The maximum height was recorded in cowpea genotype G13 (36.31 cm) which was statistically at par with G15 (35.38 cm), G5 (33.97 cm), G10 (33.71 cm), G12 (32.73 cm), G3 (32.59 cm) and G2 (32.43 cm). The minimum plant height was observed in G6 (24.15 cm) which was statistically at par with G1 (25.26 cm), G7 (25.66 cm), G8 (27.46 cm), G9 (28.02 cm) and G14 (28.36 cm).

Effect of aluminium concentration and interaction effect of genotypes and aluminium concentration on plant height at 3rd week after planting was found to be non-significant.

4.1.4 Plant height at 4th week after planting (cm)

The effect of different genotypes on plant height at 4th week after planting was found significant (Table 4.4). The maximum plant height was recorded in G13 (47.13 cm) which was statistically at par with G15 (46.00 cm) whereas, the minimum plant height was found in G7 (30.11 cm).

Table 4.3 Effect of aluminium concentration on plant height (cm) of cowpea genotypes after three weeks of planting.

Genotype	A0	A25	A50	A100	Mean G
G1	22.92	28.03	22.07	28.01	**25.26**
G2	32.00	30.51	32.24	34.97	**32.43**
G3	28.79	35.46	32.36	33.75	**32.59**
G4	27.81	29.91	30.35	32.39	**30.11**
G5	31.06	33.84	34.21	36.77	**33.97**
G6	28.36	25.74	21.08	21.42	**24.15**
G7	28.81	18.86	29.93	25.07	**25.66**
G8	27.71	23.57	29.75	28.83	**27.46**
G9	29.95	26.48	27.71	27.93	**28.02**
G10	34.98	34.25	31.26	34.34	**33.71**
G11	25.23	24.86	29.36	24.63	**26.02**
G12	31.65	36.09	27.75	35.45	**32.73**
G13	37.19	36.99	31.68	39.38	**36.31**
G14	31.21	33.47	27.75	21.01	**28.36**
G15	38.22	33.62	34.36	35.31	**35.38**
Mean A	**30.39**	**30.11**	**29.46**	**30.62**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	4.06	2.03	1.43		
Factor (A)	-	-	-		
Factor (GxA)	-	-	-		

Table 4.4 Effect of aluminium concentration on plant height (cm) of cowpea genotypes after four weeks of planting.

Genotype	A0	A25	A50	A100	Mean G
G1	30.07	30.40	27.64	32.84	**30.23**
G2	36.00	33.92	36.66	38.22	**36.20**
G3	34.96	38.28	35.77	37.48	**36.62**
G4	32.55	34.15	32.92	36.72	**34.08**
G5	34.44	38.32	39.79	50.15	**40.67**
G6	34.02	31.50	30.08	28.50	**31.02**
G7	34.80	25.44	32.06	28.16	**30.11**
G8	32.50	27.57	37.03	34.63	**32.93**
G9	36.43	30.04	30.97	30.90	**32.08**
G10	51.27	36.90	40.66	38.53	**41.84**
G11	30.24	27.38	34.71	28.58	**30.23**
G12	37.35	45.56	38.12	41.08	**40.53**
G13	44.89	51.36	38.70	53.56	**47.13**
G14	36.47	37.98	32.60	29.58	**34.16**
G15	43.49	45.59	50.78	44.15	**46.00**
Mean A	**36.63**	**35.62**	**35.90**	**36.87**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	4.80	2.40	1.70		
Factor (A)	-	-	-		
Factor (GxA)	-	-	-		

While assessing the response of aluminium levels and the interaction effect of genotype and aluminium concentration, they were found non-significant.

4.1.5 Plant height at 5th week after planting (cm)

Plant height at 5th week was found to be significantly affected by genotypes of cowpea and presented (Table 4.5). The plant height was found maximum in G13 (62.69 cm) which was statistically at par with G15 (57.71 cm) and G12 (56.52 cm). Whereas, the minimum plant height was recorded in G11 (33.61 cm) which was statistically at par with G7 (34.35 cm), G1 (36.17 cm), G9 (36.47 cm), G6 (37.18 cm), G8 (37.21 cm), G4 (38.07 cm), and G2 (39.49 cm).

But the influence of aluminium concentration on plant height was found statistically non-significant. However, interaction effect of genotypes and aluminium concentration was highly significant. The maximum plant height was found in G13 (83.09 cm) at highest aluminium concentration which was statistically at par with G12 at A25 (72.44 cm), whereas genotypes G1 showed minimum plant height at control level.

4.1.6 Root length (cm)

From the analysis of variance study, it was revealed that the root length was significantly influenced by different genotypes and aluminium treatments. The highest root length was recorded in genotype G13 (23.24 cm) and G6 (21.50 cm) which was significantly superior as compared to other genotype. The lowest root length was recorded in G7 (9.97 cm) which was statistically at par with G9 (9.98 cm), G8 (11.74 cm) and G14 (11.95 cm) (Table 4.6).

With respect to the aluminium treatments, the maximum root length was recorded at 25 µM (19.57 cm) which was significantly higher than the control and other

Table 4.5 Effect of aluminium concentration on plant height (cm) of cowpea genotypes after five weeks of planting.

Genotype	A0	A25	A50	A100	Mean G
G1	37.80	36.09	33.54	37.26	**36.17**
G2	40.79	39.50	37.99	39.67	**39.49**
G3	39.88	40.80	38.54	43.00	**40.55**
G4	37.13	38.42	36.75	40.00	**38.07**
G5	42.38	42.09	42.80	62.34	**47.40**
G6	36.21	35.49	38.75	38.27	**37.18**
G7	40.25	31.43	34.75	31.00	**34.35**
G8	36.50	31.59	40.71	40.03	**37.21**
G9	38.89	33.44	37.15	36.40	**36.47**
G10	61.25	42.52	57.50	43.59	**51.21**
G11	35.12	30.17	38.37	30.79	**33.61**
G12	47.19	72.44	49.25	57.19	**56.52**
G13	53.34	67.99	46.34	83.09	**62.69**
G14	41.64	44.50	39.77	37.73	**40.91**
G15	50.17	60.19	69.00	51.50	**57.71**
Mean A	**42.57**	**43.11**	**42.75**	**44.79**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	6.79	3.40	2.40		
Factor (A)	-	-	-		
Factor (GxA)	13.58	6.79	4.80		

Table 4.6 Effect of aluminium concentration on root length (cm) of cowpea genotypes.

Genotype	A0	A25	A50	A100	Mean G
G1	14.44	18.00	11.67	11.25	**13.84**
G2	18.09	20.00	14.88	14.45	**16.85**
G3	13.92	17.42	10.92	9.33	**12.89**
G4	15.09	17.33	11.17	8.38	**12.99**
G5	18.38	21.92	17.59	11.50	**17.34**
G6	23.68	26.56	20.45	15.33	**21.50**
G7	11.09	14.29	8.37	6.13	**9.97**
G8	13.84	14.83	9.92	8.40	**11.74**
G9	10.34	11.81	9.32	8.46	**9.98**
G10	14.25	15.92	11.50	8.42	**12.52**
G11	21.89	24.67	15.92	13.49	**18.99**
G12	18.00	25.09	15.79	11.02	**17.47**
G13	23.09	27.42	22.38	20.09	**23.24**
G14	13.93	18.09	9.83	5.94	**11.95**
G15	14.67	20.17	13.89	11.92	**15.16**
Mean A	**16.31**	**19.57**	**13.57**	**10.94**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	2.29	1.14	0.81		
Factor (A)	1.18	0.59	0.42		
Factor (GxA)	**-**	**-**	**-**		

high concentrations. In comparison to control, root length was increased at 25 µM (3.26 cm) but it was decreased at 50 µM (-2.74 cm) and 100 µM (-5.37 cm) (Table 4.10). However, the interaction effect of genotype and aluminium was found non-significant.

4.1.7 Biomass (g plant^{-1})

The plant biomass was significantly influenced by the different genotype, aluminium concentration and interaction of these factors (Table 4.7). The maximum value of biomass was recorded in genotype G13 (3.81 g) while the lowest biomass was found in genotype G7 (0.93 g) and G9 (1.13 g).

The biomass of cowpea genotypes was found to be highest at aluminium concentration 25 µM (2.51 g) followed by control (2.47 g). Whereas the lowest biomass was recorded at highest concentration of aluminium (1.89 g). The biomass was increased slightly at 25 µM (0.04 cm) in comparison to control, but decreased at 50 µM (-0.39 cm) and 100 µM (-0.58 cm) (Table 4.10). Interaction effect of genotypes and aluminium concentration revealed that, the highest value of biomass was obtained under the treatment G13 at A25 (4.16 g) which was statistically at par with treatments G13 at A0 (3.87 g) and G5 at A0 (3.87 g). The lowest value of biomass was obtained under G7 at A100 (0.61 g) which was statistically at par with treatments G9 at A100 (0.65 g), G9 at A50 (0.82 g), G7 at A50 (0.85 g), G15 at A100 (0.85 g), G7 at A0 (1.03 g), G8 at A100 (1.03 g), G1 at A100 (1.09 g), G15 at A50 (1.20 g), G7 at A25 (1.22 g), G8 at A50 (1.30 g), G15 at A25 (1.33 g), G10 at A100 (1.40 g) and G9 at A0 (1.45 g). The biomass of the cowpea genotypes were in general more than the control at 25 µM aluminium concentration which decreased as the concentration increased from 25 µM to 100 µM (Fig 4.1).

Table 4.7 Effect of aluminium concentration on biomass (g plant^{-1}) of cowpea genotypes.

Genotype	A0	A25	A50	A100	Mean G
G1	1.62	1.82	1.59	1.09	**1.53**
G2	3.02	3.29	2.75	2.46	**2.88**
G3	2.57	3.01	2.24	2.10	**2.48**
G4	2.36	2.60	1.84	1.45	**2.06**
G5	3.87	3.51	3.41	3.20	**3.49**
G6	3.49	2.80	2.61	2.51	**2.85**
G7	1.03	1.22	0.85	0.61	**0.93**
G8	1.78	2.16	1.30	1.03	**1.57**
G9	1.45	1.62	0.82	0.65	**1.13**
G10	1.70	1.60	1.57	1.40	**1.57**
G11	2.39	2.81	2.22	2.24	**2.41**
G12	3.19	3.01	2.75	2.71	**2.91**
G13	3.87	4.16	3.70	3.51	**3.81**
G14	3.13	2.76	2.45	2.60	**2.73**
G15	1.62	1.33	1.20	0.85	**1.25**
Mean A	**2.47**	**2.51**	**2.08**	**1.89**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	0.21	0.11	0.08		
Factor (A)	0.11	0.05	0.04		
Factor (GxA)	0.43	0.21	0.15		

Table 4.8 Effect of aluminium concentration on shoot dry matter (g plant^{-1}) of cowpea genotypes.

Genotype	A0	A25	A50	A100	Mean G
G1	0.20	0.23	0.17	0.13	**0.18**
G2	0.31	0.32	0.23	0.20	**0.26**
G3	0.25	0.25	0.31	0.26	**0.26**
G4	0.23	0.17	0.24	0.15	**0.20**
G5	0.36	0.35	0.22	0.17	**0.27**
G6	0.22	0.23	0.16	0.10	**0.18**
G7	0.15	0.21	0.13	0.13	**0.15**
G8	0.26	0.23	0.23	0.16	**0.22**
G9	0.21	0.21	0.15	0.12	**0.17**
G10	0.19	0.24	0.19	0.20	**0.20**
G11	0.20	0.30	0.18	0.13	**0.20**
G12	0.25	0.28	0.25	0.20	**0.24**
G13	0.35	0.35	0.29	0.20	**0.29**
G14	0.31	0.25	0.21	0.18	**0.23**
G15	0.33	0.28	0.25	0.18	**0.26**
Mean A	**0.25**	**0.26**	**0.21**	**0.17**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	0.04	0.02	0.01		
Factor (A)	0.02	0.01	0.01		
Factor (GxA)	0.08	0.04	0.03		

Fig 4.1 Reduction in biomass of cowpea genotypes in comparison to control at various aluminium concentration.

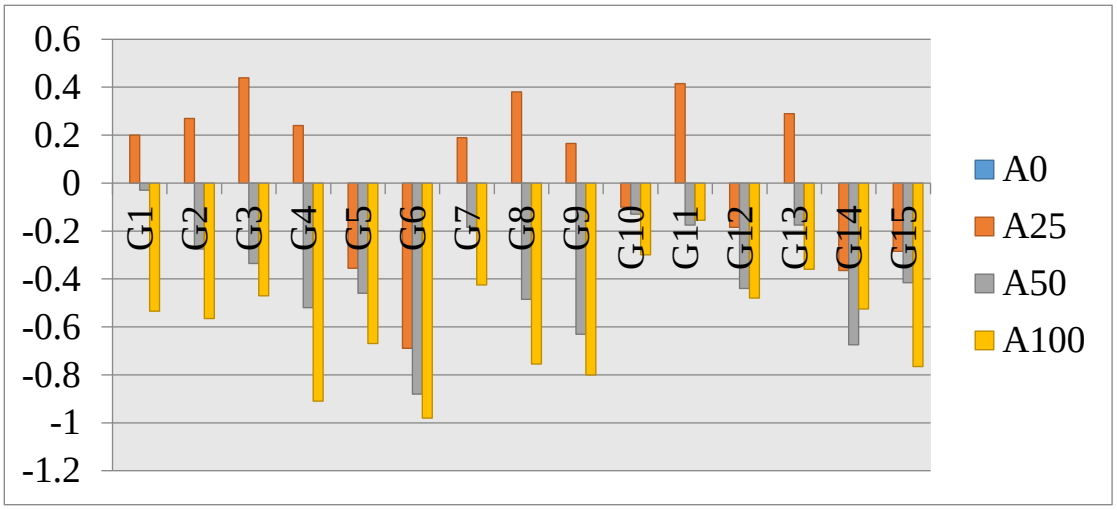


Fig 4.2 Reduction in Shoot dry matter in comparison to control at various aluminium concentration.

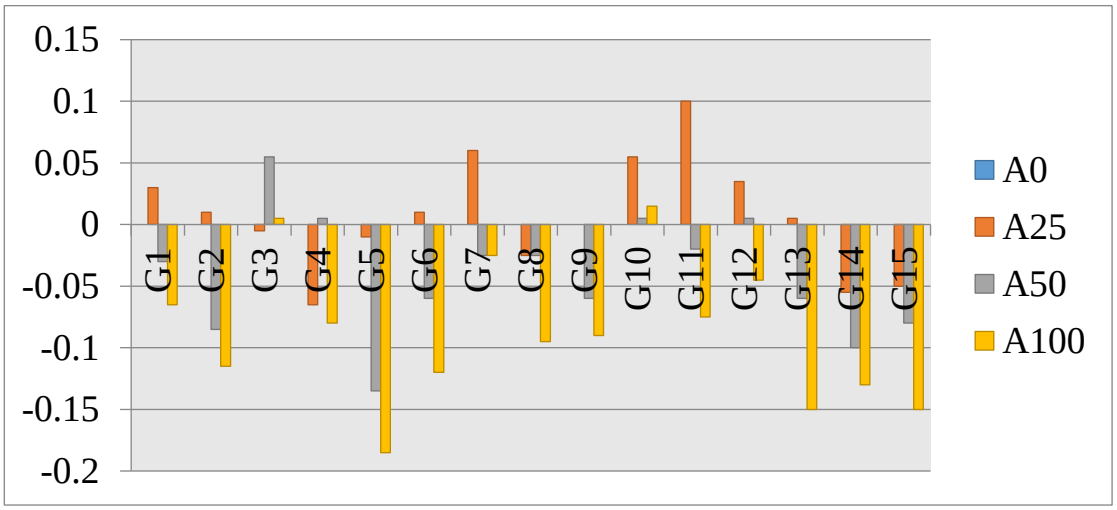
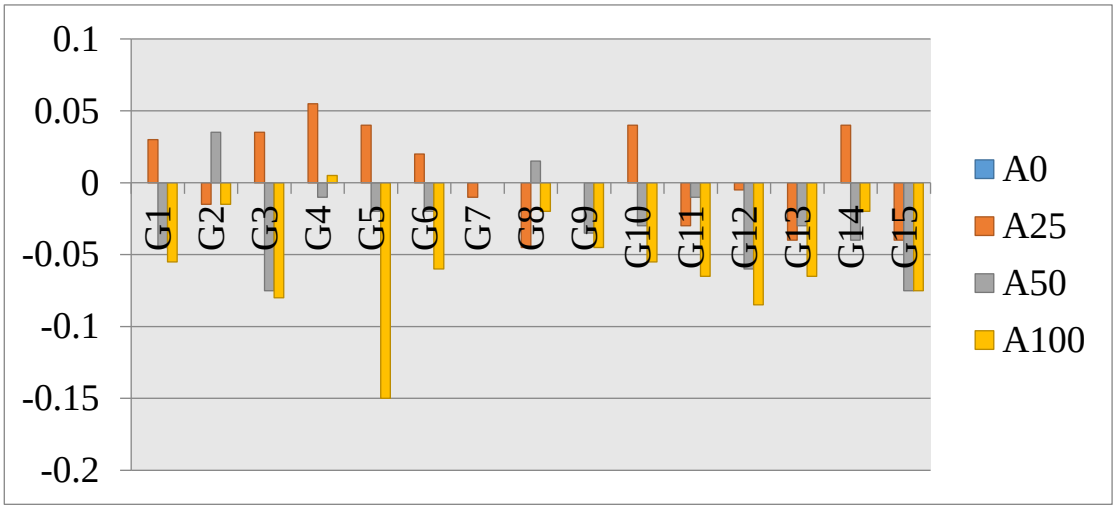


Fig 4.3 Reduction in Root dry matter in comparison to control at various aluminium concentration



4.1.8 Shoot dry matter (g plant^{-1})

It was observed that shoot dry matter was significantly influenced by genotypes and aluminium treatment (Table 4.8). The highest shoot dry matter was recorded in G13 (0.29 g) which was statistically at par with G5 (0.27 g), G2 (0.26 g), G3 (0.26 g) and G15 (0.26 g) and lowest value was recorded in G7 (0.15 g), G9 (0.17 g), G6 (0.18 g) and G1 (0.18).

With respect to the response to aluminium stress, the highest amount of shoot dry matter (0.26 g) was recorded at aluminium level of 25 µM which was at par with control (0.25 g). Shoot dry matter gradually decreased as the concentration of aluminium increased from 25 µM to 100 µM and shoot dry matter was lowest (0.17 g) at 100 µM. There was reduction of -0.04 g at A50 and -0.08 g at A100 but it increased at A25 by 0.01 g in comparison to control (Table 4.10). The interaction effect of genotypes and aluminium was also found to be significant. The highest shoot dry matter was found in G5 at A0 (0.36 g) and lowest at treatment G6 at A100 (0.10 g). Shoot dry matter decreased at A25, A50 and A100 in most of the genotypes (Fig. 4.2).

4.1.9 Root Dry Matter (g plant^{-1})

Root dry matter was significantly influenced by genotypes and aluminium treatment (Table 4.9). The highest root dry matter was observed in genotype G13 (0.27) which was at par with G2 (0.25 g) and G5 (0.24 g) and lowest value was recorded in genotype G7 (0.09 g).

Among all level of aluminium treatment highest root dry matter was recorded at control (0.20 g) and 25 µM level (0.20 g) and lowest at 100 µM (0.15 g). The root dry matter was reduced by -0.03 g at A50 and -0.05 g at A100 (Table 4.10). The interaction of genotypes and aluminium level was found to be significant.

Table 4.9 Effect of aluminium concentration on root dry matter (g plant^{-1}) of cowpea genotypes.

Genotype	A0	A25	A50	A100	Mean G
G1	0.16	0.19	0.12	0.11	**0.14**
G2	0.25	0.24	0.29	0.24	**0.25**
G3	0.26	0.29	0.18	0.18	**0.23**
G4	0.14	0.19	0.13	0.14	**0.15**
G5	0.28	0.32	0.26	0.13	**0.24**
G6	0.16	0.18	0.14	0.10	**0.15**
G7	0.10	0.09	0.10	0.10	**0.09**
G8	0.20	0.15	0.21	0.18	**0.18**
G9	0.16	0.16	0.13	0.12	**0.14**
G10	0.16	0.20	0.13	0.11	**0.15**
G11	0.16	0.13	0.15	0.09	**0.13**
G12	0.23	0.22	0.17	0.14	**0.19**
G13	0.30	0.26	0.27	0.24	**0.27**
G14	0.21	0.25	0.17	0.19	**0.21**
G15	0.26	0.22	0.19	0.19	**0.21**
Mean A	**0.20**	**0.20**	**0.17**	**0.15**	
Factors	**CD**	**SE(d)**	**SE(m)**		
Factor (G)	0.03	0.02	0.01		
Factor (A)	0.02	0.01	0.01		
Factor (GxA)	0.07	0.03	0.02		

Table 4.10 Reduction in different plant characteristic at various level of aluminium across fifteen cowpea genotypes compared to control.

