IN THE NAME OF

ALLAH

THE MOST BENEFICENT THE MOST MERCIFUL
SALT RESISTANCE OF *HALOPELIS PERFOLIATA*: A COASTAL SALT MARSH STEM SUCCULENT HALOPHYTE

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UNIVERSITY OF KARACHI
KARACHI, PAKISTAN
January, 2016
SALT RESISTANCE OF *HALOPEPLIS PERFOLIATA*: A COASTAL SALT MARSH STEM SUCCULENT HALOPHYTE

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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Ascorbic acid</td>
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<td>Ascorbate peroxidase</td>
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<td>Catalase</td>
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<td>$\delta^{13}C$</td>
<td>$^{13}$ Carbon isotope discrimination</td>
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<td>Carboxylation efficiency</td>
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<td>Chlorophyll</td>
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<td>Glutathione reductase</td>
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<td>CE*</td>
<td>Gross carboxylation efficiency</td>
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<td>Guaicol peroxidase</td>
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<td>Hour</td>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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Halopeplis perfoliata is a perennial species of flowering plant in the family Amaranthaceae. It is native to parts of the Middle East and is known for its tolerance to saline conditions. In the experimental setup, the plants were grown in a saline environment with a concentration of NaCl set at 300 mM. The leaves of the plants were harvested and analyzed for chlorophyll content. The results showed a decrease in chlorophyll a and b content in the NaCl-treated plants compared to the control group. The photosynthetic efficiency of the plants was also measured using chlorophyll fluorescence analysis. The results indicated a decrease in the quantum yield of photosynthesis in the NaCl-treated group. These findings suggest that Halopeplis perfoliata is capable of withstanding high salinity conditions but at the cost of decreased photosynthetic efficiency.
Summary

*Halopeplis perfoliata* Forssk. is a C₃ perennial halophyte found in coastal salt marshes of Saharo-Sindian, Mediterranean and Western Irano-Turanian regions. The plant has several ecological and economic usages but little is known about its salt resistance strategies. Therefore, salt tolerance mechanism at seed germination and growth stages of *H. perfoliata* were investigated. Increases in salinity concentration decreased seed germination at all temperature regimes, while some seeds could still germinate in 0.6 mol L⁻¹ NaCl (equivalent to seawater salinity). Hyper-saline conditions and complete darkness induced conditional dormancy. Among salts tested, sea-salt inhibited seed germination more than isotonic NaCl treatments. Among anions, chloride salts inhibited germination more than isotonic sulfate salts. Dry storage of seeds for 1-year at room temperature increased their tolerance to moderate salinities (0.2-0.3 mol L⁻¹ NaCl) and complete darkness, but decreased seed tolerance to higher temperature (25/35 °C) as compared to freshly collected seeds. All dormancy regulating chemicals (except for proline) alleviated dark-enforced dormancy under non-saline conditions. *Halopeplis perfoliata* grew optimally at 0.15 mol L⁻¹ NaCl treatment and with increasing salinity (0.3 and 0.6 mol L⁻¹ NaCl) treatments plant biomass was comparable to non-saline controls. Lower (more negative) shoot osmotic potentials and Na⁺ accumulation indicated capacity for osmotic adjustment with increasing salinity. In general, photosynthetic CO₂ fixation, light harvesting ability of PSII and cation (K⁺, Ca²⁺ and Mg²⁺) concentrations were unchanged with increasing salinity treatments. Higher CAT activity and H₂O₂ and the less negative carbon isotope ratios in photosynthetic shoots indicated high rates of photorespiration under saline conditions. *Halopeplis perfoliata* displayed unique adaptations at seed germination and growth to survive under saline conditions.
Halopepis perfoliata
Chapter 1
General Introduction
Introduction

Salt marshes are highly productive yet threatened ecosystems

Coastal salt marshes are transition zones between marine and terrestrial ecosystems (Teixeira et al., 2014). They inundated diurnally by seawater therefore marsh sediments have high salinity (equivalent to $\geq 0.6$ mol L$^{-1}$ NaCl) (Aziz et al., 2001; Khan et al., 2005). Salt marshes in sub-tropical regions often lack sufficient rainfall which leads to even higher soil salinity (Böer, 1996; Boorman, 2003). Most, salt marshes are characterized by dense stands of few highly salt-tolerant species (Anjum et al., 2014). In marshy habitats, halophytes perform key role in carbon cycling (Gribsholt et al., 2003; Sousa et al., 2010) by serving as a net sinks of carbon from atmosphere and produce between 100-1000 g carbon m$^{-2}$ y$^{-1}$ (McLusky and Elliot, 2004; Sousa et al., 2010) which makes the marsh environment highly productive. However, these habitats face various natural and anthropogenic threats (Yasseen and Al-Thani, 2007; Ramadan et al., 2013). According to a recently published t of United Nations report, the global climate changes are causing global warming and mean sea level which could have drastic effects on these habitats (IPCC, 2014). In addition, intense construction activities for resorts, hotels and picnic points in coastal areas are also a serious threat to these important habitats. Hence, coastal marshes and their vegetation require special attention both in research and conservation/protection activities.

Salt marsh vegetation of coastal areas is under serious threat

Common plant species of warm coastal salt marshes can be categorized into perennials, succulents, shrubs, stolonifers and hemicryptophytes (Ghazanfar, 2006) and include many highly salt tolerant succulent species such as *Arthrocnemum macrostachyum*, *Halocnemum strobilaceum*, *Halopeplis perfoliata*, *Haloxylon persicum*, *Salicornia europea*, *Zygophyllum mandavielli*, *Salsola imbricata*, *Sesuvium verrucosum*, *Suaeda maritima* and *Suaeda vermiculata*. (Gairola et al., 2015). These salt marsh plants provide habitat for the coastal fauna, beside many other ecological and economic benefits (Barbier, 2013). However, anthropogenic activities such as installation of reverse osmosis facilities, construction of excursion points and buildings are posing serious threat to these salt marshes. Therefore, salt marshes have received special attention both
from researchers and Government, which is evident from a growing body of research works (Mahmoud et al., 1983, Böer, 1991, Al-Zaharani and Hajar, 1998; Ghazanfar, 2006; Anjum et al., 2015 and Meirland et al., 2015). Most of these research deals with species composition, edaphology and threats to salt marshes. However, information on eco-physiology and salt resistance mechanisms of the plants is missing.

Multiple factors regulate seed germination responses of marsh halophytes

Seed germination is the most sensitive stage in the life cycle of halophytes and is influenced by a number of environmental factors, of which salinity is considered foremost (Ungar 1995; Katembe et al., 1998; Gul et al., 2013). Insufficient rainfall in sub-tropical regions results in high salinity in salt marsh sediments (Böer, 1996; Boorman, 2003). Therefore, salinity of upper soil layers in such salt marshes may reach 44 ppt (equivalent 70 dS m\(^{-1}\)) (Mandora et al., 1987). Seeds of most of the coastal plants deposited in highly saline soils, hence they have to endure highly hostile conditions (Ungar, 1978; Gul and Khan, 1998; Gul et al., 2013).

Seeds of most succulent halophytes of salt marshes are generally high salt tolerant and can germinate in salinities higher than seawater salinity e.g. *Salicornia herbacea* (1.7 mol L\(^{-1}\); Chapman 1960), *Salicornia europaea* (0.6 mmol L\(^{-1}\) NaCl; Keiffer and Ungar, 1997), *Arthrocnemum macrostachyum* (1.0 mol L\(^{-1}\) NaCl; Khan and Gul, 1998), *Halosarica pergranulata* (0.8 mol L\(^{-1}\) NaCl; Short and Colmer, 1999), *Halocnemum salicornicum* (0.8-0.9 mmol L\(^{-1}\) NaCl; El-Keblawi and Al-Shamsi, 2008) and *Sarcocornia ambigua* (45 g NaCl L\(^{-1}\) ~ 770 mmol L\(^{-1}\) NaCl; Freitas and Costa, 2014). In addition, seeds of these succulent halophytes can endure hypersaline conditions by undergoing enforced dormancy (yet maintaining their viability) and show high recovery of seed germination when salinity of the salt marshes is reduced following rainfall (Ungar, 1995; Khan and Ungar, 1997; Gul et al., 2013). Seeds of most non-succulent salt marsh halophytes such as the *Atriplex*, *Crithmum* and *Limonium* species on the other hand could germinate at or below seawater salinity (Katembe et al., 1998; Zia and Khan, 2008; Atia et al., 2009).

Seed germination of marsh halophytes is not only influenced by total salinity, but also by different salts (Ryan et al., 1975; Khan and Gul, 2006). For example, germination of stem succulent salt marsh halophytes *Arthrocnemum macrostachyum*, *Halocnemum strobilaceum*, *Sarcocornia fruticosa* and *Salicornia ramosissima* varied under similar
levels of Cl⁻ and SO₄²⁻ salts, and was linked to differential osmotic rather than ionic effects of these salts (Pujol et al., 2000). Furthermore, comparison of NaCl and sea-salt on salt marsh halophyte *Arthrocnemum indicum* indicated that NaCl reduced germination more than the sea-salt (Saeed et al., 2011). However, little information is available on the effects of different salts and seawater on salt marsh halophytes in comparison to the effects of NaCl.

Beside salinity, many other factors such as light, temperature, storage, and levels of phyto-hormones also influence seed germination of halophytes (Baskin and Baskin, 1994; Khan and Gul, 2006). According to Baskin and Baskin (1998) out of 91 halophytes, seed of 23 species were germinated in the presence of light. Similar responses were observed for various other stem succulent halophytes like *Salicornia pacifica* (Khan and Weber, 1986), *Allenrolfia occidentalis* (Gul et al., 2000), *Arthrocnemum indicum* (Saeed et al., 2011), *Halocnemum strobilaceum* and *Halopeplis perfoliata* (El-Keblawy and Bhatt, 2015). In addition, temperature also affects seed germination of halophytes (Bewley and Black 1994; Gul and Weber 1999; Khan et al., 2001; Zheng et al., 2004, 2005). Seeds of salt marsh stem succulent halophytes generally germinate best at the temperature 20/30 °C. For example *Allenrolfia occidentalis* (Gul and Weber, 1999), *Salicornia rubra* (Khan et al., 2000) and *Arthrocnemum indicum* (Saeed et al., 2011) germinated best at 20/30 °C. According to Mahmoud et al. (1983) germination success in salt marshes of Arabian Peninsula depends on temperature. However, this kind of information on the marsh halophytes of Arabian-Peninsula is limited to just few studies (Gul et al., 2013).

Growth hormones are reported to enhance the seed germination under salinity stress condition (Ahmed et al., 2014; Li et al., 2014). Alternation in endogenous dormancy regulating compounds at the appropriate salinity, temperature and light could facilitate seed germination (Ahmed and Khan, 2010; El-Kablawy et al., 2010). It has been reported that ethephon, fusicoccin, GA₃ and kinetin enhanced germination in stem succulent halophytes such as *Salicornia utahensis* (Khan and Weber, 1986; Gul and Khan, 2003), *Allenrolfia occidentalis* (Gul and Weber, 1998), *Arthrocnemum indicum* (Khan et al., 1998) and *Salicornia rubra* (Khan et al., 2002). However, this information on the salt marsh halophytes of Arabian-Peninsula is largely missing.
Most salt marsh halophytes have “obligate” salt requirement for optimal growth

Succulent halophytes particularly those of Salicornioideae are reported to dominate coastal salt marshes (McKee et al., 2012). Halophytes of Salicornioideae are supposed to have higher salt tolerance than other halophytes. For example, *Arthrocnemum macrostachyum* (up to 1.0 mol L\(^{-1}\) NaCl, Khan et al., 2005), *Salicornia europaea* and *S. persica* (up to 0.6 mmol L\(^{-1}\) NaCl, Aghaleh et al., 2009) and *Tecticornia pergranulata* (syn. *Halosarcia pergranulata*) (up to 0.8 mol L\(^{-1}\) NaCl, Colmer et al., 2009). A number of Salicornioideae members are in fact “obligate halophytes” (Flower and Colmer, 2008; Rozema and Schat, 2013) which require some salt for their optimal growth (Flowers and Colmer, 2008). For instance, *Sarcocornia natalensis* (300 mmol L\(^{-1}\) NaCl, Naidoo and Rughunanan, 1990), *Salicornia bigellovii* (0.2 mol L\(^{-1}\) NaCl, Ayala and O’Leary, 1995), *Salicornia persica* (Aghaleh et al., 2009) and *Salicornia dolichostachya* (0.3 mol L\(^{-1}\) NaCl, Katschnig et al., 2013) required salt for optimal growth. Hence, salt marsh halophytes are well-adapted for surviving highly hostile marsh conditions. Owing to their obligate halophyte nature, they could be interesting organisms for studying salt tolerance mechanisms.

Salient features of salt tolerance mechanisms in salt marsh halophytes

Salt marsh halophytes are mostly “includers” which accumulate large amounts of salt in their tissues and show tissue tolerance to increasing salinity (Aziz and Khan, 2001). Salt tolerance is a highly complex phenomenon that incorporates a number of cellular/molecular as well as whole plant level processes (Flowers and Colmer, 2008). Generally, we can classify salt tolerance mechanisms of halophytes into four groups: 1) osmotic adjustment, 2) ion homeostasis, 3) protection of metabolic activities such as photosynthesis and 4) oxidative stress management (Jitesh et al., 2006; Flowers and Colmer, 2015).

Osmotic adjustment in halophytes helps to absorb and retain water in tissues under hypersaline conditions (Shabala and Mackay, 2011; Flowers and Colmer, 2015). This is achieved by compartmentalizing salts (mostly as Na\(^{+}\) and Cl\(^{-}\)) in the apoplast and cell vacuoles, with concomitant accumulation of organic osmolytes such as glycine betaine, proline, free amino acids, sugars and sugar alcohols in cell cytosols (Flowers and Colmer, 2008; Slama et al., 2015). Sequestration of salts in vacuoles is energetically less costly than synthesis of organic osmolytes as vacuoles occupy the bulk of the cells’
internal volume (Flowers and Colmer, 2008). Organic osmolytes in cytoplasm provide additional advantage by protecting macromolecules under stress, also referred as ‘osmoprotection’ (Slama et al., 2015). Plant growth may be compromised for osmotic adjustment with salinity increments due to the high energy cost of organic osmolytes (Flowers and Colmer, 2008).

Salt accumulation in halophytes for osmotic adjustment is a tightly regulated multi-facted phenomenon (Shabala and Mackay, 2011). Salt exclusion at the level of root, restrict its entry into the xylem stream (Flowers and Colmer, 2015) and most of the woody halophytes of tidal marshes are reported to exclude 90 to 99% of the external Na⁺ (Reef and Lovelock 2015). Stomatal size and density as well as number reduces transpiration rates which may also help in restricting Na⁺ entry into the shoots/leaves (Shabala and Mackay, 2011). Some non-succulent salt marsh halophytes particularly grasses get-rid of excess salts by secreting them via salt glands/hairs (Flowers et al., 2010; Céccoli et al., 2015). At the cellular level, using a complex machinery of membrane transporter proteins, halophytes keep Na⁺ and Cl⁻ within tolerable ranges along with efficient acquisition of K⁺ and Mg²⁺ (Mansour, 2014; Flowers and Colmer, 2015). High external salinity may however results in ion imbalance which leads to metabolic disruption causing tissue injury. Effective osmotic adjustment and ion-homeostasis for plant survival under saline conditions would require the ability to maintain significant photosynthetic CO₂ gain at minimum water loss (Álvarez et al., 2012; Naidoo et al., 2012).

Salinity is known to affect photosynthesis due to stomatal limitation (Lawlor and Cornic, 2002; Flexas et al. 2004; Lambers et al., 2008). A decrease in stomatal conductance reduces the influx of CO₂ which ultimately reduces photosynthetic rate (Maricle et al., 2007). However, many succulent halophytes of salt marshes are reported to maintain significant photosynthetic rates under high salinities (Maricle et al., 2007). Unlike photosynthetic CO₂ assimilation, light-harvesting mechanisms of halophytes are generally more resilient. For example, photochemistry was generally unaffected by salinity in succulent halophytes Suaeda salsa (James et al., 2002) and Sarcocornia fruticosa (Redondo-Gómez et al, 2006). Under decreased CO₂ assimilation such as in response to salinity, use of alternate electron sinks become essential for the survival of plants (Taiz and Zeiger, 2010). Non-photochemical quenching (NPQ), which is the dissipation of surplus energy mainly by heat production is considered an important route
in plants (Taiz and Zeiger, 2010; Lambrev et al., 2012). For example, NPQ increased with increases in salinity in *Atriplex centralasiatica* (Qiu et al., 2003) and *Cakile maritima* (Megdiche et al., 2008). Rise in NPQ is a photo-protective mechanism that minimizes the incidence of electron flow from photosystems to oxygen (O₂) which otherwise accelerates the formation of cytotoxic reactive oxygen species (ROS) (Jithesh et al., 2006; Taiz and Zeiger, 2010; Demidchik, 2015). Accumulation of ROS can cause oxidative damage to cell components such as membrane lipids, proteins, chlorophyll and nucleic acids (Müller et al., 2001, Jithesh et al., 2006; Möller et al., 2007; Hameed and Khan, 2011; Demidchik, 2015). Therefore, the antioxidant defense system, which comprises of both enzymatic and non-enzymatic components, plays an important role in defending cell components from oxidative damage (Jithesh et al., 2006). Superoxide dismutase (SOD), catalase (CAT), and enzymes of Foyer-Halliwell-Asada pathway are important antioxidant enzymes, while ascorbate (ASA) and glutathione (GSH) are key antioxidant substances, which are implicated in protecting cells from salinity-induced oxidative stress (Jithesh et al., 2006). However, efficacy of antioxidant system in protecting plants from salinity effects may vary considerable with species and the magnitude of salinity imposed, among other factors (Hameed and Khan, 2011).

