Physiological and Genetic Deciphering of Water, Salinity and Relative Humidity Stress in Chickpea (*Cicer arietinum* L.)

A THESIS

submitted

for the award of the Degree of

DOCTOR OF PHILOSOPHY

in

PLANT BIOTECHNOLOGY

Submitted by

R Pushpavalli

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Bharathidasan University
Tiruchirappalli - 620 024
Tamil Nadu,
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Under the guidance of

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This thesis is dedicated to

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This is to certify that the Ph.D. thesis entitled "**Physiological and Genetic Deciphering of Water, Salinity and Relative Humidity Stress in Chickpea (*Cicer arietinum* L.)** " is the record of bonafide research carried out at ICRISAT as per the MOU of Bharathidasan University and in the Department of Plant Science, Bharathidasan University, Tiruchirappalli, by Ms R Pushpavalli, under my guidance and also co-guidance of Dr Vincent Vadez, Principal Investigator, Crop physiology laboratory, *International Crops Research Institute for the Semi-Arid Tropics* (*ICRISAT*), Patancheru 502 324, Telangana. This is submitted for the award of DOCTOR OF PHILOSOPHY in Plant Biotechnology at Bharathidasan University, Tiruchirappalli, Tamil Nadu.

I, further, certify that the research work is original and the data presented in the thesis are based on her own observations and no portion thereof has been submitted elsewhere in part or full for any other degree, diploma, associateship of fellowship of any other university.

6.07.2015

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ICRISAT
Science with a human face

This is to certify that the Ph.D. thesis entitled **"Physiological and Genetic Deciphering of Water, Salinity and Relative Humidity Stress in Chickpea (*Cicer arietinum* L.)"** is the record of bonafide research carried out at ICRISAT as per the MOU of Bharathidasan University and in the Department of Plant Science, Bharathidasan University, Tiruchirappalli, by Ms R Pushpavalli, under my guidance and also co-guidance of Dr Vincent Vadez, Principal Investigator, Crop Physiology Laboratory, *International Crops Research Institute for the Semi-Arid Tropics* (*ICRISAT*), Patancheru 502 324, Telangana. This is submitted for the award of DOCTOR OF PHILOSOPHY in Plant Biotechnology at Bharathidasan University, Tiruchirappalli, Tamil Nadu.

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DECLARATION

I hereby declare that the work embodied in this thesis has been originally carried out by me under the guidance of Dr M V Rao, Honorary Professor, Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India and co-guidance of Dr Vincent Vadez, Principal Investigator, Crop physiology laboratory, Assistant Director, Dryland Cereals, *International Crops Research Institute for the Semi-Arid Tropics* (*ICRISAT*), Patancheru 502 324, Telangana, India. I, further, assure that the work presented in this thesis, to the best of my knowledge and belief, is original except as acknowledged in the text. I declare that I have not submitted this material, either in whole or in part, for a degree, diploma, associateship or fellowship at this or any other institution.

Place: Tiruchirappalli Signature of the candidate

Date: (R Pushpavalli)

CONTENTS

ABBREVIATIONS

-1	:	Per
%	:	Percentage
<	:	Lesser than
>	:	Greater than
ABA	:	Abscisic Acid
ADM	:	Aboveground Dry Matter
ADP	:	Adenine Di Phosphate
ANOVA	:	Analysis of Variance
BGM	:	Botrytis Gray Mold
BLAST	:	Basic Local Alignment Search Tool
C. reticulatum L.	:	*Cicer reticulatum* L.
Ca^{2+}	:	Calcium ion
CaLG	:	*Cicer arietinum* Linkage Group
CaM	:	*Cicer arietinum* Marker
CEC	:	Cation Exchange Capacity
CKAM	:	Kaspar Marker
Cl^-	:	Chloride ion
cM	:	centi Morgan
Corp.	:	Corporation
DABS	:	Decolorized Aniline Blue Solution
DAS	:	Days After Sowing
DF	:	Days to Flower
DM	:	Days to Maturity
DTI	:	Drought Tolerance Index
EC	:	Electrical Conductivity
et al	:	Et alia (and others)

Et	:	Ethylene
FC	:	Field Capacity
FTSW	:	Fraction of Transpirable Water
G	:	Gram
GO	:	Gene Ontology
H^2	:	Heritability
HI	:	Harvest Index
i.e	:	That is
ICCM	:	ICRISAT Chickpea Marker
ICRISAT	:	International Crops Research Institute for the Semi-Arid Tropics
ID	:	Identification
IST	:	Indian Standard Time
JA	:	Jasmonic Acid
K^+	:	Potassium ion
kg	:	Kilogram
kPa	:	Kilo Pascal
LSD	:	Least Significant Difference
L	:	Litre
M	:	Meter
MAB	:	Marker Assisted Breeding
Mha	:	Million Hectare
mM	:	milliMolar
mM	:	milliMeter
Mt	:	Million tonnes

Na^+	:	Sodium ion
Na_2PO_4	:	Sodium Di Phosphate
NaCl	:	Sodium Chloride
NTR	:	Normalized Transpiration Rate
PCR	:	Polymerase Chain Reaction
PVE	:	Phenotypic Variation Explained
Px	:	Number of Pixels for a given Temperature
Pxt	:	Total Number of Pixels for Range of Temperatures
QTL	:	Quantitative Trait Loci
RBD	:	Randomized Block Design
RH	:	Relative Humidity
RILs	:	Recombinant Inbred lines
ROS	:	Reactive Oxygen Species
S	:	Sensitive
SNP	:	Single Nucleotide Polymorphism
SPS	:	Sucrose Phosphate Synthase
SS	:	Sucrose Synthase
SSR	:	Simple Sequence Repeats
t	:	Tonne
T	:	Tolerant
TILLING	:	Targeting Induced Local Lesions IN Genome
Tr	:	Transpiration Rate
TTSW	:	Total Transpirable Soil Water

UK	:	United Kingdom
UniProt KB	:	The UniProt Knowledge Base
VPD	:	Vapor Pressure Deficit
WS	:	Water Stressed
wt.	:	Weight
WW	:	Well Watered
×	:	Cross
/	:	Per

Abstract

Chickpea (*Cicer arietinum* L.), an important cool-season, food legume crop, is known to be sensitive to several abiotic stresses: drought, salinity and heat. The yield losses caused by these stresses are accounted to 6.4 million tonnes (t)/ year on global production. To improve any existing cultivar and harness the genetic regions involved in the tolerance it is important to understand the genetic and physiological mechanisms that underlie any tolerance. The objectives of this study were to (i) understanding the effect of either water deficit or salt stress on the reproductive biology of genotypes know to contrast for either salt or drought stress and (ii) construction of genetic map and identification of QTLs and candidate genes for salinity tolerance in 188 RILs derived from the ICCV 2 × JG 11 cross.

In the water deficit study conducted in two consecutive years, ten genotypes with contrasting yields under terminal drought stress in the field were exposed to a gradual, but similar, water stress in the glasshouse. Nine parameters related to yield were recorded in well-watered plants (WW) and in water-stressed plants (WS) when the level of deficit was mild (phase I), and when the stress was severe (phase II). The WS treatment reduced seed yield, seed and pod number, but not flower + pod + seed abortion percentage or 100-seed weight. The controlled drought imposition in glass house conditions revealed genotypic differences in the sensitivity of the reproductive process to drought. The

seed yield differences in chickpea were largely related to the capacity to produce a large number of flowers and to set seeds, especially when the degree of water deficit was mild.

In the salinity experiments, fourteen genotypes of chickpea (*Cicer arietinum* L.) were used to study yield parameters, and eight genotypes were selected for ion analysis after being grown in soil treated with 0 mM and 80 mM NaCl, to assess any possible relationship between salt ion accumulation in different plant tissues and yield reduction. Salinity delayed flowering and the delay was greater in sensitive than tolerant genotypes under salt stress. Filled pod and seed numbers, but not seed size, were associated with seed yield in saline conditions, suggesting that salinity impaired reproductive success more in sensitive than tolerant lines. The delay in flowering was associated with higher concentrations of Na^+ in the laminae of fully expanded young leaves (R^2=0.61) and old green leaves (R^2=0.51). Na^+ accumulation in leaves was associated with delayed flowering that in turn could have played a role of the lower reproductive success in the sensitive lines.

In QTL mapping for salinity tolerance, yield and components were assessed in 188 recombinant inbred lines (RILs) derived from cross ICCV 2 × JG 11, in soil treated with either 0 mM NaCl (control) or 80 mM NaCl (salinity) over two consecutive years. Salinity significantly ($P<0.05$) affected almost all traits across years. The mean yield reduction under salinity compared to control was around 40% across years. A genetic map was constructed using 56 (SSR, SNP) polymorphic markers. The

QTL analysis revealed two key genomic regions on CaLG05 (28.6 cM) and on CaLG07 (19.4 cM) that harboured QTLs for salinity tolerance associated traits. Two major QTLs for higher yield in the salinity treatment (explaining 12 and 17% of the phenotyping variation) were identified within the two key genomic regions. Comparison with already published chickpea genetic maps showed that these regions conferred salinity tolerance across two other populations and the markers can be deployed for enhancing salinity tolerance in chickpea. Based on gene ontology annotation 48 putative candidate genes responsive to salinity stress were found. Most of them were believed to be involved in achieving osmoregulation under stress conditions.

In the relative humidity stress study, five genotypes that contrasting for yield under heat stress were studied. The plants were grown in three different vapor pressure deficit conditions (2.5, 3.0, 3.4 kPa) where the temperature was maintained constant (30°C) and the RH varied as 40, 30, 20% respectively. Genotypic variation found for almost all traits across treatments. The traits seed number and seed weight differentiated tolerant and sensitive group significantly at VPD conditions 2.5 and 3.0 but not in 3.4 kPa. Seed size was unaffected under 2.5 and 3.0 kPa VPD regimes but did get reduced upto 45% under 3.4 kPa treatment compared to 2.5 kPa treatment. The lowest RH treatment, even under fully well-watered condition, as any other abiotic stress reduced yield. Thus, it is important to consider the effect of low RH and the mechanisms behind its tolerance and sensitivity in future heat tolerance

studies. The pollen viability or pollen *in vivo* germination was unaffected in this study.

All the four studies have revealed that certain parameters can be used for achieving water deficit, salinity and relative humidity stress tolerance in future chickpea improvement programs.

General Introduction

Chickpea (*Cicer arietinum* L.) is one of the seven crops that were domesticated from Neolithic age (10, 000 years ago) (Vander Maesan, 1972; Berger *et al.* 2005). It is known for its high proteinaceous nature and also rich in other nutrients such as carbohydrates, micro nutrients and ash. The crop was believed to be originated in Turkey and had been cultivated in more than 52 countries (Berger *et al.* 2003). Chickpea production in world accounted for 13.1 million tonnes in 13.5 million hectares annually. India is the largest producer of chickpea with 75% global production. In India, it is mostly grown in the states of Madhya Pradesh, Uttar Pradesh, Rajasthan, Maharashtra and Andhra Pradesh, whereas the largest producer is Madhya Pradesh with 6 million tonnes (40%) followed by Uttar Pradesh (16%) (http://www.crnindia.com/commodity/chickpea.html). In Australia, Canada and in parts of Africa chickpea has gained a significant importance as rotation crop because of its ability to fix nitrogen and due to higher demand in global market. The Indian imports figure around 3-4 lakh tons i.e. 30% of the total world imports. The countries which exports chickpea to India are Canada, Australia, Iran, Myanmar, Tanzania, Pakistan, Turkey and France. This indicates large gap in the demand and supply for chickpea in India.

Evolution of cultivated chickpea crop has four major bottlenecks in achieving its highest yield potential and adaptation (Abbo *et al.* 2003). They are,

(i) Limited distribution of the wild progenitor *Cicer reticulatum* L.

(ii) Founder effect associated with domestication

(iii) Sowing shift from winter to spring in early crop history had made the crop to grow in residual soil moisture instead of rainy season in most of regions of the world and

(iv) Replacement of landraces by elite cultivars as a result of modern plant breeding programmes.

In addition to these, the yield loss of the crop by biotic and abiotic stress had accounted to be 4.8 and 6.4 million tonnes annually (Ryan, 1997). It is reported that under optimum condition yield potential of chickpea is 6t/h which is higher than the current global yield average of 0.8 t/h. The drought stress was found to be the harshest among abiotic stress as it accounts upto 40-50% of yield loss in chickpea followed by salinity (8-10%) and heat (exact % yield loss is still unavailable in chickpea). Among the abiotic, the stresses drought, heat and cold was experienced in all crop growing regions, whereas salinity and nutrient deficiencies were particular to certain regions of the world. Efforts to breed to achieve high level of tolerance for each stress were carried out for several years. Most of the time the only focus is yield and very few works had focused in understanding the reason for tolerance/ sensitivity. In past 15 years, in India there was major shift of chickpea growing regions from northern India to central and southern India. Since flowering, the crop experience a combination of terminal drought, salinity and heat stress. Parameters like flower abortion, pod abortion, loss of function of pistil under drought condition in two genotypes (Fang *et al.* 2011), accumulation of higher Cl^- in shoot in one genotype (Samineni *et al.*

2010) and Na^+ in shoot under salinity, loss of pollen viability, improper anther dehiscence in 4-6 genotypes under heat stress found to have role in decreasing yield (Devasirvatham *et al.* 2010). It is reported that the water stress may accelerate the flower initiation (Fang *et al.* 2010), whereas salinity and heat stress may delay the initiation of flower initiation and thus maturity in chickpea (Pushpavalli *et al.* 2015; Devasirvatham *et al.* 2012).

Thus, it becomes important to consider all the three stresses and what happens or what are the parameters/ traits that confer tolerance to the crop under various stresses. Before understanding the effect of combined stress on the crop, it becomes important to understand how the crop responds to each stress separately. In new breeding strategies, not only yield but it is considered intelligent to breed as many surrogate traits along with yield to achieve higher tolerance under any stress conditions.

In the present study, four major objectives were set to understand,

- Effect of progressive water stress treatment at flowering on chickpea reproduction in glass house condition.
- Effect of salinity (NaCl) & Na^+, Cl^-, K^+ ion accumulation on yield.
- Mapping quantitative trait loci and identification of candidate genes for salinity tolerance in ICCV 2 (Salt-sensitive) and JG 11 (Salt-tolerant) derived 188 recombinant inbred chickpea lines.
- Effect of atmospheric drought imposed by low relative humidity on yield, pollen viability, pollen *in vivo* germination and canopy temperature.

Based on the results obtained from this study, we would be able to identify the traits that are important to be considered under particular stress condition (water deficit, salinity, relative humidity) in future chickpea research programme. Through this study we predicted to find any similarities/ dissimilarities across three stresses. Also this study may help to widen the knowledge on abiotic stress tolerance in chickpea.

General Review of Literature

Origin

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n = 2x = 16) annual legume belong to the section monocicer, family *Fabaceae* (Van der Maesan, 1987). Chickpea is one of the seven crops that were reported to be domesticated since Neolithic age (10,000 years) (Abbo *et al.* 2003). Chickpea is believed to be originated in southeastern Turkey (Ladizinsky and Adler, 1976) and had spread from west and south via Kabuli. Four centers of diversity have been identified in the Mediterranean, Central Asia, the Near East and India, as well as a secondary center of origin in Ethiopia. Morphologically chickpeas are divided into two different types - "Kabuli" (large round seeds of white or pale cream-color) and "*Desi*" (smaller, angular seeds with sharp edges).

Nutrition

Chickpea is a highly nutritious grain legume crop and is one of the cheapest sources of protein for people who can't afford to non-vegetarian food or vegetarian by choice. It can be eaten raw, roasted or boiled. Mature seeds of chickpea are an important source of energy, protein and soluble and insoluble fiber. Mature chickpea grains contain 20-30% of protein which is higher than any other pulse crop. It is reported that the mature total dry seed mass of chickpea constitutes 80% of carbohydrates + protein (19-20%) rich in and only 2.7-6.48% fat. It is also a good source of vitamins (especially B vitamins) and minerals like calcium, magnesium, iron, potassium, zinc and phosphorus (Jukanti *et al.* 2012). Chickpea contains

isoflavones like biochanin A (5, 7-dihydroxy-40-methoxyisoflavone) and formononetin (7-hydroxy-40-methoxyisoflavone) (phytochemicals that helps in preventing diseases like cardiovascular, cancer etc.,) but not in high amounts as in soybeans (USDA-ARS 2004). Flavonoids such as naringenin and daidzein found to have role in induction of nod genes in chickpea.

They provide more beneficial carotenoids such as *β* carotene than genetically engineered "Golden Rice". Unlike other legumes, chickpea seeds have no anti-nutritional or toxic compounds. Through symbiotic nitrogen fixation, the crop meets up to 80% of the soil's nitrogen needs which is more beneficial for farmers.

Nitrogen fixation

Grain legumes, in rotation with cereals, will provide additional source of income for farmers and provide a net input to soil N. Chickpea is one of the important legumes in the farming systems in India, Europe and Mediterranean basin. Agronomic factors such as climate, crop management, plant nutrition, soil characteristics (mainly soil moisture and pH) are all of major importance for legume growth, nodule formation, and maximum N_2 fixation. It is also important to confirm that rhizobia inoculation is effective under field conditions. The scale of nitrogen fixed and economic benefits from growing a legume is determined by grain yield, the amount of legume biomass and the C: N ratio of the legume residue. Removal of the residue from the field can often result in a net depletion of soil N. For instance, the northern grain growers of Australia sowed about 450,000 hectares of chickpeas and 30,000 hectares of faba beans in 2012, resulting in the

fixation of about 35,000 tonnes of nitrogen (N) worth $55 million in fertiliser N equivalence.

(http://www.grdc.com.au/Resources/Factsheets/2013/07/Nitrogen-fixation-and-N-benefits-of-chickpeas-and-faba-beans-in-northern-farming-systems).

Cultivation and adaptation

Chickpea is grown either as a rain fed, post rainy season, or a winter crop in subtropical south Asia, parts of Africa and Australia (Plate 1, 2). In the temperate and Mediterranean regions it is grown as a spring season crop. In Asia and Africa, chickpea was traditionally grown as an intercrop, but in recent years it is mostly cultivated as a sole crop. In low input traditional production systems chickpea has been a preferred crop because of its minimal dependence on monetary inputs of N and P-containing fertilizers, irrigation, and agrochemicals in general.

Changes in phenological traits under stress

Large variation for the phenological traits, flowering and maturity (early, medium and late) exist in chickpea germplasm. The flowering in early maturity may be around 28-35 days and from then it took 4-6 days for the formation of small pod followed by pod wall reaching its complete size and seed filling. The maturity (the pods completely get dried in plant itself) happens at around 85-110 days (Plate 3, 4, 5 and 6).

The phenological traits were found to be conserved under optimum condition, but tend to undergo changes under stress conditions. For

instance in early maturity varieties, the flowering and maturity got accelerated in the case of drought conditions in medium maturing genotypes (in which flowering/maturity happens in 40-55/ 109-115 days, whereas it got accelerated by 38-51/83-98 days under severe stress). In saline conditions the flowering tends to get delayed, whereas the maturity gets accelerated in some cases and delayed in few other cases. In the case of heat stress, both flowering and maturity delayed and plants tend to have longer vegetative phase (Krishnamurthy *et al.* 2010; Pushpavalli *et al.* 2015; Personal communication- L Krishnamurthy, ICRISAT).

Bottlenecks

There is a large gap in the potential (around 3.0 to 5.0 t ha^{-1}) and realizable yield (world average 0.7 t ha^{-1}) of chickpea. In 1988, Dr Kenneth J Frey from Iowa State University called chickpea as a "recalcitrant" crop species meaning it was not very amenable to genetic improvement despite of several breeding efforts for three decades from 1960-1990.

The large gap between obtained and potential yield was mainly due to,

- Limited genetic diversity
- Change of sowing season from autumn to spring
- Biotic stresses
- Abiotic stresses

Limited genetic diversity is a common bottleneck not only for chickpea but also for several domesticated crops. The sowing season shift from autumn to

spring happened in early Bronze Age (3300–2100 BC) to get rid of *Aschochyta blight* epidemics (Berger *et al.* 2005).

Biotic stresses

Chickpea is prone to several soil borne diseases. Biotic stresses reported to reduce 4.8 million tonnes the overall chickpea yield annually (Ryan, 1997). Fungal disease followed by viral and bacterial disease found to cause overall reduction. Around 67 fungi, 22 viruses, 3 bacteria and 80 nematodes have been reported on chickpea (Nene *et al.* 1996), but only few among them cause diseases that lead to severe economic losses. Blight and wilt are the most devastating diseases affecting chickpea in temperate and tropical regions, respectively; while in the Mediterranean countries, Ascochyta blight, Fusarium wilt, Botrytis gray mold (BGM), stem rot, stunt and root rot are the most commonly occurring diseases (Haware,1998).

Abiotic stresses

Chickpea is exposed to various abiotic stresses like drought, cold, heat and salinity. Very often not a single stress but a combination of abiotic stresses affected the plant growth at various growth stages. The yield losses due to abiotic stresses were accounted to be more than 6.8 million tonnes which are higher than biotic stresses (Ryan, 1997). Chickpea growing countries were greatly concerned by the economic losses of 1.3 billion, 186 million and 354 million US dollars due to drought/heat, cold and salinity, respectively (Ryan, 1997).

Changing climatic scenarios tend to increase the severity of these stresses in chickpea growing regions. Several breeding efforts were made to attain

tolerance to each stress. Abiotic stress tolerance exhibits complex quantitative inheritance, in addition influenced by number of genetic and environmental interactions. Complex genetic architecture, phenotypic plasticity, continuously changing environments etc., made breeding for abiotic stresses tolerance a challenging task (Jha *et al.* 2014).

Drought stress

Terminal drought stress i.e., the drought that occurs mostly from reproductive stage till end of the crop growth. There is no chance of getting water in between this period. This drought alone accounted for 50% yield loss in chickpea annually. Drought can be intermittent as well as terminal based on the areas where chickpea is grown. Largely, the crop is grown in stored soil moisture which gradually depletes as the plant grows. The water demand during reproductive phase is higher compared to the vegetative phase of the plant. As a result the water depletes completely in the middle of reproductive phase reflected in termination of growth, flower, pod, seed abortion, interruption in seed formation and filling. Continuous efforts are being made by physiologists, breeders, geneticists and molecular scientists in achieving higher tolerance towards drought along with higher yield. As an outcome of these efforts several varieties that escape or tolerate drought and traits that confer drought tolerance and high yield were identified. Few such traits that confer drought tolerance in chickpea are proper functioning of pistils, less flower pod, seed abortion, high seed number, increased root volume, better use of available water, deep rooting system, early flowering, high drought tolerance index (DTI), high proline content, cell membrane

stability and high rate of partitioning associated with DTI (Jha *et al.* 2014). Physiological traits such as genotypes having high water use efficiency, high transpiration efficiency were also found to be beneficial in chickpea.

Several molecular tools such as dense genetic maps, quantitative trait loci, molecular markers associated with drought tolerance, candidate genes for 100-seed weight, hot spot regions for root traits were identified that are associated with drought tolerance related traits (Jaganathan *et al.* 2015). Chickpea draft genome is now been sequenced opening new windows to use quick molecular methods in breeding (Varshney *et al.* 2013).

Though there are efforts made for improving and identifying materials and traits, there are still several traits that have direct and indirect link with high yield and drought tolerance need to be explored. Efforts in identifying a genotype that has several positive traits towards drought tolerance, stacking several surrogate traits that confer drought tolerance through different disciplines is of great importance in future chickpea breeding programmes.

Salinity stress

Salinity is a problem that can be consistent in few chickpea growing areas like in Australia or it occurs in association with drought and heat when there is change in water balance in soil. Genetic variability for salinity tolerance was reported by Vadez *et al.* (2007) in chickpea. Chickpea is generally considered a sensitive crop towards saline conditions. Salinity and its effects were reviewed in detail by Flowers *et al.* (2010). Yield loss due to salinity was reported to be 8 to 10% in overall production of chickpea. Higher saline condition could even inhibit germination. Salinity has negative

effect on germination, vegetative phase and severely in reproductive phase. Imposing salinity in soil and evaluation of its effect is more critical than any other abiotic stress.

Drought, salinity, extreme temperatures (cold and heat) and oxidative stress are interrelated in natural environment of chickpea growing areas. These conditions separately or in combination induce cellular damage. These stress stimuli are complex in nature and may induce responses that are equally, if not more, complex in nature. Severe drought during critical growth phases may directly result in mechanical damage, changes in the synthesis of macro molecules, and low osmotic potential in the cellular settings. In addition, almost all of these abiotic stresses lead to oxidative stress and involve the formation of reactive oxygen species (ROS) in plant cells. Plants have mechanisms to reduce their oxidative damage by the activation of antioxidant enzymes and the accumulation of compatible solutes that effectively scavenge ROS. However, if the production of activated oxygen exceeds the plant's capacity to detoxify it, deleterious degenerative reactions may occur with the typical symptoms being loss of osmotic responsiveness, wilting and necrosis. The sensing of biotic and abiotic stress induces signaling cascades that activate ion channels, kinase cascades, production of reactive oxygen species, accumulation of hormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA). These signals ultimately induce expression of specific sub-sets of defense genes that lead to the assembly of the overall defense reaction (Fraire-Velázquez *et al.* 2011).

As in drought, several salinity tolerant lines and traits were identified after several years of breeding and pre-breeding efforts. Several physiological and biochemical traits such as lower Na^+ concentration in roots, exclusion of Na^+ in roots avoiding them in phloem pathway, better ion homeostasis and thus achieving osmoregulation, early flowering, large number of tertiary branches, higher flower number, early flowering etc., were identified that conferred salinity tolerance. Recently it is found that the higher Na^+ ion concentration solely affects the yield but not the Cl^- ion concentration in chickpea. In fact, the presence of higher Cl^- and K^+ concentration found to be beneficial under saline condition. When the plants are exposed to a level of salinity where yield loss is 50% in sensitive genotypes compare to control, the pattern of ion accumulation in different tissues highly varied. The vegetative tissues tend to accumulate or compartmentalize the toxic ions and letting least amount of these ions reaching reproductive tissues. Such several underlying mechanisms that are responsible for sensitivity/ tolerance to salinity in chickpea are yet to be explored.

QTL mapping for salinity tolerance

Mapping of quantitative trait loci (QTLs) for salinity tolerance can enable dissection of the genetic control of each tolerance mechanism, opening up the possibility of future efforts to develop varieties with improved salinity tolerance by precisely transferring QTLs into popular varieties and pyramiding multiple relevant QTLs for a particular stress-prone environment. A number of mapping studies have identified QTLs associated with salinity tolerance in rice QTLs controlling tolerance traits, including

major QTLs for shoot K^+ concentration on chromosome 1 ($qSKC^{-1}$) and shoot Na^+ concentration on chromosome 7 . The SKC1 gene was subsequently cloned and found to encode a sodium transporter that helps control K^+ homeostasis under salt stress (Thomson *et al.* 2010). In soybean, a genomic region harbouring single dominant gene *Ncl* (Qi *et al.* 2014), responsible for chloride concentration in leaves found to confer salinity tolerance. In chickpea, there are only one study reported major QTLs for salinity tolerance (Vadez *et al.* 2012). Availability of dense genetic maps, candidate genes for salinity tolerance in chickpea do not exist and QTLs reported for traits that confer salinity tolerance is very few compared to drought, Aschochyta blight and Fusarium wilt in chickpea.

Relative humidity stress

Heat stress is often more destructive in reproductive stage of chickpea growth. Heat stress studies gained importance in recent years due to changing and predicted climatic change and shift of chickpea growing areas from cooler northern states to hotter central and southern states (Plate 7). Few traits such as early maturity, higher filled pod number, high rate of portioning, cooler canopy, maximum extraction of soil water, higher rate of viability of pollen were found to confer higher heat tolerance in chickpea.

In chickpea, compared to research on other stresses, the heat tolerance related research is in its budding stage. It is found that the sensitive genotypes tend to loss the pollen viability at a maximum temperature of 33°C and the tolerant genotypes can maintain pollen viability till 40°C. In this particular experiment, not much importance was given in maintaining

relative humidity (Devasirvatham *et al.* 2012). When we consider heat, many times it is referred to maximum temperature. But, in reality, it is the compounded effect of maximum temperature and lower humidity in atmosphere and in soil. Considering the chickpea growing regions in India, there are several days where the relative humidity dropped below 15% during summer season when the heat studies were conducted. In any future heat tolerance study, it is important to focus on both temperature and humidity effects/ heat tolerance should be seen through the perspective of high vapour pressure deficit and not merely temperature while selecting parent material for breeding. Large scale high throughput phenotyping studies, developing large mapping populations, high throughput genotyping are required in understanding the physiological, biological and genetic mechanisms behind heat stress tolerance in chickpea.

Drought alone reduces yield significantly, compounded by high sensitivity to heat and salinity. As this situation is predicted to become more severe under predicted climate change scenarios, specific breeding and selection for tolerance to drought, heat and salinity are in priority to be addressed in chickpea.

Chapter 3- Effect of Water Stress on Flowering and Seed Set

Introduction

Chickpea, a cool-season food legume, has a global production of 10.9 Mt grown on 11.9 Mha (FAOSTAT, 2013), second only to soybean. In India, chickpea ranks second next to soybean with total production area of 6897×10^3 ha with 5597×10^3 Mt production, 811kg/ha yield

(http://www.icrisat.org/tropicallegumesII/pdfs/BTL1020111029.pdf).

It is grown either on stored soil moisture after the rainy season (South Asia, Eastern Africa, North-Eastern Australia), or in the rainy season itself (Canada and Mediterranean-climatic regions) (Berger *et al.* 2004). Whether grown on stored soil moisture or current rainfall, chickpea is exposed to terminal water shortage during the reproductive phase (Siddique *et al.* 2000; Turner 2003). In India, where 75% of the world's chickpeas are grown, the crop experiences severe drought during the reproductive phase (at flowering and podding that occurs in January to March in India) as a result of the cultivated region having been displaced from the cooler, longer-season in the north to the hotter, dryer, and shorter-season in the south and east of the country (Gowda *et al.* 2009). Identification and understanding parameters that result in improved drought tolerance is important in selection of parental lines for drought-prone areas.

The effect of water deficits at both the vegetative and reproductive phases of chickpea has been studied in both the field and the glasshouse conditions (Leport *et al.* 1998, 1999; Davies *et al.* 1999; Zaman-Allah *et al.* 2011b; Fang *et al.* 2010, 2011). Davies *et al.* (2000) and Leport *et al.* (2006) showed

that flower and pod abortion, along with reduced pod production, limited chickpea seed yield during terminal drought stress. In most of the above mentioned studies, one to six genotypes were used and it is not clear whether all genotypes were exposed to similar rates of drying. Therefore a question remains whether the observed effects were actual sensitivity differences in the reproductive phase, or whether they were consequences of differences in the soil water status during the drying phase. In the current study, the application of a controlled slow progressive water deficit was imposed to ensure an equal availability of soil water for all genotypes throughout the stress treatment using a protocol (dry-down method) used earlier in chickpea (Zaman-Allah *et al.* 2011a). This protocol minimizes differences in water use due to differences in leaf area, conductance, or rooting. It also allowed us to distinguish between a phase of mild stress when the water loss of the water-stressed plants was between 100% and 50% of that in the fully irrigated plants, and a subsequent phase of severe stress when the water loss of the water stressed plants was less than 50% of that in the fully irrigated plants.

The objective of the study was to test whether there is genetic variation in the sensitivity of reproductive biology to a controlled water stress, and, if so, to assess the variables that lead to greater reproductive success under controlled water stress. The work was conducted on ten chickpea genotypes that were previously observed to contrast for seed yield under terminal stress in the field) where sensitive genotypes produced less than 1000 kg/ha and tolerant genotypes had yield higher than 2000 kg/ha (Krishnamurthy *et al.* 2010). The specific objectives were: (i) to assess flower and pod number

and their abortion along with yield components of the chickpea genotypes during a phase of mild stress and the subsequent phase of severe water stress in comparison with the same genotypes given adequate water, (ii) to test whether tolerant genotypes have different responses to the slow soil drying from sensitive genotypes during either or both of the two stress phases, and (iii) to determine whether any parameter is linked directly or indirectly to higher yields in the water-deficit treatment.

Materials and methods

This study was conducted in the glasshouse at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India (17°30'N; 78°16'E; altitude 549 m). Ten chickpea genotypes from ICRISAT's mini-core and reference collections (Upadhyaya and Ortiz 2001; Upadhyaya *et al.* 2008) were selected based on observed differences in yield under terminal drought conditions in the field at ICRISAT (Krishnamurthy *et al.* 2010) – five drought-sensitive (S) and five drought-tolerant (T) - ICC8058 (S), ICC4814 (S), ICC3776 (S), ICC7184 (S), ICC7323(S), ICC3325 (T), ICC867 (T), ICC8950 (T), ICC14799 (T) and ICC2263 (T). The genotypes came from five different countries and had comparable times to flowering and maturity (Table 1).

Plant growth

One hundred and fifty 275-mm diameter pots filled with 9 kg of vertisol (fine montmorillontitic isohyperthermic typic pallustert) were sown with four seeds per pot after the seeds were treated with Thiram® (Sudhama Chemicals Pvt. Ltd., Gujarat, India) to control seed-borne infections. Since,

native rhizobia bacteria were available in the soil collected from farm, no separate inoculation of bacteria was carried out. This applies to all the experiments. In the first week of December 2009 and in the third week of December 2010, 15 pots were randomly assigned to each genotype. The maximum day/night temperature and relative humidity was 28.5/ 15°C and 40/90% in 2009 whereas in 2010 it was 32/15°C and 35/85%. The maximum and minimum VPD ranged between 0.2- 2.3 kPa in 2009 and 0.3-3.1 kPa in 2010.

All the pots were thinned to two plants per pot 14 days after sowing (DAS) and maintained well watered (WW) until the first flower had opened in all genotypes. Two plants were kept to have bigger canopy that facilitate transpiration measurements. Among the 15 pots per genotype, 12 pots with healthy similar sized plants were selected by visual observation for the experiment.

Exposure to water deficit

In this study, dry down methodology, a method to achieve progressive soil moisture deficit, where the soil dries over period as described by Vadez and Sinclair (2001) was used. At flowering (50 DAS), the plants were exposed to two watering treatments: six replicate pots in each genotype were kept well watered (WW) and six replicate pots were water stressed (WS). All pots were watered to excess and allowed to drain overnight to field capacity. The following morning each pot was enclosed in a transparent plastic bag that was wrapped around the base of the stem to prevent evaporation from the soil and subsequently weighed at 100% field capacity. Thereafter, all pots

were weighed each morning at 09.00 h Indian Standard Time (IST) and the rate of daily transpiration was calculated as the difference in weight between successive days. The experimental design was a randomized block design (RBD) with two treatments, WW and WS as the main factor, and genotypes as the sub factor randomized in each main block.

To avoid waterlogging, the WW plants were maintained at 90% field capacity throughout the experiment. The WS treatment was imposed gradually by allowing the plants to lose only a maximum of 70 g of water per pot each day to avoid a too rapid imposition of water stress. Water loss greater than 70 g pot^{-1} day^{-1} was added back, as described by Vadez and Sinclair (2001). In this way, all the plants in the water-stressed treatment were reset to the same soil moisture content on each day of the experiment, regardless of their size and water use, until the water loss was less than 70 g pot^{-1} day^{-1}. In fact, all the pots lost more than 70 g per day, so that the soil moisture declined at a very similar rate. It was only at later stages (phase II) that there were slight differences in the daily water losses but not sufficient to lead to major FTSW differences.

Estimation of normalized transpiration ratio

In order to compare the transpiration rate of the WS plants to the fully irrigated (WW) controls, the transpiration data was subjected to two normalizations (see Vadez and Sinclair, 2001). First the transpiration ratio was calculated:

TR*ij* (daily transpiration ratio) = Transpiration rate of plant *i* in genotype *j* **/** Mean transpiration rate of WW of genotype *j*

Then to minimize the differences in plant size within a genotype, the transpiration ratios of each day for each individual plant was divided by the mean TR of the first 3 days, i.e. before there was any stress. The normalized transpiration ratios, NTRi, were then:

$$NTR_i = TRi \text{ day } n / \text{Mean TRi days 1-3.}$$

Once the transpiration rate had started to decrease compared to the fully irrigated controls, that is when the NTR values fell below 1.0, the water stress treatment was divided into two phases based on the NTR values: phase I was when the NTR values were between 1.0 and 0.5 (100% and 50% of the irrigated controls), *i.e.* a mild stress, and phase II was when the NTR values were between 0.49 and 0.10 (49% to 10% of the irrigated controls), *i.e.* a severe stress. Based on a typical dry down curve (data not shown), the transition from the non-stress to phase I (NTR falls below 1) was when the fraction of transpirable soil water (FTSW, see below for calculation) was between 50 and 60%. The transition from phase I to phase II occurred at a FTSW between 20 and 30% FTSW. By definition of the NTR at the end of phase II there was no more transpirable soil water in the pots and therefore the FTSW was down to zero. No leaf water potential was taken as in this study the soil moisture was used as stress index rather than the plant water status. The exposure to water stress was terminated for each genotype when the NTR value of the WS plants fell below 0.1, i.e. when the transpiration of

WS plants was less than 10% of that in WW plants and when it was assumed that stomata were fully closed (Sinclair and Ludlow, 1985). Once the NTR values of the WS plants had dropped below 0.1 (79-85 DAS in 2009 and 81-87 DAS in 2010), they were given one amount of 400 mL of water to stop the stress treatment, whereas no water was given to WW plants. This amount represented 18% of the water required to bring the soil to 100% FC and insufficient to allow any regrowth or flowering. The purpose was not to attempt a recovery phase but to let the plants mature and completely dry before harvest 15-21 days later in 2009 and 11-17 days later in 2010. In any case, at this stage flowering had ceased and this period of the crop cycle had no bearing on the final yield results.

Estimation of fraction of transpirable soil water (FTSW) and relationship with transpiration

The fraction of transpirable soil water (FTSW) was back-calculated for each day of the experiment after the water stress was relieved. FTSW is the fraction of water available in the soil for plant transpiration between the stage when the soil is at field capacity and that when transpiration has become negligible and when it was considered there was no longer any water available for transpiration, i.e, when the NTR was below 0.1. Therefore the FTSW is set at 1 when the soil is at field capacity (100%) and 0 when NTR falls below 0.1. The difference in pot weight between field capacity (the first weighing of pots) and FTSW=0 (the pot weight when NTR reaches 0.1) provides the total transpirable soil water (TTSW) of the pot. TTSW is then used to calculate daily FTSW on any day 'n' such as:

$$FTSW_n = 1 - (Weight_{FC} - Weight_{day\ n})/TTSW$$

Where $Weight_{FC}$ and $Weight_{day\ n}$ are the pot weight at field capacity and on every single day 'n'.

For each plant of a genotype, the daily NTR values were plotted as a function of FTSW value. A plateau regression procedure (Ray and Sinclair, 1998) was used to determine the FTSW threshold (t) when NTR began to decrease i for each genotype. The plateau regression procedure carried out iterations of the NTR data starting at FTSW=1 (wet soil) and fitted them to *y*=1 equation. When *y*=1 was no longer the best fit for the response of NTR to FTSW, data were fitted to a linear decline equation. The FTSW threshold (with confidence interval) at which NTR began to decrease was taken as the intersection between the plateau (*y*=1) and the linear decline equation.

Parameters measured

The parameters measured in this study were flower number, flower+ pod+ seed abortion, pod number, pod weight, seed number, seed weight, harvest index (HI) and 100-seed weight across phases and treatments. Each flower that developed was identified daily with a short piece of wool thread placed at the node; different color threads were used in Phase I and Phase II in the WS treatment. When 90-95% plants of a given genotype in the WS treatment entered into phase II, the color of the thread was also changed in the WW plants of the corresponding genotype thus dividing the WW treatment into

phase I and phase II for comparison of each phase across water regimes. Identification of flowers with the threads was discontinued in the WW treatment at the end of phase II. The plants were harvested at maturity after complete drying (101 DAS in 2009-2010, 104 DAS in 2010-2011) by cutting the plants at soil level and oven dried at 65°C for 48 h prior measuring the plant components. The stem + leaf weight was separated for each plant per pot and the mean calculated per pot. Flowers were counted from the number of threads in each phase. At harvest, the threads and corresponding pod were put into one of the two categories: (i) threads where no pod was present or the pods were empty or had only undeveloped seeds, and (ii) threads with large pods containing one or two seeds (chickpea has a maximum of two seeds per pod). Category (i) represented the flower + pod+ seed abortion, and category (ii) represented the fertile pods with complete seed development. The seed number was recorded by hand threshing the fertile pods in category (ii) and subsequently weighed to obtain seed weight from each pot of each genotype and treatment. The seed weight obtained from flowers and pods identified in phase I, phase II and their sum are mentioned as 'phase I seed yield', 'phase II seed yield' and 'total seed yield' respectively. Except for the stem + leaf weight and the HI, the seed and pod parameters were measured separately for phase I and phase II. All the parameters that were evaluated separately in phase I, phase II and their sum (phase I + phase II) were prefixed with the terms phase I, phase II and total. The 100-seed weight was calculated by dividing the seed yield by the seed number and multiplied by 100 (total, phase I and II). The HI at maturity was calculated from the ratio of total seed yield to total

aboveground plant dry weight. The total flower + pod + seed abortion percentage was calculated by dividing threads in category (i) where no pods or pods with no or small pods were recorded by total thread (total flower) number multiplied by 100.

Statistical analysis

The data were analyzed using GENSTAT 12.0 (VSN International Ltd., Hemel Hempstead, UK) where an Unbalanced Analysis of Variance was carried out for all observed parameters. To calculate FTSW threshold SAS 9.2 software (SAS Institute, Cary, NC, USA) was used. Differences between mean values of treatments were evaluated using the least significant difference (LSD) at 0.05 significance level. The data were plotted and linear regressions were fitted using Microsoft Excel 2007 (Microsoft Corp. 1985).

