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Article

Comparative Phenotype and Transcriptome Profiling in Some Grapevine Cultivars in Response to Drought Stress

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Abstract

Drought is one of the main stress factors significantly affecting the growth, development and yield of agricultural crops. This study investigated the impact of drought stress on 30 grapevine genotypes. The cultivars were then ranked according to their degree of drought tolerance and classified as drought-tolerant, intermediately tolerant or sensitive. Phenotypic characteristics (NL, NR2 and LR2) were identified that can be used to determine the stress threshold. Using transcriptomic data from five cultivars with different levels of tolerance, differentially expressed genes (DEGs) were identified in control plants and in plants under stress, as well as DEGs between different varieties when exposed to 2% mannitol. General patterns under drought stress were subsequently identified, including the activation of antioxidant defense systems and changes in the metabolism and biosynthesis of glucan, cellulose, polysaccharides, monocarboxylic acids, fatty acids and metal transport and splicing processes. It is hypothesised that drought tolerance is determined by increased expression of genes associated with glutathione metabolism and methylation processes.

Keywords: drought tolerance; grapevine; mannitol; phenotyping; RNA-seq; *Vitis*

1. Introduction

Grapevines rank first in the world in terms of production volume among fruits and berries and seventh among all agricultural crops. Global production of this crop reaches 70 million tons, occupying more than seven million hectares of land for harvesting [1], with most of its production taking place in temperate areas with a Mediterranean-type climate [2]. As precipitation becomes more sporadic in these areas, grapevines are often affected by drought stress, which would seriously affect the yield and quality of grapevines and then restrict the development of the grapevine industry [3], impacting the economic sustainability of viticulture [4]. Grapevines generally shows an avoidance strategy to drought stress, as it tends to maintain stem water potential (Ψstem) above −1.5 MPa under non-irrigated conditions [5]. However, there is variability in drought responses between *Vitis* cultivars, and drought tolerant cultivars can serve as donors of drought resistance for the creation of elite, high-quality berry cultivars to improve their performance under drought [6]. Adaptation to climate change is an important step in the future of viticulture, which depends heavily on weather and climatic conditions [7]. Quite a few studies have looked at mechanisms by which rootstocks improve grapevine drought tolerance [8–13], and how plant-water relations are affected by rootstocks that maintain transpiration and root water uptake even to the point of risking hydraulic failure [9,14].

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Yet, a better molecular mechanistic understanding of grapevine tolerance to drought is necessary to improve crop management and development of new cultivars [15].

In order to maintain the water content of cells and protection from drought stress plants were increasing the content of soluble substances such as proline, soluble sugar, betaine and polyamines [16]. Besides, reactive oxygen scavenging systems (ROS) in plants, including catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and other antioxidant enzymes, could also work together to maintain the balance of ROS level and avoid oxidative damage to plants [17].

In response to different stresses, plants have been protected by complex regulatory networks in which transcription factors play a central role by regulating target gene expression leading to downstream physiological level changes [18]. The complexity of the processes involved in developing plant stress tolerance stems from the fact that the implementation of the genetic program is determined by a large number of external and internal factors. Various transcription factors (such as WRKY, MYB, ERF, and the NAC transcription factor family) are involved in modulating the transcription of genes sensitive to abiotic stress. In the current situation, only complete transcriptomic data can shed light on the complex molecular mechanisms regulating plant resistance to drought stress.

2. Results

2.1. Clustering of Grapevine Cultivars According to Drought Resistance

After one month of exposure, thirty grapevine genotypes showed different responses to drought. Nine phenotypic traits were examined in the presence of mannitol at concentrations ranging from 1% to 6%. The parameters studied were divided into two groups: biometric (RF, NL, NR1, NR2, LR1 and LR2) and biochemical (chlorophyll a and b, proline and carotenoids). Changes in the biometric characteristics of each cultivar were analysed in relation to different concentrations of mannitol (Figure 1). ANOVA revealed significant variations in biometric indicators across all levels of drought stress, when compared to the control group.

The number of first-order roots gradually decreased for all cultivars as the mannitol concentration increased, while the number of second-order roots dropped sharply by 2%. At mannitol concentrations above 3%, the number of second-order roots was almost zero for all cultivars. In contrast to NR1, the length of the first-order roots decreased more sharply with increasing stress levels, reaching close to zero for almost all cultivars at a mannitol concentration of 6%. Different levels of drought stress affected the length of second-order roots and the number of new leaves in the same way as the number of second-order roots; at a mannitol concentration above 3%, the number of second-order roots and new leaves became zero for almost all cultivars. It should be noted that risogenesis efficiency was the only biometric parameter that did not change significantly at low stress levels (mannitol concentrations of 1% and 2%). At this level of drought stress, risogenesis efficiency decreased for only a few cultivars, such as Akademik Avidzba, Malbec and Riesling. Most cultivars maintained sufficiently high risogenesis efficiency (>60%) even at a mannitol concentration of 5%. Genotypes such as Aligote, Ferkal, Kober 5BB, Livia and Syrah retained 100% risogenesis efficiency under maximum stress.

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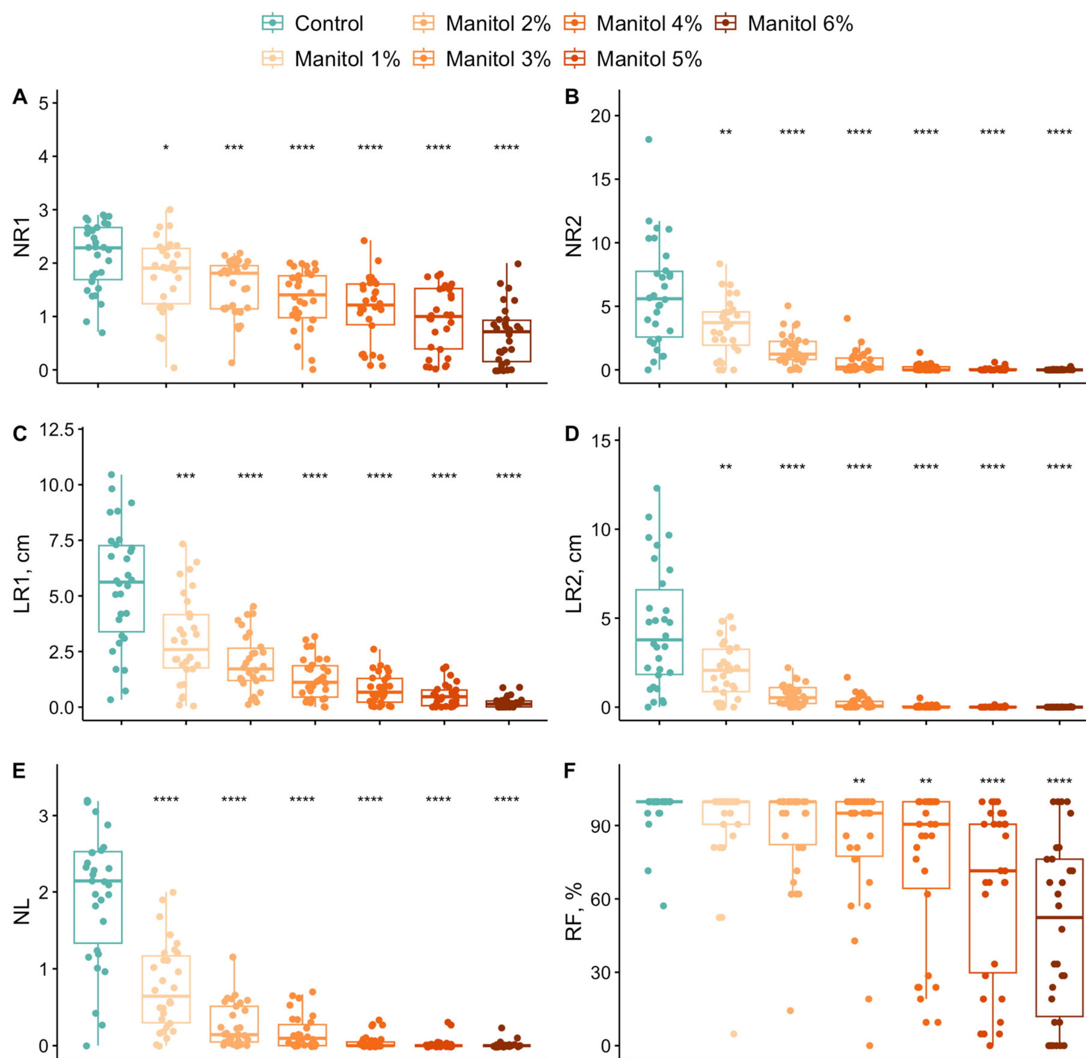

Figure 1. Biometric characteristics of each grapevine cultivar in relation to different concentrations of mannitol, where the number of first-order roots (NR1) (**A**), the number of second-order roots (NR2) (**B**), the length of first-order roots (LR1) (**C**), the length of second-order roots (LR2) (**D**), the number of new leaves (NL) (**E**) and the risogenesis efficiency (RF) (**F**) for thirty grapevine genotypes under control and drought-stress conditions. *, **, *** and **** indicate significance levels at $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ according to the t-test respectively. The results are presented with a boxplot (middle bar: median; box limits: upper and lower quartiles).

