In vitro studies of salt and drought tolerance
at the physiological, cellular and molecular
levels in *Medicago truncatula*

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Abbreviations

2,4-D	2, 4-Dichlorophenoxyacetic acid
A	Adenine
ANOVA	Analysis of variance
At	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
BLAST	Basic local alignment search tool
BY-2	Tobacco Bright Yellow var.2 cell line.
ΔCt	Mean of cycle threshold
CCS52	Cell- cycle switch 52
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CKI	CDK Inhibitor
DAPI	4, 6 diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTP	Dexyribonucleotide triphosphate
DTT	Dithio-1,4-threitol
DW	Dry weight
ECR	Embryo conversion and rooting medium

EDM	Embryo development medium
EDTA	Ethylenediamine tetra-acetic acid
EST	Expressed sequence tag
FCM	Flow cytometry
FDA	Fluoroscein diacetate
FW	Fresh weight
g	Gram(s)
G1	Gap1 of cell cycle
G2	Gap2 of cell cycle
mg	Milligrams
MI	Mitotic index
mM	Millimolar
µg	Micrograms
µM	Micromolar
mRNA	Messenger RNA
MS	Murashige and Skoog basal salts
Mt	*Medicago truncatula*
NAA	α-Naphthaleneacetic acid
P5C	pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
P5CR	P5C reductase
P5CS	Δ^1- pyrroline-5-carboxylate synthetase

PCR	Polymerase chain reaction
PDH	Proline dehydrogenase
PEG	Polyethylene glycol
PI	Propidium iodide
poly A	polyadenylated
RGR	Relative growth rates
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SDS	Sodium docecyl sulphate
SE	Standard error
SERK	Somatic embryogenesis receptor-like kinase
SOS	Salt overly sensitive
T	Thymine
UV	Ultraviolet light
WC	Water content

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Abstract

My aim was to acclimate calli of *Medicago truncatula* to be NaCl tolerant and discover the extent to which various traits could act as markers of improved NaCl tolerance *in vitro*. I developed a new tissue culture protocol incorporating multi-step up acclimation (Phase I) followed by two rounds of culture ± NaCl (Phases II and III) over 0 to 350 mM exogenous NaCl. Following Phases I, II and III, calli were tolerant at 150 mM and embryogenically competent at 100 mM NaCl. Remarkably, in Phase III, calli grew faster in the 100 and 150 mM NaCl treatments compared with the 0 mM controls. There were positive and negative linear relationships between Na^+ and K^+ uptake and exogenous NaCl concentration, respectively that intercepted each other at 160 mM suggesting a Na^+/K^+ homeostasis at this NaCl concentration. Proline level and vacuolar size peaked at 100/150 mM whilst highest osmolarity and lowest water content were at 250/350 mM NaCl. The concentration of water soluble sugars was positively related to 0 to 250 mM NaCl. Flow cytomerty was used to examine the extent to which cells underwent to endoreduplication. From 50 to 250 mM NaCl, cells were observed in G1 (2C), G2 (4C) or a state of endopolyploidy (8C). Hence, callus growth and embryogenesis occurred regardless of endoreduplication. Expression of genes linked to growth (*WEE1*), *in vitro* embryogenesis (*SERK*), salt tolerance (*SOS1*), proline synthesis (*P5CS)* and ploidy level (*CCS52* and *WEE1)* peaked at 100/150 mM NaCl. Hence, these genes and various physiological traits except sugar levels, served as useful markers for NaCl tolerance. To my knowledge, this is the first report of multi-step acclimation conferring salt tolerance in leaf-derived calli of *Medicago truncatula*. A pilot study of drought tolerance, mimicked by polyethylene (PEG), demonstrated tolerance to 10% PEG increases in gene expression of *WEE1*, with proline and sugar accumulation.

Chapter 1: General Introduction

1.1. General Introduction

Drought and soil salinity are considered major abiotic stresses for agricultural production in arid and semi-arid regions such as North African countries that lie along the southern coast of the Mediterranean Sea (Munns 2002; Munns et al., 2005). In these harsh environmental conditions, water availability is a major problem that severely limits crop productivity (Dragiiska et al., 1996; Lutts et al., 2004; Tounekti et al., 2011). Hence, most agriculture in these regions depends totally on irrigation from underground water in the coastal strip that runs along this region. This practice has led to increases in sea water level in these coastal areas and consequently has resulted in increased salinity of underground water to a level that has become unsuitable for irrigation for most crops (Zalidis et al., 2002). Ecological constraints are strong limiting factors for agricultural production. The major abiotic stresses, are over- or under- availability of water (drought and flooding), low or high temperature, salinity, toxic metals and soil structure (homogenous, heterogeneous, sandy soil, clay soil) (Barnabas et al., 2008). Generally it is a combination of, or interaction between, these abiotic stresses that strongly affects plant growth, development and finally crop yield.

Plant drought and/or salt resistance are complex traits, depending on the action and interaction of different morphological, physiological and biochemical traits that normally regulate plant growth. For example, a high level of drought or salinity can lead to water deficit which in turn can perturb the plant's physiology with a consequent reduced capacity for carbon assimilation (Abdul-Jaleel et al., 2009). Clearly, this can limit photosynthetic activity or even induce tissue damage.

As shown by Bailey and Mittler (2006), Reactive Oxygen Species (ROS) can affect: growth, development, responses to abiotic stresses such as salinity and drought and can regulate cell death (Zsigmond et al., 2012). For example an accumulation of stress-induced ROS in plastids and mitochondria can result in irreversible cellular and tissue damage (Bailey and Mittler, 2006). Nevertheless, some plants have developed detoxification mechanisms to withstand these harsh environments. Tolerance to a stress such as salinity has occurred genetically through adaptation (heritable morphological traits) or physiologically by exposing plants/plant cells to gradual increases in concentration of the abiotic stress (acclimation) (Farooq et al., 2009). Some well-known responses of plants that are stress-tolerant include an overproduction of many organic solutes (osmoprotectants or osmolytes) such as sucrose, betaines and proline. They help in osmotic adjustment and protection of subcellular structure and metabolism (Silva-Ortega et al., 2008).

About 20% of global agricultural land (Samineni, et al., 2011), comprising about 800 million hectares, is affected by salinity (FAO, 2008). Moreover, this is a worsening problem from the accumulation of the salt over long periods of time in arid and semi-arid zones (Rengasamy, 2002). At the same time, agriculture in North Africa tends to be concentrated on cereal crops like wheat and barley. However, intensive applications of herbicide and "over-farming" have also led to soil erosion (Zalidis et al., 2002). Therefore, looking for alternative forage and grain legumes could be extremely useful because of their important characteristics / traits such as salinity tolerance, soil improvement through fixing atmospheric nitrogen and producing grain with high protein content (Rubio et al., 2002). *Medicago truncatula* is the recognized model species for legumes (Sagan et al., 1995). Therefore, manipulation of *Medicago truncatula* to become tolerant to drought and salinity would be of use for

agricultural research and planning in this region. My general aim was to improve *M. truncatula*`s tolerance to salinity and drought using plant tissue culture techniques.

Tissue culture techniques have been useful to elucidate the cellular mechanisms involved in salt tolerance by using selected NaCl-tolerant cell lines as study systems (Ochatt et al., 1999; Gu et al., 2004; Queiros et al., 2007). Moreover, tissue culture media supplemented with sodium chloride (NaCl) or polyethylene-glycol (PEG) have long been used to regenerate plants that are tolerant to salinity and drought (Bressan et al., 1981, Dragiiska et al., 1996). The first example of tolerance to osmotic stress was demonstrated using tobacco cell cultures (Heyser and Nabors, 1979). Several publications followed that examined abiotic stress *in vitro* (Erickson and Alfinito, 1984; Basu et al., 1997; Ochatt et al., 1999; Tao and Van Staden, 2000; Shi et al., 2002; Merchan et al., 2003; Veatch et al., 2004; Machuka et al., 2008; Siahpoosh et al., 2012; Alet et al., 2012). For example, tissue culture has been used in the selection of salt and drought tolerant cell lines. These lines have been used to regenerate plants resistant to harsh environmental conditions in several crops such as potato, tomato and wheat (Miki et al., 2001; Queiros, 2007). Therefore, plant cells cultured *in vitro* are suitable to separate tolerant clones from non-tolerant populations and to study acclimative mechanisms whereby making it possible to regenerate plants that can withstand more adverse environments than non-acclimatised plants of the same species (Ochatt et al., 1999).

1.2 *Medicago truncatula*

1.2.1 Biodiversity and agronomic traits of *Medicago* species

Generally *Medicago* species evolved from perennial ancestors during the tertiary era (Lesins and Lesins, 1979). They have a high agronomical potential as forage legume crops and excellent tolerance to salinity or drought and can grow in soils under harsh environmental conditions (Lesins and Lesins, 1979). Several varieties of *Medicago truncatula* and *Medicago sativa* (alfalfa) are used commercially and are grown on more than 32 million hectares world wide (Iantcheva et al., 2001; Iantcheva et al., 2005) especially in Australia and France (Prosperi, 1989). From 1985, there has been selection of different ecotypes of *Medicago truncatula* in North African countries. For example, Libya has provided / categorised 21.3% of all natural populations of the genus, *Medicago*, representing the largest stock among all North African countries. In percentage terms, these ecotypes include, 7.2% of *Medicago truncatula*, 52.3% of *Medicago littoralis* and 10.9% of *Medicago italica* (Magalie et al., 2007). In addition, Libyan scientists/breeders have collated 413 out of 5,700 accessions of *Medicago truncatula* worldwide (Magalie et al., 2007). The relative ease with which *M. truncatula* can be studied *in vitro*, and its use as a model legume, encouraged me to choose this plant for my studies of salinity and drought stress.

1.2.2 *Medicago truncatula* as a model plant

Genetic studies of legumes such as alfalfa (*Medicago sativa*), soybean (*Glycine max*) and pea (*Pisum sativum*) are difficult due to their comparatively large genome sizes (Table 1-1). Moreover, two of these species (soybean and alfalfa) are tetraploids (Table 1-1) and

protocols have yet to be developed that would enable efficient transformation and regeneration of these polyploid species. Diploid autogamous legumes such as *Medicago truncatula* and *Lotus japonicus* have emerged as better model legumes (Cook et al., 1997; Handberg and Stougaard, 1992).

Table 1-1. Ploidy level, chromosome number and genome size in various model and crop species.

Plant species	Ploidy	Genome Size (Mbp[1]/1C[2])	References
Arabidopsis thaliana	2n=2x=10	145	Arumuganathan and Earle, 1991.
Glycine max (soybean)	2n =4x=40	1,115	
Lotus japonicus	2n =2x=12	450	Arumuganathan and Earle, 1991;
Medicago sativa (alfalfa)	2n=4x =32	1,350	Bennett and Smith, 1976.
Medicago truncatula	2n=2x =16	450	
Pisum sativum (pea)	2n=2x =14	5,000	

[1] Mbp/1C = megabase pairs per 1C. [2] 1C is defined as the amount of nuclear DNA in the unreplicated haploid genome of a gamete, where n = ploidy level and x = multiples of the haploid ancestral no. of chromosomes per species. E.g. *Medicago sativa* has 32 chromosomes per diploid genome, 16 chromosomes per haploid genome and 8 chromosomes per ancestal genome making this species tetraploid.

Approximately 30 years ago, a group of researchers in INRA-France interested in plant-rhizobium symbiosis began to search for a model system to facilitate genetic studies of this interaction. As Arabidopsis could not be used, the researchers turned to the genus *Medicago* of the family *Leguminosae* (Lesins and Lesins, 1979) and screened a collection of annual *Medicago* species for several characters including genome size and regenerability in

tissue culture. They decided to focus on *Medicago truncatula*, an autogamous species with a relatively small genome size (Table 1-1). In the 1990s, *Medicago truncatula* was chosen as one of two model legumes, the other being *Lotus japonicus* (Rose, 2008). Suitable *Medicago truncatula* genotypes (principally A17 Jemalong [J5], and R-108) were selected, and a series of genetic and genomic resources were developed (Table 1-2). In parallel, the suitability of *Medicago truncatula* was evaluated as a model for numerous pathogen, symbiont and abiotic stress studies (Cook, 1999). Many groups have exploited a large collection of accessions of *Medicago truncatula,* and other annual *Medicago* species, held in Montpellier (France) (http:// www1.montpellier.inra.fr/BRC-MTR/, curator Jean-Marie-Prosperi) and at SARDA Adelaide (Australia) (http://ressources:ciheam.org/om/pdf/c45/00600172.pdf).

Table 1-2. Development of genomics resources for *Medicago truncatula*

Resource	Publication
Mutant Collection	
Gamma-ray mutant collection	Sagan et al. (1995)
EMS mutant collection	Penmetsa et al. (2000)
T-DNA insertion mutants	Scholte et al. (2002)
Tnt1 transposon insertion	Tadegeet al. (2008)
TILLING mutant collection	Javotet al. (2007) Le Signor et al. (2009)
Gene Expression	
Proteome reference maps (suspension cell culture)	Lei et al. (2005)
Proteome references maps (seeds)	Gallardo et al. (2007)
Transcriptomics: Gene expression atlas	Benedito et al. (2008)
Ontogeny of embryogenic callus	Wang et al. (2011)
Genome Sequencing	
A sequence-based genetic map of *Medicago truncatula*.	Choi et al. (2004)
Sequencing the genespaces of *Medicago truncatula* and *Lotus japonicus*	Young et al. (2005)
Three sequenced legume genomes and many crop species: rich opportunities for translational genomics	Cannon et al. (2009)

1.2.3 Advantages of *Medicago truncatula* as a model species

The advantages of *M. truncatula* as a model species can be summarized as follows:

1- Diploid genome (2n=2x=16)

2- Closely related to *Medicago sativa* (2n=4x=32) which is an agronomically important forage legume. Several traits of *Medicago truncatula* make it suitable as a model plant for legume biology and symbiosis research;

3- Small genome size (4.5×10^8 bp. Blondon et al., 1994);

4- Annual and self-fertile growth habits;

5- Short life cycle and rapid seed production;

6- Ease of transformation by *Agrobacterium tumefaciens* and regeneration by somatic embryogenesis (Chabaud et al., 1996; Barker et al., 1990);

7- Symbiosis with the nitrogen-fixing soil bacterium *Rhizobium meliloti.*

Features 3 to 6 are comparable to the most widely used dicot model plant, *Arabidopsis thaliana* (Fig. 1-1). However, the 7th listed feature is distinctive for *Medicago truncatula* as a legume.
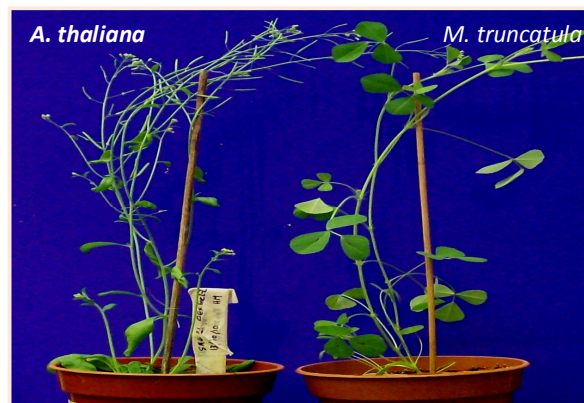
Fig 1-1. Morphological comparison between the model plants, *Arabidopsis thaliana* and *Medicago truncatula*

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1.2.4 *Medicago* as an economically important legume

All perennial *Medicago* (medics) species, including *Medicago truncatula,* originate from the Mediterranean regions. Agronomically they are very important because they show phenotypic plasticity in establishing a perennial habit that is well acclimatised to the various

climatic conditions in North African countries directly facing the Mediterranean. Within the *Medicago* genus, is the capacity to associate with soil bacteria, essentially of the genus *Rhizobium*, forming specialized organs, nodules, at the surface of their roots. The bacteria housed in the nodules reduce atmospheric nitrogen to ammonia which can enable legumes to grow in nitrogen-poor soils (Long, 1989). Understanding the processes which control this symbiosis is an important objective for reducing the amount of fertilizers applied to the soil and to increase forage yield, especially in areas characterized by drought and salinity.

1.3. Sodium chloride (NaCl): a widespread soluble salt responsible for soil salinity

Salinity is typically caused by sodium chloride which accumulates in soil top-layers (Queiros et al., 2007; Feki et al., 2011). Soil is classified as saline when the electrical conductivity (EC) is 1.30 mM/cm^{-1} or more which amounts to 40 mM NaCl, a level of salt that dramatically decreases the productivity of many crops.

Sodium chloride is the most widespread salt in Libyan soils and commonly accumulates to toxic levels. For example, in Libya, of all salts measured in soils of different salinity, NaCl was the predominant one, reaching levels between 55 to 200 mM at 13 different sites (Achuthan et al., 2005). Salt tolerant ecotypes have developed mechanisms to control its accumulation and to select against it in favour of other elements normally present at low levels e.g. potassium (K^+) and nitrate (NO_3^-) (Munns, 2005). Most species are more sensitive to the toxic effects of Na^+ than Cl^-. Thus, most research has focused on sodium exclusion, vacuolar sequestration and control of sodium transport within the plant.

Nevertheless some plants such as soybean and citrus are more sensitive to Cl^- (Storey and Walker, 1999).

1.4 Effects of salinity on physiological and metabolic acclimation to salt stress.

Plants can be classed as glycophytes or halophytes depending on their capacity to grow on salt-contaminated land. Glycophytes are NaCl tolerant up to an external concentration 40-100 mM NaCl. However, halophytes grow in highly saline soils and are able to maintain tolerance at higher levels of salinity than glycophytes. Flowers and Colmes (2008) defined a halophyte having an ability to complete its life cycle in NaCl concentration of at least 200 mM. For instance, the halophyte, *Hordeum marinum* (sea barley grass), can withstand an exogenous concentration of 450 mM NaCl (Garthwaite et al., 2005).

Salinity can cause severe toxicity, since Na^+ is not easily sequestered into vacuoles in non-halophytes. High salt concentrations (e.g. NaCl at 40 mM) can generate an imbalance of native cellular ions causing ion toxicity and osmotic stress (USDA-ARS. 2008). One consequence of this type of perturbation is that it can lead to an increase of reactive oxygen species (ROS) which can prevent photosynthesis (see above) (Hasegawa et al., 2000). Ultimately, this cascade of events can lead to plant death (Hernandez et al., 1999; Abebe et al., 2003). Yet higher concentrations of NaCl (up to 100 mM) can have differential effects depending on the stage of plant development and can also result in differential stresses such as water deficit, ion toxicity and nutrient imbalance (Vinocur and Altman, 2005).

Some plant species have become tolerant to salt stress and exhibit phenotypic changes that enable tolerance (Sairam and Tyagi, 2004). These changes include modifications to lipids that govern membrane permeability (regulating NaCl uptake), and can lead to stabilization of proteins that in turn are able to regulate an increased antioxidant capacity (Vinocur and Altman, 2005; Surjus and Durand, 1996). For example, endogenous lipids, amino acids and organic acid-composition can combine to stabilize membrane biosynthesis. One feature of the development of tolerance at the physiological level is an increase in proline, glycine and betaine content, all of which are indicators of tolerance to salt stress (Trinchant et al., 2004). Therefore, the compatible osmolytes generally found in higher plants are low molecular weight sugars, organic acids and amino acids. For example, there is strong evidence that glycinebetaine (quaternary ammonium compound) and proline (amino acid) play an adaptive role in mediating osmotic adjustment and protecting subcellular structures through exclusion or vacuolar sequestration of Na^+ ions (Feki et al., 2011). Aydi ei al (2010) also reported that, salt stress tolerance of *M. trucatula* was correlated to nodule osmotic adjustment due to both sequestrstion of Na^+ and accumulation of soluble sugar and amino acids, together with an adequate nitrogen metabolism due to relatively high glutamine synthetase activity. In many studies, a positive correlation exists between the accumulation of these two osmolytes (glycinebetaine and proline) and stress tolerance in plants (Yang et al., 2003; Parida and Das, 2005; Feki et al., 2011). In this context, I have examined the extent of proline and sugar accumulation in calli of *M. truncatula* acclimated to various concentrations of NaCl (Chapter 4).

1.5 Genes involved in stress responses.

Responses to abiotic stress factors, such as salinity and drought, involve the plant responding by altering the expression of thousands of genes, so that a range of cellular and physiological possesses will be modified (Cushman and Bohnet, 2000; Sreenivasulu et al., 2004). However little is known about cell cycle genes in plants exposed to high levels NaCl stress. In Arabidopsis, a negative regulator of mitosis, *WEE1,* is strongly expressed in reponse to abiotic stress (De Schutter et al., 2007). This gene has also been studied extensively in the Cardiff lab but not in the context of salinity or drought. *WEE1* together with *CCS52*, a regulator of ploidy levels, were incorporated in my study.

In highly saline conditions (e.g. 100 mM NaCl), tolerant ecotypes exhibit mechanisms that accumulate NaCl in vacuoles or exclude Na^+ (Feki et al., 2011; Zsigmond et al., 2012). In this regard the *SALT OVERLY SENSITIVE (SOS)* gene family is important in maintaining ion homeostasis and in conferring salt tolerance. It was recently reported by Tang et al. (2010) in *Arabidopsis thaliana* that *sos1, sos2 and sos3* mutants were hypersensitive to 60 or 120 mM NaCl. Root growth of all three mutants was highly suppressed compared with 0 mM NaCl controls. *SOS*1 encodes a membrane bound antiporter that is regulated by a SOS2/3 kinase complex (Tang et al., 2010).

Another example of tolerance at the molecular level, is the induction of *P5CS* that encodes Δ^1-pyrroline -5-carboxylate synthetase involved in proline biosynthesis. This gene is highly expressed in salt-and drought-tolerant plant species (Choudhary et al., 2005). Data of Somboonwatthanaku et al. (2010) demonstrated high expression of *OsP5CS* occurring in friable callus of rice (*Oryza sativa* L.) on medium supplemeted with 250 mM NaCl. Twenty four hours following the expression of *OsP5CS,* callus accumulated proline more rapidly in

this salt treatment compared with 0 mM NaCl. The effect of over-expression of these genes and their effect in inducing tolerance tend to be species-specific and, within the plant, seem to be related to a particular stage of development.

Many physiological and molecular studies have been undertaken to try and determine or evaluate the toxic effects of NaCl (Trapp at al., 2008). For example, there is published evidence showing a primary effect of salinity of inducing osmotic and ionic stresses that were confined to root systems (e.g. Tester and Davenport, 2003; Tester and Leigh, 2001; Kim et al., 2010; Alet et al., 2012). Despite numerous papers which document changes in gene expression in response to drought or salinity, there is insufficient knowledge for a complete molecular and cellular understanding of the mechanisms involved either *in vivo* or *in vitro*. In part, I hope to contribute new information to this debate with specific reference to salt tolerance in *Medicago truncatula*. The genes that I examined were chosen as potential markers of salinity at the molecular level (Chapter 6). A more complete survey of the genes *WEE1, CCS52, P5CS*, *SOS1* and *SERK* is provided in the Introduction of chapter 6.

1.5.1 Transcriptomics of gene expression analysis in *Medicago truncatula*.

Medicago truncatula is closely related to other members of the legume family, which include important crops such as pea, faba bean, chickpea, pigeonpea and lentil. The extensive genomic synteny within this group allows for the rapid transfer of knowledge acquired in *Medicago truncatula* to these species. Over two decades, genomic resource development has been increasingly ambitious. Whilst in 1998 a collection of 900 ESTs (transcripts) was reported, in 2008, incorporating genomic sequence information, a 55,000 EST collection was

used to create an Affymetrix chip The resulting transcriptomic data has been gathered in the *Medicago truncatula* Gene Expression Atlas (http://bioinfo.noble.org.gene-atlas/vs/), a public database regrouping expression data for 55,000 genes obtained for more than 50 different conditions. Recently, a study of transcriptional profiling of root for *Medicago truncatula* under 180 mM NaCl using microarray technology (Daofeng et al., 2011) exhibited novel transcription factor gene named *MtCBF4* which belongs to the AP2-EREBP transcription factor family, that novel transcription factor gene (*MtCBF4)* played an important role between primary and secondary metabolism pathways in response to salt stress and is a good candidate gene for genetic improvement to obtain salt tolerant plants (Daofeng et al., 2011). Among legumes *M. trucatula* is found in a wide range of habitats with varying environmental stress, notably soil salinity (Badri et al., 2007).Therefore, a large diversity of genotypes present to explore environmental adaptation mechanisms (Badri et al., 2007; www.noble.org/medicago/ecotypes.html). The several genetic tools obtained for the *Medicago truncatula* model legume (Zahaf et al., 2012) will then serve to identify genes that could improve crops to abiotic stress tolerance.

1.6 Genetic changes *in vitro*

Plant tissue culture is a powerful technique for studying plant development. It can also enable regeneration of whole plants from cells, tissues, or organs cultured under suitable culture media (Vogel, 2005). However, during *in vitro* culture clonally propagated plants can exhibit somaclonal variation (Anjanasree et al., 2012), occurrence of either genetic or epigenetic changes exhibited by somaclones, (Schaffer, 1990). Somaclonal variation is

characterised by changes in ploidy level and DNA sequence (s) together with changes and elements jumping to other chromosomal sites. Epigenetic changes sach as DNA methylation and histone modification also reportedly occur during in *vitro* culture (Kaeppler et al., 2000). Both somaclonal and epigenetic changes can be induced by abiotic factors (e.g. salt, drought and cold) and are very prone to occur *in vitro* and especially over long periods of culture (Anjanasree et al., 2012). Somaclonal variation and epigenetic changes and indeed viral infection are suppressed by using the technique, micropropagation (the culture of shoot apical meristems *in vitro*). Most probably the lack of somaclonal variation is because the shoot apical meristem is genetically stable eventually providing gametes for the next generation (Berdasco et al., 2008). In some species, somaclonal variation has been exploited to develop new varieties (de novo) of important crops (Jain 2001) with desired traits such as disease resistance (Sotirova et al., 1999) and salinity or drought stress tolerance (Lu et al., 2007). For example, Chen et al. (2011) obtained five salt tolerant lines from callus of *Zoysia matrella* L. over six years *in vitro* culture on medium supplemented with 250 and 350 mM NaCl. The presence of transposable elements leading to somaclonal varation can occur in *Medicago truncatula* tissue-cultures (Rakocevic et al., 2009). In this paper, *Medicago RetroElements1-1 (MERE1-1)* are described as insertion elements in the *NSP*2 gene, a low-copy number family in the sequenced *Medicago truncatula* genome. In *Medicago truncatula* this transposable element is only active in tissue culture of the limes, J5 and R108) under salt stress (Rakocevic et al., 2009).

The range of DNA instability *in vitro* depends on many biotic factors: age of cell culture, genotypes, type of explants, choice of hormones used in the culture medium, and abiotic factors such as exogenous salt, cold, or drought etc). However, both hypotheses genetic and/or epigenetic changes are predictable when the plants / cells alter during

acclimation treatments *in vitro*. However, some of changes observed will be caused by acclimation treatments but not adaptation (Farooq et al., 2009). Some of the changes observed *in vitro* culture might be caused by non-inheritable changes (acclimation). These changes are facilitated by re-programming of gene expression patterns responses to the stress such as receptor kinases (Nolan et al., 2009; Nolan et al., 2006), transcription factors (Liu et al., 1999), enzymes and structural proteins that play a pivotal role for modify physiological and metabolism leading to acclimation to abiotic stress (Farooq et al., 2009). However, some of this metabolic adjustment latter might become inheritable epigenetic changes that depends on many abiotic factors (Anjanasree et al., 2012). For example, Ochatt et al. (1999) working with potato cultures identified salt tolerant calli in 20 month-old cultures   on MS (Murashige and Skoog, 1962) medium supplemented by 10.7 µg ml^{-1} NAA, 0.46 µM Kinetin and 120 and 150 mM NaCl. Then optimum concentrations for good callus growth were used of: 120 and 150 mM NaCl.

1.7 Somatic embryogenesis

Somatic embryogenesis is one of the most important prerequisites for genetic manipulation and propagation of plants (Zimmerman, 1993). Essentially it comprises an alteration in the competence of sub-populations of somatic cells to change fate and become somatic embryos in an entirely *in vitro* environment (Williams and Maheswaran, 1986). Somatic embryos produced in this way can serve as reliable models to study embryo development. Clearly, for somatic embryogenesis to be induced from callus, differential gene expression is necessary so that somatic cells are redirected to form embryonic roots and

shoots (Santos et al., 2005; Zimmerman, 1993). Zygotic embryos and somatic embryos are more similar during globular and torpedo stages of embryogenesis whereas, somatic embryos do not have any desiccation stage imposed by dormancy, but rather continue development into fully differentiated plantlets (Fig. 1-2).
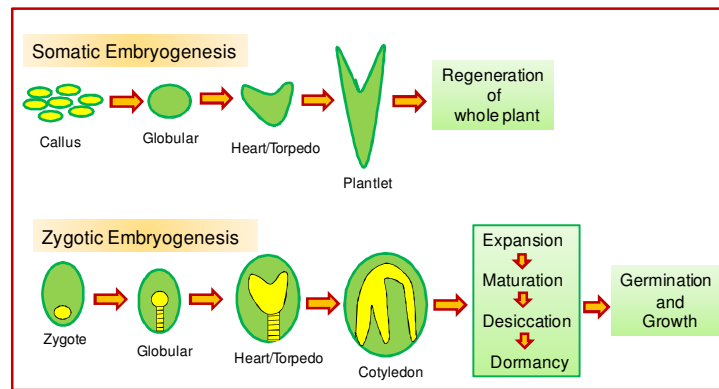
Fig 1-2 Somatic and zygotic embryogenesis stages in plants (adapted from Zimmerman, 1993).

1.7.1 Somatic embryogenesis in *Medicago truncatula*

The first *in vitro* regeneration protocol for *Medicago truncatula* that enabled indirect somatic embryogenesis was developed by Nolan et al. (1989). Subsequently, many protocols for inducing indirect somatic embryogenesis for this species have followed (Chabaud et al., 1996; Hoffmann et al., 1997; Trinh et al., 1998; Wang et al., 2011). More recently, direct somatic embryogenesis in *Medicago truncatula* has been achieved in both liquid and solid media (Iantcheva et al., 1999; Iantcheva et al., 2001; Iantcheva et al., 2005). The type of explant that is used is an important factor for successful somatic embryogenesis. Indirect somatic embryogenesis of *Medicago truncatula* has been generated widely from explants

such as leaves hypocotyls, cotyledons, petioles and flower parts (Nolan et al., 1989; Chabaud et al., 1996; Trinh et al., 1998). Direct somatic embryogenesis in *M. truncatula* is mainly induced from root material (Duque et al., 2006).

1.7.2 Hormonal composition of media leading to somatic embryogenesis in *Medicago truncatula.*

In *Medicago truncatula*, the induction and maintenance of somatic embryogenesis is achieved mainly on media supplemented with combinations of auxins (2,4-D, dichlorophenoxyacetic acid), NAA, (α-naphthalenacetic acid) or Picloram and cytokinins (BAP, 6-benzylaminopurine or kinetin) (Nolan et al 1989; Trinh et al., 1998; Pintos et al., 2002; Elmagrabi and Ochatt, 2006). There are many combinations of hormones that have been used by different researchers for embryo induction and development as summarized in Table 1-3. In general, the synthetic auxin, 2,4-D is well known to generate callus for indirect embryogenesis in legumes, particularly in the *Medicago* genus (Trinh et al., 1998; Zafar et al., 1995; Denchev et al., 1991). Also the concentration of the auxins , 2,4-D or NAA, play an essential role in the outcome of *in vitro* culture (Vergana et al., 1990). For example, 2,4-D at a concentration of 4 mg/L can induce a high frequency of direct somatic embryo formation in cell suspension liquid media while at 11 mg/l, it induces somatic embryogenesis but with a lower frequency of embryo formation; 40 mg/l blocks embryo induction in *M. truncatula*. The auxin, NAA, is also very important for somatic embryogenesis initiation for some species such as *M. truncatula* (Nolan et al., 1989) and *M. polymorpha* (Scarpa et al., 1993). Production of callus tissue followed by embryo formation has been achieved in *Medicago truncatula* on solid medium supplemented with the cytokinin, BAP (6-benzylaminopurine) (Trinh et al., 1998). However, induction of somatic embryogenesis by a single cytokinin is

relatively rare with legumes; different cytokinins (e.g. BAP, Kinetin, Thidiazuron) are usually required (Chabaud et al., 2003). The molecular process activated in the induction and development of embryogenesis are still poorly understood. However, recently, a gene from *M. truncatula*, *SOMATIC EMBRYOGENESIS RECEPTOR, KINASE* (*MtSERK1*) has been cloned and its expression evaluated on defined culture media. (Nolan et al., 2003; Wang et al., 2011). Its expression is strongly linked to somatic embryogenesis. *MtSERK1* was therefore incorporated into my study.

In *Medicago truncatula* a lowering, or complete removal, of auxin is essential for embryo development and maturation beyond the globular stage (Trinh et al., 1998; Iantcheva et al., 2001; Iantcheva, 2005). However moderate reductions in cytokinin concentrations (e.g. from 0.5 mg/l to 0.02 mg/l) are also essential for the normal progress of embryogenesis *in vitro* (Chabaud et al., 1996; Iantcheva et al., 2001). Removal of hormones from the culture medium (auxin and cytokinin) is often the only way to enable the *in vitro* embryo to develop into a robust plantlet. Moreover, the stage of embryo maturation at which auxins are removed is critical for subsequent development of an embryo into a vigorous plantlet. Other changes in the composition of the medium can also have a dramatic effect on embryogenesis *in vitro*. For example, a combination of growth factors, in the form of casein hydrolysate or yeast extract or in combination with cytokinin led to a high rate of embryo formation (Chabaud et al., 1996).

Table 1-3. Different combinations of plant growth hormones added to media of different composition that have been used to induce embryogenesis in *Medicago truncatula* : (MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), SH (Schenk and Hidebrand,1972)

Initial Name	Basal Media	Embryo induction	References
B5EI	B5 Gamborg et al., 1968	1mg/L 2,4D 0.2mg/Ll Kinetin 1mg/L adenine 500mg/L casein hydrolyte 500mg/L myo-insosital 3% sucrose 7g/L agar	Iantcheve et al., 2005.
EID	MS Murashige and Skoog 1962	1mg/L 2,4D 0.2mg/L BAP 1g/L casein hydrolyte 3% sucrose 7g/L agar	Iantcheve et al., 2005a.
B5,4	B5	4mg/L 2,4D 0.2mg/ L Kinetin 1mg/L adenine 500mg/L casein hydrolyte 3% sucrose 7g/L agar	Iantcheve et al., 2001.
MS4	MS	4mg/L 2,4D +0.2mg/LBAP 3% sucrose 7g/L agar	Svetoslavova et al., 2005.
PCI-4	SH Schenk and Hidebrard 1972	4mg/L 2,4D +0.2mg/LBAP 3% sucrose 7g/L agar	Hoffmann et al., 1997.
R2B5	B5	5.71µM IAA 23.23 µM Kinetin 1g/L casein hydrolyte 3% sucrose 7g/L agar	Basu et al., 1997
MANA	MS	2mg/L NAA 0.5 mg/L BAP 3% sucrose 7g/L agar	Ochatt et al., 2000
MPIC	MS	0.5 mg/L Kinetin +0.2mg/L Picloram 3% sucrose 7g/L agar	Ochatt et al., 2000

1.8 Limitations of use of *in vitro* methods in model legumes such as *Medicago truncatula.*

Generally contamination is considered the biggest problem in maintaining cells in tissue culture. Therefore aseptic technique is vital as is developing protocols that lead to plantlet regeneration *in vitro*. In the long term, plantlets may have to acclimatised. This might also be necessary when plantlets are transferred from *in vitro* to *in vivo* conditions. For example Chen et al (2011) found that callus regeneration to plants decreased sharply with the age of calli which means the older the callus the less is regenerative capacity. Another limitation of *Medicago truncatula in vitro* culture is that *Rhizobium* interaction with the roots is an important symbiosis for N_2 fixation that leads to growth of the plant under harsh environments (salt, drought, lack fertility, etc) (Mhadhbi and Aouani, 2008). However, rhizobium interaction is absent in *in vitro* cultures. Thus data which were obtained from *in vitro* studies may not translate to performance in the field when under abotic stress.

1.9 Experimental Aims

My review of the literature has demonstrated progress made in understanding the effects of abiotic stress at various levels. Indeed, there has been progress in using tissue culture as a tool to generate cells and plantlets *in vitro* that are tolerant to toxic levels of NaCl. However, my review also demonstrates many aspects of acclimation to NaCl and associated changes at the molecular level that remain poorly understood particularly in relation to legume crops grown in Libya. The efficient exploitation of biotechnology-derived plants for breeding for abiotic stress in legume species encompasses the need for reliable identification of all regenerants obtained from different explants on different media *in vitro* and, in particular, their characterization as compared to the control or mother plant. This is required to check on any deviation from the original phenotype. Hence, using *Medicago truncatula* as a model, the aims of the work reported in this thesis, were:

1. Evaluate various explants (leaf, hypocotyl and cotyledon) of *Medicago truncatula* (A17) for somatic embryo induction on four different culture media that differ in the composition of added plant growth regulators.

2. Use this information to optimise somatic embryogenesis.

3. Study the effects of different concentrations of NaCl and 10 w/v PEG on cultured explants of *M. truncatula*. In particular, the response of cultured cells to multiple courses and long term exposure to a range of NaCl concentrations (50-350 mM) or to regular step-up concentrations (50 though to 350 mM). Note that the concentration range was set to span either side of the "average molarity" of sea water (150 mM).

4. Define biochemical and cytological markers of acclimation to salt and drought stress.

5. Study the expression of genes related to somatic embryogenesis (*SERK1*) and abiotic stress (*SOS* and *P5CS*), cell cycle checkpoint gene (*WEE1*), and the regulator of ploidy level, (*CCS52*) in the treatments outlined above.

Chapter 2: General Materials and Methods

2.1   *In vitro* culture

2.1.1   Plant material

Medicago truncatula (2n=2x=16, 1C value = 0.48 pg) cv. Jemalong line J5 (A17) (provided by S. Ochatt, INRA Dijon, France) was used as the source of material for these studies this genotype is mycorhizogenic (Myc$^+$) and nodulating (Nod$^+$) (Sagan et al., 1995).

2.1.2   Surface sterilisation of seeds and *in vitro* culture

Dry seeds were immersed in sulphuric acid (14M H_2SO_4) (6 min) to remove the seed coat and were surface-sterilised in 70% (v/v) ethanol (1 min) sodium hypochlorite (10%) for 5 min, followed by at least four rinses in sterile deionised water. They were then cultured in hormone-free MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and adjusted to pH 5.8 before addition of 0.9% agar. The medium was autoclaved for 20 min at 121ºC /1bar. For germination, seeds were placed at 24/22 ºC with a 16h/8 h light/dark cycle at 90 $\mu E/m^2s^{-1}$ from warm white fluorescent tubes, as reported elsewhere (Elmaghrabi and Ochatt, 2006).

2.1.3 Phases of salt acclimation protocol

As shown in Figure 2-1, 400 calli (8 calli per dish) were grown on MANA medium supplemented with different concentrations of NaCl in three phases (I, II, III). Phase I (salt step-up): 80 calli of 50 mM NaCl (6 months), 80 calli of 100 mM NaCl (5 months), 80 calli of 150 mM NaCl (4 months), 40 calli of 250 and 40 calli of 350 mM NaCl (3 months). At the same time, another set of calli was maintained on 50 mM NaCl for 6 months. In Phase II, calli were then placed on MANA medium without NaCl for two months to enable callus growth in normal conditions. All calli were then grown for four months on the same NaCl concentration as they were during Phase I. Phase III, (is an exact repeat of Phase II) (Fig. 2-1). For example in an attempt to acclimate at 350 mM NaCl, calli were initially cultured on 0 mM NaCl (5 months). Then: 50 (1 month), 100 (1 month), 150 (1 month) then 350 (3 months) mM NaCl, followed by 0 mM (2 months), 350 mM (4 months) followed by 0 (2 months) and finally 350 (4 months) NaCl (Fig. 2-1). Note age of callus is from the start of the protocol (Table 2-1)

Table 2-1. Age of callus from start of the protocol.

Phases	Time (months)
Pretreatment	5
Phase I	11
Phase II	17
Phase III	23

Fig. 2-1 400 calli (8 calli per dish) were induced during a 5 month pretreatment on MANA medium. Following this treatment, non-embryogenesis calli were removed.

Phase I. 320 calli (8 calli per dish) were either stepped up to 50 mM NaCl or 80 calli remained in 0 mM NaCl. One month later, 240 calli were either stepped up to 100 mM NaCl or 80 calli remained on 50 mM for 6 months. One month later, 160 calli were either stepped up to 150 mM, or 80 calli remained on 100 mM for 5 months. One month following transfer to 150 mM, 40 calli were either stepped up to 250 or 40 calli to 350 mM, or 80 calli remained on 150 mM NaCl for 4 months. Following transfer to 250 or 350 mM calli remained on these concentrations of NaCl for 3 months.

Phase II. Following Phase I, all calli were subcultured onto MANA medium minus NaCl for two months. This was followed by sub culture to MANA supplemented with same concentration of NaCl used during acclimation.

Phase III. A repeat of Phase II. In parallel, calli remained on 0 mM NaCl, for a total period of 23 months.

2.1.4 Embryogenic or, organogenic calli treated with PEG.

Leaf-derived mature callus was obtained after culture on MANA medium for five months. During that time, the callus was observed by light microscopy for signs of embryogenic, and/or organogenic or non-regenerative patterns. Then, either embryogenic or organogenic calli were transferred to Petri dishes (10 cm diameter x 2 cm) with 25 ml MANA medium supplemented with 10% w/v 6000 Polyethylene glycol (PEG) (Biswas et al., 2002) for six months. Thirty two replicates were apportioned to eight Petri dishes. All of these calli were subcultured monthly on the same medium with 10% w/v PEG treatments and were maintained in an incubator with the same conditions used for seed germination and callus induction. Callus growth was estimated as increase in fresh weight per unit time (FW2 – FW1 divided by time (month)), where, FW1 is the fresh weight of the callus at the beginning and FW2 is the fresh weight of the callus at the end of the test period.

2.1.5 Cell suspension establishment under salinity stress

For initiation of cell suspension cultures, after 5 months of embryogenic callus induction on MANA medium pieces of leaf callus of at least 0.5g fresh weight, were transferred into 250 ml Erlenmeyer flasks with 100 ml BY-2 tobacco liquid medium (Nagata et al., 1992) which is a modified MS medium enriched with phosphorus and supplemented with 0.2 mg/l^{-1} 2,4 D, 1 mg/l^{-1} thiamine HCl, 100 mg/l^{-1} myoinositol and 200 mg/l^{-1} KH$_2$PO$_4$. The cultures were shaken at 130 rpm and maintained under conditions as stated in Section 2.1.2 Cell suspensions were sub cultured every 14 days. After four subcultures, when suspension cells were of sufficient quantity (turbidity), cells were transferred into the same medium

supplemented with varying concentrations of NaCl (0, 50, 100, 150, 250 or 350 mM) for four months and maintained as above with subculture occurring every two weeks.

2.2   Microscopy and imaging

The viability of the cell suspension cultures was tested regularly by Hoechst staining (1µL of a 10 mg ml^{-1} stock of Bisbenzimide H, 2µL Triton x-100 and 97mL sterile distilled H$_2$O), and images captured using an Olympus BH-2 compound microscope equipped with UV epi-fluorescence. Following the first through to the fourth month of culture under 0-350 mM NaCl, cell and nuclear size were measured using Sigmascan– Pro, (objective: DPlan Apo 20 UV, 0.70, 160/0.17).

2.3 Statistical analysis

Data from all experiments was analyzed using Minitab software. Statistically significant differences between treatments were assessed using one-way ANOVA with Tukey`s multiple comparison test at $P \leq 0.05$ level of significance.

Chapter 3: A new *in vitro* acclimation protocol that led to increased callus growth and continued maintenance of embryogenic potential in *Medicago truncatula*: evidence of tolerance at 150 mM NaCl

3. 1. Introduction

Plant tissue and cells, if cultured appropriately, can become morphogenetic and, again under ideal conditions, can be cloned with relative ease. In some, but by no means all species, explants from their tissues/organs can be induced to make calli (masses of proliferative cells that lack morphogenetic potential). Induction of callus is normally obtained from explants including: embryos, leaves, cotyledons, or roots. However there is an enormous and complex literature that reports on the highly species-specific media used to induce plantlet formation *in vitro* (Ochatt et al., 1999; Elmaghrabi and Ochatt, 2006). Callus can respond to exogenous hormones in a number of ways. For example, callus can be made to be embryogenic and will produce very large numbers of cloned embryos. In such cases, seeds or dissected embryos are the source of callus induction. In some cereals, explanted embryos are grown on a medium supplemented with the synthetic auxin, 2, 4 dichlorophenoxyacetic acid (2, 4-D) and after about 2-3 months the callus is tested for its embryogenic potential by subculturing the callus onto the same medium but removing 2, 4-D (Chai et al., 2011). Subsequently, embryogenic callus will begin to form embryos on the surface of the callus. This protocol often leads to somatic embryogenesis, or more correctly, indirect somatic embryogenesis. In some cases, embryos will form directly on the explant in the absence of callus formation. This is known as

direct embryogenesis, a popular choice for cloning embryos in many cereals and grasses (Santos et al., 2005; Wang et al., 2011).

