

REVIEW: PART OF A SPECIAL ISSUE ON REACTIVE OXYGEN AND NITROGEN SPECIES

Seed birth to death: dual functions of reactive oxygen species in seed physiology

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- **Background** Reactive oxygen species (ROS) are considered to be detrimental to seed viability. However, recent studies have demonstrated that ROS have key roles in seed germination particularly in the release of seed dormancy and embryogenesis, as well as in protection from pathogens.
- **Scope** This review considers the functions of ROS in seed physiology. ROS are present in all cells and at all phases of the seed life cycle. ROS accumulation is important in breaking seed dormancy, and stimulating seed germination and protection from pathogens. However, excessive ROS accumulation can be detrimental. Therefore, knowledge of the mechanisms by which ROS influence seed physiology will provide insights that may not only allow the development of seed quality markers but also help us understand how dormancy can be broken in several recalcitrant species.
- **Conclusions** Reactive oxygen species have a dual role in seed physiology. Understanding the relative importance of beneficial and detrimental effects of ROS provides great scope for the improvement and maintenance of seed vigour and quality, factors that may ultimately increase crop yields.

Key words: Reactive oxygen species (ROS), seed physiology, germination, seed dormancy, signalling, embryogenesis, programmed cell death (PCD).

INTRODUCTION

Reactive oxygen species (ROS) are natural products of metabolism. They originate from the incomplete or partial reduction of oxygen, which leads to the formation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and ultimately the hydroxyl radical ($HO\cdot$). In contrast, singlet oxygen (1O_2) is produced by direct energy transfer from triplet chlorophyll to oxygen (Apel and Hurt, 2004; Ray *et al.*, 2012; Diaz-Vivancos *et al.*, 2013). It has been assumed for a long time that ROS accumulation is detrimental to seed viability because of oxidative stress imposed during seed desiccation or seed ageing (Bailly, 2004). However, recent studies on seed physiology have shown that ROS also fulfil crucial roles in cell signalling that underpins seed germination, and the breaking of seed dormancy, as well as in protection against pathogens. ROS play important roles at all stages of seed life, from germination (embryogenesis) to cell death [programmed cell death (PCD)]. Evidence from proteomics and transcriptomic studies has disproved the concept that ROS are detrimental to the seed. The dual function of ROS in facilitating cell growth and development at low concentrations while triggering cell death at higher concentrations is of key importance in seed physiology (Moller *et al.*, 2007). Reviews that have discussed the dual role of ROS in seed physiology are listed in Table 1.

Seed stages

Seeds are classified into orthodox and recalcitrant types based on their ability to germinate following storage under dry

conditions (Roberts, 1973). In orthodox seeds, major changes in moisture content and metabolism occur during the seed life cycle. For example, during embryogenesis, high metabolic activity precedes cell division. While an ample supply of water is indispensable for the translocation of metabolites to the seed during filling, extensive dehydration occurs during maturation and shedding. The low seed moisture content is associated with adaptive mechanisms that are required for seed survival. Orthodox seeds germinate upon subsequent rehydration (El-Maarouf-Bouteau and Bailly, 2008). The desiccative and hydrated stages of seed development require that ROS production and accumulation are tightly controlled to ensure seed survival. ROS act as signalling molecules that break dormancy and facilitate seed germination. Moreover, they act as important signals that trigger cellular defences against biotic and abiotic stresses. At higher concentrations they can trigger molecular genetic programs leading to cell death. Thus the regulation of ROS is indispensable for seed survival (Moradshaban *et al.*, 2013).

ROS production and signalling pathways in seeds

The production and accumulation of ROS depends on the metabolic and physiological state of the seed. In the hydrated state (that occurs following imbibition), mitochondria, glyoxysomes and plasma membrane NADPH oxidases act as major sources for ROS, whereas in dry seeds ROS production may result from Amadori and/or Maillard reactions and associated lipid peroxidation mechanisms (Zhang *et al.*, 1995; Lamb and Dixon, 1997; Mc Donald, 1999; Leubner-Metzger, 2005).

