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SALINITY TOLERANCE IN SEVEN *Trifolium* SPECIES

by

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In The Name of God (Allah),

The Beneficent, The Merciful

To

My Brother, Martyr

Akbar Babagolzadeh

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ABSTRACT

The genus of *Trifolium* includes most of the annual and perennial forage legumes, and is distributed all over the world. Clovers are the predominant legume of pastures and are most frequently grown in combination with various grass species. Salinity tolerance of these species is very important, because clovers, which are of low tolerance to salinity (salt-sensitive), do not grow or tend to die out on saline soils as more tolerant plants (grasses) become the predominant vegetation. Loss of clover from pasture mixtures significantly reduce the nutritional value of the pasture.

With increasing salinisation of irrigated soils in arid semi-arid areas, salinity tolerance of plants that can be grown on such soils is of considerable importance. Examination of salinity tolerance in seven *Trifolium* species has been the aim of the work described in this thesis, because selection and breeding are two rapid and economically cost-effective techniques to overcome problems caused by soil salinity.

Seedling shoot and root lengths of 10 accessions from *T. alexandrinum*, *T. resupinatum*, *T. repens*, *T. subterraneum*, *T. pratense*, *T. ambiguum*, and five accessions of *T. fragiferum* were measured after 14 days growth in 7 levels ($EC_{(25°C)}$ dSm^{-1}) of saline solution, NaCl + $CaCl_2$ in equal amounts by weight. Increasing salinity caused significant reductions in shoot and root lengths both between and within species. Considerable variation in salinity tolerance was found within and between accessions within each species.

Analysis based upon the non-linear least square inversion method from shoot and root data, showed significant differences among and within species in the estimated salinity threshold (C_t), the concentration at which growth root and shoot begin to decline, the salinity level at which the growth decreases by 50% (C_{50}), and the concentration at which growth becomes zero (C_0). According to those three parameters *T. resupinatum* and *T. alexandrinum* were the most tolerant to salinity, *T. repens* and *T. fragiferum* were the least tolerant, the other species having intermediate tolerance.

The response to increasing salinity of the four *Trifolium* species, *T. alexandrinum* (2 accessions), *T. resupinatum* (3 accessions), *T. ambiguum* (1 accession), and *T. pratense* (1 accession) was assessed in a sand culture experiment, based upon the

performance of the whole plant. Increasing NaCl + $CaCl_2$ concentration significantly decreased shoot and root dry and fresh weight in all accessions examined. At the highest concentration, EC 26 dSm^{-1}, *T. resupinatum* was the most tolerant and *T. pratense* was the least tolerant, the other two species having intermediate tolerance (*T. resupinatum* > *T. alexandrinum* > *T. ambiguum* > *T. pratense*). Comparison of data gained from seedling shoot growth of two-weeks-old seedlings in solution culture and adult plants in sand culture experiment under saline conditions, showed very good correlations for all four species.

Selection and improvement of salinity tolerance from 7,500 seeds of each of the two most tolerant species (*T. resupinatum* and *T. alexandrinum*) and one of the most valuable and nutritious forage species (*T. repens*) was followed over three generations. It was shown that improvement in tolerance in the three species examined was possible, especially in *T. resupinatum*. Salinity tolerance in those three species had broad sense heritability (h^2_B) estimates for both soot and root lengths with the range of 0.23 to 0.71, 0.26 to 0.84, and 0.26 to 0.68 respectively.

Interspecific hybridisation between the aforementioned three species used pollination by hand, and bees, and hybrid plants were obtained from *T. alexandrinum* (2n = 16) and *T. repens* (2n = 32), The other two combinations of crosses were not successful. The viability of pollen grain of the hybrid progenies was examined, and showed that they have highly stainable pollen grain. Analysing the data shown, the hybrid plants have a similar behaviour to *T. repens* in some characters, some characters were intermediate, and the other characters were superior those in both parents.

The overall conclusion from this work is that the genus of *Trifolium* varies considerably in salinity tolerance, and increased tolerance could probably be achieved through selection and breeding. It might be possible to transfer or combine the useful characters (salinity tolerance, grazing quality, and perenniality) through inter/intra-specific hybridisation.

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CHAPTER ONE

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CHAPTER ONE

GENERAL INTRODUCTION

The early agriculture developed in three distinct areas, namely South America, East Asia, and Central Asia, over the same period 6,000 to 9,000 B. C. (Higgs and Jarman 1972, Protsch and Beger 1973). Iran, like the rest of the Near East, advanced rapidly into the Bronze Age, while Europe was still in the later stages of Palaeolithic culture (Cameron 1936). The cradle of Old World plant husbandry was located within an arc stretching from the western foothills of the Zagros Mountains (west of Iran), the Taurus mountains (southern Turkey), to the Galilean uplands in northern Palestine (Helbaek 1959).

Iran was initially identified by Vavilov (1951) and later on by Harlan (1971) as a part of The Near Eastern Centre of the origin of many grains, several legumes, vegetables, fruits and nuts, and subsequently by Cox and Atkins (1979) for a diverse assortment of all of those. In the early 7th millennium B. C. people descended to the plain from the highlands and established communities in sufficient numbers, and techniques adequate to turn the waters of the rivers to their use. As a result, Khozistan developed an agricultural civilisation based upon river irrigation (Diakonoff 1985). The region shows an extensive climatic and ecological diversification due to its wide physiographic variations, but is semi-arid to arid in its overall climate (Cox and Atkins 1979).

Iran covers an area of some 1,648,000 km^2. Approximately 71% of the whole area is arid or semi-arid (Turner 1955). Precipitation is low, and although it is distributed

throughout the year, it is heaviest during winter, but even so, the rainfall is unreliable. The strong winds and higher temperatures that occur during summer create a high level of evaporation and evapotranspiration as well (Cox and Atkins 1979).

In general, winter is the rainy season in Iran, and although there are some areas that receive their maximum rainfall in spring or autumn, it is during the winter months that more than two thirds of the surface area of the country receives more than half of its annual precipitation. Summer is a dry season all over Iran except in the areas surrounding the Caspian sea (Jafari 1990).

With conservation, protection, and improvement of pasture however, useful, economic results may be obtained from rangelands. The main outcome expected of rangelands is to produce optimum productions of forage plants with a high nutritive value. However, this proves to be very difficult in arid zone areas. A further problem has arisen because an expansion of dry-land farming has increased the grazing pressure on the remaining areas and has reduced the area of good rangelands (Nemati 1986). Probably the greatest impact upon agriculture in arid and/or semi-arid zones is salinity, which is a consequence of too many causes, mainly the mis-management of farmers.

Aridity is associated with salinity, saline water sources, and saline soils. Under these conditions precipitation is insufficient to leach away the soluble salts and to keep them in dilute solution until they are washed away. Water is essential for crop production, of course, and an excess of soluble salts is detrimental to successful agriculture. Nearly 10% of the total land surface is covered with different types of salt-affected soil. The distribution of salt-affected soils in the world is shown in Table 1 (Kovda

and Szabolcs 1979). Salt affected soil can be divided into the five following groups (Szabolcs 1994):

1) Saline soil that develops under the influence of electrolytes of sodium salts with nearly neutral reaction (mainly NaCl and Na_2SO_4). Arid and semiarid regions, a major part of all salt affected soils of the world are in this group.

2) Alkali soils that develop under the influence of electrolytes of alkaline hydrolysis (mainly Na_2CO_3 and $NaHCO_3$). This group extends to practically all climatic regions from the humid tropics to beyond the polar circles, but the total salt content is usually lower than that of saline soil.

3) Gypsiferous soils develop in the presence of $CaSO_4$ or rarely $CaCl_2$, can be found mainly in the arid and semi-arid regions.

4) Salt affected soil that develops under the influence of magnesium salts. This group occurs in arid and semi-arid regions and especially the soils that have a heavy texture.

5) Acid sulphate soils whose salt content is composed mainly of $Al_2(SO_4)_3$ and $Fe_2(SO_4)_3$. This type of salt affected soil is extensive in seashores of all the continents.

The major solutes comprising dissolved mineral salts are the cations Na, Ca, Mg, and K, and the anions Cl, $S0_4$, HCO_3, and NO_3 (Tanji 1990). Various units have been used for assessment of salinity, expressed in SI metric units such as mol m^{-3}, mol l^{-1} equivalents per litre or mg l^{-1} (ppm) for major solutes, and $\mu g\ l^{-1}$ for trace elements. Because of the strong relationship between the electrical conductivity (EC) of the soil extracts and the soil's salt concentration, the salt content of a given soil is commonly

expressed by the EC, measured at a reference temperature of 25°C. The EC is nowadays expressed in deci-Siemens per meter (dSm^{-1}). To judge by many papers devoted to the physiology of the salt relations of plants, salinity is equated with NaCl, or a mixture of NaCl and $CaCl_2$. The widespread occurrence of these potential hazards hinders the development of agricultural production. Thus, there is a global need for protective and remedial action. Prognosis is needed where arable and potentially salt affected soils exist.

Table 1.1. Distribution of salt affected soils in the world (FAO/UNESCO 1974, Ponnamperuma 1984, Szabolcs 1994).

Continent and Subcontinent	Area (million hectare)
North America	15.7
Mexico and Central America	2.0
South America	129.2
Africa	80.5
South Asia	87.6
North and Central Asia	211.7
South east Asia	20.0
Australia	357.3
Europe	50.8
Total	954.8

Problems of soil salinity are widespread throughout the world. Nearly one-third of the world's arable land is already saline, and by the end of the century nearly half of the world's irrigated arable land is expected to have problems associated with salinity. Estimates of saline soils ranged from 400 to 950 $\times$ 10^6 ha according to Shannon in 1984.

There are many ways for saline soils to form (Brady 1974 and Pessarakli 1991). Firstly, soil salinity may result from the weathering of parent rock materials. Secondly salts may be brought in by wind, originating in sea-spray or in dry blow of salt from other saline soils. Thirdly, salts may rise to the surface by capillary action from mineralised ground-waters. Fourth, salt accumulation may be caused by irrigating with water of poor quality, especially when this is combined with poor irrigation practices (Hoffman *et al.* 1990).

From the perspective of plant productivity, salinity problems accentuate year after year as a result of repeated irrigation and the concentration of salts in the soil in which these crops are grown by evaporation and concentration processes and in the tissues of crops themselves through the process of transpiration. Soil salinity may be quantified from assessment of the total amount of exchangeable cations that a soil retains, designated the cation exchange capacity. It is often convenient to express the relative amounts of various exchangeable cations present in a soil as a percentage of the cation exchange capacity. The soluble cations which give saline soils their characteristics are calcium, magnesium, sodium, and potassium. The predominant anions are bicarbonate, carbonate, sulphate and chloride. Depending on which of these factors is/are present, soils can be divided broadly in to saline and sodic as follows (Fitzpatrick 1980). Saline, Electrical Conductivity (EC) of a saturated extract > 4

mmhos cm^{-1}, exchangeable sodium < 15%, and pH < 8.5. Sodic soils have an EC of a saturated extract < 4 mmhos cm^{-1}, exchangeable sodium > 15%, and pH > 8.5. Nevertheless, some workers, for example Milijikovic (1965), as tabulated by Fitzpatrick (1980), have provided a separate scale for salinity and alkalinity as follows:

a) Degree of salinity

Slightly saline EC 2 - 4 dSm^{-1}

Moderately saline EC 4 - 8 dSm^{-1}

Strongly saline EC 8 - 15 dSm^{-1}

Very strongly saline EC > 15 dSm^{-1}

b) Degree of alkalinity

Slightly alkaline < 20% exchangeable sodium

Moderately alkaline 20 - 50% exchangeable sodium

Strongly alkaline > 50% exchangeable sodium

Populations in the developing countries of the arid and semi-arid regions of the world are growing so quickly that the land and water are unable to sustain them. In most developing countries, prime farmland and fresh water are already fully utilised. Although irrigation can be employed to bring land in arid areas into production, this often leads to salinisation (Shay 1990). The problem of secondary salinisation is more serious in arid and semi-arid regions (Flowers *et al.* 1977). It is estimated that more than 50% of all irrigated lands of the world were affected by secondary salinisation and/or sodication and water-logging almost 20 years ago (Massoud 1977, and Zahran and Abdel Wahid 1982). This has no doubt, increased considerably since then.

Because of the continued degradation of irrigated lands due to salt accumulation, successes and gains of the green revolution which depended to large extent on exploitation of irrigated lands and becoming losses, according to Wyn Jones and Gorham (1986). Although in general the destructive effect of soil salinity is explicable in terms of loss of crop yield, the extent of loss varies from farm to farm due to differences in regional water quality and management practices. Gates and Grismer (1989) noted that the most effective program for the long-term amelioration of the saline soil involves managing irrigation and water quality to fit crop needs, and leaching of salts deep into the soil profile using large volumes of water. An appropriate drainage system designed to remove the resulting saline leaching water to minimise the return of salts to the root zone by capillary movement is also a crucial requirement. The use of chemicals such as gypsum to facilitate the mobility of salt and water in the soil is another viable option. The effectiveness of these methods depends largely on the availability of good quality water which in many areas is a source of potential competition between urban and agricultural demands. Poor quality brackish water is on the other hand inappropriate for such purposes (Wyn Jones and Gorham 1986).

The physical approaches to mitigate saline soils has been successful in small scale, although it has proved to be costly and labour consuming. Thus making such endeavours to be economically not feasible on the one hand (Shannon 1984). On the other hand as more and more agricultural lands lose their productivity.

The world population continues to increase and the needs for food, energy, fuels, chemicals, fertilisers, fibres and medicinal compounds increase concurrently, more and more agricultural lands lose their productivity due to salinity problems. The possibility of alleviating the salinity problem using chemical and physical methods is

extremely limited and quite often impossible because of expense, and therefore such methods are unlikely to be used on are large scale in the near future.

The genetic modification of crop plants by exploiting genetic variability both within and between species to provide salinity tolerant plants capable of growing under saline conditions is still the most feasible solution to the soil salinity problem (Epstein *et al.* 1980, Shannon 1984). Salt tolerant plants can provide a logical alternative for many developing countries. Saline farmland may be used without costly remedial measures, and successful reclamation of degraded land is usually preferable, in terms of resource conservation, to opening new land. Low quality water, too saline for irrigating conventional crops, may be used to irrigate salt-tolerant plants. Even the thousands of kilometres of coastal deserts in developing countries may serve as new agricultural land, with the use of sea water for irrigation of salt tolerant plants. These plants can be grown using land and water not suitable for conventional crops and can provide food, fuel, fodder, fibre, resins, essential oils, and pharmaceutical feed-stocks (Shay 1990). Accordingly, agriculture in saline soils are essentially needed, especially in arid and semi-arid regions. Domestication of the salt tolerant plants currently growing in saline soil or water will introduce them as non-conventional crops to be cultivated under environmental stresses induced by salinity and aridity.

Soil salinity is a major environmental stress that drastically affects crop productivity. Salinity poses a severe threat for cultivation of crops. Because of the continuous build-up of salinity in the soil, millions of hectares of usable lands have now become unsuitable for cultivation. It is estimated that every year more than a million hectares of land is subjected to salinisation. Soil salinity is thus threatening civilisation by persistently reducing the area for crop cultivation. To achieve optimal food production

in saline regions, the most appropriate, logical choice is growing salt-tolerant varieties best suited for these regions. Progress in developing salt-tolerant crop varieties has been very slow not because of our limited knowledge of the mechanism of salt damage and the complex nature of salt tolerance. It is because, whilst considerable amounts of money have been put into physiological studies of mechanisms of tolerance, only minute amounts have been put to support traditional selection and breeding for improved crop tolerance. Different varieties of a particular species may exhibit different tolerance behaviours. Salinity affects seed germination, plant growth, nutrient uptake, and metabolism due to osmotic inhibition of water availability, toxic effects of salt ions, and nutritional imbalance caused by such ions (Levitt 1972). In the life cycle of plants, germination, seedling, and flowering stages are more critical for salt damage.

As the problems of salinity for agriculture become more severe every year, the possibility of growing alternative plants and crops suited to moderately saline conditions has been investigated. The first option pertaining to the choice of the new agricultural plants is to introduce under exploited, salt tolerant minor crops and plants (Läuchli 1984 and Muller *et al.* 1990). Another favourable option is to select new plants from conventional plant breeding programmes for increased tolerance to salt stress (Johansen *et al.* 1990 and Zahran 1991). A third alternative is to modify traditional crops by molecular biology techniques so that they are made to tolerate higher salinity (Saxena *et al.* 1993). Molecular manipulation can apply such techniques as somaclonal variations, mutagenesis, somatic hybridisation, and genetic engineering (Saxena *et al.* 1993).

Sustained and profitable production of crops on salt affected soils is possible if on farms, appropriate management decisions are made. To be successful, growers require an understanding of how plants different stages of growth, and how different soil and environmental conditions affect salt stressed plants (Francois and Maas 1994).

Plants growing in saline soils face two problems, high salt concentrations in the soil solution (i.e., high osmotic pressure and correspondingly low soil water potential) and high concentrations of potentially toxic ions, such as Cl^- and Na^+. Salt exclusion minimises ion toxicity but accelerates water deficit in plants, whereas salt absorbtion facilitates osmotic adjustment but can lead to ion toxicity and nutritional imbalance (Günes *et al.* 1996).

Local land races of particular crops which have evolved over long periods of time in diverse locations traditional farming systems possess vast genetic diversity. These populations constitute a very valuable source of genetic diversity for plant breeding programmes (Frankel 1977). Not enough attention has been paid to the immense diversity of gene complexes determining adaptation and productivity, assembled and incorporated over long periods of cultivation in differing environments (Frankel and Bennett 1970), and co-adapted gene complexes of crucial importance in the adaptation of various populations to their particular environments (Dobzhansky 1970). Some of this genetic variability has been included in those plants from land races. However, much more variability is to be exploited, some of which grants adaptations to extreme conditions, such as salt tolerance, which, as discussed above, is a character of great importance in the world. Furthermore Vavilov (1926), emphasised that the combination of parents adapted to a wide range of different environments may confer opportunities for major advances through the combination of different adaptive complexes. Likewise, Moeljopawiro and Ikehashi (1981) noted that the success of

breeding for salt tolerance depends largely on the cumulative tolerance obtained from combining of genetic material from different sources.

Although, gaps in our knowledge of the origin of our crops exist, sources including archaeology, anthropology and ethnology, plant geography, climatology, ecology, and cytogenetics, and experimental evolution has rendered much information on the evolutionary relations of crops and their wild ancestors. Further understanding into the evolution of crops may increase the appreciation of wild progenitors and other related species as potentially valuable sources for the improvement and even revolutionary restructuring of crop species (Frankel 1977). It has been shown that some wild ancestors of some cultivated plants contain large gene pools for salinity tolerance. For example, in a number of crops there are indications that salt tolerance is associated with more efficient Na^+ or Cl^- exclusion, which in *Aegilops squarrosa* (the putative source of the D genome of *Triticum aestivum*), is a common character (Wyn Jones *et al.* 1984). The moderate salt tolerance of *T. alexandrinum* is credited to the mechanisms such as retranslocation of Na^+ and Cl^- out of young leaves, redirection of Na^+ absorbed by basipetal into the root medium, maintaining a high K^+: Na^+ ratio in younger leaves and the restriction of Cl^- from roots to shoots (Winter 1982a, b; Winter and Läuchli 1982; Winter and Preston 1982). Likewise the highly salt tolerant wild rice, *Oryza coarctata*, can survive up to 30 to 40 dSm^{-1} salinity and this species may be used as a parent for developing more and truly salt tolerant rice varieties (Bal and Dutt 1986). Rush and Epstein (1981) made a successful interspecific cross in tomatoes between the wild *Lycopersicon cheesmanii* and the cultivated *L. esculentum* to transfer salt tolerance from the wild into the cultivated species. Many generations of back

crossing, selected salt-tolerant lines which completed their life cycle when grown in sandy soil irrigated with 70% sea water.

Selection and breeding for resistance to a given environmental stress depends basically on two factors; genetic variability with respect to the particular stress involved, and selection following exposure of genetically variable material to that stress, thus detecting identification of individuals approaching or containing the desired phenotypes. A reliable method of quantifying variability within a species for the stress resistant characters in question is also valuable. Previous studies provide sufficient evidence about the occurrence of variation in salt tolerance between a considerable number of plant species (Epstein and Norlyn 1977, Norlyn 1980, Norlyn and Epstein 1984, Verma and Yadava 1986). Variation in salt tolerance has also been found between different wild populations within the same species were this occurs naturally in saline and non-saline environments. For example, Hannon and Bradshaw (1968) found significant differences in salt tolerance between different populations of both *Agrostis stolonifera* and *Festuca rubra.*

The extent of evidence about the genetic basis of salinity tolerance is not great, but evidence from four grass species (Ashraf *et al.* 1986a), lucerne (Noble *et al.* 1984, Ashraf *et al.* 1987, Al-Khatib *et al.* 1993), sorghum (Azhar and McNeilly 1989) and millet (Kebebew and McNeilly 1995) suggest that both additive and non-additive genetic effects are involved. An extensive understanding about the genetic make up of salinity tolerance in crop species in which tolerance is to be improved in essential to achieve effective and speedy improvement through selection and breeding.

To overcome the problems of salinity Epstein *et al.* (1980) pointed out the inadequacy and expense of the traditional engineering approaches and emphasised the need for developing salt tolerant plants to solve this problem. The significance of breeding salt tolerant plants as an energy-efficient approach that could be applied to land and water management alternatives was highlighted by Shannon (1984). The hitherto unusable saline soils could be put into productive use, if the economically important plants acquired the necessary level of salt-tolerance. Leguminous plants have an ability to fix atmospheric nitrogen in symbiotic association with *Rhizobium* bacteria present in soil and with their consequent independence from fertiliser nitrogen, they should be actively sought for propagation in marginal saline lands.

The effect of salinity on the most important crops has been examined by many workers, for example (Ayers *et al.* 1952, Pearson and Bernstein 1959, Bernstein 1964b, 1975, George and Williams 1964, Maas *et al.* 1983, Francois *et al.* 1986, Francois *et al.* 1989, Maas 1990, Chauhan and Singh 1993, Maas *et al.* 1994, Kebebew and McNeilly 1995, Beshir 1996, Rao 1997, Safarnejad *et al.* 1997, Shannon *et al.* 1998).

Plant populations vary if the environment varies and differentiation between them follows the pattern of the environment. Selection is very effective because it can cause this evolutionary differentiation to follow the pattern of the environment most meticulously (Jain and Bradshaw 1966). A considerable amount of studies of salt tolerance in a considerable number of species have been carried out, and they have shown that in at least a number of samples, salinity tolerance is present at different stages of growth. For example, using different methods, Dobrenz *et al.* (1981) increased salt tolerance at germination of lucerne (*Medicago sativa*) by selection, and

Dewey (1962) evaluated the salt tolerance of 60 strains of *Agropyron desertrorum*, and suggested a recurrent selection and breeding programme for increasing salt tolerance.

One of the most important genera of the *Fabaceae* family is *Trifolium*, both for its agricultural value and in the number of species within it, amounting to 237 (Zohary 1972a, Zohary and Heller 1984), of which, about 25 species are of significance as food for grazing animals and of these, about 10 are agriculturally important (Evans 1984). About one third of clover species is perennials the remaining species are annuals. One third of the species of the genus is self pollinated, the remaining being cross pollinated mainly by bees (Taylor *et al.* 1980). In general, clover inhabits temperate regions of the world. It will grow on many different soils, but its distribution depends mainly on climatic conditions (Taylor 1985).

The origin of clovers been speculated by Zohary (1972b) as being in western North America, and from there he suggested that it spread into the rest of world. However, Taylor *et al.* (1980), concluded that the Mediterranean area is more likely to be its centre of origin, basing his suggestion on area of diversity, and chromosomal studies.

Legumes of the genus *Trifolium* are of major importance in animal husbandry agriculture, both in arid-zones and in wetter climates. This is due to their high digestibility and protein/carbohydrate ratios, as a cover of soils to protect against soil erosion, and their ability to fix atmospheric nitrogen, an element that is low in quantity in most soils. They are generally considered as being salt sensitive, and most species do not grow in soil with high salinity (Richards *et al.* 1954), *T. alexandrinum* (Winter and Läuchli 1982, Läuchli 1984, and Noble and Rogers 1994), *T. hirtum* All., and *T. fragiferum* are moderately salinity tolerant (Gauch and Magistad 1943, Kaddah 1962,

Russell 1976, West and Taylor 1981), *T. hybridum* L. *T. pratense*, and *T. repens* have low salinity tolerance.

The present study was carried out to assess the variability in salt tolerance within and between species in a range of *Trifolium* species using simple and convenient selection techniques. In the age of modern technology there is a need for rapid and successful selection and breeding methods. The project reported in this thesis was undertaken to:

1. Determine the variability within and between species in salinity responses of two-week-old seedlings of seven species of *Trifolium*, using the solution culture technique which has previously been shown to provide acceptable estimates of salinity tolerance in number of genera and species.

2. Assessment of the effects of seven levels of salinity applied at adult stages. Determine the relationship between shoot length of two-week-old seedling from four Trifolium species in solution culture, and shoot dry weight after 16 weeks growth of the same species grown in seven salinity levels of sand culture, and also determining of the correlation between adult and seedling stages.

3. Examine heritability of variation for salinity tolerance of three *Trifolium* species, *T. alexandrinum*, *T. resupinatum*, and *T. repens*, and three cycle of selection pressure to improve salinity tolerance in those species.

4. Determine the cross ability between the two most tolerant species, *T. alexandrinum* and *T. resupinatum*, and the most important species agriculturally *T. repens* to providing information about the possibility of developing a perennial, high quality, salinity tolerant clover.

CHAPTER TWO

VARIATION IN RESPONSE TO SALINITY OF SEVEN *Trifolium* SPECIES

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CHAPTER TWO

VARIATION IN RESPONSE TO SALINITY OF SEVEN *Trifolium* SPECIES

2.1. INTRODUCTION

A salt-affected soil is defined as one that has been adversely affected to the extent that it is no longer suitable for the growth of most crops, due to the presence or action of soluble salts. This group of soils includes both saline and sodic soils. James *et al.* (1982) defined a saline soil as one that contains a sufficient quantity of soluble salts to interfere with the growth of most crops. A sodic soil possesses enough exchangeable sodium to also have an adverse effect on the growth of most plants. A saline-sodic soil contains both soluble and exchangeable Na at levels that imposes stress on plant growth. Jones (1968) and Meiri and Poljakoff-Mayber (1970) reported that, when Na^+ was present in high concentration in the solution, the transpiration rate of peas was reduced in proportion to salinity. Porath and Poljakoff-Mayber (1964) found that Na^+ also affected the respiratory pathway of roots. High Na^+ in soil solution also has an antagonistic effect on Ca^{2+} and Mg^{2+} uptake (Poljakoff-Mayber and Gale 1975).

Salt-affected soils are a common feature of arid and semi-arid landscapes, and the loss of plant productivity from the excess of salinity is a world-wide problem. Where salinity is a problem, an effective use of soil and water resources dictates the production of agricultural crops. Numerous laboratory and field experiments have been conducted to determine plant growth and yield response to various levels of soil salinity. For example, Shalhevet *et al.* (1969) found that the yield of peanuts grown in artificially salinised plots was reduced to 20% at EC_e of 3.8 dSm^{-1} and by 50% at EC_e

of 4.7 dSm^{-1}. Additionally, these investigators reported that salt tolerance was much higher during seed germination than during subsequent growth stages, a 50% reduction in germination occurring at EC_e = 13 dSm^{-1}. Shalhevet and Yaron (1973) reported a 10% yield reduction in tomatoes for every 1.5 dSm^{-1} increase in EC_e. Above 2.0 dSm^{-1} soil salinity clearly affects plants, but the adverse effects of soil salinity on plant growth and productivity vary with the particular plant being grown. Clearly there are significant effects of salinity on non-halophytes, whereas the effects are, as would be expected, much less on halophytes.

In studies of the differences between halophytes and non-halophytes in their response to salinity, the following properties have been determined in halophytes.

1. Ability to accumulate or to exclude ions selectively (Läuchli 1984).
2. Control of ion uptake by the root and control of transport to the shoot and leaf (Flowers *et al.* 1977).
3. Selectivity in xylem release (Jeschke 1984).
4. Role of accumulated ions in osmotic adaptation (Flowers *et al.* 1977, and Bernstein 1961).
5. Compartmentation of ions at the cellular and at the whole-plant level (Flowers *et al.* 1977).
6. Accumulation of so-called compatible solutes and their role in salt tolerance (Pollard and Wyn Jones 1979).

Nevertheless, none of these characteristics could be used as markers for breeding salt tolerant crops, and in fact, none of these factors alone can be a basis even for a definition of a halophyte. The difficulty arises from the fact that expression of such traits changes with the age of the plant, its physiological stage of development, and

under changing environmental conditions (Poljakoff-Mayber and Lerner 1994). As an alternative it has been suggested that because of the high correlation between salt tolerance and vigour in wheat and barley addition lines, breeding for agricultural traits may be more productive than breeding for physiological traits (Forster *et al.* 1990).

Escalating costs of reclamation, or drainage, or water control, make these means of reducing the extent and spread of saline soils in arid and semi-arid regions unattractive prospects for governments. An alternative biotic approach to the problem, namely, the breeding of salt tolerant crop varieties for such soils as proposed by Epstein *et al.* (1980), is an attractive possibility. In combination with appropriate management programmes this would allow exploitation of saline soils for agriculture.

The salinity tolerance of crop plants can be defined as their ability to survive and produce economically acceptable yield under adverse conditions caused by soil salinity. It is normally expressed in terms of the yield decrease associated with a given level of soil salinity as relative crop yield in saline compared with non saline soils (Maas and Hoffman 1977). A yield decrease or growth reduction of 50% is usually considered a critical level for evaluating the relative salt tolerance of crops (Maas and Hoffman 1977, and Johansen *et al.* 1990).

Salinity plays an important role in determining the type of vegetation cover, and it is a world wide problem. The distribution of salt-affected lands is closely related to environmental factors, in particular arid and semi-arid climates. The extent of the increase of salinity in arid and semi-arid lands that has occurred recently has become a problem of great concern in agriculture, salinity and aridity are the two oldest enemies of agriculture. Recent estimates put the area of salt affected soils at some 9.5 million

km^2 on a world scale (Szabolcs 1989), much of it due to inadequate irrigation practices in arid and semiarid regions, and the consequent loss of agricultural production is enormous. It is believed that about 7% of the total surface area of the world is salt affected (Sen and Mohammed 1994), and approximately 10% of the world's 7×10^9 ha arable land surface consist of saline or sodic soils (Francois and Maas 1994). The percentage of cultivated lands affected by salinity is even greater. Of the 1.5×10^9 ha cultivated lands, 23% are considered as being saline and another 37% are sodic, It has been estimated that one-half of all irrigated lands (about 2.5×10^8 ha) are seriously affected by salinity or water logging (Rhoades and Loveday 1990a). Crop species growing on salt affected soils are subjected to toxic effects of sodium and chloride ions (Flowers 1985) and the dehydrating impact of salt, (Steponkus 1980), the combined effects of which adversely affect the physiological activities of the plant to such an extent that plant growth becomes severely restricted, or impossible. The result is at least considerable loss, but frequently a total loss of yield.

Because of increases in human population and the amount of land that is becoming non-productive due to soil salinity, considerable efforts are on the one hand being made to improve naturally salt tolerant wild species, and on the other hand to produce varieties of crop species which can be used to exploit salt affected soils, both for forage production and arable cropping (Maas and Hoffman 1977, Epstein *et al.* 1980, Shannon 1984).

The principal criteria to determine irrigation water quality are salinity, sodicity, and specific ion concentrations. However, the effects on crops of a given water are not determined solely by its solute composition. These water quality factors should be considered in relation to the specific conditions under which the water is to be used

(Bernstein 1967, and Rhoades 1972, 1994), that is, soil properties, irrigation methods, cultural practices, climatic conditions, and the crop to be grown.

The extent of permissible water depletion for a given crop is determined by the maximum acceptable salt concentration for that crop (Bernstein 1974). When additional water depletion occurs and no irrigation water is applied to recharge the root zone and dilute this concentrated soil water, yield is reduced. Therefore, increased irrigation frequency is generally required under saline conditions (Rhoades and Loveday 1990b). With shorter irrigation intervals, the concentrating effect for evapotranspiration on soil salinity is minimised (Bernstein and Francois 1973, and Hoffman *et al.* 1983). Evidence indicates that plants respond primarily to the soil salinity in that part of the root zone with the highest total water potential (Bernstein and Francois 1973 and Francois 1981). With more frequent irrigation, this zone corresponds primarily to the upper part of the root zone, where soil salinity is influenced primarily by the salinity of the irrigation water. With infrequent irrigation, the zone of maximum water uptake becomes larger as the plant extracts water from increasingly saline solutions at greater depths.

In soils that are not well drained, the frequency and amount of irrigation water must be closely monitored. Application of excess water over that required for the crop and for leaching should be avoided. Not only are valuable nutrients lost with over irrigation, but flooded or poorly drained soils suffer from poor aeration, which may affect the crop's response to salt stress (Francois and Maas 1994).

The early seedling stage of growth is the most salt sensitive for most crops (Ayers *et al.* 1952, and Pearson *et al.* 1966). Although salt stress delays germination and emer-

gence, most crops are capable of germination at higher salinity levels than they would normally tolerate at the vegetative or reproductive stages of growth (Maas and Grieve 1992). However, this high tolerance is of little benefit when the plants are much less tolerant during the following growth stages. It is generally agreed that after the seedling stage, most plants become increasingly tolerant as growth proceeds through the vegetative, reproductive, and grain-filling stages. Rice may be an exception (Francois and Maas 1994), and Pearson and Bernstein (1959) showed that rice yields were significantly reduced if salt stress was imposed at either the seedling stage or during pollination and fertilisation.

There are three major approaches to the problem available for improving the salinity tolerance of existing crop species which have the potential, unlike some of the more "in vogue" techniques of genetic engineering, of producing useful advancement in tolerance in the relatively short term. Firstly, variation within existing crop cultivars can be examined, and promising lines/genotypes can be selected (Srivastava and Jana 1984, Kingsbury and Epstein 1984). Secondly, variable material, produced by artificial crossing of self-pollinated species or which occurs naturally in out-crossing species, can be screened, and again the most promising lines multiplied for further selection (Ashraf *et al.* 1986b). Finally the tolerance of a crop may be improved if genes from a wild relative (if present) can be transferred to the cultivated species either by conventional crossing techniques, or if possible through genetic engineering. A useful contribution to information about breeding potential in any crop can then be made through an examination of variability existing at the cultivar level (Ashraf and McNeilly 1987).

Virtually all commercially grown varieties of world crops are developed under non-saline conditions, and as a consequence are not bred to endure salt stress. Therefore, their relative tolerances to salinity are often similar and difficult to measure. In addition, many cultivars developed in the past were derived from a narrow genetic base and thus contain limited genetic variability. Currently, developed cultivars are from a much more diverse genetic base, and may therefore possess a wider range of genetically based variability within them, the variability including possibly, salinity tolerance. Among the crop species that already show some diversity in salt tolerance are Bermuda grass, bromegrass, creeping bent grass, wheat, barley, soybean, alexandrian berseem clover, squash, muskmelon, strawberry (Bernstein 1964b, Bernstein *et al.* 1966), sorghum (Azhar and McNeilly 1987), maize (Ashraf and McNeilly 1989), lucerne (Al-Khatib *et al.* 1994), millets (Kebebew and McNeilly 1995, and rice (Tsuchiya *et al.* 1994, Shannon *et al.* 1998).

Legumes are generally considered as being sensitive or only moderately tolerant to salinity (Maas and Hoffman 1977, Läuchli 1984, and Saxena *et al.* 1993). However, considerable variability in salinity tolerance among crop legumes has been reported (Table 2.1). Among the cultivated legumes, *Sesbania cannabina* and *Lupinus luteus* have been shown to be particularly tolerant to salinity (Keating and Fisher 1985, and Läuchli 1984), and some of the tree legumes, such as *Prosopis* and *Acacia* spp., are also highly tolerant to salinity, with their tolerance levels approaching that of sea water (Felker *et al.* 1981, and Rhoades and Felker 1988). On the other hand, grain legumes, such as *Phaseolus vulgaris, Vigna radiata*, and *Cicer arietinum*, are highly sensitive to

Table 2.1. Relative Tolerance of Different Legumes to Salinity

Species	EC_e (dSm^{-1}) at 50% yield	Reference
Sesbania cannabina	13.2	Keating and fisher (1985)
Lens esculenta	12.8	Rai (1983)
Trifolium subterraneum	11.1	Hopmans *et al.* (1984)
Medicago sativa	10.2	Russell (1976)
Pisum sativum	10.0	Cerda *et al.* (1982)
Sesbania bipinosa	8.4	Giridhar (1987)
Trifolium alexandrinum	8.3	Russell (1976)
Vigna aureus	8.3	Balasubramanian and Sinha (1976)
Medicago scutillata	8.2	Russell (1976)
Trifolium hirtum	8.1	Russell (1976)
Medicago truncatula	7.8	Russell (1976)
Vicia faba	6.8	Maas and Hoffman (1977)
Glycine max	6.7	Keating and fisher (1985)
Trifolium fragiferum	6.5	Russell (1976)
Cliteria turnatea	6.4	Keating *et al.* (1986)
Trifolium repens	6.2	Russell (1976)
Lablab purpureus	5.5	Russell (1976)
Psolarea tenax	5.3	Keating *et al.* (1986)
Vigna mungo	5.0	Russell (1976)
Trifolium semipilosum	4.2	Russell (1976)
Phaseolus vulgaris	3.6	Maas and Hoffman (1977)
Vigna radiata	3.5	Keating and fisher (1985)
Cicer arietinum	3.0	Saxena (1987)

salinity, with a 50% reduction in growth occurring at 3-4 dSm^{-1} salinity. Most of these species are grown for grain or fruit, which are generally low in Na^+ and Cl^- unlike many forage species. Considering legumes, two features are relevant: There is variability in salt resistance among legumes, and most of them respond to saline conditions by salt exclusion, that is, exclusion of sodium and / or chloride from the leaves (Läuchli 1984). Winter and Läuchli (1982) showed that some legumes exclude both Na^+ and Cl^- (e.g. soybean cv. Lee, *Lupinus angustifolius* and *Trifolium alexandrinum*). Beans and alfalfa, however, are only Na^+ excluders. More to the point is the question of whether there is a widespread, positive correlation in legumes between Na^+ or Cl^- exclusion and relative salt resistance. Such a positive correlation indeed exists in soybean varieties when Cl^- exclusion is considered (Abel and MacKenzie 1964, Abel 1969, and Läuchli and Wieneke 1979). Also, *T. alexandrinum*, a major forage crop in the Middle East, India, and Pakistan, was found by Winter and Läuchli (1982) to be more salt resistant and a more efficient Na^+ and Cl^- excluder than the moderately salt resistant *T. pratense* and salt sensitive species *T. repens*.

Selection for improvement of crop salt tolerance may be practised at a number of stages during the plant life cycle. However, selection at the seedling stage would clearly be easier and more economical, provided it also conferred tolerance in the mature plant. Information about salt tolerance at the seedling stage and its relevance to crop improvement is limited (Ashraf 1986).

The objective of the work described in this chapter was to examine within and between species variability in salinity responses of two-week-old seedlings of seven species of

Trifolium, using the solution culture technique which has previously been shown to provide acceptable estimates of salinity tolerance in number of genera and species (Azhar and McNeilly 1988, Ashraf and McNeilly 1992, Al-Khatib *et al.* 1994, Kebebew and McNeilly 1995).

2.2. MATERIALS AND METHODS

2.2.1. Plant material

Seeds of seven *Trifolium* species used in this experiment were obtained from the following sources: *T. alexandrinum* from Egypt, Iran, and Pakistan, *T. resupinatum* from Iran, *T. repens* from Iran and United Kingdom, *T. subterraneum* from Australia and Iran, *T. pratense* from Iran and UK, *T. ambiguum* from Australia, Iran, and UK, *T. fragiferum* from Iran (see Table 2.2).

2.2.2. Methods of testing

Responses to salinity were examined using eight $EC_{(25)}$ levels in 1/2 strength nutrient solution (Appendix 2.1), the stock solution being that used by, and described in, Hewitt (1966). Salinity (EC dSm^{-1}) was imposed using NaCl and $CaCl_2$ in equal (1:1) amounts by weight (Ashraf and McNeilly 1991), the salinity levels being at $EC_{(25°C)}$ 4, 8, 10, 14, 18, 22 and 26 dSm^{-1}. Unamended nutrient solution was used as a control which had an EC 0.32 dSm^{-1}.

The experiments were carried out in a controlled environment growth room at a temperature of 24 ± 1°C with 16 hours of day length at a light intensity of 27 Wm^{-2}, and relative humidity of 75%.

Seed samples of all the accessions from *T. repens*, *T. alexandrinum*, *T. resupinatum*, *T. ambiguum*, *T. subterraneum*, *T. pratense*, and *T. fragiferum*, were surface sterilised in 0.5% (v/v) sodium hypochlorite for 2 minutes, and then washed three times with deionised water. The accessions from *T. subterraneum* were treated to overcome hard coat dormancy by puncturing the seed coat with a sterilised needle,

after which the seeds were sterilised using the same procedure as described above. Approximately forty five seeds of each accession were sown on a five layer deep raft of black alkathene beads floated on the surface of the appropriate salinised solution culture in 300 cm^3 plastic beakers.

The experiments were set up as completely randomised designs with three replicates. The beakers were placed in 54 × 54 cm plastic trays and covered by clear plastic chambers to reduce evaporation of the solutions, and to maintain as far as possible, constant humidity. Solutions were not changed or aerated because of the limited growth period, and the small amount of growth occurring.

After 14 days, root and shoot lengths of ten seedlings sampled randomly from each of the three replications of each accession were measured. Shoot measurements were made from the base of the hypocotyl to the top of the shoot, and root measurements were made from the base of the hypocotyl to the tip of the longest root.

Table 2.2. List of seeds used in experiment.

Species	Number of accessions	Seeds supplied from:
T. alexandrinum	10	Iran, Egypt, Pakistan
T. resupinatum	10	Iran
T. repens	10	Iran, UK
T. subterraneum	10	Iran, Australia
T. pratense	10	Iran, UK
T. ambiguum	10	Iran, UK, Australia
T. fragiferum	5	Iran

2.2.3. Data analysis

Absolute shoot and root length values of 30 seedlings of each accession of each *Trifolium* species in each concentration were subjected to nested analysis of variance The pattern of variability in the salinity responses of the 5 accessions of each species was also examined on the basis of the relative tolerance of seedlings within each accession at 7 EC dSm^{-1} levels. The relative tolerance was calculated using the formula:

$$\text{Relative tolerance} = \frac{\text{Individual seedling shoot / root length in saline solution}}{\text{Individual seedling shoot / root length in control solution}} \times 100$$

A non linear least squares method was used to assess and present the data for response of accessions to salinity based upon the methods of Van Genuchten and Hoffman (1984).

The model for the determination of (i) absolute yield in non-saline conditions (Y_m), (ii) threshold concentration at which yield starts to decrease (C_t), (iii) the concentration at which yield equals to zero (C_0), and (iv) the average root zone salinity during growing season (C).

The absolute yield curve is given from NOPT 5 by the following equation:

$$Y = \begin{cases} Y_m & 0 \leq C \leq C_t \\ Y_m - Y_m s\,(C - C_t) & C_t < C \leq C_0 \\ 0 & C < C_0 \end{cases}$$

Where: Y = absolute yield;
Y_m = absolute yield in under non saline conditions;

s = absolute value of the slope of the response function between C_t and C_0;

C = mean root zone salinity during the growing season;

C_t = threshold concentration at which yield starts to decrease;

C_0 = concentration at which yield equals zero.

The salinity beyond which the yield becomes zero (C_0) is given by:

$$C_0 = C_t + \frac{1}{s}$$

The sigmoid-form curve is given from NOPT 12 by the following equation:

$$Y = \frac{Y_m}{1 + \left(\frac{C}{C_{50}}\right)^p}$$

Where: p = an empirical constant that specifies the steepness of the curve.

C_{50} = salinity at which yield decreases by 50%;

The computer programme, 'SALT' (Van Genuchten 1983) was used to carry out these computations. This programme, applied to the shoot and root length of 14 day-old seedlings, provides estimates for C_t, C_0 and 's' (NOPT 5), and C_{50} and 'p' (NOPT 12), as well as the fitted response curve.

The C_t, and C_{50} values generated from the replicates were subjected to analyses of variance. The analyses of variance for within and between accessions of each species for absolute shoot and root length, and for C_t, and C_{50} values were made using the Statistical Analysis System (SAS) computer programme.

Maas and Hoffman (1977) proposed this technique which is based on a simple linear regression equation described in general terms as y = a - b(x), where y = absolute yield at EC 14 dSm^{-1}, x = absolute yield at control, and b = the rate of decline in yield from most tolerant to most sensitive species.

2.3. RESULTS

2.3.1. Between species variation

This experiment was designed to provide information about the general pattern of shoot and root growth inhibition of the seven species in response to increasing salinity in culture solution, with the secondary aim of determining at which EC level screening for increased salinity tolerance might be possible.

The results for relative tolerance from this experiment are presented in Figures 2.2 and 2.4, and the nested analyses of variance in Tables 2.3 and 2.5.

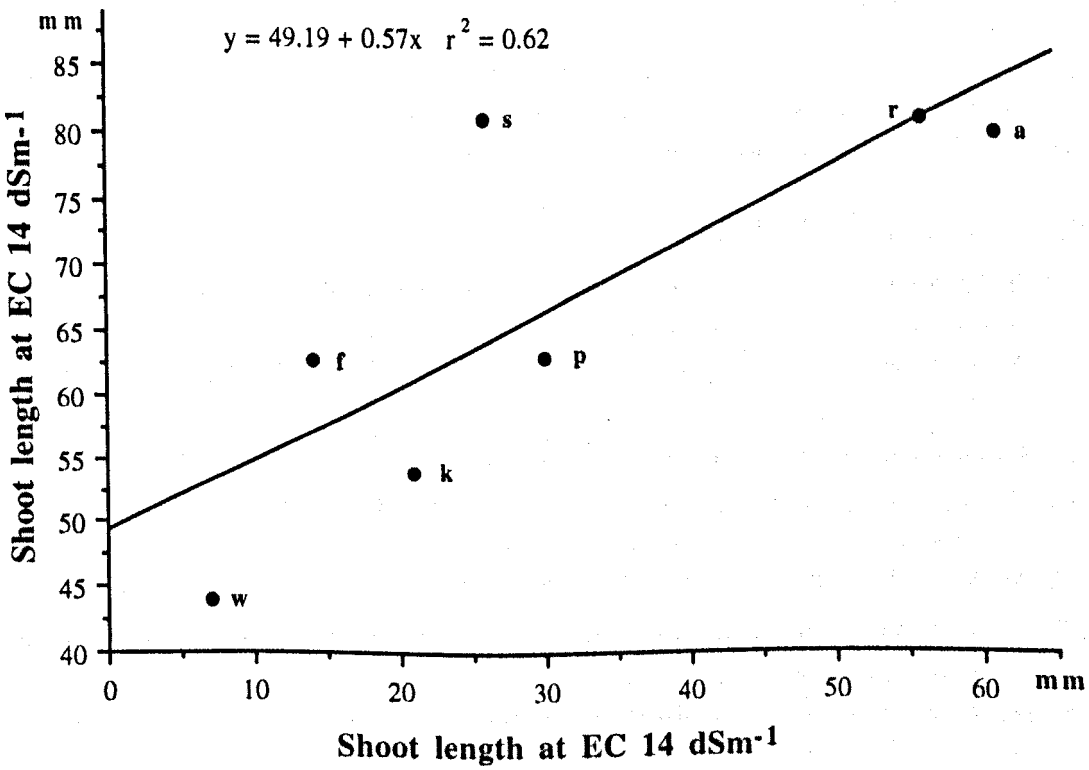
Increasing EC caused a decrease in mean shoot and root lengths in all the accessions in the 7 species examined ($P \leq 0.0001$), but the different accessions in the different species reacted to increasing NaCl + $CaCl_2$ concentration in different ways, the interaction between species × concentrations (SPP × EC), and accessions (species × concentrations) being significant at $P \leq 0.0001$ (Tables 2.3 and 2.5).

Examination of Tables 2.4 and 2.6, shows considerable increases of shoot and root lengths in most of accessions at low concentration, e.g. at EC 4 dSm^{-1}, relative root length value of *Trifolium resupinatum* was 136% of its control. It suggests that low concentration of NaCl + $CaCl_2$ might act as fertiliser for these accessions.

At EC 18 dSm^{-1} shoot and root growth was severely inhibited in all species except *T. alexandrinum* and *T. resupinatum* in which mean relative shoot lengths were 42% and 51% respectively, and relative root lengths were 52% and 45% respectively, (Tables 2.4 and 2.5). The inhibition of *T. repens* was particularly marked at EC 14 dSm^{-1}.

The species can be examined in more detail by simple linear regression analysis. To apply the technique to the data collected here mean shoot and root length of all seven *Trifolium* species at EC 14 and their control were calculated. The results are presented graphically in Figures 2.1 and 2.4, and show clear and substantial differences between species at this EC level. Thus, for example, at a concentration of EC 14, *T. alexandrinum* and *T. resupinatum* were the most and *T. repens* and *T. fragiferum* the least tolerant (Figures 2.1 and 2.4).

Figure 2.1. Comparison of 7 *Trifolium* species from absolute data at EC 14 dSm^{-1} and their control (Note: control axes begin at 40 mm).



a = *T. alexandrinum* r = *T. resupinatum* w = *T. repens* s = *T. subterranean*
p = *T. pratense* k = *T. ambiguum* f = *T. fragiferum*

2.3.1.1. Shoot length

From the analyses of variance of relative shoot length (Table 2.3 and Appendix 2.2) the interaction between species and concentration was highly significant, indicating that there were significant differences in species responses.

From the Duncan Multiple Range Test (DMRT) of relative shoot length of *T. ambiguum* at EC 4 dSm^{-1} with the 127% of control (Duncan's group A) was significantly ($P \leq 0.05$) greater than that of the other species, and shoot lengths of *T. fragiferum* and *T. repens* with relative values of 102% and 97% respectively were more affected by solution EC than the other species. The other species were intermediate. At EC 8 dSm^{-1} the shoot length of 5 species was in the same group (Group A), However percentage-wise they are higher than their respected controls except *T. repens* and *T. fragiferum* with shoot length approximately 80% of control (Group B). At EC 10 shoot lengths of *T. alexandrinum* and *T. pratense* were approximately the same as control (EC 0.32 dSm^{-1}), whereas the relative shoot value of *T. fragiferum* was in the lowest group at 56% of control. As shown in Table 2.4 and Appendix 2.4 the critical solution conductivity in this experiment was EC 14, shoot lengths of four of the species, namely *T. repens*, *T. subterraneum*, *T. ambiguum*, and *T. fragiferum*, being reduced by 50% of each control. At EC 18 not only is there inhibition of germination in most species, but also only a very small number of individuals in each of the five species grew except for *T. alexandrinum* and *T. resupinatum* which had relative shoot lengths of 42 and 51% respectively, these two species being the most tolerant species by a considerable margin. By contrast, *T. repens* (4%) was the most susceptible to this degree of salinity.

Table 2.3. Mean Squares and significance from nested analysis of variance of relative shoot length of 5 accessions of 7 *Trifolium* species grown in solution cultures of 8 EC levels for 14 days.

Sources	DF	Mean Squares	P > F
EC	7	72.29***	0.0001
ACC (SPP)	28	0.62***	0.0001
SPP	6	6.62***	0.0001
SPP × EC	42	0.76***	0.0001
ACC (SPP × EC)	196	0.08***	0.0001
Residual	8120	0.008	

DF = degree of freedom

EC = Solution conductivities dSm^{-1}

ACC = Accessions

SPP = Species

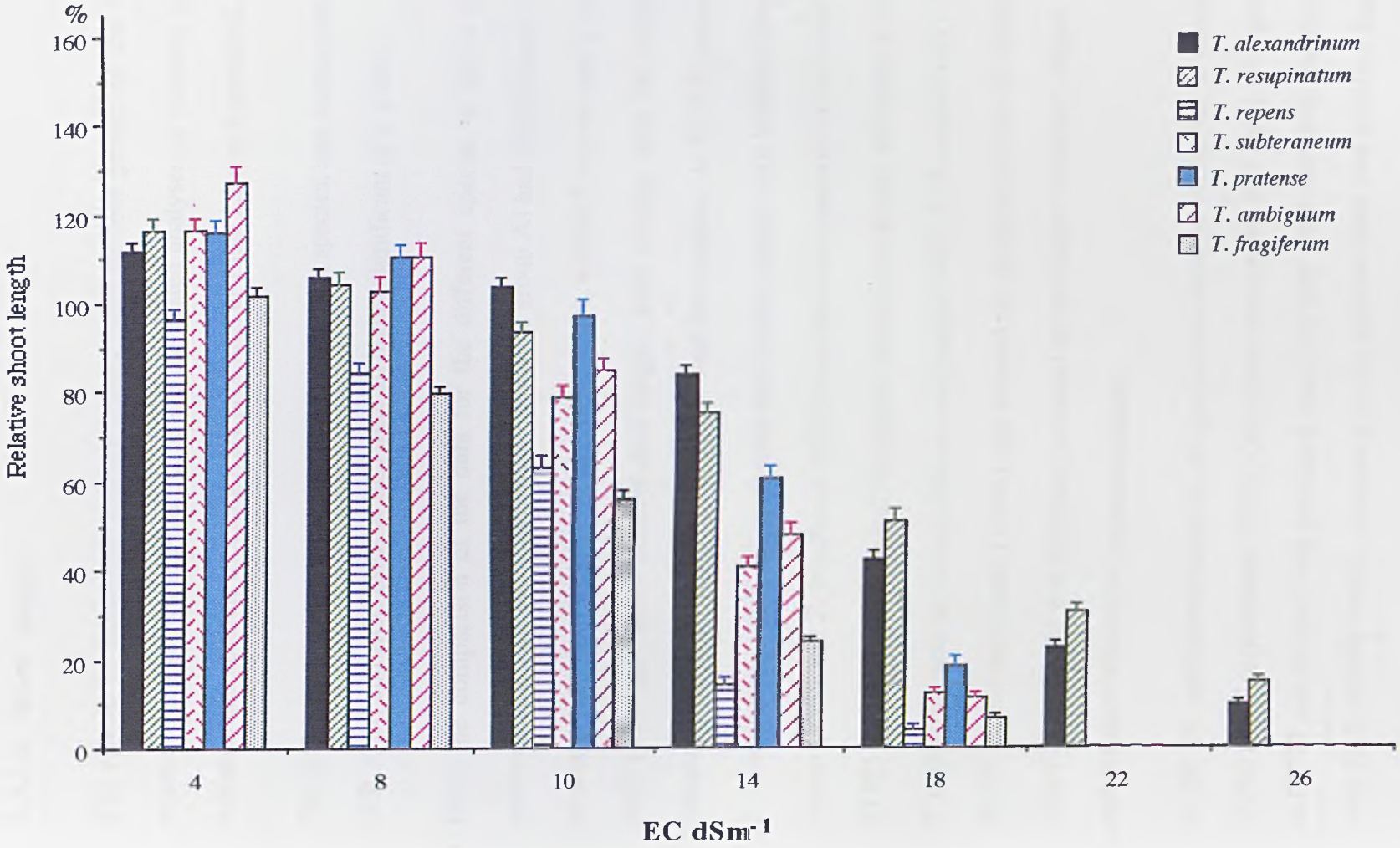
Table 2.4. Mean relative shoot lengths (%) of two-week-old seedlings of 5 accessions from 7 *Trifolium* species, grown in solution culture at 7 EC levels of salinity.