In vitro cultures are very useful in basic biochemical, molecular and physiological studies (Deb and Imchen, 2010; Siahpoosh et al., 2012). For example, tissue cultures have been used to study the factors involved in the establishment of calli of rice that are salt tolerant (Miki et al., 2001). However the genetic basis of tolerance via cell and tissue culture protocols is not well understood.

The aim of the work reported in this chapter, was to evaluate various explants of *Medicago truncatula* (A17) for their competence for NaCl tolerance and somatic embryo induction. I assessed various organ explants grown on four different culture media that enable me to:

- optimize the best explant
- optimise the medium for the best explant
- examine growth of calli on a CONTINUOUS and specific concentration of salt , or, to test, at each sub-culture, the effectiveness of STEP UPs to a more concentrated NaCl in the range: 0 to 350 mM
- assess embryo induction

3.2. Materials and Methods

3.2.1 Callus growth assessment

Callus growth was monthly measured of each phase (I, II and III) during full protocol of acclimation. Data were obtained tested for exponential growth by assessing whether log transformation of y axis coordinates could be linearised. Such exponentially growing calli were then measured in terms of relative growth rates in grams per gram per month (RGR g g^{-1} month^{-1}):

$$\frac{\ln B - \ln A}{t}$$

where A and B are callus fresh weight at two sampling times within the exponential phase and t is time (months).

Data conforming to linear growth, were analyzed by regressions and growth (g month^{-1}) determined according to the gradient of the straight line (y = mx+c). Statistically significant regressions were then used to calculate growth (g m^{-1}). Where growth data conformed neither to exponential nor linear growth, absolute callus growth per month was estimated as:

$$\frac{(FW2 - FW1)}{t}$$

Where, FW1 is the fresh weight of the callus at the beginning, FW2 is the fresh weight of callus at the end of treatment and t is the interval spanning FW1 to FW2 (months). Montlhy growth can be ascertained by (FW2 – FW1) ÷1.

3.2.2   Proliferation of calli

To examine the effect of different combinations of exogenous hormones and choice of explant material of *Medicago truncatula*, aseptically grown 6 week old plants were used. Hypocotyls, cotyledons or green leaves were explanted to tissue culture. Leaves that were too old or insufficiently developed, were discarded (Fig. 3-1). Each leaflet and cotyledon was cut with a scalpel to ensure contact between the medium and tissues. To test for regeneration, all explants (leaf, cotyledon and hypocotyls) were cultured with four different media MANA, MPIC, EID and MS4 as detailed in Table 3-1, in multi-well dishes consisting of 5 X 5 replicates with 2 ml of medium per well. All experiments were repeated twice, and included a minimum of 10 replicates per medium. Dishes were kept at 24/22 ºC, with a 16/8 h (light/dark) photoperiod of 90 μE/m^{-2}s^{-1} from warm white fluorescent tubes. After four weeks, explants were sub-cultured on the four different media and the frequency of callus initiation assessed.

Table 3-1. Media based on MS (Murashige and Skoog, 1962). Key: MANA= France Nam medium MS+NAA (Ochatt et al., 2000), EID= Embryo induction development, MS4= MS + 4 mg/L 2,4D, MPIC= France Nam medium MS+Picloram (Ochatt et al., 2000).

	MANA	EID	MS4	MPIC
Growth Regulators (mg/L)	0.5 BAP 2.0 NAA	0.2 BAP 1.0 2,4D 1000 Casein hydrolysate	0.2 BAP 4.0 2,4D	0.5 Kinetin 0.2 Picloram
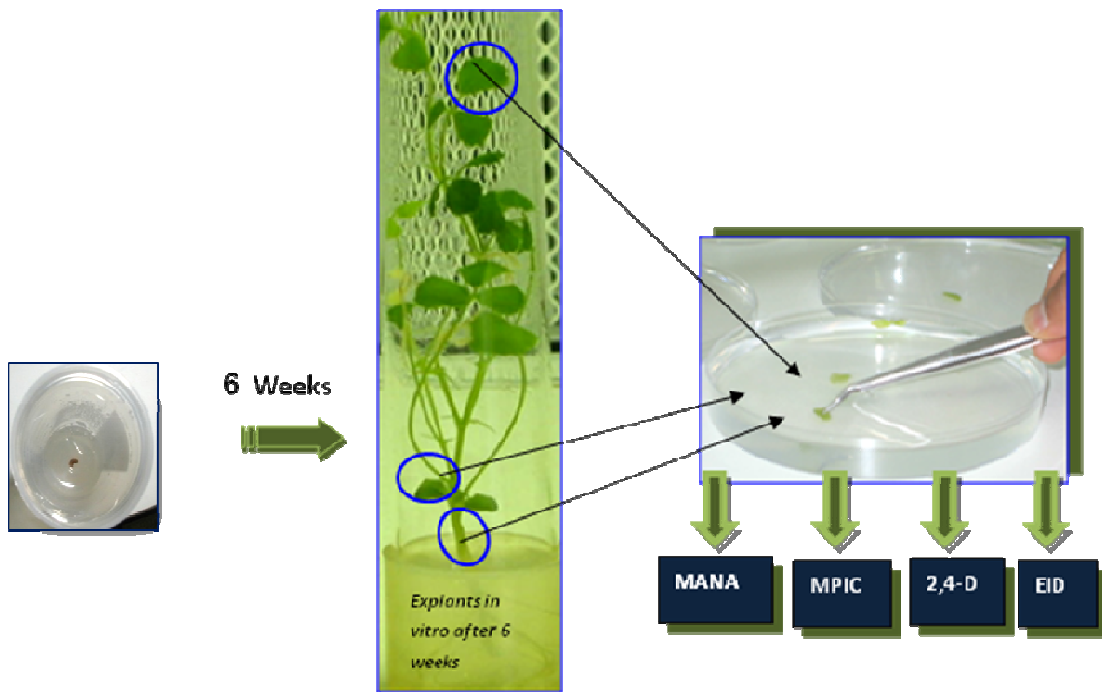
Fig 3-1. Seeds were germinated aseptically on MS medium, then after 6 weeks were sub-cultured, using three different explants on four different media

3.2.3 The effectiveness of acclimation on somatic embryogenesis

3. 2.3.1 Preliminary protocol.

Leaf-derived mature callus was obtained after culture on MANA for five months. During that time, the callus was observed by light microscopy for signs of embryogenesis and organogenesis (embryogenic and/or organogenic). Eight samples of calli were transferred to Petri dishes (10 cm diameter x 2 cm) with 25 ml MANA medium supplemented with 50 mM

NaCl for one month. A step-wise protocol of increasing NaCl concentrations was then devised. Leaf-derived callus was grown on MANA (0 mM NaCl) for 5 months. Then at monthly intervals, callus was sub-cultured from 0 to 50, 50 to 100, 100 to 150 mM, or, 150 to 250, or 150 to 350 mM NaCl enabling an assessment of growth in this step up method compared with callus that remained continuously on 50 (4 months) 100 (3 months) 150 (2 months) or 250 or 350 mM NaCl (1 month;) (Fig. 3-2).
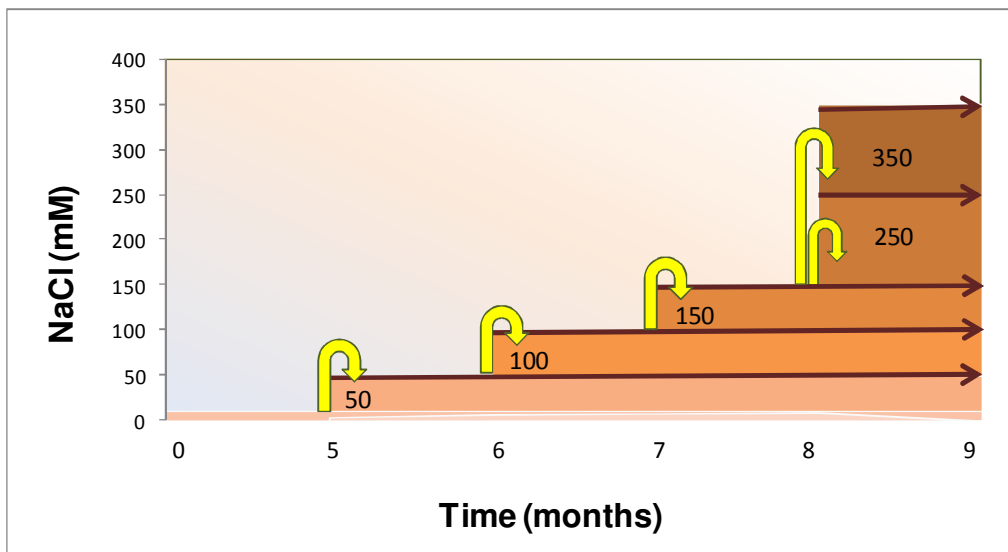
Fig 3-2. Preliminary protocol for callus acclimation to salt stress. Five month old leaf-derived callus growing on 0 mM NaCl was subcultured to 50 mM for one month and then 100 mM for one month, 150 mM for one month and then either to 250 or to 350 mM NaCl for one month (see curled arrows). At the same time other cultures were left on 50 mM for four months, 100 mM for three months or 150 mM for two months (horizontal arrows).

3. 2.3.2 Full Protocol (Phases I, II and III)

The full protocol of acclimation under NaCl stress consisted of 23 months total period *in vitro* culture including 18 months comprising three phases (I, II and III) under various concentrations of NaCl (Fig 3-3). This was expained in Fig. 2-1 and is repeated here for clarity.

Fig. 3-3 400 calli (8 calli per dish) were induced during a 5 month pretreatment on MANA medium. Following this treatment, non-embryogenesis calli were removed.

Phase I. 320 calli (8 calli per dish) were either stepped up to 50 mM NaCl or 80 calli remained in 0 mM NaCl. One month later, 240 calli were either stepped up to 100 mM NaCl or 80 calli remained on 50 mM for 6 months. One month later, 160 calli were either stepped up to 150 mM, or 80 calli remained on 100 mM for 5 months. One month following transfer to 150 mM, 40 calli were either stepped up to 250 or 40 calli to 350 mM, or 80 calli remained on 150 mM NaCl for 4 months. Following transfer to 250 or 350 mM calli remained on these concentrations of NaCl for 3 months.

Phase II. Following Phase I, all calli were subcultured onto MANA medium minus NaCl for two months. This was followed by sub culture to MANA supplemented with same concentration of NaCl used during acclimation.

Phase III. A repeat of Phase II. In parallel, calli remained on 0 mM NaCl, for a total period of 23 months.

3.2.4 Testing morphogenetic potential

Following the completion of Phase III, 12 replicates of calli (4 per petri dish) of each concentration (0, 50, 100 and 150 mM NaCl) were sub-cultured to EDM (embryo development medium) supplemented with the the same [NaCl] concentration used in Phase III, for one month. Then, the calli were sub-cultured to ECR (embryo conversion and rooting medium) supplemented with the same NaCl concentration used previously, for two months (Fig 3-4). Calli were then examined via a dissecting microscope to determine the extent of embryo and organ formation at each concentration of NaCl.

Minus NaCl	Continuous NaCl	EDM MS (Murashige and Skoog 1962) + 0.05 mg l^{-1} BAP + 250 mg l^{-1} casein hydrolysate + 3% sucrose and 7% agar (Iantcheva et al., 2005a)	ECR MS (Murashige and Skoog 1962) + 0.1 mg l^{-1} NAA + 250 mg l^{-1} casein hydrolysate + 1% sucrose and 7% agar (Iantcheva et al., 2005a)
0 →	150 →	150 →	150 →
0 →	100 →	100 →	100 →
0 →	50 →	50 →	50 →
0 →	0 →	0 →	0 →
(2M)	(4M)	(1M)	(2M)
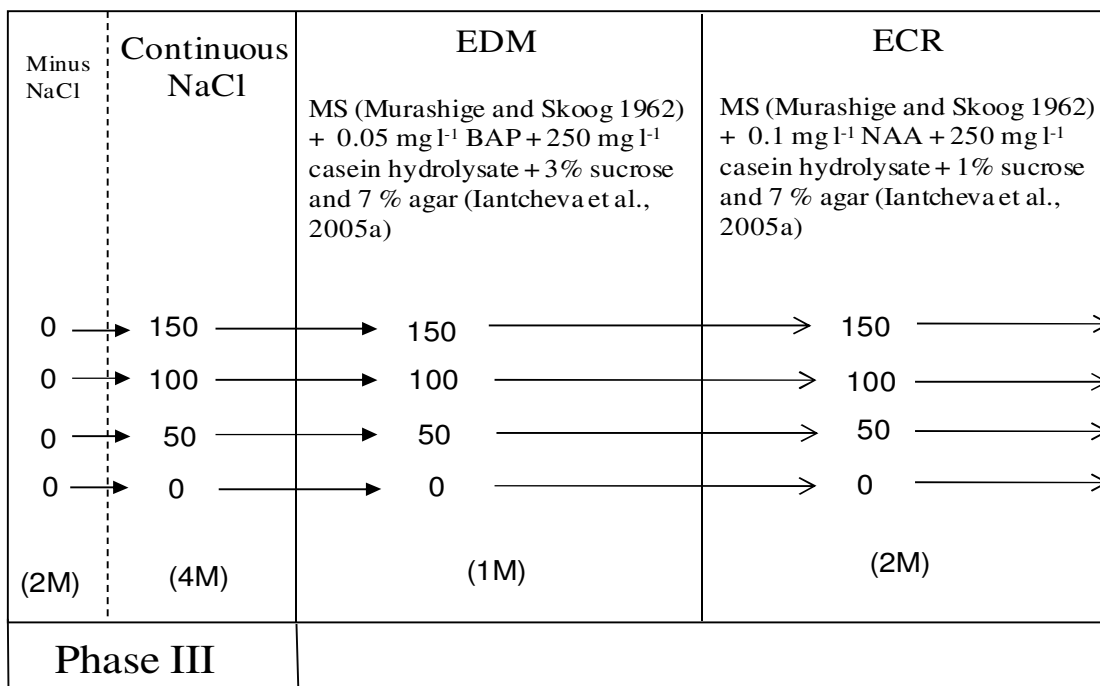
Phase III

Fig 3-4. Protocol for testing for embryogenesis / organogenesis. Following Phase III, calli were subcultured continuously on EDM for one month, followed by ECR for two months, supplemented with 0, 50, 100 or 150 mM NaCl. Following the start of the protocol, calli were 24 and 26 months old at the end of EDM and ECR, respectively.

3.3 Results

3. 3.1 Assessment of explants on different media.

Unless otherwise stated, plotted data were anlaysed between treatments while the same data in tabulated format were used to demonstrate within treatment differences. For eight week old callus, MANA was optimal for leaf and cotyledon-derived callus (Fig. 3-5) while the percentage callus induction was slightly less on EID and MS4 medium, although not to a significant extent (Fig. 3-5). Note that leaf- and cotyledon-derived callus induction dropped significantly on MPIC medium compared with the other three media (MANA, EID and MS4) (Fig. 3-5, Table. 3-2 $P \leq 0.05$). Conversely, with hypocotyls, the highest percentage initiation of callus was observed on MS4 medium, while EID and MPIC yielded slightly lower (though not significantly) percentages but callus induction decreased significantly on MANA medium (Fig. 3-5). When the same data are plotted as a function of the explant response on the different culture media, a highly significant reduction in callus formation from hypocotyl explants was observed (Fig. 3-5, Table. 3-2).

Table 3-2. The mean percentage ± SE of explants that initiated callus on the various culture media (n = 8).

Explants	MANA	EID	MS4	MPIC
Leaves	96 ± 4.7^d	85.3 ± 9.9^d	81.5 ± 9.8^b	60.8 ± 9.5^c
Cotyledons	77.7 ± 6.7^d	71.8 ± 5.5^d	69.8 ± 5.1^b	51.8 ± 5.1^c
Hypocotyls	10.8 ± 1.2^a	18.7 ± 3.8^a	25.5 ± 8.5^a	17.5 ± 3.8^a

Means (±SE) are given a super-scripted letter [a] through to [d] within that column. Those given the same letter were not significantly different within medium treatment but different letters denote statistically significant within medium treatment differences ($P \leq 0.05$).
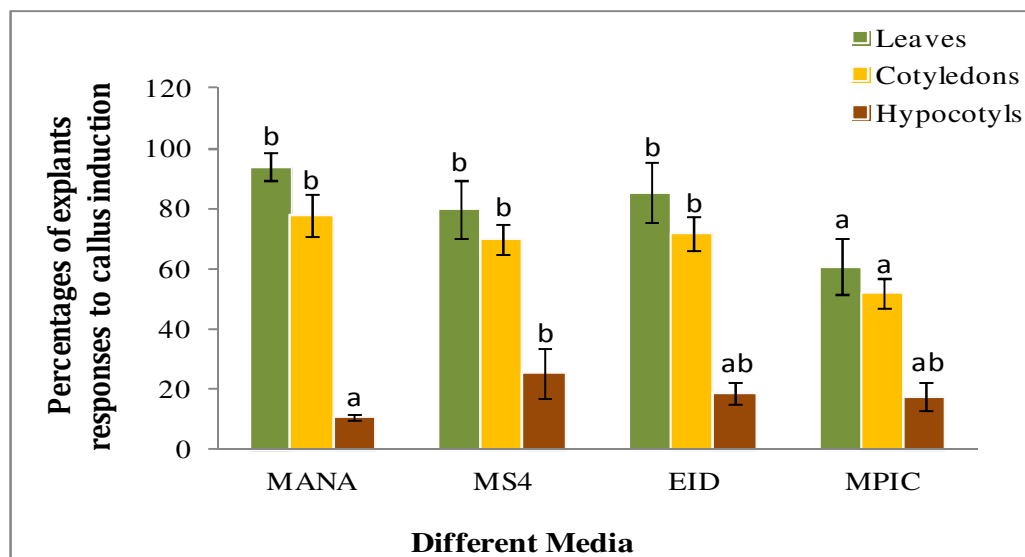
Fig 3-5. A composite of responses of the organ explants on the various media. Mean percentage induction (± S.E.) of callus derived from leaves, cotyledons and hypocotyls, grown on MANA, MS4, EID or MPIC for eight weeks. Note, different letters on the bars of the same colour indicate significant differences between treatments ($p \leq 0.05$). (n=8).

In summary, the frequency of callus formation, in descending order of responsiveness, was: leaves, cotyledons and finally, hypocotyls. In general, leaf-derived calli were more homogenous and grew faster than cotyledonary ones (Fig. 3-7). In the screening of different media, and in optimizing the ideal source of explant, leaf explants gave the best regenerative responses and, in general, MANA was the best for callus-induction of leaf explants. Leaf derived calli on MANA were used for the remainder of my study unless otherwise indicated.

Fig 3-7. Callus profile from various explants on different media after eight weeks.

3.3.2 Morphogenetic potential during pretreatment

Five months following the start of leaf culture on MANA solid medium, morphological differences in the patterns of callus development could be distinguished in terms of organogenic and/ or embryogenic potential, Differential patterns of callus responses were observed (Fig. 3-8), varying from embryogenic to non-regenerative callus, in line with results observed previously with this species (Elmaghrabi and Ochatt, 2006).
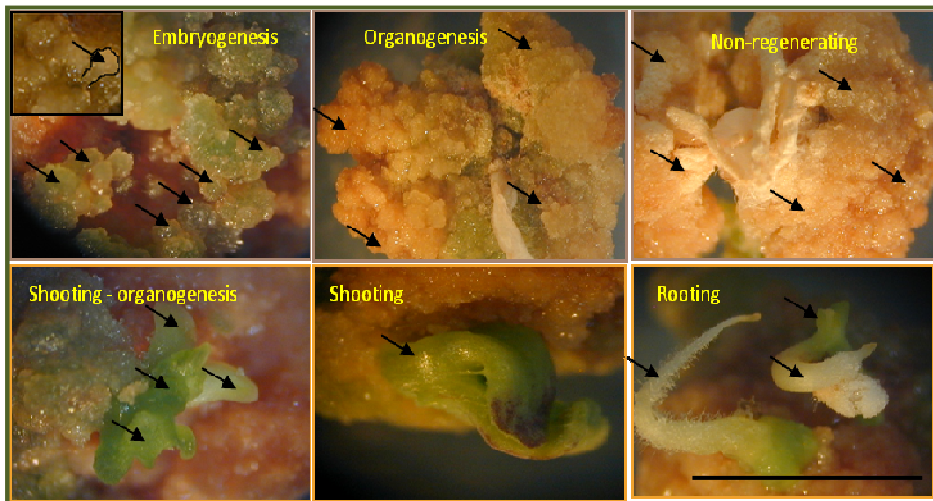
Fig 3-8. Morphogenetic responses after five months of embryogenic induction on MANA medium. Bar = 10mm.

3.3.3 Initial salt stress treatment.

Mature embryogenic and/or organogenic calli were obtained from leaf explants after five months on MANA medium and were then sub-cultured to medium supplemented with different concentrations of sodium chloride (0, 50, 100 or 150 mM). The fresh weight of salt-

stressed and salt tolerant calli was determined monthly for a three-month period to provide a measurement of callus growth and an indication of the extent of salt tolerance. After three months of culture, callus growth on 50 mM NaCl was not significantly different from the calli that were maintained on medium minus NaCl ($P \leq 0.05$) (Fig. 3-9). Hence, over the three months of culture, calli were growing at comparable rates ± 50 mM NaCl, which is consistent with calli beginning to acquire salt tolerance at this concentration.
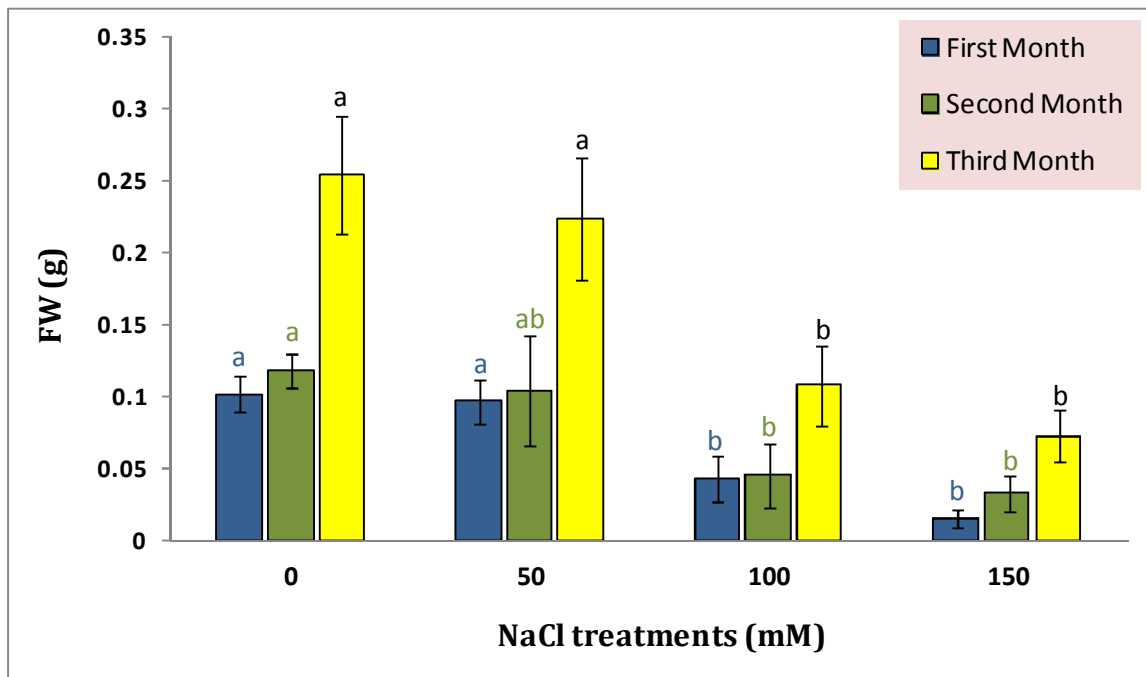
Fig 3-9. Composite mean (± S.E.) callus growth during a three-month continuous exposure to varying concentrations of NaCl. Callus FW was recorded monthly. Note that above each bar, the lower case letters of the same colour indicate significant between treatment differences ($P \leq 0.05$) (n= 12 replicates).

However, over the same period, callus growth was significantly impaired at ≥ 100 mM NaCl compared with the control. Within treatment, callus growth was significantly higher during

the third month compared with the first and second months for all NaCl concentrations (Table 3-3).

Table 3-3. Comparison within treatments of means (± S.E.) callus growth (g/month) between three-month continuous exposures to varying concentrations of NaCl.

Treatments mM NaCl	First month	Second month	Third month
0	0.101^a	0.117^a	0.253^c
50	0.096^a	0.104^a	0.222^b
100	0.043^a	0.045^a	0.110^b
150	0.016^a	0.033^a	0.073^b

Means were given a super-scripted letter [a] through to [c] within the rows (within treatments) those given the same letter were not significantly different from each other whilst different letters show significant differences within treatment at $P \leq 0.05$ (n= 12 replicates).

Green callus continued to be observed at each NaCl concentration, consistent with growth, despite the increase in salinity up to 100 mM (Fig. 3-10). However, at 250 and 350 mM, the callus turned brown suggesting increased cell death at these very high salt levels (images not shown). Between treatments, exposure to 100 and 150 mM NaCl, growth was significantly less compared with the zero control over the three months indicating a relative lack of tolerance at 100 and 150 mM (Fig. 3-9). Embryogenic calli obtained on 50 mM NaCl were characterised by the appearance of small shoots during the last month of this study whereas they did not develop in the control (0 mM NaCl) (Fig. 3-10).
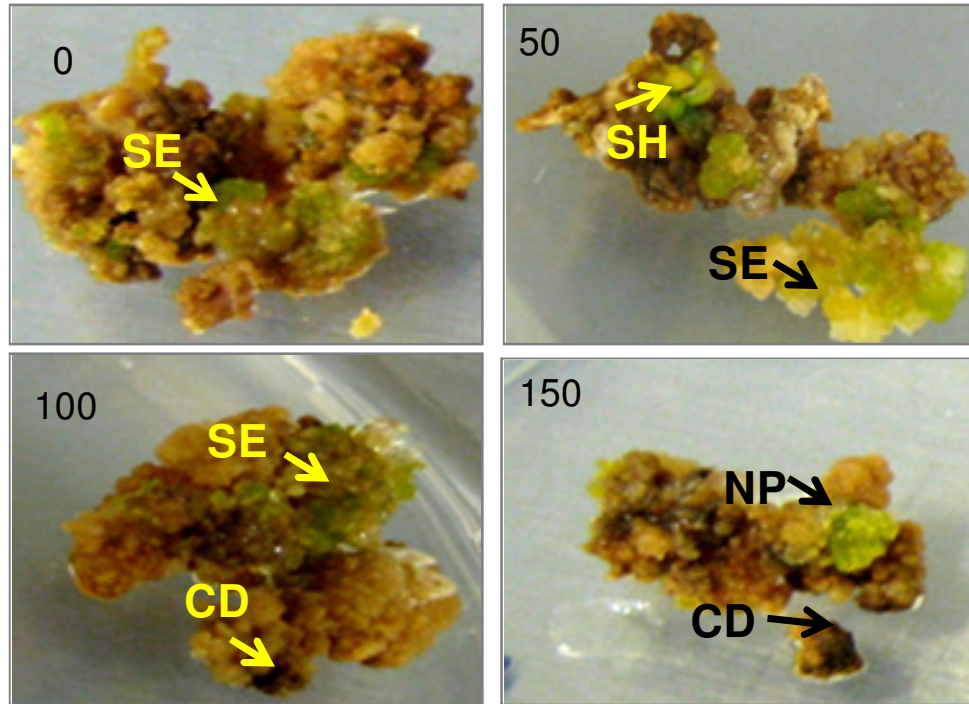
Fig. 3-10 Morphogenetic responses at various NaCl concentrations. SE=somatic embryogenesis SH= shoot formation, NP= new proliferation; CD= probable cell death

In addition to a continuous culture period of three months on the various NaCl concentrations, a second type of protocol was performed. Callus was first exposed to 50 mM, for one month and then successively to 100 for one month and then from 100 to 150 mM for one month (see Fig. 3-11). At 150 mM NaCl, some of the cultures were left at this concentration for a further two months. Following 1 month on 150 mM, callus was then subcultured from 150 to 250 mM or from 150mM to 350mM for one month. At the same time, calli were cultured continuously minus NaCl for four months.
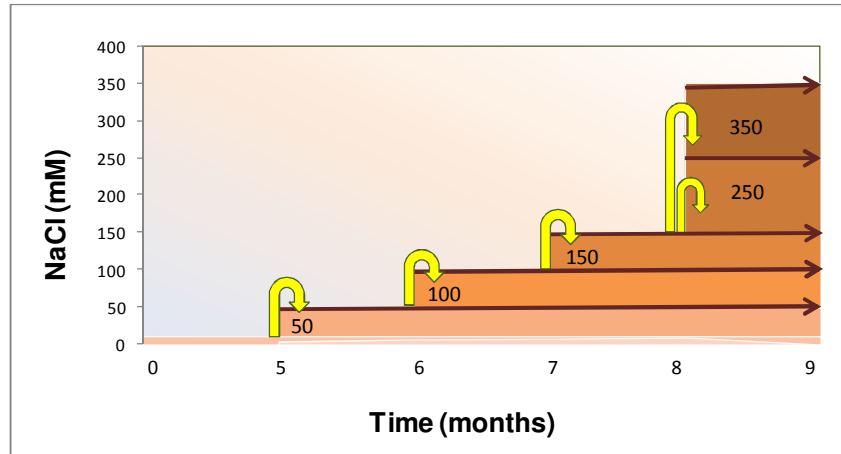
Fig 3-11. Initial protocol testing callus acclimation to salt stress. Five month old leaf-derived callus growing on 0 mM NaCl was subcultured to 50 mM for one month and then 100 mM for one month, 150 mM for one month and then either to 250 or to 350 mM NaCl for one month (see curled arrows). At the same time other cultures were left on 50 mM for four months, 100 mM for three months or 150 mM for two months (horizontal arrows) compared with controls that remained continuously on 0 mM NaCl.
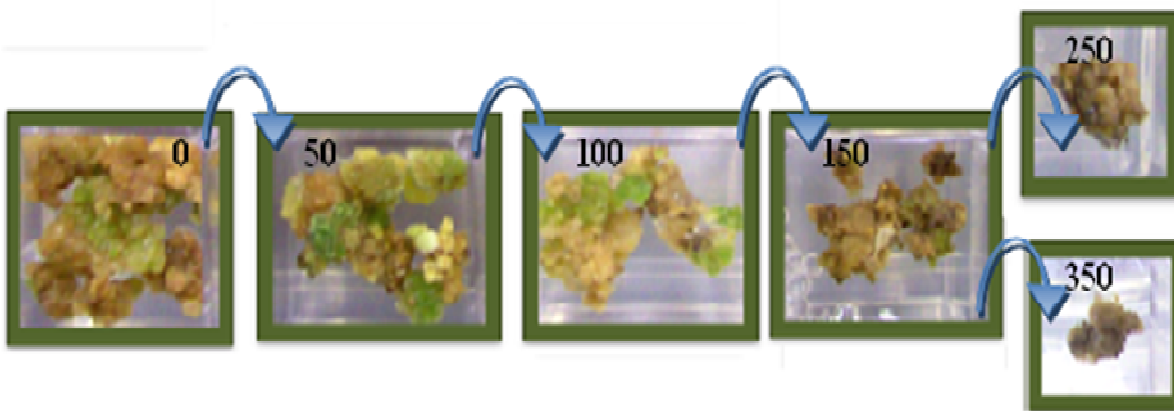
Fig 3-12. Callus patterns during step-up for acclimation to different concentration from 0 to 350 mM NaCl.

There was no significant difference between step up to 100 mM compared with continous culture at this concentration (Table 3-4) suggesting that calli were becoming tolerant to 100 mM NaCl. The higher concentrations of NaCl (250 and 350 mM) were lethal to the vast majority of cells, and only small clusters survived whereas at 50 and 100 mM NaCl green and

healthy callus tissues were observed (Fig 3-12). More salt-tolerant callus was obtained when calli were stepped up to increasing concentrations of NaCl: from 100 to 150 to 250 and 350 mM NaCl compared with calli cultured continuously at these concentrations (Fig. 3-13; Table 3-4). Indeed, calli obtained via the step-up protocol were greener, and generally free from necrosis compared with callus cultured continuously at one NaCl concentration. However, the step from 0 to 50 mM and then maintenance of calli at 50 mM NaCl, resulted in the best growth response. Thus, the gradual (monthly) increase in salt concentration provided preliminary data that suggested acclimation led callus to become more salt tolerant than calli that were maintained constantly at a particular NaCl concentration for four months. However, continous culture at 0 mM was not included in this experiment.
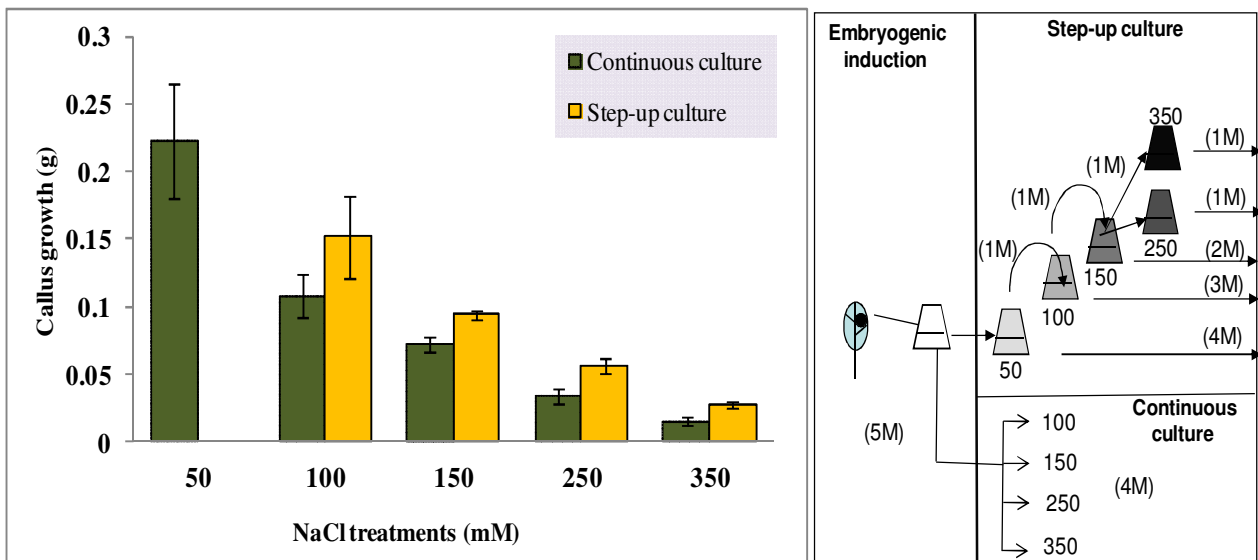
Fig 3-13. Mean (± S.E.) callus growth (FW at beginning and ending as a function of time (monthly for four months) either under a continuous exposure to one of five different concentrations of NaCl for four months or following the step up protocol at 100, 150, 250 or 350 mM NaCl. Note that only a green bar appears for the 50 mM treatment because it is both a part of the step up protocol and to the continuous exposure to this treatment.

Table 3-4. callus growth (mg) by either continuous culture on a given NaCl concentration (Single step), or by monthly step-ups in concentration (see text for details of Step-up) protocols on 100, 150, 250 and 350 mM NaCl. % increase in growth in step-up compared with continous culture.

mM NaCl	Continuous culture	Step-up	% increase in growth within treatments
100	107.5[a]	151.4[a]	40.9
150	72.50[a]	94.00[b]	29.7
250	33.75[a]	56.00[b]	65.9
350	15.00[a]	28.00[b]	86.7

Mean growth for continuous and step ups at 100 to 350 mM NaCl. The data are superscripted with [a] or [b.] Within treatments in the same rows with the same superscript indicates NSD but [a] to [b] denotes a significant difference in growth ($p \leq 0.05$).

3.3.4 Phase I Modification of the initial protocol comprising increased acclimation and increased sampling times

Given the preliminary indication that acclimation can improve salt tolerance, the protocol was extended to provide monthly assessment up to six months following callus induction

3.3.4.1 Phase I

This treatment comprised firstly, growing leaf explants for five months on control medium followed then by transfer to 50 mM. After one month at this concentration, some of the callus

was transferred to 100 mM NaCl, which was then left for five months. One month into this treatment, some of the callus was transferred to 150 mM NaCl for four months. Following one month of this treatment, some callus was transferred to either 250 or 350 mM NaCl for three months (Fig. 3-14). In parallel, some calli were left continuously at 0 mM NaCl for 6 months. Hence-forth, graphed data illustrate between treatment differences, whilst the same tabulated data illustrate within treatment differences unless stated otherwise.

There were no significant between treatment differences in callus growth in the 0 to 50 mM step up (Fig 3-14 (B)) compared with the 0 mM treatment ($P \leq 0.05$). This was not so in the step up from 50 to 100 mM NaCl (Fig 3-14 (C)) in which month by month resulted in significant decreases in growth compared with the monthly data of the control (0 mM NaCl). This was also the case for the step–up to 150 mM (Fig. 3-14 (D))
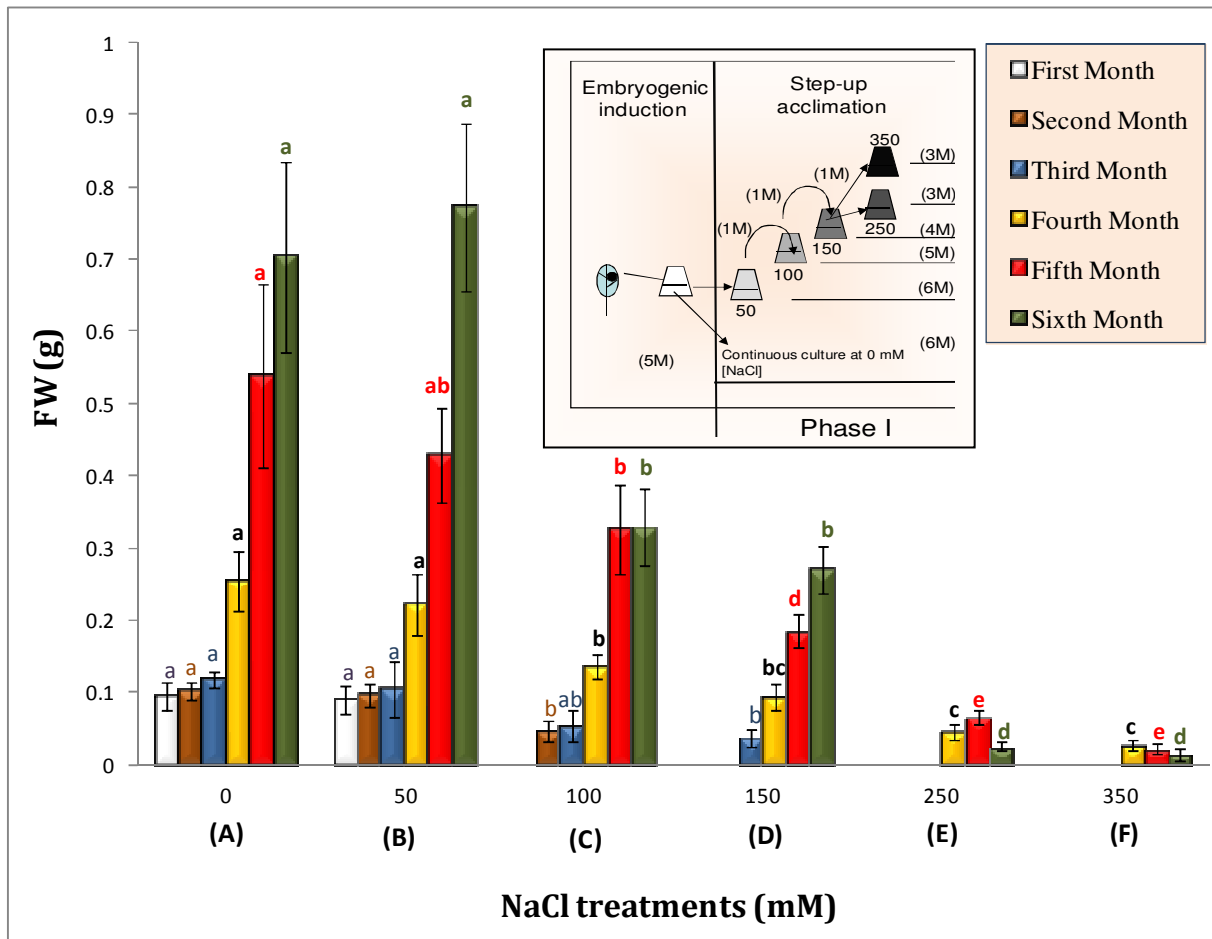
Fig 3-14. The effect of acclimation to NaCl on callus growth, at monthly intervals for a maximum of six months. All calli were initially grown on MANA without NaCl for five months. Following this pretreatment, one batch of calli remained on zero NaCl **(A)** for six months. However at the same time, calli were stepped up from 0 to 50 mM NaCl and measured monthly for six months **(B).** Once again, after one month of this treatment, one batch of callus was stepped up from 50 to 100 mM and measured monthly for five months **(C).** One month into this treatment, callus was stepped up from 100 to 150 mM and measured monthly for four months **(D).** Finally, after one month of this treatment calli were stepped up to either 250 or 350 mM NaCl and growth measured monthly for three months **(E and F** respectively**).**

Note: different letters on bars with the same colour indicate significant differences between treatments at $P \leq 0.05$ (n=12)

Indeed, the pattern of increase in growth in 150 mM step-up was not significantly different from that in the 100 mM step up at most of the time points (i.e. between treatments). However, within the 100 mM NaCl treatments, the amount of callus growth was significantly higher after four to six months compared with two months (Table 3-5). Both 150 to 250 and 150 to 350 step ups resulted in highly significant decreases in growth compared with the control (Fig. 3-14 E and F) at all time points. The major finding, is that callus grew at the same rate $\pm$ 50 mM NaCl and hence were tolerant at this concentration but this was not so at $\geq$100 mM.

Table 3-5. Comparisons within treatments (0-350 mM NaCl) of Phase I. Mean monthly callus growth for cultures grown (A) continuously minus NaCl for 6 months. (B) callus transferred from 0 to 50 mM NaCl for 6 months. (C) callus transferred from 50 to 100 mM for 5 months. (D) callus transferred from 100 to 150 mM 4 months (E and F) callus transferred from 150 to 250 or 350 mM NaCl respectively for 3 months.

Time (Months)	mM NaCl Treatments					
	(A)	**(B)**	**(C)**	**(D)**	**(E)**	**(F)**
	Control	**50**	**100**	**150**	**250**	**350**
1	0.094^a	0.09^a	-	-	-	-
2	0.101^a	0.096^a	0.046^a	-	-	-
3	0.12^{ab}	0.10^a	0.052^a	0.038^a	-	-
4	0.25^b	0.22^b	0.14^b	0.094^b	0.046^a	0.028
5	0.54^c	0.43^c	0.34^c	0.19^c	0.065^b	0.022
6	0.70^d	0.77^d	0.33^c	0.27^d	0.025^a	0.014

Means were given a super-scripted letter ^{a} through to ^{d} within that column (<u>within treatment</u>) those given different letters were significantly different within treatment $P \leq 0.05$.

Within treatment, callus growth at $\leq$ 150 mM NaCl was exponential enabling kinetic measurements of relative growth rates (RGR) and Absolute growth rate (AGR) (Table 3-6).

Table 3-6. Within treatment mean $\pm$ S.E. relative growth rates (RGR g g^{-1}month^{-1}) and absolute growth rate (g month^{-1}) calculated using the data presented in Table 3-5 and Fig. 3-14. Appearance of different superscript letters indicate a significant difference between treatments ($P < 0.05$). n=12.

NaCl concentration (mM)	RGR (g g^{-1}month^{-1})	Absolute growth rate (g month^{-1})
0	0.45 ± 0.06^a	$0.12\pm.01^a$
50	0.42 ± 0.06^a	0.12 ± 0.02^a
100	0.45 ± 0.1^a	$0.05\pm.01^b$
150	0.82 ± 0.05^b	$0.06\pm.01^b$
250	ND	ND
350	ND	ND

In the 0, 50, 100 and 150 mM treatments exhibited exponential growth. Hence, relative growth rates (RGR) could be calculated. The RGR in the 50 and 100 mM NaCl treatments were not significant different compared with the 0 mM control. There also a significantly faster RGR in the 150 mM compared with the 0 mM control (Table 3-6). However, Absolute growth rate was significantly slower in the 100 and 150 compared with 0 mM NaCl control. These data are consistent in sharing a lower yield of FW (AGR) in the 100 and 150 mM treatments but a similar, or a doubling of RGR, compared with the 0 mM NaCl control. Hence, in terms of AGR the single step up to 50 mM NaCl resulted in salt tolerance.

