12-2016

Quantitative Trait Loci (QTL) for Salt Tolerance in Soybean and Physiological Response to Salt Stress During Early Growth Stage

Cindy Massiel Lopez Ramirez
University of Arkansas, Fayetteville

Quantitative Trait Loci (QTL) for Salt Tolerance in Soybean and Physiological Response to Salt Stress During Early Growth Stage

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Crop, Soil, and Environmental Sciences

by

Cindy Massiel Lopez Ramirez
Universidad Nacional de Colombia
Bachelor of Science in Agronomic Engineering, 2011

December 2016
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Dr. Pengyin Chen
Thesis Director

Dr. Ken L. Korth
Committee Member

Dr. Esten Mason
Committee Member

Dr. David Miller
Committee Member

OVERALL ABSTRACT

Soybean is a major cash crop used as a source of high–quality protein and oil. Salt stress is one of the main abiotic stresses causing significant yield losses in soybean, which is considered a moderately salt–sensitive crop. Breeding selection is a promising strategy to improve salt tolerance as soybean germplasm display wide variation in response to salinity stress. However, the physiological and genetic mechanisms for salt tolerance are not quite clear. The discovery of novel QTL/genes associated with salt tolerance facilitates the development of tolerant cultivars through marker-assisted selection (MAS). The objectives of this study were: 1) identify/confirm QTL associated with salt tolerance, and 2) evaluate progressive shoot ion accumulation in sensitive/tolerant genotypes and leaf physiological changes induced by salt stress during early growth stage. For the first objective, QTL mapping was performed using an $F_{2:3}$ population from Jake (tolerant) x Ozark (sensitive). A major QTL was found on chromosome 3 linked to four SNP loci in the same genomic region previously reported, explaining 37% to 49% of the phenotypic variation in LSS, PDP, leaf chlorophyll and leaf chloride content. Additionally, a new minor QTL linked with two SNP markers was identified on chromosome 19 explaining 5% of leaf chlorophyll variation. These QTL and linked SNP markers will be useful in MAS for salt tolerance. For the second objective, two sensitive (Desha, Ozark) and two tolerant (Jake, Lee) cultivars were treated with NaCl and KCl at 80 mM and 120 mM from stage V1 to V5. The most adverse effects on tolerant and susceptible varieties, was caused by KCl compared to NaCl stress. Under KCl treatment, the tolerance capacity of the excluders was severely inhibited causing early death, while under NaCl stress, tolerant varieties were able to accumulate up to 2.3 and 3.8 times less leaf Cl^- and leaf Na^+, respectively, than the sensitive ones. Plant death

occurred when shoot ion concentration reached 80,000 mg/kg and 18,000 mg/kg of Cl^- and Na^+, respectively, under 120 mM NaCl. Under 120 mM KCl, plants died when leaf Cl^- content reached 120,000 mg/kg and leaf K^+ content was over 100,000 mg/kg.

AKNOWLEDGEMENTS

I would like to express my deepest gratitude to my family and friends in Colombia and here, in Fayetteville, for the continuous support and encouragement provided during all my master's study.

I would like to thank my academic advisor Dr. Pengyin Chen for giving me the opportunity to join his soybean breeding program, for his guidance, encouragement, and for teaching me many valuable aspects about the art and science of plant breeding.

I want to give special thanks to all my committee members Dr. Mason, Dr. Korth and Dr. Miller for their time, guidance, and effort dedicated to contribute on this thesis project. In addition, I want to thank Dr. Mason and Dr. Korth for the knowledge and professional input provided during their interesting and very useful classes of Plant Breeding and Biotechnology in Agriculture.

I am deeply grateful to Dr. Gbur and Dr. Shi for their instructions and advice during all my academic program; to Dev Crippen, Jonh Guerber and Jessica Kivett for their help with the greenhouse research.

I am sincerely grateful to all the past and current members of the soybean breeding team (Razorbeans), for their understanding, advice, support and great team effort dedicated during all the time we have shared. Special thanks to my dear friends Mirta Dalzotto, Laura Lara, Liliana Florez, Maria de Oliveira, and Ali Alsalman.

Finally, I would like to thank all the staff of the Crop, Soil, and Environmental Sciences Department for their kindness and generous help throughout the time I have been part of this academic community.

DEDICATION

To God and my beloved family, who have always motivated me, supported me, and inspired me all my life to go forward and beyond.

TABLE OF CONTENTS

Chapter III. Study of soybean physiological response under salt stress during early growth stages

LIST OF TABLES

Chapter I

Chapter II

Chapter III

LIST OF FIGURES

Chapter II

Chapter III

CHAPTER I. Introduction and literature review

Soybean production and uses

Soybean [*Glycine max* (L.) Merr.] is one of the most important cash crops worldwide used as a source of high–quality seed protein (40%) and oil (20%) for animal and human consumption. This crop has the highest protein and vegetable oil content among the cultivated species in the world (Singh, 2010). It is also used as raw material for the production of biodiesel and nutraceutical/pharmaceutical and industrial products (Phang *et al.*, 2008; Salimi, 2013). The total soybean world production registered for 2014 – 2015 is 319.73 million metric tons occupying a land area of 118.40 million hectares. The United States ranks first in world soybean production with 106.8 million metric tons followed by Brazil, Argentina, China, and India with 97.20, 61.40, 12.5, and 8.71 million metric tons respectively (Soy Stats, 2013; USDA 2016).

The origin of soybean cultivation is China about 5000 years ago, where the most extensive distribution and diversity types have been registered. Soybean production rapidly developed in the USA during the 1950s. The demand for soybean products is continuously increasing. Some of the traditional non-fermented soybean products for human consumption are: milk, soybean sprouts, tofu (bean curd), and edamame. The most traditional fermented soybean products are natto, tempeh, miso, and soybean sauce. The seed is pressed to obtain oil and soybean meal. Oil is frequently used to produce edible oil, printing ink, and biodiesel while soybean meal is used as a main protein source for animal feed for farming. Compounds with high nutritional value can also be obtained from soybeans: soy peptide, isoflavones, saponins, phosphatides, and oligosaccharides are usually purchased as functional health foods (Singh, 2010).

Salinity and its effects on soybean

More than 800 million hectares of land across the world are salt-affected due to salinity and sodicity, which is equivalent to more than 6% of the worldwide land area (Arzani, 2008) and about 20% of the arable land (Sairam and Tyagi, 2004). The FAO reported in 2000 a global salt-affected area of 831 million hectares including saline and sodic soils (soils having natric horizons with an exchangeable sodium percentage (ESP) above 15). In order to supply the increasing food demand to feed the growing world population, it is necessary to increase yield per unit of land by improving crop productivity. Most of the suitable area for agricultural practices is already in use, and the expansion of land, in most of the cases, is considered neither feasible not desirable (Rengasamy, 2006).

The process of salinization occurs when water-soluble salts accumulate in the first horizons of the soil. A saline soil is characterized by an electrical conductivity of its saturation extract higher than 4dS m^{-1}, causing a diversity of negative impacts in agriculture, economy and environment. The main causes of salinization are rainfall, rock weathering, seawater intrusion, as well as inappropriate drainage, irrigation, and fertilization practices (Rengasamy, 2006; White and Broadley, 2001).

Sodium salts are found to be predominant in saline soils; however, the presence of soluble salts containing such ions as calcium, magnesium, potassium, iron, boron, sulphate, carbonate and bicarbonate are present in certain areas (Rengasamy, 2006).

A plant is considered salt tolerant if it is able to grow and complete its life cycle in a rooting medium with high concentrations of soluble salts. Plants exposed to salt stress conditions can use protection mechanisms to either exclude salt from the cells or tolerate them inside, such as the

selective accumulation or exclusion of ions, ion uptake control from roots to shoots, synthesis of compatible solutes, changes in photosynthetic pathways, and induction of enzymes and hormones, among others (Parida and Das, 2005; Phang *et al*., 2008). These mechanisms are classified as low–complexity mechanisms when they involve changes in biochemical pathway, and high–complexity when the mechanisms involve protection of major processes such as photosynthesis, respiration, cell wall and membrane integrity, and chromosome/DNA structure changes (Parida and Das, 2005).

Salt stress is one of the main abiotic stresses that produce significant yield losses in soybean. Soybean is considered a moderately salt–sensitive crop. Currently, plant breeding is a promising strategy to improve salt tolerance as soybean germplasm display wide variation in response to salinity stress.

Luo *et al.* (2005) found that soybean was more susceptible to Cl^- than Na^+ after being exposed to saline solutions in the first stages of development. However, it is still unknown whether Na^+ or Cl^- play the most critical role in the response of the plants to salt stress. Additionally, there is a differential inclusion and exclusion of these ions in roots and leaves among soybean cultivars, defining their tolerance capability (Phang, 2008).

In the soil solution, chlorine is present as the chloride ion (Cl^-), which is generally considered to be a conservative tracer of water, flows in soils. It is osmotically active in the vacuoles regulating cell turgor, being involved in the function of some enzymes, membrane regulation, and intracellular pH gradients. Chloride ion is mobile within the plant and it is an essential micronutrient required for the water–splitting reaction during photosynthesis. Its transport occurs at the tonoplast and is regulated by its content in the roots. Crops are classified according to their

salt tolerance level, depending on their response to high Cl^- concentrations and other ions present in the soil solution.

The capacity of the cultivars to withstand high levels of chloride is related to the capacity of the plant to restrict the movement of the ion from the roots. In *Glycine max*, this trait has been found to be heritable and controlled by one gene: *Ncl* (White and Broadley, 2001).

Salt stress leads to decreases in productivity and ultimately to plant death. Major processes such as photosynthesis and the metabolism of protein, energy, and lipids are affected under salt stress conditions. Leaf surface expansion and growth are reduced. Saline soils limit water uptake and induce osmotic and nutritional imbalances in the plants. Salt stress interferes in the uptake, transport and use of mineral elements such as N, K, P and calcium. Soybean growth, development, yield and seed quality are the result of the interaction between genetic potential and environmental conditions (Essa, 2002; Parida and Das, 2005; Ghassemi-Golezani and Taifeh-Noori, 2011).

Mechanisms for salt tolerance in plants

Numerous studies have been performed to understand the salt tolerance mechanisms in different types of plants and crops (Muns and Tester, 2008; Parida and Das, 2005; Tester and Davenport, 2003; Zhu 2001, 2002, 2003) including soybean (Abel and MacKenzie, 1964; Abel, 1969; De Souza *et al.*, 1997; Guan *et al.*, 2014; Pantalone *et al.*, 1997; Luo *et al.*, 2005; Phang *et al.*, 2008; Ping *et al.*, 2002; Shereen *et al.*, 2001). Phang *et al.* (2008) summarized four main mechanisms involved in soybean response to salt stress: 1) maintenance of ion homeostasis, 2) osmotic adjustment by osmoprotectants, 3) restoration of oxidative balance, and 4) other metabolic and

structural adaptations. The level of response of soybean using these mechanisms varies according to the efficiency of the genotypes in coordinating them.

1) Maintenance of ion homeostasis: Sodium chloride is one of the most common salts present in the soil. Even though the accumulation of Cl^- in the leaves is associated with salt–induced damage in soybean, it remains unclear which of the ions, Na^+ or Cl^-, plays the most critical role in sodium chloride mortality. It has been suggested that salt tolerant soybeans have a more energetic system for the transportation of ions and exchange between Na^+ and K^+ in xylem tonoplasts mediated by Na^+/H^+ (NHX exchangers) and K^+/H^+ antiporters, which sequester excess of cytosolic Na^+ into the vacuoles. This way soybean roots can pump out Na^+ from the transpiration stream in exchange with K^+ using energy from H^+–ATPase (Allen *et al.*, 1995; Parida and Das, 2005).

2) Osmotic adjustment by osmoprotectants: Salt excess leads to osmotic stress due to low water potential in the environment dropping stomatal conductance, similar to what it happens in drought stress. In response to this phenomenon, several metabolites (compatible solutes) are produced to lower the osmotic potential and protect metabolic reactions (Hasegawa *et al.*, 2000). These metabolites are grouped in four categories: onium compounds, polyols/sugars, amino acids, and alkaloids. The role of proline, another type of osmoprotectant, remains controversial due to the discrepancy among studies when using germplasm of different background in different experimental conditions; however, proline may contribute to the reduction of the plant osmotic potential to enhance water uptake.

3) Restoration of oxidative balance: Salt and osmotic stresses interfere in the scavenging of reactive oxygen species (ROS), a byproduct generated by several metabolic pathways mainly

localized in mitochondria and chloroplasts due to their active electron transport. ROS accumulation causes adverse effects in plants by inhibiting enzymes, promoting chlorophyll degradation, lipid peroxidation and damage in nucleic acids leading to cell death (Halliwell and Gutteridge, 1985; Fath *et al.*, 2001). It is proposed that antioxidant components can minimize cellular damage by restoring oxidative balance. Some of these components include: the enzyme SOD (superoxide dismutase), oxidases, glutathione (GSH), ascorbic acid (AsA), and acid phosphatases encoded by the gene GmPAP3. Salt tolerant genotypes produce higher amounts of antioxidants than sensitive cultivars as a mechanism to protect cell metabolism (Yu and Liu, 2003).

4) Structural adaptations: Cellular structure modifications are thought to play an important role in soybean adaptation to salt stress environments. One of them is the presence of salt gland–like cells in the epidermis of leaves and stems, which contain large vacuoles. Likewise, the presence of glandular hairs on wild soybean leaves helps with the secretion of Na^+ and Cl^- excess (Li *et al.*, 2003). Similarly, wall and membrane cell modifications support plant adaptation. A proline–rich cell wall protein encoded by SbPRP3 increases under high salt concentrations to modify wall structure. Under salt stress phospholipid content is reduced and the saturated fatty acid contents increased in plasma membrane in order to enhance salt tolerance (Huang, 1996).

Effect of salt stress in soybean growth and development

High salinity severely affects growth and development by affecting metabolic processes such as CO_2 assimilation, protein and oil synthesis (Ghassemi-Golezani and Taifeh-Noori, 2011). Salt stress produces damage in the whole soybean cycle, causing decreases in seedling growth, nodulation, height, seed weight, leaf size, biomass, pod number, and yield (Abel and MacKenzie,

1964; Chang *et al.*, 1994, Essa, 2002; Katerji *et al.*, 1998; Serraj *et al.*, 1998; Wang and Shannon, 1999; Katerji *et al.*, 2003).

The first stages of soybean are greatly affected by salt stress. Soybean germination is delayed under low salt concentrations and seedling stage is considered to be more sensitive to salt stress than germination (Shao *et al*, 1993; Phang *et al*., 2008). After plants are subjected to salt stress environments, the first physiological reaction is the reduction of water entry in the roots, followed by reduced height and development of small dark green leaves (Abel and MacKenzie, 1964).

Several studies have reported wide variation in soybean salt tolerance during germination and plant growth. Even though soybean generally behaves as a sensitive cultivar, the duration of this sensitivity varies among cultivars (Abel and MacKenzie, 1964; Lauchli and Wieneke, 1979; Essa and Al–Ani, 2001; Essa, 2002; Luo *et al.*, 2005; Ghassemi-Golezani and Taifeh-Noori, 2011; Kondetti *et al*, 2012).

Parker *et al.* (1983) reported that salt–sensitive soybean cultivars reached a 37% lower yield than tolerant cultivars and displayed clear foliar symptoms such as chlorosis, browning, scorch and abscission of leaves. The soybean cultivar Lee, considered moderately salt tolerant, displayed a seed yield reduction of 50% when the electrical conductivity of the soil was raised up to 9 millimhos/cm (Abel and MacKenzie, 1964). Under salt stress net photosynthesis is significantly correlated with grain yield. Higher chlorophyll fluorescence in varieties with higher salt tolerance is another factor that contributes to better photosynthetic traits (He *et al*, 2016).

Salinity reduces the amount of chlorophyll and the efficiency of photo–system II and increases the amount of proline, an amino acid involved in the lowering of osmotic potential of vacuoles.

The more tolerant the plant is to adverse salinity conditions, the less chlorophyll degradation occurs (Kummar *et al*., 2003). Photosynthesis in leaves is reduced under salt stress conditions and induces premature senescence, primarily due to toxic accumulations of Na^+ and Cl^- and/or depletion of K^+ or Ca^{2+} (Saquib *et al.,* 2012). Salt accumulation reduces the potassium roll as an osmotic regulator in leaves. Thus, Na^+ and K^+ increase is one of the main causes of growth decrease (Sofalian *et al.,* 2013).

Chen *et al.* (2013) found that salt stress reduced photosynthesis rate in cultivated and wild soybean by reducing stomatal conductance as NaCl concentration increased from 50 to 200 mM. With concentrations higher than 300 mM, a decrease in photosynthesis was due to other non–stomatal factors.

In soybean, salt induced damage is associated with chloride accumulation in the aerial part; however, it has been found that tolerant cultivars accumulate less Na^+ in leaves (Li *et al.,* 2006). Additionally, there is a differential inclusion and exclusion of Na^+ and Cl^- in soybean cultivars with contrasting tolerance response. For example, in a study performed by An *et al.* (2002) to study the role of the root system in salt tolerance and compare ion accumulation between the tolerant cultivar ´Dare´ and the susceptible cultivar ´Tachiyutaka´. It was found that ´Dare´ had lower Na^+ contents in roots and higher water uptake rate than ´Tachiyutaka´, which in contrast performed as a Na^+ includer in roots. Therefore, Dare either take up less Na^+ ions or exclude more Na^+. Valencia *et al.* (2008) reported that in general soybean roots tend to contain higher amount of Na^+ and Cl^- than leaves, and between them, Cl^- is predominant. In addition, Cl^- excluders (tolerant genotypes) contained greater amount of this element in roots than Cl^- includers (susceptible lines) in all treatments evaluated (NaCl 40, 80, 120 mM).

Also, it is reported that cultivated soybean (*Glycine max*) is more susceptible to Cl^- than wild soybean (*G. soya*). Wild soybean is more susceptible to Na^+ and its tolerance level depends on how genotypes successfully retain Na^+ in roots (Luo *et al.,* 2005). Tolerant cultivars have the capacity to not only restrict the mobility and accumulation of Na^+ and Cl^- in the aerial parts, but also to assimilate more K^+, thus being able to maintain a more desirable Na^+ / K^+ ratio, especially in leaves (Essa, 2002; Chen *et al*., 2013).

Ghassemi-Golezani and Taifeh-Noori (2011) evaluated three soybean genotypes in the field under three concentrations of NaCl solution (3, 6, and 9 dS/m) using hydroponics. Plants were observed over a period of approximately seven weeks in order to evaluate chlorophyll content and fluorescence, protein, oil, proline content, and seed yield. They found that both proline and grain yield were significantly affected by salinity. Chlorophyll content index and protein yield decreased in all cultivars as salinity increased due to disturbance in nitrogen absorption and metabolism. In contrast, proline, and oil content increased with increasing salinity. Crop production was severely limited because high salinity lowers water potential, inducing ionic stress, which leads to secondary oxidative stress.

Salt stress also reduces growth and yield components by affecting endogenous growth hormones. Hamayun *et al.* (2010) performed a study to evaluate the effects of NaCl induced stress on phytohormones and growth attributes on a soybean cultivar using saline solution 70 mM and 140 mM before and after flowering. Results showed that endogenous gibberellins (GA) were significantly reduced under high salt concentration, while abscisic acid (ABA) and jasmonic acid (JA) content in leaves significantly increased in both stages. The decrease in growth and chlorophyll content was higher when the stress was applied in pre–flowering stage. Under saline conditions Na^+ competes with K^+ for uptake through common transport systems. The increase of

Na^+ / K^+ ratio due to elevated cytosolic Na^+, exerts ion toxicity by competition of these ions for enzyme binding sites and displaces Ca^{+2} from plasma membranes affecting their permeability.

Salt tolerance screening in soybean

The discovery of new genotypes is essential in the aim of finding new sources of salt tolerance resistance. Although soybean is a moderately salt–sensitive crop, differential response among genotypes has been found, suggesting that there is genetic variability for this trait (Abel and MacKenzie, 1964; Pantalone *et al.,* 1997; Shannon, 1997; Lee *et al.,* 2004). Different screening methods have been proposed to evaluate and select salt–tolerant soybean genotypes in the field and greenhouse (Yang and Blachar, 1993; Pantalone *et al*., 1997; An *et al*., 2002; Lee *et al*., 2004; Valencia *et al.,* 2008; Lee *et al.,* 2008). Field screening evaluations of soybean genotypes planted in soils with high salt concentration have been sometimes considered unsuccessful due to the variability of salt levels across the soil and the changing environmental conditions. Another method was proposed by Valencia *et al.,* 2008 using hydroponics, in which a NaCl solution was supplied to the plants in a greenhouse controlling nutrition and environment. However, it becomes expensive and inefficient since nutrient solutions need to be constantly changed. A simpler screening called PC method (plastic cone-tainers) was tested with good results. In this method, sandy soil was used as a growth medium instead of a nutrient solution (Lee *et al*., 2008). Measuring traits as plant biomass, leaf scorch, and ion concentration in plant tissue has led to the discovery of clear differences in salt tolerance among genotypes. Valencia *et al.* (2008) found that soybean plants treated with 120 mM NaCl displayed the most effective and consistent salt tolerance data based on visual foliar symptoms evaluating a set of differential genotypes. Additionally, there is negative correlation between root dry weight and Cl^- content in leaves and

screening for Cl^- sensitivity using root visual evaluation for symptoms was not as effective as the visual leaf evaluation.

Ledesma *et al.* (2016) also proposed a greenhouse salt screening method using sandy soil and 120 mM NaCl and measuring visual leaf scorch score (LSS, scale 1–9) and chloride content in leaves as parameters to evaluate salt tolerance in a large set of soybean cultivars with differential response to salt stress. After the development of a set of experiments and the validation of the methodology, a positive and strong correlation was found between LSS and ion content in roots and leaves validating the accuracy of the visual ratings; however, the lowest correlation was found between LSS and ion content in roots indicating that ion content in roots is not a consistent indicator for the evaluation of salt tolerance response. Differently, the strongest correlation was found between LSS and chloride content in leaves. These parameters provided the clearest statistical differences among all growing media and NaCl concentrations.

Genetics of salt tolerance in soybean

Salinity control using environmental amendments like the use of improved irrigation techniques is usually very expensive and constitute a short–term solution to mitigate the process of soil salinization. The development of salt tolerant crops widening plant-breeding programs is a very effective way to develop cultivars able to produce economic yields under saline conditions. Salt tolerance is a complex trait of polygenic nature that responds to cellular osmotic, ionic, and oxidative stresses, which operate at the cellular level. Glycophytes have special cellular mechanisms to overcome salt stress (Hasegawa *et al.*, 2000). The development of salt tolerant plants has been achieved via cell and tissue culture, and molecular breeding using molecular markers and genetic engineering (Arzani, 2008).

The first genetic studies for soybean salt tolerance report the existence of a single gene pair, which is responsible for the regulation of the exclusion and inclusion of chloride in soybean leaves and stems denominated *Ncl* and *ncl*, respectively (Abel, 1969). This QTL have been since mapped by several researchers to the same region in Chromosome 3 (linkage group N) using distinct crosses between cultivated and/or wild soybean parents (Shao *et al*., 1994; Lee *et al*., 2004; Chen *et al*., 2008; Hamwieh and Xu, 2008; Hamwieh *et al*., 2011). These consistent results found in this region of chromosome 3, have led to the hypothesis that a conserved gene or several genes control salt tolerance in diverse germplasm (Qu *et al.*, 2015).

Recent studies have reported that *Ncl* gene simultaneously controls Cl^-, Na^+ and K^+ transport and accumulation in soybean. Do *et al.* (2016) found that the expression of this gene in the soybean variety FT–Abyara under salt stress, is higher in roots when the accumulation of these ions in shoots diminishes.

Marker assisted selection and quantitative trait loci (QTL) mapping for salt tolerance in soybean

Important traits in crops such as yield, quality, and resistance are considered quantitative since many genes with a low effect, or polygenes, regulate them. A QTL is a region (loci) within the genome, which contains genes associated with a particular quantitative trait (Collard *et al.*, 2005). Marker assisted selection constitutes a powerful tool to identify QTL associated with salt tolerance using specific DNA markers associated with this trait, given that conventional phenotypic evaluation is not enough for gene discovery and linkage mapping (Hamwieh *et al*, 2011). A linkage map is a representation of the linkage groups (chromosomes) of a species used to identify genomic regions that contain genes controlling either simple traits (single gene), or

complex traits using QTL analysis (process known as QTL mapping). QTL mapping is based on the principle that genes segregate during meiosis through chromosome recombination, which can be analyzed in the progeny considering the fact that genes with narrow distance between them (tightly–linked genes) are going to be inherited together with higher frequency in following generations (Collard *et al.,* 2005).

Mapping of QTL related to abiotic stresses, as salt tolerance, is very important in soybean breeding programs for the application of map–based cloning and marker assisted selection (Chen *et al.,* 2008)

Several studies have examined salt tolerance inheritance in soybean. Abel (1969) used different parent combinations to study chloride exclusion in soybean, finding that there is a single dominant gene, *Ncl*, controlling salt tolerance in the cultivar Lee (S100 x CNS), S–100 being the source of tolerance. Lee *et al.* (2004) identified a major QTL on linkage group N (chromosome 3) associated with salt tolerance, after the evaluation of 106 $F_{2:5}$ RILs (recombinant inbred lines) coming from the cross S–100 x Tokyo. The estimated position of this QTL was in the interval between the SSR markers Sat091, Satt339, Satt237 explaining 41% (field), 60% (greenhouse) and 79% (combined environments) of the genetic variance, and it was concluded that it could be the *Ncl* locus previously found. Hamwieh and Xu (2008) studied wild soybean salt tolerance inheritance using an F_2 population derived from the cross between the cultivar Jackson (sensitive) and the wild accession JWS156–1 (tolerant) based on visual symptoms (scorching) and chlorophyll content. A major salt–tolerant QTL, accounting for 68.7 % of the total scorch rating variance, was located in a similar QTL interval region (covering markers Satt339, Satt237) previously reported by Lee *et al.* (2004). Hamwieh *et al.* (2011) found the same QTL using NILs developed from the FT–Abyara (tolerant) x C01 (sensitive) population. Chen *et al.* (2008)

screened under greenhouse and field conditions 184 $F_{7:11}$ RILs coming from the cross between Kefeng No.1 (tolerant) x Nannong 1138–2 (sensitive). A different major QTL was localized on linkage group G (chromosome 18) between the markers Sat_164 and Sat_358 explaining 11 and 18% of the phenotypic variation in field and greenhouse, respectively. Overall, they found seven new QTLs in six different linkage groups. Three QTL were detected in field experiments explaining 7.1–19.7% of the phenotypic variance for salt tolerance in linkage groups G (Chr. 18) and M (Chr. 7). Under greenhouse conditions six QTLs were detected explaining 7.8–19.2% of the phenotypic variation in linkage groups B1 (Chr. 11), B2 (Chr. 14), D1b (Chr. 2), G (Chr. 18), K (Chr. 9), and N (Chr. 3).

The salt tolerance mechanism in wild soybean genotypes is different than the mechanism used by cultivated soybean in terms of Na^+ and Cl^- susceptibility and exclusion (Luo *et al.*, 2005). Lee *et al.* (2009) reported the existence of a tolerant gene on linkage group N in the wild soybean PI483463, designated as *Ncl2*. This PI was crossed to the sensitive cultivar Hutcheson to perform an allelism test and study the inheritance of this trait. The gene *Ncl2* was localized between Sat_91 and BARC-016485-02069. In a more recent study performed by Ha *et al.* (2013), the QTL conferring salt tolerance in PI483463 was mapped within a 658-kb region between SSR03_1335 and SSR_1359. This region was 658-kb and contained 80 annotated genes, including two genes (*Glyma03g32890* and *Glyma03g32900*) belonging to the sodium/hydrogen exchanger family. Subsequently, Qi *et al*. (2014) mapped the candidate causal gene underlying *GmCHX1* (counterpart of *Glyma03g32900* in Williams 82) in the wild soybean accession W05 using whole genome sequencing. Similarly, Guan *et al* in 2014 identified the candidate causal gene *Glyma03g32900 (GmSALT3)* underlying the QTL in chromosome 3 in the Chinese soybean cultivar Tiefeng 8, which limits sodium accumulation, by using fine mapping.

The use of *Ncl* locus by gene transfer or MAS could contribute to sustainable soybean production in saline soils. Do *et al.* (2016) isolated the *Ncl* gene from the soybean cultivar FT–Abyara using map–based cloning. They found that the gene not only regulated Na^+ and K^+ as expected (since the gene belongs to the Na+/H+ antiporter gene family), but was also responsible for the control of Cl^- accumulation. Additionally, the allele *Ncl* helped to achieve sustainable yields in a set of NILs obtaining 3.6–5.5 higher yields than lines without the allele. This represented a 28% vs. 80% yield loss for tolerant lines and susceptible lines, respectively.

A study to evaluate alkaline salt tolerance (tolerance to excess of sodium) revealed the existence of a major QTL on linkage group D2 (Chr. 17) between the markers Satt669 and Sat_300, which accounted for 50.2 and 13.0% of the total variation for scorch scoring in F_6 and F_2 populations respectively. These populations were derived from the cross JWS156-1 (tolerant *G. soja* to NaCl and alkaline salt stress) x Jackson (sensitive *G. max*) (Tuyen *et al.*, 2010).

