Thesis Report

COMBATING ABIOTIC STRESS USING TREHALOSE

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Cross-protection in tissue culture of *Arabidopsis thaliana*

Robert Stolker

June 2010

PLANT BREEDING
WAGENINGEN UR

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By Robert Stolker
Reg.Nr. 870204-808-070
Master Thesis Plant Breeding (PBR-80433)
MSc. student Plant Sciences, Specialization: Breeding and Genetic Resources
Wageningen University & Research Centre

Supervisors
Dr. Geert-Jan M. de Klerk*
Msc. Laura I. Rojas Martinez*
Prof. Dr. Richard G.F. Visser*
* Wageningen UR Plant Breeding

Examination
Dr. Geert-Jan M. de Klerk
Prof. Dr. Richard G.F. Visser

Wageningen UR Plant Breeding
Droevendaalsesteeg 1
6708 PB Wageningen

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Abstract

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Cross-protection in tissue culture of Arabidopsis thaliana (June 2010)

Robert Stolker, Wageningen University & Research

Plants subjected to abiotic stress use various defense mechanisms to cope with the stress. A common strategy is the synthesis and accumulation of osmoprotectants or compatible solutes like proline, glycine betaine, polyamines or trehalose. Tolerance to abiotic stresses can be acquired by pre-treatment with such a protective compound. Our results show an increased survival of *Arabidopsis* seedlings when drought and high salinity stress are preceded by a treatment with trehalose while in heat stress this is not the case. After pre-treatment with trehalose, the relative electrolyte leakage (REL) was significantly decreased in both drought and heat stress, suggesting that the protective action of trehalose might be through its preservative action on membranes by scavenging of reactive oxygen species (ROS). After heat stress, seedlings are more sensitive to light than after drought stress. The differential response to heat stress on the one hand and drought and high salinity stress might be explained by the type of damage they cause. The protective effect of trehalose seems to be primarily caused by its direct function as a protectant against damage to lipids and membranes. Relatively high levels of trehalose are necessary to significantly increase the tolerance to drought and high salinity stress. If trehalose would have a signaling function, one would expect to see the effect of trehalose already at the lower trehalose concentrations. The increased tolerance to drought and high salinity stress after trehalose treatment could be the result of shared steps in the stress response pathways of these stresses. Our results do not show the protective function of trehalose in heat stress, indicating that there is no cross-talk between drought and high salinity stress on the one hand and heat stress on the other hand. For future research, gene expression analyses could be used to see if and subsequently what genes are differentially expressed after a trehalose pre-treatment. Such an experiment could result in a better understanding of the exact function of trehalose in the signal transduction pathway of the different abiotic stresses. Understanding the stress response of plants to different types of abiotic stress is the first step in obtaining stress tolerant plants.

Abbreviations

CMS	cell membrane stability
DAB	di-amino benzidine
HAT	hot air treatment
HO•	hydroxyl radical
HWT	hot water treatment
H_2O_2	hydrogen peroxide
NBT	nitroblue tetrazolium
$^1O_2$	singlet oxygen
O_2^{-}•	superoxide radical
REL	relative electrolyte leakage
ROS	reactive oxygen species
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose 6-phosphate synthase
T6P	trehalose 6-phosphate

1 Introduction

During their life cycle, plants are frequently exposed to stress. In biology, stress is described as a major deviation from the normal environmental conditions, which has a major impact on the organism and can result in injury. Therefore, a biological stress has been defined as "any unusual environmental factor imposing a –potentially injurious-strain" (De Klerk, 2007a). There are two types of stresses, those caused by biotic and those caused by abiotic factors. Biotic stress is the result of damage by other living organisms, for example herbivores, weeds, viruses, fungi and harmful insects. Abiotic stress comprises non-living environmental factors that can have a negative effect on the plant. Examples are drought, chilling, freezing, high temperature, salinity, strong light and flooding. Abiotic stresses can delay growth and development, reduce productivity and can even lead to death of the plant (Chen and Murata, 2002). Many stresses that plants face in natural growing conditions are not encountered when they grow in tissue culture. However, in the tissue culture vessels, the relative humidity is close to 100%. This results in a poor development of the epicuticular wax layer and stomata that do not have their normal function for evaporation (Chen, 2004). When these tissue-cultured plants are acclimatized to ex-vitro conditions, they are exposed to drought stress (De Klerk and Wijnhoven, 2005). Other stresses might be encountered in tissue culture when the plants receive special treatments like thermotherapy, cryopreservation and protoplast preparation (De Klerk and Pumisutapon, 2008). It has been suggested that because of various extreme, unnatural conditions, tissue culture itself is a stress (De Klerk, 2007b).

1.1 Reactive Oxygen Species (ROS)

When plants are exposed to abiotic stress, this triggers many common responses like cellular dehydration and an increase in Reactive Oxygen Species (ROS). Examples of cellular ROS are the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the hydroxyl radical (HO•). These ROS cause oxidative damage to multiple cellular components like proteins, DNA, RNA and lipids. One of the effects of lipid peroxidation is an increase in the leakage of electrolytes since membrane permeability to electrolytes increases (Garg and Marchanda, 2009). All types of abiotic stresses, for example high salinity, chilling, freezing and drought stress, induce oxidative stress in plant cells (Chen and Murata, 2008; Groppa and Benavides, 2008; Pastori and Foyer, 2002). ROS are produced continuously, even under non-stress conditions, and are scavenged by antioxidant defense systems. Under stress conditions, the balance between the production of ROS and the antioxidant defense system is disturbed (De Klerk and Pumisutapon, 2008). Since all types of abiotic stresses result in the rapid accumulation of ROS in plant cells, this might indicate that the oxidative stress defense responses are an essential component in cross-protection. Before, ROS

were seen as toxic by-products of aerobic metabolism but in recent years is has become evident that they also play an important signaling role in controlling the response to abiotic stress and programmed cell death (Garg and Marchanda, 2009 and references therein). At low concentrations, ROS induce defense genes while at high concentrations cell death is initiated (Bhattacharjee, 2005). Although the role of ROS in the defense against abiotic stress is well accepted, it is not known what its targets and molecular functions are in the biochemical and transcriptional changes (Garg and Marchanda, 2009).

1.2 Compatible solutes

A common strategy of plants to cope with various abiotic stresses is the synthesis and accumulation of osmoprotectants or compatible solutes (Miranda *et al.*, 2007; Chen and Murata, 2002). These compatible solutes are protective low-molecular weight compounds like proline (an amino acid), glycine betaine (a quaternary amine), putrescine (a polyamine) and trehalose (a sugar) (De Klerk and Pumisutapon, 2008). These compounds are called compatible since they, even at high concentrations, do not interfere with normal metabolic reactions (Shen *et al.*, 1997). With high cellular concentration, many of the compatible solutes also act as osmoprotective compounds by significantly reducing the osmotic potential. Some of these compounds do not accumulate to high amounts in response to osmotic stress but have a protective function even at low concentrations (Mackenzie *et al.*, 1988). This indicates that compatible solutes may have a specific protective role, rather than acting as an osmoprotectant (Bohnert and Shen, 1999). These solutes can be active in non-osmotic mechanisms like radical oxygen scavenging and the stabilization of proteins, protein complexes and membranes under environmental stress. Trehalose is one of the most effective protectants against extreme environmental stress among the different classes of osmoprotectants (Miranda *et al.*, 2007).

1.3 Trehalose

Trehalose is a non-reducing disaccharide (1,1 α-D glucopyranosyl α-D-glucopyranoside) and occurs in a broad range of organisms (Miranda *et al.*, 2007; Elbein *et al.*, 2003). In yeast, bacteria and certain fungi, it is an important storage carbohydrate and stress protectant (Elbein *et al.*, 2003; Wingler, 2002). In higher plants, trehalose is synthesized using a pathway that is common to most organisms (Iordachescu and Imai, 2008). This pathway consists of two steps. First, trehalose 6-phosphate (T6P) is synthesized from glucose-6-phosphate and uridine diphosphate (UDP)-glucose. This step is catalyzed by trehalose 6-phosphate synthase (TPS). The free sugar trehalose is generated by the action of trehalose-6-phosphate phosphatase (TPP) (Bae *et al.*, 2005b).

There is much debate about the function of trehalose in higher plants. Although the involvement of trehalose metabolism in stress tolerance is indisputable (Iordachescu and Imai, 2008), some research supports the hypothesis that trehalose is a putative signaling molecule in higher plants while others support the hypothesis that trehalose functions as a direct stress protectant. There is evidence for both hypotheses.

1.3.1 Direct protection

Throughout all biological domains, there are adult organisms that can survive without water (anhydrobiotic organisms) even when they have lost 99% of their water content (Iturriaga *et al.*, 2009; Elbein *et al.*, 2003). In all these organisms (for example fungi and nematodes) trehalose is known to accumulate to high concentrations to survive complete dehydration by preserving the membranes and preventing the denaturation of proteins during drought stress (Drennan *et al.*, 1993; Crowe *et al.*, 1984). Substantial amounts of trehalose were identified in two so-called resurrection plants *Myrothamnus flabellifolia* and *Sporobolus stapfianus* (Phillips *et al.*, 2002). It is proposed that in these plants the accumulation of trehalose prevents damage to intracellular structures due to anhydrobiosis (Lunn, 2007). There is convincing evidence from both *in vitro* and *in vivo* experiments that trehalose protects membranes against lipid peroxidation. *In vitro* observations show trehalose interacts directly with the parts of unsaturated fatty acids that are sensitive to oxidation, hereby protecting them from auto-oxidation (Oku *et al.*, 2003). Recently, *in vivo* protection of the membrane by external trehalose has been demonstrated for the first time (da Costa Morato *et al.*, 2008).

There are different mechanisms to explain the direct function of trehalose in protecting membranes and macromolecules: water replacement, glass formation and chemical stability (Luo *et al.*, 2008; Lun, 2007). According to the water replacement theory, all biological macromolecules are normally stabilized by water by formation of hydrogen bonds around the macromolecules. It has been proposed that, during dehydration, trehalose replaces the water molecules, thereby preventing the denaturation of proteins and the fusion of membranes (Clegg, 1985). Another theory is the glassy state hypothesis: in the dry state trehalose forms glasses, this may be required for the stabilization of dry macromolecules. The third theory is the chemical stabilization hypothesis: even at elevated temperatures and at low pH trehalose remains stable and does not undergo Maillard browning with proteins (Wingler, 2002). These mechanisms may all contribute to the protective function of trehalose and do not exclude one another. The chemical stability and the water replacement theory have been subject of debate while the glassy state hypothesis is more widely accepted (Sussich *et al.*, 2001). Sussisch *et al.* (2001) propose an alternative anhydrobiosis mechanism of trehalose which includes the nucleation and formation of layered epitaxial crystals of dihydrate trehalose on the surface of cellular membranes. Instead of replacing the water molecules, in this theory the dihydrate trehalose captures the water molecules in the same hydrogen bonding network as in the solvated trehalose. When dehydration

continues, slowly more dihydrate is formed at the membrane surface without disrupting the cellular structure. Finally, slow dehydration of the layered crystals produces anhydrous trehalose. This process would protect life functions during dehydration by preserving active molecular conformations and membrane structure.

