TMT-based proteomic analysis of the causes of reduced seed viability in Allium mongolicum under accelerated aging

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Research Article

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Abstract

Background: Seeds are the most basic production material in agricultural production and occupy an important position in the whole process of agricultural production. However, aging begins to occur after seed harvested, serious aging will affect the conservation of germplasm resources. Seed propagation is the most commonly used method in the production of *A.mongolicum*seeds, but with the prolongation of storage time, *A.mongolicum* seeds undergo different degrees of aging. Therefore, we used an integrated approach of Tandem Mass Tag(TMT) labeling in conjunction with liquid chromatographytandem mass spectrometry (LC-MS/MS) to capture the differential protein expression profiles of *A.mongolicum*seeds under unaged (CK), lightly aged (T1), and severely aged (T4) treatments to provide evidence of protein expression levels for revealing the aging mechanism study of *A.mongolicum*seeds.

Results: A total of 4336 proteins were identified in this study, of which 4318 total proteins could be quantified. There were 291 diferentially expressed proteins (DEPs) identifed after accelerated aging treatments. Compared with CK, 36 DEPs (16 up-regulated and 20 down-regulated) were found in T1; and 139 DEPs (64 up-regulated and 75 down-regulated) in T4. Compared with T1, 116 DEPs (51 up-regulated and 65 down-regulated) were found in T4. A number of candidate proteins associated with seed aging were screened, for example, ATP-dependent RNA helicase-like protein DB10(Unigene0060546) β-D-glucan exohydolase(Unigene0008772) Histone H1.2 partial(Unigene0049278) defensin Ec-AMP-D2(Unigene0042879) NAD(P)H dehydrogenase (quinone) FQR1(Unigene0047137) glutathione S-transferase(Unigene0000863) oleosin S1-2(Unigene0011542) probable inactive purple acid phosphatase 2(Unigene0010352) glutathione S-transferase U8-like(Unigene0006910) glutathione S-transferase U17-like(Unigene0051938).

Conclusions: This study was the first to determine the proteome of *A.mongolicum* seeds, these data provided references for further study of seed aging mechanism of *A.mongolicum*. The results showed that the related proteins were significantly enriched in glutathione metabolism and other pathways after accelerated aging. In addition, among the candidate proteins, PAP2 is down-regulated after aging, which was speculated to be the main reason for limiting radicle growth.

Background

Allium mongolicum is a perennial herbaceous dry plant, which is resistant to wind erosion, drought and infertile. However, the wild *A.mongolicum* is severely damaged by climate change and overgrazing, etc. It is urgent to develop and utilize it rationally through artificial cultivation and domestication, and understanding its seed characteristics is the primary work for artificial domestication and cultivation. Seed propagation is the most commonly used method in the production of *A.mongolicum* seeds, but with the prolongation of storage time, *A.mongolicum* seeds undergo different degrees of aging, such as reduced vigor, decreased germination rate and uneven seedling emergence, thus causing germplasm resources cannot be preserved for a long time [1]. Previous research found that its seed life is up to 2-4 years [1], and the germination ability of *A.mongolicum* seeds during storage aging was significantly and positively correlated with antioxidant enzyme activity[2]. Protein synthesis is a necessary biological response for seed germination, as inhibition of protein translation can completely block Arabidopsis seed germination, and a significant correlation has been found between protein translation capacity and seed viability [3,4]. However, proteomic studies on seed aging in *A.mongolicum* have not been reported.

Seed aging is a complex biological process involving various processes of cellular, physiological and biochemical metabolism, and gene regulation changes that ultimately lead to seed death [5,6]. At the protein level, the main causes of seed aging are attributed to two aspects:on the one hand, changes in proteins in dry seeds, and on the other hand, the inability of low-activity seeds to express their normal proteome during germination. The key mechanisms that maintain seed viability include seed translocation capacity, seed storage mobilization and antioxidant efficiency [7]. In addition, some late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) play an important role in maintaining seed viability [7]. Similar to the loss of genetic integrity due to natural aging, accelerated aging may also have a negative impact on genetic integrity. High temperatures and high seed water content may lead to accelerated aging, which mimics stress conditions leading to high respiration rates and reserve depletion, resulting in seed loss of viability usually within days or

weeks [8]. As seeds age, seed viability decreases, leading to severe economic losses and loss of germplasm resources during seed marketing [9].

In recent years, proteomics has become a new technique to resolve seed characteristics e.g. seed germination [10], seed development [11,12], seed drought tolerance [13], etc. Chu et al. used proteomics to resolve the variation in the viability of membrane linked proteins associated with heat tolerance and germination vigor in lotus seeds [14]. Li et al. revealed by proteomics techniques that the differences in seed vigor in maize are due to the influence of growing sites on the seed development process [15].Yan et al. analyzed the differentially expressed proteins (DEPs) of wheat seed embryos during germination mainly related to carbohydrate metabolism, protein metabolism, and amino acid metabolism using proteomic techniques [16]. Rajjou et al. found that loss of Arabidopsis seed vigor was associated with protein changes in dry seeds by proteomic analysis and the inability of low viability seeds to produce a normal proteome during germination [17]. Li et al. found that calcium channel blockers affect radicle prominence during rice seed germination by proteomic analysis [18]. In conclusion, proteomics technology provides a powerful tool in seed aging studies. Therefore, we used an integrated approach of Tandem Mass Tag(TMT) labeling in conjunction with liquid chromatographytandem mass spectrometry (LC-MS/MS) to capture the differential protein expression profiles of *A.mongolicum* seeds under unaged (CK), lightly aged (T1), and severely aged (T4) treatments to provide evidence of protein expression levels for revealing the aging mechanism study of *A.mongolicum* seeds, and for propagation renewal of *A.mongolicum* germplasm resources and This study will provide a theoretical basis for the propagation, renewal and safe storage of *A.mongolicum* germplasm resources, and lay the foundation for related gene mining.

Results

A general overview of protein identification in *A.mongolicum* seeds under accelerated aging

A total of 401747 secondary spectra, 29560 matched spectra, 19076 identified peptides and 4336 identified proteins were obtained by MS analysis; the total number of quantifiable proteins for all samples was 4318 (Table 1). The results of principal component analysis showed that these 9 samples were clearly distinguished into three major categories and are circled with different colours (Fig.1); each circle corresponded to the three replicate samples within the treatment, indicating that protein expression had clear biological reproducibility.