Recently, Guan *et al.* (2014 a, b) mapped and validated *GmSALT3 (Glycine max* Salt Tolerance-associated gene on chromosome 3*)*, a dominant gene for salt tolerance to a 209 Kb region on linkage group N found in the salt–tolerant cultivar Tiefeng 8. The authors treated an $F_{2:3}$ population derived from the soybean cultivars Tiefeng 8 (tolerant) and 85–140 (sensitive) with 200 mM NaCl to evaluate salt tolerance, obtaining a 1:2:1 segregation ratio. *GmSALT3* was localized within the markers QS08064 (SCAR) and Barcsoyssr_3_1301 (SSR) using map–based cloning. This gene encodes a protein associated with cation/H^+ exchange in root cells present in xylem and phloem, leading to lower sodium accumulation in shoots. The expression of this candidate gene in the cultivar Tiefeng was higher in roots compared to shoots. Probably, the reduction of salinity selection pressure on soybean led the gene *GmSALT3* to have mutations resulting in a loss or reduced function of the gene product, producing a loss of salt tolerance (Qu

et al., 2015). It has been hypothesized that the variation of the response in cultivated and wild soybean to salt tolerance can be explained by loss-of-function mutation in *Ncl* occurred before soybean domestication from ancestral wild species (Do *et al.*, 2016).

Qi *et al.* (2014) used whole–genome–sequencing and genotyping–by–sequencing based genetic mapping in a recombinant inbred population to identify novel genes associated with salt tolerance in wild soybean. The RI population was obtained "by crossing the *de novo*–sequenced W05 (high–tolerance wild soybean accession) with the re–sequenced cultivated soybean accession C08 (sensitive)". A 338 Kb novel transporter gene (*GmCHX1*) was identified. This major salt tolerant locus confers salt tolerance by lowering the Na^+/K^+ ratio in leaves.

The constantly detected QTL on chromosome 3 associated with soybean salt tolerance is likely to be the *Ncl* locus. The genes *Glyma03g32890* and *Glyma03g32900*, belonging to the Na^+/H^+ exchanger family have been found in this QTL region in wild soybean accessions. Similarly, the candidate causal genes *GmCHX1* in Williams 82 and *GmSALT3* in the wild soybean accession W05, are counterparts of Glyma*03g32900* and are associated with limiting the accumulation of sodium (Do *et al.*, 2016). Further studies are needed to find genes associated with Cl^- transport and accumulation and its relationship to Na^+ and K^+ regulation in the efficiency of the salt tolerance response of soybean genotypes.

Table 1. Texture and chemical properties of the sandy loam soil used for the salt tolerance screening in the greenhouse.

pH	EC (µmhos/cm)	----------mg/kg----------												Texture %		
		P	K	Ca	Mg	S	Na	Fe	Mn	Zn	Cu	B	$NO_3N\&NO_2N$	NH_4-N	Sand	Silt	Clay
8,3	125	7.8	55	2083	107	4.5	4.9	89	46	1.7	0.6	0.02	16.2	2.4	71	24	5

Table 2. Summary of molecular markers reportedly associated with soybean salt tolerance

Chr.	Marker type	% V	Marker	Parents (T x S)	Authors
3	SSR	45	Satt237-Sat_091	S-100 x Tokyo	Lee *et al*. (2004)
3	SSR	68.7	Satt339, Satt237, Satt255	JWS156-1 x Jackson	Hamwieh and Xu (2008)
3	SSR, SNP	56.5	Satt255, BARC-038333-10036	PI 483463 x Hutcheson	Ha *et al.* (2013)
3	SSR	44	Sat_091, Sat_304	FT-Abyara x C01	Hamwieh *et al*. (2011)
3	SSR	47.1	Sat_091	Jin dou No.6 x 0197	Hamwieh *et al*. (2011)
3	CAPS	–	QS100001, QS1119	Tiefeng 8 x 85-140	Guan *et al.* (2014)
7	SSR	7-19.7	Satt702-Satt728, Satt655-Satt210	Nannong1138-2 x Kiefeng	Chen *et al.* (2008)
17	SSR	50.2	Satt447	JWS156-1 x Jackson	Tuyen *et al.* (2010)
18	SSR	10.8	Sat_164-Sat_358	Nannong1138-2 x Kiefeng	Chen *et al.* (2008)

REFERENCES

Abel, G.H. and A.J. MacKenzie. 1964. Salt tolerance of soybean varieties (*Glycine max* L. Merrill) during germination and later growth. Crop Sci. 4: 157–161.

Abel. G.H. 1969. Inheritance of the Capacity for Chloride Inclusion and Chloride Exclusion by Soybeans. Crop Sci. 9: 697–698.

Allen G.J, R.G Wyn Jones, R.A Leigh. 1995. Sodium transport in plasma membrane vesicles isolated from wheat genotypes with differing K+/Na+ discrimination traits. Plant Cell Environ. 18: 105–115.

An, P., S. Inanaga, Y. Cohen, U. Kafkafi, and Y. Sugimoto. 2002. Salt tolerance in two soybean cultivars. J. Plant Nutr. 25: 407-423.

Arzani, A. 2008. Improving salinity tolerance in crop plants: a biotechnological view. In Vitro Cell. Dev. Biol. Plant. 44: 373-383.

Chang, R.Z., Y.W. Chen, G.H. Shao and C.W. Wan. 1994. Effect of salt stress on agronomic characters and chemical quality of seeds in soybean. Soybean Sci. 13, 101–105.

Chen H., S. Cui, S. Fu, J. Gai, and D. Yu. 2008. Identification of quantitative trait loci associated with salt tolerance during seedling growth in soybean (*Glycine max* L.). Aust. J. Agric. Res. 59: 1086–1091.

Chen, P., C.H. Sneller, J.C. Rupe, and R.D. Riggs. 2004. Registration of 'Ozark' Soybean. Crop Sci. 44:1872-1873.

Chen, P., H. Wang, X. Tang, M. Brestic, and H. Shao. 2013.Comparative physiological study between cultivated and wild soybean species under salt stress. Jokull J. 63: 114–124.

Chen, P., M. Orazaly, J.C. Rupe, D.G. Dombek, T. Kirkpatrick, R.T. Robbins, C. Wu, and P. Manjarrez. 2014. Registration of _UA 5213C_ soybean. J. of Plant Reg. 8: 150-154.

Chinnusamy, V., A. Jagendorf and J.K. Zhu. 2005. Understanding and Improving Salt Tolerance in Plants. Crop Sci. 45: 437–448.

Collard, B.C.Y., M.Z.Z. Jahufer, JB Brouwer, and E.C.K. Pang. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142: 169-196.

De Souza, P.I, D. Egli, and W. Bruening. 1997. Water Stress during Seed Filling and Leaf Senescence in Soybean. Agron. J. 89: 807-812.

Do, T. D. *et al.* 2016. *Ncl* Synchronously Regulates Na+, K+, and Cl– in Soybean and Greatly Increases the Grain Yield in Saline Field Conditions. Sci. Rep. 6, 19147. 10 p.

Doyle, J.J. and J.L. Doyle .1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Essa, A. T., and D. H. Al-Ani, 2001: Effect of salt stress on the performance of six soybean genotypes. Pakistan. J. Biol. Sci. 4: 175–177.

Essa, T.A. 2002. Effect of salinity stress on growth and nutrient composition of three soybean [*Glycine max* (L.) Merr.] cultivars. J. Agron. Crop Sci. 188: 86–93.

Fath A, P.C Bethke, M.V Belligni, Y.N Spiegel, and R.L Jones. 2001. Signaling in the cereal aleurone: hormones, reactive oxygen and cell death. New Phytol. 151: 99–107.

Ghassemi-Golezani K. and M. Taifeh-Noori .2011. Chapter 34: Soybean Performance under Salinity Stress. Soybean - Biochemistry, Chemistry and Physiology. Ed. InTech. 654 p.

Guan, R. Y. Qu, Y. Guo, L. Yu, Y. Liu, J. Jiang, J. Chen, Y. Ren, G. Liu, L. Tian, L. Jin, Z. Liu, H. Hong, R. Chang, M. Gilliham, and L. Qiu. 2014 (a). Salinity tolerance in soybean is modulated by natural variation in *GmSALT3.* The Plant J. 80: 937–950.

Guan, R., J. Chen, J. Jiang, G. Liu, Y. Liu, L. Tian, L. Yu, R. Chang, and L. Qiu. 2014 (b). Mapping and validation of a dominant salt tolerance gene in the cultivated soybean (*Glycine max)* variety Tiefeng 8. The Crop J. 2: 358–365.

Ha, B. K., T. Vuong, V. Velusamy, H. Nguyen, J.G Shannon, J. Lee. 2013. Genetic mapping of quantitative trait loci conditioning salt tolerance in wild soybean (*Glycine soja*) PI 483463. Euphytica 193: 79–88.

Halliwell, B. 1982. The toxic effects of oxygen on plant tissues. In: Oberly, L.W. (Ed.), Superoxide Dismutase, vol. I. CRC Press, Boca Raton, FL, p. 89–123.

Hamayun, M., S.A. Khan, A. L. Khan, Z. K. Shinwari, J. Hussain, E. Sohn, S. Kang, Y. Kim, M. A. Khan, and I. Lee. 2010. Effect of salt stress on growth attributes and endogenous growth hormones of soybean cultivar Hwangkeumkong. Pak. J. Bot., 42: 3103-3112.

Hamwieh, A., D. D. Tuyen, H. Cong, E. R. Benitez, R. Takahashi, and D. H. Xu. 2011. Identification and validation of a major QTL for salt tolerance in soybean. Euphytica 179: 451–459.

Hasegawa P.M, R.A Bressan, J.K Zhu, H.J Bohnert. 2000. Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51: 463–499.

He, Y., Y. Chen, C. L. Yu, K.X. Lu, Q. S. Jiang, J.L. Fu, G. M. Wang, and D.A. Jiang. 2016. Photosynthesis and yield traits in different soybean lines in response to salt stress. Photosynthetica 54. 6 p.

Huang C.Y. 1996. Salt-stress induces lipid degradation and lipid phase transition in plasma membrane of soybean plants. Taiwania. 41: 96 – 104.

Huang, L. 2013. Genome-wide association mapping identifies QTLs and candidate genes for salt tolerance in soybean. M.S thesis. University of Arkansas. 129 p.

Jamil, M., Rehman, S., Lee, K. J., Kim, J. M., Kim, H. S. and Rha, E. S. 2007. Salinity reduced growth PS II photochemistry and chlorophyll content in radish. Sci. Agric. 64: 1-10.

Kondetti, P., J. Narendra, S. K. Apte, and M. G. Shitole. 2012. Salt tolerance in Indian soybean (*Glycine max* (L.) Merill) varieties at germination and early seedling growth. Ann. Biol. Res. 3: 1489-1498.

Kummar S.G., A. Matta, C. Sudhakar. 2003. NaCl effects on proline metabolism in two high yielding genotypes of mulberry with contrasting salt tolerance. Plant Sci. 165: 1245–1251.

Lauchli, A., and J. Wieneke. 1979. Studies on growth and distribution of Na, K and Cl in soybean varieties differing in salt tolerance. Z. Pflanzenernahr. Bode- nkd. 142: 3–13.

Ledesma, F., C. Lopez, D. Ortiz, P. Chen, K. L. Korth, T. Ishibashi, A. Zeng, M. Orazaly, and L. Florez-Palacios. 2016. A simple greenhouse method for screening salt tolerance in soybean. Crop Sci. 56: 585-594.

Lee, G.J., R.H. Boerma, R.M. Villagarcia, X. Zhu, T.E. Carter, Jr., Z. Li, and M.O. Gibbs. 2004. A major QTL conditioning salt tolerance in S-100 and descendent cultivars. Theor. Appl. Genet. 109: 1610-1619.

Lee, J.D., S.L. Smothers, D. Dunn, M. Villagarcia, C.R. Shumway, T. Carter, Jr., and J.G. Shannon. 2008. Evaluation of a simple method to screen soybean genotypes for salt tolerance. Crop Sci. 48: 2194-2200.

Li G, G.Y Wang, J.Z Son, H. Gao, J.M Lu. 2003. Salt-resistant structures of leaves from Glycine max cultivar "Fendou 16". J. Northeast Normal Univ. 4: 109–111.

Li, X., P. An, S. Inanaga, E. Eneji, and K. Tanabe. 2006. Salinity and defoliation effects on soybean growth. J. Plant Nutr. 29: 1499–1508.

Luo, Q., B. Yu and Y. Liu. 2005. Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. J. Plant Physiol. 162: 1003–1012.

Munns, R. and M. Tester. 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59: 651–681.

Parida, A.K., and A.B. Das. 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60: 324–349.

Pantalone, V.R., W.J. Kenworthy, L.H. Slaughter, and B.R. James. 1997. Chloride tolerance in soybean and perennial *Glycine* accessions. Euphytica 97: 235–239.

Parker M.B., G.J. Gascho, and T.P. Gaines. 1983. Chloride toxicity of soybean grown on Altantic coast flatwoods soils. Agro. J. 75:439-443.

Phang, T.H., G.H. Shao and H.M. Lam. 2008. Salt tolerance in soybean. J. Integr. Plant Biol. 50: 1196–1212.

Ping, A., S. Inanaga, Y. Cohen, U. Kafkafi and Y. Sugimoto. 2002. Salt tolerance in two soybean cultivars. J. Plant Nutr. 25: 407–423.

Plank, C.O. 1992. Plant analysis reference procedures for the southern region of the United States. Southern Cooperative Series Bull. 368. Univ. of Georgia, Athens.

Qi, X., M. Li, M. Xie, X. Liu, M. Ni, G. Shao, C. Song, A. Yim, *et al*., 2014. Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. Nat. Commun. 5: 4340. 11 p.

Qu, Y., R. Guan, L. Qiu, and M. Gilliham. 2015. Improving the Salinity Tolerance of Soybean. ISB News Report. Available in: http://www.isb.vt.edu/news/2015/Apr/QuGuanQiuGilliham.pdf. Last visit: June 2016.

Rengasamy, P. 2006. World salinization with emphasis on Australia. Journal Exp. Bot. 57: 1017–1023.

Tester, M. and R. Davenport. 2003. Na^+ Tolerance and Na^+ Transport in Higher Plants. Ann. Bot. 91: 503-527.

Tuyen, D.D., S.K. Lal, and D.H. Xu. 2010. Identification of a major QTL allele from wild soybean (*Glycine soja* Sieb. & Zucc.) for increasing alkaline salt tolerance in soybean. Theor. Appl. Genet. 121: 229–236.

Sairam, R.K., and A. Tyagi. 2004. Physiology and molecular biology of salinity stress tolerance in plants. Curr. Sci. 86: 407–421.

Saquib, Z., Zulfiqar A. Saqib, J. Akhtar, M. A. Ul-Haq, and I. Ahmad. 2012. Salt induced changes in leaf phenology of wheat plants are regulated by accumulation and distribution pattern of Na^+ ion. Pak. J. Agri.Sci. 49: 141–148.

Slabu, C., D.P. Simioniuc, F. D. Lipşa , V. simioniuc. 2010. Physiological response to water and salt stress of some white lupine cultivars (*Lupinus Albus* L.). Lucrări Ştiinţifice. 53: 64–68.

Shannon, M.C. 1997. Adaptation of plants to salinity. Adv. Agron. 60: 75-120.

Shannon J.G., J.A. Wrather, D.A. Sleper, R.T. Robbins, H.T. Nguyen, and S.C. Anand. 2007. Registration of 'Jake' Soybean. J. Plant Regist. Vol 1. May–June. p.29.

Shereen, A., R. Ansari, and A. Soomro. 2001. Salt tolerance in soybean (Glycine max L.): Effect on growth and ion relations. Pak. J. Bot. 33: 393–402.

Shao G.H., J. Z Song, and H. L Liu. 1986. Preliminary studies on the evaluation of salt tolerance in soybean varieties. Acta Agron. Sin. 6: 30– 35.

Shao G.H, C.W Wan, R.Z Chang, and Y.W Chen. 1993. Preliminary study on the damage of plasma membrane caused by salt stress. Crops 1: 39–40.

Shao G.H., R.Z. Chang, Y.W. Chen, and S.R. Yan. 1994. Study on inheritance of salt tolerance in soybean. Acta Agron. Sin. 20: 721–726.

Singh, G. 2010. The Soybean: Botany, production and uses. CABI. 494 p.

Singh, A. K. and Dubey, R.S. (1995). Changes in chlorophyll a and b contents and activities of photosystems 1 and 2 in rice seedling induced by NaCl. Photosynthetica, 31: 489-499.

Sofalian, O., P.B Miandoab., A. Asghari, M. Sedghi, and A. Eshghi. 2013. Relationship between salt tolerance related physiological traits and protein markers in soybean cultivars (*Glycine max* L.). Cercet. Agronon. Mold. 46:47–56.

USDA– United States Department of Agriculture. 2016. World Agricultural Production. Foreign Agricultural Service. Circular Series WAP 6-16 June 2016. Available in: http://apps.fas.usda.gov/psdonline/circulars/production.pdf

USDA, ARS, National Genetic Resources Program. Germplasm Resources Information Network (GRIN).URL: http://www.ars-grin.gov/npgs/searchgrin.html. Last visit: January, 2014.

Valencia, R., P. Chen, T. Ishibashi, and M. Conaster. 2008. A rapid and effective method for screening salt tolerance in soybean. Crop Sci. 48:1773-1779.

Vile, D., E. Garnier, B. Shipley, G. Laurent, M. Navas, C. Roumet, S. Lavorel, S. Diaz, J. Hodgson, F. Lloret, G. Midgley, H. Poorter, M. Rutherford, P. Wilson, I. Wright. 2005. Specific leaf area and dry matter content estimate thickness in laminar leaves. Ann. Bot. 96: 1129–1136.

Virginia Crop Improvement Association. Glenn Soybean Variety Description. URL: http://www.virginiacrop.org/glenn_soybean_variety.htm (January, 2014).

Wang J, M. van Ginkel M, D. Podlich, G.Ye, R. Trethowan, W. Pfeiffer *et al*. (2003). Comparison of two breeding strategies by computer simulation. Crop Sci. 43: 1764–1773.

Wheal, M.S., and L.T Palmer. 2010. Chloride analysis of botanical samples by ICP- OES. J. Anal. At. Spectrom 25: 1946-1952.

White, P., and M. Broadley. 2001. Chloride in Soils and its Uptake and Movement within the Plant: A Review. Ann. Bot. 88: 967-988.

Yang, J., and R.W. Blanchar. 1993. Differentiating chloride susceptibility in soybean cultivars. Agron. J. 85: 880-885.

Yu, B.J., Y.L. Liu. 2003. Effects of salt stress on the metabolism of active oxygen in seedlings of annual halophyte Glycine soja. Acta Bot. Boreal.-Occident. Sin. 23: 18–22.

Zhu, J.K. 2001. Plant salt tolerance. Trends Plant Sci. 6: 66-71.

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53: 247–273.

Zhu, J.K. 2003. Regulation of ion homeostasis under salt stress. Curr. Opin. Plant Biol. 6: 441–445.

CHAPTER II. Identification of Quantitative Trait Loci in Soybean for Salt Tolerance

ABSTRACT

Soybean growth and yield is adversely affected by soil salinity. The identification tolerance genes that improve soybean performance in soils with problems of salinization is a very effective way to use and develop soybean genotypes with high salt tolerance response. Early studies have revealed that salt tolerance in soybean is regulated by a single dominant gene, *Ncl*, located in chromosome 3, mainly responsible for Cl^- exclusion. Recently, additional studies have repeatedly reported a major QTL associated with salt tolerance in chromosome 3 close to the markers Satt 255 and Sat_091, and other minor QTLs have been also reported. The main objective of this study, was to identify/confirm QTL associated with salt tolerance using an $F_{2:3}$ linkage mapping population from the cross Jake (salt tolerant) x Ozark (salt- sensitive). This population was screened in the greenhouse using 120 mM NaCl. After two weeks, leaf scorch score (LSS), percentage of dead plants (PDP), leaf chlorophyll, and leaf chloride content were measured. Genotyping was performed using SoySNP 6K chip. Two QTLs were found in this study, a major QTL on chromosome 3 (linkage group N) and a minor QTL on chromosome 19 (linkage group L). The QTL identified on chromosome 3, is located in the same genomic region previously reported. Four SNP markers were highly linked to this QTL mapped to chromosome 3. The SNP Gm03_41020834_T_C is associated with LSS, PDP, and leaf chlorophyll content explaining 37 to 48.9 % of the phenotypic variance; Gm03_40600088_A_G is associated with PDP and leaf chlorophyll accounting for 37% and 43.5% of the variation, respectively, and the markers Gm03_40270199_T_C and Gm03_40663609_G_A explained 41% to 43% of the leaf chloride variation. The new minor QTL identified on chromosome 19, is associated to leaf chlorophyll content and linked to the SNP markers Gm19_40508288_C_T and

Gm19_42246131_A_G, which explained approximately 5% of the phenotypic variation of the trait. The markers linked to the identified QTLs associated with the evaluated traits, can be of valuable use for future marker assisted selection.

INTRODUCTION

Soybean is a traditional cash crop cultivated in different types of environments being exposed to many biotic and abiotic stresses. Salt stress is considered a major limitation to soybean production in several regions around the world, affecting all the crop development stages and yield components. Sodium salts dominate in many saline soils around the world; Na^+ and Cl^- are major ions responsible for salinization. Salt damage in soybean is expressed by the accumulation of chloride and other ions in stems and leaves causing toxicity, followed by the presence of leaf chlorosis and necrosis leading to a reduction in plant biomass. It has been reported that the soybean salinity threshold is 5.0 dS m^{-1}. Soybean yield is reduced up to 20 % and 56 % under a soil electrical conductivity of 4.0 dS m^{-1} and 6.7 dS m–1, respectively (Lee *et al.*, 2008; Katerji *et al.*, 2003).

Soybean germplasm displays a wide spectrum in the response of phenotypes to salt stress, suggesting that there is genetic diversity and natural variation of this crop to salt tolerance.

Several studies have reported U.S varieties and breeding lines to be chloride tolerant based on visual ratings and the measurement of leaf chloride content. A high correlation between low leaf scorch visual ratings and low leaf chloride accumulation has led to the use of the common description of tolerant genotypes as chloride excluders (Lee *et al.*, 2004). Chloride resistance in soybean cultivars is controlled by the single dominant gene *Ncl*, identified by Abel (1969) in the cultivar 'Lee' (CNS x S-100), and it has been confirmed that the cultivar S-100 is the source of the major chloride exclusion allele. Several research groups have mapped this QTL to the same region in chromosome 3 (linkage group N), using parents from both, cultivated and wild soybeans. The same region has been highlighted using different genetic sources, which has led to

the hypothesis that a conserved gene or several few genes control salt tolerance in diverse soybean germplasm.

Plant breeding is considered to be the major strategy to improve salt tolerance in soybean, however, substantial effort is needed to achieve this goal due to the close linkage between abiotic stress loci and undesirable traits (Phang *et al.*, 2008; Wang *et al.*, 2003). Several studies based on shoot chloride accumulation have provided evidence to conclude that soybean genotypes may be dominated by a single or few major loci suggesting that salt tolerance is an inheritable qualitative trait. However, other studies have reported the existence of salt tolerance mechanisms regulated by minor genes when soybean plants are rated on salt–induced chlorosis in leaves (Luo *et al.*, 2004). These variable results are probably associated with the genetic background of the parental germplasm selected and the use of different parameters for the evaluation of the salt stress response (Phang *et al.*, 2008).

The lack of salinity selection pressure in soybean (e.g. when plants are grown in soils with low salinity levels) can either cause no significant change in conserved salt tolerance genes, or produce some gene mutations, which eventually can reduce the function of the gene products lowering salt tolerance (Qu *et al.*, 2015). Additionally, salt tolerance genes may be lost after following several recombination events through natural selection and domestication (Guan *et al.*, 2014 (a)).

Making use of soybean natural variation with the application of conventional breeding and marker assisted selection, it is possible to identify genes that improve soybean salt tolerance obtaining stable yields under saline conditions, and at the same time, gaining effectiveness in soil utilization for agriculture to make a contribution in present and future food security challenges.

The objective of this study was to identify and / or confirm QTL associated with salt tolerance using linkage mapping.

MATERIALS AND METHODS

Parental material and population development

The F1 segregating population Jake x Ozark was selected to perform this study. Jake is tolerant (excluder) and Ozark susceptible (includer) to salt stress. After subjecting these soybean genotypes to a 120 mM NaCl treatment during two weeks, Jake reached a maximum LSS (leaf scorch score: 1–10 scale) of 4, while Ozark displayed a LSS of 7 (Ledesma *et al.*, 2016). Jake comes from the cross S94-1867 × 'Anand', tracing back to S-100, a salt-tolerant cultivar. Jake was developed and released by the University of Missouri in 2006 due to its high yield potential (50 bu/ac average) and broad nematode resistance (SCN, reniform, and southern root knot). It has purple flowers, tawny pubescence, tan pods and black hilum seed (Shannon *et al.*, 2007; USDA–GRIN). Ozark (Holladay x Delta Pine DP 415) is a cultivar released by the University of Arkansas in 2004 with high yield potential (61.6 bu/ ac). It has purple flowers, grey pubescence, tan pods and buff/imperfect black hilum (Chen *et al.*, 2004; USDA–GRIN). The crosses for the development of this population were made in the field in 2012, in the Agricultural Experiment Station of the University of Arkansas (Fayetteville, AR). The F1 plants were grown and confirmed as true hybrids in 2013. The F_2 generation was advanced in the greenhouse in 2014; 300 seeds were planted and seeds from 269 plants were harvested individually to create the $F_{2:3}$ mapping population. Subsequently, the seed coming from each $F_{2:3}$ family (line) was planted in the greenhouse to be screened for salt tolerance in order to obtain the phenotypic data to perform the QTL analysis.

Phenotyping: Salt tolerance screening

The $F_{2:3}$ families obtained from Jake x Ozark were screened for salt tolerance in the greenhouse. The parents, two salt–tolerant (S–100, Lee 68) and two salt–sensitive (Clark, Dare) checks were included in the experiment. The checks were used to have an additional reference for the initiation of salt response evaluation of the population. This experiment was performed in the Rosen Center at the University of Arkansas, maintaining plants under 14 hours of light /day at 25 ± 2 °C.

Twelve to ten seeds of each genotype were planted per pot (3.5 x 3.5 ") using sandy loam soil (Table 1). After emergence, 8–10 seedlings per pot were maintained for further data collection. Pots were placed in plastic trays (17 3/4" x 25 1/2" x 1") to be irrigated from the bottom (Figure 1). When plants reached stage V1 (first trifoliate leaf expansion) the salt treatment was initiated pouring inside the tray 4 L of 120 mM NaCl solution every day. The solution was left standing for two hours daily and the treatment was applied during two weeks. Right after the 2-h treatment, the solution was immediately removed from the trays and no other type of irrigation was provided. The experiment was a split– plot design with two replications (2 pots per treatment), where the main plot was salt level (NaCl 0, 120 mM) and the sub–plot the genotypes. This experiment was repeated to confirm results obtaining a total of four replications. Plants were fertilized once a week with the application of water–soluble fertilizer Miracle-Gro® All Purpose Plant Food (The Scotts Miracle-Gro Company, Marysville, Ohio) to avoid nutritional deficiencies following the manufacturer´s instructions. The fertilizer was dissolved in both, the saline solution used for the salt treatment application and the irrigation water for the control.

In order to evaluate soybean salt stress response, four variables were measured: leaf scorch score (LSS), percentage of dead plants (PDP), and leaf chlorophyll and leaf chloride content. All these variables were measured at the end of the experiment, when the salt–sensitive checks showed symptoms of necrosis. The LSS is based on a 1–9 scale where: 1= healthy dark green leaves/ no chlorosis, and 9= necrotic leaves (Figure 2). Percentage of dead plants was calculated counting the number of completely necrotic plants observed over the total number of plants contained in each pot (replication). Leaf chlorophyll content was evaluated using a chlorophyll meter Konica Minolta SPAD–502 plus, measuring chlorophyll in three fully developed (mature) leaves in the upper part of the plant. Chloride concentration was analyzed in the Altheimer Laboratory (University of Arkansas. Fayetteville, AR) using 0.1 g of ground oven–dried (70°C for 3 days) leaf tissue, then estimated using a spectrophotometer model ARCOS ICP (Spectro Analytical Instruments Inc., Mahwah, NJ). The dried tissue was extracted using hot water with the addition of 40 mL of 4% nitric acid (HNO_3) for matrix matching (Wheal and Palmer, 2010).

Genotyping: DNA extraction and marker screening

For DNA extraction, young trifoliate leaves were collected separately from each one of the 269 individual plants of the F_2 population (Jake x Ozark) and then stored at -80 °C. The protocol used for the DNA extraction was based on the cetyltrimethyl ammonium bromide (CTAB) buffer method (Doyle and Doyle, 1990). Ground tissue samples were mixed and incubated at 65°C for an hour in an extraction buffer that contained 5M NaCl, 1M Tris HCl pH 8.0, 4% (w/v) CTAB, 0.5 mM EDTA pH 8.0, and β-mercaptoethanol followed by chloroform: isoamyl alcohol (24:1) to remove proteins. DNA was centrifuged and washed in cold 95% ethanol and then 75 % ethanol for DNA precipitation. Samples were left overnight, then the pellet was dissolved in 200 µl deionized sterilized water. The DNA concentration and purity was measured using a

NanoDrop™ spectrophotometer. Values of the absorbance ratios 260/280 and 260/230 greater than 1.8 are suitable for analysis (pure DNA preparations have an 260/280 ≥ 1.8); lower 260/280 values may indicate protein contamination.

