PHYSIOLOGICAL AND GENETIC CHARACTERIZATION OF SALT TOLERANCE IN TOMATO (*LYCOPERSICON ESCULENTUM*)

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ABSTRACT

PHYSIOLOGICAL AND GENETIC CHARACTERIZATION OF SALT TOLERANCE IN TOMATO (*LYCOPERSICON ESCULENTUM*)

Plant growth is limited by different environmental conditions. Salt stress is one of these conditions that affects plant growth. Elimination of salt from the soil is time consuming and very expensive. The most effective way to eliminate salinity effects is to produce salt tolerant crops. Both transgenic applications and molecular marker technology are of importance in producing salt tolerant plants.

In this study, responses to salt stress of tomato were studied during the germination and vegetative stages of the life cycle. Inbred Backross Lines (IBLs) from a cross between salt-sensitive *L. esculentum* and a salt-tolerant *L. pimpinellifolium* were used for evaluation of salt tolerance during seed germination and QTL mapping. At the end of the germination study, it was observed that the IBLs have some degree of salt tolerance. *L. esculentum* alleles provided improved total percent germination on salt, however, *L. pimpinellifolium* alleles provided an improved rate of germination on salt. Thus, different parameters of salt tolerance are controlled by different mechanisms during seed germination. *L. pennellii* introgression lines (ILs) generated by crossing *L. pennellii* (LA716) to *L. esculentum* cv. M82 were used to map antioxidant traits related to salt tolerance at the vegetative stage of tomato. Plants of cultivated tomato (M82) and 32 IL lines were grown in aerated Hoagland solution in the greenhouse. At the seven-true leaf stage salt treatment was started and was achieved with the gradual addition of NaCl to the nutrient solution until 150 mM NaCl was reached. Superoxide dismutase (SOD) and catalase (CAT) activities were measured in the leaf tissues of these plants. Enzyme activities of the ILs were compared with M82 and QTLs associated with SOD and CAT activity under control and salt conditions were mapped.

ÖZET

DOMATES'TE (*LYCOPERSICON ESCULENTUM*) TUZA DAYANIKLILIĞIN FİZYOLOJİK VE GENETİK KARAKTERİZASYONU

Bitki büyümesi farklı çevresel şartlarla sınırlıdır. Tuz stresi, bitki büyümesini etkileyen şartlardan biridir. Toprağın tuzdan arındırılması uzun zaman gerektiren ve çok pahalı bir işlemdir. Tuzluluğun etkisini ortadan kaldırmak için uygulanacak en etkili yöntem, tuza dayanıklı ürün yetiştirmektir. Hem transgenik uygulamalar hem de moleküler işaretleyici teknolojisi tuza dayanıklı bitkilerin üretilmesinde önem taşımaktadır.

Bu çalışmada, domatesin tuz stresine tepkisi, yaşam döngüsünün çimlenme ve vejetatif dönemlerinde çalışılmıştır. Tuza dayanıklı bir tür olan *L. pimpinellifolium* ve tuza duyarlı bir tür olan *L. esculentum* arasında yapılan melezlemeden türetilen kendilenmiş geri melez hatları (Inbred Backross Lines) tohum çimlenmesi dönemindeki tuza toleransın değerlendirilmesi ve QTL haritalaması için kullanılmıştır. Çimlenme dönemi çalışmalarının sonunda, IBL'lerin belli dereceye kadar tuza toleranslı oldukları gözlemlenmiştir. *L. esculentum* alleleleri tuzda toplam çimlenme yüzdesini artırırken, *L. pimpinellifolium* alleleleri tuzda çimlenme oranında bir iyileştirme sağlamıştır. Buda, tuz toleransının değişik parametrelerinin, çimlenme döneminde farklı mekanizmalar tarafından kontrol edildiğini göstermektedir. Tuza dayanıklı bir tür olan *L. pennellii* (LA716) ve tuza duyarlı bir tür olan *L. esculentum* cv M82 arasında yapılan melezlemeden türetilen, *L. pennellii* hatları, domatesin vejetatif evresindeki tuz toleransıyla ilişkili olan antioksidan özelliklerin haritalanması için kullanılmıştır. Kültür domates çeşidi M82 ve 32 IL hatlarına ait bitkiler, serada havalandırılmış Hoagland çözeltisi içerisinde yetiştirilmiştir. Yedi gerçek yaprak safhasında, tuz uygulaması başlatılmış ve bu uygulama 150 mM NaCl konsantrasyonuna ulaşıncaya kadar besin çözeltisine kademeli olarak NaCl'nin eklenmesi ile gerçekleştirilmiştir. Süperoksit dismutaz (SOD) ve katalaz (CAT) aktiviteleri, bu bitkilerin yapraklarından ölçülmüştür. IL'lerin enzim aktiviteleri, M82'ninkilerle kıyaslanmış, tuz ve kontrol koşullarındaki SOD, CAT aktivitesi ile ilişkili QTL'ler haritalanmıştır.

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CHAPTER 1

INTRODUCTION

1.1. Definition of Salinity

Salinity is one of the major limitations of plant growth and productivity all over the world. Damage caused by high salinity to plants is observed as either loss of plant productivity or plant death. Water sources on earth contain 30g of sodium chloride per litre and from this data it can be said that earth is a salty planet. Soil salinity occurred long before humans and agriculture, however, the problem is increasing at a rate of 10% annually (Flowers, 2004, Foolad, 2004).

Soil salinity is defined as a measurement of the total amount of soluble salt in soil. Soil with an electrical conductivity of saturation extracts above 4 dSm^{-1} is called saline soil (Marschner and Termaat, 1995). Soil salinity has been increasing due to many factors: low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, entry of sea water into freshwater, and poor cultural practices (Foolad, 2004). Among other things, the accumulation of salts because of irrigation extensively affects agriculture. The soil can lose its pure water as a result of evaporation and transpiration and it becomes enriched with solutes. The problem is worsened, if irrigation is done with water that has a high solute concentration. Moreover, many factors interact with salinity such as humidity, temperature, light and soil fertility. The effect of salinity is altered when they present.

According to the FAO Land and Plant Nutrition Management Service, over 800 million hectares of land throughout the world are salt-affected (Munns, 2005). This number includes over 6% of the world's land area. There are 14 billion ha of available land on earth for farming. Arid and semi-arid regions compose 6.5 billion ha of this farm land and 1 billion ha of these regions are saline soils. With the increase of saline soils over the years, it is expected that by 2050, more than 50% of the available land for agriculture will be lost because of salinity. Salinity negatively affects crop productivity and quality. So, it is an important limitation to food supply. Although food supply is currently enough for the world's population, today more than 800 million people are undernourished. The human population will increase to 9.3 billion by 2050, so it is

inevitable that the world will face a huge food scarcity problem in the near future (Flowers, 2004).

According to Epstein *et al*. (1980) two types of methods can be applied to cope with negative effects of high salinity in agriculture. One is using high quality water for irrigation. Despite effectiveness in some areas, this method is not an answer to the salinity problem due to its high costs and limited applicability. The second method is generating crops that can tolerate high levels of salt. This approach has been found to be more promising for growing plants in saline soils and can be used in combination with the first method (Epstein *et al*., 1980).

1.2. Effects of Salt Stress on Plants

Plants' main requirements for their life cycle are mineral nutrients (elements) and energy from sunlight. There are certain elements called essential mineral nutrients and it has been determined that plants need them to grow and develop. These elements are of importance in numerous biological functions and each has its own functions in the cell. Energy storage, structural integrity and roles in redox reactions are some examples of the biological functions of these nutrients. Although essential mineral nutrients are imperative for plant survival, excessive soluble salts in the soil have deleterious effects on most plants. In addition, plant growth is more influenced by salt than other toxic substances (Xiong and Zhu, 2002). According to their response to high salt concentrations, plants can be divided into two groups (Flowers *et al*., 1977). Glycophytes are sensitive to high salinity. Halophytes have tolerance to saline soils and they comprise a wide spectrum of families. Halophytes are experimentally of importance since they have the ability to cope with salt stress and studies of them have led to the discovery of salt tolerance mechanisms (Flowers, 2004).

The main environmental factor for plants growing in saline soils is the high concentration of salts. As a result of high salt concentration, soil water potential decreases. Consequently, the plant water potential is lowered (Flowers *et al*., 1986). With the entry of sodium ions into the plant cells, plant solute content is also altered. The mechanism of uptake of Na^+ into plant cells is not clear due to the lack of a specific transport system. Na^+ entry occurs via passive transport, since the amount of Na^+ in the soil solution is much higher than in the cytosol of root cells. It has been reported that

Na^+ enters the root cells through different cation channels. Voltage-dependent cation channels and voltage-independent cation (VIC) channels fall into the two main categories. VIC channels are thought to be the major way for Na^+ to move into plant cells (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999; White, 1999). Molecular mechanisms of VIC channels are not yet clear, on the other hand, voltage-dependent cation channels have been partially elucidated. Potassium channels are thought to be one route for Na^+ entry to root cells. Since Na^+ and K^+ have the same charge (Blumwald *et al*., 2000), it is possible that these channels can also be used to move NaCl.

Salinity causes four detrimental effects on plants. The first type of negative effect is osmotic stress. Salt stress alters the water potential in the environment and this causes osmotic stress to plants. Because of high salinity, plants lose their turgor (Xiong and Zhu, 2002). Another negative effect of salinity is nutrient deficiency. As a result of decreasing water uptake from the soil, the entry of essential minerals such as phosphorus, potassium, nitrate, and calcium, for plant growth is lowered (Xiong and Zhu, 2002). Ion cytotoxicity is another detrimental effect of salinity. Injurious concentrations of Na^+, Cl^- and SO_4^{2-} cause ion toxicity (Xiong and Zhu, 2002). Oxidative stress occurs as a secondary effect of salinity and it is caused by excessive reactive oxygen species (ROS). Hydrogen peroxide, hydroxyl radicals and superoxide anions belong to ROS. In fact generation of ROS usually involves normal cellular reactions. However, when plants are subjected to stress, the amount of ROS in the cells increases (Xiong and Zhu, 2002).

Salinity generally affects growth rate and it results in plants with smaller leaves, shorter stature and sometimes fewer leaves by reducing growth rate. Osmotic stress forms the initial and primary effect of salinity at low to moderate concentrations (Munns and Termaat, 1986; Jocoby, 1994). Salinity changes the roots' structure by reducing their length and mass, therefore roots may become thinner or thicker (Shannon and Grieve, 1999). The osmotic effects of salinity result in reduced growth rate and altered leaf color and changes in developmental characteristics including root/shoot ratio and maturity rate. Timing of development is affected by salinity. Onion plants flower early under salt stress, but flowering of tomato plants is delayed by salinity (Pasternak *et al*. 1979). Moreover, ionic effect of salinity are generally seen in leaf and meristem damage or typical nutritional disorder symptoms. Burning of leaves is an effect that is caused by both salinity and nutritional disorders (Shannon and Grieve,

1999). In spite of negative effects, salinity may have some good effects on yield, quality and disease resistance. For example, Osawa (1963) worked on spinach at low to moderate salinity and found that salinity may have a positive effect on yield increase. Sugar content of carrot and starch increase in the presence of salinity (Bernstein, 1959).

1.3. Salt Tolerance of Plants

Salt tolerance can be defined as the ability of plants to survive and maintain their growth under saline conditions. A wide variety of plant salt tolerance is seen depending on several factors such as the species of the plant. A continuous spectrum of plant tolerance to saline conditions is found ranging from glycophytes that are salt sensitive to halophytes that are salt tolerant (Volkmar *et al*., 1998). Many important crops are glycophytes and show susceptibility to soil salinity. Among crop species, different threshold tolerances (EC_e) and different reduction rates of yield have been seen and this indicates that there is variation for salt tolerance mechanisms (Chinnusamy and Zhu, 2005). Salinity tolerance of common crops is summarized in Table 1.1 (Maas, 1990). The first three plants, bean, eggplant and onion, are very sensitive to low EC_e values. Tomato and rice are moderately sensitive to salt stress. Barley, cotton and wheat show tolerance to high EC_e values.

Table 1.1. Salinity Tolerance of Common Crops.

Crop	**Threshold salinity** dSm^{-1}	**Decreased in yield** Slope % per dSm^{-1}
Bean (*Phaseolus vulgaris* L.)	1.0	19.0
Eggplant (*Solanum melongena* L.)	1.1	6.9
Onion (*Allium cepa* L.)	1.2	16.0
Pepper (*Capsicum annuum* L.)	1.5	14.0
Corn (*Zea mays* L.)	1.7	12.0
Sugarcane (*Saccharum officinarum* L.)	1.7	5.9
Potato (*Solanum tuberosum* L.)	1.7	12.0
Cabbage (*Brassica oleracea* var. *Capitata* L.)	1.8	9.7
Tomato (*Lycopersicon esculentum* Mill.)	2.5	9.9
Rice, paddy (*Oriza sativa* L.)	3.0	12.0
Peanut (*Arachis hypogaea* L.)	3.2	29.0
Soybean [*Glycine max* (L.) Merr.]	5.0	20.0
Wheat (*Triticum aestivum* L.)	6.0	7.1
Sugar beet (*Beta vulgaris* L.)	7.0	5.9
Cotton (*Gossypium hirsutum* L.)	7.7	5.2
Barley (*Hordeum vulgare* L.)	8.0	5.0

Plants have three types of mechanisms to tolerate high salt concentrations: cellular homeostasis which includes ion homeostasis and osmotic adjustment, detoxification and growth regulation (Zhu, 2001). These mechanisims are described in the following sections.

1.3.1. Homeostasis

Establishment of ion homeostasis is important for the response of plant cells to salinity. One mechanism of coping with the ionic stress imposed by high salinity is avoidance of salt accumulation in plant parts where they are harmful. This can be done by increasing the Na^+ efflux at the plasma membrane, passive exclusion of ions from the cytoplasm by a permeable membrane or dilution of ions in the plant tissue. Another metabolic process for protection from ionic stress is the storage of Na^+ in the vacuoles. This is an ideal way of preventing Na^+ toxicity in the cytosol. Therefore, Na^+ provides

osmotic adjustment to plant cells (Allen *et al*., 1994). Halophytes, naturally salt tolerant plants, generally use this strategy (Flowers *et al*., 1977). Mimura *et al*. (2003) have worked on increase of vacuolar volume under salt stress. They found that rapid increase in vacuolar volume is an adaptive mechanism of plant cells to salinity.

Potassium is one of the essential elements for plant growth and it has important functions. K^+ is necessary to maintain osmotic balance. K^+ also has a role in opening and closing of stomata. Moreover it is an essential co-factor for many enzymes such as pyruvate kinase (Mahajan and Tuteja, 2005). Because of K^+'s importance, salt tolerant plants must maintain a high level of potassium in their cells (Volkmar *et al*., 1998). Under salt stress, potassium deficiency occurs because of the similar structures of potassium and sodium, which impedes discrimination between the two ions (Maathius and Amtmann, 1999). The Na^+ toxicity problem mainly results from this lack of discrimination. Because there is competition between Na^+ and K^+ for uptake by Na^+- K^+ cotransporters, Na^+ blocks K^+ acquisition. Moreover, Na^+may block K^+ uptake through K^+ specific transporters in root cells. A high cytosolic K^+/ Na^+ ratio is an essential requirement for plant growth in high salt concentrations (Zhu, 2003). Determination of which transporter(s) can block Na^+ influx is an important aim of salt tolerance studies (Zhu, 2001). Wide genetic variation is found for Na^+ accumulation and cell K^+ /Na^+ discrimination in plants (Munns, 2005). Ion transport systems in the plasma and vacuolar membranes are important for determining the ratio of sodium and potassium. Na^+/H^+ antiporters have a role in the compartmentation of Na^+ in the vacuole and the extrusion of Na^+ from the cell (Blumwald *et al*., 2000). H^+ pumps in the plasma membrane and tonoplast are the driving force behind this secondary transport of ions. Na^+ extrusion is an active process. In higher plants, the plasma membrane H^+-ATPase pumps H^+ out of the cell via generating a gradient of electrochemical potentials of H^+ across the plasma membrane. During this procedure, H^+-ATPase uses the energy of ATP hydrolysis. The potential of the electrochemical H^+ gradient maintains most of the ionic gradients across membranes of higher plants (Tazawa and Okazaki, 1987). This Na^+/H^+ antiporter also allows the transportation of Na^+ out of the cell depending on the electrochemical gradient. The activity of these H^+ pumps is increased by salt treatment.