*Halopeplis perfoliata* is a common halophyte of coastal salt marshes

*Halopeplis perfoliata* Forssk. is a succulent C₃ perennial shrub found in coastal salt marshes of Arabian peninsula (Böer, 1996; Zahran and Ansari, 1999; Al-Oudat and Qadir, 2011), saline desert habitats along Arabian Gulf coast (El-Keblawy and Bhatt, 2015) and also occupies the first zone (near coast) of the many Red Sea salt marshes (Migahid, 1978). There are many ecological and economic usages of this halophyte. For example, *H. perfoliata* is used for soap manufacture by local communities (Al-Oudat and Qadir, 2011). It can also be used for sand dune stabilization (Zreik, 1990). *Halopeplis perfoliata* is an important primary producer of intertidal coastal zones and provides habitat for wild life (Pilcher et al., 2003). However, little is known about the seed germination (Mahmoud et al., 1983; El-Keblawy and Bhatt, 2015) and growth (Al-Zaharani and Hajar, 1998) of this halophyte. Furthermore, underlying mechanisms for salt tolerance of halophytes from the Arabian Peninsula have seldom been studied. *Halopeplis perfoliata* is an interesting plant, owing to its naturally high salt tolerance and economic potentials.
Fig. 1.1. *Halopepis perfoliata* A) plants and B) in the natural environmental conditions.
Research questions

The following question were addressed in the present study:

1. What is the salt tolerance limit of *Halopeplis perfoliata* during seed germination stage under various photoperiod, temperature, and storage environments?
2. Can salinity-induced seed germination inhibition be mitigated by the exogenous application of various dormancy regulating chemicals?
3. Are seeds of *Halopeplis perfoliata* equally tolerant to all types of salts?
4. What is the salt tolerance limit of *Halopeplis perfoliata* at growth stage?
5. What are different physiological and biochemical adaptations which enable *Halopeplis perfoliata* to tolerate high salinity?
References:


Chapter 2

Effects of environmental factors, seed storage conditions, and exogenous chemical treatments in modulating seed germination of *Halopeplis perfoliata*
Abstract

*Halopeplis perfoliata* is a coastal marsh halophyte with several ecological and economic usages. Information about seed germination ecology of this plant is scanty. Therefore this study was conducted to investigate the germination, recovery and viability responses of *H. perfoliata* seeds to photoperiod (light/dark), temperature, salinity, hyper-salinity, long-term storage and dormancy regulating chemicals (DRCs). Seeds were small and non-dormant, and displayed highest germination in distilled water irrespective of incubation temperatures. Seeds germinated better in light (12-h photoperiod) than in 24-h dark. Increases in salinity decreased seed germination; however some seeds could germinate in 0.6 mol L\(^{-1}\) NaCl (equivalent to seawater salinity) under 12-h photoperiod. High salinity (≥ 0.3 mol L\(^{-1}\) NaCl) and darkness imposed conditional dormancy in seeds and they showed high germination recovery when transferred to distilled water and light respectively. Seeds of *H. perfoliata* could endure hyper salinity (≥ 1.0 mol L\(^{-1}\) NaCl) by entering into a state of conditional dormancy. However, high incubation temperatures resulted in higher seed mortality under hyper-saline condition. Dry storage of seeds for one year at 25 °C increased their tolerance to moderate salinity and dark, but substantially decreased tolerance to high temperature compared to fresh seeds. All DRCs (except proline) alleviated dark-enforced dormancy but were generally ineffective in reversing inhibitory effects of salinity. Hence it appears that individual effects of photoperiod, salinity and temperature influenced seed germination more than their interactive effects.
Introduction

*Arabian Peninsula salt marshes are facing various natural and anthropogenic threats*

Coastal salt marshes are among the most productive ecosystems of the world and harbor unique halophytic vegetation bearing specialized adaptations to produce enormous biomass despite high seawater salinity (Teixeira et al., 2014). Their location at the interface of sea and land makes them ecologically important. These habitats serve as an important carbon and nitrogen sink, thereby help in CO$_2$ sequestration and prevention of eutrophication in coastal areas, beside many other ecosystem services (Seitzinger, 1988; Valiela and Cole, 2002; Teixeira et al., 2014). However, these habitats are facing various natural and anthropogenic threats (Yasseen and Al-Thani, 2007; Ramadan et al., 2013). Sub-tropical salt marshes especially those along the coastline of Arabian Peninsula often face lack of sufficient rainfall which leads to increased sediment salinity in salt marshes (Böer, 1996; Boorman, 2003). Salinity of upper soil layer in such salt marshes could be as high as 44 ppt (equivalent 70 dS m$^{-1}$) (Mandora et al., 1987) which could be detrimental for the prevailing vegetation (Ungar, 1991; Böer, 1996). Since seeds of most coastal plants are deposited in top soil layers, which have substantially higher salinity than lower layers, therefore conditions for seeds are harsher in comparison to mature plants (Ungar, 1978; Gul and Khan, 1998; Gul et al., 2013).

*Seed germination responses of salt marsh halophytes are highly variable*

Salinity is one of the main factors influencing vegetation zonation in coastal salt marshes mainly via its effects on seed germination (Bertness et al., 1992; Pujol et al., 2000; Hameed et al., 2006; Elsey-Quirk et al., 2009; Gul et al., 2013). Seed germination of halophytes generally decreases with increases in salinity (Khan and Gul, 2006). However, seeds of most salt marsh halophytes could germinate in as high as seawater (≥ 0.6 mol L$^{-1}$ NaCl) or even higher salinity (Gul et al., 2013). For instance, seeds of salt marsh halophytes *Arthrocnemum indicum* (1.0 mol L$^{-1}$ NaCl, Khan and Gul, 1998; *Ruppia tuberosa* (90 g L$^{-1}$ sea-salt or ~1.5 mol L$^{-1}$ NaCl, Kim et al., 2013) and *Sarcocornia ambigu*a (0.77 mol L$^{-1}$ NaCl, Freitas and Costa, 2014) showed high salinity tolerance during germination. Seeds of most salt marsh halophytes can even endure hyper-saline conditions (Gul et al., 2013) in which they although do not germinate but maintain their viability and hence quickly germinate (germination recovery, sensu Khan and Ungar, 1997) following sufficient rains which dilute soil/sediment salinity (Khan
and Gul, 2006). Salinity tolerance of halophyte seeds also varies with changes in ambient temperature and presence/absence of light (Gul et al., 2013). Sub/supra-optimal temperatures and dark (mainly due to burial) decrease both germination as well as salinity tolerance of halophyte seeds (Baskin and Baskin, 2001; Zia and Khan, 2004; El-Keblawy and Al-Rawai, 2006; Hameed et al., 2013). However, large variations exist in responses of the seeds of salt marsh halophytes to temperature and photoperiod changes. For example, seeds of only four out of eight salt marsh halophytes responded to changes in temperature and light/dark during germination (Noe and Zedler, 2000). Seeds of another salt marsh halophyte *Arthrocnemum indicum* responded to changes in temperature but not to light/dark (Saeed et al., 2011). Changes in temperature and light/dark also influence seed viability and dormancy of halophytes (Gulzar et al., 2013; Hameed et al., 2013). However, information about the seed germination of salt marsh halophytes is limited (Saeed et al., 2011; Gul et al., 2013).

Salinity tolerance of halophyte seeds, as discussed above, is a variable trait and is also influenced by storage conditions and duration (El-Kablawy, 2013a; Cao et al., 2014). Recently El-Keblawy (2013a) studied effect of various storage conditions on seed germination of two succulent halophytes *Haloxylon salicornicum* and *Salsola imbricata*. He found that the seed storage for three months significantly increased germination but longer than nine month storage at room and warm temperatures caused significant reduction or complete inhibition in the germination of the aforementioned halophytes. While seeds of some halophytes such as *Allenrolfea occidentalis* (Gul and Weber, 1999), *Salicornia rubra* (Khan et al., 2000), *Kochia scoparia* (Khan et al., 2001) and *Salsola iberica* (Khan et al., 2002) could maintain viability and germinability after 20 days of high salinity (1.0 mol L⁻¹ NaCl) exposure. In another study, Keiffer and Ungar (1997) found variable viability and germination responses of the seeds of *Atriplex prostrata, Hordeum jubatum, Salicornia europaea, Spargularia marina*, and *Suaeda calceoliformi* to storage under high salinity for different time periods. Hence, these data indicate that seed responses of halophytes to storage under high salinity could be variable and species specific.

*Dormancy regulating compounds can improve seed germination under stress conditions*  
Germination and salinity tolerance of the seeds of halophytes could be improved by exogenous application of certain chemicals (Mehrun-Nisa et al., 2007; Atia et al., 2009;
Ahmed et al., 2014). GA₃, kinetin and thiourea are widely used chemicals, which can mitigate salinity effects and improve seed germination of halophytes (Khan and Gul, 2006; Gul and Khan 2008; El-Keblawy et al., 2010). Recently, Ahmed et al. (2014) reported that thiourea could significantly improve seed germination of three salt playa halophytes under saline condition, while GA₃ and kinetin had species-specific effects. Hence, information about effects of chemicals on salinity tolerance and germination of halophyte seeds appears inconclusive and could be species and/or chemical specific (Gul and Khan 2008; El-Keblawy et al., 2010; Ahmed et al., 2014).

Seed germination eco-physiology of *H. perfoliata* is largely unstudied

*Halopeplis perfoliata* Forssk. is a succulent halophyte from family Amaranthaceae, that occupies coastal salt marshes of Arabian peninsula (Al-Oudat and Qadir, 2011). This species has many ecological and potential economic uses. For instance, it can be utilized for sand dune stabilization in coastal deserts (Zreik, 1990) and also in soap industries (Al-Oudat and Qadir, 2011). This plant is an important intertidal/terrestrial producer and provides habitat structure for wild life (Pilcher et al., 2003). Seeds of this plant could germinate up to 0.25 mol L⁻¹ NaCl treatment (Mahmoud et al., 1983). However, little is known about the seed germination eco-physiology or the two other members of genus *Halopeplis*. Therefore detailed studies on salinity tolerance mechanisms of *H. perfoliata* were performed to understand how its seeds withstand highly saline salt marsh conditions. The objectives of this research were to find out: 1) salinity tolerance limit and optimal germination conditions (temperature and light/dark regimes) for *H. perfoliata* seeds, 2) viability and germination response of *H. perfoliata* seeds to storage under hyper-saline conditions, 3) germination characteristics after long-term (1-year) dry-storage in laboratory condition, and 4) salinity tolerance and germination responses to exogenous application of various dormancy regulating chemicals (DRCs).
Materials and Methods

Study site and Species description
Mature inflorescence of *Halopeplis perfoliata* were collected from the coast of Jizan, Saudi Arabia in 2012. Study site was a salt marsh with frequent inundation, where mean ambient temperature reportedly ranges between 36 and 21 °C (Alfarhan et al., 2005). Mean annual rainfall of the area is 169 mm and humidity ranges between 39 and 86% (Alfarhan et al., 2005). Seeds were collected from large number of plants randomly to ensure adequate representation of the genetic diversity. Seeds were separated from their inflorescence husk manually and brought to the laboratory in Pakistan. Seeds were surface sterilized using 1% bleach solution for 1 min, followed by thorough rinsing with distilled water and air-drying. Sterilized seeds were then used in experiments.

Seed characteristics
Fresh weight (F\(_W\)) of 1000 seeds was measured using an analytical balance (0.0001 g). Dry weight (D\(_W\)) of the seeds was determined by placing 1000 seeds in an oven at 105 °C for 48-h. Moisture content was then calculated as difference between F\(_W\) and D\(_W\). Ash or inorganic content of the seeds was determined by igniting oven dried seeds in a furnace at 550 °C for 3-h. Size of the seeds was measured using photographs of about randomly chosen 100 seeds with the help of ImageJ software (http://imagej.nih.gov/ij/images/). Seed texture was determined by observing seeds under a mini-microscope (Dini-Lite digital Microscope, ANMO Electronics Corporation). Seed color was matched against the catalog of Ralcolors (www.ralcolors.com).

Experiment 1: salinity tolerance limit and optimal germination conditions
Germination was carried out in 50 x 9 mm (Gelman No. 7232) tight-fitting plastic petri-plates with 5 ml of test solution. Seven concentrations of NaCl solution (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mol L\(^{-1}\)) were used. Petri plates were placed in a germination chambers set at temperature regimes of 10/20, 15/25, 20/30 and 25/35 °C, where low temperature coincided with 12-h dark and high temperature with 12-h light (~25 µmol m\(^{-2}\) s\(^{-1}\), 400-750 nm, Philips cool-white fluorescent lamps). Under similar temperature and salinity treatments seeds were also incubated in complete darkness (24-h dark) by using dark photographic envelops. Four replicates with twenty-five seeds per petri-plate were used for each treatment. Petri-plates placed under 12-h photoperiod were monitored at 2 days
intervals for 20 days and percent seed germination were noted. Seeds were considered to be germinated with the emergence of the radical (Bewley and Black, 1994). After 20 days of incubation, rate of germination was estimated by using a modified Timson’s index of germination velocity given below:

\[
\text{Germination velocity (\%)} = \frac{\sum G}{t} \times 100
\]

Where \( G \) is the percentage of seed germination at 2d intervals and \( t \) is the total germination period (Khan and Ungar, 1985). The greater the value, the more rapid was germination. While, germination of seeds incubated in dark was noted once after 20 days. After 20 days light exposed (12-h) un-germinated seeds were transferred to distilled water, while un-germinated seeds from dark incubation were exposed to light in order to study the recovery of germination. Seed germination during recovery experiment was recorded at 2 days intervals for another 20 days period. The recovery percentage was calculated by using following formula given below:

\[
\text{Recovery (\%)} = \left( \frac{A-B}{C} \right) \times 100
\]

Where, \( A \) is total number of seeds germinated after being transferred to distilled water, \( B \) is the number of seeds germinated in saline solution and \( C \) is total number of seeds. The seeds which did not germinate were further tested for their viability using 1\% (w/v) 2,3,5-triphenyle-tetrazolium chloride solution (MacKay, 1972; Bradbeer, 1998).

**Experiment. 2: germination and viability responses of seeds to hyper-saline storage**

To study the tolerance of *H. perfoliata* seeds to hyper-saline conditions, seeds were exposed to four high salinity treatments (0.5, 1.0, 1.5 and 2.0 mol L\(^{-1}\) NaCl) at two temperature regimes (20/30 and 25/35 °C) under 12-h light: 12-h dark photoperiod for different time periods (20, 40 and 60 days). Seeds were also incubated for 24-h in dark. Germination, recovery and viability of the seeds were examined according to the methods described above.
**Experiment. 3: germination characteristics after long-term dry storage**

Seeds of *H. perfoliata* were stored at room temperature (~25 °C) for 1-year and then tested for germination, recovery and viability responses to salinity, temperature and light/dark treatments, as described in experiment 1.

**Experiment. 4: salinity tolerance and germination responses to dormancy regulating chemicals**

To study if salinity tolerance of *H. perfoliata* seeds could be improved using different dormancy regulating chemicals (DRCs), seeds were germinated in different salinity treatments (0, 0.3 and 0.6 mol L⁻¹ NaCl) in light (12-h photoperiod) as well as dark under optimal temperature (20/30 °C) in presence and absence of different DRCs. Betaine (1 mmol L⁻¹), ethaphon (10 mmol L⁻¹), fusicoccin (5 µmol L⁻¹), kinetin (0.05 mmol L⁻¹), proline (0.1 mmol L⁻¹) and thiourea (10 mmol L⁻¹) were used. Germination and recovery were noted, as described above.

Statistical analyses

Germination data were arcsine transformed before statistical analysis. Analyses of variance (ANOVAs) were used to determine if treatments (photoperiod, temperature, salinity, hyper-saline storage, dry storage and DRCs) had significant effect on seed parameters. While, a Post-hoc Bonferroni test was carried out to compare individual mean values for significant (*P* < 0.05) differences. SPSS Version 11.0 (SPSS, 2011) was used for data analysis. Student t-test (*P* < 0.05) was performed to compare mean values for experiment 3 and 4.
Results

Seed characteristics

Seeds of *H. perfoliata* were ellipsoid, small (0.28 mm ø) and rough textured (Fig. 2.1). Fresh weight of 1000 seeds was 92.86 mg and they had about 4.88% moisture. There were no perianth or hairs attached to the fully mature seeds.

![Morphology of a) un-germinated and b) germinated seeds of *Halopeplis perfoliata* as seen under a light microscope.](image)

**Fig. 2.1** Morphology of a) un-germinated and b) germinated seeds of *Halopeplis perfoliata* as seen under a light microscope.

**Table 2.1.** Characteristic features of *Halopeplis perfoliata* seeds collected from a coastal salt marsh in Jizan, Saudi Arabia.

<table>
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<tr>
<th>Characteristic</th>
<th>Description</th>
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<td>Color</td>
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<td>Texture</td>
<td>Rough</td>
</tr>
<tr>
<td>Shape</td>
<td>Ellipsoid</td>
</tr>
<tr>
<td>Size (ø, mm)</td>
<td>0.28</td>
</tr>
<tr>
<td>FW (mg 1000⁻¹ seeds)</td>
<td>92.86</td>
</tr>
<tr>
<td>DW (mg 1000⁻¹ seeds)</td>
<td>88.33</td>
</tr>
<tr>
<td>Moisture (% of FW)</td>
<td>4.88</td>
</tr>
<tr>
<td>Ash (% of FW)</td>
<td>3.87</td>
</tr>
</tbody>
</table>

*RAL-8028, www.ralcolors.com*
Experiment 1: salinity tolerance limit and optimal germination conditions

Three way Analysis of variance (ANOVAs) indicated significant ($P < 0.001$) effect of temperature, photoperiod, salinity and their interaction on mean final germination (MFG), rate of germination ($G_{Rate}$), recovery of germination from salinity ($S_R$) and recovery of germination from dark ($D_R$) of *H. perfoliata* seeds (Table 2.2).

Table 2.2. Three-way ANOVA indicating effects of photoperiod (Phot.), temperature (Temp.), salinity (Salt) and their interactions on mean final germination (MFG), rate of germination ($G_{Rate}$), recovery from salinity ($S_R$) and recovery from dark ($D_R$) of *Halopeplis perfoliata* seeds. Numbers are $F$-values and asterisks in superscript are significance levels at $P < 0.05$ (*** = $P < 0.001$).

