1 Prolog

1.1 Orthodox seeds - survival in a dry state

1.1.1 Evolutional aspects of seeds

Unlike animals or humans plants cannot escape from their surroundings, therefore they have adapted themselves to their changing environments over thousands of years ago. One of the evolutionary most successful adaptation was the appearance of seed plants (*Spermatophyta*) which comprises the gymnosperms (*Acrogymnospermae*) with nowadays about 800 species and the angiosperms or flowering plants (*Angiospermae*) with about 250,000 species (Cantino *et al.* 2007, Linkies *et al.* 2010). Especially flowering plants seemed to evolve abruptly in the mid-cretaceous and spread in a highly accelerated rate of diversification which was named by Charles Darwin as 'an abominable mystery' (Friedman 2009). The reasons for this development are widely hypothesised but mainly accepted is the mutually beneficial animal-plant relationship including the remarkable co-evolution of the angiosperms and the pollination/dispersal animal agents. High speciation rates, among the annual growth form, homeotic gene effects, asexual/sexual reproduction, the propensity for hybrid polyploidy, as well as a low extinction rate, or broad ecological tolerances have to be shown empirically as additional advantages (Crepet & Niklas 2009).

The major points of the fast dominance of angiosperms are the seeds which are, in contrast to gymnosperms, enclosed inside an ovary. Seeds are the dispersal unit of the angiosperms and consist of one or more mature seeds in the same ovary which are protected by a pericarp or fruit coat that can contain additional flower parts (Linkies *et al.* 2010). A further differentiator is the double fertilization, that is, in addition to the egg cell fertilization, a second fertilization of a central cell nucleus which leads to the formation of diploid or triploid endosperm (Friedman 1995, Friedman & Ryerson 2009). It is assumed to enhance the fitness of the embryo, serves a nutrient source during seed development and is, in some cases, also involved in the control of germination by being a barrier for the growing radicle (Linkies *et al.* 2010).

Seed of about 90% of the angiosperms species is desiccation tolerant (Hoekstra 2005, Alpert 2006) and termed 'orthodox' (Colville & Kranner 2010). This is remarkable because these organisms are able to tolerate an absolute water content of less than 10% and regain their normal function after rehydration. The achieved water content is equivalent to equilibration with air of 50% relative humidity at 20°C or to dropping to a water potential of -100 MPa and corresponds to a point at which enzymatic reactions and thus metabolism probably stops (Alpert 2005). In this state organisms are able to overcome periods of unfavourable conditions such as drought, high-energy radiation or prolonged exposure to very low temperatures (França *et al.* 2007). In contrast, desiccation sensitive seeds which are termed 'recalcitrant' do not undergo maturation drying and remain metabolically active after they are shed from the parent plant. These kind of seeds are adapted to germinate immediately and cease in viability as soon as the water content declines (Berjak & Pammenter 2008).

The ability of seeds to remain viable after dehydration is thought to be an ancient trait. It was lost during the evolution of structural and morphological modifications of vegetative tissue in association with internal water transport (Alpert 2005, Illing *et al.* 2005). The subsequent successful distribution of flowering plants was probably a consequence of the evolution of desiccation tolerance in seeds, in parallel, which re-evolved desiccation tolerance in vegetative tissue again and allowed greater control of water status (Oliver *et al.* 2000).

The anhydrobiotic character which identifies desiccation tolerant species enables most seed to extend their life span from months to decades under ambient conditions (Priestley *et al.* 1985, Nagel & Börner 2010) and can even exceed to 2,000 years (Sallon *et al.* 2008) as shown in Tab. 1. However, the absolute seed life spans are the results of environmental conditions in combination with intrinsic properties which we want to refer to during the next chapters.

Tab. 1: Exceptional seed longevities discovered during the last two decades.