Three complementation groups of *sos* (salt overly sensitive [*sos1-sos3*]) mutants of *Arabidopsis* have been screened to identify key genes and cellular processes involved in plant salt tolerance. As a result of these studies the *sos* genes were positionaly cloned and characterized (Liu and Zhu, 1998). SOS1, SOS2 and SOS3 are the elements of a

signaling pathway important in controlling salt tolerance and ion homeostasis. The functions of these three genetically linked loci are Ca^{2+} dependent. The molecular genetic analysis of *sos* mutants of *Arabidopsis* also gives important insights into the identification of a plasma membrane Na^+/H^+ antiporter. The SOS1 gene encodes a Na^+/H^+ antiporter and its transcriptional level is upregulated under salinity. Mutations in SOS1 make *Arabidopsis* plants hypersensitive to salt stress. Shoot Na^+ content is lowered by the overexpression of SOS1 (Shi *et al*., 2000 & 2002). According to Shi *et al*. (2003) these plants show enhanced salt tolerance. A SOS3-SOS2 protein kinase complex controls the sodium efflux through SOS1 under salinity. A shematic representation of the SOS signaling pathway for ion homeostasis is summarized in Figure 1.1.

The SOS3 family of Ca^+ binding proteins in *Arabidopsis* are Ca^+ sensors with three calcium binding EF hands and an N-myristoylation motif (Liu and Zhu, 1998; Ishitani *et al*., 2000). It was shown by Ishitani *et al*. (2000) that a loss of function of SOS3 gene makes *Arabidopsis* mutant plants hypersensitive to salinity. SOS2 activity is Ca^{2+} dependent (Halfter *et al*. 2000) because of the interaction of SOS3 with SOS2 kinase (Liu *et al*. 2000). SOS2 is composed of an autoinhibitory FISL motif at the C-terminal regulatory domain (Liu *et al*., 2000). This FISL motif keeps the enzyme in an off state under normal conditions and when it is deleted, SOS2 becomes activated (Guo *et al*., 2001). SOS3 binds to the FISL motif of SOS2 and under salt stress the activation of SOS2 occurs (Halfter *et al*., 2000). As a result of this signaling pathway, SOS1 (the plasma membrane Na^+/H^+ antiporter) is phosphorylated and activated. One example of how the SOS3 $\rightarrow$ SOS2 $\rightarrow$ SOS1 signal pathway works is described by Guo *et al*., (2004). They showed that the overexpression of an active form of SOS2 could overcome the salinity hypersensitivity in *sos2* and *sos3* transgenic *Arabidopsis* mutants.

The compartmentation of Na^+ into the vacuoles is an important mechanism to prevent negative effects of Na^+ in the cytosol. It is also a cost-effective strategy, since Na^+ contributes osmotic adjustment to plant cells. Positive turgor should be maintained for cell growth and stomatal opening. Under saline conditions, the water potential of the soil decreases because of osmotic stress and this causes turgor loss in cells. To take up water, plants have an osmotic adjustment mechanism which maintains turgor under osmotic stress. Inorganic ions (Na^+ and K^+) or organic compatible solutes (proline, betaine, soluble sugars and polyols) are used as osmotica. A Na^+/H^+ antiporter is responsible for the transport of Na^+ into the vacuoles. The electrochemical gradients of

protons maintains the activity of the Na^+/H^+ antiporter. Tonoplast H^+-ATPase and H^+-PPiase generate the proton gradient for the Na^+/H^+ antiporter (Blumwald, 1987). Gaxiola *et al*. (2001) showed that the overexpression of the vacuolar H^+-pyrophosphatase AVP1 in *Arabidopsis* increased the sequestration of salt in vacuoles. This work points out an important difference between salt-sensitive and salt-tolerant plants. Salt-sensitive plants mainly exclude the Na^+ ions out of the plasma membrane, however salt-tolerant plants use the Na^+ compartmentation strategy.

Besides maintaining osmotic adjustment by Na^+ compartmentation and K^+, the synthesis and accumulation of organic compatible solutes/osmoprotectants are another adaptation mechanism to tolerate osmotic stresses. These solutes help plants to enhance osmotic adjustment and maintain turgor, otherwise dehydration will occur (Chinnusamy and Zhu, 2003). Water flows from a region with high water potential to one of low water potential. With the help of these accumulated osmolytes, the water potential inside the cell is maintained at a low level and intracellular water loss is prevented. These osmoprotectants are nontoxic and are compatible with metabolic activity. Therefore, these solutes can increase to high concentrations in the cytosol without any side effects (Jones, *et al*., 1977). The term osmoprotectant was given due to their ability to stabilize membrane proteins in order to maintain growth at high salinity (Yancey *et al*.,1982). Moreover, organic compatible solutes have a role in detoxification of reactive oxygen species (Chinnusamy and Zhu, 2003). These solutes contain the amino acid proline, sugar alcohols such as sorbitol and mannitol, sugars such as sucrose and raffinose, polyols, guaternary amino acid derivatives such as glycine betaine, proline betaine and □-alanine betaine. It was reported by Zhu (2002) that the genes related to osmoprotectant biosynthesis are up-regulated under salt stress and there is a correlation between concentrations of accumulated osmoprotectants and osmotic stress tolerance.

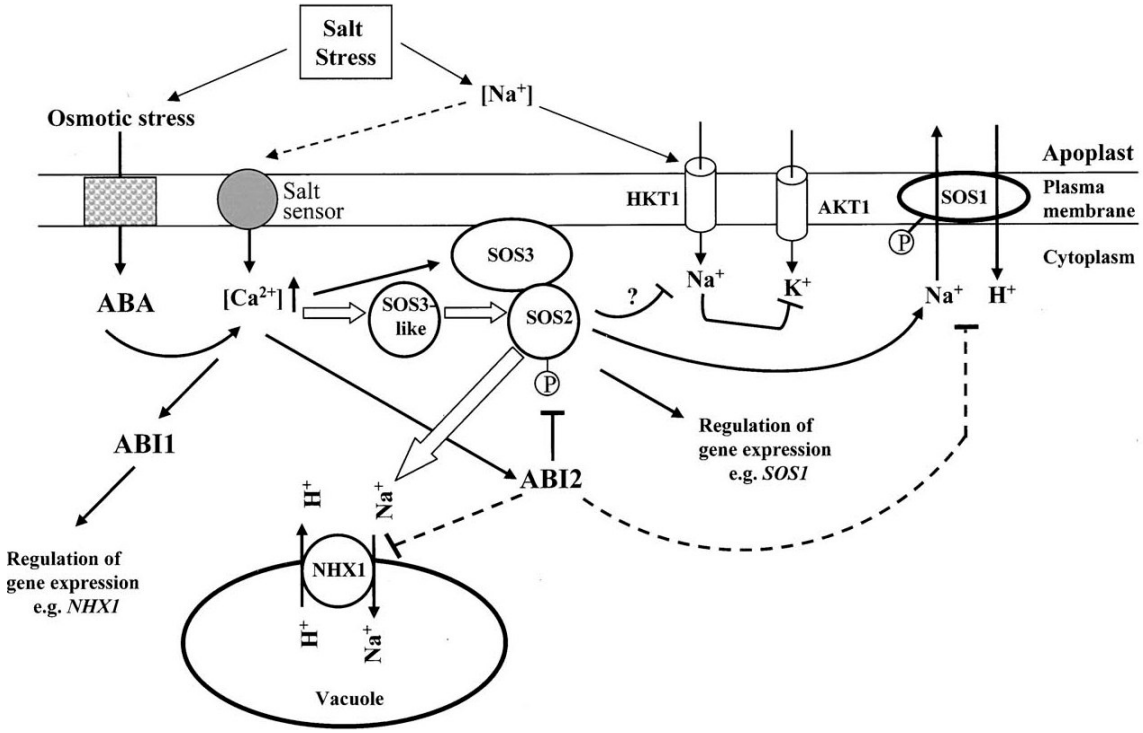

Figure 1.1. SOS signaling pathway for ion homeostasis under salt stress in *Arabidopsis*. Salt stress elicited Ca2_ signals are perceived by SOS3, which activates the protein kinase SOS2. Activated SOS2 phosphorylates SOS1, a plasma membrane Na_/H_ antiporter, which then transports Na out of the cytosol. The transcript level of SOS1 is regulated by the SOS3-SOS2 kinase complex. SOS2 also activates the tonoplast Na_/H_antiporter that sequesters Na_ into the vacuole. Na_ entry into the cytosol through the Na_ transporter HKT1 may also be restricted bySOS2. ABI1 regulates the gene expression of NHX1, while ABI2 interacts with SOS2 and negatively regulates ion homeostasis either byinhibiting SOS2 kinase activity or the activities of SOS2 targets. Double arrow indicates SOS3-independent and SOS2-dependent pathway (Zhu *et al*., 2005).

1.3.2. Detoxification

Oxidative stress is an important aspect of salinity stress in plants in addition to water stress and ionic stress. The formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) is a normal function of aerobic metabolism. The types of cellular activities that generate ROS include photorespiration, □-oxidation of fatty acids, and mitochondrial and chloroplast electron transport. However, ROS production increases under abiotic stresses including salinity (Xiong and Zhu, 2002). Under normal conditions the negative effects of ROS can be eliminated. However under stress,

induced production of reactive oxygen species strikes a balance between oxidants and antioxidants. The excessive ROS can damage proteins, lipids and nucleic acids (Halliwell and Guteridge, 1985). Hence, the alleviation of this oxidative damage is important in defence against abiotic stresses. Antioxidant compounds (nonenzymatic antioxidants) such as ascorbic acid, glutathione, thioredoxin, carotenoids and ROS scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) are employed by plants to eliminate ROS. The activity and expression level of the genes that encode the ROS scavenging enzymes are increased under abiotic stresses. There are many studies describing the correlation between oxidative stress and detoxifying mechanisms.

Superoxide dismutase catalyses the conversion of superoxide anions to hydrogen peroxide and water and is the first step of the defence mechanism (Figure 1. 2). SOD plays a crucial role in the defence against oxidative stress. If the superoxide anion is not neutralized at this step, it joins to Fe and Cu, causes oxidation and a hydroxyl radical occurs. There is no elimination mechanism for $OH^{\cdot}$**.** Catalases and several classes of peroxidases catalyse the conversion of H_2O_2 to oxygen and water (Figure 1.2). Transgenic plants containing higher levels of ROS scavenging enzymes, such as SOD (Alscher *et al*., 2002), APX (Wang *et al*., 1999), and GPX (Roxas *et al*., 2000) showed more tolerance to abiotic stresses.

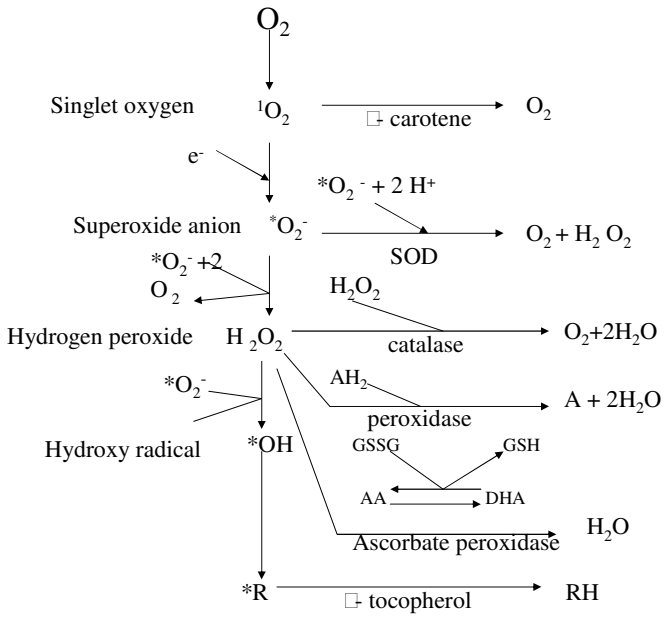

Figure 1.2. Mechanisms of reactive oxygen species formation and ROS/antioxidant signaling pathways.

1.3.3. Growth Regulation

Plant growth is inhibited by salt stress like other abiotic stresses. Plants can adapt to stress conditions via slower growth, since it allows the limited use of multiple resources for building blocks and energy under stress. Slower growth rate results from inadequate photosynthesis due to stomatal closure, limited carbon dioxide uptake and inhibition of cell division and expansion. There is a connection between stress signaling and cell division and expansion. Some plants are tolerant to stress and they almost stop growing when stress occurs. However, some plants are sensitive and they continue growing, which can cause plant death (Zhu, 2001) . Thus, differences in percent biomass production in saline and control conditions can be used as an assessment of salt tolerance. For example, sugarbeet as a salt-tolerant species might show a 20% reduction in dry weight, cotton as a moderately salt-tolerant species might show a 60% reduction and soybean as a sensitive species might be dead in 200 mM NaCl (Greenway and Munns, 1980). A halophyte such as *Suaeda maritima* may grow at its optimum rate under salt stress (Flowers *et al.* 1986). High salt concentrations trigger increases in levels of abscisic acid (Thomas *et al*. 1992) and the accumulation of abscisic acid might inhibit cell division (Zhu, 2001). Furthermore, the concentration of plant growth promoting hormones such as auxin, cytokinin, gibberellins and brassilonides, which have roles in cell elongation, are reduced (Zhu, 2001).

1.4. Developing Salt-Tolerant Crop Plants

Soil salinity is one of the major constraints to agricultural productivity throughout the world. There is a growing need to produce salt-tolerant crops and the possible methods for producing salt tolerant crops have been extensively tested. Two main approaches are employed to improve salt tolerance. One of them is the use of natural genetic variation by using direct selection in stressful environments or mapping quantitative trait loci and subsequent marker-assisted selection. Another approach is the generation of transgenic plants (Yamaguchi and Blumwald, 2005). There is limited success in attempts to improve salt tolerance of crops under salinity because of the necessity for complete understanding of the fundamental stress tolerance mechanism in plants. Thus, only a few examples of salt tolerant crops that have been developed exist

in salinity research literature. The physiological and genetic complexity of the trait causes this difficulty (Foolad, 2004). Salt-tolerant plants (halophytes) and salt-sensitive plant species (glycophytes) that show different salt tolerance levels are evidence that salt response is based on a genetic foundation. Studies with barley, citrus, rice and tomato indicate that salt tolerance has the characteristics of a multigenic trait involving the functions of many genes (Flowers, 2004). Furthermore, the functions of these genes are influenced by several environmental factors. Another important feature of salinity tolerance in plants is that it appears to be a developmentally regulated, stage specific phenomenon. Salinity tolerance varies according to the stage of plant development. There seems to be no correlation between tolerance of different ontogenic stages. For example, salt tolerance in tomato, barley, corn, rice and wheat increases with the age of the plant (Foolad, 2004). Different QTLs for salt tolerance at germination stage and at the early stage of growth were identified in barley (Mano and Takeda, 1997), in tomato (Foolad, 2004) and in *Arabidopsis* (Quesada, *et al*., 2002).

