

Protection of in-vitro grown *Arabidopsis* seedlings against abiotic stresses

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Abstract Severe abiotic stresses may cause permanent damage leading to death. In *Arabidopsis* seedlings germinating in vitro, we examined whether stress-related damage could be reduced by addition of protective low-molecular-weight compounds (trehalose and putrescine), addition of a specific signal molecule (acetylsalicylic acid), culture in the dark before and/or after the stress, and hardening mild-stress pretreatments. All four tested protective procedures increased survival after exposure to drought, warm air, warm water or salinity, often from ca. 15% in the control to ca. 85% in the treated seedlings. These protective measures may be usable to reduce the negative effects of abiotic stresses related to tissue culture.

Keywords Heat stress · Drought stress · Priming · Salinity · Trehalose · Hot-water treatment · Hot-air treatment

Abbreviations

HWT Hot-water treatment
HAT Hot-air treatment
MS Murashige-Skoog

LEA Late-embryogenesis abundant
HSPs Heat shock proteins
ROS Reactive oxygen species

Introduction

Plants have developed a range of mechanisms to overcome damage by adverse environmental conditions. To survive predictable climatic stresses such as hot dry summers or cold winters, plants generate survival organs with specific morphological and biochemical properties, in particular dormant seeds and buds. To overcome other stresses, plants synthesize protective low-molecular-weight compounds, viz., proline, glycine betaine, polyamines or trehalose, and protective proteins, viz., late-embryogenesis abundant proteins (LEA-proteins; unknown function, Tunnacliffe and Wise 2007) and heat shock proteins (HSPs; chaperone-function, Wang et al. 2003). Since under severe stress conditions, the balance between the production of reactive oxygen species (ROS) and the antioxidant defense system is disturbed, increased antioxidant activity also occurs in stressed plants (review in Wang et al. 2003).

To obtain genotypes with improved stress tolerance, plants have been transformed with genes that confer the synthesis of protectants (trehalose, Garg et al. 2002; polyamines, Kasukabe et al. 2004), protective proteins (HSPs, Malik et al. 1999; LEA-proteins, RoyChoudhury et al. 2007) and antioxidative

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enzymes (McKersie et al. 1999). Plants may be rendered resistant to severe stress in several other ways. Direct application of protectants results in resistance to stress (de Klerk 2002; Bae et al. 2005; Zeid and Shedeed 2006). Addition of signal molecules that form part of the signal transduction pathway for stress, may bring about protection (Lopez-Delgado et al. 1998). A foregoing moderate stress may harden against severe stress because the moderate stress sets off protective mechanisms (Larkindale et al. 2005). Finally, since photosynthesis is a major source of ROS, culture in darkness may also reduce the negative effects of stress.

Tissue-cultured plants are exposed to stress when they are acclimatized to ex-vitro conditions (de Klerk and Wijnhoven 2005) and during various techniques such as protoplast preparation, thermotherapy etc. Moreover, it has been suggested that several “unnatural” conditions inherent in tissue culture, e.g., mixotrophy, high levels of plant growth regulators and very high relative humidity (close to 100%, Chen 2004) also impose major stresses in sensitive genotypes and cause typical tissue-culture problems such as hyperhydricity, recalcitrance and variation (review in de Klerk 2007). Since often cross-protection occurs (Sabehat et al. 1998), damage by typical in-vitro stresses may be reduced by treatments that confer resistance to other abiotic stresses. Thus, the protective measures mentioned above may reduce these tissue-culture problems. In this article, we explore the effectiveness of various protective treatments in *Arabidopsis* seedlings germinating in vitro and exposed to common stresses (drought, heat and salinity).

