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Potential Biochemical Markers Affecting Aging and "the Compensatory Effects" of Canola (*Brassica napus* L.) Seeds Stored in Deep Underground

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Abstract: Understanding the impact of deep underground environment on seed storage is an essential prerequisite for realizing the idea of "deep underground agriculture". To explain the changes in seed germination performance after deep underground storage, we examined the biochemical properties of envelope-packed canola seeds stored for three different durations (66, 90, and 227 days) in four different depths (0, 240, 690, and 1410 m) of a gold mine. Results showed that deep underground storage duration was the leading cause of biochemical properties changes of canola seed, while storage depth exacerbated such changes. Deep underground environment significantly suppressed seed superoxide dismutase (SOD) and soluble sugar (SS), which could be the main reason for the accelerated loss of seed vigor. The appearance of the "compensatory effect" was mainly attributed to the increase in seed growth hormone and the decrease in abscisic acid (ABA) content. The most significant enhancement in growth hormone content was observed in seeds stored at a depth of 1410 m for 66 days, where indoleacetic acid (IAA), gibberellin (GA), and cytokinin (CTK) increased by 63.37%, 21.77%, and 79.36%, respectively. In this study, short-term deep underground storage could enhance seedling growth, but the recommended storage duration for canola seeds should not be longer than 90 days.

Keywords: deep underground environment; seed storage; storage depth; storage duration; canola seeds

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1. Introduction

Global food security issues are now undergoing severe challenges. In 2020, about 800 million people faced hunger worldwide, while almost one-third of the population did not have adequate food [1]. Climate change and increased extreme weather are the main external drivers of food insufficiency [2]. Meanwhile, with the development of human society and the rapid depletion of the Earth's resources, the above-ground environment would probably not be able to meet human demand in the future. Therefore, underground spaces with a more stable environment have entered the vision of agricultural practitioners. Many reports have documented growing plants in shallow underground areas with stable and easily controlled environmental factors [3]. However, continuous climate change or other large-scale disasters such as nuclear wars would cause the surface and shallow subsurface unsuitable for crop growth and human survival. Therefore, using the deep underground as a new space for human existence may be a more viable option, and for the sustainability of human habitation, agriculture is an essential component. Conducting agricultural activities in deep underground environments, which we call deep underground agriculture [3,4], is not only an exploration of the possibilities of

sustainable agriculture but also an efficient use of exploited underground spaces, such as numerous abandoned mines [5]. In addition, the deep underground environment offers the possibility for agriculture to be completely free from dependence on the weather since all environmental factors are fully controllable.

Previous studies mainly focused on the impact of deep underground environment on the growth and metabolism of prokaryotic and eukaryotic cells, as well as on the longevity and reproductive characteristics of organisms, such as fruit flies [6]. Still, the effects on plants, particularly crop plants, were not well documented. Seeds carry all of the plant genetic information, and successful germination with a proper seedling establishment is crucial for plants [7,8]. Therefore, providing appropriate storage conditions during agricultural production is imperative, as the quality of seeds is directly related to the final yield and quality [9]. In general, temperature, relative humidity (RH), and duration are the factors that most affect seed quality during storage [10]. In the deep underground, the depth affects the environmental temperature, RH, background radiation, and air pressure [6]. Thus, storage depth and duration are perhaps the most significant factors affecting seed quality. Therefore, understanding the response of seed quality stored in deep underground to depth and duration is an indispensable prerequisite for deep underground agriculture activities.

Since the chemical attributes of seeds are relevant for successful seedling establishment [11,12], an exploration of the biochemical changes could better evaluate and predict changes in the quality of seeds stored in deep underground spaces. Biochemical indicators associated with seed quality include proteins and sugars, oxidation-related indicators, and hormones. Of these, proteins and sugars are essential energy sources in the early stages of seed germination [13], and both may also be involved in forming intracellular glasses, thus better preserving seed vigor during seed storage [14,15].

Reactive oxygen species (ROS) are produced and accumulated during seed storage due to incomplete or partial oxygen reduction [16]. Although ROS is involved in cellular messaging [17], excessive ROS accumulation in seeds can cause oxidative damage and vigor loss, which is considered the leading cause of seed aging [18,19]. Consequently, seeds can only germinate if ROS content remains at a certain level. An enzymatic defense system in seeds consisting of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) can maintain a stable balance between ROS production and scavenging [20]. The relationship between dynamic changes in antioxidant enzyme activity and seed aging has been documented [21], and such changes can be considered one of the potential indicators of seed aging [22]. The accumulation of proline (PRO), a compatible osmolyte, can also eliminate ROS and improve a plant's ability to survive under stress [23–25]. In addition, the accumulation of malondialdehyde (MDA), one of the leading products of lipid peroxidation of the seed cell membrane, disrupts the antioxidant system and leads to a decrease in seed viability [26,27], making the content an evaluation indicator for the extent of cell membrane damage.

Endogenous plant hormones control plant growth and mediate responses under biotic or abiotic stress, which can be functionally categorized as growth hormones and stress hormones [27,28]. Plant growth hormones in seeds, such as indoleacetic acid (IAA), gibberellin (GA), and cytokinins (CTK), are responsible for regulating seed germination and play an essential role in the growth of plants after germination [29]. In addition, abscisic acid (ABA), known as a stress hormone, is one of the fundamental causes of seed dormancy, inhibiting the germination process and also responsible for seed longevity and viability [30–32]. Therefore, measuring the content of phytohormones in seeds can help us evaluate the germination potential of seeds and interpret the seed behavior after sowing.

Canola (*Brassica napus* L.) is the fourth largest oilseed crop worldwide and is also an industrial raw material, livestock feed, and a source of vegetable protein [33,34]. As a cruciferous crop, canola seeds have a relatively short seed life even under excellent storage conditions, making it a critical model for studying seed aging in dicotyledonous crops [22]. The previous paper [4] reported that deep underground storage of canola seeds could

compensate for the reduced germination rate by increasing seedling hypocotyl length and biomass accumulation, which was defined as the "compensatory effect." Indeed, the vigor of seeds is not only reflected in their high germination rate and ability to germinate quickly and uniformly under ideal situations but also in their ability to emerge under unfavorable conditions [35]. The "compensatory effect" of deep underground storing may enhance the ability of seeds to emerge under adverse conditions while possibly increasing the final crop yield, but the mechanism by which this occurs is unclear. The objectives of this study were (1) to investigate the effects of different storage depths and durations on the biochemical parameters of seeds stored deep underground and (2) to explain the endogenous mechanism of morphological compensatory effect illustrated by changes in biochemical indicators.

