IN THE NAME OF ALLAH
THE MOST BENEFICENT MOST MERCIFUL
The Effect of Salinity on Morphological, Physiological and Biochemical Responses of *Panicum turgidum* Forssk

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The Effect of Salinity on Morphological, Physiological and Biochemical Responses of *Panicum turgidum* Forssk

A thesis submitted to the Faculty of Science, in fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

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2014
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**THESIS APPROVED**

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Summary

Agriculture productivity particularly in the semi-arid and arid zones of the world is declining and one of the major reasons is the increase in soil salinity. The aim of the present study was to investigate the physiological, biochemical and morphological aspects of salt resistance in *Panicum antidotale*, a local salt desert grass. The plants were grown at green house in a gravel culture of quick check system under 0, 125, 250, 375 and 500 mM NaCl. Plant growth was promoted at low salinity (125 mM NaCl) but was inhibited with increase in salinity with little or no mortality. Plant survival under saline conditions is due to its ability to maintain water level through reduction in its water potential by utilizing Na\(^+\) as an osmoticum. High Na\(^+\) in substrate had little effect on the absorption of K\(^+\) and Ca\(^{++}\) which not only maintain ion homeostasis but also reduce the harmful effect of Na\(^+\). Decreased rate of photosynthesis was concomitant with declined growth. Unchanged internal CO\(_2\) concentration along with increasing stomatal resistance showed the reduction in utilization of CO\(_2\) which is also supported by the decline in Rubisco protein at higher salinities. This suggests biochemical rather than stomatal limitation of photosynthesis. Salt resistance demands high energy to boost defense (e.g. biosynthesis of carbohydrate, proline and destruction against ROS) and to compensate for increased dark respiration which would lead to reduced biomass production. Oxidative stress indicators (ROS; H\(_2\)O\(_2\), electrolyte leakages, and MDA) do not indicate any stress at low salinity but increased with a further increase in salinity up to 500 mM NaCl. Antioxidant defense system, enzymatic (SOD, CAT, APx, GR) and non-enzymatic (AsA and DAsA) of *P. antidotale*, was up-regulated with increasing salinity treatments which seemed to minimize the toxic effects of ROS. Increase in AsA and DAsA which in turn was well correlated with APx activity. Redox homeostasis, calculated as a ratio of AsA/DAsA was maintained in *Panicum* under salt stress. This study suggests that *P. antidotale* produce considerable biomass at low salinity by effectively managing all parameters of salt resistance however at high salinity production of various chemicals to achieve salt resistance substantially reduce biomass production. This suggests that *P. antidotale* is a valuable fodder crop for low saline conditions.
دریا کے شکر اور دوسرے غار کی میکس کے ساتھ تیز پیچھا اور کی ایک اچھی تجربہ کے قیام کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسلہ کے ساتھ بھی دوسرے غار خاص کے ساتھ "Panicum antidotale" پر انسداد مالاکی سلسل
CHAPTER 1
GENERAL INTRODUCTION
1.1. Background

More than 95% of water on earth is saline which is due either to natural causes or through mal-practices such as over-irrigation of crops (Adolf et al., 2013; Munns and Tester, 2008) leading to massive reduction in crop production. Lack of fresh water resources (FAO, 2011) and increasing soil salinity (Bennett et al., 2009; Gleick et al., 2011) reduce crops production, threatens food security, livelihoods and human health (Shi and Wang, 2005; Sobhanian et al., 2010). Available water is seriously decreasing in the developing countries like Pakistan, where basic economy relies on agriculture. About 22.4 M ha area of Pakistan is under conventional crop production of which more than 18 M ha has already become salinized (Qureshi et al., 2008). Soil salinity in Pakistan is also increasing due to addition of 150 million tons of salts annually in the soil-matrix (Kahlown et al., 2002). By 2050 world population is anticipated to increase about 9 billion which will lead to a 70% increase in food requirement (FAO, 2011). These global issues collectively demands scientific research on proposed aspects to seek some sustainable alternative options like: (1) to increase the salt resistance or water use efficiency of conventional crops (Apse and Blumwald, 2002) (2) to increase fresh water availability and (3) to evaluate wild plants of arid and semiarid regions as alternate potential cash crops that can be grown on saline ground water (Khan et al., 2009; Koyro et al., 2011).

1.2. Halophytes

Halophytes are the plants that have specialized mechanisms to counter harmful effects of soil salinity and can complete their life cycles in saline habitats (Flowers and Colmer, 2008; Munns et al., 2010). These plants are better adapted to the following three major constraints caused by salinity: (a) water deficit arising from the low water potential, (b) ion toxicity on the basis of excessive uptake of Na$^+$ or Cl$^-$ (c) oxidative
stress and (d) nutrient imbalance disturbing the uptake of essential nutrients (Flowers and Colmer, 2008). Many of these halophytes can be utilized as non-conventional alternate source of food, fodder, wood, medicine, oil, biofuel, industrial raw material, land reclamation and ornamental purposes in salinity affected lands (Abideen et al., 2012; Khan et al., 2009; Qasim et al., 2010; Weber et al., 2007). Therefore, the detailed study of their salt resistance/tolerance mechanisms would enable us to utilize their full potential in a sustainable manner.

1.3. Mechanisms of salinity tolerance

1.3.1. Osmotic effects

Salinity causes osmotic stress that lead to water deficit (Krasensky and Jonak, 2012) and plants may develop various strategies to absorb water. Water loss is controlled by reducing leaf area and number of leaves which limits light exposure and maintain leaf turgidity in an acceptable range. Additionally, grasses increase stomatal resistance to minimize the transpiration on the expense of a reduced CO₂/H₂O exchange rate and the subsequent reduction in biomass production (Ahmed et al., 2013; Koyro et al., 2013). Plant decreases more water potential as compared to hyperosmotic saline substrate and this mainly achieved by reducing leaf water content and by an increased accumulation of ions. Salt resistant gramineaes use inorganic ions, mainly Na⁺ and K⁺ for osmotic adjustment (Ahmed et al., 2013; Marcum, 2008; Zhou and Yu, 2009). Na⁺ accumulation probably facilitates osmotic adjustment to minimize drastic effects of water deficit under salinity.

1.3.2. Ionic effects

1.3.2.1. Ion regulation and homeostasis

Increased Na⁺ in the medium disturbs the uptake and transport of K⁺ (Marcum, 2008; Zhou and Yu, 2009) leading to K⁺ starvation and/or [K⁺]/[Na⁺] imbalance in the plant
(Ahmed et al., 2013). Na⁺ toxicity or K⁺ deficiency in the cytoplasm and within organelles can affect growth negatively (Sobhanian et al., 2010). Under saline conditions, K⁺ leakages created via K⁺ outward-rectifying (KOR) efflux channels are also reported (Bafeel, 2013; Lan et al., 2010). Therefore, ability to maintain K⁺ content under highly saline conditions seems to be a promising feature of plant salt resistance. High Na⁺ levels disturb the cellular metabolic processes by influencing enzymatic activities (Guo et al., 2012) which could be minimized through compartmentalization of Na⁺ in the vacuole with the help of Na⁺/H⁺ antiporters and non-selective cation channels. However, this ionic compartmentalization reduces cytoplasmic water potential, which is counterbalanced by the accumulation of compatible organic solutes for stabilization of macromolecules like proteins, protein complexes, membranes and scavenging ROS are also reported (Cuin and Shabala, 2007; Lee et al., 2007; Marcum, 2002).

1.3.2.2. Photosynthesis under saline conditions

The yield of a plant mainly depends on the ability to maintain high carbon assimilation reactions with minimal water loss. Therefore, the biomass production is always seen to link with energy utilization and CO₂/H₂O exchange. Ion toxicity and imbalance cause by high soil salinity inhibits photosynthesis (Munns and Tester, 2008). There are several limiting factors for photosynthesis like; (1) opening and closing of stomatal guard cells, (2) resistance of CO₂ diffusion in to leaf, (3) photosynthetic pigments, (4) regeneration of RubP and (5) carboxylase activity of Rubisco (Lawlor and Cornic, 2002; Naumann et al., 2007). Reduction in CO₂ fixation also disturbed the flow of electron which led to induction in oxidative stress (Foyer and Noctor, 2009). Electrons may be transported to photosystem II and to make it
over reduced state that ultimately caused photo-inhibition (Koyro et al., 2013; Yang et al., 2012).

This deficiency of electron and proton acceptors causes plants facing excessive light to release surplus amount of energy as heat and chlorophyll fluorescence (Martínez-PeñaIver et al., 2011). Usually, energy dissipation by such process is only about 2-5% but even this small change in fluorescence values protect and remarkably influence plant photochemistry (Baker, 2008). Therefore measurement of chlorophyll fluorescence as the function of photosynthesis is a powerful technique to evaluate the performance of photosynthetic apparatus (Maxwell and Johnson, 2000). The electron transport in PSII results ATP synthesis and NADP formation which further used for carbon harvesting. Such quenching is termed as photochemical quenching (qP). At the same time, plant converts energy to heat and this process is known as non-photochemical quenching (NPQ). Later process dissipates excessive energy from the system and reduced the risk of ROS formation (Yang et al., 2012). These above said parameters are very informative to assess the photosynthesis specially the state of PSII during stress condition. Although these processes are relatively well known whereas heterogeneity of leaf development make them more complex rather than generalized. Therefore, there is a need to explore the dynamics of stress at both spatial and temporal level by taking advantage of non-invasive chlorophyll fluorescence technique. Monocot leaves (i.e. test species) have a gradient for tissue differentiation, with young undifferentiated tissues to be found near the leaf base while mature or senescing tissues are at the leaf apex.

1.3.2.3. Oxidative stress under salinity

The balance between electron excitation and acceptance among various compounds disturbed when plant facing abiotic stresses. For example, NAD$^+$ and NADP$^+$ through
electron transport chains (mitochondria, peroxisomes, and chloroplasts), where O₂ liberation and most of the electron transport occurs (Foyer and Noctor, 2009). As a result, these organelles are common site for the origin of reactive oxygen species (ROS), which may deteriorate the cellular components and damage the physiological and biochemical processes of the cell. Among these sites of ROS production, chloroplast has great distinction when plants are under consideration. The over-excited chlorophyll molecule - named as excited triplet (chl*) in the chloroplast formed due to excessive light energy can be balanced by inversion of electron spin and thus acquires its inactive form by energy transfer to a triplet O₂ and changed it as a singlet oxygen (¹O₂*) which have only a few nano-seconds half-life (Foyer and Noctor, 2009). When the photosynthetic apparatus cannot protect itself from produced ROS, the light harvesting complex of PSII surrounded by accessory pigments avoid photo-inhibition (Miller et al., 2010).

Fig 1.1. A schema of salinity causing oxidative stress in plants.
In result superoxide ($O_2^{−}$) radical formed if acceptor molecules failed to compete with molecular oxygen, for example NADP$^+$ are not freely accessible, or when the stomata are closed which concentrated cellular oxygen (Kocsy et al., 2013). Several antioxidant enzymes can detoxify ROS like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX) and glutathione reductase (GR) along with non-enzymatic antioxidant substances like nitrate, ascorbate, glutathione, carotenoids and tocopherols known as ROS scavengers (Ahmad et al., 2010; Hernández et al., 2009). Ascorbic acid (AsA) and glutathione (GSH) are main antioxidant substrates and play important role to detoxify ROS in Halliwell-Asada pathway. AsA is a electron donor during ascorbate peroxidase activity and convert $H_2O_2$ to water, forming mono-dehydro-ascorbate, which either dissociates into ascorbate and dehydro-ascorbate. GSH provides electrons for dehydro-ascorbate reduction, and it reduced by the glutathione reductase. A strong antioxidant system is of vital for plants for survival with low soil water potential, hyperosmotic salinity and nutrient imbalance (Jithesh et al., 2006).

1.4. Present study

Salt tolerance of the test species has been assessed by the wide range of morphological, physiological and biochemical responses when growing in saline substrate. The precise eco-physiological knowledge of salt resistance abilities in plants are enabling to grow them in saline areas. Therefore this study was conducted on a potential halophytic grass *Panicum turgidum* Forssk (it was revised taxonomically as *Panicum antidotale*) which produced about 60,000 kg/ha/year fresh biomass on saline soils with brackish water irrigation (Khan et al., 2009). Before modeling the fluxes of salt and water in plant it is essential to study the integrated response of *P. antidotale* to salinity on eco-physiological, biochemical, cellular and
molecular level to find comprehensive parameters indicating the degree of the current debit. The understanding of these mechanisms during salt stress will develop arguments regarding the salt resistance of *Panicum* up to levels of seawater salinity. Therefore following hypotheses were tested:

1. *P. antidotale* survives in up to sea water salinity.
2. Optimum growth can be obtained at moderate salinity (125 mM NaCl).
3. Gas exchange (stomatal conductivity) correlates with morphometric parameters such as the size and number of stomata on the leaf surface.
4. Decline in photosynthesis under salt stress is due to reduction in the levels of Rubisco.
5. Salt induced injury is higher at the tip of the leaf lamina.
6. Salinity increases the accumulation of Na\(^+\) and K\(^+\).
7. Proline acts as an ROS scavenger under salt stress.
8. Oxidative stress increase with the increase in substrate salinity.
9. Antioxidant system can cope with low salinity stress.
References


CHAPTER 2

PHOTOSYNTHESIS AND GROWTH RESPONSES OF PANICUM ANTIDOTALE TO NACL: A PERENNIAL HALOPHYTIC GRASS
2.1. Abstract

Several halophytes – plants which complete their life cycle in saline environment - have considerable economic potential as oil source, fodder, wood, ornamental and medicinal plants. They may also serve as model plants to get insight in the mechanisms of salt resistance and with the future aim to develop halophytic crops.