According to Zhu (2001), trehalose and other compounds like glycine betaine, proline and mannitol are active in the scavenging of ROS. This is confirmed by Luo *et al.* (2008) who found a direct role of trehalose in eliminating H_2O_2 (hydrogen peroxide) and $O_2^{-\bullet}$ (superoxide anion) in a concentration-dependent manner with the maximal scavenging rate of H_2O_2 and $O_2^{-\bullet}$ at 50 mM trehalose (95% and 78%, respectively). In addition to this antioxidant activity, trehalose also showed an indirect protection against oxidative stress by slightly protecting superoxide dismutase activity. Whether direct or indirect, through scavenging of ROS, trehalose is thought to protect plants from the detrimental effects of different types of abiotic stress. Since all stresses result in the accumulation of ROS, the scavenging of these oxygen radicals may be the main explanation for the protective role of trehalose and other protective compounds.

1.3.2 Signaling function

Although substantial amounts of trehalose were identified in some plant species, in general most plant species do not accumulate trehalose to detectable amounts (Wingler, 2002). *Arabidopsis thaliana* can accumulate trehalose (Vogel *et al.*, 2001), but naturally the in vivo accumulated trehalose level is too low to play a role as a stress protectant. Many higher plants, including Arabidopsis, have trehalase activity, which might result in rapid degradation of any trehalose synthesized (Bartels and Sunkar, 2005; Muller *et al.*, 2001). Attempts have been made to obtain transgenic plants that accumulate trehalose. A yeast chimaeric gene coding for a bifunctional TPS-TPP enzyme was expressed in Arabidopsis (Miranda *et al.*, 2007). The resulting plants accumulated trehalose at low concentration, did not show morphological alterations and were tolerant to multiple abiotic-stress conditions like drought, salinity, freezing and heat.
In the Arabidopsis genome, eleven TPSs and ten TPPs were identified of which TPS1, TPS6, TPPA and TPPB have been proven to be functional using yeast complementation (Iordachescu and Imai, 2008; Leyman *et al.*, 2001). Over-expression of the AtTPS1 in Arabidopsis led to drought tolerance, however the transgenic plants accumulated only low amounts of trehalose, not sufficient to play a role as osmoprotectant (Avonce *et al.*, 2004). The level of trehalose in these transgenic plants does not correlate well with the level of tolerance, indicating that trehalose may have a function other than osmoprotectant (Iordachescu and Imai, 2008). Since trehalose is hardly detectable in most plants Schluepmann *et al.* (2004) conclude that it is therefore not a stress protectant. Bae *et al.* (2005b) are somewhat less conclusive and state that because of its low concentration, the function of trehalose in higher plants remains unclear. When trehalose is supplied exogenously to plants, this leads to accumulation of trehalose-6-phosphate (T6P, the phosphorylated precursor). Accumulation of T6P

inhibits plant growth, but when sucrose is supplied simultaneously, growth on high trehalose is restored (Schluepmann *et al.*, 2004). Findings of Bae *et al.* (2005b) suggest that trehalose and its derivatives are important regulators of plant gene expression since trehalose treatment altered the expression of several stress-related genes. Rather than accumulation of trehalose as an osmoprotective compound, an important role of the trehalose biosynthesis pathway could be the synthesis of small amounts of T6P and/or trehalose as signaling molecules (Iturriaga et al., 2009).

1.4 Cross-protection

An interesting phenomenon in the stress response of plants is cross-protection or cross-tolerance. In plants, common pathways and components are used in the stress-response relationship (Pastori and Foyer, 2002). This allows plants to adjust to different stresses after exposure to one specific stress. Sabehat *et al.* (1998) defines cross-protection as "Exposure of tissue to moderate stress conditions (that) often induces resistance to other stresses" and Knight and Knight (2001) as "any instance of two signaling pathways from different stressors that converge". By applying a moderate stress, for example a moderate heat stress, a plant is more tolerant to another type of (severe) stress that would have resulted in death if the moderate stress had not been applied. De Klerk and Pumisutapon (2008) gave 5 day-old Arabidopsis seedlings a mild hot water pre-treatment ($1^1/_2$ h at 35°C) followed, after 4 h, by a major stress in the form of a severe hot water treatment (2 h at 40°C). The survival after pre-treatment increased from ca. 10% to ca. 80%. At the same time, a moderate drought pre-treatment did protect the plant against a severe hot water treatment, indicating cross-protection. Next to the application of a moderate stress, an interesting approach to identify mechanisms determining cross-tolerance would be to apply protective chemicals. There are two types of such compounds. First there are signal molecules that initiate a protective action by the tissue. Horvath *et al.* (2007) found that the results published so far indicate that salicylic acid, a signal molecule, causes an enhanced tolerance towards most kinds of abiotic stresses primarily due to increased antioxidative capacity. Second, protective compounds can have a direct protective function, e.g. by stabilizing vulnerable macromolecules. Hayashi *et al.* (1998) applied glycine betaine exogenously to germinating seeds and found that the compound was effective in protecting the seedlings from the harmful effects of salt stress.

Although there is much debate concerning the role of trehalose in plants, promising results have been obtained by both introduction of trehalose biosynthesis genes in Arabidopsis and by exogenous application of trehalose to plants in vitro. De Klerk and Pumisutapon (2008) grew Arabidopsis seedlings in vitro on trehalose medium and found that a concentration of 100 mM resulted in high protection against drought. Accumulation of trehalose was shown to provide protection against freeze and heat stress in transgenic *Arabidopsis* plants (Miranda *et al.*, 2007). Increased tolerance to heat

stress is also found by Almeida *et al.* (2005) who transformed tobacco with the TPS1 gene from *Arabidopsis*. The trehalose biosynthesis pathway and many trehalose biosynthetic genes in plants are tightly regulated by multiple stresses. This indicates the importance of trehalose in stress tolerance. However, the understanding of how exactly trehalose and T6P are involved in the stress pathway is far from complete (Iordachescu and Imai, 2008).

1.5 This research

This project focuses on cross-protection in tissue culture of *Arabidopsis thaliana* seedlings by treating seedlings with the compatible solute trehalose and monitoring the effect on the recovery from different types of abiotic stress (drought, high salinity and heat stress). The effect of trehalose on stress tolerance was analysed using survival rates, measurements of Relative Electrolyte Leakage (REL) and visualization of ROS accumulation in the seedlings by nitroblue tetrazolium (NBT) and di-amino benzidine (DAB) staining. The outcome of these experiments will give us some insight in the complex network of responses that mediates cross-protection and the involvement of ROS in these responses.

2 Materials and methods

2.1 Plant material and growing conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. Ecotype Columbia were obtained from Lehle Seeds (Round Rock, TX USA). 25 seeds were sown on a nylon gauze, 3x3 cm in size, in Petri dishes (Ø 50 mm) containing 8 ml of medium composed of ½ MS (Murashige & Skoog, 1962), 1.5% [w/v] sucrose and 0.7% [w/v] micro-agar. The pH was adjusted to 5.5 before addition of the micro-agar. This medium is further referred to as standard medium. The nylon gauzes were boiled in demi-water for 1 h to release plasticizers present in the nylon. Some droplets of Tween 80 were added to remove any possible contaminations from the nylon gauze. After boiling, the nylon gauzes were rinsed with demi-water before being autoclaved. After sowing, seeds were vernalized at 4°C for at least 2 days before transfer to the climate room (Fig. 1). Growing conditions were 21°C, with a 16 h/8 h day/night rhythm.

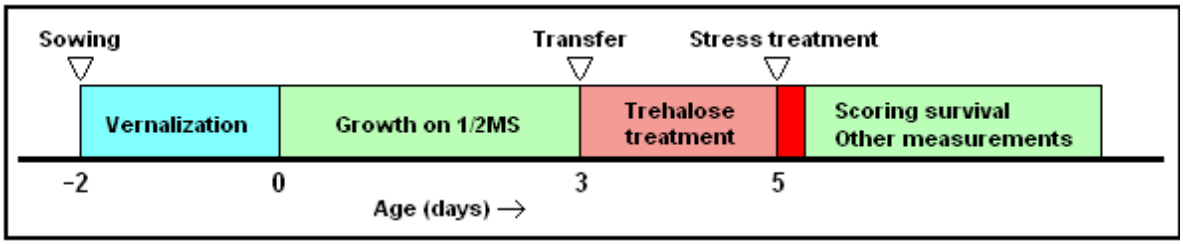

Figure 1. Plant life from sowing to scoring of results. Vernalization: two days at 4°C; Growth: three days on ½ MS medium, two days on trehalose medium. Stress is applied on 5-day old seedlings.

2.2 Sterilization of seeds

Two methods have been used for the sterilization of the Arabidopsis seeds: a 'dry' and a 'wet' method.

2.2.1 Vapor-phase sterilization

Arabidopsis seeds were sterilized in eppendorf tubes using vapor-phase sterilization in a dessicator jar. Three ml of concentrated hydrochloric acid (HCl) was added to 100 ml of concentrated (~12%) bleach. This brings about the formation of chlorine gas. After 3 h the dessicator jar was opened and tubes were closed immediately to prevent escape of the chlorine gas. After transfer to a laminar flow cabinet, the tubes were opened to ventilate and remove the rest of the chlorine gas.

2.2.2 Sterilization using ethanol and hypochlorite solution

The Arabidopsis seeds were packed in filter paper and placed in a 70% ethanol solution for 5 minutes. Subsequently the bag was transferred to a 1.5% (w/v) hypochlorite solution with a drop of Tween 20 for 10 minutes. After this, seeds were rinsed 5 times in sterile water. Seeds were sown directly after the sterilization procedure.

2.3 Trehalose treatment

After three days in the climate room, nylon gauzes with seedlings were transferred to ½ MS medium supplemented with trehalose in different concentrations (0, 20, 60 and 100 mM). Age of the seedlings is counted from the time when they are put in the climate room. After two days growing on trehalose medium, the stress treatments were applied on five-day old seedlings. After the stress treatment, seedlings were transferred back to the standard medium and returned to the climate room for scoring of survival or subjected to other measurements.

2.4 Stress treatments

Several stress treatments have been applied in the course of the research. These include drought stress, high salinity stress and heat stress (both a hot water treatment and a hot air treatment).

2.4.1 Drought stress

Drought stress is applied by treatment with silica gel. The high surface area of silica results in a high capacity to absorb water and this property makes it useful as a desiccant. The nylon gauze with seedlings is transferred from the medium to a Petri dish containing blue (anhydrous) silica gel. On top of the silica gel a sterile piece of filter paper is placed and the nylon gauze with the seedlings is put in the Petri dish (Fig. 2). The dish is closed using household foil and seedlings are kept in the dish for 2 h. After the drought treatment, the seedlings are transferred back to the standard medium and returned to the climate room.

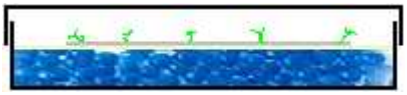

Figure 2. Drought treatment. A nylon gauze with seedlings is placed on top of sterile filter paper and anhydrous blue silica gel.

2.4.2 Salinity stress

Seedlings are subjected to salinity stress by transferring the nylon gauze to a Petri dish containing standard medium with 400 mM NaCl. The seedlings are kept on this medium 4 h and are then transferred back to the standard medium after 5 seconds on a piece of sterile filter paper to remove NaCl solution. After the stress treatment the plates are returned to the climate room.

2.4.3 Hot Air Treatment (HAT)

Hot Air Treatment (HAT) was given by placing the Petri dishes with seedlings in a low temperature incubator (Model LTIM10, Labcon). Temperature of the growth medium reached the set temperature in approximately 1/2 h after had been put in the incubator (Fig. 3). After the HAT the Petri dishes were allowed to cool down in the laminar flow cabinet before the seedlings were transferred to the standard medium and returned to the climate room.