Species	EC 4 dSm^{-1}		EC 8 dSm^{-1}		EC 10 dSm^{-1}		EC 14 dSm^{-1}		EC 18 dSm^{-1}		EC 22 dSm^{-1}		EC 26 dSm^{-1}	
	Mean	D G[a]	Mean	D G	Mean	D G	Mean	D G	Mean	D G	Mean	D G	Mean	D G
T. alexandrinum	111.81	B	105.61	A	103.36	A	83.70	A	42.17	B	22.14	B	9.48	A
T. resupinatum	116.36	B	103.88	A	93.28	B	74.93	B	50.63	A	30.33	A	14.80	A
T. repens	96.52	C	84.33	B	63.16	D	14.27	G	4.22	F	0.00	C	0.00	C
T. subterraneum	116.11	B	102.54	A	78.58	C	40.47	E	11.98	D	0.00	C	0.00	C
T. pratense	115.67	B	109.98	A	97.15	A B	60.28	C	18.41	C	0.00	C	0.00	C
T. ambiguum	126.82	A	110.14	A	84.89	C	47.92	D	10.97	D E	0.00	C	0.00	C
T. fragiferum	101.68	C	79.76	B	56.04	E	23.70	F	6.78	E F	0.00	C	0.00	C

a. D G = Duncan's Multiple Range Test (DMRT) Grouping

In the high NaCl + $CaCl_2$ concentrations (EC 22 and 26 dSm^{-1}) there was no germination for any but the two most tolerant species, of which *T. resupinatum* was significantly ($P \leq 0.05$) more tolerant than that of *T. alexandrinum*. Based on the absolute and relative data for shoot lengths, *T. repens* and *T. fragiferum* are least tolerant species, *T. alexandrinum* and *T. resupinatum* are most tolerant, and the other three species are intermediate in response to NaCl + $CaCl_2$ concentrations (Table 2.4 and Figure 2.2).

Figure 2.2. Relative shoot mean values (persentage of control) of 5 accessions of 7 *Trifolium* species grown in solution cultures at 7 EC levels of salinity.



2.3.1.2. Root length

The effects of increasing solution conductivity on root growth of the 7 species examined are shown in Figure 2.3, and the nested analyses of variance of relative tolerance and absolute root length are presented in Table 2.5 and Appendix 2.3.

The differences between concentrations, accessions, species, and interactions between SPP × EC and ACC (SPP × EC) were all highly significant ($P \leq 0.0001$).

From the comparison of the data for the different species at EC 4 dSm^{-1}, *T. subterranean* with 147% of control (Duncan's group A) had significantly ($P \leq 0.05$) greater relative root length than the other species, and the *T. repens* and *T. fragiferum* with 113% and 109% relative root length, were smaller than the other species (Duncan's group D). The other 4 species were intermediate. At EC 8 *T. alexandrinum*, *T. resupinatum*, and *T. subterraneum* had similar relative root lengths (Group A). *T. repens* (91%) and *T. fragiferum* (91%) were the most sensitive to increased salinity. At EC 10 *T. alexandrinum* had the highest relative root length, and again *T. repens* and *T. fragiferum* had the lowest relative root lengths (64%, 61% respectively). At EC 14 dSm^{-1} *T. alexandrinum*, (113%) still showed no significant effect of salinity and *T. resupinatum* (84%) was only partly affected by this degree of salinity. Again *T. repens* was the most affected by increased salinity.

At EC 18, seed germination of all species was reduced except for *T. alexandrinum* (52%) and *T resupinatum* (45%). Only these two species had seeds with germinating at EC 22 and 26, and both produced roots, but they were markedly reduced at these very high salinity levels. According to the relative value and absolute data of root

Table 2.5. Mean Squares and significance from nested analysis of variance of relative root length of 5 accessions of 7 *Trifolium* species grown in solution cultures of 8 EC levels for 14 days.

Sources	DF	Mean Squares	P > F
EC	7	81.68***	0.0001
ACC (SPP)	28	0.92***	0.0001
SPP	6	8.92***	0.0001
SPP × EC	42	0.77***	0.0001
ACC (SPP × EC)	196	0.12***	0.0001
Residual	8120	0.01	

DF = degree of freedom

EC = Solution conductivities dSm^{-1}

ACC = Accessions

SPP = Species

Table 2.6. Mean relative root lengths (%) of two-week-old seedlings of 5 accessions from 7 *Trifolium* species, grown in solution culture at 7 EC levels of salinity.

Species	EC 4 dSm^{-1}		EC 8 dSm^{-1}		EC 10 dSm^{-1}		EC 14 dSm^{-1}		EC 18 dSm^{-1}		EC 22 dSm^{-1}		EC 26 dSm^{-1}	
	Mean	D G[a]	Mean	D G	Mean	D G	Mean	D G	Mean	D G	Mean	D G	Mean	D G
T. alexandrinum	124.71	C	127.31	A B	142.27	A	112.97	A	51.68	A	25.24	A	9.97	A
T. resupinatum	136.45	B	124.92	A B	113.88	B	84.02	B	45.10	B	21.44	B	11.26	A
T. repens	112.80	D	90.51	D	63.91	D	13.34	F	2.10	D	0.00	C	0.00	B
T. subterraneum	147.21	A	129.64	A	107.43	B	58.89	C	17.09	C	0.00	C	0.00	B
T. pratense	119.06	C D	111.33	C	89.10	C	53.03	C	14.68	C	0.00	C	0.00	B
T. ambiguum	126.18	B C	118.53	B C	86.62	C	43.69	D	7.29	D	0.00	C	0.00	B
T. fragiferum	109.08	D	90.60	D	61.01	D	24.50	E	5.52	D	0.00	C	0.00	B

a. D G = Duncan's Multiple Range Test (DMRT) Grouping

lengths, again *T. repens* and *T. fragiferum* were the least tolerant species, *T. alexandrinum* and *T. resupinatum* were the most tolerant, and the other three species were intermediate to response NaCl + $CaCl_2$ concentrations (Table 2.6 and Figures 2.3 and 2.4).

Overall, examination of the data presented in Tables 2.7a and b clearly shows considerable differences in species response to salinity. However their response to increasing salinity varies with the character used to assess that response. Thus if threshold salinity (C_t) is taken as an estimate of tolerance, *T. alexandrinum* and *T. resupinatum* were the most tolerant, *T. repens* and *T. fragiferum* were the least tolerant, and the three remaining species were intermediate. However, if C_{50} estimates are taken as estimates of tolerance, again *T. alexandrinum* and *T. resupinatum* were most tolerant, *T. pratense* was moderately tolerant, and the other species were the least tolerant.

Plate 2.1. Effect of varying levels of salinity on two-week-old seedlings shoots of *T. alexandrinum.*

Plate 2.2. Effect of varying levels of salinity on two-week-old seedlings shoots of *T. pratense.*

Figure 2.3. Relative root mean values (persentage of control) of 5 accessions of 7 *Trifolium* species grown in solution cultures at 7 EC levels of salinity.

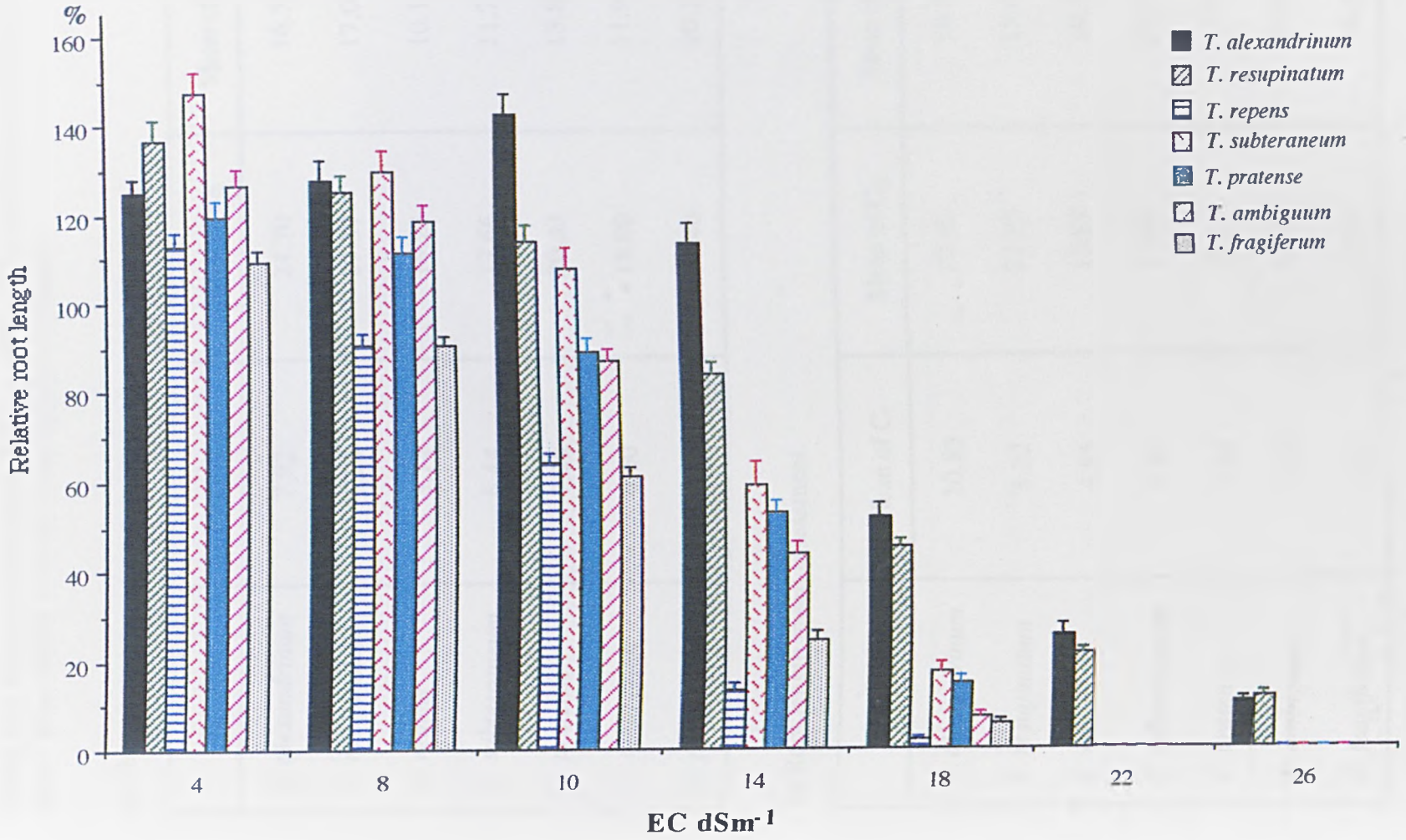


Table 2.7. Means of calculated $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12 of the seven species examined.

a) Shoot length based assessment.

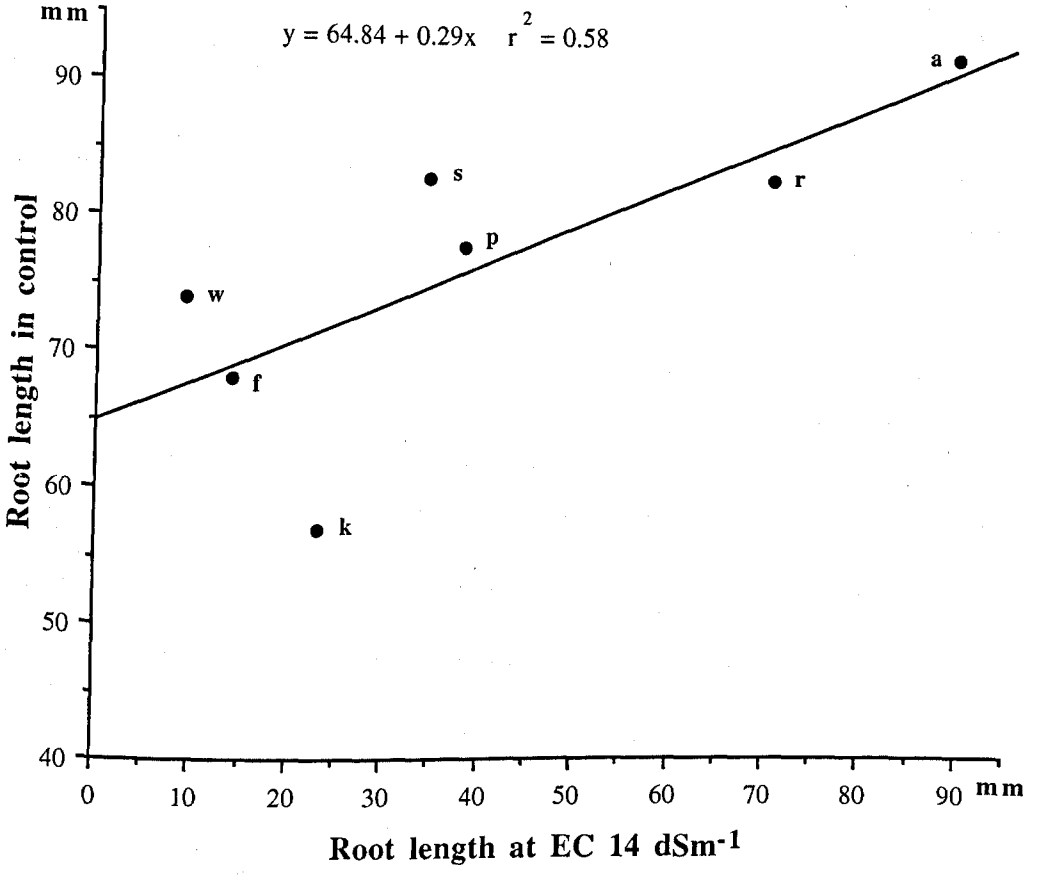
Species	Mean of C_t	Mean of C_0	Mean of C_{50}
T. alexandrinum	7.92	24.70	16.53
T. resupinatum	7.24	27.37	17.04
T. repens	3.74	16.23	10.17
T. subterraneum	6.44	17.66	11.78
T. pratense	7.29	20.33	13.80
T. ambiguum	6.60	18.00	11.98
T. fragiferum	3.81	17.46	10.37

b) Root length based assessment.

Species	Mean of C_t	Mean of C_0	Mean of C_{50}
T. alexandrinum	10.83	22.62	16.52
T. resupinatum	8.22	23.85	15.65
T. repens	4.85	15.87	10.25
T. subterraneum	7.55	17.81	12.23
T. pratense	7.64	19.10	13.05
T. ambiguum	7.80	17.75	12.51
T. fragiferum	4.81	16.63	10.58

Figure 2.4. Comparison of 7 *Trifolium* species from absolute data at EC 14 dSm^{-1} and their control (Note: control axes begin at 40 mm).

Root



a = *T. alexandrinum* r = *T. resupinatum* w = *T. repens* s = *T. subterranean*
p = *T. pratense* k = *T. ambiguum* f = *T. fragiferum*

2.3.2. Within species variation

This experiment was designed to provide information about the general pattern of shoot and root growth inhibition of the species in response to increasing NaCl + $CaCl_2$ concentrations in culture solution, with the aim of determining a sodium chloride + calcium chloride concentration at which screening for salinity tolerance might be possible.

The tables from analyses of variance, using absolute values for shoot and root measurement data from solution culture of the 7 species assessed, in 8 levels of NaCl + $CaCl_2$ concentrations, are presented in Appendices 2.6 to 2.12. The tables of means of three replications of C_{50} (concentration at which growth decreases by 50%) and C_t (threshold concentration at which growth starts to decrease) for 't Grouping' from analysis of variance procedure 't' test (LSD $P \leq 0.05$) for shoot and root length values derived from absolute shoot and root length data, were carried out separately for the 7 species and presented in Tables 2.15 to 2.18, and Appendices 2.13 to 2.19.

2.3.2.1. *T. alexandrinum*

Shoot and root length were significantly reduced ($P \leq 0.001$) with increasing NaCl + $CaCl_2$. Differences between accessions for shoot and root lengths were highly significant ($P \leq 0.001$). The interaction between accessions and different salt concentrations was also highly significant (accessions × EC interactions at $P \leq 0.001$), indicating that, as expected, increased salinity adversely affected shoot and root length to a different extent in different accessions (Appendix 2.6).

2.3.2.1.1. Shoot data

The evidence for inter-accession variability was most clearly seen at higher $EC_{(25)}$ levels. Thus, some accessions failed to produce shoots at $EC_{(25)}$ above 20 dSm^{-1}. e.g. Accession 6. Others e.g. Accession 3, grew up to $EC_{(25)}$ = 24 dSm^{-1}, whilst Accession 1 grew at $EC_{(25)}$ = 26 dSm^{-1} (Table 2.8 and Figure 2.5).

C_{50} estimates differed significantly ($P \leq 0.05$) between accessions of *T. alexandrinum*. Between accessions C_t estimates also differed significantly ($P \leq 0.05$) for shoots (Tables 2.15 and 2.17).

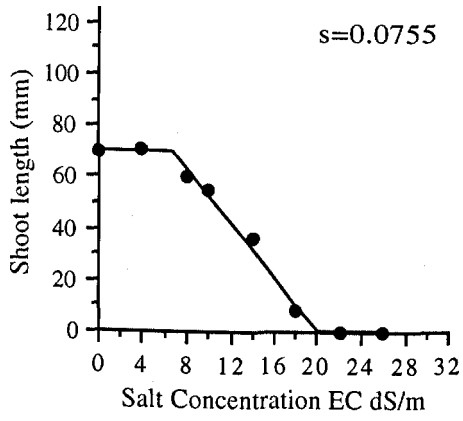
To facilitate data interpretation, the accessions have been ranked on simple arbitrary scales, from the most tolerant to the most susceptible of I, II and III for tolerance of the estimates of C_t, C_0, and C_{50} in Table 2.8.

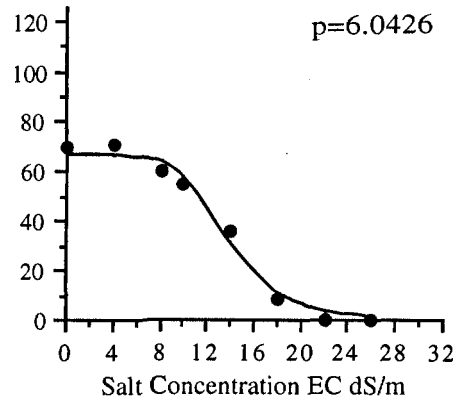
Examination of the data presented in Table 2.8, Figure 2.5 and Appendix 2.6 clearly show considerable differences in accession responses to salinity. If the threshold value (C_t) is the scale for salinity tolerance or sensitivity (Martinez *et al.* 1987), some accessions of *T. alexandrinum* such as Accession 1 is the most tolerant, Accession 9 is moderately tolerant, and Accession 10 being relatively sensitive. However if C_{50} estimates are taken as an estimate of tolerance, Accession 1 is relatively tolerant, Accessions 3, 8 and 9 are moderately tolerant, and Accession 6 is relatively sensitive (Tables 2.8, 2.15 and 2.17). However according to the C_0 estimation the Accessions 1 and 6 are the most and least tolerant respectively.

In Appendix 2.6. the analysis shows that there were no significant differences ($P > 0.05$) between the replicates in absolute shoot length.

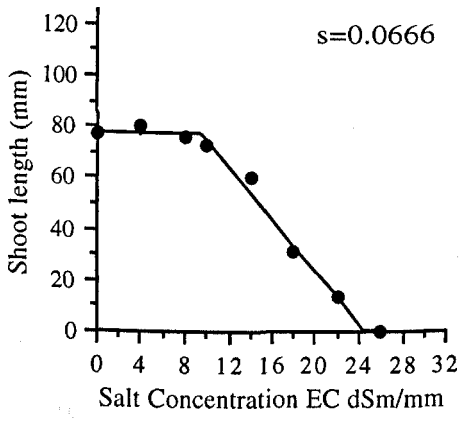
Figure 2.5. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. alexandrinum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle and bottom are least, middle, and most tolerant accessions respectively.

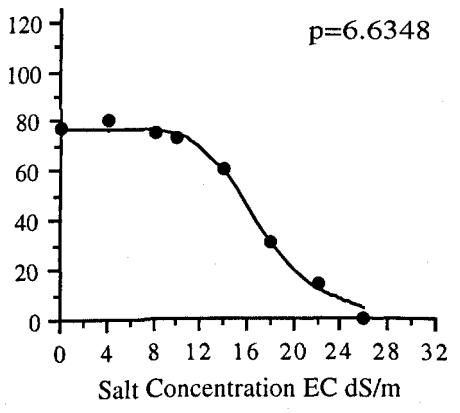
Accession 6.



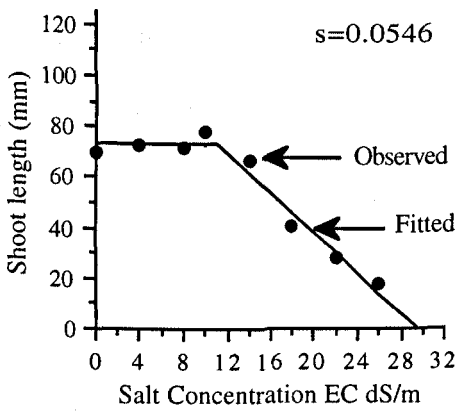


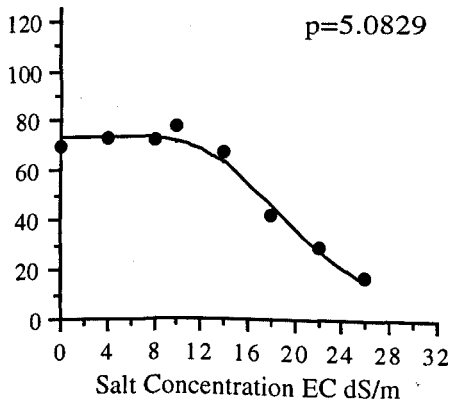
Accession 3.





Accession 1.





2.3.2.1.2. Root data

Accessions 6 and 10 failed to produce roots at $EC_{(25)}$ above 18 dSm^{-1}, whereas Accession 1 grew even at EC = 26 dSm^{-1}, with the estimated C_0 being 28.52 dSm^{-1}, and the other accessions fall in between (Table 2.8).

C_{50} estimates differed significantly ($P \leq 0.05$) between accessions of *T. alexandrinum*. Between accessions C_t estimates also differed significantly ($P \leq 0.05$) for roots (Tables 2.16 and 2.18).

Examination of data presented in Table 2.8, and Figure 2.6, clearly show considerable differences in accession responses to salinity. If the threshold value (C_t) is the scale for salinity tolerance or sensitivity, some accessions of *T. alexandrinum* such as 1 with C_t estimated value of 14 dSm^{-1} is the most tolerant, Accession 9 with C_t estimated value of 11.1 dSm^{-1} is moderately tolerant, and Accession 2 with C_t value of 5.3 is relatively sensitive. However, if C_{50} estimates are taken as an estimate of tolerance, Accession 1 is relatively tolerant, Accessions 3, 8, and 9 are moderately tolerant, whilst Accession 6 is relatively sensitive (Tables 2.8, 2.16 and 2.18). However, according to C_0 estimations, Accessions 1 and 6 are the most and least tolerant respectively, as well as for shoot based data.

There were significant differences ($P \leq 0.01$) between replicates for absolute root length data.

Table 2.8. *T. alexandrinum.* Calculated, for 10 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	11.20	I	29.50	I	19.73	I
2	9.81	I	25.20	II	17.66	II
3	9.53	I	24.54	II	16.93	II
4	6.73	III	24.51	II	15.36	III
5	7.10	III	23.18	III	15.04	III
6	6.83	III	20.08	III	13.74	III
7	7.13	III	23.11	III	15.72	III
8	6.80	III	26.81	I	17.67	II
9	8.03	II	27.29	I	16.91	II
10	6.01	III	22.76	III	16.49	III
Mean	7.92		24.70		16.53	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	14.00	I	28.52	I	20.97	I
2	5.34	III	22.81	II	13.38	III
3	11.63	I	22.29	II	16.50	II
4	11.23	I	22.46	II	16.56	II
5	7.39	III	23.44	II	16.41	II
6	10.97	II	18.48	III	14.44	III
7	10.51	II	19.81	III	15.03	III
8	13.31	I	22.67	II	17.73	II
9	11.01	II	26.78	I	18.36	II
10	12.95	I	18.93	III	15.81	III
Mean	10.83		22.62		16.52	

2.3.2.2. *T. resupinatum*

The absolute shoot and root lengths of the 10 accessions, and interactions between treatment and accessions (EC × ACC) were highly significant ($P \leq 0.001$), showing that the differences between inter-accession or within species is high (Appendix 2.7).

2.3.2.2.1. Shoot data

There were highly significant differences between 8 treatment levels ($P \leq 0.001$), indicating the negative correlation between concentrations and shoot lengths, after the shoot lengths threshold point had been reached at 4.20 $EC_{(25)}$ to 11.57 $EC_{(25)}$.

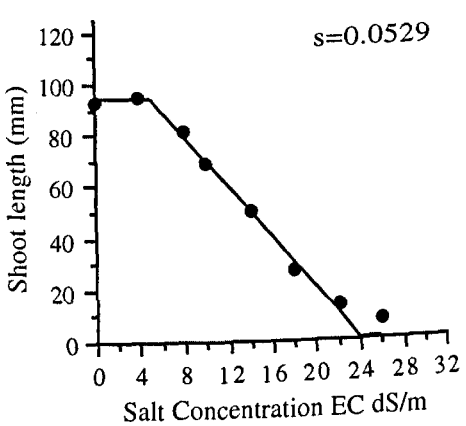
Accession responses were examined using 'SALT' programme (NOPT 5 and 12) as used by Van Genuchten (1983) and ranked in order (Table 2.9). To simplify shoot data presentation and interpretation, a sub sample of 3 accessions including the most tolerant (Accession 9 rank I), moderately tolerant (Accession 1 rank II), and the least tolerant (Accession 5 rank III), have been extracted from the 10 accessions of *T. resupinatum* species and given in (Figure 2.6).

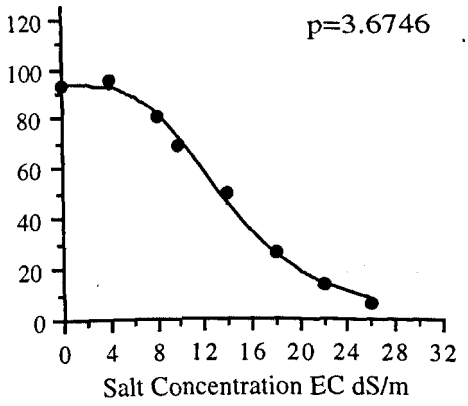
The shoot growth of the least tolerant accession, Accession 5, ceased above EC 22 dSm^{-1}, but the most tolerant, Accession 9, produced a shoot at EC 26 dSm^{-1}. Based upon the data in Tables 2.15 and 2.17 the mean of shoot lengths of Accession 9 in 't Grouping' of C_{50} (shoot mean C_{50} = 22.01 dSm^{-1}), and C_t (shoot C_t = 10.06 dSm^{-1}) was the most tolerant and Accession 5 was significantly less tolerant than the Accession 9. The other accessions were of intermediate tolerance.

There were no significant differences ($P > 0.05$) between replicates (Appendix 2.7).

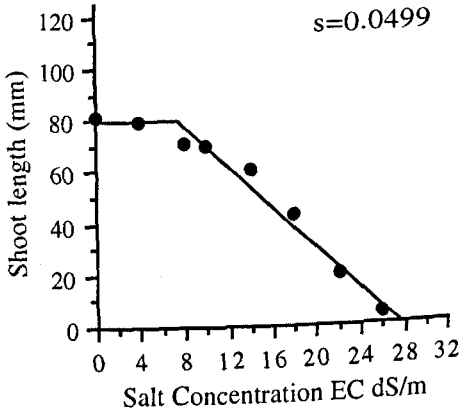
Figure 2.6. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. resupinatum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle and bottom are least, middle, and most tolerant accessions respectively.

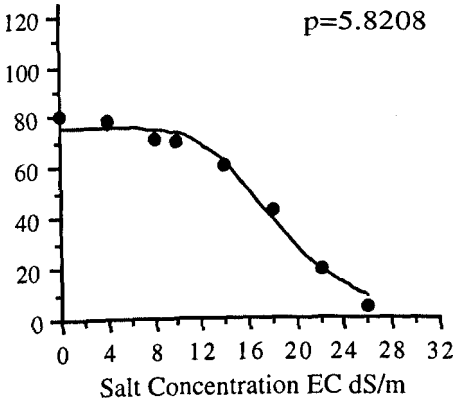
Accession 5.



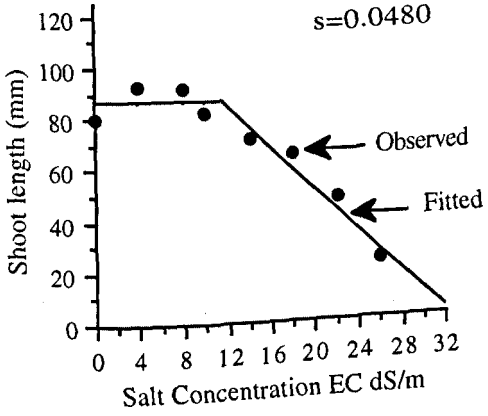


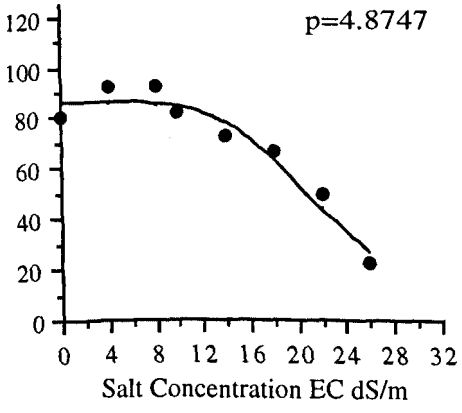
Accession 1





Accession 9.





2.3.2.2.2. Root data

There were highly significant differences between the 8 treatment levels ($P \leq 0.001$), with the clearly expected negative correlation between concentrations and root lengths, after the shoot length threshold points, had been reached, ranging from 1.3 dSm^{-1} to 11.6 dSm^{-1}.

In order to again simplify shoot data presentation and interpretation, a sub sample of 5 accessions including the Accession 9 the most tolerant, Accession 1 a moderately tolerant accession, and Accessions 5 the least tolerant, have been extracted from the 10 accessions of *T. resupinatum* species and are presented in Figure 2.6, and Table 2.9.

The root growth of the least tolerant accession, Accession 5, terminated above EC 22 dSm^{-1}, but the root of the most tolerant accession, Accession 9 grew at EC 26 dSm^{-1}, and from the data in Tables 2.16 and 2.18 it can be seen that the mean of shoot lengths of Accession 9 in 't Grouping' of C_{50} (root mean C_{50} = 19.31 dSm^{-1}) C_t (root C_t = 11.29 dSm^{-1}) is in the most tolerant group, but Accession 5 is significantly less tolerant than the Accession 9. The other accessions are of intermediate tolerance.

There were again no significant differences ($P > 0.05$) between replicates (Appendix 2.7).

Table 2.9. *T. resupinatum.* Calculated, for 10 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	7.53	II	27.59	II	18.19	II
2	6.50	III	29.31	II	18.27	II
3	9.57	I	28.14	II	18.59	II
4	5.16	III	24.50	III	13.82	III
5	5.12	III	24.02	III	13.84	III
6	6.56	III	26.88	II	16.08	III
7	4.20	III	27.66	II	15.30	III
8	7.30	II	29.06	II	18.24	II
9	11.57	I	32.42	I	22.01	I
10	8.88	II	24.08	III	16.10	III
Mean	7.24		27.37		17.04	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	11.55	I	19.71	III	15.49	II
2	5.87	II	24.43	II	15.05	II
3	11.29	I	27.31	I	18.86	I
4	9.41	I	19.99	III	14.27	II
5	1.25	III	22.95	II	11.37	III
6	10.07	I	23.67	II	16.67	I
7	4.00	III	26.55	I	14.76	II
8	7.99	II	26.21	I	16.57	II
9	11.06	I	28.38	I	19.30	I
10	9.68	I	19.29	III	14.20	II
Mean	8.22		23.85		15.65	

2.3.2.3. *T. repens*

There were highly significant differences ($P \leq 0.001$) between growth at different NaCl + $CaCl_2$ concentrations (EC dSm^{-1}), and between accessions, for absolute shoot and root length, and the interaction accessions × concentration (ACC × EC) of *T. repens* was also significant (Appendix 2.8).

2.3.2.3.1. Shoot data

C_{50} and C_t estimated for Accessions 4 and 9 were ranked I, these accessions being the most tolerant, but shoot growth of these two accessions however stopped (C_0) at EC 20 dSm^{-1}. The most sensitive accession was Accession 2 which ranked III for C_{50} and C_t, and growth ceased at EC 14 dSm^{-1}, again ranking III. The remaining accessions were intermediate. For five of the Accessions, 1, 3, 7, 8, and 10, ranking was not consistent across the three characters estimated, C_t, C_0, and C_{50}, and the higher values, indicating the greatest tolerance varied across these characters (Table 2.10, Figure 2.7, and Appendix 2.8).

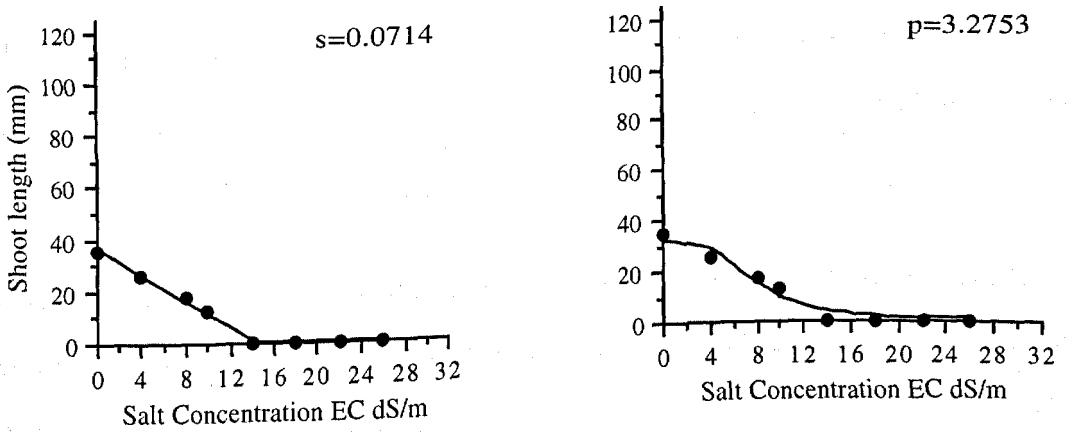
The threshold point of Accessions 2, 5, 6, 7, 8, and 10 were very low, being less than 4 dSm^{-1}. The threshold point of the 4 remaining accessions were $C_t > 7$ dSm^{-1} (Tables 2.15 to 2.18). There were no significant differences ($P > 0.05$) between three replicates.

2.3.2.3.2. Root data

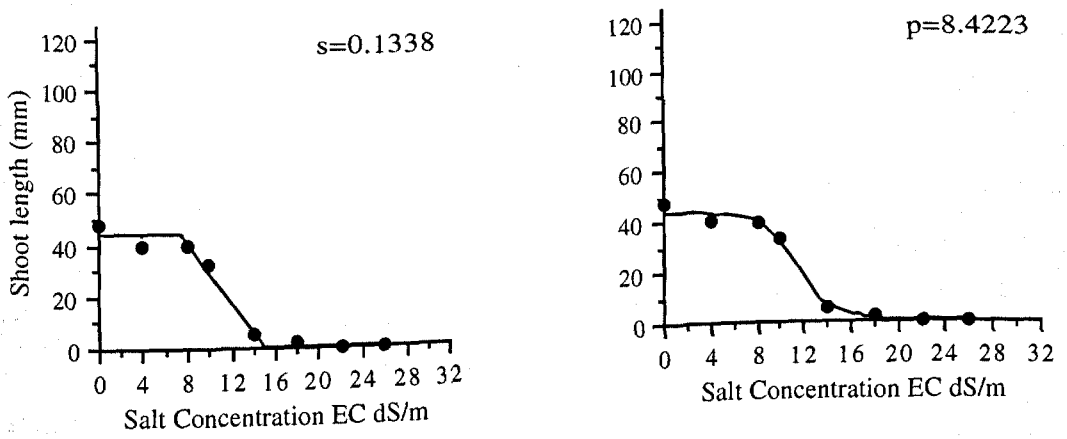
Accessions 4 and 9 were the most tolerant when compared using C_{50} and C_t, ranking I, and the root length of these two accessions ceased (C_0) at EC 18 dSm^{-1}. The most sensitive was Accession 2 which was ranked III for C_{50} and C_t, and the growth of this

Figure 2.7. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. repens* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle and bottom are least, middle, and most tolerant accessions respectively.

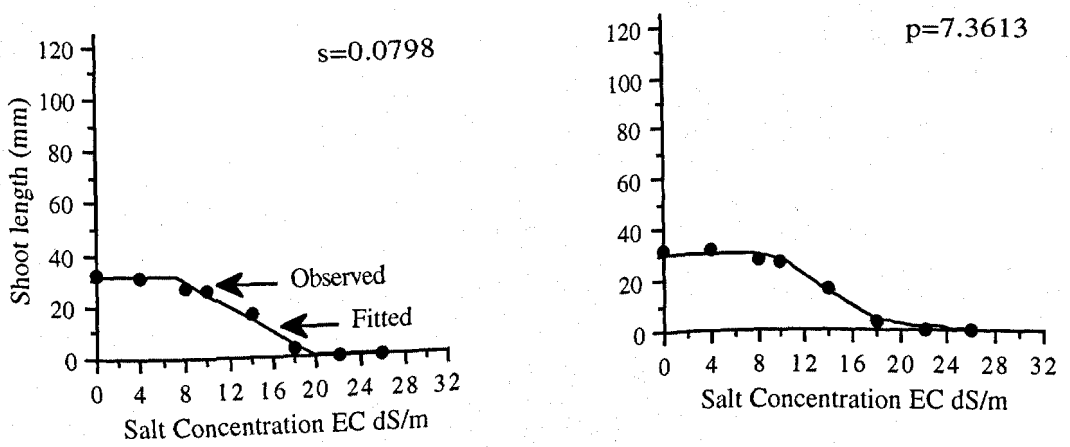
Accession 2.



Accession 1.



Accession 4.



accession ceased at EC 14 dSm^{-1}, interpreted as C_0 = 14.17. The other seven accessions were intermediate (Table 2.10 and Figure 2.7).

The threshold point of Accessions 2, 5, 6, 8, and 10 were less than 4 dSm^{-1}. The threshold point of the 5 remaining accessions was $C_t > 6$ dSm^{-1}, and statistically they are of the same group (Tables 2.15 to 2.18). There were significant differences ($P \leq 0.01$) between replicates of root length data.

As for the data for shoot growth, a number of accessions, in this case seven of them, do not have the same ranking across the three characters, C_t, C_0, and C_{50}, Accessions 3, 7, and 10 showing the most differences.

Table 2.10. ***T. repens.*** Calculated, for 10 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	7.55	I	15.02	III	11.23	II
2	0.40	III	14.40	III	7.70	III
3	7.11	I	14.67	III	10.59	II
4	7.25	I	19.77	I	14.02	I
5	2.41	III	14.88	III	8.96	III
6	2.32	III	14.36	III	9.08	III
7	2.97	II	14.76	III	9.49	III
8	0	III	16.73	II	7.87	III
9	7.42	I	19.02	I	12.80	I
10	0	III	18.73	I	9.94	II
Mean	3.74		16.23		10.17	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	8.74	I	14.66	II	11.35	I
2	3.27	III	14.17	III	8.91	III
3	6.62	I	14.49	III	10.18	II
4	6.79	I	18.29	I	12.46	I
5	3.27	III	15.06	II	9.59	II
6	3.85	II	13.35	III	7.57	III
7	6.54	I	12.15	III	9.31	II
8	1.22	III	19.25	I	10.55	II
9	6.73	I	18.19	I	12.05	I
10	1.45	III	19.07	I	10.54	II
Mean	4.85		15.87		10.25	

2.3.2.4. *T. subterraneum*

Analysis of variance of absolute data for shoot and root length of 10 accessions of the species *T. subterraneum* in response to increasing concentrations of NaCl + $CaCl_2$ are given in Appendix 2.9. There were highly significant differences ($P \leq 0.001$) between concentrations, and accessions, and the interaction concentrations and accessions (EC × ACC) was also significant. The analyses fro C_{50}, C_t and C_0 of NOPT 5 and NOPT 12 for shoot and root are presented in Tables 2.15 and 2.18.

2.3.2.4.1. Shoot data

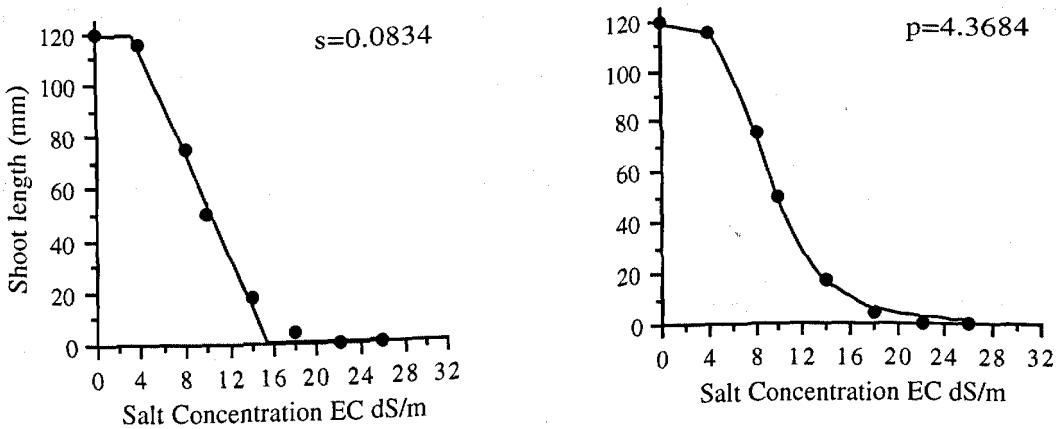
Increasing concentrations of salt caused reduction in shoot growth of all 10 *T. subterraneum* accessions. The toxicity of the higher salinities was reflected in their effects in reducing shoot growth. Comparing C_{50} means for shoot lengths of the 10 accessions, it can be clearly seen that Accession 9 was the most tolerant and Accession 3 was the least tolerant. The remaining 8 accessions show varying degrees of tolerance. According to the threshold concentration (C_t) value of shoot data the Accession 10 with threshold values of 9.4 dSm^{-1} was the most tolerant, and Accession 2 with a threshold value of 2.5 dSm^{-1} was the least tolerant accession. The 8 remaining accessions had threshold values which varied from 3.5 to 7.6 dSm^{-1}. (Tables 2.11, 2.15 and 2.18, and Figure 2.9).

2.3.2.4.2. Root data

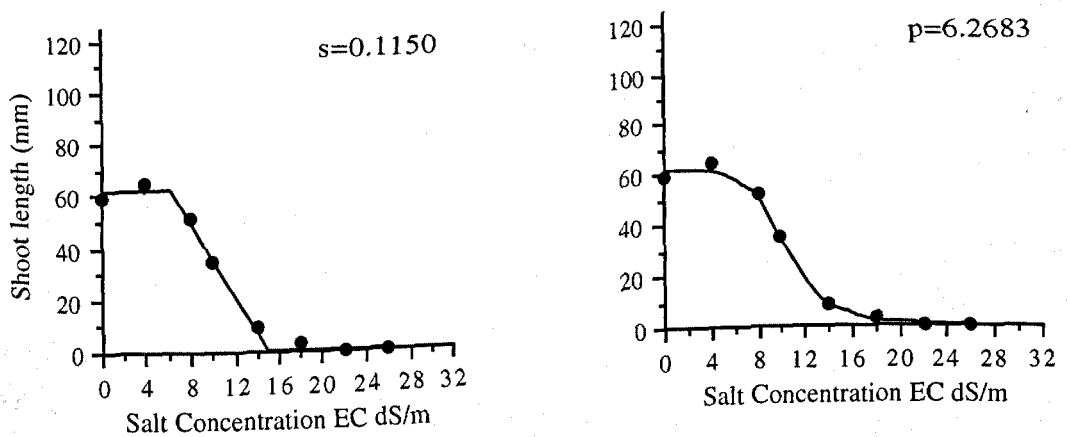
Increasing salt treatment causes reduction in root growth of all 10 accessions. The toxicity of higher salinity levels was reflected as its effect in reducing root lengths of all accessions. According to the C_{50} value estimates. Accession 1 was the most tolerant, with a C_{50} value of 14.7 dSm^{-1}. Accession 3 with the C_{50} value of 9.0 dSm^{-1}

Figure 2.8. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. subterraneum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle and bottom are least, middle, and most tolerant accessions respectively.

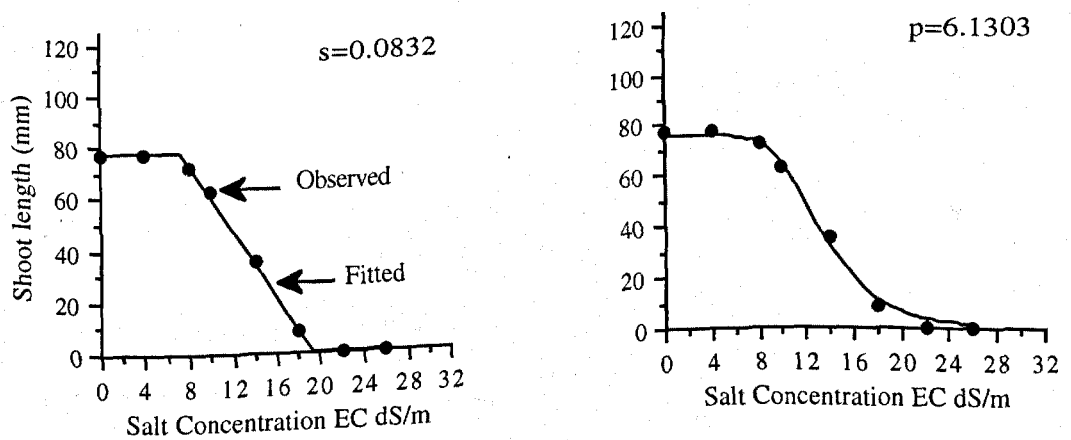
Accession 3.



Accession 6.



Accession 9.



was the least tolerant, the other accessions showing variable C_{50} values. According to the threshold concentration (C_t) estimate Accession 1 with a threshold value of 11.0 is again classed as the most tolerant, and again Accession 3 with the threshold value of 4.0 dSm^{-1} is the least tolerant, the remaining accessions having threshold values ranging from 5.6 to 7.8 dSm^{-1} (Tables 2.11, 2.16 and 2.18).

There were no significant differences ($P > 0.05$) between replicates.

Table 2.11. ***T. subterraneum.*** Calculated, for 10 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	6.10	II	19.80	I	12.56	I
2	2.48	III	20.31	I	11.10	II
3	3.46	III	15.45	III	9.15	III
4	7.38	I	16.76	III	12.25	I
5	7.75	I	18.40	II	12.00	I
6	6.35	II	15.05	III	10.33	III
7	6.58	II	18.91	I	12.30	I
8	7.61	I	16.78	III	12.11	I
9	7.35	I	19.37	I	13.28	I
10	9.38	I	15.78	III	12.70	I
Mean	6.44		17.66		11.78	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	11.04	I	20.07	I	14.68	I
2	7.94	II	19.81	I	12.92	I
3	3.98	III	15.01	III	9.02	III
4	7.05	II	18.20	II	12.40	II
5	8.14	II	18.56	I	12.85	I
6	7.05	II	15.05	III	10.40	III
7	7.69	II	19.38	I	13.56	I
8	7.45	II	16.98	II	12.23	II
9	7.77	II	19.27	I	12.97	I
10	7.37	II	15.73	III	11.31	II
Mean	7.55		17.81		12.23	

2.3.2.5. *T. pratense*

Sum of squares and significances from analyses of variance of absolute shoot and root data of the 10 accessions of *T. pratense* grown in 8 levels of NaCl + $CaCl_2$ are presented in Appendix 2.10.

2.3.2.5.1. Shoot data

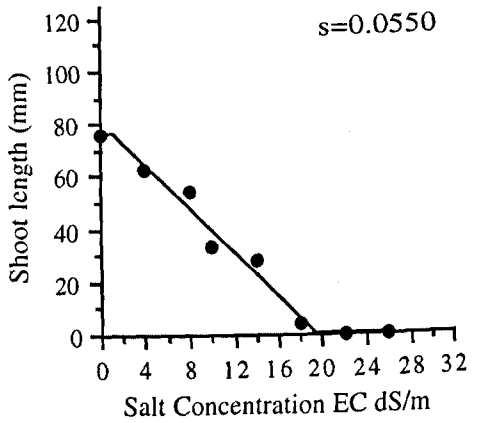
There were significant differences ($P \leq 0.05$) in the analysis of variance due to increased salinity treatments, accessions, and the between accession × concentrations interactions (ACC × EC) was also significant ($P \leq 0.05$). With an increasing concentration salt treatment, shoot and root length of 10 accessions of *T. pratense* decreased, accession differed over all concentrations and accessions responded differently to increased salinity.

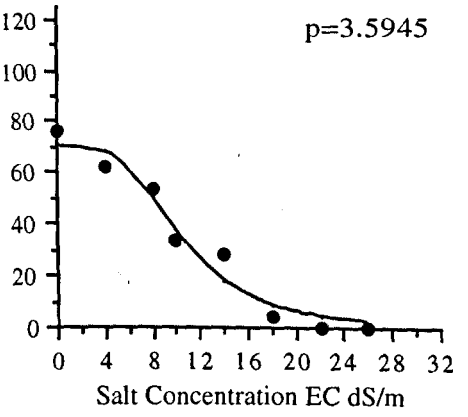
Analysis of variance of these data, and calculated mean values and arbitrary ranking of $EC_{(25)}$ values of C_t dSm^{-1} and C_0 dSm^{-1} from NOPT 5, and C_{50} dSm^{-1} from NOPT 12 of 10 accessions are presented in Tables 2.12, 2.15, and 2.17, respectively, and an Figure 2.9. As it shown in Table 2.15 from C_{50} the Accession 1 appears to be the most salinity tolerant and Accession 10 is the most sensitive. Based upon C_t data Accessions 1, 4, and 6 are superior with threshold concentrations of more than 9.5 dSm^{-1}. Accession 10 with a threshold estimated at 1.2 dSm^{-1} is the most sensitive to salinity, the remaining accessions with threshold concentrations varying from 5.9 to 7.8 dSm^{-1}.

There was a significant difference ($P \leq 0.05$) between replicates (Appendix 2.10).

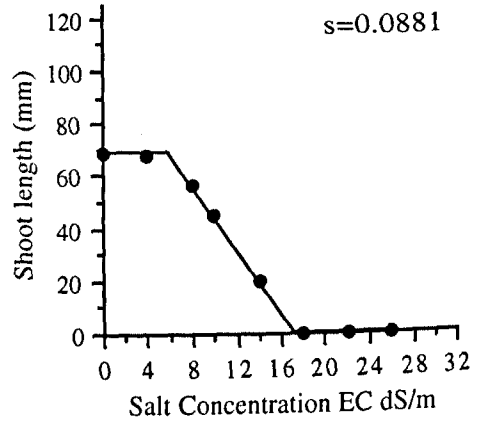
Figure 2.9. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. pratense* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle and bottom are least, middle, and most tolerant accessions respectively.

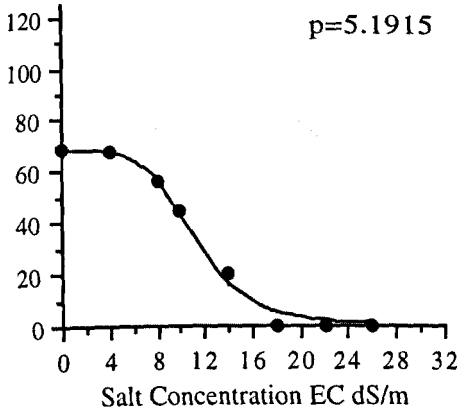
Accession 10.



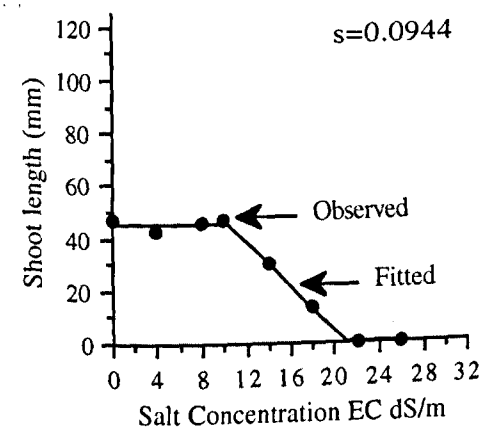


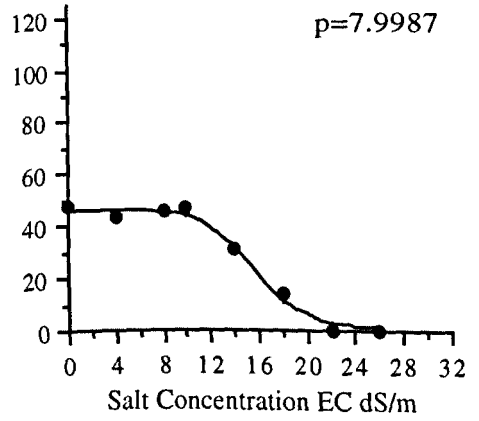
Accession 3.





Accession 1.





2.3.2.5.2. Root data

Increasing EC reduced root length of all 10 accessions significantly ($P \leq 0.001$), and the accessions × concentrations indication item was also significant ($P \leq 0.001$) indicating different responses to EC increase from different accessions.

Accession 1 was again the most tolerant based upon shoot data, and was ranked I for C_t and C_{50}. Accession 8 has the higher concentration which prevents root growth, C_0 = 22.08, and Accession 1 has an only slightly lower C_0 estimated value, 20.26. Threshold values C_t for the remaining accessions, are all considerably less, than that for Accession 1, and a similar picture is seen for C_{50} estimates. C_0 ranking is less diverse than the other two characters with 6 accessions having values over 19 (Table 2.12).

