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Oxidant and Antioxidant Profiling in *Viscaria alpina* Seed Populations Following the Temporal Dynamics of an Alpine Climate

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Abstract: The adaptability of seed metabolism to different environmental conditions represents a crucial aspect to understand the effects of climate change on plant populations in wild environments. Among the indicators of stress and repair in seeds, tocopherols and malondialdehyde have been related to membrane stability in seed deterioration. Alpine plants constitute an interesting system to understand stress response dynamics because of the relevant climate variations challenging seed viability in alpine environments. This study considered five accessions of *Viscaria alpina* seeds collected over five years, highlighting significant correlations between environmental parameters such as precipitations and temperature, and several indicators of the oxidative stress response. These provide new insights on how changes in indicators of the seed stress response can reflect annual variations in temperature and precipitations affecting their parental plants, with possible implications on the current understanding of seed persistence in alpine environments threatened by climate change and on the effects of seed storage.

Keywords: seed storage; reactive oxygen species; malondialdehyde; tocopherols; quercetin; correlation analyses; *Viscaria alpina*

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1. Introduction

Alpine plant species are highly threatened by the rapid climatic change happening drastically in mountain environments, which are considered true climate change hotspots [1,2]. Climate change has been particularly marked in European mountains such as the Alps, Apennines, and Pyrenes, leading to an intensification of temperatures extremes and drought episodes, along with modifications in the duration of snow cover [3–5]. The impact of these changes on critical plant life stages, such as seed germination and seedling establishment, is receiving particular attention [6–8], as it may significantly affect plant growth, survival, and distribution [9]. For example, a recent meta-analysis study showed that increasing temperatures and water stress decreased the number, size, and germination of seeds in alpine species, suggesting an overall decrease in regeneration fitness with global warming [7].

From an ecological point of view, seeds play a crucial role in plant population dynamics, being the main vehicle for plant regeneration, persistence, and expansion [10–13]. In this context, seed quality is an essential trait positively correlated with seed germination, namely high-quality seeds lead to high germination rates. Hence, seed quality describes the potential performance of a seed lot. Seed vigor and germination are important features of seed quality. Seed vigor is defined by ISTA (International Seed Testing Association) as "the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments" [14]. Therefore, the impact of the

environment on seed germination is well-defined and can affect the quality of a seed lot based on different indicators. Among these indicators, oxidative stress and activation of antioxidant responses can be envisaged as important tools to determine or predict seed quality and germination behavior [15–17].

Reactive oxygen species (ROS), chemically reactive species containing oxygen, are constantly produced during all phases of seed development, having different implications on seed quality and longevity [16,18–20]. ROS accumulation in seeds has been well documented in multiple species, including seeds from native environments [21,22]. ROS production is a side effect of different metabolic pathways (e.g., lipid oxidation, peroxisome reactions, mitochondria and chloroplast electron transport chains) taking place under physiological and stress conditions [23–25]. If ROS accumulate excessively, it can cause oxidative damage and subsequently compromise seed viability [26,27]. Nevertheless, positive functions are also related to the pre-germinative metabolism, as ROS production promotes signaling cascades leading to dormancy release, storage reserves' mobilization, and radicle elongation [28,29].

Because of this duality, ROS levels need to be kept under tight control by antioxidant defenses. Even during the quiescent state of orthodox seeds, oxidative damage can happen mainly through lipid peroxidation [30] and Amadori–Maillard reactions [31]; hence, detoxification systems are required to prevent extensive cellular injury. Because in dry quiescent seeds the activity of antioxidant enzymes is substantially reduced, many orthodox seeds rely on non-enzymatic systems to scavenge ROS [32]. For instance, polyphenols are among the most common non-enzymatic antioxidants that can be found in the seed coat, endosperm, or embryo as a result of developmental signals, and their depletion was linked with the loss of seed longevity [32–34]. Other antioxidant molecules such as tocopherols can protect membrane lipids from non-enzymatic oxidation during dry storage. This was demonstrated in *Arabidopsis thaliana* plants where impaired tocopherol biosynthesis was associated with reduced seed longevity [35].

