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Enhanced Antioxidant Activity and Secondary Metabolite Production in Tartary Buckwheat under Polyethylene Glycol (PEG)-Induced Drought Stress during Germination

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Abstract: Drought stress is one of the key factors impeding agricultural productivity worldwide. This experiment aimed at investigating the polyethylene glycol (PEG)-induced drought stress effects on seed germination, physiology, and biochemical mechanisms in Tartary buckwheat genotypes. Four PEG-induced stress conditions (0%, 10%, 20%, and 30%) were applied to 14 selected genotypes at the germination stage to evaluate their stress tolerance capacity. Significant differences were obtained in germination percentage, relative water content (RWC), and all growth parameters among the studied 14 genotypes. Based on the stress tolerance index (STI), XiNong 9943, XiNong 9940, and QianKu-5 were found to be tolerant, and QuanKu-4 was susceptible. These cultivars were selected for further physiological and biochemical characterization. The results demonstrated that the activity of enzymes was significantly increased with the increase in PEG dose. SOD (superoxide dismutase), POD (peroxidase), CAT (catalase), and APX (ascorbate peroxidase) levels obtained at 30% PEG in the XiNong 9943 genotype were 2.01, 2.19, 4.92, and 4.46 times higher, respectively, than the normal growth condition (T_0). Moreover, the secondary metabolite content also increased with the increase in PEG dose. At 30% PEG, the genotype XiNong 9943 yielded phenols, flavonoids, polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL) levels that were higher by 131%, 95%, 154%, and 164%, respectively, than T_0 condition. From both the findings of the activity of enzymes and the secondary metabolite content, the genotypic response to drought was ranked in the following order: XiNong 9943 > XiNong 9940 > QianKu-5 > QianKu-4, which supported the STI selection system. Assessing the overall performance, the genotype XiNong 9943 shows drought tolerance, which can be useful material for future buckwheat breeding programs.

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Citation: Hossain, M.S.; Li, J.; Wang, C.; Monshi, F.I.; Tabassum, R.; Islam, M.A.; Faruquee, M.; Muktadir, M.A.; Mia, M.S.; Islam, A.K.M.M.; et al. Enhanced Antioxidant Activity and Secondary Metabolite Production in Tartary Buckwheat under Polyethylene Glycol (PEG)-Induced Drought Stress during Germination. *Agronomy* **2024**, *14*, 619. https://doi.org/10.3390/agronomy14030619

Received: 17 February 2024
Revised: 12 March 2024
Accepted: 15 March 2024
Published: 19 March 2024

Keywords: drought; polyethylene glycol; germination; antioxidant; secondary metabolite

1. Introduction

Drought stress is one of the predominant abiotic factors that affects regular plant growth by disturbing its prevailing vegetative, reproductive, and metabolic processes [1–3]. Depending on the intensity, time, and nature of the crops, drought can reduce the yield by more than 50%, which can have a great impact on global food security [4,5]. Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn), a member of the Polygonaceae family, is well adapted and cultivated in arid and semi-arid regions and its consumption propensity is increasing due to its innate nutritional and biological properties [6]. It has wide genetic diversity with some advantageous qualities, including quick growth rates, low agronomic expenses and great adaptability even in infertile soil [7,8]. Basically, buckwheat seeds are used as a prime source of traditional medicines and now they are used in daily food intake, such as bread, noodles, tea, sprouts, and vinegar [6,9], because its seeds contain a wide range of bioactive phytochemicals, amino acids, dietary fiber, vitamins, minerals, and trace elements [9,10]. Thus, considering several health-promoting benefits, increasing production per unit area in the ever-changing climatic conditions is imperative to gaining global food security.

Unfavorable soil moisture conditions during sowing can often impede buckwheat seed germination, which leads to irregular seedling emergence [11,12]. Drought stress reduces water potential and hampers water uptake, thereby impeding seed germination and seedling growth [13–15]. Polyethylene glycol (PEG)-induced drought stress is the most popular screening technique used to evaluate the drought tolerance of various crop varieties during the seed germination stage [16,17]. Determining alterations in radicle and plumule properties, including radical length, plumule length and biomass of seedlings exposed to drought stress gives significant insight regarding these traits [14,15,18].

From a physiological and biochemical standpoint, drought stress triggers excessive generation of reactive oxygen species (ROS) like $O_2^{\bullet -}$, leading to oxidative damage in plants [19,20]. Wang et al. [21] suggested that PEG or NaCl can enhance the enzyme activity in the plumules and radicles of alfalfa. Phenolics also play a key role in the plant's defense against ROS-induced damage by eliminating free radicals [22,23]. Drought stress also influences the formation of secondary metabolites, including phenols and flavonoids which enhance plant defense mechanisms [24–26]. Phenylalanine ammonia-lyase (PAL), a key regulating enzyme, promotes the synthesis of phenolic compounds in plant tissues [27–29]. Moreover, the production of PAL and PPO increases during stress, bolstering plant defense mechanisms [30,31]. In addition, water-stressed plants synthesize low-molecular-weight, water-soluble organic compounds [32,33]. The resultant lower osmotic potential helps to maintain the cellular turgor and volume which are crucial for metabolic processes [34–36].

The physiological activities of various crops, including chickpea [37], cowpea [38], lentil [3], and walnut [39], etc., under PEG-induced drought stress at the germination stage have been demonstrated. However, no research has been performed so far on Tartarian buckwheat at seed germination stage under PEG-induced drought stress. This is the first study in Tartary buckwheat to look at PEG-6000-induced drought stress at the germination stage to identify tolerant and susceptible genotypes. The results will be helpful in selecting the most tolerant Tartary buckwheat genotypes for future breeding programs.

2. Results

2.1. Effect of PEG-Induced Drought Stress on Seed Germination and Seedling Morphology

2.1.1. Germination Percentage

The germination percentage of all the tested genotypes steadily decreased with the increase in PEG-6000 concentration (Figure 1A). The highest germination reduction was found at 30% PEG (T_3) in QianKu-4 (96%) followed by XiQiao-3 (93%), whereas the lowest

was in QianKu-5 (39%) followed by XiNong 9943 (43%) in contrast to its normal growth condition (T_0) (Figure 1A). In addition, among the treatments, the germination percentage at T_3 treatments was significantly affected compared to the control.