Al toxicity level	Plant height at 1st week (cm)	Plant height at 2nd week (cm)	Plant height at 3rd week (cm)	Plant height at 4th week (cm)	Plant height at 5th week (cm)	Root length (cm)	Biomass (g)	Shoot dry matter (g)	Root dry matter (g)
A0	0	0	0	0	0	0	0	0	0
A25	2.33	1.97	-0.28	-1.01	0.54	3.26	0.04	0.01	0
A50	-0.76	0.2	-0.93	-0.73	0.18	-2.74	-0.39	-0.04	-0.03
A100	0.81	1.77	0.23	0.24	2.22	-5.37	-0.58	-0.08	-0.05

The highest amount of root dry matter was found in G5 at A25 (0.32 g) and lowest value was obtained under the treatment combination of G11 at A100 (0.09 g). Root dry matter decreased in seven genotypes whereas, increased in remaining genotypes at A25. It was decreasing at A50 and A100 in most of the genotypes (Fig. 4.3).

4.2 Principal component analysis and Cluster analysis

Based on the principal component analysis, at control PC1 and PC2 together contributed 80.47% of total variance within the dataset. Characters Root length (RL), Biomass (BM), Shoot dry matter (SDM), Root dry matter (RDM) and Plant height at 2nd week (PH2) showed positive values for both PC1 and PC2 while Plant height at 1st (PH1), 3rd (PH3), 4th (PH4) and 5th week (PH5) of growth showed positive values for PC1 and negative PC2 values (Fig.4.4).

The PC analysis and cluster analysis resulted in four group of cowpea genotype based on their aluminium tolerance. Group one had G10, G13 and G15 having higher values for PH1, PH3, PH4 and PH5 and moderate value of PH2, RDM, SDM, RL and BM. Genotypes G14, G5, G2 and G12 clustered as a second group based on the high value of PH2, RDM, SDM, RL and BM and moderate values for remaining growth response under study. A third group comprised of G6 and G11 with moderate values for all the growth parameters under study could be distinguished. The fourth group consisted of G1, G3, G4, G7, G8 and G9. This group of genotype had lower values for all the traits under the present study.

PC analysis and cluster analysis of genotypes at the aluminium level of 25 µM grouped into four groups where PC1 and PC2 contributed cumulative 78% of the total variance present in the dataset. At 25 µM, RL, SDM, BM, PH4 and PH5 had positive value for PC1 and PC2 both and remaining characters had positive values for the PC1 and negative value for PC2 (Fig. 4.5).

Fig. 4.4 PCA and two-way cluster analysis of plant morphophysiological characters of cowpea genotypes at A0

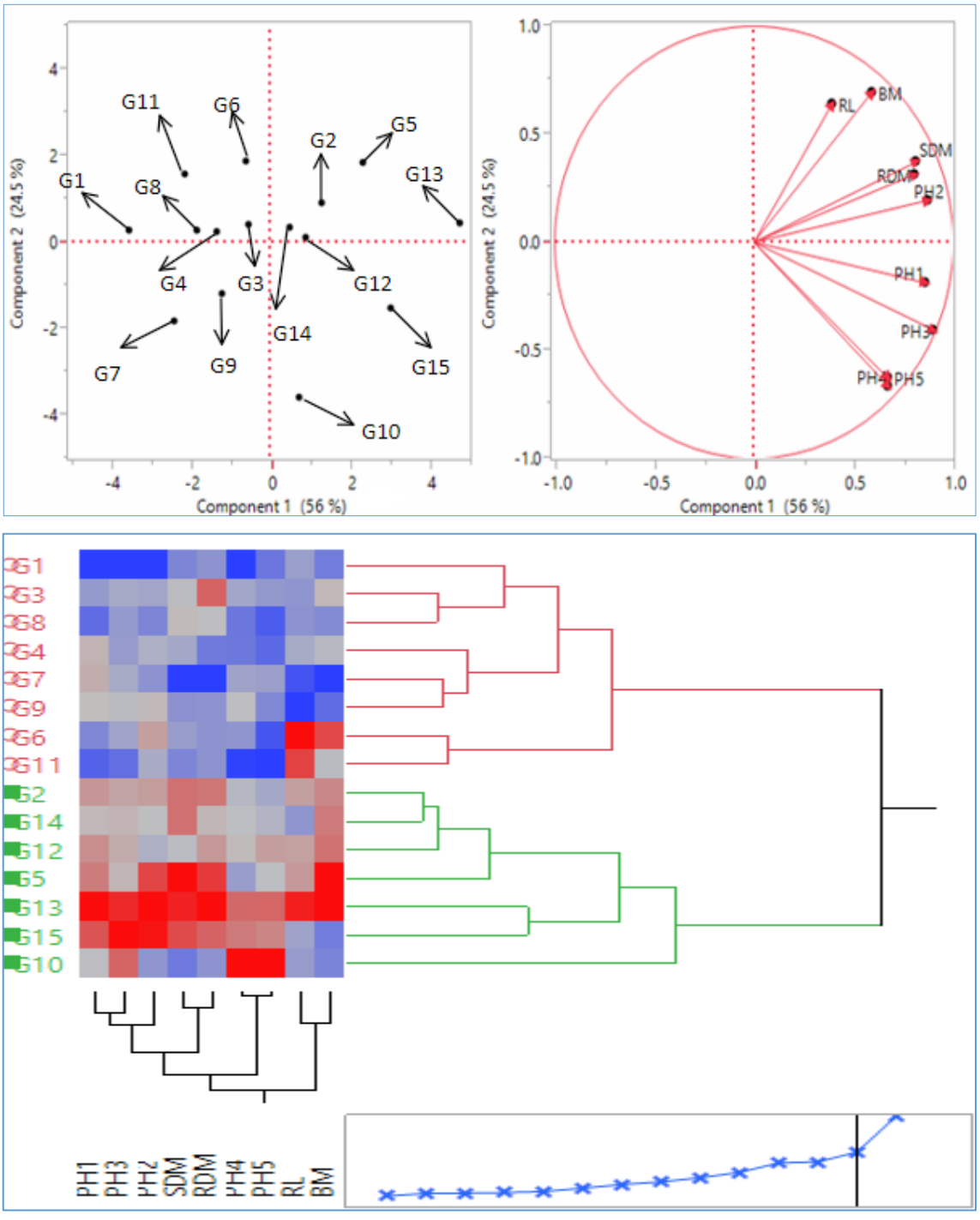
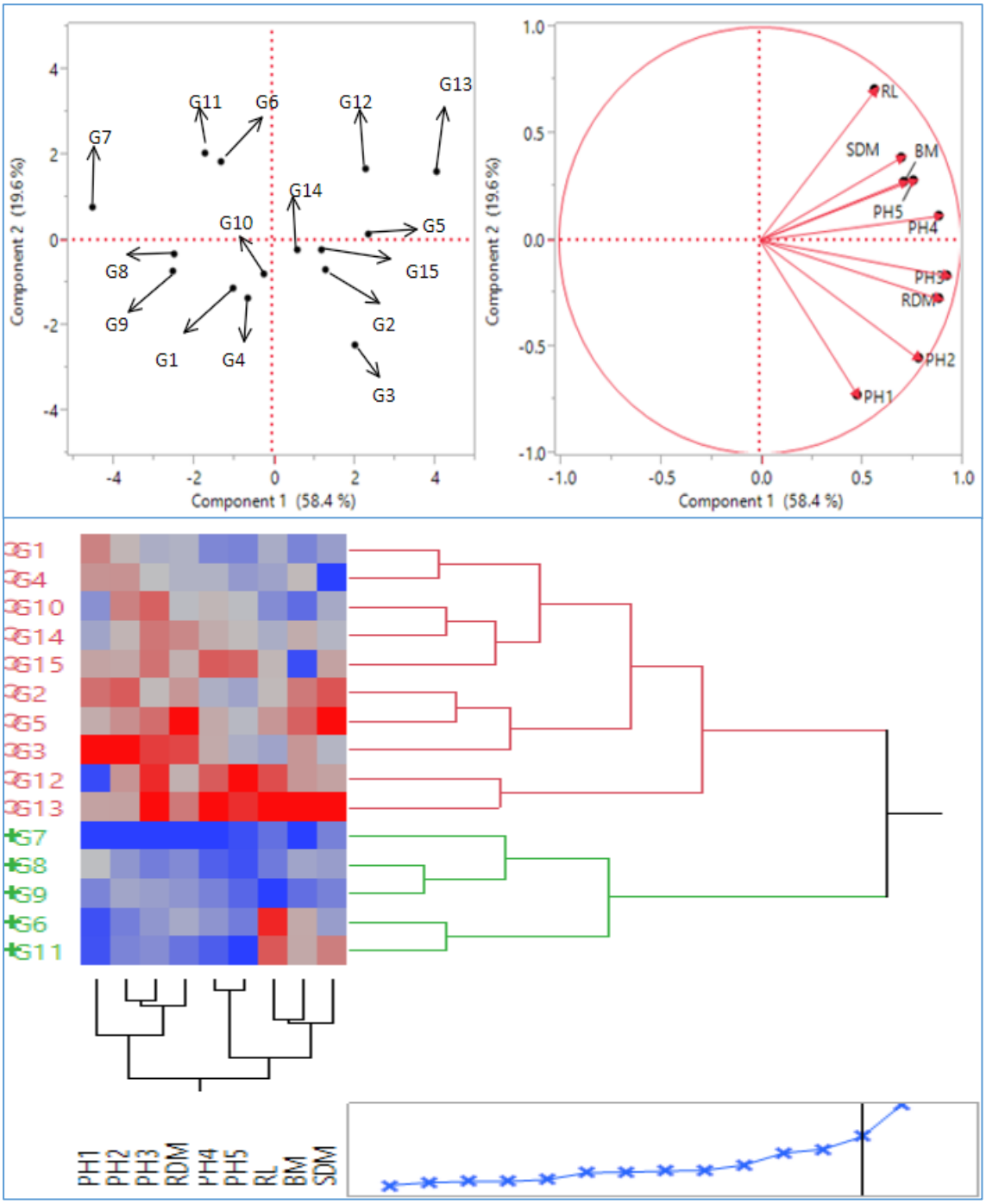


Fig. 4.5 PCA and two-way cluster analysis of plant morphophysiological characters of cowpea genotypes at A25



The first group comprised of G12 and G13 which had high value for the characters *viz.* RL, SDM, BM, PH5 and PH4 and moderate value for character PH3, PH2, PH1 and RDM while G5, G2 and G3 which made into second group had moderate value for characters PH3, PH2, PH1, RDM and high for RL, SDM, BM, PH4 and PH5. Genotypes G7, G8, G9, G6, and G11 grouped as the third group had moderate values for all characters under study. The fourth group consisted genotypes G1, G4, G10, G14 and G15 had lower values for all the characters under study.

At the aluminium level of 50 µM, PC1 and PC2 both contributed 67.4% of total variance present in the dataset. All the genotypes could be grouped into four clusters based on PC analysis and cluster analysis (Fig. 4.6). At this level of aluminium treatment RL, BM, RDM, SDM, PH5 and PH4 had a positive value for both PC1 and PC2 while, PH3, PH2 and PH1 had positive PC1 and negative PC2 values.

In the first group of genotypes G1, G9 G12, G11, G14 and G6 had moderate values for all the character under study. Genotypes G2, G13, G5 grouped as the second group had higher values for characters RL, BM, RDM, SDM, PH5 and PH4 and moderate values for PH3, PH2 and PH1 while, G10 and G15 clustered as another group. The last group comprised of G3, G4, G7 and G8 had lower values for all the characters under study.

PC and cluster analysis for the aluminium level of 100 µM had revealed that genotypes under study could be categorised into four groups where PC1 and PC2 cumulatively contributed 73.6% of total variance of the dataset. Characters BM, RL, RDM, PH5 and PH4 having positive values for both PC1 and PC2 while SDM, PH1, PH2 and PH3 have positive value for PC1 and a negative value for PC2 (Fig. 4.7).

Fig. 4.6 PCA and two-way cluster analysis of plant morphophysiological characters of cowpea genotypes at A50

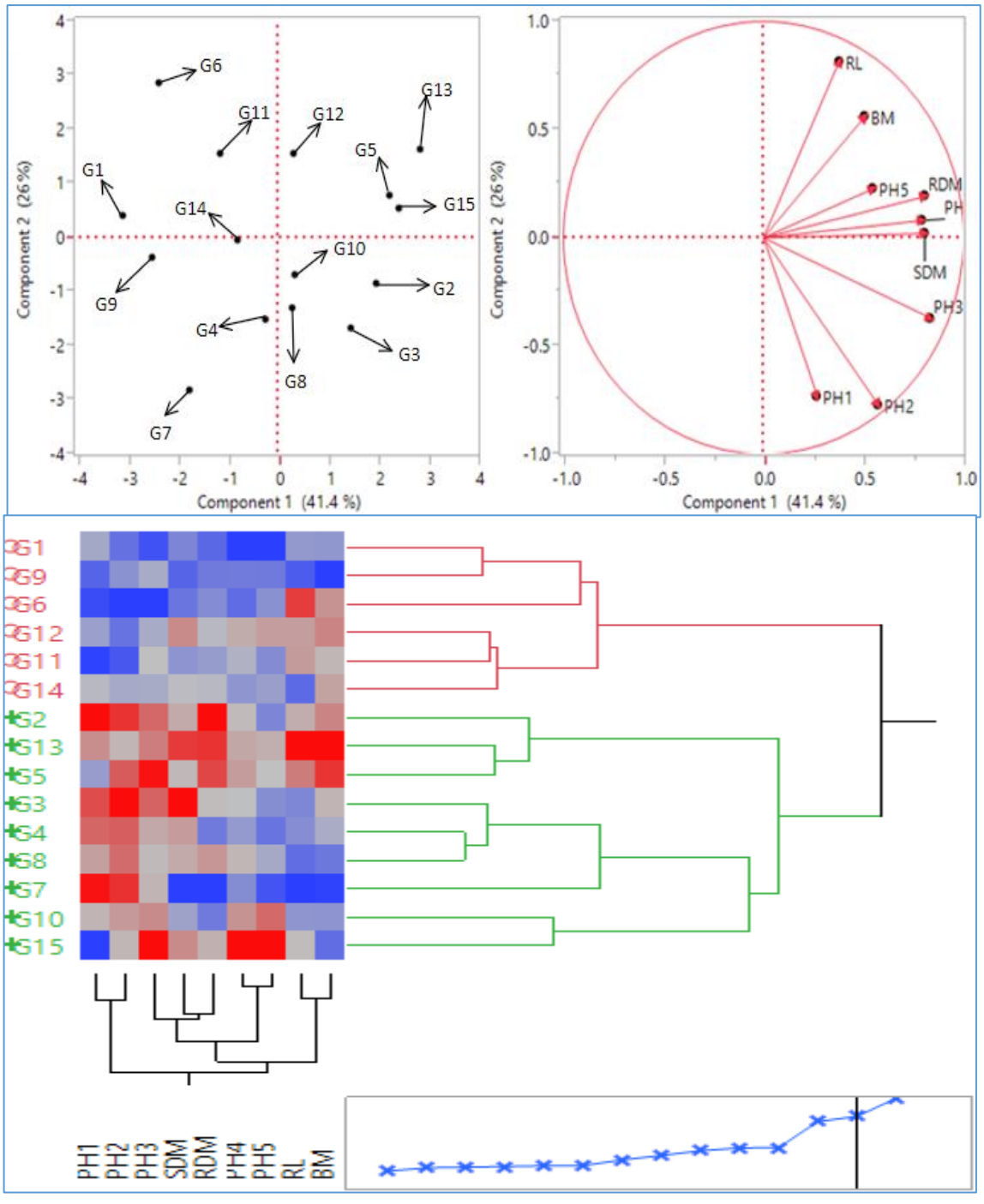
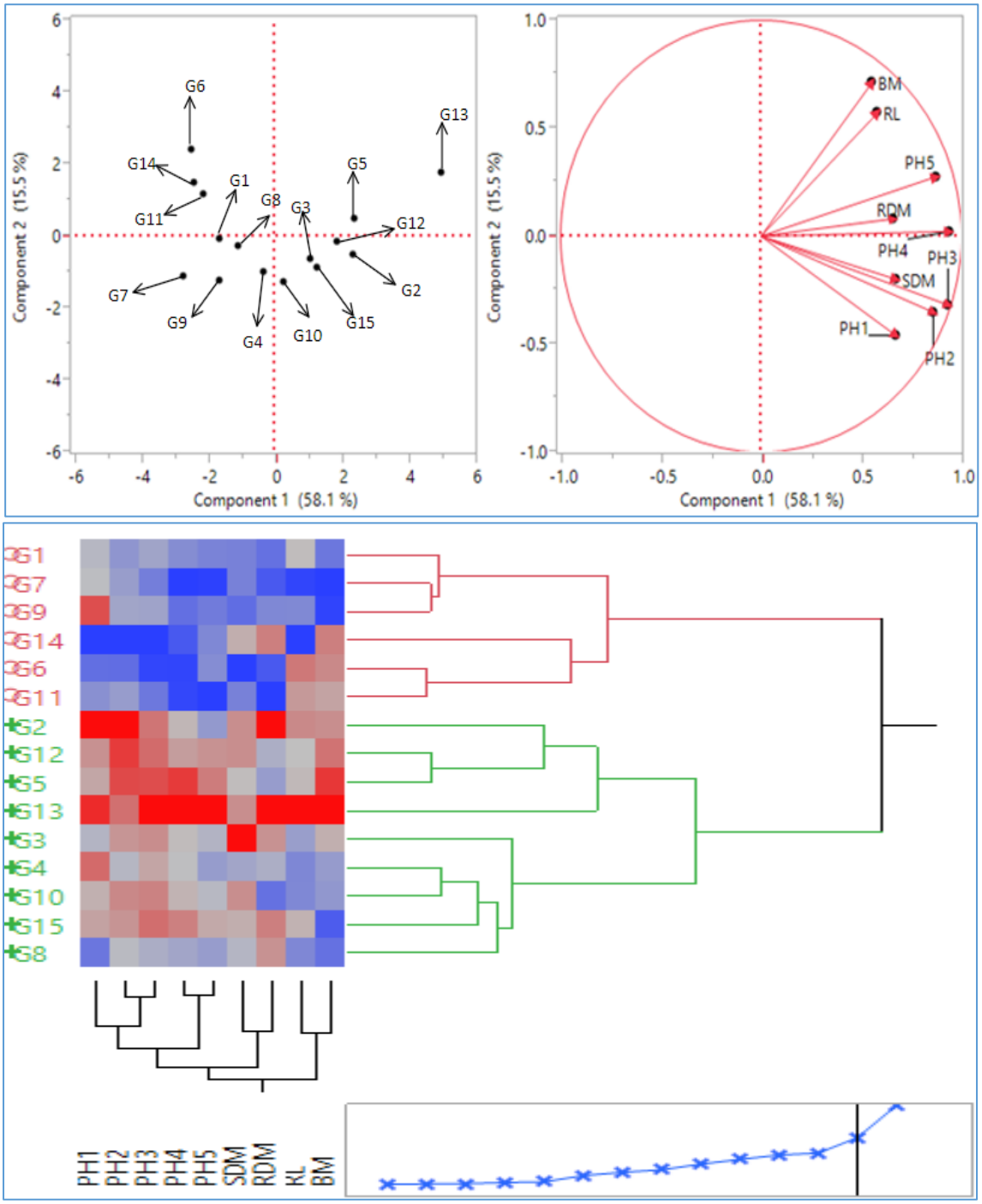


Fig. 4.7 PCA and two-way cluster analysis of plant morphophysiological characters of cowpea genotypes at A100



Genotypes G12, G13 and G5 could be grouped under one group. BM, RL, RDM, PH5 and PH4 growth responses were higher for this group of genotypes while SDM, PH1, PH2 and PH3 were moderate for this group. Genotypes G14, G6 and G11 which had moderate values for all the growth responses formed the second group. Genotypes G3, G4, G8, G10 and G15 with moderate value for BM, RL, RDM, PH5 and PH4 and lower values for characters SDM, PH1, PH2 and PH3 clustered as another group. The fourth group consisted of genotypes G1, G7 and G9 which have lesser value for all the character under study.

Hence, based on the above obtained results we could classify G13 as most tolerant and G7 as most susceptible genotype across fifteen cowpea genotypes tested. All the fifteen genotypes could be categorised into four groups according to their performance towards aluminium tolerance.