TABLE 1. Published reviews on the dual role of ROS in seed physiology

ROS molecule	Physiological trait	Reference
Hydrogen peroxide (H ₂ O ₂)	Alleviation of seed dormancy	Wang <i>et al.</i> (1995)
Hydrogen peroxide (H ₂ O ₂)	Somatic embryogenesis	Cui <i>et al.</i> (1999)
Superoxide (O ₂ ⁻)	Plant defence response	Wisniewski <i>et al.</i> (1999)
Superoxide (O ₂ ⁻)	May play roles in survival and germination of recalcitrant seeds	Roach <i>et al.</i> (2010)
Hydrogen peroxide (H ₂ O ₂)	Response to wounding	Oroczo-Cardenas <i>et al.</i> (2001)
Hydrogen peroxide (H ₂ O ₂)	Promoted seed germination by reducing endogenous ABA levels	Barba-Espin <i>et al.</i> (2010)
Hydroxyl radical (OH [·])	Breakdown of polysaccharides	Fry (1998); Schweikert <i>et al.</i> (2002)
Superoxide (O ₂ ⁻), hydroxyl radical (OH [·])	Cell growth by auxin	Schopfer and Liskay (2002)
Hydroxyl radical (OH [·])	Cell wall loosening during seed germination and seedling growth	Müller <i>et al.</i> (2009)
Hydrogen peroxide (H ₂ O ₂)	Lateral root formation	Chen <i>et al.</i> (2013)
Hydrogen peroxide (H ₂ O ₂)	Promoted seed germination by resurgence of reducing power and the pentose phosphate pathway	Barba-Espin <i>et al.</i> (2011)
Hydrogen peroxide (H ₂ O ₂)	Programmed cell death	de Jong <i>et al.</i> (2002)
Superoxide (O ₂ ⁻)	Cell death	Doke <i>et al.</i> (1994)

Unlike ·O₂ and H₂O₂, hydroxyl radicals (·OH) and singlet oxygen (¹O₂), are highly reactive and cause damage to DNA and proteins (Sharma *et al.*, 2012).

The ROS signalling pathway in seeds is not yet fully understood. An intriguing question concerns how seeds sense the ROS in the hydrated (germination) and desiccated (maturation, shedding) states. During germination, seeds imbibe water, which results in a low cytoplasmic viscosity and free water availability. Due to differences in the reactivity of different ROS, some ROS (·OH) can react only with surrounding molecules but others (H₂O₂) may move over longer distances in the cell (Moller, 2001). Some ROS forms can activate established signal transduction pathways such as mitogen-activated protein kinase (MAPK) cascades, or inhibit protein phosphatases, and activate Ca²⁺ channels or Ca²⁺-binding proteins. This interplay between redox and other signalling pathways may form the basis of coordinated regulation (Apel and Hurt, 2004; Mittler *et al.*, 2004; Brock *et al.*, 2010; Xu *et al.*, 2010; Barba-Espin *et al.*, 2011; Diaz-Vivancos *et al.*, 2013). In contrast, in the dry state, the cytoplasmic viscosity is high and the absence of free water availability may hinder the mobility of the less reactive ROS forms such as H₂O₂. Several hypotheses have been proposed to describe ROS mobility and their sensing in desiccated seeds. One hypothesis considers that desiccated or dry seeds have restricted hydrated zones, which allow a limited mobility of H₂O₂, which can participate in the cell signalling in hydrated areas. Another hypothesis suggests that H₂O₂ can accumulate during the dry stage and then, upon imbibition, this pool acts to signal changes in gene expression (El-Maarouf-Bouteau and Bailly, 2008).

Several reviews to date have concerned ROS production, signalling and physiological functions in abiotic stress responses (Gill and Tuteja, 2010) during seed germination and dormancy (Bailly, 2004; El-Maarouf-Bouteau and Bailly *et al.*, 2008; Diaz-Vivancos *et al.*, 2013). However, few comprehensive reviews have considered the dual roles of ROS in seed physiology because of the perception of ROS only in cytotoxic roles. Hence, the aim of this review is to consider the dual roles of ROS in seed physiology: (1) as positive signalling molecules in seed germination, the alleviation of seed dormancy and in pathogen-induced protection; and (2) as cytotoxic or detrimental molecules.

