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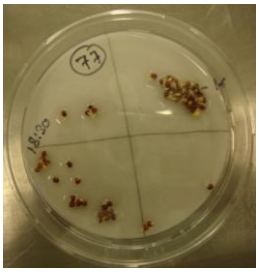
Identification of Quantitative Trait Loci for Seed and Seedling Vigor under Salt Stress Condition in a *Brassica rapa* Double Haploid Population



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June, 2011

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for partial fulfillment of ***Master of Sciences in Plant Sciences***
Specialization ***Plant Breeding and Genetic Resources***

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ABBREVIATIONS AND ACRONYMS

ACS	Automatic cofactor selection
ANOVA	Analysis of variance
AUC	Area under curve
AFLP	Amplified fragment length polymorphism
CMS	Cytoplasmic male sterility
cM	centiMorgan
DAG	Days after germination
DH	Doubled haploid
DNA	Deoxyribonucleic acid
DPI	Dots per inch
GW	Germination in water
Gmax	Maximum germination
HCl	Hydrochloric acid
IM	Interval Mapping
LR	Lateral root
LOD	Likelihood of Odds
MQM	Multiple QTL Model mapping
MGT	Mean germination time
NaCl	Sodium Chloride (Natrium)
NaOCl	Sodium hypochloride
NILs	Near isogenic lines
PCA	Principal Component analysis
PC	Principal Component
PVE	Phenotypic explained variance
QTL	Quantitative trait loci
Rdwt	Root dry weight
Rfwt	Root fresh weight
RILs	Recombinant inbred lines
RL	Root length
SI	Self incompatibility
SL	Shoot length
Sdwt	Shoot dry weight
Sfwt	Shoot fresh weight
SSR	Simple sequence repeats
T_{50}	Time required having 50% germination
OSR	Osmotic sensitivity response
u7525	Uniformity: time between 25% and 75% germination
WUR	Wageningen University and Research Centre

ABSTRACT

Seed vigor is a key determinant of seed quality influencing germination and seedling growth. Seedling vigor plays role in crop establishment under varying environmental conditions. *Brassica rapa* L. is a leading vegetable and oil crop and its growth and development is largely affected by salt stress. Salt stress is being a major constraint to increase production by affecting on crop establishment. Genetic improvement is an important strategy to develop salt tolerance, the genetic control of seed and seedling vigor under salinity conditions was studied through quantitative trait loci (QTL) mapping approach using a double haploid (DH) population consisting of 170 DH lines. The population was evaluated for 15 quantitative traits related to seed germination and seedling vigor at 0, 25 and 50mM NaCl concentrations. In total, 322 genetic markers were used for QTL mapping for all those traits. Root length and root weight were more sensitive to salinity than shoot and germination. Transgressive segregation was observed in both germination and seedling growth traits that indicate for the higher variation in traits and presence of quantitative control. In total, 83 QTLs were identified for all those studied traits in which 28 QTLs were at 50mM NaCl and followed by 17 QTLs at 25mM NaCl and 22 QTLs at control. The number of QTLs detected for root length, shoot length, fresh and dry weight, lateral roots and germination are 23, 14, 14, 3 and 5 respectively. Eleven major QTLs with explained variance greater than 14.0% were identified for germination parameters, root length; shoot length, lateral root and fresh and dry weight. In addition, six major QTLs were observed for salt sensitivity in root length. QTLs were widely distributed across 10 linkage groups with small effects suggesting traits as complex and polygenic nature. QTLs for seedling growth co-localized with respect to different salinity level and time points. Stronger QTL were obtained at 50mM NaCl and more pronounced in shoot length. It exhibited few co-localizations of QTLs in stressed and non-stressed condition. But QTL for germination traits and seedling growth traits were rarely coincided. It indicates that different genetic loci might regulate seed and seedling vigor traits. For some time points of root length and T_{50} of germination and shoot fresh weight QTL were identified in the same syntenic regions, where QTL for those traits were reported in *Arabidopsis thaliana*. This study identified hotspots for root length, shoot length, fresh as well as dry weight according to salinity levels. Thus, this study will be useful to find the candidate genes in future for seed and seedling vigor traits in *Brassica rapa*.

Keywords: *Brassica rapa*, double haploid (DH) population, seed and seedling vigor, salt stresses, quantitative trait loci (QTL)

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Chapter 1: INTRODUCTION

1.1 Background

1.1.1 *Brassica rapa*

Brassica rapa L. is a member of family *Brassicaceae* and genus *Brassica* comprises economically important diploid and amphidiploid species with wide range of morph-types. The genus *Brassica* has significant contribution with regard to economic and nutritional aspects thus are widely cultivated for vegetable, oil, fodder and industrial purposes (Ashraf and McNeilly, 2004). *Brassica* with diploid genome are *B. rapa* (AA, 2n=20), *B. nigra* (BB, 2n=16) and *B. oleracea* (CC, 2n=18) (Figure 1) from which other amphidiploid species *B. napus* (AACC, 2n=38), *B. juncea* (AABB, 2n=36) and *B. carinata* (BBCC, 2n=34) genomes have been developed from interspecific hybridization between these three common diploid species (Ashraf and McNeilly, 2004). *Brassica rapa* regarded as diploid species includes vegetable turnips, Chinese cabbage, Pak Choi, oilseed turnip rape and sarsoon (Teutonico and Osborn, 1994; Gomez Campo, 1999). *Brassica* seeds are basically used for the production of vegetable oil for consumption and propagation. Oil seed species of *Brassica* holds third position among the oilseed crops and important source of vegetable oils (Nazir *et al.*, 2001). The widely cultivated and common *Brassica* oilseed crops for industrial uses are rape-seeds, *Brassica campestris*, and *B. napus* as well as mustards, *B. juncea* and *B. carinata*.

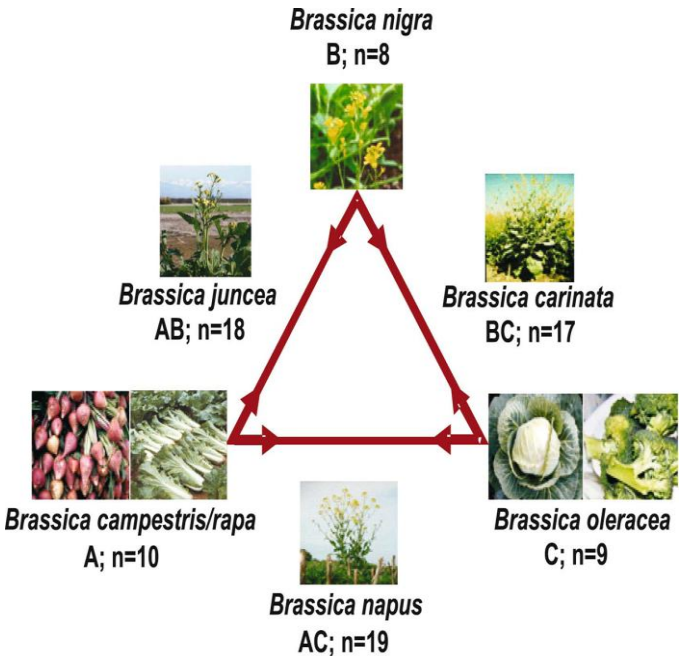


Figure 1. Genomic relationship between six *Brassica* species shown in "Triangle of U" diagram (Murphy, 1994).

Brassica rapa has been considered as first domesticated *Brassica* species from the ancient times. Due to long history of cultivation and breeding techniques employed, it has wide genetic diversity among the *Brassica* species (Zhao, 2007). Based on information obtained from the morphological, geographical and molecular studies, two centers of origin have been considered. Europe is assumed as a center of origin for oil and turnip type, and East Asia as center of origin for Indian types and Chinese leafy vegetables (Gomez Campo, 1999).

1.1.2 Seed and seedling vigor traits

Seeds are considered as fundamental basis for the propagation of crops and major source of nutrition for human beings and livestock feeds (Joosen *et al.*, 2010). Reduced crop yield from oilseeds is attributed to many factors, one of them being the quality of seed that is pre requisite for the crops (Ghassemi-Golezani *et al.*, 2010). Seed vigor determines seed quality, which directly leads to enhanced yield due to proper establishment of crop under detrimental environmental conditions (Ghassemi-Golezani *et al.*, 2010).

The seed quality is an important concern for both propagation and nutrition. The seed quality includes seed health, physical appearance, purity, seed and seedling vigor and size (Ellis, 1992). The nutritional value of seed is largely dependent on storage products that accumulate during seed filling phase of seed development; this is more significant in determining the quality of seed. Seeds are complex structures and the development is regulated by many unknown regulatory pathways. Other most important trait associated with seed quality is seedling vigor that is reflected by germination and subsequently seedling emergence and growth (Perry, 1978). Thus, the seed germination is a very complex characteristic governed by many genes, thus quantitative trait loci (QTL) analysis is essential (Bettey *et al.*, 2000).

Since germination is a crucial and complex phenomenon, salinity affects germination, radicle growth, seedling emergence and establishment (Joosen *et al.*, 2010). Most of the plant growth stages right from germination to fruit development are largely influenced by salinity resulting into reduced production and quality (Neumann, 1995; Sairam and Tyagi, 2004).

Germination and seedling vigor characteristics have greater significance for an early establishment under the stress conditions. Judicious selection of cultivars tolerant to salt stress that produce profuse germination could fulfill this requirement (Mahdavi and Seyed, 2007). Canola (*Brassica napus* L.) is one of the leading oil seed crop for which salinity regarded as one of the important abiotic stress affecting on seed germination and seedling emergence, growth and development eventually resulted in poor production.

The result of study on different salt concentrations (0, 5, 10, 15 and 20 $dS.m^{-1}$) on different canola cultivars revealed that there was reduced germination and also germination period was extended for all the cultivars due to increased salt concentration. The decreased germination rate resulted into poor seedling emergence and growth which ultimately affected the potentiality of canola to counteract the adverse environmental stress like cold stress (Bybordi and Tabatabaei, 2009).

It is an evident fact that faster and profuse germination governed by good seed quality that gives vigorous and robust seedlings consequently resulting in higher yield due to better plant performance. The seed vigor is controlled by basically three factors; genetic, environment during seed formation and accumulated storage substances in endosperm. The vigor also influenced by various abiotic factors like soil nutrients, water, temperature, humidity and harvesting stage (Sun *et al.*, 2007). The germination and seedling growth process are highly

dependent on the environmental conditions and seed quality itself particularly in small seeded crops such as *Brassica* species (Gusta *et al.*, 2004).

1.1.3 Major abiotic factors and salinity tolerance

Abiotic factors like high or low temperature, drought, high salinity create substantial stress to the plants during the growth and development and cause significant yield reduction (Purty *et al.*, 2008). The unfavorable environmental conditions like high or low temperatures, high salinity and drought conditions are responsible for affecting the germination by inhibition or by prolonging the duration of time of germination and growth. This is determined not only by the extent and duration of stress condition but also genetic constitution of the crop as well. Occurrence of salt or drought stress at the time of germination prevents seedling establishment of direct seeded crops (Ashraf and McNeilly, 2004; Foolad *et al.*, 2007). Globally, 50% annual yield loss have been observed for most of the crop plants that are attributed to drought, high salinity, high or low temperature and water logging (Ouyang *et al.*, 2007).

Among the various environmental stresses, salinity is more prominent and thus influence of this factors to the performance of crops is profound (Bartels and Sunkar, 2005). Salt stress adversely affect metabolic processes inside plants resulting into water deficit increasing osmotic stress (Bartels and Sunkar, 2005).

Soil salinity can be defined as the amount of salt present in the soil which can measured in terms of the electrical conductivity (EC) of an extracted soil solution (Flowers *et al.*, 1997). Soil salinization is generally associated under conditions where evaporation is greater than precipitation and in coastal areas subject to tides (Flowers *et al.*, 1997). Adverse effect of salinity in plant growth and development are caused by osmotic imbalance and high sodium ions which leads to toxicity. The effect of salinity is observed in early stage of development (Fageria, 1985). Salinity causes four detrimental effects on plants, namely osmotic stress, nutrient deficiency, ion toxicity and oxidative stress. (Xiong and Zhu, 2002).

Salinity, in general, arrests the germination due to development of osmotic potential (Khajeh-Hosseini *et al.*, 2003) and osmotic and ionic stress accompanied by oxidative stress (Zhu, 2000), upward movement and evapotranspiration of soil solution (Ashraf and McNeilly, 2004) which hinders absorption of water and nutrients due to the harmful effect of sodium and chloride on the seeds. There are many mechanisms related to salt tolerance reported (Yeo and Flowers, 1983). Firstly, salt exclusion: plants do not take up excess salt by selective absorption. Second, salt re-absorption: tolerant varieties absorb high salt but it is reabsorbed from the xylem and Na^+ is not trans- located to the shoot. Third, root-shoot translocation: salt tolerance is associated with high electrolyte content in the roots and a low content in the shoot. Fourth, salt translocation: tolerant plants have the ability to translocation a less proportion of Na^+ to the shoot. Fifth, salt compartmentation: excess salt is transported from younger to older leaves. Sixth, tissue tolerance: plants absorb salt but are transported and stored in vacuoles within leaves in order to lower the harmful effects on plant growth.

Seventh, salt dilution: plants dilute salt by fast growth rate and high water content in the shoot (Yeo and Flowers, 1983).

Salinity tolerance is a complex trait controlled by many genes (Zhu, 2000; Ouyang *et al.*, 2007; Joosen *et al.*, 2010). Thus such a complex nature of salt stress is posing great threat to breeding for salt tolerance due to lack of knowledge on salt tolerance mechanism of plant. (Zhu, 2000). *Brassica rapa* is also affected by salinity stress as consequences plant growth, seed and oil production has been reduced over years (Ashraf and McNeilly, 2004). The effect of salinity is more pronounced during the germination and early seedling stage than later stages of growth (Mohammadi, 2009). Moreover, it was reported that the amphidiploid species of *Brassica* were more tolerant to the salt stress as compared to the diploid species. (Ashraf, 2001).

The effect of salinity is apparent in *Brassica* species during the onset and termination of the process which reflects that the rate of germination was inhibited with higher salt treatment. Similar to the germination, the root and shoot emergence was sharply delayed with higher salt treatment which is more abundant in root growth as compared to the shoot growth (Jamil *et al.*, 2006; Bybordi and Tabatabaei, 2009). This effect was attributed due to the Na^{+} toxicity and imbalance in nutrient uptake (Bybordi and Tabatabaei, 2009). Salinity is known to affect the growth of hypocotyl and radicle. It has been found that salt stress has prominent impact on hypocotyl growth with higher concentration (Mahdavi and Seyed, 2007).

A study was also undertaken on six *Brassica* species to evaluate salt tolerance with three salt treatments 0, 100 and 200 $molm^{-3}$ demonstrated that the shoot growth was declined with increased salt concentration (Ashraf, 2001).

1.1.4 Genetic studies on QTL analysis

Segregating populations such as double haploid (DH) and recombinant inbred lines (RILs) are essential for the genetic study to identify variations in natural populations through QTL mapping approach. We also used DH which has been largely used in these kinds of genetic studies. Doubled haploid plants are obtained from the anther, microspores cultures and also from unfertilized ovules of the F1 progeny (Pink *et al.*, 2008). DH involves only one round of recombination which fixed favorable alleles present in the parents and there is no selection (Pink *et al.*, 2008). The advantages of DH populations are its reproducibility.

Quantitative trait loci (QTL) mapping approach is being an important approach to identify the genomic regions that control the traits, this approach has largely been used in *Brassica rapa* also to identify genomic regions associated with morphological and physiological traits (Zhao, 2007). However, very limited QTL studies are carried out on seed and seedling vigor traits. In a study in *B. oleracea*, QTL on seed vigor and pre-emergence of seedling growth traits were reported (Bettey *et al.*, 2000).

Salt tolerance study performed in natural population of *Arabidopsis thaliana* also identified a major QTL (76.6% percent variation explained, PVE) for green seedling, and four QTL for

root growth under 120mM NaCl concentration supplemented with MS medium cloned for germination, root growth and other traits (Ren *et al.*, 2010). They also found the co-localization of QTL for those traits. Two QTL in RIL population, Sha X Ler and four QTL in Sha. X Col population were found on germination rate (T_{50}) (Galpaz and Reymond, 2010).

Five QTLs reported for germination percentage which is co-localized with osmotic and salt sensitivity responses in Arabidopsis RIL population from a cross of Bay and Sha accessions. In addition, two QTL loci (SSR1 and SSR2) for salt sensitivity responses and four QTL loci (0SR1-0SR4) for osmotic sensitivity response were also identified in that study (Vallejo *et al.*, 2010). In Rice, a many minor and one major QTL *saltol* identified and largely studied for its gene expression. They have observed that most of genes for salt tolerance are down regulated at increased salt concentration (Alam *et al.*, 2011).

The study on salinity tolerance of plants during early stage of development has received the attention because very little information was available in the past efforts. Most of studies were focused at later developmental stages when the seedlings have already established. Few studied were done in *Arabidopsis* and rice during germination and early growth stage. However, information on salinity tolerance of *Brassica* particularly on *B. rapa* are very limited.

1.1.5 Genome organization of *B. rapa* in relation to *Arabidopsis thaliana*

Brassica rapa has genome size of 529Mb per haploid genome (Zhao, 2007), has close phylogenetic relation with *Arabidopsis thaliana,* also member of same family *Brassicaceae*. *Arabidopsis* is considered as model plant whose genome has been completely sequenced due to its reduced genome size and chromosome number (n=5) (Schranz *et al.*, 2006). It has been reported that *Arabidopsis* and *Brassica* diverged almost 14.5-20.4 million years ago (mya) from a common ancestor (Schranz *et al.*, 2006). Comparative genomic studies have demonstrated that the *Brassica* genome has undergone genome triplication, followed by deletions after divergence from a common ancestor shared with *Arabidopsis thaliana* (Schranz *et al.*, 2006; Yang *et al.*, 2006). Compared to *Arabidopsis*, *Brassica* species have undergone genome and chromosome rearrangements. The *B. napus* genome consists of 19 linkage groups (N1-19) that correspond with ten chromosome of *B. rapa* (N1-10) and nine chromosome of *B. oleracea* (11-19). Twenty one synteny genomic units were identified in *A. thaliana* genome that after triplication and rearrangement lead to evolution of present *B. napus* genome (Parkin IA *et al.*, 2005).

However, twenty-four conserved chromosomal synteny blocks reported in *B. rapa*, indicated as (A-X) with naming, order, orientation that share synteny with *Arabidopsis thaliana* (Schranz *et al.*, 2006) (Figure 2).

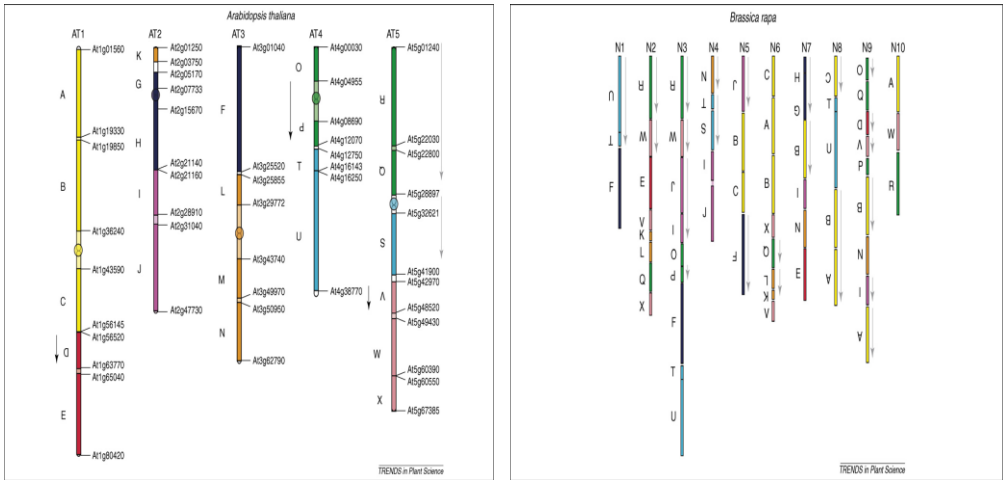


Figure 2. Genome blocks shown for Arabidopsis thaliana (n=5) and Brassica rapa (n=10) (Schranz *et al.*, 2006)

B. rapa genome possesses excess of repetitive DNAs with transposable elements over *A. thaliana* in connection with genome triplication attributed to the genome expansion (Lim *et al.*, 2006).

Salinity stress during germination and seedling growth has become one of the limiting conditions for the crop establishment, thereby higher production, the understanding of the genetics of salinity tolerance in *B. rapa* is essential to improve salt tolerance and crop loss. Thus, we determined to use QTL mapping approach for the study of genetic control of seed germination and seedling vigor under the different levels of salinity stress conditions.

1.2 Research hypothesis and objectives

The main objective of this investigation is to explore the influence of salt (NaCl) stress on germination and early seedling growth traits. The research will be mainly concentrated on identification of QTLs for seed germination and seedling vigor under control and salinity condition. Our hypothesis in this study was, we will be able to understand the genetic control of germination and seedling vigor traits under salinity stress in *Brassica rapa* with QTL mapping approach in double haploid population.

Research objectives:

1. To know the effect of different salt stresses on seed germination and seedling growth in *B. rapa*.
2. To identify the QTLs for seed germination and seedling growth under different salinity levels.
3. To compare the QTL for seed germination and seedling vigor in *B. rapa* to QTL in other Brassica species and *Arabidopsis* based on map synteny.
4. To compare QTLs for seed germination and seedling vigor under stress with non-stress conditions.

Chapter 2: MATERIALS AND METHODS

2.1 Plant Materials

The study was conducted on seed material of *B. rapa* DH68 population produced in late spring season of 2010. The population has been developed from a cross between a female parent Yellow Sarsoon YS143 (R500, USA, an annual oil crop with yellow seeds) that is self-compatible and a male parent Pak Choi PC175 (HKG Nai Bai Cai, China, a leafy vegetable with black seeds) that is self-incompatible. The DH68 is developed from three different F1 plants harvested from the same YS143 female parent plant consisting together of 170 DH lines obtained through microspore culture.

2.2 Seed preparation and sterilization

Seed from 126 lines out of 170 DH lines were used in this study because of seed availability. Among 126 DH line, six lines had no germination or very little germination. In total, only 120 DH lines including two parents were used for seed and seedling vigor assessment under normal and different salt stress levels. Seeds were sterilized overnight with fumes of sterilization solution. The sterilization solution was prepared by mixing 20ml demi water with 80 ml sodium hypochloride (NaOCl, 12%) and 3ml fuming hydrochloric acid (HCl, 37%) in a jar. Sterilization was done in exicatus in the fume hood overnight. The seed were kept in open eppendorf tubes and put on the jar overnight. The seeds were exposed to the open air at least before 3 hours when germination assay to be undertaken but no rinsing was done.