<table>
<thead>
<tr>
<th>Factor</th>
<th>MFG</th>
<th>$G_{Rate}$</th>
<th>$S_R$</th>
<th>$D_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phot.</td>
<td>1746.58***</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Temp.</td>
<td>80.37***</td>
<td>87.85***</td>
<td>24.80***</td>
<td>24.70***</td>
</tr>
<tr>
<td>Salt</td>
<td>404.20***</td>
<td>656.80***</td>
<td>284.46***</td>
<td>62.43***</td>
</tr>
<tr>
<td>Phot. * Temp.</td>
<td>41.94***</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phot. * Salt</td>
<td>215.50***</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Temp. * Salt</td>
<td>14.28***</td>
<td>19.34***</td>
<td>7.790***</td>
<td>5.08***</td>
</tr>
<tr>
<td>Phot. * Temp. * Salt</td>
<td>12.03***</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Seeds were non-dormant and germinated maximally (~90%) in distilled water irrespective of incubation temperature (Fig. 2.2) however increase in salinity (NaCl concentration) decreased MFG at all temperature regimes and there was > 10% MFG in 0.6 mol L$^{-1}$ NaCl treatment. Comparatively higher MFG and $G_{Rate}$ values were observed in > 0.5 mol L$^{-1}$ NaCl treatments at 20/30 °C (Fig. 2.2 and 2.3). There was substantially low MFG in dark in comparison to 12-h photoperiod in all salinity and temperature treatments (Fig. 2.2). Salinity and temperature treatments had a similar effect on $G_{Rate}$ as in case of MFG (Fig. 2.3).

When un-germinated seeds from salinity and 12-h photoperiod were transferred to distilled water and incubated at the respective temperature for another 20 days, almost
Fig. 2.2. Germination and recovery percentages of *Halopeplis perfoliata* seeds after different salinity treatments (0, 0.1, 0.2 0.3, 0.4, 0.5 and 0.6 mol L\(^{-1}\) NaCl); temperatures (10/20, 15/25, 20/30 and 25/35 °C) and light (12-h photoperiod and complete darkness) regimes. Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments; Bonferroni test; \(P < 0.05\).
all seeds were germinated ($S_R$), hence total germination (MFG under saline condition + $S_R$) approach to the level of MFG in distilled water. Likewise, when un-germinated seeds from dark incubation were exposed to 12-h photoperiod for 20 days, they germinated ($D_R$) and total germination (MFG in dark + $D_R$) was comparable to MFG in respective salinity treatments under 12-h photoperiod (Fig. 2.2).

**Fig. 2.3.** Rate of germination responses of *Halopeplis perfoliata* seeds after different salinity treatments (0, 0.1, 0.2 0.3, 0.4, 0.5 and 0.6 mol L$^{-1}$ NaCl); temperatures (10/20, 15/25, 20/30 and 25/35 °C) and light (12-h photoperiod and 24- darkness) regimes. Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments; Bonferroni test; $P < 0.05$. 
Experiment 2: germination and viability responses of seeds to hyper-saline storage

A three-way ANOVA indicated significant individual effects of duration, temperature, hyper-salinity and their interaction on MFG, \( S_R \), viability and mortality of the seeds of \( H. \) perfoliata (Table 2.3). Maximum (85%) germination occurred in distilled water within 20 days and no seed germinated after this period (Fig. 2.4). Some seeds were germinated (>20%) was noted in 0.5 mol L\(^{-1}\) NaCl treatment and no seed could germinate in hyper-salinity treatments (1.0, 1.5 and 2.0 mol L\(^{-1}\) NaCl) even after 60 days (Fig. 2.4). Comparatively higher MFG was found in distilled water and 0.5 mol L\(^{-1}\) NaCl treatments at 20/30 °C than at 25/30 °C. About 70% of the un-germinated seeds from hyper-salinity germinated when transferred to distilled water (\( S_R \)) at 20/30 °C, even after 60 days of exposure. Exposure of seeds to hyper-salinity for 60 days caused significant \( (P < 0.05) \) reduction in \( S_R \) value at warmer incubation temperature 25/35 °C (Fig. 2.4). About 10-15% seeds were found dead in all salinity treatments at 20/30 °C after various exposure times. High temperature (25/35 °C) treatments resulted in mortality of about 50% seeds after 60 days under saline condition (Fig. 2.4).

Table 2.3. Two-way ANOVA indicating effects of storage time (Days) temperature (Temp.), hyper-salinity (Salt), and their interactions on mean final germination (MFG), recovery from salinity (\( S_R \)), viability (Viab.) and mortality (Mort.) of Halopeplis perfoliata seeds. Numbers are \( F \)-values and asterisks in superscript are significance levels at \( P < 0.05 \) (ns = non-significant, \* = \( P < 0.05 \), \** = \( P < 0.01 \) and \*** = \( P < 0.001 \)).

<table>
<thead>
<tr>
<th>Factor</th>
<th>MFG</th>
<th>( S_R )</th>
<th>Viab.</th>
<th>Mort.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>2.127**</td>
<td>8.45***</td>
<td>25.32***</td>
<td>8.70***</td>
</tr>
<tr>
<td>Temp.</td>
<td>8.55**</td>
<td>58.41***</td>
<td>35.99***</td>
<td>26.07***</td>
</tr>
<tr>
<td>Salt</td>
<td>416.19***</td>
<td>143.92***</td>
<td>6.48***</td>
<td>1.85ns</td>
</tr>
<tr>
<td>Days * Temp.</td>
<td>2.13ns</td>
<td>1.493ns</td>
<td>9.01***</td>
<td>2.50ns</td>
</tr>
<tr>
<td>Temp. * Salt</td>
<td>3.30*</td>
<td>9.70***</td>
<td>5.41**</td>
<td>3.04*</td>
</tr>
<tr>
<td>Days * Salt</td>
<td>0.83ns</td>
<td>3.00*</td>
<td>1.52ns</td>
<td>1.04ns</td>
</tr>
<tr>
<td>Temp. * Days *Salt</td>
<td>3.28**</td>
<td>1.70ns</td>
<td>2.27*</td>
<td>1.04ns</td>
</tr>
</tbody>
</table>
Germination, recovery, viability and mortality percentages of *Halopeplis perfoliata* seeds after prolonged exposure (20, 40 and 60 days) in hyper-saline (0.5, 1.0, 1.5 and 2.0 mol L\(^{-1}\) NaCl) treatment, temperatures (20/30 and 25/35 °C); and light (12-h photoperiod) regimes. Values are means of 4 replicates.
**Experiment. 3: germination characteristics after dry storage**

A three-way ANOVA showed significant individual effect of storage, photoperiod, temperature, salinity, and their interaction on seed germination of *H. perfoliata* (Table 2.4). Maximum germination of both fresh and stored seeds occurred in distilled water and increase in salinity decreased germination of both fresh and stored seeds at all temperature regimes under 12-h photoperiod (Fig. 2.5). Relatively higher germination was observed in stored seeds as compared to fresh ones under moderately saline (0.30-0.50 mol L$^{-1}$ NaCl) conditions at 10/20, 15/25 and 20/30 °C. However, stored seeds did not germinate at 25/35 °C under 12-h photoperiod. There was no difference in germination of fresh and stored seeds under dark at low (10/20 and 15/25 °C) and warm (25/35 °C), while stored seeds germinated better under dark at moderate (20/30 °C) temperature (Fig. 2.5).

**Table 2.4.** Four-way ANOVA showing effects of dry storage (Stor.) photoperiod (Phot.), temperature (Temp.), salinity (Salt) and their interactions on mean final germination (MFG) of *Halopeplis perfoliata* seeds. Numbers are *F*-values and asterisks in superscript are significance levels at $P < 0.05$ (* = $P < 0.05$ and *** = $P < 0.001$).

<table>
<thead>
<tr>
<th>Factor</th>
<th>MFG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stor.</td>
<td>21.07***</td>
</tr>
<tr>
<td>Phot.</td>
<td>2302.68***</td>
</tr>
<tr>
<td>Temp.</td>
<td>303.30***</td>
</tr>
<tr>
<td>Salt</td>
<td>240.28***</td>
</tr>
<tr>
<td>Stor. * Phot.</td>
<td>5.80*</td>
</tr>
<tr>
<td>Stor. * Temp.</td>
<td>44.70***</td>
</tr>
<tr>
<td>Phot. * Temp.</td>
<td>125.28***</td>
</tr>
<tr>
<td>Stor. * Salt</td>
<td>6.86***</td>
</tr>
<tr>
<td>Phot. * Salt</td>
<td>100.60***</td>
</tr>
<tr>
<td>Temp. * Salt</td>
<td>19.40***</td>
</tr>
<tr>
<td>Stor. * Phot. * Salt</td>
<td>27.00***</td>
</tr>
</tbody>
</table>
Fig. 2.5. Comparison germination percentages of fresh and 1-year stored seeds of *Halopeplis perfoliata* in salinity treatments (0, 0.1, 0.2 0.3, 0.4, 0.5 and 0.6 mol L⁻¹ NaCl); temperatures (10/20, 15/25, 20/30 and 25/35 °C) and light (12-h photoperiod and 24- darkness) regimes. Different alphabets show significant differences among salinity treatments; Bonferroni test; *P* < 0.05. Asterisks (*) shows significant differences in fresh and stored seeds by Student’s t-test; *P* < 0.05.
**Experiment. 4: salinity tolerance and germination responses to dormancy regulating chemicals**

There was a significant effect of dormancy regulating chemicals (DRCs), photoperiod, salinity and their interaction on MFG of *H. perfoliata* (Table 2.5). Ethephon improved MFG in high salinity (0.6 mol L\(^{-1}\) NaCl) only; kinetin was inhibitory, while betaine, fusicoccin, proline and thiourea had no effect on MFG of *H. perfoliata* in distilled water as well as in saline condition under 12-h photoperiod (Fig. 2.6). However, all DRCs (except proline) improved MFG under complete darkness in distilled water as well as under saline condition (Fig. 2.7). There was no effect of DRCs on recovery (both S\(_R\) and D\(_R\)) responses of seeds (data not given).

Table 2.5. Three-way ANOVA showing effects of dormancy regulating chemicals (DRCs), photoperiod (Phot.), salinity (Salt) and their interactions on mean final germination (MFG) of *Halopeplis perfoliata* seeds. Numbers are *F*-values and asterisks in superscript are significance levels at *P* < 0.05 (** = *P* < 0.01 and *** = *P* < 0.001).

<table>
<thead>
<tr>
<th>Factor</th>
<th>MFG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRC(_S)</td>
<td>4.07**</td>
</tr>
<tr>
<td>Phot.</td>
<td>1119.73***</td>
</tr>
<tr>
<td>Salt</td>
<td>470.31***</td>
</tr>
<tr>
<td>DRC(_S) * Phot.</td>
<td>15.59***</td>
</tr>
<tr>
<td>DRC(_S) * Salt</td>
<td>3.65 ***</td>
</tr>
<tr>
<td>Phot. * Salt</td>
<td>105.73***</td>
</tr>
<tr>
<td>DRC(_S) * Phot. * Salt</td>
<td>3.78***</td>
</tr>
</tbody>
</table>
**Fig. 2.6.** Germination percentages of *Halopeplis perfoliata* treated with different dormancy regulating chemicals (DRCs) under different salinity treatments (0.3 and 0.6 mol L$^{-1}$ NaCl) at 20/30 °C temperature and 12-h photoperiod. Vertical bars are means ± S.E. Asterisks (*) show significant differences among salinity treatments within each chemical treatment by Student’s t-test; $P < 0.05$. 
Fig. 2.7. Germination percentages of *Halopeplis perfoliata* treated with different dormancy regulating chemicals (DRCs) under different salinity treatments (0.3 and 0.6 mol L\(^{-1}\) NaCl) at 20/30 °C temperature and complete darkness. Vertical bars are means ± S.E. Asterisks (*) show significant differences among salinity treatments within each chemical treatment by Student’s t-test; \( P < 0.05 \).
Discussion

Seed size and moisture content of Halopeplis perfoliata

Seeds of *H. perfoliata* were small (> 1 mm in diameter) like *H. amplexicaulis* and *H. pygmaea* seeds (Shepherd et al., 2005; Anonymous, 2014) and also had low (> 5%) moisture like many other halophytes such as *Limonium stocksii* and *Suaeda fruticosa* (Hameed et al., 2014). In absence of hairs and perianth, small size could be an effective adaptation for dispersal and also reduces their predation (Fenner, 1985; Leishman et al., 2000; Shepherd et al., 2005). Stromberg and Boudell (2013) found that small seed size was associated with adaption to disturbance and wet environments. Low moisture content on the other hand is reportedly essential for prolonged longevity of the seeds (Tompsett, 1986; Ellis, 1991; Rajjou and Debeaujon, 2008). Hence, small size and low moisture content of the seeds are important adaptations of *H. perfoliata* for survival in salt marsh, as reported for another subtropical halophyte *Arthrocnemum macrostahyum* (Gul and Khan, 1998).

Seed germination responses of *H. perfoliata* are influenced by salinity, temperature and photoperiod

Innate dormancy is generally absent in perennial halophytes (Gul et al., 2013), which may be an adaptive strategy to take advantage of water availability after rain (Song et al., 2005). Similarly, mature seeds of *H. perfoliata* were non-dormant and germinated maximally (> 90%) in distilled water under 12-h photoperiod irrespective of incubation temperature. Increases in salinity decreased both rate and final seed germination of *H. perfoliata*, however a few seeds could also germinate in 0.6 mol L\(^{-1}\) NaCl (equivalent to seawater salinity) under 12-h photoperiod. Some other Salicornioideae species such as *Arthrocnemum indicum* (1.0 mol L\(^{-1}\) NaCl, Khan and Gul, 1998) and *Sarcocornia ambigua* (0.77 mol L\(^{-1}\) NaCl, Freitas and Costa, 2014) also showed high salinity tolerance during seed germination. When transferred to distilled water from saline solutions, ungerminated seeds showed a high recovery of germination, as reported for most other halophytes (Gul et al., 2013). This enforced or conditional dormancy (inability to germinate yet maintaining their viability) due to salinity is an important adaptive feature of halophyte seeds that distinguishes them from glycophytes and is a key to constitute persistent soil seed bank so that chance for seedling recruitment only after sufficient rain (Khan and Ungar, 1984; Khan and Gul, 2006; Cao et al., 2014).
Changes in temperature affect a number of processes regulating the seed germination such as membrane permeability, activity of membrane-bound proteins and cytosol enzymes (Gul and Weber, 1999; Bewley et al., 2013). Change in temperature changes may also act as a germination timing sensor (Ekstam et al., 1999). Hence, changes in incubation temperature also influence germination and salinity tolerance of halophyte seeds (Hameed et al., 2013; Gul et al., 2013). However, seeds of some halophytes are more sensitive to temperature changes than others (Khan and Gul, 2006). In this study, relatively higher germination and salinity tolerance of *H. perfoliata* seeds was observed at 20/30 °C in comparison to other temperature regimes used. Recently Gul et al. (2013) reviewed that seed of most subtropical halophytes germinate better at 20/30 °C, which resembles the post-rain ambient temperature in many subtropical areas. Higher (25/35 °C) temperature was more inhibitory for the seed germination of *H. perfoliata* than lower (10/20 and 15/25 °C) temperatures, as reported for another salt marsh halophyte *Arthrocnemum indicum* (Saeed et al., 2011). Changes in temperature also influence recovery of germination of halophyte seeds (Khan and Ungar, 1997). Recovery of germination was also inhibited significantly at higher temperature regime, as in case of *A. indicum* (Saeed et al., 2011). Hence, higher temperature and salinity appear to act as regulatory factors by preventing germination of *H. perfoliata* in summer when there is high salinity in marsh sediments owing to no/low precipitation and high evapotranspiration due to high ambient temperatures.

Light has also been recognized as an important factor regulating seed germination of many plant taxa including most halophytes (Pons, 2000; Baskin and Baskin, 2001; Flores et al., 2006; Kettenring et al., 2006; Gul et al., 2013). Halophytes vary in their light requirement for seed germination (Gul et al., 2013). Baskin and Baskin (1995) reported that out of 41 halophytes, seed germination of 20 species was promoted by light, 10 germinated in dark, and 11 species germinated equally well in light or dark. However, most light requiring (positive photoblastic) seeds are small (Grime et al., 1981). Seeds of *H. perfoliata* are also small and germinated better in presence of light (12-h photoperiod) than in dark. In addition un-germinated (conditionally dormant) seeds from dark treatment showed recovery of germination when exposed to 12-h photoperiod, thus indicating their positive photoblastic nature. Milberg et al. (2000) suggested that light requirement for seed germination is a co-evolved adaptation of small seeds to ensure germination only when close to the soil surface. Light requirement is important
for small seeds because they contain too little food reserve to support seedling emergence from deep burial in soil (Winn, 1985; Bonfil, 1998; Seiwa, 2000). In our test species, light requirement would favor germination of seeds at/near soil surface, to ensure light access to nascent seedlings, thereby maximizing their survival in marsh environment.

*Seeds of Halopeplis perfoliata can endure hyper-salinity*

Seeds of most halophytes can even withstand hyper salinity (equivalent to 1.0 mol L\(^{-1}\) NaCl or higher) by undergoing conditional dormancy, in which although they do not germinate but remain viable and germinate quickly when salinity is diluted/alleviated (Keiffer and Ungar, 1997; Gul et al., 2013). Seeds of the salt marsh halophyte *H. perfoliata* also exhibited tolerance to hyper salinity by entering into a state of conditional dormancy and showed high (80%) recovery when transferred to distilled water after 60 days of exposure to 2.0 mol L\(^{-1}\) NaCl at 20/30 °C. Similarly, seeds of another chenopod halophyte *Salsola affinis* showed 37% recovery of germination, after 14 days of exposure to 2.0 mol L\(^{-1}\) NaCl salinity (Wei et al., 2008). Seeds of some other halophytes *Suaeda depressa*, *Salicornia europaea*, *Suaeda linearis* and *Spergularia marina* could also withstand 0.85 mol L\(^{-1}\) NaCl treatment for 30 days in state of conditional dormancy and showed substantial recovery in distilled water (Ungar, 1995). Hence, germination inhibition in halophytes including our test species appears a consequence of osmotic effect rather than ionic toxicity. However, high (25/35 °C) incubation temperature was detrimental to seed viability and about 50% seeds of *H. perfoliata* seeds died after 60 days of exposure to 2.0 mol L\(^{-1}\) NaCl at 25/35 °C as compared to 20/30 °C, at which there was only ~20% seed mortality. High storage temperatures reportedly reduce longevity of seeds, especially under moist condition (Egley, 1990; Rajjou and Debeaujon, 2008).

