

Soil salinization constitutes a severe and increasing constraint that impairs crop production worldwide and has led to attempts to understand the genetic basis of salt stress responses and adaptive mechanisms in seed plants (Richardt *et al.*, 2010). Soil salinity is one of the major wide spread environmental stresses that limits growth and development of plant. Salt stress adversely affects plant growth through osmotic and ion toxicity. A change in endogenous levels of phytohormones under stress condition has been considered as a step towards adaptation to the adverse conditions. The obstructions and dysfunctions in plant life form under stress may be related to disturbed endogenous levels / balances of phytohormones which can be minimised through exogenous supply of plant growth regulators, particularly when the developmental window is open. The exogenous application of plant growth regulators may be quite effective in alleviation of stress responses and use of exogenous plant growth regulators to mitigate the stress effects is of practical significance (Bagdi and Afria, 2008).

In this context, in the present study attempts were made with the objective of studying physiological and biochemical impacts of salt tolerance along with deriving a suitable stress mitigating strategy for groundnut plant (*Arachis hypogaea* L.) affected by salinity stress. In order to alleviate the salinity stress, use of foliar spray of plant growth regulating chemicals and nutrients were attempted.

Selection of the Crop for the Study

All plants are subjected to a multitude of stresses throughout their life cycle. The two major environmental factors that currently reduce plant productivity are drought and salinity (Serrano, 1999) and these two stresses create similar reactions in plants by inducing water deficit. Environmental stresses come in many forms yet salinity remains as one of the world's oldest and the most serious environmental problems, especially in arid and semiarid areas where poor crop establishment is the major limiting factor in crop production (Khan *et al.*, 2001).

Plate 1

Five different varieties of groundnut chosen for the study



VRI2



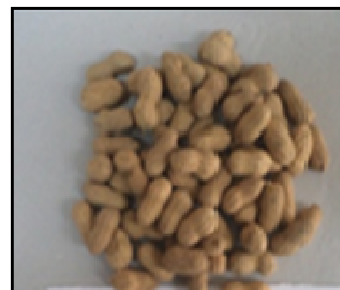
VRI3



TMV7



CO3



CO4

Saline soil, which causes reduction in yield, is one of the important abiotic constraints to groundnut production. Pulses in general are sensitive and have inadequate control over ion uptake, which leads to high internal salt concentrations and plant injury. However, tremendous variability exists regarding salt tolerance among different species/cultivars in all pulses. Consequently there is differential reduction in growth and yield in salt affected soils (Mensah *et al.*, 2006).

Groundnut is an important commodity in many developing countries, particularly in India where the nitrogen rich crop residues are also used as fodder. The production of groundnut in India needs to be increased from the current 8 million to about 14 million tonnes by 2020 to meet the increasing demand of the oil and confectionery industries (Girdhar, 2004). This increase will have to be

practically achieved by growing groundnut in lands considered so far as unsuitable for agriculture, including salt affected soils.

Information on salt tolerance of local groundnut (*Arachis hypogaea* L.) varieties are scanty. Therefore, the objective of the present study was to screen five varieties of groundnut commonly cultivated by the local farmers to determine the variety that could tolerate saline environments and thus help the farmers for their cultivation.

The seeds of five bunch type groundnut varieties namely, VRI2, VRI3, TMV7, CO3 and CO4 were obtained from Tamil Nadu Agricultural University, Coimbatore with seed maturity duration ranging between 105 and 115 days (Plate 1).

The study was conducted in four phases:

Phase 1: Screening of five selected varieties of groundnut (*Arachis hypogaea* L.) seeds namely VRI2, VRI3, TMV7, CO3 and CO4 for tolerance under different salinity levels in laboratory condition and pot culture

Phase 2: Study of phenotypic and genotypic variance among the five varieties of groundnuts chosen for the study and analysis of fatty acid composition in groundnut (*Arachis hypogaea* L.) varieties under salt stress by High Pressure Thin Layer Chromatography and Gas Chromatography

Phase 3: Field trial of the groundnut (*Arachis hypogaea* L.) varieties (identified as salinity tolerant and susceptible in pot culture study) with selected plant growth regulators namely brassinolide, salicylic acid and nutrient mixture.

Phase 4: Analysis of expression of heat shock protein-70 (hsp-70) gene in the variety of groundnut that was found to be tolerant and search for sequence similarity for hsp70 gene in groundnut with other plant species by *in silico* methods

Phase 1

3.1 Screening of five selected varieties of groundnut (*Arachis hypogaea* L.) seeds for tolerance under different salinity levels in laboratory condition and pot culture

3.1.1. Screening of Groundnut varieties for Tolerance under various levels of Salinity Stress

The seeds of the five groundnut varieties were screened for tolerance to various levels of salinity stress as per the protocol set up by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (Vadez *et al.*, 2005), based on germination percentage, seedling growth and vigour index. The seeds were allowed to germinate in petri dishes.

Petri dishes were sterilized using 0.01% mercuric chloride and 70 % ethanol and finally washed with distilled water. Petri dishes were cleaned with cotton swab before placing the germination sheet. 15 seeds from each variety were surface sterilized using 70% ethanol and placed in each petri dish. Sodium chloride solution at the concentration of 50, 100 and 125 mM were prepared by maintaining the pH at 7.4, 8.0 and 8.1 and Electrical Conductivity (EC) at 5.0, 10.0 and 12.5 dSm⁻¹ respectively for imposing three levels of salinity stress. Distilled water was used for maintaining the control and the seeds were allowed to germinate by sprinkling the salt solution of 10 ml each on alternate days.

3.1.2. Biometric Profile of the Seedlings

3.1.2.1. Germination percentage

Germination is a complex phenomenon involving many physiological and biochemical changes leading to the activation of embryo. Seed germination is the most critical and sensitive stage to salinity / sodicity stresses. Poor germination in saline soils leads to poor crop stand and productivity. Germinability of the groundnut seeds under salt stress condition is an index of salt tolerance (Vadez *et al.*, 2005).

The germinability was recorded on the fifteenth day after sowing (DAS) and number of seeds germinated was expressed as percentage.

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Number of seeds kept for germination}} \times 100$$

3.1.2.2. Seedling root length

Singh and Prasad (2009) explained that the growth inhibition in plant under salt stress was due to disturbed balance of hormones through alteration in osmotic system. In order to study the growth progress of the plant, the length of root and shoot were measured.

On the 15th day after sowing, seedlings from each replication were carefully removed at random and the length of the root of the seedlings was measured from the base of the stem to the tip of the longest root and expressed in cm.

3.1.2.3. Seedling shoot length

The length of the shoot was measured from the collar region to the tip of the longest leaf and expressed in cm.

