GENERAL INTRODUCTION

1. Abiotic stress and plants

Abiotic stress is one of the major causes of crop loss worldwide and restricts certain areas from productive agriculture and even less severe stress makes plants more susceptible to diseases and pests. Plants grow in almost any part of the world under diverse climatic conditions differing in temperature, light quantity and quality and availability of water. Plants that grow in a specific environment are adapted to those conditions, and can also cope with changes that might be adverse for their growth and development. Adaptation is required because plants cannot escape unfavorable conditions due to their sessile growth habit. This implies that species differ genetically in their adaptation and resistance to abiotic stresses. Abiotic stress is in fact the principal cause of the loss of crop productivity worldwide, reducing average yields for various crops by more than 50%. Abiotic stresses cause losses worth hundreds of millions of dollars each year due to reduction in crop productivity and crop failure. Any external constraint that reduces the ability of a plant to develop to its genetically predetermined level is called stress. Plant species are highly variable in their optimum environments and tolerance to abiotic stress conditions. Principal abiotic stresses that can affect growth and productivity of the plant include the following:

i. Cold (chilling and frost)

ii. Heat (high temperature)

iii. Salinity (salt)

iv. Drought (water deficit condition)

v. Excess water (floodling)

vi. Radiations (high intensity of ultraviolet and visible light)

vii. Chemicals and pollutants (heavy metals, pesticides, and aerosols)
viii. Oxidative stress (reactive oxygen species, ozone)

ix. Wind (sand and dust particles in wind)

x. Nutrient deprivation in soil

2. Agricultural productivity affected by abiotic stress

Population growth, changed lifestyle, competition for fresh water between farmers and cities, and possible global environmental changes have led to alarming projections that seem to argue additional strategies by which food supply can be guaranteed (Miflin, 2000). Today, in a world of 7 billion people, agriculture is facing great challenges to ensure a sufficient food supply while maintaining high productivity and quality standards. In addition to an ever increasing demographic demand, alterations in weather patterns due to changes in climate are impacting crop productivity globally. Abiotic stresses are serious threats to agriculture and the environment which have been exacerbated in the current century by global warming and industrialization. According to FAO statistics, more than 800 million hectares of land throughout the world are currently salt-affected, including both saline and sodic soils equating to more than 6% of the world’s total land area. Continuing salinization of arable land is expected to have overwhelming global impact, resulting in a 30% loss of agricultural land over the next 25 years and up to 50% loss by 2050.

Overall, it has been estimated that the world is losing at least 3 hectare of arable land every minute due to soil salinity. Some of the most serious effects of abiotic stresses occur in the arid and semiarid regions where rainfall, high evaporation low, native rocks, saline irrigation water, and poor water management all contribute in agricultural areas. Unfavorable climate not only causes changes in agro-ecological conditions, but indirectly affects growth and distribution of incomes, and thus increasing the demand for agricultural production (Schmidhuber and Tubiello, 2007). Adverse climatic factors, such as water scarcity (drought), extreme temperatures (heat, freezing), photon irradiance, and contamination of soils by high ion concentration (salt, metals), are the major growth stressors that significantly limit productivity and quality of crop species worldwide. As
has been pointed out, current achievements in crop production have been associated with management practices that have degraded the land and water systems (FAO, 201). Soil and water salinity problems exist in crop lands in China, India, the United States, Argentina, Sudan, and many other countries in Western and Central Asia. Globally, an estimated 34 million irrigated hectares are salinized (FAO, 2012), and the global cost of irrigation-induced salinity is equivalent to an estimated US$11 billion per year (FAO, 2005).

2. The Effects of Abiotic Stresses on Plants

Plants encounter a wide range of abiotic stresses such as drought, high soil salinity and extremes of temperature, which limit their growth and development (Sachs and Ho, 1986). Under extreme conditions, the plants might even die. For agricultural crops, potential yields may be drastically reduced (Altman, 2003; Tester and Bacic, 2005) thus affecting the world’s food supply. Worldwide, abiotic stresses are the primary causes of crop losses (Vinocur and Altman, 2005), reducing the potential yields by more than 50% (Boyer, 1982). By 2030, climatic changes are projected to reduce cereal production in Africa by about 2 to 3% (Food and Agriculture Organization of the United Nations, 2002).

Drought and salinity stresses are most prevalent in semi-arid regions and/or regions that experience low rainfall patterns and thus dependent on irrigation (Denby and Gehring, 2005). Soil salinization in particular is projected to affect more than 50% of all arable lands by the year 2050 (Vinocur et al., 2003). With the increase in the world’s population coupled with the global climatic changes, there is a growing need to develop food crops that are well adapted to these extreme environmental conditions (Flowers, 2004; Vinocur and Altman, 2005). It is of great importance therefore, to fully understand the underlying plants’ tolerance mechanisms to drought and salinity in cereal crops such as sorghum, which are relatively more drought and salt tolerant than maize, the most cultivated cereal. The knowledge gained would be useful for the genetic improvement of stress tolerance of agriculturally important crops in order to improve food security (Sachs and Ho, 1986; Mundree et al., 2002; Seki et al., 2003; Denby and Gehring, 2005).
3. **The Complex Nature of Plant Response Mechanisms to Abiotic Stresses**

In order to survive, reproduce and complete their life cycles, plants have naturally developed a wide range of adaptive and tolerance mechanisms to abiotic stresses. These mechanisms range from morphological, developmental, biochemical, molecular and cellular changes that occur once the plant is exposed to a specific stress condition. Understanding the nature of these response mechanisms in totality is complicated by several reasons. Firstly, a single stress may occur at multiple stages of the plant’s development (Chinnusamy et al., 2004), and thus interacts uniquely with that particular developmental stage. Secondly, more than one stress may simultaneously affect the plant (Chinnusamy et al., 2004) or be separated in time (Knight and Knight, 2001), thus resulting in complex physiological and molecular response (Mittler, 2006). Thirdly, plants under different stresses may share common response mechanisms, perception and signal pathways as protective action against the stresses (Chinnusamy et al., 2004). This being due to the fact that under certain conditions the two stresses cannot be distinguished from one another (Knight and Knight, 2001). The complexity of studying and understanding plant response mechanisms to abiotic stresses is further compounded by the fact that a primary stress is capable of producing a secondary or even a tertiary stress (Levitt, 1980). In some cases, different primary stresses may produce the same secondary or tertiary stress. For example, primary stresses such as drought, salinity, cold, heat and chemical pollution are interconnected; all producing secondary stresses such as osmotic and oxidative stress (Wang et al., 2004). Therefore, the response to a particular primary stress may result in a complex network of response mechanisms against effects of all the secondary and tertiary stresses that are induced by a specific primary stress (Levitt, 1980). Nevertheless, molecular studies on plant responses to abiotic stresses have indicated that gene expression may be altered resulting in an increase, decrease, induction or total suppression of some proteins. Some stress-induced proteins of plants under stress may allow plants to make biochemical and structural adjustments (Sachs and Ho, 1986; Ho and Sachs, 1989; Kasuga et al., 1999), which are necessary for conferring stress tolerance. Abiotic stress inducible genes can thus be classified into two groups. The first group encodes proteins that directly protect the plant cells against the stresses such as enzymes involved in the biosynthesis of various osmoprotectants, late-embryogenesis
abundant (LEA) proteins, chaperones and detoxification enzymes. The second group encodes for proteins that regulate gene expression and signal transduction in abiotic stress responses such as transcription factors and protein kinases (Seki et al., 2003; Shinozaki et al., 2003).

Figure Shows a diagrammatic illustration of the complexity of plant response to abiotic stresses. As plants are exposed to a range of abiotic stresses, signals are relayed to the intracellular machinery for the activation of gene expression. As gene expression increases, stress responsive mechanisms are activated thus resulting in the restoration of cellular homeostasis, and the protection and repair of stress damaged proteins and membranes (Wang et al., 2004).
4. Need for halophytes use as experimental plants:

Although they represent only 2% of terrestrial plant species, halophytes are present in about half the higher plant families and represent a wide diversity of plant forms. Despite their polyphyletic origins, halophytes appear to have evolved the same basic method of osmotic adjustment: accumulation of inorganic salts, mainly NaCl, in the vacuole and accumulation of organic solutes in the cytoplasm. Halophytes are defined as plants that naturally inhabit saline environments and benefit from having substantial amounts of salt in the growth media. Halophytes grow in a wide variety of saline habitats, from coastal regions, salt marshes and mudflats, to inland deserts, salt flats and steppes. They occur across a wide range of plant families, with the Chenopodiaceae being dominant (Flowers and Colmer, 2008). The evolution of salt tolerance is interesting for several reasons. First, since salt-tolerant plants (halophytes) employ several different mechanisms to deal with salt, the evolution of salt tolerance represents a fascinating case study in the evolution of a complex trait. Second, the diversity of mechanisms employed by halophytes, based on processes common to all plants, sheds light on the way that a plant’s physiology can become adapted to deal with extreme conditions. Third, as the amount of salt-affected land increases around the globe, understanding the origins of the diversity of halophytes should provide a basis for the use of novel species in bioremediation and conservation.

Kerstiens et al. (2002) claimed that there has been least search for salt-tolerant cultivars and that the focus of attention in future should be upon halophytes, to discover how they are able to maintain production under saline condition the aim being to ‘define a minimal set of adaptations required in tolerant germplasm’. This is by no means a simple objective because halophytes display huge diversity, having apparently evolved independently in many different families and genera. Glenn et al. (1991) stated that it is important to discover any particular traits that are so essential for salt tolerance that they appear in many different halophytes as a result of convergent evolution.

Halophytes are naturally ‘salt-loving’ plants that outcompete any existing traditional crop when grown in hostile saline environments. Importantly, there is nothing unique that halophytes possess that is not found in crop species. Instead, halophytes are doing everything ‘a bit better’ and possess a set of highly complementary and well-orchestrated mechanisms in place to deal with salinity stress. However, the rapid progress
in molecular biology and development of various ‘omics’ has somewhat overshadowed
the importance of in-depth physiological studies on regulation and co-ordination of the
mechanisms. The general acceptance of the lack of a ‘silver bullet’ to improve the
salinity trait, hundreds of papers are submitted describing attempts to manipulate one
specific gene in the hope of tackling salinity stress tolerance. In light of the above, this
approach is erroneous by default. Also, for many years, efforts of breeders have been
predominantly aimed at the trait involving Na⁺ exclusion from uptake (Munns and Tester,
2008). This is not what halophytes are doing. At the same time, many key features of
halophytes, such as using trichomes for external Na⁺ sequestration, reducing stomatal
density or regulating efficiency and timing of xylem Na⁺ loading, have never been
manipulated by breeders. This opens up novel and previously unexplored possibilities for
improving salinity tolerance in crops.
Objectives of the present investigations
The general aim of the present study was to investigate some of the intimate bioactive compound of the halophyte *Sesuvium portulacastrum*, effect of salt on seed germination, abiotic stress effect on physiological and oxidative status, and identification of salt responsible protein as well as microbial diversity in halophyte rhizosphere essential for growth of halophyte. The specific objectives of the present study were as following objectives:

- To evaluate bioactive compounds of *S. portulacastrum*
- Adaptation of halophytes present near our institute in greenhouse environment to provide abiotic stress conditions and for protein profiling
- To analyze the effect of salt on seed germination of *S. portulacastrum*
- To evaluate physiological and oxidative status of halophyte in hydrophonic condition with different abiotic stress.
- To study the relationship between plant and microbes through pyrosequencing.
- Establishing protein extraction protocols and two dimensional electrophoresis technique for the optimal extraction and separation of proteins from halophyte
- Identifying differences in the protein expression patterns of *Sesuvium portulacastrum* control to salt treated through MALDI TOF MS
2. DESCRIPTION OF STUDY AREA

The vellar river originates from Serivarayan Hills in the Salem district of Tamilnadu and runs through the coleroon basin and merges in the Bay of Bengal through Vellar estuary (Lat.11° 24’ N; Long.79° 46’ E). Vellar estuary is a tropical, shallow, barbuilt estuary having an average depth of 2.5m. The maximum width of the estuary is about 200 m. The Vellar estuary is influenced by semidiurnal tides and tidal amplitude extends up to a distance of about 15kms upstream. During the monsoon season (October –December), it receives large quantity of fresh water from various sources. Compared to other estuaries of south India, the Vellar estuary is highly productive and forms a good nursery ground for fin and shell-fishes. Dense populations on mangrove plants and its associate plants occur on the bank of Vellar estuary.
TAXONOMIC DESCRIPTION

The genus *Sesuvium* includes twelve different species with a world wide tropical distribution (Hartmann, 2001). *Sesuvium portulacastrum* L. (seapurslane) is one of the fast growing, herbaceous, perennial, dichotomous halophyte. In India, it grows in the eastern, western and southern coastal sides as a mangrove associated plant (Lokhande et al., 2009a). The plant has remarkable ability to survive under different stress conditions. The genus name derives from the old name for a Houseleek or succulent and the specific epithet refers to this species’ resemblance to members of the related genus *Portulaca* and the Latin name for wild.

Kingdom : Plantae
Subkingdom : Tracheobionta
Superdivision : Spermatophyta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Caryophyllidae
Order : Caryophyllales
Family : Aizoaceae
Genus : Sesuvium L.
Species : *Sesuvium portulacastrum* L.

*Sesuvium portulacastrum* L.
**Common Names:** Sea Purslane, Cenicilla, Shoreline Purslane.

**Foliage:** The plant exhibits evergreen, alternate, simple succulent leaves of about 2” long and more or less cylindrical or flattened oblong to narrowly oblanceolate, tapering to point; bases tend to clasp the stems; in cool weather. When plants are under stress, the foliage takes on a reddish cast, but is otherwise a strong dark green color.

**Flower:** It is small in size of ¾ "diameter, solitary star-shaped, borne on a long peduncle (¼") in the axil of the leaves. The appearance of the flower is white to pinkish purple or pale purple; numerous stamens surround a single pistil. Flowering occurs whenever temperature conditions permit.

**Fruit:** It is aesthetically small (¼”). Long conical capsules develop with seeds that are shaken out by the wind after the lid-like top of the capsule matures and is lost.

**Seed:** Seeds are very small, about 1.2–1.5 mm long, black, smooth and lustrous; on germination produce seedlings of which each of the pair of cotyledons is fleshy and oblong.

**Stem:** Stems are snaky, long, rope-like, and mostly prostrate or pendent, although some genotypes are erect for brief periods of time prior to being weighted down to the ground as they grow. It is thick and succulent, nearly round in cross-section and light green, green, or reddish on new stems, these typically mature to a dark green as stems age.
**Bud** — It is small and look like similar color of stems.

**Habit:** It is an extremely rapidly growing coastal trailing vine with sparsely set internodes that overlaps to create a dense evergreen herbaceous perennial groundcover. The stems often root at the nodes. Apart from survival in the saline habitat, proliferation of *Sesuvium* is hindered because of insufficient planting material and unfavourable environmental conditions like high salinity, high wind velocity, temperature variation and muddy anaerobic soil (Kathiresan *et al.*, 1997).

**Native Habitat:** *S. portulacastrum* is widely distributed in the pantropical, including some subtropical. It is unclear as to this species' original native distribution due to extensive naturalization in various tropical and subtropical coastal locations around the globe.

**Cultural Requirements:** Its culture is easy if plants are given a sunny to regularly sunny location and reasonable water availability; although they heat and wind tolerant. *S. portulacastrum* is only moderately drought tolerant but it will grow on a wide range of soils. Plants are responsive to increased fertility also.

**Ornamental Assets:** Although the flowers are interesting, they are too small to be of much value. The primary aesthetic reason for planting this species is its succulent coarse textured foliage. Some taxa have a stronger reddish flush than others and variable degrees of erect versus pendent foliage are present.

**Utilization:** *S. portulacastrum* contains secondary metabolites which display a great potential as a substitute for some synthetic raw materials in the food, perfumery, cosmetic and pharmaceutical industries (Lis-Balchin and Deans, 1997). This plant is used in traditional medicine as a remedy for fever, kidney disorders and scurvy (Rojas *et al.*, 1992) by the indigenous people in Africa, Latin America and in Asian countries such as India, China, Pakistan and Japan. The plant is used on the Senegal coast as a haemostatic; its decoction is considered to be the best known antidote for stings of venomous fish. Leaves have acidulous flavor of sorrel as well as antiscorbutic (Anonymous, 2009).

*S. portulacastrum* occasionally cultivated as vegetable for cooking purpose in India and South East Asia (Hammer, 2001). It has a great potential food value and also utilized as a wild vegetable crop in the southern India because of its salty taste and fleshy
nature (Kathiresan et al., 1997). After thorough washing, the plants has to be boiled and subsequently residued salt can be removed by repaid wasing. The plant is also used as a fodder crop for the cattle and domestic animals (sheep and goats) in the coastal area. It provides a good salty pasturage for camels in Mauritania. Plant is favorite food for crabs and also serves as bait in crab-traps (Anonymous. 2009).

**Landscape Utilization:** The most obvious use of this species is in coastal dune stabilization, but it may also serve as a fast temporary summer groundcover in colder climates; greenroofs or living walls in regions with little frost are other possibilities. It can be grown in containers, either as a coarse textured spiller or very long trailing hanging basket plant. It can make an interesting curtain effect when grown hanging over walls, cutouts, or arches.

**Limitations & Liabilities:** Under favorable reproductive conditions, this species can be weedy and the vegetative tissues can overwinter so there is a potential for this species to spread rapidly.

The present interest in this species lies in its importance as “salt accumulator” plants which accumulate high salt concentration in their cells and tissues and overcomes salt toxicity by developing succulence. This halophyte is used as a fodder for animals and has an ornamental value since it blooms throughout the year in the barren areas (Lokhande et al. 2009b). Due to its survival in adverse environmental conditions, the plant is recognized as a promising candidate for the environmental protection (Ghnaya et al. 2005, 2007; Lokhande et al., 2009b, 2010a; Rabhi et al., 2009, 2010; Moseki and Buru, 2010; Zaier et al., 2010a, b). The experiments have presented the optimum growth potential of the plant in the presence of salt concentrations of NaCl (200 mM) (Lokhande et al. 2010b; Moseki and Buru, 2010). Recently, preliminary experiments have also shown that the pre-plantation of *S. portulacastrum* on saline soil has the capacity to remove excess salts from the soil and helps to regain the fertility of arable lands for the production of crop plants (Rabhi et al., 2010).
PART 1

Bioactive compound analysis of halophyte Sesuvium portulacastrum L.

1. Introduction

Foods from plant origin provide our diet with large amounts of antioxidants, mostly phenolic compounds such as proanthocyanidins, flavonoids and phenolic acids (Aron and Kennedy, 2008; Rodrigues et al., 2011). Plant phenolic compounds are synthesised via the phenylpropanoid pathway and play a role in plant defence mechanisms against biotic and abiotic stresses (Rodrigues et al., 2010). Salinity is a major environmental factor limiting plant growth and productivity (Paridaa and Das, 2005). It generates oxidative stress in plant tissues (Amor et al., 2005; Bartosz, 1997; Rout and Shaw, 2001) and induces the generation of cytotoxic reactive oxygen species (ROS), such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals (Gossett et al., 1994). These ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein, and nucleic acids (Fridovich, 1986; Imlay and Linn, 1988; Paridaa and Das, 2005), which leads to cellular damage, tissue injuries, metabolic disorders, and senescence processes (Ksouri et al., 2009).

Halophytes grow in a wide variety of saline habitats, from coastal sand dunes, salt marshes and mudflats to inland deserts, salt flats and steppes (El Shaer, 2003). These plants are characterized by a high physiological plasticity not only for their salt tolerance limits, but also for the climatic zone from which they originate (Tipirdamaz et al., 2006). Environmental stresses (salinity, drought, heat/cold, luminosity and other hostile conditions) may trigger oxidative stress in plants, generating the formation of reactive oxygen species (ROS), leading to cellular damage, metabolic disorders, and senescence processes (Wang et al., 2004). The halophytes are able to withstand and quench these toxic ROS, since they are equipped with a powerful antioxidant system that includes enzymatic and non-enzymatic components. The ability of halophytes to tolerate salt-triggered oxidative stress is governed by multiple biochemical mechanisms that facilitate retention and/or acquisition of water, protect chloroplast functioning, and maintain ion homeostasis. Most essential traits include the synthesis of osmolytes, specific proteins,
and antioxidant molecules (Ksouri, 2008). A large flora of medicinal halophytes exhibit higher content of bioactive compounds as stress metabolites mainly polyphenols. The excellent medicinal properties of these halophytes are mainly attributed to their antioxidant constituents (Maisuthisakul et al., 2007).

Polyphenols constitute the main powerful compound, owing to their multiple applications in food industry, cosmetic, pharmaceutical and medicinal materials (Maisuthisakul et al., 2007). Structurally, phenolics comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1998). In addition to their role as antioxidant, these compounds exhibit a wide spectrum of medicinal properties, such as anti-allergic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effects (Balasundram et al., 2006). In plants, polyphenol synthesis and accumulation is generally stimulated in response to biotic/abiotic stresses (Naczk and Shahidi, 2004), such as salinity (Navarro et al., 2006), secondary metabolites may play a role in the adaptation of halophytic species to this constraint (Ksouri et al., 2007). Previous studies have shown that the amount of polyphenolics in plants, and antioxidant activities, depend on biological factors (genotype, organ and ontogeny) (Lisiewska et al., 2006).

Furthermore, the solubility of phenolic compounds is governed by the type of solvent (polarity) used, the degree of polymerization of phenolics, and their interaction (Zhao, 2006). Polyphenols from halophytes such as Mesembryanthemum edule are potentially useful as traditional remedy against fungal and bacterial infections and sinusitis, diarrhoea, infantile eczema and tuberculosis (Van Der Watt and Pretorius, 2001) and Tamarix gallica as astringent, detergent, diuretic, expectorant, and laxative (Ksouri et al., 2009). Different extracts isolated from halophytes like aqueous extract from Suaeda fruticosa, responsible for hypoglycaemic and hypolipidaemic activities(Bennani-Kabchi et al., 1999; Benwahhoud et al., 2001), furthermore methanolic extracts of Suaeda pruinosa were used to detect antimicrobial activity (El-Hagrasi et al., 2005).

Besides, polyphenolic constituents of various halophytes have been reported to contain several biological properties. In fact, these polyphenol-type compounds with
several -OH groups, show chemopreventive role towards cardiovascular and degenerative
diseases (Halpern et al., 1998), cardioprotective, anticancer, antibacterial and antiviral
activities (Okuda, 2005; Esposito et al., 2002). Although it is probably not the sole
mechanism involved, their beneficial effect is likely to be linked to their antioxidant
activity protecting the cells against oxidative stress (Okuda, 2005). The antioxidant and
radical scavenging activities of proanthocyanidins depend on their molecular structure
(Aron & Kennedy, 2008), and are also influenced by their conformation and their
intramolecular and intermolecular interactions (Zielinska et al., 2008).

Sesuvium portulacastrum L., belongs to the family Aizoaceae, commonly known
as “sea purslane”, is a sprawling perennial herb that grows in coastal areas and grows
naturally in the sub-tropical, mediterranean coastal and warmer areas around the
world (Robert and Frank, 1997; Rabhi et al. 2010). The plant has a long history of use in
folk medicine, for the treatment of epilepsy, conjunctivitis, dermatitis, haematuria,
leprosy and purgative and also used to cure toothache (Bandaranayake, 1998). This plant
is being used to treat various infectious diseases and kidney problems by the traditional
healers in Zimbabwe and South Africa (Magwa et al, 2006). S. portulacastrum expresses
fatty acid methyl esters (FAME extract) which can be used in medicine as a potential
antimicrobial and antifungal agent (Chandrasekaran et al. 2011). The essential oil from the
fresh leaves of S. portulacastrum exhibited antibacterial, antifungal and antioxidant
activity (Michael et. al, 2006). To my knowledge, few studies have been conducted on
the other biological capacities of this halophyte. Thus, the aim of the present work was to
quantify phenolic compounds of S. portulacastrum and to evaluate its antioxidant
capacity as well as its phytochemical constituents with the help of FT-IR and Gas
Chromatography-Mass Spectrum (GC-MS) technique.
2. Materials and Methods

2.1. Plant material and extract preparation

Fresh leaves and stems of *S. portulacastrum* were collected from Vellar estuary Parangipettai. The collected specimens were identified based on the manual by Kathiresan K. (2002). Withered leaves and stems of *S. portulacastrum* were rinsed under running tap water to eliminate dust. After that samples were washed several times with distilled water and air-dried at 25-30°C for about 3-5 days. The dried samples were ground to fine powder using mortar and pestle. The powder was passed through a sieve of 22 mm mesh size. The powder sample was kept in a clean, dried, air tight amber glass container to protect it from sunlight. Twenty gram of ground *S. portulacastrum* was extracted using three fold volumes of solvents of different polarity in order of increasing hydrophilic property (i.e. hexane, dichloromethane (DCM), ethyl acetate and methanol respectively) for 48 h on an orbital shaker to make the extracts(Gülçin et al. 2010; Tohma and Gülçin 2010). This procedure was repeated for two more times. Finally, the extracts were concentrated using a rota-evaporator (IKA- RV 10, USA) at a reduced pressure at ≤40°C. The resulting extracts were then dissolved in dimethylsulfoxide (DMSO) and kept at 4°C until further use.

2.2. Determination of Total phenolic content

Phenolic contents of all crude extracts in 10 mg/ml of DMSO were estimated by the method of Taga et al., 1984. Briefly, 100 µl aliquot of sample was mixed with 2.0 ml of 2% Na₂CO₃ and allowed to stand for 2 min at room temperature. After incubation, 100 µl of 50% Folin-Ciocalteau’s phenol reagent was added, and the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. Absorbance of all the solutions was measured at 720 nm using LAMBDA 25 UV/Vis Spectrophotometers (PerkinElmer). Phenolic contents were expressed as gallic acid equivalent per gram (GE/g).

2.3. Reducing power ability
The reducing power of all crude extracts obtained *S. portulacastrum* was determined through the transformation of Fe$^{3+}$ to Fe$^{2+}$ inducing was determined by the method prescribed by Oyaizu, 1986. Briefly, 1.0 ml of different solvent extracts containing different concentrations of sample (50, 100, 250, 500 and 1000 µg) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). Reaction mixture was incubated at 50°C for 20 min. After incubation, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged at 650 g for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl$_3$ (0.1%). Absorbance of all the solutions was measured at 700 nm using LAMBDA 25 U/V is Spectrophotometers (PerkinElmer). Increased absorbance is indicated increased reducing power.

### 2.4. Determination of Total antioxidant activity

Total antioxidant activities of all crude extracts (10 mg/ml of DMSO) were determined according to the method of Prieto *et al.*, 1999. Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min in water bath. Absorbance of all the sample mixtures was measured at 695 nm using LAMBDA 25 UV/Vis Spectrophotometers (PerkinElmer). Total antioxidant activity is expressed as the number of equivalents of ascorbic acid.

### 2.5. Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

The radical scavenging ability was determined as described by Mensor *et al.*, 2001 with little modifications. Briefly, one ml from 0.3 mM methanol solution of DPPH was added to 2.5 ml from the samples with different concentrations of all *S. portulacastrum* extract. The samples were kept at room temperature in the dark and after 30 min the optic density was measured at 518 nm. The antiradical activity (AA) was determined by the following formula:

\[ \text{AA}\% = 100 - \left\{ \frac{(\text{Abs. Sample} - \text{Abs. Empty Sample}) \times 100}{\text{Abs. control}} \right\}, \]
Where empty samples = 1 ml ethanol + 2.5 ml from various concentrations of all 
*S. portulacastrum* extract; control sample = 1 ml 0.3 mM DPPH + 2.5 ml methanol.

The optical density of the samples, the control and the empty samples were measured in comparison with methanol.

**2.6. Detection of hydroxyl radicals by deoxyribose assay**

The assay was performed as described by Halliwell *et al.*, 1987 with minor
changes. All solutions were freshly prepared. One millimetre of the reaction mixture 
contained 100 µl of 28 mM 2-deoxy-D- ribose (dissolved in KH$_2$PO$_4$–K$_2$HPO$_4$ buffer, 
 pH 7.4), 500 µl solution of various concentrations of the all *S. portulacastrum* extract, 
200 µl of 200 µM FeCl$_3$ and 1.04 mM EDTA (1:1 v/v), 100 µl H$_2$O$_2$ (1.0 mM) and 100 
µl ascorbic acid (1.0 mM). After an incubation period of 1 h at 37$^\circ$C the extent of 
deoxyribose humiliation was measured by the TBA reaction. 1.0 ml of TBA (1% in 50 
mM NaOH) and 1.0 ml of TCA were added to the reaction mixture and the tubes were 
heated at 100$^\circ$C for 20 min. After cooling, the absorbance was read at 532 nm against a 
blank (containing only buffer and deoxyribose). All tests were performed three times. 
Ascorbic acid was used as a positive control. Percent inhibition in hydroxyl radical was 
calculated by the following expression:

$$I (\%) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100,$$

Where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the sample. 
The data obtained at each point was the average of three measurements.

**2.7. Hydrogen peroxide radical scavenging assay**

The ability of halophytes *S. portulacastrum* extract to scavenge hydrogen 
peroxide was determined according to the method described by Gülçin *et al.*, 2004. 
Different concentrations of extracts were added to 2 ml solution of 10 mM hydrogen 
peroxide (H$_2$O$_2$) in 0.1 M phosphate buffer, pH 7.4. After 10 min, absorbance of 
H$_2$O$_2$ was recorded at 230 nm against blank solution without H$_2$O$_2$. Percent inhibition in 
H$_2$O$_2$ was calculated using the following formula:
\[
\text{(%)} \text{ inhibition} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100,
\]

Where \(A_0\) is the absorbance of the control and \(A_1\) is the absorbance of the sample.

**2.8. Fourier transform infrared spectrophotometer (FT-IR) spectroscopy**

Fourier transform infrared spectrophotometer analysis is used to predict functional groups present in a molecule based on their frequencies of vibration between bonds of the atoms. All crude extracts (10 mg/ml) were characterized using Fourier transform infrared spectrophotometer (FT-IR; IR Affinity-1, Shimadzu, Tokyo, Japan) for FT-IR spectra measurement in the frequency range of 400 to 4,000 cm\(^{-1}\).

**2.9. Identification of phytochemicals through GC-MS analysis**

GC-MS technique was used in this study to identify the components present in the extract. GC-MS technique was carried out at VIT University, Vellore, Tamil Nadu. GC-MS analysis of this extract was performed using a Perkin Elmer GC Claurus 680 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-5MS column (30.0m, 0.25mmID, 250\(\mu\)m df). For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.99\%) was used as the carrier gas at a constant flow rate of 1ml/min. and an injection volume of 1\(\mu\)l was employed (Split ratio of 10:1). Injector temperature was 250\(^\circ\)C. The oven temperature was programmed from Initial temp 60\(^\circ\)C for 2 min, ramp 10\(^\circ\)C/min to 300\(^\circ\)C, hold 6 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 50 to 600 Da. Total GC running time was 32 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbomass Ver5.4.2.