3.3.4. 2 Effect of the addition of Phase II

Following acclimation, or continuous 0 mM NaCl (Phase I), calli from all treatments were placed in MANA medium without NaCl for 2 months followed by a return of these calli to the same NaCl treatments that terminated Phase I, for four months (Phase II) (Fig. 3-3). The rationale for this was that, following Phase I, the calli will be a mixture of salt tolerant and physiologically adapted cells, while upon the return of calli to the NaCl component of Phase II, only tolerant cells can proliferate whereas those that were only physiologically (and expand transiently) adapted will die.
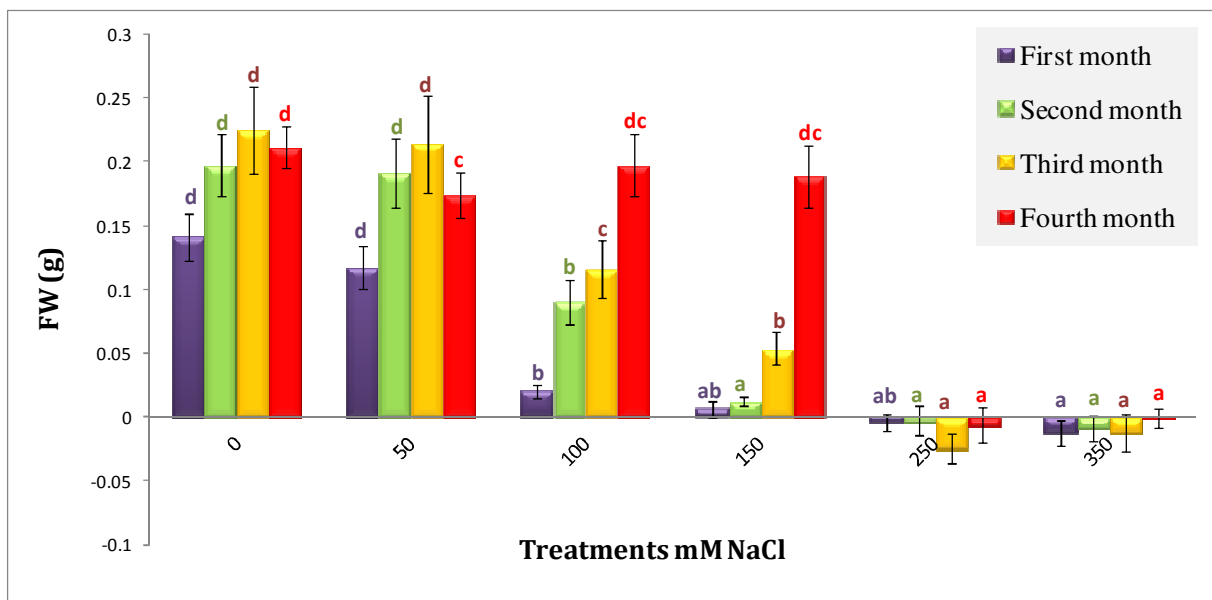
Fig. 3-15 Mean (± S.E. callus growth (g month^{-1}) following Phase I and Phase II.
Phase II comprised sub–culture of all calli onto 0 mM NaCl for two months followed by returning each batch of calli to the same NaCl concentration that they finished on in Phase I. Growth was measured monthly following the start of Phase II in all treatments.
Note: different letters on bars with the same colour indicate significant differences between treatments at each month of culture at P ≤ 0.05 (n=12)
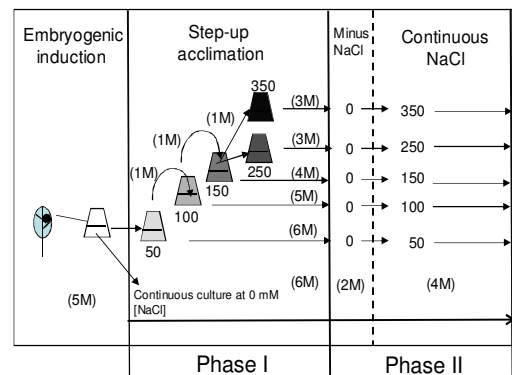
Comparisons between treatments indicate a remarkably similar growth response for the 0 and 50 mM treatments during the 4 months of continuous culture (i.e. the second component of Phase II). This parallels the virtually identical growth responses for these treatments measured during Phase I. Hence following Phase II, there were no between treatment differences in callus growth in 50 compared with zero mM NaCl treatments for the first three months of culture (Fig 3-15). During the first three months of Phase II, between treatment differences in growth were significantly lower in both 100 and 150 mM compared with zero control ($P \leq 0.05$). However, between the third and four month following the start of the second component of Phase II, there was a dramatic spurt of growth in both the 100 and 150 mM NaCl treatments such that neither were significantly different compared with the fourth months of the 0 mM NaCl control (Fig. 3-15). Hence the addition of Phase II resulted in an increase in tolerance to 100 / 150 mM NaCl and resulted in a three fold increase in tolerance compared with Phase I. Note that the step up to 250 or 350 followed by Phase II repressed callus growth both within and between treatments.

Table 3-7. Comparisons within treatments (0-350 mM NaCl) of phase II followed phase I. Mean ± S.E.callus growth (g month^{-1}) at each NaCl concentration of phase II followed phase I.

Time (Months)	mM NaCl Treatments					
	Control	**50**	**100**	**150**	**250**	**350**
1	0.1413^a	0.117^a	0.02^a	0.0088^a	-0.0014^a	-0.013
2	0.198^b	0.191^b	0.09^b	0.0129^a	-0.0029^a	-0.0086
3	0.255^c	0.214^b	0.116^b	0.0538^b	-0.025^b	-0.0125
4	0.211b^c	0.174^b	0.198^c	0.189^d	-0.006^d	-0.0012

Means were given a super-scripted letter ^{a} through to ^{d} the same letter indicates no within treatment differences but different letters within treatment indicate statistically differences at $P \leq 0.05$.

Within 0 and 50 mM treatments, the data plateaued from the 2nd to the 4th month of continuous culture following the minus salt component of Phase II (Table 3-7). Hence further growth analysis was not possible for these treatments. However, the 100 mM data were linear ($y=0.56x – 0.034$) resulting in a rate of growth of 0.56 g month^{-1}. The 150 mM data were exponential and led to a RGR of 0.13 g g^{-1} m^{-1}. Thus although the 4th month data at 150 mM were no different from the 0 control, the RGR for the Phase II 150 mM treatment is 3.0 fold slower than the corresponding rate following the 150 mM treatment in Phase I . Consistent with the improved growth exhibited in the 100 and 150 mM salt treatments following Phase I+II, was the appearance of green and healthy callus (Fig. 3-16). Moreover, these cultures exhibited somatic embryogenesis. However, the shrinkage of callus and lack of embryogenesis in the 250 and 350 mM NaCl treatments suggested that these concentrations were lethal for callus growth (Fig. 3-16).
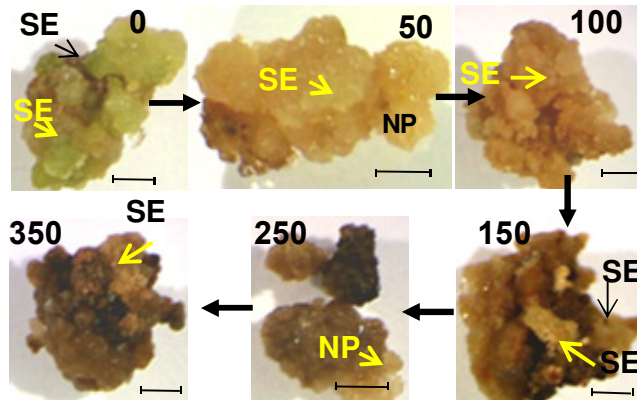
Fig 3-16. Appearance of callus, following Phase I and II on different concentrations of NaCl (mM). SE= Somatic embryo. NP= New proliferation. Bars= 5.0 mm

3.3.4.3 The addition of Phase III

Given that cultures were able to grow at the same rate ± 100 mM NaCl, I tested whether the addition of Phase III (which is a repeat of Phase II) to the protocol led to any increased or sustained tolerance.

During and following the completion of continuous NaCl treatment, callus growth at 0 and 50 mM NaCl was minimal at each sampling time and there were no significant differences between treatments after the first month of culture (P ≤ 0.05) (Fig. 3-17). However, callus growth in the 100 mM treatment significantly was faster when compared with the zero controls at each sampling time (P ≤ 0.05). This was also the case in the 150 compared with the 0 mM control. Indeed after one, two, three or four months of the continous NaCl component of Phase III, callus growth was 2.17 to 3.3-fold faster in the 100 compared

with the 0 mM treatment and, remarkably in the 150 mM treatment growth was 4.63 to 6.8-fold faster compared with the 0 mM control. The 250 and 350 mM NaCl treatments of the full protocol suppressed callus growth sharply following Phases I II, and III (Fig. 3-17; Fig. 3-18) which might be consistent with ionic stress.
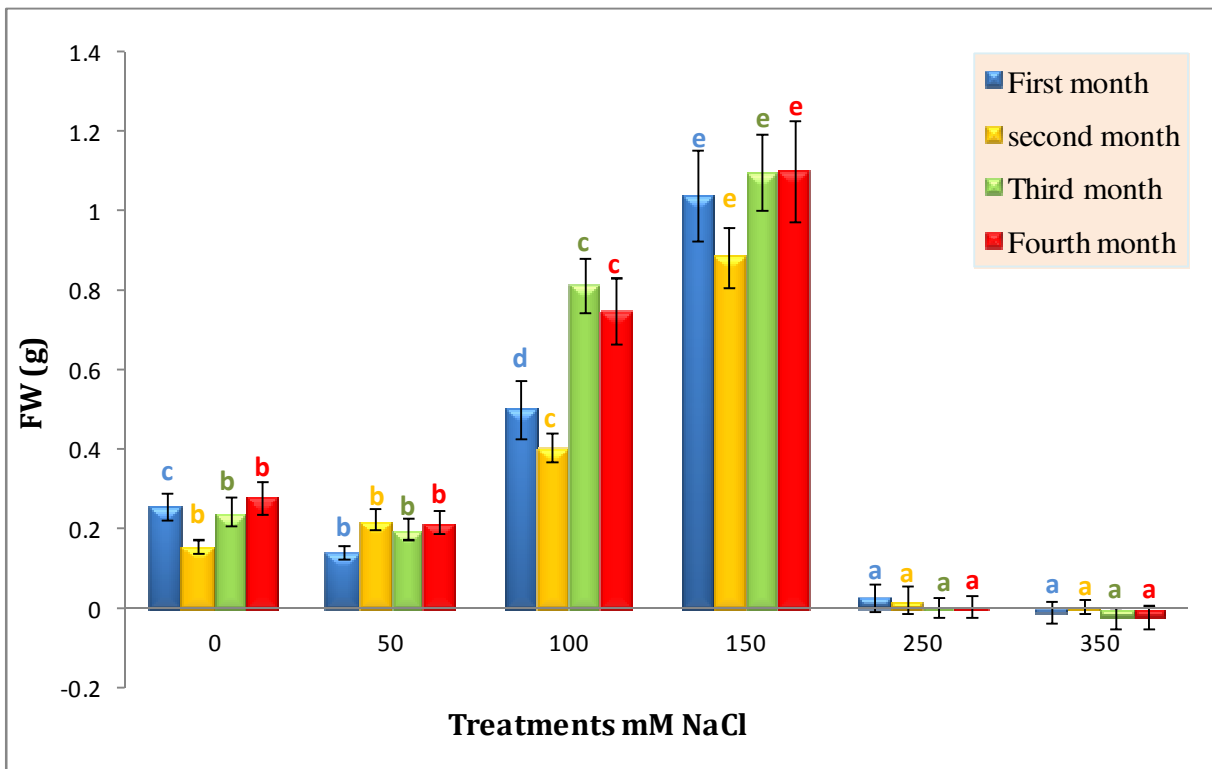
Fig 3-17. Mean (± S.E.) growth of calli following Phase I, Phase II and Phase III (during the 4 months of the continuous NaCl treatment of the second component of Phase III) Different letter combinations between the same coloured bars indicate significant differences between treatments (P ≤ 0.05). (n=12).
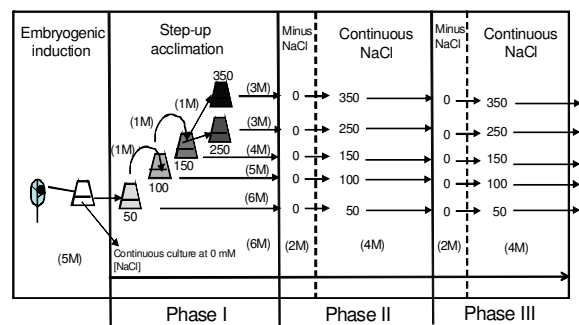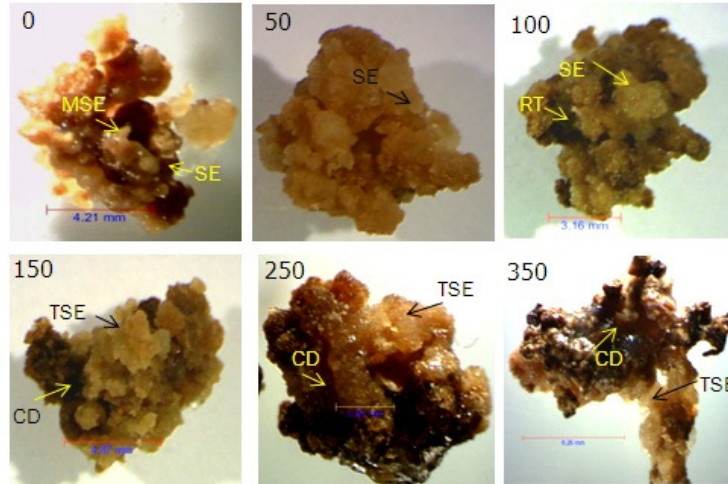
Fig 3-18. Appearance of callus after Phase III (23 month old calli) on different concentrations of NaCl. SE= Somatic embryo. CD= Probable cell death, MSE= Mature somatic embryo, RT= Root, TSE Tolerant somatic embryogenesis.

Within treatment, callus growth was minimal for the 0 and 50 mM treatments whilst within the 100 mM treatment there was significantly more growth at 3-4 compared with 1-2 months treatment. There were no significant differences in growth within the 150 mM NaCl treatment (Table 3-8).

Table 3-8. Mean callus growth (g month^{-1}) following Phase III, different superscript letters denote a significant difference within treatment at the $P \leq 0.05$ level. n=12.

Time (month)	Treatments mM NaCl					
	Control	**50**	**100**	**150**	**250**	**350**
1	0.25^a	0.14^a	0.5^a	1.04^{ab}	0.03	-0.011
2	0.16^b	0.23^b	0.4^a	0.89^b	0.02	0.004
3	0.24^a	0.2^b	0.81^b	1.10^a	0.002	-0.023
4	0.26^a	0.22^b	0.76^b	1.10^a	0.005	-0.022

Thus, the addition of Phase III extended tolerance to 150 mM. Note that tolerance increases in successive phases: 50 (Phase I), 100/150 (Phase II) and 150 mM (Phase III).

3.3.5 Regeneration from calli

At the end of Phase III, calli were sub-cultured onto the embryo development medium (EDM) for 1 month, followed by rooting medium (ECR) for two months again supplemented with 0, 50, 100 or 150 mM NaCl. Following these additional sub-cultures on EDM and ECR, 100 mM NaCl was clearly able to sustain embryogenesis in cultures that were healthy as indicated by green colouration throughout the callus whereas callus was colourless at 150 mM and turned brown in the 0 and 50 mM NaCl treatments (Fig. 3-19a). When sub cultured to ECR medium, a clear rooting response in the callus was only observed in the 100 mM NaCl treatment (Fig 3-19b). Hence, in my study following Phases I-III, EDM and ECR, 100 mM NaCl is able to sustain embryogenesis and organogenesis in 24 and 26 month-old calli, respectively whilst 150 mM resulted in fastest callus growth.

Fig 3-19. a = Representaive appearance of somatic embryos taken from the entire tissue culture protocol and subcultured to either (a) EDM for one month or (b) ECR for two months both supplemented with 0, 50, 100 or 150 mM NaCl. Arrows point to embryogenesis. Age of calli from the start of the protocol was 24 and 26 months, in (a) and (b), respectively.
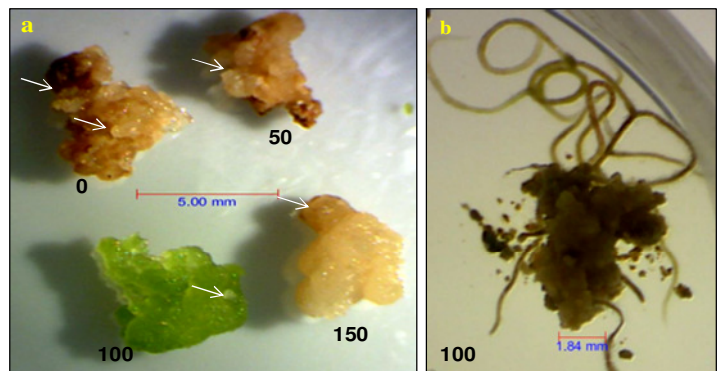
3.4 Discussion.

3.4.1 Assessment of explants on different media

Previous work on accessions of *M. trucatula* showed that optimal callus formation/somatic embryogenesis from callus occur on media supplemented with an auxin (NAA or 2,4-D) alone or in combination with cytokinins; optimal levels of plant growth regulators was species- and explant-dependent (Nolan et al., 1989; Trinh et al., 1998; Svetoslavova et al., 2005; Sivanesan et al., 2011). These observations were confirmed by my results using MS4 and EID media, which are supplemented with a combination of 2,4-D and BAP; these treatments induced embryogenesis in about 80-85% of leaf-derived and 69-71% of cotyledon derived callus (Table 3-2). Combinations of 2,4-D and BAP were used to induce somatic embryogenesis and were also species-dependent (Prado et al., 2010; Zdravkovic-Korac et al., 2010; Rodriguez-Sahagun et al., 2011; Kong and von Aderkas., 2011). The auxin, NAA, used in MANA medium is regarded as essential for somatic embryogenesis in most genotypes of *Medicago* (Nolan et al., 1989; Denchev et al., 1991). Recently it was reported that NAA activated *MtSERK1* expression while its expression was much higher when both NAA and BAP were present in the medium (Nolan et al., 2009; Steiner et al., 2012). In addition, Trinh et al. (1998) observed in *M. truncatula* that the effect of cytokinin (BAP) appears to be more promotive of somatic embryogenesis and for the production of callus tissues followed by embryo formation. As reported in this study, when MANA medium was supplemented with a combination of NAA and BAP, the percentage of callus induction from leaf- and cotyledonary derived explants increased significantly (Table 3-2).

3.4.2 Acclimation to increasing NaCl concentration (Phase I)

Initial experiments showed, that the first step up of Phase I resulted in fastest callus growth in 50 compared with $\geq$ 100 mM (Table 3-4 and Fig. 3-13). However, calli obtained via the step-up protocol were greener, and generally free from necrosis compared with callus cultured continuously at one specific NaCl concentration. There is evidence that gradual exposure to successively higher concentrations of NaCl can lead to increased salinity tolerance (Chen et al., 2011). For example, Queiros et al (2007) reported that salt-tolerant calli of potato, established *in vitro* on LM medium were obtained by using a "step-up" NaCl protocol: from 50 to 100 mM NaCl (one month), from 100 to 150 mM NaCl (one month) and from 150 to 200 mM NaCl (one month). The callus obtained by step-ups to 150 and 200 mM NaCl grew faster than calli exposed to 150 and 200 mM NaCl for four months. Also, the calli on step-ups were greenish and without any necrotic areas (Queiros et al., 2007). Salt tolerant embryogenic and organogenic callus have been produced by this method for many species (Ochatt et al., 1999; Shankhdhar et al., 2000; Miki et al., 2001). However, to my knowledge, this is the first report that acclimation through a step-up procedure results in long term increased NaCl tolerance in *Medicago truncatula* J5.

3.4.3 Increase in NaCl tolerance following Phases I and II

Acclimation *in vitro* can lead to greater tolerance to abiotic stress by supplementing the medium with auxin and/or cytokinin and this type of protocol is an effective tool in relation to plant breeding (Predieri, 2001; Prado et al., 2010; Liu et al., 2011; Sivanesan et al., 2011). For example, Chen et al. (2011) obtained five salt tolerant lines from callus of *Zoysia matrella* L. over six years *in vitro* culture on medium supplemented with 250 and 350 mM NaCl when combined with ^{60}C γ irradiation. In this study they evaluated the effect of 250 and 350 mM NaCl on growth rate over six years. They found that acclimated callus grew faster compared with a 0 mM NaCl control in *in vitro* culture. However, in my work step up to 150 mM NaCl, followed by Phases II and III resulted in greater growth compared with the 0 mM NaCl control whilst step up to 100 mM NaCl resulted in sustained competence for embryogenesis (Fig. 3-17 and Table. 3-8).

A recent study on *Zoysia matrella* (Chai et al., 2011) showed that callus could be maintained *in vitro* for more than five years on MS medium supplemented with 2 mg l^{-1} 2,4D and 0.02 mg l^{-1} Kinetin (KT) and could be acclimated to grow at non-optimal temperatures (e.g. 4°C). Remarkably, significant increases in the extent of embryogenesis were found between an acclimated line which was stable at 4°C compared with a control cultured under normal lab conditions (Chai et al., 2011). Furthermore, long-term potato calli were grown on MS (Murashige and Skoog, 1962) medium with 10.7 µg ml^{-1} NAA, 0.46 µM Kinetin supplemented with 60, 90, 120, 150, 300 or 450 mM NaCl for 20 months. The optimum concentrations for good callus growth were: 120 and 150 mM NaCl while 300 and 450 mM

NaCl inhibited callus growth (Ochatt et al., 1999). These data represent a single step from 0 mM NaCl to each of the test concentrations of mM NaCl. This is in line with data observed in my study when I used MANA medium (MS with 2 mML^{-1} NAA + 0.5 mML^{-1} BAP) and single step up to 50 mM NaCl in Phase I. However, I employed multiple step ups over the 0 to 350 mm range that ultimately, led to faster growth in 23-month old callus on 150 mM NaCl compared with the 0 mM NaCl control (section 3.3.4.1.3). This kind of long-term acclimation or adaptation protocol for salt stress has been described for cell lines of rice (Miki et al., 2001), potato (Queiros et al 2007), and *Zoysia matrella* L (Chen at al., 2011). In my work 250 and 350 mM mM NaCl were toxic and resulted in shrunken and brown coloration of callus (Fig. 3-16 and 3-18). However, it was remarkable that some cells/ calli of *M. truncatula* showed signs of callus growth and embryogenic competence at these high NaCl concentrations (Fig. 3-18).

3.4.4 Assessment of embryogenesis

Although embryogensis was abundant following Phases I-III followed by EDM and ECR at 100 mM NaCl, shoots did not form. These media (EDM and ECR) have been used to regenerate plantlets of *Medicago truncatula* from callus (Iantcheva et al., 2005a) but in my work, long-term maintenance of callus *in vitro* had a negative effect on shoot development. Nevertheless, 100 mM NaCl did lead to embryogenic calli and rooting. This result is similar to that obtained from maintained *in vitro* for six years for callus cultures of *Zoysia matrella* L. reported by Chen et al. (2011). Their treatment (s) led to the regeneration of plantlets from

calli which were maintained over six years *in vitro* on MS medium supplemented with 250 and 350 mM NaCl. Only a small percentage of calli formed plantlets after six years *in vitro* but a greater percentage of calli formed plantlets following 6 weeks *in vitro* (Chen at al., 2011; Chai et al 2011). Clearly, this suggests the longer calli were cultured on 250 or 350 mM NaCl the lower was their competence to form plantlets, (Chai et al., 2011). However, there was insufficient time available for me to discover whether 23-month old callus could be further manipulated to form plantlets. The excellent level of embryogenic callus particularly following the 100 m M NaCl treatment suggests that plantlets could be regenerated and then tested for salt tolerance. This would be an experiment for future work.

3.5 Summary

In this chaper I present quantitative and qualitative results following a protocol, which to my knowledge, has not been used before. My protocol involves a pretreatment to induce embryogenesis followed by three phases, Phase I including a step up acclimation component, Phase II a minus and plus salt combination in series and Phase III, a repeat of Phase II. The entire protocol was used for the first time with *Medicago truncatula*. Remarkably, the 100 and, in particular, 150 mM NaCl treatments resulted in faster callus growth than controls cultured in the absence of NaCl. Note also that the highest concentration of NaCl that resulted in the same or faster growth compared with 0 controls, changed from 50 mM following Phase I, to 100/150 mM following Phase II and 150 mM NaCl following Phase III. Hence acclimation to 100 and 150 mM NaCl, resulted, in the long term, in profuse embryogenesis and fastest growth, respectively. The data reported in this chapter are summarised as follows:

- Of the various types of media and organ explants used to culture *M. truncatula*, leaf explants and the MANA medium were optimal.

- The auxin, NAA in combination with the cytokinin BAP (0.5mg/l) was optimal for inducing embryogenesis and organogenesis for the A17 genotype of *M. truncatula*.

- Gradual step-wise increases in NaCl concentration up to 350 mM NaCl was a better method for establishing salt tolerant callus than continuous culture at one concentration.

- Calli established from *M. truncatula* and acclimated in Phase I followed by minus and the plus NaCl (Phases II and III) proved to be very tolerant to 150 and 100 mM

NaCl but, surprisingly, some cells could also withstand very high concentrations of NaCl up to 350 mM.

- Somatic embryogenesis continued to be supported in the 100 mM treatment after approximately two years *in vitro*.

Chapter 4: Calli responses to NaCl: physiological analyses.

4.1 Introduction

In vitro culture can provide a controlled and uniform system for studying the physiological effects of drought or salt stress at the cellular level (Ahmed et al., 2007; Lokhande et al., 2011). Plant acclimation under abiotic stresses such as salinity or drought has been examined through physiological and biochemical analyses. This has led to much information through studies of embryos or plants subjected to salt and "drought" treatments (Munns, 2002; Piza et al., 2003; Santos et al 2011). The general picture is that tolerant plants / cells make osmotic adjustments in response to abiotic stress. For example, highly soluble compounds like sugars and the amino acids, proline, serine, leucine, alanine, arginine, polyols and glycine accumulate in higher plants under salinity stress (Ashraf, 1994a). They serve as organic compatible solutes, which besides causing osmotic adjustments, play a role in stabilization of proteins, protection of membrane integrity and help regulate ionic sequestration (Patade et al., 2011). Predictably, many studies have been initiated to study populations of cells *in vitro* in response to abiotic stress (Cui et al., 2010; Lokhande et al., 2011; Sun et al., 2010). For example, *in vitro* cultures have provided systems for studying the effects of salt and drought stress at the biochemical and physiological levels (Ahmed et al., 2007; Lokhande et al., 2011).

4.1.1 Proline.

Proline often accumulates in large quantities in plant cells under salt stress (Ali et al., 1999; Ashraf, 1994b; Abraham at al., 2003). In addition, proline accumulation also occurs in response to water availability (Choudhary et al., 2005; Fulda et al., 2011); water loss will of course increase the concentration of water soluble molecules in the cell. Proline accumulation, which is osmotically very active (Ashraf 1994b), contributes to stabilization of membranes, which protect the plant against osmotic stress (Gadallah, 1999; Ashraf and Fooled, 2007) and reduce the impact of sodium chloride on cell membrane disruption (Mansour, 1998; Ji-Bao et al., 2009). Maggaio et al. (2002) suggest that proline may have a role in intracellular signaling as part of the response of the plant to unusually high levels of NaCl. Overall, cytoplasmic proline accumulation either directly or indirectly, regulates the vacuolar sequestration of sodium (Na^+) and chloride (Cl^-) ions.

Proline accumulation is one of the better known characteristics of many plant species growing under saline conditions (Guo-An et al., 2009; Ji-Bao et al., 2009). For example, its accumulation coincides with improved growth of barley embryos *in vitro* when the medium is supplemented with high levels of NaCl (100-200 mM) (Lone et al., 1987). Proline accumulation also occurred in response to salt stress in: sorghum (Jogeswer et al., 2006), potato (Himida-Sayari et al., 2005), wheat (Colmer et al., 1995; Vendruscolo et al., 2007), rice (Lutts et al., 1996), mulberry (Kumar et al., 2003), green gram (Misra and Gupta, 2005), tobacco (Yamchi et al., 2007) and *Medicago sativa* (Petrusa and Winicov, 1997; Miller et al., 2005).

4.1.2 Soluble sugars.

There have been many studies that have attempted to relate the extent to which soluble carbohydrate accumulation is linked to salinity tolerance. Water soluble carbohydrates tend to accumulate in plant tissues as a response to abiotic stresses such as salt and drought (Murakeozy et al., 2003). Cram (1976), in his study of glycophytes, showed that the accumulation of water soluble carbohydrates had a substantial input to the regulation of osmotic potential. Indeed, many plant species show increases in carbohydrate levels in response to salinity. For example, sucrose levels rise in response to salinity in *Hordeum vulgare* (Gauch and Eaton, 1942), *Glycine max* (Rathert, 1985), *Lepidium crassifolium* (Murakeozy et al., 2003) and *Saccharum officinarum* (Patade et al., 2011). However accumulation of water soluble sugars in response to salinity stress is not universal among all higher plants. The concentration of soluble sugars accumulated in five accessions of sunflower that varied in salt tolerance, (Ashraf and Tufail, 1995). One accession was salt tolerant and exhibited an increased concentration of soluble sugars (30 mg/g^{-1}FW) while another accession was salt tolerant without any major changes in water soluble carbohydrates. In other lines that were salt sensitive, soluble sugar concentrations were much the same as the tolerant lines (Ashraf, and Fatima 1995). Hence the role of sugars in a plant's response to salt or drought is controversial. The consensus view is that soluble sugar accumulation can be important but is not a universal component of a plant's acquisition of salt or drought tolerance.

4.1.3 Ionic stress

As reviewed in the main Introduction (see p. 10), over accumulation of Na^+ leads to toxicity in NaCl sensitive species whereas Na^+ sequestration / elimination occurs in NaCl tolerant species. In addition, when $[K^+]$ drops below a specific threshold in response to increasing [NaCl], Na^+/K^+ homeostasis is perturbed and Na^+ is then toxic (Sun et al., 2010; Tang et al., 2010; Misic et al., 2012).

The aim of the work reported in this chapter was to assess the extent to which various physiological traits can be used as markers of salinity in *Medicago truncatula*. In callus derived from leaf explants of *Medicago truncatula* under NaCl stress, I examined changes in the levels of:

- proline

- water soluble sugars

- osmolarity

- Na^+ and K^+

- water content

4.2 Materials and Methods.

4.2.1 Measurement of Sodium (Na^+) and Potassium (K^+) accumulation.

To determine the sodium accumulation in the callus, 100 mg fresh weight was collected from three replicates following Phases I, II and III in calli that were 11, 17, and 23 months-old, respectively (see Fig. 2-1). The fresh calli from each replicate were placed into 50 ml pyrex beakers and incubated in an oven at 80 ºC for 24 h; then the dry weight was calculated for each sample. Ten ml of 15.8 M HNO_3 (Fisher-MOS) was added to each beaker. Then, all the beakers were placed on a sand bath at 240 ºC in a fume cupboard for 2-3 h until the digests started to clear (more acid was added as necessary to prevent the digest from drying up). After the samples had cooled for 15 min at room temperature, they were filtered into 25 ml volumetric flasks and made up to 25 ml with ultra pure water. The measurement of Na^+ accumulation for each treatment was carried out using an atomic absorption spectrophotometer (Varian- Spectr AA-100, version 2.00 software) and the calculation of $[Na^+]$ was processed according to the formula:

Ppm value ($\mu g/cm^3$) x volume x dilution factor / Dry weight = $\mu g/g$ sample material.

This methodology was also used to measure K^+ accumulation 23 months following the start of the protocol.

4.2.2 Measurement of proline content.

Proline content was evaluated according to Troll and Linsley (1955) and Boukel and Houassine (1997). One hundred mg of callus tissue per sample per treatment was examined over the concentration range: 0, 50, 100, 150, 250 and 350 mM NaCl. All treatments were repeated three times. The samples were collected and placed in 30 ml tubes. Two ml of 40% ethanol was added to each tube, then they were heated in a water- bath at 85° C for 60 min. After cooling the samples for 2 h at room temperature, 1 ml of the extract of each sample was transferred to a tube containing 1 ml of acetic acid and 1 ml of ninhydrin reagent. Ninhydrin was prepared as follows: (120 ml of distilled water + 30 ml of acetic acid + 80 ml orthophosphoric acid (density 1.7) + 25 mg of ninhydrin). The mixture (sample and reagent) was boiled for 30 min. After cooling at room temperature, 5 ml of benzene was added to each tube. Two layers appeared; the upper layer yellow- coloured band indicated the presence of proline (Boukel and Houassine, 1997).

The determination of the optical densities of different samples was measured using a spectrophotometer (LNICAM: uv-vis spectromery) at a wavelength of 528nm. Standard solutions of proline were prepared from a stock solution (125 mg proline in 100ml 0.3 M HCl). The dilutions were made with HCl (0.3M) to obtain concentrations of 0.1, 0.2 and 0.4 mg ml^{-1} for the calibration used to determine the proline concentration of different samples.

4. 2.3 Determination of soluble sugars

The determination of soluble sugars was carried out by the anthrone method (Plummer, 1989). The extraction of soluble sugars was performed on callus from three samples of 100 mg tissue from each salt stress treatment of 0, 50, 100, 150, 250 and 350 mM NaCl. Each sample was immersed in 3 ml of 80% ethanol and left for 48 h, then, they were heated in a water bath at 70 °C for 30 min. Two ml were taken from the extract solution from each sample and diluted 10 times with 80% ethanol to which was added 4 ml of reagent consisting of: 0.2 g anthrone dissolved in 100 ml of sulphuric acid (18M H_2SO4). The reagent was prepared at least 4 h in advance. After shaking, the tubes were placed in a water bath at 92 °C for 8 min, then cooled for 30 minutes on ice in darkness. The soluble sugar content was read spectrophotometrically (LNICAM: uv-vis spectromery) at a 585 nm wavelength, and the data were converted to mg l^{-1} using the calibrations established prior to the assay (Appendix. A1).

4.2.4 Osmolarity measurements.

MANA medium and intracellular osmolarity were evaluated at the end of step-up acclimation (Phase I). These measurements were made using a Wescor (model VAPRO 5520, USA) vapour pressure micro-osmometer. Measurements were performed on a minimum of three 10-µl samples for each treatment. For assessments at the intracellular level, 2 g fresh weight of callus were collected from all the salt stress treatments of 0, 50, 100, 150, 250, and 350 mM NaCl after callus terminated Phase I. Calli were crushed in an Eppendorf tube with a pestle

and then centrifuged (1000g, 10 min, 4ºC). Finally, the supernatant was employed for measurements of osmolarity after calibration of the equipment with three standard solutions. The three standard solutions used were: 100 ± 0.3 mM/Kg (equivalent to osmosed deionized water osmolarity), 290 ± 0.5 mM/Kg (equivalent to milliQ water osmolarity) and 1000 ± 1.4 mM/Kg (equivalent to sea water osmolarity) (Ochatt at al., 2008).

Results, (mM/kg) are the mean ± S.E of measurements performed at least three times. For all parameters assessed, results were statistically analysed by ANOVA (P ≥ 0.05) using Minitab.

4.2.5 Water Content (WC).

Water content was determined according to Yazici et al. (2007). About 0.3g fresh weight of callus was obtained at the end of Phase I (11 month old calli). Three replicates were taken from each treatment of NaCl: 0, 50, 100, 150, 250 and 350 mM. Fresh weight was determined for each replicate (FW) and the dry weight (DW) was also determined after oven drying at 70 ^{o}C for 72 h. The water content (WC) was evaluated according to the formula:

WC (%) = FW- DW/ FW X 100 (Smart and Bingham, 1974).

4.3 Results

4.3.1 Osmolarity and water content.

Osmolarity was measured in the media used during the acclimation process and in the calli themselves. For reliability, sensitive apparatus set to show minimum variation was used with specific reference to calibration standards.

The osmolarity of the medium increased significantly relative to the increase in NaCl concentration up to 350 mM NaCl (Fig. 4-1). Intracellular osmolarity of callus that passed through Phase I (6 month old calli) showed a more-or-less linear relationship with external salt concentration so that at each concentration, it was significantly higher than at the previous one ($P \leq 0.05$) although osmolarity at 100 and 150 mM were the same and at 350 mM was no different from that at 250 mM NaCl (Fig. 4-1). Notably, the highest osmolarities were recorded for callus on the 250 and 350 mM NaCl media, treatments known to significantly repress callus growth. It is of further interest that the osmolarity of medium/callus at 150 mM NaCl was very close to that expected from sea water whilst 250 and 350 mM NaCl exhibited osmolarites much greater than in sea water (1393.5 and 1196.33 mM/Kg) respectively (Fig. 4-1).

	0 NaCl	50 NaCl	100 NaCl	150 NaCl	250 NaCl	350 NaCl
■ Osmolarity of calli	409	546	725.5	761.25	1393.5	1196.33
■ Osmolarity of medium	338	409.4	561.6	635.4	710.6	853.2
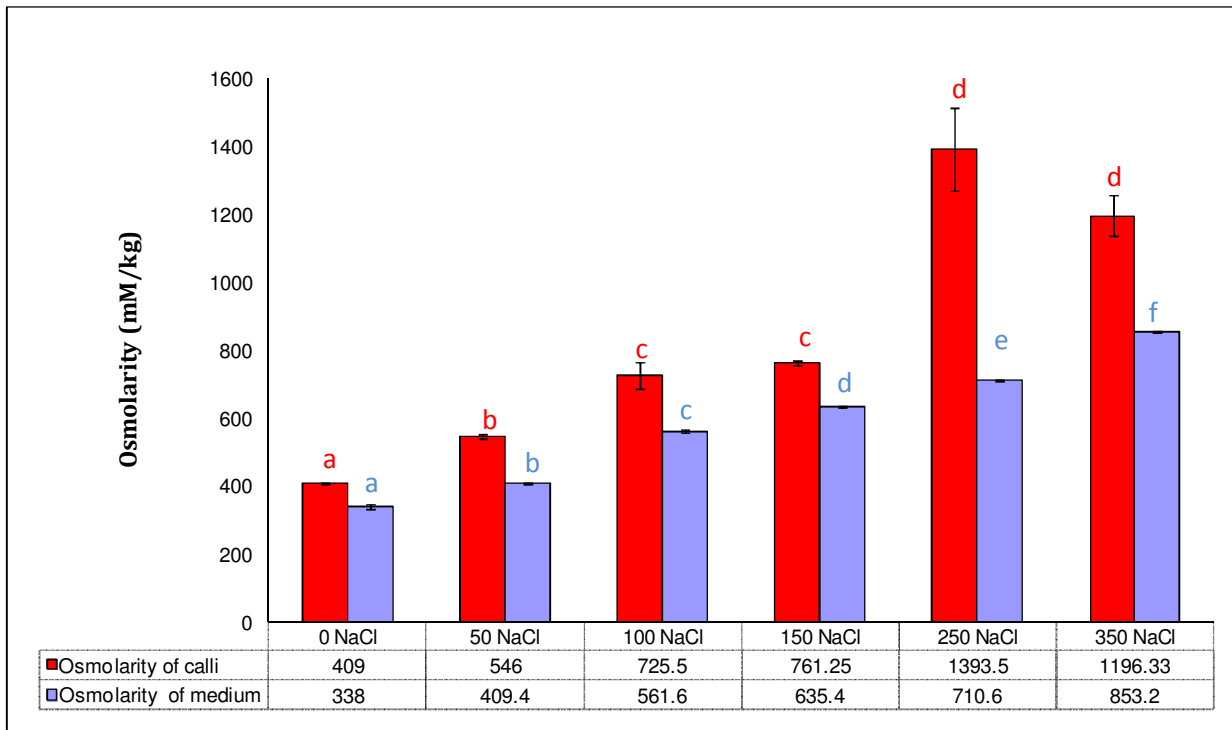
Fig 4-1. Comparison between treatments of mean (± S.E.) osmolarity in the callus tissues and in the culture medium after six months step-up acclimation on various NaCl concentrations (Phase I). Different letter combinations between the same coloured bars indicate significant differences between treatments and the 0 mM NaCl control (P ≤ 0.05) (n=3).

The next step was to examine how the water potential was altered in the cells of calli grown on 0-350 mM NaCl (following Phase I). Cellular water content declined between treatments in an almost linear fashion from 50 to 350 mM NaCl although there were fewer, significant differences between NaCl treatments compared to osmolarity (Fig. 4-2). Hence, the consistent rise in intracellular osmolarity was matched by a predictable and consistent fall in water content along the NaCl gradient. The calli grown on 250 or 350 mM NaCl, were water-deficient and were suffering from severe NaCl toxicity.
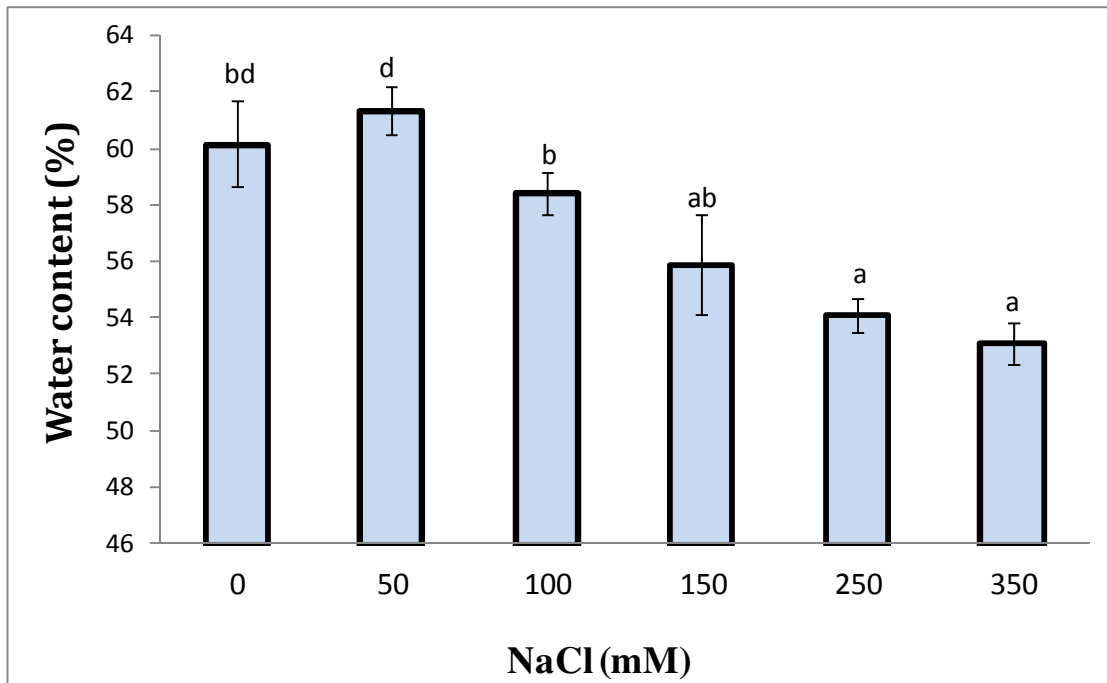
Fig 4-2. Mean percentage (± S.E.) water content in the callus tissues after 6 months step-up acclimation on the various NaCl concentrations (Phase I). Note different letters indicate between treatment significant differences compared with the 0 mM NaCl control (P ≤ 0.05) (n=3).

4.3.2 Soluble sugar content.

Water-soluble sugars were measured after callus terminated Phase III (23 month old calli) on 0-350 mM NaCl. The 150 and 250 mM NaCl treatments induced a significantly higher level of water-soluble sugars in the callus compared with the 0 mM control (Fig. 4-3). The accumulation of sugars in the 150 mM salt treatment might be linked to the increased tolerance of callus exposed to this level of NaCl. However, soluble sugar continued to rise in the 250 mM NaCl a treatment that suppressed growth, albeit not significantly.
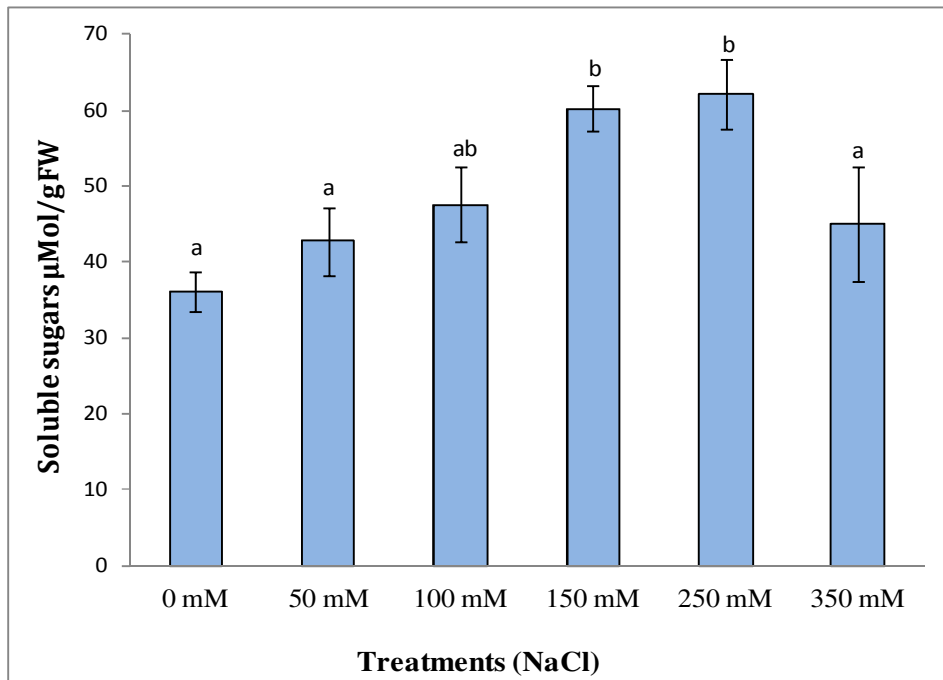
Fig 4-3. Mean (± S.E.) soluble sugar content in callus tissues at the end of Phase III (18 months culture on NaCl). Note different letters indicate significant differences between treatments (P ≤ 0.05) (n=3). Linear regression of 0 to 250 mM data: y = 0.11x + 38, P ≤ 0.001.

