Responses of *Suaeda maritima* to flooding and salinity

By

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Declaration

The contents of this thesis are the original work by the author, except where otherwise stated.

I hereby declare that this has not and will not be submitted in whole or in part to any other university for the award of any other degree.

Signature…….Gazala Alhdad

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RESPONSES OF SUAEDA MARITIMA TO FLOODING AND SALINITY

ABSTRACT

Suaeda maritima is an annual halophyte commonly found in salt-marshes. Its salt tolerance has been well studied, though there is little published on the effect of simultaneous waterlogging. The effects of saline waterlogging on growth, antioxidants (glutathione and total polyphenolic compounds, antioxidant activity) and oxidative damage were investigated with simulated tides in a controlled glasshouse and on plants collected from the field. Flooded shoots possessed higher levels of antioxidants than those from plants growing in well-drained situations, in the glasshouse and the field. The effects of hypoxia, (simulated in nutrient solution by flushing with nitrogen in a solution containing a low concentration of agar, which limits convection within the solution and so the transport of oxygen from the air) were determined on growth and trace metal concentrations, in plants grown in different concentrations of artificial seawater (100 and 350 mM Na^+ at low pH, > pH 5.5), in sand/mud irrigated with half-strength fresh seawater (at high pH, ca 7-8) and in different concentrations of manganese and iron in solution culture. High salt concentration reduced accumulation of trace metals in plants. Optimal growth occurred in 14 μM Fe and 1 mM Mn. Accumulation of trace metals was reduced at high pH, with more accumulating in the roots than the shoots. Hypoxia increased soluble sugars in shoots and roots, and this was affected by the salt concentration. Hypoxia also caused adventitious root development in hydroponic experiments, while in sand, adventitious root development was greater in drained than flooded conditions. Hypoxia significantly reduced shoot sodium concentration, sodium flux and bypass flow, at low and high salt concentrations. In high salt conditions, *S. maritima* reduced its transpiration rate and improved its water use efficiency. It was also shown that the roots contained high lactate concentrations under aerated and hypoxic conditions. *S. maritima* demonstrated many adaptations for tolerating extreme hypoxia.

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Contents

Declaration ..... **ii**
ABSTRACT ..... **iii**
Acknowledgements ..... **iv**
List of publications ..... **vi**
List of Figures ..... **xii**
List of Tables ..... **xviii**
Abbreviations xxi
Chapter 1 : General introduction 1
1.1.2 The effects of salinity on plants with an emphasis on halophytes 2
1.1.2.1 Growth 2
1.1.2.2 Osmotic effects 4
1.1.2.2.1 Osmotic adjustment, ion compartmentation 5
1.1.2.2.2. Compatible solutes 5
1.1.2.3 Effects on mineral nutrition 6
1.1.2.4 Antioxidants 8
1.1.2.5. The pathways of Na^+ uptake in halophytes 9
1.1.2.5.1 Apoplastic pathway (Bypass flow) 10
1.1.2.5.2 Symplastic pathway 11
1.2 Tolerance to flooding 13
1.2.1. Adventitious root 13
1.2.2 Aerenchyma and internal O_2 movement 14
1.2.3 Anoxia tolerance 17
1.3 The effects of combination of waterlogging and salinity on plants with an emphasis on halophytes 18
1.3.1 The halophyte *Suaeda maritima* 18
1.4 Research objectives 20
Chapter 2 : The effect of combined salinity and waterlogging on the halophyte *Suaeda maritima*: the role of antioxidants 23
2.1. Introduction 23
2.2 Materials and Methods 25
2.2.1. Plants materials and culture conditions 25
2.2.2 The tidal flow tank system 26
2.2.3 Glutathione Analysis 30

2.2.3.1. Glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) 31
2.2.4. Lipid peroxidation 32
2.2.5. Preparation of plant extracts for determination of total phenolic compounds and antioxidant activities 32
2.2.5.1. Determination of total phenolic content 33
2.2.5.2. DPPH radical scavenging activity 33
2.2.5.3 Superoxide anion radical scavenging assay 34
2.2.6 Analysis of data 35
2.3 Results 35
2.3.1 Dry weight 35
2.3.2. Glutathione content 35
2.3.3. MDA 36
2.3.4. Polyphenol content 37
2.3.5. DPPH and superoxide anion scavenging activity 37
2.4 Discussion 38
Chapter 3 : Uptake of trace metals under a combination of waterlogging and salinity in *Suaeda maritima* 43
3.1 Introduction 43
3.2 Materials and Methods 48
3.2.1 Germination and growth of seedlings 48
3.2.2 Experiment 1: The effect of different concentrations of artificial seawater on ion uptake, in aerobic and hypoxic conditions, under controlled conditions in a growth cabinet 48
3.2.3 Experiment 2: The effect of tidal movements in the tank system on ion uptake in *S. maritima* plants in the glasshouse 49
3.2.3.1`Soil investigations 49
3.2.4 Experiments 3 and 4: The response of *Suaeda maritima* to varying concentrations of iron and manganese under aerobic and hypoxic conditions 49
3.2.2.1 Physiological parameters 52
3.2.2.1.1 Fresh and dry weight determination 52
3.3 Statistical analysis 52
3.4. Results 52
3.4.1. Trace metals in *Suaeda maritima* plants grown under aerated and hypoxic conditions, in different concentrations of artificial seawater in a growth cabinet 52

3.4.1.1 Shoot and root dry weight 53
3.4.1.2 Shoot and root manganese 54
3.4.1.3 Shoot and root iron 55
3.4.1.4 Shoot and root zinc 57
3.4.1.5 Shoot and root copper 58
3.4.1.6 Root:Shoot ratios of trace elements 60
3.4.2 Trace metals in *Suaeda maritima* grown under drained and flooded conditions in the greenhouse 61
3.4.2.1 Redox values in the growth medium 61
3.4.2.2 Mn, Fe, Zn, and Cu concentrations in the shoot 62
3.4.3. The response of *Suaeda maritima* to varying concentrations of iron under aerobic and hypoxic conditions:- 63
3.4.3.1 Shoot dry weight 63
3.4.3.2 Total chlorophyll 64
3.4. 3.3 Mn, Fe, Zn, and Cu concentrations in the shoot 64
3.4.3.4 Mn, Fe, Zn, and Cu concentrations in the root 66
3.4.4 The response of *Suaeda maritima* to varying concentrations of manganese under aerobic and hypoxic conditions 68
3.4.4.1 Shoot and root dry weight 68
3.4.4.2. Total chlorophyll 69
3.4.4.3 Shoot and root manganese 69
3.4.4.4 Shoot and root iron 71
3.4.4.5 Shoot and root zinc 71
3.4.4.6 Shoot and root copper 72
3.4 Discussion 73
Chapter 4 : Mechanisms of waterlogging tolerance in *Suaeda maritima*: morphological and metabolic adaptations under hypoxic and drained conditions, in varying concentrations of artificial seawater 86
4.1 Introduction 86
4.2 Materials and Methods 89
4.2.1 Growth of plants 89
4.2.2 Sugar extraction 90
4.2.3 External standards 91
4.2.4 Calculation of tissue sugar concentrations 91

4.2.5 Root morphology and anatomy 91
4.2.5.1 Morphological characteristics 91
4.2.5.2 Preparation of root sections from treatments with different concentrations of salinity in aerated and hypoxic conditions 92
4.2.6 Lactate production by roots of *Suaeda maritima* grown in different salt concentrations under hypoxic and aerated conditions 93
4.3 Results 96
4.3.1 Oxygen concentration in the growth media 96
4.3.2 Root dry weight 97
4.3.3 Intercellular space area in the cortex 98
4.3.4 Root length 99
4.3.5 Root area (root hair sample) 100
4.3.6 Root lactate concentration 101
4.3.7 Root sodium and potassium 103
4.3.8 Leaf sugar concentration 104
4.3.9 Root sugar concentrations 104
4.5 Discussion 105
Chapter 5 : The importance of controlled transpiration in regulating sodium accumulation in *Suaeda maritima* 110
5.1 Introduction 110
5.2. Materials and Methods 113
5.2.1 Plant materials and growth conditions 113
5.2. 1.1 Experiment 1 - The effect of oxygen concentration in the root medium on gas exchange and sodium and potassium uptake in *S. maritima* plants. 113
5.2.1.2 Experiment 2 - The contribution of bypass flow to sodium uptake in *S. maritima*, under different saline conditions, in aerated and hypoxic conditions 114
5.2.3. Fresh and dry weight determination 115
5.2.4 Extraction of plants and ion analysis 115
5.2.5 Transpiration and Bypass Flow 115
5.2.5.1 Determination of transpiration 115
5.2.5.2 Determination of bypass flow 116
5.3.2.2.4 Ion fluxes 117
5.2.6 Gas exchange measurements 117
5.2.7 Stomatal measurements 117

5.2.8 Leaf area 118
5.2.9 Data analysis 118
5.3 Results 118
5.3.1 Experiment 1 118
5.3.1.1 Shoot and root dry weight 119
.5.3.1.2 Leaf area 121
5.3.1.3 Number of stomata 121
5.3.1.4 Stomatal measurements 121
5.3.1.5 Shoot and root sodium 122
5.3.1.6 Shoot and root potassium 122
5.3.1.7 Gas exchange 122
5.3.1.8 Water use efficiency 122
5.3.2 Experiment 2 123
5.3.2.1 Water loss (J_V) 123
5.3.2.2 PTS content in shoots 124
5.3.2.3 Sodium content in shoots 124
5.3.2.4 Shoot sodium and potassium concentrations 124
5.3.2.5 Root sodium and potassium concentrations 125
5.3.2.6 Ion fluxes 125
5.3.2.7 Gas exchange and water use efficiency 126
5.3.2.7.1 Stomatal conductance 126
5.3.2.7.2 Transpiration and photosynthesis 126
5.3.2.7.3 Water use efficacy (WUE, mmol CO_2 m^{-2}/mmol H_2O m^{-2} s^{-1}) 126
5.3.2.8 Leaf area and number of stomata 127
5.3.2.8.1 Leaf area 127
5.3.2.8.2 The number of stomata 127
5.3.2.9 Stomatal aperture 127
5.4 Discussion 128
Chapter 6: Summary and conclusions 138
References 144
Appendices 176

List of Figures

Figure 1.1 The chloroplast ascorbate-glutathione cycle (adapted from Foyer and Halliwell (1976). **9**

Figure 1.2 A diagram showing the shift from aerobic respiration in (mitochondria) to anaerobic respiration in (cytosol) (modified from Hossain and Uddin 2011). **18**

Figure 1.3 Photograph shows *Suaeda maritima* plants in Cuckmere Haven salt-marsh taken by T. J. Flowers. **19**

Figure 1.4 Photograph of Shoreham-by-Sea salt-marsh on the east bank of the River Adur taken by A. M. Wetson. A distinct lower and high *Suaeda maritima* can be seen.**20**

Figure 2.1 Diagrammatic representation of the simulated tidal flow tank system for creating fluctuation waterlogged conditions in pots of *Suaeda maritima.*..... **27**

Figure 2.2 Shows photograph of the tanks with *Suaeda maritima* plants..... **27**

Figure 2.3 Photograph showing flooded plants of *Suaeda maritima* in the glasshouse tidal flow tank system. **28**

Figure 2.4 Photograph showing drained pots of *Suaeda maritima* in the glasshouse tidal flow tank system. **28**

Figure 2.5 Shows glutathione GSH chromatography for glutathione by reversed-phase HPLC **31**

Figure 2.6 Photograph shows purple colour of DPPH reagent was bleached by plant extract..... **34**

Figure 2.7 concentration of GSH (dark bars), GSSG (white bars) and EGSSG/2GSH (closed dots) in *S. maritima* grown under drained and flooded conditions, in the field and a glasshouse..... **36**

Figure 2.8 Relationship between total phenolic content and antioxidant activity (DPPH) of methalonic extracts from shoots of *S. maritima*..... **40**

Figure 2.9 Relationship between total phenolic content and antioxidant activity (superoxide anion radicals) of methalonic extracts from shoots of *S. maritima.*..... **41**

Figure 3.1 A diagram of the different treatments of Fe **50**

Figure 3.2 A diagram of the different treatments of Mn..... **51**

Figure 3.3 Shoot dry weight (A) and root dry weight (B) of *Suaeda maritima* plants after 8 weeks growth, under different levels of oxygen: aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na+) in a growth chamber..... **54**

Figure 3.4 Shoot manganese (A) and root manganese (B) concentrations of Suaeda maritima plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawate (100 & 350 mM Na+), in a growth chamber.. ..... **55**

Figure 3.5 Shoot iron (A) and root iron concentrations of *Suaeda maritima* plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and Stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+), in a grow chamber. **57**

Figure 3.6 Shoot zinc (A) and root zinc (B) concentrations of *Suaeda maritima* plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and Stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+), in a growth chamber.. ..... **58**

Figure 3.7 Shoot copper (A) and root copper (B) concentrations of *Suaeda maritima* plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+), in a growth chamber.. ..... **59**

Figure 3.8 Shoot manganese (A), iron (B), zinc (c) and copper (D)concentrations of Suaeda maritima plants (12 weeks old at harvest) grown for 8 weeks under controlled conditions in the glasshouse. Plants were grown in mud and 50% sand at two heights with half-strength fresh seawater in tanks subjected to flooding twice daily.. ..... **62**

Figure 3.9 Shows shoot dry weight of *Suaeda maritima* plants after 10 days growth, under different concentrations of iron in Aerated nutrient solution (Aerated), and Stagnant agar solution (hypoxic), in the growth chamber. **63**

Figure 3.10 Total chlorophyll concentration of *Suaeda maritima* plants after 10 day growth, under different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic) in the growth chamber. **64**

Figure 3.11 Show shoots manganese (A). Iron (B), zinc (C) and copper (D) concentrations of *Suaeda maritima* plants after 10 days growth, in different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic), in the growth chamber., ... **65**

Figure 3.12 Root manganese (A), iron (B), zinc (C) and copper (D) concentrations of Suaeda maritima plants after 10 days growth, in different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic), in the growth chamber.. ..... **67**

Figure 3.13 Shows mean shoot (A) and root (B) dry weight of *Suaeda maritima* plants (7 weeks old at harvest), after 3 weeks growth in hydroponic solutions containing 350 Na+ mM (artificial seawater and half strength Arnon & Stout culture solution), in varying Mn concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic).. ..... **68**

Figure 3.14 Shows total chlorophyll of S*uaeda maritima* plants (7 weeks old at harvest), after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic).. ..... **69**

Figure 3.15 Shows the concentration of manganese in shoots (A) and roots (B), in *Suaeda maritim*a shoots after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic)..... **70**

Figure 3.16 Shows mean shoot iron (A) and root iron (B) concentrations in Suaeda maritima shoots, after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated), and stagnant agar solutions (Hypoxic).. ..... **71**

Figure 3.17 Shows mean shoot zinc (A) and root zinc (B) concentrations in Suaeda maritima shoots, after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated), and stagnant agar solutions (Hypoxic). **72**

Figure 3.18 Shows mean shoot copper (A) and root copper (B) concentrations in Suaeda maritima plants, after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrat concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic).. ..... **73**

Figure 3.19 Relationship between shoot manganese and shoot dry weight (A), shoot iron and shoot dry weight (B), shoot manganese and total chlorophyll (C) and shoot iron and shoot dry weight in *S. maritima* plants, after 3 weeks growth in 350 mM Na+ artificial seawater, in varying concentrations of manganese and iron. **76**

Figure 3.20 Relationship between total chlorophyll and shoot dry weight and total chlorophyll and shoot dry weight in *S. maritima* plants, after 3 weeks growth in 350 mM Na+ artificial seawater, in varying concentrations of manganese and iron..... **77**

Figure 3.21 Shows the relationship between root manganese concentration and shoot dry weight, root iron and shoot dry weight, root manganese concentrations and root dry weight and root dry weight and shoot dry weight in *S. maritima* plants, after 3 weeks growth in 350 mM Na+ artificial seawater, in varying concentrations of manganese and iron. **78**

Figure 3.22 Shows the relationship between shoot manganese concentrations and water content, hoot iron concentration and the mean shoot water content and root manganese concentration and water content in S. *maritima* plants, after 3 weeks growth in 350 mM Na^+ artificial seawater, in varying concentrations of manganese and iron. **79**

Figure 3.23 Roots of *S. maritima* grown under different manganese concentrations, in aerated on the right hand side of each treatment and hypoxic conditions on the left hand side. **84**

Figure 3.24 *S. maritima* shoots (light green) grown in aerated conditions, at high manganese concentrations in the growth medium 5 mM Mn (B) and 10 mM Mn (A) .. **84**

Figure 4.1 Diagram shows roots excised to upper and lower root in roots grown under aerated conditions (A) and adventitious root (upper root) and lower root under hypoxic conditions (B). **92**

Figure 4.2 Photograph shows the adventitious roots under hypoxic conditions. Arrows indicate adventitious root arising from the stems of four plants, a, b, c and d, which were suspended with non-absorbent cotton wool through holes in a lid. **94**

Figure 4.3 Illustrates the system for incubating root tissue under anoxic conditions in a bath of Argon. The tissue from aerated and hypoxic low and high salt (100 and 350 Na+ mM artificial seawater) roots (adventitious and seminal root in hypoxic samples) and (upper and lower seminal root in aerated samples)) was incubated for 4 h in vials (approx. 20 ml) contained in a tank flushed with Argon. Argon was delivered from a cylinder through a tube which divided into two conical flasks containing water so that the rate of bubbling could be monitored and adjusted. Argon was used as it is heavier than nitrogen and so anoxic conditions were assured (Tavzes et al., 2001). The tank was contained in a fume-hood to evacuate excess Argon. **96**

Figure 4.4 Root dry weight of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: aerated nutrient solution (A), and stagnant agar solution

(AGN), in different concentrations of artificial seawater (100 & 350 mM Na+) in a growth chamber..... **98**

Figure 4.5 Mean intercellular space area in the cortex (µm2 per 1000 µm2) roots of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber..... **99**

Figure 4.6 Root length of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+) in a growth chamber. **100**

Figure 4.7 Upper root hair area and lower root hair area per root hair of *Suaeda maritima* plants, after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber..... **101**

Figure 4.8 Upper and lower root lactate concentration of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber..... **102**

Figure 4.9 The whole root lactate concentration of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber..... **103**

Figure 5.1 Shoot (A) and root (B) dry weight of *Suaeda maritima* plants (9 weeks old at harvest), after 5 weeks treatment in hydroponic solutions under different concentrations of oxygen, in artificial seawater 100 mM Na+ (aerated and hypoxic): Aerated nutrient solution without agar (A), aerated nutrient solution with agar (AGA), nitrogen-flushed nutrient solution without agar (N), nitrogen-flushed nutrient solution with agar (AGN). Error bars are (n = 10). **120**

Figure 5.2 Leaf areas of *Suaeda maritima* plants (9 weeks old at harvest) after 5 weeks treatment in hydroponic solutions under different concentrations of oxygen in artificial seawater, 100 mM Na+ (aerated and hypoxic): Aerated nutrient solution without agar (A), aerated nutrient solution with agar (AGA), nitrogen-flushed nutrient solution without agar (N), nitrogen-flushed nutrient solution with agar (AGN).. ..... **121**

Figure 5.3 Relationship between shoot and root dry weight and mean oxygen concentration in the growth medium for Experiment 1. ..... **130**

Figure 5.4 The relationship between stomatal conductance and water loss (A), the degree of stomatal opening on the leaf surface and gravimetric water loss (B). **132**

Figure 5.5 The relationship between water use efficiency per leaf and water use efficiency per plant. **133**

Figure 5.6 Shows a lack relationship between stomatal conductance and photosynthesis in *S. maritima*. **134**

Figure 5.7 The relationship between transpiration and bypass flow in the *S. maritima* plant. **137**

List of Tables

Table 1.1 Shows some halophytic species and whether or not their roots contain aerenchyma **15**

Table 2.1 MDA concentration in *S. maritima* grown under natural and controlled conditions. In the natural conditions, 'high marsh' plants grew at an elevation 0.6 m above those of the 'low marsh' plants, while under the controlled conditions of the glasshouse, plants were grown in mud and 50% sand at two heights in tanks subjected to flooding twice daily. On harvesting, plants were directly frozen in liquid nitrogen and stored at -80 °C for determination of MDA. **37**

Table 2.2 The total polyphenolic concentration and free-radical scavenging activity in *S. maritima* grown under natural and controlled conditions. The growth conditions were as described in Table 1. Plants were dried at room temperature for 4 weeks before being analysed for phenolic compounds and antioxidant activity.. ..... **38**

Table 3.1 The effect of pH on the solubility of manganese, iron, zinc, and copper 'Low' pH is an acidic solution with a pH lower than 7, and 'high' pH is an alkaline solution with a pH higher than 7. ..... **45**

Table 3.2 Shows the oxygen concentration before and after renewing the growth medium, pH, temperature, and electrical conductivity (EC) for *S. maritima* plants grown under different concentrations of oxygen: aerated medium (A), and hypoxic medium (AG N), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth chamber at 20^0C. **53**

Table 3.3 Shows the ratio between root and shoot concentrations for manganese, iron, zinc and copper, and shoot and root dry weight of *S. maritima* plants grown under different concentration of oxygen: aerated medium (A), and hypoxic medium (AG N), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth chamber. **60**

Table 3.4 Mean Eh values (mV) recorded at three depths for 30 seconds, after one hour of flooding, in growth medium composed of a mixture of sand and estuarine mud, in which *Suaeda maritima* was grown at the top, middle and base of the growth medium, in drained and flooded pots, in the tidal flow glasshouse tank system.. ..... **61**

Table 3.5 Shows the comparison between trace metal concentrations for *S. maritima* shoots grown at low salt concentrations (100 mM Na^+) and high salt concentrations (350 mM Na^+) in a growth cabinet. **82**

Table 3.6 Shows comparison of trace metal concentrations in *S. maritima* shoots, grown in 350 mM Na^+ artificial seawater, at ~pH 5.5 in a growth cabinet, and in half strength fresh seawater at ~pH 8, in greenhouses under flooded and drained conditions **83**

Table 4.1 Shows the oxygen concentration before and after renewing the growth medium (twice a week) for *S. maritima* plants grown under different concentrations of oxygen: aerated medium (A), and hypoxic medium (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth chamber at 20^0C. **97**

Table 4.2 Sodium and potassium concentrations, in roots of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks growth in hydroponic solutions, at 100 and 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution). **103**

Table 4.3 The sucrose, glucose, fructose , and total sugar concentrations in *S. maritima* leaves after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. **104**

Table 4.4 The sucrose, glucose, fructose, and total sugar concentrations (µmol g FW^{-1}) in *S. maritima* roots after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber..... **105**

Table 5.1 Shows the oxygen concentration before and after renewing the growth medium, for *S. maritima* plants grown under different concentrations of oxygen in 100 mM Na+ artificial seawater, in a growth chamber at 20^0C..... **119**

Table 5.2 Shows the oxygen concentration before and after renewing the growth medium (every day) for *S.maritima* plants grown under different concentrations of oxygen: aerated medium (A), and hypoxic medium (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth cabinet at 20^0C..... **123**

Table 5.3 Na+, PTS uptake and bypass flow (JVB) by *S. maritima* plants in different salt concentrations, under aerated and hypoxic conditions. **124**

Table 5.4 Sodium and potassium concentrations, and sodium and potassium ion fluxes in shoots and roots of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks growth in hydroponic solutions, at 100 and 350 mM Na+ (artificial seawater and half strength Stout and Arnon culture solution).. ..... **125**

Table 5.5 Photosynthesis, stomatal conductance, transpiration and water use efficiency per leaf and plant of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks

growth in hydroponic solutions at 100 and 350 mM Na+ (artificial seawater and half strength Stout and Arnon culture solution). **126**

Table 5.6 Number, length, width and Stomatal aperture area on the abaxial and adaxial leaf surfaces of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks growth in hydroponic solutions, at 100 and 350 mM Na^+ (artificial seawater and half strength Arnon & Stout culture solution)..... **128**

Abbreviations

A: Aerated nutrient solution

ABA: Abscisic acid

ADH: Alcohol dehydrogenases enzyme

AGN: Stagnant agar solution with culture

AKT1: Inwardly rectifying potassium channel

ATP: Adenosine triphosphate

β-NAD: β-nicotinamide-adenine dinucleotide

CAT: Catalase

(pH_{Cyt}): Cytoplasmic pH

D: Day

DF: Dilution factor

dH_2O: Distilled wate

DPPH: 2,2-diphenyl-1-picry-hydrazyl-hydrate

DTT: Dithiothreitol

DW: Dry weight

E°′: Standard half-cell reduction potential

EC: Electrical conductivity

EDTA: Ethylenediaminetetraacetic acid Fe (III) sodium salt

Eh: Redox potential

ETC: Photosynthetic electron transport chain

EVAP: Evapotranspiration

F: The Faraday

GAE: Gallic acid

GB: Glycinebetaine

GC-MS: Gas chromatography–mass spectrometry

GS: Stomatal conductance

GSH: Reduced glutathione

GSSG: Oxidized Glutathione

H: Hour

H_2O_2: Hydrogen peroxide

HKTs: High-affinity K^+ transporters

HMDS+TMCS : Hexamethyldisilazane and trimethylchlorosilane

HPLC*:* High-performance liquid chromatography

ICP-MS: Inductively coupled plasma mass spectrometry

IMS: Industrial Methylated Spirits

LDH: Lactate dehydrogenase enzyme

mBBr: Monobromobimane

MCTs: Monocarboxylate transporters

MDA: Malonyldialdehyde

MIPS: Membrane Intrinsic Proteins

MSA: Methanesulfonic acid

N: Kelvin

NAD: Oxidised form of nicotinamide adenine dinucleotid

NADP: Nicotinamide adenine dinucleotide phosphate

NADPH: Reduced form of NADP (Nicotinamide adenine dinucleotide phosphate)

NBT: Nitro Blue Tetrazolium

NEM: N-ethylmaleimide

NHX: Na^+/H^+ exchanger or Na^+/H^+ antiporter

NIPS: Nodulin 26-like intrinsic proteins

NSCCs: Non-selective cation channels

^{O}C : Degrees Celcius

$O2^-$: Superoxide anions

OH: Hydroxyl radicals

$^1O_2$: Singlet oxygen

PAR: Photosynthetically active radiation

PMF: Proton motive force

PMS: Phenazine methosulphate

Pn : Photosynthetic rate

PTS: Trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid

PDC: Pyruvate decarboxylase

QACs: Quaternary ammonium compounds

R: Gas constant

RRF:	Relative Response Factor
RF:	Response Factor
RH:	Relative humidity
ROS:	Reactive Oxygen Species
SOD:	Superoxide dismutase
SS:	Sucrose synthase
T:	Temperature
TBA:	Thiobarbituric acid
TCA:	Trichloroacetic
$TEA^{+:}$	Tetraethylammonium
TEA^{+}:	Tetraethylammonium
UHP H_2O:	Purity water
WC:	Water content
WUE:	Water-use efficiency

Chapter 1 : General introduction

1.1 Salinity

1.1.1 Salinity, saline environments and saline soils

Salinity, one of the most significant biotic stresses for plants, causes an extensive problem in agriculture in many areas around the globe. About 7% of the world land surface is affected by salt seawater, creating soils with soluble salt concentrations that have negative effect on growth of most plant species (e.g. Greenway and Munns, 1980). Sodic soils are typified by an excess of bicarbonate ($HCO3^-$) and carbonate ($CO3^{2-}$), while saline soils are subject to high concentrations of sulphate ($SO4^{2-}$) and especially chloride (Cl^-): sodium (Na^+) and Cl^- dominate the majority of salt-affected soils. Soils are classified as saline when the electrical conductivity (EC) is 4 dS/m or greater (Ghassemi et al., 1995); 4 dS/m is equivalent to approximately 40 mM NaCl.

Salinisation, the increase in soluble salts in soil, may happen due to natural causes or as a result of human activities. Primary salinisation (natural) results from weathering of parent rock, precipitation of salts in rainwater from sea spray blown inland, and ingress of seawater into low lying coastal regions. Regular tidal flooding creates salt marshes, whose soils are dominated by those ions present in seawater. Seawater includes about 460 mM Na^+ and 540 mM Cl^- with 28 mM SO_4^{-2} and 50 mM Mg^{2+}. The electrical conductivity (EC) of seawater is approximately 58 dS m^{-1}. In the next 500 years it is expected that the sea level will increase by 1.0 m due to the melting of the polar ice caps so that more land will become salt affected.

The most important problem for agriculture is secondary salinisation that is induced by human activities as a result of irrigation and forest clearance (Flowers and Flowers, 2005; Munns and Tester, 2008). Irrigation of semi-arid regions with water of low salt content to supplement rainfall insufficient for crop cultivation is becoming an increasing problem. Although only about 17% of the world's cropland is irrigated, this land provides over 33% of the world's food (Munns and Tester, 2008) and much of this land is threatened by salinisation. Salinity affects a quarter of the irrigated land in some

countries. About 80 million ha of irrigated farm land has some degree of salinisation and about 1.5 million ha of irrigated land is lost each year to waterlogging and salinity (FAO 2008). Excessive irrigation, beyond that required for plant growth, increases the water table, allowing capillary rise of the soil solution, taking salt in the soil to the surface. Consequently, the soils become waterlogged and the salinity is increased as a result of evaporation of water, leaving the salts behind in the soil.

The loss of agricultural land to salinity and flooding necessitates consideration of how plant growth is affected by the interaction of salinity and waterlogging with a view to selecting tolerant genotypes for agricultural use. Responses to combined waterlogging and salinity are physiologically complex but screening for genetically tolerant strains in both crop plants and naturally tolerant species is important for plant breeders in attempts to transfer traits for waterlogging and salt-tolerance to existing crop species.

'Waterlogging', of cultivated land is "the state of land in which the subsoil water table is located at or near the surface with the result that the yield of crops commonly grown on the land is reduced", and for uncultivated land is "[land that] cannot be put to its normal use because of the high subsoil water table" (FAO 2008). Much saline land is waterlogged (Barrett-Lennard, 2003), and these two conditions, waterlogging and salinity, are becoming an increasing problem for terrestrial habitats. In waterlogged soil, the saturation with water causes the loss of the air-filled pores normally present. As a result, the plants are subjected to salinity and hypoxia, or even anoxia, simultaneously, and aerobic root metabolism subsequently becomes an issue.

1.1.2 The effects of salinity on plants with an emphasis on halophytes

1.1.2.1 Growth

Soil salinity has a negative effect on growth in plant species in three ways: via decreasing the water potential leading to osmotic stress as a result of water deficit, via the absorption of toxic amounts of Na^+ and Cl^-, and via decreasing the bioavailability of essential nutrients (Waisel, 1972; Flowers et al., 1977; Greenway and Munns, 1980; Gorham, 1996; Flowers and Flowers, 2005). Glycophytes are plants that only tolerate relatively low salt levels in the soil, though they may be able to adjust to slightly increased salt content, by absorbing salts into their leaves (Suzaki et al., 1989). The term 'glycophyte' is used to contrast those plants that are typically found in non-saline

soils with those typically found in saline environments, 'halophytes'; the key difference between them is the extent to which growth is inhibited by increasing salinities. Halophytes are plants that are able to survive and complete their life cycle under highly saline conditions of around 200 mM NaCl or more, conditions that are similar to those of their natural environment. Halophytes have a wide range of mechanisms that enable them to adapt to salinity. These include the control of Na^+ uptake; regulation of Na^+ delivery and allocation to particular parts of the shoot (e.g. older leaves); control of transpiration by stomata, secretion of salts on the leaf surface by salt glands, accumulation of organic solutes to alleviate osmotic stress and antioxidant defence mechanisms (see Flowers and Colmer, 2008).

In general, halophytes are characterized by their ability to survive with high concentrations of salt in their environments, which are normally dominated by NaCl , particularly in seawater (at about 500 mM). There are also sodic soils with pH values > 8.5, where the predominant anions are HCO_3^- and CO_3^{2-}. The exact dividing line between 'salt-tolerant' halophytes, and 'salt-sensitive' glycophytes is largely a matter of judgement and the division has ranged from the equivalent of about 75 mM NaCl to 200 mM (Aronson, 1989; Flowers and Colmer, 2008). Halophytes can also be distinguished from glycophytes by their ability to accumulate ions to high concentrations in the leaf cells (Flowers et al., 1977; Flowers and Colmer, 2008). Many dicotyledonous halophytes demonstrate optimal growth in concentrations of 50 to 250 mM NaCl (Flowers et al., 1986; Flowers and Colmer, 2008); for example, *Suaeda maritima* shows maximal growth at about 200 mM NaCl (Yeo and Flowers, 1980; Clipson and Flowers, 1987), while halophytic grasses generally grow better in non-saline solutions. There are exceptions to this generalisation, some grasses and less-tolerant dicotyledonous plants (dicots) show optimal growth at lower salinities (25 to 100 mM) NaCl (Ungar, 1991). Changes in the quantity of water per unit dry mass make a huge contribution to changes in the fresh weight of many halophytes, particularly succulent halophytes, growing under saline conditions and accumulation of massive quantities of ions to the dry weight. In succulent dicotyledonous species, nearly half the dry mass of the shoots may be comprised of 'ash content'. Consequently, some have advocated using organic dry mass to measure growth: this parameter also shows an optimum; for example, Yeo and Flowers (1980) reported that at 340 mol m^{-3} NaCl, *S. maritima* had a greater organic dry mass than when grown in a standard culture medium (13 mol m^{-3} NaCl).

The causes of reduction in growth at supra-optimal salinities have not been clearly identified by Flowers and Colmer (2008), although there are, as for glycophytes, several options. For example, the reduction could be due to a decrease in C fixation, alterations in the allocation of biomass between roots, leaves and stems and changes in the balance between respiration and photosynthesis (Ball, 1988; Lovelock and Ball, 2002). A fall in turgor is another possibility for the decrease in growth (Clipson et al., 1985; Rozema, 1991; Balnokin et al., 2005), or a change in the elasticity of the cell walls (Tomos and Wyn Jones, 1982; Touchette, 2006). Further possibilities are those associated with osmotic adjustment: perhaps a lack of ability to distribute and/or accumulate enough nutrients or synthesize enough organic solutes, the energy-consuming futile cycling of ions (Britto and Kronzucker, 2006), or the additional energy-consumption needed for compartmentation of ions (Yeo, 1983).

1.1.2.2 Osmotic effects

Seawater salt concentration has a major effect on the plant as a result of osmosis causing water stress. Water flows through a plant down a water potential gradient from the soil solution through the root to the xylem sap and then via the leaf to the atmosphere. In order to take up water, a plant must have a more negative water potential than that in external growth medium. The water potential in non-saline soil is about -0.1 MPa which requires only a small osmotic adjustment if the plant is to maintain a positive turgor pressure. In contrast, halophytes that live in seawater-flooded soil should have a water potential in their roots lower than that in seawater (about -2.3 MPa) (Harvey, 1966) This requires a greater degree of osmotic adjustment than required by glycophytes.

Plants generate low water potential in their tissue by accumulation of inorganic ions from their external growth medium and synthesis of organic solutes. Halophytes are distinguished from glycophytes by their ability to accumulate ions to high concentrations in living leaf cells (Flowers et al., 1977; Colmer and Flowers, 2008). Halophytes commonly use Na^+ and Cl^- for osmotic adjustment, but to varying degrees in different families. For example, there is an obvious difference in Na^+ and K^+ balance quantified by the net potassium, sodium selectivity ($S_{K,Na}$). The grasses show a much higher $S_{K,Na}$ values than, for example, the chenopods; the Brassicaceae and the

Chenopodiaceae, have lower net $S_{K:Na}$ at saline concentrations under 200 mM than the Asteraceae. The Plumbaginceae possess salt glands, and have higher values of net $S_{K:Na}$ than the Tamaricaceae (Flowers and Colmer, 2008).

1.1.2.2.1 Osmotic adjustment, ion compartmentation

In order to maintain water balance during abiotic stress, halophytes accumulate inorganic ions in the vacuoles (Flowers et al., 1977; Flowers and Colmer, 2008). The osmotic balance between the cytoplasm and the vacuole, is maintained by organic solutes, such as proline and glycinebetaine in the cytoplasm (Flowers et al., 1977; Wyn Jones et al., 1977; Glenn et al., 1999), as a high K^+/Na^+ ratio (100-200 mM K^+, <1mM Na^+ (Tester and Davenport, 2003) is needed in the cytoplasm to maintain the activity of salt-sensitive cytosolic enzymes (Glenn et al., 1999). The first evidence of the compartmentalisation of ions in the vacuoles of higher plants to maintain turgor, without an increase in ion concentration in the cytoplasm, was reported by Flowers (1972) and Greenway and Osmond (1972).

1.1.2.2.2. Compatible solutes

Production of several kinds of compatible organic solutes in plants is the most common response to stress (Serraj and Sinclair, 2002). These solutes include proline, sucrose, polyols (sorbitol, mannitol, pinitol and inositol), trehalose and quaternary ammonium compounds (QACs) such as glycinebetaine (GB), alaninebetaine, prolinebetaine, choline *O*-sulfate, hydroxyprolinebetaine, and pipecolatebetaine (Flowers et al., 1977; Wyn Jones et al., 1977; Rhodes and Hanson, 1993; Ashraf and Foolad, 2007; Flowers and Colmer, 2008). Among the carbohydrates, sucrose, fructose and glucose have been noted to accumulate in plants found in coastal salt marshes (Briens and Larher, 1982), such as *Juncus maritimus, Atriplex hastata* and *Aster tripoliun*. According to their capacity to accumulate carbohydrates and/or nitrogenous solutes, halophytic higher plants can be divided into three main groups: (1) species producing high levels of soluble carbohydrates only, (2) species accumulating both carbohydrates and nitrogenous compounds, (3) species producing more nitrogenous solutes than soluble carbohydrates. The compatible solutes are highly soluble, low molecular weight compounds that are metabolically harmless even at high cellular concentrations. The compatible solute theory states that maintaining osmotic equilibrium under high salinities involves the synthesis of these organic compounds, as well as partial exclusion

of NaCl and uptake of potassium ions (Gagneul et al., 2007). In general, these solutes defend plants from stress in several ways, including contributing to the detoxification of reactive oxygen species, osmotic adjustment in the cell, membrane integrity protection, and the stabilisation of enzymes and proteins (Yancey et al., 1982; Bohnert and Jensen, 1996) Proline in particular, which can be found at high concentrations in the cytosol, chloroplasts and vacuoles, without compromising enzyme activity, has proved to contribute significantly to osmotic adjustment. Proline also acts as a signalling molecule, stabilises proteins, and acts as an antioxidant (Szabados and Savourè, 2010).

1.1.2.3 Effects on mineral nutrition

The availability and uptake of nutrients (both macro and micro nutrients) by plants is affected by (1) nutrient ionic activity in the solution of soil (2) the relative concentrations of the other ions present, where they control uptake of nutrients by the root; and (3) environmental factors such as pH and redox potential (Eh), which influence the ionic strength of the nutrients. Both the rate of absorption and the distribution of particular elements within the plant (i.e. across the whole plant, within organs, and within the cell), may vary between plant species. Salinity might disrupt mineral nutrient uptake by plants in two ways: the most common mechanism is competition from the major ions (Na^+ and Cl^-), which reduces the bioavailability of other ions, frequently causing Ca^{2+} or K^+ deficiencies (induced by Na^+) or reduced NO_3^- accumulation (through competition with Cl^-) (Torres and Bingham, 1973; Greenway and Munns, 1980), possibly by accelerating its reduction to NH_4 (Hu and Schmidhalter, 1997). Secondly, salinity can alter the ionic strength of ions, further influencing uptake and translocation.

Halophytes have not received the interest glycophytes have, regarding the relationship between nutrient uptake and salinity. It is generally unknown whether halophytes experience mineral deficiencies in saline conditions. If they occur, the imbalance of nutrients might be for any of the above reasons. Okusanya and Ungar (1984) reported that under saline conditions, the growth of halophytes was increased as result of nutrient applications (such as calcium nitrate and calcium chloride), indicating that the salinity was only moderately growth limiting. In contrast, for glycophytes grown in saline conditions, applications of nutrients did not improve the growth of the plants, indicating that for glycophytes salinity was extremely growth-limiting. Many researchers have

shown that salinity reduces the K^+ concentration in plant tissue, (e.g. Okusanya and Ungar, 1984; Cramer et al., 1985; Janzen and Chang, 1987; Subbarao et al., 1990) and this includes halophytes. This occurs due to competition with Na^+ ions, regardless of whether the Na^+ salts are NaCl or Na_2SO_4. (Yeo and Flowers, 1980). Yeo and Flowers (1986) reported that growth of *S. maritima* at high concentrations (340 mM) was maximal in NaCl and the effects of other salts ranged between this and toxicity in KC1. Toxicity of high KC1 concentrations, in contrast to a beneficial effect at low concentrations, has also been found for *Atriplex inflata* and *A. nummularia* (Ashby and Beadle, 1957), *Halogeton glomeratus* (Williams, 1960) and for *Salicornia europea* (Eijk, 1939). Potassium chloride was toxic for the halophilic alga *Dunalliela viridis* (Johnson et al., 1968).

Micronutrient concentrations in soil solutions are very low (µM range) and their availability depends upon the chemical and physical features of the soil, as well as the release of organic molecules from the plant. The bioavailability of most micronutrients is dependent on the pH and redox potential (Eh) of the soil solution, in addition to the types of particle surfaces and their binding sites (both organic and inorganic; see Chapter 3 for more details). The levels of micronutrients in plant shoots may decrease, increase or remain unaffected (depending on the plant type, tissue, micronutrient concentration) as salinity changes. However, as for macronutrients, the relationship between salinity and mineral nutrition, of halophytes has been subjected to much less research than for glycophytes. Studies on horticultural crops have found that salinity decreases the concentration of Mn in the plant, although other reports on the effect of salinity on the concentration of other ions, such as Zn and Fe have been contradictory (Grattan and Grieve, 1998). Saline conditions can also modulate the uptake of certain trace elements by halophytes. Mn uptake by salt-marsh plants decreased upon the addition of NaCl to the growth medium (Singer and Havill, 1985), while halophytes grown in salt marshes have been reported to have lower Fe and Mn contents than hydrophytes grown in freshwater marshes (Gorham and Gorham, 1955). Mn and Fe uptake is stimulated in response to waterlogging in *Juncus gerardii* and *Agrostis stolonifera*, and uptake was higher without salt (Rozema and Blom, 1977). *Spartina alterniflora* grown in an upper marsh had lower Fe levels than when grown in a lower marsh site (Adams, 1963).

1.1.2.4 Antioxidants

The levels of Reactive Oxygen Species (ROS) increase in plants as a result of stressful conditions, such as salinity or waterlogging. ROS are defined as any species containing one or more unpaired electrons (Halliwell, 2006), such as superoxide anions (O_2^-). ROS are typically produced at low levels during photosynthesis (Lesser, 2006), but their concentrations have been shown to increase when plants experience stress (Jithesh et al., 2006; Bansal and Srivastava, 2011). Seawater can also contribute to ROS production. A review by Lesser (2006) concluded that in marine conditions, organic matter can be dissolved by ultraviolet radiation, causing the production of ROS which damage cell membranes through hydrogen peroxidation. Salt stress also causes stomata to close, decreasing the CO_2:O_2 ratio in the leaf tissue. This inhibits CO_2 fixation (Hernandez et al., 1999), causing over-reduction of the photosynthetic electron transport chain (ETC), resulting in ROS production, as previously mentioned. The production of malondialdehyde (MDA) from added thiobarbituric acid is used as an indicator of the presence of ROS and many researchers report that MDA production is increased under salt in halophytes suggesting that salinity causes an increase in oxidative stress (Parida et al., 2004; Taji et al., 2004; Wang et al., 2004; Zhang et al., 2005).

Plants are able to prevent high levels of ROS occurring and protect cells using enzymes and metabolites. There are many scavenging enzymes involved, such as catalase (CAT), superoxide dismutase (SOD), peroxidase and gluthathione peroxidase, glutathione-S-transferase, phospholipid-hydroperoxide and ascorbate peroxidase. The low molecular weight antioxidants include ascorbate (vitamin C), glutathione, and tocopherols (vitamin E) (Blokhina et al., 2003). Other important metabolites include phenolic compounds, tannins, flavonoids, lignin, esters and hydroxycinnamate and are often the most abundant of secondary metabolites in plant tissue (Grace and Logan, 2000). Phenolic compounds are ideal for scavenging free radicals, as they can donate an electron or hydrogen atom and thereby detoxify ROS (RiceEvans et al., 1997; Falleh et al., 2012); polyphenols are more effective antioxidants than either ascorbate or tocopherols (Blokhina et al., 2003). Halophytes as other plants, utilise antioxidant enzymes for protection against damaging ROS (Jithesh et al., 2006). Glycophytes under salt stress have also been reported to show increased antioxidant activity (Hernández et al., 1993; Gueta-Dahan et al., 1997; Shalata and Tal, 1998).

There is a strong relationship between glutathione and ascorbate. Since an early hypothesis that glutathione and ascorbate had potential roles in detoxification, in isolated chloroplast preparations, ascorbate and glutathione have been found to be involved in the oxido/reduction of NADP/ NADPH (Foyer and Halliwell, 1976, 1977), and a simple metabolic scheme has been proposed (Fig 1.1). The suggested role of glutathione and ascorbate in metabolism of H_2O_2 in chloroplasts led to the identification of ascorbate peroxidise, both thylakoid-bound and soluble stromal forms (APX; Groden and Beck, 1979; Kelly and Latzko, 1979). The ascorbate-glutathione or "Foyer-Halliwell-Asada" pathway is now known to be important in H_2O_2 metabolism in both animals and plants, and components of this pathway have been found in numerous organelles, including chloroplasts, mitochondria, peroxisomes and the cytosol (Edwards et al., 1991; Mittler and Zilinskas, 1991; Jimenez *et al*., 1997).

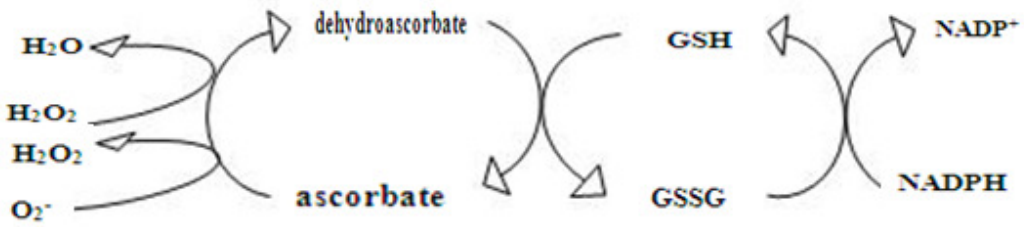


Figure 1.1 The chloroplast ascorbate-glutathione cycle (adapted from Foyer and Halliwell (1976).

In waterlogged conditions, hypoxia induces oxidative stress. The role of antioxidants has had little attention in halophytes, under combinations of salinity and waterlogging. The work of this thesis includes biochemical studies of shoots from plants grown under such a combination of flooding and salinity (reported in Chapter 2).

1.1.2.5. The pathways of Na^+ uptake in halophytes

There are two main pathways for ion transport in plants. The apoplastic pathway takes place in spaces external to the plasma membranes, through the xylem and cell walls, while the symplastic pathway takes place via the linked protoplasts of cells, including those of the root cortex, the phloem and leaf parenchyma (Jachetta et al., 1986). Na^+

uptake pathways in plants have been regularly reviewed (Amtmann and Sanders, 1999; Maathuis and Amtmann, 1999; Blumwald et al., 2000; Tester and Davenport, 2003; Apse and Blumwald, 2007; Flowers and Colmer, 2008; Plett and Moller, 2010; Zhang et al., 2010; Shabala and Mackay, 2011); net transport occurs by both apoplastic and symplastic pathways.

1.1.2.5.1 Apoplastic pathway (Bypass flow)

When Na^+ moves in, from outside, through cell walls, and then through the intercellular space to the xylem, this is called 'bypass flow' (also known as the apoplastic pathway) (Yeo et al., 1987; Yeo, 1992; Singh and Flowers, 2010). Bypass flow, which is generally only a few percentage (around 1% Munns et al., 1983) of the total ion transport to the shoot, occurs in all plants but only delivers small quantities of ions under non-saline conditions. However, where the external concentration of any ion is high, bypass flow can deliver a significant proportion of the total net transport to the shoot. For example, for Na^+ uptake in rice under saline conditions bypass flow is an important component of net ion transport (Yeo et al., 1987; Yeo, 1992; Yadav et al., 1996; Garcia et al., 1997; Yeo et al., 1999; Tester and Davenport, 2003; Bridges et al., 2005; Gong et al., 2006; Krishnamurthy et al., 2009; Singh and Flowers, 2010). Faiyue et al.(2010) reported that in rice, even at high relative humidity (90%), the Na^+ delivered to the shoots through bypass flow accounted for 22% of that accumulated in the shoots. For halophytes, limiting bypass-flow is likely to be an important component of their tolerance of saline conditions (Hajibagheri et al., 1985; Shabala and Mackay, 2011). More details on bypass flow in halophytes are available in Chapter 5 (Sections 5.1 and 5.2.1.2). The effectiveness of limitations of bypass flow are intimately linked to the net flux of water through the plant; to the rate of transpiration, the vehicle of net transport.

Generally, the rate of transpiration is decreased by increasing rhizospheric salinity in both glycophytes and halophytes. This might be as a result of lower water potential in the roots and/or effects of abscisic acid (ABA). ABA plays an essential role in root-to-shoot cellular signalling to regulate stomatal[1] conductance (Robinson et al., 1997; Redondo-Gómez et al., 2006; Munns and Tester, 2008). Stomata have a basic role in

[1] The numerical measure of the highest rate of passage both carbon dioxide and water vapour through the stomata

control of transpiration and photosynthetic rates (Willmer and Fricker, 1996) and are sensitive to change in external water potential, thus stomatal closure often accompanies salinity, drought and waterlogging (Kozlowski, 1997; Karlberg et al., 2006; Maricle et al., 2007; Munns and Tester, 2008). Stomatal closure is likely to be an important feature of salt tolerance in halophytes (Robinson et al., 1997; Vèry et al., 1998). In some species of halophytes, stomata close to limit transpiration and thus the transport of salts to the leaves (Vèry et al., 1998). For example, *Hibiscus tiliaceus* (Youssef, 2007) and *Aster tripolium* do not have specific morphological adaptations to salt and respond by preventing excessive sodium accumulation inside the shoot through regulating the rate of transpiration (Vèry et al., 1998).

Exclusion of ions such as Na^+ from the xylem is an important adaptation against salt stress and a factor in salt-tolerance. Control of salt exclusion operates at the whole plant level. It depends on selectivity of uptake at the entry into roots, at xylem loading, at distribution from root to shoot by the unloading of xylem and the loading of phloem and to any loss from the plant through salt glands (Munns, 2002). Munns et al. (2000) concluded that in halophytes and glycophytes, at 200 mM Na^+, approximately 97% of the Na^+ existing in the root should be rejected. Exclusion of Na^+ and Cl^- from the xylem sap in several salt-marsh species has been shown (Rozema et al., 1981) and a high degree of exclusion of these$^-$ ions from xylem sap of *S. maritima* has been reported by Yeo and Flowers (1986). Exclusion appears to be greatest in species that do not excrete salt. The endodermis is generally thought to be the barrier to exclusion and the means by which low xylem ion concentrations are maintained. Flow of water and ions across the root by the apoplastic pathway is impeded by the suberised Casparian strip of the endodermis where there is controlled entry into the xylem. Evidence for sodium exclusion at the endodermis in has been presented by Hajibagheri *et al* (1985) who reported differentiation of the endodermal Casparian strip much closer to the root tip in plants of *S. maritima* grown in saline conditions than non-saline conditions.

1.1.2.5.2 Symplastic pathway

Movement of Na^+ through plasma membranes is mediated by membrane transporter proteins called carriers and channels (Maathuis and Amtmann, 1999; Taiz and Zeiger, 2006; Maathuis, 2007). The solute specificity of channels is primarily based on the size

and charge of the solute (Taiz and Zeiger, 2006), and although transport is rapid (~10^6 – 10^8 ions per second (Maathuis and Amtmann, 1999; Taiz and Zeiger, 2006; Maathuis, 2007), it is always passive. Carriers mediate a much slower rate of transport, due to the conformational change required during transport, but they are capable of mediating active transport (Maathuis and Amtmann, 1999; Taiz and Zeiger, 2006; Maathuis, 2007).

Evidence suggests that Na^+ entry into the cell generally occurs via low-affinity non-selective cation channels (NSCCs) (Amtmann and Sanders, 1999). However, Wang et al. (2007) provided evidence (based on studies that showed that for *S. maritima*, increasing the concentration of Ca^{2+} had no significant effect on Na^+ influx, while the inhibitors TEA^+ and Cs^+ significantly decreased Na^+ influx and net Na^+ uptake) for the existence of two distinct low-affinity pathways for Na^+ uptake in *S. maritima*, one which may involve an AKT1-type channel in saline conditions, and for low salinites, a high-affinity K transporter proteins (HKT). Little is known about the regulation of proteins that mediate K^+ and Na^+ movement across the plasma membrane.

Halophytes have mechanisms to maintain cytosolic Na^+ concentrations during Na^+ accumulation in leaf tissues for osmotic adjustment. These mechanisms include the control of Na^+ transport at tonoplast and plasma membrane (Shabala and Mackay, 2011), and possibly the control of proton motive force (PMF)-generating enzymes (Flowers and Colmer, 2008), as the PMF, which drives ion transport into vacuoles, has been linked to an increase in Na^+/H^+ antiporter activity (Qiu et al., 2007). The role of pinocytosis in the transport of Na^+ and Cl^- remains under investigated.

In halophytes, vacuolar Na^+ sequestration, achieved by the tonoplast Na^+/H^+ antiporters, is an important physiological feature (Barkla et al., 1995; Glenn et al., 1999; Flowers and Colmer, 2008). Tonoplast Na^+/H^+ antiporters have been found to be stimulated, in several halophyte species, in response to salt, species such as *Salicornia bigelovii* and *Mesembryanthemum* (Parks et al., 2002). Pinocytotic vesicles have been reported in many halophytes, such as *S. altissima* and *Climacoptera lanata* (Kurkova and Balnokin, 1994; Kurkova et al., 2002; Balnokin et al., 2007), Pinocytotic vesicles may be essential for the removal of toxic ions from the apoplast and their transport into the vacuoles of

shoot cells, in halophytes (Kurkova and Balnokin, 1994). The high concentrations of ions within the vacuoles, which may be 4 to 5 times higher than in the cytosol, mean that Na^+ could leak back into the cytosol through cation channels; however, Maathuis et al. (1992), reported that in the vacuoles of *S. maritima*, tonoplast cation channels were closed.

1.2 Tolerance to flooding

Under flooded conditions, flooding-tolerant plants have several mechanisms that increase their ability to survive, by maintaining a good supply of oxygen to their inundated parts (Kozlowski, 1997). Mechanisms of flooding tolerance in plants involve the ability to develop a large adventitious root system (Jackson and Drew, 1984), aerenchyma formation and related features, for internal aeration (Armstrong, 1979; Colmer, 2003), and anoxia tolerance in tissues (Gibbs and Greenway, 2003).

1.2.1. Adventitious root

Under flooded conditions, two types of morphological adaptation have been reported; increases in root branching and the production of adventitious roots which grow horizontally and close to the water surface (Hook and Crawford, 1978).

The production of adventitious roots is a general morphological adaptation of wetland plants and may be essential in flooding tolerance of some species (Jackson and Drew, 1984). Some plants growing in wetland produce aquatic adventitious roots from the stem, roots that stay suspended in the water column. In the flood-tolerant species *Meionectes brownii* (Rich et al., 2010; Rich et al., 2012), *Cotula coronopifolia* (Rich et al., 2012) and also in the flood-tolerant halophyte *Tecticornia pergranulata* (Rich et al., 2008), these adventitious roots contain chloroplasts in the cortical cells, and are able to carry out photosynthesis, providing a source of oxygen to the waterlogged plants. Some authors have suggested that adventitious roots compensate physiologically for the failure of rotted portions of the original root system under waterlogging, as adventitious root production and flood tolerance are frequently related (Clemens et al., 1978; Senagomes and Kozlowski, 1980a, 1980b). Adventitious roots in numerous wetland species dominate root biomass; for example, in wetland halophytes such as *Sporobolus*

virginicus (Naidoo and Mundree, 1993), *T. pergranulata* (*Halosarcia pergranulata*) (Pedersen et al., 2006) and also in glycophytic *Rumex spp* (Laan et al., 1989).

The effect of the interaction of salinity and waterlogging on the production of adventitious root has been little considered. The growth of adventitious roots is negatively affected by the salinity of the flood water in some plant species (Colmer and Flowers, 2008). Naidoo & Mundree (1993) reported that in *Sporobolus virginicus* grown under high salinity (400 mM NaCl), the aquatic roots were unaffected. Adverse effects of salinity (49-60 ds m^{-1}) were noted on a halophytic tree *Melaleuca ericifolia* (Salter et al., 2007), and a salt-tolerant semi-xerophytic tree *Eucalyptus camaldulenis* (van der Moezel et al., 1988), while a study on a halophytic perennial grass (*Sporobolusvirginicus* showed no adverse effect on adventitious root development (Naidoo and Mundree, 1993). Van Der Moezel et al. (1988) reported that *Casurina obese* produced adventitious root in both saline water (42 dS m^{-1}) and non-saline water, although in saline water adventitious root growth was reduced as compared with adventitious root growth in non-saline water.

1.2.2 Aerenchyma and internal O_2 movement

One of the most significant anatomical adaptations to waterlogging is the development of aerenchyma, which increases the transfer of oxygen from the aerial parts to the roots, and, via radial loss of oxygen, may also create an oxidized zone around the roots (Armstrong et al., 1994; Jackson and Colmer, 2005). In natural conditions, shoots containing aerenchyma can transport much-needed oxygen to the roots (Mustroph and Albrecht, 2007) and so shoots have an important role to play in supporting root tolerance of oxygen deficiency (Schluter and Crawford, 2001; Mustroph and Albrecht, 2007), as has been known for many years (Hook and Crawford, 1978; Armstrong, 1979; Justin and Armstrong, 1987). Hypoxia or ethylene can stimulate the collapse or death of a proportion of root cortical cells, leading to the formation of aerenchyma (Drew et al., 2000; Gunawardena et al., 2001). Stelzer and Läuchli (1977) reported that salt regulation and growth were both improved under the interaction of salinity and waterlogging, by the development of aerenchyma in *Puccinellia peisonis.* Although there are many halophytic species that use aerenchyma to tolerate waterlogging, there are waterlogging tolerant species that do not have aerenchyma as shown in Table 1.1.

Table 1.1 Shows some halophytic species and whether or not their roots contain aerenchyma

Species	Aerenchyma	References
Spartina alterniflora	YES	Maricle and Lee, 2002
Spartina anglica	YES	Maricle and Lee, 2002
Corispermum puberulum	NO	XIN et al., 2002
Salsola komarovii	NO	XIN et al., 2002
Dianthus chinensis L.	NO	XIN et al., 2002
Cynanchum thesioides	NO	XIN et al., 2002
Suaeda glauca	YES	XIN et al., 2002
Puccinellia peisonis	YES	Stelzer and LÃ¤uchli, 1977.
Spartina patens	YES	Burdick, 1989
Sporobolus virginicus (L.) Kunth	YES	Donovan and Gallagher, 1985
Tecticornia pergranulata	YES	Rich et al., 2008; English and Colmer, 2011
Tecticornia indica	NO	English and Colmer, 2011
Tecticornia mellaria	NO	English and Colmer, 2011
Juncus bufonius	Increased root porosity under flooding	Justin and Armstrong, 1987
Rumex maritimus	Ditto	Justin and Armstrong, 1987
Lysimachia nummularia	Ditto	Justin and Armstrong, 1987
Ranunculus acris	Ditto	Justin and Armstrong, 1987

A large survey of the development of root aerenchyma was conducted by Justin and Armstrong (1987), in a study of 91 species including non-wetland, intermediate, and wetland plants, grown in pots under non-saline flooded conditions. Justin and Armstrong (1987) found that in halophytic plants, the root porosity was ~2 to 4% in *Lepidium latifolium, Suaeda maritima, Halimione portulacoides* and *Juncus bufonius*, ~3 to 33% in *Salcornia europaea, Spartina anglica, Cochlearia anglica*, and *Puccinellia maritima*; and in *Phragmites australis*, it was 52%. Colmer and Flowers (2008) concluded that from the data available; glycophytes have a similar range of porosity to halophytes.

Aerenchyma is only able to deliver oxygen in anaerobic conditions over short distances. Armstrong (1979) identified a chain of correlations between root porosity, the maximum length of an internal aerobic pathway, and oxygen utilization. The correlation suggests that in cereal roots, the rate of oxygen utilization at 15% porosities would be sufficient for oxygen transport to 10-20 cm within roots (Armstrong, 1979). A review by Barrett-Lennard (2003) concluded that in deep-rooted plants, aerenchyma formation is not likely to be of great advantage when plants are exposed to sudden flooding.

Little data is available, however, on the interaction of high and low salt with waterlogging. Donovan and Gallagher (1985) reported that when *Sporobolus virginicus* was grown under hypoxic, fresh water conditions, the roots contained more aerenchyma (32%) than roots grown under hypoxic, salt water (~410 mM Na^+) conditions (28%). In the same species, the air spaces in the culm were reduced in response to salinity; the aerenchyma declined from 30–32% to ~22% when salinity was increased from 0 or 100 mM to 200 or 400 mM NaCl (Naidoo and Mundree, 1993). This is in contrast to *Spartina alterniflora* and *S. patens*, which were grown in various salinities, from 25 to 325 mM NaCl, and the root porosity was not effected (Naidoo et al., 1992). It has been suggested that other characteristics may also improve the diffusion of oxygen in roots, such as the number of laterals, root diameter, relative stele volume, and barriers to radial oxygen loss (ROL) (Armstrong, 1979; Colmer, 2003). The work of this thesis includes both morphological and anatomical studies of roots from plants grown in low and high salinity, and hypoxic and aerated conditions, for long periods of time, as well as biochemical investigations into the production of lactate and sugar (reported in Chapter 4).

1.2.3 Anoxia tolerance

In salt-marsh plants flooded in seawater for periods of hours, in middle and low marsh soils, aerenchyma cannot provide sufficient oxygen to the portion of root under extremely reduced conditions (Mendelssohn et al., 1981; Burdick and Mendelssohn, 1990). Armstrong (1979). Armstrong et al. (2000) concluded that some roots tissue, such as the stele and apices, as well as deeper portions of roots, may experience a lack of oxygen while other portions remain aerated. Under these conditions, tolerance of anoxia might be essential during periods of flooding. Upon root exposure to anoxia, the level of ATP decreases by approximately 65-97%, as a result of the inhibition of oxidative phosphorylation, as compared with roots grown in aerated conditions (Gibbs and Greenway, 2003). Many researchers have suggested that the ability of plants to survive under anoxic conditions depends on the species, the organ and the carbohydrate supply (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003). Anaerobic catabolism of carbohydrates, combined with the regulation of adenosine triphosphate (ATP) consuming processes, allows survival during anoxic conditions (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003).