2.3 Preparation of agar medium and plates

For seedling vigor tests, agar medium was prepared by dissolving 8 grams micro-agar (0.8%, gel strength of >900 g/cm2) in a litter of demi-water with final pH of medium maintained at 5.8. The mixture was poured in glass bottle and was shaked well then autoclaved for 18 minutes at 121^0C temperature and 1.5 bar pressure. The solution after autoclaving was put in the thermo room for temperature normalization to 55^0C. The agar medium (80-90ml) poured into the rectangular plates (12x12x1.7cm) after shaking agar-solution to ensure well mix of agar. After approximately 30 minutes, gels were solidified. The one third portion of the agar in a plate was removed from the plate to create a space to deposit seeds on the vertical plate and to have space for shoot growth.

2.4 Pilot study for the selection of NaCl concentrations

A pilot study was conducted to know NaCl concentrations to give different levels of salt stresses so that there will be enough variation on germination, root and shoot traits among the DH lines in this population. Pilot study was done in 5 NaCl concentrations; 25, 50, 75, 100 and 150mM NaCl (Sodium chloride, molecular weight, 58.44), where mili-Molar (mM) is equivalent to $molm^{-3}$ (Personal Communication: Dr. Gerard van der Linden, Wageningen University and Research Centre, the Netherlands). In pilot study, there were no seedling growth at 75, 100 and 150mM NaCl concentrations, we chose 25mM, 50mM and control (0 mM) for phenotyping germination, root growth and shoot growth in DH population. The

amounts of salt for respective salt treatments were directly added to the agar medium in the bottle before autoclaving. Similarly, for the germination test the amounts of salt were added to the normal tap water and dissolved by shaking before application.

2.5 Seed germination and seedling growth test

Germination test was carried out for three levels of NaCl: 0mM **(**control), 25mM and 50mM. For germination test, Petri-dish (94x16mm) without vents was used. The filter paper was soaked in 5-10 ml NaCl solution and seeds were spread onto the filter paper. Two filter papers (88mm) were used in a Petri-dish so that the solution remains for longer time. Five to six Petri dishes were piled in a stack and the stacks were tightly pressed with the plastic tape to reduce the chances of evaporation. The Petri dishes were placed in the climate room with continuous light and temperature regime (21^0C) throughout the experiment. Thirty seeds were taken for the germination study from each line per treatment. The germination counting was performed in five time points in a day in three hours interval (9.00h, 12.00h, 15.00h, 18.00h and 21.00h). When there was radicle protrusion on seed, we considered as germinated seed.

The seedling growth study was performed under three NaCl concentrations (0, 25 and 50mM NaCl) with three replications in a randomized complete block design (RCBD). Germinated seeds within the same time period were transferred to the agar plates in the same day. In case of difference in radicle length, seed with uniform radicle length (seeds of same line that germinated in the same time period) were transferred into agar-plate for seedling growth experiment. Five seedlings were placed in each plate at an equal distance and covered with the plastic tape. The plates were randomized within replication and arranged in the tray in a slanting position in climate chamber at the constant temperature of 21^oC and continuous light. The seedlings were grown for ten days and root growth measurements were undertaken five times with 1, 3, 5, 7 and 9 days after germination (DAG). The root length was measured manually on 1, 3 and 5 DAG, while image analysis was done for 7 and 9 DAG. But shoot length was measured only on 1, 3 and 5 DAG manually because of time constraint and not enough variation based on visual observation. The pictures were taken for image analysis at one day intervals. It was carried out with standard digital camera (Nikon D 80) fixed to repro stand connected to computer with control pro software version 2.0 (Joosen *et al.,* 2010). On either side of camera two fluorescent tubes were fixed vertically. The setting option of Nikon under the format JPEG with F- stop(aperture) value of f/18, exposure time of one second, ISO-speed-400, exposure composition +2Ev as well as flash composition 0Ev having picture dimension of 4288x2848 and vertical/horizontal resolution of 300 dpi.

The image analysis was done with EZ- Rhizo software package to measure root length and count number of lateral roots (Armengaud *et al.,* 2009). This software combines windows-operated semi-automated computer model to analyze the root system parameter that are grown in solid (agar) medium. Before analysis the pictures were converted from JPEG format to *bmp* format. While converting to *bmp* picture settings and DPI (dot per inch) value 220 were fixed based on comparison of software results and manual observations, where both observations were approximately identical. This EZ-Rhizo software gives the measurement on root architecture traits such as main root path, main root angle and main root vector,

number of lateral roots, apical basal zone, and total root size and root depth in text file format. The result text file was converted to the Excel file format using EasyPHP database software (Armengaud *et al.*, 2009).

After 10 DAG, seedlings were taken out from the plates carefully to avoid breakage on main and lateral roots. The seedlings were washed with water to remove the agar from the roots. Root and shoot were separated and pressed in the white roll paper for two hours to absorb water before taking fresh weight of the sample. The samples were dried by putting in electric dryer at temperature of 70^0C for 16 hours, and dry weight of root and shoot were taken separately in weighing balance.

2.6 Data analysis

The statistical analysis were carried out for summery statistics and to calculate area under curve (AUC) for root growth, shoot growth and germination, and one way- analysis of variance (ANOVA) for germination, seedling vigor traits (root and shoot length), number of lateral roots and fresh as well as dry weight of root and shoot. Box plot and histogram were drawn to know the distribution of traits. The statistical analysis was carried out using R package (Venables *et al.*, 2009) and Genstat 13th edition (Payne *et al.*, 2009).

2.6.1 Summary statistics, Box plot and Histogram

The summary statistics was calculated for all traits measured in this experiment. The summary statistics includes mean, median, standard deviation, variance and range of each trait. The summary statistics also gives the general information about the nature of data, growth pattern of root and shoot over the time periods and distribution pattern of trait at different time points. Summary statistics were calculated using Genstat 13th edition (Payne *et al.*, 2009).

The box plot applied generally for the checking the distribution pattern of the observed values and also the normality of the data as well. This is one of the ways of graphically depicting the numerical data. Thus it is an essential measure to know distribution or skewness of data in the population. Histogram is also a tool for graphical representation for distribution pattern of the data. It is an estimate of probability distribution and provides the overview on the distribution of mean and variance of traits and also used to know the distribution of data such as normality or skewness. Box plot and histogram were made using Genstat 13th edition (Payne *et al.*, 2009).

2.6.2 Area under curve (AUC)

Area under curve (AUC) was estimated for germination and roots and shoot growth under different NaCl concentrations. AUC was calculated for curve of each DH line based on the trapezoid area formula. First, area of each trapezoid considered based on each time points were calculated and later summed the area of all trapezoids made on each curve of DH line. AUC value measure the speed of root or shoot growth or germination of each DH line, which

also one of the important parameter to measure seed or seedling vigor. AUC calculation was done by using R software.

2.6.3 Germinator curve-fitting script

Germinator package (Joosen *et al.*, 2010), a specialized software package to measure different traits related to seed germination, was used to calculate maximum germination (G_{max}), time to reach 50% germination (T_{50}), uniformity: time between 25% and 75% germination (u7525), AUC and Mean germination time (MGT). From the curve fitting procedure of Germinator package, germination curves were made based on seed germination of each DH line over the time points. It is essentially applicable to the cumulative germination data from all the plant species.

2.6.4 Salt sensitivity response (SSR)

It was derived from the phenotypic difference on root traits under two consecutive treatments divided by the phenotypic data obtained at control (non-stress) conditions. This method was mainly applied in case of QTL mapping. It gives an idea about how large is effect of increasing salt stress with respect to plant response.

2.6.5 Principal Component Analysis (PCA)

PCA is a multivariate data visualization technique that is commonly used to identify the grouping pattern of samples, in this case DH lines, and variables such as root, shoot and germination traits. It is a dimension reduction technique by making new latent variables (also called principle components, PC), where first latent variables retains the largest amount of variation present in data. Similarly, second latent variables retains second largest amount of variation and other subsequent latent variables retains direction of greatest variability (Holland, 2008). PCA was performed for the root and shoots growth traits and germination separately using "princomp" function available in R-software.

2.7 Construction of genetic linkage maps

The pre-requisite step to perform the QTL analysis is construction of genetic linkage map which was done with JoinMap 4 (Ooijen, 2009). The markers used in the mapping function were mainly AFLP; Myb targeted profiles, SSRs and gene-targeted markers involved in flowering time, tocopherol and carotenoids pathway and glucosinolates pathway. In total, 120 DH lines and 322 marker loci were utilized for map construction using JoinMap 4.

2.8 QTL analysis and visualization of QTL on map

The QTL analysis was performed to identify genomic regions controlling traits of interest using interval mapping (IM) and Multiple QTL model mapping (MQM) approach in MapQTL version 6.0 (Ooijen, 2009). To carry out the QTL mapping three types of data files are needed as input files. The genotype file, called loc file, contains marker data of the segregating population representing genotype locus. The second input file is called map file which provides information about map position of each marker genotype and third one is quantitative data file (qua file) which presents all phenotypic trait value.

QTL analysis carried out with the interval mapping (IM) method that gives putative position of QTL in the linkage group, and then MQM was performed to include co-factor to avoid false QTL caused by co-factors. The co-factor markers were selected based on automatic cofactor selection (ACS). Automatic cofactor selection takes into account the backward elimination by reducing the effects of other markers present within or beyond the linkage groups (Ooijen, 2009). The markers that were at peak position of QTL regions or possible QTL in different groups were considered for co-factor analysis. LOD refers to logarithm of the odds (to the base 10). Generally, LOD value of three or above is considered that illustrates two loci of genes in a chromosome are located close each other. LOD score of three refers to the odds are a thousand to one in case of genetic linkage (Ooijen, 2009). To determine the significant QTL, genome-wide LOD value at 95 percentile was chosen as a threshold after permutation test (1000 counts) and map interval of 5cM for both IM and MQM approach. The QTL mapping was carried out for germination related parameters (AUC, T50, MGT and u7525), root and shoot growth, number of lateral roots and fresh and dry weight of root and shoot, AUC for root and shoot growth and salt sensitivity response.

Since QTL analysis was done on many traits under normal and different salt stress treatment, we used Map Chart version 2.2 (Voorrips, 1999 - 2006) to visualize QTL of all the traits in single linkage map. 2-LOD support interval was drawn for each QTL by dropping 2 LOD from the peak position of that particular QTL. Different colors used to mark QTL according to traits.

2.9 QTL comparison between *Brassica rapa* and *Arabidopsis thaliana*

QTL results of different traits under different treatments were compared with *Arabidopsis* results obtained from literatures. Based on comparison of *B. rapa* and *Arabidopsis* genome, 24 genomic blocks identified in *Arabidopsis* were triplicated in *B. rapa* (Schranz *et al.*, 2006). In this study, we compared linkage group of *B. rapa* and chromosome of *Arabidopsis* having QTL for the same trait and identified the common genomic blocks based on genome comparison (Schranz *et al.*, 2006).

Chapter 3: RESULTS

3.1 Germination

Germination experiments were done in 0mM (control), 25mM and 50mM NaCl stresses. Time to reach 50% germination (T_{50}) and mean germination time (MGT) were significantly different between control and 50mM NaCl ($P<0.05$). Area under curve (AUC), maximum germination (G_{max}) and uniformity of time between 25% and 75% germination (u7525) between treatments were not significantly different (Table 1). However, the T_{50} and MGT had increasing pattern according to levels of NaCl concentrations, while AUC had decreasing pattern (Table 1). The T_{50} (22.20 hours) and MGT (22.25 hours) were earlier in control than T_{50} (24.22 hours) and MGT (24.47 hours) at 50mM NaCl. Similarly, maximum germination (G_{max}) was 98% at control, 96% at 25 mM NaCl and 97% at 50 mM NaCl.

Table 1. ANOVA results for germination traits of *B. rapa* DH68 population in different NaCl (0mM, 25mM and 50mM) concentrations.

Treatments	*T_{50} (hr)*	*MGT (hr)*	*G_{max} (%)*	*AUC*	*u7525 (hr)*
Control	22.20 a	22.25 a	0.98ns	40.23 ns	4.04 ns
25mM	23.22 ab	23.45 ab	0.96ns	38.30 ns	3.95 ns
50mM	24.22 b	24.47 b	0.97ns	37.74 ns	4.32 ns

Note: For each trait, mean values with different letters indicates for significant difference ($P<0.05$) and ns for non-significant difference.

The mean germination percentage of DH lines was observed higher in control in all time points measured while in germination percentage remains almost close in 25 and 50mM NaCl concentrations (Figure 3). The germination pattern of the parents and the DH lines were shown in graph (Figure 5). The graph showed that parent YS143 germinates earlier than PC175 in terms of T_{50} at all the NaCl level. Both parents germinated early as reflected by (T_{50}) in control and 50mM than 25mM NaCl. The histogram showed transgressive segregation for the germination traits as depicted in Appendix 14.

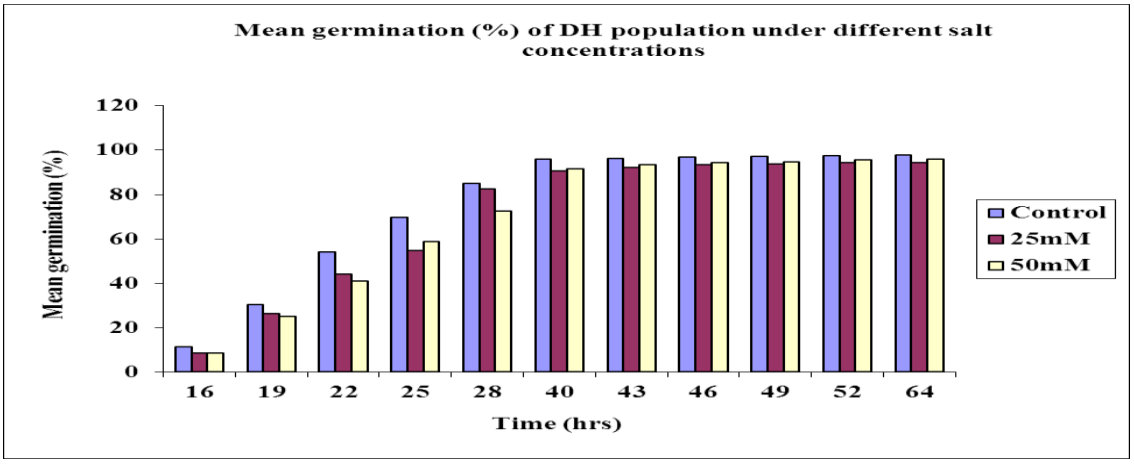


Figure 3. Mean germination percentage of DH population and parents in different salt concentrations (0, 25 and 50mM NaCl).

The PCA biplot based on germination test indicates that the different germination parameters were not grouped according to levels of NaCl treatments. The parents were very close which indicates similar sensitivity to salinity stress. It also clearly indicates that both T50 and MGT were highly correlated under all salinity level and more correlation was observed between 25 and 50mM NaCl concentrations. These germination traits were not correlated (Figure 4).
The biplot in Figure 5 depict that u7525 had higher variation as compared to AUC,T_{50} and MGT parameters which was more pronounced at 25mM NaCl.

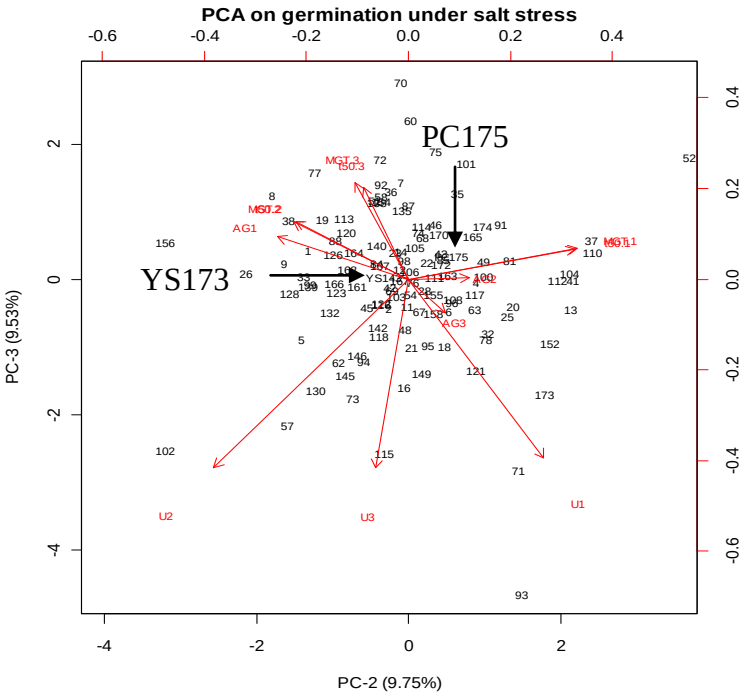


Figure 4. PCA biplot based on germination traits of DH population and parents at different treatments (0, 25 and 50mM NaCl). The traits names are indicated in red color where AG denotes area under curve for germination, u for u7525, t50 for time to 50% germination, MGT for mean germination time figure after decimal represents treatments (1= control, 2= 25mM NaCl and 3= 50mM NaCl).

The wider variation in uniformity of germination time (u7525) at 25mM NaCl as compared to control and 50mM might be because of different response of DH lines at 25mM NaCl thus different genetic influence. This DH line seems outlier which might be either more sensitive on levels of salt stress.

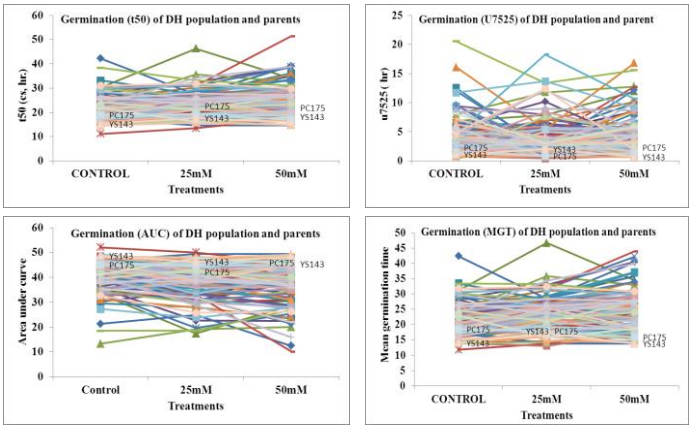


Figure 5. Germination parameters of DH population and parents in different treatments. T_{50}, u7525, AUC and MGT.

3.2 Root length

The statistical analysis showed that NaCl treatments had a highly significant effect (P<0.001) on root length measured at 1, 3, 5, 7 and 9 days after germination (DAG) for the DH population (Table 2). The final mean root length observed in control, 25mM and 50mM were 3.21cm, 2.63cm and 1.459cm respectively. The patterns of the root growth of two parents and DH population in all treatments were depicted in the graph (Figure 6). The graph shows that the longest root lengths observed in YS143 were 7cm, 5.1cm and 2.5cm in control, 25mM and 50mM NaCl respectively as compared to 2.4cm, 2.0cm and 1.7cm in PC175 (Figure 6). The mean root length of the population was decreasing with the higher levels of NaCl and the difference was bigger at later stages of growth (Table 2).

Table 2. ANOVA results for root length of *B. rapa* DH and parent in different NaCl (0, 25 and 50mM) concentrations

Treatments	*Root length (cm)*				
	1DAG	*3DAG*	*5DAG*	*7DAG*	*9DAG*
Control	0.56 a	1.27 a	2.29 a	2.75 a	3.21 a
25mM	0.40 b	0.87 b	1.56 b	2.07 b	2.63 b
50mM	0.34 c	0.53 c	0.89 c	1.22 c	1.46 c

Note: For each trait, mean values with different letters indicates for significant difference (P<0.05) and ns for non-significant difference.

It showed that mean, standard deviation, variance of the DH population for root length in control is 3.21cm, 1.17cm, and 1.37cm respectively at 9 DAG. Similarly, the highest mean root length after 9 DAG at 25mM was 2.62cm with standard deviation 0.93 and variance 0.87. The mean root length at day 9 in 50mM NaCl condition was 1.46cm with standard deviation 0.56 and variance 0.31 (Table 2). The graph depicts that some DH lines exhibit transgressive segregation pattern in all growing conditions (Appendix 11). The root growth patterns of DH population as well as two parents were decreasing with the increased levels of NaCl (Appendix 1). The summary statistics for root growth under different treatments is presented in Appendix 4.

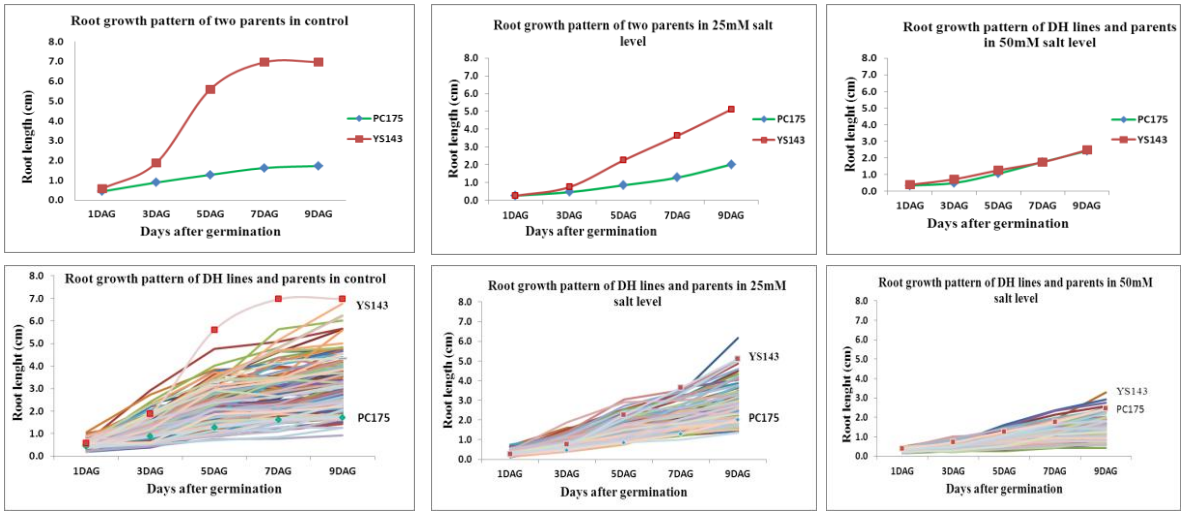


Figure 6. Root growth pattern of DH population and two parents in different NaCl (0, 25 and 50mM) concentration

The PCA on root growth showed that the effect of NaCl on root growth was distinctly visible on root length at different time points. There was less correlation between the levels of NaCl treatments. PC175 was grouped towards 50mM NaCl thus assumed as salt tolerant in while YS143 as salt sensitive in terms of root growth (Figure 7).