**2.10. Identification of Components**

Interpretation on mass spectrum of GC-MS was conducted using the data base of National Institute Standard and Technology (NIST) and Wiley spectra Libraries. Spectrum of the unknown component was compared with the spectrum of known components stored in the NIST Library having more than 62,000 patterns. The molecular
weight, molecular formula and the number of hits used to identify the name of the compound from NIST and Wiley spectra Libraries were recorded.

3. **Results and Discussions**

3.1. **Extraction and yield of extract**

Four different solvents (with different polarity) were used in this study, since a wide range of extract holds a better chance for the extraction and isolation of biologically active molecules for general screening of bioactivity (Kumar *et al.*, 2008). The average percentage yield of each extracting solvent was based on triplicate analysis of samples. Results showed that extraction with hexane (0.6 ± 0.03 gm) and DCM (0.5 ± 0.02 gm) showed the highest yield. The hexane solvent yielded the highest amount of extract indicating that the leaves and shoots of *S. portulacastrum* contain mostly of lipophilic compounds such as waxes, chlorophyll, and fatty acids.

3.2. **Total polyphenol content (TPC)**

There are number of recent studies showing that phenolic compounds are commonly found in plants and have been reported to possess several biological activities including powerful antioxidant compounds (Ksouri *et al.*, 2008; Maisuthisakul *et al.*, 2007). For that, total polyphenolic contents of all the four extracts of *S. portulacastrum* were assessed. Principal results showed that significant difference was found in TPC among the different solvents used (Fig.1.). The TPC in hexane, dichlomethane, ethyl acetate and methanol were found as 6.75, 24.25, 20.06 & 40.75 mg/g dry weight (DW) GAE equivalent respectively. The highest amount of TPC was observed for methanol extract. Moreover, total phenolic content was significantly higher in *S. portulacastrum* shoot and leaf as compared to other medicinal halophytes like *Suaeda fruticosa* (31.8 mg GAE/g DW) (Samia Oueslati *et al*, 2012) as well as *Salsola kali* which belong to the same family (17.23 mg GAE/g DW) and in a glycophytic species such as the aromatic and medicinal plant *Nigella sativa* L. (10.04 mg GAE/g DW) (Bourgou *et al.*, 2008).
These observations indicated the richness of crude extract of *S. portulacastrum* on phenolic content, for that the in vitro estimation of biological activities was determined.

![Figure 1. Total phenolic content of total extract from *S. portulacastrum*. The data are the mean ± SD of 3 replicates](image)

3.3. Reducing power ability

In case of reducing power, antioxidants in the sample reduce ferric (III) to ferrous (II) in a redox-linked colourimetric reaction (Li *et al.*, 2006) that involves single electron transfer. The reducing power indicates that the antioxidant compounds are electron donors and reduce the oxidized intermediate of the lipid peroxidation process, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). In this assay, reducing power ability of *S. portulacastrum* extracts steadily increased with increasing concentration all the samples (Fig. 2). Same trend has also been reported by Kumaran and Karunakaran, (2007) in methanol extracts of higher plants. The maximum (0.350 ± 0.009) reducing power value was observed in dichloromethane extracts and minimum (0.239±0.009) was obtained from hexane extracts. This property is associated with the presence of reductions that are reported to be terminators of free radical chain reaction (Duh, 1998). Similar observation was seen in case of other halophytes like *Cakile*
maritima, Limoniastrum monopetalum, Mesembryanthemum crystallinum, M. edule, Salsola kali, and Tamarix gallica also with different solvent extracts of their different parts (Ksouri et al., 2008).

3.4. Total antioxidant activity

The total antioxidant activity of leaves & shoot extracts using four different solvents (hexane, dichlomethane, ethyl acetate and methanol) was assessed. In the case of phosphomolybdenum method, molybdenum VI (MoVI) is reduced to form a green phosphate/MoVII complex. In figure 3, results indicated that dichlomethane extract showed the highest antioxidant activity of 112.95 ± 12.95 mg/g DW ascorbic acid equivalent, followed by ethyl acetate (83.56 ± 112.95 mg/g DW ascorbic acid equivalent.). While in contrast, in case of methanol and hexane extracts, this activity was less significant. However, a total antioxidant activity has been reported in higher plant extracts of 245 – 376 mg ascorbic acid/g (Kumaran and Karunakaran, 2007). In comparison to brown seaweed (Sargassum pallidum) highest total antioxidants activity (30.50 µmol FeSO₄/mg) in ethanol extract (Ye et al., 2009). Higher activity in fractions may be due to the interferences of other compounds present in crude extract; and, it has

![Figure 2. Reducing power of total extract from S. portulacastrum. The data are the mean ± SD of 3 replicates](image-url)
also been reported that solvents used for extraction have dramatic effect on the chemical species (Yuan et al., 2005).

Results represented a large and significant variability between S. portulacastrum organs for their antioxidant activity, aerial parts (i.e. leaves and stems) presenting the strongest activities for almost all the tested assays. Such an organ-related variability in antioxidant activity was informed previously in other halophytes (Ksouri et al., 2008). Similar result was observed by Matkowski et al., (2008) for the total antioxidant activity of different organs of Salvia spp.

![Graph](image.png)

Figure 3. Total antioxidant activity of total extract extract from S. portulacastrum. The data are the mean ± SD of 3 replicates.

### 3.5. Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

It is well identified that the antioxidant activity of plant extracts having polyphenol components is due to their ability to be donors of hydrogen atoms or electrons and to capture the free radicals. DPPH has been used widely as a free radical to assess reducing substances(Cotelle et al., 1996) and is a valuable reagent for inspecting the free radical scavenging activities of compounds (Duan et al., 2006). The DPPH radical scavenging activity of S. portulacastrum extracts increased in a concentration dependent manner. The comparison of % DPPH radical scavenging activity of all the four solvent extracts obtained results are shown in figure 4. It was observed that the extracts containing high level of TPC were also potent DPPH radical scavenger, suggesting that halophytic polyphenols may be the principle constituents responsible for the antiradical properties of the extracts. The similar results were obtained by some researchers that
change in solvent’s polarity alters its efficacy to extract a specific group of antioxidant compounds and influences the antioxidant properties of the extracts (Zhou and Yu, 2004). Among all extracts, maximum effective scavenging ability was exhibited by methanol (75.10%), followed by dichloromethane (52.30%). The other extracts showed relatively weak scavenging potentials.

These findings may be related to the higher polyphenol contents in *S. portulacastrum*. Many other authors have reported a positive and significant relationship between the antioxidant components including phenols and polyphenols with the DPPH radical scavenging capacity (Connan *et al.*, 2006, Huang *et al.*, 2005).

![Graph showing scavenging effects of S. portulacastrum extract on DPPH radical. The data are the mean ± SD of 3 replicates.](image)

**Figure 4.** Scavenging effects of *S. portulacastrum* extract on DPPH radical. The data are the mean ± SD of 3 replicates.

### 3.6. Detection of hydroxyl radicals by deoxyribose assay

Hydroxyl radical is an extremely responsive free radical formed in biological systems and has been involved as a highly damaging species in free radical pathology (Li *et al.*, 2008). This radical has a capability to join nucleotides in DNA and cause component breakage that contributes to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz *et al.*, 2002; Manian *et al.*, 2008; Duan *et al.*, 2007). Ferric-EDTA was incubated with H$_2$O$_2$ (Fenton reaction) and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-D-ribose into fragments that on heating with TBA at low pH form a pink chromogen (Halliwell *et al.*, 1987; Aruoma *et al.*, 1989). The hydroxyl radical scavenging activity of
different fractions of *S. portulacastrum* extract added to the reaction mixture removed the hydroxyl radicals from the sugar and prevented it from degradation. These results are shown in figure 5. It was observed that DCM fraction has the strongest percentage inhibition (85%) against hydroxyl radical at 1000 µg/mL concentration in comparison to other extracts. Hydroxyl radical scavenging activity of the different extracts was in this order: DCM > ethyl acetate > hexane > methanol.

![Figure 5](image.png)

**Figure 5.** Inhibition of the radical degradation of 2-deoxy-D-riboza of *S. portulacastrum* extract. The data are the mean ± SD of 3 replicates

### 3.7. Hydrogen peroxide radical scavenging assay

Hydrogen peroxide is a weak oxidizing agent and can deactivate a few enzymes directly, usually by oxidation of necessary thiol (-SH) groups. It can cross cell membranes rapidly and enter inside the cell. H$_2$O$_2$ probably reacts with Fe$^{2+}$ and possibly Cu$^{2+}$ ions to form hydroxyl radical which may be the origin of many of its toxic effects (Nagavani *et al.*, 2010). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is getting accumulated inside the cells. The scavenging activity of the different fractions of *S. portulacastrum* is shown in figure 6. In this study, all fractions exhibited appreciable scavenging activity. The maximum scavenging activity (83%) was exhibited by DCM fraction.
3.8. Functional groups identification

The FTIR spectrum was used to identify the functional groups of the active components present in extract based on the peaks values in the region of IR radiation. When the extract was passed into the FTIR, the functional groups of the components were separated based on its peaks ratio. The results of FTIR analysis confirmed the presence of alcohol, phenol, alkanes, aldehyde, aromatic compound, secondary alcohol, aromatic amines and halogen compound (Fig-7, and table-1 to 4).
Table 1: FTIR peak values and functional groups of Hexane extracts of *S. portulacastrum*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>603.72</td>
<td>Alkyl halide</td>
<td>12.</td>
<td>2108.2</td>
<td>Alkyne</td>
</tr>
<tr>
<td>2.</td>
<td>692.44</td>
<td>Alkyl halide</td>
<td>13.</td>
<td>2148.7</td>
<td>Alkyne</td>
</tr>
<tr>
<td>3.</td>
<td>950.91</td>
<td>Alkene</td>
<td>14.</td>
<td>2335.8</td>
<td>Unknown</td>
</tr>
<tr>
<td>4.</td>
<td>1035.77</td>
<td>Ester</td>
<td>15.</td>
<td>2447.67</td>
<td>Unknown</td>
</tr>
<tr>
<td>5.</td>
<td>1211.3</td>
<td>Ether, Acid, Ester</td>
<td>16.</td>
<td>2594.26</td>
<td>Acid</td>
</tr>
<tr>
<td>6.</td>
<td>1315.45</td>
<td>Amine</td>
<td>17.</td>
<td>2711.92</td>
<td>Acid</td>
</tr>
<tr>
<td>7.</td>
<td>1423.47</td>
<td>Aromatic</td>
<td>18.</td>
<td>2918.3</td>
<td>Aldehyde</td>
</tr>
<tr>
<td>8.</td>
<td>1512.19</td>
<td>Nitro</td>
<td>19.</td>
<td>3001.24</td>
<td>Aromatic</td>
</tr>
<tr>
<td>9.</td>
<td>1658.78</td>
<td>Alkene</td>
<td>20.</td>
<td>3431.36</td>
<td>Amine</td>
</tr>
<tr>
<td>10.</td>
<td>1909.53</td>
<td>Unknown</td>
<td>21.</td>
<td>3846.06</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
### Table 2: FTIR peak values and functional groups of Dichloromethane extracts of *S. portulacastrum*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
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<td>Unknown</td>
<td>16.</td>
<td>1917.24</td>
<td>Unknown</td>
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<tr>
<td>2.</td>
<td>520.78</td>
<td>Alkyl halide</td>
<td>17.</td>
<td>1984.75</td>
<td>Unknown</td>
</tr>
<tr>
<td>3.</td>
<td>570.93</td>
<td>Alkyl halide</td>
<td>18.</td>
<td>2108.2</td>
<td>Alkyne</td>
</tr>
<tr>
<td>4.</td>
<td>651.94</td>
<td>Alkyl halide</td>
<td>19.</td>
<td>2345.44</td>
<td>Unknown</td>
</tr>
<tr>
<td>5.</td>
<td>690.52</td>
<td>Alkyl halide</td>
<td>20.</td>
<td>2439.95</td>
<td>Unknown</td>
</tr>
<tr>
<td>6.</td>
<td>756.1</td>
<td>Alkyl halide</td>
<td>21.</td>
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<td>Unknown</td>
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<tr>
<td>7.</td>
<td>950.91</td>
<td>Alkene</td>
<td>22.</td>
<td>2592.33</td>
<td>Acid</td>
</tr>
<tr>
<td>8.</td>
<td>1037.7</td>
<td>Alcohol</td>
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<td>2698.41</td>
<td>Acid</td>
</tr>
<tr>
<td>9.</td>
<td>1153.43</td>
<td>Alcohol, Ester</td>
<td>24.</td>
<td>2916.37</td>
<td>Acid</td>
</tr>
<tr>
<td>10.</td>
<td>1211.3</td>
<td>Ester</td>
<td>25.</td>
<td>2999.31</td>
<td>Acid</td>
</tr>
<tr>
<td>11.</td>
<td>1315.45</td>
<td>Amine</td>
<td>26.</td>
<td>3435.22</td>
<td>Amine</td>
</tr>
<tr>
<td>12.</td>
<td>1423.47</td>
<td>Aromatic</td>
<td>27.</td>
<td>3726.47</td>
<td>Alcohol</td>
</tr>
<tr>
<td>13.</td>
<td>1514.12</td>
<td>Nitro</td>
<td>28.</td>
<td>3851.85</td>
<td>Unknown</td>
</tr>
<tr>
<td>14.</td>
<td>1658.78</td>
<td>Alkene</td>
<td>29.</td>
<td>3921.28</td>
<td>Unknown</td>
</tr>
<tr>
<td>15.</td>
<td>1855.52</td>
<td>Ketone</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3: FTIR peak values and functional groups of Etyl acetate extracts of *S. portulacastrum*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>459.06</td>
<td>Unknown</td>
<td>21.</td>
<td>1992.47</td>
<td>Unknown</td>
</tr>
<tr>
<td>2.</td>
<td>513.07</td>
<td>Alkyl halide</td>
<td>22.</td>
<td>2050.33</td>
<td>Unknown</td>
</tr>
<tr>
<td>3.</td>
<td>559.36</td>
<td>Alkyl halide</td>
<td>23.</td>
<td>2094.69</td>
<td>Unknown</td>
</tr>
<tr>
<td>4.</td>
<td>603.72</td>
<td>Alkyl halide</td>
<td>24.</td>
<td>2140.99</td>
<td>Alkyne</td>
</tr>
<tr>
<td>5.</td>
<td>657.73</td>
<td>Alkyl halide</td>
<td>25.</td>
<td>2194.99</td>
<td>Alkyne</td>
</tr>
<tr>
<td>6.</td>
<td>700.16</td>
<td>Alkyl halide</td>
<td>26.</td>
<td>2345.44</td>
<td>Unknown</td>
</tr>
<tr>
<td>7.</td>
<td>759.95</td>
<td>Alkene</td>
<td>27.</td>
<td>2443.81</td>
<td>Unknown</td>
</tr>
<tr>
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<td>862.18</td>
<td>Alkene</td>
<td>28.</td>
<td>2590.4</td>
<td>Acid</td>
</tr>
<tr>
<td>9.</td>
<td>952.84</td>
<td>Alkene</td>
<td>29.</td>
<td>2696.48</td>
<td>Acid</td>
</tr>
<tr>
<td>10.</td>
<td>1039.63</td>
<td>Amine</td>
<td>30.</td>
<td>2748.56</td>
<td>Acid, Aldehyde</td>
</tr>
<tr>
<td>11.</td>
<td>1157.29</td>
<td>Alcohol, Ester</td>
<td>31.</td>
<td>2858.51</td>
<td>Alkane, Acid</td>
</tr>
<tr>
<td>12.</td>
<td>1209.37</td>
<td>Ether, Acid, Ester</td>
<td>32.</td>
<td>2916.37</td>
<td>Aldehyde</td>
</tr>
<tr>
<td>13.</td>
<td>1255.66</td>
<td>Ether, Acid, Ester</td>
<td>33.</td>
<td>2999.31</td>
<td>Aromatic, Alkene</td>
</tr>
</tbody>
</table>
Table 4: FTIR peak values and functional groups of ethyl acetate extracts of S. portulacastrum

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
<th>S.No.</th>
<th>Peak values</th>
<th>Functional Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.</td>
<td>1313.52</td>
<td>Amine</td>
<td>34.</td>
<td>3101.54</td>
<td>Acid</td>
</tr>
<tr>
<td>15.</td>
<td>1423.47</td>
<td>Aromatic</td>
<td>35.</td>
<td>3427.51</td>
<td>Amine</td>
</tr>
<tr>
<td>16.</td>
<td>1510.26</td>
<td>Aromatic</td>
<td>36.</td>
<td>3728.4</td>
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<tr>
<td>17.</td>
<td>1660.71</td>
<td>Alkene</td>
<td>37.</td>
<td>3842.2</td>
<td>Unknown</td>
</tr>
<tr>
<td>18.</td>
<td>1793.8</td>
<td>Carbonyl</td>
<td>38.</td>
<td>3855.7</td>
<td>Unknown</td>
</tr>
<tr>
<td>19.</td>
<td>1853.9</td>
<td>Ketone</td>
<td>39.</td>
<td>3921.28</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

3.9. Identification of phytocemicals through GC - MS analysis

The more precise information in qualitative analysis can be obtained by gas-chromatography coupled with mass spectrometry. For quantitative determination, gas-chromatography with flame ionization detector (GC-FID) and GC-MS are preferred (Lampronti et al., 2006; Haznagy et al., 2007). The compounds present in the methanolic extract of *Sesuvium portulacastrum* were identified by GC-MS analysis (Fig. 8).

On comparison of the mass spectra of the constituents with the NIST library, thirteen peaks were obtained out of which twelve phytocemients were characterized and identified. The active principal Molecular Weight (MW), concentration (%), molecular Formula (MF), and retention time (RT) is presented in table 5.

Among the twelve compounds identified after GC-MS, one of compounds Hentriacontane, a saturated hydrocarbon, the major compound was identified has been isolated from *Scabiosa comosa*. It is reported to be responsible for its uptake in the soil.
by plant and shown to be involved with stimulation of fungal spore germination. Hentriacontane has also been isolated from spinach leaves, and discovered to be unsaponifiable and shown to have possible anti-tumour activity (McGinty et al., 2010). Other compound phenol, 2, 4-bis (1, 1-dimethylethyl), their anti-inflammatory activity, in comparison with indomethacin and vitamin E (Costantino et al., 1993). L-(+)-ascorbic acid, 2-6-dihexadecanoate which is a derivative of ascorbic acid, vitamin C, is present in the essential oil. Vitamin C is an antioxidant and belongs to the class of compounds identified to enhance sperm quality and prevent sperm agglutination, thus making them more motile with forward progression and hence promote male fertility (Glenville, 2008; Dawson et al., 1992). L-(+)-ascorbic acid, 2-6-dihexadecanoate has also been isolated from Ipomoea pes-caprae (L.) R.Br leaves (arun et al., 2014).

They were identified as three major phytochemical constituent’s mass spectra are presented in figure 9 - 11. They were identified as Hentriacontane, L-(+)-ascorbic acid, 2-6-dihexadecanoate and phenol, 2, 4-bis (1, 1-dimethylethyl).

Figure 8. GC-MS pattern of Phytoconstituents obtained from Sesuvium portulacastrum
**Table 5:** Phytoconstituents identified in the methanolic extracts of the whole plant of *sesuvium portulacastrum* by GC-MS

<table>
<thead>
<tr>
<th>S. No</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular Formula</th>
<th>MW</th>
<th>Peak Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>13.158</td>
<td>PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-</td>
<td>C14H22O</td>
<td>206</td>
<td>18.571</td>
</tr>
<tr>
<td>2.</td>
<td>16.904</td>
<td>PYRROLO[1,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(2-METHYLPROPYL)-</td>
<td>C11H18O2N2</td>
<td>210</td>
<td>6.789</td>
</tr>
<tr>
<td>3.</td>
<td>17.279</td>
<td>BUTANOIC ACID, PYRROLIDIDE</td>
<td>C8H15ON</td>
<td>141</td>
<td>3.799</td>
</tr>
<tr>
<td>4.</td>
<td>17.865</td>
<td>L-PROLINE, N-VALERYL-, HEXADECYL ESTER</td>
<td>C26H49O3N</td>
<td>423</td>
<td>3.403</td>
</tr>
<tr>
<td>5.</td>
<td>18.030</td>
<td>PYRROLO[1,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(2-METHYLPROPYL)-</td>
<td>C11H18O2N2</td>
<td>210</td>
<td>6.853</td>
</tr>
<tr>
<td>6.</td>
<td>18.140</td>
<td>L-(+)-ASCORBIC ACID 2,6-DIHEXADECANOATE</td>
<td>C38H68O8</td>
<td>652</td>
<td>33.988</td>
</tr>
<tr>
<td>7.</td>
<td>18.295</td>
<td>HEPTACOSYL HEPTAFLUOROBUTYRATE</td>
<td>C31H55O2F7</td>
<td>592</td>
<td>4.114</td>
</tr>
<tr>
<td>8.</td>
<td>19.975</td>
<td>OCTADECANOIC ACID</td>
<td>C18H36O2</td>
<td>284</td>
<td>9.943</td>
</tr>
<tr>
<td>9.</td>
<td>22.221</td>
<td>PYRROLO[1,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-</td>
<td>C14H16O2N2</td>
<td>244</td>
<td>5.751</td>
</tr>
<tr>
<td>10.</td>
<td>24.887</td>
<td>HENTRIACONTANE</td>
<td>C31H64</td>
<td>436</td>
<td>1.723</td>
</tr>
<tr>
<td>11.</td>
<td>25.588</td>
<td>HENTRIACONTANE</td>
<td>C31H64</td>
<td>436</td>
<td>1.911</td>
</tr>
<tr>
<td>12.</td>
<td>26.263</td>
<td>HENTRIACONTANE</td>
<td>C31H64</td>
<td>436</td>
<td>1.605</td>
</tr>
</tbody>
</table>

**Figure 9:** The mass spectrum analysis and structure of Hentriacontane
Figure 10: The mass spectrum analysis and structure of L-(+)-ascorbic acid, 2-6-

Figure 11: The mass spectrum analysis and structure of phenol, 2,4-bis(1,1-
PART -2

Effect of salinity stress on seed germination in halophyte

*Sesuvium portulacastrum* L.

1. Introduction

Salinity is the process of accumulation of soluble slats, by which saline soils are produced. The composition of salts in large amounts mostly is calcium, sodium, Magnesium, chloride and sulphate ions and in relatively small amounts are potassium, carbonates, bicarbonates, borate and lithium salts (Zhu, 2001). Accumulation of these salts increases the osmotic pressure of the soil solution because of restricted water intake by plants (Cramer et al., 1999). The ability of plant induced systems to tolerate severe levels of stress signifies the importance of stress proteins (Uma et al., 1995). Soil salinity is one of the most important constraints that limit crop production in arid and semiarid areas. The excess salt in soils and water has injurious effect on crop yields and results in substantial losses of arable soil especially in the arid and semiarid regions (Kennedy and de Filippis, 1999; Cayuela et al., 2001). More than 900 million hectares of land worldwide, approx. 20% of the total agricultural land, are affected by salt, accounting for more than 6% of the world’s total land area. NaCl is the predominant salt causing salinization, and it is not surprising that plants have evolved mechanisms to regulate its accumulation (Munns and Tester, 2008). Seed germination is a critical stage in the history of plants and salt tolerance during germination is crucial for the establishment of plants that grow in saline soils.

Halophytes are potentially useful for ecological applications, such as landscaping, or rehabilitation of damaged ecosystems. They may also present economic interest as food, forage, or for production of metabolites (Single et al., 1996; Lieth, 1999). One such potential candidate is *Salicornia bigelovii*, which produces oil rich in unsaturated fatty acids, amenable to commercial oilseed extraction methods (Glenn et al., 1991). Coastal halophytes are exposed to frequent fluctuations in salinity levels, with respect to seasons, depending on their distance from the sea. Such variations greatly determine their establishment ability and geographical distribution (Weber and d’Antonio, 1999). Investigating the impact of salinity on physiological and metabolic processes in
halophytes is quite complex since their response depends on salt type and level, plant genotype and growth stage (Meneguzzo et al., 1999; Houle et al., 2001).

Seed germination is an important and vulnerable stage in the life cycle of terrestrial angiosperms, which determines the seedling establishment and plant growth. Despite the importance of seed germination under salt stress (Ungar, 1995), the mechanism(s) of salt tolerance in seeds is relatively poorly understood, especially when compared with the amount of information currently available about salt tolerance, physiology and biochemistry in vegetative plants (Hester et al., 2001; Hu et al., 2005; Garthwaite et al., 2005; Kanai et al., 2007). In vegetative parts, salt stress causes reduced cell turgor and depressed rates of root and leaf elongation (Fricke et al., 2006), suggesting that environmental salinity acts primarily on water uptake. Furthermore, high intracellular concentrations of both Na\(^{+}\) and Cl\(^{-}\) can inhibit the metabolism of dividing and expanding cells (Neumann, 1997), retarding germination and even leading to seed death.

Seeds of halophytes can tolerate high salinity and remain dormant in the soil and germinate when conditions are conducive for their germination (Al-Khateeb, 2006; Khan & Gul, 2006). However, during germination, seeds are more sensitive to salinity and germinate better under non-saline conditions (Khan & Gul, 2006). Salinity impairs seed germination, reduces nodule formation retards plant development and reduces crop yield (Greenway and Munns, 1980).

Halophyte seeds have the ability to remain viable for long periods during exposure to hyper saline conditions and then germinate when salinity is reduced (Keiffer and Ungar 1995). Several studies have shown that halophytes are particularly salt sensitive during seed germination and seedling emergence (Pujol et al., 2000; Debez et al., 2001; Khan et al., 2002), while exhibiting a higher resistance to salt when autotrophic (Wilson et al., 2000; Khan et al., 2000).

Present study was carried out to determine the effect of salinity stress on germination and seed establishment in *Sesuvium portulacastrum*. 
2. **Material and Methods**

2.1. **Seed material and treatment**

Mature flowers of *Sesuvium portulacastrum* were collected from Vellar estuary, Parangipettai, Tamilnadu, India. Seeds were separated from mature ovary, cleaned, and stored in dry conditions at room temperature. Seeds were disinfected in 10% sodium hypochlorite solution and then washed 3 times using sterilized distilled water. Six NaCl concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl) were used for salt tolerance of the species. Seeds were germinated in two folds of Whatman number 1 filter paper placed in 90 mm glass petri dish with 5 mL of test solution. To prevent evaporation, plates were sealed using parafilm and kept in the humidity chamber (Labline) under controlled conditions (25 °C Temperature, 70–90% relative humidity in dark). Four replicates of 25 seeds each were used for each treatment and observed for 14 days.

2.2. **Germination percentage**

Germination of the seeds was determined by using ‘between papers’ method (ISTA 1985). One hundred seeds in four replications of 25 seeds each were placed between two layers of moist germination papers using distilled water in control and respective saline solution in other treatments. These were wrapped in a sheet of wax paper to reduce surface evaporation of moisture and placed in saline solutions of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in the germination incubator at 25 °C in an upright position. After 14 days, seeds were evaluated for normal, abnormal seedling, dormant and dead seeds. Germination percentage was worked out on the basis of normal seedling only.

2.3. **Rate of germination (GR):**

Seeds were placed in Petri dish on a filter paper moistened with different concentrations of saline solutions and incubated in dark in a germinator at 25 °C. A daily germination count of the incubated seeds was taken until no more seeds germinated, and the speed of germination was calculated by following Maguire’s method/formula (1962).

\[ GR = \frac{\sum_{i=1}^{n} S_i}{D_i} \]
Where,

GR: Germination Rate (number of germinated seed in each day)
Si: number of germination seeds in each numeration
Di: number of days till nth numeration.
n: number of numeration times.

2.4. Seedling growth and vigour index

Ten normal seedlings from each replicate were randomly selected to measure shoot and root length. They were subsequently dried at 80 °C in an oven and weighed together to obtain seedling dry weight. Seedling vigour was determined by calculating the vigour index as described by Abdul-Baki and Anderson (1973) as given below:

\[
\text{Vigour index} = \frac{\text{germination percentage} \times \text{means of seedling length (radicle + plumule) cm}}{100}
\]

2.5. Protein extraction by BPP method

The protein extraction method using phenol was modified from a published protocol (Wang et al., 2007), 100 mg of frozen lyophilized tissue powders was resuspended in 1 mL ice-cold extraction buffer (table 1.1). After the sample was vortexed for 5 min at room temperature, two volumes of Tris saturated phenol (pH 8.0) were added and then the mixture was further vortexed for 10 min. After centrifugation (4 °C, 15 min, 15,000 × g), the upper phase was transferred to a new centrifuge tube. Equal volume of extraction buffer was added into the new tube, the mixture was then vortexed for 10 min, followed by centrifugation at the same condition. The upper phase was then transferred to a new centrifuge tube. Proteins were precipitated by adding five volumes of ammonium acetate saturated-methanol, and incubating at -20 °C for at least 6 h. After centrifugation as described above, the protein pellet was re-suspended and rinsed with ice-cold methanol followed by ice-cold acetone twice, and spun down at 15,000 × g for 5 min at 4 °C after each washing. Finally, the washed pellet was air-dried, then recovered with rehydration buffer (table 1.2) and stored at -80°C for further use.
The extraction buffer used in the BPP protocol was modified based on the recipe for extracting soluble proteins from trees by Tian and co-workers (Tian et al., 2003) with slight modifications as described below. Triton X-100 is a nonionic detergent that is efficient in breaking lipid-lipid and lipid-protein interactions (Shaw and Riederer, 2003; Vincent et al., 2006; Valcu and Schlink, 2006). In this method, borax was used to remove the interfering compounds such as polysaccharides, polyquinones, and phenolic compounds. Ascorbic acid (vitamin C) and β-mercaptoethanol were used as strong reducing agents, which can inhibit the phenolic oxidation efficiently (Kim et al., 2001). Phenol serves as a strong protein solvent while having little activity in dissolving nucleic acids.

### Table 1.1: Preparation of Protein Extraction Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris pH 8.0, w/v</td>
<td>10.00 ml</td>
</tr>
<tr>
<td>100 mM EDTA pH 8.0, w/v</td>
<td>20.00 ml</td>
</tr>
<tr>
<td>50 mM borax, w/v</td>
<td>0.02 gm</td>
</tr>
<tr>
<td>50 mM vitamin C, w/v</td>
<td>0.88 gm</td>
</tr>
<tr>
<td>1% Triton X-100, v/v</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>2% β-mercaptoethanol, v/v</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>30% sucrose, v/v</td>
<td>30.00 gm</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>100.00 ml</td>
</tr>
</tbody>
</table>

### Table 1.2: Preparation of Protein Rehydration Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 M urea, w/v</td>
<td>5.45 gm</td>
</tr>
<tr>
<td>2% CHAPS, w/v</td>
<td>0.20 gm</td>
</tr>
<tr>
<td>13 mM DTT, w/v</td>
<td>0.02 gm</td>
</tr>
<tr>
<td>1% IPG buffer v/v</td>
<td>0.10 ml</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>10.00 ml</td>
</tr>
</tbody>
</table>
acids and polysaccharides (Carpentier et al., 2005). The Phenol extraction step allows us to efficiently solubilize proteins while removing salt ions to certain degrees (Saravanan and Rose, 2004; Li et al., 2006). Ammonium acetate saturated methanol was used to precipitate the proteins.