3.1.2.4. Vigour index of the seedlings

The vigour index of the seedlings was calculated using the following formula proposed by Abdul-Baki and Anderson (1973) and expressed as percentage.

$$\text{Vigour Index} = (\text{Shoot length} + \text{Root length}) \times \text{Germination percentage}$$

3.1.2.5. Stress tolerance index

Stress tolerance index was calculated using the following formula proposed by Dhopte and Livera (1989) and expressed as percentage.

$$\text{Stress tolerance index} = \frac{\text{Vigour index of the treated seedling}}{\text{Vigour index of the control seedling}} \times 100$$

3.1.3. Pot culture Screening for Salinity Tolerance of the Groundnut (*Arachis hypogaea* L.) varieties selected for the Study

The five varieties of groundnut (*Arachis hypogaea* L.) after their laboratory screening were further evaluated by pot culture for their morpho-physiological characters besides yield, subjecting them to two different levels of salinity stress. In order to study the differences in varieties of groundnuts under salt stress,

the treatment was focused only in two different concentrations namely 50 mM and 100 mM sodium chloride solutions.

3.1.3.1. Experimental details

Table 1

Experimental details of the pot culture carried out for the screening of salinity tolerance of the groundnut varieties selected for the study

Design	Factorial Complete Randomised Design
Replications	Three
Treatments	T1: Control T2: 50mM NaCl T3: 100mM NaCl
Groundnut varieties tried	V1: VRI2 V2: VRI3 V3: TMV7 V4: CO3 V5: CO4

3.1.3.2. Preparation of the pots

Sandy clay loam soil was collected for pot culture experiment. Each of the pots were filled with 10 kg soil containing mixture of sand, soil and farm yard manure in the ratio of 3:2:1. Four plants were maintained in each pot. The control pots were irrigated with tap water (one litre per pot) on alternate days and similarly the experimental pots were irrigated with 50 mM and 100 mM salt solutions (one litre per pot) on alternate days. Salt stress treatments were imposed ten days after sowing, to avoid a rapid build up of salt in the soil.

Quality of irrigation water

The quality of tap water irrigated without any salt treatment (control) was tested for its pH and electrical conductivity, which were found to be 7.4 and 2.5 dSm⁻¹ respectively.

pH and EC of the irrigation water were determined using Elico digital pH meter and Electrical Conductivity meter respectively (Jackson, 1973).

3.1.3.3. Assessment of selected morphological, physiological and biochemical characteristics of the groundnut plant

Selected morphological, physiological and biochemical characteristics namely plant height (cm), leaf area (Leaf Area Meter-LICOR, Model LI 3000), total dry matter production, total chlorophyll content, soluble protein, proline and number of seeds per plant were analyzed at different growth stages of the five varieties of groundnut plants starting from 25 days after sowing in an interval of 15 days upto the harvest by selecting three representative samples at random from each replication. After harvest, the number of pods per plant, oil content of the seeds and the fatty acid profile of the groundnut varieties were also analysed.

Salt stress has toxic effects on plants and lead to metabolic changes, like loss of chloroplast activity, decreased photosynthetic rate and increased photorespiration rate which then leads to an increased reactive oxygen species (ROS) production (Parida and Das, 2005).

Proline accumulation is a universal response of plants to various stresses. Proline acts as an osmolyte and helps the plants to maintain tissue water potential under all kinds of stresses. Proline, as an osmoprotectant, is largely confined to the cytoplasm and is mostly absent from the vacuole (Zhu *et al.*, 2008).

Several salt-induced proteins have been identified in plant species. Stress proteins could be used as important molecular markers for improvement of salt tolerance using genetic engineering techniques. However, proteins produced under salt stress are not always associated with salt tolerance. Using proteins as salt tolerance indicator depends on the nature of the plant species or cultivar. In this regard, proline is a compatible solute known to accumulate in plants subjected to unfavourable environmental conditions. The concentration of this amino acid has been used in experiments as a measure of the stress imposed on test plants grown at different NaCl concentration in in vitro culture. It protects folded protein structures against denaturation, stabilizes cell membranes by interacting with phospholipids, functions as a hydroxyl radical scavenger, or serves as an energy and nitrogen source (Pareek *et al.*, 2010).

Table 2

Details of the assessment of selected morphological, physiological and biochemical characteristics in the groundnut plant (*Arachis hypogaea* L.)

Parameters analysed	Method of analysis	Reference	Appendix
Plant height	-	-	-
Leaf area	Leaf Area Meter (LICOR, Model LI 3000)	-	-
Total dry matter production	Oven dried at 80 °C for 48 hrs	-	-
Total chlorophyll content	Spectrophotometry	Yoshida <i>et al.</i> (1971)	1
Soluble protein	Spectrophotometry	Lowry <i>et al.</i> (1951)	2
Proline content	Spectrophotometry	Bates <i>et al.</i> (1973)	3
Number of pods	-	-	-
Number of seeds	-	-	-
Oil content in groundnut seed	Soxhlet Extraction	Cox and Pearson (1962)	4

3.1.3.4. Assessment of stomatal changes in leaves

The morphology and size of cells change during growth and development of a plant. This is due to metabolism of cell wall components, mainly polysaccharides by cell wall hydrolases (Cosgrove, 2005; Martin and Dopica, 2005; Minic and Jouanin, 2006).

The reduction in the growth of plant under salinity is due to changes in the anatomy of different tissues. Generally cell shape, size and decrease of intercellular spaces are seen in the plants under salt stress. In comparison to leaves there are only few reports regarding study of anatomical changes in root and shoot tissue under salinity stress. Effects of salt stress on the anatomical changes in groundnut seedlings were studied by observing stained ultra thin transverse sections under light microscope (Singh and Prasad, 2009).

One cm piece from the leaf centre of fully mature old leaf along the midrib was taken. For stomatal studies same leaf surface was scratched from both adaxial and abaxial surfaces to expose epidermis. The material was kept in FAA (formalin 5%, acetic acid 10%, ethyl alcohol 50% and distilled water 35%) solution for 48 h for fixation. The material was then readily transferred in acetic alcohol solution (one part acetic acid and three parts ethyl alcohol) for long-term preservation. Free-hand sectioning was used for preparing permanent slide of leaf transverse sections by using double-stained standard technique (safranin and fast green) as suggested by Ruzin (1999).