Results

Development of water stress

Phase I (mild stress) and phase II (severe stress) (Plate 8) were longer in 2010 than in 2009 (Table 2). In 2009, the mean duration across genotypes of phase I and phase II was 11 days, whereas in 2010, it was 17.4 days (Table 2). The FTSW threshold values when NTR began to decrease varied between 0.50 and 0.83 in 2009 and 0.67 to 0.86 in 2010 except ICC4814 (0.44). There was no significant difference in the threshold values between the putatively tolerant group and putatively sensitive group of genotypes from the field evaluation and no significant correlation between the threshold values and drought tolerance (relative yield in the glasshouse) among the genotypes in either 2009 (R^2=0.12) or in 2010 (R^2=0.09).

Comparisons across years and stress phases

When averaged across the ten genotypes, phase II flower number, phase II seed number and phase II seed yield were lower than in phase I in the WW treatment in 2010 and in the WS treatment in both the years, indicating that values of the reproductive stage parameters, except the total 100-seed weight, decreased with time, even under WW conditions (Fig. 1). There was also a year effect in these results. However the differences between phase I and phase II were greater in the WS treatment, indicating a specific effect of the severe stress treatment on parameters (flower number, seed number and seed yield), particularly in 2010 (Fig. 1). The phase I flower + pod + seed abortion percentage was greater in the WS treatment than in the WW treatment only in 2009 (Fig. 1).

Genotype and genotype × year interaction

Although the experiments were conducted in a controlled-environment glasshouse in both years, there were significant genotype × year interactions for most parameters (Table 3), indicating that the conditions in the two years of experiment were very different. In the WW treatment, the genotype and genotype × year interactions were significant for all parameters in both phases except for phase II 100-seed weight, and the phase II seed yield varied among genotypes under WW condition in both the years (Table 3). The *F* statistic value suggests that the interaction effect between genotype and year had an effect that was equal or larger than the effect of genotype on phase I and phase II flower + pod + seed abortion percentage and phase I and phase II flower number in the WW treatment, indicating a strong

influence of the year on these parameters. For the other yield components in phase I and phase II (pod and seed number, pod weight and seed yield, but not 100-seed weight) the genotype × year interactions were also significant, but the *F* statistic values were larger for the G component indicating these variables were strongly affected by the genotype. In summary, while indeed the two year offered different conditions to the trials, which had effect on some aspects of the experimentation, it was mostly the genotypic effects that ruled the variations.

In the WS treatment, the genotypes differed significantly for all parameters except phase II flower number and phase I and phase II flower + pod + seed abortion percentage. The interaction between genotype × year was not significant for phase I and phase II 100-seed weight and for phase II flower number and phase II flower + pod + seed abortion number. The *F* statistic values suggested strong genotype × year interaction effects on the phase I and phase II flower + pod + seed abortion percentage and on the phase I flower number. The *F* statistic value suggests that the relative stem + leaf weight, relative total seed number, relative phase I pod weight were determined by genotype × year interaction effects, whereas relative total pod weight, relative HI, relative phase I flower number and relative phase II flower + pod + seed abortion percentage were determined largely by the genetic component (Table 4).

Genotype and genotype × treatment interaction

The genotypes varied significantly for all parameters in phase I in both years and the WS treatment has a significant effect on all parameters except

phase I flower number in 2009. However, the genotype × treatment interaction was not significant in 2009 for any parameter, and in 2010, although the genotype × treatment interaction were significant (Table 5), the *F* statistic values were higher for the genotype component, indicating that variation in the different parameters was strongly driven by the genetic component.

As the genotypes varied significantly under both WW and WS treatments in both years and no or limited genotype × treatment interaction was found, the variation in the WS treatment might be the result of differences in the WW treatment. Therefore, we calculated the relative values (WS/WW) of the parameters to remove possible differences among the genotypes under WS conditions that might arise from differences in the WW treatment. The absolute (in the WS treatment) and relative values of total seed yield (Table 6) and phase I and phase II seed yield (Table 7) were ranked in the order of high to low seed yield. This was to assess whether the high yielding genotypes in the WS treatment had high potential yields (high yields in the WW conditions) and whether they showed any similarity to the drought tolerance scores found in the field and which were used to classify the genotypes into drought tolerant and drought sensitive genotypes in this study. The ranking of the total seed yield in the WS treatment did demonstrate that the group of putatively drought tolerant genotypes had higher total seed yield than the putatively drought sensitive genotypes in 2009 and in four of the five genotypes in 2010. However, the ranking of the relative values of total seed yield, phase I seed yield and phase II seed yield

did not match the ranking of the absolute seed yield values in either of the years (Table 6, 7).

Seed yields and seed yield components

In the WW treatment, the phase I and phase II seed yields varied significantly among most of the genotypes in both years (Fig. 2) and this genetic variation was also apparent in the phase I and II seed number in both years, in phase I flower number and in phase I flower + pod + seed abortion percentage, but not in phase II flower number and in phase II flower + pod + seed abortion percentage in both years (Tables 5 and 6). Likewise, in the WS treatment, there were significant differences among the genotypes in total seed yield and in phase I and phase II seed yield (Fig. 3). There was also genetic variation in phase I and phase II seed number except in phase II, 2010, and in phase I flower + pod + seed abortion percentage, 2009 (Table 8, 9).

Relative total values of parameters to relative total seed yield

The relative total seed number i.e. the ratio of the total seed number in the WS treatment to that in the WW treatment was found to be closely related to the relative total seed yield in both the years (R^2 = 0.83, R^2 = 0.79) (Fig. 4). By contrast, the relative total 100-seed weight was not related to the relative total seed yield in both the years (R^2 = 0.008, R^2 = 0.057). The relative total flower number was found to be significantly associated with relative total seed yield in 2010 (R^2 = 0.23), but not in 2009 (R^2 = 0.031), but the relative total flower + pod + seed abortion percentage was not associated with the relative total seed yield (drought tolerance) in either year (R^2 = 0.002, R^2 =

0.009) (Table 10). The relative HI also showed significant correlation to relative total seed yield in both the years (R^2 = 0.39, R^2 = 0.53). The relative total pod number and relative total pod weight were also significantly correlated (R^2 = 0.22 and 0.59, R^2 = 0.97 and 0.94, 2009 and 2010) with relative total seed yield in both years (Appendix 1).

Relative values of parameters in phase I and II to relative total seed weight

In 2010, the relative phase I pod number (R^2 = 0.41), relative phase I flower number (R^2 = 0.42), were found to be significantly and positively associated to relative total seed yield. The relative phase I seed number was associated with high relative total seed yield in both years (R^2 = 0.46, 2009, R^2 = 0.45, 2010) and in phase II (R^2 = 0.31, in 2009), but not in 2010 as very few seeds were set. Similarly, the relative total pod weight (i.e. the ratio of the pod weight in the WS treatment to that in the WW treatment) was more closely related to the relative phase I pod weight (R^2 = 0.56, 2009, R^2 = 0.45, 2010), than in phase II (R^2 = 0.44, in 2009). The relative phase I seed number significantly contributed to relative phase I seed yield in both the years (R^2 = 0.78, 2009, R^2 = 0.46, 2010) and a similar trend followed in phase II (R^2 = 0.89, 2009, R^2 = 0.19, 2010). This suggests that the setting of a high seed number was important in the production of high seed yields when the stress was both mild and severe. The contribution of phase II seed yield to total seed yield was still significant, indicating that the number of seeds under severe stress conditions also contributed substantially to the total seed yield. The relative phase I flower number contributed more significantly (R^2 =

0.26, 2009, R^2 = 0.46, 2010) to relative phase I seed yield compared to the case in phase II (R^2 = 0.12, 2009, R^2 = 0.09, 2010) (Table 11) suggesting that the production of a high flower number under mild stress led to higher seed yield. However in both years, the relative total seed yield was more closely related to the relative phase I seed yield than to the relative phase II seed yield, indicating that most of the genetic variation for the sensitivity of the reproductive phase to water stress was expressed under a mild water stress (Table 10, 11).

Discussion

Comparison of drought tolerance/sensitivity in this glasshouse study with previous measures of drought tolerance/sensitivity in the field

In the WS treatment, the total seed yield of the genotypes that were observed to be higher yielding when exposed to terminal drought in the field remained higher than most of the sensitive genotypes in the present study (Fig. 3). This was also true for the phase I seed yield, but was not the case when measured in the pods produced in phase II when the stress was more severe. It is noted that several of the sensitive genotypes had also a poor yield under WW conditions, suggesting that part of the differences under WS conditions was explained by differences in the yield potential (Fig. 2, 3). Thus the relative values were calculated for each parameter. The ranking for relative total seed yield differed from the ranking of absolute yields in the field in both years indicating that the yield potential of genotypes explained in part the performance of the genotypes under stress conditions. Therefore,

we used the relative values to analyse the possible causes for reproductive success in the drought-tolerant genotypes (Table 6).

Effect of drought on reproduction (flower, seed and pod numbers)

The maintenance of a high flower, pod and seed number led to high seed yield in this study (Fig. 4). There was a very significant and strong positive relationship between the relative total seed number and relative total yield in both years (Fig. 4). As relative seed size did not vary, the role of seed number in determining yield was clearly important. The variation in relative seed number among genotypes was largely the result of the variation in the relative flower number. There was a positive relationship between the relative total seed yield and the relative total flower number at least in one year (2010) (Table 7), and also clear relationships between the flower number produced in phase I and the relative seed yield produced in that phase in both years. By contrast, there was no association between the relative total seed yield and the relative total flower + pod + seed abortion in either of the years. Therefore, the genotypes that achieved high yields in the water stress treatment were those producing a large number of flowers, but not those that aborted fewer flowers + pods + seeds. The ability to produce a high number of flowers was particularly important in the phase I (the mild stress) (Table 7) and this led to higher phase I seed yield and thus overall. Chickpea as an indeterminate crop that continues to produce flowers, pods and set seeds while water is available (Croser *et al.* 2003). When the plants undergo water shortage, our results suggest that continuing to produce a high number of flowers and pods during the initial phase of mild water stress was a key factor in enhancing yield (Table 8). Production of a large

number of flowers was also found to be a key yield determinant under saline stress in chickpea (Vadez *et al.* 2012). These findings could be somewhat counter intuitive – we would expect that a lower seed / pod abortion rate would be more favorable. The results may therefore suggest that the key step in the reproductive process is to successfully fertilize flowers and generate a young fertile embryo. The production and maintenance of many flowers would indeed increase the chances of success of that key step. This would of course need further research.

The fact that there was only a weak association between the relative phase II seed yield and the relative phase II flower number indicated that much of the discriminatory effect of drought took place under mild stress conditions. Ontogenetic effects on flower production observed in both the WW and WS treatments presumably contributed to the poor association between flower number and seed yield in phase II. Moreover, in the present study, the later formed flowers/pods had a 20% higher abortion percentage than the early formed ones in water-limited environments in both the years (Table 9) in agreement with Fang *et al.* (2010). The ability of flowers to set pods is influenced by a number of environmental factors in addition to cultivar, and a large proportion (50-80%) of flowers do not develop pods even under WW conditions (Clarke and Siddique, 1998; Fang *et al.* 2010). As the environmental conditions in the glasshouse and the development of the water stress in phase I was similar in all genotypes, the differences in flower and seed production was largely genetic and this genetic variation in flower production in response to mild stress is clearly worthy of further investigation. In this study in year 2010 the sensitive genotypes showed

higher decrease in flower number (44%) than the tolerant genotypes (27%) but it is not the case in year 2009.

The 100-seed weight in both the mild and severe WS was not affected by the WS treatment compared to the WW treatment (Fig. 4), indicating that the seed filling was maintained under the controlled water stress conditions of this study. The seed number of tolerant and sensitive genotypes decreased by 51, 41% in year 2009 and by 38, 34% in year 2010 under mild stress treatment. This suggests that once the seed enters the phase of rapid dry weight accumulation, the young seeds already developed had priority for assimilates rather than the small seeds early in their development. Also, during seed development, the decrease in source: sink ratio may increase the abortion of young pods, but not the rate of seed growth in filling pods (Turner *et al.* 2005).

Therefore, reproductive success in a water-limited environment appears to be linked to the capacity to produce a large number of flowers and retain a high number of fertile seeds like in ICC3325 which was able to have more than 10-20 seeds on an average across years under mild water stress treatment, while maintaining the filling of these seeds. These results are very similar to the finding of Vadez *et al.* (2012) where the salinity tolerance was determined by maintenance of relatively large number of seeds in early and late- flowering chickpea genotypes. A similar trend of increased pod number under mild stress was observed as in seed number in tolerant genotypes. During mild stress, the seed yield reduced by 35, 57% and 28, 59% in tolerant, sensitive group in year 2009 and 2010.

Genotypic variation in threshold values – value of the dry down method

Previously water deficit and terminal drought studies have been carried out at both the vegetative and reproductive stages (Krishnamurthy *et al.* 2010; Zaman-Allah *et al.* 2011b; Leport *et al.* 2006; Fang *et al.* 2010, 2011). In this study, the water stress was gradually imposed to ten genotypes from the onset of flowering. The slow dry down by re-watering to the same soil water level in all genotypes overcomes the drawback of simply imposing a water deficit by withholding water when plants vary in leaf area and rates of transpiration and are then exposed to different rates of development of water deficit (Harb *et al.* 2012). The fact that we found a different ranking in the relative seed yield in these experiments and in the field indicate that we revealed specific sensitivities of genotypes to a controlled water stress imposed during reproduction. For instance, field tolerant ICC867 had ranking values close or equal to those of the sensitive genotypes, indicating that this genotype must have its reproductive stage particularly sensitive to the application of a water stress. On the contrary, field sensitive ICC7184 had high relative seed yield values, indicating that the low yield of this genotype in the field was not related to the sensitivity of its reproduction to drought.

The FTSW threshold values, measured here during the reproductive phase, tended to be slightly higher than those of Zaman-Allah *et al.* (2011b), measured in the vegetative phase of chickpea. In our study, although we found higher levels of genotypic variation among genotypes in *t,* the threshold values did not differ between the putatively tolerant and putatively sensitive genotypes either based on absolute or relative total seed yield as

also observed under glasshouse conditions by Zaman-Allah *et al.* (2011b). While the rates of development of water deficit were similar among genotypes within years in the present study, they were not identical across years, in part because of plant size differences across years, probably caused by differences in environmental conditions such as radiation or vapour pressure deficit that were not controlled in the glasshouse. However, these environmental differences had little effect on yield, which was mostly driven by genotypic variation, but had a substantial interaction effect on the parameters, seed number and flower number. As chickpea a legume crop, in this study as well the nitrogen fixing capacity might be reduced in water stress condition as reported by Serraj *et al.* (1999) due to physiological response on nitrogenase activity and involving mechanisms like carbon shortage, oxygen limitation or feedback regulation by nitrogen accumulation. This needs to be studied further in case of selected genotypes whether there is variation in their sensitivity to nitrogen fixation.

Conclusions

This study in a way shows the usefulness of dry down methodology to impose slow water deficit effect in controlled environments. Also, it shows that the flowers and seeds developed and maintained during mild water stress holds importance than that formed during severe stress conditions.

Chapter 4- Effect of Salinity on Phenology and Yield Parameters

Introduction

Salinity affects an arable land area of 100 million ha worldwide, and this area is increasing (Rengasamy, 2006). Chickpea is considered very sensitive to salinity (Flowers *et al.* 2010), but variation in salinity tolerance has been observed among chickpea accessions (Vadez *et al.* 2007; Krishnamurthy *et al.* 2011; Turner *et al.* 2013). However, little is known about the mechanisms of salt tolerance in chickpea. Adverse water relationships, excess Na^+ accumulation (Munns and Tester, 2008), interference with K^+ homeostasis, production of ROS in plant tissues are reportedly causes for crop sensitivity under exposure to salinity, but the influence of shoot Na^+ concentration in chickpea sensitivity/tolerance is equivocal (Abogadallah, 2010; Bose *et al.* 2014; Pottosin *et al.* 2014). Although shoot Na^+ concentration was low and was not associated with yield under saline conditions in a study with 263 accessions (Vadez *et al.* 2007), higher Na^+ concentrations in the youngest fully-expanded leaves were associated with lower yields under saline conditions in a second study with several of the same genotypes (Turner *et al.* 2013). The salt-sensitive genotypes also had higher concentrations of Na^+ in the seed than salt-tolerant genotypes (Turner *et al.* 2013). Salt sensitivity was not significantly associated with the accumulation of Na^+ in other tissues or the accumulation of Cl^- in any vegetative or reproductive tissues (Turner *et al.* 2013). Moreover, based on apparent critical concentrations for Cl^- and Na^+ in chickpea shoots (as reported in the literature), Samineni *et al.* (2011) hypothesized that Cl^- toxicity might be of importance in chickpea. On exposure to stresses like

drought and salinity, the plant cells are affected by osmotic stress and osmotic adjustment takes place to maintain normal turgor pressure by uptake of inorganic ions (Wyn Jones and Pritchard, 1989; Bohnert *et al.* 1995). Shabala and Lew (2002) showed that turgor recovery, along with increased uptake of K^+, Cl^- and Na^+, occurred in *Arabidopsis* root cells within a few minutes after a hyperosmotic stress treatment.

Yield per plant of chickpea in saline soil has been associated with more tertiary branches and flowers, as well as the capacity to maintain filled pods (Vadez *et al.* 2007, 2012). However, seed size was maintained under salinity, suggesting that seed set was more sensitive than the rate of seed filling under salinity (Vadez *et al.* 2007, 2012). Although pollen viability and germination were not affected by salinity, pod abortion was higher in sensitive genotypes (Turner *et al.* 2013), suggesting that reduced seed numbers may be due to failed fertilization or early seed development (Samineni *et al.* 2011; Turner *et al.* 2013).

Tissue concentrations of Na^+ and Cl^- in chickpea increased under saline conditions (Samineni *et al.* 2011; Turner *et al.* 2013). In white clover and white lupin, adverse changes in tissue ion homeostasis led to cellular damage, cessation of growth and tissue death, and eventually to plant death (Munns and Termatt, 1986; Manchanda and Sharma, 1989; Zhu, 2001). On the other hand, exposure of plants to salinity has been shown to induce osmotic adjustment by uptake of ions (Bernstein, 1961, 1963; Shabala and Lew, 2002) and synthesis of organic solutes (Greenway and Munns, 1980), result in increased production of abscisic acid (Wolf *et al.* 1990) and other

hormones, and increase ROS and activate antioxidant defense mechanisms (Bose *et al.* 2014; Pottosin *et al.* 2014). In their review, Munns and Tester (2008) concluded that exclusion of Na^+ and Cl^- by the roots and sequestering the ions in old tissues helped to avoid ion toxicity in young leaves and reproductive organs. Turner *et al.* (2013) found an association between higher Na^+ concentration in young leaves and seeds and salt sensitivity in chickpea, but no association in older tissues. This finding suggests that limiting ion accumulation in young tissues is important for salt tolerance, but may not relate to storing of salt ions in older tissues. In addition, there was no association under saline conditions between yield and the accumulation of Cl^- in leaves or pod shells and accumulation in the seed (Turner *et al.* 2013). This result for a larger number of genotypes suggests that Cl^- does not play a major part in salt tolerance/sensitivity in chickpea, and this finding furnishes an important broader understanding of the earlier physiological work of Samineni *et al.* (2011) on only one variety. Our present hypothesis is that reproductive success is the key factor in attaining a higher yield under salinity and that this linkage may relate to a particular pattern of ion accumulation in both reproductive and vegetative tissues.

In the present study, seven reportedly tolerant and seven reportedly sensitive genotypes (Krishnamurthy *et al.* 2011) were exposed to salinity, and their salinity tolerance, based on yield or relative yield, was confirmed. In an adjacent experiment, four salt-sensitive and four salt-tolerant genotypes were sampled for a systematic analysis of Na^+, K^+ and Cl^- concentrations in leaves, stems, floral and seed tissues during reproductive development. We focused on (i) confirming yield-related traits that

discriminate tolerant and sensitive genotypes for salinity, and (ii) determining whether Na^+, Cl^- and/or K^+ concentrations in vegetative and reproductive tissues were associated with salt tolerance/sensitivity among genotypes. The results of these investigations could yield a better understanding of salt tolerance in chickpea. The analysis of Na^+, K^+, and Cl^- concentrations in different vegetative and reproductive tissues helps to test the hypothesis that higher accumulation of the above-mentioned ions, in particular in reproductive tissues, under saline conditions compared with non-saline conditions will cause a disturbance of ion homeostasis and thus affect plant growth and yield. Salinity treatment found to delay flowering in chickpea in study by Krishnamurthy *et al.* (2010) but not in Vadez *et al.* (2007). In previous studies in chickpea yield has been affected by 30-50% by salinity.

Materials and Methods

Plant material, growth and treatment conditions

This study was conducted in pots buried in the field at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India (17°30'N; 78°16'E; altitude 549 m); the system enables soil salinity treatments to be imposed in outdoor conditions but with controlled soil salinity. Fourteen chickpea genotypes, contrasting for sensitivity to salinity based on yield, were selected from a larger study (Krishnamurthy *et al.* 2011). The seven sensitive (S) genotypes had low yields (ranging 1.9-3.1 g $plant^{-1}$) when exposed to salinity (ICC3421, ICC6263, ICC7315, ICC15510, ICC10755, ICC13283, ICC15518), and the seven tolerant (T) genotypes had

high yields (10.7-12.5 g pot^{-1}) when exposed to salinity (ICC11121, ICC1431, ICC4495, ICC8950, ICC456, ICC9942, ICC12215) (Krishnamurthy *et al.* 2011). The first four tolerant and sensitive genotypes listed were also used for ion analyses of different vegetative and reproductive tissues.

The experiment was carried out during November 2011-March 2012. Seeds were sown on November 15th 2011 and plants were harvested in March 2012. The average maximum and minimum air temperatures ranged from 29°C to 36.5°C and 12°C to 20°C, respectively. Pots (0.27 m diameter) containing 7.5 kg of a vertisol (fine montmorillontitic isohyperthermic typic pallustert) soil were buried in the soil so that the outer rim of each pot and outside soil surface were at the same level to avoid direct heating of the pots by solar radiation. The vertisol soil (pH = 8.1, cation exchange capacity (CEC)/clay ratio = 0.87, electrical conductivity (EC) = 0.1 mM) (El-Swaify *et al.* 1985) was taken from the ICRISAT farm and fertilized with di-ammonium phosphate at a rate of 300 mg kg^{-1} soil. One-half of the pots were artificially salinised with 1.17 g NaCl kg^{-1} soil, equivalent to 80 mM NaCl in sufficient volume (1.875 L) to wet the vertisol to field capacity. The control pots received tap water containing no significant amounts of NaCl, in the same volume to bring the soil to field capacity. Subsequent watering of both treatments was performed with tap water. The bottoms of the salinised pots were sealed to avoid any salt leaching.

In both treatments, six seeds were planted in each pot and later (14 DAS) thinned to four similar-sized plants per pot. The plants for the evaluation of Na^+, Cl^- and K^+ concentrations in vegetative and reproductive tissues were

adjacent to those for evaluation of yield and yield components. The experimental design was a RBD with two treatments, a non-saline control (0 mM NaCl) and a saline treatment (80 mM NaCl) as main factors and genotypes as sub-factors with four replications per treatment (each replicate was a single pot containing four plants).

Measurements

The parameters observed/calculated in this study include days to flowering, days to maturity, total above ground dry matter (shoot biomass), filled pod number, empty pod number, seed number, pod weight, seed weight, 100-seed weight. In the plants used for yield and yield components, time (days) to first flower (two plants per pot had commenced flowering) and to maturity (all plants in the pot had yellowed) was recorded. At maturity, all plants were harvested and oven dried at 65°C for 48 h. After drying, the number of filled pods, empty pods and seeds were counted, and total shoot dry matter, pod weight and seed yield (seed weight) were measured on a pot basis and calculated on a per plant basis. The 100-seed weight (seed size) was calculated from seed yield/seed number per pot.

When plants used for ion analysis reached the mid-podding stage (60–65 DAS for the genotypes used), tissue samples were collected for analyses of Na^+, Cl^-, and K^+. The tissues were: (i) old green leaves from the bottom 2–3 nodes, (ii) laminae of the youngest fully-expanded leaves, (iii) petioles of the youngest fully-expanded leaves, (iv) other leaves, i.e., all leaves between the oldest green leaves and the youngest fully-expanded leaves, (v) unopened flower buds from the top nodes, and (vi) seeds at the filling stage (developing

seeds). At maturity, mature seeds and pod shells were also sampled for ion analyses. Each tissue sample was placed into a paper envelope and oven dried at 60°C for 48 h. Tissues were weighed, ground and transferred (with appropriate export/import and quarantine permissions) to the laboratory at The University of Western Australia, Perth, Australia. Each sample was extracted in 0.5 M nitric acid in 10 mL tubes placed on a shaker for 48 h (Munns *et al.* 2010). The samples were then diluted as appropriate and analysed for Na^+ and K^+ on a Sherwood flame photometer (Model 410, Sherwood Scientific, Cambridge, UK), and Cl^- was measured using a chloridometer (SLAMED, model 50CL l-50, Frankfurt, Germany). Reference plant tissue with known ion concentrations was measured along with the samples and showed that the analyses recovered 95% of the Na^+, 98% of the Cl^- and 83% of the K^+; no adjustments were made to the measured values.

Statistical analysis

The data were analyzed using GENSTAT 12.0 (VSN International Ltd., Hemel Hempstead, UK). An unbalanced analysis of variance was performed for all observed parameters individually. As the number of genotypes differed for ion analysis and yield components, the two data sets were analyzed separately. Differences between mean values of treatments were evaluated using a LSD test at a 0.05 probability level. Linear regressions were fitted using Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA). A cluster analysis was performed using PAST software (version 1.9).

Results

Agronomic assessment

All parameters differed significantly for genotype, treatment and genotype × treatment interaction at the 5% level of significance except the interaction for total shoot dry matter (Table 12). In the control treatment, genotypes differed significantly ($P<0.001$) for days to flower, days to maturity, filled pod number, empty pod number, 100-seed weight, seed number and seed yield, but not for total shoot dry matter (Fig. 5, Table 13).

The salinity treatment (80 mM NaCl) induced a delay in flowering and maturity compared with the control. However, the delay in flowering and maturity in sensitive genotypes varied more (12 to 20 days for flowering except for ICC10755 (4 days); 1 to 23 days for maturity) than in tolerant genotypes (1 to 3 days for the delay in flowering; 1 to 5 days for the delay in maturity). When compared to control, salinity reduced total shoot dry matter by 30% in tolerant and 38% in sensitive genotypes (Table 13). The salt treatment reduced pod number per plant less in tolerant (59–96%) than in sensitive genotypes (78–99%) (Table 13). Similarly, the salt treatment reduced filled pod number per plant by 13–43% in tolerant and 48–89% in sensitive genotypes except for ICC10755 (S) and ICC15510 (S), which increased by 15% and 18%, respectively. Empty pod number was less in the salt treatment compared with the control plants (Table 12, 13), reflecting that salt-treated plants produced smaller numbers of pods. At 80 mM NaCl, the seeds of the tolerant genotypes were similar in size to those in the

controls, while several sensitive genotypes had smaller seeds than the controls (Table 13).

Yield in the 80 mM NaCl treatment varied more than 10-fold among the genotypes, ranging from 0.36–4.1 g per plant; all sensitive genotypes had lower yields than tolerant genotypes (Fig. 5). Salinity decreased seed number and seed yield per plant compared with the non-saline control, although the reduction was less in tolerant genotypes (23–45% for seed number, 10–46% for seed yield) than in sensitive genotypes (34–90% for seed number, 52–90% for seed yield) except for seed number in ICC10755 (S), which decreased by 14%; in ICC15510 (S), it increased by 8% (Fig. 5). These results could be related to the relatively low seed number in the control treatment of both of these sensitive genotypes. The highest seed number and seed yield in both treatments was recorded in ICC4495 (T) and the lowest in ICC15518 (S). The delay in flowering under 80 mM NaCl compared with 0 mM NaCl treatment was significantly associated with the reduced relative yields (R^2 = 0.21) (Fig. 6).

Seed number, filled pod number (Table 13) and seed yield (Fig. 5) in the 80 mM NaCl treatment clearly discriminated tolerant from sensitive genotypes. Even under the 0 mM NaCl treatment, sensitive genotypes had significantly fewer seeds compared to tolerant genotypes, but the seed yield of tolerant and sensitive genotypes did not differ in the non-saline treatment because the low seed number in sensitive genotypes was compensated for by a larger seed size. Thus, to account for the variation in seed parameters in the 0 mM NaCl treatment, parameters were expressed as relative values, calculated as

the ratio of values in 80 mM NaCl to the mean value of the trait under 0 mM NaCl for each genotype. The relative values were calculated only for the yield parameters but not for the ion concentrations as no significant relationship was found between ion concentration under control and saline treatment. Relative filled pod number (R^2 = 0.93) and relative seed number (R^2 = 0.96), but not relative seed size (R^2 = 0.028), were associated with relative yield (Fig. 7). Several replicates of both tolerant and sensitive genotypes had higher yields in saline pots than in non-saline controls, and this outcome was always associated with higher pod and seed numbers (Fig. 7).

Ion concentrations in various tissues

The concentration of Cl^- in tissues differed between the 0 and 80 mM NaCl treatments, but although salt treatment increased the Cl^- concentration in all tissues (Fig. 8) no genotypic differences between tolerant and sensitive group were observed for any ion concentration in the assessed tissues (Appendix 2, 3, 4 and box-and-whisker plots in Fig. 8, 9 and 10 show the ranges of tissue ion concentrations for the eight genotypes measured). In both treatments, old green leaves had the highest concentration of Cl^-, followed by other leaves, with the lowest in mature seeds; the increase more than two-fold in old green leaves and other leaves compared to the control.

The K^+ concentration was highest in the petioles, stems and laminae of fully expanded young leaves and lowest in mature seeds in both saline and non-saline treatments (Fig. 9). In no tissue was the K^+ concentration able to significantly discriminate the tolerant from the sensitive genotypes under saline conditions (Appendix 3). The concentration of Na^+ increased markedly

in tissues of plants in 80 mM NaCl, in the stems, in the laminae and petioles of fully-expanded young leaves, in seeds at the mid-pod filling stage and in mature seeds (Fig. 10). In most of the tissues, the Na^+ concentration was higher in the sensitive genotype ICC3421 than in all other genotypes (Appendix 4). However, the Na^+ concentrations in the various tissues did not discriminate the group of tolerant and sensitive genotypes, except for the old green leaves which contained higher Na^+ in the sensitive genotypes.

Relationships between tissue ions and seed yield in the 80 mM NaCl treatment

There were only a few associations between ion concentrations in tissues and seed yield. The accumulation of Cl^- in mature seeds, of K^+ in seeds at the filling stage and a higher K^+/Na^+ ratio in the laminae of fully-expanded young leaves under 80 mM NaCl treatment were positively associated with higher seed yield. The accumulation of Na^+ in old green leaves under saline treatment was negatively correlated with seed yield. Additionally, the mean Na^+ concentration in old green leaves differed between the tolerant (79 µmol g^{-1} dry mass) and sensitive (117 µmol g^{-1} dry mass) genotypes at $P<0.01$ (LSD = 20.50) except for ICC8950 (T), where the accumulation difference was not significant (Fig. 11).

Relationship between accumulation of ions and delay in flowering

The accumulation of Na^+ in laminae of fully-expanded young leaves (R^2 = 0.61), of K^+ in old green leaves (R^2 = 0.57), and of Na^+ in old green leaves (R^2 = 0.51) were all significantly ($P<0.05$) correlated with delayed flowering. The

more the Na^+ and K^+ accumulated in corresponding tissues, the longer the delay in flowering (Fig. 12).

Discussion

The main findings from the present study are as follows: (i) exposure to 80 mM NaCl delayed flowering to a greater extent in sensitive genotypes than tolerant ones, was related to lower seed yield, and was positively correlated with the accumulation of K^+ and Na^+ in leaf tissues, (ii) yield of chickpea under saline stress was determined by seed number, but not seed size, (iii) in none of the tissues did ion accumulation discriminate between tolerant and sensitive genotypes except for the slight increase in Na^+ concentration in old green leaves in the 80 mM NaCl treatment, (iv) the accumulation of Cl^- in mature seeds and K^+ in developing seeds, was positively associated with seed yield as was the K^+/Na^+ ratio in the laminae of fully-expanded young leaves, while the accumulation of Na^+ in old green leaves was negatively associated with the seed yield.

Agronomic traits

Effect of salinity on yield and yield components

Reduced seed yield under salinity was highly correlated with a reduction in filled pod and seed numbers, in agreement with previous reports (Vadez *et al.* 2007; Vadez *et al.* 2012; Samineni *et al.* 2011; Turner *et al.* 2013) (Fig. 7). The greater reduction in filled pod number in sensitive genotypes could be associated with higher levels of pod abortion than those that occur in tolerant genotypes. Similarly, in tomato, Albacete *et al.* (2014) showed that fruit set and development was affected under salinity and in turn caused

yield reduction. While the reason for the pod abortion under saline treatment is not clear, salinity could decrease sink activity and impaired sucrose metabolism by reducing the apoplastic and cytoplasmic sucrose cleaving enzyme activity. These changes could be mediated by changes in carbon supply or hormone concentration. Increased cytokinin concentration and/or metabolic activity have been linked to increase fruit sink strength, growth and yield in tomato under salinity (Albacete *et al*. 2014). In chickpea salinity could impair sucrose metabolism, increase abscisic acid production and/or decrease the production of cytokinins, and/or influence other metabolic factors (Flowers *et al*. 2010). More research is needed to ascertain a possible role of hormonal changes or carbon supply in pod abortion under salinity.

Effect of salinity on phenological development

Exposure to salinity delayed flowering and delayed flowering to a greater extent in the sensitive than tolerant genotypes (Fig. 6). The plants adjust their physiology to survive under salt stress, drought, high temperature and extending darkness by accelerating the vegetative growth combined with leaf senescence and enters rapidly into the reproductive phase i.e. flowering and podding (Allu *et al*. 2014). In contrast, in our study, though we found stunted growth in plants under saline treatment earlier leaf senescence was not observed. High salinity has been observed to delay the onset of flowering in many plant species (Van Zandt and Mooper, 2002). We are not aware of any reported delay in flowering arising from salinity in chickpea. Indeed, Turner *et al*. (2013) reported that salinity did not affect the time to first

flower. The delay in flowering was much shorter in tolerant genotypes (1–3 days) than in sensitive genotypes (12–20 days) (Fig. 6) and this difference in the delay of flowering could have been the cause of the higher reproductive failure of the sensitive genotypes in this late-sown trial. Indeed, a negative relationship between time to flowering and seed yield under salinity was found earlier, although exclusively in late-sown trials, at the same location as this current work (Krishnamurthy *et al.* 2010). In such a situation, a delay in flowering would result in pods and seeds developing in warmer conditions, particularly in the short-season southern Indian environment where the study was conducted. Later flowering in the sensitive genotypes would have forced pod and seed development into a period of increasing temperatures at the beginning of summer when heat stress shortens the period of flower production, induces pod and seed abortion, and reduces yields.

The delay in flowering was associated with the accumulation of Na^+ in the laminae of fully-expanded young leaves and the accumulations of Na^+ and K^+ in old green leaves, and this association was stronger in sensitive than in tolerant genotypes (Fig. 12). However, whether the greater reduction in seed yield in the sensitive genotypes was associated with the greater delay in flowering *per se* or the greater Na^+ concentrations in the leaves in the sensitive than tolerant genotypes is not clear. Further study is needed to determine the causes of the delay in flowering. There is a possibility that salinity could have impaired the N nutrition of the crop by impairing symbiotic N_2 fixation, which could have delayed flowering in plants as a result of N deficiency (Nord and Lynch, 2008). Saline treatment with 100

mM NaCl found to increase the level of compatible solutes but diminish the nodulation, nitrogen fixation and photosynthesis (Soussi *et al.* 1998). Therefore, more research would be needed to test the hypothesis of a higher N_2 fixation impairment in sensitive lines, then leading to a delayed flowering and a lower yield, itself potentially related to two negative influences: (i) a delayed flowering that would expose flower to warmer temperature in the conditions of the trials reported here; (ii) more pod abortion related to less N availability.

Ion concentrations and the association with yield

Salinity reduced yield and there were clear genotypic differences among genotypes for seed yield and relative seed yield under salinity. Ion accumulation in plant tissues has been proposed as a simple explanation for the deleterious effect on yield under salt stress. The accumulation of Na^+ or Cl^- in leaves may lead to dehydration of cells; the accumulation of these ions in the cytoplasm could inhibit enzymes in metabolism; and accumulation in the chloroplast may exert a direct toxic effect on photosynthetic processes (Munns and Tester, 2008). Cl^- accumulated to higher concentrations compared to Na^+, but the greater accumulation of Cl^- in the seed was associated with greater seed yield (Fig. 8, 9, 10). By contrast, Na^+ accumulation in the old green leaves was associated with lower seed yields in the sensitive genotypes (Fig. 11). The toxic effects of Na^+ are related to its competition with K^+ for binding sites of over 50 enzymes (Tester and Davenport, 2003), whereas the effects of Cl^- on metabolism have been found to be much smaller. In this study it is also possible that the Cl^-

preferentially accumulated in the epidermal cells of leaves, thus reducing the Cl^- toxicity in the mesophyll cells that play an important role in photosynthesis (Teakle and Tyerman, 2010). These results contrast with previous findings. For instance, Manchanda and Sharma (1989) reported that accumulation of Cl^- concentration beyond 5% w/w dry mass in tissues, equivalent to 1410 μmol g^{-1} dry mass, disturbed plant metabolic processes, nutrient absorption and its utilization, thus decreasing chickpea yield. Dua, (1998) observed higher Na^+ concentrations in roots than shoots in sensitive genotypes, but similar amounts in tolerant genotypes; in our study, the Na^+ concentration in old green leaves was negatively associated with seed yield with the tolerant genotypes having lower Na^+ concentrations and higher yields than the sensitive genotypes. The higher accumulation of Na^+ in sensitive genotypes may have induced necrosis in older leaflets and thus shortened the lifetime of individual leaflets and in turn affected the yield (Tester and Davenport, 2003).

Salinity decreased total shoot dry mass. This finding might be explained by reduced photosynthesis and higher leaf necrosis (Maliro *et al.* 2008; Dua and Sharma, 1997) resulting from the destruction of chlorophyll in cells due to the increased accumulation of Na^+ or Cl^- in leaves. However, the decrease in shoot weight did not differ between tolerant and sensitive lines and we observed little accumulation of Cl^- and Na^+ in reproductive tissues relative to vegetative tissues. Interpretations of ion concentrations against critical concentrations in tissues derived from other studies can be relatively crude, as these thresholds may vary with the type of plant tissue and with various other growth conditions. Nevertheless, in view of the critical concentrations

in tissues from other studies (Lauter and Munns, 1987; Reuter and Robinson, 1986) albeit in vegetative tissues, the data reported here support the idea that none of the ions analyzed here (Na^+, K^+ and Cl^-) reached toxic concentrations that could have explained the reproductive failure.

The lower Na^+ concentration in the lower old green leaves of tolerant genotypes compared with sensitive genotypes may be a result of a reduced Na^+ uptake rate (Ding and Zhu, 1997). If so, this finding would justify an investigation of possible differences in Na^+ exclusion in root tissues in chickpea. In almost all of the tissues, the Na^+ concentration was much lower than those of K^+ and Cl^-. In addition, the level of Na^+ may be lower in shoots if Na^+ is sequestered in the roots, as less Na^+ would then enter the xylem and reach the shoot (Munns and Tester, 2008). K^+/Na^+ homeostasis was maintained in the laminae, but a higher retention of K^+ was observed in seeds at the filling stage. This outcome may be a result of better Na^+ exclusion, helping to avoid Na^+ toxicity and improve yield (Zepeda-Jazo *et al.* 2008). The concentration of Na^+ was only 10–40% that of Cl^- in tissues, except in pod shells, seeds at the filling stage and mature seeds (Fig. 8). This finding suggests that the exclusion of the cation Na^+ is better regulated than that of the anion Cl^- in ion translocation to reproductive tissues, possibly because Cl^- is an essential micronutrient that regulates enzyme activities in the cytoplasm, is an essential co-factor in photosynthesis, acts as a counter anion to stabilize membrane potential and is involved in turgor regulation (Teakle and Tyerman, 2010). Ion transport across cellular membranes is largely determined by membrane potential, and root Na^+ uptake results in a massive membrane depolarization. From this point, a concurrent uptake of

negatively charged Cl^- may be essential to attenuate (or completely overcome) this salt-induced plasma membrane depolarization (Anschutz *et al.* 2014). Therefore, the role of Cl^- here, initially thought to be harmful, could actually have a beneficial role to play. In addition, the beneficial effect of chloride ion could have been in terms of osmotic adjustment to maintain turgor pressure and growth and development processes, as it has been shown to be responsible for 30% of the osmotic adjustment under salt treatment (Shabala and Lew, 2002). Indeed, it is known that a drought effect hastens flowering in chickpea (Soltani *et al.* 2001). Therefore, a delay in flowering would suggest that our salt treatment did not create any osmotic effect on the crop, possibly because of the higher accumulation of chloride ions playing the role of osmoticum here.

The present study showed that higher K^+ retention in laminae of young leaves and seeds at the filling stage, and higher accumulation of Cl^- in the mature seed were all associated with higher grain yield. A recent report by Wu *et al.* (2014) showed that higher retention of K^+ in leaf mesophyll cells in barley was found to be an important trait that was closely associated with higher levels of salinity tolerance. High cytosolic K^+ level were reported to be important to suppress activity of caspase- like proteases and endonucleases and loss of cytosolic K^+ homeostasis leads to programmed cell death. In present study, as we measured the whole leaf tissues, differentiation of cytosolic and vacuolar compartmentation of ions was not possible. The higher yield/ salinity tolerance in the present study may be due to higher retention of cytosolic K^+ and thus better K^+/Na^+ homeostasis (Anschutz *et al.* 2014). Shabala and Lew (2002) showed that accumulation of Cl^- in

Arabidopsis can be beneficial under saline conditions and also showed in direct single-cell pressure-probe measurements that 30% of total root osmotic adjustment was achieved solely by increased Cl^- concentration. In sugarcane, Gandonou *et al.* (2011) showed that genotypes that accumulated more Cl^- and maintained higher K^+ concentration in young leaves had higher levels of tolerance under saline conditions.