2.6. Quantification of Proteins:

Protein concentration was determined by Bicinchoninic Acid protein Assay kit (BCA, Sigma) following the protocol supplied along with the kit using the LAMBDA 25 UV/Vis Spectrophotometers (PerkinElmer). BSA was used as the standard (100µg/µl) and the absorbance was measured at 562nm.

2.7. One-Dimensional (1D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein extracts were separated on 1D SDS-PAGE as previously described (Laemmli, 1970).

One dimensional (1D) gels were cast on 16 cm (width) x 19 cm (height) spacer glass plates mounted with 1 mm thick spacers using the Midi-protein Electrophoresis system (Biotech, Hitech, R & D laboratories, Salem). Resolving gels of 10% (v/v) were prepared from a 30% Acrylamide/Bis stock solution using a 1.5 M Tris-HCl gel buffer, pH 8.8 as indicated in Table 1.3. Twenty millilitres of the prepared resolving gel mixture was poured into cast plates, overlaid with 1 ml of water saturated n-butanol and left to polymerise for 1hr at room temperature. After polymerisation, the water saturated n-butanol overlay was rinsed off with distilled water and gel surfaces were blotted dry with filter paper. Stacking gels of 5% (v/v) were prepared from 30% Acrylamide/Bis stock solution using 0.5 M Tris-HCl gel buffer, pH 6.8 as indicated in Table 1.3. Five milliliters of the prepared stacking gel was poured over the resolving gel. Immediately thereafter, 1 mm thick 13 well combs were placed in the stacking gels to form sample-loading wells. After the polymerization of the stacking gel, the comb was carefully removed. The slots were cleaned by rinsing with 1X Tris-Glycine-SDS Buffer (TGS) presented in table 1.6.
Protein samples were mixed in a ratio of 1:1 with 2X SDS sample loading buffer (60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 200 mM DTT, 0.025% (w/v) bromophenol blue) and denatured by heating on an AccuBlock digital dry bath (Labnet) at 95 °C for 5 min. Samples were pulse centrifuged before loading onto the gels. Total protein quantities of 10 µg and 20 µg per well were typically loaded onto 13 gels respectively along with wide range molecular weight Protein marker (Sigma Aldrich, USA). Electrophoresis was carried out in 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS) on a Midi protein Electrophoresis Cell using a PS 300-B Universal Power supply (Hoefer). The run was initiated at 100 V for the first 30 min and then 120 V until the bromophenol blue tracking dye reached the bottom of the gel plates. The gels were removed from the gels plates, stained with Coomassie Brilliant Blue (CBB) and destained.

### Table 1.3.: Preparation of resolving and stacking gels for SDS-PAGE.

<table>
<thead>
<tr>
<th>Component</th>
<th>10% Resolving gel buffer</th>
<th>5% Stacking gel buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled water</td>
<td>8.00 ml</td>
<td>3.64 ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bis acrylamide stock solution</td>
<td>6.60 ml</td>
<td>0.83 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>5.00 ml</td>
<td>--</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>--</td>
<td>0.33 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.20 ml</td>
<td>--</td>
</tr>
<tr>
<td>20% SDS</td>
<td>--</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.20 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>8.0 µl</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.00 ml</td>
<td>5.00 ml</td>
</tr>
</tbody>
</table>

### 2.8. Coomassie Brilliant Blue Staining in SDS-PAGE Gels

Proteins separated by either 1D or 2D SDS-PAGE were routinely detected using a modified CBB G-250 staining protocol. After electrophoresis, the gels were dismounted from the gel plate assembly and immersed firstly in CBB G-250 staining solution (table 1.4) [5% aluminium sulphate (w/v), 1% ethanol (v/v), 0.02% (w/v) CBB G-250 and
orthophosphoric acid (2.35% v/v)] and kept on a gel rocker (Tarsons) for 2-3 hours with shaking at room temperature until the protein bands or protein spots (in 1D or 2D gels, respectively) were developed. After staining, the gels were immersed in destaining solution (table 1.5)[10% (v/v) ethanol and 2% (v/v) orthophosphoric acid] with shaking at room temperature until the protein bands or protein spots (in 1D or 2D gels, respectively) were visibly distinct against a clear background. The gels were imaged using a Molecular Imager AlphaImager EC (Alpha Innotech).

**Table 1.4: Preparation of CBB G-250 staining solution for SDS-PAGE**

<table>
<thead>
<tr>
<th>Components</th>
<th>Weight/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled water</td>
<td>193.30 ml</td>
</tr>
<tr>
<td>Aluminium sulphate (5% w/v)</td>
<td>10.00 gm</td>
</tr>
<tr>
<td>Ethanol (1% v/v)</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>CBB G-250 (0.02% w/v)</td>
<td>0.04 gm</td>
</tr>
<tr>
<td>Orthophosphoric acid (2.35% v/v)</td>
<td>4.70 ml</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>200.00 ml</td>
</tr>
</tbody>
</table>

**Table 1.5: Preparation of destaining solution for SDS-PAGE**

<table>
<thead>
<tr>
<th>Components</th>
<th>Weight/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled water</td>
<td>88.00 ml</td>
</tr>
<tr>
<td>Ethanol (2% v/v)</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>Orthophosphoric acid (10% v/v)</td>
<td>10.00 ml</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>100.00 ml</td>
</tr>
</tbody>
</table>
Table 1.6: Preparation of 10X Tris-Glycine-SDS Buffer (TGS)

<table>
<thead>
<tr>
<th>Components</th>
<th>Weight/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>SDS</td>
<td>1 g</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>100.00 ml</strong></td>
</tr>
</tbody>
</table>

The stock was diluted to 1X TGS before use.
3. Results and discussion:

Plants display great diversity with regard to soil salinity tolerance. Species distribution and survival mainly depend on the seed ability to complete germination and the seedling ability to develop successfully under unfavorable conditions (Živković et al., 2007). Seed germination takes the most important part in life cycle of plants (Khan et al., 2000). Most seeds are located near the soil surface. Salt concentration in the soil surface changes over time. Continuous water evaporation causes surface salt deposition (Ungar, 1991), while rain dissolves and washes away salt deposits and provides enough water for germination. In the course of evolution, seeds have adapted to such changes, staying viable at high soil salinity and being able to germinate under appropriate external conditions (Khan and Ungar, 1997). In many plant type, germination and seedling growing phase is very sensitive to salt stress. Germination is described as the rootlet’s coming out of the testa (Coopland and McDonald, 1995). Seeds’ germination begins with water intake but it is decreased by the salt (Othman, 2005).

The groups of plants that are well adapted to saline habitats are called halophytes. Their seeds germinate well in freshwater and the germination is similar to that of seeds of non-adapted species. However, they differ from them in their ability to germinate at higher salt concentrations in the soil. Germination of halophytes in the field is controlled by several environmental factors, in particular light (Huang and Gutterman, 1998), temperature (Badger and Ungar, 1989) and salinity (Ungar, 1995; Khan et al., 2002).

3.1. Germination Percentage

Seed germination of halophytic species is regulated by factors such as water, temperature, light, soil salinity, and their interactions (Noe and Zedler, 2000); however, each species responds to the abiotic environment in a unique manner. Halophytes vary greatly in their ability to tolerate salt. The germination percentage of halophyte was strongly affected by higher salt concentration (0.5 M). On the fourteenth day after sowing, the final germination percentage for each of the different concentrations of NaCl was calculated. Germination percentage was higher in distilled water (control) than in any of the saline treatments. This could be attributed to the fact that salinity is inversely correlated with germination (Khan et al., 2000). Higher germination percentage of
80.33% was observed in seeds treated with distilled water and the seeds treated with 0.1, 0.2, 0.3, 0.4 and 0.5M NaCl exhibited 80.34, 47.05, 39.97, 36.66, 22.58, and 17.36% germination respectively (figure 1). In general, the highest germination percentage occurs under non-salty conditions and it decreases with the ascending salt concentrations (Khan et al., 2000). Similar results were reported in a wide range of halophytes (Joshi et al., 2005; Khan et al., 2006).

Figure 1. Seeds were germinated in petri dishes on filter paper. The values shown are the means of germination percentage determined at different concentration of NaCl for replicates with 25 seeds per treatment of *S. portulacastrum*. The data are the mean ± SD of 3 replicates.

In general, many researchers have concluded that salinity is inhibitory to the germination of halophyte seeds in two ways: (i) causing a complete inhibition of the germination process at salinities beyond the tolerance limits of a species and (ii) delaying the germination of seeds at salinities that cause some stress to seeds but do not prevent germination (Ungar, 1995). The germination responses of seeds of annual halophytes to salinity are highly variable and species specific (Ungar, 1995). According to Rivers and Weber (1971), the seeds of *Salicornia bigelovii* could germinate in up to 1369 mM NaCl. Khan and Weber (1986) described that the germination of the Great Basin halophyte *Salicornia pacifica* var. *uathensis* reduced from 55% in distilled water to 3% germination at 856 mM NaCl. Increase in salinity progressively inhibited germination, and few seeds
of *H. mucronatum* germinated above 300 mmol/L NaCl. These results are concordant with the present study which indicated that high salinity would have a negative impact on seed germination.

3.2. Germination Rate:

The germination rate of seeds at different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5M) of NaCl is illustrated in figure 2. The rate of seed germination followed a concentration dependent trend with respect to salinity. Germination rate decreased with increasing salinity. It was found to be higher (9.90) in the control followed by 0.1, 0.2, 0.3 and 0.4M NaCl. Lowest germination rate of 1.23 was observed in seeds treated with 0.5M NaCl. This could be attributed to the presence of higher concentrations of anions and cations, which interfere with the physiological processes associated with seed germination. (Singah *et al.*, 1988; Khan *et al.*, 2002).

Similar trends have been found in several species of *Atriplex* and in some annual halophytes (Debez *et al.*, 2001). The decreasing tendency of rate of germination due to increasing salinity is in concordance with the reports of others (Khan *et al.*, 1997). Heenan *et al.* (1988) have reported that seed germination shows a negative correlation with osmotic stress induced by high salt concentrations.
3.3. Seedling length (radicle + plumule):

The effect of salinity on the seedling length is illustrated in figure 3. The length of radicle and plumule of seeds decreased on being subjected to stress. With increasing salinity, radicle and plumule length drastically reduced. Maximum seedling length was observed in the control group (4.01cm) and a minimum of 0.82cm was observed in the group treated with 0.5M NaCl. Munns and Termaat (1986) suggested that there exists an inverse relation between salinity and radicle-plumule growth. The growth of the radicle and plumule decreases when salinity is increased. Increase in salinity results in osmotic pressure, which negates plant cell division and differentiation, further leading to reduced water absorbance and hence significance decrease in plumule and radicle length. Saline environment also shows a deterrent impact on the embryo tissue appearance (Khan and Ungar, 1997). Similar results were observed in many plants grown under saline conditions (Amin et al., 1996).

![Figure 3. Effect of different levels of NaCl on Seedling length (radicle + plumule) of S. portulacastrum. The data are the mean ± SD of 3 replicates](image)

In addition, Hajar et al. (1996) noted a gradual decrease in the root growth of *Nigella sativa* L. as the concentration of NaCl increased from 150 mM to 300 mM. Studies have also shown that germinated seeds in saline environments have short root and shoot length, and NaCl has an extreme deterrent effect on the development of embryo rather than other saline elements (Katergi, 1994). It was observed by Younis et al. (2008) that enhancing salinity leads to growth reduction. It also reduces germination amounts and seedling weight. In concurrence with the above reports, reduction in growth (plumule
and radicle length) due to salinity was a common observation in the present study. The reduction may be due to water shortage and ionic toxicity created by salinity (Iqbal et al., 1998). Sodium chloride is well known to inhibit the emergence and elongation of embryonic structures due to accumulation of Na$^+$ and Cl$^-$ (Marcar, 1987) to toxic levels in the germinating seedlings.

3.4. Seed Vigour index

The seed vigour of *S. portulacastrum* decreases in a concentration dependent manner in response to NaCl treatment (figure 4). A maximum of 3.27 seed vigour was observed in control as compared to other groups showing 1.67, 1.33, 1.14, 0.3 and 0.2 when treated with 0.1, 0.2, 0.3, 0.4 and 0.5M NaCl respectively. Generally, rate and percentage of germination and seed vigor index are affected by different ions and reduction of environmental water potential in the presence of salinity. The findings of Kader and Jutzi (2004) showed that considerable increase in salinity (reduction of environmental osmotic potential) leads to decrease in seed characteristics.

![Figure 4. Effect of different concentration of NaCl on seed vigour of *S. portulacastrum*. The data are the mean ± SD of 3 replicates](image)

3.5. Protein profiling from Seedling of *S. portulacastrum*:

The total soluble protein content of seedling decreased in *S. portulacastrum* as a result of NaCl treatment, but without any changes in untreated controls (figure 5). The total protein content of leaf gradually decreased with increasing concentration of NaCl.
This decrease in protein content might be due to the increasing activity of acid and alkaline proteases. As earlier reported, in *B. parviflora* levels of free amino acid increase as a result of salt stress (Parida *et al.*, 2004).

Total proteins were extracted from seedling of control and NaCl treated plants after fourteen days of treatment and analyzed by SDS-PAGE. As visualized from SDS-PAGE gel, two peptides of molecular weight ~40 kDa and ~19 kDa were up-regulated in response to 0.4 M NaCl treatment (figure 6).

Figure 6. Profiling of Seedling protein from *S. portulacastrum* by SDS-PAGE showed differential expression after treatment with varying concentration of NaCl. Lane M: protein marker; lane C: control seed; lane 2-5: increasing concentration of NaCl (100mM, 200mM, 300mM, and 400mM) harvested after 14 days. All lanes were loaded with 10μg protein. An arrow indicates the up-regulation of protein ~40 kDa and ~19 kDa after NaCl treatment.
As discussed by Khajeh-Hosseini et al. (2003), high salinity leads to reduced osmotic potential which prevents the water uptake necessary for the mobilization of the nutrients required for germination. It can also be due to the toxic effects of Na\(^+\) and Cl\(^-\) ions on the germination process.

The present results were consistent with those reported for other species (Ungar, 1991), and indicate that the effect of salinity on initiation of seed germination is variable and cultivar specific. The rapidity in seed germination is particularly important in adverse climatic conditions, where salinity is aggravated by scarce and erratic precipitation. Seeds that germinate without delay can make use of the limited soil water pool to establish seedlings before adverse climatic conditions overcome.

Similar observation about germination speed was made by Akbari et al. (2012), who found that wheat cultivars have relatively rapid germination at moderate salt concentrations. The decrease in seed germination percentage may be attributed to osmotic retention of water or toxicity to the embryo or both (Zekri, 1993; Jamil et al. 2005). They reported that germination of Brassica species (cabbage, cauliflower, canola) decreased as the salinity concentration increased. Other studies on tomato genotypes (Doğan et al., 2008) and varieties (Kemer, Pala and Aydin Siyahı) of eggplant (Akıncı, 2004) showed that the highest germination was observed in control and 50 mM salt applications, however it considerably decreased and with the 100–150 mM salt treatment. In another study, 100 mM salt concentration caused 75% reduction in germination (Jones, 1986) in Lycopersicon esculentum seeds and 150 mM salt concentration caused 90% reduction in cucumber seeds (Passam and Kakouriotis, 1994). The results of another study indicate that salinity stress decreases the cumulative seed germination and delays initiation of germination processes, and that considerable variations exist among the studied cultivars in their ability to avoid or tolerate the damaging effect of saline conditions.

Salinity results in poor stand due to decrease in the rate of seed germination. Presumably, the osmotic effect due to salinity was the main inhibitory factor that reduced germination as indicated by Akbar and Ponnamperuma (1982). According to Etesami and Galeshi (2008), salinity is the cause of reduction in germination percentage, rate and homogeneity of germination and dry weight of barley (Hordeum vulgare) seedling.
Massai et al. (2004) reported that salinity is delaying plant growth under reduction of photosynthesis effects, it is cause of closing stomata and reduction of water entrance into the plant and so that it cause duplicate reduction in plant weight. Redman et al. (1994) observed that the reduction in dry weight of plumule and radicle resulting from increased salinity is a normal phenomenon and probably it is the result of low water absorbance by germinated seeds.

In order to adapt to or tolerate salt stress, plant cells may alter their gene expression resulting in an increase, decrease, induction or total suppression of some stress responsive proteins (Sekiet et al., 2003; Shinozaki et al., 2003). To establish whether or not our salt stress treatment was within the physiological range of this study experimental system, investigation on the effect of salinity on the expression of stress responsive protein is warranted and studies of this type are essential for selection of cultivars with seed characteristics acceptable for plantation establishment in salt-affected area.
PART-3

Diversity of bacterial communities associated with the halophyte,
*Sesuvium portulacastrum* L.

1. Introduction

One of the biggest challenges in agriculture nowadays is to increase yield and sustainability of crop production as the global population is booming and is going to reach nine billion people by 2050 (Evans, 2013). According to projections of the Food and Agriculture Organization of the United Nations (FAO), the demand for cereals will increase by approx three fourth of the present demand, and will double in developing countries in the coming years (FAO. World agriculture, 2013). To increase the yield of basic food grains, additional inputs for crop production are needed and new technologies are essential for managing soil nutrients as well as crop pests and diseases.

Soil quality is believed to be an integrative indicator of environmental quality, food security, and economic viability (Herrick, 2000). Soil quality is defined as “the ability of a specific kind of soil to function, within natural succeeded ecosystem boundaries, to sustain plant and animal efficiency, maintain or improve water and air quality, and maintain human health and habitation” (Karlen *et al.*, 1997). Therefore, the evaluation of soil quality will be closely associated with the role for which the soil was selected. Thorough soil exploitation particularly for agriculture has led to physical, chemical, and biological changes. Soil erosion is cooperating with plant health and sustainability of crops. Soil quality has been decreasing dramatically due to the exacerbated anthropogenic disruption that accelerates the degradation and desertification of soils (Lal, 2001).

Another problem is soil deficiencies, which are becoming increasingly common and may reduce significantly plant growth and crop yields (White and Brown, 2010). Generally plants require at least 17 different minerals for adequate nutrition. There are many factors including soil, plant species, microbial interactions, and environment that can affect the acquisition of these nutrients.

In case of microbial interactions, microorganisms numerically dominate terrestrial biodiversity, and play important biochemical and geochemical roles in the environments
they inhabit. Microorganisms are a great source of genetic diversity, still far from completely known and explored (Prosser et al., 2007). To understand structure and function of complex ecosystems, it is necessary to identify primary drivers of microbial diversity and its community structure. In nature, bacteria are mainly found in association with different species, composing bacterial communities. These communities inhabit all terrestrial niches, colonizing environments such as soil, water, air, plants and animals. Bacteria are the main portion of biomass on Earth, and are responsible for essential processes for life such as cycling of carbon, nitrogen and sulfur. Hence, additional to bacterial species diversity, there is intra-specific diversity. The bacterial genome is characterized by the total number of genes found in strains, which can be divided into two groups:

(i) The core, composed of the group of genes found in at least 95% of strains and are essential for the cell’s life cycle; and

(ii) The auxiliary group, found in a maximum of 5% of strains, and responsible for species adaptation in different environments (Konstantinidis et al., 2006).

The core is maintained in species by speciation and vertical transmission, while the auxiliary group does not identify the species, since it is different in every strain. This last group of genes is also transmitted from strain to strain and even between species by horizontal gene transfers (Boucher et al., 2001).

This concept clearly demonstrates that bacterial diversity is not static, due to the high reproduction capacity associated with the short life cycle and high cell multiplication rates, which leads to the high adaptation value, and fast responses to environmental change (Abby and Daubin, 2007; Konstantinidis et al., 2006).

Historically, bacterial sampling has been analyzed indirectly via the Limulus Amebocyte Lysate Endotoxin Assay (LAL) and the Recombinant Factor C (rFC) endotoxins assay. The culture method has been a long-standing gold standard for direct enumeration. However, the culture method is restricted to viable cultivation; the researcher is limited to selected media and is unable to culture the majority of microorganisms (Nonnenmann 2010). Therefore, traditional means of microbial quantification are limited and non-culturable methods have been recommended (Nonnenmann 2010; Poole 2010).
A wide diversity of bacteria can interact with plants, composing bacterial communities with important roles in plant development and health status (Hallmann et al., 1997). These interactions can vary according to the host plant in a process similar to those widely known for pathogenic microorganisms (Salvaudon and Shykoff, 2008).

This wide distribution is driven by plant development that carries bacteria over the plant tissues (Prieto and Mercado-Blanco, 2008). Chi et al. (2005) presented that similar bacteria were distributed over the rice plant, from roots to leaves. However, the abundance of bacterial types along the different niches can differ, mainly due to differences in these niches in nutrient supply, atmospheric conditions and competitiveness with other components of these communities (Rao et al., 2006). The behavior of these populations and how they colonize plants is determined by environmental conditions, like formation of biofilms that help bacteria fix to cell walls, avoiding the migration driven by sieve transportation. Similarly, in the parenchyma region, being single-celled can enable better contact with cells and so better nutritional supply for the bacterium.

The rhizosphere, commonly described as the soil portion is directly influenced by root exudates; however, an updated definition of rhizosphere considers it as the soil compartment influenced by the root, including the root itself (Hartmann et al., 2008). The rhizosphere is one of the most complex environments with thousands of interactions that play vital role in plant health. Plants secrete up to 40% of photosynthates that reach the roots into the rhizosphere (Berendsen et al., 2012). Because most of the soils are carbon deficient, these hot spots of carbon increase the microbial densities from 10 to 1000 times, compared to bulk soil (Smalla et al., 2006). The elevated concentration of microorganisms in this particular region is due to an exchange of nutrients between the plant and the different communities surrounding the root, which allows different types of associations (Figure 1). A number of factors have been shown to influence the quantity and quality of root exudates including plant species (Dennis et al., 2010), soil type (Bulgarelli et al., 2012; Berg and Smalla, 2009), developmental stage (Houlden, 2008), and nutritional status (Carvalhais, 2011). If specific elements associated to the release of such exudates are better understood, novel approaches to enhance beneficial microbial communities could be proposed.
Bacterial populations are distributed in the rhizosphere as epiphytic and endophytic communities. Epiphytic and endophytic bacteria are characterized by the colonization of surface and inner tissues of plants, respectively. There is an ongoing discussion toward a better definition of these microorganisms; a commonly used definition of endophytes is those whose isolates form on surface-disinfected plant tissues (Hallmann et al., 1997). However, in addition to these definitions is the separation of endophytes according to their essentiality in niche occupations. In that case, the endophytic community is divided into “passenger” endophytes, i.e. bacteria that eventually invade internal plant tissues by stochastic events and “true” endophytes, those with adaptive traits enabling them to strictly live in association with the plant (Hardoim et al., 2008).

The cells in the rhizosphere, plant-surface or endophyte communities are variable. A superficial analysis of these communities could lead to the conclusion that there is a strict specificity for niche colonization. However, a more realistic scene is represented by the gradient of population distribution along plants. If a didactic approach is applied to explain bacterial communities associated with plants, it would divide these bacteria into distinct communities, with separation between epiphytic and endophytic communities in accordance with plant organs, such as roots, stems and leaves. However, in nature the gradient of distribution will prevail over separation. It is important to note that bacteria in the rhizosphere are often similar to those in the endophytic community and on leaf surfaces.

Microbial societies play an important role in nutrient cycling by mineralizing and decomposing organic material, which are released into the soil as nutrients that are needed for plant growth. These communities can influence nutrient accessibility by solubilization, chelation, and oxidation/reduction processes. In addition, soil microorganisms may affect nutrient uptake and plant growth by the discharge of growth stimulating or inhibiting materials that influence root physiology and root structural design. It has been suggested that microbial inoculants are promising components for integrated solutions to agro-environmental problems because inoculants possess the capacity to promote plant growth (Compant et al., 2010; Lugtenberg and Kamilova,
enhance nutrient availability and uptake (Adesemoye and Kloeper, 2009; Yang et al., 2009), and improve plant health (Berendsen et al., 2012).

Recent studies have revealed that plants are able to shape their rhizosphere microbiome (Berendsen et al., 2012; Lundberg et al., 2012; Badri and Vivanco, 2009). Some plant species have been demonstrated to host specific communities and attract protective microorganisms to suppress pathogens in the rhizosphere (Mendes et al., 2011). Soil physical, chemical, and biological properties will also play an important role in the establishment of such plant-microbe interactions (Berendsen et al., 2012). Although pathogens can severely affect plant health, certain beneficial bacteria and fungi that also thrive in the rhizosphere, or inside plant tissues, also known as endophytes, can compete with these pathogens for space and nutrients, therefore exert an antagonistic effect on them (Nihorimbere et al., 2011; Raaijmakers et al., 2009). Root-associated beneficial soil bacteria are generally known as Plant Growth Promoting Rhizobacteria (PGPR).

PGPR grow in, on, or around root plant tissue and enhance plant growth, increase yield, protect plant against pathogens, and/or reduce abiotic or biotic stress (Vessey, 2003). Growth promotion can be achieved directly by the interaction between the microbe and the host, as well as indirectly, due to antagonistic activities against plant pathogens. Various interacting microbes produce phytohormones, which have been shown to inhibit or promote root growth, protect plants against biotic or abiotic stress, and improve nutrient acquisition by roots (Davies, 2010; Berg, 2009). PGPR represent an environmentally sustainable alternative to increase crop production and plant health as they have the potential to at least partially replace chemical fertilizers and pesticides and their use may then be reduced (Figure 1). An interesting example of the role of microbial communities in plant nutrition and health is the interaction between rhizosphere fluorescent pseudomonas and plants (Vivian et al., 2013).
Plants reduce soil iron (Fe) availability by acquiring iron and releasing exudates which attract the rhizosphere microbes that also utilize Fe. In Fe-stressed environment, siderophore-producing bacterial populations are enriched, which then suppress pathogens such as fungi and oomycetes through competition for Fe. The plants, however, are able to utilize siderophores-bound iron, which enhances their growth (Lemanceau et al., 2013). Another instance applied to plant disease suppression is the ability of resident microbiota in suppressive soils or compost to prevent pathogen infection (Hadar, 2012). In a soil suppressive to the fungal pathogen, *Rhizoctonia solani*, Proteobacteria, Firmicutes, and Actinobacteria were prominent taxa found to be involved in disease suppression (Mendes et al., 2011). There is also evidence to suggest that plants may use microbial communities to their own benefit to avoid infections (Mendes et al., 2011).
The presence of potentially toxic compounds, low availability of essential minerals and pathogens in the soil often restrict crop production. To address these issues, numerous studies have focused on specific genes from plants and microbes that are involved in nutrient uptake and defense against pathogens (Damiani et al., 2012; Dechorgnat et al., 2012; Hann and Boller, 2012). Different molecular techniques have been used to conduct these studies. These methods range from DNA-based techniques (López-Fuentes et al., 2012; Doornbos et al., 2011), microscopic observation of labeled microorganisms colonizing roots (Compant et al., 2011; Lecomte et al., 2011), and incorporation of labeled nutrient substrates (Pellegrini et al., 2012; Marschner et al., 2011; Jones et al., 2009).

Using molecular methods to address research questions in soil environments is often challenging given the intrinsic characteristics of soil samples. The most common problems include presence of enzyme-inhibiting organic compounds such as humic and fulvic acids, as well as low extraction yields due to adsorption of nucleic acids to soil particles, incomplete cell lysis, and DNase and RNase contamination (Opel et al., 2010; Arbeli and Fuentes, 2007). Extraction methods using bead-beating are the most used and they are shown to be so far the most efficient to overcome the problem of adsorption of nucleic acids to soil particles (Lakay et al., 2007). RNA-based studies are even more challenging because of the higher instability of RNA molecules compared to DNA. The ubiquity and stability of RNases make it difficult to obtain RNase-free environments. In addition, often mRNA is fragmented even before cell lysis, because of simultaneous transcription and translation occurring in archaeal and bacterial cells (Proshkin et al., 2010). For approaches that focus on mRNA, such as microarray and metatranscriptomics analysis, often an rRNA subtraction step is advised as only up to 5% of extracted total RNA is comprised of mRNA. For this purpose, several methods have been developed and a comprehensive review describing methods and alternatives to deal with most methodological problems can be found elsewhere [Carvalhais et al., 2012].

Recent developments in non-culture analytical tools have greatly expanded bacterial quantification and identification. Of those molecular based methods, pyrosequencing has proven a novel non-culturable technology that could be used to not only measure the biodiversity of microorganisms, but also to characterize exposure to
these microorganisms in occupational settings (Nonnenmann, 2010). Pyrosequencing has broken the barrier of sequence limitations in study of bacterial diversity. With the ability to generate megabases of sequences in a few hours, it allows deep exploration of species in any environmental sample (Edwards et al., 2006). This technique differs from Sanger methodology, which is based on incorporation and further detection of fluorescently labeled dNTP (dideoxynucleotide triphosphate). During pyrosequencing, only one dNTP (deoxynucleotide triphosphate) is available at a time, and incorporation of this nucleotide generates the signal detected by the equipment (Margulies et al., 2005; Ronaghi et al., 1996). The signal is emitted once complementarity is achieved and the base is incorporated; then a reaction is catalyzed and pyrophosphate is released, activating ATP sulfurlase, producing energy to the luciferase to convert luciferin into oxyluciferin, releasing the light signal. The bacterial tag-encoded flexible (FLX) amplicon pyrosequencing (bTEFAP) approach utilizes the ribosomal DNA 16s gene for phylogenetic analysis. These highly conserved structures can be used to identify individual genera or species of bacteria from varied and diverse samples.

In addition to being an exceptionally sensitive method, pyrosequencing is relatively low in cost and allows for a relatively quick turnaround in the identification, distribution and concentration of bioaerosols (Nonnenmann, 2010). However, reliable sampling methods in use with pyrosequencing have yet to be investigated. This technique has been used to describe bacterial communities in different environments. The deep ocean biosphere was described by pyrosequencing of samples collected at different depths (Sogin et al., 2006), and soil bacterial communities (Roesch et al., 2007) were similarly investigated. Both studies showed that diversity of these organisms was extremely high, and although 30,000 sequences were obtained, the complete description of species and sequences in both environments were not completed.