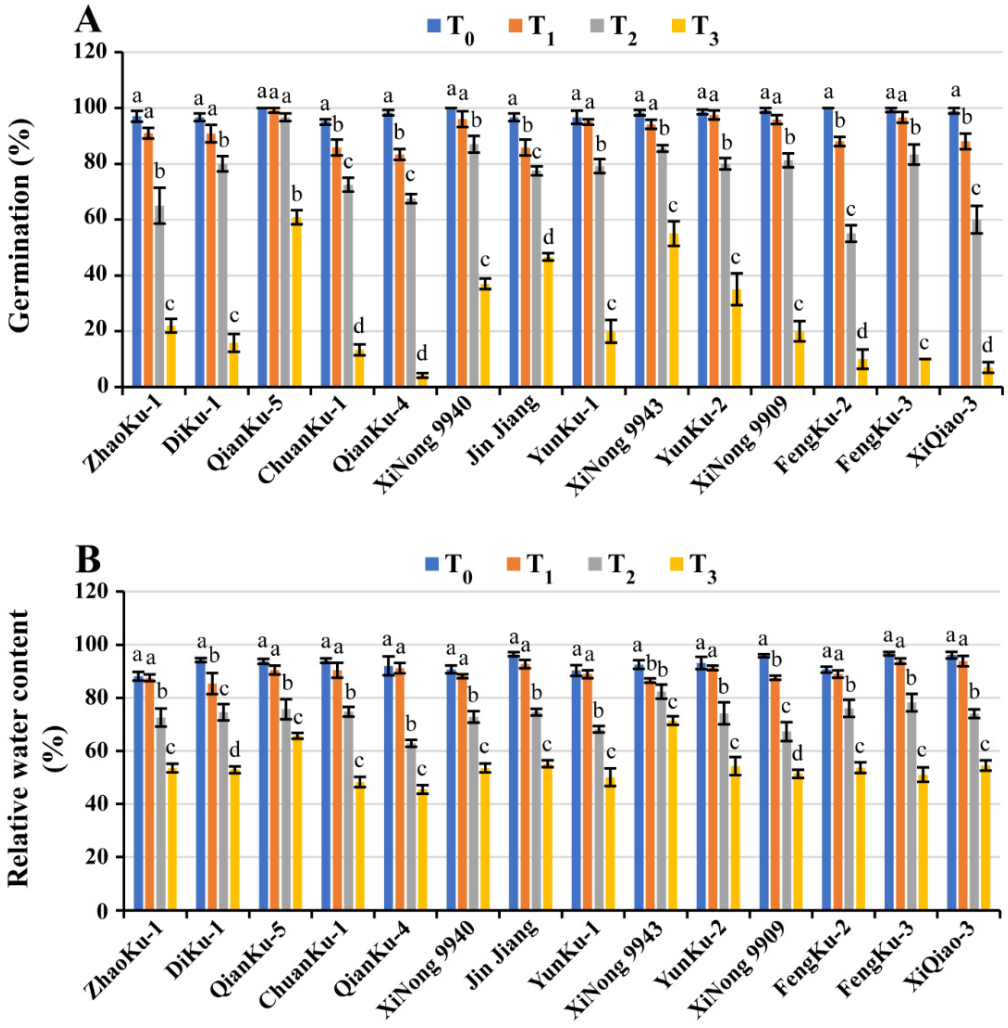

Figure 1. Effect of PEG-induced drought stress on germination (**A**) and relative water content (**B**) in different Tartary buckwheat genotypes at germination stage. T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. Different letters indicate that the mean values of the presented data are significantly different according to Duncan's test at the $p < 0.05$ level.

2.1.2. Relative Water Content

In the case of relative water content (RWC), all the genotypes showed a decreasing trend with increasing PEG concentration and eventually, in all cases, the highest reduction of RWC was reported in QianKu-4 (51%) followed by ChuanKu-1 (49%) at T_3 treatment compared to the respective control (Figure 1B). Genotype XiNong 9943 showed the lowest reduction of RWC (23%) compared to other genotypes at T_3 treatment. This result suggested that severe water stress significantly reduced the RWC of Tartary buckwheat at its germination stage.

2.1.3. Shoots and Roots Length

Both plumule and radical length of Tartary buckwheat genotypes were reduced with an increment in PEG percentage (Figure 2). Among the treatments, the highest reduction

of both parameters was observed in T_3 as compared to the normal condition (T_0). It is suggested that the growth length of shoots and roots was severely affected by the imposed drought level. But lower levels of drought stress were not significantly affected by growth of shoots and roots. In addition, among all the genotypes, the highest reduction of shoot length was observed in QianKu-4 (94%) and XiQiao-3 (94%), along with their root reduction at 95%. The lowest shoot-root length reduction was found in QianKu-5 (69% and 54%), followed by XiNong 9943 (61%) genotypes, respectively, at T_3 treatment compared to control.

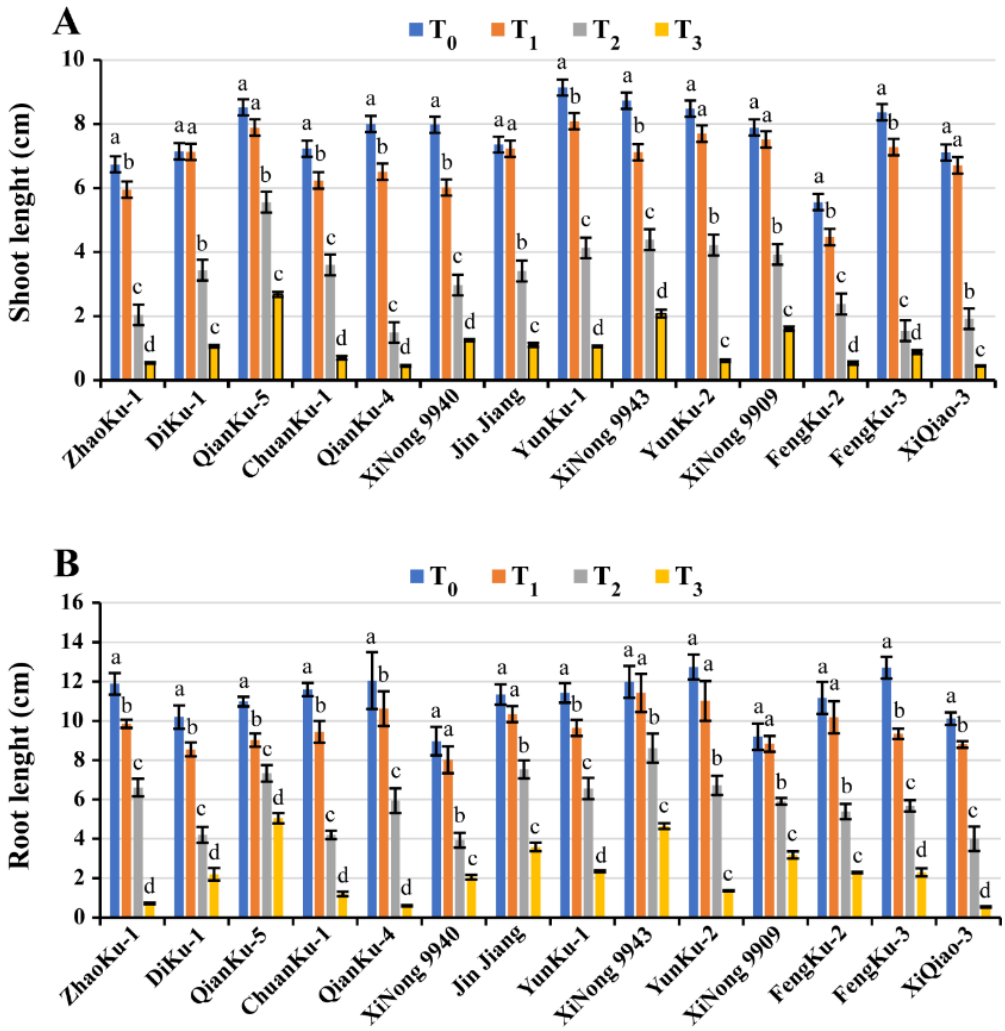

Figure 2. The PEG-induced drought stress effect on (**A**) shoot length and (**B**) root length in different Tartary buckwheat genotypes at the germination stage. T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% FC PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. Different letters indicate that the mean values of presented data are significantly different according to Duncan's test at the $p < 0.05$ level.

2.1.4. Fresh and Dry Weight of Shoots and Roots

All 14 genotypes of Tartary buckwheat revealed a common trend in reduction rate in fresh and dry weight of both shoots and roots with increasing PEG-6000 concentration due to osmotic stress. Among the treatments, the highest reduction in fresh and dry weight of shoots and roots was observed in T_3 treatment as compared to control (Figures 3 and 4). Among the genotypes, the lowest shoot fresh weight was estimated in ChuanKu-1 (6.54 mg) followed by QianKu-4 (6.63 mg), and shoot dry weight in ChuanKu-1 (1.19 mg) followed by XiQiao-3 (1.45 mg). Similarly, the lowest root fresh weight was reported in QianKu-4

(1.55 mg) followed by ZhaoKu-1 (1.56 mg) while root dry weight was in XiQiao-3 (0.18 mg) and QianKu-4 (0.19 mg) at the 30% PEG treatments.

2.2. Measurement of Stress Tolerance Index Based on Seed Germination and Seedling Morphology

Drought stress is a significant growth-limiting condition that impedes plant growth at any point in its life cycle. We calculated stress tolerance index (STI) for all 14 genotypes and selected three tolerant and one susceptible genotype for further biochemical investigation (Table 1). In our study, genotype XiNong 9943 performed better than other genotypes in terms of STI for most of the tested shoots and roots-related parameters. For germination, genotype QianKu-5 had the highest mean STI value (85.56) followed by XiNong 9943 and the lowest was in FengKu-2. Shoots and roots lengths are both tightly linked with stress tolerance in the seedling stage. For example, deep root systems easily harvest moisture from deeper soil. The current study demonstrated a clear decrease in both shoots and roots length during the PEG-drought-induced condition. Among the genotypes, QianKu-5 had the highest STI for shoot length, which was 63.11, and the lowest was in the genotype QianKu-4 (35.21).