There are two fundamental biochemical adaptations to flooding, as described by Crawford (1989). The first is adaptation to hypoxia or brief anoxic conditions, and requires the regulation of glycolysis and ethanol formation, as well as the accumulation of malate, due to anaerobic respiration. The second is adaptation to periods of prolonged flooding, which requires an increase in glycolysis, ethanol or lactate formation, and ATP synthesis (Fig 1.2). An example is the flooding-tolerant plant *Nyssa sylvatica* var. *biflora* which increases ADH activity of the enzyme alcohol dehydrogenase (ADH) in order to remove toxic concentrations of acetaldehyde and replenish the NAD^+ needed for glycolysis; these changes are accompanied by increases its storage of starch for use in growth and anaerobic respiration (Angelov et al., 1996). Such adaptations do not allow plants to tolerate complete anoxia for very long, and the lack of oxygen has both direct and indirect effects on all physiological processes (Hook and Crawford, 1978) (see Chapter 4 for more information on anoxia tolerance).

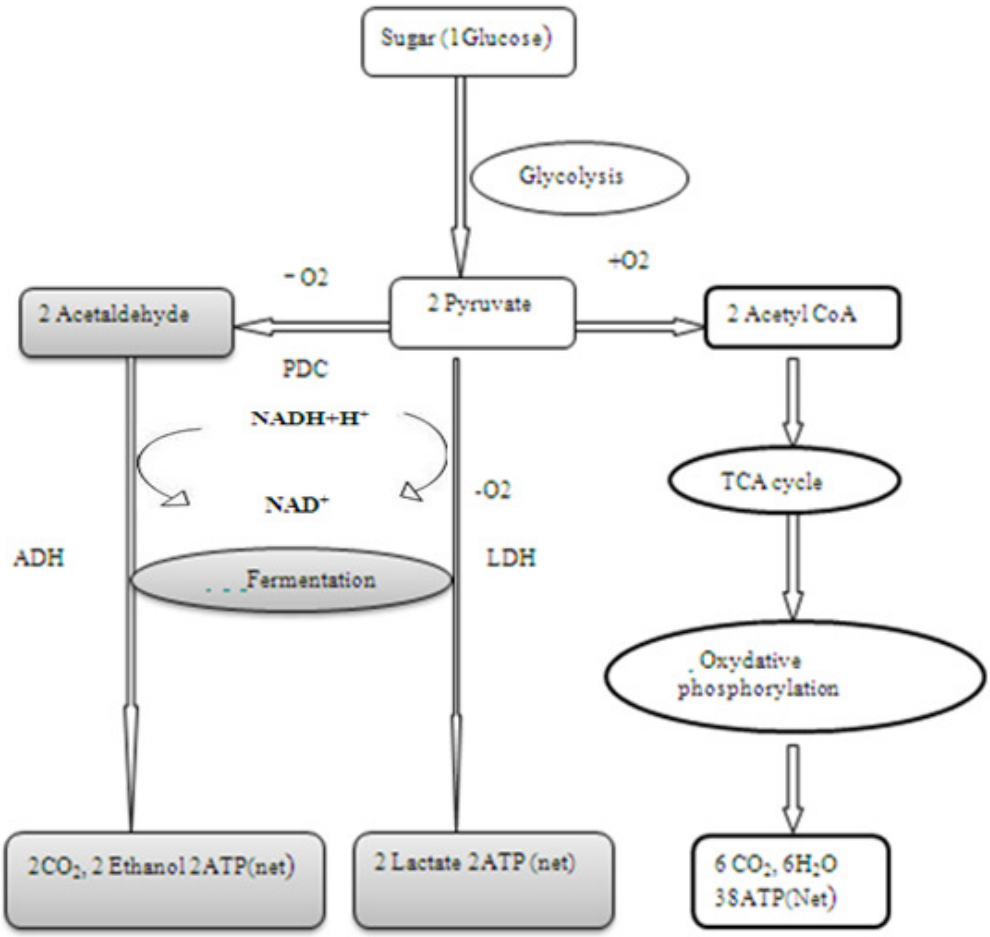


Figure 1.2 A diagram showing the shift from aerobic respiration in (mitochondria) to anaerobic respiration in (cytosol) (modified from Hossain and Uddin 2011).

1.3 The effects of combination of waterlogging and salinity on plants with an emphasis on halophytes

1.3.1 The halophyte *Suaeda maritima*

The halophyte *Suaeda maritima* (L) Dum. or annual sea-blite, it is a member of the Amaranthaceae, an extensively occurring family, including many halophytes which survive in "the saltiest niches in moist salines" (Waisel, 1972). The genus *Suaeda* consists of about 110 species in Wikipedia and 57 accepted names in 'The Plant List' (http://www.theplantlist.org/), ranging from the smaller annuals (10-30 cm in height) typically found in wet, saline habitats of the northern hemisphere (coastal or inland), to the larger (up to 4 m) perennials such as *S. monoica*, found in the tropical saline regions of the Middle East.

Suaeda maritima is a 'hairless annual, prostrate to erect, and its height ranges from 10 to greater than 30 centimetres' (Clapham et al., 1959). It is often purple or reddish, with cylindrical, narrow shoots, thick alternate leaves, and greenish, small, inconspicuous hermaphrodite flowers in the axils of the leaves. The seeds are shiny and black, biconvex in shape, and approximately 1.1-2 mm in diameter. The plant is common on the salt marshes of Britain and northwestern European, between low and high tidal movements, flowering from June to October (Clapham et al., 1959).

The genus *Suaeda* may have some economic potential; it has in the past been used as a source of food (Moerman, 1998), and may be a source of unsaturated fats, suitable for human consumption (Weber et al., 2004). *S. maritima* has been used to study mechanisms of salt tolerance (see Appendix.1.1), though only a few studies have been published on the effect of waterlogging and salinity combined (Wetson et al., 2008; Wetson and Flowers, 2011; Zhang et al., 2010; Colmer et al., 2012; Wetson. et al., 2012)

Figure 1.3 Photograph shows *Suaeda maritima* plants in Cuckmere Haven salt-marsh taken by T. J. Flowers.

Figure 1.4 Photograph of Shoreham-by-Sea salt-marsh on the east bank of the River Adur taken by A. M. Wetson. A distinct lower and high *Suaeda maritima* can be seen.

1.4 Research objectives

Chapter 2 the effect of salinity and waterlogging combined, on the halophyte *S. maritima*: the role of antioxidants

Chapter 2 reports tests of the hypothesis that:

- Antioxidant capacity is greater in plants of flooded with saline water as opposed to those growing in well-drained conditions, as a means of defence against high levels of ROS brought about by flooding.

Data were obtained from growth experiments conducted in a simulated tidal flow system in a glasshouse, where variation of the environmental conditions could be minimised between treatments to differences in flooding, and from plants collected from their natural habitat. The shoot dry weight, the levels of reduced glutathione (GSH), disulphide glutathione (GSSG), malonyldialdehyde content (MDA), polyphenolic compounds, 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and superoxide anion radical scavenging activity were measured in shoots from plants growing in drained and flooded conditions.

Chapter 3 uptake of trace metals under a combination of waterlogging and salinity in *S. maritima*

The aim of this investigation was to determine the effect of the interaction of salinity and waterlogging on concentrations of heavy metals (Mn, Fe, Cu and Zn) in leaves, roots and root morphology of *S. maritima*, using four different methods: (1) plants grown with different Fe concentrations in the growth medium, (2) with different concentrations of Mn in the growth medium, (3) in the green house with tidal flow, and (4) in the growth chamber under different salt concentrations, in hypoxic and aerated conditions.

Chapter 3 reports tests of the hypotheses that:

- accumulation of Mn, Fe, Zn, and Cu in *S. maritima* plants is higher in hypoxic than in aerated conditions, particularly at low pHs in hydroponic experiments, which prevents the precipitation of micronutrients;
- the concentrations of heavy metals in leaves could be expected to be higher under high concentrations of Mn and Fe in the growth medium;
- high concentrations of transition metals could be accumulated in the roots under hypoxic conditions, preventing the build-up of toxic levels in the leaves;
- lower concentrations of transition metals might be expected in plants grown at high pH (ca 7-8) than at low pH (pH >5.5), and in those grown under high rather than low salt concentrations in the growth chamber experiments.

Chapter 4 mechanisms of waterlogging tolerance in *suaeda maritima*: morphological and metabolic adaptations under hypoxic and drained conditions, in varying concentrations of artificial seawater

Chapter 4 tested the hypotheses that:

- flooding would lead to an increase in the sugar content of the leaves and the roots, and an increase in the concentration of lactate in the roots;
- prolonged hypoxia would lead to the growth of adventitious roots and the development of aerenchyma in the root tissue.

Detailed examinations of root and shoot soluble sugar (sucrose, glucose and fructose) and root lactic acid concentrations, and the anatomical characteristics of the roots for plants grown under different concentrations of artificial seawater, in hypoxic and aerated conditions in the growth chamber are presented.

Chapter 5 the importance of controlled transpiration to regulate sodium accumulation in *S. maritima.*

The aim of this study was to achieve more extensive data on the importance of transpiration rate in the control of sodium uptake, growth and ion accumulation in *S. maritima*, in growth chamber conditions and at different salt concentrations, in aerated and hypoxic conditions.

Chapter 5 tested the hypothesis that:

- plants reduce transpiration rates under hypoxic (low and high salt) conditions as compared with aerated (low and high salt) conditions, thereby preventing the accumulation of high levels of Na^+ in their shoots, by lowering bypass flow and diminishing stomatal opening, at the same time increasing water use efficiency as a mechanism of salinity tolerance.

Chapter 6 summary and conclusions

This Chapter draws conclusions from the results of all Chapters and suggests areas of future research.

Chapter 2 : The effect of combined salinity and waterlogging on the halophyte *Suaeda maritima*: the role of antioxidants

2.1. Introduction

Apart from their fundamental tolerance to salinity, some halophytes are also adapted to live in areas flooded with saline water, such as coastal salt marshes (Colmer and Flowers, 2008). Tides flood some areas seasonally, monthly, or daily with flood duration and depth that depends on elevation (Armstrong *et al.*, 1985). In low-lying areas soil may be anoxic due to frequent flooding with seawater over long periods of time, while in higher areas of a salt marsh the soils are well aerated (Wetson, 2008), although salinity may differ from the lower elevations depending on rainfall. Plants have a variety of mechanisms to cope with flooded conditions, including the development of large adventitious roots (Section 1.2.1), the formation of aerenchyma (Section 1.2.2) and increasing the activity of enzymes involved in fermentation and glycolysis (Section1.2.3) (Colmer and Flowers, 2008). Plants can decrease their oxygen demands by lowering their respiration rate, increasing the availability of soluble sugars for metabolism and growth, and significantly improving antioxidant protection through the synthesis of phenolic compounds and glutathione (Colmer and Voesenek, 2009; Noctor and Foyer, 1998; Parida et al., 2004b; Perata et al., 2011).

S. maritima is a common species on saline areas of the northern hemisphere. It grows in both inland and coastal wetland, such as British salt marshes, between high and low tides, from April to October (Clapham and Warburg, 1959; Wetson and Flowers, 2011) and commonly shows optimal growth between 170 and 340 mM NaCl (Flowers, 1972; Thiyagarajah et al., 1996). Although much work has been done on the effect of salinity on growth of *S. maritima*, there is less information on its response to the combination of salinity and waterlogging. *S. maritima* is able to grow under a combination of salinity and waterlogging, with a high level of fermentative enzyme activity (Wetson. *et al.*, 2012), despite increased net uptake of Na^+ to the shoots and decreased net uptake of K^+ and shoot growth at low oxygen levels (Wetson and Flowers, 2011). Recent studies of Song et al. (2011) have found that inland populations of *Suaeda salsa* adapt to the combination of waterlogging and salinity by producing adventitious root. However, the

response of the antioxidant system to combined salinity and waterlogging has received little consideration in any species of *Suaeda*.

Antioxidants can control the levels of reactive oxygen species (ROS; Section 1.1.2.3). During deep floods, the rate of photosynthesis can be limited by lowered light levels and decreased access to CO_2 (Colmer and Flowers, 2008). As a result, the photosynthetic electron transport chain (ETC) becomes over-reduced, causing the generation of several ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$) (Hoshida et al., 2000). ROS are also generated by over-reduction of the photosynthetic ETC in response to salt stress, when stomatal closure decreases the CO_2:O_2 ratio in the leaf tissue and inhibits CO_2 fixation (Hernandez et al., 1999).

Although ROS are important signalling molecules in the response to biotic and abiotic stresses, excessive levels cause oxidation of macromolecules such as lipids, proteins and nucleic acids resulting in a loss of cell function (Kranner et al., 2010). For example, lipid peroxidation, which causes a loss of membrane integrity and cell function (and can be measured as malondialdehyde, MDA, formation from added thiobarbituric acid) has been shown to increase during oxidative stress (Bailly et al., 1996). In some halophytes, a low concentration of MDA has been associated with salt tolerance (for example Sekmen et al., 2007). Stress tolerance is therefore a balancing act in which ROS are maintained at sufficient levels by antioxidants and other ROS-processing enzymes for signalling, but not so that they become deleterious to the plant (Kranner et al., 2010).

Production of glutathione is known to be induced in many plant species in response to environmental stresses, including salinity (Parida and Jha, 2010) and waterlogging (Goggin and Colmer, 2005). In cells, glutathione exists in a reduced form (GSH) and can donate electrons to detoxify free radicals, forming glutathione disulphide (GSSG); GSSG can be re-reduced back to GSH by the enzyme NADPH-dependent glutathione reductase. A reducing cellular environment, as provided by the maintenance of GSH levels, is vital to prevent oxidative damage to cells during waterlogging (Lesser, 2006) and is essential for cellular development under salinity (Zagorchev et al., 2012). For concentration-dependent redox couples such as the GSSG/2GSH couple, the redox state can be more accurately defined by the glutathione half-cell reduction potential

($E_{GSSG/2GSH}$), which is a better indicator of cell viability than concentration of GSH (or GSSG) alone in animal, plant and fungal cells (Kranner et a*l.*, 2006). When values of $E_{GSSG/2GSH}$ reach -180 mV to -160 mV, the buffering ability of the glutathione system fails, allowing ROS to attack molecules thereby leading to cell death (Kranner et a*l*., (2006). Phenolic compounds are also free radical scavengers and a relationship between polyphenolic compounds and antioxidant activity, as demonstrated by the ability to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion radicals, has also been reported in plants, including halophytes (for example, Ksouri et al., 2007).

The present study examines the hypothesis that antioxidant capacity is greater in plants of *S. maritima* flooded with saline water as opposed to those growing in well-drained conditions as a means of defence against high levels of ROS brought about by flooding. Plants were collected from a salt marsh and also grown in a glasshouse where variation of the environmental conditions could be minimised between treatments to differences in flooding. The levels of GSH, GSSG, MDA, polyphenolic compounds, DPPH and superoxide anion radical scavenging activity were measured in shoots of *S. maritima* from plants growing in drained and flooded conditions in both the natural and controlled conditions.

2.2 Materials and Methods

2.2.1. Plants materials and culture conditions

Plants of *S. maritima* were collected from two elevations with a difference in height of 0.6 m at a salt marsh at Shoreham-by-Sea, located on the East bank of the River Adur, West Sussex (UK) (TQ206060) in August 2010. Plants were also grown from seeds collected from Cuckmere Haven, East Sussex, (UK) (TQ515978). Seeds were germinated in sand and irrigated with a culture solution (see below) for 4 weeks before being transferred to plastic pots (9 cm deep and 9 cm diameter at the top) containing mixed (1:1, v/v) sand and estuarine mud collected from the Shoreham marsh. Plants were grown in one of six tanks

2.2.2 The tidal flow tank system

Six fibreglass tanks, each with a cross-sectional area of 1 m^2 and a depth of 20 cm, were supported on frames1 m above the ground and positioned in a block inside a glasshouse. A reservoir tank (280 L) was located under each fibreglass tank. Each fibreglass tank had a high overflow pipe 18 cm from one corner and a central inlet pipe connected to the reservoir tank beneath (Fig. 2.1 and Fig 2.2). The fibreglass tanks (upper tanks) were filled with washed pea gravel to a depth of 9 cm and the reservoir tanks (lower tanks) were filled with 280 L of culture solution (see below) made up in filtered diluted seawater. The seawater was collected from the English Channel. at 50 degrees 48 minutes North, 8 degrees 5 minutes West, and stored at the Brighton Aquarium (East Sussex, UK), The salinity required for the experiments (equivalent to half-strength fresh seawater, about optimal for the growth of *S. maritima*) was obtained by diluting the fresh filtered seawater with de-ionised water. A submersible pump placed in each reservoir tank pumped seawater up, through the central inlet pipe, into the tank of washed pea gravel above, up to a water depth of 18 cm. Afterwards, water would flow into the overflow tube, into the reservoir tank beneath, and from there would be re-circulated. In order to replace water lost through evaporation, deionised water was added to the tanks.

The system set up ensured that the gravel-filled tanks would be filled with water up to a maximum of 18cm, but not less than 9 cm deep, with circulating water. The pumps were set using a twenty four hour electrical timer, turned on for one hour twice daily to simulate tides; this was calculated as being the average length of time that plants growing at high-tide level would be waterlogged each day. The tanks took about 20 minutes to flood to the maximum level (Fig 2.3) and about 20 minutes to drain to the minimum level (Fig. 2.4).

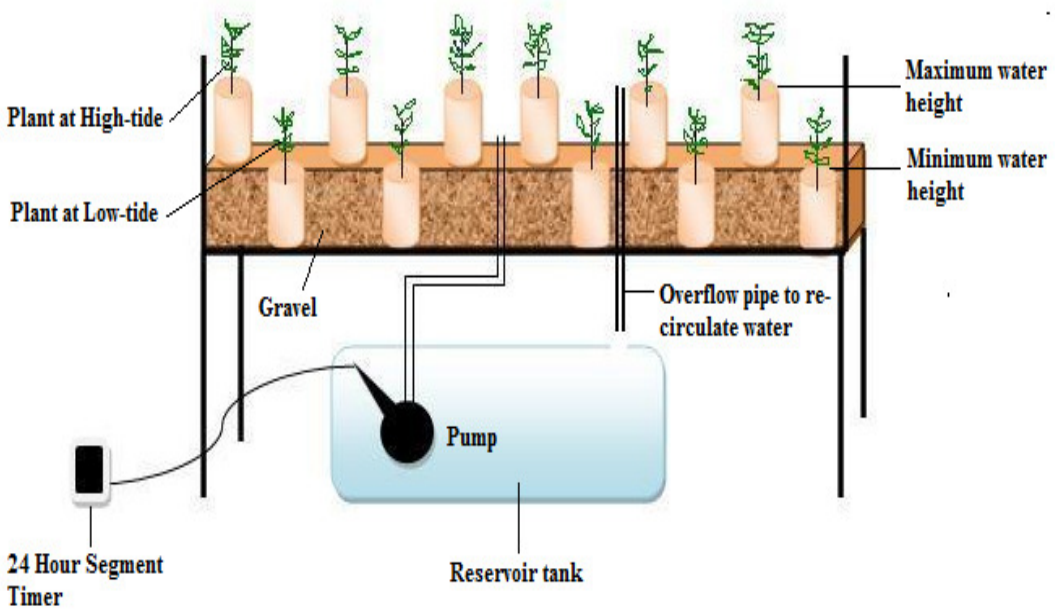


Figure 2.1 Diagrammatic representation of the simulated tidal flow tank system for creating fluctuation waterlogged conditions in pots of *Suaeda maritima.*

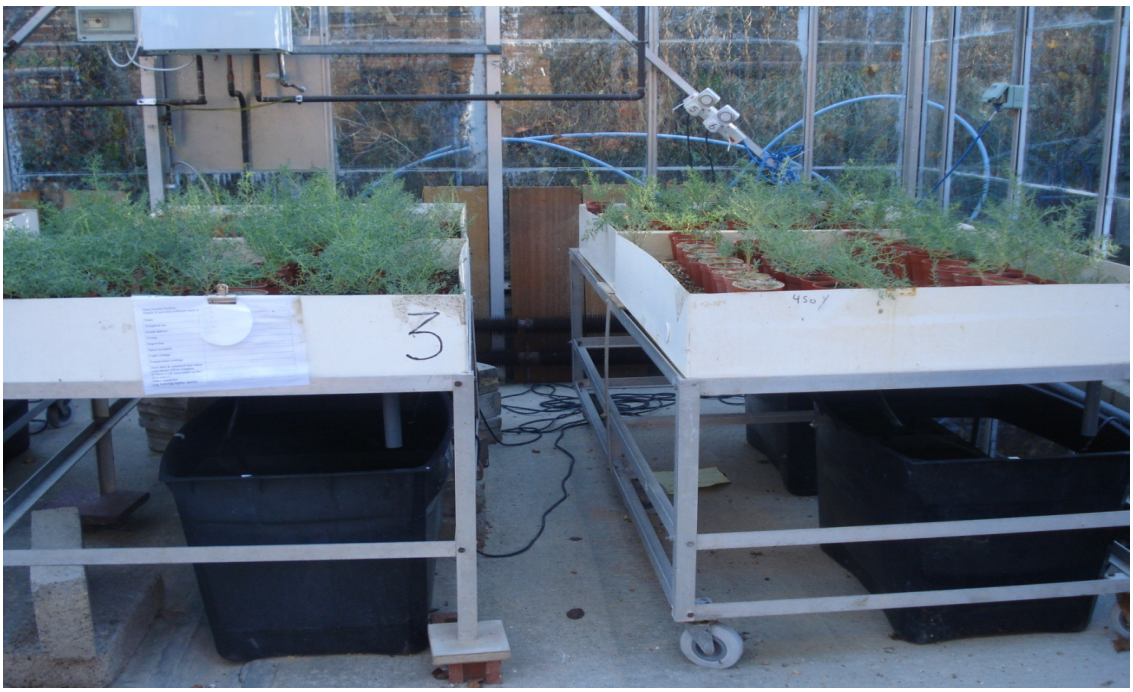


Figure 2.2 Shows photograph of the tanks with *Suaeda maritima* plants.

Figure 2.3 Photograph showing flooded plants of *Suaeda maritima* in the glasshouse tidal flow tank system.

Figure 2.4 Photograph showing drained pots of *Suaeda maritima* in the glasshouse tidal flow tank system.

The culture solution used was that of Stout & Arnon (1939) (for the Stout and Arnon solution, see Appendix 2.1) made up in filtered seawater. The filtered seawater was diluted to half its original concentration with water, then the Stout and Arnon salts were added.

The plastic pots, each containing one *S. maritima* plant growing in a sand/mud mix (see below), were 9 cm deep, with a top diameter of 9 cm. They each had a basal drainage hole, and were filled with growth medium. The plants to be 'flooded' were positioned within the gravel, at the base of the tank, so that they were in constantly waterlogged conditions. The other plant pots were positioned on the gravel surface, where they would be flooded only when the tanks were full of water (see Fig. 2.1). The 'high-tide' plants were therefore flooded for approximately 2 h per day, simulating the condition of plants at the high tide of a salt marsh. The flooded, 'low-tide' plants were constantly waterlogged, to simulate the condition of plants at the low tide of a salt marsh. All the pots were positioned at equal horizontal distances from each other.

Estuarine mud was collected from the Shoreham marsh. The estuarine mud was mixed with equal volumes of half-strength seawater and washed silver sand in a large trough. After thorough mixing by hand and removal by hand of any large matter, such as shells or debris, pots were filled with this mixture and left to drain overnight before the *Suaeda* seedlings were transplanted.

Plants were grown under controlled conditions in the glasshouse (with minimum temperatures of 24.0 in the day and - 17.0 °C at night, 60-75% relative humidity, 16/8 h light/darkness) for 8 weeks. Stout and Arnon (1939) culture solution, made up in half strength fresh seawater, was pumped for one hour twice daily to simulate tides. Electrical conductivity (EC) and pH in the tanks was measured every 2 d: the average EC was 29.6 ±0.03 dS m^{-1} and the average pH 8.2.±0.01

After collection from the greenhouse and the field, samples were either directly frozen in liquid nitrogen and stored at -80°C for determination of MDA and subsequently freeze-dried for glutathione content, or dried at room temperature for 4 weeks before being analysed for phenolic compounds and antioxidant activity.

2.2.3 Glutathione Analysis

Glutathione and glutathione disulphide were determined following the procedure of Kranner & Grill (1996), in which low molecular weight thiols were separated after derivitisation using reversed-phase HPLC. Five replicates of freeze-dried shoot (50 mg each) were ground in a pestle and mortar with liquid nitrogen to obtain a fine powder, to which was added 1 ml of 0.1 M HCl with 0.5% (v/v) Triton X-100 and polyvinylpolypyrrolidone (30 mg) followed by centrifugation at 13 000g for 40 minutes at 4 °C.

For the analysis of total thiols, 2-(N-cyclohexylamino) ethanesulfonic acid (CHES; 180 µl of 200 mM) buffer was added to the extract (120 µl), so that the combined pH was pH 9.3. Analysis of disulphides, unlike thiols, requires slightly different pHs, depending on species, and even tissue type. Before analysis of the plant samples, the ideal pH for glutathione analysis was determined for each species and tissue type. For plants grown in the glasshouse (drained and flooded), the ideal pHs for glutathione analysis were pH 8.3 and 9.7, respectively, and for plants grown in the field (high and low tide), the ideal pHs were pH 8.0 and 8.2, respectively. The pH of the CHES buffers used for glutathione analysis was adjusted using [2]HCl, DTT (30 µl of 3 mM) was then added and the mixture incubated for 60 minutes at room temperature, to reduce the disulphides to their corresponding thiols. The thiols were then labelled with monobromobimane (mBBr; 20 µl of 15 mM) for 15 minutes in the dark, and the reaction was stopped by the addition of 0.25 % methanesulfonic acid (MSA, 250 µl).

To analyse the thiol disulphides, extract (400 µl) was combined with CHES (600 µl, pH as mentioned above) and N-ethylmaleimide (NEM, 30 µl of 50 mM) to block the free thiol groups. After 15 minutes of incubation at room temperature, excess NEM was removed by toluene extraction (five times of equal volumes of toluene). After this, NEM-treated extract (300 µl) was combined with DTT (30 µl of 3 mM) to reduce the disulphides to thiols and labelled with mBBr as before. Prior to analysis on the HPLC, samples were centrifuged for 20 minutes.

[2]The objective was to achieve pH 8-8.3 when 400 µl extract and 600 µl of CHES buffer (or 120 µl extract and 180 µl CHES buffer) were mixed together. For plants grown in the glasshouse (drained and flooded), the pH of the CHES buffer needed to be pH 8.3 and 9.7, and for plants grown in the field (high and low tide), the pH of the CHES buffer was pH 8.0 and 8.2, respectively.

To separate low-molecular-weight thiols, reversed-phase HPLC on a HiQsil RP18 column (150 x 2.1 mm i.d., 3 µm particle size; KyaTech, Japan) was used, and thiols were detected fluorimetrically (emission: 480 nm; excitation: 380 nm) using a gradient elution of 0.25% (v/v) acetic acid in dH_2O at pH 3.9/methanol. Glutathione was separated from other low- molecular-weight thiols, γ-glutamyl-cysteinyl, cysteinyl-glycine and cysteine (Fig 2.5). Different concentrations of these molecular weight thiols (Sigma Aldrich, UK) were prepared to construct calibration curves.

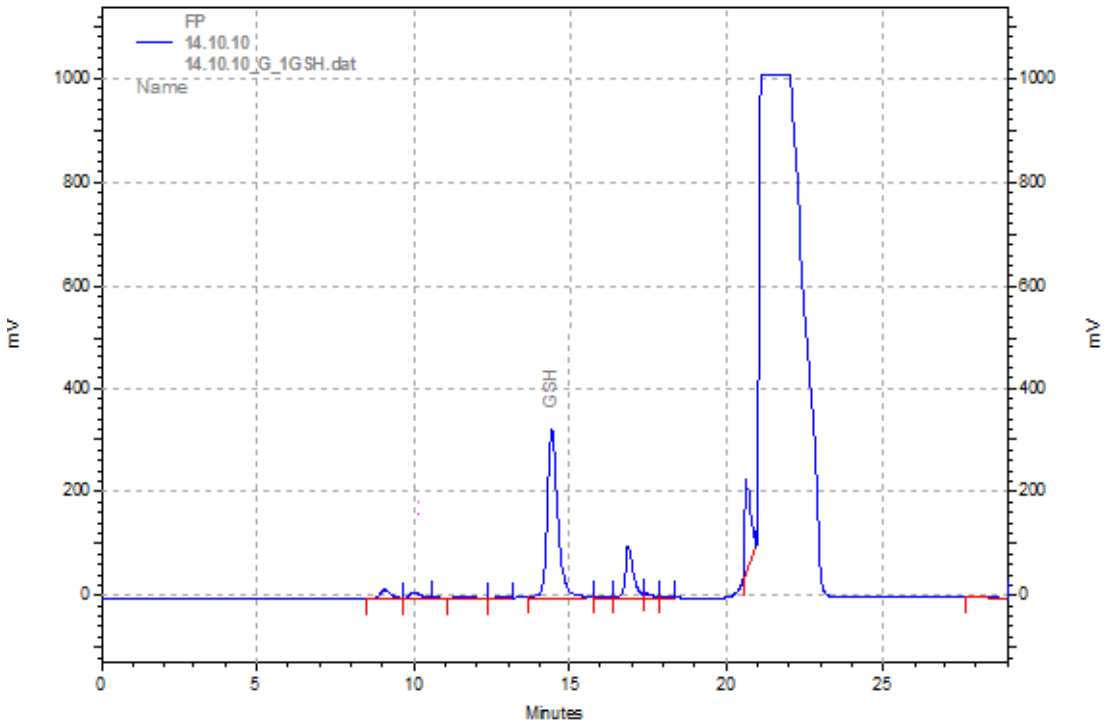


Figure 2.5 Shows glutathione GSH chromatography for glutathione by reversed-phase HPLC

2.2.3.1. Glutathione half-cell reduction potential ($E_{GSSG/2GSH}$)

Glutathione half-cell reduction potential was calculated using the following Nernst equation (Schafer and Buettner, 2001; Kranner et al., 2006): with a slight modification to the calculated value of the standard $E_{GSSG/2GSH}$ (see below):

$$E_{GSSG/2GSH} = E^{0'} - \frac{RT}{nF} \ln \frac{[GSH]^2}{[GSSG]}$$

Where R represents the gas constant (8.314 J K^{-1} mol^{-1}); T, temperature in degrees Kelvin; n, number of transferred electrons; F, the Faraday (9.6485 x 10^4 C mol^{-1}); [GSH] and [GSSG] are molar concentrations of GSH and GSSG, estimated using the shoot water content (WC) and assuming the density of water to be approximately 1 g ml^{-1}; E°′, standard half-cell reduction potential at pH 7.4 [$E°'_{GSSG/2GSH}$ = -264 mV];. The value of E°′ was taken at pH 7.4 based on data from salinized barley (Carden et al., 2003).

2.2.4. Lipid peroxidation

Lipid peroxidation was estimated spectrophotometrically by determining the amount of MDA formed in the leaves, using the thiobarbituric acid method of Hernandez and Almanas (2002). Leaves (200 mg FW) were ground with trichloroacetic acid (TCA, 2 ml of 0.1%) and after centrifugation (10 min at 15000xg, at 4 °C), the supernatant (0.5 mL) was mixed with thiobarbituric acid (1.5 ml TBA, 0.5% in 20% of TCA) and incubated for 20 min at 90 °C. An ice bath was used to stop the reaction, the samples were centrifuged (5 min at 10000xg) and the absorbance was measured at 532 nm and 600 nm. The MDA concentration (five replicates per treatment) was determined using the extinction coefficient of 155 mM^{-1} cm^{-1} in the following equation: MDA (nmol g^{-1} FW) = ((A_{532} - A_{600}) x 1000 x DF) / 155 x FW x $10^{-3.}$

Where FW is fresh weight, A_{532} and A_{600} are the sample's absorbances at 532 and 600 nm, respectively and DF is the dilution factor.

2.2.5. Preparation of plant extracts for determination of total phenolic compounds and antioxidant activities

Harvested plants from the field and glasshouse were dried by moving them twice a day at room temperature accordance with the practice described in the publications in the literature (Ksouri et al., 2007; 2008) for 4 weeks. Dry shoot material (1 g) was weighed and homogenized with methanol (10 ml), stirred for 30 min, then kept at 4 °C for 24 h. The extract was filtered through Whatman filter paper No.4, before being evaporated to dryness under vacuum, and then stored at 4°C until required (Mau et al., 2001).

2.2.5.1. Determination of total phenolic content

The total phenolic content of *S. maritima* samples (0.1 g mL^{-1} methanolic extract) was evaluated using Folin-Ciocalteu reagent and gallic acid (GAE) as a reference, as described by Dewanto et al. (2002). Extract (125 µL of a 10-fold diluted sample) was added to Folin-Ciocalteu reagent (0.125 ml) and deionized water (0.5 ml). The mixture was shaken and left to stand for 6 min, then Na_2CO_3 solution (7%, 1.25 ml) was added. The mixture was then diluted with de-ionized water to obtain a 3 ml final volume and mixed thoroughly. The mixture was incubated at 23 °C for 90 min, and the absorbance was read at 760 nm, against a blank. The total phenol content of the sample was determined as mg gallic acid equivalents (GAE) g^{-1} DW, using a calibration curve with gallic acid. The range of the calibration curve was 50-400 µg ml^{-1} (R^2=0.99). Samples were analysed in triplicate. The results were expressed as milligrams of GAE equivalents per gram of sample (DW, dry weight).

2.2.5.2. DPPH radical scavenging activity

The method for determining radical scavenging activity was that commonly used to estimate a plant extract's ability to scavenge the free radicals produced by DPPH (Chung et al., 2006; Mhamdi et al., 2010). This is achieved by measuring the bleaching of the purple DPPH solution, as described by Hatano et al (1988). Different amounts of plant material (1, 10, 50, 100, 1500, 2000 and 2500 µg ml^{-1}) were prepared by addition of pure methanol to the dried sample extract. DPPH (methanolic solution, 0.5 ml of 0.2 mM) was added to 2 ml of extract. The mixture was vigorously shaken and placed in the dark for incubation, for 30 min at ambient temperature. The absorbance of samples was measured at 517 nm and related to the extract's ability to bleach the purple DPPH, to the yellow-coloured diphenylpicryl-hydrazine (Fig 2.6).

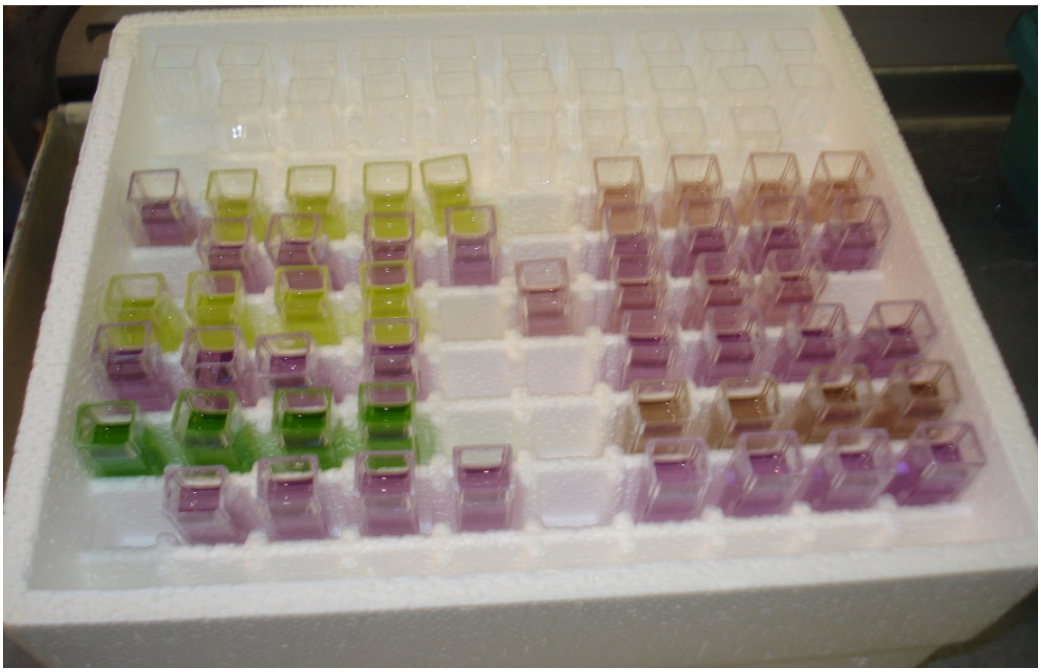


Figure 2.6 Photograph shows purple colour of DPPH reagent was bleached by plant extract

The expression of antiradical activity was as IC_{50} (mg ml^{-1}), the antiradical amount required to cause 50% inhibition. A higher antioxidant activity results in a lower IC_{50} (Patro et al., 2005). The sample's ability to scavenge DPPH radicals was calculated using the following formula (Ksouri et al., 2007):

DPPH scavenging effect (%) = $(A_0-A_1)/A_0$ x 100

Where A_0 is the absorbance of the control at 30 minutes (the control contained DPPH only, without plant extract) and A_1 is the absorbance of the sample at 30 min. The results were expressed as IC_{50} (mg ml^{-1}), the concentrations of plant extract required to cause a 50% inhibition of DPPH radical activity, which was obtained by plotting the concentrations of plant extract against DPPH scavenging %.

2.2.5.3 Superoxide anion radical scavenging assay

Superoxide anion radical scavenging was assayed spectrophotometrically, using phenazine methosulphate (PMS), according to the method of Duh et al. (1999). The reaction mixture contained sample (0.2 ml) in a methanolic extract (1, 10, 50, 100, 200 and 400 μg ml^{-1}), NADH (0.2 ml of 677 μM), PMS (0.2 ml of 60 μM) and Nitro Blue Tetrazolium (NBT, 0.2 ml of 144 μM), all in a phosphate buffer (0.1 mol L^{-1}, pH 7.4

made fresh on the day of assay). The addition of PMS marked the beginning of the reaction, after which the mixtures were incubated at room temperature for 5 min, and the absorbance recorded at 560 nm against a control (the same reaction mixture without the extract), and the Correction tube (the same reaction mixture without NADH; the absorbance of this tube is due to superoxide anions present in the extract). The IC_{50} values were calculated using a method similar to that described for DPPH. Eliminate initially

2.2.6 Analysis of data

Statistical analysis was carried out by two-way ANOVA using SPSS version 18 (SPSS, Inc., Chicago, IL). Means are reported with their standard errors; different letters indicate significant difference of the mean from post-hoc Tukey tests.

2.3 Results

2.3.1 Dry weight

Plants grown in drained conditions had a significantly greater dry weight than those in the flooded conditions, in both the natural and controlled environments ($P < 0.001$). In their natural habitat, the dry weight of plants growing at the lower elevation (2.6 ± 0.2 g per plant) was 32% lower than that of plants growing 0.6 m higher up the salt marsh (3.8 ± 0.1 g per plant). Under controlled conditions, the dry weight of the flooded shoots (0.7 ± 0.04 g per plant) was 67% lower than that of shoots grown in the drained conditions (2.1 ± 0.1 g per plant).

[

2.3.2. Glutathione content

As shown in Fig. 2.7, plants grown in flooded conditions, in both natural and controlled environments, showed higher total glutathione ([GSH+GSSG]) and [GSH] than in drained conditions and more negative $E_{GSSG/2GSH}$ values ($P < 0.001$). The [GSSG] (Fig. 1) was lower in the flooded plants than in those growing under drained conditions ($P < 0.001$).

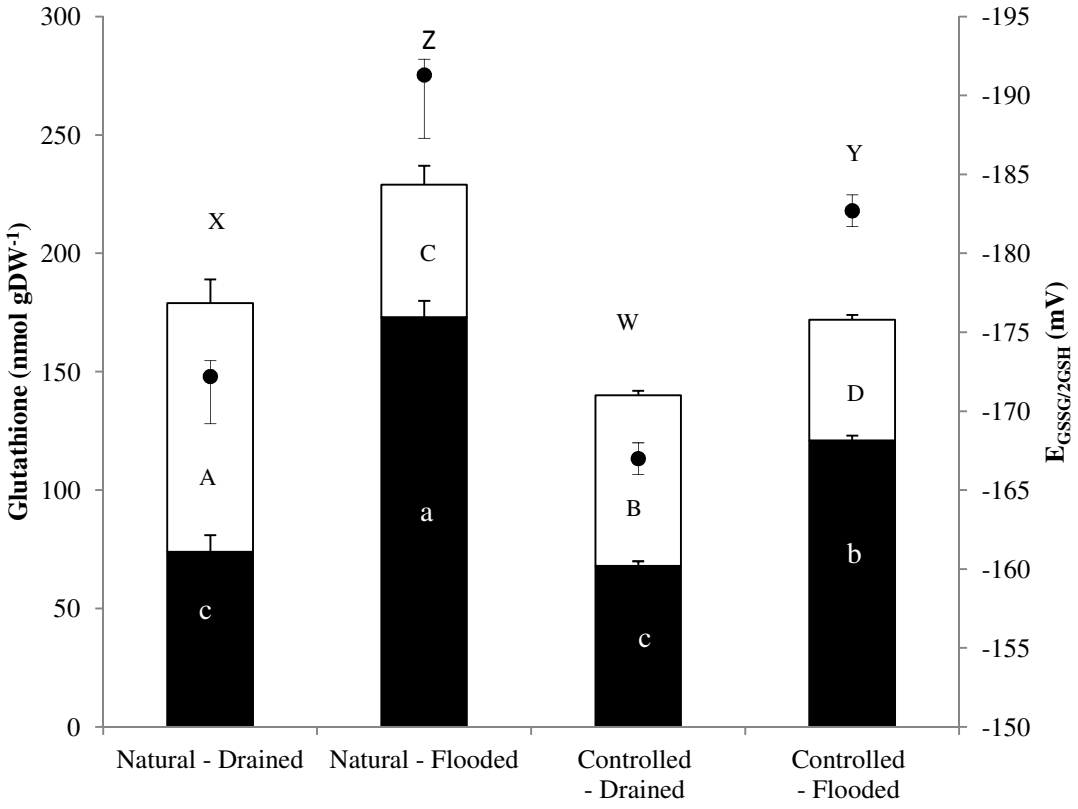


Figure 2.7 concentration of GSH (dark bars), GSSG (white bars) and EGSSG/2GSH (closed dots) in *S. maritima* grown under drained and flooded conditions, in the field and a glasshouse. Values are mean ± standard error (n = 5). Different letters indicate significant difference of the mean from post-hoc Tukey tests ($P < 0.05$).

2.3.3. MDA

MDA levels showed a similar pattern to [GSSG] in response to flooding (Table 2.1). MDA concentrations were lower in plants growing under flooded than well drained conditions in both natural and controlled environments ($P < 0.001$).

Table 2.1 MDA concentration in *S. maritima* grown under natural and controlled conditions. In the natural conditions, 'high marsh' plants grew at an elevation 0.6 m above those of the 'low marsh' plants, while under the controlled conditions of the glasshouse, plants were grown in mud and 50% sand at two heights in tanks subjected to flooding twice daily. On harvesting, plants were directly frozen in liquid nitrogen and stored at -80 °C for determination of MDA. Values are mean ± standard error (n = 5). Different letters indicate significant difference of the mean from post-hoc Tukey tests ($P < 0.05$).

Conditions	Elevation or Treatments	MDA (nmol g^{-1} DW)
Natural	High marsh (Drained)	97 ± 2 a
	Low marsh (Flooded)	67 ±.3 b
Controlled	Drained	48 ± 3 c
	Flooded	30 ± 1 d

2.3.4. Polyphenol content

Flooded and drained conditions had a significant effect on polyphenol concentration (Table. 2.2). In the natural habitat, the polyphenol concentration in shoots of flooded plants was 80% of that from drained plants, whereas under controlled conditions, polyphenol in shoots from flooded plants was almost 147% of that of drained shoots.

2.3.5. DPPH and superoxide anion scavenging activity

Antioxidant activity was evaluated by the ability of the samples to quench DPPH and superoxide anion radicals. Flooding significantly decreased IC_{50} values of superoxide ($P < 0.001$) under natural and controlled conditions and IC_{50} of DPPH ($P < 0.05$) in the controlled environment of the glasshouse (Table 2.2).

Table 2.2 The total polyphenolic concentration and free-radical scavenging activity in *S. maritima* grown under natural and controlled conditions. The growth conditions were as described in Table 1. Plants were dried at room temperature for 4 weeks before being analysed for phenolic compounds and antioxidant activity. The data for polyphenolic concentration are means of 12 replicates and for DPPH and superoxide anion activities of 4 replicates (± standard error). Different letters indicate significant difference of the mean from post-hoc Tukey tests ($P < 0.05$).

Conditions	Treatments	Total polyphenolic concentration	Free-radical scavenging activity	
		(mg GAE g^{-1} DW)	DPPH IC_{50} (µg ml^{-1})	Superoxide anion IC_{50} (µg ml^{-1})
Natural	High marsh (Drained)	230 ± 2.0 a	180 ± 7 a	6 ± 0.8 a
	Low marsh (Flooded)	183 ± 1.0 b	160 ± 0 a	2 ± 0.2 b
Controlled	Drained	74 ± 1.0 c	835 ± 68 b	14 ± 0.8 c
	Flooded	109 ± 3.0 d	666 ± 41 c	5 ± 0.5 a

2.4 Discussion

In this study, the antioxidant capacity in plants of the halophyte *S. maritima* flooded with saline water was compared to that of plants in well-drained conditions, under both natural (salt marsh) and controlled (glasshouse) conditions. Under flooded conditions, plants were able to complete their life cycle and demonstrated tolerance to saline flooding, as previously reported by Wetson (2008). However, flooded plants had lower shoot biomass, most likely due to oxygen deprivation in the root medium (Colmer *et al*., 2012; Wetson *et al*., 2012). Under waterlogged conditions, oxygen deficiency restricts respiration of the roots, thereby decreasing the activity of H-ATPase in the membranes, ultimately inhibiting Na^+ exclusion and K^+ uptake (Barrett-Lennard, 2003). The deleterious effect can be seen in plants on some salt marshes, where tidal flooding and the resultant decrease in oxygen levels leads to lower biomass of halophytes in low marsh areas relative to upper marsh (Wetson, 2008) and can influence the distribution of

species (Armstrong et al., 1985). Although *S. maritima* is able to grow under a combination of salinity and waterlogging, with a high level of lactate dehydrogenase activity (Wetson. *et al.*, 2012), the interaction between waterlogging and salinity has a clear effect on plant biomass.

Increasing stress, including under salinity and low oxygen concentrations, causes excessive formation of ROS (Blokhina et al., 2003; Kranner et al., 2010) and has been shown to induce raised antioxidant capacity in numerous plant species, including halophytes such as *Salicornia brachiata, Bruguiera parviflora* and *Aegiceras corniculatum* (Parida *et al*., 2004a; Parida *et al*., 2004b; Parida and Jha, 2010). The antioxidant defence system is a key component of stress tolerance due to its ability to maintain ROS at safe levels. Glutathione is a major intracellular water-soluble antioxidant and production of glutathione is known to be induced in many plant species in response to environmental stresses, including salinity (Parida *et al*., 2004a; Parida and Jha, 2010) and waterlogging (Arbona *et al*., 2008; Goggin and Colmer, 2005). In plants of *S. maritima*, saline flooding induced significantly higher concentrations of total glutathione and GSH, a lower GSSG concentration and more negative values of $E_{GSSG/2GSH}$. At both elevations, $E_{GSSG/2GSH}$ was lower than -160 mV which is considered the threshold above which plant stress generally becomes lethal (Kranner *et al*., 2006). As far as I am aware, there are no other data on $E_{GSSG/2GSH}$ nor glutathione concentration in the shoots of halophytes grown under combination of salinity and waterlogging with which to compare our findings. But, there is some data on the effects of salinity on glutathione concentrations in halophytes. In *Salicornia brachiata and Cakile maritima,* raised concentrations of [GSH+GSSG], [GSH] and the ratio of GSH:GSSG were reported with increasing and/or prolonged exposure to salinity above the optimum concentration required for growth (Ben Amor et al., 2007; Parida and Jha, 2010). In these halophytes, as found in our study, raised concentrations of glutathione were also associated with decreased biomass.

Raised antioxidant capacity under saline flooding was also seen in the concentrations of polyphenolic compounds and scavenging activity. Dry plant material was used to determine the levels of polyphenolic compounds and their antioxidant activity because this method is that most commonly reported in the literature. Lower DPPH and superoxide anion IC_{50} values relate to a higher concentration of total phenolic

compounds and free radical scavenging activity (Djeridane et al., 2006; Wong et al., 2006; Zainol et al., 2003), since phenolic compounds can donate an electron or hydrogen atom to detoxify ROS (Michalak, 2006; RiceEvans et al., 1997), and thus higher concentrations of phenolics can be associated with higher free radical scavenging activity as measured by the consumption of DPPH and superoxide anions. *S. maritima* shoots showed a significant positive correlation between total phenolic concentration and ability to scavenge DPPH (R2 = 0.97) (Fig 2.8) and superoxide anion (R2 = 0.98) (Fig 2.9). Similar relationships were previously found in *Cakile maritima* (Ksouri *et al*., 2007); in mangrove and halophyte species living in different saline habitats (backwater, low saline inland and marsh; Agoramoorthy et al., 2008); and in *S. maritima* leaves collected from mangrove forests in India (Thirunavukkarasu et al., 2010).

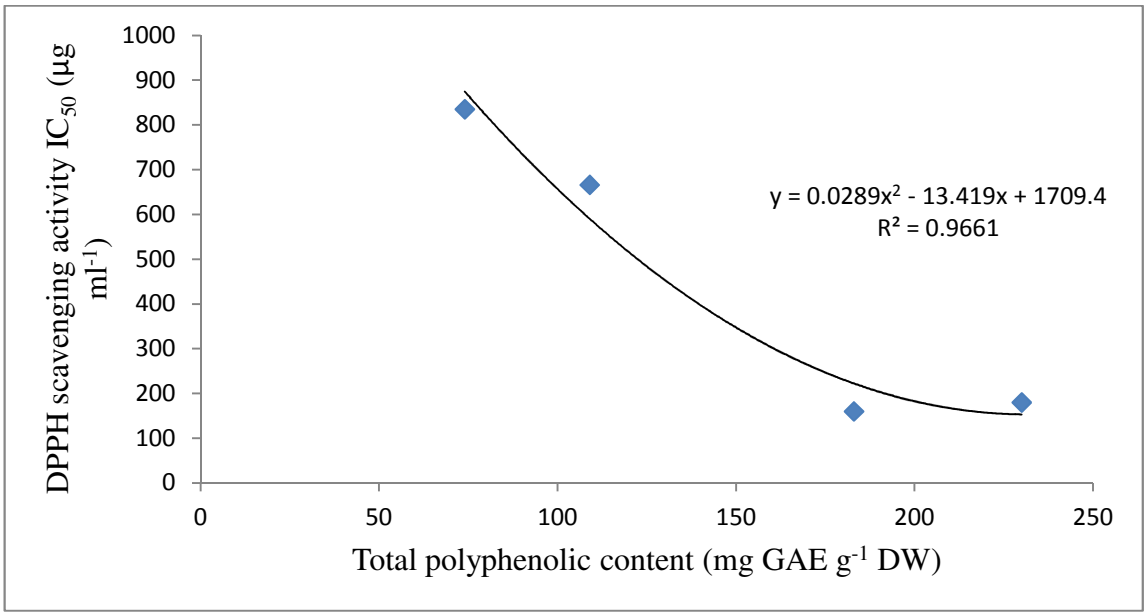


Figure 2.8 Relationship between total phenolic content and antioxidant activity (DPPH) of methalonic extracts from shoots of *S. maritima*

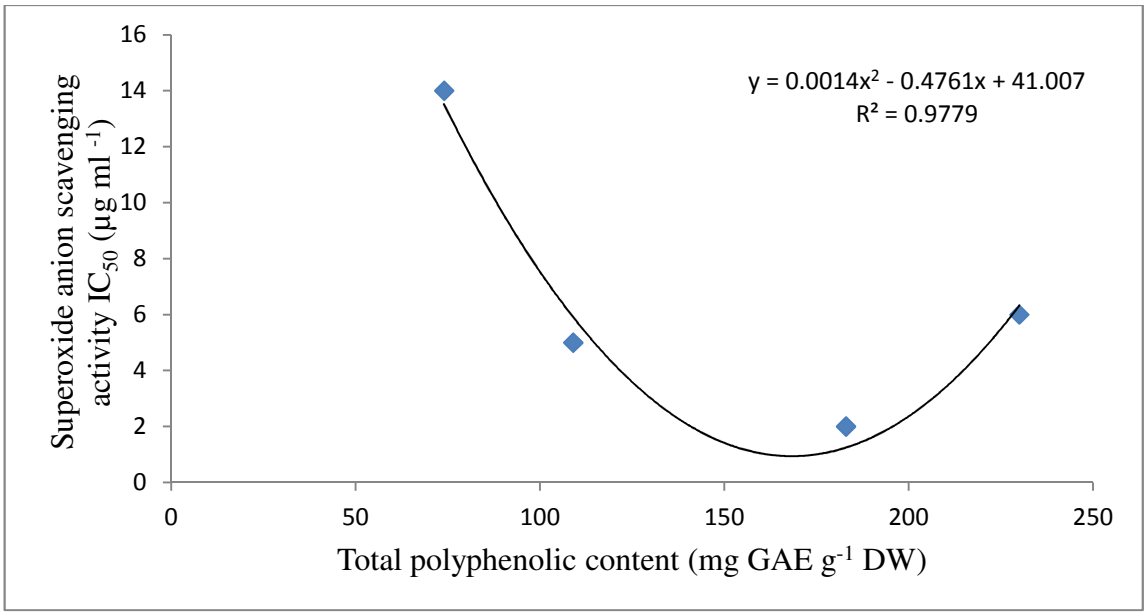


Figure 2.9 Relationship between total phenolic content and antioxidant activity (superoxide anion radicals) of methalonic extracts from shoots of *S. maritima.*

The higher antioxidant capacity and more reducing cellular conditions of *S. maritima* plants under saline flooding may be a result of a lower respiration rate which plants under flooding use to decrease their oxygen demand (Perata et al., 2011). Under the well-drained conditions, a higher rate of respiration, enhanced growth and lower antioxidant capacity may all contribute towards higher levels of ROS. ROS cause damage to macromolecules and can result in a loss of cell function; for example, lipid peroxidation compromises membrane integrity and is associated with tissue damage and even seed death (Berger et al., 2001; Sattler et al., 2004). In some halophytes, a low concentration of MDA has been associated with salt tolerance (Boughalleb and Denden, 2011; Li, 2008; Sekmen et al., 2007). Here, the higher antioxidant capacity in *S. maritima* plants grown under flooded conditions was associated with a lower concentration of MDA. However, the level of lipid peroxidation observed does not appear to be detrimental to growth of *S. maritima* plants since those with higher MDA had a higher biomass than those with the lower MDA concentrations. The increased antioxidant capacity appears to be an important response to saline flooding stress in *S. maritima*, and may contribute towards the plant completing the life cycle, but the combination of salinity and waterlogging is more detrimental to biomass accumulation than salinity alone.

In addition to examining the plants growing in natural conditions, I also investigated whether growth of plants under controlled conditions, where the variation of the environmental conditions was minimised, would have an effect on the antioxidant capacity under flooding. The antioxidant capacity was higher in flooded and drained plants grown in the natural habitat compared to controlled conditions, in terms of glutathione concentration, polyphenol concentration and free-radical scavenging activity, although the levels of MDA were also higher in these plants. These differences may reflect variations in environmental factors between the glasshouse and field (such as salinity, water stress, temperature and light intensity) and/or the plant growth stage (Ashraf et al., 2010; Ghasemi et al., 2011). A previous study on Tunisian halophytes in their natural habitat with differences in the growing conditions (such as latitude, longitude, altitude, salinity, temperature, water stress and light intensity), found IC_{50} values of DPPH were approximately 610 μg ml^{-1} in *Cakile maritima* from the Jerba region (arid bioclimatic region, Tunisia) and 940 μg ml^{-1} in *C. maritima* from the Tabarka region (humid bioclimatic area, Tunisia; Ksouri *et al*., 2008). Such studies in addition to my own, highlight that caution is needed when comparing plants of different growth conditions or translating experimental results from the laboratory to the field and vice versa. However for plants of *S. maritima*, the biological response to saline flooding (i.e. increased antioxidant capacity) was the same irrespective of growth conditions.

In conclusion, the results showed a clear difference between the flooded and drained shoots of *S. maritima.* Plants grown in their natural habitat showed some differences to plants grown under controlled conditions, suggesting unknown environmental factors may have an impact on the antioxidant capacity. These results suggest that *S. maritima* shows a significant increase in glutathione concentration and polyphenolic compounds as a protective strategy against ROS under these conditions and that tolerance towards flooding favours increasing antioxidant capacity for detoxifying ROS. However, these adaptations carry a yield penalty as plant biomass was lower under flooded than drained conditions.

Chapter 3 : Uptake of trace metals under a combination of waterlogging and salinity in *Suaeda maritima*

3.1 Introduction

Trace elements, such as manganese (Mn), iron (Fe), copper (Cu) and zinc (Zn), are important for plant growth. Such micronutrients are needed for many essential functions of plants, including electron transport, carbon dioxide assimilation in photosynthesis and chlorophyll synthesis (Marschner, 1995; Demirevska-Kepova et al., 2004; Boojar and Goodarzi, 2007; Mou et al., 2011). Mn, Fe and Cu are examples of transition metals, elements whose atoms have an incomplete d orbital, and whose compounds can often exist in several different oxidation states. Consequently, their ionic form can vary with the oxidation state of the soil and this may influence their bioavailability. Although plants require these elements in very small quantities, they can be harmful when they exist in soil at high concentrations, causing growth inhibition, a decline in biomass and plant death (Michalak, 2006).

The bioavailability to plant roots of micronutrients in soil depends on their concentration, which is affected by factors such as redox potential (Eh), water content, salinity, organic matter and pH (Harmsen and Vlek, 1985; David and Joel, 2012). The solubility of Mn, Fe and Zn has been shown to be higher at low than at high pH values, as shown in Table 3.1 (Srivastava and Dubey, 2011). Bruemmer et al (1986) also reported that neutral and alkaline conditions in the soil are often linked to low solubility of metal compounds and the solubility of Fe, Zn, and Cu specifically has been shown to be lower with high than low pH (Harmsen and Vlek, 1985). For Cu, it has been reported that soil pH can affect its solubility, as well as complexation, speciation, and adsorption (Payne and Pickering, 1975; Msaky and Calvet, 1990; Reddy et al., 1995). However, other research has shown little effect of soil pH on Cu concentration in the soil (Jeffery and Uren, 1983; McGrath et al., 1988; Sauve et al., 1997). Cu has a high affinity for organic matter (Norvell, 1991), therefore this variable may have a more significant effect on its solubility than soil pH (Reichman., 2002).

The availability of micronutrients is affected by waterlogging, as the periodic and prolonged flooding of soil results in biological and chemical processes that are very different from those that happen in well-drained and aerated soils. When soil is flooded, oxygen diffuses from the air into the soil around 10,000 times more slowly, as air-filled pores are submerged, and the concentration of oxygen quickly decreases to very low levels (Ponnamperuma, 1972). Under flooded conditions, the redox potential of the soil is greatly decreased, altering the elemental profile of the soil. Once oxygen is depleted, respiring soil microbes use nitrates as electron acceptors, followed by oxides of Mn, then Fe and then sulphate. The end result is a significant increase in soluble Fe^{2+} and Mn^{2+}, which may exceed toxic levels in the soil (Ponnamperuma, 1972; Marschner, 1991). The conversion of Mn (IV) and Fe (III) oxides to Mn (II) and Fe (II) oxides, increases the solubility of both elements (Ponnamperuma, 1972; Armstrong et al., 1991; Otero et al., 2009), with a sharp decline in redox potential (Shabala, 2011). Redox conditions seem to have more influence on the availability of Mn than on the availability of Cu and Zn. Most of the Cu and Zn present in soil are in the divalent form due to the instability of the monovalent forms (Whitehead, 2000), therefore under low redox conditions, these metals are not significantly reduced (Moraghan and Mascagni, 1991; Whitehead, 2000). On the other hand, flooded soils have been reported to be deficient in Zn, possibly due to the reducing conditions causing precipitation in compounds such as $ZnFe_2O_4$ (Sajwan and Lindsay, 1986; Moraghan and Mascagni, 1991), while oxidization of the sediment, or a decrease in pH results in a substantial increase in the availability of Zn (Gambrell et al., 1991).

Table 3.1 The effect of pH on the solubility of manganese, iron, zinc, and copper 'Low' pH is an acidic solution with a pH lower than 7, and 'high' pH is an alkaline solution with a pH higher than 7.

Trace Elements	pH	Solubility	References
Manganese	acidic	Increase	Bortner, 1935; Löhnis, 1951; Mahmoud and Grime, 1977; Fisher et al., 2001
	alkaline	Decrease	Etherington, 1982; Harmsen and Vlek, 1985; Bruemmer et al., 1986; Davy et al., 2011
Iron	acidic	Increase	Fisher et al., 2001.
	alkaline	Decrease	Harmsen and Vlek, 1985; Bruemmer et al., 1986; Liu and Millero, 2000.
Zinc	acidic	Increase	Sanders and Adams, 1987
	alkaline	Decrease	Harmsen and Vlek, 1985; Bruemmer et al., 1986.
Copper		No significant change'	McGrath et al., 1988; Sauve et al., 1997.

When waterlogged soils are drained, the redox potential quickly rises, and there is a possibility of the soil becoming supersaturated in Fe and Mn oxides and of co-precipitation of metals with Fe and Mn. The pH may also decrease due to the oxidation

of metal sulphides, causing an increase in the solubility of metals (Harmsen and Vlek, 1985).

The concentration of micronutrients in soils can also be influenced by salinity. Johnson et al (1997) reported that in seawater, Fe can form Fe(II) and Fe(III), and can complex with both organic and inorganic ligands, or exist freely, while Liu and Millero (2000) reported a decrease in Fe(III) solubility as seawater pH increased. Chloride complexation appears to increase the solubility and mobility of Cu by decreasing its adsorption to sediment; chloride also decreases the adsorption of Zn to sediment (Bourg, 1988). Bioavailability may also be affected by the relative amounts of elements, for example, low concentrations of Zn, or Fe or sulphate may alter the toxicity of Cu (Chaney, 1988). In a marine environment, sulphide formation is the most important reductive process, so free metal ions precipitate, and as long as the conditions are reducing, this decreases the availability of metal ions.

Soil salinity and waterlogging have been shown to affect the uptake of trace elements by plants (Zurayk et al., 2001). Studies on the effect of salinity on horticultural crops have found it to decrease the concentration of Mn in the plant, although other reports on the effect of salinity on the concentration of Mn, Zn and Fe have been contradictory, showing increased concentrations in some cases, and decreased in others (Grattan and Grieve, 1998). Grattan and Grieve (1998) reported that the effect of salinity on Cu accumulation varied, while Roussos et al (2007) reported that increasing salinity caused Cu concentrations to rise. Cooper (1984) reported that the root dry weight of *Plantago maritima* and the root and shoot dry weights of *Salicornia europaea* and *Aster tripolium* decreased at high Mn concentrations. Saline conditions can also reduce the phytotoxicity of certain trace elements, such as Mn, whose uptake by salt-marsh plants was decreased upon the addition of NaCl to the growth medium (Singer and Havill, 1985), while Gorham & Gorham (1955) reported that halophytes grown in salt marshes had lower Fe and Mn content than hydrophytes grown in freshwater marshes. It has also been found that Mn and Fe uptake is stimulated in response to waterlogging in *Juncus gerardii* and *Agrostis stolonifera* and that uptake was higher in plants grown without salt (Rozema and Blom, 1977). *Spartina alterniflora* growing in an upper marsh had lower iron levels than those growing in a lower marsh (Adams, 1963).