In the present study, the microbial biodiversity in wild soil, as well as in the greenhouse soil, which was more tightly root-adhering soil, the rhizosphere of halophyte Sesuvium portulacastrum L. were analyzed. To this end two soil samples were collected for analyzed physico-chemical parameter as well as total DNA extracted and used for a PCR-based 16S rDNA gene diversity survey of microbial communities.
2. Material and Methods

I. Physico-Chemical Parameters

2.1. Sample collection and Analysis

The soils strongly adhering to the roots and are within the space explored by the roots are considered as the rhizosphere soil (García et al., 2005). In this study, the rhizosphere soil samples of the halophyte *Sesuvium portulacastrum* were collected from the greenhouse condition (S1) and the wild (S2) during 2012. For the wild rhizosphere, soil samples were collected in fresh polythene bags using pre-sterilized spatula. The sample was transported in an ice box to the laboratory as soon as possible without any contamination for further analysis. Each soil sample was divided into two parts, one for the estimation of its physico-chemical parameters and the other for the study of microbial diversity through pyrosequencing.

2.2. Determination of salinity

The salinity alone was immediately tested from soil samples. One gram of soil from each sample was weighed into a clean sterile 50 ml centrifuge tube and 20 ml of Millipore Milli-Q Ultrapure water (Merck) was added to the tubes. The tubes were kept in a shaker at 140 rpm for one hour at room temperature. 20 ml of water from each sample tube was transferred to a beaker and the salinity was measured with Extech EC400 Water proof ExStik Salinity Meter. The salinity, TDS (total dissolved solids) and conductivity for all sediment samples were recorded and displayed.

2.3. pH determination

One gram of soil sample was weighed into a clean sterile 15 ml centrifuge tube and 1 ml of water from a Millipore Milli-Q® Ultrapure Water Purification System (Merck) was added to each tube and shaken well for 10 mins. pH tutor Tester (Eutuch instrument) was used to determine the pH of the soil sample. pH probe was placed into the centrifuge tube to obtain a pH reading from each sample. Duplicate readings were acquired for samples to determine if there were significant differences between each reading.
2.4. Ion and metal analysis

Ions were extracted from the desiccated leaves tissues with 0.5% (v/v) nitric acid. The concentration of Cd, Cu, Zn, Ca$^{2+}$, Na$^+$ and K$^+$ were measured by inductively coupled plasma optical emission spectrometer (Optima 2000DV, Perkin Elmer, Germany). Ion content was determined via the method described by Shukla et al., (2011). Plant tissue (0.2 g) was digested in 4 ml of a solution of perchloric and nitric acids (3:1). The solution was dried on a hot plate at 90 °C and reconstituted to 25 ml with Millipore Milli-Q Ultrapure water (Merck) before filtration through a 0.2 μm microfiber filter and transferred into clean vial and aspirated into a metal analyzer.

II. Culture-Independent bacterial communities from Rhizosphere soil associated with halophyte

2.5. Extraction of eDNA from rhizosphere soil

Extraction of eDNA from soil sample was extracted according to the procedure described by Shivaji et al., (2004). One gram of the soil was taken in a conical flask containing 5 ml of 120 mM sodium phosphate buffer (pH 8) and incubated for 2 hrs at 150 rpm on a shaker at room temperature. The slurry was spun at 6000 rpm for 10 min and the pellet washed with phosphate buffer twice. The washed pellet was re-suspended in 5 ml of lysis buffer (0.15 M NaCl, 0.1 M EDTA, pH 8) containing 30 μg/ml lysozyme and incubated at 37°C for 2 hr in a shaking water bath. Subsequently, 2 ml of 0.1 M NaCl prepared in 0.5 M Tris-HCl (pH 8) buffer and 10% SDS was added such that the final concentration of SDS was 1%. The contents were then subjected to five freeze-thaw cycles by freezing the samples in liquid nitrogen and thawing at 65°C, so as to release the DNA from the bacterial cells in the soil. De-proteinization was done once with tris-saturated phenol and then with chloroform: isoamyl alcohol mixture (24:1, v/v). The aqueous phase was collected and DNA was precipitated with 2% sodium acetate and 5 ml of isopropanol. The DNA pellet was recovered by centrifugation at 13000 rpm for 20 min at room temperature, washed with 70% alcohol, air dried at room temperature and dissolved in 50 μl of DNase and RNase free water (Life technology, Invitrogen).

2.6. Purification of eDNA

The eDNA was purified by PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, USA) following the protocol given along with the kit. This clean-Up Kit
was utilized for cleaning up isolated genomic DNA. Starting DNA may be amber to brown in appearance; an indicator of PCR inhibiting substances, particularly humic and polyphenols. Even samples that appear colorless may contain PCR inhibitors which can be cleaned up with this kit. This Clean-Up Kit removes the brown color as well as any PCR inhibiting substances, such as heme, polysaccharides, polyphenols fulvic acids and dyes. This kit was validated with DNA isolated from a variety of problematic soils and also with artificially spiked DNA samples with commercial humic acids.

2.7. Bacterial tag-encoded amplicon 454 Pyrosequencing

Tag-Pyrosequencing was performed in the Research and Testing Laboratory (Lubbock, TX, USA). Samples were amplified for pyrosequencing using a forward and reverse fusion primer, and the amplicons were sequenced using Roche 454 Titanium chemistry, producing reads from the forward direction from 939F. Sequences were quality trimmed according to Acosta-Martínez et al. (2008).

Targeted DNA in each sample was sequenced using the Roche 454 Life Sciences sequencer for the identification of the bacterial species present. The forward and reverse primers were used to detect and amplify the target sequence in each sample. The samples were differentiated from each other when the run was performed on the 454 sequencer by a "tag", a unique identifying sequence attached to the forward primer implemented when the targeted sequence is amplified using this PCR procedure. The forward primer was constructed with the Roche A linker (5’-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3’), and reverse fusion primer was constructed with a biotin molecule, the Roche B linker (5’-CCTATCCCTGCTGCTTGAGTGACGCTCAG-3’). Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1µl of each 5 µM primer, and 1 µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, California) under the following thermal profile: 95 °C for 5 minutes, 35 cycles of denaturing at 94°C for 30 sec, annealing at 54 °C for 40 sec, 72 °C for 1 minute, extension of 10 minutes at 72 °C and 4 °C hold(on completion). Amplification products were visualized under eGels gel documentation system (Life Technologies, Grand Island, New York).
After the sample DNA was amplified and each sample had its own unique sequence tag, the sample DNA was pooled together for downstream applications. Each sample was pooled in different amounts into a single tube based on the band strength determined when running the samples on the eGels (Life Technologies, Grand Island, New York). An equal ratio of DNA was necessary for quality control to ensure that the varying sequences of each sample attached to their unique tag was present at an equal ratio when amplified a second time during emulsion PCR (emPCR) and that the samples which had a strong amplification band do not have more reads than the weak amplified samples when they were placed on the 454 Life Sciences Sequencer (Branford, Connecticut).

The pooled DNA was purified by removing small fragments with a size less than 200 base pairs like primer dimmers using Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York) and Ampure beads. The Ampure beads help to eliminate fragments of DNA that are not between 550 to 650 base pairs in length that could be amplified in emPCR and any contaminants that are not bound to a tag. After washing and drying of the DNA bound beads, the DNA was eluted from the Ampure beads into a buffer and was implemented in downstream applications.

Each region with approximately 150ng of DNA was added to the immobilization beads. This amount of DNA was proportional to the amount of immobilization beads utilized. DNA bound to the immobilization beads was made single-stranded by using a "melt" solution. One strand of the double-stranded DNA remain bound to the immobilization beads, the second strand being reconstituted in the melt solution. The melt solution had a basic pH, and the neutralization solution was added to return the DNA in solution to a neutral pH. The Qiagen mini-elute kit was used for purifying the single stranded DNA. The single stranded DNA was measured with fluorometer for downstream applications. The dilutions made in this procedure were used to add the appropriate amount of DNA to the amount of beads used in emPCR.

The single stranded DNA attached to the DNA Capture beads was amplified during emPCR and the beads were recovered for downstream applications. The beads for each region to be sequenced by the Roche 454 Sequencer were removed from the plate using several washes with isopropanol and enhancing fluid (Titanium Bead Recovery
Reagents). Once collected, the DNA attached to the DNA capture beads accumulated in filter were washed several times. Ideally, each DNA capture bead attached several copies of one strand of sample DNA to it. After bead recovery, the DNA capture beads were treated with melt solution to make the captured double-stranded DNA into single-stranded DNA. The addition of an enrichment primer attached the DNA capture beads with DNA to enrichment beads. The enrichment beads have magnetic property, allowing for a Magnetic Particle Collector to separate the DNA capture beads with amplified DNA from DNA capture beads that have incomplete amplified DNA or no bound DNA. A second addition of melt solution was removing the enrichment beads from the DNA capture beads bound by DNA. The DNA was single stranded, attached to the DNA capture beads, and ready for sequencing.

The PicoTiter plate and DNA bound capture beads were prepared before a sequencing run. The PicoTiter plate was allowed to sit in the designated buffer before loading with the different sequencing layers for at least 10 minutes to allow for all of the wells on the plate to be filled. The measured beads for each region were also prepared by the addition of Control Beads and Packing Beads. The Control Beads was added to the DNA bound capture beads in a constant quantity. The Packing Beads hold the DNA and Control beads in the wells on the plate. The Enzyme and Ppiase beads were components in the layers that pack the DNA in each region. The Enzyme Beads contain the enzyme components in each region to carry out sequencing. The Ppiase beads reduce cross contamination between wells during the nucleotide flow.

2.8. Analysis of Pyrosequencing data

2.8.1. SILVAngs pipeline analysis

The SILVAngs pipeline analysis is primarily used for the analysis of large scale small and large subunit (LSU/SSU) ribosomal RNA (rRNA) gene, tag sequencing projects but can also be used for meta-genomic studies. Each project normally includes thousands to millions of reads from many different samples produced by massive parallel high-throughput “next generation” sequencing (NGS) technologies. Each read is aligned, quality checked, and classified based on the SILVA Reference alignment and taxonomy. Intuitive graphical outputs are provided for statistical information about the taxonomical
distribution of the reads within and across samples. Interactive tax breakdowns are available for detailed inspection of the diversity in the samples.

The sequences reads were processed by the NGS analysis pipeline where the sequences were classified in SILVA rRNA gene database project (SILVAngs 1.2) (Quast et al., 2013). Each read was aligned using the SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). Reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations and artifacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream analysis.

After these initial steps of quality control, identical reads were identified (dereplication), the unique reads were clustered into operational taxonomic units (OTUs), on a per sample basis, and the reference read of each OTUs was classified. Dereplication and clustering was done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (Li and Godzik, 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 119; http://www.arb-silva.de) using blastn (version 2.2.28+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Camacho et al., 2009).

The classification of each OTUs reference read was mapped onto all reads that were assigned to the respective OTUS. This yields quantative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as, multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function “(sequence identity + alignment coverage)/2” did not exceed the value of 93, remain unclassified. These reads were assigned to the meta group “No Relative” in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011).
2.8.2. **VITCOMIC Genera level Identification**

The bacterial populations were classified at their appropriate taxonomic level by BLASTN analysis performed through the web tool **VI**sualization tool for T**axonomic CO**mpositions of **MI**crobial **CO**mmunity, VITCOMIC (Mori *et al*., 2010). The total BLAST score was calculated against each sequence of the reference database ([http://mg.bio.titech.ac.jp/vitcomic/](http://mg.bio.titech.ac.jp/vitcomic/)) to identify the nearest relative of the sample sequence in order to classify at species level. In the **VITCOMIC web based classification of sequence**, each species name in the reference database is placed in circles with ordered phylogenetic relatedness. Physical distances between nearest species in the plot indicate genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80%, followed by 85, 90, 95 and 100 % identity to the database sequence). Each dot represents average similarities of each sequence against the nearest relative species in the reference dataset. The size of these dots indicates the relative abundance of sequences in the sample. The VITCOMIC plot contains four categories of dot size that indicate the relative abundance of the sample sequence. The reads represented by the largest dot.
3. Results and Discussions:

The rhizosphere microbial diversity of soil surrounding a plant root is influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. Root exudates include amino acids, organic acids, carbohydrates, sugars, vitamins, mucilage and proteins. The exudates act as messengers that stimulate biological and physical interactions between roots and soil organisms. They modify the biochemical and physical properties of the rhizosphere and contribute to root growth and plant survival. High levels of moisture and nutrients in the rhizosphere attract much greater numbers of microorganisms than elsewhere in the soil. The composition and pattern of root exudates affect microbial activity and population numbers which, in turn, affect other soil organisms that share this environment. Roots progressing in soil introduce labile carbon and nutrients while creating water ways and deposits of antimicrobial compounds and hormones (Brimecombe et al., 2001; Bringhurst et al., 2001; Hawkes et al., 2007) in time (hours or days) (Lubeck et al., 2000). As many soil microbes exhibit limitations to carbon (Paul and Clark, 1996), they could be expected to respond quickly to root induced changes, by modifying their activity (Heijnen et al., 1995; Herman et al., 2006).

The microbial communities of the soil perform a fundamental role in cycling nutrients, in the volume of organic matter in the soil and in maintaining plant productivity. Thus it is important to understand the microbial response to environmental stress, such as high concentrations of heavy metals or salts, fire and the water content of the soil. Stress can be detrimental for sensitive microorganisms and decrease the activity of surviving cells, due to the metabolic load imposed by the need for stress tolerance mechanisms (Schimel et al, 2007; Yuan et al., 2007, Ibekwe et al., 2010; Chowdhury, 2011).

3.1. Salinity

The salinity of rhizosphere soil of *Sesuvium portulacastrum* varied in both the greenhouse (S1) and wild (S2) conditions. The maximum salinity (29.12 ppt) was recorded in the S2 whereas it was very low (0.5 ppt) in S1. The result is plotted and displayed in figure 2.
The detrimental influence of salinity on the microbial soil communities and their activities has been reported earlier in majority of studies (Rietz & Haynes, 2003; Sardinha et al., 2003). Wong et al. (2008) evaluated the effects of salinity on the microbial biomass which exhibited high rates of respiration in soils with low salinity and vice versa. The composition of the microbial community may be affected by salinity (Gennari et al., 2007; Llamas et al., 2008; Chowdhury et al., 2011) since the microbial genotypes differ in their tolerance of a low osmotic potential (Mandeel, 2006; Llamas et al., 2008).

3.2. Determination of pH

The pH value of greenhouse rhizosphere soil (9.19 ± 0.015) was higher than the wild rhizosphere soil (8.89 ± 0.045). The duncon’s test illustrates a significant difference in pH (Figure 3) of the samples collected from the above mentioned site.
The soil pH in the rhizosphere of both conditions was alkaline in nature (ranging from 8.89 to 9.19), with slightly higher pH (9.19) in the greenhouse soil. Augusto et al. (2002) found the basification of soil is due to the halophyte plants. This trend was observed by Jobbágy and Jackson (2003) and Sinha et al. (2009) and has been attributed to release of H\textsuperscript{+} ions from the respiration of plant roots/soil microorganisms (Hinsinger et al., 2006) or release of acidic exudates in the rhizosphere (Hagen-Thorn et al., 2004). The soil characteristics like pH may be the most important factor determining the dominant bacterial populations in soil (Felske and Akkermans, 1998; Kowalchuk et al., 2000), while the microbial communities found in the rhizosphere may be considered.

### 3.3 Ion and metal analysis

Both the samples were subjected to three ions (Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}) and three metals (Cd, Cu, Zn) analyses employing ICP spectrometry. The result showed higher concentration of calcium than other ions and metals in both samples; the concentration was 118.03 ppm in S2 and 59.78 ppm in S1. The second highest concentration was sodium which was 50.65 ppm in S2 and was 13.17 ppm in S2. The potassium concentration was 33.84 ppm in S2 and 18.4 ppm in S1. Other metals like zinc, copper and cadmium were recorded in less concentration in both samples and shown in Figure 4.

![Figure 4. Heavy metal concentration in the greenhouse (S1) and wild (S2) samples in that Ca concentration is more in wild rather than greenhouse and very low concentration was Cu.](image-url)
The microbes of the rhizosphere soil is playing a key role in the availability of nutrients. Lin et al. (2006) observed that the bacteria, actinobacteria and fungi increased 2.3, 4.3 and 71 times, respectively during the investigation with drip irrigation after planting *Suaeda salsa* L. in coastal saline soil.

### 3.4. Isolation of Environment DNA (eDNA)

The isolated eDNA was checked in 1% Agarose gel electrophoresis (Fig. 5.a.). Both the samples (S1 and S2) showed clear bright bands in ~23 kb region corresponding with negative control. The quality and quantity of eDNA were analyzed in Nanodrop ND1000 which showed 280.6 ng for S1 and 311.38 for S2.

### 3.5. Purification of eDNA

The purified eDNA samples were analyzed in 1% Agarose gel electrophoresis (Fig. 5.b.). They were quantified in Nanodrop ND1000 of the ratio of 260/280 and 230/260. The result indicated less impurities as shown in figure 5.b. The quantity of the eDNA recovered was 198.6 ng in S1 and 264.97 ng in S2.

**Figure 5. a.** 1% Agarose gel shows the presence of eDNA isolated: Lane1-1 kb Marker, Lane 2- eDNA isolated from greenhouse (S1), Lane 3- eDNA isolated from wild (S2) and Lane 4- Control. **Figure 5. b.** 1% Agarose gel showing Purified eDNA band: Lane 1- Marker Lane 2- greenhouse (S1) purified eDNA, Lane 3- wild (S2) purified eDNA, Lane 4- Control.
3.6. Data analysis of 454 Pyrosequencing through SILVAngs pipeline

The sequences reads were processed by the NGS analysis pipeline where the sequences were classified in SILVA rRNA gene database project (SILVAngs 1.2) (Quast et al., 2013). The analysis reveal a total of 4415 sequences (reads) were obtained from the 2 samples after thorough quality check and chimera check, of which only five numbers (0.11%) of sequences were not matched. The aligned sequences had a total minimum length of 243 bp and maximum of 613 bp and subjected to reference analyses (Fig.6 & 7). The information on clustering depends on operational taxonomic units (OTUs). The exhibited OUT was 47.95% OTUs in S1 and S2; 43.92% clustered sequences and 8.02% replicates. The sequences were submitted to the NCBI SRA and accession numbers (SRS735121 and SRS735123) were obtained. More than 91.73 % sequences were classified as bacteria and 8.15% has “No Relative” sequences; so they were not classified as bacteria. The read numbers were uneven, ranging from 2176 for S1 and 2239 for S2. The reads were classified in silva-classifier to assign lowest possible systematic position accompanied with bootstrap-like confidence values (Wang Q et al., 2007). Overall, 21 phyla in S1 and 23 phyla in S2 were recorded using this analysis.

Figure 6. The total aligned sequence length of greenhouse (S1) and wild (S2)
The total number of sequences, total sequence quality (OTUs-References), total sequences alignment quality, alignment quality in OTUs-References, total number of identified aligned sequences and total identified aligned sequences in OTUs were presented in figures 8 to 13.
Figure 9. The total sequence quality of greenhouse (S1) and wild (S2) on basis of OTUs

Figure 10. The total quality of aligned sequences of greenhouse (S1) and wild (S2)
Figure 11. The total quality of aligned sequences of greenhouse (S1) and wild (S2) on the OTUs References

Figure 12. The total number of aligned identically similar sequences of greenhouse (S1) and wild (S2)
3.7. Bacterial community composition

3.7.1. Silva Classifier

The Silva classifier revealed a total of 21 phyla in S1 and 23 phyla in S2 showed the bacterial diversity on S1 was dominated with the phylum Proteobacteria (40%), followed by Acidobacteria (30%), Verrucomicrobia (8%), Bacteroidetes (5%), Planctomycetes (5%), Chloroflexi (4%) and Actinobacteria (2%). Other phyla included Spirochaetae, SM2F1, Nitrospirae, Gemmatimonadetes, Firmicutes, Elusimicrobia, Cyanobacteria, Fibrobacteres, Candidate division WS3, Candidate division TM7, Candidate division OD1, BD1, and Armatimonadetes which represented the load of ≤1%. The percentage of phylum abundance represented in Krona chart (Ondov et al., 2011) is been presented in figure 14.

The S2 sample showed slightly different patterns of diversity and distribution of phyla compared to S1 sample. From the total of 2239 sequences, 23 phyla were identified using silva classifier. The dominant phylum in S1 was Proteobacteria (55%) followed by
other phyla like Bacteroidetes (14%), Chloroflexi (7%), Verrucomicrobia (6%), Gemmatimonadetes (5%), Actinobacteria (3%), Planctomycetes (3%), Acidobacteria (2%) and Cyanobacteria (2%). In addition other phyla including Spirochaetae, SM2F11, Nitrospirae, NPL-UPA2, Lentisphaerae, Firmicutes, Fibrobacteres, Deinococcus-Thermus, Deferribacteres, Candidate division WS3, Candidate division TM7, Candidate division OD1, Candidate division BRC1 and BD1 were also present (≤1%). The identified phyla percentages determined with Krona chart (Ondov et al., 2011) are showed in figure. 15. The identified taxonomic fingerprint at Phylum Level of the samples S1 and S2 are given in figure 16 and for class basis it was presented in figure 17 for S1 sample and figure 18 for S2 sample.

The majority of the bacteria fell under the phylum, Proteobacteria followed by Acidobacteria, Verrucomicrobia and Bacteroidetes. Previous studies have also demonstrated that the bacterial community in the rhizosphere is generally represented by these four phylotypes (Chowdhury et al., 2009) and Proteobacteria represent the largest and metabolically diverse group of soil microbes (Anne et al., 2009).

The application of cultivation-independent techniques are used as routine tools to depict the overall microbiome composition in environmental samples which infer the factors influence the abundance and distribution of specific microbial taxa (Fisher and Triplett, 1999). Similarly, bacterial distribution in 33 phyla distribution were previously witnessed in the hypersaline mat systems of Guerrero Negro, Mexico (Ley et al., 2006), Eilat, Israel (Sorensen et al., 2005) and Salin-de-Giraud, France (Mouné et al., 2003). The Proteobacteria are commonly observed in waters and sediments from other saline and alkaline lakes (Demergasso et al., 2004; Grant et al., 2004). In the rhizosphere niche, 21 phyla in sample S1, and 20 phyla in sample S2, were recorded, which were similar to that reported in agricultural and forest soil samples (Roesch et al., 2007; Fulthorpe et al., 2008; Uroz et al., 2010).

In a complete review of the dominant soil bacterial taxa using 16S rRNA gene libraries (Janssen, 2006), members of the phylum Proteobacteria represented an average of 39% (range, 10–77%) of libraries constructed from soil bacterial communities, and the members of the phylum Acidobacteria made up an average of 20% (range, 5–46%). The phylum Proteobacteria, which is metabolically versatile and genetically diverse,
comprises the largest fraction of the bacterial community in soil ecosystems, including the rhizosphere (Filion et al., 2004; Sanguin et al., 2006). Even in other natural and human-made ecosystems (e.g. marine, freshwater, wastewater, hot spring microbial mats, and the oral cavity), the phylum Proteobacteria is more dominant than the phylum Acidobacteria (LaPara et al., 2000; Layton et al., 2000; Sievert et al., 2000; Smit et al., 2001; Paster et al., 2002; Martiny et al., 2003; Polymenakou et al., 2005; Penn et al., 2006).

In present study, phylum Acidobacteria (30%) was second dominant after Proteobacteria in the sample S1 but in case of sample S2 it was present at a very low percentage as those regions are conducive for the growth of halophytes. Sang-Hoon Lee et al. (2008) suggested that phylum Acidobacteria might be numerically dominant as well as metabolically active in the soil sample, implying that this phylum might be highly involved in the biogeochemical cycles of the rhizosphere soil.
Figure 14. Bacterial community profile in greenhouse (S1) 16S rRNA matching the silva classifier. Matching pie chart plotted in Krona. Phyla abundance was represented in percentage.

Figure 15. Bacterial community profile in wild (S2) 16S rRNA matching the silva classifier. Matching pie chart plotted in Krona. Phyla abundance was represented in percentage.
Figure 16. Bacterial taxonomic fingerprint of greenhouse (S1) and wild (S2) 16S rRNA matching the SILVA ngs classifier. Matching taxonomic fingerprint at Phylum Level of the samples abundance (S1 and S2) was represented in percentage of reads.
Figure 17. Family composition of rhizosphere soil Pryosequence of S1 library. There was 100% presented in phylum level because it’s not having any classes.

Figure 18. Family composition of rhizosphere soil Pryosequence of S2 library. There was 100% presented in phylum level because it’s not having any classes.
3.7.2. Classification of genera

Overall, 208 genera were documented in the sample S1 and 218 genera in sample S2. The total BLAST score was calculated against each sequence from reference database (http://mg.bio.titech.ac.jp/vitcomic/) to identify the nearest relative. Each species name in the reference database is placed in circles with ordered phylogenetic relatedness. Using VITCOMIC, the overall taxonomic compositions within and between S1 and S2 samples could be clearly distinguished (Figure 19 and 20). In Vitcomic map, large circles indicate boundaries of BLAST average similarities where innermost circle (i) corresponds to 80–85%, followed by (ii) 85–90 %, (iii) 90–95 %, (iv) 95–100%. Large colored dots indicate relatively abundant taxa in each sample (relative abundance >10%). Small dots that are located at the most lateral circle indicate closely related strains whose genome were sequenced previously. The large colored dots exhibited different abundance percentage genera within the sample S1 and S2. There were five different VITCOMIC clade regions calculated for most abundant percentage of the genus. The most abundant (>10%) genera including Candidatus solibacter, Acidobacterium capsulatum, Candidatus koribacter and Elusimicrobium minutum were presented in S1 as compared to S2 which exhibited only >=1% (Figure 21.a and 21.b).

The second region of VITCOMIC chat showed that bacterial genera including Beijerinckia indica, Xanthobacter autotrophicus, Azorhizobium caulinodans, Mesorhizobium loti, Bartonella bacilliformis, Bartonella Quintana, Bartonella henselae, Bartonella grahamii, Bartonella tribocorum, Ochrobactrum anthropic, Brucella canis, Brucella ovis, Brucella abortus, Brucella suis, Brucella melitensis, Parvibaculum lavamentivorans, Paracoccus denitrificans, Rhodobacter sphaeroides and Dinoroseobacter shibae were present in abundant (10%) in sample S2. However, some genera like Beijerinckia indica, Xanthobacter autotrophicus, Azorhizobium caulinodans, Mesorhizobium loti and Bartonella bacilliformis were present in similar numbers in S1 sample as shown in Figure 21.c and 21.d.

In third clade regions, Thioalkalivibrio sp., Halorhodospira halophile, Alkalilimnicola ehrlichii, Coxiiella burnetii, Saccharophagus degradans, Marinobacter aquaeolei, Hahella chejuensis, Azotobacter vinelandii and Pseudomonas fluorescens were the most abundant (>10%) genera in S2 but in S1 their abundance was only >=1%
In case of the fourth group, genera like *Heliobacterium modesticaldum*, *Desulfitobacterium hafniense*, *Natran aerobius thermophilus*, *Symbiobacterium thermophilum*, *Syntrophomonas wolfei* and *Carboxydothermus hydrogenoformans* were abundantly (>10%) present in the sample S1 as compared to S2 where the percentage of abundance was ≥1% (Figure 21.g and 21.h). In case of fifth region, *Akkermansia muciniphila*, *Opitutus terrae* and *Methylacidiphilum infernorum* were found to be distributed almost identically with a relative abundance of >10% between S1 and S2 (Figure 21.i and 21.j).

**Figure 19. Species presence and abundance in sample greenhouse (S1), each species name in the reference database is placed in circles with ordered phylogenetic relatedness. Physical distances between nearest species in the plot indicate genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80 %, followed by 85, 90, 95 and 100 % identity to the database sequence).**
Figure 20. Species presence and abundance of wild (S2), each species name in the reference database is placed in circles with ordered phylogenetic relatedness. Physical distances between nearest species in the plot indicate genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80 %, followed by 85, 90, 95 and 100 % identity to the database sequence).
Figure 21. (a-j). VITCOMIC web based classification of sequence which shows shift in abundance percentage of different genera. There was five different VITCOMIC clade regions calculated for most abundant percentage of the genus.
3.7.3. OTUs finder Bacterial diversity and richness

Species richness was represented in rarefaction curves and was measured based on 4415 sequences and the number of operational taxonomic units (OTUs) using a cut-off of 97% for sequence similarity, a commonly known level for comparative analysis of whole and partial 16S rRNA sequences (Konstantinidis et al., 2006). Rarefaction analysis was used to compare bacterial richness between the wild and greenhouse rhizosphere samples. The results showed that as the number of sequences in the samples increased, the number of OTUs tended to level.

OTUs were identified at genetic distances of 3% by using 4415 randomly selected and denoised sequences per sample. About 1086 and 1106 OTUs were detected in S1 and S2 respectively using 3% cutoff using Silva Cluster analysis. At 3% sequence divergence, most rarefaction curves pointed exponential and not reached half saturation, indicating that the surveying effort has not covered almost the full extent of taxonomic diversity (Ondov et al., 2011). Figure 22 was a rarefaction curve based on best match for each sequence of 16S rDNA genes and their frequency of recovery. Comparison of the rarefaction analyses with the number of OTUs determined by Chao1 and ACE richness estimators revealed that 97% (3% genetic distance) of the estimated taxonomic richness was covered by the surveying effort (Table 1). At 3% genetic distance, the rarefaction curves were saturated and the richness estimators indicated 97% of species coverage (Table 1). Thus, this survey obtained >97% of the taxonomic diversity in both the samples at 3% genetic distance cutoff. The comparison of rarefraction curve, the mean Chao1 richness estimates showed all showed similar values at genetic distances of 3% (1086 OTUs and 1106 OTUs, in both S1 and S2 respectively). The richness showed that the species richness was higher in S2.

Table 1. Rhizosphere soil bacterial diversity, richness derived from multiple diversity estimators for individual sediment samples 3%, OTUs, Shannon and Chao1 index of the samples

<table>
<thead>
<tr>
<th>Vertical Location Sample ID</th>
<th>Sequence library</th>
<th>Number of OUTs(^a)</th>
<th>Chao I richness</th>
<th>ACE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>2176</td>
<td>1086</td>
<td>861.5</td>
<td>861.5</td>
</tr>
<tr>
<td>S2</td>
<td>2239</td>
<td>1106</td>
<td>922.45</td>
<td>922.45</td>
</tr>
</tbody>
</table>
**Figure 22.** Rarefaction analysis for greenhouse (S1) and wild (S2) rhizosphere soil. Rarefaction curves representing the richness of the Pyrosequencing read with distance values. The vertical axis shows the number of OTUs that would be expected to be found after sampling the number of sequences shown on the horizontal axis. Rarefaction is shown for OTUs with differences that do not exceed 3%.

In the present study, overall results are similar to those of Kowalchuk and colleagues (2000). Using culture independent techniques, they showed that, wild plant species were able to influence the composition of bacterial diversity in the rhizosphere. In their specific study, they compared the influence of *Cynoglossum officinale* (hound’s tongue) and *Cirsium vulgare* (spear thistle) on soil-borne bacterial communities and found differences in the corresponding microbial communities of the rhizosphere.