4.3.3 Sodium (Na$^+$) and Potassium (K$^+$) content of calli grown on NaCl

In this experiment, Na$^+$ accumulation was measured in callus after Phase I (11 months), Phase II (17 months) and Phase III (23 months). These calli were either acclimated to each of the step up NaCl concentrations: 0, 50, 100, 150, and 250 or 350 mM NaCl or grown continuously at each of these concentrations (see Fig. 2-1).

The data show a positive linear relationship between [Na$^+$] uptake and exogenous [NaCl] concentration (Fig. 4-4) following Phases I, II and III. Hence, the higher the exogenous [NaCl] was, the greater was endogenous [Na$^+$]. Within the 0, 50, 100 and 150 mM NaCl treatments [Na$^+$] fluctuated but not greatly (Fig. 4-4). However, within the 250 and 350 mM NaCl treatments, [Na$^+$] fell dramatically following Phase III compared to levels at the end of Phases I and II (Fig. 4-4 and Table 4-1).
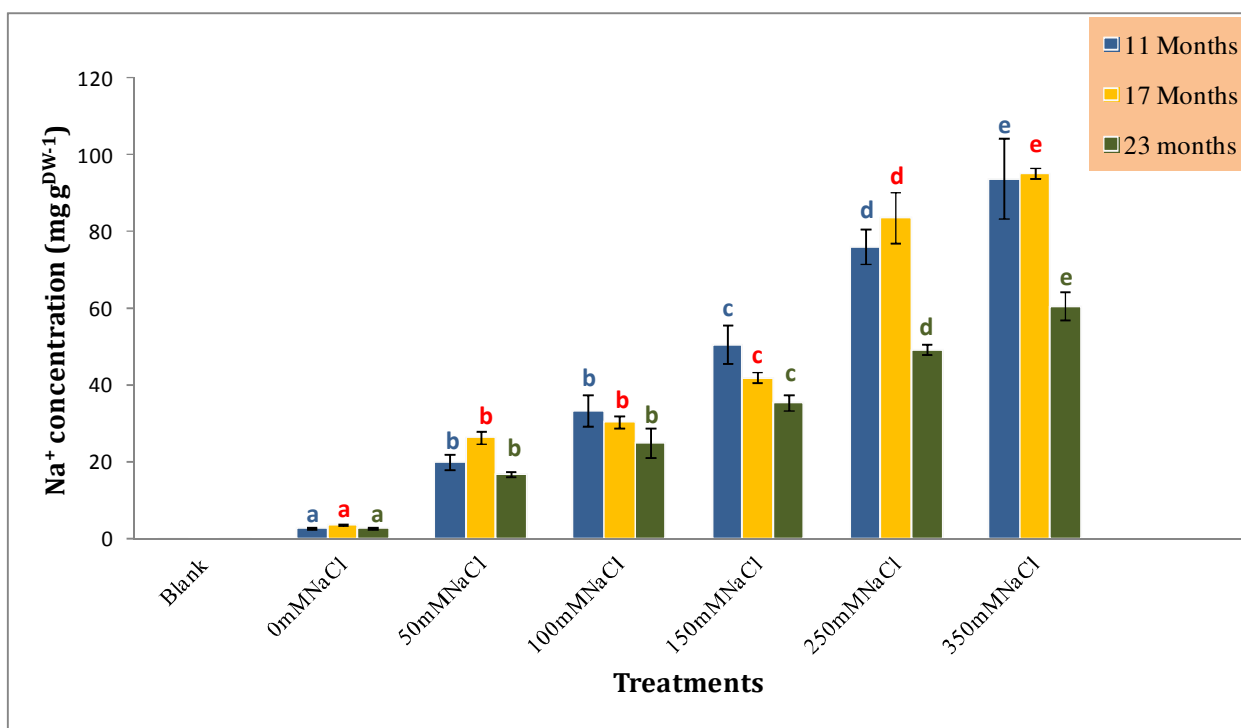
Fig 4-4. Mean (± S.E.) Na^+ content in callus tissues after the start of the protocol Phase I (11 months including 6 months of step-up acclimation), Phase II (17 months) and Phase III (23 months). Note different letters between the same coloured bars indicated significant differences between treatments (P ≤ 0.05) (n=3). Age of calli is from the start of the protocol
Linear regression of Phase I. y = 0.26x + 6.97 (P <0.001).
Linear regression of Phase II. y =0.26x + 7.01 (P <0.001).
Linear regression of Phase III. y = 0.16x + 7.50 (P <0.001).

Table 4-1. Comparison within treatments of mean (± S.E.) sodium accumulation in callus tissues following completion of: Phases I, II and III. Note, different letters in the same column indicate significant within treatment differences (P ≥ 0.05) (n=3).

Phases	NaCl treatments (mM)					
mM NaCl	0	50	100	150	250	350
I (11M)	2.8±0.2[a]	20.1±2.0[a]	33.5±4.0[a]	50.8±5.0[c]	76.3±4.6[b]	93.9±10[b]
II (17M)	3.7±0.3[a]	26.3±1.7[b]	30.5±1.3[a]	42.1±1.3[b]	83.7±6.6[b]	95.2±1.5[b]
III (23M)	2.9±0.2[a]	16.8±0.5[a]	25.0±3.8[a]	35.5±2.1[a]	49.3±1.2[a]	60.8±3.7[a]

Potassium (K^+) accumulation, which was determined following Phase I, II and III (23 month old calli), decreased linearly as the concentration of NaCl in the medium increased (Fig. 4-5), with the lowest concentration of K^+ in the 250 and 350 mM NaCl treatments, although statistically there was no significant diffence between 100 and 150 mM or 150 and 250 mM NaCl treatments.
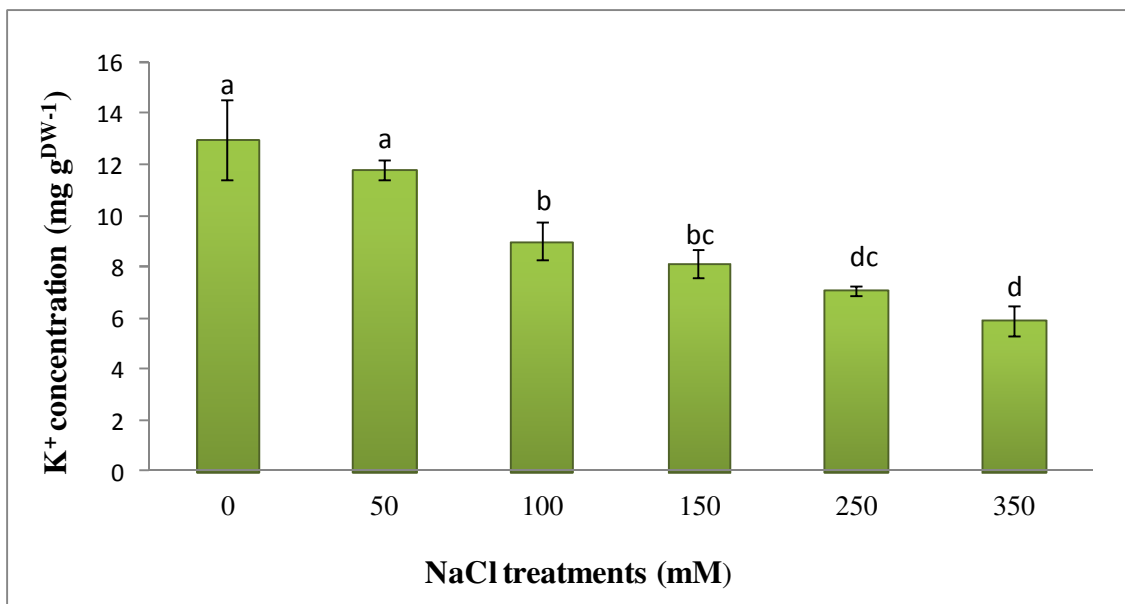
Fig 4-5. Mean (± S.E.) [K^+] in callus exposed to the various NaCl treatments for 23 continuous months of exposure to NaCl. Different letters indicate significant differences (P ≤ 0.05) (n=3). Linear regression of data: y = 12.2 - 0.02x, P = < 0.001.

Na^+ and K^+ accumulation between treatments showed an inverse linear relationship (Fig. 4-6) in that between treatments, [Na^+] increased whilst [K^+] decreased which fits a model that excessive cytoplasmic Na^+ accumulation can prevent potassium (K^+) uptake from the media (Tang et al., 2010 and Misic et al., 2012). The cross-over point for these results occurred at

150 mM NaCl whilst linear regressions crossed at ~ 160 mM (Fig. 4-6). Interestingly, following Phase III fastest growth was also at 150 mM NaCl (see Fig. 3-17. Chapter 3).
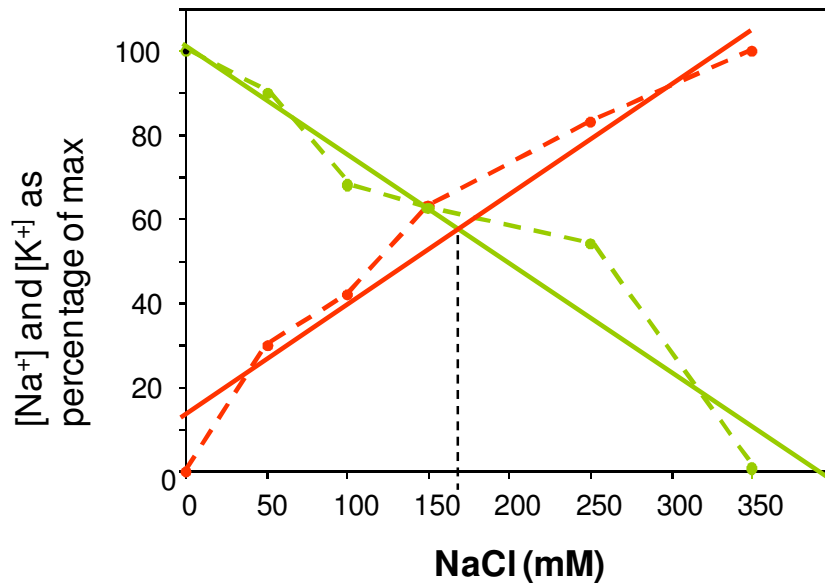
Fig. 4-6 Mean concentration of [Na+] (green) and [K+](red) as percentage of maximum, in calli following Phase III (23 month old calli). The regression equations for [Na$^+$] and [K$^+$] are y = 0.273x + 12.072 (red), and y = 101-0.258x, respectively (green) (P < 0.001). n=12. Intercept of regressions occurs at ~ 160 mM

4.3.4. Proline accumulation

Proline content was determined for calli following Phases I and II (17 month-old) and following Phases I, II+ III (23 month-old). Proline content increased significantly in salt treatments compared with the 0 mM NaCl treatment (Fig. 4-7). Within treatments, there were significant increases in proline amount following Phase III compared with following Phase II

at $\geq$ 100 mM with the highest proline amount recorded following Phase III in the 100 and 150

mM treatments (Table 4.2). Again it is important to note that following Phase III, growth was

fastest in the 100 and 150 mM treatments compared with 0 controls (see Fig. 3-17. Chapter 3).
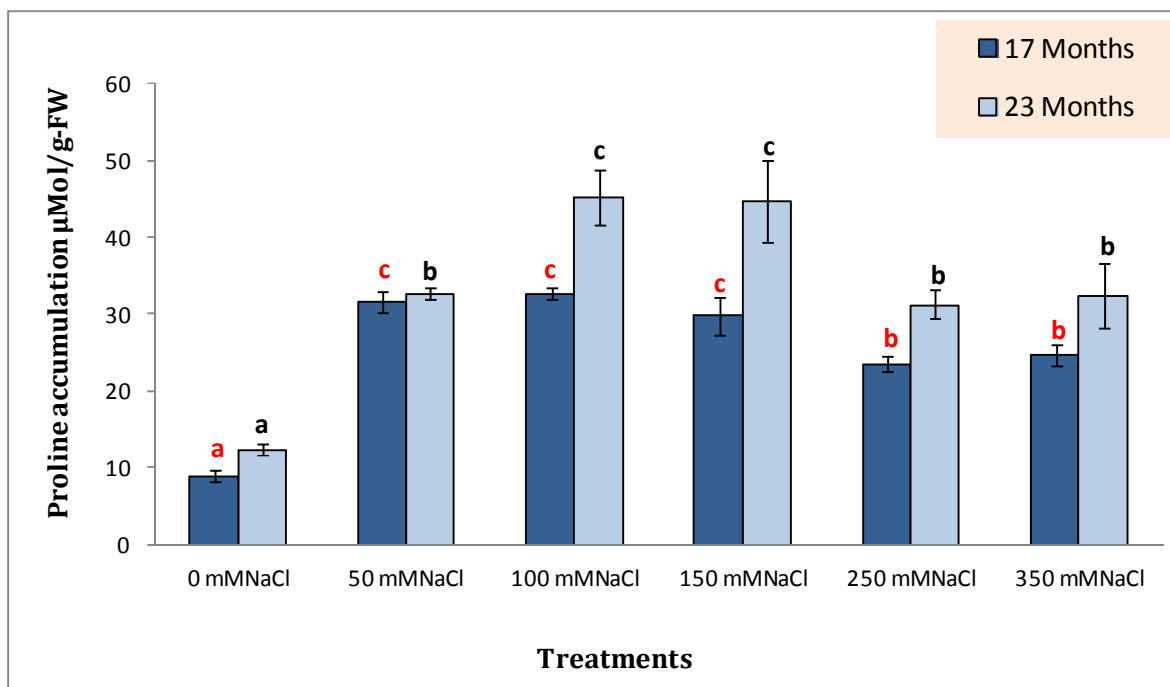
Fig 4-7. Mean ($\pm$ S.E.) proline accumulation in callus tissues after Phase I+II (17 months) or Phase III (23 months) on 0-350 mM NaCl. Note, different letters between treatments on the same colour columns represent significant differences (P $\geq$ 0.05) (n=3).

Table 4-2. Mean ($\pm$ S.E.) proline amount (μ mol g FW-1) upon completion of: Phases I + II, and Phases I+II+III across the 0 – 350 mM NaCl treatments. Different superscript letters denote within treatment differences. (n=3). P $\leq$ 0.05

Phases	NaCl treatments (mM)					
	0	50	100	150	250	350
II (17M)	9.0 ± 0.8^a	31.6 ± 1.3^a	32.2 ± 0.8^a	29.9 ± 2.5^a	23.5 ± 0.9^a	24.6 ± 1.3^a
III (23M)	12.3 ± 1.3^a	32.7 ± 0.8^a	45.2 ± 3.7^c	44.8 ± 5.3^c	31.3 ± 1.8^b	32.5 ± 4.2^b

4.4 Discussion

In this chapter, physiological data have been collated from calli of *Medicago truncatula* following the partial or complete tissue culture protocol.

The linear decreases in potassium [K^+] in the 250 and 350 mM NaCl treatments resulted in calli that were unable to support growth, or the linear increases in [Na^+] in these treatments were unable to be sequestered and became toxic. In fact, both the [K^+] decrease and [Na^+] increase could be responsible for poor growth in the 250 and 350 mM treatments. In other studies, excessive cytoplasmic Na^+ accumulation prevented potassium [K^+] uptake in calli derived from several species (Zhu, 2003; Volkov et al., 2004; Sun et al., 2010; Tang et al., 2010; Misic et al., 2012). For example when eight week-old root tips of *Centaurium spicatum* were cultured on BM medium supplemented with 0 or 200 mM NaCl endogenous [K^+] decreased significantly from the 68 µmol g^{-1} DW (control) to 45 µmol g^{-1} DW (Misic et al., 2012). Hence I suggest that in *Medicago truncatula* excessive Na^+ uptake might also prevent K^+ uptake.

Interestingly, the two sets of data (Na^+ and K^+) intercepted each other at 150 mM NaCl, the highest NaCl concentration that supported increased callus growth compared with 0 mM NaCl (Fig. 4-6). This type of K^+/Na^+ homeostasis at 150 mM NaCl was also reported in calli derived from a salt tolerant line of olive (*Olea europaea*) (Sun et al., 2010). As I mentioned above, my full protocol resulted in faster growth only in phase III at 150 compared with 0 mM NaCl suggesting strongly that potentially harmful Na^+ ions are sequestered, presumably

into a vacuolar compartment or were excluded thereby reducing cytoplasmic [Na^+] at $\leq$ 150 mM NaCl. This type of mechanism has been resolved in *Arabidopsis thaliana*; (Chen et al., 2007), barley (Shi et al., 2002) and *Centaurium maritimum* L. (Misic et al., 2012) in maintaining optimal cytosolic K^+/Na^+ homeostasis. I also detected a positive relationship between [NaCl] and osmolarity, proline and soluble sugar accumulation (Fig. 4-3) as reported by many authors (e.g. Raven, 1985; Hariadi et al., 2011). For example, Summart at al. (2010) showed that in rice cultures exposed to 250 mM NaCl, [proline] increased to 230µg/g FW compared with 80 µg/g FW 250 mM NaCl compared to a control [0 mM NaCl]. Hence my data support the idea that in salt-stressed calli derived from *M trucatula*, proline and water soluble sugars accumulate to protect the cells against osmotic perturbation that would otherwise occur in response to NaCl. However, although in my study 150 and 250 mM NaCl treatments induced significantly higher levels of water soluble sugars in the callus compared with the control (Fig. 4-3); the 250 mM NaCl treatment suppressed growth. So, in this instance, the level of water soluble carbohydates was not a sensitive marker of salt tolerance. The accumulation of soluble sugars in plant tissues has been widely confirmed as a response to salt and /or drought tolerance. For example, an increase in the level of sucrose occurred in several genotypes under conditions of salinity stress: *Hordeum vulgare* (Gauch et al., 1942), *Zygophyllum album* (El-shourbagy et al., 1975), *Glycine max* (Rathert, 1985) and *Lepidium crassifoium* (Murakeozy et al., 2003). However as discussed above a tight relationship between increased soluble sugars and salt tolerance has not been found universally.

Mean proline content peaked in the 100/150 mM treatments both following Phase II and III (Fig. 4-7). This type of result has been demonstrated for many plant species, such as tobacco (Banua et al., 2009) and *Pancratium maritimum* L. under salt stress (Khedr et al., 2003). The proline plays a role in mediating osmotic adjustment and protecting the subcellular structures in stressed plant tissues (Yang et al., 2003, Ashraf and Foolad, 2007; Santos et al., 2011; Patade et al., 2011) and abiotic stresses such as salinity and drought induce enzymes involved in proline biosynthesis (Ashraf and Foodal, 2007; Hasegawa et al., 2000; Banua et al., 2009). Therefore, the highest amount of proline accumulation reported here for the salt treatments, 100 and 150 mM, may serve to protect the callus from salt toxicity. It is interesting that 150 mM NaCl is the highest concentration to permit substantial callus growth during a treatment that includes acclimation as a key component (Phase I). Following Phases I-III, somatic embryos that were initiated in medium supplemented with 150 mM NaCl, clearly benefited from an acclimation component in Phase I and during the entire culture protocol they may have benefited further from increased levels of proline. In particular, the 150 mM NaCl step up, suggests that they were acclimated and made competent to over produce proline, contributing to salt tolerance. Notably, calli on 150 mM NaCl were green and healthier than the 0 control (Fig. 3-18). Overall, the data reported here indicate a positive relationship between salt tolerance and [proline]. However, water soluble carbohydrates were highest in the 250 mM treatment: since this NaCl concentration in habited growth, this was not a sensitive marker of NaCl tolerance. Hence, I concluded that such calli were unable to synthesise sufficient proline to deal with either 250 or 350 mM NaCl, consistent with poor growth, browning and shrinkage of the calli as reported in Chapter 3.

4.5 Summary

The data reported in this chapter are summarised as follows:

- Na^+ accumulation and osmolarity in all callus cultures increased significantly and linearly, proportionate to the concentration of exogenous 0-350 mM NaCl.

- K^+ accumulation decreased linearly over the exogenous 0-350 mM NaCl concentration range.

- When plotted as % maximum over 0-350 mM NaCl, regressions for $[Na^+]$ and $[K^+]$ intersected at approximately 160 mM NaCl. I therefore suggest Na^+/K^+ homeostasis at this exogenous NaCl concentration.

- Water soluble sugar concentration increased linearly over the 0-250 mM range of NaCl. Since 250 mM NaCl suppressed growth, soluble sugar concentration is not a useful marker of NaCl tolerance.

- Proline accumulation increased significantly over the range 0-150 mM NaCl, the latter treatment resulted in fastest growth. Hence [proline] was a good marker of NaCl tolerance.

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Chapter 5: Cellular responses to salinity

5.1 Introduction

In general cytological changes in tissue culture are affected by many factors such as type of media, extent of subculture, choice of plant hormones and responses to abiotic factors such as salinity (Bairu et al., 2008; Perez et al., 2009; Jeong et al., 2009). Cells *in vitro* can exhibit various ploidy levels and chromosomal instability (Dhooghe et al., 2010; Kumar and Mathur, 2004). The cellular instabilities can be particularly acute in long term tissue cultures. Alteration of plant cell structure has already been observed under abiotic stress (Skirycz et al., 2010). These changes at the cytological level include: cell and vacuolar size, cell wall thickness and DNA ploidy level (diploidy, haploidy, mixoploidy, endoreduplication etc.). For example, Lema-Ruminska (2011) noted extensive endoreduplication (truncated cell cycles that omit mitosis) when calli derived from cacti were acclimated to be drought or salt tolerant suggesting that these truncated cell cycles operate in tissue (s) under abiotic stress. Another example, is from calli of *Medicago truncatula* J5, which showed extensive endoreduplication when grown on callus induction medium (CIM) for 6 months (Elmaghrabi and Ochatt, 2006).

5.1.1 Flow cytometry for assessment of ploidy levels in *Medicago truncatula*

Flow cytometry is a popular technique used to measure the relative frequency of cells in different phases of the cell cycle (principally G1, S-phase and G2). It does this through analysis of the relative nuclear DNA amounts quantified by the flow cytometer. The DNA is made to fluoresce by staining with Hoechst or DAPI for A-T and Propidium Iodide or Chromomycine A3 for G-C. A flow cytometer can be considered as a fluorescence microscope capable of analyzing moving particles (e.g. cell suspensions) which exit a source of UV light and that in turn emit an epifluorescence which is filtered via a set of dichroic mirrors (Ochatt, 2006). The flow cytometer then quantifies these signals into one or more fluorescent peaks (Robinson, 2006). The flow data reported on in this chapter were obtained using a Partec PAS-II flow cytometer equipped with HBO-100 W mercury lamp and a dichroic mirror (TK420) (Elmaghrabi and Ochatt 2006; Ochatt, 2008).

Flow cytometry is the most common way to study ploidy levels. Indeed flow cytometers can measure very large populations of cells rapidly (e.g. 3000 cells in 1 minute). As such it as an invaluable tool to determine relative nuclear DNA content and ploidy of cellular populations. In so doing, the flow cytometer generates integrated peaks from which cells can be assigned to G1 (2C), S-phase (2-4C), G2 (4C), followed by one or more peaks that indicate the presence of polyploid cells as multiples of 4C where 1C is the amount of nuclear DNA in a gamete (Bergounioux et al., 1988; Ochatt, 2008; Elmaghrabi and Ochatt 2006). Fluorescent signals are transformed by the inbuilt algorithm of the cytometer into a series of peaks (where the position is measured in abscissa in channel units) and are taken to correspond, from left to

right, as the proportion of cells with nuclear DNA amounts of 2C, 4C, or multiples thereof,

Unlike microdensitometry, where the densities of prophase (4C) and half telophase (2C) can

be recorded on fixed monolayers on microscope slides, and used as internal standards to

integrate interphase nuclear DNA amounts, flow cytometry relies on an assumption that

stained nuclei moving through a fluorescence gate will generate multiple peaks that conform

to 2, 4, 8C etc. Hence flow data are interpreted on a comparative basis.

Several genotypes of *Medicago truncatula* have been analysed by flow cytometry,

enabling measurement of: true-to-type aneuploidy (less than diploid amount of DNA),

haploidy (n = x (diploidy 2n= 2x), and polyploidy. Flow cytometer profiles that have a long

tail extending to zero, indicate severe perturbation of cells that are likely to be anueploid or

senescent. However, it should be pointed out, that flow cytometry is unable to resolve a G2

(4C) from an endo G cell in its first round of endoreduplication (Fig. 5-2). Within a callus the

most likely mechanism for polyploidy is by endoreduplication, where a 4C nucleus bypasses

mitosis but enters S-phase for a second time to yield 8C nuclei. In the complete absence of

mitosis such cells are undergoing true endoreduplication (Fig. 5-2). A less common endo

cycle is endomitosis where a mitotic cell reaches late prophase / early metaphase of mitosis

but then exits into its own endo cycle (Fig. 5-2). Such cells are more common in insects than

any other eukaryotes but they have been observed in terminally differentiated cells of floral

organs (Nagl et al., 1985). For *M. truncatula* (leaf explants), the percentage of cells in each of

the component phases of the cell cycle comprise: 80 to 75% in G1 phase, 15 to 20% in S

phase and 5 to 10% G2 phase with a 2% mitotic index (Fig. 5-1) (Ochatt, 2008; Ochatt, 2006).

Any variation of this profile might indicate that the cells/ tissues sampled were from plants or tissue cultures undergoing abiotic stress (Elmaghrabi and Ochatt, 2006).

The aim of the work reported here was to study the extent to which cellular instability and endoreduplication is occurring in calli of *M. truncatula* exposed to various concentrations of NaCl for various time intervals.
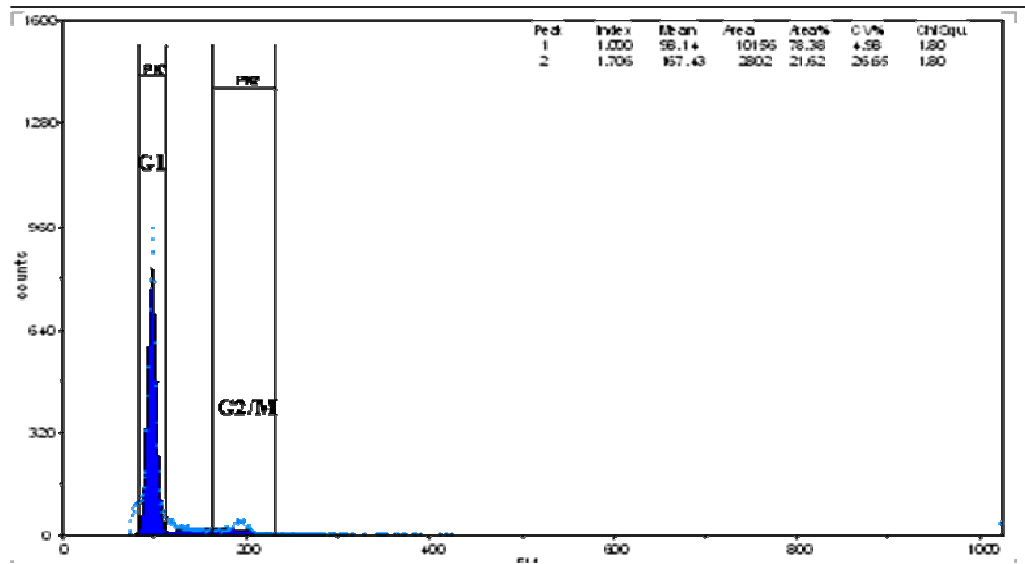
Fig 5-1. Flow cytometry analysis of leaves of seedling plants of *Medicago truncatula* A17 showing a major peak, that represents a large G1 population and a much smaller peak representing a much smaller G2 population (n = 5000). (Elmaghrabi and Ochatt, 2006). MI=2.4%
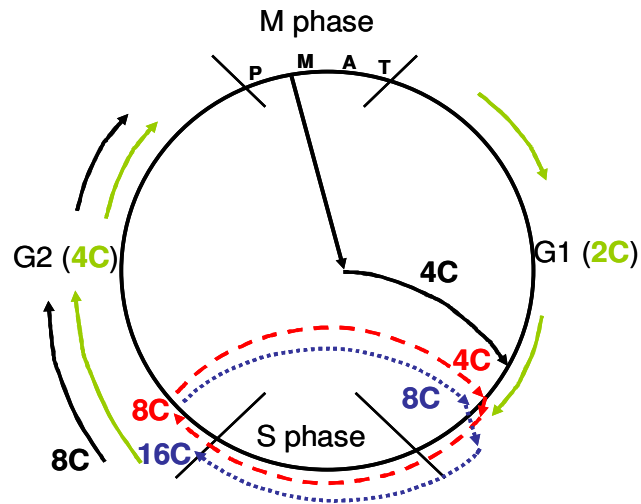
Fig. 5-2 Different routes to polyploidy.
 In the normal cycle, nuclear DNA amounts alternate between 2C and 4C via DNA replication in S-phase (green). In the first round of true endoreduplication, nuclei bypass mitosis and the 4C nucleus replicates leading to 8C (red). In the second round, the 8C nucleus replicates leading to 16C (blue). In endomitosis, the 4C nucleus exits mitosis in late prophase/early metaphase, and replicates leading to an 8C nucleus (black) where 1C is the nuclear DNA amount in a gamete.

5.1.2 Mortality under salt stress.

There is no doubt that salinity stress has severe impacts on plant cell development (Li et al., 2007). Therefore, plant cells should cope with altering environmental conditions by acclimating to salt stress by different molecular, biochemical and physiological processes. A high salinity condition in media lead to three different types of stress: ionic, osmotic, and

oxidative stress (Zhu, 2002, Leshem et al., 2007). This salt stress increase significantly the ratio of cell mortality causing cell death of those cells could not acclimate under salt stress (Huh et al., 2002). For example Huh et al. (2002) shown, that high salinity leads to programmed cell death in higher plants which could be regarded as a salt adaptation process. Programmed cell death is also obtained by various biotic or abiotic stresses in algae and higher plants such as hypersensitive response during pathogen attack (Greenberg and Yao, 2004) and heat (Zuppini et al., 2007). Therefore, I measured the rate of mortality cell under various concentration of mM NaCl (0-350) in order to investigate that salt stress have severe effect of *Medicago truncatula* cell growth development.

5.2 Materials and Methods.

5.2. 1 Flow cytometry analysis

Ten replicates of nuclear DNA content of callus derived from *M. truncatula*, under different saline treatments (0, 50, 100, 150, 250 and 350 mM NaCl) was measured by flow cytometry, at the end of Phase I on 11 month old calli.

Nuclei were mechanically isolated from about 0.2 g of calli or from a single leaf of *M. truncatula* grown in green house conditions(Ochatt, 2008). Tissue (s) were chopped roughly with a sharp razor in 400µl of nuclei extraction buffer and 1.6 ml of staining buffer (Partec®) (Ochatt, 2008). The suspension derived contains not only nuclei but also cytoplasm, vacuoles and various debris (mitochondria, chloroplasts, etc). The suspension was filtered through a 20 µm nylon mesh, and 4, 6 diamidino-2-phenylindole (DAPI) and a specific fluorochrome, was added to the filtrate to a final concentration of 1 µg/ml (Ochatt et al., 2006). As mentioned in the introduction DAPI, binds to the A-T bases of DNA and when the nucleus passes through UV light it fluoresces in blue. Then, the software in the flow cytometer converts the fluorescent signal into nuclear DNA content (Elmaghrabi and Ochatt, 2006). The mitotic index is normally measured microscopically by observing mitotic cells alongside interphase ones at random. In *Medicago truncatula*, mitoses are very difficult to observe mainly because of its very low genome size. An alternative method for estimating the mitotic index involves the formula:

$$MI = 4 \times 4C/ \Sigma \, 2C + 4C$$

Where 2C and 4C correspond to the mean integrated value of nuclei in G1 phase and G2, respectively (Ochatt, 2008).

5.2. 2 Mortality measurements.

Three stock solutions were prepared for dual propidium iodide (PI) and flouroscein diacetate (FDA) viability staining and stored at 4ºC (Table, 5-1)

Table 5-1. Stock solution composition.

Stock solution name	Composition	Concentration
PI	40µl of a concentrated stock of 25 mg of PI in 1ml water	1mg ml^{-1}
FDA	0.1g of FDA powder in 10 ml acetone	1% FDA
Sucrose solution	3g of sucrose in 100 ml distilled water	3%

5.2.2.1 Dual staining solution.

This staining solution consists of 240µl of PI stock solution (1mg ml^{-1}) and 40µl of FDA stock solution (1%) mixed together on ice and then, a sucrose stock solution (w/v, 3%) was added to complete a volume of 1ml. Then, the staining solutions were kept on ice.

5.2.2.2 Staining Procedure

A volume (75µl) from the cell suspension was added to an Eppendorf tube on ice and mixed with 75µl of dual staining solution (final concentration = 200 µg ml^{-1} FDA and 120 µg ml^{-1} PI). They were then incubated on ice for 20 min; 50µl of this mixture were added to a microscope slide and a coverslip applied gently. Live or dead cells were counted by using an Olympus BH2 fluorescent microscope at 20 x magnification. Approximately 300 cells were scored as either living (green) or dead (red). The calculation of mortality index was measured by the equation:

Mortality index = number of red cells / number of total cells (green and red) X 100.

5.2.3 Vacuolar measurements

After calli terminated Phase III   (23 months ± acclimation) in the 0, 50, 100, 150, 250 and 350 mM NaCl treatments (see Fig. 3-3 from Chapter 3). The vacuoles were measured by placing 50 mg callus in small vials containing a 1% (w/v) aqueous solution of Lucifer yellow –dipotassium salt (Sigma) for two hours. Lucifer yellow is a trace; it stains the apoplast and accumulates in the pro-vacuole, it cannot penetrate membranes but, it can penetrate the tonoplast (Oparka, 1991). Stained vacuole size was measured by image analysis using Sigmascan–pro (objective: DPlan Apo 20 UV, 0.70, 160/0.17).

5.3 Results

5.3.1   Cellular behaviour

After 5 months of callus induction on MANA medium, pieces of callus were used to establish cell suspensions on BY-2 medium supplemented with 0, 50, 100, 150, 250 or 350 mM NaCl and cultured for four months. Cell and nuclear area, and mortality were scored as indicated above.

There were no significant differences in nuclear or cell size in the 50 mM treatment compared with the control following 1, 2, 3 or 4 months continuous culture (Fig. 5-3 and Table 5-1) suggesting strongly that there was no osmotic stress ± 50 mM NaCl during four months of culture. In the 100mM treatment nuclear and cell size were significantly smaller following 1, 3 and 4 months ($P < 0.02$) but not so following 2 months of culture ($P > 0.05$). A very similar pattern was observed in the 150mM compared with 0 mM control. Cell and nuclear size was also significantly smaller in the 250 and 350 mm compared with the 0 mM NaCl control after the third and fourth months of culture (Fig. 5-3b and Table 5-2).

Cellular morphology was examined in these cell cultures. The 50, 100, 150, 250 and 350 mM NaCl treatments resulted in the migration of the nucleus from the centre toward the outside of the cell (Fig. 5-4).
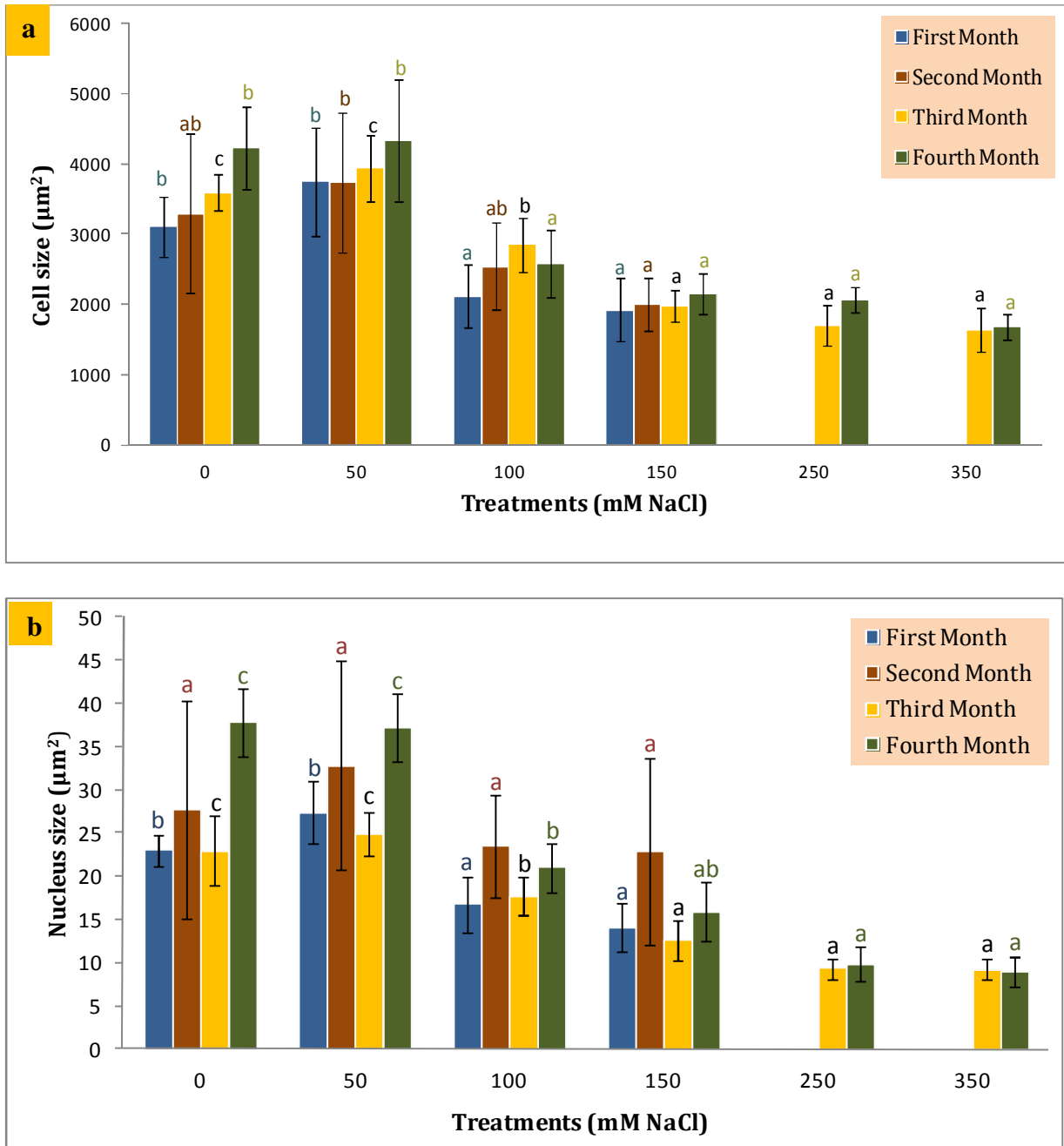
Fig 5-3. Mean ± S.E. (a) nuclear and (b) cell size (μm^2) in cell suspensions of *M. truncatula* exposed to various NaCl concentrations (mM) measured either at the first, second, third or fourth month following the start of treatment. Different letter combinations between the same coloured columns indicate significant differences between treatments compared with the 0 mM NaCl control at $P \leq 0.05$ (n = 12).

Table 5-2. Mean cell size and nuclear size (μm^2) after first, second, third and fourth months on the various salt treatments (n=12).

NaCl mM	Cell size(μm^2)				Nuclear size(μm^2)			
	First month	Second month	Third month	Fourth month	First month	Second month	Third month	Fourth month
0	3203[b]	3541[ab]	3597[c]	4226[b]	22.89[b]	25.45[a]	22.94[c]	37.88[c]
50	3749[b]	4093[b]	3950[c]	4337[b]	27.43[b]	35.50[a]	24.86[c]	32.01[c]
100	2113[a]	2546[ab]	2849[b]	2574[a]	17.50[a]	23.52[a]	17.73[b]	20.73[b]
150	1924[a]	1952[a]	1974[a]	2149[a]	14.10[a]	23.04[a]	12.61[a]	15.91[ab]
250	-	-	1700[a]	2073[a]	-	-	9.29[a]	9.74[a]
350	-	-	1642[a]	1682[a]	-	-	9.24[a]	9.03[a]

[a-c] Means between treatments of the same column which are followed by the different letters are statistically different at $P \leq 0.05$.
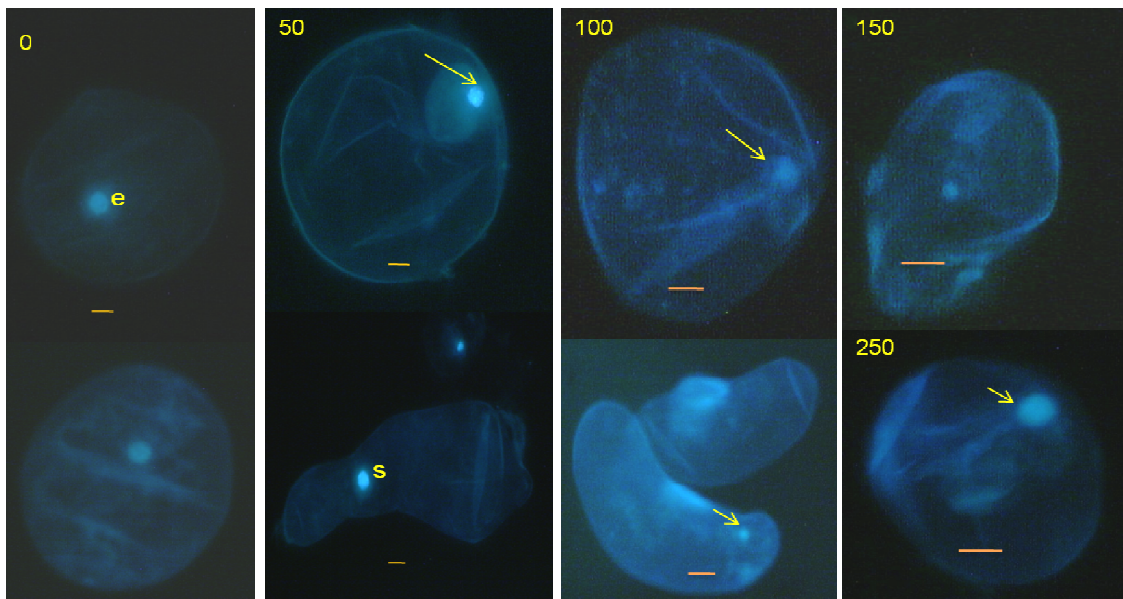
Fig 5-4. Cell and nuclear patterns at 50 cells were analysed under different concentrations of salt (mM NaCl). Arrows show migration of the nuclei to the cell wall in response to salt stress and elongation of cells in the 50 and 100 mM NaCl treatments. e points to a circular nucleus, and s points to septa forming along the presumptive cell plate of a cell undergoing cytokinesis. Bar = 10 μm

5.3.2 Viability and mortality tests.

Cell viability was determined at the end of Phase II (0, 50, 100, 150, 250 and 350 mM NaCl) using fluorescein diacetate (FDA) staining. In living cells, the esterases in the cell membrane cleave the FDA molecule so that fluorescein enters the cell and, if the membrane is intact, it cannot escape. Therefore, cells fluorescing yellow-green under the UV are metabolically active and hence viable (Fig. 5-5). In contrast propidum iodide (PI) can only penetrate dead cells (damaged membranes etc) and they stain red. Up to 300 cells were scored and percentage viablities were calculated on the basis of frequency of bright green to red cells. As the concentration of NaCl increased, the % viability decreased so that at the highest concentration (350 mM) only one fifth of the cells were viable (Table 5-3). However, it is interesting that the 150 mM treatment sustained callus growth and this was achieved with a viability of 39% (Table 5-3). Note that cell viability in the 0 mM NaCl treatment was 68%. In other words, in growing and embryogenic callus of *M. truncatula* at least a 30% level of cell death is sustained. In *M. truncatula* cells in suspension in the BY-2 medium the level of mortality was similar to the data obtained from calli (Appendix B).

Fig 5-5. a= FDA–stained and b= PI-stained cells of *Medicago truncatula* callus after 17 months (end of Phase II) of culture under 150 mM NaCl (bright green images are of living cells, dull red images are of dead/dying cells). 300 cells were scored and scale is in μm.
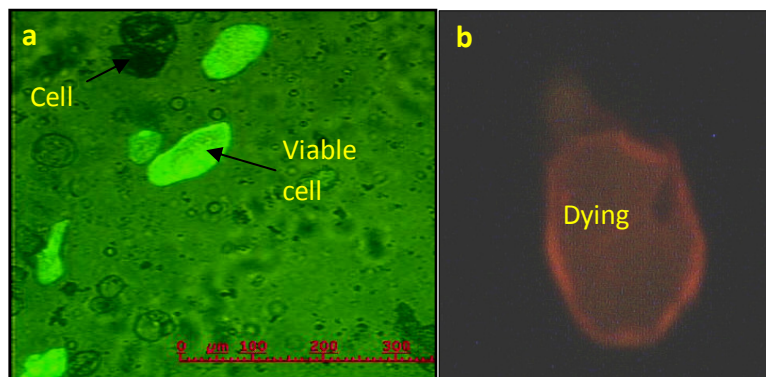
Table 5-3. Percentages (%) of callus viability at the end of Phase II (17 month old calli) on MANA medium with various concentrations of NaCl (n=3).