For the purpose of genotyping and genetic map construction, DNA samples from the 269 individuals of the population and the parents were screened using 5402 SNPs (Single Nucleotide Polymorphism). The DNA samples were used with a concentration between 50–100 ng/ µl. This analysis was performed using the Illumina Infinium® HD Beadchip Genotyping (SoySNP6k iSelect BeadChip) and the fluorescence of the samples were read by the Illumina iScan (Illumina, San Diego, CA) in Michigan State University (East Lansing, MI). The alleles found in each SNP marker locus were analyzed by Illumina's GenomeStudio software. For each SNP marker, the possible genotypes were recorded as AA (homozygote), BB (homozygote) or AB (heterozygote).

Data analysis

The statistical analysis of the phenotypic data (LSS, PDP, leaf chlorophyll and chloride content) was conducted in SAS 9.4 performing an analysis of variance (ANOVA) and using PROC GLM procedure at 5% of significance in order to determine the overall differences of the evaluated traits among genotypes, batches (runs) and blocks (replications). Given that the population under study is an early generation ($F_{2:3}$), broad sense heritability (H^2) of all the traits was calculated estimating the ratio of total genetic variance to total phenotypic variance using the following equation (Nyquist, 1991): $H^2 = \sigma^2_g / [\sigma^2_g + (\sigma^2_{gy}/y) + (\sigma^2/ry)]$.

Where, σ^2_g is the total genetic variance, σ^2_{gy} is the genotype by year (batch) interaction variance, σ^2 is the error variance, r is the number of replications (blocks) and y is the number of

environments/years (in this experiment this corresponds to batches or runs).

Join Map 4.1 was used for the construction of the linkage groups with a LOD (logarithm of odds) of 2.5. Haldane function was used to perform regression mapping for each one of the linkage groups, which corrects for double crossovers. QTL detection was made using WinQTL Cartographer 2.5 comparing the functions of single marker analysis (SMA) and composite interval mapping (CIM) with a $P<0.05$ threshold. Composite interval mapping was performed using 1000 permutations for threshold calculation with a walk speed of 1 cM. MapChart was used to create the LOD plots for detected QTLs combining the data obtained from Join Map 4.1 and WinQTL Cartographer 2.5.

RESULTS

Phenotypic data

The analysis of variance (ANOVA) performed for all the traits of this study displayed an R^2 of 0.83 for leaf scorch score (LSS) (Table 1) and leaf chlorophyll content (Table 2), and 0.81 for leaf chloride content (Table 3) and percentage of dead plants (PDP) (Table 4). This indicates that the model applied for the experimental design and analysis explained appropriately the data variation of the evaluated traits.

Significant differences were found between treatments (salt vs. control) and among genotypes for all the traits, as expected. There was also a significant variation between batches (two experiment runs) and blocks (reps) for leaf chloride and leaf chlorophyll. Most of the phenotypic variation in all the traits was explained by the genotype, while the effects of batches, blocks, and treatment x genotype interaction were very small compared to the variation accounted for the experimental error. Based on the variance components for LSS, PDP, leaf chlorophyll, and leaf chloride

variation, the calculated broad sense heritability *(H^2)* was 93.07%, 91.63%, 91.72%, and 82.75%, respectively, suggesting that these traits are highly heritable under salt stress and phenotypic selection should be effective.

The Shapiro-Wilk test with a significance of 0.05 was used to test normality (data not shown). None of the phenotypic data sets for the studied traits distributed normally, however, they all show a bimodal distribution suggesting that salt tolerance is a trait controlled by one or a few major genes/QTLs and probably multiple genes with small effects (modifying genes) (Fehr, 1991) (Figure 4 A-D).

The tolerant parent 'Jake' did not show any plant mortality, and exhibited a lower LSS (2.9 vs 6.9), lower leaf chloride (30,127 mg/kg vs. 67,663 mg/kg), and higher leaf chlorophyll content (40 vs. 26) than the susceptible parent 'Ozark'. In order to classify genotypes as tolerant, intermediate, or sensitive, the mean of the tolerant parent and the susceptible parent plus/minus two standard deviations was used as a criterion for all the traits. Therefore, genotypes grouped into the range of ($\text{Mean}_{\text{(tolerant parent)}} - 2\text{SD}$, $\text{Mean}_{\text{(tolerant parent)}} + 2\text{SD}$) were classified as tolerant; genotypes grouped into the range of ($\text{Mean}_{\text{(sensitive parent)}} - 2\text{SD}$, $\text{Mean}_{\text{(sensitive parent)}} + 2\text{SD}$) were classified as sensitive. The rest of the genotypes falling in between susceptible and tolerant, were classified as intermediate (Table 5). Transgressive segregation for leaf chloride content was found in the population after comparing the phenotypes observed in both parents. Similarly, families that displayed higher LSS and lower chlorophyll content than the susceptible parent (Ozark), were observed in the phenotypic distribution of these traits (Figure 4).

The phenotypic data for LSS and PDP in the population closely fit in a 1:2:1 ratio after using chi-square test (data not shown). Most of the families tend to be grouped close to the tolerant parent

mean in all traits, showing a tendency of having a higher proportion of tolerant families than susceptible in the observed bimodal distribution. This can be an indicator of the existence of at least one major gene (dominant allele) and probably the existence of recessive alleles controlling the trait as previously described (Lee *et al.*, 2009; Walker and Rapley, 2008).

Genotypic data

The 269 families of the F_2 mapping population and the parents were genotyped with 5402 SNP markers covering the 20 soybean chromosomes (Table 6). A total of 1156 polymorphic markers were mapped to the 20 chromosomes represented by 23 linkage groups (Figure 5A-C). The linkage map covered 2356.5 cM.

Single marker analysis showed between 35- 43 highly significant markers at $p < 0.0001$ and 3-10 markers with a significance at $p < 0.001$ associated to all the evaluated traits on chromosome 3. On chromosome 19, 12 markers were associated to leaf chloride content and LSS at $p < 0.05$, and 10 markers were significantly associated to leaf chlorophyll content at $p < 0.01$. Another set of 9 markers significant at $p < 0.001$, 30 markers significant at $p < 0.01$, and 10 markers significant at $p < 0.05$ were associated to all the traits in chromosome 6 (Table 7).

In the composite interval mapping analysis, a previously reported major QTL associated with all the traits was identified on chromosome 3 and a new minor QTL associated with leaf chlorophyll content was found in chromosome 19 (Figure 6, 7). The QTL on chromosome 3 is linked to eight SNP loci. Four markers explained between 37- 49 % of the phenotypic variance in all the evaluated traits and the other four markers explained between 3-8 % of this variation (Table 8). Among the identified SNPs with larger effects, the SNP marker Gm03_41020834_T_C was associated with LSS, PDP, and leaf chlorophyll content accounting for 48.8%, 37%, and 46.5%

of the phenotypic variation of these traits, respectively. The marker Gm03_40600088_A_G explained 37-43% of PDP and leaf chlorophyll content phenotypic variation; and the markers Gm03_40270199_T_C and Gm03_40663609_G_A explained approximately 42 % of the leaf chloride content variation. These markers are located within a10 cM region (between 81-90 cM) in chromosome 3, in which the marker Gm03_41020834_T_C, displays the maximum LOD value (98.17) observed among all the linked markers found in this QTL region (Figure 6 A-D). In addition, the markers Gm03_41984976_T_C, Gm03_38415618_T_G, Gm03_37902930_C_T and Gm03_38469714_C_T were associated with leaf chlorophyll content and leaf chloride content explaining between 2.8% - 7.7% of the phenotypic variation of these traits with a LOD value between 6 to 8.74.

The minor QTL detected in chromosome 19, is linked to the SNP markers Gm19_40508288_C_T, and Gm19_42246131_A_G explaining about 4.8% of the leaf chlorophyll phenotypic variation, with an LOD value of 8.6 and 7, respectively. These markers are located flanking the region between 27 – 33.8 cM (Table 8, Figure 7).

DISCUSSION

The genotypic results and the phenotypic distribution observed in the $F_{2:3}$ evaluated population coming from Jake x Ozark, suggests that soybean phenotypes under salt stress may be dominated by one/few major loci and multiple genes with small effects. Therefore, salt tolerance can be defined as a highly heritable trait controlled by multiple genes with major and minor effects, as previously reported in linkage and association mapping studies (Huang, 2013). However, it has been frequently remarked that salt tolerance is dominated by a single gene (usually referring to the gene *Ncl*) due to the repeatedly reported major QTL in chromosome 3 in *Glycine max* and

Glycine soja (Abel, 1969; Lee *et al*.,2004; Hamwieh and Xu, 2008; Lee *et al.*,2009; Hamwieh *et al.,* 2011). Other studies report the effect of multiple minor QTLs on soybean salt tolerance (Luo *et al.*, 2004; Chen *et al.*, 2008). Differences among these research findings can be explained by the use of diverse germplasm or populations coming from different background, the variables/traits measured, and the type and conditions of the salt screening method applied. The most used traits to evaluate soybean salt tolerance are visual scorch in leaves and leaf chloride content. In this study LSS (leaf scorch score), PDP (% dead plants), leaf chlorophyll, and leaf chloride content were measured during the salt screening process of the population finding that all these traits had a high heritability and were very effective for salt tolerance phenotypic and genotypic characterization. Measuring multiple traits increases the power for QTL detection.

In this study, the major QTL mapped to chromosome 3 is linked to significant SNPs with major (R^2 = 37- 49%) and minor (R^2 = 3-8%) effects. The physical position of the SNP markers with the largest effects on the evaluated traits is flanked by the markers Gm03_40270199_T_C and Gm03_41020834_T_C (38256161 bp - 39009305 bp) within a 10 cM region, approximately, based on Williams 82 physical map (Table 8). The QTL region defined by these markers are within a relatively narrow region compared to other previously reported. This QTL is located within the same genome region found by Lee *et al.* (2004) in the soybean linkage group N (chr.3). They used a $F_{2:5}$ population from the cross S-100 (tolerant) x Tokyo 9 (sensitive) which was subjected to NaCl stress under field and greenhouse conditions. This major QTL was discovered close to the SSR markers Sat_091 and Satt 237 within a 3.6 cM interval, accounting for 60% of the LSS phenotypic variation (in greenhouse) with an LOD value of 7.2. The very approximate region of this QTL is near the region 38284805 bp – 40972200 bp (soybase.org). Similarly, the QTL identified on chromosome 3 in Jake x Ozark population is near the genomic

region reported by Hamwieh and Xu (2008) (R^2 = 68.7%, LOD= 33- 43, position = 36.57 – 39.42 Mbp;); Hamwieh *et al.* (2011) (R^2 = 44-47%, LOD=12, position = 38.30 – 39.87 Mbp); and Ha *et al.,* 2013 (R^2 = 56%, LOD=18.8, position = 37.32 – 39.87 Mbp), which correspond to the same region of the *Ncl* locus, involved in chloride exclusion and inclusion. Likewise, the novel transporter genes *GmCHX1* (338 Kb) and *GmSALT3* (17.5 Kb), which encode proteins from the cation/ H^+ exchange family and regulate Na^+/K^+ ratio, are located in the same region on chromosome 3 (Guan *et al.*, 2014; Qi *et al.*, 2104). This suggests that the salt tolerance gene *Ncl* may be involved not just in Cl^- regulation, but also, in the transport and accumulation of Na^+ and K^+ as found by Do *et al* (2016).

Among the markers linked to the major QTL mapped to chromosome 3 in the present study, Gm03_40600088_A_G (38.6 Mbp) and Gm03_41020834_T_C (39.0 Mbp) could be of special value for marker assisted selection considering the relatively narrow distance among them and the fact that they displayed the largest effect on the evaluated traits with the highest LOD values. For example, 37 % of the PDP variation was explained by these markers with an LOD value over 97. For this trait, the additive effect of the tolerant parent (Jake) decreased the percentage of dead plants by approximately 42%. Overall, the favorable alleles of the evaluated markers for all the evaluated traits come from the tolerant parent (Table 8).

Most likely, the favorable allele source for salt tolerance in this population comes from the soybean cultivar S-100 considering the fact that Jake traces back to the soybean cultivar S-100 (Figure 3), and the major QTL found is close to the region reported by Lee *et al.* (2004).

There is not a previous report of a QTL associated with salt tolerance on chromosome 19 (linkage group L). The new minor QTL on chromosome 19 identified in this study, associated

with leaf chlorophyll content, is linked to markers that have close physical proximity (40701199 bp – 42447208 bp, equivalent to 6.8 cM in the genetic map) suggesting that they can be useful for marker-assisted selection.

Patil *et al* (2016) developed and validated SNP markers using SOYSNP50K in a set of diverse soybean lines and a F8 population from PI483463 x Hutcheson measuring leaf scorch, leaf chlorophyll content and leaf Na^+ accumulation. They found a major locus on chromosome 3, corresponding to the previous characterized *GmCHX1* gene. The large number of functional SNP markers found in this study and the use of other reported SNP markers associated with salt tolerance, constitute a valuable tool in the application of high throughput genotyping technologies offering several advantages over other type of molecular markers.

Understanding the role of mapped major and minor QTLs will bring more chances of developing new cultivars with improved salt tolerance. In addition, the selection of diverse and informative traits related to salt tolerance in soybean is very important to perform an accurate phenotyping in the aim of mapping QTLs for this abiotic stress. In this study all the evaluated traits (leaf chlorophyll content, leaf chloride content, LSS and PDP) were very good salt-response descriptors.

CONCLUSIONS

The use of soybean germplasm variation to identify genes that increase soybean production under saline conditions is a very useful tool for meeting food security challenges in areas where soil salinization is becoming moderate to severe. Numerous and consistent research results have reported a major QTL on the same region in chromosome 3 (linkage group N) using diverse genetic sources; other studies report the existence of minor QTLs in several linkage groups. In

this study two QTLs were identified. A major QTL was found in chromosome 3, validating the major salt tolerance QTL previously reported. This QTL is linked to four SNP markers with large effect. The SNP Gm03_41020834_T_C is associated with LSS, PDP, and leaf chlorophyll content explaining 37 to 48.9 % of the phenotypic variance; Gm03_40600088_A_G is associated with PDP and leaf chlorophyll accounting for 37% and 43.5% of the variation, respectively, and the markers Gm03_40270199_T_C and Gm03_40663609_G_A explained 41% to 43% of the leaf chloride variation. Additionally, a new minor QTL was identified on chromosome 19 (linkage group L) associated with leaf chlorophyll content being linked to the SNP markers Gm19_40508288_C_T and Gm19_42246131_A_G, which explained about 5% of the phenotypic variation of the trait. The markers linked to the QTLs found in this study can be used as an additional tool for marker assisted selection in the process of targeting salt tolerance traits into new soybean germplasm to facilitate the breeding of salt-tolerant cultivars.

REFERENCES

Abel. G.H. 1969. Inheritance of the Capacity for Chloride Inclusion and Chloride Exclusion by Soybeans. Crop Sci. 9: 697–698.

Chen, H., S. Cui, S. Fu, J. Gai and D. Yu. 2008. Identification of quantitative trait loci associated with salt tolerance during seedling growth in soybean (*Glycine max* L.). Aust. J. Agric.Res. 59: 1086–1091.

Do, T. D. *et al.* 2016. *Ncl* Synchronously Regulates Na^+, K^+, and Cl^- in Soybean and Greatly Increases the Grain Yield in Saline Field Conditions. *Sci. Rep.* **6**, 19147. 10 p.

Fehr, W.R. 1991. Principles of cultivar development. Theory and technique. Vol. 1. 536 p.

Guan, R., J. Chen, J. Jiang, G. Liu, Y. Liu, L. Tian, L. Yu, R. Chang, and L. Qiu. 2014 (b). Mapping and validation of a dominant salt tolerance gene in the cultivated soybean (*Glycine max)* variety Tiefeng 8. The Crop Journal 2: 358–365.

Katerji, N., J.W. Hoorn, A. Hamdy, and M. Mastrorilli. 2003. Salinity effect on crop development and yield, analysis of salt tolerance according to several classification methods. Agric.Water Manage. 62: 37–66.

Ha, B. K., T. Vuong, V. Velusamy, H. Nguyen, J.G Shannon, J. Lee. 2013. Genetic mapping of quantitative trait loci conditioning salt tolerance in wild soybean (*Glycine soja*) PI 483463. Euphytica 193: 79–88.

Hamwieh, A., D. Xu. 2008. Conserved salt tolerance quantitative trait locus (QTL) in wild and cultivated soybeans. Breed. Sci. 58: 355-359.

Hamwieh, A., D. D. Tuyen, H. Cong, E. R. Benitez, R. Takahashi, and D. H. Xu. 2011. Identification and validation of a major QTL for salt tolerance in soybean. Euphytica 179: 451–459.

Huang, L. 2013. Genome-wide association mapping identifies QTLs and candidate genes for salt tolerance in soybean. M.S thesis. University of Arkansas. 129 p.

Ledesma, F., C. Lopez, D. Ortiz, P. Chen, K. L. Korth, T. Ishibashi, A. Zeng, M. Orazaly, and L. Florez-Palacios. 2016. A simple greenhouse method for screening salt tolerance in soybean. Crop Sci. 56: 585-594.

Lee, G.J., R.H. Boerma, R.M. Villagarcia, X. Zhu, T.E. Carter, Jr., Z. Li, and M.O. Gibbs. 2004. A major QTL conditioning salt tolerance in S-100 and descendent cultivars. Theor. Appl. Genet. 109:1610-1619.

Lee, J., G. Shannon, T. Vuong, and H.T. Nguyen. 2009.Inheritance of Salt Tolerance in Wild Soybean (Glycine soja Sieb. and Zucc.) Accession PI483463. J. Heredity 100: 798–801.

Luo, Q.Y., B.J. Yu, Y.L. Liu, Y.M. Zhang, Y.L. Xue and Y. Zhang. 2004. The mixed inheritance

analysis of salt tolerance in cultivars of *Glycine max*. Soybean Sci. 23: 239–244.

Nyquist, W.E. 1991. Estimation of heritability and prediction of selection response in plant populations. Crit. Rev. Plant Sci. 10:235-322.

Patil, G., T. Do, T. Vuong, B. Valliyodan, J. Lee, J. Chaudhary, J. Shannon, and H. Nguyen. 2016. Genomic-assisted haplotype analysis and the development of highthroughput SNP markers for salinity tolerance in soybean. Sci. Rep. 6, 19199. 13 p.

Qi, X., M. Li, M. Xie, X. Liu, M. Ni, G. Shao, C. Song, A. Yim, *et al*., 2014. Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. Nat. Commun. 5:4340.

Walker J.M and R. Rapley. 2008. Molecular Biomethods Handbook. Humana Press. Second edition. 1124 p.

Table 1. Analysis of variance for leaf scorch score (LSS) of $F_{2:3}$ population derived from Jake x Ozark evaluated in greenhouse under 120 mM NaCl treatment

Source	D.F[Ŧ]	Mean Square	Variance estimate	% Variance[¥]	p-value	R^2
Model	539	3.597			<0.0001	0.831
Genotype	268	6.431	1.4113	64.758	<0.0001	
Batch	1	0.204	0.0034	0.156	0.5972	
Block(batch)	2	2.019	0.0047	0.216	0.0645	
Genotype*Batch	268	0.786	0.0268	1.230	0.2478	
Error	536	0.733	0.7331	33.640		

[Ŧ] Degrees of freedom. Analysis of variance using PROC GLM in SAS 9.4 for 269 $F_{2:3}$ families
[¥] Percent of LSS variation explained by each source in the model, using PROC VARCOMP in SAS 9.4

Table 2. Analysis of variance for leaf chlorophyll content of $F_{2:3}$ population derived from Jake x Ozark evaluated in greenhouse under 120 mM NaCl treatment

Source	D.F[Ŧ]	Mean Square	Variance estimate	% Variance[¥]	p-value	R^2
Model	539	73.34			<0.0001	0.830
Genotype	268	121.72	26.18	57.003	<0.0001	
Batch	1	1723.91	2.61	5.682	<0.0001	
Block(batch)	2	317.55	1.12	2.438	<0.0001	
Genotype*Batch	268	16.98	0.96	2.090	0.1246	
Error	536	15.06	15.06	32.787		

[Ŧ] Degrees of freedom. Analysis of variance using PROC GLM in SAS 9.4 for 269 $F_{2:3}$ families
[¥] Percent of leaf chlorophyll content variation explained by each source in the model, using PROC VARCOMP in SAS 9.4

Table 3. Analysis of variance for leaf chloride content of $F_{2:3}$ population derived from Jake x Ozark evaluated in greenhouse under 120 mM NaCl treatment

Source	D.F[Ŧ]	Mean Square	Variance estimate	% Variance[¥]	p-value	R^2
Model	539	805765605.8			<0.0001	0.809
Genotype	268	1219191342.5	252237453.0	47.870	<0.0001	
Batch	1	14646030021.0	6803068.1	1.291	<0.0001	
Block(batch)	2	18286810501.0	67270777.0	12.767	<0.0001	
Genotype*Batch	268	210241532.5	9635065.6	1.829	0.1776	
Error	536	190971401.4	190971401.0	36.243		

[Ŧ] Degrees of freedom. Analysis of variance using PROC GLM in SAS 9.4 for 269 $F_{2:3}$ families
[¥] Percent of leaf chloride content variation explained by each source in the model, using PROC VARCOMP in SAS 9.4

Table 4. Analysis of variance for percentage of dead plants (PDP) of $F_{2:3}$ population derived from Jake x Ozark evaluated in greenhouse under 120 mM NaCl treatment

Source	D.F[Ŧ]	Mean Square	Variance estimate	% Variance[¥]	p-value	R^2
Model	539	2884.76			<0.0001	0.814
Genotype	268	5227.24	1165.30	62.091	<0.0001	
Batch	1	1423.70	2.02	0.108	0.1428	
Block(batch)	2	431.24	0.85	0.046	0.5212	
Genotype*Batch	268	566.05	47.52	2.532	0.9252	
Error	536	661.08	661.08	35.224		

[Ŧ] Degrees of freedom. Analysis of variance using PROC GLM in SAS 9.4 for 269 $F_{2:3}$ families
[¥] Percent of dead plants variation explained by each source in the model, using PROC VARCOMP in SAS 9.4

Table 5. Descriptive statistics for the traits evaluated in 269 genotypes from the $F_{2:3}$ population (Ozark x Jake) under greenhouse conditions.

Trait	P1	P2	Min	Max	Mean	SD[Ŧ]	2SD	Salt stress response[¥]	No. genotypes	Range
PDP	0	100	0	100	36.8	12.54	25.08	Tolerant	60	0
								Intermediate	162	-
								Sensitive	47	100
LSS	2.9	6.9	2.75	7.75	4.8	0.43	0.86	Tolerant	56	2.0 - 3.8
								Intermediate	144	3.9 - 6.0
								Sensitive	69	6.1 - 8.0
Chlorophyll	39.7	26	16.5	39.6	30.7	1.98	3.96	Tolerant	48	36 - 44
								Intermediate	126	31-35
								Sensitive	95	22 - 30
Chloride	30,127	67,663	17,583	91,082	54,496	7,025	14,050	Tolerant	89	16,077 – 44,176
								Intermediate	55	44,177 – 53,612
								Sensitive	125	53,613 – 81,713

Ŧ Standard deviation

¥ Salt response was calculated using the means of the tolerant parent (P1: Jake) and the susceptible parent (P2: Ozark) ± two standard deviations. Genotypes with a score < 3.8 (2.9 – 0.86, 2.9 + 0.86) were classified as tolerant; genotypes with a score > 6.1 (6.9 – 0.86, 6.9 + 0.86) were classified as sensitive; and genotypes with a score between 3.9 - 6.0 were classified as intermediate. Same criteria were used for the other traits.

Table 6. Single Nucleotide Polymorphic (SNP) markers used in the screening of the $F_{2:3}$ mapping population (Jake x Ozark)

Chr.[₮]	LG[¥]	Length (cM)	No. polymorphic SNPs	Avg.[₲] distance (cM)
1	D1a	57.78	55	1.05
2a	D1b	58.55	53	1.10
2b	D1b	51.56	35	1.47
3	N	133.98	68	1.97
4	C1	61.00	32	1.90
5	A1	155.7	39	3.99
6	C2	161.63	116	1.39
7	M	84.40	27	3.13
8	A2	93.00	63	1.50
9	K	157.04	34	5.06
10	O	96.06	22	4.36
11	B1	154.30	48	3.21
12	H	149.19	73	2.04
13a	F	113.28	81	1.4
13b	F	15.198	20	0.75
14	B2	135.69	65	2.08
15	E	97.85	33	2.96
16	J	102.38	37	2.77
17	D2	134.22	57	2.35
18a	G	97.51	99	0.98
18b	G	16.02	21	0.76
19	L	83.33	29	2.87
20	I	146.83	49	2.99
Average		102.45	50.26	2.26
Total		2356.49	1156	

[₮] Chromosome
[¥] Linkage group
[₲] Average distance between SNP loci in cM

Table 7. Single Marker Analysis summary for LSS, percentage of dead plants, leaf chlorophyll content, and leaf chloride concentration in 269 $F_{2:3}$ derived families from the cross Jake x Ozark evaluated in the greenhouse for salt screening.

Chr.	Marker No.	Name	Position (cM)	Physical position (bp)	p-value	R^2	Trait
3	snp757	Gm03_6459920_A_G	22.19	6459920	<0.05	0.02	ALL
	snp758	Gm03_6631189_A_G	22.38	6631189	<0.05	0.02	
	snp593	Gm03_30809885_T_C	31.91	30809885	<0.001	0.05	
	snp595	Gm03_30955790_A_G	31.91	30955790	<0.001	0.05	
	snp597	Gm03_31711487_A_G	32.09	31711487	<0.001	0.05	
	snp645	Gm03_37902930_C_T	62.12	37902930	<0.0001	0.31	
	snp646	Gm03_37963252_A_G	63.52	37963252	<0.0001	0.35	
	snp647	Gm03_38069022_A_G	64.09	38069022	<0.0001	0.36	
	snp648	Gm03_38121627_T_C	64.84	38121627	<0.0001	0.39	
	snp649	Gm03_38173815_A_C	65.97	38173815	<0.0001	0.42	
	snp653	Gm03_38415618_T_G	66.91	38415618	<0.0001	0.44	
	snp654	Gm03_38469714_C_T	68.24	38469714	<0.0001	0.44	
	snp661	Gm03_39351009_C_T	75.13	39351009	<0.0001	0.53	
	snp667	Gm03_39796778_T_G	79.87	39796778	<0.0001	0.61	
	snp669	Gm03_39843152_T_C	79.87	39843152	<0.0001	0.61	
	snp670	Gm03_39945298_T_C	79.87	39945298	<0.0001	0.61	
	snp666	Gm03_39998708_A_G	79.87	39998708	<0.0001	0.61	
	snp671	Gm03_40052612_T_C	80.05	40052612	<0.0001	0.62	
	snp674	Gm03_40197155_A_C	80.99	40197155	<0.0001	0.65	
	snp680	Gm03_40270199_T_C	83.47	40270199	<0.0001	0.61	
	snp678	Gm03_40417269_A_G	86.34	40417269	<0.0001	0.69	
	snp673	Gm03_40600088_A_G	87.66	40600088	<0.0001	0.66	
	snp675	Gm03_40613405_T_C	89.17	40613405	<0.0001	0.65	
	snp677	Gm03_40663609_G_A	89.36	40663609	<0.0001	0.66	
	snp676	Gm03_41020834_T_C	89.36	41020834	<0.0001	0.66	
	snp686	Gm03_41605831_A_C	95.73	41605831	<0.0001	0.54	
	snp689	Gm03_41984976_T_C	98.46	41984976	<0.0001	0.48	
	snp688	Gm03_42148379_T_G	100.95	42148379	<0.0001	0.55	
6	snp1348	Gm06_16133328_G_A	97.58	16133328	<0.01	0.03	ALL
	snp1349	Gm06_16207402_T_C	97.58	16207402	<0.01	0.03	
	snp1358	Gm06_16853739_A_C	103.91	16853739	<0.0001	0.05	
	snp1357	Gm06_16923935_T_C	103.91	16923935	<0.0001	0.05	
	snp1359	Gm06_17188046_A_G	104.48	17188046	<0.001	0.05	

Table 7. Single Marker Analysis summary for LSS, percentage of dead plants, leaf chlorophyll content, and leaf chloride concentration in 269 $F_{2:3}$ derived families from the cross Jake x Ozark evaluated in the greenhouse for salt screening (Cont.)

Chr.	Marker No.	Name	Position (cM)	Physical position (bp)	p-value	R^2	Trait
19	snp5006	Gm19_39686084_T_C	24.96	39686084	<0.05	0.02	LSS,
	snp5016	Gm19_39807274_C_A	29.83	39807274	<0.05	0.03	Chloride,
	snp5018	Gm19_40508288_C_T	30.58	40508288	<0.05	0.02	
	snp5019	Gm19_41192542_G_T	30.77	41192542	<0.05	0.02	
	snp5020	Gm19_41343324_G_A	30.96	41343324	<0.05	0.02	
	snp5021	Gm19_41381563_A_C	31.14	41381563	<0.05	0.02	
	snp5024	Gm19_41420857_A_G	31.33	41420857	<0.05	0.02	
19	snp5006	Gm19_39686084_T_C	24.96	39686084	<0.01	0.02	Chlorophyll
	snp5030	Gm19_41638742_G_T	33.81	41638742	<0.01	0.02	
	snp5031	Gm19_42089062_C_T	33.81	42089062	<0.01	0.02	
	snp5032	Gm19_42143190_T_C	33.81	42143190	<0.01	0.02	

Table 8. Composite Interval Mapping for LSS, percentage of dead plants (PDP), leaf chlorophyll content (Chlorop.), and leaf chloride concentration in 269 $F_{2:3}$ derived families from the cross Jake x Ozark evaluated in the greenhouse for salt screening.