Table 1. Protein Identification Information Statistical Summary

Total spectra	Matched spectrum	Peptide	Identified protein	ALL
401747	**29560**	**19076**	**4336**	**4318**

Quantification and annotation of differentially-expressed proteins (DEPs) in *A.mongolicum* seeds under accelerated aging

To understand in detail the differences between different aging treatments and controls, we compared T1and T4 with CK (i.e. 'T1 vs. CK', 'T4 vs. CK', and 'T4 vs. T1'). Compared with CK, 36 DEPs (16 up-regulated and 20 down-regulated) were found in T1; and 139 DEPs (64 up-regulated and 75 down-regulated) in T4. Compared with T1, 116 DEPs (51 up-regulated and 65 down-regulated) were found in T4 (Fig.2A.B.C).Venn diagram showed that 21 differential proteins were significantly expressed in T1 and T4 treatments compared with CK, of which, Histone H1.2 partial (Unigene0049278) and defensin Ec-AMP-D2 (Unigene0042879) were significantly up-regulated after T1, but down-regulated after T4. The expression trend of the other 19 differential proteins was consistent between T1 and T4 (Fig.2D). Obviously, compared with CK, the number of significant differences in T4 protein than T1 more significant differences in seed protein, protein quantity and lower expression than raising expression, speculation in the accelerated aging treatment, the more protein involved in regulation and some protein activity become low to outside bad environment, make the seed survival.

GO enrichment analysis of DEPs in *A.mongolicum* seeds under accelerated aging

To resolve the response mechanisms of *A.mongolicum* seeds to different degrees of accelerated aging, we used GO analysis of the identifed proteins (Fig. 3, Table S1). We found that the number of proteins in seeds varied for each GO entry from T1 to T4. Compared with CK, the difference of T1 proteins are mainly involved in biological processes(BP) and molecular function(MF), the biological process involved are mainly in response to wounding and vacuolar transport; the molecular functions involved are mainly carbon-sulfur lyase activity enzyme inhibitor activity acetyl-CoA carboxylase activity serine-type endopeptidase inhibitor activity and O-methyltransferase activity (Fig.3A).

Compared with CK, the differential proteins in T4 are assigned to various classifications and participate in the embryo development and ceramide metabolic process in terms of biological process. From the molecular function analysis, Mainly for acid phosphatase activity, hydrolase activity acting on the ester bonds, endonuclease activity, and adenylosuccinate synthase activity. From the analysis of cell components, It mainly includes monolayer-surrounded lipid storage body and intracellular non-membrane-bounded organelle (Fig.3B).

Compared with T1, the differential proteins in T4 are involved in various categories. in biological processes, it is mainly involved in embryo development, cellular amide metabolic process and ceramide metabolic process process, translation, response to oxidative stress, etc.; The main molecular functions involved are carbon-sulfur lyase activity, heme binding, lyase activity and structural constituent of ribosome, adenylosuccinate synthase activity, phosphatidylserine decarboxylase activity, phosphatase activity, mRNA guanylyltransferase activity, alanine-tRNA ligase activity. The cellular components involved mainly include intracellular non-membrane-bounded organelle and ribosome (Fig.3C).

KEGG pathway enrichment of DEPs in *A.mongolicum* seeds under accelerated aging

To further clarify the metabolic and signal transduction pathways involved in aging and development-related DEPs of *A.mongolicum* seeds under accelerated aging and in CK treatment, and to gain insight into the biological functions of DEPs, the DEPs obtained were subjected to KEGG metabolic pathway enrichment analysis (Fig.4, Table S2). Compared with CK, 18 metabolic pathways were involved in T1, with most proteins involved in Biosynthesis of unsaturated fatty acids Glutathione metabolism Spliceosome Fatty acid biosynthesis Phenylpropanoid biosynthesis (Fig.4A); 28 pathways were involved in T4, with most proteins involved in Glutathione metabolism Ribosome RNA transport Phenylpropanoid biosynthesis Starch and sucrose metabolism Endocytosis (Fig.4B); Compared with T1, 22 pathways were involved in T4, with most proteins involved in Ribosome Spliceosome Glutathione metabolism RNA transport Glycerophospholipid metabolism Glycerolipid metabolism Ribosome biogenesis in eukaryotes RNA degradation Oxidative phosphorylation (Fig.4C). From above it could be seen that the number of proteins involved in the same metabolic pathway increased with increasing accelerated aging.

Analysis of candidate differential proteins of *A.mongolicum* seeds after accelerated aging

As shown in Fig.5, a total of 21 differential proteins were significantly enriched during the process from T1 to T4, which were mainly divided into three categories(Table S3). The first category of proteins was significantly up-regulated expression after aging treatment, such as ATP-dependent RNA helicase-like protein DB10(Unigene0060546) β-D-glucan exohydolase(Unigene0008772) prostaglandin reductase-3(Unigene0003044) aladin isoform X2(Unigene0048082) trans-resveratrol di-O-methyltransferase-like isoform X2(Unigene0042829); the second class of proteins is in significantly up-regulated after T1 and significantly down-regulated afterT4, such as Histone H1.2 partial(Unigene0049278) defensin Ec-AMP-D2(Unigene0042879) the third category of proteins is significantly up-regulated after aging treatment down-regulated expression, such as aldehyde dehydrogenase family 2 member C4-like(Unigene0049013) NAD(P)H dehydrogenase (quinone) FQR1(Unigene0047137) tetraketide alpha-pyrone reductase 1-like isoform X3(Unigene0045869) glutathione S-transferase(Unigene0000863) inhibitor of trypsin and hageman factor-like isoform X2(Unigene0005112) isoflavone reductase-like protein(Unigene0053605) vacuolar protein sorting-associated protein 32 homolog 2(Unigene0042292) polyadenylate-binding protein-interacting protein 11(Unigene0047075) receptor protein kinase TMK1-like(Unigene0053916) oleosin S1-2(Unigene0011542) mitogen-activated protein kinase kinase 1 isoform X1(Unigene0045171) probable inactive

purple acid phosphatase 2(Unigene0010352) glutathione S-transferase U8-like(Unigene0006910) glutathione S-transferase U17-like(Unigene0051938).