A significant decrease in carotenoids and total chlorophyll was observed as stress levels increased compared to control growing conditions (Figure 2). At the same time, a sharp decrease in carotenoid content occurred at the first levels of stress (1–2% mannitol), and at a mannitol concentration of more than 3%, carotenoid content decreased for all cultivars. In the control groups, the carotenoid content ranged from 574.8 to 5932.6 ng/100 mg of fresh weight (fw), whereas at 3% mannitol, this range was 15.3–1441.3 ng/100 mg fw. Total chlorophyll a and b decreased steadily, reaching values of 0.3–86.1 ng/100 mg fw at a mannitol concentration of 6%. In control plants, this indicator ranged from 70.2 to 255.1 ng/100 mg fw. No significant changes in proline concentration, an indicator of rapid stress, were detected 12 hours after plant incubation began under stress conditions.

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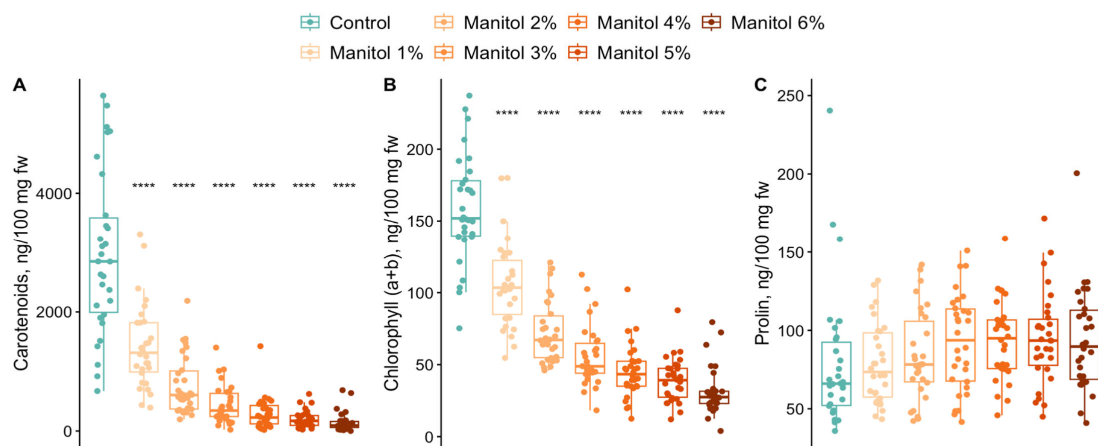

Figure 2. Biochemistry characteristics of each grapevine cultivar in relation to different concentrations of mannitol, where the contents of carotenoids (**A**), the contents of chlorophyll (a+b) (**B**) and the contents of prolin (**C**) for thirty grapevine genotypes under control and drought-stress conditions. *, **, *** and **** indicate significance levels at $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ according to the t-test respectively. The results are presented with a boxplot (middle bar: median; box limits: upper and lower quartiles).

An analysis was conducted to determine the levels of drought stress experienced by different grapevine cultivars. This analysis showed that phenotypic parameters respond differently to mannitol concentrations (see Table 1). The number of first-order roots is the only parameter that differs for each stress level (groups a–g). The length of the first-order roots varies across the stress levels up to 4% mannitol (groups a–e), but a further increase in mannitol concentration does not lead to significant changes (groups e, ef and f). The number and length of second-order roots exhibit a consistent pattern across groups under control conditions and at 1%, 2%, and 3% mannitol (groups a–d), with no significant differences observed at higher concentrations (group d). A decrease in the number of new leaves occurs at 1% (group b), then at 2–3% (group c) and at >4% (group d). The effectiveness of rhizogenesis gradually decreases and can only be separated into groups d and e at 5% and 6% mannitol respectively. Stress levels of 1%, 2% and >3% mannitol can also be distinguished based on analysis of carotenoid and chlorophyll a+b content (groups a-c and a-d respectively). So, for all phenotypic parameters except proline, a mannitol concentration of 1% is stressful and causes a decrease in these parameters. At mannitol concentrations of more than 3%, no significant changes are observed and virtually all cultivars are in a state of maximum stress.

A tolerance index (STI) was calculated for each cultivar and phenotypic trait: the higher the index, the more resistant the genotype (Table S2). This enabled us to cluster cultivars according to their drought stress resistance. As no significant changes in proline were observed when the genotypes were exposed to mannitol, this parameter was excluded from the analysis. Therefore, PCA was only performed for the STI of eight phenotypic characteristics. Based on the contribution of STI values to the main components, the data were divided, with biochemical indicators (carotenoid and chlorophyll a+b content) contributing significantly to the second main component and biometric indicators (LR1, NR2, LR2 and RF) to the first (see Figure 3b). Cluster analysis of cultivars using the FANNY method revealed an optimal number of clusters k=3 (Figure 3a). These clusters correspond to drought-sensitive genotypes (cluster 1), drought-resistant genotypes (cluster 2), and genotypes with intermediate resistance (cluster 3).

The spread of cultivars within the selected clusters varies. Sensitive cultivars form a more compact cluster, whereas resistant cultivars have a greater spread. This is because biometric and biochemical indicators react differently to stress. For instance, the Aligoté cultivar has an STI of 0.52 and 0.43 for carotenoid and chlorophyll a+b content respectively. Meanwhile, the biometric indicators NR1 and RF have an STI of 0.89 and 1.06, respectively. The STI for the remaining parameters (NL,

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LR1, NR2 and LR2) varies between 0.089 and 0.43, which is associated with a sharp decline when the mannitol concentration is increased to 4% (see Figure 1b,d,e). Such index values are observed for most resistant cultivars. However, some resistant cultivars differ significantly from the main group. The cultivar Syrah has high STI values for both biochemical parameters (STI = 1.0). The cultivar Bastardo has low index values for the biochemical parameters (0.01 and 0.012 for carotenoid and chlorophyll a+b content, respectively) but high STI values for the number and length of second-order roots compared to other cultivars (0.96 and 0.4).

Sensitive cultivars are characterized by low tolerance index values for all phenotypic traits. The mean STI value is 0 for the number and length of second-order roots, 0.019 for the length of first-order roots, and 0.089 for the number of new leaves. All sensitive cultivars demonstrate low rhizogenesis efficiency. Figure 3c shows the clustering of 30 grapevine cultivars into sensitive, intermediate and drought-resistant groups.

Table 1. Response of phenotypic parameters to drought stress experienced by different grapevine cultivars.

Condition	NR1, pcs	LR1, cm	NR2, pcs	LR2, cm	NL, pcs	RF, %	Car.	Chlor.	Prol.
							ng/100 mg of fresh weight		
Control	2.14^{a}	5.46^{a}	5.97^{a}	4.44^{a}	1.97^{a}	96.8^{a}	2983.7^{a}	159.0^{a}	79.1^{a}
Manitol 1%	1.79^{b}	3.01^{b}	3.44^{b}	2.04^{b}	0.77^{b}	90.3^{ab}	1420.4^{b}	106.1^{b}	78.6^{a}
Manitol 2%	1.56^{c}	1.96^{c}	1.63^{c}	0.68^{c}	0.27^{c}	88.9^{ab}	767.9^{c}	72.5^{c}	83.7^{a}
Manitol 3%	1.34^{d}	1.28^{d}	0.58^{d}	0.23^{d}	0.17^{c}	83.2^{bc}	437.0^{cd}	55.8^{d}	92.5^{a}
Manitol 4%	1.15^{e}	0.82^{e}	0.15^{d}	0.04^{d}	0.05^{d}	75.1^{cd}	296.1^{d}	45.0^{de}	93.6^{a}
Manitol 5%	0.94^{f}	0.52^{ef}	0.06^{d}	0.01^{d}	0.02^{d}	63.7^{d}	198.9^{d}	39.4^{e}	94.0^{a}
Manitol 6%	0.65^{g}	0.19^{f}	0.02^{d}	0.002^{d}	0.01^{d}	47.9^{e}	144.0^{d}	31.7^{e}	94.8^{a}

The mean values of morphometric and biochemical parameters were separated using the least significant difference (LSD) test at $p \leq 0.05$. Bonferroni p-value adjustment method. Different letters in superscript indicate the significant treatment effect.