Characters such as fruit color and shape are determined by a single pair of alleles. These qualitative Mendelian characters show dominant-recessive inheritance. On the other hand, characters such as grain color in wheat, cob length in maize and yield in all crops are influenced by many genes and are quantitative traits. The regions of the genome containing a gene or genes encoding a quantitative character are called quantitative trait loci (QTLs). Poehlman (1987) listed the characteristics of multiple gene inheritance: (1) contribution of the quantitative character is caused by a number of genes at different loci, (2) each of the multigenes can have a small effect on the phenotype, (3) the quantitative characters show continous variation because of the additive effect of multigenes, (4) interaction between genotype and environment forms the phenotype and (5) transgressive segregation (offspring beyond the range of the parental phenotypes) can occur.

Genetic transformation, marker mapping and quantitative trait locus (QTL) analysis have enabled a better understanding of the genetic, physiological and biochemical bases of plant salt tolerance. Plants with improved salt tolerance can be developed with the help of these molecular genetic techniques. The identification of genes, enzymes or compounds which have significant effects at the organismal or cell level of plant salt tolerance are key for progress in developing salt tolerant plants (Shen *et al*., 1997; Winicov, 1998; Apse *et al*., 1999; Grover *et al*., 1999). Molecular marker technology is also effective in the identification, characterization and comparison of

QTLs with significant effects on plant salt tolerance at different developmental stages (Ellis *et al*., 1997; Foolad and Lin, 1998; Foolad and Lin, 2001). The detection of QTL related to salt tolerance has been possible by crossing two parents that show differences in salt tolerance and analysing the genotype and phenotype of their offspring (Flowers, 2004). Genetic markers contribute to a better understanding of the genetic basis of complex traits like salt tolerance and also contribute to improved efficiency of selection for QTLs controlling the traits. With the advent of molecular marker technology, the number, chromosomal locations and individual and interactive effects of QTLs can be detected. A process known as marker-assisted selection (MAS) allows the subsequent introgession of useful QTLs into desirable genetic backgrounds. Furthermore, identification of QTLs with significant effects on salt tolerance is of importance to understand the genetic basis of plant salt tolerance.

The response of plants to salt stress is controlled by many genes and the functions of these genes cause a wide variety of biochemical and physiological changes. Compartmentalization of toxic ions in the vacuole, activation of detoxifying enzymes, synthesis of late embryogenesis abundant (LEA) proteins and accumulation of compatible solutes are examples of the roles of these genes. This polygenic feature of plant's response to salt stress makes it difficult to transfer individual genes using traditional plant breeding or MAS. Transgenic approaches have been employed to obtain genetically modified plants that are tolerant to salt stress. Overexpression of genes involved in tolerance related physiological mechanisms is the main approach used in genetic engineering of this trait (Bajaj *et al*. 1999; Serrano *et al.* 1999; Rontein *et al*. 2002). Osmotic compatible solutes provide osmotic adjustment and these solutes are helpful in improving plant stress tolerance (Rathinasabapathi, 2000; Rontein *et al*. 2002). The genes encoding enzymes that have roles in enhancing the synthesis of compatible solutes were engineered. For example, Thomas *et al*. (1995) worked on mannitol, Lilius *et al*. (1996) worked on glycine betaine, Zhu *et al*. (1997) worked on proline and Galston *et al*. (1997) worked on polyamines. Overexpression of different vacuolar antiport proteins, which provide the exclusion of toxic ions from the cell cytosol were also studied and transgenic plants have been produced with enhanced stress tolerance (Apse *et al*. 1999; Serrano *et al*. 1999; Zhang and Blumwald, 2001; Zhang *et al*. 2001). Moreover, detoxifying enzymes, which decrease the harmful effects of oxidative stress were studied and transgenic plants have been produced (Tanaka *et al*. 1999). The transgenic approach is of importance both to produce salt tolerant plants and

to understand stress tolerance mechanisms better (Foolad, 2004). Transgenic tomato plants overexpressing Na^+/H^+ antiport protein were developed. In this study a single gene (*AtNHX1*) coding Na^+/H^+ antiport protein was isolated from *Arabidopsis thaliana* and transformed into the tomato genome (Zhang and Blumwald, 2001).

1.5. Tomato and Salinity

The cultivated tomato, *Lycopersicon esculentum* Mill., is in the family Solanaceae and is a widely distributed annual vegetable crop. Tomato has high nutritional quality. It is rich in vitamin C, lycopene and different phenolic compounds (Scalfi *et al*. 2000). Tomato has the ability to reduce risks of many cancers thanks to its nutritional content. Tomato is also valuable for breeding studies, because making crosses between wild and cultivated tomato plants is simple and its wild relatives provide a rich germplasm pool. Currently, the tomato crop is grown in a wide variety of climates ranging from the tropics to within a few degrees of the Arctic Circle (Foolad, 2004). In spite of its broad distribution, the production of tomato is concentrated in a few warm and dry areas (Mediterranean Sea, southern and western parts of America and Mexico). Tomato production has been limited by a high level of salinity in the soil or irrigation water. Tomato is sensitive to moderate levels of salinity like most crop plants. All stages of plant development including seed germination, vegetative growth and reproduction show sensitivity to salt stress and economic yield is reduced under salt stress (Jones *et al*. 1988; Maas, 1986; Bolarin *et al*. 1993). Rick, (1979) reported that the potential source of useful genes for salt tolerant breeding are present in several wild *Lycopersicon* species, on the other hand the cultivated species contributes limited variation for salt tolerance. Lyon (1941) was the first person who identified gene resources for salt tolerance in tomato. He improved salt tolerance of tomato by using introgression of genes from *L. pimpinellifolium* (Jusl.) Mill. in 1941. Salt tolerance in tomato appears to be a developmentally regulated, stage-specific phenomenon. There is often no correlation in salt tolerance at different plant developmental stages and tomato salt tolerance increases with plant age. Because of these reasons each stage such as germination and emergence, seedling survival and growth, and vegetative growth and reproduction should be studied separately. Furthermore, each of these stages may need a different screening procedure for salt tolerance studies (Foolad and Lin, 1997a).

Several QTL mapping studies were conducted in order to determine the number, genomic locations and individual effects of QTLs affecting salt tolerance. The research of Breto *et al*., (1994) was the earliest example for salt tolerance analysis. In this study, a cross between the cultivated tomato, *L. esculentum* and a wild relative, *L. pimpinellifolium*, were used and QTLs were found for fruit yield under saline conditions. After this finding, Foolad *et al*., (1999) and Monforte *et al*., (1997) indicated that such QTL related yield characteristics were affected by different environmental conditions. Foolad *et al*. (1997b) worked on mapping QTLs associated with salt tolerance during seed germination in tomato. In this study, the germination response of an F_2 population derived from *L. esculentum* (salt-sensitive) and *L. pennellii* (salt-tolerant) was tested. It was reported that chromosomes 1, 3, 9 and 12 contained salt tolerance loci with favorable alleles from the salt-tolerant parent. In the same manner, chromosomes 2, 7 and 8 contained loci with favorable alleles from the salt-sensitive parent. To map QTLs for salt tolerance during vegetative and reproductive stages in tomato, various tolerance related characteristics were used. For example, a BC_1F_1 population of a cross between salt-sensitive and salt-tolerant lines was evaluated by using an aerated hydrophonics system and five QTLs for salt tolerance were identified via scoring survival rates of tomato lines under salinity (Foolad and Chen, 1999).

The purpose of this study was the determination of salt tolerance differences among cultivars and wild species of tomato by using morphological, physiological and biochemical parameters and the identification and mapping of genes related to salt tolerance mechanisms during the germination and vegetative stages of tomato.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant Materials

For preliminary testing of salt tolerance at the germination stage, several tomato lines were tested at different salt concentrations. The lines were *L. esculentum* cv (SC2121), *L. esculentum* cv. (M82), *L. esculentum var. cerasiforme* (LA1310), *L. esculentum* cv. (E6203), *L. pimpinellifolium* (L3708), *L. peruvianum* (LA2744), *L. peruvianum* (LA2172), *L. peruvianum* (LA1278), *L. pennellii* (LA716), and *L. hirsutum* (LA1223). All seeds except for SC2121 were obtained from the Tomato Genetics Resource Center at UC Davis, CA, USA. SC2121 was provided by Ege Tarımsal Araştırma Enstitüsü.

The inbred backcross lines (IBLs) were used as plant material at the germination stage of this project. *L. pimpinellifolium* (LA1589), the wild relative of tomato, and *L. esculentum* cv. E6203 were hybridized in order to obtain these lines (Doğanlar *et al*. 2002). F_1 progeny were generated by using *L.pimpinellifolium,* as the donor parent and *L. esculentum* cv. E6203 as the recurrent parent. One F_1 hybrid was backcrossed to this line to produce BC_1F_1 families. By backcrossing BC_1F_1 with the recurrent parent, BC_2F_1 families that were fixed in *L. esculentum* characteristics were obtained. Each of these BC_2F_1 plants was then selfed pollinated for seven generations to increase homozygosity of each line.

The *L. pennellii* introgression lines (ILs) were used to map antioxidant traits related to salt tolerance at the vegetative stage of plant development. These lines were generated by crossing *L. pennellii* (LA716), the wild green-fruited species, to a red fruited tomato variety, *L. esculentum* cv. M82 (Eshed and Zamir, 1995). This set of *L. pennellii* introgression lines (ILs) covers the entire genome in the genetic background of *L. esculentum* M82 and each IL line contains a single introgression from *L. pennellii* such that the population provides complete coverage of the wild species genome. This population is very good for QTL analysis due to the presence of only a single introgressed DNA segments from the wild species in each plant line.

2.2. Preliminary Experiments

Preliminary experiments were done to determine the salt tolerance of different tomato species and cultivars. In this preliminary experiment, the role of $CaCl_2$ on tomato germination was also determined. In the first preliminary experiment, several *L. esculentum* cultivars and wild tomato species were tested at different salt concentrations. For this purpose, two types of control medium were made, sterilized and poured in 100 x 15 mm petri plates. Both contained 0.8% agar and one was supplemented with 17.5 mM $CaCl_2$. The sterile salt treatment media contained different salt concentrations (100 mM, 125 mM, 150 mM and 175 mM), 0.8% agar and 17.5 mM $CaCl_2$. Because of limited seed supplies, not all treatments were tested on all of the plant material. The treatment and tomato accession combinations that were tested are shown in Table 2.1. In general, a total of 20 seeds were used for each treatment (10 seeds on each of 2 petri plates) with some exceptions due to limited seed supply. Thus, only 10 seeds were used for LA2744, LA2172 and LA1278 and only 5 seeds were used for LA1310. In the second preliminary experiment, the germination response of *L. esculentum* cv. M82 was tested on control medium and two different levels of salt stress (100 mM and 175 mM) with and without $CaCl_2$ (17.5 mM). Germination response of *L. pennellii* LA716 was tested in a similar way. However, because of limited seed availability only 175 mM NaCl+17.5 mM $CaCl_2$ could be tested. For this experiment 10 seeds of each line were placed on petri plates containing each treatment medium and germination response was monitored. Each of the preliminary experiments was conducted for 30 days and petri plates were incubated at 20°C in the dark. The rate of seedling germination was determined by counting the number of germinated seeds each day and percent germination on each day was graphed. Final percentage germination for each accession and treatment was also calculated.

Table 2.1. Tomato accession and salt treatment combinations that were tested.

Genotype	Treatment
SC2121	1, 2, 3, 4, 5 and 6
L3708	1, 3 and 6
M82	1, 2, 3, 4, 5 an 6
LA2744	1 and 6
LA2172	1 and 6
LA1278	1 and 6
LA1310	1 and 6
LA1589	1, 2, 3, 4, 5 and 6
LA1223	1, 3 and 6
TA496	1, 2, 3, 4, 5 and 6

* The numbers used in grouping treatment type; 1 refers to control
2 refers to 17.5 mM CaCl2
3 refers to 100 mM NaCl+17.5 mMCaCl2
4 refers to 125 mM NaCl+17.5 mMCaCl2
5 refers to 150 mM NaCl+17.5 mMCaCl2
6 refers to 175 mM NaCl+17.5 mMCaCl2

2.3. Germination Stage Salt Tolerance Screening of IBLs

The response of the IBLs to salinity was tested using three different salt concentrations (100 mM**,** 125 mM and 150 mM) and control (non-salt) medium. Fifty seeds of the parental and each IBL line were surface sterilized with 70% ethanol for 2 minutes. Following the ethanol treatment, the seeds were surface sterilized with 10% Domestos plus 0.1% Tween-20 for 10 minutes. The seeds were then rinsed with sterile distilled water three times. Twenty five seeds (half of the seeds) of each line were sown on germination media without NaCl and the other 25 were sown on germination medium with NaCl under aseptic conditions. The Petri dishes were tightly sealed with parafilm to prevent evaporation of water and minimize changes in salt concentration. Then petri plates were placed in an incubator maintained in the dark at 20°C. To assess germination response, plates were monitored for 30 consecutive days. While scoring, radicle protrusion was taken as an indication of germination. The collected data were used to calculate several parameters in order to determine salt stress response of the

parents and each line. These parameters included percent germination for each line which was calculated for both the control and salt treatments. Difference in percent germination was also calculated for each line using the formula: (salt % germination/ control % germination) X 100. This calculation allowed a correction for lines which did not show 100% germination on control medium. More tolerance to salt stress was represented by higher values for this parameter, since salt tolerant lines could germinate more completely on saline media. To determine the germination delay due to salt stress, the number of days that passed before the first seeds on saline and control medium germinated was recorded. This was used to calculate germination delay in days. In addition, the number of days needed for each line to reach complete germination on both control and saline medium was determined in order to assess the delay to full germination because of salt stress. The delay to 50% germination for each treatment was calculated in the same way. The 100 mM and 125 mM salt treatment experiments were performed once, on the other hand, the 150 mM salt treatment experiment was repeated twice (three replicates).

2.4. Growth Conditions of Plants Used in Vegetative Stage Salt Tolerance Screening

The 32 *L. pennellii* segmental introgression lines and their parental line *L. esculentum* cv. M82 were used as plant material. The salt tolerance experiment was carried out in a greenhouse. Tomato seeds were germinated in peat with an EC of 0.4 on 15 May 2006. Fifteen-day-old seedlings at the second-true leaf stage were transferred to 4 litre plastic pots containing nutrient solution in a hydroponic system. The solution was comprised of: $3x10^3$ M $Ca(N03)_2$, $1x10^3$ M K_2SO_4, $1x10^3$ M $MgSO_4$, $0.2x10^3$ M KH_2PO_4, $1x10^5$ M H_3BO_3, $1x10^6$ M $MnSO_4$, $1x10^7$ M $CuSO_4$, $1x10^8$ M $(NH_4)_6Mo_7O_{24}$, $1x10^6$ M $ZnSO_4$ and $1x10^4$ M Fe EDTA. The day on which plants were transferred into this system, the density of nutrient solution was 10% less than described above. The nutrient solution reached its final density in one week. The internal surface of the pots was covered with sponge. Nutrient solution was renewed every seven days, at which time salt stress treatment was initiated. On 15 June 2006, the plants were at the seven-true leaf stage and salt treatment was started. Salt treatments were achieved with the gradual addition of NaCl to the nutrient solution. The first increment of salt was 25 mM

and additional increments of 25 mM NaCl were added daily until the salt concentration reached the final treatment level of 150 mM NaCl (electrical conductivity 15.45 dS/m). The electrical conductivity of the identical nutrient solution without NaCl was 1.30 dS/m. Each of the tomato lines was treated with non-saline nutrient solution for controls. The plants were grown for 15 days after the final salt concentration reached 150 mM.