2. Materials and Methods

2.1. Plant Material and Deep Underground Seed Storage

The canola (*Brassica napus* L.) seeds used in this study were "Dexing Oil No. 12" (Chengdu Damei Seed Co., Ltd., Chengdu, China) provided by Sichuan Academy of Agricultural Sciences. Before stored in deep underground, the seeds were transferred from their original commercial packaging into paper envelopes designed to fully expose to the deep underground environment. In October 2018, we placed the canola seeds in the horizontal mine channel at four different depths (i.e., 0 m, 240 m, 690 m, and 1410 m under the rock) at the Jiapigou gold mine in Huadian, Jilin Province ([42°52′36″ N, 127°30′46.2″ E]). Seeds stored at 0 m were located inside the horizontal tunnel at the entrance of the gold mine, approximately 30 m from the top of the hill, and considered at a depth of 0 m in this study. According to the experimental design and gold mine safety requirement, we retrieved the seeds at specific durations (as shown in Table 1) and sent them to the Irrigation and Drainage Laboratory, College of Water Resources and Hydropower, Sichuan University. The control check (CK) seeds were kept in their original commercial packaging (with breathing hole) and stored in the lab (temperature, RH, air pressure, and CO_2 concentration were 17.4 ± 0.5 °C, 55.7 ± 2.3%, 473 ± 7 ppm, and 950.60 ± 7.6 hPa, respectively).

All seeds for testing were transported and stored under four °C to ensure that their properties remained unchanged and were tested for biochemical parameters after the last collection. The collected seed of each group was randomly divided into three parts as three replicates for subsequent testing.

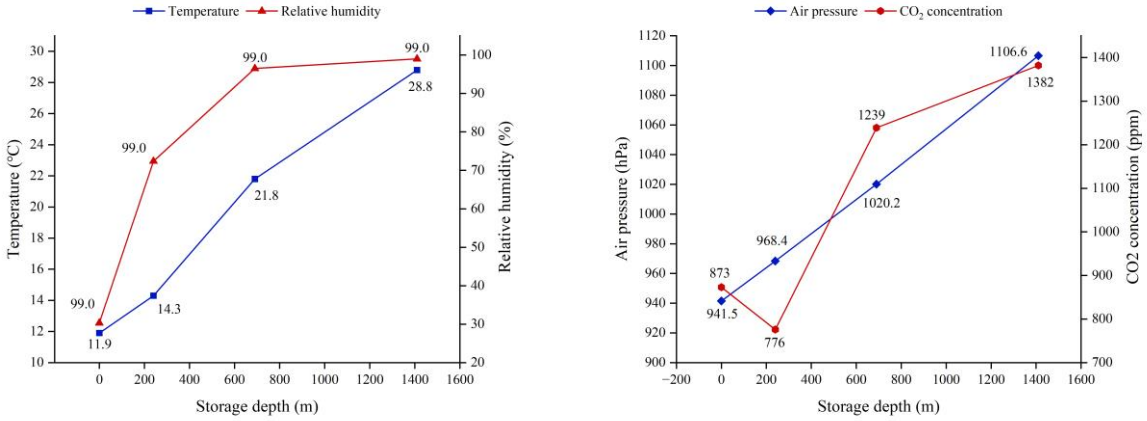
Table 1. Durations of seed storage at different depths in the deep underground.

	Storage Duration			
CK	42 d	66 d	90 d	226 d
0 m	42 d	66 d	90 d	226 d
240 m		42 d	66 d [1]	
690 m	42 d	66 d	90 d	226 d
1410 m	42 d	66 d	90 d	226 d

[1] 90 d and 226 d samples were damaged by mineworkers.

2.2. Environmental Parameter Measurement

Environmental parameters were measured at each depth between September 2018 and June 2019, with a minimum of three measurements at each point. The measured environmental parameters included temperature, RH, air pressure (Testo 480; Testo, AG, Schwaben, Germany), and CO_2 concentration (AR8100; SMART SENSOR, Hong Kong, China). The average values of the environmental parameters are shown in Figure 1 [4]. The deep underground environment was fairly stable, where the fluctuations of the measured data were less than 0.3 °C, 0.5%, 20 ppm, and 2.5 hPa, respectively.



(**a**) (**b**)

Figure 1. The relationship between environmental parameters and the storage depth. (**a**) Temperature and relative humidity with storage depth. (**b**) Air pressure and CO_2 concentration with storage depth. Adapted from Reference [4]. 2022, Springer Nature.

2.3. Protein Content and Soluble Sugar Content Measurement

The protein content was determined using the Coomassie Brilliant Blue G-250 method [36]. The protein in the seeds was extracted with phosphate-buffered saline (PBS) and centrifuged at 2500 rpm for 10 min. Supernatant (50 μL) was mixed with Bradford reagent (750 μL). The absorbance of the samples were measured at 595 nm, and bovine serum albumin (BSA) was used to apply the standard curve.

Soluble sugar (SS) content was measured by using anthrone colorimetry with dry samples [37]. After homogenization, the soluble sugar was extracted with 80% ethanol and centrifuged at 2800 rpm for 10 min. Diluted soluble sugar extract (100 μL) was mixed with 0.2% anthrone sulfuric acid solution (500 μL). The absorbance of the samples were measured at 620 nm and the soluble sugar content was calculated by plotting the standard curve.

2.4. Extraction and Measurement of Antioxidant Enzymes

The SOD (EC 1.15.1.1) assay was carried out according to Beauchamp and Fridovich [38]. Xanthine and xanthine oxidase reaction systems can produce superoxide anion (O^{2-}), which reduces nitroblue tetrazolium (NBT) to formazan. As SOD can remove O^{2-} from the reaction solution, the higher the absorbance measured, the lower the SOD activity, and vice versa. The CAT (EC 1.11.1.6) activity was assayed following the method of Aebi et al. [39]. Since CAT is able to decompose H_2O_2, the activity of CAT can be calculated from the rate of change of absorbance of the H_2O_2 solution. The APX (EC 1.11.1.11) activity was determined according to the method of Nakano et al. [40]. APX catalyzed the oxidation of ascorbic acid (AsA) by H_2O_2, and the APX activity was calculated by measuring the oxidation rate of AsA.

To extract the antioxidant enzymes, 0.1 g of fresh seeds were ground in liquid nitrogen, and homogenized in ice bath with PBS (1 mL). The supernatant was then centrifuged at 10,000 rpm for 20 min at 4 °C and placed on ice for measurement. The activities of SOD, CAT, and APX were finally determined using commercial assay kits from Beijing Solarbio Science & Technology Co., Beijing, China.