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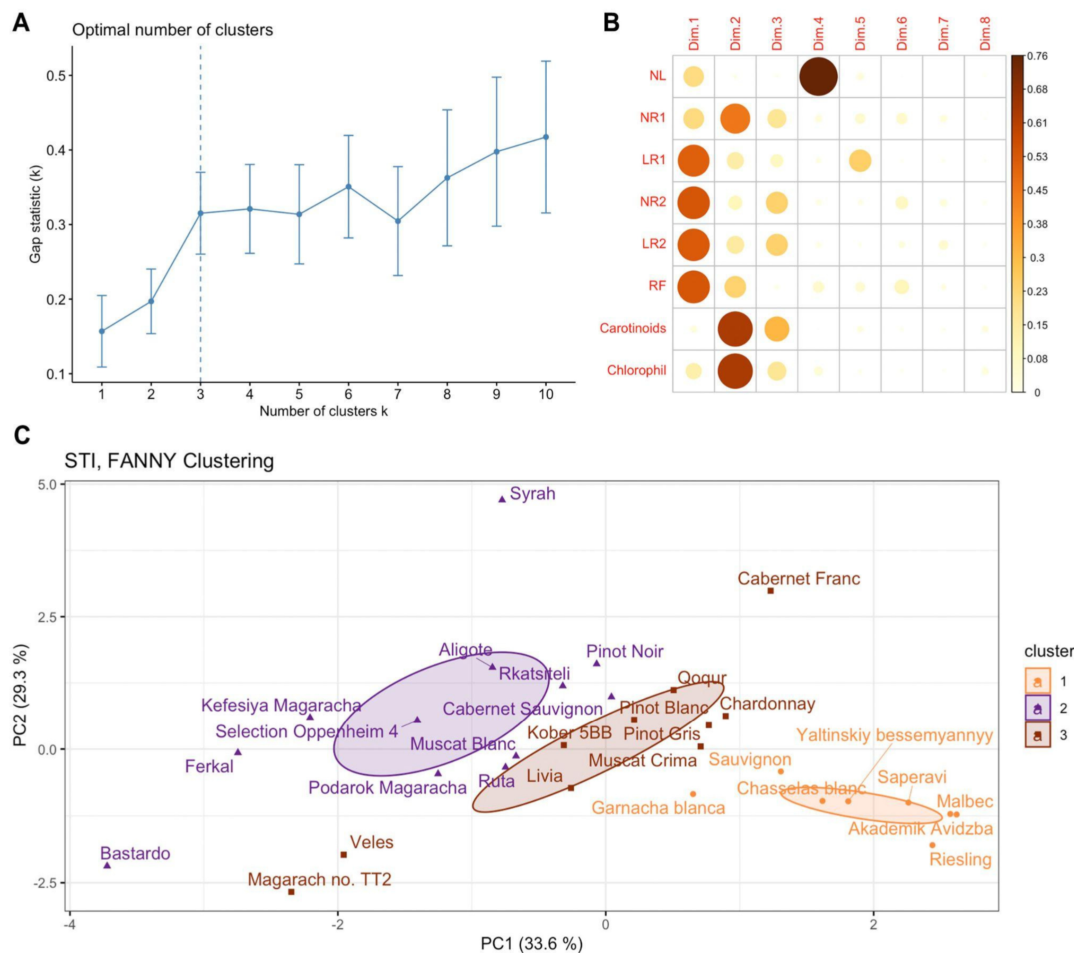

Figure 3. PCA and clustering of thirty grapevine cultivars according to the stress tolerance index (STI) to drought stress. (**A**) Gap statistic plot to determine the optimal number of clusters. (**B**) Contribution of the variables to the principal components. (**C**) Clustering of thirty grapevine cultivars. The colour indicates the cluster group. The size of the concentration ellipses notes the normal probability (0.95).

2.2. Phenotypic Response of Cultivars with Varying Levels of Resistance to Drought Stress

Cultivars with different levels of tolerance to drought stress showed distinct responses to mannitol at various concentrations. The correlation matrices for phenotypic traits differ between the sensitive, intermediate and drought-resistant groups (see Figure 4). However, there are also similarities. For example, a strong positive correlation was observed for carotenoid and chlorophyll a+b content ($r = 0.97–1.0$, $p < 0.01$), as well as between NR2 and LR2 ($r = 0.97–1.0$, $p < 0.01$), in all groups of cultivars. Other phenotypic traits showed a shift in the correlation matrix pattern in groups of cultivars with different levels of resistance. In resistant group, the parameters NR2 and LR2 showed a strong positive correlation with the number of new leaves (NL) ($r = 0.86$ and $r = 0.87$, respectively, $p < 0.01$), as well as a positive correlation with LR1 ($r = 0.75$ and $r = 0.76$, respectively, $p < 0.01$). The correlation of second-order root characteristics with other phenotypic traits is weak ($r < 0.7$, $p < 0.01$) (Figure 4C). As drought resistance in plants decreases, the degree of correlation between NR2 and LR2 with other characteristics decreases; there is no correlation in sensitive cultivars ($r = 0.35–0.56$, $p < 0.01$) (Figure 4A). The opposite situation is observed for carotenoid and chlorophyll a+b content: in resistant cultivars, a positive correlation is shown only with LR1 ($r = 0.72$ and $r = 0.73$ respectively, $p < 0.01$), which increases with other parameters as drought resistance decreases. Similarly, RF and NR1 in resistant cultivars show a positive correlation only with LR1 ($r = 0.73–0.77$, $p < 0.01$). In sensitive cultivars, all these parameters (carotenoid and chlorophyll a+b content, RF, NR1

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and LR1) form a cluster due to a strong positive correlation between them (r = 0.68–0.97, p < 0.01) (Figure 4A).

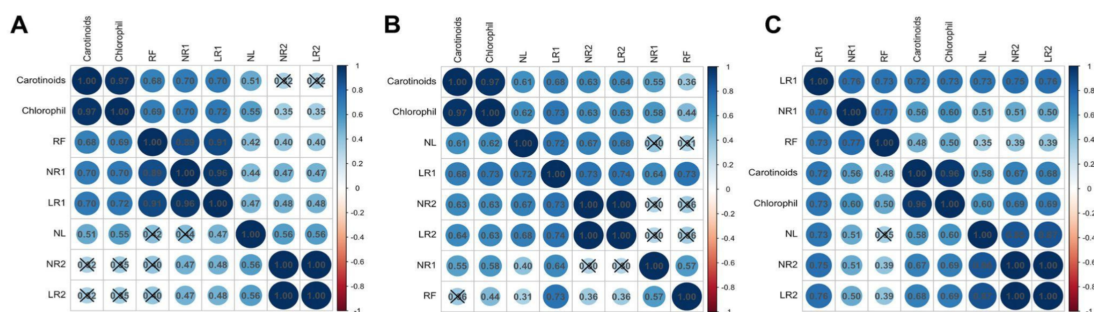

Figure 4. Spearman's correlation coefficient between phenotypic traits in drought-sensitive genotypes (**A**), genotypes with intermediate resistance (**B**) and drought-resistant genotypes (**C**). The high and low intensity of colour represents strong and weak relationships (blue for positive and red for negative) between the two variables, respectively. Values closer to one indicate a strong correlation, and a value closer to zero indicates a weaker relationship between the two variables. Crossed-out values indicate insignificant correlation between pair of parameters (significant level p<0.01). The hierarchical clustering order was chosen for the correlation matrix. Spearman's coefficients above the diagonal are given with Bonferroni correction for multiple comparisons.

To further study the response of grapevines to drought, the following contrasting genotypes were selected for assessment: resistant (Ferkal, Podarok Magaracha and Aligote), intermediate (Kober 5BB) and sensitive (Saperavi). As resistant cultivars exhibit different tolerance indices for various phenotypic parameters, three such cultivars were selected. It was shown that drought stress significantly affected shoot height, formation of new internodes and leaves, the number of roots and their length and functionality (Figures 5 and 6).

Genotypes showed different responses. Under the influence of the stress factor, the height of shoots, the number and length of roots decreased sharply. Thus, in the sensitive Saperavi cultivar, after 30 days ofanosis of leaves (except for the Aligote cultivar), a complete absence of second-order roots, and a decrease in the length of formed first-order roots (significant in the Kober 5BB rootstock and almost complete in the Saperavi cultivar) were observed in all genotypes (Figures 5 and 6B,C). The influence of the stress factor (mannitol) a significant or complete reduction in the growth processes of both the root and the shoot, partial or complete necrosis of the leaves in all the vines studied, regardless of the degree of resistance of the genotype were observed (Figure 5). Cultivation on a medium with the addition of 1% mannitol, complete inhibition of shoot growth and a decrease in the number and length of formed roots were observed. At the same time, the drought-resistant (Ferkal, Podarok Magarach, Aligote) and intermediate (Kober 5BB) genotypes did not differ from the control groups in terms of biometric parameters.