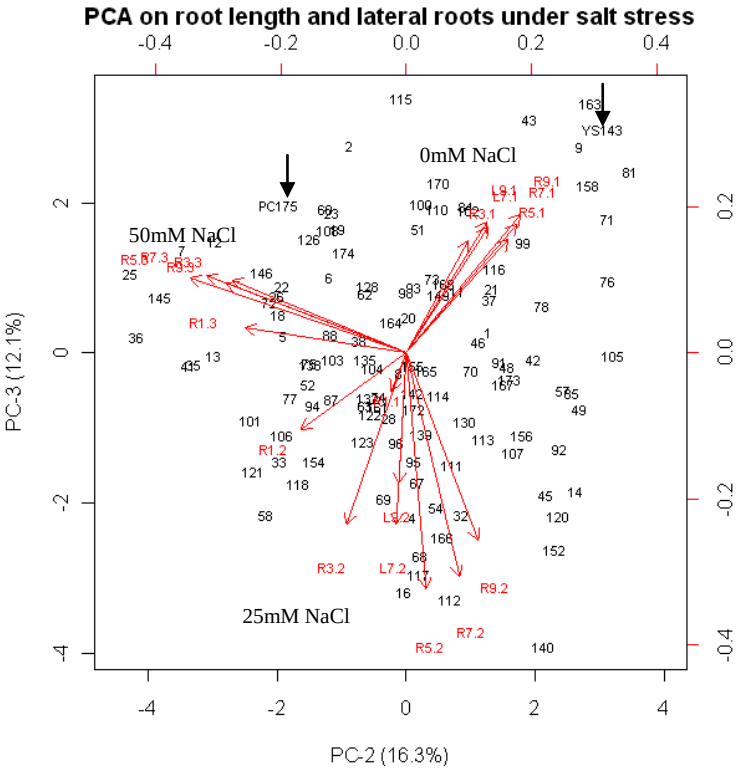


Figure 7. PCA Biplot on root growth pattern of DH population and parents in different treatments (0, 25 and 50mM NaCl). The traits names are indicated in red where R denotes root length; L for lateral roots, figures followed by these letters indicate days after germination and last figure after decimal represents treatments (1= control, 2= 25mM NaCl and 3= 50mM NaCl).

3.3 Shoot length

Shoot length was significantly different according to levels of NaCl concentrations at all time points considered in this study (P<0.001)(Table 3). The result revealed that mean shoot length of the population was decreasing with higher NaCl levels for all the time points. The mean shoot lengths of the DH population with parents after 5 DAG were 1.03cm, 0.71cm and 0.51cm in control, 25mM and 50mM NaCl concentration respectively (Table 3, Appendix 5). The shoot growth of the two parents and DH population at three different NaCl levels showed decreasing pattern (Figure 8). Some DH lines show transgressive segregation in all the treatments (Appendix 12).

Table 3. ANOVA result for shoot growth of *B. rapa* DH population in different NaCl (0, 25, 50mM) concentrations

Treatments	*Shoot length (cm)*		
	1DAG	*3DAG*	*5DAG*
Control	0.33 a	0.58 a	1.03 a
25mM	0.20 b	0.50 b	0.71 b
50mM	0.26 c	0.38 c	0.51 c

Note: For each trait, mean values with different letters indicates for significant difference (P<0.05) and ns for non-significant difference.

YS143 had higher shoot length of 1.45cm, 0.9cm, and 0.65cm than PC175 with 0.87cm, 0.8cm and 0.61cm at 5DAG in control, 25mM and 50mM NaCl respectively (Figure 8).

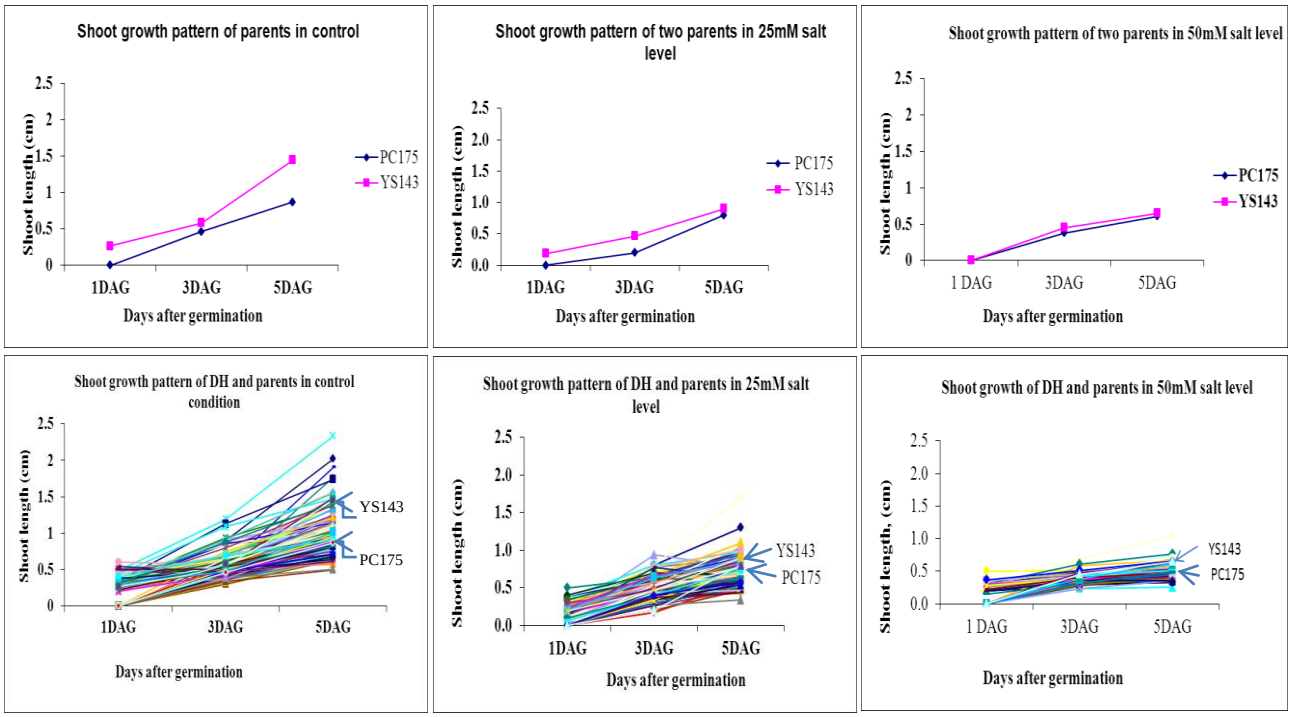


Figure 8.Shoot growth patterns of DH population and parents in different (0, 25 and 50mM) NaCl concentration

In PCA biplot based on shoot length, there were not distinct groupings of shoot length at day 1, 3 and 5 according to different NaCl concentrations. PC175 is observed as more salt

tolerant to 50mM NaCl because it is grouped towards 50mM NaCl as compared to other parent YS143. The traits were more correlated under 50mM NaCl as compared to control and 25mM NaCl (Figure 9).

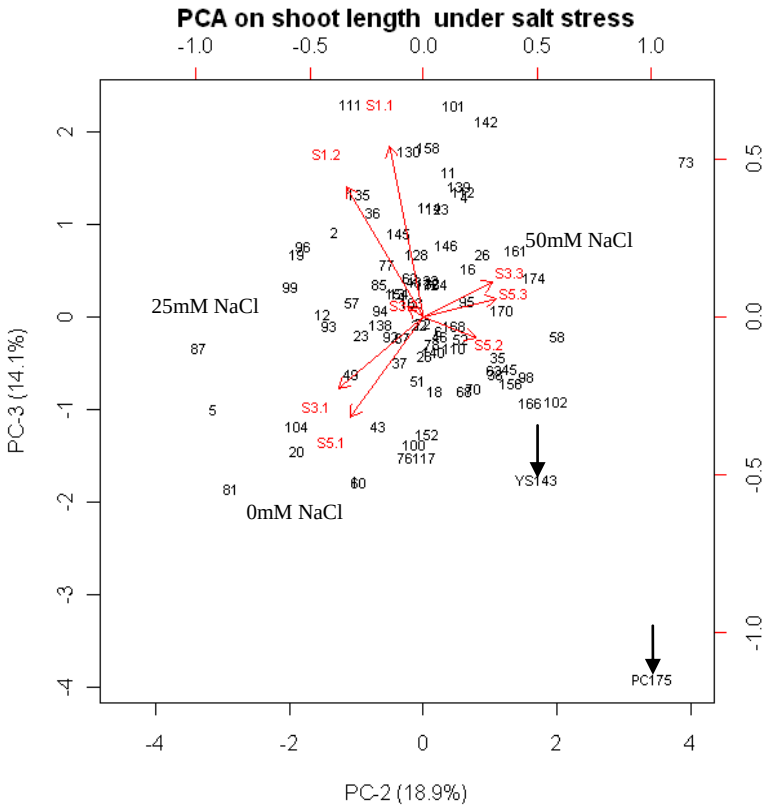


Figure 9. PCA Biplot of shoot growth pattern of DH lines and parents in different treatments (0, 25 and 50mM NaCl). The traits names are indicated in red where S denotes shoot length figures followed by letter indicate days after germination and last figure after decimal represents treatments (1= control, 2= 25mM NaCl and 3= 50mM NaCl).

3.4 Fresh and dry weight

Root Fresh and dry weight of DH population were significantly different (P<0.001) between all the treatments. However, shoot fresh and dry weight were not significant different between control and 25mM NaCl, but both are found significantly different with 50mM NaCl. The mean fresh and dry weights of both root and shoot were decreasing with application of higher NaCl concentrations. The reduction in biomass and dry matter upon salt stress observed higher in root than that of shoot, and consequently the difference was more pronounced in 50mM NaCl (Table 4). The fresh and dry weight distribution also showed transgressive segregation pattern as depicted in Appendix 13.

Highly significant differences (P<0.001) on root to shoot ratio were found for both fresh and dry weight (Table 5). Highest root to shoot ratios in control were 0.14 and 0.35 for both fresh and dry weight respectively, and it has decreasing trend with increased salinity.

Table 4. ANOVA result for fresh and dry weight of *B. rapa* DH population in different NaCl (0, 25 and 50mM) concentrations.

Treatments	*Root fresh weight (mg)*	*Root dry weight (mg)*	*Shoot fresh weight (mg)*	*Shoot dry weight (mg)*
Control	62.6 a	13.69 a	428.3 a	40.55 a
25mM	55.0 b	10.70 b	430.7 a	37.41 a
50mM	13.7 c	4.21 c	227.9 b	32.66 b

Note: For each trait, mean values with different letters indicates for significant difference (P<0.05) and ns for non-significant difference.

The result reflected that reduction in root to shoot ratio for fresh weight is more pronounced than in dry weight. The ratio was higher in dry weight than in fresh weight (Table 5).

Table 5. ANOVA results for root to shoot ratio of fresh and dry weight of *B. rapa* DH population in different NaCl (0, 25 and 50mM) concentrations.

Treatments	*Fresh weight Root/Shoot*	*Dry weight Root/ Shoot*
Control	0.14 c	0.35 c
25mM	0.13 b	0.29 b
50mM	0.06 a	0.16 a

Note: For each trait, mean values with different letters indicates for significant difference (P<0.05) and ns for non-significant difference.

The pattern of root to shoot ratio for fresh and dry weight are graphically presented in the Figure10, DH lines showed large variation for this trait where transgressive segregation was observed.

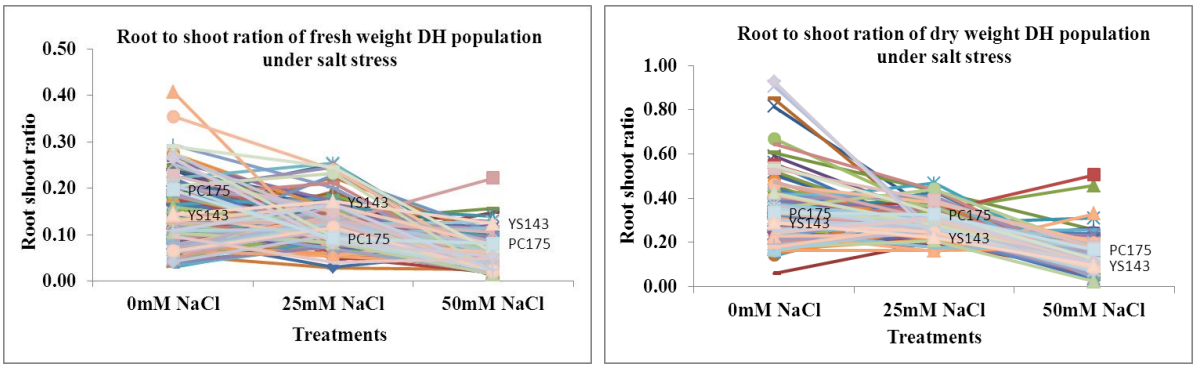


Figure 10 Root to shoot ratio of fresh and dry weight of parents and DH population in different NaCl (0, 25 and 50mM) concentrations

The fresh and dry weight of YS173 is higher than PC175 except shoot fresh weight in 25mM NaCl. The fresh and dry weight of some of DH lines is observed higher than parents (Figure 11) that reflected transgressive segregation pattern (Appendix 13). The summary statistics for the fresh and dry weight distribution of both root and shoot is presented in Appendix 6.

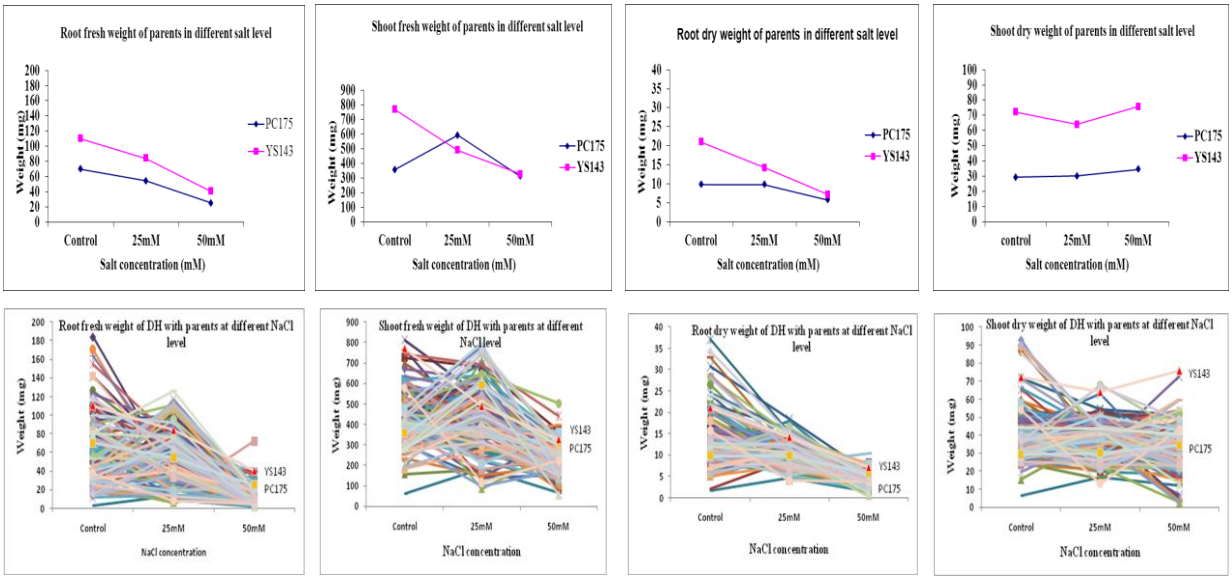


Figure 11. Fresh and dry weight of DH population and parents in different NaCl (0, 25 and 50mM) concentrations

3.5 QTL Mapping

The main aim of this study is to unravel the genetic regulation of seed germination and seedling vigor traits in *Brassica rapa* DH68 population under salinity of 0, 25 and 50mM NaCl concentrations by QTL mapping approach. QTLs are detected in almost all of the linkage groups. Map position and details of QTLs associated with different salinity and time points are presented below (Table 6-12, Figure 12). The name of QTLs are presented in abbreviation form initiated with the QTL symbol (q), NaCl level with 0, 25 and 50mM), trait name followed by time point (1-9). The digits after the symbol (–) indicates number of QTLs for that time point. For example, q0RL3-1. A total of 83 QTLs were detected in 10 linkage groups for all the traits measured under three different situations. Out of them 22, 17 and 28 were identified for the control, 25mM and 50mM NaCl stress respectively and remaining of 16 were detected for salt sensitivity response between three treatments. The QTLs are classified in three categories; <10, 10-14 and >14 % based on variance explained by QTL. The QTL with >14% explained variance was considered as major QTL.

3.5.1 Germination

QTL mapping was also carried out for seed germination traits of the DH68 population in control and salt stress conditions. The result showed one QTL, q0AG-1 for area under curve (AUC) in control, two QTLs; q0u7525-1 and q50u7525-1 for (u7525) each in control and 50mM NaCl. One QTL each q50T_{50}-1 and q50MGT-1 detected for T_{50} and MGT at 50mM NaCl (Table 6). QTLs for germination are mapped in linkage group A02, A05 and A10. One major QTL; q50MGT-1 for MGT was identified at 50mM NaCl with 14.2% explained variance. It was observed that QTLs q50T_{50}-1 and q50MGT-1 were co-localized in linkage group A02. LOD value and explained variance of QTLs present in this trait are 3.44 to 4.5 and 10.2 to 14.2% respectively (Table 6).

Table 6. List of QTLs detected for AUC germination (AG), T50, MGT and u7525 at 0, 25 and 50mM NaCl concentration with LOD score and phenotypic variance explained (PVE, %) . The column with cM (centiMorgan) indicates the map position at highest LOD point.

QTL Name	*Treatment*	*Traits*	*LG*[1]	*cM*	*Region*	*LOD*	*PVE*[2]	*Total PVE*
q0AG-1	Control	AG	A10	82.9	Bottom	4.5	13.7	13.7
q0u7525-1	Control	u7525	A05	16.6	Top	3.44	10.7	10.7
q50u7525-1	50mM	u7525	A02	34.1	Middle	3.49	10.2	10.2
q50T_{50}-1	50mM	T_{50}	A02	107	Bottom	3.68	11.7	11.7
q50MGT-1	50mM	MGT	A02	107	Bottom	4	14.2	14.2

3.5.2 Root length

For root length, 23 QTLs were detected for all treatments at five time points (1, 3, 5, 7 and 9 DAG). In control condition, eight QTLs for all time points were mapped at linkage groups A01, A05, A08 and A09. Linkage group A08 contains five QTLs (q0RL1-2, q0RL3-2, q0RL5-1, q0RL7-1 and q0RL9-1) of which later four QTLs map into the same position (Table 7). Similarly, seven QTLs detected in 25mM NaCl during 1, 3 and 5 DAG were observed in A02, A04, A06, A08 and A09. On the other hand, eight QTLs found in 50mM NaCl treatment were detected during 1, 3, 5 and 7 DAG in A05, A06, A07 and A09. Linkage group A09 co-localized with four QTLs at 1 and 3 DAG time point under all 0, 25 and 50 mM NaCl salinity levels (Table 7). The explained phenotypic variance and LOD score ranged from 8-15.1% and 3.08 to 6.66 respectively. Two major QTLs q25RL1-1 (25mM NaCl) and q50RL5-1 **(**50mM NaCl) explained 14.2 and 15.1% of phenotypic variance respectively. The QTLs for root length were distributed in many linkage groups except A03 and A10.

Table 7. List of QTLs detected for root length (RL) at 0, 25 and 50mM NaCl concentrations with LOD score and phenotypic variance explained (PVE, %) . The column with cM (centiMorgan) indicates the map position at highest LOD point.

QTL Name	*Treatment*	*DAG*[3]	*LG*	*cM*	*Region*	*LOD*	*PVE*	*Total PVE*
q0RL1-1	Control	1	A01	42.49	Middle	4.85	12.6	20.8
q0RL1-2	Control	1	A08	88.53	Bottom	3.25	8.2	
q0RL3-1	Control	3	A09	77.3	Middle	4.46	11.8	29.3
q0RL3-2	Control	3	A08	104.1	Bottom	3.68	9.5	
q0RL3-3	Control	3	A05	24.19	Top	3.11	8	
q0RL5-1	Control	5	A08	102.3	Bottom	4.44	12.4	12.4
q0RL7-1	Control	7	A08	102.1	Bottom	3.79	11.1	11.1
q0RL9-1	Control	9	A08	102.3	Bottom	3.08	8.9	8.9
q25RL1-1	25mM	1	A09	75.82	Middle	6.66	14.2	32.2
q25RL1-2	25mM	1	A02	50.58	Middle	4.61	9.3	
q25RL1-3	25mM	1	A06	99.44	Bottom	4.27	8.7	
q25RL3-1	25mM	3	A09	82.24	Middle	3.87	10	2.83

[1] LG stands for linkage group
[2] PVE stands for Phenotypic variance explained (%)
[3] DAG stands for days after germination

q25RL3-2	25mM	3	A08	71.24	Middle	3.85	10.1	
q25RL3-3	25mM	3	A04	29.80	Middle	3.18	8.2	
q25RL5-1	25mM	5	A04	28.80	Middle	3.67	10.8	10.8
q50RL1-1	50mM	1	A07	93.33	Bottom	4.04	11.8	11.8
q50RL3-1	50mM	3	A05	10.91	Top	5.65	13.2	31.5
q50RL3-2	50mM	3	A06	39.03	Middle	4.05	9.2	
q50RL3-3	50mM	3	A09	82.24	Middle	4.04	9.1	
q50RL5-1	50mM	5	A07	86.73	Bottom	4.68	15.1	24.9
q50RL5-2	50mM	5	A05	4.573	Top	3.11	9.8	
q50RL7-1	50mM	7	A05	4.573	Top	3.65	11.5	21.9
q50RL7-2	50mM	7	A07	85.41	Bottom	3.32	10.4	

3.5.3 Lateral roots

Three QTLs are detected for lateral roots (q0LR7-1, q0LR9-1 and q25LR9-1), two of them in control condition at 7 and 9 DAG and one in 25mM NaCl at 9 DAG (Table 8). All three QTLs present in linkage group A08. One major QTL; q0LR7-1 observed with variance explained and LOD score of 16.2% and 6.21 respectively.

Table 8. List of QTLs detected for number of lateral roots (LR) at 0mM, 25mM and 50mM NaCl concentrations with LOD score and phenotypic variance explained (PVE, %). The column cM (centiMorgan) indicates the map position at highest LOD point.