2.4.4 Hot Water Treatment (HWT)

From each Petri dish an about equal number of seedlings was transferred to an Eppendorf tube containing 1 ml of liquid standard medium. The tubes were placed in a water bath for a duration of 2 h. The remaining seedlings were removed from the plates to prevent mixing of treated and non-treated seedlings. After the HWT, the treated seedlings were dried on sterile filter paper for a few seconds before being transferred back to the standard medium.

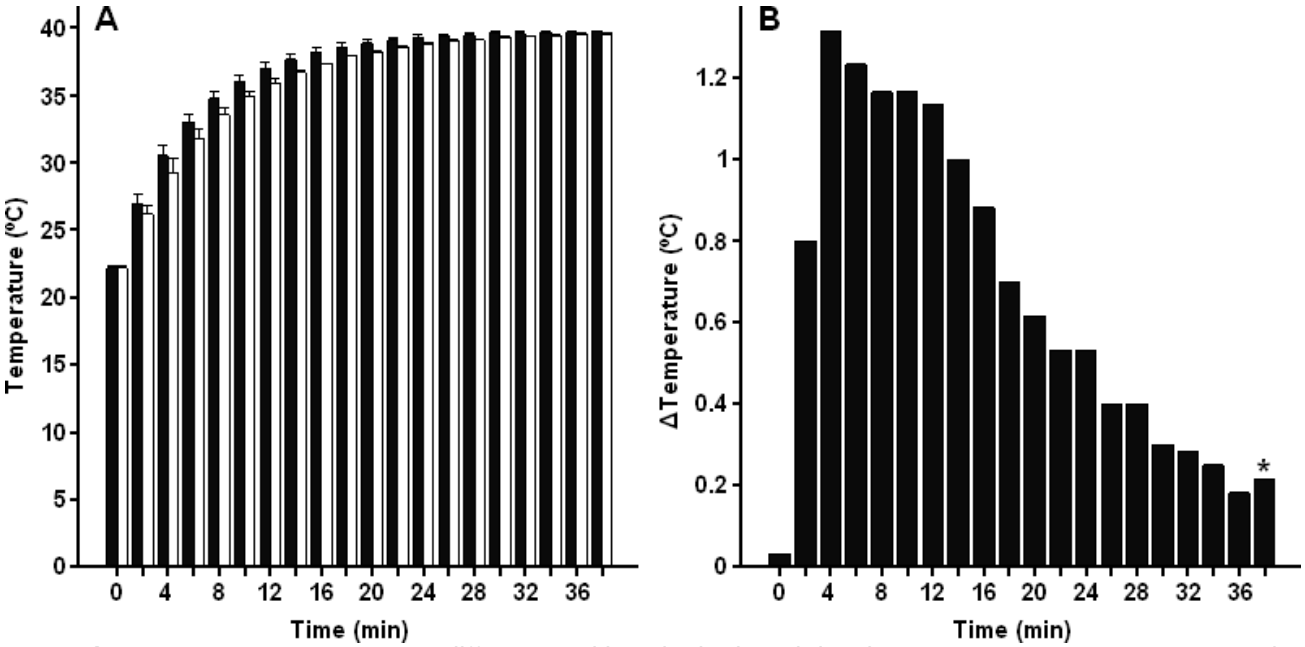

Figure 3. Temperature on two different positions in the hot air incubator. Temperature was measured in the left top (■) and right bottom (□). Indicated are absolute temperature (A) and ΔT of ■ minus □ (B). Data are means ± standard error of three(■) or two(□) replicates. * Indicates a significant difference in temperature between the two positions in the incubator ($P < 0.05$).

2.5 Relative Electrolyte Leakage (REL) and Cell Membrane Stability (CMS)

Electrolyte leakage was measured using a handheld conductivity measuring instrument (Handheld meter Cond 315i, Wissenschaftlich-Technische Werkstätten GmbH). Before measuring, roots were removed from the seedlings, so electrolyte leakage from the cotyledons and hypocotyl was measured. The derooted seedlings were washed in de-ionized water (MilliQ) for 20 seconds to remove electrolytes present on

the surface of the plant. Leaves were placed in tubes with 15 ml of deionized water at room temperature. The electrical conductivity of the water was determined at t=180 min. Subsequently, the samples were heated at 100°C for 20 min. to completely lyse the plant tissue and release all electrolytes. After cooling down to room temperature, the conductivity in the bathing solution was measured again. Cell Membrane Stability (CMS) was expressed according to the method of Blum and Ebercon (1981) using the following calculation:

$$CMS\ (\%) = \{[1-(T_1/T_2)]/[1-(C_1/C_2)]\}*100$$

The Relative Electrolyte Leakage (REL) is expressed as the percentage leakage at t=180 min. of the total electrolyte leakage after lysis:

$$EL(\%) = (C_1/C_2)x\ 100$$

or

$$EL(\%) = (T_1/T_2)x\ 100).$$

T and C refer to the stress and control samples, respectively; the subscripts 1 and 2 refer to the initial and the final conductance measurement, respectively (Fig. 4).

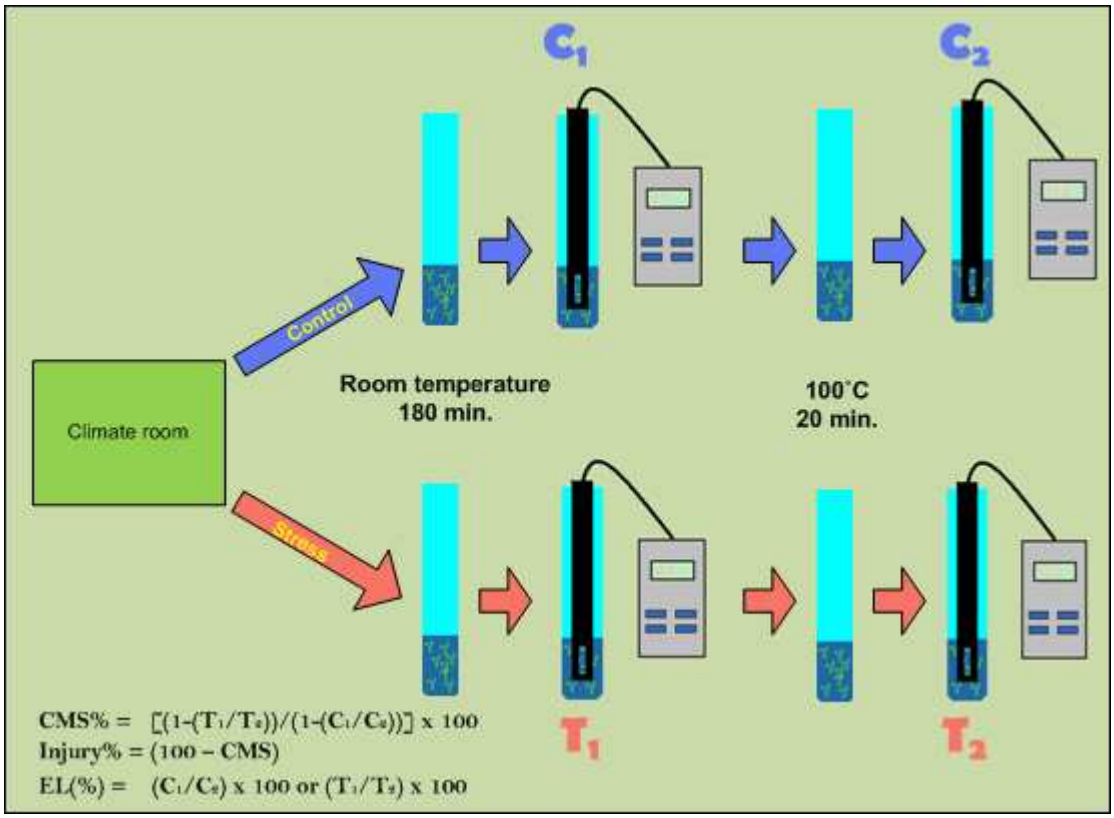

Figure 4. Measuring electrolyte leakage. CMS = Cell Membrane Stability; EL = Electrolyte Leakage; T = stressed samples; C = control samples; $_1$ = initial conductance reading at t=180 min; $_2$ = final conductance reading after lysis of plant material.

2.6 Staining of Reactive Oxygen Species (ROS)

There are different methods to visualize ROS accumulation in Arabidopsis seedlings. First, the 2,2′-bis(4-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene) ditetrazolium chloride or nitroblue tetrazolium (NBT) staining that is specific for the superoxide radical ($O_2^{-\bullet}$). When incubating seedlings in a NBT solution, the reaction of NBT with superoxide results in the production of a dark blue insoluble formazan compound (Tyburski *et al.*, 2009; Fryer *et al.*, 2002). This method has been successfully applied to the cotyledons of Arabidopsis plants that received a chilling treatment (Einset *et al.*, 2007b). Second, the di-amino benzidine (DAB) staining that allows the detection of H_2O_2 in leaves. When DAB reacts with H_2O_2, it forms a deep-brown polymerization product (Fryer *et al.*, 2002). It is likely that in vivo, other radical species are quickly converted to the more stable H_2O_2. This method is thought to provide an integral assay for several ROS (Rodriguez *et al.*, 2002).

2.6.1 Nitroblue tetrazolium (NBT) staining of superoxide

For detection of superoxide whole seedlings were vacuum infiltrated with 6 mM nitroblue tetrazolium in 25 mM HEPES buffer, pH 7.6. Samples were incubated in the dark at room temperature for 0.5 h. To verify the specificity of the NBT staining, control seedlings were incubated in a water solution of the antioxidant ascorbic acid (10 mM) for 60 min before the NBT staining was performed. NBT is difficult to dissolve, vigorous shaking for 30 min is required to dissolve all the NBT in the concentration used here.

2.6.2 3,3'diaminobenzidine (DAB) staining of H_2O_2

For detection of H_2O_2 whole seedlings were vacuum infiltrated with 0.5% (w/v) 3,3'diaminobenzidine in 50 mM NaAc buffer, pH 3.6. Samples were incubated in the dark at room temperature for 0.5 h. In control treatments to verify the specificity of the method, 10 mM ascorbic acid was added to the buffer containing DAB.

2.6.3 Chlorophyll extraction and Imaging

After incubation, chlorophyll was extracted from the seedlings by immersion in a solution of ethanol, lactic acid and glycerol (4:1:1 by volume) followed by heating in a water bath at 80ºC for 25 min. After chlorophyll extraction, cotyledons were detached from the seedlings and imaged using a bright field imaging microscope (SteREO Discovery.V8, Carl Zeiss B.V., Sliedrecht). Shutter time and illumination have been kept the same for all cotyledons to make it possible to compare the results. Images have been taken with a resolution of 150 dpi.

2.7 Statistical analysis

Data were analyzed by appropriate Student's t-tests or ANOVA, in which case significant differences between individual treatments were determined by Tukey's test. Correlation coefficients and significance levels were determined using the correlations function. All statistical analyses have been performed using the statistical software GenStat 12th edition (VSN International, UK) and PASW Statistics (SPSS Inc., Chicago, Illinois, USA).