*Storage influences seed germination of Halopeplis perfoliata*

Long term storage of seeds is often reported to affect their germination and viability (Kibinza et al., 2006; Rajjou and Debeaujon, 2008; Zhang et al., 2011). Studies on crop seeds showed that germination requirements may become less specific after dry storage (Probert, 2000). However, little is known about effects of long term storage on stress tolerance of the seeds of non-crops such as halophytes (El-Keblawy, 2013a; Gul et al., 2013). Dry storage of the seeds of two succulent halophytes *Haloxylon salicornicum* and
Salsola imbricata for three months significantly increased germination but longer than nine month storage at room temperature caused significant reduction or complete inhibition of germination (El-Keblawy, 2013a). Similarly, another study showed that the light requirement for germination reduced significantly in Prosopis juliflora seeds after 8 months of dry-storage at room temperature (El-Keblawy and Al-Rawai, 2006). In this study, 1-year dry storage enhanced tolerance of H. perfoliata seeds to moderate salinity and dark, but substantially decreased high temperature tolerance, in comparison to fresh seeds. The ecological advantage of reduction in light requirement for seed germination after storage although seems unknown, however increase in germination under moderate salinity but not at high temperature could be to provide chance to seeds stored in distal dry marsh sediments to germinate after winter rains but to prevent germination after light summer rains which follow fast soil drying due to high temperatures.

Application of dormancy regulating chemicals can mitigate dark but not salinity effects
Various dormancy regulating chemicals (DRCs) such as betaine, ethephon, fusicoccin, kinetin, proline and thiourea are often reported to increase germination and stress tolerance of halophyte seeds (Khan et al., 1998; Gul et al., 2000; Khan et al., 2004; Mehrun-Nisa et al., 2007; Atia et al., 2009; El-Keblawy et al., 2010; Zehra et al., 2013; Ahmed et al., 2014). However, actions of these DRCs could be both species and stress specific (Gul et al., 2000; Ahmed et al., 2014). In this study, different DRCs generally had little/no effect on salinity tolerance of H. perfoliata but all (except proline) improved MFG under complete darkness as compared un-treated seeds. Ethephon and thiourea (Gul and Weber, 1998), betaine and kinetin (Gul et al., 2000) and fusicoccin (El-Keblawy et al., 2010; El-Keblawy, 2013b; Zehra et al., 2013) and could also substitute for light requirement for the seed germination of many other halophytes. As in this study, proline did not improve salinity as well as dark enforced dormancy in two other halophytes Halogeton glomeratus and Peganum harmala (Ahmed et al., 2014).
Conclusions

*Halopeplis perfoliata* seeds were non-dormant and germinated quickly in distilled water irrespective of incubation temperature but required light for germination. Seeds showed high tolerance to salinity and some seeds could germinate in as high as seawater salinity (0.6 mol L\(^{-1}\) NaCl) under 12-h photoperiod. High salinity (\(\geq 0.3\) mol L\(^{-1}\) NaCl) and dark imparted enforced/conditional dormancy in seeds, as they showed high recovery of germination when transferred to distilled water and light. Seeds of our test species could withstand hyper salinity (\(\geq 1.0\) mol L\(^{-1}\) NaCl) by entering a state of enforced dormancy. However, high incubation temperature enhanced seed mortality under hyper-saline condition. Dry storage for 1-year increased tolerance of *H. perfoliata* seeds to moderate salinity and dark, but substantially decreased high temperature tolerance, in comparison to fresh seeds. Whereas, dormancy regulating chemicals could compensate for light requirement but had no effect on salinity-enforced dormancy. Hence, it appears that salinity enforced dormancy has osmotic, while dark enforced dormancy has biochemical basis.

*Fig. 2.8.* Regulation of seed germination in *Halopeplis perfoliata* by light, temperature, salinity and chemicals (DRCs).
References:


reducing water uptake and ascorbate dependent antioxidant system. 

*Environmental and Experimental Botany, 107*, 32-38.


Chapter 3

Comparison of osmotic and ionic effects of various salts on germination inhibition and recovery responses of *Halopeplis perfoliata* seeds
Abstract

Salinity affects seed germination of halophytes by inducing ionic toxicity, osmotic constraint or both. Information about the effects of salinity on seed germination of a large number of halophytes exists, but little is known about the basis of this salinity-induced germination inhibition. In order to partition salinity effects, we studied seed germination and recovery responses of a coastal salt marsh halophyte *Halopeplis perfoliata* to different isotonic treatments ($\psi_s$: -0.5, -1.0, -1.5, -2.0 and -2.5, MPa) of various salts and polyethylene glycol (PEG) under two light regimes (12-h light photoperiod and 24-h complete darkness). Highest seed germination was observed in distilled water under 12-h light photoperiod and reduction in osmotic potential of the solution decreased seed germination. However, some seeds of *H. perfoliata* could germinate in as low as -2.5 MPa (~0.6 mol L$^{-1}$ NaCl), which is equivalent to seawater salinity. Sea-salt treatment was more inhibitory than isotonic NaCl at the lowest osmotic potential ($\psi_s$ -2.5 MPa). Generally, chloride salts inhibited germination more than the isotonic sulfate salts. Comparable germination responses of seeds in NaCl and isotonic PEG treatments as well as high recovery of germination in un-germinated seeds after alleviation of NaCl salinity indicated prevalence of osmotic constraint. Hence seed germination inhibition in *H. perfoliata* under different salts is a combination of osmotic constraint and their differential ionic effects.
Introduction

**Seeds of salt marsh halophytes are under direct influence of seawater**

Coastal salt marshes are transition between marine and terrestrial ecosystems, characterized by highly salt tolerant halophyte vegetation and offer a number of ecological and economic services to mankind (Böer, 1996; Pennings and Bertness 2001; Lee et al., 2006; Gedan et al., 2009; Teixeira et al., 2014). These habitats are however under threat due to various natural and anthropogenic reasons (Yasseen and Al-Thani, 2007; Gedan et al., 2011; Ramadan et al., 2013). Therefore, salt marsh ecosystems warrant special attention in research, which would eventually help in their protection and conservation. Establishment of plants in salt marshes is reportedly dependent on the seed germination responses which are influenced by a number of factors especially salt concentrations in the marsh sediments (Pujol et al., 2000; Khan et al., 2001; Hameed et al., 2006; Gul et al., 2013). However, data on seed germination responses of succulent halophytes of subtropical salt marshes is scanty (Saeed et al., 2011; Gul et al., 2013). Seeds of salt marsh halophytes are directly influenced by seawater owing to diurnal and seasonal inundations (Gul and Khan, 1998; Gul et al., 2013; Teixeira et al., 2014). NaCl is although dominant but other chloride and sulfate salts and their interactions may also play a significant role in germination, radical emergence and seedling growth of coastal halophytes (Gul and Khan, 1998; Khan, 2003; Zia and Khan, 2008). Most research on the seed germination of halophytes has focused on responses to NaCl (reviewed by Gul et al., 2013), with few studies on comparison of the effects of NaCl and sea-salt (Zia and Khan, 2002; Atia et al., 2006; Hameed et al., 2006; Liu et al., 2006; Saeed et al., 2011; Shaikh et al., 2013) and even fewer studies on effects of different salts (Ungar, 1991; Egan et al., 1997; Panuccio et al., 2014). Likewise, investigations to partition osmotic and ionic constraints of various salts (Munns et al., 1995) by comparing their effects on seed germination with germination responses in non-ionic solutes such as polyethylene glycol (PEG) are also limited and inconclusive (Tobe et al., 2000; Sosa et al., 2005; Hameed et al., 2013).

**Salt marsh halophyte Halopeplis perfoliata is largely unstudied**

*Halopeplis perfoliata* Forssk. (Amaranthaceae) is a succulent halophyte of coastal salt marshes along Arabian Peninsula (Al-Oudat and Qadir, 2011), where it acts as an important primary producer and provides shelter to marine life (Pilcher et al., 2003). A
previous report showed that the seeds of *H. perfoliata* can germinate in up to 0.25 mol L⁻¹ NaCl treatment (Mahmoud et al., 1983). This study was therefore designed to comparatively investigate the effects of 1) sea-salt 2) various chloride and sulfate salts and 3) polyethylene glycol 6000 (PEG-6000) on germination and recovery of the seeds of *H. perfoliata*.

**Materials and methods**

*Study site and seed collection*

Seeds of *Halopeplis perfoliata* were collected from the coast of Jizan, Saudi Arabia in 2012. Seeds were separated from inflorescence husk manually; surface sterilized using 1% bleach solution for 1 min, followed by rinsing with distilled water and air-drying in the laboratory.

*Treatments and experimental conditions*

Germination experiments were carried out in clear-lid plastic petri-plates (50 mm diameter x 15 mm depth) with 5 ml of test solution. Seeds were immersed in distilled water (0 MPa) and isotonic solutions of various salts (NaCl, Na₂SO₄, KCl, K₂SO₄, MgCl₂, MgSO₄, CaCl₂ and Sea-salt) and polyethylene glycol 6000 (PEG). Five isotonic levels (Ψₛ: -0.5, -1.0, -1.5, -2.0 and -2.5, MPa) were used. The osmotic potential of the above solutions was measured with a vapor pressure osmo-meter (5520 Wescor, Inc.). There were four replicates of 25 seeds each per treatment. The petri-plates were placed in programmed incubators maintained at 20/30 °C (optimum temperature for the seed germination of sub-tropical halophytes, Gul et al., 2013), where low temperature coincided with 12-h dark and high temperature with 12-h light (–25 μ mol m⁻² s⁻¹, 400-750 nm, Philips cool -white fluorescent lamps). Seed germination percentage (radicle emergence, Bewley and Black, 1994) was recorded at 2 days intervals for 20 days. Rate of germination (G_rate) was estimated by using a modified Timson index of germination velocity given below:

\[
\text{Germination velocity (\%) } = \frac{\sum G}{t} \times 100
\]

Where G is the percentage of seed germination at 2 days intervals and t is the total germination period (Khan and Ungar, 1985). The greater the value, the more rapid is germination. After 20 days un-germinated seeds were transferred to distilled water for
another 20 days and recovery of germination ($S_R$) from various osmotic treatments of solutes (salts and PEG) was recorded. The recovery percentage was determined by counting the recovered seeds from total number of seeds. The recovery percentage was calculated by using given formula:

$$Recovery (\%) = \frac{(A - B)}{C} \times 100$$

Where, A is total number of seeds germinated after being transferred to distilled water, B is the number of seeds germinated in saline solution and C is total number of seeds. A parallel set of experiment was conducted by placing petri-plates in dark-envelops under aforementioned temperature and osmotic treatments for 20 days. Germination of this experiment was noted once after 20 days. After 20 days un-germinated seeds were exposed to light in order to study the recovery of germination from dark ($D_R$).

Statistical analyses
Data were statistically analyzed using SPSS version 11.0 (SPSS, 2011). Analysis of variance (ANOVA) was performed to determine if various treatments affected the seed germination parameters significantly. A Bonferroni post-hoc was conducted to indicate significant ($P < 0.05$) differences among mean values.

Results
A four-way ANOVA indicated significant effects of osmotic potential ($\Psi_S$), photoperiod, anions and but not of cations on mean final germination (MFG) of *H. perfoliata* seeds (Table 3.1).

*Comparitive effects of NaCl and Sea-salt*
Seeds were non-dormant and germinated maximally (~90%) in distilled water under 12-h photoperiod (Fig. 3.1). Effects of NaCl and sea-salt were comparable in up to -1.0 MPa, wherein there was maximal (~90%) MFG as in distilled water under 12-h photoperiod (Fig. 3.1). While, in lowest (-2.5 MPa) $\Psi_S$ treatment, sea-salt (MFG = ~10%) was found more inhibitory than NaCl (MFG = ~25%). Rate of germination in -2.5 MPa was also lowest in sea-salt compared to NaCl (Fig. 3.2). Un-germinated seeds from low $\Psi_S$ treatments of both NaCl and sea-salt, showed recovery ($S_R$) when transferred to distilled
water and almost all un-germinated seeds recovered (Fig. 3.1). Dark inhibited seed germination of test species in both NaCl and sea-salt treatments (Fig. 3.3). When un-germinated seeds from dark were transferred to 12-h photoperiod for another 20 days, a significant recovery (DR) was seen in either salt type. Still un-germinated seeds from low \( \Psi_S \) treatments of both NaCl and sea-salt showed complete recovery (SR) when transferred to distilled water (Fig. 3.3).

**Comparison of the effects of different salts**

A decrease of up to -1.0 MPa (equivalent to 0.215 mol L\(^{-1}\) NaCl) for chloride salts and -1.5 MPa (equivalent to 0.4 mol L\(^{-1}\) NaCl) for sulfate salts had no inhibitory effect on seed germination of test species (Fig. 3.1). \( K_2SO_4 \) was less inhibitory, in which over 70% seeds germinated in lowest (-2.5 MPa) \( \Psi_S \) treatment. On the other hand, \( CaCl_2 \) and NaCl reduced MFG to ~50% in -1.5 MPa treatments (Fig. 3.1). Inhibitory effects of chloride salts were in following order: \( CaCl_2 > NaCl > KCl > MgCl_2 \). While, MFG inhibition among sulfate salts was as follows: \( Na_2SO_4 > MgSO_4 > K_2SO_4 \). Rate of germination also decreased with decreases in \( \Psi_S \) of salts (Fig. 3.2). However, there was a significant decrease in rate of germination in -1.0 MPa for chloride salts and in -1.5 MPa for sulfate salts. While similar to MFG, NaCl and \( CaCl_2 \) had higher inhibition and \( K_2SO_4 \) caused less inhibition to the rate of germination (Fig. 3.2). Almost all un-germinated seeds showed recovery of germination (SR) when transferred to distilled water and generally highest SR was observed from the lowest (-2.5 MPa) \( \Psi_S \) treatments of all salts (Fig. 3.1).

Dark caused substantial inhibition to the MFG of *H. perfoliata*, irrespective of the salt and \( \Psi_S \) treatments (Fig. 3.3). When un-germinated seeds from dark were exposed to 12-h photoperiod after 20 days, a significant recovery of germination (DR) was observed. The DR was highest in distilled water and there was decrease in DR in solution of low \( \Psi_S \) (Fig. 3.3). When still un-germinated seeds from low \( \Psi_S \) treatments were transferred to distilled water for another 20 days, most of them showed recovery of germination (SR), irrespective of salt used (Fig. 3.3).

**Comparison of the effects of NaCl and PEG**

Effects of NaCl and PEG on MFG and germination rate were generally comparable at higher osmotic levels and these germination parameters decreased with decreases in \( \Psi_S \) under 12-h photoperiod (Fig. 3.4 A and 4 C). Un-germinated seeds from low \( \Psi_S \)
treatments of both NaCl and PEG showed high recovery ($S_R$; Fig. 3.4 B) complete darkness caused substantial inhibition to MFG (Fig. 3.4 D). Un-germinated seeds from the dark showed high recovery ($D_R$) upon transfer to 12-h photoperiod condition (Fig. 3.4 E). However, relatively higher $D_R$ was observed in moderately low (-1.0 to -1.5 MPa) PEG compared to isotonic NaCl treatments. Remaining un-germinated seeds from low $\psi_S$ treatments of both NaCl and PEG germinated ($S_R$) when transferred to distilled water (Fig. 3.4 F).

**Table 3.1.** One-way ANOVA of the effects of salinity on growth, water and ion relations, pigments, photosynthesis, isotope ratios, stress markers, antioxidant enzymes and substrates of *Halopeplis perfoliata* grown under salinity treatments (0, 1.50, 0.30 and 0.60 mol L$^{-1}$ NaCl) for 30 days. Numbers are $F$-values and asterisks in superscript are significance levels at $P < 0.05$ (ns = non-significant, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

<table>
<thead>
<tr>
<th>Factors</th>
<th>MFG</th>
<th>$G_{Rate}$</th>
<th>Rec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phot.</td>
<td>4341.52***</td>
<td>372.55***</td>
<td>-</td>
</tr>
<tr>
<td>$\psi_S$</td>
<td>98.50***</td>
<td>4.02**</td>
<td>500.63***</td>
</tr>
<tr>
<td>Anion</td>
<td>13.63***</td>
<td>0.53ns</td>
<td>28.65***</td>
</tr>
<tr>
<td>Cat.</td>
<td>2.16ns</td>
<td>1.96ns</td>
<td>5.71**</td>
</tr>
<tr>
<td>Phot. $\times$ $\psi_S$</td>
<td>97.76***</td>
<td>189.41***</td>
<td>-</td>
</tr>
<tr>
<td>Phot. $\times$ Ani</td>
<td>14.39***</td>
<td>45.74***</td>
<td>-</td>
</tr>
<tr>
<td>Phot. $\times$ Cat.</td>
<td>2.35ns</td>
<td>9.08***</td>
<td>-</td>
</tr>
<tr>
<td>$\psi_S$ $\times$ Ani</td>
<td>4.65***</td>
<td>5.45***</td>
<td>9.31***</td>
</tr>
<tr>
<td>$\psi_S$ $\times$ Cat.</td>
<td>0.78ns</td>
<td>1.58ns</td>
<td>4.29***</td>
</tr>
<tr>
<td>Ani $\times$ Cat.</td>
<td>2.46ns</td>
<td>9.20**</td>
<td>10.58***</td>
</tr>
<tr>
<td>Phot.$\times$Ani$\times$ Cat.</td>
<td>2.26ns</td>
<td>3.51*</td>
<td>-</td>
</tr>
<tr>
<td>Phot. $\times$ $\psi_S$ $\times$ Ani</td>
<td>4.50**</td>
<td>3.13**</td>
<td>-</td>
</tr>
<tr>
<td>Phot. $\times$ $\psi_S$ $\times$ Cat.</td>
<td>0.74ns</td>
<td>1.45ns</td>
<td>-</td>
</tr>
<tr>
<td>$\psi_S$ $\times$ Ani$\times$ Cat.</td>
<td>2.91**</td>
<td>2.91**</td>
<td>16.72***</td>
</tr>
<tr>
<td>Phot. $\times$ $\psi_S$ $\times$ Ani$\times$ Cat.</td>
<td>2.95**</td>
<td>1.99*</td>
<td>-</td>
</tr>
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</table>
Fig. 3.1. Effects of NaCl, Na₂SO₄, KCl, K₂SO₄, MgCl₂, MgSO₄, CaCl₂ and sea-salt on mean final germination (MFG) and germination recovery in distilled water (S₀) at 20/30 °C temperature and 12-h photoperiod. Vertical bars are means ± S.E. Different alphabets show significant differences among isotonic treatments within each parameter; Bonferroni test; $P < 0.05$. 
Fig. 3.2. Effects of NaCl, Na$_2$SO$_4$, KCl, K$_2$SO$_4$, MgCl$_2$, MgSO$_4$, CaCl$_2$ and sea-salt on rate of germination at 20/30 °C temperature and 12-h photoperiod. Vertical bars are means ± S.E. Different alphabets show significant differences among isotonic treatments within individual salt; Bonferroni test; $P < 0.05$. 