The present study proposed to investigate if the accumulation of ROS and the contribution of several non-enzymatic antioxidants can be linked with seed performance and climatic conditions in *Viscaria alpina* seeds collected from alpine areas during five consecutive years. *V. alpina* is an arctic-alpine plant growing in the northern hemisphere. It is an insect-pollinated perennial herb with a life span of up to 10 years, having a strong taproot that allows the plant to resist frost and snow cover, and the seeds (0.5–0.8 mm long) are passively dispersed in autumn [36]. Despite its widespread distribution, in Italy *V. alpina* is classed as vulnerable according to the International Union for Conservation of Nature (IUCN) criteria [37]. For this reason, a recent study has also evaluated the suitability of storing *V. alpina* seeds under gene bank conditions as backup for future climate changes [38].

2. Materials and Methods

2.1. Seed Material and Climate Data

Seeds of *Viscaria alpina* (L.) G.Don, an arctic-alpine plant whose populations are distributed in Europe, Greenland, and North America [36], were used in this study. It is worth mentioning that, until recently, this species was known as *Silene suecica* (Lodd.) Greuter & Burdet. The seeds were collected at the time of natural dispersal (August) for several years (namely the period between 2014–2018) from a population located on the summit of Monte Prado in the Northern Apennines, Italy (44°14′ N 10°24′ E, 2054 m above sea level) and subsequently processed and stored under genebank conditions at the Lombardy Seed Bank, Botanical Garden, University of Pavia. For the collected seeds, the seed longevity in experimental storage and seed mass data were collected and reported in a previous study [38]. The mass of 1000 seeds was determined in ten replicates of 100 seeds. Seed longevity was determined using a standard rapid ageing protocol [39], namely 45 °C and 60% relative humidity. The optimum conditions were used to assess germination following the aging experiment: 25 °C, with a 12/12 light/dark photoperiod.

Overall, 50 seeds were sown into one single plate at each ageing interval, as recommended by the protocol [39]. Seeds were sown Petri dishes containing 1% agar (Sigma-Aldrich, Milan, Italy), placed in a temperature and light-controlled incubator (LMS 250A, LMS Ltd., Sevenoaks, UK) at conditions previously found to be optimal for germination [40]. Plates were checked weekly for germination and seeds were scored as germinated once the radicle had reached approximately 2 mm in length. The germination data from the seed storage (ageing) experiments were analyzed by probit analysis, estimating the time for viability to fall to 50% under the storage conditions used (p_{50}) as previously described [41]. Maximal germination was also tested at 15 °C and 20 °C to infer the level of dormancy at suboptimal temperatures. In these germination tests, three replicates of 50 seeds each were used. Since the materials used in this study represent a fraction of the materials described by White et al. [38], Table 1 summaries these relevant parameters.

Table 1. Biometrical parameters recorded for the *V. alpina* accessions collected over the 2014–2018 timespan; p50, the time necessary to reduce germination to 50% as assessed by probit analysis. s.e., standard error; C.I., confidence interval.

Accession	Storage Time (y)	Seed Mass (mg)	p50 (d ± s.e.)	Maximal Germination (% ± C.I.)	
				15 °C	20 °C
Y2014	6.00	7.65	7.77 ± 0.5831	41.67 ± 8.82	55.96 ± 3.04
Y2015	5.00	7.48	12.28 ± 0.689	5.48 ± 0.26	60.09 ± 7.46
Y2016	4.00	7.03	10.07 ± 0.7256	20.18 ± 5.62	59.20 ± 7.64
Y2017	3.00	6.94	13.01 ± 0.7697	58.52 ± 5.96	50.26 ± 8.21
Y2018	2.00	5.94	10.88 ± 0.7938	32.95 ± 5.89	65.00 ± 10.13

The climate data at the collection site were obtained from an online database provided by the Emilia Romagna Region (https://arpaeprv.datamb.it/dataset/erg5-eraclito, accessed on 1 June 2023; [42]). The minimum and maximum daily temperatures and total precipitation data collected for the 2014–2018 interval are presented in Table 2.

Table 2. Average temperature and precipitations registered for years (Y) 2014-2018 at the seed collection site. s.e., standard error. Data were retrieved from https://arpaeprv.datamb.it/dataset/erg5-eraclito (accessed on 1 June 2023).