SIGNALLING ROLE OF ROS IN SEED PHYSIOLOGY

Seed germination

The process of seedling emergence from the seed embryonic axis is known as germination (Bewley and Black, 1994). Seed germination is a complex system regulated by extrinsic and intrinsic factors. Several reports have described ROS involvement in the early imbibition period, together with their signalling roles during seed germination. For example, H₂O₂, ·O₂ and ·OH are involved in seed germination (Puntarulo *et al.*, 1991; Caro and Puntarulo, 1999; Schopfer *et al.*, 2001). The probable site of ROS production might be either the mitochondria or the NADPH oxidases of the plasma membrane. It is proposed that the inhibition of NADPH oxidase leads to delayed germination, which indicates a putative function for the NADPH oxidases (Sarath *et al.*, 2007). It is suggested that after imbibition, the resurgence of mitochondrial respiration in the seed might result in electron donation to oxygen as an electron acceptor, leading to ROS production (El-Maarouf-Bouteau and Bailly, 2008). Accumulation of H₂O₂ may increase the rates of protein carbonylation and protein turnover, as well as decreasing the electron pressure in the mitochondrial electron transport chain, allowing the provision of reducing equivalents (NADPH) to the thioredoxin (Trx) system (via the pentose phosphate pathway), which is involved in the regulation of seed germination and seedling development (Lozano *et al.*, 1996; Job *et al.*, 2005; Oracz *et al.*, 2007; Barba-Espin *et al.*, 2011). In addition, H₂O₂ accumulation may influence the hormone balance by increasing gibberellins (GAs) and decreasing abscisic acid (ABA) and ethylene via 1-aminocyclopropane-1-carboxylic acid (ACC). This re-modelling of hormone signalling may lead to the commencement of metabolic activity that is essential for seed germination and seedling emergence (Barba-Espin *et al.*, 2010, 2011; Bahin *et al.*, 2011; Diaz-Vivancos *et al.*, 2013). A schematic model of the signalling role of H₂O₂ during seed germination is provided in Fig. 1.

Endosperm weakening

In some seed species (arabidopsis, tobacco), seed germination is hindered by the micropylar endosperm. In order to

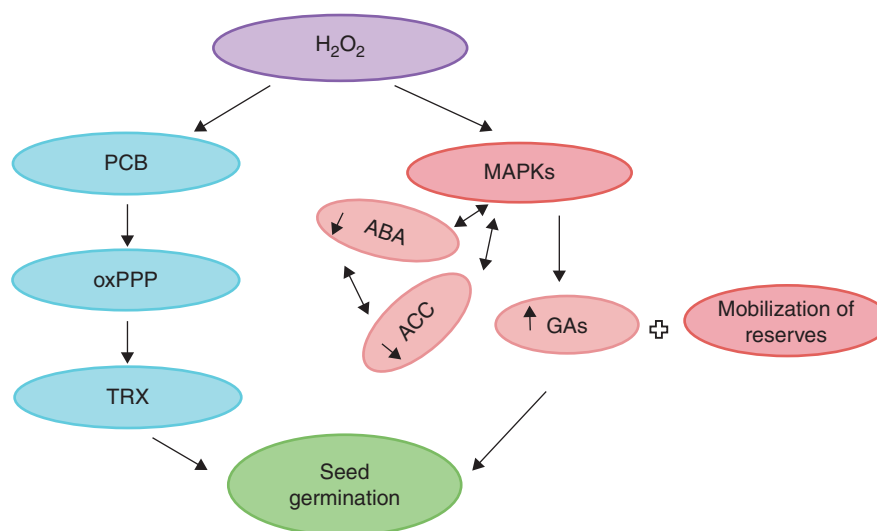


FIG. 1. Signalling role of H_2O_2 in the seed germination process. During the process of germination, H_2O_2 triggers seed storage protein carbonylation (PCB) that induces the oxidative pentose phosphate pathway (oxPPP) which supplements NADPH to thioredoxin (TRX) for seed germination. Furthermore, it stimulates an increase in GAs and a decrease of abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) through mitogen-activated protein kinase (MAPK) (Lozano *et al.*, 1996; Job *et al.*, 2005; Oracz *et al.*, 2007; Barba-Espin *et al.*, 2010, 2011; Bahin *et al.*, 2011; Diaz-Vivancos *et al.*, 2013). Upon mobilization of reserves, seed germination takes place with resurgence of metabolism. For dormancy alleviation, the ABA content should be high to maintain the dormant state after fruit ripening and, in the non-dormant state, GAs could be high, leading to germination (Liu *et al.*, 2010).

germinate, the mechanical barrier imposed by the endosperm has to be weakened. Plant growth regulators such as GA_3 and ABA regulate hydrolases that in turn regulate endosperm loosening. Several reports have shown that ROS are involved in endosperm loosening (Müller *et al.*, 2009; Barba-Espin *et al.*, 2011). It is suggested that $\cdot OH$ is generated from $\cdot O_2$ produced by NADPH oxidases, and H_2O_2 in Fenton reactions in the cell walls of growing organs. Since cell walls have little or no antioxidant buffering capacity, $\cdot OH$ produced by Fenton chemistry can break polysaccharides by oxidative scission, resulting in weakening of the endosperm. In addition, it is probable that the physiological role of auxin in promoting cell division and growth) is highly dependent on its ability to promote the generation of $\cdot O_2$ radicals (Kawano, 2003).