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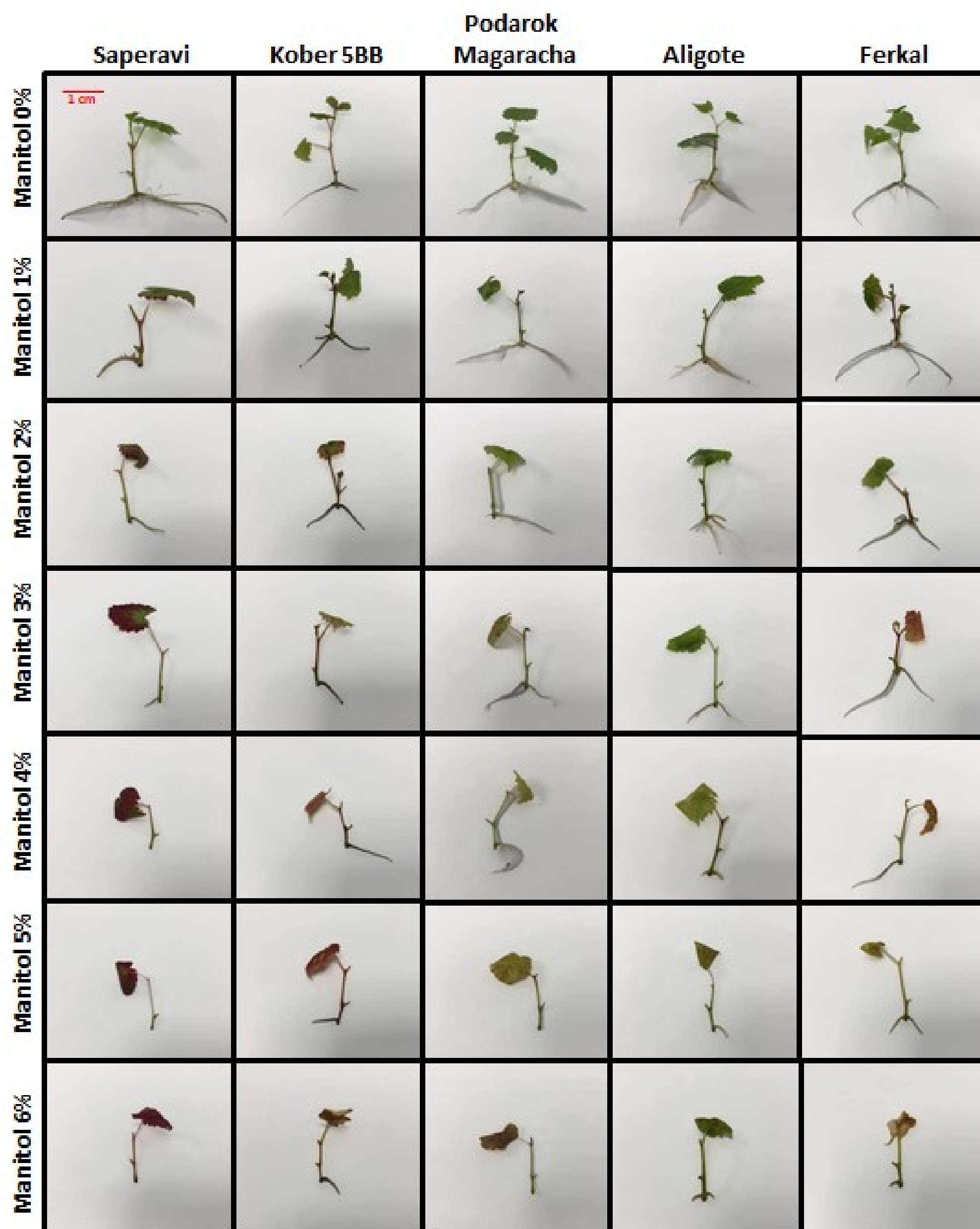

Figure 5. The effects of drought stress on the genotype when adding mannitol to the culture media at a concentration of 0 to 6% in 1% increments.

After 30 days of cultivation on a medium with the addition of 2% mannitol, the Saperavi cultivar showed significant inhibition of rhizogenesis (an even greater reduction in the number and length of formed roots, a complete absence of second-order roots), partial necrosis and anthocyanosis of the leaves. In the Kober 5BB rootstock, after 30 days of cultivation on a medium with the addition of 2% mannitol, partial inhibition of rhizogenesis (a decrease in the number and length of formed roots) and partial anthocyanosis of the leaf blade were also noted. Drought-resistant (Ferkal, Podarok Magaracha, Aligote) genotypes demonstrated only partial inhibition of shoot growth. After 30 days of cultivating explants on a medium with the addition of 3% mannitol, inhibition of shoot growth, partial necrosis and anthocyanosis of leaves (except for the Aligote cultivar), a complete absence of second-order roots, and a decrease in the length of formed first-order roots (significant in the Kober 5BB rootstock and almost complete in the Saperavi cultivar) were observed in all genotypes. A further increase in the content of mannitol in the culture medium led to a significant or complete reduction

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in the growth processes of both the root and the shoot, partial or complete necrosis of the leaves in all the vines studied, regardless of the degree of resistance of the genotype.

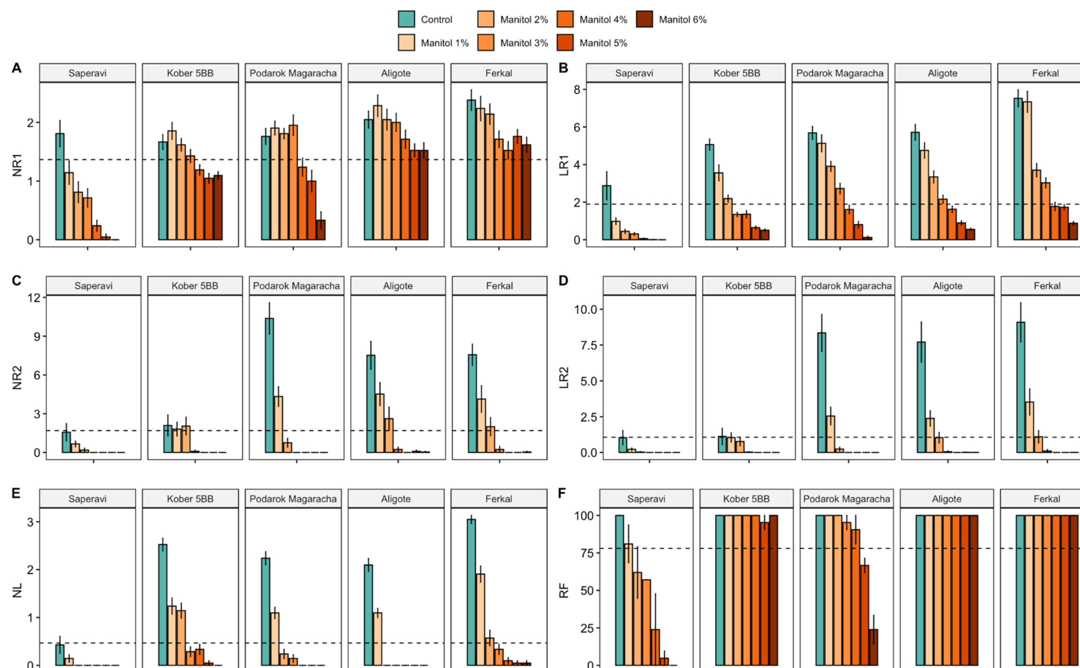

Figure 6. The statistics significant effects of drought stress in the study cultivars, where the number of first-order roots (NR1) (**A**), the length of first-order roots (LR1) (**B**), the number of second-order roots (NR2) (**C**), the length of second-order roots (LR2) (**D**), the number of new leaves (NL) (**E**) and the risogenesis efficiency (RF) (**F**) under control and drought-stress conditions after 30 days. The vertical bars represent mean of 21 replicates ± SE. The dotted line indicates the mean of the parameter for all cultivars.

Taking the biometric and biochemical data for all 30 cultivars into account, a threshold value was calculated for each parameter, which is defined as the mean value for that parameter across all cultivars. Even under slight drought stress, all biometric parameters for the sensitive Saperavi cultivar typically fall below the mean values for all cultivars (Figure 6). Meanwhile, rhizogenesis efficiency remains high for cultivars with intermediate and high resistance. The number of new leaves (NL), the length of first-order roots (LR1), the number of second-order roots (NR2) and the length of second-order roots (LR2) all decrease with an increasing mannitol concentration in all cultivars. For all the resistant cultivars (Ferkal, Podarok Magaracha and Aligoté), the NR2 and LR2 values in the control group and at a 1% mannitol concentration are higher than the average values for these parameters across all the cultivars (Figure 6C,D). The Saperavi cultivar, which is sensitive, and the intermediate Kober 5BB rootstock have average biometric parameter values below the threshold, even in the control groups.

Biochemical indicators such as carotenoid and chlorophyll (a+b) content change similarly across all cultivars, indicating stress in the plants (Figure 7). These parameters decrease as the mannitol concentration increases, crossing the threshold at 1% or 2%.

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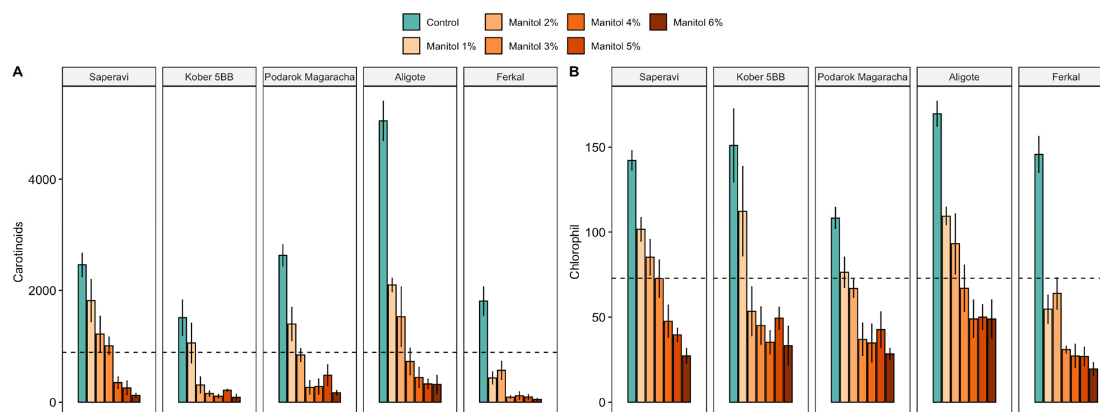

Figure 7. The statistics significant effects of drought stress in the study cultivars, where the contents of carotenoids (A) and the contents of chlorophyll (a+b) (B) under control and drought-stress conditions after 30 days. The vertical bars represent mean of 21 replicates ± SE. The dotted line indicates the mean of the parameter for all cultivars.