Accession	Average Temperature (°C ± s.e.)				Total Precipitations (mm)			
	Current Year		Previous Year		Current Year		Previous Year	
	Annual	Growth	Annual	Growth	Annual	Growth	Annual	Growth
Y2014	7.19 ± 0.29	13.38 ± 0.31	6.40 ± 0.38	16.44 ± 0.39	2659.00	217.90	2209.40	67.00
Y2015	7.65 ± 0.36	14.79 ± 0.36	7.19 ± 0.29	13.38 ± 0.31	1369.30	349.30	2659.00	217.90
Y2016	6.85 ± 0.34	15.15 ± 0.39	7.65 ± 0.36	14.79 ± 0.36	1927.20	139.80	1369.30	349.30
Y2017	7.32 ± 0.39	16.50 ± 0.35	6.85 ± 0.34	15.15 ± 0.39	1741.90	97.10	1927.20	139.80
Y2018	7.13 ± 0.39	15.64 ± 0.28	7.32 ± 0.39	16.50 ± 0.35	1689.00	152.20	1741.90	97.10

2.2. Measurement of Reactive Oxygen Species

ROS levels were quantified in *V. alpina* seed samples collected and stored for the periods indicated in Table 1. The measurements were carried out by using the fluorogenic dye 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Milan, Italy). The dye is converted to a nonfluorescent molecule after a deacetylation reaction mediated by esterases and later oxidized by ROS into the fluorescent compound 2,7-dichlorofluorescein (DCF), which is then detected by fluorescence spectroscopy with an excitation and emission spectra of 495 nm and 529 nm. The assay was carried out as described by Pagano et al. [16], with the following modifications. Seed samples were incubated for 30 min with 70 µL of DCFH-DA (50 µM), and subsequently, the fluorescence sensor (at 517 nm) of the Rotor-Gene 6000 PCR

apparatus (Corbett Robotics, Brisbane, Australia) was used with a specific program setup for one cycle of 30 s at 25 °C. Five replicates containing three seeds were used for each experimental set. Additionally, control samples containing only DCFH-DA were used as a negative control to subtract the baseline fluorescence. The data were expressed as relative fluorescence units (RFU).

2.3. Determination of Malondialdehyde

Malondialdehyde (MDA) levels were quantified as previously described [43,44], with the following modifications. About 500 mg of seeds (50–100 seeds) were ground using a mixer mill type MM200 (Retsch, Haan, Germany) for 30 s with the frequency set at 1/30 to obtain fine powdery flour. Each sample was mixed with 5 mL of an H_2O: 0.5 M $HClO_4$ solution (4:1) with a few drops of 2% BHT (butylated hydroxytoluene, Sigma-Aldrich, Milan, Italy) in ethanol, to precipitate the proteins. After centrifuging at 4 °C for 10 min, samples were then filtered with Whatman No. 1 paper (Whatman Limited, Buckinghamshire, UK), and MDA level was determined as TBARS (thiobarbituric acid reactive substance) following its reaction with thiobarbituric acid (TBA) at high temperature, which results in a colored compound, which can be determined spectrophotometrically. For each sample, 100 µL aliquots were mixed with 100 µL TBA in 1 mL H_2O and heated in a water bath at 95 °C for 60 min. The test tubes were then cooled at room temperature (RT) and the absorbance was measured at 532 nm with a UV spectrophotometer (UV-1800, Shimadzu, UK). The standard MDA (Sigma-Aldrich, Milan, Italy) solution (100 µL, in a range of 0.025–0.1 mg/mL) used was added to a 1 mL test tube and mixed with TBA (100 µL). All the analyses were performed in triplicates.

2.4. Determination of Tocopherols

The tocopherols extraction procedure was performed as previously reported [45,46] with the following modifications. About 500 mg of ground seed powder from each sample, obtained as described in the previous paragraph (see Section 2.3), was added to 5 mL of ethanol containing 0.1% butylated hydroxytoluene (BHT), and the mixture was incubated for 10 min at 85 °C. Samples were subjected to saponification by adding 150 µL of 80% potassium hydroxide (KOH) and incubating for 10 min while vortexing. Subsequently, 3 mL H_2O was added, and the samples were placed in an ice bath for 3 min after which 3 mL of pure hexane was added. After shaking for 10 min at 800 rpm and centrifuging at 12,000 rpm, the upper layer was transferred into a separate test tube, and the pellet was re-extracted twice more using 2 mL of hexane. The hexane fractions were washed with 3 mL of deionized dH_2O, vortexed, centrifuged for 10 min and transferred into a new test tube. The collected hexane fractions were dried using a vacuum evaporator (Rotavapor R-300, Buchi), and the residue dissolved in 150 µL of acetonitrile:methanol:dichloromethane (45:20:35 $v/v/v$) solution, before injection into the HPLC system (Kontron Instrument 420 system, Kontron Instruments, Munich, Germany) equipped with a C18 column (Zorbax ODS column 250 × 4.6 mm, 5 µm, Agilent Technologies, Milan, Italy). The isocratic mobile phase consisted of acetonitrile:methanol (60:40 v/v), the flow rate was 1.0 mL/min at RT, and absorbance was measured at 220 nm. As standard, α- and γ-tocopherol (Sigma-Aldrich) were used for a calibration curve (in a range of 0.2–1 mg/mL) and identified in the chromatogram. All the analyses were performed in triplicates.