2.5. Determination of Malondialdehyde

To extract the MDA, 0.2 g of seeds were ground with 5 mL of 10% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 15 min at 4 °C. MDA was determined using the colorimetric method [41]. The supernatant was mixed with 0.67% thiobarbituric acid and kept at 95 °C for 30 min, followed by centrifugation at 10,000 rpm for 5 min at 25 °C. The MDA content was calculated from the absorbance of the samples at 450 nm, 532 nm, and 600 nm.

2.6. Determination of Proline Content

The proline content was measured using the ninhydrin colorimetric method [42]. Firstly, 0.2 g of ground seeds were extracted in 3% 5-sulphosalicylic acid (10 mL) for 15 min and mechanically homogenised. The clear supernatant was heated at 100 °C for 30 min with concentrated formic acid (200 μL) and 3% ninhydrin in 2-methoxyethanol (400 μL). After the mixture cooled, the absorbance was measured at 520 nm.

2.7. Analyses of Hormone Contents

Seed endogenous phytohormones IAA, GA, CTK and ABA were analyzed with enzyme-linked immunosorbent assay (ELISA) technique [43]. The hormone ELISA kits were provided by Huabiaoce Testing Technology Co., Ltd., Chengdu, China. Extraction, purification, and determination methods of endogenous hormones were performed according to the manufacturer's instructions.

2.8. Statistical Analysis

All chemical analysis data were statistically analyzed using the SAS 9.4 software PROC MIXED program (SAS Institute, 2015, Cary, NC, USA). The least square mean separation for each treatment during the sampling period was at a significance level of $p = 0.05$. Principal components analysis (PCA) and correlation analysis (CA) were performed using OriginPro 2022b (OriginLab Corporation, 2022, Northampton, MA, USA) to examine the relationship between biochemical indicators of canola seeds and to evaluate the correlation between biochemical indicators and indicators tested in germination tests, respectively. OriginPro 2022b was also used for plotting, and Microsoft Excel 2019 (Microsoft, 2019, Seattle, WA, USA) was applied to produce tables.

3. Results

3.1. Principal Components Analysis (PCA) and Correlation Analysis (CA)

PCA results converted the similarity between samples and also showed the correlations of the biochemical indicators and their contribution to the differentiation of samples, with the first two principal components accounting for 68.3% of the total variance (Figure 2). According to the loading plot (Figure 2a), PC1 explained 47.5% of total variation and mainly was positively loaded on growth hormones. Meanwhile, PC1 was positively associated with the antioxidant enzymes CAT and SOD while negatively loading on MDA and ABA. PC2 explained 20.8% of the total variance and was mainly positively loaded on SS, protein, and APX. In general, PC1 represents hormones and oxidation-related indicators, while PC2 can mainly represent protein and SS associated with energy. Additionally, growth hormone scores were generally consistent, and significant negative correlations were found between growth hormone and ABA ($r = -0.77$, -0.67 & -0.79, respectively, $p < 0.0001$; Figure S1). The strongest negative correlation among antioxidant enzymes with MDA was SOD ($r = -0.59$, $p < 0.0001$; Figure S1).

The score plot (Figure 2b) revealed that seeds stored for 66 days and 227 days in the deep underground environment were separated from the CK group located in the second quadrant, which means significantly distinct from CK on PC1-related and PC2-related indicators, respectively. On the other hand, the score for seeds stored for 90 days was essentially indistinguishable from that of CK, and was basically unaffected by storage depth.

In contrast, storage depth significantly affected samples stored for other durations in the deep underground, particularly for seeds stored for 227 days.

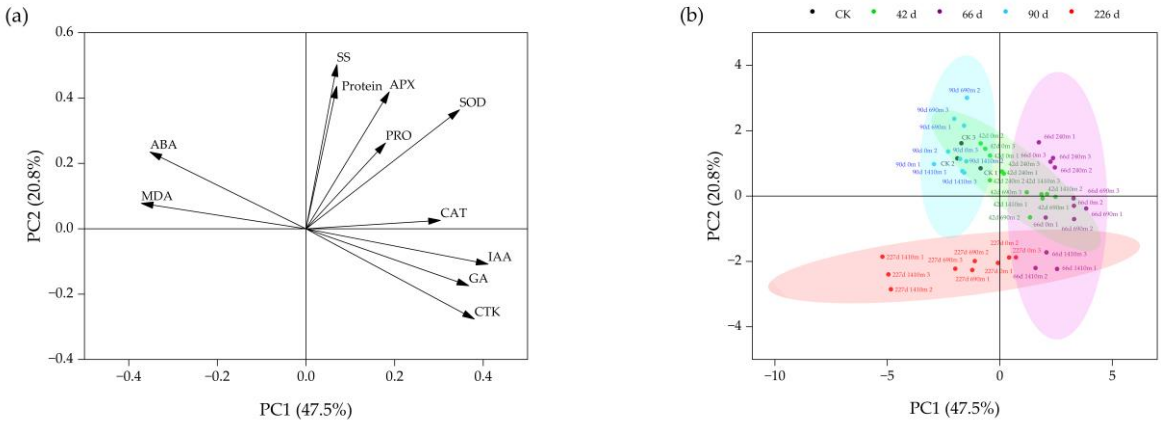


Figure 2. (**a**) Loading plot of principal components analysis (PCA) on biochemical indicators of deep underground stored canola seeds. Protein content, mgprot/mL; SS: Soluble sugar, mg/g; SOD: superoxide dismutase, mg/g; CAT: catalase, mg/g; APX: ascorbate peroxidase, mg/g; MDA: malondialdehyde, nmol/g; PRO: proline, μg/g; IAA: indoleacetic acid, ng/g; GA: gibberellin, ng/g; CTK: cytokinins, ng/g; ABA: abscisic acid, ng/g. (**b**) Score plot of PCA. Each dot represents one replication, and the different colored areas are 95% confidence ellipse of different treatments stored for different durations. In the two plots, the positions of the dots and arrows indicate the composite principal component score for each treatment and indicator, respectively.

The correlation plot (Figure 3) showed the correlation between the biochemical traits of canola seeds and the indicators measured in the germination test. Protein, SS, antioxidant enzymes, PRO, and growth hormones were positively correlated with seed germination indicators. On the contrary, MDA and ABA contents were negatively correlated with germination indicators. In particular, SS, SOD, and growth hormones were significant and strongly correlated with the parameters measured in the germination test.

