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Age-dependent loss of seed viability is associated with increased lipid oxidation and hydrolysis

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Abstract

The accumulation of reactive oxygen species has been associated with a loss of seed viability. Therefore, we have investigated the germination ability of a range of seed stocks, including two wheat collections and one barley collection that had been dry-aged for 5–40 years. Metabolite profiling analysis revealed that the accumulation of glycerol was negatively correlated with the ability to germinate in all seed sets. Furthermore, lipid degradation products such as glycerol phosphates and galactose were accumulated in some seed sets. A quantitative analysis of nonoxidized and oxidized lipids was performed in the wheat seed set that showed the greatest variation in germination. This analysis revealed that the levels of fully acylated and nonoxidized storage lipids like triacylglycerols and structural lipids like phospho- and galactolipids were decreasing. Moreover, the abundance of oxidized variants and hydrolysed products such as mono-/diacylglycerols, lysophospholipids, and fatty acids accumulated as viability decreased. The proportional formation of oxidized and nonoxidized fatty acids provides evidence for an enzymatic hydrolysis of specifically oxidized lipids in dry seeds. The results link reactive oxygen species with lipid oxidation, structural damage, and death in long-term aged seeds.

KEYWORDS

ageing, germination, hydrolysis, lipid, oxidation, seed, seed longevity, storage, *Triticum aestivum* (wheat), viability

1 | INTRODUCTION

Ageing is the process of becoming older and, unless immortal, leads to the death of an organism. The life span of plants in the form of dried seeds is highest among beings without clonal reproduction cycle. For example, seeds aged 1,300 (Shen-Miller, Mudgett, Schopf, Clarke, & Berger, 1995) and 2,000 years (Sallon et al., 2008) were still able to germinate. The placental tissue of 32,000-year-old seeds found below the permafrost was still viable and formed plants (Yashina et al., 2012).

Though seeds are a resilient form of life, they cannot completely defy ageing. Seed viability and longevity are affected by interspecific and intraspecific genetic variation (Bentsink et al., 2000; Nagel et al., 2016; Probert, Daws, & Hay, 2009; Sasaki et al., 2015; Christina Walters, Wheeler, & Grotenhuis, 2005) and environmental influences during seed development and storage (Ellis, Osei-Bonsu, & Roberts, 1982; Roberts, 1961). Survival during long-term storage of dry seeds is important for the conservation of valuable genetic resources stored in gene bank collections (Li & Pritchard, 2009). Furthermore, the

frequency and uniformity of seed germination are important determinants of crop yields and quality and, thus, are of high-economic relevance. High temperature, high humidity (Harrington, 1963; Roberts, 1960), high oxygen vapour pressure (Groot, Surki, de Vos, & Kodde, 2012), and long storage intervals (Steiner & Ruckenbauer, 1995; Telewski & Zeevaart, 2002) are known to reduce the germination of orthodox (desiccation tolerant) seeds.

Seeds exposed to artificial ageing regimes (typically high temperature and humidity for a short period) produce reactive oxygen species (ROS), malondialdehyde, and 4-hydroxynonenal, the latter products of lipid oxidation, as shown for Arabidopsis, rice, or legumes (Chen et al., 2016a; Colville et al., 2012; Petla et al., 2016). Yet biochemical changes in response to these artificial conditions may not reflect the situation in naturally or long-term dry aged seeds (Galleschi et al., 2002; Roach, Nagel, Börner, Eberle, & Kranner, 2018). Oenel et al. (2017) have found that singly oxidized triacylglycerols and fatty acids (FAs) were elevated in two long-term aged Arabidopsis seed batches whose germinability was lower than the control seeds. Oxidized lipids from other major lipid classes were not reported in this study. However, hundreds of different singly and multiply oxidized lipids of various classes are formed in long-term stored wheat seeds (Riewe, Wiebach, & Altmann, 2017).

To investigate the occurrence of lipid oxidation and degradation during long-term dry storage (seed water content <10%) and its relation to seed viability, we analysed 90 differently aged wheat seed stocks that vary in genotype, storage period, and storage conditions. Central metabolites (e.g., sugars, amino acids, organic acids and other polar intermediates from essential plant metabolic pathways) and lipids correlated with germinability were identified using gas chromatography-mass spectrometry (GC-MS) and a recently developed liquid chromatography-mass spectrometry (LC-MS) method for the large-scale quantitative detection of oxidized and nonoxidized lipids (Riewe et al., 2017). We have found that germination frequencies were positively correlated with concentrations of nonoxidized lipids, such as triacylglycerols (TAGs), phospho-, and galactolipids, and were negatively correlated with oxidized lipids and lipid hydrolysis products such as oxidized diacylglycerols (DAGs), lysophospholipids (lysoPLs), FAs, and glycerol. These results suggest that the occurrence of lipid oxidation triggers the hydrolysis of storage and structural lipids and results in the loss of seed viability. Moreover, the GC-MS analyses of further sets of long-term cold stored wheat (176 genotypes) and barley (170 genotypes) seed stocks confirmed the aforementioned observations.

2 | MATERIAL AND METHODS

2.1 | Seed storage conditions

Seeds (Table S1) were obtained from plants grown in the field in Gatersleben, Germany, and stored in the Federal *Ex situ* Gene Bank, according to contemporary Genebank standards (FAO, 2014). For collection "wheat 1," seeds were harvested in 1998, 2000–2006, and 2008. Every year, five wheat accessions were harvested and stored. The seeds were dried at 20°C/20% relative humidity (RH) for 4 months and then threshed, cleaned, and further dried at 20°C/13% RH for 2 weeks. After drying, the moisture content was approximately 6%. Then, stocks were split: One batch of seeds was transferred into glass jars covered with silica gel and stored at 0°C until 2008 and then at −18°C thereafter. Following the cold storage, the equilibrate relative humidity was 17.4%, which corresponds to a moisture content of approximately 6%. The other batch was stored in paper bags at ambient conditions (20.3 ± 1.3°C/50.5 ± 6.3% RH), corresponding to moisture contents of around 10%, according to the Kew Seed Information Database (Royal Botanic Gardens Kew, 2019). In collections "wheat 2" and "barley," the seeds of 176 wheat and 170 barley accessions were harvested in 1974 and then threshed and dried in the same manner as the wheat 1 set. Seeds were initially stored in paper bags at ambient conditions. In 1978, the seeds were transferred to glass jars with silica gel and kept at 0°C, and from 2008 onwards, they were kept at −18°C. In 2013, aliquots of wheat 1 seeds were subjected to germination assays, and other aliquots were shock frozen for metabolite analysis. In 2014, aliquots of wheat 2 and barley seeds were subjected to the same procedure.