Longevity (years)	Species	Place of discovery	Source
2,000	Date palm (*Phoenix dactylifera* L.)	Herodian fortress	Sallon *et al.* (2008)
1,300	Lotus (*Nelumbo nucifera* Gaertn.)	Chinese dry lakebed	Shen-Miller *et al.* (1995)
200	*Liparia* sp., *Acacia* sp., *Leucospermum* sp.	British Archives/ Tower of London	Daws *et al.* (2007)
150	*Acacia* sp.	Swedish Museum	Leino & Edqvist (2010)
110	Barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), weed seeds	Haberland's experiment in Vienna	Steiner & Ruckenbauer (1995)

1.1.2 Glassy state

The ability of orthodox seed to resist complete dehydration is due to dramatic decrease of molecular mobility and the transition of cytoplasm to the glassy or vitreous state (Sun & Leopold 1997, Buitink & Leprince 2004). A glass is an amorphous, solid state with an extremely high viscosity which is comparable to crystalline materials having defined structures, stoichiometric compositions and melting points. At the same time it is also an amorphous matrix including random position of molecules with restricted mobility like a super-cooled liquid (Sun 1997, Buitink *et al.* 1998, Buitink & Leprince 2004) equipped with 'holes' and open spaces which enable small molecules to diffuse through (Walters 1998). Its flow rate is in the order of 10^{-14} m·s^{-1} compared to a typical liquid of 10 m·s^{-1} (Franks *et al.* 1991). A key feature of characterisation is the glass transition temperature (T_g) which describes the temperature when a substance transforms from glass into liquid (Buitink & Leprince 2004).

When water is removed, the packing density of the phospholipids head groups in membranes increases and results in stronger van der Waals forces among hydrocarbon chains (Crowe *et al.* 1992). Consequently, the phase transition temperature increases significantly and dry lipids turn into a gel phase at room temperature (Hoekstra 2005).

Carbohydrates are important players by forming sugar glasses which is related with their ability to replace the hydrogen (OH-) bonding function of water. This property enhances the

maintenance of the native structure of membranes and biopolymers (Hoekstra 2005). The molecular packing of several mono-, di- and tri- saccharides, such as glucose, trehalose and raffinose showed that monosaccharide glasses exhibited a stronger OH-bonding network than glasses made of higher molecular weight sugars. These suggest that smaller monosaccharide units can be packed more tightly in the glassy state (Buitink & Leprince 2004, Wolkers *et al.* 2004, Hoekstra 2005). Especially the non-reducing disaccharide trehalose is one of the most effective osmoprotectant sugars (Crowe *et al.* 1992, Iturriaga *et al.* 2009). It is thought to prevent damage by replacing the water shell around molecules (Patist & Zoerb 2005) and due to its high T_g it leads to stable glass formation (Iturriaga *et al.* 2009). There is growing evidence that trehalose, additionally, acts as an antioxidant by reducing intracellular oxidation and lipid peroxidation during dehydration (Pereira *et al.* 2003).

In accordance with different studies it is accepted that intracellular glasses are not only made of sugar molecules alone. The phase diagram of glass transitions for embryo tissues is distinctively different from that of carbohydrate mixes (Sun & Leopold 1995, Buitink *et al.* 2000, França *et al.* 2007). The addition of peptides (Wolkers *et al.* 1998), Late Embryogenesis Abundant (LEA) proteins (Wolkers *et al.* 2001) or tricarboxylic acid salts (Kets *et al.* 2004) enhanced further the hydrogen bonding strength and increased the T_g. In particular, LEA proteins and also heat shock proteins (HSP) facilitate glass formation (Buitink & Leprince 2004) and act as molecular shields which prevent molecular interactions, membrane fusion and protein aggregations. Improved glass characteristics are only achieved in cytoplasmic glasses where compounds, such as amino acids, ions and salts contribute to a denser OH-bonding network (Hoekstra 2005). In this state the activity of enzymatic and structural integrity remains, the secondary structure of proteins appears to be very stable and deteriorative chemical reactions are slowed down over several years of storage (Buitink & Leprince 2004).

A direct relationship between intermolecular hydrogen bonding strength in the glassy cytoplasm or the T_g and the life span could not be proved (Hoekstra 2005) but a relationship between seed longevity and the glass transition kinetics itself has been hypothesised by Sun (1997). His study indicated that the rate of seed viability loss during storage was affected by the water content which was correlated with the plasticization effect of water on intracellular glasses. Conditions, as for example higher water content and temperature, bring anhydrobiotes above their T_g and result in a linear increase of activation energy and rotational mobility of molecules in the cytoplasm. Buitink *et al.* (2000) could demonstrate that the stability of *Arabidopsis* seeds correlates with the density of the molecular matrix.