Discussion

Effect of accelerated aging treatment on the viability of *A.mongolicum* seeds

Seeds are the most basic production material in agricultural production and occupy an important position in the whole process of agricultural production. Seed vigour is the most important index of seed quality, which refers to the comprehensive performance of the seeds in germination and seedling emergence during the activity intensity and characteristics. High vigor seeds germinate early, emerge neatly and quickly, and have a strong ability to resist adverse conditions, with obvious growth advantages and production potential; low vigor seeds can germinate under suitable conditions, but germination is slow, and seedlings do not emerge neatly under adverse environmental conditions, or even no seedlings. The seeds are affected by external environmental conditions at every moment from the formation and development until they mature from the mother plant. Seed aging occurs when the seed reaches its maximum physiological maturity and dry weight, when the seed has the highest vigor, and then decreases as the seed continues to age and progresses toward death.

It was found that aging seeds germinate slowly and with little radicle growth compared to highly viable seeds [19]. In a previous study, it was found that the viability as well as the germination rate of *A.mongolicum* seeds showed a decreasing trend with increasing aging time, and at T4 treatment, the seeds almost completely lost viability (Fig.6) [1]. Similar results were found for rice and sequoia seeds [20,21]. This may be related to the accelerated metabolism due to high temperature and humidity [21].

Effects of accelerated aging treatments on starch and sucrose metabolism in *A.mongolicum* seeds

Seeds can lose viability and undergo genetic changes during long-term storage, and genetic integrity can be disrupted [22]. Seed deterioration is an inevitable and irreversible process [23] that begins immediately after seed maturation. During degeneration, the decline in vigor precedes the decline in germinability and ends with the death of the seed [24]. In recent years, proteomics approaches for overall plant expression analysis and protein identification have become very efficient [25]. Proteins are key performers of cellular activities and studying their altered abundance and modifications in various biological processes can greatly help in understanding their functions [26]. Stored proteins in seeds are not only an important source of amino acids in the early stages of germination, but are also important for energy production [27]. In Arabidopsis, proteins also undergo early degradation and rapidly resume metabolic activity through seed uptake. After seed uptake, the three major nutrients (sugar, protein, and fat) begin to interconvert to provide energy and substrate for germination. The chemical and physiological changes in the embryo, endosperm and seed coat and their interactions contribute to the successful germination of seeds [28]. Starch is an important carbohydrate reserve and energy supplier. The metabolic activity of starch in germinating seeds is associated with several enzymes [29]. β-amylase and starch phosphorylase are responsible for the metabolism and degradation of starch [30]. starch is converted to UDP-glucose and fructose by β-amylase, which is important for storage function and metabolism [31]. Sucrose synthase, a key class of enzymes in sucrose metabolism, is widely present in living organisms and mainly catalyzes the synthesis and breakdown of sucrose, but also acts as a signaling molecule to regulate the growth and developmental processes in plants [32]. It plays an important role in the respiration, carbohydrate biosynthesis and utilization of plants [33]. In this experiment, β-glucosidase was significantly up-regulated in "starch and sucrose metabolism" after aging treatment, and one protein encoding sucrose synthase (Unigene0063235) was significantly down-regulated after severe aging (T4) treatment, while five proteins encoding sucrose synthase (Unigene0001700, Unigene0063235) were significantly down-regulated after light aging treatment. Unigene0001700, Unigene0053027, Unigene0063235, Unigene0050027, Unigene0050026) were up-regulated in expression after light aging treatment.

Effects of accelerated aging treatment on proteins associated with energy metabolism in *A.mongolicum* seeds

The ability to produce ATP largely reflects the metabolic activity of the seed, and the amount of ATP during seed germination correlates with seed viability.The ATPase family may act as a proton pump, which is the primary mechanism for lowering intracellular pH, altering membrane potential, and is used as a powerhouse for protein hydrolysis within seed cell tissues [34].ATP synthesis is mediated by ATP synthases, and studies have found that aging The expression of six ATP synthase subunits was found to be down-regulated in the seeds of maize, resulting in a decrease in ATP [35]. In contrast, the expression of the ATP synthase subunit beta (Unigene0024535) was up-regulated after aging treatment in this experiment, and the expression was significantly up-regulated at T4. This may be a result of a "vicious circle" between high metabolic activity and ATP production to maintain a low level of metabolism or to defend against the external environment, resulting in a low ATP supply during the germination stage of aging seeds.

It was shown that there are 29 purple acid phosphatases (PAPs) in the model plant Arabidopsis with different spatio-temporal expression patterns [36]. AtPAP15 may be involved in the mobilization of Pi reserves during seed and pollen germination [37]; AtPAP9 and AtPAP5 may be involved in the defense against plant pathogens [38]; while targeting plastids and mitochondria AtPAP2 is involved in plant growth [39]. It has been shown that embryo elongation growth requires cell expansion in specific regions of the radicle and hypocotyl during early seed germination, while cell division occurs mainly in post-emergence seedling growth [40]. In this experiment, PAP2, PAP22-like, and PAP29 were down-regulated after aging treatment, while PAP1 and PAP20 were up-regulated in expression. Among them, PAP2 was down-regulated in expression after aging treatment, which may be the main reason for the slowdown of radicle growth due to aging.

Effect of accelerated aging treatment on glutathione metabolism-related proteins in *A.mongolicum* seeds

Glutathione metabolism plays an important role in antioxidant [41], and glutathione and glutathione disulfide are essential for the reduction of various peroxides [42]. Glutathione S-transferases play a direct role in reducing oxidative damage as well as toxic damage by foreign substances in response to external environmental stimuli [43,44]. Our results showed that after accelerated aging, three proteins enriched in the glutathione metabolic pathway encoding glutathione S-transferase (glutathione S-transferase U8-like, glutathione S-transferase, glutathione S-transferase U17- like) were significantly down-regulated in expression and at T4 treatment, ascorbate peroxidase (Unigene0050520) was significantly down-regulated, possibly affecting ascorbate to dehydroascorbate synthesis and also responding to the reduced antioxidant capacity of low viability seeds. This suggests that the external environment (accelerated aging) diminishes the antioxidant capacity of seeds and reduces seed activity.