The leaves of individual plants of both salt stress and nonstress treatments were harvested and treated with liquid nitrogen for storage at -80°C. Measurements of superoxide dismutase (SOD), catalase (CAT) and determination of total protein amount were then performed.

2.5. Preparation of Tomato Leaf Tissues

To determine antioxidative enzyme activity and total protein amount, 1 gram of fresh weight of tomato leaf tissues was homogenized for two minutes in 20 ml of cold 100 mM potassium phosphate buffer (pH 6.8) containing 0.1 g PVPP using a Waring blender. The homogenate was filtered through four layers of nylon cloth and samples were centrifuged at 35.000 X G for 20 minutes with all steps performed at +4°C. The supernatant was collected as clear phase into a tube and was kept in an ice bath.

2.6. Enzyme Assays

SOD activity was determined by measuring the extract's ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), as described by Giannopolitis and Ries (1997). Each 3 ml reaction mixture contained 10 mM potassium phosphate buffer (pH 6.8), 13 mM methionine, 2 µM riboflavin, 75µM NBT, 100 nM EDTA and 200 µl enzyme extract. Reactions were carried out in 3 ml disposable cuvets at 25 °C under illumination of a 15 W fluorescent lamp in an incubater. Riboflavin was added last and cuvets were shaken. The reaction was started by switching on the light. After 10 minutes the light was switched off in order to stop the reaction and absorbance of the reaction mixture was read at 560 nm. Each measurement was repeated three times for each enzyme extract. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of NBT. SOD activity values are given in units per g of protein.

CAT activity was determined according to Lester *et al*. (2004). The reaction mixture consisted of 10 mM potassium phosphate buffer (pH 6.8) and enzyme extract. The reaction was initiated by the addition of 1.0 mM H_2O_2. Decomposition of H_2O_2 was monitored at 240 nm for 6 minutes. Each measurement was repeated three times for each enzyme extract. One unit of CAT activity was expressed as the amount of H_2O_2 spent (µmol per minute).

All protein concentrations were determined according to Bradford (1976) by using BSA (Bovine Serum Albumin) as a standard reference. The reaction was carried out in 15 ml falcon tubes and consisted of 0.1 ml enzyme extract and five ml of protein reagent (Coomassie Brilliant Blue G-250). The contents were mixed by vortexing and after 2 minutes absorbance at 595 nm was measured. Each measurement was repeated five times for each enzyme extract.

2.7. Analysis of SOD and CAT Activities in the ILs

In order to analyze SOD and CAT activity in the ILs, two types of calculations were done. For the first calculation, the enzyme activity of each IL was compared with M82 under control conditions. The formula used for calculation of this trait was SOD or CAT activity in IL/ SOD or CAT activity in M82 x 100. In this way, IL enzyme activity under control conditions was expressed as a percentage of M82 activity. For the second calculation, IL enzyme activity under salt conditions was compared with M82 enzyme activity under salt conditions. First, M82 and each IL salt SOD or CAT activity were standardized to control enzyme activity using the formula: (SOD or CAT activity on salt/ SOD or CAT activity on control) X 100. That value was then expressed as a percentage of M82 activity under salt using the formula: standardized IL salt enzyme activity/standardized M82 salt enzyme activity x 100. Both sets of values were then graphed. QTL were then mapped by comparing enzyme activities of individual ILs to the control M82 genotype for both the control and salt graphs. A 30% increase or decrease of an IL line compared to M82 was used to declare the presence of a QTL in a given IL.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Studies to Determine the Optimal Conditions for Salt Stress Germination Experiments

This work aimed to determine the effect of salinity on cultivated and wild tomato accessions. Each tomato line tested in the preliminary tests was selected for its reported salt tolerance or for use as controls. *L. esculentum* M82 and TA496 were used because they are the parents of several mapping populations. *L. esculentum* SC2121 was tested because of its wide cultivation in the Aegean region. All of the other wild species including *L. pimpinellifolium*, *L. peruvianum* and *L. hirsutum* were selected due to their reported tolerance to some degree of salt stress. As given in Figures 3.1 to 3.7, almost all lines including the cultivars were able to germinate even under salt stress. For example, SC2121 seeds could germinate at even the highest NaCl level, 175 mM (Figure 3.1). M82 and TA496 showed tolerance to moderately high NaCl concentrations (up to 125 mM). Tolerance to salinity was also detected within wild tomato species. Among these wild lines, *L. peruvianum* LA2744 was the most tolerant one with a germination percentage of 78% on 175 mM NaCl. The first day of germination and the day when the tomato lines reached full seed germination were also important parameters to determine salt tolerance. For example, highly salt tolerant SC2121 reached full germination in 10 days on control medium whereas a total of 21 days were required for fulll germination on 100 mM NaCl. Seeds of TA496 first germinated seven days after sowing on 100 mM NaCl. However, seeds of M82 needed seven more days for first germination. Full germination on 175 mM NaCl for LA2744 required 18 days. On the other hand, it took only 9 days for LA1589 to reach full germination.

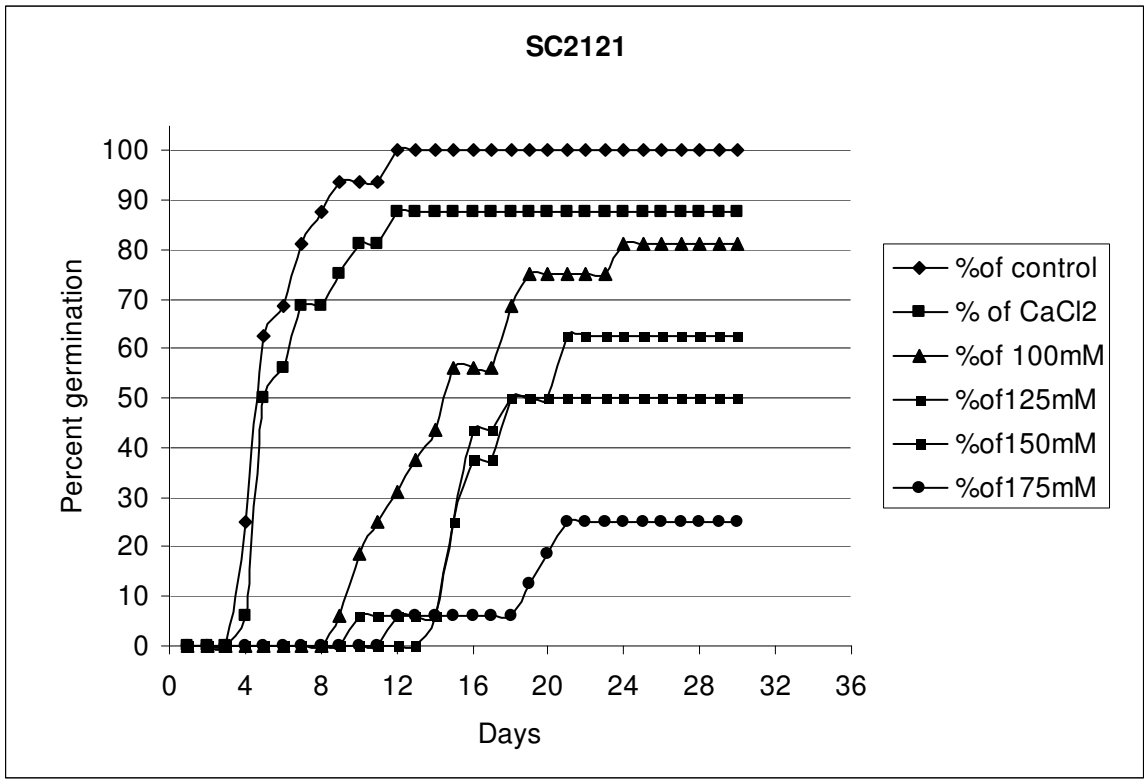

Figure 3.1. Germination response of *L. esculentum* cv. SC2121 with and without salt stress.

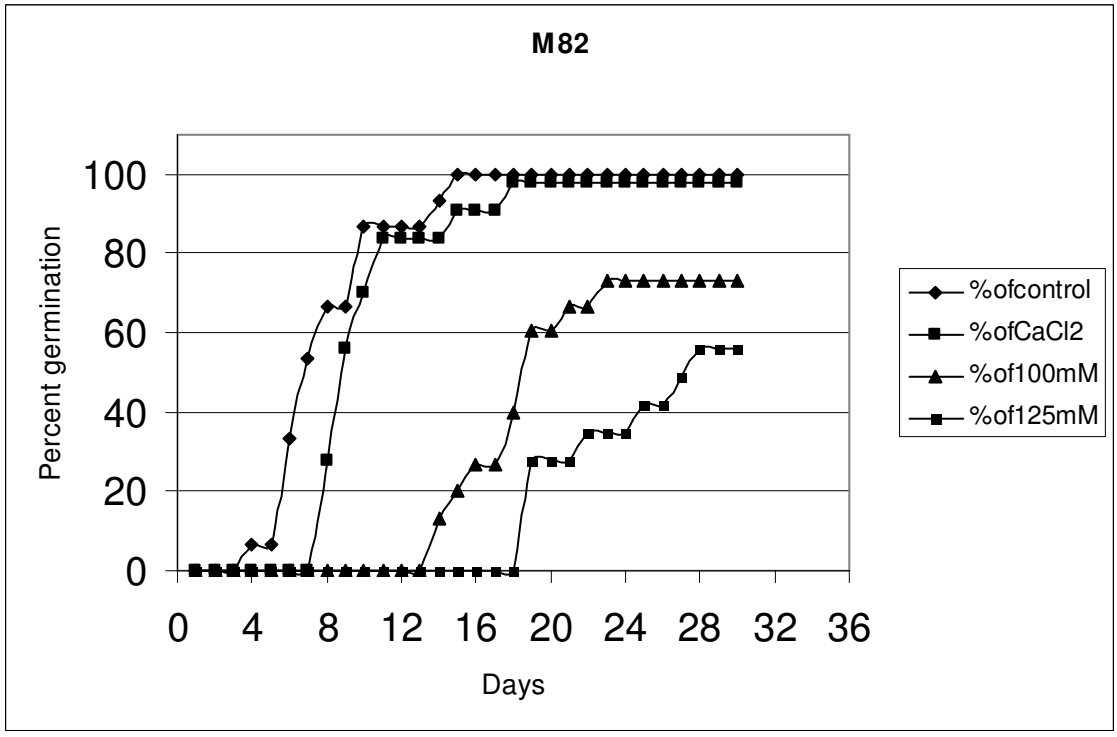

Figure 3.2 Germination response of *L. esculentum* cv. M82 with and without salt stress.

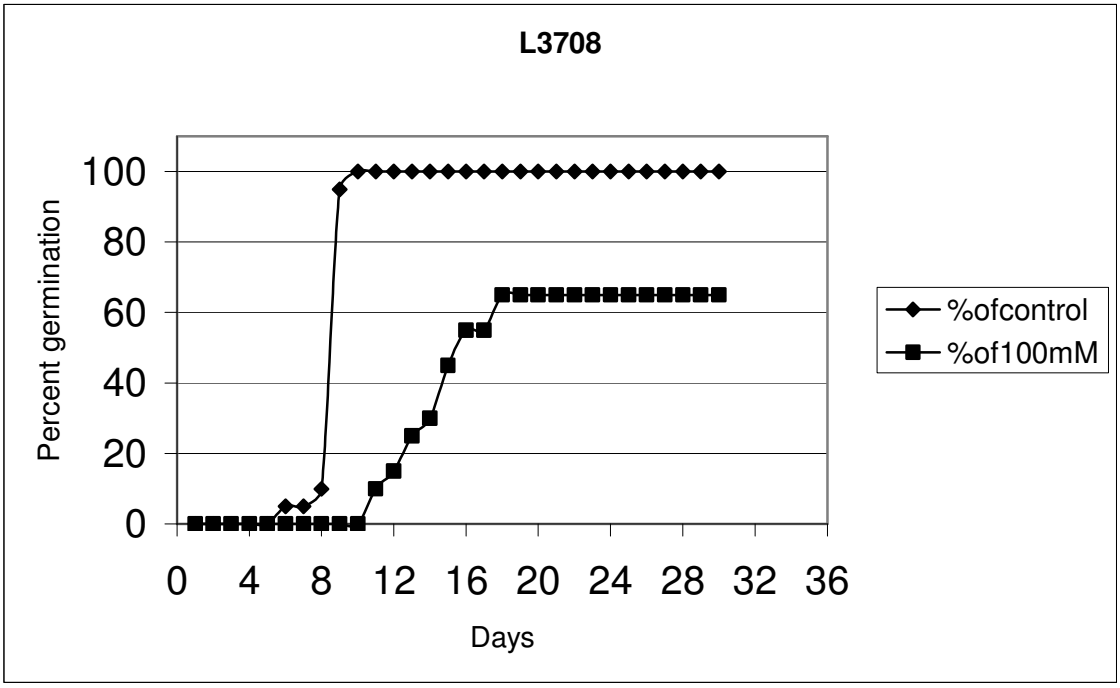

Figure 3.3 Germination response of *L. pimpinellifolium* L3708 with and without salt stress.

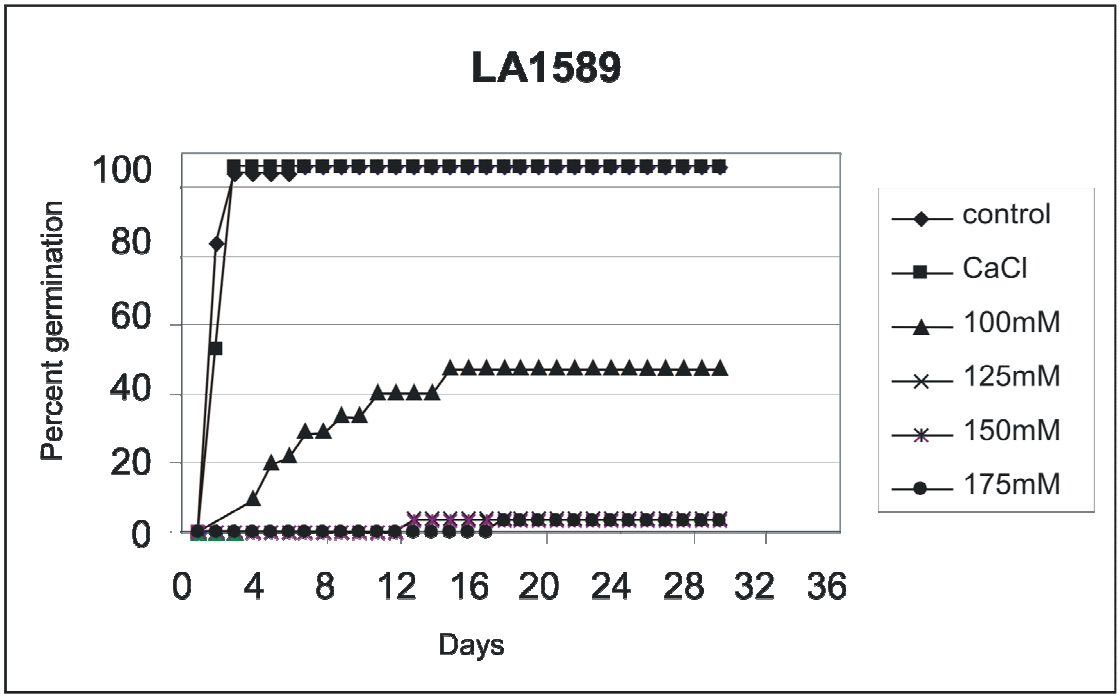

Figure 3.4 Germination response of *L. pimpinellifolium* LA1589 with and without salt stress.