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Fig. 3.3. Effects of isotonic treatments of NaCl, Na$_2$SO$_4$, KCl, K$_2$SO$_4$, MgCl$_2$, MgSO$_4$, CaCl$_2$ and sea-salt on mean final germination (MFG), recovery from Salinity (S$_R$) and recovery from Dark (D$_R$) at 20/30 °C temperature and complete darkness. Vertical bars are means ± S.E. Different alphabets show significant differences among isotonic treatments within each parameter of individual salt; Bonferroni test; $P < 0.05$. 

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Fig. 3.4. Comparative effects of NaCl and PEG on mean final germination (MFG), Germination rate (GRate); recovery from salinity (SR) and recovery from dark (DR) under 12-h photoperiod at 20/30 °C temperature and complete darkness. Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments within each parameter;Bonferroni test; *P < 0.05. Asterisks (*) show significant differences in germination within an isotonic treatment of NaCl and PEG by student’s t-test; *P < 0.05.
Discussion

*Sea-salt is more inhibitory for germination than NaCl*

Seeds of *H. perfoliata* were non-dormant and showed maximum germination (~90%) in distilled water under 12-h photoperiod, as reported for many other marsh halophytes such as *Aeluropus lagopoides* (Khan and Gulzar, 2003), *Limonium stocksii* (Zia and Khan, 2004) and *Arthrocnemum indicum* (Saeed et al., 2011). Recently, Gul et al. (2013) reviewed that lack of innate dormancy in most subtropical perennial halophytes is probably a common adaptation to take advantage of brief water availability after rains. In addition, seeds of *H. perfoliata* displayed a high salt tolerance, as some seeds could germinate in as high as seawater (-2.5 MPa NaCl) salinity. Seeds of many other Salicornioideae halophytes such as *Arthrocnemum indicum* (Khan and Gul, 1998) and *Sarcocornia ambigua* (Freitas and Costa, 2014) also showed tolerance to seawater or higher salinity during seed germination. Furthermore, un-germinated seeds of *H. perfoliata* showed a high recovery of germination when transferred to distilled water from saline solutions like many other perennial halophytes, indicating that salinity treatments resulted in enforced/conditional dormancy and were no detrimental to seed viability (Khan and Gul, 2006; Cao et al., 2014). Hence, it appears that seeds of *H. perfoliata* are well-adapted for saline environment. However, whether these seed responses to NaCl based salinity are comparable to seawater salinity, have seldom been studied. Most studies on effects of salinity on seed germination of halophytes are based on NaCl treatments and generally little is known about effects of seawater on seed germination of halophytes (Zia and Khan, 2002; Atia et al., 2006; Hameed et al., 2006; Liu et al., 2006; Saeed et al., 2011; Shaikh et al., 2013). In this study, effects of NaCl and sea-salt were comparable in up to -1.0 MPa but at lowest (-2.5 MPa) $\Psi_S$ treatment sea-salt was more inhibitory than isotonic NaCl. Many studies showed that the sea-salt inhibits seed germination more in comparison to NaCl. For instance, higher concentrations (or lower $\Psi_S$ treatments) of sea-salt were more inhibitory for the seed germination of *Limonium stocksii* (Zia and Khan, 2002), *Aeluropus lagopoides, Desmostachya bipinnata* and *Suaeda fruticosa* in comparison to NaCl (Hameed et al., 2006). However, NaCl inhibited seed germination of *Hipophae rhamnoides* (Tirmizi et al., 1993) and *Crithmum maritimum* (Atia et al., 2006) more as compared to sea-salt. Effects of NaCl and sea-salt on seed germination of *Haloxylon stocksii* comparable
(Hameed et al., 2006) also indicated that the effects of NaCl and seawater on seed germination of halophytes are species-specific.

*Sulfate salts are less detrimental than chloride salts for seed germination of Halopeplis perfoliata*

Seawater is composed of many salts, of which NaCl is the major (> 85%) fraction (Murray, 2004; Khan and Gul, 2006). Other notable cations of seawater in order of magnitude are Mg$^{2+}$ > Ca$^{2+}$ > K$^+$ and SO$_4^{2-}$ is second major anion after Cl$^-$ (Murray, 2004). Typically, six ions constitute > 99% of the seawater (http://oceanplasma.org/documents/chemistry.html). Therefore, I examined the effects of these ions on seed germination of coastal marsh halophyte *H. perfoliata* found significant effect of anions but not of cations on seed germination of test species. A decrease of up to -1.0 MPa (equivalent to ~0.2 mol L$^{-1}$ NaCl) for chloride salts and -1.5 MPa (equivalent to 0.4 mol L$^{-1}$ NaCl) for sulfate salts had no inhibitory effect on seed germination of test species but a further decrease in $\Psi_S$ of solutions was inhibitory. Likewise, seed germination of three perennial salt marsh halophytes *Arthrocnemum macrostachyum*, *Juncus acutus* and *Schoenus nigricans* was inhibited more in chloride than sulfate salts of sodium and magnesium (Vicente et al., 2007). Seed germination characteristics of *Kalidium capsicum* (Tobe et al., 2002) *Haloxylon ammodendron* (Tobe et al., 2004) and *Halostachys caspica* (Assareh et al., 2011) were also more sensitive to chloride than sulfate salts. These data including the present study indicate that sulfate salts are comparatively less detrimental to seed germination of most halophytes than isotonic chloride salts. However eco-physiological significance of this differential effect is yet to be determined by further research.

*Halopeplis perfoliata seeds show differential response to isotonic NaCl and PEG treatments*

Salinity affects seed germination either by reducing soil water potential (i.e. compromising imbibition an osmotic effect) and/or by excessive entry of Na$^+$ which is toxic to metabolism (ionic toxicity) (Khan and Gul, 2006; Kranner and Seal, 2013; Hameed et al., 2014). Uptake of salt could lower seed’s water potential thereby facilitating imbibition, however ionic toxicity may overshadow such beneficial osmotic effects (Collis-George and Sands, 1962; Khan et al., 1987; Song et al., 2005; Gul et al., 2013). In order to partition osmotic and ionic components of salinity, often seed
germination response in a salt solution is compared with that in an isotonic solution of a non-ionic solute such as polyethylene glycol (Hardegree and Emmerich, 1990; Bajji et al., 2002; Song et al., 2005; Hameed et al., 2013). In this study, effects of NaCl and PEG on seed germination of *H. perfoliata* were generally comparable, especially under high osmotic levels. Similar effects of NaCl and non-ionic solutes (PEG or mannitol) were observed on seed germination of *Chrysothamnus nauseosus* (Dodd and Donovan, 1999) and *Atriplex halimus* (Bajji et al., 2002). Greater incidence of osmotic constraint of salinity on seed germination than ionic toxicity was also observed in this study. High recovery of un-germinated seeds in this study after alleviation of salinity also supports this assumption. On the other hand, salinity caused ionic toxicity in some species like *Artemesia ordosica* (Tobe et al., 1999), *Aristida adscensionis* and *Prosopis strombulifera* (Sosa et al., 2005) and *Suaeda heterophylla* (Hameed et al., 2013).

**Conclusions**

*Halopeplis perfoliata* seeds appear highly tolerant to salinity, as some seeds could germinate in NaCl treatments as low as -2.5 MPa (~0.6 mol L\(^{-1}\)), which is equivalent to seawater salinity. However, a sea-salt treatment of -2.5 MPa inhibited seed germination more than isotonic NaCl treatment (Fig. 3.5). Furthermore, chloride salts were generally more inhibitory to seed germination than isotonic sulfate salts (Fig. 3.5). Un-germinated seeds from low osmotic potential treatments of all salts showed high recovery of germination when transferred to distilled water, indicating osmotic rather than ionic effect of salts (Fig. 3.5). Similar germination response of *H. perfoliata* seeds in isotonic NaCl and PEG treatments at higher osmotic levels also indicate osmotic effect rather than ionic effect of NaCl salinity.
Fig. 3.5. Differential inhibitory effects of iso-osmotic treatments with NaCl, seasalt, and various chloride (Cl⁻) and sulphate (SO₄²⁻) salts on seed germination of *Halopeplis perfoliata*.

References:


Chapter 4

Growth and physio-chemical adaptations of *Halopepis perfoliata* under saline conditions
Abstract

*Halopeplis perfoliata*, a C$_3$ salt marsh halophyte with succulent stems, was grown in a greenhouse to study its adaptive responses for ion regulation, antioxidant defense system, photosynthesis and growth after one month exposure to saline conditions. Plants were grown in sand culture with and without 0.15, 0.3 and 0.6 mol L$^{-1}$ NaCl with Hoagland’s nutrient solution and sampled for measurements of biomass, osmotic adjustment, cations, photosynthetic pigments, Rubisco content, CO$_2$ gas exchange, chlorophyll fluorescence and antioxidant enzymes and substrates. After four weeks of exposure to salinity, biomass was highest in the 0.15 mol L$^{-1}$ NaCl treatment but similar to non-saline controls at higher salinities. There was little difference in the effect of salinity on the water relations, as indicated by the constitutively high shoot succulence, osmotic potential and relative water content except for the highest salinity (0.6 mol L$^{-1}$ NaCl) treatment where decreased succulence and more negative values of $\Psi_S$, indicated signs of water stress. Plants were able to maintain adequate K$^+$ levels even at 0.6 mol L$^{-1}$ NaCl where Na$^+$ accumulation was highest. Photosynthetic rates were unaffected by salinity whereas, transpiration (E) rates and stomatal conductance (gs) increased in low and moderate salinity treatments. Decreased E and gs induced higher WUE in the 0.6 mol L$^{-1}$ NaCl treatment. The larger fall in the value of carbon isotope discrimination measured in photosynthetic shoots at 0.6 mol L$^{-1}$ NaCl also indicated greater transpiration efficiency when exposed to high saline conditions. Chlorophyll fluorescence measurements failed to indicate damage to photochemical pathways. However, decreased ETR/(P$_N$+R$_L$+R$_D$) ratios indicated a down regulation of photochemistry under saline conditions and little need for alternative electron sinks for ROS detoxification. Increased H$_2$O$_2$ production and CAT activity, higher CO$_2$ compensation point and lower carbon isotope discrimination indicated increased photorespiration rates at the highest salinity to avoid photo-inhibition. These data indicate that *H. perfoliata* is an obligate halophyte that can grow up to seawater salinities by down regulating its photochemistry and growth, high osmotic adjustment, an efficient ion homeostasis and antioxidant defense system.
Introduction

Growth
Halophytes distributed in salt marsh environments have developed specialized adaptations for growing in high light, salinity and inundation regimes (Flowers et al., 2015; Flower and Colmer, 2015). Extreme variations in these abiotic conditions results in additional stress for plants. Characterization of the salt resistance traits could help in predicting and improving plant performance under increasing salinity. Succulent eu-halophytes such as *Arthrocnemum macrostachyum* and *Suaeda fruticosa* are reported to endure up to 1.0 mol L\(^{-1}\) NaCl salinity during vegetative growth (Gul and Khan, 2006; Hameed et al., 2012). Growth of these succulent halophytes appears to be promoted between 0.2-0.4 mol L\(^{-1}\) NaCl in contrast to succulent xero-halophytes such as *Haloxylon recurvum* syn. *Haloxylon stocksii* (Khan et al., 2000), *Atriplex halimus* (Khedr et al., 2011) and *Zygophyllum xanthoxylum* (Yue et al., 2012). Little is known about the eco-physiology of salt resistant Chenopods (Amaranthaceae) of the warm Saharo-Sindian region which may vary considerably among species (Moinuddin et al., 2014).

Water and ion relations
Salinity affects the plant growth via osmotic or ionic stress. Osmotic stress affects the plants growth by limiting the water availability. Halophytes adapt to seawater or higher salinity through osmotic adjustment via ion compartmentalization in cell vacuoles, by accumulating compatible organic solutes, increased succulence, and by means of salt-secreting glands or bladders (reviewed by Shabala, 2013). Flowers and Colmer (2015) also highlighted the role of ion exclusion besides excretion from photosynthetic tissues which would otherwise result in the accumulation of salts to precipitate in the xylem stream in a plant growing under seawater salinity. Ion exclusion from the xylem involves membrane transporter proteins to maintain tissue levels of Na\(^+\) and Cl\(^-\) within tolerable range with efficient acquisition of K\(^+\) and Mg\(^{++}\) (Mansour, 2014; Flowers and Colmer, 2015). In addition, loading of excess salt in old senescing leaves also contribute to management of excess ions (mainly Na\(^+\) and Cl\(^-\)) at the whole-plant level (Flower and Colmer, 2008; Shabala, 2013; Reef and Lovelock, 2015). Na\(^+\) and Cl\(^-\) retained in growing plant tissues are actively compartmentalized in cell vacuoles which minimize metabolic toxicity and contribute towards osmoregulation to acquire and retain water. Accumulation of organic solutes such as glycine betaine, proline and sugars in the
cytoplasm may be involved in osmotic adjustment and osmoprotection (Munns and Tester, 2008; Hameed and Khan, 2011). Higher amount of organic solutes were mostly found in Chenopods such as *Atriplex nummularia*, *Halocnemum strobilaceum* and *Salicornia europaea*, (reviewed by Lokhande and Suprasanna, 2012).

**Photosynthesis and photorespiration**

At the leaf level, growth is driven by CO₂ fixation which is more sensitive to salinity than light harvesting ability of photosynthetic machinery (Osmond 1994; Maricle et al., 2007; Taiz and Zeiger, 2010). Increasing salinity generally decreases CO₂ assimilation by reducing mesophyll CO₂ availability as a result of stomatal closure to minimize water loss (Álvarez et al., 2012; Naidoo et al., 2012). Stomatal closure in salt-stressed leaves has been shown to result in enhanced rates of photorespiration as reviewed by Wingler et al. (2000). At the biochemical level, photosynthetic efficiency could be limited under saline conditions due to reduced rubisCo activity, rubisCo activase, or chlorophyll content (Rivelli et al., 2002; Koyro et al., 2013). At higher leaf Na⁺ accumulation under saline conditions, C₃ wheat responded to reduced internal CO₂ concentration by increasing water use efficiency (lower carbon isotope discrimination) (Rivelli et al., 2002).

δ¹³C values can indicate whether C₃ or C₄ pathways are used for carbon fixation (Zhu and Meinzer, 1999). The use of C-isotope discrimination in plant biomass is an efficient tool for determining changes in carboxylation efficiency of photosynthetic machinery under salinity stress (Michener and Lajtha, 2008). In C₃ plants, δ¹³C enrichment of biomass with increasing salinity treatments is attributed to either low Ci values by stomatal closure and/or increased rates of photorespiration (higher oxygenation/carboxylation) (Ivlev et al., 2013). Under both the above conditions, δ¹³C values tend to become less negative (greater ¹³C enrichment in biomass) (Michener and Lajtha, 2008; Ivlev et al., 2013). Atmospheric nitrogen has a δ¹⁵N value of zero. High δ¹⁵N values are found in salt marsh halophytes such as *Spartina* sp. (6%) (Wigand et al., 2007). A number of pot culture studies have reported significant discrimination between plant tissues and the N in solution, there is general agreement that discrimination is only observed when plant N demand is low compared with N supply. Proteins are generally δ¹⁵N enriched relative to the bulk δ¹⁵N of the plant cell, while secondary products such
as chlorophyll, lipids, amino sugars and alkaloids are depleted in $\delta^{15}N$ (Michener and Lajtha, 2008).

**Chlorophyll Fluorescence**

Although salinity stress causes damage to the light harvesting machinery of most plants, many halophytes show no evidence of photo-inhibition (Qiu et al., 2003). Growth under higher salinity could ultimately lead to over-reduction of photosynthetic electron transport chains resulting in higher demands for alternative sinks to prevent photo-inhibition of PSII (Demmig-Adams and Adams, 1992). Dissipation of excess energy in form of heat via xanthophyll cycle at PSII acts as an efficient front-line defense in this regard (Demmig-Adams and Adams, 1996; Müller et al., 2001; Terashima et al., 2009). Whereas, water-water cycle contributes in protection of PSI by quenching reactive oxygen species (ROS) produced by electron leakage to di-oxygen from over-reduced PSII (Mehler, 1957; Asada, 1999). However, the magnitude and efficiency of these mechanisms in protecting photosynthetic machinery may vary among species (Driever and Baker, 2011; García-Plazaola et al., 2012).