1. Very susceptible: G7 and G9
2. Susceptible: G1, G8, G3, G4, G10
3. Tolerant: G11, G2, G6, G12, G14, G15
4. Highly tolerant: G13 and G5

4.3 Ionomic profile of the cowpea genotypes under aluminium stress

4.3.1 At control level

In order to reveal the effect of aluminium on the distribution of elements in cowpea genotypes ionomic profiling was performed. Based on the PCA analysis of ionomic profiling of cowpea genotypes at control PC1 and PC2 together contributed 56.2% of total variance within dataset (Fig 4.8). Elements Al, Pb, Mn, Ca, Ba, B, Mg, Li, and Co were having positive value of PC1 and PC2 while, Ni, Ag, Mo, Na, K, Zn and Rb were having positive values for PC1 and negative for PC2. Only Cu was having

Fig 4.8 PCA and two-way cluster analysis of ionome profiling of cowpea genotypes at A0

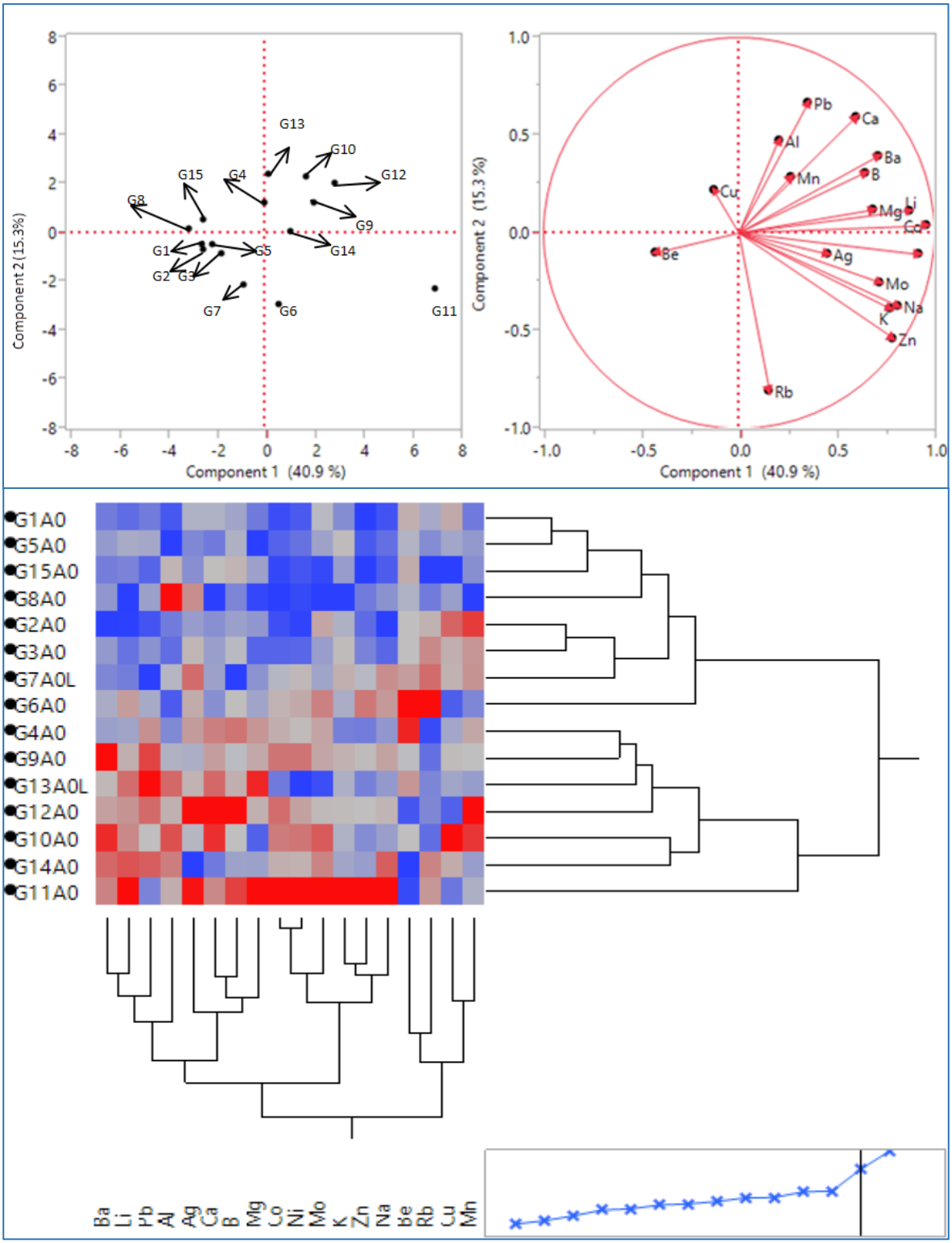
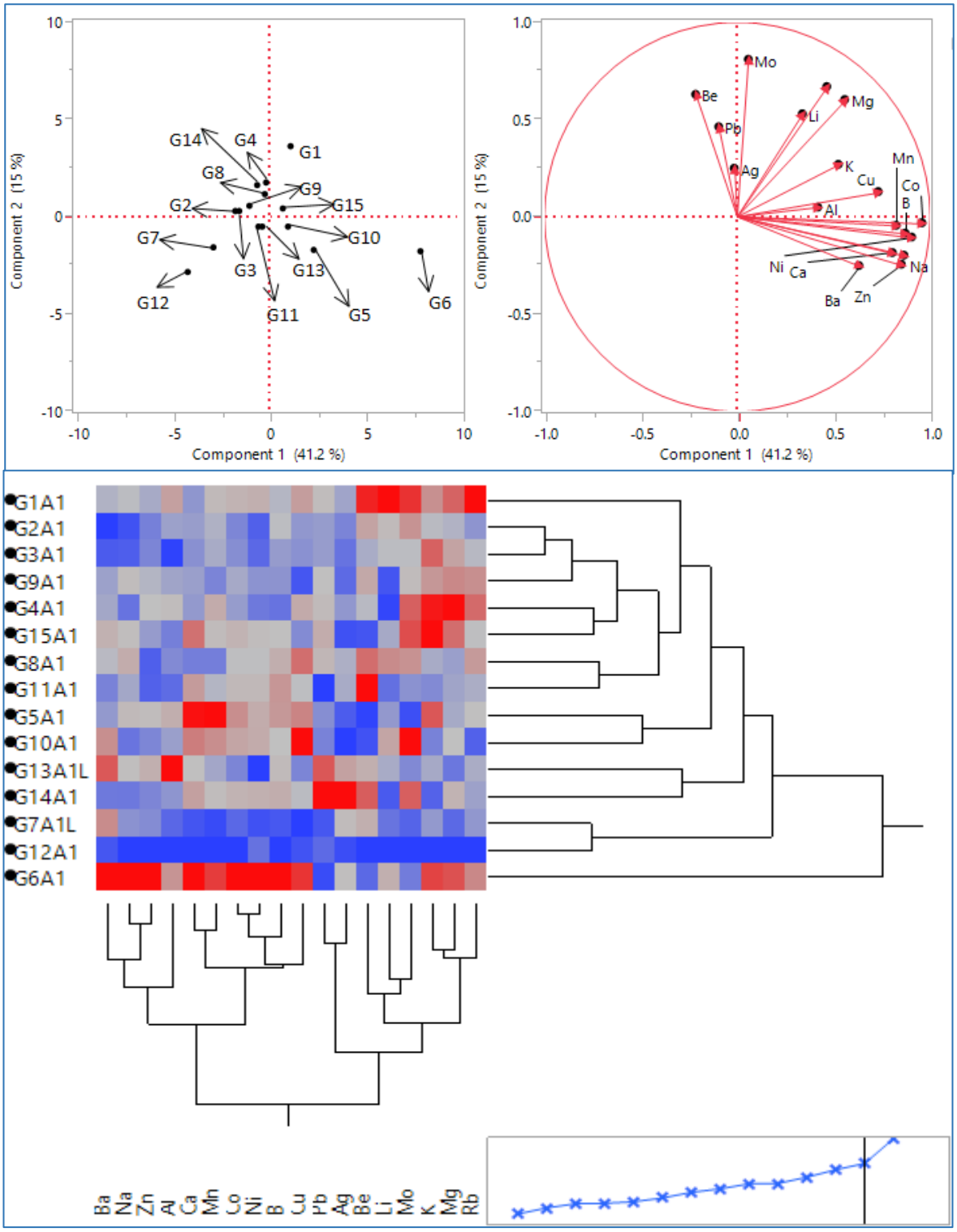


Fig 4.9 PCA and two-way cluster analysis of ionome profiling of cowpea genotypes at A25



negative value for PC1 and positive for PC2 whereas, Be was having negative value for both PC1 and PC2. The principal component analysis and cluster analysis categorize genotypes in to four groups. First group comprised of genotypes G4, G9, G10, G12, G13 and G14. In second group genotypes G11 and in third group G6 were there. The fourth group comprised of genotype G1, G2, G3, G5, G7, G8 and G15.

4.3.2 At A25 level

PC analysis and cluster analysis of genotypes at the 25µM concentration aluminium, grouped them into four groups where PC1 and PC2 contributed cumulative 56.2% of the total variance present in the dataset (Fig. 4.9). At 25µM concentration, elements Mo, Li, Rb, Mg, K, Cu and Al had positive values for both PC1 and PC2. While, elements Mn, Co, B, Ni, Ca, Ba, Zn and Na had positive values for the PC1 and negative value for PC2 and elements Be, Pb and Ag had positive value for PC2 and negative for PC1. The first group comprised of G6 while, second group comprised of genotype G7 and G12. Third group consisted of genotypes G5 and G10 and remaining genotypes clustered as fourth group.

4.3.3 At A50 level

At the aluminium level of 50 µM concentration both PC1 and PC2 contributed 62.4% of total variance present in the dataset. All the genotypes grouped into four clusters based on PC analysis and cluster analysis (Fig. 4.10). At this level of aluminium concentration in the medium elements Ag, Pb, Al, Ba, K, Mg, Li, and B had a positive value for both PC1 and PC2 while, Mn, Ca, Na, Cu, Zn, Co and Ni had positive values for PC1 and negative for PC2. Elements Mo had positive values for PC2 and negative for PC1 and element Be and Rb had negative values for both PC1 and PC2. The first cluster comprised of genotypes G13 while, genotype G12, G15 and G11 grouped as

Fig 4.10 PCA and two-way cluster analysis of ionome profiling of cowpea genotypes at A50

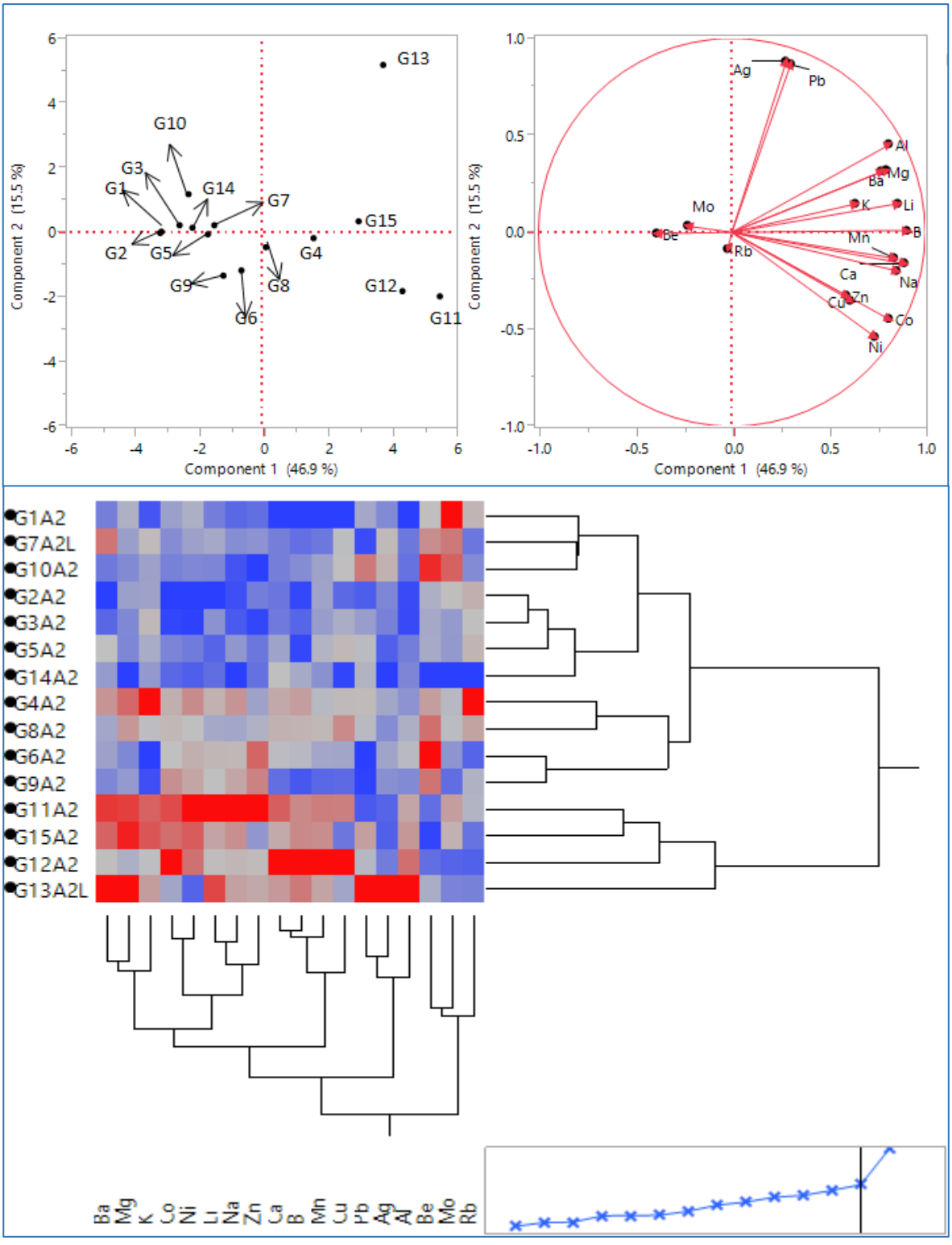
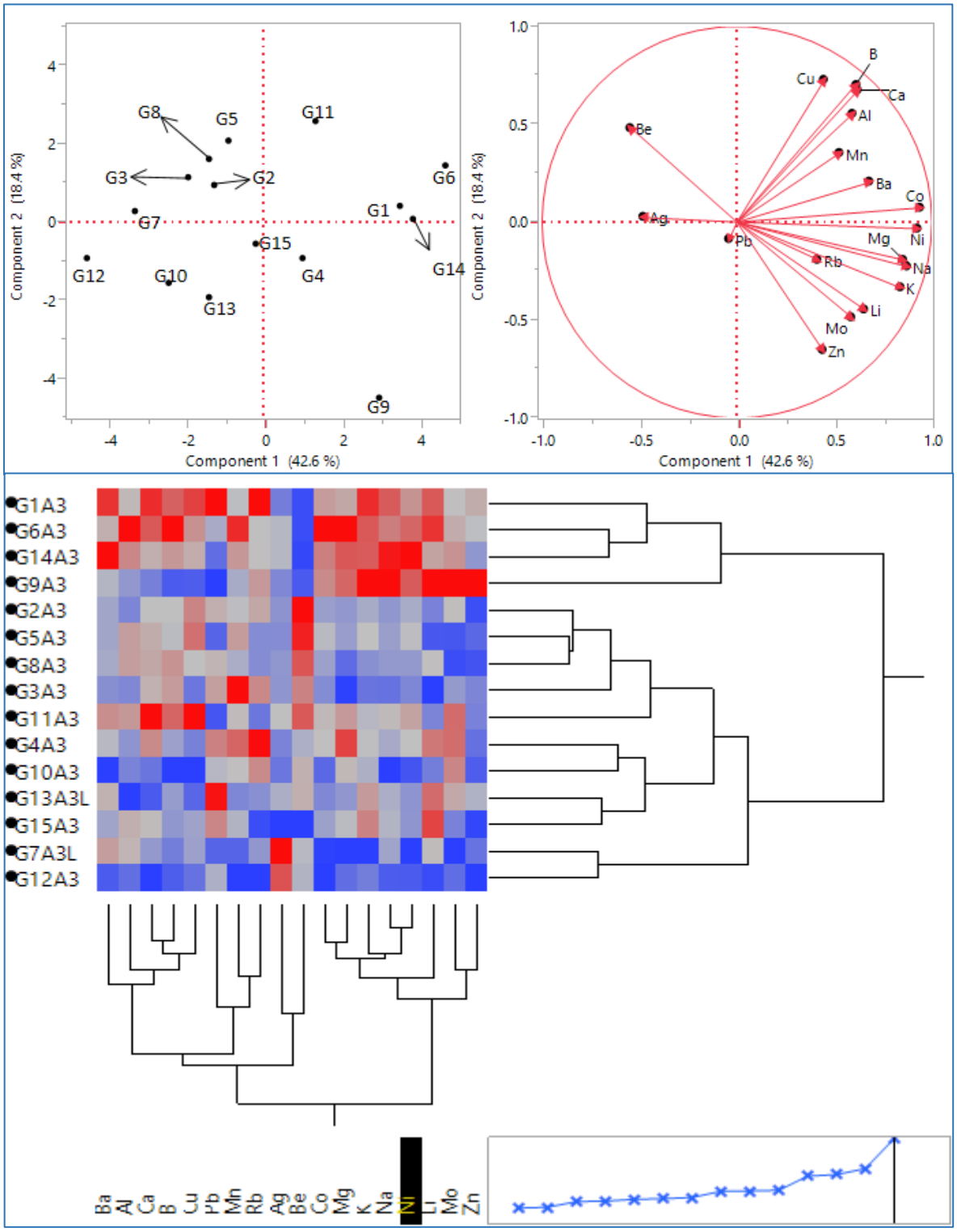


Fig 4.11 PCA and two-way cluster analysis of ionome profiling of cowpea genotypes at A100



second group. Group third comprised of G1, G2, G3, G5, G7, G10 and G14 and the last group comprised of G4, G6, G8 and G9.

4.3.4 At A100 level

PC and cluster analysis for the aluminium level 100 µM concentration had revealed that genotype under study could be categorized into four groups where PC1 and PC2 cumulatively contributed 61% of total variance of the dataset (Fig 4.11). Elements Cu, Al, B, Ca, Mn, Ba and Co had positive values for both PC1 and PC2 whereas Pb had negative values for both PC1 and PC2. A positive PC1 and negative PC2 values were found for elements Ni, Mg, Na, Rb, K, Li, Mo and Zn, while Be, and Ag had negative values for PC1 and positive for PC2. Genotypes G1, G6, G9 and G14 grouped under one group. The second group was formed from genotypes G2, G3, G5, G8 and G11 while, genotypes G4, G10, G13 and G15 grouped as third group. The last and fourth group consisted of genotypes G7 and G12.

4.3.5 Ionome profile of roots of tolerant and susceptible genotypes of cowpea at control and A100 level of aluminum

Comparison of root ionome profile of tolerant and susceptible cowpea genotypes through PCA analysis had revealed that the PC1 and PC2 cumulatively contributed 82.1% of total variance present in the dataset. Tolerant genotype at highest aluminum level was having higher concentration of elements Ba, Co, Mg, Na and Al, moderate concentration of K, Zn, Ni, Mn, Mo, Rb and Pb and lower concentration of Ag, Ca, Cu, Be, Li and B. While, at control level it was having higher values of K, Ag, Mo, Ca, Mn, Zn, Ni, Be, Li and B, moderate value of Na, Mg, Cu and lower values of Ba, Co, Al, Pb and Rb. Susceptible genotype at highest level of aluminium had lower value of all the elements except Al and Be which were in moderate concentration.