The third biochemical parameter, proline content, characterises the rapid response to stress. Therefore, it was measured 12 hours after the beginning of the stress period. For all cultivars, proline content tended to increase as stress levels rose (Figure 8). The sensitive Saperavi cultivar exceeded the threshold even in the control group. Proline content values remained unchanged for the two resistant cultivars (Ferkal and Podarok Magaracha). The Kober 5BB rootstock and the Aligoté cultivar crossed the threshold as the concentration of mannitol increased. Overall, none of the cultivars showed significant changes after 12 hours of drought stress. However, differences were observed between cultivars with varying degrees of resistance.

Analysis of all biometric and biochemical parameters showed that a 1% mannitol concentration is insignificant and does not exert a strong stress effect. Significant changes were observed in almost all parameters across all cultivars when the mannitol concentration was increased to 2% (Figures 6–8).

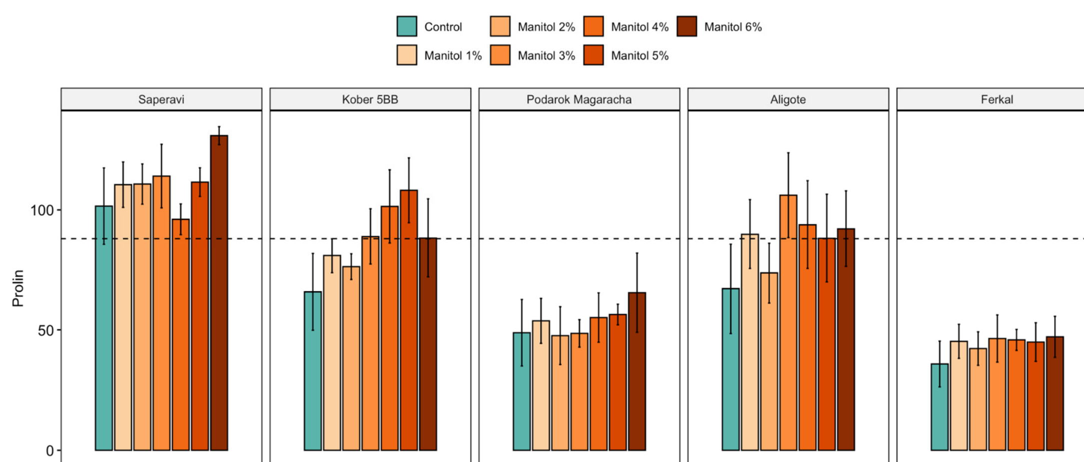

Figure 8. The statistics significant effects of drought stress in the study cultivars, where the contents of prolin under control and drought-stress conditions after 12 hours. The vertical bars represent mean of 21 replicates ± SE. The dotted line indicates the mean of the parameter for all cultivars.

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2.3. Transcriptome Profile of Cultivars With Varying Levels of Resistance Exposed to Drought Stress

To reveal the molecular mechanism of grapevine response to drought stress 20 cDNA libraries of the above five grapevine genotypes with different resistance at control and drought stress were constructed and sequenced using the DNBSeq G400 platform (BGI). 712.11 million reads were produced by 150 nt paired-end sequencing from twenty libraries. Quality control included the removal of adapters and low-quality reads. Raw reads were mapped to the reference sequences for major grapevine viruses and the human genome to eliminate potential contamination (the alignment rate was 0.66–3.55% for human genome and was 0.13–3.10% for grapevine viruses with an average of 1.65% and 0.86% respectively). The cleaned dataset ranged from 23,926,502 to 44,695,307 reads with the 44% GC content.

In order to understand the common molecular mechanisms underlying cultivars with different levels of tolerance, differentially expressed genes (DEGs) were identified in two groups of plants comprising all five cultivars: a control group and a group that was treated with 2% mannitol for 12 hours. This approach identified only those genes exhibiting the same changes in expression levels in response to drought stress, regardless of the plants' degree of resistance.

Only 55 DEGs were found for 5 libraries from control group vs 5 libraries from stressed group, in which genes 33 were up-regulated and 22 were down-regulated. Cluster analysis of DEGs revealed a clear separation of plants into control and stress-exposed groups (Figure 9A). However, no clustering of cultivars according to their degree of drought tolerance was observed within each group.

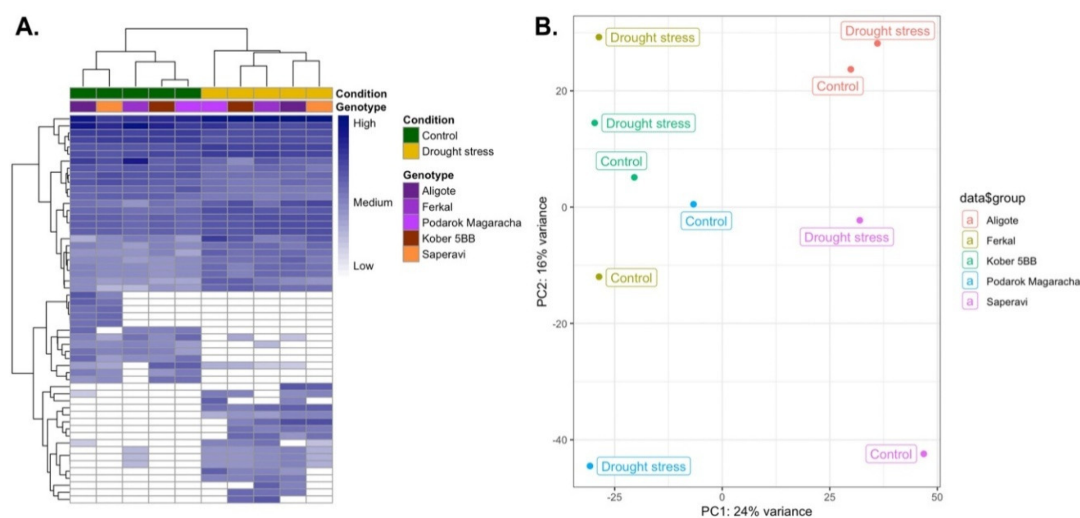

Figure 9. Transcriptome analysis of differentially expressed genes (DEGs) in five grapevine cultivars under drought stress. (**A**) Hierarchical cluster analysis of DEGs. Columns and rows in the heatmaps represent samples and genes, respectively. (**B**) Principal component analysis (PCA) of transcriptome data.

A principal component analysis (PCA) was performed based on gene expression DESeq level (Figure 9B). In the PCA scores plot, the first principal components (PCs) accounted and second PCs accounted for 24% and 16% of the total variance among the two groups, respectively. Interestingly, principal component analysis of the differentially expressed genes didn't reveal a clear distinction among the control and stressed plants. In this context, two types of cultivars can be identified based on PCA. For the Aligote cultivar and Kober 5BB rootstock, the control and stressed plants cluster quite closely together. However, for the Ferkal, Podarok Magaracha and Saperavi cultivars, the control and stressed samples differ significantly.

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2.4. Functional Prediction and Pathway Enrichment Analysis of DEGs

All DEGs were further subjected to Gene Ontology (GO) analysis. Matched DEGs were divided into three categories: biological processes, molecular functions and cellular components (Figure 10 A, B). It should be noted that there is no overlap in GO terms between up-regulated and down-regulated DEGs across all three categories, and it is possible to identify biological processes that are activated and downregulated in response to drought stress. The down-regulated DEGs in the biological process category were associated with transitional and transmembrane metal transport. It is likely that the beta-glucan, cellulose and polysaccharides metabolic processes are also inhibited as well as the biosynthesis of macromolecule, cell-wall beta-glucan, cellulose and polysaccharide (Figure 10A). In contrast, the up-regulated DEGs in the biological process category were protein-RNA and protein-containing complex organization, intra-Golgi vesicle transport, hydrogen peroxide and rRNA metabolic process, biosynthesis of monocarboxylic and fatty acid (Figure 10B). The down-regulated and up-regulated DEGs according to the molecular function and cellular component also differ in response to drought stress (Figure 10A,B).