Conclusions

This study reports for the first time that delay in flowering could differentiate the tolerant and sensitive genotypes under salinity. Also the delay in flowering and seed yield reduction was associated with disturbance in Na+ concentration in old green leaves.

Chapter 5- Quantitative Trait Loci Mapping for Salinity Tolerance

Introduction

Chickpea ranks second after soybean (*Glycine max* L.) among the pulses that are consumed (FAOSTAT, 2013), and is subjected to various biotic and abiotic stresses during its life cycle. The yield loss in chickpea due to salinity has been estimated to be approximately 8-10% of total global production (Flowers *et al.* 2010). Chickpea is known to be sensitive to salinity (Flowers *et al.* 2010) at both the vegetative and reproductive stages (Samineni *et al.* 2011), which affects the productivity of the crop across the chickpea growing areas (Rengasamy, 2006). Despite the sensitivity of the crop to salinity, there is a large variation for salinity tolerance (Vadez *et al.* 2007; Krishnamurthy *et al.* 2011; Turner *et al.* 2013). In order to understand the complex phenomenon of salt tolerance, it is important to understand the genetic and physiological basis of salinity tolerance in order to improve existing crop cultivars. Several studies have been carried out to understand the molecular basis of salt tolerance in other crops and quantitative trait loci (QTLs) for traits associated to salinity tolerance have been identified in cereals like bread wheat (Genc *et al.* 2013), barley (Nguyen *et al.* 2013), and in legumes such as *Medicago truncatula* (Arraouadi *et al.* 2012), and soybean (Hamwieh and Xu, 2008). In chickpea, the development of molecular markers in recent years has paved the way to dissect the possible underlying tolerance mechanism for various stresses (Winter *et al.* 1999; Varshney *et al.* 2009; Nayak *et al.* 2010; Gujaria *et al.* 2011; Thudi *et al.* 2011; Hiremath *et al.* 2012). In chickpea, although several mapping studies have been conducted to identify loci for biotic tolerance (Millàn *et al.*

2006; Winter *et al.* 2000), and drought tolerance (Varshney *et al.* 2014), only two studies have reported the presence of QTLs for salinity tolerance (Samineni, 2010; Vadez *et al.* 2012). Till date no report was found on putative candidate genes that would confer salinity tolerance in chickpea. One of the objectives of the present study was to construct a linkage map and to identify QTLs for phenological development (days to flower (DF) and days to maturity (DM)), yield, and yield-related traits (aboveground dry matter (ADM), stem + leaf weight, pod number, seed number, pod weight and 100-seed weight and HI. Also, effort was made to identify putative candidate genes for salinity stress tolerance from QTL harboring genomic regions.

Another objective was to carry out a physiological analysis of the traits contributing to increasing yield in the control and saline treatment in order to highlight the importance of certain QTLs in influencing yield. The trait mapping used composite interval mapping in an intra-specific mapping population derived from ICCV 2 (sensitive) and JG 11 (tolerant). The parents of the mapping population were selected from a previous study based on their contrasting yield on exposure to an 80 mM NaCl solution in a soil-based evaluation (Vadez *et al.* 2007).

Materials and Methods

Plant material and treatment conditions

A total of 188 recombinant inbred lines (RILs) were derived from the salt-sensitive parent ICCV 2 and salt-tolerant parent JG 11. The RILs were in the F_8 generation and obtained from the chickpea breeding programme at

ICRISAT. The study was conducted in pots buried in the ground at ICRISAT, Patancheru, India (17°30'N; 78°16'E; altitude 549 m). This system enables soil salinity treatments to be imposed in outdoor conditions, but sheltered from the rain (Vadez *et al.* 2007; Krishnamurthy *et al.* 2011).

Two experiments were carried out between October and February in two consecutive growing seasons (2010-2011 and 2011-2012) with a saline treatment and a control treatment in both growing seasons. In 2010-2011, the seeds were sown on October 30th 2010 and plants were harvested in 1st week of February 2011. In 2011-2012, the seeds were sown on October 25th, 2011 and plants were harvested between January 19th and February 6th, 2012 in the saline pots and between February 6 and 25th, 2012 in the control pots. Hereafter, the year of sowing, 2010 and 2011, will be used to indicate the 1st and 2nd experiment, respectively. Maximum temperatures during the growing season ranged from 22 to 32°C in 2010 and 25 to 36°C in 2011, while minimum temperatures ranged from 6 to 22°C in 2010 and 8.6 to 22°C in 2011 with relative humidities of 46-86% during the day in 2010 and 41-79% in 2011.

Pots (0.27 m diameter) containing 7.5 kg of a vertisol (fine montmorillontitic isohyperthermic typic pallustert) soil were buried in the soil so that the outer rim of each pot and outside soil surface were at the same level to avoid direct heating of the pots by solar radiation. The vertisol soil (pH = 8.1, cation exchange capacity (CEC)/clay ratio = 0.87, EC_e = 1 dS m^{-1}) (El-Swaify *et al.* 1985) was taken from the ICRISAT farm and fertilized with di ammonium phosphate at a rate of 300 mg kg^{-1} soil. One-half of the pots

were artificially salinized with 1.17 g NaCl kg^{-1} soil, equivalent to 80 mM NaCl in sufficient volume (1.875 L) to wet the vertisol to field capacity. The control pots received tap water containing no significant amounts of NaCl in the same quantity to bring the soil to field capacity. Subsequent watering of both treatments was performed with tap water. The bottoms of the salinized pots were sealed to avoid any salt leaching. In both treatments, six seeds were sown in each pot and later thinned to four similar-sized plants per pot. The experimental design was a RBD with two treatments, a control (0 mM NaCl) and a saline treatment (80 mM NaCl) as main factors and genotypes as sub-factors with four replications per treatment (each replicate was a single pot containing four plants) (Plate 9).

Parameters evaluated

The RIL population along with parents was phenotyped for days to 50% flowering and maturity [DAS and recorded when at least two plants per pot commenced flowering or reached maturity]. At maturity, all plants were harvested and oven dried at 65°C for 48 h. After oven-drying, seven yield-related traits - aboveground dry matter g $plant^{-1}$ (including stem, leaves left at maturity and the pods) (ADM), stem+leaf weight g $plant^{-1}$, total pod number $plant^{-1}$, seed number $plant^{-1}$, yield (seed weight) g $plant^{-1}$ were recorded. HI was calculated by dividing yield by aboveground dry matter. The100-seed weight was calculated by dividing yield by seed number and multiplied by 100. In 2011, along with above-mentioned traits, the number of filled pods $plant^{-1}$ and number of empty pods $plant^{-1}$ was counted. Any pod that had no or nonviable seeds was considered as an empty pod. The

filled pod number was the difference between the total pod number and the empty pod number. All parameters were measured on a pot basis and calculated on a per plant basis.

PCR and marker analysis

The DNA isolation from the leaf samples collected at 12 DAS and the polymorphic markers identification was done as mentioned in previous study (Nayak *et al.* 2010) (Appendix 5). For genotyping the mapping populations, 66 polymorphic markers from that included 36 simple sequence repeats (SSRs) and 30 single nucleotide polymorphic (SNPs) markers were used for genotyping on population (Thudi *et al.* 2011; Hiremath *et al.* 2012) (Table 14, 15). PCR analysis for all SSR markers were performed in 5 µl reaction volume employing GeneAmp® PCR system 9700 DNA thermal cycler (Applied Biosystems, CA, USA). The genotyping for SSRs (ICCM, CaM markers) and SNPs (CKAM series) was carried out as mentioned in earlier studies (Nayak *et al.* 2010; Thudi *et al.* 2011; Hiremath *et al.* 2012).

Construction of genetic maps and QTL analysis

To construct the genetic maps, the genotypic data from 66 polymorphic markers on the 188 RILs of the mapping population were used. The linkage-based mapping was performed using Join Map v 4.0 (www.kyazma.nl/index.php/mc.JoinMap) (Van Ooijen, 2006). In order to find the QTLs responsible for the salinity tolerance, composite interval mapping (CIM) was employed using Windows QTL Cartographer version 2.5 (Wang *et al.* 2010). To carry out the comparison of the QTL position, the

markers in each linkage group in the present and previous two studies (Samineni, 2010; Vadez *et al.* 2012,) were compared with the already existing genetic maps on chickpea (http://cmap.icrisat.ac.in/cgibin/cmap_public/viewer). Hereafter, the different chickpea genetic maps that were used for comparison were collectively referred as published maps.

Identification of genes in genomic regions on CaLG05 and CaLG07

In order to identify candidate genes present in the genomic regions that harboured QTLs for salinity tolerance associated traits, the markers either present in these QTL regions/close proximity whose physical position was already known were selected. They were subjected to BLAST against chickpea genome assembly (Varshney *et al.* 2013). From that, the corresponding UniProt IDs were retrieved. For functional categorization of the genes, the UniProt IDs of the genes were mapped onto UniProt KB database (http://www.uniprot.org/).

Statistical analysis

The data were analyzed with GENSTAT 14.0 (VSN International Ltd., Hemel Hempstead, UK). An unbalanced analysis of variance was performed for all observed parameters individually. Differences between mean values of treatments were evaluated using a LSD test at a 0.05 significance level. Linear regressions were fitted using Microsoft Excel 2007 (Microsoft Corp. 1985, Redmond, Washington, USA). Genotypic and phenotypic components were obtained from unbalanced Analysis of Variance (ANOVA) which was used to calculate the broad sense heritability (H^2).

Results

The detailed results obtained from the ANOVA for the phenotyping data, such as mean performance of parental lines, range of trait values (i.e., maximum and minimum mean values for each trait) across RILs, broad sense heritability values (H^2), *F* probability values and least significant difference (LSD) of traits across two years and treatments, are provided in Table 16 and 17.

Variance analysis

In both years and treatments the RILs but not the parents showed significant variation for DF and DM except parents showed variation for DM in the saline treatment in both the years. In 2010, with control treatment, no significant variation was observed between the two parents for all the yield and yield-related traits, whereas in the saline treatment they differed significantly except for the traits stem+leaf weight and HI (Table 16). In 2011, both the control and saline treatments did not differentiate the parents for any traits except for filled pod number and empty pod number in the control treatment (Table 17).

The combined unbalanced ANOVA on two years data, in the both treatments revealed that the traits DF, DM and 100-seed weight were significantly influenced by both genotype and environment, but largely affected by the genetic potential rather than the environment (larger *F* statistic value for genotype than for genotype × year). All the other traits were influenced significantly by the genotype, but not by the environment.

Heritability

Heritability estimates were categorized into low (5-10%), medium (10-30%), high (30-60%) and very high (>60%) by Robinson, (1966). In 2010 in the control treatment, the broad sense heritability (H^2) of DF, DM, HI and 100-seed weight was high, whereas all other yield and yield-related traits had medium heritability (Table 16). In the saline treatment, the heritability of DF, DM, 100-seed weight, stem+leaf weight was high, whereas heritability of ADM, yield, pod number, seed number and HI had medium heritability values. In 2011, in the control treatment, the traits DF, DM and 100-seed weight had high heritability values, whereas all other traits had medium heritability values (Table 17). In saline treatment, the traits ADM and yield had medium heritability, whereas all other traits had high to very high heritability values (Table 17). In summary, the phenological traits had high, whereas the yield and yield-related traits had moderate-to-high heritability values in the saline treatment.

Relationships of yield and yield-related traits variables

The seed yield in the saline treatment correlated significantly to control treatment in both the years (R^2 = 0.23; 0.21). Similarly, means of all other traits in the saline treatment significantly correlated with control mean of the corresponding trait in both the years (Table 18). To understand the importance of the QTLs identified, the mean value of traits for which QTLs were found was correlated with the mean yield in both the treatments and across years (Table 19). Except for DM in the control treatment in 2010 and

DF under salinity in 2011, all the other traits for which QTLs were identified showed significant correlations with yield. In the saline treatment, the ADM, pod number, and seed number explained up to 76%, 75%, and 76% of the variation in yield, respectively. In the control treatment, the stem+leaf weight, filled pod number and seed number explained up to 51%, 56% and 49% variations in yield. Though, HI and 100-seed weight were significantly correlated to seed yield they explained less than 12% of the yield variation in both treatments.

As all the traits showed significant correlations between the control and saline treatments, indicating that the yield in the saline treatment was influenced by the potential yield in the control treatment (Table 20), the traits were expressed as relative values, calculated as the ratio of values in saline treatment to the mean value of the trait in the control treatment for each RIL. In 2010 and 2011, the relative ADM (R^2 = 0.86, R^2 = 0.76), relative stem+leaf weight (R^2 = 0.52, R^2 = 0.27), relative pod number (R^2 = 0.85, R^2 = 0.64 and relative seed number (R^2 = 0.89, R^2 = 0.89) showed significant correlations with relative yield. This indicates that these traits were important in determining higher yield under salinity in chickpea. By contrast the relative values of phenological traits, 100-seed weight and HI were not significantly related to the relative seed yield (Table 21).

Linkage mapping and QTL analysis for salinity tolerance-related traits

The intra-specific map based on ICCV 2 × JG 11 spanned 329.6 cM with 56 markers mapped in 7 out of 8 linkage groups. No markers were mapped on CaLG02. The genetic maps developed and phenotyping data generated were

analyzed for identification of major and minor QTLs to understand the genetic and molecular basis of salinity tolerance.

In the mapping population derived from ICCV 2 × JG 11, a total of 46 QTLs were identified that included 19 QTLs for phenological traits (7 for DF; 12 for DM) and 27 QTLs for yield and yield-related traits across years and treatments. The QTL analysis for seven (2010) and nine (2011) yield and yield-related traits detected 23 major QTLs across treatments for all traits (3 for ADM; 1for seed number; 1 for pod number; 3 for yield; 2 for stem+leaf weight; 9 for-HI; 4 for 100-seed weight) except for filled pod number and empty pod number (Table 22). In the saline treatment a few minor QTLs were identified for HI on CaLG04d in 2010, while in the control treatment minor QTLs were identified for yield, pod number, filled pod number and seed number on CaLG07 in 2011 (Table 22).

In case of one of the flanking markers was common to more than one QTL, that region was considered as a single genomic region that contained two or more QTLs. By following this criterion, the 46 QTLs identified were present in 9 genomic regions (Fig. 13). QTLs that contributed >10% of the phenotypic variation explained (PVE) were considered as major QTLs (Varshney *et al.* 2014). The PVE by QTLs, in this study, ranged from 6 to 67%. If in a particular treatment, the QTL for a given trait appeared in the same genomic region in more than one year, the QTL was considered stable QTL (Varshney *et al.* 2014). A total of 14 stable QTLs for five different traits in control treatment were identified (Fig. 13).

QTLs for phenological traits

In 2010, for DF neither in control nor in the saline treatment major QTL was identified but in 2011, six major QTLs, three QTLs in the control and three in the saline treatment, for DF were identified and explained up to 40% of the PVE. Therefore, no stable QTL was found for DF in any treatment. In 2010 no major QTL for DM in the saline treatment was identified but four major QTLs (up to 67% PVE) for DF were identified in the control treatment. In 2011, in the saline treatment, four major QTLs were identified for DM (up to 67% PVE) and in the control treatment; three QTLs (up to 65% PVE) were identified. Four stable QTLs for DM in control treatment were detected, two each in CaLG05 (with flanking markers CaM0463-ICCM272) and in CaLG08 (CKAM1903-CKAM0343) (Table 22). In any case, since there was no relationship between phenological development and yield either in the control or saline treatments, these QTLs were not considered important for the primary purpose of this study.

Yield and biomass

Four yield QTLs, three major and one minor QTL, were identified across two years and treatments. In 2010, in the saline treatment one major QTL was identified located on CaLG07 that explained 17% of the PVE. In 2011, one major QTL in the saline treatment that explained 12% PVE was also identified on CaLG05, while one major QTL (16% PVE) and one minor QTL (8% PVE) were identified on each of CaLG05 and CaLG07 in the control treatment. The two major QTLs identified in the control and saline

treatments in 2011 were located at the same position on CaLG05 with flanking markers, CaM0463 and ICCM272.

In the saline treatment, one major QTL for ADM that explained 12% PVE was identified in 2011. In the control treatment, two major QTLs for ADM that explained up to 27% PVE were identified across years. All the three QTLs for ADM were found at the same loci of CaLG05 (CaM0463-ICCM272). Thus two stable QTLs for ADM in control treatment were identified. In the saline treatment, no QTL for stem+leaf weight was identified, whereas in the control treatment two major and stable QTLs for stem+leaf weight were identified on CaLG05 (CaM0463-ICCM272) across years (Table 22).

QTLs for pod number, filled pod number and seed number

In the saline treatment in 2010, one major QTL for pod number (25% PVE) was found on CaLG07 (CaM2031-CKAM0165) while in the control treatment in 2011, one minor QTL (8% PVE) was found on CaLG07 (ICCM0034-CaM0906). In the control treatment, one more minor QTL for filled pod number (8% PVE) was found on CaLG07. Again on CaLG07, in the saline treatment in 2010, one major QTL for seed number with 17% PVE and in the control treatment in 2011, one minor QTL (9% PVE) was identified for seed number. These QTLs were of great interest since the correlation analysis above also showed a close relationship between seed and pod number and yield across treatments (Table 22).

QTLs for HI and 100-seed weight

The QTL analysis identified nine QTLs for HI across years and treatments. In 2010, in the saline treatment a minor QTL (6% PVE) for HI was identified on

CaLG04d while in the control treatment two major QTLs for HI were identified, one each on CaLG05 (46% PVE) and CaLG08 (10% PVE). In 2011, in the saline and control treatment, three major QTLs per treatment for HI explaining PVE of 30-49% and 32 to 56%, one each on CaLG05, CaLG04d and CaLG08 were identified. Four stable QTLs for HI under control treatment was identified. Four major QTLs for 100-seed weight, one each per treatment and per year, were identified on CaLG05. Three of the four QTLs for 100-seed weight were identified at the same locus of CaLG05 (CaM0463-ICCM272) and explained PVE up to 40%. Two stable QTLs for 100-seed weight under control treatment was identified. The fourth QTL was also identified on CaLG05, but at a different position which explained 17% of the PVE. Again, although these QTLs were significant, they had limited importance for the primary scope of this study since there was only limited or no significant relationship between 100-seed weight or HI and yield in any of the treatments, especially under salinity.

Important genomic regions for salinity tolerance

The genomic region of CaLG05 flanked by markers CaM0463 and ICCM272 contained 17 major QTLs for seven different traits (DF, DM, ADM, stem+leaf weight, 100-seed weight, HI and yield) across treatments (Fig. 13). Furthermore, one major QTL for DF, DM, ADM, HI, 100-seed weight and yield in the saline treatment was found in this region. Another genomic region, on CaLG07, harboured seven QTLs, out of which five QTLs were identified in the saline treatment for five different traits (DF, DM, seed number, pod number and yield), but none of these QTLs were stable (Fig.

14). A genomic region on CaLG08 harboured eight QTLs (six in the control treatment and two in the salinity treatment) for three traits, DF, DM and HI. Out of these three genomic regions, the genomic regions on CaLG05 and CaLG07 were of greatest interest as they hold QTLs for traits that were significantly related to yield under salinity, but the one on CaLG08 had less interest because the traits it harboured were unrelated to yield (Fig. 13).

Reassigning linkage group number as per published maps

Each linkage group of three populations was compared with published maps. A feature search for set of markers in each linkage group and population was performed against published maps

(http://cmap.icrisat.ac.in/cgi-bin/cmap_public/feature_search). This provided the details about the marker's position and its location on linkage groups across chickpea genetic maps. The number of markers and the linkage group where they were predominantly located on published maps were noted. Based on these results, the linkage group numbers was reassigned. The number of common markers that existed between linkage groups of particular population and published maps was given (Table 23). Also, each linkage group in present study and Vadez *et al.* (2012) were linked to the linkage groups in other published studies in cmap server (http://cmap.icrisat.in).

Mining candidate genes in salinity stress responsive genomic regions

An effort was made to mine candidate genes in the genomic regions on CaLG05 and CaLG07 on chickpea genome map. The sequences of SSR markers on CaLG05 and CaLG07 that were present close to genomic regions

were aligned to the chickpea genome (Varshney 2013). This resulted in locating them into a distance of 11.7 Mb (33.1Mb to 44.8Mb) and 12.5 Mb (starting at 36.3 Mb and ending at 48.9 Mb) respectively on the chickpea genome. Genome annotation of these regions on CaLG05 and CaLG07 has identified a total of 1129 and 440 genes respectively. All the identified 1569 genes were functionally categorized based on gene ontology (GO) descriptions (UniProt database, 151 UniProt-GO), and all could be assigned to at least one GO term. The genes were further assigned to three functional categories: (i) molecular function, (ii) cellular component and (iii) biological processes.

The sum of genes assigned to different functional categories (2710) were higher than the total number of genes (1569), as a given gene may fall in more than one category. In the molecular function category, the highest number of genes fell into binding (575) followed by catalytic activity (501). Under cellular component category, the highest number of genes fell into cell part (765) followed by membrane (335). Similarly, in the biological processes category, a maximum number of genes fell into metabolic process (747) followed by cellular process (727) and biological regulation (336).

Based on GO annotation, from 1569 genes, 48 putative candidate genes were found to be up-regulated, down-regulated or induced in response to salinity stress (31 on CaLG05 and 17 on CaLG07). These 48 genes were located in a distance of 11.1 Mb (33.6 Mb to 44.7 Mb) and 8.2 Mb (starting at 37.9 Mb and ending at 46.1 Mb) on CaLG05 and CaLG07 respectively (Table 24 A, B).

Discussion

Comparing the loci of QTLs for salinity tolerance with previous studies

No common markers existed between the genetic map of the present study and the genetic maps derived from two other populations (Samineni, 2010; Vadez *et al.* 2012) that were developed for studying salinity tolerance in chickpea (Table 23). The markers on each LG were compared with published maps and a standard LG number was assigned. For example, nine markers were mapped on LG 5 in Vadez *et al.* (2012). When we searched for the position of these nine markers in published maps, we found that seven out of nine markers were located on LG 7 in the published maps (Thudi *et al.* 2011; Varshney *et al.* 2014; Hiremath *et al.* 2012). Thus, the LG 5 was re-assigned to LG 7 to coincide with the published maps. Re-assigning LG numbers was done for each LG group in the three populations (Table 23). By doing this, we were able to compare the key genomic regions identified in the present study with those in the other two studies and this comparison helped us to identify whether a particular LG contained QTLs for salinity tolerance related traits across populations. In the present study, we identified two key genomic regions for salinity tolerance using a composite interval mapping approach.

Genomic region on CaLG05 in the present study (CaM0463- ICCM272)

CaLG05 in the present study, LG 7 in Samineni (2010) and LG 7 in Vadez *et al.* (2012) corresponded to LG 5 on the published maps (Fig. 14). In the present study on CaLG05, two major QTLs were identified for yield, one in the saline treatment (12% PVE) and another in the control treatment (16%

PVE). The genomic region on CaLG05, flanked by CaM0463 and ICCM272 markers spanning the distance of 28.6 cM, harboured at least one QTL for six different traits per treatment (control, salinity) other than the QTL for yield. So, this locus clearly not only harboured salinity-tolerant QTLs, but also had a highly significant effect on enhancing yield and its related traits across environments in this particular population. Moreover, the favorable allele for yield and the QTLs for 6 different traits on CaLG05 were from ICCV 2, the sensitive parent. In another study, by Samineni, (2010), a minor QTL for yield that explained 8% PVE was located on LG 7 of ICC 1431× ICC 6263 genetic map. In Vadez *et al.* (2012), in the saline treatment the LG 7 of the ICCV 2 × JG 62 mapping population harboured one QTL for seed weight, pod number, HI and100-seed weight. So after standardizing the LG number of three populations, it is clear that the LG 5 of the published maps harboured several important QTLs for salinity tolerance in chickpea (Table 22, Fig. 13). Thus, the genomic region found on CaLG05 in present study (LG 5 in the published maps), is considered to be important genomic region for future MAB for salinity tolerance in chickpea (Fig. 14).

Genomic region on CaLG07 in the present study (CaM2031-CKAM0165)

CaLG07 in the present study and LG 5 in Vadez *et al.* (2012) corresponded to LG 7 in the published maps (Fig. 15). The major QTL that contributed 17% PVE to yield in saline treatment was identified on CaLG07 using a composite interval mapping approach. In the control treatment a minor QTL (8% PVE) for yield was also found on CaLG07. Vadez *et al.* (2012) identified two major QTLs for aboveground dry matter on LG 5 (LG 7 as per published maps) with 27% and 20% PVE and also QTLs for HI and DF under saline

conditions. In this study, the loci flanked by the markers CaM2031-CKAM0165 on CaLG07 that spanned the distance of 19.4 cM contained one QTL per treatment for yield and pod number.

Unlike on CaLG05, on CaLG07 the QTL for yield that contributed the highest PVE (17%) was found in the saline treatment, whereas the QTL in the control treatment had a low PVE (7%). The QTL for yield in the saline treatment in CaLG07 co-maps (at the same position 15.91cM) with the QTL for pod number and seed number, indicating that this particular loci could be particularly responsible for enhanced yield in saline stress environments in chickpea. Moreover, the allele for the loci is from the salinity-tolerant parent, JG 11 (Fig. 15).

Key traits to impart salinity tolerance

The QTLs for DF and DM were located on CaLG01, CaLG05, CaLG04d, CaLG07 and CaLG08, indicating these traits may be controlled by polygenes present on different chromosomes (Fig. 13). Though the phenological traits had high heritability values across treatments and years, the QTLs can't be used in breeding. Unlike the study in soybean by Liu *et al.* (2007), the phenological traits had no role in determining yield in the ICCV 2 × JG 11 mapping population, this might be due to the fact that both genotypes were early maturing and the range of variation in phenology was small. This was different from an earlier QTL study by Vadez *et al.* (2012), in which the two parental lines (one was ICCV 2) had large phenological and yield variation, so that the related QTLs, had to be analyzed through the lens of flowering

time differences. The QTLs for phenological development and HI was harboured in CaLG04d and CaLG08 (Fig. 13).

The yield-related traits such as aboveground dry matter (R^2= 0.85 in the control treatment, R^2 = 0.75 in the saline treatment), stem+leaf weight (R^2 = 0.51 in the control treatment), total pod number (R^2 = 0.28 in the control treatment; 0.75 in the saline treatment) and seed number (R^2 = 0.49 in the control treatment; 0.76 in the saline treatment) were found to be significantly and linearly related to yield across treatments. Also the mean values of above-mentioned traits in the saline treatment were significantly explained by the control treatment (Table 18). So in the mapping population, ICCV 2 × JG 11 used in the present study, QTLs found in the control treatment also holds significant importance in enhancing salinity tolerance. The co-mapping of QTLs for traits like aboveground dry matter, stem+leaf weight, total pod number, filled pod number and seed number along with the yield QTL makes the two major genomic regions on LG 5 and LG 7 (as per the published maps) promising targets for future breeding of salinity tolerant chickpea.

Candidate genes identification and its association with salinity tolerance

In plant response pathways to stresses, the membrane receptors, ion channels, histidine kinase etc., perceive the extracellular stress signal and in turn activate complex signaling cascade at intracellular level (Tuteja, 2007). This is followed by generation of secondary signal molecules such as Ca^{2+}, inositol phosphates; ROS and ABA that transduce stress responsive

genes and lead to plant adaptation to stress tolerance directly or indirectly. The stress induced genes involve in the generation of regulatory molecules like ABA, salicyclic acid and ethylene resulting in second round of signaling. These molecules were found to cross talk in stress signaling pathways (Tuteja, 2007).

The putative candidate genes found in this study were also experimentally demonstrated for their role in salinity stress response by several studies in different plants (Table 24 A, B). Across CaLG05 and CaLG07, ten candidate genes that encode for proteins ABA-insensitive 5 like protein, UBP16, HVA22-like, HDA6, and beta glucosidase 24, transcription factors Myb 44, ATHB 5, and GTE10 were identified. These genes were found to have vital role in ABA biosynthesis, metabolism, and ABA dependent signaling pathways (Table 24 A, B). In soybean, novel ion transporter gene *GmCHX1* was reported to confer salinity tolerance by achieving ion homeostasis (Qi *et al.* 2014). In present study, on CaLG05, three putative candidate genes that encode proteins potassium channel AKT1 (involved in regulating K^+/Na^+ ratio), ubiquitin carboxyl-terminal hydrolase 16 and probable inactive poly [ADP-ribose] polymerase SRO2 (regulates plasma membrane antiporter activity) were reported to confer salinity stress tolerance in *Arabidopsis* (Zhou *et al.* 2012; Jaspers *et al.* 2009).

Genes involved in biosynthesis of methionine proved to have salt tolerance in yeast and osmolytes like Gly betaine had found to be mitigate cold stress damage in chickpea, were also identified on CaLG05 and CaLG07 which may have important role in chickpea as well (Nayyar *et al.* 2005). Among 48

putative candidate genes, most of the genes were found to play a direct or indirect role in osmoregulation that helps the plants to cope up not only with salinity stress but also with other abiotic stresses (Table 24 A, B). Identification of putative candidate genes for salinity tolerance on CaLG05 and CaLG07 made the genomic regions on them more promising which can be exploited for improving abiotic stress tolerance through MAB.

Conclusions

The present study for the first reports two potential genomic regions and candidate genes responsible for saline response. The genomic region can be further used for fine mapping for salinity tolerance in chickpea.

Chapter 6- Effect of Atmospheric Drought Imposed by Low Relative Humidity on Yield and Pollen Germination

Introduction

Chickpea is sensitive for high temperature but large variation exists for heat tolerance in field conditions based on yield. Studies on effect of heat gained importance in recent years due to reasons like continuously changing climatic conditions and change in the areas where chickpea is grown in India (Gowda *et al.* 2011). The heat studies were done in both field conditions and in controlled environments of growth chambers (Devasirvatham *et al.* 2012a, b; Kaushal *et al.* 2014). Both in field and growth chamber conditions, the parameter like pod set was measured when the mid-day temperature was high.

However, while much of the focus has been on the temperature, much less emphasis has been given to relative humidity. During the months of January to April, which correspond to part of the summer season in ICRISAT, Patancheru, India for past 40 years there were 24% days and 40-80% days that had less than 20% and 30% relative humidity (RH) recorded. It is, therefore, unclear whether the relative humidity conditions have a role to play in what is seen as a "heat stress effect". Both higher and lower RH were found to have impact on the plants in different ways and affected on overall yield. High RH% increased leaf area and accelerated leaf emergence (Hirai *et al.* 1996). Cotton plants grown under lower RH% had higher transpiration rates, lower leaf temperatures and lower stomatal conductance, and this led to reduced biomass (Barbour and Farquhar,

2000; Grantz, 1990). Similarly at low RH% rice had smaller shoot and root biomass than plants grown in higher RH% (Hirai *et al.* 1996).

In arid and semi-arid tropics, a high VPD condition is mainly due to combination of high temperature and low RH. To understand the effect of heat on yield and yield components, studies were made in different legume crops like soybean (Herzog and Chai-Arree, 2012), field pea (Sadras *et al.* 2013) and chickpea (Devasirvatham *et al.* 2012a, b). Various studies had been carried out to understand the effect of high VPD in legume crops like cowpea (Belko *et al.* 2012), chickpea (Zaman Allah *et al.* 2011). Very few studies consider the overall effect of high temperature, air humidity and wind velocity in heat study (Matsui *et al.* 1997).

In pearl millet, heat tolerant genotypes that can withstand high temperature upto 42°C have been reported (Gupta *et al.* 2015). In this study, a great emphasis was given to maximum temperature and high VPD associated with maximum temperature. If we look into the VPD it is 6.3 kPa i.e. if temperature was 42°C, the RH would be 22%. The sites where these particular studies were conducted, the crop material had encountered low RH (10-20%) several consecutive days. It is highly possible that the genotypes selected for high temperature might be tolerant to high temperature/ low RH or both.

Genotypes that showed the ability to conserve water through less transpiration under high VPD conditions were considered water deficit/drought tolerant, whereas it is vice-versa in the case of heat tolerance. During months of January to April / May, a high VPD exist in

most of the chickpea growing areas in India during day time. In India, these are the months when the heat tolerance studies were carried out in chickpea (Devasirvatham *et al.* 2012a; Kaushal *et al.* 2014) and pearl millet (Gupta *et al.* 2015) and several other crops in field conditions. During these months, in field conditions the effect of high temperature and low humidity is inseparable. In chickpea, the possible mechanisms underlying heat tolerance was discussed by Devasirvatham *et al.* (2012a, b), Kaushal *et al.* (2014) in field and growth chamber conditions. In the present study, we focused on understanding the effect of low (20%) to medium (30%, 40%) RH on chickpea growth, yield and its components keeping the temperature constant.

One of the objectives of this study is to assess the traits under three different VPD conditions where the temperature was kept constant but the RH was changed between 40 to 20% and to understand the underlying mechanisms. The specific objectives of this study is to understand the effect of three VPDs 2.5, 3.0 and 3.4 kPa when the temperature was kept constant at 30°C and RH was different (40%, 30% and 20%) on

- Seed weight (yield) and yield related components.
- Pollen viability and pollen-pistil function which may lead to understanding of how the tolerant and sensitive groups cope up with minimum RH.
- Canopy temperature and its influence in tolerance mechanisms.

Materials and Methods

This study was conducted in the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India (17°30'N; 78°16'E; altitude 549 m). Five chickpea genotypes from ICRISAT reference set, contrasting for sensitivity to heat stress based on yield and heat tolerant index (HTI), were selected from previous field studies. The two sensitive (S) genotypes had low yields when exposed to heat stress (ICC10755, ICC15294), and the three tolerant (T) genotypes had high yields when exposed to heat stress in field conditions (ICC9942, ICC4495 and ICC92944) (Personal communication - L. Krishnamurthy and Devasirvatham *et al.* 2012a, 2013).

Plant growth

One hundred and fifty five and two hundred and thirty 220 mm diameter pots were filled with 5 kg of vertisol (fine montmorillontic isohyperthermic typic pallustert) and four seeds were sown per pot in the first to second week of November 2012 and 2013 respectively. The seeds were treated with Thiram (Sudhama Chemicals Pvt. Ltd, Gujarat, India) to control seed – borne infections. For experiment1, in 2012, 30 and in 2013, 30 pots were randomly assigned to each genotype. In glass house, in both the years, the maximum day/night temperature and RH ranged between 27-28°C/12-18°C and 40-45%/80-90%. All the pots were thinned to two plants per pot 14 DAS and maintained well watered (WW) until the first flower had opened in all genotypes. Among the thirty pots per genotype, 20 pots with healthy similar-sized plants were selected for the experiment. The sowing was done in different days to synchronize flowering in all genotypes (Plate 10, 11).

Experiment 1

To study the yield and component traits under different vapour pressure deficit (VPD) conditions

After flower initiation in all genotypes, five pots per genotype were selected and moved to two different growth chambers (Model No. E15, Control Environments Limited, Canada) with VPD-2.5 and 3 kPa in 2012 and to three different growth chambers with VPDs 2.5, 3.0 and 3.4 kPa in 2013. The day/night temperature was kept constant across chambers but the relative humidity changed which brought the change in VPD values. Keeping the temperature constant and changing relative humidity facilitated us to solely study the effect of relative humidity on yield and its components. To achieve 20% RH within the growth chamber, a dehumidifier (Model: WDE301, White-Westinghouse international company, Pittsburgh, USA) that was set to 30% RH was used. The plants were kept for 30 days in growth chamber when the period of peak flowering happen in chickpea. The plants were kept well watered throughout the experiment. Each flower that developed was identified daily with a short piece of wool thread placed at the node; Identification of flowers with the threads and watering was discontinued at the end of 30 days and the pots were moved back to glass house condition.

The plants were harvested at maturity 10-13 days after it had moved to glass house from growth chamber in both the years by cutting the plants at soil level and oven-dried at 65°C for 48 h before measurement of the plant components. The stem + leaf weight was separated for each plant per pot

and the mean calculated per pot. Flowers were counted from the number of threads. At harvest, the threads and corresponding pod were put into one of the two categories: (i) threads where no pod was present or the pods were empty or had only undeveloped seeds, and (ii) threads with large pods containing one or two seeds (chickpea most of the time has a maximum of two seeds per pod). Category (i) represented the flower + pod+ seed abortion, and category (ii) represented the fertile pods with complete seed development.

The seed number was recorded by hand threshing the fertile pods in category (ii) and subsequently weighed to obtain seed weight from each pot of each genotype and treatment. The number of flowers, filled pods and empty pods were counted and pod weight, seed weight, flower + pod + seed abortion percentage and seed weight (yield) were measured on a pot basis and calculated on a per plant basis in both the years. The 100-seed weight (seed size) and HI were also calculated.

Experiment 2

Assessment of pollen viability; pollen *in vivo* germination and canopy temperature measurement

For experiment 2, 25 pots per treatment were randomly assigned to each genotype. After flower initiation five pots per genotype per treatment were moved to each growth chamber that was maintained at 2.5, 3.0 and 3.4 kPa i.e. 30°C and 40%, 30% and 20% RH as in experiment 1. The plants were kept for four days in the growth chambers. From these plants, canopy

temperature was recorded and anther and pistil samples were collected to measure pollen viability and pollen *in vivo* germination.

Determination of canopy/tissue temperature

On the third day, 10-15 pictures/ genotype/ treatment were taken using the precision infrared thermal camera (Fluke thermography, USA) (Plate 12). The thermal images were analysed using colour analysis function of the image analysis software Smart View 2.3 (Fluke Thermography, USA) to estimate the canopy temperature of the plant canopy. The canopy temperature was estimated by two different ways- by spotting method and by histogram method. In spotting method, on each image 80-100 spots were made on the image and the temperature recorded in each spot was averaged which gave the canopy temperature of the particular plant.

Smart View 2.3 provides a histogram of the distribution of pixels related to temperature in the thermal image. The temperature distribution of the canopy should follow a normal distribution, and a temperature threshold was fixed beyond which pixels of higher temperature was considered as background. Based on distribution of thermal image pixels compared with target canopy temperatures, an average canopy temperature was calculated as,

$$T_{canopy} = \text{Sum}((Ti * Pxi)/ Pxt),$$

Where, Pxi is number of pixels for a given temperature Ti, and Pxt is total number of pixels for range of temperatures covering the whole canopy.

Pollen viability

Six to eight flower buds were collected on third day to assess the pollen viability during pre-anthesis. The non-dehiscent anthers were stained with Alexander's stain. The samples were examined under a Olympus compound microscope. The fertile pollen grains inside the anthers were red in colour whilst the sterile pollen grains were green. The chemical composition and pH of the stain differentiated the fertile and sterile pollen grains depend on the pollen wall thickness. Malachite green stained the pollen grain wall. Therefore, sterile pollen grains appeared green in colour. The protoplasm in the pollen grain was stained by acid fusion used in the Alexander's stain and hence it coloured the fertile pollen grain red to deep red (Alexander, 1969) (Appendix 6).

Pollen *in vivo* germination

The anthers were emasculated in 8-12 buds on second day of experiment from the plants in growth chamber and in glass house. On third day, the emasculated bud was hand pollinated with pollen from control plants that were maintained under glass house condition and vice versa. Previously pollinated pistils were excised from flowers 24 h after pollination and fixed in 70% ethanol for 24 hours and then cleared with 8mM sodium hydroxide (NaOH) solution for 48 hours and thoroughly rinsed before being stained with decolorized aniline blue solution (DABS). The DABS was prepared by dissolving 0.2 g of aniline blue powder in 108mM Na_2PO_4 solution (Turner *et al.* 2013) (Appendix 7). The preparation was observed and photographed under the same Olympus fluorescence microscope as above, linked to a Dell

computer with progress capture software to view images under the fluorescence microscope.

Statistical analysis

The data were analysed using GENSTAT 15.0 software (VSN International Ltd., Hemel Hempstead, UK). An unbalanced analysis of variance was performed for all observed parameters individually. Differences between mean values of treatments were evaluated using a LSD test at a 0.05 significance level. Linear regressions were fitted using Microsoft Excel 2007 (Microsoft Corp. 1985).

Results

Effect of 40% RH and 30°C (VPD- 2.5 kPa)

The results represented here are from two years data (2012 and 2013). In 2012, all the traits except shoot weight, seed number, empty pods and flower + pod + seed abortion percentage showed significant genotypic variation at 5% level of significance. The mean value of traits- flower number, pod number, pod weight, filled pod number and HI significantly differentiated the sensitive and tolerant group. The mean shoot weight of sensitive group remained higher compared to the mean tolerant group. The percentage reduction in sensitive group ranged from 42 to 74% for all traits excluding shoot weight, 100-seed weight and flower + pod + seed abortion% compared to tolerant genotypes (Table 25). The seed weight of mean sensitive group was 1.74 g and the mean tolerant group's was 3.67 g and the percentage reduction was around 53% in sensitive genotypes. In 2013, the genotypic variation was found for traits- shoot weight, flower number,

seed number, filled pod number, 100-seed weight and flower + pod + seed abortion%. All traits differentiated the sensitive group from tolerant group significantly at 5% level of significance. The percentage reduction varied between 17 to 78% among traits. The reduction of seed number and seed weight was 61 and 44% in sensitive group compared to the tolerant group. As in year 2012, the mean shoot weight of sensitive group remained higher than the mean tolerant group. The tolerant group had 1.8 fold higher seed weight than the sensitive group (Table 26).