There were no significant differences between replicates (Appendix 2.10).

Table 2.12. *T. pratense.* Calculated, for 10 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	10.47	I	21.06	II	15.56	I
2	6.58	II	19.40	II	12.83	II
3	5.83	II	17.18	III	11.13	III
4	9.76	I	21.18	I	15.42	I
5	7.24	II	22.54	I	15.60	I
6	9.93	I	20.71	II	15.31	I
7	6.55	II	19.61	II	12.80	II
8	7.33	II	23.01	I	15.17	I
9	7.96	I	19.17	II	13.68	II
10	1.23	III	19.42	II	10.46	III
Mean	7.29		20.33		13.80	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	11.93	I	20.26	I	16.45	I
2	6.39	III	18.53	II	12.21	III
3	6.57	III	16.23	III	10.93	III
4	9.35	II	19.25	II	14.05	II
5	7.65	III	19.51	II	13.41	II
6	8.76	II	18.72	II	13.44	II
7	5.93	III	19.66	II	12.17	III
8	5.62	III	22.08	I	13.29	II
9	6.73	III	19.06	II	12.79	II
10	7.42	III	17.70	III	11.79	III
Mean	7.64		19.10		13.05	

2.3.2.6. *T. ambiguum*

Increasing NaCl + $CaCl_2$ reduced shoot and root growth of the 10 accessions of *T. ambiguum* examined, as in pervious species significantly ($P \leq 0.001$). The interaction accession × concentrations was also significant ($P \leq 0.001$), indicating significant different responses to EC increase from different accessions (Appendix 2.11).

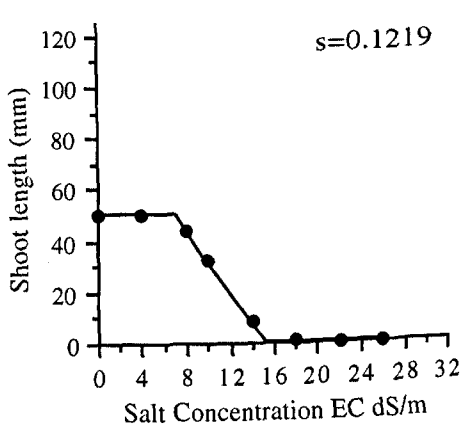
Transformation of the data whereby shoot and root lengths expressed as C_t, C_{50} and C_0 by the 'SALT' programme in NOPT 5 and NOPT 12 are shown in Tables 2.13, 2.15 to 2.16, and Figure 2.10.

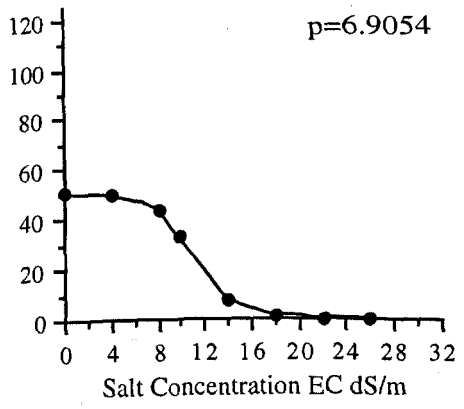
2.3.2.6.1. Shoot data

It is clear that shoot length decreased significantly ($P \leq 0.001$) in response to increasing salinity in all 10 accessions, decreasing occurred approximately at EC 8 dSm^{-1}. There were clear signs of variability in C_t estimates between individual accessions. Accessions 3 and 5 were the least sensitive and Accession 7 was the most sensitive to salinity. According to LSD estimates from analyses of variances, for value C_{50}, Accessions 2, 3, 4, 5, and 10 were in the same group whilst Accessions 1, 6, 8 and 9 were in the lowest group E in shoot lengths. This shows that there is significant genotypic variation ($P \leq 0.05$), but it is not very great.

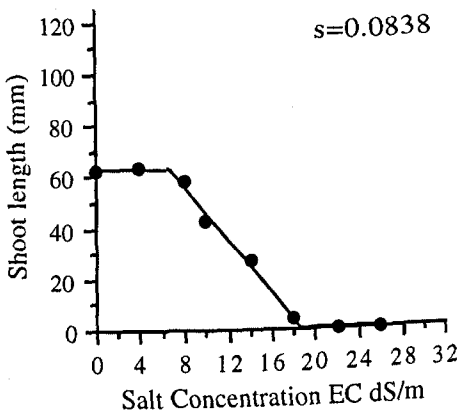
Figure 2.10. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. ambiguum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle, and bottom are least, middle and most tolerant accessions respectively.

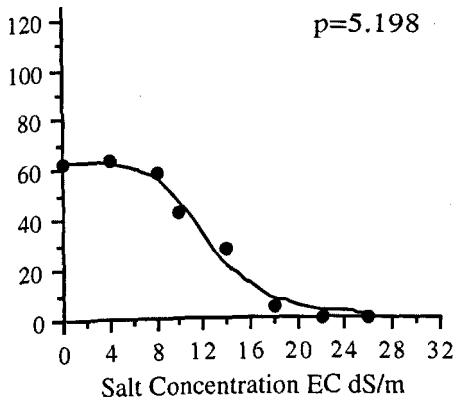
Accession 6



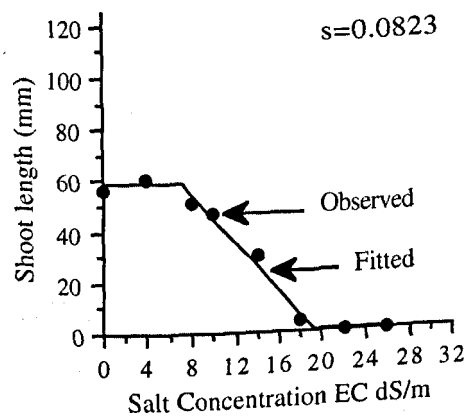


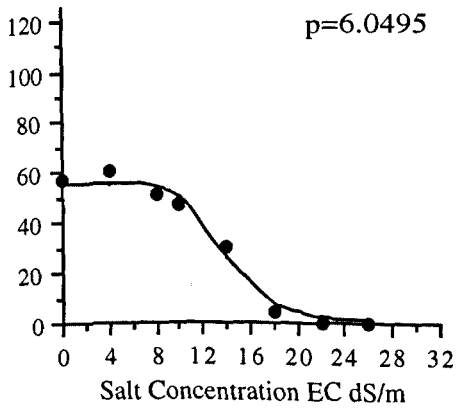
Accession 2.





Accession 3.





2.3.2.6.2. Root data

Significant differences occurred between accessions, and individual accessions had different responses to increasing NaCl + $CaCl_2$ concentrations ($P \leq 0.001$). From the LSD grouping of C_t value, Accession 8 is the most tolerant and Accession 6 is least tolerant, the other accessions having intermediate but similar rankings. From data for the C_{50} value, Accessions 7 is the most and Accession 6 is the least tolerant, and the remaining accessions are of intermediate tolerance (Table 2.16).

According to the three parameters (Ct, C_{50}, and C_0) for both shoot and root, Accession 7 is the most tolerant and Accession 6 the most sensitive (Table 2.13).

There were no significant differences between replicates.

Table 2.13. *T. ambiguum.* Calculated, for 10 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	6.08	III	15.82	III	10.49	III
2	6.77	II	18.71	I	12.29	II
3	7.59	I	19.36	I	13.72	I
4	6.79	II	18.82	I	12.38	II
5	7.58	I	18.80	I	12.91	I
6	7.00	I	15.20	III	10.85	III
7	5.47	III	19.68	I	11.94	II
8	6.88	II	15.72	III	11.49	III
9	5.92	III	18.13	II	11.29	III
10	6.33	II	19.76	I	12.45	II
Mean	6.60		18.00		11.98	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	7.72	I	14.89	III	10.27	III
2	8.32	I	18.30	I	13.12	I
3	8.02	I	18.37	I	13.12	I
4	8.00	I	18.55	I	12.93	II
5	6.81	II	18.59	I	12.18	II
6	3.60	III	17.34	II	10.90	III
7	9.28	I	19.03	I	14.20	I
8	9.52	I	14.61	III	11.99	II
9	8.07	I	18.52	I	12.75	II
10	8.61	I	19.28	I	13.66	I
Mean	7.80		17.75		12.51	

2.3.2.7. *T. fragiferum*

The analyses of variance of absolute shoot and root length of 5 accessions from *T. fragiferum* are presented in Appendix 2.12, and C_t, C_{50} and C_0 estimates calculated from means of shoot and root length with arbitrary ranking, and analyses of variance of C_t, C_{50} and C_0 for both shoot and root lengths are presented in Tables 2.14 and 2.15 to 2.18 respectively.

2.3.2.7.1. Shoot data

Increasing salinity caused a decrease in mean shoot length in the five accessions examined ($P \leq 0.001$). However, the different accessions reacted differently to increasing salinity in different ways, indicated by the accession × concentration interaction being significant at $P \leq 0.001$, (Appendix 2.12).

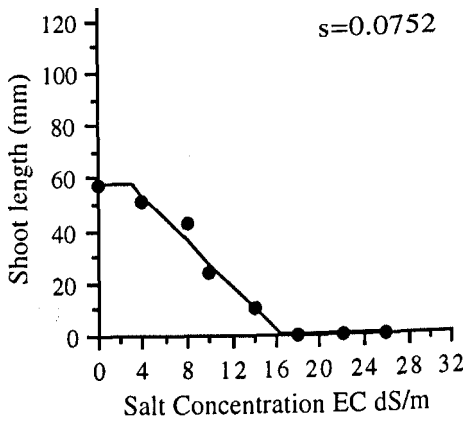
At EC 8 dSm^{-1} shoot growth was severely inhibited in all accessions except Accession 5 in which (Table 2.14), the shoot length threshold concentration (C_t) was 6.2. The C_{50} values from 5 accessions did not significantly differ ($P > 0.05$). Clearly in this small sample of this species there is not enough variability to allow selection based upon C_{50} value, and there is little variation in C_0 estimates (Tables 2.15 and 2.17, and Figure 2.11).

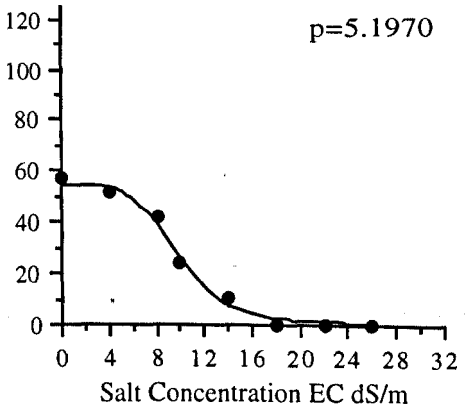
2.3.2.7.2. Root data

Reduction in root length due to increasing NaCl + $CaCl_2$ concentrations followed the same pattern as the shoot data, and the accession × concentration interaction item was also significant ($P \leq 0.001$).

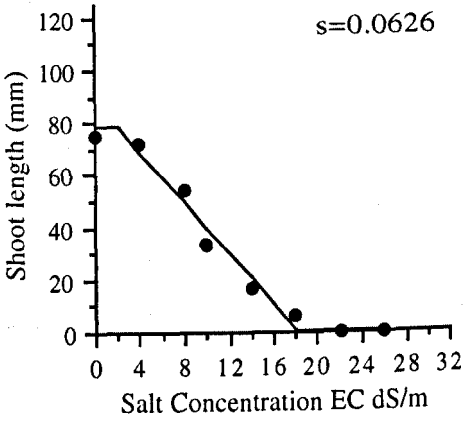
Figure 2.11. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. fragiferum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods). The top, middle and bottom are least, middle, and most tolerant accessions respectively.

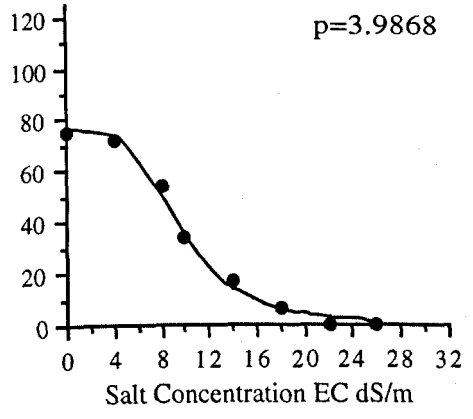
Accession 2.



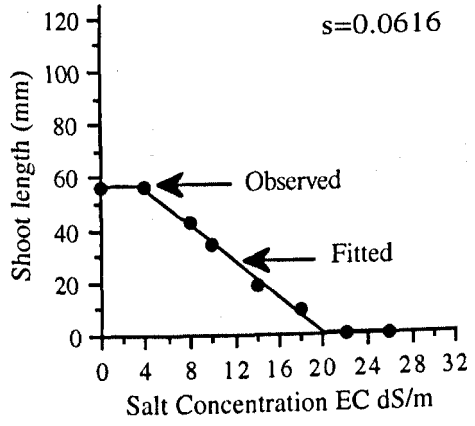


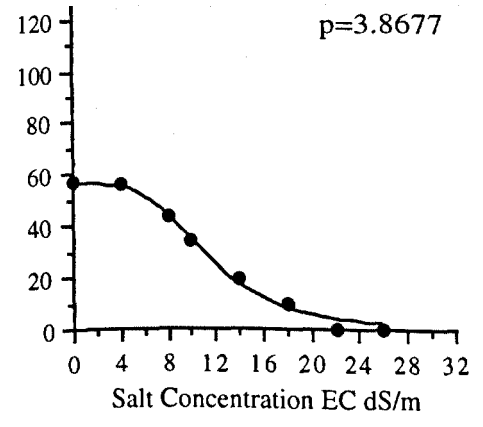
Accession 4.





Accession 1.





At EC 8 dSm^{-1} root growth was severely inhibited in all accessions except Accession 5 for which both root and shoot grew, (Table 2.14). Differences between values for the root length threshold concentration (C_t) estimated for the 5 accessions were not significant ($P > 0.05$) except for Accessions 2 and 5. Accessions 1 and 5 also had greater C_{50} estimates than the three other accessions as was also seen for shoot growth (Tables 2.14, 2.16, and 2.18).

Table 2.14. ***T. fragiferum.*** Calculated, for 5 accessions, $EC_{(25)}$ values of C_t and C_0 from NOPT 5, and C_{50} dSm^{-1} from NOPT 12.

Shoot length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	3.90	II	20.14	I	11.24	I
2	3.11	III	16.40	III	9.81	III
3	3.69	II	16.04	III	10.05	III
4	2.19	III	18.18	II	9.59	III
5	6.16	I	16.52	III	11.14	I
Mean	3.81		17.46		10.37	

Root length based assessment.

Acc. No.	C_t	Ranking	C_0	Ranking	C_{50}	Ranking
1	2.63	III	20.18	I	11.41	I
2	2.50	III	15.96	III	9.57	III
3	6.25	I	15.09	III	10.30	III
4	5.63	I	14.89	III	9.58	III
5	7.02	I	17.01	II	12.02	I
Mean	4.81		16.63		10.58	

Table 2.15. Means of C_{50} (Threshold concentration at which growth starts to decrease) and "t" Grouping, from Analysis of Variance procedure LSD (Least Significant Difference Alpha = 0.05 df = 18) for shoot length of 7 *Trifolium* species.

Species	LSD[1]	Mean[2] / t G[3]	Acc. 1	Acc. 2	Acc. 3	Acc. 4	Acc. 5	Acc. 6	Acc. 7	Acc. 8	Acc. 9	Acc. 10
T. alexandrinum	1.87	Mean	19.67	14.82	16.76	16.20	14.96	13.83	15.69	17.62	16.92	14.75
		"t" Grouping	A	E F	B C D	B C D E	D E F	F	C D E F	B	B C	E F
T. resupinatum	1.07	Mean	13.74	18.22	18.54	13.88	13.86	16.03	15.37	18.32	22.01	16.09
		"t" Grouping	D	B	B	D	D	C	C	B	A	C
T. repens	1.98	Mean	11.31	8.25	10.77	13.88	8.90	9.05	9.49	8.12	12.85	10.16
		"t" Grouping	B C	E F	C D	A	D E F	D E F	C D E F	F	A B	C D E
T. subterraneum	0.87	Mean	12.59	11.09	9.12	12.28	11.99	10.37	12.24	12.36	13.30	12.89
		"t" Grouping	A B C	D	E	B C	C	D	B C	B C	A	A B
T. pratense	1.64	Mean	15.65	12.89	11.19	14.94	15.59	15.33	12.77	15.06	13.35	10.47
		"t" Grouping	A	C	D E	A B	A	A	C D	A	B C	E
T. ambiguum	1.35	Mean	10.55	12.27	13.52	12.37	12.95	10.87	12.13	11.56	11.31	12.55
		"t" Grouping	E	A B C	A	A B C	A B	D E	B C D	C D E	C D E	A B C
T. fragiferum	0.88	Mean	11.29	9.74	10.03	9.58	11.10					
		"t" Grouping	A	B	B	B	A					

1. LSD = Least Significant Difference.
2. Mean = Means of three replications of Threshold concentration (EC dSm^{-1}) at which growth starts to decrease.
3. t G = t Tests grouping it means the same letter are not significantly different.

Table 2.16. Means of C_{50} (Threshold concentration at which growth starts to decrease) and "t" Grouping, from Analysis of Variance procedure LSD (Least Significant Difference Alpha = 0.05 df = 18) for root length of 7 *Trifolium* species.

Species	LSD[1]	Mean[2] / t G[3]	Acc. 1	Acc. 2	Acc. 3	Acc. 4	Acc. 5	Acc. 6	Acc. 7	Acc. 8	Acc. 9	Acc. 10
T. alexandrinum	1.87	Mean	20.93	13.36	16.42	16.52	16.42	14.44	15.03	17.69	18.33	15.79
		"t" Grouping	A	F	C	C	C	E	D E	B	B	C D
T. resupinatum	1.07	Mean	15.42	15.21	18.83	14.23	14.13	16.47	14.58	16.58	19.31	14.12
		"t" Grouping	B C	B C	A	C	C	B	C	B	A	C
T. repens	1.98	Mean	11.31	10.03	8.94	12.46	9.05	8.78	9.27	10.28	12.07	10.46
		"t" Grouping	A B	B C D E	D E	A	D E	E	C D E	B C D	A	B C
T. subterraneum	0.87	Mean	14.36	12.89	9.03	12.41	13.05	10.44	13.65	12.31	12.89	11.34
		"t" Grouping	A	B C	F	B C D	B C	E	A B	C D	B C	D E
T. pratense	1.64	Mean	15.76	12.39	10.90	14.03	13.30	13.32	12.75	13.42	12.72	11.59
		"t" Grouping	A	C D	E	B	B C	B C	B C D	B C	B C D	D E
T. ambiguum	1.35	Mean	10.46	13.13	12 78	12.87	12.13	10.92	13.88	12.04	12.76	13.59
		"t" Grouping	E	A B C	B C D	A B C D	C D	E	A	D	B C D	A B
T. fragiferum	0.88	Mean	11.53	9.48	10.23	9.53	12.02					
		"t" Grouping	A	B	B	B	A					

Table 2.17. Means of C_t (Threshold concentration at which growth starts to decrease) and "t" Grouping, from Analysis of Variance procedure LSD (Least Significant Difference Alpha = 0.05 df = 18) for shoot length of 7 *Trifolium* species.

Species	LSD[1]	Mean[2] / t G[3]	Acc. 1	Acc. 2	Acc. 3	Acc. 4	Acc. 5	Acc. 6	Acc. 7	Acc. 8	Acc. 9	Acc. 10
T. alexandrinum	1.87	Mean	11.77	7.71	9.68	10.33	7.02	6.82	7.59	6.91	8.34	5.08
		"t" Grouping	A	B C	A B	A B	B C	B C	B C	B C	A B C	C
T. resupinatum	1.07	Mean	4.50	6.43	10.28	5.10	4.96	6.50	4.71	7.06	10.06	7.68
		"t" Grouping	C	B C	A	B C	B C	B C	B C	B C	A	A B
T. repens	1.98	Mean	8.12	0.93	6.51	7.28	2.04	3.41	3.31	0.12	7.53	0.00
		"t" Grouping	A	C	A B	A	C	B C	BC	C	A	C
T. subterraneum	0.87	Mean	6.05	3.11	3.42	7.21	7.55	6.32	6.51	8.53	7.49	9.37
		"t" Grouping	D	E	E	B C D	B C	C D	C D	A B	B C D	A
T. pratense	1.64	Mean	10.57	6.56	5.89	10.27	7.36	10.16	6.54	8.46	7.78	1.25
		"t" Grouping	A	C	C	A B	C	A B	C	A B C	B C	D
T. ambiguum	1.35	Mean	5.92	6.74	7.59	6.80	7.58	6.92	5.57	6.91	6.18	6.32
		"t" Grouping	B C	A B C	A	A B C	A	A B C	C	A B C	A B C	A B C
T. fragiferum	0.88	Mean	3.75	3.14	4.44	2.77	5.67					
		"t" Grouping	AB	AB	AB	B	A					

Table 2.18. Means of C_t (Threshold concentration at which growth starts to decrease) and "t" Grouping, from Analysis of Variance procedure LSD (Least Significant Difference Alpha = 0.05 df = 18) for root length of 7 *Trifolium* species.

Species	LSD[1]	Mean[2] / t G[3]	Acc. 1	Acc. 2	Acc. 3	Acc. 4	Acc. 5	Acc. 6	Acc. 7	Acc. 8	Acc. 9	Acc. 10
T. alexandrinum	1.87	Mean	14.05	5.27	10.91	11.25	8.54	10.79	10.85	13.20	11.28	12.87
		"t" Grouping	A	D	B C	A B C	C	B C	B C	A B	A B C	A B
T. resupinatum	1.07	Mean	11.31	5.80	10.73	8.69	7.90	9.74	5.13	9.03	11.29	9.34
		"t" Grouping	A	C D	A	A B	B C	A B	D	A B	A	A B
T. repens	1.98	Mean	8.68	4.36	5.68	6.82	3.26	4.82	5.13	2.70	6.91	1.20
		"t" Grouping	A	B C D	A B C	A B	B C D	A B C D	A B C	C D	A B	D
T. subterraneum	0.87	Mean	10.56	7.14	4.00	7.14	8.51	5.59	7.81	7.78	7.38	7.48
		"t" Grouping	A	B C	D	B C	A B	C D	B	B	B C	B C
T. pratense	1.64	Mean	11.22	6.24	6.61	9.66	7.37	8.32	6.47	5.85	6.72	5.53
		"t" Grouping	A	C	B C	A B	B C	A B C	C	C	B C	C
T. ambiguum	1.35	Mean	7.70	8.90	7.90	8.06	6.38	5.05	8.27	9.75	8.74	8.61
		"t" Grouping	A B	A	A B	A B	B C	C	A B	A	A	A B
T. fragiferum	0.88	Mean	3.03	2.10	5.22	5.63	6.89					
		"t" Grouping	A B	B	A B	A B	A					

2.4. DISCUSSION

Whether selection based upon the root measurement technique produces individuals which are salt tolerant in all physiological attributes or not has been questioned (Rozema and Visser 1981) and is not considered in this chapter. It has nonetheless been demonstrated that shoot and root growth is one manifestation of salt tolerance.

A major aim of this work was to identify tolerance to NaCl + $CaCl_2$. The requirements for adaptation to salinity stress include resistance to Na^+ and Cl^- toxicity, and to osmotic stress, which are different from the requirement of resistance to specific ion toxicity as in the case of metal tolerance (Rozema and Visser 1981).

The potential for selecting and breeding plants for tolerance to high levels of mineral elements in the soil must be similarly dependent (Humphreys and Bradshaw 1976). Various researchers have reported that the responses of species to selection for salinity tolerance and for heavy metal tolerance are clearly different. Selection for metal tolerance has shown that metal tolerant individuals (Gartside and McNeilly 1974, Walley *et al.* 1974, Symeonidis *et al.* 1985) occur only in a limited number of species, those which naturally occur on metal contaminated soils and have metal tolerant populations. By contrast it is clearly possible to detect accessions which have increased shoot and root growth in NaCl + $CaCl_2$ solutions which markedly reduce, or prevent root growth i.e. salinity tolerance. Two species, *Trifolium alexandrinum* and *T resupinatum*, have this potential to survive increased salinity in their rooting medium (Babagolzadeh and McNeilly 1995).

It is not surprising that selection readily produces individuals with increased NaCl tolerance in *T. alexandrinum*, because the plant has previously been shown to be

tolerant of moderate salinity in solution culture experiments (Winter and Läuchli 1982), and ion analysis in previous studies with *T. alexandrinum*, indicated the presence of some mechanisms controlling salt distribution within the plant (Winter and Läuchli opp. cit. Winter 1982a,b; Winter and Preston 1982).

Mean relative data of five accessions (Tables 2.4 and 2.6), *T. alexandrinum* and *T. resupinatum* showed markedly high tolerance to salinity especially at the higher concentrations. For example, at EC 14 shoot length of *T. alexandrinum*, *T. resupinatum*, and *T. pratense* were more than 50% of the control shoot length, whereas the other species in this level of salinity had more than 50% shoot length reductions. At EC 18 dSm^{-1}, the relative shoot length values were *T. resupinatum* (51%) > *T. alexandrinum* (42%) > *T. pratense* (18%) > *T. subterraneum* (12%) > *T. ambiguum* (117%) > *T. fragiferum* (7%) > *T. repens* (4%), this is due mainly because this degree of salinity caused a 50% reduction in shoot length of *T. resupinatum*, whereas in *T. fragiferum* and *T. repens* only a few seeds germinated, all having very short roots.

Greater salt tolerance in the annual species *T. resupinatum* than in a salt sensitive cultivar of *T. repens* was reported by Zawadzka (1976). The data presented here and that of many researchers, show that *T. repens* is normally a salt sensitive pasture legume (Gauch and Magistad 1943, Zawadzka 1976, Smith and McComb 1981, Gonzalez-Murua *et al.* 1985), and Läuchli (1984) categorised it as a moderately salt sensitive species. However Ab-Shukor *et al.* (1988), found moderate salt tolerance in a natural population of *T. repens* growing on the margins of the salt marshes in South Wales. It may occur in the margin of salt marshes in other areas, assuming that genes for salinity tolerance are present elsewhere in this species. The *T. repens* material from

the site examined by Ab-Shukor, had been collected to be grown in order to provide seed for NaCl tolerance assessment, but it did not flower, and no seed was obtained from it. This could have been a very useful source of tolerance in that species.

Carlsson (1994) reported that it has been possible to cultivate forage legumes such as *T. fragiferum* that tolerate saline conditions. However, according to the data presented here (from 5 accessions), it is one of the least tolerant species. There was no germination above EC 18 dSm^{-1} of any of the five accessions examined. In spite of the fact that all the accessions examined here had very poor growth in salinity levels above 10 dSm^{-1}, it must be possible, from Carlssons (1994) observations that tolerant material within this species also can be detected if sufficient numbers of accessions can be assessed as has been shown for *T. repens*. Clearly a sample of five accessions is extremely small, and therefore consistent poor growth of them in saline conditions may be exploited. *T. fragiferum* performs here as overall a salinity sensitive species, with salinity similar to that of *T. repens*.

Taken overall, it is not possible to say that species which contain variability in salt tolerance in their normal populations are always to be found exploiting saline habitats by the formation of salt tolerant ecotypes. Their ecology may be such that they never come in content with saline soils even though they carry resistance gene(s), or they simply do not have the necessary salinity tolerance at the species level. Although variability in salt tolerance is important in allowing species to exploit saline habitats, other factors are also involved which may prevent this exploitation. What is interesting is that the data from these few accessions of each species suggests that variability in salt tolerance is widely available in plant species, and these could therefore be selected artificially for exploitation in plant breeding programmes.

T. alexandrinum has been reported as moderately tolerant, and according to Läuchli (1984), *T. pratense* is intermediate in salt tolerance compared to other legumes. This is confirmed here, in that growth occurred at EC values up to 18 dSm^{-1}, but thereafter neither shoots or roots appeared at the greater EC values (Appendices 2.4 and 2.5, Figures 2.2 and 2.3). *T. pratense* is clearly more sensitive to salt than *T. alexandrinum* as was shown by Winter and Läuchli (1982), and Babagolzadeh and McNeilly (1995), and the *T. resupinatum* accessions examined here.

However, of the 65 accessions assessed in this experiment for C_t, C_0 and C_{50} values, Accession 1 from *T. alexandrinum*, Accessions 3 and 9 from *T. resupinatum*, Accessions 1, 4 and 9 from *T. repens*, Accession 10 from *T. subterraneum*, Accession 1 from *T. pratense*, Accessions 3 and 5 from *T. ambiguum*, and Accession 5 from *T. fragiferum* have relatively high shoot length threshold values (C_t) (Table 2.17). These accessions were the most salt tolerant among the 65 accessions of seven species. However, mean relative tolerance of some accessions e.g. Accession 1 from *T. subterraneum*, Accession 5 from *T. pratense*, and Accession 1 from *T. fragiferum* were not confirmed as being tolerant by the C_t parameter. For most of the accessions their degree of tolerance based upon C_0 and C_{50} estimates provide good estimates of tolerance, especially in the least tolerant group with very low threshold estimates. No general consistency for tolerance was found between these three parameters (C_t, C_0 and C_{50}), for example Accessions 1 from *T. repens* with the highest threshold value had relatively poor values for C_0 and C_{50}. In a study examining 24 barley cultivars at the stage of germination, Martinez-Cob *et al.* (1987) assessed tolerance using threshold salinity (C_t) as a reference parameter, and showed that some cultivars, Mari, Viva, and Kim, proved to be highly tolerant to salinity. From this work C_t was

suggested that the most appropriate parameter for determining salinity tolerance. Kebebew and McNeilly (1995) also found that estimates of C_t, C_0 and C_{50} differed considerably both between accessions, and within the three species pearl millet, finger millet and tef at the germination stages. They also concluded that C_t is a useful parameter for assessing salinity tolerance for three species. In study of response function from relative grain yield of wheat varieties Steppuhn *et al.* (1996) showed that a sigmoid-shape function described responses much better than either the two-piece linear or the exponential response function. The responses of the 7 species examined to increasing salinity, presented in Figures 2.5 to 2.11, and Appendices 2.12 to 2.19 using the NOPT 12 model, very clearly follow sigmoid patterns. A total 65 accessions have thus be examined. From both *Trifolium* and wheat data it can be concluded that C_{50} (from NOPT 12) estimates could be useful to compare salinity responses because they provide useful assessment of salinity tolerance.

Of all the seven species assessed in this chapter for C_t, C_0, and C_{50} estimated values, only *T. repens* and *T. fragiferum* have low threshold values (C_t) (Table 2.7), and they are clearly the most salt sensitive among the species examined. For most of the species examined C_{50} and C_0 estimates provide good estimates of tolerance, especially where the plants being assessed fall in the salt sensitive group with low threshold (C_t) estimates. No general consistency for tolerance was found between these three estimates. For example, *T. pratense* has a higher shoot threshold value than *T. resupinatum*, but low values for C_{50} and C_0. In a similar study of 24 barley cultivars at the germination stage, Martinez-Cob *et al.* (1987), assessed their tolerance based upon their threshold estimates (C_t) and identified three cultivars as highly tolerant to salinity, and they concluded that C_t was the most appropriate parameter for

determining salt tolerance. Estimates of C_t, C_{50} and C_0 also differed considerably both between and within pearl millet, finger millet, and tef (Kebebew and McNeilly 1995) and they also concluded that C_t was a useful parameter for assessing salinity tolerance. Using the two parameters C_{50} and C_0 for shoot and root lengths, the species responses to salinity were: *T. resupinatum* > *T. alexandrinum* > *T. pratense* > *T. ambiguum* > *T. subterraneum* > *T. fragiferum* > *T. repens*, from the most to the least tolerant.

Salt tolerance of plants not only varies considerably among and within species but also depends on the cultural conditions under which the crop is grown. Many plant, soil, water, and other environmental factors interact to influence the salt tolerance of a plant. Consequently, plant responses to known salt concentrations cannot be predicted on an absolute basis. Nevertheless, plants can be compared on a relative basis to provide general salt-tolerance guidelines (Maas 1986). Plant tolerance to salinity is usually assessed in one of three ways,

i - plant survivability on saline soils,

ii - yield or absolute plant growth, on saline soil,

iii - the relative growth or yield of the plant on saline soil as compared with that on non-saline soil.

Although plant survival on saline soils is also of interest to physiologists and ecologists, this criterion has limited use for agriculturists because it often bears little relation to yield reduction within commercially acceptable limits (Maas 1986).

Measurements of root length of plants at the seedling stage in saline solution culture have been successfully used in discriminating between salt tolerance and salt

sensitivity in wild grass species of which populations could be found growing on salt marsh soils, and also an adjacent non-salt affected pasture soils (Hannon and Bradshaw 1968, and Ashraf *et al.* 1986a). The technique was subsequently adapted and slightly modified to assess salt tolerance in seedlings of several crop species with the aim of using it to select within them for improved salt tolerance (e.g. sorghum, Azhar and McNeilly 1987; maize, Ashraf and McNeilly 1989, and Mishra *et al.* 1994; lentil, Ashraf and Waheed 1990; grasses, Jafari 1990; pearl millet, Ashraf and McNeilly 1992; lucerne, McNeilly 1990 and Al-Khatib *et al.* 1993; millet, Kebebew and McNeilly 1995; and cotton, Lin *et al.* 1997). Previous experiences of selecting for improved salinity tolerance in these species have shown that selection at the seedling stage based upon 14-day-old seedling root length differences is effective in producing individuals which are predominantly more tolerant at all subsequent growth stages than unselected control individuals (Ashraf and McNeilly 1992, Al-Khatib *et al.* 1994).

There is no consistent relationship between the root length of seedlings grown in non-saline solution, and the same material grown in saline solutions, and similar information has been reported for different crop species. It has been suggested by some seedlings having long roots in non-saline conditions will also have long roots in saline conditions. Data for the 14-day-old seedlings examined here show no consistent relationships between these two parameters, and this has been reported for several other species e.g. sorghum (Azhar and McNeilly 1987), millets (Kebebew and McNeilly 1995), and mayz Rao (1997). As shown in Figures 2.2 and 2.3, at low concentration of NaCl + $CaCl_2$, the relative root lengths of all seven species were greater than control, especially for *T. alexandrinum* even at EC 14 dSm^{-1}. In contrast the shoot length of some species significantly decreased as salinity increased. Based

upon this evidence, measurement of shoot length is more suitable and reliable than root lengths of *Trifolium* species, for estimating salinity tolerance/susceptibility.

The tolerance of all seven species with different accessions examined here showed considerable variability between and within species. Overall the data obtained suggests that Accession 1 from *T. alexandrinum* (relatively tolerant species), Accessions 3 and 9 from *T. resupinatum* (relatively the most tolerant species), and the relatively tolerant Accession 1 from *T. repens* (relatively the least tolerant species) might be useful for selection and breeding to establish forage vegetation that can be exploited in saline regions.

The data that have been obtained are encouraging from a plant breeding point of view, since it appears that sufficient variation exists both between and within species, to make selection for improved salt tolerance feasible, provided a link can be established between tolerance of seedlings (as estimated in this chapter), and tolerance of those selected seedlings at adult plant stage, which is examined in Chapter 3.

CHAPTER THREE

VARIATION SALINITY RESPONSE AT THE WHOLE PLANT LEVEL; FOUR *Trifolium* SPECIES

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CHAPTER THREE

VARIATION IN SALINITY RESPONSE AT THE WHOLE PLANT LEVEL; FOUR *Trifolium* SPECIES

3.1. INTRODUCTION

The need to produce crops with enhanced tolerance to high salt levels in soils has been emphasised by many workers (Dewey 1962, Shannon 1979, Epstein 1985, and Zahran *et al.* 1992). The success of this approach depends upon the occurrence of appropriate genetic variability in crop species, and the ability to exploit such variability using appropriately convenient selection techniques.

It would be greatly advantageous if selection at one stage of the life cycle for increased tolerance would confer this tolerance upon the remaining stages. It has been argued however, (Shannon 1979), that plant response to salinity varies at different stages of the life cycle. In some species, tolerance exhibited at all growth stages are highly correlated, as for example in *Medicago sativa* L. (Noble 1983). In others this may not be the case.

Variability in salinity tolerance within species has been reported with increasing frequency in recent years; however, the choice of criteria by which tolerance is measured has not been consistent among investigators (Rush and Epstein 1976, Shannon 1978, Pasternak *et al.* 1979, Norlyn 1980, Shannon *et al.* 1983). Plant response to salinity may change with age, and salt stress increases as the plant continues to grow and transpire under saline conditions due to increased salt load on the root as along time gradient (Blum 1988). A plant response, and consequently its

effective salt tolerance, is influenced by its ontogenic stage, and salinity effects have been shown to vary depending upon the growth stage at the time of stress (West and Taylor 1981, Smith *et al.* 1981, Pearson *et al.* 1966, Ashraf and Waheed 1990), suggesting that the ability of plants to respond to salt stress depends upon those genes that are functioning at the developmental stage during which the stress occurs (Shannon 1985).

Lack of sufficient information with respect to the effect of plant age on salinity resistance (Blum 1988) does not allow the development of general speculation. Therefore, for varietal improvement in salinity tolerance to be effective, availability of information about the effects of salinity on all phases of plant growth are essential, and equally worthwhile would be identification of the life stage most susceptible to the effect of salinity in order to maximise selection efficiency (Azhar and McNeilly 1989).

Many reviewers of salinity studies who have examined the sometimes considerable differences in the reactions of some crop species to salinity during germination and during early seedling growth stage, agreed that plants become increasingly more tolerant with maturation (Bernstein 1964b, Kaddah 1963, Pearson *et al.* 1966, Pearson and Bernstein 1959, Meiri and Shalhevet 1973). The desired adaptive response would therefore be one in which plants become more resistant with age, either as a function of age *per se*, or as a function of hardening (Blum 1988). The early seedling stage of growth is the most salt sensitive for most crops (Ayers *et al.* 1952, and Maas *et al.* 1986). For example, barley, wheat, and maize were shown to be more sensitive during emergence and the seedling stage than during germination or adult stages (Ayers *et al.* 1952, Ayers 1953, Greenway 1965, Kaddah and Ghowail 1964, Maas *et al.* 1983), whereas sugar beet and sunflower (Ayers and Hayward 1948,

Francois and Bernstein 1964), tomato (Pasternak *et al.* 1979), lucerne (Ayer and Hayward 1948, Forsberg 1953, Chang 1961), sorghum (Lall and Sakhare 1970, Taylor *et al.* 1975, Ratanadilok *et al.* 1978, and Maas *et al.* 1986) were observed to be relatively sensitive to salinity at the germination or seedling stage. It is possible therefore that the salt tolerance of species at germination and as seedlings are not closely correlated with that of later stages (Maas 1986, Ashraf and McNeilly 1989).

For a successful breeding program, it is crucial to consider a single selection criterion rather than a set of characters (Ashraf 1994). Root growth does not appear to be a useful criterion in some leguminous and other dicotyledonous species, although Ab-Shukor *et al.* (1988) successfully distinguished salt tolerant and salt sensitive natural populations of *Trifolium repens* on the basis of root growth tests. Ashraf *et al.* (1986a,b) and Al-Khatib *et al.* (1994) successfully used shoot length measurements for assessment of tolerance. In other crops, selection based on whole plant performance provided a means of selecting for salt tolerance such as in rice (Akbar *et al.* 1986), millet (Ashraf and McNeilly 1987), and wheat (Ashraf and McNeilly 1988). Such procedures may well be applicable to other crop species.

This Chapter describes an experimental assessment of the effects of various levels of salinity applied at adult stages, on the shoot and root fresh and dry weight of seven accessions of four *Trifolium* species.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Seed grown plants from of seven accessions, 3 from *T. resupinatum*, 2 from *T. alexandrinum*, and 1 each from *T. ambiguum* and *T. pratense* were used in this experiment.

3.2.2. Methods

Seeds of the seven accessions were germinated in washed river sand and irrigated with 1/2 strength nutrient solution (following Rorison in Hewitt 1966) in 20 × 30 cm plastic trays. Three one-week-old seedlings of similar size were transplanted into standard 17.5 cm plastic pots containing washed river sand. Drainage holes in the base of the pots were covered using polyester cheesecloth at the base of the pots for the prevention of sand loss. To retain leachate solution, plastic saucers were placed under each pot. Before starting the experiment, the sand in each pot was washed three times per day for three days with tap water, and subsequently with 1/2 strength nutrient solution for two days.

Three one-week-old similar sized seedlings from each accession were transplanted into separate pots, with three replications. The seedlings were irrigated every two days for two weeks with normal Rorison nutrient solution.

After two weeks, the salinity stress treatments were commenced. Nutrient solution with NaCl + $CaCl_2$ in equal amounts by weight (Ashraf and McNeilly 1991) was added. NaCl + $CaCl_2$ was added at a concentration of $EC_{(25°C)}$ = 2 dSm^{-1} initially, and increased by 4 every 7 day until the appropriate salinity treatment was reached.

The salinity levels were $EC_{(25°C)}$ 4, 10, 14, 18, 22, and 26 dSm^{-1}. The controls were nutrient solution without NaCl + $CaCl_2$, with an $EC_{(25°C)}$ = 0.32 dSm^{-1}. Every week the salinity levels of each treatment were checked using an electrical conductivity meter, to minimise any fluctuations in salinity concentration.

The experiment was set up as a completely randomised design with six treatments, three replicates, and seven accessions. The daytime temperature of the glasshouse ranged from 20°C to 35°C; night temperatures, ranged from 12°C to 26°C. Relative humidity varied from 40 to 80% during the day and remained at approximately 70% during the night. A day length of 16 hours in the glasshouse was provided from natural day light, supplemented using 400 W mercury vapour lamps.

The three month old plants were harvested 4 weeks after the appropriate salt treatment was reached. Plant roots were removed carefully from the sand, shoots and roots were separated, and washed with de-ionised water. Plant shoot and root materials were weighed as a fresh weight, and then dried at 65°C for three days and re-weighed.

3.2.3. Statistical analysis

Data for both absolute and relative values for the seven *Trifolium* accessions for shoot/ root fresh and dry weight measured were subjected to analysis of variance and the relative values expressed as percentages of values for plants grown as controls. Correlation coefficients between six different measurements (shoot and root lengths of two-week-old seedlings, and shoot /root fresh and dry weight of adult plant) for four species were calculated using the formula below (Russel 1996):

$$\text{cov}_{xy} = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{n-1}$$

cov = covariance

x and y = means of variables

n = number of variables

$$\text{Correlation coefficient} = r = \frac{\text{cov}_{xy}}{s_x\, s_x}$$

s_x = standard deviation of x

s_y = standard deviation of x

3.3. RESULTS. Species comparison

Duncan Multiple Range Test (DMRT) using a Statistical Analysis System (SAS). Salinity responses of the accessions has been appraised on the basis of absolute growth (Dewy 1960) and their relative salt tolerance (Maas 1985).

3.3.1. Salt Tolerance: Absolute data

The information obtained from the analyses of variance of absolute values for the mean of shoot fresh and dry weight, and the mean of root fresh and dry weight are presented in Tables 3.1a to 3.2b.

The results of these analyses show that increasing salt concentrations significantly ($P \leq 0.0001$) reduce mean shoot fresh weight, and that the species differed significantly ($P \leq 0.0001$) in shoot fresh weight. The species shoot fresh weight also differed significantly in different NaCl + $CaCl_2$ concentrations (interaction SPP × EC significant at $P \leq 0.05$) for shoot fresh and dry weight, and also for absolute root dry

weight, significant at $P \leq 0.001$. There were no significant interactions for root fresh weight values between species and concentrations (EC × SPP) at $P > 0.05$.

There were no significant differences ($P > 0.05$) between replicates for absolute shoot and root fresh and dry weight data.

Table 3.1. Mean Squares and significances from analysis of variance of absolute shoot weight of 4 *Trifolium* species grown in sand cultures of 7 EC levels.

a) Shoot fresh weight

Sources	DF	Mean Squares	P > F
Replications	2	8.13 **ns**	0.20
EC (dSm^{-1})	6	1187.72***	0.0001
Species (SPP)	3	811.04***	0.0001
SPP × EC	18	10.27**	0.01
Residual	117	5.02	

b) Shoot dry weight

Sources	DF	Mean Squares	P > F
Replications	2	0.15 **ns**	0.66
EC (dSm^{-1})	6	44.89***	0.0001
Species (SPP)	3	16.45***	0.0001
SPP × EC	18	1.70***	0.0001
Residual	117	0.36	

Table 3.2. Mean Squares and significances from analyses of variance of absolute root weight of 4 *Trifolium* species grown in sand cultures of 7 EC levels.

a) Root fresh weight

Sources	DF	Mean Squares	P > F
Replications	2	6.26 **ns**	0.07
EC (dSm^{-1})	6	311.57***	0.0001
Species (SPP)	3	55.68***	0.0001
SPP × EC	18	2.14 **ns**	0.54
Residual	117	2.28	

b) Root dry weight

Sources	DF	Mean Squares	P > F
Replications	2	0.10 **ns**	0.11
EC (dSm^{-1})	6	7.70***	0.0001
Species (SPP)	3	0.87***	0.0001
SPP × EC	18	0.10**	0.005
Residual	117	0.04	

3.3.1.1. Absolute shoot fresh weight

As can be seen from Table 3.3a and Figure 3.1a, increasing salt concentrations significantly reduced mean shoot fresh weight in all species. In *T. alexandrinum* (moderately tolerant) the reduction began from EC 4 dSm^{-1}, and continued to EC 26 dSm^{-1}. There were no significant differences in fresh weight between EC 10 and 14, and EC 14 and 18 dSm^{-1}. In *T. resupinatum* (most tolerant), the fresh weight reduction began at EC 10 dSm^{-1}, and the mean fresh weight at EC 18 and 22 dSm^{-1} was not significantly different. In *T. ambiguum*, and *T. pratense* (least tolerant) the shoot growths were severely reduced at EC 14 dSm^{-1}. There were no statistical differences between mean fresh weight at EC 18 and 22, and EC 22 and 26 for *T. ambiguum*, but fresh weight at EC 14 differed from that at EC 10, and EC 18 dSm^{-1}. For *T. pratense* at EC 0.32 and 4, and between EC 18, 22, and 26 dSm^{-1}, there were no significant differences.

The absolute shoot fresh weight of *T. resupinatum* was significantly ($P \leq 0.05$) greater than that of the other three species at all 7 concentrations except for *T. alexandrinum* at EC 0.32. Absolute shoot fresh weights of *T. ambiguum* and *T. pratense* were not statistically different, and all were significantly smaller than those of *T. alexandrinum* and *T. resupinatum* except at control EC. *T. alexandrinum* was moderately tolerant at all EC levels. Above EC 10 the shoot growths of *T. ambiguum* and *T. pratense* were seriously inhibited. According to this evaluation, *T. resupinatum* is the most salt tolerant species at all salinity levels, *T. ambiguum* and *T. pratense* the least tolerant species, and did not differ from each other at all 7 EC levels. *T. alexandrinum* is moderately tolerant to NaCl + $CaCl_2$ concentrations, but does not differ significantly from the other three species in control conditions.

3.3.1.2. Absolute shoot dry weight

The mean shoot dry weight of the experiment, and DMRT grouping of different treatments of the 4 species are presented in Figure 3.1b and Table 3.3b, indicating shoot dry weight of all species being reduced by increasing salinity. For *T. alexandrinum* the reduction started when the treatment began, but at EC 14 dSm^{-1}, the mean dry weight did not differ from EC 10 and 18 dSm^{-1}, and the mean dry weight at EC 22 was similar to that at EC 18 and 26 dSm^{-1}. For *T. resupinatum* shoot dry weight, there were no significant differences between EC 0.32 and 4, and none also between EC 18 and 22 dSm^{-1}, but absolute shoot dry weight differed from each other at the other EC levels. For *T. ambiguum* root growth reduction occurred at all EC levels, but the differences between EC 14 and 18, 18 and 22, and 22 and 26 were not significant. In the case of *T. pratense* absolute mean shoot dry weight values, there were no significant differences between EC 0.32 and 4, between 10 and 14, and between 14, 18, 22, and 26 dSm^{-1}.

At EC 0.32 dSm^{-1} (control) mean shoot dry weight of *T. pratense* was significantly ($P \leq 0.05$) greater that of *T. resupinatum* and *T. ambiguum*, but not different from *T. alexandrinum*, whilst it and *T. resupinatum* did not differ significantly from one another. At EC 4 dSm^{-1}, *T. ambiguum* had the smallest dry weight, the other three species having similar absolute dry weights. For *T. ambiguum* and *T. pratense*, EC 10 dSm^{-1} was as a critical concentration because shoot dry weight dropped from 3.70 and 6.2 g in control, to 1.9 g and to 2.9 g at EC 10 dSm^{-1} respectively, indicating that *T. ambiguum* was the more sensitive species. Shoot dry weight of *T. alexandrinum* and *T. resupinatum* did not differ from each other at EC 10 dSm^{-1}, *T. alexandrinum* also did not differ in shoot dry weight from *T. pratense*. At higher concentrations ($EC \geq 10$

dSm^{-1}) mean shoot dry weights of *T. alexandrinum* and *T. resupinatum* were consistently significantly greater than those of *T. ambiguum* and *T. pratense*. Based upon these shoot dry weight values *T. resupinatum*, was the most tolerant species in response to salinity at the highest level used, i.e. EC 26 dSm^{-1}.

Table 3.3. Mean absolute shoot weight (g) for 4 *Trifolium* species grown at 7 EC levels, 0.32 dSm^{-1} being control. (Comparisons based on Duncan's Multiple Range Test [DMRT 5%]).

a) Shoot fresh weight

Species→	*T. alexandrinum*		*T. resupinatum*		*T. ambiguum*		*T. pratense*	
EC dSm^{-1}	Mean	DMRT	Mean	DMRT	Mean	DMRT	Mean	DMRT
0.32	25.36	A B a	28.62	A a	22.21	B a	21.22	B a
4	21.14	B b	28.51	A a	16.94	C b	19.74	B C a
10	16.23	B c	21.94	A b	13.85	B C c	11.47	C b
14	14.24	B c d	18.43	A c	7.43	C d	6.24	C c
18	11.74	B d	15.37	A d	4.70	C e	2.07	C d
22	9.06	B e	13.38	A d	2.84	C e f	1.19	C d
26	4.89	B f	8.59	A e	2.10	C f	0.81	C d

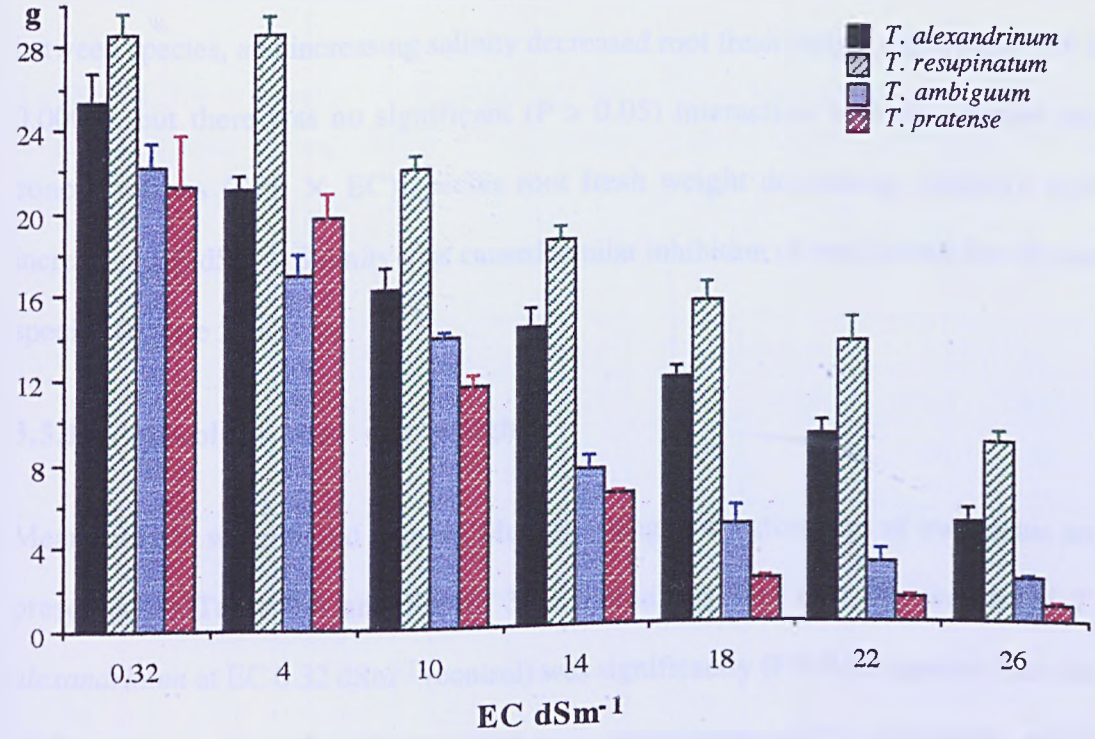
b) Shoot dry weight

Species→	*T. alexandrinum*		*T. resupinatum*		*T. ambiguum*		*T. pratense*	
EC dSm^{-1}	Mean	DMRT	Mean	DMRT	Mean	DMRT	Mean	DMRT
0.32	5.65	A B a	4.75	B C a	3.70	C a	6.16	A a
4	4.65	A b	4.62	A a	3.04	B b	5.61	A a
10	3.57	A B c	4.02	A b	1.89	C c	2.89	B b
14	2.87	A c d	3.50	A c	1.06	B d	1.49	B b c
18	2.45	A d e	2.47	A d	0.95	B d e	0.89	B c
22	1.83	A e f	2.31	A d	0.62	B e f	0.57	B c
26	1.18	B f	1.74	A e	0.47	C f	0.28	C c

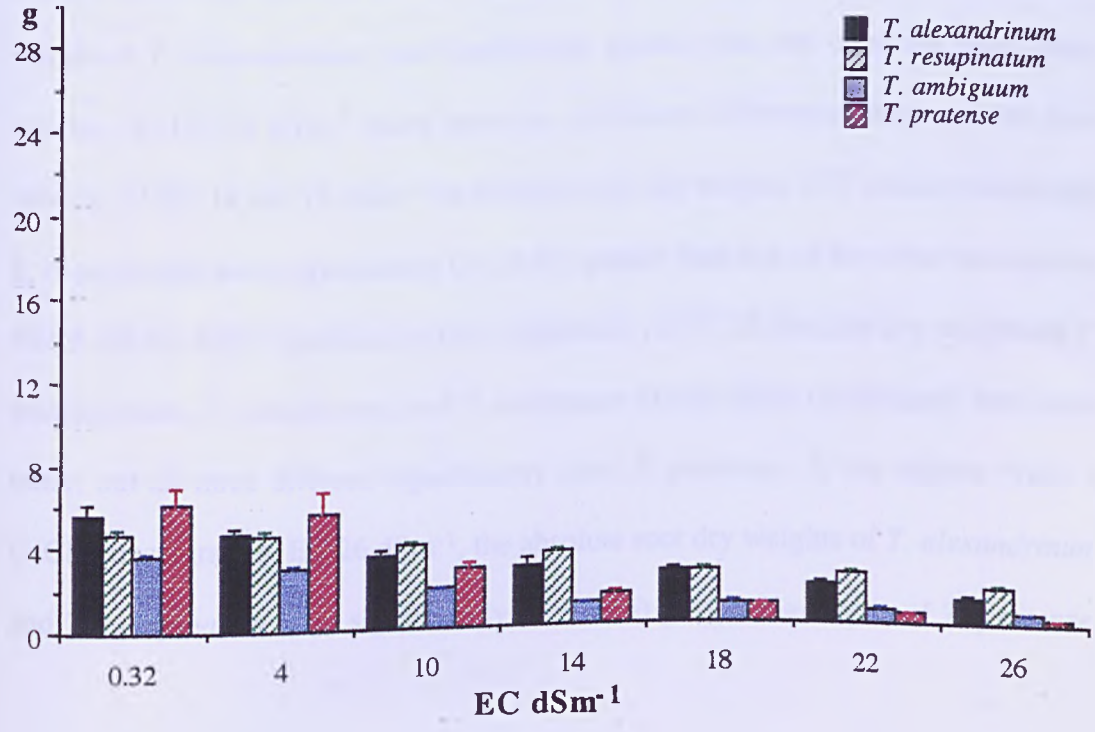
Means with the same capital letter are not significantly different between species.
Means with the same lower case letters are not significantly different between salinity levels.