2.2 | Germination assay

Four replicates of 50 seeds were placed on moistened filter paper and kept in an incubator at 20°C, 60% RH for 8 hr of light. Physiological germination was assessed as radicle emergence of at least 2 mm. In accordance with the International Rules for Seed Testing (ISTA, 2013), the percentage of germination was recorded for wheat after 8 days and for barley after 7 days.

2.3 | GC-MS analysis

Central metabolites were extracted and analysed as described previously (Riewe et al., 2012; Riewe et al., 2016). Fifty deep frozen seeds per stock were pooled in scintillation vials containing three steel balls with 8 mm diameters and ground in a ball mill (5 × 2 min at 50 Hz at −70°C) using a cryogenic robot (Labman, North Yorkshire, UK). Fifteen milligrams deep frozen material was extracted with 1 ml ice-cold methanol: chloroform: water (2.5:1:1, containing 2 µg internal standard) to inhibit enzymatic conversion of metabolites. After an addition of 400 µl water and centrifugation, 100 µl of the polar phase were dried and stored under Ar. The dried metabolites were in-line derivatized and analysed using an Agilent 7890 gas chromatograph (Agilent, Santa Clara, CA, USA) coupled to a Pegasus HT mass spectrometer (LECO, St. Joseph, MI, USA). The data were normalized using the internal standard L-[2,3,3,3-d_4] alanine (www.sigmaaldrich.com) and exact weight (Table S2).

2.4 | LC-MS analysis

The LC-MS analysis of the wheat 1 set was described in detail recently (Riewe et al., 2017). Twenty-five milligrams of homogenized deep frozen seed material was extracted with 750 µl ice-cold methanol:methyl *tert*-butyl ether:water (1:3:0.5) to inhibit the enzymatic conversion of lipids. After an addition of 487.5 µl methanol: water (1:3) and

centrifugation, 80 µl of the apolar phase were dried and stored under Ar. The samples were resolubilized prior to analysis in 50 µl acetonitrile: isopropanol (7:3). All 90 samples were quantitatively analysed in positive and negative modes using high-resolution LC-MS and were structurally analysed in the positive mode using LC-MS/MS. The LC-MS spectra were deconvoluted to identify pseudospectra/base peaks of nonredundant analytes (Kuhl, Tautenhahn, Bottcher, Larson, & Neumann, 2012). In the positive mode, oxidized lipids were annotated by accurate mass (1.5 ppm tolerance), and lipids of major classes were cross-validated by MS/MS spectra. In total, 624 annotations with this level of confidence were made for nonoxidized and oxidized TAGs, DAGs, monoacylglycerols (MAGs), phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylglycerols (PGs), phosphatidylinositols, lysoPCs, lysoPEs, lysoPGs, lysophosphatidylinositols, monogalactosyldiacylglycerols, and digalactosyl-diacylglycerols (DGDGs). In the positive mode data, 154 (oxidized) FAs were annotated based on accurate mass (1.5 ppm) and filtering procedures (Riewe et al., 2017). Six (oxidized) phosphatidic acids (PAs) and four lysophosphatidic acids were quantified in the negative mode as [M-2H + NH_4] adduct with 5 ppm tolerance if they were detected in the positive and negative modes ("Bothmode," "Adduct," and "Retention filter" = TRUE, Table S3).

2.5 | Statistical information

Data processing and analysis were performed using RStudio (R Development Core Team, 2018; RStudio Team, 2016). All *P* values were false discovery rate (FDR) adjusted according to Benjamini and Hochberg (1995) and referred to as FDR. Significant means FDR < 0.05.

2.6 | Accession numbers

Accession numbers of the 436 wheat and barley accessions are provided in the first column ("ACCENUMB") of Table S1. Of the 436 accessions, 416 are currently available at the IPK gene bank (http://www.ipk-gatersleben.de/en/gbisipk-gaterslebendegbis-i/informationordering/).

3 | RESULTS

3.1 | Germination variation in a genetically diverse wheat population after long-term dry storage at different conditions

To study molecular processes associated with seed deterioration, we compiled long-term stored seed stocks from globally collected wheat accessions with large variations in germination. The set comprised 45 wheat genotypes stored at ambient or cold storage conditions for 5–15 years and formed 90 individual seed stocks (wheat 1, Table S1). The range of germination was 0–99%, and more stocks had a germination over 50% (Figure 1a). Germination was typically more varied between seeds from the same genotype that were harvested in the same year but stored at different temperatures than those within different genotypes harvested in the same year and stored at the same temperature (Table S2). This finding suggests that the storage environment has a large effect on seed viability. The observation that seeds harvested in 2008 display a lower germination than expected indicates that seed production, not storage, may have additionally lowered germination in this case.

3.2 | Glycerol accumulates in seeds with lower germinability

Central metabolism is hub, donor, and acceptor of matter for all specialized pathways, such as cell wall synthesis and specialized or lipid metabolism (Buchanan, Gruissem, & Jones, 2000). Changes in distant pathways with effects on seed viability are likely to be reflected in central metabolism as well. In wheat 1 seeds using GC-MS, 89 known and 277 unknown metabolites were quantified (Table S2). We identified 7 and 67 metabolites with positive or negative correlations to germination (Figure 1b), ranging from R = −.82 to.55. All correlation coefficients of known and unknown central metabolites are provided in Table S2. The top 12 negatively correlated metabolites of known chemical identity are displayed in Table 1. The three most strongly correlated metabolites are glycerol (R = −.82, Figure 1c), glycerol-3-phosphate (R = −.80), and glycerol-2-phosphate (R = −.78), and they are not topped by other compounds of unknown chemical structure. The only significantly positively correlated identified metabolite was glutamine, and this correlation was weak (R = .30, FDR = 0.023).