3 Results

3.1 Drought stress

3.1.1 Effect of trehalose on survival after drought stress

After 3 days germination on standard medium, seedlings were transferred to medium containing 0, 20, 60 or 100 mM trehalose for 2 days. Subsequently, seedlings were drought-treated and transferred back to the standard medium. Trehalose increased survival after the drought treatment from 36.1% (control) to 42.6%, 84.4% and 98.7% for the 20, 60 and 100 mM trehalose treatment, respectively. Data are means ± standard error of 5 independent experiments with 1-3 replicates per experiment. The increased survival in the 20 mM treatment is not significant compared to the control. The 60 and 100 mM treatment show a highly significant increase in survival ($P \leq 0.001$) compared to the control treatment (Fig. 5A).

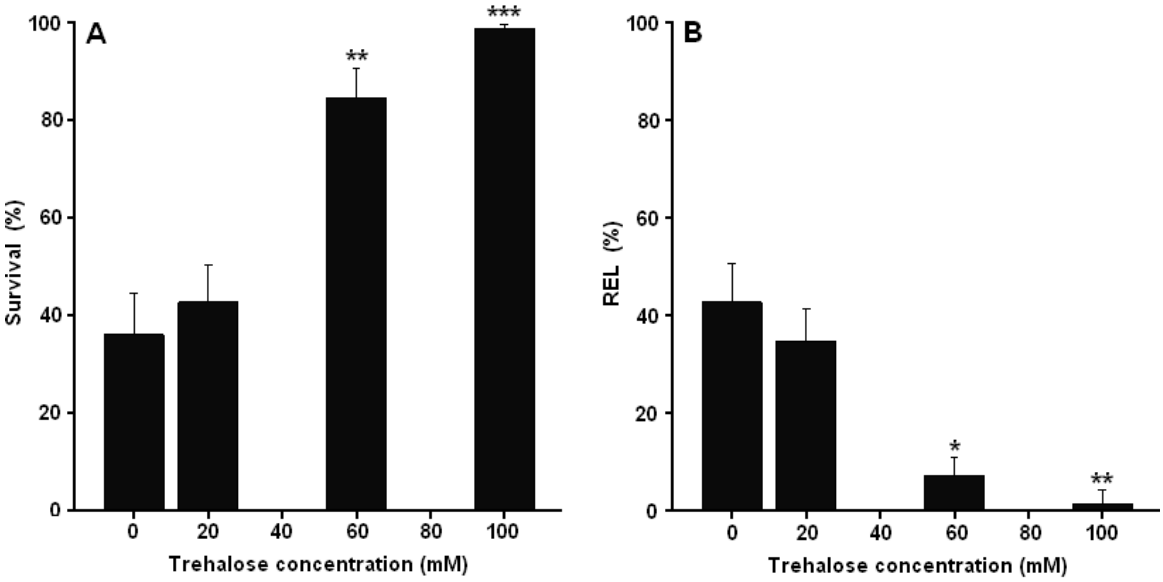

Figure 5. (A) Survival and (B) Relative Electrolyte Leakage (REL) of Arabidopsis seedlings after drought stress. The seedlings were germinated for 3 days on standard medium and after that for 2 days on medium with 0, 20, 60 or 100 mM trehalose. After the trehalose treatment, seedlings were subjected to drought stress for 2 h and (A) transferred back to the standard medium or (B) used in the REL-assay. Survival data are means ± standard error from 5 independent experiments with 1-3 replicates per experiment. REL data are means ± standard error of three independent experiments with 3 replicates per experiment. *,**,*** Survival and REL values significantly different from the control (0 mM trehalose) at $P <$ 0.05, 0.01, or 0.001, respectively.

3.1.2 Effect of trehalose on Relative Electrolyte Leakage (REL) after drought stress

After 3 days germination on standard medium, seedlings were transferred to medium containing 0, 20, 60 or 100 mM trehalose for 2 days. After the trehalose treatment, seedlings were subjected to drought stress and subsequently analyzed for electrolyte leakage. Trehalose treatment decreased the electrolyte leakage after the 2 h drought treatment from 42.7% (control) to 34.7%, 7.3% and 1.4% for the 20, 60 and 100 mM trehalose treatment, respectively. Data are means ± standard error of three independent experiments with 3 replicates per experiment. Compared to the control treatment, there is a lower REL in the 20 mM treatment. However, this difference is not significant. Both the 60 and 100 mM treatment show a significant decrease in REL ($P < 0.05$ or 0.01, respectively) compared to the control treatment (Fig. 5B).

3.1.3 Cell Membrane Stability (CMS) assay

CMS is calculated using the REL of both stressed and non-stressed seedlings (Fig. 4). Since stress results in the loss of cell membrane integrity, it is associated with an efflux of solutes. Therefore, the REL from seedlings after stress can be used as a measure of cellular damage (Blum and Ebercon, 1981). The CMS increased with the trehalose concentration from 55.1% (control) to 62.7%, 92.0% and 98.4% for the 20, 60 and 100 mM trehalose treatment, respectively. The difference between the 20 mM treatment and the control is not significant whereas the CMS in the 60 and 100 mM treatments are significantly different from the control treatment (Fig. 6).

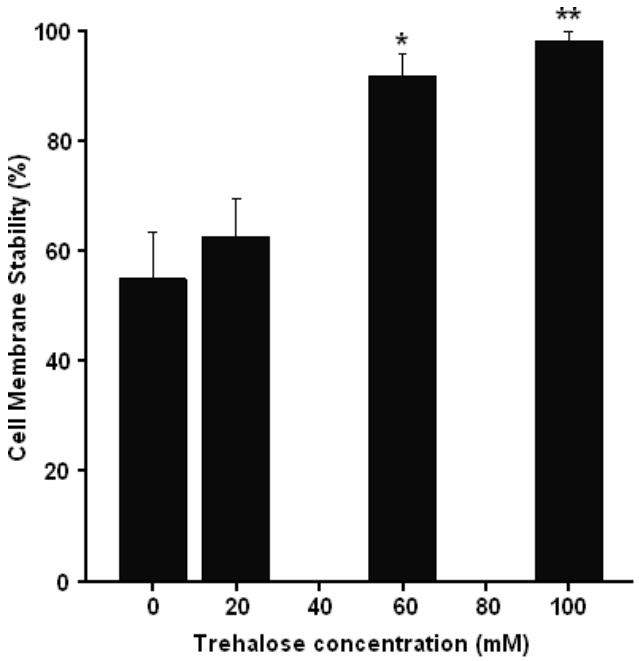

Figure 6. Cell Membrane Stability (CMS) of Arabidopsis seedlings after drought stress. CMS is calculated using the REL data of both stressed and non-stressed plants. CMS data are means ± standard error of three independent experiments with 3 replicates per experiment. *,** CMS values significantly different from the control at $P < 0.05$ or 0.01, respectively.

3.2 Heat stress

3.2.1 Hot Air Treatment (HAT) temperature range

The goal of this research is to see the effect of a trehalose treatment on the survival after a stress treatment. In this case, the aim is to have a survival of around 50% in the control samples so that both positive and negative effects of a treatment can be optimally determined. Based on previous research, the seedlings were subjected to a Hot Air Treatment (HAT) of 45ºC for 2 h. The survival rate was extremely low, with only one out of 61 seedlings surviving in the 100 mM trehalose treatment. In the 0, 20 and 60 mM trehalose treatment no seedlings survived the HAT treatment. To find the optimal temperature for the HAT, a temperature range from 40 to 44.5ºC was applied with intervals of 1.5ºC (Fig. 7). A HAT of 40°C or 41.5°C results in 100% survival in the control treatment, indicating the stress is not severe enough. The HAT at 44.5°C is too severe since none of the seedlings in the control survived the heat treatment. A HAT 43°C seems to give a good response with survival of 77% of the seedlings. Keeping in mind the variable survival rates in the HAT, the standard error of the control treatment at 43°C is 11.6%, a temperature of 43°C was selected as temperature to be used in the HAT.

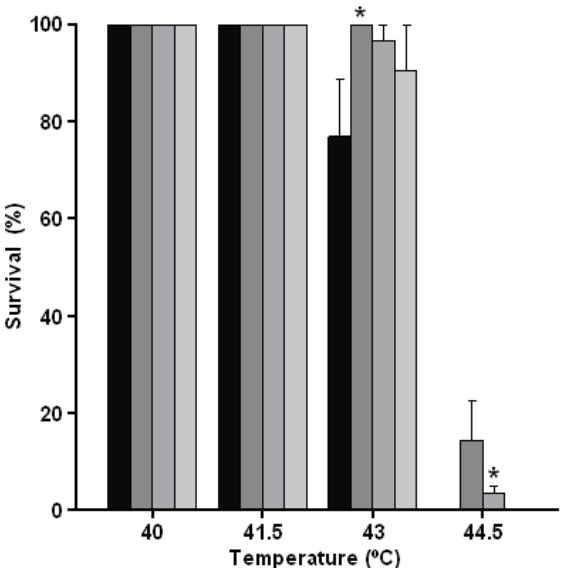

Figure 7. Survival of Arabidopsis seedlings after a HAT temperature range. After treatment with 0, 20, 60 or 100 mM trehalose, seedlings were subjected to a HAT of 40, 41.5, 43 and 44.5ºC for 2 h and transferred back to the standard medium. Survival data are means ± standard error of 3 replicates per trehalose concentration. * Survival values significantly different from the control (0 mM trehalose).

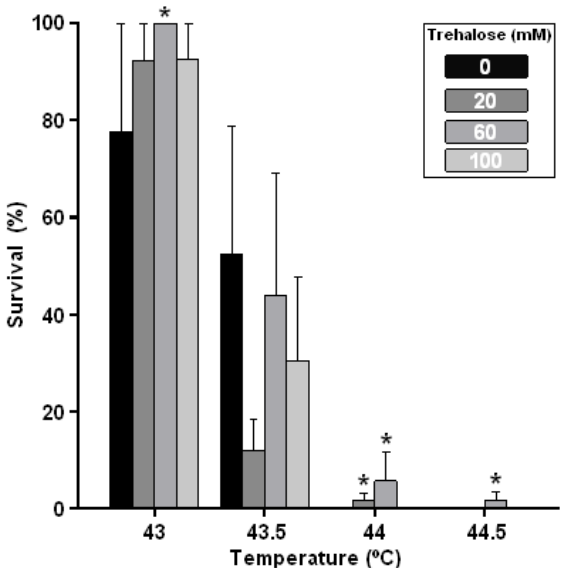

Figure 8. Survival of Arabidopsis seedlings after a HAT temperature range. After treatment with 0, 20, 60 or 100 mM trehalose, seedlings were subjected to a HAT of 43, 43.5, 44 and 44.5ºC for 2 h and transferred back to the standard medium. Survival data are means ± standard error bars of 3 replicates per trehalose concentration. * Survival values significantly different from control (0 mM trehalose).

3.2.2 Effect of trehalose on survival from HAT

In the 43ºC treatment, trehalose increased survival after the HAT from 73.3% (control) to 92.4%, 87.9% and 82.6% for the 20, 60 and 100 mM trehalose treatment, respectively (Fig. 9A). Data are means ± standard error of four independent experiments with 3 replicates per experiment. Although the observed survival rates in the 20, 60 and 100 mM treatment are higher than the control, the differences are not significant. In the 44 and 44.5ºC treatment, the only survival is observed in the 20 and 60 mM trehalose treatment (Fig. 8). Trehalose does not seem to have a clear effect on the survival from heat stress (HAT), within one treatment the observed survival rates are very variable.