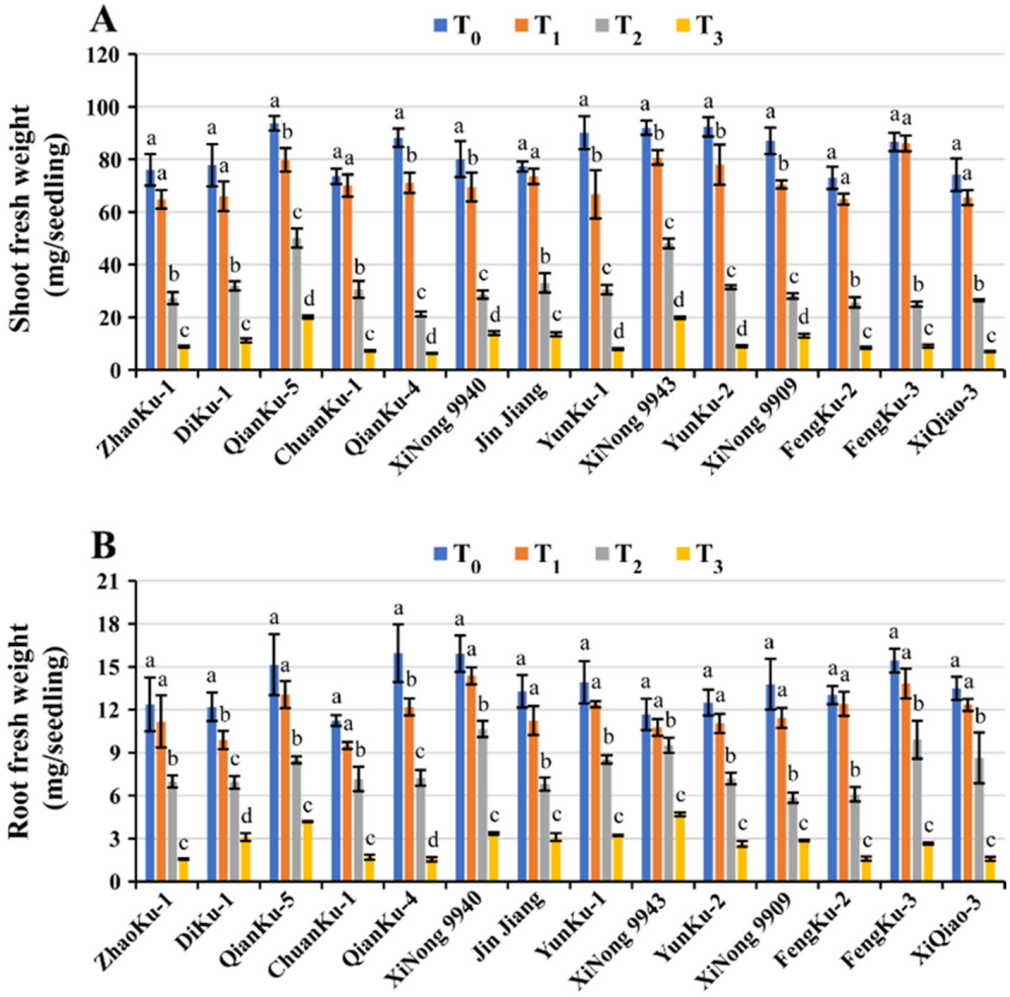

Figure 3. The effect of PEG-induced drought stress on (**A**) shoot fresh weight and (**B**) root fresh weight in different Tartary buckwheat genotypes at the germination stage. T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% FC PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. According to Duncan's test at the $p < 0.05$ level, different letters denote statistically different mean values of the reported data.

We found the highest STI for root length was in genotype XiNong 9943 (68.67) and the lowest was in ChuanKu-1 (42.68). We also tested STI for shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), and root dry weight (RDW) and found that the highest STI for SFW, SDW, RFW, and RDW was in the genotype XiNong 9943 followed by QianKu-5, and the lowest for QianKu-4. On top of these morphological traits, we also calculated STI for RWC. The mean STI for all 14 genotypes ranges from 79.93 to 89.75. Like shoots and roots traits, XiNong 9943 had the highest STI for RWC followed by QianKu-5 and the lowest was found in XiNong 9909. In the overall summation index for all the traits, STI ranked highest in XiNong 9943 followed by QianKu-5 and XiNong 9940. The lowest STI summation index was observed in QianKu-4.

2.3. Effect of PEG-Induced Drought Stress on Physiological and Biochemical Mechanisms

Based on the STI summation index, three tolerant (XiNong 9943, QianKu-5, and XiNong 9940) and one susceptible (QianKu-4) cultivars were hypothetically selected to investigate the impact of drought stress on physiological and biochemical activities at the germination stage of Tartary buckwheat.

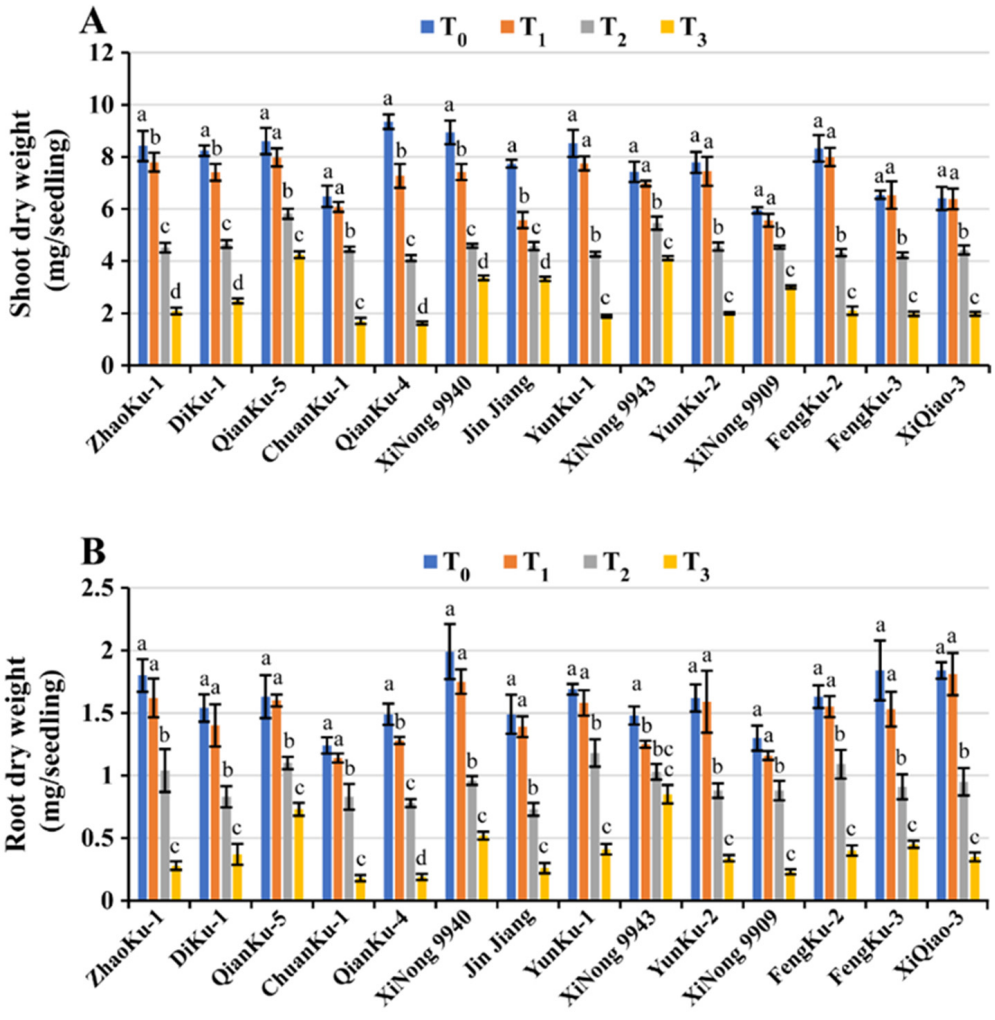

Figure 4. The PEG-induced drought stress effect on (**A**) shoot dry weight and (**B**) root dry weight in different Tartary buckwheat genotypes at the germination stage. T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% FC PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. Using Duncan's test at the $p < 0.05$ level, different letters denote statistically significant differences in the mean values of the reported data.

Table 1. Stress tolerance index performance based on germination percentage and morphological characters at germination stage.