Phase 2

3.2 Study of phenotypic and genotypic variance among the five varieties of groundnuts chosen for the study and analysis of fatty acid composition in groundnut (*Arachis hypogaea* L.) varieties under salt stress

3.2.1. Heritability Analysis

Plant breeders want to improve the yields of their crops to the greatest degree they can. They must choose the parents of the next generation on the basis of this generation's yield and so they are continually performing selection experiments. Breeders run into two economic problems. They cannot pick only the very best to be the next generation's parents as they cannot afford to decrease the size of a crop by using only a very few select parents and they must avoid inbreeding depression, which occurs when plants are self fertilised. After frequent inbreeding, too much homozygosity occurs and many genes that are slightly deleterious begin to show themselves, depressing vigour and yield. So an index for potential response to select and to get the greatest amount of selection with lowest risk of inbreeding depression for the breeders is the study of heritability (Tamarin, 2002). Heritability is the proportion of phenotypic variation in a population that is genetically inherited among individuals. Phenotypic variation among individuals may be due to genetic and/or environmental factors. Heritability analyses estimate the relative contributions of differences in genetic and non-genetic factors to the total phenotypic variance in a population. Often measured empirically, heritability is an important notion in quantitative genetics, particularly in selective breeding, but

less so in the general theory of population genetics (<http://jhered.oxfordjournals.org/content/98/4/382.full>).

Breeders often calculate a heritability estimate, a value that predicts to what extent their selection will be successful. Heritability measures the fraction of phenotype variability that can be attributed to genetic variability, and not the fraction by which the phenotype is caused by genetics. For example, if both genes and environment have the potential to influence intelligence, but if a given sample of individuals shows very little genetic variation and a great deal of environmental variation, then the contribution of genetic variability to phenotype variability in that sample will be lower than if the sample showed greater genetic variability (<http://jhered.oxfordjournals.org/content/98/4/382.full>).

Heritability is specific to a particular population in a particular environment, but the extent of the dependence on environment is also a function of the genes involved. Individuals with the same genotype can exhibit different phenotypes through a mechanism called phenotypic plasticity, which makes their heritability difficult to measure in some cases. Recent insights in molecular biology have identified changes in transcriptional activity of individual genes associated with environmental changes. However, there are a large number of genes whose transcription is not affected by the environment (Raj *et al.*, 2008).

The genetic analysis was based on those parameters related to the variations in concentrations of electrical conductivity/salinity effects. The mean squares at the treatment levels were taken as the phenotypic variance (PV). To obtain the genotypic variance (GV) caused by variations in genes the mean square at the error level was subtracted from their corresponding phenotypic variance for all the treatments used.

The Genetic Gain (GG) was calculated in terms of the Genetic Advance (GA) expressed as percentage of the population mean \bar{X} . All these calculations were carried out using IRRISAT-Mixed Model Analysis software- Residual Maximum Likelihood (ReML) under randomized Complete Block Design (RCBD).

The genetic parameters analysed in the present study were:

(i) Heritability (Ho) according to Allard (1999):

$$Ho = \frac{\delta^2g}{\delta^2ph} \times 100$$

where δ^2g = genotypic variance and δ^2ph =phenotypic variance

(ii) Genetic Advance (GA) and Genetic Gain (GG) values were determined following the methods of Johnson *et al.* (1955):

$$GA = \frac{\delta^2g}{\delta ph} \times K$$

where K=2.06 (selection differential at 10%), δ^2g =genotypic variance and δph =square root of phenotypic variance.

3.2.2. Analysis of Fatty Acid Composition in Groundnut (*Arachis hypogaea* L.) varieties under Salt Stress

Arachis oil (groundnut oil) is derived from groundnuts (seed of *Arachis hypogaea* L.). Groundnut oil is a vegetable oil which contains only a small proportion of non-glyceride constituents. Its fatty acid composition is complex including saturated fatty acids. Vegetable oils generally primarily consist of triglycerides but several other compounds are also present. The importance of analyzing vegetable oils cannot be overemphasized because in analysing vegetable oil, major feature influencing the physical and chemical properties, their application and uses are got (Aluyor *et al.*, 2009).

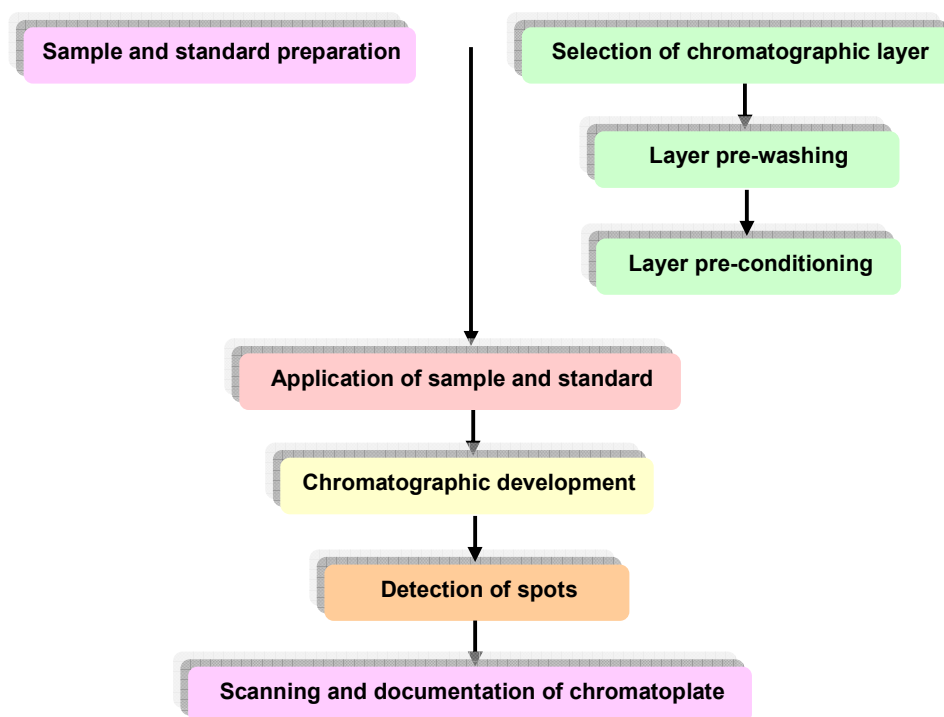
Salinity affects plant growth, development and some seed characteristics, usually oil content, and also influences nutrient uptake (ikisan.com). Based on these facts the oil extracted from the five varieties of groundnut were subjected to HPTLC as well as GC method of analysis so as to identify the fatty acid profile of the oil and also the changes that might take place in it because of salt stress and the steps involved in the HPTLC method is given in Figure 5.

Automation is useful to overcome the uncertainty in droplet size and position when the sample is applied to the TLC plate by hand. One recent approach to

automation has been the use of piezoelectric devices and inkjet printers for applying the sample (Morlock *et al.*, 2010).

The significant popularity of HPTLC in the analytical testing of pharmaceutical, bulk drugs and herbal, lends its fame to the attributes (Sethi, 1997; Green, 1996).

Figure 5
Steps involved in the HPTLC method to study the fatty acid profile



In order to analyse the fatty acid profile of the five groundnut varieties harvested in the pot culture, the oil samples were extracted by using soxhlet apparatus and were run in HPTLC and the procedure for the same is given in Appendix 5.