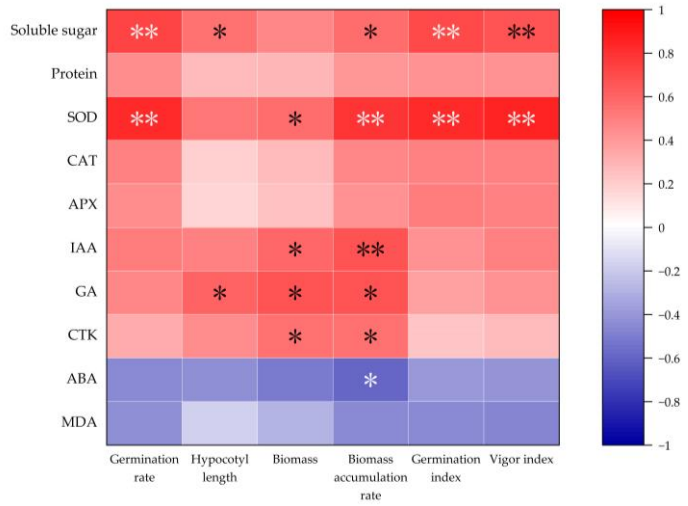


Figure 3. Correlation plot of biochemical indicators (vertical axis) with germination test results (horizontal axis). The germination test results adapted from Reference [4]. 2022, Springer Nature. Red areas indicate positive correlation, blue areas indicate negative correlation, darker colors indicate that the absolute value of the correlation coefficient between the two indicators is closer to 1. Asterisks indicate significance, * Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$.

According to the PCA and CA results, SOD, growth hormones, and SS of seeds stored deep underground were affected by storage duration and depth, significantly affecting seed germination. On the other hand, protein, MDA, CAT, and APX were not found to have a significant effect on germination, but still appeared to change significantly under different storage conditions. Therefore, this study focuses on analyzing changes in the two types of indicators mentioned above.

3.2. Biochemical Indicators Related to Germination Performance

Among the indicators measured in the germination test, the vigor index is a comprehensive evaluation of seed germination rate, speed, and biomass accumulation. Therefore, the vigor index was considered the emphasis of the joint analysis with SS and SOD activity in the subsequent analysis. SOD activity and growth hormones were analyzed and compared with seed biomass and biomass accumulation rate (BAR), respectively. The ABA content was co-analyzed with growth hormone to facilitate the analysis. The SS content, SOD activity, and hormone content are shown in Table 2. The vigor index, biomass, and BAR of seeds are shown in the previous article [4].

Table 2. Biochemical indicators related to germination performance.

		CK	42 d	66 d	90 d	227 d
SS content	CK		58.84 ^{A}	58.84 ^{A}	58.84 ^{A}	58.84 ^{A}
(mg/g)	0 m	58.84 ^{a}	58.57 ^{Aa}	53.68 ^{Bb}	52.12 ^{Cb}	50.69 ^{Bb}
	240 m	58.84 ^{a}	48.39 ^{Cc}	53.91 ^{ABb}	.	.
	690 m	58.84 ^{a}	53.89 ^{Bb}	42.50 ^{Cc}	56.52 ^{ABab}	44.33 ^{Cc}
	1410 m	58.84 ^{a}	49.01 ^{Cc}	45.34 ^{Cd}	53.99 ^{BCb}	33.53 ^{De}
SOD activity	CK		4643.20 ^{B}	4643.20 ^{C}	4643.20 ^{B}	4643.20 ^{A}
(mg/g)	0 m	4643.20 ^{c}	5021.53 ^{Aab}	5298.94 ^{ABa}	4712.83 ^{Bbc}	4421.29 ^{Ac}
	240 m	4643.20 ^{c}	4918.85 ^{ABb}	5441.04 ^{Aa}	.	.
	690 m	4643.20 ^{c}	5014.73 ^{Ab}	5374.84 ^{Aa}	5035.27 ^{Ab}	3845.37 ^{Bd}
	1410 m	4643.20 ^{c}	5264.95 ^{Aa}	4945.67 ^{BCb}	4603.82 ^{Bc}	2888.44 ^{Cd}
ABA	CK		225.56 ^{A}	225.56 ^{A}	225.56 ^{A}	225.56 ^{A}
(ng/g)	0 m	225.56 ^{a}	212.42 ^{ABa}	177.99 ^{Bb}	219.81 ^{Aa}	164.69 ^{Bb}
	240 m	225.56 ^{a}	194.22 ^{BCb}	173.35 ^{BCb}	.	.
	690 m	225.56 ^{a}	181.00 ^{Cb}	155.79 ^{Cc}	215.13 ^{Aa}	187.71 ^{Bb}
	1410 m	225.56 ^{a}	183.70 ^{Cb}	178.38 ^{Bb}	213.85 ^{Aa}	224.26 ^{Aa}
IAA	CK		10.10 ^{C}	10.10 ^{C}	10.10 ^{AB}	10.10 ^{C}
(ng/g)	0 m	10.10 ^{cd}	11.28 ^{BCc}	17.70 ^{ABa}	8.19 ^{Bd}	14.48 ^{Ab}
	240 m	10.10 ^{c}	12.42 ^{Bb}	14.90 ^{Ba}	.	.
	690 m	10.10 ^{c}	16.33 ^{Ab}	19.03 ^{Aa}	11.12 ^{Ac}	11.86 ^{Bc}
	1410 m	10.10 ^{b}	16.5 ^{Aa}	18.92 ^{Aa}	11.25 ^{Ab}	5.71 ^{Dc}
GA	CK		267.56 ^{B}	267.56 ^{D}	267.56 ^{AB}	267.56 ^{B}
(ng/g)	0 m	267.56 ^{bc}	283.21 ^{Bb}	363.57 ^{Ba}	251.72 ^{Bc}	339.78 ^{Aa}
	240 m	267.56 ^{b}	286.10 ^{Bab}	300.10 ^{Ca}	.	.
	690 m	267.56 ^{b}	346.23 ^{Aa}	340.67 ^{Ba}	196.93 ^{Cc}	279.23 ^{Bb}
	1410 m	267.56 ^{c}	325.81 ^{Ab}	420.66 ^{Aa}	287.96 ^{Ac}	155.72 ^{Cd}
CTK	CK		95.92 ^{C}	95.92 ^{C}	95.92 ^{A}	95.92 ^{B}
(ng/g)	0 m	95.92 ^{c}	101.14 ^{BCc}	194.13 ^{Aa}	69.86 ^{Bd}	154.98 ^{Ab}
	240 m	95.92 ^{b}	114.56 ^{Bb}	141.68 ^{Ba}	.	.
	690 m	95.92 ^{d}	177.29 ^{Ab}	194.07 ^{Aa}	58.19 ^{Be}	150.75 ^{Ac}
	1410 m	95.92 ^{d}	172.04 ^{Ab}	201.58 ^{Aa}	110.57 ^{Ac}	61.61 ^{Ce}

Note: ANOVA analysis for $p = 0.05$. Different capital letters indicate significant differences between different storage depths for the same storage duration. Different lower-case letters indicate significant differences between storage durations for the same storage depth.