Figure 3.1. Absolute tolerance values of 4 *Trifolium* species grown in sand culture, and subjected to 6 salinity levels plus control.

a) Shoot fresh weight



b) Shoot dry weight



3.3.1.3. Absolute root fresh weight

As shown in Table 3.2a, there were highly significant differences ($P \leq 0.0001$) between species, and increasing salinity decreased root fresh weight significantly ($P \leq 0.0001$), but there was no significant ($P > 0.05$) interaction between species and concentrations (SPP × EC) species root fresh weight decreasing similarly with increasing EC dSm^{-1}. Salinity thus caused similar inhibition of root growth for all four species (Figure 3.2a).

3.3.1.4. Absolute root dry weight

Mean root dry weights and Duncan Multiple Range Test groupings of treatments are presented in Table 3.4 and Figure 3.2b. Mean absolute root dry weight of *T. alexandrinum* at EC 0.32 dSm^{-1} (control) was significantly ($P \leq 0.05$) greater than that of *T. pratense* only, but similar to those of *T. resupinatum and T. ambiguum*, which did not differ significantly from *T. pratense*. At EC 4 dSm^{-1}, the absolute root dry weight of *T. alexandrinum* was significantly greater than that of all the other three species. At EC 10 dSm^{-1} there were no significant differences between the four species. At EC 14 and 18 dSm^{-1} the absolute root dry weights of *T. alexandrinum* and *T. resupinatum* were significantly ($P \leq 0.05$) greater than that of the other two species which did not differ significantly from eachother. At EC 22 absolute dry weights of *T. alexandrinum, T. resupinatum*, and *T. ambiguum* did not differ significantly from each other, but all three differed significantly from *T. pratense*. At the highest NaCl + $CaCl_2$ concentration, EC 26 dSm^{-1}, the absolute root dry weights of *T. alexandrinum* and *T. resupinatum* were significantly ($P \leq 0.05$) greater than those of *T. pratense*,

whilst *T. ambiguum*, and *T. pratense* had not different significantly in root dry weights ($P > 0.05$).

Table 3.4. Mean absolute root weight (g) for 4 *Trifolium* species grown at 7 EC levels, 0.32 dSm^{-1} being control. (Comparisons based on Duncan's Multiple Range Test [DMRT 5%]).

Root dry weight

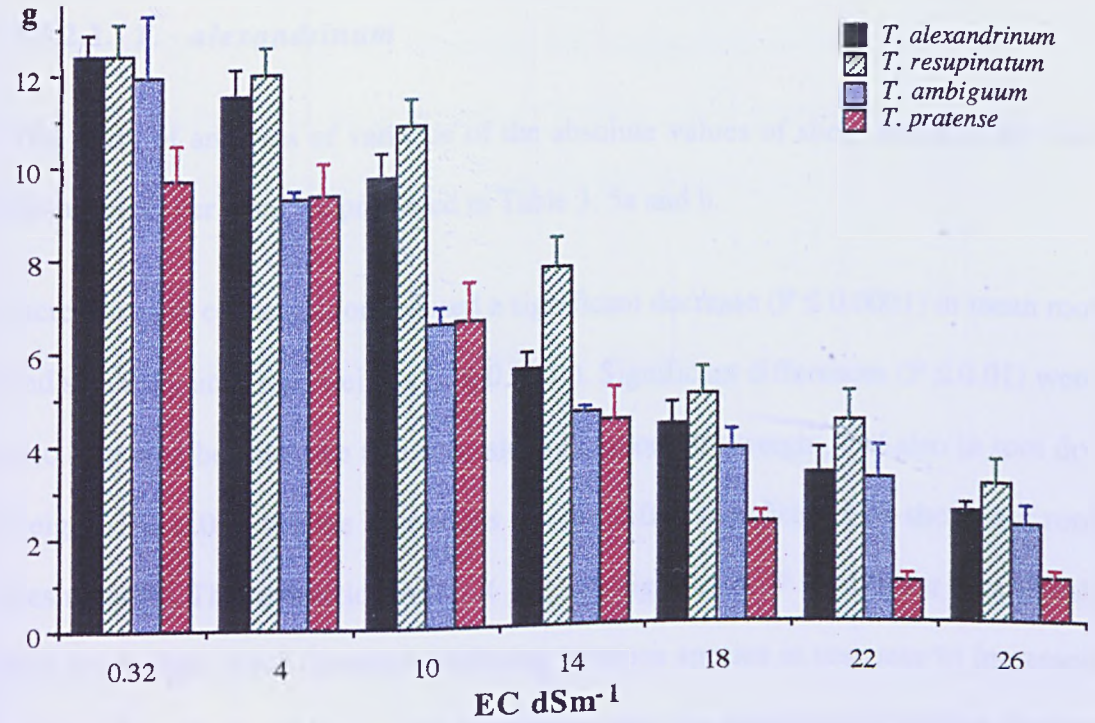
Species→	*T. alexandrinum*		*T. resupinatum*		*T. ambiguum*		*T. pratense*	
EC dSm^{-1}	Mean	DMRT	Mean	DMRT	Mean	DMRT	Mean	DMRT
0.32	2.29	A a	1.98	A B a	2.15	A B a	1.90	B a
4	2.17	A a	1.82	B a	1.73	B b	1.85	B a b
10	1.69	A b	1.74	A a	1.29	A c	1.51	A b
14	1.49	A b	1.32	A b	0.95	B d	0.97	B c
18	1.01	A c	1.08	A c	0.75	B d e	0.58	B d
22	0.70	A d	0.79	A c d	0.71	A e	0.22	B d
26	0.55	A d	0.66	A d	0.40	A B f	0.24	B d

Means with the same capital letter are not significantly different between species.

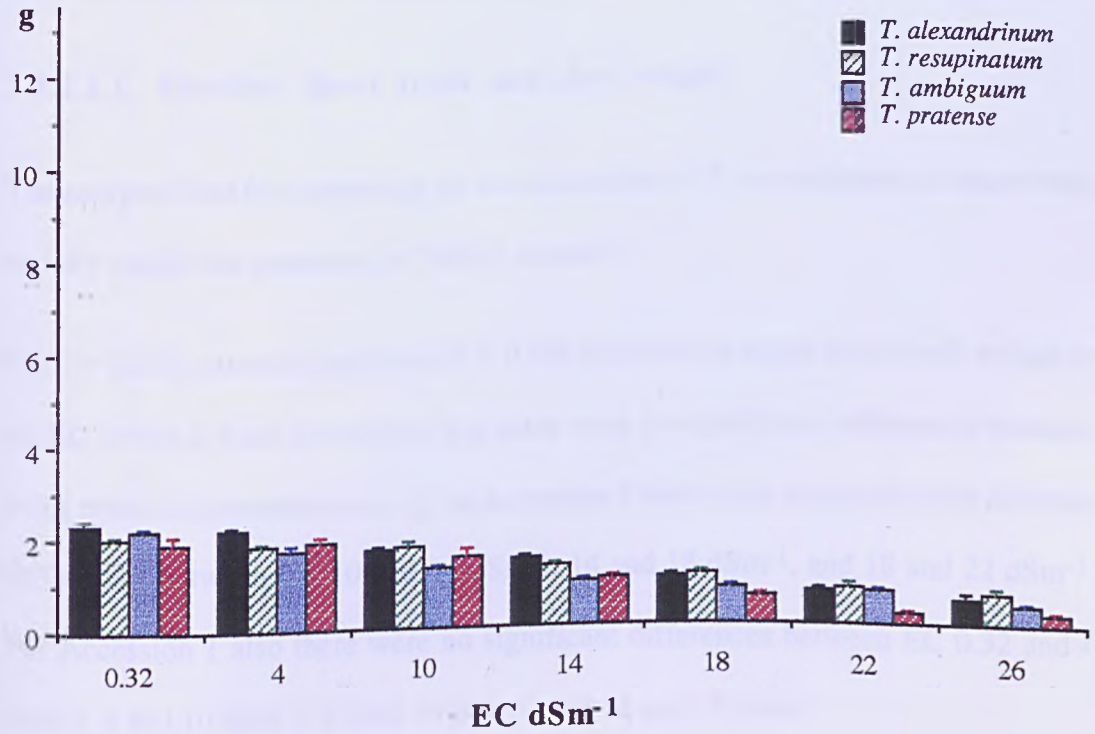
Means with the same lower case letters are not significantly different between salinity levels.

Figure 3.2. Absolute mean values of 4 *Trifolium* species grown in sand culture, and subjected to 6 salinity levels plus control.

a) Root fresh weight



b) Root dry weight



3.3.2. Comparison of absolute salt tolerances of accessions within species

3.3.2.1. *T. alexandrinum*

The result of analyses of variance of the absolute values of shoot and root dry and fresh weight per plant are presented in Table 3. 5a and b.

Increasing salt concentrations caused a significant decrease ($P \leq 0.0001$) in mean root and shoot dry and fresh weights ($P \leq 0.0001$). Significant differences ($P \leq 0.01$) were also observed between the two accessions for shoot dry weight, and also in root dry weight ($P \leq 0.05$), but the accessions did not differ significantly in shoot and root fresh weight. The interaction ACC × EC was significant ($P \leq 0.05$) for shoot fresh and dry weight, these characters differing between species in response to increased salinity. Root fresh and dry weight interactions were not significant, indicating that the two accessions responded similarly to increasing salt concentration.

3.3.2.1.1. Absolute shoot fresh and dry weight

The analysed data for comparing the two accessions of *T. alexandrinum* in shoot fresh and dry weight are presented in Table 3.6a and b.

NaCl + $CaCl_2$ caused significant ($P \leq 0.05$) decreases in mean shoot fresh weight at all EC levels in both accessions, but there were no significant differences between some levels of concentrations, e.g. in Accession 2 there were no significantly different differences between EC 10 and 14 dSm^{-1}, 14 and 18 dSm^{-1}, and 18 and 22 dSm^{-1}. For Accession 1 also there were no significant differences between EC 0.32 and 4 dSm^{-1}, 4 and 10 dSm^{-1}, 10 and 14 dSm^{-1}, and 14 and 18 dSm^{-1}.

At EC 0.32 and 4 dSm^{-1}, the mean values of shoot fresh and dry weights of both accessions did not differ significantly. At EC 10 and 14 dSm^{-1} Accession 1 had significantly greater ($P \leq 0.05$) shoot fresh and dry weights than Accession 2. At EC 18, 22, and 26 dSm^{-1} the two accessions had similar reduced weights in response to high salinity for shoot fresh weight. Accession 1 had greater shoot growth than Accession 2 at EC 18 and 26 dSm^{-1}, but the two accessions did not differ at EC 22 dSm^{-1}.

3.3.2.1.2. Absolute root fresh and dry weight

As shown in Table 3.5b, increasing NaCl + $CaCl_2$ concentration affected and decreased similarly root fresh and dry weight of the two accessions of *T. alexandrinum* (interaction ACC × EC not significant at $P > 0.05$). There were no significant differences ($P > 0.05$) in root fresh weight between the two accessions, but a significant difference was observed in root dry weight at $P \leq 0.05$, Accession 1 having the larger dry shoot weight. Overall, Accession 1 was the more tolerant of the two accessions examined.

Table 3.5. Mean Squares and significances from analysis of variance of absolute shoot fresh and dry weights from 2 accessions of *T. alexandrinum* responses in sand culture to 7 salinity levels.

a) Absolute shoot values

Sources	D F	Shoot fresh weight		Shoot dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	5.51 **ns**	0.19	0.14 **ns**	0.67
EC (dSm^{-1})	6	94.36***	0.0001	14.83***	0.0001
Accessions (ACC)	1	8.31 **ns**	0.12	2.84**	0.008
ACC × EC	6	9.49*	0.03	1.02*	0.02
Residual	26	3.29		0.34	

b) Absolute root values

Sources	D F	Root fresh weight		Root dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	3.08 **ns**	0.17	0.03 **ns**	0.44
EC (dSm^{-1})	6	101.07***	0.0001	2.84***	0.0001
Accessions (ACC)	1	0.44 **ns**	0.61	0.20*	0.02
ACC × EC	6	0.45 **ns**	0.94	0.03 **ns**	0.44
Residual	26	1.61		0.03	

Table 3.6. Absolute shoot weights (g) of 2 accessions of *T. alexandrinum*, and comparisons of means based on Duncan's Multiple Range Test (DMRT 5%).

a) Shoot fresh weight

EC dSm^{-1}	*T. alexandrinum*			
	Accession 1		Accession 2	
	Mean	DMRT	Mean	DMRT
0.32	24.05	A a	26.67	A a
4	20.34	A a b	21.93	A b
10	18.14	A b c	14.31	B c
14	15.98	A c d	12.50	B c d
18	12.75	A d	10.73	A d e
22	8.76	A e	9.36	A e
26	5.74	A e	4.04	A f

b) Shoot dry weight

EC dSm^{-1}	*T. alexandrinum*			
	Accession 1		Accession 2	
	Mean	DMRT	Mean	DMRT
0.32	5.50	A a	5.79	A a
4	4.33	A b	4.97	A a
10	4.06	A b	3.08	B b
14	3.80	A b	1.95	B b c
18	2.78	A c	2.11	B b c
22	2.01	A d	1.67	A c
26	1.54	A d	0.83	B c

Means with the same capital letters are not significantly different between accessions.
Means with the same lower case letters are not significantly different between salinity levels.

3.3.2.2. Comparison of accessions within *T. resupinatum*

3.3.2.2.1. Absolute shoot fresh and dry weight

Analyses of variance for absolute shoot fresh and dry weight of three accessions of *T. resupinatum* are presented on Table 3.7a.

There were highly significant differences ($P \leq 0.0001$) in EC affects on fresh and dry weights of shoots, increasing concentrations of NaCl + $CaCl_2$ causing reduced mean plant shoot fresh and dry weights in the three *T. resupinatum* accessions. There were highly significant differences ($P \leq 0.0001$) between the three accessions in all parameters. However, the responses in fresh and dry weight production of shoots of the three accessions grown at the varying salt concentrations (ACC × EC) did not differ significantly ($P > 0.05$), indicating that all three of them suffered similar depression in growth due to increased salinity.

3.3.2.2.2. Absolute root fresh and dry weight

Analyses of variance from absolute root fresh and dry weight of three accessions of *T. resupinatum* are presented on Table 3.7b.

There were highly significant differences ($P \leq 0.0001$) in EC affects on fresh and dry weights of roots. Increasing EC reduced mean root fresh and dry weights in the three *T. resupinatum* accessions. There were highly significant differences ($P \leq 0.0001$) between accessions in root fresh weight, but not for root dry weight. The responses in fresh and dry weight production of root of the three accessions grown at the varying salt concentrations (ACC × EC) did not differ significantly ($P > 0.05$), indicating that

all three accessions had similar reduction responses in growth with increasing salinity levels.

Plate 3.1. *T. resupinatum* Accession 3 grown in sand culture with 7 EC levels of salinity plus control.

Plate 3.2. *T. resupinatum* Accession 9 grown in sand culture with 7 EC levels of salinity plus control.

Table 3.7. Mean Squares and significances from analysis of variance of absolute shoot fresh and dry weights from 3 accessions of *T. resupinatum* responses in sand culture to 7 salinity levels.

a) Absolute shoot values

Sources	D F	Shoot fresh weight		Shoot dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	1.20 **ns**	0.77	0.08 **ns**	0.60
EC (dSm^{-1})	6	516.73***	0.0001	12.70***	0.0001
Accessions (ACC)	2	45.33***	0.0003	2.51***	0.0001
ACC × EC	12	8.22 **ns**	0.07	0.15 **ns**	0.53
Residual	40	4.46		0.16	

b) Absolute root values

Source	D F	Root fresh weight		Root dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	2.36 **ns**	0.36	0.07 **ns**	0.33
EC (dSm^{-1})	6	136.11***	0.0001	2.43***	0.0001
Accessions (ACC)	2	47.93***	0.0001	0.14 **ns**	0.11
ACC × EC	12	0.56 **ns**	0.99	0.04 **ns**	0.82
Residual	40	2.25		0.06	

3.4.1. Salt tolerance: Relative values

An assessment of salt tolerance for any given character at certain level of salinity can be expressed as a relative value (Simpson *et al.* 1960) which presents treatment values as percentages of control values from non-saline conditions (Maas and Hoffman 1977, Maas 1986). The results from analyses of variances for percentage of relative fresh and dry, shoot and root data are given in Tables 3.8 and 3.9.

There are highly significant differences ($P \leq 0.0001$) between different salt treatments (EC dSm^{-1}), and between species, which significantly differed for all four parameters ($P \leq 0.0001$). The significant interactions, species and concentrations (SPP $\times$ EC) for relative shoot fresh weight ($P \leq 0.001$), relative shoot dry weight ($P \leq 0.01$), and absolute root dry weight showed that the effects of increasing salinity were different for these four species in the two different characters. However, based upon relative root fresh weight data, the interaction between species and concentrations (SPP $\times$ EC) was not significant ($P > 0.05$), indicating that all four species had similar depressions in salinity tolerance. On the other hand, the interaction between species and concentrations (SPP $\times$ EC) for relative root dry weight was significant ($P \leq 0.01$). There were no observed differences between replications except in the case of relative root fresh weight ($P > 0.05$).

3.4.1.1. Relative shoot fresh weight

Relative percentage data for mean fresh shoot weights of the four *Trifolium* species are presented in Figure 3.3a, and Duncan Multiple Range Test (DMRT) for each level of salt concentrations and also for each level of salt concentration per species are shown in Table 3.10a.

Table 3.8. Mean Squares and significance from analysis of variance of relative shoot weight of 4 *Trifolium* species grown in sand cultures of 7 EC levels.

a) Shoot fresh weight

Sources	DF	Mean Squares	P > F
Replications	2	195.94 **ns**	0.18
EC (dSm^{-1})	6	18763.22***	0.0001
Species (SPP)	3	4730.50***	0.0001
SPP × EC	18	342.94***	0.0002
Residual	117	112.70	

b) Shoot dry weight

Sources	DF	Mean Squares	P > F
Replications	2	62.53 **ns**	0.70
EC (dSm^{-1})	6	17668.82***	0.0001
Species (SPP)	3	6256.16***	0.0001
SPP × EC	18	397.80**	0.005
Residual	117	177.69	

Table 3.9. Mean Squares and significance from analysis of variance of relative root weight of 4 *Trifolium* species grown in sand cultures of 7 EC levels.

a) Root fresh weight

Sources	DF	Mean Squares	P > F
Replications	2	571.87*	0.03
EC (dSm^{-1})	6	22605.20***	0.0001
Species (SPP)	3	1637.48***	0.0001
SPP × EC	18	185.29 **ns**	0.33
Residual	117	163.27	

b) Root dry weight

Sources	DF	Mean Squares	P > F
Replications	2	161.74 **ns**	0.20
EC (dSm^{-1})	6	17931.88***	0.0001
Species (SPP)	3	1657.00***	0.0001
SPP × EC	18	214.73**	0.006
Residual	117	97.85	

Table 3.10. Relative shoot fresh weight (percent of control) of 4 *Trifolium* species. From Duncan's Multiple Range Test (DMRT 5%).

a) Shoot fresh weight

Species→	*T. alexandrinum*		*T. resupinatum*		*T. ambiguum*		*T. pratense*	
EC dSm^{-1}	Mean %	DMRT	Mean %	DMRT	Mean %	DMRT	Mean %	DMRT
4	85	A B a **ns**	100	A a **ns**	76	B b *	96	A B a **ns**
10	66	A B b	77	A b	63	A B c	55	B b
14	58	A b c	65	A c	33	B d	30	B c
18	47	A c d	54	A d	22	B e	10	B d
22	37	A d	47	A d	13	B f	6	B d
26	19	B e	30	A e	9	C f	4	C d

b) Shoot dry weight

Species→	*T. alexandrinum*		*T. resupinatum*		*T. ambiguum*		*T. pratense*	
EC dSm^{-1}	Mean %	DMRT	Mean %	DMRT	Mean %	DMRT	Mean %	DMRT
4	86	A B a b **ns**	99	A a **ns**	82	B b *	97	A B a **ns**
10	66	A B b c	86	A b	51	A B c	47	B b
14	53	A c d	75	A c	29	B d	25	B b c
18	45	A d	53	A d	26	B d	15	B c
22	34	A d e	49	A d	17	B e	10	B c
26	21	B e	37	A e	13	C e	7	C c

Means with the same capital letter are not significantly different between species.
Means with the same lower case letters are not significantly different between salinity levels.
ns = Non significant differences with their control
* = Significantly different ($P \leq 0.05$) with their control

Shoot fresh weight of *T. resupinatum*, the most tolerant species, at EC 22 dSm^{-1}, was reduced to 47%, less than 50% of its control, whereas in contrast, shoot fresh weights of *T. ambiguum* and *T. pratense* (least tolerant) at EC 14 dSm^{-1}, were reduced to 33 and 30% respectively. *T. alexandrinum* (moderately tolerant) at EC 18 dSm^{-1}, was reduced to 47%, lees than 50% its control.

At EC 4 dSm^{-1} the relative shoot fresh weight of 100% for *T. resupinatum* was greater than the other three species, *T. ambiguum* with 76% being in the lower group (group B). *T. alexandrinum* and *T. pratense* were intermediate. At EC 10 dSm^{-1}, *T. resupinatum* was again the higher tolerance in group A and *T. pratense* was in group B and the two remaining species were in group AB differing from neither of these species. At higher NaCl + $CaCl_2$ concentrations (EC 14, 18, and 22 dSm^{-1}) *T. alexandrinum* and *T. resupinatum* had the higher shoot fresh weights, and *T. ambiguum* and *T. pratense* the lowest shoot fresh weights. At the highest concentration in this experiment (EC 26 dSm^{-1}), the mean fresh weight of *T. resupinatum* was 30% of control, and it was most tolerant, whilst, *T. ambiguum* (9%) and *T. pratense* (4%) were the least tolerant, and *T. alexandrinum* with 19% of control was moderately tolerant.

3.4.1.2. Relative shoot dry weight

The 50% reduction point was, for *T. resupinatum* at EC 22, *T. alexandrinum* at EC 18, and *T. ambiguum* and *T. pratense* between 10 and 14 dSm^{-1} (Table 3.10a).

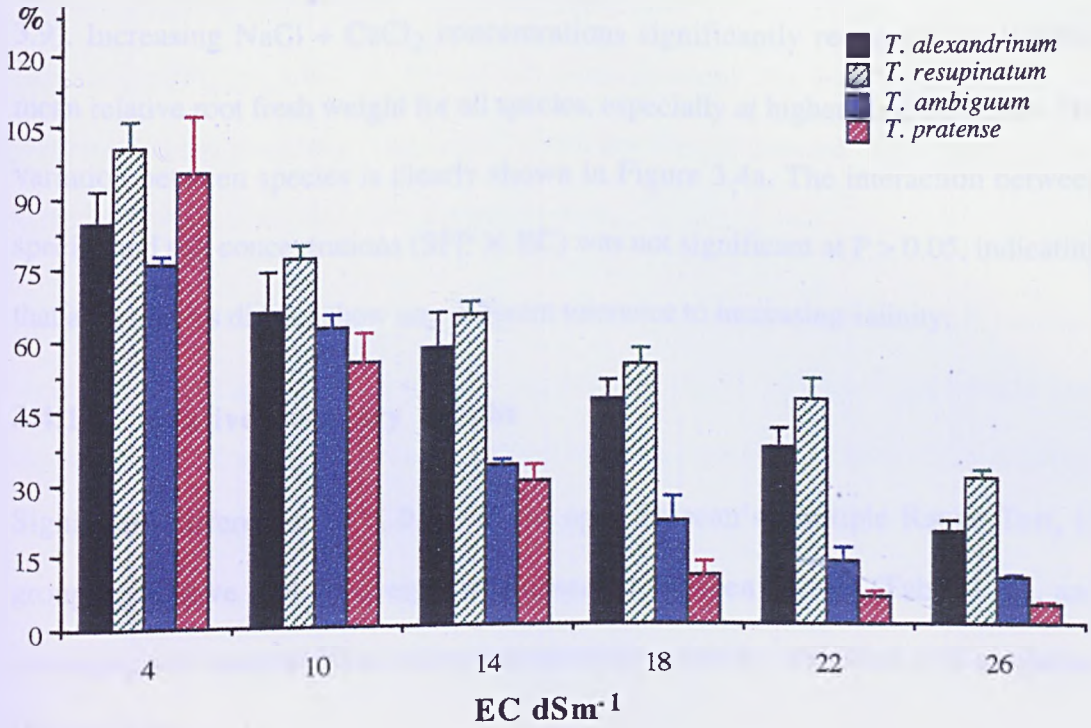
These relative shoot dry weight data (Figure 3.3a) showed that *T. resupinatum* had greater relative shoot dry weight at all 6 salinity levels and this was the most tolerant species. In contrast, *T. pratense* showed a very sensitive response and had the lowest

relative shoot dry weight above EC 4 dSm-1 being the least tolerant species. In this experiment, at EC 4 dSm^{-1} there were no significant differences ($P > 0.05$) between *T. alexandrinum*, *T. resupinatum*, *T. ambiguum* and *T. pratense* and the relative shoot dry weights of them varied between 82 to 99% of control. At EC 10 dSm^{-1}. *T. resupinatum*, was the most tolerant, *T. ambiguum* and *T. pratense* were the least tolerant, and *T. alexandrinum* showed intermediate tolerance. At EC 14, 18, and 22 dSm^{-1}, the relative salt tolerance of *T. resupinatum* was again significantly greater ($P \leq 0.05$) than that of the other species, *T. ambiguum* and *T. pratense* were smaller, and *T. alexandrinum* was again intermediate. Finally at EC 26 dSm^{-1} *T. resupinatum* with a value of 37% of control was again most tolerant, and *T. pratense* with 7% the least tolerant, the other two species being of intermediate tolerance.

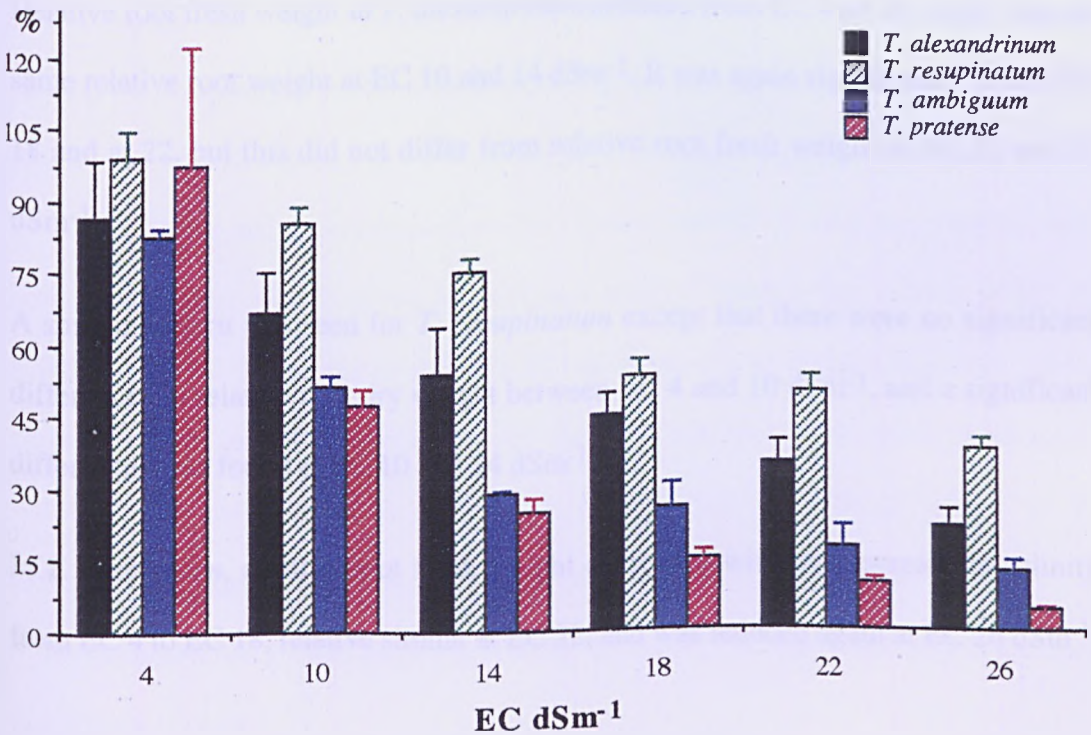
Comparison of 7 accessions on both characters (mean seedling shoot length and mean shoot dry weight of sand culture experiment) at EC > 18 dSm^{-1} shows, the shoot growth of *T. ambiguum* and *T. pratense* seriously inhibited in both experiments, whereas the accessions of *T. alexandrinum* (Accession 1, 2) and *T. resupinatum* (Accessions 1, 2, 3) had high values. The mean shoot length of two-weeks-old seedling in solution culture showed a linear relationship with shoot dry weight of plants in sand culture experiment with, $r^2 > 0.70$. However, only *T. alexandrinum* produced a weak relationship with mean shoot length and mean shoot dry weight with $r^2 = 0.58$ (Figure 3.5).

Figure 3.3. Relative tolerance values (percentage of control) of 4 *Trifolium* species grown in salinised sand culture.

a) Shoot fresh weight



b) Shoot dry weight



3.4.1.3. Relative root fresh weight

Species differed significantly ($P \leq 0.0001$) in relative root fresh weight data (Table 3.9). Increasing NaCl + $CaCl_2$ concentrations significantly reduced ($P \leq 0.0001$) mean relative root fresh weight for all species, especially at higher concentrations. The variation between species is clearly shown in Figure 3.4a. The interaction between species and salt concentrations (SPP × EC) was not significant at $P > 0.05$, indicating that all 4 species did not show any different tolerance to increasing salinity.

3.4.1.4. Relative root dry weight

Significant differences ($P \leq 0.05$) based upon Duncan's Multiple Range Test, in grouping relative root dry weight was observed between species (Table 3.11), and increasing salt concentrations reduced mean relative root dry weight of all four species (Figure 3.4b).

Relative root fresh weight in *T. alexandrinum* reduced from EC 4 to 10, which had the same relative root weight at EC 10 and 14 dSm^{-1}. It was again significantly less at EC 18 and at 22. but this did not differ from relative root fresh weight at EC 22 and 26 dSm^{-1}.

A similar pattern was seen for *T. resupinatum* except that there were no significant differences in relative root dry weight between EC 4 and 10 dSm^{-1}, and a significant differences was found at EC 10 and 14 dSm^{-1}.

In *T. ambiguum*, relative root fresh weight decreased with less increase in salinity from EC 4 to EC 18, relative similar at EC 22, and was reduced again at EC 26 dSm^{-1}.

The pattern was similar for *T. pratense*, except that decline was from EC 4 to EC 22 continuously, but it did not fall further from EC 22 to EC 26 dSm^{-1}.

The salt sensitive species were clearly separated from the salt tolerant species by these parameters (Figure 3.4b). At higher salt concentrations, the most salt sensitive, *T. pratense* having markedly lower relative root dry weight than the other three species, especially when compared with *T. resupinatum.*

According to this parameter (relative root dry weight), *T. resupinatum* and *T. pratense* were again most and least salt tolerant respectively. At EC 18 dSm^{-1}, the relative root dry weight of all species had been reduced by more than 50% of their control, except *T. resupinatum* the most tolerant species, which had 55% of control root dry weight. At EC 22 dSm^{-1} all 4 species showed the same tolerance except *T. pratense*, the least tolerant.

In Figure 3.5, the differences between accessions on root length of seedling measurement, and root dry weight of sand culture experiments at EC 18 dSm^{-1} clearly shown, the Accession 1 of *T. alexandrinum* had the highest and *T. ambiguum* had the lowest root length, and root dry weight in this sand culture experiment, Accession 3 of *T. resupinatum* and *T. pratense* had the highest and the lowest dry weight value respectively, and also had the longest and shortest roots when grown at EC 18 solution salinity. The mean root length of two-week-old seedling in solution culture showed a linear relationship with root dry weight of plants in sand culture experiment. All four species showed similar linear relationship, only Accession 2 of *T. alexandrinum* again had a week relationship ($r^2 = 0.53$), Meanwhile the Accession 1 of *T. resupinatum* had a maximum relationship ($r^2 = 0.88$) between root length and root dry weight (Figure 3.5)

At the highest concentration in this experiment EC 26 dSm^{-1}, *T. resupinatum*, with a relative salt tolerance of 33% was the most tolerant, whilst *T. pratense* with 13% of control was the most sensitive. At this level *T. alexandrinum* was moderately tolerant, and *T. ambiguum* was moderately sensitive to salinity (Table 3.11).

Table 3.11. Relative root dry weight (percent of control) of 4 *Trifolium* species. From Duncan's Multiple Range Test (DMRT 5%).

Root dry weight

Species→	*T. alexandrinum*		*T. resupinatum*		*T. ambiguum*		*T. pratense*	
EC dSm^{-1}	Mean %	DMRT	Mean %	DMRT	Mean %	DMRT	Mean %	DMRT
4	96	A B a **ns**	93	A a b **ns**	81	B a*	98	A B a **ns**
10	75	A B b	88	A b	60	A B b	80	B b
14	65	A b	67	A c	44	B c	52	B c
18	44	A c	55	A d	35	B d	31	B d
22	31	A d	41	A e	34	B d	12	B e
26	24	B d	33	A e	19	C e	13	C e

Means with the same capital letter are not significantly different between species.

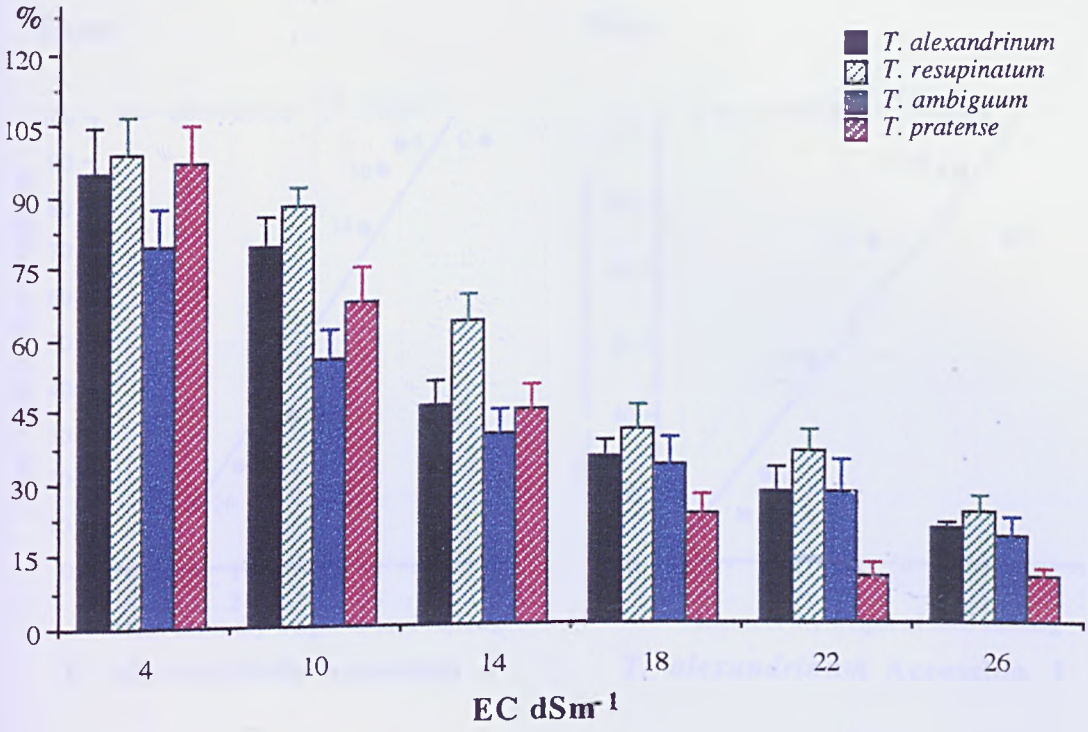
Means with the same lower case letters are not significantly different between salinity levels.

ns = Non significant differences with their control

* = Significantly different ($P \leq 0.05$) with their control

Figure 3.4. Relative tolerance values (percentage of control) of root weight of four *Trifolium* species grown in salinised sand culture.

a) Root fresh weight



b) Root dry weight

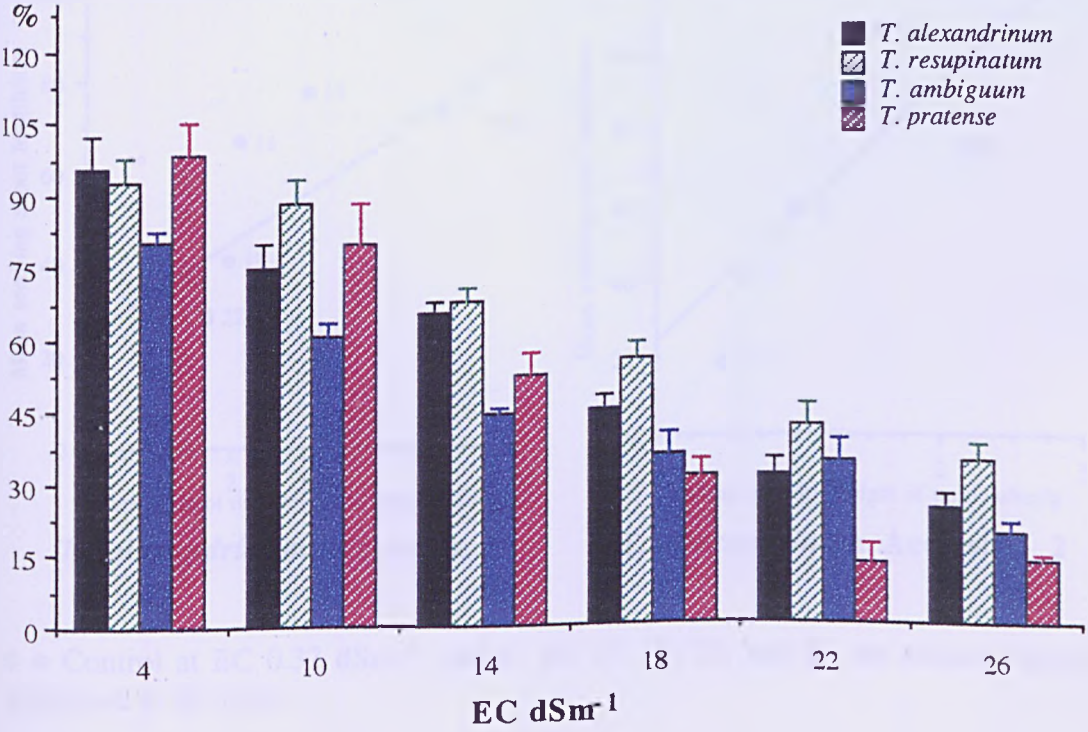
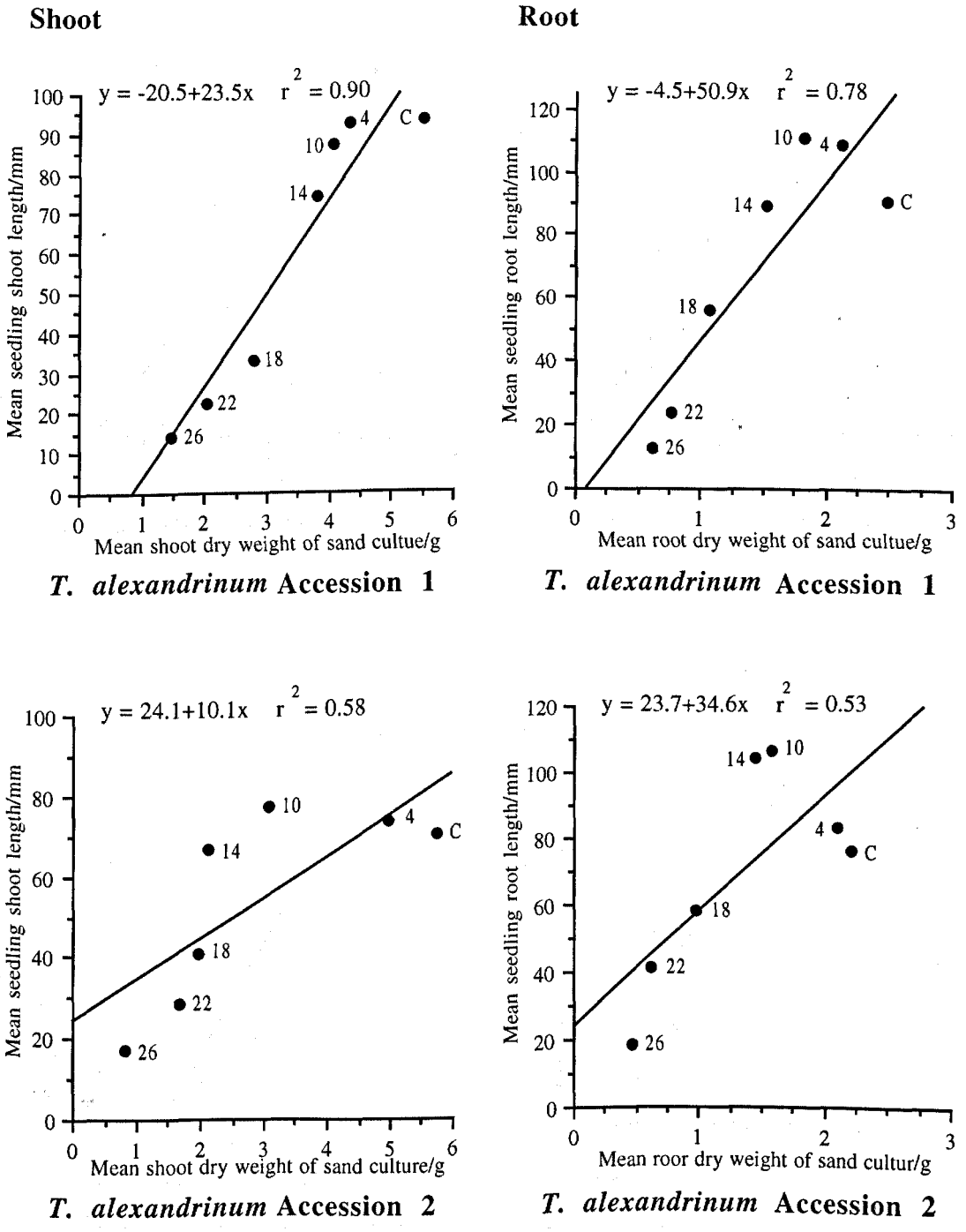


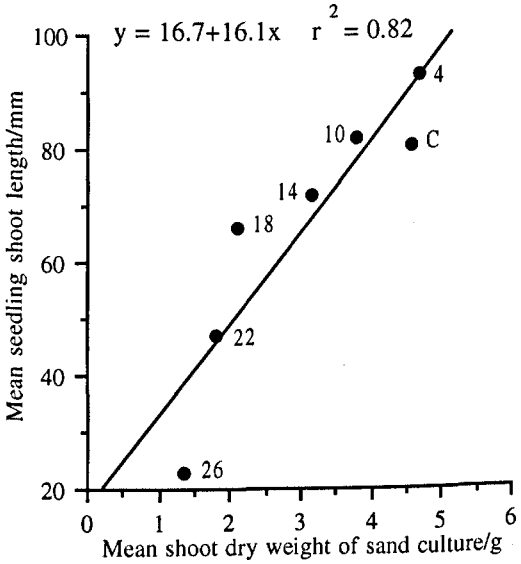
Figure 3.5. Relationship between shoot dry weight after 16 weeks growth of 4 *Trifolium* species in sand culture in 7 salinity level, and shoot length of two-week-old seedlings of the same species grown in 7 salinity level of solution culture.



c = Control at EC 0.32 dSm^{-1}, and 4, 10, 14, 18, 22, and 26 are concentrations expressed in EC dSm^{-1}.

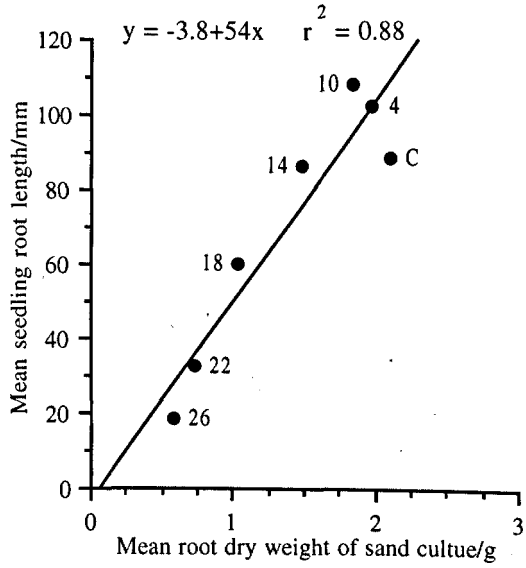
Figure 3.5. Continued.

Shoot

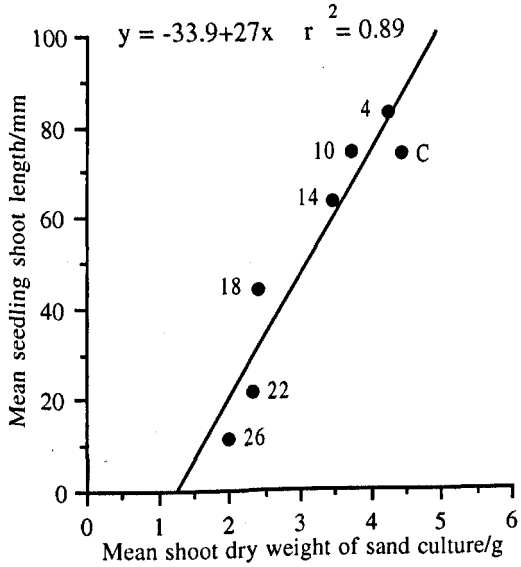


***T. resupinatum* Accession 1**

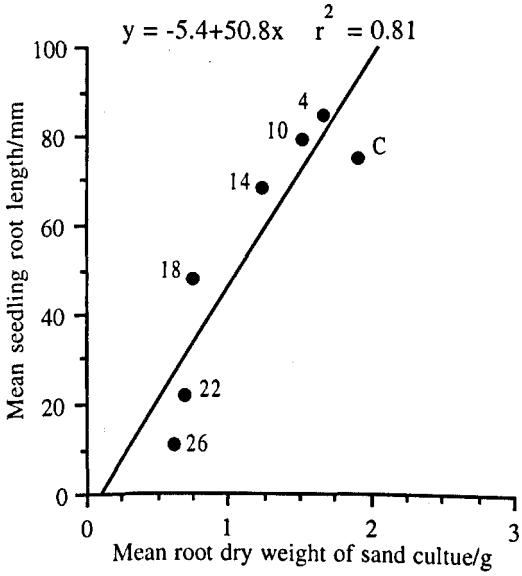
Root



***T. resupinatum* Accession 1**



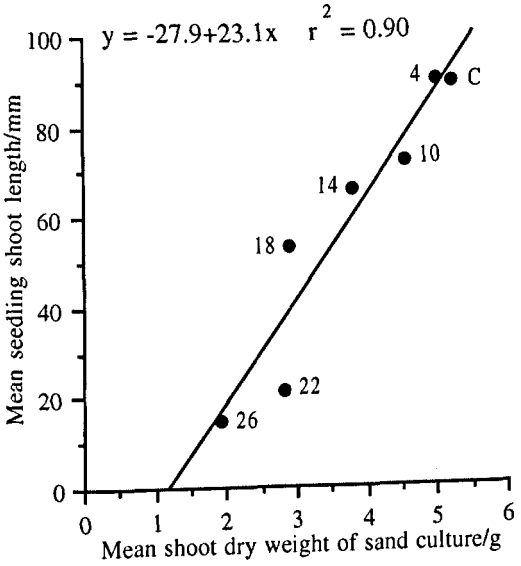
***T. resupinatum* Accession 2**



***T. resupinatum* Accession 2**

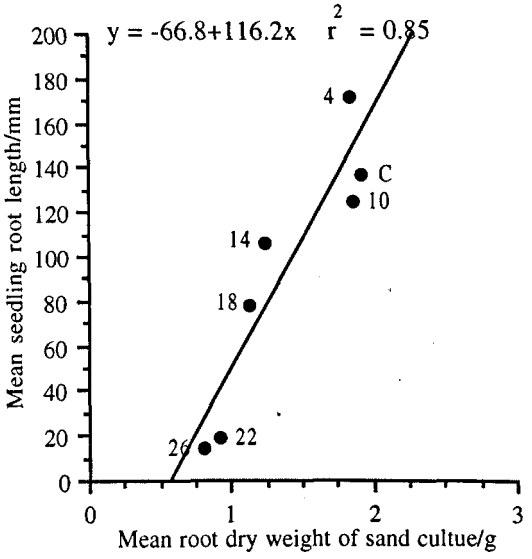
Figure 3.5. Continued.

Shoot

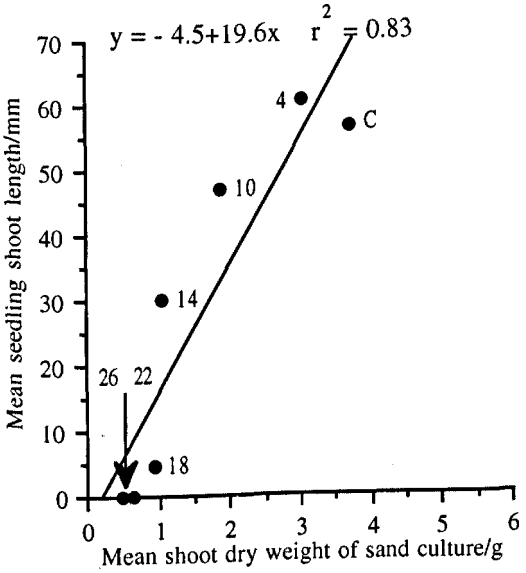


***T. resupinatum* Accession 3**

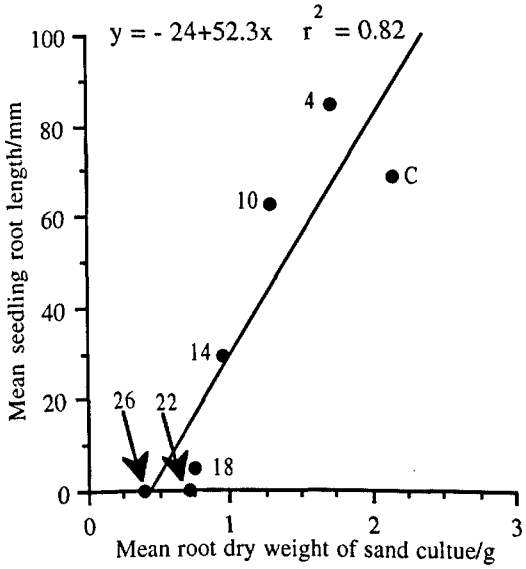
Root



***T. resupinatum* Accession 3**

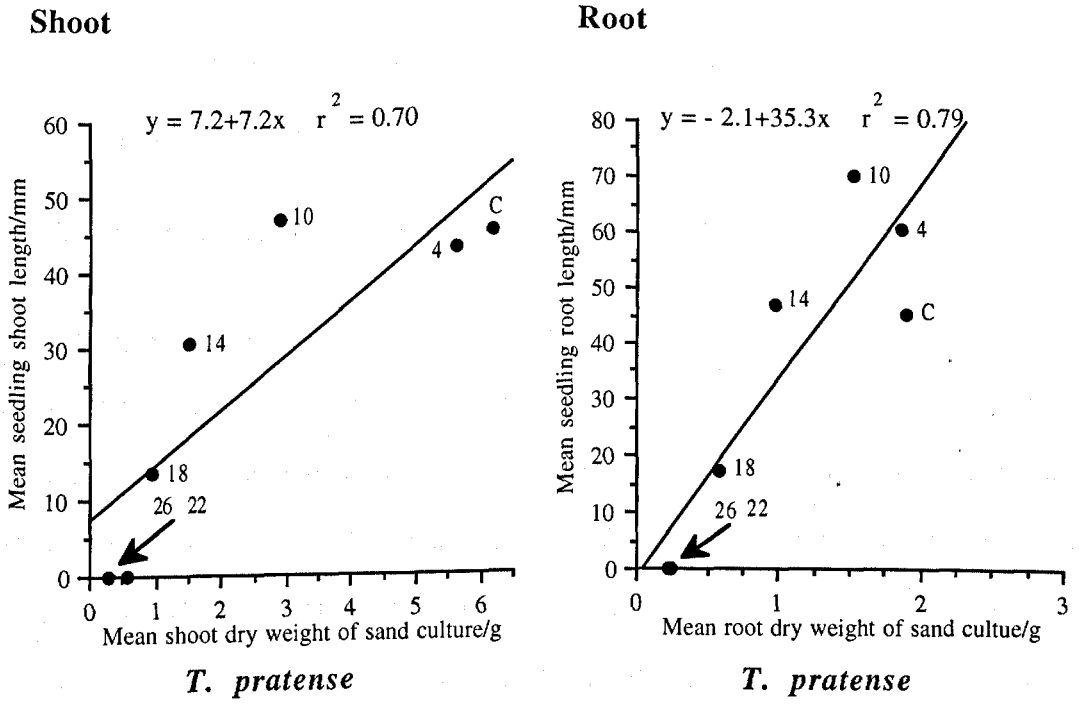


T. ambiguum



T. ambiguum

Figure 3.5. Continued.



3.4.2. Relative salt tolerance within species

3.4.2.1. *T. alexandrinum*

The results of analyses of variance of the relative values of shoot and root dry and fresh weight per plant are presented in Tables 3.12a and b.