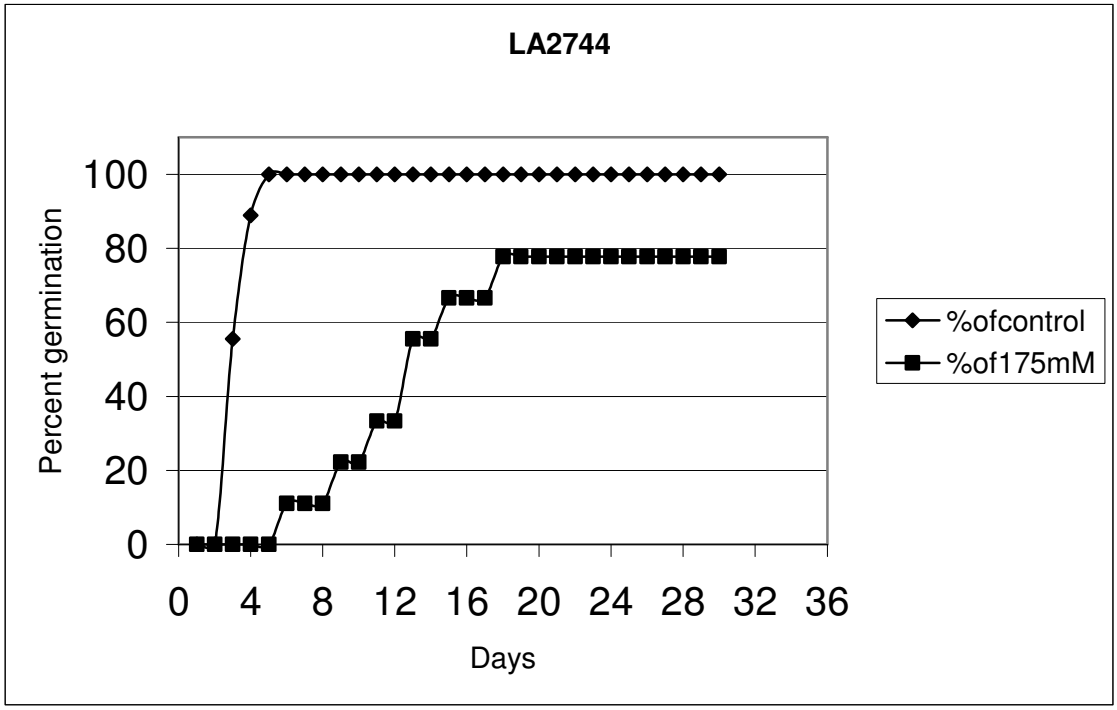

Figure 3.5 Germination response of *L. peruvianum* LA2744 with and without salt stress.

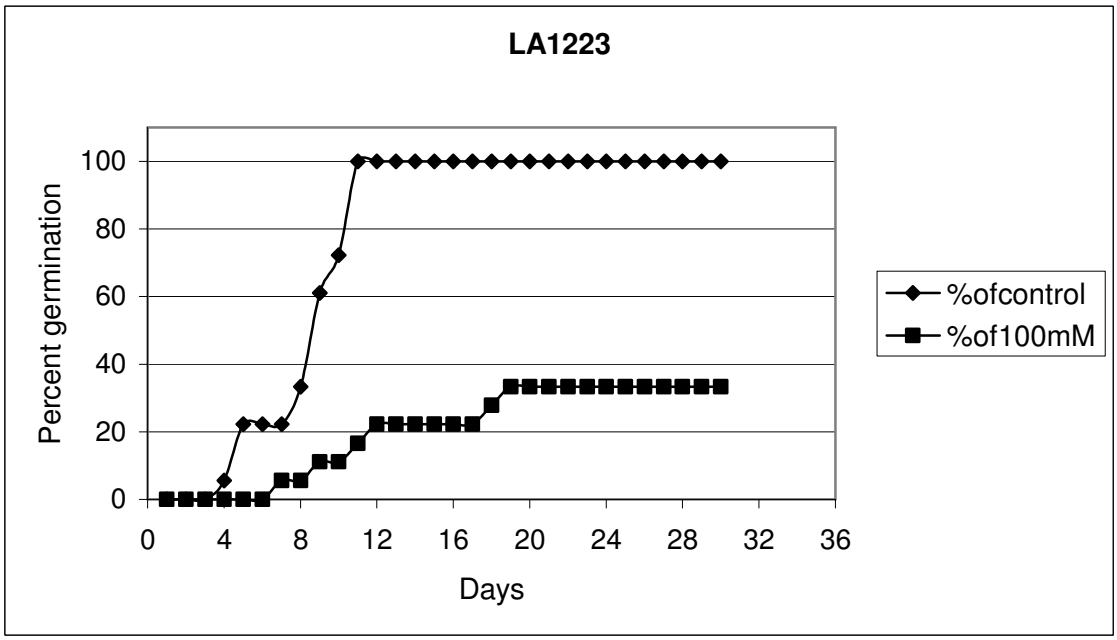

Figure 3.6 Germination response of *L. hirsutum* LA1223 with and without salt stress.

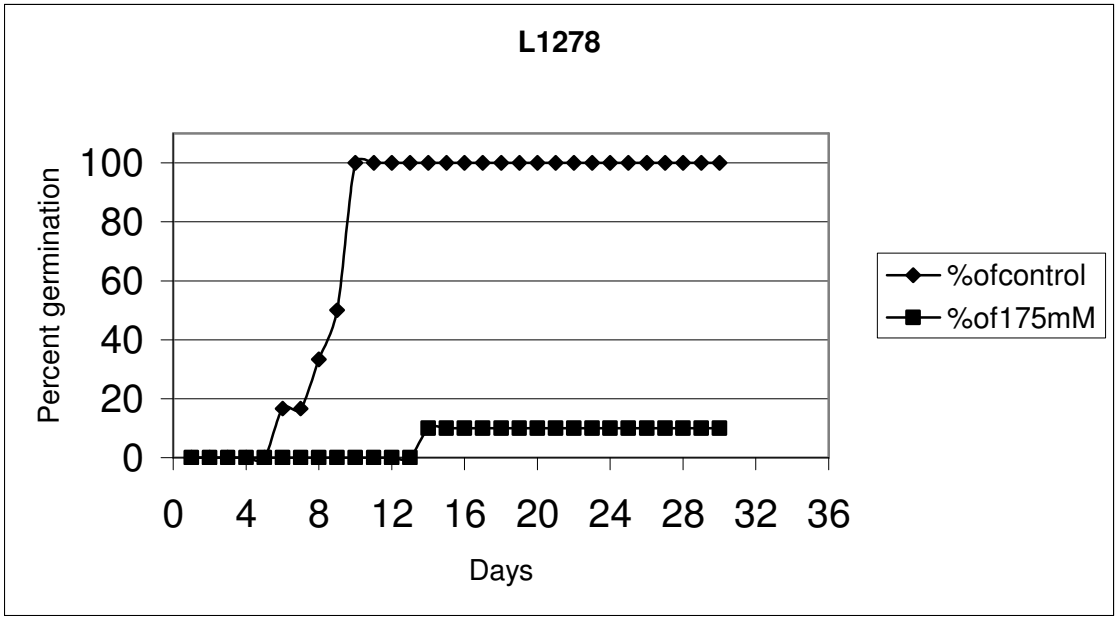

Figure 3.7 Germination response of *L. peruvianum* L1278 with and without salt stress

Figure 3.8 shows the germination response of *L. esculentum* cv. M82 on control medium (water agar) and on two levels of salt stress (100 and 175 mM NaCl) with and without $CaCl_2$ (17.5 mM). In addition, the response of *L. pennellii* LA716 on control and 175mM NaCl +17.5mM $CaCl_2$ is shown in this figure. Seed germination of M82 decreased, while NaCl concentration increased. There was a seemingly unexpected result in final germination for M82 for the control and 100mM NaCl treatments because more seeds germinated on the salt medium than on control medium. This, however, can be explained by variability in the viability of M82 seeds. Seeds of *L. esculentum* germinated less than seeds of *L. pennellii* on 175mM NaCl with $CaCl_2$. $CaCl_2$ increased the level of stress for the salt treatments and delayed the time of first germination. For example, M82 seeds first germinated after 10 days on 175mM NaCl. However, they first germinated after more than 10 days on 175mM NaCl with $CaCl_2$.

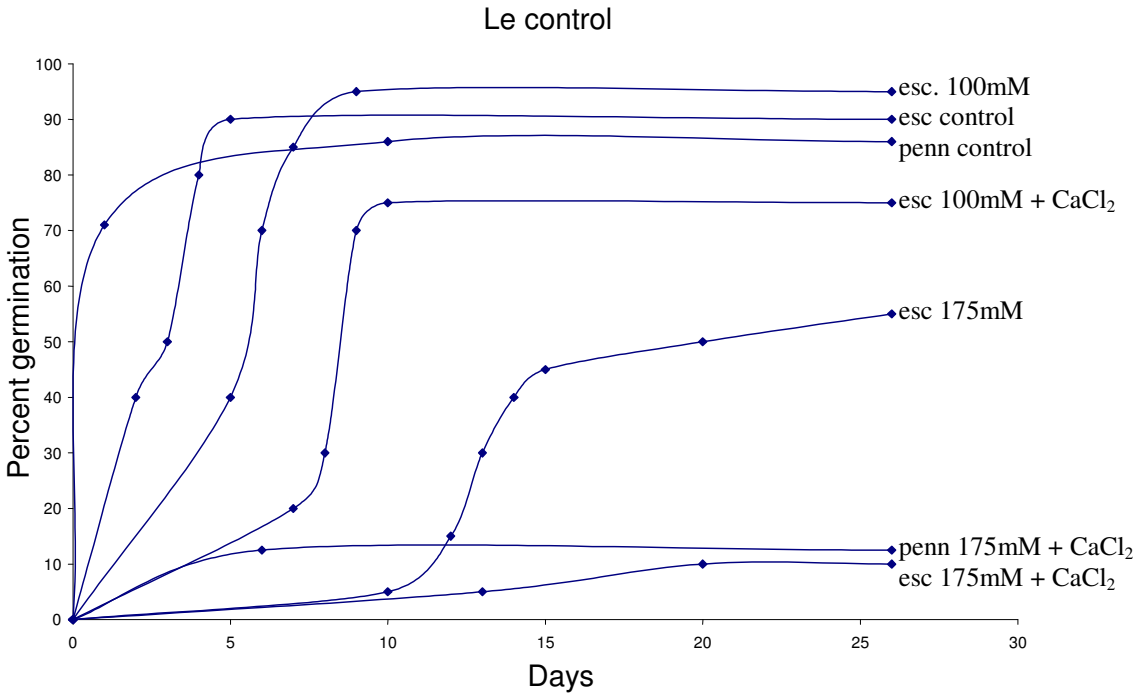

Figure 3.8. Response of *L. esculentum cv* M82 and *L. pennellii* LA716 on control and salt medium (100 and 175 mM NaCl) with and without $CaCl_2$.

3.2. Germination Response of the Parental Lines and IBL population

Three different salt concentrations were used for germination experiments with the IBLs. These different salt concentrations (100 mM, 125 mM and 150 mM) were selected based on the preliminary tests.

In the first IBL germination test, a salt concentration of 100 mM NaCl was used. Figures 3.9 and 3.10 show the germination response of the *L. pimpinellifolium* and *L. esculentum* parents on control and 100 mM NaCl treatments. Total percent germination of *L. esculentum* TA496 was not affected at 100 mM NaCl because germination of this accession achieved 100% (Figure 3.10). On the other hand, total germination of *L. pimpinellifolium* LA1589 was decreased to 84% by salinity. Mean values for eight response parameters for the parental and inbred backcross lines are summarized in Table 3.1. Parental lines and IBLs showed delays in germination for all parameters in response to salt stress. The delay of *L. esculentum* TA496 was longer than *L. pimpinellifolium* LA1589. For example, full germination of TA496 was delayed by 11 days while full germination of LA1589 was only delayed by 8 days. IBLs showed a delay in germination similar to parental lines. According to the distribution histogram of

the difference in percent germination, a total of 75 IBLs showed complete germination on 100 mM NaCl (Figure 3.11).

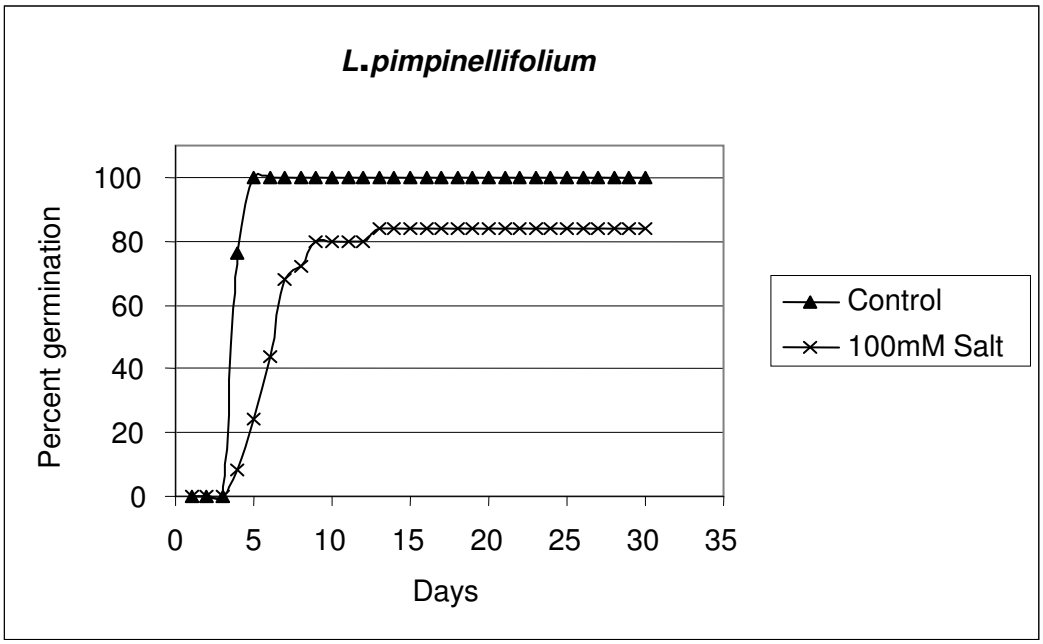

Figure 3.9. Germination responses of *L. pimpinellifolium* LA1589 on control and 100 mM NaCl media.

