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Exploring Reactive Oxygen Species Accumulation in *Allium fistulosum* L. Seeds Exposed to Different Storage Conditions

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Abstract: The purpose of this work was to investigate the production of reactive oxygen species (ROS) in *Allium fistulosum* seeds stored under different conditions. Optimized seed storage conditions are essential to maintain seed viability, otherwise accumulation of ROS-induced oxidative damage can lead to seed aging. The *A. fistulosum* seed lots used in this study have been selected based on their breeding background and reproduction site. Seed samples were stored up to 22 months under six different conditions of temperature (25, 10, and 7.5 °C) and relative humidity (RH) (25% and 45% RH). A germination test and ROS quantification assay were performed on the samples collected after 12 and 22 months of storage, respectively. Within a time-window of 10 months, the tested seed lots evidenced a decrease in the germination rate associated with increased ROS levels. Correlation analysis also showed that ROS production was influenced by genotype. The reported data showed that ROS accumulation was dependent on the storage condition and genotype. Some of the tested seed lots appeared to be prone to ROS accumulation, independent of storage conditions. On the other hand, specific condition storages (25 °C, 25% RH; 25 °C, 45% RH; 10 °C, 25% RH; 7.5 °C, 25% RH) resulted in a lower impact on seed aging.

Keywords: *Allium fistulosum*; seed storage; reactive oxygen species; 2,7-dichlorofluorescein diacetate

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1. Introduction

The genus *Allium* includes approximately 850 species used for food and medicinal purposes [1,2]. The group includes onion (*Allium cepa* L.), garlic (*Allium sativum* L.), wild leek (*Allium ampeloprasum* L.), chive (*Allium schoenoprasum* L.), shallot (*Allium ascalonicum* L.), Welsh onion (*Allium fistulosum* L.), Chinese chive (*Allium tuberosum* Rottl. ex Spr.), leek (*Allium porrum* L.), and Jiaotou (*Allium chinense* G. Don), with all of them facing seed quality issues, including poor viability under storage conditions [3–5].

The Welsh onion (*Allium fistulosum* L.) (also called stone leek, Chinese onion, Chinese spring onion or Japanese bunching onion), less known than onion, is commercially produced in Japan, China and South Korea [6]. Recent studies have highlighted the added value of Welsh onion for applications in the context of plant disease protection. For instance, Tang et al. [7] demonstrated the ability of the mixed cropping of apple trees with *A. fistulosum* L. to alleviate apple replant disease. Similarly, Bian et al. [8] showed the beneficial effects of *A. fistulosum* planted with ginseng cultivation as well as the positive impact of and *A. fistulosum* cultivation in reducing soil salinity. When considering seed

quality issues, to date there is still poor attention on Welsh onion. The germination rate and vigor index were significantly improved by applying smarming [9,10]; however, strong efforts are needed to overcome the current gap of knowledge on the cellular and molecular mechanisms underlying seed quality, and to find effective strategies to enhance germination and seedling resilience as well as seed longevity [11].

Long-term storage under sub-optimal conditions affects seed storability since progressive accumulation of oxidative damage impairs the function of lipid membranes and proteins, as well as compromising genome integrity. Genotoxic stress resulting from uncontrolled ROS accumulation determines DNA damage that needs to be repaired during the early phase of germination, which otherwise will fail [12]. The age-dependent dynamics of chromosomal instability were correlated with the germination of Welsh onion seeds stored for six years under room temperature (14–28 °C) and low temperature (4–9 °C), respectively, highlighting a temperature-dependent damage response [13,14]. A parallel study investigated the frequency of chromosome aberrations in root meristem cells of differently aged Welsh onion seeds subjected to γ-irradiation, underlining that the third year of storage was critical for genome integrity maintenance and seed longevity [15]. The use of accelerated ageing has provided useful information about the dynamics of Welsh onion seed deterioration, highlighting improved seed longevity under the following temperature and gas atmosphere conditions: 4 °C, argon/carbon dioxide; −6 °C, air/argon/nitrogen/carbon dioxide; and −18 °C, air [16].

Oxidative damage results from ROS accumulation; however, free radicals also play an essential role as signaling molecules at the onset of germination [17]. Given such a crucial role, ROS homeostasis can be monitored under different conditions in dry and imbibed seeds using dedicated assays [18,19]. In the present work, germination and ROS levels were investigated in *A. fistulosum* seed lots selected based on their breeding background and reproduction site and stored for 22 months under six different conditions of temperature and relative humidity relevant to the seed horticultural trade.