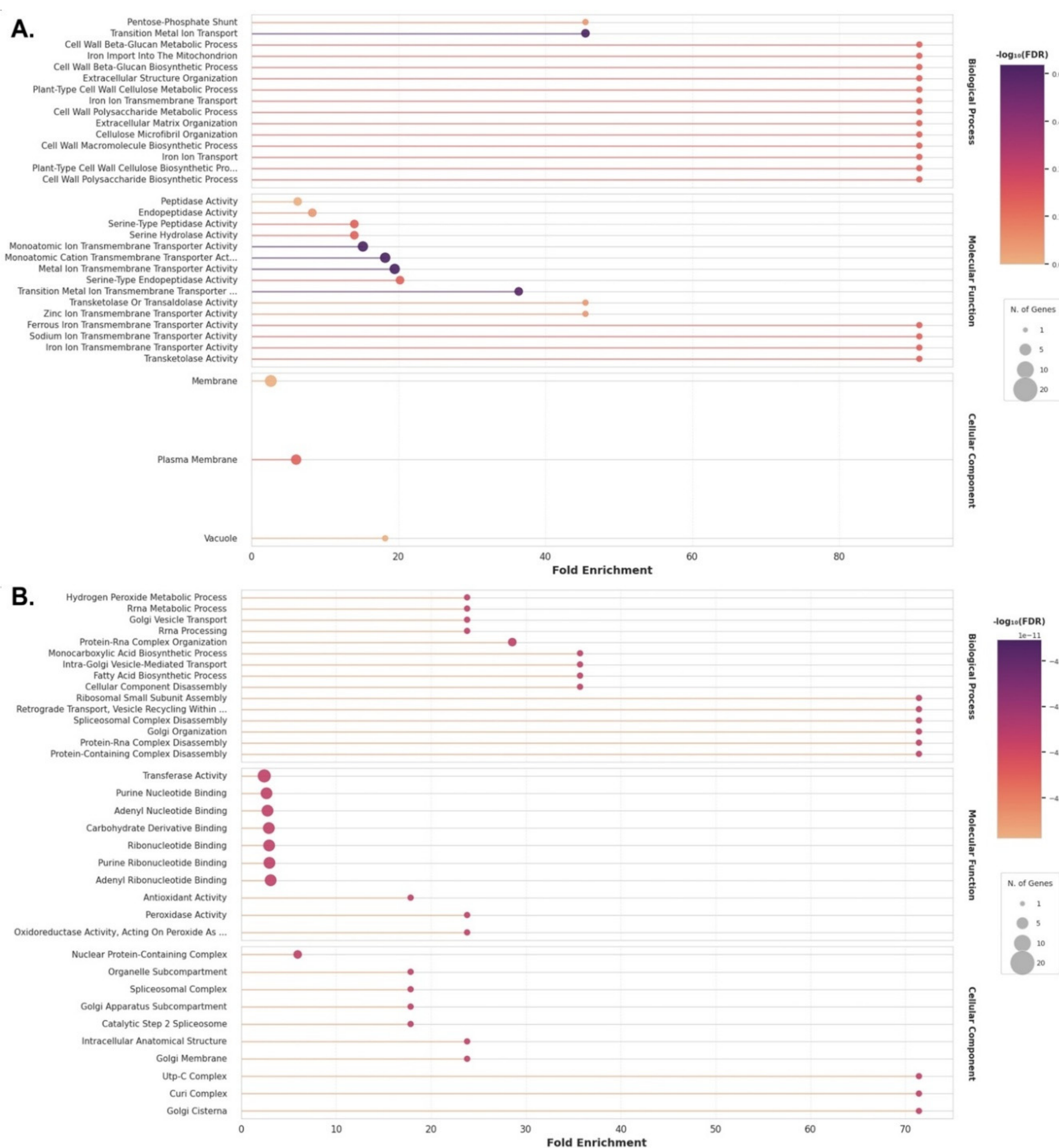

Figure 10. Gene ontology (GO) enrichment analysis of DEGs across control and stressed groups. GO enrichment analysis highlights enriched terms with $p < 0.05$. The color intensity and size of each dot represent the -log10 (p-

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value) and the number of genes, respectively. (**A**) GO enrichment of up-regulated genes, (**B**) GO enrichment of down-regulated genes.

2.5. Identification of DEGs Between Cultivars with Different Drought Tolerance Under Drought Stress

To assess the differences in the way different grapevine cultivars respond to drought, depending on their level of tolerance, additional libraries were sequenced after the plants had been exposed to 2% mannitol for six hours. A search was then conducted for DEGs between the cultivars. The DEGs analyses was carried out for the next comparison groups: Kober 5BB_vs_Fercal, Kober 5BB_vs_Aligote, Kober 5BB_vs_Podarok Magaracha, Kober 5BB_vs_Saperavi, Fercal_vs_Aligote, Fercal_vs_Podarok Magaracha, Fercal_vs_Saperavi, Aligote_vs_Podarok Magaracha, Aligote_vs_Saperavi and Podarok Magaracha_vs_Saperavi. These analyses indicated that the expression patterns of DEG genes in all ten comparison groups were significantly different. The significantly up-regulated and down-regulated genes were identified with RankProd analyses (pfp<0.05) (Figure 11A). When the DEGs of these comparison groups are represented by a Venn diagram, it was clear that both the unique and shared DEGs appeared between different pairs (Figure 11B–F).

The number of DEGs identified in the paired groups ranged from 2 to 73, with an average of 25. For some pairs of cultivars, the number of DEGs was low, indicating minor differences in gene expression patterns. For instance, only two DEGs were identified when comparing gene expression following exposure to 2% mannitol for the Kober 5BB_vs_Aligote group. In contrast, the Aligote_vs_Podarok Magaracha comparison group had the highest number of DEGs (73 in total) (Figure 11A).

When comparing the identified DEGs across paired groups under stress, practically no common DEGs were found, and many DEGs were unique to individual comparison groups (Figure 11B–F). For example, only 37 DEGs were identified for the Fercal_vs_Podarok Magaracha group; however, when identifying common DEGs in the Ferkal and other cultivars, 34 out of 37 were not identified in other comparison groups (Figure 11D).

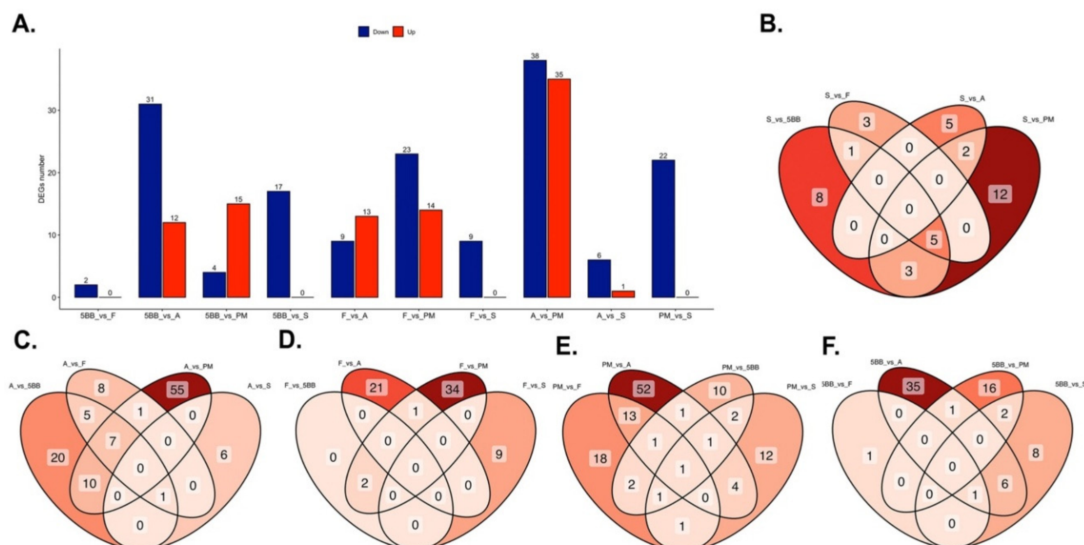

Figure 11. Differentially expressed genes (DEGs) between tested samples under stress condition identified using RankProd with pfp<0.05. (**A**) Numbers of DEGs compared between two samples are shown in red (up-regulated) and blue (down-regulated). (**B**) Venn diagram of the DEGs between Saperavi and other grapevine cultivars. (**C**) Venn diagram of the DEGs between Aligote and other grapevine cultivars. (**D**) Venn diagram of the DEGs between Ferkal and other grapevine cultivars. (**E**) Venn diagram of the DEGs between Podarok Magaracha and other grapevine cultivars. (**F**) Venn diagram of the DEGs between Kober 5BB rootstock and other grapevine

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cultivars. The DEGs analyses was carried out for the comparison groups: 5BB_vs_F, 5BB_vs_A, 5BB_vs_PM, 5BB_vs_S, F_vs_A, F_vs_PM, F_vs_S, A_vs_PM, A_vs_S and PM_vs_S (where 5BB is Kober 5BB rootstock, F – Fercal, A – Aligote, PM – Podarok Magaracha and S – Saperavi).

No significantly up-regulated genes were detected in the Saperavi cultivar when it was compared with the more drought-tolerant plants (Figure 11A). However, genes that are more strongly expressed in the tolerant cultivars than in Saperavi were identified: 17 for Kober 5BB, nine for Ferkal, six for Aligote and 22 for Podarok Magaracha. At the same time, the Venn diagram revealed no common genes for these groups (Figure 11B). Functional gene analyses and GO enrichment were performed for all of the identified genes. For the Fercal_vs_Saperavi group, genes involved in the following biological processes were more strongly expressed in the more resistant Ferkal rootstock: the ubiquitin-dependent protein catabolic process; the sulphur compound metabolic process; the modified amino acid metabolic process; and the glutathione metabolic process. For Kober 5BB_vs_Saperavi, the following were identified: modified amino acid metabolic process, glutathione metabolic process and methylation. For Aligote_vs_Saperavi, only methylation was found to be more expressed. For Podarok Magaracha_vs_Saperavi, the following processes were: modified amino acid metabolism, glutathione metabolism and methylation. Thus, although no common genes were identified for these comparison groups, genes involved in the same processes were more strongly expressed in the resistant cultivars under stress.

3. Discussion

Drought is one of the main factors causing stress to plants and, consequently, reducing crop yields [3–6]. Current climate changes, such as global warming and reduced rainfall, are leading to increasingly prolonged droughts worldwide [5,6]. This is precisely why transcriptomics methods are being used to investigate the molecular mechanisms of drought tolerance in various plants.

