Environmental and Experimental Botany 156 (2018) 8–15

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Changes in tocochromanols and glutathione reveal differences in the mechanisms of seed ageing under seedbank conditions and controlled deterioration in barley

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ARTICLE INFO

Keywords:
antioxidant
seedbank
lipid peroxidation
oxygen
ROS
vitamin E

ABSTRACT

"Orthodox" seeds are desiccation tolerant, enabling dry storage for agricultural and conservational purposes. Protocols of "controlled deterioration" (CD) using elevated temperatures and seed water contents (WCs) are frequently used to study mechanisms of seed ageing. However, the relevance of using CD to understand how seeds age at lower water contents and temperatures has been disputed. Here, comparisons in antioxidant changes are drawn between barley seed stored for up to 15 years in a seedbank with seeds aged using CD (45 °C, 75% RH; WC = 13% FW) under normoxia (21%) or elevated (75%) O_2. Regardless of ageing method, glutathione (GSH) levels decreased concomitantly with an oxidative shift in the glutathione redox state ($E_{GSSG/2GSH}$) as viability was lost. An earlier oxidative shift in $E_{GSSG/2GSH}$ and more rapid loss of seed vigour was found in seeds aged by CD under 75% O_2, compared to 21% O_2, but longevity was not shortened by elevated O_2. Seedbank-aged seeds contained more glutathione disulphide (GSSG) levels, leaked more electrolytes upon imbibition and contained less tocochromanols than non-stored seeds. In contrast, in response to CD, levels of GSSG fell, electrolyte leakage did not increase and tocochromanol levels increased (21% O_2), or remained stable (75% O_2). In a further set of 24 barley genotypes stored for up to 15 years at 20 °C (WC = 11% FW) and 0 °C (WC = 8% FW), tocochromanol levels positively (r = 0.42) correlated with total germination, which ranged between 1-78% (20 °C) and 65-97% (0 °C). Of all tocochromanol species, α-tocopherol correlated most with total germination (r = 0.49) and γ-tocotrienol least (r = 0.08). It is concluded that CD is not a perfect model for understanding mechanisms of ageing that occur under seedbank conditions.

1. Introduction

Seed ageing comprises various molecular processes that result in damage to seed macromolecules, affecting germinability and seed vigour. Seed deterioration is governed by storage temperature, seed water content (WC) and can also be influenced by O_2 contents in the storage environment (Groot et al., 2014; Harrington, 1963; Ibrahim et al., 1983; Roberts, 1973). At room temperature, the aqueous domain of the seed becomes 'glassy' when seed WC decreases below a certain threshold, typically 0.1 g H_2O g^{-1} dry mass, restricting metabolism (Buitink et al., 1998; Fernandez-Marin et al., 2013) and extending seed longevity (Vertucci, 1989; Walters et al., 2005). Glass formation and weakening are also temperature dependent. Protocols of "artificial ageing" are frequently applied by seed scientists and industry to assess seed longevity upon storage. Two commonly used methods of artificial ageing are "controlled deterioration" (CD) and "accelerated ageing". CD involves seed exposure to elevated temperatures (e.g. 30 to 45 °C) and relative humidities (RH), such as 60–75%, that achieve seed WCs that

Abbreviations: CD, controlled deterioration; DW, dry weight; EPPO, elevated partial pressure of oxygen; $E_{GSSG/2GSH}$, glutathione half-cell reduction potential; FW, fresh weight; GSH, glutathione; GSSG, glutathione disulphide; HGGT, homogentisate geranylgeranyl transferase; HPT1, homogentisate phytyltransferase; LMW, low-molecular-weight; mBBr, monobromobimane; PSSG, protein-bound glutathione; PUFA, polyunsaturated fatty acid; r, Pearson's correlation coefficient; R^2, coefficient of determination; RH, relative humidity; ROS, reactive oxygen species; TG, total germination; VTE1, tocopherol cyclase; VTE2, vitamin E2; VT3, vitamin E3 or methyl trasferase; WC, water content