Effects of accelerated aging treatments on LEA and HSP proteins in *A.mongolicum* seeds

The role of specific proteins in maintaining seed viability or longevity has been verified. Two groups of late embryogenesis-rich proteins (LEA) (dehydrin/RAB group) were shown to contribute to Arabidopsis seed viability by controlled denaturation assays and proteomic analyses [45]. LEA proteins are highly hydrophilic and accumulate in large amounts during the final stages of seed maturation [46]. Mitochondria-specific "late embryo development-rich" (LEA) proteins contained in seeds were found to play a key role in protecting these enzymes [47,48], as well as stabilizing cell membranes and preventing seed dehydration [49,50]. In this experiment five proteins encoding LEA (Unigene0016207 , Unigene0016537, Unigene0013380, Unigene0037631, Unigene0048194) were upregulated after mild aging, and eight proteins (Unigene0030390 , Unigene Unigene0010414, Unigene0032477, Unigene0011984, Unigene0047667, Unigene0050372, Unigene0007117, Unigene0000326) were down-regulated, but the differences were not significant, while six proteins encoding LEA were down-regulated after the heavy aging treatment (Unigene0016537, Unigene0013380, Unigene0037631, Unigene0048194). six proteins (Unigene0007117, Unigene0016537, Unigene0011984, Unigene0047667, Unigene0013380, Unigene0037631) were significantly down-regulated in expression after severe aging treatment. It indicates that LEA function is reduced under severe aging and loses its ability to protect against enzymes.

Heat shock proteins are involved in a variety of cellular mechanisms, including regulation and prevention of protein folding, transport, activity regulation, degradation, and irreversible protein aggregation [51], associated with seed development, protein translocation, reserve synthesis and mobilization [52], and cellular defense [53].

HSP proteins can play a crucial protective role in the process of antioxidant damage of seed proteins and can enhance the resistance of seeds to senescence [54]. hsp70 and hsp90 are important chaperone proteins in eukaryotic cells, heat shock protein 70 is involved to the plasma membrane fluidity of the cell membrane [55], heat shock protein 90-1- like is involved in stress response processes, mainly involved in cell activity regulation and apoptosis, and the expression of this protein has a positive effect on the stress response [56]. The results of this experiment showed that in T1 treatment, the expression of each heat shock protein was not significantly different from CK, while in T4 treatment, heat shock protein 70-2 (Unigene0053814) and Heat shock protein 60, mitochondrial (Unigene0067445) were significantly upregulated in expression, suggesting its essential role in protein biogenesis in response to accelerated aging.

Conclusion

The present study focused on the problem that *A.mongolicum* seeds would be aged during storage and could not be preserved for a long time. By proteomic analysis of unaged (CK), lightly aged (T1) and heavily aged (T4) seeds, a total of 4336 proteins were identified, of which 4318 total proteins could be quantifed. There were 291 diferentially expressed proteins (DEPs) identifed after accelerated aging treatments. Compared with CK, 36 DEPs (16 up-regulated and 20 down-regulated) were found in T1; and 139 DEPs (64 up-regulated and 75 down-regulated) in T4. Compared with T1, 116 DEPs (51 up-regulated and 65 down-regulated) were found in T4.

A number of candidate proteins associated with seed aging were screened, for example, ATP-dependent RNA helicase-like protein DB10(Unigene0060546) β-D-glucan exohydolase(Unigene0008772) Histone H1.2 partial(Unigene0049278) defensin Ec-AMP-D2(Unigene0042879) NAD(P)H dehydrogenase (quinone) FQR1(Unigene0047137) glutathione S-transferase(Unigene0000863) oleosin S1-2(Unigene0011542) probable inactive purple acid phosphatase 2(Unigene0010352) glutathione S-transferase U8-like(Unigene0006910) glutathione S-transferase U17-like(Unigene0051938) and the functional analysis of these proteins needs to be further investigated.

Material And Methods

Plant culture and accelerated aging treatments

Wild *A.mongolicum* seeds harvested in 2019 were used as test material (thousand grain weight of 2.32 g and initial moisture content of 5.18 %). The *A.mongolicum* seeds were artificially aged using the high temperature and high humidity method according to the International Seed Inspection Protocol [57], with a treatment temperature of 50 °C, relative humidity (RH) of 100 %, and treatment times of 0 h, 5 h, 15 h, 25 h, and 35 h, denoted as CK, T1, T2, T3, and T4, respectively. Finally, they were stored in a sealed bag in a 4℃ refrigerator for backup. Fig.1 shows the germination of the *A.mongolicum* seeds after different aging, in which the values of all germination indexes in the *A.mongolicum* seeds almost tended to 0 at T4 treatment, indicating that the *A.mongolicum* seeds almost reached the state of complete death [1]. For this reason, the materials of CK (unaged), T1 (lightly aged), and T4 (severely aged) treatment groups were selected for this experiment.

Total protein extraction from *A.mongolicum* seeds

The *A.mongolicum* seeds samples were ground into powder at low temperature in a grinder (purchased from Shanghai Jingxin/ 24-well), and quickly transferred to a liquid nitrogen pre-cooled centrifuge tube. The seed protein was extracted using standard methods [58].

TMT labeling and peptide separation

Proteolytic solution was added to each protein sample (100 μL), trypsin was added and 100 m M TEAB buffer, mixed well and then digested at 37°C for 4h. More trypsin was added and $CaCl_2$ to digest overnight. Formic acid was then added to adjust the pH to less than 3, mixed well and centrifuged at 12000 g for 5 min at room temperature. The supernatant was slowly passed through a C18 column for desalting, and the filtrate collected and vacuum freeze dried. To each 100 μL of 0.1 M TEAB buffer was added to dissolve the peptide and 41 μL of TMT labeling reagent, mixed well, and the reaction allowed to proceed at room temperature for 2 h. Afterwards, 8% ammonia was added to terminate the reaction, mixed with an equal volume of labeled sample, desalinate, and freeze-dry under vacuum [59].