One common approach is to compare contrasting genotypes and identify differentially expressed genes (DEGs) in stressed plants versus unstressed plants. However, the resistance mechanisms identified in model organisms cannot necessarily be applied to other [13]. For instance, a study of two contrasting soybean cultivars revealed that the expression of genes involved in calcium and MAPK signalling pathways is enhanced in the drought-tolerant cultivar. It was hypothesised that it responds to drought stress by regulating cell wall remodelling [19]. A study of contrasting wheat cultivars under drought conditions identified DEGs mainly involved in flavonoid biosynthesis, plant hormone, phenolamide and antioxidant pathways [20]. This indicates that different adaptive mechanisms have emerged in various groups of plants as a result of evolution. Studies on grapevines have also been conducted to investigate drought tolerance mechanisms. These studies either compared two contrasting cultivars [21,22] or examined the effects of drought on specific tissues within a single cultivar [23–27].

In this study, we adopted a different approach involving the investigation of phenotypic changes and transcriptome profiles in several cultivars with varying degrees of drought stress resistance, using different concentrations of mannitol. Analysing biometric and biochemical parameters enabled us to rank 30 *V. vinifera* genotypes according to their level of drought tolerance (Figure 3). Regardless of their tolerance level, all 30 genotypes exhibited certain common patterns in their response to stress. Thus, certain phenotypic traits decreased equally for all cultivars after a certain level of stress (see Figures 1 and 2). For example, the length of primary roots (LR1) and carotenoid content decreased equally in all cultivars at mannitol concentrations of over 4%, while the number of leaves (NL) decreased at concentrations of over 3%. The most sensitive biometric characteristics were the number and length of second-order roots (NR2 and LR2), which decreased equally in all cultivars at concentrations of over 2% and were practically absent at concentrations of over 4%. Other parameters, such as chlorophyll content, the number of first-order roots (NR1), and rhizogenesis efficiency (RF), showed significant variation among the cultivars, particularly under maximum stress conditions.

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A shift in correlations among the parameters was observed for susceptible and resistant genotypes (Figure 4). For resistant cultivars, the correlation increases between the number of leaves and the number and length of second-order roots (NL, NR2 and LR2), while for sensitive cultivars, the correlation shifts towards rhizogenesis efficiency and the number and length of first-order roots (RF, NR1 and LR1). It should also be noted that, in the sensitive cultivar without stress exposure, the NL, NR2 and LR2 characteristics were below average for all cultivars (Figure 6), while the proline content was above average (Figure 8). This may indicate that sensitive cultivars have limited reserves for a compensatory response in the event of stress. Therefore, these phenotypic traits (NL, NR2 and LR2) can be used to evaluate not only the impact of stress on plants, but also the drought tolerance of grapevine cultivars.

An analysis of transcriptomic data from five genotypes with varying levels of resistance (Kober 5BB rootstock, cv. Aligoté, cv. Saperavi, cv. Podarok Magaracha and Ferkal rootstock) identified stress-induced differentially expressed genes in response to 2% mannitol. We had expected to identify patterns among cultivars with similar resistance levels, but none were found. Thus, cultivars that are quite similar based on phenotyping, such as Kober 5BB rootstock and cv. Aligote and cv. Podarok Magaracha (Figure 3), do not cluster into a single group based on either hierarchical cluster analysis of DEGs or principal component analysis of transcriptome data (Figure 9). Furthermore, grape genotypes under normal conditions and those subjected to stress do not cluster into groups based on PCA either. These results are consistent with variation in phenotypic characteristics across different cultivars (Figures 1 and 2) and likely reflect the compensatory potential mentioned above.

As significant differences in transcriptomic profiles were observed even among cultivars with similar tolerance levels, only a small number of DEGs were identified for the five *V. vinifera* genotypes under drought stress. These genes probably reflect the general patterns of grapevine response to this type of stress in both sensitive and drought-tolerant cultivars (Figure 10). As in other studies [21,22,24,27,28], we have demonstrated that antioxidant activity is activated in all cultivars under drought conditions, regardless of their degree of drought tolerance. Along with the increase in antioxidant activity, genes associated with peroxidase and oxidoreductase activity are induced, indicating a cellular defense mechanism against excess ROS formed under stress. For the first time, it has been demonstrated that there is a decrease in the expression of genes associated with transitional and transmembrane metal transport, alongside an increase in intra-Golgi vesicle transport. Shifts also occur in biosynthesis and metabolism. The biosynthesis of cell-wall beta-glucan, cellulose and polysaccharides is inhibited, while the biosynthesis of monocarboxylic and fatty acids increases. This was also observed in a study of the transcriptomic responses of the developing buds of the Merlot grapevine cultivar to drought-induced cell wall modification [27]. Another biological process that changes under drought stress in the five cultivars studied, which have varying levels of tolerance, is spliceosome complex disassembly. It has previously been suggested that alternative splicing plays a key post-transcriptional regulatory role in response to single and combined stressors. In a study in which cv. Cabernet Sauvignon cuttings were subjected to drought stress, differentially expressed genes (DEGs) were enriched in the spliceosome pathway [25]. Another study investigated the leaves and roots of *V. vinifera* cv. Shine Muscat and cv. Thompson Seedless, it was found that the retained intron was the predominant type of differential alternative splicing event under drought stress [28]. Thus, the main biological processes affected by drought in the five cultivars have been demonstrated, and these can likely be regarded as general patterns.

It should be noted that the biological processes identified above do not address the specific characteristics of defense mechanisms in different cultivars, nor do they explain the principles of drought tolerance. To this end, we investigated differential gene expression in various cultivars under drought stress. Our results confirmed significant differences in the molecular defense mechanisms of cultivars with varying degrees of drought tolerance. These differences were observed even between cultivars with similar levels of tolerance, as evidenced by the varying numbers of DEGs identified, as well as the small number of genes shared according to the Venn diagram (Figure 11). Previous studies have demonstrated the different drought resistance mechanisms in the two

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grapevine cultivars *V. vinifera* cv. Shine Muscat and *V. vinifera* cv. Thompson Seedless [28]. When comparing resistant and intermediate resistant cultivars with the susceptible Saperavi cultivar, it was shown that two processes are most frequently induced in resistant cultivars: glutathione metabolism and methylation.

Glutathione (GSH) is a small intracellular thiol molecule regarded as a potent non-enzymatic antioxidant. It acts as a substrate for both glutathione peroxidase and glutathione S-transferase, playing a role in the second phase of the detoxification of xenobiotics and cytotoxic molecules [29]. It is well established that glutathione and related antioxidants reduce osmotic stress in plants, the primary manifestation of drought [30]. The signaling function of GSH in *Arabidopsis thaliana* under drought stress conditions has also been demonstrated. The authors concluded that GSH transmits information about drought as perceived by the roots to the leaves during the early stages. This study compared the drought tolerance of wild-type plants with normal GSH expression levels with that of GSH-deficient mutants with reduced GSH expression levels. Wild-type plants demonstrated higher drought tolerance [31]. Another study investigated modified poplar plants with PtrGSTU23 overexpression and found that they had higher GST activity and lower reactive oxygen species (ROS) accumulation, resulting in increased drought tolerance [32].

DNA methylation is an epigenetic regulatory mechanism that dynamically interacts with plant responses to abiotic stresses, modulating gene expression and developmental processes. DNA methylation is known to play a significant role in drought stress in crops, including the dual regulation of gene expression via DNA methylation and the RNA-dependent DNA methylation pathway, as well as alternative splicing and long non-coding RNAs. While many studies have observed significant shifts in methylation levels across the genome or in gene promoters in drought-stressed plants, identifying the specific genes and pathways involved remains challenging [33–35]. For example, drought-induced hypermethylation was observed in the promoter of the cytokinin-oxidase gene in barley [36]. Studies of genomic differences in DNA methylation patterns between rice varieties with contrasting responses to drought and salinity revealed an interplay between DNA methylation, gene expression, and small RNA content. These studies also suggest a more extensive role for DNA methylation in rice's adaptation to abiotic stress [37]. Drought priming is a promising strategy for enhancing wheat's tolerance to recurring drought. The results showed that light or moderate priming intensity had a positive effect on the drought-sensitive YM16 cultivar. Analysis revealed that the demethylation of TaP5CS and TaBADH, which are involved in the accumulation of osmolytes, contributes to the enhanced drought tolerance induced by priming [38].