Chr.	Name	Traits	Position (cM)	LOD ₮ Score	R^2	P1	P2	Diff.	Add.¥	Dom. ₠
3	Gm03_41020834_T_C (39009305 bp)*	LSS	83.5	97.12	0.49	3.6	6.3	-2.7	-1.5	-0.6
		PDP		98.17	0.37	6.4	80.4	-74.1	-42.2	-24.4
		Chlorop.		94.63	0.47	35.8	24.3	11.4	6.3	2.7
	Gm03_40600088_A_G (38585840 bp)	PDP	90.4	97.47	0.37	7.4	87.3	-79.9	-42.3	-24.6
		Chlorop.		89.62	0.43	35.5	23.4	12.1	6.3	2.8
	Gm03_40270199_T_C (38256161 bp)	Chloride	81	65.41	0.41	38,580	74,257	-35677	-18,239	-7,102
	Gm03_40663609_G_A (38651529 bp)	Chloride	86.3	70.9	0.43	38,661	75,680	-37019	-18,823	-7,299
	Gm03_41984976_T_C (39973490 bp)	Chlorop.	102	8.74	0.08	35.4	24.5	11.0	0.5	2.3
	Gm03_38469714_C_T (36449357 bp)	Chloride	71.3	6.42	0.06	42,432	72,686	-30254	-1,310	-6,975
	Gm03_38415618_T_G (36395079 bp)	Chlorop.	66.9	7.01	0.03	35.0	24.6	10.3	0.2	1.8
	Gm03_37902930_C_T (35876483 bp)	Chloride	62.2	6.01	0.03	45,280	70,565	-25285	-20.4	-6,402
19	Gm19_40508288_C_T (40701199 bp)	Chlorop.	27	8.64	0.05	32.3	29.3	3.0	1.7	-0.2
	Gm19_42246131_A_G (42447208bp)	Chlorop.	33.8	7.034	0.05	31.8	29.3	2.5	1.7	-0.2

₮ LOD = logarithm of the odds.
¥ Additive effect
₠ Dominance effect
*Equivalent position in Williams82, assembly

A.  B. 

Figure 1. A. View of the phenotyping screening in the greenhouse and B. Plastic tray with pots submerged in NaCl solution 120 mM (Photo by author).

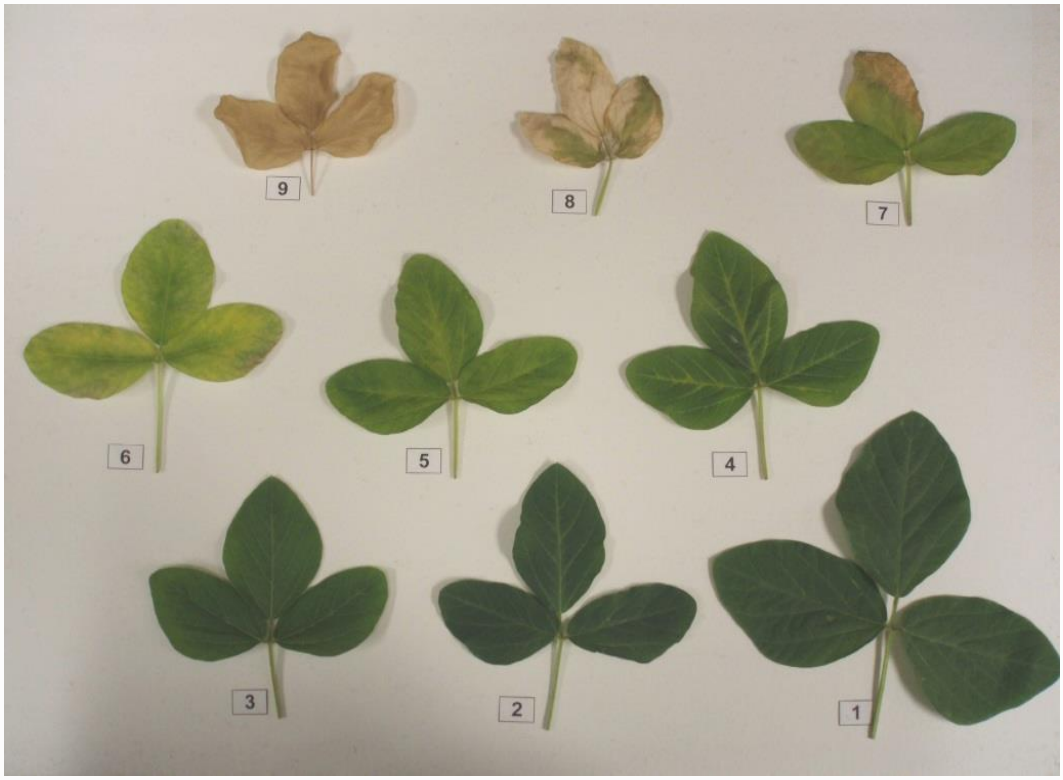

Figure 2. Leaf scorch score (LSS) system for evaluating soybean for salt tolerance (1=no chlorosis to 9=necrosis) (Ledesma *et al.*, 2016).

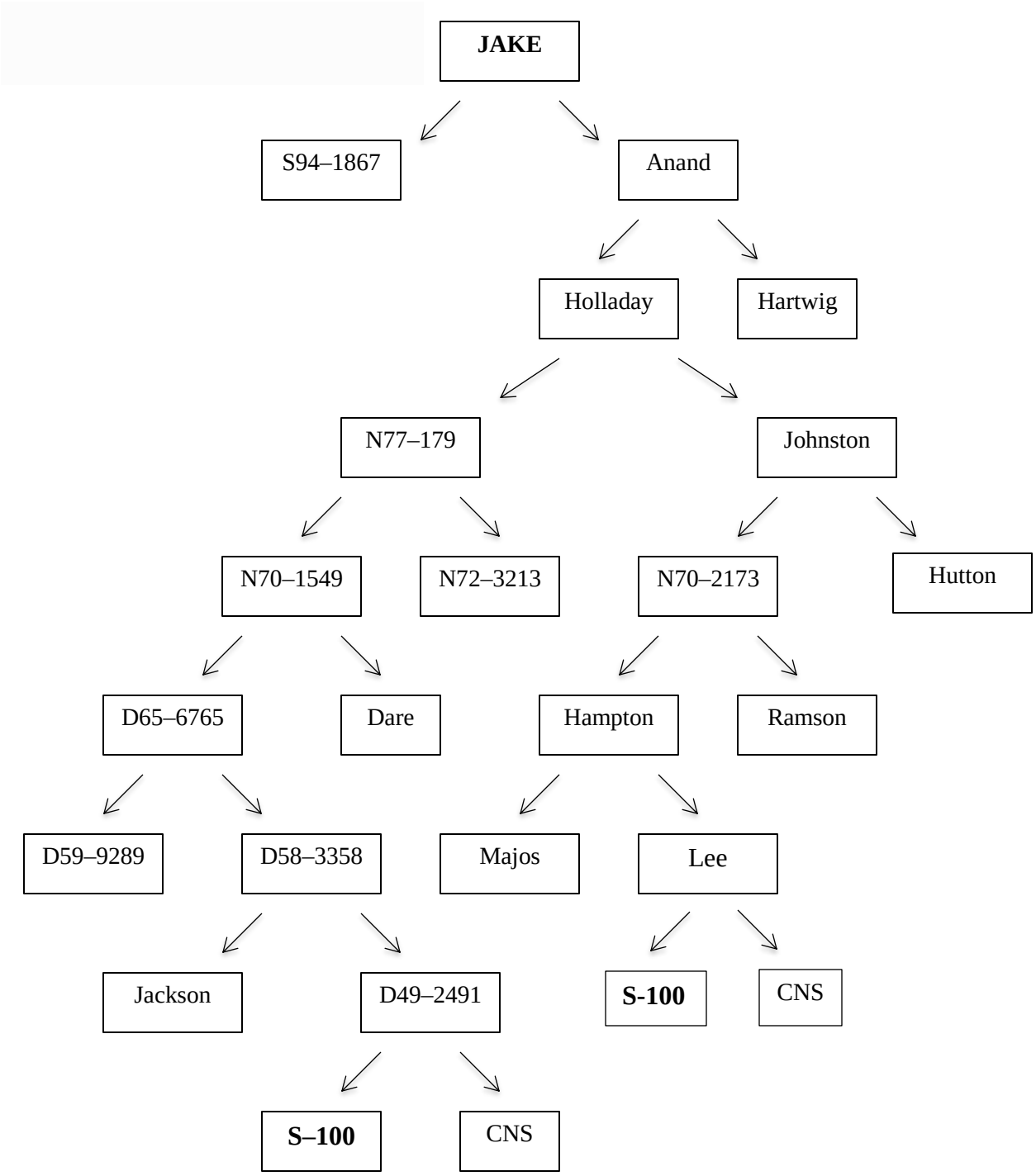

Figure 3. Pedigree of the soybean variety Jake

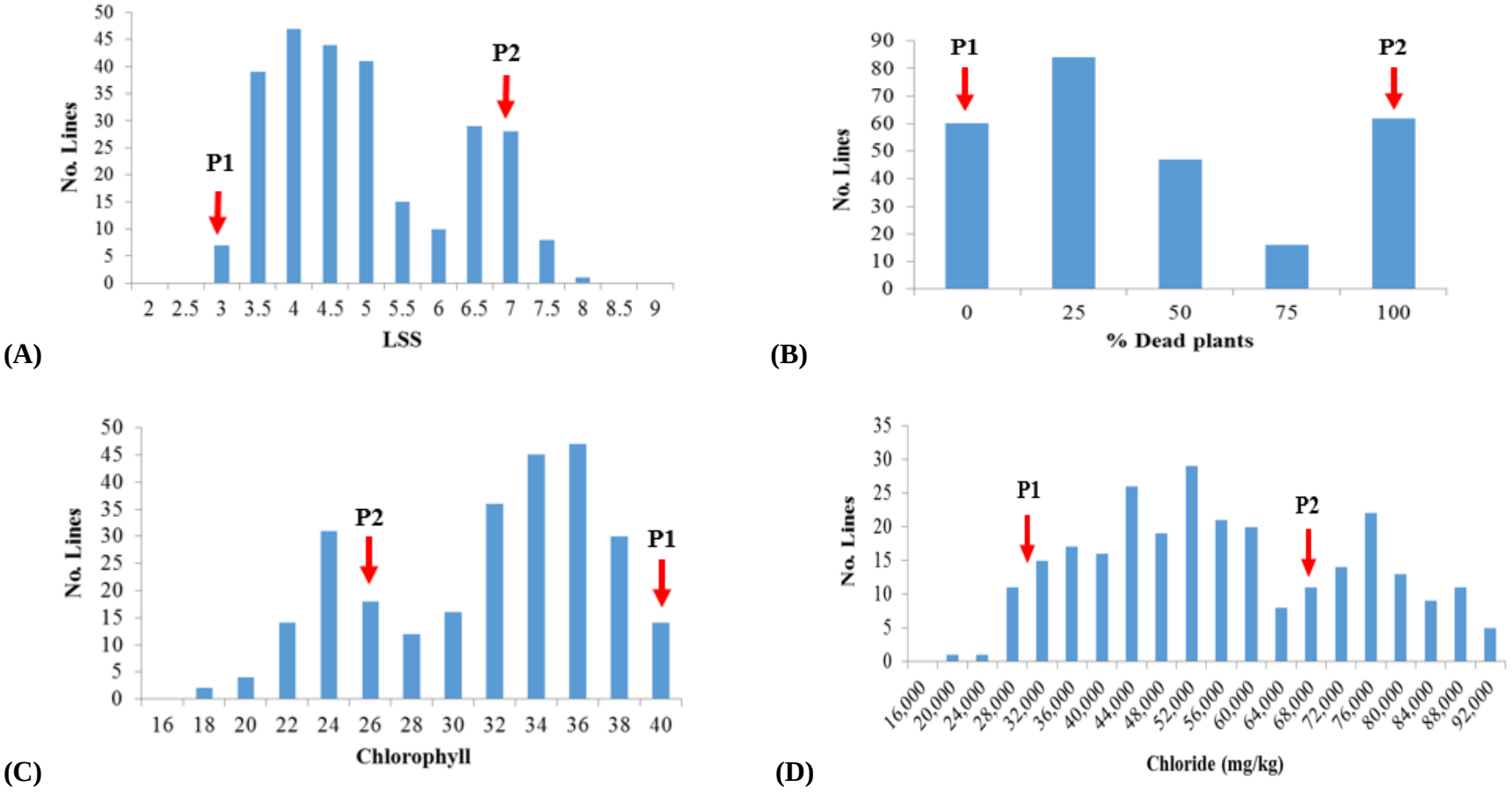

Figure 4. Frequency distribution of the phenotypic data in the 269 families from the $F_{2:3}$ population evaluated under greenhouse conditions for the traits: (A) LSS (leaf scorch score), (B) PDP (percentage of dead plants), (C) Leaf chlorophyll content, and (D) Leaf chloride content. P1 stands for the tolerant parent (Jake) and P2 is the sensitive parent (Ozark).

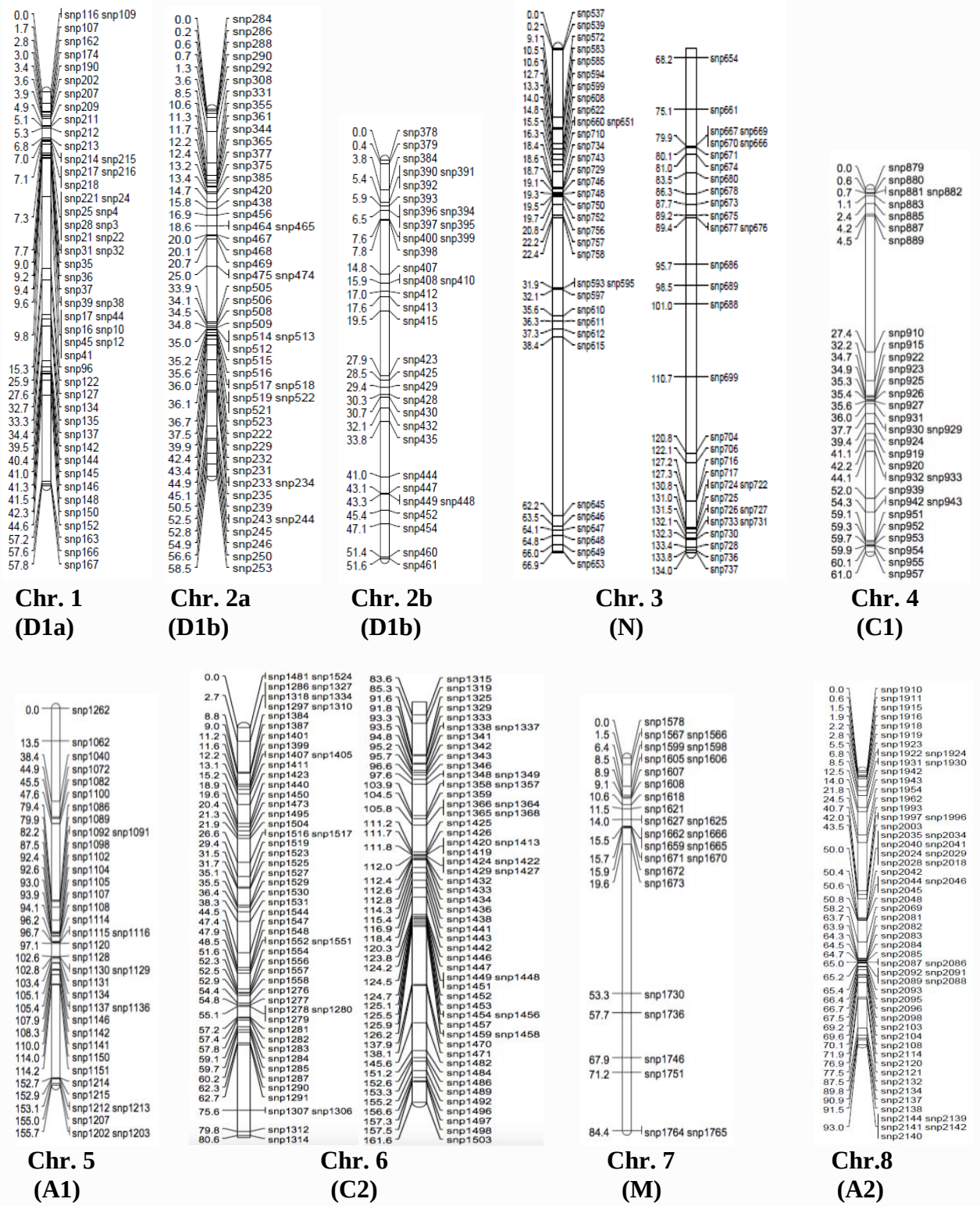

Figure 5A. Genetic map constructed for chromosomes 1–8 using the $F_{2:3}$ mapping population derived from Jake and Ozark. A total of 1156 SNP polymorphic markers were mapped to the soybean genome (20 chromosomes).

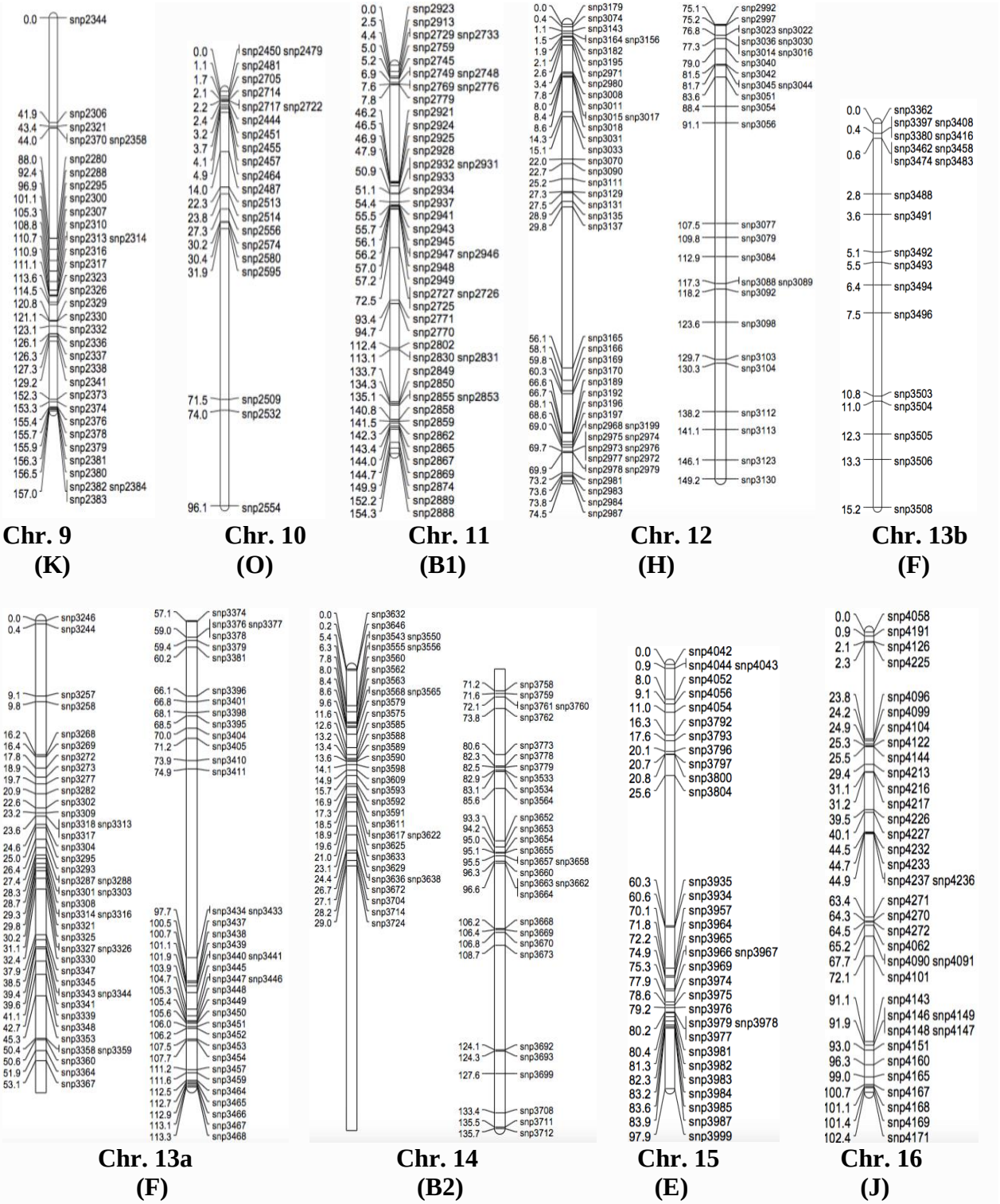

Figure 5B. Genetic map constructed for chromosomes 9–16 using the $F_{2:3}$ mapping population derived from Jake and Ozark. A total of 1156 SNP polymorphic markers were mapped to the soybean genome (20 chromosomes).

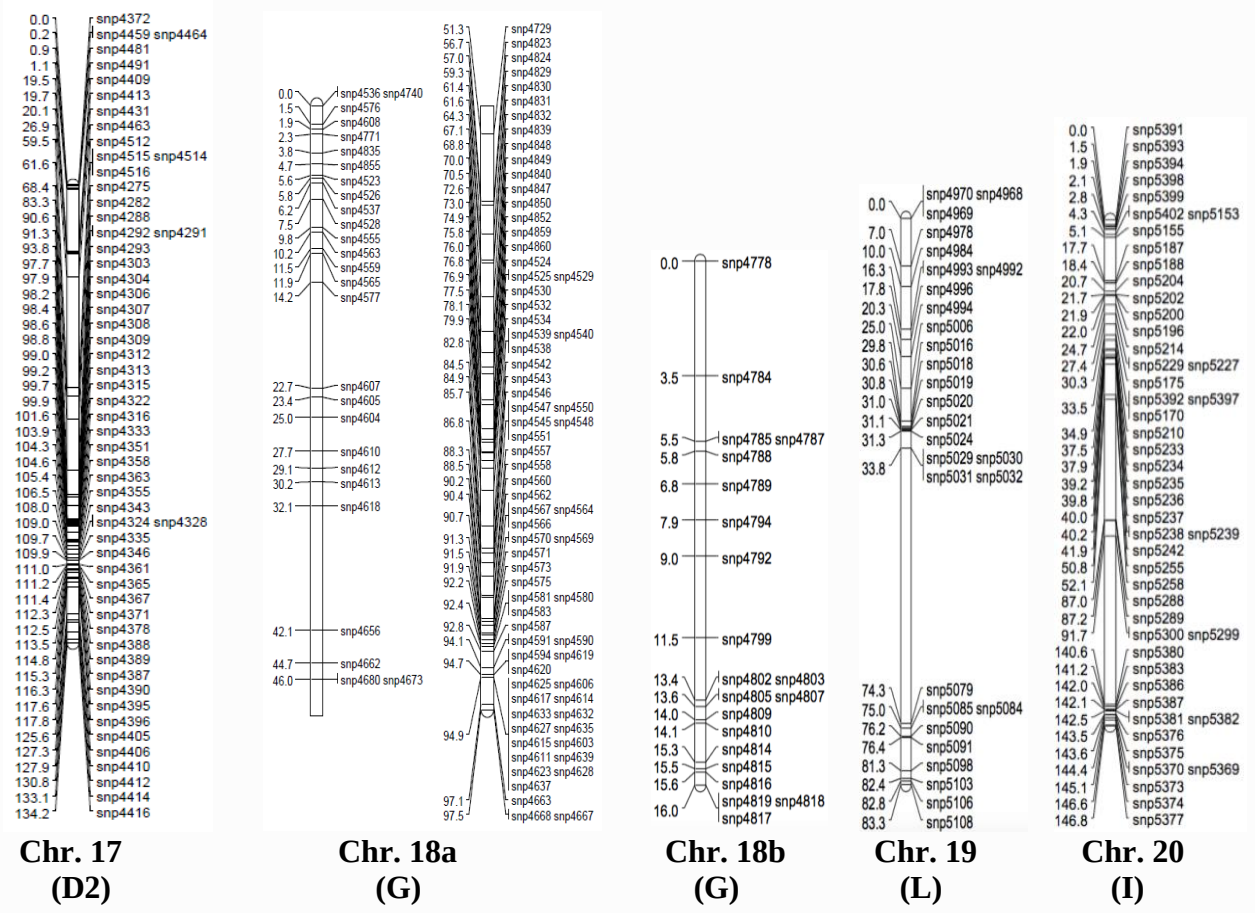

Figure 5C. Genetic map constructed for chromosomes 17–20 using the $F_{2:3}$ mapping population derived from Jake and Ozark. A total of 1156 SNP polymorphic markers were mapped to the soybean genome (20 chromosomes).

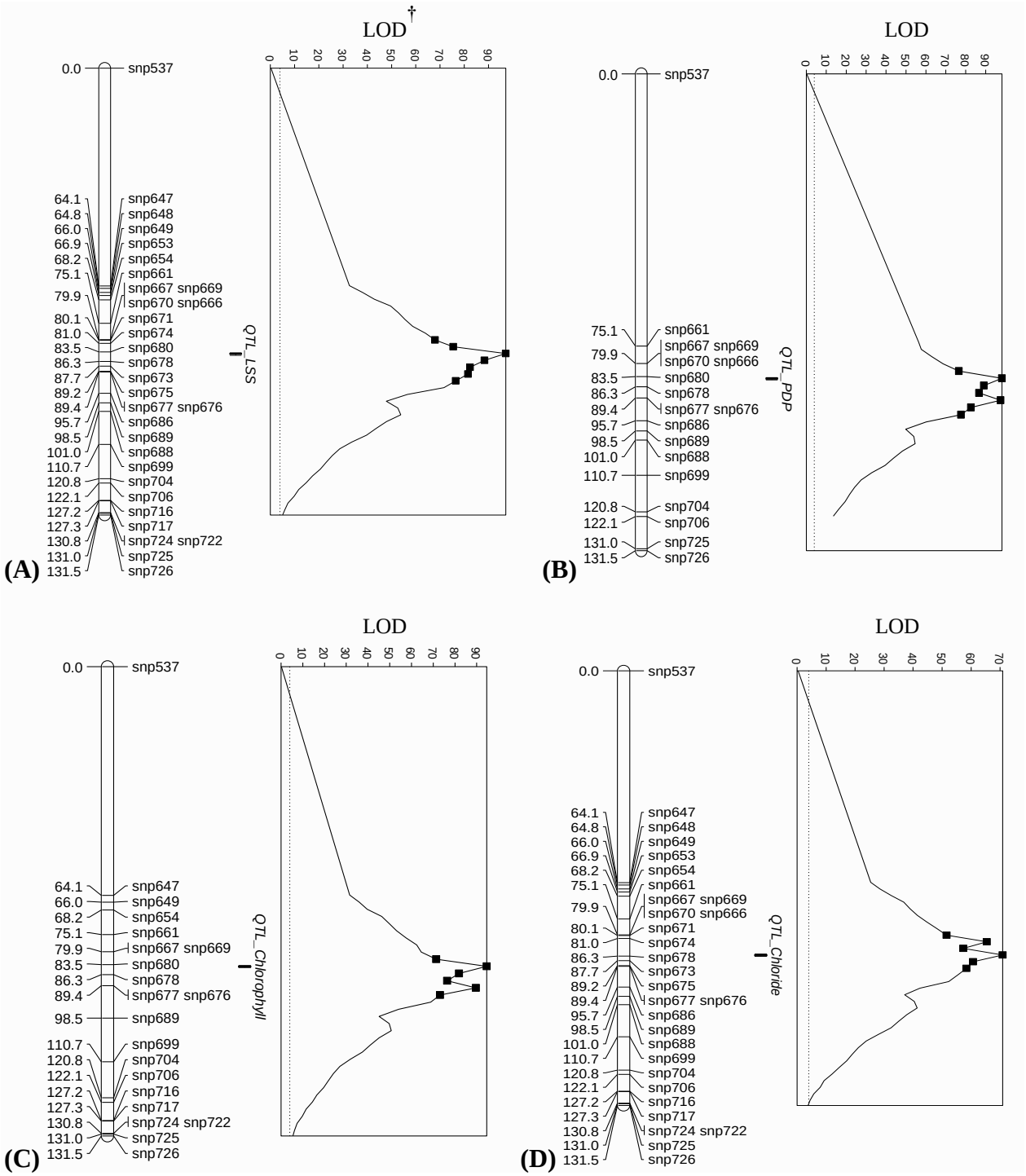

Figure 6. Composite interval mapping using SNP markers on chromosome 3 for QTL detection in 269 $F_{2:3}$ derived families from the cross Jake x Ozark for the traits: (A) LSS (leaf scorch score), (B) PDP (percentage of dead plants), (C) Leaf chlorophyll content, and (D) Leaf chloride content.

† LOD = logarithm of the odds.

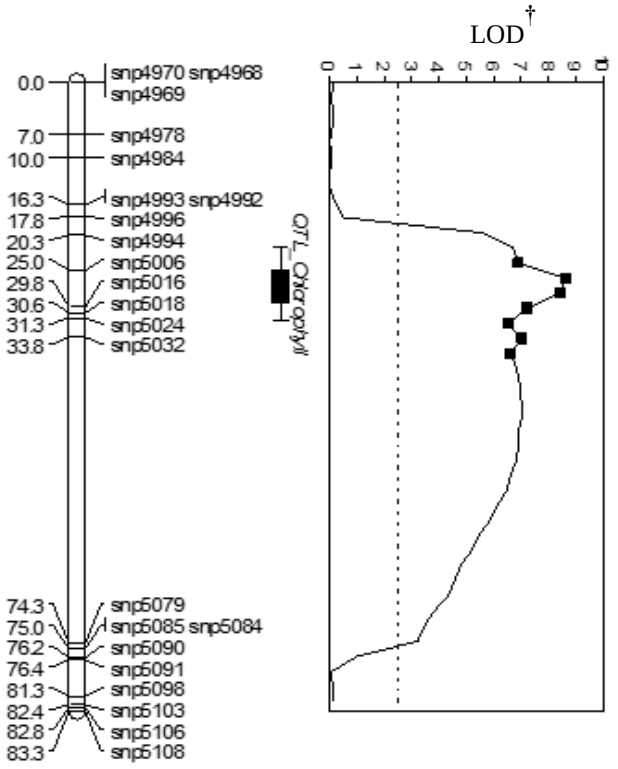

Figure 7. Composite interval mapping using SNP markers on chromosome 19 for QTL detection in 269 $F_{2:3}$ derived families from the cross Jake x Ozark for leaf chlorophyll content.
† LOD = logarithm of the odds.