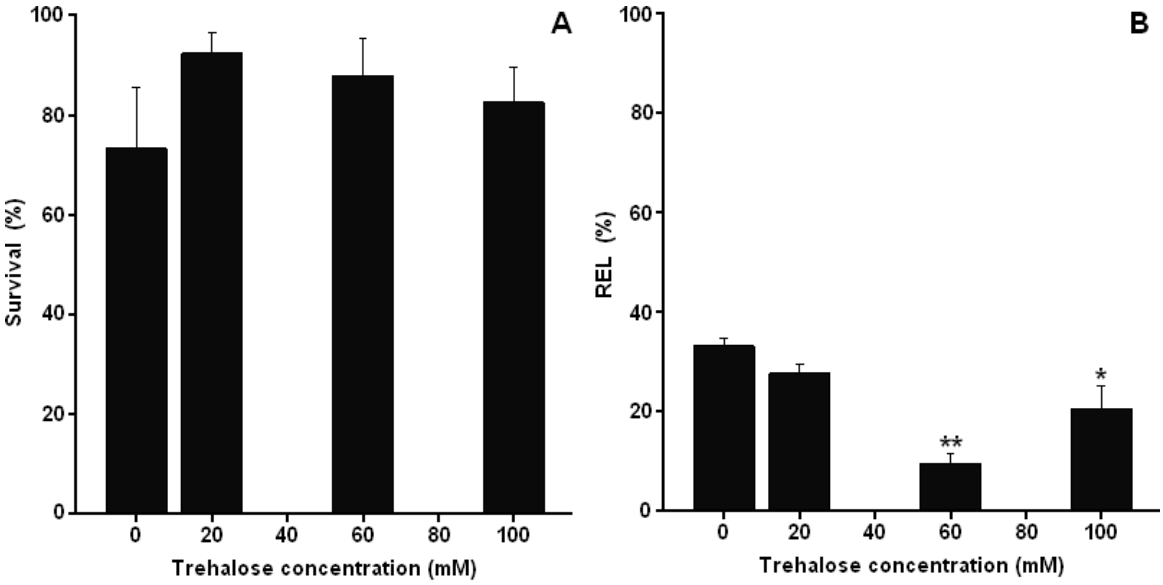

Figure 9. (A) Survival and (B) Relative Electrolyte Leakage (REL) of Arabidopsis seedlings after a HAT of 43ºC. After treatment with 0, 20, 60 or 100 mM trehalose, seedlings were subjected to a HAT of 43ºC for 2 h and (A) transferred back to the standard medium or (B) used in the REL-assay. Survival data are means ± standard error from 4 independent experiments with 3 replicates per experiment. REL data are means ± standard error of 3 replicates per trehalose concentration. *,** REL values significantly different from the control at $P < 0.05$ or 0.01, respectively.

3.2.3 Effect of trehalose on the REL after HAT

Trehalose treatment decreased the electrolyte leakage after the 2 h HAT at 43ºC from 33.2% (control) to 27.7%, 9.6% and 20.6% for the 20, 60 and 100 mM trehalose treatment, respectively. Data are means ± standard error of 3 replicates per trehalose treatment. The REL after treatment with 60 and 100 mM trehalose is significantly different from the control ($P < 0.01$ or 0.05, respectively), while the 20 mM treatment does not show a significant difference (Fig. 9B). There is an increase in REL from the 60 to the 100 mM trehalose treatment, however this difference is not significant ($P = 0.086$).

3.2.4 Cell Membrane Stability (CMS) Assay

The REL is measured on both heat-stressed and non-stressed seedlings. The resulting RELs are used to calculate the CMS (Fig. 4). The CMS increases from 58.8% for the 0 mM to 65.6, 88.1 and 74.5% for the 20, 60 and 100 mM trehalose treatment respectively. The difference between the 20 mM treatment and the control is not significant whereas the CMS in the 60 and 100 mM treatments are significantly different from the control treatment (Fig. 10). There is a decrease in CMS from the 60 to the 100 mM trehalose treatment, however this decrease is not significant ($P = 0.086$).

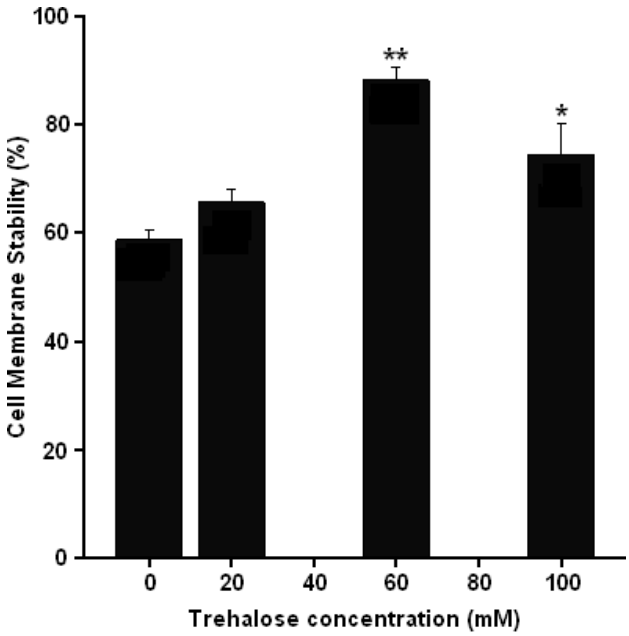

Figure 10. Cell Membrane Stability (CMS) of Arabidopsis seedlings after a HAT of 43ºC. CMS is calculated using the REL data of both stressed and non-stressed plants (see Fig. 4). CMS data are means ± standard error of 3 replicates per trehalose concentration. *,** CMS values significantly different from the control at $P < 0.05$, 0.01, respectively.

3.2.5 HAT temperature range (II)

In general, the survival rates in the 43ºC HAT were high with a survival that often reaches 100% in the control treatment. Next to this, the survival dropped dramatically from 77% to no survival in the 43 to 44.5ºC treatments (Fig. 7). Therefore, a second temperature range was applied from 43 to 44.5ºC with intervals of 0.5ºC (Fig. 8). In the control treatment, the percentage survival decreased from 78% to 52%, 0% and 0% for the 43, 43.5, 44 and 44.5°C HAT respectively. The HAT at 43.5°C seems to give a good

response with an average survival of 52% of the seedlings. This temperature was used in the remainder of the thesis research.

3.2.6 Effect of a temperature pre-treatment on the survival after HAT

In the previous experiments, seedlings have been treated with trehalose to examine the protective effect when exposed to different abiotic stresses. Although the involvement of trehalose metabolism is indisputable (Iordachescu and Imai, 2008), there are many other factors involved in the acquirement of stress tolerance. Exogenous application of trehalose mimics part of the response but to see how the effect of trehalose relates to the protective effect of a pre-treatment, seedlings were subjected to a 90 min hot air pre-treatment of 38ºC. Subsequently, seedlings were allowed to recover for 3 h in the climate room before being subjected to a severe HAT of 43, 43.5, 44 and 44.5ºC respectively for 2 h. The survival rate in all treatments was 100% (Fig. 11), indicating that the acquired thermotolerance by the pre-treatment is very effective compared to a trehalose treatment (Fig. 8).

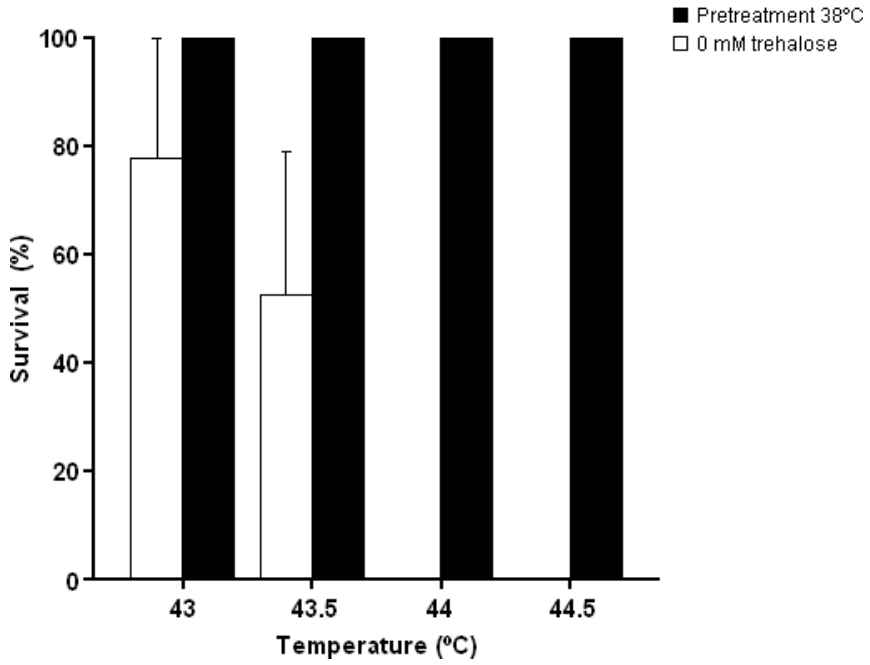

Figure 11. Survival of Arabidopsis seedlings after a HAT temperature range. Seedlings received a pre-treatment of 38ºC for 90 min (■) or no pre-treatment (□). Subsequently, seedlings were subjected to a HAT of 43, 43.5, 44 and 44.5ºC for 2 h and transferred back to the standard medium. Survival data are means ± standard error of 3 replicates (no pre-treatment) or 12 replicates (pre-treatment 38ºC) per stress treatment.

3.2.7 Level and duration of HAT

The survival of a plant after a heat treatment depends on both the temperature and the incubation time, so on the duration and level of heat stress (Luthe *et al.*, 2000). Plants have an optimum temperature for growth and development and Arabidopsis seedlings are reported as being relatively heat sensitive (Binelli and Mascarenhas, 1990). To see the effect of the level of heat stress on Arabidopsis, seedlings were subjected to a hot air treatment with a temperature range from 43 to 44.5°C with steps of 0.5°C (Fig. 8). The survival rates dropped rapidly in a ΔT of only 1ºC. While the survival at a HAT of 43ºC ranged from about 80 to 100% in the different trehalose treatments, at a HAT of 44°C the survival dropped to almost zero in all trehalose treatments. This shows that the seedlings are very sensitive to small changes in temperature. To study the effect of the duration of a HAT on survival, non-pretreated seedlings were subjected to heat stress at 42.5 and 43.5°C for 1, 1.5, 2 and 2.5 h (Fig. 12). At 42.5°C, the survival was 100% over all the durations (data not shown). At 43.5°C, differences were observed between the different durations. While after 1 h almost all seedlings survived, the survival dropped to 58.5, 20.1 and 3.5% survival with respectively 1.5, 2 and 2.5 h in the hot air incubator. This drop is more gradual than the dramatic drop in survival in the temperature range where a ΔT of as little as 1°C makes the difference between survival or death of the seedling.