The rhizosphere is more nutrient-rich niche than bulk soil (Grayston *et al*., 1998). The ability of plants to alter microbial diversity and distribution in their rhizosphere may be due to their ability to create a microenvironment that is rich in carbohydrates, carboxylic acids and amino acids, and therefore differences in plant exudates may be behind this discrimination (Molina *et al*., 2000; Uroz *et al*., 2010).
Previous studies have shown several degrees of a ‘rhizosphere effect’ using either culture-dependent (Miller et al., 1989; Germida et al., 1998; Grayston et al., 1998) or culture-independent strategies (Marilley and Aragno, 1999; Miethling et al., 2000; Duineveld et al., 2001; Smalla et al., 2001; Sanguin et al., 2006). The general results of these studies suggest that different plant species differ in the degree and manner in which they influence microbial community structure in the rhizosphere, as was indeed the case when microbial populations of oil seed rape were compared with those of strawberry (Duineveld et al., 2001; Smalla et al., 2001; Berg et al., 2005; Berg and Smalla, 2009). The effect of different plant species on soil microbial communities has been demonstrated for rhizosphere (Grayston et al., 1998; Söderberg et al., 2002; Iovieno et al., 2010). The influence of plants on the soil microbial community has even been found for different genotypes of the same species (Grayston et al., 1998; Schweitzer et al., 2008).

In this regard, the present study carried out detailed analyses of the relative distributions of the genera, class and phyla between microbes in the wild and greenhouse in the *S. portulacastrum* rhizosphere. These analyses are based on partial 16S rRNA sequence analyses and their location in phylogenetic trees based on the SILVAngs programme.

Analysis of Proteobacteriain wild rhizosphere revealed that Proteobacteriarepresent 55% of total sequences with *alphaproteobacteria* being the most prevalent (~50% of total Proteobacteria), followed by *gamma* (~33%), *delta* (~13%) and *beta* (~1%). In the greenhouse rhizosphere, analysis of the *Proteobacteria* phylum showed that there were significantly more *alphaproteobacteria* (~56%) followed by *beta* (~25%) *gamma* (~11%), and *delta* (~8%). This contrasts with studies of the rhizosphere of grape in which there were significantly more *Betaproteobacteria* in the rhizosphere (Sanguin et al., 2006; Haichar et al., 2008). In a recent meta-analysis of 19 libraries of bacterial clones associated with the roots of 14 plant species, over 1200 distinguishable taxa from 35 different taxonomic orders were described (Hawkes et al., 2007). Proteobacteriadiominated the rhizosphere in 16 of the 19 studies included, presumably because of their relatively rapid growth rates (Atlas and Bartha, 1998). The present study observations that *Proteobacteria* are frequent in rhizosphere soils are in agreement with
studies carried out with microarrays to detect soil bacteria by Sanguin and colleagues (2006). García-Salamanca et al., (2012) also observed that Proteobacteria are abundant in rhizosphere soils. Present data also showed that the proportion of Acidobacteria found in rhizosphere soil is independent of the plant species. This finding is in agreement with the results by Acosta-Martínez and colleagues (2008), who found that levels of Acidobacteria were similar regardless of the type of plantation (grass or wheat) and land management practice.

Bacterial communities are acknowledged as one of the major components of soil function, playing a key role in niche maintenance. Pseudomonas spp. are well known root colonizers (Molina et al., 2000) which are able to proliferate using plant-secreted amino acids such as proline, lysine, phenylalanine, glutamate and others (Vílchez et al., 2000; Espinosa-Urgel and Ramos, 2001; Herrera and Ramos, 2007). Present results showed that nitrogen-fixing microbes are highly abundant in this soil and it plays a key role in the mobilization of nutrient in rhizosphere soil; instead, microbes capable of metabolizing inorganic nitrogen are present, which is consistent with the historical use of inorganic nitrogen sources at this field site.

The present study provided data on how certain bacterial populations become dominant in the rhizosphere soil through a mechanism that is most likely due to the microenvironment created by the presence of S. portulacastrum exudates and bacterial chemotaxis towards nutrients in the exudates. In general, this main conclusion in a soil with a relatively high pH is in agreement with studies that suggest that soil characteristics may be the most important factor determining the dominant bacterial populations in rhizosphere soil (Felske and Akkermans, 1998; Kowalchuk et al., 2000), while the microbial communities found in the rhizosphere are, to a greater extent, plant-driven.
PART 4

Effects of abiotic stress on the physiological and oxidative status of halophyte

1. Introduction

In both natural and agricultural conditions, plants are frequently exposed to environmental stresses. Some environmental factors, such as air temperature, can become stressful in just a few minutes; others, such as soil water content, may take days to weeks, and factors such as soil mineral deficiencies can take months to become stressful. It has been estimated that because of stress resulting from climatic and soil conditions (abiotic factors) that are suboptimal (Boyer 1982). In addition, stress plays a major role in determining how soil and climate limit the distribution of plant species. Thus, understanding the physiological processes that underlie stress injury and the adaptation and acclimation mechanisms of plants to environmental stress is of immense importance to both agriculture and the environment.

Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant. The concept of plant stress is often used itself with environmental or abiotic factors that produce stress in plants, although biotic factors such as weeds, pathogens, and insect predation can also produce stress. In most cases, stress is measured in relation to plant survival, crop yield, growth (biomass accumulation), or the primary assimilation processes (CO$_2$ and mineral uptake), which are related to overall growth. The concept of stress is intimately associated with that of stress tolerance, which is the plant’s fitness to cope with an unfavorable environment. The term stress resistance is often used interchangeably with stress tolerance, although the latter term is preferred.

With an expected population of over 9 billion by 2050 and added abiotic and biotic plant stresses due to climate change, there is an increased demand for agricultural production on marginal lands (Wang, 2003; Munns, 2005). Abiotic stress may be caused by numerous factors such as drought (Simova-Stoilova et al., 2009), cold (Van Heerden et al., 2003), high temperature (Reynolds- Henne et al., 2010), salinity (Meloni et al., 2003), heavy metals (Smeets et al., 2009) and ultraviolet radiation (Gao and Zhang, 2008). Among the various abiotic stresses to which plants are constantly exposed, salt
stress is the one that most affects growth and productivity of plants around the world (Vaidyanathan et al., 2003; Veeranagamallaiah et al., 2007), reaching more than 800 million land hectares around the globe (FAO, 2005).

Abiotic stresses cause considerable loss to agricultural production worldwide. Abiotic stress conditions such as drought, salinity, heat, or heavy metal, etc., have been the subject of intense researches. In plants, osmotic stress generated by either drought and/or salinity or heavy metal stress represents the most common environmental hazard for the plant's growth and productivity. In addition, it has been reported that when combined, multiple abiotic stresses may interfere with nutrient accumulation, thereby further contributing to growth inhibition. Consequently, it seems a prerequisite for plant species selected for their use in these areas to have adaptations that confer abiotic stress resistance through notably the optimized utilization of water and nutrients. However, in nature, tea and other crop plants are routinely subjected to a combination of different abiotic stresses. Recent studies have pointed out that drought may exacerbate the adverse effects of abiotic stress on the plant nutrient status, but only a few attempts have been made to quantify the combined effect of drought, mineral nutrients, and other abiotic stress.

Since plants are sessile, they have developed mechanisms that enable them to sense stressful environmental conditions and to elicit complex interactions between signaling molecules and pathways to adapt to various stresses. Soil salinity is one of the major abiotic stresses responsible for reduced crop productivity worldwide (Food and Agriculture Organization, 2002). Salt stress affects a wide range of physiological parameter such as reduces growth, especially in the aerial part (Syversten et. al., 1988; Lloyd and Howie, 1989); increases leaf succulence (Cerda et.al., 1977; Zekri, 1991); reduces photosynthetic rate and stomatic conductance (Banuls and Primo-Millo, 1992; Banuls et.al., 1997; Gomez-Cadenas et.al., 1998); decreases root hydraulic conductivity, affecting nutrient transport ( Zekri and Persons, 1989). Importantly, when the accumulation of chloride ions in leaves reaches toxic levels for the cellular metabolism, premature abscission occurs (Banuls and Primo-Millo, 1992; Gomez-Cadenas et.al., 1998).

Biochemical and physiological processes in plants are known to be affected by exposure to environmental pollutants such as heavy metals. Heavy metals are common
pollutants of coastal saline area and halophytes can be used to phytoremediate heavy metal pollution (Sharma et al., 2010). Mangroves possess high tolerance to heavy metal pollution and act as a sink for their removal (Macfarlane and Burchett, 2001). Contaminants, such as cadmium (Cd), copper (Cu), lead and zinc, enter the environment through industrial waste, mill tailings, and landfill runoff. Cd is a toxic trace pollutant for humans, animals, and plants which enters to the environment mainly from industrial processes and phosphate fertilizers and then is transferred to the food chain (Wagner, 1993). Cd is one of the major industrial pollutants that show phytotoxicity even at low doses (Chakravarty and Srivastava, 1992; Das et al., 1997).

However, Cu is an essential metal for plants at low concentrations, but can become toxic at higher concentrations (Hattab et al., 2009a). Zinc is an essential element for all living organisms. Its metabolic functions are based on its strong tendency to form tetrahedral complexes with N\(^-\), O\(^-\) and S\(^-\) ligands and it therefore plays both a functional and a structural role in enzyme reactions. Zinc excess in the environment, however, frequently occurs as a result of anthropic emissions (Callender and Rice 2000; Reck et al., 2006). It may accumulate at high concentrations in estuarine sediments where it compromises the ecosystem stability and constitutes a serious threat for human health (Sáinz et al., 2002; Morillo et al., 2004).

Among many other physiological alterations, heavy metals trigger oxidative stress in plants leading to the oxidation of proteins and membrane lipids (Schützendübel and Polle, 2002), even after a short-term exposure (Ortega-Villasante et al., 2005). Copper is a redox active metal, known to intervene through Fenton reactions in the accumulation of oxygen free radicals, leading to the alteration of cell membranes by peroxidative degradation of polyunsaturated fatty acids Weckx and Clijsters, 1996; Fry et al., 2002, Schützendübel and Polle, 2002). The major symptoms of Zn toxicity in plants, photosynthesis inhibition, modification in the plant water status and oxidative stress are frequently reported (Vaillant et al. 2005; Tewari et al. 2008; Lefe`vre et al. 2010; Redondo-Go´mez et al. 2011).

Halophytes serve as model system for investigating the adaptation mechanism of saline environment. Analysis of gene expression pattern of halophytic plants is one of the approach for studying stress-specific signaling and transcriptional responses. There have
been reported from several plant species and known to be involved in abiotic/metal stresses and developmental processes, there are a few reports on halophytes under heavy metal stress (Kholodova et al., 2010). Among many other physiological alterations, heavy metals trigger oxidative stress in plants leading to the oxidation of proteins and membrane lipids (Schützendübel and Polle, 2002), even after a short-term exposure (Ortega-Villasante et al., 2005).

Reactive oxygen species (ROS) are produced constantly under normal aerobic metabolism, and plant cells possess a well-equipped anti-oxidative defense system to maintain a redox homeostasis (Bhattacharjee, 2005; Foyer and Noctor 2005). Indeed, enzymes such as superoxide dismutases (SOD) are able to scavenge superoxide (O2―) radicals, whereas catalases (CAT) and peroxidases (POXs) have a role in quenching hydrogen peroxide (H2O2). Among the latter, ascorbate peroxidases (APX) are ubiquitous to several cellular parameters, are known to be a major component of the antioxidant network in several plant species (Davletova et al., 2005). Many of the indicated antioxidant enzymes use the soluble antioxidants ascorbate and glutathione (GSH) (Noctor and Foyer, 1998). In particular, the cellular thiol status apparently plays a central role in redox homeostasis and cell function, in which the concentration of GSH and the balance with its oxidized counterpart (GSSG) is kept in a constant level (Noctor, 2006).

In this study, we examined the interactions between salinity, growth, lipid peroxidation and antioxidant enzyme activities in three species with contrasting tolerance to salt (cotton, sorghum and cowpea) and aiming a better understanding of these salt tolerance indicators in these species.

1.1. Analysis of peptide through MALDI-TOF-TOF MS

Over the past decades, the rapid development of mass spectrometry (MS) has made it an indispensable tool in biological research. The introduction of soft ionization techniques such as electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) has made it possible to characterize relatively large and labile biomolecules including peptides and proteins using MS. MS-based protein identification typically involves two different steps: (1) analysis of peptides generated by
proteolytic digestion; and (2) database searching (Fig. 1). Digestion of protein can be achieved using various proteases, but the most popular one has been trypsin which hydrolyzes the C-terminal side of lysine and arginine (unless the subsequent amino acid in the sequence is a proline). Peptides produced by tryptic digestion normally possess two basic sites, i.e., the basic lysine or arginine residual at the C terminal and the basic amine group at the N terminal. This allows peptides to be protonated efficiently and hence enhances ionization of mass spectral sensitivity.

The general principle of MALDI-TOF-TOF MS revolves around the rapid photovolatilization of a sample embedded in a UV-absorbing matrix followed by time-of-flight mass spectrum analysis. Several chemical and physical pathways have been suggested including gas-phase photoionization, ion-molecule reactions, disproportionation, excited state proton transfer, energy pooling, thermal ionization, and desorption of preformed ions (Zenobi and Knochenmuss, 1998).

MALDI-TOF-TOF MS can more definitively identify and characterize proteins by isolating and fragmenting a molecular ion of interest and measuring the fragment ion masses. MALDI-TOF-TOF MS identification of proteins is carried out by the so-called peptide mass mapping or peptide mass fingerprinting technique. This highly effective approach of protein identification is based on the accurate mass measurement of a group of peptides derived from a protein by sequence-specific proteolysis. After proteolysis with a specific protease (e.g. trypsin), proteins of different amino acid sequence produce a series of peptides masses, this can be detected by MALDI MS. The spectrum of identified peptide masses is unique for a specific protein and is known as a mass fingerprint. Searching the selected masses from the fingerprint against databases of known protein sequences (e.g. SwissProt- TrEMBL, NCBI) enables the identification of most proteins.
Figure 1. Basic Components of a Mass Spectrometer
2. Materials and methods

2.1. Plant culture and treatments

Mature seeds of *Sesuvium portulacastrum* were collected in 2012 from Vellar estuary coasts, Parangipettai. The seeds were germinated and grown in soil mixture containing inert sand and vermiculated manure in a 1:1 ratio in plastic pots (12-cm diameter and 8-cm depth) and watered daily with distilled water. Then, one month old seedlings were transferred to hydroponically condition, using ½ MS basal medium pH 5.8 (Murashige and Skoog, 1962) without sucrose and organic components (Himedia). An initial harvest was made on 10 days old plants. The plants were treated with different abiotic stresses, NaCl (300 mM); drought; dark; heat (45 °C); cold and metal stress (Zn++, 0.5 mM; Cu++, 0.2 mM and Cd++, 0.1 mM). The experiments were performed in humidity chamber (Labline) under controlled conditions (20–25 °C Temperature, 70–90% relative humidity, 16 hr day & 8 hr night–photoperiod, and 200 mmol m⁻² s⁻¹ photosynthetic active radiation, PAR).

Sixteen plants per treatment were used: 5 for measuring the physiological parameters, 5 for the antioxidative enzyme assays, 3 for the determination of malonyldialdehyde (MDA) contents and 3 for the study of protein. The final harvest occurred for 72 hrs of abiotic stress treatment. For the determination of the fresh weight, dry weight and turgid weight, leaves, shoots and roots were separated from plants. Besides, fresh leaves, shoots and roots samples from each plant were immediately frozen in liquid nitrogen and stored at -80 °C for further analysis.

2.2. Effect of salinity on growth and water status

The fresh weight (FW) and dry weight (DW) of leaves and roots were measured at each sampling. The plant samples were dried in a forced-ventilation oven at 65 °C until constant dry weight. FW and DW of leaves were measured at each sampling (Gao, 2000).

\[
\text{Dry matter (DM)} = \frac{\text{DW}}{\text{FW}} \times \text{FW}
\]

\[
\text{Tissue water content (TWC)} \% = 100 \times \frac{\text{FW} - \text{DW}}{\text{FW}}
\]

\[
\text{Relative water content (RWC)} \% = 100 \times \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}
\]

Where,

TW is the turgid weight measured after 24 hr of saturation on deionized water (when leaf weight reached a constant value) at 4 °C in the dark.
2.3. Ion content analysis

Ions were extracted from the desiccated leaves tissues with 0.5% (v/v) nitric acid. The concentration of Cd, Cu, Zn, Ca\(^{2+}\)Na\(^{+}\)and K\(^{+}\) were measured by inductively coupled plasma optical emission spectrometer (Optima 2000DV, Perkin Elmer, Germany). Ion content was determined via the method described by Shukla et al., 2011. Plant tissue (0.2 g) was digested in 4 ml of a solution containing perchloric and nitric acids (3:1). The solution was dried on a hot plate at 90 °C and reconstituted to 25 ml with Millipore Milli-Q Ultrapure water (Merck) before filtration through a 0.2 \(\mu\)m microfiber filter and transferred into clean vial and aspirated into a metal analyzer.

2.4. Estimation of Chlorophyll Content

The chlorophylls are the essential components for photosynthesis, and occur in chloroplasts as green pigments in all photosynthetic plant tissues. They are bound loosely to proteins but are readily extracted in organic solvents such as acetone or ether. Chlorophyll a, chlorophyll b, and total chlorophyll were extracted and estimated from fresh leaves, following the standard method of Arnon, 1949. Chlorophyll was extracted by homogenizing 0.1 g of fresh leaves in 10 ml of 80% acetone. Homogenates were centrifuged at 4°C for 15 min (3000 rpm). Supernatants were used for the analysis of chlorophyll pigments. Chlorophyll content was determined via a LAMBDA 25 UV/Vis spectrophotometer (PerkinElmer) from the acetone extract at 645, 652 nm and 663 nm, as described by Witham et al. (1971).

The amount of chlorophyll were calculated and presented in the ‘extract mg chlorophyll per gram tissue’ using the following equations:

Chlorophyll a, b and total chlorophyll content as ‘mg in 1 gram of plant tissue’ were calculated.

\[
\text{mg Chlorophyll a/g tissue} = [12.7 (A_{663}) - 2.69 (A_{645})] \times (V/1000 \times \text{Weight})
\]

\[
\text{mg Chlorophyll b/g tissue} = [22.9 (A_{645}) - 4.68 (A_{663})] \times (V/1000 \times \text{Weight})
\]

\[
\text{mg total Chlorophyll/g tissue} = [20.2 (A_{645}) + 8.02 (A_{663})] \times (V/1000 \times \text{Weight})
\]
Where,

\[ A = \text{absorbance at specific wavelengths} \]
\[ V = \text{final volume of chlorophyll extracts in 80\% acetone} \]
\[ W = \text{fresh weight of tissue extracted} \]

2.5. Determination of lipid peroxidation

Lipid peroxidation is a well-established mechanism of cellular injury in plants. It is used as an indicator of oxidative stress in plant cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. The measurement of MDA and HAE has been used as an indicator of lipid peroxidation (Esterbauer et al., 1991). This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA and HAE at 45°C. One molecule of either MDA or HAE reacts with 2 molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 532 nm.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid method described by Madhava Rao and Sresty (2000). For MDA extraction, 0.5 g of different abiotic stress treated leaves samples were homogenized with 2.5ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged for 10 min at 10,000 \( \times \) g. For every 1ml of the aliquot, 4ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added. The mixture was heated at 95 °C for 30 min and then cooled quickly on an ice bath. Afterwards, the mixture was centrifuged for 15 min at 10,000 \( \times \) g and the absorbance of the supernatant was measured in a LAMBDA 25 UV/Vis spectrophotometer (PerkinElmer) at 532 nm. The concentration of analyte in each unknown was calculated from the net A586 of the sample:

\[
[\text{MDA}] = \frac{A_{586} - b}{a} \times \text{df}
\]

Where:

\[ [\text{MDA}] = \text{concentration of MDA in the sample in } \mu\text{M} \]
\[ A_{586} = \text{Net absorbance of the sample at 586 nm} \]
\[ \text{df} = \text{dilution factor} \]
a = regression coefficient (slope)

b = intercept

2.6. Determination of Proline (Pro) content

Proline is a basic amino acid found in high percentage in basic protein. Free proline is said to play a role in plants under stress conditions. Though the molecular mechanism has not yet been established for the increased level of proline, one of the hypotheses refers to breakdown of protein into amino acids and conversion to proline for storage. Many workers have reported a several-fold increase in the proline content under physiological and pathological stress conditions. Hence, the analysis of proline in plants has become routine in pathology and physiology division of agricultural sciences. During selective extraction with aqueous sulphisalicylic acid, proteins are precipitated as a complex. Other interfering materials are also presumably removed by absorption to the protein-sulphosalicylic acid complex. Proline content was determined according to Bates et al. (1973). For proline extraction, 0.5 g fresh leaves were homogenized in 10 ml of 3% sulfosalicylic acid and the homogenate was filtered through Whatman No. 1 filter paper. 2 ml of filtrate extract obtained reacted with 2 ml of ninhydrin acid and 2 ml glacial acetic acid. The mixture was heated at boiling water bath for 1 hr and then the reaction was terminated by placing the tube in ice bath. After that, 4 ml toluene was added to reaction mixture and stirred well for 20-30 sec. Then the toluene layer was separated and solution was kept at room temperature. Afterwards, proline concentration was measured in a LAMBDA 25 UV/Vis spectrophotometer (PerkinElmer) at 520 nm absorbance.

Proline content was calculated per unit of fresh weigh (FW) according to:

$$\text{µmoles per gm tissue} = \frac{\text{µg proline/ml x ml toluene}}{115.5} \times \frac{5}{\text{gm sample}}$$

Where,

115.5 is the molecular weight of proline
2.7. Antioxidant enzymatic activities

2.7.1. Enzyme extractions from leaves of *S. portulacastrum*

For enzyme determination, the newly originaed leaves of *S. portulacastrum* were freshly sampled and treated with different abiotic stresses for 72 hrs. 0.3 gm of leaves tissue was ground with liquid nitrogen. The ground powder was collected into a 1.5 ml or 2 ml microtube with a spatula and 1 ml extraction buffer was added to it (Table 1), then it was vortexed for a few seconds. The complete extraction procedures were carried out at 4 °C. The homogenate were centrifuged at 10, 000 x g for 15 min at 4 °C and the supernatants were collected in new centrifuge tubes and the crude extract were kept on ice. The crude extract was used for the assays of enzyme activity.

<table>
<thead>
<tr>
<th>Table 1: Enzyme extraction buffer</th>
</tr>
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<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>50 mM sodium phosphate buffer pH 7.5, w/v</td>
</tr>
<tr>
<td>1 mM EDTA pH 8.0, w/v</td>
</tr>
<tr>
<td>8 % polyvinylpyrolydone (M.W. 40000), (w/v)</td>
</tr>
<tr>
<td>0.01 % (v/v) Triton X-100</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
</tr>
</tbody>
</table>

2.7.2. Total Catalase (CAT, EC 1.11.1.6) activity:

Total catalase activity was measured spectrophotometrically according to the method of Luck (1965), by monitoring the crude extract’s decline in absorbance at 240 nm as H₂O₂ was consumed. 3 ml reaction mixture contained 66 mM sodium phosphate buffer (pH 7.0), to which 30% (v/v) H₂O₂ was added. The reaction was initiated by adding 100 µl of leaves extract to this solution. The time ∆t required for a decrease in the absorbance from 0.45 to 0.4 is used for CAT activity calculations. CAT activity was expressed as unit per mg of protein. A unit is the amount of an enzyme which liberates
half the peroxide oxygen from a hydrogen peroxide solution of any concentration in 100 s at 25°C. One CAT unit was equivalent to 1 µmol of H₂O₂ consumed per minute.

2.7.3. **Total peroxidase (POD, EC 1.11.1.17) activity:**

Peroxidase (POD) includes a group of specific enzymes such as NAD-Peroxidase, NADP- Peroxidase, fatty acid peroxidase etc. as well as a group of very non-specific enzymes from different sources which are simply known as POD (donor: H₂O₂-oxidoreductase). POD catalyses the dehydrogenated organic compounds such as phenols, aromatic amines, hydroquinones etc. POD occurs in animals, higher plants and other organisms (Putter, 1974).

Peroxidase activity was determined according to Tatiana et al. (1999) with minor modifications. The reaction mixture contained 0.05 M sodium phosphate buffer (pH 5.5), 2% H₂O₂, 0.05 M guaiacol, and 100 µl enzymes extract in a final volume of 5 ml. The reaction was started by the addition of enzyme extract. The formation of tetraguaiacol was measured at 470 nm. One unit of enzyme was defined as the amount of enzyme to decompose 1 µmol of H₂O₂ per min.

2.7.4. **Total superoxide dismutase (SOD, EC 1.15.1.1) activity:**

The cell generates a variety of molecules during its metabolic processes. Environmental stresses like high/low temperature, water stress, air pollution, UV light, heavy metals and chemicals results in excess production of active oxygen species such as super oxide, hydrogen peroxide and hydroxyl radicals. Unless these toxic molecules are eliminated, damage to the macromolecules such as DNA/tissue is imminent. Superoxide dismutase(SOD) was assayed spectrophotometrically as the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT). SOD was conveniently assayed using a slightly modified procedure (Madamanchi et al., 1994) originally described by Beauchamp and Fridovich (1971). The 3 ml reaction mixture consisted of 50 mM Na-phosphate buffer (pH 7.8), 13 mM L- methionine, 75 µM NBT, 10 µM EDTA-Na₂, 2.0 µM riboflavin, and 0.3 ml leaves enzyme extract. The test tubes containing the reaction mixture were kept for 10 min under 4000 lux at 35°C. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm.
2.8. Protein Profiling of *Sesuvium portulacastrum* treatment with different abiotic stresses

2.8.1. Protein Extraction from leaves of *S. portulacastrum*

Protein isolated from leaves of *S. portulacastrum* was separately ground in liquid nitrogen using pestle and mortar. The BPP method was used for extraction of protein with slight modification and this method was described in chapter 2 section 2.6.

2.8.2. Quantification of Proteins:

Protein concentration was determined by Bicinchoninic Acid protein Assay kit (BCA, Sigma) following the protocol supplied along with the kit using the LAMBDA 25 UV/Vis Spectrophotometers(PerkinElmer). BSA was used as the standard (100μg/μl) and the absorbance was measured at 562nm.

2.8.3. One Dimensional Protein Profiles of *Sesuvium portulacastrum* Leaf with different abiotic stress treated

One dimensional (1D) gel electrophoresis (chapter 2 section 2.7.) was carried out to evaluate the quality and loading quantities of the protein extracts.

2.8.4. Tryptic Digestion of Protein Bands

Tryptic digestion of protein band was performed as described below. Protein band was excised from the gel by manual cutting, the of protein bands of interest were carefully cut with sterilized surgical blades in small pieces (0.2 to 0.5 mm) and transferred into sterile microcentrifuge tubes. Gel pieces were first equilibrated in 50 µl of 25 mM ammonium bicarbonate (ABC) and proteins were reductively alkylated with 10 mM DTT and 100 mM iodoacetamide. Gel slices were dehydrated with 50µl of solution A [2:1 mixture of acetonitrile [(ACN) MS grade, Sigma Aldrich] : 50mM ABC] for 5 min. Supernatant was removed and 50µl of 25mM ABC added for 2 min. Gel slices were dehydrated with 50µl of solution A for 5 min. Supernatant was removed and 50µl of 25mM ABC was added for 2 min. Trypsin was prepared by adding 1ml of ice-cold 25mM ABC to a standard 20 μg trypsin vial (Proteomics Grade, Sigma Aldrich) and stored on ice. The gel slices were rehydrated with trypsin, (about 10-20 µl). Gel slices were incubated on ice for 20-30 min until trypsin was absorbed. After gel slices were completely rehydrated, 25mm ABC was added just enough to cover gel slices in tube and
incubated at 37°C for at least 6 hrs, typically overnight. The gel slices were periodically checked to make sure that they were still covered with 25mM ABC or added more if needed.

After digestion, supernatant was removed and gel slices were vortexed with 2X extractions of 100µl 20% ACN, 1% Formic Acid (MS grade, Sigma Aldrich), these extractions were added to supernatant. Mixture was lyophilized until volume was reduced to approx 10-20 µl. The digested proteins were then stored at 4°C until further analysis.

2.8.5. Matrix preparation
The matrix α-cyano-4-hydroxy-cinnamic acid is suitable for peptides (<10 kDa). 10 mg of α-cyano-4-hydroxy-cinnamic acid (matrix) was added in 2 ml of matrix diluent (50% acetonitrile, 0.1% TFA, 10 mM ammonium acetate).

2.8.6. Nano-LC Instrument and Automated MALDI Spotter
Liquid chromatography (LC) and capillary electrophoresis (CE) are high efficiency separation techniques, and are among the primary methods that have been hyphenated to mass spectrometers (Metzger et al., 2009; Mirgorodskaya et al., 2005). Not surprisingly, the interface between LC and MALDI-MS plays an important role and has been studied intensively (Pes and Preisler, 2010; Lee et al., 2009).

The LC instrument used to test the microfluidic interface was the NanoLC Ultra® Systems (Eksigent, SINGAPORE) consisting of a degasser, an autosampler (NanoLC AS-2), a thermostated flow manager module, a UV flow cell and Micro-pumps. The mobile phases used for the reverse phase separation were Buffer A: 0.1% trifluoroacetic acid v/v and Buffer B: 0.1% TFA and 90% acetonitrile v/v. The trypsin digested peptide samples were loaded on to a PepMap™ 300 µm×5 mm C_{18} reverse phase trapping column and then eluted into the PepMap™ 75 µm×150 mm C_{18} analytical separation column in back flush mode at a flow rate of 0.4 µl/minute. The separation was performed across a gradient of 0–60% Buffer B and Buffer A and completed in 30 minutes. The peptides eluted off the analytical column were initially passed through the UV flow cell to ascertain resolution between peaks and then run into the Probot™ spotter.
Detection was carried out at 220 nm. The length of capillary joining the analytical separation column to the UV flow cell was approximately 62 cm and the length of capillary connecting the flow cell to the spotter was 72 cm. Once required separation resolution was obtained, the sample was run directly into the spotter through the UV flow cell. The length of capillary connecting the separation column to the automated spotter was 97 cm, while in the case of droplet interfaced Nano LC-MS, the droplet generation micro device is attached 12 cm from the analytical separation column, and therefore totally 85 cm of continuous fluidic conduit was replaced with the droplet transferal.