Seed protection from pathogens

Several studies have demonstrated that ROS play key signalling roles in abiotic and biotic stresses (Kotchoni *et al.*, 2006; Gomes and Garcia, 2013). During their development and lifespan, seeds may be exposed to biotic stress. ROS accumulation would safeguard seeds from pathogen attack. It is well known that ROS may directly affect pathogens or act indirectly by prompting the hypersensitive reaction leading to PCD (Grant and Loake, 2000). During infection, $\cdot O_2$ produced by the activation of plasma membrane NADPH oxidase is converted to H_2O_2 either by spontaneous dismutation or by superoxide dismutase (SOD) activity (Dietz *et al.*, 2010). The H_2O_2 produced by this process kills the local infected cells and thus inhibits pathogen entry. In addition, it has a substantial role in resisting the cell infection, implicating a substrate role in cell wall peroxidation (Levine *et al.*, 1994). Moreover, it has been demonstrated that supply of ROS from germinated seeds has an inhibitory role against pathogens (Murphy *et al.*, 1998). In order

to validate the hypothesis of pathogen-induced protection through ROS, much more research is needed.

Alleviation of seed dormancy

Seed dormancy is a phenomenon which delays germination even under favourable conditions. Seed dormancy can be imposed either by the seed coat or by any surrounding structures, whereas in embryo dormancy the embryo is inherent and devoid of any influence of the surrounding structures. Seed dormancy is regulated mostly by ABA and GAs, and the alleviation of dormancy results from the alteration of the balance of their ratio. For example, it is suggested that a high ABA/GA ratio culminates in dormancy maintenance whereas a low ABA/GA ratio leads to dormancy release. In other words, GAs favour dormancy release but ABA maintains seed dormancy. In a recent study, where H_2O_2 was added exogenously, it was observed that the ABA levels were decreased with a subsequent increase of GAs, that triggers the release of seed dormancy (Liu *et al.*, 2010). Recent studies further substantiate that the cross-talk of ROS with GAs, ethylene and ABA demonstrates the pivotal role of ROS in alleviation of seed dormancy (Finkelstein *et al.*, 2008). For instance, it has been demonstrated that the interaction of ROS and ABA or GAs alleviated seed dormancy of barley (Bahin *et al.*, 2011). The probable mechanism of ROS in this process might involve oxidation of reserve proteins during after-ripening, showing that seed dormancy accompanies protein mobilization. To corroborate this hypothesis, dormant sunflower seeds were imbibed and treated with hydrogen cyanide (HCN) which is a dormancy-ameliorating compound. Interestingly, H_2O_2 was produced in response to cyanide, which triggered the ethylene pathway and protein carbonylation (Oracz *et al.*, 2009). In contrast, a ROS-generating compound, such as methylviologen, activated protein

carbonylation with concomitant seed dormancy alleviation (Bailey *et al.*, 2008). These studies corroborate the putative role of ROS and protein oxidation in seed dormancy alleviation which needs further validation with other dormant seed species.

SEED DETERIORATION

Possible routes of seed deterioration during the ageing process

To obtain a higher yield and productivity, viable seed is indispensable. Conventionally, stored seed has been used to produce crops, but improper storage leads to poor germination and loss of seed viability. Seed longevity is a paramount trait playing a significant role in conserving the germplasm (*ex situ*). Seed ageing, occurring either by natural or accelerated ageing (which can be achieved by increasing the relative humidity and moisture content), has been studied by several researchers. Studies revealed that the ageing process resulted in deterioration of seed viability mainly due to the production and progressive accumulation of ROS. In order to maintain seed viability, regulation of ROS by enzymatic and non-enzymatic (antioxidant activities) and DNA repair mechanisms should be at an appropriate level in the embryo (Dona *et al.*, 2013).