3.4.2.1.1. Relative shoot fresh and dry weight

Increasing salt concentrations caused a significant decrease ($P \leq 0.0001$) in relative shoot fresh and dry weights ($P \leq 0.0001$). Significant differences ($P \leq 0.01$) were also observed between the two accessions for shoot fresh weight, but this difference in shoot dry weight was not significant at $P > 0.05$. The interaction between accessions and NaCl + $CaCl_2$ (ACC × EC) was not significant for both shoot fresh and dry weight, the salinity effect being similar for both accessions. Replications differed significantly in relative shoot fresh weight, but not for shoot dry weight.

3.4.2.1.2. Relative root fresh and dry weight

The analyses of variance for comparing the two accessions at 7 EC levels of *T. alexandrinum* in root fresh and dry weight are presented in Table 3.12b.

The salinity treatments caused significant ($P \leq 0.0001$) decreases in mean root fresh and dry weights. There were no significant differences between accessions, and the interaction between accessions and salinity concentration (ACC × EC) was also not significant, showing again that the two accessions responded similarly to increased salinity. There were not significant differences between replicates.

Table 3.12. Mean Squares and significance from analysis of variance of relative value from 2 accessions of *T. alexandrinum* grown in sand cultures of 7 EC levels.

a) Relative shoot value

Sources	D F	Shoot fresh weight		Shoot dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	585.55*	0.01	642.03 ns	0.10
EC (dSm^{-1})	6	4616.72***	0.0001	4743.23***	0.0001
Accessions (ACC)	1	1278.97**	0.003	1018.34 ns	0.06
ACC × EC	6	136.74 ns	0.34	308.90 ns	0.34
Residual	26	114.27		257.07	

b) Relative root value

Sources	D F	Root fresh weight		Root dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	130.38 ns	0.54	68.04 ns	0.41
EC (dSm^{-1})	6	6735.75***	0.0001	5501.98***	0.0001
Accessions (ACC)	1	5.17 ns	0.88	204.11 ns	0.11
ACC × EC	6	26.23 ns	0.99	92.93 ns	0.31
Residual	26	205.79		74.46	

3.4.2.2. *T. resupinatum*

3.4.2.2.1. Relative shoot fresh and dry weight

The results of the analyses of variance are summarised in Table 3.13 and as expected show that NaCl + $CaCl_2$ concentrations has a significant effect on shoot fresh and dry weight ($P \leq 0.0001$).

The non-significant ($P > 0.05$) interaction term, accessions × concentrations showed that the three accessions did not differ in their response to NaCl + $CaCl_2$ treatments. However, a significant differences in shoot fresh weight between accessions was found ($P \leq 0.01$), but the three accessions did not differ significantly in shoot dry weight. Replicates were not significantly different, for both fresh and dry weight.

3.4.2.2.2. Relative root fresh and dry weight

The result obtained from the analyses of variance of relative value for the three *T. resupinatum* (Table 3.13b) show that root fresh and dry weight declined over 7 concentrations for all accessions ($P \leq 0.0001$). The relative root fresh weight of the three accession also differed significantly ($P \leq 0.01$), but for root dry weight differences were not significant ($P > 0.05$).

Accessions did not show different responses to increased salinity, interaction ACC × EC was not significant, increasing salt concentration affecting similarly root fresh and dry weight of the three accessions.

Table 3.13. Mean Squares and significance from analysis of variance of relative value from 3 accessions of *T. resupinatum* grown in sand cultures of 7 EC levels.

a) Relative shoot value

Sources	D F	Shoot fresh weight		Shoot dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	120.05 **ns**	0.24	192.95 **ns**	0.18
EC (dSm^{-1})	6	6367.98***	0.0001	5700.76***	0.0001
Accessions (ACC)	2	609.82**	0.002	173.92 **ns**	0.22
ACC × EC	12	100.96 **ns**	0.28	64.53 **ns**	0.83
Residual	40	80.39		107.37	

b) Relative root value

Sources	D F	Root fresh weight		Root dry weight	
		Mean Squares	P > F	Mean Squares	P > F
Replications	2	656.04*	0.04	101.14 **ns**	0.51
EC (dSm^{-1})	6	9200.08***	0.0001	6241.35***	0.0001
Accessions (ACC)	2	1117.14**	0.006	346.80 **ns**	0.11
ACC × EC	12	66.61 **ns**	0.98	66.72 **ns**	0.93
Residual	40	194.52		147.07	

3.4.3. Comparison of tolerance assessment at seedling and adult stages

To estimate any relation between shoot and root growth data from seedlings, and shoot/root dry and fresh weight at adult stage, correlation analysis of the data were calculated, and are presented in Table 3.14.

All correlations are significant, and at the same level. For all 4 species correlation significant, and seedling shoot and root lengths also correlate significantly with all weight values from plants grown in saline sand culture experiment for 13 weeks. This indicates that impact to salinity on these adult plant, can be predicted using two week of seedling growth in saline conditions.

Table 3.14. Correlation coefficients of six different measurements adult (A) and at 14 day old seedling (S) stages of four *Trifolium* species in salt treatment experiments.

Species	Characters	Shoot fresh weight (A)	Shoot dry weight (A)	Root fresh weight (A)	Root dry weight (A)	Shoot length (S)
T. alexandrinum	Shoot fresh weight (A)	-				
	Shoot dry weight (A)	0.96	-			
	Root fresh weight (A)	0.87	0.83	-		
	Root dry weight (A)	0.90	0.88	0.93	-	
	Shoot length (S)	0.81	0.77	0.83	0.89	-
	Root length (S)	0.58	0.52	0.66	0.72	0.90
T. resupinatum	Shoot fresh weight (A)	-				
	Shoot dry weight (A)	0.95	-			
	Root fresh weight (A)	0.87	0.92	-		
	Root dry weight (A)	0.85	0.87	0.91	-	
	Shoot length (S)	0.88	0.86	0.84	0.83	-
	Root length (S)	0.80	0.74	0.71	0.70	0.87
T. pratense	Shoot fresh weight (A)	-				
	Shoot dry weight (A)	0.69	-			
	Root fresh weight (A)	0.95	0.94	-		
	Root dry weight (A)	0.93	0.91	0.99	-	
	Shoot length (S)	0.85	0.80	0.90	0.93	-
	Root length (S)	0.71	0.68	0.79	0.83	0.93
T. ambiguum	Shoot fresh weight (A)	-				
	Shoot dry weight (A)	0.98	-			
	Root fresh weight (A)	0.96	0.97	-		
	Root dry weight (A)	0.97	0.97	0.98	-	
	Shoot length (S)	0.93	0.90	0.87	0.90	-
	Root length (S)	0.92	0.90	0.85	0.88	0.97

All correlations positive, and significant at $p < 0.001$.

3.5. DISCUSSION

It has been argued that in some species, tolerance at the seedling stage may not confer that to the adult stage (Akbar and Yabuno 1974, Shannon 1979). On the other hand, the seedling stage has been found to be more sensitive than the adult stage by other workers (Pasternak *et al.* 1979, Noble 1983), and it has also been argued that increasing tolerance at the sensitive stage is one important facet of improving the salt tolerance of any plant species (Noble 1983).

The expression of salt tolerance in a crop species is a complex trait, the manifestation of many plant characters, both physiological and morphological (Shannon 1984). In addition, information is needed about the effect of different levels of salinity at various stages of plant development for different crops. Identifying the response of those growth stages to salinity would help the breeder in determining target characters for improvement through selection and breeding. When a specific and readily quantifiable physiological mechanism conferring salt tolerance is not available, the assessment of plant material according to the amount of salt injury reflected in partial or complete necrosis, or the measurement of other plant characters of importance, yield of green matter, and or grain yield, appear to be practical alternative methods (Noble *et al.* 1984). Information about crop sensitivity to salinity at different life stages is thus essential if improvement in salinity tolerance is to be effected through selection and breeding.

Salinity is known to affect plant growth during all developmental stages, and it affects crop responses to salinity which vary during ontogeny (Maas and Hoffman 1977, Shannon 1985, Maas *et al.* 1986, Maas and Poss 1989). Because of these differences

in salinity tolerance during ontogeny, some studies have been concerned with selecting for tolerance through the different stages of the plant life cycle. For example, in rice, salt treatment was commenced at the early tillering, late tillering, and heading stages (Pearson and Bernstein 1959), in maize and pigeon pea during different growth stages (Ashraf and McNeilly 1988), during maize tasseling, and grain filling stages (Maas *et al.* 1983), in sorghum during vegetative, reproductive, and maturation periods (Maas *et al.* 1986), in tomato and barley during all growth stages (Epstein *et al.* 1980). In wheat (Maas and Poss 1989, and Maas and Grieve 1992), and in sorghum (Azhar and McNeilly 1989), seedling and early vegetative growth stages appear to be most sensitive, the growth of shoots often being more suppressed than that of roots (Feigin 1985).

Salinity tolerance of clovers is also highly dependent upon the growth stage at which salinity is first imposed (West and Taylor 1981), while other workers reported that in white clover (*T. repens* L.), seedling emergence was significantly more sensitive in its response to salinity (NaCl) than was germination (Rogers *et al.* 1995). In strawberry clover (*T. fragiferum* L.), germination tolerance to salinity was not correlated with plant growth in either saline, or in non-saline irrigation treatments (Rumbaugh *et al.* 1993). What does appear from the data presented by these authors is that different parts of the plant are not equally affected by salinity.

The results from the experiment presented in this chapter describe the responses of 4 *Trifolium* species under salinity and for accession within species for the two most tolerant species, *T. alexandrinum* and *T. resupinatum*. From these data it is clear that there is a need for precise determination of the environment in which the plant germinates and grows and that there is a need to understand the reaction of the whole

plant to salinity at the adult stage of development. It is also necessary to consider the importance of both absolute and relative yield (so-called "salt tolerance") for each particular case (growth stages) under study.

This experiment clearly reconfirms the existence of considerable inter-species variability in response to NaCl + $CaCl_2$ concentrations. It is also clear that such variability could be exploited through further selection and breeding to increase markedly the salinity tolerance of both *T. alexandrinum* and *T. resupinatum*. In particular, it also shows variation in tolerance within species, and that some accessions/populations are of very high tolerance, and that the tolerance shown at the seedling stage is also expressed at the adult plant stage.

In the experiment conducted here, relative shoot and root fresh and dry weight reduction by 50% did not occur below EC 18 dSm^{-1} for *T. resupinatum*, and also the relative mean shoot length at seedling stage (Table 2.4 in Chapter 2) was above 50% of control, but the relative root length was about 45% of control. By contrast, in *T. alexandrinum* 50% of reduction occurred above EC 14 dSm^{-1}. In *T. ambiguum* and *T. pratense* 50% reduction in shoot and root weights and in total plant weight occurred above EC 10 dSm^{-1}, except that relative root dry weight value in *T. pratense* did not being to decline until EC 14 dSm^{-1} was reached. Shoot fresh weights of *T. ambiguum* and *T. pratense* were reduced by 77 and 70% respectively at EC 14 dSm^{-1}. By contrast the accessions of *T. resupinatum* and *T. alexandrinum* produced more than 47% and 37% shoot fresh weight even at the very high concentration of 22 dSm^{-1} (Table 3.10a).

Comparing the salinity treatments, the data clearly shows that, most of the accessions here more sensitive at the seedling stage than at the mature plant, e.g. *T. ambiguum*

and *T. pratense* seed did not germinate in the higher salinity level, EC 22 and 26 at the seedling stage, but they produced shoot and root in the same level of salinity in the sand culture experiment. Kebebew and McNeilly (1995), in pear millet, showed that four accessions, 221726, Kitui L., 93661, 93614 which had been shown to be tolerant at seedling stage, were more tolerant at maturity than equivalently assessed NaCl sensitive accessions. In contrast Rao (1997), showed that of five maize accessions tested, two, C 12338 and ZEA 671, which were tolerant at the seedling stage were not more tolerant than the non-tolerant accessions ICI 49 and C 12373.

Based upon the results of previous chapters suggesting that the accessions in *T. resupinatum* chosen were particularly tolerant, and they have been to shown to be tolerant as adult plants. *T. resupinatum* accessions and *T. alexandrinum* are known to be moderately tolerant, but the *T. resupinatum* accessions showed high tolerant to $NaCl + CaCl_2$.

Ultimately variation in whole plant reaction to salinity must provide the best means of selection for salinity tolerance. While based on somewhat limited experimental data, in three accessions of *T. resupinatum* and two accessions of *T. alexandrinum*, no single accession was however found to be significantly superior ($P \leq 0.05$) to the others at the adult growth stage. Most importantly the responses of the accessions examined in this chapter appeared to be in great part, consistent with performance assessed after two weeks of growth in solution culture (Chapter 2). It was clear in Chapter 2 that based upon mean overall values of C_t, C_0, and C_{50}, three accessions of *T. resupinatum* and two accessions of *T. alexandrinum* were considerably more tolerant than that as yet found in other *Trifolium* species.

In this sand culture experiment the aforementioned accessions were again the most tolerant, based on four whole plant measurements, namely shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight. Where data are included for two-week-old seedling shoot and root growth data, *T. resupinatum* and *T. alexandrinum* were the most tolerant accessions assessed (Chapter 2), and it became very clear that, *T. pratense* and *T. ambiguum* were the most sensitive accessions in C_t, C_0, and C_{50} (Tables 2.12 and 2.13 in Chapter 2, and Tables 3.3 and 3.4 in Chapter 3). Future work in improving salinity tolerance in clover species through selection could be based upon the growth of two-weeks-old seedlings in solution culture, having in this experiment provided a very good correlation with performance of the adult plant under saline conditions (Table 3.14). The relationship between shoot dry weight in the sand culture experiment at salinity level EC 18, and shoot length of two-week old grown at same salinity level in solution culture, clearly indicated that, *T. pratense* and *T. ambiguum* were the more sensitive than the other species, but in root dry weight from sand culture experiment, all the accession were not affected as the two-weeks-old seedling stage (Figure 3.5). Even if only used as an initial screening procedure, much of the time and effort involved in screening at the adult stage would be saved, most importantly, the number of accessions that can be assessed in this short time is considerable.

There are two important findings from this experiment, firstly the finding of high tolerance accessions which grow well at high salinity, and secondly that the seedling test is capable of detecting tolerant material after 15 days growth.

The 10 accessions of *T. resupinatum*, and *T. alexandrinum*, have been shown to be considerably more tolerant than the other accessions in the other species. This was

indicated in Chapter 2. Clearly the possibility of increasing salinity tolerance through selection is there, and combining this with quality in grazing and quality factors, may lead to the development of very useful lines in these two species, at least as salinity tolerant as the tolerance accepted currently in *T. alexandrinum*.

CHAPTER FOUR

SELECTION TO IMPROVE AND HERITABILITY OF SALINITY TOLERANCE IN THREE *Trifolium* SPECIES

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CHAPTER FOUR

SELECTION TO IMPROVE AND HERITABILITY OF SALINITY TOLERANCE IN THREE *Trifolium* SPECIES

4.1. INTRODUCTION

The basis of plant evolution in nature, and that controlled by man (plant breeding) is fundamentally the same, presence of variation, selection process, useful and genetically based variation, in attempting to produce salinity tolerant crop materials. This should be considered and clearly addressed (McNeilly 1990).

In classic plant breeding programmes, selection is carried out on large populations normally under field conditions and to a remarkable degree, they have produced and improved germplasm. Crop plants exhibit salt tolerance at germination and at later stages of growth (Maas 1986). Some of the most salt tolerant agricultural crops (e.g. sugarbeet, barley, and cotton) are more sensitive during germination or early seedling growth than they are at later growth stages. In contrast, maize, peas, and beans are more sensitive during later stages of development (Subbarao and Johansen 1994).

It has been argued that, usually, mature plants are the most salt tolerant. Germinating seeds and seedlings may require less saline conditions for the initial development of the plant (Poljakoff-Mayber and Gale 1975, Pasternak *et al.* 1979, and Nobel 1983). For example, seedlings produced under non-saline conditions can subsequently be transplanted into saline soils (National Research Council 1990). It has been found that clover is more sensitive to salinity stress at germination and early seedling growth than at later growth stages (West and Taylor 1981, Nobel *et al.* 1984, and Rogers and Noble 1991). Assessment of the salinity response at germination and early seedling

growth would be easier, provided there is a clear relationship between early seedling growth and whole plant response. The use of simple measures of root growth or shoot length have revealed genetically based variation in response to salinity (Ashraf *et al.* 1987, McNeilly 1990, and Al-Khatib *et al.* 1993), and these authors have demonstrated that using this approach of screening large numbers of seeds, successful selection leading to tolerant adult plants can be effected. Other more frequent selection used seedlings or plants grown in the presence of high concentrations of sea water or NaCl, followed by selection to select and maintain the survivors (Moshe 1985). Any genetic expression of salt tolerance was determined either by analyses of the tolerance to salt of the vegetative or seed progeny of the survivors, by crossing selected and unselected plants and examining their progeny (Norlyn 1980), or using quantitative genetics procedures such as the North Carolina Model II of Comstock *et al.* (1949), used by Al-Khatib *et al.* (1993) for examining the genetic basis of salinity tolerance in alfalfa.

Because of the consistent growth at different salinity levels within accessions it seems reasonable to conclude that differences between accessions are probably genetically based to some degree. Based upon this assumption, selection for enhanced salinity tolerance should be possible in these species.

Evidence about the existence of variability for salt tolerance in seven species of *Trifolium* germplasm shown in the proceeding chapter has fulfilled the first prerequisite. Clearly for effective progress in improving the salinity tolerance by exploiting the available variability in the species, information about the second i.e. genetic component, is necessary. Previous studies on the genetic basis of salt tolerance in clover, as well as in other crop species relative to information about the occurrence

of variation in salt tolerance, are relatively few in number. The available evidence, for example, in citrus rootstocks (Furr and Ream 1969), sorghum (Ratanadilok *et al.* 1978 and Azhar 1988), rice (Moeljopawiro and Ikehashi 1981), millet (Kebebew 1994), and corn (Rao 1997), reveals that salt tolerance in these species is predominantly under genetic control. In *Medicago sativa* salt tolerance is highly heritable (Noble *et al.* 1984) and significant improvement was made after two generations of selection, whilst Allen *et al.* (1985) working with the same species found broad sense heritability of the character at the germination stage to be 0.50. Increased salt tolerance appears also to be possible through selection in seven grass and four forage species (Ashraf *et al.* 1986b, 1987) due to the genetic basis of variability.

The work reported in this chapter was therefore to examine heritability of variation for salt tolerance of three *Trifolium* species reported in Chapter 2, and undertaken to assess to what extent selection could further improve the salinity, of *T. alexandrinum* and *T. resupinatum*, and to increase tolerance in *T. repens*.

4.2. MATERIALS AND METHODS

4.2.1. Heritability estimates for variation in salinity tolerance

The data used for the present investigations are those used for the assessment of salinity response of 30 *Trifolium* accessions to seven NaCl + $CaCl_2$ increasing concentrations, measuring the characters reported in the preceding chapter.

For the estimation of heritability of salt tolerance, the indicator of response was a measurement of the shoot and root lengths of two-week-old seedlings.

Estimates of broad sense heritability were made following Falconer and Mackay (1996), based on between and within accessions of the three species in both shoot and root lengths. The data of 30 seedlings of the 30 accessions assessed under each salinity concentration were analysed using an analysis of variance which partitioned total variances. Broad sense heritability (h^2_B) was estimated from:

$$h^2_B = V_G / V_P$$

$$V_G = V_P - V_E$$

V_G = Genetic Variance
V_P = Between accession variances comprised genetic and environmental components
V_E = Within accession variance comprised an environmental component

The heritability can range from 0 to 1. A broad-sense heritability of 0 indicates that none of the variation in phenotype among individuals results from genetic differences. A heritability of 0.5 means that 50 percent of the phenotypic variation arises from genetic differences among individuals, and a heritability of 1 would suggest that all the phenotypic variance is genetically based.

4.2.2. Experiment 1. Selection for increased salinity tolerance

Seed of three species *Trifolium resupinatum*, *T. alexandrinum*, and *T. repens* kindly supplied by the Institute of Forests and Rangelands through the Ministry of Jihad Sazandegi, Islamic Republic of Iran, were used in these experiments. They had been identified as the most tolerant accessions of each species, Accession 1 from *T. alexandrinum*, Accession 9 from *T. resupinatum*, and Accession 4 from *T. repens* in the screening experiment reported in Chapter 2.

Seed samples of the three species were surface sterilised in 5% v/v sodium hypochlorite (BDH) for two minutes. Approximately 2,500 seeds of each species were then sown on four layer deep rafts of black alkathene beads in three replications of clear plastic containers 23 cm × 23 cm × 10 cm deep. This gave a total of 7,500 seeds per species. Each container contained 2.5 l of half strength nutrient solution following Rorison in Hewitt (1966). The salinity levels of the NaCl + $CaCl_2$ solution chosen for selection were also based upon results given in Chapter 2, namely those giving maximum inhibition of shoot and root growth. *T. alexandrinum* and *T. resupinatum* were therefore screened in solutions with $EC_{(25)}$ = 26 and 29 dSm^{-1}, whereas *T. repens* was screened in solutions with $EC_{(25)}$ = 18 and 20 dSm^{-1}. Control seeds were sown and grown in non-saline solution $EC_{(25)}$ = 0.32 dSm^{-1}. Solutions were not changed during the experiment because it would be impossible to do so since the seedlings would become tangled amongst beads, and would not re-float with refilling the containers. The small amount of shoot and root growth occurring in such concentration is such that their impact upon solution and salt concentrations would be negligible.

The experiment was set up as a completely randomised block design in a controlled environment room at 23 ± 1°C with 16 hour day length at light intensity of 27 Wm^{-2} with relative humidity of approximately 80%.

After two weeks, the 15 largest seedlings from each replication of each of the three species, (giving a total of 45 seedling per species) were then selected (S_1 generation genotypes) and grown in the same growth room conditions as the screening experiment in non-saline nutrient solution for two weeks with aeration to promote growth. After a further two weeks growth, each of the 45 plants of each species were transferred to John Innes potting soil in 15 cm plastic pots, one plant per pot, and were grown in a glasshouse with a 16 hour photoperiod, natural daylength being supplemented using 400 Watt mercury vapour lamps, and a temperature range of 16-30°C.

When the selected plants commenced flowering, plants of each species were isolated in separate white muslin net chambers (150 cm × 90 cm × 100 cm) to avoid unwanted pollination by insects. The parent plants from each species were randomly inter-crossed by hand. Hand pollination was effected using a small triangular piece of cardboard to retain the pollen from tripped flowers /inflorescences and transferring it to the stigma of the other non-tripped flowers on different plants per each species separately. The crossed inflorescences were covered immediately with small non moisture-proof glassine bags for at least three days to avoid contamination by extraneous pollen and to allow the fertilisation process to be completed (Sayers and Murphy 1966). The bags were then removed to allow seeds to mature (about 10 weeks). The parent plants were grown in the same glasshouse conditions until seeds

were mature. Seed from each of the plants of the three species was harvested separately.

The S_1 generation progeny seeds obtained from these crosses were then used in two further experiments, Experiment 2, to assess response of the selected material to salinity, and Experiment 3, a second cycle of selection for increased salinity tolerance.

4.2.3. Experiment 2. Response to salinity of progeny: Screening test (S_1 generation).

Approximately 450 seeds collected from the individuals selected in Experiment 1 from each species were surface sterilised as in Experiment 1, and again as in Experiment 1, sown in 300 ml plastic beakers containing half strength Rorison nutrient solution, with three replications. There were two EC treatments for *T. alexandrinum* and *T. resupinatum*, namely EC = 26 and 29 dSm^{-1}, and two for *T. repens* $EC_{(25)}$ = 18 and 20 dSm^{-1}. Seeds were also sown on control non-saline solution $EC_{(25)}$ = 0.32 dSm^{-1}. The experiment was again a completely randomised block design and the conditions in the growth room were as in Experiment 1. Again, after two weeks growth, shoot and root lengths of 10 plants per species in each EC treatment in each replication were measured, and the shoot and root lengths are shown in Figures 4.1 to 4.3.

4.2.4. Experiment 3.

4.2.4.1. Second cycle of selection

5,000 seeds of *T. alexandrinum*, and 4,000 seeds of *T. resupinatum*, from the polycross progeny of S_1 adults resulting from Experiment 1 selected at $EC_{(25°C)} = 29$ dSm^{-1}, were sown on alkathene beads in clear rigid plastic containers as used in Experiment 1, with two replicates. There were two treatments, EC 29, and EC 32, with standard control EC 0.32 dSm^{-1}.

3,400 seeds of *T. repens* selected at EC 20 in Experiment 1 were sown following the same procedure as for *T. alexandrinum* and *T. resupinatum* but with 1,700 seeds in each of the two replicates, and treatment levels of EC 20, and EC 22 dSm^{-1} again with the control at $EC_{(25)} = 0.32$ dSm^{-1}.

T. alexandrinum and *T. repens* produced roots and shoots at EC 29 and 20 respectively, but they failed to grow at EC 32 and EC 22 dSm^{-1} respectively. After two weeks the 40 tallest seedlings of *T. resupinatum* were selected at EC 32 dSm^{-1}, 20 from each replicate and placed in aerated nutrient solution for 1-2 weeks in the same growth room conditions as Experiment 1 and 2.

35 of these seedlings were then transferred into John Innes potting compost in 15 cm pots, and grown on to maturity in glasshouse conditions as described for Experiment 1. When flowering began, each plant was isolated by white muslin cloth from its neighbours, in chambers to avoid unwanted pollination by bees. Random polycross pollinations were subsequently made manually, and a total of 2,720 seeds were produced.

4.2.4.2. Screening test S_2 seeds

Again approximately 450 seeds of *T. resupinatum* from Experiment 3 were used for screening test, as described in Experiment 1 and 2. There were two EC treatments for *T. resupinatum* namely $EC_{(25)}$ = 29 and 32 dSm^{-1}. Seeds were also sown on control non-saline solution $EC_{(25)}$ = 0.32 dSm^{-1}. The experiment was designed, grown in conditions, and grown as described for Experiments 1 and 2, and harvesting made again as in Experiments 1 and 2.

4.3. RESULTS

4.3.1. Estimation of broad sense heritability (h^2_B)

The mean squares of all three species from different salinity levels (Tables 4.1, 4.3, and 4.5) show that increasing NaCl + $CaCl_2$ concentration had deleterious effects on both shoot and root lengths. There were highly significant ($P \leq 0.0001$) differences among the accessions in all three species. Increasing EC levels in culture solution produced a range of responses reflecting varying tolerances between species to salinity. The accessions of *T. repens* did not survived in the EC 22 and 26 dSm^{-1} (Table 4.5).

Broad sense heritability (h^2_B) was estimated from analysis of variance over all salinity levels. For *T. alexandrinum* (Table 4.2), the highest estimates of h^2_B value were at EC 26 for both shoot 0.81 and root 0.84, and the lowest values were at EC 10 dSm^{-1} for shoot length 0.35, and at EC 14 for root length 0.26. For *T. resupinatum* (Table 4.4), the data suggest that a reasonable proportion of differences to salinity are genetically determined at the range from 0.71 at EC 22, to 0.23 at EC 8 for shoot length, and 0.64 at EC 18 to 0.43 at EC 14. For *T. repens* (Table 4.6), the highest h^2_B value was at EC 10 for both shoot 0.68 and root 0.63, and the lowest was at EC 18 dSm^{-1} for both shoot 0.30, and root 0.26.

Table 4.1. Mean squares (MS) from analysis of variance of absolute shoot and root length of individual seedling of *T. alexandrinum* at each NaCl + $CaCl_2$ levels.

Items		Df	Control	EC = 4	EC = 8	EC = 10	EC = 14	EC = 18	EC = 22	EC = 26	Expected MS
Shoot	Between accessions	9	2664.06***	1195.20***	3154.92***	1821.22***	3347.46***	3326.99***	2072.36***	1317.94***	$V_w + 30V_b$
	Within accessions	290	183.24	161.39	107.57	104.51	128.42	101.54	31.50	10.18	V_w
Root	Between accessions	9	12602.48***	24829.41***	15551.05***	5660.81***	4110.44***	7463.17***	4449.80***	1340.51***	$V_w + 30V_b$
	Within accessions	290	491.06	555.22	507.88	373.55	359.90	201.18	51.01	8.74	V_w

Table 4.2. Components of variance, and broad sense heritabilities (h^2_B) of salinity tolerance in *T. alexandrinum* at each salinity levels.

Components		Control	EC = 4	EC = 8	EC = 10	EC = 14	EC = 18	EC = 22	EC = 26
Shoot	$V_b = V_G$	82.69	344.60	101.58	57.22	107.30	107.52	68.03	43.59
	$V_P = V_b + V_w$	265.93	505.99	209.15	161.73	235.72	209.06	99.53	53.77
	$h^2_B = V_G / V_P$	**0.31**	**0.68**	**0.49**	**0.35**	**0.46**	**0.51**	**0.68**	**0.81**
Root	$V_b = V_G$	403.71	825.81	501.44	176.24	125.02	242.07	146.62	44.39
	$V_P = V_b + V_w$	894.78	1381.03	1009.32	549.79	484.92	443.25	197.63	53.13
	$h^2_B = V_G / V_P$	**0.45**	**0.60**	**0.50**	**0.32**	**0.26**	**0.55**	**0.74**	**0.84**

Table 4.3. Mean squares (MS) from analysis of variance of absolute shoot and root length of individual seedling of *T. resupinatum* at each NaCl + $CaCl_2$ levels.

Items		Df	Control	EC = 4	EC = 8	EC = 10	EC = 14	EC = 18	EC = 22	EC = 26	Expected MS
Shoot	Between accessions	9	2753.08***	2333.97***	2577.15***	2472.06***	4286.63***	6929.49***	3188.82***	933.18***	$V_w + 30V_b$
	Within accessions	290	235.05	213.50	258.51	174.32	114.16	110.53	41.89	44.69	V_w
Root	Between accessions	9	16249.95***	45516.01***	37216.78***	18345.66***	8607.86***	9815.06***	1678.22***	766.91***	$V_w + 30V_b$
	Within accessions	290	430.30	880.84	724.76	549.17	370.06	182.19	40.74	32.11	V_w

Table 4.4. Components of variance, and broad sense heritabilities (h^2_B) of salinity tolerance in *T. resupinatum* at each salinity levels.

Components		Control	EC = 4	EC = 8	EC = 10	EC = 14	EC = 18	EC = 22	EC = 26
Shoot	$V_b = V_G$	83.93	70.68	77.29	76.59	139.08	227.30	104.89	29.62
	$V_P = V_b + V_w$	318.98	284.18	335.80	250.91	253.24	337.83	146.79	71.31
	$h^2_B = V_G / V_P$	**0.26**	**0.25**	**0.23**	**0.31**	**0.55**	**0.67**	**0.71**	**0.42**
Root	$V_b = V_G$	527.32	1487.84	1216.40	593.22	274.59	321.10	54.58	24.49
	$V_P = V_b + V_w$	957.62	2368.68	1941.17	1142.39	644.65	503.29	95.32	56.80
	$h^2_B = V_G / V_P$	**0.55**	**0.63**	**0.63**	**0.52**	**0.43**	**0.64**	**0.57**	**0.43**

Table 4.5. Mean squares (MS) from analysis of variance of absolute shoot and root length of individual seedling of *T. repens* at each $NaCl + CaCl_2$ levels.

Items		Df	Control	EC = 4	EC = 8	EC = 10	EC = 14	EC = 18	EC = 22	EC = 26	Expected MS
Shoot	Between accessions	9	4175.12***	2496.81***	3264.33***	3674.06***	1821.94***	226.96***	0	0	$V_w + 30V_b$
	Within accessions	290	78.22	41.81	55.01	57.69	40.36	16.35	0	0	V_w
Root	Between accessions	9	2940.54***	5691.99***	6726.92***	8860.63***	3060.53***	191.07***	0	0	$V_w + 30V_b$
	Within accessions	290	228.13	206.82	230.71	184.73	76.51	16.69	0	0	V_w

Table 4.6. Components of variance, and broad sense heritabilities (h^2_B) of salinity tolerance in *T. repens* at each salinity levels.

Components		Control	EC = 4	EC = 8	EC = 10	EC = 14	EC = 18	EC = 22	EC = 26
Shoot	$V_b = V_G$	136.56	81.83	106.98	120.55	59.39	7.02	-	-
	$V_P = V_b + V_w$	214.78	123.64	161.98	178.24	99.75	23.37	-	-
	$h^2_B = V_G / V_P$	**0.64**	**0.66**	**0.66**	**0.68**	**0.60**	**0.30**	**-**	**-**
Root	$V_b = V_G$	90.41	182.84	216.54	289.20	99.47	5.81	-	-
	$V_P = V_b + V_w$	318.54	389.66	447.24	473.93	175.98	22.50	-	-
	$h^2_B = V_G / V_P$	**0.28**	**0.47**	**0.48**	**0.63**	**0.57**	**0.26**	**-**	**-**

4.3.2. Results Experiments 1 and 2

Increasing [NaCl + $CaCl_2$] concentration caused, as expected, a significant reduction ($P \leq 0.001$) in shoot and root growth of the three species, (Table 4.7 and Figures 4.1 to 4.3). There were highly significant differences in shoot and root growth ($P \leq 0.0001$) between control and $EC_{(25)}$ = 26, and 29 dSm^{-1} for *T alexandrinum* and *T. resupinatum*, and there were significant differences ($P \leq 0.0001$) between the control and treatment i.e. EC 18 and 20 dSm^{-1}, for *T. repens*. These of course were expected.

Growth of *T. alexandrinum* and *T. resupinatum* was stopped at solution conductivity EC 29 dSm^{-1} in the experiment described in Chapter 2, but in this experiment it was possible to select 45 (the largest) seedlings from screening approximately 7,500 seeds at EC 29 dSm^{-1}.

The 45 plants per species were transplanted into John Innes compost, and grown in normal glasshouse conditions. Random polycrosses were made by hand to produce seeds for a further cycle of selection.

The most tolerant accession of the sensitive species, *T. repens*, was subjected to the same procedure as used for *T. alexandrinum* and *T. resupinatum*, but the salinity levels were $EC_{(25)}$ = 18 and 20 dSm^{-1}. 450 seeds were again used for screening for salt tolerance in Experiment 2. As in shoot and root lengths shown in Table 4.8, there were highly significant differences ($P \leq 0.001$) between concentrations of EC 18, 20 dSm^{-1} and control ($EC_{(25)}$ = 0.32 dSm^{-1}) of *T. repens*. The remaining seeds from Experiment 1 were used for Experiment 2.

The frequency distribution of shoot lengths of 30 seedlings subsamples at EC 26 and 29 dSm^{-1} from *T. alexandrinum* and *T. resupinatum*, indicate the presence of variation in salinity tolerance within these two species. The most interesting finding in these data is that at EC 29 dSm^{-1} *T. resupinatum* produced shoot and root lengths from 5 to 35 mm, and *T. alexandrinum* produced from 5 to 15 mm grown in the same concentration (Figures 4.1 and 4.2).

Table 4.7. Mean squares and significances from the analyses of variance of shoot and root lengths of seedlings of *T. alexandrinum* and *T. resupinatum* seedlings grown for 14 days at three salinity levels in solution culture.

a) Shoot length

Sources	DF	Mean Squares	P < F
Blocks	2	21.74 **ns**	0.80
EC dSm^{-1}	2	71465.41***	0.0001
Species (SPP)	1	2993.09***	0.0001
SPP × EC	2	107.67 **ns**	0.34
Residual	172	99.33	

b) Root length

Sources	DF	Mean Squares	P < F
Blocks	2	37.76 **ns**	0.70
EC dSm^{-1}	2	71377.17***	0.0001
Species (SPP)	1	8080.20***	0.0001
SPP × EC	2	3142.62***	0.0001
Residual	172	105.06	

Table 4.8. Mean squares and significances from the analyses of variance of shoot and root lengths of seedlings of *T. repens,* seedlings grown at three salinity levels in solution culture for 14 days.

a) Shoot length

Sources	DF	Mean Squares	P < F
Blocks	2	17.91 **ns**	0.71
EC dSm^{-1}	2	23128.74***	0.0001
Residual	85	51.70	

b) Root length

Sources	DF	Mean Squares	P < F
Blocks	2	6.71 **ns**	0.92
EC dSm^{-1}	2	376556.81***	0.0001
Residual	85	77.75	

Figure 4.1. Frequency distribution of shoot and root length of 30 two-weeks-old seedlings of *T. alexandrinum* (S_1) at two salinity levels compared with control. Mean of shoot and root lengths differed significantly across salinity levels ($P \leq 0.05$).

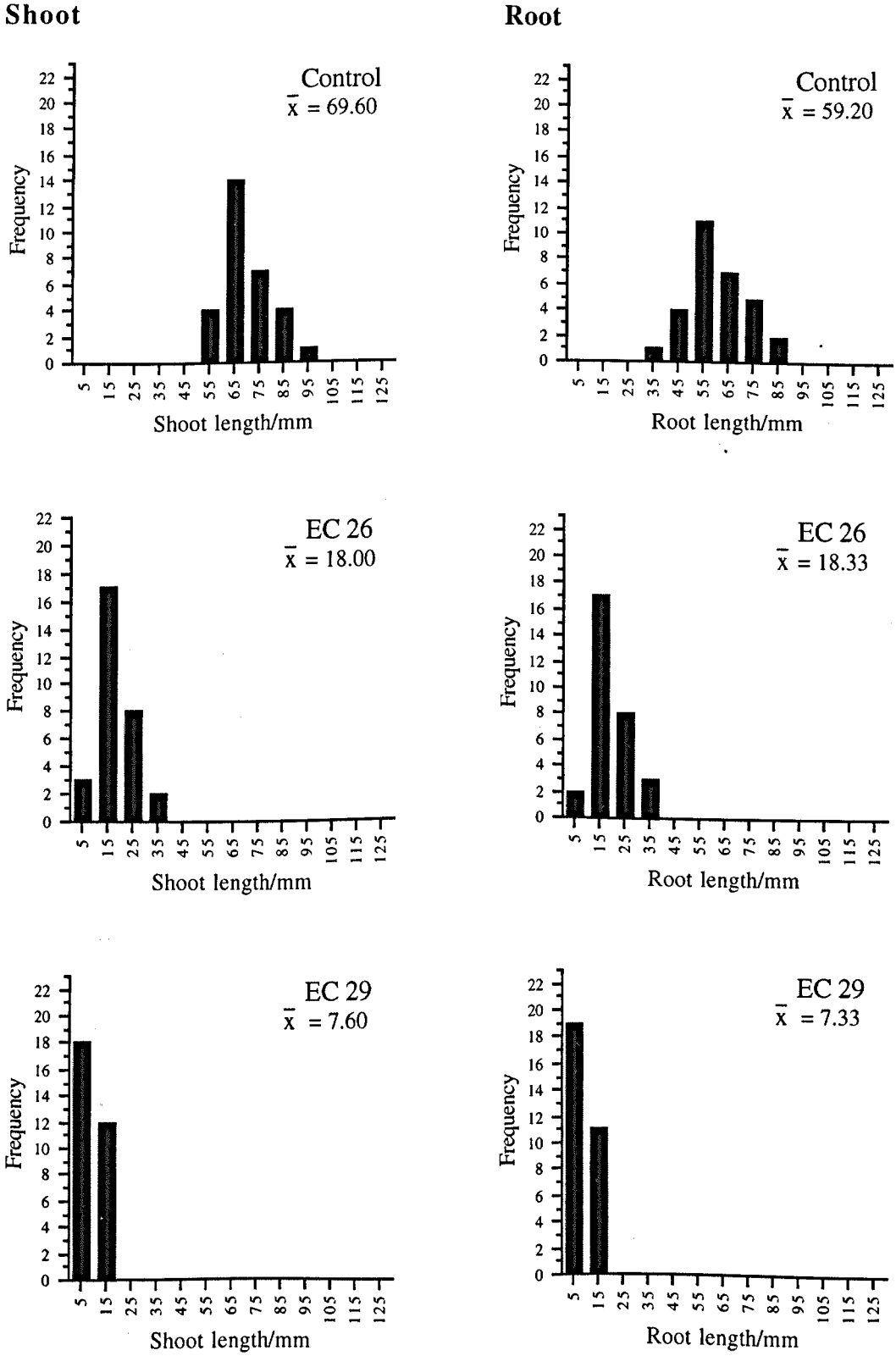


Figure 4.2. Frequency distribution of shoot and root length of 30 two-weeks-old seedlings of *T. resupinatum* (S_1) at two salinity levels compared with control. Mean of shoot and root lengths differed significantly across salinity levels ($P \leq 0.05$).

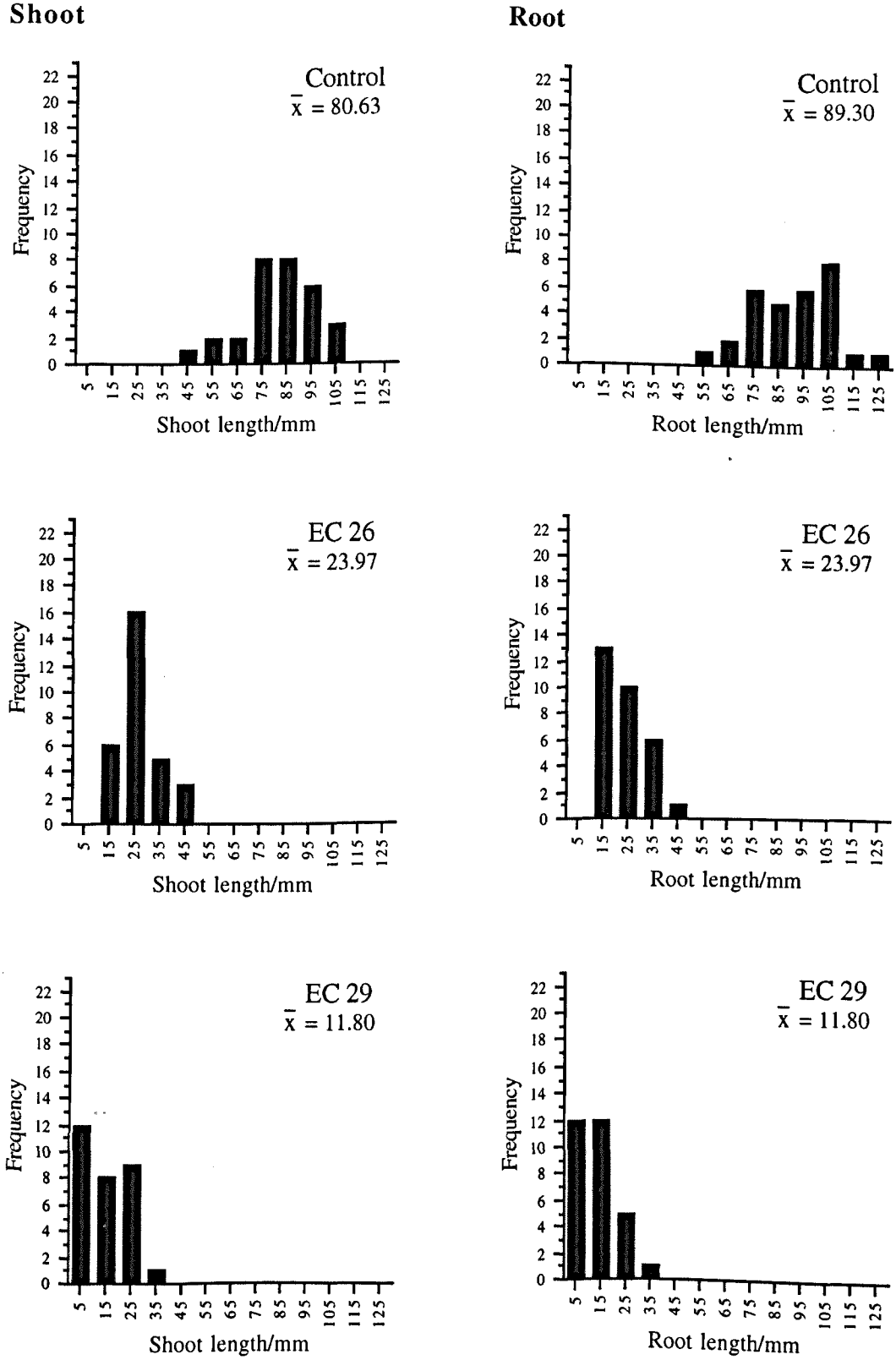
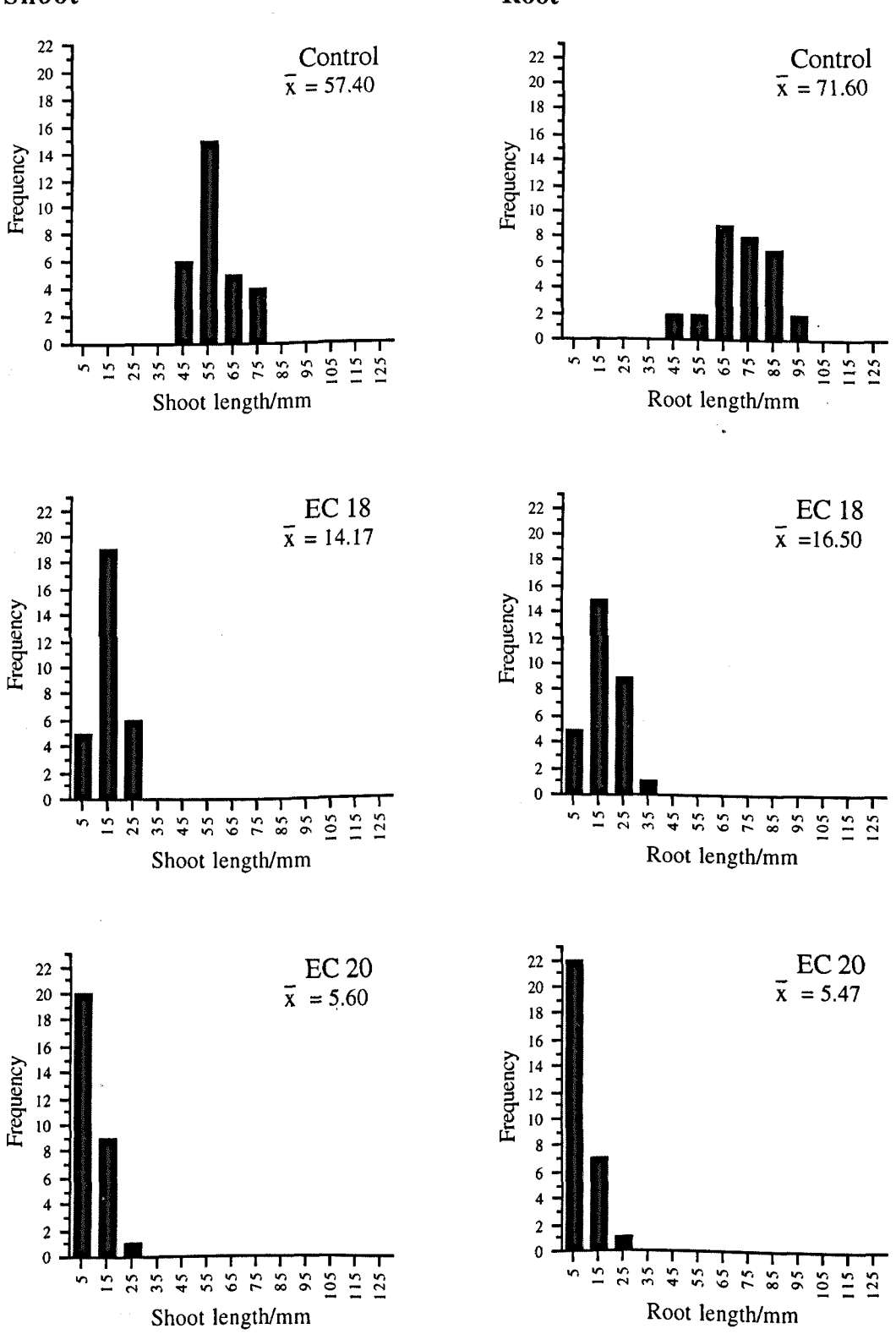


Figure 4.3. Frequency distribution of shoot and root length of 30 two-weeks-old seedlings of *T. repens* (S_1) at two salinity levels compared with control. Mean of shoot and root lengths differed significantly across salinity levels ($P \leq 0.05$).



4.3.3. Results Experiment 3

The result of analyses of variances using shoot and root length data from *T. resupinatum* (S_2 generation) are presented in Table 4.9, and mean shoot and root lengths of original and two generations selected for increased salinity tolerance in three species are also presented in Figures 4.7 to 4.9.

Shoot growth of the S_1 generations of *T. alexandrinum* occurred at EC 29, and shoot growth of *T. repens* occurred at EC 22 dSm^{-1}. Shoot growth of *T. alexandrinum* and *T. repens* was completely inhibited at EC 32 and EC 20 dSm^{-1} respectively. For the S_2 generation of *T. resupinatum* however, shoot growth continued at both EC 29 and EC 32 dSm^{-1}. Treatments had significant effects ($P \leq 0.001$) on shoot growth, reducing it markedly. There were no significant differences ($P > 0.05$) between control values in Experiments 2 and 3 for *T. resupinatum* but there were highly significant differences ($P \leq 0.01$) between mean shoot lengths and root lengths at EC 26 dSm^{-1} for S_1 and S_2 generations. This experiment has shown that it was possible to select for increased salinity tolerance in *T. resupinatum*, exploiting the genetic variability within the accessions examined.

Table 4.9. Mean squares and significances from the analysis of variance of shoot and root lengths of *T. resupinatum* seedlings (S_2) grown at EC 29 and 32 dSm^{-1}.

Accession	Sources	DF	Mean Squares	P < F
Shoot	Concentration	2	45476.40 ***	0.0001
	Error	87	123.72	
Root	Concentration	2	61184.41 ***	0.0001
	Error	87	120.82	

Figure 4.4. Frequency distribution of shoot and root lengths of 30 two-weeks-old S_2 generation seedlings of *T. alexandrinum* grown at two salinity levels plus control.

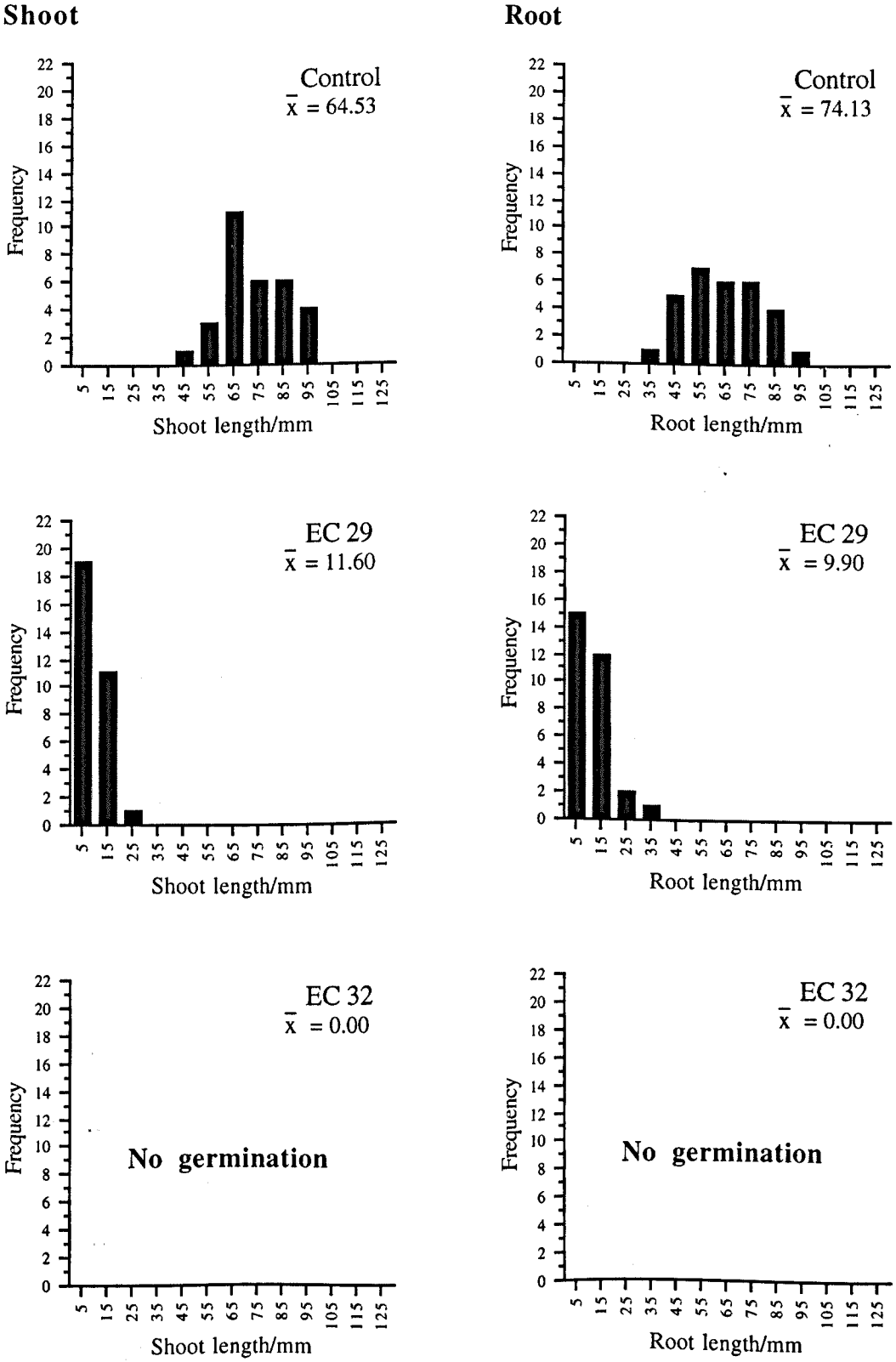


Figure 4.5. Frequency distribution of shoot and root lengths of 30 two-weeks-old S_2 generation seedlings of *T. resupinatum* grown at two salinity levels plus control.