2. Materials and Methods

2.1. Materials, Storage Conditions and Germination Tests

The *Allium fistulosum* seed lots used in this study are listed in Table 1. They have been selected taking into consideration two main factors: the breeding background and the reproduction site. The first aspect included different genotypes identified on the seed market as cultivars, hybrids and open pollinated, or inbred breeding lines. The second aspect referred to the seed production site, both in the North and Southern Hemisphere, considered as optimal for Welsh onion seed multiplication [6]. Genotypes 271227, 251051, 250609 and 251533 are open pollinated (OP) cultivars developed by a Japanese breeding company with seed production located in Italy and Chile. Seed production for the two OP cultivars 1240694 and 1240695, originated by a Dutch breeding company, was localized in Italy and South Africa. The seeds of the only genotype bred by a South Korean company were produced in South Africa. All hybrid genotypes have been sourced through Japanese breeding companies and seed production has been carried out in Italy and Chile. The 17TSITGH03 seed lot was derived from a parental inbred male sterile (MS) line, used to obtain a hybrid cultivar and produced in Italy under greenhouse conditions. Once seeds of all the *A. fistulosum* genotypes reached the final stage of maturity, they were hand-harvested and subjected to artificial drying to reduce the seed moisture content to a range of 7–8% on a fresh weight basis. The samples showed a high germination percentage (>90%), as confirmed by preliminary germination tests. For storage, seed samples were placed into natural textile sacks, used in standard seed storage conditions. Samples were kept in six climatic chambers (CB-CS series, FDM-Environment Makers®, Rome, Italy). The range of controlled conditions spanned from 7.5 °C to 25 °C. The RH values tested in this work correspond to the optimal conditions (25% RH), recommended for vegetable seeds, and to the highest level (45% RH) that could be used. Seed samples were stored for 22 months under six different conditions, as shown in Table 2. Germination tests were conducted

according to the International Seed Testing Association (ISTA) protocol, as follows. Seeds were previously pre-chilled at 5 °C for 4 d. Then, 4 replicates of 50 seeds (each placed in one 9 cm Petri dish) were placed on 6 layers of blotting paper and tested at 20 °C with a 16 h light and 8 h dark photoperiod. The first and the final counts were performed after 6 d and 12 d, respectively (Figure 1).

Table 1. List and specific features of *A. fistulosum* seed lots used in this study. OP, open pollinated. MS, male sterile. F1, Filial 1.

Seed Lot	Seed Production Site	Breeding Site	Genetic Background
271227 (BO01a)	Italy	Japan	OP
251051(BO02a)	Chile	Japan	
251533 (BO03a)	Italy	Japan	
250609 (BO04a)	Chile	Japan	
270446 (BO05a)	Chile	Japan	F_1
270341 (BO06a)	Italy	Japan	
261286 (BO07a)	Italy	Japan	
270322 (BO08a)	Chile	Japan	
17TSITGH03 (BO09a)	Italy	Japan	Inbred line (MS)
18STC35 (BO10a)	Chile	Japan	F_1
1240694 (BO11a)	South Africa	Netherlands	OP
170403214(BO12a)	South Africa	Korea	
1240695 (BO13a)	Italy	Netherlands	
1801030128 (BO14a)	Chile	Korea	F_1

Table 2. List of storage conditions applied to *A. fistulosum* seed lots used in this study.

Condition	Temperature	Relative Humidity (RH)
A	25 °C	25%
B	25 °C	45%
C	10 °C	25%
D	10 °C	45%
E	7.5 °C	25%
F	7.5 °C	45%