S. maritima is a plant that grows in both the upper and lower regions of salt marshes. Recent studies (Wetson and Flowers, 2011) have found that plants were larger on the upper than the lower marsh, although the reasons for this growth difference are unclear. Lower growth under hypoxic rather than normoxic conditions could reflect lower ATP production when oxygen is in poor supply, although *S. maritima* accumulates lactate enzyme activity in both normoxic and hypoxic conditions (Wetson. et al., 2012). A further possibility to explain poor growth on the lower marsh is that hypoxia influences the bioavailability and accumulation of metal ions, leading to deficiency or toxicity. Research using both soil-based and hydroponic methods may improve our understanding of metal bioavailability to *S. maritima* plants under flooded and drained conditions, and elucidate the strategies and mechanisms employed by *S. maritima* to adapt to various conditions.

The purpose of the experiments detailed in this Chapter was to examine the hypothesis that the accumulation of Mn, Fe, Zn, and Cu in *S. maritima* plants is higher in hypoxic than in aerated conditions, particularly at low pH in hydroponic experiments which prevents precipitation of micronutrients. The levels of heavy metals in leaves could be expected to be higher under high concentration of Mn and Fe in the growth medium. High concentrations of transition metals could be accumulated in the roots under hypoxic conditions so preventing the build-up of toxic levels in the leaves. Lower concentrations of transition metals might be expected in plants grown at high pH (ca 7-8) than at low pH (> pH 5.5) and in those grown under high than low salt concentration in growth chamber experiments.

The problem with growing plants under a high pH with hydroponics, is that many micronutrients precipitate from solution at high pH values (seawater has a pH of around 8.3), so that the solution has to be changed daily or other ways found of supplying micronutrients, such as by foliar spray (Singh et al., 2002). Since in the majority of previous research on *S. maritima* plants have been grown hydroponically at pH values below 7, this practice was continued in the experiments described in this Chapter. In order to investigate the effects of anoxia solutions containing 0.1% or more, as opposed to the 0.01% agar/water were used [as opposed to deoxygenated water (Armstrong, 1967); with wheat for example (Wiengweera et al., 1997), and *Suaeda maritima* (Wetson. et al., 2012); tests concluded that the 0.1% agar stagnant solution more

effectively simulated the situation in waterlogged soils and in the rhizosphere, as compared to N_2 flushed or non-flushed semi-stagnant agar-free nutrient solutions]. Experiments were also conducted in a medium based on a natural salt-marsh soil for comparative purposes.

3.2 Materials and Methods

3.2.1 Germination and growth of seedlings

S. maritima seeds from Cuckmere Haven were germinated in plastic trays of silver sand with half-strength nutrient solution (Stout & Arnon, 1939) for four weeks, in a growth chamber. Conditions in the growth chamber (Weiss 1) were, a 16 h photoperiod at 200 μmols m^{-2} s^{-1}at 22 ^{o}C and 60% relative humidity; during the dark period, the temperature was 17 ^{o}C at 70% relative humidity.

3.2.2 Experiment 1: The effect of different concentrations of artificial seawater on ion uptake, in aerobic and hypoxic conditions, under controlled conditions in a growth cabinet

Plants (Cuckmere seeds) were germinated as previously described (Chapter 2 Section 2.2.1), with full-strength nutrient solution (Stout and Arnon, 1939); in 100 and 350 Na^+ mM concentrations of artificial seawater (as described in Appendix 3.1). Plants were transplanted at 4 weeks into nutrient solution (Stout and Arnon 1939) in different strength artificial seawater (Harvey, 1966) under aerated and hypoxic conditions in black plastic-lidded pots of 500 ml capacity (5 pots per treatments). Each plant was suspended through a hole in the lid and held in place with non-absorbent cotton wool. 3 plants per pot. The pot (500 ml, 15 cm high and 7 cm diameter). Compressed air was bubbled through to obtain good aeration in the solution (pre-bubbled). Stagnant agar-nutrient solution was prepared by dissolving 10 g of agar (Sigma, Plant Cell Culture A 1296) in 2 litres of distilled water and autoclaving at 120°C for 15 minutes. After cooling, this solution was added to 7.390 and 2.140 L of full artificial seawater solution, then distilled water was added to make a final volume of 10 litres of 350 and 100 Na^+ mM artificial seawater, respectively, with 0.1% w/v agar and stirred thoroughly to avoid lumps of agar forming. Nitrogen gas was bubbled through to obtain reduce oxygen to less than 0.5 mg L^{-1} in the solution (pre-bubbled with nitrogen in 100 and 350 mM Na^+).

Plants were harvested after eight weeks of treatments.

Four treatments were imposed:

(a) Aerated nutrient solution with 100 mM Na^+ (100, A).

(b) Aerated nutrient solution with 350 mM Na+ (350, A).

(c) Stagnant agar solution with 100 mM Na^+ (100, AGN).

(d) Stagnant agar solution with 350 mM Na^+ (350, AGN).

3.2.3 Experiment 2: The effect of tidal movements in the tank system on ion uptake in *S. maritima* plants in the glasshouse

Plants were grown in the green-house in the tank system under controlled conditions, as described in the previous experiment (Chapter 2 Section 2.2.2). Plants were grown for 8 weeks in treatments with full-strength Stout & Arnon culture solution and in half-strength fresh seawater.

3.2.3.1`Soil investigations

Direct measurements of the degree of oxygenation of soil cannot reliably be made with a simple oxygen sensor because of the likelihood of damage to the delicate membrane by the pressure of soil particles during its insertion into the soil (Wetson, 2008). Consequently, redox potential (Eh) was used as an index of soil oxygenation. Readings were taken using a Combined Redox Electrode with a platinum rod joined to a Calomel reference electrode ORP meter (CMPTRII/DWGI806, Thermo Electron Corporation, Fife, Scotland) attached to a portable microcomputer/pH meter (HI 9025 HANNA Instruments). The redox state (Eh) of the growth medium surrounding the roots was measured at three depths: 1 cm, ~4 cm and ~8 cm.

3.2.4 Experiments 3 and 4: The response of *Suaeda maritima* to varying concentrations of iron and manganese under aerobic and hypoxic conditions

These experiments were performed to compare the effect of good aeration and hypoxia at different concentrations of ethylenediaminetetraacetic acid Fe(III) sodium salt (EDTA) (13.6, 263.6, 513.6 µM, and 1.01 mM of Fe), and manganese (Mn) (3.35 and 250 µM, 1, 5, and 10 mM $MnSO_4$) in half-strength Stout & Arnon nutrient solution, with 350 Na^+ mM of artificial seawater (as described in Appendix 3.1). under aerated and hypoxic conditions as described in above (Section 3.2.2).

Four-week-old plants were transferred to black plastic boxes, (2 L; 10 plants per box and 3 boxes per treatment) and suspended with non-absorbent cotton wool through holes in a lid. The solutions were changed at weekly intervals; pH values and EC were recorded weekly before and after solutions were renewed. These experiments were carried out in a controlled environment chamber (Weiss 2400E/+5 JU-Pa-S growth cabinet; Weiss Technik, Gmbh, Reiskirchen-Lindenstruth, Germany) in the same conditions as those in which germination was carried out.

Eight treatments were used for the Fe experiment (see also Fig.3.1:

(a) Aerated nutrient solution with 13.6 µM Fe (13.6 µM, A).

(b) Aerated nutrient solution with 263.6 µM Fe (264 µM, A).

(c) Aerated nutrient solution with 513.6 µM Fe (514 µM, A).

(d) Aerated nutrient solution with 1.01 mM of Fe (1.01 mM, A)

(e) Stagnant agar solution with culture 13.6 µM Fe (13.6 µM, AGN).

(f) Stagnant agar solution with 263.6 µM Fe (264 µM, AGN).

(g) Stagnant agar solution with 513.6 µM Fe (514 µM, AGN)

(h) Stagnant agar solution with 1.01 mM of Fe (1.01 mM, AGN).

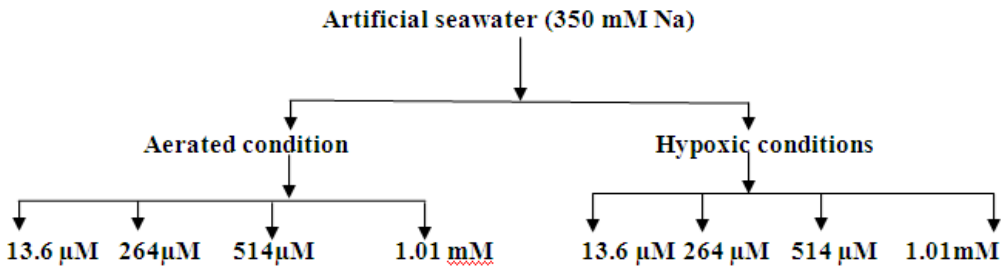


Figure 3.1 A diagram of the different treatments of Fe

Ten treatments were used for the Mn experiment (see Fig. 3.2):

(a) Aerated nutrient solution with 3.35µM (3.35µM, A).

(b) Aerated nutrient solution with 250 µM Mn (250 µM, A).

(c) Aerated nutrient solution with 1 mM Mn (1 mM, A).

(d) Aerated nutrient solution with 5 mM Mn (5 mm, A).

(e) Aerated nutrient solution with 10 mM Mn (10 mM, A).

(f) Stagnant agar solution with 3.35 µM (3.35µM, AGN).

(g) Stagnant agar solution with 250 µM Mn (250 µM, AGN).

(h) Stagnant agar solution with 1 mM Mn (1mM, AGN).

(i) Stagnant agar solution with 5 mM of Mn (5mM, AGN).

(j) Stagnant agar solution with 10 mM of Mn (10 mM, AGN).

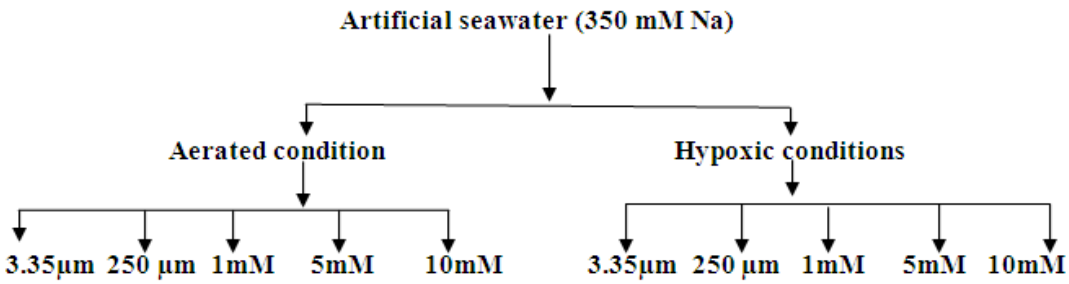


Figure 3.2 A diagram of the different treatments of Mn.

The boxes were topped up to a constant level with distilled water to replace evapotranspiration losses throughout the experiment. For the hypoxic boxes, water was bubbed with nitrogen gas before use. Plants were harvested after Fe and Mn toxicity were appeared; for Fe after 10 d of treatment, and for the Mn experiments, the plants were harvested after three weeks. In some cases, the roots were very small and could not be removed from the paper bags used for drying, so that root dry weight data are not available.

3.2.2.1 Physiological parameters

3.2.2.1.1 Fresh and dry weight determination

After harvesting the plants, the shoots and roots were washed with distilled water, patted dry with paper towels and quickly weighed for determination of fresh weight. The lengths of the longest root and shoots in Experiments 3 and 4 were measured. Dry mass was determined after drying in an oven (Ohaus) at 80^{o}C for 72 h, then weighing using a sensitive Metler AND (HR-60) balance.

3.2.2.2.1.2 Chlorophyll

Leaves (250 mg fresh weight) were collected, immediately frozen with liquid nitrogen and stored at –80°C until analysis. Frozen leaf samples were ground; chlorophyll was extracted with 25 ml of 85% (v/v) acetone and filtered. Absorbency was determined at 645 and 663 nm, and levels of chlorophyll a and b were estimated using the equations of (Arnon, 1949).

3.2.2.2.1.3 Nutrient analysis

Leaf and root samples were collected and dried at 80°C for 24 h for elemental analysis. Leaves and roots (50 mg DW of each) were ashed at 550°C for 4 h, dissolved in 0.5 ml of 70 % concentrated nitric acid, heated for five minutes and diluted with distilled water to a final volume 20 ml. All ions were measured by ICP-MS, performed on an Agilent 7500ce ICP-MS; the data was acquired in Helium gas collision mode with a He flow of 4.5 ml min^{-1}. RF Power was 1500W and the spray chamber was cooled to 2°C.

3.3 Statistical analysis

Data were analysed by ANOVA using SPSS v 18. Different letters above the bars on the graphs indicates a significant difference in means from post-hoc Tukey tests.

3.4. Results

3.4.1. Trace metals in *Suaeda maritima* plants grown under aerated and hypoxic conditions, in different concentrations of artificial seawater in a growth cabinet

In this experiment, the growth of *S. maritima* was investigated in nutrient solutions where conditions ranged from good aeration by pre-bubbling with air, to severe hypoxia

using pre-bubbled stagnant agar solution, at different concentrations of artificial seawater (100 and 350 mM Na^+). The mean oxygen concentrations are shown in Table.3.2: Trace elements were investigated, as they are important for plant growth in small quantities, while excessive accumulation may be damaging under flooded conditions.

Table 3.2 Shows the oxygen concentration before and after renewing the growth medium, pH, temperature, and electrical conductivity (EC) for *S. maritima* plants grown under different concentrations of oxygen: aerated medium (A), and hypoxic medium (AG N), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth chamber at 20^0C. Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level**.**

			Treatment			
			A100	AGN100	A350	AGN350
Oxygen concentration	mg L^{-1}	After	8±0.14a	0.35±0.04a	8.1±0.11a	0.4±0.04a
		Before	4±0.02b	0.36±0.02a	2.3±0.01b	0.3±0.01a
EC	dS m^{-1}		14.5±0.2a	14.7±0.4a	41.6±0.6b	41.5±0.6b
pH			5.4±0.03a	5.5±0.01a	5.0±0.08b	5.6±0.02a

3.4.1.1 Shoot and root dry weight

Analysis of shoot and root dry weight showed significant effects of salt concentrations in the growth medium ($P < 0.001$), an effect of oxygen concentration in the medium on shoot dry weight ($P < 0.001$), an interaction between salt concentration and oxygen concentration on root dry weight ($P < 0.05$), but no significant effect of the interaction on shoot dry weight ($P = 0.070$) or of oxygen concentration on the root dry weight ($P = 0.568$) was recorded. As far as the shoots were concerned, the data (Fig. 3.3A) showed that shoot dry weight was 1.3 times greater in aerated conditions than in the hypoxic conditions at both salt concentrations, and that shoot dry weight was 2.3 times higher in

plants grown in high salt concentrations than those grown in low salt concentrations, again in both aerated and hypoxic conditions.

Root dry weight was also significantly affected by salt concentration; in aerated conditions at high salt concentrations it was twice that at low salt concentrations and in hypoxic conditions 1.3 times higher. At low salt concentrations, root dry weight was 0.7 times lower in aerated than hypoxic conditions, while at high salt concentrations, it was not significantly affected (only 1.1 times higher in aerated conditions).

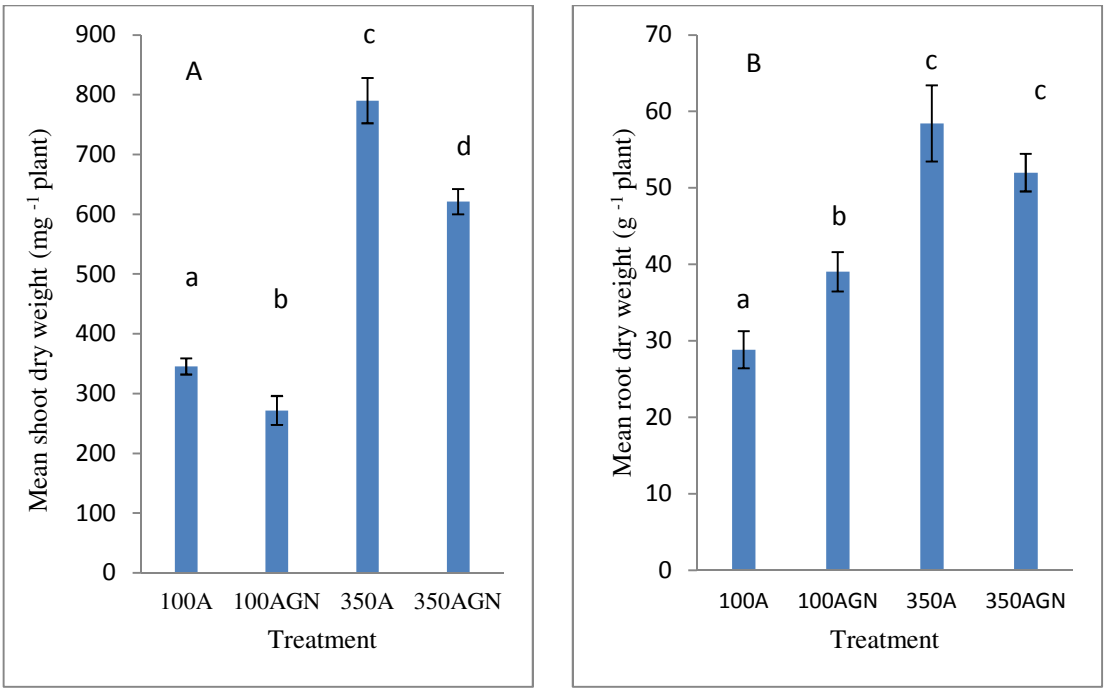


Figure 3.3 Shoot dry weight (A) and root dry weight (B) of Suaeda maritima plants after 8 weeks growth, under different levels of oxygen: aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na+) in a growth chamber..Error bars are SE (n = 14). Data were analysed by 2-way ANOVA. Shoot dry weight; Salt concentration in the medium F (1, 52) = 237.870, $P < 0.001$, oxygen level F (1, 52) = 22.179, $P < 0.001$, the interaction F (1, 52) = 3.428, $P = 0.070$. Root dry weight; Salt concentration in the medium F (1, 52) = 41.964, $P < 0.001$, oxygen level F (1, 52) = 0.330, $P = 0.568$, the interaction F (1, 52) = 6.410, $P < 0.05$. Letters above bars indicate significant difference in means from post-hoc Tukey tests.

3.4.1.2 Shoot and root manganese

Analysis by ANOVA showed that shoot (Fig 3.2 A) and root (Fig 3.2 B) Mn concentrations were significantly affected by salt concentrations in the growth medium ($P < 0.001$), oxygen concentration ($P < 0.001$), and their interaction ($P < 0.001$). Post-

hoc tests showed that shoot Mn concentrations (Fig 3.4A) were 1.8 and 1.4 times higher in hypoxic than well aerated conditions, at low and high salt concentrations, respectively. In aerated conditions, shoot Mn concentrations were 2.6 times greater in low salt than high salt concentrations, and in hypoxic conditions, there 3.2 times greater in low salt than high salt concentrations.

Root Mn concentrations were also significantly affected by salt concentration and oxygen level (Fig 3.4B). The root Mn concentration was higher in hypoxic conditions than aerated, by 22 times at low salt concentrations, and by 13 times at high salt concentrations. In addition, the root Mn concentration was higher at low salt concentrations, by 3 times in hypoxic conditions, and twice in aerated conditions

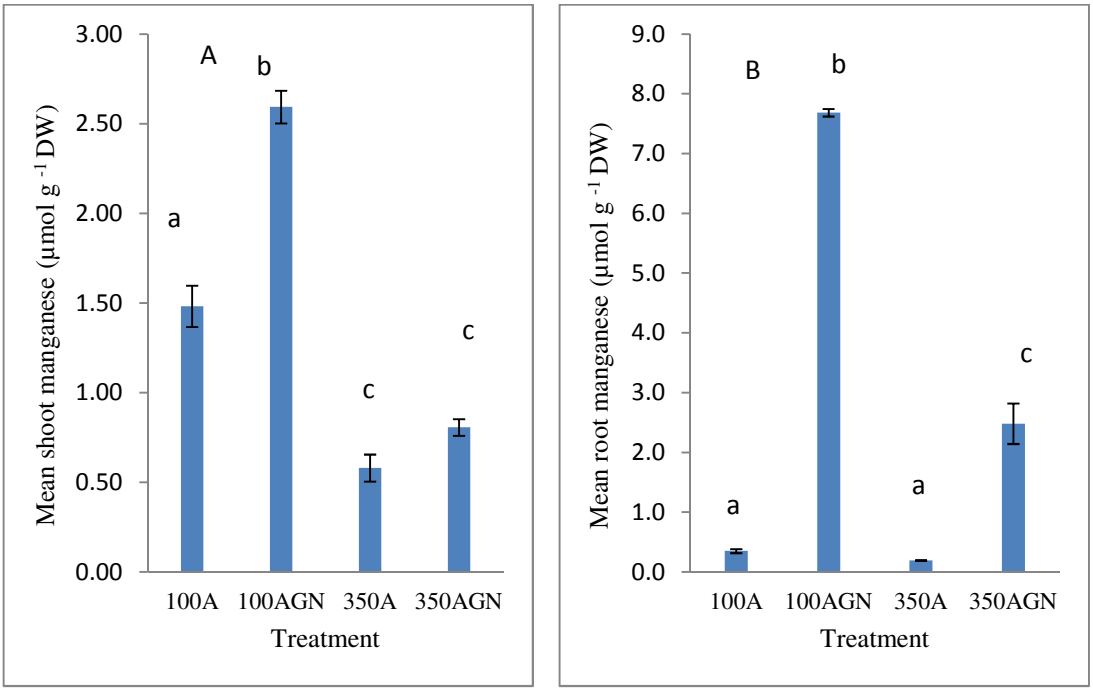


Figure 3.4 Shoot manganese (A) and root manganese (B) concentrations of *Suaeda maritima* plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawate (100 & 350 mM Na+), in a growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot manganese concentration; salt concentration in the medium F (1, 20) = 245.667, $P < 0.001$, oxygen level F (1, 20) = 60.853, $P < 0.001$, the interaction F (1, 20) = 26.631, $P < 0.001$. Root manganese concentration; salt concentration in the medium F (1, 20) = 238.856, $P < 0.001$, oxygen level F (1, 20) = 769.661, $P < 0.001$, the interaction F (1, 20) = 211.552, $P < 0.001$. Letters above bars indicate significant difference in means from post-hoc Tukey tests.

3.4.1.3 Shoot and root iron

Analysis of the Fe concentration in shoots (Fig 3.5A) showed that there was a significant difference between plants grown in low and high salt concentrations ($P <$

0.001). Shoot Fe concentration was 3.6 times higher in aerated, low salt conditions than in aerated, high salt conditions, and was also 1.3 times higher in hypoxic, low salt conditions, than in hypoxic, high salt conditions. The shoot Fe concentration was also affected by oxygen concentration in the growth medium. Post-hoc tests showed that there was a significant difference between plants grown in aerated and hypoxic conditions; it was 1.4 times greater in shoots grown in aerated, low salt conditions, than in those grown in hypoxic, low salt conditions, whereas it was 0.5 times lower in plants grown in aerated, high salt conditions. There was a significant effect of the interaction between salt and oxygen concentration in the growth medium on shoot Fe concentration ($P < 0.001$).

Analysis of the Fe concentration in roots also showed that there was a significant difference between plants grown in low and high salt concentrations under aerated conditions ($P < 0.001$). Root Fe concentrations were 1.6 times higher in aerated, low salt concentrations than in aerated, high salt concentrations (Fig 3.5B), but were not significantly different in hypoxic conditions. The oxygen concentration in the growth medium also had a significant effect on the root Fe concentration ($P < 0.001$). It was 1.9 and 1.3 times higher in the plants grown in aerated conditions, at both low and high salt concentrations, than in those grown in hypoxic conditions, at low and high salt concentrations, respectively. There was a significant effect of the interaction between salt and oxygen concentration in the growth medium on roots Fe concentrations ($P < 0.001$).

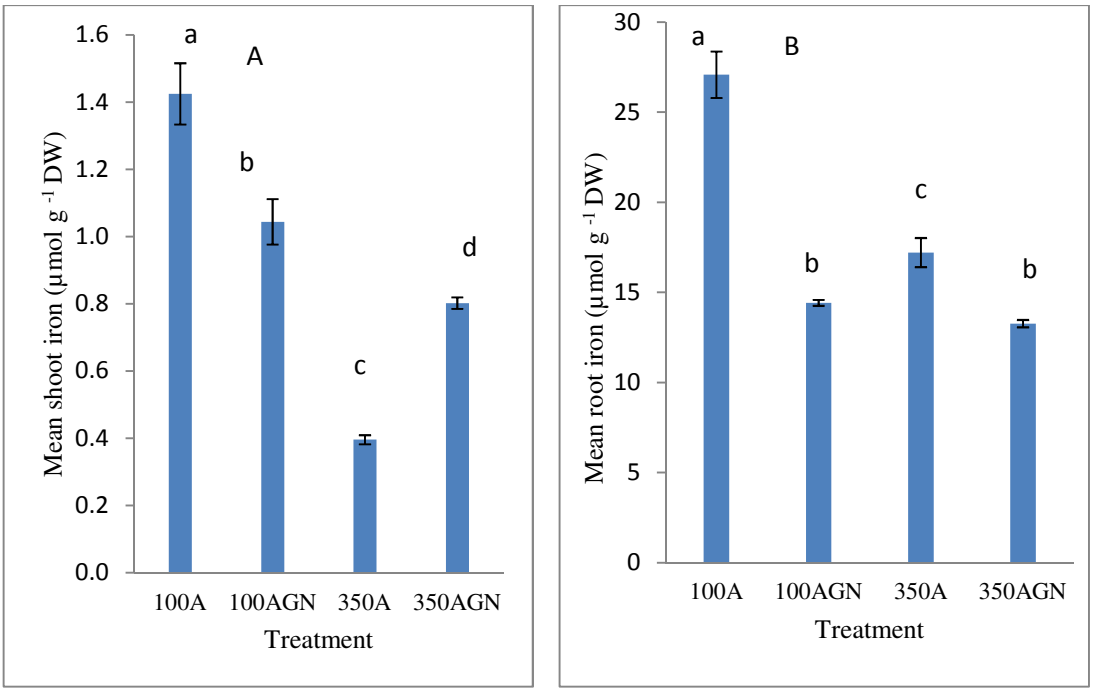


Figure 3.5 Shoot iron (A) and root iron concentrations of Suaeda maritima plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and Stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+), in a grow chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot iron concentration; salt concentration in the medium F (1, 20) = 120.484, $P < 0.001$, oxygen level F (1, 20) = 0.049, $P = 0.827$, the interaction F (1, 20) = 46.174, $P < 0.001$. Root iron concentration; salt concentration in the medium F (1, 20) = 50.894, $P < 0.001$, oxygen level F (1, 20) = 115.613, $P < 0.001$, the interaction F (1, 20) = 31.903, $P < 0.001$. Letters above bars indicate significant difference in means from post-hoc Tukey tests.

3.4.1.4 Shoot and root zinc

Shoot Zn concentrations (Fig 3.6A) were analysed by ANOVA, and showed a significant effect of salt concentration in the growth medium, only under aerated conditions ($P < 0.05$). Shoot Zn concentration was 1.3 times higher in shoots grown in aerated, low salt concentrations, than in those grown in aerated, high salt concentration. However, there was no significant effect of salt concentration under hypoxic conditions. Oxygen concentration in the growth medium also had a significant effect on shoot Zn concentration ($P < 0.001$): it was 0.5 and 0.8 times lower in shoots grown in hypoxic, low and high salt concentrations, than in shoots grown in aerated, low and high salt concentrations, respectively.

Root Zn concentration (Fig 3.6B) was analysed by ANOVA, and also showed a significant effect of salt concentration in the growth medium ($P < 0.001$). In contrast to the situation in the shoots, root Zn concentration was 1.3 times higher in roots grown in

aerated, low salt concentrations, than in those grown in aerated, high salt concentrations, and it was 1.7 times higher in roots grown in hypoxic, low salt concentrations, than in those grown in hypoxic, high salt concentrations. Root Zn was 1.9 and 1.4 times greater in hypoxic, low and high salt concentrations, than in aerated, low and high salt concentrations.

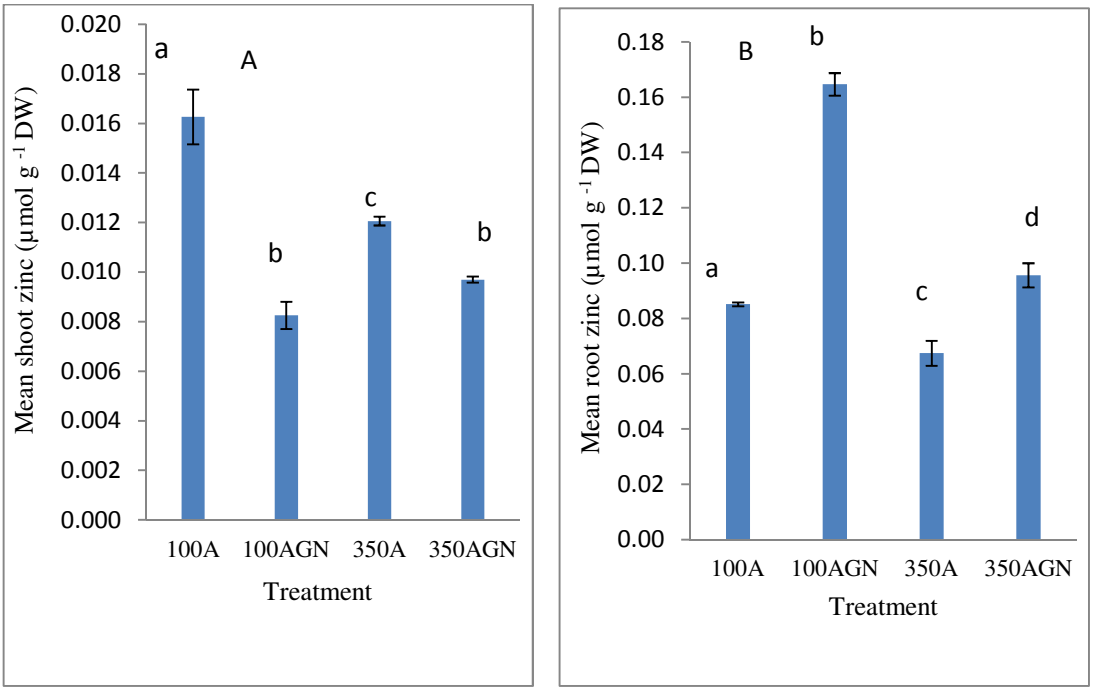


Figure 3.6 Shoot zinc (A) and root zinc (B) concentrations of Suaeda maritima plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and Stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+), in a growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot zinc concentration; salt concentration in the medium F (1, 20) = 4.859, P = 0.039, oxygen level F (1, 20) = 68.770, $P < 0.001$, the interaction F (1, 20) = 20.366, $P < 0.001$. Root zinc concentration; salt concentration in the medium F (1, 20) = 132.811, $P < 0.001$, oxygen level F (1, 20) = 204.974, $P < 0.001$, the interaction F (1, 20) = 46.556, $P < 0.001$. Letters above bars indicate significant difference in means from post-hoc Tukey tests.

3.4.1.5 Shoot and root copper

Salt concentrations in the growth medium had a significant effect on shoot Cu, ($P <$ 0.001), which was 1.3 and 1.2 times higher in shoots grown in aerated and hypoxic conditions, at low salt concentrations, than in those grown in aerated and hypoxic conditions, at high salt concentrations, respectively (Fig 3.7A). Oxygen concentration was also shown to significantly affect shoot copper concentrations ($P < 0.001$). Shoot Cu concentration was 1.4 times greater in plants grown in hypoxic, low salt concentrations, than in plants grown in aerated, low salt concentrations. It was also 1.5

times higher in shoots grown in hypoxic, high salt concentrations, than in those grown in aerated, high salt concentrations. The interaction did not have a significant effect (P = 0.604).

Salt concentrations did not have a significant effect on root Cu concentration (P = 0.538). However post-hoc Tukey tests indicate a significant difference under aerated conditions (Fig 3.7B). Root Cu concentration was 1.3 times higher in roots grown in aerated low salt than in aerated high salt conditions, but in hypoxic conditions, salt concentrations had no significant effect. Oxygen concentration was shown to significantly affect root Cu concentration under high salt concentrations ($P < 0.05$). Root Cu concentration was 1.5 times higher in roots grown in hypoxic, high salt concentrations, than in those grown in aerated, high salt concentrations. The effect of the interaction was significant ($P < 0.05$).

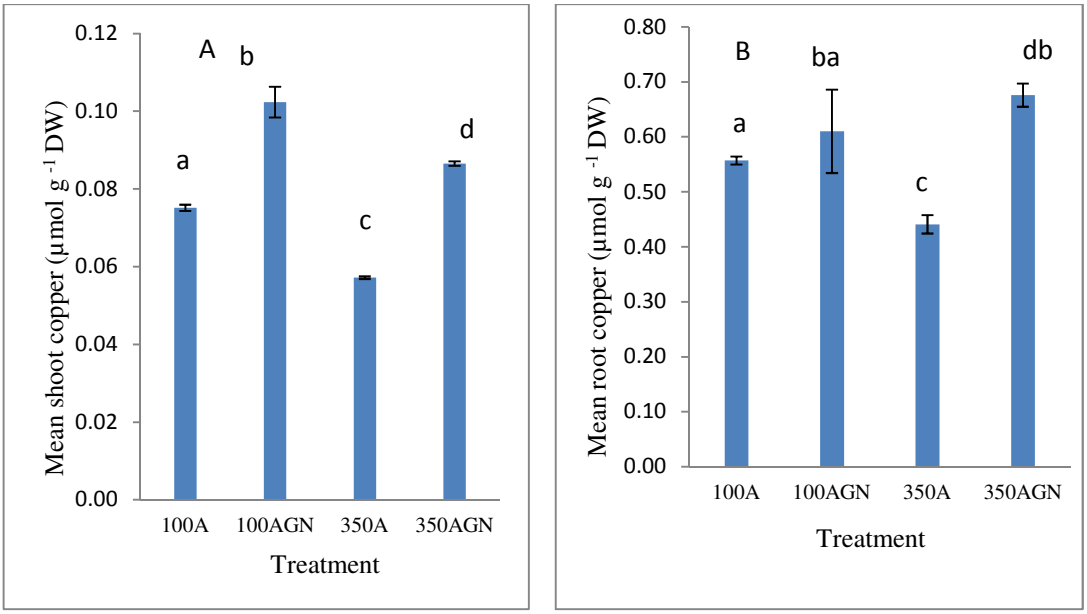


Figure 3.7 Shoot copper (A) and root copper (B) concentrations of Suaeda maritima plants after 8 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+), in a growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot copper concentration; salt concentration in the medium F (1, 20) = 67.352, $P < 0.001$, oxygen level F (1, 20) = 189.019, $P < 0.001$, the interaction F (1, 20) = 0.278, P = 0.604. Root copper concentration; salt concentration in the medium F (1, 20) = 0.393, P = 0.538, oxygen level F (1, 20) = 12.702, $P < 0.05$, the interaction (F (1, 20) = 5.064, $P < 0.05$). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

3.4.1.6 Root:Shoot ratios of trace elements

As shown in table 3.3, the ratio of root to shoot Mn and Zn concentrations was lower in plants grown in aerated low and high salt conditions, than in those grown in hypoxic low and high salt conditions. However, the ratio of root to shoot Fe was higher in aerated high salt conditions than in hypoxic high salt conditions, while at low salt concentrations, in both aerated and hypoxic conditions, the ratio of the root to shoot Fe concentration was not significantly affected. The ratio of the root to shoot Cu was not significantly affected in any treatment. The shoot to root ratio for dry weight was higher in aerated low salt conditions than in hypoxic low salt conditions, but in high salt conditions there was no significant effect.

Table 3.3 Shows the ratio between root and shoot concentrations for manganese, iron, zinc and copper, and shoot and root dry weight of *S. maritima* plants grown under different concentration of oxygen: aerated medium (A), and hypoxic medium (AG N), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth chamber. Letters indicate significant difference in means from post-hoc Tukey tests

		Treatment			
		100A	100AGN	350+A	350AGN
Mn	Root: Shoot	0.24±0.02a	2.9±0.1b	0.36±0.04a	3.01±0.3b
Fe		19.3±1a	14.09±1a	43.7±3b	16.5±0.01a
Zn		5.3±0.3a	20.2±0.9b	5.6±0.4a	9.9±0.6c
Cu		7.4±0.1a	6.1±1a	7.7±0.3a	7.8±0.2a
DW	Shoot: Root	13.2±1.3a	6.8±0.3b	14.7±1.3a	12.1±0.3a

3.4.2 Trace metals in *Suaeda maritima* grown under drained and flooded conditions in the greenhouse

In order to evaluate whether the effects seen at pH 5.5-6.0 in culture solution were similar to those at the pH at which *S. maritima* normally grows, growth was determined in a medium composed of salt-marsh mud and sand (50%, necessary to adjust the hydraulic conductivity) at two heights with half-strength fresh seawater in pH 8, in tanks subjected to flooding twice daily.

3.4.2.1 Redox values in the growth medium

Mean redox values (Eh) showed the contrasting redox state of the growth medium in drained and flooded conditions (Table 3.4). As would be expected the redox values fell with increasing depth in the pots and with the degree of waterlogging from high to low-tide position. In the flooded growth medium the Eh values were more negative at all depths, especially at the base of the growth medium, than in the drained growth medium. Negative Eh values lower down in the flooded and drained pots showed that the medium became hypoxic and reducing conditions developed from the in all pots.

Table 3.4 Mean Eh values (mV) recorded at three depths for 30 seconds, after one hour of flooding, in growth medium composed of a mixture of sand and estuarine mud, in which *Suaeda maritima* was grown at the top, middle and base of the growth medium, in drained and flooded pots, in the tidal flow glasshouse tank system. Means are ± SE (n = 18), and also Electrical conductivity (EC) and pH in the tanks was measured every 2 days.

Depth in growth medium	Tidal position of pot in tank		Electrical conductivity (EC) and pH in the tanks	
	Drained mV	Flooded mV	EC	pH
Top	- 63.3 ±3.5	- 116.6±4		
Middle	- 183.4±4	- 286.6±4.1	29.6±0.1	8.2±0.02
Base	- 276.2±8.3	- 380.5±7.3		

3.4.2.2 Mn, Fe, Zn, and Cu concentrations in the shoot

As shown in Figs 3.11 to 3.14, shoot Mn (Fig 3.8A), Fe (Fig 3.8B), and Cu (Fig 3.8D) concentrations were significantly increased by flooding; shoot Mn and Fe were 3 times greater in flooded shoots than in shoots grown in drained conditions, while shoot Cu was 2.3 times higher in flooded shoots than in drained shoots. However, shoot Zn was 5 times higher in drained shoots than in flooded shoots (Fig 3.8C).

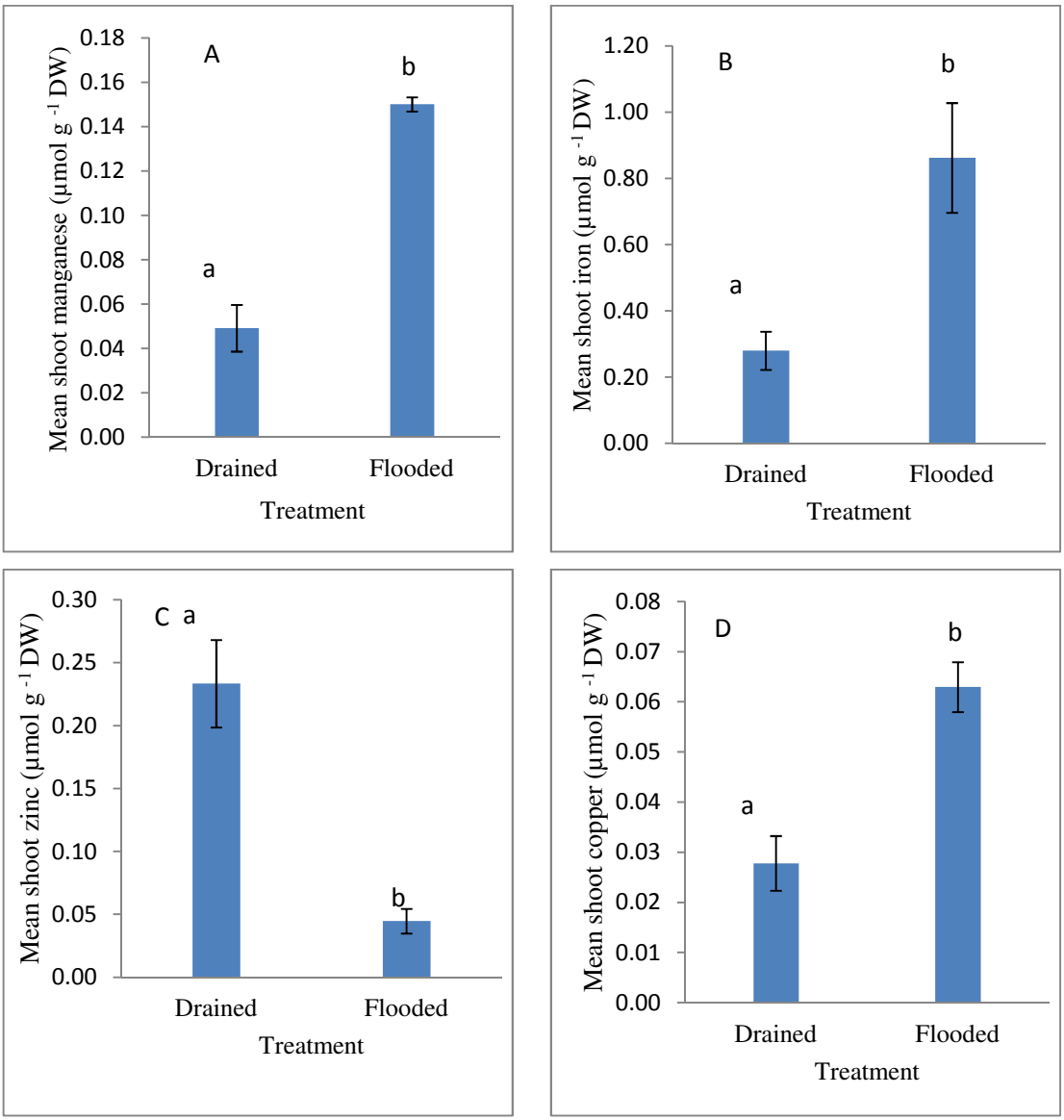


Figure 3.8 Shoot manganese (A), iron (B), zinc (c) and copper (D)concentrations of Suaeda maritima plants (12 weeks old at harvest) grown for 8 weeks under controlled conditions in the glasshouse. Plants were grown in mud and 50% sand at two heights with half-strength fresh seawater in tanks subjected to flooding twice daily. Error bars SE (n = 16). Data were analysed by one-way ANOVA. Shoot manganese concentration (A); F (1, 30) = 84.052, $P < 0.001$. Shoot iron concentration (B); F (1, 30) = 11.014, $P < 0.05$. Shoot zinc concentration; F (1, 30) = 27.341, $P < 0.001$.and Shoot copper concentration; F (1, 30) = 22.678, $P < 0.001$. Letters above bars indicate significant difference in means from post-hoc Tukey tests.

3.4.3. The response of *Suaeda maritima* to varying concentrations of iron under aerobic and hypoxic conditions:-

Since both previous experiments showed that Fe concentrations were increased by flooding (in hydroponics under hypoxic high salt as compared to aerated high salt and also in the greenhouse in the sand and mud mixture), in this experiment the growth of *S. maritima* was investigated in nutrient solutions where conditions ranged from good aeration by pre-bubbling with air, to severe hypoxia using pre-bubbled stagnant agar solution, in 350 mM Na^+ artificial seawater, at different concentration of Fe [ethylenediaminetetraacetic acid Fe(III) sodium salt (EDTA); 13.6, 263.6, 513.6 µM, and 1.01 mM Fe].

3.4.3.1 Shoot dry weight

Shoot dry weight decreased with increasing Fe concentration in the growth medium (Fig 3.9) and under hypoxia was lower than under aerated conditions at13.6 and 513.6 µM Fe.

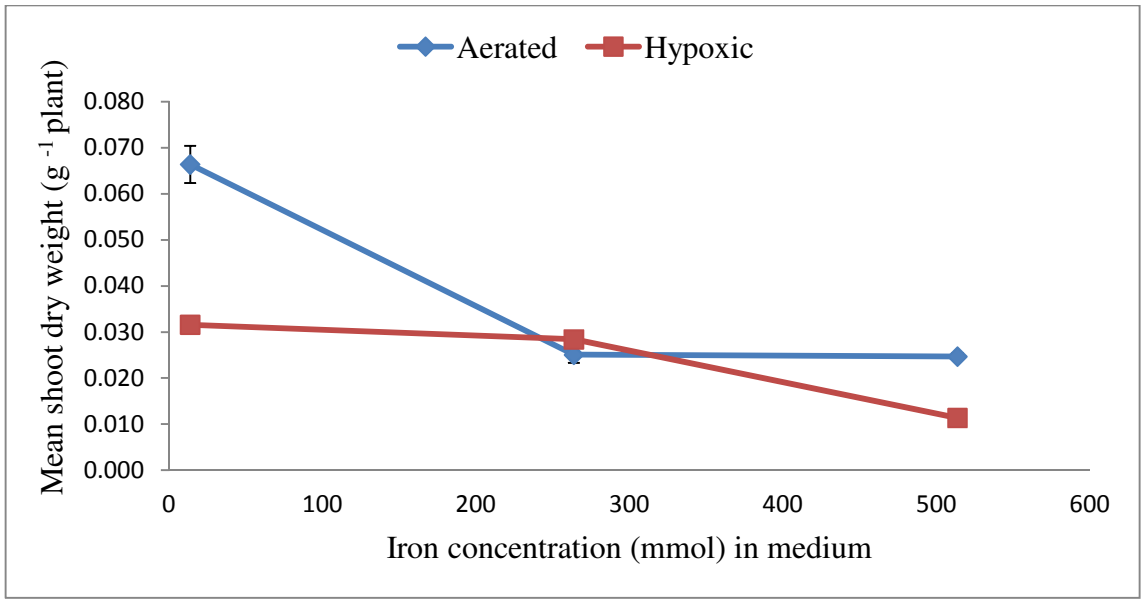


Figure 3.9 Shows shoot dry weight of Suaeda maritima plants after 10 days growth, under different concentrations of iron in Aerated nutrient solution (Aerated), and Stagnant agar solution (hypoxic), in the growth chamber. Error bars are SE (n = 14). Data were analysed by 2-way ANOVA. Oxygen concentration in the medium; F (1, 78) = 78.552, $P < 0.001$. Iron concentration in the medium; F (2, 78) = 119.879, $P < 0.001$.The interaction between oxygen concentration and iron concentration in the medium; F (2, 78) = 42.920, $P < 0.001$.

3.4.3.2 Total chlorophyll

Under both flooded and drained conditions, the total chlorophyll concentration decreased with increasing Fe concentration in the growth medium and was higher in drained than in flooded conditions in all Fe concentrations, whether expressed per unit dry (Fig 3.16) or fresh (see Appendix 3.2) weight.

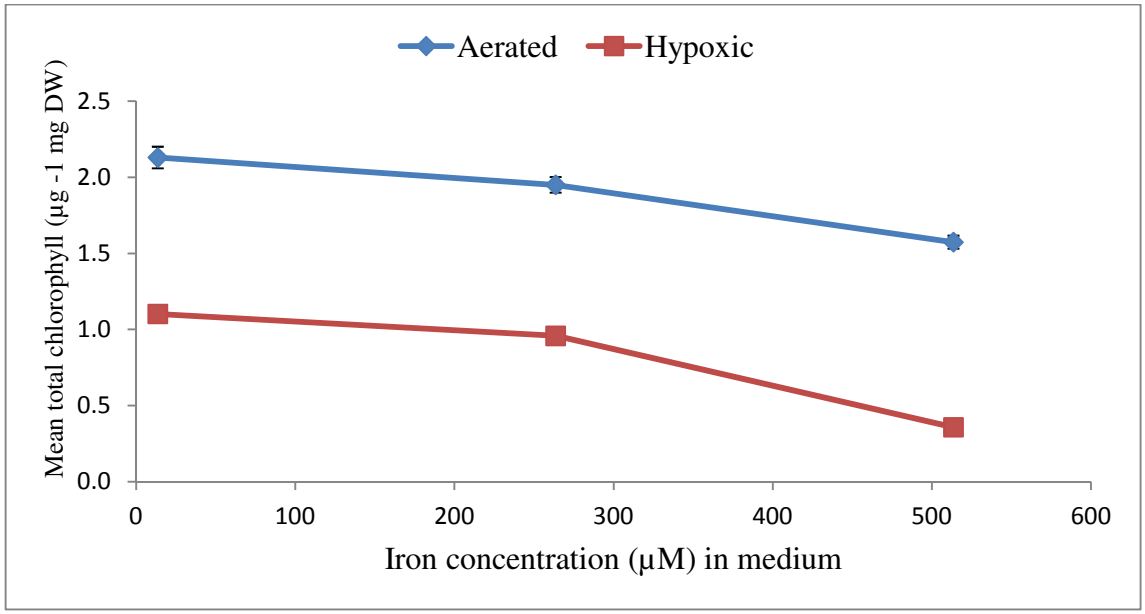


Figure 3.10 Total chlorophyll concentration of Suaeda maritima plants after 10 day growth, under different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic) in the growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Oxygen concentration in the medium; F (1, 30) = 924.552, $P < 0.001$, iron concentration in the medium; F (2, 30) = 121.540, $P < 0.001$, the interaction between oxygen concentration and iron concentration in the medium F (2, 30) = 3.839, $P < 0.05$

3.4. 3.3 Mn, Fe, Zn, and Cu concentrations in the shoot

Analysis of the data showed that shoot Mn, Fe, Zn, and Cu concentrations were significantly affected by Fe concentration in the growth medium ($P < 0.001$). Oxygen concentration in the root medium also significantly affected shoot Mn, Fe and Cu concentrations ($P < 0.001$) but shoot Zn was not affected ($P = 0.291$). In addition, the interaction between external Fe concentration and oxygen also significantly affected shoot Mn, Fe and Cu concentrations ($P < 0.001$), but not shoot Zn (P = 0.285). Under hypoxic conditions, the shoot Mn (Fig. 3.11A), Fe (Fig. 3.11B), Zn (Fig. 3.11C) and Cu concentrations (Fig. 3.11D) were higher than in those shoots grown in the drained conditions. All shoot trace elements were increased with increasing Fe concentration in the root medium, with the exception of Mn in flooded shoots.

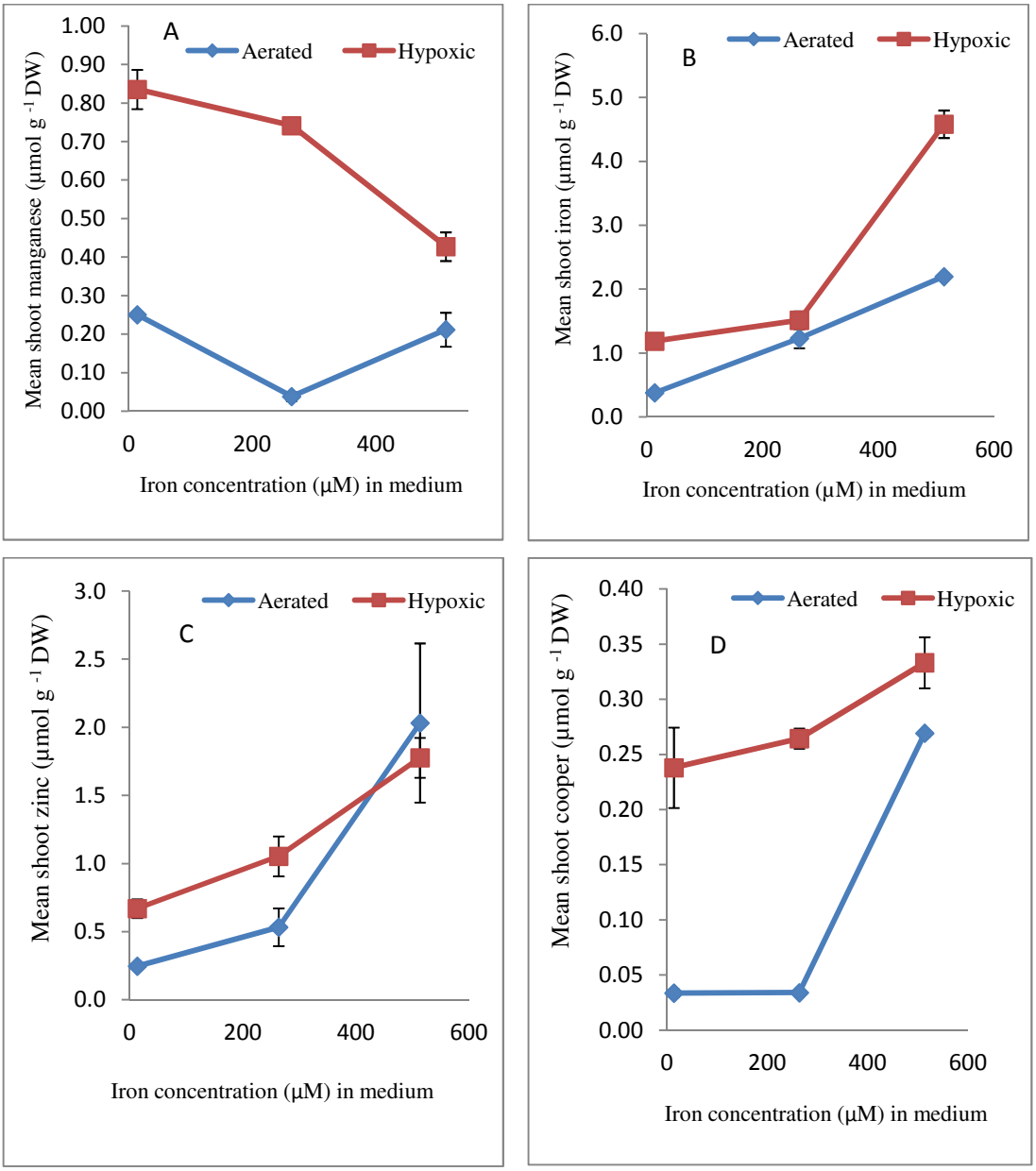


Figure 3.11 Show shoots manganese (A). Iron (B), zinc (C) and copper (D) concentrations of Suaeda maritima plants after 10 days growth, in different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic), in the growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Oxygen concentration in the medium F (1, 30) = 354.897, $P < 0.001$, iron concentration in the medium F (2, 30) = 24.565, $P < 0.001$, the interaction between oxygen concentration and iron concentration in the medium F (2, 30) = 30.444, $P < 0.001$. Shoot iron concentration; Oxygen concentration F (1, 30) = 133.491, $P < 0.001$, iron concentration in the root medium F (2, 30) = 247.571, $P < 0.001$, the interaction F (2, 30) = 39.637, P <0.001. Shoot zinc concentrations;. oxygen concentration F (1, 30) = 1.157, $P = 0.291$, the interaction F (2, 30) = 1.309, $P = 0.285$. Iron concentration in the root medium F (2, 30) = 16.794, $P < 0.001$. Shoot copper concentrations;. Oxygen concentration in the root medium F (1, 30) = 125.930, $P < 0.001$, iron concentration in the root medium F (2, 30) = 51.331, $P < 0.001$, the interaction F (2, 30) = 12.183, $P < 0.001$.

3.4.3.4 Mn, Fe, Zn, and Cu concentrations in the root

The Fe concentration in the root medium had a significant effect on root Mn (Fig. 3.12A), Fe (Fig.3.12B), Zn (Fig.3.12C) ($P < 0.001$) and Cu (Fig.3.12D) concentrations ($P < 0.05$). Oxygen concentration also had a significant effect on root Mn ($P < 0.001$) and Fe concentrations ($P < 0.05$), though root Zn and Cu concentrations were not significantly affected. The root Mn, Fe and Zn concentrations were significantly affected by the interaction between root Fe and aeration ($P < 0.001$); however, root Cu was not significantly affected. Root Fe concentration was increased with increasing Fe concentration in the growth medium under both aerated and hypoxic conditions, whereas root Mn was slightly decreased with increasing Fe concentration in the growth medium, under both aerated and hypoxic conditions.

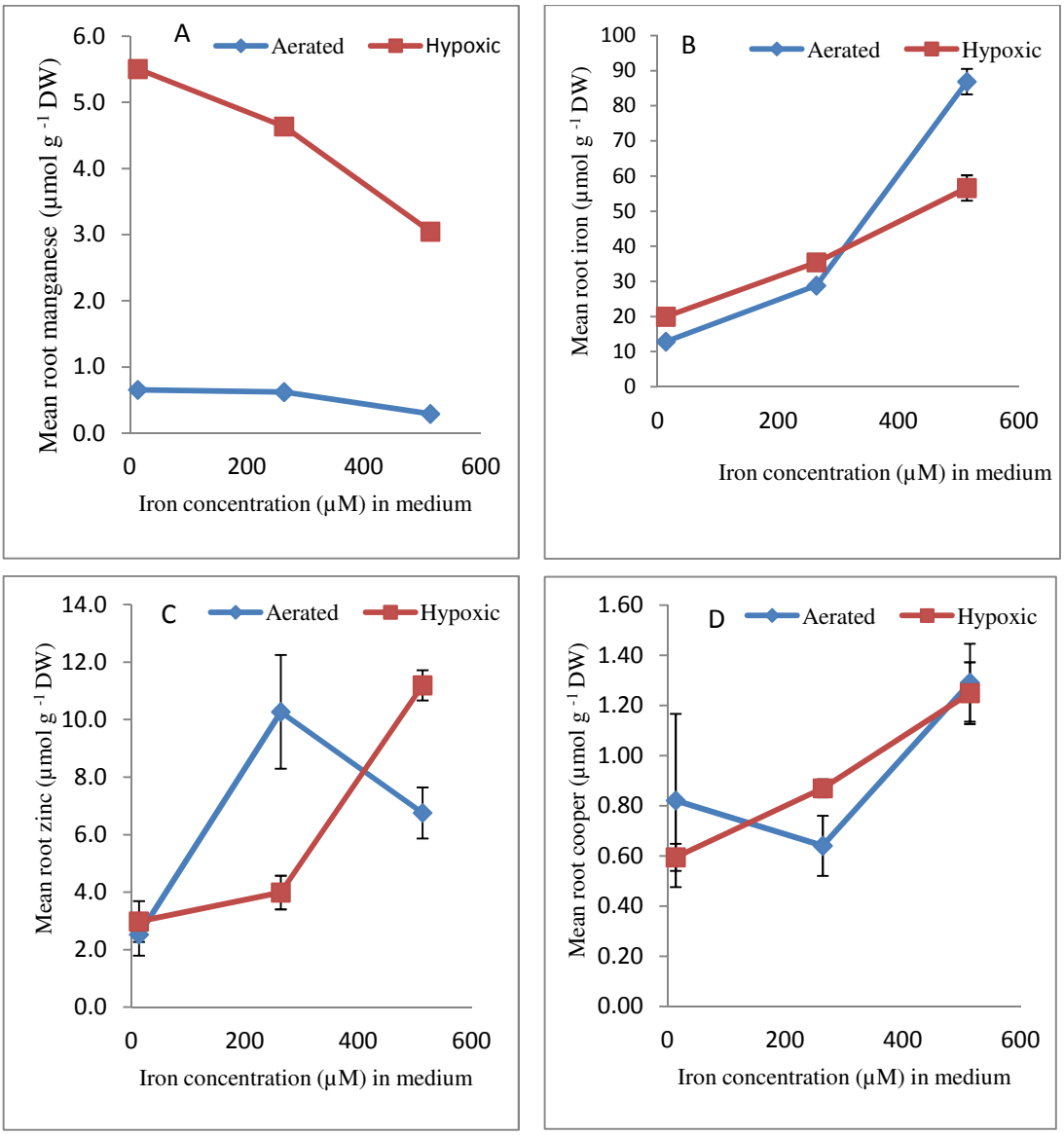


Figure 3.12 Root manganese (A), iron (B), zinc (C) and copper (D) concentrations of Suaeda maritima plants after 10 days growth, in different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic), in the growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Root manganese concentration (A); oxygen concentration in the medium F (1, 30) = 13977.589, $P < 0.001$, iron concentration in the medium F (2, 30) = 645.013, $P < 0.001$, the interaction between oxygen concentration and iron concentration in the medium F (2, 30) = 345.122, $P < 0.001$. Root iron concentrations (B); oxygen concentration in the root medium F (1, 30) = 9.499, $P < 0.05$, iron concentration in the root medium F (2, 30) = 339.731, $P < 0.001$, the interaction F (2, 30) = 48.034, $P < 0.001$. Root zinc concentration (C); iron concentration in the root medium F (2, 30) = 19.294, $P < 0.001$, the interaction F (2, 30) = 13.863, $P < 0.001$, oxygen concentration in the growth medium F (1, 30) = 0.302, $P = 0.586$. Root copper concentration (D); iron concentration in the root medium F (2, 30) = 6.592, $P < 0.05$, oxygen concentration in the growth medium F (1, 30) = 0.009, $P = 0.927$, the interaction F (2, 30) = 0.891, $P = 0.421$.

3.4.4 The response of *Suaeda maritima* to varying concentrations of manganese under aerobic and hypoxic conditions

In this experiment, the growth of *S. maritima* was investigated in nutrient solution where conditions ranged from good aeration (pre-bubbled with air) to severe hypoxia (pre-bubbled stagnant agar solution) in 350 Na^+ mM concentration in artificial seawater at different concentration of $MnSO_4$ (3.35 and 250 µM, 1, 5, and 10 mM).

3.4.4.1 Shoot and root dry weight

Analysis of the data showed that shoot dry weight and root dry weight were significantly affected by Mn concentration ($P < 0.001$), oxygen concentration; shoot dry weight ($P < 0.001$), root dry weight ($P < 0.05$), and their interaction, ($P < 0.001$). As shown in figures 3.25 and 3.26, shoot and root dry weight increased with increasing Mn concentrations in the growth medium, up to 1 mM then decreased. Under aerated conditions, plants had a greater shoot dry weight than those grown in the hypoxic conditions.