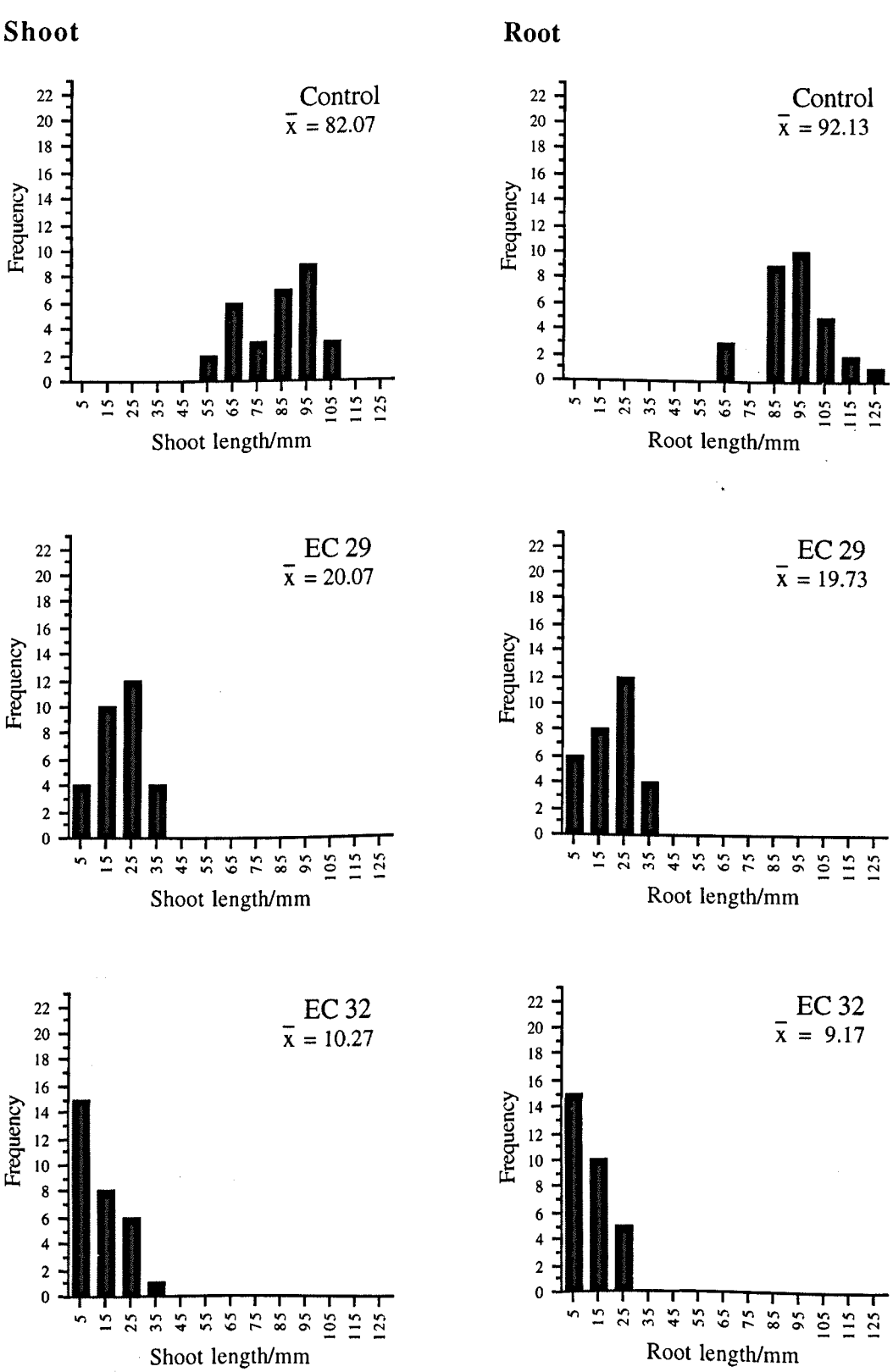


Figure 4.6. Frequency distribution of shoot and root lengths of 30 two-weeks-old S_2 generation seedlings of *T. repens* grown at two salinity levels plus control.

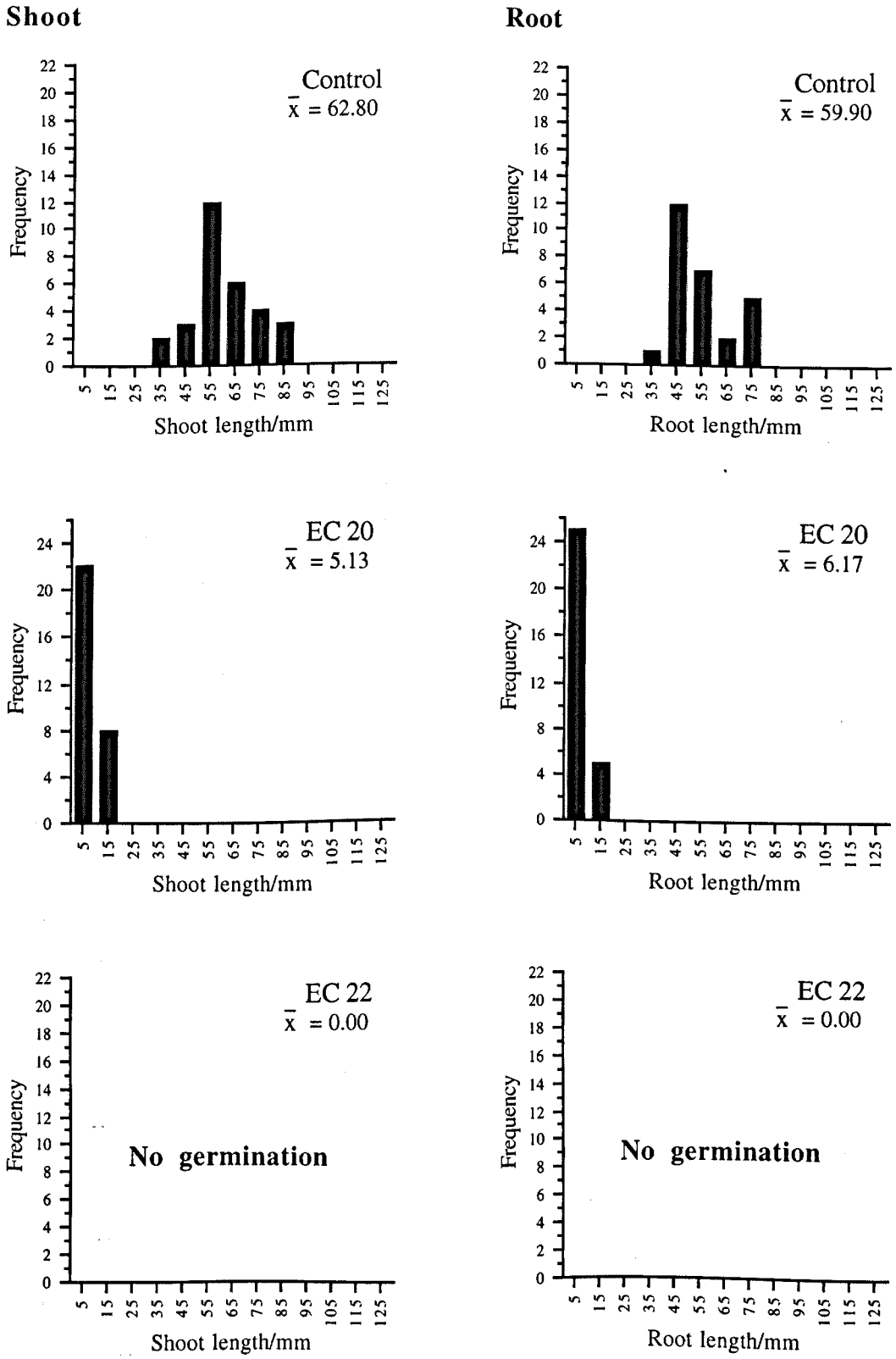
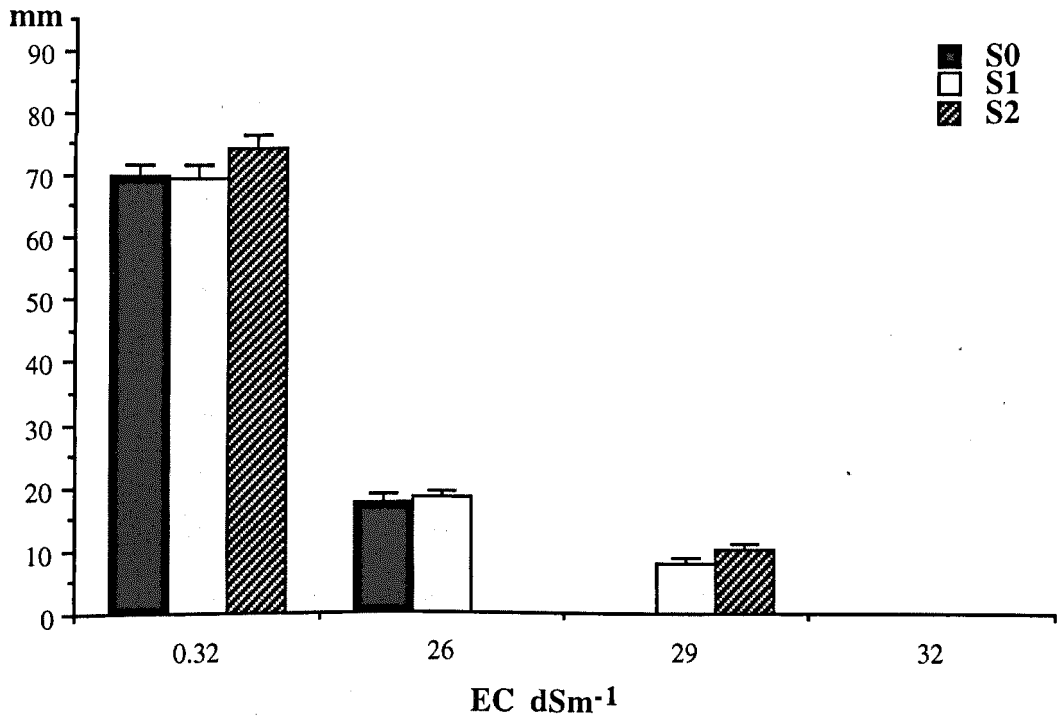


Figure 4.7. Absolute means for shoot and root length values of original, S_0, and selected S_1 and S_2 generations receptively, for increased salinity tolerance in *T. alexandrinum*

a) Shoot length



b) Root length

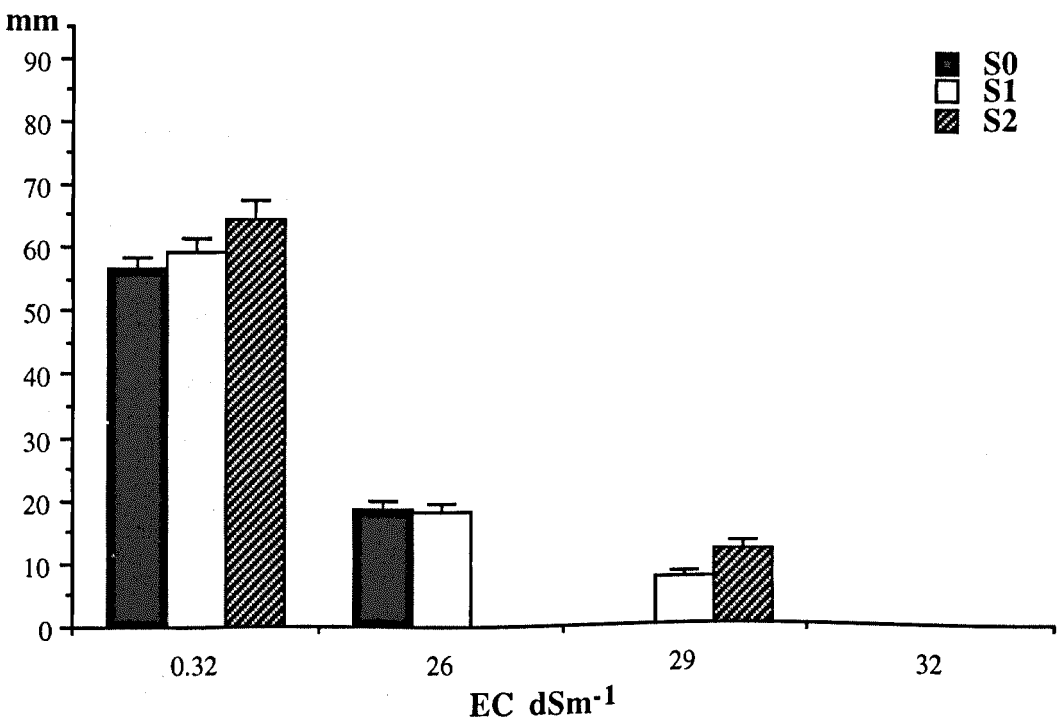
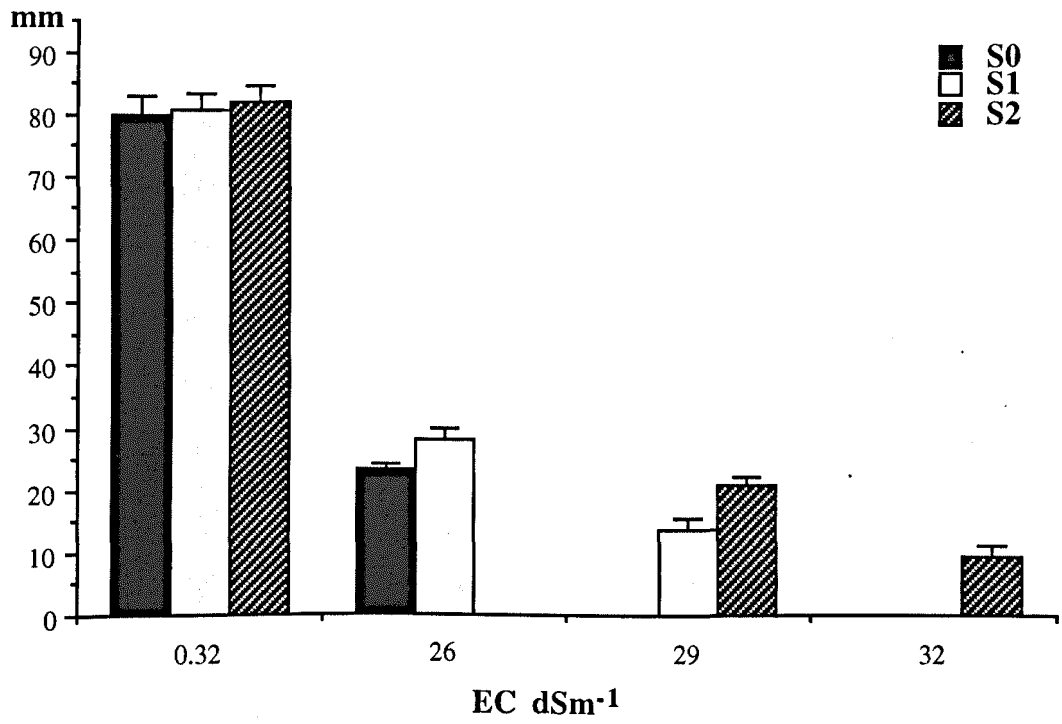


Figure 4.8. Absolute means for shoot and root length values of original, S_0, and selected S_1 and S_2 generations receptively, for increased salinity tolerance in *T. resupinatum*

a) Shoot length



b) Root length

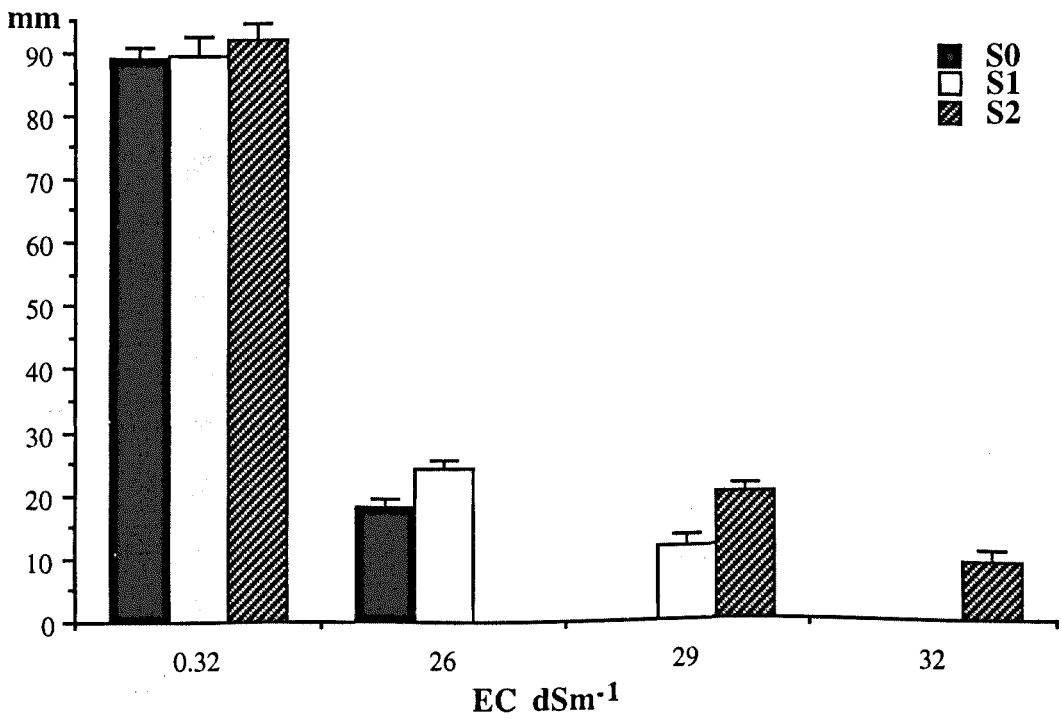
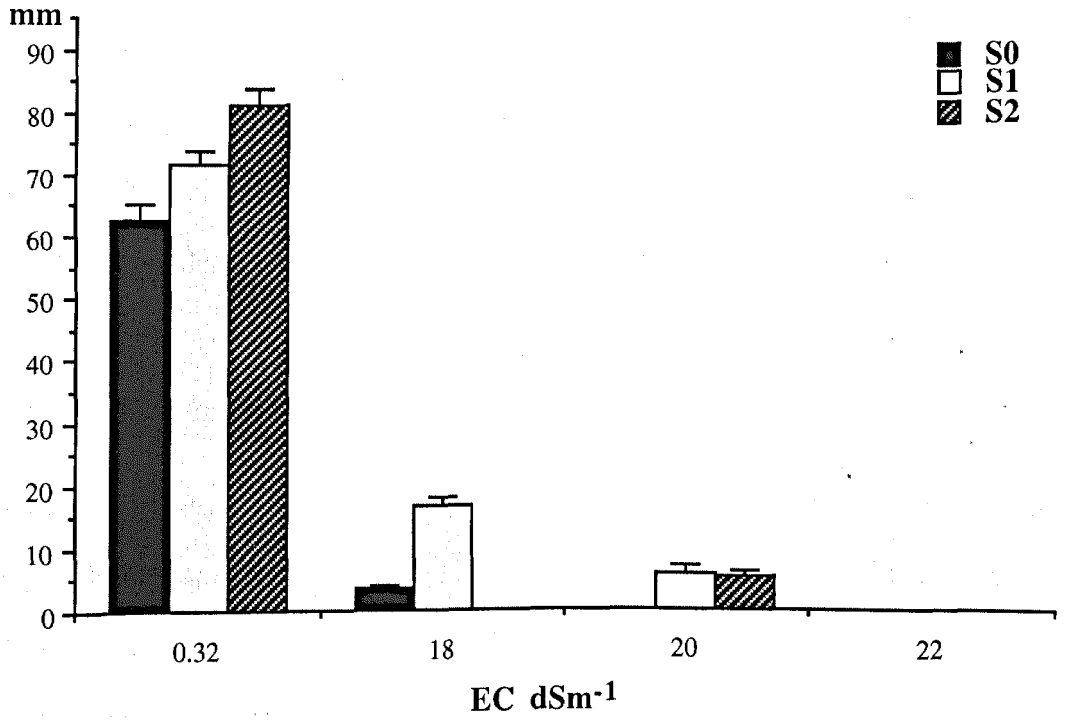
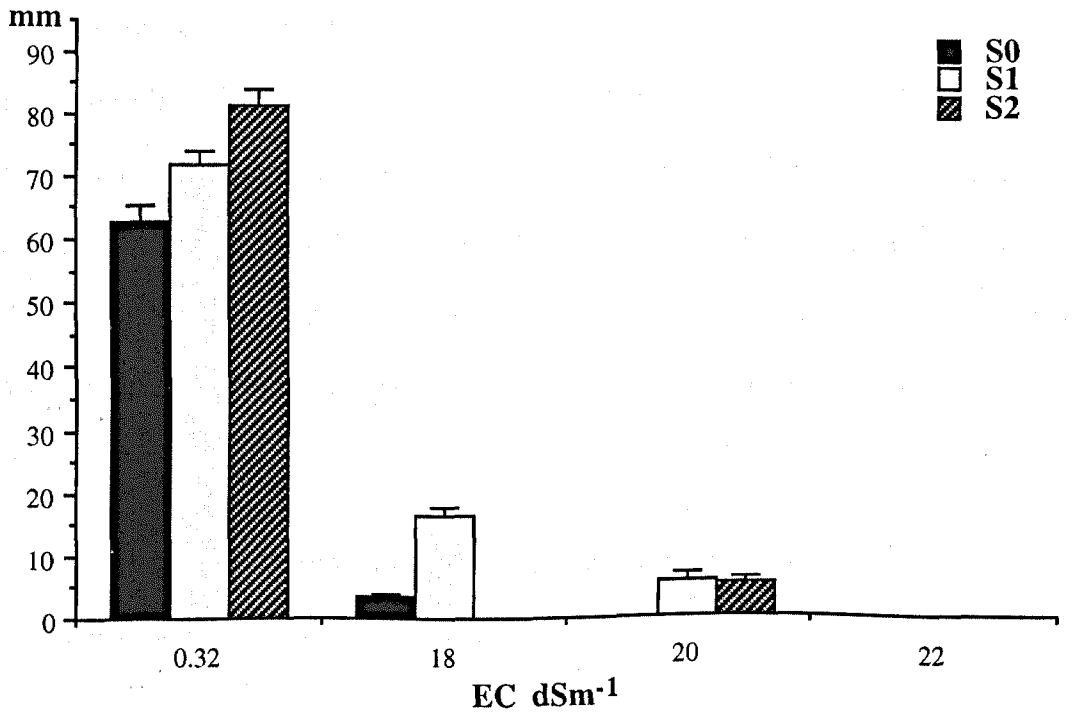


Figure 4.9. Absolute means for shoot and root length values of original, S_0, and selected S_1 and S_2 generations receptively, for increased salinity tolerance in *T. repens.*

a) Shoot length



b) Root length



4.4. DISCUSSION

Although saline soils are predominantly affected by Na^+ or Cl^- ions, cations of other salts, particularly of Ca^{2+}, are also of frequent occurrence (Shannon 1984). This investigation was carried out to determine whether within the accessions from, the most tolerant of the three species, namely *T. alexandrinum*, *T. resupinatum* and *T. repens* (Chapter 2), selection for increased salinity tolerance could be successful, and whether their degree of tolerance may be increased to allow this material to be used as a source for development of commercially viable salinity tolerant lines for growing at higher concentrations under field conditions.

It has been argued that selection for salt tolerance at the seedling stage may not confer equivalent tolerance to the adult plant (Shannon 1979, Kingsbury and Epstein 1984). In contrast however, Greenway (1965) and Blum (1985) consider that seedling response to salinity is highly predictive of adult plant response to salinity, and Norlyn (1980) and Kingsbury and Epstein (1984), working with barley and wheat respectively, screened seedlings of these two species and obtained fully salt tolerant adult individuals from that selection. For a number of species, seedling and early vegetative growth stages have been shown to be the most sensitive, with subsequent stages showing increased tolerance (Maas 1985 and 1986, Maas and Poss 1989, Azhar and McNeilly 1989). It would seem however that there are differences between species as to whether seedling salinity tolerance is conferred upon the mature growing plants.

More recently in the 1990's, attention has be given to the possibility of exploiting seedling selection for salinity tolerance.

Simple measurements such as root and shoot length and dry weight, germination percentage and germination rate, percentage of dead and live leaves, and seedlings survival, have been used successfully as selection criteria in a number of crop plants, e.g. alfalfa (Thamir *et al.* 1992, Al-Khatib *et al.* 1993), rice (Yeo *et al.* 1990, Lutts *et al.* 1995), sorghum (Azhar and McNeilly 1987, Abdel-Hamid *et al.* 1993), maize (Ashraf and McNeilly 1990), and wheat (Prakash and Sastry 1992, Farida *et al.* 1992, Reggiani *et al.* 1995). From such work, and the results reported in this chapter, it appears that high intensity selection for salt tolerance is a practical method of detecting individuals within forage legume species that are able to grow in moderate to highly saline soils. The effectiveness of a screening procedure is measured by transfer of tolerance to the progeny (Shannon 1985).

This was approached in this chapter in two ways. First to determine of genetic basis of variation observed in shoot and root lengths by estimates of heritabilities, and secondly by establishing the presence of tolerance in the progeny of the selected individuals, and assessing tolerance in the seed progeny of those selected individuals.

For selection to be successful it is of course necessary that the variability observed in shoot and root growth is of genetic basis. In clover this has been shown to be the case (Ashraf 1986), and in lucerne (Al-Khatib 1991), based upon estimates of realised heritabilities. Estimates of broad sense heritability (h^2_B) in other crop species with marked differences have been reported, e.g. alfalfa with h^2_B of 0.5 (Allen *et al.*, 1985), seven grass and four forage species with estimates ranging from 0.2 to 0.8, and 0.3 to 0.6 respectively (Ashraf *et al.* 1986b, 1987), lucerne with estimates ranging from 0.2 to 0.6 (Al-Khatib 1991), finger millet and tef estimates ranging from 0.2 to 0.6, and 0.7 to 0.3 respectively (Kebebew 1994), and in *Zea mays* estimates ranging

from 0.1 to 0.5 (Rao 1997). Thus, it has been suggested that a considerable advance in salinity tolerance in these species may be possible using high artificial selection pressures (Ashraf *et al.* 1986b, 1987), as was elegantly shown by Lerner (1985). The relationship between selection progression and character heritability was examined. Lerner compared the effect of disruptive selection after one generation of selection in hens for egg numbers laid in one month, and egg weights. The former had a narrow heritability of 0.25, and the latter 0.75. Mean egg number did not change after one generation of selection, whereas selection for egg weight resulted in significantly different mean egg weight. In the present study although the heritability estimates are broad sense heritability estimates, for *T. alexandrinum*, *T. resupinatum*, and *T. repens* across seven salinity concentrations, h^2_B = 0.26 to 0.84, 0.23 to 0.71, and 0.26 to 0.68 respectively, suggest that prospects of improving the character through selection and breeding are considerable, provided the genetic system controlling the variation is predominantly affected by genes with additive effects. Unfortunately no evidence is available for this at present.

The data from Figures 4.7 to 4.9 show, that improvement in tolerance in the three species examined was possible, especially in *T. resupinatum*. However more cycles of selection, and selection involving all aspects of the saline environment, are necessary before this can be firmly established. The presence of such variation in seed populations has provided the basis for selection for salt tolerance these three species, and the same procedure was used previously for alfalfa (Allen *et al.* 1985), grass species (Ashraf *et al.* 1986a), all based upon seedling selection. Such a procedure has resulted in all these cases in increase in salinity tolerance, and it would seem to have significance in breeding for exploitable salinity tolerance. When seed progeny of the

selected plants were germinated/grown and the seedlings exposed to salt, there were wide variations in response in all three species, but particularly in *T. resupinatum* at EC 29 and 32 dSm^{-1}, where a number of individuals grew at these high salinities. This suggests that the character, salinity tolerance is under polygenic (q.t.l.) control and these selection cycles exploit transgressive segregation for tolerance, suggesting that a number of genes control tolerance.

It thus seems possible, based upon the data presented here, that further significant advances in salinity tolerance in these forage legumes may be achieved by further cycles of selection, with a great probability that this may be achieved if a more diverse genepool could be assessed for tolerance to salinity.

The most important information is the evidence reported in Chapter 3, that these tolerance estimates based upon 14 days growth in solution culture, are maintained in plants grown in saline soil or conditions.

CHAPTER FIVE

INTERSPECIFIC HYBRIDISATION BETWEEN THREE *Trifolium* SPECIES

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CHAPTER FIVE

INTERSPECIFIC HYBRIDISATION BETWEEN THREE *Trifolium* SPECIES

5.1. INTRODUCTION

White clover (*Trifolium repens* L.), is one of the most nutritious and widely distributed forage legumes of the world. History and the presence of diverse forms in the areas indicate that white clover originated in the eastern Mediterranean countries or in Asia Minor. Its spread to other continents was rapid, and apparently was associated with early colonisation by man and the presence of domesticated grazing animals. Historical records indicate that it was one of the first forage plants to form dense stands in pastures that followed cultivated crops or cleared forests. At present, white clover is extensively and effectively used in mixtures or alone. Its contribution to agriculture is threefold.

(1) As a forage legume. It provides a highly nutritive feed as pasture, hay, and silage for livestock and poultry. Although it is usually grown in a mixture with grasses for grazing, it may be seeded alone, particularly for poultry and pigs.

(2) By fixing atmospheric nitrogen. If white clover is effectively inoculated with symbiotic bacteria, the amount of nitrogen made available for associated plants may range from 56 to 280 kg ha^{-1} per year (Gibson and Hollowell 1966). The amount of nitrogen fixed depends on density of stand, growth produced, length and nature of growing season, soil fertility, and degree of effective inoculation.

(3) As a cover crop. The growth of stolons soon after seedlings are well established, provides a ground cover that promotes soil stabilisation and reduces erosion (Gibson and Hollowell 1966).

Berseem clover (*T. alexandrinum* L. 2n = 16) is an important crop throughout the Middle East (Whyte *et al.* 1953) and Iran (Attaran 1989 and Karimi 1990). Berseem flowers are essentially self sterile, although self fertile plants were reported in the cv. Fahl by Putiivsky and Katznelson (1970). Cross pollination is accomplished as in *T. repens* by honeybees (Dennis and Massengale 1962).

Persian clover (*T. resupinatum* L.) is a glabrous, often course, forage crop and an excellent grazing plant (Knight 1985 and Karimi 1990). Britten (1963) recorded that Persian clover had a chromosome count of 2n = 16.

Trifolium is a genus previously thought to exhibit complete cross-incompatibility between species (Keim 1953b). However attempts to produce interspecific hybrids in the genus *Trifolium* have been largely unsuccessful (Wexelsen 1928, Guravich 1949, Trimble 1951, Keim 1952). Natural occurring hybrids of *Trifolium* species have been reported (Ascherson and Graebner 1906-10, Hegi 1925), although the hybrids were not investigated or verified cytogenetically. Several investigators reported successful experimental hybridisation between different species of *Trifolium*, e.g. Wexelsen (1928), Guravich (1949), Trimble (1951), Brewbaker and Keim (1953), and Marshall *et al.* (1995), *T. repens* and *T. nigrescens*; Keim (1953b) *T. ambiguum* and *T. hybridum*; Williams (1978, 1990), Williams and Verry (1981), Yamada and Fukuoka (1985, 1986), and Meredith *et al.* (1995), *T. ambiguum* and *T. repens*.

White clover, *T. repens* is a tetraploid (2n = 32 chromosomes) perennial species that is normally self-incompatible (Ahlgren and Hill 1940). The presence of successful interspecific hybridisation, *T. repens* × *T. nigrescens* Viv. and *T. repens* × *T. uniflorum* L., has opened the door for the plant breeder to exploit variation beyond that which exists within *T. repens* (Pandey 1957, Hovin 1962, and Gibson *et al.* 1971). Other successful hybrids have been obtained from an interspecific cross between *T. alexandrinum* and *T. resupinatum*, but the cross was successful in one direction only (Selim *et al.* 1977).

For commercial seed production, several species of bees and other insects pollinate white clover (Bohart 1960), and Honey and other bees are used in cages to effect pollination to produce the amounts of seed needed in any breeding program.

A number of characteristics of white clover that affect the choice of techniques and methods to be used in developing improved varieties are as follows:

(1) Plants are highly self-incompatible controlled by a series of S alleles.

(2) Plants are highly heterozygous as a result of enforced cross-pollination.

(3) Selfing is possible by using either the S_f gene, or the pseudo-self-compatibility characteristic.

(4) Plants normally flower and set seed every year.

(5) Flowering for making crosses may be induced easily under glasshouse conditions.

(6) Hand pollinations are easy to make and result in good seed set.

(7) Vegetative propagation to the extent needed in a breeding programme is easily done by producing rooting stolons.

(8) Plants of clones may be grown and maintained in glasshouse conditions in 15 cm pots or smaller containers (Gibson and Hollowell 1966), so that large populations, clones, may be produced.

Exploitation of the considerable variation between and within species, can be made possible using within species polyploidy to increase the possibility of fertile interspecific crosses within the genus *Trifolium* between species. For example, a fertile F_1 from the cross 8x = 64 *T. repens* × 4x = 32 *T. nigrescens* was obtained by Brewbaker and Keim (1953), and Hovin (1962). Although these crosses did not contribute to an improved variety, it did provide proof that interspecific crosses were possible in the genus *Trifolium.*

Interspecific hybridisation was attempted by Trimble and Hovin (1960) between *T. repens* and the other three diploid (2n = 16) *Trifolium* species *T. alexandrinum*, *T. arvense*, and *T. hybridum*, and from approximately 100 pollinations in each cross, respectively 52, 8, and 19 seeds were harvested. The development of improved techniques for handling excised embryos has offered greater possibilities for making wide crosses and for obtaining desired characters from other species (Keim 1953a).

Because of the effective self-incompatibility in the genus *Trifolium*, emasculation is not necessary in much of the crossing included in a breeding programme. However it is considered that emasculation should be practised in making critical crosses, or obviously when working with self-compatible plants. Several emasculation techniques

have been successfully used, the two most common being emasculation by removal of the anthers with forceps or by suction.

One of the most rapid method involves removing all open florets from a young inflorescence, then removing all florets above the whorl of florets that are about to open; the corolla is then seized on the underside at a point midway between the tip of the calyx and the tip of the standard, using only the tip of a pair of forceps. The entire corolla together with the staminal tube, and all anthers are withdrawn by pulling slowly and steadily. The stigma is then left exposed ready for pollination. By using this technique, emasculation and pollination can be performed as quickly as pollination without emasculation (Gibson and Hollowell 1966). To reduce the chance of selfing as a result of anthers dehiscing during emasculation, the stigmas may be atomised with water immediately after emasculation and pollination delayed until the stigmas have dried (Williams 1954).

In order to provide information about the possibility of developing a perennial, high quality, salinity tolerant 'clover', the work carried out and reported in this chapter was to determine the cross ability between the two most tolerant species *T. alexandrinum* (2n = 16) and *T. resupinatum* (2n = 16) and the most important species agriculturally *T. repens* (2n = 32).

5.2. MATERIALS AND METHODS

5.2.1. Plant material

Two-week-old seedlings obtained from the work reported in Chapter 4 which tolerated the highest concentration of salinity were used, and consisted of *T. alexandrinum*, Accession 1 which grew at EC 29 dSm^{-1}, *T. resupinatum* Accession 3 which survived at EC 32 dSm^{-1}, and *T. repens* Accession 4 which grew at EC 20 dSm^{-1}.

5.2.2. Methods

The 30 most vigorous plants of each species were selected at their highest salinity tolerance, and transferred to non-saline aerated nutrient solution for two weeks in a growth room (conditions as given on page 27). After two weeks, the 10 healthiest and strongest seedlings from each species were transferred to John Innes potting compost in 15 cm plastic pots, one plant per pot, and all were transferred to, and grown in, a glasshouse. Daytime glasshouse temperature ranged from 20 to 32°C, night time temperatures, from 16 to 25°C. Relative humidity ranged from 40 to 85%. Sixteen hours natural daylength was provided, natural daylight being supplemented using 400 Watt mercury vapour lamps.

When the plants began flowering, individual plants of each species were isolated in separate white muslin net chambers (150 cm × 90 cm × 100 cm) to avoid unwanted pollination by bees or other insects. Pollinations were made either by hand or by enclosed bumble bees.

5.2.2.1. Pollinations by hand

At first 100 single flowers in different inflorescences were self pollinated by hand in all three species. Only three seeds out of 100 pollinations were produced in one genotype of *T. repens*. The other genotypes of *T. repens*, and all the other genotypes of the other species produced no seed.

Pollinations were made by manually tripping individual flowers using a small triangular piece of cardboard. For one genotype of *T. repens* which has produced several seeds from selfing, emasculation was carried out by removing the anthers and sterilising the flowers using 65% ethanol (v/v) for 10 seconds and washed, using deionised water, for 10 seconds and the remaining water was then removed by tissue paper, the pollen grains of the male plant were placed in the top of stigma. The pollinated inflorescences were covered by small plastic bags to protect unwanted pollen grains, and preventing moisture loss. After five days the plastic bags were removed (Michaelson-Yeates, personal communication).

5.2.2.2. Pollinations by bees

Bee cages were used to effect inter-species pollinations. Five plants of the same clone of each species were selected and placed in a muslin net chamber as male parents, and the female parent was placed in a separate chamber. Artificial lighting was set to extend natural daylight to 16 hour. This resulted in a flush of flowers before and during the time the bees were used for pollination. Whilst letting plants grow until enough flower buds were formed on plants of the clones, bees were moved into the cages until plants of the clones had enough open flowers. After three days flowering the female plant

was placed in the middle of the cage, and was surrounded by five of the aforementioned flowering male plants. Reciprocal crossing was carried out by the same procedure. In the event of bumble bees death, replacement was carried out by means of transferring other bees, each after two times sterilisation using warm water in a test tube, to eliminate any alien pollen grain. Seeds were harvested approximately 30 days after pollination.

5.2.2.3. Distinguishing of viable and non-viable pollen grain in parents and progenies

10 g of carmine stain per 500 ml of 45% acetic acid (v/v) was slowly boiled and simmered for 30 minutes in a water bath, and filtered through Whatman filter paper after cooling at room temperature.

Inflorescences were cut and placed in water, and the pollen was rescued and placed on a microscope slide, to which one drop of aceto-carmine 2% (Anderson *et al.* 1991) was added. The material was then covered by coverslip applying gentle pressure. Viable pollen grains (spherical shape/plump with pink to yellowish colour), and non-viable pollen grains (shrunken and colourless) were counted.

5.2.2.4. Morphological features

The plants were measured from base node producing roots to tip of the shoot excluding tip lives for plant height. The petiole lengths of the parents and hybrid plants were measured from the base of petiole on the stems to the base of leaflets (foliates). Ten leaflets were chosen randomly per pot in all (maximum) ten parents, and hybrid plants. The leaflet was measured from the base of leaflet to the tip of the leaflet as a

leaflet length, and breadth was measured across the middle of the leaflet as a leaflet breadth. Ten inflorescences were also chosen randomly; number of flowers per inflorescence were counted separately, and the diameter of those inflorescences were measured as inflorescence diameter. The lengths of sepal and petal were measured from those same flowers as calyx and corolla lengths respectively. The data were analysed by Statistical Analyses System (SAS) programme by computer and they are presented in Table 5.4 and Figures 5a-h.

5.2.2.5. Estimates of Heterosis

"Complementary to the phenomenon of inbreeding depression is its opposite, hybrid vigour or heterosis. In general terms the fitness lost on inbreeding tends to be restored on crossing. The heterosis on crossing should be equal to the depression on inbreeding" (Falconer 1997). Heterosis has been quantified on the basis of mean of the parents for the eight morphological characters described above, using the following formula given by Falconer and Mackay (1997).

$$H_{F1} = M_{F1} - M_{\bar{P}}$$

H_{F1} hybrids stand for heterosis for the F_1 hybrids, M_{F1} and $M_{\bar{P}}$ are the mean values for F_1 hybrids and mid-parents respectively. Mid-parent was calculated as follows:

$$M_{\bar{P}} = \frac{M_{P1} + M_{P2}}{2}$$

M_{P1} is the mean of *T. alexandrinum* genotypes, and M_{P2} is the mean of *T. repens* genotypes, the means being for each of the measured characters.

$$M_{\bar{P}}H = \frac{H - M_{\bar{P}}}{M_{\bar{P}}} \times 100$$

$M_{\bar{P}}H$ = Mid-Parent heterosis

$$H_PH = \frac{H - H_P}{H_P} \times 100$$

H_PH = High parent heterosis.

This is used in examining believed hybrid material.

5.3. RESULTS

The data presented in Table 5.1 show the successful results of hybridisation between *T. alexandrinum* ♀ × *T. repens* ♂ from direct (*T. alexandrinum* as a female parent) and reciprocal (*T. repens* as a female parent) crosses. From hand pollination of 450 florets, 6 seeds were obtained, and from 376 reciprocal pollinations 11 plump seeds were obtained. Pollination by bumble bees resulted 56 seeds between those species. The seeds that were obtained in this work were placed on moist Whatman filter paper in sterile petri-dishes. 21 out of the 56 seeds obtained did not germinate. The remaining 35 two-week-old seedlings were transferred into sterile soil. After 4 weeks they were transferred to a glasshouse, (conditions were as Chapter 4). 11 seedlings were weak and died at the seedling stage.

As shown in Table 5.1, the cross between *T. repens* and *T. resupinatum* produced no seeds in either direction, either from hand or from bee pollination.

Table 5.1. Interspecific hybridisation between 3 *Trifolium* species (*T. resupinatum*, *T. alexandrinum*, and *T. repens*).

Crosses by hand	No. of florets	No. of seeds
T. alexandrinum ♀ × *T. repens* ♂	450	6
Reciprocal	378	11
T. repens ♀ × *T. resupinatum* ♂	450	0
Reciprocal	450	0
T. alexandrinum ♀ × *T. resupinatum* ♂	450	4*
Reciprocal	211	17**
Cross by bee pollination	Number of seeds produced	
T. alexandrinum ♀ × *T. repens* ♂	14	
Reciprocal	42	
T. repens ♀ × *T. resupinatum* ♂	0	
Reciprocal	0	
T. alexandrinum ♀ × *T. resupinatum* ♂	6*	
Reciprocal	3*	

♀ = The parent as female

♂ = The parent as male

* = The seeds did not germinate

** = Only two seeds germinated, but all failed to grow.

5.3.1. Viability of pollen-grains

Investigation of viable/non-viable pollen grains in the parents and progenies from direct ands reciprocal crosses of *T. alexandrinum* and *T. repens* were carried out and the data compared using analysis of variance (Table 5.2). Percentages of non-viable pollen grains are presented in Table 5.3.

Significant differences in viability were found between the different genotypes used as parents within species, and their progenies, and also the interaction parents × progeny indicating that viability of pollen from different parent crosses differed significantly ($P \leq 0.0001$). The mean percentage of non-viable pollen grain from the hybrid plant was higher than that of their parents. As shown in Table 5.3, the hybrid plants produced relatively high numbers of stainable pollen grains in all except Genotypes 8 and 9, which both produced less than 50% fertile pollen grains, and direct crosses between *T. alexandrinum* and *T. repens* gave the same results except Genotypes 1, 18, and 19, which produced 71%, 98%, and 99% of non-viable pollen grain respectively. The calculation of total percentages of sterile pollen grains of *T. alexandrinum* with 4% of the 45,000 pollen grains had the least non-viable pollen grains, and the hybrid genotypes of *T. repens* ♀ × *T. alexandrinum* ♂ with 28% of the 55,000 pollen grain had the most non-viable pollen grains. *T. repens* and the progenies between *T. alexandrinum* ♀ × *T. repens* ♂ with 14% and 23% had intermediate non-viable pollen grains.

The hybrid plants from crosses between *T. alexandrinum* and *T. repens* in both direction crosses were perennial with stolons (Plates 5.1, 5.2, and 5.5).

Table 5.2. Mean Squares and significance from analysis of variance of non-viable pollen grain of 2 *Trifolium* species and their progenies

Sources	DF	Mean Squares	P > F
Inflorescence	4	102.07 **ns**	0.20
T. alexandrinum	17	222.13***	0.0001
T. repens	10	256.43***	0.0001
T. alexandrinum ♀ × *T. repens* ♂ F_1 hybrids	14	1549.85***	0.0001
T. repens ♀ × *T. alexandrinum* ♂ F_1 hybrids	21	3191.16***	0.0001
Parents × ($2F_1$ hybrids)	3	1441.47***	0.0001
Residual	260	67.30	

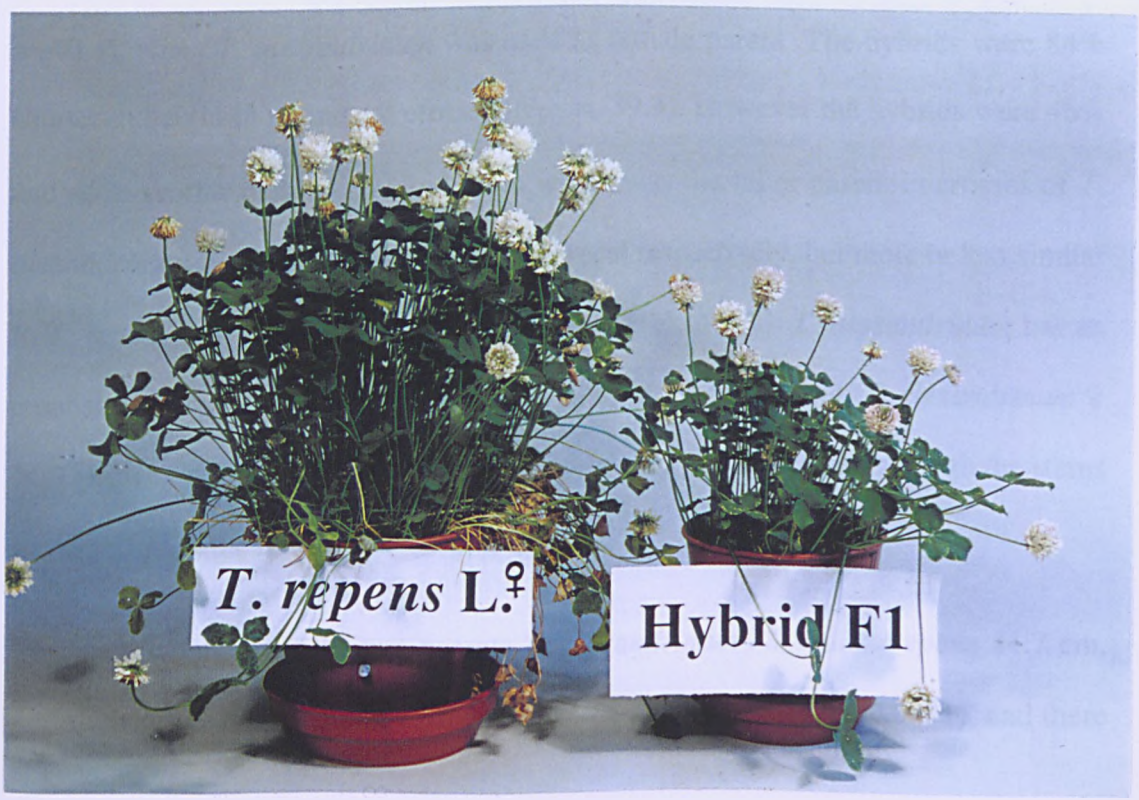
DF = degree of freedom

Table 5.3. Percentages of non-viable pollen grains in samples of 2,500 pollen grains from two *Trifolium* species and their progenies.

Genotypes	Percentage of non-viable pollen grains			
	T. alexandrinum	*T. repens*	*T. alexandrinum* ♀ × *T. repens* ♂	*T. repens* ♀ × *T. alexandrinum* ♂
1	26	13	71	13
2	2	2	13	29
3	3	4	13	36
4	24	8	25	47
5	4	17	13	20
6	6	25	7	31
7	6	3	8	5
8	10	8	23	54
9	8	12	26	55
10	3	18	15	8
11	4	5	14	13
12	2	-	15	35
13	9	-	16	41
14	5	-	8	24
15	9	-	41	10
16	7	-	8	-
17	1	-	22	-
18	2	-	98	-
19	-	-	99	-
20	-	-	18	-
21	-	-	24	-
22	-	-	32	-
Total	4	14	23	28

Plate 5.1. F_1 hybrid. A cross between *T. alexandrinum* ♀ × *T. repens* ♂

Plate 5.2. F_1 hybrid. A cross between *T. repens* ♀ × *T. alexandrinum* ♂



5.3.2. Morphological features

The results from the analysis of variance for mean of plant height, petiole length, leaflet length and breadth, number of flowers per inflorescence, inflorescence diameter, calyx length, and corolla length from parents and both direct and reciprocal crosses hybrids are presented in Table 5.4, and from parents and hybrids four different qualitative characters are described in Table 5.5. The data from the two parents and their progenies for eight characters are given in the form of histograms in Figures 5.a-h. Calculation of heterosis (Falconer and Mackay 1997) for all eight characters are presented in Table 5.6.

T. alexandrinum was significantly ($P \leq 0.0001$) taller than *T. repens* as expected, and was significantly taller than the hybrid progenies. As shown in Figure 5.a., plant height of *T. repens* (as male and female parent) and the F_1 hybrid, did not differ significantly ($P > 0.05$). The hybrid was 87% shorter in height than mid-parent (H_{F1} = -40.4), when *T. alexandrinum* was used as female parent. The hybrids were 84% shorter in height in reciprocal crosses (H_{F1} = -39.3). However the hybrids were 46% and 45% shorter than *T. alexandrinum* which was the taller parent in crosses of *T. alexandrinum* ♀ and *T. repens* ♂, and reciprocal respectively, but more or less similar to *T. repens* (no significant differences at the level of 5%). *T. alexandrinum* has an erect stem, while, *T. repens* has no such type of stem. The hybrid (*T. alexandrinum* ♀ × *T. repens* ♂ and reciprocal) has the same type of stem as *T. repens* with the stems having roots at the nodes.

The petiole lengths of the two parents *T. alexandrinum* 2.5 cm, *T. repens* 11.7 cm, and their progenies 14.5 and 12.8 cm, differed significantly ($P \leq 0.0001$), and there

were no significant differences between the direct and reciprocal hybrids. The petiole lengths of hybrids were 7.4 and 5.6 cm longer than the mid-parent, 104 and 79% longer than mean of the parents, and 63 and 48% longer than that of *T. repens* (Figure 5.b).

Figure 5.c shows that *T. alexandrinum* and *T. repens* had significantly ($P \leq 0.05$) long (32.2 mm) and shortest (18.8 mm) leaflet, but the hybrids have intermediate (25 and 25.6 mm) leaflets. The leaflet length of hybrid were 2 and 1.4 mm shorter than the mid-parent. Leaflet length of hybrids were 7 and 5% shorter than mid-parent, and 6 and 4% shorter than larger leaflet bearing parent (*T. alexandrinum*).

The leaflet breadth of *T. alexandrinum* was 11.3 mm significantly shorter than that of *T. repens*, 17.2 mm. The two hybrids, direct and reciprocal were, receptively, 18.9 and 18.4 mm, both being significantly longer than both parent. The hybrids show (Figure 5.d and Table 5.6) greater leaflet breadth than the mid-parent (4.7 and 4.1 mm), but also than that of *T. repens* (27 and 24%). The leaflet shape of *T. alexandrinum* was oblong and *T. repens* was obovate (Plate 5.3), but the hybrid plants were elliptical to obovate (Table 5.5, Plate 5.4). Several abnormalities on hybrids (Plate 5.4) have been found, many leaflets (4-8), excessively long leaf-stalks, flower on the leaf. The same abnormalities were reported by Starzycki (1969) from hybrids between *T. repens* and *T. pratense*.

Significant differences ($P \leq 0.05$, Table 5.4, and Figure 5.e) between parents and progenies were found for number of flowers per inflorescence. There were no significant differences between *T. alexandrinum* and the hybrid in which it was the female parent, and the same result was found between *T. repens* and the hybrid when

T. repens was the female parent. The mean number of flowers per inflorescence of the hybrids from crosses between *T. alexandrinum* ♀ and *T. repens* ♂, and reciprocal crosses were 7.3 and 2.5, more than mid-parent respectively.

The mean inflorescence diameter of *T. alexandrinum* was 13.8 mm, and did not differ significantly ($P > 0.05$) to *T. repens*, 14.7 mm. The two hybrids, direct (*T. alexandrinum* as a female) and reciprocal were, respectively, 18.9 and 20.1 mm, both being significantly greater than both parents. These hybrids had 4.7 and 5.9 mm greater diameter than the mid-parent, and 32 and 40% larger than that of the larger *T. repens* parent (Figure 5.f and Table 5.6).

Highly significant differences ($P \leq 0.0001$) in calyx lengths were found between *T. alexandrinum* (6.5 mm), *T. repens* (3 mm), and the hybrids between them (3.1 and 3.4 mm). The calyx lengths in both direction crosses were not different from those of *T. repens* (Figure 5.g). The calyx of *T. alexandrinum* has small hairs (Stace 1991), but the hybrid had a smooth non-hairy calyx like that of *T. repens* (Table 5.5).

The corolla length of *T. alexandrinum* was 10.6 mm (Figure 5.h), significantly longer than that of *T. repens*, 7.7 mm. The two hybrids, direct and reciprocal were, respectively 8.1 and 8 mm, and did not differ from each other and from those of *T. repens*, but highly significant differences ($P \leq 0.0001$) in corolla lengths were, found between *T. alexandrinum* and the hybrids. The hybrids were 9 and 11% shorter than the longer parent (*T. alexandrinum*). The corolla of *T. alexandrinum* is of cream colour, whereas *T. repens* has a white to pale pink coloured corolla. The hybrid plants however had mixed colours of white, pale pink, and pink (Table 5.5).

Table 5.4. Analysis of variances of eight different characters of *T. alexandrinum*, and *T. repens*, and their progenies.

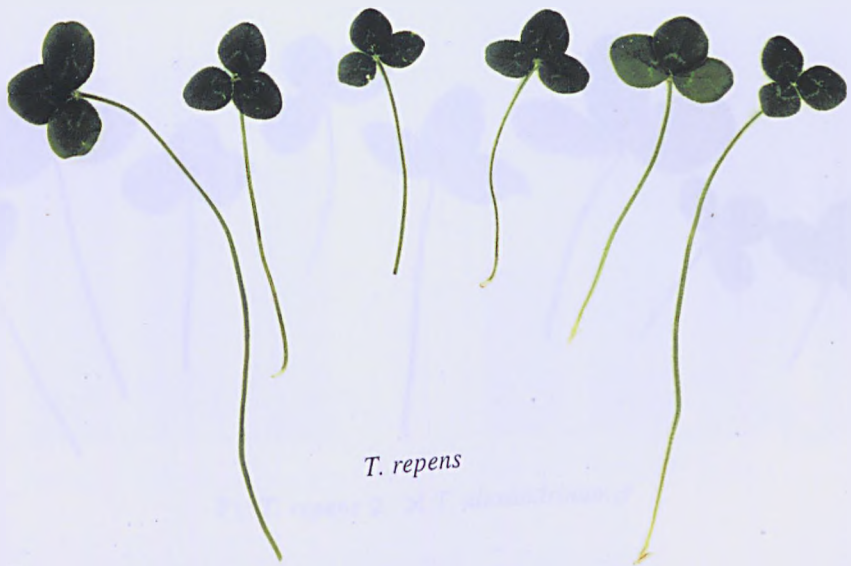
Characters	Df	Mean squares	P > F
Plant height	3	71527.04	0.0001
Petiole length	3	1278.33	0.0001
Leaflet length	3	1626.17	0.0001
Leaflet breadth	3	560.84	0.0001
Number of flowers / Inflorescence	3	1476.11	0.036
Inflorescence diameter	3	368.10	0.0001
Calyx length	3	108.87	0.0001
Corolla length	3	70.23	0.0001

Table 5. 5. Qualitative four different characters of *T. alexandrinum*, and *T. repens*, and their progenies.