*Panicum antidotale* is a C₄ perennial halophytic grass, widely distributed in saline and arid areas of Pakistan and with a considerable potential as a fodder crop because of its high palatability and nutritional values. The aim of this work was to determine growth, physiological and biochemical responses of *P. antidotale* by subjecting plants to varying concentrations of salinity (0, 125, 250, 375 and 500 mM NaCl) and to relate these data to photosynthetic parameters.

When grown at low salinity (125 mM NaCl) fresh and dry biomass (shoot and root) were similar to non-saline control. Photosynthetic parameters such as net photosynthesis rate, transpiration rate, water use efficiency, Fv/Fm and electron transport rate correlated with growth response. High salinity led to a significant decrease of water use efficiency mainly because of an over proportional reduction of carboxylation rate arising from non stomatal factors such as decreasing Rubisco content and chlorophyll content. Reduction of carboxylation rate at higher salinity facilitated evolution of oxidative stress, electrolyte leakage, high malondialdehyde and H₂O₂ levels. Under severe salt stress *Panicum* exhibited photo-inhibition and xanthine cycle dependent heat release. However, with increasing levels of salt stress these protective mechanisms could not sufficiently save from ROS toxicity. *Panicum* seems to be a promising cash crop halophyte at moderate salinity. Its sustainable use can also help in desalinizing and reclaiming degraded land as well as sequestering CO₂. The data reported herein suggest that this species produces considerable biomass
at low quality soils with low quality water. It therefore has a high economic potential as a fodder crop in arid and saline areas.
2.2. Introduction

Global environment is rapidly changing due to increase in CO₂ concentration leading to higher ambient temperatures. Expected reduction of agricultural production will cause serious problems. These threats are aggravated by limited freshwater resources (Lieth et al., 1999) and impending soil salinization. Irrigated agricultural production already has decreased 20 – 35% due to increasing levels of salinity (FAO 2008; http://www.fao.org/ag/agl/agll/spush/; Hu and Schmidhalter, 2005). Fast growing population is suffering from severe shortage of water and food which will aggravate with time (Türkan and Demiral, 2009).

These problems could be partially alleviated especially in arid regions by utilization of low quality irrigation water such as saline groundwater or seawater on appropriate wastelands for production of adapted crops. Sine qua non it is necessary to develop sustainable biological production systems which can tolerate water with considerable amount of salinity because most of the conventional crops are sensitive to salinity. The development of suitable halophytic crops has been considered for the production of food, forage, oil, wood, timber, ornamental, medicine and biofuel (Koyro et al., 2008, 2011). Halophytes are extremophiles and are equipped with physiological and biochemical mechanisms enabling them to cope with high soil salinity. A candidate for an economic and ecologic sustainable production system at arid conditions could be Panicum antidotale Forssk. (Khan et al., 2009). This xerohalophyte is a perennial tussock-grass, commonly found in the salt deserts of southern Pakistan (Khan and Qaiser, 2006) but also in other arid areas.

Panicum antidotale produces considerable biomass and can be used as food and fodder plant but also as medicine (http://www.fao.org/ag/AGP/AGPC/doc/gbase/DATA). Recently Khan et al., (2009)
have reported tremendous potential of *P. antidotale* to replace maize as cattle feed and produce about 60,000 kg/ha/year fresh biomass on saline soils with brackish water irrigation. The present study determines the physiochemical responses of this member of Poaceae when subjected to different levels and up to seawater salinity. It has been undertaken with the intention to utilize this grass on saline and arid lands in sustainable manner without the risk of land degradation or destruction.

Before model the fluxes of salt and water in plant, it is essential to study the integrated response of *P. antidotale* to salinity on eco-physiological, biochemical, cellular and molecular level (Munns and Tester, 2008) to find comprehensive parameters indicating the degree of the current debit. In plants, particularly suitable for this purpose are processes of photosynthesis in correlation with the particular growth response.

Biomass production mainly associated with the ability to maintain high carbon assimilation and low water loss. Therefore, plant biomass formation has to be seen in relationship with energy utilization and CO₂/H₂O exchange (for example, water use efficiency (WUE)). A critical point for the plant is reached if CO₂-fixation falls below the level of CO₂-release (CO₂ compensation point). One crucial parameter is therefore, to study the correlation between growth reduction and net photosynthesis (Campbel et al., 2005; Hausler et al., 1999; Schulte et al., 2003).

Salinity hinders photosynthesis and growth by inducing alterations in photosynthetic tissues, disturbing water balance and developing ionic toxicity (Galmes et al., 2011; Munns and Tester, 2008). Suppression of photosynthesis in saline environment is generally due to limitation of stomatal conductance, uptake of carbon dioxide, photosynthetic capacity, carboxylase activity of Rubisco, regeneration of RubP and chlorophyll content (Lawlor and Cornic, 2002). Salinity induced
restrictions of above mentioned factors consequently lead to a reduction in plant growth (Naumann et al., 2007) but also to the danger of oxidative stress. Reduced availability of the electron and proton acceptor CO₂ can retrovert the over-reduction of the reaction centers of photosystem II (PSII) and can lead to photoinhibition and/or photodamage by production of reactive oxygen species (ROS) (Foyer and Noctor, 2005) if plant failed to dissipate excess energy as heat that can measure as non-photochemical quenching (NPQ) which is the part of xanthophyll cycle (Yang et al., 2007). The possible effect of photo damage by reactive oxygen species was studied on membrane level. Therefore electrolyte leakage, development of H₂O₂ and lipid peroxidation of membranes (development of malondialdehyde (MDA) were measured.

Additionally, the chlorophyll fluorescence measurement in vivo to identify cellular responses to salinity and degrees of salt stress in leaves was explored. Parallel measurement of gas exchange and chlorophyll fluorescence is widely accepted tool to explore performance of photosynthesis under salinity and other environmental stresses. Measured parameters provide some perceptive of the physiological status of a plant, which should correlate to its growth under stress conditions.

The aim of the present study was to evaluate the effects of elevated NaCl levels (0 to 500 mM) on P. antidotale growth and photosynthesis. The specific objectives were: (1) determine the minimum, maximum and optimum of growth response and the limit of salt resistance; (2) determine simple nondestructive parameters for the diagnosis of the current debit of the plant; (3) determine and link the changes in chlorophyll fluorescence parameters, chlorophyll content and gas exchange; (4) examine the specific role of Rubisco, (5) correlate gas exchange parameters such as the stomatal conductivity with morphometric parameters such as
the size and number of stomata on the leaf surface; and (6) search for indices of photo-damages on membrane level.
2.3. Materials and methods

2.3.1. Growth conditions and salinity treatment

Seeds were germinated in soil type LD 80 (Faarchut, Vechta, Germany) in an environmentally controlled green house (Giessen, Germany). After 2 weeks young seedlings were transplanted to a soil less (gravel/hydroponics) quick check system (Koyro, 2006). The plants were irrigated with a basic nutrient solution as modified by Epstein (1972) under photoperiodic (16 h light/8 h dark) conditions. Temperatures were 27 ± 2°C during the day and 17 ± 2°C during the night. Relative humidity ranged from 45 to 65%. Irradiation intensity was in the range of 190 µE m⁻² s⁻¹ at the plant level. NaCl concentrations were increased stepwise daily at the start of experiment by 50 mM NaCl (25 mM each at the beginning and at the end of the daily light period) until the final concentration was achieved: 0 (control), 125, 250, 375 and 500 mM NaCl. Water provided to quick check system to irrigate plants every 4 h for 30 min early at mid-night, 4h, 8h, 12h and 20h daily using timer and allow the saline solutions to drain freely from the pots. Solutions were recycled and changed every 2 weeks to maintain nutrient levels. The experiment was conducted for a total period of 12 weeks.

2.3.2. Growth measurements

At the end of the experiment, plants were carefully removed from the substrate and were separated in to shoot and root while fresh weight was recorded immediately. Aliquots were dried in an oven at 70 °C until constant weight of shoot and root was obtained.

2.3.3. Stress marker; electrolyte leakage, MDA content and H₂O₂ determination

Electrolyte leakage was measured on leaf discs (1 cm dia., 3 replicates) after being incubated in 3 ml of deionized water in a desiccators under a vacuum of ≈10⁻¹ bar for
Fig.2.1. Growth culture of *Panicum antidotale* (n = 10) growing in the green house under various NaCl concentrations (0, 125, 250, 375 and 500 mM).
60 minutes. Conductivity of each sample was measured and referred to as electrolyte leakage. Total conductivity measured after boiling the sample at 95 °C for 2h. The electrolyte leakage was calculated as percentage of total conductivity (Dionisio-Sese and Tobita, 1998).

Malondialdehyde (MDA) content as an indicator for the degree of lipid peroxidation of membranes was measured using thiobarbituric acid (TBA) assay (Cakmak and Marschner, 1992). Fresh leaf (0.1g) was ground with pre chilled pestle in a solution of 0.5% TBA in 20% trichloroacetic acid (TCA) followed by heating at 95 °C for 30 minutes. Samples were cooled at room temperature before centrifuging it for 5 minutes at 3000 rpm. The absorbance of supernatant was recorded at 532 nm (Original OD), OD of the non-specific absorbance was taken at 600 nm and subtracted from the original OD (Cakmak and Marschner, 1992).

Plant material (0.03 g) was homogenized with pre-chilled TCA (3.5 %) and centrifuged at 18,000 x g for 20 minutes at 4 °C (Loreto and Velikova, 2001). The supernatant was mixed with 1M potassium iodide in a ratio 2:1 (v/v). The hydrogen peroxide content was measured at 390 nm and was determined using a standard curve.

2.3.4. Gas exchange, chlorophyll estimation and chlorophyll fluorescence
CO₂/H₂O gas exchange measurements were carried out by Li-COR 6200 (LI-COR 6200, Lincoln, NE, USA) at ambient CO₂ partial pressure, temperature of 28–32 °C and natural relative humidity of the air. The light response curve was measured from 0 to 2000 µmol photon m⁻²s⁻¹ of photosynthetically active radiation (PAR). Dark respiration rate (RD), compensation irradiance (Ic) and saturation irradiance (Is) was calculated according to Schulte et al., (2003). Net photosynthetic rate (A), stomatal resistance (rs), transpiration (E), internal CO₂ (Ci) and water use efficiency (A/E) were measured on the fully emerged leaf (third and fourth node) blades at saturated
irradiation of each treatment under constant light source. At the same time chlorophyll content was estimated by using SPAD-502 (Konica Minolta, Japan).

Pulse modulated chlorophyll fluorescence meter (Junior PAM, Walz, Germany) was used to determine chlorophyll a fluorescence on the same leaf on which CO₂/H₂O gas exchange measurements were made. The minimal fluorescence (Fo) value was measured after applying modulated light (< 0.1μmol photon m⁻² s⁻¹) on dark adapted (25 min) leaf, while the maximal fluorescence (Fm) value was obtained by imposing a saturating pulse of 10,000 photons (μmol m⁻² s⁻¹ for 0.6 s. Fo and Fm values were used to calculate maximum photochemical quantum yield of PSII (Fv/Fm = Fm-Fo/Fm) in predawn and noon by the method of Kitajima and Butler (1975) and photo-inhibition was calculated as described by Dodd et al., 1998:

\[ \text{Photoinhibition (\%)} = 100 - \left( \frac{F_v}{F_m} \frac{\text{noon}}{\text{Predawn}} \right) \times 100 \]

Subsequently, the leaves were continuously illuminated with actinic light, which was equivalent to the actual growth light of plants in order to measure steady-state (Fs) and maximal fluorescence (Fm’) in light-adapted leaves. The minimal fluorescence level in light-adapted leaves (Fo’) was estimated following the method of Baker and Rosenqvist (2004). Effective photochemical quantum yield of PSII was calculated as Fm’-Fs/Fm’ (Genty et al., 1989). Non-photochemical quenching of fluorescence (NPQ) which is proportional to the rate of constant heat dissipation (Bilger and Björkman, 1990), was calculated as NPQ = Fm/Fm’-1. The coefficient of photochemical quenching (qP) was calculated as (Fm’-Fs) / (Fm’-Fo’) (Schreiber et al., 1986). PSII is used for calculation of the linear electron transport rate (ETR; Krall and Edwards 1992) as ETR = PSII * PPFD * 0.5 * 0.84, where Photosynthetic Photon Flux Density (PPFD) incident on the leaf; 0.5: factor that assumes equal distribution
of energy between the two photosystems; 0.84: assumed leaf absorbance. ETR/A ratios in entire range of salt treatment were also calculated by using the ETR from fluorescence and net photosynthesis from gas exchange measurement.