Fig 4.12 PCA analysis of root ionome profiling of tolerant and susceptible cowpea genotypes

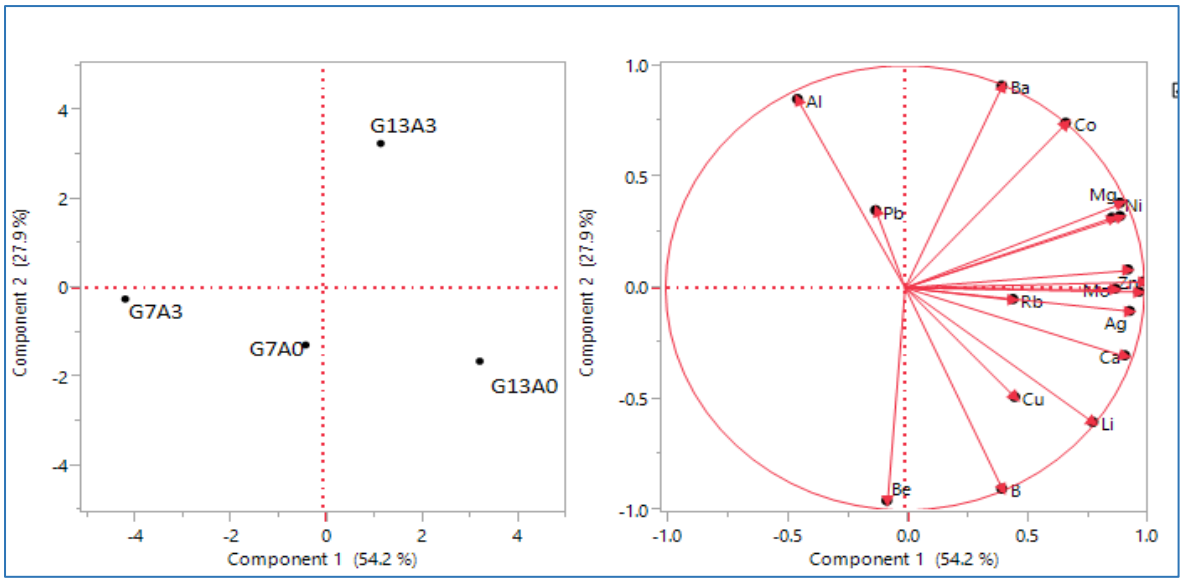


Table 4.11 Root ionome profile of tolerant and susceptible cowpea genotypes at control and A100 aluminium level.

Elements (mg $100g^{-1}$)	T7A0	T7A100	T13A0	T13A100
Ba	7.173	6.029	7.413	14.005
Pb	3.704	1.126	0.231	2.831
Ag	0.177	0.135	0.325	0.232
Al	52.458	229.424	50.141	266.055
Be	0.038	0.042	0.050	0.008
Ca	4.384	1.872	9.803	4.840
Co	0.237	0.208	0.265	0.327
Cu	950.412	49.867	572.982	284.621
K	37.569	15.956	41.290	39.622
Li	0.732	0.527	0.858	0.594
Mg	11.060	6.340	25.216	25.704
Mn	7.234	5.141	11.375	9.092
Na	22.867	15.179	23.443	25.494
Ni	14.505	12.997	21.798	21.100
Mo	3.573	3.512	5.547	4.567
Rb	0.974	0.693	0.815	0.856
B	39.004	16.086	46.987	3.103
Zn	2.227	1.179	3.406	2.800

While, at control level Pb, Cu and Rb were in higher concentration and K, Na, B were in moderate amount and rest were in lower concentration.

Based on the above presented result of PCA analysis following conclusion can be made:

- Concentration of **Ba, Pb, Co, Mg, Na and Rb** increased in aluminium tolerant genotype and decreased at stress condition in aluminium susceptible genotype compared to control.
- Increased elemental concentration of **Al** in both aluminium tolerant genotype and aluminium susceptible genotype at A100 level compared to control was observed.
- Decreased elemental concentration of **Ag, Ca, Cu, K, Li, Mn, Ni, B and Zn** in both aluminium tolerant genotype and in aluminium susceptible genotype at A100 level compared to control was recorded.
- **Be** concentration decreased in aluminium tolerant genotype at high Al stress compared to control but in susceptible cultivar it was at par in stress and control condition.
- **Mo** concentration increased in aluminium tolerant genotype at high Al stress compared to control but in susceptible cultivar it was at par in stress and control condition.

4.4 Proteome profiling of root proteins of tolerant and susceptible cowpea genotypes

4.4.1 Aluminium susceptible genotype (Control vs High stress treatment)

Comparison of protein profile of susceptible genotype in control and highest stress condition, a total 85 protein were found. Among that 48 proteins were found to

Table 4.12 Upregulated protein with their accession no, peptide count, fold changes and mass in aluminium susceptible genotype (control vs treated)

Accessions	Peptide count	Unique peptide	Fold Changes	Mass	Description
A0A151FX57	3	3	1.92	20245.44	Peptide synthetase
Q43683	3	3	3.74	26668.31	Acidic chitinase class 3
A0A151FIM0	7	7	1.54	15735.87	Uncharacterized protein
A0A0K1R0Z8	8	1	2.14	18653.03	ATP synthase beta subunit
A0A0K1R0W1; A0A0K1R0Y8; A0A0K1R120	8	1	1.69	16713.91	ATP synthase beta subunit (Fragment)
A0A0K1R0U9; A0A0K1R1H7	9	2	1.96	17229.53	ATP synthase beta subunit (Fragment)
Q43685	15	3	1.76	35310.92	Chitinase class 1 (Fragment)
P93700	20	20	2.29	35932.91	CPRD14 protein

Table 4.13 Downregulated protein with their accession no, peptide count, fold changes and mass in aluminium susceptible genotype (control vs treated)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
A0A151FEB0	2	2	6.07	13352.89	Calcium-binding protein
A0A151FEP6	3	3	2.42	17874.07	(2Fe-2S)-binding protein
A0A151FYX1	3	2	1.77	22771.28	DNA ligase
A0A151FFL2	6	4	2.60	41787.6	Amidohydrolase
A0A151FTR9	7	5	2.27	42077.91	Amidohydrolase
M9RSB0;M9RTP5;M1RLI9	14	14	1.59	39279.16	Glutamine synthetase
O24548	17	17	1.57	40106.6	Type IIIa membrane protein cp-wap13
Q5NT85	18	18	2.18	50561.35	Apyrase

Table 4.14 Upregulated protein with their accession no, peptide count, fold changes and mass in aluminium tolerant genotype (control vs treated)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
A0A151FZR5	1	1	3.24	11322.66	Uncharacterized protein
A0A075TMV4	1	1	2.29	11948.02	RNA-dependent RNA polymerase (Fragment)
A0A151FII1	1	1	1.54	17079.3	Polyketide cyclase
A0A151FHD4	1	1	2.38	7644.321	Uncharacterized protein
A0A151FYX1	3	2	2.52	22771.28	DNA ligase
A0A151FR23	5	5	2.97	20801.89	Ribosome-recycling factor
A6H5B1; A6H5B0	6	6	1.50	22124.64	Putative cathepsin B-like cysteine protease putative
Q41713	6	6	2.48	56163.38	Aspartic proteinase
A0A151FFL2	6	4	1.61	41787.6	Amidohydrolase
A0A151FIM0	7	7	2.29	15735.87	Uncharacterized protein
A0A0K1R0Z8	8	1	1.75	18653.03	ATP synthase beta subunit (Fragment)
F5C0D3	10	10	1.55	22225.23	2-cys-peroxiredoxin (Fragment)
B4X941	14	14	2.36	17712.12	17.7 kDa class I small heat shock protein
Q072J9	14	13	1.79	54216.89	Glutathione reductase
Q43685	15	3	3.69	35310.92	Chitinase class 1 (Fragment)
Q5NT85	18	18	2.00	50561.35	Apyrase
P93700	20	20	2.19	35932.91	CPRD14 protein
Q41712	20	20	1.95	27090.71	Cytosolic ascorbate peroxidase
Q9SXX8	20	13	1.55	16402.53	Cowpea pathogenesis-related protein 3 (CpPR3)

Table 4.15 Downregulated protein with their accession no, peptide count, fold changes and mass in aluminium tolerant genotype (control vs treated)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
P05045	1	1	1.87	29405.97	Seed lectin subunit I
Q43686	1	1	324.94	27731.79	Chitinase class 4 (Fragment)
A0A151FUI8	2	2	6.23	16910.32	Uncharacterized protein
A0A151FEB0	2	2	13.57	13352.89	Calcium-binding protein
A0A151FRR1	2	2	3.06	7182.95	Uncharacterized protein
A0A151FH78	3	3	1.53	20943.32	MarR family transcriptional regulator
A0A151FKF4	4	3	1.83	29424.78	Uncharacterized protein

be significant based on ANOVA (p) value. Among 48 significant proteins, 21 were having >1.5 fold change. 11 proteins (fold changes ranges from 3.74-1.54) were upregulated in Al stress condition and 10 proteins were downregulated (fold changes 6.07-1.57). These upregulated proteins were peptide synthetase, acidic chitinase class 3 protein, uncharacterized protein, 6 different ATP synthase beta unit, Chitinase class I, CPRD14 protein (Table 4.12). Like, that downregulated proteins were calcium binding protein, iron sulphur binding protein, DNA ligase protein, 2 amid hydrolase protein, 3 glutamine synthetase protein, type IIIa membrane protein cp-wap13 and apyrase (Table 4.13).

4.4.2 Aluminium tolerant genotype (Control vs treatment)

Protein profiling of aluminium tolerant genotype at control and stressed condition had yielded 85 proteins. In that, 42 proteins were found significant based on ANOVA (p) value. Among these significant proteins, 27 were having fold changes >1.5. From these, 20 proteins (fold changes 3.69-1.50) were found to be upregulated while, 7 (fold changes 324.94-1.53) were downregulated. Upregulated proteins were listed in Table 4.14 which were 3 uncharacterized protein, RNA-dependent RNA polymerase protein, polyketide cyclase, DNA ligase protein, ribosome-recycling factor, 2 putative cathepsin B-like cysteine protease putative, aspartic proteinase, amidohydrolase, ATP synthase beta subunit protein, 2-cys-peroxiredoxin, 17.7 kDa class I small heat shock protein, glutathione reductase, chitinase class 1, apyrase, CPRD14 protein, cystolic ascorbate peroxidase and cowpea pathogenesis related protein 3. The down regulated proteins were seed lectin subunit I, chitinase class 4, calcium-binding protein, MarR family transcriptional regulator and 3 uncharacterized protein (Table 4.15).

4.4.3 Aluminum tolerant and susceptible genotypes at control condition

A total 85 protein were found while comparing aluminium tolerant genotype with susceptible genotypes at controlled condition. Among that 47 proteins were found significant based on ANOVA (p) value. Among significant proteins, 33 were having 1.5 fold changes in which 23 proteins (3.49-1.56 fold changes) were more in tolerant genotype and 10 proteins (infinity- 1.76 fold changes) were more in susceptible genotype. The proteins RNA-dependent RNA polymerase, MarR family transcriptional regulator protein, ribosome-recycling factor, 2 amidohydrolase protein, 13 ATP synthase beta subunit, glutathione reductase, cytosolic ascorbate peroxidase, 2 uncharacterized protein and cowpea pathogenesis -related protein 3 (CpPR3) were more in tolerant genotype (Table 4.16). While, chitinase class 4, calcium binding protein, peptide synthetase, acidic chitinase class 3, 2 AraC family transcriptional regulator, Apyrase protein and 3 uncharacterized protein were more in susceptible genotype (Table 4.17).

4.4.4 Aluminum tolerant and susceptible genotypes at treated high stress condition

At treated condition, a total of 85 proteins were found, in which 59 were found to be significant based on ANOVA (p) value. Among that, 38 proteins were having >1.5 fold change. 28 proteins (fold changes 1.55-26.51) were found more in tolerant genotype in treated condition. These proteins were RNA-dependent RNA polymerase (fragment), (2Fe-2S)-binding protein, Acyl-CoA dehydrogenase, DNA ligase, Ribosome-recycling factor, 2 putative cathepsin B-like cysteine protease putative, aspartic proteinase, 2 amidohydrolase, 2-cys-peroxiredoxin (fragment), leghaemoglobin reductase, 17.7kDa classI small heat shock protein, 2 glutamine synthetase, ATP synthase subunit alpha, glutathione reductase, chitinase class I, type IIIa membrane protein cp-wap13, apyrase, class I chitinase, CPRD 14 protein, cytosolic ascorbate peroxidase, cowpea

pathogenesis related protein 3 and 4 uncharacterized protein. Likewise, 10 proteins (fold changes ranges from 1.58-∞) are found more in susceptible genotype which were 4 different uncharacterized protein, seed lectin, chitinase class 4, calcium binding protein, peptide synthetase, acidic chitinase class 3, ATP synthase beta subunit. (Table 4.19).

Table 4.16 Changes in protein fold (positive) in controlled condition (tolerant vs susceptible genotype)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
A0A075TMV4	1	1	2.32	11948.02	RNA-dependent RNA polymerase
A0A151FUI8	2	2	1.93	16910.32	Uncharacterized protein
A0A151FH78	3	3	2.65	20943.32	MarR family transcriptional regulator
A0A151FR23	5	5	3.49	20801.89	Ribosome-recycling factor
A0A151FFL2	6	4	1.81	41787.6	Amidohydrolase
A0A151FIM0	7	7	1.65	15735.87	Uncharacterized protein
A0A151FTR9	7	5	1.83	42077.91	Amidohydrolase
A0A0K1R0W1;A0A0K1R0Y8;A0A0K1R120	8	1	3.06	16713.91	ATP synthase beta subunit
A0A0K1R0U9;A0A0K1R1H7	9	2	2.38	17229.53	ATP synthase beta subunit
Q072J9	14	13	1.68	54216.89	Glutathione reductase
A0A151FQ06;A0A0K1R0V5;A0A0K1R0Z2;A0A0K1R0Z4;A0A0K1R103;A0A0K1R115;A0A0K1R124;A0A0K1R1H1	17	11	1.56	51020.05	ATP synthase subunit beta
Q41712	20	20	1.69	27090.71	Cytosolic ascorbate peroxidase
Q9SXX8	20	13	1.82	16402.53	Cowpea pathogenesis-related protein 3 (CpPR3)

Table 4.17 Changes in protein fold (negative) in controlled condition (tolerant vs susceptible genotype)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
A0A151FEB1	1	1	Infinity	10688.00	Uncharacterized protein
Q43686	1	1	1.84	27731.79	Chitinase class 4 (Fragment)
A0A151FJP2	1	1	80.18	11274.73	Uncharacterized protein
A0A151FEB0	2	2	26.21	13352.89	Calcium-binding protein
A0A151FX57	3	3	2.04	20245.44	Peptide synthetase
Q43683	3	3	8.70	26668.31	Acidic chitinase class 3
A0A151FR10	3	3	2.13	18609.80	Uncharacterized protein
A0A151FG35; A0A151FKK4	5	4	1.76	33488.97	AraC family transcriptional regulator
Q5NT85	18	18	2.27	50561.35	Apyrase

Table 4.18 Changes in protein fold (positive) in treated condition (tolerant vs susceptible genotype)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
A0A151FZR5	1	1	4.71	11322.66	Uncharacterized protein
A0A075TMV4	1	1	5.57	11948.02	RNA-dependent RNA polymerase
A0A151FRP2	1	1	26.51	7921.043	Uncharacterized protein
A0A151FEP6	3	3	2.17	17874.07	(2Fe-2S)-binding protein
A0A151FRV1	3	3	2.30	45589.82	Acyl-CoA dehydrogenase
A0A151G057	3	3	1.87	10542.76	Uncharacterized protein
A0A151FYX1	3	2	4.33	22771.28	DNA ligase
A0A151FR23	5	5	13.37	20801.89	Ribosome-recycling factor
A6H5B1; A6H5B0	6	6	1.88	22124.64	Putative cathepsin B-like cysteine protease_putative
Q41713	6	6	4.93	56163.38	Aspartic proteinase
A0A151FFL2	6	4	7.56	41787.6	Amidohydrolase
A0A151FIM0	7	7	2.45	15735.87	Uncharacterized protein
A0A151FTR9	7	5	5.73	42077.91	Amidohydrolase
F5C0D3	10	10	1.80	22225.23	2-cys-peroxiredoxin (Fragment)
Q9SPB1	11	11	1.76	56065.62	Leghemoglobin reductase
B4X941	14	14	3.64	17712.12	17.7kDa classI small heat shock
M9RSB0;M9RTP5	14	14	1.90	39279.16	Glutamine synthetase
A0A151FQT6	14	9	2.13	55318.44	ATP synthase subunit alpha
Q072J9	14	13	3.75	54216.89	Glutathione reductase
Q43685	15	3	1.55	35310.92	Chitinase class 1 (Fragment)
O24548	17	17	3.02	40106.6	Type IIIa membrane protein cp-wap13
Q5NT85	18	18	1.92	50561.35	Apyrase
A0A0A0U6G1	19	2	2.12	32636.74	Class I chitinase (Fragment)
P93700	20	20	1.69	35932.91	CPRD14 protein
Q41712	20	20	4.47	27090.71	Cytosolic ascorbate peroxidase
Q9SXX8	20	13	2.29	16402.53	Cowpea pathogenesis-related protein 3 (CpPR3)

Table 4.19 Changes in protein fold (negative) in treated condition (tolerant vs susceptible genotype)

Accession	Peptide count	Unique peptides	Max fold change	Mass	Description
A0A151FEB1	1	1	Infinity	10688	Uncharacterized protein
P05045	1	1	3.35	29405.97	Seed lectin subunit I
Q43686	1	1	426.65	27731.79	Chitinase class 4 (Fragment)
A0A151FJP2	1	1	129.43	11274.73	Uncharacterized protein
A0A151FUI8	2	2	4.73	16910.32	Uncharacterized protein
A0A151FEB0	2	2	58.65	13352.89	Calcium-binding protein
A0A151FRR1	2	2	3.45	7182.946	Uncharacterized protein
A0A151FX57	3	3	2.76	20245.44	Peptide synthetase
Q43683	3	3	33.57	26668.31	Acidic chitinase class 3
A0A0K1R1I3	8	1	1.58	17154.42	ATP synthase beta subunit

CHAPTER- 5

DISCUSSION

Chapter- 5

Discussion

The present investigation entitled "Ionome and Proteome Assisted Characterisation of Aluminium Tolerance in Cowpea [*Vigna unguiculata* (L.) Walp.]" was carried out at Department of Horticulture, Sikkim University, Gangtok, Sikkim to screen the fifteen cowpea genotypes for their aluminium tolerance and to find out the ions and proteins responsible for the aluminium tolerance in cowpea. In this chapter, the findings of the present investigation along with the results obtained have been discussed under the following heads:

i) Screening of cowpea genotypes against Al stress based on physiological parameters

ii) Ionome profiling of the cowpea genotypes for aluminium tolerance

iii) Proteome profiling of the cowpea genotypes for aluminium tolerance

5.1 Screening of cowpea genotypes against Al stress based on physiological parameters

The present study examined the variation among cowpea genotypes at different aluminium toxicity levels for their suitability to become potential genotypes in offering best possible performance under acid stress soil condition as well as the effect of aluminium stress on the physiological traits of cowpea genotypes. We found substantial variation among the genotypes for various plant characteristics.

5.1.1 Plant height at weekly interval

At the fifth week of growth, plants of G13 (62.69 cm) were the tallest, followed by G15 (57.71 cm) and G12 (56.52 cm) and were significantly at par with

G13. Aluminium treatments influenced growth of cowpea plants and early growth of untreated plants expressed shorter height as compared to treated one (1st two weeks). Further, at third, fourth and fifth week of growth, effect of aluminium concentration became non-significant.

While observing the interaction effect of the aluminium and cowpea genotypes it was found that plant height at first and second week of growth was highest at A20 in most of the genotypes which decreased at A50 and A100. Variation in height may also be due to differential level of aluminium tolerance potential of the genotype which was an indication of diversity in cowpea germplasm. At fifth week of growth, plant height was at par with all level of aluminium concentration in most of the genotype. Stimulatory and inhibitory roles were obvious at successive growth periods. George and Carolyn (2002) reported similar stimulatory effects of Al on the growth of sugar maple seedlings.

Present findings of aluminium response to cowpea genotypes are in line with the report of Akinrinde *et al.,* (2004) who observed no genotypic difference in cowpea response to 20 μM Al treatment though there was strong Al induced inhibition of growth in two genotypes Epace 10 and Santo Inacio. Present observation were also in agreement of Thornton *et al.,* (1989) who observed enhanced growth of sugar maple seedling by low levels of aluminium (2.7 and 13.5 mg l^{-1}) and inhibited growth at higher level >27 mg l^{-1}. A stimulatory effect of Al has been attributed to alleviation of hydrogen ion toxicity (Kinraide, 1993 and Kinraide, 1997) and stimulation of iron and phosphorus uptakes (Foy, 1984). In peanut, root and shoot growth were enhanced at Al concentrations in the nutrient solution between 49 and 20.4 μM due to reduced Zn

uptake and shoot Zn concentrations which were in the toxic range in plants without Al supply (Asher, 1991).