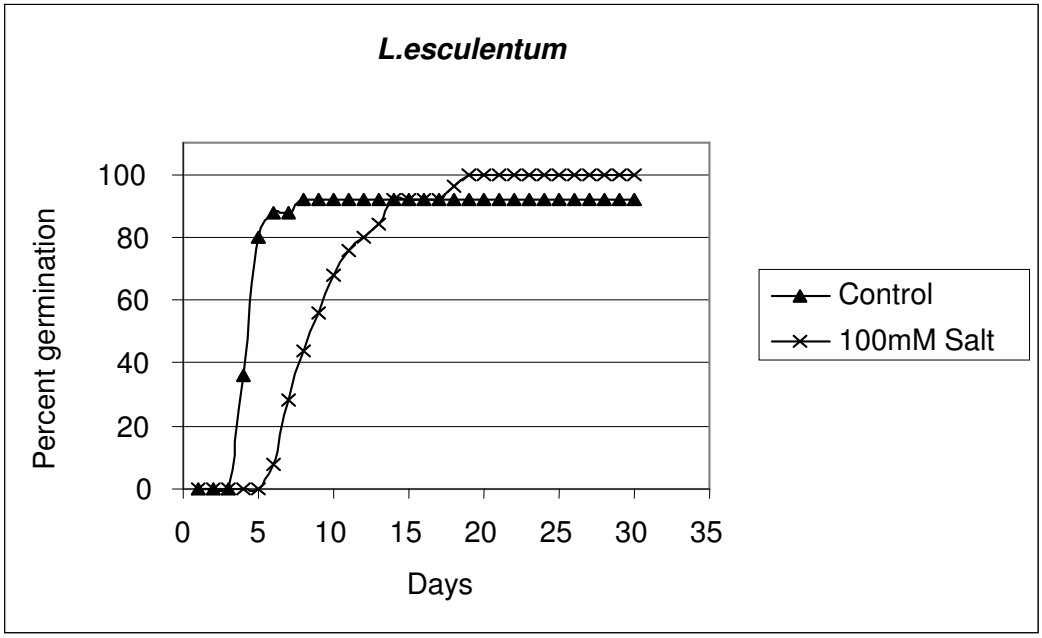

Figure 3.10. Germination responses of *L. esculentum* TA496 on control and 100 mM NaCl media.

Table 3.1. Germination response of the parental lines and IBLs on control and 100 mM NaCl media. Values for IBLs are averages of 131 lines.

Trait	Treatment	TA496	LA1589	IBLs
% germination	control	92%	100%	85.6%
	salt	100%	84%	75.1%
difference % germ	-	0%	84%	87.6%
# days to 1st germ	control	4	4	4.2
	salt	6	4	6.2
delay to 1st germ	-	2	0	2.1
# days to full germ	control	8	5	15.0
	salt	19	13	20.2
delay to full germ	-	11	8	5.9
# days to 50% germ	control	5	4	6.5
	salt	9	7	11.0
delay to 50% germ	-	4	3	5.1

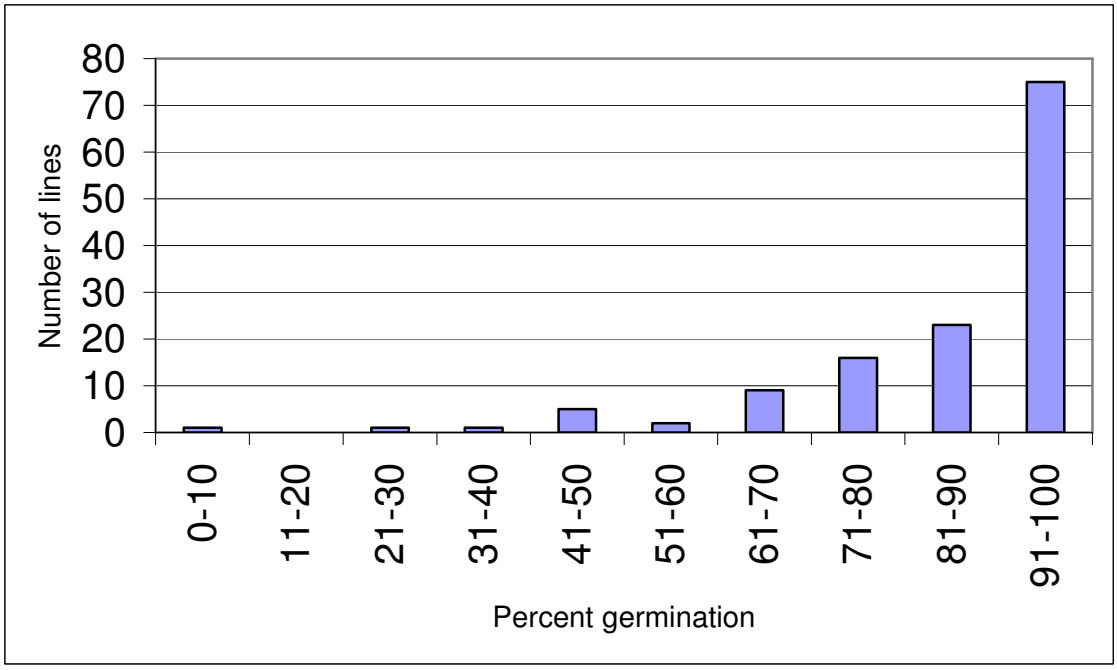

Figure 3.11. Distribution histogram of difference in percent germination of IBLs for the 100 mM salt treatment.

In the second screening of the IBLs, salt concentration was increased from 100 mM to 125 mM because the germination responses of TA496 and LA1589 to 100 mM NaCl were quite similar. At 125 mM, germination of TA496 reached a maximum of 92% germination (Figure 3.13). Germination of LA1589 decreased to 52% (Figure 3.12,

Table 3.2). Percent germination of the IBLs decreased by 17% on the 125 mM NaCl. The IBLs reached full germination on control and salt medium later than both of the parents. First day of germination on salt and control medium for the IBLs were between the two parents. At 125 mM NaCl most of the IBLs showed 50-100% difference in percent germination.

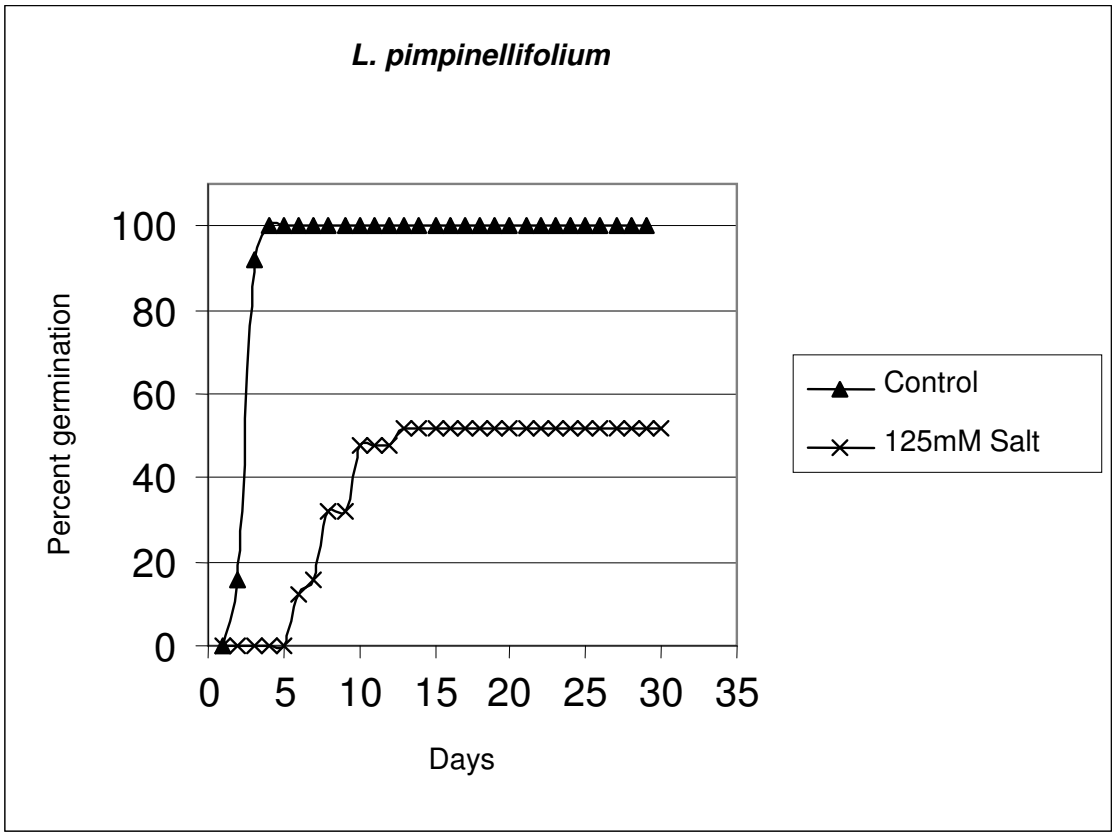

Figure 3.12. Germination responses of *L. pimpinellifolium* LA1589 on control and 125 mM NaCl media.

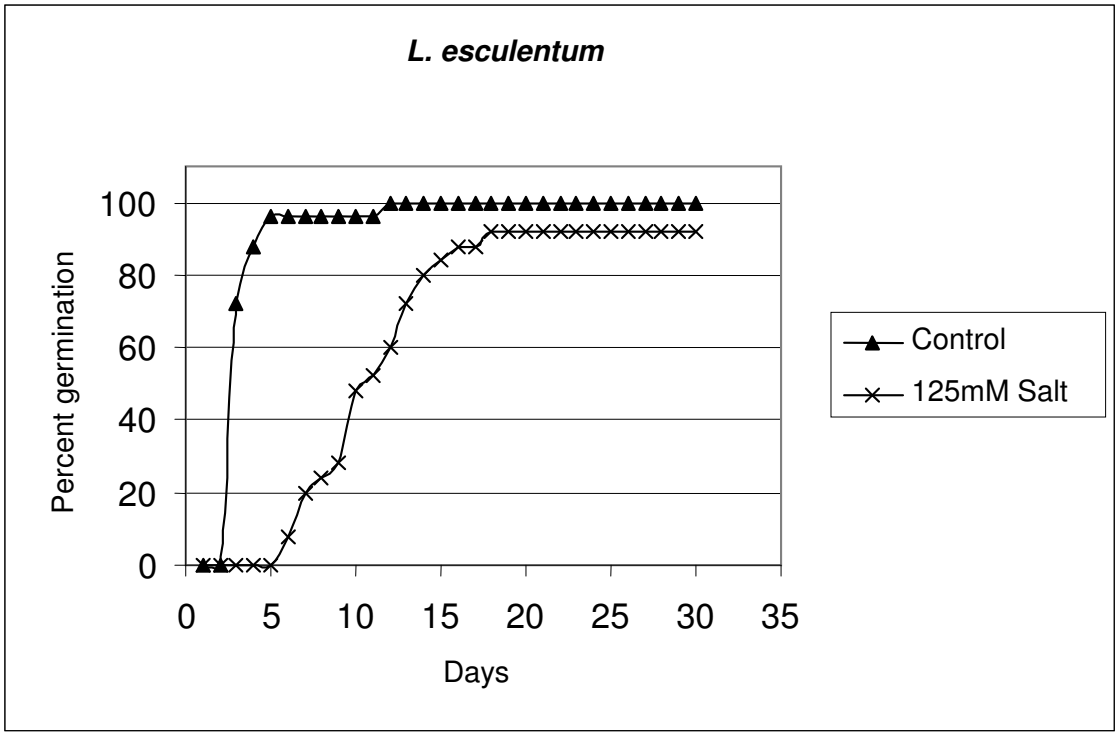

Figure 3.13. Germination responses of *L. esculentum* TA496 on control and 125 mM NaCl media.

Table 3.2. Germination response of the parental lines and IBLs on control and 125 mM NaCl media. Values for IBLs are averages of 131 lines.

Trait	Treatment	TA496	LA1589	IBLs
% germination	control	100%	100%	85.2%
	salt	92%	52%	68.2%
difference % germ	-	92%	52%	78.2%
# days to 1st germ	control	3	2	2.1
	salt	6	6	5.3
delay to 1st germ	-	3	4	3.0
# days to full germ	control	12	4	14.2
	salt	18	13	23.5
delay to full germ	-	6	9	10.2
# days to 50% germ	control	3	3	5.3
	salt	11	13	13.8
delay to 50% germ	-	8	10	9.1

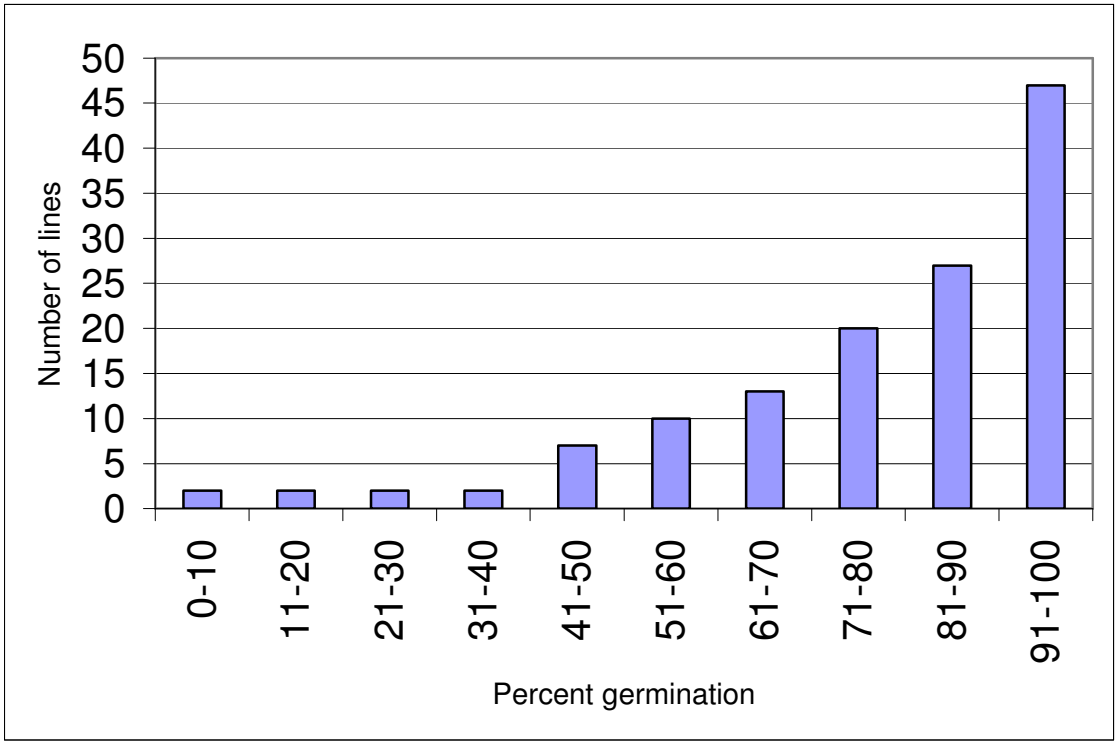

Figure 3.14. Distribution histogram of difference in percent germination of IBLs for the 125 mM salt treatment.

Germination response of the IBLs was also tested at 150 mM NaCl concentration. The purpose of using this high level of NaCl was to provide more dramatic differences in germination responses from the control treatment and IBL lines. The 150 mM NaCl test was repeated twice and the germination data from these three replicates were combined together. From these experiments, it was found that germination of TA496 on 150 mM NaCl decreased to 80% (Figure 3.16). LA1589 could germinate well on 150 mMNaCl, however the length of time to reach final germination was long (Figure 3.15). In general, germination of IBLs at 150 mM NaCl was lower than for control medium with a 40% reduction obtained. The 150 mM treatment markedly delayed seed germination. There were differential responses to 150 mM within parental and the IBL lines. For all parameters, the delay in germination for TA496 was much longer than that for LA1589 and the IBLs showed an intermediate delay between the two parental accessions. For example, full germination of TA496 was delayed 23 days by salt treatment while full germination of LA1589 was delayed only 3 days (Table 3.3). Figure 3.17 shows the distribution histrogram of the IBLs at 150 mM NaCl. Percent difference of the IBLs showed wide variation.