Materials and methods

Plant material and growth conditions

Arabidopsis ecotype Columbia (Lehle Seeds, Round-rock, TX) was used. Seeds were surface-sterilized for 5 min in 70% ethanol followed by 10 min in 1.5% (w/v) hypochlorite + 0.02% Tween 20. They were rinsed 5 times with water, dried and stored under sterile conditions at 4°C. Seeds were sown in a 60-mm Petri dish with 8 ml nutrient medium composed of ½ MS (Murashige and Skoog 1962), 1.5% sucrose and 0.7% (w/v) agar (micro-agar, Duchefa, Haarlem, Netherlands), vernalized at 4°C for 2 day in the dark to

synchronize growth, and then kept at 20°C and a 16 h photoperiod ($30 \mu\text{E m}^{-2} \text{s}^{-1}$, Philips TL 33). Per Petri dish, 20 seeds were germinated. The germination percentage was close to 100. The seeds were sown on a nylon gauze (3.5×3.5 cm) on the medium. For transfer, e.g., for the drought treatment, the gauze with the seedlings was transferred. To remove excess of liquid, the gauze was kept for 5 s on filter paper.

Stress treatments

Heat stress was given in an incubator (hot-air treatment, HAT) or in a hot-water bath (hot-water treatment, HWT). In the latter case, 20 seedlings were in an Eppendorf tube in 1 ml liquid medium unless indicated otherwise. Drought stress was imposed in a sealed 60-mm Petri dish on top of blue silica gel. For salinity stress, seedlings, grown for 5 day at 20°C, were exposed to nutrient medium with 600 mM NaCl for 4 h and then transferred back to standard medium.

Statistical analysis

Survival was scored 12 day after the stress. For each determination six Petri dishes with 20 seedlings each were used. In the figures and tables, means \pm SEs are shown.

Results and discussion

Development of test systems for drought and heat

For drought stress, various experimental setups may be chosen (Verslues et al. 2006). Plantlets may be cultured on medium with low water potential obtained by adding a high-molecular-weight solute such as polyethylene glycol 6000. Alternatively, plantlets may be air-dried. The latter may be done by taking the seedlings from the nutrient medium and leaving them on the laminar flow bench. Preferably, though, drought stress should be given under controlled conditions. In our experiments, seedlings were removed from the nutrient medium, kept constantly for 2½ h in a sealed 60-mm Petri dish on filter paper on top of blue silica gel and then transferred back to the nutrient medium. Survival was scored 12 days after the stress. Only ca. 15% of the seedlings survived the drought-stress treatment. Detached leaves of *Arabidopsis* plants lose 50% FW in 6 h

(Verslues et al. 2006) whereas in-vitro grown Arabidopsis seedlings lose 50% FW in ½ h of drought stress (de Klerk 2007). After 2½ h, the seedlings have lost 85% FW. The rapid loss of FW of in-vitro grown seedlings is for the greater part likely due to the specific tissue culture conditions which strongly decrease the water retention capacity (de Klerk and Wijnhoven 2005).

Heat stress may be given simply by transferring Petri dishes into an incubator. This is referred to as “hot-air treatment” (HAT). However, because thermal conduction of air is poor, heat treatment may be applied in hot water in a small tube in a water bath or in a thermoblock. A hot-water treatment (HWT) was given by Larkindale and Knight (2002). We compared both methods. After a HWT far more seedlings died than after a HAT (Table 1).

Various differences between HWT and HAT may be responsible for the much higher level of damage during HWT. We assume that poor gas exchange leading to anaerobiosis is a major factor (Jackson 1985; Dolferus et al. 2003). To examine the effect of low oxygen, the number of seedlings per Eppendorf tube was increased so that per seedling even less oxygen would be available. Figure 1 shows that for tubes with five or ten seedlings survival was ca 40%, but when the HWT was applied to tubes with 40 seedlings, survival was close to 0% (Fig. 1). We also added an oxygen donor, H₂O₂. This resulted in a somewhat increased survival (10% to 20%; data not shown). In horticulture, HWTs are being used routinely to render seeds, bulbs and fruits free from micro-organisms (Grondeau and Samson 1994). This effect of HWT is likely related to the synthesis of pathogenesis related proteins during the HWT (Van Loon et al. 2006).