2.5. Determination of Quercetin and Quercetin-3-Rutinoside

By using the HPLC system described above, identification and measurement of the quercetin and quercetin 3-rutinoside flavonoids (rutin) were performed. Chromatographic analyses were carried out with a 0.8 mL/min flow rate, setting the detector at 280 nm. The mobile phases consisted of 5% acetic acid (A) and pure methanol (B), and the chromatographic gradient conditions are summarized in Table 3. Quercetin and quercetin-3-rutinoside were identified and quantified using a calibration curve obtained using a standard solution (Merck, Milan, Italy) in a range of 0.01–0.1 mg/mL.

Table 3. HPLC mobile phase gradient used to quantify quercetin and quercetin-3-rutinoside in *Viscaria alpina* seeds.

Time (min)	5% *v*/*v* Acetic Acid	5% *v*/*v* Pure Methanol
1	90	10
5	90	10
7	80	20
8	80	20
10	75	25
15	70	30
20	20	80
25	50	50
28	70	30
30	90	10

2.6. Statistical Analyses

Pearson's correlation analysis was performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/, accessed on 1 June 2023) [47]. The dataset utilized for ANOVA and Pearson's correlation analyses did not contain outliers and were assessed to meet the necessary statistical assumptions (linearity, normality, homoscedasticity, independence). The used variables included the climatic data (temperature, precipitation) and biometrical (seed storage, mass, germination) and biochemical (ROS, MDA, tocopherols, quercetin) parameters. The values are represented as Z-scores, a numerical measure that describes the relationship of a value with the mean of a group of values.

3. Results and Discussion

3.1. Different ROS and MDA Accumulation Patterns in V. alpina Seed Accessions

ROS production was estimated by DCFH-DA assay to provide an insight into the oxidative status of the five *V. alpina* seed accessions in response to the site-specific climate throughout five years of storage under controlled conditions. As shown in Figure 1A, ROS levels were stable in the accessions from 2014 to 2016, whereas a significant increase (3.02-fold) was detected in the 2018 accession, followed by a further increase (1.27-fold) in the 2019 accession. The nature of ROS homeostasis in seed metabolism is multifaceted and involves several biochemical players, including antioxidant enzymes and ROS scavenging compounds keeping ROS levels within the physiological ranges that prevent oxidative damage to biological macromolecules while allowing for their functions in regulating dormancy and germination, where they act as signal molecules and promoters of cell wall plasticity [48].

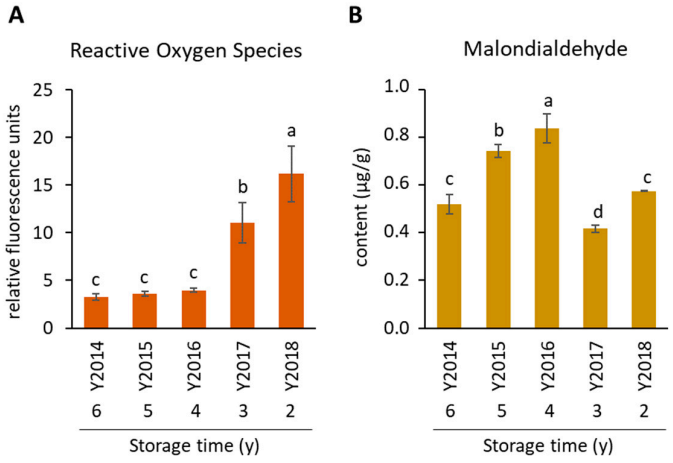


Figure 1. Reactive oxygen species (ROS) and malondialdehyde (MDA) accumulation in *V. alpina* seed accessions from 2014 to 2018. (**A**) ROS production levels as assessed by DCF-DA assay. (**B**) MDA content

per gram of fresh seed tissue. Values without common letters are significantly different ($p < 0.05$) as analyzed by one-way ANOVA and Tukey's post hoc test.