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https://doi.org/10.1016/j.envexpbot.2018.08.027
Received 12 July 2018; Received in revised form 22 August 2018; Accepted 22 August 2018
Available online 23 August 2018

do not change during CD, and is often used as a proxy for seedbank ageing. Accelerated ageing involves treatment of dry seeds with high RH (close to 100%) and temperatures (such as 45 °C), and seed WCs change dramatically during the treatment, which is often used to quickly (days to weeks) produce seed lots with low viability. Although it has been established that seed WC and temperature affect rates of seed ageing, the precise underlying biochemical processes of seed deterioration under various conditions are yet to be resolved. In addition, it is believed that reactive oxygen species (ROS) are involved in seed deterioration upon ageing (Bailly et al., 2008; Kranner et al., 2010), but how storage conditions influence the susceptibility of the hydrophilic and hydrophobic (i.e. membranes / storage lipids) domains of seeds to ROS is unclear.

In 15 year-stored wheat seeds, several hundred different oxidised lipid-associated species were identified using high resolution LC-MS/MS (Riewe et al., 2017). The accumulation of oxygenated triacylglycerols upon ageing in Arabidopsis seeds was shown to be mainly based on non-enzymatic oxidation of seed storage lipids (Oenel et al., 2017). Non-enzymatic oxidation can be initiated by free radical attack of a polyunsaturated fatty acid (PUFA), which readily reacts with O_2, forming a peroxyl radical that can attack another PUFA, leading to a self-propagating process known as lipid peroxidation (McDonald, 1999). Peroxyl radicals can be scavenged by tocochromanols, the collective name for lipid-soluble tocopherols, tocotrienols and tocomonoenols, which are structurally distinguished by the presence of three trans-double bonds in the hydrocarbon tail, forming four isomers, α, β, γ and δ (Munné-Bosch and Alegre, 2002). Tocochromanol synthesis is shared by plastoglobules and the chloroplast envelope, with leaf plastids predominantly storing α-tocopherol (DellaPenna and Pogson, 2006; Esteban et al., 2009; Vidi et al., 2006). γ-Tocopherol has been reported to be the dominant tocopherol in the seeds of many eudicot species (Falk and Munné-Bosch, 2010; Fernández-Marín et al., 2017) alongside a small fraction (typically < 2.5%) of tocomonoenols (Pellaud et al., 2018). Tocotrienols have been almost exclusively found in seeds, particularly in the endosperm of caryopses (Falk et al., 2004; Horvath et al., 2006). Tocochromanol synthesis involves homogentisate phytyltransferase (HPT1 or VTE2), tocopherol cyclase (VTE1), methyl transferase (VTE3) and homogentisate geranylgeranyl transferase (HGGT) (Dormann, 2007). In most eudicot seeds and monocot embryos α- or γ-tocopherol predominate (Falk and Munné-Bosch, 2010; Matthaus et al., 2016), whereas tocotrienols accumulate in the seed endosperm of monocots (Horvath et al., 2006; Moreau et al., 2007).

Seeds of *vte1* Arabidopsis and *hpt* or *hggt*-knock-out tomato mutants had reduced longevity and suffered more lipid peroxidation than wild-type seeds (Chen et al., 2016; Mène-Saffrané et al., 2010; Sattler et al., 2004). A rice mutant over-expressing the *OsVTE2* gene also had improved seed longevity (Hwang et al., 2014), supporting a role for tocochromanols in seed longevity. However, a decrease in tocochromanols does not always occur during seed ageing (Lee et al., 2017; Morscher et al., 2015; Nagel et al., 2015; Priestley et al., 1980; Seal et al., 2010b). Electrolyte leakage from imbibing seeds, and the consequential increase in leachate conductivity, is used as an indicator of seed quality (ISTA, 2010), whereby aged seeds leak more electrolytes due to damaged membranes. Since tocochromanols are involved in protecting membranes from lipid peroxidation, electrolyte leakage could be associated to ageing-induced losses of tocochromanols.