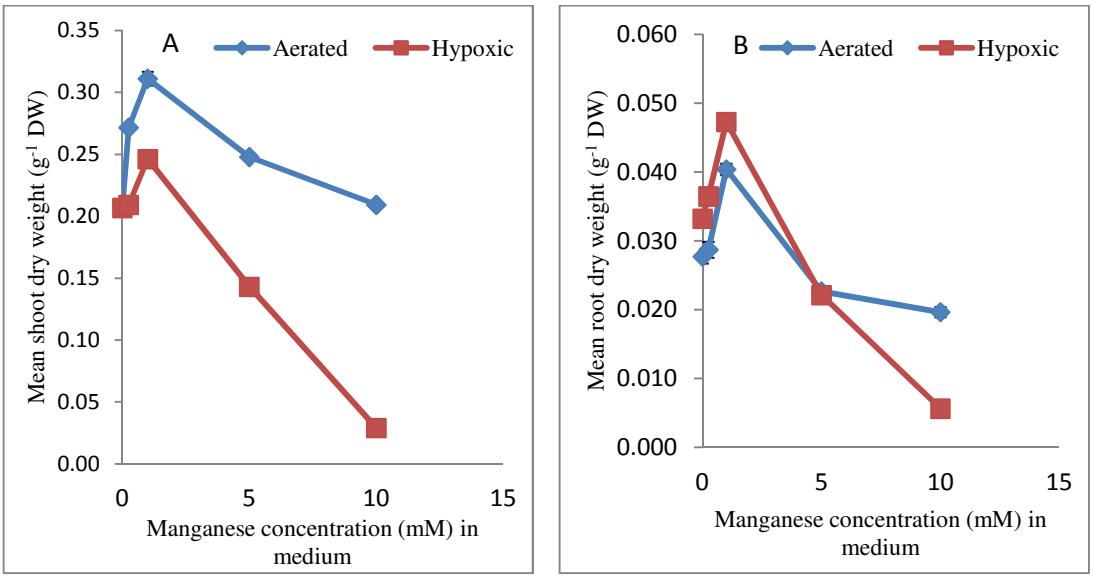


Figure 3.13 Shows mean shoot (A) and root (B) dry weight of Suaeda maritima plants (7 weeks old at harvest), after 3 weeks growth in hydroponic solutions containing 350 Na+ mM (artificial seawater and half strength Arnon & Stout culture solution), in varying Mn concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic). Error bars are SE (n = 14). Data were analysed by 2-way ANOVA. Managanese concentration F (4, 130) = 852.952, $P < 0.001$, oxygen concentration F (1, 130) = 2067.736, $P < 0.001$, and the interaction F (4, 130) = 263.250, $P < 0.001$. Root dry weight; Manganese concentration F (4, 130) = 373.316, $P < 0.001$, oxygen concentration F (1, 130) = 4.307, $P < 0.05$, and the interaction F (4, 130) = 56.237, $P < 0.001$.

3.4.4.2. Total chlorophyll

Total chlorophyll was analysed by ANOVA, showing that total chlorophyll was significantly affected by Mn concentration ($P < 0.001$), oxygen ($P < 0.001$), and their interaction ($P < 0.001$). Total chlorophyll increased with increasing Mn in the growth medium under both hypoxic and aerated conditions, up to 1 mM Mn, then decreased (flooded) or remained constant). At high Mn concentrations (5 and 10 mM), total chlorophyll was higher in hypoxic conditions than in aerated conditions (Fig.3.14) and also whether expressed per unit dry fresh (see Appendix 3.3) weight.

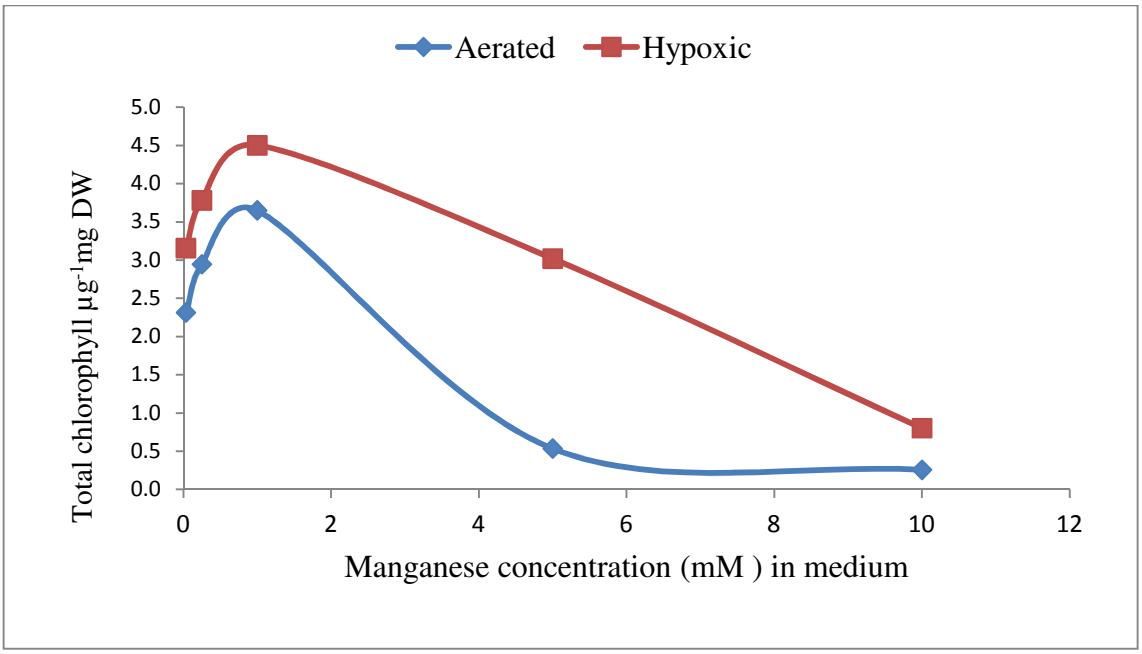


Figure 3.14 Shows total chlorophyll of Suaeda maritima plants (7 weeks old at harvest), after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic). Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Analysis with ANOVA showed a significant effect on the chlorophyll concentration by manganese concentration F (4, 50) = 925.708, $P < 0.001$, oxygen concentration F (1, 50) = 382.161, $P < 0.001$, and the interaction F (4, 50) = 87.086, $P < 0.001$.

3.4.4.3 Shoot and root manganese

Manganese and oxygen concentrations in the growth medium, as well as the interaction, had a significant effect on shoot and root Mn concentrations ($P < 0.001$). Under both aerated and hypoxic conditions, shoot with increasing Mn in the root medium (Fig 3.15A) Mn increased significantly in both aerated and hypoxic conditions. Root Mn

(Fig 3.15B) increased up to an external concentration of 5 mM, but not beyond: in these high external Mn concentrations root Mn was higher in aerated than hypoxic conditions.

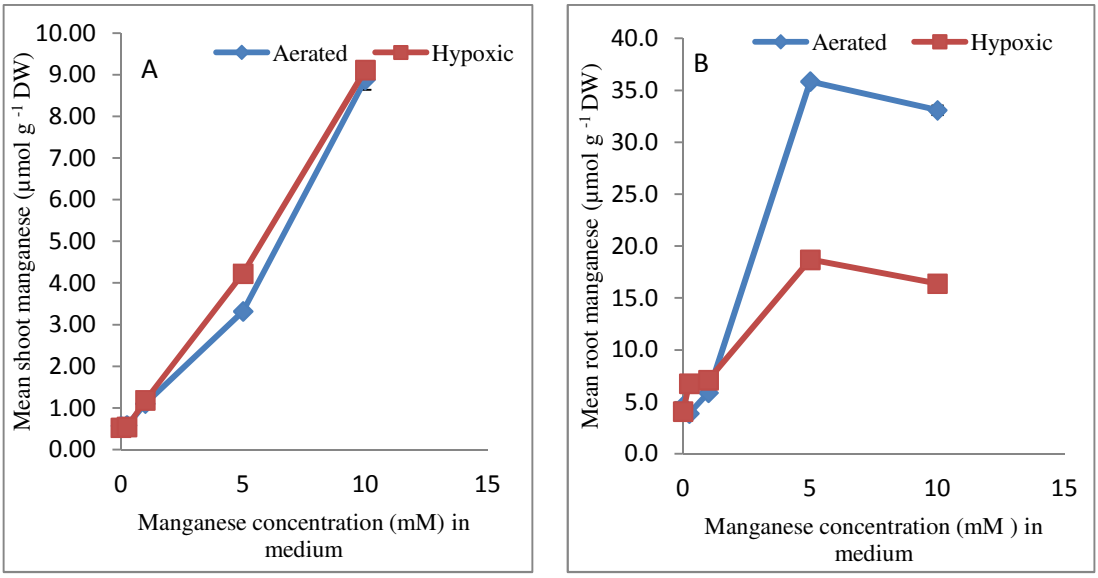


Figure 3.15 Shows the concentration of manganese in shoots (A) and roots (B), in Suaeda maritima shoots after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic). Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot manganese (A); manganese concentration in the growth medium F (4, 50) = 3025.092, P < 0.001, oxygen level F (1, 50) = 14.931, P < 0.001, and the interaction F (4, 50) = 9.132, P < 0.001. Root manganese concentration (B); manganese concentration in the root medium F (4, 50) = 4688.770, P < 0.001, oxygen concentration F (1, 50) = 1670.100, P < 0.001, and the interaction F (4, 50) = 909.008, P < 0.001.

3.4.4.4 Shoot and root iron

Analysis of the data in (Fig 3.16 A and B) showed that Mn and oxygen concentrations in the root medium, and the interaction, had a significant effect on shoot and root Fe concentrations ($P < 0.001$). Under hypoxic conditions, shoot and root Fe concentrations were higher than those in aerated shoots and roots, especially above 1 mM Mn concentrations in the growth medium.

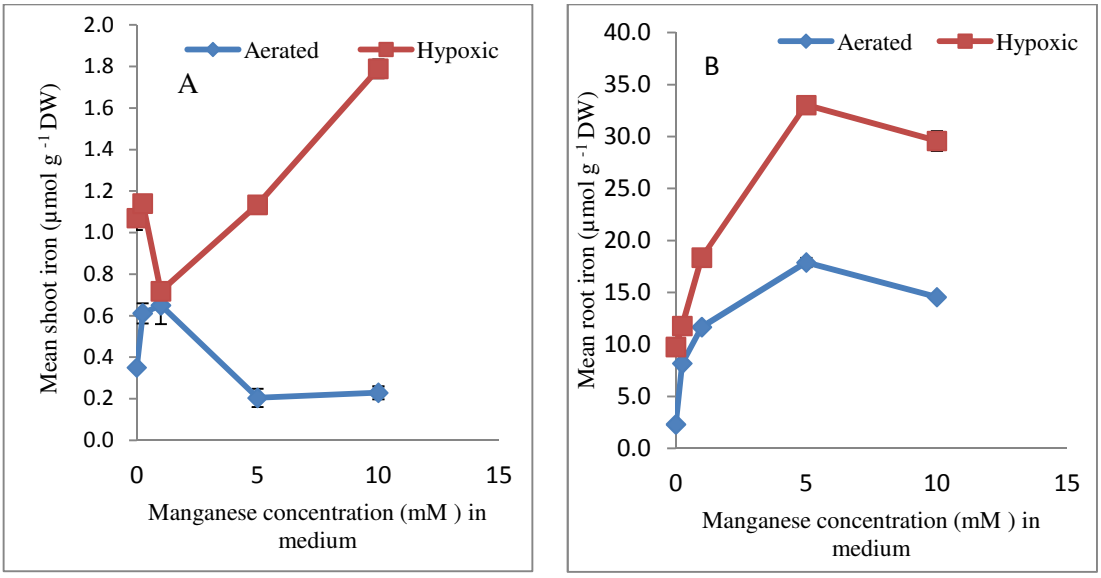


Figure 3.16 Shows mean shoot iron (A) and root iron (B) concentrations in Suaeda maritima shoots, after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated), and stagnant agar solutions (Hypoxic). Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot iron (A); manganese concentration F (4, 50) = 20.950, $P < 0.001$, oxygen concentration F (1, 50) = 693.541, $P < 0.001$, and the interaction F (4, 50) = 72.062, $P < 0.001$. Root iron (B); manganese concentration F (4, 50) = 631.357, $P < 0.001$, oxygen concentration F (1, 50) = 1105.001, $P < 0.001$, and the interaction F (4, 50) = 65.676, $P < 0.001$.

3.4.4.5 Shoot and root zinc

Shoot and root Zn concentrations were significantly affected by Mn concentration in the root medium and their interaction ($P < 0.001$). Oxygen concentration had a significant effect on shoot Zn ($P < 0.001$), but no significant effect on root Zn ($P = 0.150$) was noticed (Fig.3.17 A & B, respectively). Shoot and root Zn concentrations increased with increasing Mn concentrations under hypoxic and aerated conditions. Hypoxic shoots had higher Zn concentrations than those in aerated shoots, and hypoxic roots also had higher Zn concentrations up to 1 mM of Mn.

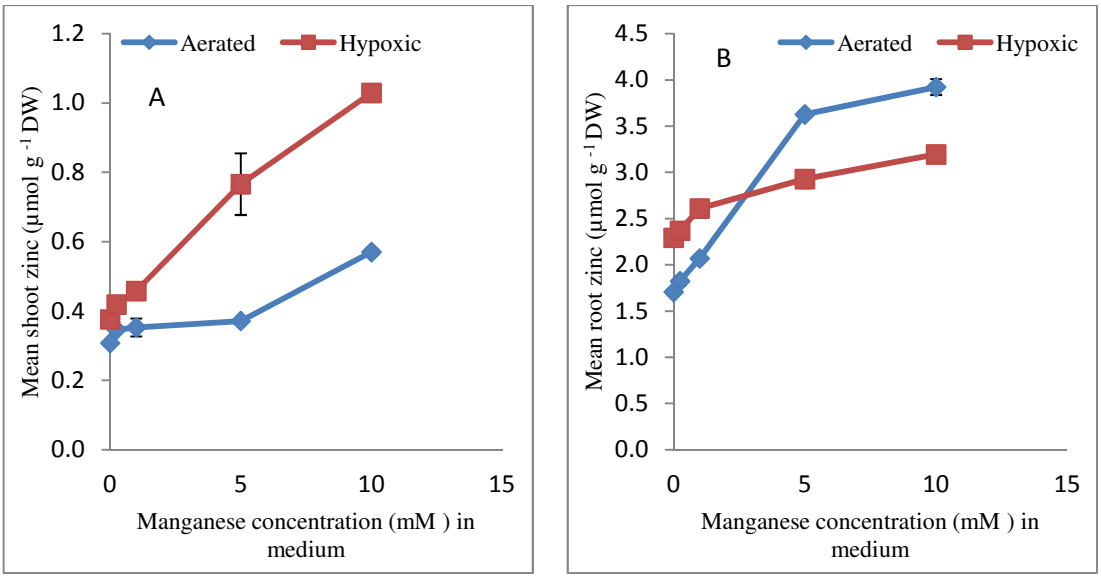


Figure 3.17 Shows mean shoot zinc (A) and root zinc (B) concentrations in Suaeda maritima shoots, after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated), and stagnant agar solutions (Hypoxic). Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot zinc concentration (A); manganese concentration F (4, 50) = 77.380, $P < 0.001$, oxygen concentration F (1, 50) = 131.405, $P < 0.001$, and the interaction F (4, 50) = 19.819, $P < 0.001$. Root zinc concentration (B); manganese concentration F (4, 50) = 369.851, $P < 0.001$, oxygen concentration F (1, 50) = 2.140, $P = 0.150$, and the interaction between manganese and oxygen concentration F (4, 50) = 87.724, $P < 0.001$.

3.4.4.6 Shoot and root copper

Shoot and root Cu concentrations were significantly affected by Mn concentration ($P < 0.001$), the interaction ($P < 0.001$), also shoot copper was significantly affected by oxygen ($P < 0.001$), while root Cu was not significantly affected ($P = 0.666$) as shown in (Fig.3.18, A & B).

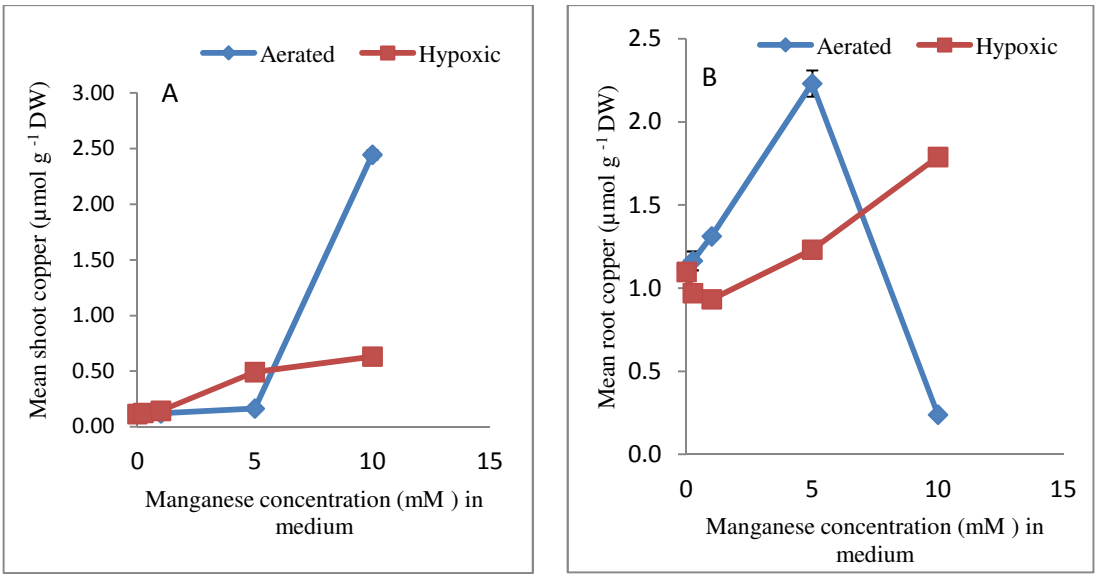


Figure 3.18 Shows mean shoot copper (A) and root copper (B) concentrations in Suaeda maritima plants, after 3 weeks growth in hydroponic solutions containing 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrat concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic). Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Shoot copper concentration (A); manganese concentration F (4, 50) = 2171.986, $P < 0.001$, oxygen concentration F (1, 50) = 607.354, $P < 0.001$, and the interaction F (4, 50) = 1061.818, $P < 0.001$. Root copper concentration (B); manganese concentration F (4, 50) = 149.878, $P < 0.001$, oxygen concentration F (1, 50) = 0.189, $P = 0.666$, and the interaction between manganese and oxygen concentration F (4, 50) = 385.337, $P < 0.001$.

3.4 Discussion

Shoot dry weight (Fig 3.9) decreased with increasing Fe concentrations in the growth medium, as did total chlorophyll (Fig 3.10), and it was higher in aerated conditions. Iron toxicity symptoms appeared in seedlings after 24 h; the seedlings died after 24 h in 1 mM Fe in the growth medium. With the other treatments, the symptoms (yellow colour) began after 7 d of high Fe concentrations in the growth medium. This can be explained as high concentrations of trace metals are known to inhibit growth, and cause a decline in biomass and plant death (Michalak, 2006). Flooded shoot Mn concentrations decreased with increasing Fe in the growth medium (Fig 3.11A). Root Mn concentrations also decreased with increasing Fe in the root medium, and were higher in hypoxic than normoxic conditions (Fig 3.12A). This result can be explained by the interaction between the Fe and Mn in the growth medium, which reduces Mn uptake by plants. The low plant uptake of Mn is caused by excessive available Fe in the root medium (Tanaka and Navasero, 1966).

Shoot and root dry weight increased with increasing Mn in the growth medium up to a concentration of 1 mM, above which it decreased, and was higher in drained conditions (Fig 3.13A and 13B). The same pattern was observed for shoot heights and root lengths (Appendix.3.6 and 3.7), they increased up to 1mM Mn, and then decreased. These results agree with those of Cooper (1984), who found that root dry weight was significantly decreased in *Plantago maritima*, as well as the shoot and root yields of *Aster tripolium* and *Salicornia europaea* at higher Mn concentrations. However in this study, at lower concentrations of Mn in the growth medium, the biomass of *S. maritima* was lower than at 1 mM of Mn. These results are also in agreement with those of Cooper (1984), who noted that in *P. maritima*, the shoot and root dry weight decreased at the lowest Mn concentrations. This was probably due to Mn deficiency, as *S.maritima* has an unusually low concentration of Mn in its tissue in comparison to other treatments. The different symptoms and the response of *S. maritima* to iron in experiment 3 and manganese in experiment 4 may be due to differences in the chemical properties of iron and manganese (Barceló and Poschenrieder, 1990).

Total chlorophyll increased (Fig.3.14) with increased manganese in the growth medium up to 1 mM, above which it decreased, while at 5 and 10 mM Mn, total chlorophyll levels were higher under flooded conditions than in aerated conditions. Mn toxicity symptoms as shown in (Fig 3.23 & 3.24) (black roots and light green shoots) were observed at 18 days and onwards, at 5 and 10 mM Mn, under aerated conditions. Rozema et al (1985) reported that Mn toxicity symptoms (drying and bleaching of the shoots and black roots) were not observable until the third week at 10 mM Mn, in *Artemisia maritima, Atriplex littoralis, Festuca rubra ssp. Litoralis* and *Elytrigia pungens*. The reduction of Mn and Fe increases their availability to plants, leading to several physiological disturbances (Foy et al., 1978), such as the inhibition of chlorophyll biosynthesis due to excess Mn, causing a decrease in the rate of photosynthesis (Foy, 1984; Houtz et al., 1988; Singer and Havill, 1993; Hauck et al., 2002; Srivastava and Dubey, 2011), and a consequent reduction in biomass (Barceló and Poschenrieder, 1990).

Manganese and Fe excess both impaired chlorophyll formation, implying that chlorophyll synthesis is dependent on both Fe and Mn. Houtz et al (1988) reported that

excess Mn may cause increased polyphenol oxidase activity in leaves as well as a decrease in photosynthesis, then a reduction in chlorophyll and protein content in the leaves. This can lead to foliar chlorosis and necrosis (Foy, 1984). Damage to plants has been ascribed to the accumulation of Fe and Mn in the aerial tissues of plants in waterlogged soil, as the increased concentrations may be phytotoxic (Drew and Lynch, 1980). See Experiment 4 (Fig.3.19A) for further details.

There is a strong negative relationship between shoot manganese and shoot dry weight in hypoxic conditions (R^2 = 0.94), and a weak negative correlation between shoot manganese and shoot dry weight in aerated conditions (R^2 = 0.25), in plants grown under hypoxic conditions. Shoot dry weight decreased with increasing manganese in the *S. maritima* shoots; it was slightly decreased in aerated conditions but under hypoxic condition was greatly decreased. The same result was observed in experiment 3 (Fig. 3.19B), where plants were grown under various iron concentrations; the shoot dry weight decreased with increasing iron in the shoot. This may be due to the damaging effects that high levels of manganese and iron can have on processes such as chlorophyll synthesis. These results can be supported by the relationship observed between total chlorophyll and shoot manganese concentration (Fig.3.19C) and also iron concentration (Fig. 3.19D); there is a negative relationship between total chlorophyll and shoot manganese concentrations in both hypoxic and aerated conditions (Fig. 3.19C) (R^2 = 0.8164 and R^2 = 0.6458, respectively).

Total chlorophyll decreased with increasing shoot Mn concentrations, in aerated and hypoxic conditions. Additionally, in experiment 3 (Fig. 3.19D), the total chlorophyll decreased with increasing shoot Fe under both aerated and hypoxic conditions. In experiment 4, a strong positive relationship was found between total chlorophyll and shoot dry weight in hypoxic conditions (Fig. 3.20A) (R^2 = 0.9426) and also in aerated conditions, though it is a weak relationship (R^2 = 0.4874). Shoot dry weight increased with increasing total chlorophyll, under both conditions, also in experiment 3 (Fig. 3.20B).

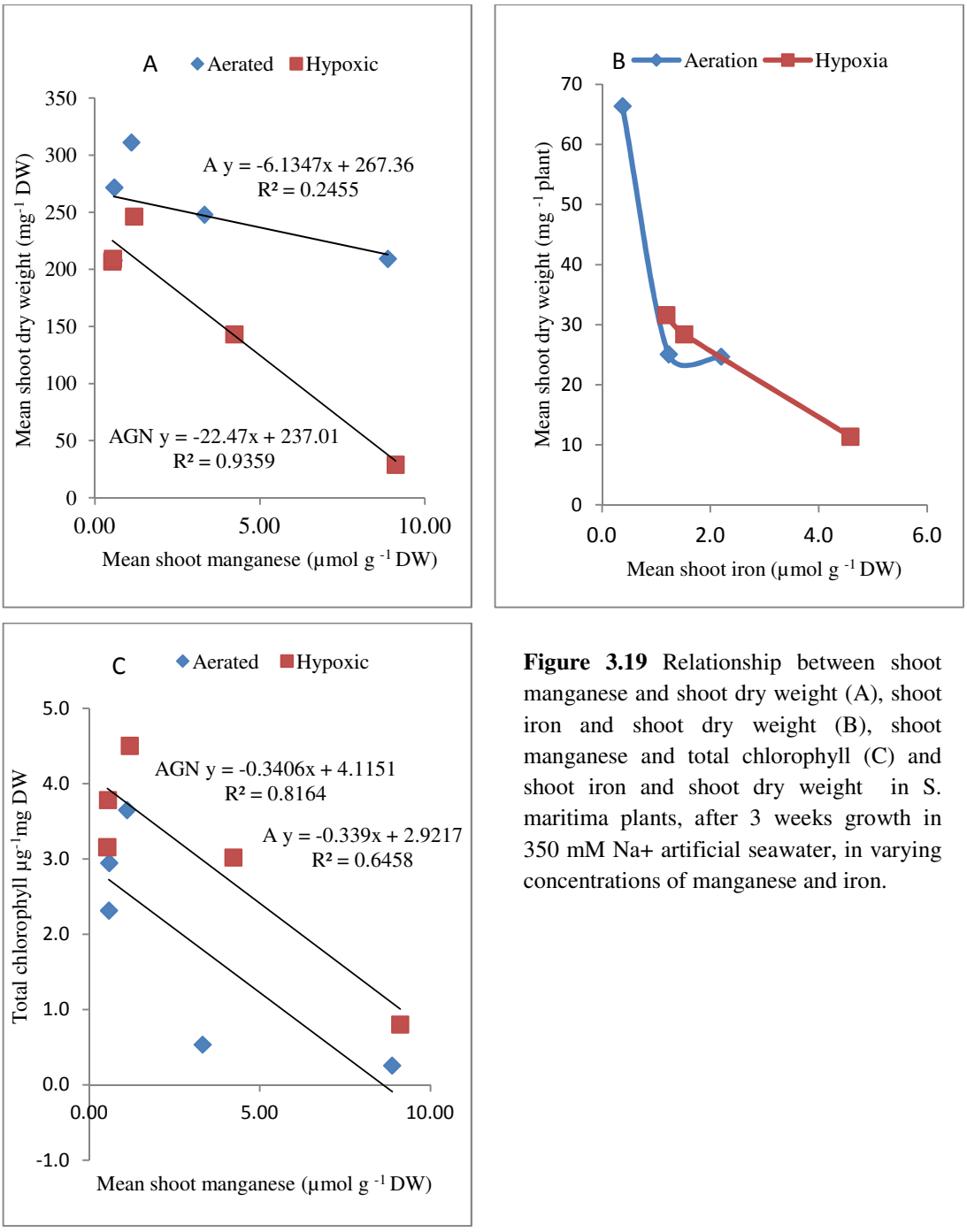


Figure 3.19 Relationship between shoot manganese and shoot dry weight (A), shoot iron and shoot dry weight (B), shoot manganese and total chlorophyll (C) and shoot iron and shoot dry weight in S. maritima plants, after 3 weeks growth in 350 mM Na+ artificial seawater, in varying concentrations of manganese and iron.

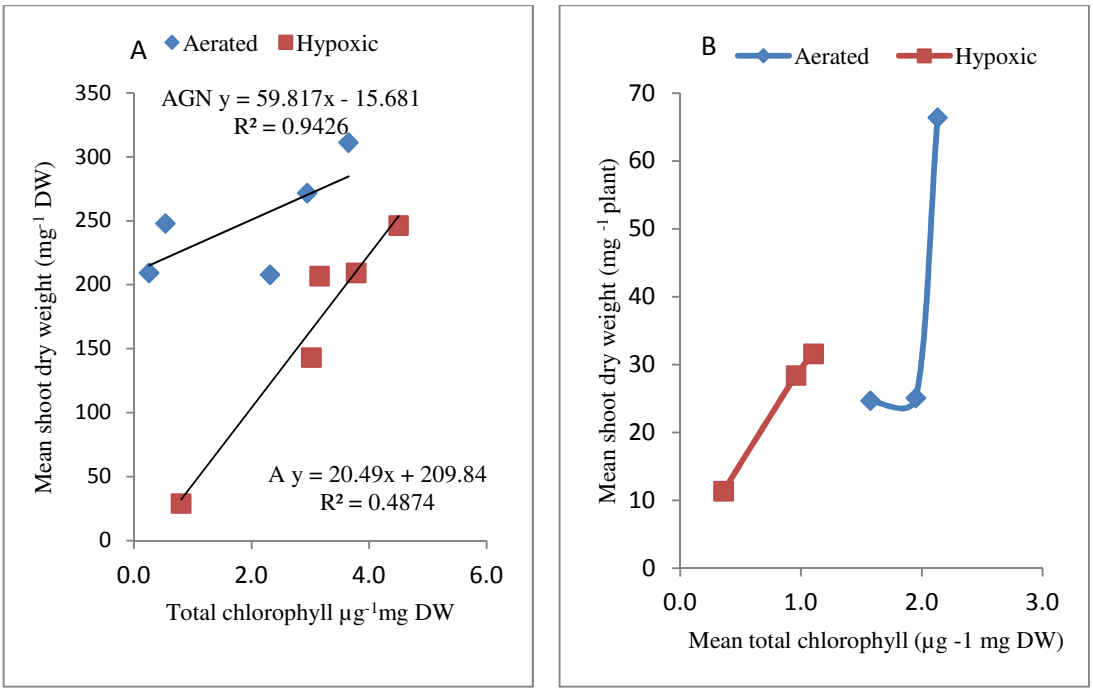


Figure 3.20 Relationship between total chlorophyll and shoot dry weight and total chlorophyll and shoot dry weight in S. maritima plants, after 3 weeks growth in 350 mM Na+ artificial seawater, in varying concentrations of manganese and iron.

There is a negative relationship between root Mn and shoot dry weight (R^2 = 0.58) under hypoxic conditions (Fig.3.21A), shoot dry weight was decreased with increasing root Mn concentration, and slightly decreased in aerated conditions (R^2 = 0.16). Shoot dry weight also decreases with increasing root Fe (Fig.3.21B).

The relationship between root Mn concentration and root dry weight is negative (Fig.3.21C); root dry weight was decreased with increasing root Mn concentration under both conditions, these results agree with those of Waldren et al (1987), who reported that in *Geum urbanum*, there was a negative relationship between root Mn concentrations, and root dry weight. This can be explained by the adverse effect of high Mn and Fe concentrations on root functions and development; high Mn concentrations can reduce important nutrients for plants, such as K, Ca, and Mg (Ohki, 1983). Also, for *Lolium perenne,* the order of toxicity in terms of root growth inhibition was as Mn (Wong and Bradshaw, 1982). There is a strong positive relationship between root dry weight and shoot dry weight (Fig. 3.21D) in both hypoxic and aerated conditions (R^2 = 0.97 and R^2 = 0.67, respectively). Shoot dry weight increased with increasing root dry weight under both conditions, which can be explained by the fact that healthy root

biomass can provide shoots with water and nutrients for growth. This result agrees with Waldren et al (1987), who found a positive relationship between root dry weight and leaf dry weight in *Geum urbanum.* The high significance of these relationships indicates that root biomass, itself determined by trace metals in the growth medium, is the determining factor for the amount of shoot biomass.

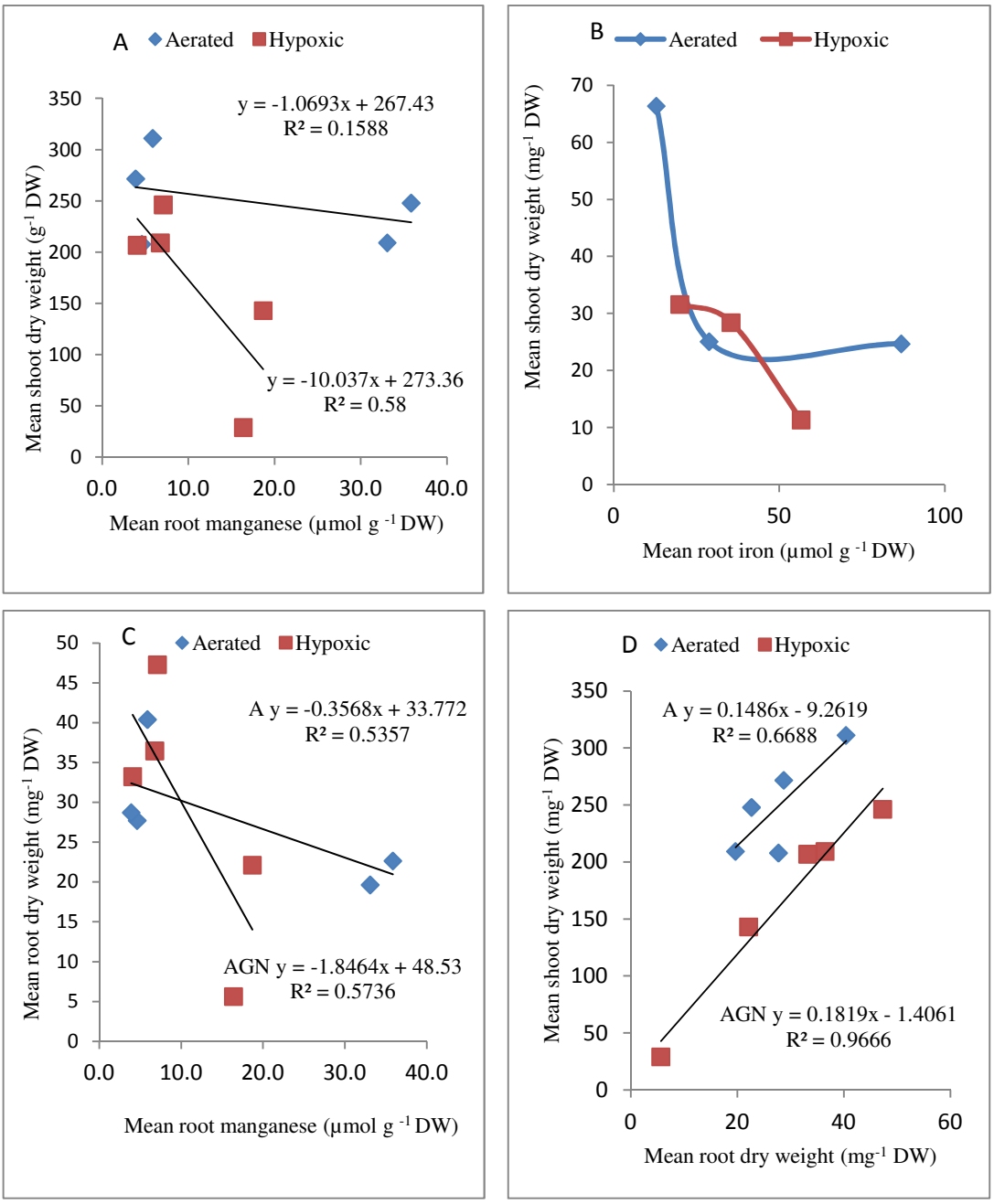
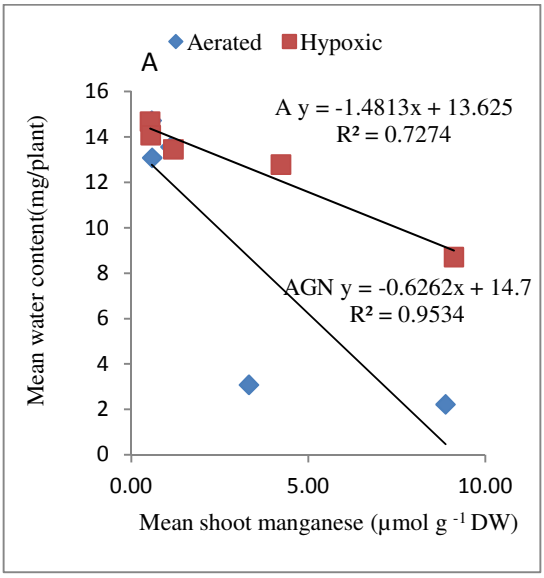
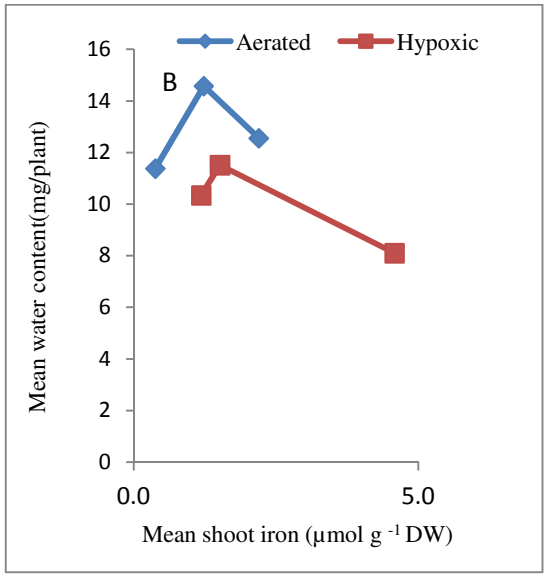


Figure 3.21 Shows the relationship between root manganese concentration and shoot dry weight, root iron and shoot dry weight, root manganese concentrations and root dry weight and root dry weight and shoot dry weight in *S. maritima* plants, after 3 weeks growth in 350 mM Na+ artificial seawater, in varying concentrations of manganese and iron.

There is a strong negative relationship between shoot Mn concentration and water content in both hypoxic and aerated conditions (R^2 = 0.95 and R^2 = 0.72, respectively) (Fig.3.22A). Water content was decreased with increasing shoot Mn concentration, under both conditions. There was also a negative correlation between root Mn and root water content, under both aerated and hypoxic conditions (R^2 = 0.9668 and R^2 = 0.5373, respectively). However in experiment 3 the relationship between shoot Fe and water content under aerated conditions was higher than in hypoxic conditions, while water content under hypoxic conditions was decreased significantly under high shoot Fe concentrations (Fig.3.22B). These results can be explained by the metal stress reducing water uptake as a result of the small size of the roots, as well as the increased resistance to water flow into and within roots (Barceló and Poschenrieder, 1990).





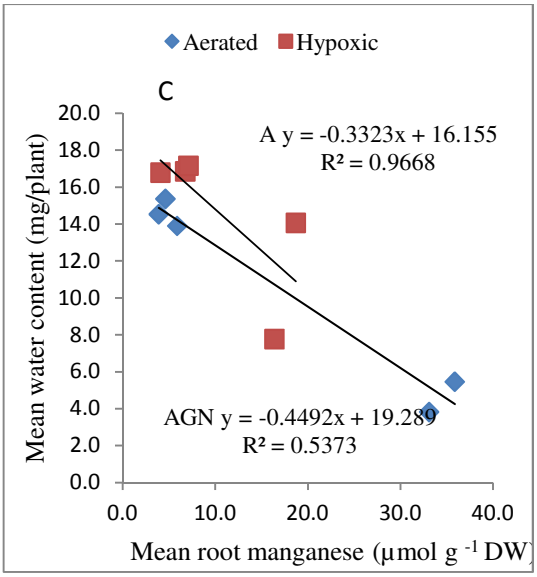


Figure 3.22 Shows the relationship between shoot manganese concentrations and water content, hoot iron concentration and the mean shoot water content and root manganese concentration and water content in S. *maritima* plants, after 3 weeks growth in 350 mM Na^+ artificial seawater, in varying concentrations of manganese and iron.

The roots of plants grown in flooded conditions (low and high salt) accumulated more Mn, Zn and Cu (Figs 3.4B, 3.6B and 3.7B)) than those grown in drained conditions. At low redox potentials, the bioavailability of these elements is increased (References in Table 3.1). Their accumulation in the roots may be a mechanism to avoid the toxicity of their accumulation in the leaves.

Roots of *S. maritima* accumulated much more trace elements than the shoots (Table 3.3), with the exception of Mn in drained conditions, which in experiment 1 was observed to accumulate in the leaves to a greater extent than the roots. These findings are in agreement with those of Reboredo (1993), who reported a higher concentration of Cu, Fe and Zn in the roots of *Spartina maritima* as opposed to the leaves, although in *Atriplex portulacoides* the concentrations of Cu and Zn in the roots and leaves were quite similar. In plant species adapted to waterlogging, Mn and Fe accumulate in root tissue, which prevents the accumulation of toxic levels in leaf tissue, as seen in waterlogged *Juncus gerardii*, where Mn and Fe levels were much higher in the roots than the shoots (Rozema and Blom, 1977). The accumulation of trace metals in the roots appears to be the most common mechanism of protecting the more sensitive shoots from the toxic effects of these metals (Horst and Marschner, 1978; Brown and Wilkins, 1985; Qureshi et al., 1985; Wheeler and Power, 1995; Zhang et al., 1998) Fe and Mn accumulation in the roots of salt marsh halophytes is very common. Mendelssohn and Postek (1982) reported that the reddish-brown deposits observed on the roots of *Spartina alterniflora* (similar deposits observed on the roots of waterlogged rice plants were only on the outer cell wall of the epidermis, and that they are mainly Fe(III) hydroxide, with some Mn. The formation of these coatings was due to the radial oxygen loss that occurs in roots growing in waterlogged soils, such roots have decreased oxidizing ability, most likely impairing their ability to prevent high levels of uptake of Fe and Mn. Rozema et al (1985) also reported higher concentrations of Mn and Fe in the roots than the shoots, for plants inundated with seawater. This may be due to the oxidising ability of roots, and the resultant decreased translocation of Fe^{2+} and Mn^{2+} to the shoots.

In experiment 3; Fe, Zn and Cu concentrations increased in shoots with increasing levels of Fe in the growth medium, in both flooded and drained conditions (Figs. 3.11B, 3.11C and 3.11D). In flooded conditions, concentrations were higher in both roots and

shoots. Root Fe concentration increased with increasing Fe in the root medium (Fig.3.12B). In experiment 3, EDTA was added to the nutrient solutions to maintain Fe availability, though the high affinity of other metals for chelates could result in the displacement of Fe from the chelate complex (Gunn and Joham, 1963; Halvorso and Lindsay, 1972). Checkai et al (1987) reported that at relatively low concentrations, the uptake of Cu and Zn is increased by the addition of EDTA. Srivastava and Appenroth (1995) have also shown that chelation increases uptake of metals, indicating that chelates may be a confounding factor in metal uptake, and as such, free-ion activity is only an estimate of plant availability. It is therefore important to add minimal amounts of chelates in studies of metal toxicity.

Salt concentration in the growth medium affected significantly shoot Mn, Fe, Cu, and Zn concentrations; in flooded and drained conditions. At low salt concentrations (Experiment 1. Table 3.3, shoots accumulated much higher concentrations of Mn, Fe and Cu than at high salt concentrations, in both flooded and drained conditions. Gorham and Gorham (1955) also suggested that ionic antagonism caused by higher ion concentrations in salt marshes, could be the cause behind the lower Mn and Fe levels in the plant tissue of salt marsh halophytes, as compared to freshwater marsh hydrophytes. Cooper (1984) found that salinity treatments resulted in significantly less Mn accumulation in the shoots of *Juncus gerardii, Salicornia europaea* and *Plantago maritima,* due to an antagonistic effect of sodium, with the opposite relationship seen in *Festuca rubra*.

Data from experiment 4 suggests that a Mn concentration of 2.6 µ µmol g^{-1} DW in *S. maritima* shoots might be toxic. This concentration was present in flooded low salt marsh in experiment 1, since in experiment 4, under flooded and aerated high Mn concentrations in the growth medium (5 and 10 mM Mn); the concentrations of Mn in the shoots were more than 3 µmol g^{-1} DW.

In experiment 3, the data suggests that Fe concentration of more than 2 µmol g^{-1} DW in the shoot is toxic. This value was observed in aerated and flooded shoots grown in high Fe concentrations in the growth medium, but in experiments 1 and 2 the concentrations of Fe in all treatments was lower than 2 µmol g^{-1} DW.

Since 1 µmol g^{-1} DW might be toxic in shoots of *S. maritima,* because this concentration was present in experiments 3 and 4, at high Mn and Fe concentrations in the growth

medium, the toxic effects of these metals were observed. In experiments 1 and 2, the concentration of Zn in the shoots was lower than this value in all treatments.
Regarding Cu, more than 0.2 μmol g^{-1} DW concentrations in the shoot may be toxic. This value was present alongside poor growth, at high concentrations of Fe and Mn in the growth medium in experiments 3 and 4, while in experiments 1 and 2 the concentration of Cu in the shoots in all treatments was lower.

It can be concluded that the reduction in growth of *S. maritima* might be due to a deficiency of Zn in the shoot, because in experiments 1 and 2, under aerated conditions in all treatments, the concentration of Zn was higher than in flooded conditions.

Table 3.5 Shows the comparison between trace metal concentrations for *S. maritima* shoots grown at low salt concentrations (100 mM Na^+) and high salt concentrations (350 mM Na^+) in a growth cabinet.

Trace metals in shoot (μmol g^{-1} DW)	Low salt		High salt	
	Drained	Flooded	Drained	Flooded
Mn	1.5±0.1	2.6±0.1	0.6±0.07	0.8±0.05
Fe	1.4±0.09	1±0.07	0.4±0.01	0.8±0.02
Cu	0.08±0.001	0.1±0.004	0.06±0.000	0.09±0.001
Zn	0.016±0.001	0.008±0.001	0.012±0.0002	0.0097±0.0001

In general, it is difficult to study the effects of trace metals on plants at the pH values at which halophytes normally grow (approximately 8.3, the pH of seawater), because as the pH increases from that of normal culture solutions, about pH 5.5 to 6.0, the solubility of the metals decreases, sometimes causing precipitation. Experiments 1, 3 and 4 were performed at low pHs in order to study the toxicity of trace metals (Gupta, 1972; Miles and Parker, 1979; Lexmond, 1980; Suresh et al., 1987; Daviscarter and Shuman, 1993; Watmough and Dickinson, 1995). In experiment 2, plants were grown in a soil-based medium in an attempt to simulate field conditions. In experiment 1, where a low pH was used (~pH 5.5), *S. maritima* shoot accumulated higher concentrations of trace metals (Mn (84% and 92% in flooded and aerated conditions, respectively), Fe (59% in aerated conditions) and Cu (60% and 67% in flooded and aerated conditions, respectively), as compared with experiment 2 (~pH 8.0), although the concentration of

Fe in flooded shoots was similar (Table 3.5). These findings are consistent with those of Bruemmer et al (1986), who also reported that neutral and alkaline conditions in the soil are often associated with low solubility of metal compounds, and the solubilities of Fe, Zn, and Cu in particular have been shown to be lower at high pHs (Harmsen and Vlek, 1985), as shown in Table 3.1. In both experiments 1 and 2 (Table 3.5), shoots grown in flooded conditions accumulated high concentrations of Mn, Fe and Cu. These findings are consistent with those of Adams (1963), who reported that the Fe uptake of plants from the lower marshes of the North Carolina coast were higher than plants from the upper marshes. Rozema and Blom (1977) also found that inundation enhanced the uptake of Fe and Mn by *J. gerardii* and *Agrostis stolonifera*. However, the shoot Zn concentrations, in both experiments 1 and 2, under drained conditions, were higher than those of flooded shoots, 1.2 times higher in drained, high salt conditions than in flooded. In experiment 2, the Zn concentration was 5 times higher in drained shoots than flooded shoots. This may be due to increased Zn solubility in drained conditions, as Gambrell et al (1991) reported an increase in soluble Zn when sediment is well oxidized. In addition, this may be due to the precipitation of Zn in flooded conditions (Sajwan and Lindsay, 1986; Moraghan and Mascagni, 1991).

Table 3.6 Shows comparison of trace metal concentrations in *S. maritima* shoots, grown in 350 mM Na^+ artificial seawater, at ~pH 5.5 in a growth cabinet, and in half strength fresh seawater at ~pH 8, in greenhouses under flooded and drained conditions

Trace metals in shoot (μmol g^{-1} DW)	Experiment 1 ~pH 5.5		Experiment 2 ~pH 8.0	
	Drained	Flooded	Drained	Flooded
Mn	0.58±0.07	0.81±0.04	0.05±0.01	0.15±0.003
Fe	0.40±0.01	0.80±0.02	0.28±0.05	0.86±0.16
Cu	0.06±0.0003	0.09±0.001	0.03±0.005	0.06±0.005
Zn	0.012±0.0001	0.01±0.0001	0.23±0.03	0.04±0.01

Figure 3.23 Roots of *S. maritima* grown under different manganese concentrations, in aerated on the right hand side of each treatment and hypoxic conditions on the left hand side.

Figure 3.24 *S. maritima* shoots (light green) grown in aerated conditions, at high manganese concentrations in the growth medium 5 mM Mn (B) and 10 mM Mn (A)

Conclusion; *S. maritima* is sensitive to high levels of Mn above 1 mM and also to high levels of Fe. The sensitivity to Fe is greater, because even small quantities of Fe can be toxic to plants. The poor biomass under flooded conditions might be a result of high concentration of trace metals in the shoots and roots, which could have an adverse effect on root growth and chlorophyll synthesis due to low shoot biomass. *S. maritima* accumulated much more trace metals at low pH and low salt than high pH and high salt concentrations. On the other hand, *S. maritima* plants can survive under flooded conditions with high levels of trace metals by accumulating these metals in their roots, to prevent toxic levels accumulating in the shoots.

Chapter 4 : Mechanisms of waterlogging tolerance in *Suaeda maritima*: morphological and metabolic adaptations under hypoxic and drained conditions, in varying concentrations of artificial seawater

4.1 Introduction

Waterlogging drastically disrupts the exchange of gases between the air and flooded soil. The aerobic metabolism of plant roots and microbes in the soil gradually uses up the available oxygen, exposing the roots to an environment that is first hypoxic and then anoxic (Jackson and Drew, 1984). Hypoxia is a term denoting an environment in which oxygen availability is the limiting factor for aerobic metabolism, typically seen in temporary flooding, while anoxia denotes a complete lack of oxygen (Morard and Silvestre, 1996), and can be observed in the deeper regions of flooded environments (Sairam et al., 2008).

In plant studies, waterlogging tolerance can be described as the ability to survive or maintain high growth rates under flooded conditions, in relation to well-drained conditions (Setter and Waters, 2003). The adaptations that enable this tolerance may be morphological, physiological or biochemical (Colmer and Flowers, 2008). Different adaptations have been observed in different species: plant species do not necessarily show all tolerance mechanism to be waterlogging tolerant and metabolic characteristics of shoots and roots combine to permit different levels of anoxia tolerance (Drew, 1983; Perata and Alpi, 1993). Morphological, anatomical and biochemical adaptations will be discussed in the present chapter, while physiological adaptations will be dealt with in Chapter 5.

Waterlogging tolerance also requires metabolic adaptations that allow for an increase in the ability to produce ATP when oxygen is in short supply (Brandle and Crawford, 1987; Brandle, 1990). Hypoxia and anoxia, caused by root waterlogging, can reduce root respiration to the point where the plant tissue experiences an energy crisis (Gibbs and Greenway, 2003), this can occur in both waterlogging-tolerant and intolerant plants (Marshall et al., 1973; Lambers, 1976; Drew, 1983; Drew, 1990). In order to cope with

this situation, plants must alter their metabolism to meet several challenges: the need for an increased rate of anaerobic respiration and the need for an increased supply of carbohydrates to fuel the anaerobic respiration (Armstrong et al., 1994; Drew, 1997; Setter et al., 1997). In hypoxic conditions, the ability of the Krebs cycle and oxidative phosphorylation to produce ATP is limited, more so in anoxic conditions, resulting in the dependence of the plant on anaerobic respiration to meet energy demands (Davies, 1980; Legnani et al., 2010). Anaerobic respiration consists of glycolysis followed by fermentation (Fig. 4.1), which recycles NADH back to NAD^+, allowing glycolysis to continue in the absence of oxygen. In anaerobic conditions, plants may use two types of fermentation, ethanolic fermentation and/or lactic acid fermentation (Legnani et al., 2010). Ethanolic fermentation involves the conversion of pyruvate to acetaldehyde and CO_2 by pyruvate decarboxylase (PDC); acetaldehyde is reduced to ethanol, with the oxidation of NADH to NAD^+ by alcohol dehydrogenase (ADH). Pyruvate may, however, also be directly reduced to lactate by lactate dehydrogenase (LDH), with the associated reoxidation of NADH. For example, Hoffman *et al*. (1986) reported the increase in LDH activity (up to 20-fold), in barley after lengthy exposure to hypoxic conditions is an adaptation to the long term lack of oxygen.

An increase in ADH activity has been found in some halophytic species, upon exposure to a combination of salinity and waterlogging. For example, in *Spartina patens*, ADH activity was greater under flooded conditions than in drained, although in *Spartina alterniflora* ADH activity was not significantly affected by flooding (Naidoo et al., 1992). Both ethanolic fermentation and lactate formation have been recorded in some species of genus *Limonium* (see below Rivoal and Hanson, 1993). However, the yield of ATP produced per molecule of glucose is dramatically lower in anaerobic respiration, than aerobic respiration (Fig. 1.2). In order to produce sufficient ATP, the rate of glycolysis must therefore be greatly increased (Jackson and Drew, 1984), and the maintenance of glycolysis and the induction of fermentation are well-known adaptations to anoxia in plants (Kennedy et al., 1992; Ricard et al., 1994; Drew, 1997; Sairam et al., 2008) supporting processes such as ion transport (Huang et al., 1997).

In order to support high rates of glycolysis, roots in hypoxic/anoxic conditions must have access to sufficient amounts of sugars, and the accumulation of sugars in the roots has been observed in waterlogging-tolerant genotypes (Setter et al., 1987; Xia and

Saglio, 1992; Sairam et al., 2009). Kumutha et al. (2008) also reported that tolerance of pigeon pea genotypes to flooding depends on high sugar levels in the roots as well as sucrose synthesis to provide a source for glycolysis; they also reported that under long-term flooding, plants with high levels of sugar were better able to survive than those with lower levels of sugar in their roots. Increased sucrose synthase (SS) activity and the resulting increase in sugar availability in the roots is believed to be one of the most important strategies for coping with waterlogging (Hossain and Uddin, 2011). However, the increase in sugars could be explained by the fact that in hypoxic conditions, root growth is inhibited, while photosynthesis in the leaves is less affected (Mustroph and Albrecht, 2003, 2007).

Anoxia not only presents challenges in terms of the maintenance of ATP production but also in the disposal of end products, ethanol and lactate, which are potentially toxic. In some cases, species that are less tolerant to flooding have been found to produce more ethanol than those that are tolerant (Crawford, 1967; Crawford, 1978; Barta, 1984), which could result from a 'self-poisoning' of the increased amounts of ethanol, although this is not invariably so (Jackson et al., 1982). In order to explain why ethanol production is favoured over lactate for short-term flood tolerance, (Davies, 1980) proposed a 'pH stat hypothesis', suggesting that lactate production causes a potentially fatal decrease in the pH of the cytoplasm (Roberts et al., 1984) in plants such as wheat, maize and barley, which are sensitive to anoxia (Menegus et al., 1989; Menegus et al., 1991). After short periods of anoxia, ethanol is actually the normal product of anaerobic metabolism in most plants (Davies, 1980; ap Rees T et al., 1987; Ricard et al., 1994), whether flooding tolerant or intolerant (ap Rees T et al., 1987).

Both ethanol and lactate, as the end products of anaerobic metabolism, do, then, have drawbacks; both lactate and ethanol are toxic. Although toxic, ethanol diffuses quickly out of cells, leading to a significant loss of carbon (Rocha et al., 2010). However, increasing ADH activity is likely essential once acetaldehyde is synthesised as it is highly toxic at low concentrations (Schaffer, 2006). Drew (1997) and Vartapetian and Jackson (1997) suggested that the transport of ethanol through the xylem could be toxic to the shoots, although Drew (1997) realized that excessively high concentrations of ethanol would be required to cause such damage. The alternative to the production of acetaldehyde from pyruvate, the synthesis of lactate also carries risk. It has been argued that the

production of lactic acid, butyric acid and malate as end products of anaerobic catabolism without a compensatory increase in cations such as K^+, or the exclusion of other acids, leads to a decline in cytoplasmic pH (pH_{Cyt}) (see for example, Roberts et al., 1992; Fox et al., 1995; Felle, 2005), which may lead to plant death.

In spite of its supposed toxicity, significant amounts of lactic acid have been observed in some plant tissues under anoxic conditions (Vartapetian and Jackson, 1997), such as in species of the halophytic genus *Limonium* (Rivoal and Hanson, 1993). For halophytes, however, the normal flux of Na^+ to the vacuoles may facilitate an accompanying flux of lactate allowing its safe compartmentation in vacuoles (Colmer et al., 2012).

S. maritima may experience periods of hypoxia during tidal flooding, so the aim of this investigation was to determine the effect of salinity and waterlogging on the morphological and anatomical characteristics of its roots, together with the sugar content in leaves and roots and lactate concentration in roots. I hypothesized that flooding would lead to an increase in the sugar content of the leaves and the roots, an increase in the concentration of lactate in the roots, and that prolonged hypoxia would lead to the growth of adventitious roots and the development of aerenchyma in the root tissue.

4.2 Materials and Methods

4.2.1 Growth of plants

Plants were grown in a growth chamber, as in previous experiments (Chapter 3, Section 3.2.2, experiment 1), for 6 weeks in half-strength Stout & Arnon (1939) culture solution and with different artificial seawater concentrations (100 and 350 mM Na^+), under aerated and hypoxic conditions; four treatments were used as described below:

(e) Aerated nutrient solution with 100mM Na^+ (A100,).

(f) Aerated nutrient solution with 350mM Na^+ (A350).

(g) Stagnant agar solution with 100 mM Na^+ (AGN 100).

(h) Stagnant agar solution with 350 mM Na^+ (AGN 350).

Oxygen concentrations in the growth medium were recorded before and after changing the nutrient solutions twice a week (Table 4.1). Plants were harvested in several groups;

the first group was used to measure lactate concentration (see Section 4.2.6), another group for measuring sugar concentration (see Section 4.2.2), and another was used for root sections (see Section 4.2.5.2) and other measurements, as described in Chapter 3 (Section 3.2.2.1.1).

4.2.2 Sugar extraction

Sugar was extracted and analysed as described by Stitt (1990). Fresh leaf and root tissue (100 mg of each) were weighed and homogenised in liquid nitrogen and extracted in 750 µl of the cold extraction solvent containing ultra-high purity water (UHP H_2O, 80 µl), methanol (400 µl), and chloroform (200 µl), to which was added 10 µl of 3 mg/ml of internal standard (phenyl β-D-glucopyranoside), to evaluate the proportion of sugar extracted. The extract was transferred to a labelled 1.5 ml pre-cooled Eppendorf tube, vortexed and left on ice for 30 minutes. UHP H_2O (400 µl) was then added, vortexed and centrifuged at 1400 rpm at 0 °C for 2 minutes (centrifuge model 5415R, Eppendorf AG 22331, Hamburg, Germany). The aqueous layer was transferred to a labelled pre-cooled 2 ml Eppendorf tube and UHP H_2O (400 µl) was added to re-extract the chloroform layer. The aqueous phases were then combined and dried overnight in a freeze-drier (Savant, model speedvac concentrator). The dried samples were then dissolved in anhydrous pyridine (850 µl), 150 µl of hexamethyldisilazane and trimethylchlorosilane (HMDS+TMCS+pyridine at a ratio of 3:1:9) for derivatization, then heated for 30 minutes in a 70 °C water bath, under nitrogen in a fume-hood.

The derivatized samples were analysed by GC-MS using a Perkin Elmer Autosystem XL GC, interfaced with a Perkin Elmer TurboMass mass selective detector. A DB5-MS capillary column (30 m x 0.25 mm internal diameter, film thickness of 0.25 µm) was used with helium as the carrier gas, at a constant flow rate of 1.3 ml/min. The injector and MS source temperatures were maintained at 250 °C. The column temperature programme consisted of injection at 60°C with a hold time of 3 minutes, then the temperature was increased by 10°C per minute to reach a final value of 325°C, followed by an isothermal hold at 325°C for 5.5 minutes. The MS was operated in electron impact ionization mode with ionization energy of 70 eV. The scan range was set from 45 to 600 Da with a total scan time of 0.3 seconds. The injector was operated in splitless mode with a splitless time of 60 seconds. The results showed several peaks, with each peak representing one sugar and the peaks were also identified using the NIST Mass Spectral Database.

4.2.3 External standards

Pure glucose (D-(+)-glucose, ACS reagent Sigma-Aldrich), fructose (D-(-)-fructose Fluka), sucrose (Sigma Ultra, >99.5% GC), and phenyl-D-glucopyranoside (13.3 mg of each) were used in individual test tubes and also as a mixed standard (where 13.3 mg each of glucose, fructose, sucrose and phenyl-D-glucopyranoside were combined in the same test tube) and dissolved in pyridine (1 ml). HMDS+TMCS+pyridine (3:1:9); 0.2 ml) was added to 1µl of the standard solutions. All samples were derivatized by heating in a water bath at 70 ºC for 30 minutes under nitrogen conditions in a fume-hood. The standard and sugar assays were conducted at the same time.

4.2.4 Calculation of tissue sugar concentrations

1) Response Factor (RF) for each compound:

RF = Peak area / Amount

2) Relative Response Factor (RRF) for each sugar:

RRF = RF for a compound / RF for Internal Standard

The RRF should be close to 1

3) Formula used for sugar calculation in a sample:

Amount = Peak area for sugar x amount of Internal Standard / Peak area for Internal Standard x RRF for sugar

4.2.5 Root morphology and anatomy

4.2.5.1 Morphological characteristics

The roots from plants grown in hypoxic conditions were separated into adventitious roots (upper) and seminal roots (lower) [seminal roots are those that emerge from the scutellar node located within the seed embryo after a few days of germination and form the main root system of most plants]. However, as plants grown in aerated conditions do not produce adventitious roots, the roots were divided into two parts as shown in (Fig 4.1), and were defined in relation to the root/shoot junction; the upper root beginning at 2cm below the root/shoot junction and the lower root could be identified by the difference in appearance, having fewer lateral branches: both samples included laterals and the main root. The lengths of roots were measured with a ruler.

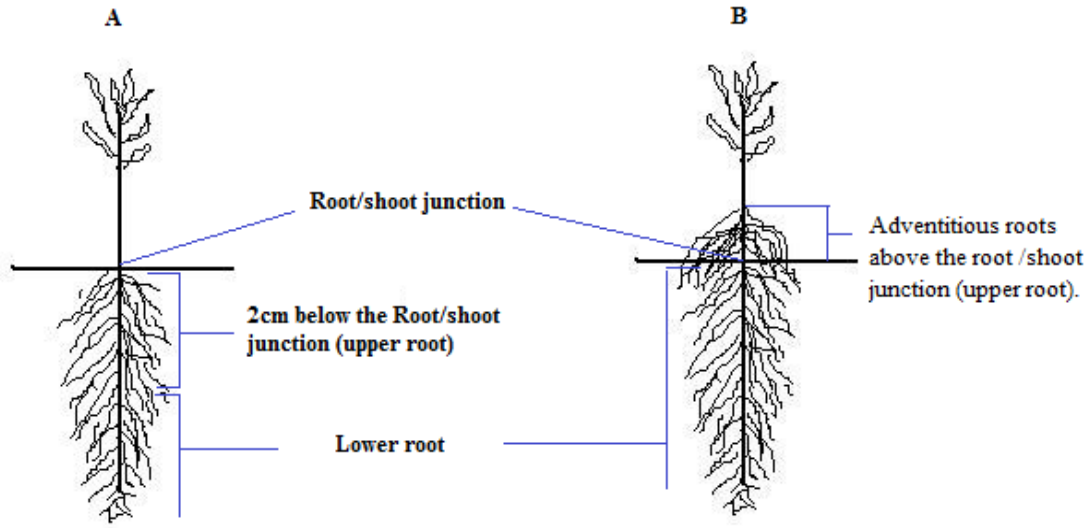


Figure 4.1 Diagram shows roots excised to upper and lower root in roots grown under aerated conditions (A) and adventitious root (upper root) and lower root under hypoxic conditions (B).