Peaks of ROS production in germinating seeds can be interpreted as a consequence of biotic or abiotic stressors, as a cause of oxidative damage, or as a proxy of an active metabolic state [48–50]. On the other hand, the intracellular glassy state of dry seeds inhibits the enzymatic sources of ROS production as well as enzymatic mechanisms of ROS removal. Nonetheless, ROS accumulation has been recurrently reported in dry seeds during natural or accelerated ageing [51]. Relevantly for dry seeds under storage, the oxidation of membrane lipids can occur non-enzymatically, opening further interpretative possibilities for ROS production patterns [52,53]. For example, *Fagus sylvatica* seeds show increasing levels of different ROS (superoxide, $\cdot O_2^-$; hydrogen peroxide, H_2O_2; hydroxyl radical, OH) over a decade-long cold storage, along with decreasing catalase activity, had increased DNA damage, and loss of germinability [54]. Differently, low hydrogen peroxide content was detected in *Trifolium* spp. seeds after long-term storage, in association with low antioxidant enzymatic activity and reduced germination [53]. The relevance of the oxidative status for seed conservation of alpine herbal species was evidenced in previous studies on *Silene vulgaris* and *Silene acaulis*, which display different resistance to artificial aging in association with contrastive profiles in non-enzymatic antioxidant activity [21]. Although cellular damage associated with unbalanced ROS homeostasis and seed water content are considered as driving factors of seed aging [27,51], the role of other contributors such as water content, storage atmosphere and temperature cannot be excluded, and their implications on the seed physical state and the ROS accumulation/scavenging dynamics remain complex to interpret and to apply to seed conservation strategies [53,55].

To provide a better insight into the oxidative status of *V. alpina* seeds in response to environmental parameters and ROS levels, HPLC analyses have been used to assess the levels of MDA as an indicator of membrane lipid peroxidation. The results evidenced increasing MDA levels from 2014 to 2016 accessions (6 to 4 years of storage, respectively) followed by a decrease in the more recent 2017 and 2018 accessions (3 and 2 years of storage, respectively) (Figure 1B). The observed MDA decrease in recent accessions following storage is compatible with the pattern observed in other species, such as *Medicago polymorpha* seed accessions collected in six different years [56]. Similar findings made MDA an established indicator of membrane lipid peroxidation in aging seeds, as these levels have been positively correlated with ROS accumulation and germinability reductions also in other herbal species such as *Lallemantia* spp., *Festuca arundinacea*, *Psathyrostaehys juncea* [57,58]. Moreover, hydrogen peroxide and MDA levels in seeds with different germination indexes have been demonstrated to be responsive to various environmental parameters, such as temperature and photoperiod, affecting seed maturation on the mother plant [58], corroborating the use of the membrane oxidation status as a proxy of phenotypic plasticity and germination performance. Although MDA has been recurrently used as a stress indicator, its biological roles as a signal molecule have been reported, particularly as a regulator of the redox state and as a mediator of the response to biotic and abiotic stress [59]. Furthermore, the contribution of other reactive carbonyl species in ROS homeostasis and stress signaling cannot be excluded while interpreting MDA accumulation patterns [60].

3.2. Tocopherols and Quercetin Content Have Comparable Patterns in V. alpina Seed Accessions

The accumulation of antioxidant compounds has been assessed through HPLC analysis. As shown in Figure 2, the accumulation of α- and γ-tocopherols follow comparable patterns, with peaks in 2015, 2016, and 2017 seed accessions (5, 4 and 3 years of storage, respectively). Nonetheless, the quantified levels of α-tocopherol were sensibly lower and below the HPLC detectability threshold in 2014 and 2018 accessions (Figure 2A). The role of tocopherols in preventing lipid peroxidation and maintaining seed viability has been demonstrated in a variety of experimental systems, including quiescent and germinating seeds, under natural and artificial aging, in the presence of abiotic stress and low-tocopherol

transgenic rice lines [19,61,62]. Nonetheless, the present work is a novel account of antioxidant profiling in alpine herbs' seeds sampled for several years. The higher content of γ-tocopherols over α-tocopherols confirms the information available in Arabidopsis [35], although opposite trends have been highlighted in other model plants, including rice [61]. The assessment of other lipophilic antioxidant compounds relevant for seed metabolism, longevity and stress response, such as lipocalins, tocotrienols, tocomonoenols, and other tocopherols [63,64], has been also reported to provide detailed insights into their specific functions in seed metabolism.