The oil samples of groundnut varieties were further analysed in GC for identifying the changes that could have taken place in fatty acid profile during salt stress.

Phase 3

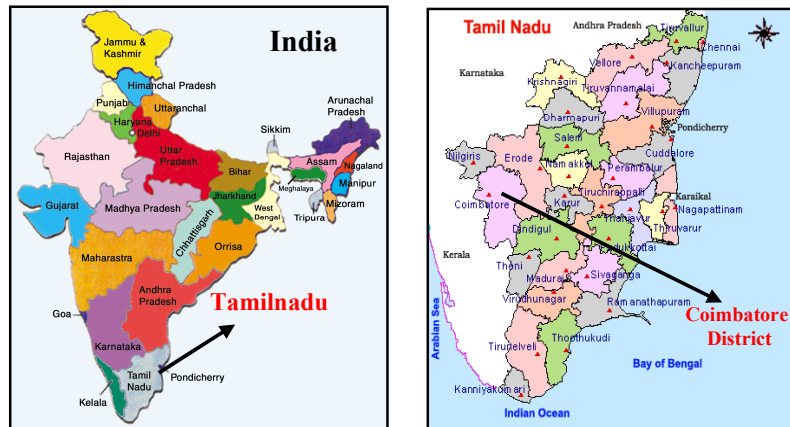
3.3 Field trial of the groundnut (*Arachis hypogaea* L.) varieties identified as salinity tolerant and susceptible in pot culture study with selected plant growth regulators

To study the effect of various plant growth regulating chemicals on alleviating the adverse effects of salt stress in groundnut, a field experiment was carried out. The two varieties identified as salinity stress tolerant and susceptible through pot culture screening, were employed for field trial in this study.

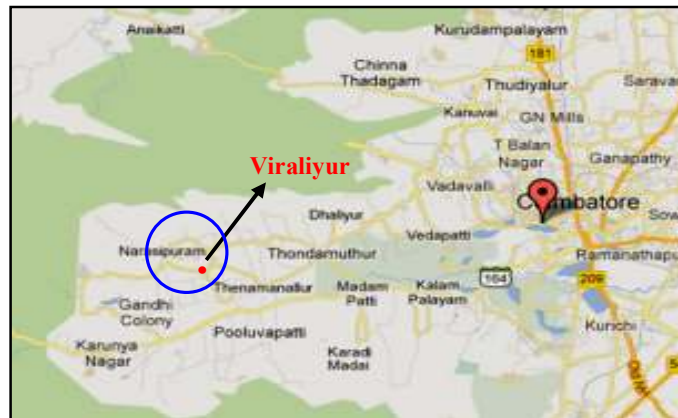
Field experiment was conducted in Viraliyur, a village 8 km from Thondamuthur, Coimbatore, Tamilnadu State, INDIA where farmers cultivate groundnut (Figure 6).

Figure 6

Area selected for the study



Coimbatore (Viraliyur, Thondamuthur)



3.3.1. Soil Characteristics of the Field chosen for trial

The mechanical compositions, physical and chemical properties of the soil were analyzed before the start of the experiment. The soil parameters analysed and their methods of analysis are given in Table 3.

3.3.2. Determination of Textural Class of the Experimental Soil using Triangular Textural Diagram given by United States Department of Agriculture (USDA)

After computing the relative percentage of different groups namely, clay, silt and sand, the textural class of the soil was determined by using the triangular textural diagram given by the United States Department of Agriculture (Mani *et al.*, 2007).

Figure 7

A soil textural triangle showing the subtle differences between the USDA (colours) and UK-ADAS (black lines) soil classes

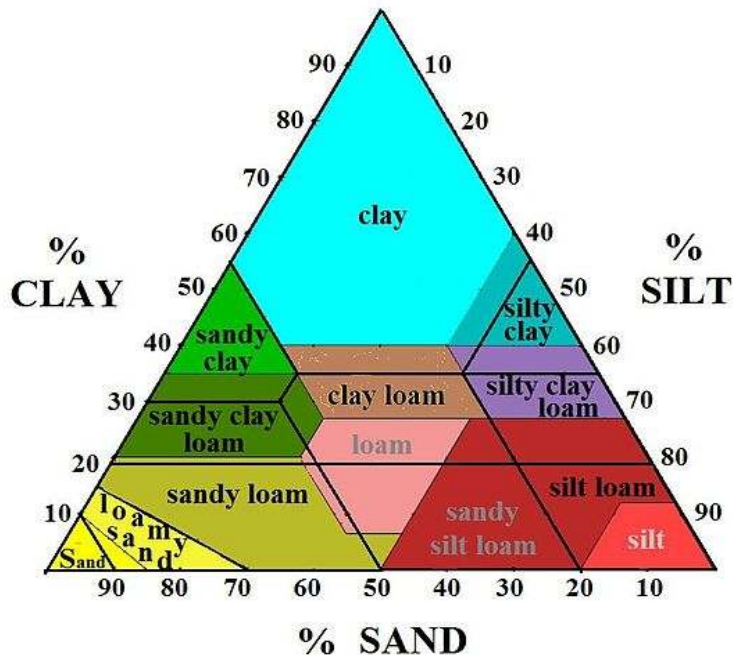


Table 3
Soil profile of the experimental field

Characters	Method	Reference	Appendix
Mechanical composition			
Coarse sand (%)	International Pipette Method/Robinson's Pipette method	Mani <i>et al.</i> (2007)	6
Fine sand (%)			
Silt (%)			
Clay (%)			
Chemical properties			
pH	-	-	-
Electrical Conductivity (EC) (dSm ⁻¹)	-	-	-
Available nitrogen (kg ha ⁻¹)	Alkaline KMnO ₄ method	Subbiah and Asija (1956)	7
Available phosphorus (kg ha ⁻¹)	Olsen's method using 0.5M NaHCO ₃	Olsen <i>et al.</i> (1954)	8
Available potassium (kg ha ⁻¹)	Flame Photometric method	Stanford and English, 1949	9

3.3.3. Amendment of the Soil in the Experimental Field with Chemical Fertilizer

Groundnut being a legume crop, can fix atmospheric nitrogen with the help of root nodule bacteria. Adequate amount of nitrogen should be made available in the early stage of growth for young seedling to get established and help in the formation of healthy and effective nodules. Groundnut which is rich in oil and protein has relatively high requirement for phosphorus. Phosphorus had a very critical role in view of the fact that lack of phosphorus resulted in the greatest reduction in nodulation and nitrogen fixation (Kausale *et al.*, 2007). Intensive land use results in nutrient deficiencies and nutrient imbalances, deteriorating soil health and resulting into consequent stagnation of crop yield (Deotale and Dhopte, 2005). On an average groundnut crop removes about 120kg nitrogen, 27 kg phosphorus and 34kg potassium from one hectare of land (ikisan.com). Based on these facts urea, single super phosphate and muriate of potash were applied at the rate of 17 kg ha⁻¹ nitrogen, 34 kg ha⁻¹ phosphorus, 54 kg ha⁻¹ potassium as fertilizer sources in the field. Nitrogen was applied in two splits, as basal (8.5 kg ha⁻¹) and at 40 days after

sowing (8.5 kg ha^{-1}) and phosphorus and potassium were applied as basal. Gypsum was applied at the rate of 400 kg ha^{-1} in two splits, one as basal (200 kg ha^{-1}) and another at 40 days after sowing (200 kg ha^{-1}). Irrigation was periodically done throughout the plant growth.