**Oxidative stress**

Halophytes possess an efficient antioxidant system that keeps cellular levels of ROS within a range, which is essential for their signaling and other roles (Jithesh et al., 2006; Ozgur et al., 2013; Uzilday et al., 2015). Antioxidant system is composed of various antioxidant enzymes such as superoxide dismutases (SODs), catalases (CATs) and Foyer-Halliwell-Asada pathway enzymes and different low-molecular weight antioxidant substances such as ascorbate (ASA) and glutathione (GSH) (Sharma et al., 2012; Ozgur et al., 2013). These enzymatic and non-enzymatic antioxidants quench excess ROS; thereby prevent oxidative damages to important cell components such as membrane lipids, proteins, chlorophyll and nucleic acids (Sharma et al., 2012). However, under highly saline conditions these protective activities become inadequate, thereby oxidative damages results on species.

*Halopeplis perfoliata* Forssk. is a stem succulent C$_3$ perennial halophyte from family Amaranthaceae, which is commonly found in the coastal salt marshes and coastal salt deserts of Arabian gulf coast (Boer and Saenger, 2006; Al-Oudat and Qadir, 2011; El-Keblawy and Bhatt, 2015). An earlier study showed that this plant can tolerate as high
as ~ 0.5 mol L\(^{-1}\) NaCl salinity by maintaining Na\(^+\) and Cl\(^-\) contents to low levels (Al-Zaharani and Hajar, 1998). Besides, nothing is known about the salt tolerance mechanisms of this halophyte. The aim of this study was therefore to understand the effects of increasing NaCl salinity on growth, water relations, ion relations, photosynthesis and the antioxidant system of *H. perfoliata* seedlings.

It has been hypothesized that (1) *Halopeplis perfoliata* will tolerate near seawater concentrations of NaCl treatments; (2) *Halopeplis perfoliata* will accumulate high shoot Na\(^+\) concentrations with increasing salinity; (3) Shoot succulence will increase with increasing shoot Na\(^+\) accumulation; (4) Plants will maintain K\(^+\) ion homeostasis across all salinity treatments; (5) Light harvesting efficiency of PSII will be unaffected in comparison with gas exchange processes; (6) *Halopeplis perfoliata* will maintain an efficient constitutive antioxidant defense system under saline conditions.

Materials and Methods

*Seed collection site*

*Halopeplis perfoliata* inflorescence were collected from a salt marsh of Jizan Island, Saudi Arabia (16\(^0\) 20` N to 17\(^0\) 40` N and 41\(^0\) 55` E to 43\(^0\) 20` E) in 2012. Study area is frequently inundated by seawater and mean ambient temperature ranges between 36 and 21 °C (Alfarhan et al., 2005). Mean annual rainfall of the area is 169 mm, while humidity ranges between 39 and 86% (Alfarhan et al., 2005). Seeds were collected from large number of plants randomly to ensure adequate representation of the genetic diversity. Seeds were separated from inflorescence husk manually and brought to laboratory in Pakistan.

*Growth experiment*

Seedlings were raised in plastic pots (19 cm \(\Phi\) x 25 cm depth) containing sandy soil through sub-irrigated with half strength Hoagland’s nutrient solution (Epstein, 1972) in a green net house (PAR ~600 umol m\(^{-2}\) s\(^{-1}\)) under semi-ambient environmental conditions. Temperature during the day-time ranged between 32 to 40 °C, while relative humidity ranged from 40 to 60%. When seedlings attained a height of ~10 cm salinity (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl) was introduced gradually (at the rate of 0.05 mol L\(^{-1}\)) NaCl after every 12-h to avoid osmotic shock) in such a manner that all final salinity concentrations were achieved on the same day. There were four replicate pots of one plant each per
treatment. Plants were harvested 5 weeks after final salinity concentrations achieved. Plant fresh weight ($F_W$) were recorded immediately after harvest. Dry weight ($D_W$) was determined after drying vegetative parts in an oven at 60 °C for 48-h.

**Fig. 4.1.** Scheme showing salinity treatment regimes administered for growth experiment on *Halopeplis perfoliata* under greenhouse conditions.


Water and ion relations

Shoot succulence

Succulence was measured on a dry weight basis by using the following formula:

\[
\text{Succulence (H}_2\text{O g g}^{-1} \text{ D}_W\) = (F}_W - D_W)/D_W
\]

Relative water content (RWC)

Shoot RWC was calculated with the help of the following formula:

\[
\text{RWC (\%)} = (F}_W - D_W)/(T}_W - D_W) \times 100
\]

Where, turgid weight (T_W) of the shoot was determined after rehydration in distilled water for 24-h at room temperature (25 °C).

Osmotic potential (\(\Psi_S\))

Shoot osmolality was measured in pressed sap with the help of vapor pressure osmometer (VAPRO-5520; Wescor Inc., Logan, UTAH, USA) (Gucci et al., 1991) and osmotic potential (\(\Psi_S\)) was calculated by using van’t Hoff equation (Kramer and Boyer, 1995).

Cations regulation

Soluble Na\(^+\), K\(^+\), Ca\(^{++}\) and Mg\(^{++}\) in shoot and root sap were determined by using atomic absorption spectrometry (AA-700; Perkin Elmer, Santa Clara, CA, USA).

Photosynthetic system

Shoot Photosynthetic pigments

Photosynthetic pigments (chlorophyll a, b and carotenoid) were extracted from freshly harvested shoots in 80% (v/v) ice-cold acetone and estimated with the method of Lichtenthaler (1987).

\(\text{CO}_2\) Gas-Exchange Measurements

Net photosynthesis rate (\(P_n\)) on intact green shoots was estimated with the help of a Li-Cor 6400XT portable gas exchange system (Li-Cor Biosciences Inc., Lincoln, Nebraska, U.S.A.) by providing \([\text{CO}_2]\) of 400 \(\mu\text{mol m}^{-2}\) with a flow rate of 300 \(\mu\text{mol m}^{-2}\ \text{s}^{-1}\) and leaf chamber relative humidity between 60-80%. A \(\text{CO}_2\) cartridge was used, and a 6400-01 \(\text{CO}_2\) Injector system controlled the \(\text{CO}_2\) partial pressure entering the cuvette. For all
salinity treatments of *H. perfoliata*, measurements were made on one photosynthetic shoot on three different plants (*n* = 3). In general, shoots in the same position as those sampled for other biochemical tests in this experiment were chosen. The mid to distal portion of individual branch was inserted in the conifer chamber for gas-exchange measurements. All the readings were recorded between 10:00 am and 2:00 pm standard time in a room with air temperature around 35 °C. Digital images of overlapping projected area of photosynthetic shoots were used to calculate the complex leaf area assuming that photosynthesis reading are predominantly dependent on surface area to exposed incident light. The ImageJ 1.43 u software (NIH, U.S.A) was used to calculate the projected shoot area. Carboxylation efficiency (CE) and gross Carboxylation efficiency (CE*) were calculated from photosynthetic gas exchange data as:

\[
CE = \frac{P_N}{PPFD}
\]

\[
CE* = \frac{P_N}{PPFD} \times \text{Leaf Absorbance}
\]

The risk of oxidative damage was indicated by the ratio of gross ETR (ETR*) (from chlorophyll fluorescence data) and the sum of photosynthesis, photorespiration and dark respiration (from gas exchange data)

\[
= \frac{ETR*(P_N+R_L+R_D)}{P_N+R_L+R_D}
\]

Where \( P_N \) = rate of photosynthesis; \( E \) = rate of transpiration; \( F \) = flow rate; \( C_s \) = sample CO\(_2\) concentration; \( C_r \) = reference CO\(_2\) concentration; \( C_a \) = ambient CO\(_2\) concentration; \( k \) = diffusion effect; \( S \) = leaf area.

**Quantification of Rubisco**

Rubisco (Large Sub Unit) in photosynthetic shoots was extracted by grinding 0.05 g plant material in liquid nitrogen and adding 0.05 g polyvinyl-polypyrroolidon (PVPP) in a buffer (100 mmol L\(^{-1}\) Imidazol; 1.25 mmol L\(^{-1}\) EDTA; pH 7.8). Proteins were denatured with sodiumdodecylsulfate (SDS) and protein content calculated on fresh weight basis (Bradford, 1976). Proteins were separated by polyacrylamide gel electrophoresis (agarose 6% (g/g) for stacking, 12.5% (g/g) for separation) according to Laemmli (1970). Rubisco content to the total protein content was calculated using the integrals of the signal strength on the gel using the image processing and analysis software ImageJ
Rubisco total amount was calculated with the protein analysis mentioned above.

δ\(^{13}\)C and δ\(^{15}\)N values

Shoot δ\(^{13}\)C values were determined relative to VPDB (Vienna Pee Dee Belemnite) and δ\(^{15}\)N values were determined relative to atmospheric N\(_2\) (Ehleringer and Osmond, 1989). Three photosynthetic shoots were sampled each from a different potted plant 30 days after salinity treatments were initiated. Shoot samples were oven dried at 60 °C for 48-h, and ground to a fine powder in a mixer mill (Retch GmbH, Haan, Germany).

Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence of intact green shoots was measured with a PAM-2500 flurometer (Heinz Walz GmbH, Effeltrich, Germany) under conditions similar to those used for the gas exchange data. Maximum quantum efficiency of PSII (Fv/Fm) and effective Quantum yield of photosystem II (PSII) was estimated by the method of Genty et al. (1989) respectively. Non-photochemical fluorescence quenching (NPQ) was estimated according to Bilger and Björkman (1990) and the coefficient of photochemical fluorescence quenching (qL) as formulated by (Oxborough and Baker, 1997). ETR was calculated according to the method of Krall and Edwards (1992) by using the shoot absorbance data with a Licor Li-1800-12 integrating sphere (Li-Cor Biosciences Inc., Lincoln, Nebraska, U.S.A.).

Oxidative stress markers

Trichloroacetic acid (TCA) extracts were prepared by homogenizing finely ground plant samples (0.5 g) in 5 ml of 3% (w/v) ice-cold TCA, followed by centrifugation at 12000\(\times\)g for 15 min at 4 °C. Supernatant was stored at -80 °C in form of aliquots prior to biochemical assays, which were conducted at 25 °C. Hydrogen peroxide (H\(_2\)O\(_2\)) was determined by the method of Loreto and Velikova (2001) with minor modifications. TCA extract (0.5 ml) was mixed with 0.5 ml of 0.5 mol L\(^{-1}\) potassium phosphate buffer (pH 7.0) and 1.0 ml of 1 mol L\(^{-1}\) potassium iodide (KI). Mixture was incubated at room temperature (25 °C) for 10 min and absorbance was recorded at 390 nm.

Malondialdehyde (MDA) contents were quantified with slightly modified method of Heath and Packer (1968). TCA extract (0.5 ml) was mixed with 0.5 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid and heated at 95 °C for 30 min in a
shaking water bath. The reaction was terminated in an ice bath, followed by centrifugation at 12000×g for 10 min at 4 °C. Absorbance of the supernatant was measured at 532 nm and 600 nm (Heath and Packer, 1968).

**Antioxidant enzyme activities**

Antioxidant enzymes were extracted by the method of Polle et al. (1994). Finely ground plant samples (0.5 g) were homogenized in 5 ml of ice-chilled 0.1 M potassium phosphate buffer (pH 7.0) containing 2% (w/v) polyvinyl-pyrrolidone, 5 mmol L⁻¹ disodium ethylene-diamine-tetra-acetic acid and 1 mmol L⁻¹ L-ascorbic acid. Homogenate was then centrifuged at 12000×g for 20 min at 4 °C. Aliquots of the supernatants were stored at -80 °C prior to antioxidant enzyme assays, which were carried out at 25 °C. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed at 560 nm according to Beauchamp and Fridovich (1971), catalase (CAT, EC 1.11.1.6) activity was measured at 240 nm by the method of Abey (1984), guaicol peroxidase (GPX, EC1.11.17) activity was determined at 470 nm using the method Zaharieva et al. (1999); ascorbate peroxidase (APX, EC 1.11.1.11) activity was estimated at 290 nm by ascorbic acid oxidation method of Nakano and Asada (1981), and glutathione reductase (GR, EC 1.6.4.2) activity was assayed at 340 nm by NADPH oxidation method of Foyer and Halliwell (1976). Enzyme activities were defined as in original methods and expressed as units of enzyme activity/mg protein which was determined according to the method of Bradford (1976).

**Antioxidant substances**

Reduced glutathione (GSH) was determined in TCA extracts according to Guri (1983). Briefly, TCA extracts (0.5 ml) were mixed with 0.5 ml of potassium phosphate buffer (pH 7.0, 200 mmol L⁻¹) and 0.1 ml of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and incubated at room temperature (25 °C) for 30 min. Absorbance was measured at 412 nm. Concentrations were estimated by using a standard curve prepared from analytical grade GSH. Reduced ascorbate (ASA) was determined in TCA extracts by following the method of Luwe et al. (1993). TCA extracts (0.2 ml) were mixed with potassium phosphate buffer (pH 7.4, 150 mmol L⁻¹) containing 10% TCA, 44% H₃PO₄, 4% bipyridyl and 3% FeCl₃ then incubated at 37 °C for 1-h and absorbance was measured at 525 nm. Concentrations were estimated by using a standard curve. Total soluble sugars (TSS) in tissue sap were analyzed by using anthrone reagent according to Yemm and
Willis (1954). Sap of different treatments were homogenized with anthrone reagent and incubated in a water bath at 100 °C for 11 min. The reaction was immediately stopped in an ice bath and absorbance was noted at 630 nm (Yemm and Willis, 1954).

Statistical Analyses

Statistical analysis of the data was carried out by using SPSS Version 11.0 (SPSS, 2011). One-way analysis of variance (ANOVA) was used to determine whether salinity had significant effects on growth and different physio-chemical parameters. A Bonferroni post-hoc test was carried out to determine if significant ($P < 0.05$) differences occurred between individual treatments.

Results

Growth

One-way analysis of variance indicated significant effects of salinity on fresh weight ($F_W$) and dry weight ($D_W$) of shoot ($P < 0.05$) and root ($P < 0.01$) and on succulence in shoot ($P < 0.001$) and root ($P < 0.01$) tissues of *H. perfoliata* (Table 4.1). Shoot biomass and succulence were highest at 0.15 mol L$^{-1}$ NaCl compared to the other saline and non-saline treatments (Fig. 4.3 A, B, C, D, E). However, root succulence increased progressively with increasing salinity (Fig. 4.3 F).
Table 4.1. One-way ANOVA of the effect of salinity on different parameters of shoot and root of *Halopeplis perfoliata*. Numbers are the *F*-values with the level of significance (ns = non-significant, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$). Details of each parameter given below are mentioned in Materials and Methods section.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Shoot</th>
<th>Root</th>
<th>Parameters</th>
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<tr>
<td><strong>Growth</strong></td>
<td></td>
<td></td>
<td><strong>CO₂ Gas-Exchange</strong></td>
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<td></td>
</tr>
<tr>
<td>$F_W$</td>
<td>8.68*</td>
<td>20.90**</td>
<td>$P_N$</td>
<td>0.42$^{ns}$</td>
<td>-</td>
</tr>
<tr>
<td>$D_W$</td>
<td>9.12*</td>
<td>15.90**</td>
<td>$D_R$</td>
<td>12.12***</td>
<td>-</td>
</tr>
<tr>
<td>Succulence</td>
<td>104.86***</td>
<td>15.85**</td>
<td>$g_s$</td>
<td>18.24***</td>
<td>-</td>
</tr>
<tr>
<td>Height</td>
<td>1.54$^{ns}$</td>
<td>1.63$^{ns}$</td>
<td>$C_i$</td>
<td>1.38$^{ns}$</td>
<td>-</td>
</tr>
<tr>
<td>Water and ion relations</td>
<td>E</td>
<td></td>
<td></td>
<td>10.68***</td>
<td>-</td>
</tr>
<tr>
<td>$Ψ_s$</td>
<td>32.60***</td>
<td>-</td>
<td>WUE</td>
<td>131.44***</td>
<td>-</td>
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<tr>
<td>RWC</td>
<td>0.27$^{ns}$</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>Cations regulation</strong></td>
<td></td>
<td></td>
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<td>-</td>
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<tr>
<td>Na$^+$</td>
<td>1157.73***</td>
<td>58.29***</td>
<td>$δ^{13}C$</td>
<td>21.14***</td>
<td>-</td>
</tr>
<tr>
<td>K$^+$</td>
<td>7.03*</td>
<td>0.86$^{ns}$</td>
<td>$δ^{15}N$</td>
<td>12.11***</td>
<td>-</td>
</tr>
<tr>
<td>Mg$^{++}$</td>
<td>8.94*</td>
<td>14.23***</td>
<td>$H_2O_2$</td>
<td>10.52***</td>
<td>14.02**</td>
</tr>
<tr>
<td>Ca$^{++}$</td>
<td>3.95$^{ns}$</td>
<td>0.75$^{ns}$</td>
<td>MDA</td>
<td>6.57**</td>
<td>14.34**</td>
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<td>Na/K ratio</td>
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<td>30.77***</td>
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<td><strong>Shoot Photosynthetic Pigments</strong></td>
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<td></td>
<td><strong>Antioxidant enzymes</strong></td>
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<td></td>
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<tr>
<td>N L/R</td>
<td>13.80***</td>
<td>-</td>
<td>SOD</td>
<td>7.04*</td>
<td>4.43$^{ns}$</td>
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<tr>
<td>K L/R</td>
<td>0.51$^{ns}$</td>
<td>-</td>
<td>APX</td>
<td>15.24**</td>
<td>4.90$^{ns}$</td>
</tr>
<tr>
<td><strong>Shoot Photosynthetic Pigments</strong></td>
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<td></td>
<td><strong>Antioxidant Substances</strong></td>
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<td></td>
</tr>
<tr>
<td>Chl. a</td>
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<td>-</td>
<td>GPX</td>
<td>10.19*</td>
<td>7.44</td>
</tr>
<tr>
<td>Chl. b</td>
<td>24.19***</td>
<td>-</td>
<td>GR</td>
<td>0.48$^{ns}$</td>
<td>2.52$^{ns}$</td>
</tr>
<tr>
<td>CAR.</td>
<td>22.27***</td>
<td>-</td>
<td></td>
<td>7.13*</td>
<td>4.02$^{ns}$</td>
</tr>
<tr>
<td>T. Chl.</td>
<td>24.34***</td>
<td>-</td>
<td>GSH</td>
<td>25.58***</td>
<td>51.88***</td>
</tr>
<tr>
<td>Chl. a/b ratio</td>
<td>3.63$^{ns}$</td>
<td>-</td>
<td>ASA</td>
<td>96.26***</td>
<td>37.63***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSS</td>
<td>20.025***</td>
<td>19.20***</td>
</tr>
</tbody>
</table>
Fig. 4.2. Comparison of *Halopeplis perfoliata* plants grown under various salinity treatments (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl for 30 days under greenhouse conditions.
Fig. 4.3. Growth analysis of *Halopeplis perfoliata* in salinity treatments (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl). Shoot \(F_W\) (A), Root \(F_W\) (B), Shoot \(D_W\) (C) Root \(D_W\) (D), Shoot succulence (E) Root succulence (F). Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments within each parameter; Bonferroni test; \(P < 0.05\).
One-way ANOVA showed significant ($P < 0.001$) effect of salinity on shoot sap solute potential ($\Psi_s$) (Table 4.1). There was a two-fold decrease in $\Psi_s$ ($P < 0.001$) under 0.6 mol L$^{-1}$ treatment compared to non-saline control (Fig. 4.4 A). RWC of shoots was unaffected by increases in salinity (Fig. 4.4 B).