CHAPTER III: Study of soybean physiological response under salt stress during early growth stages

ABSTRACT

Salt stress cause detrimental effects in soybean growth reducing grain yield. High concentrations of Na^+ and Cl^- cause a negative effect in soybean growth and a K^+/Na^+ imbalance cause multiple metabolic problems when there are high concentrations of cytosolic Na^+. Salt tolerance is usually associated with the regulation of ion transport; salt tolerance in soybean has been defined by the capacity of efficiently exclude toxic ion concentrations (mainly Cl^-) from leaves/shoots. The main objective of this study was to evaluate the Cl^-, Na^+, and K^+ accumulation pattern in two salt tolerant/excluder (Jake, Lee) and two salt sensitive/includer varieties (Ozark, Desha) during early growth stage, and their differential response to NaCl and KCl stress. The experiment was performed during 30 days, subjecting soybean varieties to NaCl and KCl treatment at 80mM and 120 mM from stage V1 to V6. Ion leaf concentration, leaf scorch score (LSS), leaf chlorophyll content, leaf area, and leaf dry weight were measured every three days. Results showed that salinity significantly reduced leaf chlorophyll content, leaf expansion, and leaf dry matter accumulation in all the varieties as salt concentration increased over time. The most adverse effects on both, tolerant and susceptible varieties, was caused by KCl in comparison with NaCl stress. Under KCl treatment, the tolerance capacity of the excluders was as inhibited as the includers causing early death, while under NaCl stress, these tolerant varieties were able to accumulate up to 2.3 less Cl^- and 3.8 times less and Na^+ in leaves than the includers, staying alive by day 30 with a slight level of chlorosis. Plant death occurred when plants reached a concentration over 80,000 mg/kg and 18,000 mg/kg of Cl^- and Na^+, respectively, under 120 mM NaCl. Under 120 mM KCl, plants died when leaf Cl^- content reached 120,000 mg/kg and leaf K^+

content was over 100,000 mg/kg. Soybean ion homeostasis seemed to be more efficient under NaCl than KCl. Future studies are needed in order to elucidate ion contributions to soybean osmotic adjustment, and if either Na^+, K^+ or Cl^- is more efficient performing this function.

INTRODUCTION

Salinity is a very common environmental stress for crop production around the world, mainly in arid and coastal areas. Agricultural practices like the use of saline irrigation water, bad drainage and excessive fertilizer application constitute some of the main factors causing the increase of soil salinity (Patel *et al.,* 2010). In soybean and many other glycophytes, the level of salinity tolerance highly depends on the root system efficiency to limit the transport of toxic ions as Na^+ and Cl^- to the shoots. An excess in the NaCl transport rate to the plant shoots causes cellular dehydration or death when the capacity of leaf cell vacuoles storage is surpassed (Shereen *et al*., 2001). High concentrations of Na^+ and Cl^- cause a negative effect in soybean growth. Potassium (K^+) is a major plant nutrient which is accumulated by roots and distributed through the plant. When there is a high accumulation of Na^+ in the cytosol, high Na^+/K^+ ratios disrupt enzymatic functions, usually activated by K^+ in plant cells (Chen *et al.,* 2014).

Shao *et al.* (1986) reported that soybean salt-tolerance response differed significantly among varieties and among different growth stages in the same variety, finding that some varieties were more susceptible during the seedling stage than during germination. Salt screening methods have been reported, suggesting the use of visual leaf scorch and leaf Cl^- content as the most accurate parameters to evaluate the differential salt-stress response among excluders and includers, offering more consistent results than measurements of Na^+ and Cl^- concentration in roots (Valencia *et al.,* 2008; Ledesma *et al.,* 2016). Several studies have been performed in order to

evaluate soybean response to salt stress in different growth stages measuring multiple growth, development, and yield components. However, the effect of salt stress on the progressive shoot accumulation of soluble ions coming from different salinity sources and its relationship to other important physiological parameters during soybean vegetative growth is not well known. In addition, it is not completely understood whether Na^+ or Cl^- plays the most critical role in NaCl induced mortality in soybeans (Phang *et al.,* 2008), and the effect of other common fertilizer sources like KCl in soybean salt stress has not been extensively studied. There is limited detailed information about the mechanisms responsible for genetic variation in salt tolerance in soybean. The understanding of the connection between physiological traits and their role in salt-stress adaptation is a key element in plant breeding research for soybean salt tolerance. Therefore, the two main objectives of this study were: 1) Evaluate and compare progressive shoot ion accumulation in soybean sensitive and tolerant genotypes over time during NaCl and KCl treatment, and 2) Study leaf physiological traits related to salt-stress response of soybean cultivars during early growth stage.

MATERIALS AND METHODS

Plant material and growth conditions

Four soybean varieties with variation in salt tolerance response were chosen for this study: Jake and Lee known as Cl^- excluders (salt-tolerant), and Ozark and Dare known as Cl^- includers (salt-sensitive) (Table 1). Eight seeds per genotype were sown equally spaced in a 6-inch pot containing sandy loam soil, then six healthy plants were selected and maintained after germination. Plants were grown in the greenhouse at the Rosen Center - University of Arkansas under 14 hours of light and 25 ± 2 °C, and were fertilized once a week using the Miracle-Gro®

All Purpose Plant Food (The Scotts Miracle-Gro Company, Marysville, Ohio). The experiment consisted in the application of five different treatments (four salt sources/solutions plus the control) during 30 days under similar conditions as the salt screening process previously described for phenotyping in chapter II. Pots were placed in trays for the purpose of watering and pouring salt treatment solutions. At the beginning of stage V1, salt treatments were applied irrigating the plants from the bottom. Two different sources of salt (NaCl, KCl) at two concentration levels (80 and 120 mM) were used. In order to evaluate the cumulative salt-stress effect in the cultivars, seven variables were measured every 3 days during 4 weeks after the initiation of the treatments: LSS (leaf scorch score), leaf ion concentration (Na^+, K^+, and Cl^-), leaf chlorophyll content, leaf dry matter, and leaf area.

Measurements

Leaf scorch score (LSS): This parameter was used as a visual measurement for tolerance evaluation. The methodology applied to score the plants was the same used for phenotyping in the salt tolerance screening, using the 1–9 range scale (1=healthy dark green leaves/ no chlorosis, and 9= necrotic leaves). All the plants contained in each pot were rated to obtain a general score. The leaf scorch is related to the level of chlorosis and necrosis associated with the reduction of chlorophyll concentration, which reduce vegetative growth and biomass production (Slabu *et al.*, 2010).

Chloride (Cl^-), Potassium (K^+), and Sodium (Na^+) content: The shoots of the plants were harvested, dried at 70°C during 5 days, and ground to measure concentrations of Na^+, K^+, and Cl^-. For chloride analysis, the extract was diluted in distilled water. Acid digestion with nitric

acid and hydrogen peroxide was used to extract Na^+ and K^+ (Plank, 1992). All the extracts were analyzed with a spectrophotometer ARCOS ICP (Wheal and Palmer, 2010).

Leaf chlorophyll content: Salt stress reduces photosynthetic capacity in plants, which is associated with chlorophyll content in leaves. Chlorophyll content decreases with salt stress due to an activity increase of the chlorophyll-degrading enzyme chlorophyllase, and the destruction of the chloroplast structure (Jamil *et al.*, 2007; Singh and Dubey, 1995). Leaf chlorophyll content was measured by using a chlorophyll meter Konica Minolta SPAD–502 plus. The data was obtained measuring the chlorophyll content of three randomly chosen mature leaves from the upper part of the canopy located in different directions. Measurements were made on three plants per pot.

Leaf area and leaf dry weight: In early phases of development, dry matter increase is closely associated with leaf area. An increase in leaf area leads to an increase in total dry matter accumulation (Echarte *et al.*, 2008). Leaf dry matter content is widely used as an indicator of plant resource use (Vaieretti *et al.*, 2007). The measurement of leaf area and leaf dry weight can provide a closer understanding on how metabolic adjustments in soybean genotypes may help to enhance assimilation and the achievement of a more efficient conservation of resources in response to stress conditions. Drought, water stress, and salinity decreases specific leaf area (defined as the ratio of leaf area to leaf dry mass), making leaves smaller and thinner than leaves under regular conditions. Leaf thickness has been used as an indicator of species´ strategies of resource acquisition and cultivar productivity. The amount of light absorbed by the leaves and the diffusion pathways of CO_2 through the tissues are partially dependent on this trait (Vile *et al.*, 2005). Leaf area and leaf dry weight were measured on three randomly selected plants per pot. Leaf area was estimated using a LI-COR LI-3100 area meter.

Experimental design and statistical analysis

The experimental design was a randomized complete block design with two factor factorial treatment structure, three replications (pots), and eleven sampling points (30 days of evaluation). Salt treatments (80 mM NaCl, 120 mM NaCl, 80 mM KCl, 120 mM KCl, and control) and varieties (four) were the two main factors, and date of evaluation was used as a block. Each date of evaluation or block consisted of five trays (salt treatments) and 3 pots of each cultivar placed in every tray (12 pots per tray). Analysis of variance (ANOVA) was performed to analyze statistical differences among the data collected for all the measured variables. The turkey test ($p= 0.05$) was used to calculate significant differences among treatments and varieties. All the statistical analyses were performed using SAS 9.4.

RESULTS

Significant differences were found among all treatments, varieties, and days of evaluation for all the variables measured during the experiment, as expected (Table 2-8). In general, leaf ion accumulation in plants under control treatment remained constantly low, while leaf ion content in plants from all varieties subjected to salt stress treatments continuously increased over time. Salinity significantly reduced leaf chlorophyll content, leaf expansion, and leaf dry matter accumulation in all the varieties. This reduction was proportional to salt concentration, finding more detrimental effects at higher salt concentrations. The most adverse effect on both, tolerant and susceptible varieties, was caused by the KCl treatment in comparison with NaCl under low and high concentrations (Figure 8). When the experiment was completed, salt-tolerant varieties subjected to NaCl treatment at both concentrations remained alive with slight chlorosis, while salt-sensitive varieties were already dead. Tolerant varieties performed differently under 120 mM

KCl treatment; by the end of the experiment the sensitive genotypes and the tolerant variety Lee were dead, and Jake stayed alive with a high level of chlorosis and some necrosis (Figure 8 D).

Leaf chloride content

Chloride content in leaves increased with increasing concentrations of NaCl and KCl over time, however, the Cl^- accumulation pattern of these salt sources were different. After 30 days of salt stress using the highest salt concentration level (120 mM), tolerant cultivars subjected to KCl treatment were able to accumulate 135% more Cl^- than tolerant cultivars under NaCl (≈105,495 mg/kg and 44,824 mg/kg, respectively). Similarly, sensitive cultivars under KCl treatment accumulated 27.7% more Cl^- than sensitive cultivars under NaCl (≈130,439 mg/kg and 102,122 mg/kg, respectively).

Under 80 mM NaCl, first clear significant differences between tolerant (Jake, Lee) and sensitive cultivars (Ozark, Desha) were found 15 days after treatment initiation (Table 9, Figure 1A). These significant differences continued until the last day of evaluation. Even though excluder varieties tended to accumulate more Cl^- under salt stress than varieties under non-stress, no significant differences were found between the tolerant cultivars (Jake and Lee) and all the varieties under control treatment throughout the course of the experiment. Lee accumulated more leaf Cl^- than Jake, and Desha significantly accumulated higher Cl^- content than Ozark by day 30 (Table 9, Figure 1A). A similar accumulation pattern was observed in 120 mM NaCl treatment (Figure 1B); however, first significant differences in Cl^- leaf content between tolerant and sensitive cultivars appeared earlier (12 days after treatment initiation) and significant differences in Cl^- accumulation between tolerant cultivars under salt stress and varieties in the control were visible from day 9 (Table 9). After 30 days of treatment, the includers Ozark (100,712 mg/kg Cl^-

) and Desha (103,533 mg/kg Cl^-) retained about 2.3 times more Cl^- in leaves than the excluders Jake (41,222 mg/kg Cl^-) and Lee (48,427 mg/kg Cl^-).

The leaf chloride accumulation pattern of the evaluated varieties under KCl treatment was similar in both concentrations, 80 and 120 mM (Figure 1 C-D). Excluder genotypes tended to reach a Cl^- leaf concentration similar to the one observed in includers, being this opposite to the results obtained under the NaCl treatments, where the Cl^- leaf content between includers and excluders kept being significantly different to each other during most of the time frame of the experiment.

The first significant differences among excluders and includers were found 15 and 9 days after KCl treatment initiation at 80 mM and 120 mM, respectively (Table 9). Towards the end of the experiment, no significant differences were found among the varieties Lee, Ozark, and Desha, which accumulated 117,273 mg/kg, 124,577 mg/kg, and 136,299 mg/kg of Cl^-, respectively. Even though Jake accumulated a high Cl^- quantity (93,718 mg/kg) after this 30-day period, it was significantly different than the Cl^- accumulation reached by the rest of the varieties.

Leaf sodium content

Sodium increase in all the varieties under the applied salt treatments was slower compared to the Cl^- accumulation pattern. No significant differences in leaf Na^+ content between varieties under salt stress and non-stress were found during the first 15-18 days of treatment (Table 10).

Leaf Na^+ content in soybean genotypes under NaCl treatments at 80 mM and 120 mM followed a similar accumulation tendency, where significant differences in sodium content between includers and excluders were observed during the last days of evaluation (from day 21 and 18 in 80 mM NaCl and 120 mM NaCl, respectively), approaching final shoot harvest (Table 10, Figure

2 A-B). Thirty days after treatment, there were no statistical differences in leaf Na^+ content neither between Jake and Lee (tolerant), nor between Ozark and Dare (sensitive). Tolerant varieties accumulated an average Na^+ content of 3,698 mg/kg and 10,271 mg/kg under 80 mM NaCl and 120 mM NaCl, respectively. Sensitive varieties accumulated an average Na^+ content of 16,252 mg/kg and 39,402 mg/kg under 80 mM NaCl and 120 mM NaCl, respectively.

Leaf sodium accumulation under the KCl treatments was slower compared to the pattern of Na^+ accumulation observed under NaCl treatments, finding significant differences between excluders and includers from day 24 and 18 under KCl at 80 mM and 120 mM, respectively (Table 10, Figure 2 C-D). The results of Na^+ content in the evaluated varieties under the highest salt concentration showed that tolerant cultivars subjected to NaCl treatment accumulated approximately 15 times more Na^+ than tolerant cultivars under KCl (≈10,271 mg/kg vs 700 mg/kg). Similarly, sensitive cultivars under NaCl treatment accumulated 36 times more Na^+ than sensitive cultivars under KCl (≈39,402 mg/kg vs 1090 mg/kg).

Maximum average leaf Na^+ accumulation in tolerant varieties at the last day of evaluation was 231 mg/kg under 80 mM KCl and 700 mg/kg under 120 mM KCl, while sensitive varieties accumulated a maximum of 361 mg/kg and 1,090 mg/kg under 80 mM KCl and 120 mM KCl, respectively.

Leaf potassium content

The leaf potassium level under NaCl treatment at 80 and 120 mM, displayed slight changes in all the varieties during most of the course of the experiment. During the last days of the experiment (starting from day 24), leaf K^+ content in sensitive genotypes was significantly different from both, the K^+ quantity accumulated in tolerant varieties and the K^+ amount accumulated in

varieties under non-stress (control) (Table 11, Figure 3 A-B). Jake and Lee had a similar accumulation pattern to the one observed in plants no subjected to salt stress.

Potassium content in leaves increased with increasing concentrations of KCl over time (Figure 3 C-D). First clear significant differences in the K^+ accumulation between excluders and includers were found between days 15-21 under 80 mM KCl and days 9-15 in 120 mM KCl (Table 11, Figure 3 C-D). Subsequently, tolerant and sensitive genotypes tended to accumulate similar amounts of leaf K^+ at final shoot harvest time (day 30); however, Jake accumulated significantly lower amounts of K^+ in comparison to Lee, Ozark and Dare under 80 mM KCl and 120 mM KCl. Similarly, Dare was the most sensitive variety accumulating the highest level of K^+ under 120 mM KCl.

Leaf scorch score

Significant differences in leaf scorch score (LSS) among cultivars under all types of salt stress were first observed 9 days after treatment initiation, when at the same time, it was possible to make a differentiation between salt treated and non-salinized plants (Table 12, Figure 4). However, the clearest significant differences between sensitive and tolerant varieties were seen between 12-15 days after salt treatment initiation under NaCl, and 9-12 days under KCl at low and high salt concentrations. Leaves of plants from all varieties under no salt stress (control) remained healthy and green during the all course of the experiment (LSS =1). In the last experiment evaluation, susceptible varieties displayed a LSS close to 9 under all salt treatments whereas tolerant varieties displayed a slight chlorosis under NaCl (LSS up to 5.7) and an advanced level of chlorosis and necrosis under KCl stress (LSS close to 9).

A chlorosis/necrosis progress was observed with increasing salt concentration levels over time under NaCl and KCl treatments, as expected. However, at the end of the experiment tolerant varieties displayed a lower LSS under NaCl stress compared to their LSS values under KCl. Under KCl stress, tolerant varieties were highly affected, reaching leaf damages similar to the ones displayed by the susceptible cultivars (Figure 8). Under NaCl stress, susceptible varieties had a LSS 1.6 times higher than tolerant varieties.

No significant differences in LSS were found between Jake and Lee under treatment with NaCl 80 and 120 mM by day 30, displaying an average LSS of 5.5. Similarly, Ozark and Desha displayed a LSS close or equal to 9 under both NaCl treatments (Table 12, Figure 4 A-B). Tolerant cultivars remained alive with a slight chlorosis level while susceptible cultivars were dead by the end of the experiment under NaCl stress (Figure 8 A-B). Under 80 mM KCl treatment, no significant differences in LSS were found between Jake and Lee (LSS ≈ 7), and Desha and Ozark (LSS ≈ 9). Contrastingly, there were significant differences in LSS between Jake and Lee under KCl 120 (7.5 vs 9.0) finding by the end of the experiment that Jake was still alive while Lee was dead (Figure 8 C-D).

Leaf chlorophyll content

Chlorophyll content in leaves decreased with increasing salt concentration over time. An earlier chlorophyll reduction was observed in both, includers and excluders, under KCl treatment compared to NaCl. Under the maximum salt level (120 mM), tolerant cultivars subjected to NaCl were able to accumulate 3 times more chlorophyll in leaves than tolerant cultivars under KCl stress by the end of the experiment, while sensitive cultivars accumulated very similar chlorophyll quantities under both salt sources (Table 13).

The tolerant varieties tended to accumulate more leaf chlorophyll than plants under non-salt treatment during the first days after treatment initiation under NaCl, this tendency was more prominent under the highest NaCl concentration (120 mM). Jake usually displayed higher leaf chlorophyll contents than Lee. Subsequently, chlorophyll content continuously decreased reaching minimum values between days 15 and 30 (Table 13, Figure 5 A–B). Sensitive cultivars displayed a progressive a decrement in chloride content over time, observing significant differences in LSS between them and tolerant cultivars by day 9 under 80 mM NaCl and 120 mM. By day 30, Ozark and Dare had reduced their leaf chlorophyll content down to 3.4 and 6.3, respectively, under the highest concentration of NaCl.

No significant differences in LSS were found between excluders and plants in control treatment under KCl during the first 12 days of evaluation. Jake and Lee had a faster leaf chlorophyll reduction under KCl stress (80 and 120mM), being more evident from day 15 to 30 after treatment initiation. However, Jake was able to accumulate a significantly higher amount of chlorophyll than Lee during the last two weeks of the experiment (Table 13 Figure 5 C-D). At the end of the experiment no significant differences in leaf chlorophyll content were found among all varieties under 80 mM KCl, ranging from 4.6 to 8.4. Under 120 mM KCl all varieties had very low leaf chlorophyll content by day 30 (from 4.7 to 10.9), however significant differences were found between Jake and the rest of the cultivars (Table 13).

Leaf area

Leaf area increased over time in all treatments being continuous in both, varieties under control and varieties subjected to salt stress. However, salinity caused a severe leaf area reduction in all

the varieties. Significant differences in leaf area accumulation were found between salt treated vs. non-salt stressed varieties beginning at day 12-15 (Table 14).

Sensitive genotypes started to decrease their leaf area 18 days after salt treatment initiation in both, NaCl and KCl (Figure 6). Salt tolerant varieties displayed a leaf area decrease between day 24 and 30 under KCl 80 and 120 mM, observing by day 30 that there were no significant differences in leaf area among all the varieties (excluders and includers). These differences were more noticeable under the highest KCl concentration, 120 mM (Table 14, Figure 6 C-D). On the other hand, significant differences between tolerant and susceptible varieties were found during the last days of salt treatment under NaCl (Table 14). Tolerant varieties performed slightly different under NaCl stress. Although no significant differences in leaf area were found between Lee and Jake under low and high concentrations of NaCl over time, Lee tended to have an increase in leaf area while Jake had a slight leaf area loss during day 24- 30 (Figure 6 A-B).

Plants under control treatment were able to expand 8 times more leaf area than excluders and includers under KCl stress at the highest concentration (120 mM). Under 120 mM NaCl, plants under control obtained 4 and 18 times more leaf area than excluders and includers, respectively.

Leaf dry weight

Leaf dry weight in all the evaluated varieties was drastically reduced by salt stress, as expected. First significant differences in leaf dry matter accumulation between varieties under salt stress and control treatment were found between days 12-15, similar to the results observed in leaf area. A slight increment in leaf dry matter accumulation was observed in excluders and includers under both KCl treatments. Although significant differences in leaf dry weight were found among tolerant and susceptible varieties over time, they all tended to accumulate similar amounts

of leaf dry matter at the time of the final evaluation (Table 15, Figure 7 C-D). Jake significantly accumulated more leaf dry weight than Lee by day 24 and day 30 under 80 mM KCl and 120 mM, respectively.

Under NaCl stress, significant differences in leaf dry weight between excluders and includers were observed from day 18 at 120 mM NaCl, and day 21 at 80 mM NaCl (Table 15, Figure 7 A-B). By the end of the experiment, Jake and Lee had accumulated statistically similar quantities of leaf dry matter and accumulated up to 3 times more leaf dry matter than sensitive varieties (Dare and Ozark).

Plants under non-stress (control) were able to accumulate 2.6 and 6.6 times more leaf dry weight than excluders and includers, respectively, under 120 mM NaCl. Similarly plants under control accumulated 4 times more leaf dry weight than excluders and includers under KCl stress at the highest concentration.

DISCUSSION

Salinity significantly reduced soybean leaf chlorophyll content, leaf dry matter accumulation, and leaf area expansion, as expected. These results are in agreement with previous studies about salt tolerance effects on soybean (Abel and MacKenzie, 1964; Chang *et al.*, 1994, Phang *et al.*, 2008; Shereen *et al.*, 2001, among others), where it has been reported that salt-induced necrosis in soybeans was associated with high Cl^- content in the aerial part, which causes alterations in the bioenenergetic processes of photosynthesis. This is usually due to changes in the K^+/ Na^+ ratio caused by the accumulation of intracellular Na^+ ions under salt stress (Sudhir and Murthy, 2004). Sodium chloride affects the permeability of the cellular membrane and increases flux of external ions and efflux of cytosolic solutes in plant cells (osmotic stress) (Allen *et al.*, 1995). In

general, salt ions interfere in the uptake of essential macro and micronutrients, additionally, salt stress disturbs cell wall flexibility and reduces water conductance through the plasmatic membrane causing problems in cell elongation (Patel *et al.,* 2010).

As NaCl concentration increased, Na^+ and Cl^- leaf contents increased significantly, while K^+ leaf content remained constant with no significant increase (between 16,000 and 30,000 mg/kg) during all the experiment time in all varieties. However, leaf K^+ content in Jake and Lee (excluders) tended to decrease over time, probably due to an exchange of vacuolar K^+ with Na^+ in order to maintain low cytosolic Na^+ (Shereen *et al.*, 2001). The opposite occurred with Ozark and Desha (includers), where a K^+ increment was observed over time. Excluders generally accumulated lower K^+ and Na^+ than includers, suggesting that tolerant varieties responded to elevated Na^+ concentrations by maintaining higher cytosolic K^+/ Na^+ ratios than the sensitive ones as a mechanism to reach ion homeostasis and avoid toxicity (Lacan and Durand, 1996), given that potassium uptake is limited by high concentrations of Na^+ and xylem translocation is restricted (Patel *et al.,* 2010).

As expected, significant differences in all the measured variables between excluders and includers were observed under salt treatment, given that tolerant varieties generally have lower shoot ion concentration than sensitive varieties (Shereen *et al*., 2001). These differences were more evident under NaCl treatment, where the excluders (Jake and Lee) clearly seemed to be more efficient than includers (Ozark and Dare) in the uptake and translocation regulation of toxic ion accumulation (primarily Cl^-) within the plant (Valencia *et al.*, 2008; Slaton *et al.*, 2014; Ledesma *et al.*, 2016). A different trend in the leaf ion accumulation pattern was observed in the evaluated varieties under KCl stress, where excluders tended to accumulate similar quantities of leaf Cl^-, Na^+, and K^+ than the includers during the last days of the experiment evaluation.

Subsequently, by day 30 the excluder Lee and the susceptible varieties were dead, and Jake had advanced chlorosis/necrosis symptoms. Potassium chloride caused a substantially higher inhibition in growth compared to sodium chloride, affecting the tolerance level of soybean genotypes. These effects have been previously reported in salt-stress studies of another species like the halophyte saltbush (Ramos *et al.,* 2004), wheat (Taware *et al.,* 2009), and banana (Shapira *et al.,* 2009). In soybean, salt tolerance response to KCl stress has not been extensively studied; however, a reason for this differential response of tolerant cultivars under KCl and NaCl can be due to the fact that K^+ is a highly mobile element in the plant and genotypes have evolved to become adapted to the presence of high Na^+, which is a more common component of saline soils than K^+. In natural environments, salt stress is usually caused by Na^+ and not by K^+ (Ramos *et al.,* 2004). Apparently, cation homeostasis in soybean seem to work more efficiently when salt-stress is caused by an excess of Na^+ in the cytosol than when it is due to K^+.

The extent of soybean salt tolerance/sensitivity varies among cultivars (Ghassemi-Golezani and Taifeh-Noori, 2011). A differential tolerance response was observed among excluders and includers under both types of salt, NaCl and KCl. Jake showed a higher tolerance level than Lee, and Ozark was less susceptible to Dare. Jake was particularly efficient at maintaining low levels of leaf Cl^-, Na^+, and K^+, while having the ability to reach higher leaf chlorophyll contents than the rest of the varieties under salt stress, and even higher chlorophyll content than plants in the control under NaCl. This was reflected on the slow progress of chlorosis and necrosis symptoms (low LSS) observed during the experiment. Tolerant genotypes use mechanisms for a higher ion metabolic efficiency, mainly, by reducing chlorophyll degradation and stabilizing water potential to avoid ionic and oxidative stress, which cause detrimental effects in the PSII, growth, and yield (Shanon, 1998).

Jake showed higher leaf greenness than Lee, and tended to expand less leaf area and accumulate more leaf dry weight during some time intervals of the experiment, suggesting that Jake could have accumulated more leaf dry weight per leaf area unit (lower specific leaf area), increasing leaf thickness as an additional protection mechanism against salt stress. However, complementary further studies are needed to confirm this hypothesis.

Based on the results obtained in this study, the best time to evaluate salt stress through foliar scoring / tissue sampling should be between 9-12 days for KCl treatment and 12-15 days for NaCl treatment, given the clear contrasting response among genotypes observed with the variables measured during this time frame. This is in agreement with previous studies in salt tolerance screening in soybean under NaCl (Lee *et al.,* 2008; Valencia *et al.,* 2008; Ledesma *et al.,* 2016), where the adequate evaluation time is two weeks after treatment initiation, when soybeans reach stage V1-V2. Also, clearest results were obtained subjecting soybean genotypes under NaCl and KCl at 120 mM, in which foliar chlorosis symptoms started to occur by day 9 when sensitive genotypes accumulated over 40,000 mg/kg of leaf Cl^- and 2,000 mg/kg of leaf Na^+, and 50,000 mg/kg of leaf $Cl^-$and 45,000 mg/kg of leaf K^+, respectively.

Soybean plants died (LSS = 8-9) when leaves accumulated over 80,000 mg/kg and 18,000 mg/kg of Cl^- and Na^+, respectively, under 120 mM NaCl. Under 120 mM KCl, plants died when leaf Cl^- content reached 120,000 mg/kg and leaf K^+ content was over 100,000 mg/kg. The 30 day-period stablished for the salt stress evaluation in soybean cultivars was long enough to observe the response of excluders and includers to NaCl and KCl during vegetative stage, from V1 to V6. However, for tolerant varieties under NaCl, it remains unknown what is the ion accumulation limit in which they would reach absolute necrosis, or if they would eventually display resilience

capacity despite of the continuous stress they are subjected to and how reproductive stages would be affected.