QTL Name	*Treatments*	*DAG*	*LG*	*cM*	*Region*	*LOD*	*PVE*	*Total PVE*
q0LR7-1	Control	7	A08	108.87	Middle	6.21	16.2	16.2
q0LR9-1	Control	9	A08	106.55	Middle	5.59	10.7	10.7
q25LR9-1	25mM	9	A08	103.09	Middle	3.72	10.7	10.7

3.5.4 QTL for salt sensitivity response (SR) on root growth

The study was also done for identifying QTLs for the salt sensitivity response at different time points considered. The result indicated that 16 QTLs were identified for sensitivity response between control, 25mM and 50mM NaCl treatments (Table 9). Four minor QTLs obtained for salt sensitivity between control and 25mM NaCl in groups A01, A02, A05 and A09. Six QTLs identified each for sensitivity response between control and50mM as well as 25mM and 50mM NaCl respectively. The result further indicated the presence of six major QTLs; q0-50R3-1; q0-50R7-1; q0-50R7-2; q25-50R5-1; q25-50R7-1and q25-50R9-1with explained variance of 14.1-18.6%. Majority of QTLs are detected in A07 and A05 and they are co-localized. Two QTLs in A08 at 5 and 7 DAG exactly map into the same position. Overall explained variance varies from 7.9 to 18.6% and 3.1 to 5.86 for sensitivity response QTLs in root length.

Table 9. List of QTLs detected for the salt sensitivity response (SSR) at 0, 25 and 50mM NaCl concentrations with LOD score and phenotypic variance explained (PVE, %). The column cM (centiMorgan) indicates the map position at highest LOD point.

QTL Name	*SS[4] level*	*DAG*	*LG*	*cM*	*Region*	*LOD*	*PVE*	*Total PVE*
q0-25R1-1	0-25	1	A01	101.332	Bottom	3.68	9.0	26.8
q0-25R1-2	0-25	1	A02	115.37	Bottom	4.00	9.9	
q0-25R1-3	0-25	1	A09	28.892	Top	3.24	7.9	
q0-25R3-1	0-25	3	A05	24.194	Top	3.07	11.2	11.2
q0-50R3-1	0-50	3	A05	8.913	Top	3.93	14.2	14.2
q0-50R5-1	0-50	5	A05	7.613	Top	3.28	10.0	35.2
q0-50R5-2	0-50	5	A07	72.405	Middle	4.12	12.5	
q0-50R5-3	0-50	5	A08	102.269	Bottom	4.13	12.7	
q0-50R7-1	0-50	7	A07	68.405	Middle	5.14	16.9	32.3
q0-50R7-2	0-50	7	A08	102.269	Bottom	4.58	15.4	
q25-50R1-1	25-50	1	A02	92.855	Bottom	3.1	8.4	32.0
q25-50R1-2	25-50	1	A06	120.299	Bottom	12.2	10.2	
q25-50R1-3	25-50	1	A09	77.637	Middle	4.74	13.4	
q25-50R5-1	25-50	5	A07	80.405	Middle	5.86	18.6	18.6
q25-50R7-1	25-50	7	A07	73.405	Middle	4.65	14.1	14.1
q25-50R9-1	25-50	9	A07	71.405	Middle	4.9	15.2	15.2

3.5.5 Shoot length

Fourteen QTLs are detected for shoot length that comprises three, four and seven in control, 25mM and 50mM NaCl respectively (Table 10). Seven QTLs for shoot length were identified at 50mM NaCl where only three and four QTLs were detected for control and 25mM NaCl respectively. Five major QTLs q0SL1-1, q25SL3-1, q50SL1-1, q50SL1-2 and q50SL3-1 identified at all NaCl levels at 1 and 3 DAG explaining greater than 14.0% variance. Later three major QTLs with PVE of 20.9, 18.5 and 14.6% observed in 50mM NaCl level in linkage group A02, A09 and A10 (Table 10). The phenotypic variance explained (PVE) and LOD score ranged from 5.8 to 20.9% and 3.0 to 5.58 respectively. QTLs were distributed in linkage groups A02, A05, A07, A08, A09 and A10 more abundantly in A02, A08 and A09. However, the linkage group A02 contains QTLs for all treatments for all the time points (1, 3 and 5 DAG).

Table 10. List of QTLs detected for shoot length at 0, 25 and 50mM NaCl concentrations with LOD score and phenotypic variance explained (PVE,%). The column cM (centiMorgan) indicates the map position at highest LOD point.

QTL Name	*Treatments*	*DAG*	*LG*	*cM*	*Region*	*LOD*	*PVE*	*Total PVE*
q0SL1-1	Control	1	A02	51.1	Middle	4.02	14.8	14.8
q0SL5-1	Control	5	A02	85.54	Middle	4.28	11.1	19.6
q0SL5-2	Control	5	A10	59.3	Middle	3.37	8.5	
q25SL3-1	25mM	3	A08	27.14	Top	4.86	15.6	23.6

[4] SS stands for salt sensitivity

q25SL3-2	25mM	3	A02	99.65	Middle	3.27	8	
q25SL5-1	25mM	5	A08	26.12	Top	4.16	10.8	19.5
q25SL5-2	25mM	5	A02	31.89	Top	3.4	8.7	
q50SL1-1	50mM	1	A10	37.83	Top	3.46	20.9	39.4
q50SL1-2	50mM	1	A09	0.00	Top	3	18.5	
q50SL3-1	50mM	3	A02	43.74	Middle	5.58	14.6	14.6
q50SL5-1	50mM	5	A09	84.85	Middle	5.27	10.1	32.6
q50SL5-2	50mM	5	A08	41.12	Top	4.52	8.5	
q50SL5-3	50mM	5	A05	61.24	Middle	4.35	8.2	
q50SL5-4	50mM	5	A07	85.41	Middle	3.13	5.8	

3.5.6 AUC for root and shoot length

Three QTLs detected for area under curve (AUC) of root length in 50mM NaCl treatment, five QTLs for AUC shoot length in all treatments consisting two each at 25 and 50mM NaCl and one in control (Table 11). Two QTLs; q50ARL-2 and q50ASL-1for AUC on root and shoot length at 50mM NaCl are detected in same linkage group A05. The result indicated that explained variance and LOD value of AUC on root and shoot length remains within the 8.0 to 12.6% and 3.09 to 4.97 respectively.

Table 11. List of QTLs detected for AUC on root length (ARL) and shoot length (ASL) at 0, 25 and 50mM NaCl concentrations with LOD score and phenotypic variance explained (PVE,%). The column cM (centiMorgan) indicates the map position at highest LOD point.

QTL Name	*Treatments*	*Traits*	*LG*	*cM*	*Region*	*LOD*	*PVE*	*Total PVE*
q50ARL-1	50mM	Root	A06	68.526	Middle	3.91	10.2	26.3
q50ARL-2	50mM	Root	A05	0.57	Top	3.14	8.1	
q50ARL-3	50mM	Root	A07	82.405	Middle	3.09	8	
q0ASL-1	Control	Shoot	A10	59.295	Middle	3.37	9.9	9.9
q25ASL-1	25mM	Shoot	A02	30.387	Top	3.25	8.9	17.6
q25ASL-2	25mM	Shoot	A03	31.26	Bottom	3.18	8.7	
q50ASL-1	50mM	Shoot	A05	62.434	Middle	4.97	12.6	23.2
q50ASL-2	50mM	Shoot	A01	42.024	Middle	4.18	10.6	

3.5.7 Fresh and dry weight

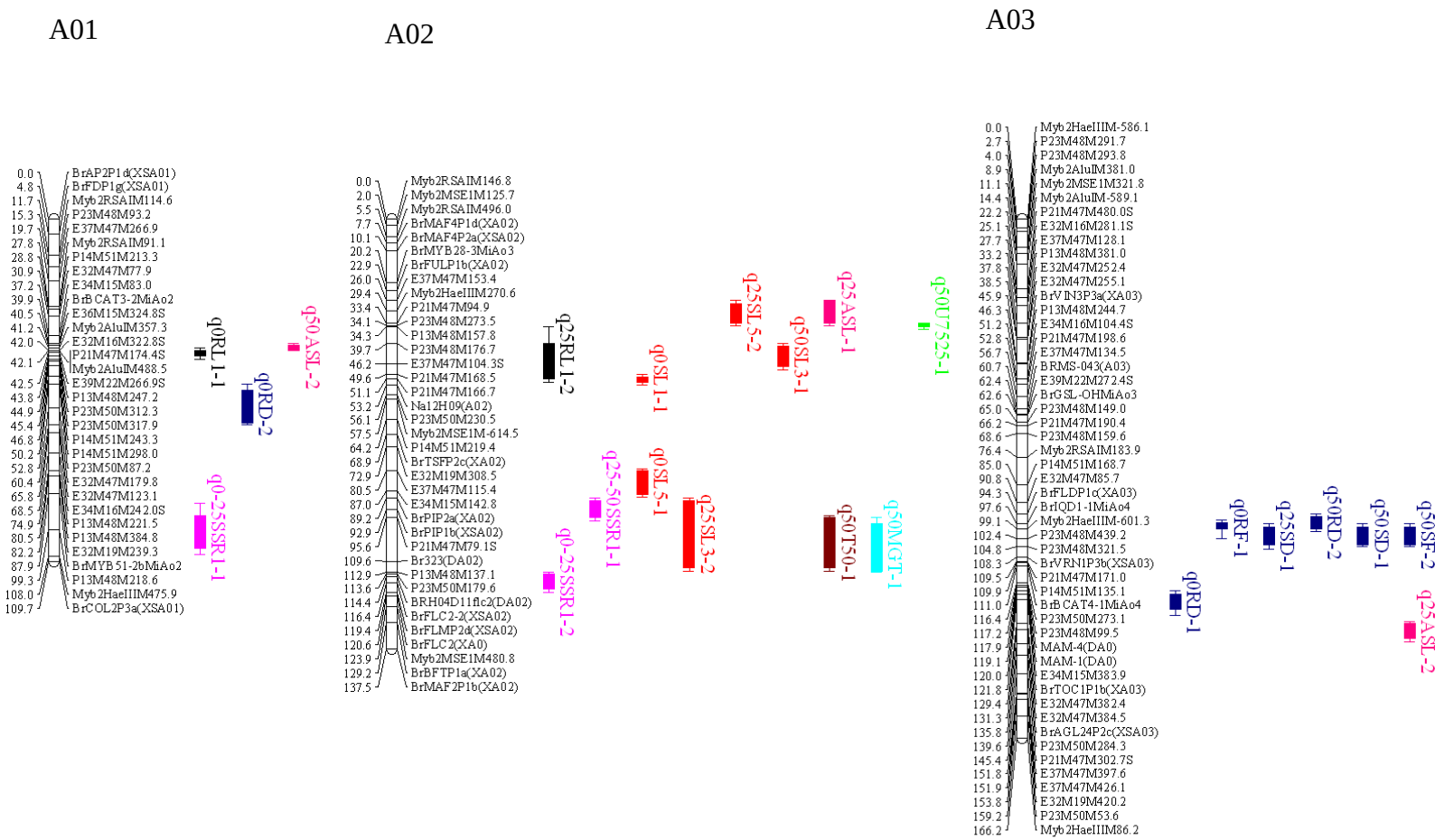
In total, fourteen QTLs identified for fresh and dry weight of root and shoot at different NaCl levels (Table 12). Five QTLs associated with root dry weight comprising two in control, one in 25mM and two in 50mM NaCl were located in linkage groups A01, A03 and A08. Likewise three QTLs each in shoot dry weight, root fresh weight and shoot fresh weight mapped into linkage groups A03, A05, A06, A08 and A10. Two major QTLs q50RD-1 and q50SD-1 were detected at 50mM NaCl with explained variance of 19.9% and 18.0% in linkage group A08 and A03 respectively (Table 12). The explained variance and LOD score lies within the interval of 8.8 to 19.9% and 3.39 to 6.13 respectively. The root fresh weight QTLs were not detected at salt stress conditions while shoot dry weight QTLs only observed

at stress conditions of 25-50mM NaCl. The result suggests that QTLs were distributed in many linkage groups and mostly abundant in linkage group A03 and A08.

Table 12. List of QTLs detected for fresh and dry weight at 0, 25 and 50mM NaCl concentrations with LOD score and phenotypic variance explained (PVE, %) . The column with cM (centiMorgan) indicates the map position at highest LOD point.

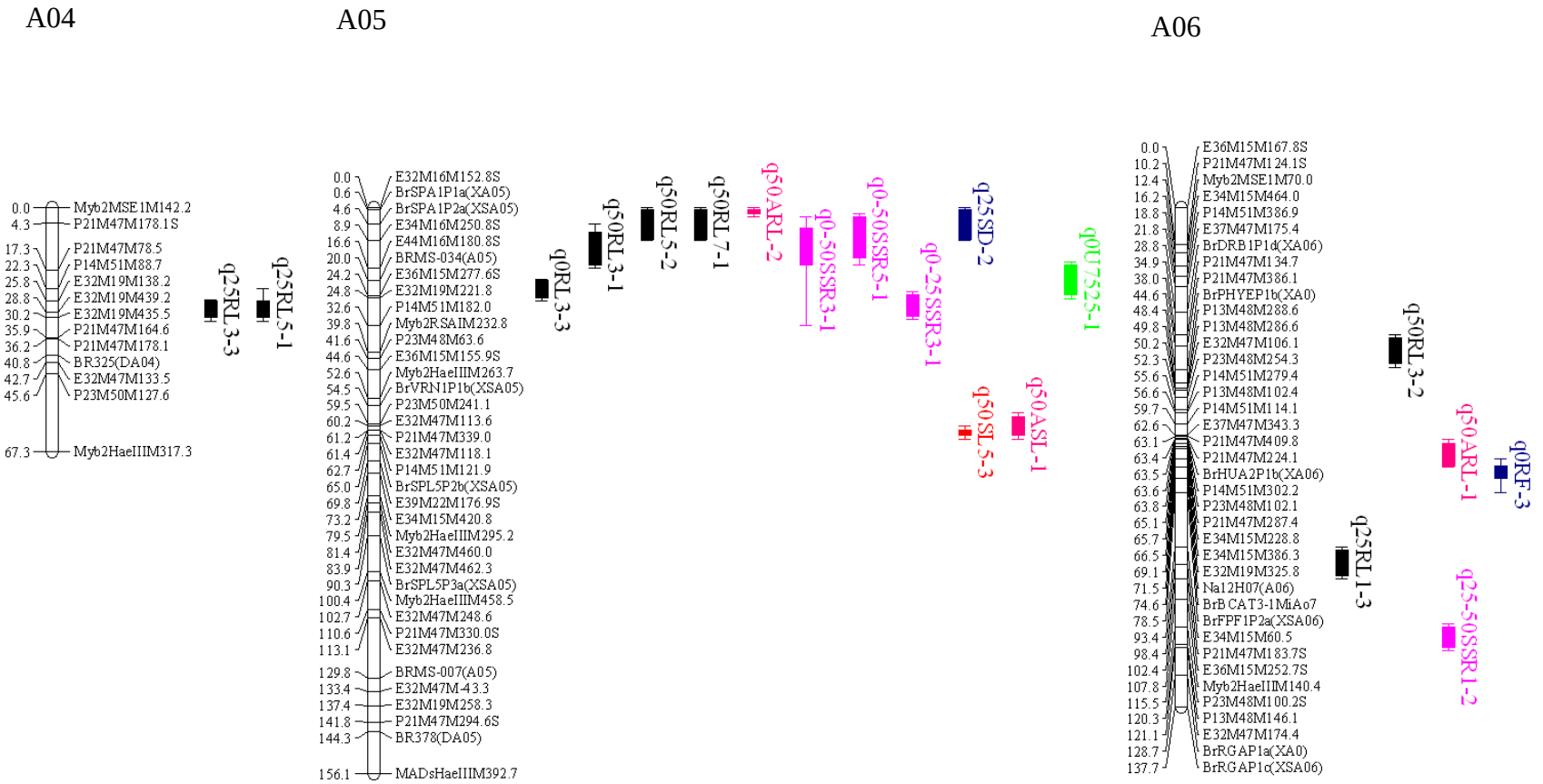
QTL Name	*Treatments*	*Trait*	*LG*	*cM*	*Region*	*LOD*	*PVE*	*Total PVE*
q0RF-1	Control	Rfwt	A03	97.58	Middle	3.96	10.7	31.5
q0RF-2	Control	Rfwt	A10	72.64	Bottom	3.9	10.8	
q0RF-3	Control	Rfwt	A06	73.49	Middle	3.68	10	
q0RD-1	Control	Rdwt	A03	121.8	Bottom	4.7	12.5	21.3
q0RD-2	Control	Rdwt	A01	58.77	Middle	3.39	8.8	
q25RD-1	25mM	Rdwt	A08	23.12	Top	4.24	12.9	12.9
q50RD-1	50mM	Rdwt	A08	19.08	Top	6.13	19.9	29.3
q50RD-2	50mM	Rdwt	A03	97.59	Middle	3.77	9.4	
q0SF-1	Control	Sfwt	A08	88.06	Bottom	3.82	9.3	9.3
q50SF-1	50mM	Sfwt	A08	22.08	Top	4.79	13.1	22.9
q50SF-2	50mM	Sfwt	A03	97.58	Middle	3.67	9.8	
q25SD-1	25mM	Sdwt	A03	102.4	Middle	3.74	9.9	19.8
q25SD-2	25mM	Sdwt	A05	5.613	Top	3.27	9.9	
q50SD-1	50mM	Sdwt	A03	101.1	Middle	5.49	18	18

Note: Rfwt- Root fresh weight, Rdwt- Root dry weight, Sfwt- Shoot fresh weight, Sdwt-Shoot dry weight



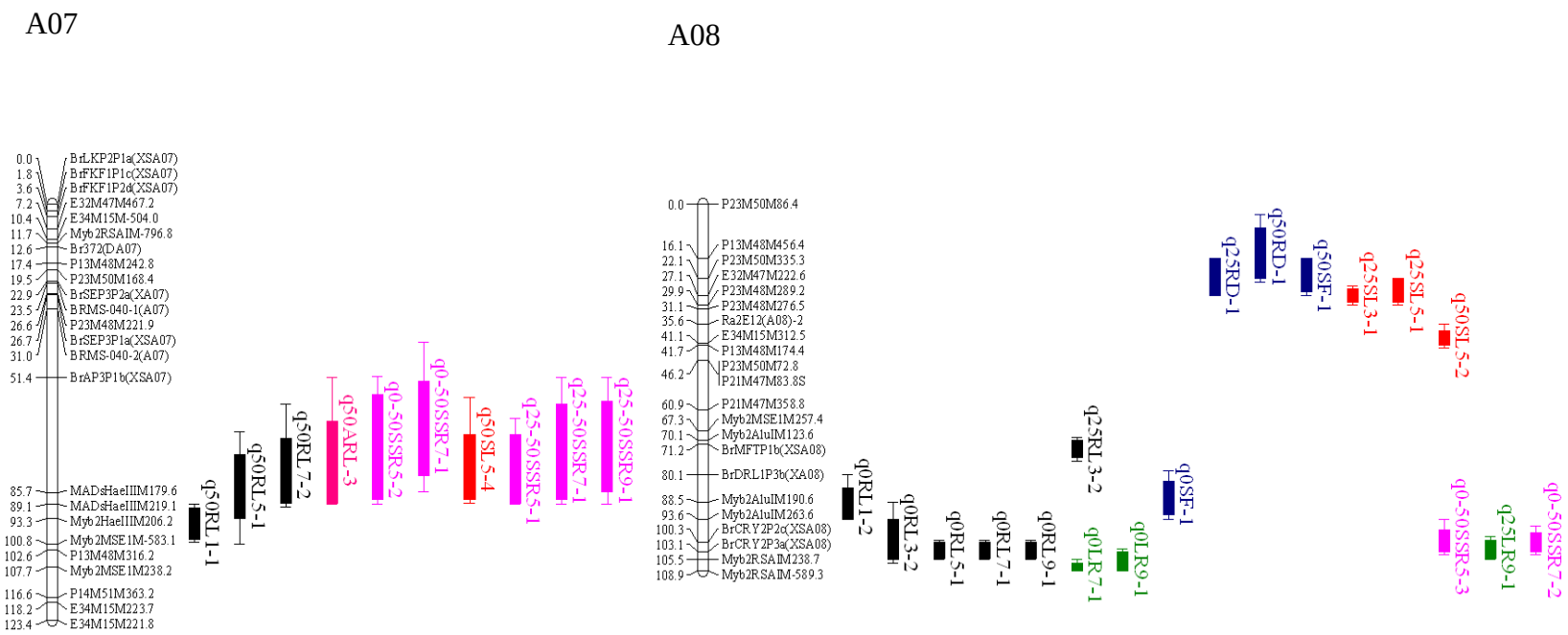
QTLs related to different traits shown in linkage groups A01-03

Figure 12. (Figure continued on next page).



QTLs related to different traits shown on linkage groups A04-06

Figure 12. (Figure continued from previous page).

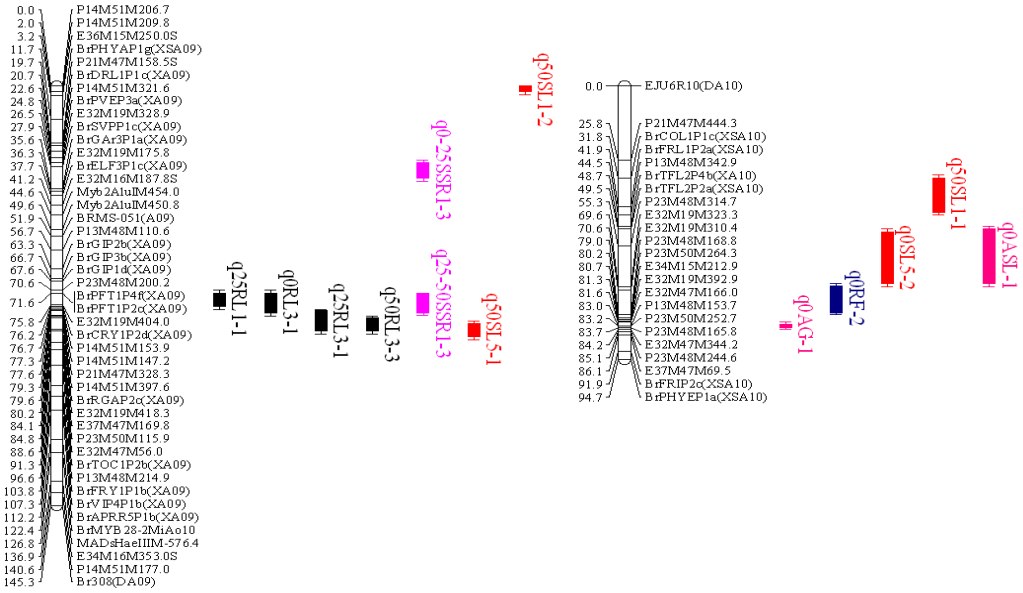


QTLs related to different traits shown on linkage groups A07-08

Figure 12. (Figure continued from previous page).