**Fig. 4.4.** Shoot osmotic potential (A) and relative water content (B) in response to salinity treatments (0, 0.15, 0.3 and 0.6 mol L$^{-1}$ NaCl). Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments within each parameter; Bonferroni test; $P < 0.05$.

Increase in salinity had significant effect on Na$^+$, Mg$^{++}$ and Na$^+$/K$^+$ ratio but not on K$^+$ and Ca$^{++}$ content in shoots and roots of *H. perfoliata* (ANOVA; Table 4.1). Shoot Na$^+$ increased linearly with increases in salinity, whereas root Na$^+$ increased only in the 0.60 mol L$^{-1}$ NaCl treatment. Shoot Na$^+$ was substantially higher than in root (Fig. 4.5 A). K$^+$ in both shoots and roots remained largely unaffected by salinity increments; however in shoots it was substantially higher than roots (Fig. 4.5 B). Ca$^{++}$ in both shoots and roots
also remained unaltered by salinity, but roots had significantly \((P < 0.05)\) higher Ca\(^{++}\) than shoots (Fig. 4.5 C). Mg\(^{++}\) in shoots increased at 0.6 mol L\(^{-1}\) NaCl compared to other treatments, but was generally lower in roots under saline conditions compared to no salinity control (Fig. 4.5 D) treatments (Fig. 4.5 E) while shoot to root K\(^{+}\) remained unaffected by salinity (Fig. 4.5 F).

**Fig. 4.5.** Na\(^{+}\) (A), K\(^{+}\) (B), Ca\(^{++}\) (C), Mg\(^{++}\) (D), Na\(^{+}\)/K\(^{++}\) ratio (E), and K\(^{+}\) shoot to root ratio in response to salinity treatment (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl). Vertical bars are means ± standard error. Different alphabets show significant differences among the salinity treatments within each parameter; Bonferroni test; \(P < 0.05\).
Salinity effects on Photosynthesis

Shoot Photosynthetic Pigments
ANOVA indicated significant effects of salinity on contents of chlorophyll a (Chl. a), chlorophyll b (Chl. b), carotenoids (CAR.) and total chlorophyll (T. Chl.) (Table 4.1). On fresh weight and dry weight basis contents of photosynthetic pigments were decreased under high salinity (Table 4.2 A and B). While on leaf area basis no significant difference was found in photosynthetic pigments between salinity treatments (Table 4.2 C).

CO₂ Gas-Exchange
Net photosynthesis (PN), measured on incident area basis, was unaffected by increases in salinity (Table 4.1; Table 4.3). Dark respiration (RD) increased with increases in salinity (Table 4.3). Stomatal conductance (gs) and transpiration rate (E) increased in up to 0.3 mol L⁻¹ NaCl and declined afterwards (Table 4.3). A transient decrease in water use efficiency (WUE) was observed under low (0.15 mol L⁻¹ NaCl) and moderate (0.30 mol L⁻¹ NaCl) salinity treatments compared to no and high (0.6 mol L⁻¹ NaCl) salinity treatments. Salinity had no effect on intercellular carbon dioxide (Ci) concentration (Table 4.3). However carboxylation efficiency (CE) and gross carboxylation efficiency (CE*) increased at optimal salinity (0.15 mol L⁻¹) as compared to other treatments. However, oxidative risk indicated by the ratio of ETR and sum of photosynthesis (PN), photorespiration (RL) and dark respiration (RD) was highest under non-saline control and lowest at optimal salinity level (0.15 mol L⁻¹ NaCl) (Fig. 4.3).
Table 4.2 Photosynthetic pigments [chlorophyll a (Chl. a), chlorophyll b (Chl. b), carotenoids (CAR.), total chlorophyll (Chl. a+b) and chl. a/b ratio of *Halopeplis perfoliata* grown in different salinity treatments (0, 1.5, 0.3 and 0.6 mol L⁻¹ NaCl) measured on (A) fresh weight (F_W) (B) dry weight (D_W) and (C) leaf area basis. Values represent means ± S.E. Different alphabets show significant difference among the salinity treatments within each parameter; Bonferroni test; *P* < 0.05.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0</th>
<th>0.15 (mol L⁻¹)</th>
<th>0.3</th>
<th>0.6</th>
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<tbody>
<tr>
<td>(A) Fresh weight basis (mg g⁻¹ F_W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl. a</td>
<td>0.23±0.03ᵃ</td>
<td>0.20±0.01ᵇ</td>
<td>0.14±0.01ᵇ</td>
<td>0.14±0.03ᵇ</td>
</tr>
<tr>
<td>Chl. b</td>
<td>0.09±0.01ᵃ</td>
<td>0.07±0.00ᵇ</td>
<td>0.04±0.00ᶜ</td>
<td>0.04±0.01ᶜ</td>
</tr>
<tr>
<td>CAR.</td>
<td>0.06±0.01ᵃ</td>
<td>0.05±0.01ᵇ</td>
<td>0.04±0.00ᵇ</td>
<td>0.04±0.00ᵇ</td>
</tr>
<tr>
<td>T. Chl. (a + b)</td>
<td>0.32±0.07ᵃ</td>
<td>0.25±0.01ᵇ</td>
<td>0.2±0.01ᵇ</td>
<td>0.23±0.01ᵇ</td>
</tr>
<tr>
<td>Chl. a/b ratio</td>
<td>2.64±0.11ᵃ</td>
<td>3.00±0.1ᵇ</td>
<td>3.7±0.2ᵇ</td>
<td>3.08±0.19ᵇ</td>
</tr>
<tr>
<td>(B) Dry weight basis (mg g⁻¹ D_W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl. a</td>
<td>7.41±0.30ᵃ</td>
<td>9.45±2.2ᵃ</td>
<td>3.58±0.02ᵇ</td>
<td>0.59±0.01ᶜ</td>
</tr>
<tr>
<td>Chl. b</td>
<td>2.90±0.02ᵃ</td>
<td>3.12±0.04ᵃ</td>
<td>1.10±0.06ᵇ</td>
<td>0.20±0.04ᵇ</td>
</tr>
<tr>
<td>CAR.</td>
<td>2.06±0.05ᵃ</td>
<td>2.72±0.6ᵃ</td>
<td>0.98±0.02ᵇ</td>
<td>0.22±0.01ᶜ</td>
</tr>
<tr>
<td>T. Chl. (a + b)</td>
<td>10.31±0.5ᵃ</td>
<td>8.14±1.5ᵃ</td>
<td>4.70±0.04ᵇ</td>
<td>0.78±0.11ᶜ</td>
</tr>
<tr>
<td>Chl. a/b ratio</td>
<td>2.645±0.11ᵃ</td>
<td>2.98±0.07ᵃ</td>
<td>3.45±0.25ᵃ</td>
<td>3.08±0.11ᵃ</td>
</tr>
<tr>
<td>(C) Shoot area basis (g m⁻²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl. a</td>
<td>0.32±0.01ᵃ</td>
<td>0.31±0.04ᵃ</td>
<td>0.23±0.03ᵃ</td>
<td>0.29±0.05ᵃ</td>
</tr>
<tr>
<td>Chl. b</td>
<td>0.12±0.01ᵃ</td>
<td>0.08±0.01ᵇ</td>
<td>0.07±0.07ᵇ</td>
<td>0.01±0.01ᵇ</td>
</tr>
<tr>
<td>CAR.</td>
<td>0.09±0.00ᵃ</td>
<td>0.09±0.01ᵃ</td>
<td>0.07±0.07ᵃ</td>
<td>0.01±0.01ᵃ</td>
</tr>
<tr>
<td>T. Chl. (a + b)</td>
<td>0.44±0.02ᵃ</td>
<td>0.41±0.05ᵃ</td>
<td>0.30±0.04ᵃ</td>
<td>0.40±0.07ᵃ</td>
</tr>
<tr>
<td>Chl. a/b ratio</td>
<td>2.75±0.04ᵃ</td>
<td>3.03±0.00ᵃ</td>
<td>3.70±0.20ᵇ</td>
<td>3.30±0.10ᶜ</td>
</tr>
</tbody>
</table>
Table 4.3. Net photosynthetic rate ($P_N$), respiration ($R_D$), stomatal conductance ($g_S$), transpiration ($E$), water use efficiency (WUE), and intercellular CO$_2$ concentration (Ci) measured on photosynthetic shoots of *Halopeplis perfoliata* in different salinity treatments (0, 1.5, 0.3 and 0.6 mol L$^{-1}$ NaCl). Values represent means ± S.E. Different alphabets show significant differences among salinity treatments within each parameter; Bonferroni test; $P < 0.05$.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0</th>
<th>0.15 (mol L$^{-1}$)</th>
<th>0.3</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_N$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>5.99±1.71$^a$</td>
<td>7.51±1.25$^a$</td>
<td>6.71±1.08$^a$</td>
<td>5.51±1.19$^a$</td>
</tr>
<tr>
<td>$R_D$ (mol m$^{-2}$ s$^{-1}$)</td>
<td>-1.49±0.01$^a$</td>
<td>-2.40±0.02$^b$</td>
<td>-2.71±0.07$^{bc}$</td>
<td>-3.21±0.04$^c$</td>
</tr>
<tr>
<td>$g_S$ (mol m$^{-2}$ s$^{-1}$)</td>
<td>0.06±0.00$^a$</td>
<td>0.11±0.00$^b$</td>
<td>0.13±0.01$^b$</td>
<td>0.08±0.01$^c$</td>
</tr>
<tr>
<td>$E$ (mol m$^{-2}$ s$^{-1}$)</td>
<td>1.43±0.05$^a$</td>
<td>2.30±0.07$^b$</td>
<td>2.78±0.44$^b$</td>
<td>1.46±0.10$^a$</td>
</tr>
<tr>
<td>Ci (µmol mol$^{-1}$)</td>
<td>286.88±18.00$^a$</td>
<td>301.18±17.90$^a$</td>
<td>295.89±17.71$^a$</td>
<td>274.85±26.08$^a$</td>
</tr>
<tr>
<td>WUE</td>
<td>69.91±3.57$^a$</td>
<td>41.31±1.51$^b$</td>
<td>34.22±1.78$^b$</td>
<td>89.35±1.17$^c$</td>
</tr>
<tr>
<td>CE</td>
<td>0.01±0.00$^a$</td>
<td>0.02±0.00$^a$</td>
<td>0.02±0.00$^a$</td>
<td>0.02±0.00$^a$</td>
</tr>
<tr>
<td>CE*</td>
<td>0.01±0.00$^a$</td>
<td>0.03±0.00$^a$</td>
<td>0.02±0.00$^a$</td>
<td>0.02±0.00$^a$</td>
</tr>
<tr>
<td>ETR/($P_N$+$R_D$+$R_L$)</td>
<td>5.06±0.88$^a$</td>
<td>3.46±0.64$^a$</td>
<td>4.16±0.54$^a$</td>
<td>3.51±0.83$^a$</td>
</tr>
</tbody>
</table>
Quantification for Rubisco

Rubisco content peaked at 0.15 mol L\(^{-1}\) NaCl with substantially lower values in all other treatments (0, 0.3 and 0.6 mol L\(^{-1}\)NaCl) used (Fig. 4.6).

![Graph showing Rubisco content vs NaCl concentration](image)

Fig. 4.6. Relative levels of Rubisco quantified on total protein basis where 50 µg protein was applied based on SDS-PAGE gel electrophoresis on photosynthetic shoots of *Halopeplis pefoliata* plants grown under different salinity treatments (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl).

δ\(^{13}\)C and δ\(^{15}\)N values

One-way ANOVA showed that significant (\(P < 0.001\)) effects of salinity on δ\(^{13}\)C and δ\(^{15}\)N in shoot tissues (Table 4.1). *H. perfoliata* exhibit a similar pattern of δ\(^{13}\)C values to other C\(_3\) species. Lower values of δ\(^{13}\)C were determined under salinity compared to control (non-saline) (Fig. 4.7 A). However δ\(^{15}\)N values increased at 0.15 mol L\(^{-1}\) and 0.3 mol L\(^{-1}\) NaCl treatments than in the non-saline control and 0.6 mol L\(^{-1}\) NaCl treatment (Fig. 4.7 B).
Fig. 4.7. Carbon and Nitrogen isotopes discrimination in salinity treatments (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl). Vertical bars are means ± standard error. Different alphabets show significant differences among salinity treatments within each parameter; Bonferroni test; \(P < 0.05\).

**Chlorophyll fluorescence**

Maximum efficiency of PSII (Fv/Fm) was not affected by low or high irradiance and salinity and it values ranged from 0.68 to 0.71 (Fig. 4.8 A). Actual yield of PSII was largely unaffected by salinity but increases in light decreased it (Fig. 4.8 B). ETR* values increased with increasing irradiance with little variation between treatments however, high salinity (0.6 mol L\(^{-1}\)) had lower electron transport rates than others (Fig. 4.8 C). Photochemical quenching (qL) gradually decreased with increasing irradiance.
however qL values were higher in non-saline control and 0.15-0.3 mol L\(^{-1}\) but lower at 0.6 mol L\(^{-1}\) (Fig. 4.8 D). Non-photochemical quenching (NPQ) increased at high salinity treatments by a factor of 0.4 - 2.0 in comparison to the control at high irradiance (~1850 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) (Fig. 4.8 E).

**Fig. 4.8.** Maximum quantum efficiency (Fv/Fm), actual yield (PSII), electron transport rate (ETR), photochemical (qL) and non-photochemical quenching (NPQ) on photosynthetic shoot of *Halopeplis perfoliata* under a range of irradiance and in salinity treatments [0 (○), 0.15 (△), 0.3 (◊) and 0.6 (□) mol L\(^{-1}\) NaCl]. Values are mean of 3 replicates with means ± S.E.

**Oxidative stress markers**

One-way ANOVA showed significant effects of salinity on endogenous H\(_2\)O\(_2\) and MDA content (Table 4.1). In the shoot, endogenous H\(_2\)O\(_2\) and MDA were higher in 0.3 and 0.6 mol L\(^{-1}\) NaCl treatments in comparison with control (Fig. 4.9 A and C). Endogenous
H₂O₂ and MDA contents of the roots decreased under high salinity compared to other NaCl treatments (Fig. 4.9 B and D).

**Fig. 4.9.** Hydrogen peroxide in shoot (A) and root (B), Malondialdehyde in shoot (C) and root (D) of *Halopeplis perfoliata* in salinity treatments (0, 0.15, 0.3 and 0.6 mol L⁻¹ NaCl). Vertical bars are means ± S.E. Different alphabets show significant differences among the salinity treatments within each parameter; Bonferroni test; *P* < 0.05.

**Antioxidant enzymes**

Salinity had significant effects on antioxidant enzyme activities (one-way ANOVA; Table 4.1). In shoots, activities of SOD (Fig. 4.10 A) and APX (Fig. 4.10 G) decreased, that of CAT (Fig. 4.10 C) increased, while those of GPX (Fig. 4.10 E) and GR (Fig. 4.10 I) remained unaffected under saline conditions as compared to non-saline controls. While in roots, SOD activity showed a transient decrease under moderately saline conditions (Fig. 4.10 B), CAT activity decreased in 0.6 mol L⁻¹ NaCl (Fig. 4.10 D), GPX activity increased with increases in salinity (Fig. 4.10 F), while activities of APX and GR transiently increases in 0.15 mol L⁻¹ NaCl (Fig. 4.10 H and J).
Fig. 4.10. Antioxidant enzyme activities in shoots and roots of *Halopeplis perfoliata* in response to salinity treatments (0, 0.15, 0.3 and 0.6 mol L⁻¹ NaCl). Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments within each enzyme activity data; Bonferroni test; *P* < 0.05.
Antioxidant substrates

Analysis of variance indicated significant effects of salinity on reduced glutathione (GSH) and reduced ascorbate (ASA) contents of the shoots and roots of *H. perfoliata* (Table 4.1). Shoot GSH content increased under saline conditions with highest values in 0.3 mol L\(^{-1}\) NaCl (4.11 A). While, root GSH content decreased with increasing salinity treatments and levels were substantially lower in 0.3 and 0.6 mol L\(^{-1}\) NaCl (Fig. 4.11 A and B). Shoot ASA content increased with increases in salinity (Fig. 4.11. C), while root ASA increased in up to 0.3 mol L\(^{-1}\) NaCl followed by a substantial decline in 0.6 mol L\(^{-1}\) NaCl (Fig. 4.11 D).