Table 1 Survival of Arabidopsis seedlings after heat stress at 42.5°C. Heat stress treatment was administered either to seedlings submerged in liquid medium (HWT = hot water treatment) or to seedlings kept on solidified medium in a Petri dish (HAT = hot air treatment)

	HWT	HAT
1 h	0 ± 0%	100 ± 0%
2 h	2.1 ± 2.1%	100 ± 0%
3 h	0 ± 0%	90.1 ± 3.2%

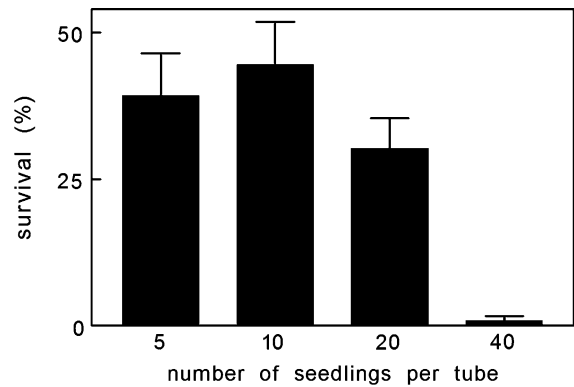


Fig. 1 Survival of Arabidopsis seedlings after a severe HWT of 2 h at 40°C. The HWT was given in an Eppendorf tube in 1 ml liquid medium. The effect of the number of seedlings per tube was determined. Survival was scored 12 day after the stress

Addition of protectants and signal molecules

For protectants, we focused on polyamines and carbohydrates. The results with polyamines have been reported previously (de Klerk 2007). Putrescine increased survival after the 2½ h drought treatment from 15% (control) to 85% (treated for 2 day with 10 mM putrescine). At the same time, though, putrescine is toxic as shown by protracted culture in putrescine-containing medium (de Klerk 2007).

With respect to protection by carbohydrates, until recently only few plant species, viz., desiccation tolerant ‘resurrection plants’ were thought to synthesize trehalose. However, most plant species accumulate sucrose during stress (Wingler 2002). Trehalose protects proteins and membranes by replacing water through hydrogen bonding to polar residues and is superior in protection because of its physical–chemical properties (Lerbret et al. 2005; Wingler 2002). We tested trehalose at a range of concentrations (10, 20, 60 and 100 mM) and found that the highest concentration tested (100 mM) resulted in high protection against drought (80% survival, Fig. 2). At this concentration, protection by other carbohydrates is less (Fig. 3).

Protectants act by direct interaction with vulnerable macromolecules or by osmoprotection. Signaling molecules act in a very different way. They evoke a protective action by the tissue, among others the synthesis of protective molecules such as the aforementioned low-molecular-weight compounds but also proteins such as LEA-proteins, HSPs and enzymes to