Effect of 30% RH and 30°C (VPD- 3.0 kPa)

The results are from two years data (2012 and 2013). Genotypic variation found for all traits except flower + pod + seed abortion% in 2012 in 3.0 kPa. All the traits except, empty pod number, HI and flower + pod + seed abortion differentiated the sensitive group from tolerant group. The percentage reduction in sensitive group ranged between 24-70% when compared to the tolerant group. But, the 100-seed weight had increased by 55% which could be due to the imbalanced source: sink ratio. The seed number and seed weight got reduced by 70 and 52% respectively (Table 27). In 2013, genotypic variation was found for all traits except empty pod number, HI and flower + pod + seed abortion%. The traits- pod number, pod weight, seed number and seed weight only differentiated the sensitive group from tolerant genotypes. The percentage reduction was more than 50% in traits pod number, pod weight, seed number, seed weight, filled pod number and empty pod number (Table 28).

Effect of 20% RH and 30°C (VPD- 3.4 kPa)

The results represented here is from one year data (2013). Genotypic variation was found only for the traits shoot weight and flower number and HI. None of the traits except shoot weight differentiated sensitive group and tolerant group. The % reduction of traits ranged between 15 to 27% except for shoots weight which was above 40%. The traits flower number and flower + pod + seed abortion% increased by 5 and 22% in sensitive group compared to tolerant genotypes. These results contrast with those one we obtained under VPDs 2.4 and 3.0 kPa in both the years. Clearly the low relative humidity affected all the parameters and no tolerance was exhibited even by the putatively heat tolerant genotypes when the RH was kept at 20% (Table 29).

Percentage reduction in mean values of traits under VPD 3.0 kPa compared to VPD 2.5 kPa

In 2012, the sensitive genotypes showed reduction in the mean values of all traits except 100-seed weight and HI under VPD 3.0 kPa compared to VPD 2.5 kPa. The reduction ranged between 11-61% across traits and the seed weight reduced by 33%. In tolerant genotypes, the reduction was between 8 to 43%. The seed weight reduction in tolerant was around 34% which is equal to the sensitive genotypes. The number of empty pods increased by 400% across all genotypes under VPD 3.0 kPa in 2012. In 2013, the trait HI increased by 31% in tolerant genotypes under 3.0 kPa compared to 2.5 kPa. In sensitive genotypes the reduction ranged between 9 to 41% and the seed weight reduced by 27% whereas in the tolerant genotypes the reduction

ranged between 3 to 37% and the seed weight reduction was only 6%. The traits HI and 100-seed weight increased by 9 and 6% under 3 kPa compared to 2.5 kPa (Fig. 16).

Percentage reduction in mean values of traits under VPD 3.4 kPa compared to VPD 2.5 kPa (2013)

In sensitive genotypes, the traits shoot weight, pod number, pod weight, seed weight, empty pod number and 100-seed weight showed reduction in their mean values under VPD 3.4 kPa compared to VPD 2.5 kPa. In contrast, the traits flower number, seed number and HI showed increased by 14, 30, 7% increase in their mean values. Despite of increased seed number, seed weight got reduced by 34% as the seed size got reduced by 45%. In tolerant genotypes, all the traits showed reduction under 20% RH to 40% RH condition. The % reduction ranged between 4 to 71% across traits. The seed weight reduced by 45% under VPD 3.4 kPa compared to VPD 2.5 kPa (Fig. 16).

Traits contributing to yield across treatments

There were few traits that had contributed to yield significantly ($P<0.05$ or 0.01) either under particular treatment or across treatments. In 2012, at 40% RH (VPD-2.5 kPa) the traits shoot weight, pod number, pod weight and filled pod number contributed to yield at $P<0.05$. In 2013, as in 2012, pod number, pod weight and filled pod number contributed to yield at $P<0.05$ and HI at $P<0.01$. Under VPD 3.0 kPa, where the RH was 30%, in 2012, five traits (flower number, pod number, pod weight, seed number and filled pod number contributed to yield at $P<0.01$. Empty pod number and 100-seed

weight was also found to have significant association with yield (P<0.05). In 2013, under VPD 3.0 kPa, four traits, pod number, pod weight, seed number and filled pod number contributed to yield at P<0.01. In 2013, under VPD 3.4 kPa, pod weight (P<0.05) and seed number (P<0.01) significantly contributed to higher level of yield. Assessment of these linear relationships revealed the fact that seed number is the most important trait that contributed to higher yield when the relative humidity was kept at 30% and 20% and this was not the case when the RH was 40% across two years (Table 30).

Pollen viability and pollen *in vivo* germination

Pollen viability was unaffected across years and treatments. All the five genotypes showed 98-100% viable pollen when stained with Alexander's stain across treatments and years. Similarly, the stigma receptivity and pollen tube growth was unaffected across three treatments even when the RH was only 20% (Fig. 17, 18).

Canopy temperature

In 2012, at VPD 2.5 the canopy temperature of the sensitive genotypes were 26.45 and 27.66°C while for the tolerant genotypes it ranged from 29.18 to 30.15°C. At VPD 3.0, the canopy temperature was 28.4 and 27.4 for sensitive and ranged between 28.42 to 29.03°C in tolerant genotypes. The sensitive genotypes showed significant increase in canopy temperature while it got reduced in tolerant genotypes by 0.8 to 1°C. In 2013, at VPD 3.4, the sensitive genotypes had mean tissue temperatures of 30.05°C and the

tolerant had 29.4°C. The genotype ICC92944 maintained least tissue temperature of 28.89°C among all genotypes (Table 31).

Discussion

Air relative humidity was identified as a key environmental factor which mediates the changes in stomatal sensitivity to CO_2 (Talbott *et al.* 2003). Reports suggested that the stomatal conductance of leaves decreases on exposure to drier air. In contrast there are also reports that the transpiration rate increases when air is dry. In the present study, we studied the effect of relative humidity in chickpea mainly for two reasons. (i) The cultivation of chickpea had shifted from cooler northern states to hotter central and southern states of India and thus breeding for heat tolerance gains important (ii) the selection for heat tolerant lines was done mostly under natural conditions in summer when the VPD was high due to both high temperature and low relative humidity. In chickpea, effect of higher temperature and possible underlying mechanisms were reported by Devasirvatham *et al.* (2012a, b, 2013) where the role of maximum temperature was greatly emphasized but RH left unconcerned. In chickpea, no study investigated the effect of relative humidity till date. So, in this study we addressed whether variation in relative humidity has any effect in yield components and to know whether it important to consider RH while selecting chickpea materials for heat tolerance.

The key findings from this study are:

(i) Genotypic variation existed among genotypes and the putatively heat tolerant and sensitive group were differentiated when the RH was kept at

40% and 30% RH (i.e. at VPDs 2.5 and 3.0 kPa) but not at 20% RH (3.4 kPa), (ii) Most of traits that contributed significantly to yield showed reduction in their mean values under VPD 3.0 and 3.4 kPa when compared to VPD 2.5 kPa, (iii) The trait seed number found to be the most important for attaining higher level of yield under minimum RH-30 and 20% (i.e. under maximum VPDs), (iv) Pollen viability and pollen *in vivo* germination was unaffected across three treatments.

In the present study the putatively heat tolerant genotypes yield higher than its sensitive counterparts when the RH was kept at 40, 30% (Table 26, 27). Decrease in yield and yield components under 20% RH compared to 40% RH showed that lower humidity has negative impact on overall plant growth like any other abiotic stress. The tolerance to least RH didn't vary among the five genotypes (Table 28). This indicates the genotypes that were selected for heat tolerance were incapable of withstanding least RH of 20%. Thus, it becomes important to study the impact of relative humidity stress and possible mechanisms that underlie the sensitivity/tolerance by genotypes. This result supports the idea that in future it is worthwhile to select genotypes that can yield better in high VPD conditions for heat tolerance.

Devasirvatham *et al.* (2013) had studied underlying heat tolerance mechanisms by increasing the day temperature from 27 to 38°C in growth chambers but not much was concentrated with respect to relative humidity values. The RH in these experiments was not controlled and was above 55%. In Devasirvatham *et al.* (2013), it was reported that the pod set had reduced ~50% at the temperature 35/20°C compared to the optimum temperature

28/16°C. In our study, at 20% RH, at 30/20°C temperature the pod weight got reduced by 41 and 49% in sensitive and tolerant group compared to 40% RH. This clearly indicates that under the high VPD imposed by lower RH reduction increased pod reduction similar to the results of Devasirvatham *et al.* (2013) observed under high temperature treatment.

Seed number

Higher seed number has been found to be a desirable trait and contributed significantly to increased yield under various abiotic stresses like water stress (Pushpavalli *et al.* 2014), salinity (Pushpavalli *et al.* 2015; Turner *et al.* 2013; Vadez *et al.* 2007) in chickpea. In the present study also the higher seed number under 30 and 20% RH had contributed to higher yield (Table 30). This depicts that the genotypes that had the ability to set more seeds would be more useful in high VPD conditions imposed by low RH.

Pollen viability and *in vivo* germination and canopy temperature

The pollen viability was affected under high temperature (Devasirvatham *et al.* 2012a, 2013) and water stress (Fang *et al.* 2010) treatments in the case of chickpea, whereas it is not the case in the present study. These results are similar to Turner *et al.* (2013) in chickpea under salinity where pollen viability or *in vivo* germination was unaffected. This means not all the abiotic stresses affect the pollen viability but still reduces the yield significantly (Fig. 17). In the several works in rice, the pollen viability decreased significantly under atmospheric drought. There are reports where few rice genotypes had capability to set seeds at a temperature of 40°C and 20% RH (Matsui *et al.* 2007). The mechanism behind the tolerance was

identified as transpirational cooling of panicle and leaves; i.e., the tolerant genotypes transpire more and thus maintained lower panicle temperature compared to the ambient temperature (Weerakoon *et al* 2008; Yan *et al.* 2010; Fukuoka *et al.* 2012). In chickpea the anthers and stigma within buds are well protected with inner keel, sepals and petals. It is possible that the tolerant genotypes have much cooler canopy compared to its sensitive counterparts. The plants were watered on daily basis to field capacity, so there was no scarcity for water to transpire. In the present study, neither the pollen viability/stigma receptivity nor *in vivo* pollen germination was affected (Fig. 17, 18). In this case, it is possible that the anthers or stigma hadn't experienced higher VPD as leaves/ enough moisture required for anthesis and fertilization was left in the micro environment of flower buds. The reproductive organ that might have really exposed and negatively impacted by higher VPD imposed by lower RH is the small pods. In chickpea, the time required for the pollen tube to reach ovary is only 15 minutes and for a pod to mature it usually take nearly 4-5 days and the process of seed filling continues as per the availability of favorable environment. If the pollination happens in morning or when the VPD was relatively low, pollen tube growth will remain unaffected. When we look into the pod to become a mature seed there are few prominent stages like ovule formation, pod wall development and seed filling could be affected.

In rice and pearl millet, the organ that is more sensitive to heat stress is panicle. In chickpea, the green pods involve in photosynthesis and fixing carbon. Reduction in function of vital enzymes like Rubisco (carbon-fixing enzyme), sucrose phosphate synthase (SPS) and sucrose synthase (SS)

(Kaushal *et al.* 2014), disturbance in hormones, transport of carbon to reproductive structures etc., that during photosynthesis may cause ovule abortion, pod abortion or seed abortion. It is possible these reproductive structures might not have achieved enough cooling under high VPD which is required for normal seed filling. So the yield reduction mentioned here may be due to ovule/ pod/ seed abortion rather than the fertilization itself. Throughout the treatments the heat tolerant genotype ICC 92944 had significantly lower canopy temperature compared to other genotypes (Table 31). This indicates that higher transpiration cooling may be achieved by heat tolerant genotypes to withstand adverse effects of dry air. As Weerakoon *et al.* (2008) emphasized while interpreting the results of heat trials from controlled and field conditions, it is important to consider not only temperature values but also the RH values.

Conclusions

This study reveals that heat tolerant genotypes were not different from sensitive genotypes at 20% relative humidity or 3.4 kPa of vapor pressure deficit condition. Pollen viability or germination was unaffected but still there is a significant yield loss at 3.4 kPa of VPD. In future, while studying heat stress effect in chickpea, effect of low RH should be also considered.

Summary and Conclusions

In the present study, effect of water stress, salinity and relative humidity stress on reproductive parameters were studied. Also, the quantitative trait loci, candidate genes for salinity tolerance were identified.

Water deficit study

The water deficit study was carried out under glass house condition with ten chickpea genotypes contrasting for drought tolerance. A progressive water stress was applied to the plants by dry down methodology. The study was conducted in the glass house, so that the other stresses may not influence the study as in field conditions. Based on normalized transpiration ratio values, a proxy for stress index, the stress period was divided into two phases: phase I (mild stress) and phase II (severe stress). To facilitate comparison between WW and WS, the WW was also divided into phase I and phase II as in WS condition. In this study, shoot dry weight, flower number, flower +pod +seed abortion rate, pod number, seed number, pod weight, seed weight, HI and 100-seed weight were observed/calculated. The parameters recorded during phase I (mild water stress) showed significant importance in this study. During mild stress, the seed yield reduced by 35, 57% and 28, 59% in tolerant, sensitive group in year 2009 and 2010. In this study, in year 2010, the sensitive genotypes showed higher decrease in flower number (44%) than the tolerant genotypes (27%) but it is not the case in year 2009. The seed number of tolerant and sensitive genotypes decreased by 51, 41% in year 2009 and by 38, 34% in year 2010 under mild stress treatment.

The slow dry down approach used in this study was a useful methodology for controlling the WS treatment imposition in pots in the glasshouse and for the imposition of similar intensities of water deficit across genotypes irrespective of plant size. While the ranking for total seed yield under WS in this work did not correspond with the total seed yield ranking in the field, particularly when differences in potential yield were taken into account. We consider that this was the result of a large genotype × environment interaction which was evident not only between the field and glasshouse, but even between years. From the relative parameter values, genotypic differences in total and phase I seed yield under WS conditions were mostly driven by the number of flowers produced and the number of seeds that were set under the mild stress conditions, but not by the total flower + pod + seed abortion percentage or the 100-seed weight. Seed size (100-seed weight) was conserved across treatments suggesting that seed set was adjusted to maintain seed size and viability. The factors that determine the genetic variation in flower production and seed set as water deficits develop requires further investigation.

Salinity and ion analysis study

In the salinity experiments, fourteen chickpea genotypes that contrast for yield potential were used to study yield parameters, and eight genotypes were selected for ion analysis and treated with 0 mM and 80 mM NaCl. This study aims at confirming the traits that differentiated tolerant and sensitive genotypes under saline condition and to assess any possible relationship between salt stress accumulation in different plant tissues and yield

reduction. In this study, 11 parameters such as above ground dry matter (shoot dry matter), pod number, filled pod number, empty pod number, pod weight, seed number, seed weight, 100-seed weight, HI were recorded/ calculated along with ion analysis (Cl^-, Na^+, K^+) of nine different vegetative and reproductive tissues. Salinity decreased total shoot dry mass across all genotypes. It decreased seed number and seed yield per plant compared with control, although the reduction was less in tolerant genotypes (23–45% for seed number, 10–46% for seed yield) than in sensitive genotypes (34–90% for seed number, 52–90% for seed yield) except for seed number in ICC10755 (S), which decreased by 14%; in ICC15510 (S), it increased by 8%.

Exposure to 80 mM NaCl throughout the life of the plant resulted in a delay in flowering and this delay was greater in the sensitive than the tolerant genotypes. **To best of our knowledge this is the first report in chickpea where delay in flowering significantly differentiated the sensitive and tolerant genotypes under saline conditions**. The delay in flowering was significantly associated with a decrease in seed yield which in turn was associated with the greater accumulation of Na^+ in the leaves. However, whether the greater increase in Na^+ in the leaves of the sensitive genotypes was the cause of the greater reduction in yield or whether the delay in flowering and consequent pod and seed development in the hotter conditions of summer was the cause of the reduction in yield in this late-sown experiment is not clear. What is clear is that filled pod number, seed number and seed yield can be used to distinguish salt-tolerant chickpea genotypes from salt-sensitive genotypes because reproductive failure clearly discriminated tolerant from sensitive entries. While ions accumulated

primarily at concentrations that might not be considered as toxic levels, the ion homeostasis disturbance (Na^+ and K^+) that occurred in certain tissues was associated with altered plant yield. Further research is needed to determine the causes of flowering delay and consequent pod abortion and lower yields; possible causes are the effect of salt stress on carbon assimilation and symbiotic N_2 fixation, changes in level of hormones involved in stomatal control and signaling pathways or seed development, and the activity of floral repressor genes.

QTL study for salinity tolerance

QTLs linked to salinity tolerance in chickpea using the data collected over two growing seasons from 188 RILs developed from two widely-grown cultivars, JG 11 and ICCV 2. The parents JG 11 and ICCV 2 were found to be salt-tolerant and salt-sensitive in previous study. This study was carried out because of two reasons, there were no yield QTL/ candidate genes reported for salinity tolerance till date. As salinity occurs frequently along with drought, it becomes important to decipher the genetic mechanisms underlying any tolerance. In this study, 11 parameters such as above ground dry matter (shoot dry matter), pod number, filled pod number, empty pod number, pod weight, seed number, seed weight, 100-seed weight and HI were recorded/ calculated. A genetic map with 56 markers was constructed. Through QTL cartographer analysis, two potential genomic regions were identified that can be used for MAB for salinity tolerance in chickpea. The genomic region on CaLG05 (LG 5 as per the published maps) spanning a distance of 28.6 cM included QTLs for six traits in the saline treatment and

seven traits in the control treatment, and was found to have a role in enhancing productivity across both control and saline environments. Not only in this population, but also in other two populations the yield-related QTLs were identified in LG 5 (as per the published maps). The genomic region on CaLG07 (19.4cM) was found to have major QTLs for yield and its related traits, mainly under salinity. **Availability of chickpea whole genome sequence allowed the identification of putative candidate genes for salinity response which is being reported for the very first time**. The reported putative candidate genes may be validated using the appropriate genetic material by functional genomics approaches like Targeting Induced Local Lesions IN Genome (TILLING), quantitative RT-PCR, over-expression, etc. Through identification of several QTLs and putative candidate genes, present study had opened a window for further fine mapping of the genomic regions on CaLG05 and CaLG07 which may lead to identification of novel genes for salinity tolerance in chickpea.

Relative humidity study

Heat stress studies in chickpea gained importance in recent years as the chickpea growing areas had shifted to hotter climatic zones and due to changing climatic conditions as a result of global warming. The two major components of heat stress are temperature and relative humidity. In arid and semi-arid zones regions often heat stress is a combination of high temperature and low RH. In recent studies in chickpea high temperature conditions were studied but not the low RH. Thus this study was solely designed to understand the effect of low RH without the effect of high

temperature. In this study, five genotypes that contrasted for heat stress tolerance were used. The plants were grown under glass house conditions and then moved to three different growth chambers where VPD 2.5, 3.0 and 3.4 were maintained with RH of 40, 30 and 20% respectively. The parameters studied in this study included shoot dry weight, flower number, flower +pod +seed abortion rate, pod number, seed number, pod weight, seed weight, HI and 100-seed weight. Also the canopy temperature, pollen viability and pollen in vivo germination were studied. When compared to VPD 2.5 and 3.0 kPa, the reduction ranged between 11-61% across traits and the mean seed weight reduced by 33% across tolerant and sensitive genotypes in 2012 whereas in 2013, the yield reduction was around 27%, 6% in sensitive and tolerant genotypes. Under 3.4 kPa treatment, almost all yield related traits showed significant decrease except the traits flower number, seed number and HI. Though the seed number got increased, the seed yield got reduced by 34% due to decrease in 100-seed weight by 45%. Overall the reduction across traits accounted upto 4-71% under 3.4 kPa compared to 2.5 kPa treatment. The least canopy temperature under 3.4 kPa treatment was recorded as 28.8°C in the genotype ICC92944 which had been released in several parts that are prone to high temperature in India and Africa.

The least RH as all other abiotic stresses reduced yield and thus need to be considered important in future chickpea heat tolerance breeding programmes. The pollen viability or pollen *in vivo* germination was unaffected in this study. The yield reduction may be because of ovule, pod or seed abortion as the % of empty pods increased significantly when the RH was reduced from 40% to 20%. The heat tolerant lines may achieve tolerance by

maintaining cooler canopy through transpirational cooling which is worth validating in future. If that is the case, while selecting genotypes for heat tolerance it is important to consider vapour pressure deficit rather than maximum temperature alone. The reasons behind decreased pod set/ increase in empty pod number under high VPD/low RH need to be studied in future.

Comparison among three stresses

Though we used different genotypes for each stress, we found certain trait(s) proved to have importance across abiotic stresses. In all the three stresses, increased seed number found to have important role in increased yield. All the stresses decreased shoot weight of the plants. The 100-seed weight remained unaffected across stresses except under highest VPD 3.4. The flower number maintenance had importance under mild water stress but not under salinity or RH studies. Certain agronomic and physiological traits particularly specific to a stress and that can distinguish tolerant from sensitive genotypes had also been identified like filled pod number, Na^+ accumulation in old green leaves under saline treatment.

Overall our study had reported significant results like importance of flower number in water stress, delayed flowering and its association with Na^+ and K^+ ions, yield during saline stress, identification of two potential genomic regions and 48 putative candidate genes for salinity response and importance for low RH in heat stress studies in chickpea. Also this study raised new questions on several aspects like what would be the important

stages/traits/mechanisms that need to be studied in abiotic stress tolerance studies in chickpea.

Table 1. Details of alternate accession identifier, Source country, Biotype and Days to maturity and their tolerance level to drought under field conditions (T-Tolerant, S- Sensitive) of ten ICRISAT chickpea (ICC) genotypes used in this study.

S.No.	ICC number	Alternate accession identifier	Origin/ Source country	Biotype	Days to flower	Days to maturity	Yield kg/ha (Observed by Krishnamurthy et al. 2010)
1	ICC 867 (T)	P 690; Larkapura 1	India	Traditional Cultivar/ Landrace	44.9	87.6	2472
2	ICC 2263 (T)	P 1857-1	Iran	Traditional Cultivar/ Landrace	48.5	90.3	2354
3	ICC 14799 (T)	RSB 172	India	Traditional Cultivar/ Landrace	48.1	90.0	2698
4	ICC 3325 (T)	P 3971; 2099 2144	Cyprus	Traditional Cultivar/ Landrace	48.1	90.0	2497
5	ICC 8950 (T)	NEC 243; BR 17	India	Traditional Cultivar/ Landrace	49.3	90.7	2277
6	ICC 7323 (S)	PI 251783	Union of Soviet Socialist Republics	Traditional Cultivar/ Landrace	55.0	100.6	952
7	ICC 7184 (S)	NEC 1554; Acc No. 32685-71	Turkey	Traditional Cultivar/ Landrace	51.8	96.3	827
8	ICC 4814 (S)	P 6540; 2863 6085	Iran	Traditional Cultivar/ Landrace	48.8	92.1	943
9	ICC 8058 (S)	NEC 2189; P 6916	Iran	Traditional Cultivar/ Landrace	45.9	95.6	973
10	ICC 3776 (S)	P 4394	Iran	Traditional Cultivar/ Landrace	47.1	92.4	813

Table 2. Number of days that the genotypes were exposed to soil drying in phase I [normalised transpiration rate (NTR) from 1.0 to 0.5] and phase II (NTR from 0.49 to 0.10) and the fraction of transpirable soil water (FTSW) threshold value in year 1 and year 2. The standard error and 95% confidence limits of FTSW are also given.

Genotype	No. of days in phase I	No. of days in phase II	FTSW threshold value	Std. Error	95% confidence limits
Year 1					
ICC 867 (T)	12	10	0.64	0.02	0.59-0.68
ICC 2263 (T)	11	10	0.64	0.03	0.58-0.69
ICC 14799 (T)	9	12	0.68	0.02	0.64-0.72
ICC 3325 (T)	10	11	0.66	0.02	0.61-0.70
ICC 8950 (T)	11	12	0.56	0.03	0.49-0.62
ICC 7323 (S)	12	11	0.64	0.03	0.57-0.69
ICC 7184 (S)	12	13	0.50	0.03	0.44-0.55
ICC 4814 (S)	12	8	0.83	0.05	0.73-0.94
ICC 8058 (S)	9	15	0.72	0.03	0.66-0.76
ICC 3776 (S)	9	10	0.72	0.03	0.67-0.78
Year 2					
ICC 867 (T)	21	15	0.67	0.03	0.60-0.73
ICC 2263 (T)	19	16	0.73	0.04	0.64-0.82
ICC 14799 (T)	18	13	0.71	0.02	0.67-0.75
ICC 3325 (T)	19	17	0.69	0.03	0.62-.75
ICC 8950 (T)	18	17	0.76	0.04	0.68-0.84
ICC 7323 (S)	19	17	0.76	0.04	0.68-0.83
ICC 7184 (S)	22	15	0.44	0.04	0.36-0.51
ICC 4814 (S)	19	16	0.86	0.04	0.78-0.94
ICC 8058 (S)	17	14	0.80	0.03	0.74-0.86
ICC 3776 (S)	19	17	0.78	0.03	0.71-0.85

Table 3. *F* probability values (at *P*<0.01) and *F* statistic values obtained with unbalanced ANOVA analysis for genotype, year and the genotype × year interaction for flower number, flower + pod + seed abortion percentage, pod number, pod weight, seed number, seed yield and100-seed weight in phase I and phase II for year 1 and year 2.

Well watered							
Phase I	Flower number	Fl + pod + seed Ab %	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Year	<.001	<.001	0.002	0.002	0.002	0.074	0.002
Genotype × Year	<.001	<.001	<.001	<.001	<.001	<.001	<.001
***F* statistic value**							
Genotype	6.35	4.50	6.68	12.2	10.47	12.89	17.39
Genotype × Year	7.63	7.42	3.77	4.09	6.62	4.01	4.29
Phase II	Flower number	Fl + pod + seed Ab %	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	0.044	0.009	0.006	<.001	<.001	<.001	<.001
Year	<.001	<.001	0.034	<.001	<.001	<.001	0.26
Genotype × Year	<.001	0.001	0.003	0.004	0.002	0.005	0.16
***F* statistic value**							
Genotype	2.09	3.09	2.93	5.47	5.55	4.46	5.91
Genotype × Year	4.05	4.32	3.17	3.14	3.45	3.04	1.13
Water stressed							
Phase I	Flower number	Fl + pod + seed Ab %	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	0.002	0.19	<.001	<.001	<.001	<.001	0.008
Year	0.005	0.02	0.002	0.001	<.001	0.007	<.001
Genotype × Year	<.001	<.001	<.001	0.004	0.001	0.005	0.25
***F* statistic value**							
Genotype	3.46	1.47	6.99	17.14	11.68	17.23	2.81
Genotype × Year	4.72	4.39	2.31	3.11	3.58	3.00	1.31
Phase II	Flower number	Fl + pod + seed Ab %	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	0.18	0.17	0.033	0.001	<.001	<.001	0.86
Year	0.04	0.72	<.001	<.001	<.001	<.001	0.05
Genotype × Year	0.18	0.24	<.001	<.001	<.001	<.001	0.49
***F* statistic value**							
Genotype	1.47	1.38	2.20	3.52	3.76	3.91	0.51
Genotype × Year	1.47	1.25	3.99	6.63	6.68	6.73	0.94

Table 4. *F* probability values (at *P*<0.01) and *F* statistic values obtained with unbalanced ANOVA analysis for genotype, year and the genotype × year interaction for relative stem + leaf wt., relative total pod weight, relative total seed number, relative total 100-seed weight, relative harvest index, relative phase I flower number I, relative phase I pod weight, and relative phase II flower + pod + seed abortion percentage.

Relative value	Stem + leaf weight	Total pod weight	Total seed number	Total 100-seed weight	Harvest index	Phase I flower number	Phase I pod weight	Phase II Flower + pod + seed abortion %
F Probability								
Genotype	0.08	<0.001	0.02	0.10	<0.001	0.01	0.01	<0.001
Year	<0.001	0.84	0.02	< 0.001	<0.001	0.03	0.68	0.01
Genotype × Year	0.001	0.01	0.01	0.09	0.02	0.02	0.001	0.01
F statistic value								
Genotype	1.82	4.57	2.50	1.74	3.98	2.95	2.83	5.03
Genotype × Year	3.17	2.63	2.87	1.75	2.39	2.35	3.16	2.89

Table 5. *F* probability values (at *P*<0.01) and *F* statistic obtained with unbalanced ANOVA analysis for genotype, treatment and the genotype x treatment interaction for flower number, flower + pod + seed abortion percentage, pod number, pod weight, seed number, seed yield and 100-seed weight in phase I and phase II for year 1 and year 2.

Year 1, Phase I	Flower number	Flower, pod abortion	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	<.001	<.001	<.001	<.001	<.001	<.001	0.001
Treatment	0.067	0.005	<.001	<.001	<.001	<.001	0.001
Genotype × Treatment	0.497	0.107	0.551	0.218	0.216	0.412	0.154
***F* statistic value**							
Genotype	3.95	3.87	3.65	7.38	5.46	7.81	3.50
Genotype × Treatment	0.94	0.90	0.88	1.37	1.38	1.05	1.54
Year 1, Phase II	Flower number	Flower, pod abortion	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	0.064	0.309	0.018	<.001	<.001	<.001	0.002
Treatment	<.001	<.001	<.001	<.001	<.001	<.001	0.551
Genotype × Treatment	0.133	0.139	0.742	0.681	0.504	0.962	0.671
***F* statistic value**							
Genotype	1.92	1.64	2.45	6.33	7.68	6.59	3.31
Genotype × Treatment	1.60	2.80	0.66	0.73	0.93	0.33	0.74
Year 2, Phase I	Flower number	Flower, pod abortion	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Treatment	<.001	<.001	<.001	<.001	<.001	<.001	0.052
Genotype × Treatment	0.025	0.040	0.006	0.001	0.006	0.002	0.196
***F* statistic value**							
Genotype	11.11	6.80	13.43	21.72	20.04	23.3	8.14
Genotype × Treatment	2.34	1.78	2.93	3.00	2.90	3.43	1.43
Year 2, Phase II	Flower number	Flower, pod abortion	Pod number	Pod weight	Seed number	Seed yield	100-seed weight
Genotype	<.001	<.001	<.001	<.001	<.001	<.001	0.134
Treatment	<.001	<.001	<.001	<.001	<.001	<.001	0.153
Genotype × Treatment	0.077	0.132	0.001	<.001	0.001	<.001	0.496
***F* statistic value**							
Genotype	3.71	3.92	5.03	8.49	6.94	7.82	1.69
Genotype × Treatment	1.85	1.82	3.65	5.20	3.64	4.27	0.93

Table 6. Ranking of genotype for mean total seed yield in the water stressed (WS) treatment and the mean relative total seed yield values obtained by dividing WS value by well watered (WW) value in year 1 and year 2 (T- Tolerant; S-Sensitive; NA- not applicable).

	Year 1			**Year 2**		
Rank	Genotype	Total seed yield (g pot^{-1}) (WS)	Relative total seed yield	Genotype	Total seed yield (g pot^{-1}) (WS)	Relative total seed yield
1	ICC8950(T)	2.22	0.76	ICC3325(T)	2.59	0.53
2	ICC14799(T)	2.21	0.59	ICC8950(T)	1.90	0.40
3	ICC2263(T)	2.08	0.64	ICC14799(T)	1.55	0.38
4	ICC3325(T)	2.01	0.49	ICC2263(T)	1.26	0.40
5	ICC867(T)	1.47	0.48	ICC7184(S)	1.16	0.63
6	ICC4814(S)	1.15	0.39	ICC3776(S)	1.06	0.41
7	ICC7184(S)	0.77	0.55	ICC867(T)	1.01	0.54
8	ICC3776(S)	0.62	0.29	ICC4814(S)	0.58	0.47
9	ICC7323(S)	0.44	0.31	ICC7323(S)	0.54	0.80
10	ICC8058(S)	0.24	0.19	ICC8058(S)	0.50	0.20
	F Probability	<.001	<.001		<.001	0.15
	LSD	0.57	0.19		0.63	NA

Table 7. Ranking of genotype for the relative values of phase I and Phase II seed yield obtained by dividing corresponding water stressed (WS) value by mean well watered (WW) values in year 1 and year 2.

	Year 1		Year 2		Year 1		Year 2	
Rank	Genotype	Relative Phase I seed yield	Genotype	Relative Phase I seed yield	Genotype	Relative Phase II seed yield	Genotype	Relative Phase II seed yield
1	ICC 8950 (T)	0.89	ICC 7323 (S)	0.88	ICC 8950 (T)	0.67	ICC 7323 (S)	0.25
2	ICC 7184 (S)	0.76	ICC 7184 (S)	0.76	ICC 14799 (T)	0.52	ICC 867 (T)	0.23
3	ICC 2263 (T)	0.71	ICC 3776 (S)	0.64	ICC 2263 (T)	0.51	ICC 8058 (S)	0.18
4	ICC 14799 (T)	0.67	ICC 867 (T)	0.63	ICC 3325 (T)	0.41	ICC 3325 (T)	0.15
5	ICC 867 (T)	0.54	ICC 3325 (T)	0.57	ICC 3776 (S)	0.24	ICC 4814 (S)	0.12
6	ICC 3325 (T)	0.52	ICC 14799 (T)	0.50	ICC 867 (T)	0.22	ICC 3776 (S)	0.04
7	ICC 4814 (S)	0.50	ICC 4814 (S)	0.49	ICC 7323 (S)	0.22	ICC 14799 (T)	0.02
8	ICC 7323 (S)	0.38	ICC 8950 (T)	0.45	ICC 7184 (S)	0.19	ICC 2263 (T)	0
9	ICC 3776 (S)	0.34	ICC 2263 (T)	0.42	ICC 4814 (S)	0.19	ICC 8950 (T)	0
10	ICC 8058 (S)	0.34	ICC 8058 (S)	0.22	ICC 8058 (S)	0.07	ICC 7184 (S)	0
	F Probability	0.02		0.60		0.12		0.70
	LSD	0.32		NA		NA		NA

Table 8. Mean flower number pot^{-1}, mean flower + pod + seed abortion (Fl + pod + seed Ab.) percentage pot^{-1}, mean seed number pot^{-1} developed in phase I (normalised transpiration rate between 1.0 and 0.50) and the aboveground harvest index (HI) of whole plants at maturity in the well watered (WW) and water stressed (WS) treatments in year 1 and year 2 (T- Tolerant; S- Sensitive; NA- not applicable).

Year 1, Phase I	**WW**				**WS**			
Genotype	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.	Total HI	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.	Total HI
ICC 867 (T)	41.6	73.0	17.9	0.4	35.5	68.0	8.80	0.37
ICC 2263 (T)	29.9	25.8	16.3	0.45	18.0	55.6	10.7	0.35
ICC 14799 (T)	20.0	29.6	13.2	0.48	20.8	49.1	8.36	0.45
ICC 3325 (T)	35.9	31.9	22.7	0.52	21.3	56.2	10.3	0.43
ICC 8950 (T)	27.9	54.8	13.7	0.23	17.5	74.9	2.63	0.16
ICC 7323 (S)	28.1	61.3	14.8	0.37	27.2	52.0	10.4	0.21
ICC 7184 (S)	37.1	41.7	20.2	0.50	28.3	67.0	8.70	0.35
ICC 4814 (S)	14.3	21.4	11.2	0.48	20.5	55.5	10.2	0.38
ICC 8058 (S)	19.3	71.6	5.10	0.32	21.4	99.0	1.69	0.10
ICC 3776 (S)	17.7	40.6	11.7	0.35	20.7	82.0	4.10	0.13
Mean tolerant	31.1	43.0	16.8	0.42	22.6	60.8	8.15	0.35
Mean Sensitive	23.3	47.3	12.6	0.41	23.6	71.1	7.02	0.23
Mean 10 genotypes	27.2	45.2	14.7	0.41	23.1	65.9	7.58	0.29
Geno (*F* prob)	0.03	0.003	0.01	<.001	0.10	0.05	0.001	<.001
LSD (Genotype)	16.8	29.1	8.09	0.10	NA	29.4	4.73	0.10
LSD (Treatment)	14.1	27.8	6.29	0.10	14.1	27.8	6.29	0.10

Year 2, Phase I	**WW**				**WS**			
Genotype	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.	Total HI	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.	Total HI
ICC 867 (T)	27.7	53.0	15.3	0.36	17.0	52.2	9.03	0.44
ICC 2263 (T)	67.2	70.5	31.6	0.37	31.0	67.4	13.3	0.40
ICC 14799 (T)	60.5	55.2	30.7	0.34	50.4	70.9	17.8	0.32
ICC 3325 (T)	56.5	55.5	29.4	0.41	44.0	56.6	23.3	0.39
ICC 8950 (T)	11.9	68.3	3.38	0.07	19.1	72.1	4.06	0.15
ICC 7323 (S)	48.9	69.5	11.3	0.14	25.5	85.2	9.63	0.19
ICC 7184 (S)	76.0	85.7	28.6	0.31	27.9	62.1	12.3	0.43
ICC 4814 (S)	79.5	59.2	32.2	0.36	47.8	73.0	21.7	0.38
ICC 8058 (S)	17.9	55.5	7.38	0.25	7.73	61.5	3.61	0.08
ICC 3776 (S)	17.7	63.1	5.85	0.27	23.9	70.2	8.67	0.33
Mean Tolerant	44.8	60.5	22.1	0.31	32.3	63.9	13.5	0.34
Mean Sensitive	48.0	66.6	17.1	0.27	26.6	70.4	11.2	0.28
Mean 10 genotypes	46.4	63.5	19.6	0.29	29.4	67.1	12.4	0.31
Geno (*F* prob.)	<.001	<.001	<.001	<.001	<.001	0.52	<.001	<.001
LSD (Genotype)	28.4	17.5	11.2	0.1	19.6	27.6	6.63	0.16
LSD (Treatment)	22.9	23.4	8.20	0.13	22.9	23.4	8.20	0.13

Table 9. Mean flower number pot^{-1}, mean flower + pod + seed abortion (Fl + pod + seed Ab. percentage) pot^{-1}, mean seed number pot^{-1} developed in phase II (normalised transpiration rate between 0.49 and 0.10) in the well watered (WW) and water stressed (WS) treatments in year 1 and year 2 (T- Tolerant; S- Sensitive; NA- not applicable).

Year 1, Phase II	**WW**			**WS**		
Genotype	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.
ICC 867 (T)	19.6	80.0	4.45	11.2	89.7	1.53
ICC 2263 (T)	19.0	46.7	8.21	15.2	72.0	5.53
ICC 14799 (T)	27.7	54.5	15.9	22.0	67.3	8.17
ICC 3325 (T)	28.5	76.8	10.5	16.7	76.5	4.07
ICC 8950 (T)	30.9	63.2	9.75	6.43	72.2	1.13
ICC 7323 (S)	23.4	72.9	9.73	14.8	74.0	1.61
ICC 7184 (S)	29.3	74.4	9.54	8.76	92.4	1.95
ICC 4814 (S)	35.4	45.3	15.3	15.8	81.1	8.33
ICC 8058 (S)	15.4	69.1	4.80	13.2	98.6	0.15
ICC 3776 (S)	25.5	74.8	12.6	10.5	81.6	1.90
Mean Tolerant	25.1	64.2	9.76	14.3	75.5	4.09
Mean Sensitive	25.8	67.3	10.4	12.6	85.5	2.79
Mean 10 genotypes	25.5	65.8	10.1	13.5	80.5	3.44
Genotype (*F* prob.)	0.1	0.18	0.01	0.1	0.03	<.001
LSD (Genotype)	NA	NA	6.11	NA	19.4	3.71
LSD (Treatment)	11.7	24.7	4.85	11.67	24.7	4.85

Year 2, Phase II	**WW**			**WS**		
Genotype	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.	Flower no.	Fl+ pod+ seed Ab. (%)	Seed no.
ICC 867 (T)	25.9	76.5	4.11	2.84	81.5	0.74
ICC 2263 (T)	32.6	86.5	2.51	3.08	96.4	0.09
ICC 14799 (T)	49.1	79.0	9.07	12.3	99.8	0.19
ICC 3325 (T)	29.6	81.3	3.08	7.32	85.1	0.83
ICC 8950 (T)	33.0	85.6	1.53	8.64	87.9	0.80
ICC 7323 (S)	45.8	96.6	2.21	15.1	74.0	0.84
ICC 7184 (S)	18.8	93.7	4.54	7.08	99.1	0.32
ICC 4814 (S)	40.4	73.8	3.08	4.58	91.2	0.08
ICC 8058 (S)	71.7	82.9	7.38	14.1	73.8	1.64
ICC 3776 (S)	49.3	76.2	15.8	23.1	82.8	3.33
Mean Tolerant	31.6	81.8	4.06	6.84	90.1	0.53
Mean Sensitive	43.3	84.6	6.59	12.8	84.2	1.24
Mean 10 genotypes	39.6	83.2	5.33	9.82	87.1	0.89
Genotype (*F* prob.)	0.01	0.14	<.001	0.29	0.17	0.4
LSD (Genotype)	26.2	NA	5.68	NA	NA	NA
LSD (treatment)	20.1	22.9	3.89	20.13	22.9	3.89

Table 10. Relationship between relative total seed yield (RTSY) and relative total values of flower number-year 1, flower number-year 2 , flower + pod + seed Ab % -year 1, flower + pod + seed Ab % -year 2, phase I seed yield-year 1, phase I seed yield-year 2, phase II seed yield-year 1, phase II seed yield-year 2 , phase I flower number-year 2, phase I pod number-year 2 for 10 genotypes. The equations are the fitted linear regressions with the correlation coefficients and level of significance (**-P<0.01; *-P<0.05; n.s.- non-significant).