Treatments mM NaCl	Percentage (%) viability of callus after 17 months on MANA medium
0 control	68.00^d ± 5.2
50	69.66^d ± 6.3
100	54.33^c ± 1.6
150	39.00^b ± 3.4
250	25.00^a ± 1.96
350	21.33^a ± 1.86

[a-d] Means of the same column which are followed by the different letters indicate statistically different compared with 0 mM NaCl control at $P \leq 0.05$.

5.3.3 Results of flow cytometry (FCM)

I used this technique to measure the proportions of cells in the cell cycle in callus following Phase I (6 months of step up acclimation) under salt treatments (0, 50, 100, 150, 250 and 350 mM NaCl). Ten replicate calli for each salt concentration were analyzed and included a minimum of 3,000 to 10,000 nuclei per run. In these studies, leaves were also analysed from *Medicago truncatula* (J5) grown under greenhouse conditions; these measurements served as references and were compared with all the treatments. Flow cytometry of leaves exhibited a diploid profile comprising two peaks, corresponding to the nuclei in G1 phase of mitosis (2C DNA) and those in G2 / (4C DNA): G1 77.39%, S 17.32% and G2.29% and a calculated mitotic index of 2% (Fig. 5-6).
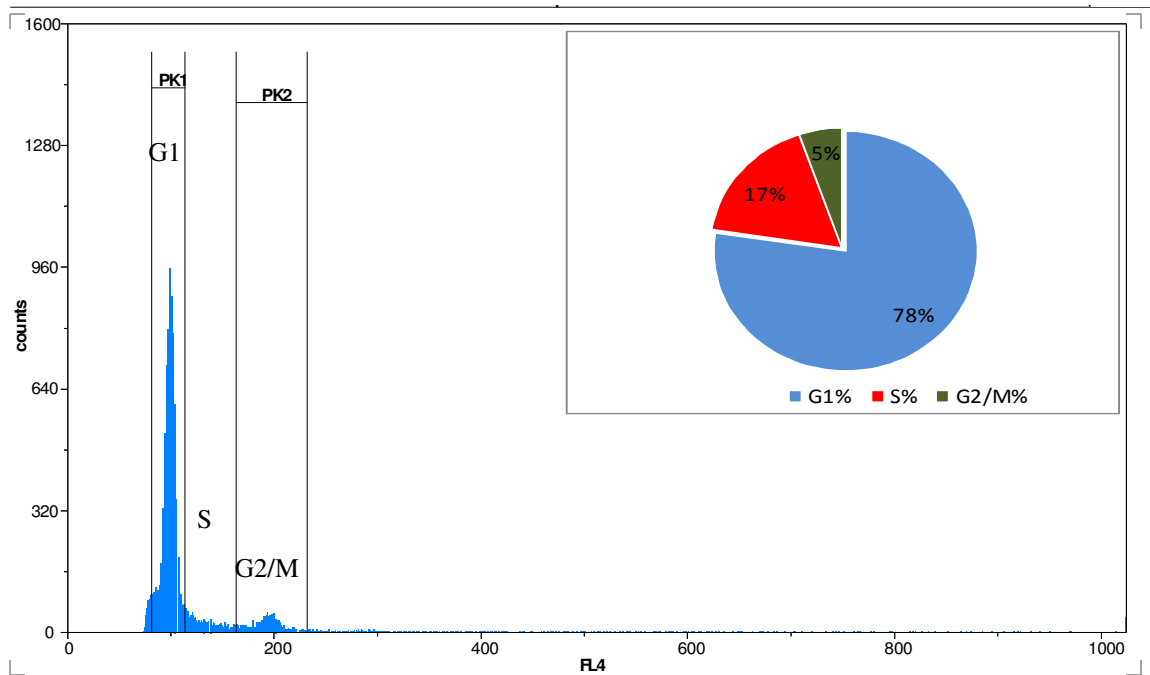
Fig 5-6. FCM profile of leaves of *Medicago truncatula* J5 (diploid normal), and percentage distribution of nuclei in G1, S, and G2 phases. All nuclei were stained with DAPI.

This type of result was also observed in control calli maintained on 0 mM NaCl. Hence, the cells from callus exhibited a very similar pattern of flow cytometric peaks to the leaves from which they were derived. In the 50 mM NaCl treatment, there were three peaks but with wider amplitudes compared with leaf-derived cells (Fig. 5-7b). The first peak is consistent with a population of cells with the 2C nuclear DNA amount, falling in exactly the anticipated location for G1 cells as shown for leaf cells in Fig. 5-7a. It then follows that the second and third peaks represent 4C and 8C, respectively in the 50 mM NaCl treatment. Note that the 4C peak will be mixture of diploid 4C cells, and 4C cells that are beginning to endoreduplicate (i.e. between 4 and 8C Fig 5-7b). The algorithm scores this peak as "S-phase" that comprises

S-phase and endo S-phase. What is clear is that the 50 mm NaCl treatment induced polyploidy and that the 8C peak has probably arisen by endoreduplication. However, given that the calculated mitotic index is 2.8% for this treatment, this might suggest that the increase in ploidy has arisen by an endomitosis, where cells enter mitosis but exit at metaphase to accumulate as 4C nuclei. However, to do this repeatedly, there would also need to be the presence of 8C endomitotic cells, a condition that is quite rare for plant systems. In the 100 mM treatment there is a normal peak of 2C cells together with an abnormally distributed peak at S-phase that then tails off with increasing amounts of nuclear DNA. Presumably, this is an indication of cells that are involved in normal S or endo S-phase (Fig. 5-7c). The same is true for the 150 mM treatment (Fig. 5-7d) where there is an even higher proportion of cells in this putative endo S phase.

In the 250 mM treatment, cells continued to be detected with the 2C and 4C amount, of nuclear DNA and in this case there was a more normally distributed population in S-phase (Fig. 5-7e). Such cells may well be undergoing endoreduplication although as mentioned above, cells continue to go through mitosis in this treatment (Fig. 5-8). Finally, in the 350 mM treatment, a single 2C peak was detected consistent with cells being in G1 of the cell cycle (Fig. 5-7f). However, from the calculated mitotic indices (Fig. 5-8) some cells continue to go through mitosis at this [NaCl] even though the majority are in G1 (Fig. 5-7a).
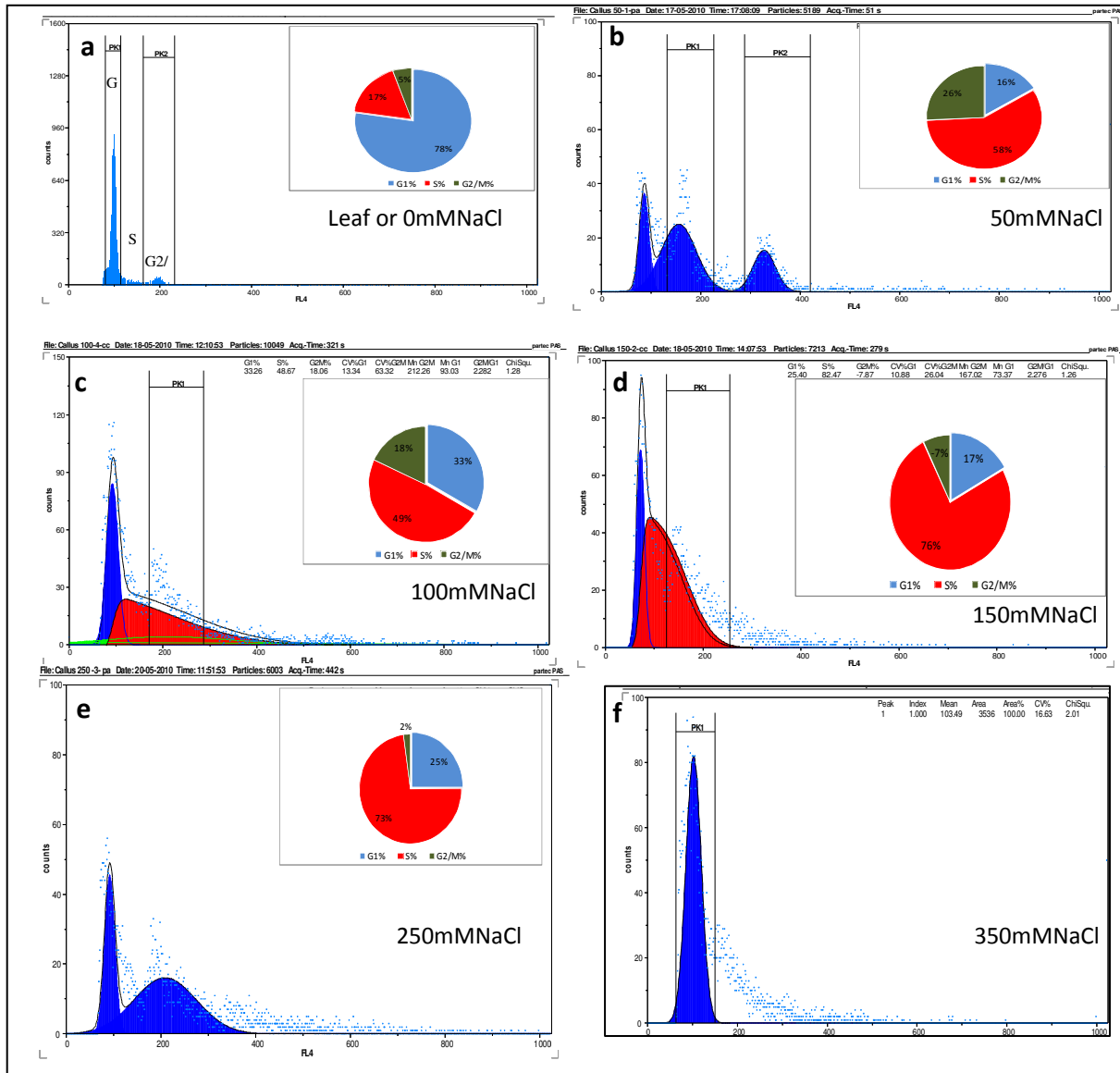
**Fig 5-7.** FCM profile of calli after termination of Phase I (step up acclimation).

a = Leaves of *Medicago truncatula* J5 at 0 mM NaCl (diploid normal), and percentage distribution of nuclei in G1, S, and G2 phases. All nuclei were stained with DAPI.

b = 50 mM NaCl treatment and percentage distribution of nuclei in G1, S, and G2/M phases. Note that the 58% calculated by the algorithm includes cells in S-phase (leading to 4C, G2 nuclei) or cells in endo S- phase.

c = 100 mM NaCl treatment, percentage distribution of nuclei in G1, S (includes endo-S) and G2.

d = 150 mM NaCl treatment and percentage distribution of nuclei in G1, S (and endo-S) and G2 phases.

e = 250 mM NaCl treatment (percentage distribution of nuclei in G1, S, and G2 phases.

f = 350 mM NaCl treatment providing a single peak of cells in G1

In the 50 through to the 350 mM NaCl treatments, the calculated mitotic index is significantly higher than in the leaf or zero NaCl treatments (Fig. 5-8). Hence despite the presence of endoreduplicating nuclei, cells continue to divide in all of the NaCl treatments compared with the 0 mM NaCl control.

Treatments	Leaf	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl	250 mM NaCl	350 mM NaCl
Mitotic index	1.999	2.27	2.776	2.768	2.808	2.75	2.65
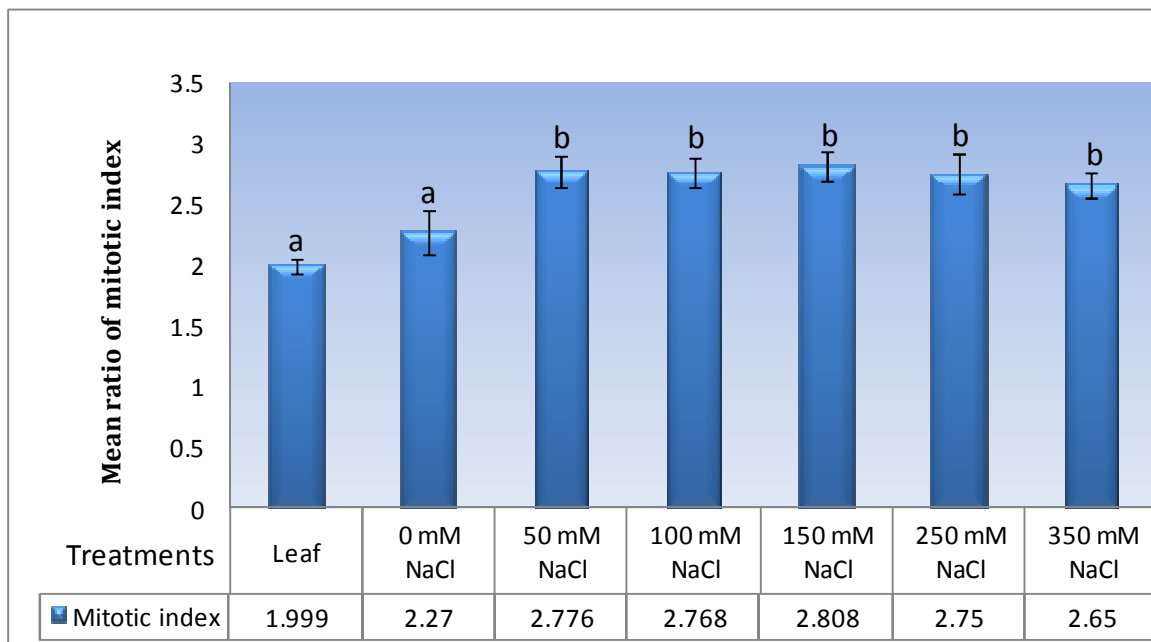
Fig 5-8. Mean (± S.E.) mitotic index   in callus tissues after calli terminated 6 months of Phase I (step- up acclimation) on mM NaCl. Note different letter indicates significant between treatment differences compared with the 0mM NaCl control. ($P \leq 0.05$) (n=5).

5.3.4 Vacuolar size is positively related to [NaCl] over the range 0-150 mM

The vacuolar size was measured following Phase III (23 months old calli). There were no significant differences in vacuolar size in the 50 mM NaCl compared with the 0 mM control. However, in the 100 and 150 mM NaCl treatments vacuolar size was significantly larger when compared with the control whilst in the 250 and 350 mM NaCl treatments, there was a significant decrease in vacuolar size compared with the control (Fig. 5-9).
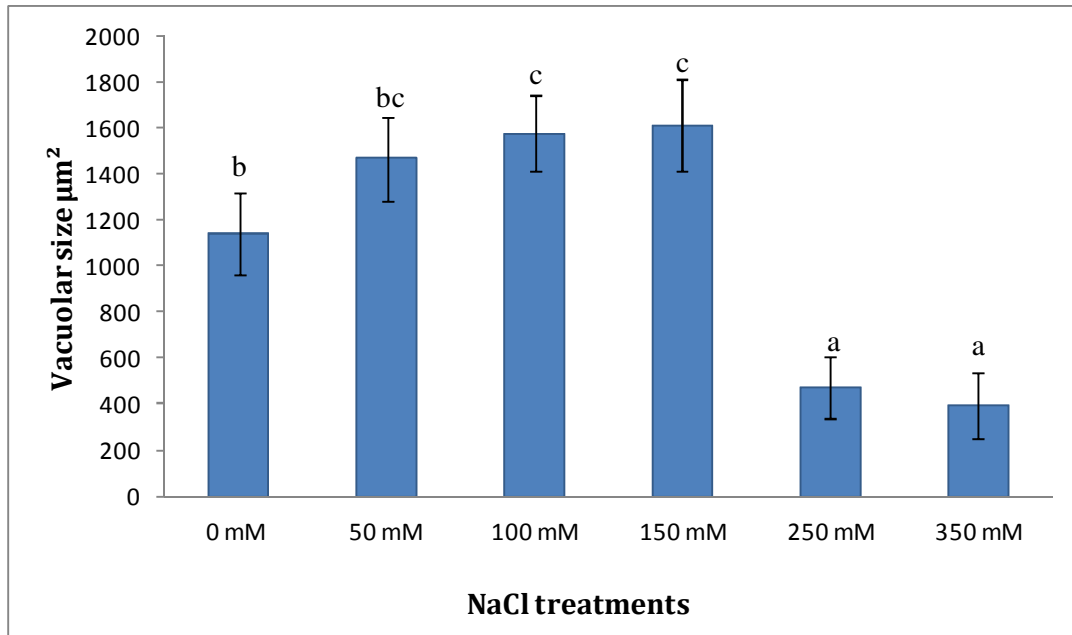
Fig 5-9. Mean ± S.E. vacuole size (μm^2) of cells from calli after 23 months (end of Phase III) under different concentrations of (0-350) mM NaCl. Note different letters indicate significant differences compared with the control ($P \geq 0.05$) (n=12)

5.4 Discussion

There is no doubt that osmotic stress has negative effects on cell division in cultured plant cells (Gu et al., 2004; Ochatt et al., 1999). Clearly, callus growth must be influenced by the extent of cell division and polyploidy that occurs during the NaCl treatments. In this chapter I reported on the extent of normal and truncated cell cycles ± NaCl treatments. Observing mitoses was virtually impossible, and might relate to the very small genome size for this species (0.48 pg; Ochatt, 2008) and perhaps to the smallness of the chromosomes in mitotic cells of *M. truncatula*. However, the presence of septa between cells and diffuse staining with Hoechst indicated cells about to undergo cytokinesis and cells in mitosis, respectively (see Fig. 5-4). Cell and nuclear size increase during the cell cycle as both cell and nuclear mass double (Kondorosi et al., 2000). Hence, for proliferating cells a constant nuclear and cell size ratio might be predicted during the cell cycle (Kondorosi et al., 2000, Ochatt, 2008). However, I wanted to discover whether cell size became uncoupled from nuclear size as a result of salt treatment. In addition, I tried to determine whether any markers of cell to nuclear size could be used as a marker in callus showing good growth or regenerative potential in culture. For example, any significant increases in cell size might be consistent with endopolyploidy and subsequent poor growth. However, careful interpretation of these cellular phenotypes is necessary because, of course, cell size increases could simply be caused by increased osmolarity in the medium. On the other hand, an increase in nuclear size *per se*, might be indicative of increased amounts of nuclear DNA arising from endoreduplication. Although there was a trend of smaller nuclear and cell sizes in the 100 and 150 mM treatments, significant decreases were not immediately obvious compared with the controls.

On the other hand NaCl treatments resulted in the migration of the nucleus from the centre toward the outside of the cell. This type of perturbation might be an effect of salt at the cellular level and may be related to negative growth responses of cells in culture to increasing concentrations of NaCl (Fig. 5-4), as they reflect typical cellular responses to osmotic stress, where various cell parameters including cell wall thickness, vacuole volume and plastid rearrangements are known to alter (Ochatt and Power, 1989).

Given the relative constancy of cell and nuclear size in the 0 - 150 mM treatments I next measured the proportion of cells in G1, S phase and G2, to test the extent of "normal" compared with "perturbed" cell cycles ± NaCl. The 50 mM NaCl treatment resulted in data that were clearly indicative of normal and endoreduplicating cell cycles. This phenomenon of endoreduplication was reported by Elmaghrabi and Ochatt (2006) in various genotypes of *Medicago truncatula* including the cultivar J5 and two mutants TRV25 and TR122 . Recently Lema-Ruminska (2011) showed that endoreduplication occurred during acclimation of somatic embryos of Cactus (*Copiapoa ternuissima* Ritt.) to drought conditions. Flow cytometry profiles presented here showed the presence of polyploid cells alongside diploid cells in the 100 and 150 mM NaCl treatments. The polyploids might well arise by endoreduplication with 76 % of nuclei in S phase in the 150 mM NaCl treatment compared with 17 % in controls (0 mM NaCl) (Fig. 5-7a). Therefore, these treatments (100 and 150 mM NaCl) where an unusual peak was observed in S-phase, could well be a mixture of cells in S-phase and cells in endo-S-phase. That 100/150 mM NaCl sustained very high rates of growths means that the calli in these treatments were able to tolerate endoreduplication or that increased cell expansion of endoreduplicating cells contributed to the fast rates of growth

observed in these NaCl treatments. However, this seems unlikely given that cell size tended to be smaller in these treatments compared with the 0 mM NaCl control. In the 350 mM treatment, there was one peak consistent with the majority of cells arresting in G1. This kind of result was also reported by Ochatt (2008), Elmaghrabi and Ochatt (2006), and Prado et al., (2010) where a single peak was obtained when the tissue / calli were very old (Ochatt 2006). Note also that viability decreased very significantly in callus in the 250 and 350 mM NaCl compared with the 0 mM control (section 5.3.2). In addition, mean callus growth in the 250 and 350 mM NaCl was very low or reduced to zero. (chapter 3, Fig. 3-14).

Mitotic index was significantly higher in the 50 -350 mM than in the leaf or zero NaCl treatments (Fig. 5-8).Whether these mitotic cells are normal or endomitotic or abberant showing chromosomal damage is unknown. This type of profile is very frequent when tissues are placed under long-term stress (Ochatt, 2008; Elmaghrabi and Ochatt, 2006).

To avoid the impacts of sodium (Na$^+$) toxicity, tolerant plants have developed mechanisms that sequester sodium into vacuoles (Wei et al., 2011). Indeed, several researchers have demonstrated that many plant species tolerant to NaCl have mechanisms that compartmentalize sodium into vacuoles, or can exclude sodium from the cell (Li et al., 2010; Blumwald et al., 2000) This sequestration process is mediated by Na$^+$/H$^+$ antiporters bound to the plasma membrane and tonoplast (Li et al., 2007; Blumwald et al., 2000). In my study, vacuolar sizes measured following Phase III were significantly larger in the 100 and 150 mM NaCl compared with the control treatment (Fig. 5-9). Hence, vacuolar size increased in these treatments (100 and 150 mM), known to sustain faster growth than the controls (see Fig 3-17

in chapter 3). Hence it may be the case, that this increase in vacuolar size was able to sequester more Na^+ ions into the vacuole. In contrast the 250 and 350 mM treatments resulted in small vacuoles that, in theory, were less able to store $[Na^+]$ correlating with very poor growth in these treatments. Enlarged vacuoles have been consistently observed in the shoot tissues of halophytes compared with small vacuoles in glycophytes, suggesting that sodium compartmentalization is more efficient in halophytes (Moghaieb et al., 2004). For example, in a succulent halophyte species (*Halostachys caspica*), vacuolar size enlarged when cells were cultured on medium supplemented with 175 mM NaCl; these enlarged vacuoles may give cells an increased capacity to sequester Na^+ (Zhu, 2003). In *M. trucatula* cells grown in BY-2 medium supplemented with NaCl (50-150 mM), nuclei migrated to the periphery of the cell compared 0 mM control. This migration may be occurring because of increased vacuolar expansion in the NaCl treatments.

5.5 Summary

The data reported in this chapter are summarised as follows:

- Nuclear and cell size shrinkage at $\geq$ 100 mM NaCl in cells grown in BY-2 cell suspension medium

- At 100 and 150 mM NaCl, a substantial fraction of cells was detected in S phase or endo S phase. Notably, at 350 mM, cells arrested in G1.

- In the 250 and 350 mM NaCl treatments cell viability decreased sharply compared with the controls.

- The largest vacuoles were observed in 100 and 150 mM NaCl, treatments, which in turn, sustained faster rates of growth compared with the 0 mM control (see Ch.3).

Chapter 6: Gene expression under salt stress at the end of Phase I (6 months), Phase II (12 months) and Phase III (18 months).

6.1 Introduction.

Thus far in this thesis, the data demonstrate increasing tolerance of cultured *M. truncatula* explants to NaCl following Phase I (50 mM), Phase II (100mM) and Phase III (150 mM) of the full protocol, first described in Chapter 3 (Fig. 3-3). Moreover, somatic embryogenesis was well sustained at 100 mM NaCl, [Na+] / [K+] homeostasis was detected at 150 (-160) NaCl, and the largest vacuoles were detected at 150 mM as was proline accumulation (chapter 4: Fig. 4-8). In this chapter, I report on patterns of gene expression following Phases I, II and III that might be linked to each of these traits. Five genes were chosen: *SERK* (somatic embryogenesis), *WEE1* (growth/cell cycle), *CCS52* (endoreduplication), *SOS1* ($[Na^+]$/ $[K^+]$ homeostasis) and *P5CS* (proline biosynthesis). In the next section I provide a brief over-view of these genes.

6.1.1 *SERK* (Somatic Embryogenesis Receptor-Like Kinase)

Somatic Embryogenesis Receptor-Like Kinase (SERK) genes encode leucine rich repeat receptor-like kinases (LRR-RLKs) and are expressed in all organs of the plant (Kwaaitaal and

de Vries, 2007). However, they are mainly activated during embryo induction and early somatic embryo development as reported in carrot and Arabidopsis (Schmidt et al., 1997; Somleva et al., 2000; Hecht et al., 2001). This gene is highly expressed in many plant species undergoing somatic embryogenesis including: *Zea mays* (Baudino et al., 2001); *Helianthus annuus* (Thomas et al., 2004); *Citrus unshiu* (Shimade et al., 2005); *Oryza sativa* (Hu et al., 2005); *Vitis vinifera* (Maillot et al., 2009); *Araucaria angustifolia* (Steiner et al., 2012) and *Pinus nigra* (Salaj et al., 2011). In *M. truncatula,* somatic embryogenesis is also correlated with high expression of *MtSERK1* (Nolan *et al*., 2003). More recent work suggests *MtSERK* contributes to a signaling pathway that regulates development *in vitro* (Nolan et al., 2009). This would be consistent with a function for this gene in regulating totipotency, where cellular reprogramming involves induction of specific genes that promote embryo formation (Thomas et al., 2004; Nolan et al., 2009). However, caution is still required in interpreting the effects of *SERK* expression. Note that there is a family of *SERK*s that may overlap in function whilst specific *SERK* genes may be unique (Albrecht et al., 2008). In addition, it is well-known that in *in vitro* expression studies, *MtSERK1* is expressed in response to a specific balance of auxin and cytokinins that then leads to somatic embryogenesis (Nolan et al., 2003; Steiner et al., 2011). On the other hand, de Lorenzo et al (2009), reported that *Leucine-Rich Receptor-Like protein Kinase (*LRR-RLKs) plays a fundamental role in responses the sensitivity of *M. truncatula* roots to salt stress. Expression of this gene is quickly and highly increased in response to salt stress in roots and promoter-β-glucuronidase *(GUS)* fusions, proposing that *Srlk* is up-regulated in the root epidermis (de Lorenzo et al., 2009).

6.1.2 Cell cycle related genes

A little is known about cell cycle genes in plants exposed to high levels NaCl stress. In Arabidopsis, a negative regulator of mitosis, *WEE1,* is strongly expressed in reponse to abiotic stress (De Schutter et al., 2007). This gene has also been studied extensively in the Cardiff lab but not in the context of salinity or drought. Therefore, I have chosen *WEE1* together with *CCS52*, a regulator of ploidy levels, were incorporated in my study in oreder to investigat the altering the expression of these genes under salt or drought stress.

The cell cycle describes the history of a proliferative cell from one mitosis to the next. In general, several different cell types are produced during development of multicellular organisms. However, all differentiated cells of the plant are descendants of proliferative cells, populations of cells that mostly reside in meristems and are continually going through the cell cycle (Potuschak and Doerner, 2001). During the cell cycle, a cell prepares for mitosis. During interphase of a proliferative cell cycle, and at the level of the chromosome, single arm chromosomes (born as a result of mitosis) undergo nuclear DNA replication to form double armed chromosomes during DNA synthetic phase (S phase), that ultimately become competent to separate once more into single arm chromosomes as a result of mitosis. Between the separation of chromatids in mitosis and the duplication of single arm chromosomes during S phase, there are two so-called gaps: G1 (pre-synthetic interphase) and, G2 (post-synthetic interphase) (Verkest et al., 2005). During interphase cell mass doubles (mostly during G1) (Eckardt, 2001) (Fig. 6-1). Cells in G2 phase possess twice the amount of nuclear DNA than those in G1. The gap phases represent intervals when the cell is preparing for DNA

replication and is becoming competent for mitosis. Key points in these transitions are G1/S and G2/M. When cells are stressed by abiotic factors that result in DNA damage or perturbation of DNA replication, a series of genes is expressed; they encode proteins that function in cell cycle checkpoints that prevent the G1/S and G2/M transitions. Only when checkpoints are satisfied can cells undergo the G1/S and G2/M transitions (Rhind and Russell, 2000).
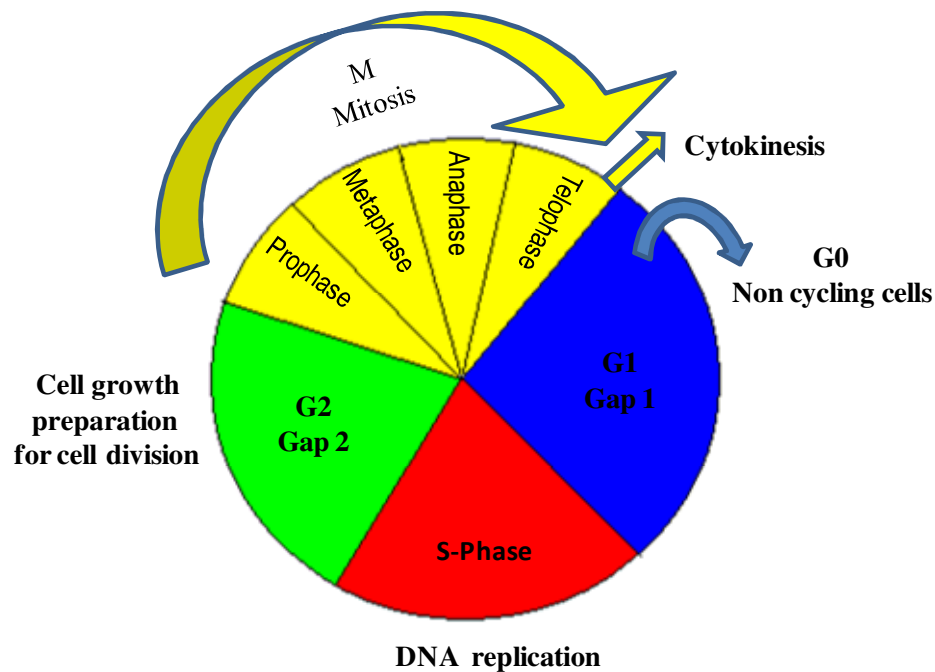
Fig 6-1. The cell cycle and its component phases. The cycle is divided into several phases an initial gap (G1), Synthesis of DNA (S), a second gap (G2) and the final mitotic nuclear and cellular division (M) that results in two identical daughter cells, or one larger and one smaller as a result of asymmetric division that is very common in higher plants . Non cycling cells that arrest in G1 are said to be in "G0"

6.1.2.1 *WEE1*

The first *WEE1* was indentified from S*chizosaccharomyces pombe (Spwee1)* where it causes a delay in mitosis by phosphorylating Cdc2 at tyrosine15 (Russell and Nurse, 1987). In most eukaryote organisms, there are both positive and negative regulators of the G1/S and G2/M transitions. WEE1 kinase consists of a catalytic domain (C-terminal) and a regulatory domain (N-terminal) (Dewitte and Murray, 2003; Inze and De Veylder, 2006). At G2/M, expression of *WEE1* from S*chizosaccharomyces pombe (Spwee1)* delays or blocks mitosis by phosphorylating another protein Cdc2 on its tyrosine15 residue and this inactivates Cdc2 (Russell and Nurse, 1987). The latter is a protein kinase that in its dephosphorylated form (at tyr 15) and bound to a partner cyclin, drives cells into mitosis (Nurse, 1990). In plants, a homologue to *wee1* was demonstrated in *Arabidopsis thaliana* (Sorrell et al., 2002), *Zea mays* (Sun et al., 1999), *Oryza sativa* (Guo et al., 2007) and *Solanum lycopersicum*, (Gonzalez et al., 2007).

Moreno (1990) reported in fission yeast that over expression of *SpWee1* and the protein phosphatase, *Spcdc25* results in short and long cell length phenotypes, respectively. Hence, both genes play an essential role in controlling cell size (Russell and Nurse, 1987). Over-expression of *ZmWEE1* or *Arath;WEE1* in S*chizosaccharomyces pombe* prevented cell division leading to a long cell phenotype (Sun et al., 1999; Sorrell et al., 2002). In Arabidopsis, its expression was confined to actively dividing regions of the plant (Sorrell et al., 2002). Furthermore, *Arath;WEE1* could phosphorylate CDKA, CDKB, and CDKD *in vitro* (Shimotohno and Umeda, 2007). *Arath; WEE1* over-expression in Arabidopsis results in

slower growth in transgenic plants (Grønlund et al., 2009). However, Arabidopsis *wee1* knock out mutants were reported to show a normal phenotype under an optimal growth environment, but they were hypersensitive to treatments that induce DNA damage (De Schutter et al., 2007). Mutants of Arabidopsis deficient in *WEE1* develop normally raising doubts about a role of WEE1 in a normal cell cycle (De Schutter et al., 2007). However, in wild type plants, *WEE1* transcription is activated in response to hydroxyurea and zeocin, substances that induce the DNA replication and DNA damage checkpoints, respectively (De Schutter et al., 2007). *WEE1* expression is also associated with endoreduplication in some species such as tomato (Gonzalez et al., 2004, 2007) and maize (Sun et al., 1999). In my study I focused on the role of *WEE1* as a possible target gene responsive to salt and drought stress under multiple step up acclimation and subsequent long-term phases of tissue culture in callus derived from *M. truncatula*.

6.1.2.2 *CCS52* (Cell-Cycle Switch 52)

CCS52 gene was later designated Cell-Cycle Switch 52 due to its proposed function in mediating the switch from the mitotic divison cycle into endoreduplication cycle (Su'udi et al., 2012). *CCS52* expression is positively related to endoreduplication (duplication of the genome without mitosis or cytokinesis) (Angel et al., 1999). Endoreduplication leads to doubling in the nuclear DNA content causing amplification of the genome; which can result in enlarged nuclear and cell size (Nagl et al., 1985). Endoreduplication in some plants is a fundamental part of development (Barow and Meister, 2003). However, the universal importance of endoreduplication for plant development must be questioned since Barrow and

Meister (2003) also listed many species of angiosperm that simply do not exhibit endoreduplication in developing tissues. In such plants, endoreduplication is restricted to terminally differentiated or short-lived cells of reproductive organs (e.g. the tapetal layer of the anther sac and in cells of the embryo suspensor). Moreover, there are monocot species that do not show any signs of endopolyploidy (El-shourbagy and Koshk, 1975). Endoreduplication may be a stress response at the level of the cell cycle (Adachi et al., 2011). Hence, examining the expression of *CCS52* in cell cultures exposed to long term salt treatments, was of interest in relation to the endopolyploidy that was observed in these cultures (see chapter 5).

6.1.3 *SOS* (Salt Overly Sensitive)

In highly saline conditions tolerant ecotypes exhibit mechanisms that accumulate NaCl in vacuoles or exclude Na^+ (Feki et al., 2011; Zsigmond et al., 2012). In this regard the *SOS* gene family is important in maintaining ion homeostasis and in conferring salt tolerance (Shi et al., 2000; Feki et al., 2011) and has an important role in preventing sodium accumulation in the cytosol (Blumwald, 2000). The SOS genes encode for Na^+/H^+ antiporters or exchangers and perform an essential role in NaCl homeostasis. It has been demonstrated that for growth in high [NaCl], by transferring excess Na^+ from the cytosol into the vacuole, or out of the cell to prevent its toxic accumulation (Fig. 6-2) (Türkan and Demiral, 2009; Smith et al., 2010). In Arabidopsis*, SOS1* and *NHX1* (Na^+/H^+ exchanger) are considered to be very important for increased sequestration of Na^+ into root and shoot vacuoles and seem to be central to salt tolerance mechanisms (Blumwald et al., 2000; Padan et al., 2001; Shi et al., 2003; Pardo et

al., 2006). Expression of *SOS1* was detected without salt treatment but was up-regulated significantly by salt stress; transcripts were more abundant in roots than in shoots (Qiu et al., 2003) which would fit with the action of a salt tolerance mechanism(s) in the roots of the plant. Their work also revealed the expression of *SOS1*-like protein kinase complex genes, *SOS3/SOS2* that encode proteins, which contribute to the tolerance pathway (Türkan and Demiral, 2009).
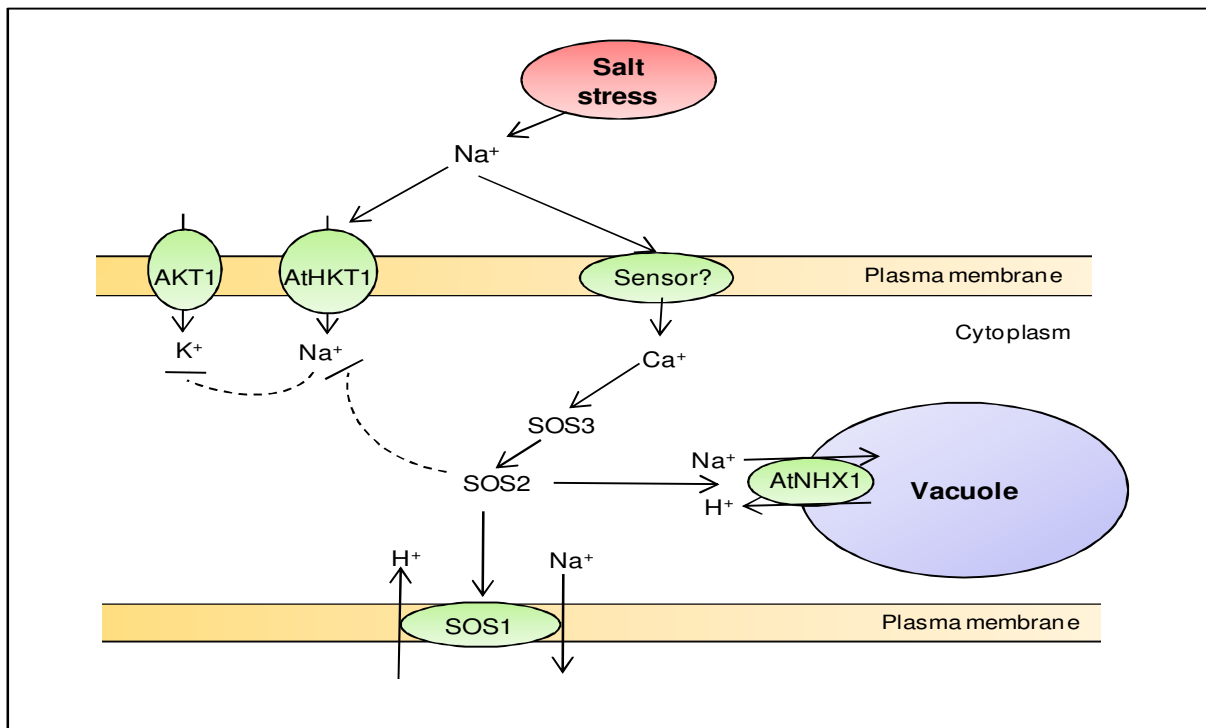
Fig. 6-2 Signaling pathways of SOS that maintain low [Na$^+$] in the cytoplasm under salt stress conditions. Excessive Na$^+$ perceived by unknown sensors induces a Ca^{2+} dependent pathway leading to a SOS 3/2 protein kinase complex, which in turn activates the membrane bound Na$^+$/H$^+$ antiporter, SOS1. SOS2 also activates the antiporter Na$^+$ in/H$^+$ out (AtNHX1) at the tonoplast, compartmenting Na+ into the vacuole and releasing H+ into the cytoplasm. Negative regulation of AtHKT1 at the plasma membrane can be imposed by SOS3/SOS2 that in turn can have a negative effect on membrane bound K$^+$ in H$^+$ out antiporter, AKT1 that normally regulates K$^+$ uptake into the cytoplasm. (Adapted from Türkan and Demiral, 2009).

6.1.4 *P5CS* (Pyrroline-5-carboxylate synthetase)

It is well-known that proline and other organic compounds (e.g lysine, betaine, glycine and arginine) accumulate under osmotic stress. In plants there are positive correlations between acclimation and increased concentration of the above mentioned amino acids (Delauney and Verma, 1993). Of these, proline accumulation in response to drought and salinity stress is very common in unrelated species such as fungi, bacteria, and many higher plants. It is synthesised from glutamic acid via pyrroline-5-carboxylate (P5C) by two successive reducing enzymes: pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), respectively and both reach high levels of activity in plant tissues and cells under abiotic osmotic stress (Delauney and Verma, 1993; Perassolo et al., 2011) (Fig. 6-3). Expression of the *P5CS* gene reflects subsequent proline accumulation in plants subject to abiotic stress (Kishor et al., 1995; Igarashi et al., 1997; Ji-Bao et al.,2009; Choudhary et al., 2005).

Fig. 6-3 General pathway of proline metabolism in plants. Key: P5C = pyrroline-5-carboxylate, P5CS = pyrroline-5-carboxylate synthetase, P5CR= pyrroline-5-carboxylate reductase, P5CDH = pyrroline-5-carboxylate dehydrogenase, PDH= proline
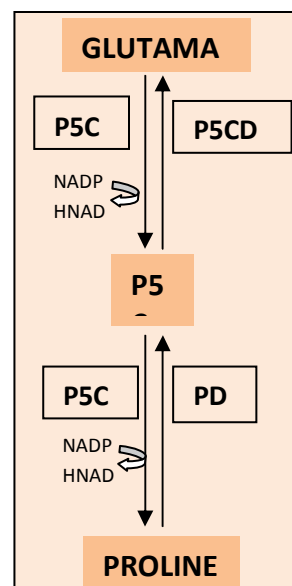
In this chapter I report on genes whose encoded proteins are known to be involved in development, regulation of the cell cycle, salt sequestration, protection against ionic stress, and protection against osmotic stress:

- *SERK*

- *WEE1*

- *CCS52*

- *SOS1*

- *P5CS*

Expression of these genes was measured by real-time PCR in callus following Phases: I, II and III.

6.2 Materials and Methods

6.2.1 RNA extraction

Total RNA was extracted from leaves of *Medicago truncatula* and also from the callus after 5 months growth on MANA medium and 3 months on different concentrations of NaCl (0, 50, 100 and 150 mM) and also 1 month on 250 mM NaCl. About 200 mg of leaf or calli was ground to a powder in the presence of liquid nitrogen using a sterile pestle and mortar (pre-chilled at -20 ^{0}C). Towards the end of grinding, 2 ml of TRI Reagent (Sigma Aldrich, UK) was added to the mix so that a homogeneous paste was formed. The paste was then transferred to a 1.5 ml Eppendorf tube, vortexed briefly (15 sec) and left at room temperature for 5 min, before being centrifuged at 12000 rpm for 10 minutes at 4 ^{0}C in a microcentrifuge (BECKMAN COULTER$_{TM}$- Allegra^{TM} 12R Centrifuge). The resulting supernatant was removed and added to 0.2 ml of chloroform, vortexed (10 sec) and left to stand at room temperature for 5 min. This was followed by centrifugation at 12000 rpm in a microcentrifuge (as above) for 15 min at 4 ^{0}C. The supernatant produced contained an aqueous layer of RNA which was carefully removed, mixed with 0.5 ml of isopropanol and left to stand at room temperature for 10 min. The mix was then centrifuged for 10 min at 12000 rpm in a microcentrifuge at 4 ^{0}C (as above). The supernatant was removed and the pellet washed in 1 ml of 75% ethanol. A quick vortex followed before another round of centrifugation for 10 min at 12000 rpm in a microcentrifuge at 4 ^{0}C (as above). The supernatant was removed and the

pellet air-dried for 30 min in a laminar flow cabinet. The pellet was then resuspended in 50 µl sterile distilled water and mixed using a pipette tip before being stored at -80 ^{0}C.

6.2.2 Purification of RNA

DNase treatment was used to remove the residual genomic DNA. Depending on the RNA concentration, from 2 to 16 µl of RNA (2µg) were added to a solution of 2 µl of RQ1 DNAase 10X buffer (Promega), 2 µl of RQ1 DNAse and sterile water to a 20 µl final volume and were incubated at 37 ^{0}C for 10 min. To stop the reaction 2 µl of RQ1 DNAse stop solution were added to the mix, and incubated at 65 ^{0}C for 10 min.

6.2.3 cDNA synthesis for testing primers

After DNAase treatment, RNA was used to synthesize the cDNA. Oligo (dT)$_{15}$ primer (1 µl of 500 µM/ ml) (Promega) was added to 19 µl of treated RNA (25 µM/ ml) and incubated for 10 min at 70 ^{0}C then followed by 10 min at 4 ^{0}C. The deoxy poly T primer anneals to the poly A tail of the RNA in the sample. All the incubation steps were performed in the GeneAmp$^®$ PCR system 2700. After this step, 6 µl 5X MLV-RT buffer (Promega), 2 µl of 0.1 M DTT (dithiothreitol-reducing agent as stabilizer) (6.6 mM/µl) and 2 µl of 10 mM dNTPs (0.66 mM/µl) were added and the sample was incubated at 42 ^{0}C for 2 min. After this step 1 µl of

M-MLV Reverse Transcriptase (Promega) was added to the reaction and incubated at 42 ^{0}C for 50 min. Then the sample was heated to 70 ^{0}C for 15 min to inactivate the enzyme. This reaction produced single stranded cDNA that was stored at -80 ^{0}C until future processing.