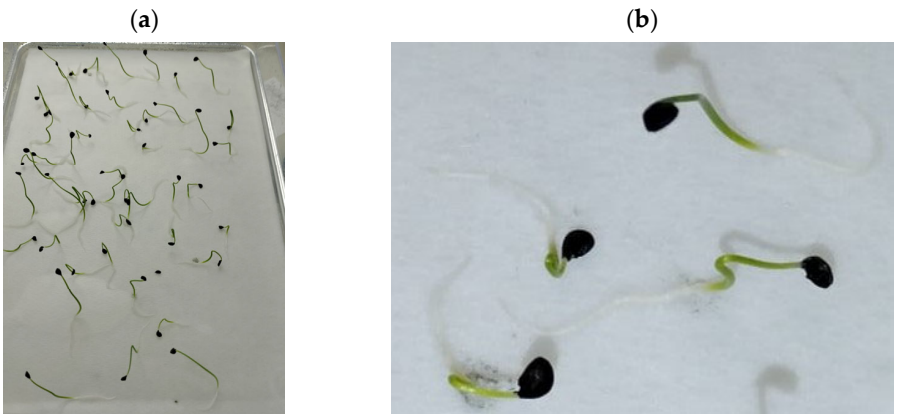

Figure 1. Results of germination test carried out on Welsh onion seeds according to ISTA rules. (**a**) Germination tray. (**b**) Welsh onion seedlings.

2.2. DCFH-DA Assay

The fluorogenic dye 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Milan, Italy) was used to measure ROS levels in seeds. The assay was carried out as described by Pagano et al. [18], with the following modifications. About 20 seeds (100 mg) were used for each sample, seed lot, and tested condition. Seed samples were incubated for 15 min with 100 mL of 10 mM DCFH-DA. Subsequently, the fluorescence sensor (at 517 nm) of the Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, QLD, Australia) was used to read the emitted fluorescence, setting the program for one cycle of 30 s at 25 °C. A sample containing only DCFH-DA was used as a negative technical control to subtract the baseline fluorescence. Data were expressed as relative fluorescence units (RFU). All measurements were performed in four technical and biological replicates for each sample/condition.

2.3. Statistical Analyses

Correlation analyses were carried out using ROS measurements (RFU) and germination percentage (%) data. The analyses were performed using the R package (version 3.5.2) [20] available online at https://gentilinidavide.shinyapps.io/correlation/ (URL accessed on 20 December 2023). The Pearson's *r* value and significance *p* values are given for the combination of factors (ROS vs. germination). Moreover, graphical representations of correlation and Principal Component Analyses (PCA) were performed using the OriginPro, Version 2023b, OriginLab Corporation, Northampton, MA, USA. Graphical representation of the different results was produced using Excel graph tools, namely the Combo design to be able to represent all variables in a single graph that includes multiple axes. Germination and ROS data have been analyzed by one-way ANOVA and Tukey–Kramer test using the online software developed by Assaad et al. [20] available at https://houssein-assaad.shinyapps.io/TableReport (URL accessed on 20 December 2023).

3. Results

3.1. Germination of A. fistulosum Seed Lots Stored for 12 Months

The tested seed lots showed a high germination percentage (>90%), as confirmed by preliminary germination tests, carried out before the long-term storage (22 months). The analyses were started using seeds stored for 12 months once they had been fully equilibrated under the indicated RH. Results of the germination tests and ROS quantification are shown in Figure 2 and Table S1. Some of the tested varieties with the lowest germination percentage showed higher levels of ROS accumulation. This was revealed mainly for the BO07 and BO12 seed lots under storage conditions A (25 °C, 25% RH), B (25 °C, 45% RH), C (10 °C, 25% RH), and D (10 °C, 45%) (Figure 2). Similar responses were also observed under storage conditions E (7.5 °C, 25% RH) and F (7.5 °C, 45% RH). Altogether, seed lots BO07 and BO12 were highly affected by all the different storage conditions taken under consideration. Subsequently, correlation analyses were performed between the investigated parameters (Table 3). When considering the different varieties, significant negative correlations were evidenced between ROS and seed germination, as a measure of viability, suggesting that ROS levels are influenced by all of the tested storage conditions.

Table 3. Correlation values grouping the different varieties for each storage condition, analyzed after 12 months of storage. The Pearson r values are indicated alongside the *p*-values. The *p*-value < 0.05 indicates significant differences, evidenced in bold. Germ, germination percentage; ROS, reactive oxygen species.