Thus, these studies confirm our findings and support the hypothesis that increased expression of genes involved in glutathione metabolism and methylation may underpin the drought tolerance of grapevine cultivars.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

To curate a research collection of grapevines in vitro, vines of thirty genotypes [twenty six cultivars: Rkatsiteli, Akademik Avidzba, Veles, Kefesiya Magaracha, Yaltinskiy bessemyannyy, Livia, Muscat Crima, Podarok Magaracha, Ruta, Qoqur, Aligote, Bastardo, Sauvignon, Syrah, Cabernet Sauvignon, Malbec, Cabernet Franc, Muscat Blanc, Pinot Blanc, Pinot Gris, Pinot Noir, Chardonnay, Riesling, Saperavi, Chasselas blanc and Garnacha blanca; three rootstocks: Kober 5BB (*Vitis berlandieri* × *Vitis riparia*), Fercal (*Vitis berlandieri* x *Vitis vinifera*) and Selection Oppenheim 4 (SO4; *Vitis berlandieri* × *Vitis riparia*); one breeding form - Magarach no. TT2 (*V. vinifera* 'Talisman' × *V. vinifera* 'Tomaisky' selected by the All-Russian National Scientific Research Institute of Vine And Winemaking "Magarach")] were collected from field-grown mother vines at the All-Russian National Scientific Research Institute of Vine And Winemaking "Magarach" (lat.: 44.850984′N, long.: 33.650112′E), vernalized for 1 month at a temperature of +4 °C, and germinated in vessels with water. All vines were checked for cultivar suitability [39,40] both ampelographically and using the VIVC

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database with next microsatellite loci: VVS2 [41]; VVMD5 [42]; VVMD25, VVMD27 and VVMD28 [43]; VrZAG62 and VrZAG79 [44] (Table S1).

Further, in the greenhouse, growing green shoots were superficially sterilized [45]. The shoots were then cut into single-node cuttings and placed in tubes on modified MS (according to Zlenko et al. [46] described as PG medium, supplemented with 1% (w/v) sucrose and 0.75% (w/v) agar (Panreac, Spain) and cultivated at pH 5.7, 25 ± 1 ° C and a light intensity of 65 μmol/m^{2}s during a 16 h day photoperiod. After 2 months' cultivation on PG medium from single-node cuttings (without signs of bacterial or fungal infection), shoots developed, which were cut off and placed in culture vessels (total volume: 500 ml) containing 50 ml agarized PG medium supplemented with 0.05 mg/l NAA, with seven plants per culture vessel [47].

4.2. Stress Treatments and Phenotyping

Stress treatments were carried out in vitro conditions by adding the mannitol at ranging from 0.0 to 6.0% (w/v) in increments of 1.0% (drought) [48]. Seven cuttings with two eyes were placed in each of the culture vessels, in three repetitions, for each variant of stress treatments.

After 6 and 12 hours of cultivation, samples were taken to analyze the content of free proline in plant tissues according to the method described by Bates et al. [49]. Determination of free proline content was carried out with a ninhydrin-based protocol with some modifications [50,51]. Proline extraction was carried out by homogenized a 100 mg leafy sample in 2 ml of 3% aqueous sulfosalicylic acid and the homogenate centrifuged (5 min at 8000g), and after cooling and transferring of 100 μl supernatant at new tubes a 0.5 ml ninhydrin reagent (1.25 g ninhydrin, 20 mL 6M H_3PO_4, 30 mL glacial acetic acid) was added and incubated at 96C during 1 hours. The reaction mixture was extracted with 1.0 ml toluene, mixed vigorously for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene for a blank. The proline content (ng/100 mg fw) was determined from a calibration curve using proline (Serva, Heidelberg, Germany).

After 4 weeks of stress treatments, biometric risogenesis frequency (RF), number of new leaves (NL), number of first (NR1) and second (NR2) orders roots, length of first (LR1) and second (LR2) orders roots] and biochemical (contents of chlorophylls a, b and carotenoids in leaves) parameters of plants were measured and calculated by the formulas:

1. The risogenesis frequency (RF) was calculated as the quotient of the number of explants with developed roots (Nr) divided by the total number of explants (No); the results are expressed as a percentage: RF = (Nr/No)×100;
2. The number of new leaves (NL) was calculated as the quotient of the number of developed new leaves on each explant (Nnl) divided by the total number of explants (No): NL = Nnl/No;
3. The number of first order roots (NR1) was calculated as the quotient of the number of developed first order roots on each explant (Nr1) divided by the total number of explants (No): NR1 = Nr1/No;
4. The number of second order roots (NR2) was calculated as the quotient of the number of developed second order roots on each explant (Nr2) divided by the total number of explants (No): NR2 = Nr2/No;
5. The length of first order roots (LR1) was calculated as the quotient of the length of developed first order roots on each explant (Nlr1) divided by the total number of explants (No): LR1 = Nlr1/No;
6. The length of second order roots (LR2) was calculated as the quotient of the length of developed second order roots on each explant (Nlr2) divided by the total number of explants (No): LR2 = Nlr2/No;
7. The photosynthetic pigment contents [chlorophylls a (Chla), b (Chlb) and carotenoids (Car)] were determined by extracting pigments from leaves with 96% ethyl alcohol [52]. The degree of

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solution absorption (optical density) for chlorophylls a, b, and carotenoids was determined using a spectrophotometer at a wavelength of 665, 649 and 471 nm, respectively.

$$C_{chl\ a} = 13.70D_{665} - 5.76\ D_{649};\ C_{chl\ b} = 25.80\ D_{649} - 7.60\ D_{665};$$

$$C_{car} = (1000D_{471} \times 2.13C_{chl\ a} - 97.64C_{chl\ b})/209$$

$$A = C \times V/1000 \times n$$

where C - pigment concentrations; D - optical density; V - extract volume; n - leaf fresh weight; and A - pigment content (ng/100 mg fw).

4.3. RNA Isolation, Library Preparation and Sequencing

RNA samples were extracted from plants (6 and 12 hours from control and stress groups) using Spectrum Plant Total RNA Kit (Sigma-Aldrich, Germany). Each sample was treated with DNAse (Thermo Fisher Scientific, USA). The quantity and quality of total RNA were analysed with Qubit 4.0 (Thermo Fisher Scientific, USA) and stained with ethidium bromide in a 1.5% agarose gel. The cDNA was synthesized using Revert Aid Minus Reverse Transcriptase (Thermo Fisher Scientific, USA) according to the manufacturer's protocol using oligo(dT) 18 primers. The libraries were synthesized with NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, USA) and paired-end sequencing as 150 nt reads was done on BGI DNBSeq G400 instrument.

4.4. Bioinformatic Analyses

Quality and length trimming of the reads was conducted using Fastp v.0.20.0 [53]. To delete contamination reads mapping of reads to the reference human genome and grape viruses database was performed using Bowtie2 software [54]. The filtered reads for each sample were aligned with the reference genome *V. vinifera* downloaded from NCBI (accession number GCF_030704535). The estimated counts were calculated using pseudo-alignment to reference genome method by Kallisto [55]. Differential expression gene (DEG) analysis was performed by using R package DESeq2 [56,57]. Differential expression genes (DEGs) were identified by the criterion ($|\log2\text{Fold Change}| \geq 1$ and FDR < 0.05). In the case of small samples, the RankProd approach was used to identify DEGs (FPF<0.05) [58].

For functional annotation of differentially expressed genes in *V. vinifera*, we used the reference genome annotation IGGP 12X.v0 [59]. GO term enrichment analysis was performed using the GOATOOLS package v1.1.6 [60]. GO term enrichment was considered statistically significant at FDR < 0.05 (Benjamini–Hochberg correction) and Fold Enrichment > 1.0. Results were categorized into three functional domains: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC).

4.5. Statistical Analysis

Analysis of variance (ANOVA) was performed for all parameters to estimate the significance of stress, cultivar, and their interaction using the library "agricolae" in RStudio v.2024.12.1 (https://www.R-project.org). The experiment design considered drought stress as the main factor and the cultivar as the subfactor. Means were separated using LSD at $p \leq 0.05$. The cultivars were characterised for each phenotypic trait using the stress tolerance index (STI) [61]. The stress tolerance index (STI) was calculated for all thirty grapevine cultivars using the formula defined by Fernandez:

$$STI = Y_C \times Y_S/(X_C)^2$$

where Y_C and Y_S - the value of phenotypic trait of a given cultivar under control and drought stress condition respectively; X_C - the mean of phenotypic trait of all cultivars under control and drought stress condition respectively.

The index was calculated for each phenotypic trait at each stress level (mannitol concentrations ranging from 1% to 6%). Then, the average index value was calculated for each phenotypic trait and cultivar. These values were then used for FANNY cluster analysis (bootstrap = 1000) and principal component analysis (PCA) performed using the library "factoextra" in R. The Spearman's correlation

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analysis was performed using the libraries "psych" and "corrplot". All graphs were constructed using the library "ggplot" in R.

5. Conclusions

Thus, an analysis of phenotypic characteristics was used to characterize the drought tolerance of 30 grape genotypes, enabling the study of the crop's response to drought stress. The study revealed that the response of different grapevine genotypes to drought is linked to the activation of antioxidant defense systems, such as the induction of peroxidase and oxidoreductase. It is also associated with changes in the metabolism and biosynthesis of glucan, cellulose, polysaccharides, monocarboxylic acids and fatty acids, as well as metal transport. Meanwhile, more resilient cultivars exhibited enhanced glutathione metabolism and methylation processes, suggesting a role for epigenetics in adaptation. Despite exhibiting common responses, each cultivar employs unique molecular mechanisms, reflecting their evolutionary diversity. Transcriptomic data confirmed variability in defense strategies, even among cultivars with similar levels of resistance.

Supplementary Materials:

Author Contributions: Conceptualization, E.V. and P.K.; methodology, I.G., V.U. and G.M.; software, I.G. and E.V.; formal analysis, E.V., A.P.; writing—original draft preparation, E.V., P.K. and I.G.; writing—review and editing, E.V., S.D. and P.K.; visualization, I.G. and E.V.; supervision, Y.P. and S.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the tables and figures in this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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