5.1.2 Root length

There was significant difference between genotypes and aluminium treatments. Root length was found highest in genotype G13 (23.24 cm) followed by G6 (21.50 cm). It showed that these genotypes tolerated higher toxicity level of aluminium and identified as promising genotypes for breeding programme of developing aluminium tolerant genotype. Root length was found longer at lowest concentration of aluminium than control *i.e.* maximum at 25 µM followed by at control level and shorter root length at A50 and A100 than control, suggesting that the experimental genotypes may have capacity to tolerate least aluminium toxicity by expanding the absorption area for more nutrients. However, at higher concentration it was reduced due to the inhibitory effect of aluminium. The interaction effect between genotypes and aluminium treatment on root length was non-significant. However, findings of present experiment was contradictory to the report of Rangel *et al.,* (2007) and Choudhary *et al.,* (2011) who observed root inhibition in aluminium treated common bean and pigeon pea respectively even at low concentration in hydroponic assays.

But in many plant species, it was reported that there was growth stimulation at lower aluminium concentration like tea, sugar beet, maize and some tropical legumes (Broadley *et al*., 2012). Root elongation might be due to the amelioration of proton toxicity in roots because the beneficial effect of aluminium was usually observed at low pH (Kinraide, 1993) which was quite reverse of increase in aluminium toxicity by higher H^+ concentration.

5.1.3 Biomass

Biomass was significantly affected by genotype, aluminium treatment and their interaction effect. In most of the genotypes biomass was found to be significantly greater at lowest aluminium concentration than control which decreased (at A50 and A100) on increasing aluminium toxicity. It may be due to that genotypes had the capacity to yield higher biomass at moderate aluminium stress condition (Kushwaha *et al.*, 2017, Akinrinde *and* Neumann, 2006 and Ezeh *et al.,* 2007). Further, in this experiment, it was reported that the genotypes produced higher root length at A25. Higher root length would have contributed for the higher biomass. Similar findings were reported by Akinrinde and Neumann (2006) and Ezeh *et al.*, (2007).

5.1.4 Dry matter per plant

Dry matter per plant for root and shoot was found significant for genotype, aluminium and their interaction effect. Genotype G13 showed highest dry matter for root and shoot, an indication of better tolerance for aluminium toxicity than other genotypes in this study. There was detrimental effect of aluminium treatment on dry matter but highest dry matter irrespective of genotypes was found at 25 µM which was at par to untreated plant (at control). The findings of present study support those of Macedo *et al.*, (1997), as the weight measurements were significant at the genotype, aluminium and genotype x aluminium treatment levels. The higher dry matter at A25 and lower at A50 and A100 across all genotypes showed that the damaging effect of aluminium on the dry matter was at higher levels. Macedo *et al.,* (1997) reported weight parameters to be better than length measurements for distinguishing toxicity thresholds for screening genotypes of rice in short-term experiments (less than 40 days). In interaction effect G5 at 25 µM for root dry matter and G5 at control and 25

µM ppm for shoot dry matter were performing better. Foy *et al.,* (1993) also found higher root and shoot dry matter at control and low level of aluminium concentration.

5.2 Principal component analysis and Cluster analysis

Principal component analysis is the most useful statistical tool for screening multivariate data with significantly high correlations. In this study, PCA was done to identify parameters that suited best to discriminate cultivars for acid tolerance using the derived eigenvectors and it helped to assess the grouping patterns caused by perceptive variables.

From the PC analysis it was observed that BM and RL at all level of aluminium treatment had higher values for PC1 and PC2. These characters are deciding factor for determination of aluminium tolerance and susceptibility (Macuha and Rychtarik, 1999). According to Delhaize and Ryan (1995) and Kochian (1995), the major aluminium toxicity symptom observed in plants was inhibition of root growth which in turn also contributed to lower biomass of the plant. Kuswantoro *et al*., (2010) reported that acid soil effects showed different root growth responses, where the tolerant genotypes had higher root growth responses and susceptible genotypes had suppression on root growth. Root length in response to Al stress in acid affected soil has been used to access Al tolerance of sorghum genotypes (Ohki, 1987), wheat (Kerridge *et al*., 1971), soybean (Hanson and Kamprath, 1979) and many other legumes (Edmeades *et al*., 1991; Mackay *et al*., 1991).

In all the treatments of aluminium, Genotype G13 showed the highest value of PC1 and PC2 except at control where it showed moderate value for PC2. Hence, G13 was found to be most tolerant among all the genotypes for aluminium tolerance. Genotypes G5 and G15 showed decreasing PC values from control to 25 µM then

increased at 50 µM and again decreased at 100 µM. This meant that this genotype can tolerate aluminium at 50 µM but it showed susceptibility at lower and higher level of aluminium. While G12 showed higher PC values at medium aluminium concentrations but lower values at control and highest (A100) concentration which indicated that this genotype could perform better in slight acidic condition. This result was in conformity with Ezeh *et al*., (2007) who also found that there was better performance of some genotypes at the slight acidic condition. Lower PC values at control, 50 and 100 µM and slightly higher value at 25 µM of G7 clearly showed that this genotype was susceptible to all the levels of aluminium treatment. Genotypes G6 and G11 had a very little deviation in their position across all the treatment which indicated their moderate tolerance towards aluminium. Hence, based on our observation G13 was most tolerant and G7 was most susceptible genotype across fifteen cowpea genotypes tested. All the fifteen genotypes could be categorised into four groups according to their performance towards aluminium tolerance.

1. Very susceptible: G7 and G9.
2. Susceptible: G1, G8, G3, G4, G10.
3. Tolerant: G11, G2, G6, G12, G14, G15.
4. Highly tolerant: G13 and G5.

5.3 Ionomic profiling for aluminium tolerance

Soil chemical factors that limit root growth in acid soils and decrease crop production include Al, Mn and various cations, and also deficiency or unavailability of Ca, Mg, P, Mo, and Si. Whereas, Mn and B could be toxic to plants. These effects are further complicated by interactions of Al with other ions in different plant genotypes under aluminium stress conditions (Foy, 1992). It is generally known that plants grown

in acid soils reduce root growth and nutrient uptake. The bio-availability of some heavy metals in soil is also strongly affected by pH.

Hence, to ascertain the effect of Al toxicity, cowpea genotypes were grown in different level of aluminium stress condition which were induced by addition of aluminium sulphate in nutrient media at different concentrations. In this study, we tried to determine the ionomic response of shoot tissues of cowpea genotypes to aluminium tolerance at different level of aluminium toxicity. Because ionomics is one the of high throughput technology which paves the way to capture information precisely, about the functional state of an organism under changed conditions. These conditions may either be determined by genetic differences, developmental differences, as well as the environmental effects including biotic and abiotic factors, since, most of the elements except carbon and oxygen, is acquired from soil by the plants (Baxter and Dilkes, 2012). The elements of interest for an ionomic study of plant samples can be divided into essential macronutrients (K, Mg, Ca), essential micronutrients (Mo, Cu, Zn, Mn, B) beneficial elements (Ni, Na, Co, Al, Ba) and others (Li, Ag, Rb, Be, Pb). Principal component analysis grouped the cultivars in to different clusters based on contributed variables.

In the present investigation PCA analysis was done after auto-scaling the data of 18 elemental parameters of fifteen cowpea genotypes. Principal component analysis (PCA) is a nonsupervised dimension reduction method. It attempts to select a small number of orthogonal coordinates (expressed as linear combinations of inputs) to maximize the overall explained variation in the data, regardless of class labels (Pearson, 1901; Johnson and Wichern, 2007). Results obtained from principal

component analysis on the correlation matrix of the traits reduce the dimensionality of the data set.

In the present study, potassium was having positive value for both PC1 and PC2 at A25 and A50. Whereas, it had positive value for PC1 and negative for PC2 at control and A100. Magnesium was having positive value for both PC1 and PC2 except at the highest level of aluminium concentration. Ca, Mn and B were having positive value for both PC1 and PC2 at control and highest concentration of aluminium while, negative value for PC2 and positive for PC1 at A25 and A50 concentration of aluminium. Element Cu showed positive PC1 at all treatments except control. Elements Zn and Na almost did not change their relative position at all the treatments which showed that their concentration was not affected so much due to presence of aluminium toxicity. Cobalt and Nickel were having positive PC1 and negative PC2 value in all treatments. Barium was possessing positive value for PC1 and PC2 except at A25 where, it had negative PC2 value. Mo maintained its position with both positive PC1 and PC2 except at A50 where it showed positive PC1 but negative PC2 value.

Furthur, at all level of the treatments element Be was having negative value for PC1 except at control. Pb and Ag clustered together except at control. Element Rb was possessing positive PC1 at all aluminium toxicity levels except A50. Similarly, Lithium had positive PC1 and PC2 value at all treatments except at A100 where it had positive PC1 and negative PC2 value.

Macro and microelements having positive PC1 in all the treatments which showed that other trace, beneficial and toxic elements were influenced more in case of aluminium stress. So, it was identified that, though the essential macro and micro

elements play a role in Al tolerance, it was other trace, beneficial and toxic elements play key role in changing the ionome profiling of cowpea genotypes.

In addition, comparing the tolerant and susceptible genotypes principal component 1 clearly separated tolerant and susceptible genotypes and PC2 separated control from treated one in tolerant genotype. Elements Ba, Pb, Al, Co, Na and were found to possess more concentration in tolerant genotype under aluminium stress compared to control. Whereas, moderate concentration of Co, K, Mg, Ni, Mo, Rb and Zn and lower concentration of Ag, Be, Ca, Cu, Li, Mn and B was observed under same condition. While, comparing susceptible genotype in stress condition with control Pb, Ag, Ca, Cu, K, Li, Mg, Mn, Na, Rb, B and Zn were in lower concentration and Ba, Be, Co, Ni, and Mo were in moderate condition. Only Al was found significantly higher than the control in treated condition. It clearly showed the tolerant genotype accumulated higher concentration of majority of the elements than the susceptible genotype which may be the responsible factor for good growth and development of tolerant genotype.

The three elements Calcium, magnesium and manganese, were important element in respect of aluminium toxicity. Ca concertation decreased in treated condition compared to control in both susceptible and tolerant genotypes but reduction percentage was more in case of susceptible genotype. So, the less reduction in accumulation of calcium elements in tolerant genotype contributed to the tolerance of tolerant genotype because calcium is well known for its ability to relieve Al toxicity (Alva *et al*., 1986). Aluminium tolerance in certain cultivars of wheat, barley, soybean and snapbean had been associated with the ability to resist Al-induced Ca deficiency (Foy *et al*., 1978). Aluminium is found to reduce Ca uptake in different plants and

reduces the retention of the Calcium in roots and tops (Fitter & Hay, 1981; Foy, 1974). Calcium treatment increased soil pH and ameliorated Al toxicity in peanut and cowpea (Chong *et al*., 1987). Ca was able to lower Al toxicity only when present in considerably higher concentrations than Al in the medium (Wagatsuma, 1983). It didn't, however, affect the rate of uptake of Al, which was related to the activity of $A1^{3+}$ in medium (Pavan, 1982). This was evident in our result where tolerant germplasm could accumulate more calcium and reduced the effect of Al. It might be the one of the reason for lesser reduction in biomass and root length of tolerant genotype in compared to susceptible genotype.

Similar to Calcium, magnesium uptake also decreased with the increase in aluminium concentration. In our study, magnesium concentration was not much influenced (1.93% increased) in case of tolerant genotype but, it was reduced up to 40% in case of susceptible genotype compared to the control. This we found as another reason for tolerance towards aluminium toxicity. Many studies reported that magnesium concentration and uptake decreased with increasing Al levels (1-10 ppm) in roots and tops of rice (Alam, 1983; Sarkunan & Biddappa, 1982), *Coffea arabica* (Pavan & Bingham, 1982), *Manihot esculenta* (De Carvalho & Cesar, 1984), potato (Lee, 1971), *Zea mays* (Gerzabek & Edelbauer, 1986), Lupinus, Secale, Vicia, and Hordeum (Horst & Goppel, 1986). Similar trend was observed in susceptible genotype in the present study, which explains the one of the reason for its susceptibility. Simon *et al*., (1994) and Lidon *et al*., (2000) reported that many plant species grown in high Al level usually had lower Ca and Mg concentrations. Our result also proved that higher Al level decreased Ca and Mg uptake by the plants. But the reduction was less in tolerant genotype

Manganese (Mn) is an essential element for plants and is involved in regulating several metabolic processes, such as photosynthesis and antioxidant activity (Marschner, 1995). In the present study aluminium treatments reduced the Mn concentration of both susceptible and tolerant genotypes compared to control. Previous reports have indicated that Al can reduce Mn accumulation in cowpea (Taylor *et al.*, 1998) and soybean (*Glycine max*) (Yang *et al.*, 2009). One explanation for this alleviation was that Al may have an antagonistic effect on Mn uptake by plant roots. However, the exact mechanism is still poorly understood (Wang *et al.,* 2015). Our result also showed the reduction in accumulation and uptake of Mn in both genotypes compared to control.

Foy, (1984) reported that aluminium tolerance has been associated with greater uptake of K in potato cultivars. In our study, K concentration decreased in the susceptible genotype compared to control due to competition of aluminium with K for root absorption site (Alam, 1983) which resulted in less K uptake with consequent dose dependent decrease in K content in plant. But, tolerant genotype was found to had less reduction of K in treated condition compared to susceptible genotype contributing for tolerance towards aluminium toxicity.

Further, Cu concentration decreased in both susceptible and tolerant genotype of cowpea in this study. Similar to K, aluminium had to compete with Cu for the same binding sites at/or near the root surface (Hiatt *et al*., 1963) and its injury caused accumulation of Cu in root tissues of potato (Lee, 1971), reduced Cu content in both roots and tops of Sorghum (Cambraia *et al*., 1983). In case of Cu also the reduction percentage was more in susceptible genotype (95%) than tolerant genotype (50%) in

comparison with control. The lesser reduction may be the one of reason for tolerance of tolerant genotype.

Other elements like B and Ag also play role in toxicity tolerance. Boron was found to be decreased in both the susceptible and tolerant genotypes compared to control. Boron affects the uptake of calcium (Djingova *et al.*, 2013). Our result supported this phenomenon as decrease in uptake of B decreased the uptake of Ca both in susceptible and tolerant genotype. But, compared to tolerant one there was less reduction in concentration of boron in susceptible genotype. Al was likely to be present as $Al(OH)_3$, which was structurally similar to $B(OH)_3$ which might be the reason for less uptake of boron in shoot and leaves and caused the less growth and development in case of susceptible genotype. Likewise, Ag was also found to be reduced in both susceptible and tolerant genotype in comparison to control, but contrary to boron it was reduced more in case of susceptible genotype compared to tolerant genotype. Element Ag interacts metabolically with Cu and changes the structure of the photosynthetic enzymes (Djingova *et al.*, 2013) which in turn reduces the photosynthesis. But, in our study there was higher concentration of Ag in tolerant genotypes than susceptible genotype and also higher biomass in case of tolerant genotype than susceptible genotype which was contradictory to the earlier report. So, the role of Ag in case of aluminium toxicity should be further investigated.

Djingova *et al.*, (2013) reported that Co is involved in increasing the growth of legume plant. In our study Co concentration increased in tolerant genotype and decreased in susceptible genotype in comparison to control that pointed towards the reason for susceptibility of susceptible genotypes. Contrary to the cobalt, beryllium was decreased in resistant variety but it was at par in susceptible variety in comparison

to control. As it was reported that Be inhibit the Ca and Mg uptake by roots (Djingova *et al.,* 2013) which supports our result of more reduction in uptake of Ca and Mg in susceptible genotype than tolerant genotype.

Apart from above discussed elements, Zn, Mo, Ni and Li were found to be reduced in both the susceptible and tolerant genotype in treated condition in comparison with control. Other elements like Ba, Pb, Na and Rb were found to be increased in tolerant genotype but decreased in case of susceptible genotype in comparison to control in treated condition. Djingova *et al.,* (2013) reported that Ba is analogous to Ca which interferes with the Ca nutrition and Na and Rb competes with the uptake of K (Djingova *et al.,* 2013). According to this report Ca and K uptake should have to decrease more in tolerant genotype than the susceptible genotype in treated condition. But in our study, opposite trend was observed which necessitates further study on role of these elements.

Based on the observed results and above made discussion it was concluded that the concentration of Ca, Mg, K and Cu as well as concentration of cobalt in tolerant genotype in treated condition compared to control was responsible for the aluminium stress tolerance in the tolerant genotype of cowpea. Besides above elements, Mn, B, Ag, Be, Ba, Na and Rb were also important in governing the tolerance towards aluminum toxicity as they influence the uptake of other elements.

5.4 Protein profiling of tolerant and susceptible genotypes

Protein profiling was done for tolerant and susceptible genotypes in control and treated condition, in which 85 prominent proteins each were profiled. In case of susceptible genotype, out of total of 85 proteins, 48 proteins were significant based on P-value. Out of 48 proteins, 21 were having more than 1.5 fold changes. 11 were

upregulated with fold changes of 1.54-3.74 in treated condition and 10 were downregulated with fold changes 1.57-6.07. While in tolerant genotype, out of total 85 proteins, 42 proteins were found significant. In 42 proteins, 27 proteins were having fold changes >1.5. 20 upregulated proteins had fold change between 1.50 -3.69 and 7 downregulated proteins had fold changes between 1.53 -324.94.

While comparing tolerant genotype with susceptible genotype in control condition, out of total 85 proteins identified, 47 were found significant and in that 36 proteins were found to be with more than 1.5 fold increase. In that, 23 were found more in tolerant genotype than susceptible genotype in control and were having 1.56-3.49 fold changes. On the other hand, 10 were found more in susceptible genotype than tolerant genotype in control and were having 1.76 to infinite fold changes. Similarly, in treated condition, a total 85 protein were observed in susceptible and tolerant genotype. Among that, 59 proteins were found significant in which 38 proteins were having more than 1.5 fold change. 28 proteins were found more in tolerant genotype than susceptible genotype in treated condition having 1.55-26.51 fold changes and 10 proteins were found more in susceptible genotype than tolerant genotype in treated condition having 1.58 to infinity fold changes.

A total of 29 types of protein were found in susceptible and tolerant genotypes. These protein were peptide synthetase, acidic chitinase class 3, chitinase class 1, chitinase class 4, ATP synthase beta subunit, ATP synthetase alpha subunit, CPRD 14, Ca-binding protein, 2Fe-2S-binding protein, amidohydrolase, glutamine synthetase, type IIIa membrane protein cp-wap 13, apyrase, RNA dependent RNA polymerase, polyketide cyclase, DNA ligase, ribosome recycling factor, putative cathepsin B-like cysteine protease putative, asparatic proteinase, 2-cys-peroxiedoxin, 17.7kDa class I

small heat shock, glutathione reductase, cytosolic ascorbate peroxidase, cowpea pathogenesis-related protein 3, seed lectin, MarR family transcriptional regulator, AraC family transcriptional regulator, acyl-CoA dehydrogenase and leghaemoglobin reductase. These proteins were classified in following categories based on their functional annotations:

Oxidoreductase activity: leghaemoglobin reductase, 2-cys-peroxiredoxin, Glutathione reductase, Cytosolic ascorbate peroxidase, Acyl-CoA dehydrogenase

Hydrolase actitivity: Apyrase, Acidic chitinase class 3, Putative cathepsin B-like cysteine protease, Aspartic proteinase, Amidohydrolase

Stress and pathogenesis related protein: 17.7kDa class I small heat shock, cowpea pathogenesis-related protein 3, CPRD 14

Binding proteins: Calcium binding, chitinase class 1, chitinase class 4, glutamine synthetase, 2Fe-2S-binding protein, seed lectin, peptide synthetase

Transmembrane and transport activity: ATP synthase beta subunit, ATP synthase subunit alpha, type IIIa membrane protein cp-wap13, RNA dependent RNA polymerase, ribosome recycling factor DNA ligase, AraC family transcriptional regulator, MarR family transcriptional regulator,

Others: polyketide cyclase

5.4.1 Oxidoreductase activity related protein

Proteins having oxidoreductase activity were having catalytic activity in the plants which saves the plant from oxidative damages. Most of the protein related to this category were upregulated in tolerant genotype in our study. Leghaemoglobin reductase protein was found to be more in the tolerant genotype in treated condition in

comparison with susceptible one by 1.76 fold. Leghaemoglobin reductase reduces ferric leghaemoglobin to ferrous leghaemoglobin which is part of nitrogen fixation activity and this process is also pH dependent (Becana and Klucas, 1990). Optimum pH for this protein is 6.5. At lower pH where acidic condition prevails and aluminium toxicity also present, this protein expresses less. In our case, it was found more in treated condition in tolerant genotype which indicated that in tolerant genotype under Al stress environment it fixed more N and in turn imparted tolerance towards aluminium toxicity.