2.3.5. Extraction and measurement of Rubisco

Mature leaves were harvested and stored at -80 °C. Protein extraction was performed according to Granier (1988). Leaves were grind to fine powder on liquid nitrogen and 0.05 g polyvinyl-polypyrrolidon (PVPP) were added. The powder was diluted in extraction buffer (100 mM Imidazol and 1.25 mM EDTA; pH 7.8). Proteins were denatured using sodium-dodecyl-sulfate (SDS). Protein was determined on fresh weight basis using 5 replicates (Bradford (1976). Protein was separated by polyacrylamide gel electrophoresis (agarose 6% (g/g) for stacking, 12.5% (g/g) for separation) according to Laemmli (1970). For calibration an internal SDS-PAGE molecular weight standard (BIO-RAD Laboratories GmbH, Munich, Germany) was used. Protein staining was performed with Coomassie brilliant R-250 blue while gels were de-stained in 10% acetic acid. Rubisco large subunit was identified by molecular weight in the range of 53 kDa (Ishida et al., 1997). Ratio of Rubisco to the total protein content was gathered using the integrals of the signal strength on the gel using the image processing and analysis software ImageJ (National Institute of Health, Maryland, USA). Rubisco total amount was calculated with the protein analysis mentioned above.

2.3.6. Stomatal morphometry

Measurement of number of stomata, opening, length and area of upper and lower surfaces of fully emerged leaves were performed by peel off method Hilu and Randall (1984). The nail varnish was applied with the brush to the upper and lower side of leaf and avoided midrib and peeled off varnish after 5-10 minutes. Placed the peeled off
piece on the slide and apply the glycerol. Count the number of the stomata at 100x under microscope and calculated length, opening and area of stomata by using Carl Zeiss Axio Vision software.
2.4. Results

Biomass showed no significant difference from control at 125 mM NaCl and it significantly decreased with an increase in salinity (Fig. 2.3).

Ion leakage, H₂O₂ and malondialdehyde (MDA) contents are well known as stress indicators at drought and excessive salinity. All three stress indicating markers increased significantly with increasing NaCl concentrations in the substrate (Fig. 2.4). The increase of all measured parameters shows a bimodal curve; no difference at lower concentration, a significant increase at 250 mM NaCl, and no significant change upon further increase in salinity (Fig. 2.4).

Increase in NaCl concentrations during growth of *P. antidotale* cause significant changes in various CO₂/H₂O gas exchange parameters. The net photosynthetic rate \( (A) \) in control plants was 10.08 ± 0.15 µmol m\(^{-2}\) s\(^{-1}\) and it increased (48% of control; \( P<0.001 \)) at 125mM NaCl but declined (54% of control; \( P<0.001 \)) as the salinity increased to the maximum level for the experiment (Table 2.1). This transient decrease of the net photosynthetic rate is strongly correlated with saturation irradiance (Is) but also with stomatal resistance (rs), transpiration rate (E) and water use efficiency (WUE). However, the internal carbon dioxide concentration (Ci) was not influenced by increase in salinity and remained far above the lower carboxylation limit of PEP carboxylase and Rubisco at all conditions (Riviere-Rolland et al., 1996). Rising NaCl concentrations led to an increase of dark respiration rate (RD) and consequently of compensation irradiance point (Ic).

Relative chlorophyll content (SPAD values) was highest (43.5 SPAD relative units) at 125 mM NaCl and decreased significantly down to 22.7 SPAD relative units at 500 mM NaCl substrate salinity (Fig. 2.5) showing a similar trend as the net photosynthesis and related parameters (Table 2.1).
Fig. 2.2. Picture showing response of *Panicum antidotale* under various NaCl concentrations (0, 125, 250, 375 and 500 mM)
Fig. 2.3. Growth of *P. antidotale* in response to 0, 125, 250, 375 and 500 mM NaCl concentration. Fresh weight of shoot and root (A and C respectively) and dry weight of shoot and root (B and D respectively. Values represent mean ± SE. Different letters indicate significant difference between treatments at P < 0.05 using Bonferroni test.
Fig.2.4. Changes in the amount of electrolyte leakage (A), malondialdehyde (MDA) (B) and hydrogen peroxide, H$_2$O$_2$ (C) induced by NaCl in leaves of P. antidotale. Each value represents the mean ±SE and different letters indicate significant difference between treatments at P < 0.05 after Bonferroni test.
Net photosynthesis (Table 2.1), PSII maximum quantum efficiency (Fv/Fm) and actual yield (PS II) showed a similar curve shape although the increase of the latter two was not significant up to moderate salinity (125–250 mM NaCl) (Fig. 2.6). However, both chlorophyll fluorescence parameters decreased significantly with a further increase of salinity. A rise in salinity from 6.5% (control) up to 18% (500 mM NaCl) also led to a steady rise in photoinhibition (Fig. 2.6). Photochemical quenching (qP) was stable at all salinities (Fig. 2.6). In contrast to qP, non-photochemical quenching (NPQ) increased by a factor of 1.5 to 2 as compared to the control at high salinity treatments.

Expression of the large subunit of Rubisco decreased with increasing salinity (Fig. 2.7). There was a relationship between Rubisco content and photosynthetic activity (net photosynthesis and ETR) in the control and high salinity treatments (from 250 to 500 mM NaCl) of *P. antidotale*.

Morphometric measurements of the stomata showed a maximum length (adaxial and abaxial surface) and opening area (adaxial and abaxial surfaces) at medium growth conditions (125 mM NaCl) (Table 2.2). The number of stomata were much lower at higher salinities as under the optimum treatment.
Table 2.1. Measurement of gas exchange of 3rd and 4th fully emerged leaf of *P. antidotale* plants at saturated light.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>A (µmol m⁻² s⁻¹)</th>
<th>Is (µmol Photon)</th>
<th>rs (mol m⁻² s⁻¹)</th>
<th>E (mol m⁻² s⁻¹)</th>
<th>WUE (A/E)</th>
<th>Ci (µmol m⁻² s⁻¹)</th>
<th>RD (mol m⁻² s⁻¹)</th>
<th>Ic (µmol Photon)</th>
<th>∆T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.08a±0.15</td>
<td>629.23a±14.49</td>
<td>6.44a±0.19</td>
<td>1.55a±0.05</td>
<td>6.53a±0.32</td>
<td>245a±20.82</td>
<td>-0.26a± 0.06</td>
<td>16.35a±2.05</td>
<td>1.50a±0.01</td>
</tr>
<tr>
<td>125</td>
<td>14.85b±0.17</td>
<td>794.90b ± 3.42</td>
<td>5.80a±0.16</td>
<td>1.95b±0.06</td>
<td>7.64b±0.24</td>
<td>230a ± 1.78</td>
<td>-0.32a± 0.11</td>
<td>13.03a±1.06</td>
<td>1.93b±0.03</td>
</tr>
<tr>
<td>250</td>
<td>8.90c±0.05</td>
<td>508.47c±10.49</td>
<td>3.39b±0.06</td>
<td>1.38ac±0.05</td>
<td>6.50a±0.19</td>
<td>329b ± 1.12</td>
<td>-0.70b± 0.10</td>
<td>18.56a±0.38</td>
<td>1.24a± 0.21</td>
</tr>
<tr>
<td>375</td>
<td>6.25d±0.05</td>
<td>458.42c±13.42</td>
<td>7.96c±0.13</td>
<td>1.38ae±0.03</td>
<td>4.55c±0.11</td>
<td>259a ± 4.49</td>
<td>-0.76b± 0.02</td>
<td>17.90a±0.33</td>
<td>1.58a± 0.02</td>
</tr>
<tr>
<td>500</td>
<td>5.47e±0.03</td>
<td>275.86d±19.42</td>
<td>9.31d±0.27</td>
<td>1.20c±0.07</td>
<td>4.60e±0.27</td>
<td>315b ± 3.42</td>
<td>-0.90e± 0.07</td>
<td>17.84a±0.21</td>
<td>2.39b±0.02</td>
</tr>
</tbody>
</table>

A: net photosynthetic rate, Is: saturation irradiance, rs: stomatal resistance, E: transpiration, WUE: ratio of net photosynthesis rate and transpiration, Ci: Intercellular CO₂, RD: dark respiration rate, Ic: compensation irradiance, and ∆T: change in temperature. Values represent mean ± S.E and different letters indicate significant difference between treatments at P < 0.05 after Bonferroni Post hoc test.
Fig. 2.5. Changes induced by NaCl solutions in the amount of chlorophyll in leaves of *P. antidotale* expressed as SPAD values. Each value represents the mean ± SE and different letters indicate significant difference between treatments at $P < 0.05$ after Bonferroni Post hoc test.
Fig. 2.6. Maximum quantum efficiency of PSII photochemistry, Fv/Fm and actual yield (PSII) (A), Photochemical (qP) and non-photochemical quenching (NPQ) (B) and electron transport rate (ETR) and photoinhibition (C) in randomly selected, fully expanded (3rd and 4th node) leaves of *P. antidotale* in response to various NaCl concentration. Values represent mean ± SE and different letters indicate significant difference between treatments at P < 0.05 after Bonferroni test.
Fig. 2.7. Rubisco large sub unit was measured on fully expanded (3rd and 4th node) leaves of *P. antidotale* in response to treatment with various NaCl concentrations in the substrate. Values represent mean ± SE and different letters indicate significant difference between treatments at $P < 0.05$ after Bonferroni test.
Table 2.2: Morphometric measurement of stomata of fully emerged leaves (3rd and 4th leaf from top) of *P. antidotale*.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Length of stomata (µm)</th>
<th>Opening area of stomata</th>
<th>Number of stomata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper</td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>0</td>
<td>16.15 ±2.01</td>
<td>17.64 ±0.11</td>
<td>232.45 ±14.94</td>
</tr>
<tr>
<td>125</td>
<td>22.88 ±1.07</td>
<td>21.68 ±1.36</td>
<td>399.15 ±19.29</td>
</tr>
<tr>
<td>250</td>
<td>14.05 ±0.17</td>
<td>10.57 ±0.54</td>
<td>218.31 ±15.97</td>
</tr>
<tr>
<td>375</td>
<td>15.36 ±0.16</td>
<td>15.67 ±0.89</td>
<td>233.92 ±5.91</td>
</tr>
<tr>
<td>500</td>
<td>14.25 ±1.95</td>
<td>8.24 ±0.79</td>
<td>301.30 ±7.06</td>
</tr>
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</table>
2.5. Discussion

As a first step of present study I intended to determine salt tolerance range of *Panicum* antidotale. Present data indicate that the tolerance threshold lies close to seawater salinity. Growth of halophytes is commonly stimulated by low level of salinity (Khan et al., 2000; Riadh et al., 2010). Like many other monocotyledonous plants (Glenn et al., 1999; Naidoo et al., 2008), *Panicum antidotale* shows similar growth performance without added salt and in presence of moderate salinity, respectively. In present experiments I did not observe significant differences between control plants and those grown in presence of 125 mM NaCl (resembling 25% seawater salinity). Trend of the data indicates that the growth may even be promoted at 100 mM like reported in the studies of Al-Khateeb (2006) who found a significant growth promotion in *P. turgidum* at 100 mM NaCl. Therefore *P. antidotale* meets the essential condition for utilization in a saline environment.

As optimal growth was found only in a narrow NaCl concentration range, it is essential for scaling up of experimental data to establish a reliable system for monitoring of plant performance under stress. Several parameters may act as bottle neck for plant growth under stress (Flowers 1985; Koyro et al., 2006). In most relevant papers limitations of photosynthesis could be aligned to observed reductions in plant growth and development. Accordingly, growth response of *P. antidotale* was found to correspond to chlorophyll content, electron transport rate (ETR), net photosynthetic rate (A), transpiration (E), water use efficiency (WUE), the temperature gradient between leaf and atmosphere (ΔT) and even the size of the stomata. These parameters provide a broad range of options for a non-destructive diagnosis at field site.
Gas exchange rate and growth at elevated CO₂ concentration vary with plant species and abiotic factors such as salinity and humidity (Ball and Munns, 1992 Drake, 1992; Geissler et al., 2009). Growth performance under salinity resembled patterns of the above mentioned parameters. This is in line with basic understanding of photosynthesis. But presented data do not allow at this stage to identify a reaction sequence leading to the observed inhibition of plant growth.

On anatomic level inhibition of plant development at increasing salinity was related to several structural changes that most probably optimize the gas-exchange (Objective 5). The increase of salinity led to a transient increase of stomata number, their size and maximum opening area with highest values at 125 mM NaCl indicating an adjustment to a particular demand of CO₂.

Stomatal conductance increased at low salinity in *P. antidotale* which is in consonance with aforementioned increase in transpiration which is proportional to higher net photosynthesis with a 17% increase in water use efficiency. Stomatal conductance was significantly reduced at higher salinities however; it did not prevent a 30% decrease in water use efficiency (WUE) because of 63% decrease in net photosynthesis. The increase in stomatal resistance is one of the main responses of plant under saline stress to minimize water loss (Kim et al., 2010) at the expense of CO₂-fixation. Consequently, changes in stomatal resistance appeared to provide an explanation for the parallel decline in photosynthetic assimilation rates.