Factor	Linear relationship
Relative total flower number-year 1 (RTFNY1)	RTSY = 0.1617x + 0.365RTFNY1, R^2 = 0.031 (n.s)
Relative total flower number-year 2 (RTFNY2)	RTSY= 0.6444x + 0.1971RTFNY2, R^2 = 0.23**
Relative total flower + pod + seed Ab % -year 1 (RFlPdAbY1)	RTSY= -0.0249x + 0.5247RFlPdAbY1, R^2 = 0.002 (n.s)
Relative total flower + pod + seed Ab % -year 2 (RFlPdAbY2)	RTSY= 0.1317x + 0.3852RFlPdAbY2, R^2 = 0.009 (n.s)
Relative phase I seed yield- year 1 (RPhISYY1)	RTSY= 0.6034x + 0.1205RPhISYY1, R^2 = 0.69**
Relative phase I seed yield- year 2 (RPhISYY2)	RTSY= 0.6868x + 0.0738RPhISYY2, R^2 = 0.76**
Relative phase II seed yield- year 1 (RPhIISYY1)	RTSY = 0.3916x + 0.3693RPhIISYY1, R^2 = 0.31*
Relative phase II seed yield-year 2 (RPhIISYY2)	RTSY = 0.066x + 0.066RPhIISYY2, R^2 = 0.009 (n.s)
Relative phase I flower number-year 2 (RPhIFNY2)	RTSY = 1.0718x + 0.1595RPhIFNY2, R^2 = 0.42**
Relative phase I pod number- year 2 (RPhIPNY2)	RTSY= 0.7219x + 0.1636RPhIPNY2, R^2 = 0.42**

Table 11. Relationship between relative phase I seed yield (RSY-Ph1) and relative values (per pot) of phase I flower number-year 1, phase I flower number-year2, phase II flower number-year 1, phase II flower number-year 2 for 10 genotypes. The equations are the fitted linear regressions with the correlation coefficients and level of significance (**-P<0.01; n.s. - non-significant).

Factor	Linear relationship
Relative phase I flower number- year 1 (RPhIFNY1)	RSY-Ph1 = 0.3424x + 0.2755RPhIFNY1, R^2 = 0.26**
Relative phase I flower number-year 2 (RPhIFNY2)	RSY-Ph1=0.5711x + 0.2588RPhIFNY2, R^2 = 0.46**
Relative phase II flower number-year 1 (RPhIIFNY1)	RSY-Ph1=0.2481x + 0.2373RPhIIFNY1, R^2 = 0.12**
Relative phase II flower number-year 2 (RPhIIFNY2)	RSY-Ph1=0.2982x + 0.0552RPhIIFNY2, R^2 = 0.093 (n.s)

Table 12. *F* probability, least significant difference (LSD) and standard error (SE) values for genotype, treatment and genotype × treatment interaction for total shoot dry matter, days to first flower, days to maturity, filled pod number per plant, empty pod number per plant, seed number, seed yield and 100-seed weight of 14 chickpea genotypes grown in soil with 0 or 80 mM NaCl. Each pot had four plants.

Parameter	Total shoot dry matter (g/plant)	Days to first flower	Days to maturity/ plant	Filled pod number/ plant	Empty pod number/ plant	Seed number /plant	Seed yield (g/ plant)	100-seed weight
				Genotype				
F probability	0.003	<0.001	<0.001	<0.001	0.045	<0.001	<0.001	<0.001
LSD	2.485	3.460	2.996	3.896	4.717	5.108	0.652	1.209
SE	1.249	1.742	1.506	1.954	2.371	2.560	0.327	0.854
				Treatment				
F probability	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD	0.939	1.308	1.132	1.473	1.783	1.931	0.246	2.402
SE	0.472	0.658	0.569	0.739	0.896	0.968	0.124	1.697
				Genotype × Treatment				
F probability	0.136	<0.001	<0.001	<0.001	0.091	0.001	<0.001	0.023
LSD	3.514	4.893	4.237	5.510	6.670	7.224	0.922	1.697
SE	1.767	2.463	2.130	2.764	3.352	3.621	0.463	0.854

Table 13. Mean values of total shoot dry matter (g/plant), days to first flower, days to maturity, filled pod number/plant, empty pod number/plant, seed number/plant and 100-seed weight. *F* probability value at the 5% level of significance and least significant difference (LSD) of 14 chickpea genotypes grown in soil with 0 or 80 mM NaCl. Each pot had four plants.

Genotype	Total shoot dry matter (g/plant)	Days to first flower	Days to maturity	Filled pod number/plant	Empty pod number/ plant	100-seed weight
			0 mM NaCl			
ICC 456(T)	8.3	46	82	22.1	6.9	10.2
ICC 1431(T)	9.3	45	77	30.2	17.3	13.9
ICC 4495(T)	12.4	44	78	38.0	5.6	13.5
ICC 8950(T)	9.3	44	80	29.8	5.3	11.0
ICC 9942(T)	10.9	36	75	20.4	5.1	12.8
ICC 11121(T)	12.3	46	80	22.3	9.9	14.6
ICC 12155(T)	9.1	40	77	23.4	5.0	13.9
ICC 3421(S)	10.9	36	85	14.3	5.5	25.5
ICC 6263(S)	14.7	35	77	15.3	4.5	26.8
ICC 7315(S)	15.0	32	86	12.7	6.9	35.8
ICC 10755(S)	12.7	35	76	2.0	4.3	56.8
ICC 13283(S)	10.1	46	87	14.8	4.7	27.7
ICC 15510(S)	7.9	32	84	4.5	5.3	38.3
ICC 15518(S)	10.8	35	78	3.4	1.7	26.8
Mean tolerant	10.2	43	78	26.6	7.9	12.8
Mean sensitive	11.7	36	82	9.6	4.7	33.9
F probability	0.08	<0.001	<0.001	<0.001	0.08	<0.001
LSD	NA	3.6	3.4	6.1	3.8	2.1
			80 mM NaCl			
ICC 456(T)	6.1	53	86	18.1	2.9	11.4
ICC 1431(T)	5.9	48	82	17.1	0.6	13.9
ICC 4495(T)	9.8	47	78	23.4	1.7	14.1
ICC 8950(T)	8.2	46	85	19.6	1.8	13.2
ICC 9942(T)	6.0	40	76	17.7	0.3	14.5
ICC 11121(T)	5.9	48	82	16.3	2.6	14.3
ICC 12155(T)	7.4	44	78	19.7	1.9	13.6
ICC 3421(S)	8.1	48	86	6.7	1.2	18.1
ICC 6263(S)	8.7	53	92	5.9	0.6	27.8
ICC 7315(S)	7.2	42	87	6.2	1.1	31.4
ICC 10755(S)	6.4	39	84	2.3	0.4	25.9
ICC 13283(S)	5.7	60	98	1.6	0.03	26.8
ICC 15510(S)	6.4	44	89	5.3	0.5	17.1
ICC 15518(S)	6.9	55	101	1.8	0.2	20.4
Mean tolerant	7.1	47	81	18.8	1.7	13.6
Mean sensitive	7.1	49	91	4.3	0.6	23.9
F probability	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD	1.6	6.08	5.11	4.13	0.42	2.7

Table 14. Polymorphic SSR markers used for genotyping the F8 RIL chickpea population of ICCV 2 × JG 11. The unlinked markers are denoted by *.

S. No	Marker name	Gene bank ID	SSR motif	Forward primer (5'-3')	Reverse primer (5'-3')
1	CaM0015	EI846874	(TTA)18(TAT)20	AAAGACTTTACGGCTACAGCTTAT	TTATATGATCGAGTGGTGGGC
2	CaM0038	EI847480	(TAA)43	CATGCTCGAATCTTATTTTGAGG	TCGATATAGCAAGGGAGAGGA
3	CaM0463	EI856964	(AAT)9n(AT)5	TGGCATGGTGGACTCATTTA	GGGCTTATGGCCTGACCTAC
4	ICCM0034	FI856651	(GA)11	TTTGTTTGCGGAGGAATAGG	TCACCTCACCACACTTCTTTTC
5	ICCM0293	FI856982	(TAA)15tg(ATA)15	AGTGATGCCACGAGAATTGC	CTGGTTCGGAATTGTCATCC
6	CaM0658	EI861468	(ATC)11	TGTTTGGGCTTTTTGCTAAC	CATTCAGCCCCAAAACCTAA
7	CaM0800	EI864573	(CT)7	CCCCTTTGCATATTCCCATT	TGATGTTTGAACAAGTGTTAGGG
8	CaM0906	EI867102	(AT)21	TGCAACAAAAACTTCTTTCCC	CATGGTTTTTGTGTTCATCCA
9	CaM1129	EI871710	(AT)6n(AT)10n(AT)8n(AT)11n(AT)10	TTCCTCTGTTTCGTTTCCTTTT	CAAAATAGTTTGAGAAGTGATGATTTT
10	CaM1158	EI872397	(AT)15	GGTTACCCTACGTGTTTGCC	GCCTGAATATAAAATACGGGCTT
11	CaM1529	EI879902	(AT)26	TGAGTTGTGAGTTTGATGCCA	TTGAAAAATTCAATCCAAATCAA
12	CaM1608	EI881730	(AT)11	CCTTCCTTCTAGTTTTCCCCC	GCTCAGAGGCTTTTTAGCCA
13	CaM2031	EI889290	(AAT)11	TCCACCTTTTTGACACTTATACACA	TTCCAGCAATCATAAAGTTCAGA
14	ICCM0178	FI856592	(AAT)13	AGTTTGGGTTTCACCGCCT	GAACGCGCTCTGTTCATAAT
15	ICCM0272a	FI856961	(A)12	TTTCCACTTGGAACAGGCTC	AATGGACGATGGTTGGGTTA
16	CaM0123	EI849604	(TA)6	AATCGGGGGATCATAACACA	CCTCGCGTTCTACGTTTCTC
17	CaM0397	EI855540	(AT)8	GCTTGTGTCGACAATCAGGA	CAGTCCACATGAATGGTTGC
18	CaM1077	EI870622	(TATT)13	TGACCTGGCCTGACCTATTC	CAAACAATTGCTATTCCTTCTAGTCA
19	CaM1301	EI875257	(AT)20n(AT)11	GGTCAACAGTTCAAGGTGCAT	TTCGAGAAATGTGGATATTGGA
20	CaM1797	EI885000	(TA)8	TGCCTTTACCTTTGACTTCG	AAAACAGAAGTAAAATTAACACCCG
21	CaM1942	EI887759	(AAT)31	TGGGAGGTTTAGGGTCTACG	AAAAATCCCTCCAACGGTAAA
22	CaM1101	EI871128	(TAA)23	CGGGTAGAATGTAACACCCAG	TTAAATGGACGTGGGTAACG
23	CaM1469	EI878861	(GA)9n(AG)5	CAAACATCGTCATTTTATGTCTGA	ACACCAGCCTTGCACAAAA
24	CaM0317	EI854030	(AAT)13	TGGCCTAAATGTCTCAGCAA	AGAGGCAAACAAGAACCGAA
25	CaM0812	EI864718	(TA)14(T)13	TTCAATGATGGATTTTGGTTCA	CAAGAGACCCGAAAGAGATAAAA

Contd…

26	CaM1742	EI884153	(AT)16	TCCTTTTCATAGAGATACGAACAAAA	TCAATACGAGGATTGGAATATGA
27	CaM2174	EI892426	(ATA)5n(AAT)23	TTTTGAGGTCATACAGGAGGA	TGACATAAATTTTGGGGACGA
28	ICCM0069	FI856558	(ATT)22	TCTTCTTTGCTATCTGTCTCGC	TGCATGTCAAACATTAGACAACTTT
29	CaM0487*	EI857438	(AT)15n(TA)20	AAGTCGCCATTTGCAAAAAC	TGGACAATAGTAAACCTGATCGAA
30	CaM0658*	EI861468	(ATC)11	TGTTTGGGCTTTTTGCTAAC	CATTCAGCCCCAAAACCTAA
31	CaM0182*	EI850929	(AT)9	AACATGTAATTTAAGTGTGGGGG	CAATCATGCCAATCCAAACA
32	CaM0643*	EI861191	(AT)10	CTCGTGCTCACAATACTCGG	TCGTCCATGTTAGTTGCTGC
33	CaM0753*	EI863632	(TTA)23	AATTGCGGCGAGAGAAGATA	TCAGTTTCTCTTTTCGATTCTTTC
34	CaM1417*	EI877674	(CAC)6	CTCCTCCGAAACCAAAAACA	GTTTTGGGGAATTTGAGGGT
35	CaM0713*	EI862793	(TAT)50	AAAAGGTTTAATTGTAGTTTTGATTCC	TTCAAAATAAGAGAGTGAGACAAAAA
36	CaM1505*	EI879462	(AT)23	ATGAAAGAAGGAGGGAGGGA	TGCGGTGAATCTTTTACGAA

Table 15. Polymorphic SNP markers used for genotyping the F8 RIL chickpea population of ICCV 2 × JG 11. The unlinked markers are denoted by *.

S. No	Marker name	Gene bank ID	Polymorphism	Left flanking sequence	Right flanking sequence
1	CKAM0044	Ca_Cap2promo	C/G	ATTCATATGTAGTATATATATATATA TAACTAATATTCATTTCCAAATAA	ACAGCAACCAAGATTCTTAAGTTAA TACAAAATAAAGGTAAGCACAGCCA
2	CKAM1131	Ca1C31106	G/A	AAATAAATATCCTAAATCGAACAA AAGCATTTACAATGTGCGACCAAAAT	TATGTACATCTACCGCAACAACCTCA TGCATAAAATTTAATTGTAATCTT
3	CKAM1902	CaSs2305	G/A	TAAGATTTGTGTTATTTTGACACTAA AATATGACTTGGAATATATCAAGT	CCGCAACTCATTAACCATTCATTAAA CATATATAACCACGAGTTTAATGA
4	CKAM1903	CD07C12	C/T	ATCGTCATAACCAANATCATCCTCA ACTTTCTCCGCAGCATTATCAGCAA	CTTATCTTCAATAAAATCAGCACCTT CCACAAGTGNAATTCCACCAANAA
5	CKAM0343	TOG898271	T/C	ATTAAACACAATCAACACATGTTTA TTTATAAGTTACAGGTTACTAAATG	ATCTGATTTGCAGTGAGCATTTCTGA GTGAGGAGAGCACATAGATTTTTA
6	CKAM0165	TOG894101	C/G	ATATCATATTAATTACATAATTTTGT GAAGGCATTCTGCTATGATGTCAC	ATTGATATTCCATTTATTTAGCTTGTA TTGCTTTTCAGCATTTTATGCAT
7	CKAM0462	TOG902768	A/G	TTTGATGAGTAAGAATCAACGCTC ACACACATTGCGCACCAAATACTTGC	CTAAACTTTGACGGTATCTACTAGAA GTTGTATATGTATTATTTTGTTTC
8	CKAM0707	Ca1773847832705	A/G	ACCTATCATTAATTTTAATAATTAT AGATATAGAATCACATTTCATCCCA	TTGTGAAACAAGTTTAAAGGTGAGT GTCAACTCTATTGTTAGCACAAAAG
9	CKAM1175	Ca1C33347	A/G	CGTCGTCTTGAACAGGGGTGTTAAA GGCTGGATTTGCGGTGCGAACTATG	AACCGTCGCTATAGACATGAAGAAC GCCACGACATTCGTCCACTAAAAGT
10	CKAM1443	Ca1C4438	T/C	ACGGCGCCATCAAGCGTCCGCCGTGTTCTCA TGAGCGCACCTCTCTCATC	GACCTCCGATCCAAATACAATGTCC GCTCCATGCCAGTCAGGAAGGACGA
11	CKAM1797	Ca1S321485_ 0320_2776	C/G	TTGTAATTCCAAATCCTATTAATGTT AAGCTCTTAAAGTTAAGTAAGTGC	ACTATATGAACCAAATTCCATCCAAA AAATATATAAAAACACAAGCNTAA
12	CKAM1894	CaSc38434	A/G	CAAGAGAATTCTTGGTTACCTTCTCA ACATTTGATATATTCCCTGGAATG	ATTTTGATGATGATAGAATTTCATAA TGTGAGACCCCAATATCTGGACAT
13	CKAM0304	TOG897326	A/G	TTCCAACATCAAAGACAATAATAGTA AATAATAATGTWCTCATAAGTAAG	GCTAGTACATTGCGATGAATCCCA AGTTCAGTAAATATTTTTTGTTAACA
14	CKAM0448	TOG902069	T/C	GAAAAAATATGAAGAAAAAGTGCTA ATGATGTTTGACACAGCATTAACTA	TAATGCACTCAAACAAATACAAATTA AGAGACTTACCAAAAAGGCTACTG

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15	CKAM0939	Ca1C21140	C/T	GGACAACCTTTAGGTTGATGCCCTAA GACTTCTTTAGTGTCAAACCTTCA	TAGTTTTGTGACAGTTTATAAAATTTT TTGTCTTAATTTATTCTTATTAC
16	CKAM0993	Ca1C23843	T/C	GAAAGTTATGTTGATGAAATTAGTT ACCAATTATGCTAGACACTGGATTC	GCATTGTGTAGTGGAACCAGTAGAC CACATTCATGTGCTTGGATCATAAT
17	CKAM1066	Ca1C27942	G/C	TATGCTTGTGTTTTTTATATATTTTTT GGATGGAATTTGGTTCATATAGT	GCACTTACTTAACTTTAAGAGCTTAC ATTAATAGGATTTGGAATTCTATT
18	CKAM1317	Ca1C39503	A/C	AATTTTTGGTGCTAAATGGACCCTG TTAAGACCGAGTAAATATAGGCTAT	CTTCAACTCAATGTGTAACCTTAA TTTCCATTGTGCTGTTTTTGGAATGC
19	CKAM1370	Ca1C41642	G/C	AGCTGAAGTAAAACTTGAACATTTT GTCTGATAATATGTGTGATTGTTTA	AAATATGGGGCGAAAAGTAGCACT CAGTTTACTGGCATCTACTAAAAAAA
20	CKAM1462	Ca1C45231	A/G	AAAAAGAAGAAGAAAAGAAAAAAA TAGATGGAAGCATGTCATGATGTTTT	TTATCATTCTTGCAACACATGCAAG AGTTCAAATTGTCATGGATGTCACC
21	CKAM0003	AGL111	T/G	AGTTGTGTACATGTTGTGTATTTT TACTATGCATGGGAATAAGTGTCTCT	CCCTGGATAATTTGTTTGTTCAGAA AAATCAGGACGTGTTAGATCCAAAC
22	CKAM1003	Ca1C242	A/T	ATTTGAATCTATGATATACTTTTG TTGATGTTTAATTCTAAATGATGAGC	GGATAGTATTTGTTTAGTTTCTGAAT TTGTCAAATTCAAAGATATTCTTA
23	CKAM1971	Mt125375	A/G	TACTAAAGAAAAATTCTTTTATTGG GGACTGTAGATTGTTAGTGGTGATG	GAAAAGGGTTAAGCGACTCAATCC ACTTCATTTCAATGAATTTAAAGACC
24	CKAM0647	TOG919502	A/G	ATCAATTGCTTGTTTTGCATGCTTC ATTGCAATATTGTTGAGTTGCTTTT	TTAGTTTTTCCAAGTTTCTTCAATTTT TATATCGTGTATTGCTTCTTGCA
25	CKAM0280	TOG896852	T/C	CTATCCAAAAATCTACCACGCCAT TCCGCCATGGCCACATTTAACAACAC	GCTCAAAATACCTGCAGTCTATCCAA ATTATTGATTTTTGTGTAAAGAAC
26	CKAM0405	TOG900323	A/G	TTGATGATCNAGAACTTATGCATG CTCAATTTCTTTTCTTGAAAGGGAAA	GGTCTCAAGGGTTTGGTTCTGTTGA AAGTTTTAGTTAGGACAATAATCTT
27	CKAM1489	Ca1C46343	A/T	CATAACGATAACTTAAACAAGTTT AACAGTAACTGAATGTATTTCAAAAG	AAATCAAAACTAAAAGTCTTGGCAT GACAAAAATAAGGAGACAAAGCAAA
28	CKAM1925	CD59-G12	C/A	TCAAAGCACTGGGCAGAAATCACA TTGCGTCAACATCCGCAGGGACCATC	CAATGCTTTGTTTTAATTAAACAGTC GGATTCCCCTTGTCCGTACCAATT
29	CKAM0234*	TOG895747	T/C	TAAAACAATAAAATTGCATAAAAA AGATATTGGCCATCATGAAGTAAAGA	GCAACACAAATAAGAAACATGCCAT GTAGATTGAGAGATCTAAGCAAATT
30	CKAM0831*	Ca1C16173	A/G	CTGTTCTAGTTAGCATTCCTTATG ACACCACACATTTGTGGCAAATTATG	GTCACACGATTAACGACAATCAAAAT TCTGTTTGTTGAACTTCTTAATAA

Table 16. ANOVA results for the parameters evaluated under control and salinity treatments in 2010. Mean values of nine parameters evaluated (two parents, maximum and minimum mean values from 188 RILs) and *F* probability, standard error (SE), least significant difference (LSD) and the heritability values under control and saline treatment, 2010.

Trait	Days to flower	Days to maturity	Above ground dry matter (g plant $^{-1}$)	Yield (g plant $^{-1}$)	Pod number plant $^{-1}$	Seed number plant $^{-1}$	Stem+leaf weight (g plant $^{-1}$)	Harvest index (%)	100-seed weight (g plant $^{-1}$)
Control, 2010									
ICCV 2 (SS)	31	84	22.47	10.86	41.43	41.78	11.61	0.48	25.93
JG 11 (ST)	33	78	24.34	14.18	54.52	60.01	10.16	0.59	23.84
Variation in RILs	23-50	73-99	9.67- 37.35	3.14-18.55	13.97-77.84	27.17-85.21	3.47-19.04	0.18-0.88	14.40-41.58
F Probability	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
SE	4.63	5.66	5.84	2.89	12.63	13.82	3.35	0.07	2
LSD	9	11	11.49	5.29	24.83	27.17	6.58	0.14	3.94
Heritability (%)	78	61	33	44	43	44	38	71	92
Salinity, 2010									
ICCV 2 (SS)	30	69	11.81	5.83	29.08	29.35	5.96	0.49	19.89
JG 11 (ST)	34	81	19.84	10.66	46.79	46.02	8.71	0.57	23.36
Variation in RILs	21-56	63-93	5.23-21.23	2.89-11.02	14.71-62.35	13.69-63.9	2.69-12.16	0.28-1.04	13.64-35.28
F Probability	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
SE	3.49	4.38	3.14	1.62	6.83	7.04	1.62	0.08	1.74
LSD	7	9	6.17	3.18	13.4	13.81	3.17	0.15	3.42
Heritability (%)	85	80	58	44	59	56	65	58	85

Table 17. ANOVA results for the parameters evaluated under control and salinity treatments in 2011. Mean values of nine parameters evaluated (two parents, maximum and minimum mean values from 188 RILs) and *F* probability, standard error (SE) , least significant difference (LSD) and the heritability values under control and saline treatment, 2011.

Trait	Days to flower	Days to maturity	Above ground dry matter (g plant $^{-1}$)	Yield (g plant $^{-1}$)	Total pod number plant $^{-1}$	Seed number plant $^{-1}$	Stem+ leaf weight (g plant $^{-1}$)	Harvest index (%)	100-seed weight (g plant $^{-1}$)
Control, 2011									
ICCV 2 (SS)	30	76	19.98	10.21	75.97	40.15	9.77	0.53	25.64
JG 11 (ST)	32	79	27.08	14.7	71.34	61.07	12.38	0.54	24.03
Variation in RILs	25-46	73-91	10.55-33.61	4.60-18.13	24.45-109.74	17.59-78.76	5.54-17.42	0.23-0.61	15.17-45.21
F Probability	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
SE	1.59	2.55	4.18	2.41	14.85	10.13	2.29	0.05	1.65
LSD	3.12	5.01	8.2	4.72	29.14	19.88	4.49	0.11	3.24
Heritability (%)	91	43	52	49	33	49	54	38	91
Salinity, 2011									
ICCV 2 (SS)	29	69	9.54	5.92	27.66	23.29	3.62	0.62	25.66
JG 11 (ST)	30	75	13.06	7.14	30.66	29.62	5.92	0.55	24.02
Variation in RILs	23-48	66-88	6.93-25.19	2.91-11.89	11.26-85.12	9.56-54.23	2.45-13.30	0.28-0.71	15.45-44.32
F Probability	<.001	<.001	<.001	0.001	<.001	<.001	<.001	<.001	<.001
SE	2.01	2.17	3.09	1.76	9.57	7.63	1.59	0.05	1.82
LSD	3.95	4.25	6.06	3.45	18.78	14.97	3.13	0.09	3.57
Heritability (%)	90	85	48	40	67	60	64	71	89

Table 18. Relationship between the nine and eleven traits evaluated under control and salinity in 2010 and 2011. All the traits were significantly correlated (P<0.001).

	2010	
Days to flower (DFCY1)	DFSY1 = 0.7664x + 5.3975DFY2	R^2 = 0.50**
Days to maturity (DMCY1)	DMSY1 = 0.7642x + 10.46DMY2	R^2 = 0.34**
Aboveground dry matter (ADMCY1)	TDMSY1 = 0.3184x + 7.0175ADMY2	R^2 = 0.22**
Stem+leaf weight (STLFWTCY1)	STLFWTSY1= 0.3396x + 3.0957STLFWTY2	R^2 = 0.26**
Total pod number (TPDNOCY1)	TPDNOSY1 = 0.4229x + 15.438TDPDNOY2	R^2 = 0.35**
Seed number (SDNOCY1)	SDNOSY1 = 0.3801x + 16.5631SDNOCY1	R^2 = 0.35**
100-seed weight (100SDWTCY1)	100SDWTSY1 = 0.6844x + 4.986100SDWTCY1	R^2 = 0.79**
Harvest index (HICY1)	HISY1 = 0.429x + 0.2997HICY1	R^2 = 0.24**
Yield (YLDCY1)	YLDSY1 = 0.2897x + 4.0806YLDCY1	R^2 = 0.23**
	2011	
Days to flower (DFCY2)	DFSY2 = 0.9081x + 1.1176DFCY2	R^2 = 0.71**
Days to maturity (DMCY2)	DMSY2 = 0.8917x + 2.2583DMCY2	R^2 = 0.46**
Aboveground dry matter (ADMCY2)	TDMSY2 = 0.2465x + 5.4841ADMCY2	R^2 = 0.25**
Stem+leaf weight (STLFWTCY2)	STLFWTSY2 = 0.3067x + 1.3624STLFWTCY2	R^2 = 0.30**
Filled pod number (FPDNOCY2)	FPDNOSY2 = 0.3144x + 10.499FPDNOCY2	R^2 = 0.27**
Empty pod number (EPDNOCY2)	EPDNOSY2 = 0.1724x + 1.3654EPDNOCY2	R^2 = 0.16**
Total pod number (TPDNOCY2)	TPDNOSY2 = 0.3204x + 8.5745TPDNOCY2	R^2 = 0.25**
Seed number (SDNOCY2)	SDNOSY2 = 0.2741x + 12.876SDNOCY2	R^2 = 0.24**
100-seed weight (100SDWTCY2)	100SDWTSY2 = 0.8892x + 3.3591100SDWTCY2	R^2 = 0.76**
Harvest index (HICY2)	HISY2 = 0.4257x + 0.3603HICY2	R^2 = 0.10**
Yield (YLDCY2)	YLDSY2= 0.9032x + 5.5458YLDCY2	R^2 = 0.21**

Table 19. Relationship between the traits for which QTLs were identified and yield. All the traits were significantly correlated either at P<0.001 or P<0.05 except for days to maturity, control, 2010 and days to flower, salinity, 2011.

Control, 2010		
Days to maturity (DMC1)	CY1 = 0.0616x + 5.2717DMC1	r^2 = 0.001 (n.s)
Aboveground dry matter (ADMC1)	CY1 = 0.4575x + 0.6915ADMC1	r^2 = 0.83**
Stem + leaf wt. (ST+LFWTC1)	CY1= 0.6142x + 3.7464ST+LFWTC1	r^2 = 0.51**
Harvest index (HIC1)	CY1 = 14.954x + 3.0064HIC1	r^2 = 0.09**
100- seed weight (100SDWTC1)	CY1 = 0.1337x + 7.1635100SDWTC1	r^2 = 0.03*
Salinity, 2010		
Days to flower (DFS1)	SY1 = 0.0671x + 4.8857DFS1	r^2 = 0.04**
Days to maturity (DMS1)	SY1 = 0.0915x + 0.2932DMS1	r^2 = 0.10**
Total pod number (TPDNOS1)	SY1 = 0.193x + 0.7443TPDNOS1	r^2 = 0.75**
Seed number (SDNOS1)	SY1 = 0.1924x + 0.6744SDNOS1	r^2 = 0.76**
Harvest Index (HIS1)	SY1 = 11.534x + 1.0604HIS1	r^2 = 0.12**
100 - seed weight (100SDWTS1)	SY1 = 0.2179x + 2.3611100SDWTS1	r^2 = 0.11**
Control, 2011		
Days to flower (DFC2)	CY2 = 0.4756x + 26.722DFC2	r^2 = 0.08**
Days to maturity (DMC2)	CY2 = 0.3687x + 75.324DMC2	r^2 = 0.09**
Aboveground dry matter (ADMC2)	CY2 = 1.6454x + 3.2286ADMC2	r^2 = 0.85**
Stem+ leaf weight (ST+LFWTC2)	CY2 = 0.6454x + 3.2286ST+LFWTC2	r^2 = 0.48**
Filled pod number (FPDNOC2)	CY2 = 3.034x + 10.336FPDNOC2	r^2 = 0.56**
Total pod number (TPDNOC2)	CY2 = 2.9113x + 33.653TPDNOC2	r^2 = 0.28**
Seed number (SDNOC2)	CY2 = 2.9747x + 15.317SDNOC2	r^2 = 0.49**
100- seed weight (100SDWTC2)	CY2 = 0.7146x + 15.12100SDWTC2	r^2 = 0.22**
Harvest index (HIC2)	CY2 = 0.0071x + 0.4364HIC2	r^2 = 0.17**
Salinity, 2011		
Days to flower (DFS2)	SY2 = 0.3838x + 27.863DFS2	r^2 = 0.012(n.s)
Days to maturity (DMS2)	SY2 = 0.6464x + 69.096DMS2	r^2 = 0.04**
Aboveground dry matter (ADMS2)	SY2 = 1.5322x + 1.2604ADMS2	r^2 = 0.76**
100 - seed weight (100SDWTS2)	SY2 = 0.5902x + 20.249100SDWTS2	r^2 = 0.04**
Harvest Index (HIS2)	SY2 = 0.0091x + 0.5234HIS2	r^2 = 0.04**

Table 20. *F* probability values (at *P*<0.01), *F* statistic values obtained with unbalanced ANOVA analysis for genotype, year, genotype × year interaction.

Parameters	Days to flower	Days to maturity	Total dry matter (g plant $^{-1}$)	Yield (g plant $^{-1}$)	Pod number plant $^{-1}$	Seed number plant $^{-1}$	Stem + leaf wt. (g plant $^{-1}$)	Harvest Index (%)	100-seed weight (g plant $^{-1}$)
Control 2 years- *F* Probability values									
Geno	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Year	<.001	<.001	0.001	<.001	<.001	<.001	0.919	<.001	<.001
Geno*Year	<.001	<.001	<.001	<.001	0.077	<.001	<.001	<.001	<.001
SE	1.88	2.57	3.481	1.901	10.79	8.313	1.931	0.0428	1.282
LSD	3.688	5.042	6.83	3.73	21.17	16.31	3.787	0.08397	2.515
***F* statistic values**									
Geno	6.26	2.43	1.89	1.95	1.61	2.04	1.92	1.76	11.97
Year	230.12	205.81	10.54	36.58	610.22	42.07	0.01	25.24	20.02
Geno*Year	7.04	2.62	1.67	1.66	1.15	1.6	1.63	1.5	9.34
Salinity 2 years-*F* Probability values									
Geno	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Year	<.001	0.037	<.001	<.001	0.049	<.001	<.001	<.001	<.001
Geno*Year	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
SE	1.943	2.357	2.201	1.187	5.886	5.161	1.136	0.04182	1.241
LSD	3.811	4.622	4.318	2.328	11.54	10.12	2.228	0.08204	2.433
***F* statistic values**									
Geno	8.09	6	1.84	1.75	2.48	2.39	2.2	2.53	10.99
Year	74.53	4.35	102.31	19.84	3.87	117.9	259.33	243.97	526.59
Geno*Year	8.45	5.82	1.89	1.51	2.38	1.94	2.45	2.27	8.71

Table 21. Relationship between relative yield in 2010 and 2011 (RY1 and RY2) and relative values of studied parameters. The equations are the fitted linear regressions with the correlation coefficients and level of significance (**-P<0.01; *-P<0.05; n.s.- non-significant).

	2010	
Relative days to flower (RDF1)	RY1 = -0.0367x + 0.9501 RDF1	r^2 = 0.003 (n.s)
Relative days to maturity (RDM1)	RY1 = 0.0355x + 0.8651 RDM1	r^2 = 0.01*
Relative ADM (RADM1)	RY1 = 0.8004x + 0.109 RADM1	r^2 = 0.86**
Relative stem + leaf wt. (RS+LWT1)	RY1 = 0.6328x + 0.2073 RS+LWT1	r^2 = 0.52**
Relative pod no. (RPDNO1)	RY1 = 0.9983x + 0.1205 RPDNO1	r^2 = 0.85**
Relative seed no. (RSDNO1)	RY1 = 0.9625x + 0.0992 RSDNO1	r^2 = 0.89**
Relative HI (RHI1)	RY1 = 0.2583x + 0.8591 RHI1	r^2 = 0.18**
Relative 100-seed wt. (R100SDWT1)	RY1 = 0.1607x + 0.7849 R100SDWT1	r^2 = 0.16**
	2011	
Relative days to flower (RDF2)	RY2 =-0.0724x + 1.0173 RDF2	r^2 = 0.004 (n.s)
Relative days to maturity (RDM2)	RY2 = 0.0174x + 0.9328 RDM2	r^2 = 0.0006 (n.s)
Relative ADM (RADM2)	RY2 = 0.6784x + 0.1137 RADM2	r^2 = 0.76**
Relative stem + leaf wt. (RS+LWT2)	RY2= 0.3812x + 0.2223 RS+LWT2	r^2 = 0.27**
Relative pod no. (RPDNO2)	RY2 = 0.8768x + 0.0514 RPDNO2	r^2 = 0.64**
Relative seed no. (RSDNO2)	RY2 = 0.8768x + 0.0514 RSDNO2	r^2 = 0.89**
Relative HI (RHI2)	RY2 = 0.1672x + 0.9433 RHI2	r^2 = 0.27**
Relative 100-seed wt. (R100SDWT1)	RY2 = 0.1672x + 0.9433 R100SDWT1	r^2 = 0.08*
Relative filled pod no.(RFPDNO2)	RY2 = 0.9064x + 0.0481 RFPDNO2	r^2 = 0.87**
Relative empty pod no.(REPDNO2)	RY2 = 0.2101x + 0.1287 REPDNO2	r^2 = 0.05**

Table 22. Summary of major and minor QTLs for various salinity tolerance related traits. The QTLs were identified using QTL Cartographer on ICCV 2 × JG 11 derived mapping population.

Trait name	Year	Treatment	QTL name	Marker interval	Linkage group	Position (cM)	LOD	PVE (R^2x100)	Additive effect
Days to maturity	2010	Control	QR9DM1	CaM0463-ICCM272	CaLG05	71.31	28.95	61.06	-36.75
Days to maturity	2010	Control	QR9DM2	ICCM0293-CKAM0707	CaLG04d	1.01	27.99	62.67	-40.76
Days to maturity	2010	Control	QR9DM3	CKAM1903-CKAM0343	CaLG08	0.01	3.6	13.24	12.64
Days to maturity	2010	Control	QR9DM4	CaM0812-CKAM0647	CaLG08	38.61	29.8	66.97	-41.45
Aboveground dry matter	2010	Control	QR9ADM1	CaM0463-ICCM272	CaLG05	71.31	3.31	26.93	-7.27
Stem +leaf weight	2010	Control	QR9STM+LFWT1	CaM0463-ICCM272	CaLG05	71.31	3.04	27.19	-3.78
Harvest Index	2010	Control	QR9HI1	CaM0463-ICCM272	CaLG05	71.31	14.56	46.03	-0.21
Harvest Index	2010	Control	QR9HI2	CKAM1903-CKAM0343	CaLG08	0.01	3.09	10.01	0.07
100-seed weight	2010	Control	QR9100SDWT1	CaM0463-ICCM272	CaLG05	71.31	8.3	39.97	-8.96
Days to flower	2010	Saline	QR9DF1	CaM2031-CKAM0165	CaLG07	20.31	2.96	6.47	3.5
Days to maturity	2010	Saline	QR9DM5	CaM2031-CKAM0165	CaLG07	20.31	4.01	7.95	5.03
Yield	2010	Saline	QR9YLD1	CaM2031-CKAM0165	CaLG07	15.91	2.67	16.99	0.86
Total pod number	2010	Saline	QR9PDNO1	CaM2031-CKAM0165	CaLG07	14.91	2.52	24.86	5.08
Seed number	2010	Saline	QR9SDNO1	CaM2031-CKAM0165	CaLG07	15.91	2.63	16.86	4.2
Harvest Index	2010	Saline	QR9HI3	CaM1077-CaM1797	CaLG04d	48.01	2.63	6.13	-0.03
100 seed weight	2010	Saline	QR9100SDWT2	CaM0038-CaM0463	CaLG05	3.01	2.65	17.42	-2.5
Days to flower	2011	Control	QR9DF2	CaM0463-ICCM272	CaLG05	72.31	8.01	26.93	-9.86
Days to flower	2011	Control	QR9DF3	CKAM0003-CKAM1003	CaLG04d	123.31	5.73	38.9	-10.4
Days to flower	2011	Control	QR9DF4	CKAM1903-CKAM0343	CaLG08	3.31	10.62	39.97	-10.84
Days to maturity	2011	Control	QR9DM6	CaM0463-ICCM272	CaLG05	72.31	19.06	42.18	-25.35
Days to maturity	2011	Control	QR9DM7	CKAM0003-CKAM1003	CaLG04d	124.31	39.19	64.34	-29.72
Days to maturity	2011	Control	QR9DM8	CKAM1903-CKAM0343	CaLG08	3.31	43.32	65.07	-30.92
Aboveground dry matter	2011	Control	QR9ADM2	CaM0463-ICCM272	CaLG05	72.31	4.94	17.17	-5.51
Stem + leaf weight	2011	Control	QR9STM+LFWT2	CaM0463-ICCM272	CaLG05	72.31	3.72	15.41	-2.84

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Yield	2011	Control	QR9YLD2	CaM0463-ICCM272	CaLG05	72.31	4.56	16.31	-2.89
Yield	2011	Control	QR9YLD3	ICCM0034-CaM0906	CaLG07	0.91	3.29	7.57	0.81
Filled pod number	2011	Control	QR9FPDNO1	CKAM0993-CKAM1317	CaLG07	74.61	3.82	8.43	3.46
Total pod number	2011	Control	QR9PDNO2	ICCM0034-CaM0906	CaLG07	0.91	3.54	7.63	4.64
Seed number	2011	Control	QR9SDNO2	CKAM0993-CKAM1317	CaLG07	74.61	4.04	9.18	3.83
100 seed weight	2011	Control	QR9100SDWT3	CaM0463-ICCM272	CaLG05	72.31	9.47	28.58	-7.61
Harvest index	2011	Control	QR9HI4	CaM0463-ICCM272	CaLG05	72.31	11.04	32.21	-0.2
Harvest index	2011	Control	QR9HI5	CKAM0003-CKAM1003	CaLG04d	123.31	20.61	52.02	-0.19
Harvest index	2011	Control	QR9HI6	CKAM1903-CKAM0343	CaLG08	3.31	27.93	55.67	-0.19
Days to flower	2011	Saline	QR9DF5	CaM0463-ICCM272	CaLG05	72.31	6.26	24.98	-9.27
Days to flower	2011	Saline	QR9DF6	CKAM0003-CKAM1003	CaLG04d	124.31	2.91	22.6	-5.85
Days to flower	2011	Saline	QR9DF7	CKAM1903-CKAM0343	CaLG08	3.31	7.75	37.75	-10.22
Days to maturity	2011	Saline	QR9DM9	CaM1301-CKAM1971	CaLG01	39.11	40.76	66.75	-34.5
Days to maturity	2011	Saline	QR9DM10	CaM0463-ICCM272	CaLG05	72.31	16.16	40.69	-27.92
Days to maturity	2011	Saline	QR9DM11	CKAM0003-CKAM1003	CaLG04d	124.31	30.34	59.95	-26.71
Days to maturity	2011	Saline	QR9DM12	CKAM1903-CKAM0343	CaLG08	3.31	31.87	56.87	-27.99
Aboveground dry matter	2011	Saline	QR9ADM3	CaM0463-ICCM272	CaLG05	72.31	2.87	12.02	-2.45
Yield	2011	Saline	QR9YLD4	CaM0463-ICCM272	CaLG05	72.31	2.76	12.21	-1.37
100-seed weight	2011	Saline	QR9100SDWT4	CaM0463-ICCM272	CaLG05	72.31	10.59	33.4	-8.69
Harvest index	2011	Saline	QR9HI7	CaM0463-ICCM272	CaLG05	72.31	8.04	29.85	-0.23
Harvest index	2011	Saline	QR9HI8	CKAM0003-CKAM1003	CaLG04d	123.31	12.23	49.13	-0.22
Harvest index	2011	Saline	QR9HI9	CKAM1903-CKAM0343	CaLG08	3.31	16.06	47.23	-0.22

Table 23. Linkage group correspondence in three studies to published maps. The linkage group number in published maps and the corresponding number in Samineni (2010), Vadez et al. (2012) and in present study were given. The numbers within parenthesis refers to the common markers identified between the linkage group in a population and reference maps. NA- Not applicable. LG 5 and LG 7 in reference maps that harbored salinity tolerance related QTLs across three population were highlighted (bold).

LG number as per published maps	Samineni (2010)	Vadez et al. (2012)	Present study
LG 1	NA	LG 1 (6)	CaLG01 (3)
LG 2	LG 2 (5)	LG 2 (4)	NA
LG 3	LG 1 (4), LG3 (2)	LG 6 (3)	CaLG03 (3)
LG 4	LG 4 (7)	LG 6 (18)	CaLG04 (3), CaLG05a (3)
LG 5	**LG 7 (8)**	**LG 7 (10)**	**CaLG02 (3)**
LG 6	LG 6 (6)	LG 3 (10)	CaLG05b (3)
LG 7	**LG 5 (6)**	**LG 5 (7)**	**CaLG07 (6)**
LG 8	LG 8 (4)	LG 4 (5)	CaLG08 (4)

Table 24A. List of putative candidate genes found to be associated with salinity stress response on CaLG05.