The sample was mixed, desalted and freeze-dried under vacuum. The sample was fractionated on a Waters BEH C18 column (4.6×250 mm, 5 μm) using an L-3000 HPLC system with the elution gradient shown in Table 2. One tube was collected every minute and divided into ten fractions, which were lyophilized and dissolved by adding 0.1% formic acid to each.

Table 2. Peptide fraction separation liquid chromatography elution gradient table

Time(min)	flow rate(nL/min)	mobile phase A(%)	mobile phase B(%)
0	1	97	3
10	1	95	5
30	1	80	20
48	1	60	40
50	1	50	50
53	1	30	70
54	1	0	100

Note: Liquid A (2% acetonitrile, 98% water, ammonia adjusted to pH = 10) and Liquid B (98% acetonitrile, 2% water).

LC-MS/MS analysis and protein identification

The liquid chromatography elution conditions and mass spectrometry parameters were set according to the method of Zhang et al [60]. The spray voltage was 2.3 kV, the temperature of the transfer tube was 320°C, the resolution of the primary mass spectrum was set to 60,000 (200 m/z), the resolution of the secondary mass spectrum was set to 45,000 (200 m/z), the mass spectrum was acquired in data-dependent mode, the threshold intensity was set to 1.2×10^5, the maximum injection time was 86 ms and the dynamic exclusion range was set to 20 s. The raw mass spectrometry data were generated (.raw). The raw data obtained by LC-MS/MS were searched using the search engine Proteome Discoverer 2.4 (PD2.4, Thermo) [60].

The database was queried with the *A.mongolicum* transcriptome database for comparison [61]. Differentially expressed proteins (DEPs) were defined according to $p < 0.05$ (Student t-test method) and the absolute value of the fold change (FC) of the protein was greater than 1.3 (up-regulated protein: FC >1.3; down-regulated protein: FC < 0.77), and the protein was considered to have undergone a significant change if the above conditions were met [62].

Protein bioinformatics analysis

Gene Ontology (GO, http://www.geneontology.org) functional annotation and classification were performed for the proteins in the experiments using interproscan software [63]. Protein functions were classified by acquiring entries and sub-category

terms, including molecular function (MF), biological process (BP) and cellular component (CC), by Fisher's Exact Test. The GO entry was considered significantly enriched when P < 0.05 by Fisher's Exact Test [64]. These proteins were further annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) functionally, and the pathway was considered significantly enriched by Fisher's Exact Test at p < 0.05 , proteins were significantly enriched [65].

Data analysis

Data were analyzed using Microsoft Excel 2010 statistical packages. A factorial experiment based on randomized complete block design was carried out with three replicates (n=3). Duncan's multiple range test($p<0.05$) was used to compare the means. Proteomic analyses were performed in three biological replicates. Volcano map analysis, cluster heat map analysis and pathway enrichment analysis for GO and KEGG were performed for DEP [66].

Abbreviations

DEP: Differentially expressed protein; FDR: False discovery rate; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS: Liquid chromatography electrospray ionization tandem mass spectrometry; TMT: Tandem Mass Tag

Declarations

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Guidelines statement

Our research complied with relevant institutional, national, and international guidelines and legislation.

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Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]. In addition the datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Contributions

SXQ helped with data analysis and wrote the manuscript. ZD and ZXY performed the experiment. ZD and ZFL made substantial contributions to conception and critically revised the manuscript. LJC and YZR designed the research. All authors contributed to manuscript revision, and read and approved the submitted version.

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Ethics declarations

Ethics approval and consent to participate

The research conducted complied with all institutional, national, and international guidelines and legislation. It also complies with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

The *A.mongolicum* seeds were collected with the consent of the local government department. The *A.mongolicum* seeds used in this study were collected from the meadows of Inner Mongolia, China and identified as the seeds by Prof. Zhongren Yang of Inner Mongolia Agricultural University.

The *A.mongolicum* seeds are kept in Inner Mongolia Key Laboratory of Wild Peculiar Vegetable Germplasm Resource and Germplasm Enhancement. Voucher specimen number: sc-seed-20191010-001.

Consent to publication

Not applicable.

Competing interests

Our research is in full compliance with the regulations of the system. Competing interests The authors declare that they have no competing interests.

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Figures

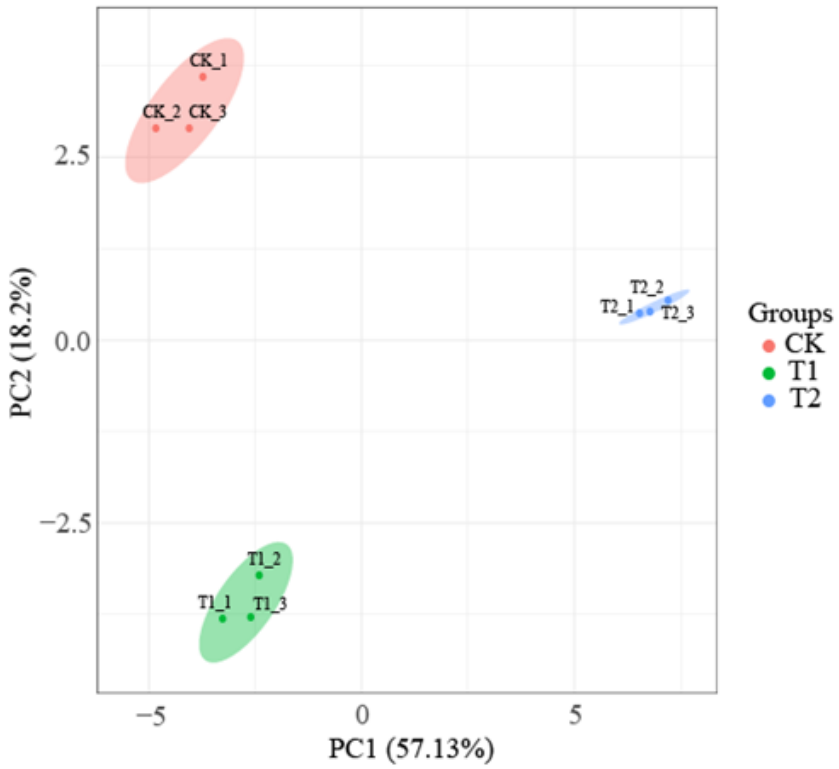

Figure 1

PCA analysis.Note: The horizontal coordinate PC1 and the vertical coordinate PC2 in the fgure indicate the scores of the frst and second ranked principal components, respectively. The scatter colour indicates the experimental grouping of the sample.