2-sys-peroxiredoxin was found to upregulated by 1.55 fold in tolerant genotype in treated condition than control and found higher by 1.80 fold in tolerant genotype from susceptible genotype in treated condition. 2-cys-peroxiredoxin is antioxidant enzyme which protects photosynthetic membrane from photo oxidative damages and also reduces hydrogen peroxide to water (Dietz *et al*., 2002). The reduction of peroxides saves the cells from oxidative damages which is prominent in case of aluminium toxicity. In our case the upregulation of 2-cys-peroxiredoxin in tolerant genotype contributed for the Al tolerance.

Glutathione reductase was upregulated by 1.79 fold in tolerant genotype in treated condition than the control. In treated condition it was found in higher amount by 3.75 fold in tolerant genotype in comparison with susceptible genotype. It showed that the susceptible was less expressing the glutathione reductase enzyme. The importance of maintaining high levels of glutathione for Al tolerance was observed in the Arabidopsis root system, in a comparative study between wild-type and transgenic lines over expressing glutathione reductase (GR) (Yin *et al.* 2010, 2017). The glutathione reductase over expressing lines contained higher levels of glutathione, which showed suppressed reactive oxygen species (ROS) (H_2O_2) production and lipid

peroxidation, and increased Al tolerance. In our study, glutathione reductase was found more by 3.75 fold in tolerant genotype than susceptible genotype in treated condition which in turn produced more glutathione in cells. Enhanced production of glutathione reductase reduced the ROS production and lipid peroxidation which were the main damaging factor in case of aluminium toxicity. So, higher expression of glutathione reductase was the one of the main factor for tolerance towards aluminium toxicity in case of tolerant genotype. In control condition also the expression of this enzyme was higher by 1.68 fold in tolerant genotype than susceptible genotype which showed that tolerant genotype generally had the potential to produce more glutathione.

Similar to glutathione reductase, cytosolic ascorbate peroxidase is also an antioxidant enzyme which was upregulated by 1.95 fold in tolerant genotype in treated condition than control. It was found higher by 4.47 fold in tolerant genotype than susceptible genotype in treated condition. In control also this protein was found more by 1.69 fold than susceptible genotype in tolerant genotype. A major hydrogen peroxide detoxifying system in plant cells is the ascorbate-glutathione cycle, in which, ascorbate peroxidase (APX) enzymes play a key role in catalysing the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor. Different APX isoforms are present in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisome, and cytosol (Caverzan *et al*., 2012). The ROS detoxification process in plants is essential for the protection of plant cells and their organelles against the toxic effect of ROS species (Apel and Hirt, 2004; Mittler, 2002). The upregulation of this cytosolic ascorbate peroxides in tolerant genotype than susceptible genotype both in treated and control condition caused more detoxification by conversion of hydrogen peroxide in to water and protected the cells from aluminium injury.

5.4.2 Hydrolase activity related protein

Apyrase protein was upregulated by two fold in tolerant genotype and downregulated by 2.18 fold in case of susceptible genotype in treated condition than control. In treated condition it was found more by 1.92 fold in tolerant genotype than susceptible genotype and in control condition reduced by 2.27 fold. Plant cells release ATP into their extracellular matrix as they grow, and extracellular ATP (eATP) can modulate the rate of cell growth in diverse tissues. Two closely related apyrases (APYs) in Arabidopsis (*Arabidopsis thaliana*), APY1 and APY2, function, in part, to control the concentration of eATP. The expression of APY1/APY2 can be inhibited by RNA interference, and this suppression leads to an increase in the concentration of eATP in the extracellular medium and severely reduces growth. In this study, the downregulation of apyrase protein in case of susceptible genotype was possible reason for lesser root length and biomass in stress condition.

Acidic chitinase III (Ac3) was upregulated in case of susceptible genotype in treated condition than control. Ac3 acts as storage protein (vegetative storage protein), induced in response to salicylic acid production, hydrolase activity, carbohydrate metabolism, lysosomal activity and expressed in case of environmental stress and heavy metal stress. Chitinase Class III gene was expressed exclusively when the plants were exposed to environmental stresses, especially salt and wound stresses (Grover, 2012). The upregulation of this protein was also reported by Duressa *et al.,* (2010) in case of Al tolerant soybean lines. But in our study this protein was upregulated only in susceptible genotype which needed further investigation.

Putative cathepsin B- like cysteine protease (CBCP) was upregulated in tolerant genotype in treated condition than control by 1.50 fold. In treated condition, compared with susceptible genotype it was found higher in tolerant genotype by 1.88

fold. Cysteine proteases are involved in almost all aspects of plant growth and development including germination, circadian rhythms, senescence and programmed cell death. They also involved in mediating plant cell responses to environmental stress (such as water stress, salinity, low temperature, wounding, ethylene, and oxidative conditions) and plant-microbe interactions (including nodulation) (Sheokand and Brewin, 2003). The increased growth of tolerant genotype than susceptible genotype was possibly due to high expression of CBCP.

5.4.3 Stress and pathogenesis related protein

CPRD 14 was upregulated by 2.19 fold in case of tolerant genotype and by 2.29 fold in susceptible genotype in treated condition than control. It was found more by 1.69 fold in tolerant than susceptible genotype in treated condition. CPRD 14 protein is a stress related protein which induces ABA synthesis, having catalytic activity and coenzyme binding activity. The upregulation of this protein in tolerant genotype conferred the tolerance towards the aluminium toxicity.

Pathogenesis related (PR) proteins were upregulated by 1.55 fold in tolerant genotype in treated condition than control. It was found more by 2.29 fold in tolerant genotype than susceptible genotype in treated condition and by 1.82 fold in control condition. PR proteins induction was related to the presence of plant signalling-molecules, e.g. ethylene, abscisic acid and salicylic acid. In general, PR-like proteins were reported to be induced by several abiotic stresses, e.g. ozone, UV irradiation, heat stress, wounding, heavy metals and during salt and cold adaptation (Fecht-Christoffers *et al*., 2003a). The upregulation of PR protein in case of tolerant genotype in all conditions showed that it's upregulation imparted the tolerance towards aluminium toxicity. Like PR proteins, CPRD 14 and 17.7kDa class I small heat shock

proteins were also involved in abiotic stress responses. They were upregulated in both tolerant genotype and susceptible genotype in treated condition than control. But they were found more in case of tolerant genotype than susceptible genotype in treated condition by 1.69 and 3.64 fold, respectively. It proved their induction was due to response towards aluminium stress and imparted tolerance to tolerant genotype.

5.4.4 Binding protein

Glutamine synthetase was found more by 1.90 fold in tolerant genotype than susceptible genotype in treated condition and downregulated by 1.59 fold in susceptible genotype in treated condition than control. Glutamine synthetase is the key enzyme of primary N assimilation, as well as ammonia reassimilation and detoxification. Al^{3+} taken up by roots from the acidic nutrient solution can reach the leaf cells and there it can increase the glutamine synthetase activity *in vivo*. This activation is because of specific binding of aluminium to the polypeptide chain of GS2, however, presence of magnesium at least on one of the metal-binding sites is essential to the active state of the enzyme (Pécsváradi *et al*., 2009). In the present study, there was higher expression of glutamine synthetase in tolerant genotype, which supported the finding that aluminium stress increased the glutamine synthetase activity and its more activity imparted aluminium toxicity tolerance in tolerant genotype.

Chitinase class I protein were upregulated by 3.69 and 1.76 fold in case of tolerant and susceptible genotype than control, respectively. Chitinase Class I were induced by ethylene or jasmonate pathway and associated with stress and wound response in plants (Kasprzewska, 2003). As chitinase class I proteins were upregulated in our study supported these findings and more induction in case of

tolerant genotype also proved their importance towards tolerance of tolerant genotype in aluminium toxicity.

Calcium binding protein was downregulated by 6.23 and 6.07 fold in tolerant and susceptible genotype than control, respectively. But its reduced concentration was more in case of treated condition (58.65 fold) than control condition (26.21 fold) in tolerant genotype than susceptible genotype. Calcium binding protein decrease in stress condition affects citrate metabolism and their less presence may release Ca^{2+} in cell and in turn they induce the genes responsible for the synthesis of citric acid which is involved in aluminium tolerance (Jiang *et al.,* 2018). The downregulation of this protein was also reported by Duressa *et al.,* (2010) and Zhou *et al.,* (2016) in case of Al tolerant soybean lines.

Seed lectin protein was downregulated in tolerant genotype by 1.87 fold than control in treated condition. Seed lectin protein is a metalloglycoprotein containing Ca, Mg, Mn, Zn and the carbohydrate galactose, glucosamine, mannose and fucose and having role in carbohydrate binding. This protein was downregulated in this study which was possibly due to reduction in concentration of the Ca, Mg, Mn and Zn.

5.4.5 Transmembrane and transport related protein

Type IIIa membrane protein cp-wap 13 was found more in tolerant genotype compared with susceptible genotype by 3.02 fold in treated condition while, downregulated by 1.57 fold in susceptible genotype in treated condition than control. Its higher expression in tolerant genotype and downregulation in susceptible genotype showed that this protein was involved in providing tolerance towards aluminium toxicity in tolerant genotype. ATP synthetase beta subunit was upregulated in case of tolerant and susceptible genotype in comparison with control. However, alpha subunit

was found more in tolerant genotype than susceptible genotype only in treated condition. RNA dependent RNA polymerase and ribosome recycling factor both were upregulated in tolerant genotype in treated condition than control. Their expression was more in tolerant genotype than susceptible genotype in both, treated and controlled condition. It showed that both of these proteins were playing important role towards aluminium tolerance in tolerant genotype.

Hence, based on the above discussion it may be concluded that various proteins related to oxidoreductase activity, hydrolase activity, stress and pathogenesis related, binding proteins, transmembrane and transport proteins were involved in tolerance towards aluminium toxicity in tolerant genotype.

5.5 Correlation of the morpho-physiological characters, ionome and proteome profiling of cowpea germplasm lines grown in aluminium stress condition.

- Aluminium in general caused the deficiency of Ca, Mg, B and Mo. Ca and Mg decreased both in tolerant and susceptible genotype but Ca and Mg concentration were reduced lesser in tolerant genotype than susceptible genotype. This trend explained more production of photo-synthates and increased root growth that was the case with tolerant genotype.

- Decreased Ca, K, B, Zn and Cu were the prime factor for toxicity effect (inhibition of root growth and low biomass) of aluminium in cowpea genotypes.

- Proteomics result showed downregulation of seed lectin protein in tolerant genotype in treated condition than control which is a metalloglycoprotein

containing Mn, Mg, Ca and Zn. These elements were also reduced in treated condition than control in tolerant genotype.

- Decrease in Ca caused the stunting of all the plant structure. Ca concentration decreased in both the genotypes compared to control. But, its reduction was more in case of susceptible genotypes. Ca being an element needed for binding with calmodulin protein its less reduction in tolerant genotype in treated condition explained the less reduction in growth parameters and biomass in tolerant genotype than susceptible genotype due to Al toxicity.
- Increased Al content activated the glutamine synthetase, which was upregulated in our study. It might have caused synthesis of glutamine which was important for plant growth and development and in turn caused the better tolerance towards aluminium tolerance.
- Glutamine synthetase activity requires Mg which was increased in case of tolerant genotype in treated condition than control. Mg in combination with glutamine synthetase might have produced more glutamine. Glutamine could have contributed for nitrogen metabolism and in turn supported growth and development which was evident from more biomass accumulation and root length in case of tolerant genotype.

CHAPTER- 6

SUMMARY AND CONCLUSION

Chapter-6

Summary and Conclusion

Legumes are the inseparable constituent in horticulture. Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important legume vegetables and an essential component of cropping system. Due to fast growing nature of the crop, it can controls erosion by covering the ground, fixes atmospheric nitrogen at faster rate, and its decomposing residues can contribute to the soil fertility.

In India, soil acidity affected 49 million hectares of area and 25 million hectare of area has pH below 5.5. About 21 million hectares of acid soil are present in North Eastern Region. In these states, soil acidity, in general, and sub-soil acidity in particular, is major limiting factor for low productivity of soils. Acid soils are mainly associated with regions of high rainfall leading to excessive base (Ca^{++}, Mg^{++} and K^{+}) leaching. The exchange complex is predominantly occupied by Al^{+++} and is released due to weathering of alumina silicate clay minerals. There is growing interest in addition of vegetable legumes in developed cropping systems on acid soils. The vegetable legumes inclusion will depends upon the mitigation of Al toxicity constraint. Keeping these points in consideration, present experiment has been planned to screen the cowpea genotypes for aluminium toxicity.

The present investigation entitled "Ionome and proteome assisted characterisation of aluminium tolerance in Cowpea [*Vigna unguiculata* (L.) Walp.]" was carried out at Department of Horticulture, School of Life Sciences, Sikkim University, Gangtok, Sikkim with the objectives of screening of cowpea genotypes for

aluminium tolerance followed by their ionome and proteome profiling in order to find out the responsible ions and proteins for aluminium tolerance.

The study was carried out on fifteen cowpea genotypes collected from Indian Institute of Vegetable Science, Varanasi, Uttar Pradesh, India. Collected genotypes were grown in hydroponics system for screening towards the aluminium tolerance. Ionome profiling of leaves of all the treatments and root of susceptible and tolerant genotypes were performed by ICP-MS. Proteome profiling of susceptible and tolerant genotypes were performed by label free analysis in LC/MS/MS condition and protein characterisation were made from Uniprot database. The salient findings of this study are summarized below:

- Aluminium treatments influenced plant height of cowpea and in first two week of growth. The untreated plants expressed shorter height as compared to treated one. Further, at third, fourth and fifth week of growth, effect of aluminium concentration became non-significant.
- Root length was found longer at lowest concentration of aluminium *i.e.* maximum at 25 µM followed by at control level and shorter root length at A50 and A100 than control.
- Biomass was found to be higher at lowest aluminium concentration than control which decreased on increasing aluminium toxicity.
- Aluminium treatment caused detrimental effect on dry matter but highest dry matter irrespective of genotypes was found at 25 µM which was at par to untreated plant (at control).
- Based on the morphophysiological characters G13 was the most tolerant and G7 was the most susceptible across fifteen cowpea genotypes tested.

- PC analysis revealed that biomass and root length were deciding factor for determination of aluminium tolerance and susceptibility.

- All the fifteen genotypes could be categorised into four groups according to their performance towards aluminium tolerance.

  1. Very susceptible: G7 and G9.

  2. Susceptible: G1, G8, G3, G4, G10.

  3. Tolerant: G11, G2, G6, G12, G14, G15.

  4. Highly tolerant: G13 and G5.

- Essential macro and micro elements plays a role in Al tolerance, but other trace, beneficial and toxic elements play key role in changing the ionome profiling of cowpea genotypes in case of aluminium tolerance.
- Ca, Mg, K and Cu as well as concentration of cobalt in tolerant genotype was responsible for the aluminium stress tolerance in the tolerant genotype of cowpea.
- Mn, B, Ag, Be, Ba, Na and Rb were also found to be important in governing the tolerance towards aluminum toxicity as they influence the uptake of other elements.
- Upregulation of proteins related to oxidoreductase activity (glutathione reductase, leghaemoglobin reductase, 2-cys-peroxiredoxin, cytosolic ascorbate peroxidase), hydrolase activity (Apyrase, putative cathepsin B- like cysteine protease), stress and pathogenesis related proteins (CPRD 14, PR proteins, heat shock protein), binding proteins (glutamine synthetase, chitinase class I) in

tolerant genotype conferred the aluminium tolerance property of tolerant cowpea genotype.

- Downregulation of Ca binding protein in tolerant genotype also played a role in tolerance towards aluminium toxicity by inducing the synthesis of citric acid.
- Decreased concentration of Ca and Mg in genotypes were due to the aluminium toxicity and in turn this caused reduction in photosynthesis and growth of the plant which reflected in the form of reduced root length, biomass and dry matter in genotypes on aluminium exposure. But lesser reduction of Ca and slight increase in Mg in case of tolerant genotype contributed to the tolerance.

- Inhibition of root growth and low biomass were also due to decreased K, B, Zn and Cu because these elements were the important factor for toxicity effect of aluminium in cowpea genotypes.

- Decrease in concentration of Mn, Mg, Ca, and Zn also caused the downregulation of seed lectin protein.
- Slight increase in Mg concentration in combination with upregulated glutamine synthetase produced more glutamine, which supported growth and development to cause more biomass accumulation and root length in case of tolerant genotype.

Hence, it can be concluded that aluminium tolerance in the tolerant genotype is due to less reduction in root length, biomass and dry matter. These less reduction in plant characters were the result of less reduction in concentration of Ca, K and Cu, increased concentration of magnesium and cobalt as well as upregulation of stress and defence related proteins, oxidoreductase proteins, hydrolase proteins and binding proteins and downregulation of Ca-binding protein.

This information can form the basis for the aluminium toxicity tolerance mechanism for the cowpea in particular and legumes in general. Source of this information could be utilized in the crop improvement programmes both in conventional breeding and biotechnological approaches.

CHAPTER- 7

BIBLIOGRAPHY

Chapter-7

Bibliography

Abdel-Hady, M.S. 2006. *In vivo* and *in vitro* selection of superior durum wheat (*Triticum durum* Desf.) genotypes. Journal of Applied Science Research. 2:830-836.

Abebe, M. 2007. Nature and Management of Acid Soils in Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia.

Akinrinde, E.A. and Neumann, G. 2006. Evaluation of differences in tolerance to Al toxicity among some tropical cowpea genotypes. Pakistan Journal of Biological Science. 9(5):954-960.

Akinrinde, E.A., Iroh, L., Obigbesan, G., Hilger, T., Romheld, V. and Neumann, G. 2004. Tolerance to soil acidity in cowpea genotypes is differentially affected by the P-nutritional status. Paper presented at Annual Conference of Deutshe Gesellschaftfuer Pflanzenernahrung, Goettigen, September 1-3, 2004 and International congress Rhisosphere 2004 - Perspectives and challenges-A tribute to Lorenz Hiltner. Munich, Neuherberg, Germany. pp: 12-17.

Akundabweni, L.S.M, Mulokozi, G. and Maina, D.M. 2010. Ionomic variation characterization in African leafy vegetables for micronutrients using XRF and HPLC. African Journal of Food, Agriculture, Nutrition and Development. 10(11):4320-4339.

Alam, S.M. 1981. Influence of Aluminium on plant growth and mineral nutrition of barley. Communication Soil Science Plant Analysis. 12:121-138.

Alam, S.M. 1983. Effect of aluminum on the dry matter and mineral content of rice. J. Sci. Tech. 7(1-2):1-3.

Alva, A.K., Edwards, D.G., Asher, C.J. and Blamey, F.P.C. 1986. Effects of phosphorus/ aluminum molar ratio and calcium concentration of plant response to aluminum toxicity. J. Soil Sci. Soc. Amer. 50:133-137.

Anitzen, C.J. and Ritter, E.M. 1984. Encyclopaedia of Agriculture Science, Vol. 4 EW Index, Academic Press.

Apel, K. and Hirt, H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol. 55:373–399.

Ardini, F., Soggia, F., Abelmoschi, M.L., Magi, E and Grotti, M. 2013. Ionomic profiling of *Nicotiana langsdorffii* wild-type and mutant genotypes exposed to abiotic stresses. Anal Bioanal Chem. 405:665–677. DOI 10.1007/s00216-012-5997-4.

Asher, C.J. 1991. Beneficial elements, functional nutrients, and possible new essential elements. WI, USA: Soil Sci. Soc. Amer. Book Series, Madison.

Bae, W. and Chen, X. 2004. Proteomic Study for the Cellular Responses to Cd^{2+} in *Schizosaccharomyces pombe* through Amino Acid-coded Mass Tagging and Liquid Chromatography Tandem Mass Spectrometry. Mol. Cell. Proteomics. 3:596-606.

Balkrishna, J. 2005. Correlation, regression and path coefficient analysis for some components in common and Tartary buckwheat in Nepal. Biotech unit, ARC Khumaltar, Nepal. 22:77-82.

Barcelo, J. and Poschenrieder, C. 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of Al toxicity resistance: a review. J. Environ. Exp. Bot., 48:75-92.

Baxter, I. 2015. Should we treat the ionome as a combination of individual elements, or should we be deriving novel combined traits? J. Exp. Bot. 66:2127–2131.

Baxter, I. and Dilkes, B.P. 2012. Elemental profiles reflect plant adaptations to the environment. Science. 336:1661-3.

Baxter, I., Hosmani, P.S., Rus, A., Lahner, B., Borevitz, J.O., Muthukumar, B., Mickelbart, M.V., Schreiber, L., Franke, R.B. and Salt, D.E. 2009. Root suberin forms an extracellular barrier that affects water relations and mineral nutrition in *Arabidopsis*. PLoS Genet. 5:e1000492.