A sample of the area of root hairs was estimated as follows: root hairs of seminal roots and adventitious roots were randomly selected, 10 hairs per root for seven plants from each treatment. This method was chosen because it was impossible to scan all the root hairs to obtain the total surface area using Delta-T SCAN software (version 2.04nc, Delta-T Devices Ltd., UK).

4.2.5.2 Preparation of root sections from treatments with different concentrations of salinity in aerated and hypoxic conditions

The harvested roots were immediately fixed in solutions of the same osmotic potential in which they had grown (using 4% formaldehyde (Sigma)), in Stout & Arnon solution, in 100 and 350 mM Na^+ artificial seawater. After 24h the roots were removed from the fixative and rinsed several times with distilled water.

Root sections were cut with a sharp razor blade. Fixed roots were selected by width and placed in 50% *Industrial Methylated Spirits (*IMS) in glass vials for at least 12 h. The tissue was then dehydrated in 70% IMS for 1 h, 95% IMS for 1 h, 100% IMS for 30

minutes and 100% IMS was repeated 3 times, for 30 min each, before immersion in *Histoclear* (R. A. Lamb,) for 30 minutes and then fresh Histoclear for at least 12 h. The Histoclear was then removed from each vial, replaced with molten wax (Histology, R. A. Lamb) at 65°C and then placed in an oven at 60 °C for 2 h. The molten wax was then replaced three times, each time the root was left in the wax for 2 h at 60 °C. The wax-impregnated roots were then poured with the molten wax into a plastic mould, the root lengths quickly aligned and the wax was left to set for at least 24 h. The wax blocks were then removed from the mould, trimmed of excess wax with a razor blade and attached to microtome chucks with wax. After 24 h, transverse sections (10 μm) were cut from the root using a hand rotary microtome (Spencer 820). Sections were mounted on slides in glycerine/albumin (3 drops in 30 ml distilled water) and then left overnight at 37 °C in an oven to dry before being placed in the oven for 20 minutes at 60^{o}C. The slides were then placed in Histoclear:wax (Dewax) for 10 minutes, fresh Histoclear (without wax) for another 10 minutes, 100% IMS for approximately 5 minutes, in fresh 100% IMS for another 5 minutes, then 95% IMS for 5 minutes, and 70% IMS for 5 minutes. Staining for cellulose was carried out with Fast Green, (0.5 % in 50 % IMS), for 15 minutes, using the methods of Hajibagheri et al. (1985) and Wetson (2008). A light microscope (ZEISS, Axiophot, Germany) with a camera (Axio cam HRC) was used to observe the sections. The area of intercellular spaces between the cells in the cortex was measured (Appendex 4.4) with software available on the light microscope (AxioVision Rel. 4.8, Carl Zeiss, Göttingen, Germany).

4.2.6 Lactate production by roots of *Suaeda maritima* grown in different salt concentrations under hypoxic and aerated conditions

Both parts (upper and lower root) from all treatments were used for the determination of lactate concentration. Roots that were incubated for 4 h under anoxic conditions were investigated to determine whether *S. maritima* roots grown in aerated conditions were able to switch from aerobic respiration to fermentation.

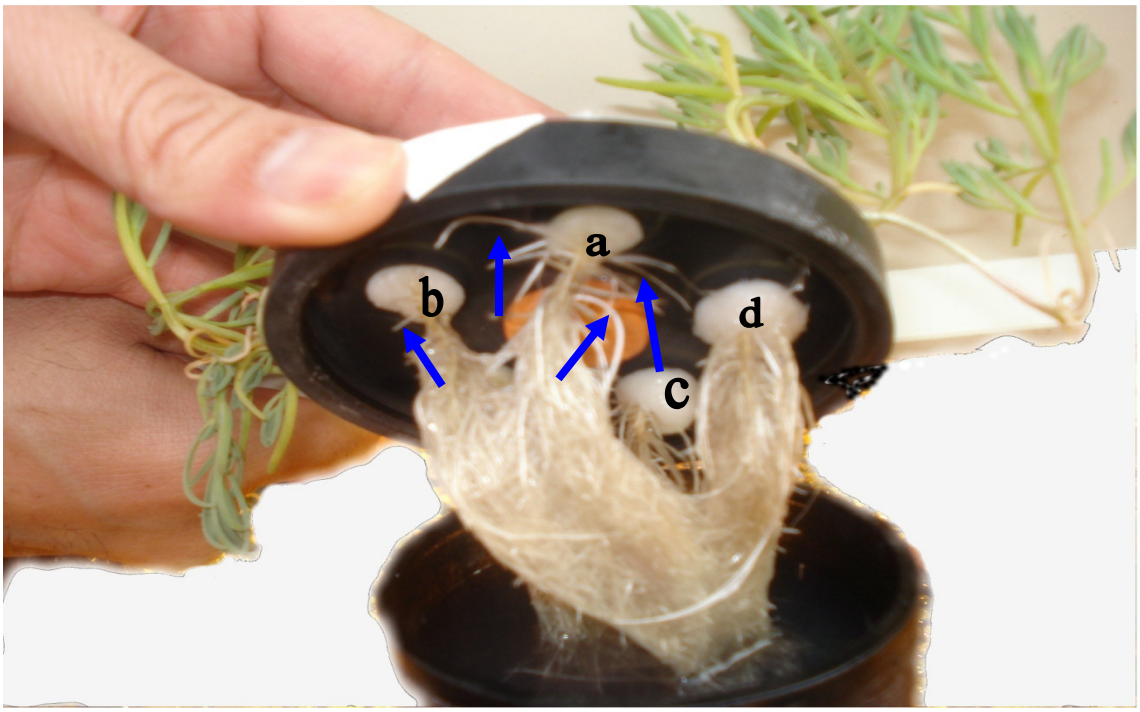


Figure 4.2 Photograph shows the adventitious roots under hypoxic conditions. Arrows indicate adventitious root arising from the stems of four plants, a, b, c and d, which were suspended with non-absorbent cotton wool through holes in a lid.

The plant roots used for incubation were washed in fresh growth medium (100 or 350 mM Na^+ artificial seawater in Stout and Arnon), containing 10 mg/l Carbenicillin, for 1 minute; followed by 1 minute in the same solution that had been autoclaved prior to the addition of Carbenicillin. The roots were then excised into upper and lower root, for incubation hypoxic conditions. Root samples grown in hypoxic conditions were washed and excised in an atmosphere of argon in the fume-hood, while the roots grown in aerated conditions were washed and excised in air. The excised roots from both aerated and hypoxic plants were transferred and incubated for 4 h, in vials (approximately 20 ml) in darkness, at 22-23 °C. The solutions in all vials were pre-flushed with argon to obtain anoxic conditions (see Figure 4.3). After 4 hours of incubation, the incubated root samples were blotted with paper towel and weighed before being frozen in liquid N_2 and then homogenised before storage at -80°C. Four replicates of initial (not incubated) root samples, from both aerobic and stagnant agar, were also excised into upper (adventitious root) and lower (seminal root) portions (as described above), weighed and frozen with liquid N_2.

Roots (100 mg FW of initial and incubated samples; aerated and hypoxic upper root, and aerated and hypoxic lower root) were ground twice in 0.5 ml of ice-cold 5% perchloric acid in a pestle and mortar, producing a volume of approximately 1 ml. The homogenates were then centrifuged at 11,000 rpm for 15 minutes. The supernatant was collected and neutralised with 80 µl 5 N K-carbonate, and re-centrifuged at 11,000 rpm for another 10 minutes at 4 °C. The supernatant was kept on ice, with 100 µl used for each assay. L-lactate concentration was measured using the procedure of Gutmann and Wahlefeld (1974); L-Lactate dehydrogenase solution, type II, from rabbit muscle (13 µl) was added to 8 ml of 25 mM phosphate buffer. The phosphate buffer contained 0.91 M KCl, 0.3 M glycylglycine, and 5 mM β-NAD, which was added just prior to use. The pH was adjusted to 9.0 with 1 N KOH prior to the addition of NAD. To the reaction tube, 500 µl of the phosphate buffer, 300 µl of deionised water, 100 µl of the enzyme, and 100 µl of supernatant that had neutralised were added, and the mixture was incubated for 30 minutes at 37 °C. Absorbance was measured at 340 nm (Pye Unicam SP 800 spectrophotometer; the zero lactate standard was used as a blank). A standard curve was prepared using 0, 40, 80, 160, and 320 µl of 5 mM L-lactate. Water was added to each to make up the volumes to 400 µl, then 100 µl of lactate dehydrogenase in phosphate buffer was added and the mixture was kept for 30 minute at 37 °C.

Figure 4.3 Illustrates the system for incubating root tissue under anoxic conditions in a bath of Argon. The tissue from aerated and hypoxic low and high salt (100 and 350 Na+ mM artificial seawater) roots (adventitious and seminal root in hypoxic samples) and (upper and lower seminal root in aerated samples)) was incubated for 4 h in vials (approx. 20 ml) contained in a tank flushed with Argon. Argon was delivered from a cylinder through a tube which divided into two conical flasks containing water so that the rate of bubbling could be monitored and adjusted. Argon was used as it is heavier than nitrogen and so anoxic conditions were assured (Tavzes et al., 2001). The tank was contained in a fume-hood to evacuate excess Argon.

4.3 Results

4.3.1 Oxygen concentration in the growth media

As shown in Table 4.1, before renewing the root media; the oxygen concentrations surrounding the roots in hypoxic conditions were slightly decreased in both salt concentrations, whereas under normoxic conditions, the oxygen concentrations were significantly decreased ($P < 0.05$).

Table 4.1 Shows the oxygen concentration before and after renewing the growth medium (twice a week) for *S. maritima* plants grown under different concentrations of oxygen: aerated medium (A), and hypoxic medium (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth chamber at 20^0C. Error bars are SE (n=70). Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level.

		Treatment			
		A100	AGN100	A350	AGN350
Oxygen concentration mgL^{-1}	After	8 ±0.1a	0.4 ± 0.01a	7.9 ±0.04a	0.5±0.02a
	Before	3.2 ±0.1b	0.3 ±0.01a	2.5 ±0.1b	0.3±0.02a
	Average	5.8 ± 0.1a	0.4 ± 0.01a	6.4. ± 0.1a	0.5 ± 0.01a

4.3.2 Root dry weight

The dry weight of the whole roots (Fig 4.4), including adventitious roots, was increased by decreasing oxygen concentration in the root medium, in low and high salt concentrations ($P < 0.001$). It was slightly higher in hypoxic than in aerated conditions ($P < 0.05$), whereas no effect was noticed using salt concentration ($P = 0.798$).

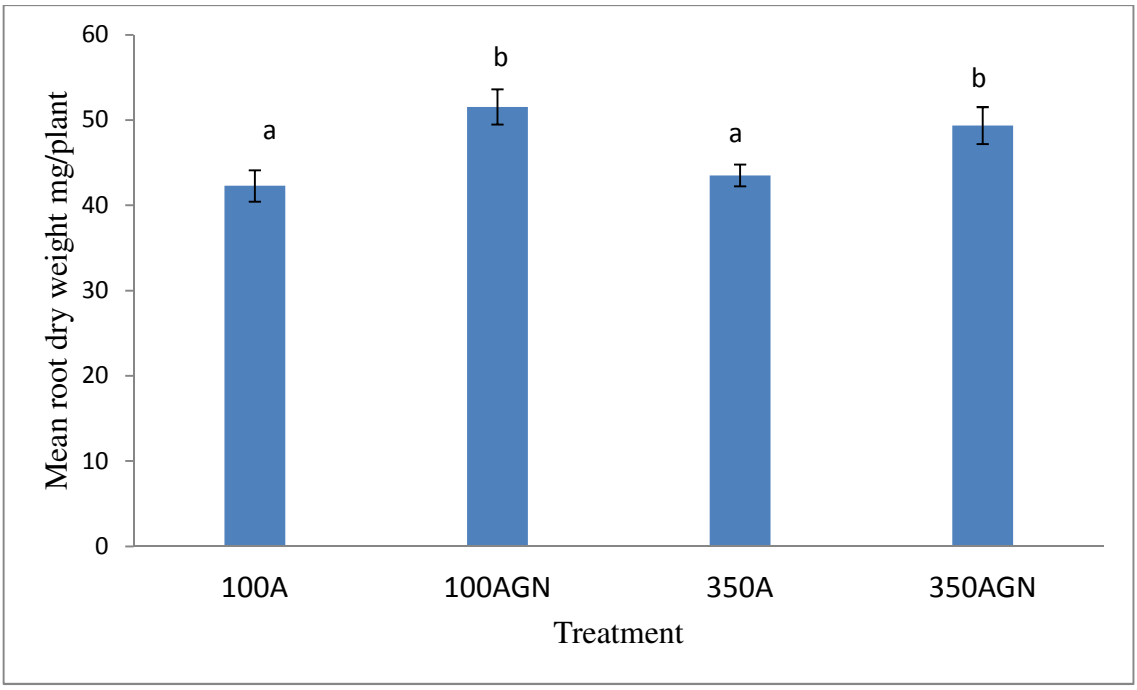


Figure 4.4 Root dry weight of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+) in a growth chamber. Error bars are SE (n=10). Data were analysed by 2-way ANOVA. Oxygen (F (1, 36) = 16.357, $P < 0.001$), salt (F (1, 36) =0.067, $P = 0.798$), the interaction (F (1, 36) = 0.0.833, $P = 0.368$). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

4.3.3 Intercellular space area in the cortex

The area of intercellular space in the cortex (Fig 4.5) was significantly increased by decreasing oxygen concentration in the growth medium, in both low and high external salt concentrations ($P < 0.001$). However, it was not affected by salt concentration in the growth medium ($P = 0.0792$).

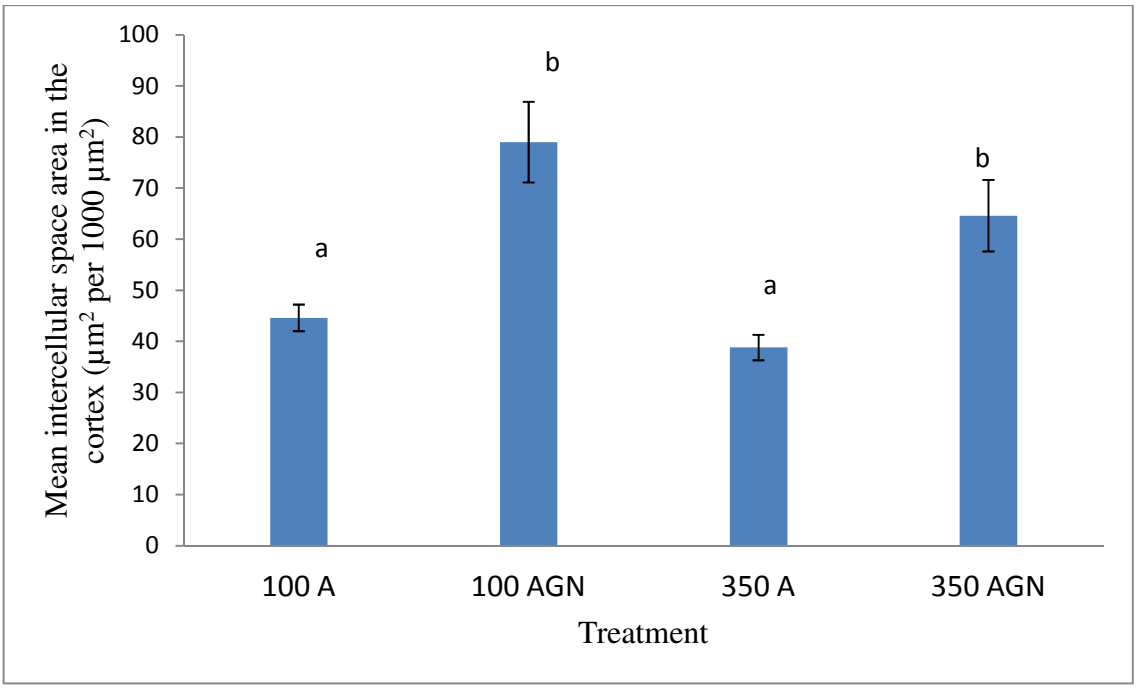


Figure 4.5 Mean intercellular space area in the cortex (µm2 per 1000 µm2) roots of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n=22). Data were analysed by 2-way ANOVA. Oxygen (*F (1, 84) = 28.119, P > 0.001*), salt (*F (1, 84) =3.165, P = 0.079*), the interaction (*F* (1, 84) = *0.589, P = 0.445*). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

4.3.4 Root length

The root length was investigated to determine whether oxygen deprivation in the root growth medium affected root elongation. The length of the root (Fig 4.6) was significantly reduced by decreasing oxygen in the external growth medium ($P < 0.001$). However, no significant results were observed with respect to salt concentration ($P = 0.518$).

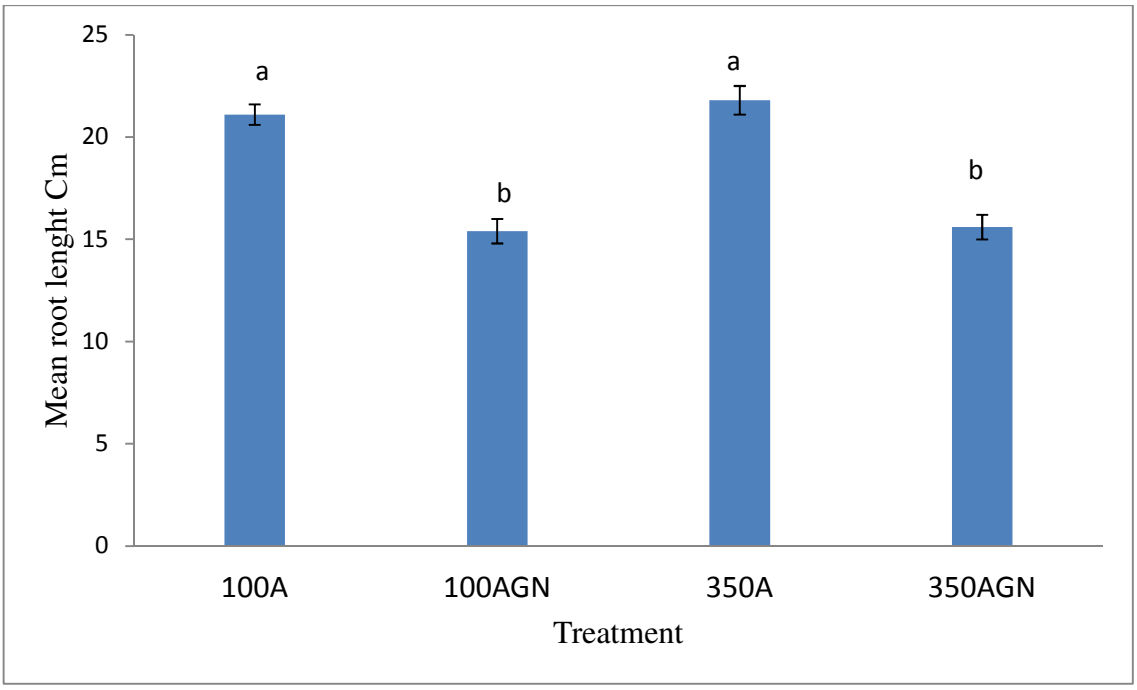


Figure 4.6 Root length of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na+) in a growth chamber. Error bars are SE (n=14). Data were analysed by 2-way ANOVA. Oxygen (F (1, 52) = 97.566, $P < 0.001$), salt (F (1, 52) =0.423, $P = 0.518$), the interaction (F (1, 52) = 0.171, $P = 0.681$). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

4.3.5 Root area (root hair sample)

The roots were divided into two parts for all treatments, as mentioned above, and the area of equivalent samples of the root hairs was determined. The area of the sample from the seminal (lower, Fig 4.7) root hairs was significantly increased by increasing external salt concentration under aerated conditions ($P < 0.001$), but was similar under hypoxic, low and high salt conditions. The oxygen concentration in the growth medium caused a significant increase ($P < 0.001$) the lower root hair area; under hypoxic, low salt concentrations, it was double that in aerated, low salt conditions, while in aerated and hypoxic high salt conditions, the effect on area was insignificant. With regards to upper root hair area, it was also significantly increased by increasing external salt concentration, under both aerated and hypoxic conditions ($P < 0.001$). The upper root hair area was also significantly increased by reducing oxygen concentration in the growth medium, under both external salt concentrations ($P < 0.001$).

In general, the root hair area in upper roots, in all treatments, was higher than in lower roots.

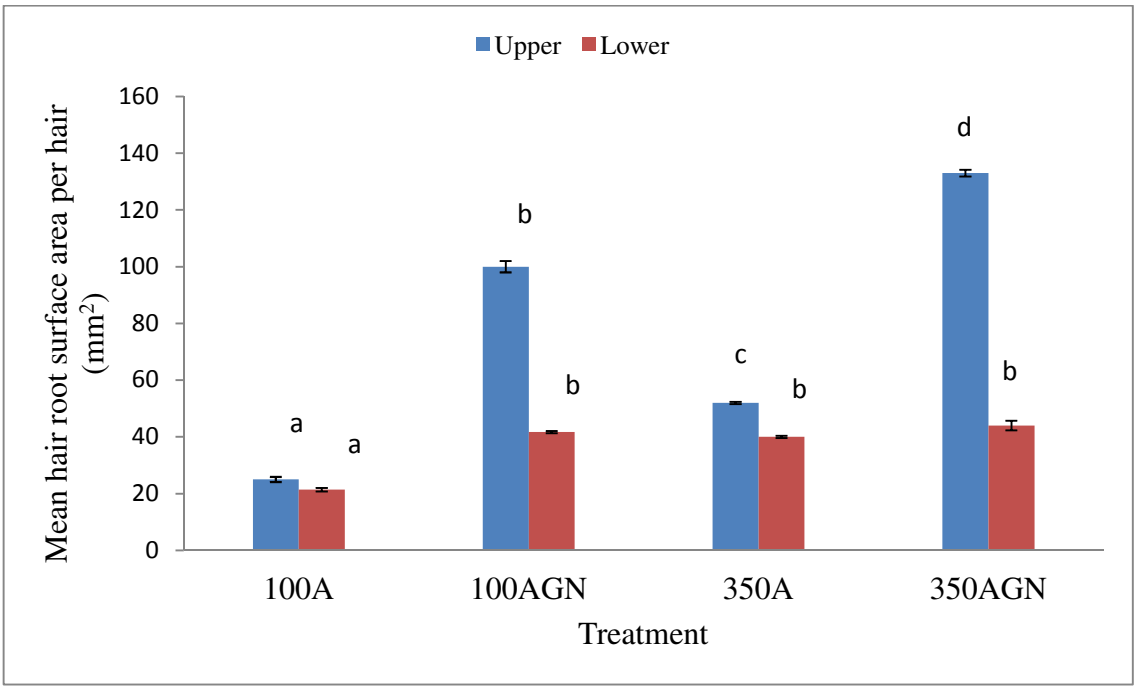


Figure 4.7 Upper root hair area and lower root hair area per root hair of *Suaeda maritima* plants, after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n=7). Data were analysed by 2-way ANOVA. Lower root area; salt (F (1, 24) = 118.400, P < 0.001), oxygen (F (1, 24) = 159.914, P < 0.001) and the interaction between salt and oxygen concentration in the growth medium (F (1, 24) = 67.805, P < 0.001). Upper root area; salt (F (1, 24) = 361.794, P < 0.001), oxygen (F (1, 24) = 3896.340, P < 0.001), the interaction salt and oxygen (F (1, 24) = 1.794, P = 0.193). Letters above bars indicate significant difference in means from post-hoc Tukey tests for upper and low root separately.

4.3.6 Root lactate concentration

There was no effect of incubation on upper or lower root lactate concentrations (appendix 4.1and 4.2). The data which are presented pertain to samples that were frozen in liquid N_2 after excision, for upper and lower root and (Fig 4.8) and the whole root (4.9).

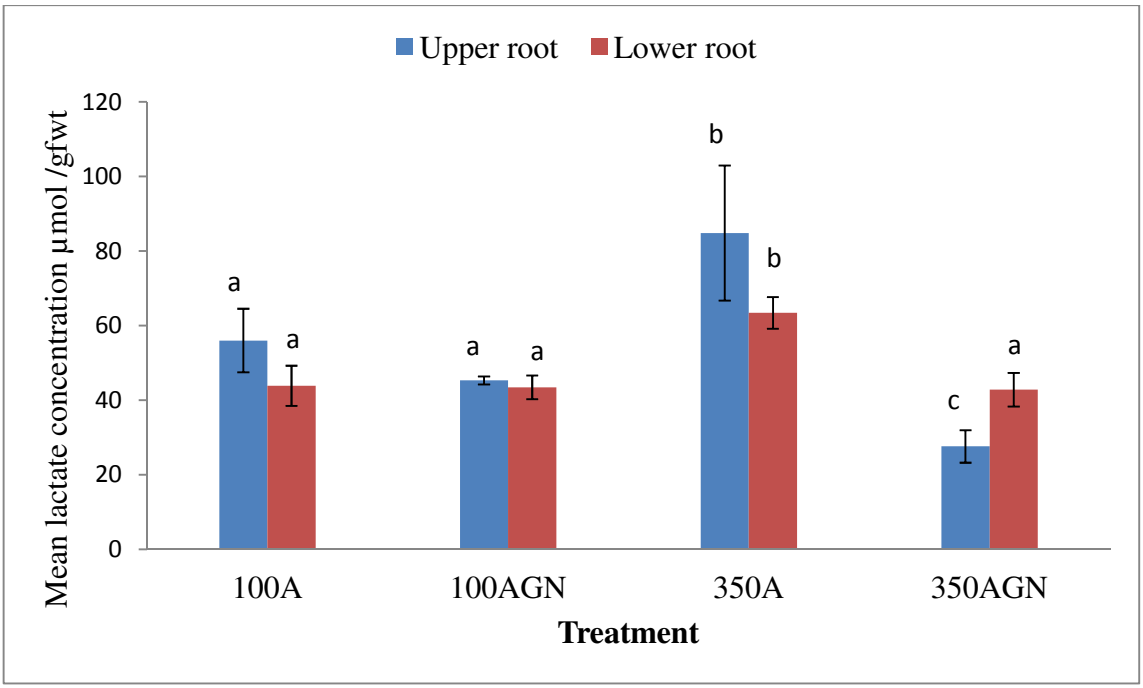


Figure 4.8 Upper and lower root lactate concentration of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n = 4). Data were analysed by 2-way ANOVA. Upper root; salt concentration F (1, 11) = 0.273, P = 0.612, oxygen concentration F (1, 11) = 10.154, P = 0.009, the interaction between salt and oxygen F (1, 11) = 4.769, P = 0.052. Lower root; Salt concentration F (1, 11) = 4.815, P = 0.051, oxygen concentration F (1, 11) = 5.936, $P > 0.05$, the interaction between salt and oxygen F (1, 11) = 5.468, P = 0.039. Letters above bars indicate significant difference in means from post-hoc Tukey tests for initial and incubated root separately.

As shown in Fig 4.8, in upper and lower roots, the lactate concentrations were significantly increased by increasing oxygen level in the root medium, under high salt conditions ($P < 0.05$). In general, upper and lower root lactate concentrations were similar and only differed in hypoxic, high salt conditions.

As shown in Fig 4.9, the whole root lactate concentration was above 50 µmol g^{-1} FW in all treatments, and the highest value was observed in aerated, high salt conditions.

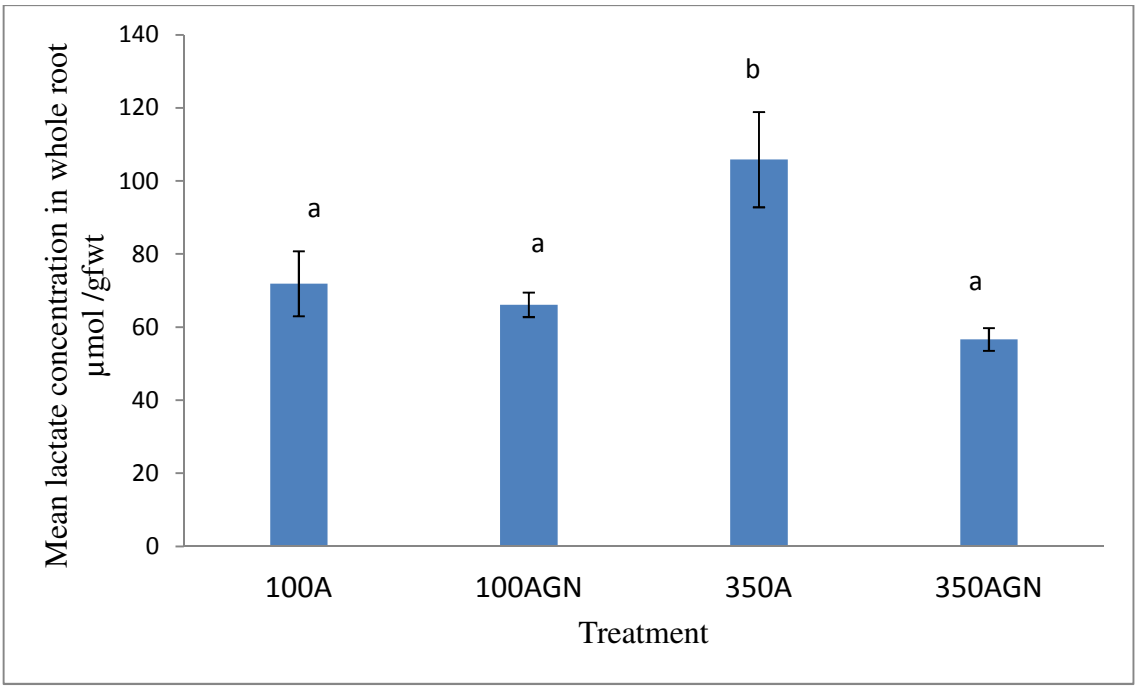


Figure 4.9 The whole root lactate concentration of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n = 4). Data were analysed by 2-way ANOVA. Upper root; salt concentration F (1, 11) = 2.204, P = 0.166, oxygen concentration F (1, 11) = 11.107, P = 0.007, the interaction between salt and oxygen F (1, 11) = 4.769, P = 0.052 Letters above bars indicate significant difference in means from post-hoc Tukey tests.

4.3.7 Root sodium and potassium

Root sodium concentration was not affected by salt concentration and by hypoxia in the growth medium. However, root potassium concentration, on the other hand, was significantly reduced by low oxygen levels in the growth medium ($P < 0.05$).

Table 4.2 Sodium and potassium concentrations, in roots of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks growth in hydroponic solutions, at 100 and 350 mM Na+ (artificial seawater and half strength Arnon & Stout culture solution). Error bars are SE (n = 14). 2-way ANOVA. Letters indicate significant difference of the means from post-hoc Tukey tests.

			100+A	100+AGN	350+A	350+AGN
Root Na^+	µmol	g^{-1}	1.1± 0.3 a	1.3± 0.1 a	1.5± 0.2 a	1.8± 0.2 a
Root K^+	DW		0.87± 0.05 a	0.84±0.03 b	1.4±0.08 c	0.85±0.1ab

4.3.8 Leaf sugar concentration

Low oxygen concentration in the growth medium significantly increased the sucrose, glucose, fructose and total sugar content in the leaves (Table 4.3), under external low and high salt concentrations in the growth media ($P < 0.001$).

Under aerated conditions, the concentrations of sucrose, glucose, fructose and total sugar in the leaves were unaffected by salt concentration (Table 4.3). However, under hypoxic conditions, increasing the salt concentration from 100 to 350 mM NaCl almost doubled the sucrose concentration (1.7 times; $P < 0.001$), while shoot glucose was 1.5 times greater in low than in high salt concentrations. No significant results was observed using salt for fructose and total sugars.

Table 4.3 The sucrose, glucose, fructose , and total sugar concentrations in *S. maritima* leaves after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n = 3). Data were analysed by 2-way ANOVA. Letters indicate significant difference of the means from post-hoc Tukey tests.

Sugar	units	100A	100AGN	350A	350AGN
Sucrose		1.7± 0.003 a	3.0±0.16b	1.5± 0.002 a	5.0±0.24c
Glucose		2.2± 0.1 a	6.2±0.9b	2.2± 0.1 a	4.1±0.2c
Fructose	µmol g	1.8± 0.02 a	5.7±0.4b	1.7± 0.1 a	6.0±0.1b
Total	FW^{-1}	5.7± 0.2 a	14.9±0.4b	5.5± 0.1 a	15.2±0.4b

4.3.9 Root sugar concentrations

In roots grown under low external salt concentrations; the soluble and total sugars were significantly increased under hypoxia ($P < 0.001$), whereas under high external salt conditions, the soluble and total sugars were decreased in hypoxia ($P < 0.05$), except in sucrose concentrations, which was increased significantly ($P < 0.05$).

Under aerated conditions, the soluble and total sugar concentration was increased by increasing external salt concentration ($P < 0.05$), however under hypoxia, it was decreased in increased external salt concentrations.

Table 4.4 The sucrose, glucose, fructose, and total sugar concentrations (µmol g FW $^{-1}$) in *S. maritima* roots after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n = 3). Data were analysed by 2-way ANOVA. Letters indicate significant difference of the means from post-hoc Tukey tests.

Sugar	100A	100AGN	350A	350AGN
Sucrose	1.65±0.04a	3.83±0.13b	2.6±0.08c	3.3±0.04d
Glucose	3.2±0.1a	5.2±0.1b	5.0±0.7b	3.3±0.04a
Fructose	2.2±0.1a	4.6±0.2b	5.4±0.1b	2.9±0.2a
Total	7.1±0.1a	13.6±0.3b	13±0.8b	9.5±0.2c

In general, the soluble of sugar (glucose, fructose and sucrose) and total sugar concentrations, high salt increased sugar concentrations under aerated conditions and decreased sugar concentrations under hypoxic conditions and low salt increased sugar concentration under hypoxic conditions and decreased sugar under aerated conditions, as shown in Table 4.4.

4.5 Discussion

The results clearly indicate that hypoxia increased root biomass at low and high salt concentrations, where the surrounding oxygen concentrations (Table 4.1), in low and high salt concentrations, was 0.4 ±0.01a and 0.5 ± 0.01a mgL^{-1}, respectively.

In hypoxic conditions, adventitious roots were developed after four weeks of treatments, which contributed to the increased in root weight (Fig 4.4). Jackson and Drew (1984) noted that some plants develop adventitious roots in response to flooding, a suggestion supported by these results (see Fig 4.2). *S. maritima* also developed adventitious roots near the stem base after a few weeks of continuous waterlogging treatment in a glasshouse (see Appendix 4.3), in plants grown in both drained and flooded conditions, but more so in drained conditions. Song et al (2011) reported that *Suaeda salsa* developed adventitious roots in inland populations under flooded conditions, although a coastal population did not develop adventitious roots. Similarly, Colmer *et al* (2012) also reported that *S. maritima* plants from the field did not possess adventitious roots.

Data on two species of *Suaeda* show it is not entirely clear what triggers adventitious root formation.

In plants grown under hypoxic conditions, the roots possess a larger intercellular space area than in aerated conditions (approximately 2-fold higher in hypoxia, in both low salt and high salt conditions). The increased spaces should enhance oxygen transport to the root as an adaptive mechanism to the lack of oxygen, although such intercellular spaces are not so wide in comparison with aerenchyma. *S. maritima* does have comparatively low (3.4-5%) root porosity (Justin and Armstrong, 1987; Wetson, 2008), so part of its tolerance to flooding may be due to shallow roots in the upper, more aerobic, soil layers. Interestingly, the ratio of root dry weight/root length was 3 in hypoxic, low and high salt conditions, and 2 in aerated, low and high salt conditions, where the oxygen concentrations were 5.8 ± 0.1 a and 6.4 ± 0.1 c, respectively, suggesting that hypoxia might induced an increase in cell wall material. The area of root hairs in the samples was also higher under hypoxic conditions than aerated conditions, which could also be an adaptation to enable a greater uptake of water and nutrients under a lack of oxygen. My general conclusion is that although hypoxia increases air spaces, the roots have low porosity, thus oxygen transport from shoot to root would be rather small and hence the need for adaptation to anaerobic metabolism.

In a review by Gibbs and Greenway (2003), it was proposed that when anoxia continues beyond 2 h, lactate production is very rare (with the exception of some species of *Limonium* (Rivoal and Hanson, 1993), in comparison to a strong shift towards ethanol production. According to Rivoal and Hanson (1993), the production of ethanol by plants experiencing anoxia around their roots, results in a loss of carbon, as the ethanol readily leaks into the surrounding medium, and this loss can be avoided by favouring lactate production an alternative hypothesis was that the basic pH of seawater causes Zn^{2+} deficiency, inhibiting the function of the Zn-requiring ADH, therefore necessitating a switch to lactate production. Lactate produced in the cytoplasm must, however, be removed in order to prevent a dangerous decrease in pH (Xia and Roberts, 1994; Dolferus et al., 2008).

In the present study, *S. maritima* roots contained levels of lactate approximately 10-fold higher than those observed in other species of halophytes (Rivoal and Hanson, 1993)

and contain little ethanol (Colmer et al 2012). Lactate production was predicted to be more important than that of ethanol because *S. maritima* roots contain 10-fold higher LDH activity than ADH (alcohol dehydrogenase, required for ethanolic fermentation) activity (Wetson. et al., 2012). In addition, Colmer et al. (2012) found that in *S. maritima* grown under half-strengh artificial seawater, under hypoxic and aerated conditions, in the 2 weeks of treatments preceding the development of adventitious root; the root tissues contained only small amounts of ethanol, while lactate concentrations in lateral root tissues were high and surprisingly, did not differ between roots from aerated and hypoxic conditions.

In this study, the production of lactate was not increased by incubation (Appendices 4.1.and 4.2) under anoxic conditions. The results also indicated lactate concentrations were above 50 µmol g^{-1} FW in all treatments, but were higher in aerated, high salt conditions (106 µmol g^{-1} FW) than in all other treatments. This concentration of lactate was higher than in other species such as in *Tabebuia cassinoides*, grown under 60 days of flooding, where the lactate concentration in the root was 7.5 µmol g^{-1} FW (Kolb and Joly, 2009), *Glycine max* under 12 h in anoxia, where the lactate concentration in the root was 0.53 µmol g^{-1} FW (Shen et al., 2006), and other studies, such as those of Arabidopsis (*Arabidopsis* C24 and *Arabidopsis* MS-LDH1), where the lactate concentrations, when treated with 24 h of hypoxia, were 3.7 µmol g^{-1} FW and 17.5 µmol g^{-1} FW, respectively (Dolferus et al., 2008) Colmer *et al* (2012) suggested that such a great quantity of lactate as found in *S. maritima* could only be present in the vacuoles, with a 200 mM sodium concentration to create sodium lactate which would avoid acidification of the cytoplasm. My data showed that in the roots of *S. maritima*, molar sodium to lactate ratios (in µmol g^{-1} FW) was between 0.1 and 0.3. Taken together, the data suggest that glycolysis followed by lactate production was used to produce ATP, with lactate being stored in vacuoles, presumably for recycling when external oxygen concentrations rises. Under aerated conditions, lactate can be recyced back to pyruvate by the enzyme lactate dehydrogenase, and then to glucose by gluconeogenesis or to produce ATP and c- skeletons via the Krebs cycle

How lactate moves in and out of the vacuoles of *S. maritima* remains uncertain. However, in *Arabidopsis* lactate can be transported with a proton by a membrane protein of the nodulin 26-like intrinsic proteins (NIPS)(Choi and Roberts, 2007), where

the expression of *AtNIP2;1* was found to increase after waterlogging. Conde *et al* (2010) found that Membrane Intrinsic Proteins (MIPS; NIPS are a type of MIP) are also involved in the transport of lactate, as well as the MIP aquaporin AQP9 that transports lactic acid. Monocarboxylate transporters (MCTs) also are able to transport lactate in the protonated form (as lactic acid). These transporters function in the kidneyof mouse, transporting lactate in exchange for a proton (Gopal et al., 2004). Interestingly, Na/lactate exchange has been reported in animal cells, for example in mouse kidney (Gopal et al., 2004), and it would be interesting to investigate whether such a transported might function in *S. maritima*.

As shown in Table 4.3, *S. maritima* leaves have shown to accumulate more sucrose, glucose and fructose under hypoxic than under aerated conditions, at both low and high salt concentrations. These results indicate that sugars were affected by oxygen level in the shoot and root medium rather than by salt concentration. The high sugar concentrations in hypoxic conditions can be explained by the dependence of fermentation on sugar, thus roots experiencing a lack of oxygen accumulate high levels of sugar as an adaptive mechanism to waterlogging (Hossain and Uddin, 2011). Also in order to support high rates of glycolysis, roots in hypoxic/anoxic conditions must have access to sufficient amounts of sugars, and the accumulation of sugars in the roots has been observed in waterlogging-tolerant genotypes (Setter et al., 1987; Xia and Saglio, 1992; Sairam et al., 2009). However, this increase in sugars could also be explained by the fact that in hypoxic conditions, root growth was inhibited, while photosynthesis in the leaves was less affected (Mustroph and Albrecht, 2003, 2007). A review by Barrett-Lennard et al (1988) concluded that high concentrations of sugars in the root may be caused by a decline in the use of sugar as a result of the inhibition of the anabolic processes normally needed for growth. In a review by Setter et al (1987), it was reported that when shoots grow slowly, this due to the accumulation of sugar in the shoot, as a result of negative or positive messages sent from the roots to the shoots (Jackson and Drew, 1984). Flowers and Hall (1978) reported that sugars concentrations in *S. maritima* shoots showed little response to different salt concentration in the root medium, and the highest value of sugar was about 8 µmol g FW $^{-1}$In contrast it was found that an increase in the incorporation of ^{14}C into sucrose, when *Suaeda macrocarpa* was grown in the presence of NaCl (Briens, 1972).

In conclusion, *S. maritima* plants are able to accumulate more soluble and total sugar in their shoots in low and high salt concentrations, as well as more sucrose in their roots, with a larger root area when there is a lack of oxygen in the growth medium. It is surprising that the roots of *S. maritima* accumulate high levels of lactate in both aerated and hypoxic conditions; this may be due to that the roots in *S. maritima* regularly undergoes temporary bouts of anoxia or severe hypoxia.

Chapter 5 : The importance of controlled transpiration in regulating sodium accumulation in *Suaeda maritima*

5.1 Introduction

Halophytes use Na^+ for osmotic adjustment in leaves, in order to allow water uptake into the plant against low external water potentials (Glenn et al., 1999; Tester and Davenport, 2003; Flowers and Colmer, 2008). The transport of ions from root epidermal cells, to the xylem, and then to the shoot is strongly affected by transpiration rate (Flowers et al., 1986), so that the salt concentration in the leaves is influenced by stomatal function; reduction in stomatal aperture prevents excessive water loss, thus reducing ion movement into the shoot during salt exposure (Lovelock and Ball, 2002). Many halophytes have morphological adaptations that may serve to limit transpiration, such as reduced leaves (Flowers and Flowers, 2005).

The response of the transpiration of halophytes to increasing salinity is somewhat variable, but in general, they show a decrease in rate at salinities above the optimum level for growth (Winter, 1979). At suboptimal growth salinities, a number of species illustrate a greater transpiration rate than at optimal salinities (e.g. *Salicornia bigelovii;* Webb, 1966, *S. maritima*; Clipson, 1984, *Suaeda aegyptica*; Eshel, 1985), although others have a lower transpiration rate at sub-optimal than at optimal salinities (e.g. *Atriplex halimus*; Gale and Poljakof.A, 1970, *Salicornia fruticosa*; Abdulrahman and Iii, 1981). In *S. maritima*, transpiration rate per unit leaf area decreased above and below the growth optimum (Clipson, 1984). Flowers (1985) concluded that a relationship existed between increased stomatal resistance, reduced stomata frequency per unit area and salinity; these factors could be an essential influence on transpiration rate. Robinson et al (1997) suggested that the closure of stomata by Na^+ provides a mechanism to regulate salt load in the shoot and so is likely to be an important feature of salt tolerance in halophytes (Robinson et al., 1997; Very et al., 1998).

Stomata are sensitive to changes in external water potential, thus stomatal closure often accompanies salinity stress (Karlberg et al., 2006; Maricle et al., 2007; Munns and Tester, 2008). In glycophytes, the function of stomata is damaged by Na^+ moving along with potassium into guard cells and this disruption prevents stomata from closing

properly (referred to as 'locked open'), which can be seen as the reason for their lack of fitness in salt conditions (Robinson et al., 1997). For example, salt-sensitivity was attributed to the inhibition of stomatal closure by Na^+ in the glycophyte *Aster amellus*, while the ability of its salt-tolerant relative *A. tripolium*, to grow in saline conditions was attributed to an capability to close stomata in the presence of high leaf apoplastic Na^+.

Overall, however, there is little information on the role of Na^+ in stomatal movements as most work has focused on K^+. Willmer and Mansfield (1969) working with *Commelina communis* reported that, in the light, stomatal apertures were the same or larger in the presence of Na^+ than those found in the presence of K^+. Under normal conditions, K^+ flows in and out of the guard cells, changing their turgor pressure. The electrical gradient, which is generated by the separation of H^+/OH^- across the plasma membrane, provides the driving force for K^+ accumulation in guard cells that is balanced by Cl^- movement or internal production of malate. Jarvis and Mansfield (1980) and Perera et al. (1997) found that in *A. tripolium*, Na^+ was much lower within the guard cells than that of other leaf cells when the plant grew under high salinity, in contrast to the glycophyte *C. communis*, where the guard cells accumulated large amounts of Na^+. This ability of the halophytic aster to close its stomata was related to an ability to down–regulate inward-rectifying K^+ channel activity in guard cells, in response to an increase in cytosolic Na^+ (Robinson et al., 1997; Vèry et al., 1998; Tester and Davenport, 2003). However in *S. maritima* grown in sodium chloride (200 mM for 30-35 days), there was a lower Na^+ concentration (94 mM Na^+) in the guard cells of closed stomata, than in the guard cells of open stomata (197 mM Na^+), while the Na^+ concentration increased in the epidermal cells surrounding the closed stomata (from 127 to173 mM Na^+ (Flowers et al., 1989). Flowers et al. (1989) proposed that the closing and opening of stomata in *S. maritima* was caused by the flux of Na^+ rather than K^+.

Most authors suggest that the reduction in stomatal conductance and transpiration rate represent adaptive mechanisms to overcome excessive salt accumulation into the leaves (Koyro, 2006) and improve water use efficiency (WUE) under salt stress. The changes in stomatal function in the presence of salinity can be accompanied by morphological and physiological adaptations, for example, Hajibagheri et al. (1983) reported that in *S. maritima*, an increase in cuticular and stomatal resistance occurred when increased salt

levels were present in the growth medium. Salt cress (*Thellungiella*) possesses a higher stomatal density than *Arabidopsis*, when growing under non-saline and saline conditions, although the transpiration rate was lower in salt cress than in *Arabidopsis*. This suggests that more succulent leaves, a double endodermis with decreased transpiration rates, together with the ability to regulate ion loading into the salt cress xylem, could limit the net rate of salt movement to the tissues of the shoot (Inan et al., 2004).

Ion transport to the leaves is a function of the rate of transpiration in the leaves and the salt concentration in the xylem. The latter is a function of both transpiration rate and the rate of ion uptake by the roots, which in turn depends on transport across membranes as well as 'bypass flow'. The latter is the flow of water and solutes to the leaves, without going through the selectively permeable plasma membranes of cells (Perry and Greenway, 1973). At low salinities, bypass-flow, considered to mainly occur through undifferentiated areas of root endodermis, contributes a small percentage of ions reaching the shoot, but at higher salinities, bypass flow can assume a greater importance. Therefore, limiting bypass-flow in halophytes could increase their tolerance of saline conditions (Hajibagheri et al., 1985; Shabala and Mackay, 2011). Shabala and Mackay (2011) suggested that flow of toxic ions through the apoplast can be prevented by the deposition of suberin in the roots, and the Caspian strip in the root endodermis. Such anatomical features can be found in certain halophytic plants, such as *Thellungiella halophila*, which contains an extra epidermal layer (Inan et al., 2004), and *S. maritma*, where the Casparian bands form earlier and closer to the root apex, and are greater in length, under saline growth than in non-salt conditions (Hajibagheri et al., 1985).

The rate of water loss from a plant is not only affected by salinity, but also by waterlogging. It has been demonstrated in many species that stomata close after flooding (Kozlowski, 1997). Stomatal closure under flooding is believed to be the result of a reduction in K^+ uptake and slower water flux from roots to shoots, thereby reducing the K^+ concentration in the guard cells (Pezeshki, 1994). Hypoxic conditions disturb xylem loading (Gibbs et al., 1998) and most likely, the retrieval of unnecessary ions such as Na^+ from the transpiration stream is also affected. However, in some species, such as *Fraxinus pennsylvanica* a fresh-water marsh plant, stomata have been shown to

reopen after approximately 15 days of flooding. This opening progressed until the stomatal apertures in flooded and non-flooded samples were approximately the same, and was shown to coincide with the development of adventitious roots. Adventitious roots appear to be an important physiological adaptive mechanism for flood tolerance: they are highly efficient at absorbing water, and their appearance is strongly correlated with the reopening of the stomata (Gomes and Kozlowski, 1980).

The purpose of the experiments detailed in this chapter was to achieve more extensive data on the importance of transpiration rate in the control of Na^+ uptake, growth and ion accumulation in *S. maritima*, under aerated and hypoxic conditions, at different salt concentrations. These experiments tested the hypothesis that *S. maritima* plants reduce transpiration rates under hypoxic (low and high salt) conditions as compared with aerated (low and high salt) conditions, in order to prevent the accumulation of high levels of Na^+ in their shoots, by lowering bypass flow and diminishing stomatal opening, at the same time increasing water use efficiency as a mechanism of salinity tolerance.

5.2. Materials and Methods

5.2.1 Plant materials and growth conditions

5.2. 1.1 Experiment 1 - The effect of oxygen concentration in the root medium on gas exchange and sodium and potassium uptake in *S. maritima* plants.

Seeds of *S. maritima* were germinated, as previously described (see Chapter 3 Section 3.2.1, Experiment 1) in sand in the growth chamber. Seedlings were then transplanted, at 4 weeks, into nutrient solution (half strength Stout and Arnon, 1939), in artificial seawater containing 100 $^+$ mM Na^+, with and without agar, in black plastic boxes of 2 liter capacity. Each plant was suspended through a hole in the lid with non-absorbent cotton wool and grown, for 5 weeks. Stagnant agar was made as described in Chapter 3 (Section 3.2.2 experiment 1).

The following treatments were carried out:-

a) A - 100 mM Na^+ artificial seawater, without agar, bubbled with compressed air before starting the experiment.

b) AGA - 100 mM Na^+ artificial seawater, plus agar, bubbled with compressed air before starting the experiment.

c) N - 100 mM Na^+ artificial seawater, without agar, bubbled with nitrogen before starting the experiment.

d) AGN - 100 mM Na^+ artificial seawater, plus agar, bubbled with nitrogen gas before starting the experiment.

The solutions were renewed weekly and the oxygen concentrations were measured before and after renewing the solutions. The growth media for all treatments were not bubbled during the experiments because Wetson (2008) reported that the bubbling of gas through the growth medium diminished plant growth as a result of the effects of turbulence on the roots.

5.2.1.2 Experiment 2 - The contribution of bypass flow to sodium uptake in *S. maritima*, under different saline conditions, in aerated and hypoxic conditions

From Experiment 1, it was concluded that there was a clear effect of different oxygen concentrations in the growth medium, on growth. However, other treatments (AGA and N) had no significant effects. From this point, Experiment 2 was carried out with A and AGN treatments for two salt concentrations (100 and 350 mM Na^+), to compare their effects, at two levels of oxygen in the growth medium: a high oxygen concentration (A, aeration) and a low oxygen concentration (AGN, hypoxia). In addition, in the present experiment, samples were harvested after three weeks of adventitious root production, (in Experiment 1, samples were harvested after only one week of adventitious root production).

Plants were grown as described in Chapter 3 (Experiment 1, Section 3.2.2), in different concentrations of artificial seawater (100 and 350 Na+ mM), under aerated and hypoxic conditions. Two weeks after plants produced adventitious root, in week six of treatments, some plants were harvested to determine shoot dry weight, and Na^+ and K^+ in shoot, while others were transferred into individual black painted tubes (50 ml), filled with growth medium (artificial seawater, culture solution and 0.4 mM PTS (200 mg l^{-1}), for one week under aerated salt (100 A and 350 A) and hypoxic (100 AGN and 350 AGN) conditions. Plants were held in the tubes with nonabsorbent cotton wool and grown in a plant growth cabinet (SANYO MLR-350HT, Osaka, Japan), in which the photoperiod was a 16 h photoperiod at 200 µmols m^{-2} s^{-1} supplied by fluorescent lamps (40 Wx15). Conditions during light and dark periods respectively were 22 °C at 60 % relative humidity and 17 °C at 70 % relative humidity. During this week, the solutions

were renewed every day and pre-bubbled with compressed air or nitrogen. The O_2 concentration and pH were measured before and after renewing the solution. Water loss was measured by weighing the plants with tubes every day, before and after changing the growth medium.

5.2.3. Fresh and dry weight determination

Plants were harvested after 7 weeks of treatment, as in previous experiments (Chapter 3 Section 3.2.2.1.1).

5.2.4 Extraction of plants and ion analysis

Dried samples of *S. maritima* were analyzed for Na^+ and K^+ concentration after grinding with a mill (Glen Creston, Model DFH48, Stanmore, London, UK). Each plant sample was ground separately, and the mill was cleaned between samples. Extracts were prepared by weighing each powdered sample (15 mg) into test tubes and extracted in distilled water (10 ml) in a water bath, maintained at a temperature of 90^{o}C. After 2 h, the solution was filtered through Ashless No.1 (90 mm) filter paper and the filtrate analyzed for Na^+ and K^+ by flame emission spectrophotometry (Eppendorf and Pye Unicam SP 90 A) at wavelengths of 589 nm and 766.5 nm, respectively.

5.2.5 Transpiration and Bypass Flow

5.2.5.1 Determination of transpiration

Whole plant transpiration was measured gravimetrically and corrected for water loss (Murray and Ayres, 1986; Yeo et al., 1999; Gong et al., 2006) every day, for one week, for plants treated with PTS (see below) under aerated and hypoxic conditions, in different salt concentrations. The individual tubes containing *S. maritima* and growth medium with PTS, as well as the tubes without plants and filled with growth medium (Control tubes) were weighed on an electronic balance (HF-300G, A&D Instruments, Oxfordshire, UK) with an accuracy of 0.001 g before and after changing the growth medium. Transpiration was then calculated using the difference in weight loss between tubes with and without (Control tubes) plants. WUE was estimated as shoot dry weight gain per unit of water transpired, for the duration of a week (Flowers et al., 1988; Yadav et al., 1996).

5.2.5.2 Determination of bypass flow

To investigate bypass flow in *S. maritima* plants, PTS (trisodium 3-hydroxy-5,8,10-pyrenetrisulphonic acid, trade name 'Pyranin", Bayer) was used (Peterson et al., 1981 and references therein ; Hanson et al., 1985; Moon et al., 1986; Faiyue et al., 2010). PTS was determined according to the method described by Yeo et al (1999). The plants were harvested, the shoots and roots were washed with distilled water, patted dry with paper towels and quickly weighed for determination of fresh weight. Dry mass was determined after drying in an oven (Ohaus) at 80^{o}C for 72 h, then weighing using a sensitive Metler and HR-60 balance, as in previous experiments (Chapter 3, Section 3.2.2.1.1). The extraction process for Na^+ and K^+ was used for PTS analyses, details in Section 5.2.4. PTS fluorescence was analyzed at $\lambda_{excitation}$ (403 nm) and $\lambda_{emission}$ (510 nm) with a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK).

The PTS content in the shoot was divided by the volume of water transpired (J_V) to give the apparent PTS concentration in the transpiration stream ([$PTS_{(xyl)}$]). Dividing this by PTS concentration in the external medium (PTS[ext]) gave the leakage of PTS ([$PTS_{(xyl)}$]/[PTS[ext]]). An empirical correction factor (7.57 Yeo et al., 1987) for the relative mobilities of PTS and water was applied to estimate the water bypass flow (J_{VB}). Bypass flow (JVB) was calculated as a percentage of the total volume flow during the experimental period, following the methods described by Flowers and co-workers (Yeo et al., 1987; Garcia et al., 1997; Gong et al., 2006), as shown in equation 1.

$$JVB\ (\%) = (PTS[cont]\ /Jv)/\ PTS[ext]\ )\ \times 7.57 \times 100 \qquad \text{Equation 1}$$

The water bypass flow (J_{VB}) multiplied by the Na^+ concentration in the external medium [$Na^+_{(ext)}$] and water transpired ($J_{V)}$, gave Na^+ delivered to the shoots via bypass flow. Comparing this with total Na^+ delivered gave the bypass Na^+ in shoots. The PTS concentration in the external medium was 0.4 mM PTS (200 mg l^{-1}), the Na concentration of the external medium was 100 or 350 mM Na^+.

The Na^+ content in the shoot was divided by the amount of water transpired, to give the apparent Na^+ concentration in the xylem (Na[xyl]). This was then divided by the

concentration of Na^+ in the external medium to give the leakage of Na^+ (Na[xyl]/Na[ext]).

5.3.2.2.4 Ion fluxes

Ion fluxes were calculated using the following equations.

Ion fluxes(J) = M2 – M1 / W (T2 – T1) Equation 2

Where

$\overline{W} = [(W2 - W1)/ \log_e (W2/W1)]$ Equation 3

M1 and M2 are the ion contents at times T2 and T1 while W is the average weight of roots or shoots between harvests (Ansari, 1982).

5.2.6 Gas exchange measurements

Transpiration rate, net photosynthesis and stomatal conductance were measured over 2 h, on three fully expanded healthy leaves of three plants of similar size, using a portable infra-red gas analyzer *(CIRAS-2*, PP systems, UK) in the growth chamber. Three plants were analysed per salt treatment for three days; one leaf was placed into the leaf cuvette [(PLC6 (U) Automatic Universal Leaf Cuvette), which had integrated temperature control and sensors for photosynthetically active radiation (PAR)] under the conditions of the photoperiod, which was a 16 h photoperiod at 200 µmols m^{-2} s^{-1}. The measurements were taken during light at 22°C and 60 % relative humidity. Transpiration, photosynthesis and stomatal conductance were calculated per unit leaf area (single surface) as described by Pour (1978) in Section 5.2.7 of this chapter.

5.2.7 Stomatal measurements

The same plants used for measuring gas exchange, were used for determining the number of stomata on adaxial and abaxial leaf surfaces. The leaves were selected at random from the plants and stomatal impressions taken using Elite fast hydrosystem impression material containing vinyl polysiloxane (VPIM obtained from 3M Dental Products, St. Paul, ISO Spec. 4823, type 3 – Category A). After curing (2 minutes), the VPIM was carefully removed from the leaf, coated with clear nail varnish and left for 5 minutes to dry. The film of nail varnish was carefully peeled from the VPIM, mounted on a microscope slide and the number of stomata counted (Reichert Microstar IV light

microscope) in 20 fields per sample at a magnification of 20x. The impressions used for counting stomata were also used to measure length and width of stomata; measurements were made of 90 stomata located at random, and five fields for each leaf at a magnification of 20x, for each treatment. The length and width of stomata were measured (Appendex 5.6) with software available on the microscope (AxioVision Rel. 4.6, Carl Zeiss, Germany).

5.2.8 Leaf area

Leaves were selected randomly from the plants to measure leaf area as a half cylinder. The length and the mean value of the width (the diameter) of the each leaf were measured using a travelling micrometer. The surface area was calculated using Equation 4.

Surface area = $D.\pi. L /2 + D. L.$ Equation 4

Where D is the mean value of the diameter, L is the length of the leaf and π is approximately equal to 3.14. (Pour 1978).

5.2.9 Data analysis

The experiments were repeated at least twice. The results presented in experiments 1 and 2 are averages and were subjected to a statistical analysis using one- and two-way ANOVA.

5.3 Results

5.3.1 Experiment 1

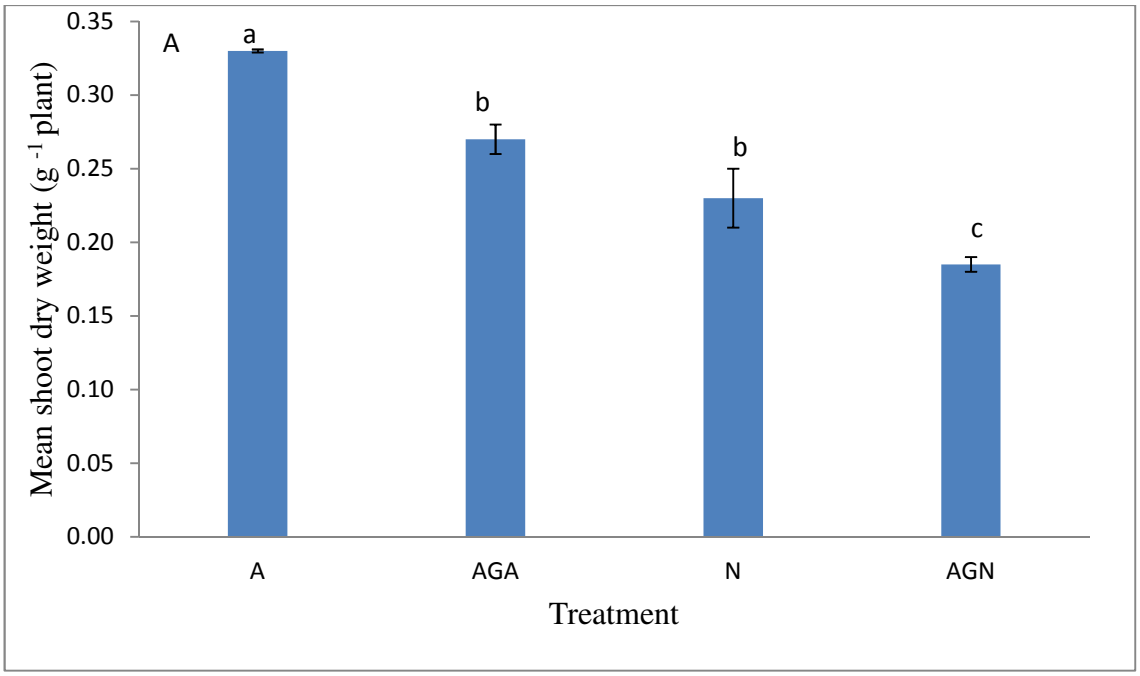
In this experiment, different methods were used to obtain different oxygen concentration in the growth medium in 100 mM Na^+ in artificial seawater (100 A, 100AGA, 100 N and 100 AGN) as described above (Section5.2.1.1); the oxygen concentrations in the growth medium are in (Table 5.1). Before changing the growth medium, the oxygen concentrations in treatments A and N treatments were similar, as were the oxygen concentrations for AGA and AGN (Table5.1). The average oxygen concentration in the growth medium was higher in A and AGA (5.3 and 4.3 mg L^{-1}, respectively) than in N and AGN (1.7 and 0.55 mg L^{-1}, respectively).

Table 5.1 Shows the oxygen concentration before and after renewing the growth medium, for *S. maritima* plants grown under different concentrations of oxygen in 100 mM Na+ artificial seawater, in a growth chamber at 20^0C. Error bars are SE (n=30). Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level.