Variety	GM (%)	Shoot Length (mm)	Root Length (mm/Seedling)	SFW (mg/Seedling)	RFW (mg/Seedling)	SDW (mg/Seedling)	RDW (mg/Seedling)	RWC (%)	STI Rank Summation Index	Ranking
XiNong 9943	79.55	51.89	68.67	53.81	71.32	74.29	70.50	89.75	1.63	1
QianKu-5	85.56	63.11	64.97	53.37	56.69	69.88	70.14	88.01	2.38	2
XiNong 9940	73.33	42.77	52.03	46.63	59.42	57.31	54.10	83.71	6.88	3
Jin Jiang	72.41	53.17	63.08	51.85	53.11	58.05	53.24	82.53	6.88	4
YunKu-2	71.86	49.25	49.92	42.73	55.63	59.95	57.82	83.62	7.00	5
XiNong 9909	66.24	55.18	65.00	42.72	48.72	73.50	58.21	79.28	7.13	6
YunKu-1	66.95	48.43	54.14	38.86	57.80	54.42	62.52	82.51	7.50	7
DiKu-1	64.36	54.22	48.90	46.75	54.43	58.86	56.28	80.25	7.63	8
FengKu-2	51.00	44.30	53.30	45.20	51.46	57.74	62.17	84.26	8.38	9
ChuanKu-1	60.23	48.59	42.68	48.89	54.42	62.92	57.80	80.09	8.75	10
XiQiao-3	52.19	42.57	43.98	44.47	55.70	66.56	56.34	82.78	8.88	11
FengKu-3	63.79	38.67	45.46	46.18	56.95	64.89	52.36	81.13	9.13	12
ZhaoKu-1	61.17	42.19	48.20	44.22	53.17	57.05	54.44	85.18	9.63	13
QianKu-4	52.54	35.21	47.47	37.24	43.85	46.38	50.34	79.93	13.25	14

STI—stress tolerance index, GM—germination, SFW—shoot fresh weight, RFW—root fresh weight, SDW—shoot dry weight, RDW—root dry weight, and RWC—relative water content.

2.3.1. ROS, MDA, and Osmotic Solutes Content

In comparison to the control, all examined cultivars underwent a progressive rise in MDA content and $O_2^{\bullet-}$ production at varying levels of PEG-induced drought stress (Figure 5A,B). The highest increasing values of MDA and $O_2^{\bullet-}$ concentration were observed in susceptible variety QianKu-4 (4.56 and 1.56 µmol g^{-1} FW) at T_3 treatment, whereas the lowest was calculated in XiNong 9943 cultivars. Meanwhile, among the treatments, T_0 and T_1 treatments did not show significant differences. However, the result suggested that the MDA content and $O_2^{\bullet-}$ generation were increased by drought stress.

Pro and soluble protein content increased significantly under PEG-induced drought stress at the germination stage. Data presented in Figure 5C,D show that Pro and soluble protein content significantly increased with increasing levels of PEG-6000 concentration and eventually, the maximum increment was observed at T_3 treatment in all genotypes. At 30% PEG, the highest Pro and soluble protein content accumulated in XiNong 9943 which was 5.55 and 6.23-fold higher than its control, QianKu-4. This result indicates that the XiXong 9943 genotype has a higher drought (PEG) tolerance capacity than the other genotypes.

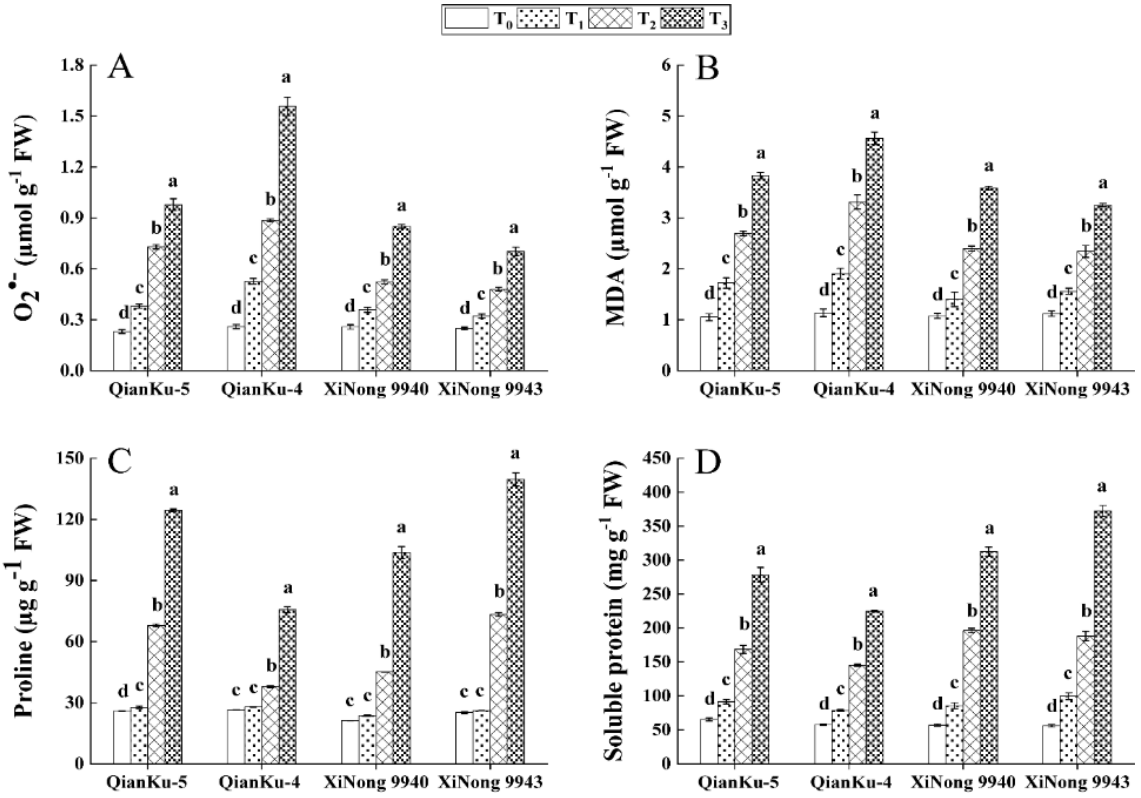

Figure 5. The effect of drought stress induced by PEG-6000 on (**A**) superoxide ($O_2^{\bullet-}$), (**B**) malondialdehyde (MDA), (**C**) proline, and (**D**) soluble protein content in different Tartary buckwheat genotypes at the germination stage. T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. Different letters indicate that the mean values of presented data are significantly different according to Duncan's test at the $p < 0.05$ level.

2.3.2. Activities of Enzymatic Antioxidants

The damaging effects of oxidative stress induced by increasing ROS content in germinated seeds of Tartary buckwheat genotypes were ameliorated by increasing activity of enzymatic antioxidants such as SOD, POD, CAT, and APX under drought stress. The activity of enzymes such as SOD, POD, CAT, and APX were elevated in all the genotypes

under varying degrees of drought stress induced by PEG (Figure 6). Ultimately, the highest increase in antioxidants across all the genotypes of Tartary buckwheat was observed at 30% PEG treatment. The maximum activities SOD, POD, CAT, and APX were recorded in the XiNong 9943 genotype and increased by 2.01, 2.19, 4.92, and 4.46 times, respectively, greater than its normal growth condition (T_0). The lowest activity of enzyme was found in the QianKu-4 genotype, which increased by 1.39, 1.45, 3.04, and 2.72 folds, respectively, compared to the control (T_0). The results showed that the activity of enzymes increased in the following order: XiNong 9943 > XiNong 9940 > QianKu-5 > QianKu-4 among the four tested genotypes.