Summarizing, there is the suggestion that ageing rate and thus life span of germplasm can be influenced by the molecular stability of the cytoplasm as an important function of intracellular glasses (Buitink & Leprince 2004). However, the lack of direct correlations between OH-bonding strength or T_g might indicate that the glassy cytoplasm is not the system that is the most sensitive to ageing (Hoekstra 2005).

1.1.3 Oxidative stress and seed protection mechanisms

The extension or reduction of seed life span has already been mentioned to be related to the glassy state influenced by the effect of water and temperature. According to Harrington's (1963) thumb rule life span is doubled by decreasing seed moisture content (smc) by one percent or cooling by 5°C. Due to the high importance of water, Walters *et al.* (2005b) categorised seed ageing processes into five hydration levels that correspond to critical water (Ψ) potential and relative humidity (RH) as shown in Fig. 1.

Hydration levels

Seeds above a water potential $\Psi \geq -1$ MPa are fully hydrated, cell division is initialised and they start to grow (Hydration Level 5). Between $-1 \geq \Psi \geq -3$ MPa (Hydration Level 4) stress response and repair mechanism are detectable which are particular advantageous for seed priming. At Hydration Level 3 ($-5 \geq \Psi \geq -15$ MPa) intensive radical production and loss of membrane integrity occurs. When the water potential decreases to less than -15 MPa, which is comparable with a water content of about 0.10 g $H_2O \cdot$(g dry mass – g lipid)$^{-1}$ at ≈ 50% RH and ≤ 25°C, seeds enter the glassy state (Hydration Level 2) and longevity extends. At a critical point ($\Psi \approx -200$ MPa, Hydration Level 1) a switch to degrading reactions including decreasing longevity is indicated (Walters *et al.* 2005b).