To validate the negative correlation between glycerol content and germination, we investigated another set of 176 wheat accessions (wheat 2) stored in the cold from 1974–2014 (Table S1). Although the seeds were older than those of wheat 1 set, 85% had a germination higher than 50% (Table S2). Furthermore, 85 known and 254 unknown metabolites were determined. Similar to wheat 1 set, more metabolites were negatively correlated to germination (83) than positively correlated to germination (23). Glycerol was ranked fourth among known germination-correlated metabolites (R = −.58, Table 3), again supporting a close link to seed longevity and the deterioration processes that occur during long-term dry storage.

3.3 | Wheat and barley show similar germination-associated central metabolite profiles

Barley seeds are similar in structure and composition to wheat seeds, and their ageing may lead to similar metabolic changes. To test this hypothesis, we assayed seed germination and central metabolites in a set of 170 barley accessions stored at cold storage for 40 years (Table S1) by following the same process conducted for wheat 2. In this case, 85 known and 253 unknown metabolites were quantified, and the correlations of their concentrations with germination were calculated (Table S2). As observed in the wheat 2 set, germination was over 50% for more than 85% of all accessions. Again, more negative (141) than positive (16) metabolite-to-germination correlations were detected. Among all detected known metabolites, glycerol was the second strongest germination-correlated metabolite (R = −.57,

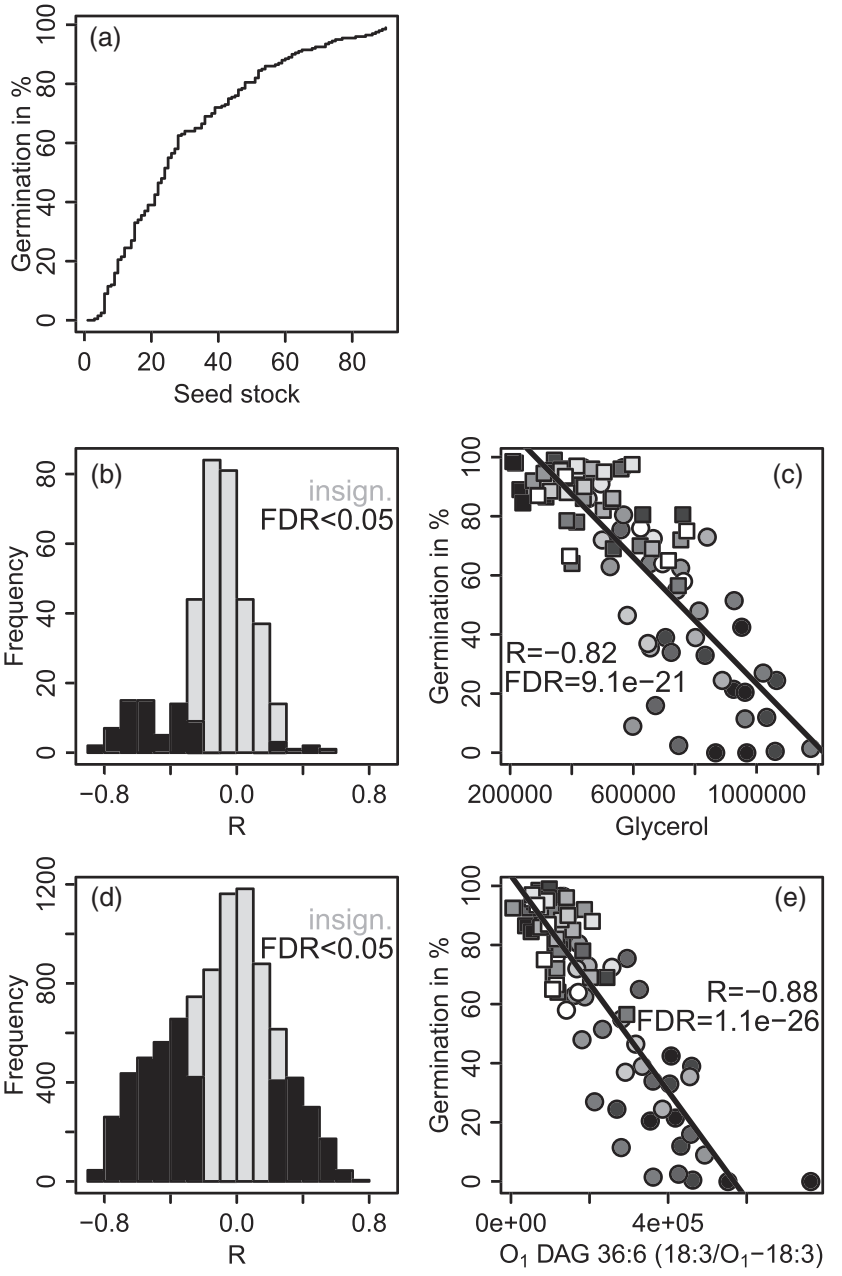

FIGURE 1 Correlation between germination and central/lipid metabolites in aged wheat seeds (wheat 1). (a) Germination frequency (ascending from left to right). (b) Frequency of measure of central metabolite-to-germination correlations; 67 negative, 292 insignificant (grey), and seven positive correlations (FDR < 0.05). (c) Glycerol-to-germination correlation (darkest symbols = 15 years of storage, lightest symbols = 5 years of storage). (d) Frequency of measure of lipid-to-germination correlations, 2,878 negative, 4,611 insignificant (grey), and 1,344 positive correlations (FDR < 0.05). (e) Negative correlation of O_1 DAG 36:6 (FA composition = 18:3/O_1-18:3, Formula ID = 7379) to germination. Glycerol and O_1 DAG 36:6 levels are displayed in mass spectral ion count (n = 90)

Table 2) in the set. Of the 12 top-ranked germination-correlated metabolites in the wheat 2 and barley sets, six were in common (Table 2), which suggests that similar metabolic processes coincide with losses in the viability of wheat and barley seeds.