Plant growth regulators used in the field study as foliar spray

In order to analyse the effect of plant growth regulators in alleviating the effect of salinity / sodicity in the field the following were used in the form of sprays

- a) Brassinolide (BR) – 0.5 ppm and 1.0 ppm
- b) Salicylic Acid (SA) – 50 ppm and 100 ppm
- c) Nutrient Mixture - Diammonium phosphate (DAP) (1%) + Potassium nitrate (KNO_3) (0.5%) + Borax (0.2%) + Naphthalene Acetic Acid (NAA) (20ppm) + Salicylic Acid (50ppm) + Brassinolide (1ppm).

Brassinosteroids are a new group of phytohormones with significant growth promoting properties (Bishop and Yokota, 2001; Clouse and Sasse, 1998). Brassinosteroids are plant hormones with pleiotropic effects, as they influence diverse physiological processes such as growth, seed germination, rhizogenesis, senescence and leaf abscission (Sasse, 1997). In addition, BRs are implicated in plant responses to abiotic environmental stresses.

Salicylic acid is (SA) an endogenous growth regulator of phenolic nature, which participates in the regulation of physiological processes in plants such as growth, photosynthesis, nitrate metabolism, ethylene production, heat production and flowering (Hayat *et al.*, 2010) and also provides protection against biotic and abiotic stresses such as salinity (Kaya *et al.*, 2002). The results obtained in the last few years strongly argue that SA could be a very promising compound for the reduction of the abiotic stress sensitivity of crops, because under certain conditions it has been found to mitigate the damaging effects of various stress factors in plants. Therefore, it was suggested that NaCl-induced reduction in the plant growth can be mitigated by the application of plant growth regulators (Javid, 2011).

3.3.4. Experimental Design of the Field Trial

Table 4 presents the experimental design of the field trial carried out at the selected target area and Figure 8 represents the layout of the experimental plot.

Table 4
Experimental details of the field trial carried out with the two varieties of groundnut in Viraliyur

Varieties	M1: TMV7 (Salt stress tolerant variety) M2: VRI3 (Salt stress susceptible variety)
Treatment	T1 : Control T2 : Brassinolides (BR) 0.5ppm T3 : BR 1.0ppm T4 : Salicylic acid (SA) 50ppm T5 : SA 100ppm T6 : Nutrient mixture [Diammonium phosphate(DAP) (1%) + Potassium nitrate (KNO ₃) (0.5%) + Borax (0.2%) + Naphthalene Acetic Acid (NAA) (20ppm) + SA(50ppm) + BR(1ppm)]
Design	Split plot
Main plot	Varieties
Sub plot	Treatments
Replications	Three
Spacing	30 cm x 10 cm
Plot size	3 m x 2 m

These treatments were imposed as foliar sprays on 25th, 55th, and 85th DAS coinciding with preflowering, pegging and pod formation stages.

Figure 8. Layout of the experimental plot

M2T2	M2T6	M2T3	M1T4	M1T5	M1T2	M2T6	M2T4	M2T2
M2T4	M2T5	M2T1	M1T6	M1T1	M1T3	M2T5	M2T1	M2T6
M2T6	M2T2	M2T4	M1T2	M1T4	M1T6	M2T2	M2T3	M2T4
M2T1	M2T3	M2T6	M1T3	M1T3	M1T1	M2T4	M2T6	M2T5
M2T5	M2T4	M2T2	M1T5	M1T2	M1T4	M2T3	M2T2	M2T1
M2T3	M2T1	M2T5	M1T1	M1T6	M1T5	M2T1	M2T5	M2T3
M1T4	M1T3	M1T5	M2T2	M2T4	M2T1	M1T4	M1T2	M1T1
M1T1	M1T2	M1T6	M2T3	M2T6	M2T5	M1T6	M1T5	M1T4
M1T6	M1T5	M1T1	M2T4	M2T2	M2T3	M1T2	M1T3	M1T6
M1T3	M1T4	M1T2	M2T1	M2T5	M2T4	M1T1	M1T4	M1T5
M1T5	M1T1	M1T3	M2T6	M2T3	M2T2	M1T3	M1T6	M1T2
M1T2	M1T6	M1T4	M2T5	M2T1	M2T6	M1T5	M1T1	M1T3

M1 and M2 – Two varieties of groundnut namely TMV7 and VRI3, T1 to T6 – Six different treatments imposed namely Brassinolide – 0.5 and 1.0 ppm, Salicylic acid – 50 and 100 ppm, Nutrient mixture of diammonium phosphate (1%), potassium nitrate (0.5%), borax(0.2%), salicylic acid(50ppm) and brassinolide(1ppm) .

3.3.5. Biometric Observations of Groundnut varieties grown in the Field

The biometric characters were recorded during different growth stages such as preflowering, flowering, pegging, pod formation and maturity. All observations were taken at 15 days interval up to harvest starting from 30th day after sowing, by selecting three representative samples at random from each replication. The methods followed in recording the observations are given in Table 5.

Table 5
Morphological and growth characters of groundnut varieties grown in the field

S.No.	Morphological and growth characters	Method	Reference	Appendix
1	Plant height	-	-	-
2	Root length	-	-	-
3	Specific leaf weight	Leaf dry weight per plant / Leaf area per plant	Pearce <i>et al.</i> (1968)	-
4	Leaf area	Leaf Area Meter (LICOR, Model LI 3000)	-	-
5	Leaf area index (LAI)	Leaf area per plant / Ground area occupied by the plant	Williams (1946)	-
6	Leaf area duration	Using the formula $*L_1+L_2 / 2 \times t_2-t_1$	Power <i>et al.</i> (1967)	-
7	Total dry matter production	Oven dried at 80 °C for 48 hrs	-	-

*where L_1 = LAI at first stage, L_2 = LAI at second stage, (t_2-t_1) = Time intervals in days

3.3.6. Biochemical Observations of Groundnut varieties grown in the Field

Proline plays a key role in the cytoplasm as a scavenger of free radicals as well as a mediator in osmotic adjustment and also increases the solubility of sparingly soluble proteins (Molazem *et al.*, 2010).