WUE is often discussed as an indicator for salt resistance (Gleick et al., 2011). Salinity led to an increase of the WUE in case of *Sarcocornia fruticosa, Beta vulgaris ssp. maritima* and same results were found in *Atriplex portulacoides* (Koyro et al., 2006; Redondo-Gómez et al., 2007) but it declined at higher salinities in *Odyssea paucinervis* (Naidoo et al., 2008). However the increase in stomatal resistance was not
accompanied by a loss of leaf water content in *P. antidotale* and therefore is likely to be the result of a controlled regulation (signaling process) rather than an effect of water loss. The stomatal resistance is highly correlated with morphometric studies which indicated that the high salt concentrations closed more stomata. However, the reduced gas-exchange did not lead to a decrease of the intercellular CO$_2$ concentration (Ci) or even to a significant increase at high salinity. It may reflect that photosynthetic decline at this condition might be caused by a reduction of the carboxylation capacity of photosynthesis and lowering of RWC (data not shown) rather than any effect on diffusion limitation. The impaired assimilation rate correlates with the Rubisco content in *P. antidotale* suggesting a reduction of the Rubisco activity while CO$_2$ starvation under salt stress was not found (Table 2.1). The internal CO$_2$ concentrations remained unchanged at all salinity treatments far above the lower limit of PEP carboxylase. If biochemical limitation occurs under stress, this is often related to the Rubisco activity (Galmés et al., 2011; Grassi and Magnani, 2005), as *Panicum antidotale* is a C$_4$ species, respective CO$_2$ affinity of PEP-carboxylase has to be taken into account. A declined in CO$_2$-assimilation reactions generally responsible to create oxidative stress due to over-reduction of reaction centers (Arneth et al., 2002; Ben Amor et al., 2005).

Interestingly the ratio of the electron transport rate (ETR) to the CO$_2$ assimilation was unaffected under NaCl treatments. But ratios calculated from this experimental data were much higher as compared to those known from unstressed C$_3$ plants (Ishida and Toma et al., 1999). The reason for this observation is that salt resistance mechanisms are often energy consuming and are reflected in *P. antidotale* by an increasing dark respiration rate (Table 2.1). This high demand of energy in
defense mechanisms (e.g. against destruction by ROS) will lead to deplete lesser amount of energy in biomass production in *P. antidotale*.

Three concrete indications of a higher net ROS production by *P. antidotale* cells under saline conditions were observed and indices of photodamage e.g. on membrane level were studied. The level of production of H$_2$O$_2$, lipid-per-oxidation (MDA content) and ion leakage are well known as stress markers (Elkahoui et al., 2005) to assess the aptitude of the oxidative damage in stress condition. High NaCl concentration induced an oxidative stress in *P. antidotale*, as evidenced by the increase in H$_2$O$_2$ concentration and led to perxoidation of polyunsaturated fatty acids of cell membrane. Many authors found (Ben Hamed et al., 2007; Koca et al., 2007; Yazici et al., 2007) positive correlation between growth inhibition with increased lipid-per-oxidation levels under salinity stress as we found in *P. antidotale* this may due to leakage in membrane after the production of ROS and peroxidation of membrane lipids.

Excessive NaCl concentrations in substrate can be toxic to plants (Debez et al., 2008; Naranjo et al., 2008) and are often attributed to an overall inhibition of photosynthesis. PSII performance was found unchanged in *P. antidotale* during salinity stress excluding 500 mM NaCl treatment, which induced significant decline in actual PSII efficiency. There is relevant information that the maximal efficiency of PSII (Fv/Fm) remained unaffected in several halophytic species at moderate salinities (Maricle et al., 2007; Naidoo et al., 2008; Qiu et al., 2003; Yıldız tugay et al., 2011), unaffected at higher salinities (Redondo-Gómez et al., 2007) and optimal at low salinities (Debez et al., 2008; Wei et al., 2006) while in salt sensitive plants it reduced even at moderate salinity levels (Netondo et al., 2004; Zhao et al., 2007).
At moderate salinity high Fv/Fm ratios in combination with increasing values of thermal dissipation (NPQ) are protecting *P. antidotale* from over reduction of the light harvesting reaction center. High salinities led to a significant reduction of Fv/Fm during the day which is indicative of photo-inhibition and allied with an over reduction of PSII centers. The decrease in Fv/Fm did not recover completely in the high salinity treatments during the night in *P. antidotale*, both PSII efficiency and net CO₂ assimilation rate declined with increasing NaCl concentrations.

In summary, I found convincing evidence at high NaCl salinity that either light harvesting or consumption of redox energy (Fig.2.6) were influenced by salt stress. Decreasing Rubisco concentration leading to reduced CO₂ fixation rate might be the main reason for the latter observation (Van den Berg and Perkins, 2007) (Figure 6). It is most probable that a concerted action of these factors resulted in the observed effects, and a reaction sequence as well as signaling and regulatory mechanisms controlling salt stress response could not be identified in present experimental approach (Tezara et al., 2003). At this state of stress I may propose that plants are under high risk of triplet state of chlorophyll formation and production of ROS that cause the damage of D1 protein of photosystem and a main precursor of photodamage. These results fit excellently with the results of membrane leakage, MDA content and H₂O₂ content (indices for photodamage). Present findings are consistent with previous literature (James et al., 2002; Moradi and Ismail, 2007; Naidoo et al., 2002; Redondo-Gómez et al., 2006) about photodamage at increasing levels of salinity.

The decreased chlorophyll content can fulfill a similar function (less energy transfer by the light-harvesting proteins). On the one hand, it reduces the assimilation rate of *A. tripolium* (Lorenzen et al., 1990), but on the other hand (and in case of *P.
*antidotale* more important), it decreases the light absorption of the leaves (Wang et al., 2003; Christian, 2005). This thesis is supported by the observation that, at moderate salinity, the reduced photosynthetic efficiency was linked to a lower light compensation point (lc). At high salinity, lc rose again, probably due to an increasing dark respiration. However at high salinity neither salt induced decrease of chlorophyll content nor increase of heat emission (see table 2.1: ΔT and figure 2.6: NPQ) reduce the flow of electrons through the photosystems sufficiently high to prevent *P. antidotale* from over excitation of the light harvesting reaction center (see photoinhibition, Fig. 5).

This study has demonstrated that *P. antidotale* adapted to saline habitats. This natural distribution of test species in saline and/or alkaline desert soils validates results present in this thesis. There appears to be a good relationship between field distribution of the species and its physiological tolerance to salinity under controlled conditions. Similar to other monocotyledonous halophytes, growth is not affected at low salinities and decreases at higher salinities. In conclusion, *P. antidotale* shows persistent growth under moderate salinities. This observation can be explained by the plant’s capacity to attain efficient control and it showed little promotion at low salinity. *Panicum* seems to be a promising cash crop halophyte. Its sustainable use can also help in desalinizing and reclaiming degraded land and developing biomass. The data reported here suggest that this species may be a potential candidate for cultivation as fodder on low quality soils irrigated with low quality water and it explains that *Panicum* is a good quality fodder in coastal areas such as in Pakistan (Kahn et al., 2009). However the range of salinity tolerance is small and although photosynthesis and growth were constant at moderate salinity, I found a pronounced decrease of growth rate reaching its limits at seawater salinity (500 mM NaCl). This
impact of high salinity on photosynthesis was mainly due to reduction in carboxylation capacity not to stomatal parameters thus leading to reduced water use efficiency with increasing salt concentrations. Results on membrane leakage, photoinhibition, H$_2$O$_2$ production, and lipid per-oxidation clearly indicate the presence of oxidative stress at high salinity. However, more research about the exact regulation of the oxidative defense system (Halliwell-Asada System, violaxanthin cycle) of P. antidotale and on the regulation of stomata size, number and regulation of their opening as well as pigment and synthesis of photosynthesis enzymes under moderate and severe saline conditions is needed.
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CHAPTER 3
ECO-PHYSIOLOGICAL ADAPTATIONS OF *PANICUM ANTIDOTALE* TO SALT STRESS: WATER AND ION RELATIONS AND ANTI-OXIDANT FEEDBACK – A POTENTIAL CASH CROP HALOPHYTIC GRASS
3.1. Abstract

Osmotic and ionic effects (primary) have been considered to play a leading role in managing salt resistance of plants. However, recent reports emphasize the importance of secondary effects like oxidative metabolism. The aim of this study was to determine the role of secondary stress along with the primary effects in the salt resistance of *Panicum antidotale*.

Five levels of salinity (0, 125, 250, 375 and 500 mM NaCl) were applied using a quick check system in a fully randomized greenhouse study for this purpose. Plant growth parameters, water relations, organic (proline and soluble sugar), inorganic osmolytes (Na\(^+\), K\(^+\), Ca\(^{++}\) and Mg\(^{++}\)) and macronutrients such as carbon and nitrogen were measured beside antioxidant enzymes activities super-oxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx) and glutathione reductase (GR) and non-enzymatic antioxidant metabolites (oxidize and reduced ascorbate).

Sodium chloride concentrations (above 125 mM) substantially inhibited the growth. This inhibition is attributed to high energy costs for osmotic adjustment, ion compartmentalization, synthesis of organic osmolytes (such as proline and sugar) and the maintenance of ionic homeostasis. Defense against oxidative stress (secondary) in addition to the effects mentioned above appears to play an important role by enhancing activities of antioxidant enzymes such as SOD, APx, GR and CAT and elevating levels of oxidized and reduced ascorbate (DAsA and AsA) at higher salinity. Especially the maintenance of a low redox ratio (parameter of oxidative stress) of ascorbate / dehydro-ascorbate at higher salinity was of major importance for the protection of *Panicum* against oxidative stress and help in its salt resistance.
3.2. Introduction

Soil salinity is one of the most threatening abiotic factors especially for arid and semiarid areas where it decreases conventional agriculture productivity (Bennett et al., 2009; Gleick et al., 2011) through reducing growth, decreasing biomass production and eventual death of plants (Shi and Wang, 2005; Sobhanian et al., 2010). Halophytes have adapted to counter harmful effects of soil salinity and are able to complete their life cycles in saline habitats (Flowers and Colmer, 2008; Munns, 2010; Wang et al., 2009). The utilization of these plants as non-conventional alternate source of food, fodder, wood, medicine, oil, biofuel, industrial raw material, land reclamation and ornamental purposes are well established (Abideen et al., 2012; Khan et al., 2009; Qasim et al., 2010; Weber et al., 2007). *Panicum antidotale* has a considerable potential to be used as cattle feed and it could be grown on salinized land with brackish water irrigation. Khan et al., (2009) have reported that *P. antidotale* produces about 60,000 kg/ha/year fresh biomass on saline soils with brackish water irrigation and can replace maize as cattle feed. This study develops arguments regarding the underlying mechanisms of salt resistance of *Panicum* (Koyro et al., 2013) up to levels of seawater salinity.

Hyperosmotic salinity limits plant growth primarily by high salt concentrations (ionic effect) and limited water availability (osmotic effect) (Krasensky and Jonak, 2012; Munns and Tester, 2008). It can also alter bioenergetics (Photosynthesis and respiration), protein synthesis, lipid metabolism and gene expression (Parida and Jha, 2010). Besides high Na\(^+\) in the medium may alter the transport and uptake of K\(^+\) (Marcum, 2008; Zhou and Yu, 2009) leading to K\(^+\) starvation and/or [K\(^+\)/[Na\(^+\)] imbalance (Ahmed et al., 2013; Hauser and Horie, 2010).
Na⁺ excess and/or K⁺ deficiency in the cytoplasm and its organelles may reduce or inhibit plant growth (Sobhanian et al., 2011) leading to secondary oxidative stress. Water deficit (osmotic effect) is a non-specific primary effect of hyperosmotic salinity that can reduce the leaf cell expansion and stomatal conductance and may lead also to secondary oxidative stress (Koyro et al., 2013; Yang et al., 2012). Halophytes reduce the osmotic potential and may avoid Na⁺ toxicity by compartmentalization in vacuole (Lee et al., 2007; Marcum, 2002). The offset and maintenance of a low cytoplasmic water potential, ionic homeostasis and the stabilization of some macromolecules such as proteins, protein complexes and membranes are ensured by the accumulation of organic solutes - amino acids (proline and citrulline) onium compounds (glycinebetaine, 3-dimethylsulfonoproprionate), carbohydrates and polyols (Kocsis et al., 1998; Subudhi and Baisakh, 2011; Verbruggen and Hermans, 2008). Proline also uses protons during its synthesis and therefore helps in reducing oxidative stress by acting as ROS scavenger (Kocsy et al., 2013). Ionic and osmotic effects, both can reduce CO₂ assimilation which is one of the main reasons of the over reduction of photosynthetic electron transport chain. It is, therefore, conceivable that under high salinity, production of active oxygen species such as superoxide and hydrogen peroxide are stimulated (Koyro et al., 2013; Krieger-Liszkay et al., 2008).

ROS (reactive oxygen species) has a high affinity to react with proteins, lipids and nucleic acids that causes the malfunctioning of these macro-molecules (Höhn et al., 2013; Kocsy et al., 2013). Several antioxidant enzymes can detoxify ROS like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX) and glutathione reductase (GR) along with non-enzymatic antioxidant substances like nitrate, ascorbate, glutathione, carotenoids and tocopherols are also known ROS scavengers (Ahmad et al., 2010; Hernándezet al., 2009; Jithesh et al., 2006). A strong
antioxidant system is of vital significance for plants survival with low soil water potential, hyperosmotic salinity and nutrient imbalance (Jithesh et al., 2006). The excessive production of ROS in *P. antidotale* at hyperosmotic salinity can lead to an increased lipid peroxidation of membranes causing membrane leakage (Koyro et al., 2013).