3.4 | Seed viability is largely correlated to lipids

Glycerol and its phosphorylated derivatives are products of TAG and phospholipid (PL) catabolism. Galactose, which was high ranking among the germination-correlated metabolites in the wheat 2 and barley sets (third and fifth positions, respectively, in Table 2), can result from galactolipid (GL) degradation. Using LC-MS, we recorded untargeted lipid profiles of the wheat 1 set and identified nonoxidized and oxidized lipids by accurate mass and MS/MS fragmentation patterns (Table S3). For details, see Riewe et al. (2017). Pseudospectra (composed by insource fragments, adducts, and isotopes from single analytes; Kuhl et al., 2012) for 8,836 nonredundant analytes were constructed by a deconvolution of all 18,556 quantitative *m/z* features of the dataset. Each pseudospectrum contains one or more features. The most abundant feature within each pseudospectrum was defined as base peak and used for the untargeted nonredundant quantification of analytes. Approximately 50% (4,222) of all lipophilic analytes were correlated to germination, and, as with central metabolites, the number of negative correlations (2,878) was higher than the number of positive correlations (1,344), although a wider range (R = −.89 to.81) was observed (Figure 1d, all correlation coefficients of known and unknown lipids are provided in Table S3). Of the 8,836 detected analytes, 771 were previously annotated as nonoxidized and oxidized

TABLE 1 Top 12 negative central metabolite-to-germination correlations in wheat 1 (n = 90, FDRs < 6.4e-7)

Central metabolite	R^a
Glycerol	−.82
Glycerol-3-phosphate	−.80
Glycerol-2-phosphate	−.78
Secologanin	−.73
Vanillic acid	−.70
Inositol-1-phosphate	−.68
Azelaic acid	−.66
Ferulic acid, trans-	−.66
Urea	−.64
Pyruvic acid	−.58
Ribonic acid	−.57
Adenine	−.53

[a]Pearson correlation coefficient.

TABLE 2 Top 12 negative identified central metabolite-to-germination correlations in wheat 2 and barley

Wheat 2	R^a	Barley	R^a
Ribonic acid	−.63	Butanoic acid, 4-amino-	−.60
Butanoic acid, 4-amino-	−.63	**Glycerol**	−.57
Galactose	−.60	Gluconic acid	−.56
Glycerol	−.58	Adenine	−.55
Adenine	−.53	Galactose	−.49
Arabinonic acid	−.48	Proline	−.48
Glyceric acid	−.46	Galactitol	−.42
Xylitol	−.46	Alanine, beta-	−.42
Lyxonic acid	−.45	Ribonic acid	−.42
Aconitic acid, cis-	−.43	Fructose	−.42
Glucose	−.42	Aconitic acid, cis-	−.40
Inositol, myo-	−.41	Valine	−.39

Note. The six highest ranking metabolites of both lists are underlined, and glycerol is bold (n = 176 for wheat 2, n = 170 for barley, FDRs < 2.4e-6).

[a]Pearson correlation coefficient.

(having one to four additional oxygen atoms) major lipids (Riewe et al., 2017). Ten annotations of (oxidized) (lyso)phosphatidic acids with lower annotation confidence were added from the negative mode recordings (Table S3) to complement this study. Of these 781 annotated lipids, 513 were correlated to germination.

3.5 | The extremal lipid-to-germination correlations are made up by known major lipids

Unlike unknown analytes, identified lipids were quantified using the dominant adduct formed per lipid class to allow for comparison within lipid classes (Collins, Edwards, Fredricks, & Van Mooy, 2016). But this

TABLE 3 Top 12 negative/positive identified lipid-to-germination correlations in wheat 1 as well as the most abundant lipid species of TAG, DAG, PC, MGDG, and DGDG in bold (n = 90, FDRs < 2.6e-5)

Lipid	Acyl composition[a]	ID[b]	R^c
O_1 DAG 36:6	(18:3/O_1-18:3)	7379	−.88
O_1 DAG 36:5	(18:2/O_1-18:3)	7380	−.88
O_1 DAG 38:4	(18:2/O_1-20:2)	7394	−.87
O_2 DAG 36:6	(O_1-18:3/O_1-18:3)	8017	−.87
O_1 DAG 36:5	(18:2/O_1-18:3)	7381	−.87
O_1 DAG 38:3	(18:3/O_1-20:0)	7397	−.85
O_1 DAG 38:5	(18:3/O_1-20:2)	7391	−.84
O_1 DAG 34:3	(16:0/O_1-18:3)	7370	−.84
O_1 DAG 34:4	(16:0/O_1-18:4)	7369	−.84
DAG 34:4	(16:1/18:3)	6591	−.84
O_1 DAG 36:4	(18:2/O_1-18:2)	7382	−.81
O_1 DAG 38:3	(18:2/O_1-20:1)	7396	−.81
DAG 36:4	**(18:2/18:2)**	**12058**	**−.62**
TAG 54:6	**(18:1/18:2/18:3)**	**19764**	**.43**
MGDG 36:4	**(18:2/18:2)**	**16888**	**.51**
PC 36:4	**(18:2/18:2)**	**16461**	**.59**
DGDG 36:2	(18:0/18:2)	6714	.62
DGDG 32:1	(16:0/16:1)	6705	.62
DGDG 32:2	(14:0/18:2)	6706	.62
DGDG 38:2	(18:1/20:1)	6719	.63
DGDG 36:3	(18:1/18:2)	6715	.64
PC 34:1	NA	630	.65
DGDG 36:5	(18:2/18:3)	6717	.65
DGDG 34:1	(16:0/18:1)	6709	.66
DGDG 34:2	(16:0/18:2)	6710	.67
O_1 DGDG 36:4	(18:2/O_1-18:2)	7495	.67
PC 36:3	NA	643	.67
DGDG 36:4	**(18:2/18:2)**	**6716**	**.69**

Abbreviations: DAG, diacylglycerols; DGDG, digalactosyldiacylglycerols; MGDG, monogalactosyldiacylglycerols; PC, phosphatidylcholines; TAG, triacylglycerols.