		Treatment			
		A	AGA	N	AGN
Oxygen concentration mg L^{-1}	After	8.3±0.1a	8.1±0.1a	0.8±0.0.01a	0.7±0.01a
	Before	2.2±0.03b	0.42±0.01b	2.6±0.01b	0.4±0.01b
	Average	5.3±0.03c	4.3±0.05c	1.7±0.03c	0.55±0.01c

5.3.1.1 Shoot and root dry weight

Only the extremes of oxygen concentration of the root medium had a significant effect on shoot and root dry weight ($P < 0.001$); post-hoc tests showed that for both shoot and root dry weights, there was no significant difference between AGA and N. However shoot and root dry weights were greater when plants were grown under the highest oxygen concentrations of the growth media (A), in contrast to plants grown under the lowest oxygen concentrations (AGN), where the shoot and root dry weights were lowest (Fig.5.1).



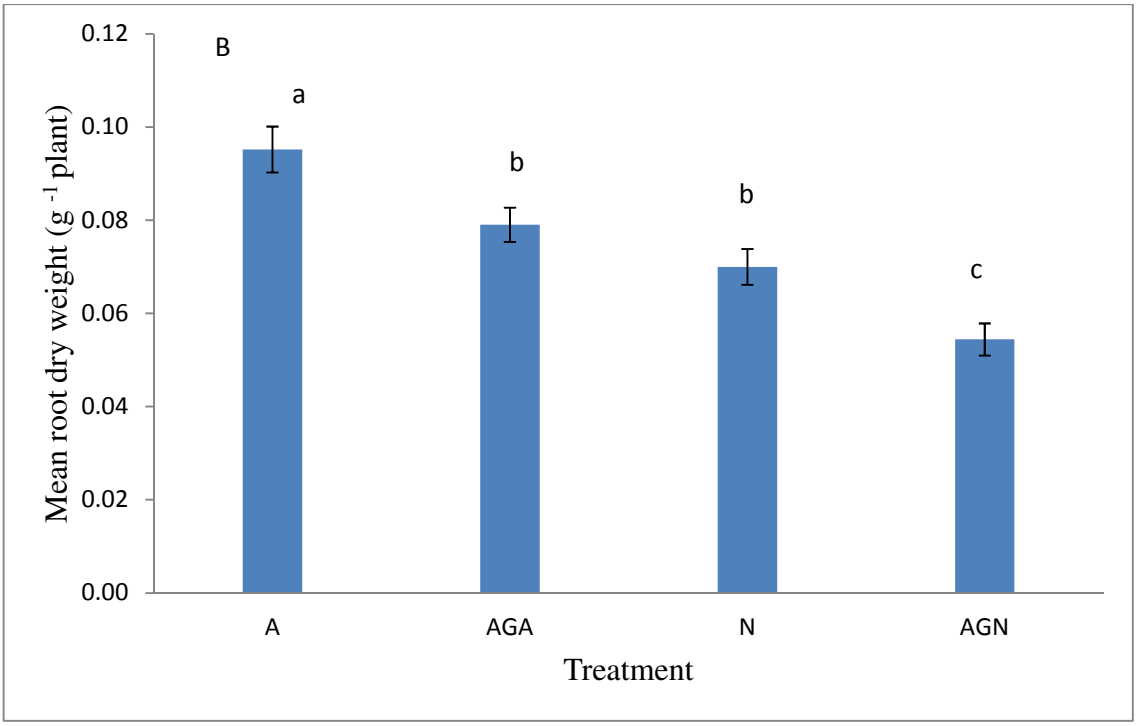


Figure 5.1 Shoot (A) and root (B) dry weight of *Suaeda maritima* plants (9 weeks old at harvest), after 5 weeks treatment in hydroponic solutions under different concentrations of oxygen, in artificial seawater 100 mM Na+ (aerated and hypoxic): Aerated nutrient solution without agar (A), aerated nutrient solution with agar (AGA), nitrogen-flushed nutrient solution without agar (N), nitrogen-flushed nutrient solution with agar (AGN). Error bars are (n = 10). Data were analysed by one-way ANOVA; shoot and root dry weight $P < 0.001$. Letters above bars indicate significant difference in means from post-hoc Tukey tests.

.5.3.1.2 Leaf area

Hypoxia decreased leaf area (Fig 5.2): leaf areas in plants grown with more oxygen (A and AGA) were higher than those grown with less oxygen (N and AGN)

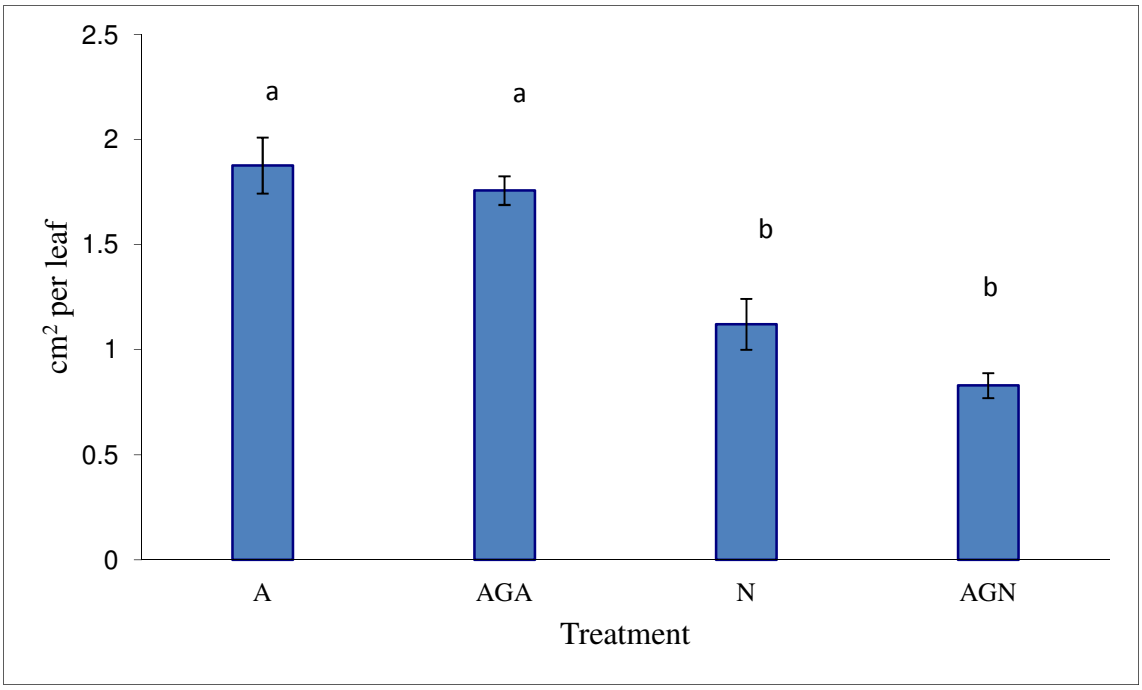


Figure 5.2 Leaf areas of *Suaeda maritima* plants (9 weeks old at harvest) after 5 weeks treatment in hydroponic solutions under different concentrations of oxygen in artificial seawater, 100 mM Na+ (aerated and hypoxic): Aerated nutrient solution without agar (A), aerated nutrient solution with agar (AGA), nitrogen-flushed nutrient solution without agar (N), nitrogen-flushed nutrient solution with agar (AGN). Error bars are (n = 25). Data were analysed by one-way ANOVA; (F (3, 96) = 4.969, $P > 0.01$). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

5.3.1.3 Number of stomata

There were between 7 and 20 % (7, 18, 15 and 20%) more stomata on the abaxial than adaxial surfaces (Appendix, Table 5.2), but post-hoc tests showed that there were no consistent trends related to the effect of oxygen supply on stomatal number.

5.3.1.4 Stomatal measurements

Post-hoc tests (Appendix, Table 5.2) showed that there were no significant differences between stomatal lengths for the A, AGA and N treatments, but length was significantly decreased in the AGN treatment, by 11 and 16% on the abaxial and adaxial leaf surfaces, respectively. Post-hoc tests showed that in the AGN treatments there were

significant decreases in stomatal width, of 33 and 39%, on the abaxial and adaxial leaf surface; they were decreased with reduced oxygen in the root medium. However, calculating the degree of stomatal opening as the ratio of width and length of stomata (Omasa and Onoe, 1984), did not reveal any significant difference between treatments, with the exception of the AGN treatment, where the degree of stomatal opening was decreased significantly on the abaxial and adaxial leaf surfaces, by 29% and 30%, respectively ($P < 0.05$; Appendix Table 5.2).

5.3.1.5 Shoot and root sodium

Reducing the oxygen supply did not have a significant effect on the shoot or root Na^+ concentration in any of the treatments (Appendix, Table 5.3).

5.3.1.6 Shoot and root potassium

Oxygen concentration in the growth medium had a significant effect on shoot and root K^+ ($P < 0.05$; Appendix, Table 5.3).; the reduced oxygen supply to the growth medium significantly reduced both shoot and root K^+, by 13 and 6% respectively, in the AGN treatment.

5.3.1.7 Gas exchange

Transpiration rate, photosynthesis and stomatal conductance were measured over 2 h, on three fully expanded, healthy leaves of three plants of similar size, using a portable infra-red gas analyzer *(CIRAS-2*, PP systems, UK) in the growth chamber. Three plants were analysed per salt treatment for three days. Hypoxia decreased transpiration, by 57% in the AGN treatment, similar to its effect on photosynthesis, which decreased by 67%, and on stomatal conductance, which was decreased by 62% (Appendix, Table 5.4). There was no significant difference between N and AGN treatments.

5.3.1.8 Water use efficiency

Values of the instantaneous WUE (mmol CO_2 m^{-2}/mmol H_2O m^{-2} s^{-1}) were calculated from data accumulated by the CIRAS-2. Water use efficiency (Appendix, Table 5.4) was only decreased significantly ($P < 0.05$) by the lowest oxygen supply, by 23% in the AGN treatment.

5.3.2 Experiment 2

In this experiment, the contribution of bypass flow to sodium uptake by *S. maritima* was determined, under different saline conditions, in aerated and hypoxic conditions. *S. maritima* plants were grown under different salt concentrations in aerated and hypoxic conditions, for 7 weeks. A large amount of data was generated in this experiment, and has been presented in Tables 5.2 to 5.6.

The oxygen concentration after renewing the growth medium (8.7 and 8.9 mg L^{-1}, in aerated low and high salt concentrations respectively) decreased rapidly after one day to about 1.0 mg L^{-1}). However in plants grown under hypoxia, average oxygen concentrations were less than on tenth of those in aerated conditions (Table 5.2).

Table 5.2 Shows the oxygen concentration before and after renewing the growth medium (every day) for *S.maritima* plants grown under different concentrations of oxygen: aerated medium (A), and hypoxic medium (AGN), in different concentrations of artificial seawater (100 & 350 mM Na^+), in a growth cabinet at 20^0C. Error bars are SE (n=70). Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level.

		Treatment			
		A100	AGN100	A350	AGN350
Oxygen concentration mg L^{-1}	After	8.7 ±0.1a	0.5 ± 0.04a	8.9 ± 0.2a	0.4±0.04a
	Before	1.0 ±0.02b	0.3 ±0.02a	0.8 ±0.01b	0.2±0.02a
	Average	4.8 ±0.03c	0.4 ± 0.02a	4.8± 0.1c	0.3 ± 0.01a

5.3.2.1 Water loss (J_V)

As shown in (Table 5.3) water loss (J_V) was significantly reduced by increasing salt concentration ($P = 0.05$) and by hypoxia ($P < 0.05$), although increasing salt concentration had no further effect under hypoxia.

5.3.2.2 PTS content in shoots

PTS content in shoots (expressed here as absorbance units, AU, per shoot) was dramatically reduced by increasing salt concentration in normoxic conditions (but not under hypoxia) and by hypoxia in both low and high salt conditions Table 5.3. PTS_{Xyl} and $PTS_{(Xyl)}/PTS_{(ext)}$, were significantly reduced by increasing external salt concentration ($P < 0.05$), in aerated conditions ($P < 0.05$), but not when the solution was hypoxic. Apoplasic bypass flow (JVB %) was thus significantly decreased by rising external salt concentrations in the growth medium ($P < 0.05$), under aerated but not under hypoxic conditions ($P < 0.05$), hypoxia decreasd J_{vb} in both salt concentrations.

Table 5.3 Na+, PTS uptake and bypass flow (JVB) by *S. maritima* plants in different salt concentrations, under aerated and hypoxic conditions. Error bars are SE (n = 14). 2-way ANOVA. Letters indicate significant difference in means from post-hoc Tukey tests

		100+A	100+AGN	350+A	350+AGN
J_V	(mg/plant)	30.7±1a	15.1±1.3b	24.2±1c	16.4±1b
PTS	(AU/plant)	1574.6±220a	202.2±130b	509.8±98c	209.4±158b
$PTS_{(xyl)}$	(AU mL^{-1})	50.1±4.5a	9.6±3.8b	21.5±4.5c	10.3±7b
$PTS_{(xyl)}/PTS_{(ext)}$	(%)	0.25±0.02a	0.05±0.02b	0.11±0.02c	0.05±0.03b
J_{VB}	(%)	1.8±0.3a	0.2±0.04b	1.1±0.2c	0.11±0.02b
Na	(µmol /plant)	2821.9±498a	535.4±80.9b	4661.1±469c	1153±207b
$Na_{(xyl)}$	(mM)	89.5±10a	42.5±8b	201.5±27c	83.03±20ab
$Na_{(xyl)}/Na_{(ext)}$	(%)	0.9±0.1a	0.4±0.08b	0.6±0.08b	0.2±0.06d

5.3.2.3 Sodium content in shoots

Na^+ content in shoots and $Na_{(xyl)}$ were significantly increased in rising external salt concentrations ($P < 0.05$; Table 5.3), and reduced by hypoxia. $Na_{(xyl)}/Na_{(ext)}$ was significantly reduced by increasing external salt concentrations ($P < 0.05$), in normoxic and hypoxic conditions, and by hypoxia.

5.3.2.4 Shoot sodium and potassium concentrations

Shoot Na^+ concentration was significantly ($P < 0.05$) increased in higher external salt concentrations, under both aerated and hypoxic conditions, and significantly reduced by hypoxia at the low and high external salt concentrations (Table 5.4). Potassium

concentration in the shoot, as shown in Table 5.4, was significantly increased by increased external salt concentration ($P < 0.05$), in aerated, high salt conditions, and reduced by hypoxia ($P < 0.05$).

5.3.2.5 Root sodium and potassium concentrations

As shown in Table 5.4, Na^+ concentration in the root was significantly increased by hypoxia ($P < 0.05$), in low and high salt conditions. However, root Na^+ was not affected by salt concentration in the growth medium. Potassium concentration in the root was not significantly affected by oxygen or salt in the growth medium.

Table 5.4 Sodium and potassium concentrations, and sodium and potassium ion fluxes in shoots and roots of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks growth in hydroponic solutions, at 100 and 350 mM Na+ (artificial seawater and half strength Stout and Arnon culture solution). Error bars are SE (n = 14). 2-way ANOVA.

		100+A	100+AGN	350+A	350+AGN
Shoot Na^+	µmol g^{-1} DW	5.1±0.1a	4.4±0.05b	8±0.2c	6.1±0.4d
Shoot K^+	µmol g^{-1} DW	1.0±0.05a	0.8±0.03b	1.2±0.1c	0.9±0.06ab
Root Na^+	µmol g^{-1} DW	1.1±0.2a	1.7±0.2b	1.3±0.1a	2.2±0.7b
Root K^+	µmol g^{-1} DW	0.8±0.2a	1.0±0.05a	0.9±0.02a	0.9±0.2a
Na Ion flux	Shoot	6.3	2.4	7.3	1.6
µmol g^{-1} DW/week	Root	1.5	2.3	1.4	2.3
K ion flux	Shoot	0.99	0.73	0.82	0.39
µmol g^{-1} DW/week	Root	1.58	1.17	0.84	0.62

5.3.2.6 Ion fluxes

Net ion fluxes were calculated from the difference in shoot or root ion contents between harvests expressed per unit (log mean) dry weight of roots and per unit of time. As shown in Table 5.4, the flux of Na^+ to the shoot was little affected by increasing the external salt concentration in but considerably decreased by hypoxia. By way of

contrast, the Na^+ flux into the roots was increased by hypoxia. The flux of K^+ into shoots and roots was decreased by hypoxia and increasing salinity.

5.3.2.7 Gas exchange and water use efficiency

5.3.2.7.1 Stomatal conductance

As shown in Table 5.5, stomatal conductance was significantly ($P < 0.05$) reduced by increasing external salt concentrations in the growth medium, in both aerated and hypoxic conditions, and also by hypoxia ($P < 0.05$), for both low and high tide.

Table 5.5 Photosynthesis, stomatal conductance, transpiration and water use efficiency per leaf and plant of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks growth in hydroponic solutions at 100 and 350 mM Na+ (artificial seawater and half strength Stout and Arnon culture solution). Error bars are SE (9). 2-way ANOVA gas exchange (n= 9) and waterloss (n= 14).

		100+A	100+AGN	350+A	350+AGN
GS	mol m^{-2} s^{-1}	54.±1a	24 ±1b	20±1.4c	14.6±0.9d
EVAP	mmol H_2O m^{-2} s^{-1}	0.5±0.003a	0.2±0.006b	0.34±0.01c	0.15±0.001d
PN	µmol CO_2 m^{-2} s^{-1}	0.8±0.07a	0.22±0.01b	0.76±0.04a	0.24±0.003b
WUE	mmol CO_2 m^{-2}/mmol H_2O m^{-2} s^{-1}	1.64±0.04a	1.1±0.04b	2.2±0.1c	1.6±0.03a
WUE	mg DW/mg water loss	17.6±0.8a	10.2±1b	25.4±1.6c	15.5±1.8a

5.3.2.7.2 Transpiration and photosynthesis

Transpiration rate (EVAP) was significantly ($P < 0.05$) reduced by rising external salt in the growth medium and by hypoxia (Table 5.5). Photosynthesis was unaffected by external salt concentrations, in either aerated or hypoxic conditions but was significantly decreased ($P < 0.05$; Table 5.5) by hypoxia, at both low and high salt concentrations.

5.3.2.7.3 Water use efficacy (WUE, mmol CO_2 m^{-2}/mmol H_2O m^{-2} s^{-1})

Instantaneous water use efficacy calculated by using CIRAS 2 data (mmol CO_2 m^{-2} / mmol H_2O m^{-2} s^{-1}) was significantly affected by salt concentration ($P < 0.001$; Table 5.5,); it was increased by increasing external salt concentration in the growth medium,

in both aerated and hypoxic conditions, and decreased by hypoxia ($P < 0.05$), in both low and high salt concentrations.

Water use efficiency was also estimated using data for whole plant growth and water loss (mg DW/mg water loss). As shown in Table 5.5, whole plant WUE in aerated and hypoxic, high salt conditions was higher than in aerated and hypoxic, low salt conditions ($P < 0.05$). Plants grown in aerated, low and high salt conditions had greater whole plant WUE than in hypoxic, low and high salt conditions ($P < 0.05$).

5.3.2.8 Leaf area and number of stomata

5.3.2.8.1 Leaf area

Leaf area was significantly increased by salt ($P < 0.05$) and reduced by hypoxia ($P < 0.05$; Table 5.6).

5.3.2.8.2 The number of stomata

The numbers of stomata on the abaxial surfaces was significantly reduced by increasing salt concentrations ($P < 0.05$) in aerated but not under hypoxic conditions (Table 5.6). A similar situation was observed on the adaxial surface under aerated conditions, but the number also decreased in hypoxic conditions. Hypoxia decreased stomatal frequency in 100 mM NaCl, but increased the number when the plants were growing in 350 mM NaCl although only significantly, $P < 0.05$, on the abaxial surface.

5.3.2.9 Stomatal aperture

The degree of stomatal opening, calculated as the ratio of the width to the maximum length of a stomatal pore (Omasa and Onoe, 1984), from data in Appendix 5.5 was reduced (Table 5.6) by hypoxia on both the adaxial and abaxial surfaces, in both salt concentrations, as well as by increasing external salt concentrations, under aerated and hypoxic conditions.

Table 5.6 Number, length, width and Stomatal aperture area on the abaxial and adaxial leaf surfaces of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks growth in hydroponic solutions, at 100 and 350 mM Na^+ (artificial seawater and half strength Arnon & Stout culture solution). Error bars are SE (n = 40). 2-way ANOVA

	position	units	100 + A	100 = AGN	350 + A	350+ AGN
Leaf area		cm	0.9±0.01a	0.7±0.01b	1.2±0.01c	0.8±0.1d
Number	Abaxial	Per unit	89±1a	85±1.7b	45±1c	84±1.5b
	Adaxial	area	92±2a	70±0.7b	52±2c	57±1c
Degree of stomatal opening	Abaxial	Ratio of	0.67±	0.64±	0.61±	0.59±
	Adaxial	width to length	0.67±	0.59±	0.59±	0.57±

5.4 Discussion

Several studies have used flushing the solution with nitrogen to maintain hypoxia (Jackson and Drew, 1984; Wiengweera et al., 1997; Wetson. et al., 2012). However, flushing the root medium with gas disrupts root growth of *S. maritima*, presumably through physical damage to the very fine roots (Wetson, 2008). To avoid disrupted root growth, treatments were imposed where solutions had been pre-bubbled with air and nitrogen in the presence or absence of agar. Agar (‘stagnant solution') was used to mimic waterlogged soils, as convection of gases through the dilute agar solution is dramatically reduced, simulating waterlogged soils. The presence of agar reduces the diffusion of air from the atmosphere to the growth medium, while the oxygen in the growth medium is taken up by the roots (Wiengweera et al., 1997; Watkin et al., 1998). The initial experiment (1) indicated a clear difference between the growth of plants in aerated conditions (A), where the average of oxygen concentration in the root medium was 5.3±0.03 mg L^{-1}, and those grown in hypoxic conditions (AGN), where the average oxygen concentration was 0.55±0.01 mg L^{-1}. This growth reduction was associated with a decrease in leaf area, photosynthesis and WUE.

The similarity in growth of the AGA and N treatments despite a large difference in initial oxygen concentration (8 and 0.8 mg L^{-1}; Table 5.1) is hard to explain, but must be a consequence of the increase in oxygen concentration in the N treatment, from 0.8 to 2.6 mg L^{-1} sufficient oxygen from the air must have diffused into the growth medium to support respiration and growth as there was no agar to prevent this. Nevertheless,

overall, the mean shoot and root dry weights (Fig 5.3) were highly correlated with the average oxygen concentration in the growth medium; the relationships were positive and strong ($Y = 0.0262x + 0.1775$, $R^2 = 0.9384$ and $Y = 0.0083x + 0.0513$, $R^2 = 0.9141$, respectively). These results are consistent with those of Wetson and Flowers (2011), who reported that growth of *S. maritima* was decreased under hypoxic conditions, as compared to aerated conditions, when plants were grown in half-strength artificial seawater for two weeks and also consist with plants grown in glasshouse under drained and flooded conditions at different fresh seawater in glasshouse (see Appendix 6.7, 6.8 and 7.1).

From this initial experiment, the addition of agar was chosen to bring about the largest differential in oxygen concentration between A (the highest) and AGN (the lowest) treatments. Experiment one was carried out on plants grown for five weeks in aerated and hypoxic treatments, at 100 Na^+ mM in artificial seawater, while Experiment 2 was on plants grown for seven weeks in aerated and hypoxic treatments. In both experiments, the dry weight was lower in hypoxic than in aerated conditions. A comparison of shoot dry weight in Experiments 1 and 2 at low salt concentration shows that in Experiment 2, where plants were harvested 3 weeks after adventitious root production, the shoot dry weight was increased in aerated conditions, as opposed to hypoxic conditions, by 27%, whereas in Experiment 1, where plants were harvested after one week from the production of adventitious roots, shoot dry weight was 74% higher in aerated than hypoxic conditions, this suggests that growth of adventitious roots in hypoxia aided growth.

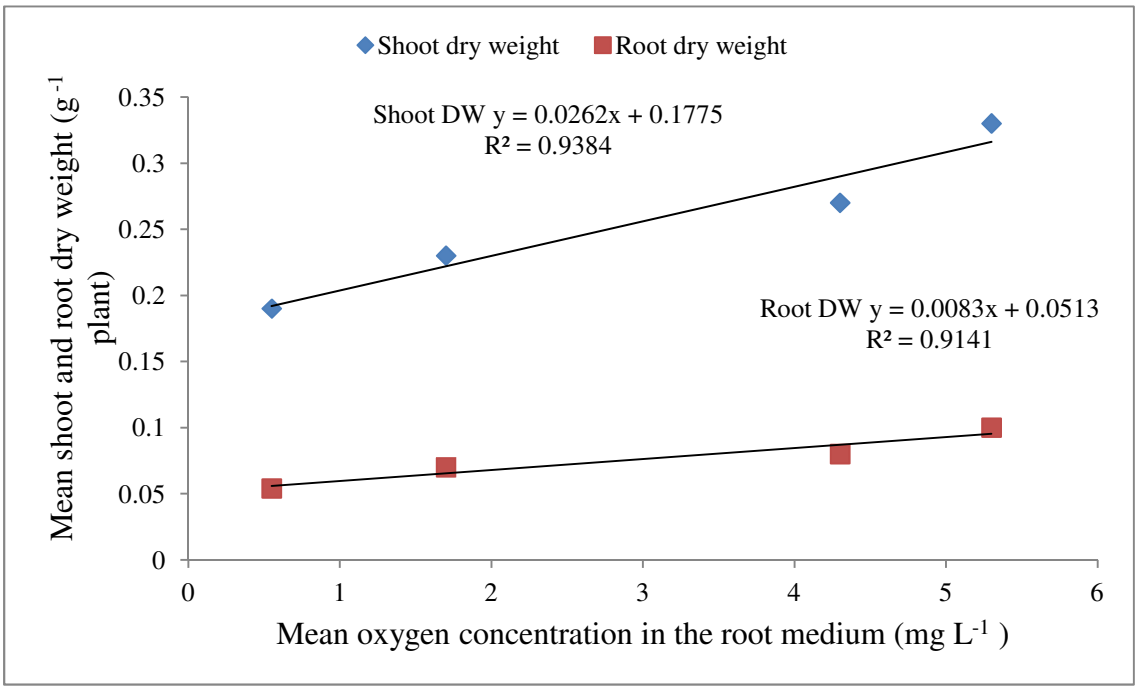
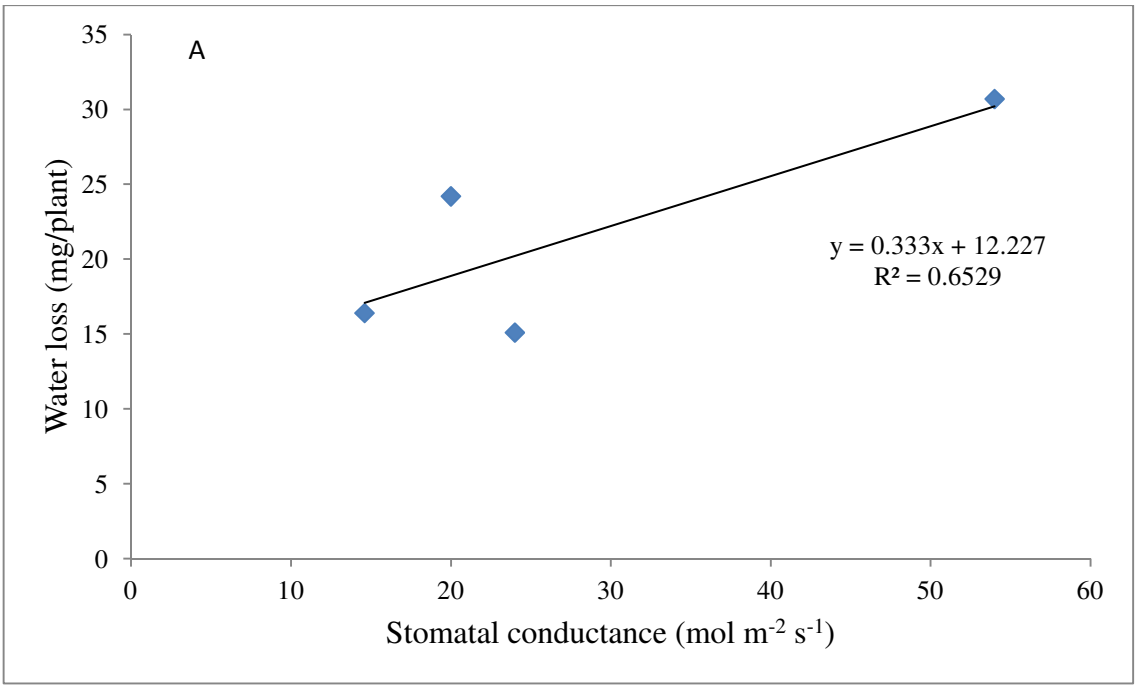


Figure 5.3 Relationship between shoot and root dry weight and mean oxygen concentration in the growth medium for Experiment 1.

The present work showed that stomatal conductance decreased in plants grown in hypoxic, low salt conditions, as compared to aerated, low salt conditions (by 62% in Experiment 1and 56 % in Experiment 2) , while in high salt conditions, the decrease was smaller (27% as compared with aerated, high salt conditions). Stomatal conductance was also decreased by increasing salt concentrations in the growth medium; it was 63% lower in aerated and 39 % lower in hypoxic, high salt concentrations, as compared to aerated and hypoxic, low salt concentrations. Similar effects of increasing salt concentration on stomatal conductance have been reported (e.g. *S. maritima;* Clipson, 1984, *Sarcocornia fruticosa*; Redondo-Gómez et al., 2006, *Atriplex portulacoides*; Redondo-Gómez et al., 2007 and *Chenopodium quinoa*; Eisa et al., 2012) as was obsorved in plants grown in glasshouse under drained and flooded conditions at different salinities (see Appendix 7.5). There is only one study that I am aware of on the effects of waterlogging, and interactions between salinity and waterlogging on gas exchange in halophytes, most studies have been done on salinity alone. Stomatal conductance and transpiration rate have been reported to be decreased in hypoxic treatments by 27% and 30% of the aerated values, in *Atriplex amnicola* (Galloway and Davidson, 1993).

Transpiration rates decreased under hypoxic conditions, in both experiments; by 57% in Experiment 1 and 60% in Experiment 2; under low salt conditions and by 56% under high salt conditions in Experiment 2. Increasing the salt in the growth medium decreased the transpiration rate by 32% and 25% in aerated and hypoxic, high salt conditions, as compared with aerated and hypoxic, low salt conditions and similar results were recorded in glasshouse (see Appendix 7.5). The reductions in transpiration rate under high salt conditions are consistent with the work of Clipson (1984), again on *S. maritima*. Flowers (1985) concluded that salinity, increased stomatal resistance, and a decrease in the numbers of stomata per unit leaf area had potentially important effects on transpiration rate, and were in fact connected. As shown in Fig. 5.4 (b), there is a positive correlation between stomatal conductance and the degree of stomatal opening on the abaxial (R^2 = 0.84) and adaxial leaf surfaces (R^2 = 0.99), as well as between stomatal conductance and gravimetric water loss Fig. 5.4 (a), (R^2 = 0.65).



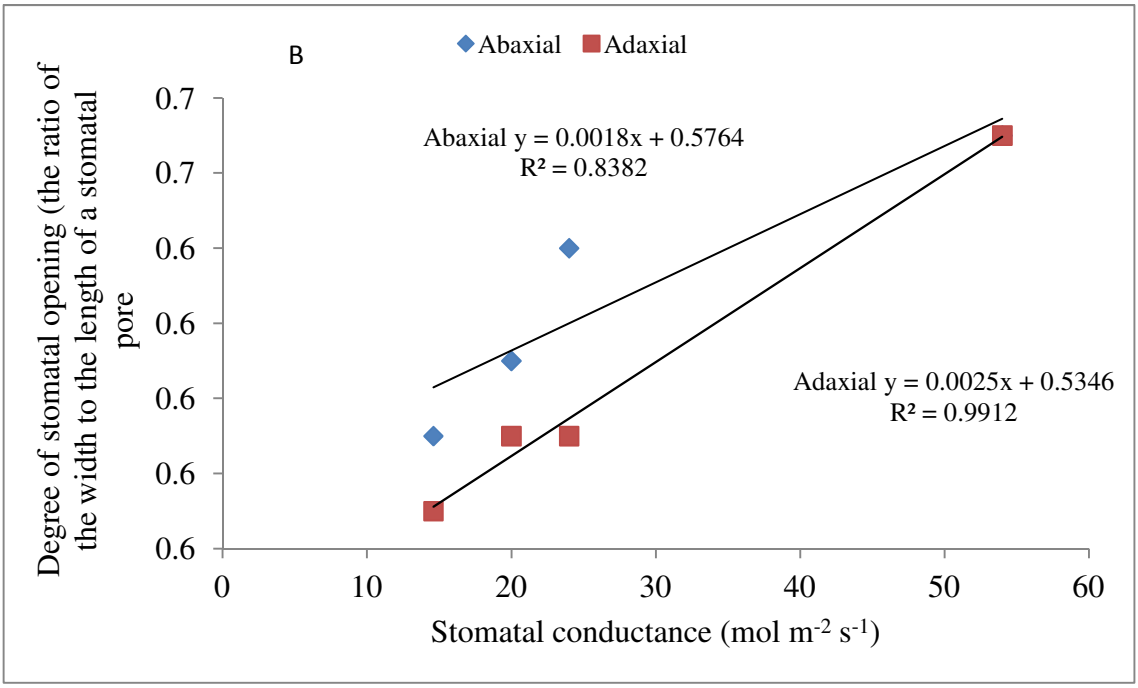


Figure 5.4 The relationship between stomatal conductance and water loss (A), the degree of stomatal opening on the leaf surface and gravimetric water loss (B).

It is widely held that the decreases in transpiration rate and stomatal conductance are both adaptive mechanisms that prevent the accumulation of excess salt in the leaves (Koyro, 2006), as well as improving water use efficiency (WUE) under salt stress. *S. maritima* plants improve their water use efficiency in response to high salt concentrations as well as hypoxia; WUE was increased by 34 and 45% in aerated and

hypoxic, high salt conditions, as compared with aerated and hypoxic low salt conditions, when using the individual leaf data obtained with the *CIRAS-2*,, while it was 44 and 52% when plotting shoot dry weight against water loss per plant. A high positive correlation is seen between instantaneous WUE obtained from measurements with the *CIRAS 2* and by weighing per plant (R^2 = 0.98) (see Fig. 5.5) and also in plants grown in glasshouse (see Appendix 6.12 and 7.7), adding confidence that the short–term measurements predict long-term behaviour. The present results do not agree with those of Pezeshki and Delaune (1993), who reported that the combination of salinity and waterlogging did not significantly affect the WUE of *Spartina patens*, a plant that grows in brackish marshes, and also that salinity, and the combination of hypoxia and salinity, both had negative effects on gas exchange.

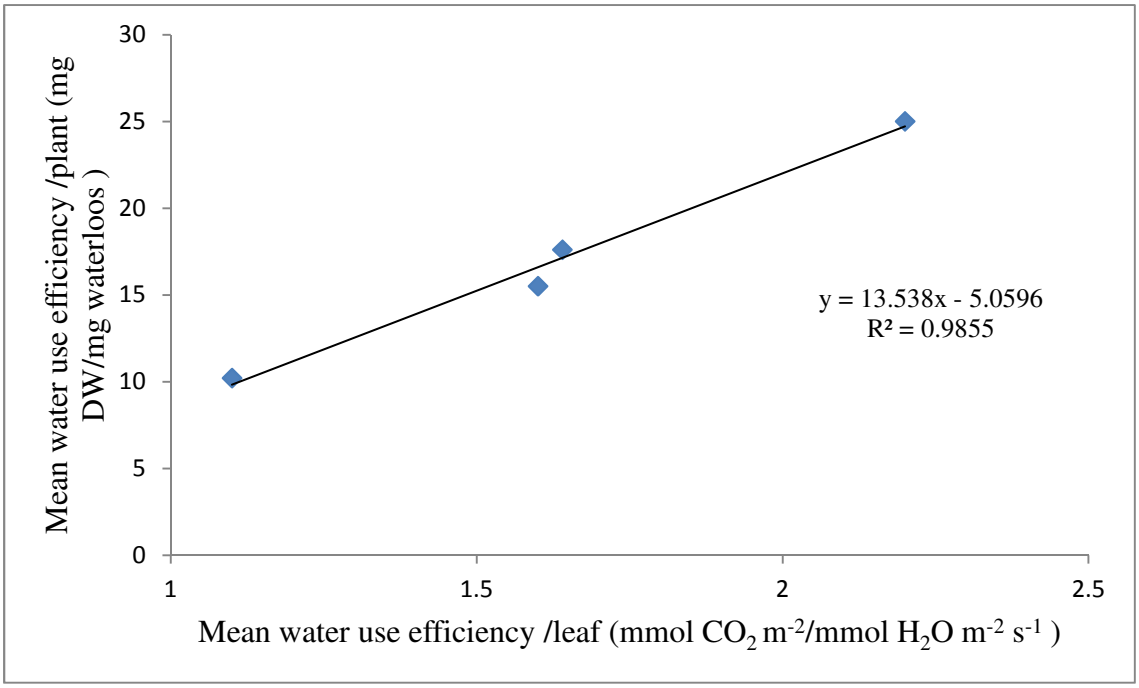


Figure 5.5 The relationship between water use efficiency per leaf and water use efficiency per plant.

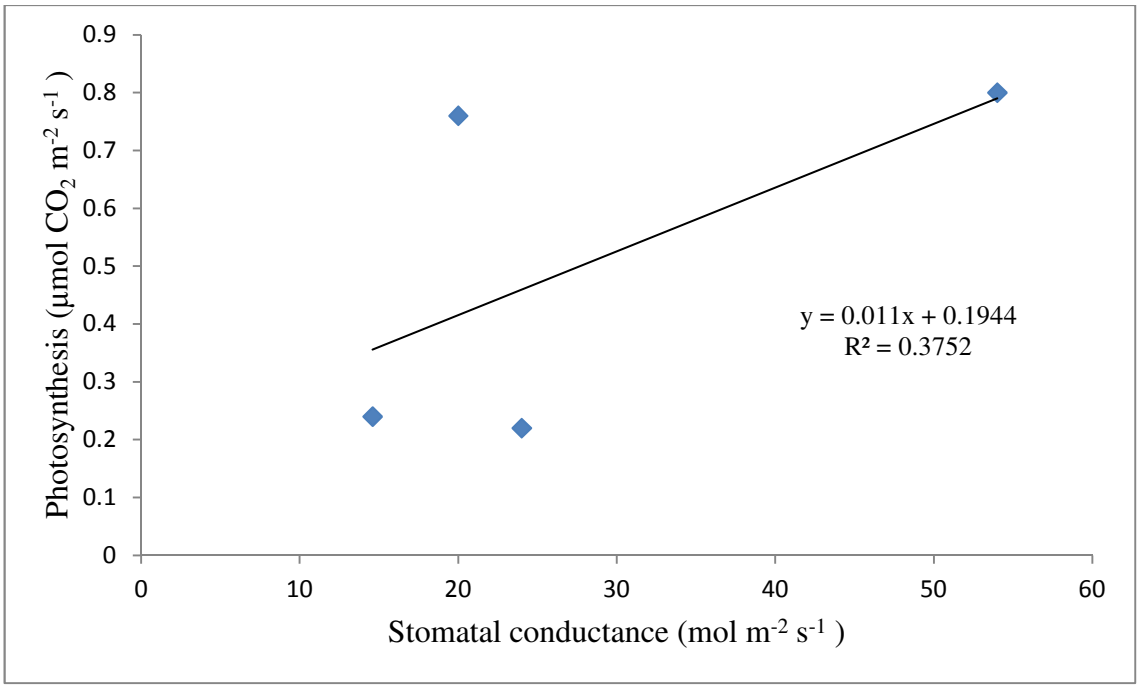


Figure 5.6 Shows a lack relationship between stomatal conductance and photosynthesis in *S. maritima*.

The shoots and roots of plants grown in higher salinities had higher Na^+ concentrations than those grown at lower salinities. This is in keeping with other studies that have been performed on halophytes which suggest that in saline conditions Na^+ is used as osmoticum, sequestered in the vacuole to maintain turgor (Flowers et al., 1977; Greenway and Munns, 1980), and that K^+ uptake is reduced due to competition with Na^+ (Aslam et al., 1988).

In saline conditions, transpiration is affected by the closure of stomata and stomatal conductance more than photosynthesis (see Fig 5.6 and Appendix 7.9) with important implications for regulating Na^+ transport and effecting salt tolerance. Under hypoxia, shoot Na^+ flux was decreased by 62 and 78% in low and high salt conditions as compared to aerated, low and high salt conditions; this is in contrast to the root, where Na^+ flux was increased. Generally, an increase in Na^+ flux is correlated with improved growth in *S. maritima* (Yeo and Flowers, 1986), and so the reduction in Na^+ transport under hypoxia in experiment 2 and also in plants grown in the glasshouse (see Appendix 6.6), at external low and high salt concentrations, correlates with decreased growth. Greenway and Gibbs (2003) suggested that increased rates of ion uptake occur due to the reduction of ATP production by anaerobic respiration of flooded roots, and the resultant disruption of the H^+ gradient, which regulates ion transport across

membranes. According to results from the present work in Experiment 2 and (Appendix 6.6), the Na^+ and K^+ concentrations observed in the shoots of plants grown under hypoxic conditions are not in accord with this. However, Yeo and Flowers (1986) explained that symplasmic and xylem loading are likely to be passive processes mediated by ion channels rather than active. On the other hand, plants grown in Experiments 1 (5 weeks in treatments at low salt concentrations, in aerated and hypoxic conditions) and also plants grown in glasshouse (see Appendix 7.6) showed no significant difference in Na^+ concentrations. These results agree with Wetson (2008), who reported that *S. maritima* plants transferred at 14 days old into half-strength artificial seawater for 2 weeks, under aerated and hypoxic conditions, showed no effect on net rate of Na^+ transport. Na^+ transport of *Atriplex amnicola* was also unaffected by 7 days of hypoxic saline conditions (Galloway and Davidson, 1993). Therefore the difference between the two experiments, in terms of shoot Na^+ concentrations, may be due to the differences in the length of time between treatment and harvest; the longer the time, the more chance to see the effects of the treatment. The shorter the time, the more the concentration is affected by what happened in the pre-treatment.

The transport of ions to the leaves is affected by the concentration of salt in the xylem, and the rate of transpiration. The xylem salt concentration is also affected by the rate of transpiration, as well as the rate and pathway by which the roots take up ions. Ions may enter the xylem by both transports across membranes, and bypass flow (Perry and Greenway, 1973).

The present study has shown that leakage of Na^+ ($Na_{[xyl]}/Na_{[ext]}$) was reduced with increasing external salt concentrations, by 33% and 50%, in shoots of plants grown in aerated and hypoxic, high salt conditions, as compared with plants grown in aerated and hypoxic, low salt conditions. Leakage was also dramatically reduced in the shoots of plants grown in hypoxic, low and high salt conditions, by 56% and 67% respectively, as compared with shoots of plants grown in aerated, low and high salt conditions. These differences correlate with differences in bypass flow. Although in aerated conditions, at both low and high salt concentrations, bypass flow was 1.8 and 1.1%, respectively, which was much higher than in hypoxic conditions (0.2 and 0.11%, respectively). This is very interesting, because the bypass flow is still very much lower (Table 5.3) when compared to that of rice (4.1%). Faiyue et al. (2010) came to the conclusion that PTS is

able to move through the cortical layers of the lateral roots, entering the stele, and from the stele it may enter the transpiration stream and reach the shoot. This agrees with the present study, which showed a positive and strong correlation between transpiration and bypass flow ($R^2 = 0.99$) (see Fig. 5.7).

The present study showed (Table 5.3) that bypass flow was significantly decreased, by salinity – by 39 % and 45%, in plants grown in aerated and hypoxic, high salt concentrations, as compared with plants grown in aerated and hypoxic, low salt concentrations. Additionally, the bypass flow was dramatically reduced by hypoxia by 89% and 90% in plants grown in hypoxic, low and high salt conditions, as compared with aerated, low and high salt conditions. Shabala and Mackay (2011) suggested that the root endodermal Casparian strip, as well as root suberin deposition, both prevent the flow of toxic ions through the apoplast. These results are supported by (Hajibagheri et al., 1985), who reported that in *S. maritima*, saline conditions caused the Casparian bands to increase in length, and to form closer to the root apex, at an earlier time.
Changes in the epidermal layer have also been reported in roots of salt cress *(Thellungiella halophila*) (Inan et al., 2004). The flow of water, solutes and fluorescent dyes has also been found to be prevented by the thick, outer sclerenchymatous cell walls in the roots of rice, as well as by suberin lamellae (Clark and Harris, 1981; Cholewa and Peterson, 2001; Armstrong and Armstrong, 2005; Krishnamurthy et al., 2009).

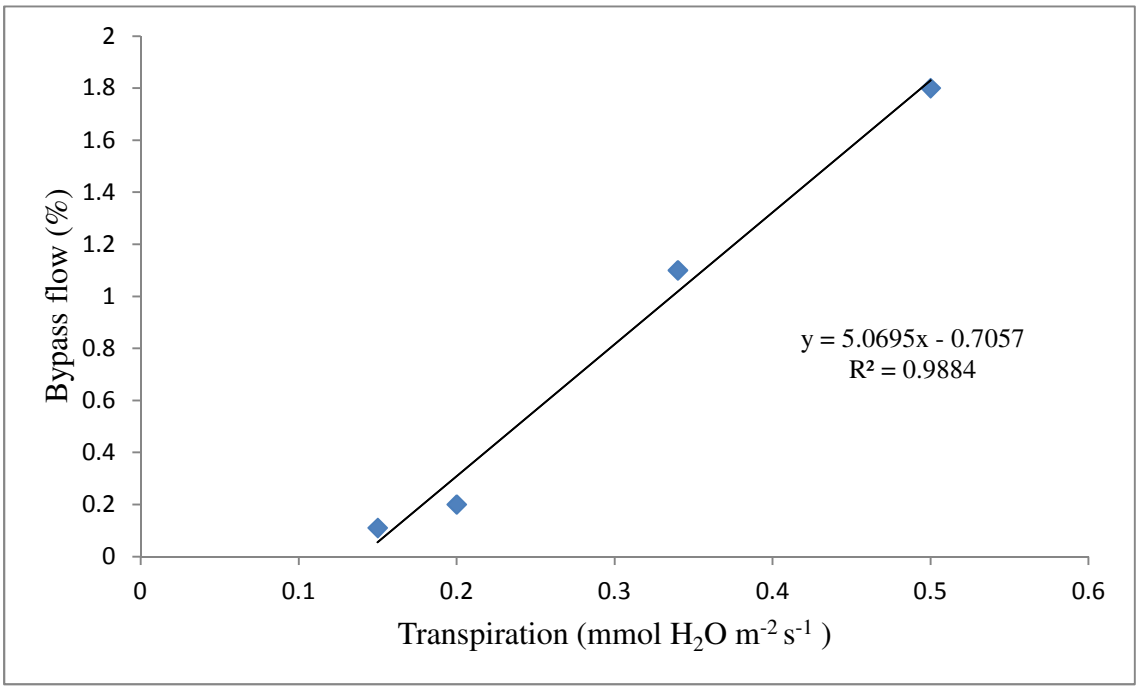


Figure 5.7 The relationship between transpiration and bypass flow in the *S. maritima* plant.

In conclusion, *S. maritima* is able to grow under a combination of salinity and hypoxic conditions by regulating transpiration rates via reducing the degree of stomatal opening and stomatal conductance and also by improving water use efficiency under aerated and hypoxic high salt conditions. It is interesting that in hypoxic conditions, under both external salt concentrations, the bypass flow was significantly reduced in shoots as compared to aerated conditions, and at the same time Na^+ ion flux in shoots and shoot Na^+ concentration were both reduced under hypoxic conditions.

Chapter 6: Summary and conclusions

The purpose of this investigation was to study the effect of the combination of flooding and salinity on the halophytic species, *S. maritima.* The general aim was to add to the current understanding of the physiology and biochemistry of this extensively occurring annual halophyte, and in particular, the adaptations of the family Amaranthaceae, of which *S. maritima* is a member, to salinity and waterlogging.

Overall, *S. maritima* has very good salinity tolerance, as has been indicated by comprehensive studies (see Appendix 6.1); *S. maritima* grows easily under laboratory conditions and is, as a result, an excellent subject for studies on salinity tolerance, flooding tolerance and the interactions between them. This thesis is, to my knowledge, the second study (following that of Wetson, 2008) on the effect of waterlogging with saline water on the growth, biochemistry and physiology of *S. maritima*. It is hoped that a study of how *S. maritima* tolerates saline waterlogging, will enable a better understanding of how other halophytes adapt to waterlogging, and may potentially be applied to the growth and breeding of crop plants.

The present study focused firstly on the effect of waterlogging and salinity on antioxidants of *S. maritima* plants grown in the field, and of *S. maritima* plants grown in a simulated tidal system. This work is reported in Chapter 2. The main objective was to investigate the effect of saline-flooding on antioxidant capacity, including the levels of MDA, glutathione and phenolic compounds (antioxidants), and to compare their concentrations and activity in shoots of *S. maritima* grown in their natural habitats against those grown in a glasshouse. The results of these findings were consistent with the hypothesis that *S. maritima* plants grown in saline, flooded conditions develop higher antioxidant capacity, as a defence against relatively high ROS levels, than plants grown in well-drained conditions, and strongly indicated that waterlogging caused an increase in [GSH+GSSG] and [GSH], and a decrease in [GSSG]. $E_{GSSG/2GSH}$ was more negative in plant shoots grown under waterlogged conditions than those grown under drained conditions. Lower DPPH and superoxide anion activity was associated with higher antioxidant concentrations (glutathione and total polyphenolic compounds). These results indicated that antioxidant molecules are used to minimise ROS.

Unexpectedly, the highest concentrations of MDA were observed under drained conditions in the field and in the glasshouse, and they were higher in the field as compared to glasshouse plants. High levels of lipid peroxidation (indicated by high concentrations of MDA) are, in the literature, generally associated with tissue damage in plants and even decreased viability in seeds (Berger et al., 2001; Sattler et al., 2004). It is not clear why the plants with the higher biomass, from the upper marsh and the drained conditions in the glasshouse, had higher levels of lipid peroxidation as compared to the flooded plants with lower biomass, unless the higher MDA could be linked to the generation of ROS associated with high rates of respiration and photosynthesis occurring under drained conditions. Under flooded conditions high concentrations of glutathione were correlated with low concentrations of MDA. Further investigations are needed in this area, to establish correlations between lipid peroxidation and growth and the role of phenolic compounds in protection against ROS. *S. maritima* shoots have an abundance of phenolics, so this plant could be used as a source of these compounds. The antioxidant capacity in the root may also play an essential role in the plant's adaptive responses to waterlogging and salinity and this also requires investigation in the future. All findings from field and laboratory investigations into antioxidant molecules (glutathione and total phenolic compounds), antioxidant activities (DPPH and superoxide anion scavenging activities) and MDA have been published (EEB-D-12-00397R1, 2012).

S. maritima is a plant that grows in both the upper and lower regions of salt marshes. Recent studies (Wetson and Flowers, 2011) have found that plants grown in drained conditions were larger than those grown in flooded conditions, but the reasons for these differences in growth were unclear. The decreased growth could reflect decreased rates of ATP production in hypoxic conditions as compared to anaerobic ones. A further possibility to explain the reduced growth on the lower marsh is that the bioavailability or uptake of metal ions could be influenced by oxygen levels, possibly causing deficiency and/or toxicity. To develop further our knowledge on the effects of flooding on metal bioavailability, and the resultant effects on, or responses of plants to, I used both hydroponics and soil-based methods of research (Chapter 3) to investigate the effects of salinity and waterlogging, on growth and trace metal uptake (Mn, Fe, Zn and Cu concentrations in the shoots). Plants were grown under drained and flooded conditions in a glasshouse, where the seawater pH was around 8.3, and hydroponically

under different salt concentrations (100 and 350 mM Na^+), at around pH 5.5 and with various concentrations of Fe and Mn. The optimal growth concentration for Mn was 1 mM in both aerated and hypoxic conditions and 13.6 µM for Fe. *S. maritima* showed greater sensitivity to Fe than Mn, since plants were able to grow under much higher concentrations of Mn (10 mM) and for longer periods (3 weeks) than for Fe, where a low concentration (1 mM) killed plants in just 24 h. Analysis of accumulation of metal accumulation showed, as expected, that *S. maritima* accumulated much more trace metals at low pH and low salt concentrations than at high pH and high salt concentrations. More Mn, Zn and Cu was accumulated in the roots under flooded conditions, in both low and high salt concentrations, reducing toxic levels accumulating in the shoots. These studies suggest that the decreased biomass observed in flooded conditions could be due to the high concentration of trace metals in the plant tissues, which could have a negative effect on the growth and function of the roots and shoots. One interesting aspect of future work might be an investigation of the effect of varying sulphate concentration in the root media of the plant, because of its role in oxido/reduction reactions in the salt marsh and also it is abundant in salt marsh soils. More investigation on Na^+, Cl^- and K^+ under different Fe and Mn concentrations and under aerated and flooded conditions is also necessary.

Since *S. maritima* plants grow in both well-aerated soils and in extremely hypoxic, even anoxic soils, various morphological and biochemical adaptations might be expected to occur in the roots of such plants, according to the degree of hypoxia. Tolerance to flooding has been previously linked to the formation of aerenchyma in roots, which increases the ability for air flow to tissues. The main objective of the work reported in Chapter 4 was to carry out more detailed investigations of the morphology, anatomy and biochemistry of aerated and hypoxic roots of *S. maritima*, grown hydroponically in different artificial seawater concentrations. Root growth and morphology as well as root and shoot sugar and lactate concentrations in the roots were examined. Although there was a higher area of intercellular space values are found in the cortex of hypoxic roots as compared with aerated roots, the area of space in both conditions was small when compared with other species, such as those of Spartina, that have comparatively high porosity and large amounts of aerenchyma,. These results supported Weston's (2008) findings, that the anatomical detail in transverse sections of roots grown in hypoxic and aerated conditions showed no evidence of aerenchyma in aerated or hypoxic roots. It is

possible that the diameter of the lateral roots is small enough that the surface area to volume ratio under hypoxic, low and high salt concentrations (0.53 and 0.67, respectively) is sufficient for adequate root aeration.

According to Justin and Armstrong (1987), in wetland species with low root porosity, flooding tolerance requires shallow rooting and there is a preference for sites with a higher level of aeration. *S. maritima* did produce adventitious roots after four weeks of hypoxia, but without aerenchyma and surprisingly, in the glasshouse experiments there were more adventitious roots in drained than flooded conditions. Studies of *Suaeda* species have not clarified the trigger for the formation of adventitious roots. Song et al. (2011), reported that inland populations of *Suaeda salsa* did produce adventitious roots under flooding, although this was not observed in a coastal population, Colmer et al. (2012) found that *S. maritima* plants taken from the field did not develop adventitious roots. The adventitious roots developed in hypoxia would remain in the upper layers of the root medium where oxygen concentrations are likely to be higher than in the deeper soil, due to diffusion of oxygen from the air into the surface. Colmer et al. (2012) reported that in the salt-marshes soil where *S. maritima* grew, three electrodes positioned in the soil all recorded the presence of some O2 before tidal submergence. Intriguingly, though 0.1 cm below the soil surface O2 was very low (~1 kPa), at 1-3 cm below the surface it was near half the partial pressure in atmospheric air. Further work is required to understand the function of adventitious root in this species.

Root cell metabolism is heavily affected by the aeration of the medium. Aerobic metabolism of glucose yields up to 38 molecules of ATP per molecule of glucose. When roots are waterlogged and deprived of oxygen, the incomplete breakdown of glucose under anaerobic conditions yields only 2 molecules of ATP per glucose molecule, a reduction of almost 95% in energy provision for the cell. Anaerobic fermentation of glucose in plant cells normally occurs by the pyruvate-ethanol pathway that leads to release of toxic ethanol. However, *S. maritima* has already been shown to be anomalous in this respect, with lactate concentrations about two orders of magnitude higher than in other species (Colmer et al, 2012). LDH activity is approximately five times higher than ADH activity, when roots are exposed to severe hypoxia, and such high levels of LDH were found in roots regardless of aeration. Since high concentrations of lactate were found in both aerated and hypoxic roots, this indicated a

pre-adaptation to anoxia, an important trait in the tolerance of varying hypoxia due to cyclical waterlogging. Further analysis of the metabolic pathways in such roots and shoots would be another aspect worthy of investigation; at present it is not known if such high lactate concentrations also occur in the shoots or how lactate is recycled. As I expected, higher soluble sugar concentrations were observed under hypoxic conditions in shoots, at all salt concentrations, and sucrose specifically was higher in hypoxic, high salt conditions. High sucrose concentrations would support high rates of glycolysis, as roots in hypoxic/anoxic conditions would require access to sufficient amounts of sugars; the accumulation of sugars in the roots has been observed in waterlogging-tolerant species. Further investigations are needed in this area, to establish whether the activity of sucrose synthase differs under different growth conditions.

The production of lactate in both aerated and hypoxic conditions requires a high level of LDH activity. Since *S. maritima* is regularly exposed to hypoxic or anoxic conditions, constitutive synthesis of high LDH may be an adaptation to cope with these constantly fluctuating conditions. Indeed, LDH appears to be sufficient to produce enough ATP to effectively control ion exchange even in hypoxic conditions, without the excessive loss of carbon associated with ethanol production. The present findings in Chapter 4 demonstrate the importance of tolerance to transient waterlogging and submergence for the halophyte *S. maritima* growing in a tidal salt marsh.

The aim of Chapter 5 was to achieve more extensive data on the importance of transpiration rate in the control of sodium uptake, growth and ion accumulation in *S. maritima*, under aerated and hypoxic conditions, and at different salt concentrations. The results indicated that stomatal conductance and transpiration rates decreased with increased salt concentration in the root media, under both aerated and hypoxic conditions, and that the water use efficiency was higher under high salt concentrations as compared to low salt concentrations. Bypass flow was lower under high salt conditions as compared with low salt conditions and was lower in hypoxic than aerated conditions, in both low and high salt concentrations. These findings support the opinion that the regulation of water flux, the mechanism of ion transport to shoots, is important for salt tolerance, as well as minimising the uncontrolled entry of ions into the transpiration stream.

In summary, *S. maritima* appears to tolerate hypoxic conditions by avoiding anoxia. The synthesis of compounds able to limit the effects of ROS, regulating ion transport to the shoot through control of transpiration and minimising apoplastic transport of ions. Reductions in growth under hypoxia may be a consequence of accumulation of toxic concentrations of Fe. Lactate metabolism appears to play a pivotal role in the adaptation to cyclical flooding and deserves further investigation in other salt marsh species occupying similar habitats. I believe that future studies should focus on identifying the genetic foundations underpinning the reported physiological adaptations of this phenotypically responsive halophyte, that allow it withstand severely hypoxic conditions. A more in-depth knowledge of halophyte physiology would engender a better understanding of plants in general.

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Appendices

Appendix 6.1

Bibliography of work on *S. maritima*.