In plants, nitrogen assimilation is regulated by the activation of the enzyme nitrate reductase. This enzyme plays a constructive role in nitrogen utilization by the plants through nitrogen metabolism. Nitrate reductase is the most affected

enzyme by salinity. The loading of nitrate into the root is also thought to be a highly sensitive step (Tischner, 2000). Salinity may strongly affect the overall nitrate assimilation process because nitrate is required to induce nitrate reductase, the key enzyme of nitrate assimilation process. Nitrate reductase activity in leaves is largely dependent on nitrate flow from roots and is severely affected by sodium chloride salt stress (Abd - El Baki *et al.*, 2000; Silveira *et al.*, 2001). Table 6 depicts the biochemical parameters assessed in groundnut leaves grown in the field.

Table 6
Details of biochemical parameters assessed in groundnut leaves grown in the field

S.No.	Physiological and Biochemical characteristics	Method	Reference	Appendix
1	Chlorophyll	Spectrophotometry	Yoshida <i>et al.</i> (1971)	1
2	Soluble protein	-do-	Lowry <i>et al.</i> (1951)	2
3	Proline	-do-	Bates <i>et al.</i> (1973)	3
4	IAA oxidase	-do-	Parthasarathy <i>et al.</i> (1970)	10
5	Polyphenol oxidase	-do-	Bateman and Daly (1967)	11
6	Nitrate reductase	-do-	Nicholas <i>et al.</i> (1976)	12
7	Peroxidase	-do-	Perur (1962) and Angelini <i>et al.</i> (1990)	13
8	Acid phosphatase	-do-	Tominaga and Takeshi (1974)	14
9	Catalase	-do-	Gopalachari (1963)	15

The inhibitory effect of Indole acetic acid oxidase on auxin and in turn, growth was demonstrated by various workers and they have reported that the enhancement of the activity of this enzyme under stress condition might be one of the reasons for stress induced stunted plant growth (Sivakumar *et al.*, 2002; Vijayalakshmi and Srinivasan, 2001; Garg and Chawla, 2002).

Plant cells possess different antioxidant enzymes such as catalase, peroxidase and superoxide dismutase which eliminates the reactive free radicals or suppress their formation. Morris *et al.* (2005) reported that PPO activity in red clover leaves converts certain phenol compounds to highly reactive quinones in the presence of oxygen. Acid phosphatase activity is known to contribute to resistance under salt stress by maintaining a certain level of inorganic phosphate (Chakraborty *et al.*, 2010).

Salt stress induces accumulation of reactive oxygen species (ROS) that are detrimental to cells as they cause oxidative damage to membrane lipids, proteins and nucleic acids (Hernandez *et al.*, 2001). Plants employ some detoxifying enzymes such as catalase, peroxidase and superoxide dismutase to scavenge the free radicals from the metabolic sites and thereby show tolerance to osmotic stresses (El-Baz *et al.*, 2003).

3.3.7. Soil Plant Analytical Development (SPAD) reading of Chlorophyll in Leaves

SPAD enables quick, easy measurement of the chlorophyll content of plant leaves without damaging the leaf. Chlorophyll content is one indicator of plant health and can be used to optimize the timing and quantity of applying additional fertilizer to provide larger crop yields of higher quality with lower environmental load. The advantages such as easy and rapid measurement, non destructive method and light weight made SPAD meters the best breeding program to improve the drought tolerance of groundnut at the ICRISAT (Kashiwagi *et al.*, 2006).

Plate 2

Chlorophyll meter (SPAD 502)



SPAD readings were recorded using Chlorophyll Meter (SPAD 502) designed by the Soil Plant Analytical Development (SPAD) section, Minolta, Japan. The data were recorded as described by Peng *et al.* (1993).

3.3.7.1. Measurement of photosynthetic efficiency in leaves

The technique of chlorophyll fluorescence has become ubiquitous in plant eco physiology studies. No investigation into the photosynthetic performance of plants under field conditions seems complete without some fluorescence data. This trend has been fuelled to a large degree by the introduction of a number of highly user friendly and portable chlorophyll fluorometers (Maxwell and Johnson, 2000). The chlorophyll fluorescence is an important measurement of photosynthetic efficiency of crops. The high ratio of variable to maximum fluorescence (F_v/F_m ratio) is proportional to quantum yield showing high degree of photosynthesis (Gitelson *et al.*, 1998). Fluorescence yield will be high when Photo System (PS) II reaction centre is least damaged by photo inhibition. This parameter provides a mean of assessing both photochemical quantum yield and photo inhibition (Krause and Weis, 1991). Chlorophyll fluorescence could be an excellent tool for the screening of large numbers of genotypes in a short time. Therefore, chlorophyll fluorescence of the leaf could be used as an indicator of photosynthetic activity (Schreiber *et al.*, 1995). This approach has been used with some apparent success in the last few years in screening several cereal crops for salinity tolerance (Belkhdja *et al.*, 1994).

Chlorophyll fluorescence measurements were recorded using Plant Efficiency Analyzer (Hansatech, UK) following the method advocated by Lu and Zhang (1998). Measurements were made on intact leaves, which were dark adapted for 30 minutes prior to measurement. The minimal fluorescence level (F_0) with all PS II reaction centers open was assessed by measuring the modulated light, which was sufficiently low ($< 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) not to induce any significant variable fluorescence. The maximal fluorescence level (F_m) with all PS II reaction centers closed was determined by a 0.8 saturating pulse at $8000 \mu\text{mol m}^{-2} \text{s}^{-1}$ in dark adapted leaves (Lu *et al.*, 2001). Using light and dark fluorescence

parameters, the maximal efficiency of PS II photochemistry in the dark adapted state, $F_v/F_m = (F_m - F_o) / F_m$ was calculated (Van Kooten and Snell, 1990).

3.3.7.2. Measurement of stomatal diffusive resistance

The stomatal diffusive resistance was measured between 11 am to 12.30 pm using a Steady State Porometer and expressed as $s\ cm^{-1}$ (Turan *et al.*, 2007).

3.3.7.3. Measurement of transpiration rate

Transpiration is a process similar to evaporation. It is a part of the water cycle, and it is the loss of water vapor from parts of plants (similar to sweating), especially in leaves but also in stems, flowers and roots. Leaf surfaces are dotted with openings which are collectively called stomata, and in most plants they are more numerous

Plate 3
Steady state porometer



on the undersides of the foliage. The stoma are bordered by guard cells that open and close the pore. Leaf transpiration occurs through stomata, and can be thought of as a necessary "cost" associated with the opening of the stomata to allow the diffusion of carbon dioxide gas from the air for photosynthesis. Transpiration also cools plants and enables mass flow of mineral nutrients and water from roots to shoots. The transpiration rate was measured between 11 am and 12.30 pm using a Steady State Porometer (Plate 3) and expressed as $\mu g\ H_2O\ cm^{-2}\ s^{-1}$.