[a]"Putative Composition" in Table S3.

[b]"Formula ID" in Table S3.

[c]Pearson correlation coefficient.

m/z feature is not necessarily the base peak of a pseudospectrum. The 20 highest positively germination-correlated quantitative features belong to six pseudospectra (Table S3 "pcgroup" = 99, 89, 22, 960, 92, and 1103), and three of these pseudospectra were identified as PCs ("Formula ID" = 613, 643, and 625). A similar observation was noted for the 20 highest negatively correlated features in the dataset. They belong to eight pseudospectra ("pcgroup" = 893, 415, 283, 8,261, 134, 6,582, 216, and 129), of which six are annotated as singly oxidized DAGs ("Formula ID" = 8019, 7379, 7397, 7380, 7394, and 7381). The closest correlations observed in this untargeted dataset

are not attributed to unknown analytes (which represent >90% of all analytes). Instead, they are formed by known major lipids and their oxides, which underline the special role of these lipids in seed integrity within the entirety of the lipidome.

3.6 | Nonoxidized membrane lipids have the highest positive correlations with seed viability

The top 12 positive lipid-to-germination correlations of identified lipids range from R = .62 to.69 (Table 3). Nonoxidized lipids are overrepresented given their frequency in the dataset. In addition, lipids typically found in membranes are overrepresented as the list only contains PCs and DGDGs with 32–38 carbon atoms in the FA moieties. The lipid with the highest positive correlation to germination, DGDG 36:4 ("Formula ID" = 6716), is also the most abundant cellular DGDG. Other high ranking positively correlated lipids also belong to the higher abundant representatives of their subclasses (Table S3 "Median intensity"). With few exceptions, the lipids consisted of FAs with 16 or 18 carbon atoms, which are typical of wheat/plant seed lipids (González-Thuillier et al., 2015).

3.7 | Singly oxidized DAGs have the highest negative correlations with seed viability

Of the top 12 negatively correlated annotated lipids (R = −.81 to −.88), 11 were oxidized (Table 3). Of these, 10 were singly oxidized, and all 12 were DAGs. The DAGs consisted mainly of FAs with 18 C atoms, such as O_1 DAG 36:6 (18:3/O_1-18:3), the lipid with the highest correlation to germination (Figure 1e), although they also contained FAs with 16 or 20 C atoms. Another lipid class with a high number of high negative lipid-to-germination correlations was oxidized and nonoxidized free FAs, typically with 18 but possibly 16 or 20 C atoms. Twelve of such FAs were among the next 20 highest negatively correlated identified lipids (Table S3).

3.8 | Lipid-to-germination correlation distribution within major lipid classes and oxidized subclasses

An analysis of enrichment of lipids that were positively or negatively correlated to germination was conducted within individual lipid subclasses to support the interpretation of the results in a broader context. A two-sided binomial test was applicable to 24 (having six or more significant positive or negative correlations to germination) of the 43 detected lipid subclasses. Of the 24 subclasses, 20 were significantly enriched in positive (7) or negative (13) correlations between individual lipids and germination frequency (Figure 2). These clear enrichments (e.g., for TAGs, O_1 TAGs, DAG, PCs, and lysoPCs) reveal that in most cases, the individual lipids of subclasses are collectively altered with progressing loss of seed viability.

3.9 | Nonoxidized TAGs are exclusively positively correlated to seed germination, and oxidized TAGs are predominantly negatively correlated to seed germination

Of the 87 nonoxidized TAGs, 52 are positively correlated to germination, and none are negatively correlated (FDR = 5.3e − 15, Figure 2a). The situation has been reversed in the detected 82 singly oxidized TAGs, where the number of negatively correlated lipids is 61, and 2 is positively correlated. The same trend was observed for twofold, threefold, and fourfold oxidized TAGs, which, in all cases, contained significantly more negative than positive correlations.

3.10 | DAGs, MAGs, and FAs are predominantly negatively correlated to germination

Nonoxidized or oxidized TAGs can be hydrolysed to corresponding forms of DAGs, MAGs, and FAs (Figure 3). Independent of the degree of oxidation, more negative (165) than positive (6) correlations were found for these glycerides/FAs (Figure 2a). The trend was significant for DAGs and FAs with up to three added oxygen atoms.

3.11 | PLs are positively correlated to germination

Of 33 nonoxidized PCs, 28 are positively correlated to germination and, similar to the nonoxidized TAGs, negative correlations were not detected (Figure 2b). In addition, the PEs and PGs display more positive correlations to germination than negative correlations to germination, and all four identified PAs were also positively correlated. The numbers of identified/correlated oxidized PLs were typically too low for enrichment analyses. Only O_2 PCs demonstrated a significant enrichment in positive correlations.

3.12 | LysoPLs are negatively correlated to germination

While 30 of 42 detected nonoxidized and oxidized lysoPLs were negatively correlated to germination, none indicated a positive correlation (Figure 2c). On the individual subclass level, correlation enrichments were significant for the lysoPCs and lysoPLs.

3.13 | GLs are predominantly positively correlated to germination

Except for two subclasses, GLs were more often positively correlated to germination than negatively correlated to germination. However, statistical testing was restricted to monogalactosylglycerols, DGDGs, and O_2 DGDGs due to low numbers of identifications/correlations in the other subclasses (Figure 2d). Nonetheless, all 21 identified DGDGs were significantly positively correlated to germination, which renders this finding a clear result. Lastly, the O_2 DGDGs are significantly enriched for positive correlations.