NO	Title	Treatment	References
1	Salt tolerance in *suaeda-maritima* (l) dum - effect of sodium-chloride on growth, respiration, and soluble enzymes in a comparative study with pisum-sativum l.	salinity	Flowers, 1972
2	Effect of salt on protein-synthesis in halophyte *suaeda-maritima*	Ditto	Hall and Flowers, 1973
3	Salt tolerance in *suaeda-maritima* (l) dum - comparison of mitochondria isolated from green tissues of suaeda and pisum.	Ditto	Flowers, 1974
4	Uptake and localization of rubidium in halophyte *suaeda-maritima.*	Ditto	Hall et al., 1974
5	Localization of chloride in leaf-cells of halophyte *suaeda-maritima* by silver precipitation	Ditto	Harvey et al., 1976
6	. Properties of membranes from halophyte *suaeda-maritima* .1. Cytochemical staining of membranes in relation to validity of membrane markers.	Ditto	Hall and Flowers, 1976
7	Properties of membranes from halophyte *suaeda-maritima* .2. Distribution and properties of enzymes in isolated membrane-fractions.	Ditto	Flowers and Hall, 1976
8	. Salt tolerance in halophyte *suaeda-maritima* - further properties of enzyme malate dehydrogenase.	Ditto	Flowers et al., 1976
9	Salt tolerance in halophyte *suaeda-maritima* - some properties of malate dehydrogenase.	Ditto	Flowers et al., 1976

10	Properties of membrane fractions isolated from the leaves of *suaeda-maritima*.	Ditto	Flowers and Hall, 1977
11	Salt tolerance in halophyte *suaeda-maritima* l dum - interaction between aluminum and salinity	Ditto	Yeo and Flowers, 1977
12	Ion localization in the halophyte *suaeda-maritima.*	Ditto	Harvey et al., 1977
13	Salt tolerance in halophyte, *suaeda-maritima* (l) dum - influence of salinity of culture solution on content of various organic-compounds.	Ditto	Flowers and Hall, 1978
14	Salt tolerance in halophyte, *suaeda-maritima* (l) dum - properties of malic enzyme and pep carboxylase.	Ditto	Flowers et al., 1978
15	Determination of the sodium, potassium and chloride-ion concentrations in the chloroplasts of the halophyte *suaeda-maritima* by non-aqueous cell fractionation	Ditto	Harvey and Flowers, 1978
16	Evidence for cytoplasmic localization of betaine in leaf-cells of *suaeda-maritima*	Ditto	Hall et al., 1978
17	Precipitation procedures for sodium, potassium and chloride localization in leaf-cells of the halophyte *suaeda-maritima*	Ditto	(Harvey et al., 1979)
18	Rubidium transport in membrane-vesicles from the halophyte *suaeda-maritima*.	Ditto	Field et al., 1980
19	Salt tolerance in the halophyte suaeda-maritima l dum - evaluation of the effect of salinity upon growth.	Ditto	Yeo and Flowers, 1980
20	Quantitative ion localization *within suaeda-maritima* leaf mesophyll-cells.	Ditto	Harvey et al., 1981
21	.The structure of the cuticle in relation to	Ditto	Hajibagheri

	cuticular transpiration in leaves of the halophyte *suaeda-maritima* (l) dum.		et al., 1983
22	Stereological analysis of leaf-cells of the halophyte *suaeda-maritima* (l) dum.	Ditto	Hajibagheri et al., 1984
23	Photosynthetic oxygen evolution in relation to ion contents in the chloroplasts of *suaeda-maritima*	Ditto	Hajibagheri et al., 1984
24	Salt tolerance in the halophyte *suaeda-maritima* l dum - the maintenance of turgor pressure and water-potential gradients in plants growing at different salinities	Ditto	Clipson et al., 1985
25	Cytometric aspects of the leaves of the halophyte *suaeda-maritima*	Ditto	Hajibagheri et al., 1985
26	Salt tolerance in *suaeda-maritima* (l) dum fine-structure and ion concentrations in the apical region of roots.	Ditto	Hajibagheri et al., 1985
27	Ion-transport in *suaeda-maritima* - its relation to growth and implications for the pathway of radial transport of ions across the root	Ditto	Yeo and Flowers, 1986
28	Salt tolerance in the halophyte *suaeda-maritima (*l) dum - the effect of salinity on the concentration of sodium in the xylem.	Ditto	Clipson and Flowers, 1987
29	Salt tolerance in the halophyte *suaeda-maritima* l dum - abscisic-acid concentrations in response to constant and altered salinity. 1388.	Ditto	Clipson et al., 1988
30	Quantitative ion localization within *suaeda-maritima* cortical-cells using the combined techniques of freeze-substitution and x-ray-microanalysis.	Ditto	Hajibagheri and Flowers, 1989
31	X-ray-microanalysis of ion distribution within root cortical-cells of the halophyte *suaeda-*	Ditto	Hajibagheri and Flowers,

	maritima (l) dum.		1989
32	Molecular markers for ion compartmentation in cells of higher-plants .2. Lipid-composition of the tonoplast of the halophyte *suaeda-maritima* (l) dum	Ditto	Leach et al., 1990
33	Sodium-chloride compartmentation in leaf vacuoles of the halophyte *suaeda-maritima* (l) dum and its relation to tonoplast permeability.	Ditto	Maathuis et al., 1992
34	Low-affinity Na+ uptake in the halophyte *Suaeda maritima*	Ditto	Wang et al., 2007
35	Do conditions during dormancy influence germination of *Suaeda maritima*?	Salinity and waterlogging	Wetson et al., 2008
36	The effect of saline hypoxia on growth and ion uptake in *Suaeda maritima*	Ditto	Wetson and Flowers, 2011
37	Glutathione half-cell reduction potential and alpha-tocopherol as viability markers during the prolonged storage of *Suaeda maritima* seeds.	Ditto	Seal et al., 2010
38	High phenotypic plasticity of *Suaeda maritima* observed under hypoxic conditions in relation to its physiological basis.	Ditto	Wetson. et al., 2012
39	Oxygen dynamics in a salt-marsh soil and in *Suaeda maritima* during tidal submergence.	Ditto	Colmer et al., 2012

Appendix 2.1

Stout and Arnon Nutrient Solution**.**

Soln	Reagent	Concentration	Use at ml $^{-1}$ L
Major elements			
(1)	KNO_3	1 M	6
(2)	$Ca(NO_3)_2$	1 M	4
(3)	$MgSO_4$	1 M	2
(4)	KH_2PO_4	1 M	1
Minor elements			
(5) A			1ml $^{-1}$L
	H_3BO_3	2.86 g L^{-1}	
	$ZnSO_4.7H_2O$	0.222 g L^{-1}	
	$CuSO_4.5H_2O$	0.079 g L^{-1}	
	$MnSO_4$	1.015 g L^{-1}	
(6) B		Each in 100 ml	1ml $^{-1}$ L
	MoO_3	17.64 mg in .88 NH_4OH	10 ml of each combined in 1 L of 0.1% NaHSO4
	NH_4VO_3	22.96 mg in H_2O	
	$CrK_2(SO_4)_4.24H_20$	99.02 mg in 5% H_2SO_4	
	$NiSO_4.6H_2O$	44.78 mg in 5% H_2SO_4	
	Co(NO3)2.6H2O	49.38 mg in 5% H_2SO_4	
	Na2WO4.2H2O	17.94 mg in H_2O	
(7) Iron		FeNaEDTA 1% aq soln	1

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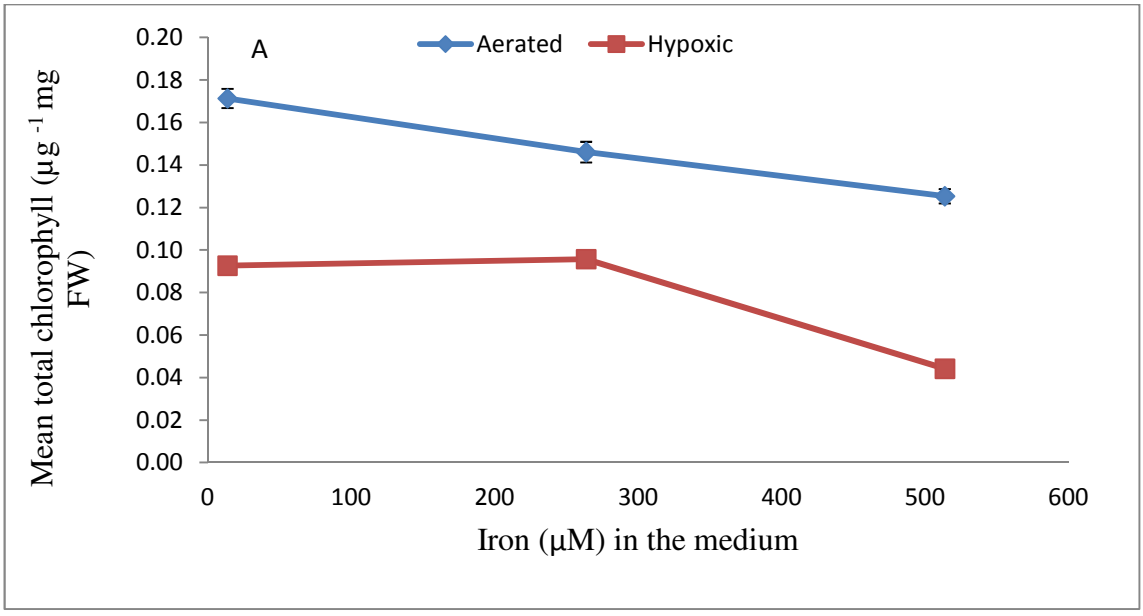
Appendix 3.1

ARTIFICAL SEA WATER.

From Harvey,H.W.: The Chemistry and Fertility of Sea Waters; Cambridge University Press, 1966. These are the major nutrients from the table on p 137. The chlorinity is 19.00% and the salinity 34.33.

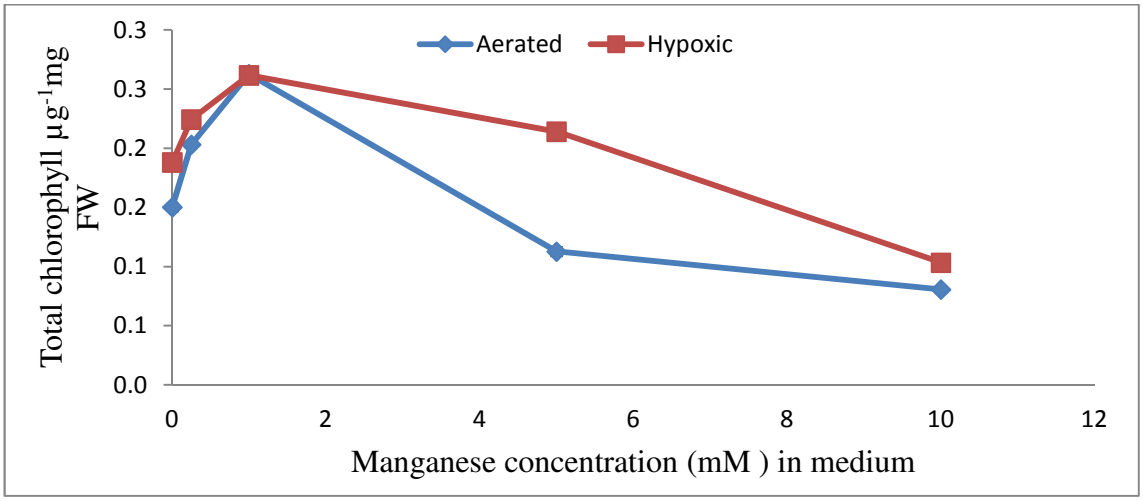
Salt	Weight per L	Molecular weight	Concentration mM
NaCl	24.017	58.44247	410.96
MgCl2,6H2O	10.881	203.30	53.52
Na2SO4	4.0072	142.0431	28.21
CaCl2,2H2O	1.4934	147.01	10.16
KCl	0.6588	74.551	8.84

Appendix 3.2



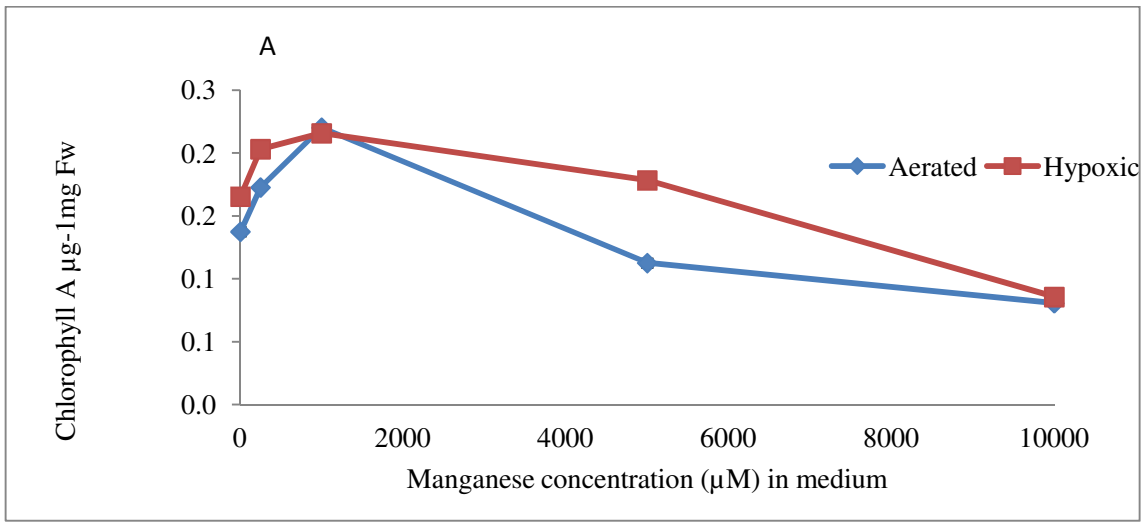
Total chlorophyll concentration of *Suaeda maritima* plants after 10 day growth, under different concentrations of iron, in aerated nutrient solution (Aerated), and stagnant agar solution (Hypoxic) in the growth chamber. Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Oxygen concentration in the medium; F (1, 30) = 624.166, $P < 0.001$, iron concentration in the medium; F (2, 30) = 103.516, $P < 0.001$, the interaction between oxygen concentration and iron concentration in the medium F (2, 30) = 12.421, $P < 0.001$.oxygen concentration and iron concentration in the medium F (2, 30) = 12.421, $P < 0.001$

Appendix 3.3



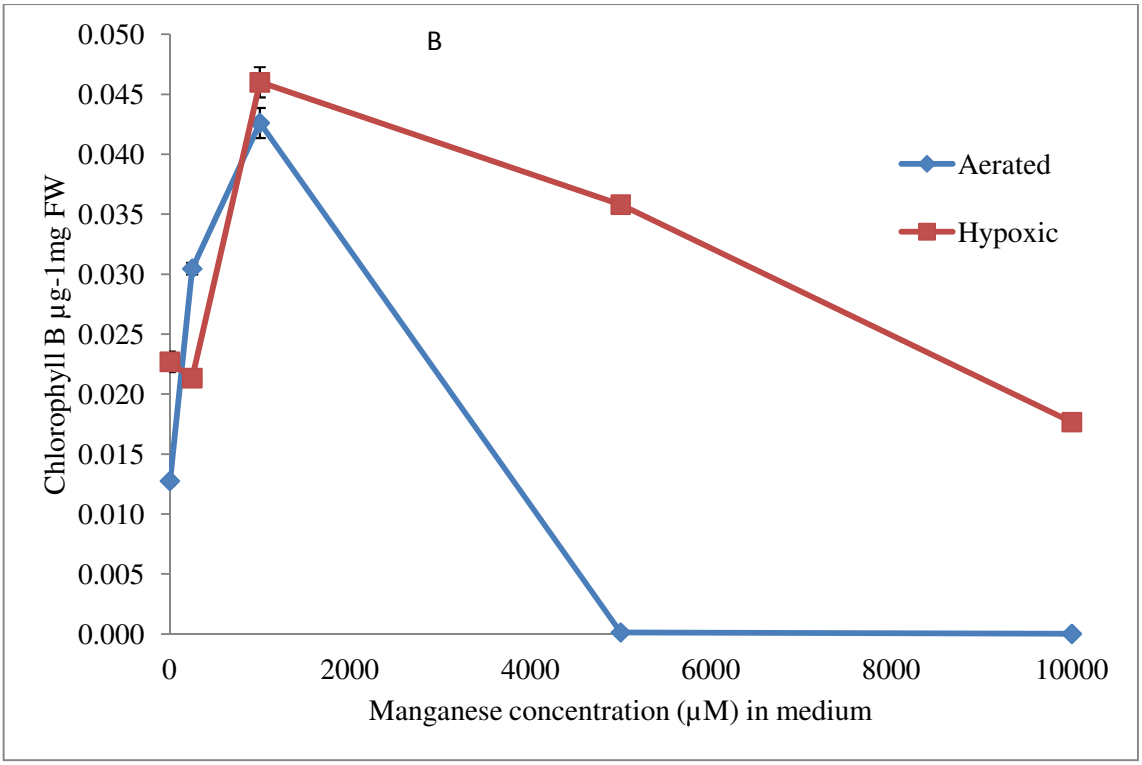
Total chlorophyll of *Suaeda maritima* plants (7 weeks old at harvest), after 3 weeks growth in hydroponic solutions containing 350 mM Na^+ (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solutions (Aerated) and stagnant agar solutions (Hypoxic). Error bars are SE (n = 6). Data were analysed by 2-way ANOVA. Analysis with ANOVA showed a significant effect on the chlorophyll concentration by manganese concentration F (4, 50) = 925.708, $P < 0.001$, oxygen concentration F (1, 50) = 382.161, $P < 0.001$, and the interaction F (4, 50) = 87.086, $P < 0.001$.

Appendix 3.4



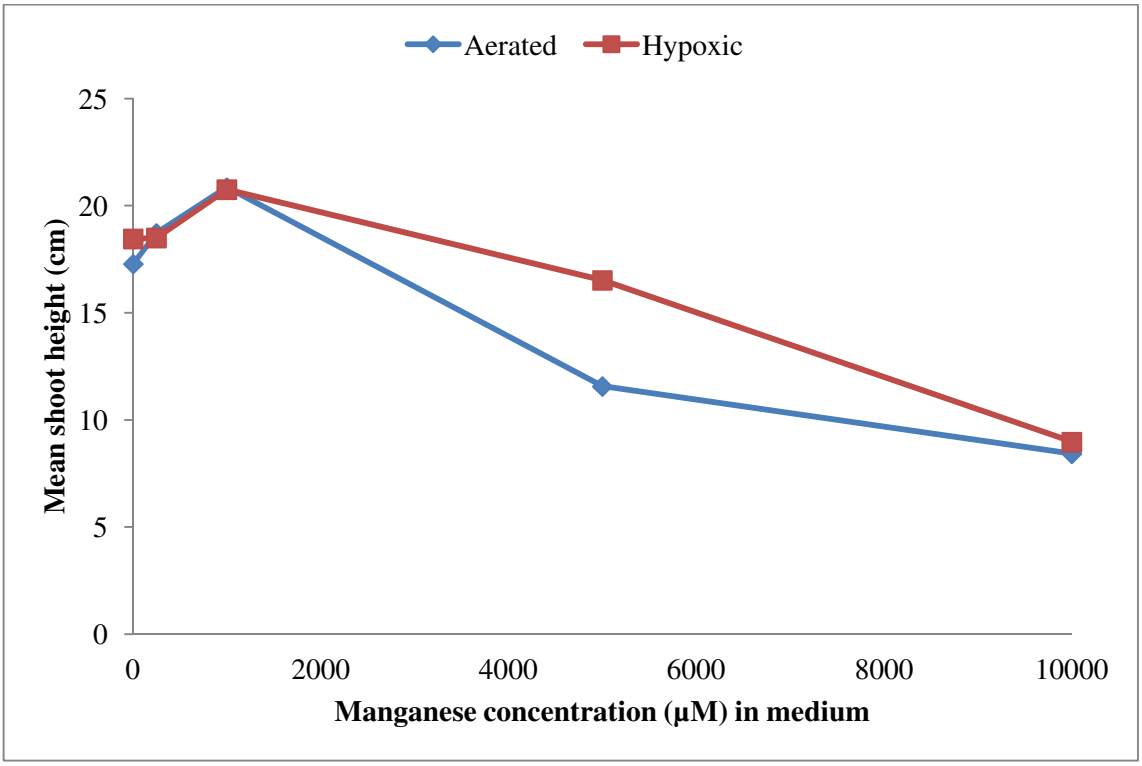
The chlorophyll (a) concentration of *Suaeda maritima* plants (7 weeks old at harvest) after 3 weeks growth in hydroponic solutions containing 350 Na^+ mM (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both oxygenated (aerated nutrient solution (aerated)) and deoxygenated (nitrogen-stagnant agar solution (Hypoxic)) solutions. Error bars are SE (n = 6) 2-way ANOVA. Analysis with ANOVA showed a significant effect on the chlorophyll (a) concentration by manganese concentration F (4, 50) = 642.215, $P < 0.001$, oxygen concentration F (1, 50) = 193.425, $P < 0.001$, and the interaction F (4, 50) = 46.154, $P < 0.001$

Appendix 3.5



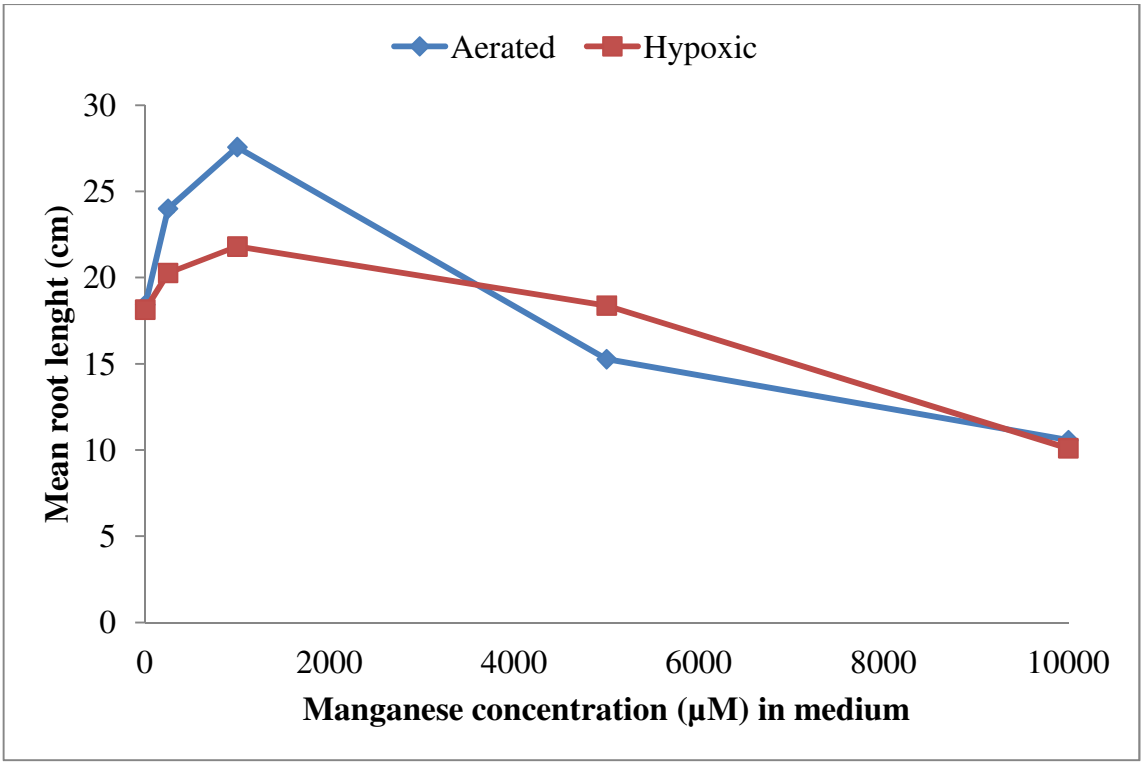
The chlorophyll (b) of *Suaeda maritima* plants (7 weeks old at harvest) after 3 weeks growth in hydroponic solutions containing 350 Na^+ mM (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both oxygenated (aerated nutrient solution (aerated)) and deoxygenated (nitrogen-stagnant agar solution (Hypoxic)) solutions. Error bars are SE (n = 6) 2-way ANOVA. Analysis with ANOVA showed a significant effect on chlorophyll (b) concentration by manganese concentration F (4, 50) = 725.036, $P < 0.001$, oxygen concentration F(1, 50) = 670.258, $P < 0.001$, and the interaction F (4, 50) = 282.474, $P < 0.001$.

Appendix 3.6



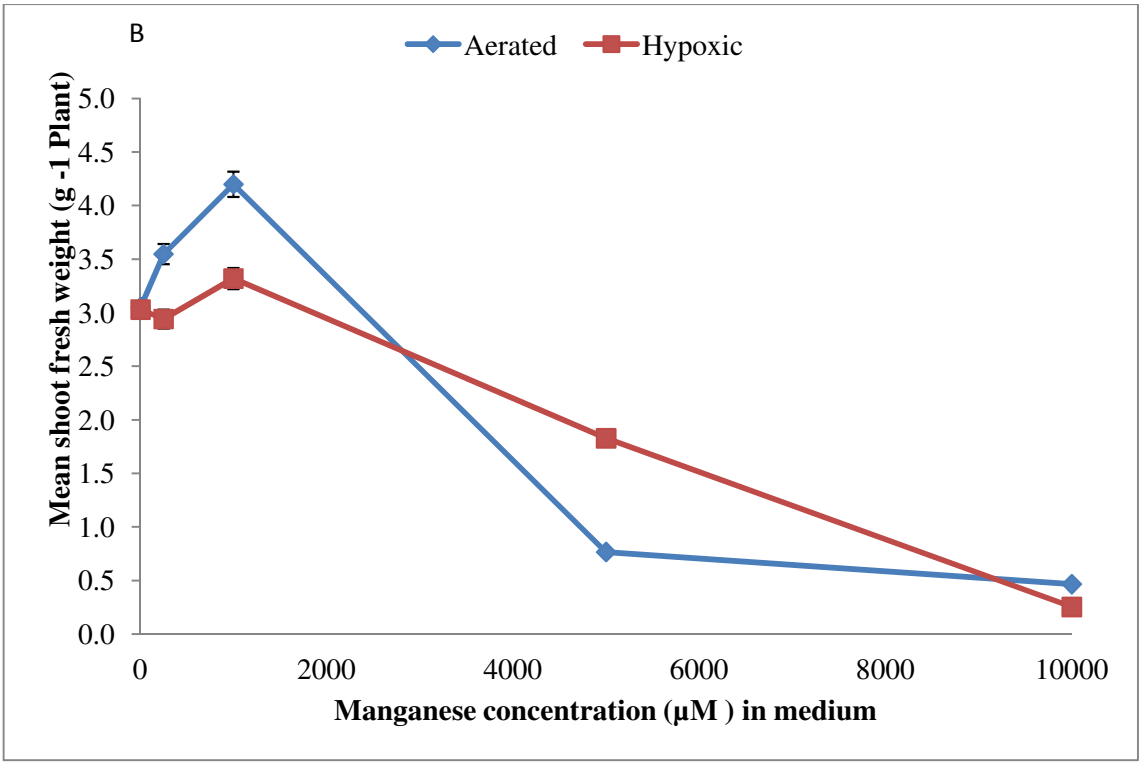
Mean shoot height of *Suaeda maritima* plants (7 weeks old at harvest) after 3 weeks growth in hydroponic solutions containing 350 Na^+ mM (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solution (Aerated) and deoxygenated (nitrogen-stagnant agar solution (Hypoxic)) solutions. Error bars are SE (n = 14) 2-way ANOVA. Analysis with ANOVA showed significant effects on shoot height by manganese concentration F (4, 130) = 1016.407, $P < 0.001$, oxygen concentration F (1, 130) = 90.558, $P < 0.001$, and the interaction F (4, 130) = 50.983, $P < 0.001$.

Appendix 3.7



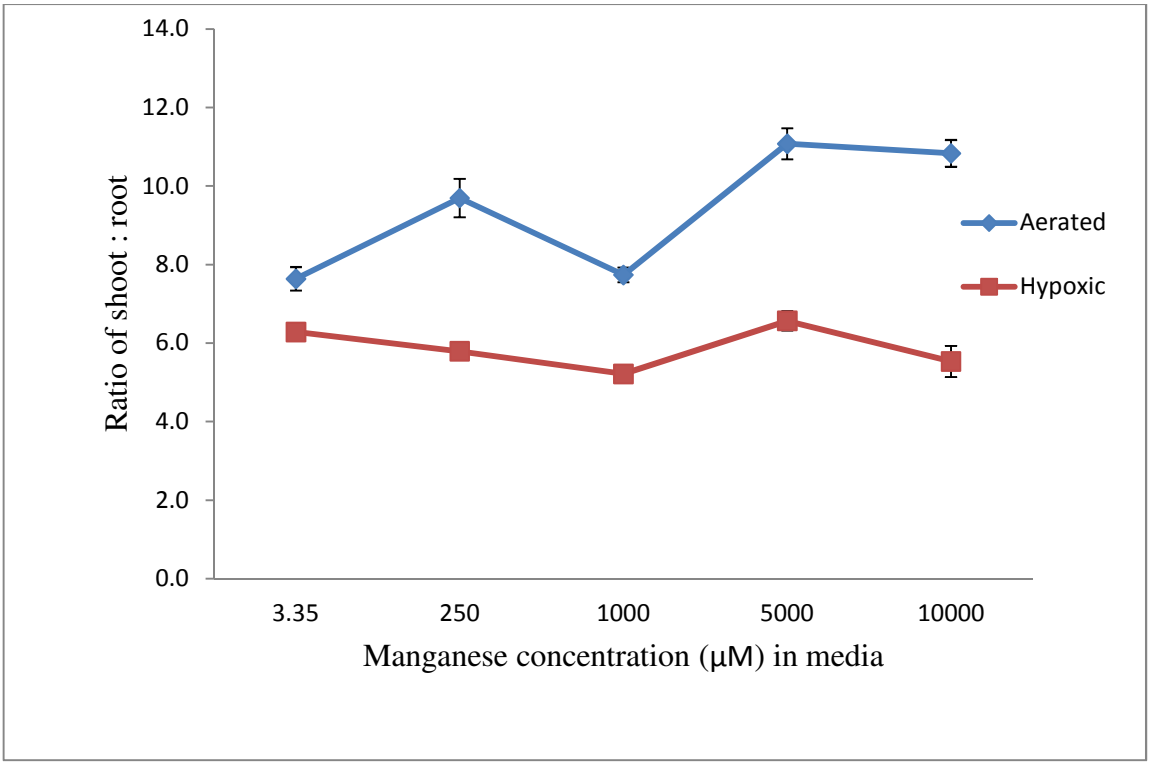
Mean root length of *Suaeda maritima* plants (7 weeks old at harvest) after 3 weeks growth in hydroponic solutions containing 350 Na^+ mM (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solution (Aerated) and deoxygenated (nitrogen-stagnant agar solution (Hypoxic)) solutions. Error bars are SE (n = 14) 2-way ANOVA. Analysis with ANOVA showed significant effects on root length by manganese concentration $F(4, 130) = 1140.453$, $P < 0.001$, oxygen concentration $F(1, 130) = 96.406$, $P < 0.001$, and the interaction $F(4, 130) = 110.620$, $P < 0.001$.

Appendix 3.7



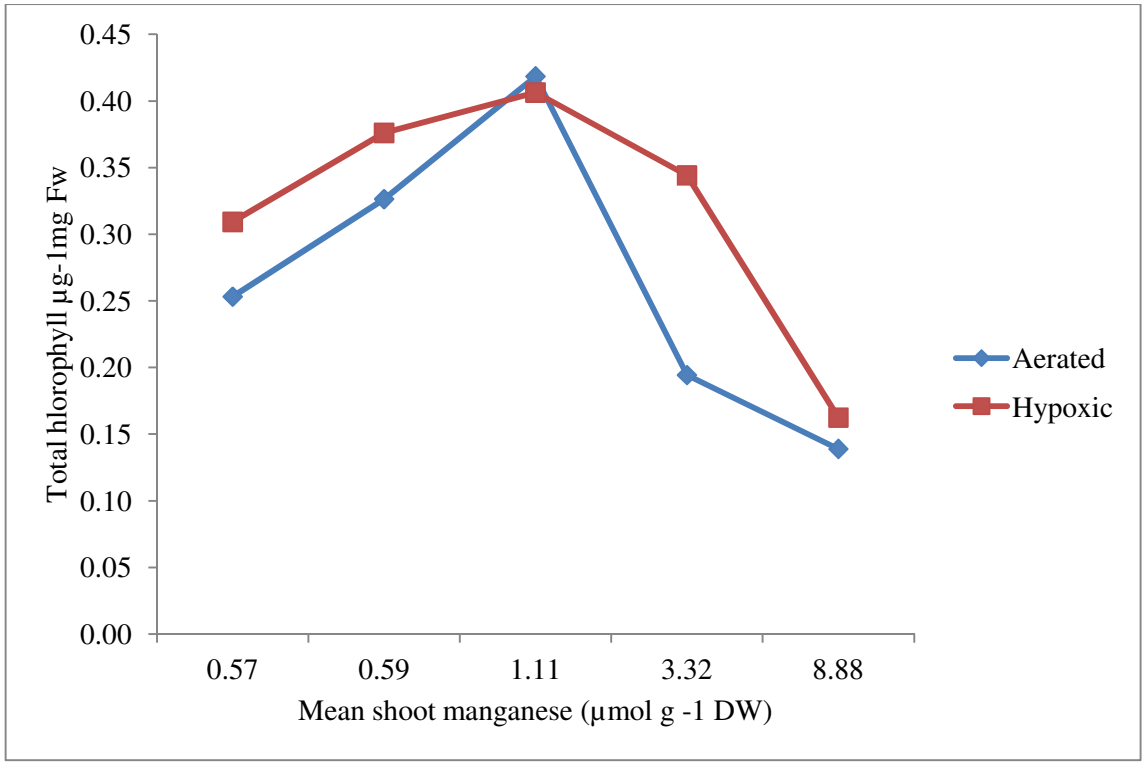
Mean shoot fresh weight of *Suaeda maritima* plants (7 weeks old at harvest) after 3 weeks growth in hydroponic solutions containing 350 Na^+ mM (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both aerated nutrient solution (Aerated) and deoxygenated (nitrogen-stagnant agar solution (Hypoxic)) solutions. Error bars are SE (n = 14) 2-way ANOVA. Analysis with ANOVA showed no significant effect of oxygen concentration on shoot fresh weight F (1, 130) =8.427, $P < 0.05$, but a significant effect by manganese concentration F (4, 130) = 806.655, $P < 0.001$, and the interaction F (4, 130) = 53.967, $P < 0.001$.

Appendix 3.8



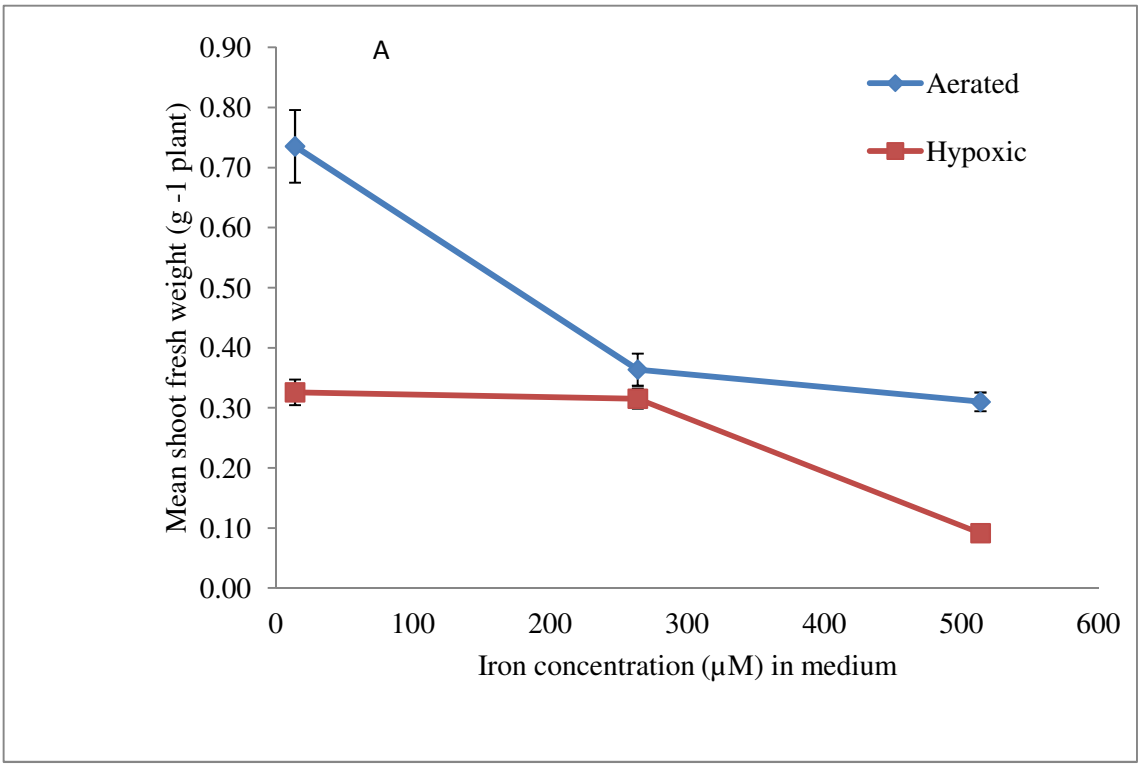
The shoot:root ratio of *Suaeda maritima* plants (7 weeks old at harvest) after 3 weeks growth in hydroponic solutions containing 350 Na^+ mM (artificial seawater and half strength Arnon & Stout culture solution) and varying Mn concentrations, in both oxygenated (aerated nutrient solution (Aerated)) and deoxygenated (nitrogen-stagnant agar solution (N)) solutions. Error bars are SE (n = 14) 2-way ANOVA. Analysis with ANOVA showed a significant effect on the shoot:root ratio by manganese concentration F (4, 130) = 19.160, $P < 0.001$, oxygen concentration F (1, 130) = 336.762, $P < 0.001$, and the interaction F (4, 130) = 13.574, $P < 0.001$

Appendix 3.9



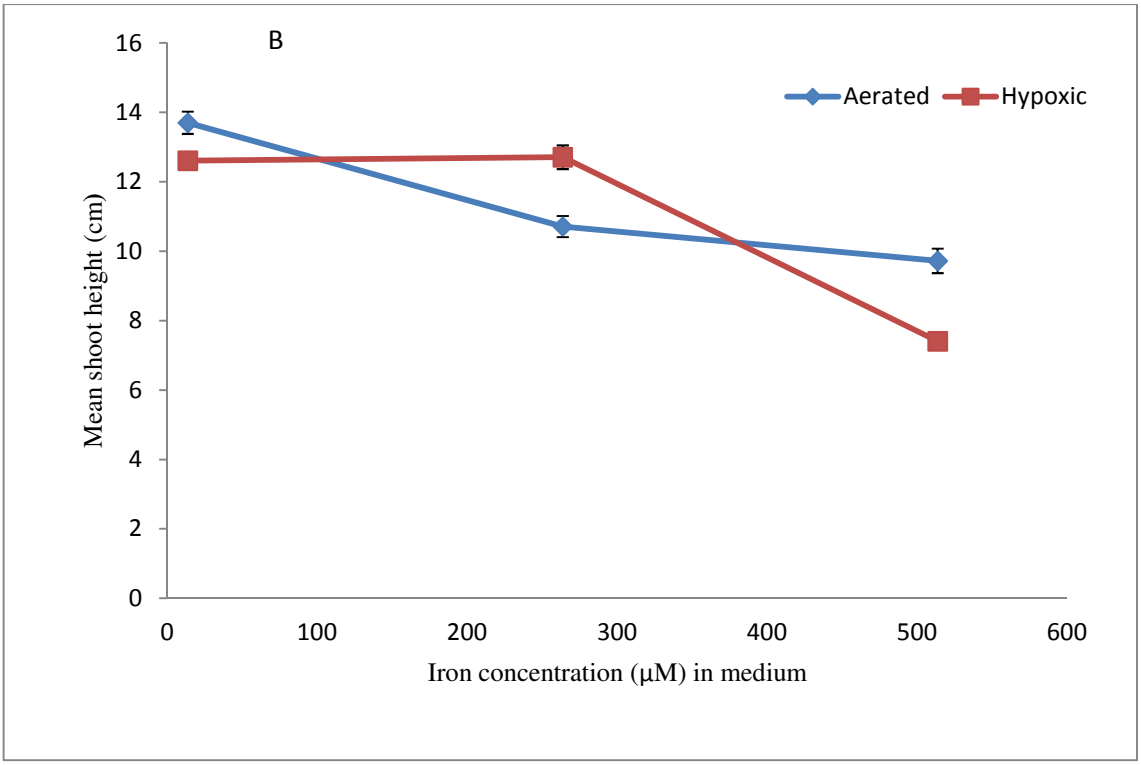
The relationship between shoot manganese concentrations and total chlorophyll in *S. maritima* plants grown for three weeks in 350 mM Na of artificial seawater,in varying concentrations of manganese.

Appendix 3.10



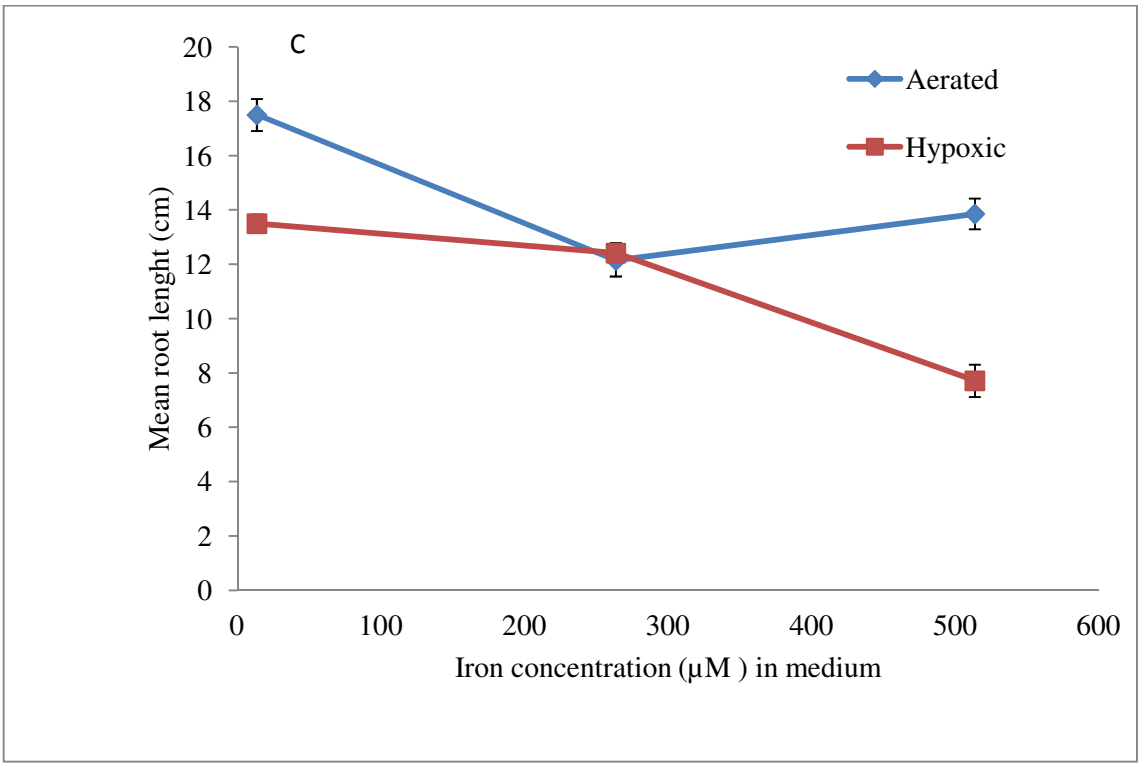
Shoot fresh weight of *Suaeda maritima* plants after 10 days growth under different concentrations of iron µM with Nitrogen- stagnant Agar Solution (hypoxic) and Aerated nutrient solution (Aerated) conditions in the growth chamber. Error bars are SE (n=14). 2-ways ANOVA; Analysing by ANOVA showed that significant effect of oxygen concentration in the root medium on the shoot fresh weight $F(1, 78) = 85.020$, $P < 0.001$, iron concentration in the root medium $F(2, 78) = 61.169$, $P < 0.001$ and the Interaction $F(2, 78) = 18.189$, $P < 0.001$.

Appendix 3.11



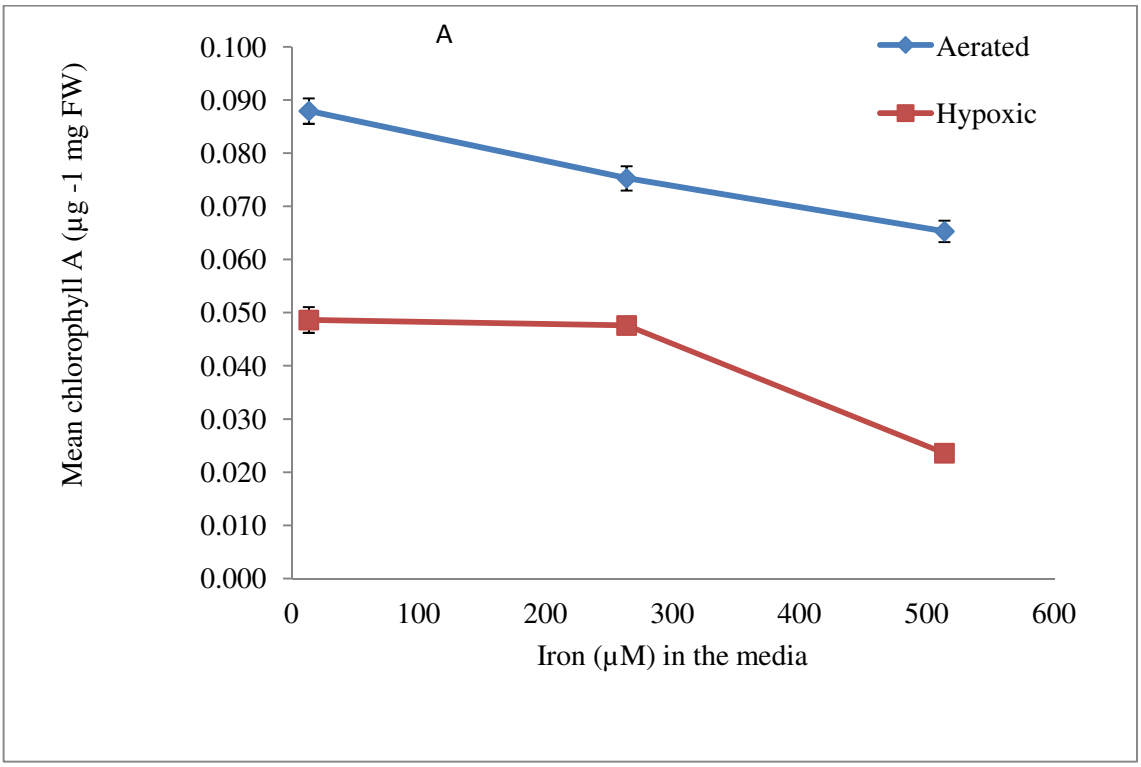
Shoot height of *Suaeda maritima* plants after 10 days growth under different concentrations of iron µM with Nitrogen- stagnant Agar Solution (hypoxic) and Aerated nutrient solution (Aerated) conditions in the growth chamber. Error bars are SE (n=14). 2-ways ANOVA; oxygen concentration in the medium $F(1, 78) = 85.020$, $P < 0.001$, iron concentration in the medium $F(2, 78) = 61.169$, $P < 0.001$, the interaction between oxygen concentration and Iron concentration in the medium $F(2, 78) = 18.189$, $P < 0.001$.

Appendix 3.12

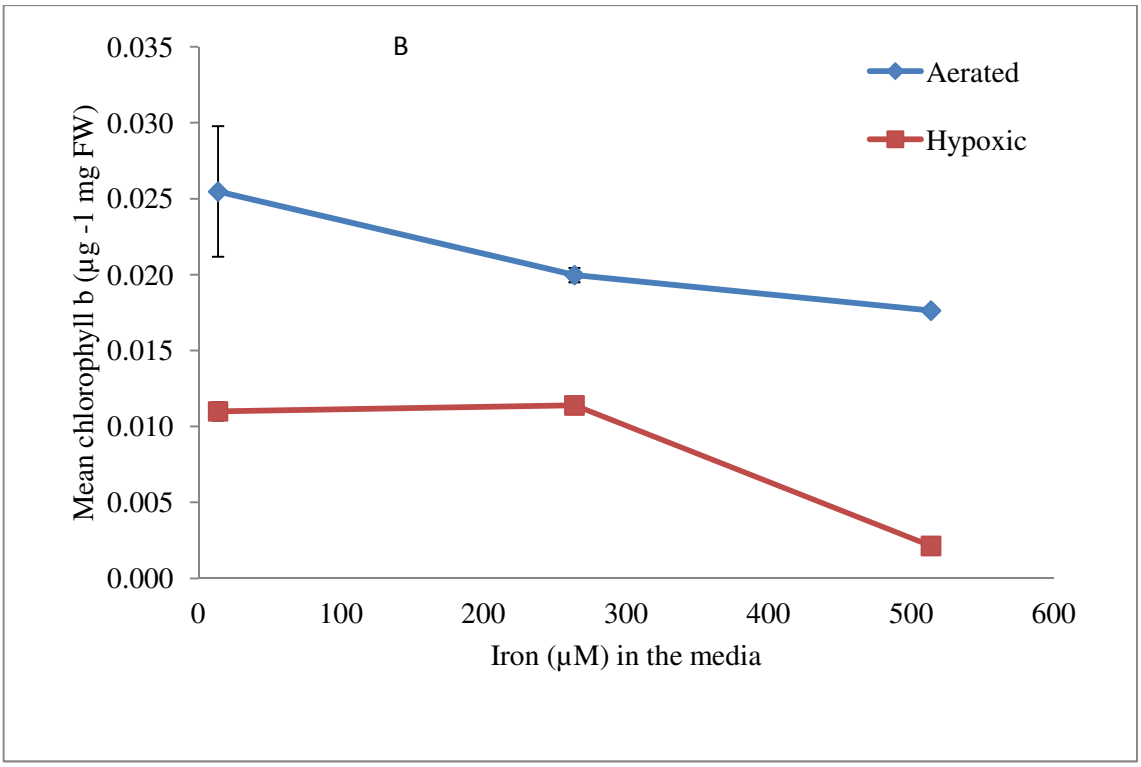


Root length (C) of *Suaeda maritima* plants after 10 days growth under different concentrations of iron µM with Nitrogen- stagnant Agar Solution (hypoxic) and Aerated nutrient solution (Aerated) conditions in the growth chamber. Error bars are SE (n=14). 2-ways ANOVA;. Analysing by ANOVA showed that significant effect of oxygen concentration in the root medium on the root length $F(1, 78) = 60.320$, $P < 0.001$, iron concentration in the root medium $F(2, 78) = 43.142$, $P < 0.001$, the Interaction medium $F(2, 78) = 19.832$, $P < 0.001$.

Appendix 3.13

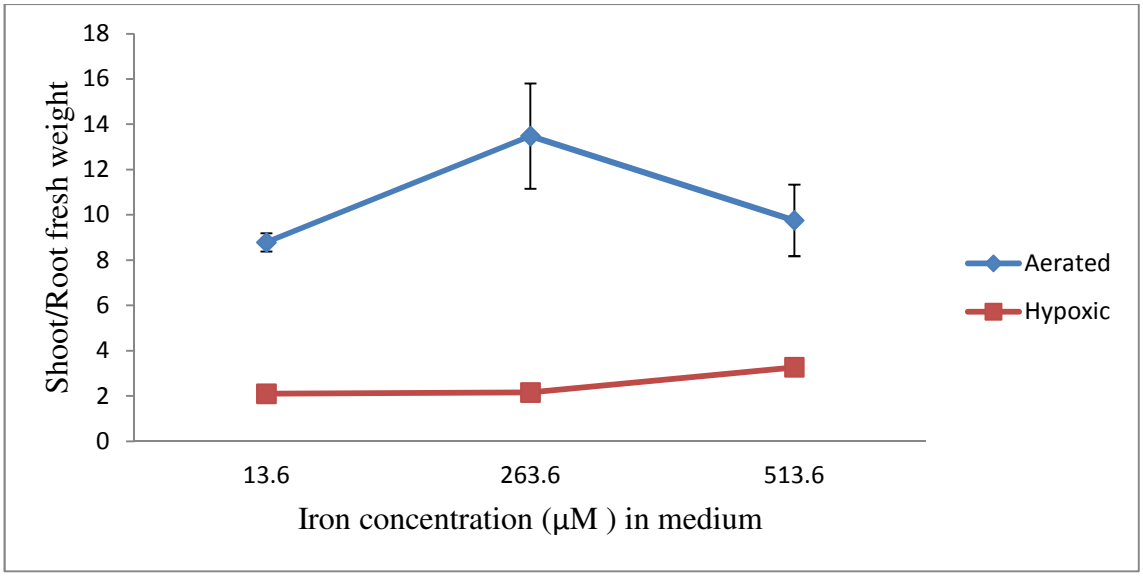


Chlorophyll (A) of *Suaeda maritima* plants after 10 days growth under concentrations of iron µM with hypoxic (N) and aerated (Air) conditions in the growth chamber. Error bars are SE (n= 6). 2-ways ANOVA; oxygen concentration in the medium $F(1, 30) = 544.895$, $P < 0.001$, iron concentration in the medium $F(2, 30) = 83.336$, $P < 0.001$, the interaction between oxygen concentration and Iron concentration in the medium $F(2, 30) = 7.811$, $P < 0.05$.



Chlorophyll (b) of *Suaeda maritima* plants after 10 days growth under concentrations of iron µM with hypoxic (N) and aerated (Air) conditions in the growth chamber. Error bars are SE (n= 6). 2-ways ANOVA;. Analysing by ANOVA showed that significant effect of oxygen concentration in the root medium on the chlorophyll (b) concentration $F(1, 30) = 77.076$, $P < 0.001$, iron concentration in the root medium $F(2, 30) = 11.405$, $P < 0.001$, and the Interaction was not significant effect on the chlorophyll (b) concentration $F(2, 30) = 2.167$, $P = 0.132$.

Appendix 3.14



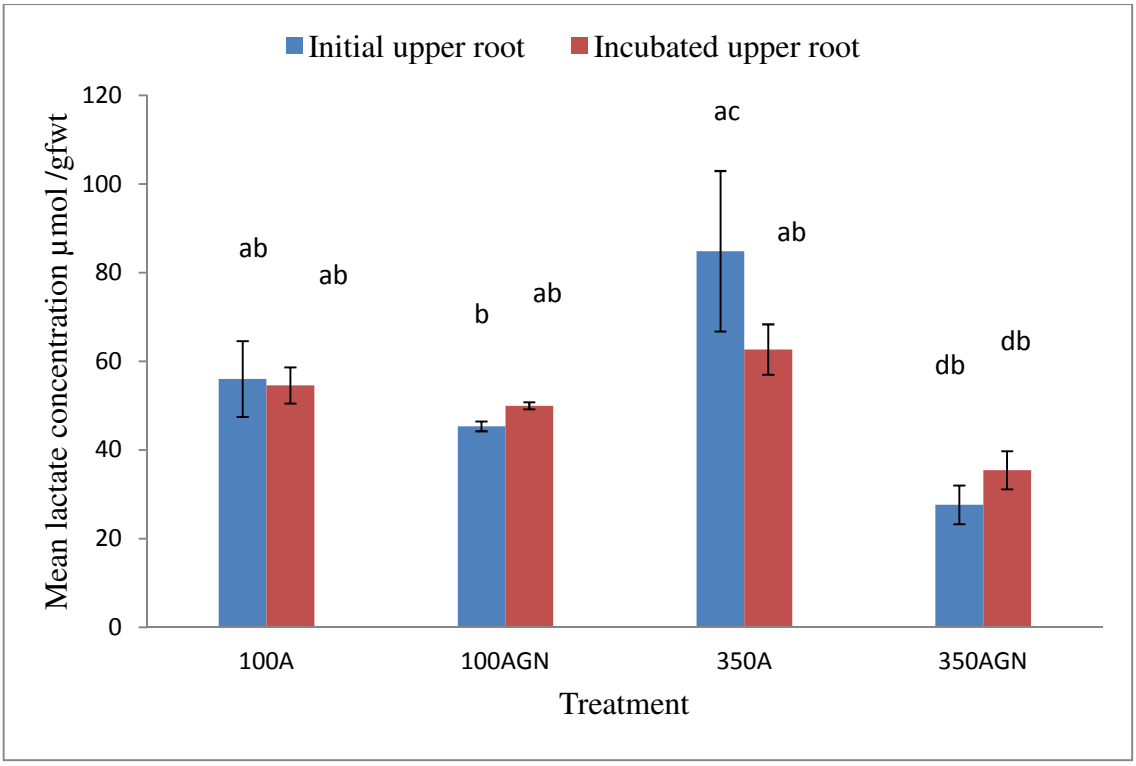
The ratio of shoot and root of *Suaeda maritima* plants after 10 days growth under concentrations of iron µM with hypoxic (N) and aerated (aerated) conditions in the growth chamber.

Appendix 3.15

The ratio of shoot to root heavy metal of *Suaeda maritima* plants 10 days growth under concentrations of iron µM with hypoxic (N) and aerated (Air) conditions in the growth chamber.

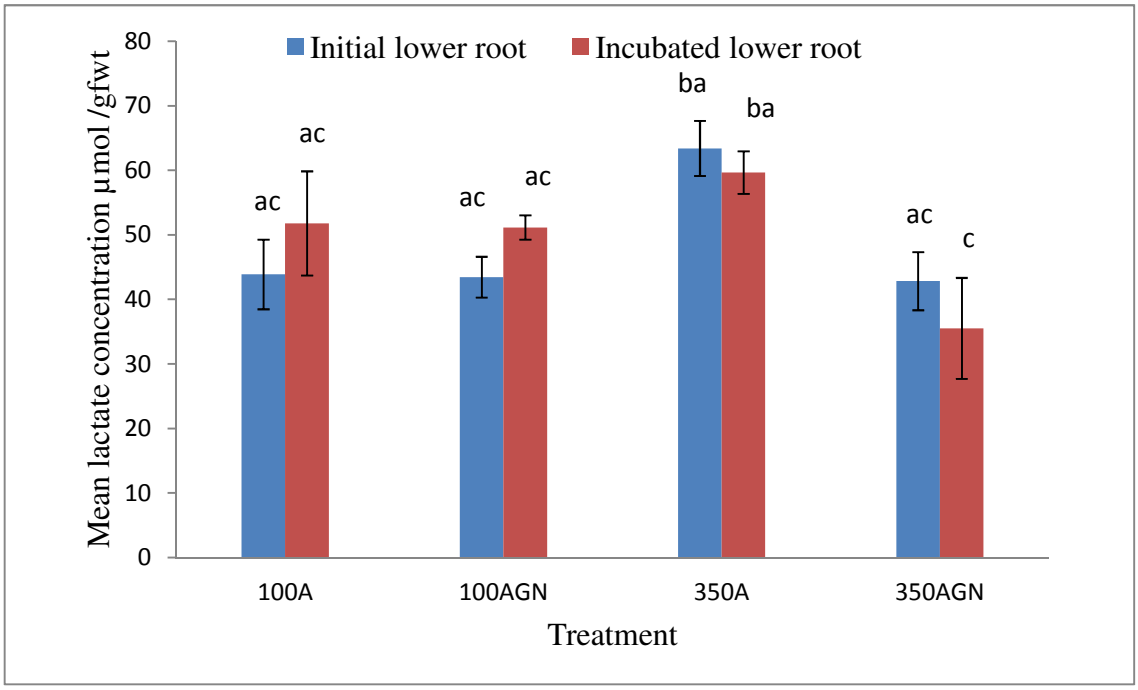
Treatment		Shoot : Root Ratio			
Oxygen concentration	Iron µM concentration	Iron	Manganese	Zinc	Copper
O2	Culture	3:97	3:7	1:9	4:96
	0.25	4:96	1:9	1:9	5:95
	0.5	2:98	4:6	2:8	2:8
N	Culture	6:94	1:9	2:8	3:7
	0.25	4:96	1:9	2:8	2:8
	0.5	7:93	1:9	1:9	2:8

Appendix 4.1



Initial and incubated upper root lactate concentration of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n = 3). Data were analysed by 3-way ANOVA. Salt concentration F (1, 24) = 0.049, P = 0.827, oxygen concentration F (1, 24) = 21.995, P = 0.000, incubation F (1, 24) = 0.276, P = 0.604, the interaction between salt and incubation F (1, 24) = 10.578, P = 0.003, the interaction between oxygen and incubation F (1, 24) = 2.876, P = 0.103, the interaction between salt, oxygen, and incubation F (1, 24) = 1.263, P = 0.272. Letters above bars indicate significant difference in means from post-hoc Tukey tests for initial and incubated root separately.

Appendix 4.2



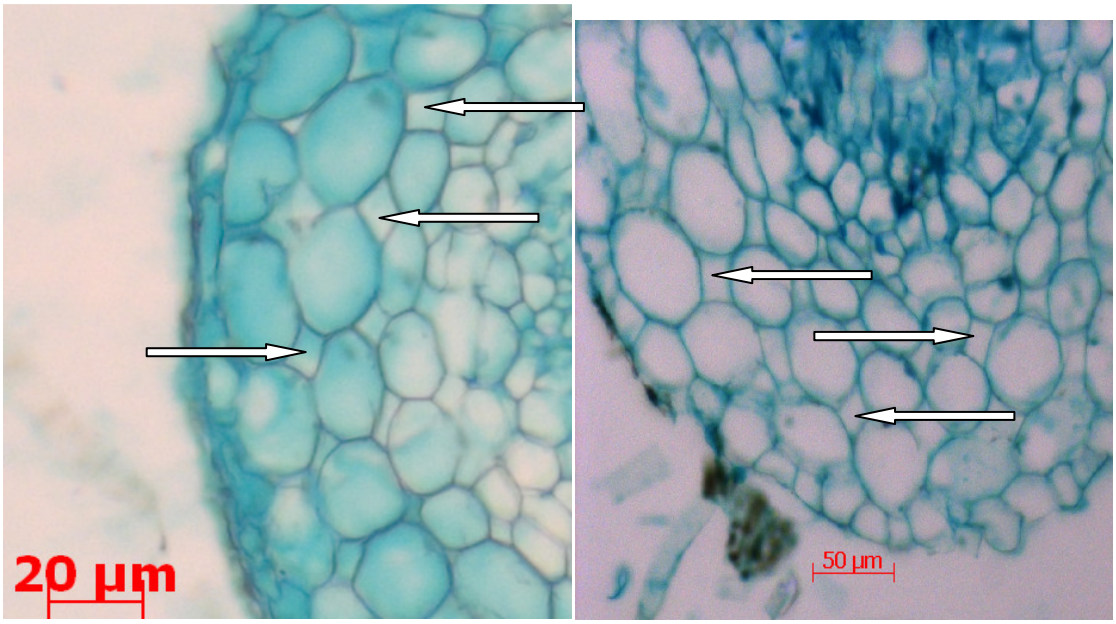
Initial and incubated lower root lactate concentration of *Suaeda maritima* plants after 6 weeks growth, under different levels of oxygen: Aerated nutrient solution (A), and stagnant agar solution (AGN) in different concentrations of artificial seawater (100 & 350 mM Na^+) in a growth chamber. Error bars are SE (n = 3). Data were analysed by 3-way ANOVA. Salt concentration in the growth medium F (1, 24) = 0.611, P = 0.442, oxygen concentrations F (1, 24) = 10.297, P = 0.004, the incubation F (1, 24) = 0.102, P = 0.752, the interaction between salt and oxygen F (1, 24) = 9.362, P = 0.005, the interaction between salt and incubation F (1, 24) = 3.491, P = 0.074, interaction between oxygen and incubation F (1, 24) = 0.071, P = 0.792, and the interaction between salt, oxygen, and incubation F (1, 24) = 0.056, P = 0.815. Letters above bars indicate significant difference in means from post-hoc Tukey tests for initial and incubated root separately.

Appendix 4.3

Adventitious roots grown under drained conditions, in a green house.

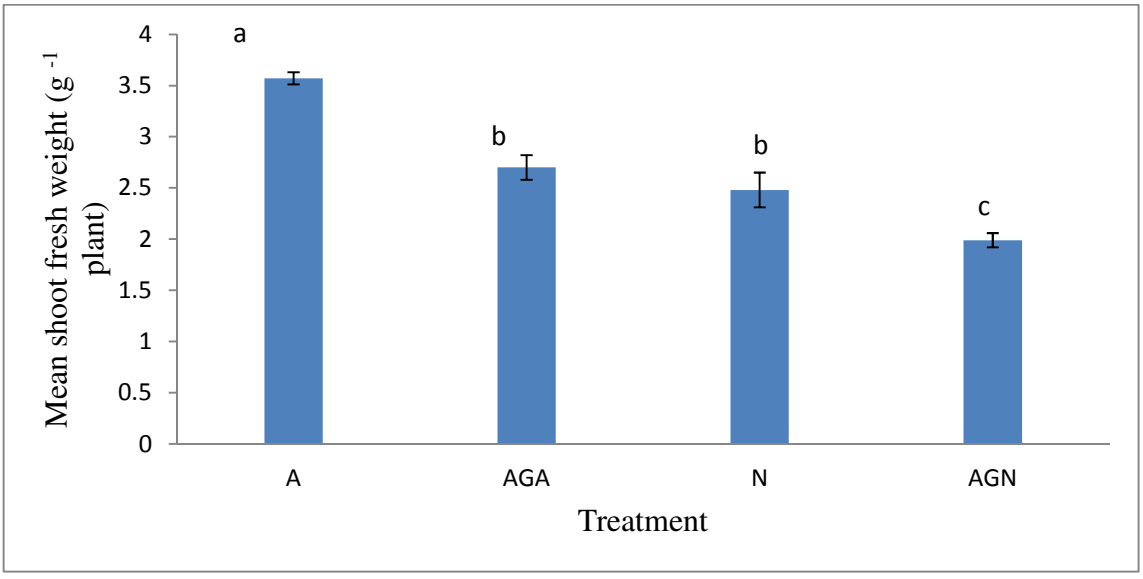
Adventitious roots grown under drained conditions, in a green house.

Appendix 4.4



Photographs cross sections from wax-imbedded *Suaeda maritima* roots of plants grown in hypoxic conditions showing intercellular spaces in the cortex (arrows).

Appendix 5.1



Shoot fresh weight of *Suaeda maritima* plants (9 weeks old at harvest), after 5 weeks treatment in hydroponic solutions under different concentrations of oxygen, in 100 mM Na^+ artificial seawater (aerated and hypoxic): Aerated nutrient solution without agar (A), aerated nutrient solution with agar (AGA), nitrogen-flushed nutrient solution without agar (N), nitrogen-flushed nutrient solution with agar (AGN). Error bars are (n = 10). Data were analysed by one-way ANOVA; shoot fresh weight (F (3, 36) = 31.666, $P < 0.001$). Letters above bars indicate significant difference in means from post-hoc Tukey tests.

Appendix 5.2

The stomatal mesurments for *S. maritima* plants grown under different concentrations of oxygen in a growth chamber at 20°C. Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level.