Correlation analysis indicated that for all three 150 mM replicates, every trait ($P \leq 0.002$) was highly correlated (Table 3.4). The highest correlated trait was percentage of germination on salt medium and the related trait diffrence in percent germination (r= 0.80-0.85). Although days to first seed germination had the lowest r values (0.29-0.33), they were still significantly correlated. All in all, high correlations among the three 150 mM replicates verify that the 150 mM seed germination tests produced repeatable results.

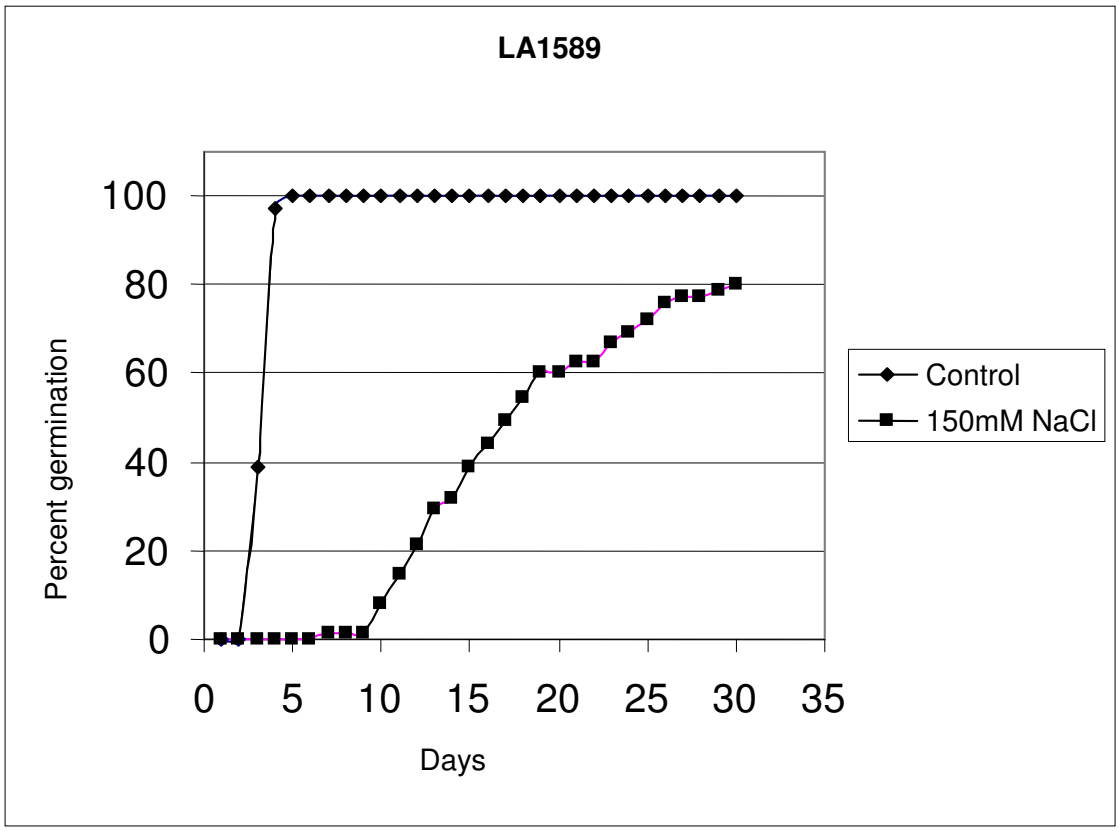

Figure 3.15. Germination responses of *L. pimpinellifolium* LA1589 on control and 150 mM NaCl media.

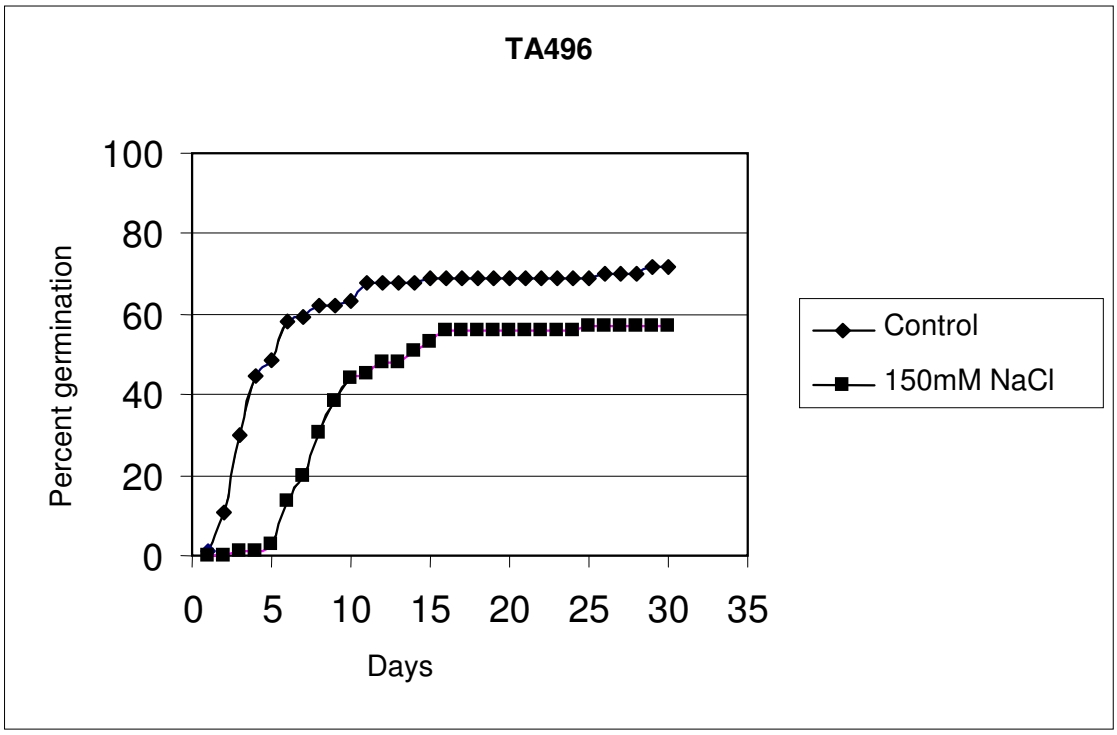

Figure 3.16. Germination responses of *L. esculentum* TA496 on control and 150 mM NaCl media.

Table 3.3. Germination response of the parental lines and IBLs on control and 150 mM NaCl media. Data are averages from 3 replicates. Values for IBLs are averages of 140 lines replicated three times.

Trait	Treatment	TA496	LA1589	IBLs
% germination	control	100±0	71.7±0.3	98.2±0.2
	salt	80.0±12.8	57.3±12.7	60.8±2.7
difference % germ	-	80.0±12.8	75.9±14.5	60.6±2.7
# days to 1st germ	control	3.0±0	1.7±0.3	2.6±0.1
	salt	9.7±1.4	4.7±0.9	7.8±0.2
delay to 1st germ	-	6.7±1.4	3.0±1.2	5.2±0.2
# days to full germ	control	5.0±0.6	23.7±4.5	7.3±0.3
	salt	28.3±1.2	17.7±3.8	21.0±0.5
delay to full germ	-	23.3±1.4	3.3±3.3	14.2±0.5
# days to 50% germ	control	4.0±0	3.7±0.3	3.2±0.1
	salt	17.3±3.0	8.3±0.3	11.7±0.3
delay to 50% germ	-	13.3±3.0	4.7±0.3	8.6±0.3

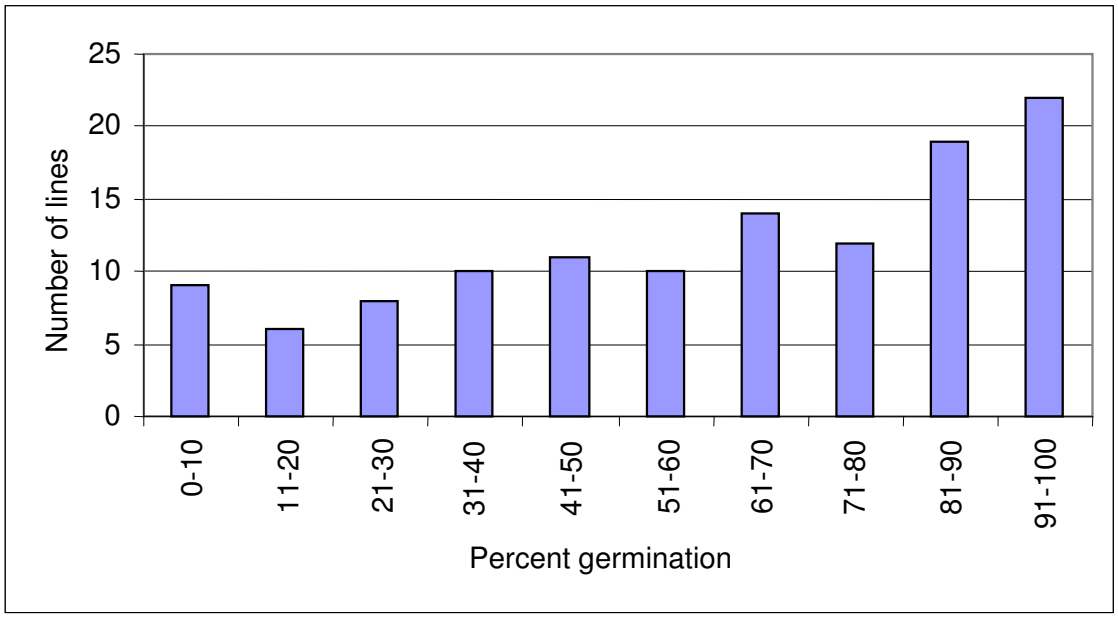

Figure 3.17. Distribution histogram of average difference in percent germination of IBLs for the 150 mM salt treatments.

Table 3.4. Correlations between the three replicates for the 150 mM NaCl germination experiment.

Trait	**Correlation (r)**	**P-value**
% germination on salt	0.81-0.85	<0.0001
difference % germ	0.80-0.81	<0.0001
# days to 1st germ on salt	0.29-0.33	≤0.002
delay to 1st germ	0.29-0.32	≤0.002
# days to full germ on salt	0.39-0.56	<0.0001
delay to full germ	0.33-0.38	<0.0001
# days to 50% germ on salt	0.36-0.50	<0.0001
delay to 50% germ	0.34-0.42	<0.0001

Comparison among the data from different germination experiments including 100 mM, 125 mM and 150 mM showed that *L. esculentum* and *L. pimpinellifolium* had different responses to higher concentrations of NaCl (Figure 3.18 and 3.19). The total germination of *L. esculentum* TA496 was affected slightly at the higher salt concentrations. However, the number of days that passed to reach full seed germination days changed according to the different NaCl concentrations. For example, 18 days were needed for full germination on the 125 mM medium, on the other hand 28 days were needed to reach full germination on the 150 mM medium. Germination of *L. pimpinellifolim* LA1589 was more affected on 125 mM NaCl than on the other two NaCl concentrations. Interestingly, there were similar delays in germination for LA1589

on 125 and 150 mM NaCl. The IBLs had responses to high NaCl concentrations intermediate to the parental lines. Total seed germination of the IBLs decreased as the NaCl concentration increased. Thus, average percent germination decreased from 87.6 to 78.2 to 59.4% at 100, 125 and 150 mM NaCl, respectively. Similarly, the delay to full germination increased with each increasing level of salt from 5.9 (100 mM) to 10.2 (125 mM) to 14.3 (150 mM) days.

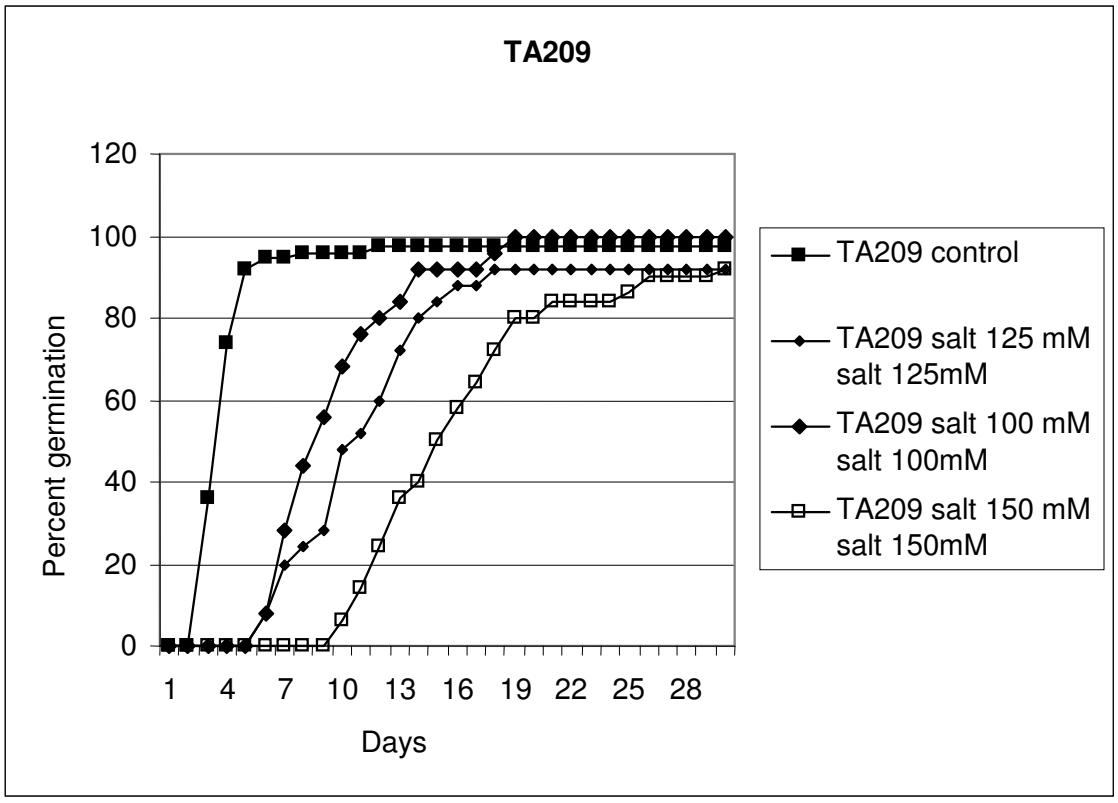

Figure 3.18. Germination of *L. esculentum* TA496 seeds over time for control and salt treatments.