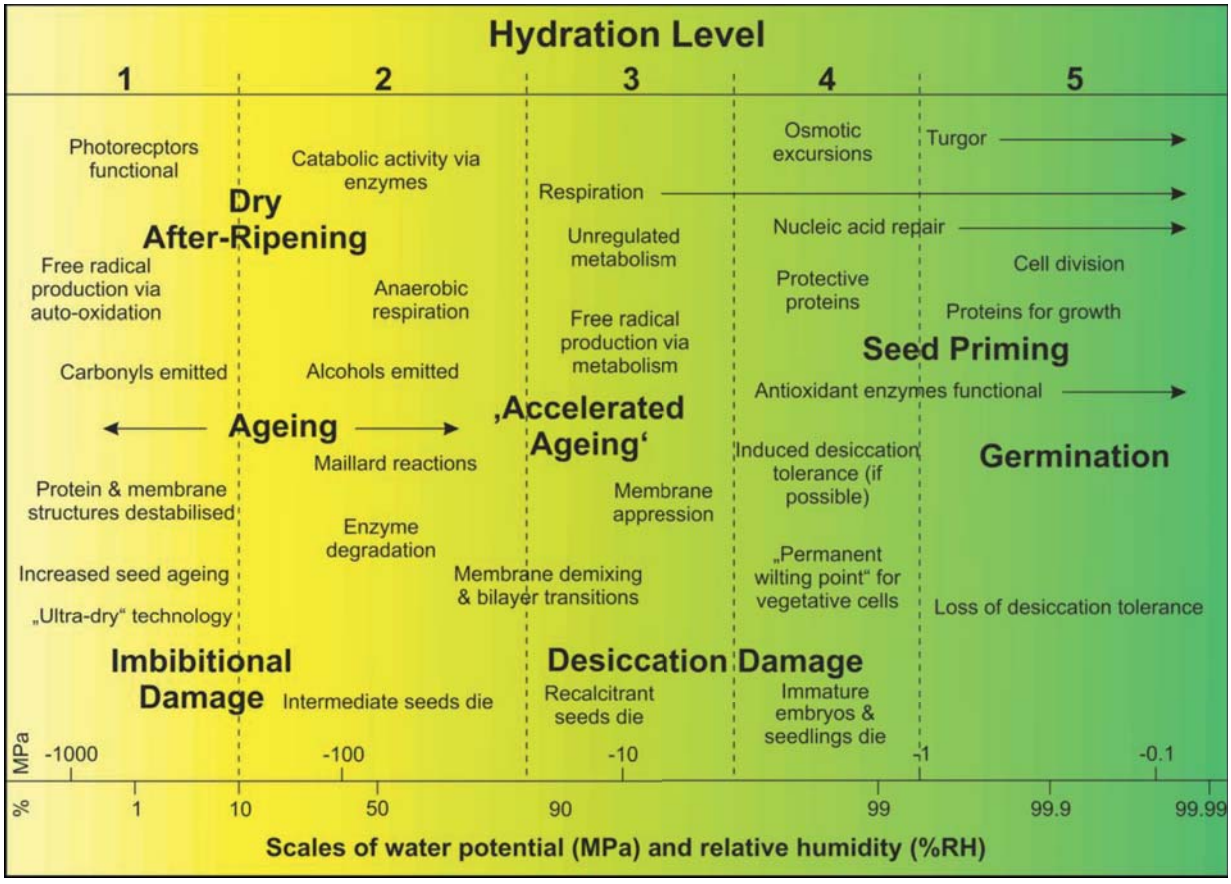

Fig. 1: Physiological activities of different seed hydration levels as a function of water potential and relative humidity. Data are adapted from Walters *et al.* (2005b) according to information of Vertucci & Roos (1990), Vertucci & Farrant (1995), Walters (1998) and unpublished data.

Stress and protection at Hydration Level 2 and lower

At Hydration Level 2 and lower seeds have entered the glassy state and metabolic processes are unlikely, although metabolic reactions cannot be totally excluded and might continue at very slow rates (Bailly *et al.* 2008, Kranner *et al.* 2010b). Within this amorphous matrix the speed of deleterious chemical processes is significantly slowed down (Sun & Leopold 1995). However, small molecules are able to penetrate holes and open space of the glassy environment and cause irreversible degradations (Walters 1998) which are even traceable in ancient barley (*Hordeum sp.*) kernels and radish (*Raphanus sativum* L.) seed (Bergen *et al.* 1997, Evershed *et al.* 1997).

These molecules are mainly reactive oxygen species (ROS) originating during abiotic and biotic stresses that enhance an impairment of the photosynthetic and respiratory electron transport (Halliwell 2006, De Gara *et al.* 2010). They encompass a variety of diverse chemical species including extremely unstable superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$) and the free diffusible and relatively long-lived hydrogen peroxide (H_2O_2) (Finkel & Holbrook 2000). Although the photosynthetic activity declines with maturation in orthodox seed (El-Maarouf-Bouteau & Bailly 2008) the metabolic ROS formation might be confined but cannot be entirely avoided and will occur through autoxidation (Colville & Kranner 2010). It is also expected that dehydration in the presence of light will accumulate free radicals (Khan *et al.* 1996).

Lipids and poly-unsaturated fatty acids (PUFAs) are one of the major targets of free radicals which often cause an extensive peroxidation (lipid peroxidation) and de-esterification of membrane lipids resulting in perturbation of membranes and water loss (Senaratna *et al.* 1987). A similar kind of molecular rearrangement under dry conditions is achieved by the reaction of carboxyl groups of sugars or aldehydes with amino groups of proteins forming complex mixtures (Amadori and Maillard reaction) which are responsible for odours and flavours (Walters 1998, Strelec *et al.* 2008). Proteins are in general targets of reactive radicals and scavenge between 50 and 70% ROS (Davies *et al.* 1999). If proteins are modified they will be possibly irreversible changed in their tertiary structure resulting in degradations and their progressive accumulation (Colville & Kranner 2010). Kibinza *et al.* (2006) showed that the occurrence of lipid peroxidation or oxidative damage of non-lipid cellular fractions (proteins, nucleic acids) are dependent on the availability of water.

Apart from proteins, damage to seed nucleic acids including DNA single-strand break caused by direct ROS attack of deoxyribose units (Bray & West 2005) will change DNA content (Sen & Osborne 1974, 1977) and lead to DNA fragmentation (Osborne 2000, Kranner *et al.* 2011). Double strand breaks result in a loss of genetic information if homologous recombination and non-homologous end-joining repair pathways are not initiated (Bray & West 2005, Waterworth *et al.* 2007). However, the DNA stability during dehydration is an important feature of desiccated seeds (Boubriak *et al.* 1997) and mainly safeguarded by a ROS scavenging system. Catalase (CAT2) and cytosolic ascorbate peroxidase (APX1) play a key role against photorespiratory-dependent H_2O_2 induced DNA damage in hydrated plant tissue (Vanderauwera *et al.* 2011).