CONCLUSIONS

In this study, four soybean cultivars were grown from stage V1 to V6 under two levels of NaCl and KCl. Visual description of foliar symptoms was more clear and effective under the highest salt concentration (120 mM) for both salt sources. Leaf chlorosis/necrosis symptoms and leaf ion accumulation increased as salt concentration increased over time with a reduction in leaf chlorophyll, leaf area, and leaf dry matter accumulation, as expected. It appeared that the leaf ion accumulation pattern in soybean under NaCl is different compared to KCl. Under NaCl at the highest concentration, includers were able to accumulate up to 2.3 and 3.8 times more leaf Cl^- and leaf Na^+, respectively, than excluders. Effects of salt stress on soybean plants were stronger under KCl compared to NaCl, observing that significant differences in LSS and ion accumulation between excluders and includers appeared earlier in KCl treatment, while leaf growth and development was being severely affected. Additionally, excluders were able to accumulate considerably higher ion amounts under KCl, reaching quantities comparable to those typically displayed by includers, diminishing their tolerance capacity and causing an early death (between V5-V6 stage). Apparently, ion homeostasis in soybean works more efficiently under NaCl than KCl. Further studies can be performed to better understand the ion accumulation pattern and tolerance limit of soybean excluders during more advanced growth stages, especially under NaCl stress. The use of visual ratings and the other leaf characteristics evaluated were very good salt-stress descriptors. This study provided useful information about soybean response to K^+, Na^+, and Cl^- accumulation in leaves when plants were subjected to salt stress during early growth stages. In addition, the according to the performance observed in all the evaluated varieties new

crosses can be design in order to create populations to find new genes. For example, the two tolerant cultivars Jake and Lee, which showed differential tolerance level to salt stress but are considered excluders, can be used to create a new mapping population.

The methods and results obtained here can be used as a basic guideline to develop future work on soybean salt screening to better elucidate the K^+, Na^+, and Cl^- contributions and mechanisms involved in soybean osmotic adjustment in response to salinity, and find which ion(s) contribute more efficiently to perform this function.

REFERENCES

Abel, G.H. and A.J. MacKenzie. 1964. Salt tolerance of soybean varieties (*Glycine max* L. Merrill) during germination and later growth. Crop Sci. 4: 157–161.

Allen G., R. Wyn Jones, and R. Leigh. 1995. Sodium transport measured in plasma membrane vesicles isolated from wheat genotypes with differing K^+/Na^+ discrimination traits. Plant Cell Envir. 18:105–115.

Chang, R.Z., Y.W. Chen, G.H. Shao and C.W. Wan. 1994. Effect of salt stress on agronomic characters and chemical quality of seeds in soybean. Soybean Sci. 13, 101–105.

Chen, H., X. Chen, H. Gu, B. Wu, H. Zhang, X. Yuan, X. Cui. 2014. GmHKT1;4, a novel soybean gene regulating Na+/K+ ratio in roots enhances salt tolerance in transgenic plants. Plant Growth Regul. 73:299–308.

Echarte, L., S. Rothstein, and M. Tollenaar. 2008. The response of leaf photosynthesis and dry matter accumulation to nitrogen supply in an older and a newer maize hybrid. Crop Sci.48:656-665.

Ghassemi-Golezani K. and M. Taifeh-Noori .2011. Chapter 34: Soybean Performance under Salinity Stress. Soybean - Biochemistry, Chemistry and Physiology. Ed. InTech. 654 p. Available in: http://www.intechopen.com/books/editor/soybean-biochemistry-chemistry-and-physiology

Lacan, D. and M. Durand. 1995. Na-K exchange at xylem/symplast boundary. Its significance in the salt sensitivity of soybean. J. Plant Physiol., 110: 705-711.

Ledesma, F., C. Lopez, D. Ortiz, P. Chen, K. L. Korth, T. Ishibashi, A. Zeng, M. Orazaly, and L. Florez-Palacios. 2016. A simple greenhouse method for screening salt tolerance in soybean. Crop Sci. 56: 585-594.

Patel P.R, S. Kajal, V.R Patel, V.J Patel, and S.M Khristi. 2010. Impact of salt stress on nutrient uptake and growth of cowpea. Braz. J. Plant Physiol. 22:43-48.

Phang, T.H., G.H. Shao and H.M. Lam. 2008. Salt tolerance in soybean. J. Integr. Plant Biol. 50: 1196–1212.

Ramos, J., M. J Lopez, and M.Benlloch. 2004. Effect of NaCl and KCl salts on the growth and solute accumulation of the halophyte *Atriplex nummularia.* Plant Soil 259: 163-168.

Shao G.H., J. Z Song, and H. L Liu. 1986. Preliminary studies on the evaluation of salt tolerance in soybean varieties. Acta Agron. Sin. 6: 30– 35.

Shanon, M.C. (1998). Adaptation of plants to salinity. Adv. Agron., 60: 75-119.

Shapira, O., S. Khadka, Y. Israeli, U. Shani, and A. Schwartz. 2009. Functional anatomy controls ion distribution in banana leaves: significance of Na^+ seclusion at the leaf margins. Plant Cell Environ. 32: 476-485.

Shereen, A., R. Ansari, and A. Soomro. 2001. Salt tolerance in soybean (Glycine max L.): Effect on growth and ion relations. Pak. J. Bot. 33: 393–402.

Slaton, N.A., R.E. DeLong, T.L. Roberts, J. Ross, M.S. Fryer, M.R. Parvej, R.J. Dempsey, J. Hedge, and S. Hayes. 2014. Soybean yield as affected by chloride rate and cultivar chloride include/excluder rating. Arkansas Soil Fertility Studies- AAES Research Series 624: 63-38.

Sudhir, P. and S.D.S Murthy. Effects of salt stress on basic processes of photosynthesis. Photosynthetica 42: 481-486.

Taware, S., A. Taware, A. Chavan, and D. Mukadam. 2009. Wheat and salinity: Response of different concentrations of NaCl and KCl. Biosc., Biotech. Res. Asia. 6: 313-316.

Vaieretti M.V., S. Diaz, D. Vile, and E. Garnier. 2007. Two Measurement Methods of Leaf Dry Matter Content Produce Similar Results in a Broad Range of Species. Ann Bot. 99: 955–958.

Valencia, R., P. Chen, T. Ishibashi, and M. Conaster. 2008. A rapid and effective method for screening salt tolerance in soybean. Crop Sci. 48: 1773-1779.

Table 1. Soybean genotypes used for performance evaluation study under NaCl and KCl stress

Genotype	Pedigree/PI	Tolerance	References
Lee	S-100 x CNS	Tolerant	Pantalone *et al.*, 1997; Huang, 2013; Ledesma *et al.,* 2016
Jake	S94-1867 × 'Anand'	Tolerant	Huang, 2013; Ledesma *et al.,* 2016
Ozark	Holladay x Delta Pine DP 415	Sensitive	Huang, 2013; Ledesma *et al.,* 2016
Desha	PI 633610	Sensitive	Huang, 2013; Ledesma *et al.,* 2016

Table 2. Analysis of variance for leaf chloride content in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates).

Source	D.F	Mean Square	F	p-value
Days	10	10,527,196,239	218.11	<0.0001
Treatment	4	27,260,427,977	564.81	<0.0001
Variety	3	12,798,086,765	265.16	<0.0001
Days x Treatment	40	1,012,135,168	20.97	<0.0001
Days x Variety	30	369,550,225	7.66	<0.0001
Treatment x Variety	12	813,487,874	16.85	<0.0001
Error	120	48,264,794	.	

Table 3. Analysis of variance for leaf sodium content in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates)

Source	D.F	Mean Square	F	p-value
Days	10	102,076,346	15.12	<0.0001
Treatment	4	436,248,587	64.63	<0.0001
Variety	3	124,517,341	18.45	<0.0001
Days x Treatment	40	46,259,882	6.85	<0.0001
Days x Variety	30	14,042,709	2.08	0.0029
Treatment x Variety	12	61,164,828	9.06	<0.0001
Error	120	6,749,943	.	

Table 4. Analysis of variance for leaf potassium content in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates).

Source	D.F	Mean Square	F	p-value
Days	10	2,826,491,710	185.75	<0.0001
Treatment	4	22,681,710,319	1490.62	<0.0001
Variety	3	1,355,925,415	89.11	<0.0001
Days x Treatment	40	1,072,030,230	70.45	<0.0001
Days x Variety	30	52,733,738	3.47	<0.0001
Treatment x Variety	12	306,509,509	20.14	<0.0001
Error	120	15,216,256	.	

Table 5. Analysis of variance for leaf scorch score (LSS) in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates).

Source	D.F	Mean Square	F	p-value
Days	10	74.02	294.85	<0.0001
Treatment	4	120.66	480.62	<0.0001
Variety	3	38.84	154.72	<0.0001
Days x Treatment	40	4.97	19.8	<0.0001
Days x Variety	30	1.67	6.67	<0.0001
Treatment x Variety	12	3.54	14.1	<0.0001
Error	120	0.25	.	

Table 6. Analysis of variance for leaf chlorophyll content in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates).

Source	D.F	Mean Square	F	p-value
Days	10	1188.95	176.4	<0.0001
Treatment	4	941.81	139.73	<0.0001
Variety	3	1203.79	178.6	<0.0001
Days x Treatment	40	63.45	9.41	<0.0001
Days x Variety	30	19.77	2.93	<0.0001
Treatment x Variety	12	95.34	14.15	<0.0001
Error	120	6.74	.	

Table 7. Analysis of variance for leaf area in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates).

Source	D.F	Mean Square	F	p-value
Days	10	29,851.0	138.63	<0.0001
Treatment	4	93,519.0	434.29	<0.0001
Variety	3	4,922.6	22.86	<0.0001
Days x Treatment	40	11,695.0	54.31	<0.0001
Days x Variety	30	1,006.9	4.68	<0.0001
Treatment x Variety	12	274.7	1.28	0.2416
Error	120	215.3	.	

Table 8. Analysis of variance for leaf dry weight in four soybean varieties under salt treatment (NaCl and KCl at 0, 80, and 120 mM) during 30 days (11 evaluation dates).

Source	D.F	Mean Square	F	p-value
Days	10	0.44	320.71	<0.0001
Treatment	4	0.50	364.67	<0.0001
Variety	3	0.10	69.54	<0.0001
Days x Treatment	40	0.08	54.72	<0.0001
Days x Variety	30	0.01	7.81	<0.0001
Treatment x Variety	12	0.01	4.54	<0.0001
Error	120	0.00	.	

Table 9. Leaf chloride content (mg/kg) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 21,434.6 mg/kg, 120 mM NaCl HSD = 17,814.9 mg/kg, 80 mM KCl HSD = 16,894.7 mg/kg, and 120 mM KCl HSD = 14,237.1 mg/kg.

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM NaCl - Chloride (mg/kg)----------										
Jake	287**a**	6,756**a**	4,737**a**	4,513**a**	5,504**ab**	9,148**a**	9,032**a**	9,372**a**	13,374**a**	11,693**a**	11,909**a**
Lee	376**a**	2,875**a**	5,668**a**	8,751**ab**	8,152**ab**	7,720**a**	11,438**a**	10,452**a**	11,536**a**	16,319**a**	21,109**a**
Ozark	542**a**	14,105**a**	16,421**a**	25,121**b**	26,005**bc**	54,57**b**	61,583**b**	58,101**b**	60,648**b**	65,253**b**	61,971**b**
Desha	361**a**	11,982**a**	20,711**a**	26,654**b**	38,326**c**	63,84**b**	67,466**b**	70,328**b**	79,538**b**	87,560**c**	90,612**c**
Jake-CK	303**a**	386**a**	302**a**	2,334**a**	344**a**	873**a**	567**a**	662**a**	889**a**	701**a**	1,321**a**
Lee-CK	356**a**	311**a**	371**a**	2,106 **a**	478**a**	1,226**a**	989**a**	2,018**a**	872**a**	772**a**	1,162**a**
Ozark-CK	656**a**	702**a**	965**a**	936 **a**	1,335**a**	3,713**a**	2,960**a**	5,745**a**	2,535**a**	2,582**a**	4,490**a**
Desha-CK	558**a**	943**a**	782**a**	1,155 **a**	1,630**a**	3,350**a**	3,072**a**	4,641**a**	2,329**a**	4,019**a**	3,816**a**
	----------120 mM NaCl - Chloride (mg/kg)----------										
Jake	356**a**	9,798**a**	10,936**a**	11,296**ab**	14,712**a**	15,511**ab**	24,050**b**	29,890**b**	38,223**b**	37,092**b**	41,222**b**
Lee	311**a**	6,038**a**	15,151**a**	20,128**b**	14,986**a**	18,071**b**	27,709**b**	37,081**b**	37,709**b**	39,536**b**	48,427**b**
Ozark	480**a**	13,751**a**	34,527**b**	40,174**c**	53,639**b**	62,979**c**	67,677**c**	84,326**c**	82,041**c**	94,137**c**	100,712**c**
Desha	394**a**	12,970**a**	31,567**ab**	46,933**c**	47,101**b**	59,593**c**	79,401**c**	82,204**c**	92,343**c**	97,645**c**	103,533**c**
Jake-CK	304**a**	386**a**	302**a**	2,334**a**	344**a**	873**a**	567**a**	663**a**	889**a**	700**a**	1,321**a**
Lee-CK	356**a**	312**a**	371**a**	2,106**a**	478**a**	1,226**a**	989**a**	2,019**a**	872**a**	772**a**	1,162**a**
Ozark-CK	656**a**	702**a**	965**a**	936**a**	1,335**a**	3,713**a**	2,960**a**	5,746**a**	2,535**a**	2,582**a**	4,490**a**
Desha-CK	558**a**	943**a**	782**a**	1,154**a**	1,630**a**	3,350**a**	3,072**a**	4,641**a**	2,329**a**	4,019**a**	3,816**a**

Table 9. Leaf chloride content (mg/kg) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 21,434.6 mg/kg, 120 mM NaCl HSD = 17,814.9 mg/kg, 80 mM KCl HSD = 16,894.7 mg/kg, and 120 mM KCl HSD = 14,237.1 mg/kg (Cont.)

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM KCl - Chloride (mg/kg)----------										
Jake	333**a**	12,405**ab**	14,383**ab**	22,850**b**	23,566**b**	26,469**b**	29,209**b**	44,757**b**	51,692**b**	64,639**b**	87,838**b**
Lee	543**a**	14,464**ab**	23,677**cb**	24,511**b**	27,120**bc**	31,781**b**	43,125**b**	51,754**b**	69,613**c**	87,860**c**	98,700**b**
Ozark	450**a**	20,058**b**	32,977**c**	33,784**b**	40,699**c**	70,823**c**	84,474**c**	87,459**c**	102,223**d**	101,783**d**	108,328**bc**
Desha	676**a**	22,359**b**	31,780**c**	34,518**b**	53,985**c**	79,418**c**	92,770**c**	92,402**c**	111,220**d**	125,529**f**	126,322**d**
Jake-CK	304**a**	386**a**	302**a**	2,334**a**	344**a**	873**a**	567**a**	663**a**	889**a**	700**a**	1,321**a**
Lee-CK	356**a**	312**a**	371**a**	2,106**a**	478**a**	1,226**a**	989**a**	2,019**a**	872**a**	772**a**	1,162**a**
Ozark-CK	656**a**	702**a**	965**a**	936**a**	1,335**a**	3,713**a**	2,960**a**	5,746**a**	2,535 **a**	2,582 **a**	4,490**a**
Desha-CK	558**a**	943**a**	782**a**	1,154**a**	1,630**a**	3,350**a**	3,072**a**	4,641**a**	2,329 **a**	4,019 **a**	3,816**a**
	----------120 mM KCl - Chloride (mg/kg)----------										
Jake	813**a**	9,291**b**	17,354**b**	27,171**b**	32,088**b**	39,659**b**	63,071**b**	82,214**b**	81,001**b**	90,735**b**	93,718**b**
Lee	608**a**	9,979**b**	15,317**b**	24,144**b**	44,439**b**	52,388**b**	73,127**b**	87,615**b**	89,259**b**	102,066**bc**	117,273**c**
Ozark	1,157**a**	21,389**bc**	50,853**d**	53,883**c**	72,153**c**	94,791**c**	104,204**c**	114,378**c**	120,329**c**	114,439**c**	124,577**cd**
Desha	1,129**a**	24,716**c**	32,196**c**	60,673**c**	71,558**c**	91,282**c**	99,438**c**	123,162**c**	137,844**d**	131,327**d**	136,299**d**
Jake-CK	304**a**	386**a**	302**a**	2,334**a**	344**a**	873**a**	567**a**	663**a**	889**a**	700**a**	1,321**a**
Lee-CK	356**a**	312**a**	371**a**	2,106**a**	478**a**	1,226**a**	989**a**	2,019**a**	872**a**	772**a**	1,162**a**
Ozark-CK	656**a**	702**a**	965**a**	936**a**	1,335**a**	3,713**a**	2,960**a**	5,746**a**	2,535**a**	2,582**a**	4,490**a**
Desha-CK	558**a**	943**a**	782**a**	1,154**a**	1,630**a**	3,350**a**	3,072**a**	4,641**a**	2,329**a**	4,019**a**	3,815**a**

Table 10. Leaf sodium content (mg/kg) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 4,317.8 mg/kg, 120 mM NaCl HSD = 9,084.9 mg/kg, 80 mM KCl HSD = 58.8 mg/kg, and 120 mM KCl HSD = 178.8 mg/kg

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM NaCl - Sodium (mg/kg)----------										
Jake	90**a**	238 **a**	323**a**	204**a**	246**a**	569**a**	480**a**	1,502**a**	1,024**a**	1,114**a**	1,698**a**
Lee	86**a**	189 **a**	27 **a**	430**a**	681**a**	461**a**	535**a**	732**a**	1,137**a**	2,113**a**	5,699**a**
Ozark	59**a**	457 **a**	56 **a**	483**a**	584**a**	1,131**a**	1,815**a**	4,114**ab**	5,032**a**	9,759**b**	15,027**b**
Desha	50**a**	196 **a**	373**a**	544**a**	1,072**a**	1,817**a**	2,426**a**	6,241**b**	10,644**b**	13,529**b**	17,477**b**
Jake-CK	63**a**	84 **a**	70**a**	44**a**	49**a**	52**a**	91**a**	55**a**	79**a**	48**a**	50**a**
Lee-CK	73**a**	98 **a**	91**a**	62**a**	52**a**	48**a**	70**a**	76**a**	85**a**	30**a**	55**a**
Ozark-CK	78**a**	102 **a**	63**a**	60**a**	61**a**	40**a**	58**a**	27**a**	74**a**	37**a**	32**a**
Desha-CK	83**a**	82 **a**	67**a**	51**a**	61**a**	53**a**	43**a**	36**a**	107**a**	52**a**	37**a**
	----------120 mM NaCl - Sodium (mg/kg)----------										
Jake	64**a**	738**a**	776**a**	933**a**	962**a**	966**a**	1,674**a**	3,196 **a**	4,349**a**	6,637**a**	13,568**b**
Lee	52**a**	671**a**	710**a**	915**a**	1,431**a**	1,655**a**	1,753**a**	3,983 **a**	4,062**a**	5,291**a**	6,974**ab**
Ozark	58**a**	995**a**	1,109**a**	1,924**a**	2,430**a**	5,923**a**	8,938**ab**	17,409 **b**	18,045**b**	20,482**b**	38,084**c**
Desha	57**a**	747**a**	1,683**a**	3,027**a**	5,053**a**	7,158**a**	14,605**b**	24,300 **b**	25,001**b**	25,203**b**	40,721**c**
Jake-CK	63**a**	84**a**	70**a**	44**a**	49**a**	52**a**	91**a**	55 **a**	79**a**	48**a**	50**a**
Lee-CK	73**a**	98**a**	91**a**	62a	52**a**	48**a**	70**a**	76 **a**	85**a**	30**a**	55**a**
Ozark-CK	78**a**	102**a**	63**a**	60**a**	61**a**	40**a**	58**a**	27 **a**	74**a**	37**a**	32**a**
Desha-CK	83**a**	82**a**	67**a**	51**a**	61**a**	53**a**	43**a**	36 **a**	107**a**	52**a**	37**a**

Table 10. Leaf sodium content (mg/kg) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 4,317.8 mg/kg, 120 mM NaCl HSD = 9,084.9 mg/kg, 80 mM KCl HSD = 58.8 mg/kg, and 120 mM KCl HSD = 178.8 mg/kg (Cont.)

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM KCl - Sodium (mg/kg)----------										
Jake	62**a**	89**a**	51**a**	48**a**	37**a**	64**a**	53**a**	62**ab**	118**a**	67**ab**	234**b**
Lee	61**a**	90**a**	44**a**	34**a**	37**a**	40**a**	64**a**	93**b**	101**a**	118**b**	228**b**
Ozark	53**a**	84**a**	90**a**	62**a**	47**a**	45**a**	60**a**	101**b**	213**b**	178**c**	304**c**
Desha	66**a**	81**a**	69**a**	43**a**	57**a**	80**a**	78**a**	141**b**	228**b**	232**c**	419**d**
Jake-CK	63**a**	84**a**	70**a**	44**a**	49**a**	52**a**	91**a**	55**ab**	79**a**	48**a**	50**a**
Lee-CK	73**a**	98**a**	91**a**	62**a**	52**a**	48**a**	70**a**	76**ab**	85**a**	30**a**	55**a**
Ozark-CK	78**a**	102**a**	63**a**	60**a**	61**a**	40**a**	58**a**	27**a**	74**a**	37**a**	32**a**
Desha-CK	83**a**	82**a**	67**a**	51**a**	61**a**	53**a**	43**a**	36**ab**	107**a**	52**a**	37**a**
	----------120 mM KCl - Sodium (mg/kg)----------										
Jake	81**a**	91**a**	41**a**	37**a**	53**a**	49**a**	85**a**	112**ab**	115**ab**	157**ab**	478**b**
Lee	62**a**	83**a**	56**a**	37**a**	81**a**	82**a**	101**ab**	106**ab**	275**ab**	232**bc**	922**c**
Ozark	79**a**	98**a**	50**a**	62**a**	119**a**	127**a**	275**bc**	283**c**	592**c**	577**c**	1,076**cd**
Desha	67**a**	68**a**	82**a**	64**a**	108**a**	133**a**	285**c**	220**bc**	432**bc**	405**c**	1,104**d**
Jake-CK	63**a**	84**a**	70**a**	44**a**	49**a**	52**a**	91**a**	55**a**	79**a**	48**a**	50**a**
Lee-CK	73**a**	98**a**	91**a**	62**a**	52**a**	48**a**	70**a**	76**a**	85**a**	30**a**	55**a**
Ozark-CK	78**a**	102**a**	63**a**	60**a**	61**a**	40**a**	58**a**	27**a**	74**a**	37**a**	32**a**
Desha-CK	83**a**	82**a**	67**a**	51**a**	61**a**	53**a**	43**a**	36**a**	107**ab**	52**a**	37**a**

Table 11. Leaf potassium content (mg/kg) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 5,208 mg/kg, 120 mM NaCl HSD = 3,158.3 mg/kg, 80 mM KCl HSD = 11,687.8 mg/kg, and 120 mM KCl HSD = 10,584.9 mg/kg

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------NaCl 80 mM - Potassium (mg/kg)----------										
Jake	23,537**b**	27,132**c**	24,240**b**	26,188**a**	25,251**a**	22,403**ab**	23,291**a**	22,232**a**	18,063**a**	18,125**a**	16,841**a**
Lee	19,822**b**	24,474**bc**	21,749**ab**	23,605**a**	21,531**a**	19,442**a**	19,101**a**	18,929**a**	17,966**a**	17,030**a**	16,822**a**
Ozark	22,292**b**	26,140**c**	20,908**ab**	24,529**a**	22,969**a**	27,316**bc**	28,194**bc**	28,135**bc**	27,213**b**	28,548**cd**	26,147**b**
Desha	21,010**b**	24,760**c**	22,135**ab**	24,501**a**	24,651**a**	30,412**c**	30,322**c**	29,878**c**	27,314**b**	29,120**d**	25,648**b**
Jake-CK	19,263**b**	19,422**ab**	21,835**ab**	25,099**a**	25,519**a**	22,549**ab**	25,912**bc**	23,524**ab**	19,437**a**	23,878**bc**	20,339**a**
Lee-CK	18,785**b**	16,722**a**	18,269**a**	23,014**a**	24,591**a**	20,285**a**	23,344**ab**	25,638**bc**	19,043**a**	20,124**ab**	20,896**a**
Ozark-CK	19,629**b**	17,092**a**	19,450**ab**	24,269**a**	23,874**a**	23,106**ab**	23,687**ab**	21,093**a**	19,549**a**	20,493**ab**	21,089**a**
Desha-CK	17,406**a**	17,853**a**	17,424**a**	25,038**a**	24,222**a**	20,923**a**	22,390**a**	21,355**a**	20,412**a**	21,621**ab**	18,208**a**
	----------NaCl 120 mM - Potassium (mg/kg)----------										
Jake	21,548**bc**	21,800**bc**	25,094**d**	25,280**abc**	23,279**a**	21,729**ab**	22,661**b**	22,426**b**	20,320**a**	20,452**a**	19,218**a**
Lee	19,676**abc**	21,455**b**	22,514**bcd**	23,056**a**	22,877**a**	19,439**a**	17,852**a**	17,891**a**	17,775**a**	19,921**a**	19,837**a**
Ozark	22,683**c**	23,910**c**	24,339**d**	26,405**bc**	26,496**bc**	25,690**c**	26,603**d**	25,718**cd**	25,476**b**	24,069**b**	24,450**b**
Desha	20,615**bc**	24,644**c**	23,644**cd**	28,265**c**	27,117**c**	25,469**c**	26,725**d**	28,034**d**	24,399**b**	28,256**c**	25,835**b**
Jake-CK	19,263**ab**	19,422**ab**	21,835**bc**	25,099**ab**	25,519**abc**	22,549**abc**	25,912**cd**	23,524**bc**	19,437**a**	23,878**b**	20,339**a**
Lee-CK	18,785**ab**	16,722**a**	18,269**a**	23,014**a**	24,591**abc**	20,285**a**	23,344**bc**	25,638**cd**	19,043**a**	20,124**a**	20,896**a**
Ozark-CK	19,629**abc**	17,092**a**	19,450**ab**	24,269**ab**	23,874**ab**	23,106**bc**	23,687**bcd**	21,093**b**	19,549**a**	20,493**a**	21,089**a**
Desha-CK	17,406**a**	17,853**a**	17,424**a**	25,038**ab**	24,222**abc**	20,923**ab**	22,390**b**	21,355**b**	20,412**a**	21,621**ab**	18,208**a**

Table 11. Leaf potassium content (mg/kg) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 5,208 mg/kg, 120 mM NaCl HSD = 3,158.3 mg/kg, 80 mM KCl HSD = 11,687.8 mg/kg, and 120 mM KCl HSD = 10,584.9 mg/kg (Cont.)