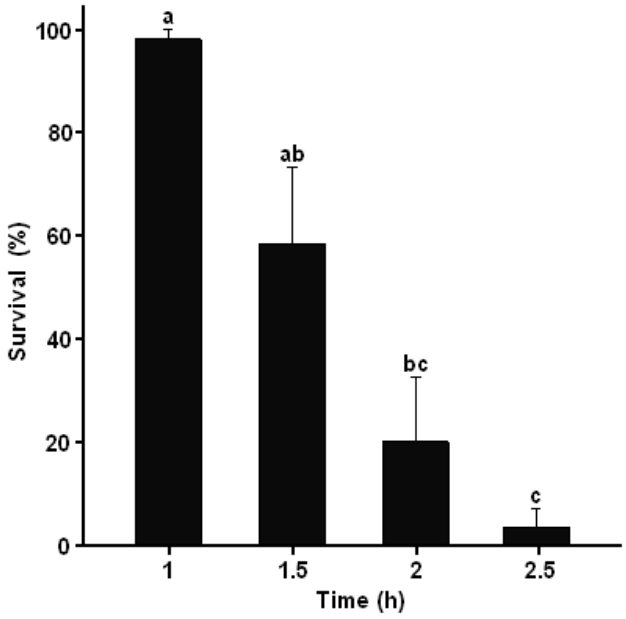

Figure 12. Effect of duration of a HAT on the survival of Arabidopsis. Seedlings did not receive a pre-treatment and were subjected to a HAT of 43.5ºC for 1, 1.5, 2 and 2.5 h. Survival data are means ± standard error of 6 replicates (1 and 2.5 h) or 7 replicates (1.5 and 2 h) per stress treatment. Different letters indicate significant differences ($P < 0.01$).

3.2.8 Hot Water Treatment (HWT) temperature range

Like with the other stresses, seedlings where germinated on standard medium for 3 days before being transferred to medium containing 0, 20, 60 or 100 mM trehalose for 2 days. After the trehalose treatment, seedlings were subjected to the stress treatment and transferred back to the standard medium. Based on experience from previous research, seedlings were subjected to a Hot Water Treatment (HWT) of 40ºC for 2 h. Almost all of the seedlings survived the treatment (data not shown), with only two out of 54 seedlings in the 100 mM trehalose treatment that did not survive. The temperature of the HWT was increased to 42ºC. The HWT at 42°C is too severe since there was no survival in any of the treatments (data not shown).

To find the optimal temperature for the HWT, a temperature range of 41, 41.5 and 42ºC was applied (Fig. 13). The experiment was hampered by problems with fungal infection. Although the infection frequency is very low, one infection results in the loss of 25 seedlings since they are growing in the same Petri dish. Preliminary results showed a survival of close to 100% in all trehalose treatments at 41°C and 41.5°C. Contrary to the previous HWT at 42ºC, the HWT at 42°C now seems to give a good response with survival of 50% of the seedlings in the control treatment. Although no definite conclusions can be made, there seems to be no effect of trehalose on the survival from HWT. Like in the HAT, the survival rates are very variable within a specific temperature-trehalose combination. To indicate the extent of variability, the survival in the 0 mM trehalose at 42ºC varies from 6.3 to 31.3, 58.8, 62.5 and 70.6%.

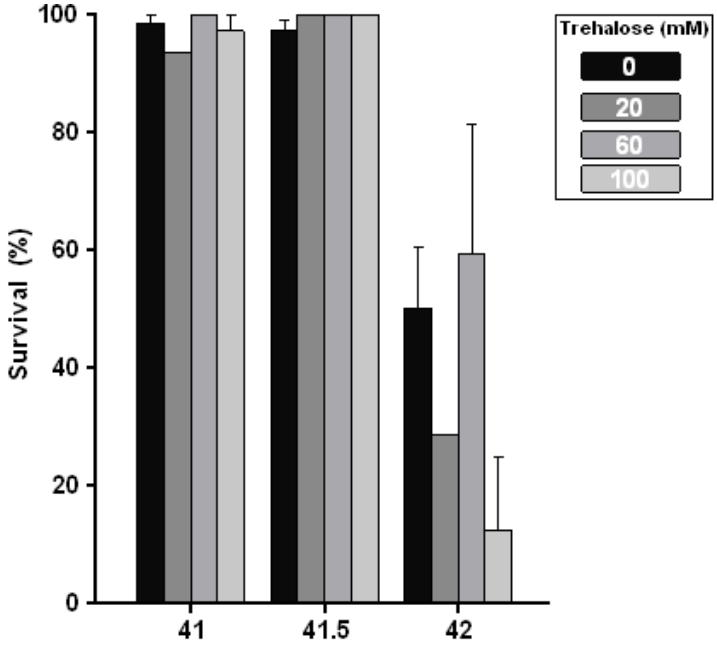

Figure 13. Preliminary results on the survival of Arabidopsis seedlings after a HWT temperature range. After treatment with 0, 20, 60 or 100 mM trehalose, seedlings were subjected to a HWT of 41, 41.5 and 42ºC for 2 h and transferred back to the standard medium. Survival data are means ± standard error of 1-6 replicates per trehalose concentration. No significant differences between means.

3.3 High salinity stress

3-day old seedlings were treated with trehalose for two days before being subjected to high salinity stress (400 mM NaCl) for 2 h. Data are means ± standard error of two independent experiments with 3 replicates per experiment. Trehalose altered the survival after high salinity stress from 51.6% (control) to 42.3%, 70.3% and 89.6% for the 20, 60 and 100 mM trehalose treatment, respectively (Fig. 14). The lower survival in the 20 mM treatment is not significantly different from the control ($P = 0.587$). Only the 100 mM treatment showed a significantly higher survival compared to the control ($P = < 0.05$).

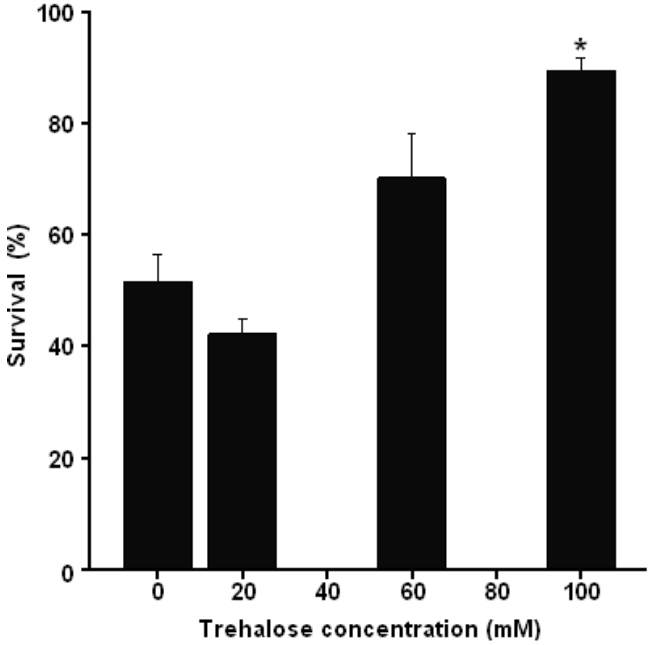

Figure 14. Survival of Arabidopsis seedlings after high salinity stress. After treatment with 0, 20, 60 or 100 mM trehalose, seedlings were transferred to ½ MS medium containing 400 mM NaCl for 2 h and transferred back to the standard medium. Survival data are means ± standard error of two independent experiments with 3 replicates per experiment. * Survival values significantly different from the control at $P < 0.05$.

3.4 Influence of light on survival

To see the influence of light on the survival from heat and drought stress, seedlings were grown on 1/2 MS medium for 5 d before being subjected to the different stress treatment. Subsequently, seedlings were allowed to recover in the dark and in the light. Results showed that in both heat and drought stress the survival increased significantly when allowed to recover in the dark after the stress treatments (Fig. 15). The relative increase in survival is higher in heat stress (76%, from 54.1 to 95.2% survival) than in drought stress (46.9%, from 31.1 to 45.7% survival). This result suggests that in heat stress, light imposes more damage to the seedlings than in drought stress.

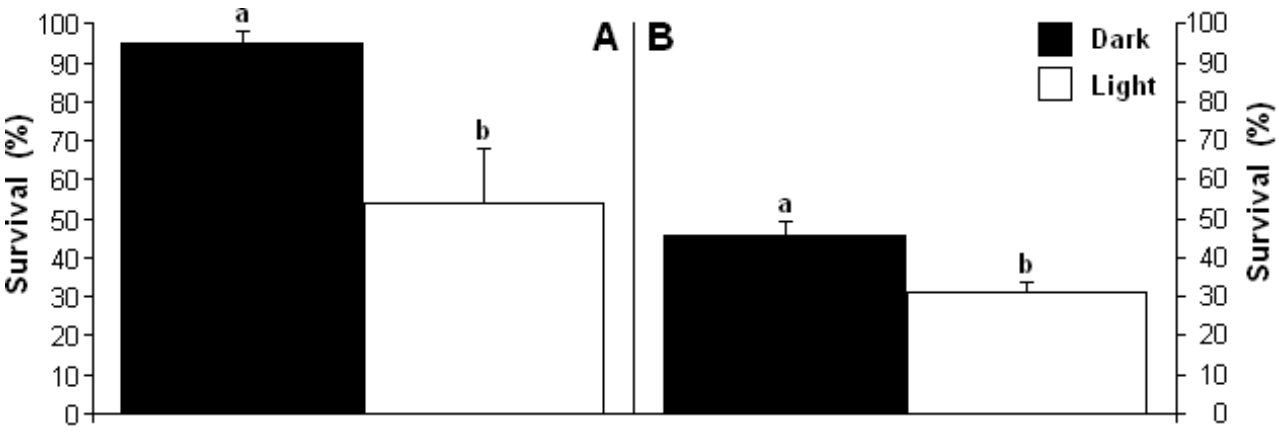

Figure 15. Survival of Arabidopsis seedlings after A) heat stress and B) drought stress. Seedlings growing for 5 days on ½ MS medium were subjected to the stress treatment. Survival data are means ± standard error bars of 9 replicates. Different letters indicate significant differences between treatments.

3.5 ROS Staining

All abiotic stresses result in the accumulation of ROS. Trehalose is found to be a direct and indirect scavenger of ROS. To verify if the protective function of trehalose is because of a reduction in level of ROS, seedlings were treated with trehalose before being subjected to drought stress. After the drought treatment, two different staining methods were performed: nitroblue tetrazolium (NBT) staining of the superoxide radical ($O_2^{-\bullet}$) and 3,3'diaminobenzidine (DAB) staining of hydrogen peroxide (H_2O_2).

3.5.1 NBT-staining

Shown in Fig. 16 are representative pictures of cotyledonary leaves after NBT staining of superoxide after drought stress. Results show there is no staining in the 0 and 20 mM trehalose pre-treatment (Fig. 16A,B). In the 60 mM trehalose pre-treatment the results are very variable, even within one plant the staining varied from almost full staining to only low level of staining (Fig. 16C). There was a consistent staining in the

100 mM treatment (Fig. 16D). Overall, the trend seems to be increasing staining with increasing trehalose concentration.