3.3.8. Nutrient Analysis in the Groundnut Plant grown in the Experimental Soil with selected Plant Growth Regulators

The uptake and distribution of different mineral elements and salts are known to have bearing on the overall growth and development processes of plants. Under stress conditions, inhibition of uptake, transport and assimilation of different

mineral elements and disturbances in their balances have been commonly observed. Osmotic constraint is the first difficulty which plants are confronted in saline medium. Soluble salts in the soil reduce the osmotic potential which becomes in a state of "physiological drought", particularly for plants that cannot adjust their osmotic potential (Mezni *et al.*, 2010). The phenomenon is more pronounced under soil stresses like salinity and alkalinity. Under such conditions, soils contain extreme ratios of sodium / calcium, sodium / potassium, calcium / magnesium, chloride / nitrate. In order to study the effect of salt status in the plant under study the nitrogen, phosphorus, potassium, calcium, magnesium and sodium contents were estimated after the plant samples were digested (Piper, 1966). Table 7 represents the nutrients that were analysed in the groundnut plant grown with selected plant growth regulators.

Table 7
Analysis of nutrients in the groundnut (*Arachis hypogaea* L.) plant grown with selected plant growth regulators

S.No	Parameter analysed	Method of analysis	Reference	Appendix
1	Nitrogen	Microkjeldahl method	Humphries, 1956	16
2	Phosphorus	Colorimetry	Jackson, 1973	17
3	Sodium and Potassium	Flame photometry	-do-	18
4	Calcium and Magnesium	Versenate titration method	-do-	19

Digestion of plant sample

Two gram of powered dry plant sample was taken in a 100 ml conical flask and to this 12 ml of triple acid mixture containing nitric acid, sulphuric acid and perchloric acids in the ratio of 9:2:1 was added and the mouth of conical flask was covered with a funnel. The content of the flask was digested over a sand bath till a clear solution was obtained. This solution was filtered through Whatman No.41 filter paper and the filtrate was collected in a 250 ml volumetric flask. The conical flask was washed with a small amount of hot water and added to the filter paper, the

residue on the filter paper was also washed with hot water. The volumetric flask containing the filtrate was cooled under tap water and the volume of the filtrate was made up to 250 ml with cooled distilled water. The triple acid extract was stored for the analysis of mineral constituents (Piper, 1966).

3.3.9. Yield Components

Fertilizer to any crop acts as a growth promoter. Its role in legumes is more conspicuous for doubling the yield. Some groundnut varieties have shown that low source strength resulted in the formulation of more unfilled pods at harvest and lowered the seed yield. A highly positive correlation between the number of flowers produced during the early reproductive phase and productivity was reported in groundnut plant and plant growth regulators were found to induce more number of flowers in lesser number of days leading to increased pod weight and yield. Fertilizer elements which are absorbed through root can also be absorbed through foliar. Foliar application supplies the nutrients to the plant directly where they are needed for production without spending energy for their transport and without any losses in transit. Foliar application of relatively small quantities of nutrients stimulates plant growth and additional nutrient uptake from soil resulting in higher yields. Foliar application of fertilizer offered considerable scope not only for better utilization of nutrients and also economy in farmer's fertilizer expenditure (Chandrasekaran *et al.*, 2008). Therefore, the selected yield components were assessed in the groundnut plant grown in the field amended with selected plant growth regulators namely Brassinolides (BR) 0.5ppm, BR 1.0 ppm, Salicylic acid (SA) 50ppm, SA 100ppm Nutrient mixture [Diammonium phosphate (DAP) (1%) + Potassium Nitrate (KNO_3) (0.5%) + Borax(0.2%) + Naphthalene Acetic Acid (NAA) (20ppm) + SA(50ppm) + BR(1ppm)].

3.3.9.1. Number of flowers per plant

Groundnut plants start flowering about 25 to 40 days after planting. Groundnuts can flower (orange yellow) over a long period (20-60 days), depending on moisture availability and temperature. The flowering period is considerably shorter in bunch type of cultivars than in spreading types (icrisat.org). Plants were selected at random and tagged in each treatment prior to flowering and the number

of flowers was counted every day from the date of commencement of flowering up to 60 DAS and the mean values were worked out.

3.3.9.2. Number of pegs per plant

After fertilization stalk of ovary elongates and forms peg which contains fertilized ovules at the tip. The pegs penetrate the soil up to a depth of 7 cm and then grow horizontally when the ovary starts developing as a pod containing seeds. Number of pegs per plant was counted in the selected plants in each replication and the mean value was expressed as pegs plant⁻¹.

3.3.9.3. Number of pods per plant

Normally 60-80 days are required for pod development from flowering to maturation in spreading types and slightly less than that (50-60 days) in bunch types. Vegetative development declines during pod filling. Number of pods per plant was counted in the selected plants in each replication and the mean value was expressed as pods plant⁻¹ (Plate 4).

Plate 4

Groundnut pods and pods in the uprooted groundnut plants



Groundnut pods

Uprooted plant with pods

3.3.9.4. Fertility coefficient

Fertility coefficient is expressed to denote the number of flowers to form pods. The ratio of flowers produced to pods formed was calculated as follows and expressed in per cent

$$\text{Fertility coefficient} = \frac{\text{Number of pods per plant}}{\text{Number of flower per plant}} \times 100$$

3.3.9.5. Pod yield per plant

Pod yield was determined by taking pod weight of randomly selected five plants from each replication and the mean value was expressed as g plant⁻¹.

3.3.9.6. Shelling percentage

After weighing, pods were shelled and the kernels were weighed. The shelling percentage was calculated as follows and expressed in per cent:

$$\text{Shelling percentage} = \frac{\text{Kernel weight}}{\text{Pod weight}} \times 100$$

3.3.9.7. Hundred seed weight

After shelling the pods, 100 kernels were randomly selected from each treatment and weighed and the mean was expressed in g.

3.3.9.8. Harvest index

Harvest index was calculated by dividing the pod yield by total dry matter produced by the plant and expressed in per cent.

$$\text{Harvest index} = \frac{\text{Economic yield}}{\text{Biological yield}} \times 100$$

Phase 4

3.4 Analysis of expression of heat shock protein- 70 (hsp-70) gene in the variety of groundnut that was found to be tolerant

Understanding the mechanisms involved in the response of plants to adverse environmental conditions is, without a doubt, the first step in the generation of crops with higher tolerance to stress. Research at the level of genes (genomics), proteins (proteomics), metabolites (metabolomics), individuals (physiology, systemic- biology) and communities (ecology) has been fundamental in the current understanding of the response of plants to stress. A fundamental step in any functional genomics study is the analysis of gene expression (Torres *et al.*, 2009).