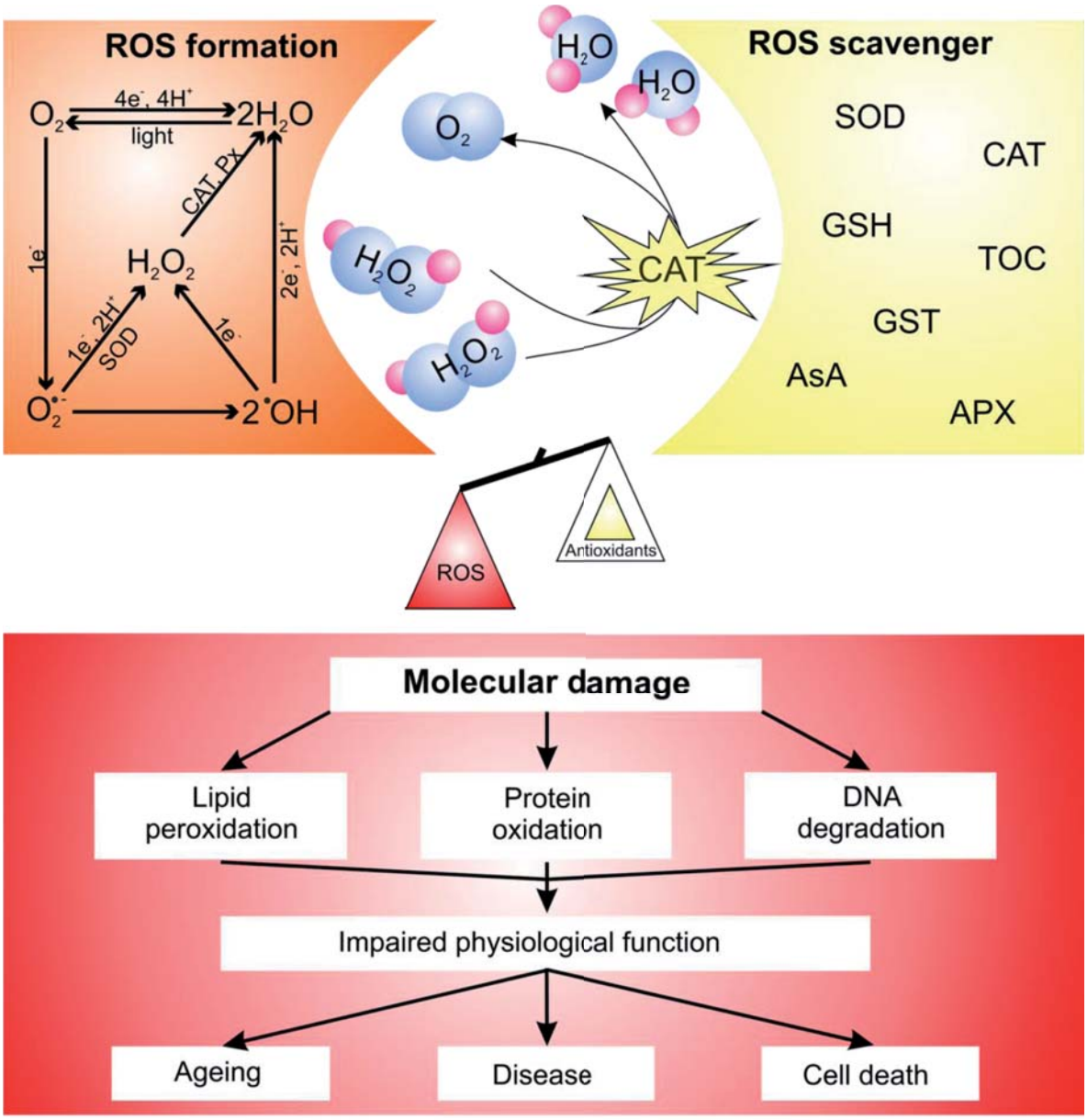

Fig. 2: Sources and cellular responses to reactive oxygen species (ROS). Oxidants are generated as a result of enzymatic and non-enzymatic reactions and are regulated by antioxidant defence systems, including catalase (CAT), superoxide dismutase (SOD), peroxidase (Px), glutathione (GSH), tocopherols (TOC), ascorbate (AsA), glutathione *S*-transferase (GST) and AsA peroxidase (APX). Figure is adapted from Franca *et al.* (2007).