Generally, it is acknowledged that seeds stored for a longer duration are prone to oxidative damage due to the cumulative increase of ROS. ROS act as a messenger at lower concentrations, and a threshold increase will damage the polyunsaturated fatty acids of phospholipids, DNA and proteins. Subsequently, loss of seed viability and reduced seed germination are the repercussions of ageing (Kranner and Colville, 2010). Hence, an important observation is that under stress conditions ROS were generated in the cell, but at the same time the cellular machinery combats the ROS by enzymatic and non-enzymatic mechanisms (antioxidants) to circumvent the problem. Therefore, a pre-requisite event for seed deterioration under ageing is generation of ROS coupled with decline of the antioxidant potential of the cell (He and Kermodé, 2010; Kibinza *et al.*, 2011). For instance, ROS formed during ageing were scavenged by glutathione *S*-hydrolase (GSH) which leads to an increase in $E_{GSSG/2GSH}$. This, in turn, splits Trx (*TRX-H;3*) from apoptosis signal-regulating kinase 1 (ASK 1). ASK 1 activates a MAPK kinase cascade that results in gene expression changes which could lead to nucleic acid damage and breakdown of structural and nuclear proteins (Chen *et al.*, 2013).

*Programmed cell death (PCD) by cytochrome *c* (apoptogenic proteins)*

Another possible mechanism for PCD is through calcium-mediated release of cytochrome *c* (apoptogenic proteins) from the mitochondria. Under the seed ageing process, calcium levels in the cytosol increase simultaneously with the release of cytochrome *c* by activation of the permeability transition pore (PTP) through the adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC). The released cytochrome *c* activates the caspases and downstream proteolytic cascade involved in PCD. Metacaspases of plants share similar structure and sequence with animal caspases but the latter are devoid of caspase-like activity. Cysteine proteases such as RD21 and papain are the main proteases activated in the

seed ageing process whose function is to degrade the nuclear and structural proteins (Lam and Pozo, 2000). In addition, the proteasome also possesses caspase-like activity. For example, the proteasome may activate the caspase cascade for the induction of PCD and degrades the misfolded proteins (ER stress) by the ubiquitin–proteasome pathway (Chen *et al.*, 2013).

An important event involved in the seed ageing phenomenon consists of endoplasmic reticulum (ER) stress. Oxidative stress perturbs the protein folding environment in the lumen of the ER, which results in accumulation of misfolded and unfolded proteins. Furthermore, the findings were corroborated by the upregulation of binding protein (BiP) which is a chaperone whose main function is to restore the misfolded and unfolded proteins (Adamakis *et al.*, 2011). Further studies have revealed the involvement of ER stress, PCDh (DNA laddering indicative of PCD) and the ubiquitin–proteasome pathways in the seed ageing process.

On the other hand, plants possess combating enzymatic and non-enzymatic (antioxidants) mechanisms to ameliorate the oxidative damage caused by increasing ROS concentrations. The ROS act as signalling molecules in response to pathogen attack, stomatal opening and PCD (Apel and Hurt, 2004). From recent gene profiling studies, it is reasoned that DNA repair mechanisms are activated at an early stage of seed imbibition (Macovei *et al.*, 2010; Balestrazzi *et al.*, 2012). Hence, the paramount feature is to withstand stress by expressing the ROS scavenging potential, concomitantly increasing seed viability and germination. Therefore, it is generally hypothesized that cells possess antioxidant systems to combat ROS attack, but oxidative injury takes place when ROS production exceeds the cell antioxidant machinery (Liu *et al.*, 2007).

CONCLUSIONS

Seeds are essential for sustainable crop production. It is widely believed that stored seeds are prone to oxidative damage because of ROS accumulation, which results in poor germination and loss of seed viability. Traditionally, farmers particularly in poor countries store seed in order to raise further generations. The accumulation of oxidative damage during storage has long been considered to be a major cause of decreased seed quality. However, a greater understanding of the functions of ROS in the processes that determine seed longevity and seed ageing is required because recent studies have shown that seed deterioration may be caused by pathways other than the accumulation of oxidative damage that lead to PCD. Conversely, the beneficial effects of ROS have generated increasing interest because of their functions in the regulation of the cellular redox state, and their putative roles in the control of seed dormancy, seed germination, and in mediating phytohormone cross-talk, particularly between GAs and ABA. It is clear that the presumption that ‘ROS are detrimental’ has to be revisited and needs further substantiation.

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