Baxter, I.R., Vitek, O., Lahner, B., Muthukumar, B., Borghi, M., Morrissey, J., Guerinot, M.L. and Salt, D.E. 2008. The leaf ionome as a multivariable system to detect a plant's physiological status. Proc. Natl. Acad. Sci. USA. 105:12081-12086.

Becana, M. and Klucas, R.V. 1990. Enzymatic and nonenzymatic mechanisms for ferric leghemoglobin reduction in legume root nodules. Proceedings of the National Academy of Sciences of the United States of America. 87(18):7295–7299. doi:10.1073/pnas.87.18.7295

Beebe, S.E., Rao, I.M., Cajiao, C. and Grajales, M. 2008. Selection for drought resistance in common bean also improves yield in phosphorus limited and favourable environments. Crop Sci. 48:582-592.

Bityutskii, N.P., Yakkonen, K.L., Petrova, A.I. and Shavarda, A.L. 2017. Interactions between aluminium, iron and silicon in *Cucumis sativus* L. grown under acidic conditions. Journal of Plant Physiology. 218:100-108.

Blair, M.W., López-Marín, H.D. and Rao, I.M. 2009. Identification of aluminium resistant Andean common bean (*Phaseolus vulgaris* L.) genotypes. Brazilian Journal of Plant Physiology. 21(4):291-300.

Bose, T.K., Som, M.G. and Kabir, J. 1986. Vegetable Crops. Naya Prokash, Calcutta, India. pp. 959.

Bot, A.J., Nachtergaele, F.O. and Young, A. 2000. Land Re- source Potential and Constraints at Regional and Country Levels. Food and Agricultural Organization of the United Nations, Rome. pp. 114.

Broadley, M., Brown, P., Cakmak, I., Ma, J. F., Rengel, Z. and Zhao, F. 2012. Beneficial Elements. In: Marschner's Mineral Nutrition of Higher Plants (Ed P. Marschner). 3rd Edition. Academic Press, San Diego. pp: 249-269.

Brunner, I. and Sperisen, C. 2013. Aluminum exclusion and aluminium tolerance in woody plants. Front. Plant Sci. 4:172.

Buresh, R.J., Sanchez, P.A. and Calhoun, F. 1996. Combined use of organic and inorganic nutrient sources for soil fertility maintenance and replenishment. In: Proceedings of an international symposium Indianapolis, Indiana. (Eds. Sanchez, P.A., Buresh, R.J. and Hatfield, J.). 6 November 1996. Soil Science Society of America (SSSA), Madison, Wisconsin, USA, pp. 193-217.

Cambraia, J., Pires, D.L.F.J., Estevao, M.D.M. and Oliva, M.A. 1983. Effects of aluminum on the levels of magnesium, iron, manganese and copper in sorghum. Rev. Ceres. 30(167):45-54.

Caverzan, A., Passaia, G., Rosa, S.B., Ribeiro, C.W., Lazzarotto, F. and Margis-Pinheiro, M. 2012. Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. Genetics and molecular biology. 35(4 (suppl)): 1011–1019.

Chaudhary, A. K., Singh, D. and Iquebal, M. A. 2011. Selection of pigeonpea genotypes for tolerance to aluminium toxicity. Plant Breeding. 130:492-495.

Chong, K., Wynne, J.C., Elkan, C.H. and Schneeweis, T.J. 1987. Effects of soil acidity and aluminum content on Rhizobium inoculation, growth and nitrogen fixation of peanuts and other grain legumes. Trop. Agric. 64(2):97-104.

Choudhary, A.K., Singh, D. and Kumar, J. 2011. A comparative study of screening methods for tolerance to aluminium toxicity in pigeonpea. Australian Journal of Crop Science. 5(11):1419-1426.

Ciamporova, M. 2002. Morphological and structural responses of plant roots to aluminium at organ, tissue, and cellular levels. Biol. Plant. 45:161-71.

Claudio, I.B., Braulio, S., Pilar, U., Felipe, A. and Marjorie, R.D. 2008. Resistance mechanisms of Aluminum (Al^{3+}) phytotoxicity in cereals: Physiological, genetic and molecular basis. J. Soil Sci. Plant Nutr., 8:57-71.

Dai, S., Yan, Z., Liu, D., Zhang, L., Wei, Y. and Zheng, Y. 2009. Evaluation on Chinese bread wheat landraces for low pH and aluminium tolerance using hydroponic screening. Agricultural Sciences in China. 8(3):285-292.

De Carvalho, L. and Cesar, P. 1984. Effect of aluminum on a cassava (*Manihot esculenta* Crantz) crop. Rev. Bras. Mandioca. 3(1):1-5.

Delhaize, E. and Ryan, P.R. 1995. Aluminium toxicity and tolerance in plants, Plant Physiol. 107:315-321.

Dietz, K.J., Horling, F., Konig, J. and Baier, M. 2002. The function of the chloroplast 2-cysteine peroxiredoxin in peroxide detoxification and its regulation. Journal of experimental botany. 53(372): 1321-1329.

Djingova, R., Mihaylova, V., Lyubomirova, V. and Tsalev, D.L. 2013. Multielement analytical spectroscopy in plant ionomics research. Applied Spectroscopy Reviews. 48(5):384-424.

Doncheva, S., Ameno´s, M., Poschenrieder, C. and Barcelo, J. 2005. Root cell patterning: a primary target for aluminium toxicity in maize. Journal of Experimental Botany. 56:1213–1220.

Duressa, D., Soliman, K. and Chen, D. 2010. Identification of Aluminum Responsive Genes in Al-Tolerant Soybean Line PI 416937. International Journal of Plant Genomics. Article ID 164862, 13 pages. doi:10.1155/2010/164862.

Duressa, D., Soliman, K., Taylor, R. and Senwo, Z. 2011. Proteomic analysis of soybean roots under aluminium stress. International Journal of Plant Genomics. 1-12. doi. 10.1155/2011/ 282531.

Edmeades, D.C., Blamey, F.P.C., Asher, C.J. and Edwards, D.G. 1991. Effects of pH and aluminium growth of temperate pasture species. I. Temperate grasses and legumes supplied with inorganic nitrogen. Australian Journals of Agricultural Research. 42:559-569.

Edwards, D.G., Kang, B.T. and Danso, K.A. 1981. Differential response of six cowpea (*Vigna unguiculata* (L.) Walp) cultivars to liming in an ultisol. Plant and Soil. 59(1):61-73.

Ehlers, J.D. and Hall, A.E. 1997. Cowpea [*Vigna unguiculata* (L.) Walp.]. Field Crops Research. 53:187-204.

Eloward, H.O.A. and Hall, A.E. 1987. Influence of early and late nitrogen fertilization on yield and nitrogen fixation of cowpea under well-watered and dry field conditions. Field Crops Research. 15:229-244.

Eswaran, H., Reich, P. and Beinroth, F. 1997. Global distribution of soils with acidity. In: Plant-Soil Interactions at Low pH. (Eds. Moniz AMCF, A.Z., Schaffert, R.E., Fageria, N.K., Rosolem, C.A. and Cantarella, H.). Brazilian Soil Science Society. pp: 159-164.

Ezeh, K.N., Omogoye, A.M. and Akinrinde, E.A. 2007. Aluminium influence on performance of some cowpea varieties on a Nigerian Alfisols. World Journal of Agricultural Science. 3(4):517-522.

Fawole, I., Gabelman, W.H., Gerloff, G.C. and Nordheim, E.O. 2001. Heritability of efficiency in phosphorus utilization in beans grown under phosphorus utilization in beans grown under phosphorus stress. Journal of the American Society for Horticultural Science. 107:98-100.

Fecht-Christoffers, M.M., Braun, H.P., Lemaitre-Guillier, C., VanDorsselaer, A. and Horst, W.J. 2003a. Effect of manganese toxicity on the proteome of the leaf apoplast in cowpea. Plant physiology. 133(4): 1935–1946. doi:10.1104/pp.103.029215.

Fecht-Christoffers, M.M., Maier, P. and Horst, W.J. 2003b. Apoplastic peroxidase and ascorbate are involved in manganese toxicity and tolerance of *Vigna unguiculata*. Physiol Plant 117:237–244.

Fitter, A.H. and Hay, R.K.M. 1981. Environmental physiology of plants. Academic Press, New York. pp: 200-231.

Foy, C. D. 1974. Effect of aluminum on plant growth. In: The plant root and its environment (Ed. Carson, E.W.). University Press of Virginia, Charlottesville. pp: 601-642.

Foy, C.D. 1984. Physiological effects of hydrogen, aluminum and manganese toxicities in acid soils. Agronomy Monogr. 12(2nd ed.):57-97.

Foy, C.D. 1992. Soil chemical factors limiting plant root growth. Adv. Soil Sci. 19:97-148.

Foy, C.D., Chaney, R.L. and White, M.C. 1978. The physiology of metal toxicity in plants. Annu. Rev. Pl. Physiol. 29:511-566.

Foy, C.D., Shalunova, L.P. and Lee, E.H. 1993. Acid soil tolerance of soybean (*Glycine max* L. Merr.) germplasm from the USSR. Journal of Plant Nutrition. 16(9):1593-1617.

Fukrei, K.P., Kumar, A., Tyagi, W., Rai, M. and Pattanayak, A. 2011. Genetic variability in yield and its components in upland rice grown in acid soils of North East India. J. Rice Res. 4(1 & 2):4-7.

George, A.S. and Carolyn, J. McQ. 2002. Stimulatory effects of aluminium on growth of sugar maple seedlings. Journal of Plant Nutrition. 25: 2583-2589.

Gerzabek, M.H. and Edelbauer, A. 1986. Aluminum toxicity in corn (*Zea mays* L.). Influence of aluminium on the yields and the nutrient contents. Bodenkultur. 37(4):309- 319.

Giller, K.E., Cadisch, G., Ehaliotis, C., Adams, E., Sakala, W.D. and Mafongoya, P.L. 1997. Building soil nitrogen capital in Africa. In: Replenishing Soil Fertility in Africa (Eds. Buresh, R.J. Sanchez, P.A. and Calhoun, F.). SSSA Special Publication. 51. SSSA Madison, WI, USA151192.

Graham, P.H. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Canadian Journal Microbiology. 38:475-484.

Graham, P.H. and Vance, C.P. 2000. Nitrogen fixation in perspective: an overview of research and extension needs. Field Crops Res. 65:93-106.

Griffin, T. J., Gygi, S. P., Ideker, T., Rist, B., Eng, J., Hood, L. and Aebersold, R. 2002. Complementary profiling of gene expression at the transcriptome and proteome level in *Saccharomyces cerevisiae.* Mol. Cell. Proteomics. 1(4):323-333.

Grover, A. 2012. Plant Chitinases: Genetic Diversity and Physiological Roles. Critical Reviews in Plant Sciences. 31(1): 57-73.

Hall, A.E. and Patel, P.N. 1985. Breeding of resistance to drought and heat. In: Cowpea research, Production and utilization (Eds. Singh, S.R. and Rachie, K.O.), Wiley, New York. pp: 137-151.

Hanson, W.D. and Kamprath, E.J. 1979. Selection for aluminium tolerance in soybeans based on seedling-root growth. Agron. J. 71:581-586.

Haynes, R. 1984. Lime and phosphate in soil plant system. Advances Agronomy. 37:249-315.

Hede, A.R., Skovmand, B., Ribaut, J.M., González-De-León, D. and Stolen, O. 2002. Evaluation of aluminium tolerance in a spring rye collection by hydroponic screening. Plant Breeding. 121(3):241-248.

Hiatt, A.J., Amos, D.F. and Massey, H.F. 1963. Effect of aluminum on copper sorption by wheat. Agron. J. 55:284-287.

Hirschi, K.D. 2003. Strike while the ionome is hot: making the most of plant genomic advances. Trends Biotechnol. 21:520-521.

Hogh-Jensen, H. and Pedersen, M.B. 2003. Morphological plasticity by crop plants and their potassium use efficiency. Journal of Plant nutrition. 26:969-984.

Horst, W.J. 1985. Quick screening of cowpea (*Vigna unguiculata*) genotypes for aluminium tolerance in an aluminium-treated acid soil. Journal of Plant Nutrition & Soil science. 148(3):335-348.

Horst, W.J. 1995. The role of the apoplast in aluminium toxicity and resistance of higher plants. Zeitschriftfur Pflanzenernahrung und Bodenkunde. 158:419-428.

Horst, W.J. and Goppel, H. 1986. Aluminum-Toleranz von Ackerbohne (*Vicia faba*), Lupine (*Lupinus luteus*), Gerste (*Hordeum vulgare*) und Roggen (*Secale cereale*). II. Mineral stoffgehaltein SproBeb und Wiirzelni n Abhangigkeitv on Aluminium - An-gebot. Z. Pflanzenernaehr. Bodenk. 149:94-109.

Huang, J.W., Grunes, D.L. and Kochian, L.U. 1992. Aluminium effect on the kinetics of Calcium uptake into cells of wheat root Apex. Planta. 188:411-421.

IFPRI. 2010. Fertilizer and soil fertility potential in Ethiopia: Constraints and opportunities for enhancing the system. International Food Policy Research Institute (IFPRI). Working paper, Washington, USA. Available at www.ifpri.org accessed on: 12 April 2011.

Jiang, C., Liu, L., Li, X., Han, R., Wei, Y. and Yu, Y. 2018. Insights into aluminum-tolerance pathways in Stylosanthes as revealed by RNA-Seq analysis. Scientific Reports. 8: 6072.

Johnson R and Wichern, D. 2007. Applied Multivariate Statistical Analysis. Pearson Prentice Hall, Old Tappan, NJ.

Kadiata, B.D. and Lumpungu, K. 2003. Differential phosphorus uptake and use efficiency among selected nitrogen-fixing tree legumes over time. Journal of Plant Nutrition. 26:1009-1022.

Kashif, M. and Khaliq, I. 2004. Heritability, Correlation and Path Coefficient Analysis for some metric traits in wheat. International Journal of Agriculture and Biology. 6:138-142.

Kasprzewska, A. 2003. Plant chitinases-regulation and function. Cell. Mol. Biol. Lett. 8:809824.

Kerridge, P.C., Dawson, M.D. and Moore, D.P. 1971. Separation of degrees of Aluminium tolerance in Wheat. Agron. J. 63:586-591.

Khatiwada, S.P., Senadhira, D., Carpena, A.L., Zeigler, R.S. and Fernandez, P.G. 1996. Variability and genetics of tolerance for aluminium toxicity in rice (*Oryza sativa* L.). Theoretical and Applied Genetics. 93:738-744.

Kinraide, T.B. 1993. Aluminium enhancement of plant growth in acid rooting media. A case of Reciprocal Alleviation of toxicity by two toxic cations. Plant Physiology. 88: 619-625.

Kinraide, T.B. 1997. Reconsidering the rhizotoxicity of hydroxyl sulphate and fluoride cations. Journal of Experimental Botany. 48:1115-11124.

Kochian, L. V., Hoekenga, O. A. and Pineros, M. A. 2004. How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annu. Rev. Plant Biol. 55:459-493.

Kochian, L. V., Pineros, M. A. and Hoekenga, O. A. 2005. The Physiology, Genetics and Molecular Biology of Plant Aluminum Resistance and Toxicity. Plant Soil. 274: 175-195.

Kochian, L.V. 1995. Cellular mechanism of Aluminium toxicity and resistance on plants. Annual Review Plant Physiology and Plant Molecular Biology. 46:237-260.

Kolawole, G.O., Tian, G. and Singh, B.B. 2000. Differential response of cowpea lines to aluminium and phosphorus application. Journal of Plant Nutrition. 23(6): 731-740.

Kumar, D. and Mahla, H.R. 2004. Cowpea – An Introduction. In: Cowpea in India (Eds. Kumar, D. and Singh, N.B.). Scientific Publisher. pp: 281.

Kushwaha, J. K., Pandey, A. K., Dubey, R. K., Singh, V., Mailappa, A. S. & Singh, S. 2017. Screening of cowpea [*Vigna unguiculata* (L.) Walp.] for aluminium tolerance in relation to growth, yield and related traits. Legume Research - An International Journal 40(3): 434-438.

Kuswantoro, H., Wijanarko, A., Setyawan, D., William, E., Dadang, A. and Mejaya, M.J. 2010. Soyabean germplasms evaluation for acid tidal swamp tolerance using selection index. International Journal of Plant Biology. 1: 1:e11/2237.

Kwapata, M.B. and Hall, A.E. 1985. Effects of moisture regime and phosphorus on mycorrhizal infection, nutrient uptake and growth of cowpeas [*Vigna unguiculata* (L.) Walp.]. Field Crops Research. 12:241-250.

Lahner, B., Gong, J., Mahmoudian, M., Smith, E.L., Abid, K.B., Rogers, E.E., Guerinot, M.L., Harper, J.F., Ward, J.M., McIntyre, L., Schroeder, J.I. and Salt, D.E. 2003. Genomic scale profiling of nutrient and trace elements in *Arabidopsis thaliana*. Nat Biotechnol. 21:1215-21.

Lee, C.R. 1971. Influence of Al on plant growth and tuber yield of potatoes. Agron. J. 63: 363-364.

Li, H., Yang, L., Qi, Y., Guo, P., Lu, Y. and Chen, L. 2016. Aluminum Toxicity-Induced Alterations of Leaf Proteome in Two Citrus Species Differing in Aluminum Tolerance. International Journal of Molecular Sciences. 17:1-19.

Liang, C., Pineros, M.A., Tian, J., Yao, Z., Sun, L., Liu, J., Shaff, J., Coluccio, A., Kochian, L. V. and Liao, H. 2013. Low pH, aluminum, and phosphorus coordinately regulate malate exudation through GmALMT1 to improve soybean adaptation to acid soils. Plant Physiol. 161:1347-61.

Lidon, F.C., Azinheira, H.G. and Barreiro, M.G. 2000. Al toxicity in maize: modulation of biomass production and nutrients uptake and translocation. J. Plant Nutr. 23(2):151-160.

Little, R. 1989. A review of breeding wheat for tolerance to Aluminium toxicity In: Fifth Regional Wheat Workshop for Eastern, Central, and Southern Africa and the Indian Ocean. (Eds. Van Ginkel, M. and Tanner, D.G.). CIMMYT, Antsirabe, Madagascar, October 5-10, 1987, pp. 83-97.

Ma, J.F. 2000. Role of organic acids in detoxification of aluminium in higher plants. Plant Cell Physiology. 41:383-390.

Ma, J.F., Zheng, S.J. and Matsumoto, H. 1997a. Specific secretion of citric acid induced by Al stress in *Cassia tora* L. Plant Cell Physiol. 38:1019-25.

Ma, J.F., Zheng, S.J., Hiradate, S. and Matsumoto, H. 1997b. Detoxifying aluminum with buckwheat. Nature. 390:569-70.

Macedo de C.C., Kinet, J.M. and van Sint, J.V. 1997. Effects of duration and intensity of aluminium stress on growth parameters in four rice genotypes differing in aluminium sensitivity. Journal of Plant Nutrition. 20:181-193.

Mackay, A.D., Caradus, J.R. and Wewala, S. 1991. Aluminium tolerance of forage speies. In: Plant-Soil Interactions at Low pH (Eds. Wright, R.J., Baligar, V.C. and Murrmann, R.P.). Kluwer Academic Publisher, Dordrecht, pp. 25-30.

Macuha, P. and Rychtarik, J. 1999. Evaluation of varieties and hybrids of winter wheat tolerance and sensibility to acid environment and Al^{3+} ions. Journal of Agricultural Sciences. 45(1):16-28.

Mandal, S.C. 1997. Introduction and historical overview. In: Acidic Soils of India (Ed. Mahapatra, I.C. *et al.*). ICAR, New Delhi. pp. 3-24.

Maréchal, R., Mascherpa, J.M. and Stainier, F. 1978. Etude taxonomique d'ungroupe complexe d'especes des genres *Phaseolus et vigna* (Papiloonaceae) sur la base de dunnees morphologiques et pulliniques, traitees pas l'analye informatique. Boissiera 28:1-273.

Marschner, H. 1995. Functions of mineral nutrients: micronutrients. In: Mineral nutrition of higher plants (Ed. Marschner, H.), 2nd ed, London: Academic Press. pp: 313–404.

Matsumoto, H., Yamamoto Y. and Rama Devi, S. 2001. Aluminium toxicity in acid soils. Plant responses to aluminium. In: Metals in the Environment (Ed. Prasad, M.), Marcel Dekker, New York, pp: 289-319.

Minella, E. and Sorellis, M. E. 1992. Aluminium tolerance in Barley: Genetic relationship among genotypes of diverse origin. Crop Science. 32:993-598.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9:490–498.

Miyasaka, S.C., Hue, N.V. and Dunn, M.A. 2007. Aluminum. In: Handbook of Plant Nutrition (Eds. Barker, A.V. and Pilbeam, D.J.). Tayler and Francis Group, Boca Raton, pp. 439-497.