A09

A10



QTLs related to different traits shown on linkage groups A09-10

Figure 12. (Figure continued from previous page). QTL detected for seed and seedling vigor traits at 0, 25 and 50 mM NaCl concentrations were shown in 10 linkage groups of *B. rapa* . QTLs identified for the traits were marked by bar plot next to each linkage group. The thick bar indicates that region is 1-LOD support interval. The thin bar indicates the region that is 2-LOD support interval. The text along the bar is the abbreviation name of the trait, and the full name of those abbreviation were mention in result section Table (6-12). The figures in the left side of the linkage map indicates marker position in centiMorgan (cM) and text on right side indicates marker name present in the linkage groups. Different colours used in the map indicates different traits given by black colour for root length, red colour for shoot length, violet colour for salt sensitivity response in root length, blue for fresh and dry weight, dark green for lateral roots, light green for uniformity of time for germination, light blue for time to 50% germination and pink colour indicates area under curve for the different traits.

Chapter 4: DISCUSSION

This study was emphasized to identify the genetic variation associated with seed and seedling vigor traits in early stage through QTL mapping approach in DH population of *B. rapa* under the salt stress and normal conditions. A pilot study was carried out at the start to determine the range of salinity levels to conduct seed and seedling vigor experiment in *B. rapa* crop. Previous studies in *Brassica* species were mainly focused on the later stages of development after seedlings establishment, where salinity stress was given in the range of 50mM to 200mM (Ashraf and McNeilly, 1990; Ashraf, 2001; Jamil *et al.*, 2006). Based on such information five different NaCl concentrations; 25, 50, 75, 100 and 150mM were considered. The preliminary testing revealed that no seedling growth (shoot and root) was observed at 75mM, 100 mM and 150 mM NaCl concentrations though few (1-2) seeds out of five were able to germinate with prolong time period (4-5 days). Hence, we decided to confine this experiment at salinity stresses of 25mM and 50mM NaCl concentrations, and control level without any salt stress.

4.1 Germination

Germination is an important parameter to measure the seed vigor. In this experiment, seed germination was observed on every three hours interval in a day during day time at 0, 25 and 50mM NaCl concentrations.

In case of germination rate (T_{50} and MGT), small delay by two hours and reduction in area under curve (AUC) was observed at 50mM NaCl as compared to control (Table 1). The delayed germination was likely caused by reduced level of water movement because of low water potential due to osmotic stress. The reduced area under curve (AUC) was observed due to delayed germination at higher salinity condition. The uniformity of germination time (u7525) was higher in 50mM NaCl as compared to control and 25mM NaCl due to less variance observed between the DH lines (Figure 5). In cabbage, no significant reduction was reported for germination rate ($1/T_{50}$) at 4.7dS/m (equivalent to 50mM NaCl) as compared to control condition (Jamil *et al.*, 2006). We observed less influence of salinity on germination hence, the extent of reduction in germination percentage was also smaller even in the increased concentrations of 25 and 50mM NaCl. These results is in line with results obtained in cereals, legumes (Noori *et al.*, 2007) and cabbage (Jamil *et al.*, 2006) and tomato (Zhang *et al.*, 2003). We found time required to reach 50% germination (T_{50}) delayed with increased application of NaCl at 50mM, which is comparable to the germination experiment in *Phaseolus* (Jeannette *et al.*, 2002). Previous studies reported the inhibition of germination caused by several reasons. The reduction in the germination rate could be triggered due to limited water movement and reduced water potential during seed imbibition (Hadas, 1977). The salt induced reduction of germination was accounted due to osmotic stress creating low water potential in seed and toxicity

caused by high salinity (Maas and Nieman, 1978; Werner and Finkelstein, 1995; Foolad *et al.*, 2007). The DH lines were also showing transgressive segregation pattern for germination in this population, which suggests continuous distribution of traits controlled by polygene as explained by histograms (Appendix 14).

Effect of NaCl on seedling growth is stronger than germination (Table 1, 2, 3). With the increasing salinity stress, the reduction in germination was marginal as compared to seedling growth, where growth inhibition was more pronounced. It indicates that effect of NaCl stress to germination and seedling growth is different in *B. rapa*, and similar results were reported in tomato (Zhang *et al.*, 2003).

4.2 Root and shoot length

The root and shoot growth measurements were done to identify the seedling vigor traits of *B. rapa* under different (0, 25 and 50mM) NaCl concentrations. Both root and shoot length showed decreasing trend with increased NaCl applications (Table 2, 3). It could likely due to adverse effect of salinity in plant growth induced by osmotic stress and sodium ions toxicity in agreement with (Fageria, 1985). It was also triggered by disrupted nutrient and water uptake through roots or flow from shoot (Hadas, 1977; Bybordi and Tabatabaei, 2009). Because of transgressive segregation in root and shoot length, these traits might be regulated by many genes with smaller effects that can be explained by the histograms for root and shoot growth (Appendix 11, 12).

In control condition, the root and shoot growth of YS143 was always higher than the PC175, hence difference was bigger at control condition (Figure 6, 8). Both root and shoot length of YS143 was decreasing rapidly with increasing salinity level while reduction was very negligible in case of PC175 for root and shoot length at higher salinity. With the increment of salinity till 50mM NaCl the both parents were very close to each other (Figure 6, 8). Similar result was also obtained for the germination where YS143 was earlier in germination than PC175. With increasing salinity the parents were showing similar germination pattern (Figure 5). From this result, it indicated that the parent YS143 was more sensitive than PC175 to higher salinity. It could be inferred that the increased root length and earlier germination of DH lines was attributed to favorable alleles of YS143 and increased salinity was contributed by PC175 in the DH population.

4.3 Fresh and dry weight

The fresh and dry weight parameters were also considered key indicator of seedling vigor. The both fresh as well as dry weight of root and shoot were affected greatly with higher NaCl stresses. The higher the NaCl concentrations, the lesser root and shoot weight. It could be caused by reduced water potential leading to imbalanced water and nutrient supply due to NaCl toxicity that hindered dry matter accumulation. The roots were more sensitive to salt stress than shoot (Table 4) which might be because of the direct contact of root with soil that results in the accumulation of the

higher salt ions in the root cells (Jamil *et al.*, 2006). The similar results were also recorded in Pak Choi type of *Brassica rapa*, where root fresh weight was more affected by salinity at 50 mM NaCl (Jamil *et al.*, 2006). Similarly, the fresh weight seems more sensitive to salinity than dry weight in case of both root and shoots (Table 4). The difference between fresh and dry weight might be because of moisture content of the plant also consistent with the result from Pak Choi (Jamil *et al.*, 2006). High Salinity more predictably reduced fresh weight as compared to the dry weight (Jamil *et al.*, 2006) which was also true in the earlier stage of seedling growth even at concentration up to 50mM in this study. Some DH lines showed transgressive segregation and produced higher fresh and dry weight even at higher salinity level than parents that was clearly illustrated by histograms shown in Appendix 13. It suggests that the fresh and dry weight trait also showed the similar continuous distribution that quantitative traits usually have. These results were in consistent with result obtained from wheat, gram, barley and mustard at 4 dS/m (~40mM NaCl) (Mer *et al.*, 2000) and in *Phaseolus* (Jeannette *et al.*, 2002) at 50mM NaCl.

The root to shoot ratio of both fresh and dry weight of population declined with increased salinity levels, which indicates that root length was more affected by salinity as compared to shoot length (Table 5). With increased salinity levels, there was reduction in root length due to NaCl toxicity that also affected emergence of lateral roots. It has been reported that the effect of salinity at early growth was found more apparent than at later stage of *Brassica juncea* in higher than 40mM (Mer *et al.*, 2000).

4.4 PCA on germination and seedling growth

Principal component analysis (PCA), a visualization tool for multivariate dataset, was carried out to observe the grouping patterns of DH lines and parents based on the germination, root and shoot traits measured under graded level salt stress. PCA showed the levels of salinity had more pronounced effects on root and shoot length than on germination (Table 1, 2 and 3). It was also reflected by distinctly visible groups of traits according to salinity levels as observed in figure 4, 7 and 9. This indicates seedlings growth were more sensitive than germination to salt stress.

PC2 and PC3 only represent the variance due to salt stress that accounted only less that 20% of proportion of variation depending upon the trait. The larger proportions of variance represented by PC1 was not contributed by variation due to salt stress, and it seems some other artifact factors such as genotype, environment and interaction between them had introducing more variation in this study.

4.5 QTLs for germination

For different traits under germination experiment, only few QTLs were detected. QTL for AUC of germination were only detected in control whereas QTLs for T_{50}, MGT are only found in higher salinity level of 50mM NaCl (Table 6). It indicates that

different germination traits might be governed by separate genetic mechanism at different salinity levels. The QTLs for MGT and T_{50} co-localized in linkage group A02 could be the same (Figure 12). It indicates that MGT and T_{50} were highly correlated traits as explained by PCA biplot (Figure 4) and the values are approximately identical for these two traits (Table 1), it suggested that both germination rate (T_{50}) and mean germination (MGT) are controlled by same genetic mechanism and similar results were reported in rice (Wang *et al.*, 2011). Most of QTLs observed at higher salinity could be due to large variation within DH population under this condition.

We found very few co-localizations of the QTLs under stress and non-stressed conditions. Similar findings reported in tomato (Foolad *et al.*, 2007) and rice (Wang *et al.*, 2011), *Arabidopsis* (Vallejo *et al.*, 2010) at control and 50mM NaCl.

For germination traits, no QTL was detected at 25mM NaCl that could be due to presence of several small effect QTLs that hinders detection of other QTLs in vicinity or unknown factors play role to mask the QTL effect to express.

Furthermore, QTL study in germination traits revealed one major QTL for MGT at 50mM NaCl and four minor QTLs at control and 50mM NaCl level (Table 6). This result was also consistent with earlier studies where several minor and few major QTLs identified at different salinity levels in *Arabidopsis* (Galpaz and Reymond, 2010), *B. oleracea* (Bettey *et al.*, 2000), tomato (Foolad *et al.*, 2007) control and stressed condition for germination rate (T_{50} and MGT).

4.6 QTLs for root length

Five QTLs co-localized for root length under control (1, 3, 5, 7, 9 DAG) in A08, two QTLs in each A09 and A04 at 25mM NaCl (1, 3, 5 DAG) and three QTL in A05 and A07 at 50mM NaCl (1, 5, 7 DAG) (Table 7, Figure 12), which suggests the presence of same QTL governing the traits. At the same time, QTLs for the lateral roots were also co-localized in the same linkage group A08 both in control and 25mM NaCl as observed for root length under control. It indicates that there might be same genetic control for root length and lateral roots. However, there were many QTLs that were detected in only one time point but not in other time points in the same genomic region. QTLs governing root growth at one stage were not consistently co-localized for the next growth stage**.** Those results indicate the possibility of having different genetic factors, which might express at different time points or at different level of salt stress, and this kind of pattern on QTLs identification was also reported in *Arabidopsis* (Galpaz and Reymond, 2010). Another likely explanation is that minor QTLs that are not always detected as they are around threshold level. And, the presence of several minor QTLs regions masks the expression of putative QTLs around co-localization region in linkage group.

We also identified six major QTLs for sensitivity of root length at increased salinity levels of 25-50mM and 0-50mM NaCl. It explained higher variation in the root length among the DH lines in DH68 population. It could also provide evidence for more sensitivity of root growth at higher NaCl concentrations.

4.7 QTLs for shoot length

Two QTLs for shoot length were co-localized in linkage group A08 at 25mM NaCl for different time points (3 and 5 DAG) (Figure 12) and many QTLs were identified in linkage group A02 at different genomic positions. However, they were not co-localized with respect to different time points and NaCl concentration levels. Furthermore, several QTLs detected in one time point and one NaCl level were not co-localized in other conditions and time points. In fact shoot growth QTLs were widely distributed along the 10 linkage groups, which means these traits might be governed by different genetic mechanisms under different growth stages and salinity levels (Table 10). Similar to root length, this pattern of QTLs detection is consistent with previous findings in Arabidopsis (Galpaz and Reymond, 2010). It could be also due to fact that QTL were not detected in some genomic regions as they were just below the QTL detection threshold level.

This study also revealed five major (1 and 3 DAG) and nine minor QTLs for shoot length (1, 3 and 5 DAG) at different salinity levels. Two major QTLs co-localized in A02 in control and 50mM NaCl level (Table 10) during early stages of growth (1 and 3 DAG). Similarly, all major QTLs were detected at the early stages (1 and 3 DAG) and majority of them obtained at 50mM NaCl. It suggests that shoot growth trait pronounced during early stages and high salinity, which could be due to large variation exhibited by the DH population at these stages in higher salinity conditions. Detection of larger effects QTLs was prominent at 1 and 3 DAG that provides NaCl tolerance during this stage. The presence of many major and minor QTLs indicates that there might exist many genetic loci controlling shoot growth with smaller to large effects providing salinity tolerance suggesting quantitative nature of trait with large variation.

4.8 QTLs for fresh and dry weight

The fresh and dry weight measurements are also important parameters for measuring seedlings vigor. Five QTLs were co-localized in A03 and three co-localized in A08 at 0, 25, 50mM NaCl for fresh and dry weight of both root and shoot (Table 12; Figure 12). It suggests that fresh and dry weight were highly correlated and likely the same QTL that governed trait under given salinity levels. Majority of QTLs identified in linkage groups A03 and A08 suggests that these genomic regions were hot spot for controlling the trait at different salinity whereas, some other QTLs were found in one salinity level but did not observe other salinity levels. Thus, it implies that QTLs were well distributed across the genome suggesting the polygenic nature of salt tolerance and seedling growth trait.

We obtained several QTLs (2 major and 12 minor) for fresh and dry weight traits. The major QTLs were observed in 50mM NaCl. The dry weight QTLs were stronger than fresh weight (Table12**)**. Detection of many and stronger QTLs indicates presence of larger genetic variation for these traits under the NaCl stress conditions.

We observed few major and several minor QTLs for root length shoot length and fresh as well as dry weight trait across the studied time points at different NaCl stresses (Table 7, 10, 12). The other studies in different crops conducted under control and different salt stress levels also reported several minor and few major QTL in sunflower (Al-Chaarani *et al.*, 2005), rice (Hossein and Atefeh, 2008), Medicago (Arraouadi *et al.*, 2010), *B. oleracea* (Bettey *et al.*, 2000) and *Arabidopsis* (Ren *et al.*, 2010). These results suggest that there might be many loci controlling the root growth with small effects and few loci with relatively higher effects.

4.9 Map synteny between *B. rapa* and *Arabidopsis thaliana*

Whenever QTL in *A. thaliana* and *B. rapa* are at syntenic position, this may be useful information to identify candidate genes in *B. rapa*. However, in this study we did not precisely defined the block positions in the *B. rapa* linkage groups based on *insilico* blasting marker sequences against *A. thaliana.* Therefore, we only observed and presented the common blocks between *A. thaliana* and *B. rapa* linkage groups that contains QTL (Table 13).

Some QTLs detected for root length in *B. rapa* were syntenic with root length QTLs in *A. thaliana* in control condition (Galpaz and Reymond, 2010). Linkage groups A01, A08 and A09 of *B. rapa* that contain QTLs (Figure 12) share many blocks O, P, T and U in chromosome-4 (At4) of *A. thaliana* (Table 13). It indicates that genomic regions already identified for controlling root growth in *A. thaliana*. Likewise, QTLs for root length detected at 50mM NaCl in *B. rapa* found at syntenic position to *A. thaliana* QTLs at salinity of 120mM NaCl (Ren *et al.*, 2010). Blocks B and N were shared many times by *B. rapa* linkage groups A05, A06, A07 and A09 to *A. thaliana* chromosomes- 1, 3 and 4 (At1, At3 and At4) for root length at QTL positions (Table 13). It implies that same genes that identified in *A. thaliana* for root length at 120mM salinity also controlling the trait in *B. rapa* at 50mM salinity. Map synteny was also observed for germination QTL (T_{50}) in *B. rapa* at 50mM and *A.thaliana* for T_{50} and germination rate at 175-250mM NaCl (Quesada *et al.*, 2002). QTL for MGT and T_{50} at 50mM NaCl share block L with Arabidopsis chromosome-3, where QTL for T_{50} was reported at 175 and 200mM NaCl (Quesada *et al.*, 2002) (Table 13). Syntenic blocks were also identified for QTLs for shoot fresh weight (A03 and A08) in *B. rapa* and *A. thaliana* (Chromosome-4) at salinity of 50mM NaCl level (Quesada *et al.*, 2002) (Figure 2). The linkage groups A03 of *B. rapa* and chromosome-4 of *A. thaliana* shared blocks O, P T and U, while linkage group A08 and chromosome-4 shared only blocks T and U between those species (Table 13). It suggests that for

those QTLs for germination rate (T50) and shoot fresh weight were governed by the same QTL in *B. rapa* and *Arabidopsis*.

Little information on QTL studies on seed and seedling vigor traits under salt stress was available in other *Brassica* species. QTL on MGT, root length, shoot length and shoot weight under control (no salt stress) condition reported in *B. oleracea*; however root and shoot length traits measured on 15 days older seedlings (Bettey *et al.*, 2000). In this study, very little coincidence of QTL was observed with respect to different levels of salt stress and growth stages. These results suggest that genetic mechanism responsible to salt tolerance during germination and seedling growth stage could be different.

Table 13. QTL comparison between *Arabidopsis* and *B. rapa* based on map synteny.

Brassica rapa			*Arabidopsis thaliana*			Reference	Common blocks[1]
Traits	Salt level	Linkage Group	Traits	Salt level	Chr.		
Root length	0mM	A01	Root length	0mM	At4	(Galpaz and Reymond, 2010)	U and T
Root length	0mM	A08	Root length	0mM	At4	(Galpaz and Reymond, 2010)	U and T
Root length	0mM	A09	Root length	0Mm	At4	(Galpaz and Reymond, 2010)	O and P
Root length	50mM	A05	Root length	120mM	At1	(Ren *et al.*, 2010)	B and C
Root length	50mM	A05	Root length	120mM	At3	(Ren *et al.*, 2010)	F
Root length	50mM	A06	Root length	120mM	At1	(Ren *et al.*, 2010)	A, B, C
Root length	50mM	A06	Root length	120mM	At3	(Ren *et al.*, 2010)	L
Root length	50mM	A07	Root length	120mM	At1	(Ren *et al.*, 2010)	B and E
Root length	50mM	A07	Root length	120mM	At3	(Ren *et al.*, 2010)	N
Root length	50mM	A09	Root length	120mM	At1	(Ren *et al.*, 2010)	A, B, D
Root length	50mM	A09	Root length	120mM	At3	(Ren *et al.*, 2010)	N
Root length	50mM	A09	Root length	120mM	At4	(Ren *et al.*, 2010)	O and P
MGT and T50	50mM	A02	T50	175 250mM	At3	(Quesada *et al.*, 2002)	L
Shoot fresh weight	50Mm	A03	Shoot fresh weight	50mM	At4	(Quesada *et al.*, 2002)	O, P, T and U
Shoot fresh weight	50Mm	A08	Shoot fresh weight	50Mm	At4	(Quesada *et al.*, 2002)	T and U
	1- Common genomic blocks between *A. thaliana* and *B. rapa*						

Similar type of findings were also reported in *Arabidopsis* (Zhu, 2000; Quesada *et al.*, 2002; Galpaz and Reymond, 2010), and *B. oleracea* (Bettey *et al.*, 2000). QTLs were very well distributed throughout the *B. rapa* genome reflecting wide genetic variation in DH population in terms of response to different salinity levels. Hence, seed and seedling vigor as well as salinity tolerance traits were complex traits showing continuous distribution suggesting polygenic control.

Chapter 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study essentially emphasized the quantitative trait loci (QTL) mapping of the seed germination and seedling growth trait of the *B. rapa* double haploid (DH68) population using of 120 DH lines under different NaCl stresses.

The statistically significant results were obtained for most of seedling growth traits while only T_{50} and MGT were significantly different between control and 50mM NaCl. It can be inferred that PC175 is more tolerant at higher salinity level of 50mM NaCl and parent YS143 is sensitive to saline conditions. It indicated that the salinity tolerance is attributed due to PC175 and better seedling growth and faster germination is governed due to YS143 in all conditions. Stronger inhibition in seedling growth was observed with increasing salinity level till 50mM NaCl than germination. But, in case of seedling growth, root growth was more affected than the shoot growth, which was shown by reduction in root to shoot ratio with increasing salinity. The osmotic and ionic stress due to NaCl toxicity caused low water potential and that might lead to more salt sensitivity in roots than in shoot.

In total, 83 QTL were detected in the DH68 population for all germination and seedling vigor traits measured on different time points under different treatments. However, some QTL found in one time point or one treatment was not obtained in other time points under the same treatment or different treatments of the same time point. Some QTLs obtained for root and shoot length was co-localized in all treatment conditions that suggest same QTL. Similarly, QTLs for T50 and MGT were also co-localized at 50mM NaCl. It is logical because there was hardly difference in germination time for T50 and MGT, and highly correlated to each other. However, there were very few co-localizations of QTLs between seedling growth and germination. In this study, total 11 major QTLs were found altogether in root length, shoot length, lateral roots, sensitivity of roots, fresh and dry weight, and germination. Besides this, six major QTLs were also observed only for sensitivity of root growth across the three NaCl concentrations. Almost all major QTLs were detected in 50mM NaCl and only one major QTL for lateral roots in control.

In most of the traits, the stronger QTLs in terms of explained variance were obtained under the higher salinity level than non-stressed condition, and this is more predominant in shoot length. It could be because of larger genetic variation with increasing salinity in this DH population. While comparing QTL results of this study with previous studies on Arabidopsis, QTL share the same genomic blocks between these two species. This comparative study hints for the confirmation of our QTL results obtained for root length, germination rate and shoot fresh weight. The synteny was observed during both stressed and non-stressed condition. The DH lines showing tolerance during germination not necessarily give tolerance at seedling. This suggests

that germination and growth stage are not related under salt stress conditions. Thus it could be concluded that the salinity tolerance and seed vigor traits are very complex and governed by many genes that showing the complex mechanism of genetic control.