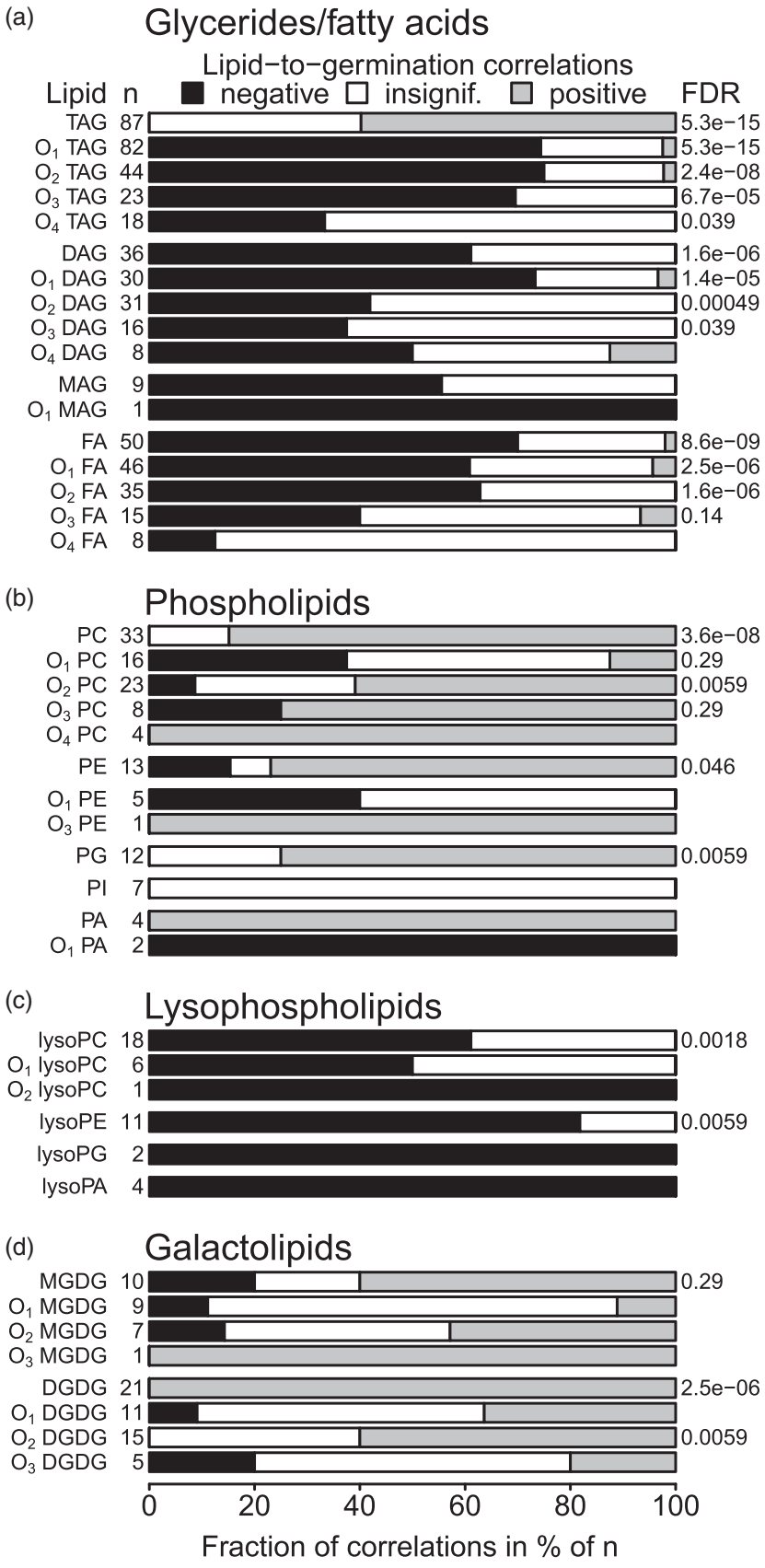

FIGURE 2 Correlation enrichment analysis. Lipid-to-germination correlations were counted for identified (oxidized) lipids belonging to (a) glycerides/fatty acids, (b) phospholipids, (c) lysophospholipids, and (d) galactolipids, and the number of lipids (*n*) was normalized to 100% (horizontal bars). Black sections represent negative lipid-to-germination correlations, white sections represent insignificant correlations, and grey sections refer to positive correlations (FDR < 0.05). FDR-corrected *P* values of a two-sided binomial test (if applicable) on enrichment of positive or negative correlations within lipid subclasses are provided on the right side. DAG, diacylglycerols; DGDG, digalactosyldiacylglycerols; FA, fatty acids; FDR, false recovery rate; MAG, monoacylglycerols; MGDG, monogalactosyldiacylglycerols; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; TAG, triacylglycerols