Mugai, E.N., Agong, S.G. and Matsumoto, H. 2002. Aluminium tolerance of four bean (*Phaseolus vulgaris* L.) varieties. Journal of Agriculture, Science and Technology. 4(1):52-65.

Naserian, B., Asadi, A.A., Rahimi, M. and Ardakani, M.R. 2007. Evaluation of wheat cultivar mutants for morphological and yield traits and comparing of yield components under irrigated and rainfed conditions. Asian Journal Plant Science. 6:214-224.

Ng, N.Q. and Marechal, R. 1985. Cowpea taxonomy, origin and germplasm. In: Cowpea research, production and utilization (Eds. Singh, S.R. and Rachie, K.O.), Wiley, New York. pp 11-21.

Nguyen, B., Brar, D., Bui, B., Nguyen, T., Pham, L. and Nguyen, H. 2003. Identification and mapping of the QTL for aluminium tolerance introgressed from the new source, *Oryza rufipogon* Griff. into indica rice (*Oryza sativa* L.). Theor. Applied Genetics. 106:583-593.

Nogueira, F., Gonçalves, F., Emmanuel, J.E., Santos, M., Costa, J., Oliveira-Neto, B.O., Soares, A., Domont, G. and Campos, A.P.F. 2007. Proteome analysis of embryogenic cell suspensions of cowpea (*Vigna unguiculata*). Plant cell reports. 26. 1333-43. 10.1007/s00299-007-0327-6.

Ogbonnaya, C.I., Sarr, B., Brou, C., Diouf, O., Diop, N.N. and Roy-Macauley, H. 2003. Selection of Cowpea Genotypes in Hydroponics, Pots, and Field for Drought Tolerance. Crop Science. 43(3): 1114-1120.

Ohki, K. 1987. Aluminium stress on sorghum growth and nutrient relationships. Plant Soil. 98: 195-202.

Oikeh, M.S.O., Maziya-Dixon, B., Welch, R. and Glahn, R.P. 2003. Genotypic differences in concentration and bioavailability of kernel-iron in tropical maize varieties grown under field conditions. Journal of Plant Nutrition. 26:2307-2319.

Ojo, G.O.S. and Ayuba, S.A. 2012. Screening of tropically adapted genotypes of soybean (*Glycine max* (L.) Merrill) for aluminium stress tolerance in short-term hydroponics. Journal of Animal and Plant Sciences. 14(2):1921-1930.

Pandey, A. and Mann, M. 2000. Proteomics to study genes and genomes. Nature 405: 837–846.

Panse, P.G. and Sukhatme, P.V. 1985. Statistical Methods for Agricultural Workers. 4th ed. I.C.A.R., New Delhi.

Pasquet, R.S. 1998. Morphological study of cultivated cowpea [*Vigna unguiculata* (L.) Walp.]. Importance of ovule number and definition of cv. group *Melanophthalmus*. Agronomy. 18:61-70.

Pasquet, R.S. 2000. Allozyme diversity of cultivated cowpea [*Vigna unguiculata* (L.) Walp.]. Theoretical and Applied Genetics. 101:211-219.

Pavan, M.A. 1982. Toxic effects of aluminum on coffee seedlings in relation to calcium nutrition. Rev. Bras. Ci. Solo. 6(3): 209-213.

Pavan, M.A. and Bingham, F.T. 1982. Toxicity of aluminum to seedlings grown in nutrient solution. J. Soil Sci. Soc. Amer. 46(5): 993-997.

Pearson, K. 1901. On lines and planes of closest fit to systems of points in space. Philos Mag 2:559-572.

Pécsváradi, A., Nagy, Z., Varga, A., Vashegyi, Á., Labádi, I., Galbács, G. and Zsoldos, F. 2009. Chloroplastic glutamine synthetase is activated by direct binding of aluminium. Physiologia Plantarum. 135: 43-50. doi:10.1111/j.1399-3054.2008.01167.x

Pineros, M., Magalhaes, J., Carvalho-Alves, V.M. and Kochian, L.V. 2002. The physiology and biophysics of an aluminium tolerance mechanism based on root citrate exudation in maize. Plant Physiol. 29:1194-1206.

Qureshi, M.I., Qadir, S. and Zolla, L. 2007. Proteomics-based dissection of stress-responsive pathways in plants. Journal of Plant Physiology. 164:1239-1260.

Ram, H.H. 1998. Vegetable breeding: Principles and Practices. Kalyani Publishers, Ludhiana, India. pp: 791.

Rangel, A.F., Rao, I.M. and Horst, W.J. 2007. Spatial aluminium sensitivity of root apices of two common bean (*Phaseolus vulgaris* L.) genotypes with contrasting aluminium resistance. Journal of experimental botany. 58(14):3895-3904.

Rao, I., Wenzl, P., Arango, A., Miles, J., Watanabe, T., Shinano, T., Osaki, M., Wagatsuma, T., Manrique, G., Beebe, S., Tohme, J., Ishitani, M., Rangel, A. and Horst, W. 2008. Advances in developing screening methods and improving aluminium resistance in common bean and Brachiaria. Revista Brasileira de Agrociência. 14(4):569-576.

Rao, I.M., Zeigler, R.S., Vera, R. and Sarkarung, S. 1993. Selection and breeding for acid-soil tolerance in crops. BioSci. 43:454-465.

Rea, P.A. 2003. Ion genomics. Nat. Biotechnol. 21:1149-1151.

Rengel, Z. and Zhang, W.H. 2003. Role of dynamics of intracellular calcium in aluminium-toxicity syndrome: Review. New Phytol 159:295-314.

Rifai, N., Gillette, M.A. and Carr, S.A. 2006. Protein biomarker discovery and validation: The long and uncertain path to clinical utility. Nat Biotechnol. 24:971-983.

Ryan, P. R., Delhaize, E. and Jones, D. L. 2001. Function and Mechanism of Organic Anion Exudation from Plant Roots. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52: 527-560.

Ryan, P.R., Shaff, J.E. and Kochian, L.V. 1992. Aluminum toxicity in roots: Correlation among ionic currents, Ion fluxes, and root elongation in aluminum-sensitive and aluminum-tolerant wheat cultivars. Plant Physiol. 99:1193-200.

Salt, D.E., Baxter, I. and Lahner, B. 2008. Ionomics and the study of the plant ionome. Annu Rev Plant Biol. 59:709-733

Samac, D.A. and Tesfaye, M. 2003. Plant improvement for tolerance to aluminum in acid soils - a review. Plant Cell, Tissue Organ Cult. 75:189-207.

Sánchez-Rodríguez, E., Rubio-Wilhelmi, M.M., Cervilla, L.M., Blasco, B., Rios, J.J., Leyva, R., Romero, L. and Ruiz, J.M. 2010. Study of the ionome and uptake fluxes in cherry tomato plants under moderate water conditions. Plant Soil. 335:339-347.

Santos, J.B.D., Daúd, M.A. and Carvalho, J.G.D. 1997. Reaction of common bean cultivars to aluminium in nutrient solution. Ciência e Agrotecnologia. 21(1):114-118.

Sarkunan, V. and Biddappa, C.C. 1982. Effect of aluminum on the growth yield and chemical composition of rice. Oryza. 19(3-4):188-190.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S.J., Ryan, P.R., Delhaize, E. and Matsumoto, H. 2004. A wheat gene encoding an aluminium-activated malate transporter. Plant J. 37:645-653.

Schlede, H. 1989. Distribution of Acid Soils and Liming Materials in Ethiopia. Ethiopian Institute of Geological Survey, Ministry of Mines and Energy, Addis Ababa, Ethiopia.

Scott, B.J., Fisher, J.A. and Spohr, L.J. 1992. Tolerance of Australian wheat varieties to aluminium toxicity. Communication in Soil Science and Plant Analysis. 23:509-526.

Sha, Z., Oka, N., Watanabe, T., Tampubolon, B.D., Okazaki, K., Osaki, M. and Shinano, T. 2012. Ionome of Soyabean seed affected by previous cropping with mycorrhizal plant and manure application. Journal of Agriculture and Food Chemistry. 60(38):9543-52.

Shahinnia, F., Rezai, A.M. and Tabatabaei, S.B.E. 2005. Variation and path coefficient analysis of important agronomic traits in two-and six rowed recombinant inbred lines of barley (*Hordeum vulgare* L.). Czech J. Genet. Plant Breed. 41:246-250.

Shamsi, I.H., Wei, K., Jilani, G. and Zhang G.P. 2007. Interactions of cadmium and aluminum toxicity in their effect on growth and physiological parameters in soybean. Zhejiang Univ Sci B. 8(3):181-188.

Sharma, U. C. and Singh, R. P. 2002. Acid soils of India: their distribution, management and future strategies for higher productivity. Fertilizer News. 47:45-52.

Sheokand, S. and Brewin, N.J. 2003. Cysteine proteases in nodulation and nitrogen fixation. Indian Journal of Experimental Biology. 41: 1133-1135.

Shibuya, T., Watanabe, T., Ikeda, H. and Kanayama, Y. 2015. Ionomic Analysis of Horticultural Plants Reveals Tissue-specific Element Accumulation. The Horticulture Journal Preview. 1-9. doi: 10.2503/hortj.MI-058.

Simon, L., Smalley, T.J., Jones, J.B. and Lasseigne, F.T. 1994. Aluminum toxicity in tomato. Part 1. Growth and mineral nutrition. J. Plant Nutr. 17(2&3):293-306.

Singh, B. 2003. Improving the production and utilization of cowpea as food and fodder. Field Crops Research. 84: 169-177.

Singh, B.B., Chambliss, O.L. and Sharma, B. 1997. Recent advances in cowpea breeding. In: Advances in cowpea research (Eds. Singh, B.B., Mohanraj, D.R., Dashiell, K.E. and Jackai, L.E.N.), IITA-JIRCAS, Ibadan. pp: 30-49.

Sobrado, M.A. 2013. Soil and Leaf Micronutrient Composition in Contrasting Habitats in Podzolized Sands of the Amazon Region. American Journal of Plant Sciences. 4: 1918-1923. http://dx.doi.org/10.4236/ajps.2013.410235

Stass, A., Kotur, Y. and Horst, W.J. 2007. Effect of boron on the expression of aluminium toxicity in *Phaseolus vulgaris*. Physiologia Plantarum. 131:283-290.

Taylor, G.J., Blarney, F.P.C. and Edwards, D.G. 1998. Antagonistic and synergistic interactions between aluminum and manganese on growth of *Vigna unguiculata* at low ionic strength. Physiology Plantarum 104: 183–194

Tazeen, M., Nadia, K. and Farzana, N.N. 2009. Heritability, Phenotypic Correlation and Path Coefficient studies for some agronomic characters in synthetic elite lines of wheat. Food, Agriculture and Environment. 7(3):278-282.

The, C., Calba, H., Zonkeng, C., Ngonkeu, E., Adetimirin, V., Mafouasson, H., Meka, S. and Horst, W. 2006. Responses of maize grain yield to changes in acid soil characteristics after soil amendments. Plant Soil. 284:45-57.

Thornton, F.C., Schaedle, M. and Raynal, D.J. 1989. Tolerance of red oak and American and European beech seedlings to aluminium. Journal of Environment Quality. 18: 541-545.

Van Breeman, N. and Moorman, F.R. 1978. Iron-toxic soils. In: Soils and Rice, International Rice Research Institute. Los Baños, Phillippines. pp. 781-801.

Vitorello, V.A., Capaldi, F.R. and Stefanuto, V.A. 2005. Recent advances in aluminum toxicity and resistance in higher plants. Brazilian J. Plant Physiol. 17:129-143.

Von Uexkull, H.R. and Mutert, E. 1995. Global extent, development and economic impact of acid soils. In: plant-soil interactions at low pH: Principles and management (Eds. Date, R.A., Grundon, N.J., Rayment, G.E. and Probert, M.E.), Dordrecht, Kluwer. pp: 5-19.

Wagatsuma, T. 1983. Characterization of absorption sites for aluminum in the roots. Soil Sci. P1. Nutr. 29(4):499-515.

Wang, J., Raman, H., Zhang, G., Mendham, N. and Zhou, M.X. 2006. Aluminium tolerance in barley (*Hordeum vulgare* L.): Physiological mechanisms, genetics and screening methods. J. Zhejiang Univ– Sci. B. 7:769-787.

Wang, W., Zhao, X.Q., Hu, Z.M., Shao, J.F., Che, J., Chen, R.F. and Shen, R.F. 2015. Aluminium alleviates manganese toxicity to rice by decreasing root symplastic Mn uptake and reducing availability to shoots of Mn stored in roots. Annals of botany. 116(2): 237–246. doi:10.1093/aob/mcv090

Wenzl, P., Chaves, A., Patin, O.G., Mayer, J. and Rao, I. 2002. Aluminium stress stimulates the accumulation of organic acids in root apices of *Brachiaria* species. Journal of Plant Nutrition and Soil Science. 165:582-588.

Westphal, E. 1974. Pulses in Ethiopia: their taxonomy and agriculture significance. Agricultural research report. Center for Agricultural Publishing and Documentation, Wageningen. pp: 815.

Yamamoto, Y., Kobayashi, Y., Devi, S.R., Rikiishi, S. and Matsumoto, H. 2002. Aluminium toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiology. 128:63-72.

Yan, S.P., Zhang, Q.Y., Tang, Z.C. and Su, W.A. 2006. Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol. Cell. Proteomics. 5:484–496.

Yang, Q., Wang, Y., Zhang, J., Shi, W., Qian, C. and Peng, X. 2007. Identification of aluminium-responsive proteins in rice roots by a proteomic approach: cysteine synthase as a key player in Al response. Proteomics. 7:737-749.

Yang, Z., Rao, I.M. and Horst, W.J. 2013. Interaction of aluminium and drought stress on root growth and crop yield on acid soil. Plant Soil. 372: 3-25.

Yang, Z.B., You, J.F., Xu, M.Y. and Yang, Z.M. 2009. Interaction between aluminium toxicity and manganese toxicity in soybean (*Glycine max*). Plant and Soil 319: 277–289.

Yin, L., Mano, J., Tanaka, K., Wang, S., Zhang, M., Deng, X. and Zhang, S. 2017. High level of reduced glutathione contributes to detoxification of lipid peroxide-derived reactive carbonyl species in transgenic *Arabidopsis* overexpressing glutathione reductase under aluminium stress. Physiol Plant, 161, 211–223. doi:10.1111/ppl.12583.

Yin, L., Wang, S., Eltayeb, A.E., Uddin, M.I., Yamamoto, Y., Tsuji, W., Takeuchi, Y. and Tanaka, K. 2010. Overexpression of dehydroascorbate reductase, but not

monodehydroascorbate reductase, confers tolerance to aluminium stress in transgenic tobacco. Planta. 231: 609–621. doi:10.1007/s00425-009-1075-3.

Yoshida, A. and Yoshida, T. 2000. Screening of Aluminium tolerant Sorghum by Hematoxyline staining and growth response. Plant production science. 3(3):246-253.

Zhen, Y., Qi, J.L., Wang, S.S., Su, J., Xu, G.H., Zhang, M.S., Miao, L., Peng, X.X., Tian, D. and Yang, Y.H. 2007. Comparative proteome analysis of differentially expressed proteins induced by Al toxicity in soybean. Physiologia Plantarum. 131:542-554.

Zhou, S., Okekeogbu, I., Sangireddy, S., Ye, Z., Li, H., Bhatti, S., Hui, D., McDonald, D.W., Yang, Y., Giri, S., Howe, K.J., Fish, T. and Thannhauser, T.W. 2016. Proteome Modification in Tomato Plants upon Long-Term Aluminum Treatment. Journal of Proteome research. 15: 1670−1684. DOI: 10.1021/acs.jproteome.6b00128.

Zhou, S., Sauve, R. and Thannhauser, T.W. 2009. Proteome changes induced by aluminium stress in tomato roots. Journal of experimental botany. 60(6):1849-1857.

Plate No.1 Cowpea genotypes after one week of growth in hydroponics system

Plate No.2 Cowpea genotypes after five weeks of growth in hydroponics system

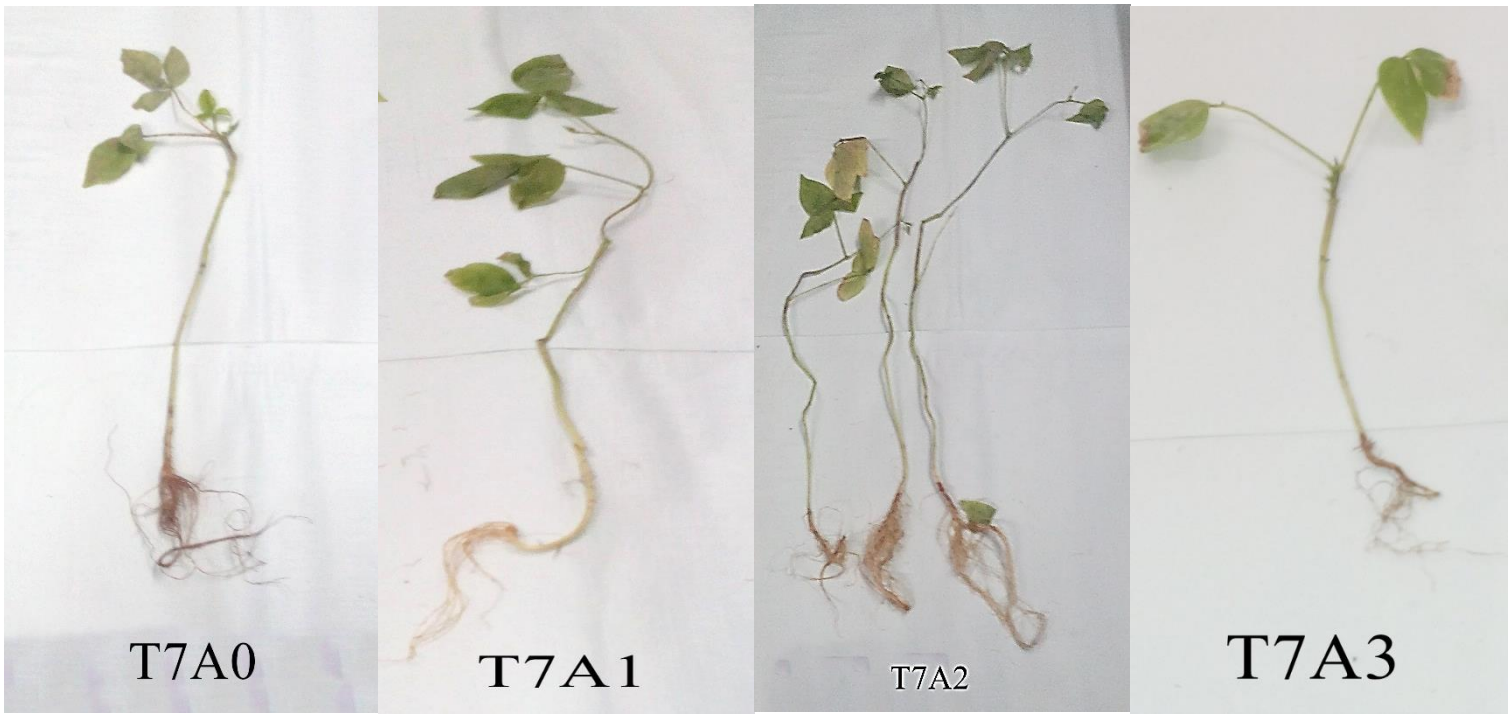


Plate No.3 Susceptible genotype of Cowpea at different aluminium concentration

Plate No.4 Tolerant genotype of Cowpea at different aluminium concentration

VITA

The author was borne on 12th August, 1988 at Nichlaul, Maharajganj district (Uttar Pradesh). He passed his High School from J.N.V. Gorakhpur with 1st division in 2004. He passed his intermediate examination in 2006 from Gorakhpur (Uttar Pradesh) with 1st division.

He has completed B.Sc. (Horticulture) degree from Narendra Dev University of Agriculture and Technology, Kumarganj, Faizabad (Uttar Pradesh) in 2012 with 1st division and M.Sc. (Horticulture) in Vegetable Science from Central Agricultural University, Imphal (Manipur) in 2014 with 1st division. Thereafter, he took admission in Department of Horticulture, Sikkim University, Gangtok (Sikkim) as Ph.D. (Horticulture) student. During the present study, he was recipient of University Grant Commission (UGC), National fellowship for students of other backward classes to pursue Ph.D. degree.

Address:

Jitendra Kumar Kushwaha

Azad Nagar, Nichlaul

Nichlaul- 273304, Uttar Pradesh

Email: jeet7622@gmail.com