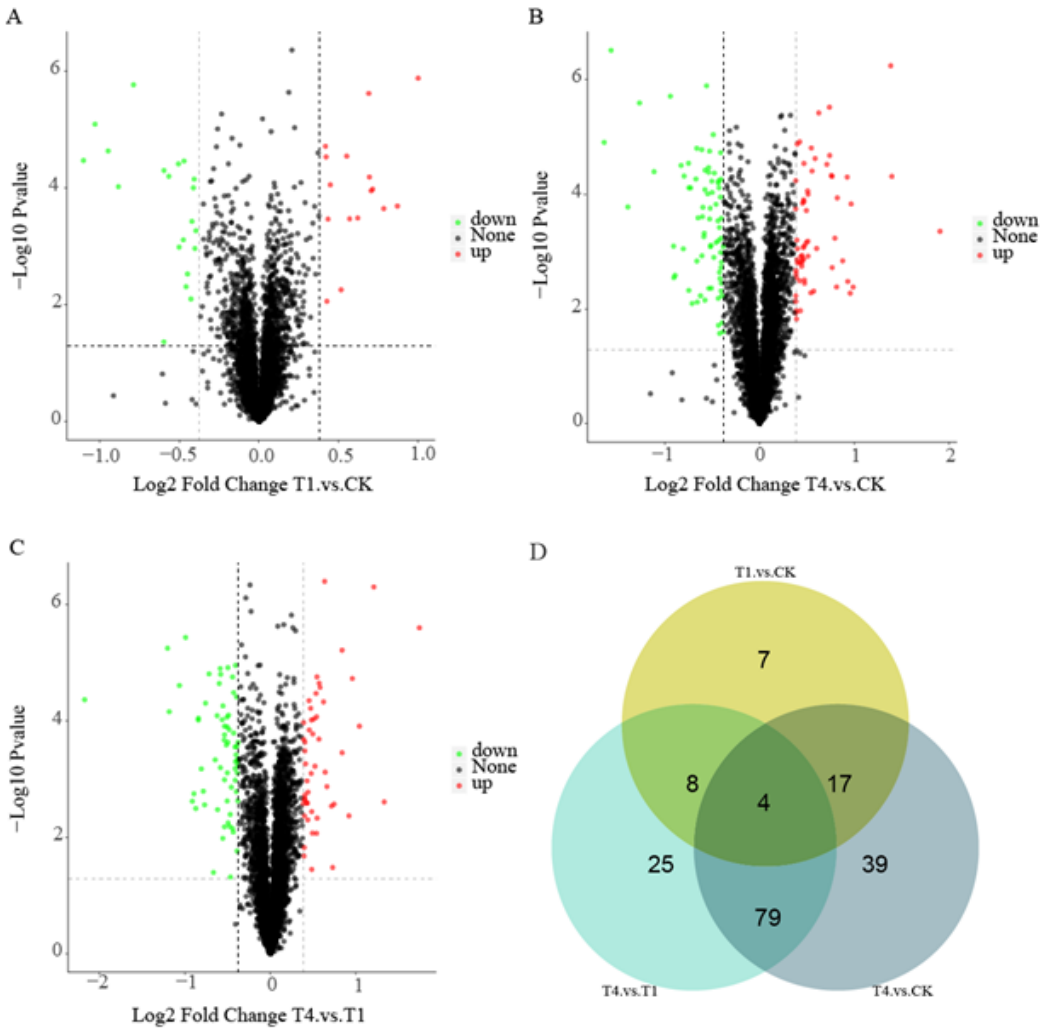

Figure 2

Volcano plot and Venn diagram of diferential protein expression in liquorice root after diferent levels of water stress. Note: a, b, c: The horizontal coordinates indicate the diference multiplicity of differential proteins (log2 value), the vertical axis indicates the P value (-log10 value), black represents proteins that are not diferentially expressed compared with the control(CK); red represents up-regulated proteins; and green represents down-regulated proteins; a: compared T1 with CK (T1 vs. CK); b: compared T4 with CK (T4 vs. CK); c: compared T4 with T1 (T4 vs. T1). d: Each circle in the graph represents a comparison group, and the numbers in the overlapping part of the circles represent the number of diferentially-expressed proteins shared between the comparison groups; the numbers in the non-overlapping part represent the number of diferentially-expressed proteins that are specifc to that group.