Gene ID	Protein name	Function	Plant/ Crop	Reference
Ca_01893	Protein EMBRYONIC FLOWER 1	Participates in Polycomb group-mediated transcriptional repression. Reduced EMF1 activity increases salinity tolerance.	Arabidopsis	Pu et al. 2013
Ca_01782	Potassium channel AKT1	Involved in inward-rectifying channels in the regulation of the K^+ to Na^+ ratio under salinity stress.	Arabidopsis	Golldack et al. 2003
Ca_01771	Cellulose synthase A catalytic subunit 1	Required for catalyzing the biosynthesis of cellulose deposited to the primary wall.	Arabidopsis	Chen et al. 2005
Ca_01586	Zinc finger protein ZAT10 (Salt-tolerance zinc finger)	Upregulated and showed enhanced tolerance of plants to drought stress, osmotic stress, and salinity.	Arabidopsis	Ciftci-Yilmaz et al. 2007
Ca_01555	Polyadenylate-binding protein 2	Functions related to protecting mRNA from degradation and regulating translation.	Arabidopsis	Tiwari, 2008
Ca_01548	ABSCISIC ACID-INSENSITIVE 5-like protein 5	Interact with the abscisic acid (ABA) receptors, and negatively regulate ABA signaling during germination.	Arabidopsis,	Rena et al. 2010
Ca_01522,Ca_03919	Transcription factor MYB44	Regulates ABA signaling to induce stomatal closure and confers drought/salinity-stress tolerance.	Arabidopsis	Seo et al. 2011
Ca_01508	Pyridoxine/pyridoxamine 5'-phosphate oxidase 1	Involved in vitamin-B6 biosynthesis, plays role in stress tolerance and photoprotection of plants.	Arabidopsis	Titiz et al. 2006
Ca_01496	Ubiquitin carboxyl-terminal hydrolase 16	Positively regulates plasma membrane Na^+/H^+ antiport activity and stabilizes serine hydroxymethyltransferase1.	Arabidopsis	Zhou et al. 2012
Ca_01423	HVA22-like protein e	Unique ABA/stress-induced protein.	Barley	Guo et al. 2008

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Ca_01414	NAC transcription factor ONAC010	Stress responsive gene and is specifically expressed in stamen and induced by drought and salinity.	Rice	Sperotto et al. 2008
Ca_01394, Ca_01625	Transcription factor ICE1	Induced by cold, dehydration and salinity. Binds to myc-cis elements and induces CBF3 expression		Chinnusamy et al. 2004
Ca_07420	BTB/POZ and TAZ domain-containing protein 2	Stress related transcription factor activated by salicylic acid.	Potato	Ginzberg et al. 2009
Ca_07429	Protein-L-isoaspartate O-methyltransferase 1	Regulated by stresses and ABA. Involves in seed longevity and germination vigor.	Arabidopsis	Verma et al. 2013
Ca_07461	Histone deacetylase 6	Involved in ABA mediated responses to drought or salinity.	Arabidopsis	Perrella et al. 2013
Ca_07490	Probable inactive poly [ADP-ribose] polymerase SRO2	RCD1 by physical interaction with SOS1, a plasma membrane Na^+/H^+ antiporter provides oxidative/ salinity stress tolerance.	Arabidopsis	Jaspers, 2009
Ca_07522	Betaine aldehyde dehydrogenase 1	Helps in the accumulation of osmolytes like Gly betaine and b-Ala betaine. Proved to enhance salinity tolerance.	Carrot	Kumar et al. 2004
Ca_07527	Two-component response regulator ARR5	Positive regulators of osmotic stress.	Soybean	Thu et al. 2014
Ca_07566	5-methyltetrahydropteroyltri glutamate—homocysteine methyltransferase 1	Involve in methionine biosynthesis. Play a significant role in amino acid metabolism.	Arabidopsis	Joshi and Jander, 2009
Ca_07579	Homeodomain transcription factor ATHB-5	Responsive to ABA and salinity stress at the seedling stage.	Arabidopsis	Ma et al. 2014
Ca_07582, Ca_11404	Glycine-rich RNA-binding protein 4	Up-regulation of this protein in response to salinity stress may stabilize mRNA and enhance synthesis of specific proteins during stress.	Rice	Dooki et al. 2006
Ca_19234	Nudix hydrolase 2	Confers enhanced tolerance for oxidative stress by maintaining NAD+ and ATP levels (nucleotide recycling from free ADP-ribose molecules) under stress conditions.	Arabidopsis	Ogawa et al. 2009

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Ca_11361	Calmodulin-binding transcription activator 4	Induced by NaCl stress. Involved in ethylene signal transduction processes regulated through Ca2 /CaM messenger system.	Arabidopsis	Reddy et al. 2002
Ca_11358	Histidine kinase 3	Cytokinin transmembrane receptor, sense osmolarity changes in cells caused by dehydration.	Maize	Javadmanesh et al. 2013
Ca_12733	Protein-tyrosine-phosphatase MKP1	Acts as a cross talk point in stress signaling pathway. Enhances resistance under salinity stress.	Arabidopsis	Ulm et al. 2002
Ca_12688	Tetratricopeptide repeat thioredoxin-like 3	Upregulated in response to osmotic and NaCl stresses.	Arabidopsis	Lakhssassi et al. 2012
Ca_12654,Ca_12655	Aldehyde dehydrogenase family 3 member H1	Encode delta-1-pyrroline-5-carboxylate dehydrogenases which play an important role in the degradation of proline to glutamate.	Grape	Zhang et al. 2012

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Table 24B. List of putative candidate genes found to be associated with salinity stress response on CaLG07.

Ca_16384,Ca_16441	BON1-associated protein 2	May have a direct role in regulating cell expansion and cell division at lower temperatures.	Arabidopsis	Hua et al. 2001
Ca_16388	COP9 signalosome complex subunit 1	Facilitates RUB deconjugation, regulates photomorphogenesis and required for removal of a ubiquitin-like protein from cullin subunits of the SCF ubiquitin-ligase.		Review: Biedermann and Hellmann, 2011
Ca_16418	Phospholipase D beta 1	Involved in the salinity-induced phosphatidic acid production.	Tomato	Laxalt et al. 2001
Ca_19579	Phosphopantothenoylcysteine decarboxylase	Overexpression of this gene increases proline biosynthesis and enhance salinity tolerance.	Tobacco	Yonamine et al. 2004
Ca_20270, Ca_20272	Annexin D3, Annexin D4	Play an important role in germination during salinity and osmotic stress.	Arabidopsis	Clark et al., 2001
Ca_17993	Beta-glucosidase 24	Plays important role in ABA metabolism, hydrolysis of conjugated gibberellins and conversion of storage forms of cytokinins to active forms.	Rice, Maize	Zörb et al. 2004
Ca_20197	Probable choline kinase 2	Upregulated under salinity and stimules phosphatidylcholine biosynthesis rate.	Arabidopsis	Tasseva et al. 2004
Ca_25034	11-oxo-beta-amyrin 30-oxidase	Catalyze C30 oxidation of β-amyrin in glycyrrhetinate biosynthesis pathway.	Medicago	Lin et al. 2013
Ca_21366	Prohibitin-3	Upregulated mainly under salinity but also by cold, UV-B stress. Enhance ethylene response plays vital role in mitochondrial function.	Arabidopsis	Aken et al. 2007
Ca_15721, Ca_15722	Transcription factor GTE10	Acts as a negative regulator in plant response to changes in environmental conditions through the control of ABA-regulated gene expression.	Arabidopsis	From UniProt database
Ca_01778,Ca_01780,Ca_01781	GTP-binding nuclear protein Ran-3	Induced by salinity stress but specific function is not known.	Arabidopsis	From UniProt database
Ca_22745	Uncharacterized protein	ER-associated ubiquitin-dependent protein catabolic process; Response to salinity stress.	Soybean	From UniProt database

Table 25. Mean values of yield and yield related traits obtained at 40% RH treatment, 2012; S-sensitive, T-tolerant.

Genotype	Shoot wt. (g/plant)	Pod no./plant	Pod wt. (g/plant)	Filled pod/ plant	Empty pods/ plant	100-seed wt.(g)	HI	Fl+ pod+ seed abortion (%)
ICC10755 (S)	8.44	5.00	1.18	4.25	0.75	10.48	0.09	63.99
ICC15294 (S)	8.69	8.25	1.80	6.25	2.00	20.50	0.14	49.33
ICC4495 (T)	6.68	29.00	6.60	24.75	4.25	14.31	0.30	56.45
ICC92944 (T)	5.63	19.00	4.78	16.25	2.75	22.12	0.36	40.50
ICC9942 (T)	6.35	22.00	3.77	20.00	2.00	12.79	0.29	52.31
Mean sensitive	8.57	6.63	1.49	5.25	1.38	15.49	0.12	56.66
Mean tolerant	6.22	23.33	5.05	20.33	3.00	16.41	0.32	49.75
F probability	0.16	0.01	0.02	0.01	0.41	0.04	<.001	0.12
LSD	2.92	13.77	3.18	11.22	3.74	8.38	0.12	17.60

Table 26. Mean values of yield and yield related traits obtained at 40% RH treatment, 2013; S-sensitive, T-tolerant.

Genotype	Shoot wt. (g/plant)	Pod no./plant	Pod wt. (g/plant)	Filled pod/ plant	Empty pods/ plant	100-seed wt. (g)	HI	Fl+ pod+ seed abortion (%)
ICC10755 (S)	6.56	3.20	0.65	1.80	1.40	21.96	0.07	90.59
ICC15294 (S)	6.69	4.25	0.80	4.25	0.00	13.43	0.06	42.5
ICC4495 (T)	5.32	9.50	1.16	6.58	2.92	9.90	0.13	80.24
ICC92944 (T)	3.76	7.00	1.01	3.90	3.10	17.16	0.16	80.48
ICC9942 (T)	5.35	10.75	1.29	7.25	3.50	11.07	0.15	78.83
Mean sensitive	6.63	3.73	0.73	3.03	0.70	17.70	0.07	66.55
Mean tolerant	4.81	9.08	1.15	5.91	3.17	12.71	0.15	79.85
F probability	0.002	0.163	0.414	0.035	0.897	0.004	0.326	<.001
LSD	1.21	3.94	0.43	2.55	2.70	4.09	0.04	7.91

Table 27. Mean values of yield and yield related traits obtained at 30% RH treatment, 2012; S-sensitive, T-tolerant.

Genotype	Shoot wt. (g/plant)	Pod no./ plant	Pod wt. (g/plant)	Filled pod/ plant	Empty pods/ plant	100-seed wt. (g)	HI	Fl+ pod+ seed abortion (%)
ICC10755 (S)	4.49	2.25	0.75	2.00	2.75	33.19	0.12	43.75
ICC15294 (S)	3.18	11.13	1.91	8.13	11.75	19.60	0.32	42.06
ICC4495 (T)	8.91	16.25	2.86	15.00	22.25	14.51	0.20	57.28
ICC92944 (T)	2.09	6.75	1.55	5.50	4.00	24.25	0.37	29.04
ICC9942 (T)	8.10	26.50	4.23	23.75	21.75	12.30	0.28	39.97
Mean sensitive	3.83	6.69	1.33	5.06	7.25	26.39	0.22	42.90
Mean tolerant	6.36	16.50	2.88	14.75	16.00	17.02	0.29	42.09
F probability	<.001	<.001	0.001	<.001	0.001	<.001	<.001	0.225
LSD	2.497	6.911	1.413	6.818	10.05	4.906	0.103	24.05

Table 28. Mean values of yield and yield related traits obtained at 30% RH treatment, 2013; S-sensitive, T-tolerant.

Genotype	Shoot wt. (g/plant)	Pod no./ plant	Pod wt. (g/plant)	Filled pod/ plant	Empty pods/ plant	100-seed wt. (g)	HI	Fl+ pod+ seed abortion (%)
ICC10755 (S)	5.17	1.50	0.26	1.17	0.33	17.08	0.07	92.61
ICC15294 (S)	5.70	4.17	0.60	3.33	0.83	13.76	0.12	81.71
ICC4495 (T)	4.51	9.00	1.26	7.13	1.88	9.76	0.18	70.32
ICC92944 (T)	2.82	4.80	0.73	2.50	2.30	19.63	0.16	76.89
ICC9942 (T)	4.97	8.20	0.90	5.60	2.60	10.96	0.14	75.70
Mean sensitive	5.43	2.83	0.43	2.25	0.58	15.42	0.10	87.16
Mean tolerant	4.10	7.33	0.96	5.08	2.26	13.45	0.16	74.30
F probability	0.002	0.004	0.006	0.002	0.103	0.001	0.315	0.025
LSD	1.287	4.14	0.51	2.99	2.07	4.90	0.11	14.39

Table 29. Mean values of yield and yield related traits obtained at 20% RH treatment, 2013; S-sensitive, T-tolerant.

Genotype	Shoot wt. (g/plant)	Pod no./ plant	Pod wt. (g/plant)	Filled pod/ plant	Empty pods/ plant	100-seed wt. (g)	HI	Fl+ pod+ seed abortion (%)
ICC10755 (S)	4.44	2.00	0.30	1.67	0.50	10.34	0.05	95.05
ICC15294 (S)	5.08	5.00	0.55	4.50	0.50	9.07	0.09	92.78
ICC4495 (T)	4.19	4.75	0.66	3.63	1.13	10.51	0.11	85.34
ICC92944 (T)	2.76	4.75	0.56	3.50	1.25	13.86	0.11	63.53
ICC9942 (T)	3.08	4.33	0.54	3.92	0.42	10.25	0.14	81.10
Mean sensitive	4.76	3.50	0.43	3.08	0.50	9.70	0.07	93.92
Mean tolerant	3.34	4.61	0.59	3.68	0.93	11.54	0.12	76.66
F probability	0.001	0.416	0.181	0.428	0.42	0.495	0.015	0.139
LSD	1.03	3.844	0.328	3.217	1.442	6.443	0.054	27.18

Table 30. Traits contributing to yield at different RH treatments.

30% RH, 2012		
Flower no. to yield	y = 0.0618x + 0.4862	R^2 = 0.95**
Pod no. to yield	y = 0.1186x + 0.4399	R^2 = 0.99**
Pod wt. to yield	y = 0.8318x + 0.0509	R^2 = 0.999**
Seed number to yield	y = 0.1025x + 0.6553	R^2 = 0.98**
Filled pod number to yield	y = 7.7293x - 4.0541	R^2 = 0.99**
Empty pod no. to yield	y = 7.6687x - 2.312	R^2 = 0.84*
100-seed wt. to yield	y = -6.997x + 34.285	R^2 = 0.87*
30% RH, 2013		
Pod no. to yield	y = 0.102x + 0.0563	R^2 = 0.97**
Pod wt. to yield	y = 0.8564x - 0.0204	R^2 = 0.99**
Seed number to yield	y = 0.0776x + 0.2176	R^2 = 0.93**
Filled pod number to yield	y = 7.2832x - 0.5751	R^2 = 0.94**
20% RH, 2013		
Pod wt. to yield	y = 0.7448x + 0.0319	R^2 = 0.82*
Seed number to yield	y = 0.0834x + 0.0724	R^2 = 0.92**
40% RH, 2012		
Shoot wt. to yield	y = -0.9637x + 9.5952	R^2 = 0.87*
Pod no. to yield	y = 0.1283x + 0.563	R^2 = 0.84*
Pod wt. to yield	y = 0.5952x + 0.5426	R^2 = 0.89*
Filled pod number to yield	y = 5.7623x - 1.2568	R^2 = 0.83*
40% RH, 2013		
HI	y = 0.0794x + 0.0226	R^2 = 0.95**
Filled pod number	y = 5.7623x - 1.2568	R^2 = 0.83*
Pod weight	y = 0.5952x + 0.5426	R^2 = 0.89*
Pod number	y = 0.1283x + 0.563	R^2 = 0.84*

Table 31. Tissue temperature at VPD 3.4 kPa when RH was 20% and temperature was 30.5°C.

Genotype	Canopy temperature at 20% RH (VPD-3.4 kPa)
ICC4495(T)	29.42
ICC9942(T)	30.35
ICC92944(T)	28.89
ICC10755(S)	30.33
ICC15294(S)	29.56
Mean tolerant	29.55
Mean sensitive	29.94

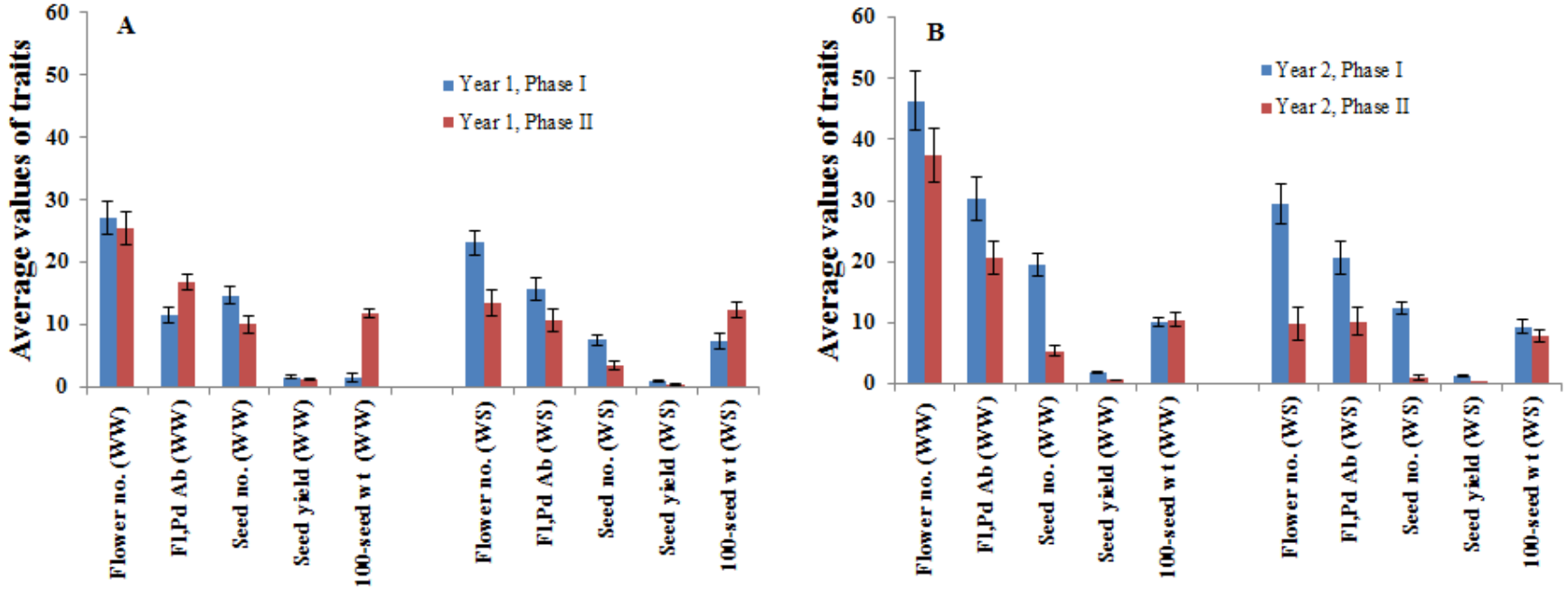


Figure 1. Mean values (of all 10 genotypes) and standard error (SE) of mean for flower number pot^{-1}, flower + pod + seed abortion number pot^{-1}(Fl, Pd Ab), seed number pot^{-1}, seed yield (g pot^{-1}), and 100-seed weight (g pot^{-1}) at two phases (Blue-Phase I, Red- Phase II) in the well watered (WW) and water stressed (WS) treatments during year 1 (A) and year 2 (B).

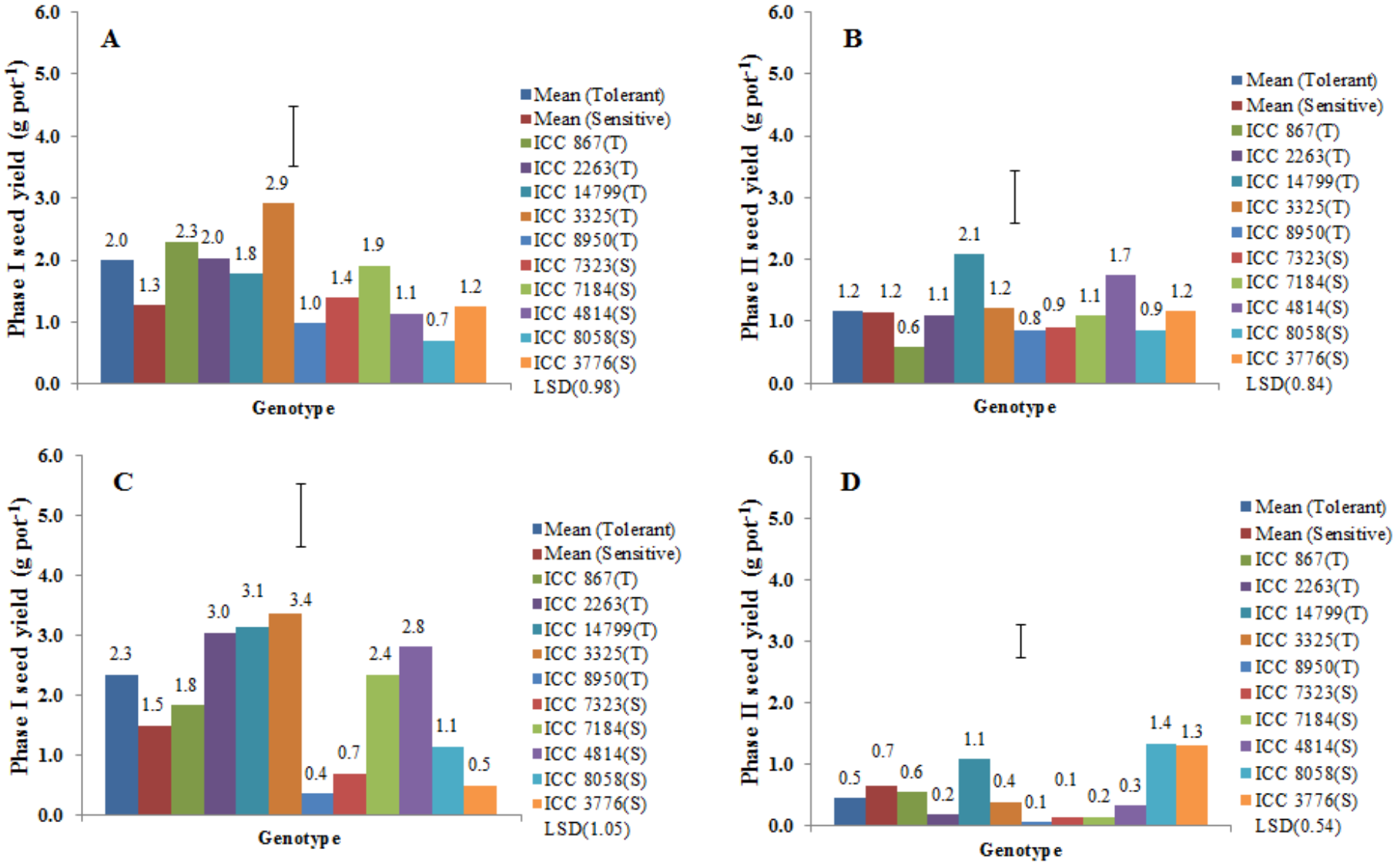


Figure 2. Mean seed yield (g pot^{-1}) of the tolerant group, and sensitive group, and of the 10 genotypes during phase I (A, C) and phase II (B, D) in year 1 (A, B) and year 2 (C, D) in the well watered (WW) treatment. Year 1 and 2 are not distinguished.

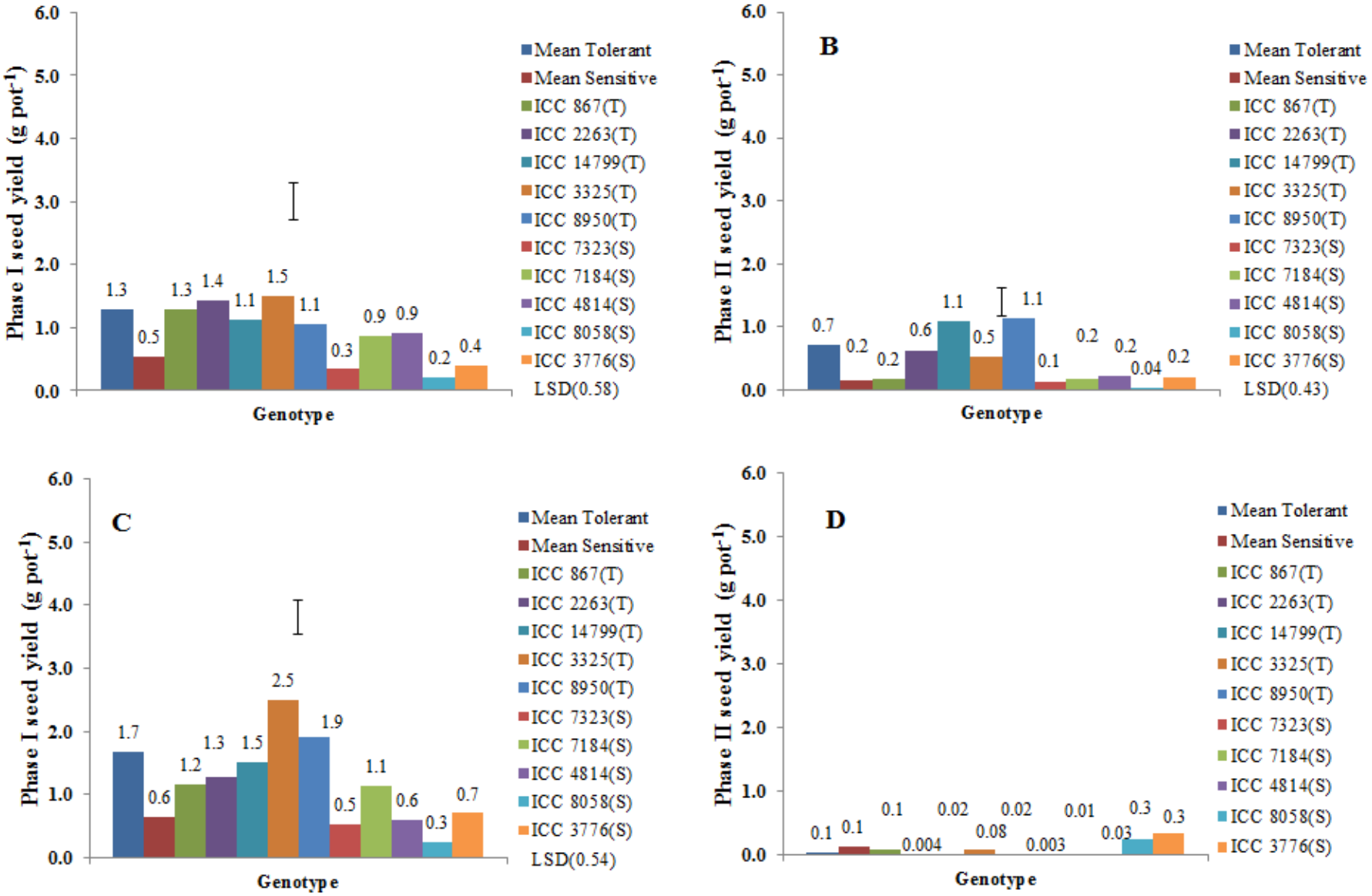


Figure 3. Mean seed yield (g pot^{-1}) in the tolerant group and sensitive group and of the 10 genotypes during phase I (A, C) and phase II (B, D) in year 1 (A, B) and year 2 (C, D) in the water stressed (WS) treatment.

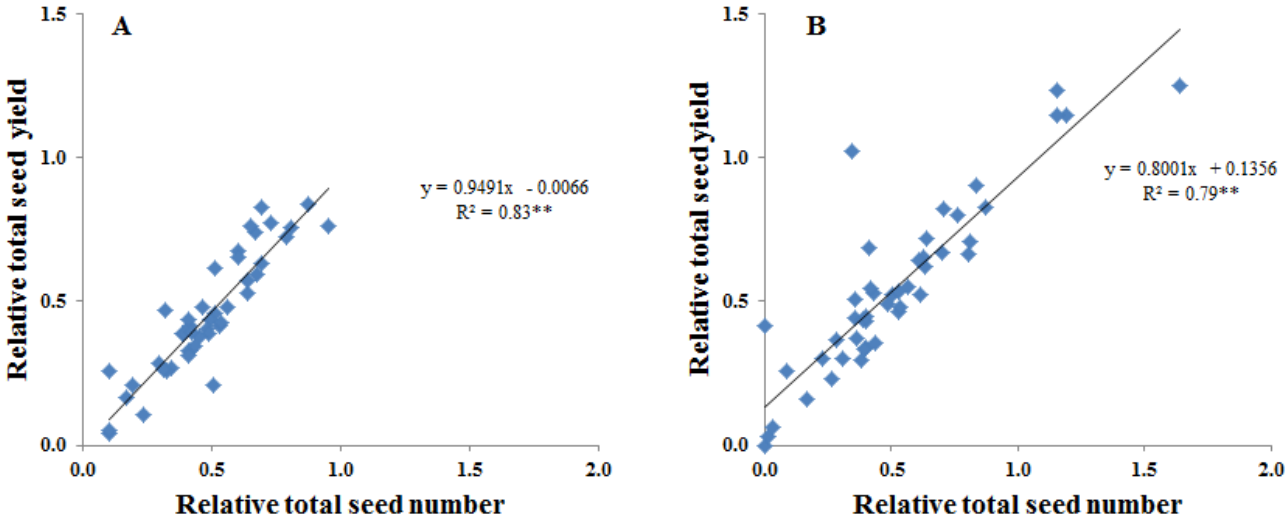


Figure 4. Relationships between relative total seed number and relative total seed yield in year 1 (A) and year 2 (B).

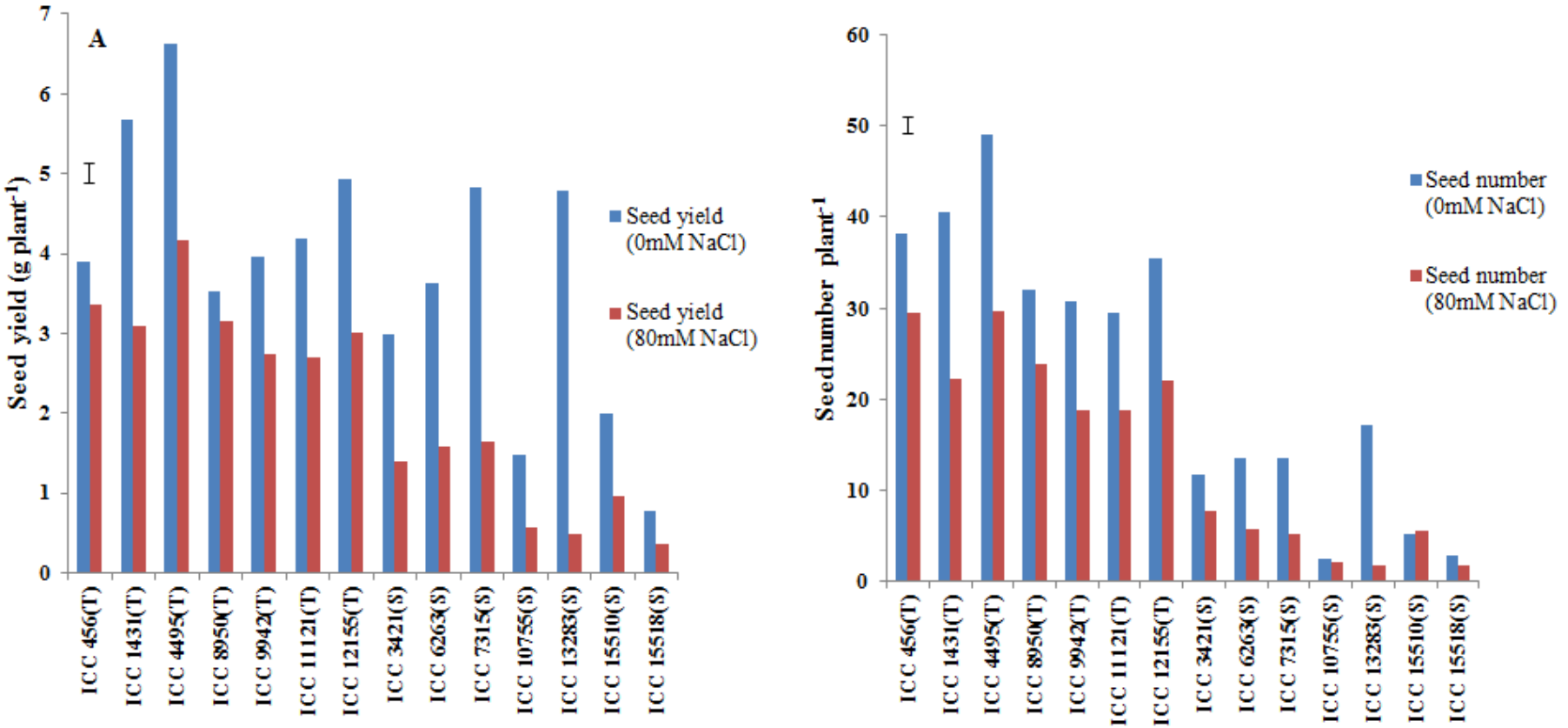


Figure 5. Seed yield and seed number per plant of 14 genotypes of chickpea, salt-tolerant (T) and salt-sensitive (S), when grown in control (0 mM NaCl, blue bars) and saline (80 mM NaCl, red bars) soil in an outdoor pot system. The bar gives the least significant difference (LSD) at $P = 0.05$ for the genotype × treatment interaction.

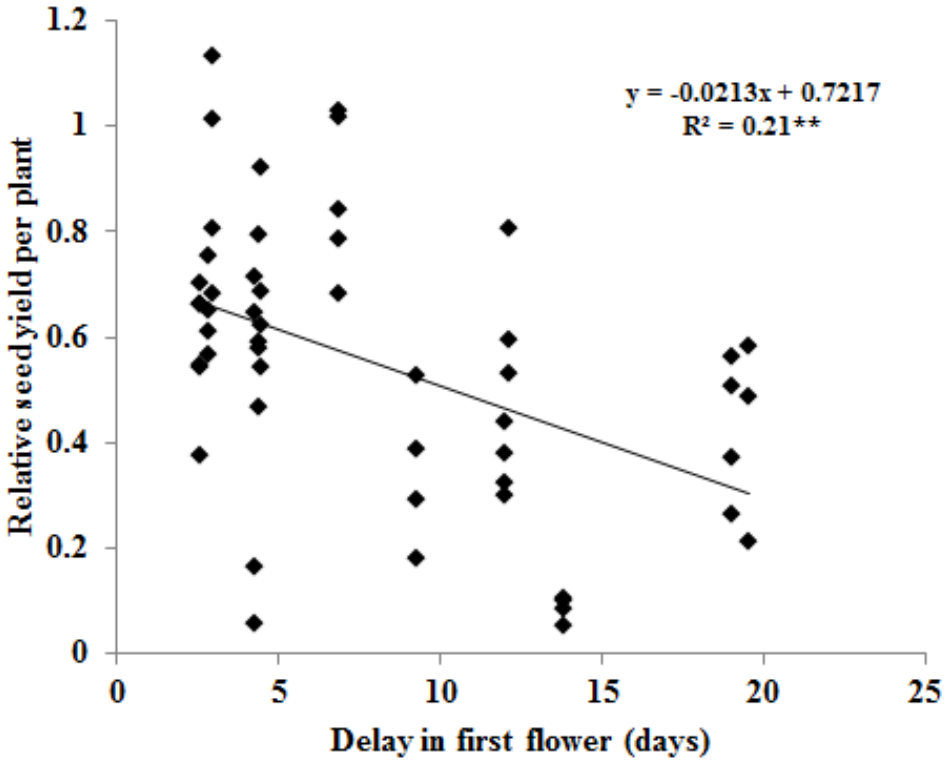


Figure 6. Relationship between delay in flowering (days) under 80 mM NaCl treatment compared to 0 mM NaCl and relative seed yield per plant (each replicate value of salt stressed in a genotype divided by corresponding non saline control mean) for 14 genotypes of chickpea. The data are values in 80 mM NaCl relative to those in 0 mM NaCl. **- significant at P<0.01.

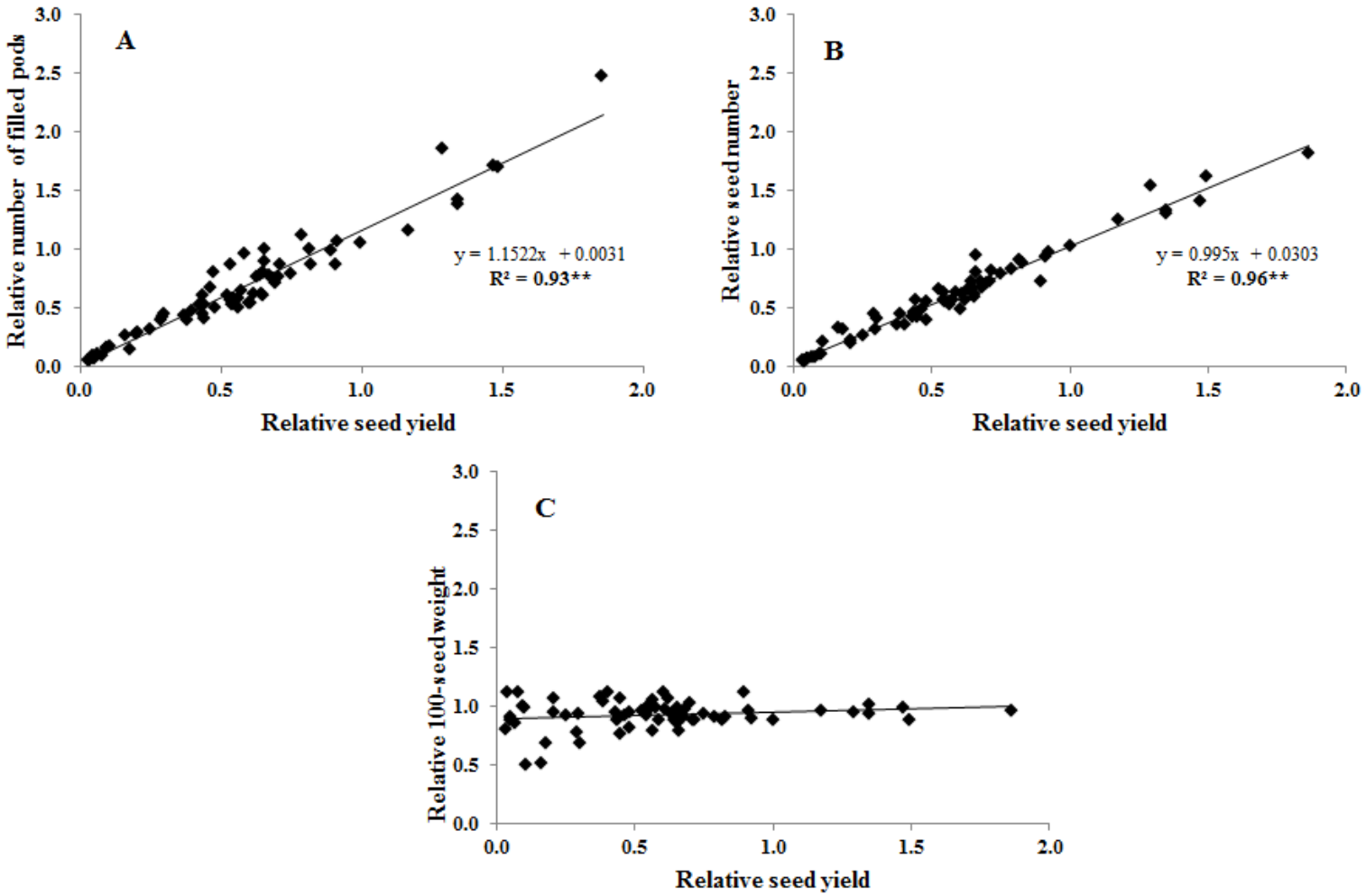


Figure 7. Relationship between relative seed yield (each replicate value of salt stressed in a genotype divided by corresponding non-saline control mean) and relative filled pod number (A), relative seed number (B), and relative 100-seed weight (C) (not significant) for 14 genotypes of chickpea. The data are values in 80 mM NaCl relative to those in 0 mM NaCl.