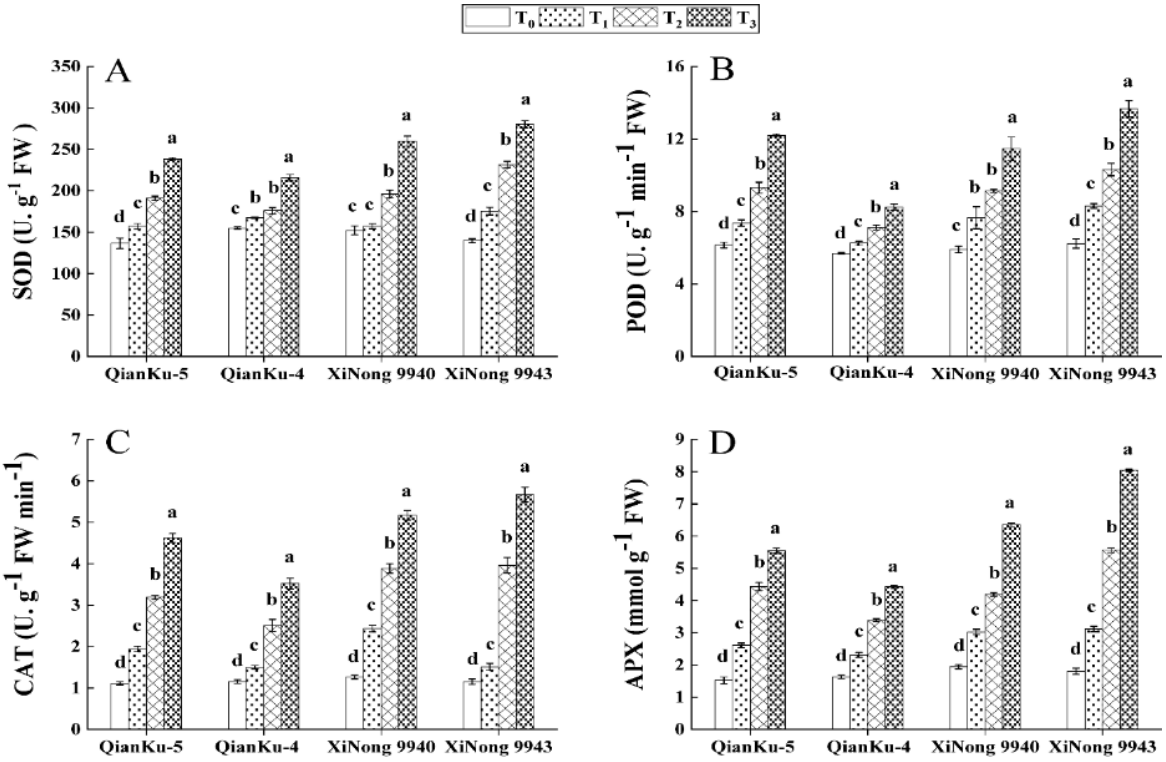

Figure 6. The impact of PEG-6000-induced drought stress on various genotypes of Tartary buckwheat at the germination stage: (**A**) superoxide dismutase (SOD), (**B**) peroxidase (POD), (**C**) catalase (CAT), and (**D**) ascorbate peroxidase (APX). T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% FC PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. According to Duncan's test at the $p < 0.05$ level, different letters denote statistically different mean values of the reported data.

2.3.3. Secondary Metabolites as Antioxidants

In comparison to the corresponding control plants, all cultivars underwent a considerable rise in the content of secondary metabolites, PAL and PPO, under varying levels of PEG concentration (Figure 7A,B). The highest increment of PAL and PPO activity was observed in XiNong 9943 (164% and 154%, respectively), followed by XiNong 9940 (158% PAL) and QianKu-5 (146% PPO) at 30% PEG treatment. On the other hand, the lowest PAL (106%) and PPO (105%) activity were observed in the QianKu-4 genotype at the same treatments.

To evaluate the effects of PEG-induced drought stress in all Tartary buckwheat genotypes, total phenol and flavonoid levels were calculated as non-enzymatic activities (Figure 7C,D). The concentration of phenolics and flavonoids was dramatically increased under PEG-induced drought stress. At 30% PEG-induced drought, the XiNong 9943 genotype yielded the highest phenolic and flavonoid content, which was 131% and 95% higher than their controls, respectively. On the other hand, the lowest was found in the QianKu-

4 genotype, which increased by 72% and 63% higher than the respective control at the same treatment.

This study also used the FRAP assay and free radical DPPH scavenging activity measurement to assess the total antioxidant capacity (Figure 7E,F). The result showed that increasing levels of DPPH scavenging and FRAP capacity gradually increased with increasing levels of PEG concentration, eventually increasing in all cases to levels that were 1.60 and 3.90 times higher at T_3 treatment than the control. The lowest activity was observed in the QianKu-4 genotype.

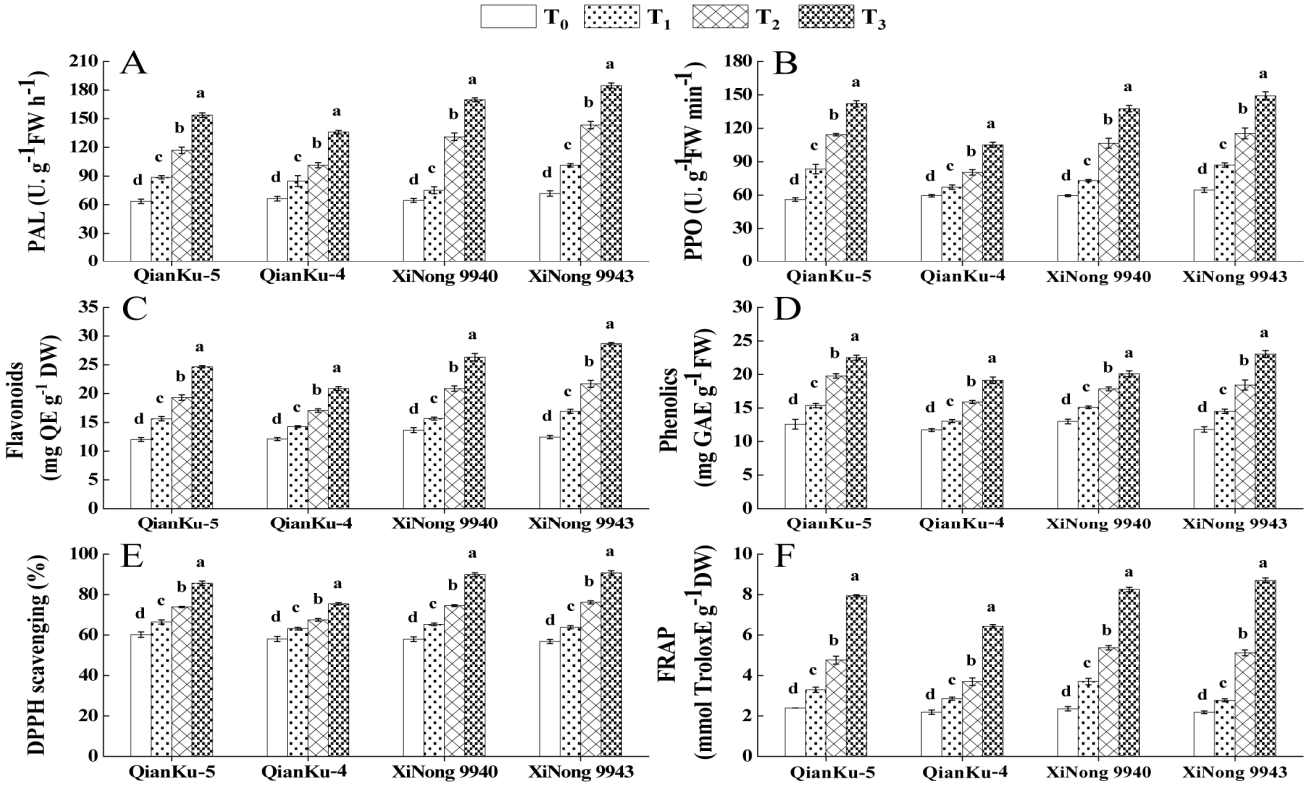

Figure 7. The impact of PEG-6000-induced drought stress on various Tartary buckwheat genotypes at the germination stage in terms of (**A**) phenylalanine ammonia lyase (PAL), (**B**) polyphenol peroxidase (PPO), (**C**) flavonoids, (**D**) phenolics, (**E**) DPPH scavenging activity, and (**F**) ferric reducing antioxidant power (FRAP). T_0: well-watered, T_1: drought stress maintained at 10% PEG-6000 concentration, T_2: drought stress at 20% FC PEG-6000, T_3: drought stress at 30% PEG-6000. Values indicate the means of four replications and the error bars represent the standard error. Different letters indicate that the mean values of presented data are significantly different according to Duncan's test at the $p < 0.05$ level.

2.3.4. Pearson's Correlation Analysis

The result of Pearson's correlation analysis illustrated that MDA and $O_2^{\bullet -}$ significantly ($p \leq 0.01$) have a positive correlation with Pro, soluble protein, SOD, POD, CAT, APX, PAL, PPO, flavonoids, phenolics, FRAP, and DPPH scavenging capacity (Table 2).

Table 2. Pearson's correlation analysis among the biochemical parameters.