Seed Lots	Comparisons	Pearson *r*	*p*-Value
BO01-14A	ROS vs. germ	−0.69	**0.006**
BO01-14B	ROS vs. germ	−0.99	**0.01**
BO01-14C	ROS vs. germ	−0.98	**0.001**

Table 3. *Cont.*

Seed Lots	Comparisons	Pearson *r*	*p*-Value
BO01-14D	ROS vs. germ	−0.95	**0.001**
BO01-14E	ROS vs. germ	−0.62	**0.017**
BO01-14F	ROS vs. germ	−0.67	**0.009**

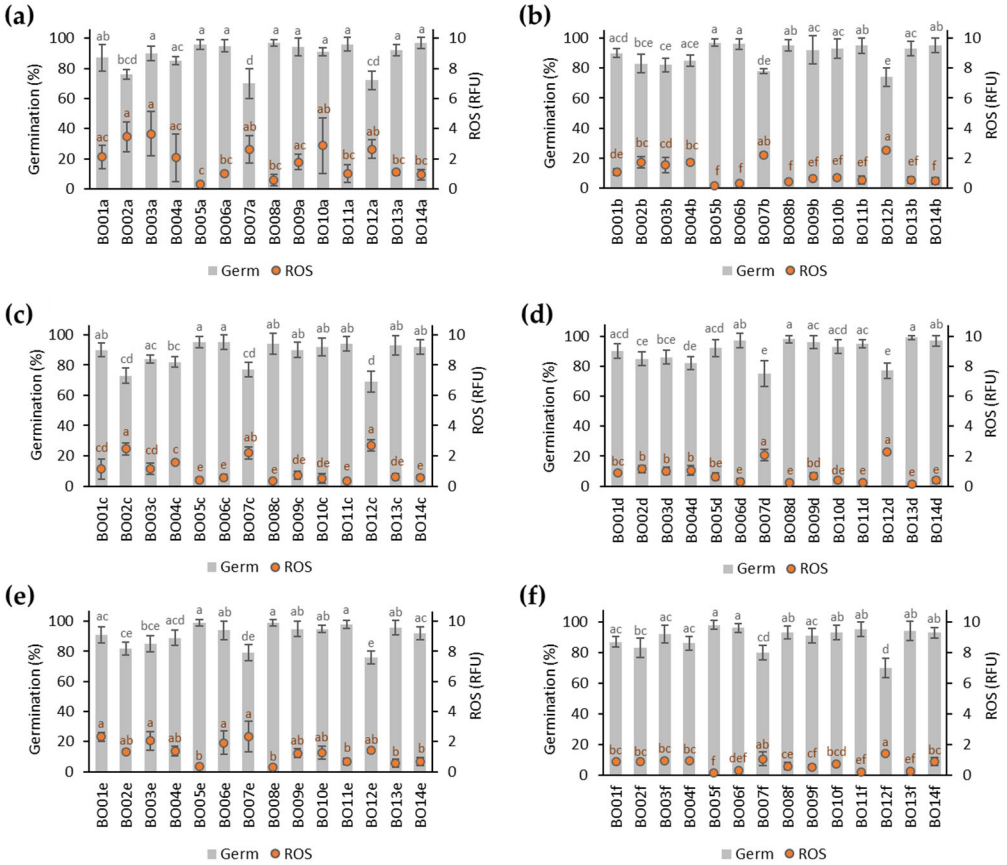

Figure 2. Graphical representation of seed germination percentage and ROS levels for each storage condition ((**a**–**f**) Table 2), taking into consideration the combination of varieties (Table 1). Analyses were carried out after 12 months of storage. Germ, germination percentage; ROS, reactive oxygen species. Values are expressed as mean ± SD of four independent replications with 50 seeds for each replication. Germination and ROS data were analyzed by one-way ANOVA and Tukey–Kramer test. Means without common letters are significantly different ($p < 0.05$).

3.2. Germination of A. fistulosum Seed Lots Stored for 22 Months

A. fistulosum seed germination and ROS levels were evaluated after 22 months of storage (Figure 3, Table S1). Similar to the 12-month data, some of the samples characterized by a low germination percentage revealed increased ROS accumulation. Enhanced ROS accumulation was observed for the BO03 samples at all of the tested conditions. Similarly, this was also the case for sample BO14, except for condition F, where the highest values were registered from sample BO07. Specifically, this sample also showed ROS accumulation under storage conditions A (25 °C, 25% RH), B (25 °C, 45% RH), C (10 °C, 25% RH), E (7.5 °C, 25% RH) and F (7.5 °C, 45% RH). Similar profiles were observed for the BO12 sample, particularly under storage conditions A, B, C, D, and E. Thus, results of the analyses carried out after 22 months of storage showed that seed lot BO07 was affected by all the different storage conditions taken under consideration. No significant ($p < 0.05$) correlations were observed between germination and ROS levels (Table 4).