Tocochromanol levels can influence levels of glutathione (GSH), a water soluble low-molecular-weight (LMW) thiol-based antioxidant. The tocopherol-deficient *vte1* Arabidopsis mutant produces more GSH in its leaves, whereas GSH levels are decreased in *vte1*-overexpressing plants (Kanwischer et al., 2005). GSH is highly abundant in all seeds and can scavenge ROS directly or via electron donation to ROS-detoxifying enzymes, such as glutathione-*S*-transferases. Thiols are prone to oxidation, ultimately leading to post-translational modifications, such as disulphide bond formation (i.e. glutathione disulphide; GSSG) or glutathionylation of protein thiol groups (PSSG). Glutathionylated proteins are protected from irreversible oxidation, and both GSSG and PSSG contents increase during seed maturation drying, and may further accumulate during seed ageing (Kranner and Grill, 1996). Glutathione is a major cellular redox buffer, in plants and animal cells alike, and the GSH/GSSG redox state can be quantified via the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) (Kranner et al., 2006; Schafer and Buettner, 2001), which has been used as a marker of seed viability (Birtic et al., 2011; Kranner et al., 2006; Morscher et al., 2015; Nagel et al., 2015; Roach et al., 2010; Seal et al., 2010a).

Barley (*Hordeum vulgare* L.) is an important crop used for food, feed, beer and whisky production, and has a fully sequenced genome (Mascher et al., 2017). Much attention is given to the prevention of unwanted lipid oxidations in barley and other grain products during processing, which lead to rancidity and undesired flavours. Moreover, α-tocopherol, referred to as vitamin E in the diet, is a commonly added food preservative (E307) to prevent rancidity. To illuminate aspects of different seed deterioration mechanisms imposed by the storage environment, we investigated the relationship between individual tocochromanol species, GSH levels and $E_{GSSG/2GSH}$ with viability of barley seed aged at different rates and methods.

2. Material and Methods

2.1. Seed material, ageing and germination conditions, and conductivity measurements

For comparing ambient storage with CD, seeds of the six-row winter barley HOR 11311 were harvested in 1) 1999 and 2) 2010 and stored under ambient conditions (50.5 ± 6.3% RH, 20.3 ± 2.3 °C) until 2014 and 3) harvested in 2016 and stored for 2 months under ambient conditions. For CD, seeds harvested in 2010 were aged by CD in 2014, identically to Morscher et al. (2015) in a hermetically sealed box (28 × 28 x 12 cm) at 45 °C and at 75% RH, as checked with a RH meter (HC2-AW-USB, Rotronic, Switzerland), after equilibration to a WC of 13.1% ± 0.5% fresh weight (FW), using non-saturated LiCl salt solution. Seeds were aged for 0, 6, 9 and 14 d under ambient O_2 atmosphere (21%) or elevated O_2 (75%). At each ageing interval, all boxes (one for each replicate) were opened to allow seed removal and gas exchange. Before returning to CD conditions, the O_2 of half the boxes were adjusted with pure O_2 gas to 75%, as measured with a fibre-optic optode (Fibox 3, PreSens, Regensburg, Germany) using temperature compensation. For each ageing interval, each replicate (n = 3) consisted of 20 seeds for germination and 20 seeds for biochemical analyses. Total germination (TG) was carried out on Whatman® seed testing paper (Grade 3644) moistened with 40 mL distilled H_2O in a sealed box 14 × 20 x 3 cm, and kept at 20 °C. Electrolyte leakage was measured by imbibing surface-rinsed desiccated seeds in 2 mL of H_2O for 0.5 h (n = 5 replicates of 6 seeds) and measuring the conductivity of the leachate with a Cond 330i (WTW Xylem Analytics, Weilheim, Germany).

Furthermore, seeds from 24 genotypes were selected according to their variable longevity after ambient storage. To avoid effects of row-type, annuity, country of origin and growth environment, two-row [*H. vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef., 17 genotypes] and six-row (*H. vulgare* L. convar. *vulgare* var. *hybernum* Viborg, seven genotypes) spring and winter barleys from 16 countries were multiplied at the IPK Gatersleben gene bank, Germany, between 1995 and 2003 (Supplemental Table S1). Freshly harvested seeds were desiccated to a WC of 7.7 ± 0.3% FW and were either stored in cold storage (0 ± 1 °C) or in paper bags at ambient storage (50.5 ± 6.3% RH, 20.3 ± 2.3 °C) and equilibrated to a WC of 10.9 ± 0.5% FW until 2009, when they were tested for TG on moistened filter paper at 25:20 °C (14:10 h, day:night), or transferred to -20 °C for use in biochemical analyses. Genotypes were regenerated again in 2008, stored under ambient conditions for 12 months (to break dormancy), before testing TG or storage at -20 °C. Seeds of each genotype were collected