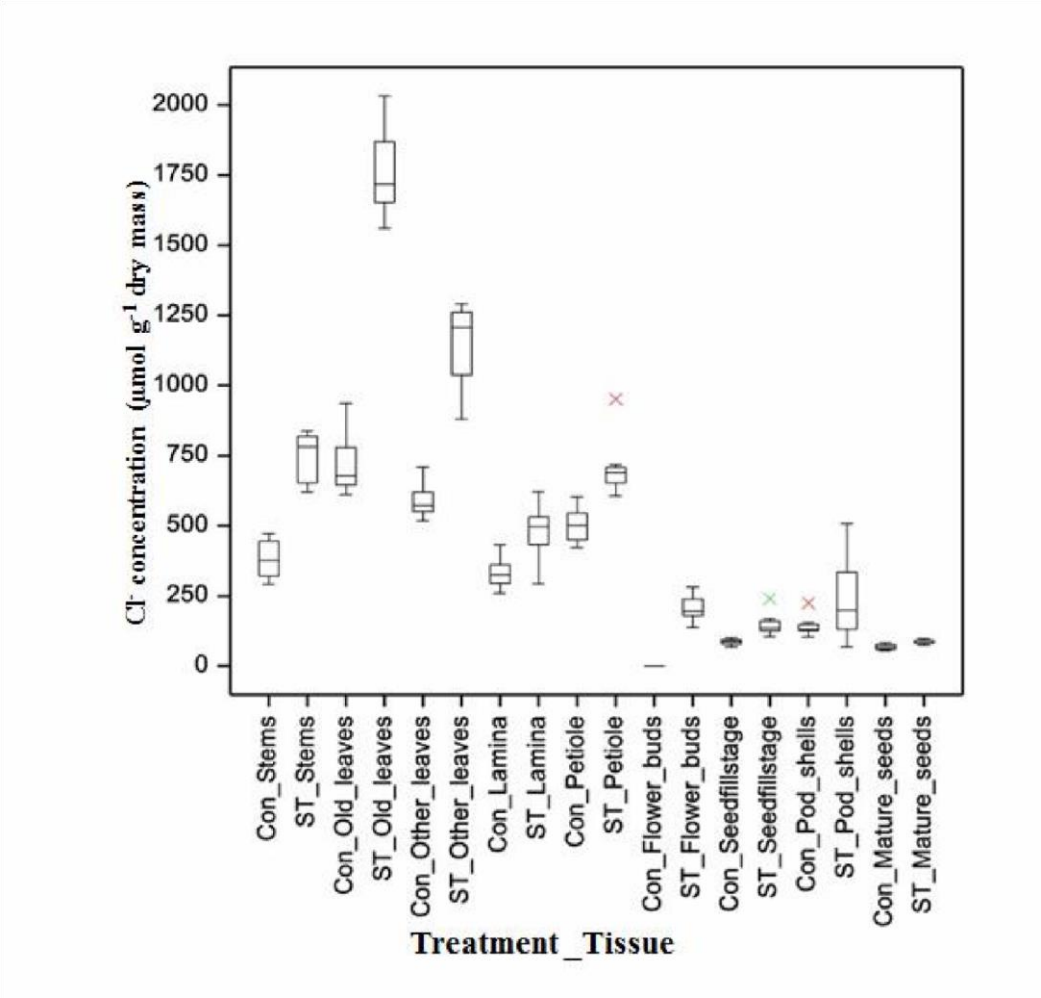


Figure 8. Chloride concentrations in nine tissues from plants grown under 0 mM NaCl (Con) and 80 mM NaCl (ST). The whiskers show the lower and upper limit of ion concentration among the eight genotypes four sensitive - ICC3421, ICC6263, ICC7315, ICC15510 and four tolerant- ICC11121, ICC1431, ICC4495, and ICC8950. The line within each box represents the median value. The upper and lower horizontal line in the box represents the quartiles 1 and 3. No flower buds under 0 mM NaCl were available for ion analysis.

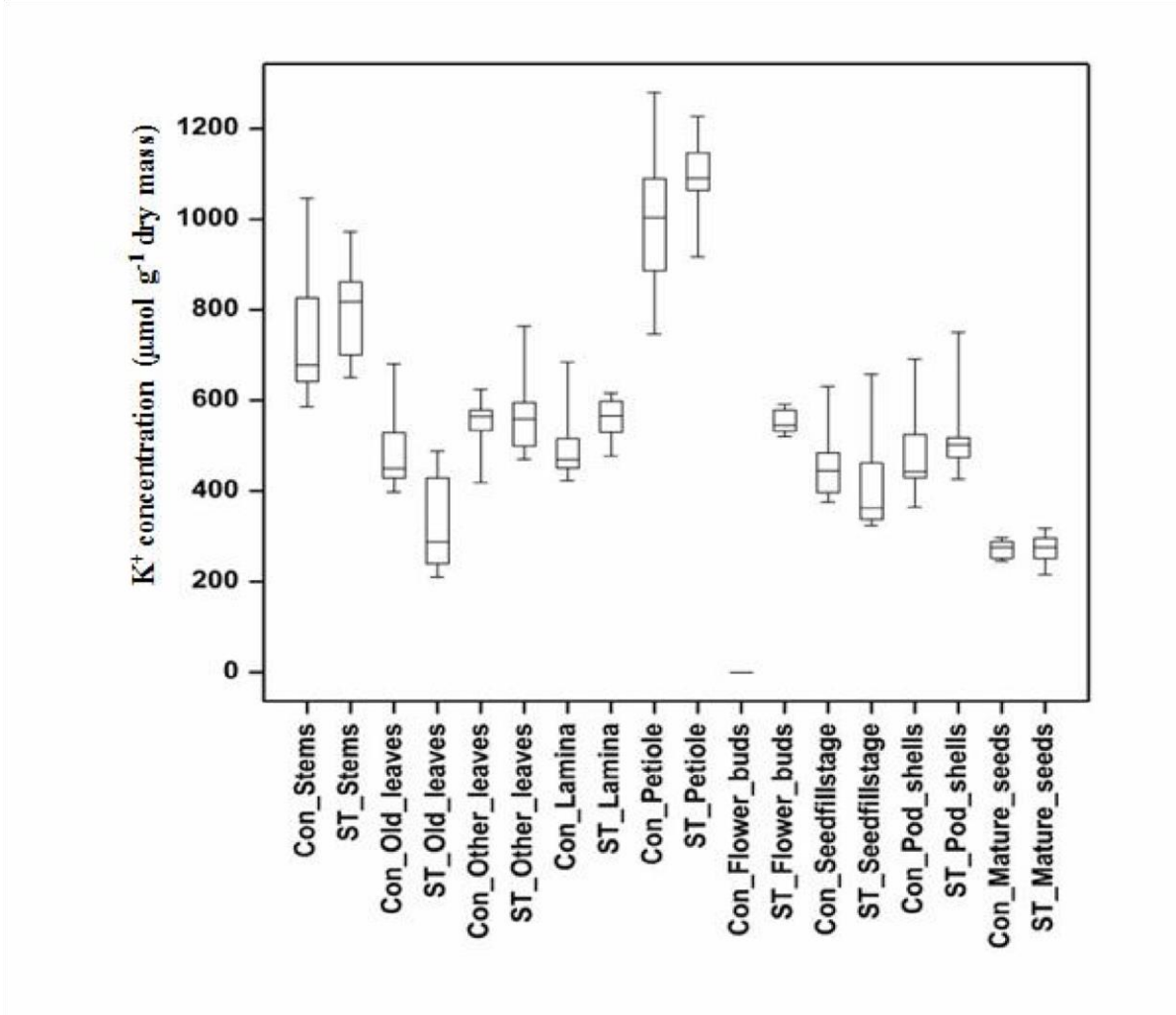


Figure 9. Potassium concentrations in nine tissues from plants grown under 0 mM NaCl (Con) and 80 mM NaCl (ST). The whiskers show the lower and upper limit of ion concentration among the eight genotypes four sensitive - ICC3421, ICC6263, ICC7315, ICC15510 and four tolerant- ICC11121, ICC1431, ICC4495, and ICC8950. The line within each box represents the median value. The upper and lower horizontal line in the box represents the quartiles 1 and 3. No flower buds under 0 mM NaCl were available for ion analysis.

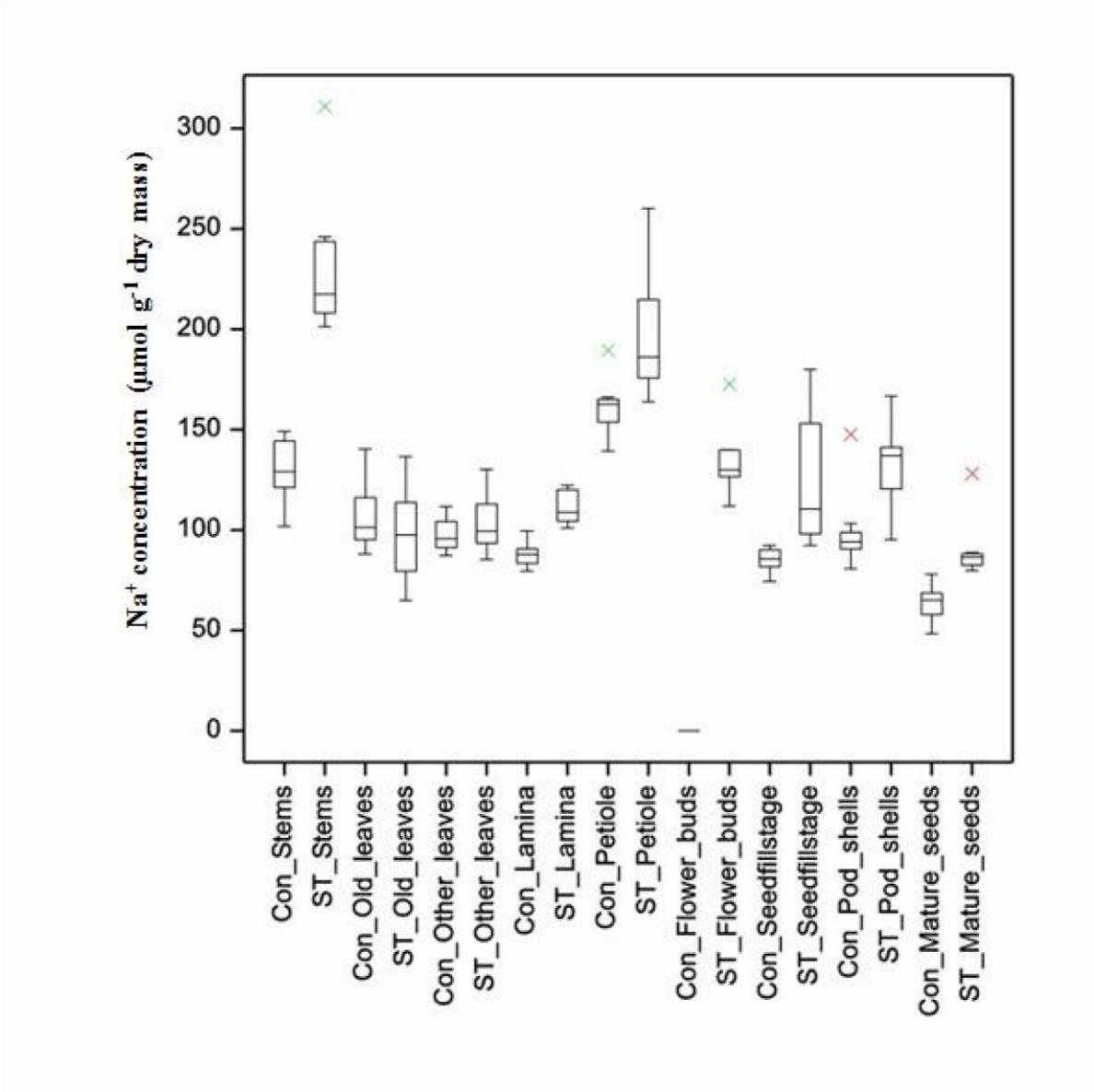


Figure 10. Sodium concentrations in nine tissues from plants grown under 0 mM NaCl (Con) and 80 mM NaCl (ST). The whiskers show the lower and upper limit of ion concentration among the eight genotypes: four sensitive - ICC3421, ICC6263, ICC7315, ICC15510 and four tolerant- ICC11121, ICC1431, ICC4495, and ICC8950. The (×) represents outliers and the line within the box represents the median. The upper and lower horizontal line in the box represents the quartiles 1 and 3. No flower buds under 0 mM NaCl were available for ion analysis.

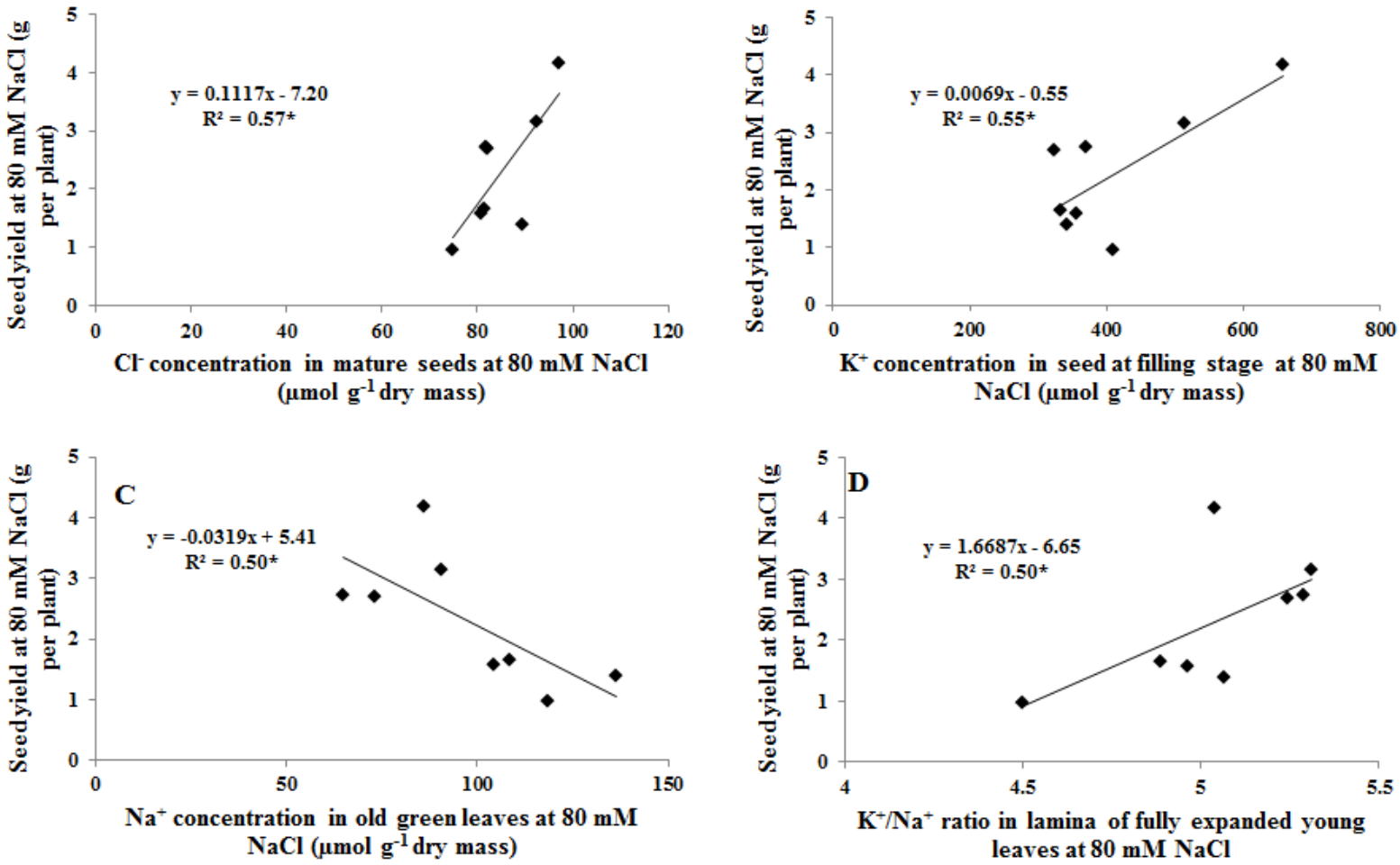


Figure 11. Relationship between Cl^- concentration in mature seeds (A), K^+ concentration in seeds at the filling stage (B), Na^+ concentration in old green leaves (C), K^+/Na^+ ratio in lamina of fully-expanded young leaves (D) and seed yield per plant (*-significant at $P<0.05$) at 80 mM NaCl treatment. The accumulation of Cl^-, K^+, and Na^+ in all other tissues had no significant effect on seed yield.

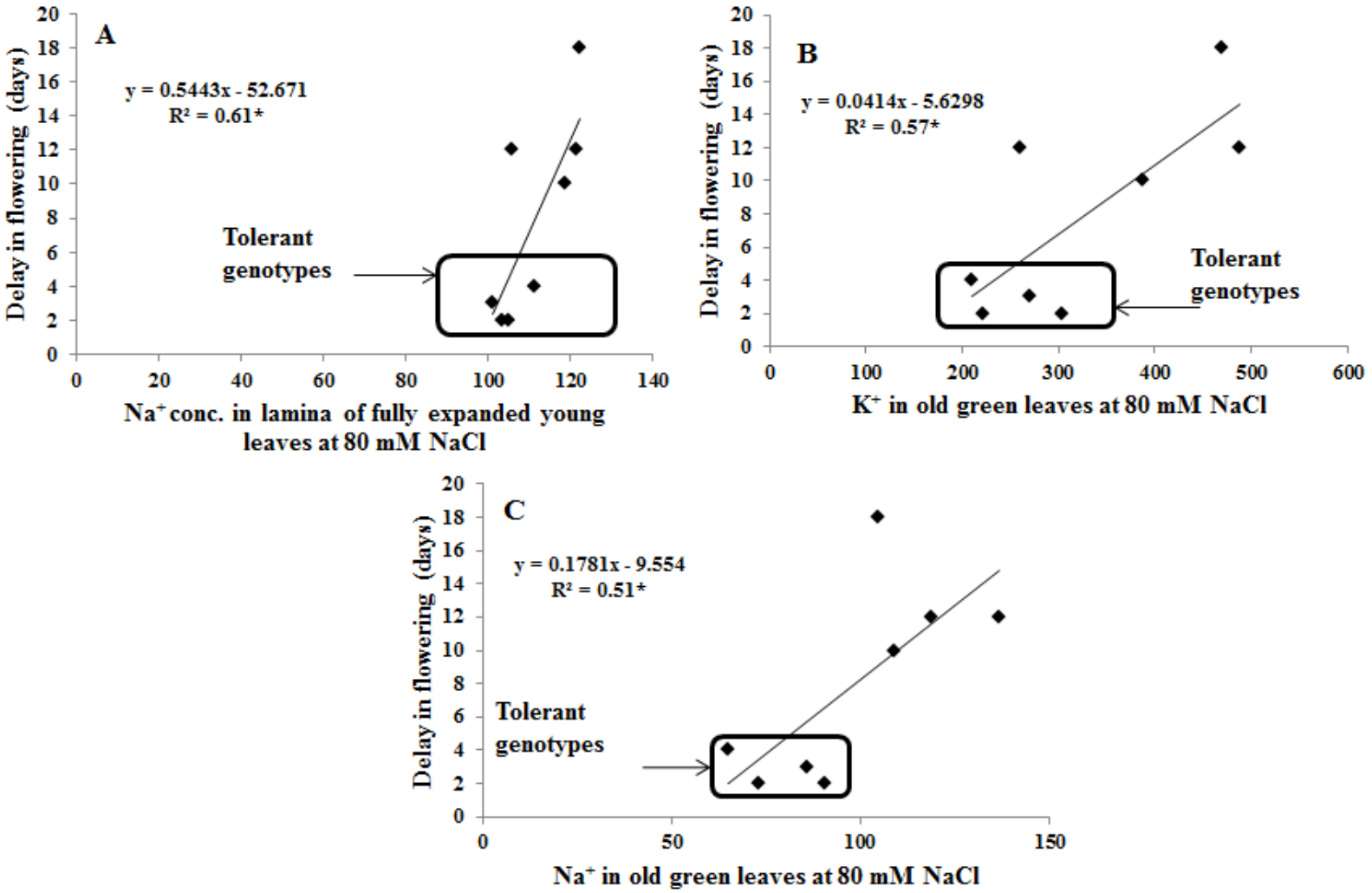


Figure 12. Relationship between the delay in 50% flowering and Na^+ concentration in lamina of fully-expanded young leaves (A), K^+ concentration in old green leaves (B), Na^+ in old green leaves (C) at 80 mM NaCl treatment. The delay in flowering at 80 mM NaCl treatment is compared to 0 mM NaCl treatment (*-significant $P<0.05$). The accumulation of Cl^-, K^+, and Na^+ in all other tissues had no significant effect on delay in flowering.

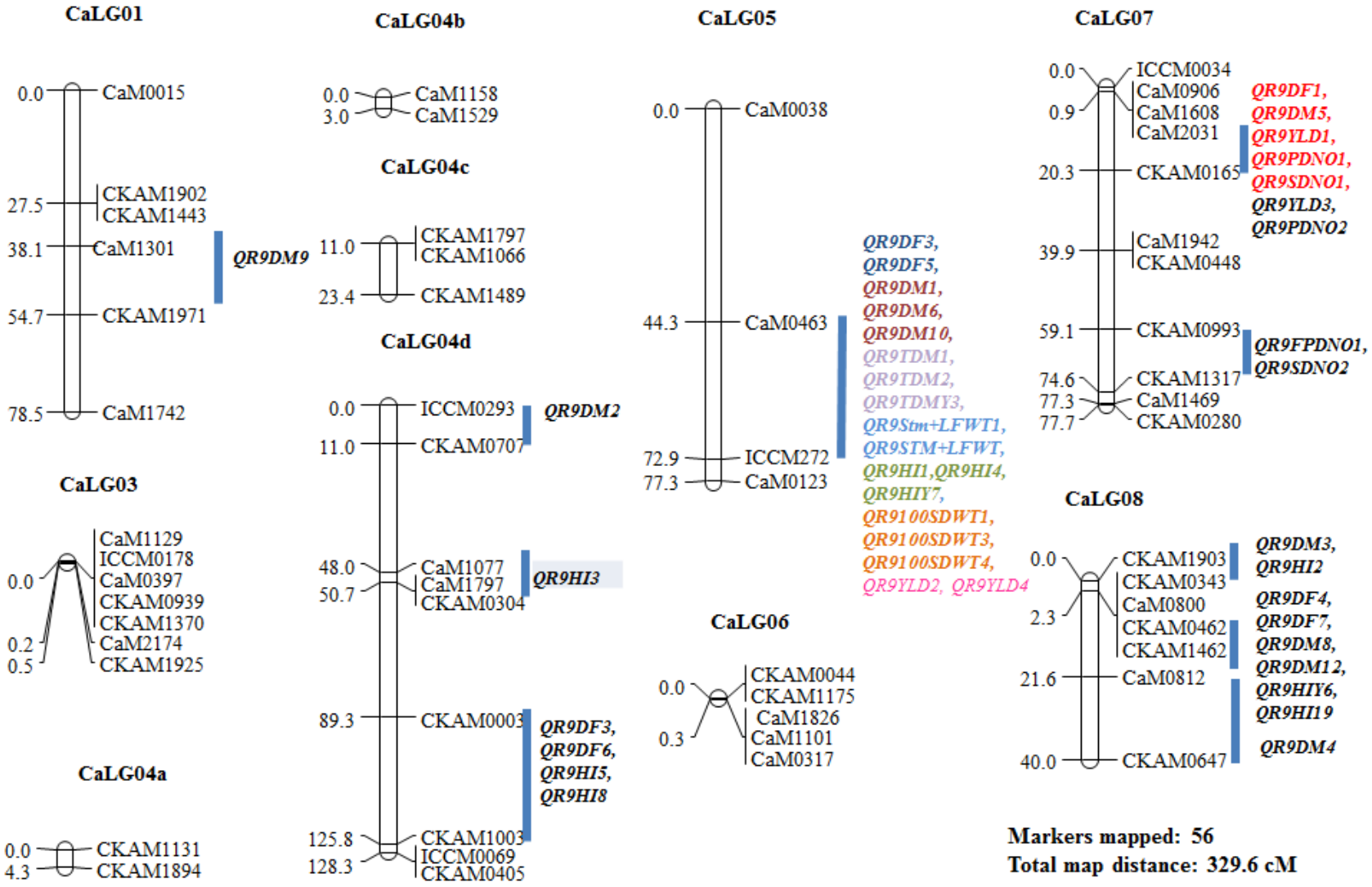


Figure 13. Genetic linkage map of chickpea (ICCV 2 × JG 11) with 56 markers on seven linkage groups.

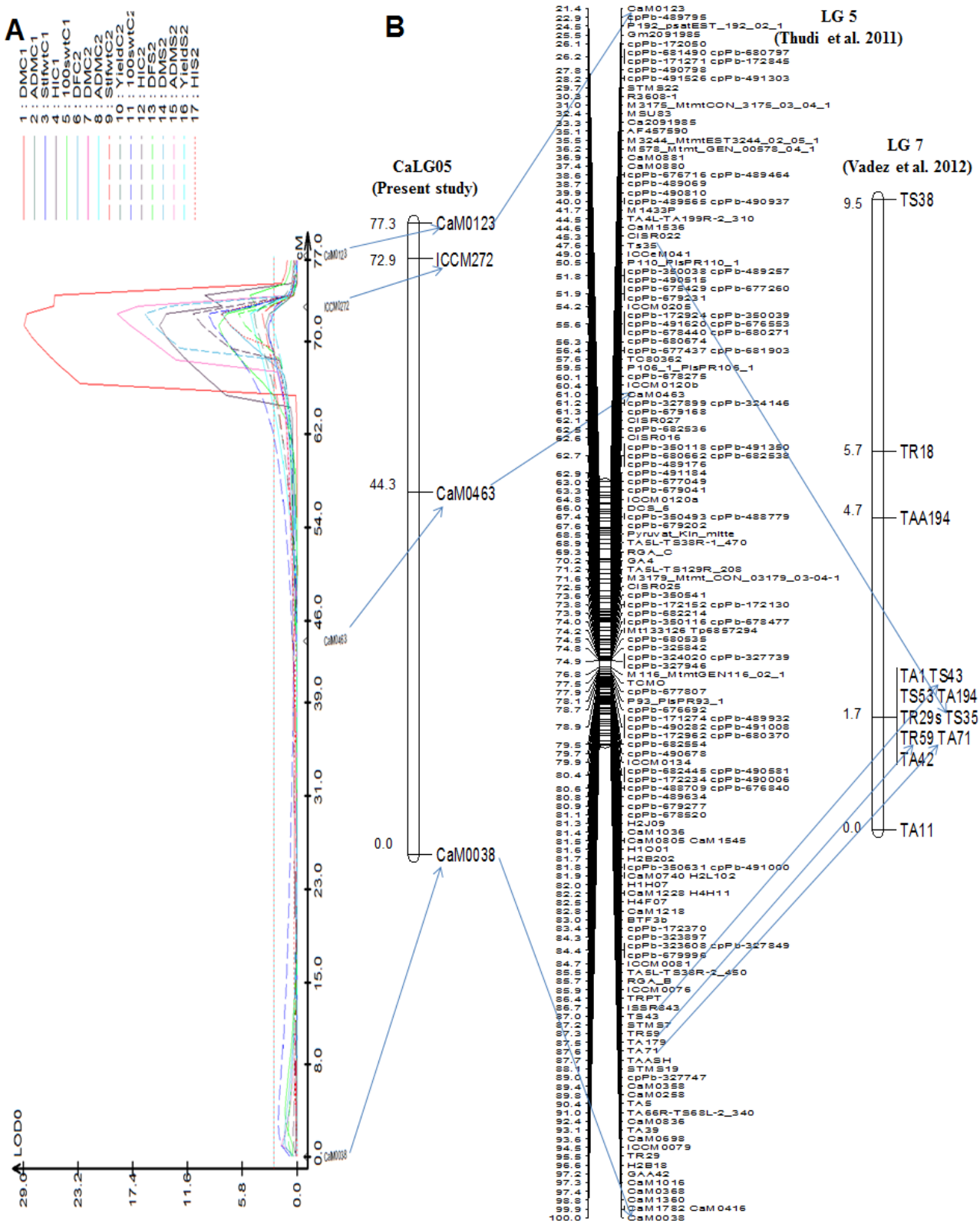


Figure 14. QTLs for seven different traits were identified across years and treatments on CaLG05. A. Genomic region on CaLG05 that harboured the 17 QTLs for traits that conferred salinity tolerance in ICCV 2 × JG 11 population were identified using QTL cartographer. B. CaLG05 in ICCV 2 × JG 11 population corresponded to LG 5 in Thudi et al. 2011 and LG7 in Vadez et al. 2012.

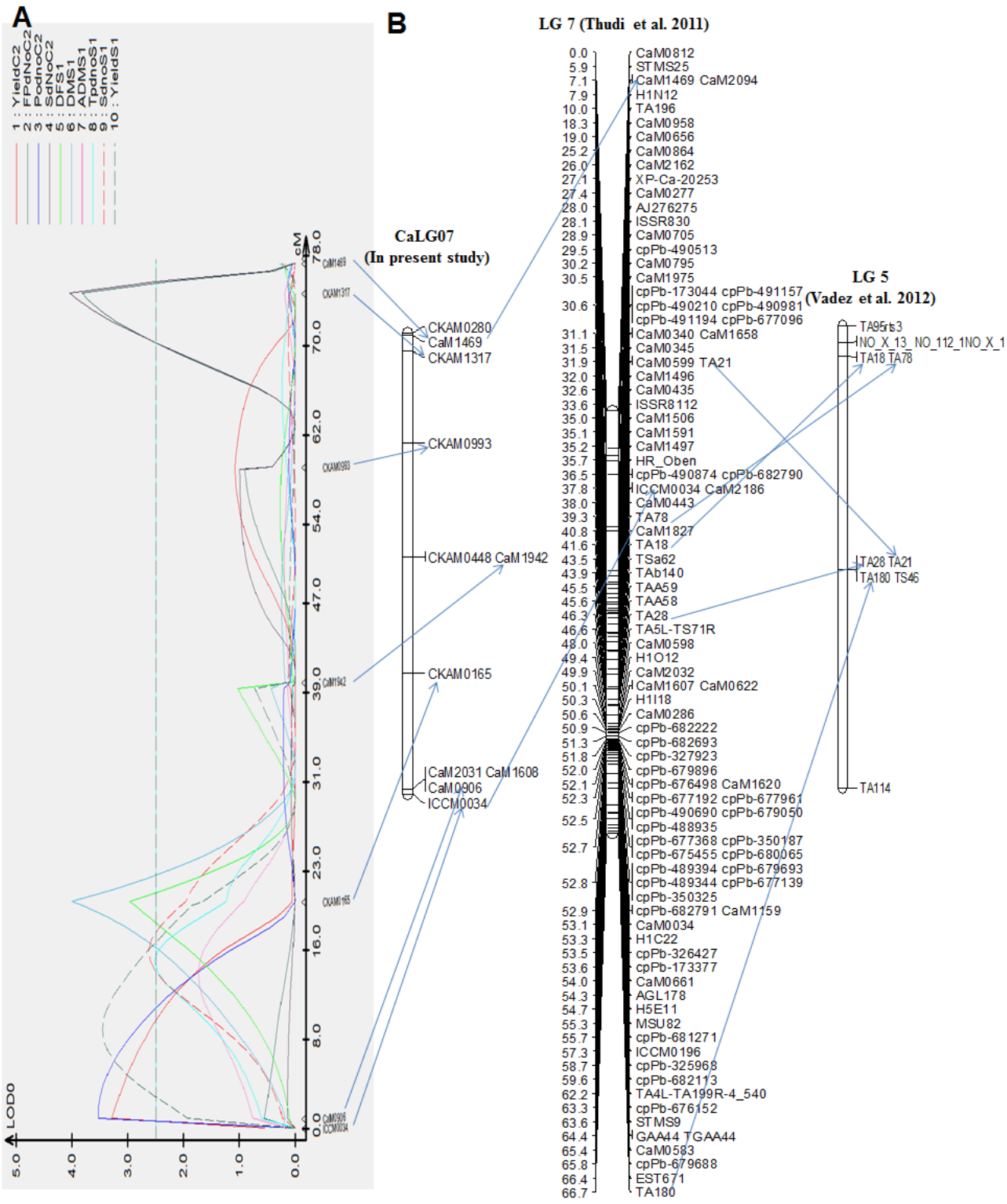


Figure 15. QTLs for five different traits were identified across years and treatments on CaLG07. A. Genomic region on CaLG07 that harboured the nine QTLs for traits that conferred salinity tolerance in ICCV 2 × JG 11 population were identified using QTL cartographer. B. CaLG07 in ICCV 2 × JG 11 population corresponded to LG 7 in Thudi et al. 2011 and LG5 in Vadez et al. 2012.

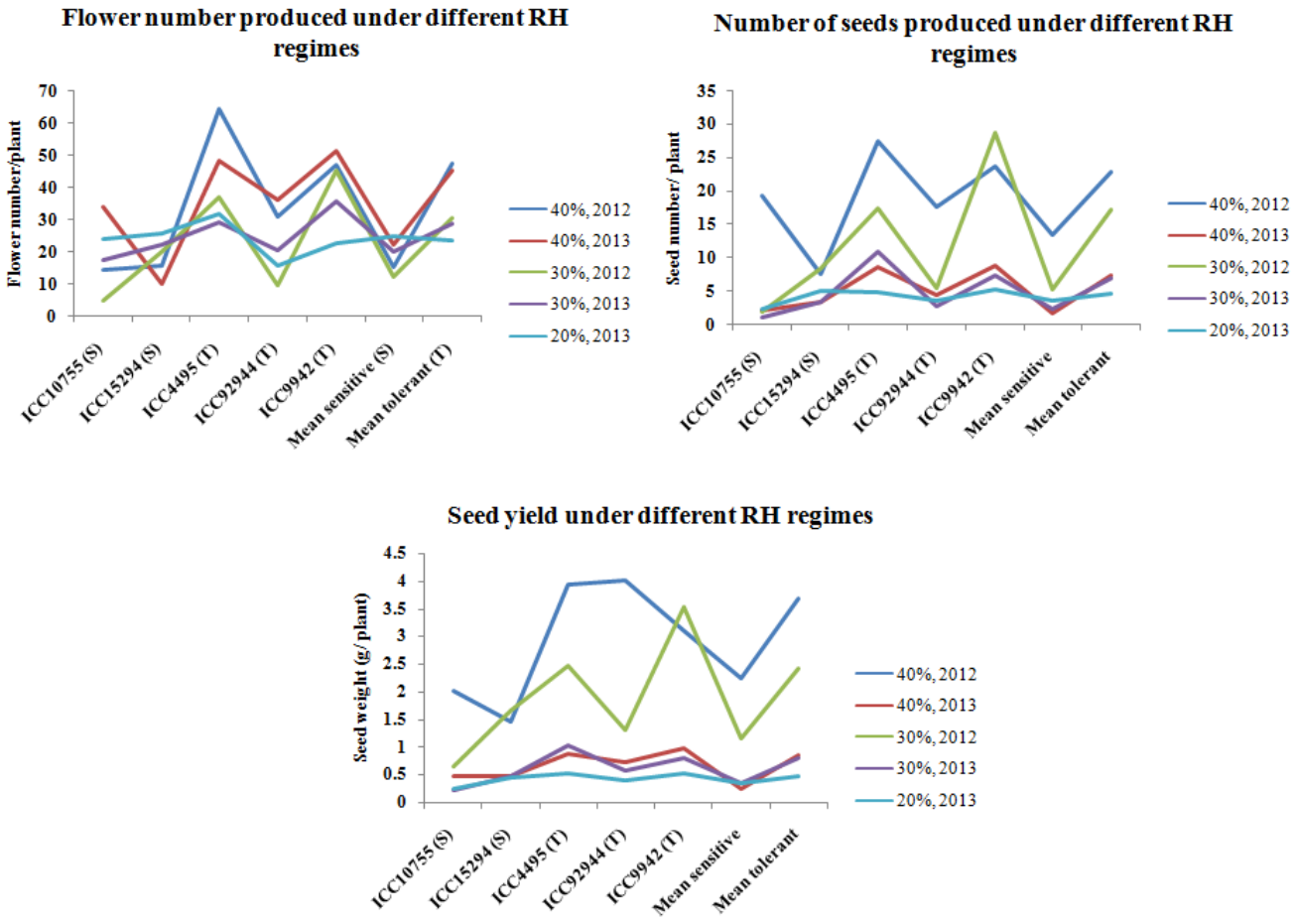


Figure 16. Number of flowers, seeds and seed yield produced under 40%, 30%, 20% RH treatments.

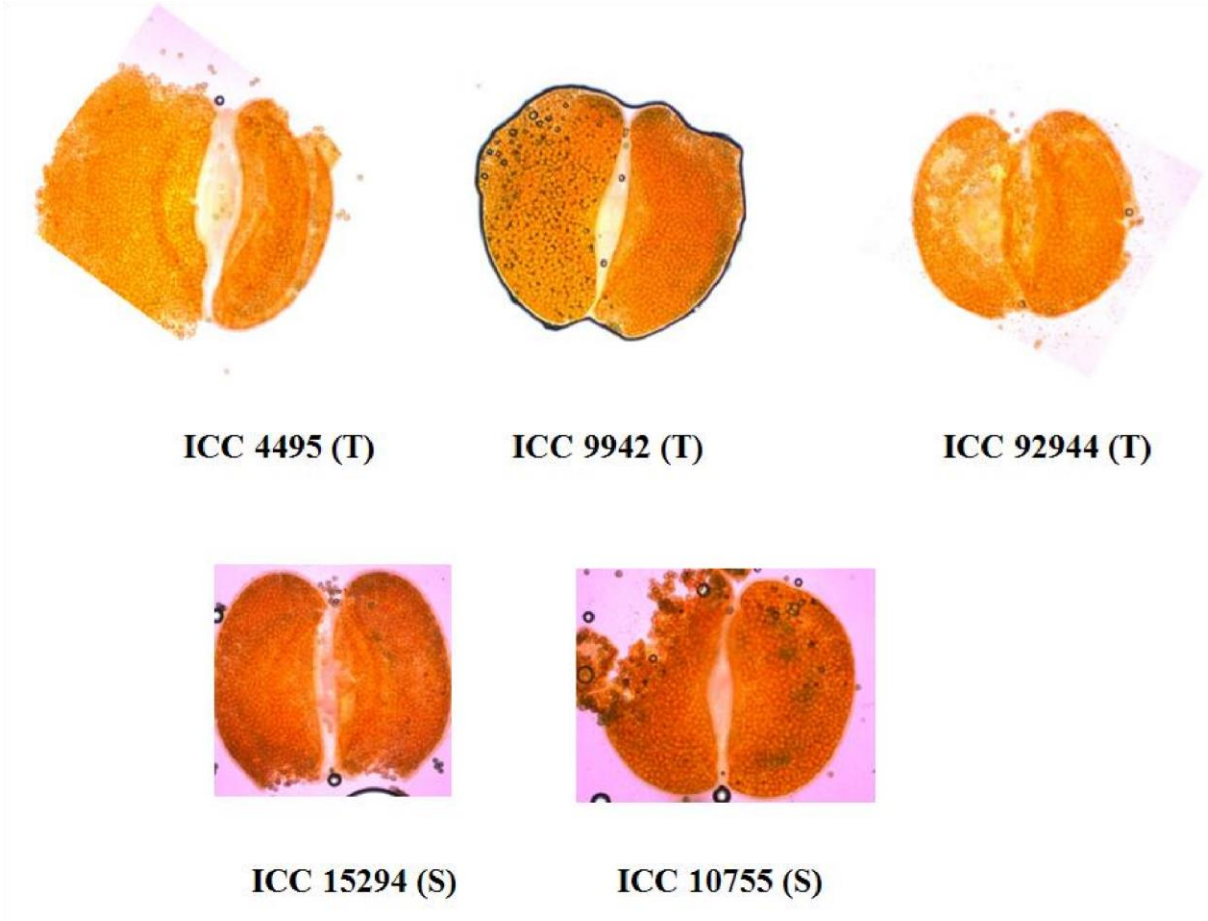


Figure 17. Pollen viability of five genotypes collected under 20% RH treatment in growth chamber condition.

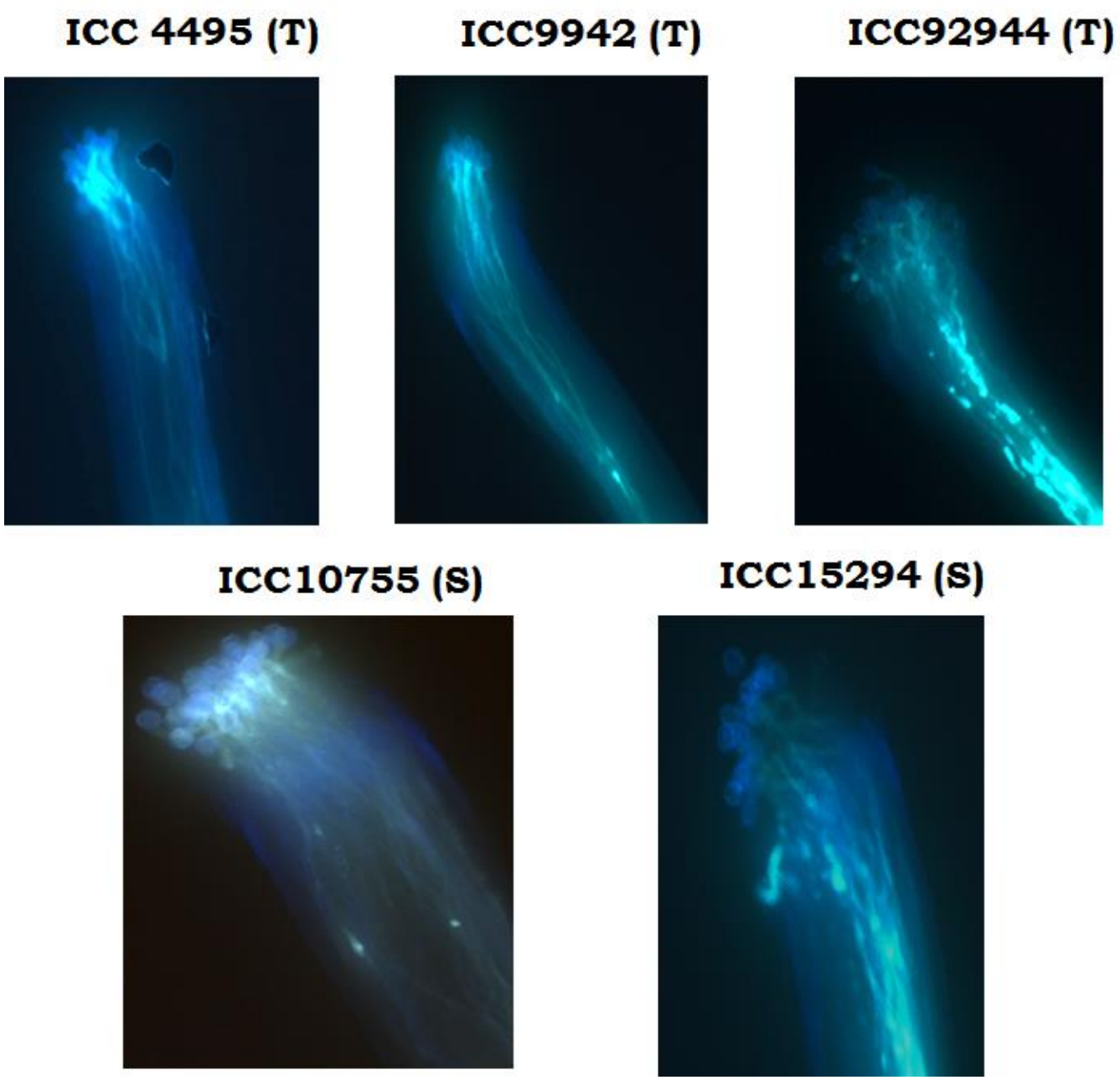


Figure 18. Pollen *in vivo* germination on stigma of five genotypes at 20% RH (3.4 kPa VPD) condition collected from growth chamber.