Parameter	$O_2^{\bullet-}$	MDA	Pro	SP	SOD	POD	CAT	APX	PAL	PPO	Flav	Phenol	DPPH	FRAP
O_2	1													
MDA	0.94 **	1												
PRO	0.611 **	0.766 **	1											
SP	0.651 **	0.815 **	0.952 **	1										
SOD	0.597 **	0.756 **	0.932 **	0.956 **	1									
POD	0.45 **	0.645 **	0.913 **	0.915 **	0.91 **	1								
CAT	0.602 **	0.773 **	0.911 **	0.963 **	0.941 **	0.919 **	1							
APX	0.556 **	0.74 **	0.928 **	0.96 **	0.962 **	0.946 **	0.963 **	1						
PAL	0.627 **	0.801 **	0.916 **	0.963 **	0.956 **	0.92 **	0.949 **	0.967 **	1					
PPO	0.58 **	0.763 **	0.929 **	0.942 **	0.93 **	0.94 **	0.939 **	0.951 **	0.953 **	1				
Flav	0.604 **	0.78 **	0.924 **	0.972 **	0.96 **	0.942 **	0.964 **	0.974 **	0.974 **	0.964 **	1			
Phenol	0.661 **	0.808 **	0.908 **	0.919 **	0.886 **	0.901 **	0.935 **	0.924 **	0.907 **	0.938 **	0.923 **	1		
DPPH	0.62 **	0.792 **	0.93 **	0.967 **	0.937 **	0.921 **	0.967 **	0.955 **	0.958 **	0.951 **	0.963 **	0.93 **	1	
FRAP	0.681 **	0.825 **	0.949 **	0.975 **	0.938 **	0.907 **	0.965 **	0.939 **	0.943 **	0.938 **	0.961 **	0.928 **	0.971 **	1

Here, $O_2^{\bullet-}$—superoxide, MDA—malondialdehyde, Pro—proline, SP—soluble protein, SOD—superoxide dismutase, POD—peroxidase, CAT—catalase, APX—ascorbate peroxidase, PAL—phenylalanine ammonia lyase, PPO—polyphenol peroxidase, Flav—flavonoids, DPPH—free DPPH radical scavenger, FRAP—ferric reducing antioxidant power, and ** indicates 1% significance ($p < 0.05$).

2.3.5. Heatmap and PCA

The heatmap hierarchical analysis revealed a gradual decline in seedling growth parameters and relative water content (RWC) with increasing drought stress levels, with the highest values observed in T_0 and T_1 treatments (Figure 8A). The most substantial enhancements were observed in T_3 treatment, while the lowest values were recorded in T_0 and T_1 treatments. Hierarchical clustering identified five distinct clusters among the sixteen treatments, with drought being the key clustering factor. T_2 and T_3 treatments of all the genotypes formed individual clusters, while T_0 and T_1 treatments clustered together due to higher germination values and growth parameters, suggesting no significant differences between them. Additionally, T_2 and T_3 treatments formed separate clusters due to increased levels of MDA, ROS, osmolytes (soluble protein and Pro), enzymatic and non-enzymatic antioxidants, and total antioxidant capacity. The clustering indices suggested that drought stress alters phytochemical parameters and establishes strong correlations among them.

Principal component analysis (PCA) was employed to assess the tolerance levels of tested genotypes and explore correlations under drought stress (Figure 8B). PCA results aligned with heatmap clustering, illustrating distinct clusters for control and PEG-induced drought stress genotypes. PC1 and PC2 explained 88.9 and 6.7% of cumulative variance, respectively, forming separate clusters, consistent with heatmap findings. PC1 indicated strong positive correlations among MDA, ROS, the activity of enzymes, and total antioxidant capacity, while PC2 showed negative correlations. The second PCA aimed to evaluate the genotypic performance (Figure 8C), revealing significant differences among the studied genotypes under severe drought stress. However, T_0 and T_1 treatments showed no significant genotype distinctions. XiNong 9943, QianKu, and XiNong 9940 genotypes showed higher tolerance to drought, characterized by elevated osmotic solutes, antioxidant capacity, and enzymatic/non-enzymatic antioxidants. Conversely, QianKu-4 showed the lowest performance due to increased MDA content and ROS levels under PEG-induced drought stress. These findings provide insight into genotype-specific responses to drought and potential markers for drought tolerance.

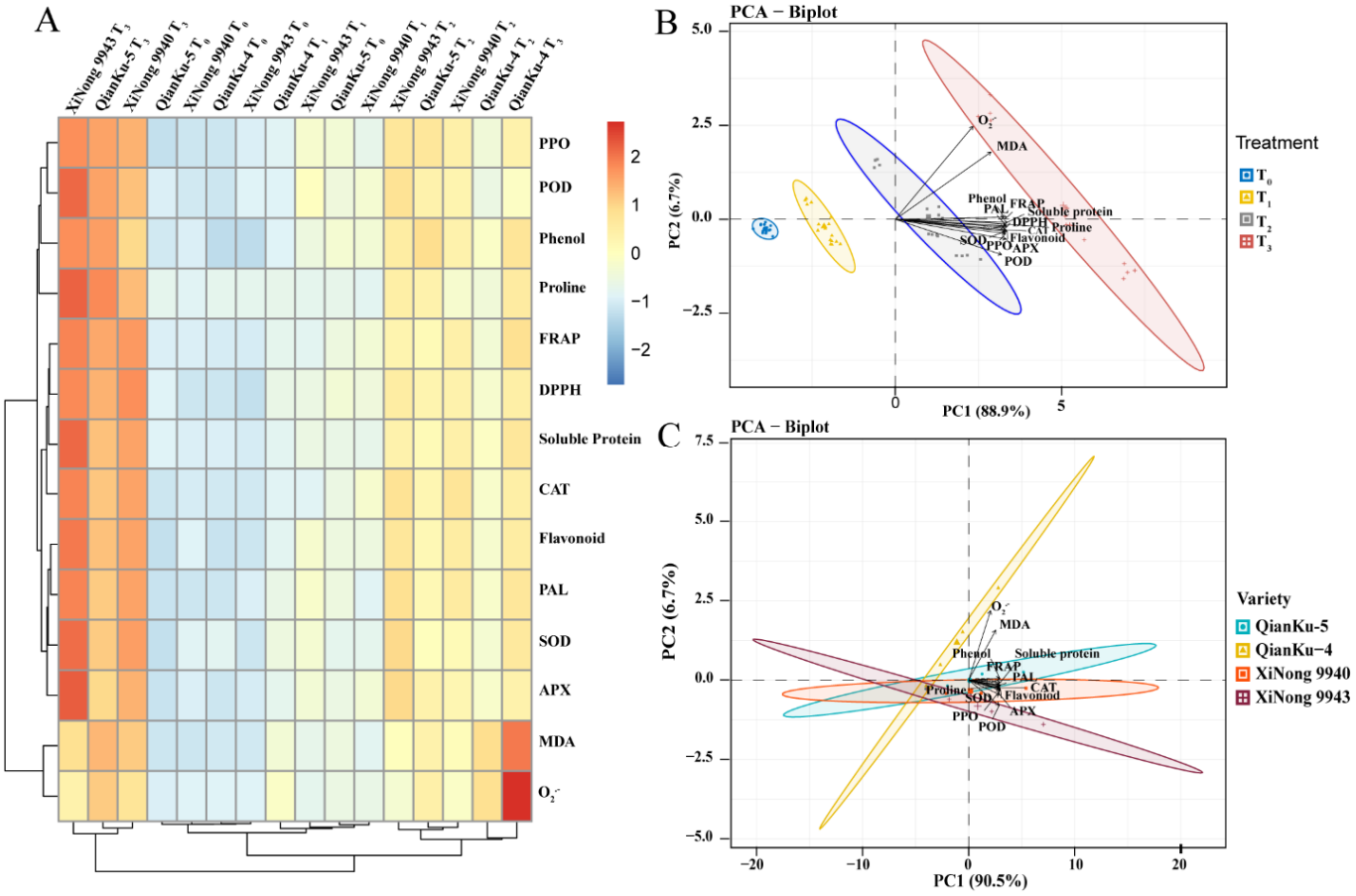

Figure 8. Drought stress impacts were assessed through hierarchical clustering analysis using various enzymatic and non-enzymatic activities, ROS, MDA, plant growth parameters, photosynthesis rate, Chl pigments, stomata properties, RWC, and osmotic solutes (**A**). Principal component analysis (PCA) was among the treatments (**B**). PCA was performed between all treatment and varieties using all trails (**C**). Here, T_0: well-watered as maintained at 80% field capacity (FC), T_1: drought stress maintained at 60% FC, T_2: drought stress at 40% FC, T_3: drought stress at 20% FC.