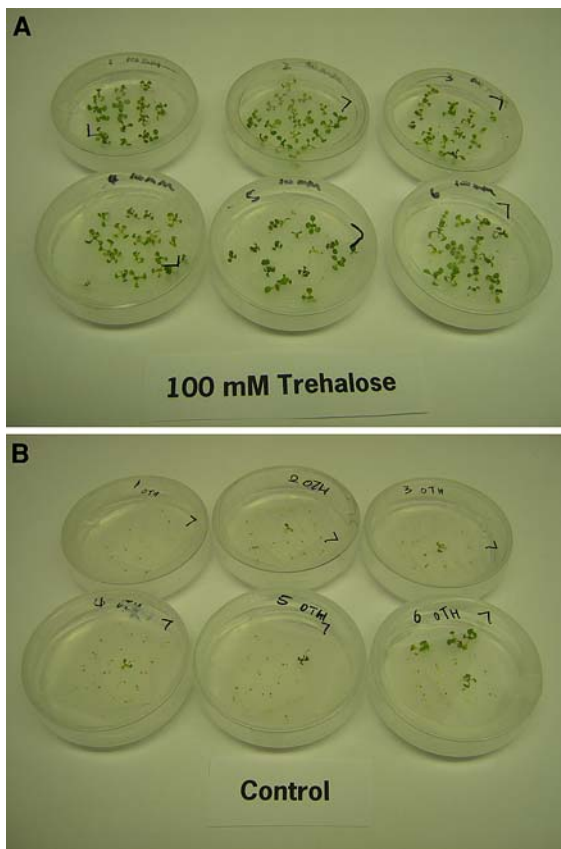


Fig. 2 Survival of Arabidopsis seedlings after severe drought stress. The seedlings were germinated for 3 day on standard medium and after that for 2 day on medium with 100 mM trehalose (a) or on standard medium (b). Then they were drought-treated for 2½ h and transferred back to standard medium. The photo was taken 12 day after the stress

remove toxic active oxygen species. We have examined salicylic acid and acetylsalicylic acid and only found a minor protective effect of acetylsalicylic acid (results not shown). Various signaling molecules have been examined by other authors and significant improvements have been obtained with salicylic acid (in contrast to our results, Lopez-Delgado et al. 1998), abscisic acid (Gómez-Cadenas et al. 2003) and jasmonates (Wasternack 2007).

Hardening by a mild prestress

Mild heat pretreatments enhance thermotolerance (Burke 1998; Larkindale and Knight 2002). Drought hardening by mild drought treatments is well known in agriculture and forestry (Lemcoff et al. 2006; Villar-Salvador et al. 2004). When 5 day-old

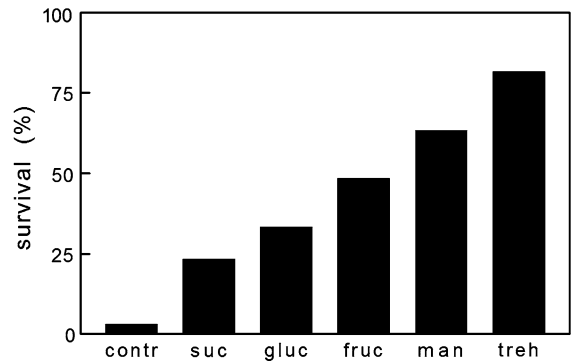


Fig. 3 Survival of Arabidopsis seedlings after severe drought stress. The seedlings were germinated for 3 day on standard medium and after that for 2 day on medium with 100 mM sucrose, glucose, fructose, mannose or trehalose. 100 mM was added in addition to 1.5% sucrose (44 mM). Then they were drought-treated for 2½ h and transferred back to standard medium. Survival was scored 12 day after the stress

seedlings were pretreated with a mild HWT (1½ h at 35°C), survival after severe HWT (2 h at 40°C) applied 4 h after the mild stress was increased from ca. 10% to ca 85%. Since maximum protection was reached some hours after the mild stress, the development of stress resistance continued for some time after the mild stress. After that, both the development of stress-resistance and the stress resistance itself decreased rapidly. After 48 h, only 50% of the maximal effect was still present (Fig. 4). Similar effects were obtained when a mild drought stress was

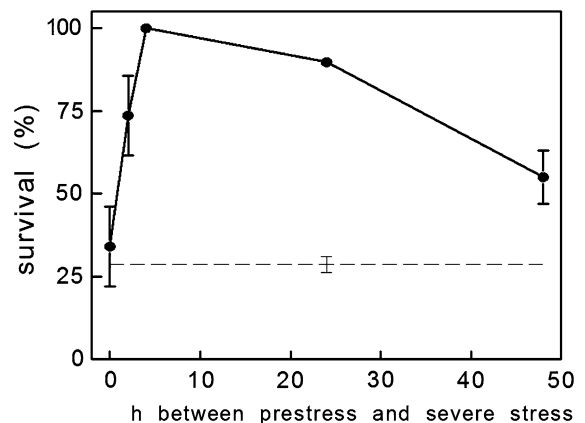


Fig. 4 Survival of Arabidopsis seedlings after a severe HWT. The seedlings were germinated for 5 day on standard medium followed by a pretreatment for 1½ h at 35°C. They were kept at standard conditions for increasing periods of time (0–48 h) and then received a severe HWT of 2 h at 40°C. Survival was scored 12 day after the stress