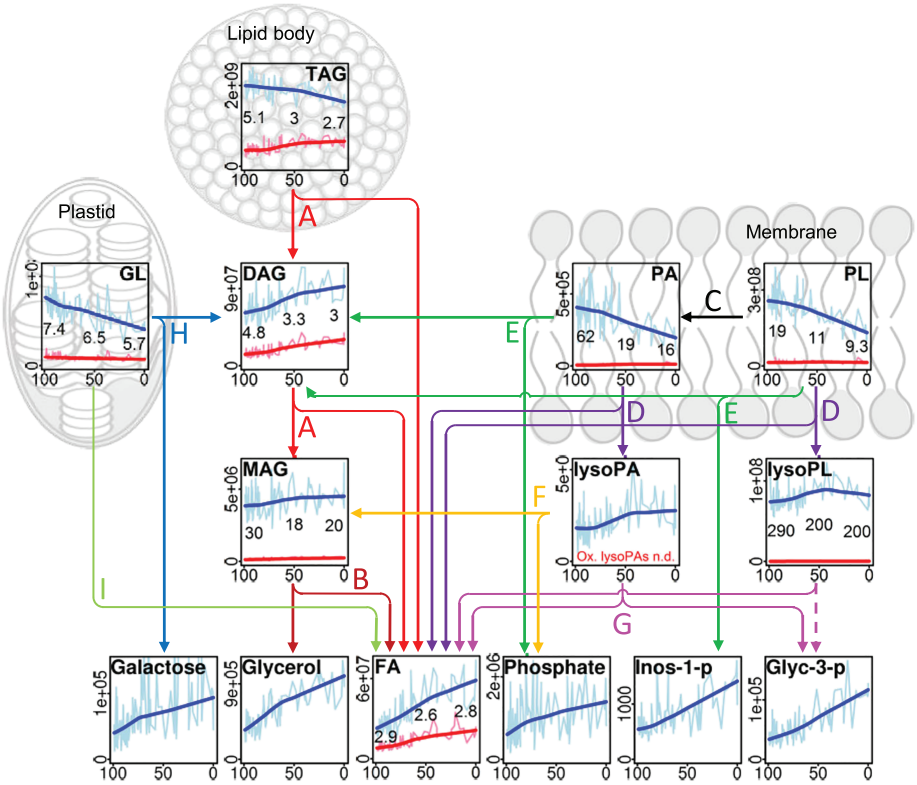

FIGURE 3 Biochemical/cellular model of lipid oxidation and degradation. For central metabolites and phosphate levels, mass spectral ion counts (Y axis) of the individual metabolites are displayed as line diagram (light blue) with moving average (dark blue) in descending order of germination (in %, X axis) of the 90 wheat 1 seed stocks. For lipid classes, the sum abundance of all nonoxidized and oxidized lipid species of the corresponding classes is displayed as the sum ion count of all nonoxidized (blue) and oxidized (red) species per class. The three numbers in each diagram containing oxidized lipid data are the ratios in abundance of nonoxidized to oxidized lipids for seeds with more than 90% (left), less than 10% (right), or 44–55% germination (middle). Known enzymatic interconversion pathways are indicated by coloured lines for (a) TAG lipase, (b) MAG lipase, (c) phospholipase D, (d) phospholipase A, (e) phospholipase C, (f) lysophosphatidic acid lipase, (g) lysophosphorylase, (h) α-galactosidase, and (i) galactolipase. Inos-1-p = inositol-1-phosphate, Glyc-3-p = glycerol-3-phophate. DAG, diacylglycerols; FA, fatty acids; GL, galactolipid; MAG, monoacylglycerols; TAG, triacylglycerols

3.14 | Cross-validation of lipid analysis

We have not used the negative mode data (with the exception of oxidized and nonoxidized lysophosphatidic acids and PAs) due to the lower degree of annotation confidence (lower mass accuracy and no validation by MS/MS spectra), lower analyte coverage, and redundancy with the positive mode data. However, the above presented results, such as the extreme correlations of major lipids to germination within the lipidomic dataset, the outstanding negative association of oxidized DAGs and FAs with germination, and the distribution of lipid-germination correlations within lipid classes, are also found in the negative mode dataset (Table S3).

4 | DISCUSSION

The ROS-caused lipid oxidation during seed ageing has been postulated to cause seed deterioration and death (Chen et al., 2016a; Hu et al., 2012; Ratajczak, Małecka, Bagniewska-Zadworna, & Kalemba, 2015). Here, we present a detailed analysis of the contents of central metabolites, lipids, oxidized lipids, and products of lipid hydrolysis of differently aged wheat and barley seeds and their relation to germination.

Several factors, including genotype, seed production year, storage time, moisture content, and storage temperature, have influenced variation in germination of seed stocks (Roberts, 1961). Factors such as storage temperature and moisture content change over time within a set. Although we cannot clearly disentangle the relative effects of these factors on ageing, our results suggest that higher temperatures and moisture content led to faster ageing, and such conditions also favour chemical/enzymatic turnover of lipids. In addition, seed viability may be reduced by storing seeds together with silica gel. More specifically, silica gel may lead to storage conditions below the critical water content, which is detrimental for seeds (C. Walters & Engels, 1998; Christina Walters, Hill, & Wheeler, 2005). However, for wheat seeds, the critical water content is estimated below 5% at temperatures of 65°C (Yan, 2017). As ERH was measured at 17.4 ± 10.0%, which corresponds to a 6% moisture content; we assume that seeds were not affected by an overdrying induced by silica gel.

Figure 3 integrates the findings of this study into a biochemical pathway of (oxidized) lipid degradation in a cellular context. Except for the production of glycerol-3-phosphate (Glyc-3-P), the complete

pathway chart is composed of known single enzymatic hydrolytic reactions and otherwise contains no gaps regarding biochemical resolution. Any cleavage/hydrolysis of headgroup or FA of fully acylated lipid classes (TAGs, GLs, and PLs/PAs) results in the formation of lipids associated with seed deterioration. Lipid degradation is potentially executed by several hydrolytic enzymes known as lipases, which hydrolyse phospho or acyl esters. Although flux information would be needed to draw conclusions on the directions of lipid conversions in the ageing wheat seed, our data does not support a substantial role for phospholipase D (conversion of PLs to PAs) in seed viability, as was the case for Arabidopsis (Devaiah et al., 2007), soybean (J. Lee et al., 2012), and recalcitrant seeds of different species (Chen et al., 2017). In this study, (oxidized) PAs were detected only in small amounts (Table S3), and ethanolamine, a product of PE to PA conversion and typically well measurable using GC-MS, was not detectable. Phospholipase A (PLA, PLs to lysoPLs and FAs) is likely to be involved in seed deterioration. LysoPLs were quantified in notable amounts, and while the substrates (PLs) of this enzymatic reaction were overall positively correlated to germination, the products (lysoPLs and FAs) displayed the opposite (Figures 2 and 3). Nonetheless, one of the two enzymes, phospholipase D or PLA, or both, must have been active for the production of Glyc-3-P from PLs. Phospholipase C (PLs to DAGs) may also contribute to the reduction of PL by removing the complete headgroup. Substrates/products show inverse correlations, supporting the course of this reaction. Independent and in support of this conversion, we found the PI specific degradation product Inositol-phosphate to be the sixth strongest negatively correlated metabolite detected in the wheat 1 GC-MS analysis (Table 1). While reductions in the levels of these phospholipids may lead to damages of cellular membranes, hydrolytic reductions of galactolipids by galactolipase (GLs to FAs) or galactosidase (GLs to DAGs) would specifically alter plastidial membranes (Dörmann, Hoffmann-Benning, Balbo, & Benning, 1995; Jarvis et al., 2000; R.-H. Lee, Hsu, Huang, Lo, & Grace Chen, 2009). The TAGs form the largest lipid pool and are concentrated in lipid bodies. Conversion of oxidized TAGs and DAGs by TAG lipase (TAGs to DAGs to MAGs and FAs) likely results from the exclusive conversion of TAGs by this enzyme and is supported by reduced TAG and elevated DAG and FA levels. Given that TAG is the principle lipid in wheat seed (Morrison, 1998), which has a lipid content of 2% (http://www.fao.org), this reaction could deliver the largest fraction of (oxidized) FAs produced in connection with the loss in seed viability.