2.8.7. MALDI-TOF-TOF Analyses

Matrix-assisted laser desorption/ionization time of flight-time of flight (MALDI-TOF-TOF) analysis was performed on AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Canada) using the following protocol. Firstly, MALDI-TOF-TOF MS analysis was performed on all the target spots using automated data acquisition and processing under the control of TOF/TOF Series Explorer™ software (version 4.1.0 ) using reflector mode, a mass range of 700-4000 m/z, 1000 total laser shots per spectrum and a laser intensity of 3300 V. Following acquisition, the TOF-MS spectra were noise corrected, peak de-isotoped and internally calibrated using the trypsin autolysis peaks 842.500 and 2211.100 m/z. The precursor ions from each spectrum were then selected by the software for fragmentation and MS-MS analysis using a 1 kV CID fragmentation method collecting 4000 laser shots per spectra with a laser intensity of 3800 over the mass range. Peak lists of ion masses were generated by denovo Explorer software version 3.6 (AB SCIEX, Canada) from the calibrated and de-isotoped MS and MS-MS spectra for each sample. Combined lists of the MS and MS-MS data were used for database searching with MASCOT (Matrix Science) against all entries in the NCBI database. Database search parameters used were; digestion enzyme trypsin, single missed cleavage allowed, fixed modification of carboxymethyl (C) and variable modification of deamidated (NQ) and oxidation (M), mass values of monoisotopic, precursor peptide mass tolerance of ± 100 ppm and fragmentation ion tolerance of ± 0.25 Da. The MASCOT outputs were generated for each sample. Candidate protein matches with MOWSE scores higher than 20 (p<0.05) were considered as positive identifications.
3. Results and Discussion

3.1. Determination of growth and water content

Under stress condition, derangement in the leaf water potential and its components takes place (Suit and DuCharme, 1953), and in the laboratory, where equipment to quantify plant water potential are not available. The determination of TWC is still a valid parameter to quantify the plant water status and its growth (Kramer, 1969). A reduction in TWC was observed with increase in abiotic stress treatment (Fig. 2). The present study observed that when plant is healthy its TWC percentage was high but in stress condition it was varies. Some study was observed by Rajaravindran and Natarajan (2012) in case of NaCl, the TWC with the increasing salinity upto 600mM in *Sesuvium portulacastrum* but at higher concentrations, the growth of plant was decreased because plants were not tolerant to the high salinity.

![Figure 2](image_url)  
Figure 2 Effect of abiotic stress on TWC (%) in the leaf of *S. portulacastrum* on 72 hrs after treatment. The data are the mean ± SD of 3 replicates.
Relative water content (RWC) is a measure of the relative cellular volume that shows the changes in cellular volume that could be affecting interactions between macromolecules and organelles. As a general rule, a RWC about 90-100% is related to closing of the stomata pore in the leaf and a reduction in the cellular expansion and growth. Contents of 80-90% are correlated with changes in the composition of the tissues and some alterations in the relative rates of photosynthesis and respiration. Abiotic stress reduced leaf RWCs in *S. portulacastrum* (Fig.3).

**Figure 3** Effect of abiotic stress on RWC (%) in the leaf of *S. portulacastrum* 72 hrs after treatment. The data are the mean ± SD of 3 replicates

### 3.2. Ion content analysis

The concentrations of Na⁺ increased in abiotic stress treated plants of *S. portulacastrum* when compared with control plants (Fig. 4.). The high levels Na⁺ level in stress treated plants and the negative correlation between RWC and Na⁺ concentrations, indicate that the capacity to prevent Na⁺ from reaching the aerial tissues and to maintain leaf water status was very limited in *S. portulacastrum*. Hernandez and Almansa (2002) observed similar changes in pea leaves after 48 hr of salt stress. Hasna et al., 2007 observed that when salt treated, this plant allows high levels of Na to be translocated to
the shoot, which suggest that efficient detoxification mechanisms by C. maritima to
tolerate the presence of Na+ inside the cells.

![Bar graph showing the effect of abiotic stress treatment on Na+, K+ Cu,Cd, and ZN concentrations in the leaves of S. portulacastrum](image)

**Figure 4. Effect of abiotic stress treatment on Na+, K+ Cu,Cd, and ZN concentrations in the leaves of S. portulacastrum**

**3.3. Chlorophyll Content**

The stress imposed at the vegetative stage, significantly decreased chlorophyll a content, chlorophyll b content and total chlorophyll content (Fig. 5). The lack of effects on the chlorophyll a/b ratio indicates that chlorophyll b is not more sensitive to stress than chlorophyll a. presented in figure 5. The results are agreement with Nyachiro et al. (2001), who described a significant decrease of chlorophyll a and b caused by water deficit in six *Triticum aestivum* cultivars. Decreased or unchanged chlorophyll level during drought stress has been reported in other species, depending on the duration and severity of drought (Kpyoorissis et al., 1995). A decrease of total chlorophyll with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygenspecies is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments (Herbinger et al., 2002).
Figure 5. Effect of abiotic stress on chlorophyll „a”, chlorophyll „b” and total chlorophyll (mg g-1 fr. wt.) of Sesuvium portulacastrum on 72 hr after treatment.

3.4. Determination of lipid peroxidation

Lipid peroxidation has been associated with the damage provoked by a variety of environmental stresses and is often used as an indicator of salt-induced oxidative damage (Elkahoui et al., 2005). Malondialdehyde (MDA) is the decomposition product of polyunsaturated fatty acids in the biomembranes. It has been used as an indicator of lipid peroxidation and tends to greater accumulation under salt stress (Zhu et al., 2008). Results showed that in leaves, lipid peroxidation was significantly influenced by abiotic stress ($P=0.5$). Leaves’ MDA was higher under all abiotic stresses, compared with control. Abiotic stresses levels at NaCl, drought, dark, heat, cold, Zn, Cu and Cd, caused respectively $39.56 \pm 0.54$, $72.19 \pm 0.76$, $44.61 \pm 0.55$, $69.59 \pm 0.45$, $73.50 \pm 0.42$, $57.84 \pm 0.44$, $71.31 \pm 0.45$and $44.67 \pm 0.55$ µmol/gm fresh weight (Fig.6). The present result showed that lipid peroxidation was influenced by abiotic stress in leaves of S. portulacastrum. Increase in MDA contents under salt stress was also found in rice (Tijen and Ismail, 2005), alfalfa (Wang and Han, 2007), cotton (Diego et al., 2003) and wheat (Sairam and Srivastava, 2002). Similar results were also observed by Sabrine Hattab et al. (2013) where Cd and Cu metals caused a clear oxidative stress detected by a remarkable increase in lipid peroxidation. Ruiming et al., observed that MDA concentration was increased it in all plant parts treated with Zn. Lin and Kao (2000) also stated that the MDA amount, which is a secondary end product of polyunsaturated fatty
acid oxidation, is widely used to measure the extent of lipid peroxidation as indicator of oxidative stress. According to Jain et al. (2001) also, the rate of lipid peroxidation in terms of MDA can be used as an indication to evaluate the tolerance of plants to oxidative stress as well as the sensitivity of plants to stress.

Figure 6. Effect of abiotic stress on Malondialdehyde (MDA) activity (µmol g-1 FW) in the leaf of Sesuvium portulacastrum on 72 hrs after treatment. The data are the mean ± SD of 3 replicates

3.5. Determination of Proline (Pro) content

Proline is a basic amino acid found in high percentage in basic proteins. Free proline is said to play role in plants under stress conditions. Proline accumulation is one of the most frequently reported modifications induced by salinity and water deficit in plants (Giridara Kumar et al., 2000). Generally, when plant species having low amounts of proline are grown in well-watered and non-saline soil, may increase the content of this amino acid upon imposition of drought or salt stresses (Teixeira and Pereira, 2007). The results revealed a significant effect of salinity on free proline content. Besides, the stress treatment caused an increase in proline content at all stress levels tested (Fig.7). The control plants maintained a lower concentration (10.87 ± 0.13) of proline in the leaves at all tested abiotic stresses concentrations. A much higher (64.21 ± 0.74) level of proline was noticed in copper treated plant. Other than copper treatment, higher proline
concentration was observed in all the treated plants compared to control. According to Lokhande et al., salt stress was coupled with the higher accumulation of proline. Proline is thought to function as osmoprotectants for proteins (Bohnert and Jenson, 1996). Jaleel et al. (2007) stated that accumulation of proline provides an environment compatible with the macromolecular structure and function and helps plants to adapt to negative consequences of salinity. Proline probably functions as osmolytes in protecting cells from dehydration (Cushman and Bohnert, 2000). Generally, in plants, there is a strong correlation between increased cellular proline levels and their capacity to survive in drought and high environmental salinity (Desingh et al., 2007). Proline is an important osmoprotectant produced in plants in response to cold stresses (Szabados et al., 2010). Recently it was shown to be increased in tall fescue cultivars under drought stress conditions (Man et al., 2011) All these observations indicated that proline accumulation may be the result instead of the cause of stress tolerance. This may also indicate that it was a contributing factor to the inhibited growth of plants under stress condition.

Figure 7. Effect of abiotic stress on proline activity (µmol g-1 FW) in the leaf of Sesuvium portulacastrum on 72 hrs after treatment. The data are the mean ± SD of 3 replicates.
3.6. Antioxidant enzyme activities in response to salt stress

Reactive oxygen species (ROS) are derived from molecular oxygen under biotic and abiotic stress such as salinity, have deleterious effects on cell metabolism. The toxic effect of ROS counteract by enzymatic as well as non-enzymatic antioxidant system. Superoxide dismutase (SOD) has a potential role for elimination of ROS. Halophytes respond to salt stress at different levels and can be a model for increasing salt tolerance in crop plants. Recent studies have shown that even under controlled conditions, salt tolerant plants display a substantially higher abundance of major antioxidant components along with an increased induction of antioxidant enzyme activity and gene expression compared to sensitive plants (Taji et al. 2004; M’rah et al. 2006).

3.6.1. Catalase (CAT) activity

The effect of abiotic stress on the catalase activity in the leaves are presented in (Fig.8 ). Indeed, several experimental observations have conclusively demonstrated the necessity of sufficient CAT activity to cope with the abundant H₂O₂ production that accompanies the high photorespiratory flux (Foyer and Noctor 2003). Rajaravindran and Natarajan (2012) observed that the enzyme activity increased with the increasing salinity upto 600mM in Sesuvium portulacastrum but at higher concentrations, the enzyme activity decreased because plants were not tolerant to the high salinity. Similar results were observed in the present study, where the enzyme activity was decreased when the halophyte was given abiotic stress conditions like drought, heat cold and copper (Fig.8 ). Enhanced activity of catalase was reported to be essential for the survival of the halophytes, Halimions portulacoides in natural saline habitats (Kalirand Poljak, 1981). The catalase activity increased with increasing concentration of NaCl upto optimum level in sand dune plant Ipomoea pes-caprae (Venkatesan and Chellappan, 1999). The catalase activity decreased with increasing concentration in Phaseolus radiatus (Saha and Gupta, 1999).
Figure 8. Effect of abiotic stress on Catalase (CAT) activity (Units g-1 FW) in the leaf of *Sesuvium portulacastrum* on 72 hrs after treatment. The data are the mean ± SD of 3 replicates

3.6.2. Peroxidase (POD) activity

Results showed that in leaves, POD was significantly influenced by abiotic stress \((P=0.5)\) presents in figure 9. Leaves’ POD was higher under all abiotic stresses, compared with control except copper treatment (1.4 ± 0.02). Abiotic stress levels at NaCl, drought, dark, heat, cold, Zn and Cd treatments, caused respectively 1.95 ± 0.03, 1.7 ± 0.15, 1.52 ± 0.25, 1.97 ± 0.25, 2.2 ± 0.04, 2.7 ± 0.05, and 2.67 ± 0.05 units min\(^{-1}\) mg\(^{-1}\) protein (Figure). Increase in peroxidase activity indicated the formation of large amount of H\(_2\)O\(_2\) which could release enzyme from membrane structure (Zhang and Krikham, 1994). Peroxidase is a scavenging enzyme which removes the toxic oxygen radicles from the cells. Manikandan and Venkatesan (2004) noticed significant increase in the peroxidase activity of the halophyte *Aegiceras corniculatum* on giving abiotic stress and same was the case in the salt tolerant varieties of *Xanthosoma sagittifolium* (Kanmegne and Omokolo, 2003). The increased peroxidase activity was mainly due to increased enzyme synthesis and might be useful for adaptation under conditions requiring prevention of peroxidation of membrane lipids (Kalir, 1984). According to Chaoui *et al.* (1997) under Cd and Zn stress conditions, the great elevation of POD activity and the induction of the new anionic isoenzyme bands could be considered as a stem’s reaction to metal-caused oxidative damage. Peroxidases are not only known to be heavy metal stress-related
enzymes (Karataglis et al., 1991) but their increased activity is also a common response to various other stress factors (Gaspar, 1985).

Figure 9. Effect of abiotic stress on Peroxidase (POD) activity (Units min\(^{-1}\) mg\(^{-1}\)) in the leaf of Sesuvium portulacastrum on 72 hrs after treatment. The data are the mean ± SD of 3 replicates.

3.6.3. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) is one of the most important antioxidants, which detoxifies superoxide radicals. The expression of SOD enzymes increases under stress conditions in salt- and drought-tolerant plants. Results showed that SOD was significantly influenced in leaves by abiotic stress \((P=0.5)\). Leaves’ SOD was higher under all abiotic stresses, compared with control. Abiotic stress levels at NaCl, drought, dark, heat, cold, Zn, Cu and Cd, caused respectively 9.0 ± 0.5, 7.6 ± 0.05, 7.51 ± 1.15, 6.85 ± 0.05, 10.13 ± 0.32, 7.5 ± 0.09, 11.14 ± 0.06 and 6.52 ± 0.025 unit mg\(^{-1}\) protein (Fig 10). Taji et al. (2004) reported that the SOD was overexpressed in unstressed conditions in Thellungiella halophile which not only made this species more tolerant to high salinity but also to oxidative stress. It was similar to SOD activity measured in salt
tolerant species such as *Najas gramina* (Rout and Shaw 2001), *Suaeda salsa* (Qiu Fang *et al.* 2005) and *Mesembryanthemum crystallinum* (Slesak *et al.* 2003). In the present study, increased SOD activity under salt stress conditions suggested that, at the primary step, leaves would have efficiently detoxified superoxide radicals. Similarly, a steep increase in the total SOD activity has been recorded in *Bruguiera gymnorrhiza* and *B. parviflora* during salt stress (Parida *et al.* 2004).

![Figure 10](image.png)

**Figure 10.** Effect of abiotic stress on *Superoxide dismutase (SOD)* activity (Units mg-1 protein) in the leaf of *Sesuvium portulacastrum* on 72 hrs after treatment. The data are the mean ± SD of 3 replicates.
3.7. **Protein profiling from Seedling of *S. portulacastrum***:

The total soluble protein concentration varied in different abiotic stress treated *S. portulacastrum*, but no changes were observed in untreated plant (Fig. 11). The total protein content of leaves decreased on being treated with different abiotic stress. This decrease in protein content might be due to the increasing activity of acid and alkaline proteases. Increased levels of free amino acid were also reported, in *B. parviflora* as a result of salt stress (Parida *et al.*, 2002).

![Figure 11: Graphical representation of total protein content isolated through BPP protocol from leaves tissues of *S. portulacastrum* under different abiotic stress treatment](image)

Total proteins were extracted from control and abiotic stress treated plants after 72 hrs of treatment and analyzed by SDS-PAGE to observe the intensity of several protein bands. One of the protein band with molecular weight ~54 kDa was highly expressed in all the stresses compared to the control (Fig. 11). That expressed protein was used for the identification through the LC-MALDI TOF-TOF MS/MS analysis.

When trypsin digested peptide was analysed through Nano-LC, several spectra were visible, among them one spectrum was quite higher compared to others (Fig. 12); that specific spectrum was considered for further analysis i.e. MALDI-TOF/TOF analysis.
Figure 12. Effect of stress treatment on protein levels in stems of *S. portulacastrum*. CBB G-250 stained 10% SDS-PAGE gel showed differently expressed proteins after treatment with different stress: lane 1 to 9: different abiotic stress treatment (dark, Drought, heat, cold, NaCl, Zn, Cu, Cd and control) and lane 10: protein marker.

Figure 13. Spectrum of trypsin digested peptide after separation through Nano LC-MS: in that high peak spectra used for MALDI analysis.
3.8. Protein identification via mass spectrometry

Analysis of protein mass fingerprinting data (PMF) of proteins derived by MS analysis using MASCOT search algorithm showed homology to Ribulose bisphosphate carboxylase large chain from Alluaudia procera.

Database Searching with MS/MS Spectra:

MS/MS spectra were used to search against the NCBI non-redundant protein database using MS/MS Ion Search Engine, a computer software program conducting protein identification based on matching the MS/MS spectra of a protein with a protein or DNA sequence database http://www.matrixscience.com/search_form_select.html. Each MASCOT search output result, the minimum score for significance level was provided, based on the absolute probability and the size of the sequence database being searched. Protein sequence with the significant homologues was retrieved from NCBI database. The consensus pattern within the homologous was detected by performing multiple sequence alignment using CLC sequence viewer version 6.0. The presence of the conserved motifs was detected using conserved domain database and Scan prosite. Sequence analysis for protein Ribulose bisphosphate carboxylase large chain with its significant homologues from MALDI-/TOF/MS was performed. Since the S. portulacastrum genome sequences are not known, a homology based search was performed.

Ribulose bisphosphate carboxylase large chain (RuBisCo) it is involved in calvin cycle. The Calvin cycle (also termed the reductive pentose phosphate pathway) is a metabolic pathway that produced pentose sugars (Heldt, 1997. RuBisCO is a multimeric enzyme with two subunits; large (50-55 kDa) and small (12-18 kDa; Andersson and Backlund, 2008). In this study, the RuBisCO proteins were observed as forming a train of band on with MW of approximately 54. This observation is consistent with results from other proteomic studies. In the pea leaf proteome, Schiltz et al. (2004) observed that RuBisCO proteins formed an abundant train of spots between pH 6-7, at approximately 50 kDa. Similarly in the maize leaf proteome, several RuBisCO large subunits were also
identified between pH 6-7 and MW of approximately 50-56 kDa (Porubleva et al., 2001). Functionally, RuBisCO proteins catalyse carbon fixation (carboxylation) reactions in the Calvin cycle of photosynthetic plants. In this process, ribulose 1,5-bisphosphate (RuBP), a 5-carbon compound serves as an acceptor molecule for CO₂ to form an unstable 6-carbon compound. The 6-carbon intermediate compound immediately breaks down, forming two molecules of 3-phosphoglycerate (3PGA; Kellogg and Juliano, 1997; Tabita et al., 2007; Andersson and Backlund, 2008). The end product of this carboxylation reaction, 3PGA, is phosphorylated by ATP to form 1,3-biphosphoglycerate and ADP. RuBP molecules are regenerated to allow the first carbon fixation step to occur. The regeneration phase is characterised by a series of enzymatic reactions that convert triose phosphate to RuBP (Heldt, 1997; Macdonald and Buchanan, 1997). RuBP is then used as a substrate by RuBisCo in the first phase of carbon fixation. Some of the triose phosphate produced in the Calvin cycle is used for sucrose and starch biosynthesis (Raines, 2003; Tamoi et al., 2005).
MALDI-TOF TOF m/z spectrum of peptides

Mascot Search Results

<table>
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<tr>
<th>Protein hits</th>
<th>Score</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>BRL_AL80</td>
<td></td>
<td>Ribulose bisphosphate carboxylase large chain (Fragment)</td>
</tr>
<tr>
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<td>BRL_AL80</td>
<td></td>
<td>Ribulose bisphosphate carboxylase large chain (Fragment)</td>
</tr>
</tbody>
</table>

Mascot Score Histogram

Ions score is -10*log(P), where P is the probability that the observed match is a random event. Individual ions scores > 44 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.
MALDI-TOF TOF m/z profile of peptides

Figure 14: Identification of proteins from *S. portulacastrum* via MALDI TOF TOF/MS. The protein Spot 1 was excised and digested with trypsin and then collected peptides were analyzed using a Bruker MALDI/TOF/ mass spectrometer. The annotated PMF spectral peaks showed the intensities of different peptide. Database searching with Mascot software against NCBI Inr database identified as Ribulose bisphosphate carboxylase large chain
PART 5

Identification of Salt Stress Responsive Proteins of the Leaf Tissues of *Sesuvium portulacastrum*

1. Introduction

1.1. Defining Proteomics

Proteomics is defined as the large-scale analysis of proteins from a particular organism, tissue or cell (Blackstock and Weir, 1999; Pandey and Mann, 2000; van Wijk, 2001), while the proteome is the expressed protein complement of the genome (Blackstock and Weir, 1999). Unlike the genome that is generally well defined and static over time, the proteome is highly dynamic and constantly changes during development and in responses to both internal and external cues (Heazlewood and Millar, 2003; Speicher, 2004; Komatsu, 2006).

The field of proteomics may be divided into three broad approaches: expression proteomics, cell map proteomics and structural proteomics (Blackstock and Weir, 1999; Ng and Ilag, 2002). Expression proteomics (also termed differential proteomics) is the study of global changes in protein expression. It offers information about specific protein changes in a biological system under specific physiological conditions and in response to various stresses. Cell-map proteomics in the study of protein-protein interactions while structural proteomics is the study of the three-dimensional structure of proteins.

1.2 Why proteomics?

Over the years, several studies on transcriptomics technologies, such as differential display, transcript imaging, serial analysis of gene expression (SAGE) and DNA microarrays (Lockhart and Winzeler, 2000; Zivy and de Vienne, 2000) have been used to measure mRNA expression profiles of different organisms under a range of experimental conditions. Although these technologies provide valuable information about gene expression (Dubey and Grover, 2001), in particular almost which genes are turned on and when (Abbott, 1999), the techniques do not always provide information about the quality and quantity of the final gene products, the proteins (Zivy and de Vienne, 2000).
Comparative analysis between mRNA and protein abundances of different biological systems using a variety of technologies have been reported previously (Carpentier et al., 2008). In the case of banana, there were lack of correlation between mRNA and protein levels from leaf extracts reported (Carpentier et al., 2008). Poor correlation between mRNA and protein levels reported in the many studies could be attributed in part to the different rates of degradation of individual mRNAs and proteins (Salekdeh et al., 2002). Furthermore, many proteins undergo (post translational modification) PTM such as phosphorylation, glycosylation, or proteolytic processing thus giving rise to several isoforms from a single gene product (Abbott, 1999). Post-translational modifications play important roles in regulating the function, subcellular localization and stability of proteins in a cell (Zivy and de Vienne, 2000; van Wijk, 2001; Kersten et al., 2009). Since, it is these post-translationally modified proteins that are functionally active in cellular processes, only the measurement of protein expression itself would thus give a better indication of gene functions at specific physiological states (Dubey and Grover, 2001) as well as an insight into subcellular protein localization (van Wijk, 2001). Although several studies have shown that there is generally a poor correlation between mRNA and protein levels, Carpentier et al. (2008) reinforces the fact that both transcript and protein measurement techniques such as SAGE and 2D gel electrophoresis (2DE) respectively, are complementary to each other as each technique focuses on a subset of proteins. For example, SAGE techniques are more biased towards low abundant and hydrophobic proteins while 2DE is more biased towards highly abundant and less hydrophobic proteins.

1.3. Plant Proteomics

The field of proteomics is increasingly gaining momentum in plant sciences with several studies having been made and reported on agriculturally important crops (; Salekdeh and Komatsu, 2007;). Although there has been major proteomic advances using several other plant species, much of the knowledge gained on plant developmental processes and stress response mechanisms has been gained from work using Arabidopsis and rice (Jorrin et al., 2007; Jorrin-Novó et al., 2009) mainly because of their completed genome sequences, which are publicly available (The Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005). In proteomics, genome
sequences are important resource tools for the identification of proteins. Where fully annotated sequences are not yet available, protein identification can be done through similarity searches of homologous proteins in closely related species (Carpentier et al., 2008). Indeed, a proteomic study on maize for instance, a partially sequenced crop, has relied largely on the limited sequence data and homology based protein identification (Porubleva et al., 2001). Alternatively, expressed sequence tags (ESTs), which represent partial gene sequences can also be used (Aebersold and Goodlett, 2001). However, for the other plant species without significant amounts of published genomic DNA or expressed sequence tags (EST) sequences, protein identification success rates are low resulting in limited proteomic data being available (Jorrin et al., 2007). Amongst the cereal crops, only rice (International Rice Genome Sequencing Project, 2005) and recently, sorghum (Paterson et al., 2009) have been fully sequenced to date. These genome sequences offer invaluable tools for the identification of genes and proteins with potential application in plant breeding approaches for both the increase in yield as well as tolerance to both abiotic and biotic stresses (Salekdeh and Komatsu, 2007).

However, only few proteomic studies on plants dealing with salt stress have been reported, which mainly focused on glycophytes, especially the model plant rice. In rice seedlings under salt stress, some stress-related proteins were identified by proteomic technique, such as auxin and salicylic acid response like protein (ASR1-like protein), ascorbate peroxidase (APX) and cafeoyl-CoA O-methyltransferase, UDP-glucose pyrophosphorylase (UDPG), cytochrome c oxidase subunit 6b-1, and glutamine synthetase, etc (Salekdeh et al., 2002; Abbasi and Komatsu, 2004; Kim et al., 2005). In another proteomic analysis targeted on pea (Pisumsativum) root under salt stress, pathogenesis-related proteins (PR10 proteins) were first identified as potential roles in salinity stress responses (Nat et al., 2004). It was hypothesized that glycophytes have most salt tolerance genes of halophytes, and use similar regulatory pathways and salt tolerance effectors (Zhu, 2000). To identify salt tolerance mechanisms of halophytes, a proteomic analysis was performed with a halophyte, Suaeda Aegyptiaca. About 27 proteins were identified in Suaeda aegyptiaca leaves treated with different salt levels. Among these proteins, cyanas is involved in cyanide detoxification, which is a new mechanism related to salt tolerance (Hossein et al., 2006). This was the first report on
proteome patterns of a halophyte plant and its response to salt treatments, which offered significant meanings on mechanisms of plant to salt tolerance. The protein patterns of a halophyte, *C. virgata* (Swartz) responding to carbonate stress by two-dimensional electrophoresis (2-DE) and MS analysis have also been reported. *C. virgata*, also known as feather finger grass, is an annual C4, herbaceous graminoid native to warm temperate regions worldwide (Hickman, 1993).

### 1.4. Challenges in Plant Proteomics

In proteomics studies, high-resolution gels with minimal spot streaking and overlap (Thelen and Peck, 2007) are essential to facilitate comparative gel analysis using 2D analysis software (Marengo *et al.*, 2005) and the downstream protein identification via mass spectrometry and database searching. However, obtaining highly resolved gels is somewhat challenging unless factors that affect their resolution are well optimized. These factors include protein extraction and solubilisation procedures, protein separation on both the first and second dimension, as well as protein detection through MS methods used (Rose *et al.*, 2004; Hurkman and Tanaka, 2007).

### 1.5. Protein separation thorough 2D gel electrophoresis

Two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) remains the method of choice for separating proteins in complex mixtures (Dunn and Gorg, 2001; Rabilloud, 2002) because of its ability to separate hundreds or thousands of proteins at a time (Gorg *et al.*, 2000) as well as different protein isoforms (Carpentier *et al.*, 2008). This electrophoresis system separates proteins in two dimensions. In the first dimension, proteins are separated on the basis of their isoelectric point (pI) by isoelectric focusing (IEF). After IEF, proteins are equilibrated in sodium dodecyl sulfate (SDS) containing buffers in preparation for the second dimension as well as to improve protein transfer from the first to the second dimension (Gorg *et al.*, 2000). In the second dimension, the isoelectric focused proteins are separated on the basis of their molecular weight (MW) by SDS-PAGE. Different gel concentrations may be used depending on sizes of the proteins to be resolved. After 2DE, separated proteins are detected in the gels using protein stains and subsequently identified by mass spectrometry (MS) and searching the selected protein against databases of known protein sequences (e.g. SwissProt, TrEMBL, NCBI).
1.6. Identification of protein using Mass Spectrometry

Mass spectrometry is defined as the accurate mass measurement of charged analytes (Patterson and Aebersold, 2003) and is widely used for the identification of proteins in proteomics. Mass spectrometers measure mass to charge ratios (m/z) of charged molecules. Mass spectrometers have three main components; an ionization source, a mass analyzer and a detector (Patterson et al., 2001) and they are named on the basis of their ionization source and mass analysers (Patterson, 2000). Two most commonly used mass spectrometers include the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and tandem mass spectrometry (MS/MS).

1.6.1. Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF)

MS

In MALDI-TOF MS (reviewed in Westermeier, 2005), the ionization source is the MALDI while the TOF is the mass analyser. Matrix-assisted laser desorption/ionization time of flight mass spectrometry is mainly used for protein identification coupled with peptide mass fingerprinting (PMF). Proteins from gel spots are digested using trypsin and the resultant peptides are mixed with a matrix such as α-cyano-4-hydroxy cinnamic acid or 2,5-dihydroxybenzoic acid (DHB). Matrices are low molecular compounds that form crystals, absorb UV and release a proton in the process (Patterson, 2004). The peptide matrix mixtures are spotted onto a metal slide, dried and then inserted into a vacuum chamber of the mass spectrometer. When high voltage is applied to the slide, the matrix molecules absorb energy and move from the plate into a gas phase together with the peptides. The generated ions are delivered to the mass analyser (TOF) which measures the time elapsed from acceleration of the charged molecules through a field-free drift region. In general, the smaller lighter ions reach the detector faster than the heavy ones and recorded flight times are used to calculate the mass to charge ratios (Westermeier, 2005).

1.7. Aim of the Research

The aim of this research project is to work on both descriptive and differential expressional proteomics of a halophyte, *S. portulacastrum* in response to salinity stress. In proteomic studies, whole plant systems offer tissue specific protein expression
profiles. For descriptive proteomics, the main objective was to extract, separate, identify and map the proteins that are expressed in the leaf tissue of *S. portulacastrum*. In differential expressional proteomics, the main objective was to identify salt (NaCl) stress responsive proteins in the leaf tissue and compared with salt stress responsive proteins of control leaf tissue (without salt), which have different levels of salt tolerance. The proteome expression data obtained in this study would be used as a pioneering *S. portulacastrum* reference tool for use in cereal proteomics. In addition, the identification of salt stress responsive proteins would lead to the identification of expressed *S. portulacastrum* genes that could possibly be used as candidate targets in genetic engineering approaches for the development of salt stress tolerant crops in future studies. The objectives of this chapter are to (i) isolate, separate, visualise and identify salt stress responsive proteins in the leaf, root and stem tissues of *S. portulacastrum*, (ii) compare the expression profile of salt responsive protein in *S. portulacastrum*. 
2. Materials and Methods

2.1. I. Protein Profiling of Sesuvium portulacastrum treatment with different concentrations of salt

A. Salt stress treatment and protein extraction from plant tissues in greenhouse condition

The halophyte S. portulacastrum collected from the Vellar estuary were planted and maintained in the greenhouse. Plants were maintained in triplicates and sequentially watered in the morning and evening with distilled water (control), 50mM, 100mM, 150mM, 200mM, 250mM, 300mM, 350mM and 400mM NaCl (Fig. 1.a.). The plant leaves were collected after three weeks and roots and stem were collected after 4 weeks of treatment, for protein extraction.