3.2.1. Soluble Sugar Content

The correlation between the SS content of canola seeds and the vigor index was 0.70 ($p < 0.01$; Figure 3). The SS content of seeds decreased with the increase of storage depth for the same storage duration (Figure 4). Exceptionally, the SS content of seeds stored deep underground for 90 days was basically the same as that of CK, while the SS of the 90 d-690 m and 90 d-1410 m treatments were significantly higher than that of the treatments stored at the same depth for 66 days. However, when the storage duration reached 227 days, the SS content of canola seeds stored at all depths showed a significant decrease compared with the CK group, and the decline increased with increasing storage depth. The largest drop occurred in 227 d-1410 m treatment group, where the SS content was only 56.98% of CK.

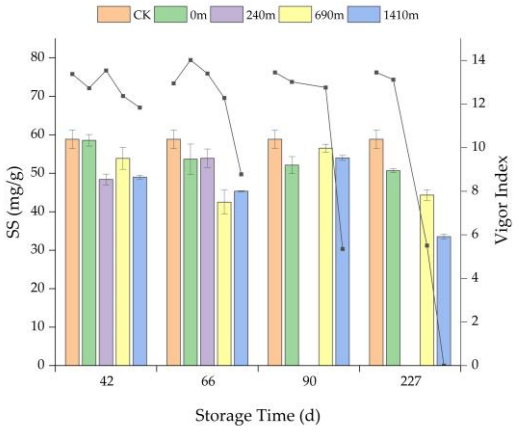


Figure 4. Variation of SS content of rapeseed seeds stored in deep underground with storage duration and depth. The bar graph showed the variation of SS content, and the line graph showed the variation of vigor index.

3.2.2. Superoxide Dismutase (SOD) Activity

The SOD activity strongly correlated with MDA content and all morphological indicators at seed germination (with vigor index r = 0.78, with BAR r = 0.78, $p < 0.001$; Figure 3). The SOD activity of canola seeds stored deep underground went upward and then downward with the increase in storage duration (Figure 5). With the exception of the treatments stored at 1410 m, the peak values of SOD activity in the whole observation period occurred at 66 days, at which time the treatments at all depths had significantly higher SOD activity than CK. The SOD activity of the 66 d-1410 m treatment was also significantly higher than that of CK but was significantly lower than that of the treatments stored at other depths in deep underground for the same duration and significantly lower compared to the 42 d-1410 m treatment, which indicates that the SOD activity of the canola seeds stored at 1410 m had already entered the decreasing stage at this time. The SOD activity of each treatment group began to decrease as the storage duration increased but was still higher (or no difference) than that of CK at 90 days. The SOD activity of all treatments eventually reduced compared to CK when the storage duration reached 227 days. Moreover, with the increased storage depth, the SOD activity showed a stepwise reduction, and the 227 d-1410 m treatment group decreased by 37.79% compared to CK. As shown in Figure 5, the changes in SOD activity were similar to the changes in seed vigor index and BAR, where storage duration and depth led to a decrement in almost all parameters, while some indicators increased in the short-term storage treatments.

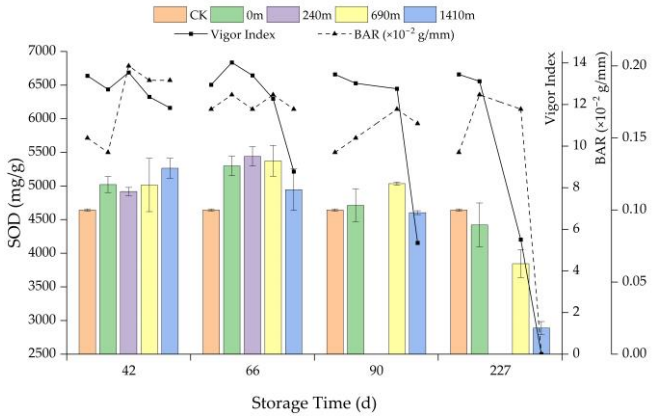


Figure 5. Variation of SOD activity of canola seeds in deep underground storage with storage duration and depth. The bar graph showed the variation of SOD activity, the solid line in the line graph showed the variation of vigor index, and the dashed line showed the variation of biomass accumulation rate (BAR).

3.2.3. Plant Hormones

The hormone content of all treatments is shown in Table 2. Plant hormones can be classified into growth and stress hormones according to their functions. In this study, the growth hormones IAA, GA, and CTK were significantly and strongly correlated with seedling biomass and BAR, and significantly and negatively correlated with ABA content in canola seeds (Figure 3). The variation patterns of the three growth hormones were generally similar, and the GA content was chosen as representative data for graphical illustration (Figure 6). After 42 days stored deep underground, the GA content of canola seeds stored at 690 m and 1410 m was significantly greater than that of CK and the treatments stored at the remained depths. When the storage duration was prolonged to 66 days, the GA content of the treatments stored at all depths increased to the maximum value during the observation period, and the GA content decreased and then increased with the increase of storage depth. The maximum value of seed GA content was observed in the 66 d-1410 m treatment at 121.77% of CK (the IAA content and CTK content were 163.37% and 179.36% of CK, respectively). When stored for 90 days, the pattern of change in GA content with storage depth observed in the 66-day treatments were retained, but the GA content of the treatments stored at each depth was lower than that of CK. The maximum GA content among the treatments stored for 90 days was still observed in the seeds stored at a depth of 1410 m but was not statistically different from CK. After 227 days of being stored deep underground, the GA content of canola seeds continued to decrease in the 227 d-1410 m treatment, where the lowest value during the observation period was observed. However, the GA contents were significantly greater in the 227 d-0 m treatment compared to CK and also increased in the 227 d-690 m treatment to CK level. A high concordance of GA content with both biomass and BAR was found in seeds stored in deep underground. The stress hormone ABA was negatively correlated with the germination indicators and had a relatively significant correlation with BAR ($r = -0.44$, $p < 0.05$; Figure 3). Deep underground storage reduced the ABA content of canola seeds, and all treatments were lower than CK. Moreover, the change in ABA content was basically opposite to the change in growth hormone, and the ABA content of the 66 d-690 m treatment group decreased to 69.07% of CK, which was the lowest value among all treatments.