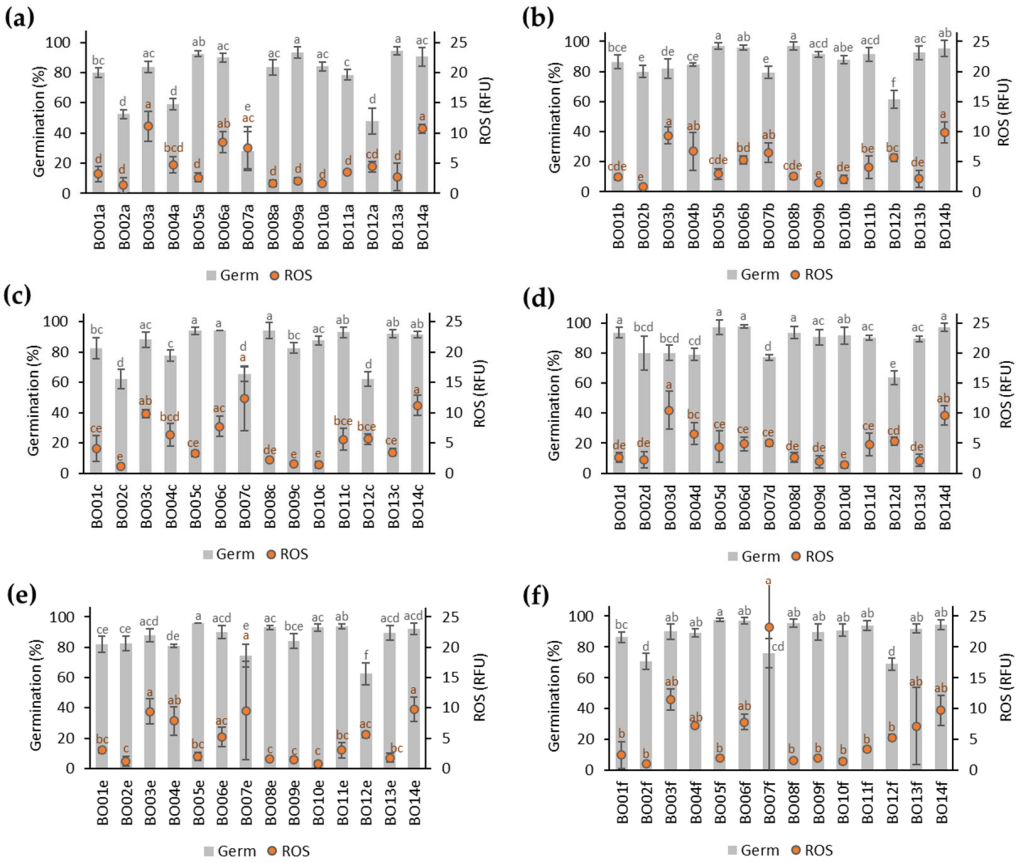

Figure 3. Graphical representation of seed germination percentage and ROS levels for each storage condition ((**a**–**f**), Table 2), taking into consideration the combination of varieties (Table 1). Analyses were carried out after 22 months of storage. Germ, germination percentage; ROS, reactive oxygen species. Values are expressed as mean ± SD of four independent replications with 50 seeds for each replication. Germination and ROS data were analyzed by one-way ANOVA and Tukey–Kramer test. Means without common letters are significantly different ($p < 0.05$).

Table 4. Correlation values grouping the different varieties for each storage condition, analyzed after 22 months of storage. The Pearson *r* values are indicated alongside the *p*-values. The *p*-value < 0.05 indicates significant differences, evidenced in bold. Germ, germination percentage; ROS, reactive oxygen species.