B. Salt stress treatment and protein extraction from the leaf, stem and root tissues of Sesuvium portulacastrum in hydroponic condition

S. portulacastrum seeds were collected from mature plants obtained from the different collection areas mentioned in the survey regions. The seeds were germinated in plastic pots containing vermiculated garden soil and the plants were grown under greenhouse conditions. After two months, the seedlings were carefully uprooted and transferred to hydroponic conditions (1/2 Murashige and Skoog medium stock) in a culture room with a dark/light cycle of 8/16 h at 37 °C for one month. The plants were treated with various concentrations of NaCl such as 300mM, (4h, 8h, 12h, 16h, 24h, 48h, 72h) 0.5M & 1M (3h, 6h, 9h) for different time intervals (Fig. 1.b). Upon completion of the treatments, the leaves, stem and root tissues were separately collected, frozen in liquid nitrogen and stored at −80°C for further analyses.

2.2. Protein Extraction from Whole Plant Systems

Leaf, stems and root tissues were separately ground in liquid nitrogen using pestle and mortar. For protein extraction, modified BPP method was followed as described in chapter 2 section 2.5.
2.3. Dialysis and clean – up of protein samples:

Dialysis of the protein samples was done using 50 μm thick dialysis membrane (Himedia) against the dialysis buffer containing 20mM Tris-cl, 100mM NaCl and 3mM DTT (pH8.0) with three times changing the buffer for an interval of 4 hours in a cold room. After dialysis, the sample was carefully transferred to an eppendorf tube and further processed with a clean – up kit (BioRad) following the protocol given in the kit for the purpose of concentrating the sample.

![Figure 1.a. Plant acclimatized greenhouse condition for 1 month](image)

2.4. Quantification of Proteins:

Protein concentration was determined using the Bicinchoninic Acid protein Assay kit (BCA, Sigma) following the protocol supplied along with the kit using the LAMBDA 25 UV/Vis Spectrophotometer(PerkinElmer). Bovine serum albumin (BSA) was used as the standard (100 μg/μl) and the absorbance was measured at 562nm.

2.5. One dimensional protein profiles of *Sesuvium portulacastrum* leaf, stems and root tissues following salt stress

One dimensional (1D) gel electrophoresis (described in chapter 2 section 2.7 & 2.8) was carried out to evaluate the quality and loading quantities of the protein extracts prior to 2D gel electrophoresis.

II. Leaf protein profile on time dependent changes in leaves of *S. portulacastrum*

2.6. Plant material and saline treatment - Time course study:
The seeds were germinated and grown in a soil mixture containing shore sand and vermiculated manure in a 1:1 ratio in small germination plastic pots. Plants cultivated in soil were watered every day and supplemented on alternate days with a $\frac{1}{2}$ Murashige and Skoog media. Two months old plantlets were carefully transferred to hydroponic conditions ($\frac{1}{2}$ Murashige and Skoog medium) and maintained in a culture room with a dark/light cycle of 8/16 h at 37 °C for two weeks. Further the plants were exposed to three different concentrations of NaCl (300mM, 0.5M & 1M) and subjected to time course study. Nutrient solution was replaced every alternate day in order to avoid depletion of nutrients. Upon completion of the treatments, the leaves, stem and root tissues were separately collected, frozen in liquid nitrogen at −80°C for proteomic analysis.

Figure 2. A. Greenhouse condition B. Hydroponic conditions

2.7. Effect of salinity on plant growth: The seeds of *S. portulacastrum* collected from mangrove associated Vellar estuary with the water salinity 26 ppt, was grown in appropriate and controlled environmental conditions in a greenhouse (Fig. 4.10. A). During hydroponic acclimatization (Fig. 4.10. B), the waxy coat of the *S. portulacastrum* was reduced due to the sudden change in growth condition, which takes at least 4 days for its gradual recovery. Plants that were treated with NaCl above their threshold levels such as 400mM and 500mM for 1, 2 and 3 days, developed several stress-related morphological phenotypes in leaves such as thickening of the leaves and wax
depositions in the cuticle. Morphological changes were observed which were rapid and showed instantaneous growth rate. Similar experiments were conducted in maize by (Cramer and Bowman, 1991), where a rapid and transient reduction in the rate of leaf expansion after sudden increase in salinity was observed. This might be due to changed water relationship and not the presence of Na\(^+\) and Cl\(^-\) (Passioura and Munns, 2000).

2.8. Two-Dimensional (2D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.8.1. Protein Load for 2D Gels
The amount of protein extracts applied on 2D gels varied with sample complexity as well as the length and pH range of immobilized pH gradient (IPG) strips used for IEF.

2.8.2. Rehydration of 7cm IPG Strips
Protein extracts (100-150 µg) were mixed with 0.8% (v/v) DTT, 0.2% (v/v) ampholytes (BIO-RAD), and a tiny pinch of bromophenol blue and made up to a final volume of 125 µl using urea buffer. The samples were mixed by vortexing for 5 min, pulse centrifuged and then placed in individual channels of an Immobiline Dry Strip Reswelling Tray (BIO-RAD). Linear, 7 cm IPG strips of pH range 3-10 (BIO-RAD) were carefully placed on top of the sample, gel side being directly in contact with the sample, avoiding trapping any air bubbles in the process. The strips were then covered with mineral oil (BIO-RAD) to prevent sample evaporation during the rehydration process and left to passively rehydrate to their original gel thickness of 0.5 mm for at least 15 hrs at room temperature.

2.8.3. First Dimension IEF of IPG Strips
After rehydration, IPG strips were briefly rinsed with distilled water to remove unabsorbed protein sample and carefully blotted with moist filter paper. The strips were then placed gel side up on the focusing platform of a PROTEIN IEF Cell (BIO-RAD). Distilled water moistened wicks (BIO-RAD) were placed at the extreme ends of both the anode and cathode ends of the IPG strips to collect excess salts and impurities from the sample during focusing. The IPG strips were then covered with mineral oil to avoid sample evaporation and carbon dioxide absorption during focusing. Isoelectric focusing
was performed in a three phase stepwise programme at 20°C as indicated in table 1. for 7 cm IPG strip.

After IEF, 7 cm IPG strips were either equilibrated (Section 2.9.4.) straight away or stored in 15 ml Falcon tubes at -20°C until use for the second dimension SDS-PAGE (Section 2.10.6).

Table 1. Isoelectric focusing programme for 7 cm IPG strips.

<table>
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<th>Step</th>
<th>Volts (V)</th>
<th>Duration (hrs)/Volt hours (Vhrs)</th>
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<td>0.15 hr</td>
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<tr>
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</tr>
<tr>
<td>3</td>
<td>4000</td>
<td>12 000 V-hrs</td>
<td>20°C</td>
</tr>
</tbody>
</table>

2.8.4. Equilibration of IPG Strips

After IEF, IPG strips were equilibrated in SDS-containing buffers in order to solubilize focused proteins and allow SDS binding prior to second dimension SDS-PAGE. The focused IPG strips were incubated gel side up in reswelling tray channels containing 2.5 ml equilibration buffer [6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8 and 20% (v/v) glycerol], initially containing 2% (w/v) DTT for 15 min followed by 2.5% (w/v) iodoacetamide for another 15 min with gentle agitation at room temperature. After equilibration, the isoelectric focused proteins were ready for separation on second dimension SDS-PAGE of mini format gels.

2.8.5. Second Dimension SDS-PAGE of Mini Format Gels

Mini format 2D SDS-PAGE gels were cast on 10.1 cm (width) x 8.3 cm (height) spacer glass plates (BIO-RAD) mounted with 1 mm thick spacers using the Mini-PROTEAN Casting Chamber (BIO-RAD) which accommodates twelve 1.0 mm glass plates. Hundred milliliters of a 10% (v/v) resolving gel solution was prepared as described in chapter 2 section 2.7, for separation of proteins (Fig. 13). The resolving gel solution was poured into the cast plates according to the instruction manual and each gel was overlaid with 1 ml of 100% isopropanol. The gels were left to polymerize for 1 hr at room temperature. After polymerisation, the isopropanol overlay was rinsed off with distilled water and gel surfaces were blotted dry with filter paper.
Equilibrated 7 cm IPG strips (above mentioned in Section 2.9.4) were gently rinsed with 1X SDS-PAGE running buffer and placed on top of mini format 10% SDS-PAGE resolving gels with the plastic backing against the spacer plate. The IPG strips were then overlaid with 1 ml of 0.5% (w/v) overlay agarose (BIO-RAD) prepared in 1X SDS-PAGE running buffer containing a tint of bromophenol blue, which was used as a migration tracking dye during electrophoresis. Electrophoresis was carried out at 100 V during the first 30 min and then at 150 V until the bromophenol dye reached the bottom of the glass plates. After the second dimension, gels were stained with CBB (Section 2.10).

2.9. Coomassie Brilliant Blue Staining in SDS-PAGE Gels
Proteins separated by 2D SDS-PAGE were routinely detected using a modified CBB G-250 staining protocol as mentioned in chapter 1, table 1.4 and distained as described in chapter 1, table 1.5).

2.10. Comparative Analysis of 2D SDS-PAGE Gels
Comparative analysis of 2D SDS-PAGE gels within defined experiments was done using the PDQuestTM Advanced 2D Analysis Software version 8.0.1 build 055 (BIO-RAD). Two-dimensional gels were initially imaged using the Molecular Imager PharosFX Plus System (BIO-RAD) and then analyzed according to the PDQuestTM Advanced 2D Analysis Software user manual (BIO-RAD). The pI and M_r of each protein was determined using 2D-PAGE markers.

2.11. Image acquisition and data analysis:
Spot detection, spot measurement, background subtraction and spot matching were performed specifically after CBB staining of the gels using PDQuest software. Following automatic spot detection, gel images were carefully edited. Before spot matching, one of the gel images was selected as the control gel. The resulting data from image analysis was transferred to PDQuest software for querying protein spots, which show quantitative or qualitative variations. The apparent M_r of each protein in gel was determined by referencing to the protein markers (Fig.14)

2.12. Protein Identification using MALDI-TOF MS
Coomassie Brilliant Blue stained gels were imaged using the Molecular Imager PharosFX Plus System (BIO-RAD) and the experimental MW and pI of proteins of interest were estimated from the gels. Protein spots were either picked manually with pipette tips or automatically using ExQuestTM spot cutter (BIO-RAD) or transferred into sterile microcentrifuge tubes. Proteins were characterised according to a method described by Shen and colleagues (2003). Briefly, individual protein spot was excised from the gel, and destained twice with 50% acetonitrile (ACN), 50mM ammonium bicarbonate at 37 °C for 30 min, dehydrated by adding 10 µl ACN, and dried. The proteins in the gel slices were reduced with 10 mM DTT in 100 mM ammonium bicarbonate for 1 h, and incubated in the solution containing 40mM iodoacetamide and 100mM ammonium bicarbonate for 30 min at room temperature. Each gel slice was minced, lyophilized, and rehydrated in 100mM ammonium bicarbonate containing 5 pmol trypsin (trypsin, modified, sequencing grade, Roche Applied Science, Penzberg, Germany) overnight at 37°C. Tryptic peptides were extracted two times with 20 µl of 50% ACN, 0.1% formic acid (FA) for 30 min at 37 °C, and 10 µl of ACN for 30 min at room temperature. After each extraction, samples were centrifuged at 1000xg for 30s (short spin) and all the supernatants were combined, vacuum-dried, and stored at -80°C until MS analysis.

Matrix was prepared by dissolving α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA, USA) in 50% ACN and 0.1% TFA. One microliter from each digested protein sample was mixed with the same volume of α-cyna-hydroxy-cinnamic acid (matrix) and spotted onto an Anchorchip target plate (600/384F, Bruker Daltonics). The dried sample on the target plate was washed with 1 mL of 0.1% TFA twice, left for 30 s before solvent removal, and dried for analysis by MALDI-TOF MS using an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics) to generate peptide mass fingerprints. The MALDI TOF was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential with optimized parameters. Peptide spectra of accumulated 1,200 shots each were automatically processed for baseline correction, noise removal and peak deisotoping. The threshold was manually adjusted to 2-8% base peak intensity. All searches were performed against the
National Center for Biotechnology Information (NCBI) and Mass spectrometry protein sequence database (MSDB) using MASCOT (http://www.matrixscience.com/search_form_select.html. Candidate protein matches with molecular weight search (MOWSE) scores higher than 66 (p<0.05) were considered as positive identifications. In addition, positive protein assignments required at least 10% sequence coverage. In cases where more than one protein satisfied the above mentioned threshold criteria, the entry with the highest MOWSE score was considered as a probable positive identification.

3. Results and Discussion:
Soil salinity, by inhibiting growth and crop yield, is a severe and increasing constraint on agricultural productivity. The study of plant salt tolerance with a view to identifying and eventually manipulating the genes involved in salt perception and responses, seems to be a promising approach to overcome this constraint (Mahajan and Tuteja, 2005; Munns, 2005). The evolution of salt tolerance represents a fascinating case study in the evolution of a complex trait and salt-tolerant plants (halophytes) employ several different mechanisms to deal with salinity stress.

The diversity of mechanisms employed by halophytes, based on processes common to all plants, sheds light on the way a plant’s physiology can become adapted to deal with extreme conditions. As the amount of salt-affected land increases around the globe, understanding the origins of the diversity of halophytes ought to provide a basis for the use of novel species in bioremediation and conservation.

To survive salt stress, plants develop complex mechanisms, including developmental, morphological, and physiological and biochemical strategies. The products of the salt responsive genes may provide salt tolerance either directly or indirectly and ultimately lead to plant adaptation and help the plant to survive and overcome the salt stress condition. Therefore, it is essential to analyse the function of stress-induced genes not only to understand the mechanism of stress tolerance but also to improve the stress tolerance of crops by manipulating such genes (Munns, 2005; Yamaguchi and Blumwald, 2005). Although the general response to salt stress is similar in all plants, ‘halophytes’ have evolved with unique mechanisms or regulatory pathways that are not found in glycophytes (Wong et al., 2006/2005).

I. Protein Profiling of Sesuvium portulacastrum on treatment with different concentrations of salt

In the above experiments, the protein content was found to be more in leaf tissues than in stem and root (Fig. 3.A & 3.B). This is due to the abundance of Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase) in leaf tissues, which makes the proteins facile to be isolated by the BPP method (Saravanan & Rose, 2004).

Protein profiling of leaves, roots and stems was carried out after the experimental duration to identify the differentially expressed proteins under different salt treatment in greenhouse condition. The figure 4 shows the protein profile of leaves where the
expression of two proteins of molecular weight ~54kDa and ~15kDa was found to be prominent in the wild plant that was taken from the native area than the control and experimental plants. Similarly in root, proteins of molecular weight ~23kDa, 30kDa & 37kDa were differentially expressed (Fig.5). In stem, not much changes in protein profile was observed, but a protein with molecular weight of 22kDa was found in 50mM NaCl treated plant, which has to be further characterized (Fig. 6).

Protein profile of the leaves, roots and stems were analysed to identify differentially expressed proteins in response to different salt concentrations under hydroponic conditions. The protein profile of the leaves exhibited the presence of a peptide of molecular weight ~54 kDa (Fig. 7). Similarly in root, ~15 kDa protein was found to be differentially expressed (Fig. 8). In stem, not much changes in protein profile was observed, but a protein with molecular weight of ~54 kDa was found similar to that of leaf in NaCl treated plant, which has to be further briefly analyzed and characterized (Fig. 9).

Protein extracted from the leaf of *S. portulacastrum* showed changes in abundance, in response to different salt stress such as 0.5M and 1M for different time intervals, when compared with the non-stressed condition at 0 hour. Maximum expression was observed in 0.5 and 1M (3h) NaCl treatment. As the treatment time increased, the expression of protein at ~54 kDa & ~15kDa showed significant reduction, which can be due to high levels of salt that makes the plant less compatible to survive in such stressful condition (Fig. 10).

*Figure 3. (A and B):* Graphical representation of total protein content isolated through BPP protocol from different tissues of *S. portulacastrum* under different NaCl treatment
A. Protein profiling from Leaves, Root, Stem of *S. portulacastrum* grown in Green house

**Figure 4.** Effect of salt treatment on protein levels in leaves of *S.portulacastrum*. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl.; lane 1: protein marker; lane 2: wild plant (*S.portulacastrum*); lane 3: control plant grown in distilled water in garden soil; lane 4: plant treated with distilled water in shore soil; lane 5 to 8: Salt treatment (50mM, 150mM, 250mM, 350mM).

**Figure 5.** Effect of salt treatment on protein expression in roots of *S.portulacastrum*. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl; lane 1: protein marker; lane 2: wild plant; lane 3: control plant (distilled water + normal soil); lane 4: plants treated with distilled water in shore soil; lane 5-7: salt treatment (50mM, 150mM & 250mM).
B. Protein profiling from Leaves, Root, Stem of *S. portulacastrum* grown in Hydroponic condition

**Figure 6.** Effect of salt treatment on protein levels in stems of *S. portulacastrum*. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl: lane 1: protein marker; lane 2: wild plant; lane 3: control plant (distilled water + normal soil); lane 4: plants treated with distilled water in shore soil; lane 5-7: salt treatment (50mM, 150mM & 250mM).

**Figure 7:** Effect of salt treatment on protein levels in leaves of *S. portulacastrum*. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl. lane M: protein marker; lane 1-7: 300mM salt treatment at different time intervals (4hr -72hr); lane 8 -10: direct salt treatment (300mM NaCl) for 4hr to 8 hr; lane C10: control plant grown in ½ MS media.
Figure 8: Effect of salt treatment on protein levels in roots of *S. portulacastrum*. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl. Lane M: protein marker; lane 1-7: 300mM salt treatment at different time intervals (4hr -72hr); lane 8 -10: direct salt treatment (300mM NaCl) for 4hr to 8 hr; lane C10: control plant grown in ½ MS media.

Figure 9: Effect of salt treatment on protein levels in Stems of *S. portulacastrum*. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl. Lane M: protein marker; lane 1-7: 300mM salt treatment at different time intervals (4hr -72hr); lane 8 -10: direct salt treatment (300mM NaCl) for 4hr to 8 hr; lane C10: control plant grown in ½ MS media.
C. Concentration dependent expression of protein in leaves of *S.portulacastrum*

![Figure 10](image)

**Figure 10.** Effect of salt treatment on protein levels in leaves of *S.portulacastrum*. CBB G-250 stained 10% SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl; lane M: protein marker; lane C1: control plant grown in ½ MS media; lane C2: control plant grown in vermiculated soil; lane 1-7: 0.5 and 1M NaCl treatment at different time point (3hr-9hr)

II. Leaf protein profile on time dependent changes in leaves of *S. portulacastrum*

![Figure 11](image)

**Figure 11.** Time dependent changes in leaf protein levels of *S.portulacastrum* under salt stress conditions. The values shown are the means of (± S.D.) of triplicate *S.portulacastrum* plants treated with 0.5M and 1M NaCl.
Time dependent analysis on leaf protein profile of *S. portulacastrum*

**Figure 12. Time dependent changes in** the protein profile of leaves of *S. portulacastrum* in 10% SDS-PAGE. Lane M: protein marker; lane C1: control plant grown in ½ MS media; lane C2: control plant grown in vermiculated soil; lane 1,3,5: 0.5 M treatment at different time point (3hr,6hr,9hr) and lane 2,4,6 : 1M NaCl treatment at different time point (3hr,6hr,9hr).

**Figure 13.** 2-DE of leaf protein profile of *S. portulacastrum* in 3-10IPG strip (A) Control plant grown in 1/2 MS media and (B) Plant treated with 300mM NaCl and proteins from leaves were extracted and separated by 2-DE. Duplicates were performed for each sample.
Six week old plants were treated with 300 mM NaCl for different time periods. The Cl⁻ content in salt treated halophytes is regulated differently in leaves and in roots with minimum and maximum accumulations occurring in the leaves (Yamanaka, 2009). Since morphological and physiological differences are prominent in leaves that may play important roles in salt tolerance, proteome analysis was performed in leaves from salt-treated *S. portulacastrum*. Total proteins were extracted after 0 h and 24 h treatment with threshold level of NaCl, 300 mM. Proteins were then separated by 2-DE, and those proteins spots showing reproducible changes were selected and their expression patterns were analysed. 2D-PAGE analysis from proteins extracted from leaves, revealed the differential expression of 59 protein spots which showed significant difference in their abundance between control and treated samples (Fig. 14). Among the 59 spots, 46 were newly expressed upon salt stress, 6 were up regulated and 7 were down regulated (Table 2.). Most distinct two different newly expressed protein spots were selected and further functional characterization was performed through MALDI-TOF/MS.
Table 2: Differentially expressed proteins from leaves of *S. portulacastrum* on treatment with 300mM NaCl

<table>
<thead>
<tr>
<th>Leaves of <em>S. portulacastrum</em></th>
<th>Differentially expressed proteins under salt treatment</th>
<th>Match between control and treated plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Up regulated</td>
<td>Down regulated</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Of a total of 150 differentially accumulated protein spots, only 44 spots were consistent from which 2 newly expressed protein spots were selected for MALDI-TOF analysis.

**Protein identification via mass spectrometry**

Analysis of protein mass fingerprinting data (PMF) of two proteins (spot 1 & spot 2) derived by MS analysis using MASCOT search algorithm showed homology to heat shock protein 70 (Hsp70) from *Cucumis sativus* and theta subunit of T-complex protein from *Coccomyxa subellipsoidea* C-169 (Table 3).

Table 3: Proteins identified from leaves of *S. portulacastrum* via MALDI-TOF/TOFMS

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Access. No</th>
<th>Protein name</th>
<th>TOF/TOF sequence</th>
<th>Mr</th>
<th>pI</th>
<th>Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>6911551</td>
<td>Heat shock protein 70</td>
<td>K.TTGQKNK.I</td>
<td>71kDa</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>384251890</td>
<td>T-complex protein, theta subunit</td>
<td>K.FDL.R.R</td>
<td>54kDa</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Two spots analysed via MALDI-TOF/MS characterized it as hsp70 of *Cucumis sativus* and T complex protein of *Coccomyxa subellipsoidea* C-169.
Database Searching with MS/MS Spectra:
MS/MS spectra were used to search against the NCBI non-redundant protein database using MS/MS Ion Search Engine, a computer software program conducting protein identification based on matching the MS/MS spectra of a protein with a protein or DNA sequence data base [http://www.matrixscience.com/search_form_select.html](http://www.matrixscience.com/search_form_select.html). A single protein having a higher score than the minimum score for the significance level \((p < 0.05)\) was judged as a significant match. In each MASCOT search output result, the minimum score for significance level was provided, based on the absolute probability and the size of the sequence database being searched. Protein sequence with the significant homologues was retrieved from NCBI database. The consensus pattern within the homologous was detected by performing multiple sequence alignment using CLC sequence viewer version 6.0. The presence of the conserved motifs was detected using conserved domain database and Scan prosite.

Sequence analysis for protein spot 1 (Fig.15), hsp70 with its significant homologues from MALDI-/TOF/MS was performed. Since the *S. portulacastrum* genome sequences are not known, a homology based search was performed. Multiple sequence alignment with their respective homologues revealed 98% consensus pattern. Functional annotation done using Scan prosite revealed the presence of three hsp70 protein signature patterns, of which penta-peptide conserved signature was present in N terminal region and the other two motifs was conserved in the central part of the sequence.

Heat shock protein 70 is a known stress responsive protein (Ndimba *et al.*, 2005), and is expressed in response to abiotic stresses such as heat, cold, drought, salinity and oxidative stress (Wang *et al.*, 2004). In this study, it was observed that *S. portulacastrum* leaves had basal levels of Hsp70 (approximately MW=77 kDa) prior to salt stress albeit in at varying levels. Following salt stress treatment using 300 mM NaCl, Hsp70 abundance levels newly synthesized in leaves tissues were with compared to control in order to resolve the Hsp70 protein and confirm their identities using MALDI-TOF MS and database searches (NCBI). The analysis showed that the protein spot 1 functions as
chaperones for newly synthesized proteins to prevent their accumulations as aggregates and folds in a proper way during their transfer to their final location (Su and Li, 2008).

Prokaryotic and eukaryotic organisms respond to heat shock or other environmental stress by the induction of the synthesis of proteins collectively known as heat-shock proteins (hsp). Hsp70 is a multifunctional protein that plays an important role in the transport of protein across membranes, in protein folding and in the assembly/disassembly of protein complexes can direct incompetent "client" proteins towards degradation.

Plant Hsp70 have identifiable motifs at their C terminus that target them to different subcellular compartments (Guy and Li, 1998). As such, Hsp70 are localized in the cytosol, chloroplast, mitochondria and the endoplasmic reticulum (Kiang and Tsokos, 1998; Sung et al., 2001; Wang et al., 2004). Structurally, Hsp70 consists of a highly conserved N-terminal ATPase domain of approximately 44 kDa and a C-terminal peptide-binding domain of approximately 25 kDa (Wang et al., 2004). Two different forms of this protein exist; the constitutively expressed heat shock cognate 70 (Hsc70) and the stress induced Hsp70. (Miernyk, 1997;). The constitutively expressed Hsc70s play a role in the folding of de novo synthesized proteins, the import and translocation of precursor proteins, as well as targeting damaged proteins for degradation ( ). On the other hand, stress induced Hsp70s prevent the aggregation of stress denatured proteins and also facilitate the refolding of proteins to restore their native biological functions (Sung et al., 2001; Wang et al., 2004).

Regarding HSPs, many researchers (Flom et al., 2006; Chan et al., 2006; Guyomarc'h et al., 2004; Kurek et al., 2002; Izhaki et al., 2001) confirmed that protein sti1 appeared to be up-regulated in response to salt stress and this protein contains two heat shock chaperonin binding motif (STI1), three tetratricopeptide repeat (TPR) and two Sti1 domains. The up-regulation of this regulatory protein may decrease the sterility of pollen during development. It is believed that Hsp interacts with TPR-containing proteins to modulate diverse cellular processes through protein–protein interaction. Studies carried out on Zea mays, showed that mitochondrial smHSPs improved mitochondrial electron transport during salt stress, mainly by protection of the NADH: ubiquinone
oxidoreductase activity (Complex I), but it failed to protect enzymes associated with Complex II.

Heat shock protein 70s, are encoded by a multi-gene family (Sung et al., 2001). Their expression patterns respond to a wide range of abiotic factors such as heat, cold, drought, salinity and oxidative stress (Wang et al., 2004). The Hsp70 functions as chaperones for newly synthesized proteins to prevent their accumulations as aggregates and folds in a proper way during their transfer to their final location (Su and Li, 2008). Furthermore, Hsp70 and sHsps primarily act as molecular chaperone and play a crucial role in protecting plant cell from the detrimental effects of heat stress (Rouch et al., 2004) and Hsp70 and sHsp17.6 might play a crucial role in the development of cross-adaptation to temperature stress induced by heat acclimation (HA) - or cold acclimation (CA) pretreatment in grape plants (Zhang et al., 2008). Cooperation in the activities of this class (folding of proteins) and small heat-shock proteins such as sHsp18.1 (prevention of aggregation of proteins) was reported in a study of P. sativum (Lee and Vierling, 2000). Hsp70 participates, also, as a part of guidance complex import (translocon) that bound to protein precursor to be transferred through the membranes into the organelles such as chloroplast (Jackson-Constan et al., 2001; Soll, 2002).

There is some indication that Hsp70B found in the stroma of chloroplasts participate in photo protection and the repairing of photosystem II during and after the photoinhibition (Schroda et al., 1999). A more recent study on A. thaliana indicated the necessity of Hsp70 found in the stroma of chloroplast for the differentiation of germinating seeds and its tolerance of heat (Su and Li, 2008).

The expression of Hsp70 genes correlates positively with the acquisition of thermo-tolerance (Lee and Schoffl, 1996/1996) and results in enhanced tolerance to salt, water and high temperature stress in plants (Cho and Hong, 2006) and for this reason Hsp70 has been proposed as potential biomarker (Ireland, 2004).

As demonstrated for most stresses taken into account, Hsp 70 is confirmed as biomarker of stress produced by NaCl in marine macroalgae and fresh water plant species (Ireland et al., 2004), which emphasized its role in protecting plants against stress.

The deduced amino acid sequence was compared with the homologues of hsp70 obtained from MALDI/TOF/TOF Mascot software for S. portulacastrum (Fig. 16).
Sequences were aligned using the CLC sequence viewer program. Gaps have been introduced to least level to optimize the alignment. Consensus amino acids are highlighted in black boxes. All three conserved hsp70 protein family motif are given in asterix. The sources of the proteins and gene bank accession numbers are as follows, *Cucumis sativus* (gi|6911551), *Ricinus communis* (gi|255575054), *Vigna radiate* (gi|45331283), *Gossypium hirsutum* (gi|211906496) and *Arabidopsis lyrata subsp. lyrata* (gi|297810345).

Similarly, the protein spot 2 (Fig. 17) which is the T-complex protein, theta subunit showed least significant among the homologues obtained via MALDI-/TOF/MS. This Protein also functions as molecular chaperon that assist in folding of proteins upon ATP hydrolysis in protecting plant cell from the detrimental effects of stress. Until now, there is not much data available on the functional specificity of theta protein on salt stress.

But many reports showed that T-complex protein (TCP) participate in the folding of newly translated proteins in the cytosol, including tubulins and actins (Stuart et al., 2011; Yaffe et al., 1992). Consistent with this finding, an actin-1 and two tubulins (alpha and beta chains) were also found to be enriched at similar levels in both rhizome tissues. Actins and tubulins have been reported to accumulate in meristematic tissues (Holmes et al., 2006) and are involved in cell elongation (Li et al., 2005; Whittaker and Triplett, 1999).
**MALDI-TOF m/z profile of peptides peak**

**Mascot Search Results**

User: Arun Kumar
Email: arumarinebiotech@gmail.com
Search title: NCBInr 20130310 (23641837 sequences; 8123359852 residues)
Taxonomy: Other green plants (998231 sequences)
Timestamp: 12 Mar 2013 at 06:50:41 GMT
Top Score: 124 for gi|6911851, heat shock protein 70 [Cucumis sativus]

**Mascot Score Histogram**

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 73 are significant (p<0.05).

**Protein Summary Report**

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Figure 15: Identification of proteins from *S. portulacastrum* via MALDI TOF/MS. The protein Spot 1 was excised and digested with trypsin and then collected peptides were analyzed using a Bruker MALDI/TOF/mass spectrometer. The annotated PMF spectral peaks showed the intensities of different peptide. Database searching with Mascot software against NCBInr database identified as a Hsp70, which corresponds to that in Table 2.
Figure 16. Comparison of deduced amino acid sequence with the homologues of hsp70 obtained from MALDI/TOF/TOF Mascot software for *S. portulacastrum*.
MALDI-TOF m/z profile of peptides peak

**Matrix Science** Mascot Search Results

- **User**: Arun Kumar
- **Email**: arunmarinebiotech@gmail.com
- **Search title**: Mascot Search Results of NCBInr 20130310 (23641837 sequences: 6123359852 residues)
- **Taxonomy**: Other green plants (992321 sequences)
- **Timestamp**: 12 Mar 2013 at 06:43:57 GMT
- **Top Score**: 76 for gi|1384251890, T-complex protein, theta subunit [Coccomyxa subellipsoidea C-169]

**Mascot Score Histogram**

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 73 are significant (p<0.05).