3. Discussion

Drought stress poses a significant challenge to agricultural productivity, particularly in arid and semi-arid regions [40,41], affecting seed germination and seedling emergence [12]. Our research showed that when PEG-induced drought stress increased, Tartary buckwheat seed germination and early seedling establishment gradually decreased. This decline in germination percentage is attributed to various factors, including reduced water availability, which hinders reserve mobilization, respiration, hormonal and enzymatic activities, and protoplasm dilution necessary for efficient embryonic growth [42]. Our findings corroborate previous research on lentils, indicating a gradual reduction in germination percentage with increasing PEG concentration [3].

Shoots and roots lengths are crucial indicators of drought resistance in plants. Our results delineated a consistent decrease in shoots and roots lengths across all the genotypes under PEG-induced drought stress, with higher reductions observed in drought-sensitive cultivars like QianKu-4. This pattern aligns with prior investigations on cowpea, *Lactuca sativa*, wheat, and maize, elucidating notable variations in shoots and roots length during germination under drought stress conditions [31,32,43]. Moreover, our analysis of fresh and dry weights of roots and shoots revealed a reduction in response to PEG-induced drought stress across the studied genotypes. While tolerant cultivars showed minimal changes in biomass accumulation, susceptible cultivars experienced significant reductions

in seedling water content and biomass accumulation. Notably, XiNong 9943, QianKu-5, and XiNong 9940 genotypes demonstrated a greater ability to withstand drought, as evidenced by their reduced shoots and roots length reductions and biomass accumulation in comparison to other cultivars. Several previous studies demonstrated that genotypes with elongated shoots and roots lengths [24,44], and lesser reductions in seedling water content and biomass accumulation [45,46] under drought stress conditions are essential for sustainable agricultural practices. These results emphasize the importance of identifying and selecting genotypes with superior drought tolerance traits to enhance crop productivity in water-stressed environments.

Our results highlight the significance of maintaining optimal leaf water status and developing osmotic adjustment mechanisms to enhance ROS detoxification, protein and enzyme stability, and cell membrane protection under PEG-induced drought stress conditions [26,27,47]. Consistent with the observations in other crops, our study demonstrated a reduction in relative water content (RWC) in Tartary buckwheat seedlings under PEG-induced drought stress [5,43]. The XiNong 9943 genotype showed higher RWC, suggesting a more effective water uptake system possibly associated with increased proline (Pro) content and osmotic solute accumulation. Malondialdehyde (MDA) levels serve as crucial indicators of membrane lipid peroxidation and damage, reflecting a variety tolerances to stress [16,48]. Under drought stress, we found increased ROS and MDA accumulation, with the susceptible genotype (QianKu-4) showing the highest concentrations and the tolerant genotype (XiNong 9943) showing the lowest. Tolerance mechanisms may mitigate $O_2^{\bullet -}$ and MDA concentrations by regulating osmotic solutes and enhancing antioxidant defenses, thereby preserving membrane permeability [49]. Compatible solutes like Pro play pivotal roles in maintaining osmotic potential and scavenging free radicals under drought conditions [37,41,50]. Our study indicated that the dramatic increases in soluble protein and Pro levels in PEG drought-stressed seedlings, with the susceptible genotype (QianKu-4) being more negatively impacted, highlighted its higher susceptibility to osmotic stress. These results corroborated past research showing elevated Pro content in response to drought stress, facilitating seed germination and seedling development [51,52].

Plants possess inherent defense mechanisms against excess ROS-induced oxidative damage by upregulating the activity of enzymes, including SOD, POD, CAT, and APX [53,54]. Our study demonstrated that PEG-induced drought stress significantly augmented the activity of these antioxidant enzymes across Tartary buckwheat genotypes, reflecting their role in mitigating oxidative stress, a finding consistent with previous research in spinach and maize [55]. With the phenylpropanoid pathway, PAL catalyzes the conversion of L-phenylalanine to trans-cinnamic acid, a precursor for phenolic compounds like phenols and flavonoids [26,32,41]. All genotypes showed an increase in PAL activity under PEG-induced drought stress, indicating PAL's function in promoting the synthesis of phenolic compounds in response to ROS exposure. Another phenolic enzyme, PPO, plays a critical role in oxidizing phenols to quinones, contributing to plant stress tolerance [56,57]. Our results revealed heightened PPO expression levels in response to drought stress, consistent with research indicating the induction of PAL and PPO activities under stress conditions to bolster phenolics and flavonoids, thus reducing oxidative damage [19,37,50]. Furthermore, secondary metabolites, including phenols and flavonoids, were found to be substantially influenced by drought stress and genotype variations, with tolerant cultivars exhibiting higher concentrations. The positive correlation between PAL activity and phenolic content underscores the significance of PAL in regulating phenolic metabolism under drought stress conditions, aligning with previous studies suggesting that drought-stressed plants increase phenolic compound production through enhanced PAL activity [58,59]. These results clarify the critical roles that phenolic and enzymatic pathways play in protecting Tartary buckwheat genotypes from oxidative stress and improving their resistance to drought.

In addition, we measured the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP) to determine the total activity

of enzymes in germination seedlings of Tartary buckwheat genotypes. The higher FRAP and DPPH activities observed in drought-exposed plants compared to drought-unexposed plants indicate enhanced total antioxidant capacity under drought stress conditions, consistent with previous research [19,60,61]. These results underscore the influence of phenolic and flavonoid levels on overall antioxidant activity, highlighting the positive relationship between PAL activity, phenolic compounds, FRAP, and DPPH scavenging activity [16,47,62]. Our results validate the hypothesis that higher PAL activity raises phenolic compound levels, which in turn increases total antioxidant activity and helps Tartary buckwheat genotypes resist drought better [6].

Heatmap hierarchical clustering, PCA, and Pearson's correlation analysis were performed to assess genotypic performance under drought stress by evaluating the interrelations among principal components and plant adaptation indexes. The correlation analysis revealed significant positive correlations among MDA, $O_2^{\bullet -}$, osmotic solutes, total antioxidant capacity, enzymatic, and non-enzymatic antioxidants, align with previous studies demonstrating the intricate network of biochemical responses to drought stress [18,63,64]. Strong correlations between physiological and biochemical indices were revealed by the heatmap hierarchical clustering, which also showed the intricate relationships between several traits across various treatments. PCA results echoed the heatmap clustering findings, observing positive correlations among traits assessing drought tolerance. The distinct clustering observed among all traits, particularly the identification of the XiNong 9943 genotype as exhibiting the highest drought tolerance. This result is consistent with prior research indicating the importance of RWC regulation, osmotic adjustment, and antioxidant defense mechanisms in conferring stress resilience [17,65–67]. Overall, the integration of these analytical approaches enhances our understanding of genotypic responses to PEG-induced drought stress, elucidating key physiological and biochemical mechanisms underlying drought tolerance in Tartary buckwheat genotypes.

4. Materials and Methods

4.1. Experimental Materials and Locations

Fourteen genotypes of Tartary buckwheat were collected from the Northwest A&F University in Shaanxi, China (34.26° N, 108.06° E). Prior to sowing, uniform seeds were properly cleaned, surface sterilized for 15 min with 0.3% (*v*/*v*) sodium hypochlorite solution, and then rinsed three times in sterile distilled water. After this, for each genotype, 25 seeds were placed on two-layer filter paper in a Petri dish (9 cm) containing 10 mL of deionized water as control (T_0) or solutions of 10% (T_1), 20% (T_2), and 30% (T_3) PEG-6000 to induce drought stress. Four replications were performed for each treatment of every genotype. The Petri dishes were arranged in a complete randomized block design within a controlled growth chamber set at 25 ± 1 °C, with a dark condition and 70% relative humidity. After 11 days, germinated seeds were collected for morphological and physiological analysis. Shoots were separated from roots, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent biochemical assays.