Seed Lots	Comparisons	Pearson *r*	*p*-Value
BO01-14A	ROS vs. germ	−0.01	0.971
BO01-14B	ROS vs. germ	−0.2	0.487
BO01-14C	ROS vs. germ	−0.04	0.891
BO01-14D	ROS vs. germ	−0.2	0.498
BO01-14E	ROS vs. germ	−0.29	0.309
BO01-14F	ROS vs. germ	−0.25	0.381

3.3. Integrative Data Analyses

To better understand the fluctuations in ROS levels and germination percentage along storage (22 months versus 12 months), data were represented as $\log_2$ of the ratio between the values recorded at 22 months and the values recorded at 12 months (Figure 4). As for ROS levels, the $\log_2$ (ROS22/ROS12) revealed a general increase over time (Figure 4a). When considering the different seeds lots, the highest $\log_2$ (ROS22/ROS12) values were

recorded for BO13 (4.944, storage condition F), BO06 (4.716, storage condition F), and BO14 (4.657, storage condition D) (Figure 4a). Overall, seed lots BO05, BO11, and BO14 showed significantly higher $\log_2$ (ROS22/ROS12) in response to all the tested storage conditions. On the other hand, BO02 revealed a significant decrease in $\log_2$ (ROS22/ROS12) under storage conditions A, B, and C, as well as BO10 under storage condition A and E (Figure 4a). When considering germination percentage, mild decreases occurred. The highest impact in terms of $\log_2$ (Germ22/Germ12) was recorded under storage condition A for seed lots BO07 (−1.322), BO12 (−0.585), and BO02 (−0.534) (Figure 4b).

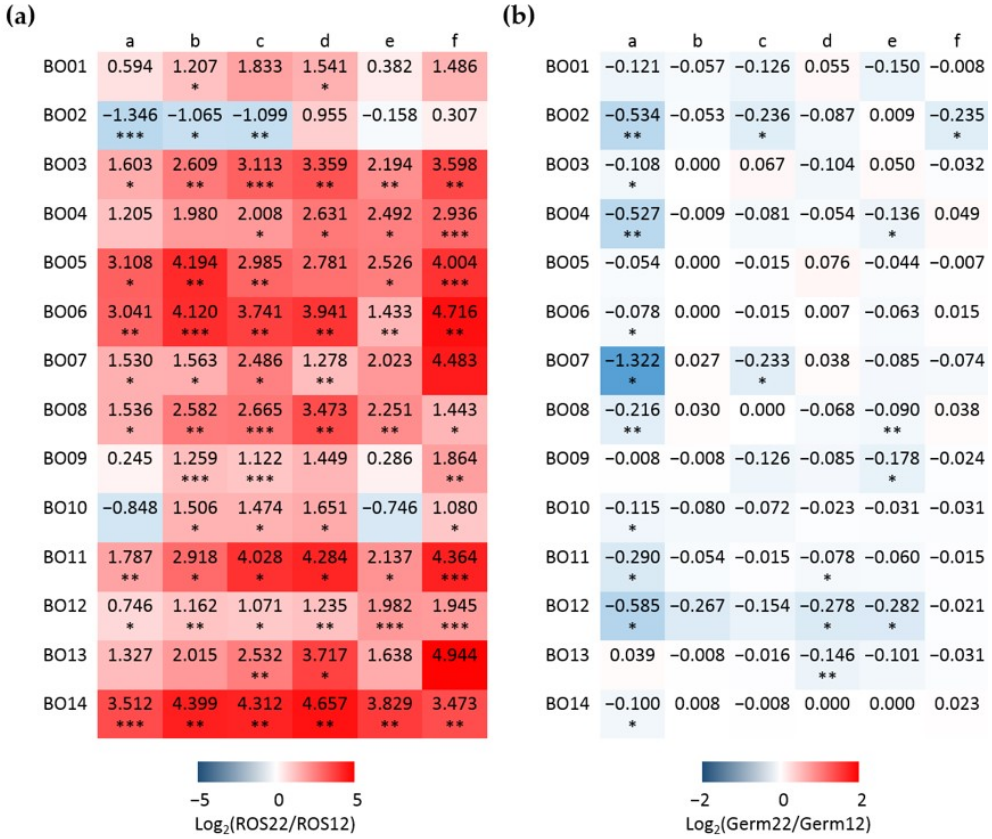

Figure 4. Changes in ROS accumulation levels (**a**) and germination percentage (**b**) observed between 12 and 22 months of storage. ROS12, ROS levels assessed at 12 months of storage. ROS22, ROS levels assessed after 22 months of storage. Germ12, germination percentage assessed at 12 months of storage. Germ22, germination percentage assessed after 22 months of storage. Values are expressed as $\log_2$ of the ratio between the values recorded after 22 months of storage and the values recorded after 12 months of storage. Storage conditions are described in Table 2. Asterisks indicate statistically significant changes as assessed by Student's t-test comparing the values recorded at the two time points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