Characters	*T. alexandrinum*	Hybrids	*T. repens*
Stem	erect	creeping rooting at the node	creeping rooting at the node
Leaflet shape	oblong	elliptical/obovate	obovate
Calyx	hairy	smooth	smooth
Corolla colour	cream	pink/pale-pink/white	white/pale-pink

Plate 5.3. Parents *Trifolium alexandrinum*, and *T. repens*.

T. alexandrinum



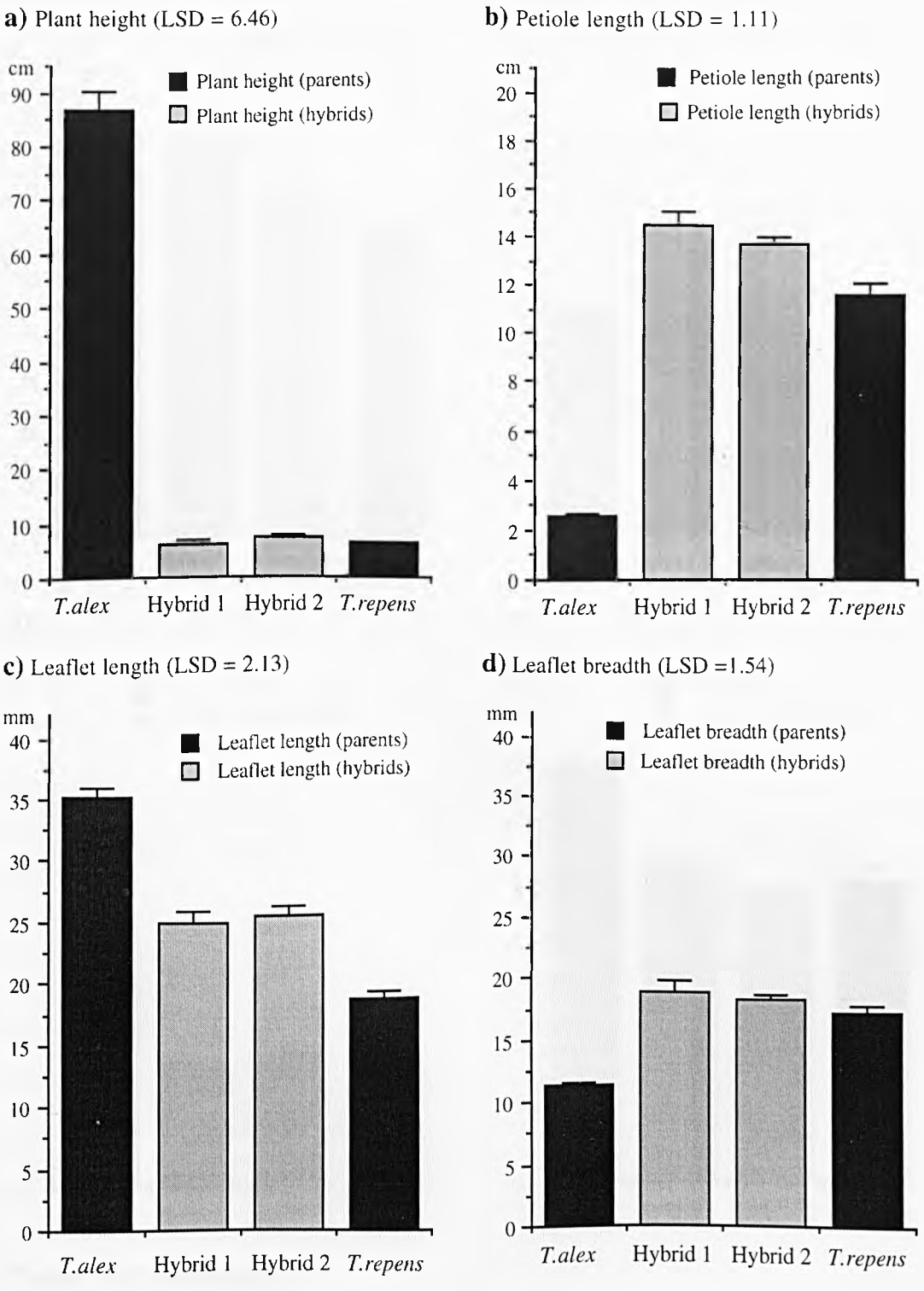
T. repens

Plate 5.4. Interspecific hybrids of *Trifolium alexandrinum*, and *T. repens*.

F_1: *T. alexandrinum* ♀ × *T. repens* ♂

F_1: *T. repens* ♀ × *T. alexandrinum* ♂

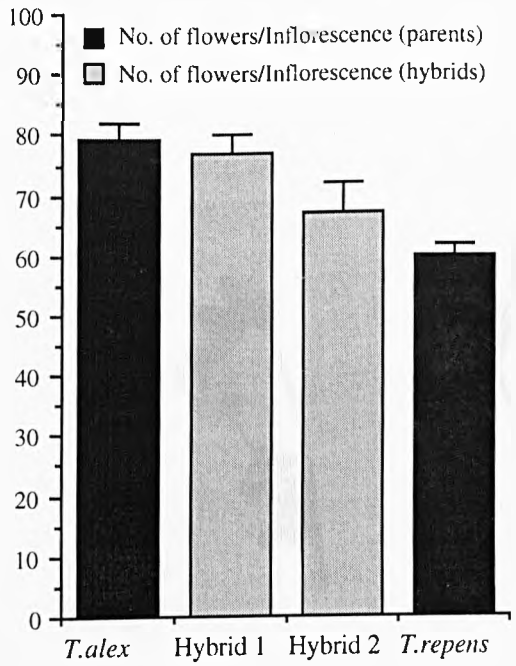
Figure 5. Comparison of mean values for eight characters of *T. alexandrinum* and *T. repens*, and their F_1 direct and reciprocal hybrids (P = 0.05).



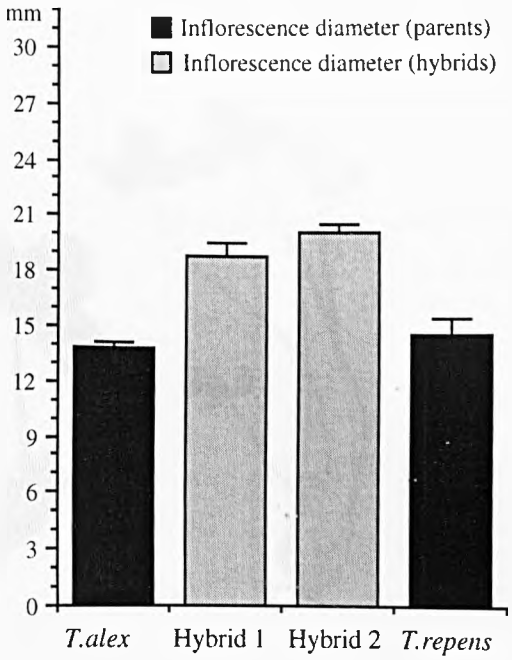
Hybrid 1 = The hybrid when *T. alexandrinum* was female parent.
Hybrid 2 = The hybrid when *T. repens* was female parent.

Figure 5. Continued

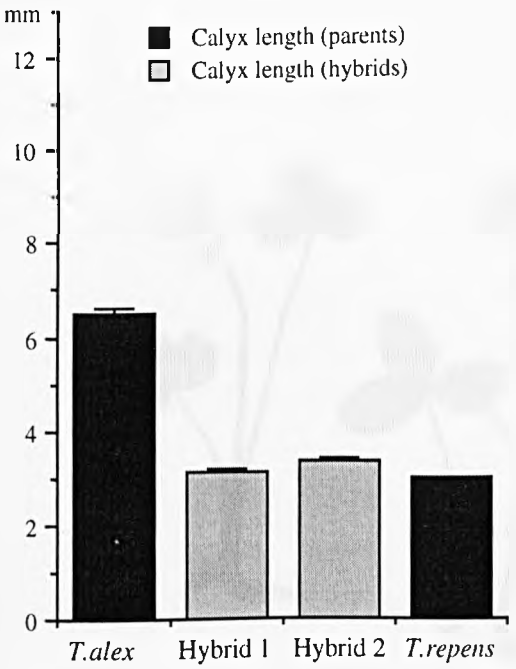
e) No. of flowers/inflorescence (LSD = 9.11)



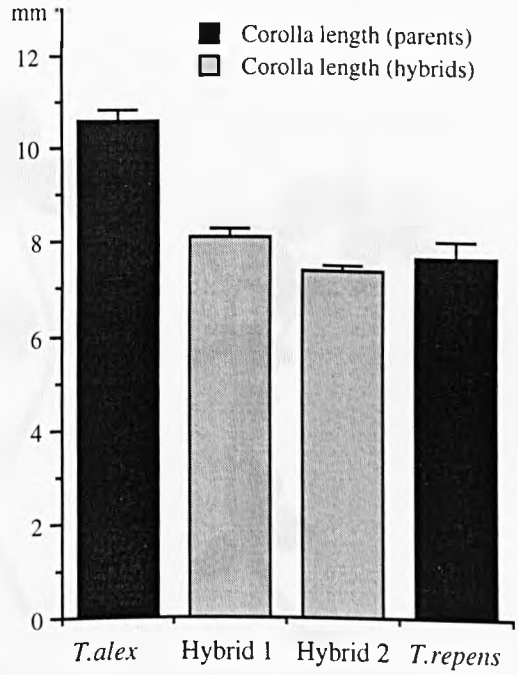
f) Inflorescence diameter/mm (LSD = 2.95)



g) Calyx length (LSD = 0.53)



h) Corolla length (LSD = 1.21)



T. alex = *T. alexandrinum*

Hybrid 1 = The hybrid when *T. alexandrinum* was female parent.

Hybrid 2 = The hybrid when *T. repens* was female parent.

Plate 5.5. Interspecific hybrids, parents *Trifolium alexandrinum*, and *T. repens*.

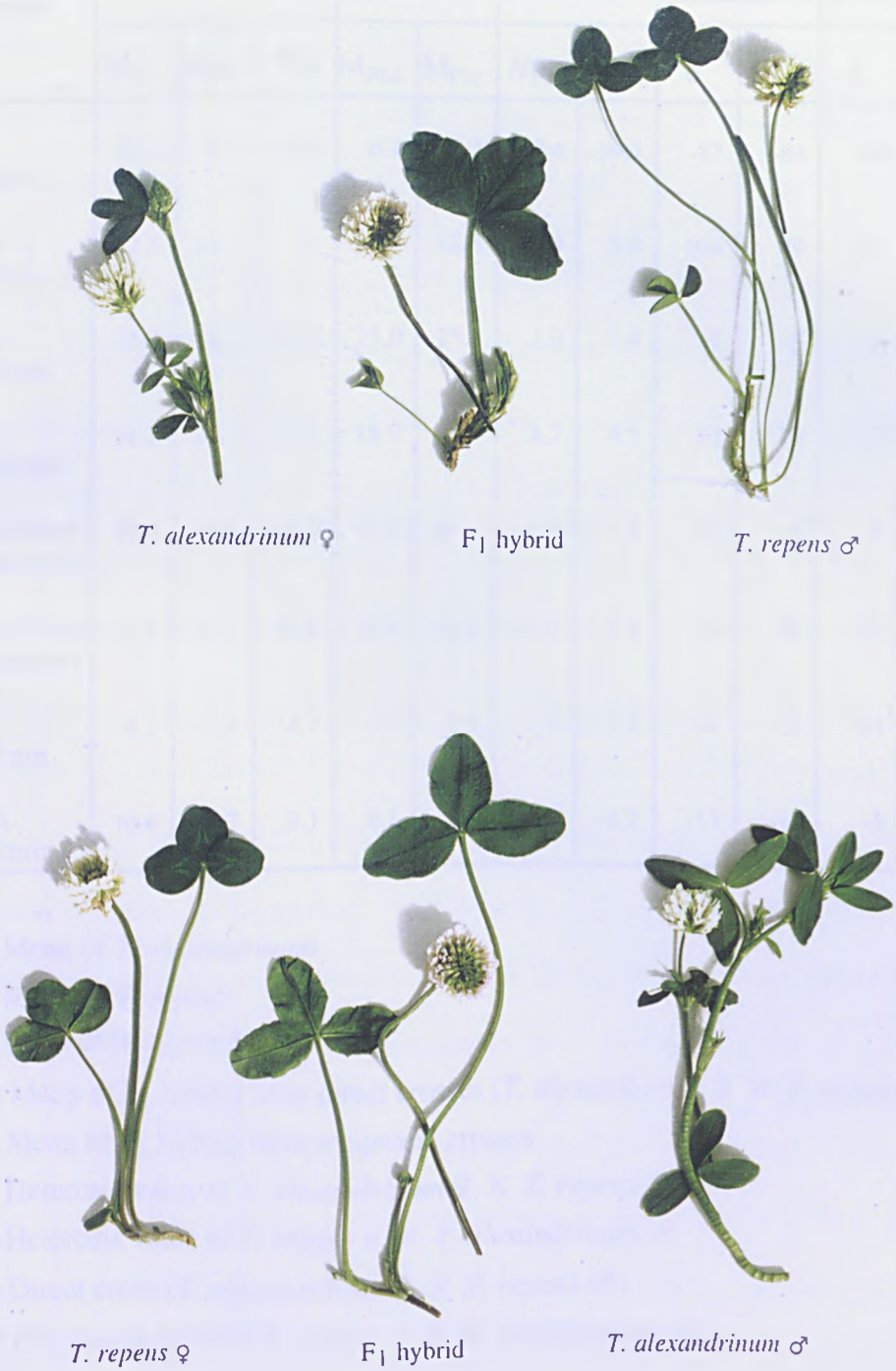


Table 5.6. Calculated values of heterosis for eight characters from F_1 hybrids of direct and reciprocal crosses between *T. alexandrinum* and *T. repens*.

Characters	Parents			Hybrids		Heterosis		Mid Parent Heterosis %		High Parent Heterosis %	
	M_{P1}	M_{P2}	$M_{\bar{P}}$	M_{F1d}	M_{F1r}	H_{F1d}	H_{F1r}	d	r	d	r
Plant height/cm	87.0	6.2	46.6	6.2	7.3	-40.4	-39.3	-87	-84	-46	-45
Petiole length/cm	2.5	11.7	7.1	14.5	12.8	7.4	5.6	104	79	63	48
Leaflet length/mm	35.2	18.8	27.0	25.0	25.6	-2.0	-1.4	-7	-5	-6	-4
Leaflet breadth/mm	11.3	17.2	14.2	18.9	18.4	4.7	4.1	33	29	27	24
No. flowers/ Inflorescence	79.4	60.0	69.7	77.0	67.2	7.3	2.5	10	4	9	3
Inflorescence diameter/mm	13.8	14.7	14.2	18.9	20.1	4.7	5.9	33	42	32	40
Calyx length/mm	6.5	3.0	4.7	3.1	3.4	-1.6	-1.4	-34	-28	-24	-22
Corolla length/mm	10.6	7.7	9.1	8.1	8.0	-1.0	-1.2	-11	-13	-9	-11

M_{P1} = Mean of *T. alexandrinum*

M_{P2} = Mean of *T. repens*

$M_{\bar{P}}$ = Mean of two parents

M_{F1d} = Mean of F_1 hybrid from direct crosses (*T. alexandrinum* ♀ × *T. repens* ♂)

M_{F1r} = Mean of F_1 hybrid from reciprocal crosses.

H_{F1d} = Heterosis value of *T. alexandrinum* ♀ × *T. repens* ♂

H_{F1r} = Heterosis value of *T. repens* ♀ × *T. alexandrinum* ♂

d = Direct cross (*T. alexandrinum* ♀ × *T. repens* ♂)

r = Reciprocal crosses (*T. repens* ♀ × *T. alexandrinum* ♂)

5.4. DISCUSSION

It is possible that the salinity tolerance of *Trifolium* species can be improved through selective breeding with inter/intra-species with higher salinity tolerance. Other possibilities include the transfer of tolerance genes from more salinity-tolerant accessions through wide inter/intra-specific hybridisation or the improvement of salinity tolerance through *in vitro* selection and regeneration of cell cultures.

Interspecific hybridisation is a means of extending widely the range of heritable variation which can be exploited by the plant breeder. Brewbaker and Keim (1953) were among the first to suggest interspecific hybridisation of *Trifolium* species as a means of improving *T. repens*. They identified potential benefits in disease and pest resistance, persistency, root development, and cold and drought tolerance. The objective of the present study was to determine the possibility of interspecific hybridisation between *T. resupinatum* (2n = 16), *T. alexandrinum* (2n = 16), and *T. repens* (2n = 32). The successful result could help to transfer salinity tolerance, one of the most important agronomic characters to extend the exploitation of saline soils, from *T. resupinatum* and *T. alexandrinum* into *T. repens*, probably the most nutritious and digestible legume for livestock and poultry, either in the green state or as hay (Miller 1958, Van Keuren and Heinemann 1958, and Koger *et al.* 1961).

Many researchers have attempted to cross *Trifolium* species using different techniques. However because of the species incompatibility most of them were not successful; for example Evans (1962) made combination crosses between several diploid species and naturally-occurring polyploids but obtained only very small numbers of seeds, 12 seeds from crosses between *T. repens* and *T. nigrescens*, 7 seeds between *T.*

hybridum and *T. ambiguum* (2x) and 9 seeds from *T. hybridum* and *T. ambiguum* (6x). The other crosses produced no seeds. Recently Marshall *et al.* (1995) produced hybrid plants from crosses between the amphidiploid *T. repens* (2n = 4x = 32) and the annual *T. nigrescens* (2n = 2x = 16) without the need of embryo culture.

As shown in Table 5.1, the crosses between *T. alexandrinum* × *T. repens* were successful and produced 6 seeds from 450 florets pollinated by hand in direct crosses, and 11 seeds from 378 florets pollinated by hand in reciprocal crosses. Using bee pollination 14 and 42 seeds were produced from direct and reciprocal crosses, respectively. These results were as successful as the work of Trimble and Hovin (1960), from which 52 seeds were harvested from 100 crosses between *T. repens* and *T. alexandrinum.*

Selim *et al.* (1977) used 10 species of *Trifolium* in a crossing programme, most of which were not successful. Crossings between *T. repens* and *T. nigrescens* were the most successful crosses. In that programme the crosses between *T. alexandrinum* (Cv. Warfir) and *T. resupinatum* produced 4 plump seeds from 18 florets pollinated.

The difficulties of successful crosses between species has been considered by Taylor (1980), and the crossing barriers are believed to be due mainly to post fertilisation phenomena. This type of barrier may be overcome successfully using embryo culture (Taylor and Smith 1979), and/or ovule culture (Keim 1952, 1953a,b, and Meredith *et al.* 1995).

At anthesis the diversity of pollen grain viability was very variable, ranging from 1 to 95%, being particularly high when *T. alexandrinum* was the mother plant. For example Hybrids 18 and 19 had very poor fertility with only 2 and 1% normal and

stainable pollen grains, the remainder being shrivelled and empty. In spite of such extreme sterility, some genotypes e.g. Hybrid 7 had a high number, (95%) of stainable pollen grains (Table 5.3). Three *T. ambiguum* × *T. repens* hybrids reported by Williams (1980) survived to flowering only to show extreme pollen sterility and these hybrids produced no F_2 or back-cross progeny, e.g. Hybrid 61, 0.1-3.6% stainable pollen; Hybrid 70, 1%; Hybrid 262, 0.3%. However, in the same experiment, 23% of the pollen grains produced by Hybrid 435 were normal, 31% of grains were undersized, and the remainder (46%) were shrivelled and empty. Meredith *et al.* (1995) produced hybrids between *T. ambiguum* and *T. repens* which were successfully established after ovule culture. The hybrids were male sterile except one of the hybrids produced a single seed when back-crossed to *T. repens*, Yamada *et al.* (1989) too, had successful back-crossing between *T. repens* and *T. ambiguum*, where the hybrid plants showed intermediate leaflet size and shape, and highly sterile pollen grains. Only one hybrid plant had about 1% stainable pollen, the other had less than 0.1% stainable pollen. These hybrid progenies might nonetheless help to produce some higher salinity tolerance material, after several cycles of back-cross or selection in the future.

Interspecific hybridisation between *T. alexandrinum* and *T. repens* was attempted with the objection to combine such good and valuable characters among the species such as salinity tolerance, perenniality, good recovery after grazing, and digestibility. *T. alexandrinum* and *T. resupinatum* are salinity tolerant with erect stems and annual habit which are not ideal for grazing purposes, whereas the sensitivity of the *T. repens* does not allow it to grow in salt affected pasture/areas. Such hybrids produced could have better agronomic characteristics with an optimum level of morphological characters and

yield of one and salinity tolerance of the other parent. *T. repens* is a perennial forage species, with very good balance between vegetative and reproductive growth. It consists of several vegetative and reproductive nodes, which are important to ensure perenniality and persistency in the sward from one to the next season. As indicated in Table 5.5, the hybrid between *T. alexandrinum* and *T. repens* from both directions are perennial with reproductive nodes and stoloniferous stems.

The F_1 interspecific hybrid has a great overall resemblance to *T. repens*, but several intermediate or vigorous characters were produced between *T. alexandrinum* and *T. repens*. Several abnormalities of hybrids (Plate 5.4) were recorded, such as high number of leaflets (4-8), excessively long leaf-stalks, leaf produced flowers. Similar abnormalities have been reported by Starzycki (1969) in hybrids between *T. repens* and *T. pratense*.

As shown in Table 5.1, the cross between *T. repens* and *T. resupinatum* produced no seeds in any direction, hand or bee pollination. Similar results have been reported by Evans (1962) and Przywara *et al.* (1996). The cross between *T. alexandrinum* and *T. repens* was successful, and hybrid plants produced highly stainable pollen grains, and also showed 'hybrid vigour' in some characters. This approach may be a means of extending the range of heritable variation useful in the breeding and selection of *T. repens* or *T. alexandrinum*. The cross between *T. alexandrinum* and *T. resupinatum* also was successful, but these hybrid seeds either did not germinate or the growth ceased for some reason at the seedling stage. The cross between *T. repens* and *T. resupinatum* was not successful in any direction. In 1996 Przywara *et al.* attempted to overcome the compatibility of interspecific hybridisation between *T. repens* and *T. resupinatum* by culturing excised embryos. Unfortunately the growth and development

of those embryos was abnormal and the embryos died within two to three weeks of culture. The new techniques like, embryo/ovule culture, *in situ* hybridisation, promise to produce many new hybrids and also to make larger samples of hybrid material from each cross.

As is shown in Table 5.5 and Figures 5a-h, the hybrid plants from both direction crosses have similar behaviour to *T. repens* in some characters, some characters were intermediate, and the other characters were superior those in both parents. Michaelson-Yeates *et al.* (1997) found a positive heterosis for dry matter production from hybrids of self fertile inbred lines of *T. repens.*

More research and investigation such as some back-crossing from the hybrids to *T. alexandrinum*, determination of salinity tolerance of the hybrids/progenies from back-cross, and analysing the hybrid cytogenetically are necessary. The achievement of this hybridisation programme shows that it might be possible to transfer salinity tolerant (genes) from tolerant species/accession to the less tolerant one.

CHAPTER SIX

DISCUSSION

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CHAPTER SIX

6.1. GENERAL DISCUSSION

About 21% of land in the world is under mineral stress, and about 34% is affected by excessive salt especially sodium (Massoud 1974). It has been further estimated that one third of the world's irrigated land has been salinised to various degrees. This salinisation results from an accumulation of salts dissolved in the irrigation water, by mismanagement, from low quality of irrigation water, to excessive use of fertiliser (Binzel and Reuveni 1994). Salinity is one of the most disastrous environmental stresses for agriculture. Two major strategies have been proposed to overcome this impediment, either changing the environment by reclamation of salt-affected land, or/and development of salt tolerant crop cultivars. The first has different ways of overcoming this magnitude of problems, e.g. desalinisation of irrigation water, removing salt from the soil by leaching, irrigation in the cool season to remove the salt from root zone to the deep ground. Soil scientists have succeeded in developing techniques for reclaiming affected soils, but these techniques cannot keep up with the increasing areas of land affected, and many of the countries most affected cannot afford the current cost of such reclamation. The second strategy would be use of already tolerant plants, and finally adaptation and breeding to improve salinity tolerance of cultivated plants.

Iran has been establishing large areas of irrigated agriculture along the basin of several rivers where the environment is favourable, for production of high yielding agricultural and/or forage crops. Mismanagement of rangeland and increased grazing by livestock has caused very serious damage to the composition of plants and

environments in Iran (Mesdaghi 1993), but recently it has undertaken considerable renewing by introducing many valuable forage species under correct management (Jafari 1995). Many wild as well as cultivated plants must thus deal with saline environments. A saline environment imposes two principal kinds of stress on plants, namely osmotic and toxicity (Jacoby 1994). The direct effect of salinity on plant growth may be a reduction in the osmotic potential of the soil solution that reduces available plant water, a deterioration in the physical structure of the soil that decreases the permeability to water and gasses, and specific ion toxicity. The common cations associated with salinity are Ca^{2+}, Mg^{2+}, and Na^{+}, and the common anions associated with salinity are Cl^{-}, SO_4^{2-} and HCO_3- (Dudley 1994).

Previous studies have suggested that improvement of salinity tolerance in many different species and cultivars can be achieved through selecting from already existing variable plant material (e.g. in barley Epstein and Norlyn 1977, in wheat Kingsbury and Epstein 1984, in forage grasses Jafari 1990, in lucerne Al-Khatib *et al.* 1993 and 1994, and in millets Kebebew and McNeilly 1995), or the exploitation of variation through hybridisation (e.g. in wheat Forster *et al.* 1987, and Ashraf and McNeilly 1991, and in *Coleus blumeii* Ibrahim *et al.* 1991). Enhanced salt tolerance in many plants is possibly the most convincing example to date that a biological fix to the problem of salt tolerance may be possible. For continued improvement in our knowledge of salinity tolerance, and exploitation of the potential available for increased salt tolerance in other crop species, further studies are essential. The work reported here about clover species may be considered to be a step forward in this direction. The basic concept underlying the investigation carried out was to develop some

understanding of the potential for the development of increased salinity tolerance in the more widespread grown forage numbers of the genus *Trifolium.*

The work described in this thesis was designed to gain an initial understanding of the relative salinity tolerances of 7 forage clover species, *T. alexandrinum*, *T. resupinatum*, *T. repens*, *T. pratense*, *T. subterraneum*, *T. ambiguum*, and *T. fragiferum*, and of the extent and nature of genetic variability within and between them, assessing material mainly of Iranian origin, with a longer term aim to exploit such variation in the development of material with considerably enhanced salt tolerance. Many plants are considered as being more sensitive to salt at germination and during early seedling stage than during later growth stages, for example in alfalfa Forsberg (1953), Chang (1961) and Rumbaugh (1990), in *T. michelianum* Rogers and Noble (1991), in millet Kebebew and McNeilly (1995). In contrast, *T. subterraneum* is more salt tolerant at germination than as a mature plant (West and Taylor 1981, Rogers and Noble 1991). The method of screening material used here seems adequate, since sixty five accessions were easily assessed at germination and during early seedling growth, their most sensitive stages. Successful screening and selection requires stable performance throughout the entire life cycle of the plants. The water culture at the germination and early seedling stage and sand culture experiments for the adult plant (Chapter 2, 3, and 4) have clearly shown that accession which were identified as tolerant at the seedling stage retained their tolerance at the adult stage. The data also showed that there were highly significant correlations between two phase of growth. At higher concentrations e.g. EC 22 and 26 dSm^{-1}, the growth of *T. ambiguum*, *T. pratense*, and *T. repens* were stopped in water culture experiment, but the growth of the same accessions of *T. ambiguum* and *T. pratense* produced shoots

and roots in the adult stage (Figure 3.5), it means these accessions were more sensitive to salinity at the germination and early seedling stages. Noble and Rogers 1994 suggest that tolerance at germination and seedling establishment are less important than tolerant during mature plant growth, since salinity at the soil surface could be overcome by irrigating with low-salinity water.

Salinity includes growth inhibition, and in many cases the shoot is affected more than the root (Poljakoff-Mayber and Lerner 1994). Al-Khatib (1991) and Al-Khatib *et al.* (1993 and 1994) showed that shoot length of alfalfa seedlings was a good indicator of salt tolerance for assessing cultivar response, and for the selection of tolerant individuals. By contrast other workers (Hannon and Bradshaw 1968, Ahmad and Wainwright 1977, Moeljopawiro and Ikehashi 1981, Wu 1981, Ashraf *et al.* 1986a,b, Ashraf and McNeilly 1991, and Ashraf and McNeilly 1992) considered root length to be a successful criterion for measuring salt tolerance. Root growth has been used as an index of tolerance for comparing lines of *T. repens* (Ab-Shukor *et al.* 1988) and considerable difference were found in this species. In this work shoot and root measuring has been carried out because of those two conflicting concepts.

From the results of the series of experiments described in Chapters 2, 3, and 4 it has become clear that there is considerable variability in both shoot and root growth in saline solution cultures in all the species examined. In general it has been found by other workers that the seedling stage is the most sensitive phase of plant development, and almost all the work on salinity tolerance in different crop species reported previously (Lall and Sakhare 1970, Taylor *et al.* 1975, Kingsbury and Epstein 1984, Norlyn and Epstein 1984, Allen *et al.* 1985, Hajibagheri *et al.* 1987, Grattan and Maas 1988, and Al-Khatib 1991) has been based upon plant assessment made at this stage.

There appears to be sufficient evidence that the genetic variability that exists among grass and legume species and cultivars offer the possibility of developing strains with higher salt tolerance (Marcar 1987, West and Taylor 1981, Noble *et al.* 1984, Läuchli 1984, Francois 1988, and Youngner *et al.*, 1967). This variation in salinity response is perhaps not surprising in view of the wide geographic diversity, origin, and distribution patterns of the species, but it seems intrinsically more probable that much of the variability in the species has developed during early transfer from place to place with developing technology. Wild relatives of crop plants may have greater levels of salinity tolerance, and these may be used in crosses to increase the range of genetic variability to salinity tolerance and/or the other desirable characters into the crop breeding programmes (Saranga *et al.* 1993, and Nevo *et al.* 1993). Thus there has been much interaction between cultivated and wild forms of plants (Doggett 1986). A part of salinity tolerance in domesticated clover species might have been the product of natural selection over generations grown in salt affected soil, and/or due to natural hybridisation with wild populations with salt tolerant ecotypes or it may be that during cultivation of some species of clover variability from wild species which may have achieved a degree of salinity tolerance which may have accumulated in cultivated forms through cross-pollination. The evidence about the occurrence of variability for many quantitative plant characters measured within the families of the highly inbreeding grass *Festuca microstachys* complex, and of wild oats (*Avena fatua*) in which the amount of out-crossing varies from 1-10% (Kannenberg and Allard 1967), supports the possibility of salt tolerance in clover.

The existence of within accession variation in the material studied here, may be a corollary of the fact that some species of clover are moderately salt-tolerant and

therefore may be grown extensively in arid and semi-arid areas. *T. repens* is a relatively salt sensitive species that cannot tolerate high salt levels (Gonzalez 1976, Zawadzka 1976, Smith and McComb 1981, Läuchli 1984, Noble and Shannon 1988, and Rogers *et al.* 1992). Nonetheless, Ab-Shukor *et al.*(1988) found a natural salt tolerant population growing on salt marshes in South Wales. Salt tolerance presumably also exists in the maritime species *T. squamosum* L. (Sea Clover), which grows in salt marsh turf in the British Isles (Clapham *et al.* 1987). Some degree of salt tolerance may occur in natural populations of other annual *Trifolium* species that commonly grow on sea-cliffs and in semi-arid habitats, such as *T. scabrum* L., *T. striatum* L., and *T. subterraneum*. Support for this view was provided by Zawadzka (1976) who found greater salt tolerance in the annual species *T. incarnatum* L. and *T. resupinatum* L. than in a salt sensitive cultivar of *T. repens*.

It is thus quite probable that some of the accessions may have evolved a degree of tolerance to the low concentrations of salt prevalent in these areas. The better response to salinity shown by Accession 9 of *T. resupinatum* and Accession 1 of *T. alexandrinum* (Figure 3.1 and 3.2 in Chapter 3) may be due to the same reason, i.e. local adaptation, because these accessions are currently being grown in areas of Iran which have been affected slightly by salinity. This condition is common in the margins of salt marshes. Two species which are widespread on normal non-saline soils in temperate regions of the world, *Agrostis stolonifera* and *Festuca rubra* also grow on the margins of salt marshes, and Hannon and Bradshaw (1968) found salinity tolerance in populations from salt marsh in North Wales.

In previous studies, salt tolerance of a plant determined using the rooting technique has shown a good correlation with the salt content of the soil from the plant's site of origin

(Hannon and Bradshaw 1968, Venables and Wilkins 1978). The work on salt tolerance reported in eight grass species by Ashraf *et al.* (1986a,b) and in millet Kebebew (1994), are based upon selecting seedlings with longest root lengths. Individuals expressing the greatest root growth in salt solutions subsequently yielded highest at the adult plant stage when grown in saline irrigated sand culture. Accessions of *T. alexandrinum* and *T. resupinatum* assessed in sand culture for whole plant response to salinity did not, in this case, originate from individual plant selection, the accessions having the longest shoot and root lengths after two weeks growth (Chapter 3) were Accession 1 and 9 respectively. They were subsequently found to have greater $NaCl + CaCl_2$ tolerance than the other accessions at the adult stage, as described in Chapter 4. Thus the use of water culture assessment methods at seedling stages seems to be a valid and worthwhile means of identifying at least initially tolerant individuals, and a means ultimately for enhancing salinity tolerance in the species studied.

The water culture technique employed in the series of experiments for assessing variability ensures that the chemical features of the root zone concentrations of individual ions and total salinity are defined, and such experiments are conducted in controlled environments in a growth room. The entire root system is uniformly exposed to a saline medium and the plants can be recovered without injury to the roots or shoots, for measurement, or transfer to other medium (Epstein *et al.* 1980). Use of simple measures of root lengths or shoot lengths of two-weeks-old seedlings has revealed genetically based variation in response to metals as well as to salinity in a range of, for example aluminium tolerance in barley and wheat varieties (Foy *et al.* 1965), copper tolerance in *Agrostis capillaris* (McNeilly and Bradshaw 1968), salt tolerance in *Agrostis stolonifera* and *Festuca rubra* (Hannon and Bradshaw, 1968), in

a number of grass species (Ashraf *et al.*, 1986a, b and Jafari 1990), in *Sorghum bicolor* (Azhar and McNeilly 1987), in millet (Kebebew 1994, and Kebebew and McNeilly 1995), and in wheat (Beshir 1996).

Usually, the mature plant is the most salt tolerant growth state. Germinating seeds and seedlings may require less saline conditions for the initial development of the plant than the adult plants (Poljakoff-Mayber and Gale 1975). For example, seedlings produced under non-saline conditions can subsequently be transplanted into saline soils (National Research Council 1990). The relationship between shoot and root dry weight in the sand culture experiment (Table 3.14 and Figure 3.5 in Chapter 3) and shoot and root length of two-week-old grown at the same salinity level in solution culture shows that the seedling test capable to detecting tolerant material. Screening a large number of accessions or genotypes on saline soil, or soils or sand culture irrigated by saline water using whole plants are not feasible using variabilities such as, salts distribution within the soil (Nieman and Shannon 1976), thus time consuming and expensive.

For successful selection it is necessary to know the genetic basis of tolerance variability, whether using shoot or root length data. A metric character heritability is one of the most important properties, because the degree of correspondence between phenotypic values and breeding values are measured by heritability (Falconer and Mackay 1996). Heritability of a certain character under a particular set of environmental conditions could supply information of a portion of the total variability that genetically controlled. In Chapter 4 estimations of broad sense heritability for salinity tolerance over seven salinity levels for 10 accessions of each three *Trifolium* species indicated that variation in shoot and root length at the seedling stage in

response to salinity was genetically controlled. Thus it should be possible to produce some higher salinity tolerant genotypes by artificial selection.

In 1978 Shannon considered that after two cycles of selection and breeding there will be an increase in the tolerance. This may lead to the production of healthy, productive plants of forage grasses. This in turn may result in an increased yield on salt affected soil. Results shown in Figure 4.7 to 4.9 in Chapter 4 coincides with Shannon's assumption, the salinity tolerance of *T. alexandrinum* improved to allow growth to extend from EC 26 to 29 dSm^{-1}, *T. resupinatum* from 26 to 32 dSm-1, and *T. repens* from 18 to 20 dSm^{-1}, after only two cycles of selection. It seems possible, that further advances in salinity tolerance in these species could be achieved by further cycles of selection and with intra/ inter-species breeding.

The other options could be the transfer of tolerance gene from tolerant accessions or wild species. Many workers have attempted inter-species crosses in the genus of *Trifolium* by different techniques, such as Trimble and Hovin (1960), Evans (1962), Kazimierski *et al.* (1972), Selim *et al.* (1977), Smith (1979), Kazimierska (1980), Taylor (1980), Przywara *et al.* (1989), Yamada (1989), Williams (1990), Marshall *et al.* (1995), Meredith *et al.* (1995), and Przywara *et al.* (1996).

The objective of Chapter 5 was to determine the possibility of interspecific hybridisation between three *Trifolium* species namely, *T. alexandrinum*, *T. resupinatum*, and *T. repens*. This could help to combine quality in grazing factors, leading to the development of very useful lines in hybrids, at least as salinity tolerant as the tolerance species *T. alexandrinum* and *T. resupinatum*. Unfortunately crosses between *T. alexandrinum* and *T. resupinatum* were not successful. Crosses between

T. alexandrinum and *T. resupinatum* produced some hybrid seeds but, no hybrid plants were achieved. The successful crosses between *T. alexandrinum* and *T. repens* produced hybrids in both direction crosses. The progenies were perennial with nodes and stoloniferous stems. The hybrids had a high percent of viable (stainable) pollen grains. They also had some superior characters from both parents (hybrid vigour), Some characters had intermediate expressions, and the other characters were similar to *T. repens*. Further work might produce cover forage of good grazing quality and salinity tolerance using high quality and productive *T. repens* varieties/cultivars.

Overall achievements from this study can be summarised as,

1) Investigations of salinity tolerance for 65 accessions of *Trifolium* species, revealed substantial variation in salinity tolerance accessions in seven species.

2) A strong correlation existed between salinity tolerance at two-week-old seedlings and adult plants grown in a sand culture experiment of accession studies.

3) *T. resupinatum* was not only similar in tolerance to *T. alexandrinum* (regarded the most tolerant species), but some accessions were more tolerant than *T. alexandrinum.*

4) Salinity tolerance of the species were strongly heritable, and thus salinity tolerance could be improved after two cycles of intensive selection pressure.

5) Interspecific hybrid of *T. alexandrinum* (tolerant species) and *T. repens* (better for grazing) with annual behaviour and stainable pollen grains was achieved, combining potentially annual and salinity tolerance characters, and perennial and herbage quality.

6.2. Future perspective

In arid and semi-arid region the salinisation of soil and water is becoming an increasingly serious constrain for crop production. For an increasing human population it is necessary to increase food production per unit area of land. One of the most serious problems of these areas is secondary salinisation which is usually associated with irrigated agriculture. About 1.5 million ha of prime farmland in the world is going out of crop production each year (The Economist 1992), the possibility of growing alternative plants suited to moderately saline conditions has been investigated. The first option could be to introduce under-exploited salinity tolerant plants (Aronson 1985 and Somers 1979). The other option could be the selection of a new salt tolerant genotypes from breeding programmes for increased tolerance to salt stress (Stutz 1983 and Epstein 1985). The third option might be to modify traditional crops by molecular biology to make higher salinity tolerance crops (Epstein and Rains 1987).

Trifolium species showed that there is some variability in salinity tolerance exist but it would be desirable to enhance tolerance further by several cycles of selection. The results of the work presented in this thesis showed that further investigation are required in number of areas in order to improve salinity tolerance or transfer genes from salinity tolerant species/accessions to sensitive ones through interspecific hybridisation.

Possible future development would require:

1) Selection of genetic sources with acceptable levels of salinity tolerance from existing accessions, as well as creating genetic variability in domestic and wild species over a wide range. For this purpose new biological techniques should be considered to strengthen the selection base material, for instance *in vitro* selection using somaclonal variation, mutagenesis, *in situ* hybridisation, somatic hybridisation, and genetic engineering or possibly recombinant DNA for salinity tolerance.

2) Embryo/ovule culture to overcome inter-species compatibility.

3) Investigation of hybrid plants by modern biotechnology techniques such as: RFLP (restriction fragment-length polymorphisms), RAPD (random amplified polymorphic DNA) to identify markers that could be used for tagging the physiological components of salinity tolerance.

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APPENDICES 255

APPENDICES

Appendix 2.1. Nutrient solution for sand and water culture experiments. based upon solution used by Rorison and outlined in Hewitt (1966).

Nutrient Sources	Concentration in Stock Solution g l^{-1}	Make up Volume		
		Full	0.5	0.1
$Ca(NO_3)_2\ 4H_2O$	472.00	1 ml l^{-1}	0.5 ml l^{-1}	0.1 ml l^{-1}
$K_2\ HPO_4$	58.00	3 ml l^{-1}	1.5 ml l^{-1}	0.3 ml l^{-1}
$MgSO_4\ 7H_2O$	123.00	2 ml l^{-1}	1.0 ml l^{-1}	0.2 ml l^{-1}
Fe - EDTA	12.50	1 ml l^{-1}	0.5 ml l^{-1}	0.1 ml l^{-1}
KCl	124.30	1 ml l^{-1}	0.5 ml l^{-1}	0.1 ml l^{-1}
Trace Elements		1 ml l^{-1}	0.5 ml l^{-1}	0.1 ml l^{-1}
$MnSO_4\ 4H_2O$	2.028			
$H_3\ BO_3$	2.863			
$(NH_4)_6\ Mo_7\ O_{24}\ 4H_2O$	0.184			
$ZnSO_4\ 7H_2O$	0.440			
$CuSO_4\ 5H_2O$	0.390			

Appendix 2.2. Mean Squares and significance from nested analysis of variance of shoot length of 5 accessions of 7 *Trifolium* species grown in solution cultures at 8 EC levels for 14 days.

Sources	DF	Mean Squares	P > F
Replication	2	195.50^{ns}	0.15
EC (dSm^{-1})	7	778407.75***	0.0001
Accessions (SPP)	28	9352.88***	0.0001
Species	6	227287.69***	0.0001
EC × SPP	42	8014.02***	0.0001
ACC (EC × SPP)	196	1602.37***	0.0001
Residual	8118	102.01	

DF = Degree of freedom
ns = No significant ($P > 0.05$)
* = significant ($P \leq 0.05$)
** = significant ($P \leq 0.01$)
*** = significant ($P \leq 0.001$)

Appendix 2.3. Mean Squares and significance from nested analysis of variance of shoot length of 5 accessions of 7 *Trifolium* species grown in solution cultures at 8 EC levels for 14 days.

Sources	DF	Mean Squares	P > F
Replication	2	70.66^{ns}	0.69
EC (dSm^{-1})	7	1330915.25***	0.0001
Accessions (SPP)	28	19124.24***	0.0001
Species	6	324791.78***	0.0001
EC × SPP	42	16779.48***	0.0001
ACC (EC × SPP)	196	5248.67***	0.0001
Residual	8118	193.80	

Appendix 2.4. Absolute shoot length means/mm of 5 accessions from 7 *Trifolium* species. [Grouping, from Duncan's Multiple Range Test (0.05%)]

Species	Mean / DG[1]	EC 0.32 dSm^{-1}	EC 4 dSm^{-1}	EC 8 dSm^{-1}	EC 10 dSm^{-1}	EC 14 dSm^{-1}	EC 18 dSm^{-1}	EC 22 dSm^{-1}	EC 26 dSm^{-1}
T. alexandrinum	Mean	80.19	83.01	78.82	76.07	61.35	30.10	15.53	6.55
	Duncan grouping	A B	A	B C	C	D	E	F	G
T. resupinatum	Mean	80.73	87.95	78.26	69.99	55.98	37.27	22.34	10.88
	Duncan grouping	B	A	B	C	D	E	F	G
T. repens	Mean	43.73	39.64	35.13	26.82	6.83	2.09	0.00	0.00
	Duncan grouping	A	B	C	D	E	F	F	F
T. subterraneum	Mean	80.68	80.81	69.61	51.88	25.90	7.56	0.00	0.00
	Duncan grouping	A	A	B	C	D	E	F	F
T. pratense	Mean	63.33	60.42	56.93	49.12	30.39	8.88	0.00	0.00
	Duncan grouping	A	B	C	D	E	F	G	G
T. ambiguum	Mean	53.81	56.17	48.78	37.70	20.63	4.47	0.00	0.00
	Duncan grouping	A	A	B	C	D	E	F	F
T. fragiferum	Mean	63.25	59.64	46.95	33.04	13.82	3.77	0.00	0.00
	Duncan grouping	A	B	C	D	E	F	G	G

1. DG = Duncan's Multiple Range Test (DMRT) Grouping

Appendix 2.5. Absolute root length means/mm of 5 accessions from 7 *Trifolium* species. [Grouping, from Duncan's Multiple Range Test (0.05%)]

Species	Mean / DG[1]	EC 0.32 dSm^{-1}	EC 4 dSm^{-1}	EC 8 dSm^{-1}	EC 10 dSm^{-1}	EC 14 dSm^{-1}	EC 18 dSm^{-1}	EC 22 dSm^{-1}	EC 26 dSm^{-1}
T. alexandrinum	Mean	91.85	105.32	105.65	115.31	88.65	39.43	16.94	6.41
	Duncan grouping	C	B	B	A	C	D	E	F
T. resupinatum	Mean	83.29	113.30	105.41	94.85	70.06	37.67	17.76	9.27
	Duncan grouping	D	A	B	C	E	F	G	H
T. repens	Mean	73.51	76.58	62.22	44.81	9.11	1.47	0.00	0.00
	Duncan grouping	B	A	C	D	E	F	F	F
T. subterraneum	Mean	83.14	100.80	86.72	68.25	33.79	9.84	0.00	0.00
	Duncan grouping	B	A	B	C	D	E	F	F
T. pratense	Mean	78.24	85.89	80.41	64.82	37.85	9.45	0.00	0.00
	Duncan grouping	B	A	B	C	D	E	F	F
T. ambiguum	Mean	56.76	68.51	63.49	46.48	23.01	4.02	0.00	0.00
	Duncan grouping	C	A	B	D	E	F	G	G
T. fragiferum	Mean	68.05	65.91	55.31	36.87	14.28	3.22	0.00	0.00
	Duncan grouping	A	A	B	C	D	E	E	E

Appendix 2.6. Sum of squares and significance from analyses of variance of absolute shoot and root data of 10 accessions of *T. alexandrinum* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of squares	P > F
Replications	2	282.00 **ns**	0.26
EC (dSm^{-1})	7	21556221.04***	0.0001
Accessions (ACC)	9	98905.23***	0.0001
ACC × EC	63	71196.00***	0.0001
Error	2318	239939.14	
Total	2399	2565943.43	

Root

Sources	DF	Sum of squares	P > F
Replications	2	3430.44**	0.005
EC (dSm^{-1})	7	4546844.42***	0.0001
Accessions (ACC)	9	166669.84***	0.0001
ACC × EC	63	517392.27***	0.0001
Error	2318	735644.63	
Total	2399	5969988.59	

Appendix 2.7. Sum of squares and significance from analyses of variance of absolute shoot and root data of 10 accessions of *T. resupinatum* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of squares	P > F
Replications	2	24.25 ns	0.92
EC (dSm^{-1})	7	1923900.61***	0.0001
Accessions (ACC)	9	127658.37***	0.0001
ACC × EC	63	830610.95***	0.0001
Error	2318	345841.88	
Total	2399	2481036.07	

Root

Sources	DF	Sum of squares	P > F
Replications	2	1187.01 ns	0.23
EC (dSm^{-1})	7	4950420.67***	0.0001
Accessions (ACC)	9	613045.38***	0.0001
ACC × EC	63	630722.65***	0.0001
Error	2318	929821.26	
Total	2399	7125196.96	

Appendix 2.8. Sum of squares and significance from analyses of variance of absolute shoot and root data of 10 accessions of *T. repens* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of Square	P > F
Replications	2	42.96 **ns**	0.55
EC (dSm^{-1})	7	705029.27***	0.0001
Accessions (ACC)	9	76632.76***	0.0001
ACC × EC	63	64300.23***	0.0001
Error	2318	83836.38	
Total	2399	929901.59	

Root

Sources	DF	Sum of squares	P > F
Replications	2	1187.37**	0.006
EC (dSm^{-1})	7	2027259.68***	0.0001
Accessions (ACC)	9	123044.47***	0.0001
ACC × EC	63	124200.69***	0.0001
Error	2318	272451.73	
Total	2399	2548143.93	

Appendix 2.9. Sum of squares and significance from analyses of variance of absolute shoot and root data of 10 accessions of *T. subterraneum* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of squares	P > F
Replications	2	289.07 **ns**	0.37
EC (dSm^{-1})	7	2235325.19***	0.0001
Accessions (ACC)	9	95807.53***	0.0001
ACC × EC	63	171232.54***	0.0001
Error	2318	340040.80	
Total	2399	2842695.12	

Root

Sources	DF	Sum of squares	P > F
Replications	2	25.02 **ns**	0.67
EC (dSm^{-1})	7	3667360.72***	0.0001
Accessions (ACC)	9	167193.25***	0.0001
ACC × EC	63	377748.01***	0.0001
Error	2318	723064.41	
Total	2399	4935616.41	

Appendix 2.10. Sum of squares and significance from analyses of variance of absolute shoot and root data of 10 accessions of *T. pratense* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of squares	P > F
Replications	2	942.37**	0.005
EC (dSm^{-1})	7	1758778.11***	0.0001
Accessions (ACC)	9	59226.92***	0.0001
ACC × EC	63	98237.40***	0.0001
Error	2318	205196.17	
Total	2399	2122380.96	

Root

Sources	DF	Sum of squares	P > F
Replications	2	232.06 ns	0.52
EC (dSm^{-1})	7	3213686.47***	0.0001
Accessions (ACC)	9	233480.99***	0.0001
ACC × EC	63	238466.06***	0.0001
Error	2318	409142.37	
Total	2399	4095007.95	

Appendix 2.11. Sum of squares and significance from analyses of variance of absolute shoot and root data of 10 accessions of *T. ambiguum* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of squares	P > F
Replications	2	123.41 **ns**	0.43
EC (dSm^{-1})	7	1273660.07***	0.0001
Accessions (ACC)	9	48401.69***	0.0001
ACC × EC	63	44957.35***	0.0001
Error	2318	167289.66	
Total	2399	1534432.19	

Root

Sources	DF	Sum of squares	P > F
Replications	2	502.85 **ns**	0.09
EC (dSm^{-1})	7	1777542.71***	0.0001
Accessions (ACC)	9	128765.91***	0.0001
ACC × EC	63	126332.78***	0.0001
Error	2318	245490.58	
Total	2399	2277734.83	

Appendix 2.12. Sum of squares and significance from analyses of variance of absolute shoot and root data of 5 accessions of *T. fragiferum* grown in 8 levels of NaCl + $CaCl_2$ concentrations, in solution culture for 14 days.