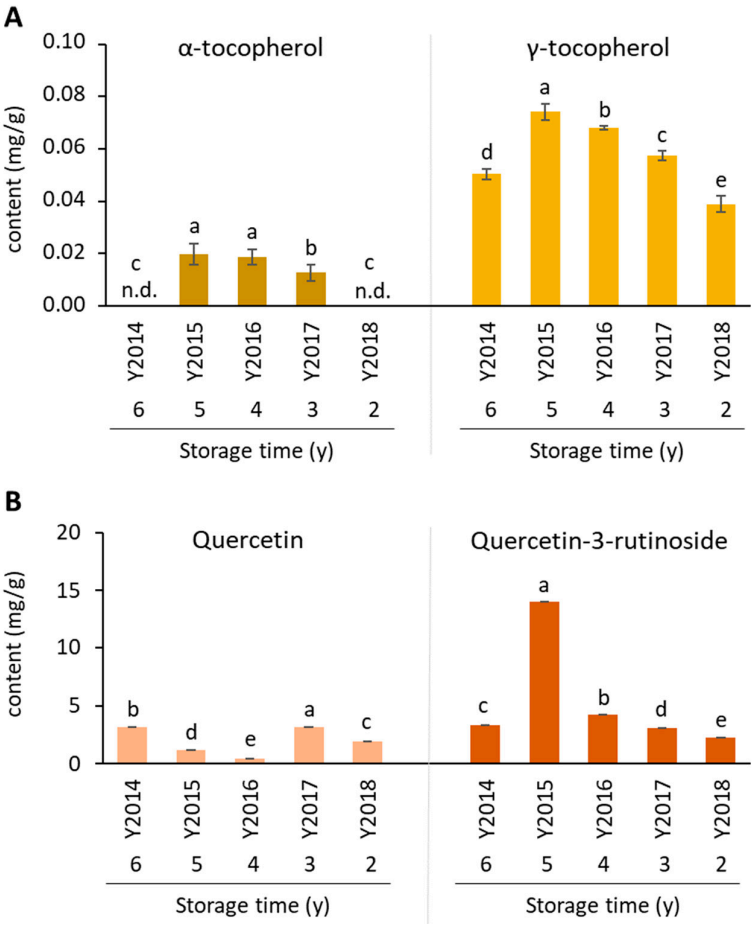


Figure 2. Tocopherol and quercetin content assessed in five *V. alpina* accessions from 2014 to 2018. (**A**) Seed α- and γ-tocopherol content per gram of fresh seed tissue. (**B**) Quercetin, quercetin 3-rutinoside and total quercetin content per gram of fresh seed tissue. n.d., not detected. Values without common letters are significantly different ($p < 0.05$) as analyzed by one-way ANOVA and Tukey's post hoc test.

Additionally, quercetin and quercetin 3-rutinoside, among the most abundant flavonoids observed by chromatographic analyses, have been quantified (Figure 2B). Quercetin levels progressively decrease from the 2014 to the 2016 accessions (6 and 5 years of storage, respectively), increasing again in the 2017 and 2018 accessions (3 and 2 years of storage, respectively). Quercetin 3-rutinoside content was comparably higher and displayed a different accumulation pattern with a peak in the 2015 accession (5 years of storage). The higher relative abundance of quercetin 3-rutinoside also determined the comparable trend of total quercetin.

Among flavonoids, quercetin is known for its medical properties, whereas its involvement in germination, development, photosynthesis, antioxidant response, and antimicrobial signaling [65–68], requires further investigation. Quercetin content has been quantified in *Fagopyrum* spp. seed accessions, revealing variations depending on geographical location [69]. Quercetin-3-rutinoside appeared to be involved in the response to artificial

aging in soybean seeds [70] as well as to several environmental parameters, including drought [67,71] and light [72] in other species. Moreover, we have previously found that in *Solanum villosum* seeds, accumulation of quercetin and rutin were associated with improved germinability during seed priming treatments [73].