This study was conducted to determine salinity induces changes of the primary stressors (ionic and osmotic relations) in *P. antidotale* plants, with consequences (1) in the growth response (2) accumulation of organic and inorganic solutes and (3) up-regulation of antioxidant system (secondary stressor) at biochemical level. This information would help to determine the particular importance of primary and secondary stressors on the overall salt resistance of this species that can be utilize as non conventional crop in arid and semi arid areas.
3.3. Materials and methods

3.3.1. Plant material and treatments

Surface sterilized seeds were germinated in soil type LD 80 (Faarchut, Vechta) in an environmentally controlled green house (Giessen, Germany). After 2 weeks young seedlings were transplanted to a soil less (gravel/hydroponics) quick check system (Koyro 2006). The plants were irrigated with a basic nutrient solution as modified by Epstein (1972) under 16 h light / 8 h dark photoperiod. Temperatures were 27 ± 2°C during the day and 17 ± 2°C during the night. Relative humidity ranged from 45 to 65%. Irradiation intensity was in the range of 190 µE m⁻² s⁻¹ at the plant level. NaCl concentrations increased daily stepwise by 50 mM NaCl (25 mM each at the beginning and at the end of the light period daily) until the final concentration was achieved: 0 (control), 125, 250, 375 and 500 mM NaCl. Plants were irrigated at 4 hours interval for half an hour every day and solutions were allowed to drain freely from the pots. Solutions were changed every 2 weeks to maintain nutrient levels. The experiment was conducted for a total period of 12 weeks.

3.3.2. Growth measurements

After 5 week of the NaCl treatment plants were harvested and were divided into three parts, leaves (juvenile leaf - upper 4 to 5 nodes and adult leaf - below 4 to 5 nodes from top), stem and root. The shoot length, leaf number, leaf area and succulence were recorded and expressed on per plant basis. Plants were dried in an oven at 70 °C until the constant weight was obtained.

3.3.3. Water relations

Water potential was measured on intact leaves by dew point method with HR-33T Dew Point Microvolt meter, using L-51-SF leaf chamber (Wescor, USA). Relative water content (RWC) measured using 3 discs of 1 cm diameter from a leaf (avoiding
margins and midrib) was taken and fresh weight (FW) was determined (Sharp et al., 1990). Discs placed in 1.5 ml of deionize water for 4 h at 4 °C to let the tissue to absorb water. Weight of the discs was termed as turgid fresh weight (TFW). Tissues were dried at 70 °C for 48 h and dry weight (DW) was determined. RWC was calculated as;

\[
RWC = \frac{FW - DW}{TFW - DW}
\]

3.3.4. Proline and sugar determination

Proline was estimated by using powdered plant material (50 mg) which was mixed with 4 ml of 3% sulphosalicylic acid and sonicated at 30 °C for 15 minutes and then centrifuged at 3000 x g. This extract was used for measuring proline content. One ml acid ninhydrin and 1 ml of glacial acetic acid added in reaction mixture which was boiled at 100 °C for 1 h. The reaction was terminated in ice and added 2 ml of toluene to the reaction mixture and vortex for exactly 30s. The absorbance of upper phase was measured at 520 nm and sample proline was estimated against standard curve using L-proline (Bates et al., 1973). Soluble sugar was determined by anthrone method (Ludwig and Goldberg, 1956). Press sap was used for measuring sugar. Samples were heated for 11 min with anthrone reagent (in 95% H₂SO₄) and reaction terminated in ice. The absorbance was recorded at 630 nm and sugar was estimated by using sucrose as a standard.

3.3.5. Cations, carbon and nitrogen analysis

200 mg of dried leaf (adult and juvenile), stem and root were ashed in an oven at 600 °C for 8 h. Ash was dissolved in 20% HNO₃ on Benson-Burner at gentle flame. Mixture was filtered through ash-less filter paper (Whatman no. 40). Na, K, Ca and Mg were measured with an atomic absorption spectrophotometer (AAs 2100, Perkin Elmer). The carbon and nitrogen content were determined in powdered dry plant
material using an element analyzer Vario MAX CNS (Elementaranalysensysteme GmbH, Hanau).

3.3.6. Protein extraction

Leaf tissues (0.5 g) were ground to a fine powder in liquid N<sub>2</sub> and then homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1mM EDTA, 1mM ascorbic acid 2% (w/v) PVPP and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar (Gossett et al., 1994). The homogenate was centrifuged at 12,000 X g for 20 min at 4 °C and the supernatants were collected and used for the assays of catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase. Protein concentrations in the enzyme extract were determined by Bradford (1976) method and bovine serum albumin (BSA) was used as standard.

3.3.7. Enzyme assays

Catalase (CAT, EC 1.11.1.6) activity was determined using assay mixture contained 50 mM potassium phosphate buffer (pH = 7.0), 25 mM H<sub>2</sub>O<sub>2</sub> and 50 µl enzyme extract in a reaction mixture of 3 ml and measuring the OD at 240 nm (Aebi 1984). This assay measures the rate of consumption of H<sub>2</sub>O<sub>2</sub> (ε = 39.4 mM cm<sup>-1</sup>). The reaction mixture for determining ascorbate peroxidase (APX, EC 1.11.1.11) activity consisted of 50 mM potassium phosphate (pH = 7.0), 0.2 mM EDTA, 0.5 mM ascorbate (ε = 2.8 mM cm<sup>-1</sup>), 2 mM H<sub>2</sub>O<sub>2</sub> and 50 µl enzyme extract with a final volume of 3 ml. The absorbance was measured at 290 nm (Nakano and Asada, 1981). Glutathione reductase (GR, EC 1.6.4.2) activity was determined following oxidation of NADPH at 340 nm (Foyer and Halliwell, 1976) in a mixture (final volume of 3 ml) contained 100 mM Tris-HCl (pH = 7.8), 0.5 mM GSSG, 0.03mM NADPH (ε = 6.2 mM cm<sup>-1</sup>), 5 mM EDTA and 100 µl enzyme extract. To determine the activity of superoxide dismutase (SOD), a reaction mixture was used which contained 10 mM of
L-methionine, 0.05 mM of nitroblue-tetrazolium salt and 0.75% of Triton X-100 in 50 mM potassium phosphate pH 7.8 (Beyer and Fridovich, 1987). One ml of the above reagent was added in a glass tube followed by 40 µL of enzyme extract and 10 µL of 0.12 mM riboflavin. After thorough mixing it was put under 40W fluorescent tube for 7 minutes before the measurement of absorbance at 560 nm against a blank kept in dark. The enzyme activity was calculated as the percentage inhibition per min.

3.3.8. Extraction and ascorbate determination

The determination of ascorbate (AsA) and dehydro-ascorbate (DAsA) was carried out by dipyridyl assay that is based on the reduction of Fe³⁺ to Fe²⁺ by ascorbic acid (Kampfenkel et al., 1995). Half g of fresh leaf material was ground to fine powder in liquid nitrogen and homogenized in 2 ml pre-chilled 6% TCA. Mixture was centrifuged at 16000x g for 20 min at 4 °C and supernatant was collected. Ascorbate was determined by adding 0.2 ml supernatant in the reaction mixture, 0.6 ml Na-phosphate buffer (pH, 7.4; 200 mM), 0.2 ml deionized water, 1 ml 10% TCA, 0.8 ml phosphoric acid (42%), 2,2- dipyridyl (4%) and finally adding the freshly prepared 0.4 ml of 3% ferric chloride immediately followed by vigorous mixing. All samples were incubated in water bath maintained at 42 °C for 40 minutes and then 10 mM dithiothertol (DTT) was added to reduce DAsA to AsA. Subsequently excess DTT was removed with 0.5% N- ethyl maleimide (NEM). Absorption of Fe⁺⁺ – dipyridyl complex recorded at 525 nm. Standard curve was prepared for the estimation of total ascorbate (with pretreatment DTT) and DAsA (subtracting AsA from total ascorbate).

3.3.9. Statistical analyses

Data were analyzed by using SPSS 11.0 for Windows and means were compared using Bonferroni test at the 5% level of significance.
3.4. Results

3.4.1. Growth measurement

Plants grown at low salinity (125 mM NaCl) had a 125% increase in leaf biomass and juvenile leaf area and all other growth parameters measured were similar to that of non-treated control (Fig. 3.1 and 3.2). Biomass production of *P. antidotale* along with leaf number, plant height and leaf area linearly declines with the increase in salinity and substantial inhibition was recorded at 500 mM NaCl (Fig. 3.1 and 3.2). Leaf succulence remained unchanged with the increase in salinity.

3.4.2. Water relations, soluble sugar and free proline

Water potential of leaf progressively decreased with increase in NaCl concentrations of the substrate solutions and was about 5 folds higher at 500 mM NaCl compared to control (Fig. 3.4). The water potential of irrigating solutions was lower in entire range of salt treatments in comparison to leaf water potential (Fig. 3.4). Relative water content (RWC) remained unaffected up to 375 mM NaCl and about 17% decrease at 500 mM NaCl was recorded (Fig. 3.4). Sugar concentration was substantially increased at or above 375 mM NaCl (Fig. 3.5). Proline concentration significantly increased in all plant parts at higher salinities and this increase was substantially higher in juvenile leaf followed by stem, adult leaf and root (Fig. 3.6).

3.4.3. Cation, carbon and nitrogen accumulation

Accumulation of cations in *P. antidotale* varied with the level of salinity and the organs (Fig. 3.7). Sodium concentration in plants was higher in all saline treatments but the differences in Na⁺ concentration among salinity treatments was not significant in shoot but progressively increased in roots (Fig. 3.7a).
Fig. 3.1. Growth of *P. antidotale* in response to 0, 125, 250, 375 and 500 mM NaCl concentration. Dry weight of adult leaf, juvenile leaf, stem and root (A) and height of the plant (B). Values represent mean ± SE. Different letters indicate significant difference between treatments at $P < 0.05$ using Bonferroni test.
Fig. 3.2. *P. antidotale* leaf area and leaf number (Adult and Juvenile leaf) changes in response to 0, 125, 250, 375 and 500 mM NaCl concentration. Each value represents the mean ± SE and different letters indicate significant difference between treatments at P < 0.05 after Bonferroni test.
Fig. 3.3. Changes induced by NaCl solutions in the water status, LMA (Leaf mass to area ratio) and succulence in adult and juvenile leaves of *P. antidotale*. Each value represents the mean ± SE and different letters indicate significant difference between treatments at $P < 0.05$ after Bonferroni test.
3.4.4. Cation, carbon and nitrogen accumulation

Accumulation of cations in *P. antidotale* varied with the level of salinity and the organs (Fig. 3.7). Sodium concentration in plants was higher in all saline treatments but the differences in Na\(^+\) concentration among salinity treatment was not significant in shoot but progressively increased in roots (Fig. 3.7a). Sodium chloride at moderate concentration had no influence on K\(^+\) concentrations which increased significantly at 500 mM NaCl in leaves and root in comparison to control plants (Fig. 3.7b). Ca\(^{++}\) and Mg\(^{++}\) showed no significant change in entire range of salt treatments in leaves and stem while it increased significantly (P<0.01) in root at 500 mM NaCl (Fig. 3.7 c and d). Nitrogen content decreased in stem and root but remained unchanged in the leaves at entire range of NaCl treatments (Fig. 3.8). Carbon content was also unchanged (Fig. 3.8). The C/N ratio of leaves was not influenced by salinity but tended to increase a little in root and significantly increased in the stem under NaCl salinity (Fig. 3.8).