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------KCl 80 mM - Potassium (mg/kg)----------										
Jake	19,263**a**	26,595**ab**	31,162**b**	34,848**bc**	34,953**ab**	38,267**b**	49,163**b**	55,235**b**	66,871**b**	72,148**b**	84,666**b**
Lee	16,475**a**	27,355**ab**	30,402**b**	36,757**cd**	39,378**bc**	43,257**b**	52,333**b**	60,619**b**	80,782**c**	93,274**c**	107,772**c**
Ozark	17,287**a**	30,324**b**	37,377**b**	47,161**d**	47,737**cd**	62,759**c**	75,447**c**	78,799**c**	92,638**d**	99,794**c**	115,324**c**
Desha	16,360**a**	29,016**b**	37,903**b**	46,279**cd**	57,020**d**	68,828**c**	81,999**c**	85,466**c**	107,529**e**	117,317**d**	114,532**c**
Jake-CK	19,263**a**	19,422**ab**	21,835**ab**	25,099**ab**	25,519**a**	22,549**a**	25,912**a**	23,524**a**	19,437**a**	23,878**a**	20,339**a**
Lee-CK	18,785**a**	16,722**a**	18,269**a**	23,014**a**	24,591**a**	20,285**a**	23,344**a**	25,638**a**	19,043**a**	20,124**a**	20,896**a**
Ozark-CK	19,629**a**	17,092**a**	19,450**ab**	24,269**a**	23,874**a**	23,106**a**	23,687**a**	21,093**a**	19,549**a**	20,493**a**	21,089**a**
Desha-CK	17,406**a**	17,853**a**	17,424**a**	25,038**ab**	24,222**a**	20,923**a**	22,390**a**	21,355**a**	20,412**a**	21,621**a**	18,208**a**
	----------KCl 120 mM - Potassium (mg/kg)----------										
Jake	20,166**a**	29,145**ab**	33,646**b**	42,605**b**	42,158**b**	44,205**b**	62,003**b**	80,264**b**	85,679**b**	84,126**b**	100,615**b**
Lee	18,137**a**	32,069**b**	36,966**b**	46,554**b**	53,752**b**	62,610**c**	73,214**c**	82,538**b**	94,599**b**	91,746**b**	119,063**c**
Ozark	18,877**a**	35,732**b**	47,969c	57,740**c**	64,356**c**	71,712**c**	86,840**d**	88,969**bc**	102,193**bc**	103,279**c**	114,683**c**
Desha	17,990**a**	36,038**b**	45,561**bc**	68,233**c**	83,438**c**	89,305**d**	102,939**e**	99,129**c**	110,757**c**	113,058**d**	134,405**d**
Jake-CK	19,263**a**	19,422**a**	21,835**a**	25,099**a**	25,519**a**	22,549**a**	25,912**a**	23,524**a**	19,437**a**	23,878**a**	20,339**a**
Lee-CK	18,785**a**	16,722**a**	18,269**a**	23,014**a**	24,591**a**	20,285**a**	23,344**a**	25,638**a**	19,043**a**	20,124**a**	20,896**a**
Ozark-CK	19,629**a**	17,092**a**	19,450**a**	24,269**a**	23,874**a**	23,106**a**	23,687**a**	21,093**a**	19,549**a**	20,493**a**	21,089**a**
Desha-CK	17,406**a**	17,853**a**	17,424**a**	25,038**a**	24,222**a**	20,923**a**	22,390**a**	21,355**a**	20,412**a**	21,621**a**	18,208**a**

Table 12. Leaf scorch score (1-9 where: 1 = dark green, healthy leaves to 9 = necrotic leaves) of our soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 1.27, 120 mM NaCl HSD = 1.75, 80 mM KCl HSD = 0.88, and 120 mM KCl HSD = 1.07

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM NaCl – LSS----------										
Jake	1.0**a**	1.0**a**	1.0**a**	3.0**a**	3.5**a**	3.8**a**	3.3**a**	3.3**a**	4.5**a**	4.0**a**	5.2**a**
Lee	1.0**a**	1.0**a**	1.0**a**	3.3**a**	4.0**ab**	4.7**a**	4.0**a**	4.3**a**	5.3**a**	5.3**a**	5.7**a**
Ozark	1.0**a**	1.0**a**	1.0**a**	4.3**b**	5.0**bc**	6.7**b**	7.0**b**	6.8**b**	7.0**b**	7.2**b**	8.3**b**
Desha	1.0**a**	1.0**a**	1.0**a**	5.7**c**	6.0**c**	7.0**b**	7.0**b**	7.0**b**	8.0**b**	7.7**b**	8.7**b**
	----------120 mM NaCl – LSS----------										
Jake	1.0**a**	1.0**a**	2.0**a**	3.0**a**	2.7**a**	2.3**a**	3.5**a**	4.0**a**	4.3**a**	3.3**a**	5.7**a**
Lee	1.0**a**	1.0**a**	2.0**a**	3.3**a**	3.3**a**	3.3**a**	4.0**a**	5.7**a**	5.3**a**	3.7**a**	5.5**a**
Ozark	1.0**a**	1.0**a**	2.0**a**	4.7**ab**	5.0**ab**	7.0**b**	7.0**b**	7.2**ab**	8.7**b**	8.8**b**	9.0**b**
Desha	1.0**a**	1.0**a**	2.0**a**	5.7**b**	6.2**b**	7.0**b**	7.5**b**	8.2**b**	9.0**b**	9.0**b**	9.0**b**
	----------80 mM KCl – LSS----------										
Jake	1.0**a**	1.0**a**	1.0**a**	3.0**a**	4.2**a**	4.8**a**	4.8**a**	4.5**a**	5.8**a**	5.3**a**	6.8**a**
Lee	1.0**a**	1.0**a**	1.0**a**	4.0**b**	5.3**b**	6.0**b**	6.0**b**	6.0**b**	6.0**a**	6.0**a**	6.7**a**
Ozark	1.0**a**	1.0**a**	1.0**a**	5.0**c**	6.0**c**	6.3**b**	6.8**bc**	7.0**c**	7.2**b**	7.0**b**	9.0**b**
Desha	1.0**a**	1.0**a**	1.0**a**	5.0**c**	6.0**c**	6.8**b**	7.0**c**	7.0**c**	8.5**c**	7.8**b**	8.8**b**
	----------120 mM KCl – LSS----------										
Jake	1.0**a**	1.0**a**	2.0**a**	3.7**a**	3.7**a**	3.8**a**	4.3**a**	5.8**a**	5.5**a**	5.2**a**	7.5**a**
Lee	1.0**a**	1.0**a**	2.0**a**	5.0**b**	4.7**a**	6.0**b**	6.0**b**	6.2**ab**	6.2**a**	6.0**a**	9.0**b**
Ozark	1.0**a**	1.0**a**	2.0**a**	5.7**b**	5.8**b**	6.8**b**	7.0**bc**	7.2**bc**	8.7**b**	7.7**b**	8.8**b**
Desha	1.0**a**	1.0**a**	2.0**a**	6.0**b**	6.0**b**	7.0**b**	7.5**c**	7.7**c**	9.0**b**	8.3**b**	9.0**b**

Table 13. Leaf chlorophyll content of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 5.8, 120 mM NaCl HSD = 6.9, 80 mM KCl HSD = 5.8, and 120 mM KCl HSD = 6.0

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM NaCl – Chlorophyll----------										
Jake	32.7**a**	41.3**a**	38.6**b**	38.8**c**	38.2**d**	35.1**c**	35.1**c**	34.0**c**	28.5**c**	27.7**c**	23.4**b**
Lee	35.0**a**	40.8**a**	34.7**ab**	34.6**c**	29.2**abc**	27.4**b**	27.3**b**	26.7**b**	22.1**b**	21.0**b**	17.9**b**
Ozark	32.6**a**	38.1**a**	33.9**ab**	28.5**ab**	27.8**ab**	17.2**a**	15.8**a**	16.5**a**	11.1**a**	12.3**a**	5.2**a**
Desha	31.2**a**	36.4**a**	31.1**a**	23.7**a**	23.6**a**	14.8**a**	16.6**a**	14.5**a**	11.4**a**	10.4**a**	3.4**a**
Jake-CK	35.2**a**	40.9**a**	34.0**ab**	33.2**bc**	29.8**abc**	30.5**bc**	32.0**bc**	33.0**c**	34.6**d**	31.1**c**	32.9**c**
Lee-CK	35.0**a**	39.7**a**	32.7**a**	32.8**b**	29.6**abc**	31.6**bc**	33.0**bc**	32.4**bc**	35.6**d**	32.8**c**	33.4**c**
Ozark-CK	34.3**a**	40.6**a**	32.8**ab**	33.4**bc**	32.9**bc**	30.9**bc**	31.0**bc**	31.4**bc**	32.2**cd**	30.1**c**	33.8**c**
Desha-CK	33.4**a**	36.8**a**	32.8**ab**	32.4**b**	34.9**cd**	32.6**bc**	33.4**c**	32.1**bc**	31.3**cd**	30.5**c**	30.2**c**
	----------120 mM NaCl – Chlorophyll----------										
Jake	34.8**a**	42.4**a**	41.8**c**	40.1**d**	40.8**c**	38.3**c**	36.8**c**	31.2**b**	35.0**c**	29.2**c**	23.4**bc**
Lee	33.2**a**	38.3**a**	38.9**bc**	38.7**cd**	40.0**c**	33.5**bc**	28.4**b**	26.7**b**	21.8**b**	21.5**b**	20.8**b**
Ozark	32.0**a**	37.9**a**	34.7**ab**	28.3**ab**	26.8**ab**	21.7**a**	16.8**a**	15.4**a**	13.2**a**	7.9**a**	6.3**a**
Desha	34.3**a**	38.4**a**	27.7**a**	21.9**a**	22.6**a**	21.7**a**	16.5**a**	13.4**a**	10.6**a**	7.3**a**	3.4**a**
Jake-CK	35.2**a**	40.9**a**	34.0**ab**	33.2**bcd**	29.8**b**	30.5**b**	32.0**bc**	33.0**b**	34.6**c**	31.1**c**	32.9**cd**
Lee-CK	35.0**a**	39.7**a**	32.7**ab**	32.8**bc**	29.6**b**	31.6**bc**	33.0**bc**	32.4**b**	35.6**c**	32.8**c**	33.4**cd**
Ozark-CK	34.3**a**	40.6**a**	32.8**ab**	33.4**bcd**	32.9**b**	30.9**b**	31.0**bc**	31.4**b**	32.2**c**	30.1**c**	33.8**cd**
Desha-CK	33.4**a**	36.8**a**	32.8**ab**	32.4**bc**	34.9**bc**	32.6**bc**	33.4**bc**	32.1**b**	31.3**c**	30.5**c**	30.2**c**

Table 13. Leaf chlorophyll content of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 5.8, 120 mM NaCl HSD = 6.9, 80 mM KCl HSD = 5.8, and 120 mM KCl HSD = 6.0 (Cont.)

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM KCl - Chlorophyll----------										
Jake	36.3**a**	42.2**a**	36.3**c**	37.2**b**	31.8**b**	28.5**b**	25.4**b**	26.5**b**	24.1**b**	16.7**b**	8.4**a**
Lee	36.8**a**	41.3**a**	30.5**bc**	32.0**b**	29.4**b**	16.5**a**	14.8**a**	14.7**a**	10.3**a**	10.6**a**	5.8**a**
Ozark	35.9**a**	37.3**a**	25.7**ab**	22.9**a**	23.5**a**	16.9**a**	14.6**a**	15.8**a**	9.2**a**	10.7**a**	6.2**a**
Desha	34.9**a**	36.5**a**	24.5**a**	21.0**a**	19.3**a**	12.2**a**	10.9**a**	12.7**a**	11.7**a**	6.8**a**	4.6**a**
Jake-CK	35.2**a**	40.9**a**	34.0**c**	33.2**b**	29.8**b**	30.5**b**	32.0**c**	33.0**c**	34.6**c**	31.1**c**	32.9**b**
Lee-CK	35.0**a**	39.7**a**	32.7**c**	32.8**b**	29.6**b**	31.6**b**	33.0**c**	32.4**c**	35.6**c**	32.8**c**	33.4**b**
Ozark-CK	34.3**a**	40.6**a**	32.8**c**	33.4**b**	32.9**b**	30.9**b**	31.0**bc**	31.4**bc**	32.2**c**	30.1**c**	33.8**b**
Desha-CK	33.4**a**	36.8**a**	32.8**c**	32.4**b**	34.9**b**	32.6**b**	33.4**c**	32.1**bc**	31.3**c**	30.5**c**	30.2**b**
	----------120 mM KCl – Chlorophyll----------										
Jake	36.3**a**	40.8**a**	36.5**c**	34.5**c**	33.6**cd**	29.7**b**	28.6**b**	24.2**b**	18.3**b**	18.7**c**	10.9**b**
Lee	38.4**a**	39.4**a**	32.4**bc**	28.0**b**	27.8**bc**	15.8**a**	13.7**a**	13.6**a**	12.0**a**	12.4**b**	3.3**a**
Ozark	36.3**a**	37.6**a**	28.1**ab**	21.7**a**	23.5**ab**	17.9**a**	16.0**a**	17.0**a**	12.5**ab**	7.1**ab**	4.1**a**
Desha	35.7**a**	35.1**a**	23.0**a**	16.5**a**	17.1**a**	14.7**a**	15.3**a**	14.1**a**	8.6**a**	4.5**a**	4.7**a**
Jake-CK	35.2**a**	40.9**a**	34.0**bc**	33.2**bc**	29.8**cd**	30.5**b**	32.0**b**	33.0**c**	34.6**c**	31.1**d**	32.9**c**
Lee-CK	35.0**a**	39.7**a**	32.7**bc**	32.8**bc**	29.6**cd**	31.6**b**	33.0**b**	32.4**c**	35.6**c**	32.8**d**	33.4**c**
Ozark-CK	34.3**a**	40.6**a**	32.8**bc**	33.4**bc**	32.9**cd**	30.9**b**	31.0**b**	31.4**c**	32.2**c**	30.1**d**	33.8**c**
Desha-CK	33.4**a**	36.8**a**	32.8**bc**	32.4**bc**	34.9**d**	32.6**b**	33.4**b**	32.1**c**	31.3**c**	30.5**d**	30.2**c**

Table 14. Leaf area (cm^2) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 40.7 cm^2, 120 mM NaCl HSD = 42.7 cm^2, 80 mM KCl HSD = 47.8 cm^2, and 120 mM KCl HSD = 43.3 cm^2

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM NaCl - Leaf area (cm^2)----------										
Jake	22.3**a**	31.9**a**	49.4**a**	59.5**a**	79.3**a**	87.5**ab**	96.5**a**	96.7**a**	115.1**b**	97.0**b**	113.2**b**
Lee	32.7**a**	43.1**a**	52.5**a**	71.4**a**	77.0**a**	87.1**ab**	111.2**ab**	100.0**a**	114.6**b**	112.5**b**	127.1**b**
Ozark	31.0**a**	32.1**a**	53.9**a**	67.7**a**	75.9**a**	98.2**ab**	100.5**a**	89.8**a**	63.3**a**	66.5**a**	38.1**a**
Desha	35.9**a**	37.5**a**	55.6**a**	77.4**a**	89.6**a**	83.9**a**	87.8**a**	77.8**a**	63.1**a**	43.9**a**	31.0**a**
Jake-CK	19.1**a**	22.0**a**	46.9**a**	72.6**a**	102.7**ab**	128.0**b**	200.0**de**	233.8**b**	312.8**c**	350.6**cd**	431.4**d**
Lee-CK	21.8**a**	29.8**a**	51.0**a**	95.1**a**	130.7**b**	121.5**ab**	217.8**e**	215.7**b**	308.0**c**	318.9**c**	368.5**c**
Ozark-CK	20.0**a**	29.2**a**	47.9**a**	69.7**a**	91.3**ab**	147.1**c**	165.4**cd**	286.1**c**	326.8**c**	330.0**cd**	354.9**c**
Desha-CK	25.8**a**	34.3**a**	45.3**a**	84.7**a**	97.3**ab**	126.6**b**	148.0**bc**	208.4**b**	303.2**c**	364.1**d**	363.1**c**
	----------120 mM NaCl - Leaf area (cm^2)----------										
Jake	25.2**a**	26.7**a**	41.2**a**	54.8**a**	69.5**a**	75.9**a**	79.3**a**	71.9**a**	105.6**b**	84.3**b**	78.9**b**
Lee	33.3**a**	39.3**a**	46.9**a**	58.1**a**	63.8**a**	71.6**a**	86.6**a**	77.3**a**	95.4**b**	116.4**b**	109.5**b**
Ozark	31.9**a**	34.6**a**	44.1**a**	60.3**a**	64.0**a**	63.8**a**	52.5**a**	41.1**a**	35.9**a**	38.5**a**	26.5**a**
Desha	36.4**a**	32.6**a**	50.3**a**	59.5**a**	60.2**a**	60.6**a**	42.0**a**	37.5**a**	37.0**a**	40.4**a**	32.1**a**
Jake-CK	19.1**a**	22.0**a**	46.9**a**	72.6**a**	102.7**ab**	128.0**b**	200.0**cd**	233.8**b**	312.8**c**	350.6**cd**	431.4**d**
Lee-CK	21.8**a**	29.8**a**	51.0**a**	95.1**a**	130.7**b**	121.5**b**	217.8**d**	215.7**b**	308.0**c**	318.9**c**	368.5**c**
Ozark-CK	20.0**a**	29.2**a**	47.9**a**	69.7**a**	91.3**ab**	147.1**b**	165.4**bc**	286.1**b**	326.8**c**	330.0**cd**	354.9**c**
Desha-CK	25.8**a**	34.3**a**	45.3**a**	84.7**a**	97.3**ab**	126.6**b**	148.0**b**	208.4**b**	303.2**c**	364.1**d**	363.1**c**

Table 14. Leaf area (cm^2) of four soybean varieties under four different salt treatments during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 40.7 cm^2, 120 mM NaCl HSD = 42.7 cm^2, 80 mM KCl HSD = 47.8 cm^2, and 120 mM KCl HSD = 43.3 cm^2 (Cont.)

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM KCl - Leaf area (cm^2)----------										
Jake	20.0**a**	32.7**a**	52.2**a**	58.1**a**	75.4**a**	98.2**a**	123.7**ab**	115.5**a**	151.5**b**	112.3**bc**	72.3**ab**
Lee	31.0**a**	42.3**a**	60.3**a**	71.5**a**	88.5**ab**	110.5**ab**	120.9**ab**	134.1**a**	135.4**b**	140.9**c**	103.5**b**
Ozark	25.5**a**	35.4**a**	55.2**a**	87.9**a**	92.6**ab**	129.1**ab**	115.7**a**	123.0**a**	70.9**a**	65.6**ab**	50.7**a**
Desha	27.4**a**	35.6**a**	60.8**a**	84.9**a**	100.3**ab**	132.1**ab**	118.3**ab**	106.6**a**	61.6**a**	56.3**a**	60.4**ab**
Jake-CK	19.1**a**	22.0**a**	46.9**a**	72.6**a**	102.7**ab**	128.0**ab**	200.0**cd**	233.8**b**	312.8**c**	350.6**d**	431.4**d**
Lee-CK	21.8**a**	29.8**a**	51.0**a**	95.1**a**	130.7**b**	121.5**ab**	217.8**d**	215.7**b**	308.0**c**	318.9**d**	368.5**c**
Ozark-CK	20.0**a**	29.2**a**	47.9**a**	69.7**a**	91.3**ab**	147.1**b**	165.4**bc**	286.1**c**	326.8**c**	330.0**d**	354.9**c**
Desha-CK	25.8**a**	34.3**a**	45.3**a**	84.7**a**	97.3**ab**	126.6**ab**	148.0**ab**	208.4**b**	303.2**c**	364.1**d**	363.1**c**
	----------120 mM KCl - Leaf area (cm^2)----------										
Jake	23.3**a**	26.8**a**	40.8**a**	58.6**a**	76.8**a**	92.6**a**	93.5**ab**	96.7**b**	125.0**b**	91.2**b**	60.3**a**
Lee	26.5**a**	32.6**a**	49.0**a**	61.6**a**	97.4**ab**	110.6**ab**	118.4**bc**	106.8**b**	108.8**b**	97.0**b**	47.3**a**
Ozark	26.2**a**	33.7**a**	51.1**a**	71.7**a**	91.6**ab**	80.0**a**	60.1**a**	57.2**a**	34.5**a**	35.2**a**	35.5**a**
Desha	31.0**a**	32.9**a**	55.1**a**	73.0**a**	85.7**a**	93.4**a**	56.0**a**	53.3**a**	43.0**a**	40.9**a**	43.9**a**
Jake-CK	19.1**a**	22.0**a**	46.9**a**	72.6**a**	102.7**ab**	128.0**b**	200.0**ef**	233.8**c**	312.8**c**	350.6**cd**	431.4**c**
Lee-CK	21.8**a**	29.8**a**	51.0**a**	95.1**a**	130.7**b**	121.5**ab**	217.8**f**	215.7**c**	308.0**c**	318.9**c**	368.5**b**
Ozark-CK	20.0**a**	29.2**a**	47.9**a**	69.7**a**	91.3**ab**	147.1**b**	165.4**de**	286.1**d**	326.8**c**	330.0**cd**	354.9**b**
Desha-CK	25.8**a**	34.3**a**	45.3**a**	84.7**a**	97.3**ab**	126.6**b**	148.0**cd**	208.4**c**	303.2**c**	364.1**d**	363.1**b**

Table 15. Leaf dry weight (g) of four soybean varieties under four different salt treatments obtained during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 0.1137 g, 120 mM NaCl HSD = 0.0985 g, 80 mM KCl HSD = 0.0857g, and 120 mM KCl HSD = 0.0465 g.

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM NaCl - Leaf dry weight (g)----------										
Jake	0.0587**a**	0.0947**a**	0.1294**a**	0.1485**a**	0.2103**ab**	0.2907**ab**	0.3261**ab**	0.3309**b**	0.4901**b**	0.5418**b**	0.5193**b**
Lee	0.0848**a**	0.1244**a**	0.1348**a**	0.1915**a**	0.2142**ab**	0.2803**ab**	0.3771**bc**	0.3478**b**	0.4940**b**	0.5065**b**	0.5799**b**
Ozark	0.0742**a**	0.0952**a**	0.1292**a**	0.1563**a**	0.1650**a**	0.2248**ab**	0.2452**a**	0.2415**ab**	0.2249**a**	0.2352**a**	0.1996**a**
Desha	0.0769**a**	0.1100**a**	0.1371**a**	0.1700**a**	0.1969**ab**	0.1910**a**	0.2129**a**	0.1981**a**	0.2409**a**	0.2104**a**	0.1633**a**
Jake-CK	0.0507**a**	0.0543**a**	0.1289**a**	0.1739**a**	0.2239**ab**	0.3379**bc**	0.4587**cd**	0.6151**cd**	0.9169**d**	1.0709**d**	1.3017**e**
Lee-CK	0.0545**a**	0.0968**a**	0.1403**a**	0.2273**a**	0.2846**b**	0.3011**abc**	0.5227**d**	0.5400**cd**	0.8381**cd**	1.0676**d**	1.1173**cd**
Ozark-CK	0.0602**a**	0.0930**a**	0.1233**a**	0.1474**a**	0.1996**ab**	0.4094**c**	0.3945**bc**	0.6528**d**	0.8722**cd**	0.9013**c**	1.0750**c**
Desha-CK	0.0571**a**	0.0995**a**	0.1148**a**	0.1875**a**	0.2127**ab**	0.3233**bc**	0.3706**bc**	0.5077**c**	0.7664**c**	0.9178**c**	1.1479**d**
	----------120 mM NaCl - Leaf dry weight (g)----------										
Jake	0.0687**a**	0.1000**a**	0.1120**a**	0.1672**a**	0.1943**ab**	0.2744**b**	0.2854**b**	0.2609**b**	0.4700**b**	0.4855**b**	0.4522**b**
Lee	0.0815**a**	0.1188**a**	0.1398**a**	0.1723**a**	0.1942**ab**	0.2799**b**	0.3491**bc**	0.3143**b**	0.4210**b**	0.5104**b**	0.4503**b**
Ozark	0.0754**a**	0.1101**a**	0.1216**a**	0.1440**a**	0.1556**a**	0.1784**ab**	0.1774**a**	0.1546**a**	0.1719**a**	0.2740**a**	0.1673**a**
Desha	0.0905**a**	0.1024**a**	0.1215**a**	0.1333**a**	0.1395**a**	0.1732**a**	0.1620**a**	0.1423**a**	0.1614**a**	0.2600**a**	0.1815**a**
Jake-CK	0.0507**a**	0.0543**a**	0.1289**a**	0.1739**a**	0.2239**ab**	0.3379**bc**	0.4587**cd**	0.6151**cd**	0.9169**d**	1.0709**d**	1.3017**e**
Lee-CK	0.0545**a**	0.0968**a**	0.1403**a**	0.2273**a**	0.2846**b**	0.3011**b**	0.5227**d**	0.5400**c**	0.8381**cd**	1.0676**d**	1.1173**cd**
Ozark-CK	0.0602**a**	0.0930**a**	0.1233**a**	0.1474**a**	0.1996**ab**	0.4094**c**	0.3945**c**	0.6528**d**	0.8722**cd**	0.9013**c**	1.0750**c**
Desha-CK	0.0571**a**	0.0995**a**	0.1148**a**	0.1875**a**	0.2127**ab**	0.3233**bc**	0.3706**bc**	0.5077**c**	0.7664**c**	0.9178**c**	1.1479**d**

Table 15. Leaf dry weight (g) of four soybean varieties under four different salt treatments obtained during 30 days of evaluation (11 sampling dates). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control). For each date of evaluation, means within columns followed by different letters are significantly different by Tukey test at 0.05 level of probability. Tukey's honest significant difference (HSD) for the treatments are: 80 mM NaCl HSD = 0.1137 g, 120 mM NaCl HSD = 0.0985 g, 80 mM KCl HSD = 0.0857g, and 120 mM KCl HSD = 0.0465 g (Cont.)

	Days										
Variety	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
	----------80 mM KCl - Leaf dry weight (g)----------										
Jake	0.0536**a**	0.0982**a**	0.1487**a**	0.1386**b**	0.1898**a**	0.3099**a**	0.3697**b**	0.3255**a**	0.4832**b**	0.4494**b**	0.3450**b**
Lee	0.0866**a**	0.1462**a**	0.1658**a**	0.1661**a**	0.2238**a**	0.3089**a**	0.3323**ab**	0.3345**a**	0.3327**a**	0.3837**ab**	0.3528**b**
Ozark	0.0635**a**	0.1142**a**	0.1319**a**	0.1860**a**	0.1987**a**	0.2667**a**	0.2768**a**	0.3298**a**	0.2490**a**	0.2903**a**	0.2517**a**
Desha	0.0700**a**	0.1112**a**	0.1414**a**	0.1637**a**	0.1982**a**	0.2666**a**	0.2714**a**	0.2841**a**	0.3132**a**	0.2623**a**	0.2699**ab**
Jake-CK	0.0507**a**	0.0543**a**	0.1289**a**	0.1739**a**	0.2239**a**	0.3379**a**	0.4587**cd**	0.6151**cd**	0.9169**d**	1.0709**d**	1.3017**d**
Lee-CK	0.0545**a**	0.0968**a**	0.1403**a**	0.2273**a**	0.2846**b**	0.3011**a**	0.5227**d**	0.5400**bc**	0.8381**cd**	1.0676**d**	1.1173**c**
Ozark-CK	0.0602**a**	0.0930**a**	0.1233**a**	0.1474**a**	0.1996**a**	0.4094**b**	0.3945**bc**	0.6528**d**	0.8722**d**	0.9013**c**	1.0750**c**
Desha-CK	0.0571**a**	0.0995**a**	0.1148**a**	0.1875**a**	0.2127**a**	0.3233**a**	0.3706**b**	0.5077**b**	0.7664**c**	0.9178**c**	1.1479**c**
	----------120 mM KCl - Leaf dry weight (g)----------										
Jake	0.0664**a**	0.0845**a**	0.1129**a**	0.1448**a**	0.1935**ab**	0.2631**ab**	0.2942**b**	0.2260**ab**	0.3601**b**	0.3188**b**	0.3566**c**
Lee	0.0677**a**	0.1102**a**	0.1349**a**	0.1532**a**	0.2308**b**	0.2594**ab**	0.2831**b**	0.2600**b**	0.3186**b**	0.3179**b**	0.2866**b**
Ozark	0.0604**a**	0.0979**a**	0.1233**a**	0.1643**a**	0.2005**ab**	0.2188**a**	0.2211**a**	0.2019**a**	0.2192**a**	0.2334**a**	0.2364**a**
Desha	0.0676**a**	0.0863**a**	0.1273**a**	0.1547**a**	0.1726**a**	0.2384**a**	0.2150**a**	0.2288**ab**	0.2293**a**	0.2194**a**	0.2402**ab**
Jake-CK	0.0507**a**	0.0543**a**	0.1289**a**	0.1739**a**	0.2239**b**	0.3379**c**	0.4587**d**	0.6151**d**	0.9169**e**	1.0709**d**	1.3017**f**
Lee-CK	0.0545**a**	0.0968**a**	0.1403**a**	0.2273**b**	0.2846**c**	0.3011**bc**	0.5227**e**	0.5400**c**	0.8381**d**	1.0676**d**	1.1173**de**
Ozark-CK	0.0602**a**	0.0930**a**	0.1233**a**	0.1474**a**	0.1996**ab**	0.4094**d**	0.3945**c**	0.6528**d**	0.8722**de**	0.9013**c**	1.0750**d**
Desha-CK	0.0571**a**	0.0995**a**	0.1148**a**	0.1875**a**	0.2127**ab**	0.3233**c**	0.3706**c**	0.5077**c**	0.7664**c**	0.9178**c**	1.1479**e**

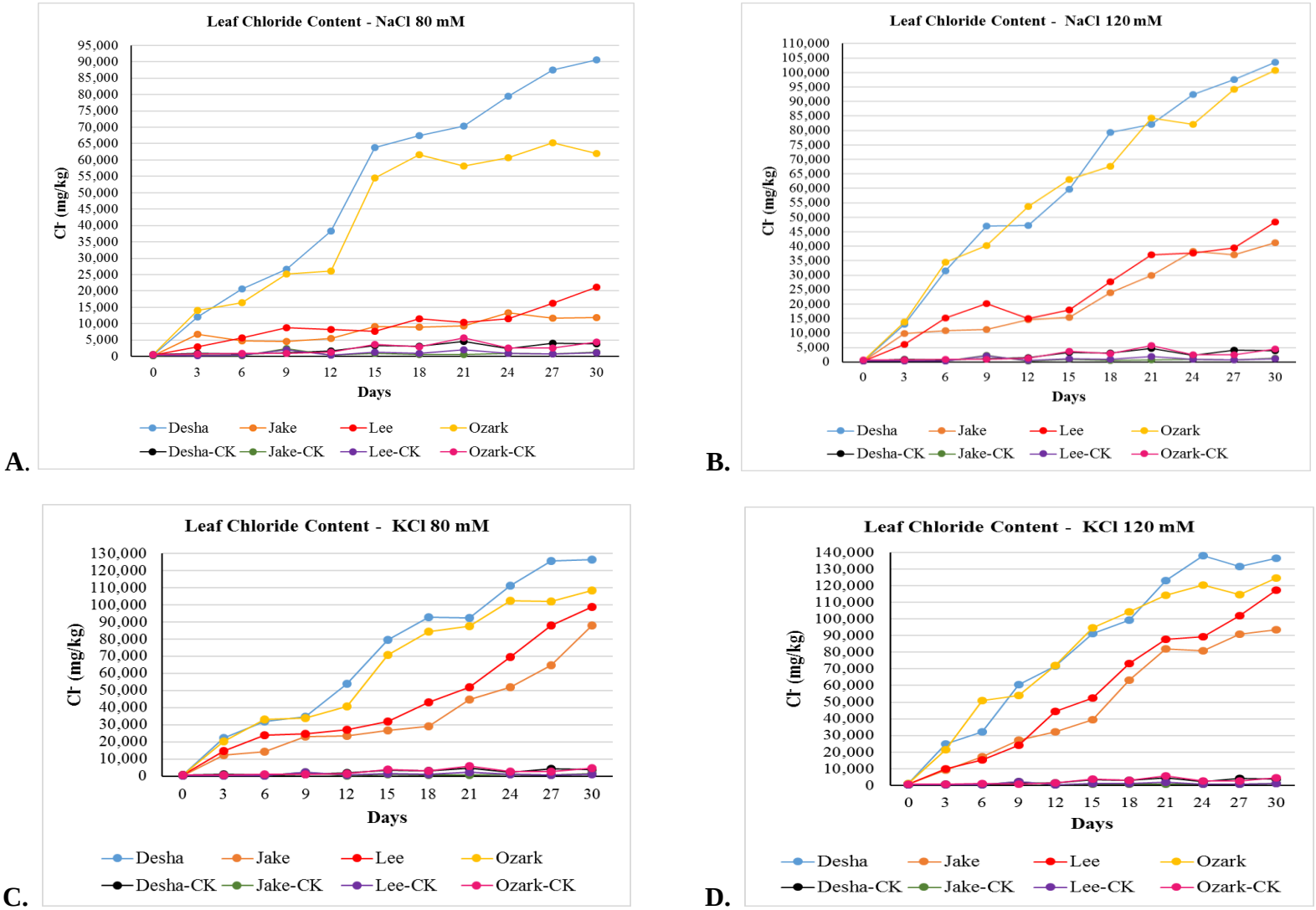

Figure 1. Leaf chloride (Cl^-) accumulation in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control).