The contribution of each of these enzymes to general lipid hydrolysis cannot be reliably specified. Moreover, other enzymes may be involved. However, the low ratio of 2.9 for nonoxidized to oxidized FAs in seeds with 90–100% germination (Figure 3, FA) suggests that lipases with higher specificity for oxidized than nonoxidized lipids as substrates are involved in lipid degradation because a higher ratio would be expected if the specificity was equal or higher for nonoxidized lipids due to their higher availability in such seeds. If these lipases specifically released oxidized or nonoxidized FA from oxidized lipids, either should accumulate relative to the other with reducing degrees of viability. Yet, they accumulate both in a proportional manner, and the ratio of nonoxidized to oxidized FAs remains constant between 2.6–2.9 in seeds with high, medium, or low germination. This constant ratio suggests that the same lipases have little product specificity. The theoretical ratio of nonoxidized to oxidized FAs obtained from the random hydrolysis of FA moieties from TAGs with a single oxidation, which is the most abundant oxidized lipid subclass in the seeds of this study (Riewe et al., 2017), would be two.

A higher specificity for oxidized over nonoxidized lipids could also explain why in many lipid classes (GL, MAG, (lyso)PA, (lyso)PL) oxidized lipids are low in abundance or are undetectable. The specific conversion of oxidized lipids due to higher affinity/turnover may also account for the highest correlations of oxidized DAGs and oxidized FAs with germination because these species would be formed at a ratio of two from singly oxidized TAGs. The twofold greater theoretical production of oxidized DAGs compared with oxidized FAs from TAGs may contribute to the observed slightly higher precision in predicting germination by oxidized DAGs compared with oxidized FAs. As a novel aspect, however, preferred conversion of oxidized lipids implies that lipid oxidation precedes lipid hydrolysis.

The seeds used in this study had moisture contents below 0.1 g H_2O g^{-1} DW^{-1} and were in a so-called glassy state wherein molecular mobility and enzyme activity were reduced severely or completely (Buitink & Leprince, 2008; Fernández-Marín et al., 2013). Our findings provide evidence for enzymatic catalysis for the reactions leading to the proportional production of oxidized and nonoxidized FAs (see above) and potentially for other reactions depicted in Figure 3. As such, diffusion limitations may be less severe for lipases. The seed-specific Arabidopsis TAG lipase gene SDP1 is expressed during late seed development. The SDP1 protein is associated with oil bodies and is enzymatically active during seed imbibitions (Eastmond, 2006). If seed lipases are in close proximity to massively concentrated amounts of their substrates and are able to diffuse in a lipophilic microenvironment, catalysis could occur in a glassy state. Lipases could lose their activity by alterations in the 3D structure under the low moisture conditions. However, lipases including wheat germ lipase are active in organic solvents with low additions of water (Caro et al., 2002; Yang & Russell, 1995), which suggests that they might remain stable and active in seeds with low moisture content.

Although we do not have direct evidence that lipid oxidation/hydrolysis precedes and is causal for the losses in viability, the high abundance of oxidized lipids and FAs in seeds with approximately 100% germination implies that these processes happen before death and not after. Additional posthumous lipid oxidation would require a mechanism to prevent/revert these processes in the dry and metabolically inert seed that would become inactive when the seed loses its ability to germinate. Lipid oxidation/hydrolysis could lead to death in many ways. For example, membrane damage may lead to uncontrolled permeability of small molecules or proteins across cells or organelles, causing cellular damage/death. The formation of pH-gradients important for energy production or transport may be impaired. Oxidized lipids/FA may inhibit enzymes or trigger fatal signal transduction. Further research is required to identify the causative ROS-induced molecular processes that lead to the termination of life.

Our present knowledge on metabolic processes in ageing seeds has been limited due to the indirect or incomprehensive analysis of oxidized lipids and other metabolites or suboptimal model systems such as controlled deterioration treatments. Here, we consider the associations of hundreds of metabolites, mostly lipids, with longevity of long-term aged cereal seeds at single metabolite, lipid class, and pathway level. Our results link ROS with losses in viability caused by lipid oxidation and hydrolysis in the longest living of all mortal life forms, the plant seed.

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AUTHOR CONTRIBUTIONS

A.B. and D.R. initiated the work. M.N. assayed germination of wheat 1 and barley, and J.W. assayed germination of wheat 2 and measured central metabolites in wheat 2 and barley. D.R. measured central metabolites in wheat 1 and designed the research. J.W. measured lipids with advice of D.R., and both analysed all data. D.R. wrote the manuscript with the support of J.W. M.N., T.A., and A.B. provided edits.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Wheat and barley accessions passport information.

Table S2. Germination, central metabolite profiles (GC-MS), and central metabolite-to-germination correlations for wheat 1, wheat 2, and barley.

Table S3. Lipid profiles (LC-MS) and lipid-to-germination correlations for wheat 1.

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