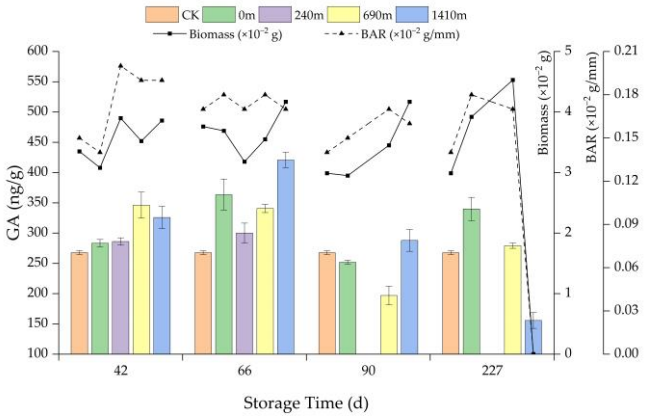


Figure 6. Variation of GA content of rape seeds stored in deep underground with storage duration and depth. The bar graph showed the change of GA content, the solid line in the line graph showed the change of biomass, and the dashed line showed the change of BAR.

3.3. Other Notable Parameters

Protein, MDA, CAT, and APX did not strongly affect the germination test indicators but were still responsive to different deep underground storage conditions (as shown in Table 3). The protein content of canola seeds appeared to increase compared to CK when the storage duration was shorter than 90 days. Moreover, the maximum value of protein content of canola seeds stored at all depths occurred when the storage duration reached 90 days. The protein content was reduced in the 227-day treatments compared to the 90-day treatments, and the protein content of treatments stored in all depth was lower than that of CK, with the lowest value occurring in the 227 d-1410 m treatment, which was 89.83% of that of CK. The MDA content decreased with increasing storage depth at storage durations shorter than 66 days, and a surge relative to CK occurred when storage duration was 90 days. While the MDA content of seeds stored for 227 days showed a decrease in magnitude at the shallower depths, it no longer decreased at 227 d-1410 m treatment, even though the 227 d-0 m treatment, which showed the greatest decrease, did not decrease below CK. The changes in CAT activity were similar to those of SOD activity, which was also stimulated at the beginning of storage, where the increase in storage depth led to an increase in seed CAT activity. The storage duration required to reach the CAT maximum in each depth treatment group was consistent with SOD viability. In addition, the storage depth also led to a decrease in CAT activity as the storage duration increased. However, compared to SOD activity, the CAT activity of deep underground stored canola seeds was maintained above the CK level even in the treatment group with the lowest content at 227 d-1410 m. In contrast, the APX activity decreased compared to CK, except for a small increase in the individual treatment groups.

Table 3. Other notable parameters.

		CK	**42 d**	**66 d**	**90 d**	**227 d**
Protein content (mgprot/mL)	CK		3.54 ^{B}	3.54 ^{B}	3.54 ^{C}	3.54 ^{A}
	0 m	3.54 ^{c}	3.76 ^{ABb}	3.58 ^{Bbc}	4.40 ^{Aa}	3.23 ^{BCd}
	240 m	3.54 ^{b}	3.96 ^{Aa}	3.85 ^{Aab}	.	.
	690 m	3.54 ^{d}	3.86 ^{Ac}	4.07 ^{Ab}	4.37 ^{Aa}	3.46 ^{ABd}
	1410 m	3.54 ^{c}	3.76 ^{ABb}	3.92 ^{Aab}	4.01 ^{Ba}	3.18 ^{Cd}
MDA content (mmol/g)	CK		24.46 ^{A}	24.46 ^{A}	24.46 ^{B}	24.46 ^{B}
	0 m	24.46 ^{bc}	21.42 ^{ABc}	14.23 ^{BCb}	38.41 ^{Aa}	27.19 ^{Bb}
	240 m	24.46 ^{a}	19.39 ^{BCb}	16.68 ^{Bb}	.	.
	690 m	24.46 ^{c}	14.16 ^{Dd}	13.60 ^{BCd}	41.26 ^{Aa}	34.39 ^{Ab}

	1410 m	24.46 ^{b}	16.21 ^{CDc}	12.29 ^{Cc}	37.66 ^{Aa}	37.37 ^{Aa}
CAT activity	CK		31.98 ^{D}	31.98 ^{C}	31.98 ^{C}	31.98 ^{B}
(U/g)	0 m	31.98 ^{b}	32.10 ^{Db}	47.97 ^{Ba}	46.11 ^{Ba}	46.75 ^{Aa}
	240 m	31.98 ^{b}	37.40 ^{Cb}	61.52 ^{Aa}	.	.
	690 m	31.98 ^{c}	46.75 ^{Bb}	68.02 ^{Aa}	48.37 ^{Ab}	43.50 ^{Ab}
	1410 m	31.98 ^{c}	52.59 ^{Aa}	43.61 ^{Bb}	34.15 ^{Cc}	31.53 ^{Bc}
APX activity	CK		0.435 ^{AB}	0.435 ^{B}	0.435 ^{A}	0.435 ^{A}
(U/g)	0 m	0.435 ^{b}	0.405 ^{BCb}	0.499 ^{Aa}	0.282 ^{Cc}	0.258 ^{Bc}
	240 m	0.435 ^{a}	0.456 ^{Aa}	0.443 ^{Ba}	.	.
	690 m	0.435 ^{a}	0.408 ^{BCab}	0.435 ^{Ba}	0.360 ^{Bb}	0.387 ^{Bc}
	1410 m	0.435 ^{a}	0.385 ^{Cb}	0.242 ^{Cc}	0.451 ^{Aa}	0.242 ^{Bc}

Note: ANOVA analysis for $p = 0.05$. Different capital letters indicate significant differences between different storage depths for the same storage duration. Different lower-case letters indicate significant differences between storage durations for the same storage depth.

4. Discussion

4.1. Deep underground Storage Accelerates Seed Aging

The environmental temperature and RH increased with depth in mines where seeds were stored. Previous studies had shown that the safe storage duration of seeds was shortened when the storage environment had a high temperature and RH, leading to increased seed moisture content [44–46]. According to the safe storage guidelines recommended by G. Sathya, canola seeds with moisture content below 10.0% should be stored at temperatures lower than 20 °C in order to ensure 15 weeks of non-deterioration [47]. Accordingly, it can be assumed that the temperature and RH in the deep underground are not conducive to preserving seed vigor. In previous germination trials, deep underground storage reduced the germination rate and vigor of canola seeds, while the increasing storage depth and duration also exacerbated seed deterioration, with canola seeds stored at 1410 m completely losing their germination capacity after 227 days of storage and seeds stored at 690 m also losing their germination rate and vigor to 73.01% and 40.97% of CK, respectively [4]. Changes in the biochemical indicators of canola seeds stored deep underground also showed similar changes to those of aging seeds.