3.3. Correlation Analyses Point at Climate-Related Patterns in Oxidant and Antioxidant Profiles

Considering the interplay of environmental, genetic, and phenotypic factors influencing seed maturation, dispersal and survival, an integrative approach can help contextualize the contribution of each player. The Pearson's correlation analysis in Figure 3 highlights significant correlations between the biochemical parameters assessed in the present study, biometrical indicators of seed mass and germination, and the environmental variables recorded at the collection sites of the five *V. alpina* accessions.

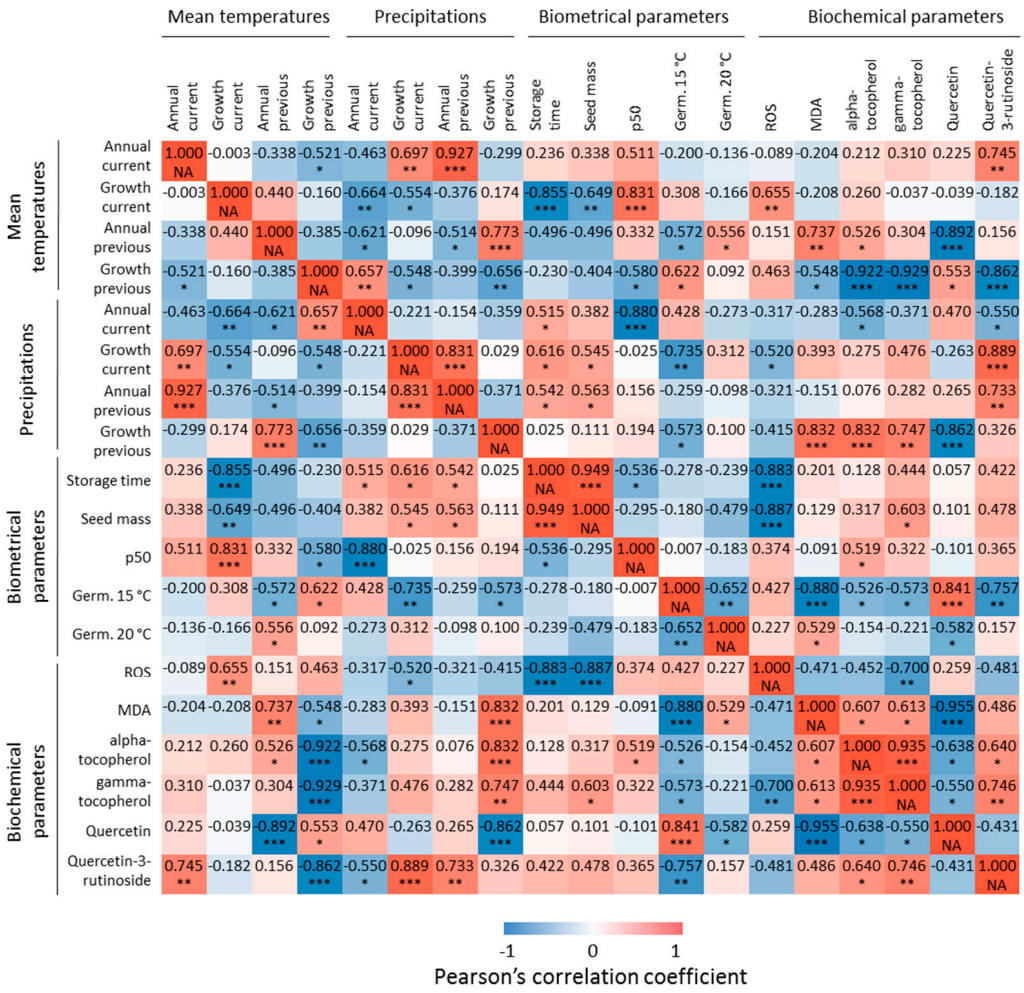


Figure 3. Correlation analysis carried out between climate, biometrical and biochemical parameters in five *V. alpina* accessions. 'Annual', average values over a given year at the corresponding growing site. 'Growth', average values recorded during the *V. alpina* growth season in the corresponding site. 'Current', condition referring to the same year of the sampling. 'Previous', condition referring to the year before the sampling. The Pearson's correlation coefficient is indicated along with its significance. '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$. NA, not applicable. ROS, reactive oxygen species; MDA, malondialdehyde; p50, time to reduce germination to 50%.