6.2.4 PCR amplification

To check the cDNA for quality and quantity it was subjected to PCR amplification using primers specific for 18S rRNA. The primers used were: PUV2 F 5`-TTCCATGCTAATGTATTCAGAG-3`: PUV4 R 5`-ATGGTGGTGACGGGTGAC-3`. The 1 µl cDNA (8 ng/µl) was amplified using a mix composed of 1 µl (10 µM) of each primer (0.4 µM/µl), 0.5 µl (10 mM) of dNTPs (0.2 mM), 2.5 µl of PCR buffer 10X (5000 mM Tris-HCl [pH9], 15 mM MgCl$_2$), 0.125 µl of Taq polymerase (Promega) and 18.9 µl sterilized water. Reactions were run on a GeneAmp$^®$ PCR system 2700 with conditions as described in Table (6-1).

Table 6-1. PCR Conditions

PCR Profiles	Time/ minutes	Temperature/ ^{0}C	Number of cycles
Denaturation	2	95	1
Denaturation	1	95	35
Annealing	1	55	35
Extension	1	72	35
Final extension	15	72	1

6.2.5 Agarose gel electrophoresis

6.2.5.1 Gel electrophoresis of RNA

A suitable tank, tray and comb were soaked in 0.1M NaOH for at least 10 min and washed in distilled water prior to use. RNA samples (8 µl) were added to 1.5 µl dedicated loading buffer (0.25% Bromophenol blue, 150 mM Tris-HCl [pH7.6], 30% glycerol in sterile distilled water) and then run on a 1% agarose gel containing 1 µg/ ml ethidium bromide, immersed in 1X TAE buffer (50X stock solution: 242g Tris-base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA [pH8]). 1Kb DNA ladder (10 µl of 50 ng/ µl) (Invitrogen) was loaded alongside the sample as an indicator of RNA fragment size and the electrophoresis was run for 30 min at 50-100V.

6.2.5.2 Gel electrophoresis of PCR products

Gel electrophoresis was as above for RNA except that the tank etc. were not treated prior to use.

6.2.6 Spectrophotometry

To determine the concentration of the RNA in the sample, 1.5 µl of RNA was analysed with a Nanodrop spectrophotometer (Nano Drop® ND-1000 spectrophotometer). Values from the spectrophotometer were compared to the gel to ensure consistency of results.

6.2.7 Primer design for *P5CS, SOS, WEE1, CCS52* and *SERK* genes of *M. truncatula*

Primers were designed based on sequences from the EST collections available (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=medicago) and checked using BLAST to verify that the gene sequences were correct (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) was used for designing the primers (Table 6-2).

Table 6-2. Primer details for Gene Expression Analysis. Note, primers in green text did not work and were replaced by redesigned primers for the name gene target (in black text).

Target gene	Primers pair	Oligonucleotide sequence	Product Size (bp)	Tm (^{0}C)	Gene function
MtSERK1	MtSERK1-F MtSERK1-R	GTTGTGGGGGATTTTGGATT AGTCGAGCAAGGTCAAAAGC	200	60	marker for cells competent to from somatic embryos
Pyrroline-5-carboxylate synthetase	Mt P5CS1-F Mt P5CS1-R Mt P5CS1-F Mt P5CS1-R	CGTAGGTCTTGCCAACAACA AGAGAAGCCCATTCCCACTT TCACGAGTACTGGTCGTTGG GTGCTCCATCGCTGAATCTT	206	60	Protective role in response to abiotic stress
SOS	SOS-F SOS-R SOS-F SOS-R	ATATCCATCTCGCGTTGAGG CCCTTTGCTCTACCAACCAA TGCTCTGACGTGAAGAACCA CTTTGTACCTCGTGCCGAAT	195	60	Related to salt stress
WEE1	WEE1-F WEE1-R	TCCGATTGAGGAAGGAGATG AATGAATGACCAGGCAGGAG	197	60	Cell cycle gene up-regulated by DNA damage
MtCC52B	MtCCS52B-F MtCCS52B-R MtCCS52B-F MtCCS52B-R	GGCCACCAATTGAACTCTGT CCCCAGCACCAGTCACTATT CTGGCCACAGCATGAGAGTA ATCTTCCATGCTTGCTGCTT	198	60	Related to endopolyploidy
Mt 18S	Mt 18S-F Mt 18S-R	TGACGGAGAATTAGGGTTCG CCTCCAATGGATCCTCGTTA	194	60	Control

6.2.8 Primer assessment

cDNA of leaf and callus growing under the salt concentrations, 0, 50, 100, 150 and 250 mM NaCl, were used to assess the primers before using them to define expression levels of target genes by real-time PCR. This assessment was done by using the Go-Taq Flexi DNA Polymerase (Promega) kit (Table 6-3). The PCR was run with a first step for 15 min at 95 ℃ and 40 cycles of: 1 min at 95 ℃, 1 min at 55 ℃ and 1 min at 72 ℃ and the last step was 15 min at 72 ℃. The gel electrophoresis was run as described in section 6.2.5.

Table 6-3. Component of Go-Taq kit

Component	Amount (µL)	Final concentrations per µL
Green buffer 5X	5	-
25 mM MgCl$_2$	1.5	1.5 mM
dNTP (10 mM)	0.5	0.2 mM
10 µM Primer F	0.5	0.2 µM
10 µM Primer R	0.5	0.2 µM
H$_2$O	15.87	-
Go-Taq	0.125	-
cDNA (0.2 µg)	1	8ng
Total volume	25	-

6.2.9 DNA extraction.

Total DNA was isolated from calli according to the method described by Edwards et al. (1991). About 50 -100 mg of callus was ground without buffer using a sterile Eppendorf

grinder for approximately 2 min in a sterile Eppendorf tube. Then, 200 µl of sterile extraction buffer (0.5 % SDS, 250 mM NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA) was added to the homogenate. The extract was centrifuged at top speed in an Eppendorf MiniSpin centrifuge for 5 min. The supernatant (150 µl) was transferred to a new sterile Eppendorf tube, mixed with 150 µl of cold isopropanol and placed on ice for 5 min. The mixture was centrifuged at top speed in an Eppendorf MiniSpin centrifuge for 10 min. The supernatant was carefully removed and the pellet was air dried at 60 ℃ for 5-10 min. The pellet was resuspended in 100 µl of TE (10mM Tris-HCl pH 8.0, 0.1mM EDTA), the DNA solution was incubated in an oven at 60 ℃ for 5-10 min to aid resuspension of the pellet.

6.2.10 Purification of DNA

The QIAquik PCR Purification Kit (Qiagen) was used to purify DNA extracts. Briefly the different steps for DNA purification in this kit were: five volumes of PB buffer were added to the sample, mixed and applied to a QIAquick spin column provided with a 2 ml collection tube. This was then centrifuged at 13000 rpm for 1 min in an Eppendorf MiniSpin centrifuge. The flow-through was discarded and the QIAquick spin column placed back into the same tube. The sample was washed with 750 µl of PE buffer which was added to the QIAquick spin column and stood for 3 min then centrifuged at 13000 rpm for 1 min as above. The flow-through was discarded and the column centrifuged again at 13000 rpm for 1 min as above. The QIAquick spin column was placed into a clean Eppendorf tube and 30 µl of EB buffer (10 mM Tris-Cl, pH 8.5) was added and stood for 1 min. The column was then centrifuged at

13000 rpm for one min as above to recover the DNA. Finally, the DNA was stored at -20 ℃. The extract (1µl) was used in 25 µl PCR reactions.

6.2.11 Real-time PCR.

6.2.11.1 RNA extraction.

RNA was extracted as described in 6.2.1 and then the concentration of RNA was measured using a nanodrop spectrophotometer (Section 6.2.6) in order to calculate the volume required for at least 2 µg RNA for use in reverse transcription.

6.2.11.2 RNA purification.

RNA was purified from residual genomic DNA as described in Section 2.3.2. To check that the RNA was purified from genomic DNA contamination, all the purified RNA samples were evaluated by PCR amplification using Go-Taq Flexi DNA Polymerase (Promega) kit (Section 2.3.8) using 18S rRNA (PUV2/PUV4) primers (Section 6.2.4). Where samples were positive, DNase treatment was repeated again and checked by PCR again.

6.2.11.3 cDNA synthesis for real-time PCR analyses

After elimination of genomic DNA from the RNA was confirmed, the volume of each sample required to obtain 2µg RNA per sample was calculated again. The retrotranscription was carried out using an Ambion kit (RETROscript® Reverse transcription for RT-PCR,): Oligo (dT) (2 µl) was added to 2 µg purified RNA in a volume of up to 10 µl and sterile distilled water added to a total volume of 12 µl. After mixing briefly, the mix was incubated for 3 min at 85 ºC then immediately placed on ice and the following were added: 2 µl 10x buffer, 4 µl of 10 mM dNTPs, 1 µl RNase inhibitor and 1 µl MMLV-RT then incubated for 1 h at 44 ºC followed by 10 min at 92 ºC. The resulting 20 µl cDNA was stored at -20 ºC. The quality and quantity of the cDNA was tested by PCR amplification of 1 µl cDNA using Go-Taq Flexi DNA Polymerase (Promega) kit and 18S primers as described above.

6.2.11.4 Real time PCR.

cDNA (2µg) of each sample was diluted 1: 20 (10µl of cDNA and 190µl distilled water). Three separate dilutions of each sample were made for each of the target genes listed in Table 6.2. (SERK, CCS52B, P5, SOS and WEE1). Mt18S primers were used as a control. The ABsolute™ QPCR SYBR® Green Mix (Thermo Scientific) kit was used in the real time PCR process. Each reaction consisted of a total volume of 25 µl per reaction which contained: 12.5µl ABsolute™ QPCR SYBR® Green Mix, 1.75µl (10µM) Forward primer, 1.75µl (10µM) Reverse primer and 4µl H₂O. This 20µl mix was loaded into a microtitre plate

(Thermo scientific-ABgene® PCR Plates sealed with optical caps-Abgene), 5µl cDNA (1:20) and the mix without cDNA were also used as blank in each experiment. The optical caps were closed carefully without touching the top of them to avoid smudges, and centrifuged at 1000 rpm for 30 s at room temperature in a BECKMAN COULTER - Allegra™ 12R Centrifuge) to eliminate air bubbles in the PCR mix. The sample was inserted into the real time PCR machine (MJ Research OPTICON™ 2) and run using the machine software (Opticon Monitor3 software), which was used to operate the real time PCR equipment for both set-up and analysis of data. To set up the run three steps were carried out according to the experiment (Appendix C1).

6.2.11.5 Analysis of real time PCR results

For determining the gene expression levels in different samples, I used relative quantification that calculates the ratio between the quantity of molecules in the target gene and in the endogenous control (Reference gene). The target molecule quantity is usually normalized with a reference gene. One of the important challenges when studying gene expression is to normalize the sample according to the number of cycle thresholds (c/t) and total amount of RNA in each sample. The gene used as a reference control should have a constant expression level for all the samples to confirm that total cDNA in each sample is the same amount. In this study Mt18s rRNA gene was used as the endogenous control to normalize the concentration of cDNA in all treatments, The expression of target genes were calculated according to the this formula:

$$\text{Relative expression} = 2^{-\Delta C(t) \text{ of target gene} - \Delta C(t) \text{ of control}}$$

6.3 Results

6.3.1 Analyses of gene expression.

6.3.1.1 RNA extraction and PCR amplification with 18S rRNA primers

The results of RNA extraction were analyzed by agarose gel electrophoresis (Fig. 6-4). RNA was successfully extracted from leaf explants and callus grown on different salt stresses 0, 50, 100, 150 and 250 mM NaCl. Two bright bands are expected due to 25S and 18S rRNA (~ 3500 and 2000 bp respectively; Dyer et al., 1982). The bands of individual mRNAs were too faint and too crowded to identify. More precise quantification of the RNA extracted was performed using a nanodrop spectrophotometer (Table 6-4). The highest concentration was obtained from leaf explants whereas the lowest concentration was from callus grown on 250 mM NaCl.

DNase digestion was carried out once or twice to remove the residual genomic DNA. cDNA synthesis was performed to produce single strand DNA and tested by RT-PCR using 18S rRNA primers and the result detected by gel electrophoresis (Figure 6-5). The expected 459 bp product was amplified successfully from all samples indicating that the RNA was of sufficient quantity and quality for RT-PCR.
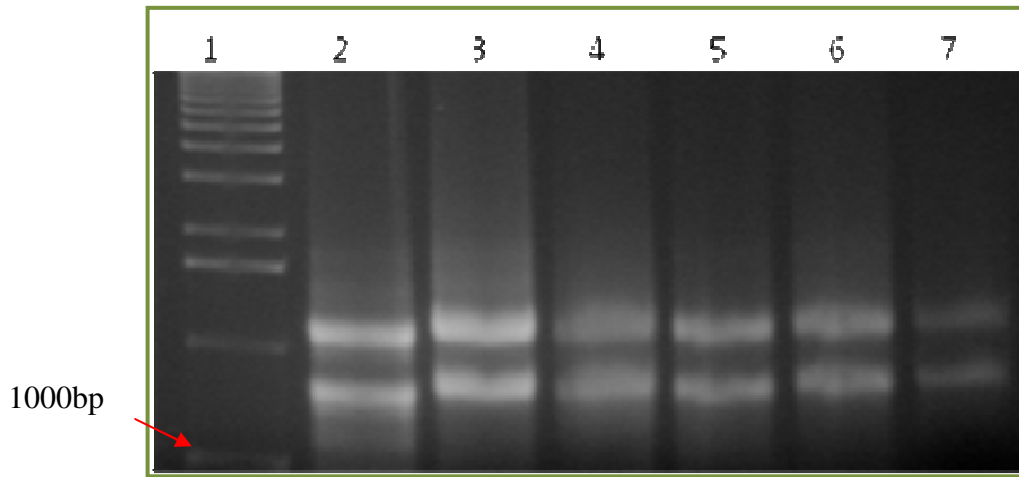
Fig 6-4. 10 µl RNA extracted from leaf and callus grown with different levels of abiotic stress. 1= 1Kb ladder, 2= leaf (0 mM NaCl), 3= callus (0 mM NaCL), 4= callus (50 mM NaCl), 5= callus (100 mM NaCl), 6= callus (150 mM NaCl), 7= callus (250 mM NaCl).
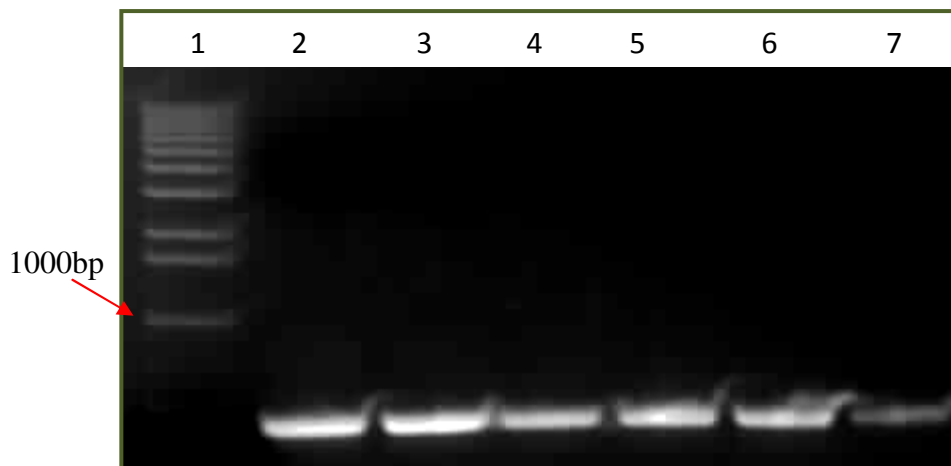
Fig 6-5. PCR with 18S PUV2 and PUV4 primers to test the quality of the cDNA. 1= 1Kb ladder, 2= leaf (0 mM NaCl), 3= callus (0 mM NaCL), 4= callus (50 mM NaCl), 5= callus (100 mM NaCl), 6= callus (150 mM NaCl), 7= callus (250 mM NaCl).

Table 6-4. nanodrop spectrophotometer result.

Sample	Concentration of RNA ng/µl	Total RNA obtained from 200 mg (µg)
leaf	1333	133
Callus (0mM NaCl)	610	49
Callus (50 mM NaCl)	443	31
Callus (100 mM NaCl)	274	19
Callus (150 mM NaCl)	556	39
Callus (250 mM NaCl)	169	12

6.3.1.2 Preliminary RT-PCR on calli derived from *Medicago truncatula* grown with NaCl.

Prior to the development of real time PCR analysis, the primers for the target genes (*MtSERK*, *MtWEE1, MtCCS52B MtSOS, and MtP5CS*) to be evaluated were checked by using RT-PCR on cDNA synthesized from RNA extracted from leaf and callus cultured for three months under 0, 50, 100 or 150 mM NaCl. The results of RT-PCR show that only *MtSERK* and *MtWEE1* were expressed in all treatments (Fig. 6-6). *SERK* is known to be expressed under saline conditions where its expression is positively related to somatic embryogenesis (Nolan et al., 2009; Nolan et al, 2003). The expression of *MtWEE1* had not been analysed previously.
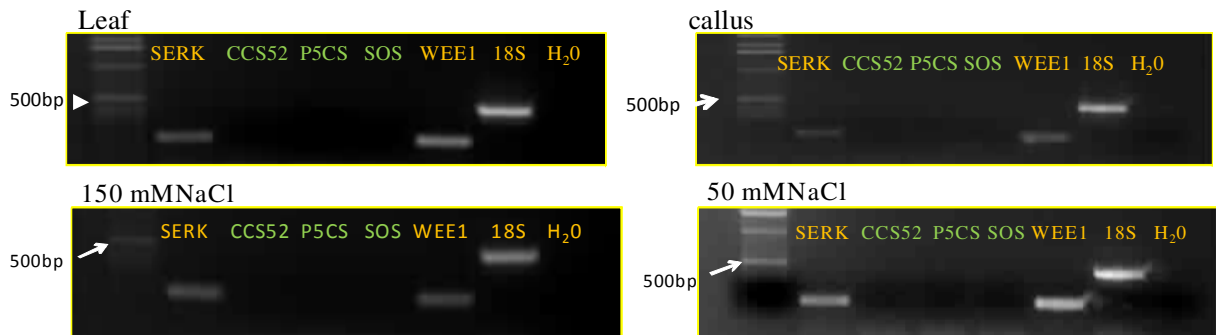
Fig 6-6. Gene expression in leaf and calli using RT-PCR after three months under salt stress. 18S positive control and H_2O negative control. Primers used are listed in Table 6-2.

Primers for *MtSOS, MtCCS52B* and *MtP5C*S that were negative on cDNA (Fig 6-6) were also tested on gDNA but no product was obtained. Alternative primers were therefore designed for these targets with product size 198-200 bp. The new primers were evaluated by RT-PCR using cDNA derived from callus grown on 150 mM NaCl and gDNA from leaves. A positive amplification was obtained (Fig. 6-7) indicating that these primers could be tested by real time PCR.
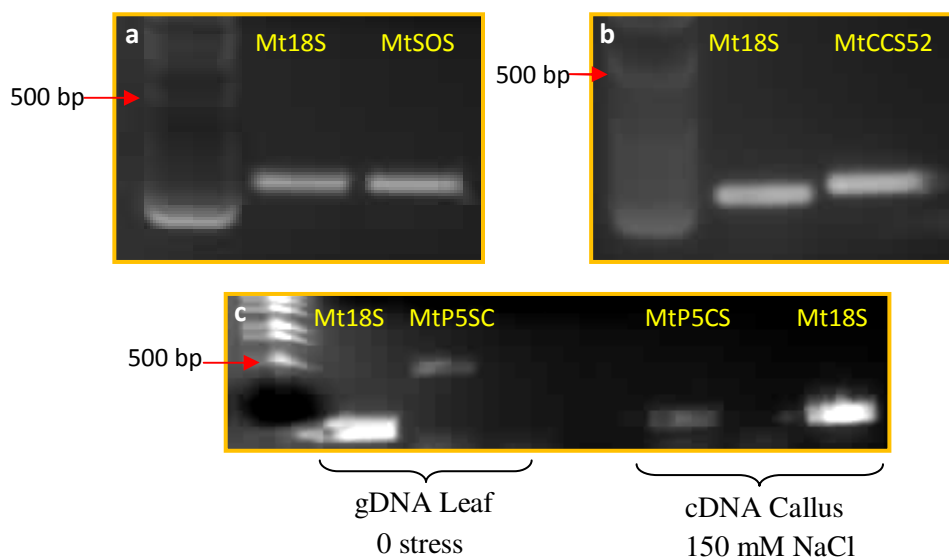
Fig 6-7. PCR amplification of cDNA template derived from callus grown for three months on 150 mM NaCl: using redesigned primers for MtSOS (a), MtCCS52 (b), and MtP5CS (c) (see Table 6-2) including also genomic DNA. *Medicago truncatula* 18S (Mt18S) was used as a positive control for all experiments.

6.3.2 Results of real time PCR

Relative expression of each of the *Medicago truncatula* genes was evaluated in calli following pretreatment (5 months) following Phase I that included an acclimation (step up) (6 months), Phase II (6 months) and Phase III (6 months by which time calli were 23 months-old) under 0, 50, 100 and 150 mM NaCl treatments; in *vitro* grown leaves were used as a reference. It proved impossible to extract sufficient RNA from the 250 and 350 mM NaCl

treatments for real time PCR. The concentration of cDNA from all the treatments was normalised by using endogenous control gene Mt18S and checking the products on agarose gels as shown in Fig 6-5. The primers were then tested by real time PCR and the results showed that the mean cycle threshold (ΔCt) for all the treatments with the 18S primers were within 2 cycles (Table 6-5). This confirms that the cDNA concentrations had been equalized. The melting curve for the 18S primers gave a sharp single peak that indicated specific amplification (Fig. 6-8). Following Phase I, all target genes were strongly expressed in the 150 mM treatment. (melting curves and quantitation curves of all primers are shown in Appendix C2.

Table 6-5. Mean of cycle threshold for endogenous control gene Mt18S in 23 month-old calli following Phase I (6months), Phased II (6 months) and Phase III (6 months) in the 0, 50, 100 and 150 mM NaCl treatments. All calli were pretreatment on MANA (5 months) and selected for embryogenesis.

Treatments	$\Delta C(t)$ number of Mt 18S		
Phases	I (6 months)	I+II (12 months)	I+II+III (18months)
Leaf	21.14 ± 0.68	-	-
0 mM NaCl	20.60 ± 1.17	20.60 ± 0.33	20.44 ± 0.32
50 mM NaCL	21.11 ± 0.44	21.10 ± 0.62	20.88 ± 0.10
100 mM NaCL	21.95 ± 0.21	21.13 ± 0.60	20.75 ± 0.40
150 mM NaCL	21.92 ± 0.66	20.26 ± 0.52	20.70 ± 0.26
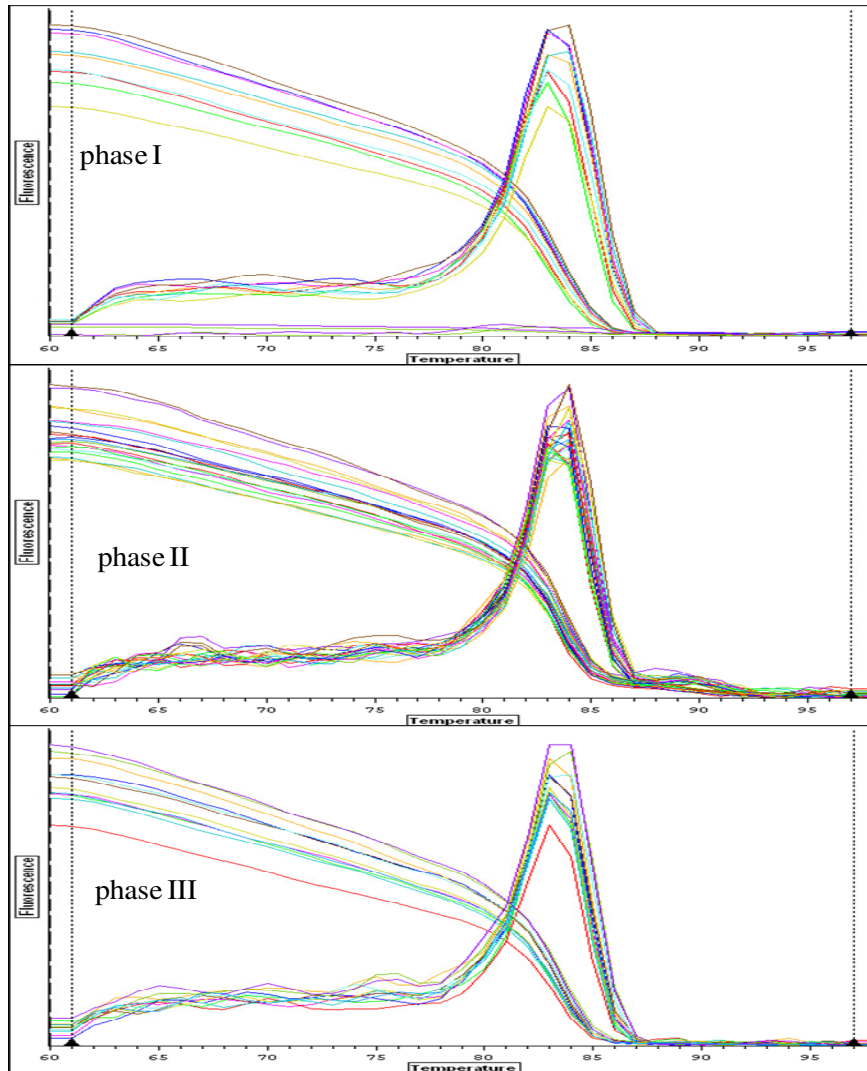
Fig 6-8. Melting curve of Mt18s gene used as control, on cDNA derived from calli following Phase I (11 months), Phase II (17 months) and Phase III (23 months) showing a sharp single peak for all treatments used in this study indicating that there are no primer-dimers or other non-specific products.

6.3.2.1 Expression of *MtSERK* (somatic embryo development-related gene)

Following Phase I (11 months), *MtSERK*, was expressed at a significantly higher level in control callus (0 mM NaCl) compared to the reference (leaf). However expression decreased significantly when the concentration of NaCl was stepped up to 50 and 100 mM NaCl. Maximal *MtSERK* expression was observed in calli cultured on 150 mM NaCl (Fig. 6-9a). However, following Phase II and III, the relative expression of *MtSERK* was maximal in the 100 mM NaCl treatment (Fig. 6-9b and 6-9c).

There was no consistency of *MtSERK* expression following each phase compared to the control. For example in callus growing on 50 mM NaCl, after Phase I, *MtSERK*, was expressed at a significantly lower level than the control (0 mM NaCl) (Fig. 6-9a). In contrast, after Phase II the expression of *MtSERK*, at 50, 100 and 150 mM NaCl increased significantly compared with 0 mM NaCl (Fig. 6-9b). However, after calli terminated Phase III, on 50 mM NaCl *MtSERK* expression decreased significantly compared with the 0 mM NaCl control, expression was significantly higher in 100 and 150 mM treatments (Fig. 6-9c). In summary, *MtSERK* peaked at 150 mM NaCl following Phase I. However, following Phase II and III *MtSERK* expression peaked at 100mM NaCl.

Fig 6-9. Relative expression of *MtSERK* after calli terminated three Phases under various concentrations of mM NaCl. (a)= Phase I (11 months), (b)= Phase I+II (17 months), (c)= Phase I+II+III (23 months) with leaf as reference. Different letters indicate significant differences (P ≤ 0.05) (n=3)

6.3.2.2 Expression of cell cycle related genes: *MtWEE1* and *MtCCS52*

The pattern of *MtWEE1* and *MtCCS52* expression was very similar to that of *MtSERK*. After the calli terminated Phase I, the relative expression of both genes increased significantly in 0 mM NaCl compared to leaf and in 50 mM NaCl compared with 0 mM NaCl (Fig. 6-10a and 6-11a). However, at the end of Phase I there was a significant decrease in expression of these genes in the 100 cf. 50 mM NaCl. Interestingly, expression of both genes was again highest in the 150 mM NaCl treatment after the step up acclimation (Phase I) (Fig. 6-10a and 6-11a). However, following Phase II the expression of both genes (*MtWEE1* and *MtCCS52*) was highest in the 100 mM NaCl treatment compared with 0, 50 and 150 mM NaCl (Fig. 10b and 11b). In contrast, following Phase III the expression of these genes was highest in the 100 and 150 mM NaCl treatment compared with 0 and 50 mM NaCl (Fig 10c and 11c). In addition, at the lowest concentration of NaCl (50 mM) expression of both genes (*MtWEE1* and *MtCCS52*) was also significantly higher than the 0 mM NaCl (control) at the end of the three Phases I, II and III (Fig 6-10 and 6-11). In summary, the pattern of alteration in *MtWEE1* and *MtCCS52* expression, was highest at 150 mM following phase I, 100 mM following phase II and 100/150 mM NaCl following Phase III.

Fig 6-10. Relative expression of *MtWEE1* after calli terminated three phases under various concentrations of mM NaCl. (a)= Phase I (11 months), (b)= Phase I+II (17 months), (c)= Phase I+II+III (23 months) with leaf as reference. Different letters indicate significant differences (P ≤ 0.05) (n=3)
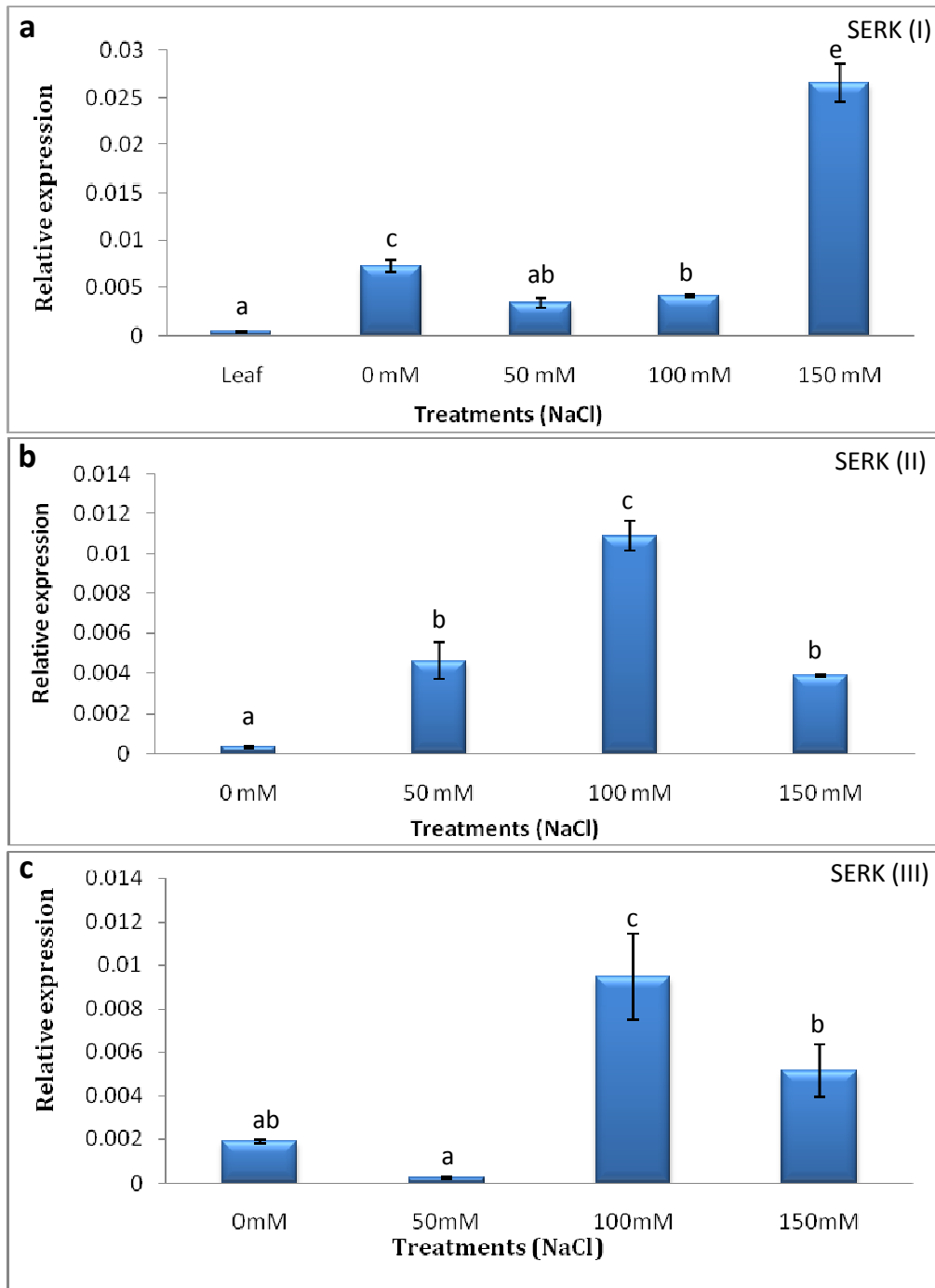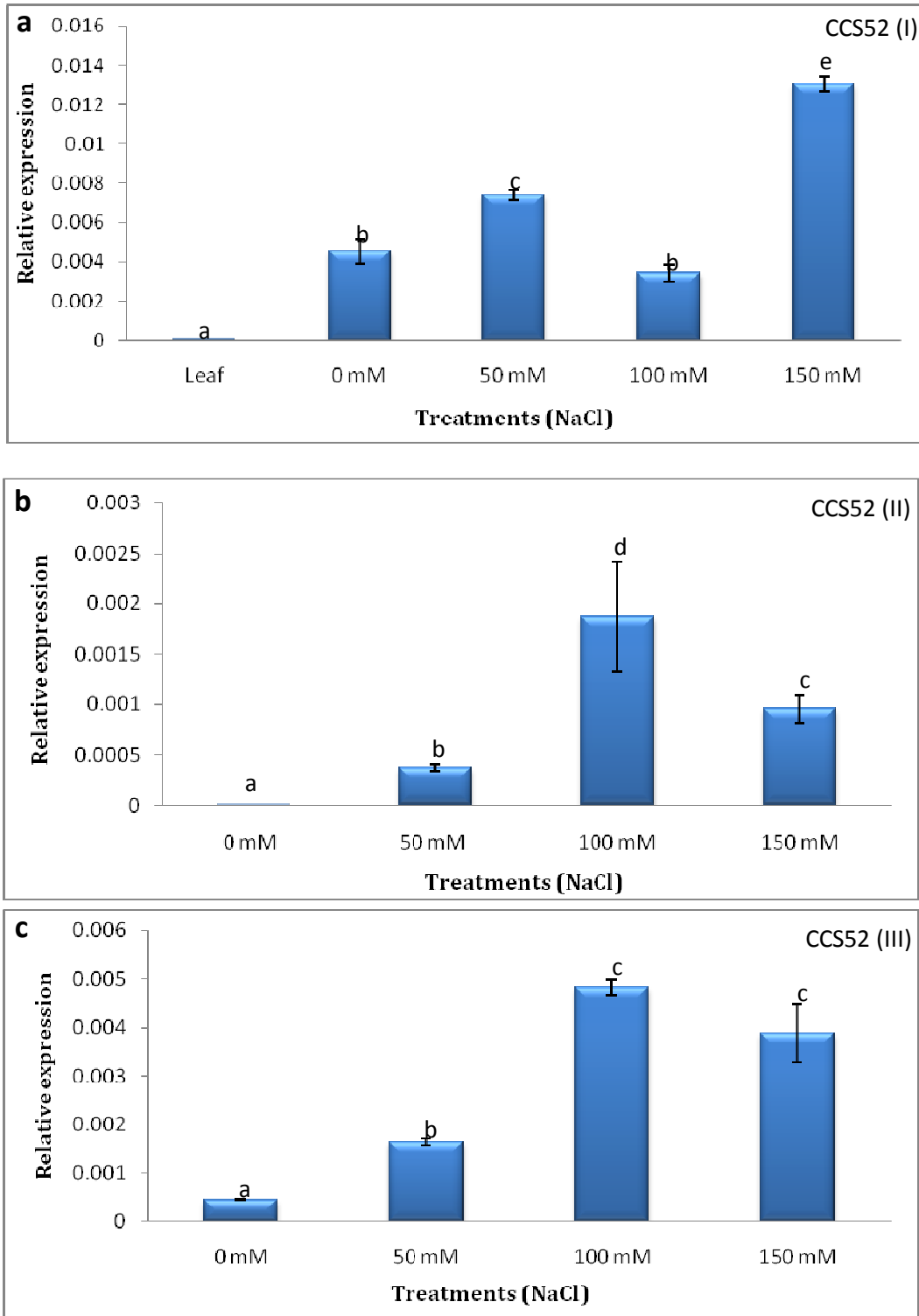
Fig 6-11. Relative expression of *MtCCS52* after calli terminated three phases under various concentrations of mM NaCl. (a)= Phase I (11 months), (b)= Phase I+II (17 months), (c)= Phase I+II+III (23 months) with leaf as reference. Different letters indicate significant differences (P ≤ 0.05) (n=3)

6.3.2.3 Expression of genes related to abiotic stress: *MtSOS* and *Mt P5CS*

MtSOS expression was highest at 150 mM NaCl following Phase I (Fig 6-12a), and in the 100 mM NaCl treatment following Phases II and III (Fig 6-12b,c). The peak of *MtP5CS* expression was at 150 mM NaCl following Phase I (Fig 6-13a). There was no significant difference in *MtP5CS* expression in the 50 and 100 mM NaCl treatments following Phase II which were the treatments that elicited the highest *MtP5CS* expression levels at the end of Phase II (Fig 6-13b). However, *MtP5CS* expression was significantly higher in the 100 compared with 150 mM NaCl treatment. Following Phase III significantly highest *MtP5CS* expression was observed in the 100 and 150 mM NaCl treatments (Fig 6-13c). In summary *MtP5CS* expression was highest at 150 mM NaCl (Phase I) 50/100 mM (Phase II) and 100/150 mM (Phase III).
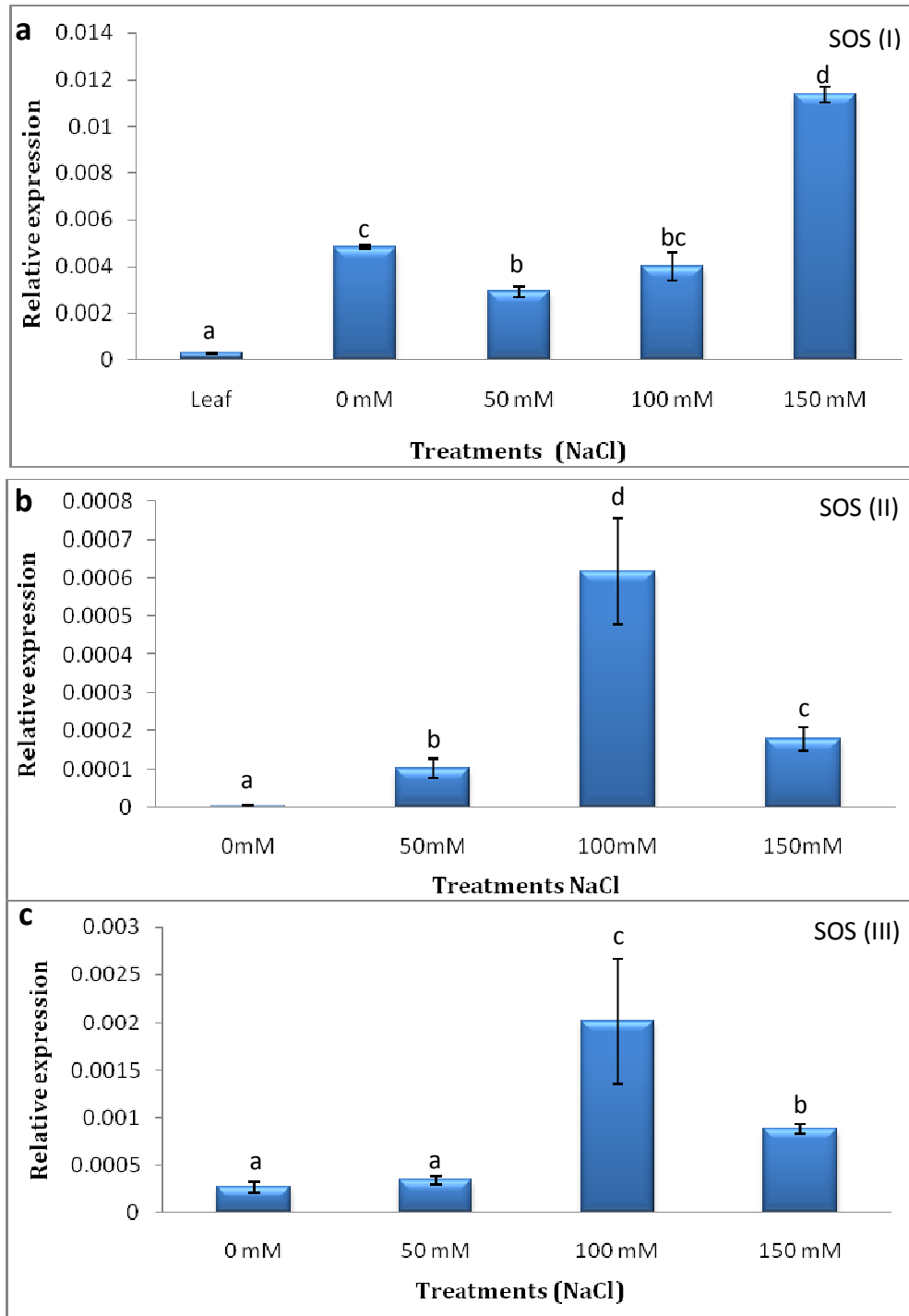
Fig 6-12. Relative expression of *MtSOS* after calli terminated three phases under various concentrations of mM NaCl. (a)= Phase I (11 months), (b)= Phase I+II (17 months), (c)= Phase I+II+III (23 months) with leaf as reference. Different letters indicate significant differences ($P \leq 0.05$) (n=3)
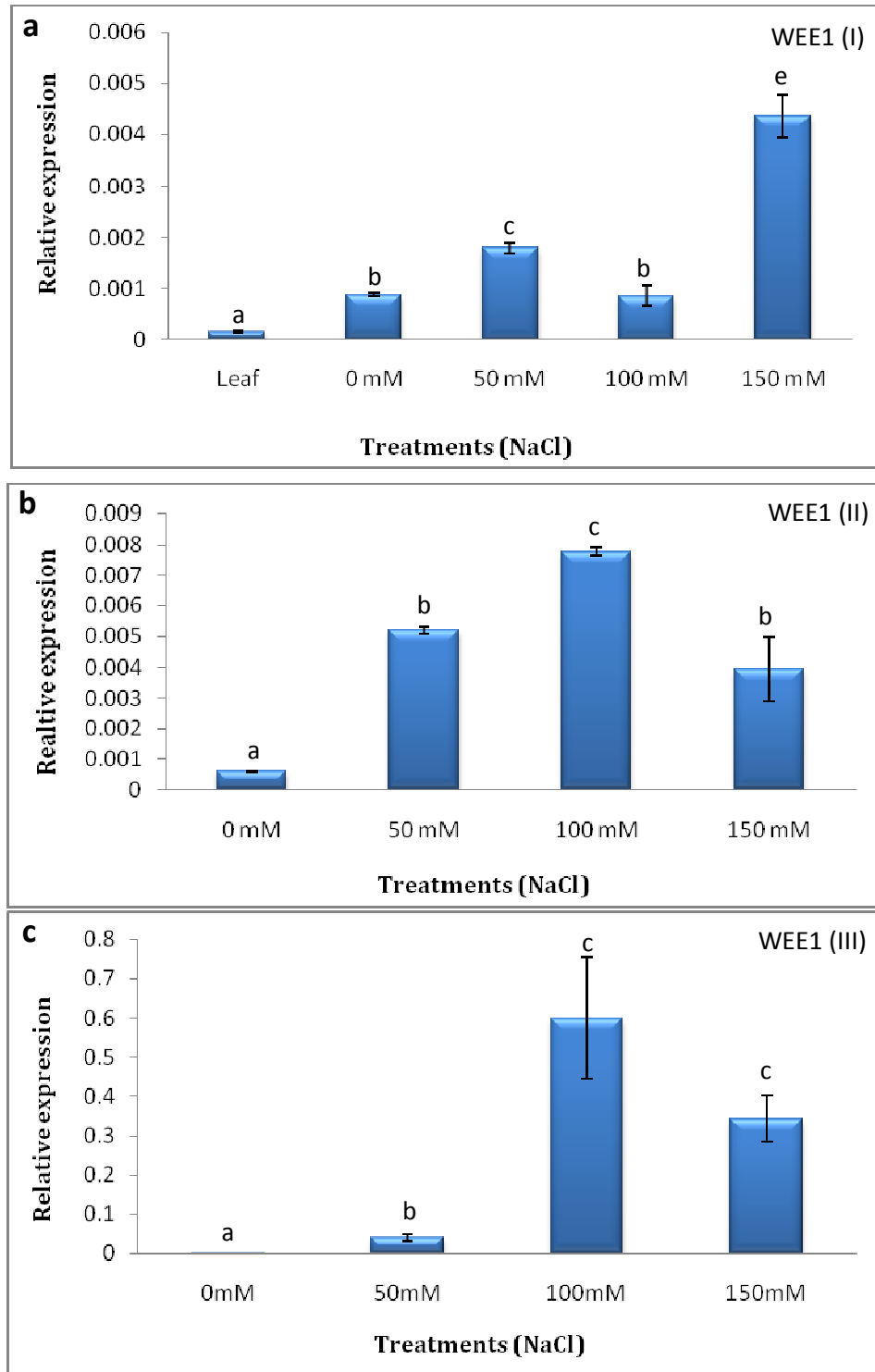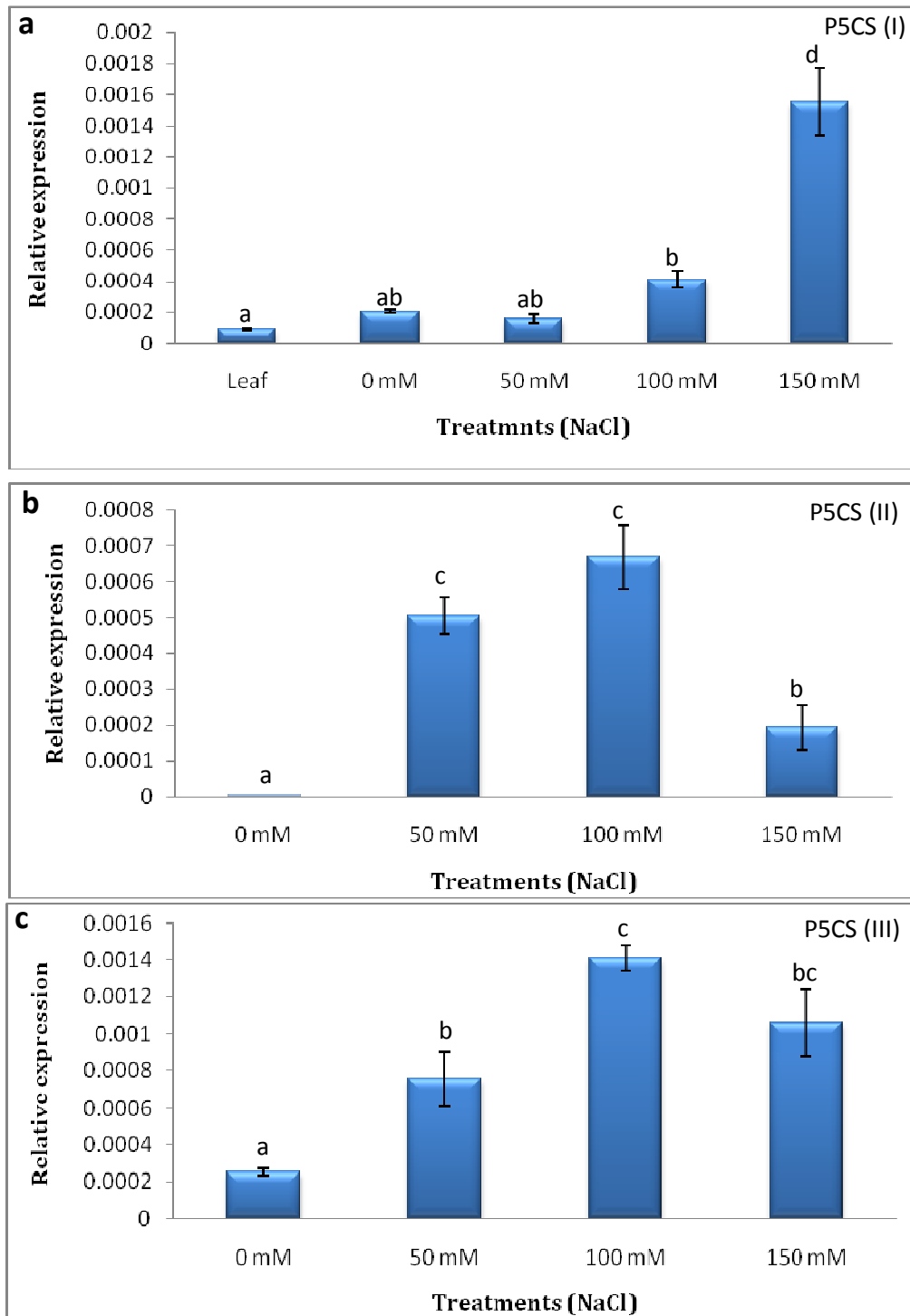
Fig 6-13. Relative expression of *MtP5CS* after calli terminated three phases under various concentrations of mM NaCl. (a)= Phase I (11 months), (b)= Phase I+II (17 months), (c)= Phase I+II+III (23 months) with leaf as reference. Different letters indicate significant differences (P ≤ 0.05) (n=3)

6.4 Discussion

Thousands of genes are involved in adjustments to the physiological processes at the cellular, tissue and whole plant levels in order to acclimate cells to salt stress (Yamaguchi-Shinozaki and Shinozi, 2005; Merchan et al., 2007). I tested the extent to which expression of five genes (*SERK, WEE1, CCS52, SOS, P5CS*) were markers of NaCl tolerance in *M. truncatula*. Also by assaying these genes, the extent to which they provided/facilitated new insight into molecular events of NaCl tolerance was examined.