To provide an overall interpretation of data analyses, correlation analysis (including both storage intervals) and PCA were carried out (Figure 5). Regarding the correlation representation (Figure 5a), it is possible to observe the negative association between ROS and germination % (blue dots). Another interesting observation visible in this representation is the positive correlation between the germination data gathered at the different storage intervals. This may indicate that the different parameters (ROS, germination) influenced the distribution of the variables (genotypes, storage conditions). The two main principal components (PC1 and PC2) extracted accounted for 64.1% and 23.9% of the total variance, respectively (Figure 5b). Interestingly, while the values relative to germination are less different over time (taking into consideration the two storage intervals), ROS values were much more divergent. The samples that most contributed to this divergence were BO07

(yellow dots) and BO12 (dark green dots), in agreement with the presented data indicating that these genotypes were more affected by all the different storage conditions.

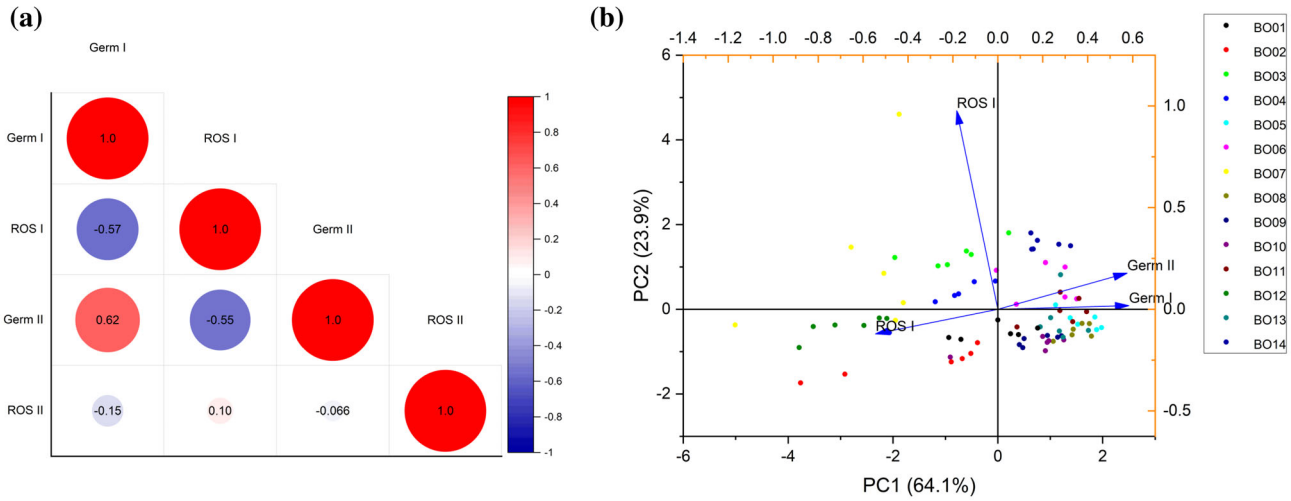

Figure 5. (**a**) Correlation analyses and (**b**) PCA plot carried out taking into consideration the contribution of ROS and germination at different storage intervals (I and II, 12 and 22 months, respectively). Storage conditions are described in Table 2. Numbers in the correlation plots indicate the *p*-values. The closer the Pearson's *r* value is to ±1, the stronger the degree of linear dependence. Differently colored dots in the PCA plot indicate data relative to the used genotypes at the different storage conditions.

4. Discussion

The aim of the present work was to investigate the response of Welsh onion seeds of various origins, subjected to different storage conditions, by combining germination with the seed ability to produce ROS. The well documented pivotal role of ROS, both as signaling molecules and toxic agents during early seed imbibition [17], makes them valuable targets to monitor the seed pre-germinative metabolism, from priming to storage [12,18]. Increased lipid peroxidation, and reduced levels of antioxidants can occur in long-term storage [12]. Ideally, Welsh onion seeds can be stored for 5 to 15 years if maintained at 2 °C or for 15 to 50 years when conserved at −18 °C [6,11]. Such storage conditions are mostly used for germplasm conservation, but they are too expensive when used for seeds intended to be commercialized [6]. The selected temperature and relative humidity conditions investigated in this study (25 °C, 25% RH; 25 °C, 45% RH; 10 °C, 25% RH; 10 °C, 45% RH; 7.5 °C, 25% RH; 7.5 °C, 45% RH) fall under conditions used in most cases by seed companies to conveniently store their commercial seed stocks. The *Allium* species are among the shortest living vegetable species, able to survive only 1–2 years under storage [21]. In the present study, no seed deterioration was detected during the first year of storage; therefore, ROS analyses were started using seeds stored for 12 months, as 'time zero'. Then, the comparison was made with seeds stored for 22 months.