6.2 Recommendations

This study generated some useful information on the genetic basis of seed and seedling vigor traits under different salinity stresses. The genomic regions that explained the observed variation in germination and seedling growth were identified in *B. rapa* linkage groups helps to further investigation of genetic control of seed and seedling vigor traits under various salinity conditions.

Inclusion of large population size would be helpful for explaining larger variation in the population due to more recombination. So during the subsequent studies it is suggested to include more DH lines and make more replicates for greater precision.

The number of plants tested per DH line in this study is also very low that produce greater amount of variance. The optimum should be around ten plants per replication in seedling vigor test to reduce the variability. The use of germination automatic scoring is essential for faster and accurate counting. Special attention to use petri-dishes without vent that restricts evaporation from then fosters germination process. The result from vitro test should be validated similar studies in greenhouse in the similar environments. Image analysis output from the EZ-Rhizo software for root architecture should be supplemented with the manual measurements for precision of data.

The QTLs could also be validated with using other mapping population in *B. rapa* and association mapping exploiting variations in natural populations. Linking *B. rapa* DH68 genetic map to block synteny in *Arabidopsis* to determine the correspondence QTLs and candidate gene mapping in the hotspot regions for seed and seedling vigor traits in *B. rapa* DH68 population.

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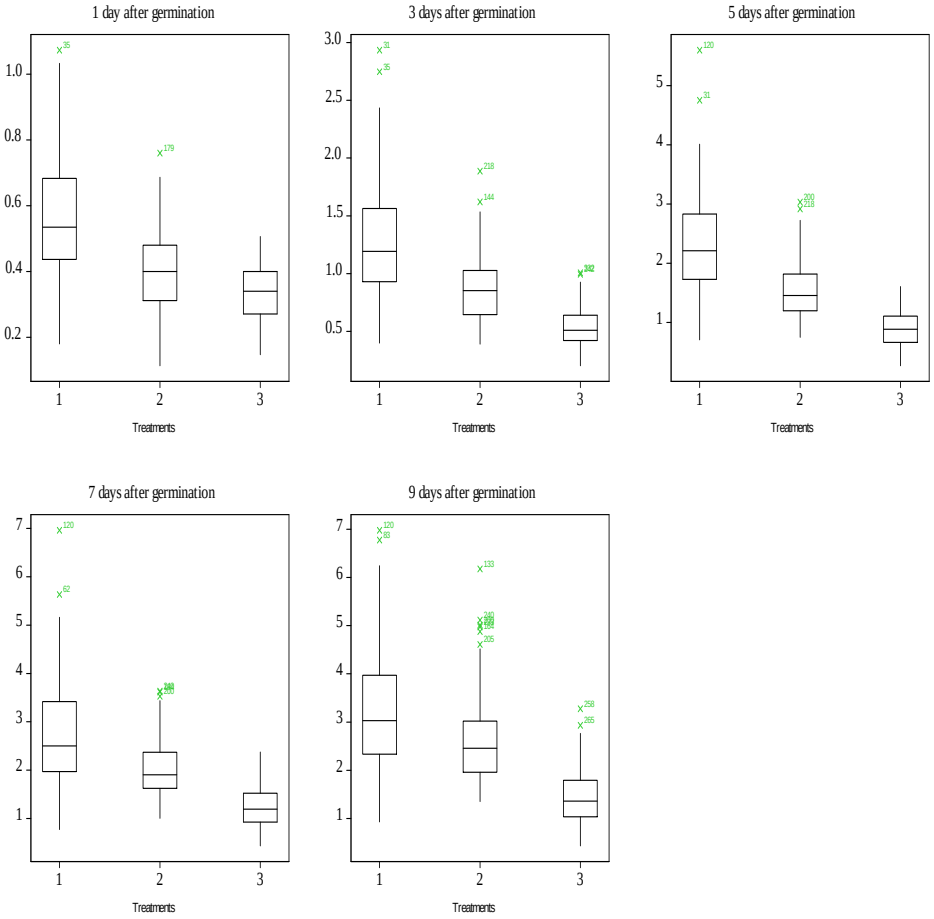
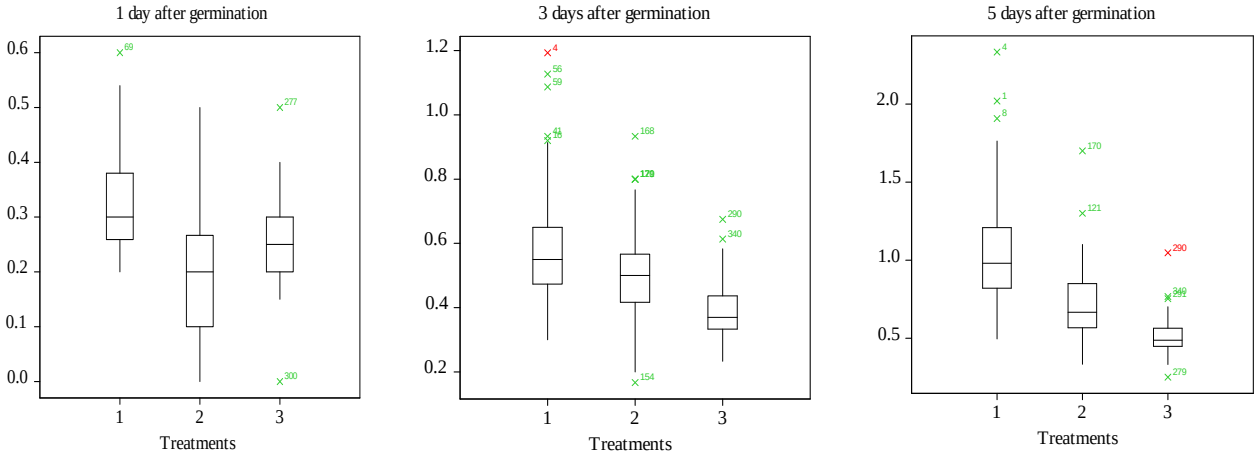
APPENDICES

Appendix 1 Boxplot showing root growth distribution of DH population and parents in different treatments (0, 25 and 50mM) NaCl concentrations[5]

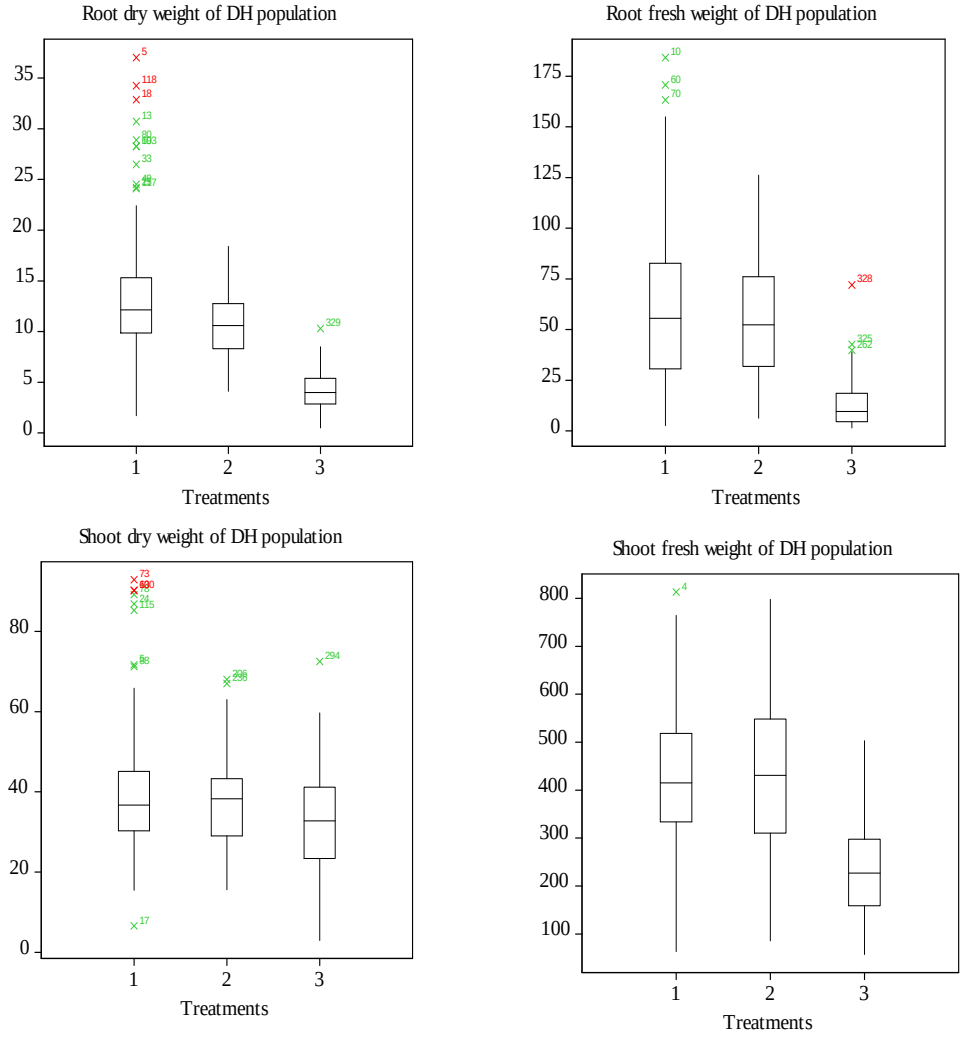


Appendix 2 Boxplot showing shoot growth distribution of DH and parents in different salt concentrations (0, 25, 50mM) NaCl concentrations



[5] The figures 1, 2 and 3 indicated in x-axis of boxplot stands for the control, 25mM and 50mM NaCl concentrations respectively.

Appendix 3 Boxplot showing fresh and dry weight distribution of DH population and parents in different (0, 25 and 50mM) NaCl concentrations



Appendix 4 Summary statistics for root growth of DH and parents in different (0, 25 and 50mM) NaCl concentrations.

Control	1DAG	3DAG	5DAG	7DAG	9DAG
Mean	0.6	1.3	2.3	2.7	3.1
Median	0.5	1.2	2.2	2.4	2.9
Std.Deviation	0.2	0.5	0.9	1.1	1.2
Variance	0.0	0.3	0.8	1.2	1.4
Minimum	0.2	0.4	0.7	0.8	0.9
Maximum	1.1	2.9	5.6	7.0	7.0
25mM	1DAG	3DAG	5DAG	7DAG	9DAG
Mean	0.4	0.9	1.4	2.1	2.6
Median	0.4	0.9	1.4	1.9	2.4
Std.Deviation	0.1	0.3	0.4	0.6	0.9

Variance	0.0	0.1	0.2	0.4	0.9
Minimum	0.1	0.4	0.8	1.0	1.4
Maximum	0.8	1.9	2.6	3.6	6.2
50mM	1DAG	3DAG	5DAG	7DAG	9DAG
Mean	0.3	0.5	0.9	1.2	1.5
Median	0.3	0.5	0.9	1.2	1.4
Std.Deviation	0.1	0.2	0.3	0.4	0.6
Variance	0.0	0.0	0.1	0.2	0.3
Minimum	0.2	0.2	0.3	0.4	0.4
Maximum	0.5	1.0	1.6	2.4	3.3

Appendix 5 Summary statistics of shoot growth pattern of DH and parents in different (0, 25, 50mM) NaCl concentrations.

Control	1DAG	3DAG	5DAG
Mean	0.33	0.58	1.03
Median	0.30	0.55	0.98
Std.Deviation	0.09	0.17	0.31
Variance	0.01	0.03	0.10
Minimum	0.20	0.30	0.50
Maximum	0.60	1.19	2.33
25mM	1DAG	3DAG	5DAG
Mean	0.12	0.50	0.71
Median	0.07	0.48	0.68
Std.Deviation	0.13	0.13	0.20
Variance	0.02	0.02	0.04
Minimum	0.00	0.25	0.43
Maximum	0.50	0.93	1.69
50mM	1DAG	3DAG	5DAG
Mean	0.10	0.38	0.51
Median	0.00	0.37	0.49
Std.Deviation	0.13	0.08	0.11
Variance	0.02	0.01	0.01
Minimum	0.00	0.23	0.25
Maximum	0.50	0.68	1.05

Appendix 6 Summary statistics for fresh and dry weight of DH population in different (0, 25 and 50mM) NaCl concentrations

Control	Rfwt (mg)	Sfwt(mg)	Rdwt(mg)	Sdwt(mg)
Mean	62.3	424.1	13.6	40.2
Median	56.5	412.5	12.0	36.5
Std.Deviation	38.2	141.3	6.5	16.1
Variance	1462.3	19978.3	42.5	258.0
Minimum	3.0	46.0	2.0	7.0
Maximum	184.0	813.0	37.0	93.0
25mM	Rfwt (mg)	Sfwt (mg)	Rdwt (mg)	Sdwt (mg)
Mean	54.6	428.1	10.6	37.2
Median	52.4	431.3	10.6	38.2
Std.Deviation	29.5	161.9	3.1	10.6
Variance	868.8	26206.8	9.7	111.4
Minimum	6.3	85.7	4.0	13.3
Maximum	126.2	798.0	18.4	68.0
50mM	Rfwt (mg)	Sfwt (mg)	Rdwt (mg)	Sdwt (mg)
Mean	13.7	227.9	4.2	32.7
Median	10.0	228.2	4.0	33.2
Std.Deviation	11.5	88.0	1.9	13.2
Variance	131.6	7745.3	3.7	174.8
Minimum	1.5	57.0	0.5	3.0
Maximum	72.0	503.0	10.3	75.7

Appendix 7 Summary statistics of seed germination of DH population and parents in different (0, 25 and 50mM) NaCl concentrations.

Control	16h	19h	22h	25h	28h	40h	43h	46h	49h	52h	64h
Mean	11	31	54	70	85	96	96	97	97	97	98
Median	0.0	17	50	83	100	100	100	100	100	100	100
Std.Dev.	20	33	37	32	25	14	13	12	11	11	10
Variance	395	1080	1361	1039	664	191	163	144	134	124	97
Minimum	0.0	0.0	0.0	3	3	10	10	13	13	17	27
Maximum	87	100	100	100	100	100	100	100	100	100	100
25mM	16h	19h	22h	25h	28h	40h	43h	46h	49h	52h	64h
Mean	9	26	44	55	83	91	92	93	94	94	94
Median	0	10	38	55	100	100	100	100	100	100	100
Std.Dev.	16	32	37	37	27	19	17	16	15	14	14
Variance	253	1016	1390	1350	710	363	300	246	230	205	205
Minimum	0	0	0	0	0	0	0	0	0	0	0
Maximum	87	100	100	100	100	100	100	100	100	100	100
50mM	16h	19h	22h	25h	28h	40h	43h	46h	49h	52h	64h
Mean	8	25	41	59	72	92	93	94	95	95	96

Median	0	7	27	63	87	100	100	100	100	100	100
Std.Dev.	17	33	38	37	33	19	16	15	14	13	12
Variance	285	1073	1478	1397	1082	361	253	220	203	176	146
Minimum	0	0	0	0	0	0	7	7	7	7	7
Maximum	87	100	100	100	100	100	100	100	100	100	100

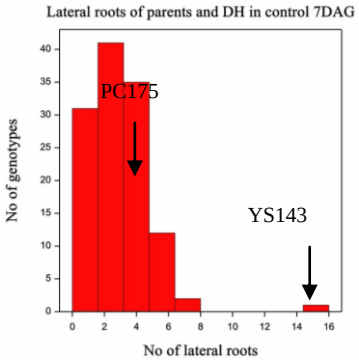
Appendix 8. Summary statistics of DH population and parents for lateral roots in control (0mM NaCl) condition

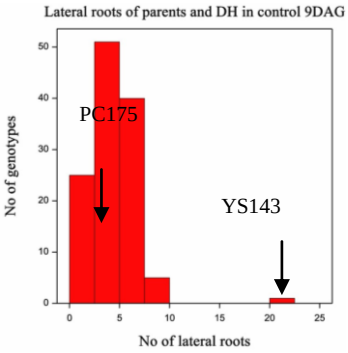
	LR7DAG	LR9DAG
Mean	3.0	4.5
Median	3.0	4.0
Std.Deviation	1.9	2.4
Variance	3.7	5.9
Minimum	0.0	0.0
Maximum	15.0	20.0

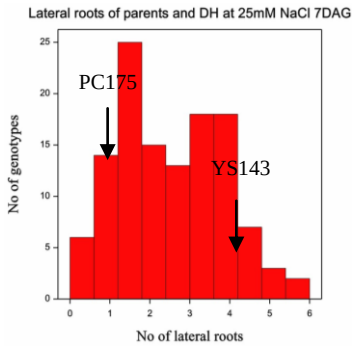
Appendix 9. Summary statistics of DH population and parents for lateral roots in 25mM NaCl concentration

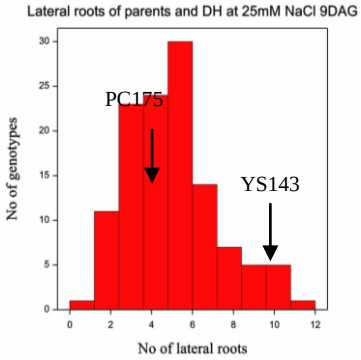
	LR7DAG	LR9DAG
Mean	2.6	5.0
Median	2.5	4.8
Std.Deviation	1.3	2.2
Variance	1.7	4.8
Minimum	0.3	0.6
Maximum	5.9	11.4

Appendix 10. Lateral root distribtion pattern of DH population in different (0 and 25mM) NaCl concentrations

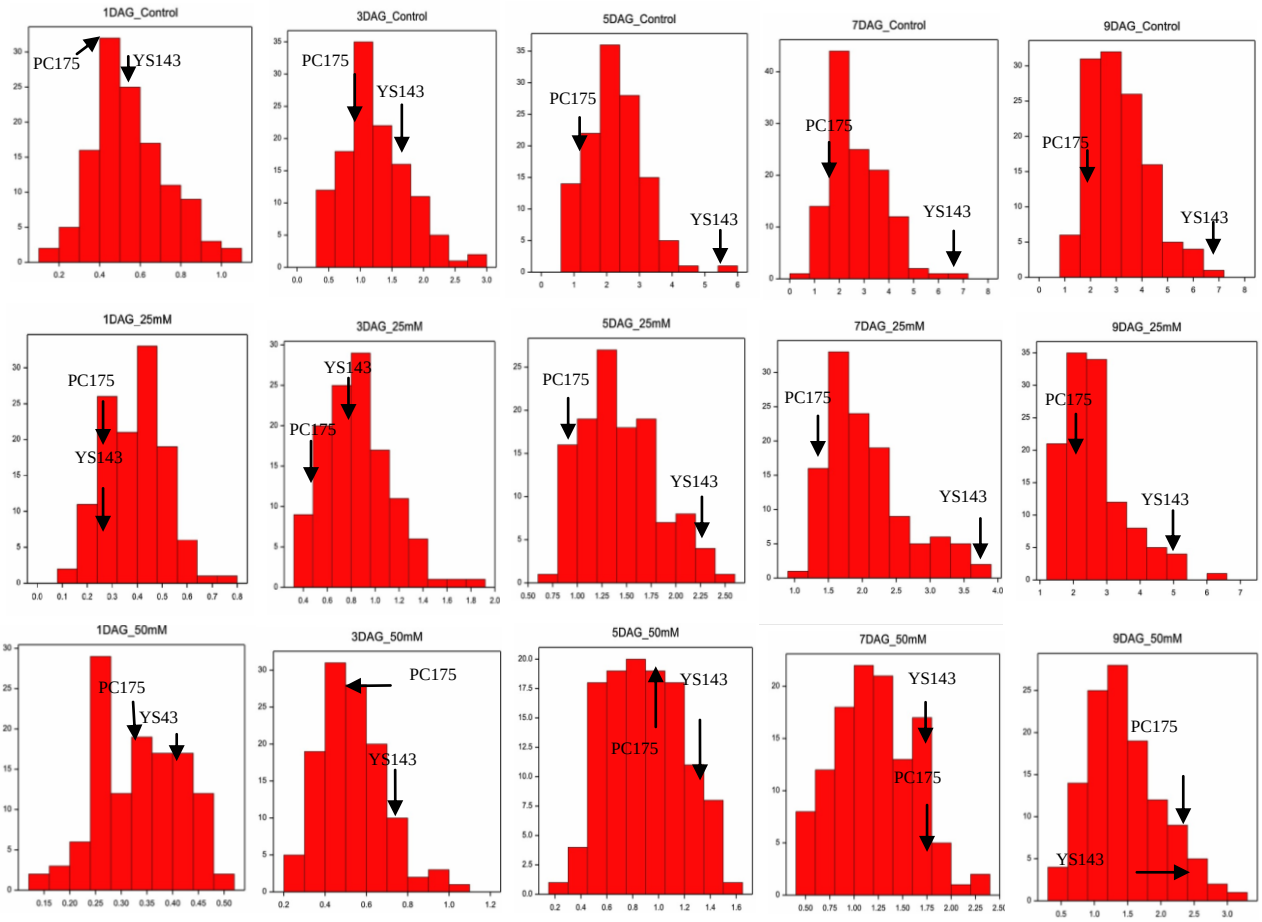






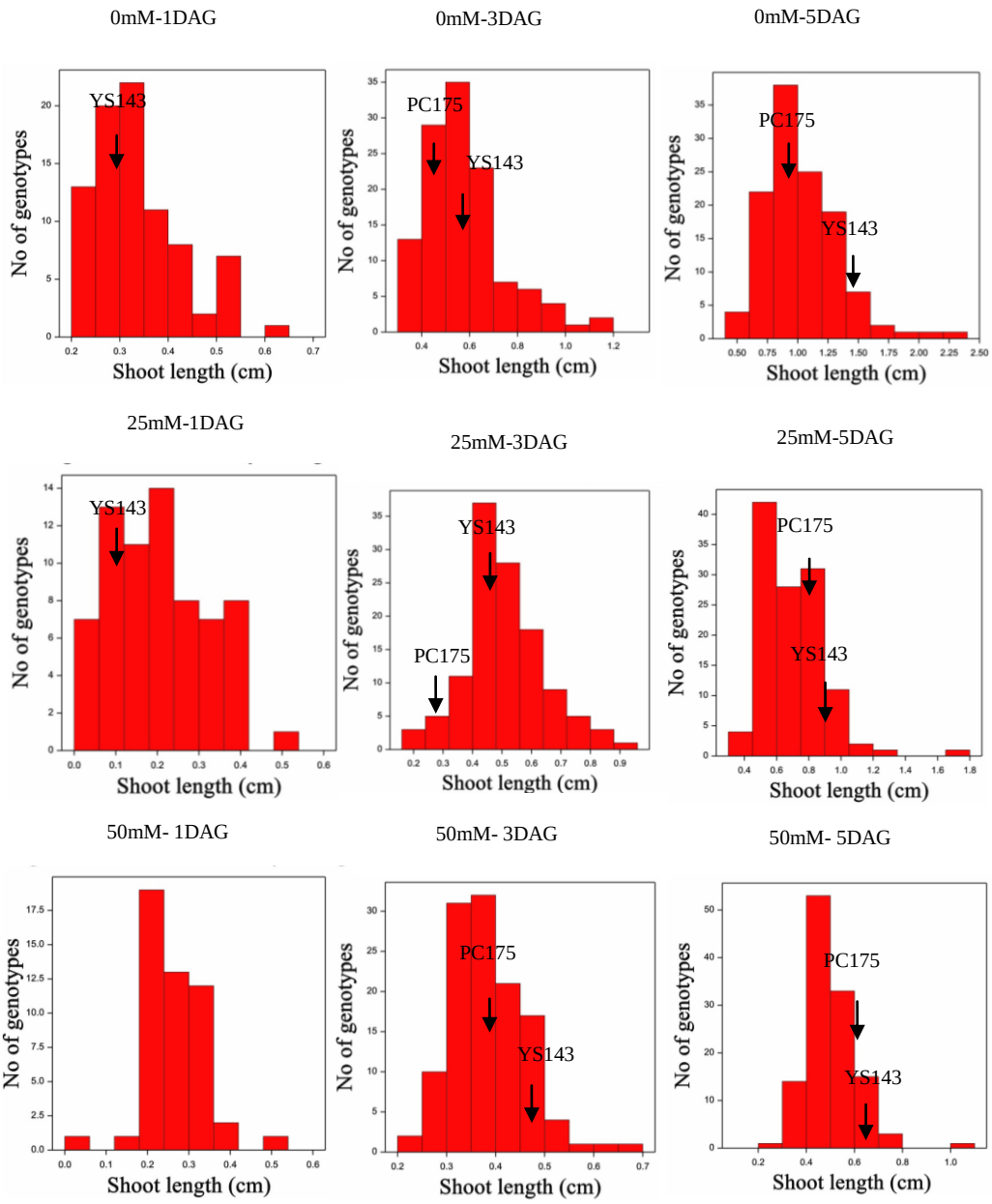


Appendix 11 Histogram of root growth pattern of parents and DH population under different (0, 25, 50mM)[6] NaCl concentrations