ROS levels positively correlate with the main temperatures recorded during the growing season of the collection year and negatively with storage time, seed mass, and γ-tocopherol. The positive correlation with temperature is consistent with previous reports [28], whereas the negative correlation with tocopherol might suggest a role of γ-

tocopherol in ROS scavenging, confirming the current knowledge [35]. These data may also indicate that in warmer year plant produce seeds with higher ROS levels. If this may be a sign of stress or an adaptation strategy to improve stress tolerance, remains to be elucidated. Additionally, it appears that bigger seeds may produce less ROS, leading to hypothesize a possible trade-off between seed size and ROS accumulation. This seems to be supported by a recent study where overexpression of specific *LEA* (Late Embryogenesis Abundant) genes resulted in an increased seed size and reduced ROS accumulation [74].

The present study records a weak negative correlation between storage time and resistance to accelerated aging (p50, duration of accelerated aging to reduce germination percentage by 50%), but does not highlight a decrease in germination performance in correlation with ROS accumulation. The correlation of ROS production patterns with biometrical indicators of seed viability and germination is helpful to define whether a given ROS production pattern falls within or outside physiological ranges compatible with seed viability over time or germination under variable environments [48]. Although several studies correlate the increase in ROS or oxidative stress indicators with susceptibility to aging, others reported lower hydrogen peroxide content after long storage, in association with low antioxidant enzymatic activity and reduced germination [53].

The MDA levels positively correlate with the mean temperatures recorded in the previous year and with the average precipitations of the previous growing season, whereas negative correlations were found with germination at suboptimal temperature (15 °C) and quercetin content. Interestingly, MDA levels did not correlate with ROS levels, which in turn did not correlate with lowered germination performance. Summarizing the ROS and MDA correlation profiles as possible indicators of oxidative stress in the context of this study, we can speculate that the entity of ROS production detectable by DCFH-DA assay may not be sufficient to induce relevant effects on germination dynamics. Compatibly, MDA levels might represent a better indicator of stress associated with reduced germination, thus confirming previous literature on herbal species [57,58].

The accumulation of α-tocopherol and γ-tocopherol displayed comparable patterns (with a positive correlation), and both negatively correlated with the mean temperatures in the previous growing season and positively with the precipitations of the previous growing season. The influence of environmental parameters such as temperature and drought on tocopherol content is consistent with previous literature [75]. As already mentioned, the accumulation of γ-tocopherol negatively correlates with ROS levels. Differently, the quercetin content negatively correlates with MDA levels, temperature recorded the previous year, and precipitations recorded during the previous growing season, whereas a positive correlation is present with germination performance. In this experimental system, the amount of quercetin-3-rutinoside was the main contributor to the amount of total quercetin detected by HPLC. Subsequently, the accumulation trends of both metabolites positively correlated with the mean temperatures of the collection year, precipitations recorded in the growing season of the collection year, and annual precipitations of the previous year, whereas negative correlations were found with the temperatures of the previous growing season. Taken together, these findings are in agreement with the reported protective roles of quercetin and quercetin-3-rutinoside as antioxidant molecules, and with their responsiveness to environmental conditions and biotic/abiotic stress [65–68,71,72].

4. Conclusions

The interplay between ROS homeostasis, oxidative damage, and accumulation of antioxidant compounds plays a prominent role in seed aging, and can be envisioned as a physiological battlefield in which environmental variables shape seed maturation on the mother plant and ultimately seed longevity. Deciphering the contribution of each player requires further integrative analyses, but is particularly relevant for those ecological niches, such as alpine environments, increasingly threatened by the effects of climate change. This study provided novel information about the combination of climate- and storage-related dynamics in *V. alpina* seed accessions collected for five years. Although ROS

levels assessed by DCFH-DA assay did not appear to correlate with MDA levels or loss in viability, MDA accumulation might reflect the stress underlying the delayed germination and confirm the protective roles of quercetin. Based on these analyses, variations of quercetin, quercetin-3-rutinoside, and tocopherol content might be interpreted as responses to climate conditions. Although correlation analyses need to be corroborated further validation to infer mechanistic explanations, these results are consistent with the current state-of-the-art and indicate possible directions for future studies aimed at understanding the molecular bases of phenotypic plasticity in alpine herbs and at devising targeted strategies for seed conservation.

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