In orthodox seed, protection against oxidative damage is ensured by a complex defence system (Fig. 2) that includes enzymes, such as peroxidases, catalases and superoxide dismutases (Sung & Jeng 1994, Kibinza *et al.* 2006, Lehner *et al.* 2008, Cakmak *et al.* 2010) and low-molecular-weight antioxidants, such as ascorbate (AsA), glutathione (GSH) and tocopherols (Kranner *et al.* 2010b). The major antioxidants AsA and GSH are water soluble and scavenge free radicals directly by acting as electron donors for ROS-detoxifying enzymes as AsA peroxidase (APX) and glutathione *S*-transferase (GST). Further both antioxidants participate in the ascorbate–glutathione cycle where GSH is needed for the reduction of dehydroascorbate (DHA) by the action of ascorbate peroxidase or by non-enzymatic reactions of ascorbate with oxidants. In these enzymatic or chemical reactions, GSH is oxidised to glutathione disulphide (GSSG) and reduced by glutathione reductase

(GR) at the expense of NADPH (Kranner *et al.* 2010b, Foyer & Noctor 2011). In the dry state AsA and GSH cannot be regenerated by reaction with ROS and the AsA/ DHA pool becomes even depleted because of the unstable DHA which is non-enzymatically broken down (De Tullio & Arrigoni 2003). GSSG on the other hand accumulates and can react with thiol groups of proteins forming protein-SSG mixed disulphides, which protect proteins against desiccation-induced oxidative injuries (Kranner & Grill 1996). The ratio between glutathione and the disulphide-glutathione (GSSG/2GSH) defines the most abundant redox couple in cells. Changes of its half-cell reduction potential $E_{GSSG/2GSH}$ appear to correlate with the biological status of the cells (Schafer & Buettner 2001) and are assumed to be part of the oxidative stress signalling cascade (Foyer & Noctor 2011) and programmed cell death (PCD) (Kranner *et al.* 2006).

Interestingly, signalling pathways are operative in desiccated seeds and are able to perceive environmental cues, such as those required for dormancy breaking (Finch-Savage *et al.* 2007). The mobility of protons might be enabled by the existence of hydrated pockets in which some metabolic activities are possible (Leubner-Metzger 2005). El-Maarouf-Bouteau & Bailly (2008) even considered that ROS could be sensed and, in particular, short-lived radicals such as $^{\bullet}OH$ react with receptor proteins close to their production site (Moller *et al.* 2007).

Thus, as demonstrated in Fig. 2, dry seeds can withstand the attack of free radicals by a complex ROS scavenging system but ongoing oxidative stress can lead to an imbalance between prooxidant and antioxidant activities (Rajjou & Debeaujon 2008). During storage the detoxification potential is strongly altered by a continuous depletion of antioxidant enzymes, low molecular antioxidants and a change of $E_{GSSG/2GSH}$ (Colville & Kranner 2010). Consequently uncontrolled oxidative attacks cause damage to macromolecules, phospholipids, PUFAs and proteins (Kranner *et al.* 2010b) resulting in a loss of membrane integrity and death (Tammela *et al.* 2003, Pukacka & Ratajczak 2007).

Stress and protection at Hydration Level 3 and higher

When accumulated damage did not result in viability loss the detoxification system can be restored by priming treatment which is the initialisation of germination events by slow water uptake and a drying to its initial moisture content (Varier *et al.* 2010). The advantageous effect is due to the re-establishment of initial GSH concentration and $E_{GSSG/2GSH}$ by rapid reduction of GSSG (Kranner *et al.* 2005), the synthesis of antioxidant enzymes (Yeh & Sung 2008) and heat shock proteins which are assumed to enhance the folding of proteins and prevent the binding to damaged proteins (Lee *et al.* 1995). Reparation of DNA is mainly required for generation of error-free templates for transcription and replication and enzymes catalysing mobilization of storage reserve proteins. Comparable reactions occur during germination except for the faster water uptake which shortens the time for repair mechanism (Varier *et al.* 2010).

Simultaneously when imbibition starts also metabolic processes are initialised and mitochondria become source of ROS by producing hydrogen peroxide (H_2O_2) (Oracz *et al.* 2007). Although these ROS produce additional oxidative stress they are supposed to play a key role as messengers or transmitters of environmental cues (Mittler 2002). As signalling