Abiotic stresses usually cause protein dysfunction. Maintaining proteins in their functional conformations and preventing the aggregation of non-native proteins are particularly important for cell survival under stress. Heat-shock proteins (hsps)/chaperones are responsible for protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membranes, and can assist in protein refolding under stress conditions. They can play a crucial role in protecting plants against stress by reestablishing normal protein conformation and thus cellular homeostasis (Wang *et al.*, 2004).

Elucidating the various mechanisms of plant response to stress and their roles in acquired stress tolerance is thus of great practical and basic importance. Much research is devoted to some of the major tolerance mechanisms, including ion transporters, osmoprotectants, free-radical scavengers, late embryogenesis abundant proteins and factors involved in signaling cascades and transcriptional control (Wang *et al.*, 2003). However, except for the hsp family, relatively little focus has been given to the role of the many other hsps/ chaperones in plant response to abiotic stress and direct support for hsp/chaperone function in plant abiotic stress tolerance is rather limited (Burke, 2000; Burke, 2001; Hong and Vierling, 2000; Hong and Vierling, 2001; Hong *et al.*, 2003). This is despite the fact that hsps/chaperones are known to be expressed in plants not only when they experience high temperature stress but also in response to a wide range of other environmental insults, such as water stress, salinity and osmotic, cold and oxidative stress (Waters *et al.*, 1996; Boston, 1996; Vierling, 1991). It is most likely, being supported by experimental data in plants and other organisms, that hsps/chaperones play a crucial role in protecting plants against stress and in the reestablishment of cellular homeostasis.

3.4.1. Assessment of the Expression of hsp 70 Gene

Based on the above said facts, in the present study the expression of heat shock protein hsp 70 gene was studied to know whether the hsp gene has been expressed in the salt tolerant groundnut variety (TMV7), by isolating RNA of the plant sample and then by doing Real Time Polymerase Chain Reaction (RT-PCR)

along with the control (untreated) and the methodology adopted for the same has been given in Appendix 20.

3.4.2. Sequence Similarity Search for Salt Tolerant Gene in Groundnut with other Plant Species using *In silico* Methods

One approach to understanding the ability of plants to tolerate salt stress has been to identify stress-induced changes of individual proteins under the assumption that stress adaptation results from alterations in gene expression (Soussi *et al.*, 2001). Large numbers of salt tolerant proteins are found in plants and possess specific role to overcome the salinity. Salt tolerant proteins are synthesized in response to salinity and the sequences of these proteins are found to be highly conserved. Bioinformatics has revolutionized the field of molecular biology. The raw sequence information of proteins and nucleic acids can convert to analytical and relative information with the help of soft computing tools. Protein prediction is important application of bioinformatics. Studies on genetic relationships and generation of evolutionary tree by comparing amino acid sequence and nucleic acid sequence have been successively carried out in many species. As single change in sequence of amino acid leads to conformational changes in protein hence the information gathered from comparison of members of same protein family can throw some light on path of their evolution (Thakare *et al.*, 2010).

3.4.2.1. BLAST

In bioinformatics, Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. Different types of BLASTs are available according to the query sequences. For example, following the discovery of a previously unknown gene in the mouse, a scientist will typically perform a BLAST search of the human genome to see if humans carry a similar gene; BLAST will identify sequences in the human genome that resemble the mouse gene based on similarity of sequence (<http://en.wikipedia.org/wiki/BLAST>).

3.4.2.2. CLUSTAL W

Clustal is a widely used multiple sequence alignment computer program. The latest version is 2.1. There are two main variations:

- ClustalW: command line interface
- ClustalX: This version has a graphical user interface. It is available for Windows, Mac OS, and Unix/Linux.

There are three main steps:

1. Do a pairwise alignment
2. Create a phylogenetic tree (or use a user-defined tree)
3. Use the phylogenetic tree to carry out a multiple alignment (<http://en.wikipedia.org/wiki/Clustal>).

3.4.2.3. DOM-PRED

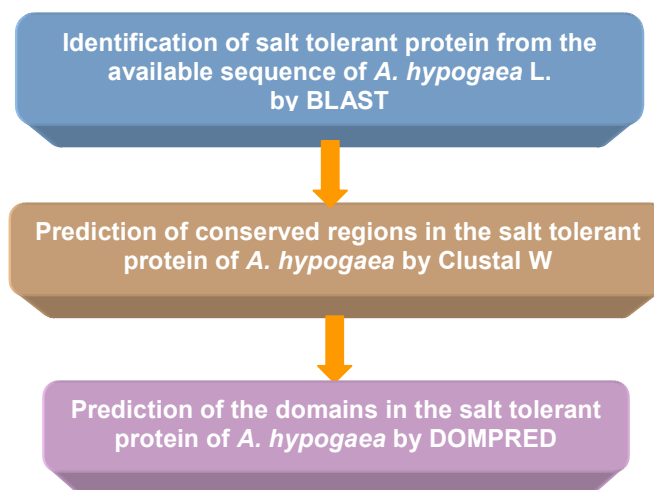
DOMPRED is a server designed to predict putative protein domains and their boundaries for a given protein sequence. The server uses several methods, from identifying obvious similarities to Pfam-A domain sequences to predicting domains using DomSSEA in cases where sequence searching has yielded no results. The delineation of protein domains within a polypeptide chain can be achieved in several ways. Methods applied by classification databases such as the Dali Domain Dictionary, CATH and SCOP use structural data to locate and assign domains. However, complete automation of domain assignment even from structural data is not a trivial problem, and obviously requires a solved protein structure. Identification of domains at the sequence level most often relies on the detection of global-local sequence alignments between a given target sequence and domain sequences found in databases such as such as Pfam. However difficulties in elucidating the domain content of a given sequence at the sequence homology level arise when searching the target sequence against sequence databases results in a lack of significant matches. In such situations, an ab initio approach to domain assignment from sequence is required.

Methods employed by the DOMPRED server are:

- Pfam-A search
- PSI-BLAST sequence alignment profile
- DomSSEA (domain identification from secondary structure element alignment) (<http://bioinf.cs.ucl.ac.uk/dompred>).

Figure 9

Methodology adopted to search the similarity of salt tolerant protein sequence of *Arachis hypogaea* L.



Based on these facts, in the present study the heat shock protein sequence which was used as the primer in the gene expression study was subjected to BLAST hits to search for any similar sequences available in the biological database. The similar sequences identified from the different plants were then run in Clustal W for predicting the conserved regions in it. Once the conserved regions were predicted, they were again run in DOMPRED software in order to identify the domains if any present in it (Figure 9).