**Protein Summary Report**

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Figure 17: Identification of proteins from *S. portulacastrum* via MALDI TOF/MS. The protein spots 2 was excised and digested with trypsin and then collected peptides were analyzed using a Bruker MALDI/TOF/ mass spectrometer. The annotated PMF spectral peaks showed the intensities of different peptide. Database searching with Mascot software against NCBInr database identified as a T complex protein, which corresponds to that in Table 2.
GENERAL DISCUSSION

Bioactive compound analysis of halophyte

*Sesuvium portulacastrum* L., belongs to the family Aizoaceae, commonly known as “sea purslane”, is a sprawling perennial herb that grows in coastal areas and grows naturally in the sub-tropical, mediterranean coastal and warmer areas around the world (Robert and Frank, 1997; Rabhi *et al.*, 2010). The plant has a long history of use in folk medicine, for the treatment of epilepsy, conjunctivitis, dermatitis, haematuria, leprosy and purgative and also used to cure toothache (Bandaranayake, 1998). This plant is being used to treat various infectious diseases and kidney problems by the traditional healers in Zimbabwe and South Africa (Magwa *et al.*, 2006). *S. portulacastrum* expresses fatty acid methyl esters (FAME extract) which can be used in medicine as a potential antimicrobial and antifungal agent (Chandrasekaran *et al.*, 2011). The essential oil from the fresh leaves of *S. portulacastrum* exhibited antibacterial, antifungal and antioxidant activity (Michael *et al.*, 2006). To my knowledge, few studies have been conducted on the other biological capacities of this halophyte. For Bioactive compound analysis of this halophyte used Four different solvents (with different polarity) were used in this study, since a wide range of extract holds a better chance for the extraction and isolation of biologically active molecules for general screening of bioactivity (Kumar *et al.*, 2008).

The average percentage yield of each extracting solvent was based on triplicate analysis of samples. Results showed that extraction with hexane (0.6 ± 0.03 gm) and DCM (0.5 ± 0.02 gm) showed the highest yield. The hexane solvent yielded the highest amount of extract indicating that the leaves and shoots of *S. portulacastrum* contain mostly of lipophilic compounds such as waxes, chlorophyll, and fatty acids.

The highest amount of TPC was observed for methanol extract. Moreover, total phenolic content was significantly higher in *S. portulacastrum* shoot and leaf as compared to other medicinal halophytes like *Suaeda fruticosa* (31.8 mg GAE/g DW) (Samia Oueslati *et al.*, 2012) as well as *Salsola kali* which belong to the same family (17.23 mg GAE/g DW) and in a glycophytic species such as the aromatic and medicinal plant *Nigella sativa* L. (10.04 mg GAE/g DW) (Bourgou *et al.*, 2008). In case of reducing power, *S. portulacastrum* extracts steadily increased with increasing concentration all the samples. Same trend has also been reported by Kumaran and Karunakaran, (2007) in
methanol extracts of higher plants. The maximum (0.350 ± 0.009) reducing power value was observed in dichloromethane extracts and minimum (0.239±0.009) was obtained from hexane extracts. This property is associated with the presence of reductions that are reported to be terminators of free radical chain reaction (Duh, 1998). Similar observation was seen in case of other halophytes like Cakile maritima, Limoniastrum monopetalum, Mesembryanthemum crystallinum, M. edule, Salsola kali, and Tamarix gallica also with different solvent extracts of their different parts (Ksouri et al., 2008). The study of TAC, Higher activity in fractions may be due to the interferences of other compounds present in crude extract; and, it has also been reported that solvents used for extraction have dramatic effect on the chemical species (Yuan et al., 2005).

DPPH radical scavenger, suggesting that halophytic polyphenols may be the principle constituents responsible for the antiradical properties of the extracts. The similar results were obtained by some researchers that change in solvent’s polarity alters its efficacy to extract a specific group of antioxidant compounds and influences the antioxidant properties of the extracts(Zhou and Yu, 2004). Hydroxyl radical is an extremely responsive free radical formed in biological systems and has been involved as a highly damaging species in free radical pathology (Li et al., 2008). This radical has a capability to join nucleotides in DNA and cause component breakage that contributes to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz et al., 2002; Manian et al., 2008; Duan et al., 2007). The FTIR spectrum was used to identify the functional groups of the active components present in extract based on the peaks values in the region of IR radiation. When the extract was passed into the FTIR, the functional groups of the components were separated based on its peaks ratio. The results of FTIR analysis confirmed the presence of alcohol, phenol, alkanes, aldehyde, aromatic compound, secondary alcohol, aromatic amines and halogen compound. Through GC-MS, there were twelve compounds are identified. Among the twelve compounds identified after GC-MS, one of compounds Hentriacontane, a saturated hydrocarbon, the major compound was identified has been isolated from Scabiosa comosa. It is reported to be responsible for its uptake in the soil by plant and shown to be involved with stimulation of fungal spore germination. Hentriacontane has also been isolated from spinach leaves, and discovered to be unsaponifiable and shown to have possible anti-tumour activity (McGinty et al.,
Other compound phenol, 2, 4-bis (1, 1-dimethylethyl), their anti-inflammatory activity, in comparison with indomethacin and vitamin E (Costantino et al., 1993). L-(+)-ascorbic acid, 2-6-dihexadecanoate which is a derivative of ascorbic acid, vitamin C, is present in the essential oil. Vitamin C is an antioxidant and belongs to the class of compounds identified to enhance sperm quality and prevent sperm agglutination, thus making them more motile with forward progression and hence promote male fertility (Glenville, 2008; Dawson et al., 1992). L-(+)-ascorbic acid, 2-6-dihexadecanoate has also been isolated from Ipomoea pes-caprae (L.) R.Br leaves (arun et al., 2014).

**Effect of salinity stress on seed germination in halophyte**

Seed germination of halophytic species is regulated by factors such as water, temperature, light, soil salinity, and their interactions (Noe and Zedler, 2000); however, each species responds to the abiotic environment in a unique manner. Halophytes vary greatly in their ability to tolerate salt. The germination percentage of halophyte was strongly affected by higher salt concentration. In general, the highest germination percentage occurs under non-salty conditions and it decreases with the ascending salt concentrations (Khan et al., 2000). Similar results were reported in a wide range of halophytes (Joshi et al., 2005; Khan et al., 2006). In general, many researchers have concluded that salinity is inhibitory to the germination of halophyte seeds in two ways: (i) causing a complete inhibition of the germination process at salinities beyond the tolerance limits of a species and (ii) delaying the germination of seeds at salinities that cause some stress to seeds but do not prevent germination (Ungar, 1995). Germination rate decreased with increasing salinity. In general, the highest germination percentage occurs under non-salty conditions and it decreases with the ascending salt concentrations (Khan et al., 2000). Similar results were reported in a wide range of halophytes (Joshi et al., 2005; Khan et al., 2006). The decreasing tendency of rate of germination due to increasing salinity is in concordance with the reports of others (Khan et al., 1997). Heenan et al. (1988) have reported that seed germination shows a negative correlation with osmotic stress induced by high salt concentrations.

The growth of the radicle and plumule decreases when salinity is increased. Increase in salinity results in osmotic pressure, which negates plant cell division and differentiation, further leading to reduced water absorbance and hence significance
decrease in plumule and radicle length. Saline environment also shows a deterrent impact on the embryo tissue appearance (Khan and Ungar, 1997). Similar results were observed in many plants grown under saline conditions (Amin et al., 1996). The seed vigour of *S. portulacastrum* decreases in a concentration dependent manner in response to NaCl treatment. Generally, rate and percentage of germination and seed vigor index are affected by different ions and reduction of environmental water potential in the presence of salinity. The findings of Kader and Jutzi (2004) showed that considerable increase in salinity (reduction of environmental osmotic potential) leads to decrease in seed characteristics. The total protein content of leaf gradually decreased with increasing concentration of NaCl. This decrease in protein content might be due to the increasing activity of acid and alkaline proteases. As earlier reported, in *B. parviflora* levels of free amino acid increase as a result of salt stress (Parida et al., 2004). As discussed by Khajeh-Hosseini et al. (2003), high salinity leads to reduced osmotic potential which prevents the water uptake necessary for the mobilization of the nutrients required for germination. It can also be due to the toxic effects of Na\(^+\) and Cl\(^-\) ions on the germination process.

**Diversity of bacterial communities associated with the halophyte**

The Diversity of bacterial communities associated with the halophyte was studied with wild rhizosphere soil with greenhouse rhizosphere soil. The rhizosphere microbial diversity of soil surrounding a plant root is influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. Root exudates include amino acids, organic acids, carbohydrates, sugars, vitamins, mucilage and proteins. The exudates act as messengers that stimulate biological and physical interactions between roots and soil organisms. They modify the biochemical and physical properties of the rhizosphere and contribute to root growth and plant survival. High levels of moisture and nutrients in the rhizosphere attract much greater numbers of microorganisms than elsewhere in the soil. The composition and pattern of root exudates affect microbial activity and population numbers which, in turn, affect other soil organisms that share this environment. Roots progressing in soil introduce labile carbon and nutrients while creating water ways and deposits of antimicrobial
compounds and hormones (Brimecombe et al., 2001; Bringhurst et al., 2001; Hawkes et al., 2007) in time (hours or days) (Lubeck et al., 2000). As many soil microbes exhibit limitations to carbon (Paul and Clark, 1996), they could be expected to respond quickly to root induced changes, by modifying their activity (Heijnen et al., 1995; Herman et al., 2006).

The microbial communities of the soil perform a fundamental role in cycling nutrients, in the volume of organic matter in the soil and in maintaining plant productivity. Thus it is important to understand the microbial response to environmental stress, such as high concentrations of heavy metals of salts, fire and the water content of the soil. Stress can be detrimental for sensitive microorganisms and decrease the activity of surviving cells, due to the metabolic load imposed by the need for stress tolerance mechanisms (Schimel et al, 2007; Yuan et al., 2007, Ibekwe et al., 2010; Chowdhury, 2011).

The detrimental influence of salinity on the microbial soil communities and their activities has been reported earlier in majority of studies (Rietz & Haynes, 2003; Sardinha et al., 2003). Wong et al. (2008) evaluated the effects of salinity of this soil on the microbial biomass which exhibited high rates of respiration in soils with low salinity and vice versa. The composition of the microbial community may be affected by salinity (Gennari et al., 2007; Llamas et al., 2008; Chowdhury et al., 2011) since the microbial genotypes differ in their tolerance of a low osmotic potential (Mandeel, 2006; Llamas et al., 2008).

Through Silva classifier found that the phylum Proteobacteria dominated. The Proteobacteria are commonly observed in waters and sediments from other saline and alkaline lakes (Demergasso et al., 2004; Grant et al., 2004). In the rhizosphere niche, 21 phyla in sample S1, and 20 phyla in sample S2, were recorded, which were similar to that reported in agricultural and forest soil samples (Roesch et al., 2007; Fulthorpe et al., 2008; Uroz et al., 2010).

In a complete review of the dominant soil bacterial taxa using 16S rRNA gene libraries (Janssen, 2006), members of the phylum Proteobacteria represented an average of 39% (range, 10–77%) of libraries constructed from soil bacterial communities, and the members of the phylum Acidobacteria made up an average of 20% (range, 5–46%). The
phylum Proteobacteria, which is metabolically versatile and genetically diverse, comprises the largest fraction of the bacterial community in soil ecosystems, including the rhizosphere (Filion et al., 2004; Sanguin et al., 2006). Even in other natural and human-made ecosystems (e.g. marine, freshwater, wastewater, hot spring microbial mats, and the oral cavity), the phylum Proteobacteria is more dominant than the phylum Acidobacteria (LaPara et al., 2000; Layton et al., 2000; Sievert et al., 2000; Smit et al., 2001; Paster et al., 2002; Martiny et al., 2003; Polymenakou et al., 2005; Penn et al., 2006).

Effects of abiotic stress on the physiological and oxidative status of halophyte

The abiotic stress effect on the physiological and oxidative status on halophyte there was many changes was found. Under stress condition, derangement in the leaf water potential and its components takes place (Suit and DuCharme, 1953), and in the laboratory, where equipment to quantify plant water potential are not available. In case of chlorophyll in stress condition it was decreasing, same trend obserbed by Nyachiro et al. (2001), who described a significant decrease of chlorophyll a and b caused by water deficit in six Triticum aestivum cultivars. Decreased or unchanged chlorophyll level during drought stress has been reported in other species, depending on the duration and severity of drought (Kpyoarissis et al., 1995). A decrease of total chlorophyll with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygenspecies is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments (Herbinger et al., 2002).

Lipid peroxidation has been associated with the damage provoked by a variety of environmental stresses and is often used as an indicator of salt- induced oxidative damage (Elkahoui et al., 2005). Malondialdehyde (MDA) is the decomposition product of polyunsaturated fatty acids in the biomembranes. It has been used as an indicator of lipid peroxidation and tends to greater accumulation under salt stress (Zhu et al., 2008). Increase in MDA contents under salt stress was also found in rice (Tijen and Ísmail, 2005), alfalfa (Wang and Han, 2007), cotton (Diego et al., 2003) and wheat (Sairam and Srivastava, 2002). Similar results were also observed by Sabrine Hattab et al. (2013) where Cd and Cu metals caused a clear oxidative stress detected by a remarkable increase
in lipid peroxidation. Ruiming et al., observed that MDA concentration was increased it in all plant parts treated with Zn. Lin and Kao (2000) also stated that the MDA amount, which is a secondary end product of polyunsaturated fatty acid oxidation, is widely used to measure the extent of lipid peroxidation as indicator of oxidative stress.

Proline is a basic amino acid found in high percentage in basic proteins. Free proline is said to play role in plants under stress conditions. Proline accumulation is one of the most frequently reported modifications induced by salinity and water deficit in plants (Giridara Kumar et al., 2000). Generally, when plant species having low amounts of proline are grown in well-watered and non-saline soil, may increase the content of this amino acid upon imposition of drought or salt stresses (Teixeira and Pereira, 2007). According to Lokhande et al., salt stress was coupled with the higher accumulation of proline. Proline is thought to function as osmoprotectants for proteins (Bohnert and Jenson, 1996). Jaleel et al. (2007) stated that accumulation of proline provides an environment compatible with the macromolecular structure and function and helps plants to adapt to negative consequences of salinity. Proline probably functions as osmolytes in protecting cells from dehydration (Cushman and Bohnert, 2000).

Catalase activity to cope with the abundant H$_2$O$_2$ production that accompanies the high photorespiratory flux (Foyer and Noctor 2003). Rajaravindran and Natarajan (2012) observed that the enzyme activity increased with the increasing salinity upto 600mM in Sesuvium portulacastrum but at higher concentrations, the enzyme activity decreased because plants were not tolerant to the high salinity. Peroxidase is a scavenging enzyme which removes the toxic oxygen radicles from the cells. Manikandan and Venkatesan (2004) noticed significant increase in the peroxidase activity of the halophyte Aegiceras corniculatum on giving abiotic stressand same was the case in the salt tolerant varieties of Xanthosoma sagittifolium (Kammege and Omokolo, 2003). The increased peroxidase activity was mainly due to increased enzyme synthesis and might be useful for adaptation under conditions requiring prevention of peroxidation of membrane lipids (Kalir, 1984). According to Chaoui et al. (1997) under Cd and Zn stress conditions, the great elevation of POD activity and the induction of the new anionic isoenzyme bands could be considered as a stem’s reaction to metal-caused oxidative damage.
Superoxide dismutase (SOD) is one of the most important antioxidants, which detoxifies superoxide radicals. The expression of SOD enzymes increases under stress conditions in salt- and drought-tolerant plants. Taji et al. (2004) reported that the SOD was overexpressed in unstressed conditions in Thellungiella halophile which not only made this species more tolerant to high salinity but also to oxidative stress. It was similar to SOD activity measured in salt tolerant species such as Najas gramenia (Rout and Shaw 2001), Suaeda salsa (Qiu Fang et al. 2005) and Mesembryanthemum crystallinum (Slesak et al. 2003). In the present study, increased SOD activity under salt stress conditions suggested that, at the primary step, leaves would have efficiently detoxified superoxide radicals. Similarly, a steep increase in the total SOD activity has been recorded in Bruguiera gymnorrhiza and B. parviflora during salt stress (Parida et al. 2004).

Total proteins were extracted from control and abiotic stress treated plants after 72 hr of treatment and analyzed by SDS-PAGE to observe the intensity of several protein bands. One of the protein band with molecular weight ~54 kDa was highly expressed in all the stresses compared to the control .That band was subjected with LC-MALDI and found as Ribulose bisphosphate carboxylase large chain. Ribulose bisphosphate carboxylase large chain (RuBisCo) it is involved in calvin cycle. The Calvin cycle (also termed the reductive pentose phosphate pathway) is a metabolic pathway that produced pentose sugars (Heldt, 1997. RuBisCO is a multimeric enzyme with two subunits; large (50-55 kDa) and small (12-18 kDa; Andersson and Backlund, 2008). In this study, the RuBisCO proteins were observed as forming a train of band on with MW of approximately 54. This observation is consistent with results from other proteomic studies. In the pea leaf proteome, Schiltz et al. (2004) observed that RuBisCO proteins formed an abundant train of spots between pH 6-7, at approximately 50 kDa. Similarly in the maize leaf proteome, several RuBisCO large subunits were also identified between pH 6-7 and MW of approximately 50-56 kDa (Porubleva et al., 2001). Functionally, RuBisCO proteins catalyse carbon fixation (carboxylation) reactions in the Calvin cycle of photosynthetic plants. In this process, ribulose 1,5-bisphosphate (RuBP), a 5-carbon compound serves as an acceptor molecule for CO_2 to form an unstable 6- carbon compound. The 6-carbon intermediate compound immediately breaks down, forming two
molecules of 3-phosphoglycerate (3PGA; Kellogg and Juliano, 1997; Tabita et al., 2007; Andersson and Backlund, 2008).

**Identification of Salt Stress Responsive Proteins of the Leaf Tissues halophyte**

In that proteomics study that there were two proteins characterized through MALDI TOF MS. Analysis of protein mass fingerprinting data (PMF) of two proteins (spot 1 & spot 2) derived by MS analysis using MASCOT search algorithm showed homology to heat shock protein 70 (Hsp70) from *Cucumis sativus* and theta subunit of T-complex protein from *Coccomyxa subellipsoidea C-169*. The analysis showed that the fist protein functions as chaperones for newly synthesized proteins to prevent their accumulations as aggregates and folds in a proper way during their transfer to their final location (Su and Li, 2008). The expression of Hsp70 genes correlates positively with the acquisition of thermo-tolerance (Lee and Schoffl, 1996) and results in enhanced tolerance to salt, water and high temperature stress in plants (Cho and Hong, 2006) and for this reason Hsp70 has been proposed as potential biomarker (Ireland, 2004). Prokaryotic and eukaryotic organisms respond to heat shock or other environmental stress by the induction of the synthesis of proteins collectively known as heat-shock proteins (hsp). Hsp70 is a multifunctional protein that plays an important role in the transport of protein across membranes, in protein folding and in the assembly/disassembly of protein complexes can direct incompetent "client" proteins towards degradation.

The second protein also functions as molecular chaperons that assist in folding of proteins upon ATP hydrolysis in protecting plant cell from the detrimental effects of stress. Until now, there is not much that available on the functional specificity of theta protein on salt stress.

There is some indication that Hsp70B found in the stroma of chloroplasts participate in photo protection and the repairing of photosystem II during and after the photoinhibition (Schroda et al., 1999). A more recent study on *A. thaliana* indicated the necessity of Hsp70 found in the stroma of chloroplast for the differentiation of germinating seeds and its tolerance of heat (Su and Li, 2008).

The expression of Hsp70 genes correlates positively with the acquisition of thermo-tolerance (Lee and Schoffl, 1996) and results in enhanced tolerance to salt, water
and high temperature stress in plants (Cho and Hong, 2006) and for this reason Hsp70 has been proposed as potential biomarker (Ireland, 2004).
SUMMARY AND CONCLUSION

As a whole in bioactive compound analysis of halophyte, these results have demonstrated that *S. portulacastrum* exhibited differential phenolic content and a remarkable antioxidant source, which are known to exhibit a wide range of biological and pharmacological, were identified for the first time in this halophyte up to my knowledge. According to my results, the antioxidative potential of this species make *S. portulacastrum* a novel natural source of antioxidants with numerous health benefits that should be considered in the fields of functional foods or nutraceuticals. Analysis of the total extracts of *S. portulacastrum* with the help of FTIR spectroscopic technique showed the presence of phenolic compound which can be isolated and further screened for different kinds of therapeutic uses. The data obtained, suggests the strong potential of this halophyte as a source of phenolic compounds with beneficial properties, and a promising source of health products for food and pharmaceutical industry.

In effect of salt on seed germination, the germination percentage of halophyte was strongly affected by higher salt concentration (0.5 M). Germination percentage was higher in distilled water (control) than in any of the saline treatments. The rate of seed germination followed a concentration dependent trend with respect to salinity. Germination rate decreased with increasing salinity. It was found to be higher (9.90) in the control followed by 0.1, 0.2, 0.3 and 0.4M NaCl. Lowest germination rate of 1.23 was observed in seeds treated with 0.5M NaCl. The length of radicle and plumule of seeds decreased on being subjected to stress. With increasing salinity, radicle and plumule length drastically reduced. Maximum seedling length was observed in the control group (4.01cm) and a minimum of 0.82cm was observed in the group treated with 0.5M NaCl. Munns and Termaat (1986) suggested that there exists an inverse relation between salinity and radicle-plumule growth. A maximum of 3.27 seed vigour was observed in control as compared to other groups showing 1.67, 1.33, 1.14, 0.3 and 0.2 when treated with 0.1, 0.2, 0.3, 0.4 and 0.5M NaCl respectively. Profiling of Seedling protein from *S. portulacastrum* by SDS-PAGE showed differential expression after treatment with varying concentration of NaCl (100mM, 200mM, 300mM, and 400mM) harvested after
14 days. All lanes were loaded with 10µg protein. There were two up-regulation of protein ~40 kDa and ~19 kDa observed after NaCl treatment.

The microbial communities of the soil perform a fundamental role in cycling nutrients, in the volume of organic matter in the soil and in maintaining plant productivity. There many factors were responsible for growth of plant and development of microbes that factors are salinity, pH, ions and metals. In this study The salinity of rhizosphere soil of *Sesuvium portulacastrum* varied in both the greenhouse (S1) and wild (S2) conditions. The maximum salinity (29.12 ppt) was recorded in the S2 whereas it was very low (0.5 ppt) in S1. The pH value of greenhouse rhizosphere soil (9.19 ± 0.015) was higher than the wild rhizosphere soil (8.89 ± 0.045).

The sequences reads were processed by the NGS analysis pipeline where the sequences were classified in SILVA rRNA gene database project (SILVAngs 1.2) (Quast *et al.*, 2013). The analysis reveal a total of 4415 sequences (reads) were obtained from the 2 samples after thorough quality check and chimera check, of which only five numbers (0.11%) of sequences were not matched. The aligned sequences had a total minimum length of 243 bp and maximum of 613 bp and subjected to reference analyses. The information on clustering depends on operational taxonomic units (OTUs). The exhibited OUT was 47.95% OTUs in S1 and S2; 43.92% clustered sequences and 8.02% replicates. The sequences were submitted to the NCBI SRA and accession numbers (SRS735121 and SRS735123) were obtained. The Silva classifier revealed a total of 21 phyla in S1 and 23 phyla in S2 showed. The bacterial diversity on S1 was dominated with the phylum Proteobacteria (40%), followed by Acidobacteria (30%), Verrucomicrobia (8%), Bacteroidetes (5%), Planctomycetes (5%), Chloroflexi (4%) and Actinobacteria (2%). Other phyla included Spirochaetae, SM2F1, Nitrospirae, Gemmatimonadetes, Firmicutes, Elusimicrobia, Cyanobacteria, Fibrobacteres, Candidate division WS3, Candidate division TM7, Candidate division OD1, BD1, and Armatimonadetes which represented the load of ≤1%.

The S2 sample showed slightly different patterns of diversity and distribution of phyla compared to S1 sample. From the total of 2239 sequences, 23 phyla were identified
using silva classifier. The dominant phylum in S1 was Proteobacteria (55%) followed by other phyla like Bacteroidetes (14%), Chloroflexi (7%), Verrucomicrobia (6%), Gemmatimonadetes (5%), Actinobacteria (3%), Planctomycetes (3%), Acidobacteria (2%) and Cyanobacteria (2%). In addition other phyla including Spirochaetae, SM2F11, Nitrospirae, NPL-UPA2, Lentisphaerae, Firmicutes, Fibrobacteres, Deinococcus-Thermus, Deferribacteres, Candidate division WS3, Candidate division TM7, Candidate division OD1, Candidate division BRC1 and BD1 were also present (≤1%).

The effect of abiotic stress on halophytes there was many changes in physical and chemical level. The present study observed that when plant is healthy its TWC percentage was high but in stress condition it was varies. Relative water content (RWC) is a measure of the relative cellular volume that shows the changes in cellular volume that could be affecting interactions between macromolecules and organelles. As a general rule, a RWC about 90-100% is related to closing of the stomata pore in the leaf and a reduction in the cellular expansion and growth. Contents of 80-90% are correlated with changes in the composition of the tissues and some alterations in the relative rates of photosynthesis and respiration. The concentrations of Na⁺ increased in abiotic stress treated plants of *S. portulacastrum* when compared with control plants. The stress imposed at the vegetative stage, significantly decreased chlorophyll a content, chlorophyll b content and total chlorophyll content. The lack of effects on the chlorophyll a/b ratio indicates that chlorophyll b is not more sensitive to stress than chlorophyll a. The leaves’ MDA was higher under all abiotic stresses, compared with control. Abiotic stresses levels at NaCl, drought, dark, heat, cold, Zn, Cu and Cd, caused respectively 39.56 ± 0.54, 72.19 ± 0.76, 44.61 ± 0.55, 69.59 ± 0.45, 73.50 ± 0.42, 57.84 ± 0.44, 71.31 ± 0.45 and 44.67 ± 0.55 µmol/gm fresh weight. The present result showed that lipid peroxidation was influenced by abiotic stress in leaves of *S. portulacastrum*. The stress treatment caused an increase in proline content at all stress levels tested. The control plants maintained a lower concentration (10.87 ± 0.13) of proline in the leaves at all tested abiotic stresses concentrations. A much higher (64.21 ± 0.74) level of proline was noticed in copper treated plant. Other than copper treatment, higher proline concentration was observed in all the treated plants compared to control. The effect of abiotic stress on the catalase activity in the leaves. The enzyme activity was decreased when the halophyte
was given abiotic stress conditions like drought, heat cold and copper. The Leaves’ POD was higher under all abiotic stresses, compared with control except copper treatment (1.4 ± 0.02). Abiotic stress levels at NaCl, drought, dark, heat, cold, Zn and Cd treatments, caused respectively 1.95 ± 0.03, 1.7 ± 0.15, 1.52 ± 0.25, 1.97 ± 0.25, 2.2 ± 0.04, 2.7 ± 0.05, and 2.67 ± 0.05 units’ min\(^{-1}\) mg\(^{-1}\) protein.

The leaves’ SOD was higher under all abiotic stresses, compared with control. Abiotic stress levels at NaCl, drought, dark, heat, cold, Zn, Cu and Cd, caused respectively 9.0 ± 0.5, 7.6 ± 0.05, 7.51 ± 1.15, 6.85 ± 0.05, 10.13 ± 0.32, 7.5 ± 0.09, 11.14 ± 0.06 and 6.52 ± 0.025 unit mg\(^{-1}\) protein. The total soluble protein concentration varied in different abiotic stress treated S. portulacastrum, but no changes were observed in untreated plant. Total proteins were extracted from control and abiotic stress treated plants after 72 hrs of treatment and analyzed by SDS-PAGE to observe the intensity of several protein bands. One of the protein band with molecular weight ~54 kDa was highly expressed in all the stresses compared to the control). That expressed protein was used for the identification through the LC-MALDI TOF- TOF MS/MS analysis.

Analysis of protein mass fingerprinting data (PMF) of proteins derived by MS analysis using MASCOT search algorithm showed homology to Ribulose bisphosphate carboxylase large chain from Alluaudia procera.

Six week old plants were treated with 300 mM NaCl for different time periods. The Cl- content in salt treated halophytes is regulated differently in leaves and in roots with minimum and maximum accumulations occurring in the leaves (Yamanaka, 2009). Since morphological and physiological differences are prominent in leaves that may play important roles in salt tolerance, we performed proteome analysis in leaves from salt-treated S.portulacastrum. Total proteins were extracted after 0 h and 24 h treatment with threshold level of NaCl (300 mM). Proteins were then separated by 2-DE, and those proteins spots showing reproducible changes were selected and their expression patterns were analysed. 2D-PAGE analysis from proteins extracted from leaves, revealed the differential expression of 59 protein spots which showed significant difference in their abundance between control and treated samples. Among the 59 spots, 46 were newly
expressed on salt stress, 6 were up regulated and 7 were down regulated. Most distinct two different newly expressed protein spots were selected and further functional characterisation was performed through MALDI-TOF/MS.

Analysis of protein mass fingerprinting data (PMF) of two proteins (spot 1 & spot 2) derived by MS analysis using MASCOT search algorithm showed homology to heat shock protein 70 (Hsp70) from *Cucumis sativus* and theta subunit of T-complex protein from *Coccomyxa subellipsoidea C-169*

High salinity is one of the most serious threats to crop production. To understand the molecular basis of plant responses to salt stress better proteomic analysis using 2 dimensional electrophoresis with mass spectrometry were combined to identify the potential important or novel proteins involved in the early stage of halophytes responses to severe salt stress. In *Sesuvium portulacastrum*, analysis of seed germination with respect to different concentration of NaCl through proteomic analysis allowed us to conclude that the germination was prompted by the expression of different regulatory proteins such as biosynthesis of germination promoting hormones, detoxifying and defence proteins. It is worthy to note that among the two salt-modulated proteins in *S. portulacastrum*, heat shock proteins (HSPs) which act as molecular chaperons, play a crucial role in protecting plants against stress by re-establishing normal protein conformations, and thus, maintain cellular homeostasis. Transgenic crops overexpressing a member of Hsp70 from the halotolerant *S. protulastrum* can be propagated. In addition, a novel protein T-complex protein, theta subunit related to chaperon family was identified during this analysis, which may surd a potential regulator to salt tolerance. This type of molecule can be exploited for transgenic crop production and propagation in saline condition.
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