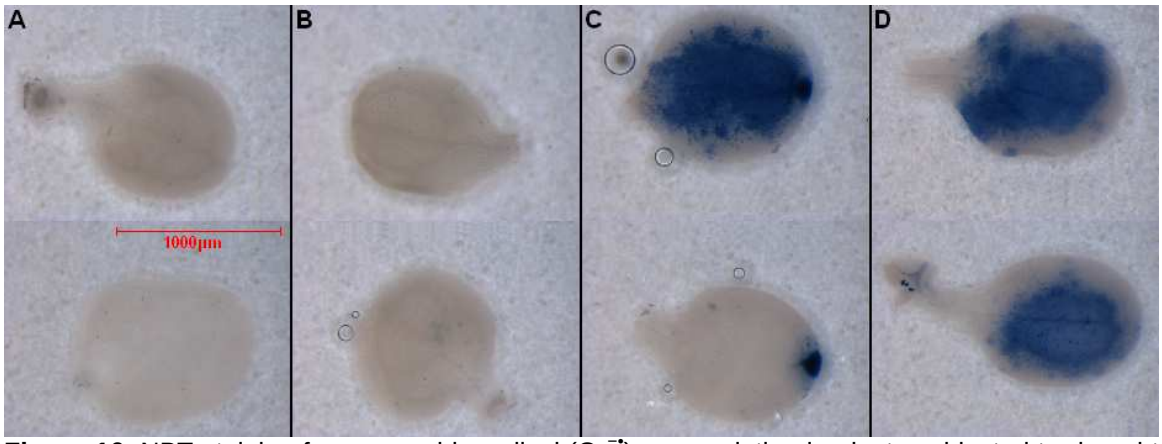

Figure 16. NBT staining for superoxide radical ($O_2^{-\bullet}$) accumulation in plants subjected to drought stress with or without trehalose pre-treatment. 3-day old Arabidopsis seedlings were pretreated with trehalose for 2 days before being transferred to drought stress conditions. After the stress treatment, superoxide in cotyledonary leaves was visualized by NBT staining. Leaf samples are (A) 0 mM trehalose pre-treatment, (B) 20 mM trehalose pre-treatment, (C) 60 mM trehalose pre-treatment and (D) 100 mM trehalose pre-treatment. Bar indicates 1000µm.

3.5.2 DAB-staining

The visualization of H_2O_2 by DAB did not result in sufficient staining to see differences between the trehalose pre-treatments (Fig. 17). Stressed seedlings that did not receive a trehalose pre-treatment showed a low level of staining of the vascular tissue. Next to this, also non-stressed seedlings treated with an antioxidant before the DAB-staining showed staining of vascular tissue. Pictures of DAB-staining found in literature, although in mature *Arabidopsis thaliana* leaves, show a stronger staining by DAB (Driever *et al.*, 2009; Fryer *et al.*, 2002).

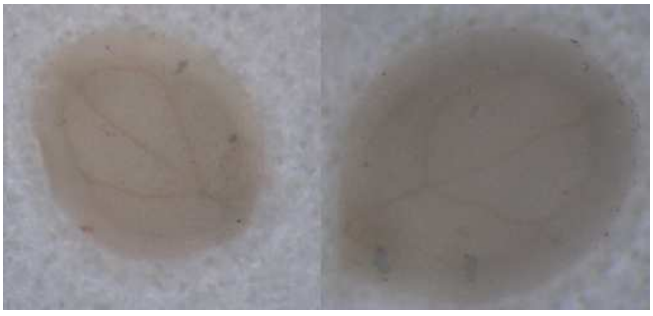

Figure 17. DAB-staining for hydrogen peroxide (H_2O_2) accumulation in plants subjected to drought stress. On the left a cotyledon of a stressed seedling non-pretreated with trehalose and on the right of a non-stressed seedling pretreated with the antioxidant ascorbic acid.

4 Discussion

4.1 Technical problems

4.1.1 Infection problems using gas method

During the course of the thesis research, some experiments were hampered by fungal infections. The infection seemed to be seed borne since all infections developed from a seed. Although the infection frequency was very low, one infection results in the loss of 25 seedlings since the seeds do not germinate individually in one container (this is usually done when cultures are initiated but not feasible in our experiments) but in batches of 25 seeds in one Petri dish. Usually, two eppendorf tubes with seeds were used for one experiment (about 1200 seeds). Seeds were taken from subsequent tubes, without mixing. Since the infections usually appeared in series, it was hypothesized that the sterilization procedure in some of the tubes was not sufficient to kill all pathogens. The number of seeds in the tubes differed. If the number of seeds is too high, it is possible that the chlorine gas does not reach the seeds at the bottom of the eppendorf tube. To test this hypothesis, the number of seeds per eppendorf tube was lowered. After the sterilization procedure, the infection frequency was lower (data not shown), however, infections did still occur. In conclusion, when using the chlorine gas method to sterilize Arabidopsis seeds, the number of seeds per tube seems to influence the infection frequency. The wet sterilization method using 70% ethanol and hypochlorite solution (See Materials and Methods) resulted in no infection but this method is more laborious.

4.1.2 Variability in survival from HAT/HWT

The survival rates in both the HAT and the HWT were very variable within one treatment. In the HAT, within one combination of trehalose and temperature, survival could vary from zero to almost 100%. For example the 0 mM trehalose treatment at 43.5°C; in this treatment the percentage survival in three replicates ranged from 0 to 72 and 85%. Like in the HAT, the observed survival rates in the HWT temperature range are also very variable within a specific temperature-trehalose combination. To indicate the extent of variability, the survival in the 0 mM trehalose at 42ºC varies from 6.3 to 31.3, 58.8, 62.5 and 70.6%. The survival rates at the 41.5 treatments were stable with survival of 91.7, 94.4 and three times 100% in the 0 mM trehalose treatment. There are two factors that explain this high variability in percentage survival.

(1) The sensitivity of seedlings to heat stress changes dramatically in a very small temperature range; a ΔT 0.5 -1°C can make the difference between survival or death of the seedling.

(2) During the stress treatment small variations in temperature occur. Temperature measurements were performed to check for the variability of temperature within the hot air incubator. There were two relevant observations. The temperature of the

incubator as a whole was fluctuating with an amplitude of 0.2°C. When the incubator was set on a temperature of 40°C, the indicated temperature of the build-in thermometer fluctuated from 38.8 to 40.2°C (data not shown). In addition to this, the temperature of the growth medium in Petri dishes was measured on two different places in the incubator, namely left top (LT) and right bottom (RB) (Fig. 3). Compared to the RB Petri dishes, there was a faster increase in temperature in the LT Petri dishes. However, only the ΔT at 38 min was significantly different from 0 ($P = 0.032$).

4.1.3 ROS Staining

It was hypothesized that the protective function of trehalose is related to ROS scavenging activity. In the 0 and 20 mM trehalose treatment there is no staining of superoxide. The staining in the 60 mM treatment is very variable; even within one plant the staining varied from almost full staining of the cotyledon to a very low extent of staining. A possible explanation for this observation is contact with the medium. Some leaves might be in contact with the medium while others are in contact with the air inside the Petri dish. Overall there is a trend for more staining with increasing trehalose concentration, or a higher level of superoxide radicals with increasing trehalose concentration. This result does not correspond to observations in other research that has shown that trehalose is active as a scavenger of ROS and promotes activity of other antioxidant enzymes (Luo *et al.*, 2008; Zhu, 2001).

4.2 Effect of trehalose

Trehalose treatment resulted in increased survival after drought and high salinity stress, however this protective effect was not present in heat stress. Previous research with transgenic *Arabidopsis thaliana* plants, transformed with constructs for increased trehalose accumulation, showed an increased tolerance to heat stress (Miranda *et al.*, 2007; Almeida *et al.*, 2005). Contrary to the survival results, trehalose decreased the Relative Electrolyte Leakage (REL) with increasing trehalose concentration in heat and drought stress. The REL data confirms the role of trehalose as a protector of cell membranes, however the survival data in heat stress did not correspond to this reduced REL (Fig. 18). While there was a highly significant correlation coefficient between the REL and survival in drought stress ($r = -0.995$, $P = 0.005$), the correlation coefficient in heat stress is lower and not significant ($r = -0.468$, $P = 0.532$). A possible explanation for this differential response to trehalose might be the type of damage caused by the different stresses. Lack of water due to drought or high salinity results in overproduction of free radicals that damage proteins and cellular membranes, leading to a loss of solutes from the cell and organelles (Gupta, 2007b,c). During heat stress as applied in this research project, the seedlings do not have water stress since they are in a Petri dish or in an eppendorf tube containing (liquid) ½ MS medium. What

differentiates heat stress from salinity and drought stress, is the fact heat stress causes, besides damaging membrane integrity, injury to the photosynthetic apparatus while high salinity and drought do not affect photosynthetic capacity (Flexas *et al.*, 2004). High temperature, among others, induces photosynthetic ion imbalance, causes disintegration of grana, affects electron transport activities and substantially decreases RuBP carboxylase enzyme activity (Gupta, 2007a). High salinity and drought stress both ultimately result in dehydration of the cell and osmotic imbalance suggesting further the possibility of cross-talk among these stresses (Tuteja, 2009). Since trehalose has its protective function both in drought and high salinity stress, this might indicate trehalose is involved in the stress response to both drought and high salinity stress (cross-talk).

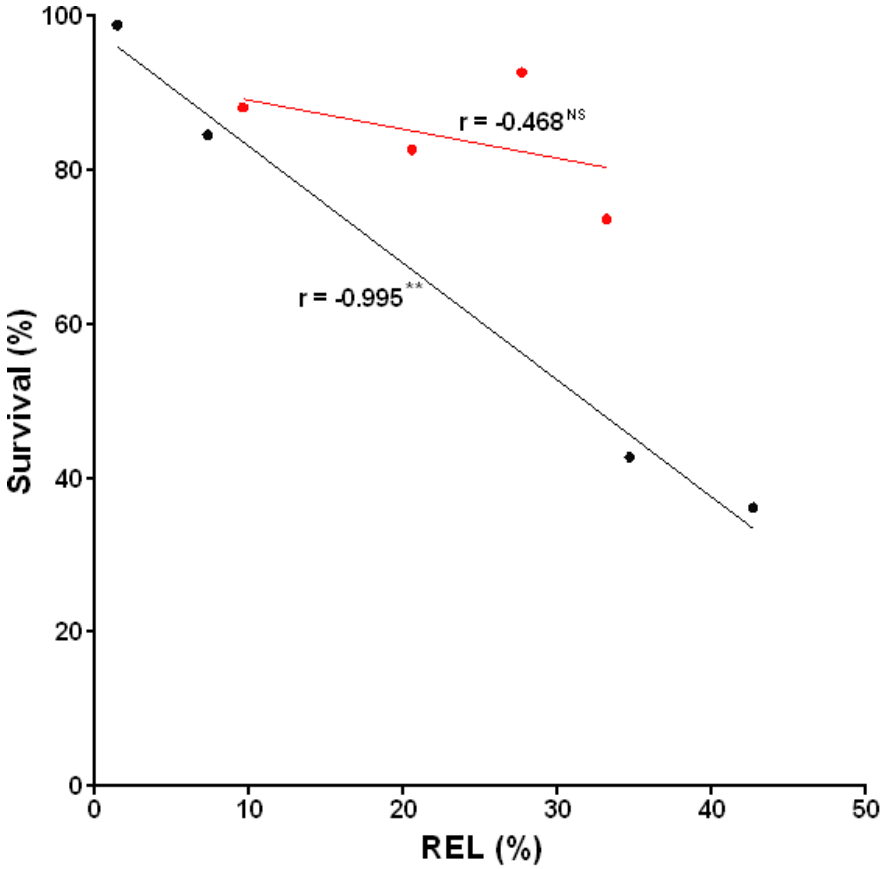

Figure 18. Correlation coefficient between Relative Electrolyte Leakage (REL) and percentage survival in drought stress (in black) and heat stress (in red). $^{NS,\ **}$ Correlation coefficient is nonsignificant or significant at $P < 0.01$.