6.4.1 *MtSERK*

SERK, which encodes a leucine-rich repeat receptor-like kinase (LRR-RLK), has an essential function in somatic embryogenesis in many plant species (Nolan et al., 2009; Nolan et al., 2003). The highest *MtSERK* expression occurred in the 150 mM NaCl treatment following Phase I. Note that after the 5 months pre-treatment, only calli with embryogenic potential were selected for Phase I. However, as judged by *MtSERK* expression, the step up (acclimation) to 150 mM NaCl of Phase I led to maximal *MtSERK* expression and possibly greater embryogenesis compared with the control and other step ups. Following Phase II and Phase III, the maximum expression of *MtSERK* was in the 100 mM NaCl treatment. As shown in Chapter 3 (Fig. 3-17), 100 mM NaCl sustained for embryogenesis suggesting that *MtSERK* expression is strongly linked to somatic embryogenesis, in *M. truncatula*. Moreveer, optimal *MtSERK* expression at 100 mM is consistent with representative high levels of embryogenesis in images of calli shown in Chapter 3 (Fig. 3-19). Note that, the calli were grown on medium

supplemented with 2mg/l NAA and 0.5 mg/l BAP; Nolan et al. (2003) reported that these hormones activated the expression of *SERK* in *Medicago truncatula* compared to other combinations of hormones. However, in my study, peak *MtSERK* expression at 150 mM NaCl was from callus that benefited from the step up, acclimation component of Phase I followed by ± NaCl of the Phases II and III respectively. To my knowledge, this is the first report of *SERK* expression in salt treatments that is clearly correlated with enhanced embryogenesis.

6.4.2 *MtWEE1 and MtCCS52*

Interestingly, expression of *MtWEE1* was also highest after the calli terminated step up acclimation to 150 mM NaCl of Phase I (11 month old calli) and then was highest in the 100 mM NaCl treatment following Phase II. Following Phase III expression was highest at 100mM NaCl but was not significantly different from expression at 150mM NaCl (Fig. 6-10). De Schutter et al. (2007) reported that inducing *Arath;WEE1* expression inhibited plant growth by arresting cells in the G2-phase of the cell cycle of *Arabidopsis thaliana*. My results show that *MtWEE1* expression is activated by high concentrations of salt (100 and 150 mM NaCl). There are at least two possible explanations. Firstly, if DNA was damaged in the 100 and 150 mM treatments, then a functional WEE1 kinase would prevent cells from dividing. However, callus growth benefited following step ups to 100 and 150 mM. Therefore it must be the case, that once toxic Na^+ was sequestered into the vacuole or prevented from entry into the cell, checkpoint genes such as *WEE1* would then be no longer necessary at elevated levels. However, this does not entirely explain why *WEE1* expression was highest in the 100 mM

NaCl treatments following Phases II and III. Secondly, if we assume a function for *WEE1* kinase in normal cell cycles, then it is hypothesized that high levels of *WEE1* expression are necessary in regulating cell size in the way described for fission yeast and animal cells (see Introduction). That Arabidopsis *wee1* knockouts behave similarly compared with wild type negates this hypothesis. However, my data show maximal *WEE1* expression in the 150 mM treatment of Phase I and 100 mM following Phase II and 100/150 following phase III. It suggests that NaCl treatment that included a multiple step up in NaCl concentration has induced significant levels of *WEE1* expression compared with controls. In future work it might be possible to measure *WEE1* at the protein and kinase levels following salt stress which in turn would require a rigorous assay for this kinase.

I also recorded the highest expression of *Mt cell cycle switch 52* (*MtCCS52*) in the 150 mM treatment of Phase I and the 100/150 mM NaCl treatment following Phases II and III. It has been reported that cell expansion increases as result of osmotic stress. This is due to vacuolar enlargement, and cell wall extension, or the endoreduplication process (Sugimoto-Shirasu and Roberts 2003). In Arabidopsis high levels of *CCS52* expression occur when cells bypass mitosis and undergo endoreduplication cycles. It is a characteristic of several different plant organs, suggesting that multiplication of the genome might contribute to the differentiation of certain cell types (Kondorosi et al., 2000; Gonzalez-Same et al., 2006; Su'udi et al., 2012). In *Medicago* nodules, endoreduplication is common leading to enlargement of cells that accommodate Rhizobia for nitrogen fixation (Kondorosi et al., 2000). These events are accompanied by the expression of *CCS52A* (Vinardell et al., 2003). Recently, in rice, *OsCCS52* expression was studied by Su'udi et al (2012). *OsCCS52* was

highly expressed in young leaves and roots that showed endoreduplcation that in turn was linked with cell elongation. In an o*sccs52* mutant in race line (IB-10423) there was very little endoreduplication consistent with decreasing cell expansion causing a small kernel and semi-dwarf phenotype (Su`udi et al., 2012). In my study, calli cultured on 50 mM NaCl induced significantly higher expression of *MtCCS52* compared to control (Fig. 6-11a). In this treatment there were 3-4 peaks of fluorescent cells (see Fig. 5-7b, in chapter 5) consistent with endoreduplication. However, as with the other genes, highest *MtCCS52* expression occurred in the 150 mM treatment following Phase I and in the 100 treatment following Phases II and III. However, following Phase III, (as was also seen for *WEE1* expression) there was no significant difference between expressions between the 100 or 150 mM treatments. Flow cytometry data obtained in these treatments showed a skewing of the data into an unusually large proportion of cells in S-phase. Perhaps cells in S-phase were either diploid or were undergoing an endo S-phase. It was only these treatments that gave this unusual skewing and it is only these treatments that led to maximal *MtCCS52* expression. However, given the maximal growth rates in the 100 and 150 mM treatments following Phase III, it is clear that these salt-tolerant cells were able to grow rapidly and they could also tolerate a population of endoreduplicating cells. It might also be the case, that peaks of *MtWEE1* expression in 100/150 mM treatments are also consistent with endoreduplication. As noted by Chevalier et al. (2011) an increase in expression of *WEE1* might contribute to the general down-regulation of mitotic CDKs that in turn would be a necessary molecular condition that leads to endoreduplication.

6.4.3 *MtSOS1*

Salt overly sensitive (SOS) genes play an essential role in maintaining ion homeostasis and conferring salt tolerance (Ren-Jie et al., 2010; Tang et al., 2010; Feki et al., 2011). Culture of *Medicago truncatula* calli at 150 mM following Phase I and 100 mM NaCl following Phases II and III induced very significant up-regulation of the expression of *MtSOS* compared to 0 mM NaCl control (Fig. 6-12). A role for this gene in responses to osmotic stress has been reported in several plant species. For example, in *Arabidopsis thaliana* the *AtSOS* pathway is responsible for Na^+ homeostasis and salt tolerance under salt stress. However *sos* mutants were hypersensitive to NaCl (Zhu, 2001; Smith et al., 2010; Tang et al., 2010). Tang et al, (2010) cultured shoot apices of two poplar genotypes, *Populus trichocarpa* and *Populus alba,* on MS medium supplemented with 0.03 mg/l^{-1} NAA for three weeks. They were then subcultured to medium supplemented with 200 mM NaCl for six months, Real time PCR results showed higher expression of *SOS*1 and *SOS*2 in shoots and roots of both genotypes compared to control 0 mM NaCl. *sos1, sos2 and sos3* mutants of *Arabidopsis thaliana* were hypersensitve to 60 or 120 mM NaCl NaCl compared with a 0 mM NaCl treatment (Tang et al., 2010). *SOS*1 encodes a plasma membrane localized Na^+/H^+ antiporter (Shi et al., 2000; Qiu et al., 2003). Therefore, the physiological and biochemical function of SOS1 is to assist removal of Na^+ from the cytoplasm and export it into to the extracellular space or into the medium or compartmentalization into the vacuole, which is regulated by a SOS2/3 complex (Blumwald et al., 2000). Thus, the strong up-regulation of *MtSOS* expression in callus acclimated during the 150 mM NaCl treatment of Phase I (Fig. 6-12a) would be consistent with cells that started to become NaCl tolerant. Note also that 150 mM

NaCl was the cross-over point of a positive relationship between [Na$^+$] and externally supplied NaCl, and a negative relationship between [K$^+$] and externally supplied NaCl (chapter 4: Fig. 4-7). From what is known about *SOS* genes in Arabidopsis, membrane bound MtSOS1 will be activated by a cytoplasmic *SOS2* that also would also promote the activity of an Na$^+$/H$^+$ antiporter bound to the tonoplast. It also seems likely that once internal [K$^+$] decreases below a particular threshold in at $\geq$ 250 mM NaCl treatment (see Fig. 4-6 in Chapter 4) SOS proteins and other antiporters are no longer able to sequester or remove [Na$^+$] that in turn leads to Na$^+$ toxicity and suppression of growth.

6.4.4 *MtP5CS*

MtP5CS encodes Δ^1-pyrroline -5-carboxylate synthetase involved in proline biosynthesis and degradation. Salt stress caused very significant expression of *MtP5CS* in callus acclimated to 150 mM NaCl after Phase I, 50-100 mM NaCl after Phase II and 100 mM following Phase III compared with the 0 mM NaCl (Fig. 6-13). Szekely et al. (2008) showed that in an Arabidopsis *p5cs* knockout there was a sharp decrease in proline synthesis and an increased accumulation of ROS and hypersensitivity to salt stress. Recently Dombrowski et al (2008) reported that a tomato *P5CS* gene was strongly induced by salt, drought and cold stress. In my work up-regulation of *MtP5CS* occurs at concentrations of salt > 50-100 mM NaCl (Fig 6-13). This is in line with results of Somboonwatthanaku et al. (2010) when they examined expression of *OsP5CS* in friable callus of rice (*Oryza sativa* L.) cultured with either

0 or 250 mM NaCl for four days. After 24 hours of NaCl treatment, expression of *OsP5CS* increased significantly compared with 0 mM NaCl. Similar data were reported by Silva-Ortega et al (2008). Nine week old seedlings of cactus pear (*Opuntia streptacantha*) were transferred to medium supplemented with 0, 75, 150, 250, or 350 mM NaCl for 11 days. After 11 days of NaCl stress the *OsP5CS* gene was significantly up-regulated in the 250 mM NaCl treatment (Silva-Ortega et al., 2008). Up-regulation of *OsP5CS* was correlated with proline accumulation which was about 15-fold higher compared with the control. It is concluded that expression of *MtP5C* and proline accumulation reflect an up-regulation of proline synthesis that contributes to cellular osmotic adjustment in calli that eventually became tolerant to 150 mM NaCl.

6.5 Summary

The data reported in this chapter on gene expression compared with controls (0 mM NaCl) can be summarized as follows:

- Real-time PCR showed that the highest significant relative expression of all target genes (SERK1, WEE1, CCS52, SOS1 and P5CS) was on 150 mM NaCl after calli terminated Phase I of step-up acclimation.

- After calli terminated Phase II and Phase III the highest relative expression of all these target genes was on 100 mM NaCl.

Chapter 7: Tissue culture responses to PEG, a treatment that mimics drought.

7.1 Introduction

Drought is an abiotc stress that can result in deterioration of crop yield world-wide particularly in arid and semi-arid ecolsystems (Bray, 1993; Gonzalez et al., 2005). As explained by Gonzalez et al. (1998) and Motan et al. (1994) *Medicago truncatula* is more drought tolerant compared to other legumes like pea and soybean. Moreover, Rubio et al. (2002) and Galvez et al. (2005) suggested that in *M. truncatula* responses to drought are similar to other species of this genus e.g. *Medicago sativa* (Naya et al., 2007). Drought resistance mechanisms can be grouped into three categories: (1) escape, which enables the plant to complete its life cycle before the most intense period of drought, (2) avoidance, which prevents exposure to water stress, and (3) tolerance, which enables the plant to withstand drought conditions (Levitt, 1972). Some resistance mechanisms are constitutive and active before exposure to drought. In other cases, plants exposed to drought alter their physiology, thereby acclimating themselves to withstand drier conditions.

Tissue culture has been used in the selection of drought tolerant cell lines using 6-15% 6000 PEG. These lines have been used to regenerate plants resistant to harsh environmental conditions in some crops such as *Medicago sativa* L, tomato, soybean and wheat (Dragiiska et al., 1996; Miki et al., 2001, Queiros, 2007; Sakthivelu et al., 2008; Guoth et al., 2010;

Mahmood et al., 2012). Transgenic approaches have been used to examine stress tolerance but its molecular basis is not understood in *Medicago truncatula* (Zhang et al., 2007). The major part of my work involved studying the effect of exogenous NaCl treatments on callus formation from leaf explants of *Medicago truncatula*. However, as was also mentioned in the Introduction of the thesis, populations of this species (and other agriculturally important species) growing in costal regions of Libya, are affected not only by NaCl but also drought. Indeed in the latter, the endogenous concentration of NaCl (and of course other ions) would be predicted to increase in the early stages of water loss caused by drought. However, and to my knowledge, very little is known about drought stress mechanisms in the leguminous *Medicago truncatula*. For these reasons, I also undertook an investigation of the effects of drought, induced experimentally by polyethylene glycol (PEG), on callus growth. Five month old calli of *M. truncatula* established on MANA medium were supplemented with 10% w/v 6000PEG for six months to order to study the physiological and molecular basis of drought stress in this species.

7.2 Material and Methods

Calli were derived from leaf explants cultured for 5 months on MANA medium followed by a further 6 months on MANA supplemented with 10% w/v PEG 6000 (see Section 2.1.4 for more details).

The mean monthly growth rate was calculated as described for the of salt treatments (section 3.2.1). The osmolarity, proline content, soluble sugars content, and water content were evaluated after six months under PEG stress as reported in the Materials and Methods of Chapter 4 (Section 4.2). Methodology for RNA extraction and gene expression studies of *MtSOS, MtP5CS*; *MtSERK, MtCCS52* and *WEE1* were as in the Materials and Methods section of Chapter 6 (Section 6.2.11).

7.3 Results

7.3.1 Morphological responses of calli grown under PEG stress.

Callus was induced by culturing leaf explants of *Medicago truncatula* on MANA medium for five months and then subcultured on the same medium supplemented with 10% w/v PEG glycol 6000 for six months (Fig. 7-1). There were no significant between treatment differences over the 6 months of culture and the linear growth rates for control and PEG treatments were 0.157 and 0.171 g month^{-1} respectively. This only represents a 1.08 fold increase in the PEG compared with the control treatment indicating very similar rates of growth in each treatment. However, the data suggest that calli were PEG-tolerant within 1 month of sub-culture and retained such tolerance throughout the experiment. Qualitative observations of callus indicated that those treated with PEG were typically bright green in colour and exhibited clear evidence of embryogenesis as did the controls that were far less green in colour (Fig. 7-2). The typical bright green coloration of calli in the PEG treatment indicate their robustness for both growth and embryogenesis regardless of the length of time in culture.
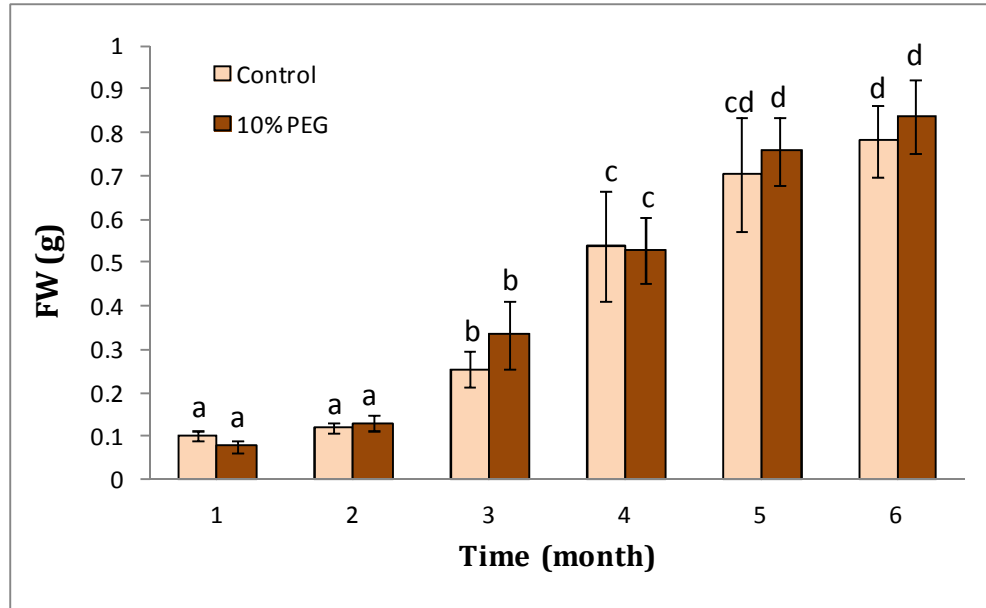
Fig 7-1. Mean (± S.E.) callus growth over 6 months on MANA medium with 10% w/v PEG compared with controls. Note different letter combinations between the same coloured columns indicate significant temporal differences (P ≤ 0.05) (n=12). Regression equations: for control, y=0.157x-0.125, PEG: y= 0.171x-0.149 (P < 0.001)
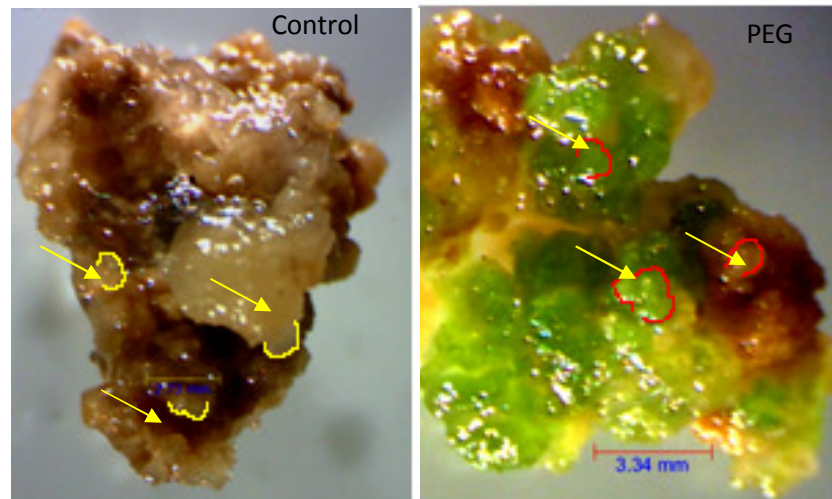
Fig 7-2. Callus pattern after 6 months on 10% w/v PEG and control, arrows indicate somatic embryos.

7.3.2 Physiological measurements

After six months under 10% PEG 6000, there was a significant increase in osmolarity of callus in the PEG treatment compared with the control (Fig. 7-3a). Proline and soluble sugars levels also increased significantly compared with the control (MANA minus PEG) (Fig. 7-3b,c). However there were no significant differences in water and Na^+ content in the PEG and control treatments (Fig. 7-3d,e).
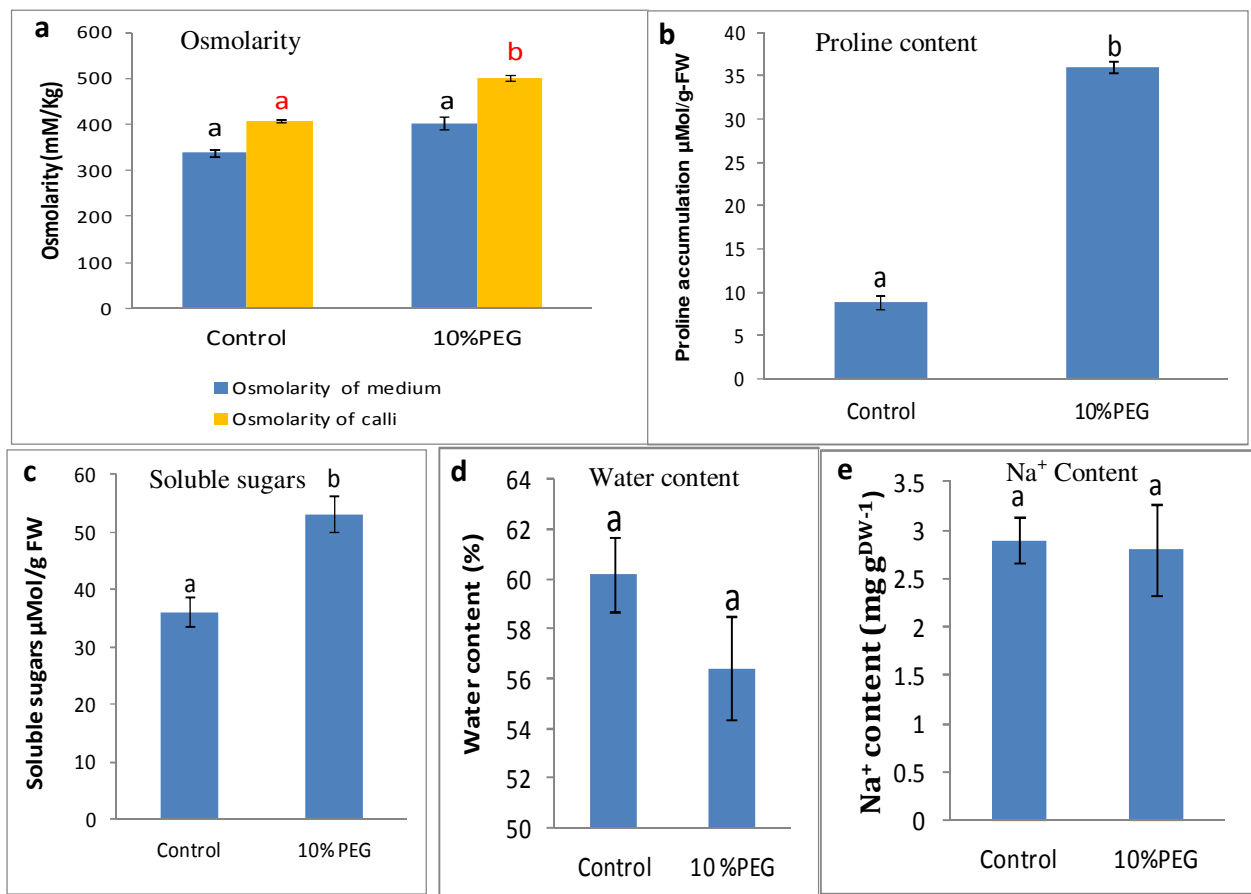
Fig 7-3. Comparisons between control and 10% PEG treatments after six months *in vitro* culture of: (a)= osmolarity, (b)= proline content, (c)= soluble sugars, (d)= water content and (e)= Na^+ content. Note different letters indicate between treatment significant differences ($P \leq 0.05$) (n=3).

7.3.3 Gene expression

Expression of those genes listed and described in Chapter 6 were measured using quantitative real time PCR after six months of callus culture in PEG (10%) and control treatments (0 % PEG). A highly significant reduction in the expression of *MtSOS1* occurred in the 10% PEG compared with the control treatments such that *MtSOS1* transcripts were virtually undetectable (Fig. 7-4a). Conversely, *MtWEE1* expression was significantly higher in the PEG compared with the control treatment (Fig. 7-4b). For *MtSERK*, *MtP5CS* and *MtCCS52*, there were no significant differences in expression in the PEG compared with the control treatment (Fig. 7-4c, d and e).

Fig 7-4. Gene expression after 6 months in 10% PEG stress and Control (0 % PEG stress), Leaf as reference. (a) = *MtSOS1*, (b) = *MtWEE1*, c = *MtSERK*, (d) = *MtP5CS* and (e) = *MtCCS52*. Note different letter indicate significant between treatment differences compared with control ($P \leq 0.05$) (n=3).
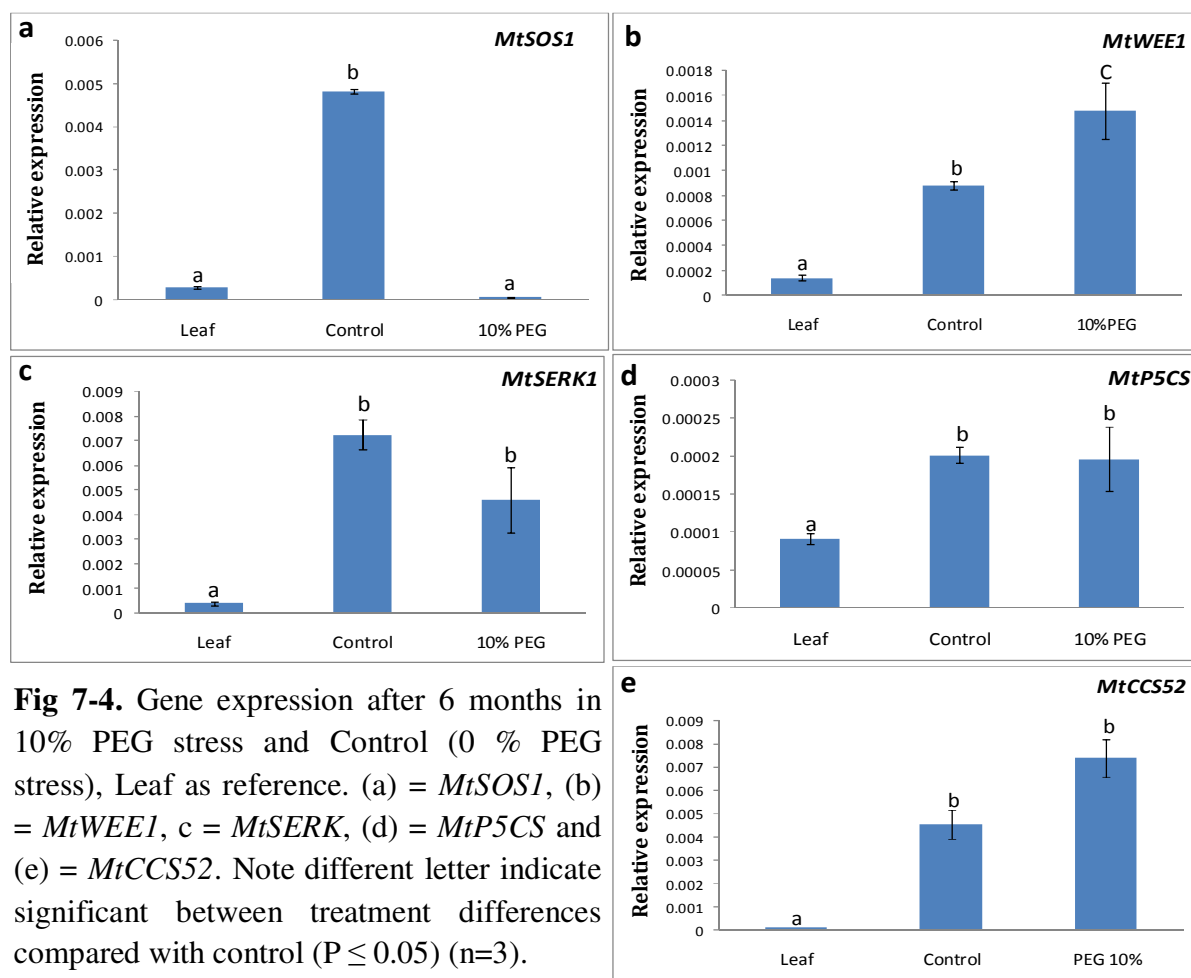
7.3.4 Flow cytometry results

In the 10% PEG treatment, four peaks of fluorescence were detected consistent with 2, 4, 8 and 16 C populations, respectively. (Fig. 7-5b). The 8 and 16 C peaks represent polyploid nuclear DNA amounts, most probably as a result of endoreduplication i.e. successive S-phase and endo G phases in the absence of mitosis (Elmaghrabi and Ochatt, 2006; Lemontey et al., 2000).
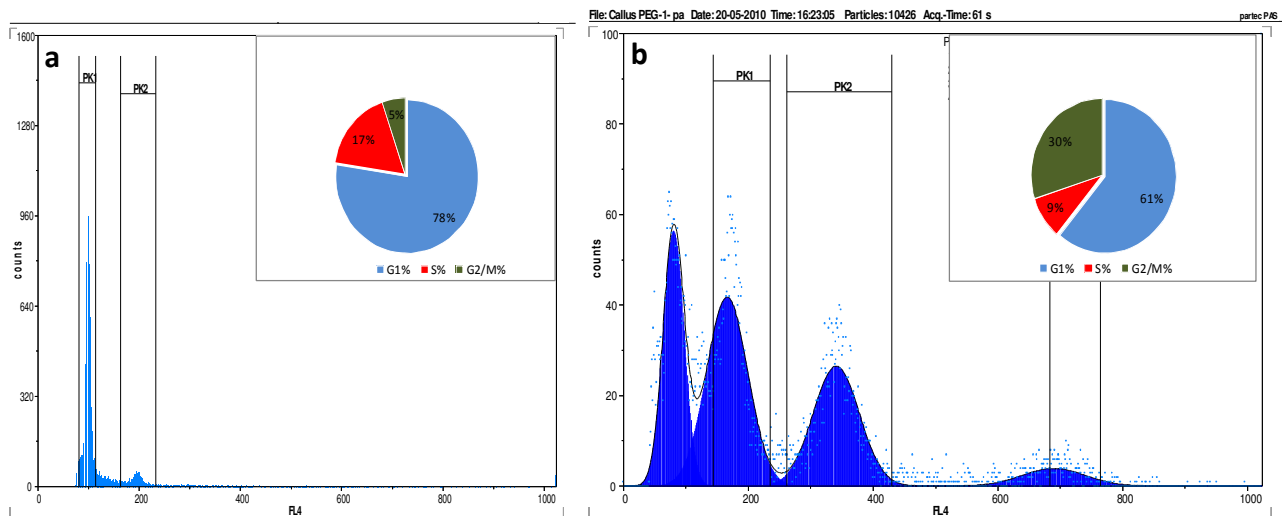
Fig 7-5. FCM profile: (a) = callus of control treatment (0 PEG stress) (diploid normal), and percentage distribution of nuclei in G1, S, and G2 phases. (b) = callus of 10% w/v PEG treatment and percentage distribution of nuclei in G1 (1st peak), G 2 phase (2nd peak) S phase (trough between 1st and 2nd peaks) and polyploid nuclei (3rd (8C) and 4th peaks (16 C), where 1C is the nuclear DNA amount of a gamete (www.insterscience.wiley.com)

7.4 Discussion

Drought or water deficit can be defined as the absence of adequate moisture necessary for a plant to grow normally and complete its life cycle (Cabuslay et al., 2002). Water deficit *in vitro* can be imposed through polyethylene glycol treatment (PEG 6000) (Romo et al., 2001; Erdei et al., 2002; Guoth et al., 2010; Yang et al 2012). PEG closely mimics soil water stress (Lu et al., 1998). It induces increases in total soluble sugars which serve as an osmoticum, or can be a source of respiratory substrates during the PEG-induced stress treatment (Handar at al., 1983; Srivastava et al., 1995). In my study, callus growth was not significantly different plus or minus 10% PEG over a period of six months, suggesting that calli on PEG acquired tolerance to drought (Fig. 7-1). The similarity in growth rates following 1 month ± PEG, suggests that tolerance was obtained relatively rapidly. This result differs from those of Biswas et al. (2002) who found that in rice, callus proliferation in the presence of PEG was greater than the controls. However in two genotypes of wheat, one drought tolerant, the other drought sensitive, water deficit decreased only slightly in the sensitive compared with the tolerant genotype under drought conditions and, it did not change significantly in either the sensitive or tolerant genotype ± 400 mM PEG 6000 (Guoth et al., 2010).

In the work reported here, the responses of calli to PEG-induced drought stress mirrored that of the results in salt stress treatments in some respects but not in others. In contrast to NaCl treatment, PEG treatment did not result in any increases in osmolarity or water content of the calli compared with the control. However soluble sugars did increase suggesting they are a useful marker of drought stress but not salt stress in this species (Fig.3-7c). PEG also

induced the highest levels of proline accumulation indicating that this is also an important component of drought tolerance in *M. truncatula* (Fig. 7-3b). The callus growth data were very similar ± PEG. Moreover, similar levels of somatic embryogenesis were observed in the PEG and control calli. However, calli in the PEG treatment were distinctly green in colour compared with the 0 control, which might be consistent with more robust embryogenic callus in the PEG compared to the control treatments (Fig. 7-2 and 7-1). That both control and PEG treatments resulted in similar levels of somatic embryogenesis was consistent with the similar expression levels of *MtSERK* in the two treatments. The expression of this gene was significantly higher in the 100/150 mM NaCl treatments compared with the control (see Chapter 6). In particular the levels of somatic embryogenesis were deemed to be higher in these salt treatments compared with controls albeit at a purely qualitative level (Chapter 6 (section 6.1.7)). Note that *SERK1* is highly expressed during embryo induction and early somatic embryo development in *M. truncatula* (Nolan et al., 2009) and in *Araucaria angustifolia* (Steiner et al., 2012). The data suggest that PEG treatment did not negate induction of somatic embryogenesis.

The accumulation of soluble sugars and proline in many species has been widely confirmed as a response to salt and/or drought tolerance and has a role to protect the cells against osmotic perturbation (Murakeozy et al., 2003; Choudhary et al., 2005; Fulda et al., 2011). For example, Cabuslay et al. (2002) showed that sugar accumulation was high in the leaf sheath in rice cultivars, IRAT9, IRT104 and IR20 under PEG stress (Cabuslay et al., 2002). Proline accumulation is a characteristic index of osmotic adjustments in plants that are exposed to drought or salt stress as reported in chapter 4 (section 4.1.1) (Valliyodan and

Nguyen, 2006). Indeed, proline accumulation was significantly higher in the PEG compared with control treatment (Fig. 7-3b). However the expression of *MtP5CS* on PEG was similar to the control (Fig. 7-4d) and hence does not correlate with increased levels of proline in the PEG treatment. This was surprising given that this gene encodes an enzyme that is central to proline synthesis. It may suggest post-translational control of enzyme activity, or that this enzyme is not a key regulatory step in proline biosynthesis under these conditions.

Na^+ concentrations were no different in PEG and control treatments treatment, indicating that PEG did not alter Na^+ homeostasis (Fig. 7-3e). Moreover, and predictably PEG did not induce *MtSOS1* expression which exhibited down-regulated compare with control. This gene is highly expressed in salt stress conditions where its encoded protein functions as a membrane-bound Na^+ antiporter contributes to Na^+ depletion in the cytoplasm (Türkan and Demiral, 2009; Smith et al., 2010). Therefore the PEG data indicate a different (non ionic) pathway leading to drought tolerance compared with NaCl tolerance.

Finally, flow cytometry analysis showed endoreduplication in calli in the PEG treatment (Fig. 7-5). These data, rather like the flow cytometry results following NaCl treatment, show that callus in the PEG treatment grew at virtually the same rate as the controls regardless of the level of polyploidy. It might have been predicted, that endoreduplicating nuclei do not contribute to growth since these cells do not divide. However, the populations of polyploid cells have larger nuclei than diploid cells and might contribute to expansion growth as the cell (s) accommodate larger nuclei formed by endoreduplication. However the expression of *MtCCS52,* was not significantly different ± PEG (Fig. 7-4e). This is another somewhat

surprising result given that in Arabidopsis this gene is a regulator of ploidy level and its expression is positively related to endoreduplication, which was also the case for the NaCl treatments reported in this thesis (see Chapter 5). However, in the PEG treatment *MtP5C* expression was not suppressed but was not elevated compared with the 0 % PEG control. Possibly in *M. truncatula* there is functional redundancy of genes that regulate ploidy level in the PEG treatment.

That *MtWEE1* expression was more highly expressed in the PEG treatment suggests that this gene may have a role in maintaining normal growth in drought stress conditions. As discussed in more detail in Chapter 6, WEE1 kinase might be necessary to regulate normal cell size in the face of ion toxicity and drought. Alternatively, as it is a gene expressed in stress checkpoints it may be induced by salinity or drought in the same way that it is highly expressed in Arabidopsis in the DNA replication and DNA damage checkpoints (De Schutter et al., 2007). However, Gonzalez et al (2004) observed that high expression of *LeWEE1* in tomato (*Lycopersicon esculentum* Mill.) which was correlated with endoreduplication during fruit development. Note that this may also be the case for *MtWEE1*.

Time did not permit a detailed analysis of the effects of a PEG concentration gradient on the traits reported on in this chapter. However, the preliminary results reported here could provide a basis for further work.

7.5 Summary

The data reported in this chapter in responses to 10% PEG compared with 0 PEG controls can be summarized as follows:

- No difference in growth rate

- Significant increases in soluble sugars and proline accumulation

- High *MtWEE1* expression

- No significant differences in *MtSERK1*, *MtP5CS* and *MtCCS52* expression but down regulation of *MtSO*S expression

- Strong evidence of endoreduplication

Chapter 8: General discussion and future directions

8.1 General discussion

Libya is one of the largest countries in the Mediterranean basin where crops on arable land suffer substantial salinity and drought stress; year by year salinity is increasing (Zalidis et al., 2002). The Biotechnology Research Center (BTRC) Tripoli-Libya, where I work, has used conventional methodology for isolating mutants using gamma irradiation (^{60}Co) and incorporating traits into crop plants through traditional breeding methods, paying particular attention to phenotypic traits such as plant height and grain size. Such programmes take from 7 to 10 years to complete and a huge area in the field is necessary to select mutants for salt or drought tolerance (Elamaghrabi et al., 2003). As outlined in the Introduction, *M. trunctula* has been used as model system. It is an important forage crop and is able to tolerate arid and semi-arid conditions. World-wide, Libya holds 7.2% of *M. truncatula* accessions (Magalie et al., 2007). Mechanisms of salt and drought tolerance have not been studied in any great detail although the use of tissue culture (with or without acclimation treatment) can be a powerful technique to investigate these problems. About 10 years ago, workers at BTRC Tripoli-Libya began tissue culture work e.g. micropropagation. However, very little work has examined the effect of *in vitro* acclimation for salt and drought tolerance in *M. truncatula.* My study on *M. truncatula* in Cardiff, began with optimizing both explant and optimal medium finding that MANA and leaf explants were optimal. Then I began to study the extent to which calli of *M.*

truncatula could be made salt tolerant using a new tissue culture protocol that I developed in the Cardiff lab. The protocol incorporated multi (monthly) step ups in exogenous [NaCl] followed by two rounds of – NaCl and + NaCl in the concentration range 0 to 350 mM NaCl (chapter 3). This range was chosen to reflect salinity levels in coastal arable soil in Libya. For example, the mean soil concentration of NaCl was 100 mM from a study incorporating 13 different sites in Libya (Achuthan et al., 2005).

The first aim was to assess the effects of acclimation on growth and embryogenesis (chapter 3). A second aim was to study the extent to which physiological traits such as Na^+ and K^+ content, osmolarity, proline and sucrose accumulation of calli cultured ± acclimation could be used as markers of salt tolerance in *M. truncatula* (chapter 4). Thirdly, I characterized cytological aspects of salt tolerance such as nuclear and cell size, endoreduplication and vacuolar size (chapter 5). Fourthly, I analysed the expression of genes related to abiotic stress (chapter 6 and 7): *MtWEE1* in relation to growth, *MtCCS52* and *MtWEE1* in relation to endopolyploidy, *MtSERK* in relation to somatic embrogenesis, *MtP5CS* in relation to proline accumulation and *MtSOS* in relation to ion uptake. In the final results chapter (chapter 7) I report preliminary data on callus responses following a PEG 6000 treatment; PEG can mimic drought conditions (Mahmood et al., 2012).

8.1.1 Growth and *MtWEE1* expression

In general acclimation has been useful in making plant cells tolerant to NaCl (Chen et al., 2011). However, as might be predicted, there are interspecific differences in the extent of tolerance brought about by either single transfer to NaCl or month-by-month acclimation to increasing concentrations of NaCl (Queiros et al., 2007) .