SS contribute to the maintenance of seed viability and cell membrane integrity during storage [48]. Earlier studies on artificially aged canola seeds showed that SS content was positively correlated with seed vigor, and it decreased as the seeds aged [49]. In the present study, the SS content of canola seeds stored deep underground positively correlated with seed vigor as well. Besides, except for seeds stored for 90 days, the SS content of seeds expressed a declining trend with increasing storage duration, with deeper storage depths leading to a more pronounced reduction.

MDA is a product of membrane lipid peroxidation and membrane damage, and its content can be used as an indicator of oxidative damage in seeds [26,50]. Although no significant correlation was observed, the MDA content of deep underground stored canola seeds negatively affected seed vigor and seedling establishment. The changes in MDA content were mainly affected by storage duration, and the severity increased with storage depth after prolonged storage, indicating that deep underground storage accelerated and intensified the oxidation and aging of seeds. The significant increase in seed MDA content is consistent with the phenomenon in artificially aged canola seeds [51]. The reduction in MDA content of seeds stored deep underground for less than 90 days was associated with the activation of antioxidant enzymes, with SOD being primarily responsible.

SOD is the first key antioxidant enzyme in the plant antioxidant system, which can convert superoxide radicals generated in seeds into H_2O_2 and O_2, maintain seed vigor and enhance seed germination performance by controlling possible oxidative damage [52–54]. For deep underground stored canola seeds, SOD activity was primarily

responsible for the changes in MDA content and was also positively correlated with seed vigor index and seedling development. The SOD activity of seeds overall showed a rising and then falling trend against the duration of storage, which was consistent with the changes in SOD observed in aging canola seeds in the previous study [49]. Short-term deep underground storage stimulated the activity of SOD and led to a decrease in MDA content. As storage duration increased, however, the antioxidant function of SOD was disrupted, and the MDA content increased uncontrollably at this time. The turning point was expected to occur near 66 days of storage, while storage depth appeared to accelerate and exacerbate the decline. Therefore, SOD activity is likely one of the most critical endogenous factors in maintaining seed vigor and post-germination growth in deep underground storage. Conversely, although activated during deep underground storage, CAT activity did not hinder the increase in MDA content, and APX was inhibited overall in the deep underground environment.

Protein, an essential source of energy for seed germination, affects seed vigor and initial seedling establishment [55], with declines in contents closely linked to seed aging [56]. Although no significant correlation was found between protein content and seed germination in canola seeds stored deep underground in this study, protein content positively affected seed germination performance. The decreasing trend in protein content controlled by storage duration was observed in seeds stored in deep underground for 227 days. Such a phenomenon of reduced seed protein viability after prolonged storage was consistent with changes in aged canola seeds [49]. However, unlike artificial aging, changes in protein content of deep underground stored canola seeds did not correlate with changes in MDA content ($r < 0.1$; Figure S1), indicating that the changes in protein content observed in this study were not mainly due to oxidative damage. In addition, the protein content slightly increased compared to CK when the stored duration was less than 90 days, which was similar to the phenomenon observed in evening primrose (*Oenothera biennis* L.) [57], but the reason for the change has not been well understood. In general, the overall amount of variation in protein content, even in completely deactivated seeds, was small. Therefore, changes in protein content may not be a major factor affecting seed viability in deep underground storage, but the pattern of variation is of interest for follow-up studies.

4.2. Speculations on Endogenous Factors of the "Compensatory Effect"

The reduced γ radiation and increased air pressure within the deep underground environment, combined with changes in temperature, RH, and gas composition, provide impactions for seeds that differ considerably from conventional storage conditions. The germination trials showed that although the deeper storage depth and extended duration in deep underground resulted in reduced seed vigor, it also promoted seedling establishment, thus compensating for the reduction in seed vigor to some extent, and the hypocotyl length and biomass of seeds stored at a depth of 1410 m for 66 days and 90 days increased by 9.98%, 25.84%, and 10.90%, 39.46%, respectively, compared to CK [4]. Changes that appeared to favor seed germination and shoot growth were also identified in this study, of which changes in plant hormones should probably be the most concern.

Plant hormones play an instrumental role in the germination and seedling of seeds. An overall negative correlation between ABA content and germination parameters was observed in canola seeds stored deep underground and was significant correlated with BAR during seedling growth. In contrast, the trends of growth hormones GA, IAA, and CTK in seeds were generally the same and significantly negatively correlated with ABA. Furthermore, growth hormone significantly affects the seedling biomass and BAR. Previous studies have shown that although ABA, an active abiotic stress hormone, induces dormancy in seeds to prolong their life span, it also inhibits the germination process [58–60]. Growth hormone GA, in turn, acts antagonistically and directly counteracts ABA, releases seed dormancy, and initiates and promotes seed germination, making it an essential hormone for seed germination [31,60,61]. In addition, CTK and IAA in seeds are not only

involved in regulating seed germination but are also required for plant seedling growth [29,62,63]. In this study, deep underground storage reduces ABA content and promotes growth hormone production in canola seeds. Studies on the hormone content changes and effects during storage were inadequate and can even lead to opposite results [22,49,64]. However, according to the hormone functions and effects on morphological parameters of germination, it is not difficult to say that the changes in hormones in deep underground storage of canola seeds, although unfavorable for seed storage, would benefit the germination of seeds, especially seedling establishment. During the observation period, this hormonal stimulation of germination was most pronounced when the storage duration reached 66 days, and the storage depth further promoted this phenomenon. With the increased storage duration in deep underground, the above phenomenon was eliminated at 90 days of storage and reappeared at 227 days. However, at this time, the storage depth was not conducive to the rise of growth hormones and the decrease of ABA content, which was probably caused by the decrease of seed vigor, as evidenced by the complete loss of vigor in the 227 d-1410 m treatment, which no longer produced hormonal changes. In general, hormonal changes in deep underground seeds can potentially promote seed germination and seedling establishment, although they are not conducive to seed storage. Therefore, hormonal changes, particularly the growth hormone increase, could be one of the main sources of the "compensatory effect" of deep underground seed storage.

Apart from hormones, the SS content also showed an increase at specific storage durations. The SS content of canola seeds stored deep underground at 690 m and 1410 m for 90 days were higher compared to seeds stored at the same depth for 66 days and increased to CK levels in the 90 d − 690 m treatment. In previous artificial aging studies on Ulmus parvifolia seeds, an increase in seed SS content after a decrease was also observed, but the increase was associated with a reduction in seed vigor and the reason for this was unknown [65]. In this study, the "rebound" of SS from seeds stored in deep underground for 90 days did not appear to have a direct effect on vigor as well. However, besides correlating with seed viability, seed SS are also an important energy source and regulator for seed germination [66,67] and are able to regulate seed germination and seedling growth by interacting with plant hormones [67]. The increase in SS content in seeds may also occur in seed oil mobilization, whereby the storage oil in the seeds gets converted into SS, thus providing fuel for seed germination [68]. It is noteworthy that the SS content of deep underground stored canola seeds was not only significantly correlated with seed germination and vigor, but also with seed hypocotyl length and BAR. Therefore, the "rebound" in SS could potentially affect seed germination and seedling growth, which requires further attention in subsequent studies.