Gene expression analysis also supports the similarity of drought and high salinity stress and shows extensive overlap with up- and down-regulated genes. In Arabidopsis, overlap has been reported for many genes that are induced by high salinity and drought stresses (Tuteja, 2007). Gene expression experiments with different abiotic stresses show that a total of 1562 genes are differentially expressed after drought stress. The

expression of about 70% of the genes that increase or decrease in the recovering period of drought stress, are also increased or decreased during high salinity stress (Wanke *et al.*, 2009). When comparing the up-regulated genes after heat stress with the genes responding to the other abiotic stresses, there is little overlap. However, a considerable number of down-regulated genes is also down-regulated during high salinity stress. Trehalose is known as a stress protectant, however, both its synthesis and degradation pathways are activated upon stress (Singer and Lindquist, 1998). Possibly, the protecting role of trehalose needs an appropriate concentration (Luo *et al.*, 2008) and high levels of trehalose are toxic because it may interference with protein refolding (Singer and Lindquist, 1998) and inhibit enzyme activity after heat stress (Sebollela *et al.*, 2004). This might explain the results obtained from measurement of REL after heat stress. The lowest REL is found in the 60 mM treatment. A higher trehalose concentration of 100 mM does not result in lower REL.
Another explanation for the differential response to trehalose between heat stress and the other two stresses is that trehalose doesn't reach the site of damage by heat stress. According to Herdeiro *et al.* (2006) trehalose is required on both sides of the lipid bilayer for protection of the membrane. Since in heat stress we found a reduced electrolytes leakage with increasing trehalose concentration, one may conclude that there was import of trehalose into the cell. Trehalose is taken up by plant cells, intracellular trehalase expression protected seedlings from toxic effect of high trehalose (Schluepmann *et al.*, 2003).

4.2.1 Cross-protection

Plants use common pathways and components in the response to different stresses (Pastori and Foyer, 2002), allowing them to adjust to different stresses after exposure to one specific stress. This phenomenon is called cross-protection or cross-tolerance. When pre-treatment with a protective compound results in increased tolerance to different stresses, this may indicate cross-protection. When combination of these stresses as a mild pre-stress and a severe stress does not result in increased tolerance, this indicates the stresses result in the same kind of damage but do not share the same stress response mechanism. Our data shows that trehalose protected against both drought and high salinity stress, this may indicate cross-protection. However, the protective function of trehalose in drought and salinity can be explained by trehalose protecting against the type of damage that is caused by these stresses (osmotic imbalance and dehydration of the cell). Work from previous students shows a trend for increased survival (from 27% to 51%, data not shown) when seedlings were subjected to a mild drought treatment (30 min) before receiving high salinity stress (400 mM NaCl for 4 h). This result suggests the occurrence of cross-talk between the signaling pathways for drought and high salinity stress. Next to this, gene expression analysis after drought and salinity stress shows a high level of overlap. This indicates trehalose might act as a signaling

component in the signal transduction pathway of these stresses since these gene expression analyses after drought and salinity show a high level of overlap.

While there are indications for cross-protection between drought and high salinity stress, This protective function is not found in the case of heat stress. Next to this, work from previous students does not show cross-protection when seedlings were subjected to a mild heat treatment (90 min at 38°C) before receiving high salinity stress (400, 600 and 800 mM NaCl for 4 h) or drought stress (2, 2.5 and 3 h). These results suggest that there is no cross-talk between the stress signaling pathways of drought and high salinity stress on the one hand and heat stress on the other hand.

These data are not conclusive about the exact function of trehalose. The possibilities are not mutually exclusive and might act at the same time. To further investigate the exact function of trehalose in the stress response of Arabidopsis seedlings, gene expression analysis should be performed to see if and subsequently what genes are differentially expressed after a trehalose pre-treatment. These experiments should make clear if trehalose functions as a signaling component in the signal transduction pathway or acts as a direct protectant against the damage produced by drought and high salinity stress.

4.2.2 Acquired thermotolerance

A temperature pre-treatment of 30 minutes at 38°C before severe heat stress of 2 h was highly protective with a survival of 100% over a temperature range of 43 to 44.5°C. This phenomenon is called acquired thermotolerance. Acquired thermotolerance is induced by pre-exposure to moderately high (but survivable) temperatures and results in tolerance to otherwise lethal heat stress (Larkindale *et al.*, 2005; Burke *et al.*, 2000). According to Larkindale *et al.* (2005) signaling pathways involving heat shock protein induction, abscisic acid, salicylic acid and ROS are critical events during acquired thermotolerance. If trehalose would have a major function in the heat response pathway, pre-treatment with this compound would partly mimic the response and result in tolerance to high temperatures. Compared to a temperature pre-treatment, the protective effect of trehalose is only very small or absent. This result suggests that trehalose does not have a function in acquiring tolerance to high temperatures.

4.3 Influence of light on survival

As shown earlier, the presence or absence of light during the stress recovery phase influenced the survival of seedlings. After both heat and drought stress, survival increased when seedlings were allowed to recover in the dark compared to recovery in the light. The increased survival in the dark is highest in heat stress. A possible explanation for this sensitivity to light is light-induced damage to photosystem II (PSII) of photosynthesis, or photoinhibition. This process is caused by an imbalance between

the rate of photodamage to PSII and the rate of repair of damaged PSII. The photochemical reaction center of PSII is inactivated by excess light that has been absorbed by chlorophyll (Murata *et al.*, 2007). PSII has two core subunits, the D1 and the D2 protein. Photodamaged PSII is repaired by the replacement of in particular the D1 protein by newly synthesized proteins (Takahashi and Murata, 2008). Damage to PSII in photosynthetic organisms is unavoidable since light energy is the driving force for photosynthesis (Murata *et al.*, 2007). Photosystem I (PSI) activity is very heat-stable: temperatures that cause complete inactivation of PSII activity do not have a significant effect on the activity of PSI. The process of photoinhibition is accelerated in the case the rate of absorption of light energy by photosynthetic pigments is higher than its consumption during the photosynthetic fixation of CO_2 in chloroplasts.

In higher plants, oxygen evolution by PSII is one of the processes that is most sensitive to heat treatment (Sharkey, 2005; Yamane *et al.*, 1998). While moderate heat stress results in increased photoinhibition by inhibiting the repair of photosystem II, direct damage to PSII often only occurs at strong heat stress (often above 45°C) by inactivating the oxygen-evolving complex directly (Murata *et al.*, 2007; Sharkey, 2005; Nash *et al.*, 1985). Photosynthesis is particularly sensitive to heat stress and photoinactivation of PSII is very rapid at high temperatures (Gupta, 2007a; Yamane *et al.*, 1998). Strong heat stress results in direct damage to PSII, and the critical temperature is often about 45°C. This might explain the rapid drop in survival from 43 to 44°C. If the critical temperature in Arabidopsis seedlings is in that range, a small increase in temperature can already result in a dramatic decrease in survival since the oxygen-evolving complex is directly inactivated. This would also explain the relatively high survival in the dark after heat stress at 43.5°C.

Abiotic stresses like drought, high salinity and moderate heat stress result in increased photoinhibition by inhibiting the repair of PSII because of suppression of the synthesis of PSII proteins and strongly limiting the photosynthetic fixation of CO_2 (Takahashi and Murata, 2008; Murata *et al.*, 2007). This limited photosynthetic CO_2 fixation is caused by inactivation of Rubisco activase at high temperatures (Feller *et al.*, 1998). Rubisco activase is indispensable for the activity of Rubisco. This reduced Rubisco activity results in limited CO_2 fixation. When CO_2 fixation is limited, electrons from PSI are transferred to molecular oxygen, resulting in the production of ROS (Larkindale and Knight, 2002; Asada, 1999). Photorespiration by Rubisco protects the photosynthetic membranes against this light-induced damage (Foyer *et al.*, 1994) but since the activity of Rubisco is limited, photorespiration is also limited.

Recovery in the dark after high salinity stress also results in an increased survival compared to recovery in the light. High salinity stress inhibits protein synthesis (Murata *et al.*, 2007) but the molecular mechanism of this inhibition is not clear. Several possible mechanisms have been proposed. First, there is direct inhibition of protein synthesis due to high concentrations of NaCl that inactivate the translational machinery in vitro. Second, there is inactivation of Rubisco by the presence of high concentrations

of NaCl. Inactivation of Rubisco results in inhibition of carbon fixation. This in turn induces the generation of ROS that inhibit protein synthesis. A third theory postulates that an increased intracellular NaCl concentration inactivates ATP synthase and decreases the intracellular level of ATP, which is essential for protein synthesis (Murata *et al.*, 2007 and references therein).

To conclude, reduced Rubisco activity after stress treatment could result in the increased survival in the dark compared to the light. In contrast to recovery in the light, there is no absorption of light when seedlings are allowed to recover in the dark. Although there is limited carbon fixation and limited photorespiration, there is also no incoming light energy. Since there is no energy absorption by the photosynthetic pigments, there is no transfer of electrons from PSI to molecular oxygen. As a result, in the dark the level of oxidative stress is limited. An interesting follow-up experiment is to see the influence of the length of the dark period after the stress treatment. Seedlings can be allowed to recover in the dark for different periods of time before being put back in the light. The results can give more evidence for the repair of the photosystem in the dark and an indication how much time is required for repair. Another interesting experiment is to see the influence of different light intensities on the survival after stress. To see the role of ROS in the reduced survival in the light, ROS can be visualized using different staining methods (for example di-amino benzidine for H_2O_2 and nitroblue tetrazolium for $O_2^{-\bullet}$). Since inactivation of Rubisco might explain the observations, it is interesting to measure Rubisco activities in the Arabidopsis seedlings after the stress treatment. The obtained results can be used to verify the hypothesis that is described above.

5 Conclusions

Trehalose treatment protected against both high salinity and drought stress, shown by increased survival rates, increased Cell Membrane Stability (CMS) and a decreased leakage of electrolytes (REL) after the stress treatment. These results support the occurrence of cross-talk between the signaling pathways for drought and high salinity stress. While there are indications for cross-protection between drought and high salinity stress, the protective function of trehalose is not found in heat stress. Trehalose does not result in increased survival after heat stress but there is an increase in CMS and decrease in REL with increasing trehalose concentration. These results support earlier results that suggested that there is no cross-talk between the stress signaling pathways of drought and high salinity stress on the one hand and heat stress on the other hand.

A possible explanation for the differential response to trehalose to drought, high salinity and heat stress is the type of damage caused by the different stresses. During heat stress as applied in this research project, the seedlings do not have water stress since they are in a Petri dish or in an eppendorf tube containing (liquid) ½ MS medium. Heat stress imposes direct damage to the photosynthetic apparatus while this is not the case with drought and high salinity stress.

The function of trehalose as a direct and indirect scavenger of ROS is only shown indirectly. Trehalose pre-treatment results in reduced electrolyte leakage after heat and drought stress. This suggests that the protective function of trehalose can indeed be through the scavenging of ROS. ROS cause lipid peroxidation in membrane that lead to increased permeability of the membrane to electrolytes. No conclusions can be drawn from the direct visualization of ROS by staining of oxygen radicals after a stress treatment.

Overall, the protective effect of trehalose seems to be primarily caused by its function as a protectant against damage to lipids and membranes by physical interaction. Relatively high levels of trehalose are necessary to significantly increase the survival after for example drought stress. If trehalose would have a signaling function, one would expect to see the effect of trehalose already at the lower trehalose concentrations.

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