Shoot

Sources	DF	Sum of squares	P > F
Replications	2	699.40**	0.005
EC (dSm^{-1})	7	747445.88***	0.0001
Accessions (ACC)	4	17045.00***	0.0001
ACC × EC	28	19278.14***	0.0001
Error	1158	75395.27	
Total	1199	859863.68	

Root

Sources	DF	Sum of squares	P > F
Replications	2	156.67 ns	0.42
EC (dSm^{-1})	7	928196.55***	0.0001
Accessions (ACC)	4	69687.19***	0.0001
ACC × EC	28	75871.96***	0.0001
Error	1158	104799.30	
Total	1199	1178621.66	

Appendix 2.13. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. alexandrinum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

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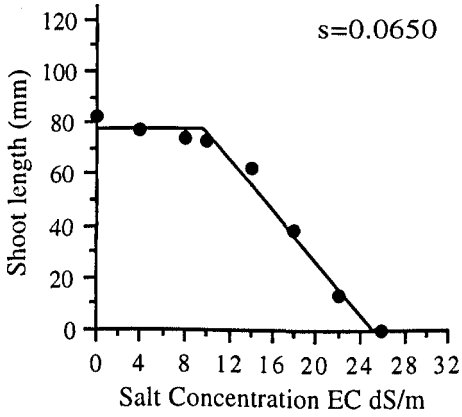

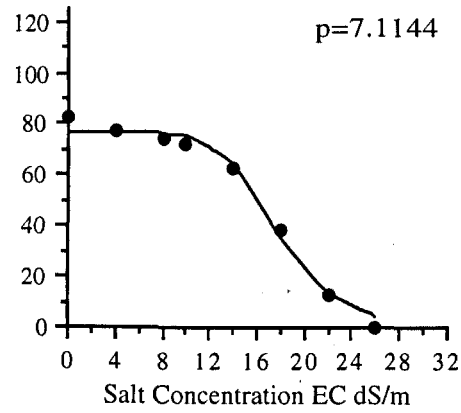

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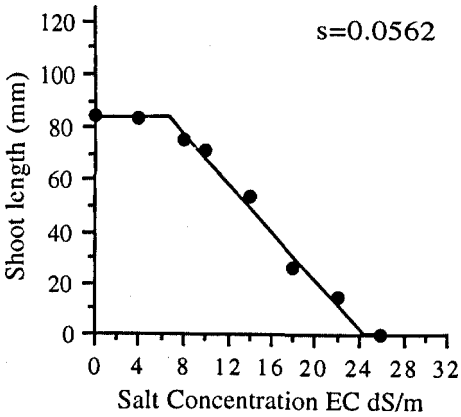

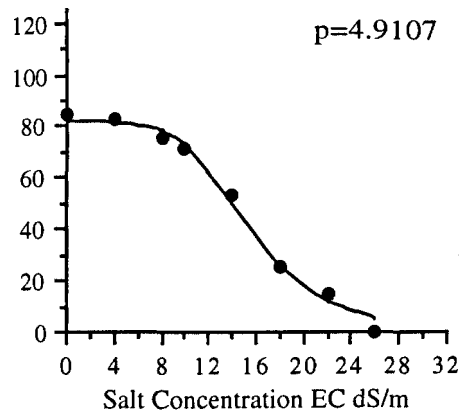

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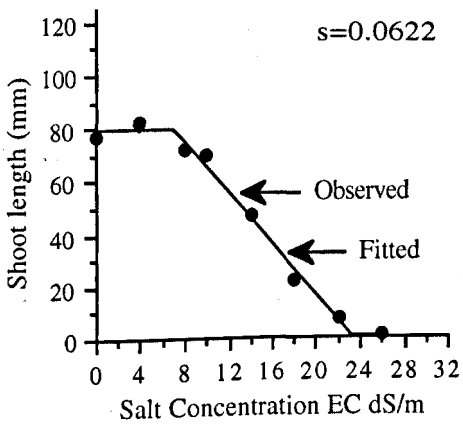

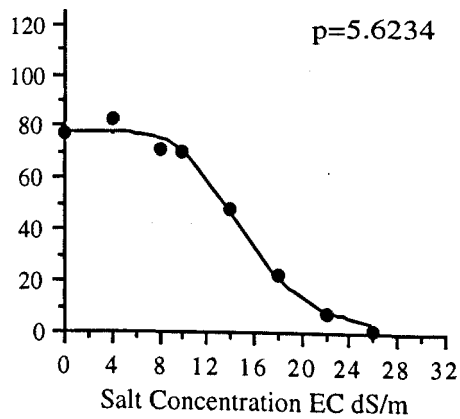

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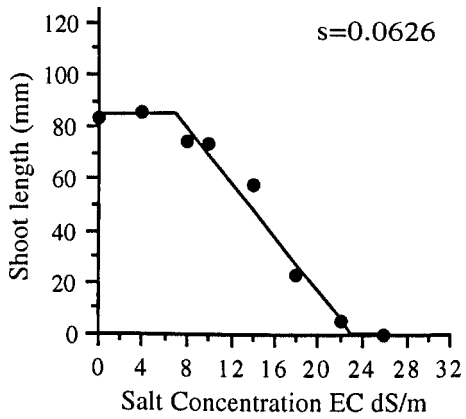

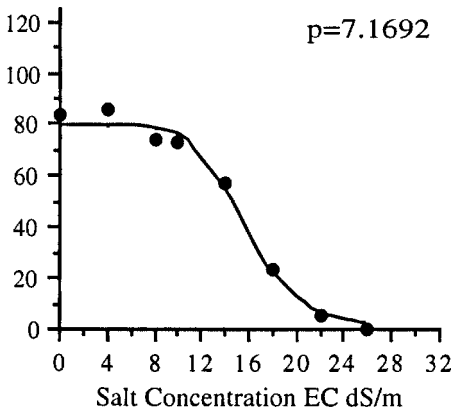

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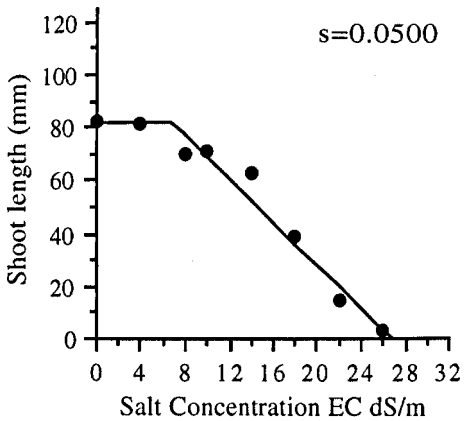

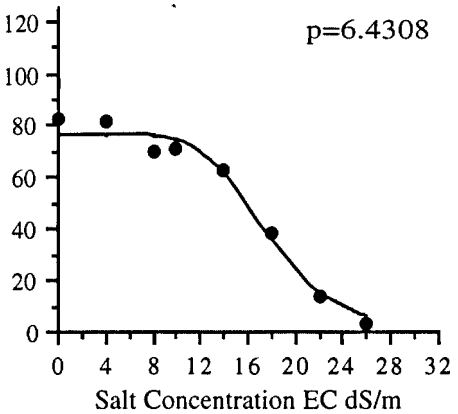

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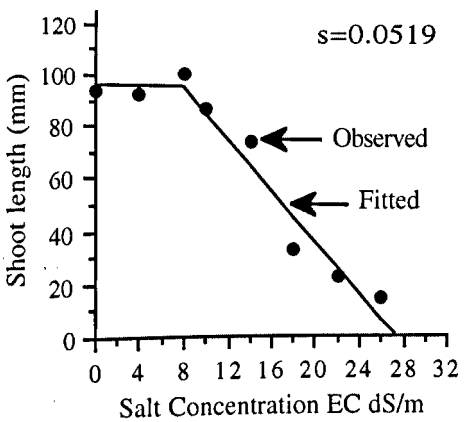

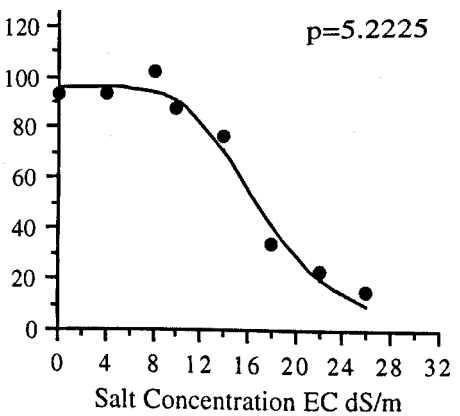

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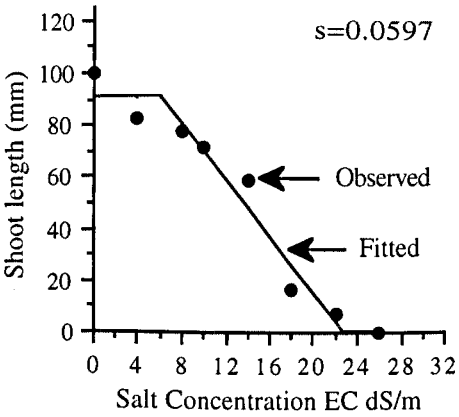

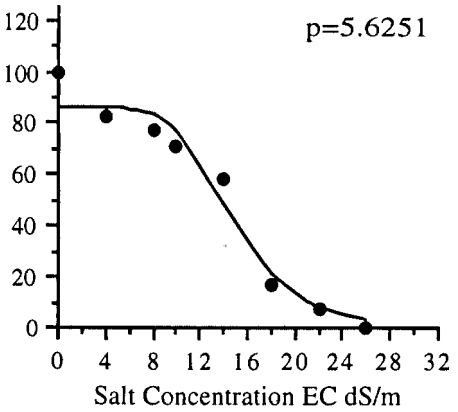

Appendix 2.14. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. resupinatum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

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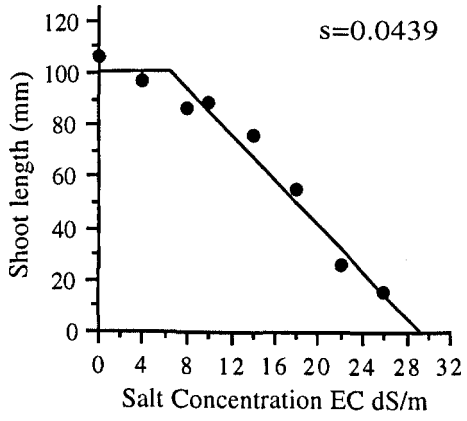

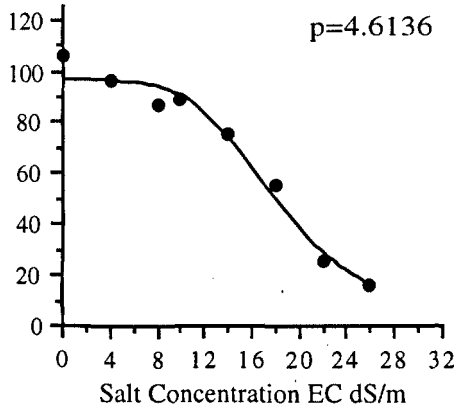

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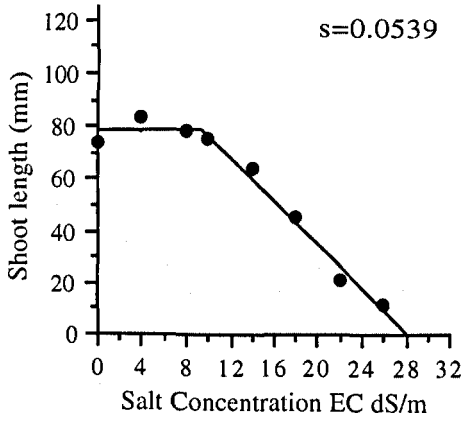

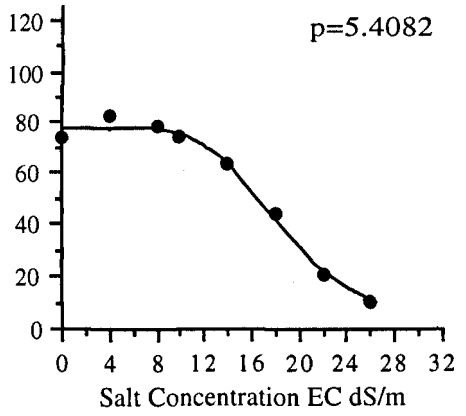

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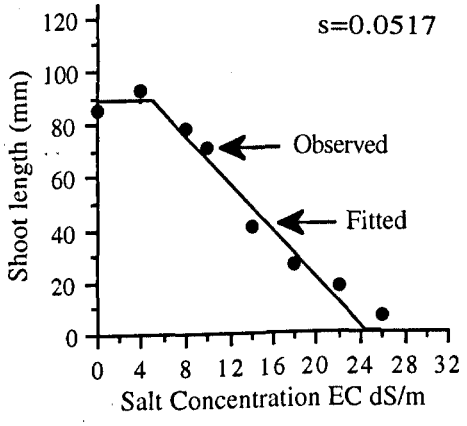

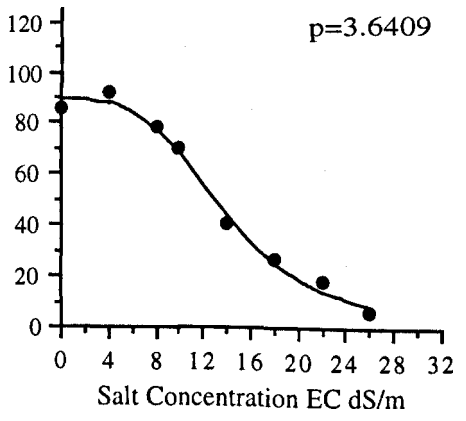

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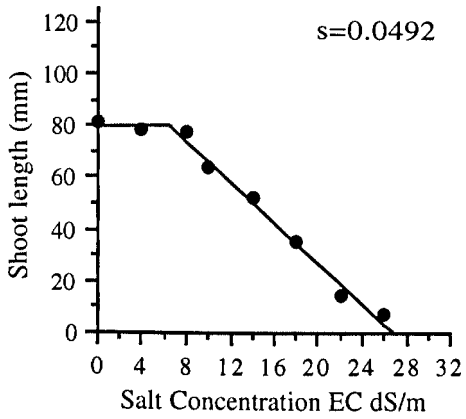

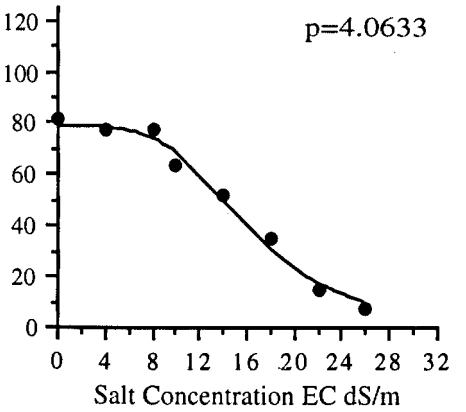

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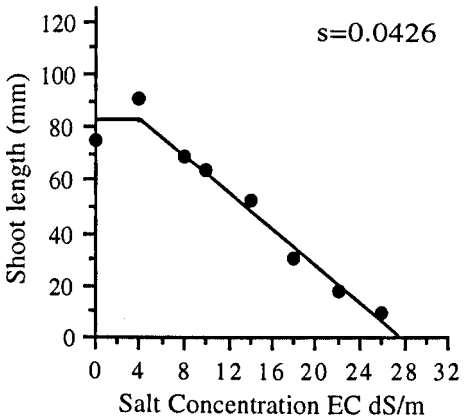

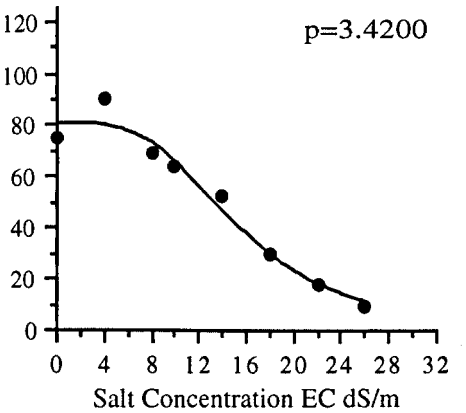

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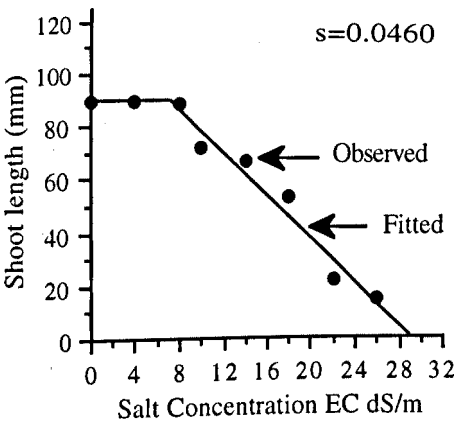

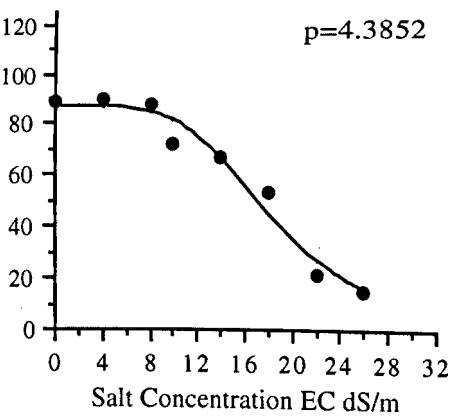

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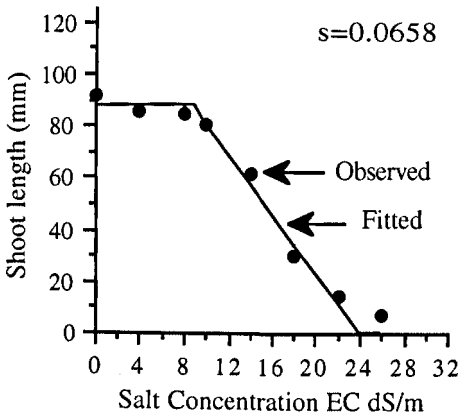

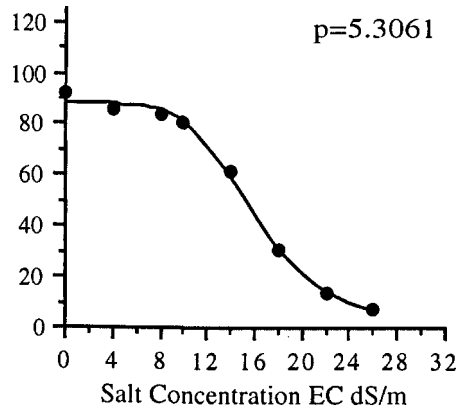

Appendix 2.15. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. repens* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

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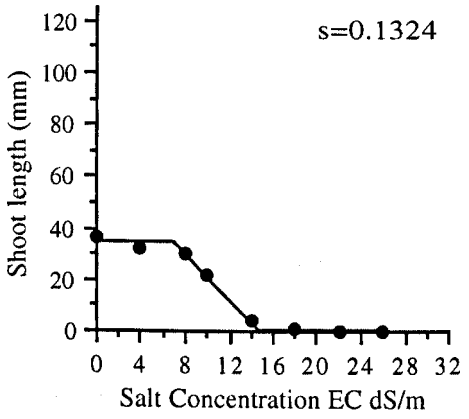

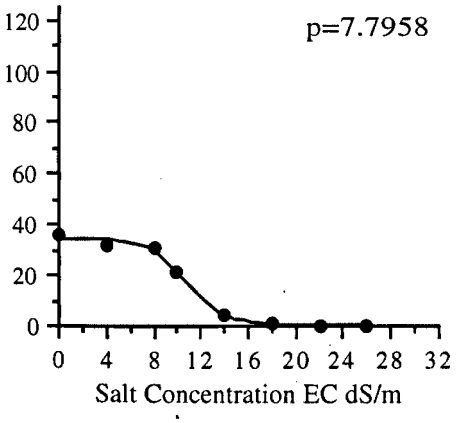

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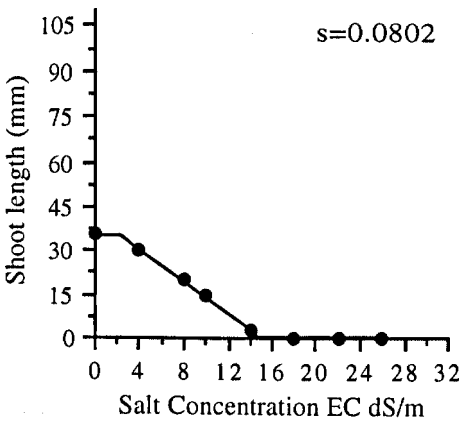

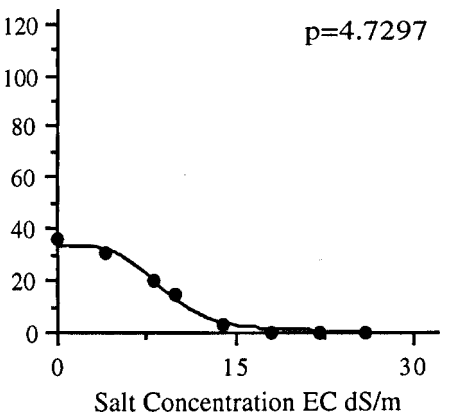

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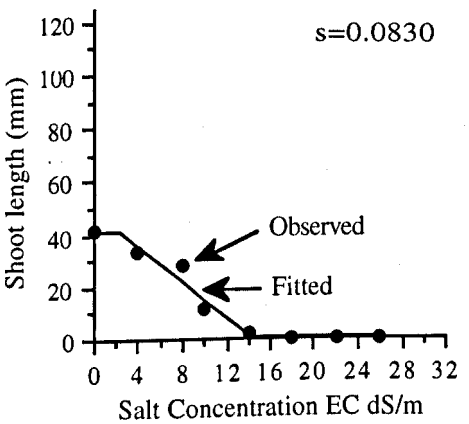

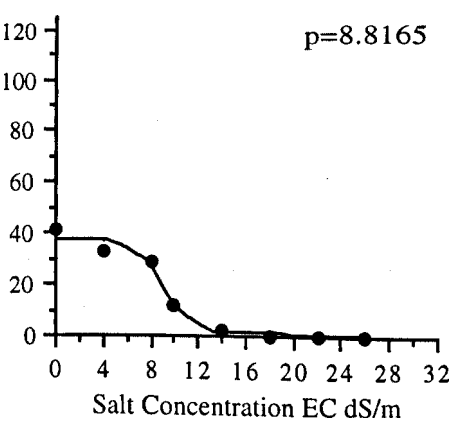

(Appendix 2.15. continued)

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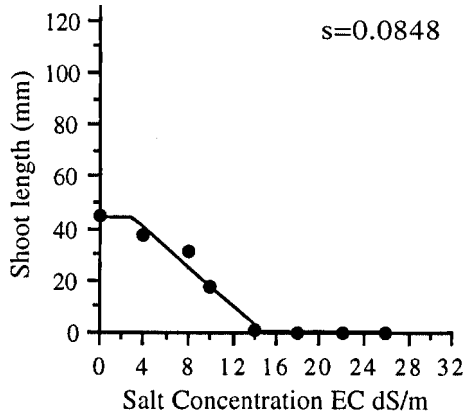

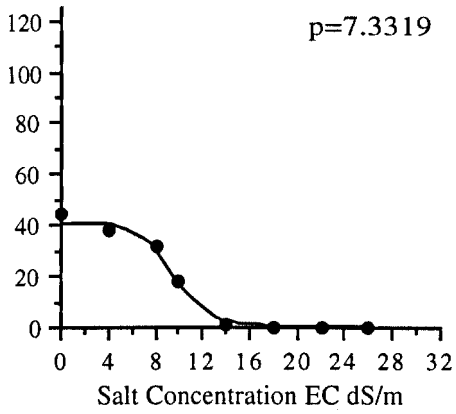

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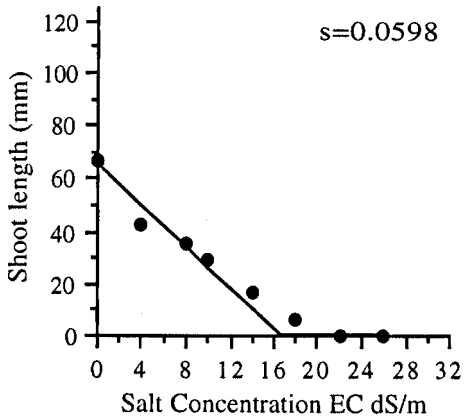

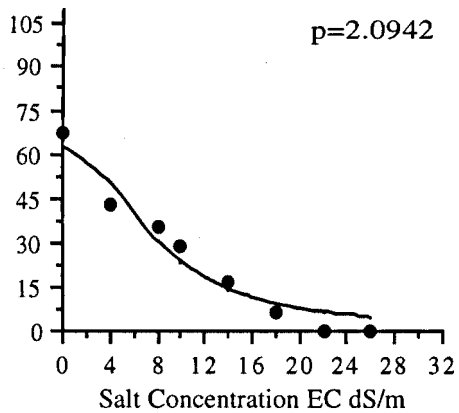

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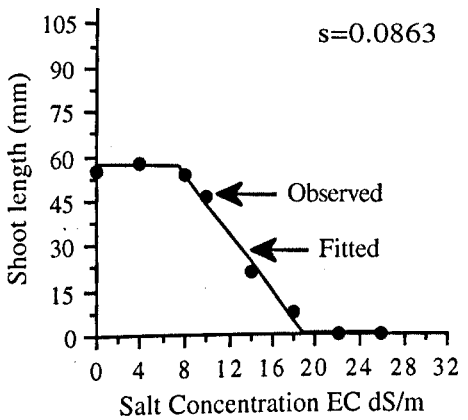

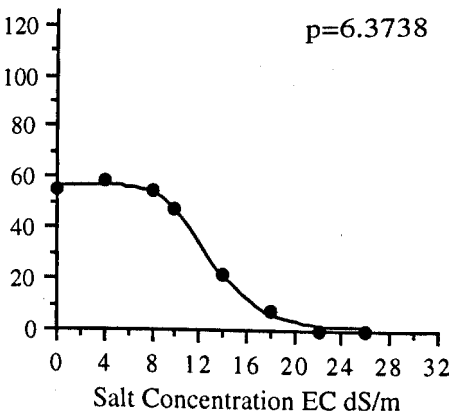

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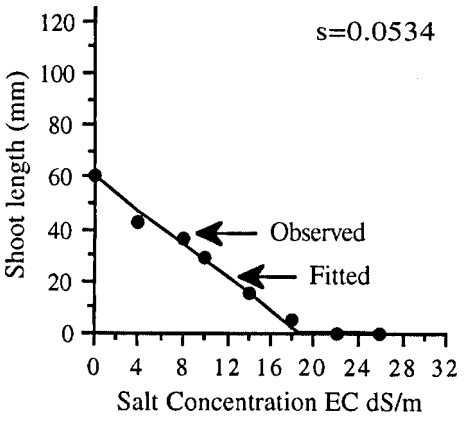
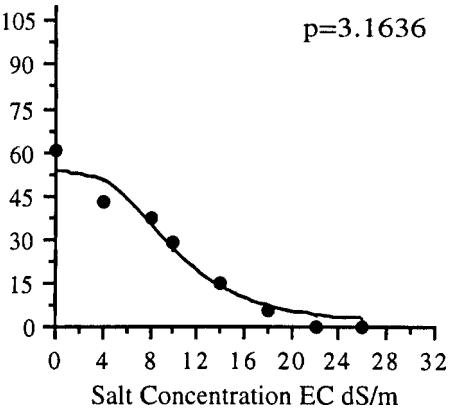
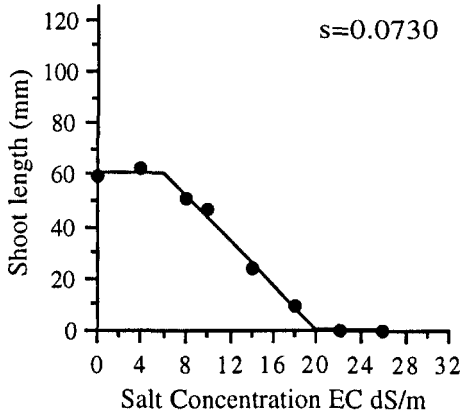
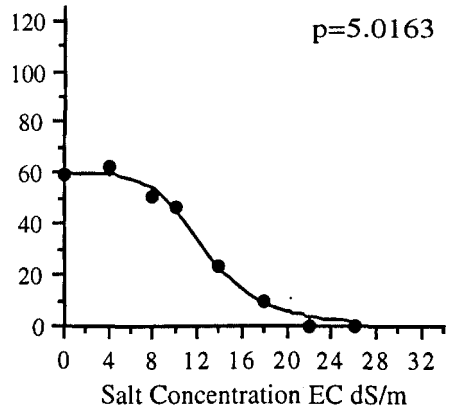




Appendix 2.16. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. subterraneum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

Accession 1.





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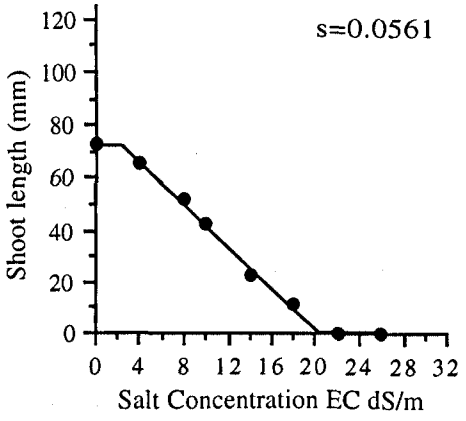

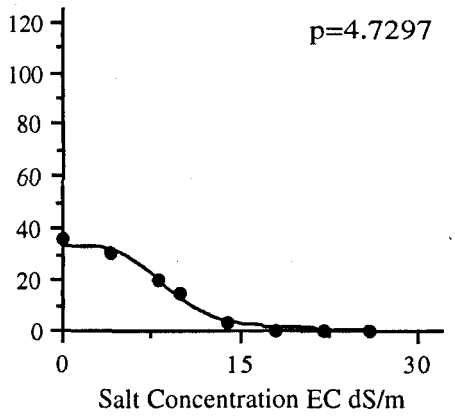

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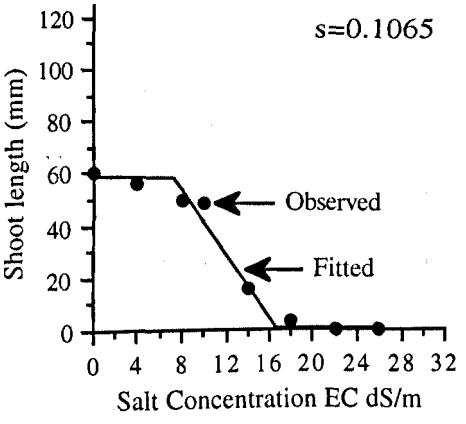
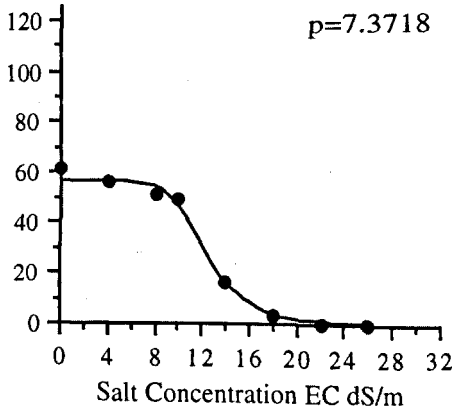
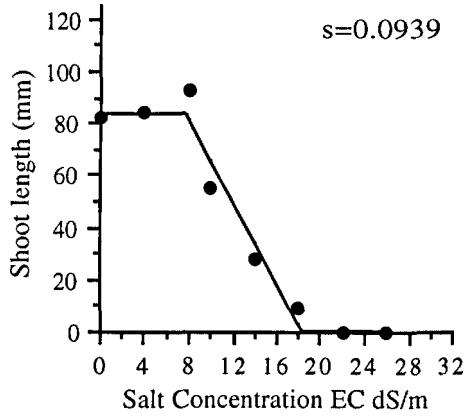
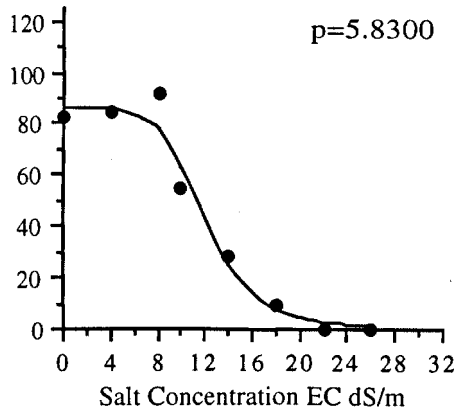




(Appendix 2.16. continued)

Accession 5.





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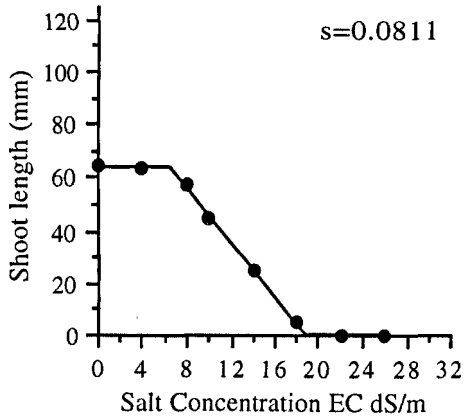

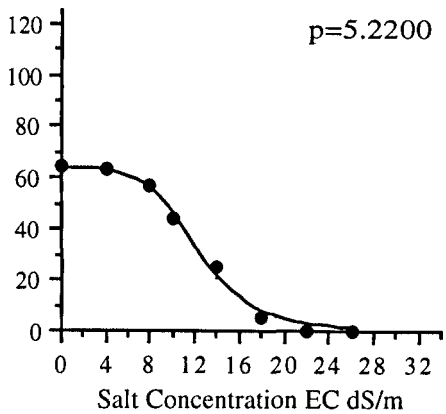

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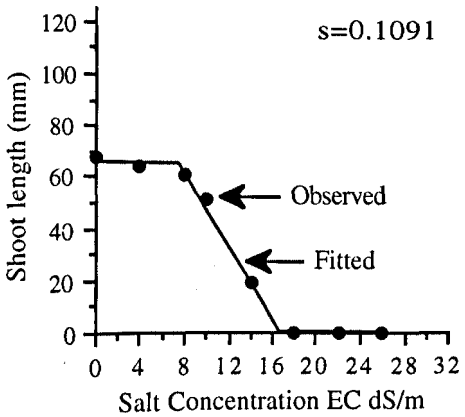
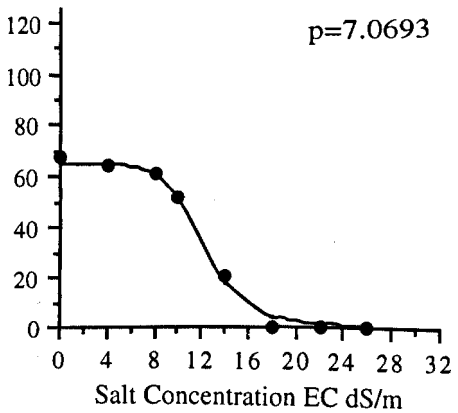
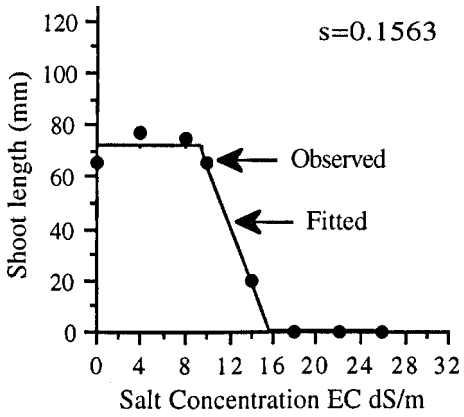
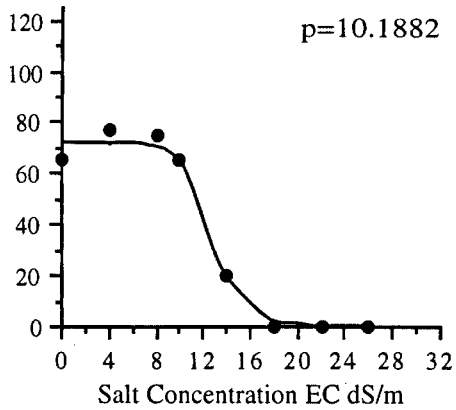




(Appendix 2.16. continued)

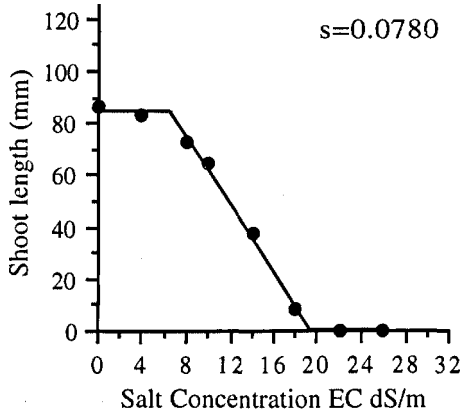
Accession 10.

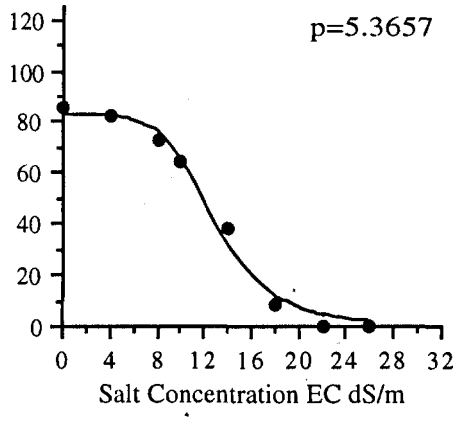




Appendix 2.17. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. pratense* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

Accession 2.





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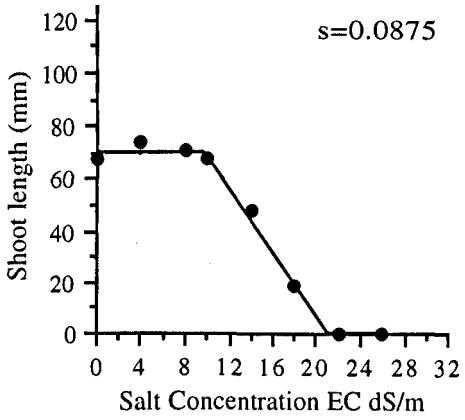

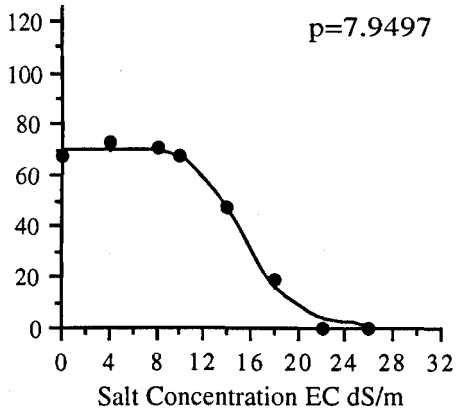

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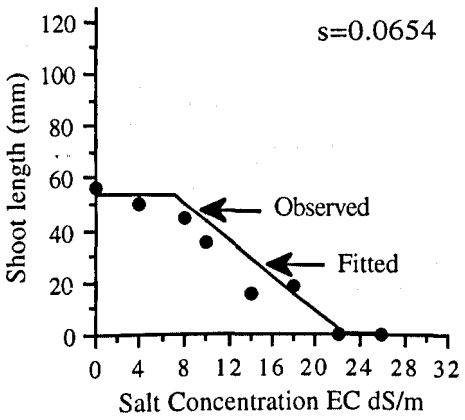

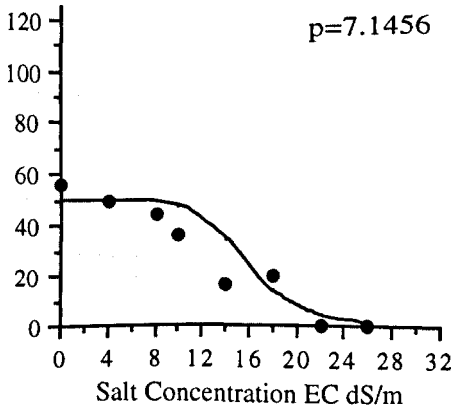

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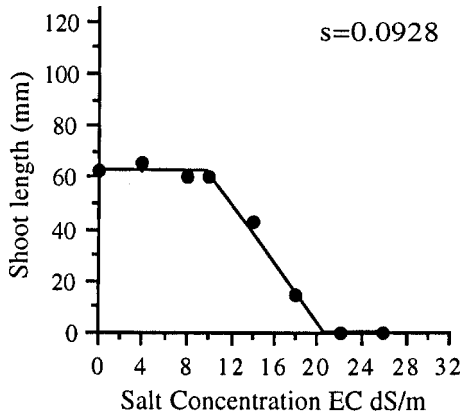

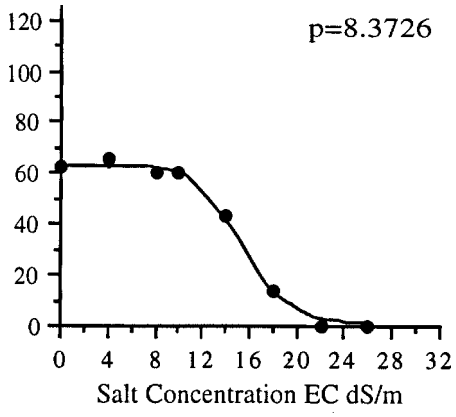

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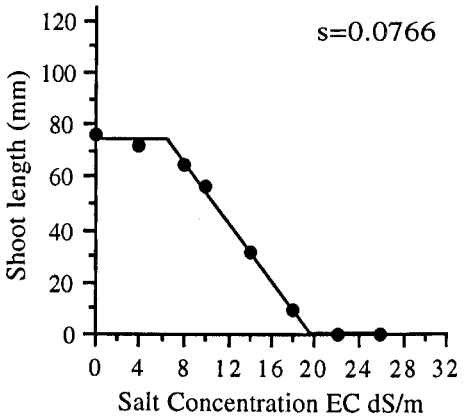

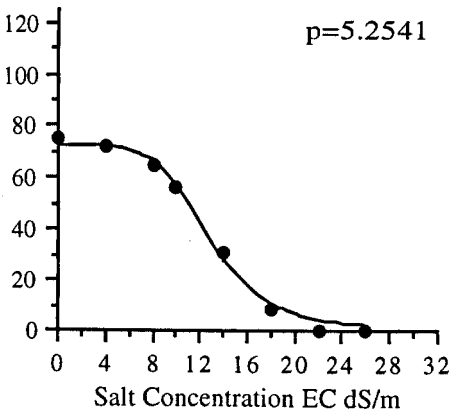

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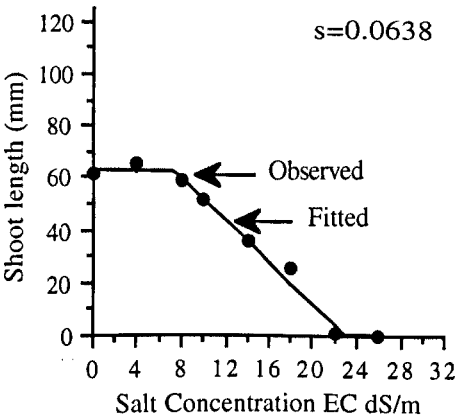
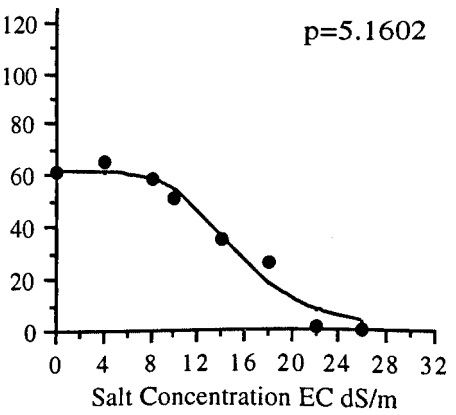
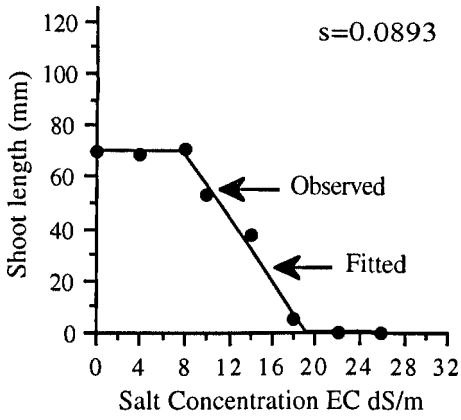
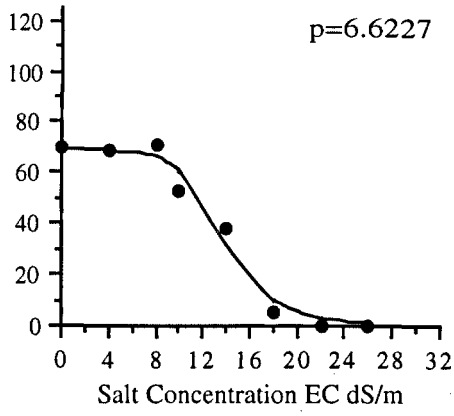




(Appendix 2.17. continued)

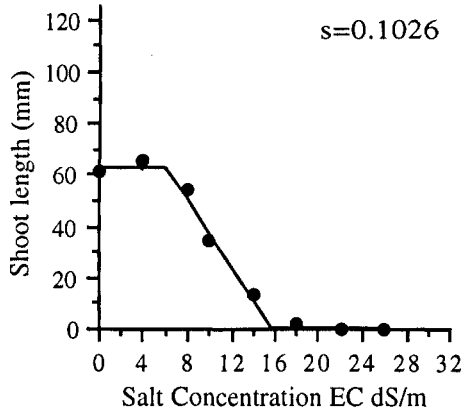
Accession 9.

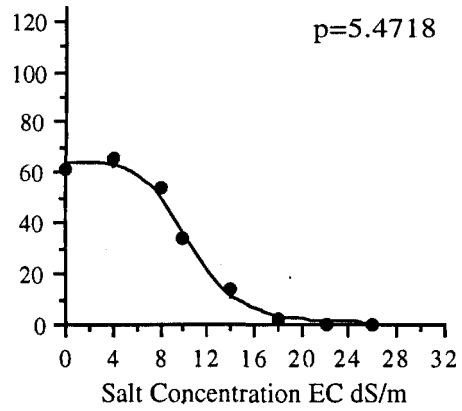




Appendix 2.18. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. ambiguum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

Accession 1.





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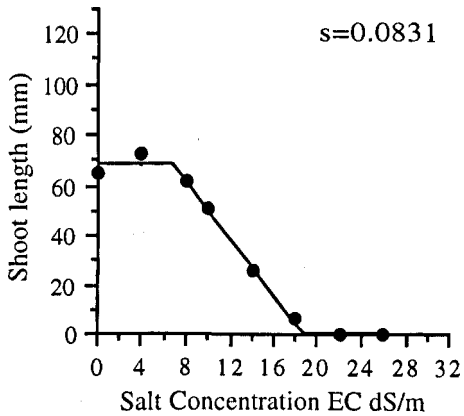

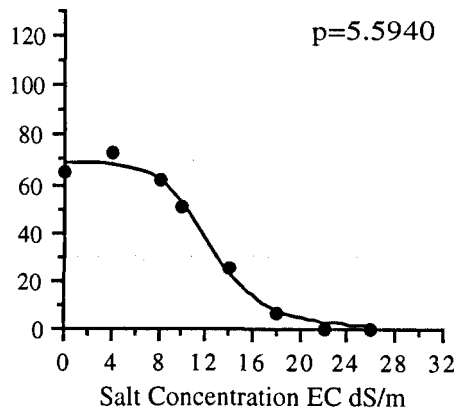

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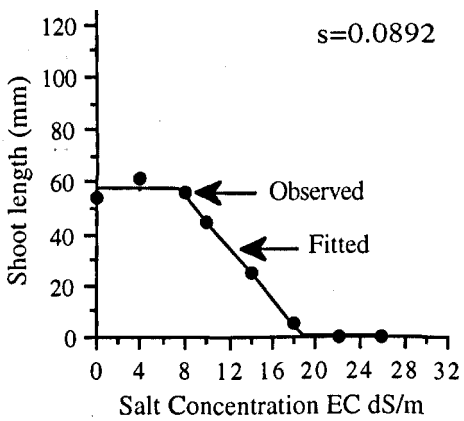

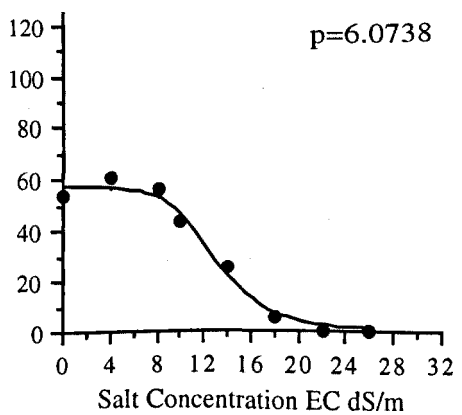

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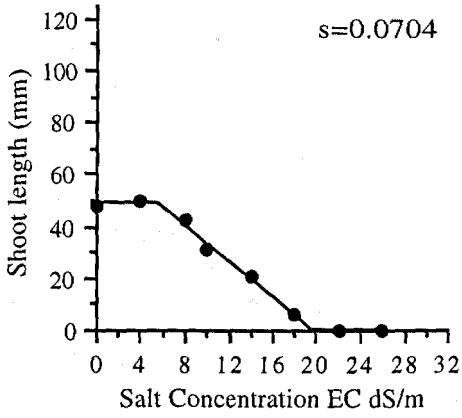

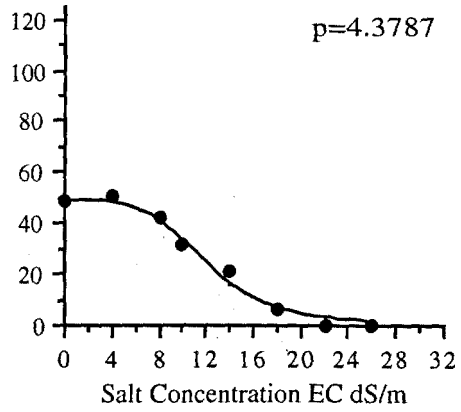

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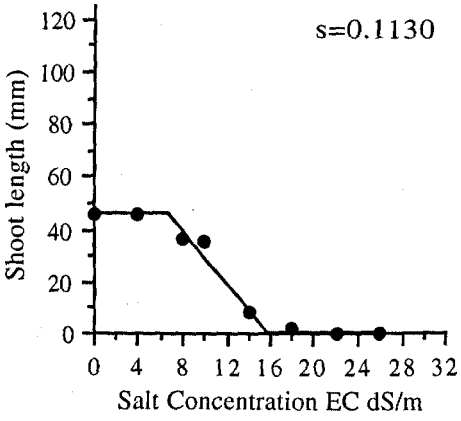

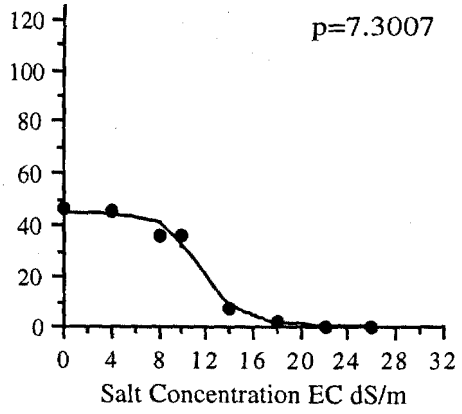

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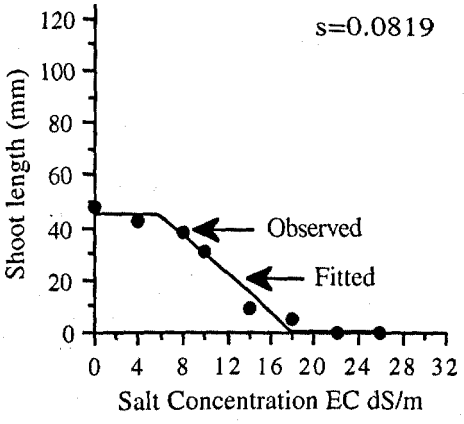
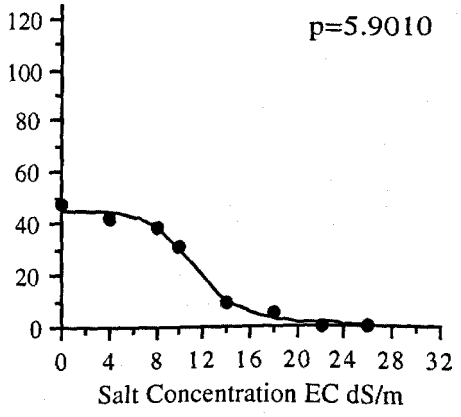
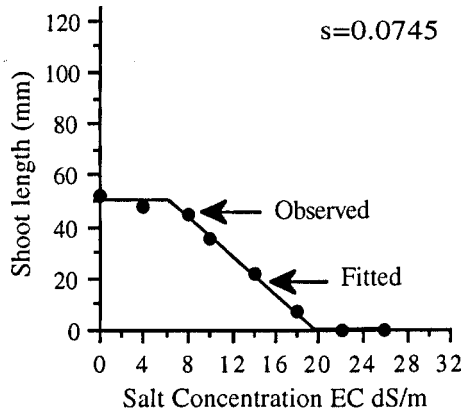
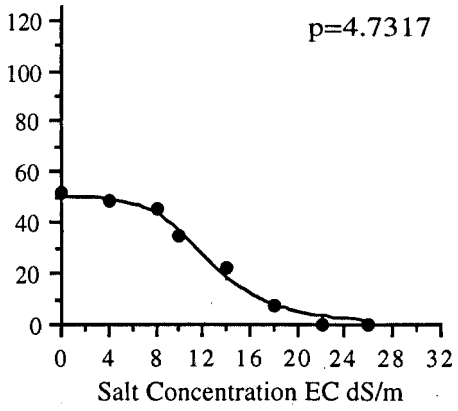




(Appendix 2.18. continued)

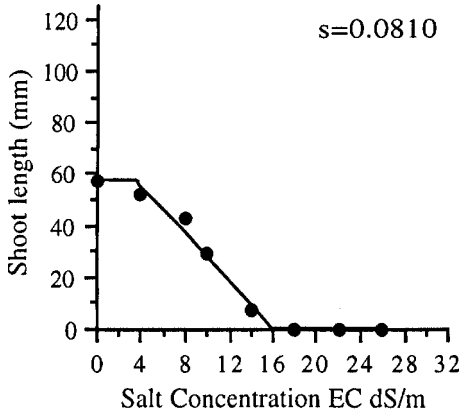
Accession 10.

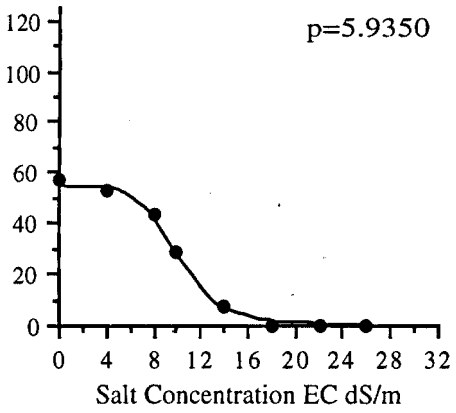




Appendix 2.19. Response functions between NaCl + $CaCl_2$ solutions (EC dSm^{-1}) and shoot length (mm) of 14 day-old seedling of *T. fragiferum* with slope, s (see NOPT 5 in materials and methods) and empirical constant, p (see NOPT 12 in materials and methods).

Accession 3.





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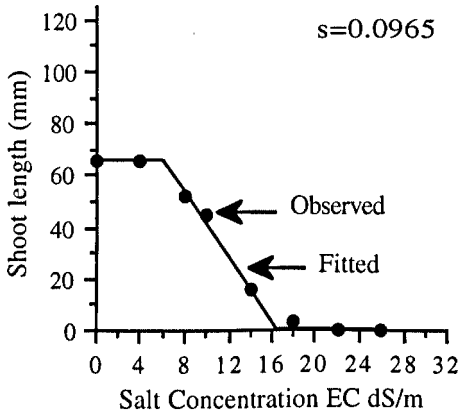

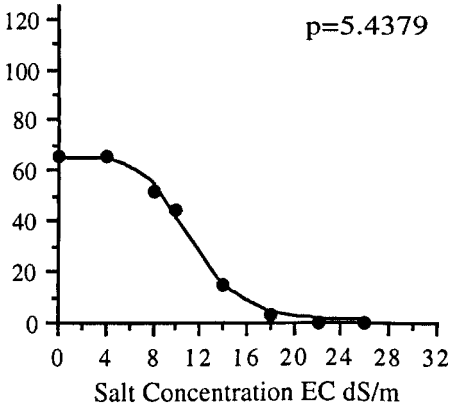

Appendix 4.1. Mean squares and significances from the analysis of variance of shoot and root lengths of *T. resupinatum* seedlings (S_2) grown at EC 29 and 32 dSm^{-1}

Accession	Sources	DF	Mean of Square	P < F
Shoot	Concentration	2	45476.40 ***	0.0001
	Residual	87	123.72	
Root	Concentration	2	61184.41 ***	0.0001
	Residual	87	120.82	

Adaptation in Plant Breeding XIV Eucarpia Congress, Jyvaskyla, Helsinki, 1995.

SALINITY STRESS TOLERANCE IN *Trifolium* SPP.

Babagolzadeh A., McNeilly T.

Department of Environmental and Evolutionary Biology, The University of Liverpool

The relative salt tolerances of two week old seedlings of several accessions of six *Trifolium* species, *T. resupinatum*, *T. alexandrinum*, *T. subterraneum*, *T. repens*, *T. pratense*, and *T. fragiferum* were examined using salinised solution culture containing NaCl and $CaCl_2$ in a ratio of 1:1 by weight. Nine salinity levels of EC 0, 4, 6, 8, 10, 14, 18, 22, 26 dSm^{-1}, were used.

Data for shoot and root length in saline solutions were assessed as percentages of control (0 EC) values. *T. subterraneum* was the most sensitive to increasing salinity, and did not grow at EC > 14. *T. resupinatum* and *T. alexandrinum* were the most salinity tolerant species, growing at EC 26 dSm^{-1}. Selection in these two species for higher salinity tolerance considerably increased tolerance. The remaining three species had intermediate tolerance, their tolerance ranked *T. fragiferum* < *T. pratense* < *T. repens*.

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