3.4.5. Antioxidant enzymes and substrates

Antioxidant enzymes appeared to play a protective role in *P. antidotale* against NaCl salinity (Fig. 3.9). All enzymes remained unchanged at 125 mM NaCl, however, their activity increased substantially with a further increase in salinity and highest activity of all enzymes were recorded at highest substrate salinity (Fig. 3.9). Total ascorbate content increased by 1.8 times in plants exposed to the 500 mM NaCl as compared to the control. Similar trends were also observed with respect to reduced and oxidized ascorbate (Table 3.1). Reduced to oxidized ascorbate ratio was slightly decreased by salinity compared to the control.
Fig. 3.4. Water relations (water potential-$\psi_w$ and relative water content-RWC) was measured on fully expanded (3rd and 4th node) leaves of *P. antidotale* in response to treatment with various NaCl concentrations in the substrate. Values represent mean ± SE and different letters indicate significant difference between treatments at $P < 0.05$ after Bonferroni test.
Fig. 3.5. Changes in the carbohydrate (CHO) induced by NaCl in adult and juvenile leaves of *P. antidotale*. Each value represents the mean ± SE and different letters indicate significant difference between treatments at $P < 0.05$ after Bonferroni test.
Fig.3.6. Proline in Leaf (adult and juvenile leaves), stem and root enhanced under various NaCl salinity (0, 125, 250, 375 and 500 mM) of *P. antidotale*. Each value represents the mean ± SE and different letters indicate significant difference between treatments at P < 0.05 after Bonferroni test.
Fig. 3.7. Changes induced by NaCl solutions in cations (Na⁺, K⁺, Ca++ and Mg++ in A, B, C and D respectively) of leaves (adult and juvenile), stem and root of *P. antidotale*. Each value represents the mean ± SE.
Fig. 3.8. Nitrogen (N), carbon (C) and C/N ratio of *P. antidotale* in response to 0, 125, 250, 375 and 500 mM NaCl concentration. C, N and C/N ratios of leaf (adult and juvenile), stem and root and expressed as % of dry weight in. Values represent mean ± SE.
Fig. 3.9. Antioxidant enzymes of *P. antidotale* in response to 0, 125, 250, 375 and 500 mM NaCl concentrations. Superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione reductase (GR) and catalase (CAT) were measured on fully expended (3rd and 4th node) leaves of *P. antidotale*. Values represent mean ± SE. Different letters indicate significant difference between treatments at P < 0.05 using Bonferroni test.
Table 3.1  Salt induced changes in the ascorbate pool

Leaves of salt stressed plants were frozen in liquid nitrogen immediately after harvesting. Concentrations of ascorbate and dehydro-ascorbate were measured in fresh extracts prepared by suspending ground frozen material in extraction buffer. Data represent analysis of 4 independent repeats. Different letters indicate significant difference between treatments at $P < 0.05$ using Bonferroni test.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>AsA (µmol g$^{-1}$FW)</th>
<th>DAsA (µmol g$^{-1}$FW)</th>
<th>Total Ascorbate (µmol g$^{-1}$FW)</th>
<th>AsA/DAsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.32 ±0.33d</td>
<td>1.42 ±0.08b</td>
<td>3.75 ±0.40d</td>
<td>1.62 ±0.15</td>
</tr>
<tr>
<td>125</td>
<td>3.15 ±0.17bc</td>
<td>2.34 ±0.22ab</td>
<td>5.49 ±0.28c</td>
<td>1.34 ±0.16</td>
</tr>
<tr>
<td>250</td>
<td>3.10 ±0.15b</td>
<td>2.24 ±0.59ab</td>
<td>5.33 ±0.65bc</td>
<td>1.38 ±0.47</td>
</tr>
<tr>
<td>375</td>
<td>3.73 ±0.12ac</td>
<td>2.79 ±0.52a</td>
<td>6.53 ±0.41ac</td>
<td>1.35 ±0.31</td>
</tr>
<tr>
<td>500</td>
<td>3.85 ±0.07a</td>
<td>2.99 ±0.30a</td>
<td>6.85 ±0.30a</td>
<td>1.28 ±0.13</td>
</tr>
</tbody>
</table>
3.5. Discussion

Growth of *Panicum antidotale* remained unchanged at low NaCl concentration (125 mM) however; leaves and height of plants were even stimulated at this concentration. Previous reports mainly indicated an absence of growth stimulation in monocotyledenous halophytes at low salinity (Ahmad et al., 2010; Li et al., 2010; Mateos-Naranjo et al., 2013; Yu et al., 2011; Zhang et al., 2012;) with the exception of a few grasses (Bell and O’Leary, 2003; Glenn, 1987; Muscolo et al., 2013; Shi and Sobhanian et al., 2010; Wang, 2005) and this increase may be smaller when compared to dicotyledonous halophytes (Rozema, 1991). Al-Khateeb (2006) recorded growth stimulation in early seedlings of *P. turgidum* under low salinities (<100 mM NaCl) at temperature regime 10-20 °C and plant could not survive in 400 mM NaCl. However, in the present study the 12 week old plants showed a different response. The adult plants were more resistant as early seedlings and survived even seawater salinity (Fig.3.1).

Primarily, salinity causes osmotic stress that leads to water deficit in plants, one of the major challenges to absorb water (Flowers and Colmer, 2008). Succulence in *P. antidotale* remained unchanged with increase of salinity due to decline in leaf area and leaf number. Plant control loss of water by reducing leaf area and number of leaves which reduces light exposure and maintain leaf turgidity in an acceptable range under salinity.

Additionally, *Panicum* also increased stomatal resistance to minimize the transpiration on the expense of a reduced CO$_2$/H$_2$O exchange rate and the subsequent reduction in biomass production similar to other salt resistant gramineaes (Ahmed et al., 2013; Koyro et al., 2013). The salt induced decrease in leaf water potential of *P. antidotale* was achieved by decrease in leaf water content and by increased
accumulation of ions, mainly $\text{Na}^+$ (Fig. 3.7). $\text{Na}^+$ appears to be one of the major osmotically active solutes and therefore of major importance for the decrease of the leaf water potential of $P. \text{antidotale}$. These results are in agreement with those observed in $\text{Sporobolus virginicus}$ (Bell and O’Leary, 2003), $\text{Odyssea paucinervis}$ (Naidoo et al., 2008), $\text{Posidonia oceanic}$ and $\text{Cymodocea nodosa}$ (Sandoval-Gil et al., 2012) and $\text{Aeluropus lagopoides}$ (Ahmed et al., 2013).

Salt resistant gramineaes commonly use inorganic ions, mainly $\text{Na}^+$ and $\text{K}^+$ for osmotic adjustment (Ahmed et al., 2013; Marcum, 2008; Zhou and Yu, 2009). $\text{Na}^+$ accumulation probably facilitates osmotic adjustment to minimize drastic effects of water deficit under salinity on $P. \text{antidotale}$. However toxic effects of $\text{Na}^+$ could not be avoided completely (membrane leakage, Koyro et al., 2013) in this species at higher salinities. High $\text{Na}^+$ concentration may also lead to imbalances in nutrient uptake and alter the transport of $\text{K}^+$ leading to starvation (Guo et al., 2012; Marcum, 2008). $\text{Na}^+$ influx is mediated through various transport system such as, non-selective cation channel ($\text{NSCC}$) family members, CBL-interacting kinase (CIPK) / calcineurin B-like (CBL) and high-affinity $\text{K}^+$ transporter (HKT) (Zhang et al., 2010; Kronzucker and Britto, 2011). $\text{K}^+$ concentration increased 7 times at high salinity in roots concomitant with increase of $\text{Ca}^{++}$. Increased concentration of $\text{K}^+$ is due to increased potassium utilization efficiencies (KUE; data not shown). Increased $\text{K}^+$ and $\text{Ca}^{++}$ concentration in plants treated with high $\text{NaCl}$ concentration suggests that HKT proteins retained their $\text{K}^+$ affinity (Bafeel, 2013; Corratgé-Faillie et al., 2010; Guo et al., 2012; Hauser and Horie, 2010; Horie et al., 2009; Kurusu et al., 2013; Mian et al., 2011).

Organic solutes like proline, sugar and polyols are produced in response to salinity and drought (Marcum, 2002; Zhou and Yu, 2009). Proline concentration
increased in Panicum treated with high salinities (Fig. 3.6). As the contribution of proline to osmotic adjustment is about 10% (Lee et al., 2008; Marcum, 2002;), therefore it may be concluded that it has other vital functions during hyperosmotic salinity, e.g. acting as a compatible solute (Sharma and Dietz, 2009; Sobhanian et al., 2011), channel regulator (Cuin and Shabala, 2007) or as ROS scavenger (Bohnert et al., 1995; Kaul et al., 2008; Mittler, 2002). Proline biosynthesis is catalyzed by pyrroline-5-carboxlate synthase and it can recycle NADP⁺, an electron acceptor in the photosynthesis reaction which may reduce ROS production and maintain redox homeostasis (Verbruggen and Hermans, 2008). Total soluble sugar (equivalent to sucrose) in leaves of P. antidotale increased linearly, up to 4 fold, in response to increasing external NaCl concentrations (from 0 to 500 mM). This response of Panicum is in agreement with other reports (Li et al., 2010; Williamson et al., 2002). Synthesis of organic osmolytes is a high energy requiring process (Ashraf and Foolad, 2007) therefore halophyte grasses use inorganic ions (below toxic level) for cheap osmolytes (Lee et al., 2007; Marcum, 2002). This suggests that carbohydrate (e.g. sucrose) synthesis is related to the ionic burden. On the other hand, it plays an important role not only as osmolyte but it also acts as a signaling molecule (Noctor and Foyer, 1998). Sucrose induces a micro RNA (miR398) that is considered to be a sucrose modulated regulator of SOD translation (Couee et al., 2006).

Carbon and nitrogen content is commonly related with contents of photosynthetic pigments and proteins (Rubisco) which can be utilized as a measure of stress index (Chapin, 1980; Ignatova et al., 2005). Present data showed that C:N-ratios were not influenced by NaCl while growth was reduced and redox status (e.g. AsA/DAsA redox couple) remained constant under saline conditions. AsA/DAsA redox couple is well known modulator of enzymes responsible for carbon assimilation.
under stress condition (Kocsy et al., 2013). Hence, in present study, this redox couple also triggers carbon assimilation in Panicum to some extent under salt stress. Therefore, the significant suppression in photosynthesis is the main cause of growth reduction in P. antidotale (Capter 2; Koyro et al., 2013).

Osmotic adjustment and compartmentalization of toxic ions are important adaptations in plants at high salinity, but salinity also induces oxidative stress (secondary stress) as a consequence of reduced fixation of CO₂ (Hernandez et al., 1999; Jubany-Mari et al., 2010; Sobhanian et al., 2011) leading to structural damage of both membranes and photosynthetic apparatus due to production of reactive oxygen species (ROS; O₂⁻, OH⁻, H₂O₂) (Koyro et al., 2013). The production of antioxidant compounds (ascorbate, glutathione etc.) as well as of antioxidant enzymes (SOD, APx, GR and CAT) belongs to a well-established antioxidant system to prevent the accumulation of ROS to toxic level in plant cells (Halliwell et al., 1995; Foyer and Noctor, 2009). Present data showed that with increasing salinity antioxidant system response increased to scavenge ROS clearly supporting the hypothesis that antioxidant defense system (e.g. enzymes) switched on under oxidative stress caused by NaCl. SOD activity increased significantly in the leaves of P. antidotale with increased salinity (Fig. 2.9). This increase in SOD level is in agreement with previous reports (Briggs et al., 2013; Hu et al., 2011; Modarresi et al., 2012; Sobhanian et al., 2011; Yang et al., 2012a) and disagree with some other reports indicating no response to hyperosmotic salinity (Sekmen et al., 2007).

About 2 fold increased concentration of H₂O₂ (Koyro et al., 2013) in high salinity is tended to support the enhanced activity of SOD in Panicum. Although H₂O₂ is a toxic molecule it also plays a very important role in signaling (Gondim et al., 2012, Maruta et al., 2012). The production of H₂O₂ due to hyperosmotic salinity
triggers also the activation of the enzymatic (catalase, ascorbate peroxidase and glutathione reductase) and/or non-enzymatic (ascorbate, glutathione, tocopherol, carotenoids etc) antioxidant system as shown in this present study. In particular, APx plays a key role in removal of $\text{H}_2\text{O}_2$ from chloroplast and cytosol (Mittova et al., 2000) and CAT responsible for $\text{H}_2\text{O}_2$ removal from peroxisomes and the cytosol (Frederick and Newcomb, 1969; Hilliard et al., 1971). In the present study, the CAT activity increased significantly with an increase in salinity (Fig. 3.9). Present results are in agreement with results reported for *Pennisetum clandestinum* (Muscolo et al., 2013), *Spartina alterniflora* (Subudhi and Baisakh, 2011), *Echinochloa crusgalli* (Abogadallah et al., 2010). But opposite results are reported from experiments with *Lolium perenne* (Hu et al., 2011); while no response of CAT activity was reported for *Aeluropus lagopoides* (Sobhanian et al., 2010). APx and GR are key enzymes in the Halliwell-Asada pathway and play a critical role in removal of indigenous $\text{H}_2\text{O}_2$ (Noctor and Foyer, 1998; Duarte et al., 2013). In this study, the APx and GR activities in *P. antidotale* were increased up to 4 and 5 folds respectively under NaCl salinity. APx uses ascorbate (AsA) as reductant substrate to convert $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and dehydro-ascorbate (DAsA) (Asada, 1992). The increased GR activity in plant under saline conditions would increase the $\text{NADP}^+$/NADPH ratio. It demonstrates that $\text{NADP}^+$ is readily present to accept electrons from PSII electron transport chain, by this means minimizing ROS accumulation in chloroplasts (Muscolo et al., 2003).

The increased activities of these keys enzymes can be interpreted as a high demand and capacity to eliminate $\text{H}_2\text{O}_2$ in the leaf of *P. antidotale* under saline conditions. Besides Halliwell-Asada pathway enzymes, ascorbate (oxidized and reduced) and glutathione (oxidized and reduced) substrate can directly scavenge superoxide, hydroxyl radicals, and singlet oxygen (Foyer and Halliwell, 1976; Foyer...
and Noctor, 2009; Skladanka et al., 2012). It can also act as an enzyme cofactor in violaxanthin de-epoxidase in xanthophyll cycle (Fedoroff, 2006; Noctor, 2006) and protect plants against oxidative stress (Fourquet et al., 2008). The increased level of ascorbate in P. antidotale is likely to participate not only as a substrate for APx but also as a ROS scavenger and signaling molecule to trigger the production of abscisic acid (ABA) (Jubany-Mari et al., 2010) and closing of stomata (Chen and Gallie, 2004). Decline in transpiration and stomatal conductance in P. antidotale (Koyro et al., 2013) is also supporting the same role of ascorbate with an increase of NaCl concentration in the soil. The decline in transpiration in often correlated with water storage in stress condition that analogous to increase in ascorbate and associated to minimize the premature senescence (Barth et al., 2004; Maggio et al., 2002). Along with all these physiological adaptations due to increase in ascorbate, redox homeostasis (e.g. AsA / DAsA) is one of the important factors to save cell from ROS toxicity (Foyer and Noctor, 2009). It can be speculated that this ratio or the availability of AsA may even be the detector of the extent of oxidative stress and regulator of the plant defense system (Kocsy et al., 2013). In the present study, Panicum kept constant AsA / DAsA ratios in the entire saline treatments (Table 3.1) but at higher salinities (Koryo et al., 2013) it is quite expensive to maintain this redox balance and, therefore, reduction in growth observed in P. antidotale.