4.2. Germination Percentage, Morphological Parameter, and Relative Water Content

To assess germination percentage, seeds were monitored daily for the first 8 days in the growth chamber, considering the emergence of the radical through the seed coat as germination. After 11 days, five seedlings were randomly selected for measuring plumule and radical length, as well as fresh and dry weights. Plumule and radical lengths were determined using a vernier scale, and the electronic balance meter was used to record the fresh and dry weights. The relative water content (RWC) of leaf samples was determined following the method of Bowman [68]. After measuring fresh weight (FW) initially, samples were then saturated with deionized water for one day in darkness. After carefully wiping away surface water with a paper towel, the turgid weight (TW)

was calculated. Subsequently, samples were oven-dried at 80 °C for 72 h, enabling the calculation of dry weights (DW). RWC was then calculated by the following formula:

$$\text{RLWC (\%)} = [\text{FW} - \text{DW}]/[\text{TW} - \text{DW}] \times 100$$

4.3. Measurement of Stress Tolerance Index

The most severe and contrasting genotypes for a thorough physio-biochemical analysis were identified by ranking the genotypes based on their total STI value. The stress tolerance index (STI) was calculated for each genotype against those traits using the formula:

$$\text{STI} = (\text{trait value under drought treatment}/\text{trait value under control}) \times 100$$

STI value ranging from 0 to 100 indicates tolerance, where a low value indicates drought tolerance among the genotypes [69].

4.4. Determination of Superoxide Anion, MDA Content, Antioxidant Activity, and Osmotic Solutes

The determination of $O_2^{\bullet -}$, MDA content, antioxidant activity, and osmotic solutes in Tartary buckwheat leaves was performed following the method described in our previous report [1]. Briefly, fresh samples (0.3 g) were homogenized with 10 mL of 0.05 M Na-phosphate buffer (pH 7.8) and subjected to two rounds of centrifugation at 12,000× *g* for 10 min at 4 °C. The $O_2^{\bullet -}$ concentration was quantified by measuring nitrite formation from hydroxylamine photometrically, with absorbance recorded at 530 nm. MDA content was assessed using thiobarbituric acid method to assess lipid peroxidation. The SOD activity was evaluated by its ability to prevent the photochemical reduction of nitro tetrazolium to blue formazan at 560 nm. POD activity was measured by guaiacol oxidation, with absorbance read at 470 nm. CAT activity was determined by changes in spectrophotometer readings at 240 nm at one-minute intervals. The activity of APX was measured based on H_2O_2-dependent oxidation of ascorbate, and the decrease in absorbance was measured at 290 nm. Secondary metabolites content: polyphenol peroxidase (PPO) activity was determined by assessing the oxidation of substrates like catechol, while phenylalanine ammonialyase (PAL) activity was measured by monitoring the production of trans-cinnamic acid, which is described in our previous studies [1]. For quantifying Pro content, fresh leaves (0.5 g) were homogenized with 10 mL of 3% sulfosalicylic acid. After centrifugation, the supernatant (2 mL) was mixed with 2 mL of acid-ninhydrin (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid) and 2 mL of glacial acetic acid, boiled, and then cooled. The reaction mixture was extracted with 4 mL of toluene, and the absorbance was recorded at 520 nm. The concentration of Pro was determined from a standard curve and calculated on a fresh weight basis as follows:

$$\mu\text{g proline/g FW} = [(\mu\text{g proline/mL} \times \text{mL toluene})/115.5\ \mu\text{g}/\mu\text{mole}]/[(\text{g sample})/5]$$

4.5. Estimation of Total Antioxidant Capacity and Non-Enzymatic Antioxidant Activity

Tartary buckwheat seedling samples were freeze-dried, then ground with a mortar and pestle and sieved through a 60 mm mesh screen to determine the amount of flavonoid, total phenol, and total antioxidant capacity. After shaking the ground samples at 200 rpm and 50 °C for two hours, 0.2 g of the samples was extracted using a 6 mL solution of 80% ethanol, then centrifuged at 4000× *g* for ten minutes. The determination of phenolic content was conducted using the Folin-Ciocalteu colorimetric method with slight modifications [70]. A mixture comprising 1 mL of the extract, 0.75 mL of Folin-Ciocalteu reagent, 0.25 mL of sodium carbonate (7.5%), and 1 mL of distilled water was incubated for 90 min at 30 °C. The absorbance was measured at 765 nm against a blank. Flavonoid content was determined using the $AlCl_3$ colorimetric method [71], where a solution of 0.3 mL of 5% $NaNO_2$ was vortexed with the extract and supernatant, followed by the addition of 2 mL of 1 M NaOH, and absorbance was measured at 510 nm. The free radical scavenging activity of ethanol

dry leaf extracts was determined using the DPPH (1,1-diphenyl-2-picryl hydrazyl) method suggested by Chen and Ho [72]. Extracts were diluted with 80% ethanol (10 to 100 g mL^{-1}), then mixed with DPPH reagent and vortexed. After 30 min of dark incubation at room temperature, the mixed sample (A_{sample}) and negative control (the mixer of DPPH reagent and ethanol extract solution without the sample, $A_{control}$) were measured at 517 nm. Trolox was used as a reference drug for free radical scavenging capacity. The DPPH-free radical discoloration percentage was estimated by using the following formula:

$$\text{Radical Scavenging Activity (\%)} = (1 - (A_{sample}/A_{control}) \times 100$$

Additionally, the antioxidant capacity was assessed following the ferric-reducing antioxidant power (FRAP) method [73]. To create a FRAP working reagent, 10 mM 2,4,6-tri-pyridyl-s-triazine, 0.3 M acetated buffer (pH 3.6), and 20 mM FeCl3 were combined in a 10:1:1 (*v*/*v*/*v*) ratio. To the mixture, 0.3 mL of FRAP reagent, 0.2 mL of extract sample, and 0.3 mL of deionized water were added. The absorbance was measured at 593 nm, and the FRAP activity was calculated using Trolox as a standard.

4.6. Statistical Analysis

For the ANOVA, SPSS version 20 was utilized. Most data are presented as mean and standard error. The statistical program SPSS 2020 performed the correlation analysis. The R program was used to perform both hierarchical cluster analysis and principal component analysis (PCA). Duncan's multiple range test ($p \leq 0.05$) was used to identify the samples' significant differences.

5. Conclusions

Drought can impede the germination and consequent growth of seedlings, impairing the establishment of crops through a higher accumulation of ROS and lipid peroxidation. Therefore, germplasm screening at germination or an earlier seedling establishment stage is a suitable approach for selecting drought tolerant genotypes. PEG induction is an easy, cost-effective, and rapid method of inducing drought that allows for screening of many germplasms. The genotype XiNong 9943 was comparatively more tolerant to drought stress conditions than all other genotypes, and this tolerance is associated with its higher germination percentage, shoots and roots length, RWC, PRO accumulations, and lower ROS and MDA concentration accumulations. Meanwhile, increased enzymatic activities such as SOD, CAT, POD, and APX, secondary metabolite enzymatic activities such as PAL and PPO, and non-enzymatic activities such as phenolics and flavonoids were beneficial to antagonize oxidative stress, as indicated by lower lipid peroxidation and ROS accumulation at the germination stage. Our results add evidence to support the view that Tartary buckwheat is a treasure trove of useful genes in response to drought stress, which is important to further understand the mechanism and identify specific genes of drought resistance and the future improvement of cultivated Tartary buckwheat. Further experiments are now required to define the molecular and cellular mechanisms underlying the acclimation more precisely of the XiNong 9943 to abiotic stresses using molecular and genetic approaches.

Author Contributions: Conceptualization and Methodology, M.S.H. and B.F.; Funding acquisition, Project administration, and Resources, B.F.; Formal analysis, M.S.H., J.L., M.A.I., and A.S.; Data curation, M.S.H., J.L., C.W., M.S.M., and M.F.; Software, M.S.H., M.A.I., and M.F.; Supervision, B.F.; Writing—original draft, M.S.H., F.I.M., M.A.M., R.T., and J.L.; Writing—review & editing, F.I.M., R.T., B.F., C.W., A.K.H., A.S., and A.K.M.M.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Minor Grain Crops Research and Development System of Shaanxi Province (grant numbers NYKJ-2021-YL(XN)40), "Two-chain" Fusion Crop Breeding Key Project of Shaanxi (grant number 2021-LLRH-07).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors would like to express their sincere gratitude to all the funding sources for their financial support, as well as to Sharmin Sultana, Nitesh Bormon, Yuan Yuhao, and Qinghua Yang for their amiable assistance with sample analysis and figure production.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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