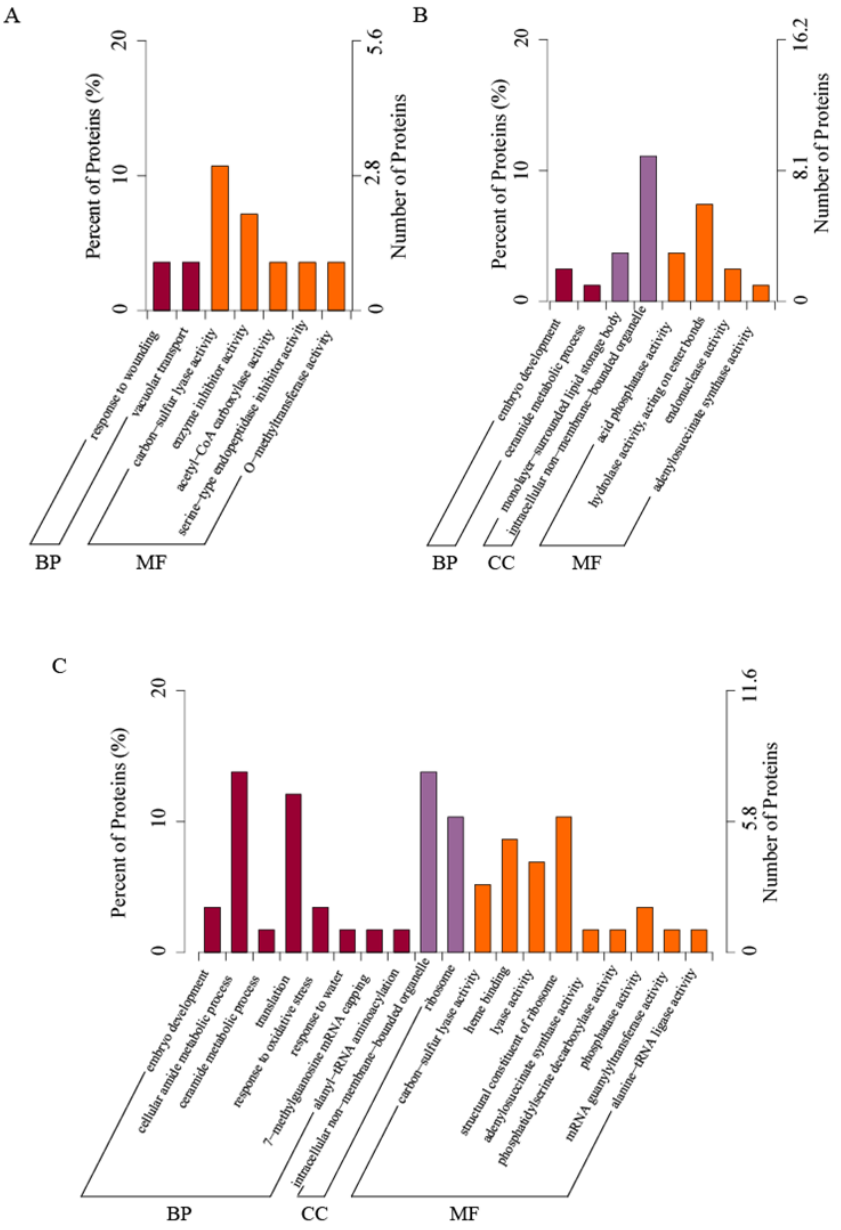

Figure 3

GO enrichment analysis of DEPs under aged *A.mongolicum* seeds of different comparison groups. Note:a: compared T1 with CK (T1 vs. CK); b: compared T4 with CK (T4 vs. CK); c: compared T4 with T1 (T4 vs. T1).

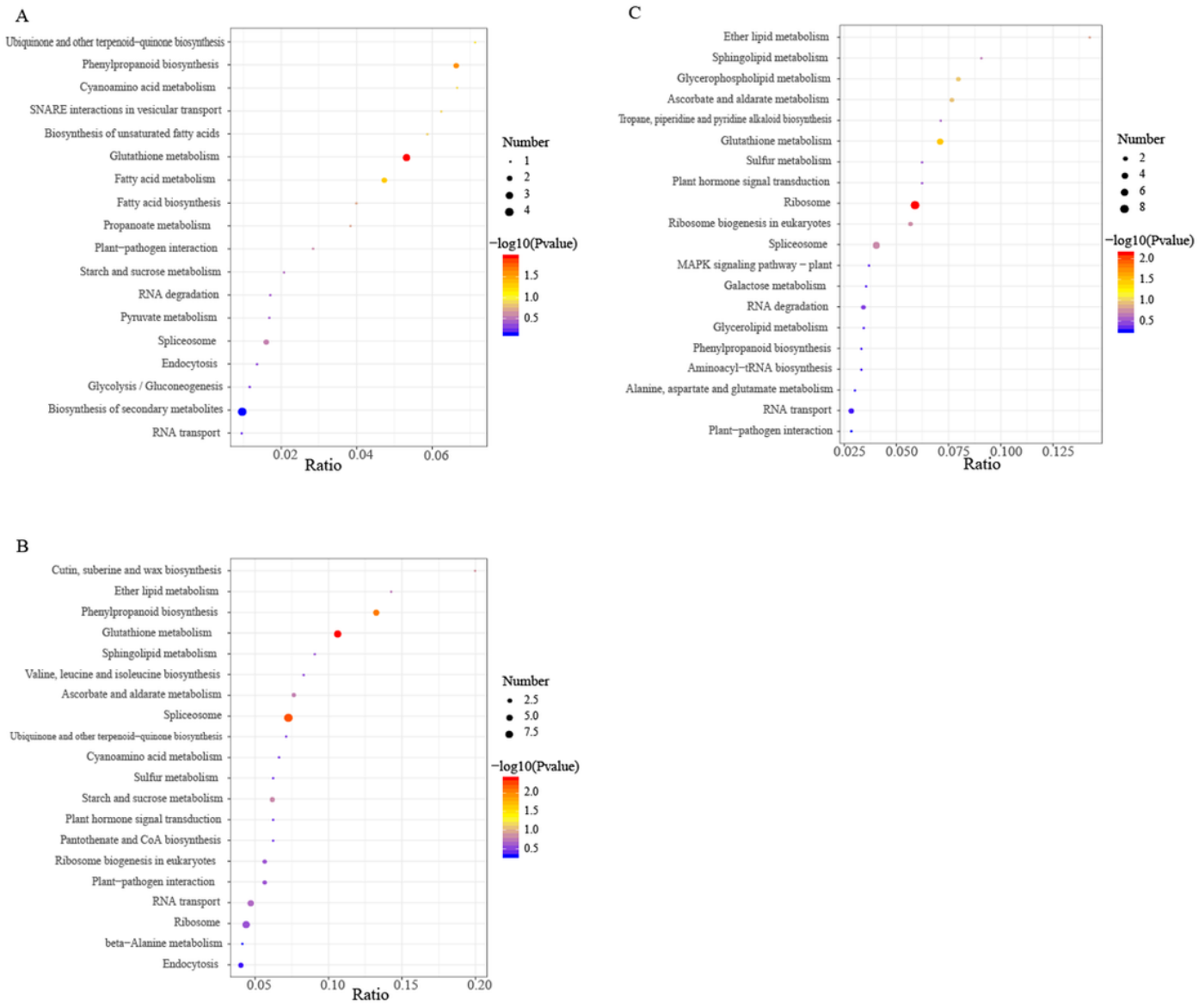

Figure 4

Bubbles representing diferent comparison groups of *A.mongolicum* seedsfollowing diferential protein KEGG enrichment. Note: a: compared T1 with CK (T1 vs. CK); b: compared T4 with CK (T4 vs. CK); c: compared T4 with T1 (T4 vs. T1). The horizontal coordinate in the graph is the ratio of the number of diferential proteins in the corresponding pathway to the number of total proteins identifed in that pathway; the larger the value, the higher the diferential protein enrichment in that pathway. The colour of the dot represents the P value of the hypergeometric test; the colours range from blue to red, the redder the colour, the smaller the value and the more reliable and statistically signifcant the test is. The size of the dots represents the number of diferential proteins in the corresponding pathway, the larger the dot, the more diferential proteins are present in the pathway.