Results clearly indicate that in present experimental conditions, the antioxidant system of P. antidotale is capable of controlling ROS stress throughout the tested spectrum of salt stress and therefore not a limiting factor. Growth inhibition is probably due to high energy cost in the synthesis of organic solutes (proline and sugars) and up regulation of the anti-oxidative system to detoxify ROS up to sea water salinity. Concentration of essential minerals, K⁺, Ca ++ and Mg ++ and homeostasis in
Na\(^+\) in the cytoplasm were maintained under saline conditions are vital for metabolic functions in *P. antidotale*. Potassium regulates the plant anti-oxidative response by increase in anti-oxidative enzymes activity and the accumulation of non-enzymatic antioxidants, especially ascorbate in well redox homeostasis. Therefore, these results recommend a sustainable utilization of *P. antidotale* in arid or semiarid regions, where salinity is often the common major abiotic stress for plants.
References


CHAPTER 4

VARIATION IN CHLOROPHYLL$_{A}$ FLUORESCENCE AT DIFFERENT POSITIONS ON THE LEAF OF *PANICUM ANTIDOTALE* WITH TIME UNDER SALINITY
4.1. Abstract

Photosynthetic apparatus showed variable response to salinity and variation in cell differentiation at various positions of leaf lamina. Monocot leaves have a gradient of cell differentiation, with young cells located closer to base while mature cells at the top. The aim of present experiment was to test a hypothesis “salt induced injury is higher at the tip of leaf lamina” on a potential cash crop Panicum antidotale, a C₄ perennial halophytic grass. Virtually leaf was divided into 5 segments and analyzed each segment separately under salt stress (400 mM NaCl) every day up to 15 days. Chlorophyll fluorescence parameters; maximum quantum yield (Fv/Fm), actual quantum yield (PSII), non-photochemical quenching (NPQ) and electron transport rate (ETR) were measured on plants grown in a greenhouse. Plants were harvested daily (up to 15 days) and biomass was recorded. Fresh and dry weight of Panicum decreased under salt stress. This inhibition of growth had become more pronounced after six days. Chlorophyll fluorescence (as a function of photosynthesis) decreased under salt stress due to change in both time and space and leaf top segments have been affected about 20 times more than base segments under salt treatment. This inhibition may result in reduction of energy flow from PSII often caused the production of oxidative stress. Therefore, MDA and ion leakage were also determined, as indicator of oxidative stress. MDA content and damage to PSII increased simultaneously under salinity. This study indicates that the change in chlorophyll fluorescence under salt stress on the leaves of P. antidotale is a function of spatial and temporal variation. Future investigation in detail mechanisms of photosynthesis with emphasis of variation in a leaf cell differentiation and time factor will be follow up of this study.
4.2. Introduction

High concentration of ions (Na\(^+\) and Cl\(^-\)) damages biochemical, physiological and photochemical process and consequently results in decline of plant biomass (Flowers and Colmer, 2008). Photosynthesis is sensitive to smaller variation in environmental factors and often the main cause of growth inhibition under salinity (Koyro et al., 2013; Redondo-Gómez et al., 2010), through the biochemical limitation (Rubisco activity and photosynthetic pigments), damage to PSII, or/and stomatal closure (Ahmed et al., 2013; Koyro et al., 2013). Photosynthetic carbon harvesting under salinity is also reduced by reduction in photosynthetic pigments (Benzarti et al., 2012; Naidoo et al., 2008) because salinity inhibits biosynthetic pathway of chlorophyll (Tezara et al., 2003; Wang et al., 2013; Le Dily et al., 1993; Soussi et al., 1998). It minimizes the risk of reactive oxygen species (ROS) production by decreasing the flow of electron through photosystems besides decline in photosynthesis (Geissler et al., 2009). Hyper-saline condition reduces efficiency of light energy absorption leading to the production of ROS (Foyer and Noctor, 2009) which may damage PSII reaction center (Redondo-Gómez et al., 2010). In this state of excessive light, plant forced to release excessive energy as heat and chlorophyll fluorescence (Martínez-Peñalver et al., 2011). This dissipation of energy is only about 2-5% but this small change in fluorescence values may remarkably influence plant photochemistry (Baker, 2008). Therefore, apart from the classical CO\(_2\)/H\(_2\)O gas exchange measurements as the photosynthetic performance, chlorophyll fluorescence has widely used method to analyze the function of photosynthetic apparatus and is a powerful tool to study plant response to abiotic stresses (Maxwell and Johnson, 2000).

Salinity induced changes in PSII level has been tested primarily without considering the heterogeneity of growing leaf anatomy (Chaerle et al., 2009). Several
authors determined chlorophyll fluorescence values; Fv/Fm (maximal PSII quantum yield), PSII (effective PSII quantum yield), qP (photochemical quenching), NPQ (non-photochemical quenching- energy dissipation) and ETR (electron transport rate) etc. under various stress conditions but these measurements have been recorded on a single fixed area of the leaf assuming that it is the response of whole leaf or a plant (Maricle et al., 2007; Naidoo et al., 2008; Zhao et al., 2007). However, there are some reports about the heterogeneity of stress index with respect to age and location of the growing plant (Ahmed et al., 2013; Chaerle et al., 2009; Martínez-Peñalver et al., 2011; Oxborough, 2004; Zribi et al., 2009). Therefore, it needs to study the dynamics of stress at both special and temporal level by taking advantage of non-invasive chlorophyll fluorescence technique. Monocot leaves have a gradient of tissue differentiation, with young undifferentiated tissues are to be found near the leaf base while mature or even senescent tissues are located at the leaf top. Leaves of *P. antidotale* therefore divided into 5 segments and the analyses were conducted on each of these segments separately at different time scales.

*Panicum antidotale* could be a potential economical candidate for a sustainable production system due its inherent ability to survive in up to sea level (500 mM NaCl) salinity (Chapters 2 & 3; Khan et al., 2009; Koyro et al., 2013). The purpose of this experiment was to establish a rationale of spatial and temporal effects considering the heterogeneity of cell differentiation and age of the *Panicum* leaves during the growth during. Therefore, non-invasive chlorophyll fluorescence parameters, Fv/Fm, PSII and ETR, were discussed along with chlorophyll and malondialdehyde (MDA) content during the growth of *Panicum* under NaCl concentration.
4.3. Materials and methods

4.3.1. Growth culture

Seeds of *Panicum antidotale* (collected from a mono-specific stand located near Hub, Pakistan, 24°58’17.28” N and 66°46’33” E) were germinated on substrate TKS1 (Floragard, Oldenburg, Germany) in growth chamber at 14h day light period at 25 °C. Ten day old seedlings were transferred to a green house and were left to acclimatize for 15 days at temperature of 24°C/20°C, humidity about 60% and light for 16 h per day to obtain a constant quantum fluence rate of 300 µmol m⁻²s⁻¹ and when necessary, additional light (sodium vapor lamps, SON-T Agro 400, Philips, Hamburg, Germany) was also supplied. Seedlings (4 leaf stage) were then transferred to plastic pots (20 cm by 25 cm) filled with sand. The plants were irrigated with a basic modified nutrient solution (Epstein, 1972). The stepwise addition of NaCl to the nutrient solution began after a period of 2 weeks by raising salinity of the solution in steps of 50 mM NaCl each day until the final concentration was achieved; 0 (control), 100, 200, 300 and 400 mM NaCl. The water was added daily to correct for evaporation. Solutions were recycled and changed every week to avoid nutrient depletion. The experiment continued for a period of 10 weeks. There were two set of experiments with the first set with salinity regime of 0 (control), 100, 200, 300 and 400 mM NaCl and contained 4 replicates per treatment for harvest after 15 days of salinity completion and the second set (spatiotemporal) which contained 0 (control) and 400 mM NaCl only with 3 replicates per treatment harvested each day after completion of salinity for 15 days.

4.3.2. Growth measurement

Plants were harvested after 15 day as well as daily on both set of experiments. Plants were divided into shoot (above ground) and root (underground). Fresh weight was measured immediately after the harvest while dry weight was determined after drying.
in oven at 70 °C till the constant weight obtained. The shoot height was recorded after 15 day in first set of experiments and daily on the spatiotemporal set of experiment and data expressed on per plant basis.

4.3.3. Chlorophyll estimation and chlorophyll a fluorescence measurement

Concentration of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll were analyzed by (Lichtenthaler, 1987) on both set of experiments. 0.1g of leaf discs were collected from the third nodes of the shoot tip. The leaf samples were homogenized in motor and pestle and added 10 ml of 80 % acetone. The concentrations of Chl a, Chl b and total chlorophyll were measured using an UV-visible spectrophotometer (Beckman Coulter - DU® 500 UV/Vis Spectrophotometer) at 663.2, 646.8 and 470 nm using quartz cuvette respectively. A solution of 80 % acetone was used as a blank. The Chl a, Chl b and total chlorophyll concentrations in the leaf tissues were calculated according to the following equations;

\[
Chla = 12.25 \times A_{663.2} - 2.79 \times A_{646.8}
\]

\[
Chlb = 21.50 \times A_{646.8} - 5.10 \times A_{663}
\]

\[
Chltotal = 7.15 \times A_{663} - 18.71 \times A_{646.8}
\]

Pulse modulated chlorophyll fluorescence meter (Junior PAM, Walz, Germany) was used to determine chlorophyll a fluorescence. The minimal fluorescence (Fo) value was measured after applying modulated light (< 0.1μmol photon m⁻² s⁻¹) on dark adapted (20-25 min) leaf, while the maximal fluorescence (Fm) value was obtained by imposing a saturating pulse of 10,000 photons (μmol m⁻² s⁻¹ for 0.6 s). Fo and Fm values were used to calculate maximum photochemical quantum yield of PSII (Fv/Fm = Fm-Fo/Fm). Subsequently, the leaves were illuminated continuously with actinic light, which was equivalent to the actual growth
light of plants in order to measure steady-state (Fs) and maximal fluorescence (Fm’) in light-adapted leaves. The minimal fluorescence level in light-adapted leaves (Fo’) was estimated following the method of (Baker and Rosenqvist, 2004). Effective photochemical quantum yield of PSII was calculated as Fm’-Fs/Fm’ (Genty et al., 1989). Non-photochemical quenching of fluorescence (NPQ) which is proportional to the rate of constant heat dissipation (Bilger and Björkman, 1990), was calculated as NPQ = Fm/Fm’-1. The coefficient of photochemical quenching (qP) was calculated as (Fm’-Fs) / (Fm’-Fo’) (Kooten and Snel, 1990; Schreiber et al., 1986). PSII is used for calculation of the linear electron transport rate (ETR) (Krall and Edwards, 1992) as 

\[
ETR = \text{PSII} \times \text{PPFD} \times 0.5 \times 0.84,
\]

where Photosynthetic Photon Flux Density (PPFD) incident on the leaf; 0.5: factor that assumes equal distribution of energy between the two photosystems; 0.84: assumed leaf absorbance. Spatial and temporal salt stress effects on chlorophyll a fluorescence in *Panicum* leaves were also measured. In this context, I took advantage of the fact that in monocot leaves there is a gradient of cell differentiation, with young undifferentiated cells preferentially to be found near the leaf base while mature or even senescent cells are located at the leaf tip. I have therefore divided (virtually) leaves into 5 segments of about 4 cm each (Fig. 3) and have analyzed these segments separately with same settings (as mentioned above) of pulse modulated chlorophyll fluorescence meter (Junior PAM, Walz, Germany).

### 4.3.4. Determination of electrolyte leakage and MDA content

Electrolyte leakage and malondialdehyde (MDA) content were measured daily on spatiotemporal experiment in the 3rd and 4th leaf from the top. Leaf discs (1 cm dia., 3 replicates) were incubated in 3 ml of deionized water in a desiccators under a negative pressure of ≈10 bar for 60 minutes. Total conductivity measured after boiling the sample at 95 °C for 2 h. The electrolyte leakage was calculated as percentage of
total conductivity (Dionisio-Sese and Tobita, 1998). Malondialdehyde (MDA) content as an indicator for the degree of lipid peroxidation of membranes was measured using thiobarbituric acid (TBA) assay (Cakmak and Marschner, 1992). Fresh leaf (0.1g) was ground with pre chilled pestle in a solution of 0.5% TBA in 20% trichloroacetic acid (TCA) followed by heating at 95 °C for 30 minutes. Samples were cooled at room temperature before centrifuging it for 5 minutes at 3000 rpm. The absorbance of supernatant was recorded at 532 nm (original OD), OD of the non-specific absorbance as taken at 600 nm and subtracted from the original OD (Cakmak and Marschner, 1992).
4.4. Results

4.4.1. Foundation experiment

*Panicum antidotale* biomass, fresh weight, height and chlorophyll content did not differ significantly compared to non-saline control but all parameters inhibited significantly (P<0.001) with increase in salinity (Figs. 4.1 & 4.2). Little change in Fv/Fm, PSII and ETR was recorded in up to 200 mM NaCl and decreased 22%, 24% and 24% respectively at 400 mM NaCl in comparison of control plants. NPQ declined with an induction of low salinity (100 mM NaCl) but it remained constant in entire range of NaCl treatments.