ROS accumulation in Welsh onion dry seeds can provide some interesting information about the way different seed lots respond to different storage conditions and genetic backgrounds. The varieties with the lowest germination percentage showed a peak in ROS accumulation mostly after 12 months of storage, and ROS levels were further enhanced after 22 months of storage. However, the complex profiles of ROS accumulation hereby evidenced make it difficult to find a clear correlation with seed germinability under different storage conditions. One possible explanation of such variegated dynamics might relate to the type of assay used in this study to measure ROS levels. Indeed, there are different types of ROS that oxidize the DCFH substrate converting the compound into the fluorescent molecule DCF [18]. They include hydroxyl radicals (·OH), nitric oxide radicals ($\cdot NO_2$), hypochlorous acid (HOCl), products generated by peroxinitrite decomposition

($ONOO^-$/ONOOH), and carboxyl ions ($CO_3^{\cdot-}$) [17]. In the presence of bicarbonate, DCF rapidly interacts with O_2 to form $O_2^{\cdot-}$ and the dismutation results in additional H_2O_2 accumulation. This can establish a redox-cycling mechanism leading to artifactual amplification of the fluorescence signal [18]. In addition, redox-active metals (Fe^{2+}) promote DCFH oxidation in the presence of O_2 and H_2O_2. The seed coat macromolecular composition must be also taken into consideration whereas additional analyses should be carried out, possibly applying other ROS quantification assays [18]. Based on the reported data on ROS accumulation profiles along storage, seed lots BO05, BO06, BO11, BO13, and BO14 showed a significant increase in ROS levels in response to all the tested storage conditions whereas seed lots BO02 and BO10 revealed a significant decrease under certain storage conditions. It is difficult to link the observed responses to the specific features of each seed lot. On the other hand, seed lots BO05, BO06, BO11, BO13, and BO14 appeared to be prone to ROS accumulation, independent of storage conditions. Seed lots BO02 and BO10 were less prone to ROS production, and this was particularly evident for storage conditions A, B, C, and E (25 °C, 25% RH; 25 °C, 45% RH; 10 °C, 25% RH; 7.5 °C, 25% RH).

The results of the present study underline the strong heterogenicity of the seed germinability as a result of both environmental and genetic components. It should be advisable to re-adjust storage conditions for the tested genotypes that were sensitive to storage while expanding these analyses to other seed lots of different production site, origin and cross type. Optimization of storage conditions currently requires combining an in-depth screening of different protocols with easy-to-manage laboratory tests that are able to provide, in short time, useful information about seed longevity. An ROS-based biochemical screening such as the one conducted in the present work might help with gaining a better understanding of the cellular mechanisms underlying storability. ROS accumulation in seeds can be also assessed by histochemical approaches, e.g., using DAB (3,3′-diaminobenzidine) staining, but the protocol is more laborious and, thus, less suitable for large-scale screening [18].

5. Conclusions

The search for effective indicators of poor seed storability, detected through assays seems like a proper strategy, while at the same time, seed technologists should continue their work to improve the current storage protocols, by refining parameters such as temperature, seed moisture content, and gaseous compositions of the storage atmosphere. Seeds from onion and onion-like varieties still represent a challenge for breeders and seed technologists, due to their storability. Given the complexity of the physiological mechanisms and molecular players involved in seed longevity, a comprehensive evaluation of these dynamics will require extensive studies at a multidisciplinary level. At the same time, efforts should be focused on exploring the impact of the storage protocols on the longevity of primed seeds, by screening hallmarks of seed deterioration such as ROS levels, lipid peroxidation and DNA damage. On the other hand, the environmental conditions during seed maturation of the mother plant dictate the initial quality of seed lots, before storage. Thus, at the company level, data concerning the origin and history of seed lots should also help operators in their management activities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/seeds3010010/s1, Table S1: Germination and ROS data from samples stored for 12 and 22 months.

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