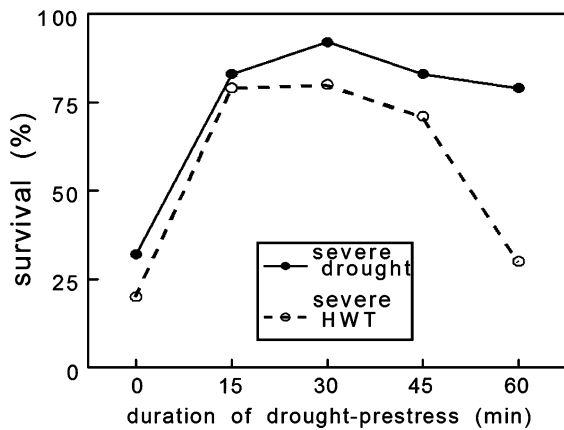


Fig. 5 Survival of *Arabidopsis* seedlings after a severe HWT or severe drought stress. The seedlings were germinated for 5 day on standard medium and then hardened by a drought treatment of increasing duration. After the drought pretreatment, the seedlings were reimplanted for 4 h under standard conditions. Then they received a severe drought treatment (2½ h drought) or a severe HWT (2 h at 40°C). Survival was scored 12 day after the stress

given before the severe drought stress. When seedlings were dried under standard conditions for a short period (15–60 min), reimplanted for 4 h and then subjected to the 2½ h drought stress, they showed strongly increased survival (Fig. 5). We examined cross-protection and found that brief exposure to drought stress protected against severe HWT (Fig. 5).

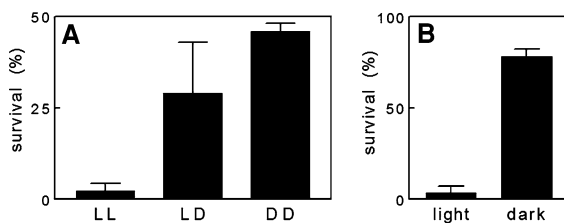


Fig. 6 Effect of darkness on survival after stress. (a) Seedlings were grown for 5 day in the dark or in the light. Then they received the severe drought stress. After that they were either kept in the light directly or after an intermediate period of 3 day darkness. Survival was scored 12 day after the stress. LL: Light-grown and cultured in the light directly after the stress. LD: Light-grown, cultured for 3 day in the dark after the stress and then in the light; DD: Dark-grown, cultured for 3 day in the dark after the stress and then in the light. (b) Seedlings were grown for 5 day under standard conditions, and then transferred for 4 h to medium with 600 mM NaCl. After the salinity stress, they were transferred back to standard medium and either kept directly in the light or after an intermediate period of 3 day transferred to the light. Survival was scored 12 day after the stress

Darkness

Photosynthesis is a major source of activated oxygen. Larkindale and Knight (2002) and Larkindale et al. (2005) report that the heat damage to *Arabidopsis* seedlings is reduced by culture in dark. In agreement with this, we observed that dark-grown seedlings have a strongly increased tolerance to drought treatment (Fig. 6a). Figure 6b shows that in darkness, the sensitivity to salinity is also much reduced. Salt stress increases photodamage to PSII (Murata et al. 2007). Similar effects were obtained with apple shoots exposed to heat or salinity stress (data not shown).

Conclusion

The aim of this research was to examine the effect of various protective measures in vitro to overcome damage by abiotic stress. Several protective procedures have been identified using seedlings of *Arabidopsis* exposed to severe abiotic stresses. In tissue culture, various instances of abiotic stress occur (see Introduction) and future experiments concern measures protective towards these alleged stresses. Positive effects of protectants on performance during acclimatization have been reported previously (de Klerk 2002).

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