4.4.2. Spatiotemporal experiment

Fresh weight of control plants increased 24 times in 15 days compared to 400 mM NaCl treated plants (Fig. 4.4) however difference in dry weight was only 9 times during the same period (Fig. 4.4). Shoot height increased 2 times on day 5 and >5 times on day 15 in control plants compared to 400 mM NaCl (Fig. 4.4).

Significant (P < 0.001) change in chlorophyll content was measured with time and salinity (400 mM NaCl; Fig.4.6). The chlorophyll content (Chl a, Chl b and total Chl) initially did not change while after day 7 the difference increased to 2 times and reached about 4 times on day 15 between control and salt treated plants. Chlorophyll fluorescence parameters declined significantly only at higher salinity (Table 4.1).

Spatial and temporal measurements of chlorophyll a fluorescence in *Panicum* leaves were affected significantly under salt stress (Fig. 5.5). Fv/Fm did not change up to day 8 and decreased significantly from the segments 4 and 5 at day 9. Fv/Fm values at leaf base segments (1 and 2) and top segments (3, 4 and 5) declined about 2 times and about 25 times respectively with further passage of time up to day 15 under saline conditions.
Fig. 4.1. Growth of *P. antidotale* in response to 0, 100, 200, 300 and 400 mM NaCl concentration. Fresh weight of shoot, root and height of shoot. Values represent mean ± SE. Different letters indicate significant difference between treatments at $P < 0.05$ using Bonferroni test.
Fig. 4.2. Photosynthetic pigments (Chl a, Chl b and total Chl) in the leaves of *P. antidotale* in response to 0, 100, 200, 300 and 400 mM NaCl concentration. Values represent mean ± SE. Different letters indicate significant difference between treatments at P < 0.05 using Bonferroni test.
Table 4.1. Fluorescence parameters (Fv/Fm, PSII, NPQ and ETR) measurements on 3\textsuperscript{rd} and 4\textsuperscript{th} node’s leaf of \textit{P. antidotale} in response to 0, 100, 200, 300 and 400 mM NaCl concentration. Values represent mean ± SE. Different letters indicate significant difference between treatments at P < 0.05 using Bonferroni test.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Fv/Fm</th>
<th>PSII</th>
<th>NPQ</th>
<th>ETR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.766\textsuperscript{a} ±0.006</td>
<td>0.446\textsuperscript{a} ±0.024</td>
<td>1.006\textsuperscript{a} ±0.047</td>
<td>53.42\textsuperscript{a} ±2.906</td>
</tr>
<tr>
<td>100</td>
<td>0.752\textsuperscript{a} ±0.001</td>
<td>0.450\textsuperscript{a} ±0.015</td>
<td>0.763\textsuperscript{b} ±0.012</td>
<td>53.85\textsuperscript{a} ±1.787</td>
</tr>
<tr>
<td>200</td>
<td>0.718\textsuperscript{ab} ±0.013</td>
<td>0.432\textsuperscript{a} ±0.026</td>
<td>0.761\textsuperscript{b} ±0.005</td>
<td>51.65\textsuperscript{a} ±3.148</td>
</tr>
<tr>
<td>300</td>
<td>0.680\textsuperscript{bc} ±0.012</td>
<td>0.356\textsuperscript{ab} ±0.048</td>
<td>0.758\textsuperscript{b} ±0.013</td>
<td>42.65\textsuperscript{ab} ±5.776</td>
</tr>
<tr>
<td>400</td>
<td>0.599\textsuperscript{c} ±0.034</td>
<td>0.338\textsuperscript{b} ±0.016</td>
<td>0.759\textsuperscript{b} ±0.004</td>
<td>40.40\textsuperscript{b} ±1.892</td>
</tr>
</tbody>
</table>
Fig. 4.3. *Panicum* leaf showing various segment positions from bottom to apex.
conditions. PSII efficiency remained constant up to day 5 on the base segments but declined significantly on the top segments of leaf. The reduction in PSII efficiency became rapid with temporal as well as spatial basis. The difference of PSII on top segments (4 and 5) was about 5 and 15 times while 2 to 3 times on the base segments (1 and 2) on day 10 and 15 respectively. The ETR reduced about 5 and 14 times at the top segments on day 10 and 15 respectively although ETR in the base portion was reduced about 3 times.

Stress markers, ion leakage and MDA were increased significantly with temporal basis under salinity (Fig. 4.7). The difference of ion leakage between control and 400 mM NaCl treated plants were 2, 3 and 6 times on days 5, 9 and 15 respectively (Fig. 4.7). MDA content was much lower and late response as compare to ion leakage that was 1.5 times and 3 times on days 9 and 15 respectively (Fig. 4.7).
Fig. 4.4. Growth of *P. antidotale* in response to NaCl (0 and 400 mM) to expressing the variation due to time (Days). Fresh and dry weight of shoot, root and height of shoot. Values represent mean ± SE.
Fig. 4.5. Fluorescence parameters (Fv/Fm, PSII and ETR) measurements on 3rd and 4th node’s leaf of *P. antidotale* in response to salinity (0 and 400 mM NaCl), time course (days) and various leaf segments (top to bottom). Values represent mean ± SE.
Fig. 4.6. Photosynthetic pigments (Chl a, Chl b and total Chl) in the leaves of *P. antidotale* in response salinity (0 and 400 mM NaCl) and time course (days). Values represent mean ± SE.
Fig. 4.7. Stress marker (Ion leakage and MDA content) in the leaves of *P. antidotale* in response salinity (0 and 400 mM NaCl) and time course (days). Values represent mean ± SE.
4.5. Discussion

Net photosynthesis is reported to decline through reduction in carboxylation capacity due to down regulated Rubisco protein in *Panicum antidotale* under saline conditions (Chapter 2; Koyro et al., 2013). This reduction of CO₂ assimilation in Calvin cycle would considerably decreased ATP and NADPH utilization. The continued flow of electron due to continuous light would have to be involved in alternate electron sink pathways as well as the production of ROS. Therefore, photoinhibition (photodamage) was also observed in *Panicum* because altering in photochemical requirements of PSII (Fv/Fm measurements) and risk of ROS production increased. Similarly, results for maximum efficiency of PSII (Fv/Fm), in present study, also confirmed the damage of PSII during NaCl treatments.

The purpose of the present experiment was to determine the effect of time and position on leaf (reflecting different degree of cell differentiation) on the chlorophyll fluorescence under NaCl concentrations. Decreased maximum PSII efficiency (Fv/Fm) was recorded after day 8 and values were also reduced progressively with passage of time. Chlorophyll contents decreased significantly during the same period with concomitant decline of PSII efficiency and electron transport (Baker, 2008). Several studies have shown similar correlation between PSII and chlorophyll content with temporal changes (Benzarti et al., 2012; Martínez-Peñalver et al., 2011).

The Fv/Fm decreased substantially at the top (segment 4 and 5) of leaf lamina along with chlorosis symptoms (Fig. 4) while delay in chlorosis as well as less destruction of PSII machinery was observed in base of the leaf cells (Donnini et al., 2013; Raschke et al., 1990; Sperdouli and Moustakas, 2012).

Actual PSII efficiency (PSII) of *Panicum* was significantly affected by both salinity and spatiotemporal variation. A strong decrease in PSII was recorded on the
top portion (segment 4 and 5) as compare to base (segment 1 and 2). Therefore, the decreased actual PSII efficiency might be due to lower photochemical quenching as a result of reduced chlorophyll content. Under stress conditions, generally, the ratio of open to closed reaction centers showed a discrepancy and therefore capacity of electrons transfer may also be diminished. Such results were also reported by other authors (Jajoo, 2013; Naidoo et al., 2012; Sperdouli and Moustakas, 2012; Zheng et al., 2009) which indicated a reduction in PSII due to increase in the amount of closed reaction centers and ultimately failure to carry out electron transport reactions.

Photochemical mechanisms like electron transport rate and quinone pool (oxidation and reduction states) are reported to affect photosynthetic rate under salinity (Kalaji et al., 2011). The electron transport rate also registered a substantial decline with time and position of Panicum leaf. The ETR suffered significant decline when recorded in old tissues (segments 4 and 5) compared to the young tissue near the base of leaf (segments 1 and 2). Reduction in ETR is highly correlated with other parameters of fluorescence causing increased accumulation of excessive energy due to reduction in flow of electrons. This excessive energy has probably negative impacts on energy trapping either by dissipating as heat/fluorescence or damaging D1 protein (Kreslavski et al., 2013; Moradi and Ismail, 2007). NPQ values did not change in leaf of Panicum as reported before (Redondo-Gómez et al., 2010; Tezara et al., 2003; Zribi et al., 2009), however studies that observed change also exist (Benzarti et al., 2012; Duarte et al., 2013; Mateos-Naranjo et al., 2008; Naumann et al., 2008). Therefore, in this case, excessive energy might be lethal as it was not dissipated as heat in Panicum during salt stress. This absorbed (but un-dissipated) energy probably harmful for the plant and can result in the formation of triplet state of chlorophyll. That was probably leading to consequent generation of ROS which ultimately damage
PSII proteins (D1 protein) and thus the oxidative stress is observed. Lipid peroxidation (malonyldialdehyde; MDA) is widely used as a measure of oxidative stress (Xu et al., 2013). Damage in cell membrane permeability (ion leakage; Fig. 8), increased during NaCl treatment in *Panicum*, indicating that the observed photoinhibition or photodamage was due to ROS production. Cell membrane damage is often associated with growth inhibition under salt stress (Huang et al., 2001; Xu et al., 2013). On temporal scale, MDA content and ion leakage were increased linearly up to 5 day after the treatment and increased up to 6 fold in NaCl (400 mM) treated plants compared to control at day 15. Similar evidence of MDA and ion leakage due to ROS production has been reported by several researchers (Ahmed et al., 2013; Hu et al., 2012; Pérez-López et al., 2010; Radić et al., 2013; Seckin et al., 2010).

In conclusion, present study indicated that highly differentiated responses of salt stress on *Panicum* leaf lamina on both temporal and spatial levels. An earlier and severe magnitude of reduced photosynthesis (fluorescence parameters) was observed at the top of the leaf as compared to the base of the leaf under NaCl salinity. Therefore, this study provokes to reconsider the experimental designs which are based only on single point study for determining stress.
References


CHAPTER 5
GENERAL CONCLUSION
*Panicum antidotale* has a considerable potential to utilize as a fodder crop in saline areas where conventional crops usually fail to grow therefore this study was designed to understand salt resistance responses of this halophytic grass. Optimal growth was observed at 125 mM NaCl indicating that *P. antidotale* has efficient mechanisms such as osmotic adjustment, ions homeostasis, ROS equilibrium and photosynthesis. Decreased growth at salinities exceeding 125 mM NaCl could be linked with high energy costs for osmotic adjustment by synthesis of organic osmolytes (such as proline and sugar) and ion homeostasis. Reduced growth is an adaptive feature of *P. antidotale* to survive in up to 500 mM NaCl because it allows plants to rely on multiple resources (e.g. energy) to avoid or reduce the stress. The ability to tolerate water deficit (due to salt) in *P. antidotale* was achieved by high stomatal resistance to minimize the transpiration rather limiting CO₂ uptake. The salt induced decrease in leaf water potential was also achieved by decreased water content and increased accumulation of ions, mainly Na⁺. This demonstrates adaptability of test species in lower soil water potential. However, the toxic effects of Na⁺ accumulation such as membrane leakage could not be avoided completely. High Na⁺ in the medium disturbs the uptake and transport of K⁺ leading to K⁺ starvation and/or [K⁺]/[Na⁺] imbalance. It can inhibit plant growth whereas in *Panicum*, K⁺ concentration increased at high salinity concomitant with increase of Ca²⁺. Increased concentration of K⁺ may be due to increased potassium utilization efficiencies (KUE) which also suggests that HKT proteins retained their K⁺ affinity. Above parameters articulate that plant has well regulated ion homeostasis under saline soil. Increased activities of antioxidant enzymes and levels of antioxidant substrates (e.g. oxidized and reduced ascorbate) helped to maintain redox status. However, with increasing NaCl concentration, these protective mechanisms seemed in-sufficient to protect against ROS toxicity which
caused photo-inhibition and ultimately damage photosynthetic machinery. Additionally, chlorophyll fluorescence measurements established a rationale of spatial and temporal variable responses considering the heterogeneity of cell differentiation and age of leaves during the growth under NaCl. By studying different eco-physiological attributes of *P. antidotale*, this thesis contributes to the insight of the salt tolerance mechanisms and the development of saline agriculture in future.