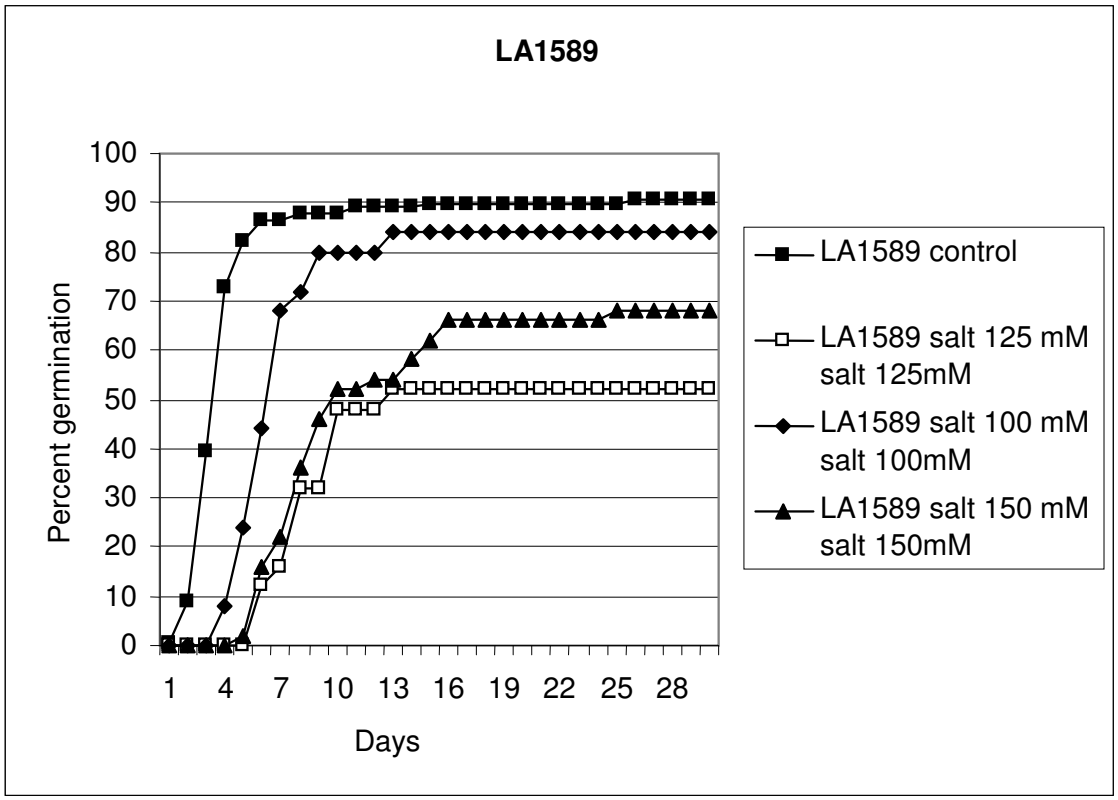

Figure 3.19. Germination of *L. pimpinellifolium* LA1589 seeds over time for control and salt treatments.

3.3. Genetic Mapping of Salt Tolerance in IBLs

The response of plants to salt stress at the seed germination stage in tomato is controlled by many genes. The QGene computer program was used to identify the quantitative trait loci (QTL) controlling salt tolerance traits for the 100, 125 and 150 mM NaCl salt stress experiments. The IBLs were previously genotyped with 127 markers including one morphological and 126 restriction length polymorhism (RFLP) markers (Doğanlar et al. 2002). Using a linkage map with these 127 markers covering all 12 tomato chromosomes, the QTLs related with salt tolerance traits were mapped in the IBLs at $P < 0.01$ for the 100, 125 and 150 mM NaCl salt stress experiments.

3.3.1. QTL Analysis Results for 100 mM NaCl Treatment

A total of 18 QTLs were identified for 7 traits (Table 3.5) and Figure 3.20 shows the most likely position of each QTL on the genetic map. There was no significant QTL

identified for the trait days to full germination. For total germination on salt medium one locus was identified. This QTL, s*pgerm2.1*, was on chromosome 2 and controlled 10% of the variance for the phenotype. For this locus, the *L. esculentum* allele was associated with better germination on salt medium. Four QTLs for difference in percent germination were identified on chromosomes 2, 6 and 10. *diffp2.1* was the most significant QTL explaining 11% (P=0.0009) of the trait phenotypic variance. Salt tolerance was increased by the *L. esculentum* (salt-sensitive parent) allele for this locus. For the number of days to first germination on salt, three QTLs were identified on chromosomes 2, 3 and 11. Among the identified QTL, *sdfirst 2.1*, (which had the alleles from *L. pimpinellifolium* (salt-tolerant parent)), was the most significant with a P value less than 0.0001. *L. esculentum* alleles were associated with salt tolerance for the other two loci. Four QTLs located on chromosomes 2, 3, 9 and 11 were identified for delay to first seed germination. The most significant locus was *delfirst2.1* and explained 29% of the phenotypic variance. Moreover, *L. esculentum* was the source of salt tolerance, in contrast to other *delfirst* QTLs. The QTL on chromosomes 2, 3 and 11 were linked to the same markers as *sdfirst*. One QTL marked by TG308 was identified for the number of days to 50% of final germination. *sdfifty2.1* located on chromosome 2 accounted for 21% of the variance in this trait. The *L. pimpinellifolium* allele was responsible for increased salt tolerance at this locus. For the delay to 50% germination, three QTLs were identified on chromosomes 2, 3 and 7. These three loci explained approximately equal phenotypic variances (8%, 8% and 12%). Three QTLs were identified for the delay to full seed germination on chromosomes 3, 6 and 10. The *L. pimpinellifolium* allele was associated with increased salt tolerance for *delfull3.1*.

Table 3.5. List of QTLs for germination stage salt tolerance identified at 100 mM NaCl. R^2 values indicate effect of each QTL on the total phenotypic variance.

Trait	QTL Symbol	Chromosome	Marker	P Value (≤0.01)	R^2 Value	AA[a]	Aa[b]	Salt Tolerance Source[c]
% Germination on Salt	*spgerm2.1*	2	TG308	0.001	10%	77	52	TA496
Difference in % Germination	*diffp2.1*	2	TG426	0.0009	11%	88	85	TA496
	diffp6.1	6	CT83	0.005	6%	89	73	TA496
	diffp10.1	10	U	0.008	5%	89	76	TA496
Number of Days to First Germination on Salt	*sdfirst2.1*	2	TG426	<0.0001	21%	6.2	5.6	LA1589
	sdfirst3.1	3	TG214	0.009	7%	5.9	7.7	TA496
	sdfirst11.1	11	TG57	0.01	7%	6.0	7.3	TA496
Delay to First Germination	*delfirst2.1*	2	TG426	<0.0001	29%	2.0	1.5	LA1589
	delfirst3.1	3	TG214	0.01	7%	1.8	3.5	TA496
	delfirst9.1	9	TG328	0.002	9%	2.0	6.0	TA496
	delfirst11.1	11	TG57	0.001	11%	1.9	3.1	TA496
Number of Days to 50% Germination	*sdfifty2.1*	2	TG308	<0.0001	21%	10.5	17.2	LA1589
Delay to 50% Germination	*delfifty2.1*	2	TG469	0.001	12%	4.7	8.2	TA496
	delfifty3.1	3	TG246	0.009	8%	4.8	8.0	TA496
	delfifty7.1	7	TG342	0.01	8%	4.9	8.8	TA496
Delay to Full Germination on Salt	*delfull3.1*	3	TG129	0.002	9%	6.3	2.1	LA1589
	delfull6.1	6	TG99	0.002	9%	5.8	14.0	TA496
	delfull10.1	10	CT95	0.01	7%	5.6	7.6	TA496

[a] AA column gives mean value for individuals homozygous for *L. esculentum* alleles at each marker
[b] aa gives mean value for individuals homozygous for *L. pimpinellifolium* alleles at each marker.
[c] The last column lists which parental alleles were associated with salt tolerance for each QTL.

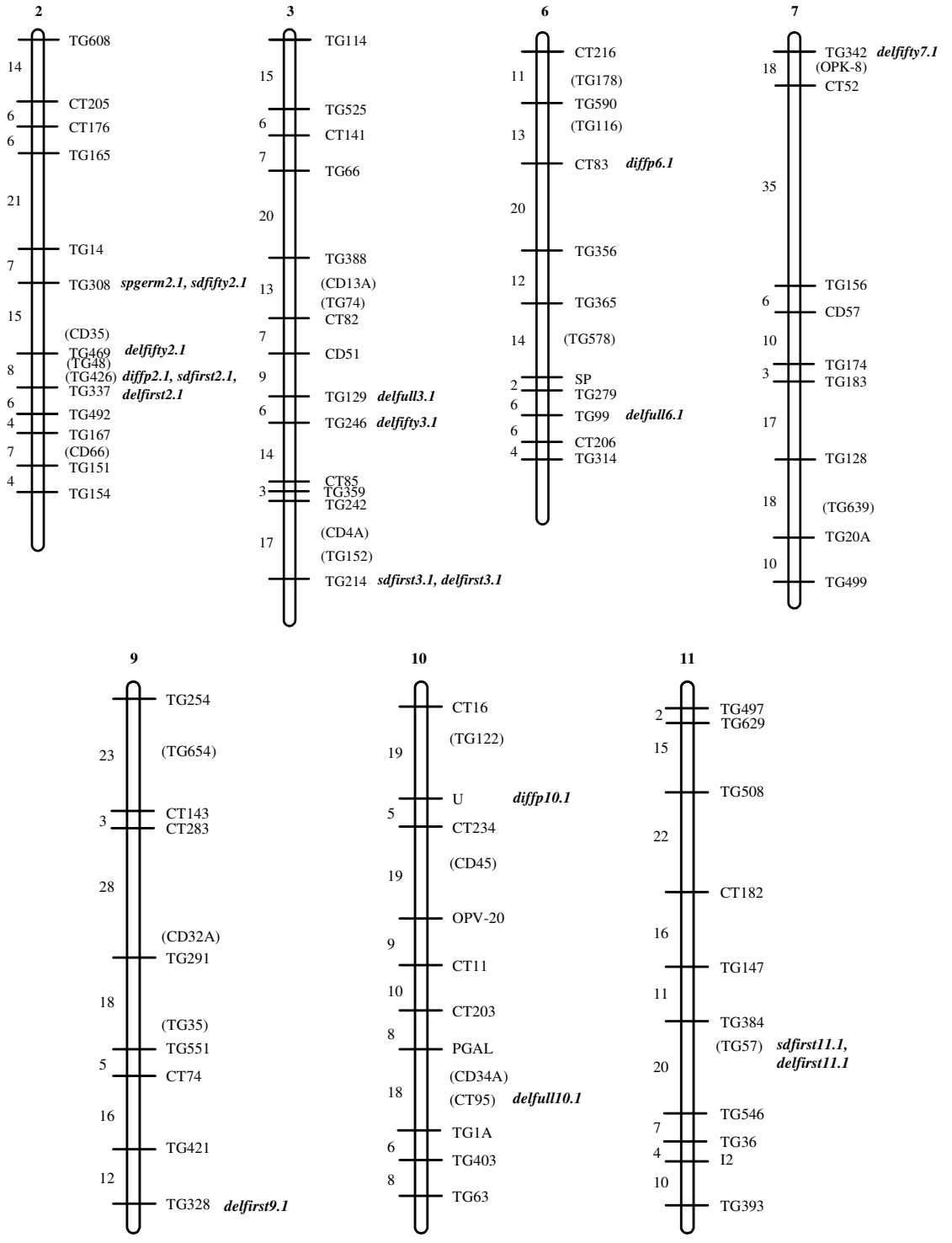

Figure 3.20. Tomato chromosome map showing locations of QTLs identified in 100 mM NaCl experiment. QTL name is placed next to most significant marker for the locus. Map distances are in centiMorgans

3.3.2. QTL Analysis Results for 125 mM NaCl Treatment

In the 125 mM NaCl screen of the IBLs, a total of 20 QTLs were identified for 6 traits (Table 3.6) and Figure 3.21 shows the most likely position of each QTL on the genetic map. For delay to full germination and delay to 50% germination, no significant QTLs were detected. Two QTLs were identified for total germination on the salt medium. These QTLs were located on chromosome 2 and 9 (*spgerm 2.1* and *spgerm9.2*). The QTL on chromosome 2 was more significant than the other and exhibited 16% of the phenotypic variance. Both QTLs had the favorable alleles from *L. esculentum*. Three QTLs associated with difference in percent germination were identified, two of which were on chromosome 2 and one of which was on chromosome 10. The QTL *diffp2.2* marked by TG308 was the most significant controlling 23% of the variance for the phenotype. For the two QTLs located on chromosome 2, *L. esculentum* alleles were the source of salt tolerance. On the other hand, the *L. pimpinellifolium* allele was the source of salt tolerance for the locus on chromosome 10. For the number of days to first germination on salt, three QTLs were identified on chromosomes 2, 8 and 10. For all three QTLs salt tolerance were contributed from *L. esculentum*. A total of 10 QTLs were identified for delay to first seed germination. Chromosomes 2, 5, 9, 12 each had two QTLs and chromosomes 10 and 11 each had one QTL for this trait. The QTL *delfirst2.1*explained 22% of the phenotypic variance and the QTL *delfirst9.1* explained 21% of the phenotypic variance. These two QTLs were the most significant loci and had favorable alleles from *L. pimpinellifolium*. In general, alleles associated with salt tolerance for this trait were equally distributed between *L. esculentum* and *L. pimpinellifolium*. Only one QTL, *dfull5.1,* was identified for the delay to full seed germination. One locus with the favorable allele from the *L. esculentum* parent controlled the number of days to 50% of final germination. This QTL, *sdfifty3.1,* explained 14% of the phenotypic variance.

Table 3.6. List of QTLs for germination stage salt tolerance identified at 125 mM NaCl.

Trait	QTL Symbol	Chromosome	Marker	P Value (≤0.01)	R^2 Value	AA[a]	Aa[b]	Salt Tolerance Source[c]
% Germination on Salt	*spgerm2.1*	2	TG308	<0.0001	16%	71.5	36.4	TA496
	spgerm9.1	9	TG654	0.008	7%	70.4	54	TA496
Difference in % Germination	*diffp2.1*	2	CT176	0.004	8%	79.2	75.8	TA496
	diffp2.2	2	TG308	<0.0001	23%	81.4	43.4	TA496
	diffp10.1	10	TG122	0.008	7%	78.6	79.1	LA1589
Number of Days to First Germination on Salt	*sdfirst2.1*	2	TG308	0.009	7.1%	5.1	6.7	TA496
	sdfirst8.1	8	TG349	0.003	8.8%	4.9	7.1	TA496
	sdfirst10.1	10	TG122	<0.0001	14.9%	5.1	5.8	TA496
Delay to First Germination	*delfirst2.1*	2	CT176	<0.0001	22%	2.8	2.7	LA1589
	delfirst2.2	2	TG48	<0.0001	19%	2.6	3.8	TA496
	delfirst5.1	5	CT93	0.001	9.7%	2.8	3.2	TA496
	delfirst5.2	5	CT118	0.002	9.7%	2.9	2.0	LA1589
	delfirst9.1	9	CT283	<0.0001	21%	2.8	2.6	LA1589
	delfirst9.2	9	TG551	0.0002	10%	2.7	6.7	TA496
	delfirst10.1	10	CT234	<0.0001	19%	2.7	2.9	TA496
	delfirst11.1	11	TG393	<0.0001	17%	2.8	2.5	LA1589
	delfirst12.1	12	CT156	<0.0001	19%	2.8	2.4	LA1589
	delfirst12.2	12	TG473	0.0001	13%	2.8	3	TA496
Delay to Full Germination on Salt	*sdfull5.1*	5	CT172	0.002	9.6%	23.7	24.7	TA496
Number of Days to 50% Germination	*sdfifty3.1*	3	TG129	0.0004	14%	12.9	19.8	TA496

[a] AA column gives mean value for individuals homozygous for *L. esculentum* alleles at each marker
[b] aa gives mean value for individuals homozygous for *L. pimpinellifolium* alleles at each marker.
[c] The last column lists which parental alleles were associated with salt tolerance for each QTL.


2
TG608
3
TG114
5
CT101
8
TG176

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