4.3. Comprehensive Evaluation and Prospects for Deep Underground Seed Storage

Based on the score plot of PCA, seeds stored deep underground for 227 days differed significantly from CK on PC2-related parameters while varying with depth in the PC1 direction (Figure 2b). In fact, the SS and protein content, which were most strongly correlated with PC2 (Figure 2a), decreased in canola seeds stored for 227 days, with the SS content decreasing significantly with storage depth. Meanwhile, the MDA content increased due to a significant drop in SOD content with depth. In addition, the growth hormone content was no longer activated with storage depth, and ABA was no longer inhibited. Overall, at 227 days of storage, although the commercial seeds for testing were far from reaching their shelf life, the energy source for germination was significantly degraded, seed vigor decreased, and germination potential was reduced. The decline in seed quality became more severe as the storage depth increased. The decrease in seed quality shown by biochemical indicators is consistent with the changes in seed vigor observed in the germination trials, particularly the 227 d-1410 m treatment, which showed the worst performance in this study with a complete loss of germination capacity in the germination trials. Thus, deep underground conditions accelerated seed aging, but simultaneously, we observed that the most significant correlation with seed viability was SOD content. While

in seed storage, the decline in seed SOD activity can be slowed down by reducing the relative humidity [27]. Meanwhile, seed SS, which was also highly correlated with seed viability, can also be better preserved in hermetic packaging [69]. Indeed, in germination tests, we found that seeds stored at a depth of 1410 m for 227 days in sealed bags still had a vigor index of 68.80% and a germination rate of 70.21%, proving that the germination capacity of the seeds had been greatly preserved [4]. Unfortunately, owing to a large number of missing samples, data on biochemical indicators for seeds in sealed packages were not measured and should be addressed in subsequent studies.

As with the seeds stored for 227 days, biochemical indicators of seeds stored deep underground were also separated from CK after 66 days of storage (Figure 2b). However, the difference was mainly in PC1, which strongly correlated with oxidation-related indicators and phytohormone (Figure 2a), while storage depth affected the scores on PC2. After 66 days of deep underground storage, the antioxidant enzyme activities of canola seeds, especially SOD, were activated, and the MDA content decreased. Meanwhile, the seed growth hormone increased to a maximum, and the stress hormone ABA was inhibited the most. These changes all contribute to the regulation of seed germination and seedling establishment, and the depth of storage makes the changes in seed hormones even more significant. Although storage depth still reduces the SS content, the germination of the seeds can be activated with a certain level of vigor, and storage depth makes this activation more pronounced. When storage duration reached 90 days, the samples did not differ from CK on the score plot. Most of the chemical properties of the seeds recovered to CK, and the MDA content increased dramatically compared to CK. This indicated that 90 days of storage could be an important turning point, with the effect of the deep underground environment on seeds turning from positive to negative. Therefore, storage in the deep underground for 66 to 90 days makes it possible to obtain the activating effect of the deep underground on the seeds, which in turn compensates for the reduction in seed vigor by increasing the biomass and hypocotyl length at germination, i.e., the "compensatory effect" observed in germination experiments. With controlled storage duration, deep underground storage can fulfill the role of pre-sowing seed treatments before planting, for instance, by hot water soaking [70] or exposure to magnetic fields [71] to improve germination rates and post-germination performance. Notably, although the seeds seemed to "activate" again when the storage duration reached 227 days, the observation was not significant due to the loss of viability of the seeds deep underground.

Considering the effects of deep underground storage on canola seeds, the duration of storage could be the main driver of changes in the chemical properties. Deep underground storage first stimulates the seeds to perform better during seedling establishment, then allows them to "recover", and finally accelerates their aging and loss of germination capacity. The depth of storage, while accelerated aging, also enhanced the "compensatory effect" of the deep underground environment. However, as the chemical properties of the seeds "recovered" with storage duration, the changes caused by storage depth were also eliminated. The present study partly explained the effect of deep underground environment on seeds through changes in biochemical indicators, but many questions still remained unanswered. Examples include the effect of changes in protein content, the effect of the "rebound" in SS, and the effect of the packaging method on biochemical parameters. Additionally, to better represent the stress status of seeds, measurement of ROS accumulation may be a valuable complement, while measurements of other plant growth-related hormones such as ethylene and brassinosteroid would complete our understanding of the hormonal effects of deep underground environments on plants. Furthermore, the essential and subsequent task is to isolate the environmental variables of the deep underground environment and to investigate the effects of temperature, RH, gas composition, radiation, and especially air pressure on seed storage and plant growth, respectively, in order to investigate the effects of environmental factors on the vegetation deep underground and to prepare for the practical application of deep underground agriculture.

5. Conclusions

Seed storage is an essential part of agricultural activities, and the storage characteristics of seeds need to be reconceptualized given the unique features of the deep underground environment, where seed storage conditions differ from those of conventional environments. Canola seeds with long-term deep underground storage (227 days) experienced increased MDA content, weakened antioxidant enzyme activity, degradation of proteins and SS, and decreased phytohormone content. All these changes led to a reduction in seed quality, which was exacerbated by the storage depth. The correlation between SS content and SOD activity was the most significant, and SOD activity was also the main antioxidant enzyme controlling the MDA content. However, after a specific period of storage in deep underground, the seed ABA content decreased, and the seed growth hormone content increased. The change in hormones positively affected seedling establishment, especially by increasing the rate of seedling biomass and BAR. This favorable change in seedling growth was most evident in seeds stored for 66 days. At the same time, the SOD activity of seeds was stimulated, and the MDA content was reduced. Therefore, deep underground storage in the short term enhanced the germination potential of canola seeds, which was more pronounced in seeds stored at deeper depths. The changes in biochemical indicators of seeds caused by deep underground storage were eliminated to a certain extent at the storage time of 90 days. In conclusion, we investigated the endogenous factors that accelerated the decline of seed vigor during deep underground storage and found that extending the safe storage time might be possible by delaying the decline in seed SOD activity. Furthermore, deep underground storage benefited seedlings, and hormonal changes partly explained the previously observed "compensatory effect," which suggested the agronomic potential of deep underground spaces.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020320/s1, Figure S1: Correlation plot of biochemical indicators.

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