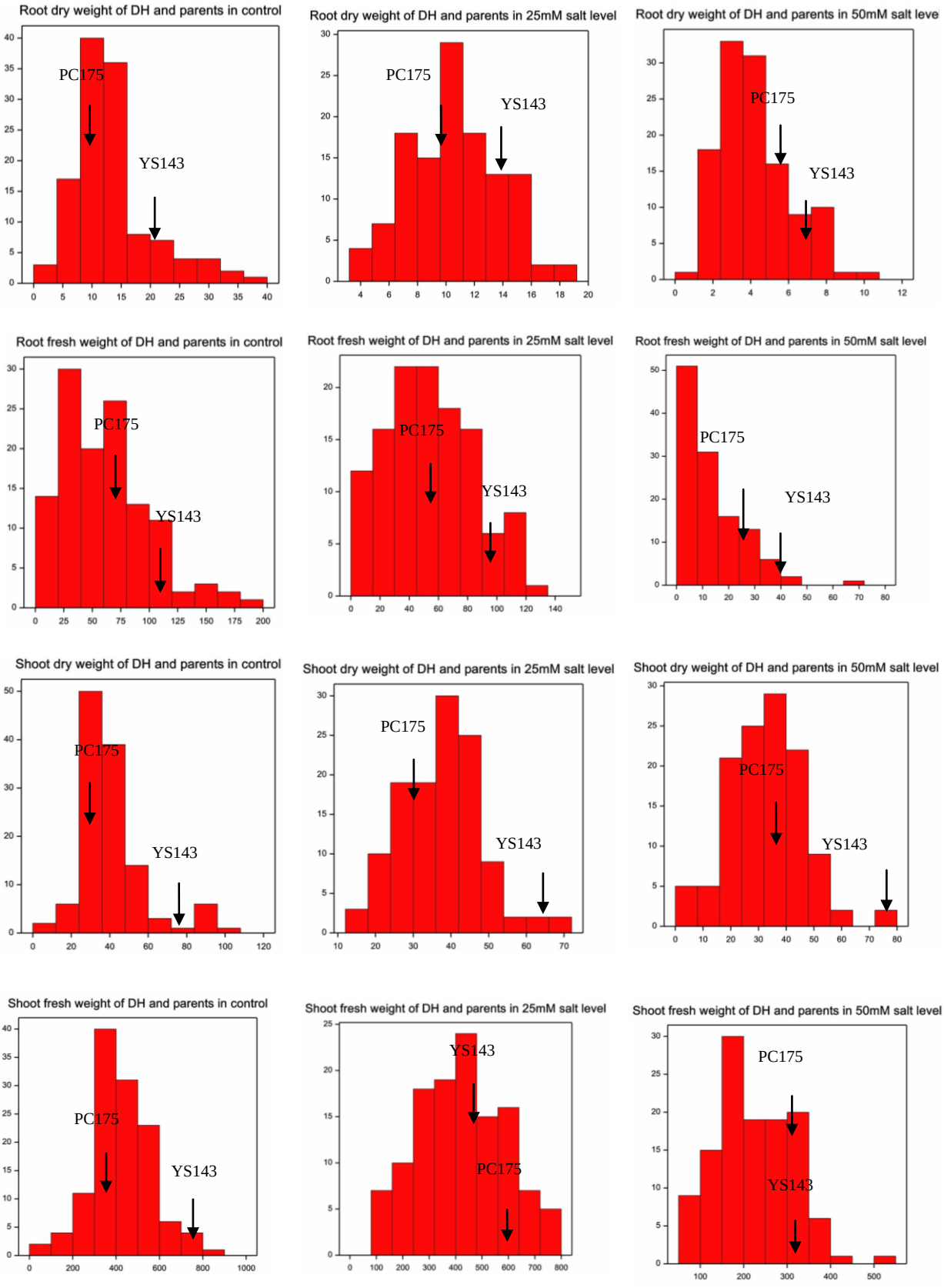


[6] Histogram showing root length on x-axis and number of DH lines in y-axis.

Appendix 12. Histogram on shoot growth pattern parents and DH population under different (0, 25, and 50mM) NaCl concentrations.

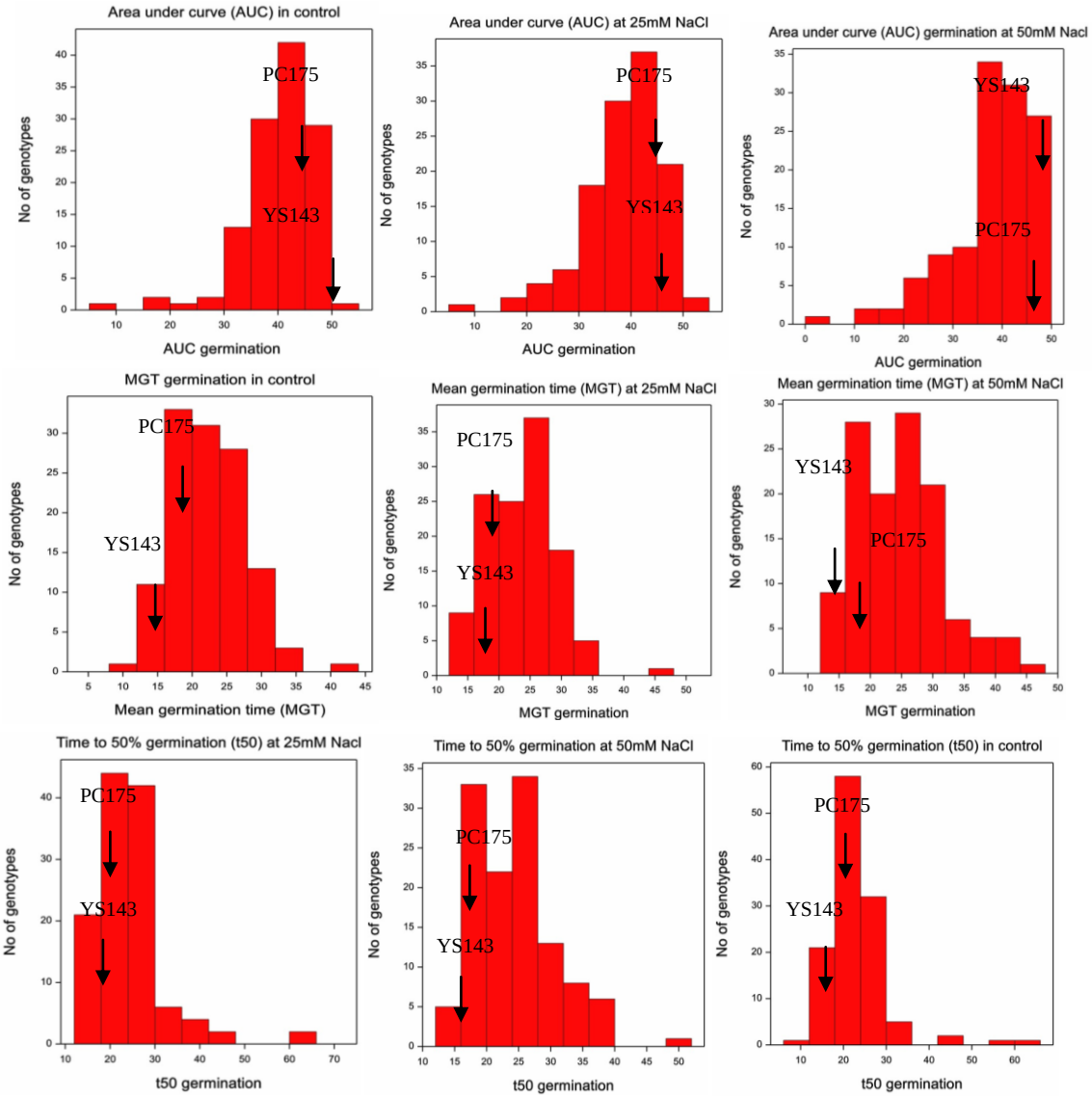


Appendix 13 Histogram on Fresh and dry weight trait of DH and its parents under different (0, 25, 50mM) NaCl concentration[7].



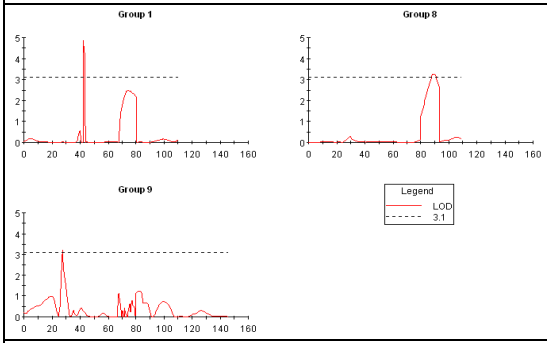
[7] Histogram showing fresh and dry weight (mg) along x-axis and number of DH lines along y-axis

Appendix 14 Histogram showing AUC, MGT and t50 under different (0, 25 and 50mM) NaCl concentrations.

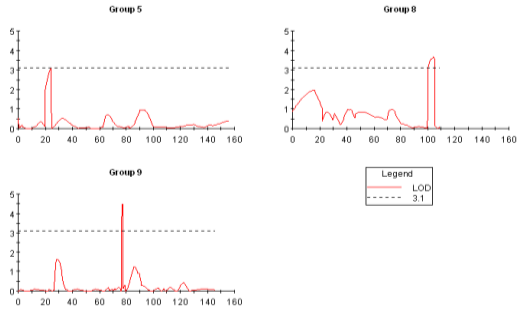


Appendix 15 QTL mapping of different traits in different (0, 25 and 50mM) NaCl concentration.

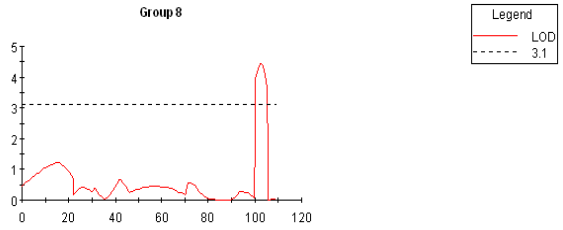
QTLs for root length found in control (0mM NaCl) at 1 day after germination.



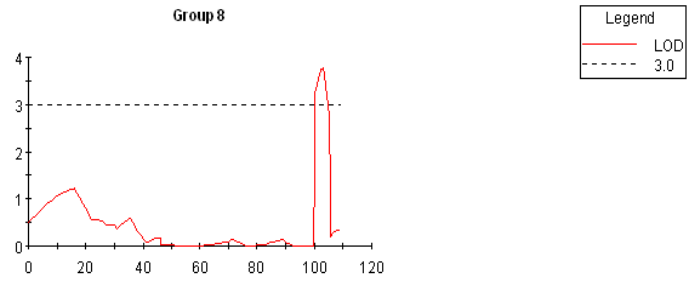
QTLs for root length found in control (0mM NaCl) at 3 days after germination



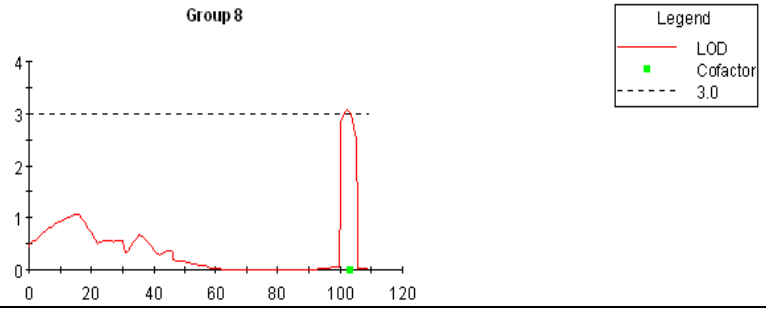
QTLs for root length found in control (0mM NaCl) at 5 days after germination



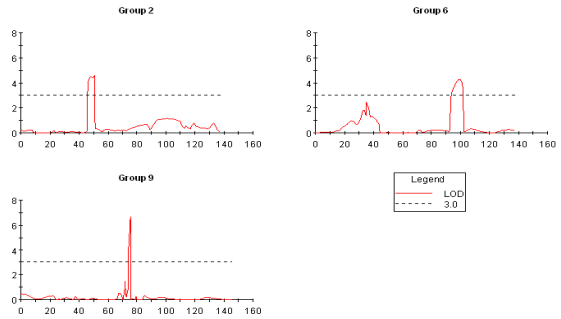
QTLs for root length found in control (0mM NaCl) at 7 days after germination



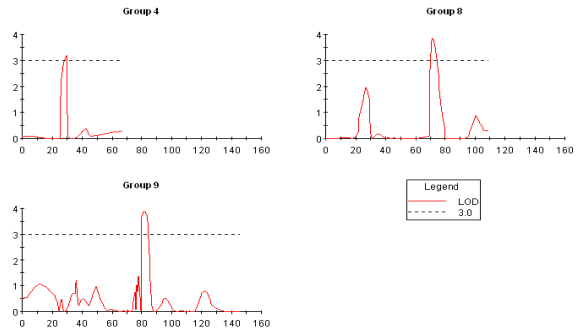
QTLs for root length found in control (0mM NaCl) at 9 days after germination



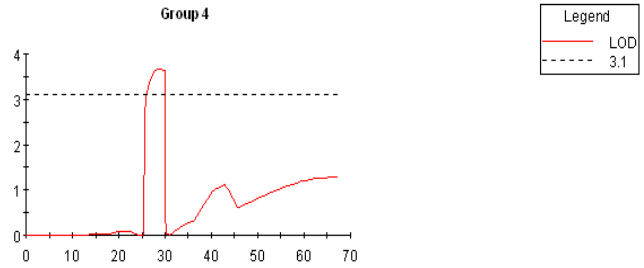
QTLs for root length found in 25mM NaCl at 1 day after germination



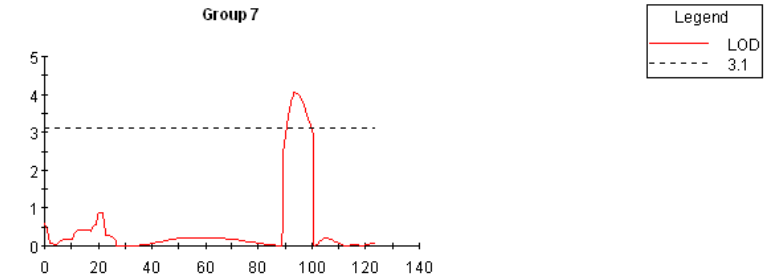
QTLs for root length found in 25mM NaCl at 3 day after germination



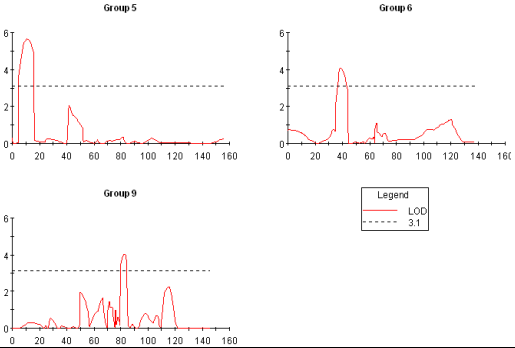
QTLs for root length found in 25mM NaCl at 5 day after germination



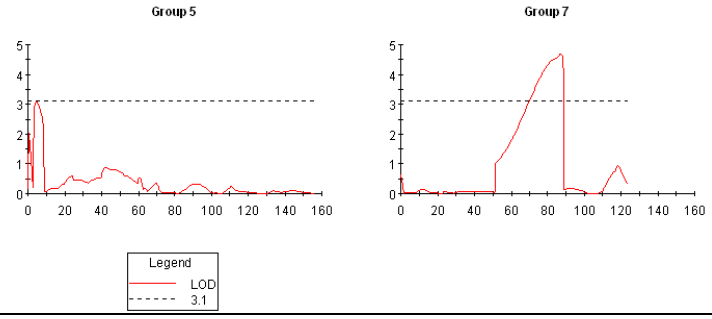
QTLs for root length found in 50mM NaCl at 1 day after germination



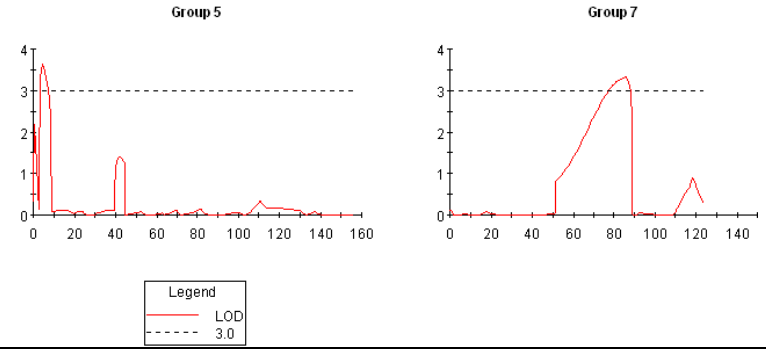
QTLs for root length found in 50mM NaCl at 3 day after germination



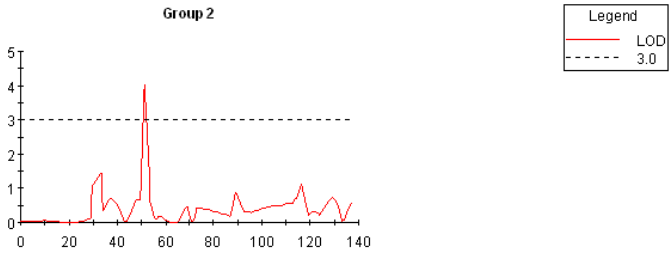
QTLs for root length found in 50mM NaCl at 5 day after germination



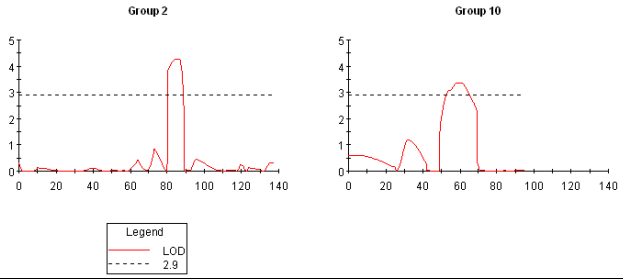
QTLs for root length found in 50mM NaCl at 5 day after germination

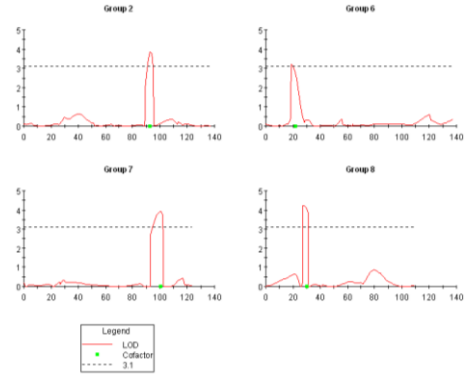


QTLs for shoot length in control (0mM NaCl) at 1 day after germination

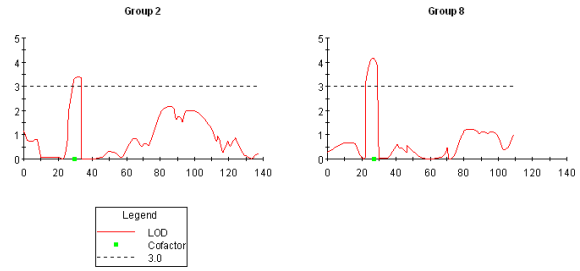


QTLs for shoot length in control (0mM NaCl) at 5 days after germination

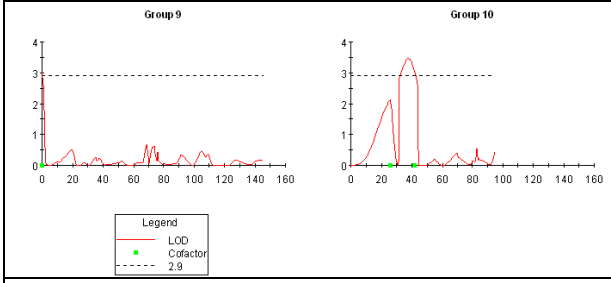




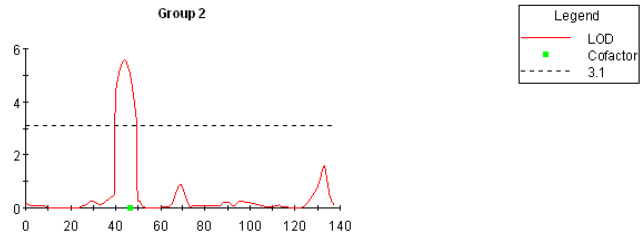
QTLs for shoot length in 25mM NaCl at 5 days after germination