![Antioxidant substrates](image)

**Fig. 4.11.** Reduced glutathione (GSH) in shoot (A), and root (B), ascorbic acid (ASA) in shoot (C) and root (D) of *Halopeplis perfoliata* in response to salinity treatments (0, 0.15, 0.3 and 0.6 mol L\(^{-1}\) NaCl). Vertical bars are means ± S.E. Different alphabets show significant differences among treatments within each parameter; Bonferroni test; *P* < 0.05.
Soluble Sugars

ANOVA table showed that salinity has significant effect on soluble sugars on shoots ($P < 0.001$) and roots ($P < 0.01$) (Table 4.1). Sugars were significantly higher in 0.3 mol L$^{-1}$ and 0.6 mol L$^{-1}$ NaCl treatments in shoots (Fig. 4.12. A). While lower in roots at the same concentrations than control and 0.15 mol L$^{-1}$ of NaCl treatments (Fig. 4.12 B).

![Soluble Sugars Graph](image-url)

**Fig. 4.12.** Total soluble sugars (TSS) in response to salinity treatments (0, 0.15, 0.3 and 0.6 mol L$^{-1}$ NaCl). Vertical bars are means ± S.E. Different alphabets show significant differences among salinity treatments in shoot and root of *Halopeplis perfoliata*; Bonferroni test; $P < 0.05$. 
Discussion

*Growth experiment indicated that Halopeplis perfoliata is an obligate halophyte*

Succulent eu-halophytes in the subfamily Salicornioideae (Amaranthaceae) could optimally grow optimally in the presence of salinity (Flower and Colmer, 2008; Rozema and Schat, 2013). For example, *Sarcocornia natalensis* (0.3 mol L\(^{-1}\) NaCl, Naidoo and Rughunanan, 1990); *Salicornia bigelowii* (0.2 mol L\(^{-1}\) NaCl, Ayala and O’Leary, 1995); *Halocnemum strobilaceum* (0.34 mol L\(^{-1}\) NaCl, Pujol et al., 2001); *Arthrocnemum macrostachyum* (0.4 mol L\(^{-1}\) NaCl, Khan et al., 2005); *Salicornia utahensis* 0.6 mol L\(^{-1}\) NaCl, Gul et al., 2009) and *Salicornia dolichostachya* (0.3 mol L\(^{-1}\) NaCl, Katschnig et al., 2013) showed optimal growth under saline conditions. Inhibition of growth above optimal salinity appears to compromise strategy to ensure survival by investing in mechanisms of salt resistance and was referred to as a “life insurance” policy under salt stress by Pujol et al. (2001). In this study, optimal growth of *Halopeplis perfoliata* at 0.15 mol L\(^{-1}\) NaCl coincided with increased shoot succulence. Succulence could help plants to avoid physiological drought, reduce ion toxicity (Sánchez et al., 2004; Touchette et al., 2009; Rozema and Schat, 2013) and buffer thermal changes (Medina, 1999).

*Osmotic adjustment and succulence maintain sufficient tissue hydration for growth under saline conditions*

Succulent halophytes such as *Sueada fruticosa* could constitutively maintain low tissue osmotic potentials for water uptake under saline conditions (Hameed et al., 2012). *Halopeplis perfoliata* also maintained low osmotic potentials up to 0.3 mol L\(^{-1}\) NaCl which was not coupled with turgidity loss (high RWC) indicating high ability for osmotic adjustment. Decreased succulence and a sudden decrease in \(\Psi_S\) at 0.6 mol L\(^{-1}\) NaCl treatment were suggestive of water stress. Vacuolar Na\(^{+}\) sequestration contributes significantly in osmotic adjustment accounting for \(~50\%\) of the sap Op (Shabala and Mackay, 2011) with parallel accumulation of organic solutes (Hussin et al., 2013; Jogaiah et al., 2014; Flowers and Colmer, 2015). Similar results were reported for *Halosarcia pergranulata* (syn. *Tecticornia pergranulata*) (Short and Colmer, 1999) *Salicornia dolichostachya* (Katschnig et al., 2013). Compromised K\(^{+}\) acquisition from soil and replacement of K\(^{+}\) by Na\(^{+}\) in plant tissues under saline condition and is deleterious for plant metabolism. However, highly tolerant halophytes of Salicornioideae
such as *Tecticornia pergranulata* (Colmer et al., 2009) and *Tecticornia indica* (English and Colmer, 2013) appeared to maintain K\(^+\) homeostasis under saline conditions which has several important roles in plants metabolism (Munns and Tester, 2008; Benito et al., 2013; Hussin et al., 2013). Several membrane-embedded transport proteins such as K\(^+\)/H\(^+\) symporters, outward rectifying K\(^+\) channels (KOR), members of high affinity K\(^+\) transporter family (KUP/HAK/KT) and inward rectifying K\(^+\) channels (KIR) are responsible for K\(^+\) homeostasis in plants (Mansour, 2014). *H. perfoliata* also maintained sufficient tissue K\(^+\) under saline conditions in all salinity treatments. Two to four fold higher shoot to root K\(^+\) indicated that K\(^+\) transport to shoot was also not compromised by salinity increments. Ca\(^{++}\) is also known to be involved in SOS pathway mediated Na\(^+\) efflux (Ji et al., 2013), inhibition of voltage independent cation channel (VIC) for Na\(^+\) influx (Maathius and Amtmann, 1999; White and Davenport, 2002). In addition, Ca\(^{++}\) regulates stress signaling (Spalding and Harper, 2011), enzyme activation (Plieth, 2005) membrane and cell wall integrity (Marschner, 1995). Shoot and root Ca\(^{++}\) of *H. perfoliata* remained unaffected by salinity increments, as reported for *Ocimum Basilicum* (Ning et al., 2015). Magnesium (Mg\(^{++}\)) is also required for activation of many enzymes, ribosome aggregation and chlorophyll synthesis (Shaul, 2002). At 0.6 mol L\(^{-1}\) NaCl peak shoot Mg\(^{++}\) levels compared to root in *H. perfoliata* compared to saline and non-saline treatments could be linked to higher transport to shoots. Similar rise in Mg\(^{++}\) under saline condition were observed in *Salicornia europaea* (McNulty, 1985) and *Limonium stocksii* (Zia et al., 2008). In sunflower leaves this rise was due to dehydration stress imposed by low water potential treatment (Rao et al., 1987). Peak shoot Mg\(^{++}\) levels at the highest NaCl treatment in *H. perfoliata* could be a consequence of water stress and/or chlorophyll degradation (Stocking and Ongun, 1962).

*Down regulation of photosynthetic machinery helps to maintain the photosynthetic efficiency*

Chlorophyll content may not always be linked with growth reduction or inhibition with increasing substrate salinity and may either increase, decrease or remain unchanged (Moinuddin et al., 2014). NaCl-induced reduction in photosynthetic pigments could be related to decrease in size and number of chloroplasts (Grigore et al., 2014). Photosynthetic pigments decreased in response to increasing salinity in members of Salicornioideae such as *Salicornia persica*, *S. europaea* (Aghaleh et al., 2009),
Arthrocnemum macrostachyum (Redondo-Gómez et al., 2010) and Sarcocornia fruticosa (Duarte et al., 2013). In H. perfoliata, changes in shoot photosynthetic pigments appeared to be masked by on a leaf area basis by variations in shoot succulence with increasing salinity. However, pigment levels decreased on fresh weight and particularly on dry weight basis indicating either chlorophyll degradation or decreased allocation of plant resources toward synthesis.

Members of Salicornioideae family utilize C3 mode of CO2 assimilation and have the ability to maintain (Salicornia europaea, Kuramoto and Brest, 1979) or enhance (Sarcocornia fruticosa; Redondo-Gómez et al., 2006 and Arthrocnemum macrostachyum; Redondo-Gómez et al., 2010) photosynthetic rates under saline conditions. This could be related to efficient storage of salt in the vacuole and dilution effect which protects the chloroplast (Kuramoto and Brest, 1979). CO2 compensation points of H. perfoliata indicated C3 function. Maintenance of CO2 assimilation rates (PN) and internal carbon dioxide (Ci) concentrations of H. perfoliata were similar to Tecticornia pergranulata with increasing salinity (Colmer et al., 2009). Decrease in WUE under moderately low and moderate salinity (0.15 and 0.3 mol L⁻¹ NaCl) could indicate fixed response similar to natural salt marsh conditions with no water stress whereas high salinity (0.6 mol L⁻¹ NaCl) stimulated stomatal closure along with decreased shoot succulence could be a preemptive protection against further dehydration. Dark respiration (RD) in H. perfoliata increased with increasing salinity such as reported by (Burchett et al., 1989; Koyro, 2006; Colmer et al., 2009), supporting the view that salinity tolerance increases energy requirements such as for ion transport processes (Yeo, 1983). Reduction in biomass alongside sustained photosynthesis in H. perfoliata also supports this assumption that most photosynthetic carbon gain was consumed in salt tolerance processes. Decreased photosynthetic rates were attributed to inhibition (synthesis or activity) of rubisco by salinity (Eisa et al., 2012; Koyro et al., 2013). Decrease in rubisco levels (per protein basis) of H. perfoliata did not correlate well with photosynthetic rates (leaf area basis) but could explain lack of plant growth at more than optimal salinity (0.15 mol L⁻¹ NaCl).
Carbon and Nitrogen isotope discrimination values indicate variations in photosynthetic efficiency and nutrient uptake

C₃ plants generally show a linear correlation of carbon isotope composition vs. salinity or close to it particularly under controlled laboratory studies for example *Puccinellia nutelliana* and *Salicornia europeae* sub sp. *rubra* (Ivlev et al., 2013). Similar results were obtained in the present study indicating a progressively higher $\delta^{13}C$ enrichment of biomass with increasing salinity treatments apparently due to increased rates of photorespiration rather than due to stomatal closure. In *H. perfoliata*, a sharp increase $\delta^{15}N$ values up to 0.30 mol L⁻¹ NaCl could be attributed to a higher demand for biomass production at 0.15 mol L⁻¹ NaCl and for nitrogen to produce osmotically active molecules at 0.30 mol L⁻¹ NaCl, as well as to a decrease in nitrate uptake at 0.60 mol L⁻¹ NaCl (Song et al., 2009).

Chlorophyll fluorescence data (Fv/Fm) for *H. perfoliata* indicated little influence of salinity on the integrity or function of light harvesting system (PSII) as reported in *Sarcocornia fruticosa* (Redondo-Gómez et al., 2006); *Salicornia veneta* (Pagliano et al., 2009) and *Arthrocnemum macrostachyum* (Redondo-Gómez et al., 2010). Decrease in ETR* under saline conditions indicates dynamic photo-inhibition, a down-regulation of the PSII activity to reduce the need for alternative electron sinks and to avoid oxidative stress (Qiu et al., 2003).

In C₃ plants, stress caused due to high light and temperature and low CO₂ and water results in increased photorespiration to avoid photo-inhibition of the photosynthetic machinery (Taiz and Zeiger, 2010). Salt stress is also known to increase photorespiration at initial stages due to stomatal reasons (similar to drought effects) i.e., by decreased stomatal conductance (lower Ci) or at later stages by non-stomatal factors, such as high Cl⁻ levels in the photosynthetic tissues (Wingler et al., 2002). In addition, zeaxanthin may also be involved in dissipating additional photon energy to avoid photo-inhibition at moderate light under saline conditions (Sharma and Hall, 1992; Duarte et al., 2013). Elevated CO₂ compensation point *H. perfoliata* indicated increased rates of photorespiration at 0.6 mol L⁻¹ NaCl possibly linked with high shoot accumulation of salts (high osmotic potential). Higher rates of photorespiration under salt stress may contribute in increased H₂O₂ production and lower CAT activity (Wingler et al., 2002).
ROS accumulation generally leads to oxidative damages to important cell components such as to membrane lipids (Jithesh et al., 2006). In this study, H$_2$O$_2$ production gradually increased with increasing salinity in photosynthetic shoots similar to previous studies such as in Arabidopsis thaliana (Ellouzi et al., 2011) and in some other plants (Corpas et al., 1993; Noctor et al., 2002; Jithesh et al., 2006; Ozgur et al., 2013). Increase in MDA content (an indicator of membrane lipids’ peroxidation) with increasing NaCl was also observed in shoots of H. perfoliata. Similarly, MDA content increased under salinity in many other halophytes (Jithesh et al., 2006; Ozgur et al., 2013; Hameed et al., 2015). The relationship between MDA levels and plant performance under stress is unresolved (Hameed et al., 2012; Alhdad et al., 2013), however, some stress signaling roles of MDA have been reported (Weber et al., 2004). Higher MDA concentrations in Suaeda fruticosa were associated with improved growth (Hameed et al., 2012). In this study, MDA contents under high salinity were not too high and absence of visible necrosis indicated that they might be involved in stress signaling.

Halophytes employ several enzymatic and non-enzymatic antioxidants to avoid oxidative damage (Jithesh et al., 2006; Ozgur et al., 2013). In this study, activity of antioxidant enzyme superoxide dismutase (SOD) declined and of ascorbate peroxidase (APX) and glutathione reductase (GR) remained unchanged under saline conditions. Unaltered photosynthesis rates under saline condition also indicate little incidence of ROS production via over-reduction of photosynthetic electron transport chain, hence enhanced activities of water-water cycle antioxidant enzyme (SOD and APX) were not needed in H. perfoliata. Furthermore, unchanged photosynthesis alongside higher CAT activity under saline condition, indicate that the rise in shoot H$_2$O$_2$ might be associated with photorespiration rather than over-reduction of photosynthetic electron transport chain. Shoot antioxidant substrates ascorbate (ASA) and glutathione (GSH) increased with increasing salinity in H. perfoliata as in Sphaerophysa kotschyan (Yildiztugay et al., 2013) and Limonium stocksii (Hameed et al., 2015). Accumulation of ASA and GSH are suggestive of an efficient antioxidant system (Foyer and Halliwell, 1976; Jithesh et al., 2006; Ozgur et al., 2013) besides their roles in photosynthesis (Foyer and Shigeoka, 2011). Increasing shoot soluble sugars could be linked with higher ASA biosynthesis under saline conditions in H. perfoliata (Nishikawa et al., 2005).
Conclusions

*Halopeplis perfoliata* could grow optimally at 0.15 mol L\(^{-1}\) NaCl treatment while with increasing salinity (0.3 and 0.6 mol L\(^{-1}\) NaCl) treatments, it could still produce biomass comparable to non-saline controls (Fig. 4.13). Lower tissue osmotic potentials along with Na\(^+\) accumulation indicated higher capacity for osmotic adjustment with increasing salinity (Fig. 4.13). In general, photosynthetic CO\(_2\) fixation and light harvesting ability of PSII remained unaltered with increasing salinity treatments (Fig. 4.13). Higher CAT activity and H\(_2\)O\(_2\) levels and also the less negative carbon isotope ratios in photosynthetic shoot tissues indicate changes in rates of photorespiration under saline conditions. Further investigations into the molecular arguments for these responses could reveal opportunities for identification of these traits.

![Ecophysiological adaptations of water and ion relations, photosynthesis and antioxidant defense system of *Halopeplis perfoliata* under high salinity concentrations. (~ = similar response; ↑ = increased response across all salinity treatments).](image)

Fig. 4.13.
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Chapter 5

General conclusions
Eco-physiological investigations into novel salt tolerance mechanisms among halophytic plants growing under saline conditions are essential for sustainable utilization of such species for conventional and non-conventional agriculture. The results obtained in this study indicate that *Halopeplis perfoliata* is an obligate halophyte with high salt tolerance at both the germination and mature vegetative stages of its life cycle. Most freshly collected seeds germinated in distilled water in the presence of light and in moderate temperature regimes indicating their non-dormant nature and their preference for less stressful environmental conditions. Non-deep conditional dormancy under hyper-saline conditions and in complete darkness was completely reversed upon transfer to non-saline conditions in the presence of light. Isotonic treatments of NaCl, PEG and sea-salt on seed germination revealed primarily an osmotic effect rather than toxic effect of salts. Among various chemicals used in this study, chloride salts proved to be more inhibitory for germination compared to isotonic sulfate salts. All seeds kept in dry storage for one year at room temperature (25 °C) were viable and showed enhanced tolerance to moderate salinities and darkness, but had lower tolerance to higher temperatures in comparison to freshly collected seeds. All dormancy regulating chemicals (except proline) alleviated dark-enforced dormancy under non-saline conditions but were generally ineffective in reversing inhibitory effects of high salinity (except for betaine, kinetin and thiourea at 0.3 mol L⁻¹ NaCl). Optimum growth of *H. perfoliata* in 0.15 mol L⁻¹ NaCl treatment indicated its obligate salt requirement (Fig. 5.1) while at higher salinities (0.3 and 0.6 mol L⁻¹ NaCl) biomass production was comparable to non-saline controls. Plants appeared to maintain water uptake by regulating succulence to achieve constant relative water content. Shoot ionic balance (K⁺, Ca²⁺ and Mg²⁺) generally remained unaltered in response to increasing salinity. Photosynthetic rates on leaf area basis were also unaffected by salinity which appear to be more than compensated for by an increase in shoot area per plant and shoot succulence per gram dry weight at low and moderate salinity treatments. This was accompanied by higher transpiration rates (E) and stomatal conductance (gs) possibly suggestive of adaptation for growth in a salt marsh environment. Decreased transpiration rates in the 0.6 mol L⁻¹ NaCl treatment resulted in higher WUE which was also indicated by less negative carbon isotope discrimination values in photosynthetic shoots. Chlorophyll fluorescence measurements ruled out any signs of damage to the photochemical machinery while decreased ETR/(Pₐ+Rₐ+R₈) ratios indicated a down
regulation of photochemistry under saline conditions and therefore reduced requirement for alternative electron sinks and ROS detoxification. In conclusion, *H. perfoliata* appears to be a high salt tolerant plant which is well adapted for growth in salt marsh habitats.

**Fig. 5.1.** Summary of eco-physiological adaptations of *Halopepis perfoliata* during seed germination and vegetative growth.