I report, for the first time, that calli established from *Medicago truncatula*, became tolerant to 150 mM NaCl following a multi-step acclimation (Phase I) followed by two passages of zero and continuous NaCl (Phases II and III). Indeed, there was a three-fold increase in tolerance following Phase III (150 mM) compared with Phase I (50 mM). Similarly, in calli of one variety (*Solanum tuberosum* L.) of potato, three "step-ups" of NaCl led to tolerance at 200 mM NaCl (Queiros et al., 2007). However, in this work on potato, NaCl-tolerant calli exhibited a slower relative growth rate and reduced water content compared with controls. In the potato variety Kennebic, several cell lines were created one of which could not grow in single transfers to ≥ 90 mM NaCl whilst in another, a tolerance was obtained with a single transfer from 0 to 150 mM NaCl (Ochatt et al., 1999). In comparison, single transfers have led to two- fold increases in tolerance in wheat (Piti et al., 1994) and rice (Miki et al. 2001). Thus acclimation can yield a greater NaCl tolerance compared with single transfers. I then tried to underpin growth responses to acclimation by measuring the expression of *WEE1*, a gene known to be expressed under stress conditions (chapter 6). *WEE1* was chosen given that in yeast and animal cells its encoded protein kinase negatively regulates CDKs both in normal and stress conditions that result in perturbation of DNA

replication or DNA damage. These so-called checkpoints utilize WEE1 kinase to suppress cell division (Rhind and Russell, 2000) and are only lifted when DNA replication is normalized or when DNA damage is repaired. WEE1 is then down regulated and subsequently cells are free to divide (Rhind and Russell, 2000). In Arabidopsis *WEE1* is highly expressed upon the induction of the DNA replication and DNA damage checkpoints (De Schutter et al., 2007). Hence *WEE1* is a feature of higher plant checkpoint pathways although its primary substrate has yet to be fully resolved (Dissmeyer et al., 2009). It is perhaps counter intuitive to predict that a negative regulator of cell division was most highly expressed in those calli showing the highest rates of growth. However, *WEE1* is only highly expressed in Arabidopsis tissues that comprise rapidly dividing cells (Sorrell et al., 2002). It might be the case, that high *WEE1* expression reported here in 100 and 150 mM NaCl, is a necessary component of growth regulation under saline conditions. Resolution of the exact function of plant WEE1 is required to test this hypothesis.

8.1.2 Endoreduplication, *MtCC52* and *MtWEE1* expression

Flow cytometric data show endoreduplication in calli of *M. truncatula* ± 50 to ±150 mM NaCl (chapter 5; section 5.3.3). Indeed endoreduplication has been reported as an adaptation factor to high salt concentration (Ceccarelli et al., 2006). As mentioned in the Introduction, *CCS52* expression is positively related to the extent of endoreduplication. Hence high expression levels of *CCS52* in the 100 and 150 mM treatments fit (chapter 6: Fig. 6-11) with the occurrence of endopolyploidy at these levels of NaCl. High *WEE1* expression reported here at 100/150 mM NaCl (chapter 6: Fig. 6-10) may also be related to endoreduplication.

Under abiotic stress, the WEE1 protein kinase might be blocking entry of cells into mitosis priming them to begin an endoreduplication cycle as was obtained in Arabidopsis (Adachi et al., 2011). However, in my study of NaCl tolerance, endoreduplication occurred regardless of NaCl treatment. Endoreduplication and high *WEE1* expression were also obtained in the 10% PEG treatment (chapter 7: Fig.7-4) compared to a 0% PEG control suggesting that tolerance to drought may be partly regulated by WEE1 kinase that blocks the G2/M transition enabling increased endoreduplication. The relevance of endoreduplication to drought tolerance clearly requires further work.

8.1.3 Embryogenesis and salt tolerance is correlated with *SERK* expression.

Both embryogenic and organogenetic calli have been observed *in vitro* for many species following a single step up in NaCl concentration (Ochatt et al., 1999; Shankhdhar et al., 2000; Chen et al., 2011). However, to my knowledge, this is the first report in *Medicago truncatula* that acclimation that involved multiple step ups led to increased embryogenic and robust calli at 100 mM. Highest expression of *SERK* occurred at 150 mM following Phase I and 100 mM NaCl following both Phases II and III. Hence profuse somatic embryogenesis and highest *SERK* expression occurred in the 100 mM treatment (chapter 6: Fig 6-9). However, the expression of *MtSERK1* on callus cultured on 10% PEG for six months was similar to the control (0 % PEG) which correlated with similar mean callus growth in the control and 10% PEG treatment (chapter 7). On a qualitative basis, embryogenesis in the PEG treatment was observed more often compared with the control.

8.1.4 Na$^+$/K$^+$ homeostasis , vacuoles and *MtSOS1* expression.

SOS1 expression was highest in the 150 mM NaCl treatment, following Phase I and then 100 mM NaCl following Phases II and III (chapter 6: Fig. 6-12). Exogenous [NaCl] had a positive and negative effect on endogenous [Na$^+$] and [K$^+$], respectively. My data conform to a model first established for Arabidopsis (Shi et al., 2003) where SOS-mediated signaling pathways maintain low [Na$^+$] in the cytoplasm under salt stress (see Fig. 6-2). Excessive Na$^+$ perceived by unknown sensors can induce a Ca^{2+} -dependent pathway leading to a SOS 2/3 protein kinase complex, which activates the membrane bound Na$^+$/H$^+$ antiporter, SOS1. SOS2 protein kinase also activates the antiporter Na$^+$ /H$^+$ (AtNHX1) at the tonoplast, compartmenting Na$^+$ into the vacuole and releasing H$^+$ into the cytoplasm. Negative regulation of AtHKT1 at the plasma membrane can also be imposed by SOS2/SOS3 that in turn can have a negative effect on the membrane- bound K$^+$ in H$^+$ out antiporter, AKT1, that normally regulates K$^+$ uptake into the cytoplasm (chapter 6: Fig. 6-2). Hence the SOS-family regulates sequestration of Na$^+$ ions into a vacuolar compartment and at the same time, suppresses Na$^+$ uptake at the cell membrane (chapter 6: Fig. 6-2). However, it can also be noted, that AKT1 at the membrane can be suppressed by SOS2 thereby reducing [K$^+$] uptake into the cytoplasm. This type of mechanism has been resolved in *Arabidopsis thaliana*; (Chen et al., 2007), barley (Shi et al., 2002) and *Centaurium maritimum* L. (Misic et al., 2012), to maintain optimal cytosolic K$^+$/Na$^+$ homeostasis. Moreover, and as mentioned in the Introduction of chapter 6, genetic evidence in Arabidopsis shows *SOS1* expression as vital for salt tolerance. Also, as reported in my study (chapter 4: Fig. 4-6), exogenous NaCl at 150 mM is a cross point of Na$^+$ accumulation and K$^+$ deficit, consistent with the idea that Na$^+$/K$^+$

homeostasis helped to maintain the highest amount of growth at this concentration of NaCl (chapter 3: Fig. 3-17). Seemingly, beyond this cross point, the 250 mM and 350 mM NaCl treatments led to an overload of [Na^+] and a severe deficit of [K^+] that led to poor growth and limited embryogenic potential (chapter 3: Fig. 3-17). Cytoplasmic [K^+] plays an essential role in osmotic adjustment during salt or drought stress (Wu et al., 1996; Cuin et al., 2012). In my work, vacuoles were largest in the 100 and 150 mM treatments and significantly larger compared to 0, 250 and 350 mM NaCl treatments. This was also reported for *Arabidopsis thaliana* under salt stress where both Na^+ and Cl^- sequestration protected the cytoplasm from the toxic effect of these ions (Wei et al., 2011). Calli that grew under 10% PEG treatment for six months showed a down regulation of *MtSOS* expression compared with control (chapter 7, Fig. 7-4a) while the Na^+ content was similar between both PEG treatments and control (Fig. 7-3e). Clearly the PEG treatment induced a different pathway that would neither require nor depend on *SOS1* expression.

8.1.5 Proline, *P5CS* expression and water soluble carbohydrates

I found a positive relationship between exogenous [NaCl] and both osmolarity and proline accumulation (chapter 4). This has been reported by many authors (e.g. Raven, 1985; Hariadi et al., 2011). For example, Summart at al. (2010) showed that in rice cultures exposed to 250 mM NaCl, [proline] increased almost three-fold compared to controls. Hence my data support the idea that in salt-stressed calli derived from *M. trucatula,* proline accumulated to protect the cells against osmotic perturbation that would otherwise occur in response to 150 mM exogenous NaCl. Note that *P5CS,* encoding an enzyme that functions en-route to proline

synthesis was most highly expressed in the 100 / 150 mM NaCl treatments (chapter 6: Fig. 6-13). There was a positive linear relationship between water soluble sugars and NaCl concentration spanning 0 to 250 mM. The 150 and 250 mM NaCl treatments also induced significantly higher levels of water soluble sugars in the callus compared with the control following Phase III (chapter 4: Fig. 4-4). Vinayak et al. (2011) reported a 75% increase in the total soluble sugars in shoots of *Sesuvium portulacastrum* cultured with 200 mM NaCl. Moreover, the accumulation of soluble sugars in plant tissues has been widely confirmed as a response to salt and/or drought tolerance (Murakeozy et al., 2003; Patade et al., 2011). However as pointed out in the Introduction of chapter 4, a positive relationship between accumulation of water soluble carbohydrates and salt tolerance is not universal. Note that my data showed the highest accumulation of these sugars was in the 250 mM NaCl treatment which could not support growth, leading me to conclude that this particular trait is not a reliable indicator of salt tolerance in calli derived from *M. truncatula.* Proline and water soluble carbohydrate content were also significantly higher in the 10% PEG treatment compared with the control. However, as mentioned in Chapter 7, a concentration gradient of PEG would be required to test the extent of accumulation of proline and water soluble carbohydrates in relation to drought.

8.1.6 Longevity

The highest level of salt tolerance was at 150 mM NaCl following Phase III when calli were 23 months old. Another recent study on *Zoysia matrella* (Chai et al., 2011), showed that callus could be maintained *in vitro* for more than five years on MS medium supplemented

with 2 mg l^{-1} 2,4D and 0.02 mg l^{-1} Kinetin; these calli could be acclimated to grow at non-optimal temperatures (e.g 4°C). Wild varieties of *M. truncatula* showed callus formation/somatic embryogenesis that occurred on media supplemented with an auxin (NAA or 2,4-D) alone or in combination with cytokinins; the optimal level of plant growth regulators was species- and explant-dependent (Nolan et al., 1989; Trinh et al., 1998; Svetoslavova et al., 2005; Sivanesan et al., 2011). However, it must be noted that total age of calli might be misleading since in my work, and that of others, calli were regularly sub-cultured, providing fresh nutrient for new sites of proliferation in addition to maintenance of pre-existing live callus. A broader conclusion is that 18 months following the start of acclimation to 100 and 150 mM NaCl, callus maintained proliferative and embryogenic competence. Likewise, long-term cultured cell suspensions of this same *M. truncatula* genotype, among others, were shown to maintain organ and embryo regeneration competence coupled with cytological stability (Elmaghrabi & Ochatt 2006), a premise for the subsequent recovery of whole plants (Ochatt et al., 2000). Overall, the work reported here represents a contribution to understanding physiological and molecular processes / mechanisms during acclimation-led abiotic stress (salinity and drought). I have summarized the results of all experiments in table (8-1) which indicates the extent of significant differences in the NaCl / PEG treatments compared with 0 mM controls. Mostly, the 100/150 treatments proved to be optimal for growth, embryogenesis, and Na^+/K^+ homeostasis. Moreover, highest expression of the five genes analysed measured in this thesis peaked in the 100/150 mM NaCl treatments. Hence my work indicates a range of physiological, cellular and molecular traits that have proved to be markers of *in vitro* NaCl tolerance in *M. truncatula*.

Table 8.1 Summary of physiological and molecular results following Phases I, II and III in the 50-350 mM NaCl treatments or 10% PEG stress (6 months) treatment compared with the control (0 mM NaCl, 0% PEG). Yellow highlighted boxes reveal key discoveries of my work.

Treatments																
mM NaCl	**50**			**100**			**150**			**250**			**350**			**PEG**
Phases	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	6M
Mean callus growth	↔	↑*	↔	↓*	↔	↑*	↓*	↔	↑*	↓**	↓**	↓**	↓**	↓**	↓**	↔
Na^+ accumulation	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑**	↑**	↑**	↑**	↑**	↑**	↔
K^+ accumulation			↔			↓*			↓*			↓**			↓**	↔
Proline content		↑*	↑*		↑*	↑**		↑*	↑**		↑*	↑*		↑*	↑*	↑*
Soluble sugars			↔			↔			↑*			↑*			↔	↑*
Water content	↔			↔			↔			↓*			↓*			↔
Osmolarity	↑*			↑*			↑*			↑**			↑**			↑*
Vacuolar size			↔			↑*			↑*			↓*			↓*	
SOS expression	↑*	↔	↔	↑*	↑**	↑**	↑**	↑**	↑*							↓*
P5CS expression	↔	↑**	↑*	↑*	↑**	↑**	↑**	↑**	↑**							↔
SERK expression	↓*	↑*	↔	↓*	↑**	↑**	↑**	↑*	↑*							↔
WEE1 expression	↑*	↑*	↑*	↔	↑**	↑**	↑**	↑*	↑**							↑*
CCS52 expression	↑*	↑*	↑*	↔	↑**	↑**	↑**	↑**	↑**							↔

KEY:

(↑*) = significant increase,

(↑**) = very significant increase,

(↓*) = significant decrease,

(↓**) = very significant decrease

(↔) = no significant difference.

P values for * (P= < 0.05) and ** (P= < 0.001).

All tests are between treatments and control (0 mM NaCl).

8.1.7 Heritable or reversible changes for physiological, cytological and gene expression during acclimatization of salt stress *in vitro* culture

Although my work focusing to the evaluation of the physiological development, cytological process and gene expression of calli of *M. truncatula* during three phases of acclimation (23 months) under various concentration of mM NaCl (0-350). However 100 and 150 mM treatments resulted in fastest calli growth compared with the controls, possibly this could be an adaptation due to heritable epigenetic changes that enable NaCl tolerance. Flow cytometry data show that endoreduplication occurred in the 50 mM NaCl treatment while in the 100 and 150 mM treatments, the majority of cells grown on 100 and 150 mM NaCl accumulated in S phase or endo S-phase (see section 5.3.3). Endoreduplication has been observed in many studies of plant cells *in vitro* (e.g. Ochatt, 2008; Anjanasree et al., 2012). Indeed Elmaghrabi and Ochatt (2006) reported that when cells of three genotypes of *M. truncatula* J5, TV25 and TR122, were grown on CIM (Callus inducing medium) for 6 months endoreduplication was observed alongside mixoploidy, aneuploidy and tetraploidy. Note that in my work, high *WEE1* and *CCS52* gene expression were obtained in 100 and 150 mM treatment; expression of both genes are strong markers of endoreduplication (Guo et al., 2007; Angel et al., 1999). Calli exhibited Na^+/K^+ homeostasis high levels of proline in the 150 mM NaCl treatment (see sections 4.3.3see section 4.3.4) and high levels of *P5CS* and *SOS* gene expression these responses are typical of Na^+ exclusion notably *P5CS* has a in proline synthesis (Patade et al., 2011). Also high *SOS*1 expression was obtained in potato callus acclimated over 23 months on 100 and 150 mM NaCl (Ji-Bao et al 2009). My data support the hypothesis that calli may be acclimated and/or adapted to heritable epigenetic changes to salt stress. However, future work is necessary to establish the extent to which these markers / genes of salt tolerance *in vitro* have a role in salt tolerant

mechanisms in plants derived from NaCl-tolerant calli. Analysis of such data could then be assessed to confirm or negate the hypotheses that calli were acclimated and/or adapted as permanent heritable genetic changes.

8.2 Future directions

Of immediate importance, is to determine whether NaCl tolerant plantlets can be regenerated from the 100 and 150 mM treatments and then to determine the extent of their NaCl tolerance under field conditions. Also it will be important to determine the extent to which the various physiological, cytological and molecular markers *in vitro* described in this thesis will continue to act as reliable indicators of tolerance to NaCl in field conditions. Six major themes should be explored through assessing:

1- salt tolerance of plantlets regenerated from NaCl tolerant calli obtained from the acclimation-led protocol that I have developed,

2- physiological features such as proline, soluble sugar, Na^+ and K^+ contents in regenerated plantlets under NaCl stress *in vivo,*

3- the extent to which vacuoles are larger in regenerated plantlets under salt stress *in vivo,*

4- expression of *WEE1, CCS52*, *SOS1, 2* and *3*, and *P5CS* in plantlets growing under NaCl stress which in turn will resolve the extent to which they can serve as useful molecular markers of NaCl tolerance *in vivo* and under field conditions

5- the effect of over-expression of key genes, such as *MtWEE1* and *MtSOS1* on salt tolerance

in vivo,

6- the effectiveness of a PEG concentration-gradient *in vitro* and whether regenerated plantlets are drought tolerant in the field.

From all these experiments (1-5), will confirmed that calli were acclimated and/or adapted for 23 months under salt stress were acquired of permanent heritable genetic changes for salt tolerance. The ease with which *M. truncatula* can be regenerated and transformed (see Araùjo et al. 2004) promises well for these future experiments. To this end, is my pledge to contribute work that helps to resolve such issues that I sincerely hope will have an impact on future breeding programs in my country.

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Appendix (A)

Table A.1. Calibration curve. A standard solution was prepared by dissolving 20 mg of sucrose in 200 ml distilled water, then four dilutions were used to create a calibration as shown in the Table below.

Dilution	Dilution 1	Dilution 2	Dilution 3	Dilution 4
contents	5ml of main solution + 5ml of distilled water	4ml of main solution + 6ml of distilled water	3ml of main solution + 7ml of distilled water	2ml of main solution + 8ml of distilled water
Concentration mg/l^{-1}	50	40	30	20

Fig. A.2 Linear regression of soluble sugar accumulation.

Linear regression of data: Reading $= 0.032_X + 0.0026$, P $= < 0.001$.
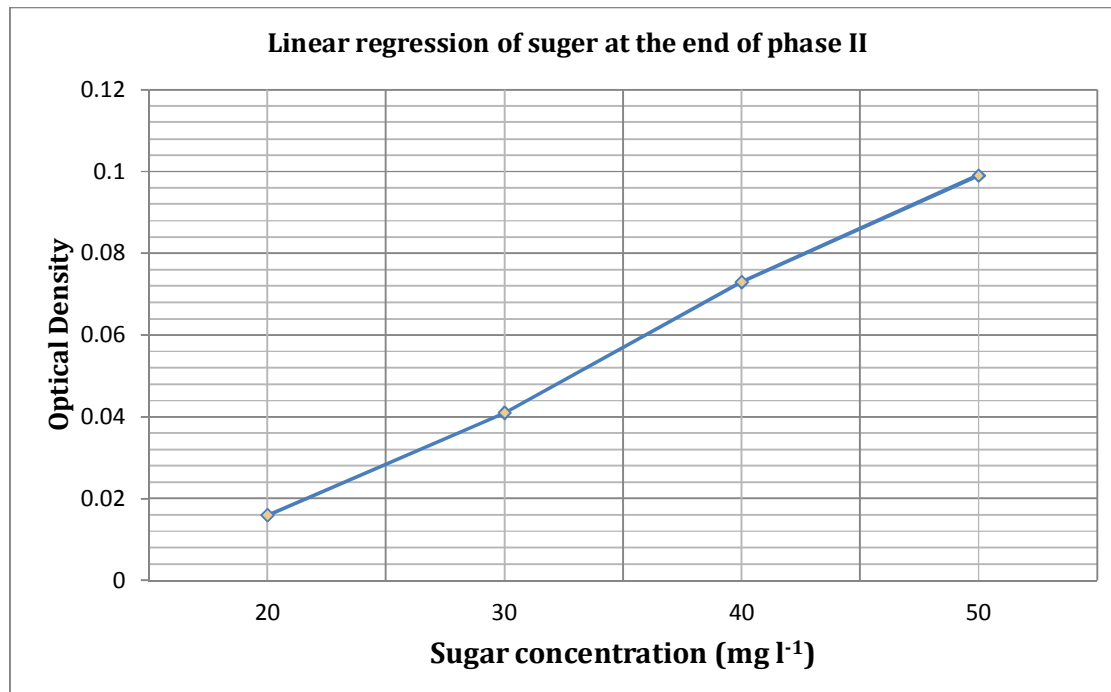
Appendix (B)

Mortality after five months of *M.truncatula* cell suspension on BY2 medium with various concentrations of NaCl (mM) and PEG (10%)

In a comparative experiment, I examined the extent to which salt and PEG affected *Medicago truncatula* the well-established cell suspension system, in BY-2 medium. The mortality index was measured after 5 months on different concentrations (mM) of NaCl or 10 % PEG. The results were obtained by measuring mortality index of cell suspensions which acclimated on various abiotic stresses. The percentage of mortality was significantly increased between each concentration of mM NaCl compared with the control (0 mM NaCl) (39.67%) to the highest concentration of 350 mM NaCl (82.67%) but not between 50 and 100 mM NaCl (Table. 1). In terms of PEG stress, as shown in Table 1 there was also a significant increase in mortality compared with control (0 mM NaCl). This result was supported by measuring the density of cells using a spectrophotometer at 600 nm after 4 and 5 months of NaCl (mM) and 10% PEG in BY2 medium. Following both 4 and months the density of cells decreased significant gradually according to the concentration of mM NaCl (Fig. 1). This significant decrease was also observed with non ionic stress (10% PEG) compared with the control 0, 50 or 100 mM NaCl (Fig. 1).

Table 1. Percentages (%) of mortality after 5 months on BY2 medium with various concentration of abiotic stress (n=3).

Treatments mM NaCl	Percentage (%) of mortality of cell suspension after 5 months on BY2 medium
0 control	$39.67^a \pm 1.47$
50	$53.57^b \pm 2.27$
100	$58.33b^c \pm 2.52$
150	$66.73^d \pm 2.09$
250	$75.33^e \pm 2.45$
350	$82.67^f \pm 1.08$
10% PEG	$62.53^{cd} \pm 0.91$

[a-f] Means of the same column which are followed by the different letters are statistically different at $P \leq 0.05$.
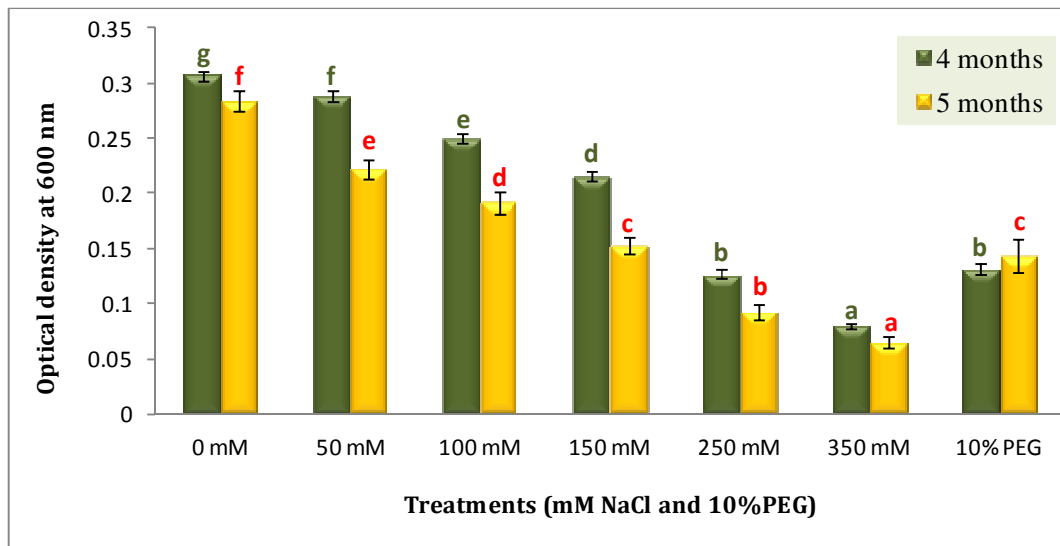
Fig 1. Mean ± S.E. density of cell suspensions of *M. truncatula* on BY2 medium exposed to various NaCl concentrations (mM) or 10% PEG for 4 or 5 months. Notes different letter combinations between the same coloured bars indicate significant differences (P ≤ 0.05) (n=4).

Appendix (C1)

Real time PCR conditions on opticon monitor 3 software

To set up the run, three steps were carried out according to the experiment. First step: a master file was set up detailing the conditions of amplification (Fig. 1) and the total volume of each microtube which was 25µl, second step: (plate setup) for that step all the samples were labeled and dye was selected from the options available with the software (SBG1) and third step: (protocol setup) the melting curve conditions were inserted: plate read from 60 ℃ to 98 ℃, read each 1 ℃, hold 01 s. Before running the result of real time PCR was already saved in a new file on the computer.
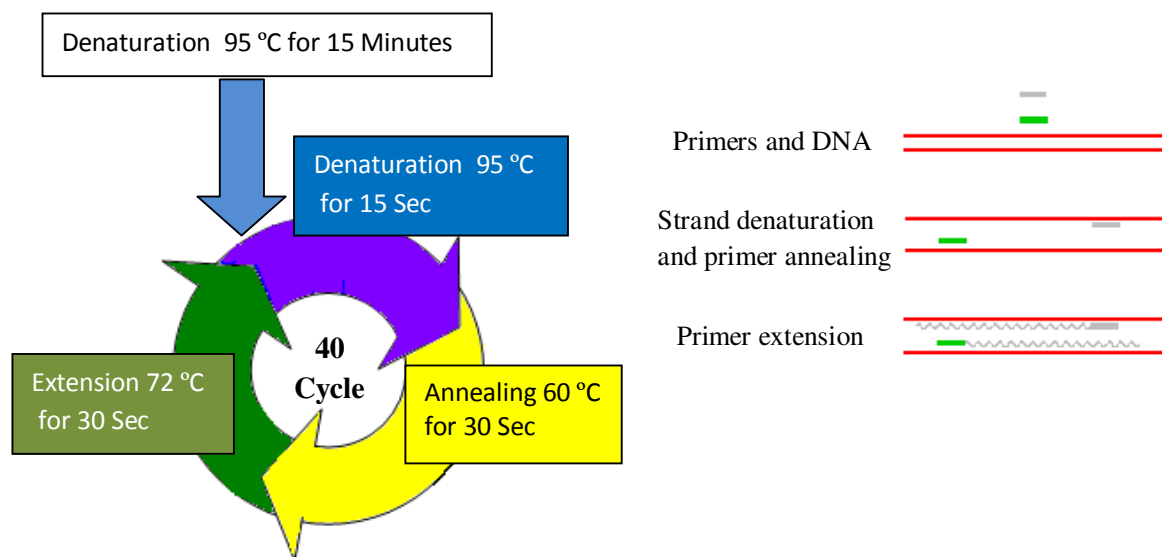
Fig 1. Cycling exponential amplification of real time PCR products.
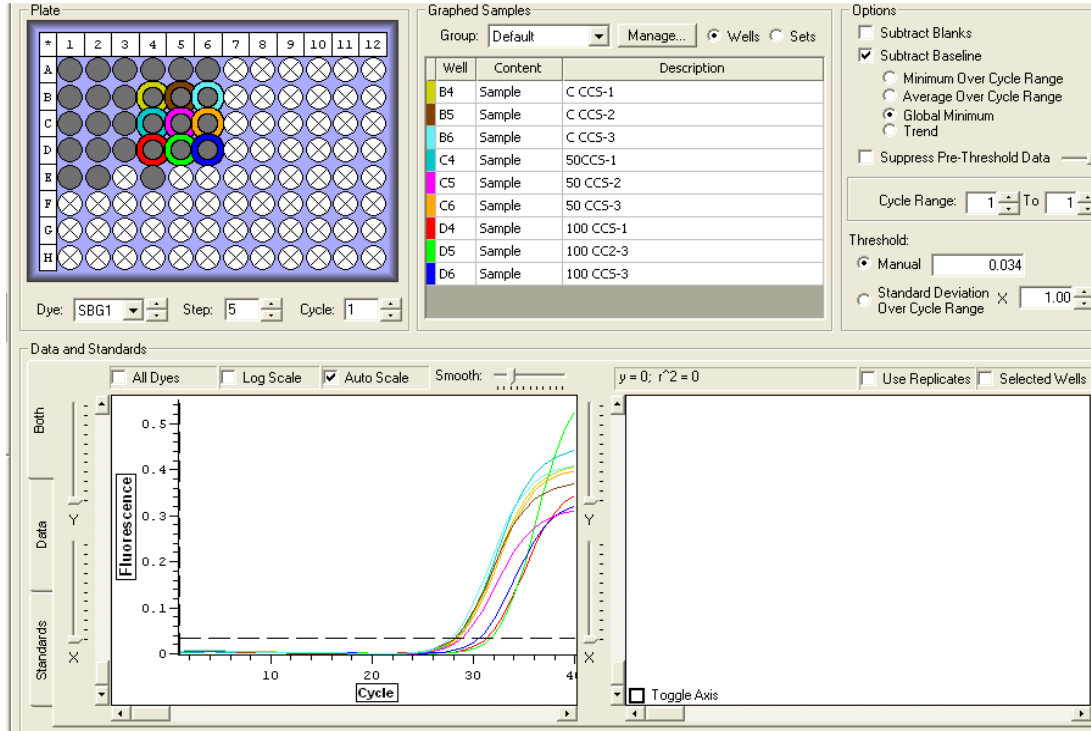
Appendix (C2)

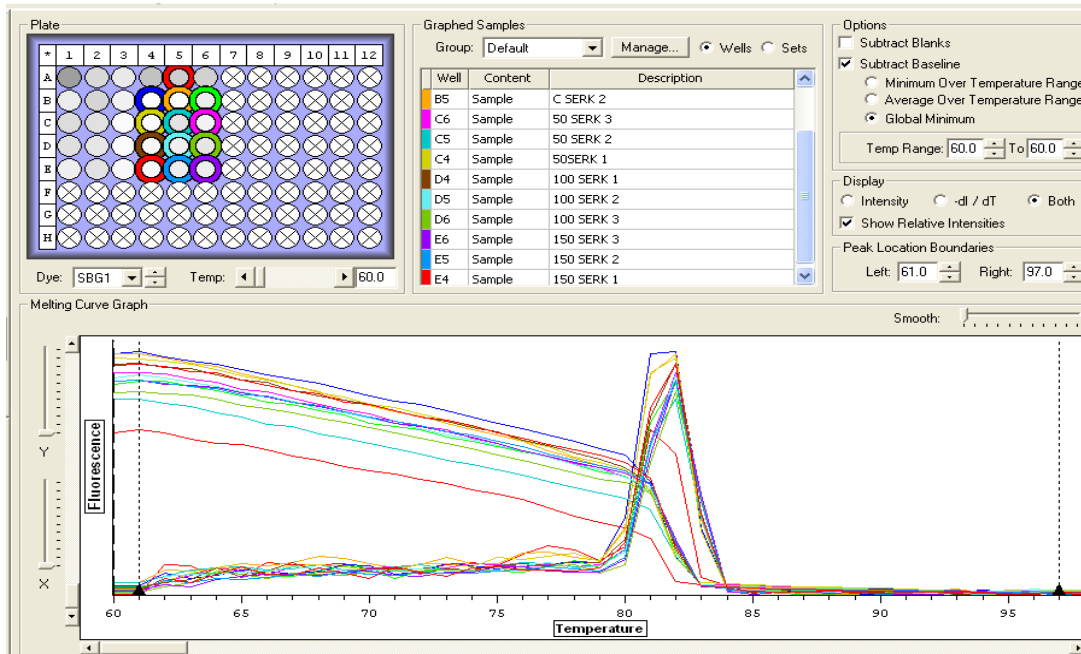Melting curve of MtSOS gene
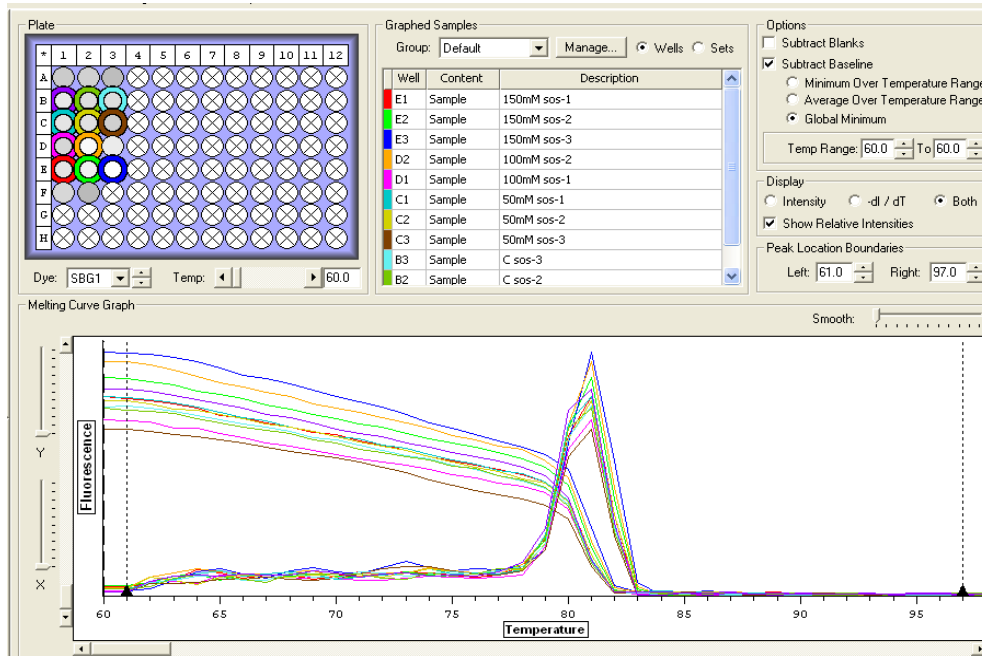
Quantitation curve MtSOS gene
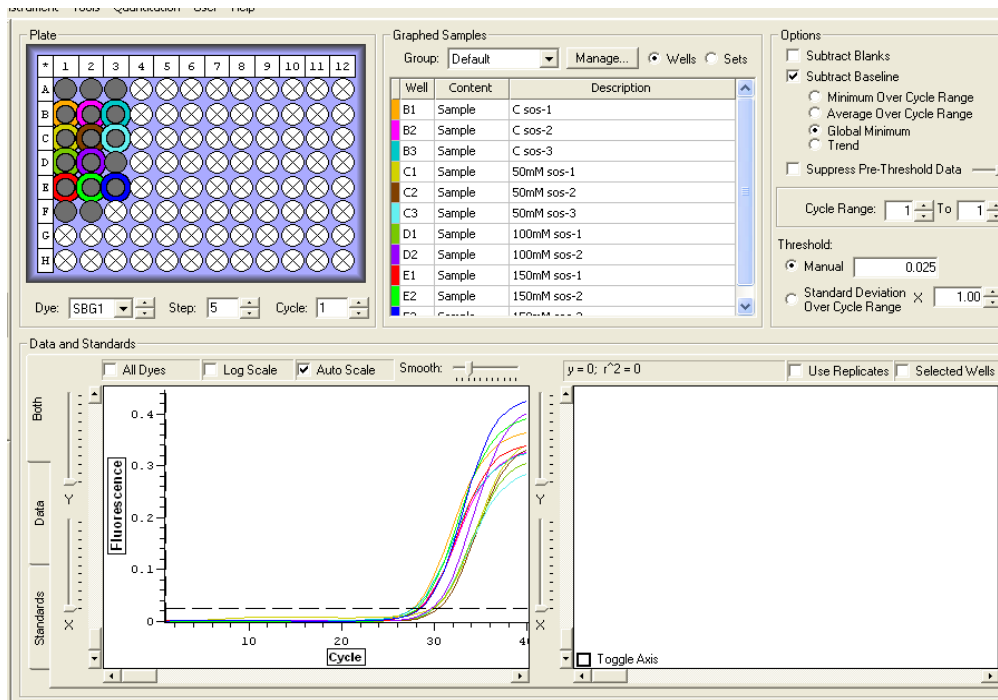
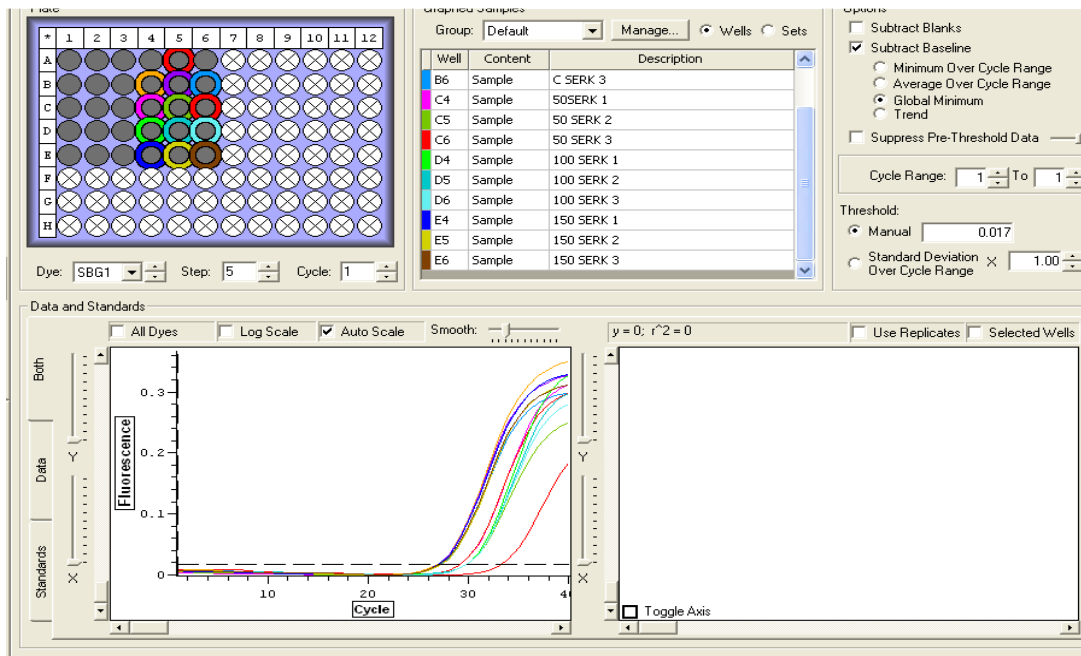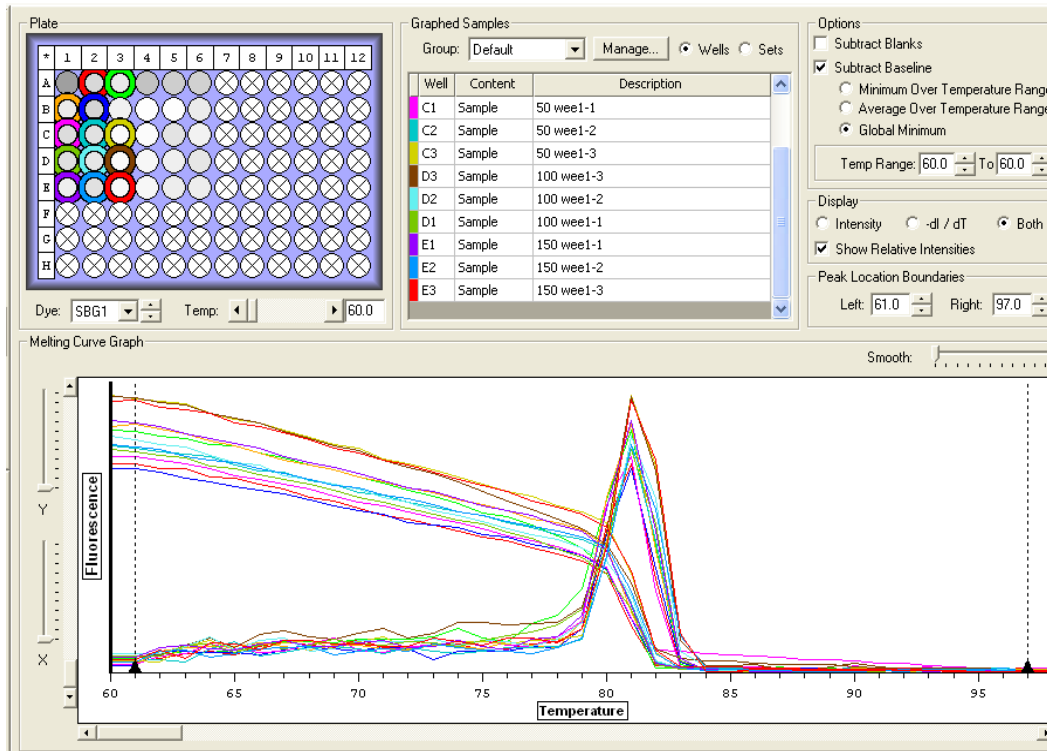Melting curve of MtCCS52 gene
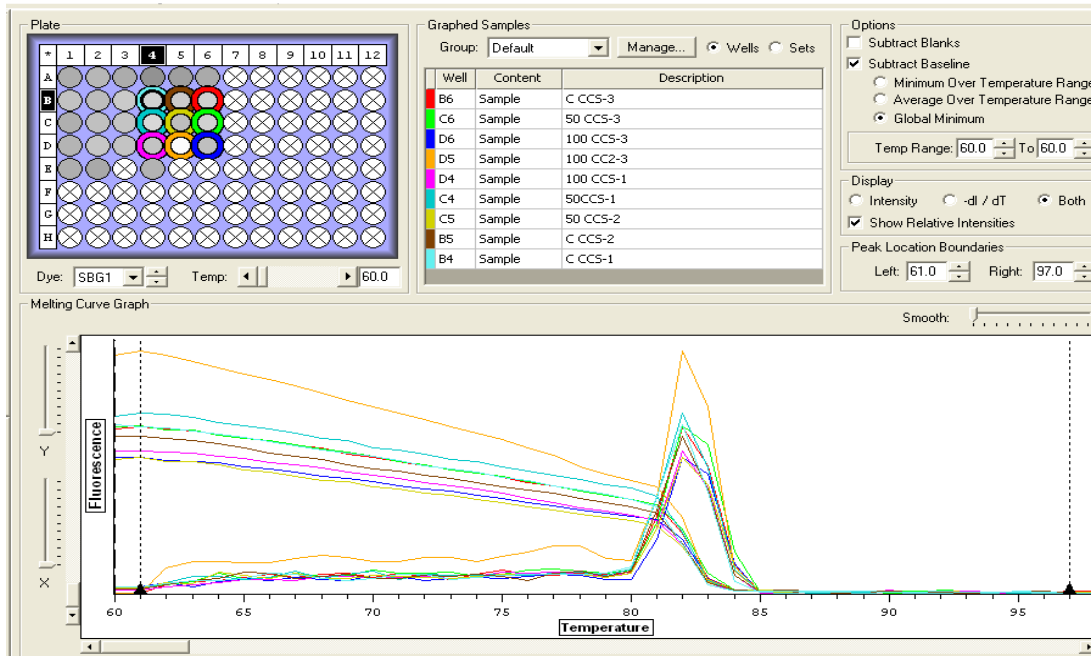
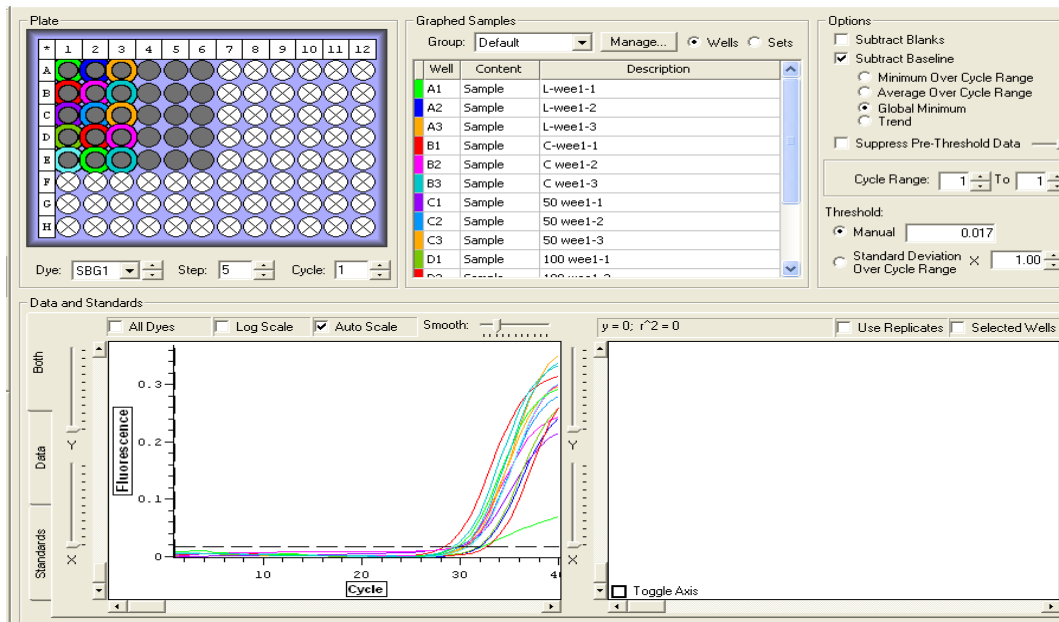Quantitation curve MtCCS52 gene

Melting curve of MtESRK gene

Quantitation curve MtSERK gene

Melting curve of MtWEE1 gene

Quantitation curve MtWEE1 gene

Appendix (D)

Publications:

- Elmaghrabi AM, Rogers HJ, Francis D, Ochatt S. (2011) Acclimation of callus to improved salt tolerance in the cellular and molecular levels in *Medicago truncatula*. Model Legume Conference, 15-19 May. Sainte-Maxime, France.

- Elmaghrabi AM, Rogers HJ, Francis D, Ochatt S. (2010) Characterisation of somatic embryogenesis and cell suspensions of *Medicago truncatula* after long-term exposure to abiotic stress. Socity for Experimental Biology annual main meeting 30th Jun- 3rd July. Prague. Czech Republic. Pp, 345.

- Elmaghrabi AM, Ochatt S, Rogers HJ, Francis D. (2009) In vitro characterisation and selection of *Medicago truncatula* explants: somatic embryogenesis on different media for studies on long-term tolerance to salinity stress. 3rd International conference of genetic engineering & biotechnology division: Biotechnology for Better life. 3-2 November. Cairo, Egypt. Pp, 63.