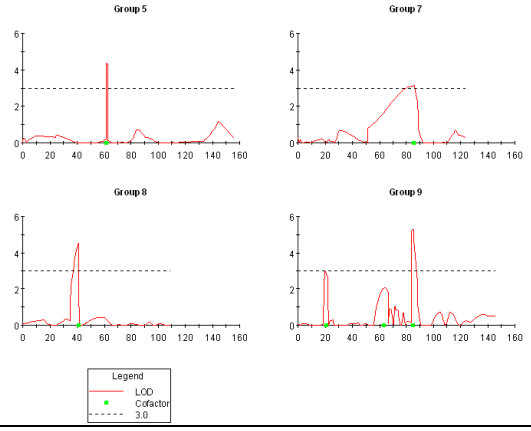
QTLs for shoot length in 50mM NaCl at 1 day after germination



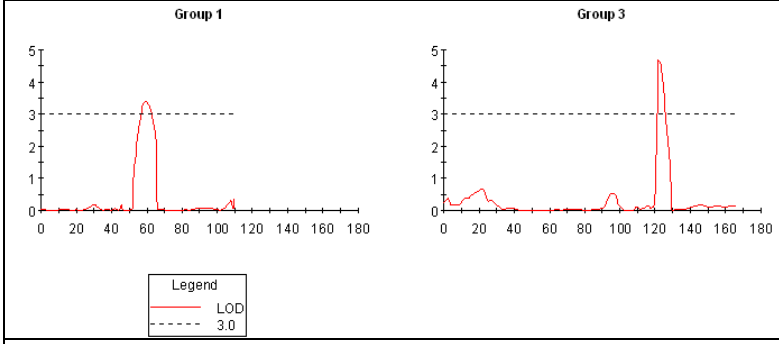
QTLs for shoot length in 50mM NaCl at 3 days after germination



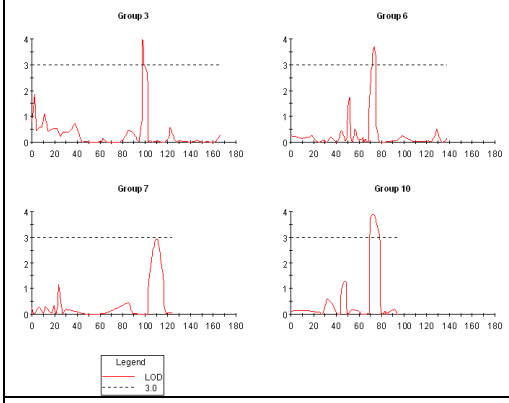
QTLs for shoot length in 50mM NaCl at 5 days after germination



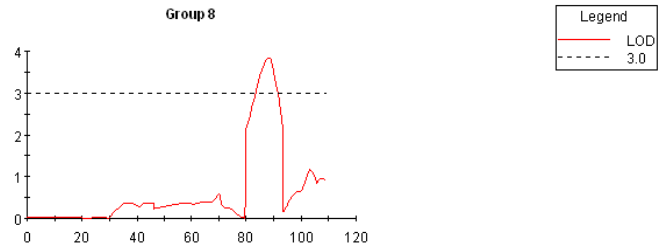
QTLs on root dry weight in control (0mM NaCl) condition



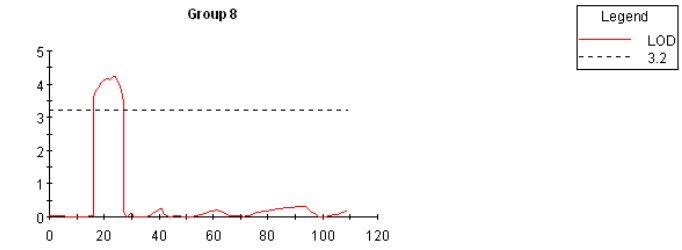
QTLs root fresh weight in control (0mM NaCl) condition



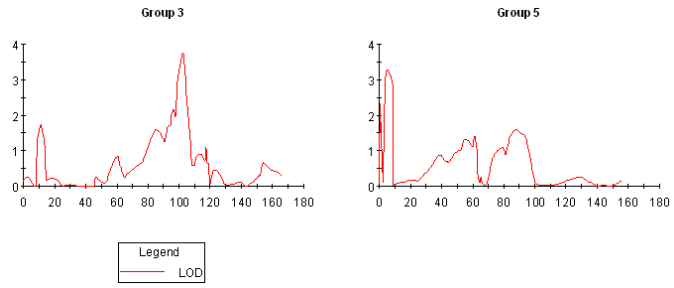
QTLs shoot fresh weight in control (0mM NaCl) condition



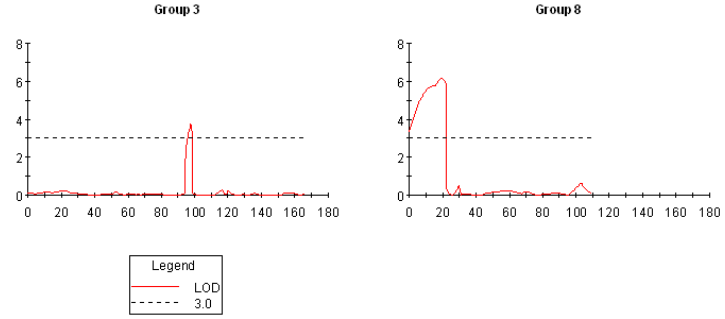
QTLs for root dry weight in 25mM NaCl concentration



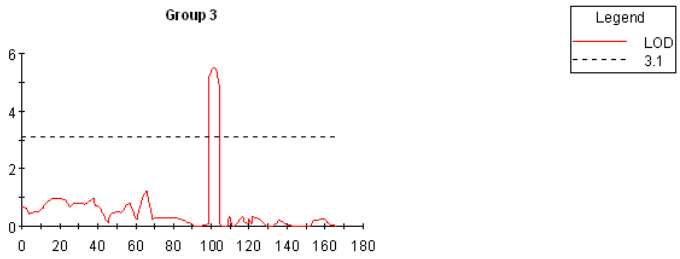
QTLs for shoot dry weight in 25mM NaCl concentration



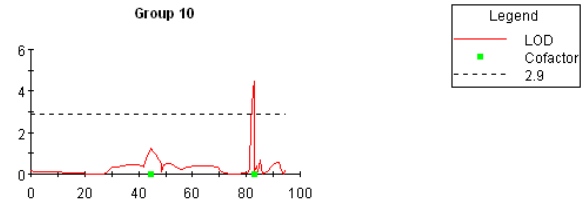
QTLs for root dry weight in 50mM NaCl concentration



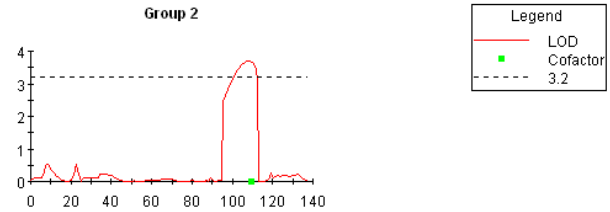
QTLs for shoot dry weight in 50mM NaCl concentration



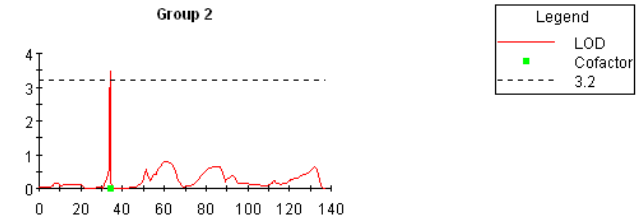
QTLs for area under curve germination in control (0mM NaCl) condition



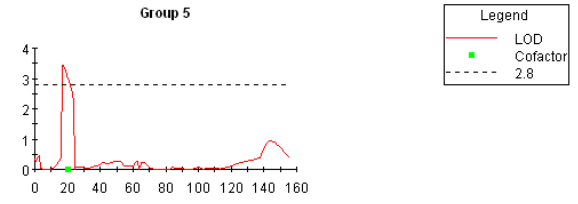
QTLs for T_{50} germination in 50mM NaCl concentration



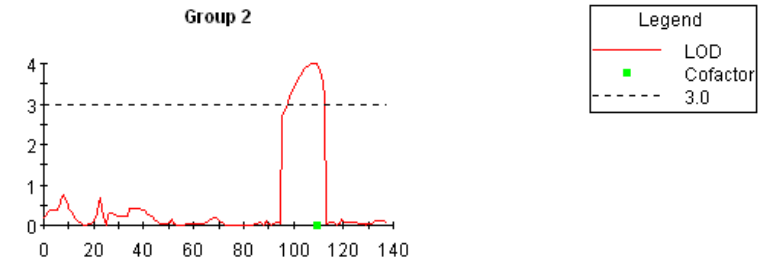
QTLs for u7525 germination in 50mM NaCl concentration



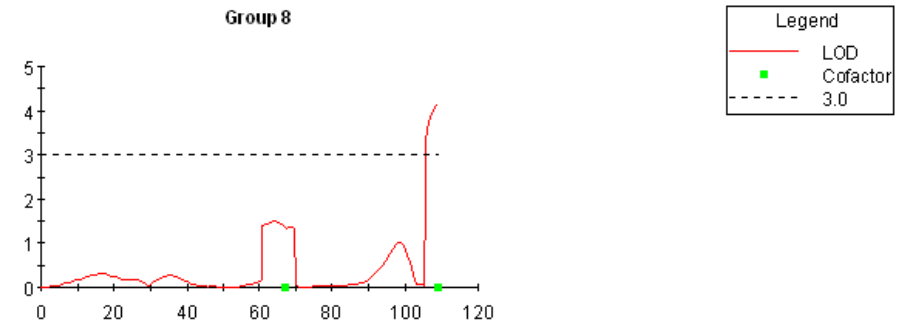
QTLs for u7525 germination in control (0mM NaCl) condition



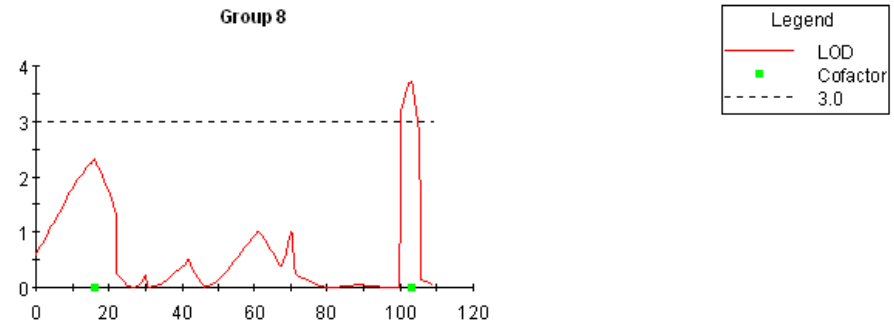
QTLs for Mean germination time (MGT) in 50mM NaCl concentration



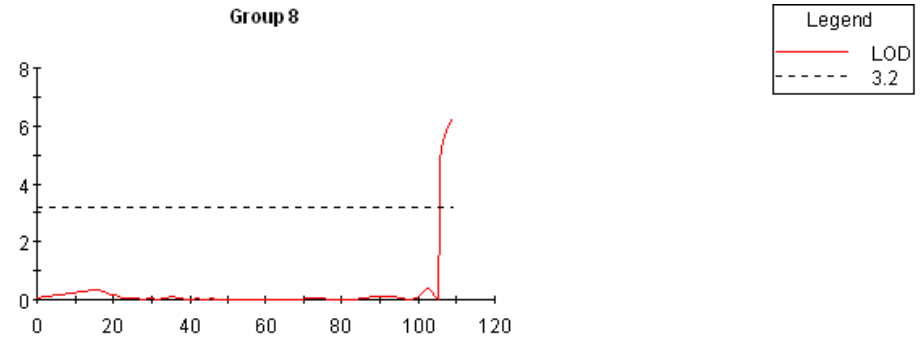
QTLs for lateral roots in 25mM NaCl at 7days after germination

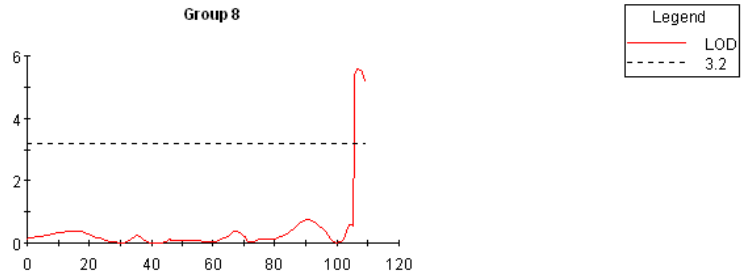


QTLs for lateral roots in 25mM NaCl at 9 days after germination

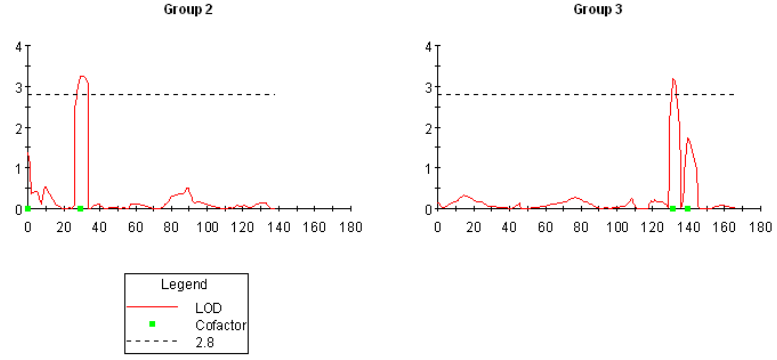


QTLs for lateral roots in control (0mM NaCl) at 7days after germination

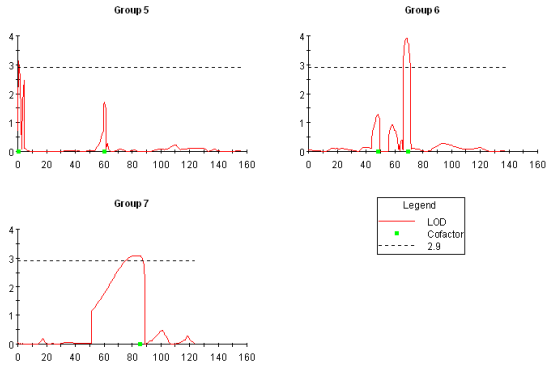




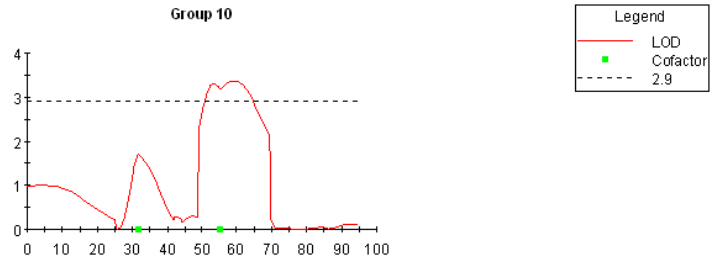
QTLs found for area under curve to root length in 25mM NaCl concentration



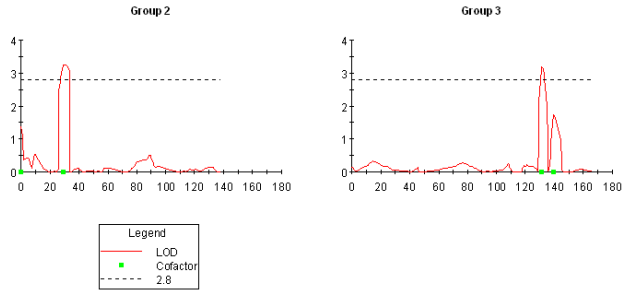
QTLs found for area under curve to root length in 50mM NaCl concentration



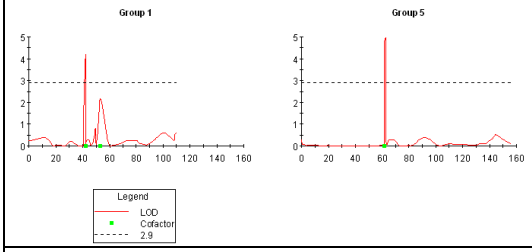
QTLs found for area under curve to shoot length Control (0mM NaCl) condition



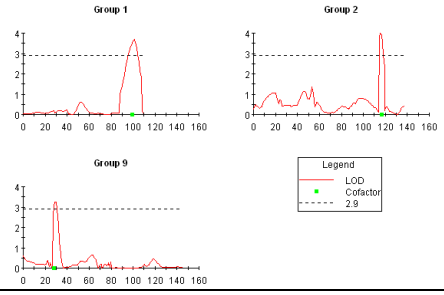
QTLs found for area under curve to shoot length in 25mM NaCl concentration



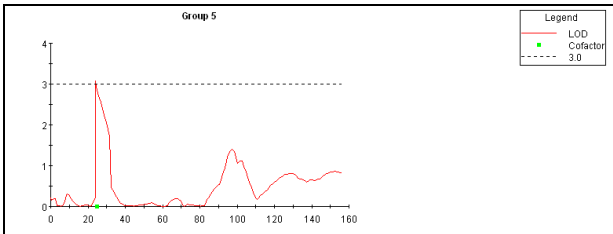
QTLs found for area under curve to shoot length in 50mM NaCl concentration



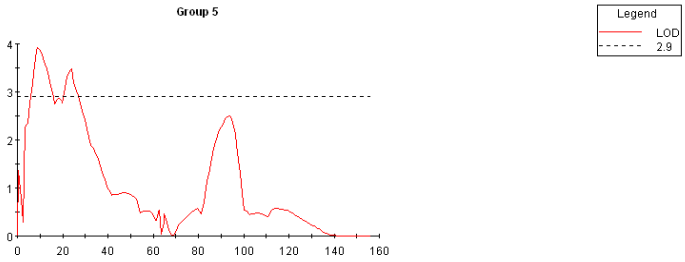
QTLs found in NaCl response on control and 25mM NaCl at 1 day after germination



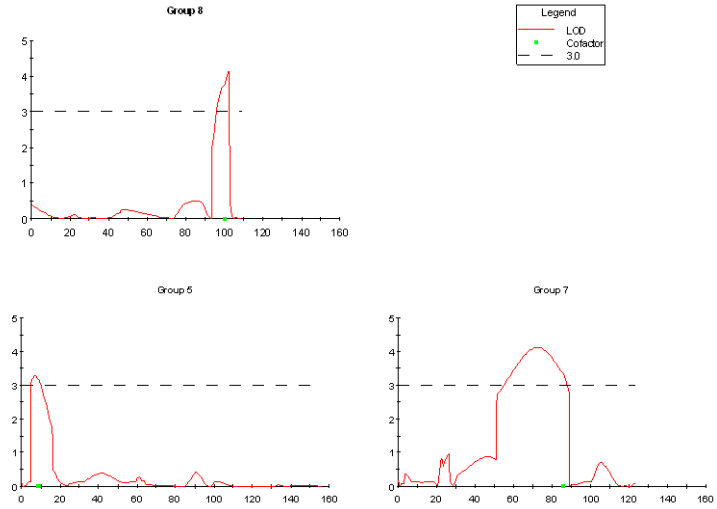
QTLs found in NaCl response on control and 25mM NaCl at 3 day after germination



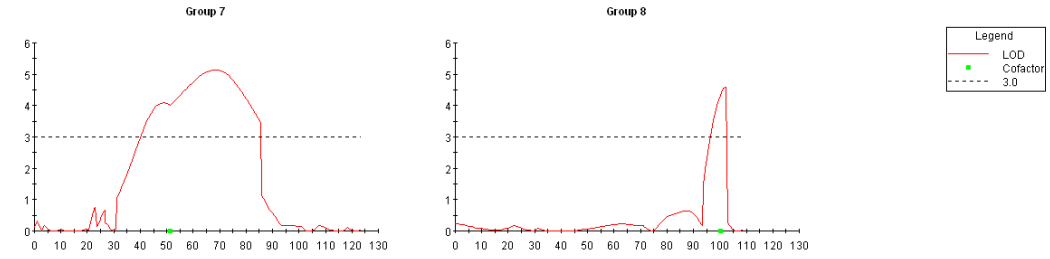
QTLs found in NaCl response on control and 50mM NaCl at 3 days after germination



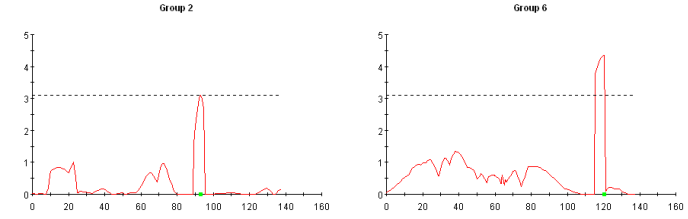
QTLs found in NaCl response on control and 50mM NaCl at 5 days after germination

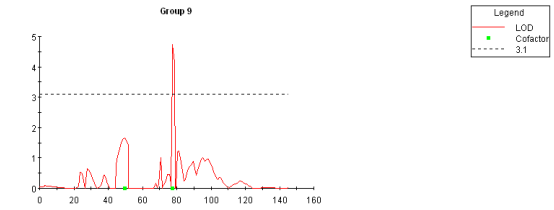


QTLs found in NaCl response on control and 50mM NaCl at 7 days after germination

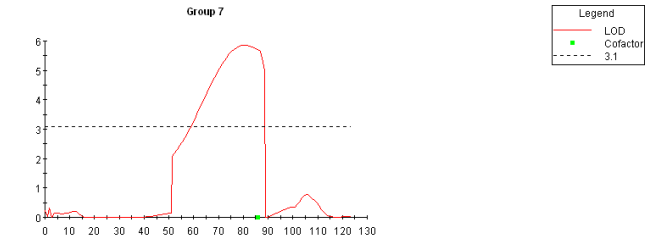


QTLs found in NaCl response on 25 and 50mM NaCl at 1 day after germination

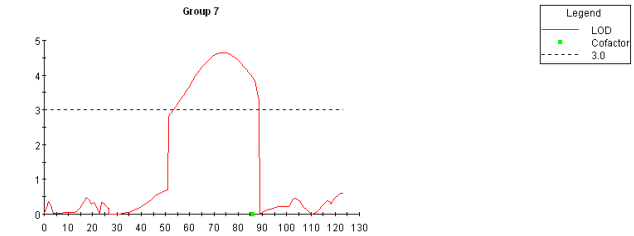




QTLs found in NaCl response on 25 and 50mM NaCl at 5 days after germination



QTLs found in NaCl response on 25 and 50mM NaCl at 7 days after germination



QTLs found in NaCl response on 25 and 50mM NaCl at 9 days after germination