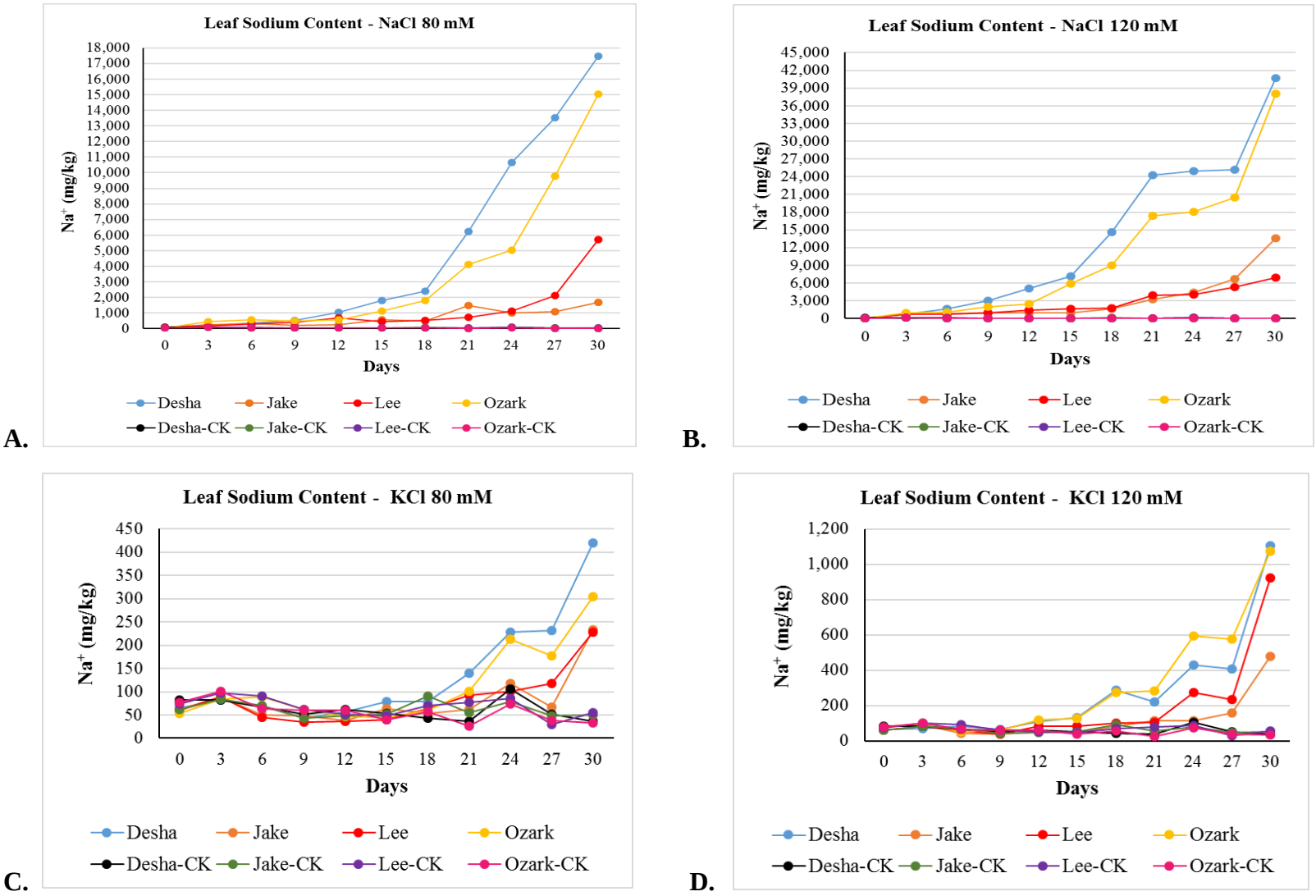

Figure 2. Leaf sodium (Na^+) accumulation in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control).

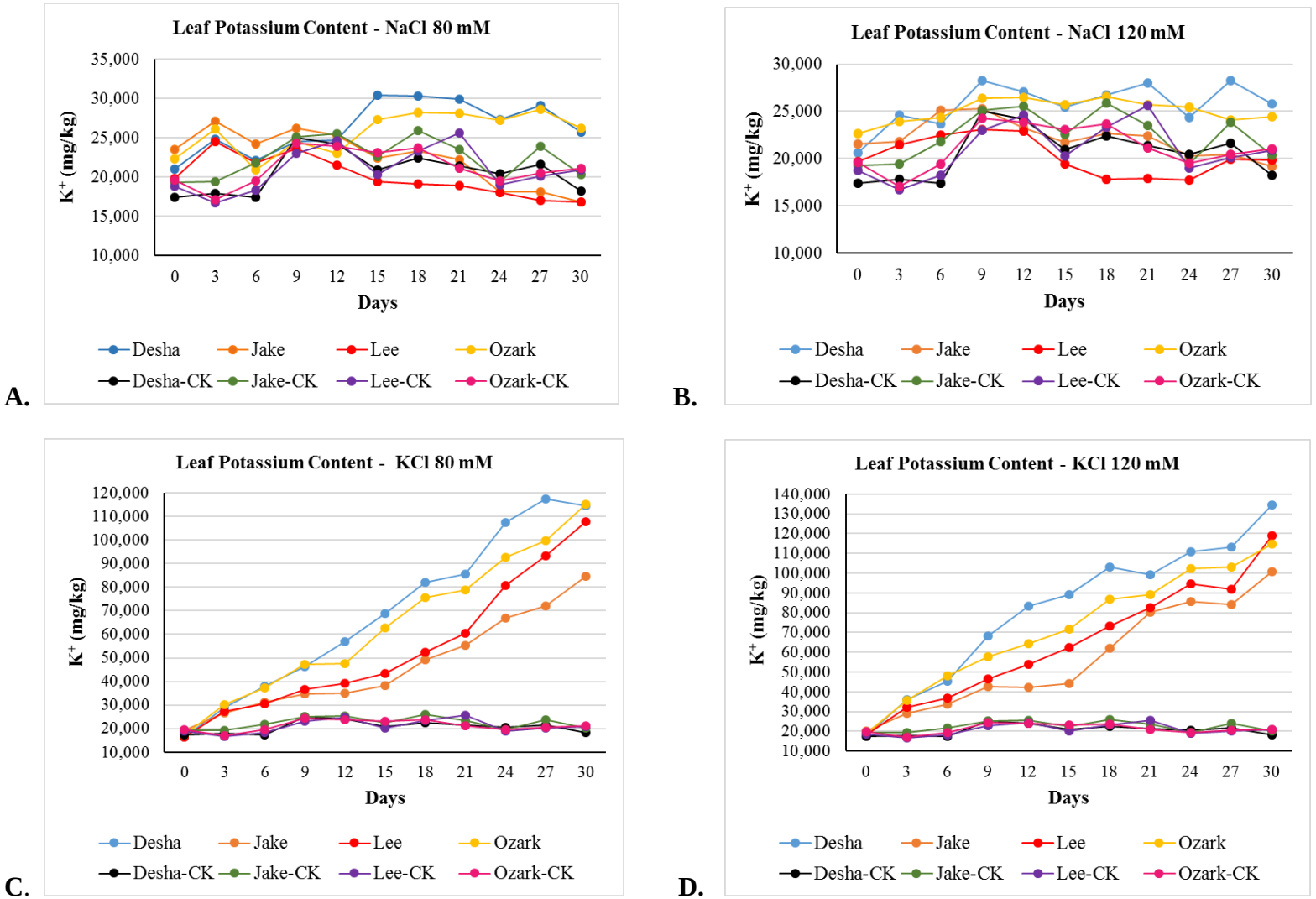

Figure 3. Leaf potassium (K^+) accumulation in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control).

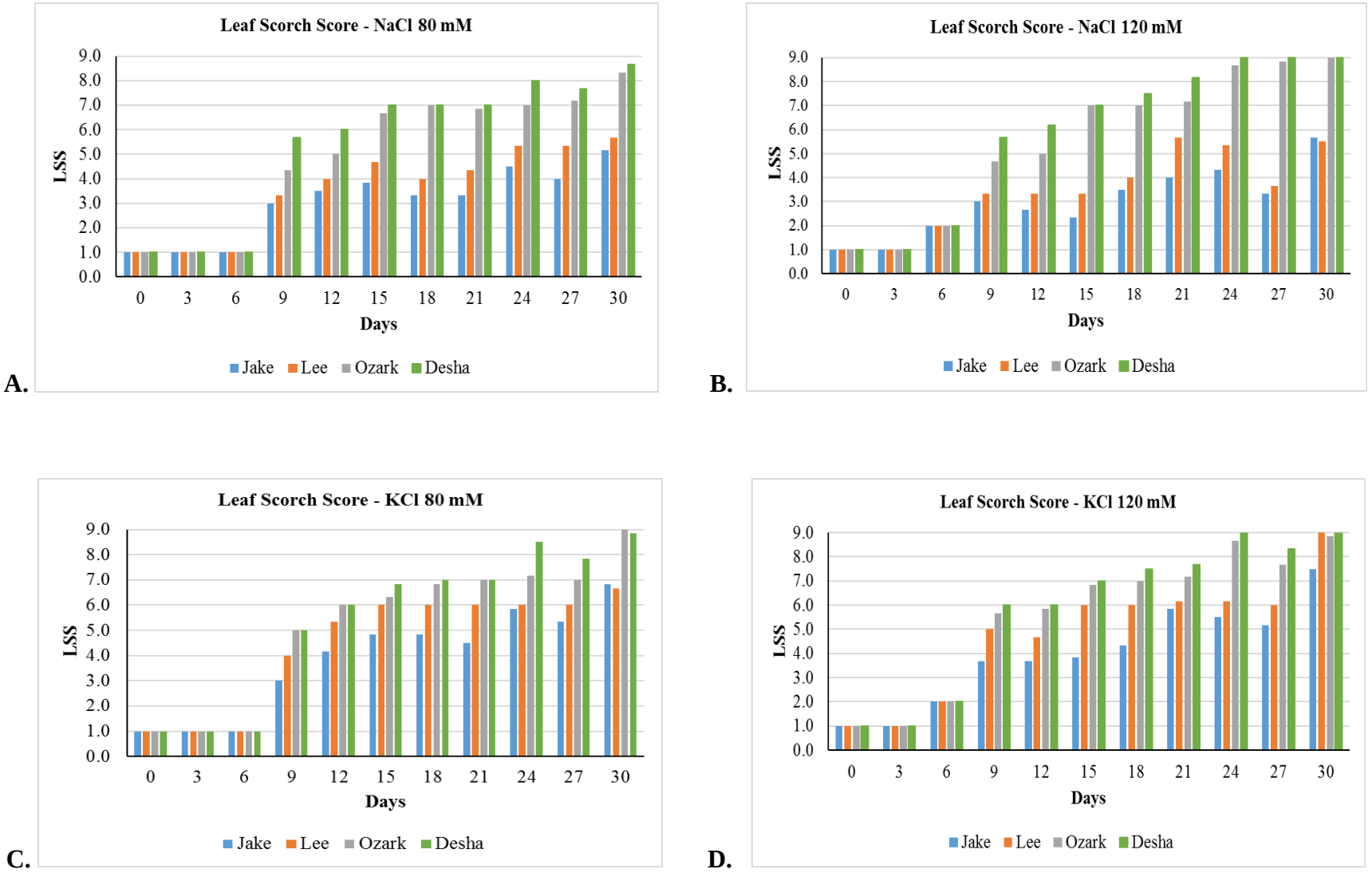

Figure 4. Leaf scorch score (LSS) (where 1= healthy dark green leaves and 9 = necrosis) in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D).

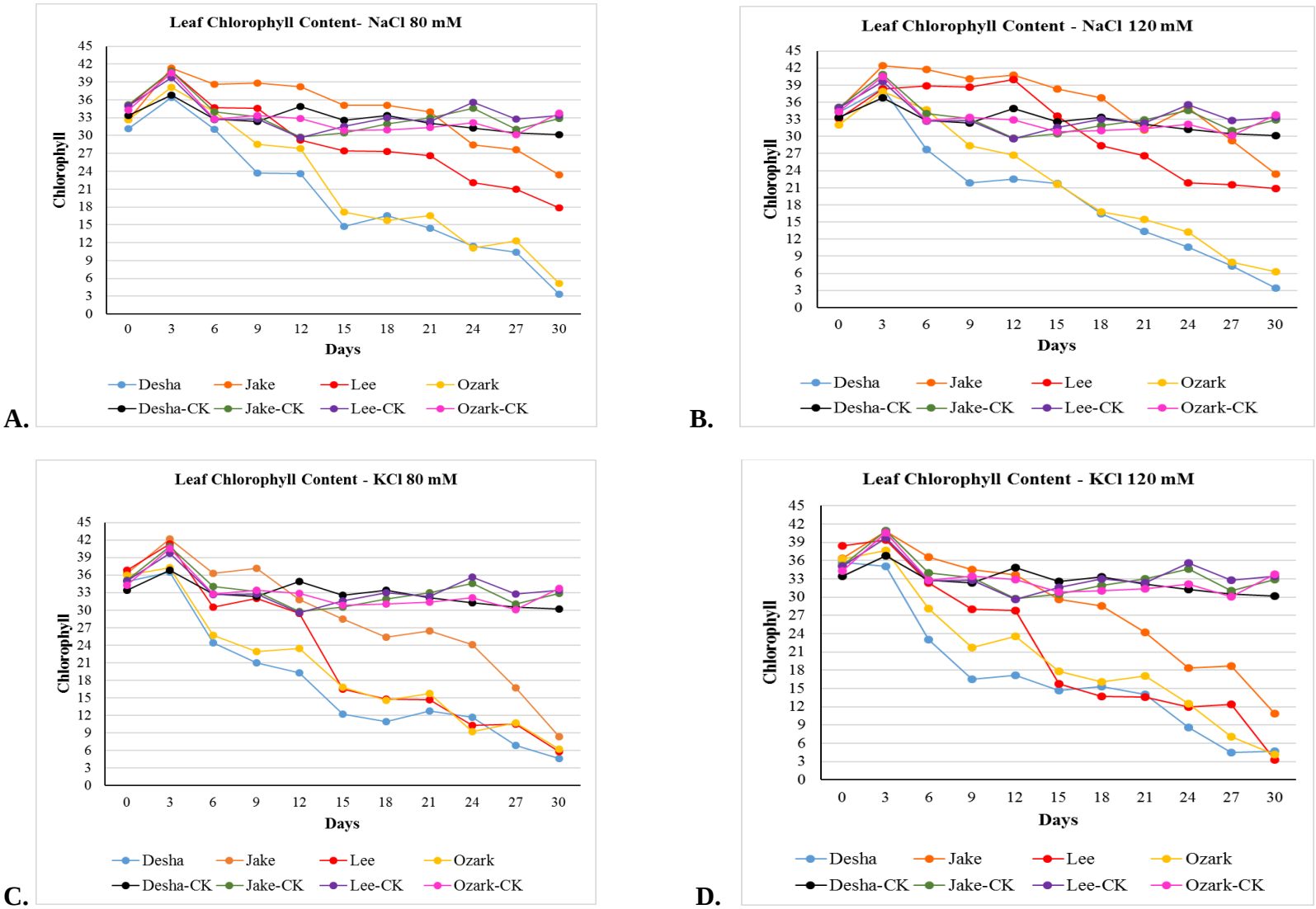

Figure 5. Leaf chlorophyll content in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control).

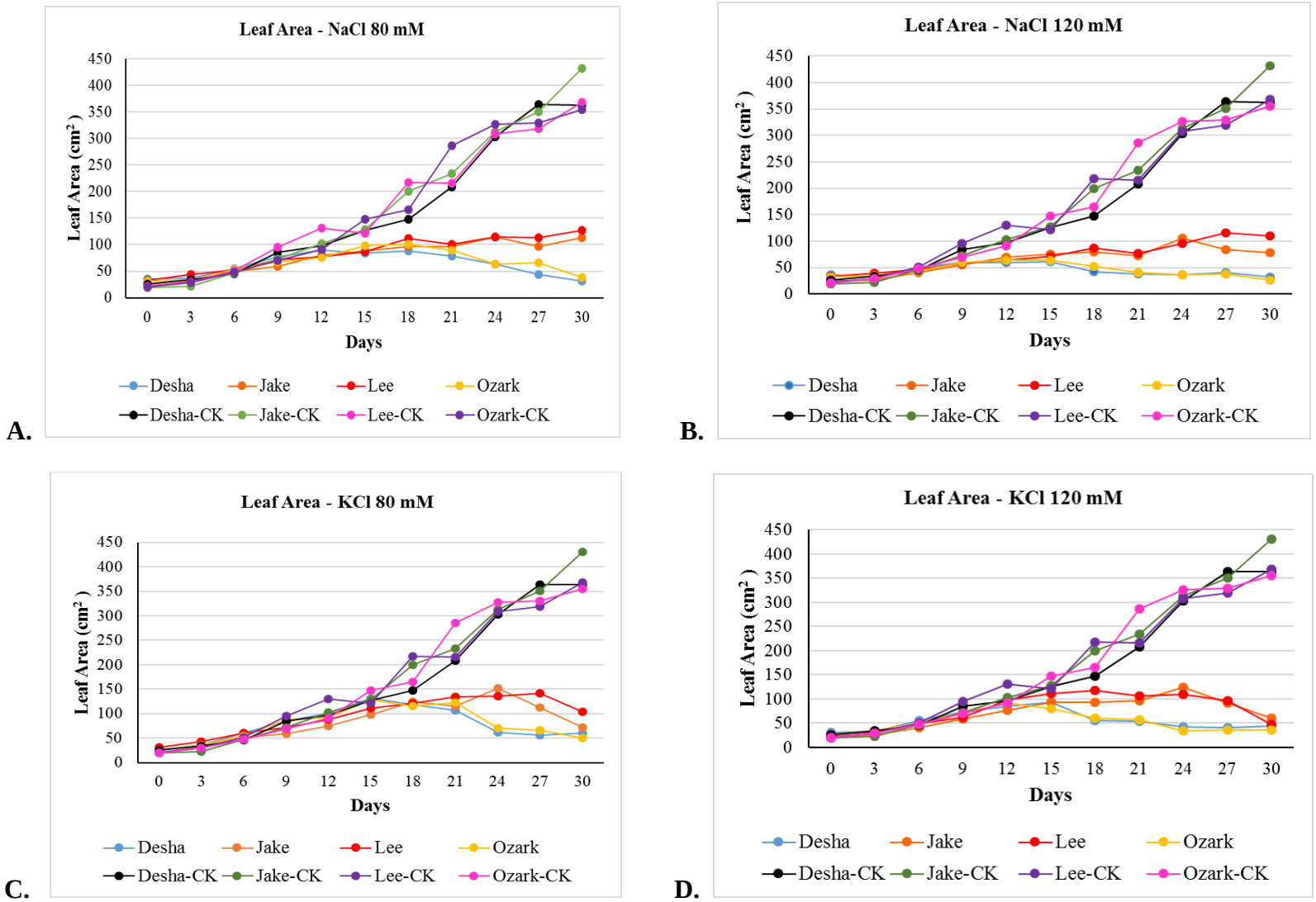

Figure 6. Leaf area (cm^2) in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control).

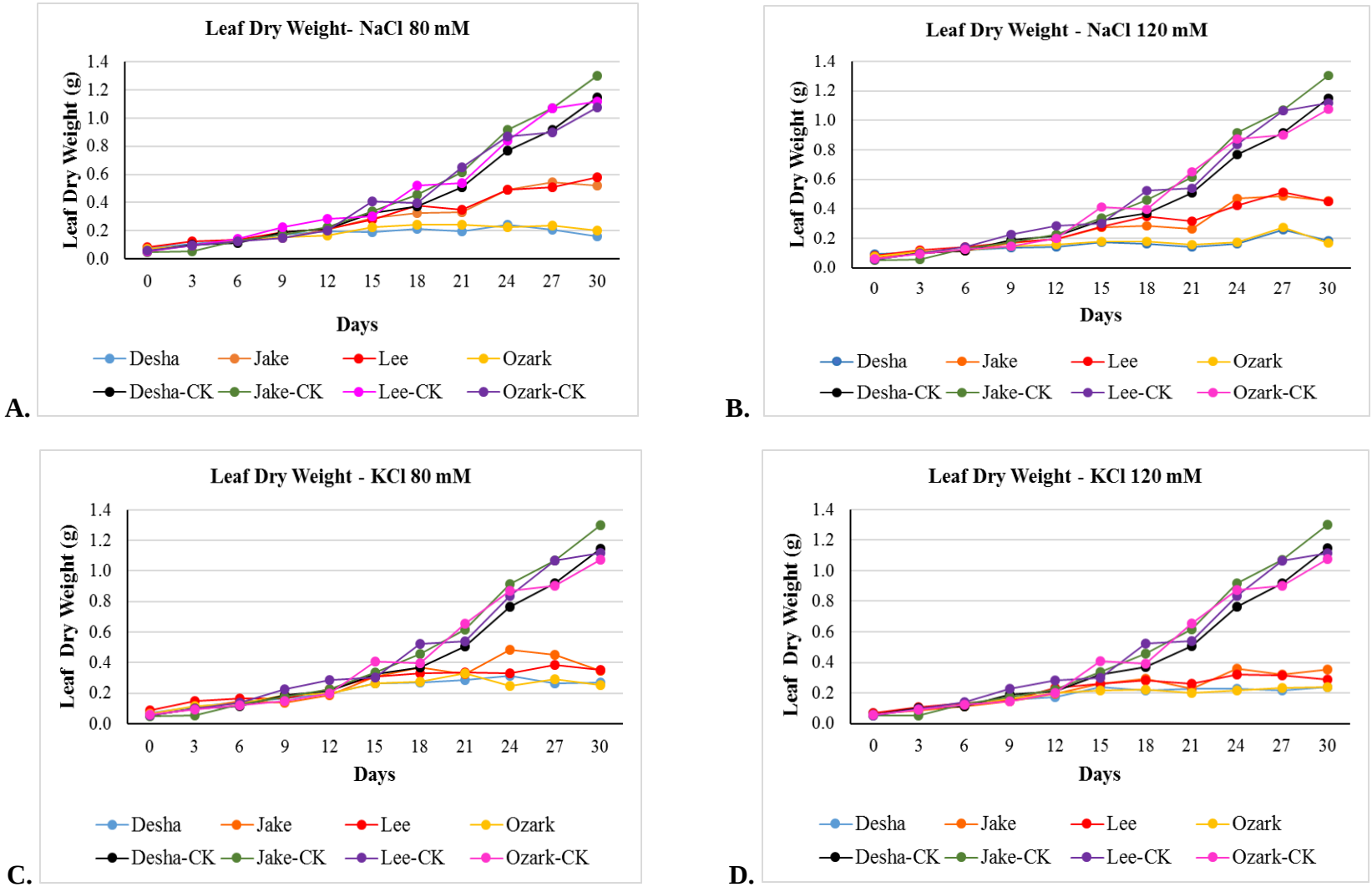

Figure 7. Leaf dry weight (g) in four soybean varieties during 30 days under four different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). Names of varieties followed by the letters 'CK' correspond to the results obtained under no salt application (control).

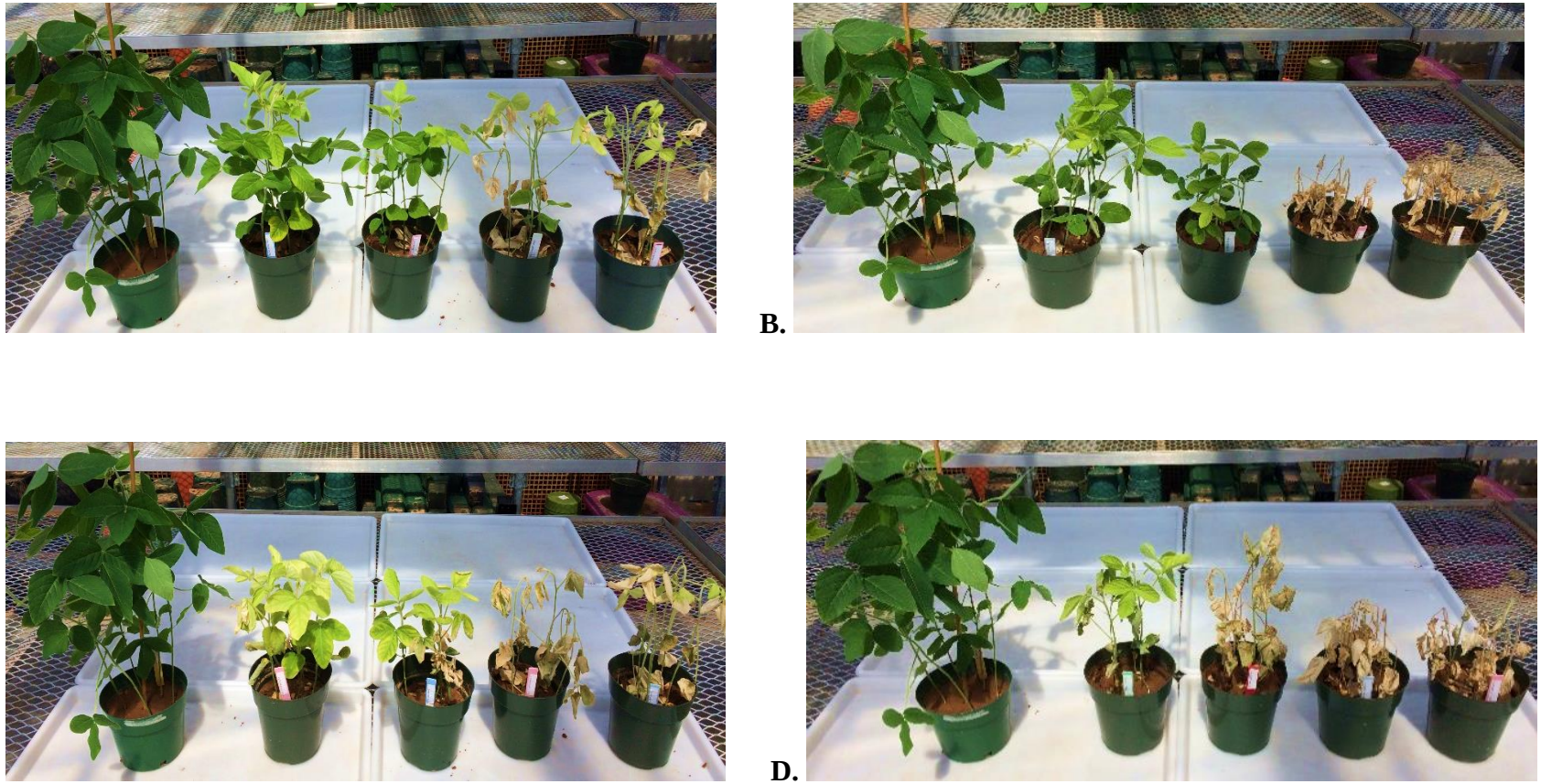

Figure 8. Chlorosis and necrosis symptoms in soybean varieties 30 days after being exposed to different salt treatments: 80 mM NaCl (A), 120 mM NaCl (B), 80 mM KCl (C), and 120 mM KCl (D). In each picture, we can observe from left to right: a plant under no salt treatment (control), Jake, Lee, Desha, and Ozark (Photo by author).

OVERALL CONCLUSION

Salinity is a major abiotic stress that adversely affects soybean crop productivity and quality. This thesis project aimed to find/confirm QTLs and molecular markers associated with traits involved in soybean salt tolerance response, in addition to evaluate the effects of the progressive ion accumulation in soybean salt-tolerant and salt-sensitive cultivars subjected to two major salt sources: NaCl and KCl. Findings from this research confirmed the presence of the major QTL on chromosome 3, which in this study was associated to all the evaluated traits (leaf scorch score, % dead plants, leaf chlorophyll and leaf chloride content), and revealed a new minor QTL associated to chlorophyll content. This highlights the essential role of that genome region in chromosome 3 in the salt tolerance response of diverse soybean germplasm, and at the same time, confirms that salt tolerance is a highly inheritable trait controlled by one or few major genes and multiple genes of small effect. In addition, after the measurement of leaf parameters, we observed that during early growth stage ion homeostasis in soybean works more efficiently under NaCl compared to KCl. Future work is needed in order to better understand the K^+, Na^+, and Cl^- contributions to soybean ion homeostasis.