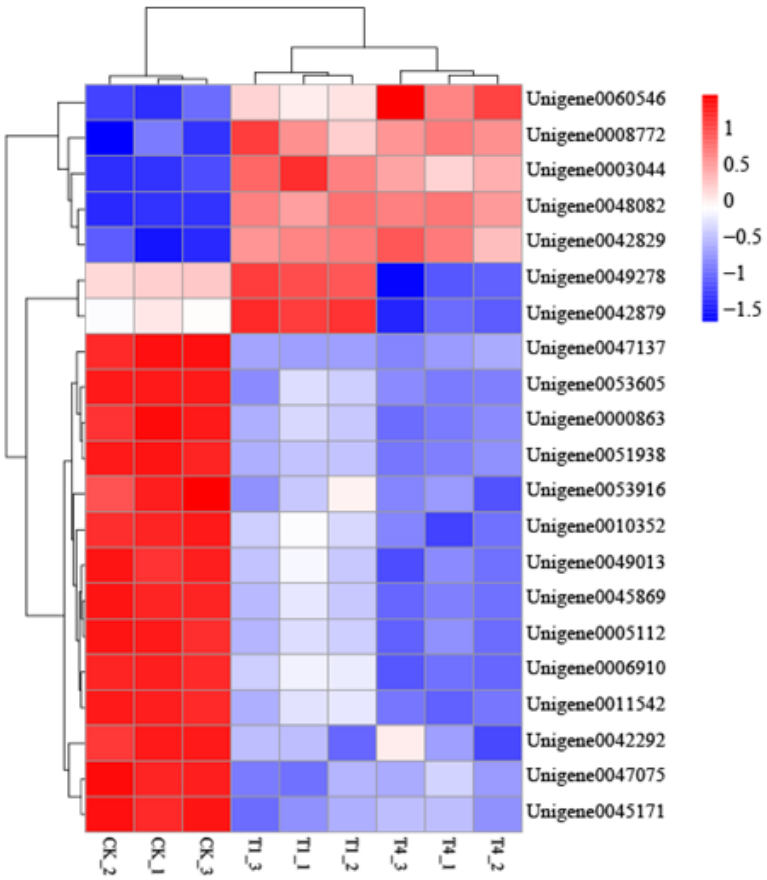

Figure 5

Heat map of clustering of co-significantly estimated differential proteins

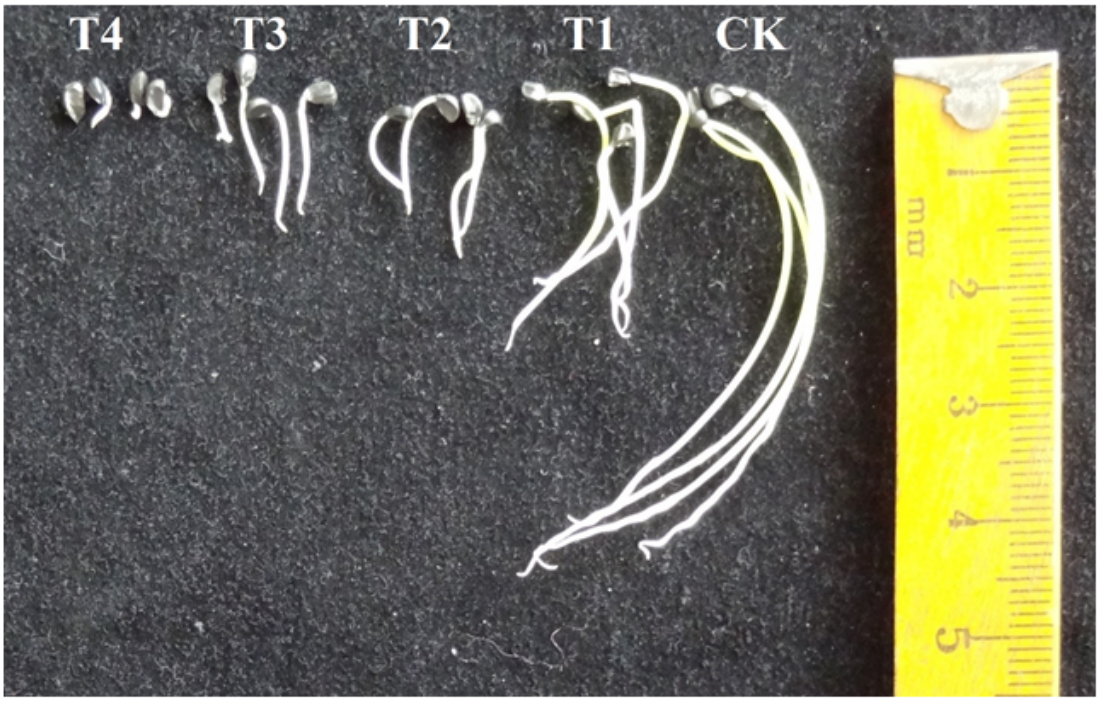

Figure 6

Embryonic root growth of *A.mongolicum* seeds after different aging treatments

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1GOenrichmentanalysisofDEPsunderagedA.mongolicumseedsofdifferentcomparisongroups..xlsx
- SupplementaryTableS2KEGGenrichmentofdifferentialproteinsindiferentcomparisongroups..xlsx
- SupplementaryTableS3ListofsomeimportantDEPs.xlsx