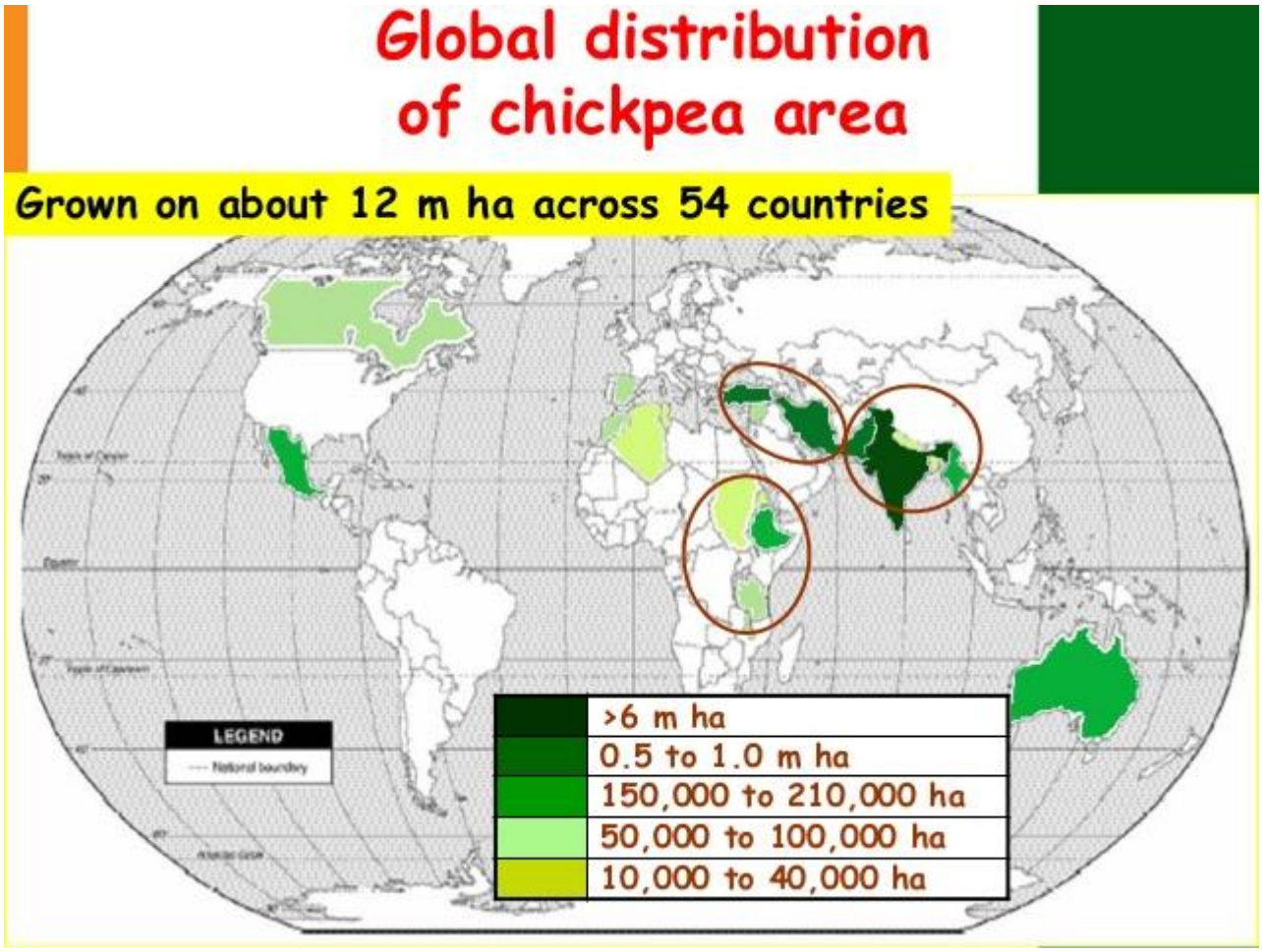


Plate 1: Chickpea growing regions worldwide.

Image courtesy: http://image.slidesharecdn.com/varshneygrmlisbon-131002051609-phpapp01/95/2013-grm-improve-chickpea-productivity-for-marginal-environments-in-subsaharan-africa-and-south-asia-rk-varshney-4-638.jpg?cb=1395191649

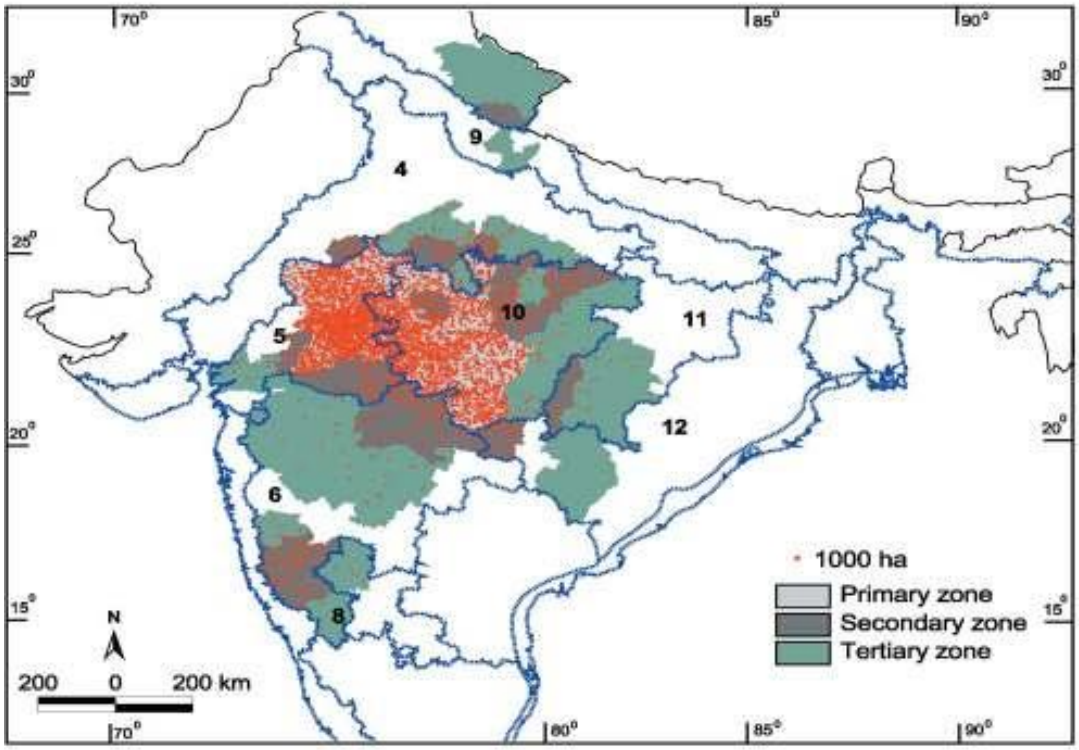


Plate 2: Chickpea growing regions in India.

Image courtesy: http://www.icrisat.org/what-we-do/satrends/feb2006.htm

Plate 3: Chickpea whole plant with shoot and root of plants collected from glass house condition (left) and from field condition (right).

Image courtesy: Dr L Krishnamurthy, Scientist, ICRISAT.

Plate 4: Chickpea plants at flowering stage.

Plate 5: Chickpea plants at podding stage.

Plate 6: Chickpea plants at maturity.

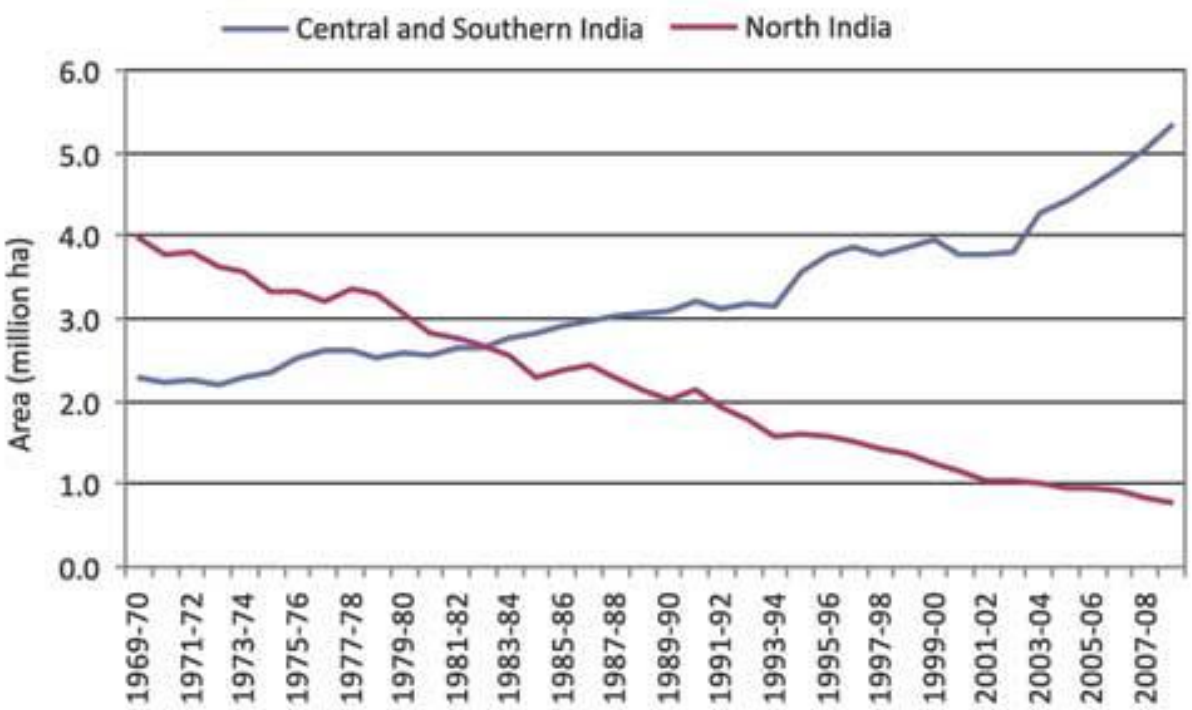


Plate 7: Regional shift in chickpea area from northern India to central and southern India.

Image courtesy: http://www.icrisat.org/crop-chickpea.htm

Plate 8: Well watered (above) and water stressed (below) chickpea plants at the end of mild water stress (Phase I).

Plate 9: High throughput phenotyping facility to conduct salinity studies in chickpea at ICRISAT.

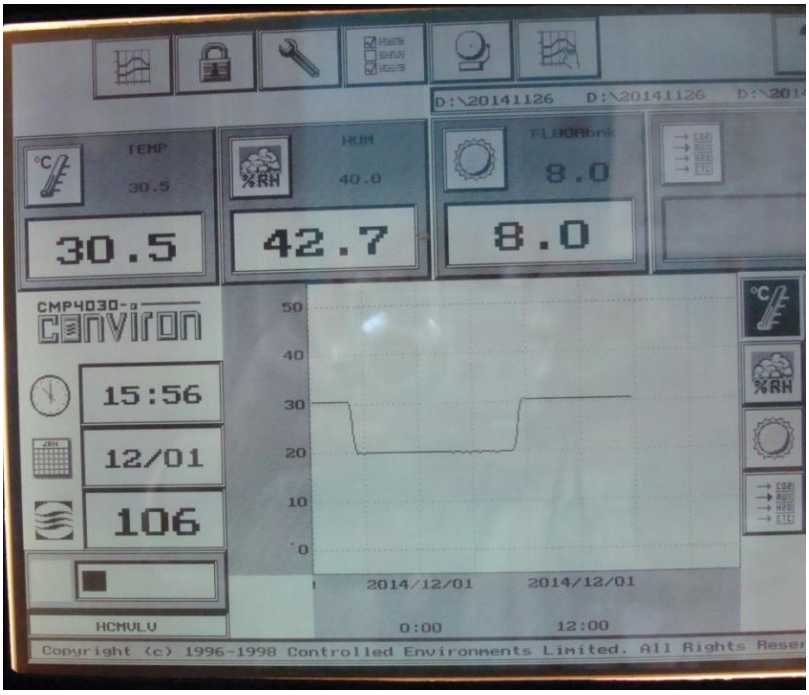


Plate 10: Growth chamber (top) and the temperature, relative humidity and light settings in growth chamber.

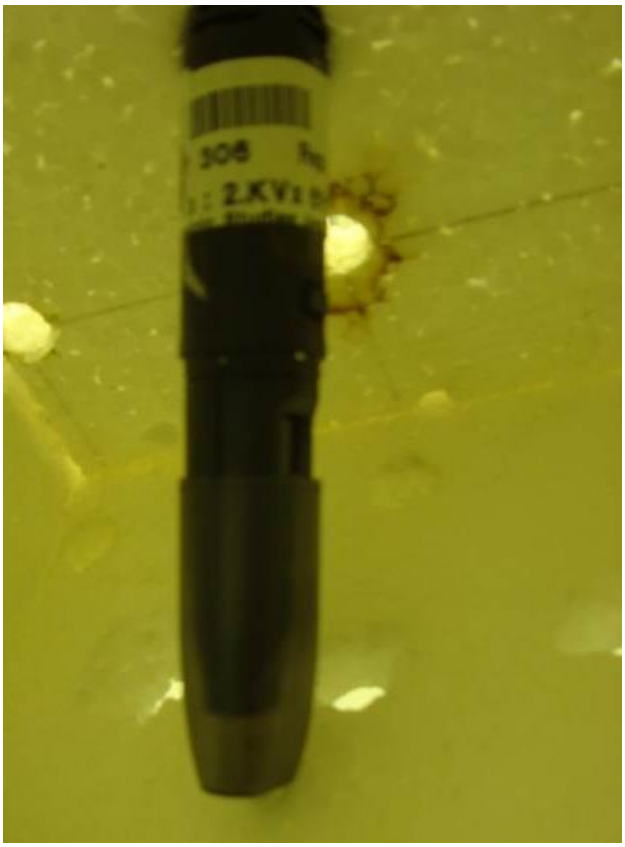


Plate 11: Data logger that were used to record temperature and relative humidity in ambient air (top), plants within growth chamber (down).

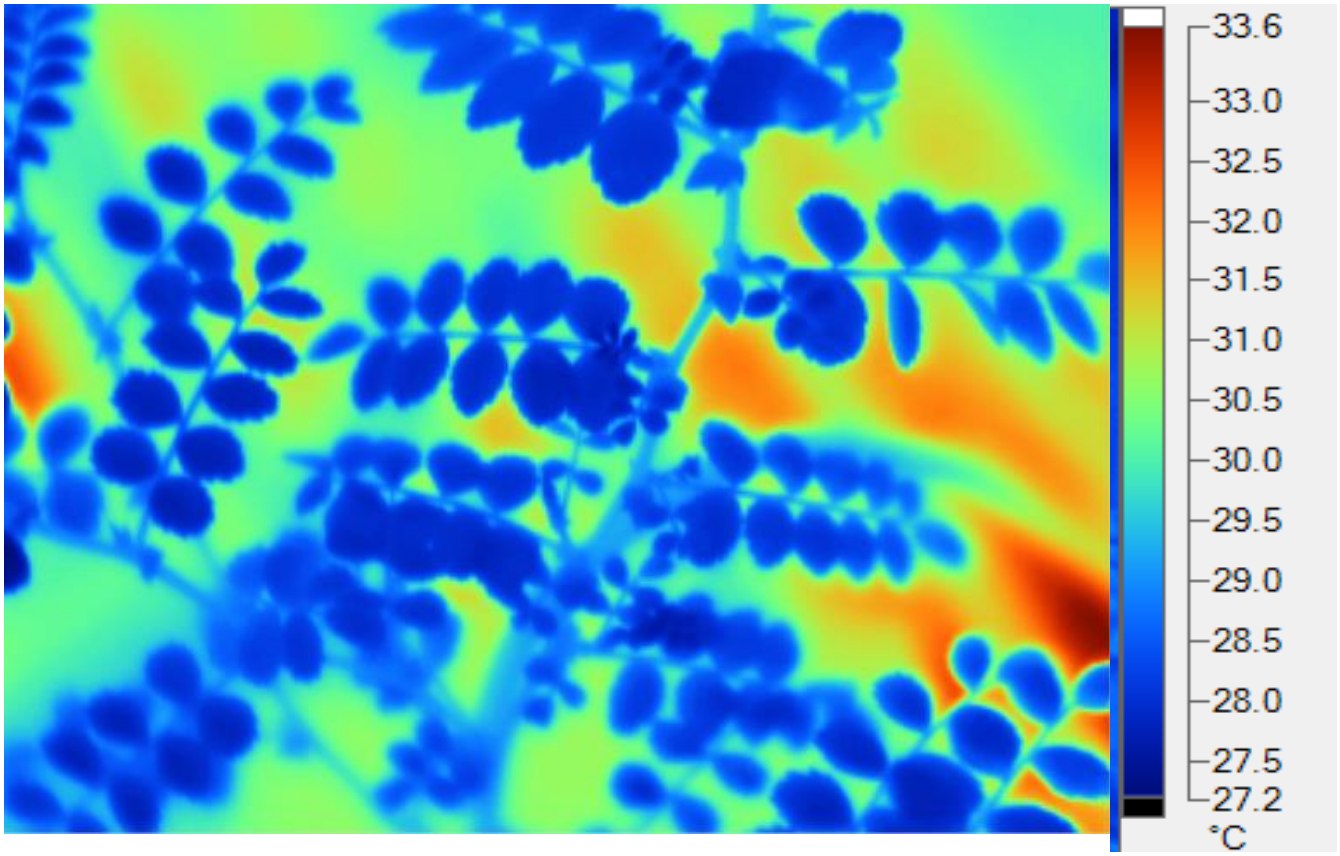
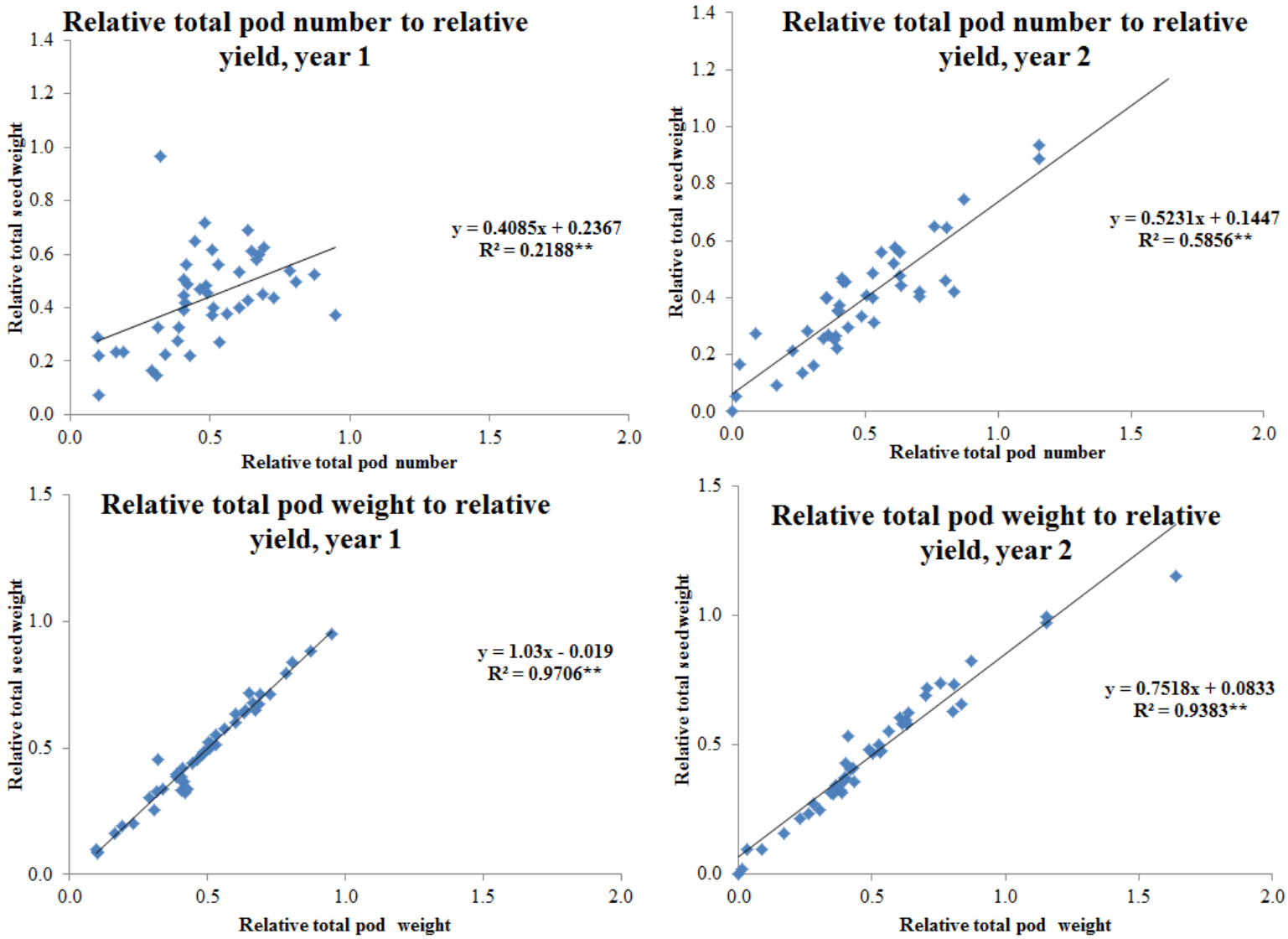
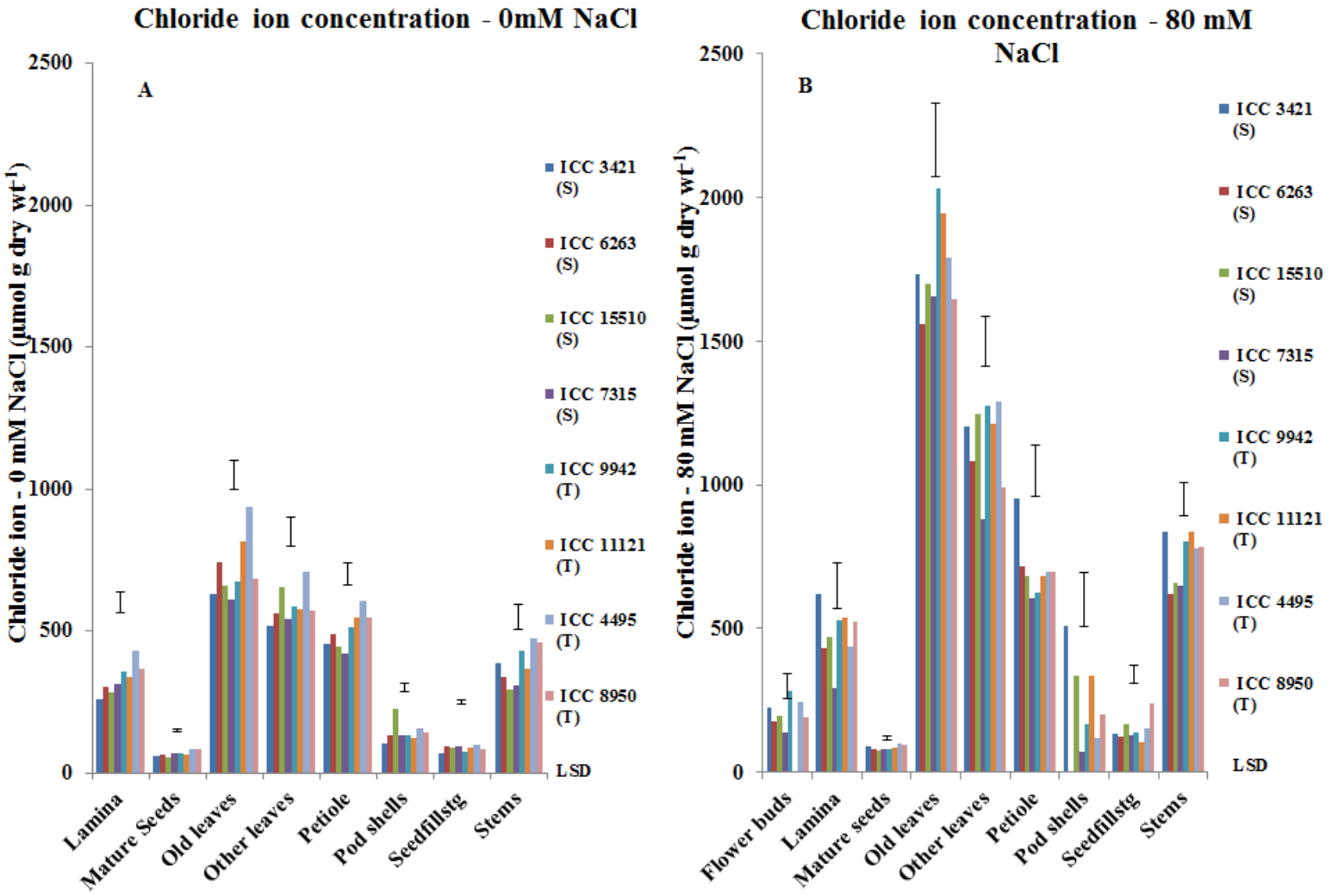


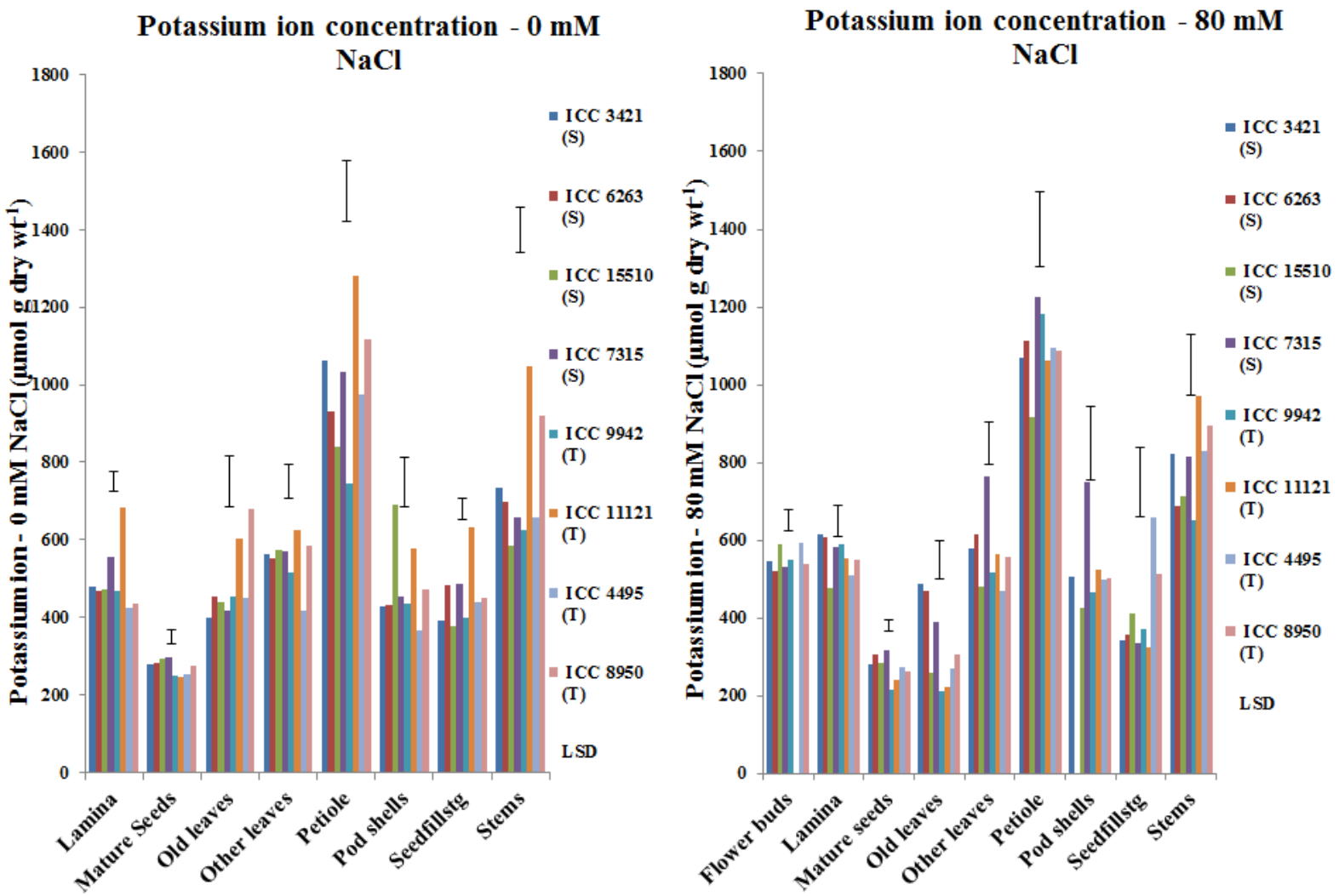
Plate 12: Infrared image of (part of) chickpea plant captured using infrared thermometer (Fluke Camera) and viewed using Smart View software.



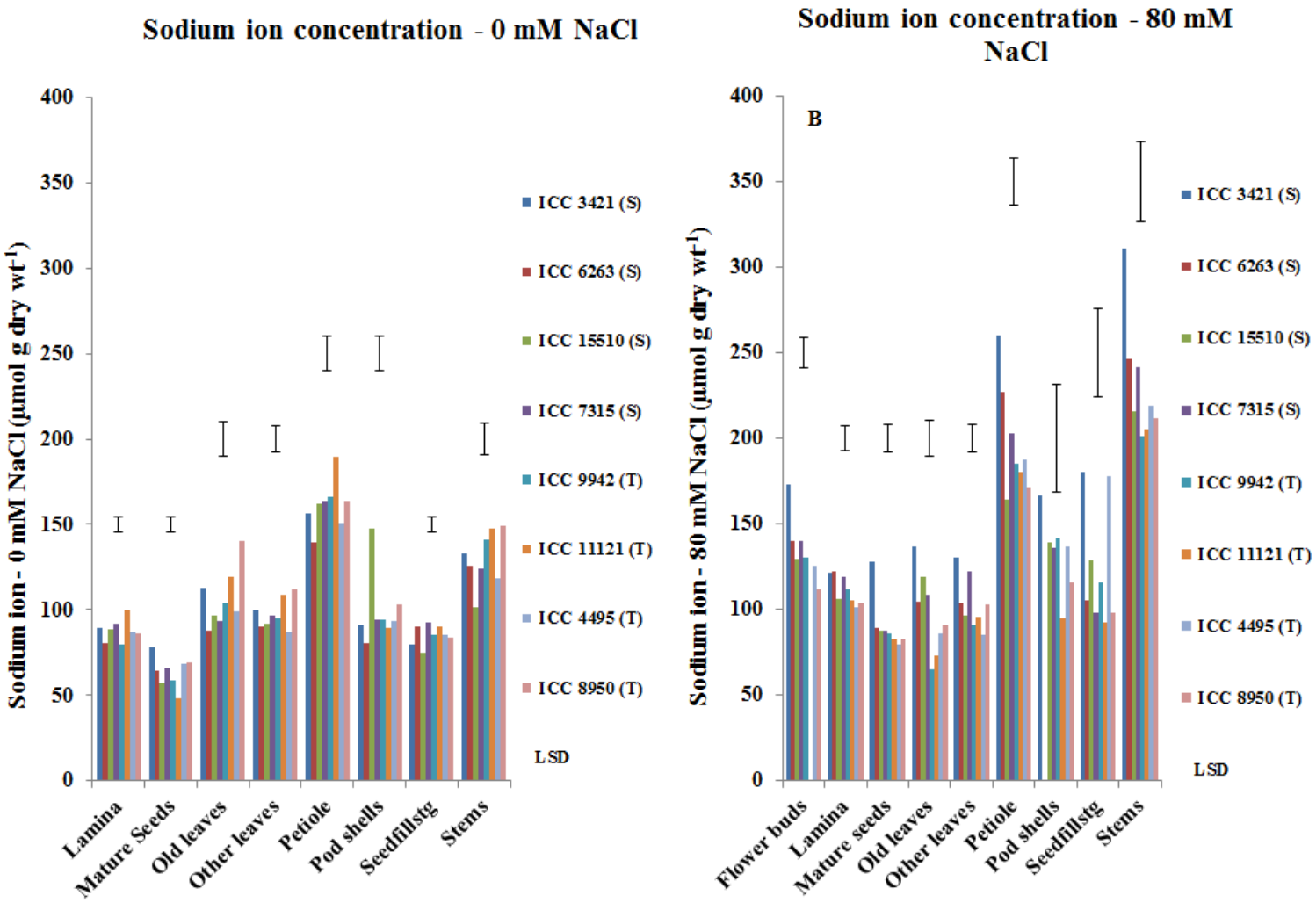
Appendix 1: Relation between total pod number, relative total pod weight to relative total seed weight across years.



Appendix 2: Chloride ion concentration across eight genotypes in eight and nine different tissues under 0 mM NaCl and 80 mM NaCl respectively.



Appendix 3: Potassium ion concentration across eight genotypes in eight and nine different tissues under 0 mM NaCl and 80 mM NaCl respectively.



Appendix 4: Sodium ion concentration across eight genotypes in eight and nine different tissues under 0 mM NaCl and 80 mM NaCl respectively.

Appendix 5

High-throughput DNA extraction

The steps involved in the DNA extraction protocol are explained below.

Sample preparation

- Leaves were harvested from 15 days old seedlings.
- Leaf tissues of 70-100 mg was placed in 12 × 8-well strip tube with strip cap (Marsh Biomarket, New York, USA) in a 96 deep-well plate together with two 4 mm stainless steel grinding balls (Spex CertiPrep, USA).

CTAB extraction

- For each sample 450 µl of preheated (at 65ºC for half an hour) extraction buffer (100 mM Tris-HCl (pH-8, 1.4 M NaCl, 20 mM EDTA, CTAB (2-3% w/v), β- mercaptoethanol) was added to each sample and secured with eight strip caps.
- Samples were homogenized in a GenoGrinder 2000 (Spex CertiPrep, USA), following the manufacturers' instructions, at 500 strokes/min for 5 times at 2 min interval.
- Plate was fitted into locking device and incubated at 65ºC for 10 min with shaking at periodical intervals.

Solvent extraction

- For each sample 450 µl of chloroform-isoamyl alcohol (24:1) was added and mixed thoroughly by inverting.

- Plate was centrifuged at 5500 rpm for 10 min. The aqueous layer (300 µl) is transferred to fresh strip tubes (Marsh Biomarket, USA).

Initial DNA precipitation

- 0.7 vol (210 µl) of isopropanol (stored at –20°C) was added to each sample and mixed thoroughly by inverting.
- Plate was centrifuged at 5000 rpm for 15 min.
- Supernatant was decanted from each sample and pellet was air dried for 20 min.

RNase treatment

- 200 µl low salt TE (10 mM Tris EDTA (pH-8)) and 3 µl RNase was added to each sample and incubated at 37°C for 30 min.

Solvent extraction

- 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each sample and mixed by inverting twice.
- Plate was centrifuged at 5000 rpm for 5 min.
- Aqueous layer was transferred to a fresh 96 deep-well plate.
- 200 µl chloroform-isoamylalcohol (24:1) was added to each sample and mixed by inverting twice.
- Plate was centrifuged at 5000 rpm for 5 min. Aqueous layer was transferred to fresh 96 deep- well plate.
- A total of 315 µl ethanol-acetate solution [30ml ethanol, 1.5ml 3 M NaOAc (pH-5.2)] was then added to each sample and placed in 20°C for 5 min.

- Plate was again centrifuged at 5000 rpm for 5 min.
- Supernatant was decanted from each sample and pellet was washed with 70% ethanol.
- Plate was centrifuged at 6000 rpm for 10 min.
- Supernatant was again decanted from each sample and samples were air dried for 1 hour.
- Pellet was re-suspended in 100 µl low-salt TE and stored at 4°C.

DNA quantification

The extracted DNA was quantified by loading the samples on 0.8% agarose gel containing 0.5 µl/10 ml Ethidium bromide (10 mg/ ml). The DNA was normalized to 5 ng/µl concentration with visual comparison by loading DNA samples with the standard λ DNA molecular weight markers (2.5 ng/µl, 5 ng/µl, 10 ng/µl) on 0.8% agarose gel.

Reagents for DNA extraction

1. **3% CTAB**

For 1 litre,

Tris-100 mM (12.1 g).

NaCl- 1.42 M (81.8 g).

EDTA- 20 mM (7.45 g).

CTAB- 3% (30 g).

2. **10X TBE**

For 1 litre,

Tris-10 g.

Boric acid- 55 g.

EDTA- 7.5 g.

3. **0.5M EDTA**

186.12 g EDTA was dissolved in 750 ml distilled water and made upto 1 litre (pH-8).

4. **5M NaCl**

292.2 g NaCl was dissolved in 750 ml distilled water and made upto 1 litre.

5. **3M sodium acetate**

246.09 g sodium acetate was dissolved in 600 ml distilled water and adjust the pH with acetic acid) and then made upto 1 litre.

6. **1M NaOH**

40 g of sodium hydroxide was dissolved in 1 litre of distilled water.

7. **RNase**

100 mg RNase was dissolved in 10 ml distilled water.

8. **$T_{10}E_1$**

1.21 g of Tris and 0.372 g EDTA (molecular weight 292.24) were dissolved in 750 ml distilled water and made upto 1 litre.

9. **1M Tris (pH 8)**

120 g Tris was dissolved in 750 ml distilled water and made upto 1 litre.

10. **High salt TE**

10 mM Tris.

1 mM EDTA.

2 M NaCl (pH 8)(Molecular weight-57.95).

11. **Chloroform : Indole acetic acid (IAA) (24:1**)

For 100 ml- Mixed 96 ml of chloroform and 4 ml of IAA.

For 1000 ml- Mixed 960 ml of chloroform and 40 ml of IAA.

12. **Phenol : Chloroform : IAA (25:24:1)**

For 100 ml: 50 ml phenol+ 48 ml Chloroform+ 2 ml IAA.

For 1 litre: 500 ml phenol+ 480 ml Chloroform+ 20 ml IAA.

Appendix 6

Preparation of Alexander's stain

To prepare Alexander's stain, added the following constituents in the order given below and finally store the solution in dark.

1. **10 ml 95% ethanol**

Mixed 95 ml ethanol (100% pure) with 5 ml of distilled water.

From this 10 ml was taken.

2. **1 ml Malachite green** (1% solution in 95% alcohol)

95% alcohol was prepared and then dissolved 0.1 g of malachite green in 10 ml of 95% alcohol was taken from it.

3. **50 ml distilled water**

4. **25 ml Glycerol**

5. **5 ml of Acid fuchsin** (1% solution in distilled water)

0.1 g of acid fuchsin was dissolved in 10 ml of distilled water and then 5 ml form it.

6. **0.5 ml Orange G** (1% solution in distilled water)

0.1 g of Orange G was dissolved in 10 ml of water and from that 0.5 ml was taken.

7. **4ml Glacial acetic acid**

8. **Distilled water** – Added 4.5 ml to make the total volume to 100 ml.

Note: When viewing under microscope along with Alexander's solution add equal amount of glycerol to view the samples much more clearly.

Appendix 7

Decolourised Aniline Blue Solution (DABS)

- 108 mM K_3PO_4 solution

  4.6 g of K_3PO_4 was dissolved in 200 ml of distilled water.

- In this solution 0.2 g of water soluble aniline blue was added and dissolved.

Note: If the stain is too dark add a drop or two of glycerol while viewing the samples under fluorescent microscope.

Peer-reviewed publications arising from this thesis

Chapter 3

R Pushpavalli, M Zaman-Allah, NC Turner, R Baddam, MV Rao, V Vadez, Higher flower and seed number leads to higher yield under water stress conditions imposed during reproduction in chickpea, Functional Plant Biology, 2015, 42 (2), 162–174.

Chapter 4

R Pushpavalli, J Quealy, TD Colmer, NC Turner, KHM Siddique, MV Rao, V Vadez, Salt stress delayed flowering and reduced reproductive success of chickpea (*Cicer arietinum* L.), a response associated with Na^+ accumulation in leaves**,** Journal of Agronomy and Crop Science, 2015, doi:10.1111/jac.12128, Online published.

Chapter 5

R Pushpavalli, L Krishnamurthy, M Thudi, PM Gaur, MV Rao, KHM Siddique, TD Colmer, NC Turner, RK Varshney, V Vadez, Two key genomic regions harbour QTLs for salinity tolerance in ICCV 2 × JG 11 derived chickpea (*Cicer arietinum* L.) recombinant inbred lines, BMC Plant Biology, 2015, doi: 10.1186/s12870-015-0491-8, Online published.

Poster presentation

Chapter 5

R Pushpavalli, M Zaman-Allah, R Baddam, MV Rao, V Vadez, High seed number is associated with tolerance to water deficit at reproductive phase in chickpea (*Cicer arietinum* L.) presented at VI international Congress on Legume Genetics and Genomics (ICLGG)- October, 2012 held at Hyderabad.

Chapter 6

R. Pushpavalli, V Vadez, NC Turner, MV Rao, Effect of atmospheric drought, imposed by low relative humidity, on the seed yield and yield components of five chickpea (*Cicer arietinum L.*) genotypes presented at Inter-drought IV- September, 2013 held at Perth, Western Australia.