	Position	Treatment			
		A	AGA	N	AGN
Number	abaxial	49± 2a	47 ± 2 a	46 ± 2a	55 ± 2b
	adaxial	46 ± 1a	40 ± 2b	40 ± 2b	46 ± 2a
Length	Abaxial	25 ± 0.5a	25 ± 0.5a	24 ± 0.7a	22.3 ± 0.4b
	Adaxial	25 ± 0.5a	24 ± 0.7a	24 ± 0.5 a	21 ± 0.6b
Width	abaxial	21± 0.5a	21 ± 0.5a	19 0.5 a	14 ± 0.7 b
	adaxial	23 ± 0.6a	21 ± 0.6 b	17 ± 0.7c	14 ± 0.7 d
Degree of stomatal opening	abaxial	0.87± 0.02a	0.85 ± 0.02a	0.83 ± 0.03 a	0.62 ± 0.03 b
	adaxial	0.96± 0.03 a	0.90 ± 0.04 a	0.71 ± 0.03 b	0.67 ± 0.04 b

Appendix 5.3

Shows ions (Na^+ and K^+) concentrations, for *S. maritima* shoot and root grown under different concentrations of oxygen in a growth chamber at 20°C. Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level.

	unite	Treatment			
	µmol g^{-1} DW	A	AGA	N	AGN
Shoot	Na+	5.9+0.1a	5.9 + 0.1 a	5.7 + 0.2a	5.4 + 0.2 a
	K	0.88 + 0.01 a	0.83 + 0.01 a	0.81 + 0.02a	0.77 + 0.004b
Root	Na+	1.3 + 0.08a	1.0 + 0.07 a	1.1 + 0.03 a	1.1 + 0.03a
	K	0.77+0.01a	0.77 +0.01 a	0.77 +0.01a	0.720.01b +

Appendix 5.4

Shows gas exchanage, in *S. maritima* plants grown under different concentrations of oxygen in a growth chamber at 20°C. Letters indicate the mean significant difference from post-hoc Tukey tests at $P < 0.05$ level.

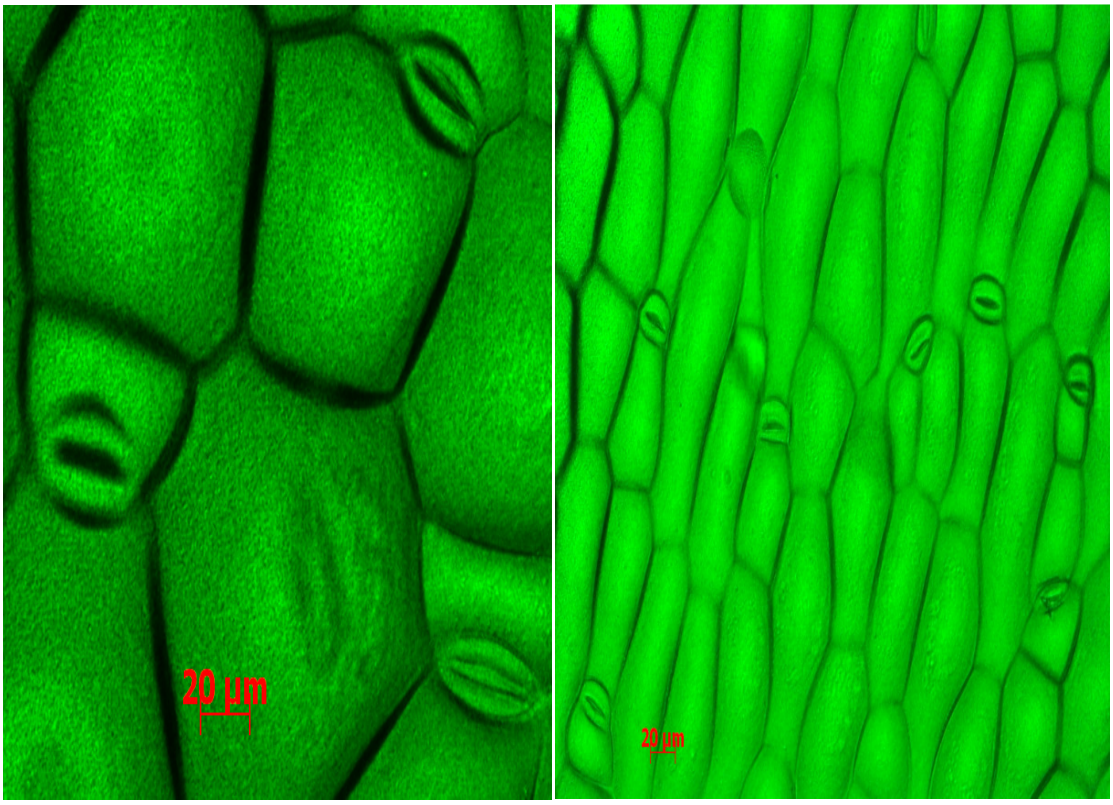
		Treatment			
		A	AGA	N	AGN
GS	mol m^{-2} s^{-1}	62±2 a	38.5±1 b	25.8 ± 0.5c	23.4 ± 0.7c
EVAP	mmol H_2O m^{-2} s^{-1}	0.7 ± 0.02a	0.4 ± 0.01 b	0.3 ± 0.01 c	0.3 ± 0.01 c
PN	µmol CO_2 m^{-2} s^{-1}	1.8 ± 0.04 a	0.9 ± 0.03 b	0.7 ± 0.02 c	0.6 ± 0.05 c
WUE	mmol CO_2 m^{-2}/mmol H_2O m^{-2} s^{-1}	2.6 ± 0.1 a	2.5 0.1 a	2.5 ± 0.1 a	2 ± 0.1 b

Appendix 5.5

Length, width on the abaxial and adaxial leaf surfaces of *Suaeda maritima* plants (11 weeks old at harvest), after 7 weeks growth in hydroponic solutions, at 100 and 350 mM Na^+ (artificial seawater and half strength Arnon & Stout culture solution). Error bars are SE (n = 40). 2-way ANOVA

	position	units	100 + A	100 = AGN	350 + A	350+ AGN
Length	Abaxial	µm	26.1±0.4a	31.8±0.4b	34±0.4c	36.8±0.4c
	Adaxial		27.4±0.5a	30.6±0.1b	26.8±0.6a	36±0.5c
Width	Abaxial	µm	17.6±0.3a	20.3±0.3b	20.7±0.3cb	21.8±0.1d
	Adaxial		18.4±0.4a	18.3±0.5a	16.3±0.3b	20.6±0.1

Appendix 5.6



Photographs of stomata in *Suaeda maritima* leaf were prepared by using Elite fast hydrosystem impression material containing vinyl polysiloxane (VPIM). This material was left on the leaves for approximately 2 minutes, then the mateiral was carefully removed from the leaf, coated with clear nail varnish and left to dry for 5 minutes. The film of nail varnish was carefully peeled from the VPIM. The impressions were used to measure the length and width of the stomata; measurements were taken at a magnification of 40x.

Appendix 6.1

Modified Yoshida culture solution

1.25 ml of stocks 1, 2, 4 and 5 were added to 1 litre of deionised water and the pH was adjusted to 4.5 using 1 N HNO3. Then 1.25 ml of stocks 3, 6 and 7 were added to the solution and the pH was readjusted to 4.5 to produce the final culture solution.

Stock no.	Reagent	g l^{-1}	Concentration in stock (mM)mM	Concentration in culture (mM)
1	Ammonium nitrate NH_4NO_3	91.4	1131	1.4
2	di-Potassium sulphate (K_2SO_4)	71.4	410	0.51
3a	Potassium di-hydrogen orthophosphate (KH_2PO_4)	46.2	339	0.42
3b	di-Potassium hydrogen orthophosphate (K_2HPO_4)	8.6	49	0.06
4	4 Calcium chloride $(CaCl_2.6H_2O$	175	799	1.0
5	Magnesium sulphate $(MgSO_4.7H_2O)$	324	1315	1.6
6	Minor Nutrients			
a	Manganese chloride $(MnCl_2.4H_2O)$	1.5	7.6	0.01
b	Ammonium molybdate $(NH_4)_6.Mo_7O_{24}.4H_2O$	0.074	0.06	0.00008
c	Boric acid (H_3BO_3)	0.93	15.04	0.02

d	Zinc sulphate ($ZnSO_4.7H_2O$)	0.035	0.12	0.0002
e	Copper (II) sulphate ($CuSO_4.5H_2O$)	0.03	0.12	0.0002
7	Ethylenediaminetetra-acetic acid ferric monosodium salt (FeNaEDTA)	10.5	28.6	0.04

Recipe is modified from Yoshida S., Forno D.A., Cock J.H. & Gomez K.A. (1972) Laboratory manual for physiological studies of rice. International Rice Research Institute, Manila.

Appendix 6.2

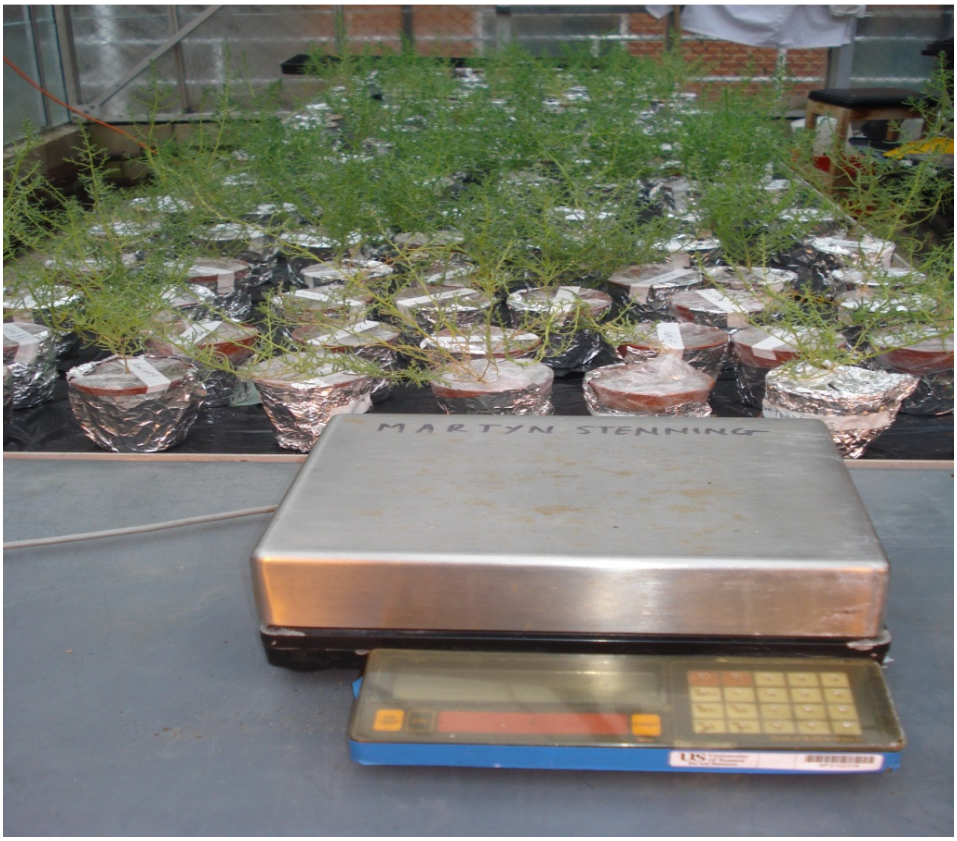
Hoagland's Solution

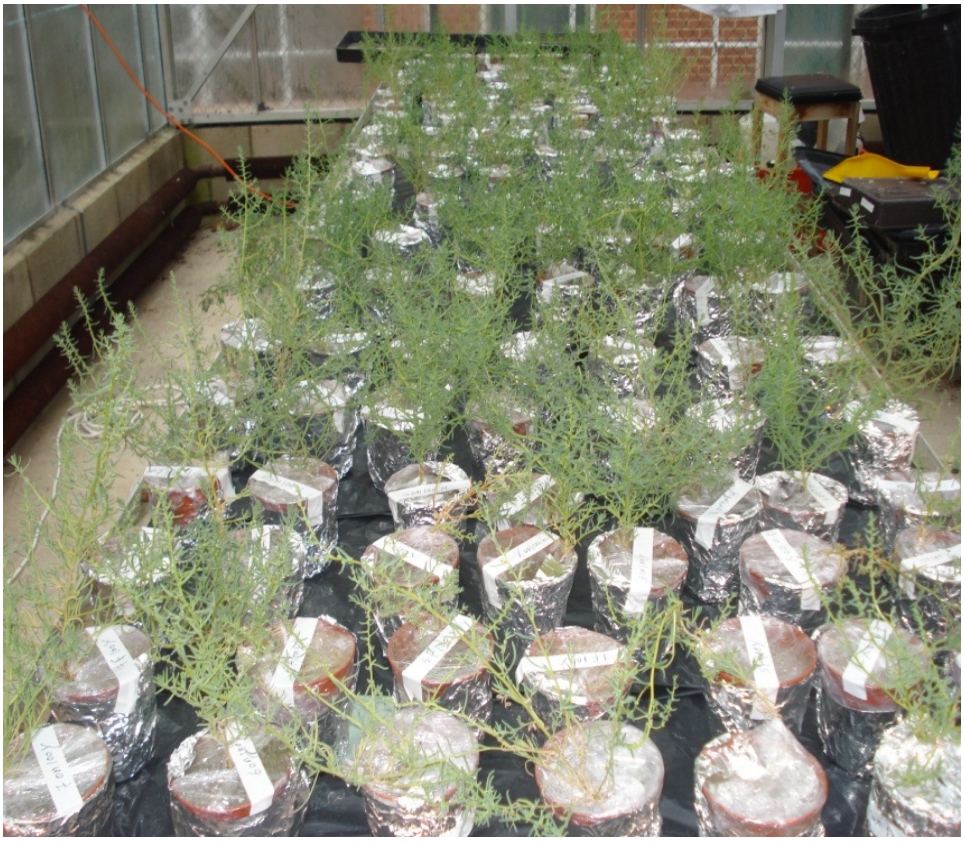
No	Soln	Reagent	Con	Use at
		g/L	mM	
1	KNO_3	60.66	600	10
2	$NH_4H_2PO_4$	11.5	100	10
3	$MgSO_4.7H_2O$	49.29	200	10
4	$CaCl_2.2H_2O$	73.51	500	10
5	minor elements		µM	10/L
	H_3BO_3	0.57	9.25	
	$MnCl_2.4H_2O$	0.36	1.83	
	$ZnSO_4.7H_2O$	0.045	0.153	
	$CuSO_4.5H_2O$	0.016	0.064	
	$(NH_4)_6Mo_7O_{24}.4H_20$	0.087	0.07	
6	FeNaEDTA	21.79		1/L

Appendix 6.3

Photograph of the tanks with *Suaeda maritima* plants in pots at the start treatments.

Appendix 6.4





Gravimetric estimation of transpiration in the glasshouse

Appendix 6.5

Shows leaf area and number of stomata on the adaxial and abaxial leaf surfaces of *S. maritima* grown under different fresh water concentrations (100, 350 and 450 mM Na^+), in Hoagland and Yoshida culture solutions, under drained and flooded conditions, for 6 weeks in a glasshouse. Number of stomata (n=40) and leaf area (n=70).

	Units	100		350		450	
		Drained	Flooded	Drained	Flooded	Drained	Flooded
Hoagland	Leaf area	0.9±0.04	0.7±0.05	1.6±0.03	1.1±0.03	1.4±0.06	1.1±0.04
Yoshida	cm	0.5±0.01	0.3±0.02	1.0±0.05	1.0±0.05	1.1±0.05	0.7±0.04
		Number of stomata					
Hoagland	Abaxial	42±1	56±2	36±1	41±1	30±1	39±1
Yoshida		53±1	56±2	49±1	40±1	39±1	39±1
Hoagland	Adaxial	41±1	50±2	34±1	43±1	32±1	36±2
Yoshida		61±2	57±2	39±1	37±1	51±1	40±1

Appendix 6.6

Shows shoot ion concentrations (sodium, potassium, magnesium and calcium) in *Suaeda maritima* shoots grown in different culture solutions, in drained and flooded conditions in the glasshouse, for (n=14)

	Units	100		350		450	
		Drained	Flooded	Drained	Flooded	Drained	Flooded
	Na						
Hoagland	µmol $^{-1}$mg	3.5±0.05	3.3±0.1	5.7±0.1	4.5±0.1	6.0±0.2	5.0±0.1
Yoshida		3.0±0.1	2.8±0.1	4.8±0.1	4.2±0.1	5.2±0.1	4.6±0.1
	K						
Hoagland	µmol $^{-1}$mg	0.55±0.02	0.57±0.02	0.44±0.01	0.38±0.01	0.48±0.01	0.34±0.01±
Yoshida		0.43±0.01	0.29±0.009	0.34±0.007	0.32±0.005	0.38±0.007	0.30±0.008
	Mg						
Hoagland	µmol $^{-1}$mg	0.44±0.01	0.45±0.02	0.63±0.01	0.55±0.01	0.64±0.01	0.61±0.01
Yoshida		0.57±0.02	0.48±0.01	0.58±0.01	0.57±0.01	0.67±0.01	0.66±0.01
	Ca						
Hoagland	µmol $^{-1}$mg	0.07±0.003	0.11±0.004	0.07±0.01	0.08±0.005	0.04±0.004	0.09±0.002
Yoshida		0.10±0.002	0.13±0.003	0.07±0.002	0.11±0.01	0.10±0.007	0.12±0.006

Appendix 6.7

Shows leaf Shoot height, fresh and dry weight of adventitious and seminal roots *S. maritima* grown under different fresh water concentrations (100, 350 and 450 mM Na^{+}), in Hoagland and Yoshida culture solutions, under drained and flooded conditions, for 6 weeks in a glasshouse (n=20).

	Unite	100		350		450	
		Drained	Flooded	Drained	Flooded	Drained	Flooded
Hoagland	Shoot height	28.6±1	25.8±1	30±1	26.4±1	24.8±1	23.6±1
Yoshida		28±2	17.8±1	25.5±1	26.2±1	24.2±1	22±0.1
Hoagland	FW g^{-1} plant	13±2	4±1	21±1	6±1	12±1	4±0.5
Yoshida		4.5±0.6	1.7±0.2	6.7±0.6	4.2±0.4	10.8±1	3.5±0.3
Hoagland	DW g^{-1} plant	1.3±0.2	0.4±0.1	1.9±0.1	0.6±0.06	1.2±0.1	0.4±0.04
Yoshida		0.6±0.07	0.3±0.03	0.6±0.06	0.5±0.04	1.2±0.1	0.4±0.04
Hoagland	Water content	10.4±0.2	10.2±0.4	11±0.2	10.7±0.2	10.3±0.2	9.8±0.2
Yoshida		7.2±0.3	6.4±0.2	10.4±0.2	8.9±0.3	9.7±0.2	9.2±0.3
Hoagland	Adventitious root mg^{-1} plant	104±9	66±15	131±11	35±8	96±9	31±4
Yoshida		90±6	25±3	60±6	36±4	98±8	29±3
Hoagland	Seminal root	11.2±1	10±1	13.7±3	10.4±1	9.9±1	8.8±1
Yoshida	mg^{-1} plant	12.6±1	8.3±1	8.0±1	8.4±1	8.1±0.5	7.0±1
Hoagland	Ratio adventitious/ seminal root	11.6±2	6.6±2	13±1	2.9±0.6	11±1	3.6±0.2
Yoshida		8±1	3.5±0.6	9.3±1	4.8±0.6	13.4±1	4.8±1

Appendix 6.8

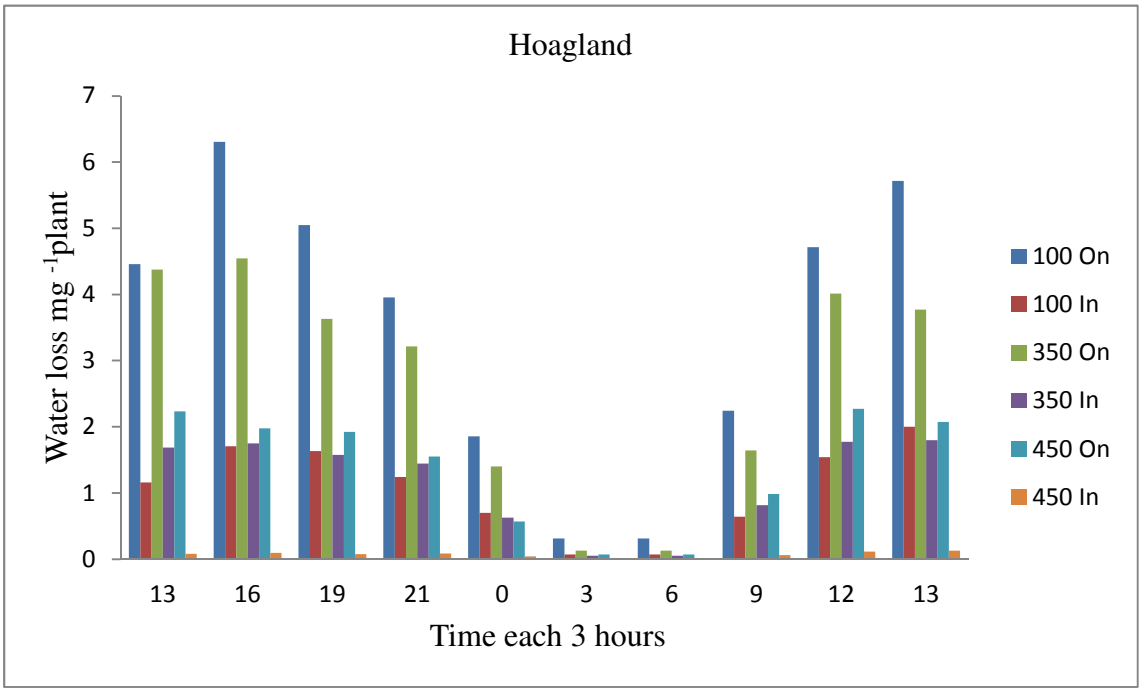
Shows the difference between *S. maritima* plants grown under aerated (on the left hand) and flooded (on the right hand) conditions.

Appendix 6.9

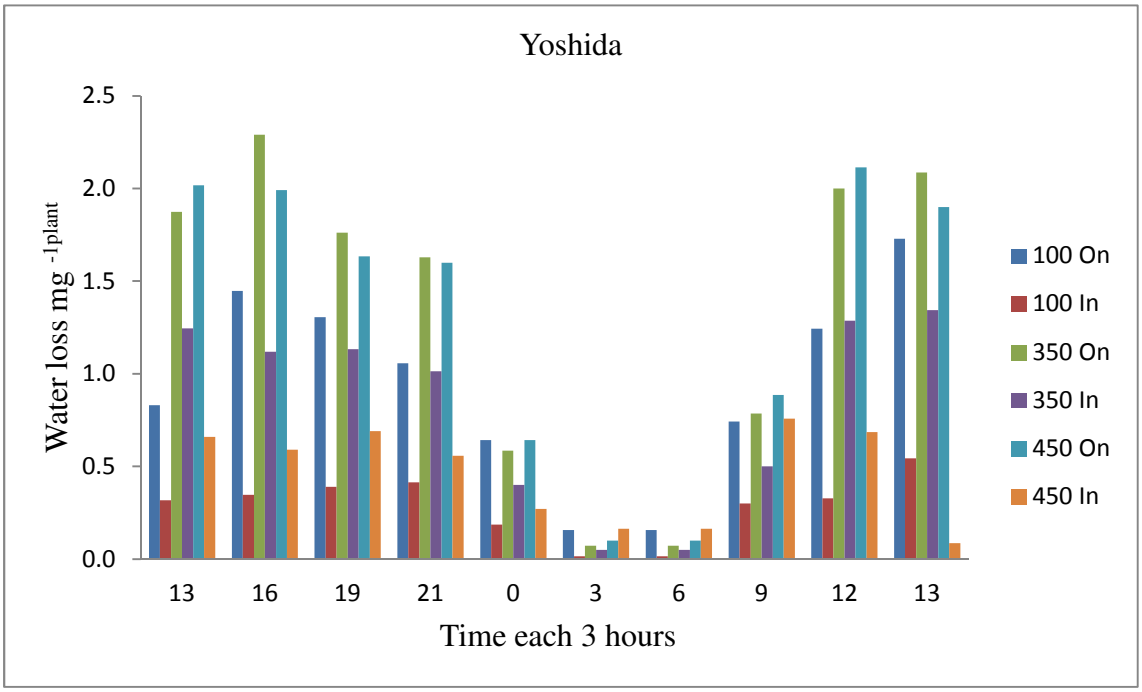
Shows Gas exchanage in *S. maritima* grown under different fresh water concentrations (100, 350 and 450 mM Na^+), in Hoagland and Yoshida culture solutions, under drained and flooded conditions, for 6 weeks in a glasshouse (n=20).

	100		350		450	
	Drained	Flooded	Drained	Flooded	Drained	Flooded
Stomatal conductance (GS) mol m^{-2} s^{-1}						
Hoagland	112±1	86±3	71±1	57±1	37±1	41±2
Yoshida	51±1	37.5±1	83±1	54±1	45±1	52±2
Transpiration (EVAP) mmol H_2O m^{-2} s^{-1}						
Hoagland	1.2±0.01	1.1±0.03	0.93±0.01	0.76±0.01	0.39±0.005	0.38±0.006
Yoshida	0.6±0.01	0.42±0.01	0.96±0.01	0.65±0.01	0.52±0.01	0.49±0.01
Photosynthesis (PN) µmol CO_2 m^{-2} s^{-1}						
Hoagland	1.2±0.04	0.85±0.05	1.2±0.03	0.8±0.03	0.72±0.01	0.57±0.01
Yoshida	0.4±0.02	0.57±0.03	1.3±0.03	0.84±0.01	0.89±0.04	0.63±0.01
Water Use Efficiency (WUE) mmol CO_2 m^{-2}/mmol H_2O m^{-2} s^{-1}						
Hoagland	1.03±0.03	0.73±0.04	1.3±0.02	1.03±0.03	1.86±0.05	1.5±0.03
Yoshida	0.68±0.03	1.4±0.08	1.35±0.03	1.31±0.03	1.74±0.08	1.28±0.02
Water Use Efficiency (WUE) mg DW/mg waterloos						
Hoagland	0.72±0.02	0.61±0.04	1.8±0.08	1.5±0.1	2.7±0.2	2.3±0.2
Yoshida	0.9±0.01	1.6±0.1	1.6±0.2	1.5±0.1	1.8±0.1	1.6±0.1

Appendix 6.10

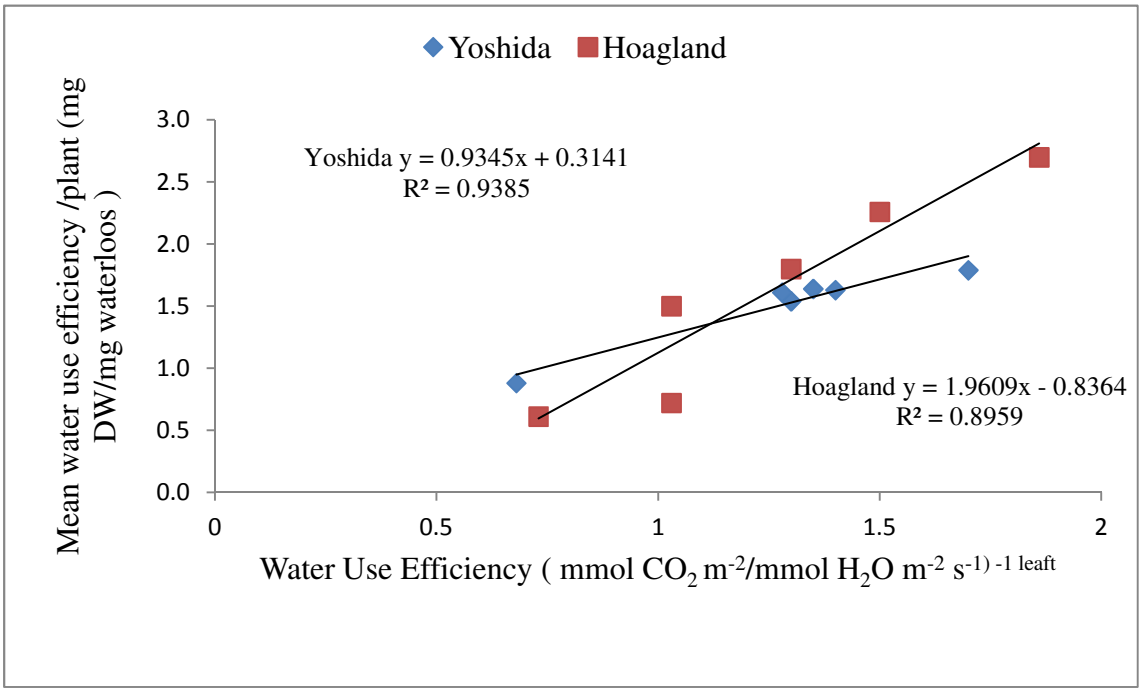


Appendix 6.11



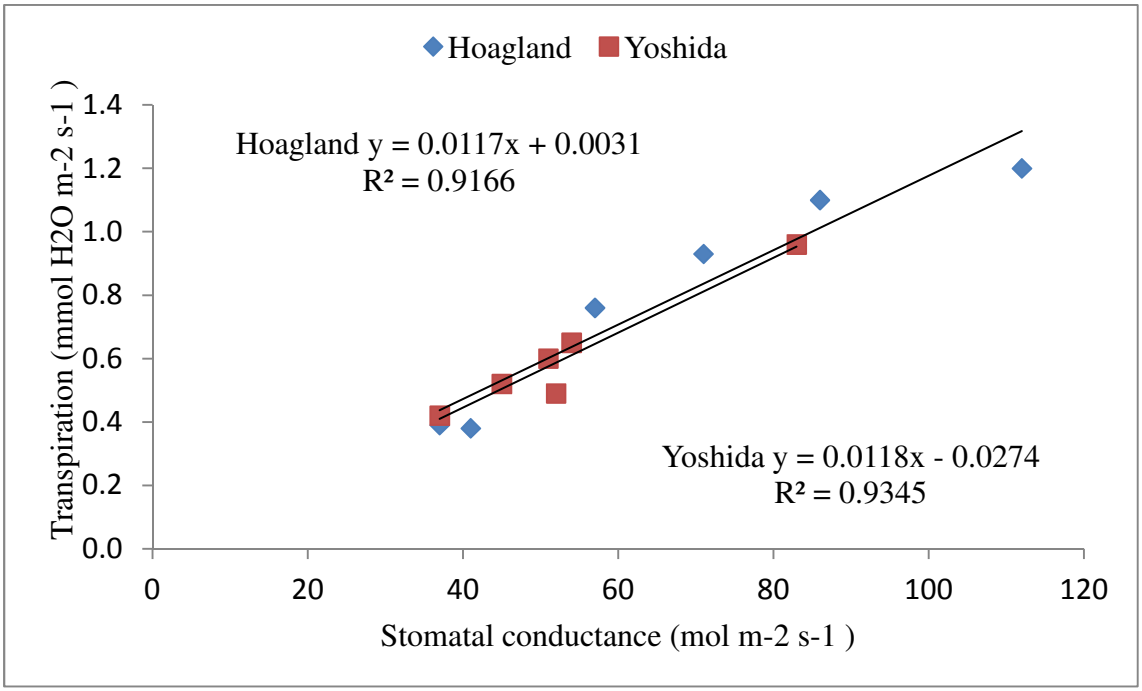
Seven plants was used to determinate water loss each treatments for over 24 hours in the green house under controlled condition

Appendix 6.12



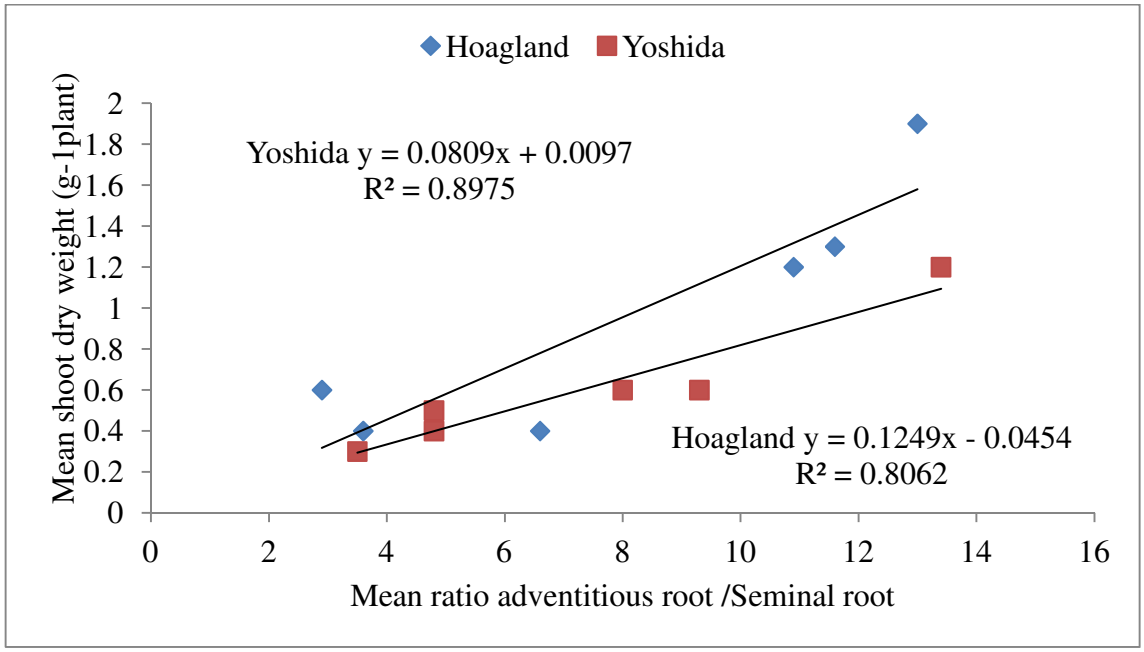
The relationship between water use efficiency/ leaf and water use efficiency / plant.

Appendix 6.13



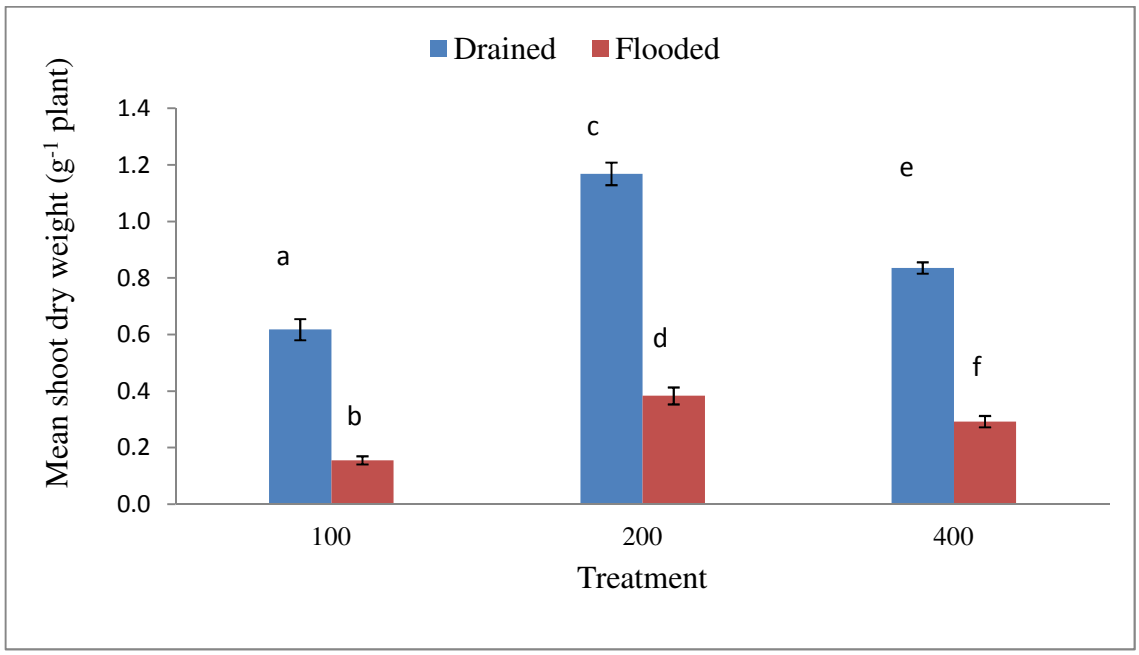
The relationship between stomatal conductance and transpiration rate.

Appendix 6.14



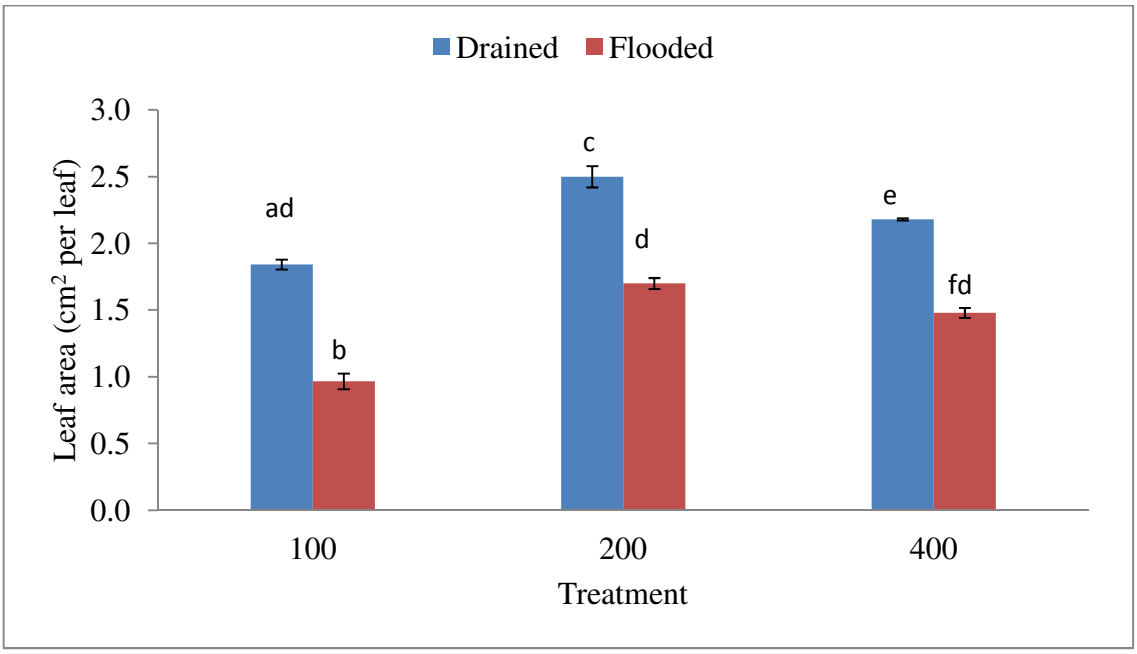
The relationship between ratio of adventitious root and seminal root with shoot dry weight

Appendix 7.1



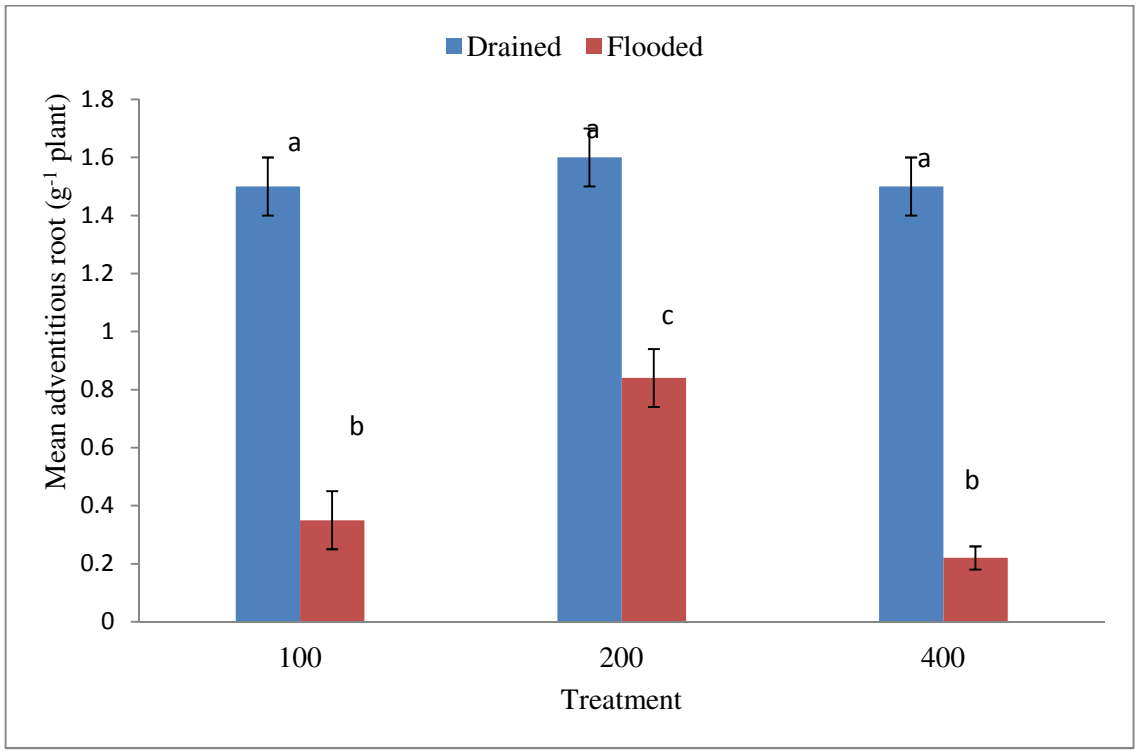
Shoot dry weight of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks treatment in sand, under different fresh water concentrations with Stout and Arnon culture solutions, under drained and flooded conditions. Error bars are (n=40). Data were analysed by two-way ANOVA showed a significant effect on shoot dry weigh by salt concentrations F (2, 214) = 76.698, $P < 0.001$, tidal flow F (1, 214) = 525.436, $P < 0.001$, and the interaction F (2, 214) = 13.985, $P < 0.001$. Letters above bars indicate significant difference in means from post-hoc Tukey tests $P < 0.05$.

Appendix 7.2



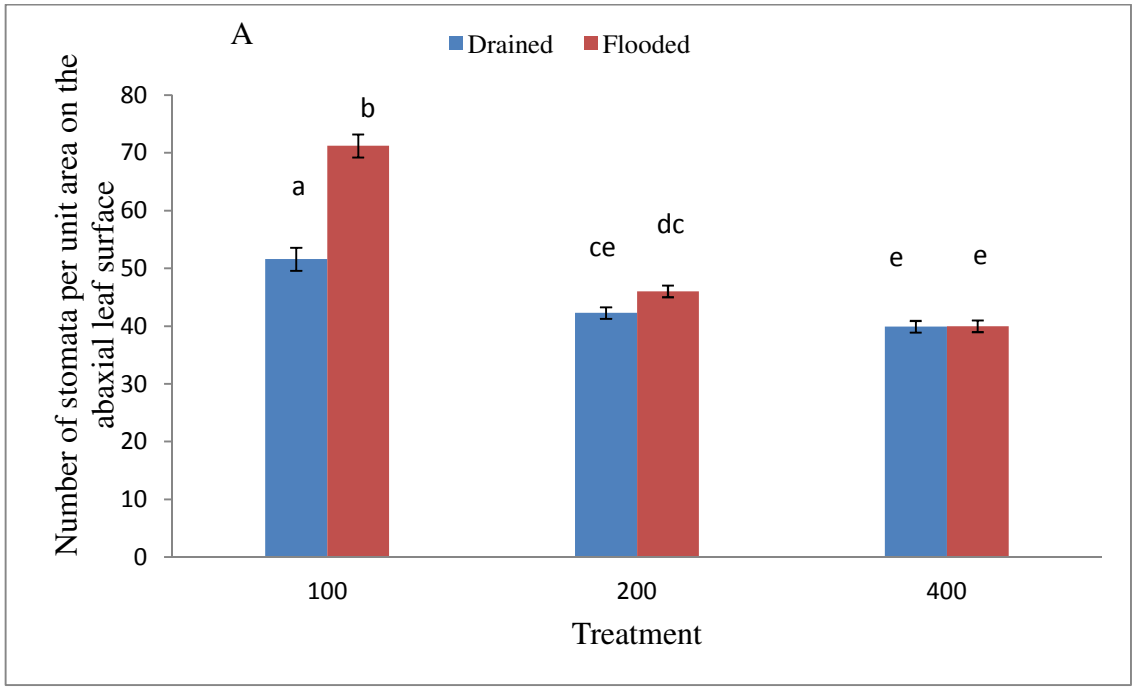
Leaf area of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks treatment in sand, under different fresh water concentrations with Stout and Arnon culture solutions, under drained and flooded conditions. Error bars are (n=30). Data were analysed by two-way ANOVA showed a significant effect on shoot dry weigh by salt concentrations F (2, 214) = 67.815, $P < 0.001$, tidal flow F (1, 214) = 227.181, $P < 0.001$, and the interaction F (2, 214) = 1.920, $P = 0.150$. Letters above bars indicate significant difference in means from post-hoc Tukey tests $P < 0.05$.

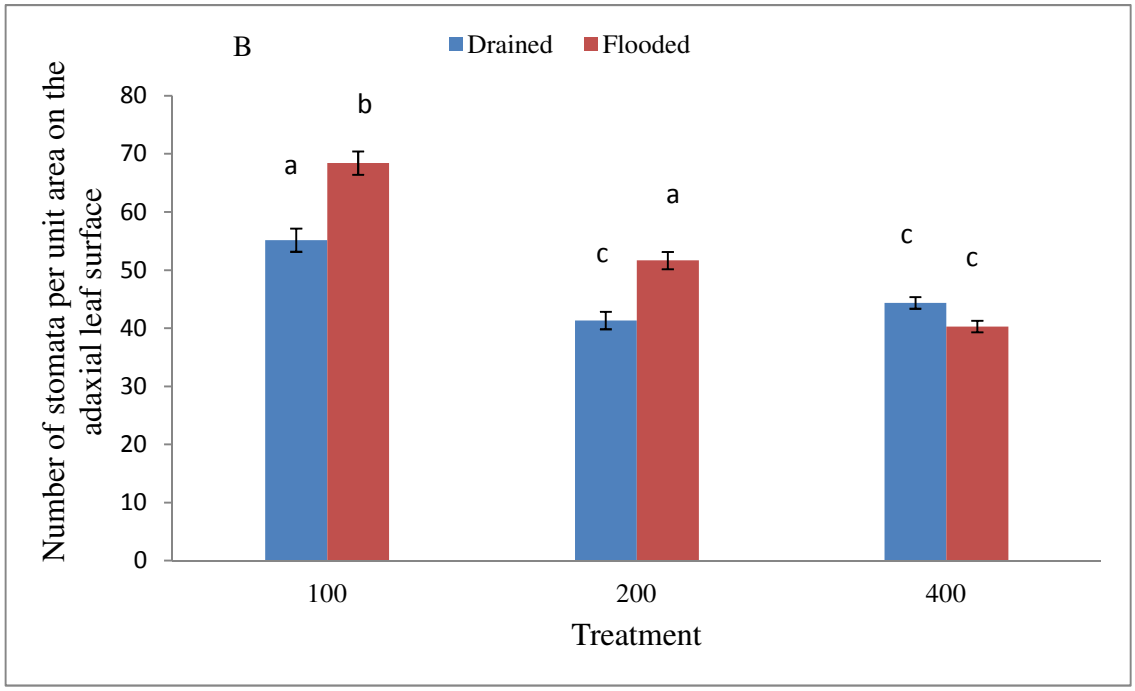
Appendix 7.3



Adventitious and seminal roots of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks treatment in sand, under different fresh water concentrations with Stout and Arnon culture solutions, under drained and flooded conditions. Error bars are (n=20). Data were analysed by two-way ANOVA showed a significant effect on adventitious root by tidal flow concentrations F (1, 107) = 121.620, $P <$ 0.001, salt F (2, 214) = 2.915, P = 0.059, and the interaction F (2, 107) = 4.946, $P < 0.05$. Seminal root; salt F (2, 107) = 29.297, $P < 0.001$, tidal flow F (1, 107) = 39.350, $P < 0.001$ And the interaction F (2, 107) = 8.248, $P < 0.001$Letters above bars indicate significant difference in means from post-hoc Tukey tests $P < 0.05$.

Appendix 7.4





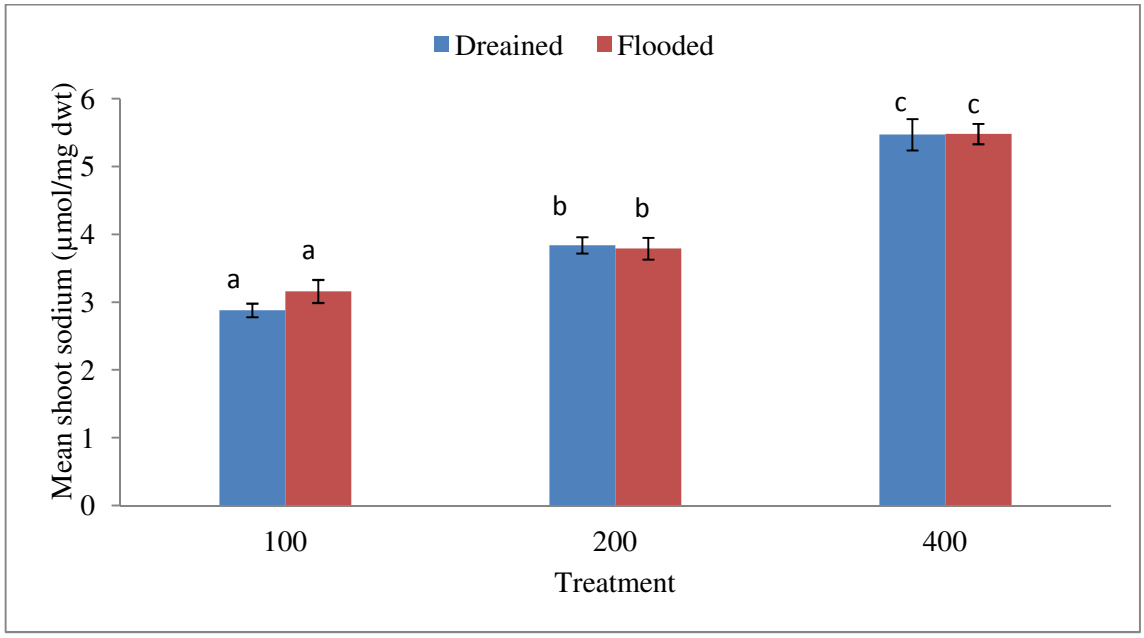
Number of stomata on the abaxial (A) and adaxial (B) leaf surface of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks treatment in sand, under different fresh water concentrations with Stout and Arnon culture solutions, under drained and flooded conditions. Error bars are (n=60). Data were analysed by two-way ANOVA showed a significant effect on number of stomata on the abaxial leaf surface by salt concentrations F (2, 350) = 133.319, $P < 0.001$, tidal flow F (1, 350) = 48.880, $P < 0.001$, and the interaction F (2, 350) = 27.217, $P < 0.001$. Number of stomata on the adaxial leaf surface; salt F (2, 350) = 95.323, $P < 0.001$, tidal flow F (1, 350) = 30.775, $P < 0.001$, the interaction F (2, 350) = 18.328, $P <$ 0.001Letters above bars indicate significant difference in means from post-hoc Tukey tests $P < 0.05$.

Appendix 7.5

Gas exchanage in *S. maritima* grown under different fresh water concentrations (100, 200 and 400 mM Na^+), in Stout and Arnon culture solution, under drained and flooded conditions, for 6 weeks in a glasshouse (n=16). Letters indicate significant difference in means from post-hoc Tukey tests $P < 0.05$.

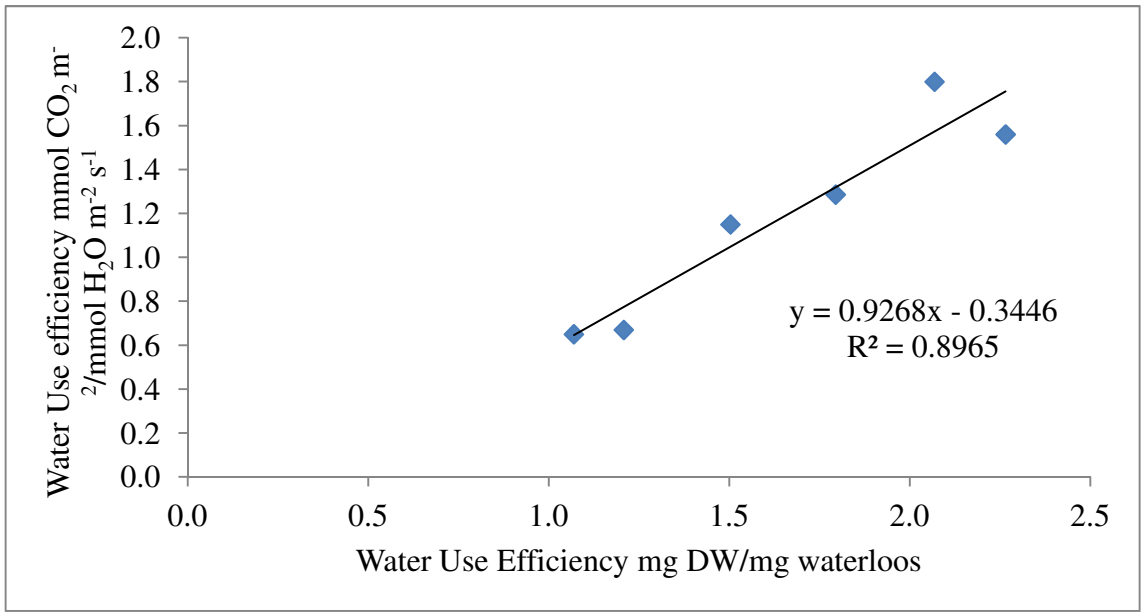
	100		200		400	
	Drained	Flooded	Drained	Flooded	Drained	Flooded
Stomatal conductance	53±4a	29.4±2b	36.1±1c	25.3±1d	28.4±2b	23.8±0.6d
Transpiration	0.7±0.02a	0.42±0.02b	0.51±0.01c	0.41±0.03b	0.39±0.01b	32±0.01d
Photosynthesis (PN) µmol CO_2 m^{-2} s^{-1}	0.5±0.05a	0.27±0.02b	0.74±0.07c	0.47±0.04a	0.72±0.02c	0.40±0.02a
Water use efficiency	0.68±0.08a	65±0.04a	1.56±0.02b	1.15±0.1c	1.8±0.06d	1.28±0.08c
Water use efficiency	1.2± 0.03a	1.1±0.03a	2.3±0.04b	1.50±0.04c	2.1±0.06db	1.8±0.07ed

Appendix 7.6



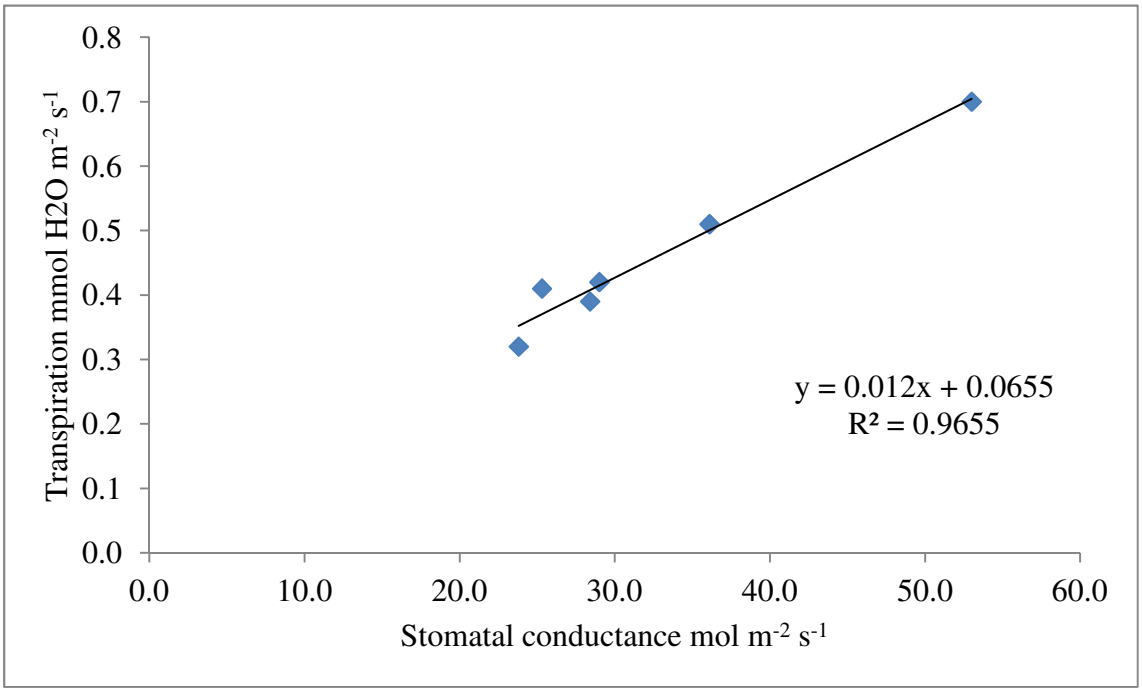
Shoot sodium of *Suaeda maritima* plants (10 weeks old at harvest), after 6 weeks treatment in sand, under different fresh water concentrations with Stout and Arnon culture solutions, under drained and flooded conditions. Error bars are (n=40). Data were analysed by two-way ANOVA showed a significant effect on shoot dry weigh by salt concentrations F (2, 210) = 179.893, $P < 0.001$, tidal flow F (1, 210) = 1.144, $P = 0.286$, and the interaction F (2, 210) = 0.657, $P = 0.520$. Letters above bars indicate significant difference in means from post-hoc Tukey tests $P < 0.05$.

Appendix 7.7



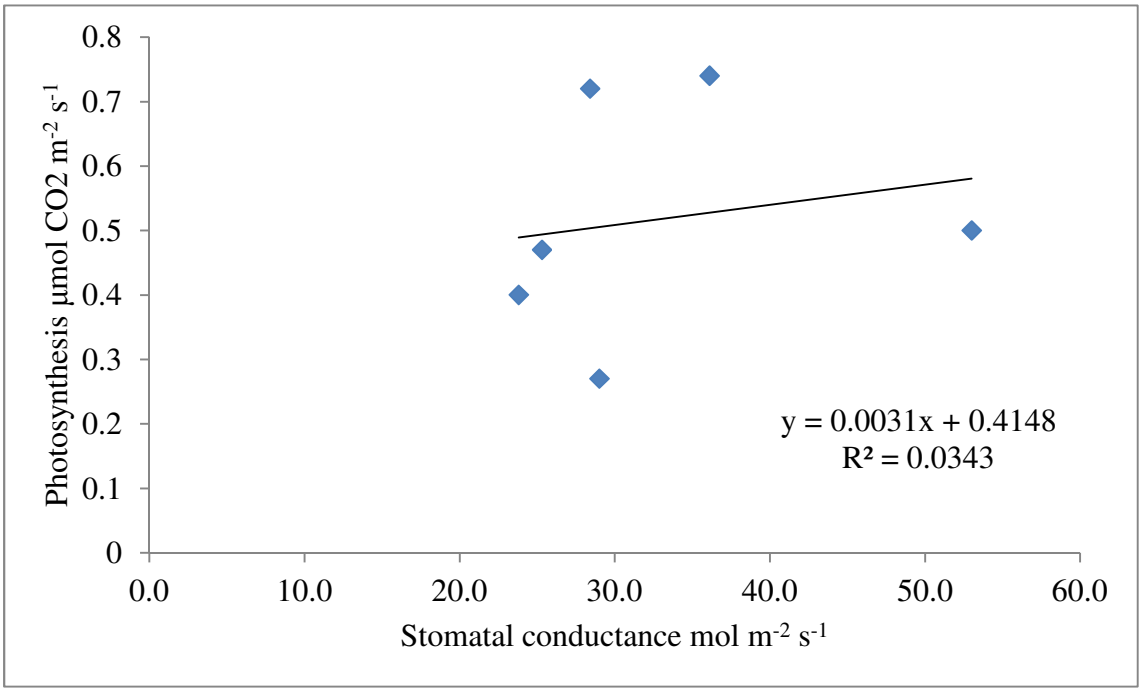
The relationship between water use efficiency/ leaf and water use efficiency / plant.

Appendix 7.8



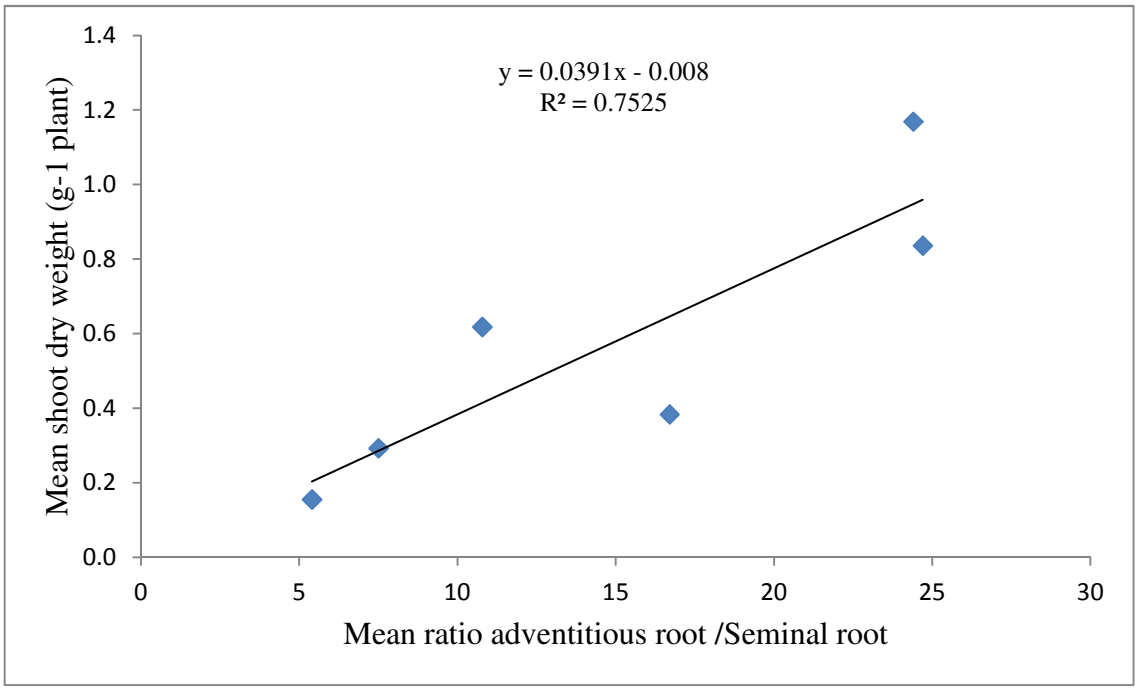
The relationship between stomatal conductance and transpitaion rate.

Appendix 7.9



The relationship between stomatal conductance and photosynthesis rate.

Appendix 7.10



The relationship between ratio of aventitious and seminal root with shoot dry weight..

Appendix 7.11

Redox potential

Mean Eh values (mV) recorded at three depths in pots of sand in which *Suaeda maritima* was growing at high-, mid- and low-tide positions in the tidal flow glasshouse tank system.

Treatment	On						In					
	Top		Middle		Bottom		Top		Middle		Bottom	
	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before
100	124±10	136±8	78±13	96±1110	23±18	61±15	101±14	87±14	36±17	10±20	-133±25	-142±25
200	79±11	107±7	11±12	58±10	-35±15	8±13	77±15	73±13	6±21	15±20	-176±32	-155±29
400	66±10	104±8	15±15	64±12	-12±18	21±15	95±10	85±12	39±15	35±16	-37±21	-36±20